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Effects of *Strobilanthes crispus* Extract and Individual Polyphenols on Lipolysis

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Acknowledgements

Abstract

Obesity is a disease that has reached epidemic proportions across the world. Many types of treatments have been used to combat obesity including using synthetic drugs such as sibutramine and orlistat. However, the high cost and potentially hazardous side-effects of anti-obesity drugs have led many researchers to turn to naturally occurring compounds obtained from fruits, vegetables, herbs and plants for the treatment of obesity. In our study, the anti-obesity effects of *S. crispus* crude extract (SCE) and individual polyphenols (EGCG, Resveratrol, Phloridzin, Quercetin and Verbascoside) found in fruits, vegetables and herbs have been investigated.

The effects of *S. crispus* extract (SCE) *in vivo* were tested on high fat-induced obese LDLr KO mice maintained on high fat diet (HFD) or switched to low fat diet (LFD). All mice were HFD for 25 weeks to induce obesity, after which half were maintained on the HFD and half switched to LFD. At the same time, mice were given normal water or 0.1% (w/v) SCE in water at Week 0-4 which was increased to 1% (w/v) at Week 5-10. Oxygen consumption (VO_2), CO_2 production (VCO_2), RER, locomotor activity (LMA) and heat production (HP) were measured at Week 0, 5 and 10. Food intake, water intake and body weight was measured weekly. Plasma glycerol (PG) and abdominal adipose tissue (AAT) weight were determined at Week 10. Mice switched to LFD lost weight ($p< 0.001$), mainly due to decreased energy intake ($p<0.001$). They also had lower AAT weight and PG concentration (all $p<0.001$). SCE had no effect at either dose on body weight, VO_2, VCO_2 or LMA, but significantly reduced respiratory exchange ratio (RER) ($p=0.034$) and increased HP at Week 4 ($P=0.048$), without altering food or water intake ($p=0.1$, $p=0.222$). PG concentration were also increased in SCE treated mice ($p=0.032$).

The effects of SCE and individual polyphenols *in vitro* were tested on rat epididymal and human omental adipose tissue explants and results were compared with with the results from the pig perirenal adipose tissue explants. SCE does not appear to have any direct effect on lipolysis in the rat epididymal adipose tissue explants and human omental adipose tissue explants. EGCG was found to consistently inhibit lipolysis in rat, human and pig adipose tissue explants and the effects were greatest at 100μM. The effects of Phloridzin in

human, rat and pig fat explants were inconsistent as it was found to either increase or decrease lipolysis with different treatments. In all experiments, when Isoproterenol (IP) was present Resveratrol inhibited lipolysis and was independent of adenosine deaminase (ADA), with greater inhibition found at 100µM compared with 50µM Resveratrol. The effects of Resveratrol on lipolysis in the human adipose tissue explants was found to be different when compared with the effects found in the pig and rat adipose tissue explants when incubated for 24 and 26hr. The effects of Resveratrol on lipolysis in the human adipose tissue itself are also dependent on the presence and absence of ADA and IP.

Subsequent experiments were carried out where basal lipolysis and effects of the presence and absence of ADA were also investigated. Basal lipolysis was found to be higher in pig adipose tissue explants (Headland, 2007) than in human adipose tissue explants, but lower than rat adipose tissue explants. This is also true for the IP stimulated lipolysis in pig perirenal adipose tissue explants, but not in the pig subcutaneous adipose tissue explants, where IP stimulated lipolysis was similar to that in human omental adipose tissue. As expected, the presence of adenosine does have an effect on the lipolysis rate in rat, human and pig adipose tissue explants, since the addition of ADA (to metabolise/remove adenosine) increased basal lipolysis. However, only in the pig perirenal adipose tissue explants was IP stimulated lipolysis found to be increased with ADA. In the human omental adipose tissue explants, we also found that although BMI and age had weak negative correlations with lipolysis, these were not statistically significant (P=0.097 for BMI, P=0.48 for age). However, the trend suggests that IP stimulated lipolysis decreased with increased BMI.

Thus, SCE appeared to induce lipolysis and body fat oxidation *in vivo* but no direct effect on lipolysis were found *in vitro*. Resveratrol is the most promising polyphenol to induce lipolysis based on the studies across the rat, human and pig species compared with quercetin, EGCG and Phloridzin. The consistent effects of EGCG on lipolysis inhibition however, might also be an anti-obesity effect through the mechanism of adipocyte apoptosis which requires further study.

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Abbreviations

°C	Celcius
μ	Micro
μg	Microgram
μM	Micromolar
4-aminoantipyrine	4-AAP
AC	Adenylyl cyclase
ACTH	Adrenocorticotropic hormone
ADA	Adenosine deaminase
ADP	Adenosine-5'-diphosphate
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
AR	Adrenoreceptor
ATGL	Adipose triglyceride lipase
ATP	Adenosine-5'-triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
BW	Bodyweight
cAMP	Cyclic adenosine monophosphate
CLAMS	Oxymax Comprehensive Lab Animal Monitoring System
CO_2	Carbon dioxide

CVD	Cardiovascular disease
DAP	Dihydroxyacetone phosphate
DG	Diglyceride
dl	Decilitre
EGCG	Epigallocatechin gallate
FA	Fatty acid
FAs	Fatty acids
g	Gram
G	Gravity
G-1-P	Glycerol-1-phosphate
Gi	Inhibiting GTP-binding protein
GK	Glycerol kinase
GPO	Glycerol phosphate oxidase
Gs	Stimulatory GTP-binding protein
H_2O	Water
H_2O_2	Hydrogen peroxide
HATE	Human adipose tissue explant
HBA	Hydroxybenzoic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High-fat diet
HPLC	High-performance liquid chromatography
hrs	Hours
HSL	Hormone-sensitive lipase
HSPG	Heparin sulphate proteoglycans

IOTF	International Obesity Task Force
IP	Isoproterenol
KCl	Kalium choride
kg	Kilogram
KO	Knockout
KRB	Krebs-ringer bicarbonate
L	Litres
LDLr	Low-density-lipoprotein receptor
LFD	Low-fat diet
m	Metres
M	Moles
mg	Miligrams
MG	Monoglyceride
MgSO4	Magnesium sulphate
min	Minutes
ml	Millilitres
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acids
nm	Nanometres
nM	Nanomolar
NPY-1	Neuropeptide Y
O$_2$	Oxygen
PATE	Pig adipose tissue explant
PBS	Phosphate buffered saline

PIA	N-6-phenyllisopropyl adenosine
PKA	Protein kinase A
POD	Peroxidase
RATE	Rat adipose tissue explant
RER	Respiratory exchange ratio
rpm	Revolutions per minute
SCE	*S. crispus* crude extract
SED	Standard error of the difference of the means
SEM	Standard error of means
TAGs	Triacylglycerols
TG	Trigylcerides
TLC	Thin layer chromatography
TNFα	Tumor necrosis factor alpha
U	Unit
UV	Ultra-violet
VCO$_2$	Carbon dioxide production
VLDL	Very-low-density lipoproteins
VO$_2$	Oxygen consumption
WHO	World Health Organization
wk	Week
α	Alpha
β	Beta
γ	Gamma

Chapter 1 - Introduction

1.1 Obesity

1.1.1 Definition and prevalence

Obesity is defined medically as a state of increased body weight, more specifically adipose tissue, of sufficient magnitude to produce adverse health consequences (Spiegelman & Flier, 2001). Bray (2004b) described obesity as a chronic disease in the same sense as hypertension and atherosclerosis. According to the World Health Organization (WHO, 2008), an adult is classified as being overweight if the Body Mass Index (BMI) is more than or equal to $25kg/m^2$ and obese if their BMI is more than or equal to $30 \ kg/m^2$. BMI is calculated as weight in kilograms divided by height in metres squared (kg/m^2). However, on the basis of available data in Asia, the WHO expert consultation (Yajnik & Yudkin, 2004) concluded that Asians generally have a higher percentage of body fat than white people of the same age, sex, and BMI. They also found that the proportion of Asian people with risk factors for type 2 diabetes and cardiovascular disease is substantial even below the existing WHO BMI cut-off point of $25kg/m^2$.

It is generally agreed that obesity has reached epidemic proportions across the world, although there is still some argument about its extent and effects (WHO, 2000, Jeffery & Sherwood, 2008; Basham & Luik, 2008). In 2005, WHO estimates that there were about 1.6 billion overweight adults aged 15years and above and at least 400 million adults are obese worldwide. The prevalence of obesity continued to rise in many parts of the world. There was an increase in the average annual change ranging from 0.2% to 18.5% in developed countries and 0.1% to 35.3% in developing countries (Low *et al.*, 2009). The UK government has recently started to consider how society might deliver a response to obesity in England over the coming decades (Butland *et al.*, 2007; Cross Government Obesity Unit, 2008) as obesity and overweight are still rising in England across all ages (Howel, 2010). According to the International Obesity Task Force (IOTF) it was estimated that the prevalence rate of obesity could reach 45% to 50% in the USA, 30% to 40% in Australia, England and Mauritius and more than 20% in

Brazil by 2025 (IOTF, 2008). Although obesity was initially most visible in developed countries, principally in the United States, it gained traction in many developing countries. As developing countries have become wealthier, adopted increasingly Westernized lifestyles characterized by increases in energy intake and reductions in energy expenditure and witnessed massive migration from rural to urban areas, obesity inevitably followed in the wake of these developments (Ford & Mokdad, 2008).

1.1.2 Health Impact and Treatments

The complications associated with obesity contribute to the 100,000 to 400,000 deaths per year (Mokdad *et al.*, 2001). This is because obesity increased the prevalence of many health hazards such as coronary artery disease, type 2 diabetes (Willet *et al.*, 1999), hypertension (Wilson *et al.*, 2002), dyslipidemia (Grundy & Barnett, 1990), osteoarthritis (Hart & Spector, 1993), obstructive sleep apnea (Gami *et al.*, 2003) and depression (Dixon *et al.*, 2003). In order to determine an individual's risk relating to obesity, WHO and the National Institutes of Health (NIH) has identified categories of BMI and the magnitude of potential health risk (Figure 1.1). Formal classification of severe obesity facilitates its conceptualization as a chronic disease.

Extensive data have demonstrated that with weight loss, obese patients can reverse the harmful health related effects attributed to excess weight (O'Brian, 2010; Klein *et al.*, 2004). Diet, exercise, pharmacotherapy, behavioural therapy, and lifestyle modification can each produce modest weight loss in the severely obese (Kissane & Pratt, 2011). Pharmacotherapy, in addition to diet and exercise, has been demonstrated to facilitate weight loss of 10% at 1 year (Eisenberg *et al.*, 2006). Behavior therapy is a useful adjunct to planned adjustments in food intake and physical activity. Specific behavioral strategies include: self-monitoring, stress management, stimulus control, problem solving, contingency management, cognitive restructuring and social support (Latner *et al*, 2002). Long-term maintenance of significant weight loss, however, continues to be the most challenging problem in the medically based treatment for obesity (Kissane & Pratt, 2011).
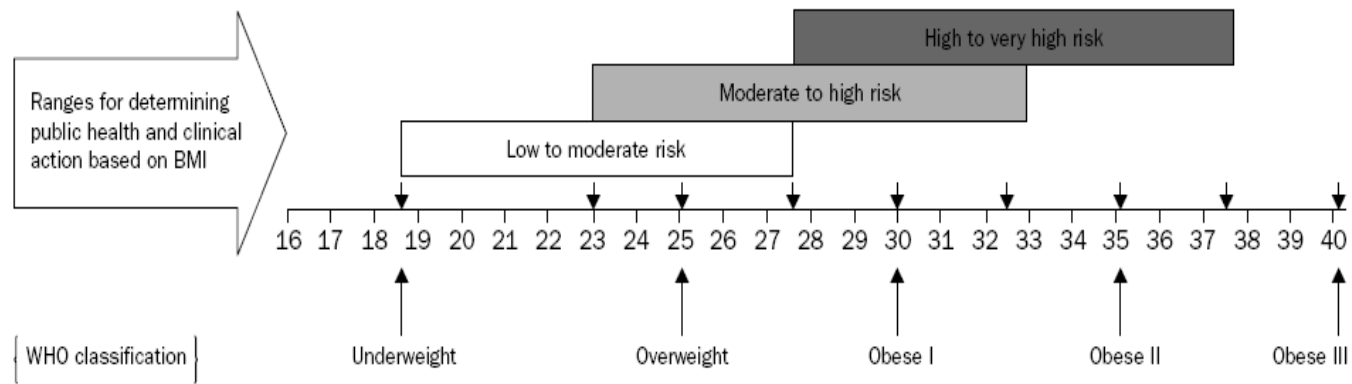
Figure 1.1 BMI cut-off points for public health action (Yajnik & Yudkin, 2004).

1.2 Adipose Tissue

1.2.1 Physiology and The Adipocyte Life Cycle

To understand more about obesity, we have to understand what is happening at the cellular concentration particularly in the adipose tissue. Adipose tissue is an organ that stores energy in the form of triglycerides synthesized from the excess of ingested fats and carbohydrates. The most important function of adipose tissue is for energy storage, mechanical and thermal protection for other organs, production of hormonal and metabolically active substances and protection against fat accumulation in other body organs (Svacina, 2008). Adipose tissue contains different cell types. Only one third of the tissue is constituted by adipocytes and the rest is represented by fibroblasts, macrophages, stromal cells, monocytes and preadipocytes (Geloen *et al.*, 1989). Hormonal activity and transcription factors are responsible for differentiation of preadipocytes to adipocytes (Farmer, 2006). The biologic events leading to obesity are characterized by changes in cell properties of adipocytes and may include an increase in the number or size or both (Flier, 1995).

Adipocytes are derived from mesenchymal stem cells, which have the potential to differentiate into myoblasts, chondroblasts, osteoblasts or adipocytes (Rayalam *et al.*, 2008a). The adipocyte life cycle includes alteration of cell shape and growth arrest, clonal expansion and a complex sequence of changes in gene expression leading to storage of lipid and finally cell death (Figure 1.2). Mesenchymal stem cells are the precursors of several different types of cells, including myoblasts, chondroblasts, osteoblasts and preadipocytes. Once pre adipocytes are triggered to mature, they begin to change shape and undergo a round of cell division known as clonal expension, followed by initiation of the genetic program that allows them to synthesize and store trigylcerides. Mature adipocytes can continue storing lipid when energy intake exceeds output, and they can mobilize and oxidize lipid when energy output exceeds input. Mature adipocytes can also undergo apoptotic cell death under certain conditions (Rayalam *et al.*, 2008a).

Figure 1.2 Adipocyte Life Cycle (Rayalam *et al*., 2008b)
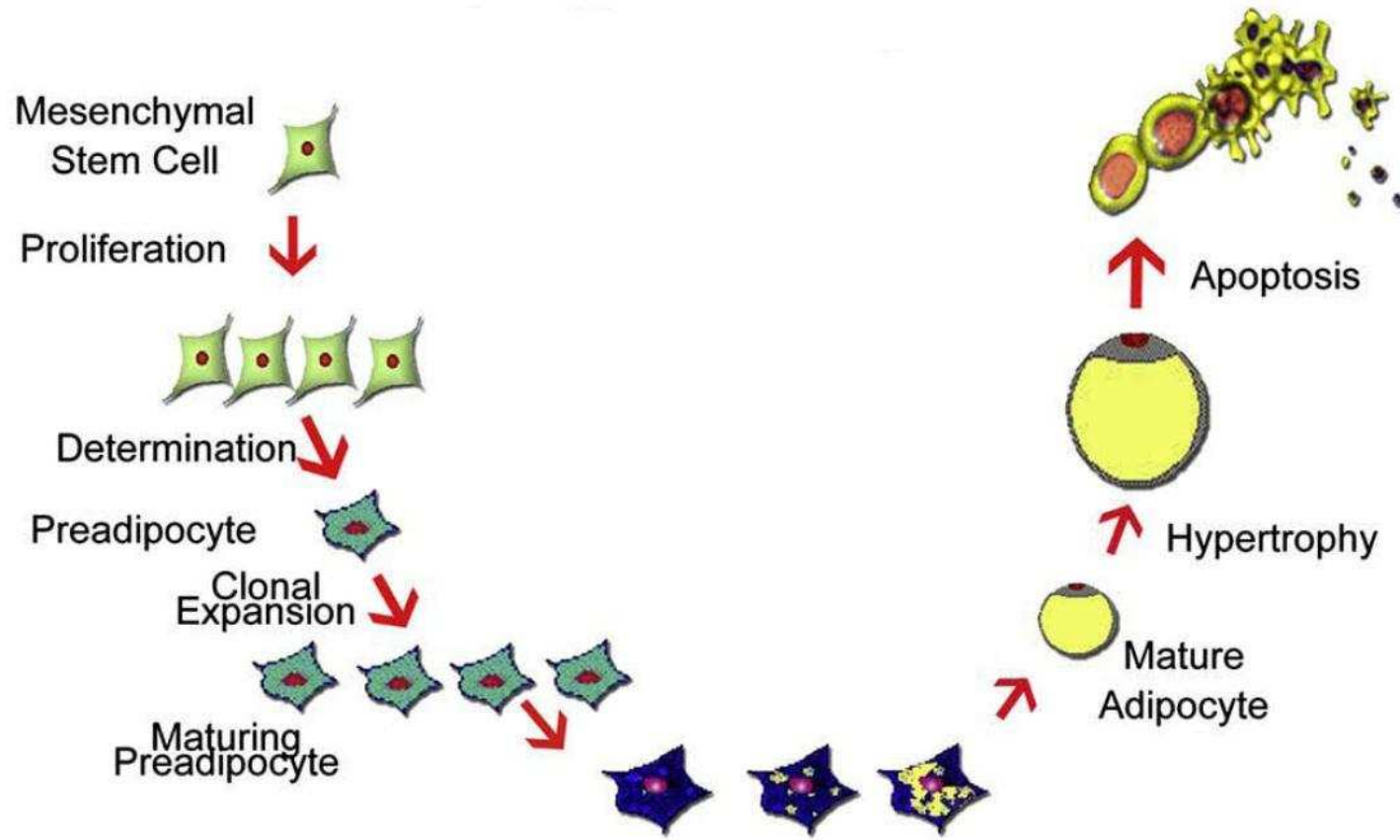
Mesenchymal stem cells are the precursors of several different types of cells, including myoblasts, chondroblasts, osteoblasts and preadipocytes. Once preadipocytes are triggered to mature, they begin to change shape and undergo a round of cell division known as clonal expansion followed by initiation of the genetic program that allows them to synthesize and store triglycerides. Mature adipocytes can continue storing lipid when energy intake exceeds output, and they can mobilize and oxidize lipid when energy output exceeds input. Mature adipocytes can also undergo apoptotic cell death under certain conditions.

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1.2.2 Endocrine Organ

Adipose tissue has been considered as an endocrine organ because of its capacity to secrete hormones and cytokines. It secretes a variety of factors in a manner dependent upon its metabolic state such as the release of tumor necrosis factor-α (TNF-α) from macrophages and the release of leptin adipocytes (Ailhaud 2006). These factors include proteins, metabolites and hormones. Thus, adipose tissue is not only known for its capacity to store excess dietary energy but also recognized as a fundamental participant in the control of energy metabolism. A growing number of adipocyte-derived factors have been described and their contribution to the pathophysiology of the metabolic syndrome, characterized by central adiposity, insulin resistance, dyslipidemia, hypertension, chronic inflammation and a prothrombotic state, is being investigated (Gimeno, 2005).

1.2.3 Lipogenesis

Most energy reserves in the human body are stored in adipocyte as triglycerides (TG) through the process of lipogenesis. Lipogenesis is the synthesis of fatty acids (FA) which form TG from carbohydrates or other energy sources in the diet (Figure 1.3). Lipid accumulation in adipose tissue depends on circulating FA uptake (Zechner *et al.*, 2000). Adipose tissue exerts a buffering activity by increasing plasma TG clearance and suppressing the release of non-esterified fatty acids (NEFA) into the circulation (Frayn, 2002). TG arise in the adipocyte from two major routes: de novo lipogenesis from non-lipid precursors or uptake of FA from the plasma. In humans, the major source for adipocyte TG comes from chylomicrons and very-low-density lipoproteins (VLDL). VLDL and chylomicron particles, too large to penetrate the endothelial lining of adipose tissue capillaries, must be processed in the luminal space (Lafontan & Langin, 2009).

TG in the lipoprotein particles are hydrolyzed by lipoprotein lipase (LPL) originating from adipocytes. LPL is synthesized and secreted by the adipocytes and is translocated into the lumen of capillaries. There, it binds to the luminal surface of endothelial cells by interacting with cell-surface glycosaminoglycans, especially heparin sulphate proteoglycans (HSPG)(Lafontan & Langin, 2009). Non-esterified fatty acids (NEFA) released from TG by LPL activity move through the endothelial lining to the adipocytes, where they are taken up, though some of them escape being trapped by the adipocytes, and are transported by albumin to other tissues. Specific NEFA-transporting proteins have been described (Frohnert

& Bernlohr, 2000; Hajri & Abumrad, 2002; Pohl *et al.*, 2004). Once taken up by the fat cells, NEFAs are esterified to TG.

1.2.4 Lipolysis

FA and TG availability in non-adipose tissues depends on the activity of lipolytic enzymes present in adipose tissue (Figure 1.3). The principal role of adipose lipolytic enzymes is to provide other tissues with FA in case of energy demand. TG stored in the lipid droplet are first hydrolyzed by the enzyme adipose triglyceride lipase (ATGL), also known as desnutrin, releasing a diacylglycerol moiety and FA (Villena *et al.*, 2004. This enzyme is induced under fasting condition and repressed after feeding and is downregulated in ob/ob and db/db mice (Villena *et al.*, 2004). There are other enzymes that can hydrolyze TG such as triglyceride hydrolase (TGH) and adiponutrin, but their role in adipocyte lipolysis is secondary (Jenkins *et al.*, 2004; Lake *et al.*, 2005; Soni *et al.*, 2004).

After hydrolysis by ATGL, diacylglycerols are then hydrolyzed sequentially by the hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL), producing FFAs and glycerol (Fredrikson *et al.*, 1986; Holm, 1986). Different lipases gain access to the lipid droplet when proteins that coat the vesicle (perilipins) are phosphorylated. Perilipin A normally prevents lipolysis of TG by surrounding the lipid droplet, preventing the access of lipases (Brasaemle *et al.*, 2000). β-adrenergic stimulation of adipocytes and the subsequent protein kinase A-dependent phosphorylation of HSL and perilipin trigger the translocation of HSL from the cytoplasm to the lipid droplet and induce neutral lipid hydrolysis (Egan *et al.*, 1992).

During fasting, glucagon and catecholamines stimulate lipolysis in the adipocytes by activating several lipases via PKA, resulting in a mobilization of FFA from the adipocyte to the circulation, which are then bound to albumin and transported to muscle, liver, heart and other tissues for oxidation or re-esterification (Lafontan *et al.*, 2000). β-adrenergic receptors induce lipolysis, whereas α_2-adrenergic receptors inhibit lipolysis. For example, visceral fat cells are more responsive to b-adrenergic receptors in comparison to subcutaneous adipocytes. This explains why visceral fat is more easily mobilized (Deshaies & Despres, 2000). In fact, moderate weight loss induced by a low-energy diet is associated with an increase

in β-adrenergic receptor sensitivity. In the postprandial state, high plasma insulin concentrations inhibit lipolytic enzymes, decreasing lipid mobilization from adipose tissue. These effects are mediated by the activation of phosphodiesterase 3B that degrades cAMP, preventing the stimulation of several lipases, and by activating protein phosphatase-1 that causes dephosphorylation of HSL, in both instances reducing the rates of lipolysis (Langin, 2006; Ragolia & Begum, 1998).

Nevertheless, during obesity, excess FA release from adipose tissue increases deposition in muscle and liver, contributing to the development of insulin resistance. Furthermore, FA release is also associated with hypertriglyceridemia, failure in insulin clearance by liver, and impaired insulin secretion by β cells in the pancreas (Cases & Barzilai, 2000).

Figure 1.3 Lipogenesis and Lipolysis (Frigolet Vásquez-Vela, 2008)
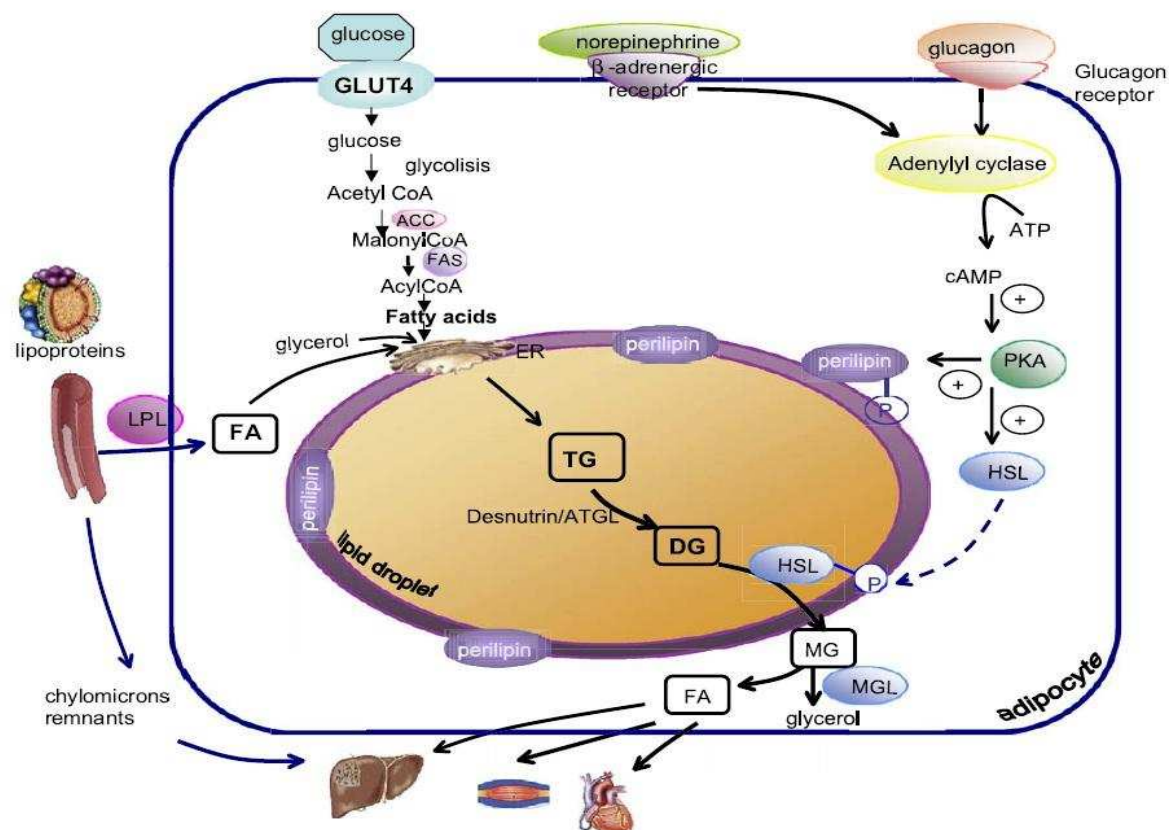
Lipogenesis and lipolysis. Glucose is transported into the adipocyte by the insulin sensitive transporter GLUT4. This glucose is oxidized via glycolysis to acetyl-CoA and then converted into fatty acids, which arethen esterified to glycerol in the endoplasmic reticulum (ER) to form triglycerides (TG). These are then translocated into the lipid droplet. Fatty acids (FA) obtained from lipoproteins, through the action of lipoprotein lipase (LPL) are also esterified into TG and stored. Under fasting conditions, lipolysis is activated by G-protein-coupled receptors resulting in an increase in cAMP. This initiates aprotein kinase cascade that results in the phosphorylation of the protein perilipin located in the membrane of the lipid droplet. This cascade also phosphorylates the hormone-sensitive lipase (HSL) and triggers its translocation from the cytoplasm to the lipid droplet this enzyme catalyses the hydrolysis of diglycerides (DG) produced by the adipocyte triglyceride lipase (ATGL) to form monoglycerides (MG). MG are then further hydrolysed to release free glycerol. The fatty acids generated are released to nonadipose tissues, mainly for energy purposes.

1.3 Medicinal plants

1.3.1 Introduction

Medicinal plants and plant extracts represent the oldest and most widespread form of medication. At least 25% of the active compounds present in currently prescribed synthetic drugs were first identified in plant sources (Balandrin *et al.*, 1985). Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands of years. The first records, written on clay tablets in cuneiform, are from Mesopotamia and date from about 2600 BC; among the substances that were used were oils of *Cedrus* species (Cedar) and *Cupressus sempervirens* (Cypress), *Glycyrrhiza glabra* (Licorice), *Commiphora* species (Myrrh) and *Papaver somniferum* (Poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation (Gurib-Fakim, 2006).

The search for obesity treatments became popular in the mid-19th century where industrialization made obesity a prevalent problem (Mermel, 2004). Vinegar and cabbage soup became one of the earliest widely touted obesity cures with the common rationale that the acidic makeup of these foods literally chewed up fat although their success as weight management agents has more to do with the psychological phenomena than any unique chemical properties of the individual foods (Mermel, 2004). Today, health functions of well-known micronutrients are studied as well as traditional ethnic plant foods and herbal extracts towards the development of functional, health promoting foods which includes the treatment of obesity. Although not designated as vitamins, there is a large group of compounds in fruits and vegetables, teas and herbal extracts which might not be essential throughout life or cause clinically manifested deficiencies, but are essential for health and well-being in adulthood and in the elderly population (Holst & Williamson, 2008). These compounds are called phytochemicals and when consumed in the diet, they may reduce the risk of age-related chronic diseases.

1.3.2 Anti-obesity Effects

At present, because of dissatisfaction with high costs and potentially hazardous side effects of anti-obesity drugs, the potential of natural products for treating obesity is under exploration, and this may be an excellent alternative strategy for developing future effective, safe anti-obesity drugs (Mayer *et al.*, 2009; Nakayama *et al.*, 2007; Park *et al.*, 2005). Although weight loss and weight control drugs are becoming extremely common in today's society, the remedies provided by the diet industry have failed in the long-term maintenance of weight loss in obese patients (Wadden, 1993). Moreover, it has been estimated that more than 90% of the people who lose weight by dieting return to their original weight within 2–5 years (Stern *et al.*, 1995). For the treatment of obesity, natural products have been known to act as lipase inhibitors, metabolic stimulants, appetite suppressants, starch blockers/nutrient absorption inhibitors, glucose/insulin metabolism regulators, lipid metabolic regulators, adipogenesis inhibitors, apoptosis inducers, lipolysis inducers and energy expenditure stimulators (Rayalam *et al.*, 2008a; Yun, 2010). During my PhD studies, I have focused on the effect of *Strobilanthes crispus* extract (SCE) as an energy expenditure stimulator and the effect of individual polyphenols as lipolysis inducers.

Numerous naturally-occurring compounds have been proposed as treatment for weight loss via enhanced energy expenditure including caffeine (Dulloo, 1993; Racotta *et al.*, 1994), capsaicin (Rayalam *et al.* b), 2008; Kawada *et al.*, 1986) and catechins such as epigallocatechin and epigallcatechin gallate (EGCG) (Wolfram *et al.*, 2006; Moon *et al.*, 2007). Natural products have also been found to have the potential for mobilizing lipids by stimulating lipolysis in adipocytes which can lead to weight loss in people with obesity. (Prins & O'Rahilly, 1997; Sorisky *et al.*, 2000). Lipolysis in adipocytes has been found to be increased by docosahexaenoic acid (DHA) in fish oil (Kim *et al.*, 2006) and raspberry ketone from red raspberry (Morimoto *et al.*, 2005).

1.4   **Strobilanthes crispus**

1.4.1 Introduction

The *Strobilanthes crispus* ZII 109 (L.) Bremek or *Saricocalyx crispus* ZII 109 (L.) Bremek (Acanthaceae) plant is native to countries from Madagascar to Indonesia, and is commonly known as 'daun picah beling' in Jakarta or 'enyoh kelo', 'kecibeling', or 'kejibeling' in Java (Sunarto, 1977). It was first recorded by Thomas Anderson (1832-1970) who classified the plant under Spermatophyta (Flowering Plants and Gymnosperma)(Brummit & Prowell, 1992).

Strobilanthes (cone head) was named from the combination of strobilos, which means flower (Plowden, 1968), and crispus, which is phyllostachyus or spike-like leaf (phyllo means leaf, and stachyus means spike)(Jackson, 1960). The conjunction of the names leads to the meaningful definition of the physical plant (Figure 1.4). This bush-like plant can be found on riverbanks or abandoned fields; some Javanese use this plant as fence hedges. The leaves are oblong-lanceolate, rather obtuse, and shallowly crenate-crispate (Backer & Bakhuizen, 1963). Top surfaces of the leaves are darker green in color and less rough compared with the below surface (Sunarto, 1977). The leaves are very scabrid on both surfaces and covered with short hairs, whereas the flowers are short, dense, and are panicled spikes (Heyne, 1987). The plant can be easily propagated by using the stacks (Sunarto, 1977).

Figure 1.4 *Strobilanthes crispus* Plant and Leaves

1.4.2 Composition

Soediro and colleagues (1983) isolated and identified an ester glycosidic compound of caffeic acid, a verbascoside in the leaves, by using thin layer chromatography (TLC) techniques. This compound is known to have analgesic effects internally, and antifungal and antibacterial effects when used externally. Later, seven phenolic acids; ρ-hydroxyl benzoic, ρ-voumaric, caffeic, vanillic, gentinic, ferulic and syryngic acid – were also identified by TLC, paper chromatography and UV spectrophotometric techniques (Soediro *et al*., 1987).

Studies done by Ismail and colleagues (2000) compared the chemical composition and total antioxidant activity between *Strobilanthes crispus* tea to Green tea, Yerbamate tea and Black tea (Table 1.1). It was found that the dried leaves of *Strobilanthes crispus* contained a high amount of minerals including potassium (51% of total mineral), calcium (24% of total mineral), sodium (13% of total mineral), iron (1% of total mineral) and phosphorus (1% of total mineral). High content of water-soluble vitamins (C, B_1 and B_2) contributed to high antioxidant activity of the leaves. Catechins of *Strobilanthes crispus* leaves showed highest antioxidant activity when compared with Yerbamate and vitamin E.

1.4.3 Health Benefits

Although there is very little record of this plant being used for medicinal purposes, an infusion of the dried leaves of *Strobilanthes crispus* have been used as an antidiabetic, diuretic, antilytic, and laxative in Indonesia (Sunarto, 1977). A study also indicated that the water extract of *Strobilanthes crispus* contained compounds with very high affinity to protein molecules, which may bind the active sites of reverse transcriptase, therefore inhibiting the proliferation of retroviruses – agents in viral diseases such as acquired immuno deficiency syndrome and adult T-cell leukemia (Kusumoto *et al.,* 1992). A recent study done by Rahmat (2006) showed that *Strobilanthes crispus* leaves may have potential effect as anticancer agent.

There are not many studies done to investigate the potential of *Strobilanthes crispus* leaves as medicinal plant. The local consumption of *Strobilanthes crispus* as tea have sparked the possibility that the leaves of this plant may have the health-promoting potentials of conventional tea especially as an anti-obesity

agent. This is supported by the fact that other types of tea has been found to have an effect in preventing a positive energy balance and obesity (Choo, 2003; Westerterp-Plantenga *et al.*, 2006; Yang *et al.*, 2001; Wang *et al.*, 2003, Khan & Mukhtar, 2007).

Table 1.1 Chemical composition of *Strobilanthes crispus*, Yerbamate, Green and Black teas (Ismail *et al.*, 2000)

	S. crispus	Yerbamate[a]	Green tea[b]	Black tea[b]
Moisture content (%)	69.3 ± 0.1	9.0	-[c]	3.9–9.5
Total ash (%)	21.6 ± 0.1	6.7	6.1–9.2	4.9-6.5
Water-insoluble ash (%)	13.8 ± 1.0	-	5.2-7.2	-
Water-soluble ash (%)	7.9 ± 1.0	2.3	1.6-2.6	3.0-4.2
Extractivities (%)	6.7 ± 0.4	33.1	33.0-45.0	30.0-50.0
Protein content (%)	13.3 ± 0.9	9.8	-	5.0-6.2
Total carbohydrate (%)	4.3 ± 0.7	-	-	-
Crude fiber (%)	13.9 ± 0.6	15.5	9.0-15.0	-
Caffeine (%)	0.01	0.6-1.4	1.5-4.3	10.0-11.0
Tannin (%)	1.0 ± 0.3	9.8	-	-
Alkaloid (%)	3.2 ± 0.6	4.4	-	-
Catechin (%)	1.18 ± 0.08	0.9	5.8	-

Each value represents the mean ± SD of 6 determinations.

a: Hart and Fisher (1971). b: Pearson (1976) c:shows no studies have been done

Value refers to values on a wet weight basis

1.5   Polyphenols

1.5.1   Introduction and Classification

Plants produce polyphenols as secondary metabolites involved in diverse processes, such as growth, lignifications, pigmentation, pollination and resistance against pathogens, predators and environmental stress (Duthie *et al.*, 2003). Edible plants provide the human diet with more than 8000 different polyphenols (Fraga *et al.*, 2010). Previously, most of the nutritional interest in polyphenolic compounds was in the effects of certain polyphenols to bind and precipitate macromolecules such as dietary protein, carbohydrate, and digestive enzymes, thereby reducing food digestibility (Bravo, 1998). However, interest in food phenolics have increased greatly because of the properties associated with some phenolics and their potential effects on human health (Rawel & Kulling, 2007; Finley, 2005; Khan & Mukhtar, 2007; Hooper & Cassidy, 2006).

The polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and the structural elements that bind these rings to one another. Distinctions are thus made between the phenolic acids, flavonoids, stilbenes and lignans (Figure 1.5). The flavonoids, which share a common structure consisting of 2 aromatic rings (A and B) bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C), may themselves be divided into 6 subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavonones, anthocyanidins and flavonols (catechins and proanthocyanidins) (Figure 1.6). The main subclasses that are important from a human health perspective are the flavones, flavonols, flavan-3-ols, isoflavones, anthocyanidins and lignins (Hooper & Cassidy, 2007).

Figure 1.5 Chemical Structures of Polyphenols (Manach *et al.*, 2004)

Hydroxybenzoic acids
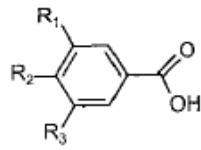
Hydroxycinnamic acids
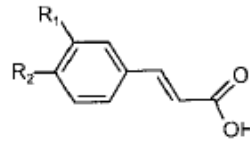
Flavonoids
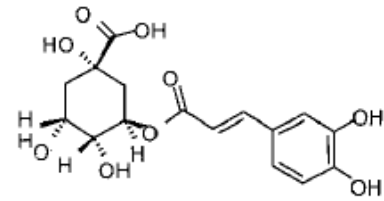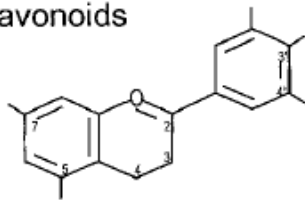
Chlorogenic acid

Stilbenes
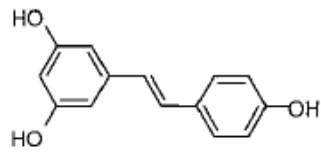
Resveratrol

Lignans
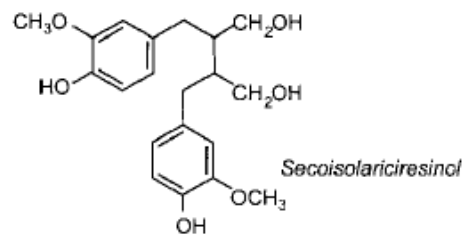
Secoisolariciresinol

Figure 1.6 Chemical Structures of Flavonoids (Manach *et al.*, 2004)
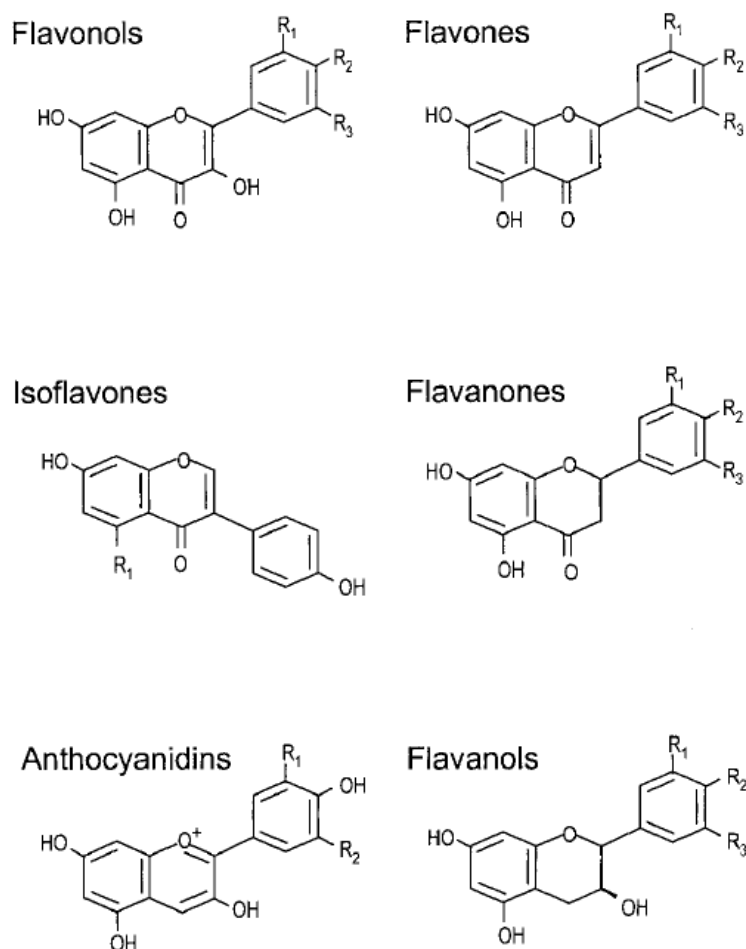
1.5.2 Anti-obesity Effects

A wealth of information indicates that there are numerous bioactive components derived from nature, which are potentially useful as obesity treatments. This includes polyphenols which have been reported to prevent and ameliorate the effect of obesity and related metabolic syndrome.

Recent research findings include the discovery of licorice flavonoid oil, which was found to decrease abdominal adipose tissue in diet-induced obese rats by decreasing the enzymatic activities of fatty acid synthase and increasing the enzymatic activity of acyl-CoA dehydrogenase, the rate limiting enzyme in the fatty acid oxidative pathway (Kamisoyama *et al.*, 2008; Aoki *et al.*, 2007). Oligonol, a new lychee fruit-derived polyphenol was found to enhance lipolysis in

primary adipocytes through the activation of the ERK1/2 pathway (Ogasawara *et al.*, 2009).

In the widely consumed green tea, epigallocatechin-3-gallate (EGCG), the polyphenol considered to be the most bioactive component of green tea (Wolfram *et al*, 2006), was found to increase weight loss and decreased body fat in obese subjects through many mechanisms (Figure 1.7) that lowered energy intake and increased energy expenditure (Thielecke & Boschmann, 2009; Rains *et al.*, 2011). Moreover, EGCG and other individual polyphenols, resveratrol and quercetin were found to activate adenosine monophosphate-activated protein kinase (AMPK) which can induce adenosine triphosphate (ATP) generation through pathways such as glycolysis and β-oxidation (Hwang *et al.*, 2009).

Most recently, aques rooibos (*Aspalathus linearis*) extract which contained 25%w/w of total polyphenol content was found to reduce adipocyte size and number as well as prevent dietary-induced hepatic steatosis in LDLr KO mice fed a high fat, high cholesterol diet (Beltrán-Debón *et al.*, 2011).

From the examples above, we can conclude that dietary polyphenols provide a wide spectrum of biological actions potentially beneficial for obesity and these biological actions, involve different mechanisms.

1.5.3 Bioavailability

To exert their biological properties, polyphenols have to be available to some extent in the target tissue. Polyphenols that are the most common in the human diet are not necessarily the most active within the body, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. Therefore, it is important to know the factors that determine their release from food, their extent of absorption in the gut (bioaccessability), metabolism, and bioavailability. A general view of the different aspects which are involved in the bioavailability of polyphenols is given in Figure 1.8. In order to reach the target tissues, the polyphenols have to pass multiple barriers and are exposed to numerous metabolic processes. The result of this is that the target tissues are exposed to not only the ingested polyphenol, but rather to a complex mixture of metabolites from various origins.

Figure 1.7 Mechanisms by which EGCG may decrease energy intake, increase energy expenditure, and reduce adipose tissue mass and prevent or treat obesity and its associated diseases, diabetes and hypertension (Thielecke & Boschmann, 2009)

↓ food intake
↓ lipid emulsification
↓ lipid digestion
↓ lipid absorption
↓ lipid transport (↓ LDL & VLDL, ↑ HDL)
↓ carbohydrate absorption
↓ insulin, IGF
↓ sex steroids
↑ CCK

↑ thermogenesis
↑ fat oxidation in lver and muscle
↑ glucose uptake in muscle (↑ GLUT4)
↓ lipid content in liver
↑ fecal lipid excretion

Energy intake ↓

Energy expenditure ↑

Adipocyte

↓ growth
↓ differentiation
↓ apoptosis
↓ lipogenic activity (↓ ACC, FAS, GPDH, SCD1)
↓ lipolytic activity (↑HSL)
↓ glucose uptake, (↓ GLUT4)

↓ adipocyte size / number → ↓ adipose tissue mass → ↓ obesity → ↓ diabetes / hypertension

Figure 1.8 Different factors influencing the bioavailability of polyphenols (Possemiers *et al.*, 2011)
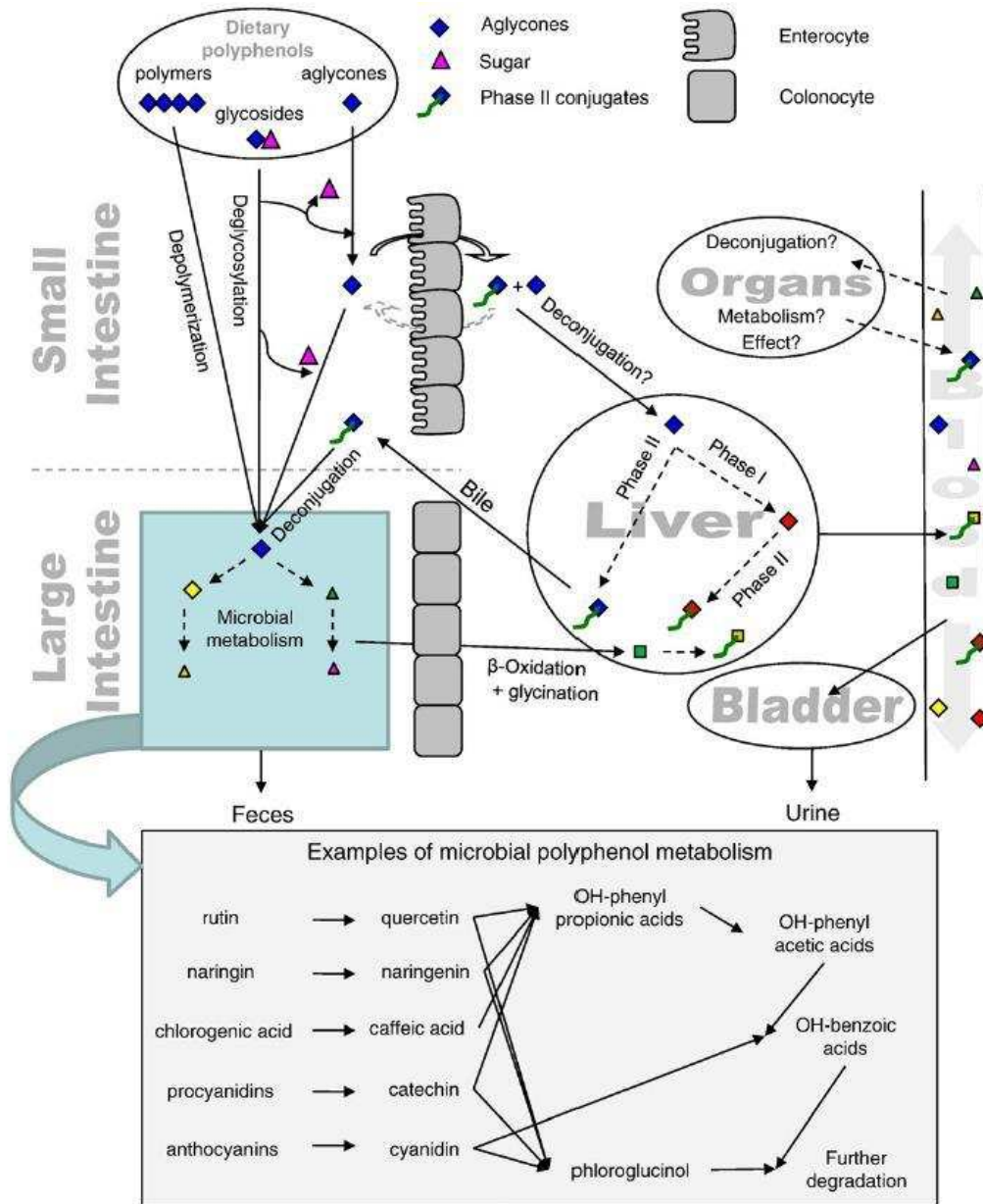
A part of the ingested fraction may be absorbed from the small intestine and may be conjugated with glucuronic acid or sulfate during absorption and in the liver. It can further be metabolized, circulate in the bloodstream or enter into enterohepatic circulation. The fraction of the ingested polyphenols which reaches the colon directly or indirectly can be exposed to the intestinal microbiota and its extensive metabolic pathway. Despite the high initial structural variations, overlapping pathways result in the production of a relatively small number of metabolites.

1.5.3.1 Bioaccessability

Bioaccessibility is defined as the amount of a food constituent that is present in the gut, as a consequence of the release of this constituent from the solid food matrix, and may be able to pass through the intestinal barrier (Saura-Calixto *et al.*, 2007). Only polyphenols released from the food matrix by the action of digestive enzymes (small intestine) and bacterial microflora (large intestine) are bioaccessible in the gut and therefore potentially bioavailable. Therefore, polyphenols have to be bioaccessible in order to be bioavailable to the cellular metabolism

Soluble aglycones or glycosides may be bioaccessible in the small intestine. The large intestine is however, an important site in the gastrointestinal tract where polyphenols become bioavailable. This is where polyphenols associated with the indigestible fraction of the food reach the colon, where they become fermentation substrates for bacterial microflora along with the non-digestible food constituents (Saura-Calixto *et al.*, 2007). The abundant microflora in the colon plays a critical role in the metabolism of polyphenols. After microbial enzyme metabolism of any polyphenols that reach the colon, there are two possible routes available, namely absorption of intact polyphenols through the colonic epithelium and passage into the bloodstream or breakdown of the original polyphenol structures into metabolites (Williamson & Manach, 2005). Also, unabsorbed polyphenols that reach the colon may counteract the effects of dietary pro-oxidants in the colon produced during colonic bacterial metabolism. Meanwhile, one crucial additional aspect of bacterial metabolism of polyphenols should be taken into account, i.e. the huge interindividual variation in the rate and extent of intestinal microbial metabolism. The consequence is therefore that the final circulating metabolite patterns can highly vary in both concentration and composition, resulting in highly varying bioactivity in different individuals (Possemiers *et al.*, 2011).

Colonic metabolism of dietary polyphenols has been extensively studied and associations between urinary excretion of simple phenolic structures, such as hippuric acid derivatives and bacterial polyphenol degradation was shown in many studies (Gao *et al.*, 2006; Gonthier *et al.*, 2003; Mulder *et al.*, 2005; Olthof *et al.*, 2003). It is clear that the metabolites are formed by microbial action, as

lower recovery was found in human and animal studies where antibiotics were dosed prior to flavonoid uptake (Kohri *et al.*, 2001; Gott & Griffiths, 1987).

1.5.3.2 Bioavailability of Catechins

One of the polyphenol groups with high bioavailability is the catechins. This is confirmed by Stalmach and colleagues (2009) after they studied the absorption of flavan-3-ol monomers which are highly available in the Choladi green tea (648µmol/500 mL) when ingested by 10 human volunteers. Unmetabolised (–)-epigallocatechin-3-gallate (EGCG) and (–)-epicatechin gallate were found in the circulatory system together with glucuronide, methyl and sulphate metabolites of (epi)catechin and (epi)gallocatechin with Cmax values ranging from 25 to 126 nM and Tmax values of 1.6–2.3 h. Cmax refers to the maximum concentration that a type of catechin achieved in the circulatory system after it has been administrated and prior to the administration of a second dose. Tmax is the time at which the plasma concentration is maximum for each dose interval. These Tmax values indicated the rate of absorption in the small intestine. The appearance of unmetabolised flavonoids in plasma is unusual compared with other polyphenols. The passage of the EGCG and (–)-epicatechin-3-gallate through the wall of the small intestine into the circulatory system without metabolic modification could be a consequence of the presence of the 3-O-galloyl moiety, as gallic acid *per se* is readily absorbed with a reported urinary excretion of 37% of intake (Shahrzad & Bitsch, 1998; Shahrzad *et al.*, 2001) .

1.5.3.3 Bioavailability of Quercetin

Quercetin which is ubiquitous in plants and plant-derived materials especially onions is present as various glycosides. The bioavailability of the flavonol quercetin was also found to be high based on the study by Hollmann and colleagues (1997). In their study, nine subjects were fed a single large dose of onions, which contained glucose conjugates of quercetin, apples, which contain both glucose and non-glucose quercetin glycosides, or pure quercetin-3-rutinoside, the major quercetin glycoside in tea. Plasma concentrations were then measured over 36 h. Results showed that the dietary antioxidant quercetin was found in the circulation after the consumption and a considerable fraction of the absorbed quercetin was present in plasma throughout the day. The bioavailability

of quercetin was confirmed later by a similar study by Krogholm and colleagues (2010). Even though the uptake of quercetin in the diet is in the form of glucosides, when it reaches the enterocyte, the glucoside is hydrolysed by intracellular cytosolic *b*-glucosidase activity which leaves quercetin to act as a bioactive compound in the cellular system (Jan *et al.*, 2010).

1.5.3.4 Bioavailability of Phloridzin

Phloridzin, the glucoside conjugate of phloretin, is the major polyphenol found in apples. Similar to quercetin glucosides, phloridzin is thought to be hydrolyzed by lactase phloridzin hydrolase, and phloretin aglycone is taken up by the intestinal cells (Boyer & Liu, 2004). When rats were fed phloridzin and phloretin, their plasma contained glucuronidated and sulfated phloretin but no phloridzin (Crespy *et al.*, 2002). This supports the theory that phloridzin is hydrolyzed prior to uptake and further glucuronidation by intestinal epithelial cells.

1.5.3.5 Bioavailability of Resveratrol

Resveratrol is another dietary polyphenol, found in grapes, red wine and peanuts (Jang *et al.*, 1997). Meng and colleagues (2004) investigated the bioavailability of resveratrol in humans after oral ingestion of grape juice preparations and found that the plasma and urine of humans yielded detectable concentrations of resveratrol and their derivatives after 24hrs. They also found that at a low dose (0.03mg/kg), more than half of the ingested resveratrol was recovered in the urine in 24hrs, whereas at a higher dose (1mg/kg), only a quarter of the administered dose could be recovered during the same period. The presence of trace amounts of unchanged resveratrol in the human plasma and urine after dietary consumption of resveratrol was also confirmed by Walle and colleagues in another study (2004).

1.6 Hypotheses and Aims

The hypotheses to be tested in this project are as follows:

- *S. crispus* extract (SCE) will reduce abdominal adipose tissue weight and improve the lipid profile of treated mice similar to the results found in a previous experiment using Sprague-Dawley rats.

- Application of SCE will result in an enhancement of lipolysis in rat and human adipose tissue explants.

- The effects of individual polyphenols on rat adipose tissue explants are similar to that found in the pig adipose tissue explants and other studies using rat adipocytes or cell lines.

- The human adipose tissue explants will have similar responses towards individual polyphenols as found in the pig adipose tissue explants (Headland K., 2007).

- Basal and stimulated lipolysis in rat, human and pig adipose tissue explants are different to each other.

The aims of the project were to:

- Investigate the anti-obesity effects of *S. crispus* crude extract (SCE) in mice *in vivo*.
- Establish a rat adipose tissue explants system for *in vitro* measurement of lipolysis.
- Establish a human adipose tissue explants system for *in vitro* measurement of lipolysis.
- Investigate the effects of SCE and individual polyphenols on lipolysis in the rat adipose tissue explants.
- Investigate the effects of SCE and individual polyphenols on lipolysis in the human tissue explants.
- Compare the effects of individual polyphenols on lipolysis between the pig (Headland, 2007), rat and human adipose tissue explants.

Chapter 2 – Analysis of *S. crispus*

2.1 Introduction

Any living organism, like plants, may be considered to be a biosynthetic laboratory for chemical constituents such as biopolymers (proteins, nucleic acids, carbohydrates and lipids), primary or basic metabolites and secondary natural substances (Nyiredy, 2004). These chemical compounds give plants their therapeutic properties.

The determination of phenolic compounds in plants is influenced by many factors. The principal factors includes: heredity (genetic composition), ontogeny (stage of development) and environment (eg. climate, soil, method of cultivation)(Nyiredy, 2004). Variables in the extraction method (sample particle size, storage time and conditions, presence of interfering substances) and the chemical nature of plant phenolics which varies from simple to highly polymerized substances that include varying proportions of phenolic acids, phenylpropanoids, anthocyanins, tannins etc. also influence the type and amount of phenolic compounds derived from plants (Naczk & Shahidi, 2004). Furthermore, phenolic compounds may also exist as complexes with carbohydrates, protein and other plant components which are also sensitive to the method of extraction used.

The *Strobilanthes crispus* Bremek or *Saricocalyx crispus* Bremek (Acanthaceae) plant is native to countries from Madagascar to Indonesia, and is commonly known as 'daun picah beling' in Jakarta or 'enyoh kelo', 'kecibeling', or 'kejibeling' in Java (Sunarto, 1977). Description and health benefits of the plant can be found on section 1.4. Extraction using Supercritical Fluid Carbon Dioxide (SFE CO_2), Soxhlet and solvent methods have been done by Md Salleh and colleagues (2007) in which they identified catechin, epicatechin, rutin, quercetin, naringin and kaempferol to be present in the *S. crispus*.

2.2 Aim

In this study, the aim was to measure the total polyphenol content in *S. crispus* extract and determine the best chloroform-extraction procedure to be used later in the *in vivo* and *in vitro* studies.

2.3 Methodology

All of the chemicals used were of analytical grade and purchased from Sigma-Aldrich (Dorset, UK), Fisher Scientific (Leicestershire, UK) or BDH (Dorset, UK) unless stated otherwise

2.3.1 Polyphenol Extraction

Phenolics are not uniformly distributed in plants at the tissue, cellular and subcellular concentrations (Naczk & Shahidi, 2004). Insolube phenolics are the components of cell walls, while solube phenolics are compartmentalized within the plant cell vacuoles (Pridham, 1960). Solubility of phenolic compunds is determined by the type of solvent (polarity) used, degree of polymerization of phenolics, as well as interaction of phenolics with other constituents and formation of insoluble complexes.

Therefore, there is no uniform or completely satisfactory procedure that is suitable for the extraction of all phenolics or a specific class of phenolic substances from plant materials. Methanol, ethanol, chloroform, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethylformamide, and their combinations are frequently used for the extraction of phenolics (Antolovich *et al.*, 2000). Prior to extraction, *S. crispus* leaves purchased from Cedar Biotea Sdn. Bhd. (Malaysia) were oven dried (40ºC, 12hours) and ground into a powder using a coffee grinder (CG100, Kenwood, Hampshire, UK).

2.3.1.1 Diethyl Ether Extract

This method was developed by D. Cook (unpublished method) from School of Biosciences, University of Nottingham. 70% methanol was used as a solvent for extraction at 30ºC. Following this, free phenolics were extracted with diethyl ether from the acidified aqueous suspension of phenolic extract. The extract was treated with anhydrous $MgSO_4$ under nitrogen to liberate insoluble-bound phenolic acids (refer to Figure 2.1). This extract was used for analysis using HPLC.

Figure 2.1 Scheme for Diethyl Ether Extract of Polyphenol from *S. crispus* (Method by D. Cook)

15g of *S. crispus* powder was transferred into a round bottomed flask. 200ml of 70% methanol (aq) was added and extraction mixture was stirred with magnetic stirrer for 2 hrs.	Extraction mixture was filtered under vacuum through Whatman GF/A paper (No. 4)	Extraction mixture was transferred quantitatively to round bottomed flask and connected to a rotary evaporater to evaporate the methanol at 30°C until there were approximately 60ml of aqueous solution.

Supernatant was transferred to a separating funnel, and 80ml of diethyl ether was added and shaken vigorously. The aqueous extract (bottom layer) was removed. This was repeated 3 times.	Solution was transferred quantitatively into 3 centrifuge tubes (50ml). Tubes were spun at 3200rpm for 15mins to separate the supernatant from pellet. Pellet was discarded.	50ml of NaOH (2N) was added and solution was left to hydrolyse overnight at room temperature.

Aqueous extract was acidified to pH 1.5 with concentrated HCl by stirring with magnetic stirrer and measuring the pH at the same time. Extract was then filtered with Whatman No. 4 filter paper	Extract was transferred to a separating funnel and 80ml diethyl ether was added. Mixture was shaken vigorously. The ether extract (top layer) was removed. This step was repeated for 3 times.	The ether extract was then added with $MgSO_4$ (anhydrous) until $MgSO_4$ is not solid. Extract was then filtered with Whatman No. 4 filter paper.	Filtered extract was then transferred to another round bottom flask and connected again to a rotary evaporator to evaporate all but a few ml of ether. This took approximately 5 minutes. Extract was then dried under a stream of N_2 and stored at -40 ºC before use.

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2.3.1.2. Chloroform-Methanol Extract

This method was developed by Maznah I (unpublished method) from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. For extraction, 100g of powdered leaves were soaked and stirred continuously with chloroform-methanol (5:3, 500 ml of chloroform and 300 ml of methanol) for 12 hours. This mixture was then filtered with Whatman filter paper No.4 and solvent mixture containing the extract was collected into a round bottomed flask. The flask was then connected to a rotary evaporator (Rotavapor R-3000, Buchi, Switzerland) with water bath set at 37ºC to remove the majority of solvents, until the volume reached approximately 50ml. To evaporate the remaining solvents, crude extract was then divided into 2 pre-weighed conical tubes and dried under a continuous flow of nitrogen gas. Further drying of the extract was done in an air oven overnight at 37ºC to make sure that all solvents had evaporated. Green coloured extract in the form of a paste was obtained. To prepare the extract in water, distilled water was added to the extract paste.

2.3.1.3 Hot Water extract

0.2g of dried *S. crispus* leaves were weighed and added to 50ml of boiling milipore water, and left to boil for 5mins. Tea infusions were then left to cool to 25-30ºC. Hot water extracts were then filtered with Whatman fiter paper (No.4) into 50ml tubes and filtered again with a syringe-filter (0.4μm) into 15ml tubes. Samples were stored at -40ºC for further analysis. The same method was done for preparation of Green tea (*Camellia sinensis*) (Boh Sdn. Bhd, Malaysia).

2.3.2 Preparation of Sample for Total Soluble Phenolic Content

Chloroform-methanol extract of *S. crispus* were subjected to different preparation conditions before the analysis of Total Soluble Phenolic Content in order to identify the best condition that provided the highest phenolic content. All samples were initially extracted using the chloroform-methanol extraction method described in section 2.2.1.2 and shaken vigorously for 2 minutes after being diluted in distilled water. After that, samples were either filtered at 0.45μm with a filter unit (MILLEX®HA from MILLIPORE, Ireland), sonicated for 15mins or centrifuged at 3000rpm for 15mins. Measurement of Total Soluble Phenolic Contents (section 2.2.3) was done in triplicate.

2.3.3 Total Soluble Phenolic Content

The total soluble phenolic assay, more commonly known as the Folin-Ciocalteu assay, was used for determination of the total phenolic content of extracts. Folin-Ciocalteu reagents are not specific and detect all phenolic groups found in extracts, including those in the extractable proteins (Naczk & Shahid, 2004). In this assay, 5mg gallic acid was dissolved in 1ml ethanol and made up to 50ml with water to give a standard stock solution with a final concentration of 0.1mg/ml. A standard curve of gallic acid ranging from 20ug/ml to 100µg/ml was prepared from the stock solution. In a cuvette, 0.25ml of water (control) or each standards or sample (all in duplicates) was mixed with 0.25ml Folin reagent (diluted to 50% with water). Finally, 0.5ml of saturated sodium carbonate was added to the mixture and left to stand at room temperature for 30 minutes. Absorbance was read immediately at 750nm using an Ultrospec III UV/VISIBLE spectrophotometer (Pharmacia LKB, USA).

2.3.4 Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) with Diode Array Detector

The equipment used for RP-HPLC was a Perkin Elmer 200 series (Supelco, Bellefonte PA, USA) equipped with a pump, an autosampler, as well as a variable wavelength diode array detector. The HPLC-system was interfaced with a personal computer powered with Perkin Elmer Totalchrom software. RP-HPLC analyses were performed using a 5 µm C18 column, 15cm long, 4.6mm internal diameter (Discovery C18, Sigma, USA) at 25℃ column temperature and an injection volume of 10 µl. The solvent system were run under the following conditions: equilibration to starting conditions 80% 0.02M sodium phosphate buffer pH 2.4: 20% methanol for 15min at 1ml/min, then changing to 80% methanol: 20% buffer on a linear gradient over 25min at 1ml/minute. Ultraviolet (UV) detection was recorded at 280 nm wavelength. For compound identification, retention time and peaks of standard individual polyphenols were compared with those found in the extract.

2.3.5 Statistical Analysis

Values for gallic acid equivalent (µg/ml) were expressed as means ± SEM. The data were analyzed using the statistical software Genstat 10.0. General Analysis of Variance with No Blocking was used. Significance was taken as $p < 0.05$.

2.4 Results

2.4.1 Total Polyphenol Content in Chloroform-Methanol Extract

Figure 2.2 shows the Total Soluble Phenolic Content (section 2.2.3) of *S. crispus* extract after the various preparations (section 2.2.2). Samples prepared with only the sonication method were found to have the highest phenolic content ($P < 0.001$) compared with other preparation methods with and without sonication. Filtering reduced the phenolic content detected ($P < 0.001$) while centrifugation did not affect the presence of phenolic content in the extract ($P = 0.557$).

Figure 2.2 Polyphenol Content of Chloroform-Methanol Extracts After Treatment with Different Conditions
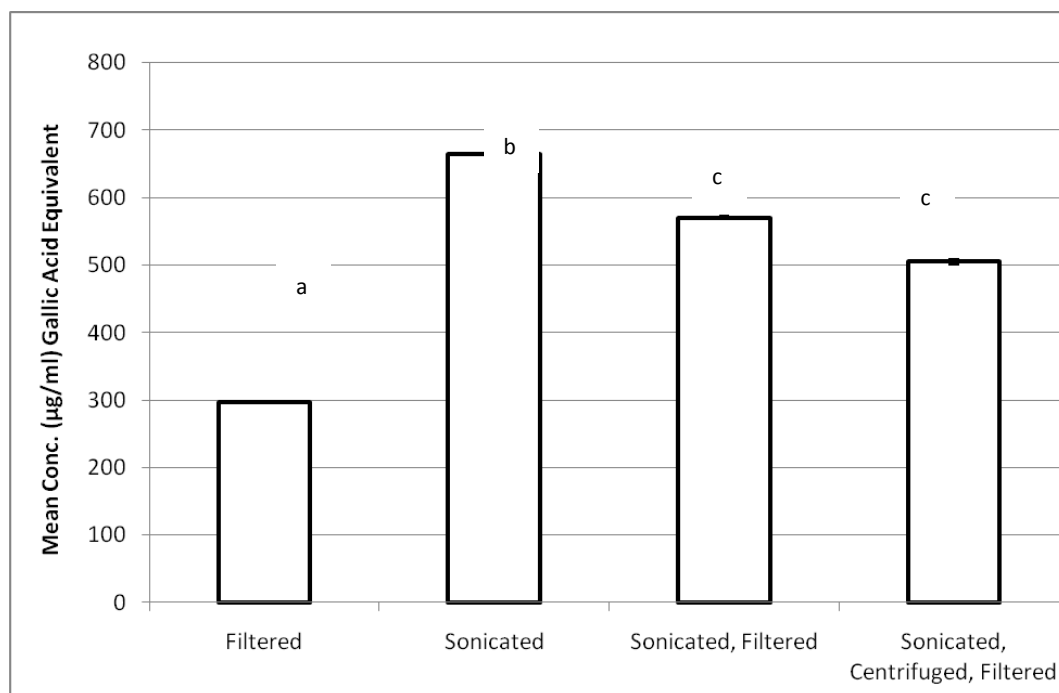
Sonication increased total phenolic content ($P < 0.001$) while filtration decreased total phenolic content ($P < 0.001$). Centrifugation did not affect total phenolic content ($P = 0.557$).

2.4.2 Total Polyphenol Content in Hot water extract

Figure 2.3 shows the total polyphenol content of *S. crispus* and Green hot water extracted using the hot water extraction method described in section 2.2.1.3. Phenolic contents were adjusted to phenolic concentration in a gallic acid standard. *S. crispus* tea was found to have a significantly lower total phenolic content than green tea ($P<0.001$).

Figure 2.3 Polyphenol Content of Hot water extracts
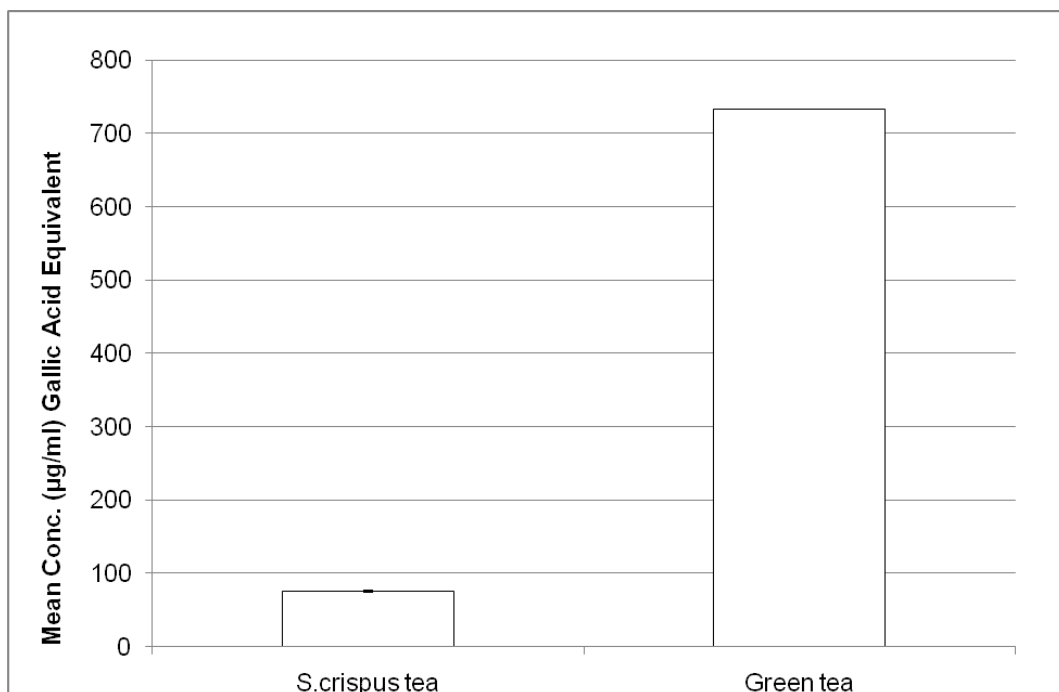
S. crispus tea has significantly lower total phenolic content compared with green tea ($P<0.001$).

2.4.3 HPLC Analysis of Various *S. crispus* Extract

S. crispus hot water extract (section 2.2.1.3), diethyl ether extract (2.2.1.1) and chloroform-methanol extract (section 2.2.1.2) were analyzed by HPLC (section 2.2.4) at 280nm and results (Figure 2.4, 2.5 and 2.6) were compared.

Figure 2.4 HPLC Analysis of *S. crispus* Hot water extract
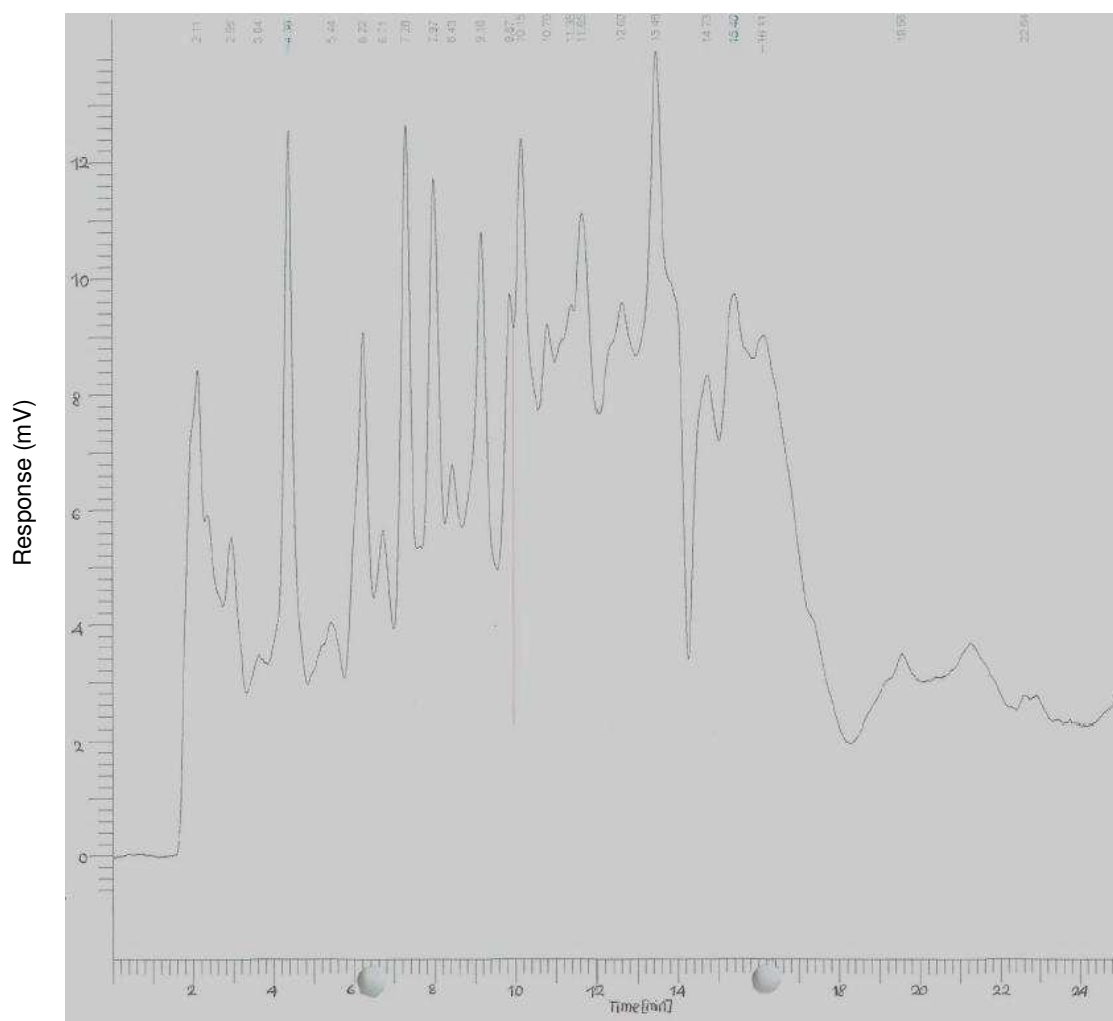
Figure 2.5 HPLC Analysis of Chloroform-methanol Extract
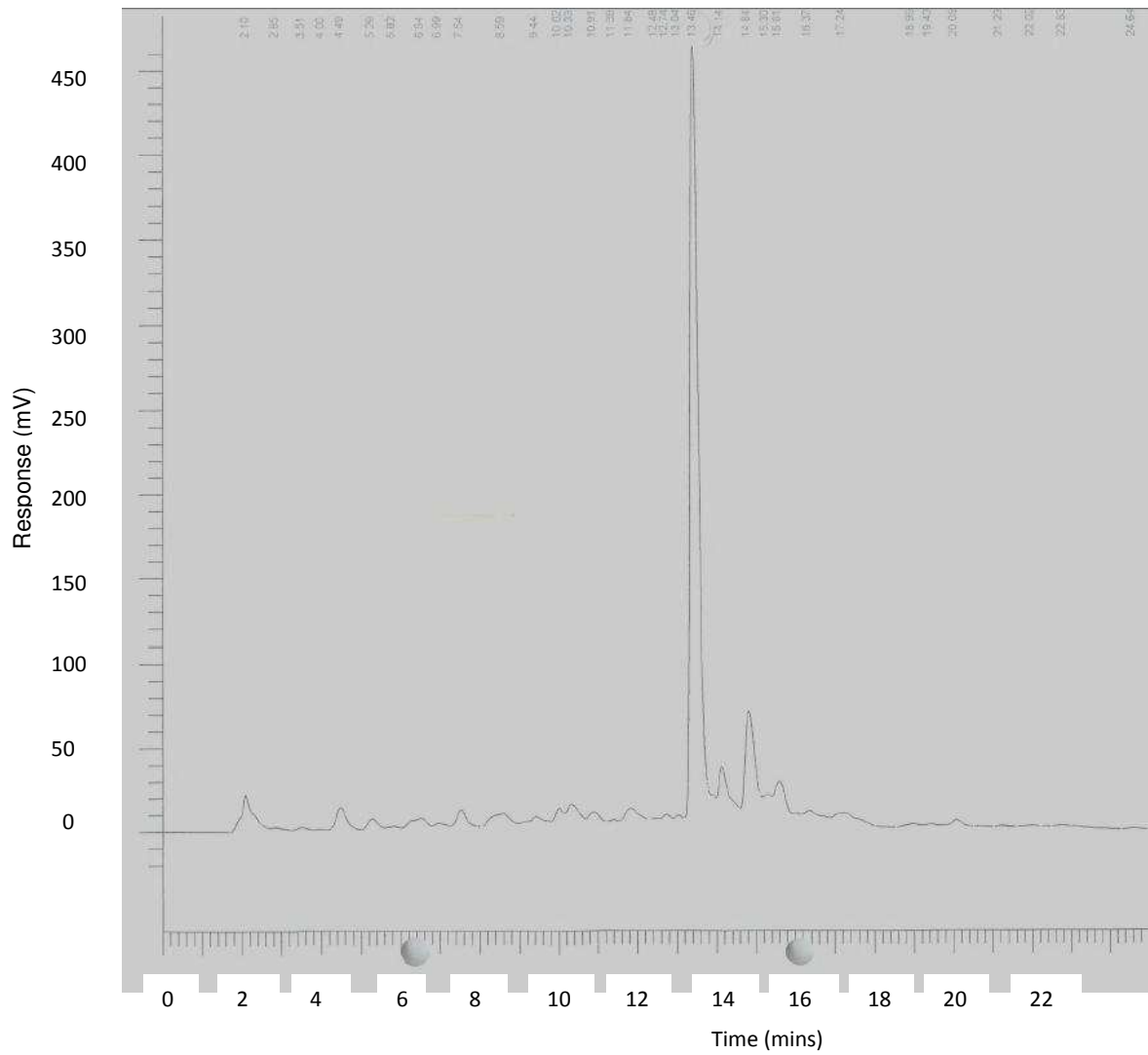
Figure 2.6 HPLC Analysis of Diethyl-ether Extract
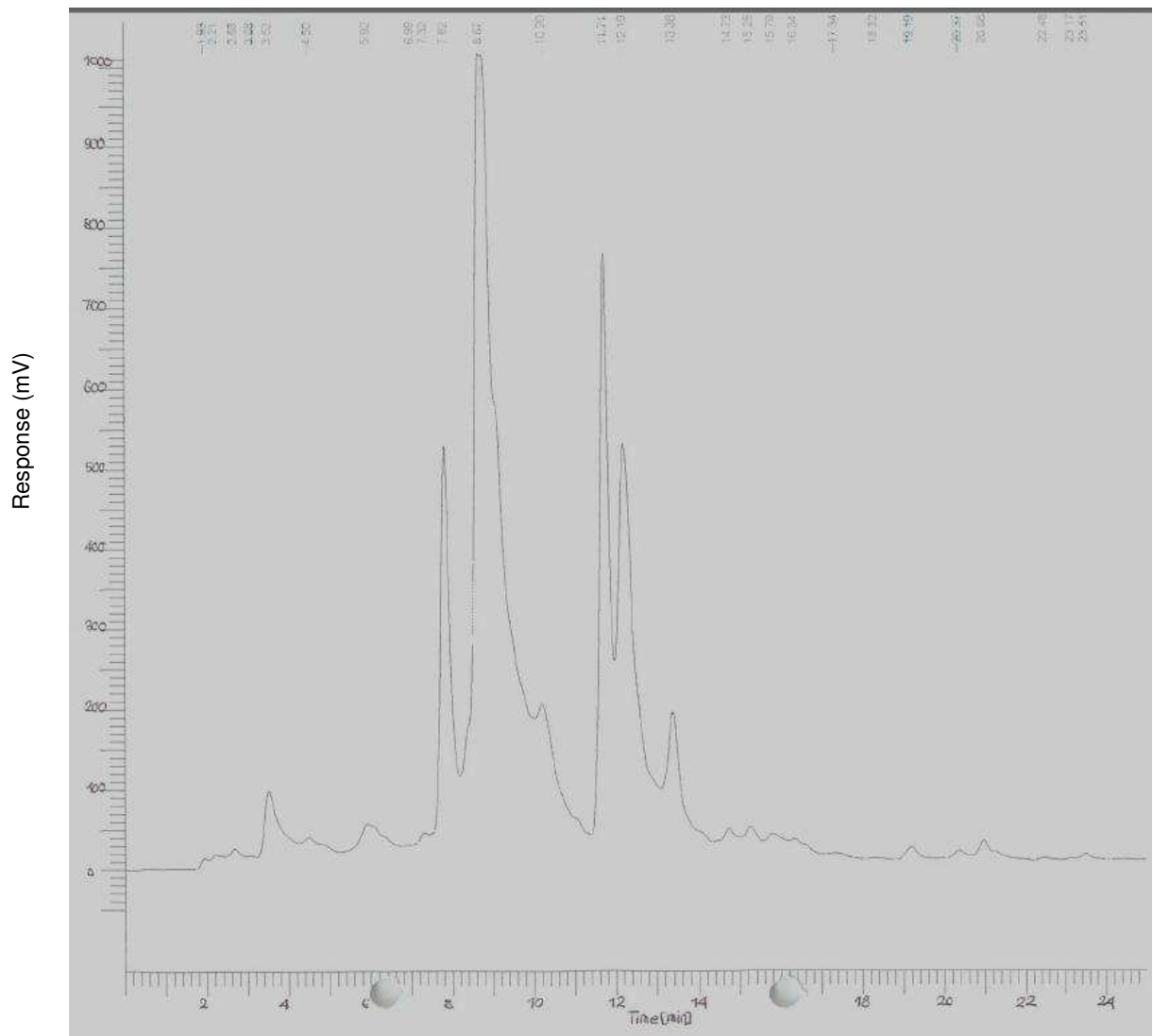
2.5 Discussion

2.5.1 Total Polyphenol Content in Chloroform-Methanol Extract

Results suggest that a chloroform-methanol extract prepared by sonication with distilled water alone had the highest total phenolic content and filtration reduced this content significantly. This indicates that most phenolic substances in the extract existed as substrates bigger than 0.45µm. Thus, this preparation method was used subsequently to prepare the extract for the *in vivo* study (Chapter 3).

2.5.2 Total Polyphenol Content in Hot water extract

S. crispus tea and green tea were prepared under the same conditions and yet the total polyphenol content in green hot water extract was 7 times higher. However, we have to note that harvest season, age of the plant, climate, environmental conditions and processing conditions are some variables that could affect the values found in our study, as mentioned by Lin and colleagues (2003). Furthermore, the most common polyphenols in the human diet are not necessarily the most active within the body, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated (Holst & Williamson, 2008).

2.5.3 HPLC Analysis of Various *S. crispus* Extract
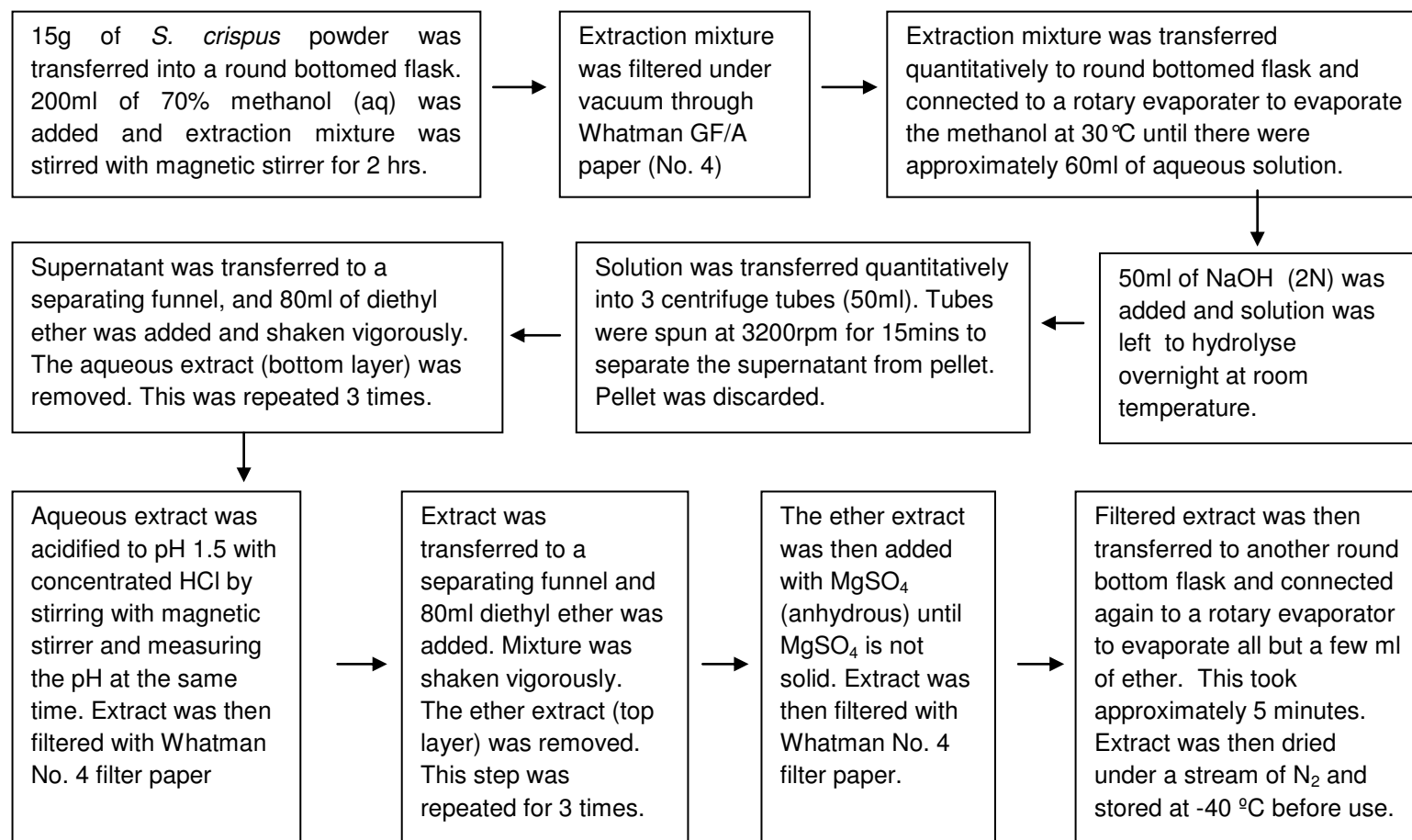
Phenolics are not uniformly distributed in plants at the tissue, cellular and subcellular concentrations. Insoluble phenolics are the components of cell walls, while soluble phenolics are compartmentalized within the plant cell vacuoles (Pridham, 1960; Beckman, 2000). Extraction of phenolic compounds from plant materials is influenced by their chemical nature, the extraction method employed, sample particle size, storage time and conditions, as well as presence of interfering substances (Naczk & Shahidi, 2004). From the results obtained in this study, we can verify that the phenolic compounds of *S. crispus* extract may vary depending on the method of extraction and sovents used. It was also noted that the separation of chemical compounds in the chloroform-methanol extract was much better compared with other extraction methods. This is because solubility of phenolic compounds is governed by the type of solvent (polarity) used, degree of

polymerization of phenolics, as well as interaction of phenolics with other constituents and formation of insoluble complexes (Naczk & Shahidi, 2004). However, the number of chemical compounds detected by chloroform-methanol extract was very low compared with other extraction methods. Therefore, there is no uniform or completely satisfactory procedure suitable for extraction of all phenolics or a specific phenolic substance in plant materials.

2.6 Conclusion

S. crispus hot water extract had a very low content of total polyphenols compared to the chloroform-methanol extract. Detection of chemical compounds using HPLC confirmed that the chloroform-methanol extract of *S. crispus* was found to clearly separate some compounds compared with other extraction methods. Furthermore, a high total phenolic content was measured particularly when the extract was sonicated in distilled water. For this reason, the chloroform-methanol extract preparation method was chosen for the subsequent study to investigate the effect of the the extract *in vivo*.

Chapter 3 – *In vivo* effects of *S. crispus* Extract (SCE)

3.1 Introduction

Previous research done during my MSc studies in Malaysia (Zawawi, 2007) showed that obese Sprague-Dawley rats fed a normal chow diet and treated with 1% *S. crispus* extract (in drinking water – approx. 1g/kg BW/day) for 14 weeks, reduced body weight, adipose tissue and liver weights and tended to lower body weight gain, plasma leptin and fasting glucose concentrations compared with control obese rats fed the same normal chow diet. They also had improved liver colour and reduced hepatic steatosis. There was no difference in food intake between the groups, suggesting that the *S. crispus* extract was not inducing a general malaise, but might be increasing energy expenditure. The lipolysis rate (plasma glycerol per gram abdominal fat) in the treated group was also found to be higher. This suggested that the extract was inducing lipolysis in the treated rats.

The first low-density lipoprotein receptor (LDLr) knockout (KO) mouse model was developed by Ishibashi and colleagues (1993). The mice were found to have moderate hypercholesterolemia (total cholesterol ~250 mg/dl) on a chow diet with lipoprotein profiles similar to humans (i.e. elevated LDL cholesterol). On a high-fat / high cholesterol 'Western-type' diet containing 21% fat and 0.15% added cholesterol, LDLr KO mice developed severe hypercholesterolemia and extensive atherosclerosis (Ishibashi *et al.*, 1993). Furthermore, when LDLr KO mice were placed on a diet with greater than 20% fat content they also became obese and insulin resistant (Wu *et al.*, 2006). This is similar to one of the most severe forms of human hyperlipidemia, familial hypercholesterolemia, which occurs due to mutations in the LDLr gene, resulting in elevated concentrations of the atherogenic LDL cholesterol (Kennedy *et al.*, 2010). Thus, the LDLr KO mouse model is useful when studying diet-induced obesity and associated diseases.

3.2 Hypothesis and Aim

The hyphothesis of this study was that the SCE will reduce abdominal adipose tissue weight and improve the lipid profile of the treated group similar to the results found in a previous experiment using Sprague-Dawley rats. Therefore, the aim was to investigate the effect of SCE on LDLr KO mice that had been made obese by feeding a high fat (60%kcal) diet and then either maintained on the high fat diet or switched to a low fat diet. The changes in feeding behaviour, physical activity and energy expenditure were measured throughout 48hr periods at Week 4 and 9 of treatment with SCE using an Oxymax Comprehensive Lab Animal Monitoring System (CLAMS).

3.3 Methodology

3.3.1 Preparation of *S. crispus* Extract in Water

S. crispus leaves (oven dried at 40ºC overnight) were bought from Cedar Biotea Sdn. Bhd. (Penang, Malaysia). Leaves were ground to powder using a coffee grinder (CG100, KENWOOD, UK). Powdered leaves were stored in glass bottles and kept at -20˚C before use. For extraction, 100g of powdered leaves was soaked and stirred continuously with chloroform-methanol (5:3, 500 ml of chloroform and 300 ml of methanol) for 12 hours. This mixture was then filtered with Whatman filter paper No.4 and solvent mixture containing the extract was collected into a round bottomed flask. The flask was then connected to a rotary evaporator (Rotavapor R-3000, Buchi, Switzerland) with water bath set at 37ºC to remove the majority of solvents, until the volume reached approximately 50ml. To evaporate the remaining solvents, crude extract were then divided into 2 pre-weighed conical tubes and dried under a continuous flow of nitrogen gas. Further drying of the extract was done by drying the extract in an air oven overnight at 37ºC to make sure that all solvents had evaporated. Green coloured extract in the form of a paste was obtained. The conical tubes containing the extract were kept at -40ºC until further use.

To prepare the extract in water, distilled water was added to the extract paste and the mixture was then sonicated to increase polyphenol content (see chapter 2). Further dilution was done with more distilled water to give the concentration

needed (0.1% or 1%), before being given as drinking water to the mice (approximate dose was 1g/kg BW/day, assuming a mouse drinks 10ml/100g/day).

3.3.2 Experimental Design

Obesity was induced in 39 LDLr KO mice by giving high-fat (HF) diet (60% kcal fat, 5.24kcal/g, Research Diets D12492) for 24wks (Week -26 to Week -2). In addition 10 LDLr KO mice were given low-fat (LF) diet (normal chow, 17% kcal fat, 3.3kcal/g Harlan Teklad 2018) as control. These were provided by RenaSci (Biocity, Nottingham, UK). At Week -2 (Baseline), 7 mice given the HF diet and 10 mice given the LF diet were culled to obtain basal organ weights, plasma lipid concentrations and liver lipid concentrations. The remaining mice were individually housed and acclimatized for one week (Refer to Figure 3.1).

At Week 0, animals were randomly allocated into one of four treatment groups: the H_2O HFD group (n=8) were maintained on the HF diet and given normal drinking water; the SCE HFD group (n=8) were maintained on the HF diet and given 0.1% *S. crispus* extract (SCE) in their drinking water for Weeks 0-5 followed by 1% SCE for Weeks 5-10; the H_2O LFD group were switched to the LF diet and given normal drinking water; and the SCE LFD group (n=8) were switched to the LF diet and given 0.1% SCE in their drinking water for Weeks 0-5, followed by 1% SCE for Weeks 5-10 (Figure 3.1).

An Oxymax Comprehensive Lab Animal Monitoring System (CLAMS) was used on Weeks -1, 4 and 9 of the study to measure 24hr profiles of oxygen consumption (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER), food intake and physical activity. Food intake, bodyweight and water intake were also measured weekly in the home cages.

All animal procedures were approved by the University of Nottingham Local Ethical Review Committee and were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

3.3.3 Comprehensive Lab Animal Monitoring System (CLAMS)

The CLAMS (Columbus Instruments, Ohio, USA) is a system for measuring oxygen consumption, carbon dioxide release, heat produced, physical activity and feeding behaviour (see Figure 3.2)

Figure 3.1 Experimental Design for *In vivo* Study
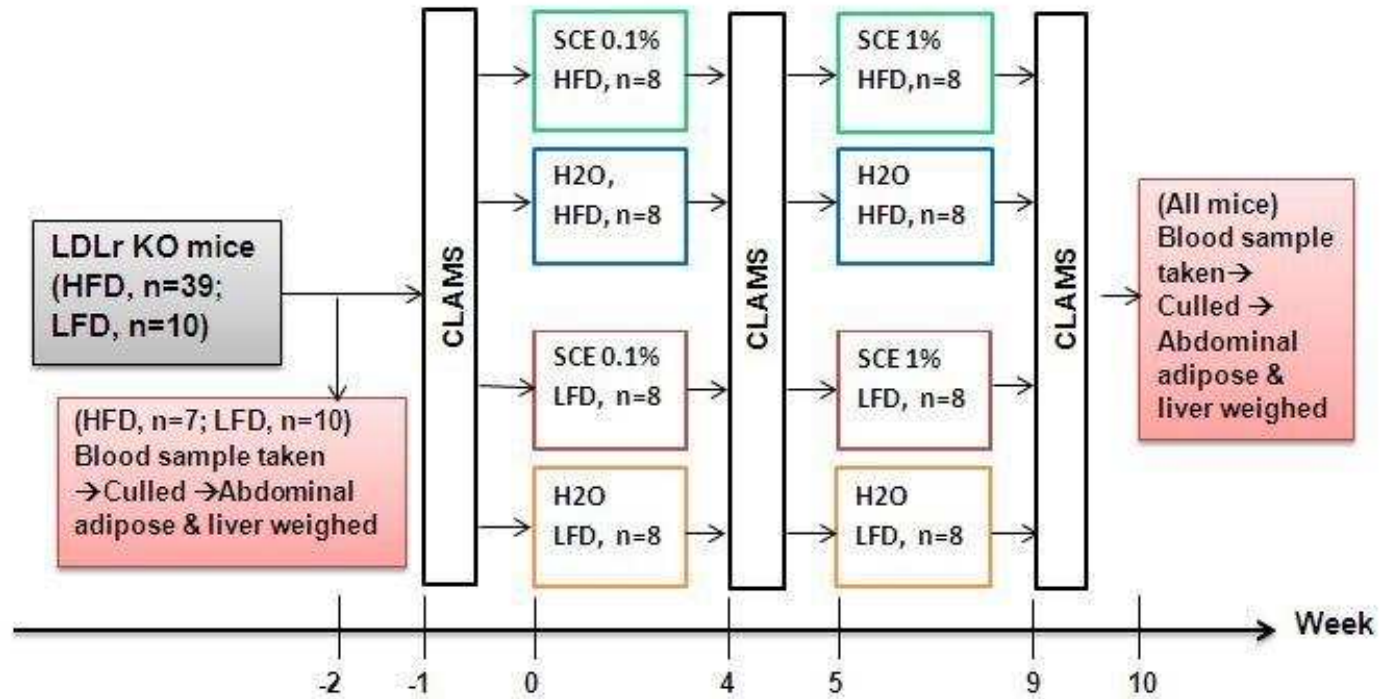
Figure 3.2 Comprehensive Lab Animal Monitoring System (CLAMS)

3.3.3.1 Oxygen Consumption and Carbon Dioxide Production

The following equations were used to calculate oxygen consumption (VO_2) and carbon dioxide production (VCO_2). The oxygen consumption was calculated by taking the difference between the input oxygen flow and the output oxygen flow. Similarly, the carbon dioxide production was calculated by taking the difference between the output and input carbon dioxide flows. The consumption and production values have the units of milliliters per hour (ml/hr).

$VO_2 = V_i O_{2i} - V_o O_{2o}$

V_i and V_o are the input and output ventilation rates (litres per minute), O_{2i} and O_{2o} are oxygen fractions at the input and output

$VCO_2 = V_o CO_{2o} - V_i CO_{2i}$

V_i and V_o are the input and output ventilation rates (litres per minute), CO_{2i} and CO_{2o} are carbon dioxide fractions at the input and output

3.3.3.2 Respiratory Exchange Ratio (RER)

The respiratory exchange ratio (RER) is simply the ratio between the carbon dioxide production and the oxygen consumption. This value is a ratio and thus does not have any units. The RER ratio was calculated before any units conversion, weight normalization, or effective mass correction.

$$RER = \frac{VCO_2}{VO_2}$$

3.3.3.3 Heat Production

Oxymax will calculate heat with one of two methods. Both methods calculate the heat before the application of any normalization or correction and, thus, reflect the exact heat of the subject. The method used here is termed Internal and will derive a calorific value (CV) based on the observed respiratory exchange ratio. This calorific value is then used with the observed oxygen consumption (VO_2) to calculate heat. The equations are:

$$Heat = CV \times VO2$$

$$CV = 3.815 + 1.232 \times RER$$

More detail explanation of the equations are described in Appendix A.

3.3.3.4 Locomotor Activity

CLAMS is configured with double axis detection of animal motion using IR photocell technology (refer to Figure 3.3). Interruption of an IR beam accrued one "count". Coverage in a single plane was implemented with IR photocells located in the X direction. The height of these beams is such that they intersect the animal midway vertically. Placement of IR photocells at a height above the animal detected rearing or jumping (Z-axis).

Figure 3.3 IR Photocells for Detecting Locomotor Activity
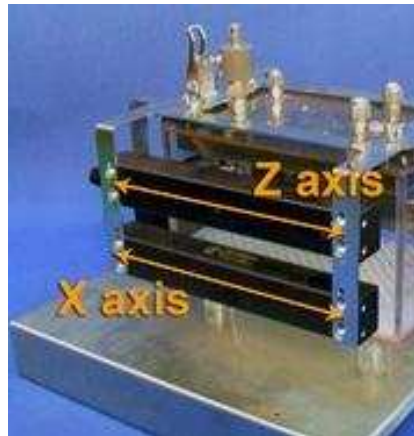
3.3.4 Animal Monitoring in the Cage

The 49 male LDL-receptor knockout (LDLr KO) mice aged 35 weeks were kindly donated by RenaSci (Biocity, Nottingham, UK) and housed individually on a 12 hr day-night cycle (lights off 19:00, on 07:00). The temperature was set to a constant 27ºC. Mice had been fed either a HF diet (n=39, 60%kcal from fat, Research Diets D12492) or a LF diet (n=10, 17%kcal from fat, normal chow, Harlan Teklad 2018) for approximately 5 months. Individual mouse food (g/day) and water/ extract (ml/day) intakes were measured once a week. The body weight of each mouse was measured once a week.

3.3.5 Sample Collections and Preparations

After 10 weeks, all mice were killed by the administration of pentobarbitone. Abdominal fat pads, heart, kidney and liver were removed and weighed, then immediately snap frozen in liquid nitrogen before being stored at -80ºC. The rest of the carcass was also kept at -80 ºC for later determination of carcass lipid content. Blood samples were collected into heparin following cardiac puncture, centrifuged at 12500rpm for 1 hour at 4 ºC and the plasma stored frozen at -80 ºC prior to determination of total cholesterol, triglyceride and glucose concentrations.

3.3.6 Liver Lipid Extraction

This method was adapted from Hara and Radin (1978). Frozen livers were crushed using a biopulverisor while maintaining freezing temperature by adding liquid nitrogen to the sample in the biopulverisor.

Crushed liver samples (300-350mg) were weighed into 15ml tubes containing 5.4ml of hexane:isopropanol (3:2, v/v) and homogenized using the Polytron Homogenizer (PT2100, Kinematica, Switzerland). Homogenized sample was then poured into another 15ml tube containing 1.6ml sodium sulphate,vortexed for 30 seconds and then centrifuged at 3000rpm for 5 minutes at room temperature. The supernatant was then transferred to another 15ml tube and dried under a stream of nitrogen on a heating block until all the solvent had evaporated. 3ml of hexane was added around the sides of the tubes to wash them and stored at -20ºC until needed. After taking the sample from storage, 150ul of each sample was removed and dried under nitrogen gas, before adding 50ul of isopropanol.

3.3.7 Triglyceride and Glycerol Quantification

Plasma and liver triglyceride and plasma glycerol were measured using a Serum Triglyceride Determination Kit (TR0100, Sigma, Missouri USA). The procedure involves enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. The glycerol produced was then measured by coupled enzyme reactions.

Triglycerides are first hydrolysed by lipoprotein lipase to glycerol and free fatty acids. Glycerol was then phosphorylated by adenosine-5'-triphosphate (ATP) to form glycerol-1-phosphate (G-1-P) and adenosine-5'-diphosphate (ADP), catalyzed by glycerol kinase (GK). G-1-P was then oxidized by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Peroxidase (POD) catalysed the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-3(3-sulfopropyl) m anisidine (ESPA) to produce a quinoneimine dye with an absorbance maximum at 540nm. A standard curve was present on each 96 well plate to enable calculation of the amount of glycerol present. 200µl of Triglyceride Reagent Blank (prepared according to manufacturers' instructions) was added to 50 µl of sample or

standard solution in each well. After incubation of the 96 well plate at 37°C for 10mins, absorbance was measured at 550nm using a microplate reader (680XR, BIO-RAD, USA).The increase in absorbance is directly proportional to the free glycerol concentration of the sample.

Figure 3.4 Triglycerides and Glycerol Assay Enzymatic Reactions

Lipoprotein Lipase

Triglycerides $\xrightarrow{\hspace{3cm}}$ Glycerol + Fatty acids

GK

Glycerol + ATP $\xrightarrow{\hspace{3cm}}$ G-1-P + ADP

GPO

G-1-P + O_2 $\xrightarrow{\hspace{3cm}}$ DAP + H_2O_2

POD

H_2O_2 + 4-AAP + ESPA $\xrightarrow{\hspace{3cm}}$ Quinoneimine dye + H_2O
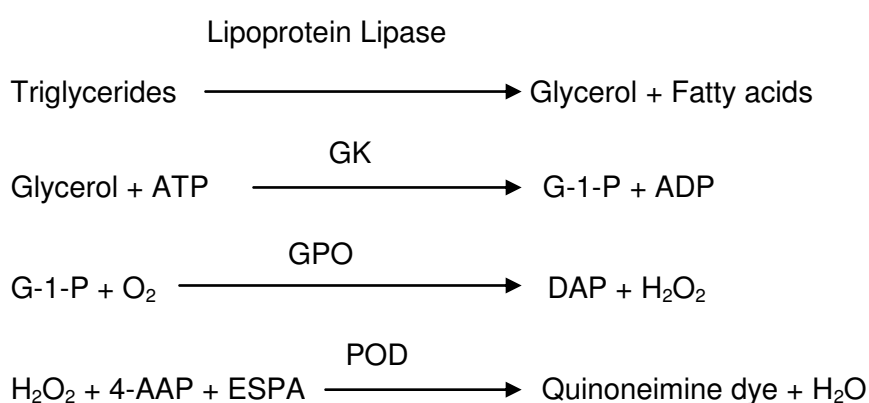
3.3.8 Cholesterol Quantification

Plasma and liver cholesterol was measured using Infinity Total Cholesterol reagent (TR13421, Thermo Scientific, UK). Cholesterol esters were enzymatically hydrolysed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol, including that originally present, was then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide combines with hydroxybenzoic acid (HBA) and 4AAP to form a chromophore (quinoneimine dye), which absorbs at 550nm. A standard curve using Cholesterol Standard (TR13923, Thermo Scientific, UK) was present on each 96 well plate to enable calculation of the amount of cholesterol present. 190µl of reagent was added to 10 µl of sample or standard solution in each well. After incubation of the 96 well plate at 37°C for 10mins, absorbance was measured at 550nm using a microplate reader (680XR, BIO-RAD, USA).

Figure 3.5 Cholesterol Assay Enzymatic Reactions

$$\text{Cholesterol Esters} \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + HBA + 4AAP \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O$$
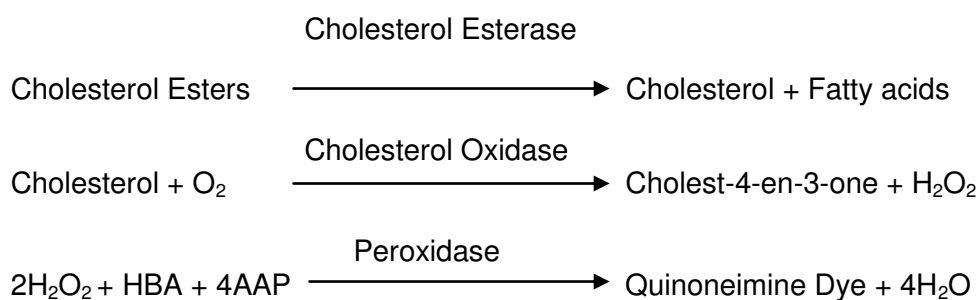
3.3.9 Glucose Quantification

Plasma glucose was measured using Infinity Glucose Oxidase reagent (TR15221, Thermo Scientific, UK). Glucose was oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. The hydrogen peroxide reacts in the presence of peroxidase with HBA and 4-AAP forming a red quinoneimine dye. The intensity of the colour formed is proportional to the glucose concentration and can be measured photometrically at 550nm. A standard curve using Glucose Oxidase Standard (TR15103, Thermo Scientific, UK) was present on each 96 well plate to enable calculation of the amount of glucose present. 250µl of reagent was added to 10 µl of sample or standard solution in each well. After incubation of the 96 well plate at 37°C for 10mins absorbance was measured at 550nm using a microplate reader (680XR, BIO-RAD, USA).

Figure 3.6 Glucose Assay Enzymatic Reactions

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$$

$$H_2O_2 + HBA + 4\text{-}AAP \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + H_2O$$
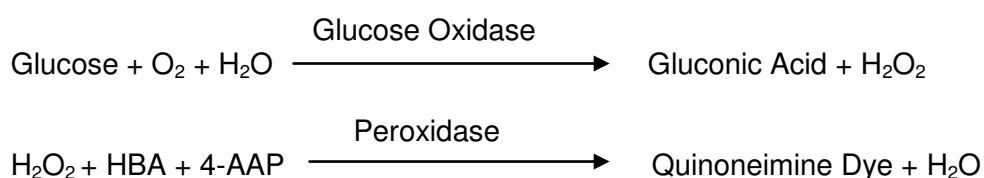
3.3.10 Total Carcass Lipid Extraction

To measure total lipid contents, individual mice carcasses were weighed in aluminium containers (with lid) and freeze dried, before frozen carcasses were put into individual clear plastic bags and crushed repeatedly with a hammer until only small pieces remained. Samples were then thoroughly mixed with a metal spoon and then transferred to smaller plastic bags before being stored at -40ºC until further extraction.

For lipid extraction, 2g of each sample was weighed onto 11cm qualitative grade filter paper, then loosely folded to fit down to the bottom of a thimble. Samples were placed into a thimble and then covered with a plug of cotton wool. A flask containing 3 anti-bump stones was weighed and 155ml of petroleum ether added. The thimble was then placed into the flask which was then fastened to a Gerhadt Soxtherm (Gerhardt, UK) with an automated Rapid Soxhlet Extraction system run according to the official method of AOAC for determination of crude fat (991.36) with some minor modifications described below.

Hot extraction process followed an AOAC approved method and consisted of 3 steps: extraction, rinsing and drying. During extraction the petroleum ether, sample and flask was heated up, then, the flask was washed with petroleum ether before being evaporated, condensed, collected beneath the condenser and transferred to the solvent tank for reuse in each cycle. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound will be concentrated in the flask. After extraction the solvent was removed by drying the flask in an oven at 103ºC for 1 hour and then the flask was cooled down in a dessicator before being weighed.

$$\text{Calculation:} \qquad \text{Total lipid (\%)} = \frac{\text{Weight of extract (g) X 100}}{\text{Sample weight (g)}}$$

3.4 Results

3.4.1 Bodyweight Changes

Figure 3.7 shows the changes in bodyweight from the start of treatment with HF diet and after treatment with SCE (0.1 and 1%) with or without the LF diet. The bodyweight of the HF diet groups significantly increased with time (P<0.001) compared with the LF diet group, such that at Week -1 the HF diet fed groups were 1.5 fold heavier than the LFD fed group. There were no significant differences (P=0.327) in bodyweights at week -1 between the groups given HF diet and then divided into H_2O, HFD, SCE, HFD, H_2O LFD and SCE, LFD groups.

At Week 0 half the mice were switched to the LF diet and this significantly decreased bodyweight, whereas the mice maintained on the HF diet continued to increase in bodyweight (P<0.001for time x diet interaction). Treatment with SCE tended to increase bodyweight, but this was not statistically significant (P=0.072). Data for Weeks 5 and 10 were not included in the statistical analysis, as bodyweights of all animals were found to be reduced after the 48hrs in the CLAMS.

Figure 3.7 Bodyweight Increase Before and After Treatment with SCE
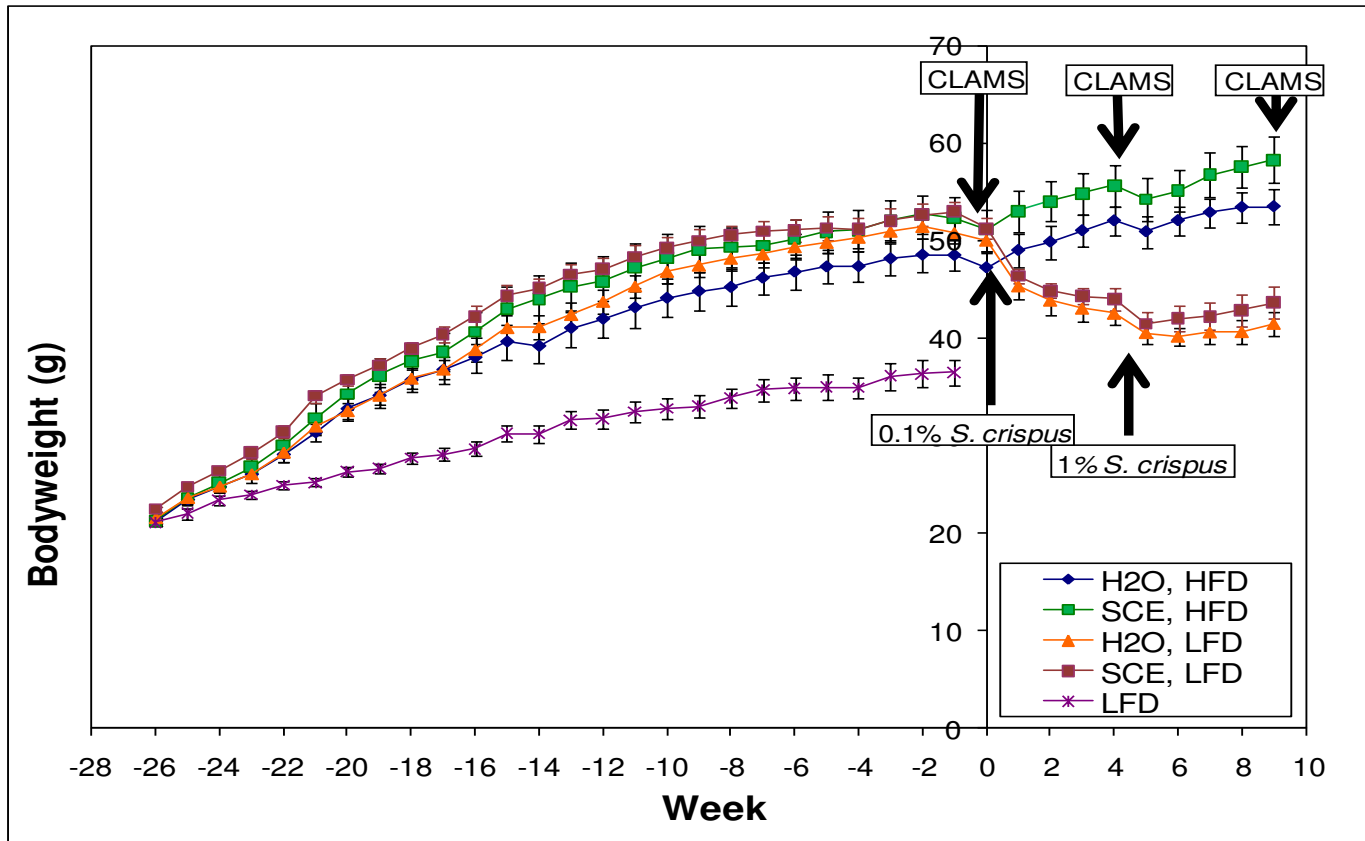
Changes in bodyweight of LDLr KO mice fed either a high (HF) or low (LF) fat diet, before and after treatment with SCE. There were no significant differences in bodyweight at week -1 between all HF diet fed groups (P=0.327). After week 0, the mice switched to the LF diet decreased in bodyweight, whereas the mice maintained on the HF diet continued to increase bodyweight (P<0.001 for diet x week interaction). SCE treatment tended to increase bodyweight (P=0.077).

3.4.2 Food Intake

Figure 3.8 shows the mean food intake (g/week) consumed in the home cages. Data at Weeks 5 and 10 was not included because after animals were housed in the CLAMS at Weeks 4 and 9, their food intake was reduced the following week. There were significant effects of Week (P<0.001) and Diet (P<0.001), but no interaction (P=0.118). Intakes of LF diet were higher than the intakes of HF diet, with mean food intake in the HFD group across all weeks being 23.28g, whereas the mean intake in the LFD group across all weeks was 27.8g. SCE treatment had no effect on food intake (P=0.13).

Figure 3.8 Mean Food Intake (g/week)
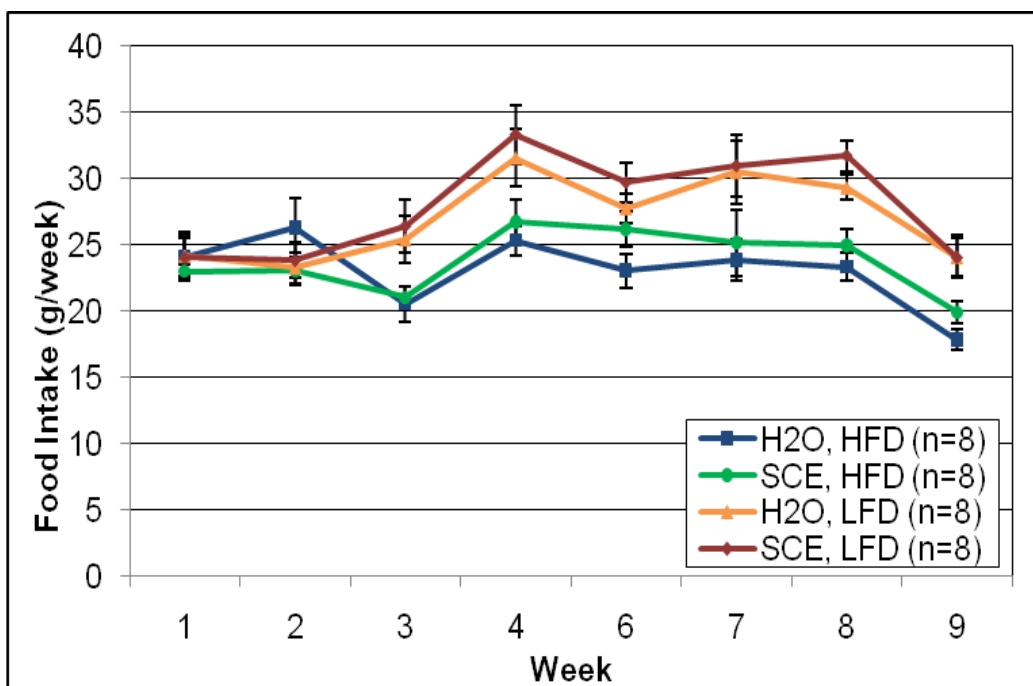
Significant effects of Diet and Week were found (P<0.001 for both), but there was no interaction (P=0.118) and no effect of SCE treatment (P=0.13).

3.4.3 Energy Intake

Figure 3.9 shows the mean energy intake (kcal/week) from diet consumed in the home cages. Data at Weeks 5 and 10 were not included because after animals were housed in the CLAMS at Weeks 4 and 9, their food intake was reduce the following week. There was a significant interaction between Week and Diet (P=0.012) where energy intake was found to be gradually increased from Week 4 to Week 8, particularly in the HFD groups, before coming down at Week 9. LF diet clearly reduces the energy intake (P<0.001). Mean energy intake in the HFD group was 17.19kcal/week, whereas the mean energy intake in the LFD group across all Weeks was 12.88kcal/week. SCE treatment tended to increase energy intake slightly (P=0.082).

Figure 3.9 Mean Energy Intake (kcal/week)
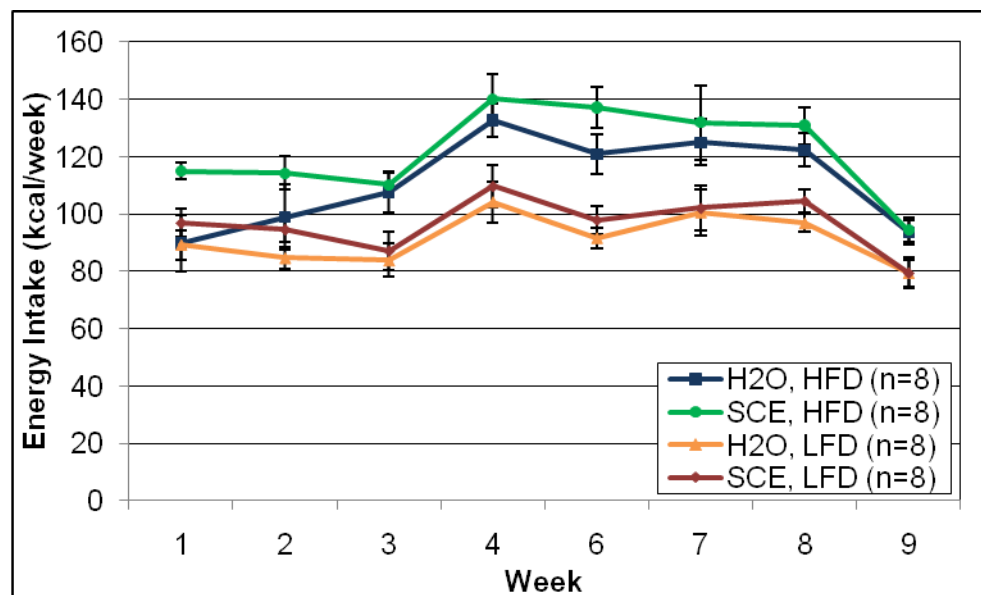
Significant interaction between Week and Diet was found (P=0.012) and SCE tended to increase energy intake (P=0.082).

3.4.4 Feed Efficiency

Figure 3.10 shows the mean feed efficiency (bodyweight gain (g)/food intake (g)) in all groups. Data at Weeks 5 and 10 was not included because after animals were housed in the CLAMS at Weeks 4 and 9, their body weight and food intake was reduced the following week. There was a significant interaction between Week and Diet (P<0.001). LF diet clearly reducedthe feed efficiency (P<0.001) particularly between Weeks 1 and 6. Mean feed efficiency across all weeks in the HFD group was 0.04g/g, whereas the average of LFD group across all weeks was -0.03g/g. SCE treatment did not have any effect on feed efficiency (P=0.701).

Figure 3.10 Mean Feed Efficiency (g/g)
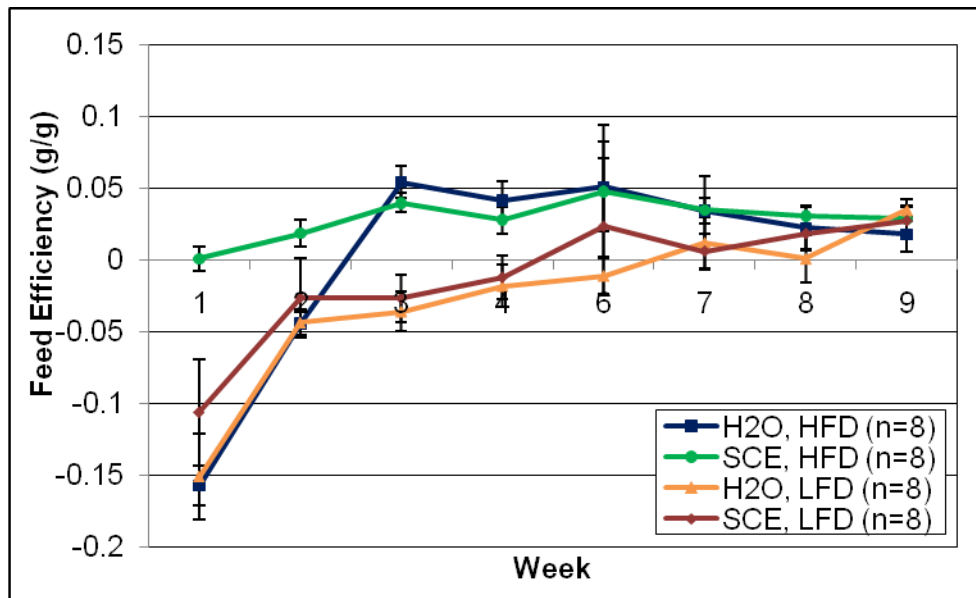
LF diet reduced feed efficiency, particularly between Weeks 1 and 6 (P<0.001 for interaction), but there was no effect of SCE treatment (P=0.701)

3.4.5 Water Intake

Figure 3.11 shows the mean SCE or water intake (ml/week) consumed in the home cages of each group. Data at Weeks 5 and Week 10 were not included because after animals were housed in the CLAMS at Week 4 and 9, their water intake was inaccurate the following week. No significant effect of Diet (P=0.495) was found on water intake. However, there was a significant Week x Extract interaction (P=0.025) on water intake, indicating that mean water intake was higher in the SCE groups at the start of the study, but lower at the end. This may cause the treated animals to receive less of the n 1%(w/w) SCE after the dose was increased at Week 5.

Figure 3.11 Mean SCE or Water Intake (ml/week)
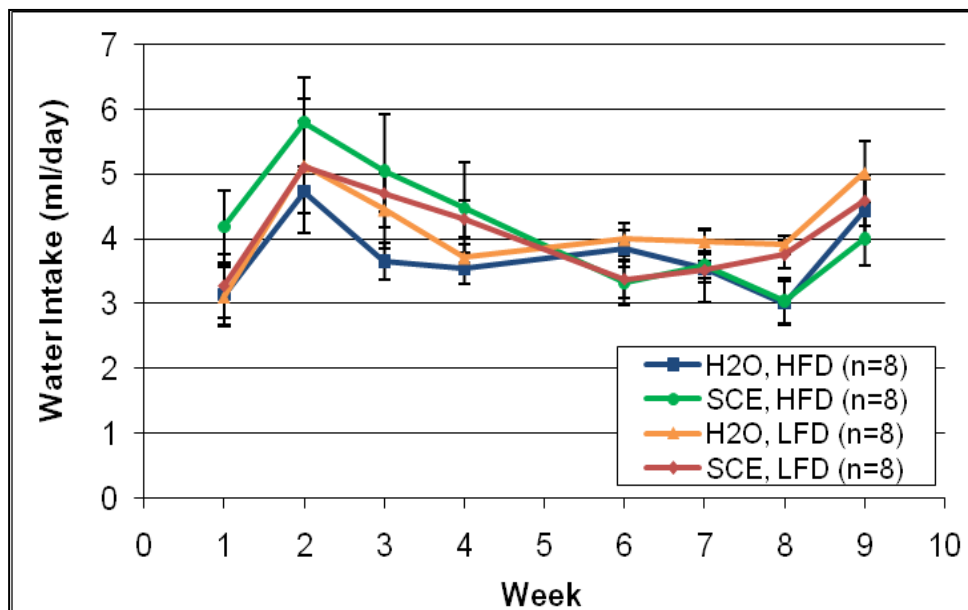
A significant interaction between Week and Extract was found (P=0.025), but there was no effect of Diet (P=0.495)

3.4.6 Oxygen Consumption in the CLAMS

Figures 3.12, 3.13 and 3.14 show the changes in oxygen consumption (VO_2, ml/kg metabolic weight/hr) at Weeks -1, 4 and 9 observed over 24hrs. No significant effect of diet or extract was found at Weeks -1, 4 and 9. There was a significant effect of time of day at all weeks ($P<0.001$), as oxygen consumption was found to be higher at night when all the mice were awake and active, indicating the expected circadian rhythm.

3.4.7 Carbon dioxide Production in the CLAMS

Figure 3.15, 3.16 and 3.17 shows the changes in carbon dioxide production (VCO_2, ml/kg metabolic weight/hr) at Weeks -1, 4 and 9 observed over 24hrs. There were no significant effects of diet or extract at Week -1, but there was a significant effect of time of day ($P<0.001$) indicating a circadian rhythm, with more carbon dioxide released at night when all the mice were awake and active. At Week 4, mice given the LF diet significantly increased ($P=0.021$) their carbon dioxide production and there was a significant time x diet interaction ($P=0.025$), indicating that at some timepoints, animals given the LF diet had higher carbon dioxide production than animals given HF diet. At Week 9, the effect of LF diet in increasing carbon dioxide production was even more significant than at Week 4 ($P<0.001$), with no time x diet interaction, indicating carbon dioxide production was higher in the LF diet group at all timepoints. No significant effects of SCE were found at Weeks 4 or 9 ($P=0.755$ and $P=0.134$ respectively).

Figure 3.12 Mean Oxygen Consumption in the CLAMS at Week -1
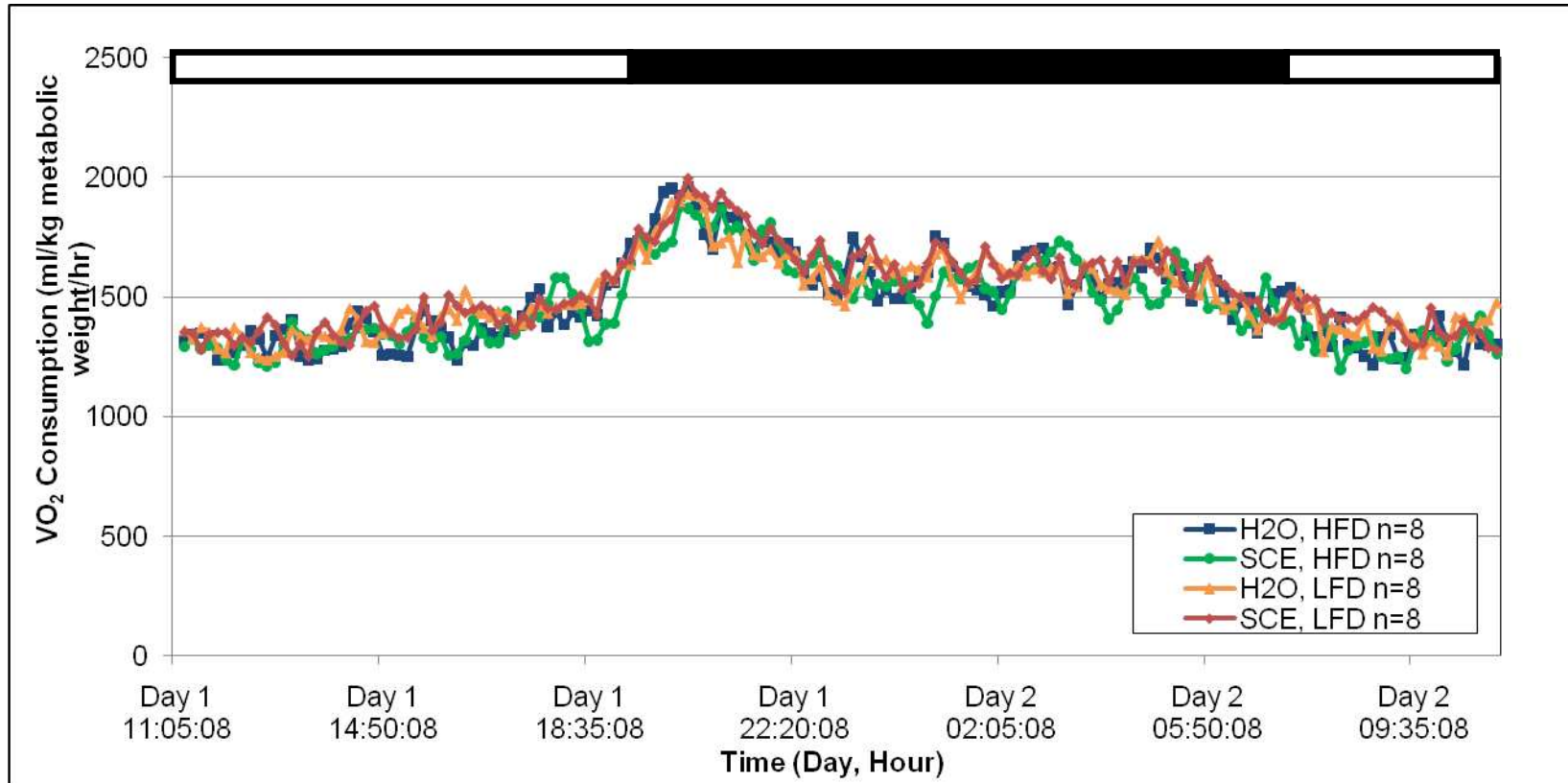
There were no significant effects of Diet (P=0.135) or Extract (P=0.984) on oxygen consumption, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.13 Mean Oxygen Consumption in the CLAMS at Week 4
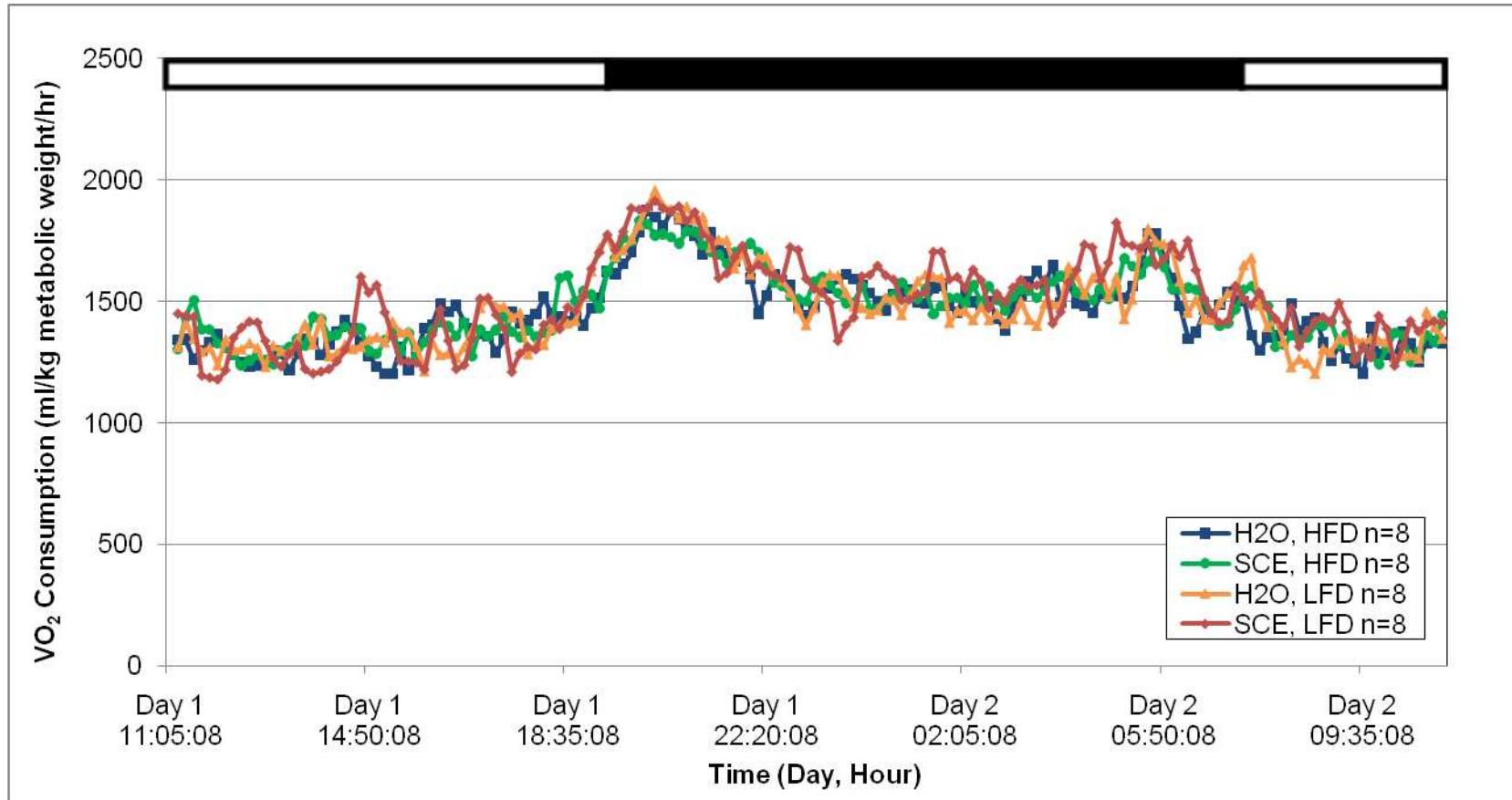
There were no significant effects of Diet (P=0.46) or Extract (P=0.356) on oxygen consumption, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.14 Mean Oxygen Consumption in the CLAMS at Week 9
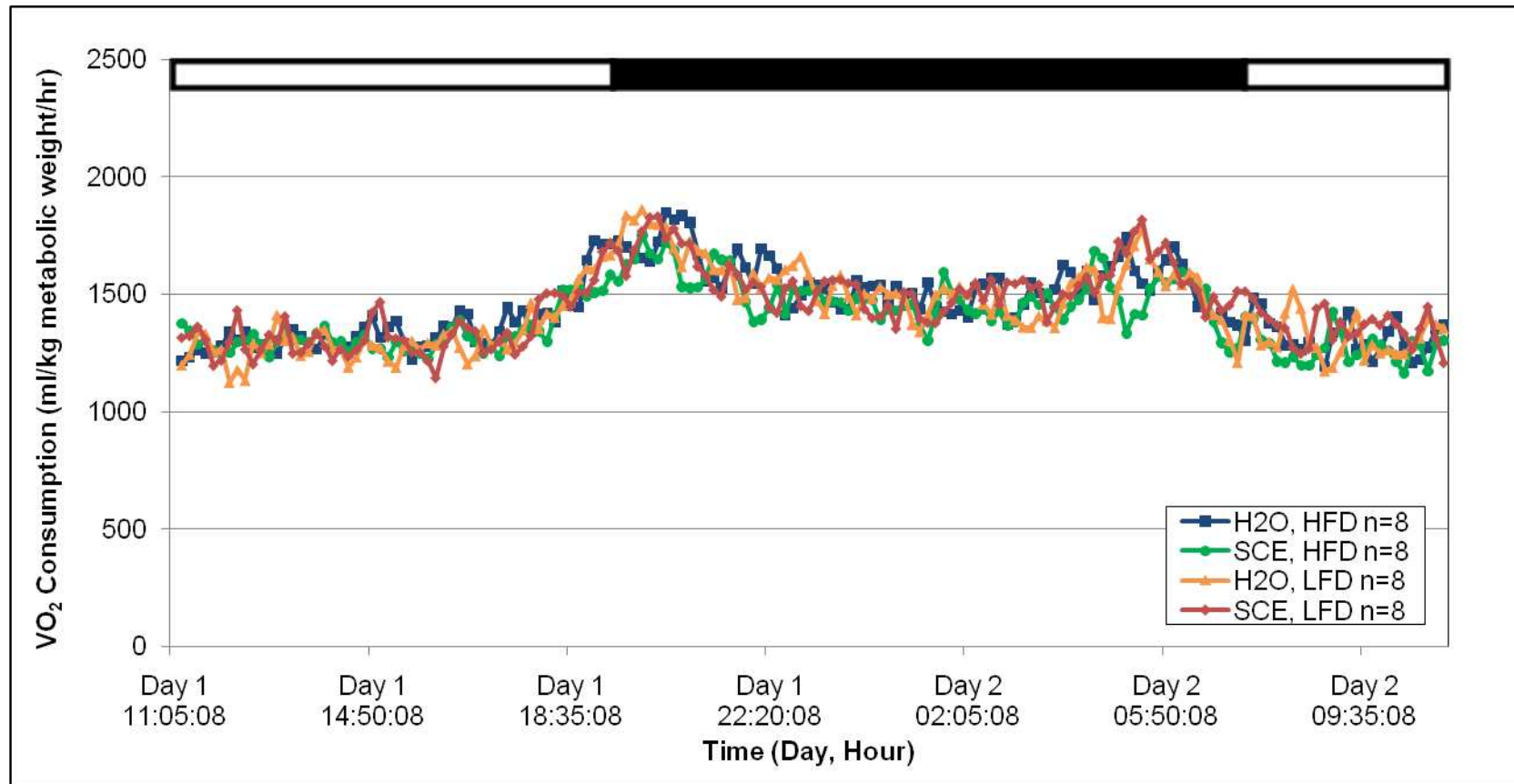
There were no significant effects of Diet (P=0.573) or Extract (P=0.574) on oxygen consumption, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.15 Mean Carbon Dioxide Production in the CLAMS at Week -1

There were no significant effects of Diet (P=0.056) or Extract (P=0.931) on carbon dioxide production , but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.16 Mean Carbon Dioxide Production in the CLAMS at Week 4
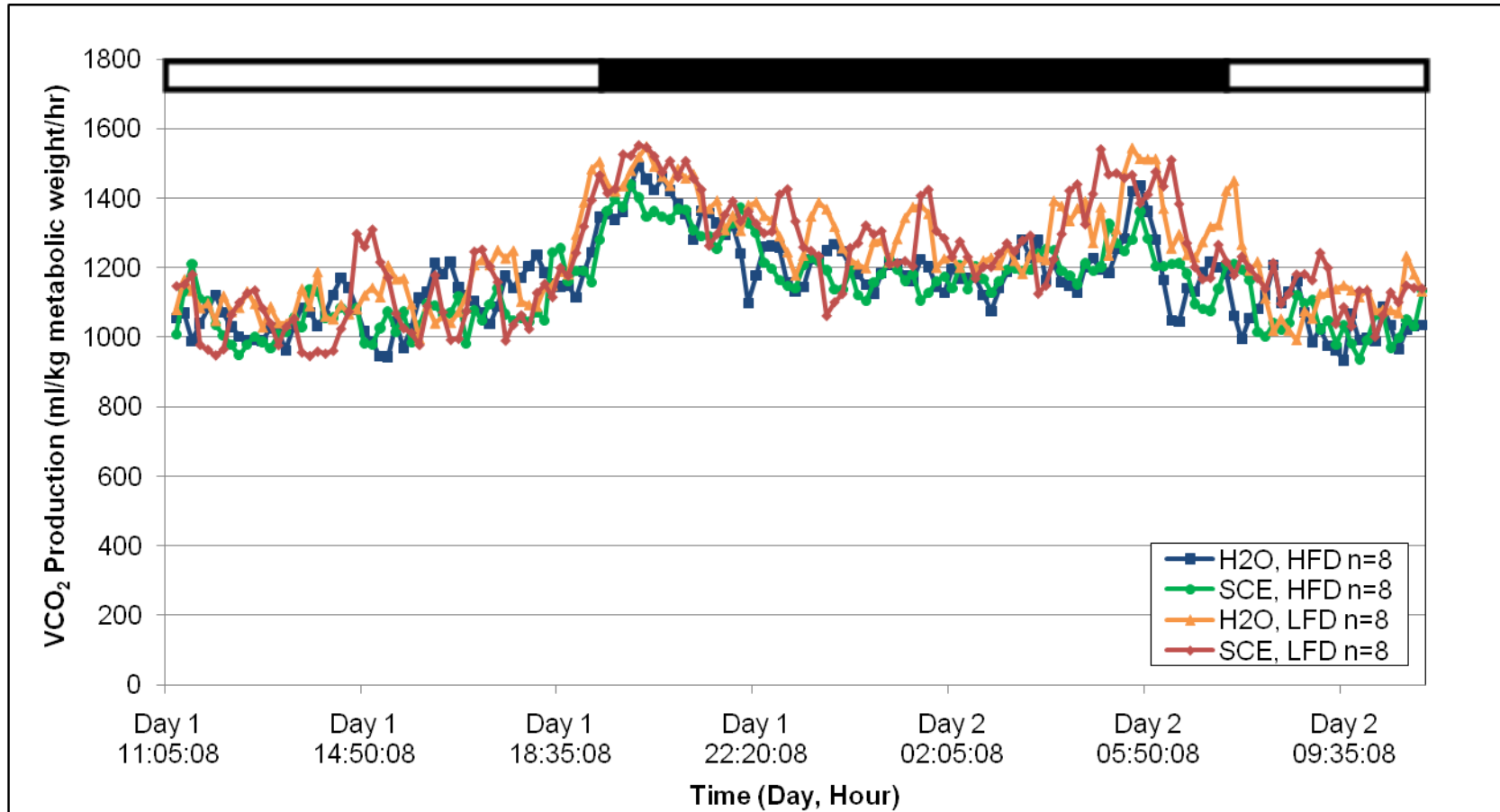
There was a significant Time of day x Diet interaction (P=0.025), but SCE had no significant effect on carbon dioxide production (P=0.755). White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.17 Mean Carbon Dioxide Production in the CLAMS at Week 9

LF diet significantly increased carbon dioxide production (P<0.001), but SCE had no significant effect on carbon dioxide production (P=0.134). A significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

3.4.9 Respiratory Exchange Ratio (RER) in the CLAMS

Figures 3.18, 3.19 and 3.20 show the changes in respiratory exchange ratio (RER) at Weeks -1, 4 and 9 observed over 24hrs. At Week -1, as expected, there were no effects of Diet (P=0.592) or Extract (P=0.693) on RER, but there was a significant effect of time of day (P=0.001). The range for RER values at Week -1 were 0.73 to 0.83 for all groups. At Week 4 there was a significant Diet x Time of day interaction (P=0.013), with LF diet increasing the RER and reducing the circadian rhythm, but no effect of extract on RER was observed at this stage (P=0.111). At Week 9, there was no longer an effect of time of day on RER (P=0.225), but there were significant effects of Diet (P<0.001) and Extract (P=0.011), with RER being increased by LF diet, but reduced by SCE.

3.4.9 Locomotor Activity in the CLAMS

Figures 3.21, 3.22 and 3.23 show the changes in locomotor activity (successive beam breaks/9 minutes) at Weeks -1, 4 and 9 observed over 24hrs. As expected, there were significant effects of Time of day at Weeks -1, 4 and 9 (all P<0.001), indicating the expected circadian rhythm as mice were more active during the dark phase. No significant effects of Diet or Extract were found at any Week (P>0.1 for all).

Figure 3.18 Respiratory Exchange Ratio in the CLAMS at Week -1
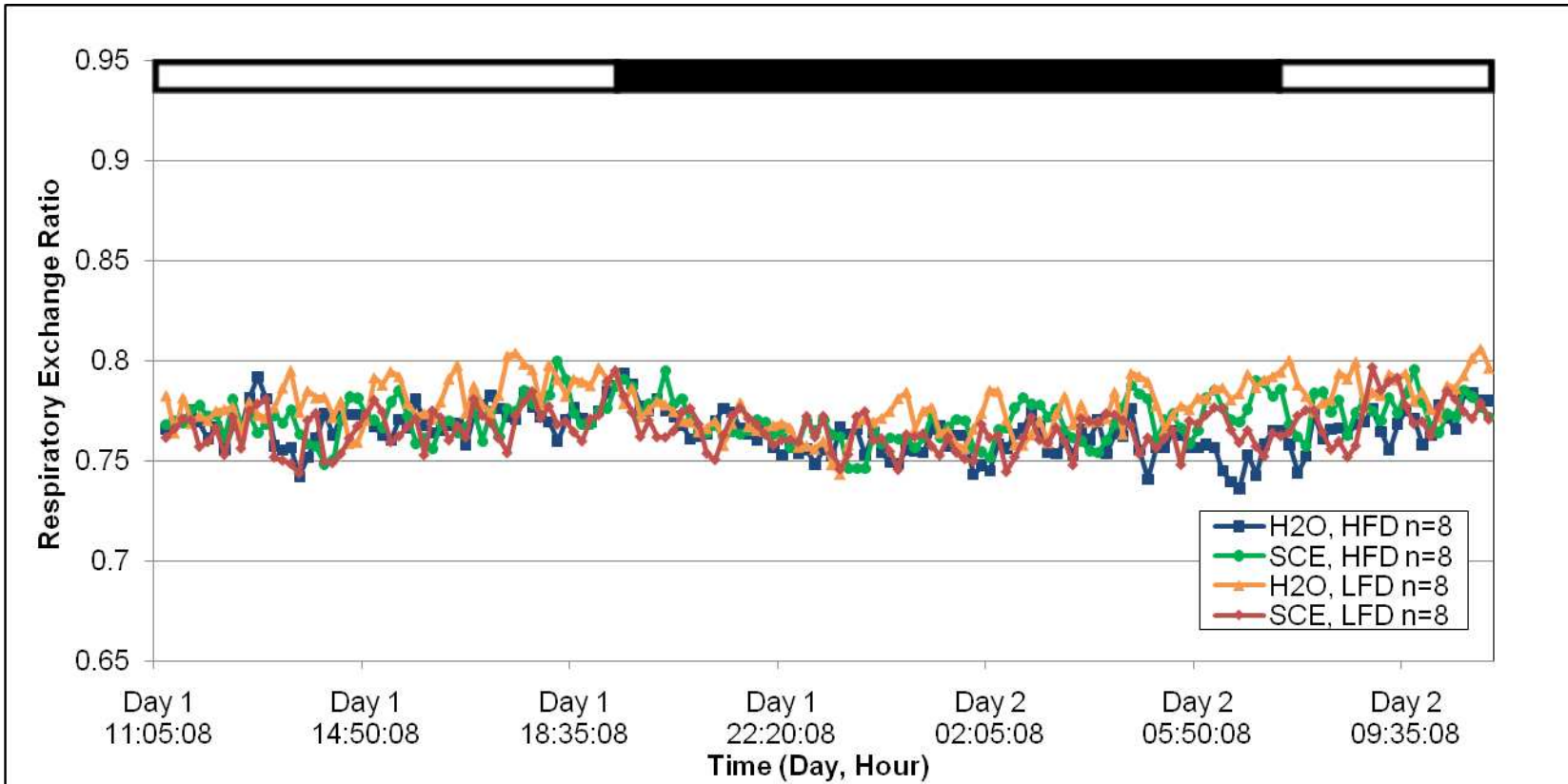
There were no significant effects of Diet (P=0.592) or Extract (P=0.693) on respiratory exchange ratio, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.19 Respiratory Exchange Ratio in the CLAMS at Week 4
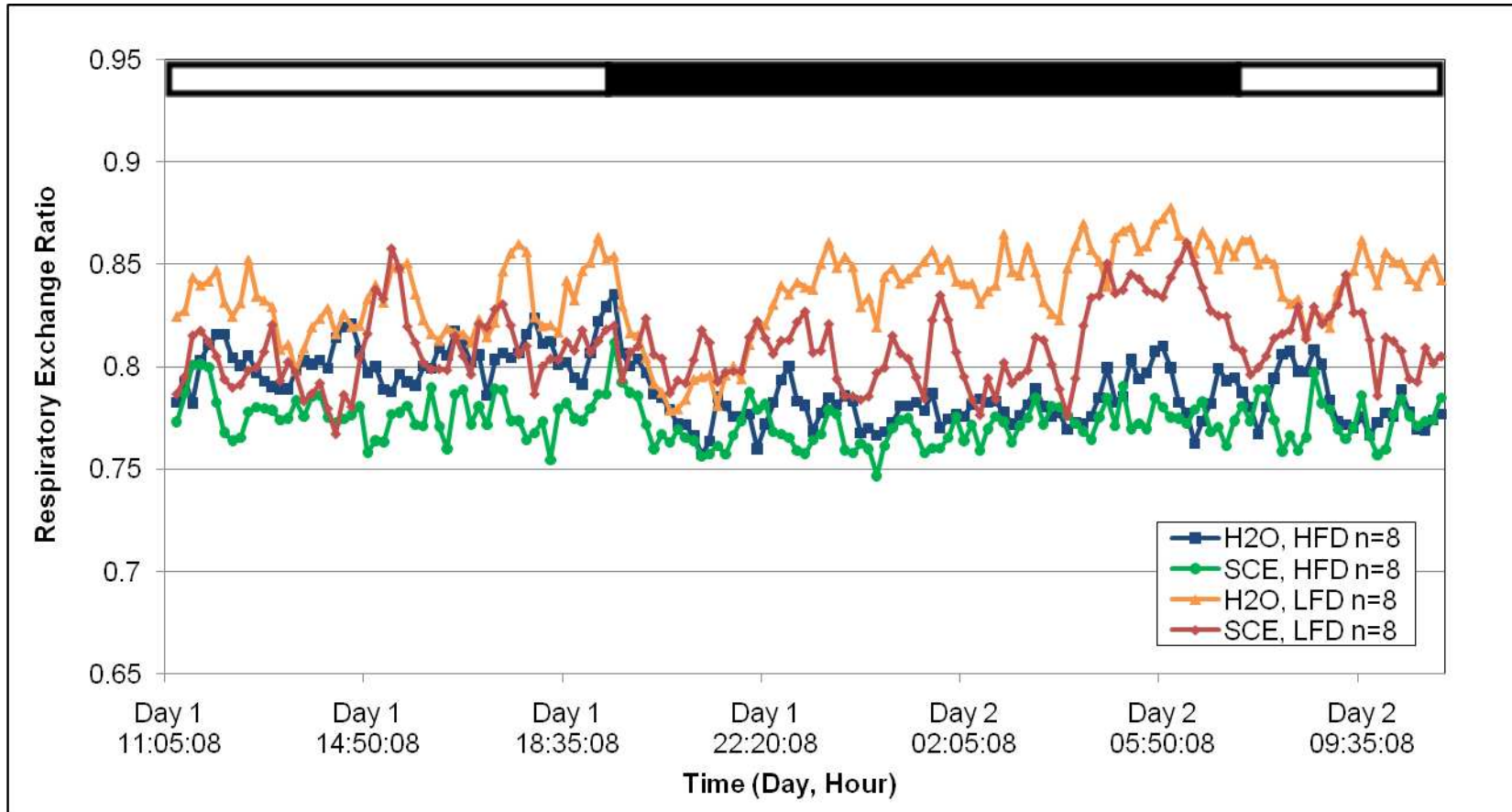
There was a significant Time of day x Diet interaction (P=0.013), but SCE had no significant effect on respiratory exchange ratio (P=0.111). White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.20 Respiratory Exchange Ratio in the CLAMS at Week 9
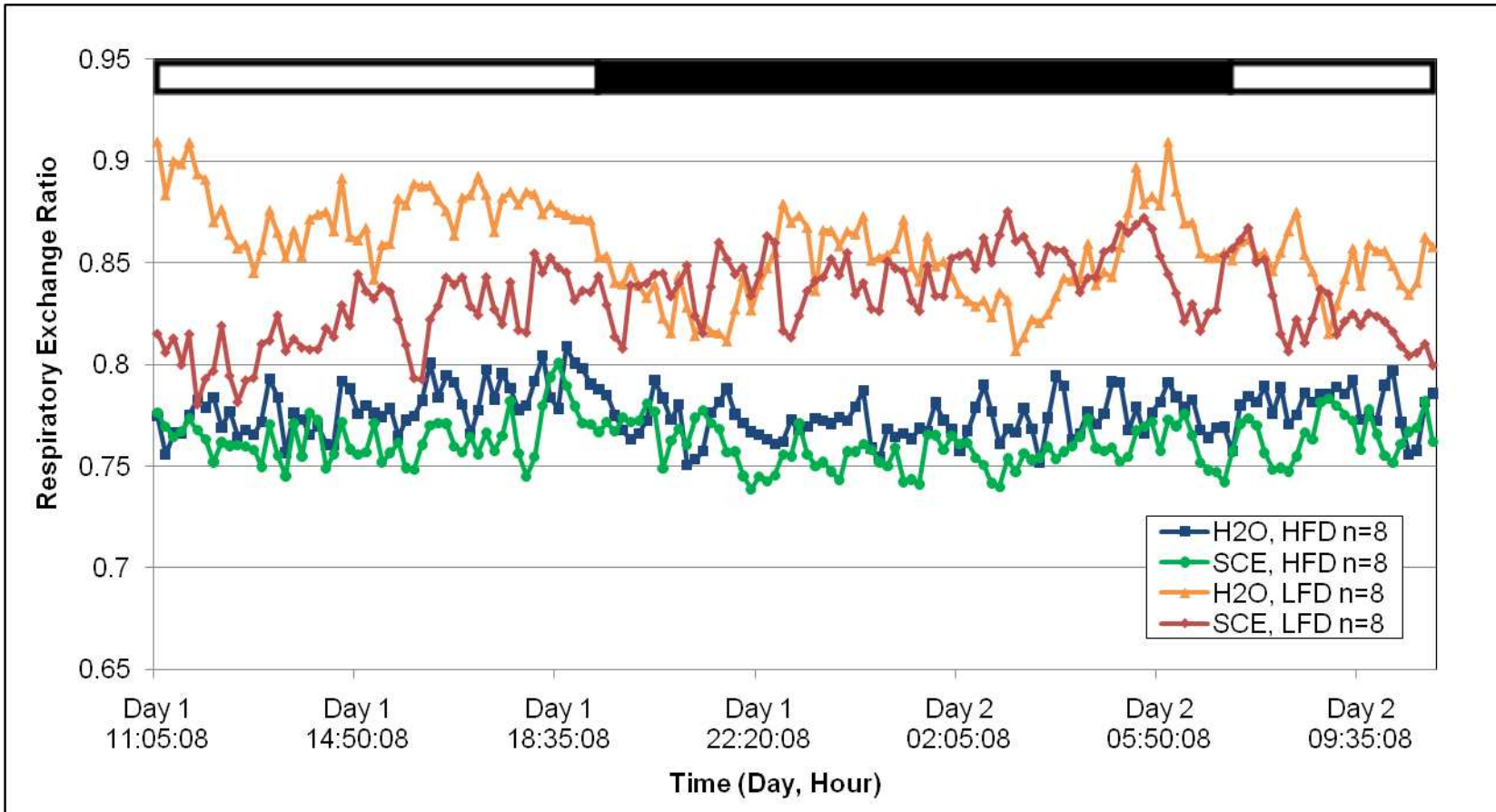
LF diet significantly increased respiratory exchange ratio (P=<0.001) and SCE significantly decreased respiratory exchange ratio (P=0.011). There was no effect of Time of day (P=0.225). White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.21 Locomotor Activity in the CLAMS at Week -1
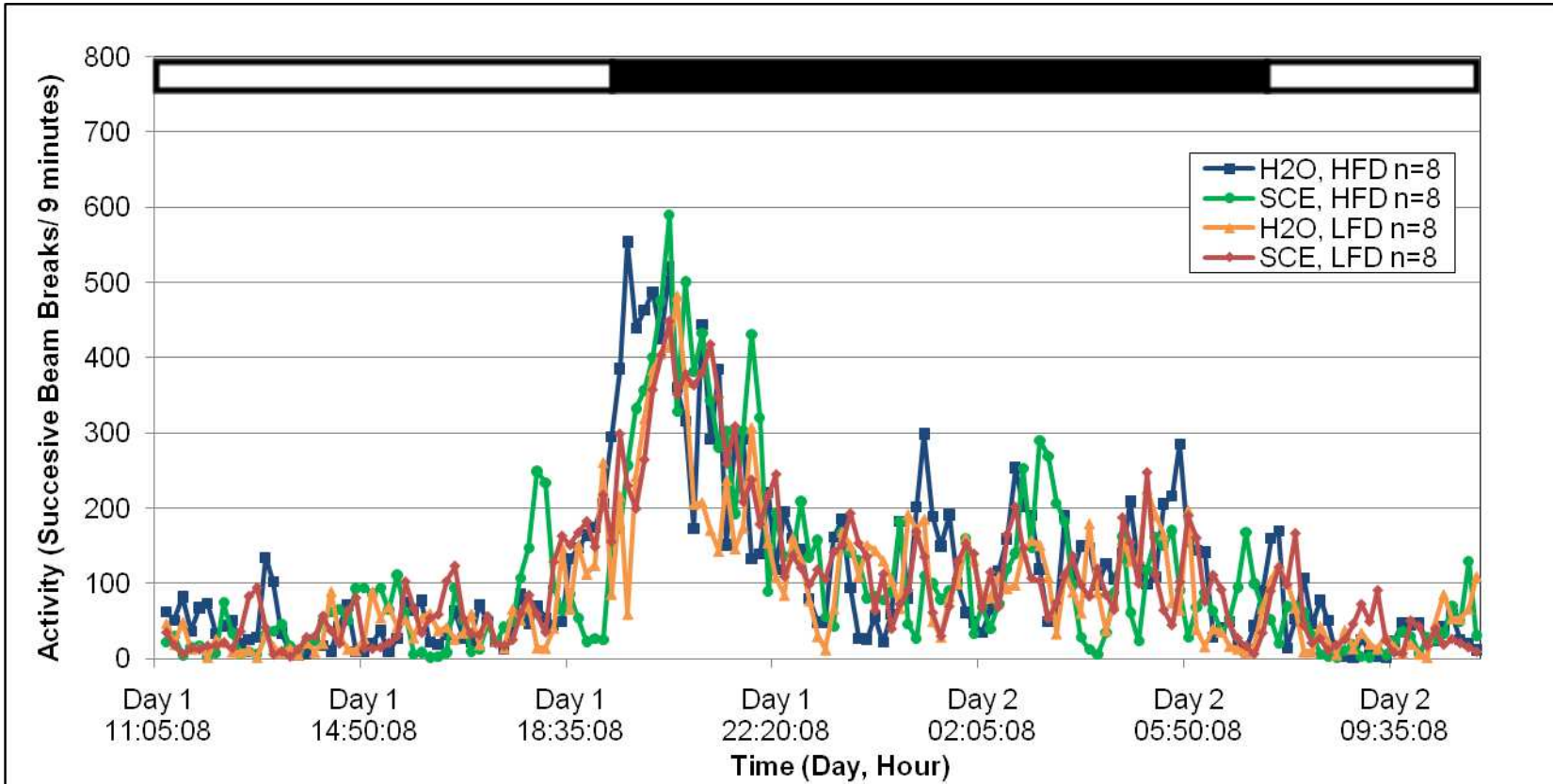
There were no significant effects of Diet (P=0.351) or Extract (P=0.824) on locomotor activity, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.22 Locomotor Activity in the CLAMS at Week 4
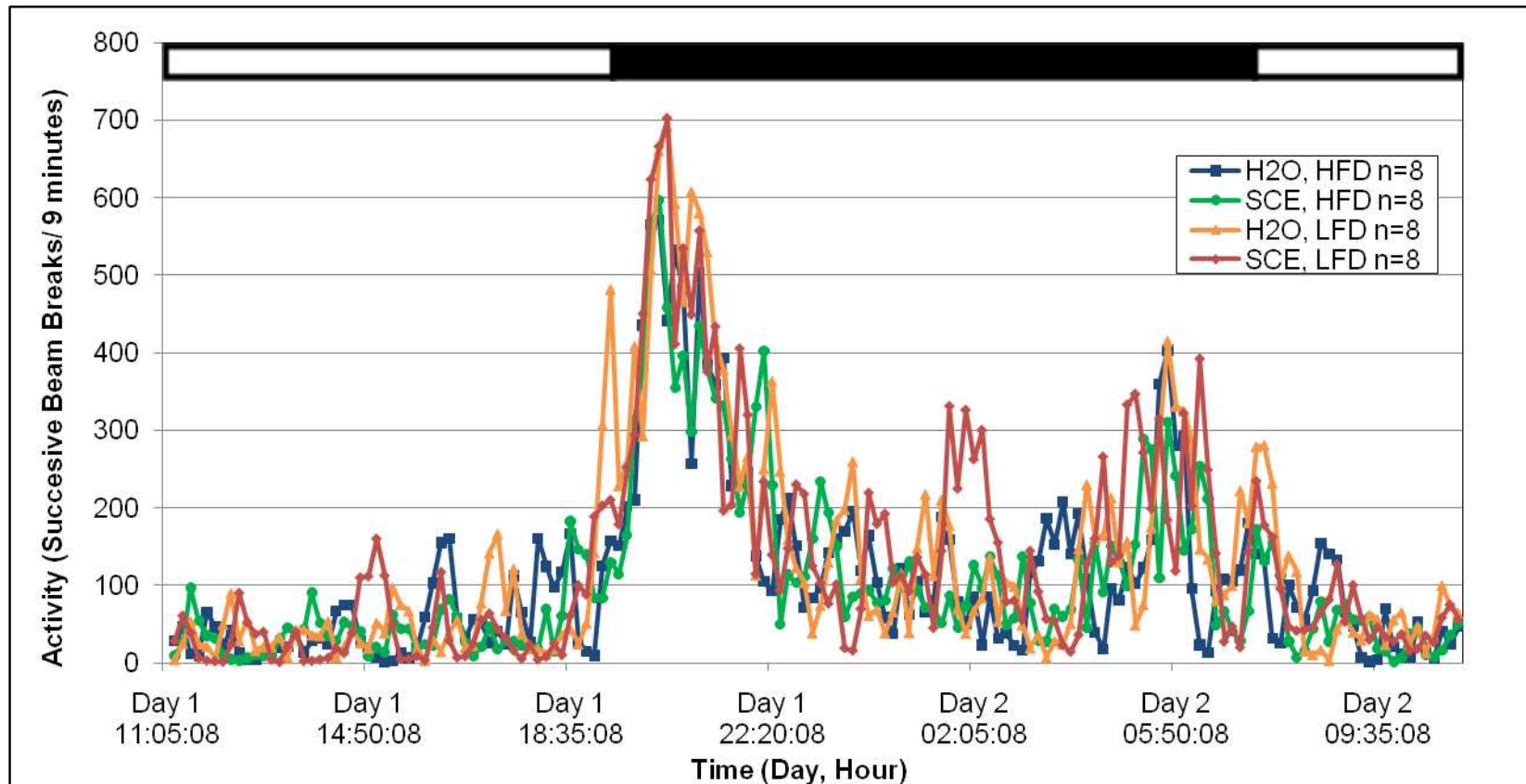
There were no significant effects of Diet (P=0.191) or Extract (P=0.843) on locomotor activity, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.23 Locomotor Activity in the CLAMS at Week 9
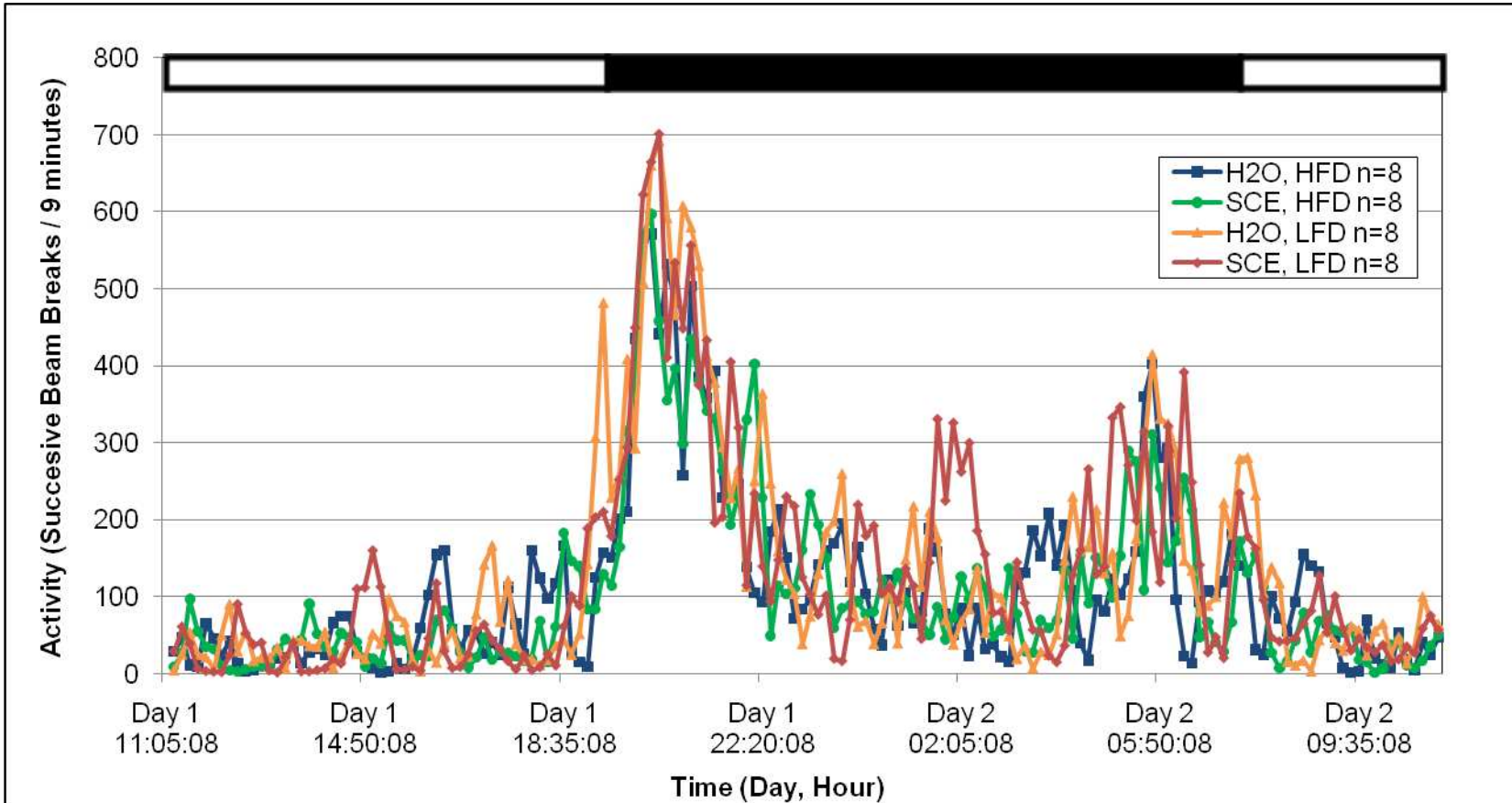
There were no significant effects of Diet (P=0.147) or Extract (P=0.846) on locomotor activity, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

3.4.10 Heat Production in the CLAMS

Figures 3.24, 3.25 and 3.26 show the heat produced in the CLAMS (kcal/hour) at Weeks -1, 4 and 9 observed over 24hrs. At Week -1, as expected, there were no effects of Diet (P=0.059) or Extract (P=0.086) on heat production, but there was a significant effect of time of day on heat production (P<0.001). The range for heat production values at Week -1 were between 0.6 to 1.05 for all groups. At Week 4 there were significant effects of Extract (P=0.048), Diet (P<0.001) and Time of day (<0.001). Extract was found to increase heat production, while LF diet decreased heat production and the effect of Time of day indicated the presence of a circadian rhythm. At Week 9, there was no longer an effect of Extract on heat production (P=0.193), but there were significant effects of Diet (P<0.001) and Time of day (P<0.001), with heat production being reduced by LF diet and a circadian rhythm being observed.

3.4.11 Meal Frequency in the CLAMS

Figures 3.27 and 3.28 shows the frequency of meals in the CLAMS measured as number of meals at Weeks -1, 4 and 9 observed for 24hrs (7hr light phase and 7hr dark phase). The number of meals was recorded in the CLAMS each time the mice returned to the feeding trap (Figure 3.2). The data for Week 4 was missing as a result of an equipment problem. There was a similar problem with the measurements at Weeks -1 and 9, such that only data for the last 24hrs was available, meaning that the data are presented slightly differently in terms of the light;dark cycle. No significant effects of diet or extract were observed at both Week -1 (P=0.199, P=0.349) and Week 9 (P=0.35, P=0.939). However, as expected, there was a significant effect of Time of day on meal frequency at Week -1 (P=0.004) and Week 9 (P<0.001), the number of meals being higher during the dark phase when the animals were awake compared with the light phase when animals were asleep or resting.

Figure 3.24 Heat Production in the CLAMS at Week -1
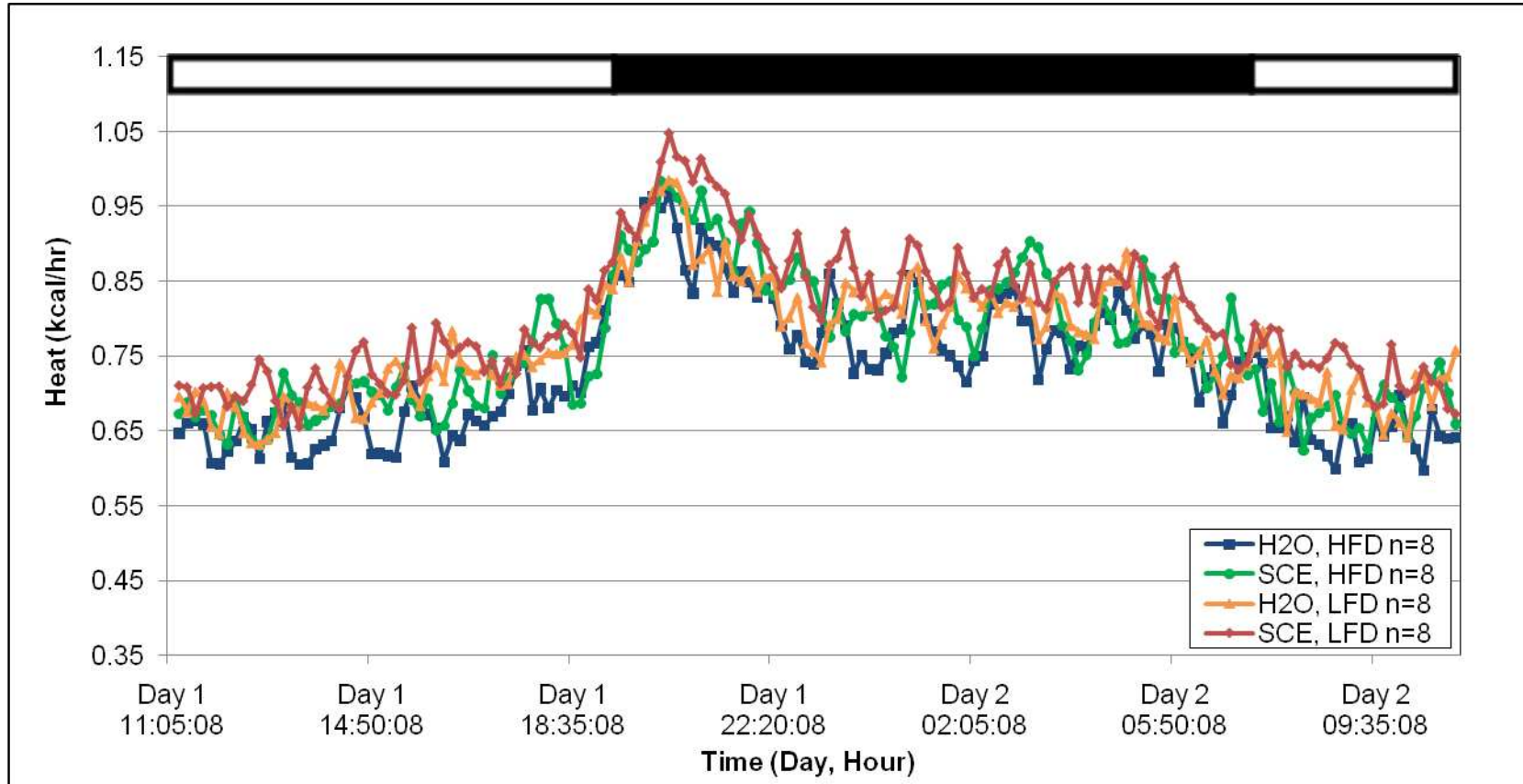
There were no significant effects of Diet (P=0.059) or Extract (P=0.086) on heat production, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.25 Heat Production in the CLAMS at Week 4
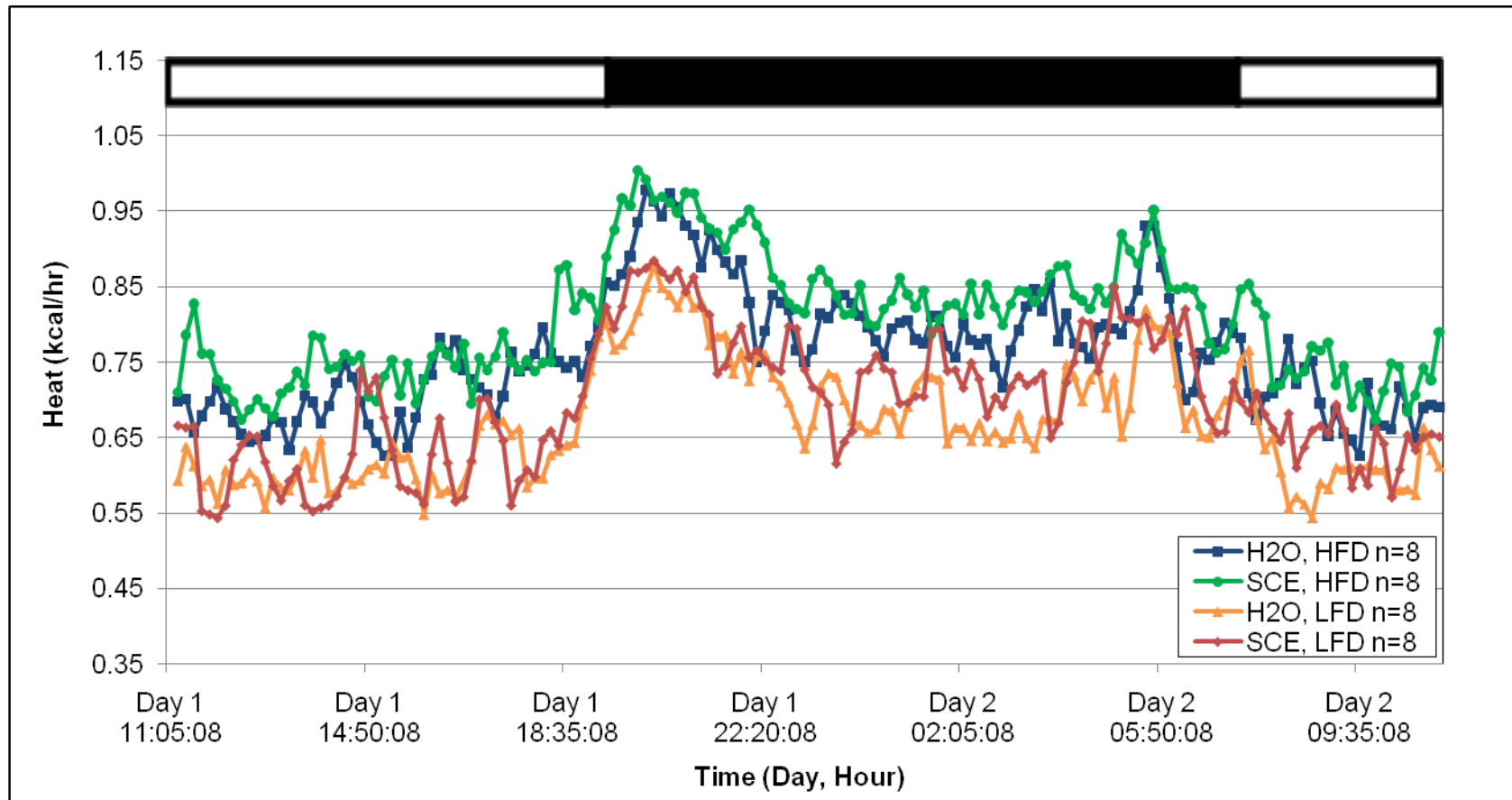
There were significant effects of Diet (P<0.001). Extract (P=0.048) on heat production. Significant effect of Time of day (P<0.001) was also found indicating the presence of a circadian rhythm. White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.26 Heat Production in the CLAMS at Week 9
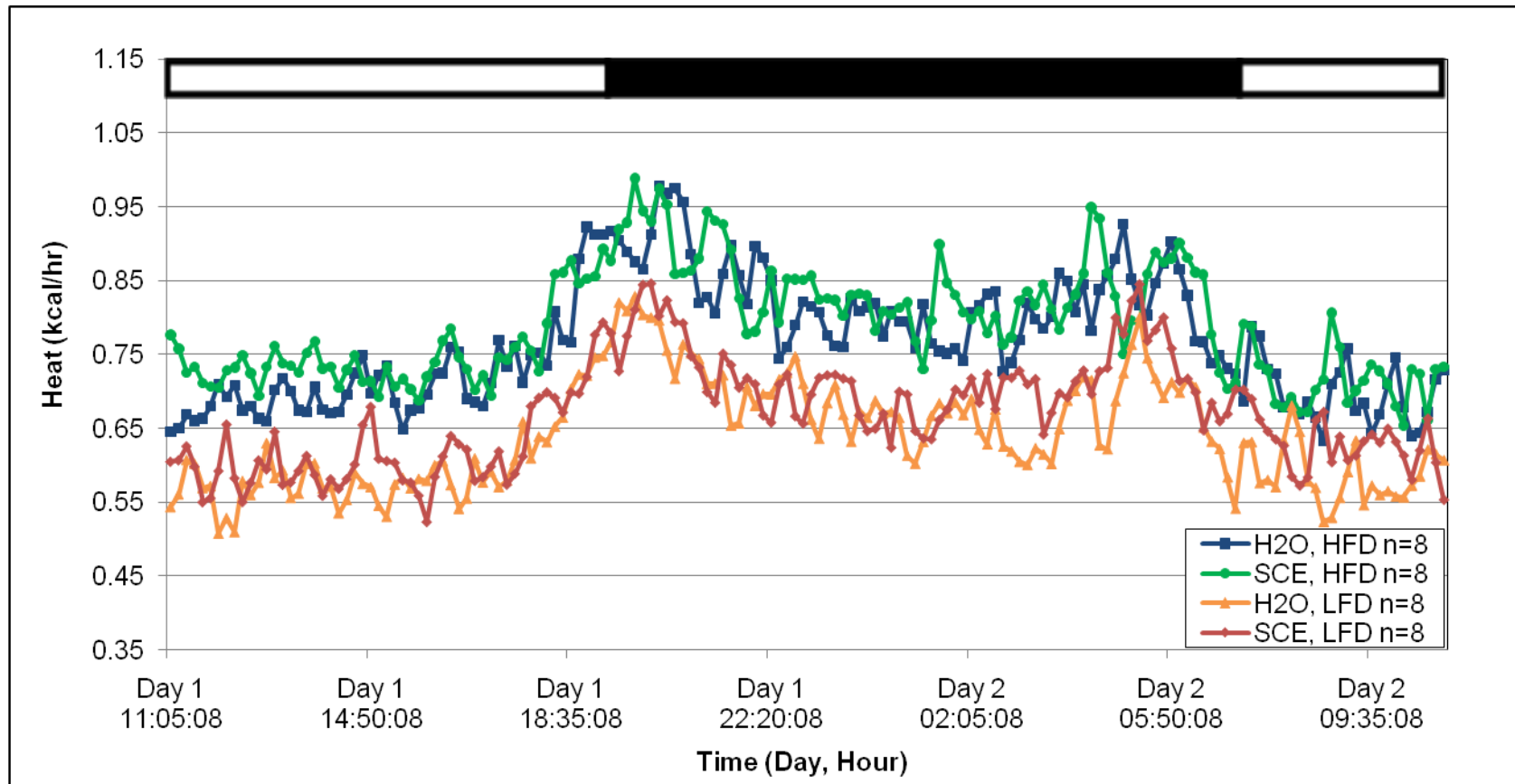
There were significant effects of Diet (P<0.001) and Time of day (P<0.001) on heat production indicating the presence of a circadian rhythm. No effect of SCE was found (P=0.193). White box indicates light phase (11:00-19:00, 8hrs and 07:00-11:00, 4hrs) and black box indicates dark phase (19:00-07:00, 12hrs).
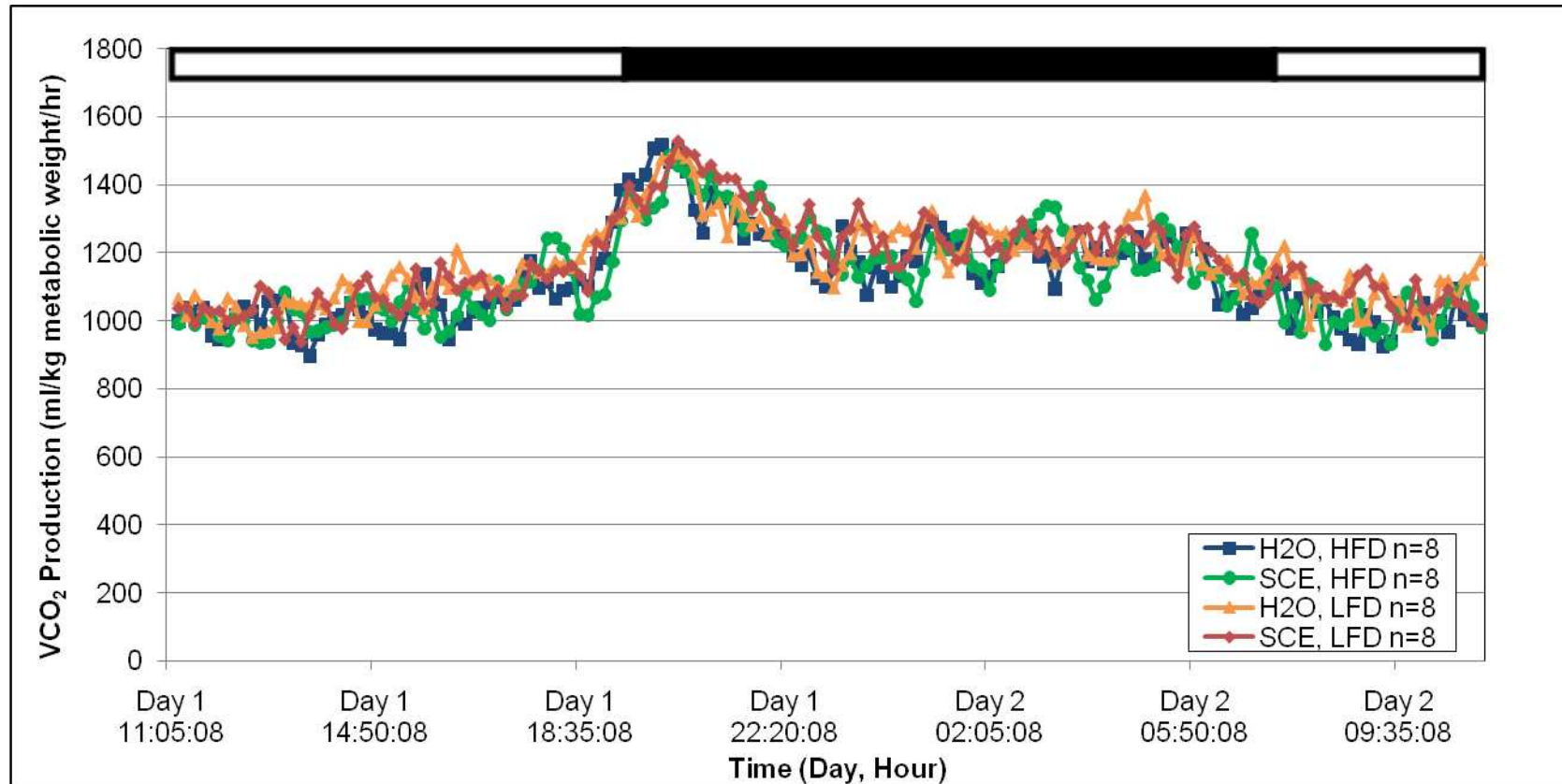
Figure 3.27 Meal Frequency in the CLAMS at Week -1
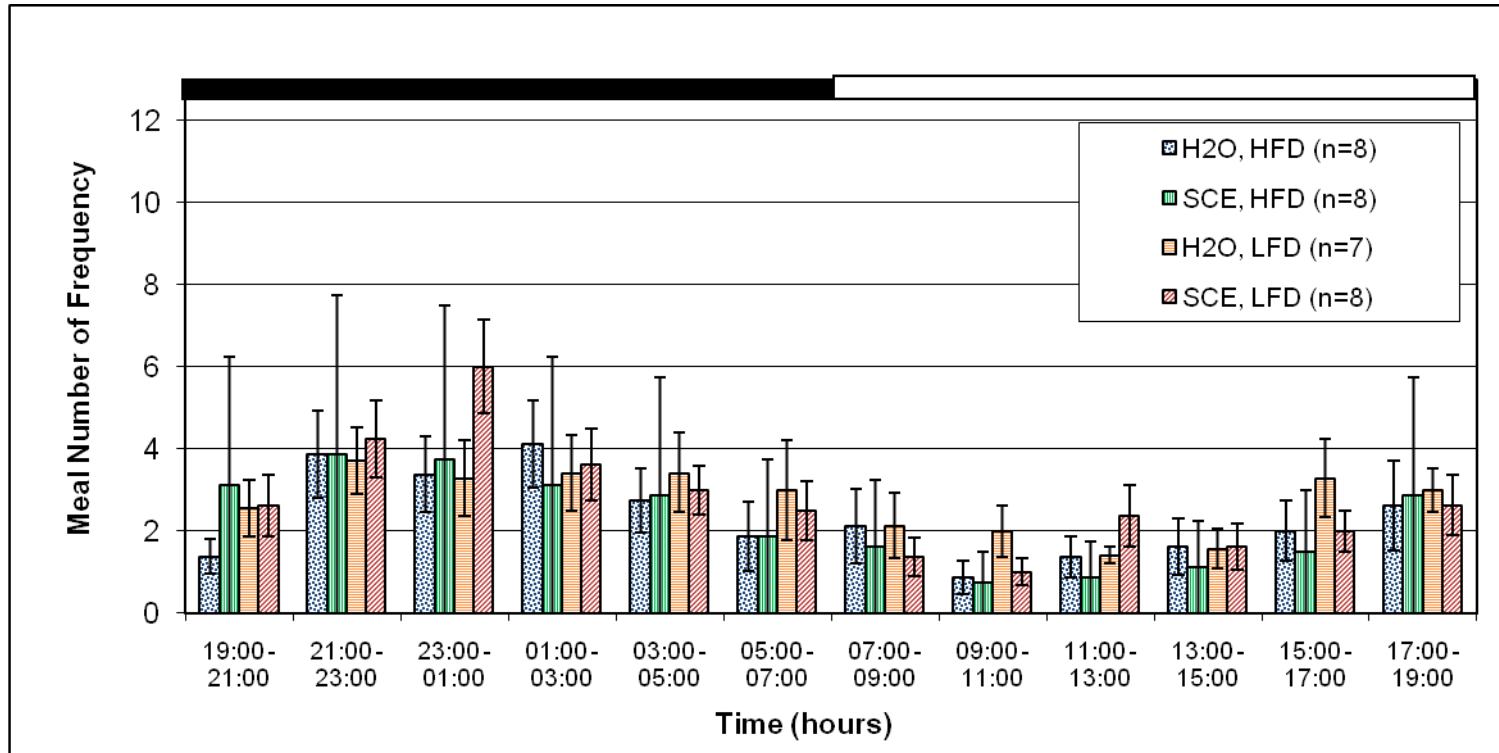
There were no significant effects of Diet (P=0.199) or Extract (P=0.349) on meal frequency, but a significant effect of Time of day (P=0.004) was found indicating the presence of a circadian rhythm. White box indicates light phase (07:00-19:00, 12hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

Figure 3.28 Meal Frequency in the CLAMS at Week 9
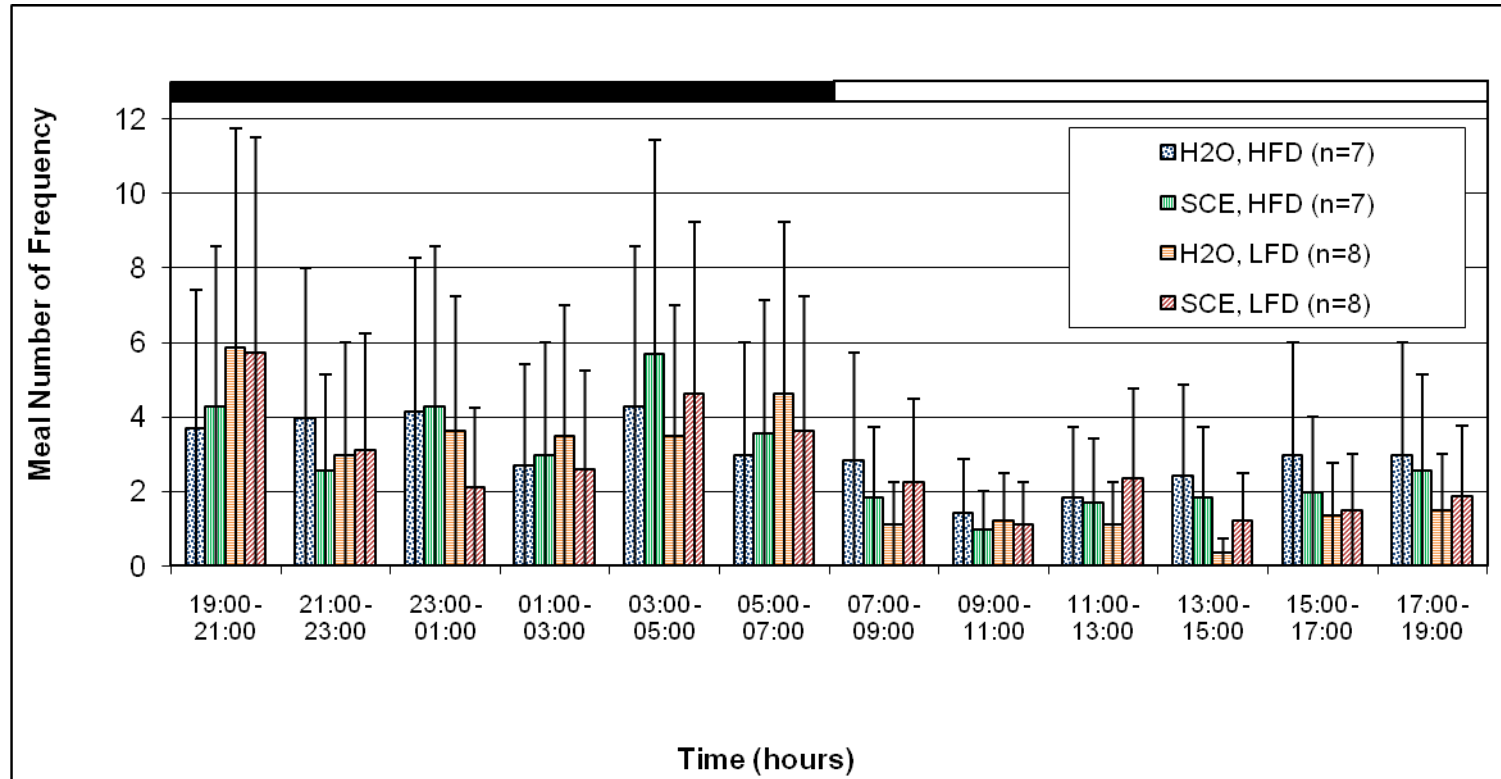
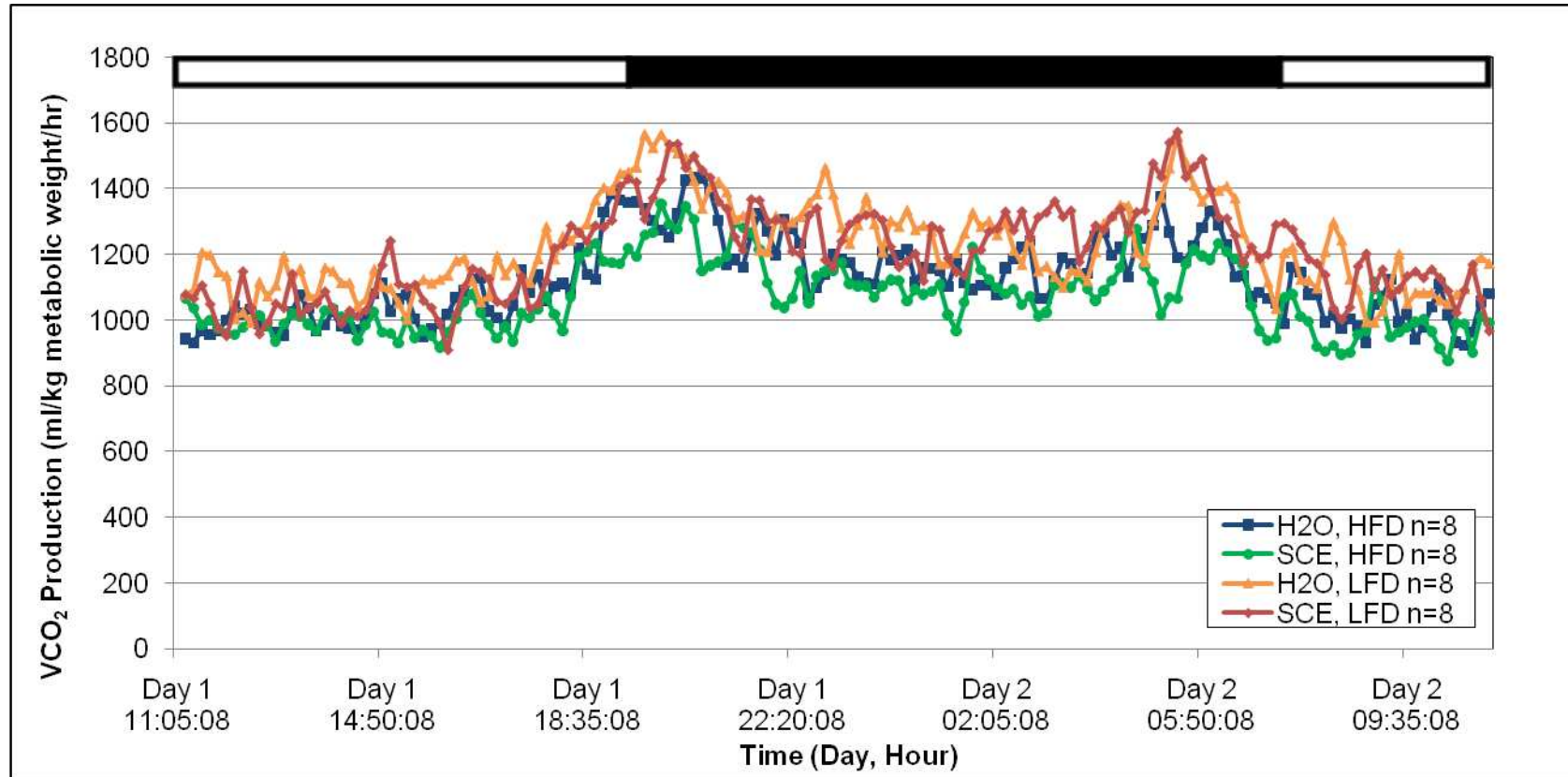There were no significant effects of Diet (P=0.35) or Extract (P=0.939) on meal frequency, but a significant effect of Time of day (P<0.001) was found indicating the presence of a circadian rhythm. White box indicates light phase (07:00-19:00, 12hrs) and black box indicates dark phase (19:00-07:00, 12hrs).

### 3.4.12 Plasma Glucose and Lipid Concentrations

Plasma glucose and lipid concentrations were analyzed from plasma obtained at Baseline (Week -2) and at the end of the study (Week 10). For data at Baseline (Table 3.1), samples were taken from control obese mice (n=7) given HF diet and control lean mice (n=10) given LF diet for 25 weeks. For data at Week 10 of treatment (Table 3.2), samples were taken from the diet and SCE treated groups.

At Baseline (Table 3.1), HF diet was found to have significantly increased plasma glucose, total cholesterol and glycerol concentrations (all P<0.001) compared with LF diet. Plasma triglyceride concentrations were numerically higher in the HF diet, but this was not statistically significant (P=0.376). At Week 10 of treatment (Table 3.2), LF diet was found to significantly reduce plasma glucose, total cholesterol, glycerol and triglyceride concentrations (all P<0.001). There was a significant interaction between diet and extract (P=0.013) on plasma total cholesterol concentrations, due to SCE increasing the total cholesterol concentrations, but only in the HF diet group. SCE also significantly increased (P=0.032) plasma glycerol concentrations in both HF and LF diet groups.

3.4.13 Liver Lipid Content

Liver lipid contents were analyzed from livers obtained at Baseline at the end of the study (week 10 of treatment), which were homogenized in isopropanol. For data at Baseline (Table 3.1), samples were taken from the livers of control obese mice (n=7) given HF diet and control lean mice (n=10) given LF diet for 25weeks. For data at Week 10 of treatment (Table 3.2), samples were taken from diet and SCE treated groups.

At Baseline (Table 3.1), significant increases in liver triglycerides and cholesterol content (all P<0.001) were found in the HF diet group and this was maintained at Week 10 of treatment (Table 3.2, all P<0.001). SCE increased liver cholesterol contents (Table 3.2), but only in the HF diet group (P=0.032 for Diet x extract interaction).

3.4.14 Carcass Lipid, Bodyweight and Organ Weight

At Baseline and at the end of the study (Week 10 of treatments), mice were weighed, culled and then their abdominal fat, liver and heart were dissected out and weighed and the remaining carcasses were homogenized and analyzed for total lipid content. For data at Baseline (Table 3.1), samples were taken from control obese mice (n=7) given HF diet and control lean mice (n=10) given LF diet for 25weeks. For data at Week 10 of treatment (Table 3.2), samples were taken from the diet and SCE treated groups.

At Baseline (Table 3.1), HF diet was found to significantly increase body weight (P<0.001), abdominal fat weight (P=0.002), carcass lipid content (P<0.001), liver weight (P<0.001) and heart weight corrected for bodyweight (P=0.022). Similarly, at Week 10 (Table 3.2), HF diet significantly increased body weight (P<0.001), abdominal fat weight (P<0.001), % abdominal fat (P<0.001), carcass lipid content (P<0.001), liver weight (P<0.001), % liver weight (P=0.001) and % heart weight (P=0.022) compared with LF diet. The only significant effects of SCE were to increase liver weight (P=0.019) and% liver weight (P=0.029), to decrease % heart weight (P=0.047) and a tendency to increase body weight (P=0.072) and abdominal fat weight (P=0.079).

Table 3.1 Plasma and Liver Contents, Carcass Lipid and Organ Weights at Baseline

Diet	High Fat (n=7)	Low fat (n=10)	SED	P-value
Weights and %				
Body weight (g)	48.84	36.48	1.859	**<0.001**
Abdominal fat wt (g)	3.43	2.29	0.312	**0.002**
% Abdominal fat (g/100g BW)	7.03	6.15	0.639	0.191
Carcass lipid (% dry wt)	71.5	54.0	2.27	**<0.001**
Liver wt (g)	2.328	1.697	0.1194	**<0.001**
% Liver wt (g/100g BW)	4.773	4.643	0.1785	0.478
Heart wt (g)	0.2023	0.1853	0.0161	0.308
% Heart wt (g/100g)	0.412	0.514	0.0398	**0.022**
Plasma concentrations				
Glucose (mg/ml)	3.386	2.389	0.158	**<0.001**
Total Cholesterol (mM)	25.1	10.8	2.77	**<0.001**
Triglycerides (μg/ml)	2637	2178	502.7	0.376
Glycerol (μg/ml)	1510	375	186.3	**<0.001**
Liver contents				
Triglycerides (μmol/g)	245.1	44.0	19.58	**<0.001**
Cholesterol (μmol/g)	15.19	10.16	1.135	**<0.001**

Table 3.2 Plasma and Liver Contents, Carcass Lipid and Organ Weights at Week 10 of Treatments

Diet (D)	High Fat		Low fat			P- values:		
Extract (E)	Cont (n=8)	SC (n=7)	Cont (n=8)	SC (n=8)	SED	D	E	D x E
Body weight (g)								
	52.8	58.4	40.03	42.53	2.63	**<0.001**	0.072	0.413
Abdominal fat wt (g)								
	3.51	3.99	2.01	2.22	0.27	**<0.001**	0.079	0.493
% Abdominal fat wt (g/100g BW)								
	6.65	6.86	4.99	5.12	0.47	**<0.001**	0.607	0.909
Carcass lipid (% dry wt)								
	71.64	78.16	59.62	61.77	4.50	**<0.001**	0.117	0.407
Liver wt (g)								
	2.96	3.81	2.03	2.23	0.298	**<0.001**	**0.019**	0.134
% Liver wt (g/100g BW)								
	5.52	6.50	5.03	5.19	0.35	**0.001**	**0.029**	0.108
Heart wt (g)								
	0.200	0.191	0.186	0.176	0.020	0.327	0.536	0.967
% Heart wt (g/100g)								
	0.382	0.323	0.464	0.420	0.035	**0.001**	**0.047**	0.761
Plasma Glucose (mg/ml)								
	3.28	3.50	2.26	2.52	0.288	**<0.001**	0.249	0.923
Plasma Cholesterol (mM)								
	27.24	36.90	11.07	12.56	2.167	<0.001	0.001	**0.013**
Plasma Triglycerides (µg/ml)								
	2463	2817	1224	1516	301.3	**<0.001**	0.141	0.884
Plasma Glycerol (µg/ml)								
	1602	2324	189	428	300.5	**<0.001**	**0.032**	0.266
Liver Triglycerides (µmol/g)								
	317	416	76	174	3807	**<0.001**	**0.001**	0.979
Liver Cholesterol (µmol/g)								
	17.64	23.81	11.68	10.58	2.276	<0.001	0.127	**0.032**

3.5 Discussion

3.5.1 HF Diet Induced Obesity in LDLr KO mice

Traditionally, leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice have been used for studies of obesity but their utility in studying atherosclerosis is limited (Coenen & Hasty, 2007). On the other hand, the LDLr KO mouse has been used extensively as a research model to investigate pathways involved with lipid metabolism and as an *in vivo* model for atherosclerosis (Kennedy *et al.*, 2009; Lewis *et al.*, 2009; Wouters *et al.*, 2005). On high fat or high cholesterol 'Western type' diets containing 21% fat and 0.15% added cholesterol, LDLr KO mice develop severe hyperlipidemia and extensive atherosclerosis (Ishibashi *et al.*, 1993). Furthermore, LDLr KO mice have also been reported to exhibit diet-induced weight gain with high fat diet feeding (Wu *et al.*, 2006) making it viable to use as a model for obesity research (Coenen *et al.*, 2007; Merat *et al.*, 1999; Bernal-Mizrachi *et al.*, 2003; Schreyer *et al.*, 2003; Towler *et al.*, 1998). Thus, in this study, the LDLr KO mouse model was used to study the effect of SCE (0.1 and 1%w/w) on diet-induced obesity in the presence of hyperlipidemia.

HF diet feeding allows the characterization of obesity development and the evaluation of anti-obesity interventions in an *in vivo* experimental setting that is pathophysiologically similar to the human disease (Buettner *et al.*, 2007). We found many attributes of obesity in the HF diet-fed group at Baseline, before any treatments were started.

Hence, the HF diet was found to induce obesity. After 25weeks of feeding the HF diet, bodyweights were found to be significantly higher (P<0.001) than the LF diet-fed control group, with the HF diet-fed group increasing their bodyweight by 127%, while the LF diet-fed group only increased their bodyweight by 70% (Figure 3.2). As a rule, experimental animals eating LF diets do not become obese (Bray *et al.*, 2004). Development of obesity in animals eating HF diets is the expected outcome (Bray *et al.*, 1990; West & York, 1998). It has also been studied by Cha and colleagues (2001) that when animals are exposed to several concentrations of dietary fat, there is a dose-response curve with a threshold at about 25% dietary fat. This suggests that the concentrations of dietary fat need to exceed 25% in the diet before obesity develops. In our case, the 34.9(% per g) or 60%kcal of fat in the HF diet successfully induced obesity.

The abdominal and carcass fat and liver weights of HF diet-fed groups had increased, and their plasma glucose, total cholesterol and glycerol concentrations were also higher than the LF diet-fed controls (Table 3.1). Liver triglycerides and cholesterol had also risen above the concentrations in LF diet-fed group (Table 3.1). In humans, these attributes contribute to health related problems such as diabetes, cardiovascular disease (CVD), non-alcoholic fatty liver disease (NAFLD), osteoarthritis and sleep apnea (Bray, 2004).

Mice have been the predominant model of energy homeostasis and obesity in humans (Thibault *et al.*, 2004), because of the attributes of diet-induced obese mice as a result of excessive energy intake and weight gain induced by the use of energy-dense foods, which is similar to human subjects. However, it must be noted that the environment of the mice studied is fully controlled (they are individually housed in cages that limit physical activity, in rooms controlled for temperature and humidity with food available nearby). Human obesity is a more complex multifactorial condition that has been related to excessive food consumption, low energy expenditure, genetics, gender, age, socio-economic status, ethnicity, educational concentration, smoking status and many other factors (Thibault *et al.*, 2004). Thus, while controlled conditions in the diet-induced obese mice were necessary to enable experimental control, they do not necessarily mimic the normal human condition.

3.5.2 Change to a Low Fat Diet Reduces Obesity in LDLr KO Mice

LF diets were given to LF groups at Week 0, after they were taken out of the CLAMS, together with the first treatment of 0.1% (w/w) SCE. Effects of treatments on bodyweight, food intake, energy intake, feed efficiency, feed energy efficiency, water intake, were measured weekly in their home cage, while effects on oxygen consumption, carbon dioxide production, respiratory exchange ratio, locomotor activity, heat production and meal frequency were measured in the CLAMS at weeks -1, 4 and 9. Finally, organ weights, plasma glucose and lipid concentrations and liver and carcass lipid contents were measured after death at the end of the experiment (week 10).

In the homecages, the bodyweights of LF groups (LFD, H_2O and LFD, SCE) immediately decreased after 1 week consumption of LF diet and continued to

decrease until Week 5 when their bodyweights became stabilized (Figure 3.2). The loss of bodyweight was as a result of reduced total body fat, liver and heart weights (Table 3.2). Even though food intake was found to be significantly increased by the LF diet (refer to section 3.3.2), the total energy intake of LF diet-fed groups was significantly lower than the HF diet-fed groups (refer to section 3.3.3) and this resulted in decreased feed efficiency (refer to section 3.3.4) which contributed to the decrease in bodyweights. LF diet was also found to improve (reduce) plasma glucose and lipid concentrations and also liver lipid contents (Table 3.2). Plasma glucose, total cholesterol, trigylcerides and glycerol concentrations were all decreased in LF diet-fed groups. Similarly, liver triglyceride and cholesterol contents were also reduced by LF diet. Only water intake was not altered by LF diet (refer to section 3.3.6).

In the CLAMS, LF diet was found to have no significant effect on oxygen consumption (refer to section 3.3.7), but carbon dioxide release was found to be increased at some timepoints at Week 4 and became significantly increased at all timepoints at Week 9 (refer to section 3.3.8). Since RER is the ratio of VCO_2 (total carbon dioxide production) to VO_2 (total oxygen consumption), obviously the increased VCO_2 at Weeks 4 and 9 lead to the significant increase of RER by LF diet. The RER value is used to indicate nutrient utilization (Westerterp, 1993). When the RER value is shifted closer to 1, this indicates reliance on carbohydrate as the major energy substrate. On the other hand, if the RER value is shifted closer to 0.7, this indicates a major reliance on fat oxidation (McNeill *et al.*, 1988). In our study, when HF diet was switched to LF diet which had 40% higher calories from carbohydrate, the RER increased indicating that the animals increased their utilization of carbohydrates. LF diet was found to have no effect on the frequency of meals taken by the animals at Week 9 even though their food intake was increased in the home cages. Unfortunately a technical problem meant that the data at Week 4 were lost.

It is well known that when obese subjects (rodents and humans) are given a LF diet ad libitum, it is effective in inducing weight loss and improves plasma lipid profile (Astrup *et al.*, 2000a; Astrup *et al.*, 2000b; Schaefer *et al.*, 1995; Harris *et al*, 1986; Uhley & Jen, 1989) and our findings confirm this. However, weight reduction does not always reverse obesity condition (Bray *et al.*, 2004). Previous

research confirmed that both mice and rats increase their number of fat cells after eating a HF diet for an extended time and these cells remain after dietary fat is reduced (Lemmonier, 1972; Faust *et al.*, 1978; Hill, 1990; Hill *et al.*, 1992; Rolls *et al.*, 1980).

Interestingly, locomotor activity did not increase after the diet was switched to LF diet although three main factors appear to participate in body-weight maintenance: metabolic utilization of nutrients, dietary habits and physical activity (Martinez, 2000). Energetic cost of physical activity was found to be one of the major components of energy expenditure that may influence body weight and composition (Tremblay & Almeras, 1996; Westerterp & Goran, 1997). In our study, after HF diet was switched to LF diet, the mice consumed less energy and their metabolic utilization changed towards carbohydrate, but their food intake increased, possibly to compensate for the reduced energy intake. However, their frequency of meals and locomotor activity were not affected, indicating that lower energy intakes were enough to decrease body weight and improve the blood lipid profile in obese LDLr KO mice.

3.5.3 *S. crispus* Extract Decreased RER Without Altering Food Intake or Other Variables in LDLr KO Mice

S. crispus leaves have been claimed to have many health benefits. In the previous chapter, we found that *S.crispus* crude extract contains polyphenols and HPLC analysis showed that chloroform-methanol extract clearly separated one particular polyphenol although the specific type of polyphenols is yet to be identified. In 1983, Soediro and colleagues discovered verbascoside (an ester glycosidic compounds of caffeic acid) and in 1987 they found 7 more phenolic acids (p-hydroxy benzoic, p-voumeric, caffeic, vanillic, gentinic, ferulic and syryngic) in extracts of *S. crispus*. In this study, we sought to find out whether a chloroform-methanol extract of *S. crispus* (SCE) had any antiobesity effect in obese LDLr KO mice.

Results from the data taken in the homecages indicate that SCE did not have any significant effect on bodyweights, food or water intake (refer to section 3.3.1, 3.3.2, 3.3.6), but was found to significantly increase the percentage of liver and heart weights and increased plasma glycerol and liver triglyceride content (refer

to Table 3.2). Plasma and liver cholesterol concentrations were also increased, but only in the HF diet fed groups. The LF diet decreased the cholesterol concentrations, with no effect of SCE observed. From these results, we suggest that SCE may induce adipose tissue lipolysis (increased plasma glycerol), though not at a high enough concentration to significantly reduce adipose tissue weights or total body fat content at the time measured. However, the increase in plasma and liver cholesterol could contribute to the development of arteriosclerosis.

In the CLAMS, SCE was found to significantly decrease the RER value at Weeks 4 and 9. The decreased RER suggests that SCE increased lipid oxidation, which may be a positive effect. Concomitantly, heat production was found to increase at Week 4 but the effect was diminished at Week 9. One possible reason for the diminished effect is tolerance towards SCE over a longer period of time. From the view of homeostatic control mechanisms, the brain is charged with maintaining body weight (or fat mass or energy content) at a given value (the set point), opposing change, and restoring it following any significant deviation (Fernstorm & Choi, 2008). Hence, when the heat production increased, the brain might sense change and oppose it by attempting to restore body weight to the set point value, perhaps by down-regulating the function of receptors involved. Although the volume intake of 1%(w/w) SCE was decreased 20-30% compared with earlier volume intake (section 3.3.5), this would not be account for the diminished effect, as the dose would only decrease between 20-30% leading to the consumption of 0.7 to 0.8%(w/w) of SCE. The reason for the decreased intake of SCE at 1%(w/w) may be due to a less acceptable taste of the more concentrated SCE.

Most weight loss agents fall broadly into two biological effect areas: those that affect energy intake (appetite suppressants and malabsorption agents) and those that affect energy expenditure (Clapham & Arch, 2011). In this case however, although the energy expenditure was increased due to the increased heat production via the decrease of RER value, these effects were not enough to reduce adiposity in the obese LDLr KO mice and thus were not sufficient to suggest its' use in treating obesity. Another comparable study which used molokheiya leaves (*Corchorus olitorius L.*) to reduce diet-induced obesity in HF diet fed LDLr KO mice had more convincing results, with body weight gains and epididymal adipose tissue and liver weights being reduced, as well as plasma glucose and triglyceride concentrations lowered and activation of beta oxidation

was detected although the experiment is not exactly similar as the mice in this study were not obese at the start of the experiment at treatments were only done for 8 weeks (Wang *et al.*, 2011).

3.6 Conclusion

Obesity was successfully induced in LDLr KO mice, with many attributes of obesity comparable with that of human obesity. LF diet successfully improved the obesity condition, by lowering energy intake but without increasing energy expenditure and heat production. SCE was found to increase lipolysis, heat production and fat oxidation in obese LDLr KO mice, but not enough to be suggested as an anti-obesity agent. However, tolerance towards heat production might be masking the real effects and so the measurements for adipose tissue weights, lipid and liver lipid contents might be different if it were to be measured at Week 4. The hypothesis was not met as effects of SCE on obese LDLr KO mice was found to be different to those found in diet-induced obese Sprague-Dawley rats (Zawawi, 2007), where adipose tissue weight was lowered and blood lipid profile was found to be improved. The effect of genetic background especially on the bioavailability of across different species and different response to different SCE doses might be responsible for the differences in the results found. *In vitro* adipose tissue explant studies will be carried out next, to establish whether the increased plasma glycerol concentrations in LDLr KO mice are due to a direct lipolytic effect of SCE on adipose tissue.

Chapter 4 - *In vitro* effects of SCE in the Rat and Human Adipose Tissue Explants

4.1 Introduction

Adipose tissue contains different cell types. Only one third of the tissue is constituted by adipocytes and the rest is represented by fibroblasts, macrophages, stromal cells, monocytes and preadipocytes (Geloen *et al*, 1989). The decrease of adipose tissue mass that occurs with weight loss may involve the mobilization of lipids through lipolysis, inhibiting adipogenesis or the loss of mature fat cells through apoptosis (Prins & O'Rahilly, 1997; Sorisky *et al.*, 2000). Preadipocytes do not have lipolytic activity until they are differentiated to mature adipocytes (Hauner *et al.*, 2001). Thus, most studies that investigate the direct effect of treatments on lipolysis will usually involve a unipotential cell line (e.g. 3T3-L1) that can only give rise to mature adipocytes (Frigolet Vasquez-Vela *et al.*, 2008). However, mature adipocytes lack other constituents found in the normal adipose tissue *in vivo*. The advantage of using whole tissue explants instead of isolated adipose cells is that the tissue contains cells at all stages of differentiation, including pre-adipocytes, immature adipocytes and mature adipocytes, which are known to secrete different hormones at different stages of differentiation. A number of studies have used cultured adipose tissue explants to more closely mimic the adipose tissue in the body and measure glycerol release into the medium as a quantitative measure of lipolysis (Lanna *et al.*, 1995; Porter *et al*, 2002; Baldwin *et al.*, 2007). Early studies often used whole epididymal fat pads from rats (Vaughan, 1961; Vaughan, 1962; Rodbell, 1960).

4.2 Hypothesis and Aim

As seen in the previous chapter, SCE induced fat oxidation and increased glycerol concentration in the blood. In this study, we hypothesized that application of SCE will result in an enhancement of lipolysis in rat and human adipose tissue explants. The aim was to optimize the method for measuring lipolysis in rat fat explants, based on the method described by Budd and colleagues (1994) and Rodbell (1960) and further modified by Headland (2007) for adipose tissue from pigs. The optimized method will then be used in the

human fat explants (if applicable). Thus, effect of SCE on lipolysis in different species will be determined.

4.3 Methodology

All work was carried out aseptically, using sterile consumables washed repeatedly with ethanol (70%) to maintain sterility. In the cell culture laboratory, all work was carried out in a sterile laminar flow hood on a heating block set to 50ºC. This was found to maintain the media temperature within the plates at 37ºC (Headland, 2007).

4.3.1 Sample Preparation

4.3.1.1 Rat Adipose Tissue

Male Sprague-Dawley rats weighing 350 – 400g were obtained from Harlan, UK and housed in pairs. Normal AIN diet was given *ad libitum* and the rats also had free access to water. The temperature of the housing was maintained at 21 ±1ºCand the rats were exposed to a 12hours light cycle (lights on at 0700hrs; lights off at 1900hrs). Rats were terminated by increasing CO_2 exposure in a closed chamber and the neck was quickly broken to ensure minimum suffering to the animals. The abdomen was aseptically wiped and opened and epididymal or perirenal adipose tissue from both sides removed.

4.3.1.2 Human Adipose Tissue

4.3.1.2.1 Selection and Withdrawal of Participants

Patients (all non-smoking) with a BMI over 40, or over 35 with a co-morbidity, referred to the obesity clinic at Derby were selected.

Enrolment

All patients approached to participate in the study had been referred to the Bariatric Clinic, Derby Royal Hospital for bariatric surgery. A Patient Information Sheet was given to each patient several weeks before consent was obtained. Patients returned for a pre-operative assessment prior to surgery, at which time written informed consent was obtained. A Unique Patient ID was allocated to all

patients in the Consent form which was used to identify their data. Patient enrolment and obtaining informed consent were carried by clinicians involved in the study including Ms Kwak, Prof Larvin, Dr Tan, Mr Leeder and Mr Ahmed.

Inclusion criteria

Patients who were undergoing bariatric surgery and were above 16 years of age were included.

Exclusion criteria

Patients who were undergoing emergency surgery were excluded, since they were unable to give informed consent and may have mental incapacity. Participants who might not adequately understand verbal explanations or written information given in English were also excluded.

4.3.1.2.2 Procedures for Obtaining Adipose Tissue Samples

During Laparoscopic (keyhole) Bariatric surgery it is often necessary to remove small segments of intra-abdominal fat in order to gain access to the operative area. This visceral adipose tissue may consist of omentum tethered in the upper abdomen, or perigastric adipose. Division of adherent fat is a routine part of surgery, and carries very little risk. It is usually affected by diathermy (electrocautery), or by scissors followed by diathermy. Adipose tissue contains small blood vessels and diathermy is usually highly effective in securing haemostasis.

Intra-operatively, after obtaining liver retraction, a small sample (up to 30 g, the weight of a typical omental adipose tissue from these patients would be several kilos) of omental fat was removed to facilitate the pars flaccida dissection. An omental adipose tissue sample may be obtained here or during the placing of the gastric band reservoir. No additional incisions were necessary for the purposes of this study.

4.3.1.2.3 Transport and Storage of Tissues

After the adipose tissue sample was collected, it was directly placed in a sterile specimen pot in warm M199 media solution, labelled with the rat or patient's identification reference allocated on their consent form. The sample was

transported immediately to the Tissue Culture Laboratory either in the Division of Nutritional Sciences, School of Biosciences (rat adipose tissue explants) or Clinical Sciences, School of Graduate Entry Medicine and Health (GEM), Derby (human adipose tissue explants).

4.3.1.3 Preparation of SCE

SCE was prepared from dried *S.crispus* leaves as described in Chapter 3, section 3.2.1 with the exception that water was replaced with Phosphate Buffered Saline solution. The concentrations used were based on the phenolic content, as measured in gallic acid equivalents (refer to the section that describes the phenolic assay).

4.3.2 Media Preparation

All media were prepared at least 1 day prior to use. Preparation was according to the manufacturer's instructions but modified slightly. Transport media and culture media were prepared as shown in Table 2.0, while Krebs-Ringer Bicarbonate (KRB) media was prepared as shown in Table 2.1. Both culture and KRB media were left to stir overnight at room temperature, after all the constituents were added and pH adjusted, in order to make sure that the bovine serum albumin (BSA) was mixed thoroughly. The next day, the media was sterile-filtered using a 0.22µm pore sized filter and kept at 4 ºC until needed.

Table 4.1 Preparation of M199 Media

	Transport Media	Culture Media
Powdered media (for 1 litre)	M199 (9.5g)	M199 (9.5g)
HEPES	25mM	-
Sodium hydrogen carbonate		2.2 g/L
Penicillin-Streptomycin	10 ml/L	10 ml/L
Gentamycin	1ml/L	1 ml/L
pH (adjusted with 1N NaOH or 1N HCl	7.4	7.4
Bovine serum albumin (fatty acid free)		1 g/L

Table 4.2 Preparation of KRB Media

	Concentration
Powdered media (for 1 litre)	KRB (with glucose, 9.5g)
Bovine serum albumin (fatty acid free)	3 g/L
pH (adjusted with 1N NaOH or 1N HCl	7.4

4.3.3 Tissue Preparation

Adipose tissue was aseptically removed from the body as a whole and immediately immersed in 20ml pre-warmed (37°C) transport media (see Table 4.1). The transport media was maintained at 37°C by keeping it in a sterile tube that was immersed in warm water (40°C) contained in a thermos flask. Following transfer to the culture lab, the tube was kept in a water bath at 37°C.

Adipose tissue was then transferred to a sterile culture dish containing 12.5ml of pre-warmed transport media (on the hot plate). The fat was then cleaned of connective tissues and blood vessels using scissors and forceps before being chopped into small pieces (approximately 2mm cubes). The tissue was then transferred to a second sterile culture dish containing pre-warmed (37°C with 5% CO_2 in air and 100% humidity) culture media (see Table 4.1) to wash.

4.3.4 Pre-incubation (if needed)

After washing, approximately 100 to 300mg of chopped tissue was placed into a 100mm culture dish containing 12.5ml pre-warmed culture media and incubated at 37°C and 5% CO_2 in air, for the amount of time studied (0 or 22 hours).

4.3.5 Acute Lipolysis Treatment

The second phase of incubations was for the measurement of lipolysis. After 22hours of pre-incubation, adipose explants were washed in plates containing 12.5ml pre-warmed KRB media (Table 4.2). Then the adipose explants were again transferred to a well on a 12 well plate containing 2ml of pre-warmed KRB media including any treatments needed for the experiment (e.g. isoproterenol,

IP). The plate was then placed in an incubator at 37°C and 5% CO_2 in air for 4 hours.

After the 4 hour incubation, aliquots of media were removed from each well separately with disposable plastic pipettes and transferred to separate and labelled 1.5ml eppendorf tubes before being stored at -40°C until required for glycerol quantification (refer to method 5.2.7). All the fat explants were removed from each well separately using forceps and transferred to another 1.5ml eppendorf tube and also stored frozen at -40°C until required for Folch Lipid Extraction (refer to method 4.2.8).

4.3.6 Chronic Lipolysis Treatment

For chronic incubations, treatments were added during pre-incubation in culture media, as well as during the 4 hours lipolysis incubation in KRB media (as in section 4.2.5).

4.3.7 Glycerol Quantification

Glycerol release into the KRB media was measured using a triglyceride assay kit (TR0100, Sigma, UK) according to the manufacturer's instructions for free glycerol quantification, but modified and optimized for use on a 96-well microplate format. Triglyceride Reagent (200µl) was added to 50 µl of sample (KRB media) in each well of a 96-well plate. A standard curve was set up on each plate to enable calculation of the concentration of glycerol present.

The free glycerol determination kit measures endogenous glycerol according to the following enzymatic reactions (see Figure 4.1). Glycerol was phosphorylated by ATP to form G-1-P and ADP, catalyzed by GK. G-1-P was then oxidized by glycerol phosphate oxidase (GPO) to form DAP and H_2O_2. POD catalysed the coupling of H_2O_2 with 4-AAP and ESPA to produce a quinoneimine dye with an absorbance maximum at 540nm. The increase in absorbance at 540nm is directly proportional to the free glycerol concentration of the sample. Absorbance was measured at 550nm as no 540nm filter was available.

Figure 4.1 Glycerol assay enzymatic reactions

$$\text{Glycerol} + \text{ATP} \quad \xrightarrow{\text{GK}} \quad \text{G-1-P} + \text{ADP}$$

$$\text{G-1-P} + \text{O}_2 \quad \xrightarrow{\text{GPO}} \quad \text{DAP} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + \text{4-AAP} + \text{ESPA} \quad \xrightarrow{\text{POD}} \quad \text{Quinoneimine dye} + \text{H}_2\text{O}$$
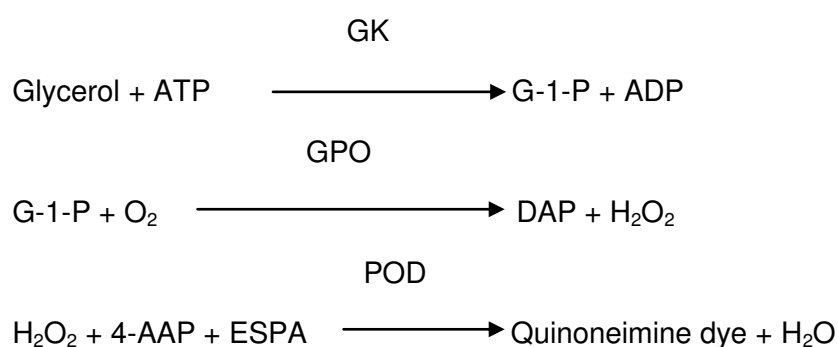
4.3.8 Folch Lipid Extraction

The amount of lipid in adipose explants from each well was determined so that glycerol concentration released could be corrected. This method was modified from the Folch extraction method (Folch *et al.*, 1957). Adipose tissue explants were placed in a 50ml conical tube and weighed. Methanol (5ml) was added to the tube and tissue was homogenized (Polytron PTA 10-35, Kinematica, Switzerland) for 30secs. Chloroform (10ml) was then added and the sample was homogenized again for 30secs followed by the addition of 3.75ml of Folch wash (0.88% KCl). The tube was then vortexed and left for 30mins at room temperature, prior to centrifugation (GS-6R, Beckman, USA) at 3000rpm for 10mins, to aid separation. The upper organic phase was removed and the lower organic phase was injected with nitrogen gas at 2.5G for 45mins. The sample was then dried in an oven at 50°C until the weight became constant. The tube was then reweighed and the total amount of lipid present was calculated.

4.3.9 Statistical Analysis

Values for glycerol release (µg per gram lipid) were expressed as means ± SEM. The data were analyzed using Genstat 10.0 statistical software. General Analysis of Variance with No Blocking was used. Significance was taken as $p < 0.05$.

4.4 Results

4.4.1 Optimization of Rat Adipose Tissue Explants

4.4.1.1 Adipose Depot Response to IP

Perirenal and epididymal adipose tissue explants response to IP was compared with identify which tissue depot to use. Figure 4.2 shows the glycerol release of epididymal and perirenal adipose tissue after incubation in KRB Media for 4 hours without pre-incubation (n=2 rats, samples in triplicate). Both epididymal and perirenal fat explants were found to respond to IP (P=0.122, ANOVA), but the baseline lipolysis was lower for epididymal, resulting in a greater-fold response to IP. Epididymal fat was therefore chosen to be used for all subsequent studies.

4.4.1.2 Linearity of Glycerol Release

To optimize the method used for fat explants, we have to determine whether glycerol concentration (as an indicator of lipolysis) was linear over time. In this experiment, we measured the glycerol released into KRB media from rat epididymal adipose tissue explants during different lengths of incubation time (2, 3 and 4 hrs) in the presence or absence of Isoproterenol (IP 10μM), without pre-incubation (n=1 rat, treatments in triplicate, see Figure 4.3). There was significant interaction between the length of the incubation and IP treatment (P <0.001, ANOVA) due to the divergence of the basal and IP stimulated response. Both basal and IP stimulated lipolysis increased over time in a linear manner and at all time points IP stimulated lipolysis was greater than basal lipolysis (P <0.001, ANOVA), but particularly at the longer incubation times. A 4 hr incubation period was adopted for all subsequent studies.

Figure 4.2 Glycerol Release in Different Adipose Depots After Treatment with IP in the Rat Adipose Tissue Explants
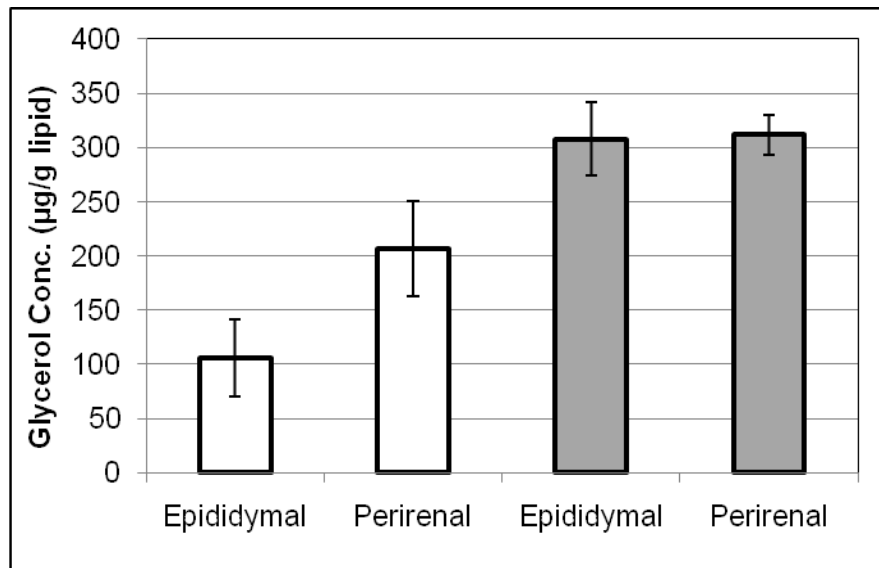
White box indicates basal (Control) and grey box indicates treatment with IP (10µM). Values are means ±sem, n=2 rats, treatments in triplicate. Depot X IP = 0.149, ANOVA. IP increased lipolysis compared with basal (P <0.001, ANOVA).

Figure 4.3 Linearity of Glycerol Release During Basal and IP Stimulated Lipolysis in Rat Epididymal Adipose Tissue Explants
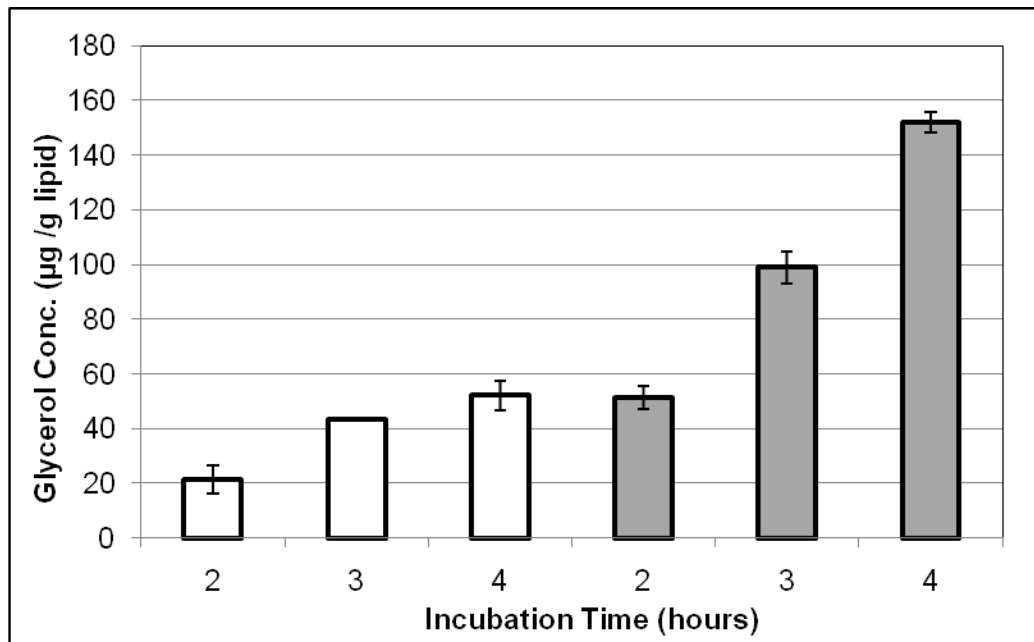
White box indicates basal (Control) and grey box indicates treatment with IP (10µM). Values are means ±sem, n=1 rat, treatments in triplicate. Time X IP = P < 0.001, ANOVA. IP significantly increased lipolysis compared with basal (P <0.001, ANOVA)

4.4.1.3 Viability of Explants in M199 for up to 48 hours

Another part of optimization is to ascertain whether explants were viable in M199 culture for up to 48hrs or not. Epididymal explants were pre-incubated for various lengths of time (0, 24 and 48hrs), followed by the standard lipolysis incubation with and without IP (10uM, 4hrs incubation, n=3 rats, treatments in triplicate, see Figure 4.4). There was no interaction between the length of time for pre-incubation and the subsequent IP treatment (P=0.66, ANOVA). There was a significant stimulation of lipolysis by IP treatment (P=0.017, ANOVA). There was no significant effect of different pre-incubation time length (P=0.274, ANOVA), but a 24hrs pre-incubation appeared to be slightly better than 48hrs. However 22hrs of pre-incubation was slightly more convenient and was therefore used for subsequent studies that required pre-incubation.

Figure 4.4 Effect of Length of Pre-incubation Basal and IP stimulated Lipolysis in the Rat Epididymal Adipose Tissue Explants
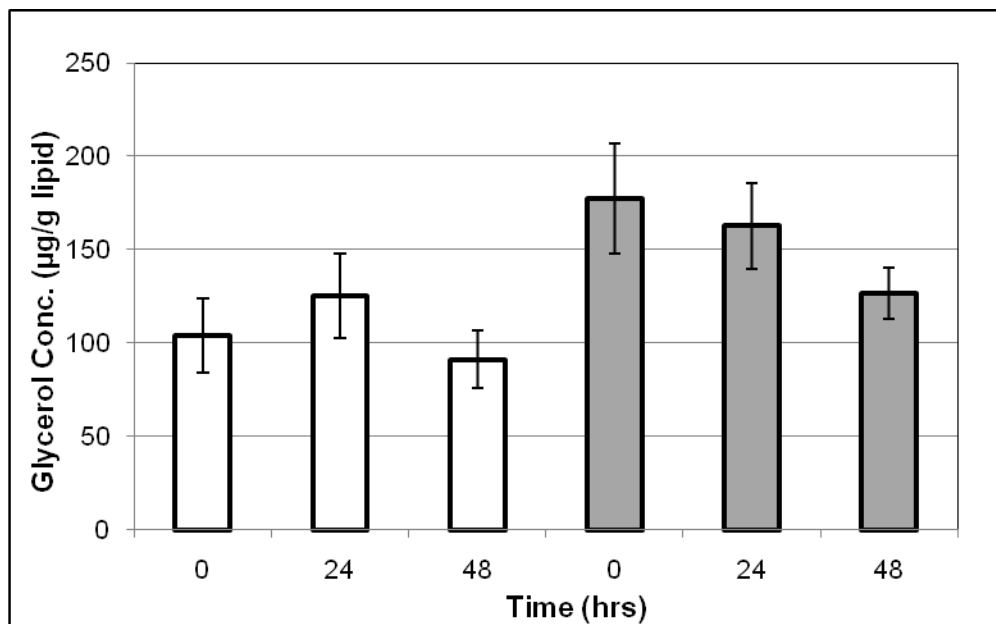
White box indicates basal (Control) and grey box indicates treatment with IP (10µM). Values are means± sem, n=3 rat, treatments in triplicate. Time X IP, P=0.66, ANOVA. IP significantly increased lipolysis when compared with basal (P=0.017, ANOVA)

4.4.2 Acute Effect of SCE in the Rat Epididymal Adipose Tissue Explants

After the experimental procedures with rat epididymal adipose tissue explants were optimized, SCE was tested in the system to investigate the direct effects on lipolysis. Figure 4.5 shows the effect of SCE (4hrs) treatment in rat epididymal fat explants without pre-incubation in the absence or presence of IP (n=2 rats, treatments in triplicate). There was a significant interaction between SCE and IP treatments ($P<0.001$, ANOVA), due to IP stimulating lipolysis and SCE decreasing lipolysis but only in the presence of IP.

4.4.3 Chronic Effect of SCE in the Rat Epididymal Adipose Tissue Explants

The chronic effects of SCE were also investigated. Figure 4.6 shows the effect of SCE during pre-incubation (22hrs) and lipolysis (4hrs) treatment on basal and IP stimulated lipolysis by rat epididymal fat explants (n=2 rats, treatments in triplicate). Once again, IP significantly increased lipolysis compared with basal ($P<0.001$, ANOVA), but there was no interaction between SCE and IP treatments ($P=0.529$, ANOVA) and there was no effect of SCE on lipolysis ($P=0.164$, ANOVA).

4.4.4 Chronic Effect of SCE in the Human Omental Adipose Tissue Explants

To further investigate direct effects of SCE on lipolysis, we also studied its effects in human omental adipose tissue explants. Figure 4.7 shows the effect of chronic treatment with SCE on basal and IP stimulated lipolysis (4hrs) (n=2 humans, treatments in triplicate). As in the rat explants, IP significantly increased lipolysis compared with basal ($P<0.001$, ANOVA), but there was no interaction between SCE and IP treatments ($P=0.693$, ANOVA) and no effect of SCE on lipolysis in the human omental adipose tissue explants ($P=0.126$).

Figure 4.5 Acute Effect of SCE in the Rat Epididymal Adipose Tissue Explants
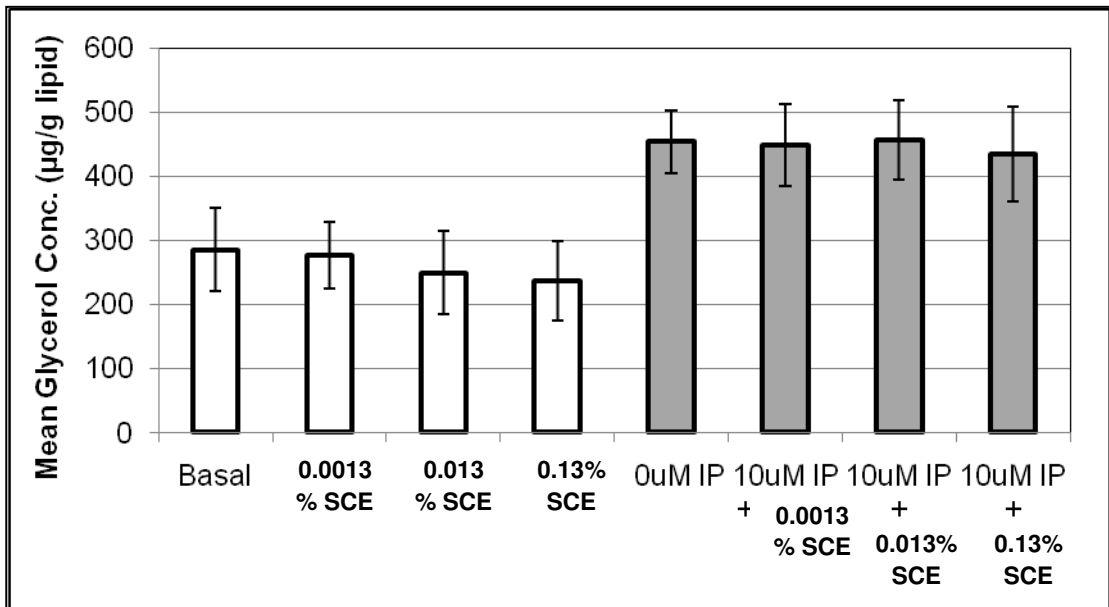
Values are means ±sem, n=2 rats, treatments in triplicate. SCE decreased lipolysis compared with control and IP (P<0.001, ANOVA). IP increased lipolysis compared with basal (P<0.001, ANOVA), SED 4.87.

Figure 4.6 Chronic Effect of SCE in the Epididymal Rat Adipose Tissue Explants
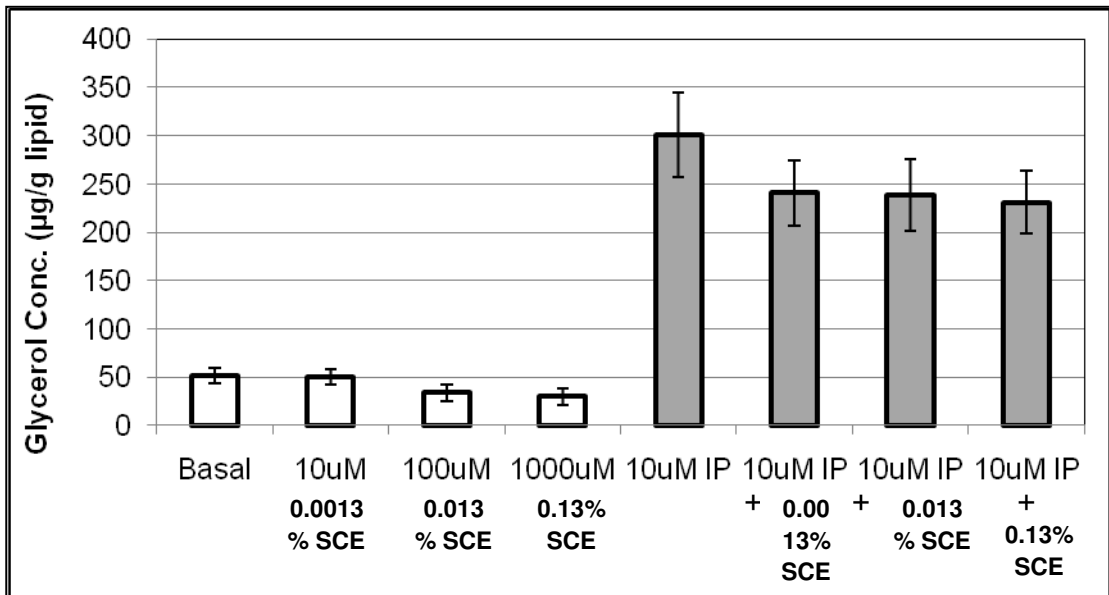
Values are means ±sem, n=2 rats, treatment in triplicate. SCE does not have any significant effect on lipolysis compared with IP (P=0.529, ANOVA). IP induced lipolysis compared with basal (P<0.001, ANOVA), SED 11.17

Figure 4.7 Chronic Effect of SCE in the Human Omental Adipose Tissue Explants
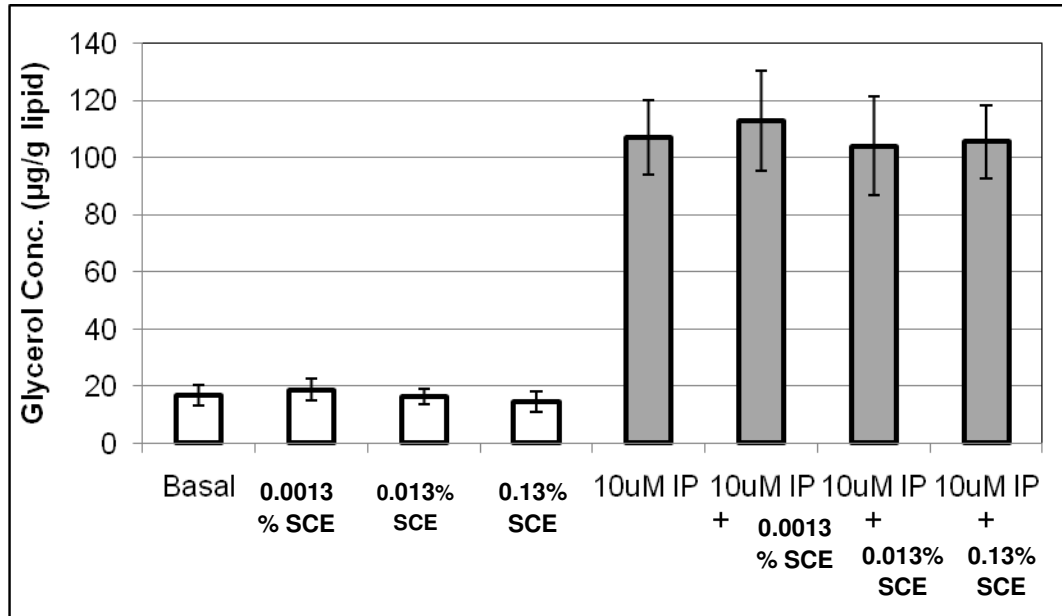
Values are means ±sem, n=2 humans, treatments in triplicate. SCE does not have any significant effect on lipolysis compared with IP (P=0.693, ANOVA). IP induced lipolysis compared with basal (P<0.001, ANOVA).

4.5 Discussion

Adipose tissue is very amenable to study *in vitro* (Vernon, 2000). The reason for developing the fat explant system instead of using the adipocyte or cell lines (which are extensively used) to study adipose tissue metabolism is because the fat explants system contains the whole array of adipose cell types ranging from immature to mature cells which are well known to discrete different types of hormones at different stages. Mature adipocytes are very fragile due to the large volume of lipid they contain, thus during the isolation process, they are verly likely to burst. Therefore, after isolation, the cell population being studied may predominantly be immature adipocytes. Another problem is the use of collagenase to yield free adipocytes which may also strip the adipocyte of the extracellular domains of membrane bound receptors. According to Vernon (2000), excessive enthusiasm with the collagenase digestion can also lead to a diminished responsiveness in adipocytes.

In these experiments, we have optimized the rat adipose tissue explants and human adipose tissue explants, based on the pig adipose tissue explants system developed by Headland (2007). When developing the rat adipose tissue explants, we had to take into consideration that the amount of fat available from a rat is a lot less than the fat available in the pig, so had to adjust the size of well and amount of fat (0.1 to 0.3g per well in a 12 well plate and maximum of 24 wells). It also appeared that the basal concentration and response to IP of adipose tissue depots in the rat and pig are different. The amount of human fat taken from an obese patient was similar to that available from rats. This is the reason for adopting the same method as the rat adipose tissue explants for the human adipose tissue explants.

4.5.1 Optimization of Rat Adipose Tissue Explants

The rat adipose tissue explants is set at the best parameters to maximize the potential of observing glycerol release in response to different treatments. To confirm this, we set out to measure the optimum number of hours of incubation in the KRB media for lipolysis, the optimum adipose tissue depot and the optimum number of hours of pre-incubation in the M199 media. We found that in the rat

adipose tissue explants, the glycerol is released into the media in a linear manner (see Figure 4.3). This is similar to pig adipose tissue explants (Headland, 2007). However, unlike in pigs, different adipose tissue depots and pre-incubation times did not seem to affect the amount of glycerol released into the media (see Figure 4.2 and 4.4). Hence, due to convenience and safety issues, a 22hrs pre-incubation followed by a 4hrs lipolysis incubation of epididymal fat explants was used for subsequent experiments.

4.5.2 SCE Regulation of Lipolysis in the Rat Epididymal Adipose Tissue Explants

To date there are no other studies investigating the *in vitro* effect of *S. crispus* extract. Results described in the previous chapter indicate that glycerol concentration was increased in the plasma of SCE treated mice. The amount of fat available in each adipose tissue depot in the mice is much too little to be studied. Thus, in this study we used adipose tissue explants from Sprague-Dawley rats, as rats are genetically similar to mice (Serikawa *et al.*, 1998). We hypothesized that SCE would have a direct stimulatory effect on lipolysis in the rat adipose tissue explants.

We investigated the glycerol release following both acute and chronic incubations with SCE. In the acute study, SCE inhibited lipolysis in the presence of IP (see Figure 4.5). However, there was no effect of SCE after chronic incubation (see Figure 4.6). These results show that SCE may have a direct, short-term inhibitory effect on lipolysis, but the effect is lost after a prolonged incubation.

4.5.3 SCE Regulation of Lipolysis in the Human Omental Adipose Tissue Explants

We also investigated the chronic effect of SCE in the human omental adipose tissue explants and found that there was no significant effect (see figure 4.7). This is consistent with the results found in the rat epididymal adipose tissue explants.

4.6 Conclusion

SCE does not appear to have any direct effect on lipolysis in the rat epididymal adipose tissue explants and human omental adipose tissue explants, other than the results following short-term incubation in the rat epididymal adipose tissue explants. This indicates that the effect of SCE on plasma glycerol concentrations found in the previous *in vivo* experiments might be due to indirect effects. As the finding is not of particular interest to obesity researchers (adipose tissue lipolysis was not increased) investigation on the SCE is now adjourned to further analyze the effect of individual polyphenols on the rat epididymal adipose tissue explants and human omental adipose tissue explants that we have developed.

Chapter 5 - *In vitro* Effects of Individual Polyphenols in the Rat Epididymal Adipose Tissue Explants

5.1 Introduction

Dietary polyphenols are the most abundant antioxidants in human diets. They represent a group of secondary metabolites which widely occur in vegetables, fruits, tea, extra virgin olive oil, chocolate and other cocoa products. Consumption of polyphenols in foodstuffs and drinks has been associated with the prevention of some diseases (Rawel & Kulling, 2007; Han *et al.*, 2007). Food and drinks containing high concentrations of dietary polyphenols such as green tea, *S. matsudana* leaves, liquorice and apple have been linked with anti-obesogenic effects (Wolfram *et al.*, 2006; Lin and Lin-Shiau, 2006; Han *et al.*, 2003 (a); Aoki *et al.*, 2007; Nakazato *et al.*, 2006).

A number of studies have been carried out to investigate the anti-obesity effects of individual polyphenols, including apigenin and luteolin (Han *et al.*, 2003b), myrisetin and quercetin (Kwon *et al.*, 2007), epigallocatechin gallate (EGCG) (Wolfram *et al.*, 2006) and kaempferol (Yu *et al.*, 2006). All the published work investigating effects of individual polyphenols on lipolysis are mainly carried out *in vivo* or using isolated adipocytes or cell lines except for the study by Headland and colleagues (2007) where pig adipose tissue explants used to test the effects of individual polyphenols (quercetin, phloridzin, resveratrol, EGCG and gallic acid). However, there appear to be no studies that compared similar effects in the rat adipose tissue explant. The molecular structures of individual polyphenols, IP and Adenosine is shown in Figure 5.9 and 5.10.

5.2 Hypothesis and Aim

The hypothesis of this study is that the effects of individual polyphenols on rat adipose tissue explants are similar to that found in the pig adipose tissue explants and other studies using rat adipocytes or cell lines. We aim to investigate the effects of a range of polyphenols on lipolysis using the rat adipose tissue explants.

5.3 Methodology

5.3.1 Preparation of Individual Polyphenols

Individual polyphenols (quercetin, phloridzin, resveratrol and EGCG) were all obtained from Sigma (UK) and verbascoside was obtained from Extrasynthese (France). Polyphenols in the form of powder were weighed and diluted with PBS to obtain the required concentration.

5.3.2 Rat Epididymal Adipose Tissue Explants

Methods used for the preparation and culture of rat fat explants are described in section 4.2. Samples were obtained as described in section 4.2.1.1.

5.3.3 Statistical Analysis

Values for glycerol release (μg per gram lipid) were expressed as means ± SEM. The data were analyzed using Genstat 10.0 statistical software. General Analysis of Variance was used with No Blocking. Significance was taken as $p < 0.05$.

5.4 Results

5.4.1 Acute Effects of Individual Polyphenols on Lipolysis in the Rat Epididymal Adipose Tissue Explants

To investigate the acute lipolytic effects of individual polyphenols, rat epididymal adipose tissue explants were pre-incubated in M199 media for 22hrs before being treated with EGCG, Phloridzin, Quercetin, Resveratrol or Verbascoside for 4hrs in the presence or absence of isoproterenol (IP) in KRB Media. As expected, treatment with IP significantly increased lipolysis in all studies ($P < 0.001$), but the effects of individual polyphenols varied.

Figure 5.1 shows an inhibitory effect of EGCG on lipolysis, particularly when IP was added (n=2 rats, treatments in triplicate). The significant interaction between EGCG and IP ($P = 0.005$) indicated that the inhibitory effect of EGCG was much more pronounced when IP was added compared with when it was absent.

Figure 5.1 Acute (4hr) Effects of EGCG on Lipolysis in the Rat Epididymal Adipose Tissue Explants
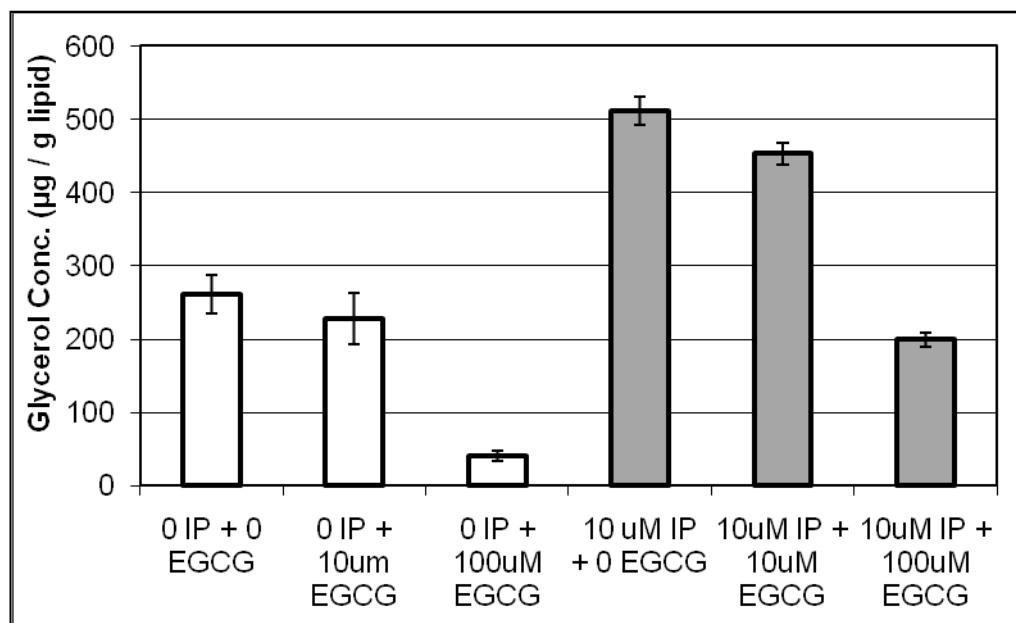
Values are means ±sem, n=2 rats, treatments in triplicate. IP increased lipolysis compared with basal ($P < 0.001$, ANOVA) whereas EGCG inhibited lipolysis particularly when IP was present ($P = 0.005$ for EGCG X IP interaction).

In another study (n=2 rats, treatments in triplicate), Phloridzin tended to inhibit lipolysis, both with and without IP (P=0.055). This can be seen in Figure 5.2.

Figure 5.2 Acute (4hr) Effects of Phloridzin on Lipolysis in the Rat Epididymal Adipose Tissue Explants
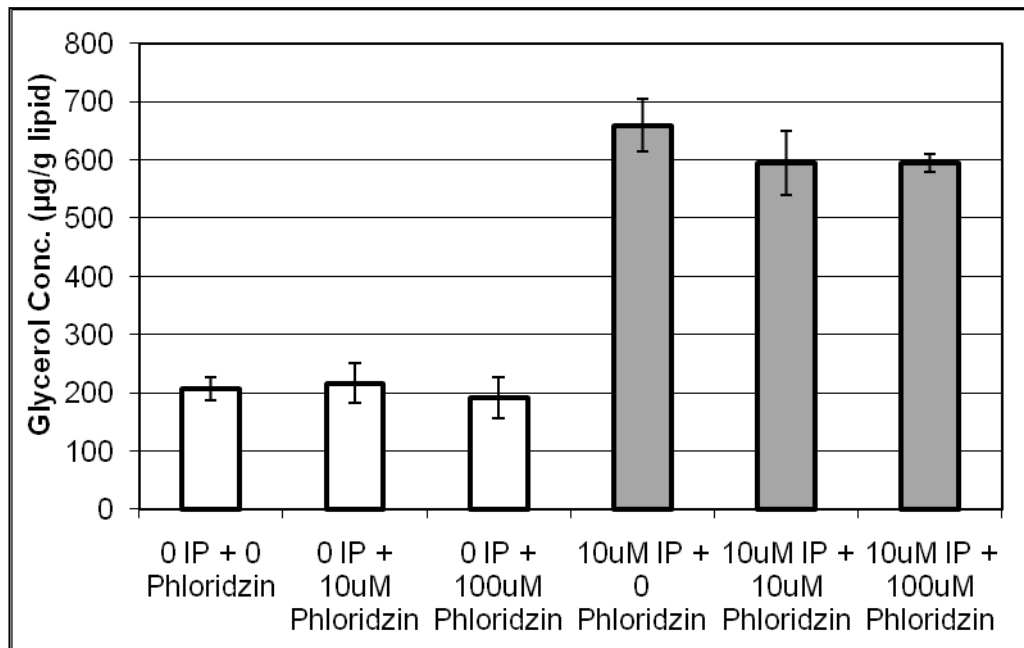
Values are means ±sem, n=2 rats, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) and Phloridzin tended to inhibit lipolysis, both with and without IP (P=0.055 for Phloridzin X IP interaction, ANOVA).

Acute treatment with Quercetin (n=2 rats, treatments in triplicate) had no significant effect on lipolysis, when IP was either added or not (P=0.773 for Quercetin and P=0.13 for Quercetin X IP interaction, ANOVA), although it appeared to result in a numerical increase in lipolysis in the absence of IP.

Figure 5.3 Acute (4hr) Effects of Quercetin on Lipolysis in the Rat Epididymal Adipose Tissue Explants
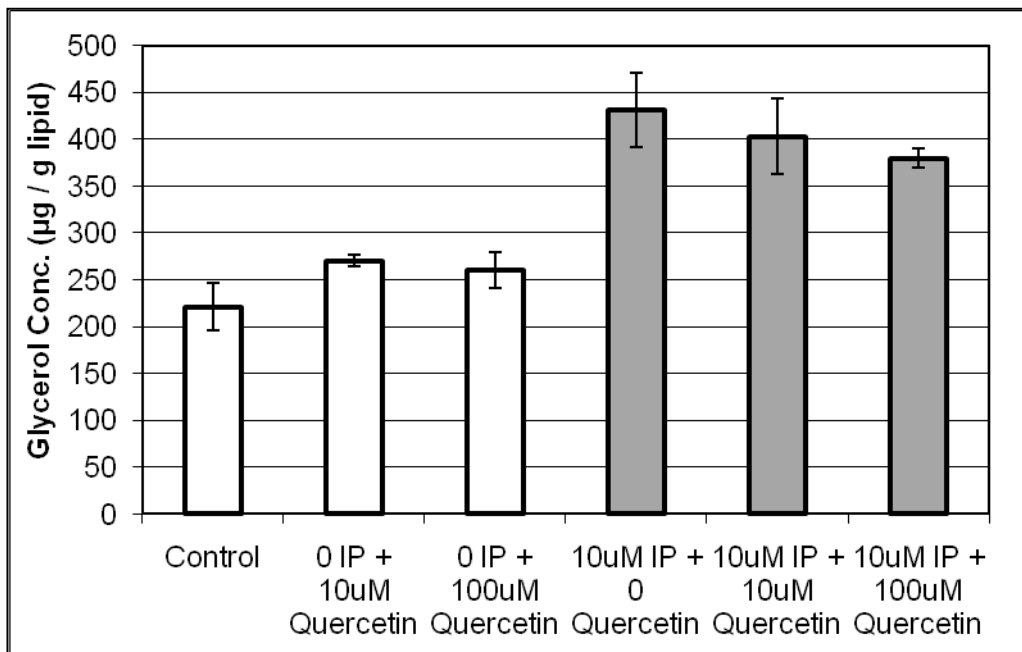
Values are means ±sem, n=2 rats, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA). Quercetin does not have significant effect on lipolysis both with and without IP (P=0.773 for Quercetin and P=0.13 for Quercetin X IP interaction, ANOVA) SED 18.98.

Interestingly, acute treatment with Resveratrol (n=2 rats, treatments in triplicate) had opposite effects on lipolysis, depending upon whether IP was added or not (P=0.013 for Resveratrol x IP interaction). Resveratrol alone (without IP) increased lipolysis, particularly at 100µM, but the effect was reversed when IP was added, with 100µM Resveratrol clearly inhibiting lipolysis.

Verbascoside (n=2 rats, treatments in triplicate) had a similar effect to EGCG, significantly inhibitinglipolysis, particularly when IP was added (P<0.001 for Verbascoside x IP interaction).

Figure 5.4 Acute (4hr) Effects of Resveratrol on Lipolysis in the Rat Epididymal Adipose Tissue Explant
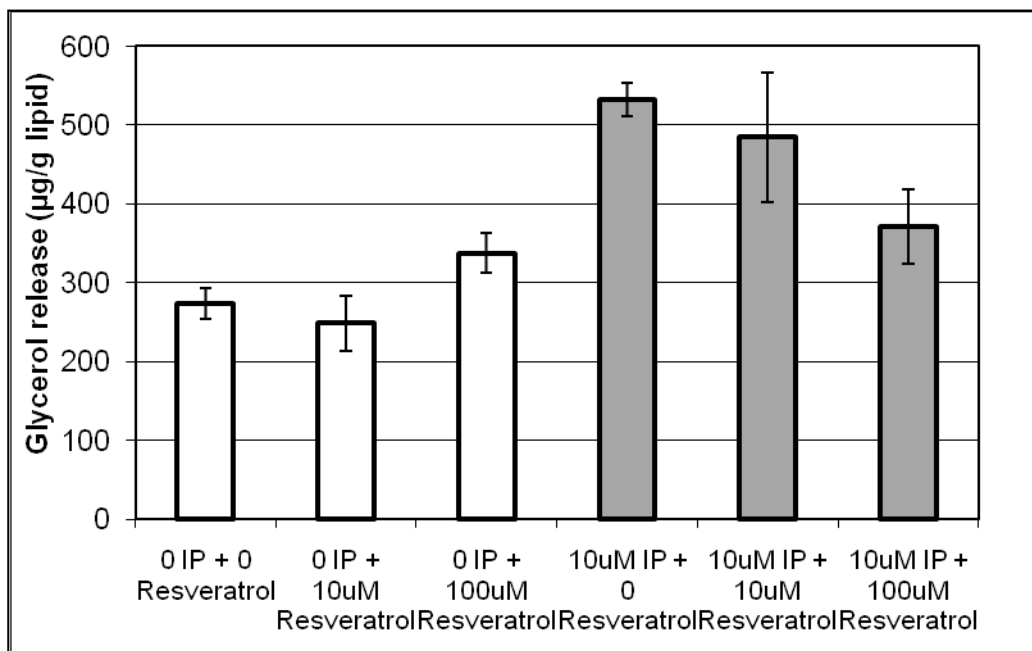
Values are means ±sem, n=2 rats, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) while Resveratrol increased lipolysis during the basal state but decreased it when IP was added (P=0.013 for Resveratrol X IP interaction, ANOVA) SED 31.3.

Figure 5.5 Acute (4hr) Effects of Verbascoside on Lipolysis in the Rat Epididymal Adipose Tissue Explants
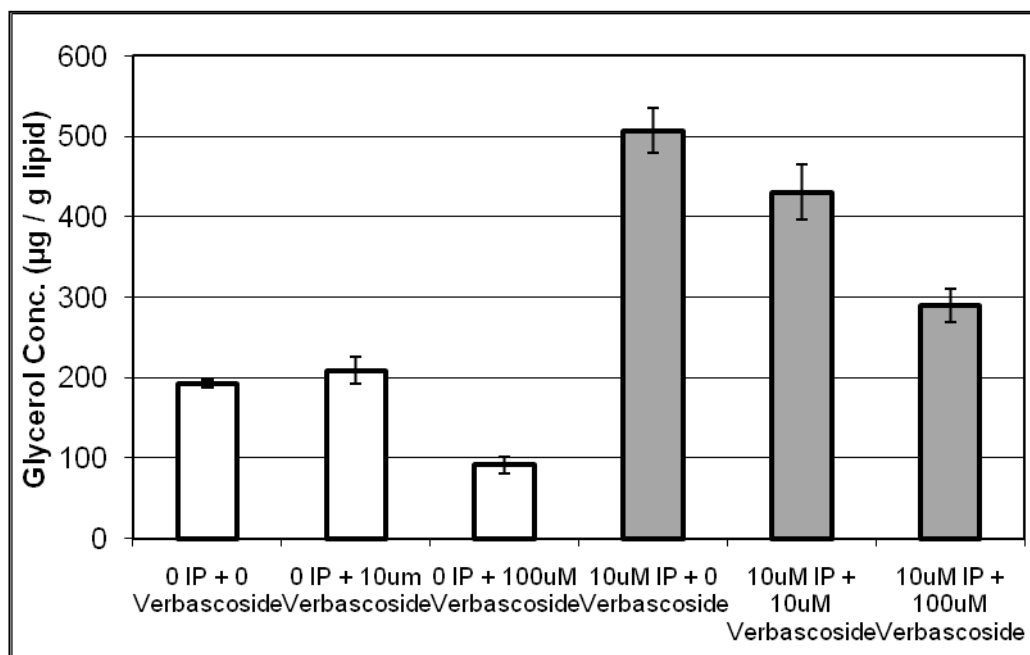
Values are means ±sem, n=2 rats, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) whereas verbascoside inhibited lipolysis particularly when IP was added (P<0.001, ANOVA), SED 8.35.

In order to further investigate the effects of individual polyphenols on lipolysis, longer incubation times (chronic effects) were used. EGCG, Phloridzin and Resveratrol were chosen because of their different acute effects on lipolysis.

5.4.2 Chronic Effects of Individual Polyphenols in the Rat Epididymal Adipose Tissue Explants

A 26hr chronic incubation of RES with EGCG, Phloridzin and Resveratrol was achieved by pre-incubating for 22hrs in the M199 media containing each polyphenol, followed by 4hrs in the KRB media. This was 22hrs longer exposure compared with the acute incubation studies described earlier. In all chronic studies, treatment with IP (for 4hrs again) significantly increased lipolysis (P<0.001). Figure 5.6 shows the chronic effects of EGCG (n=2 rats, treatments

in triplicate) which were similar to the acute effects, with EGCG significantly inhibiting lipolysis (P<0.001), but this time there was no significant interaction between EGCG and IP (i0.739). The greatest inhibitory effect was observed with 100µM EGCG.

Figure 5.6 Chronic (26hr) Effects of EGCG on Lipolysis in the Rat Epididymal Adipose Tissue Explant
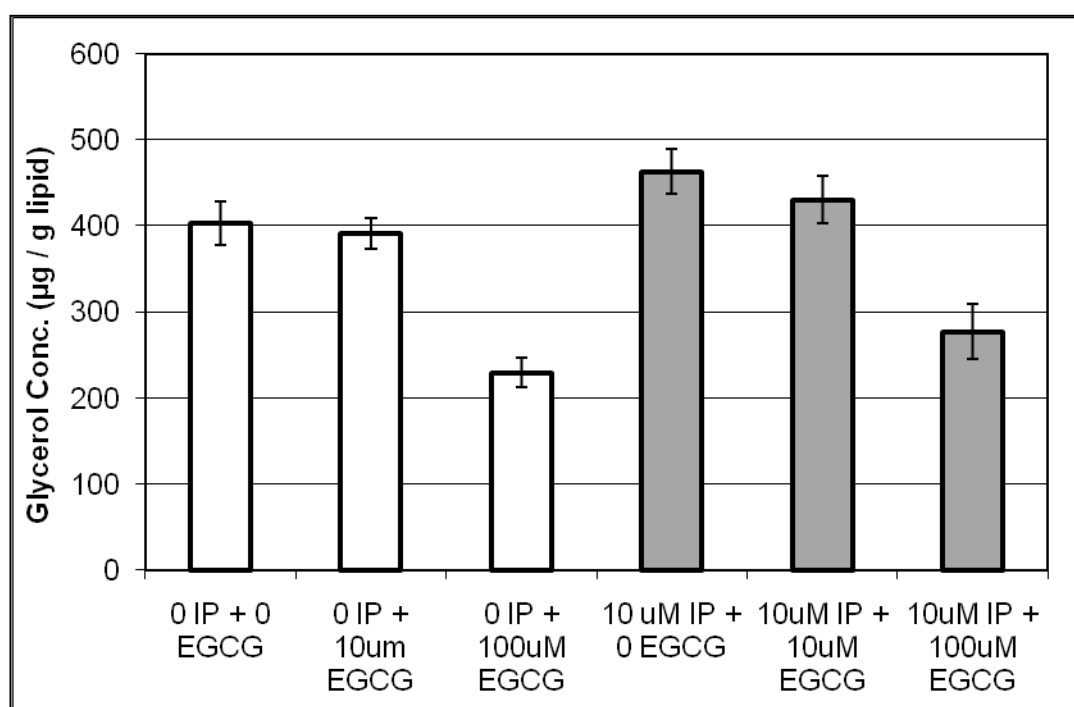
Values are means ±sem, n=2 rats, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) while EGCG significantly inhibited lipolysis, both with and without IP (P<0.001, ANOVA).

Unlike in the acute study where Phloridzin tended to inhibit lipolysis (Figure 5.2), chronic incubation with Phloridzin (n=2 rats, treatments in triplicates) had no significant effect (Figure 5.7, P=0.3 and PxIP=0.785).

Resveratrol, which increased lipolysis at the highest EGCG concentration after acute incubation in the absence of IP, had an inhibitory effect (P<0.001) on lipolysis following chronic incubation (Figure 5.8), both with and without IP, with 100µM inducing the greatest effect.

Figure 5.7 Chronic (26hr) Effects of Phloridzin on Lipolysis in the Rat Epididymal Adipose Tissue Explant
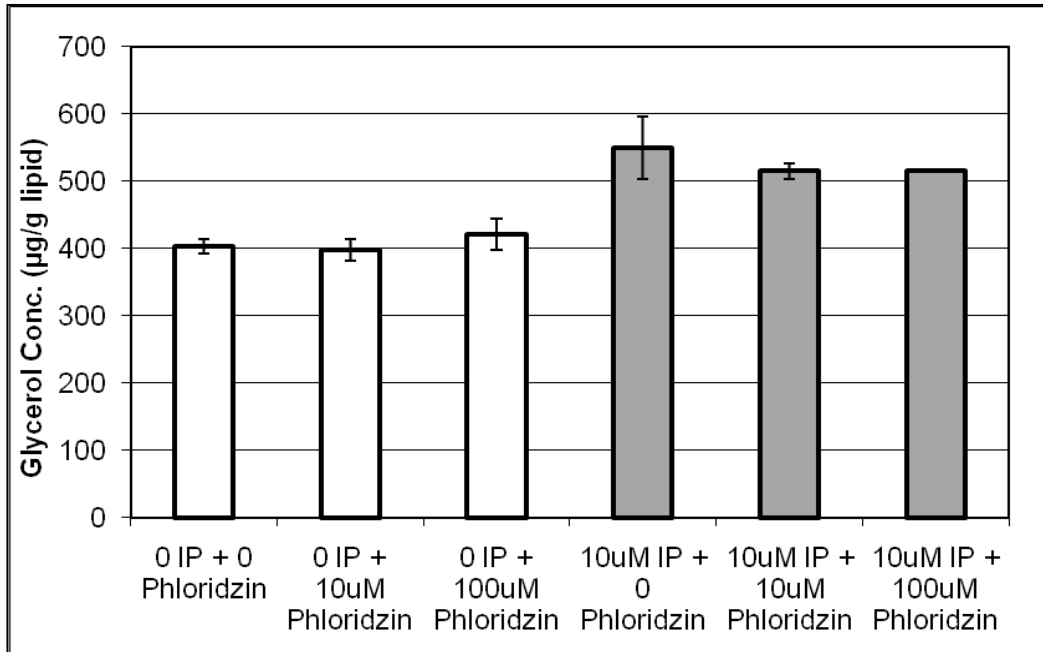
Values are means ±sem, n=2 rats, treatments in triplicate. Phloridzin does not have any significant effect on lipolysis (P=0.3, ANOVA). IP increased lipolysis compared with basal (P<0.001, ANOVA), SED 16.62.

Figure 5.8 Chronic (26hr) Effects of Resveratrol on Lipolysis in the Rat Epididymal Adipose Tissue Explant
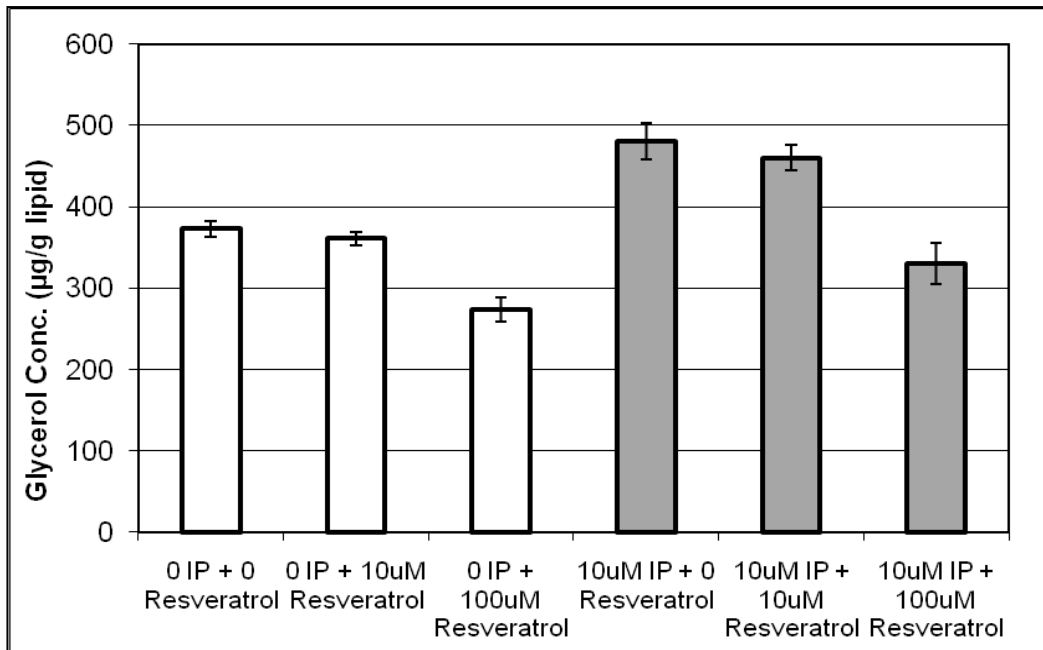
Values are means ±sem, n=2 rats, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) whereas Resveratrol inhibits lipolysis both with and without IP (P<0.001, ANOVA), SED 9.5.

Figure 5.9 Polyphenol Structures
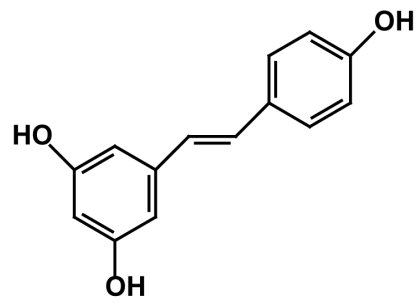
Resveratrol
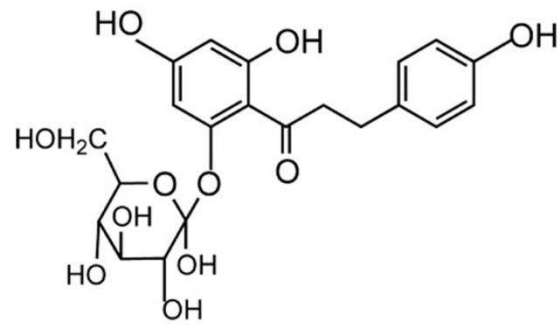
Phloridzin
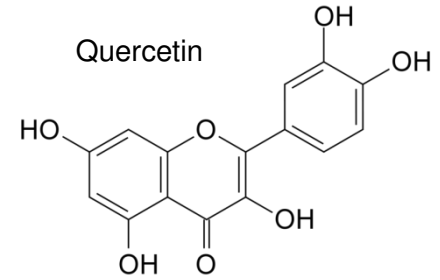
Quercetin
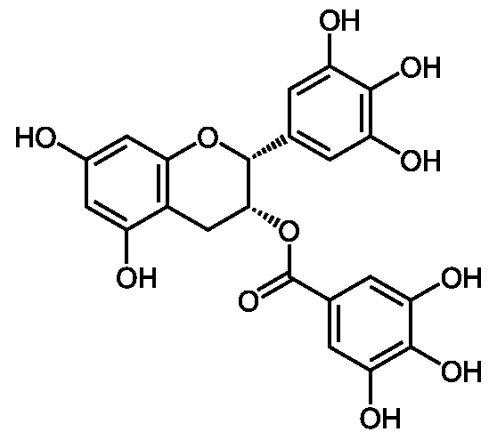
EGCG
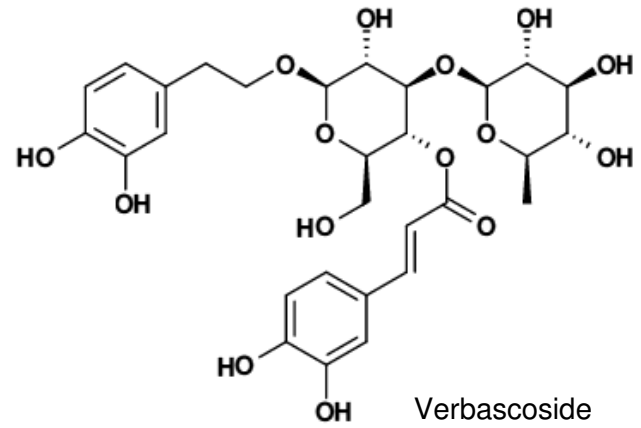
Verbascoside

Figure 5.10 Isoproterenol and Adenosine Structures
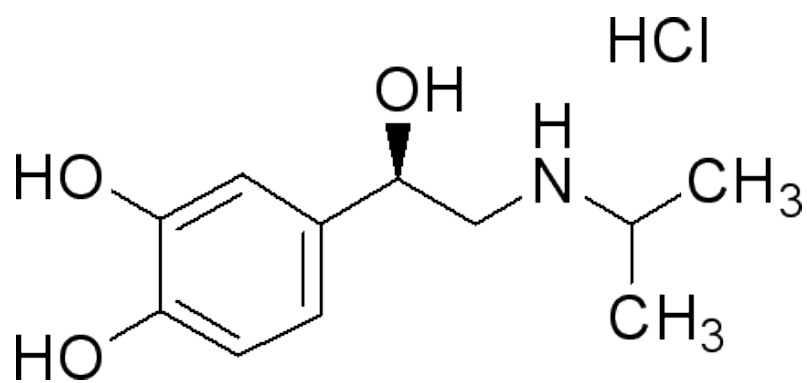
Isoproterenol
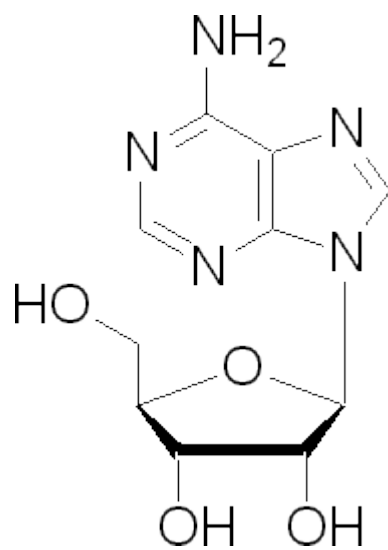
Adenosine

5.5 Discussion

5.5.1 Effects of EGCG on Lipolysis in the Rat Epididymal Adipose Tissue Explants

EGCG is a major polyphenolic compound found in green tea which is produced from dried fresh leaves of the plant *Camellia sinensis* L. Ktze. (Bettuzzi *et al.*, 2006; Demeule *et al.*, 2002). The molecular structure of EGCG is shown in Figure 5.9. Other catechins found in lower abundance in green tea include (-)-epicatechin-3-gallate (ECG), (-)epigallocatechin (EGC), (-)-epicatechin (EC) and (+)-catechin. However, EGCG is the main catechin in green tea which is thought to be responsible for many of the anti-obesity effects observed *in vivo* and *in vitro* (Nagle *et al.*, 2006; Wolfram *et al.*, 2006b). However, all of the *in vitro* studies investigating direct effects of EGCG on lipolysis were done in isolated adipocytes.

In this study, we investigated the direct effect of EGCG on lipolysis in the rat adipose tissue explant during acute (4hr) and chronic (26hr) incubation. In all experiments, EGCG was found to consistently inhibit lipolysis and the effects were greatest at 100µM. More than 50% inhibition was found following the acute (4hr) incubation compared with approximately 50% following the chronic (26hr) incubation. Comparisons of results with other fat explant studies is only possible for pig adipose tissue explants (Headland, 2007) but we have to note that all studies done in the pig adipose tissue explants has been done in the presence of adenosine aminase (ADA), an enzyme that remove adenosine in the explants and thus increase basal lipolysis.

Since no other studies appear to have been done using adipose tissue explants, studies using isolated rat adipocytes and cultured adipocytes (3T3-L1 and C3H10T1/2) will also be included as a comparison. Interestingly, studies done in adipocytes have come to different conclusions on the effect of EGCG on lipolysis, with EGCG found to inhibit (Kimura *et al.*, 1983 a&b; Wolfram *et al.*, 2006b), increase (Lee *et al.*, 2009) or have no effect at all (Mochizuki & Hasegawa, 2004). The findings of these studies are compared with below.

EGCG has previously been shown to inhibit lipolysis in pig fat explants after a chronic (24hr) incubation in both perirenal and subcutaneous fat explants, particularly at 100µM (Headland, 2007). Studies by Kimura and colleagues (1983

a&b) and Ying Wang and colleagues (Wolfram *et al.*, 2006a) in adipocytes support the findings in our study. In the study by Kimura and colleagues (1983a), isolated adipocytes from the epididymal adipose tissue of young male Wistar-King strain rats were treated with 0.5µg/ml adrenaline (as positive control) or pure EGCG (5, 20 and 100µg/ml equal to 10.91, 43.63 and 218.15µM) for 2 hours (acute incubation). Results were then compared with control (0.5µg/ml adrenaline). No effects were found after treatment with 5 and 20µg/ml EGCG, but 100µg/ml significantly inhibited (include p-value) lipolysis by approximately 50%.

A similar study was carried out by Kimura and colleagues (1983b), but this time using adrenocorticotropic hormone (ACTH) as the positive control instead of adrenaline. Once again, 100µg/ml EGCG significantly inhibited lipolysis, but this time by approximately 20%.

Ying Wang and colleagues (Wolfram *et al.*, 2006a) also demonstrated inhibition of lipolysis in differentiated C3H10T1/2 preadipocyte cultures, after 5 (acute) and 24hrs (chronic) incubation with 50µM EGCG.

In contrast, Lee and colleagues (2009) found that 3T3-L1 adipocytes treated with 10µM EGCG for 24hrs (chronic incubation) significantly increased lipolysis by 27% ($P < 0.05$). However, no effect was observed by Mochizuki and Hasegawa (2004) when 3T3-L1 adipocytes were treated with higher concentrations of EGCG (21.8 and 43.6µM) for 4 hrs (acute incubation). Hence there are inconsistent effects of EGCG described in the literature, particularly when comparing isolated primary adipocytes, differentiated 3T3-L1 adipocytes and cultured fat explants. Why this should be isn't clear.

Thus, the results observed here in fat explants would suggest that the mechanism for the reduction of white adipose tissue (WAT) observed in *in vivo* studies (Bose *et al*, 2008; Huang *et al*., 2009; Klaus *et al*., 2005; Wolfram *et al*., 2005) was not mediated via a direct increase in lipid mobilization. Instead, the reduction could be mediated via induction of adipocyte cell apoptosis as suggested by Wolfram and colleagues (2005) and Hwang and colleagues (2005).

5.5.2 Effects of Phloridzin on Lipolysis in the Rat Epididymal Adipose Tissue Explants

Phloridzin (phloretin 2'-O-glucose) is the glucosidic form of the flavonoid, phloretin. Phloretin is found exclusively in apples and in apple-derived products (Crespy *et al.*, 2002); its concentration is highly dependent on the variety of apple (Escarpa & Gonzalez, 1998). The molecular structure of Phloridzin is presented in Figure 5.9. It is sometimes referred to as phlorizin, phlorrhizin, phlorhizin or phlorizoside (Gosch *et al.*, 2010). Since its discovery in 1835, phloridzin has been used for a variety of purposes in experimental medicine and biology. Most studies relate to diabetes, obesity, stress hyperglycemia, antioxidative activity, membrane permeability, beverages, food additives, pharmaceuticals and cosmetics (Ehrenkranz, 2005 & 2006; Gaudout *et al.*, 2006; McCrimmon *et al.*, 2002; Rezk *et al.*, 2002; Sukhorukov *et al.*, 2001 and Valenta *et al.*, 2001).

Two previous studies investigated the effects of Phloridzin on lipolysis (Headland, 2007 and Kuppusamy & Das, 1992). Headland (2007) studied the effect of a chronic 24hr incubation of Phloridzin in pig fat explants from two different adipose tissue depots. In the subcutaneous adipose tissue, Phloridzin was found to increase lipolysis at 25µM but had no significant effect at 100µM. In contrast, although it appeared to increase lipolysis numerically, Phloridzin had no statistically significant effect on lipolysis in the perirenal adipose tissue. The results in the pig perirenal adipose tissue explants agree with what we have found in the rat epididymal adipose tissue, following both acute and chronic incubation with Phloridzin. In another study, Kuppusamy and Das (1992) investigated the effect of an acute (10mins) incubation of isolated adipocytes with 250µM of Phloridzin and found that there were no significant effects. This result also supports our findings. Despite appearing to have an inconsistent effect in pig subcutaneous fat explants and no effect in pig perirenal fat explants, rat epididymal fat explants and isolated rat adipocytes, Ehrenkraz and colleagues (2005) described an anti-obesity effect of Phloridzin. The findings from *in vitro* studies simply indicate that the mechanism of action for Phloridzin is not via a direct effect on lipolysis in the WAT.

5.5.3 Effects of Quercetin on Lipolysis in the Rat Epididymal Adipose Tissue Explants

Quercetin is one of the most abundant flavonol-type flavonoids present in several components of the human diet (Bravo, 1998). In the acute (4hr) incubation in rat adipose tissue explant, there was no significant effect of quercetin when IP was added, but it appeared to numerically increase lipolysis in the absence of IP, but this was not statistically significant (P=0.773). Using adipocytes isolated from epididymal adipose tissue of male Wistar rats Kuppusamy and Das (1992) found that treatment with 250µM quercetin and epinephrine for 10 mins (acute effect) increased lipolysis by nearly 198% when compared with control. Kuppusamy and Das (1994) suggest that quercetin has some structural feature which leads to it having a synergistic effect on lipolysis with epinephrine. In a subsequent study (Kuppusamy and Das, 1993), they suggest that quercetin may be acting as a β-adrenoceptor agonist, thereby increasing lipolysis through the stimulation of cAMP.

However, chronic (24hr) incubation with Quercetin shows different results found in the pig adipose tissue explants and cultured adipocyte cell lines. In the study by Headland (2007), quercetin especially at 100µM was found to significantly inhibit lipolysis both in the subcutaneous and perirenal pig adipose tissue explant after a chronic incubation (24hr). In another chronic incubation (24, 48 and 72hr) with 100µM Quercetin, Hsu and Yen (2006) found that Quercetin potently induced cell apoptosis in 3T3-L1 pre-adipocytes and effect was increased with longer incubation time. Quercetin was also found to have the highest inhibition of cell population growth when compared with Naringenin, Rutin, Hesperidin, Resveratrol and Naringin.

A consistent inhibition affect was observed after chronic (≥24hr) incubation of cultured 3T3L-1 cells and pig adipose tissue explants compared with acute (10min and 4hr) incubation. Thus, it is doubted whether quercetin may be acting as a β-adrenoceptor agonist as suggested by Kuppusamy and Das (1994) or whether they are acting as a β-adrenoceptor antagonist instead.

5.5.4 Effects of Resveratrol on Lipolysis in the Rat Epididymal Adipose Tissue Explants

Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring polyphenol found in red wine, grape juice and the root of *Polygonum cuspidatum* (Siemen and Creasy, 1992; Nonomura *et al.*, 1963). Moreover, the pure compound is now available in tablets and is suggested as a dietary supplement (Szkudelska *et al.*, 2009a). In our studies, acute (4hr) incubation with Resveratrol was found to increase lipolysis in the absence of IP, but to inhibit lipolysis in the presence of IP. However, chronic (26hrs) incubation with Resveratrol resulted in a consistent inhibition of lipolysis both in the absence and presence of IP. Different results were found in pig adipose tissue explant by Headland (2007). Although 24hr treatment with 10, 50 or 100μM Resveratrol numerically increased lipolysis in both subcutaneous and perirenal adipose tissue explants, the differences were not statistically significant.

The observed effects in rat adipose tissue explant agree with those of Skudelska and colleagues (2009a), who showed that 10 and 100μM resveratrol significantly enhanced lipolysis (approximately 900nmol/10^6 cells/90min at 10μM and 1200nmol/10^6 cells/90min at 100μM) in isolated rat adipocytes incubated for 90mins with epinephrine. Incubations of adipocytes for 30min with 0.5μM epinephrine and 1, 10 and 100μM Resveratrol also resulted in enhanced accumulation of cAMP compared with cells exposed to epinephrine alone. The increase in cAMP found in adipocytes incubated with resveratrol is proposed to be responsible for enhanced lipolytic rate (Skudelska *et al.*, 2009a).

However, in another study where a lower concentration of Resveratrol (25μM) was used, no significant effect was found on lipolysis in 3T3-L1 adipocytes treated for 24 and 48hrs chronic incubation (Rayalam *et al.*, 2007). Moreover, 100μM Resveratrol was also found to significantly increase adipocyte apoptosis by 76% and decreased lipid accumulation in maturing pre-adipocytes by 43% after a 48hrs chronic incubation (Rayalam *et al.*, 2008a).

Thus, these results suggest that the effects of Resveratrol on lipolysis are inconsistent, particularly if chronic (≥24hr) incubation with high concentration (250μM) was followed. Instead, an acute incubation with a medium concentration (100μM) gives more consistent results on lipolysis.

5.5.5 Effects of Verbascoside on Lipolysis in the Rat Epididymal Adipose Tissue Explants

Verbascoside is a polyphenol found in the *S. crispus* extract (Abou Muamer *et al.*, 2003, unpublished). Even though we found that direct incubation of rat fat explants with SCE had no significant effect on lipolysis (see previous chapter), we set out to investigate whether an individual active component of SCE had any effect. Interestingly, after an acute (4hr) incubation, verbascoside was found to significantly inhibit lipolysis, but there appear to be no published studies with which to compare. Our theory is that verbascoside might inhibit lipolysis through the same mechanism as EGCG since the total of hydroxyl group in the verbascoside structure is nine, just one less than eight in the EGCG structure.

5.6 Conclusion

These studies investigated the effects of various individual polyphenols on basal and IP stimulated lipolysis in rat adipose tissue explant and then compared the findings with similar studies in our lab using pig adipose tissue explants where individual polyphenols were incubated with ADA (Headland, 2007), as well as published studies using isolated rat adipocytes and cultured adipocyte cell lines (3T3-L1 and C3H10T1/2).

Interestingly, there was both consistency and inconsistency dependent upon the polyphenol being studied and the length of incubation. Hence, the effects of an acute (<4hrs) incubation with EGCG, Phloridzin or Resveratrol on lipolysis in rat adipose tissue explant are similar to those observed in isolated rat adipocytes subjected to the same polyphenols and length of incubation. Conversely, the effects observed after an acute incubation with EGCG or chronic incubation with EGCG or Resveratrol on lipolysis in rat adipose tissue explant are completely different to those observed in cultured adipocyte cell lines.

Thus, these findings are contrary to the hypothesis as our rat adipose tissue explant system shows similar responses to individual polyphenols as isolated adipocyte, but different to cultured adipocyte cell lines and the pig adipose tissue explants. Adipose tissue explants and isolated adipocytes will contain adipocytes at more mature stages of development than the cultured adipocyte cell lines, which may then account for some of the apparent differences in lipolytic responsiveness.

It is worth noting that the effects of the individual polyphenols in the rat fat explants appear to show some structure-activity relationship. The polyphenols (EGCG and Verbascoside) with the highest numbers of hydroxyl groups on the benzene rings (8 or 9) were found to inhibit lipolysis, while polyphenols with 5 to 7 hydroxyl groups (Phloridzin and Quercetin) had no significant effect and the polyphenol with just 3 hydroxyl groups (Resveratrol) increased lipolysis, although the effect was only observed in the acute (4hr) study. Although the main focus of these studies was the effect of individual polyphenols on adipose tissue lipolysis, we should not ignore their effects on adipogenesis and lipid accumulation (lipogenesis) as these effects may also be important in treating obesity. Further studies should compare the different lipolytic effects across species, especially to compare with human adipose tissue/ cells.

Chapter 6 - *In vitro* Effects of Individual Polyphenols in the Human Omental Adipose Tissue Explants

6.1 Introduction

In humans, fat mobilization is regulated by various mechanisms. It is acutely stimulated by catecholamines (epinephrine and norepinephrine) and natriuretic peptides. Human fat cells express β_1 β_2 and α_2-adrenergic receptors (Lafontan M., 2008). Acting through binding to β_1- or β_2 adrenoreceptors, catecholamines stimulate adenylate cyclase and promote cAMP production from ATP (see Figure 6.1). Conversely, selective stimulation of fat cell α_1-adrenergic receptors inhibits lipolysis as activation of the Gi-coupled receptors leads to a decrease in intercellular cAMP concentration (Lafontan M., 2008; Langin D., 2006). The activation of Gi protein is the best-known mechanism that mediates lipolytic activation (Carmen & Victor, 2006). Several other antilipolytic pathways including α_2-adenosine-receptors, EP3-prostaglandin, E2 receptors and neuropeptide Y/peptide YY (NPY-1) receptors have all been identified in human fat cells (Lafontan, 2008). These receptors are also coupled to adenylate cyclase through Gi inhibitory GTP-binding proteins, and their stimulation reduces cAMP concentrations and inhibits lipolysis.

A possible explanation for the existence of two opposite mechanisms that affect lipolysis in the same cell may be that, in this way, the α_2 receptors exert a permanent basal inhibition of lipolysis (Lafontan *et al.*, 1997). Adenosine is another lipolysis inhibitor that has a Gi protein coupled receptor in the adipocyte (Schwabe *et al.*, 1975). Adenosine is released from adipose tissue and acts on four G protein-coupled receptors: the adenosine α_1 receptor, the adenosine α_{2A} receptor, the adenosine α_{2B} receptor and the adenosine α_3 receptor (Fredholm *et al.*, 2001 a&b). There is pharmacological evidence that the receptor responsible for the antilipolytic effect is the adenosine α_2 receptor (Fatholahi *et al*, 2006; Schoelch *et al.*, 2004; Xu *et al.*, 1998) and basal concentrations of endogenous adenosine are sufficient to cause inhibition of lipolysis (Lönnroth *et al.*, 1989). However, lipolysis can also be maximally stimulated by lowering media

adenosine concentrations with ADA (Frühbeck *et al.*, 2001). An activation of lipolysis may also take place through TNFα mediated decrease of Gi concentration coupled to adenosine receptor which blocks the continuous lipolysis inhibition signal (Gasic *et al.*, 1999).

6.2 Hypothesis and Aim

Since the development of adipose tissue cells of humans are more similar to the pig than to the rat, in this experiment, the hypothesis is that the human adipose tissue explants will have similar responses towards individual polyphenols as found in the pig adipose tissue explants (Headland K., 2007). We aim to treat the human adipose tissue explants with individual polyphenols in the presence and absence of IP and ADA and later compare the results across human, rat and pig.
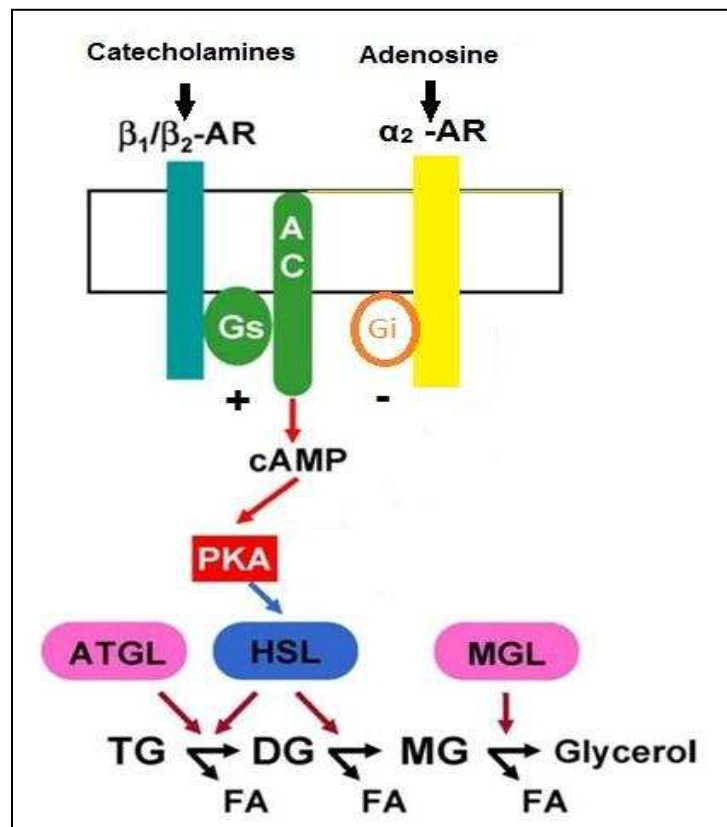
Figure 6.1 Model for stimulatory pathways in human adipose tissue lipolysis. AC, adenylyl cyclase; AR, adrenoreceptor; ATGL, adipose triglyceride lipase; DG, diglyceride; FA, fatty acid; Gs, stimulatory GTP-binding protein; Gi, inhibiting GTP-binding protein; HSL, hormone-sensitive lipase; MG, monoglyceride; MGL; monoglyceride lipase; PKA, protein kinase A; TG, triglyceride (Figure modified from Langin D., 2006)

6.3 Methodology

6.3.1 Preparation of Individual Polyphenols

Individual polyphenols: phloridzin, resveratrol and EGCG were all obtained from Sigma (UK). Polyphenols in the form of powder were weighed and diluted with PBS to obtain the concentration needed.

6.3.2 Human Omental Adipose Tissue Explants

Methods used for isolation and culture of explants in these experiments were explained in detail in section 4.2. Samples were obtained as explained in section 4.2.2.2.

6.3.3 Statistical Analysis

Values for glycerol release (µg per gram lipid) were expressed as means ± SEM. The data were analyzed using Genstat 10.0 statistical software. General Analysis of Variance with No Blocking was used. Significance was taken as $p < 0.05$.

6.4 Results

6.4.1 Chronic Effect of Individual Polyphenols with ADA

A 26hr chronic incubation of human omental adipose tissue explants with EGCG, Phloridzin and Resveratrol was achieved by pre-incubating for 22hr in the M199 media, followed by 4hr incubation in the KRB media. ADA was added in the KRB media to remove adenosine. In all chronic studies, treatment with IP (for 4hrs again) significantly increased lipolysis (P<0.001). Figure 6.2 shows an inhibitory effect of EGCG on lipolysis, particularly when IP was added (n=2 humans, treatments in triplicate). The significant interaction between EGCG and IP (P<0.001) indicated that the inhibitory effect of EGCG was much more pronounced when IP is not added compared with when it was present. The greatest inhibitory effect was observed with 100µM EGCG.

Figure 6.2 Chronic (26hr) Effects of EGCG on Lipolysis in the Human Omental Adipose Tissue Explants
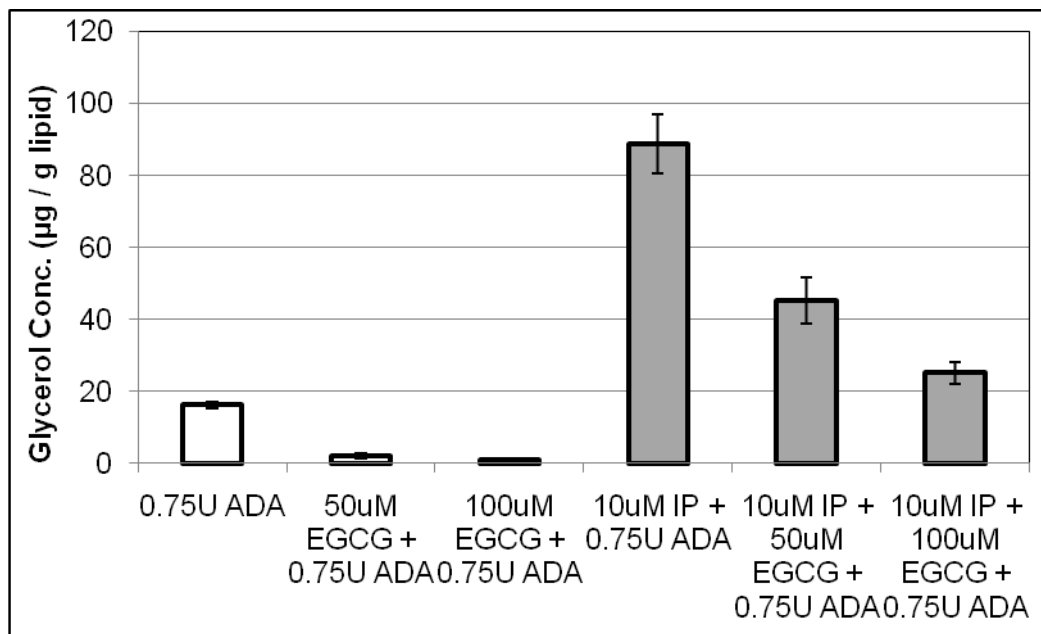
Values are means ±sem, n=2 humans, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) whereas EGCG inhibited lipolysis particularly when IP was present (P<0.001 for EGCG X IP interaction).

In another study (n=2 humans, treatments in triplicate), there was a significant interaction between IP and Phloridzin (P=<0.001). Phloridzin was found to slightly increase basal lipolysis at 100µM (Figure 6.3), but inhibit lipolysis when IP was added.

Interestingly, chronic 26hr treatment with Resveratrol (n=2 humans, treatments in triplicate) had opposite effects on lipolysis (Figure 6.4), depending upon whether IP was added or not (P<0.001 for Resveratrol x IP interaction). Resveratrol alone (without IP) increased lipolysis, particularly at 50 and 100µM, but the effect was reversed when IP was added, with 100µM Resveratrol clearly inhibiting lipolysis.

Figure 6.3 Chronic (26hr) Effects of Phloridzin on Lipolysis in Human Omental Adipose Tissue Explants
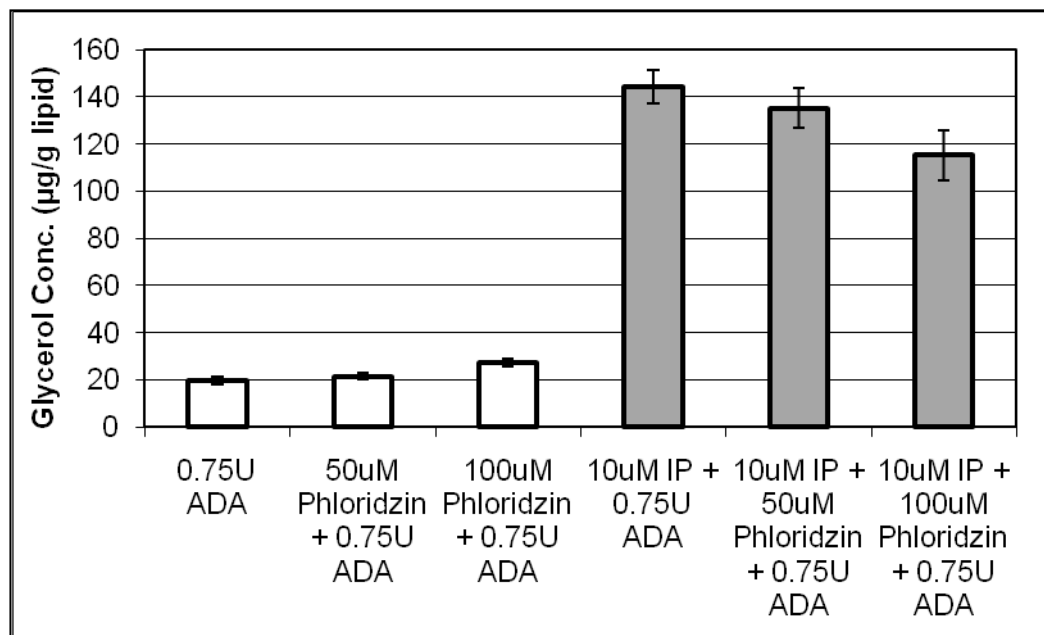
Values are means ±sem, n=2 humans, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) while Phloridzin increased lipolysis during the basal state but decreased it when IP was added (P<0.001 for Resveratrol X IP interaction, ANOVA) SED 6.09.

Figure 6.4 Chronic (26hr) Effects of Resveratrol on Lipolysis in Human Omental Adipose Tissue Explants
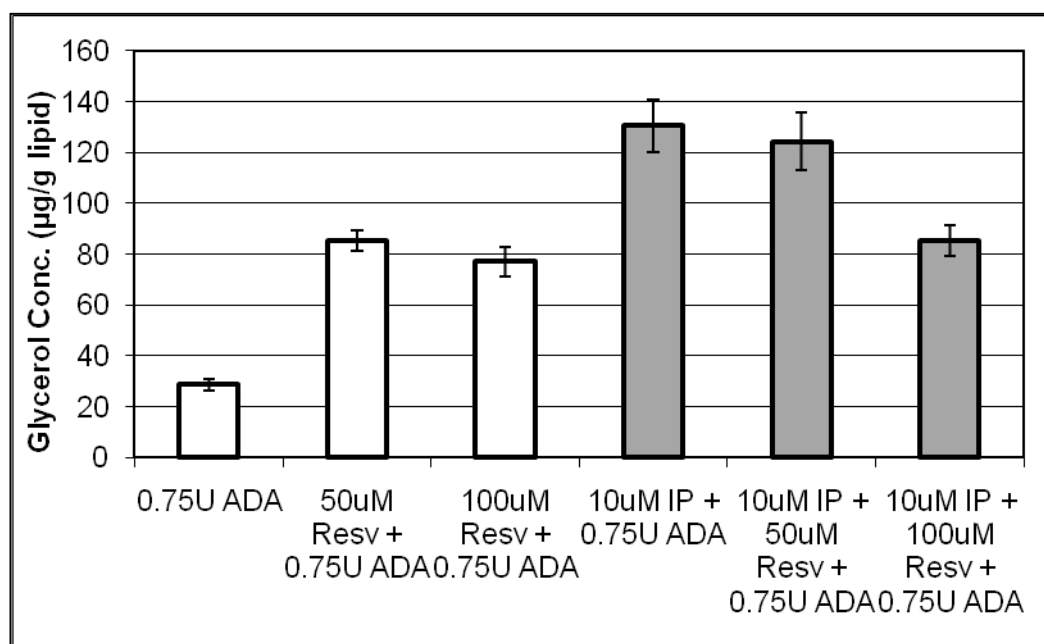
Values are means ±sem, n=2 humans, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) while Resveratrol increased lipolysis during the basal state but decreased it when IP was added (P=<0.001 for Resveratrol X IP interaction, ANOVA) SED 6.09.

6.4.2 Chronic Effects of Individual Polyphenols With and Without ADA

To check whether adenosine is involved in the effects found with the chronic 26hr incubation with individual polyphenols, the human adipose tissue explants were incubated with Phloridzin or Resveratrol, with and without IP and ADA. As before, IP and ADA were added during the 4hr incubation in the KRB media. In all experiments, treatment with IP significantly increased lipolysis (P<0.001).

Figure 6.5 shows that Phloridzin tended to inhibit lipolysis (P=0.049) whether or not IP and/or ADA was added (n=2 humans, treatments in triplicate). This is different from the results found in the previous chronic 26hr incubation with Phloridzin in the presence of ADA where it was found to significantly increase lipolysis. However, the inhibiting effect in the presence of IP continued to be consistent and was independent of ADA.

Human adipose tissue explants were also incubated with Resveratrol at 50 and 100µM in the presence or absence of IP and ADA. Experiments were done separately for each concentration as the amount of adipose tissue obtained from a single patient is only enough to analyze 8 different treatment groups (in triplicate). Each experiment was done on human adipose tissue from 2 individual patients and treatments were done in triplicate. In all experiments, IP significantly increased lipolysis ($P<0.001$).

Figure 6.6 shows the effect of 50µM Resveratrol on lipolysis. There was a significant ADA x IP x Resveratrol interaction ($P=0.006$). Resveratrol was found to increase basal lipolysis (without IP) and lipolysis was induced even more when ADA was present. However, when IP was added to the media, Resveratrol inhibited lipolysis, with no apparent effect of ADA. Similar effects were also found when 100µM Resveratrol was added (Figure 6.7).

As before, there was a significant ADA x IP x Resveratrol interaction ($P=0.002$), with Resveratrol increasing basal lipolysis (without IP) and ADA further increasing lipolysis. Once again, when IP was added to the media, Resveratrol inhibited lipolysis, with no apparent effect of ADA. Interestingly, 50µM Resveratrol on its own seems to stimulate basal lipolysis more than 100µM Resveratrol, whereas there was a bigger inhibitory effect on IP-stimulated lipolysis with 100µM Resveratrol.

Figure 6.5 Chronic (26hr) Effects of Phloridzin on Lipolysis in Human Omental Adipose Tissue Explants in the Presence and Absence of ADA and IP.
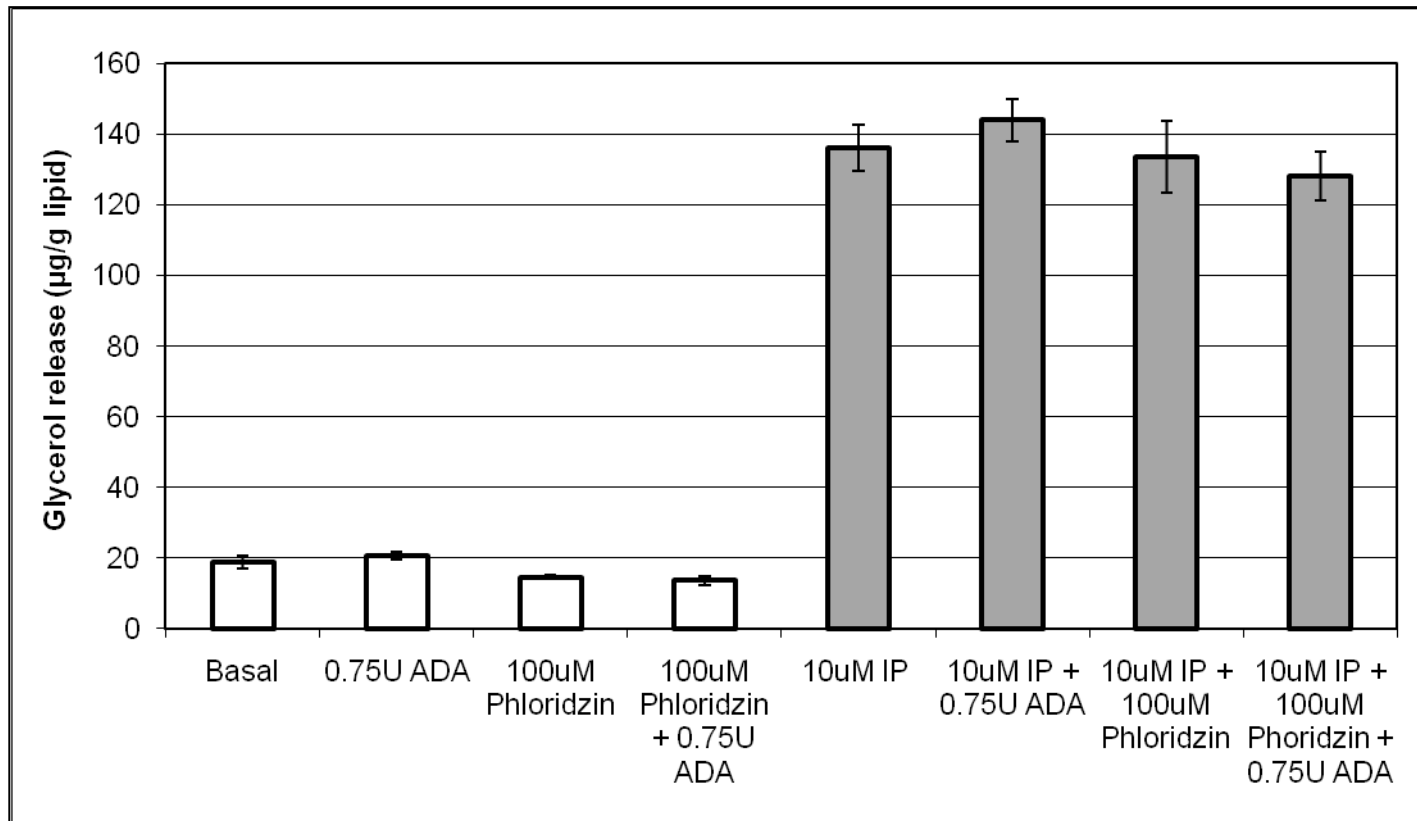
Values are means ±sem, n=2 humans, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA) while Phloridzin significantly inhibited lipolysis independent of IP and ADA (P=0.049, ANOVA).

Figure 6.6 Chronic (26hr) Effects of 50µM Resveratrol on Lipolysis in the Human Omental Adipose Tissue Explant in the Presence and Absence of ADA
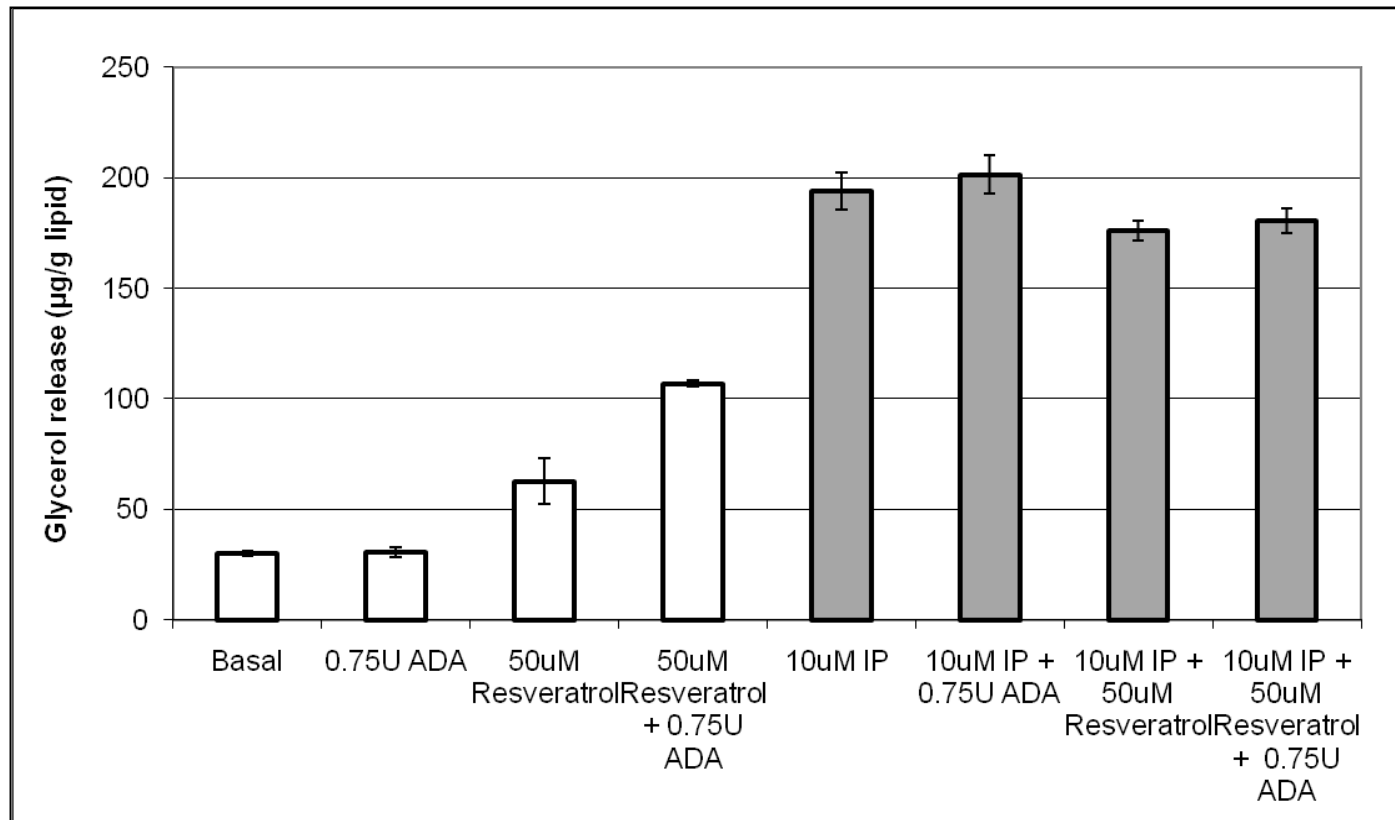
Values are means ±sem, n=2 humans, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA). Resveratrol increased lipolysis during the basal state especially when incubated with ADA but decreased it when IP was added (P=0.006 for Resveratrol X IP X ADA interaction, ANOVA) SED 7.97.

Figure 6.7 Chronic (26hr) Effects of 100μM Resveratrol on Lipolysis in the Human Omental Adipose Tissue Explant in the Presence and Absence of ADA
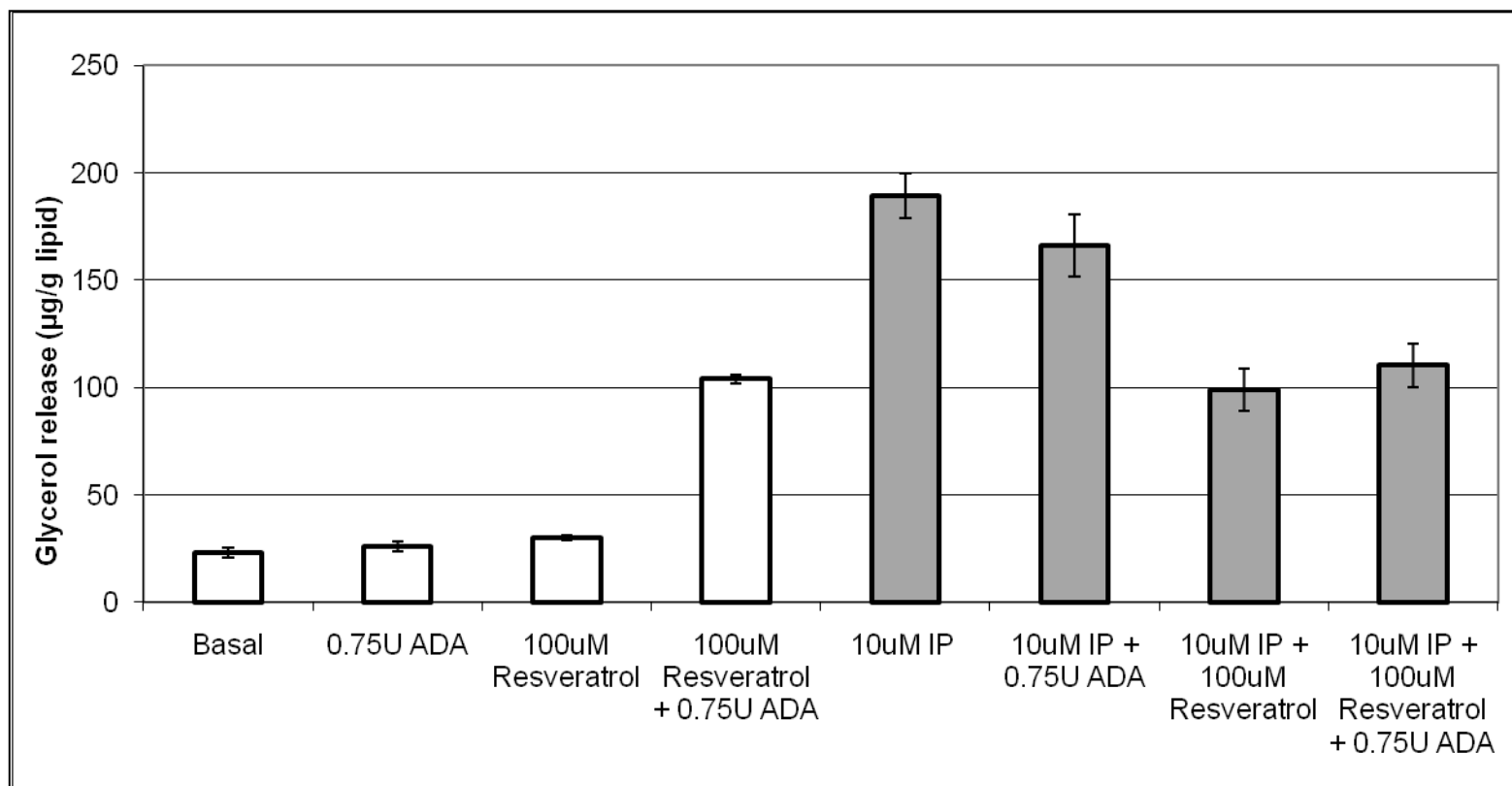
Values are means ±sem, n=2 humans, treatments in triplicate. IP increased lipolysis compared with basal (P<0.001, ANOVA). Resveratrol increased lipolysis during the basal state especially when incubated with ADA but decreased it when IP was added (P=0.002 for Resveratrol X IP X ADA interaction, ANOVA) SED 5.48.

6.5 Discussion

In all experiments, ADA was included to remove adenosine and prevent the tonic inhibition of lipolysis in adipose tissue explants. The addition of ADA also makes the lipolysis results comparable to results obtained previously in our lab in pig adipose tissue explants (Headland, 2007).

6.5.1 Effects of EGCG on Lipolysis in the Human Omental Adipose Tissue Explants

In this study, we investigated the direct effect of EGCG on lipolysis in human adipose tissue explants during chronic (26hr) incubation. Similar to the results previously found in the rat adipose tissue explants (26hr without ADA) and pig adipose tissue explants (24hr with ADA) by Headland (2007), EGCG was found to consistently inhibit lipolysis and the effects were greatest at 100µM. In the human adipose tissue explants, more than 50% inhibition was found following the chronic (26hr) incubation both with and without IP added.

EGCG has previously been shown to inhibit lipolysis in pig fat explants after a chronic (24hr with ADA) incubation in both perirenal and subcutaneous fat explants, particularly at 100µM (Headland, 2007). In the present study, 100µM EGCG inhibited lipolysis more than 50µM EGCG when IP was added, but when IP was not present both 50 and 100µM EGCG inhibited lipolysis to the same concentration. This is probably because inhibition is less pronounced during basal lipolysis, which is much lower than IP stimulated lipolysis.

Thus, the results observed here in human fat explants would suggest that the effect of EGCG on lipolysis inhibition is consistent in pig, rat and human fat explants. The reduction of WAT observed in *in vivo* studies could be mediated via induction of adipocyte cell apoptosis and inhibition of of adipocyte differentiation, as suggested by Wolfram and colleagues (2005) and Hwang and colleagues (2005) when the effect of EGCG was analyzed in an *in vitro* cultured adipocytes assay.

6.5.2 Effect of Phloridzin on Lipolysis in the Human Omental Adipose Tissue Explants

Compared with EGCG, Phloridzin has a rather inconsistent effect on lipolysis in the human adipose tissue explants. When the effect of chronic (26hr) incubation with Phloridzin was investigated the first time (in the presence of ADA), it was found to stimulate lipolysis when IP was not present, but decrease lipolysis when IP was present. However, in another experiment to investigate the effect of chronic (26hr) incubation in the presence and absence of ADA and IP, Phloridzin was found to significantly inhibit lipolysis both with and without IP added. It appears that the induction of basal lipolysis found at 50 and 100µM in the earlier experiment, was not repeated when 100µM Phloridzin was used subsequently, although the inhibitory effect of Phloridzin when IP was present was consistent in both experiments.

The results found in the human adipose tissue explants do not agree with previous findings in pig and rat adipose tissue explants. In pig subcutaneous adipose tissue explants, 24hr incubation with Phloridzin was found to increase lipolysis at 25µM, but had no significant effect at 100µM (Headland, 2007). Moreover, although it appeared to increase lipolysis numerically, Phloridzin had no statistically significant effect on lipolysis in pig perirenal adipose tissue explants. A similar result was found in rat epididymal adipose tissue explants (see chapter 4), where no significant effects were found following both acute (4hr) and chronic (26hr) incubation with Phloridzin.

Thus, the effects of Phloridzin in human, rat and pig fat explants were inconsistent. Although it is not known why the effect is different across species, it must be noted that the effect of Phloridzin in inducing and inhibiting lipolysis in the human adipose tissue explants was not greater than 40% when compared with control.

6.5.3 Effect of Resveratrol on Lipolysis in the Human Omental Adipose Tissue Explants

More interesting and consistent results were found in this study when Resveratrol was incubated for 26hr in the presence of ADA in the first experiment, and when 50µM or 100µM Resveratrol (26hr incubation) was used in the presence and absence of ADA and IP in subsequent experiments.

During the first study, Resveratrol at 50 and 100µM was found to increase lipolysis when IP was not present, but significantly decreased lipolysis when IP was present, especially at 100µM. Subsequent experiments were carried out where effects of the presence and absence of ADA were also investigated.

Interestingly, Resveratrol (at both 50 and 100µM) induced greater lipolysis when ADA was present, but only when IP was absent. When ADA and IP was not present (basal conditions), 50µM Resveratrol was seen to induce lipolysis to a greater extent than 100µM Resveratrol. In all experiments, when IP was present Resveratrol inhibited lipolysis and was independent of ADA, with greater inhibition found at 100µM compared with 50µM Resveratrol.

The results in this study are not supported by the findings in the rat (26hr) or pig (24hr) adipose tissue explants (Headland, 2007), but do agree with results found in the acute (4hr) incubation with 100µM Resveratrol in rat adipose tissue explants (refer to Chapter 5), where Resveratrol was found to increase lipolysis in the absence of IP, but inhibit lipolysis in the presence of IP.

Chronic (22hrs) incubation with Resveratrol resulted in a consistent inhibition of lipolysis both in the absence and presence of IP in the rat fat explants. In pig adipose tissue explants, there was no significant effect of treatment with 10, 50 or 100µM Resveratrol on lipolysis in subcutaneous and perirenal adipose tissue explants (Headland, 2007).

The effects of Resveratrol on lipolysis in the human adipose tissue explants was found to be different when compared with the effects found in the pig and rat adipose tissue explants when incubated for 24 and 26hr. The effects of Resveratrol on lipolysis in the human adipose tissue itself are also dependent on the presence and absence of ADA and IP.

Thus, it is unclear whether Resveratrol is acting as a beta-adrenergic receptor agonist or as an adenosine receptor antagonist, both of which could result in the induction of lipolysis. Thus, we suggest that Resveratrol may have a mixed agonist/ antagonist effect, although whether this relates to beta-adrenergic or adenosine receptors (or both?) is far from clear.

6.6 Conclusion

These studies investigated the effects of EGCG, Phloridzin and Resveratrol on basal and IP stimulated lipolysis in human omental adipose tissue explants in the presence and absence of ADA and then compared the findings with similar studies in our lab using the rat epididymal adipose tissue explants (results in the previous chapter 5) and pig fat explants (Headland, 2007).

With similar concentrations and incubation times, only EGCG was found to have consistent effects across the species, whereas Phloridzin and Resveratrol showed different effects in different species. These results suggest that individual polyphenols may act through different mechanisms in adipose tissue across different species. Thus, the hypothesis was not supported. However, we have to note that the age of rat, pig and human were not the same and this might affect the composition of adipocytes (pre-adipocytes, adipocytes and mature cells) in the adipose tissues resulting in the different effects on lipolysis found.

Chapter 7 – Regulation of Lipolysis in Rat, Human and Pig Adipose Tissue Explants: A Comparison

7.1 Introduction

A lot of experimental medical research into the regulation of lipolysis were carried out using laboratory animals, based on the assumption that factors regulating their metabolism are identical or at least similar to those present in humans. However, findings from numerous studies have established that species differences exist, particularly for adipose tissue responsiveness to certain hormones, such as insulin (Steinke *et al.*, 1965; Di Girolamo & Rudman, 1966; Gries & Steinke; 1967). It is also well known that adipose tissue of animals of various classes responds to lipolytic and anti-lipolytic substances in different ways, as described for ruminants (Menahan, 1966; Khachadurian *et al.*, 1966; Metz & van den Bergh, 1972) and for birds (Carlson *et al.*, 1964; Goodridge, 1968; Langslow, 1972).

Moreover, in animals of the same order, and even within the same family, the effect of lipolytic hormones was found to vary (Rudman & del Rio, 1969; Boberg *et al.*, 1970). For example, catecholamines were shown to be lipolytic in rat and dog adipose tissue, but not in rabbit or guinea pig adipose tissue (Mersmann *et al.*, 1974). The lipolytic response to other hormonal substances such as adrenocorticotropin, thyrotropin, melanocyte-stimulating hormone, vasopressin and glucagon, was shown to also be species specific (Rudman & DiGirolamo, 1967).

The differences found in the regulation of lipolysis between species may be attributed to the properties of adipocytes *in situ*. For example, in contrast to mice, human adipocytes possess α_2-adrenergic receptors and natriuretic peptide receptors, and some receptors for thyrotropin (Wang *et al.*, 2008). Conversely, human adipocytes have a negligible response to β_3 adrenergic agonists (Rosenbaum *et al.*, 1993), in contrast to the large response seen in rodent adipocytes. Growth hormone provokes intense lipolysis in mice but has little or no effect in humans (Arner, 2005; Djurhuus, 2004). Furthermore, differences

between omental and subcutaneous fat depots are less marked in rodents compared with humans (Arner, 2005).

7.2 Hypothesis and Aim

The response to treatment with individual polyphenols was found to be different between human, rat and pig fat explants. In this study, the hypothesis was therefore, that basal and stimulated lipolysis in rat, human and pig adipose tissue explants are different to each other. The aim was to analyze all the basal and stimulated lipolysis data taken from the previous chapters. Note that all the pig adipose tissue explant studies were performed by a previous PhD student in the Division (Headland, 2007). Additionaly, in the human adipose tissue explants, we investigated whether patients' BMI or age impacted upon lipolysis, when basal and IP-stimulated concentrations of lipolysis were correlated with BMI and age of the patients.

7.3 Methodology

7.3.1 Adipose Tissue Explants

Methods used for isolation and culture of adipose tissue explants in this experiment have been explained in detail in Chapter 4, section 4.2 for rat and human adipose tissue explants and in Chapter 2, section 2.1 in the PhD thesis written by Headland (2007) for pig adipose tissue explants.

7.3.3 Statistical Analysis

Values for glycerol release (μg per gram lipid) were expressed as means ± SEM. The data were analyzed using Genstat 10.0 statistical software. General Analysis of Variance with No Blocking was used. Significance was taken as $P < 0.05$.

7.4 Results

7.4.1 Basal Lipolysis in the Rat, Human and Pig Adipose Tissue Explants

Figures 7.1, 7.2, 7.3, 7.4 and 7.5 show the basal and IP stimulated lipolysis in rat epididymal adipose tissue explants (n=18 rats, treatments in triplicate), rat perirenal adipose tissue explants (n=2 rats, treatments in triplicate), human omental adipose tissue explants (n=18 humans, treatments in triplicate), pig perirenal (n=3 pigs, treatments in triplicate) and subcutaneous (n=3 pigs, treatments in triplicates) adipose tissue explants.

Epididymal rat and omental human adipose tissue explants were pre-incubated for 22hrs in M199 media and then for 4hrs in the KRB media with or without 10µM IP. Pig perirenal and subcutaneous adipose tissue explants were pre-incubated for 20hrs in M199 media and then for 3hrs in the KRB media with or without 10µM IP. All adipose tissue explants were derived from intra-abdominal adipose tissue depots except for the pig subcutaneous adipose tissue. Data for pig perirenal and subcutaneous adipose tissue explants were obtained from the work previously done by Headland (2007) in our lab.

In all experiments, 10µM IP significantly increased lipolysis ($P < 0.001$, ANOVA) but the concentrations and percentage increase from basal varied between species and adipose tissue depot. The highest concentrations of IP-stimulated lipolysis were in the rat epididymal adipose tissue explants (425.2µg glycerol/g lipid), followed by the pig perirenal (353.6µg/g lipid), rat perirenal adipose tissue explants (311.56 µg glycerol/g lipid), human omental (136.7µg/g lipid) and lastly, pig subcutaneous (133.6µg/g lipid) adipose tissue explants.

A slightly different order was observed for the basal lipolysis concentrations. Rat perirenal was found to be the highest with mean glycerol release of 206.38 µg/g lipid, followed by rat epididymal at 195.1µg/g lipid, pig subcutaneous at 54.151 µg/g lipid, pig perirenal at 44.31 µg/g lipid and lastly human omental at 19.33 µg/g lipid. The highest % response to IP stimulation was found in the pig perirenal (698% increase), followed by the human omental (607% increase), pig subcutaneous (164.7% increase), rat epididymal (117.9% increase) and rat perirenal (51%) adipose tissue explants.

Figure 7.1 Basal and IP-stimulated Lipolysis in Human Omental Adipose Tissue Explants (n=18)
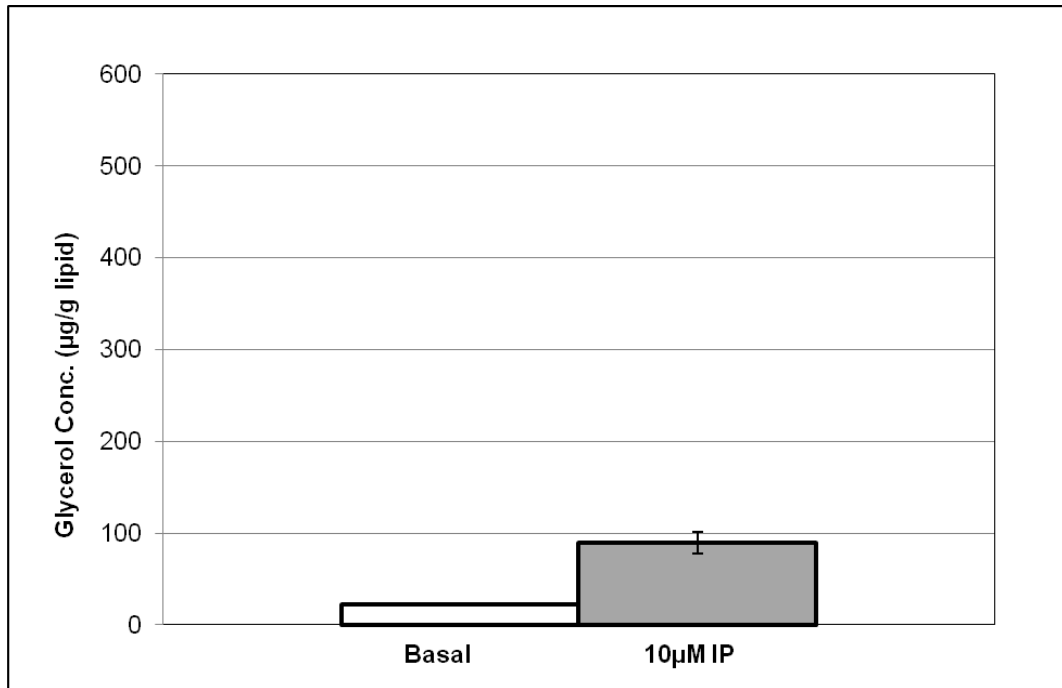
IP significantly increased lipolysis (P<0.001, ANOVA)

Figure 7.2 Basal and IP-stimulated Lipolysis in Rat Epididymal Adipose Tissue Explants (n=18)
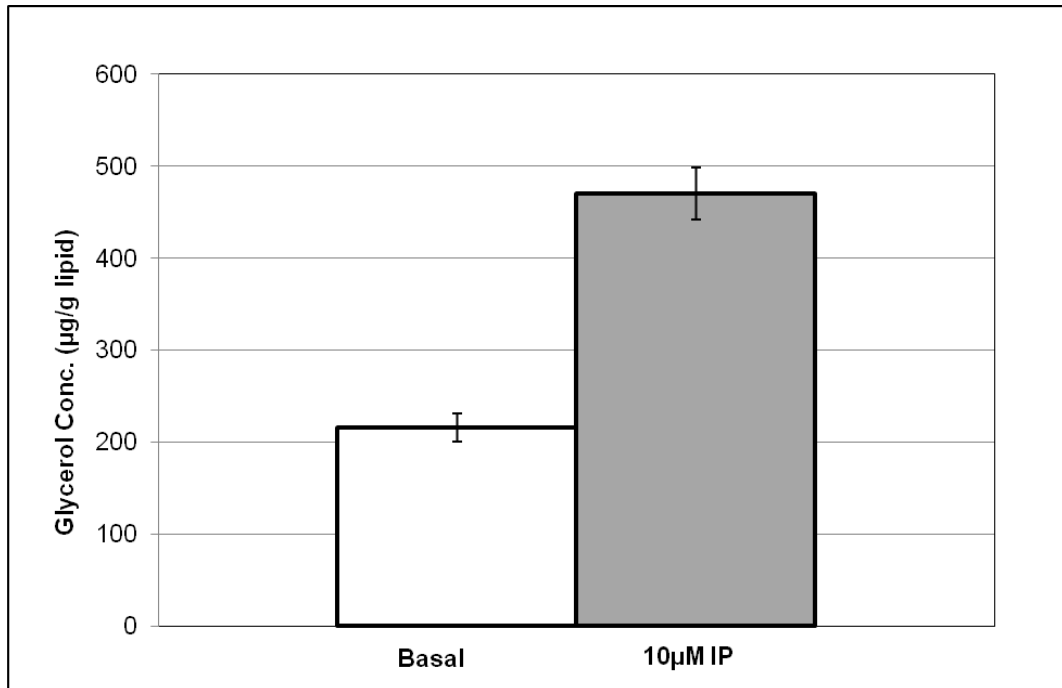
IP significantly increased lipolysis (P<0.001, ANOVA)

Figure 7.3 Basal and IP-stimulated Lipolysis in Rat Perirenal Adipose Tissue Explants (n=2)
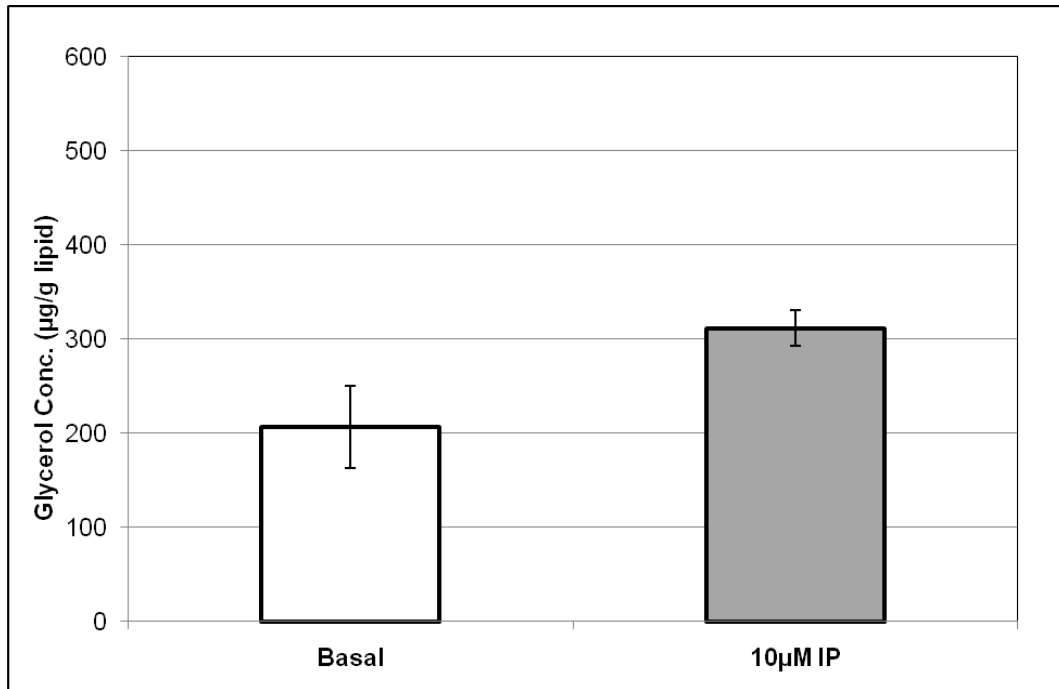
IP significantly increased lipolysis (P<0.001, ANOVA)

Figure 7.4 Basal and IP-stimulated Lipolysis in Pig Perirenal Adipose Tissue Explants (n=3)
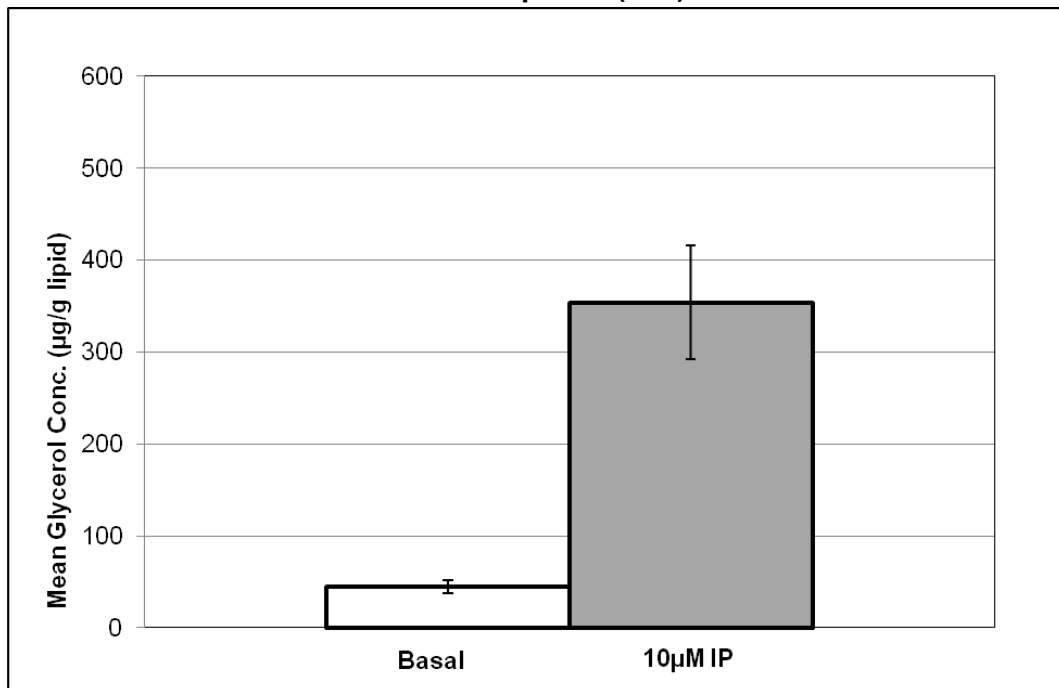
IP significantly increased lipolysis (P<0.001, ANOVA)

Figure 7.5 Basal and IP-stimulated Lipolysis in the Pig Subcutaneous Adipose Tissue Explants (n=3)
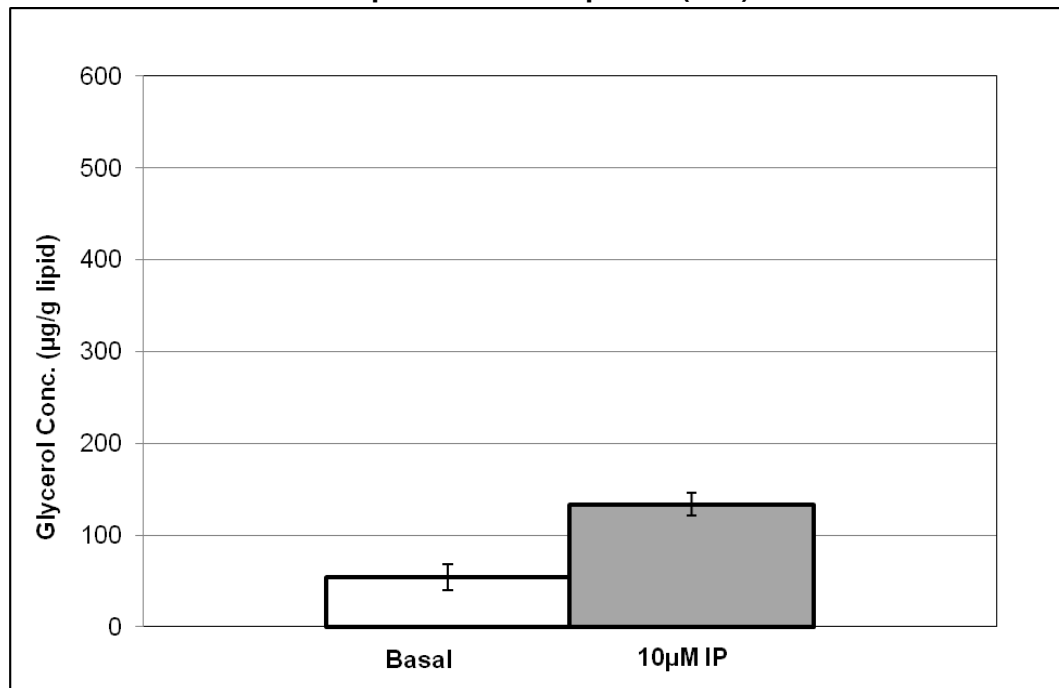
IP significantly increased lipolysis (P<0.001, ANOVA)

7.4.2 Adenosine Regulation of Lipolysis in the Rat Epididymal, Human Omental and Pig Perirenal Adipose Tissue Explants

Adenosine is an inhibitor of lipolysis via binding to adenosine receptor (Figure 6.1) which results in subsequent inhibition of adenylate cyclase and phosphorylation of hormone sensitive lipase. In this experiment, we investigated the effects of 0.75U adenosine deaminase (ADA) to remove adenosine, and the non-metabolisable adenosine analogue, N-6-phenyllisopropyl adenosine (PIA, 100nM), both separately and combined in the presence or absence of 10μM IP, when added to rat epididymal (n=2 rats, all treatments in triplicate), human omental (n=2 humans, all treatments in triplicate) or pig perirenal (n=3 pigs, all treatments in triplicate) adipose tissue explants. Data for pig perirenal adipose tissue explants were again taken from the work done previously by Headland (2007) in our lab. Results are shown in Figures 7.6, 7.7 and 7.8.

In all experiments, IP significantly increased lipolysis (P<0.001, ANOVA). As expected, lipolysis also increased with the addition of 0.75U ADA, but in all species this was only in the absence of IP, with no additional effect of combining IP and ADA (P<0.001, and P=0.025, for ADA x IP interaction in the rat and human respectively). Interestingly, PIA significantly reduced lipolysis in the human and pig adipose tissue explants (P<0.006 in human and P=0.008 in pig), but had no effect in the rat adipose tissue explants (P=0.298).

Figure 7.6 Adenosine Regulation of Lipolysis in Rat Epididymal Adipose Tissue Explants
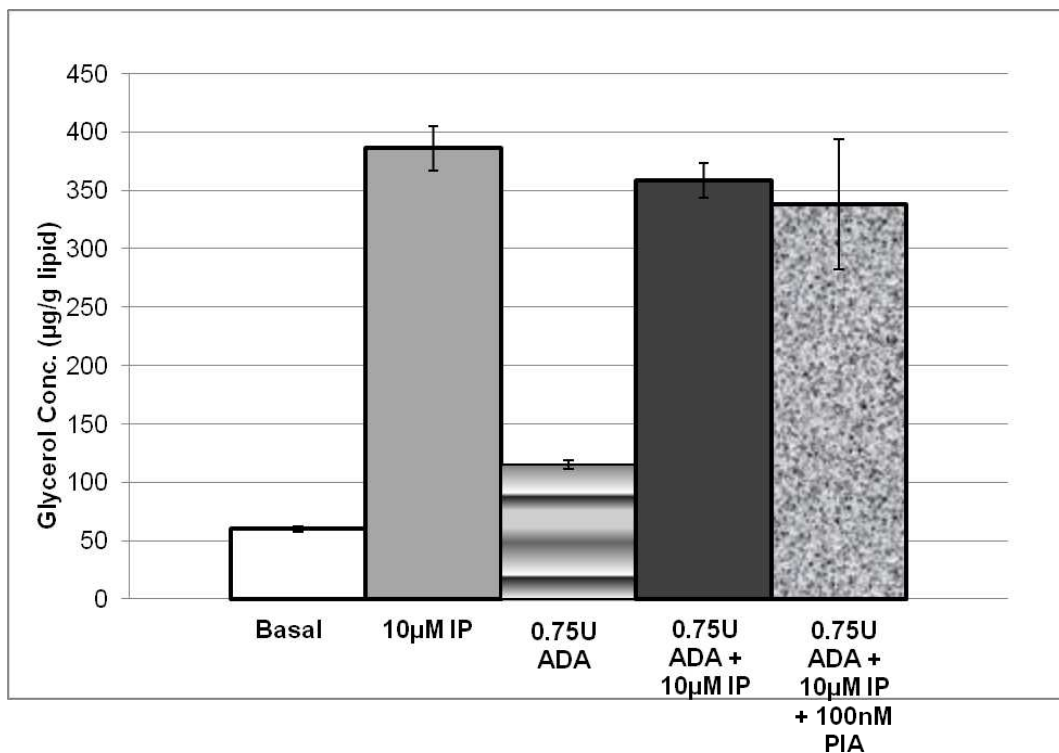
Values are means ±sem, n=2 rats, treatments in triplicate. Effect of ADA is dependent on IP (ADA X IP interaction, P<0.001, ANOVA). IP significantly increased lipolysis (P<0.001, ANOVA). PIA does not have any significant effect on lipolysis (P=0.298, ANOVA).

Figure 7.7 Adenosine Regulation of Lipolysis in the Human Omental Adipose Tissue Explants
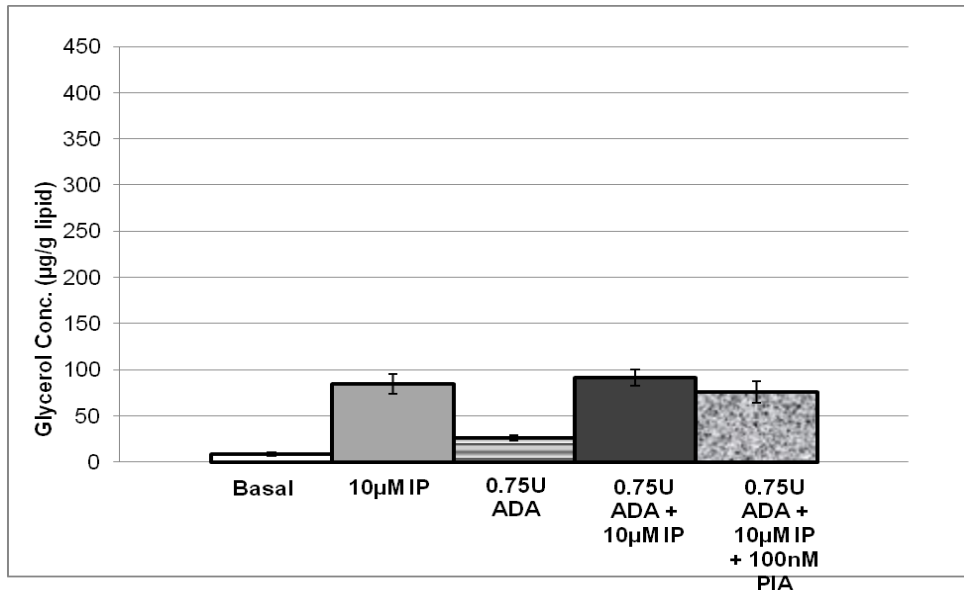
Values are means ±sem, n=2 humans, treatments in triplicate. Effect of ADA is dependent on IP (ADA X IP interaction, P=0.025, ANOVA). IP significantly increased lipolysis (P<0.001, ANOVA). PIA significantly inhibits lipolysis (P<0.006, ANOVA).

Figure 7.8 Adenosine Regulation of Lipolysis in the Pig Perirenal Adipose Tissue Explants
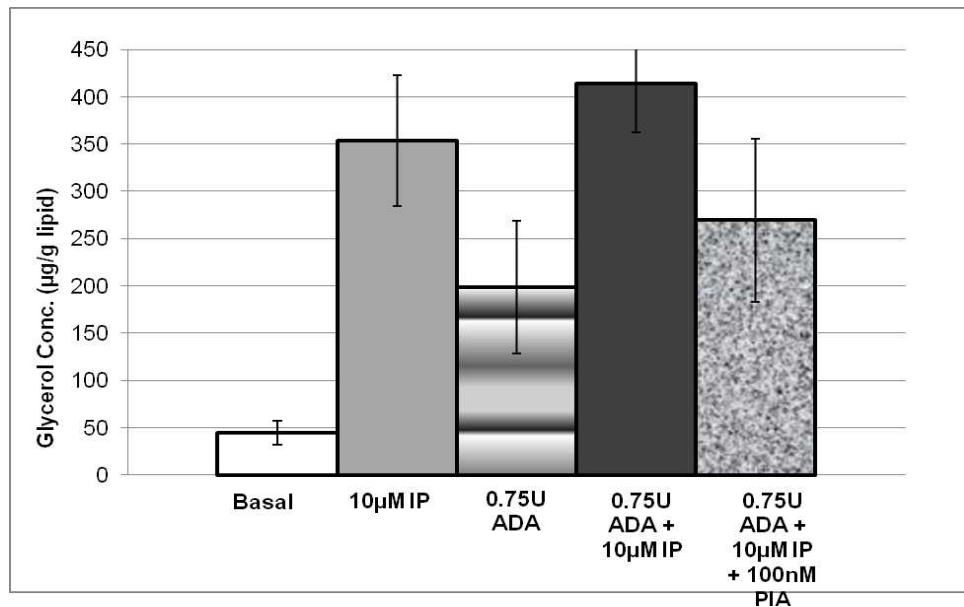
Values are means ±sem, n=3 pigs, treatments in triplicate. Both ADA and IP significantly increase lipolysis (P<0.001, ANOVA for both). IP significantly increased lipolysis (P<0.001, ANOVA). PIA significantly inhibits lipolysis (P=0.008, ANOVA).

7.4.3 Lipolysis in the Human Omental Adipose Tissue Explants According to BMI and Age

In all experiments, omental adipose tissue explants (n=18, treatments in triplicate) were pre-incubated for 22hrs in M199 media and then treated with 10µM IP for 4hrs in the KRB media. Lipolysis results were then compared with non-treated group (basal) and the effect of BMI and age were measured using a scatter plot shown in Figures 7.9 and 7.10.

From the Figure 7.9, we can conclude that BMI and glycerol released in the media have a very weak negative correlation (F=0.999). Basal lipolysis has a stronger negative correlation compared with IP stimulated lipolysis. In all samples, IP increased lipolysis significantly (P<0.001, ANOVA) but there are no significant effect of BMI on lipolysis (P=0.097, ANOVA).

Figure 7.10 shows that age also had a weak negative correlation (F=0.998). A stronger correlation was also found in the basal compared with IP stimulated lipolysis. Age did not have any effect on lipolysis (P=0.48, ANOVA). IP significantly increased lipolysis (P<0.001, ANOVA).

Figure 7.9 Changes in basal and IP-stimulated Lipolysis in the Human Omental Adipose Tissue Explants with increasing BMI.
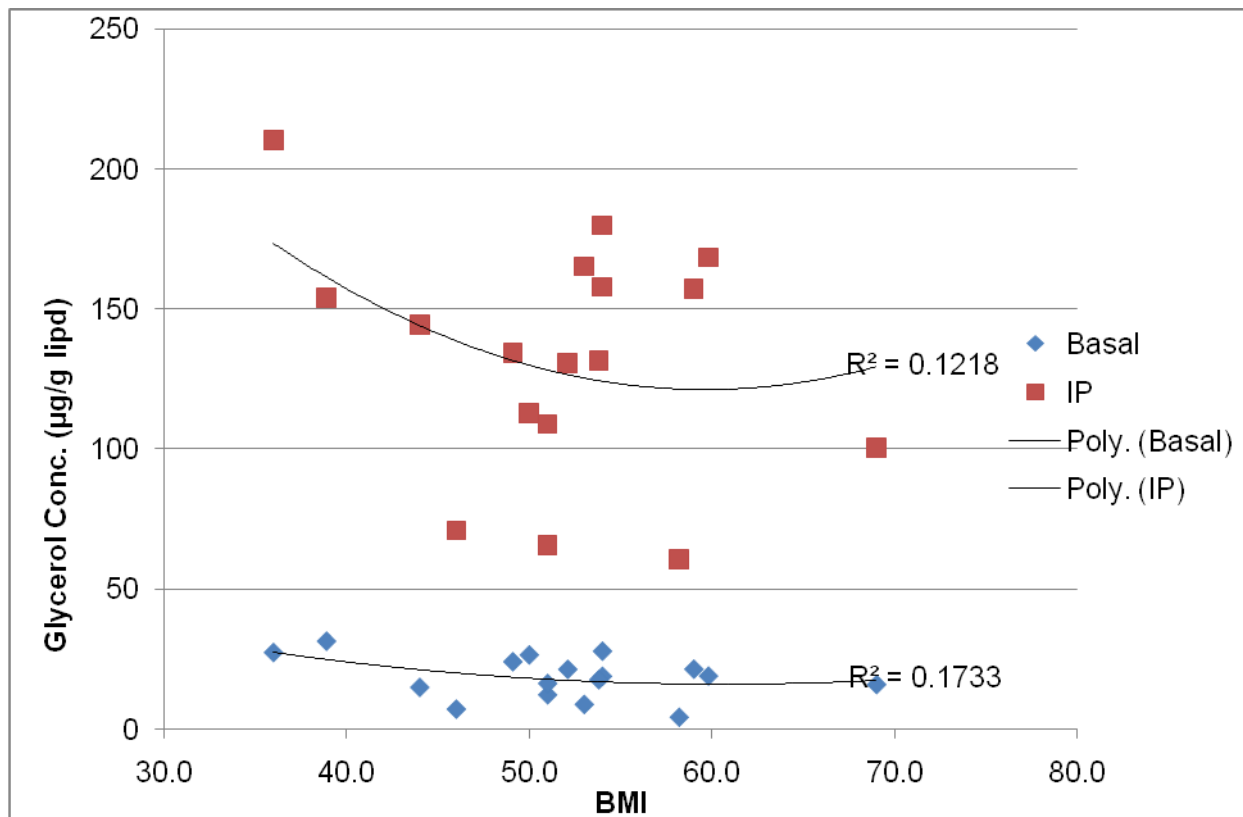
BMI and Mean Glycerol have a weak negative correlation (F=0.999). IP significantly increased lipolysis (P<0.001, ANOVA). There was a tendency for IP-stimulated lipolysis to decrease slightly with increased BMI (P=0.097, ANOVA). Values are means of triplicate measures from, n=18 humans.

Figure 7.10 Changes in basal and IP-stimulated Lipolysis in the Human Omental Adipose Tissue Explants with increasing Age
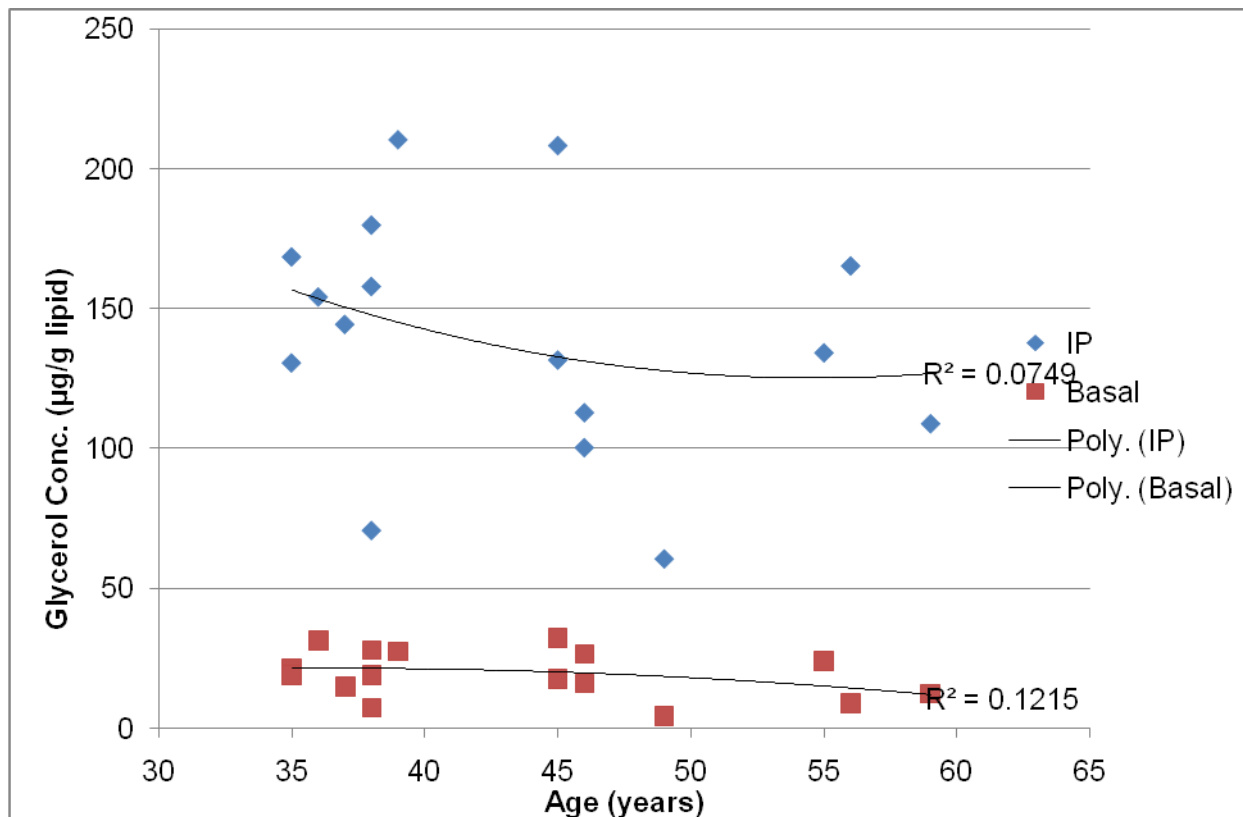
Age and Mean Glycerol have a weak negative correlation (F=0.998). IP significantly increased lipolysis (P<0.001, ANOVA). Age did not effect lipolysis (P=0.48, ANOVA).

Values are means of triplicate measures from n=18 humans.

7.5 Discussion

7.5.1 Basal Lipolysis in the Rat, Human and Pig Adipose Tissue Explants

Isoproterenol is a pure β-adrenoreceptor agonist (Mersmann, 1984a) in the adrenergic control of adipose tissue lipolysis. Lipolysis stimulated by a β-adrenoreceptor agonist was chosen to measure lipolytic response across rat, human and pig rather than an α-adrenoreceptor antagonist, because rat adipocytes are unique in that they possess β-adrenoreceptors but are deficient in functional α-adrenoreceptors compared with human and pig adipocytes which have both α and β-adrenoreceptors (Carpene *et al.*, 1983). In our studies, we found that IP significantly (P<0.001) increased lipolysis in rat, human and pig adipose tissue explants. Although the magnitude of the response to IP varied, this was expected as there are marked species differences in the response of adipose tissue to various lipolytic agents (Mersmann *et al.*, 1974; Mersmann, 1984a & b; Herberg *et al.*, 1974).

From these studies, we conclude that basal lipolysis in the rat adipose tissue explants is higher than in the human and pig adipose tissue explants (Figures 7.1, 7.2 and 7.3). Herberg and colleagues (1974) compared basal lipolysis in isolated adipocytes from four strains of mice and compared with isolated adipocytes from human subcutaneous adipose tissue. They found that basal release of glycerol by subcutaneous adipocytes from Swiss albino mice, C57BL/6J-ob/+ mice, New Zealand obese mice and Australian mice (aged 6-8 weeks and weighing around 26g) were all significantly higher than human adipocytes. Similarly, theophylline stimulated glycerol production was significantly lower in human adipocytes than in the mice adipocytes. It is also worth noting that the lipolytic activity (both basal and stimulated) was generally higher in subcutaneous than in epididymal adipose tissue explants from all strains of mice (Herberg *et al.*, 1974). Similar studies by Mosinger and colleagues (1965) and Carlson and Hallberg (1968) on human omental and subcutaneous adipose tissue explants have shown that subcutaneous adipose tissue is relatively insensitive to lipolytic stimulation. The effect of cell size on basal lipolysis should also not be underestimated. A positive correlation was found between the basal rate of lipolysis and the fat cell size in rat and human (Holm *et al.*, 1975; Arner, 1988)

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Basal lipolysis was found to be higher in pig adipose tissue explants (Headland, 2007) than in human adipose tissue explants, but lower than rat adipose tissue explants. This is also true for the IP stimulated lipolysis in pig perirenal adipose tissue explants, but not in the pig subcutaneous adipose tissue explants, where IP stimulated lipolysis was similar to that in human omental adipose tissue.

However, these results are not supported by a similar study by Mersmann (1984b) using isolated pig subcutaneous adipocytes (n=21) and rat epididymal adipocytes (n=10). In that study, when pig and rat adipocytes were stimulated with 1µM IP, fatty acid release (µmol $120min^{-1}$ g $tissue^{-1}$) was higher in the pig than in the rat. It is interesting to note that the number of pigs used in the Mersmann (1984b) study was 7 times higher than in Headland (2007) study. This and other variables such as the adipocyte composition, breed, age and weight of the pig might contribute to the differences in IP response.

7.5.2 Adenosine Regulation of Lipolysis in the Rat Epididymal, Human Omental and Pig Perirenal Adipose Tissue Explants

Adenosine is known as a major regulator of adenyl cyclase which leads to lipolysis in the adipose tissue (Chaves *et al.*, 2011). Recently, it was shown that removal of endogenous adenosine with adenosine deaminase causes lipolysis in adenosine α_1 receptor (+/+) but not adenosine α_1 receptor (-/-) adipocytes (Johansson *et al.*, 2008) and that incubation of rat isolated adipocytes with an adenosine α_1 receptor antagonist induces an increase in lipolytic activity (Szkudelski *et al.,* 2009). These findings provide strong evidence that the antilipolytic effect of adenosine involves impairment of PKA activation. In this study, as expected, the presence of adenosine does have an effect on the lipolysis rate in rat, human and pig adipose tissue explants, since the addition of ADA (to metabolise/remove adenosine) increased basal lipolysis. However, only in the pig perirenal adipose tissue explants was IP stimulated lipolysis found to be increased with ADA. We suggest that IP stimulation in the rat and human adipose tissue explants was already maximal (based on the average IP stimulated lipolysis in all experiments including the previous chapter 4, 5 and 6) and thus, lipolysis could not be increased further with the addition of ADA. It was also found that the non-metabolisable form of adenosine (PIA) reduced lipolysis in the presence of IP and ADA in the human and pig adipose tissue explants, but not in

the rat adipose tissue explants. The reason for this is unknown but one possible reason is that the concentration of PIA needed to be an effective adenosine receptor agonist.

7.5.3 Lipolysis in the Human Omental Adipose Tissue Explants According to BMI and Age

Human adipose tissue displays significant regional differences in adipocyte size, basal metabolic activities and hormonal responsiveness (Lafontan & Langin, 2009). Regional differences in regulation of lipolysis have been shown *in vitro* and *in vivo* (Jensen, 1997; Lafontan & Langin, 2009). However, the interpretation of the studies is complex and generates equivocal data since it also depends on gender, fat depot (intra-abdominal vs peripheral depots), BMI, and the choice of lipolysis units used eg. glycerol per gram lipid or glycerol per esterified fatty acids or cell number (Lafontan & Langin, 2009; Rodbell, 1964; Tchernof *et al.*, 2006; Van Harmelen *et al.*, 1997; Reynisdottir, 1994). Age, physical activity, nutrition and genetic variance are also important factors for lipolysis regulation (Arner, 2005).

In this study, we found that although BMI and age had weak negative correlations with lipolysis (Figure 7.9), these were not statistically significant (P=0.097 for BMI, P=0.48 for age). However, the trend suggests that IP stimulated lipolysis decreased with increased BMI. A study by Leijonhufvud and colleagues (2010) also showed that lipolysis was decreased with the increase of BMI, but there was no change with the increase in age. In their study, Leijonhufvud and colleagues (2010) constructed and evaluated a simple index of lipolytic activity (ratio of fasting plasma glycerol and body fat %) in population based samples of 316 teenagers (BMI 16-51) and 3039 adults (BMI 16-70). They found that in the adults, waist circumference and BMI but not age, plasma insulin, plasma noradrenaline or waist-to-hip ratio contributed independently and inversely to lipolytic activity. The same analysis in teenagers showed that only BMI contributed independently and inversely to lipolytic activity.

Our study agrees with Leijonhufvud and colleagues (2010), indicating no effect of age on lipolysis (Figure 7.10). However, we have to note that age has previously been suggested to have important effects on lipolysis related to the action of catecholamines and insulin (Gregerman, 1994; Herrera & Amusquivar 2000; Toth

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& Tchernof (2000). In previous study on elderly subjects, there was a decrease in catecholamine-induced lipolysis, mainly due to impaired function of the protein kinase A-HSL complex (Arner, 2005). The antilipolytic effects of insulin also decrease with aging, which was, at least in part, ascribed to loss of cell-surface insulin receptors (Arner, 2005).

7.6 Conclusion

The rates of lipolysis in cultured adipose tissue explants appears to differ between rat, human and pig. This confirmed the hypothesis. Other variables such as age, cell size, BMI and specific adipose tissue depot are likely to be important contributory factors in these apparent differences. It is not clear that either animal species is a "better" model for humans, therefore future studies aimed at understanding human physiology and lipid metabolism should be done in human adipose tissue explants rather than using adipose tissue from animal models.

Chapter 8 – General Discussion

Obesity increases the risk of medical illness and premature death (Kopelman PG., 2000) and thus imposes an enormous economic burden on the health care system (Katzmarzyk & Janseen, 2004). Obesity is also associated with a reduced quality of life resulting from substantial limitations and restrictions in activities of daily living (Visscher *et al.*, 2004). Obese individuals are less likely to obtain insurance, employment or promotion or enjoy personal relationships (Puhl & Brownell, 2001). Prevention and treatment of obesity is therefore now widely recognized as a priority for most health care systems. A variety of natural products, including crude extracts and isolated compounds from plants, can induce body weight reduction and prevent diet-induced obesity. Therefore, they have been widely used in treating obesity (Han *et al.*, 2005; Moro and Basile, 2000; Rayalam *et al.*, 2008b). A wealth of information indicates numerous bioactive components from nature are potentially useful in obesity treatments. A good example of such is the polyphenols. These show strong anti-obesity activity and include apigenin, genistein, and the catechins (Rayalam *et al.*, 2008a; Thielecke and Boschmann, 2009; Wolfram *et al.*, 2006).

During my PhD studies, I have focused on the effect of *Strobilanthes crispus* extract (SCE) as an energy expenditure and lipolysis stimulator and the effect of individual polyphenols as lipolysis inducers. I have first optimized the chloroform-methanol extraction method for SCE. Chloroform-methanol was the same solvent used for SCE extraction during my Masters studies, where SCE was found to decrease abdominal fat and improved plasma lipid profile in HF diet induced obese rats. In Chapter 2, it has been determined that the chloroform-methanol extraction SCE contains polyphenols.

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The SCE was then investigated in an *in vivo* study using mice where the hypothesis was that it will reduce abdominal adipose tissue weight and improve the lipid profile of the treated groups. The results showed that the SCE had no significant effect on bodyweight or abdominal adipose tissue weight but significantly increased plasma glycerol and liver triglycerides contents. Plasma and liver cholesterol concentrations were also increased but only in the HF diet

fed groups. The results from the lipid profile clearly indicated that the SCE was atherogenic. Concommitantly, SCE was found to decrease the RER value of treated groups at Weeks 4 and 9 and increased heat production at Week 4 although this effect was found to be tolerated at Week 9. The decrease in RER value at Weeks 4 and 9 would seem to indicate lipids were being more preferentially used as a source of energy but the energy expenditure between treated and non-treated groups, on week 9, was similar as the heat production value was not changed. Lipid oxidation and energy expenditure appear to be a positive effect of SCE, although this effect was insufficient to reduce adiposity in the obese LDLr KO mice. However, tolerance towards heat production might be masking any effects and so the measurements for adipose tissue weights, lipid and liver lipid contents might be different if these were to be measured earlier (Week 4). Additionally, longer treatment time with SCE might also decreased the RER value further, or long enough to see a change in adiposity as the decrease in RER was only starting to be significant at Week 10.

The different outcomes found when either LDLr KO mice or Sprague-Dawley rats were treated with SCE may be related to their different genetic background. The bioavailability of polyphenols through the actions of gut microbiota could also be different between LRLr KO mice and Sprague Dawley rats because of the different diets used in each case which may result in different microbiota. In a human study, obese people with higher *Bacteroidetes* in the fecal gut microbiota were found to lose bodyweights at a higher rate compared to obese people with higher *Firmicutes* although their calorie intake was the same (Rastmanesh, 2011). The intestinal microbial transformation of polyphenols is subject to a wide inter-individual variability (Gardana *et al.*, 2009). *In vitro* and humans studies support the hyphothesis that changes in the microbiota ratio might affect the normal food digestion process (Berlanga *et al.*, 2009; Connoly *et al.*, 2010; Koropatkin & Smith, 2010; Laparra *et al.*, 2010; Salazar *et al.*, 2009; Samuel *et al.*, 2008; Sonnenburg *et al.*, 2010) and this affects the amount of polyphenols absorbed in the gut lumen. Similarly in the LDLr KO mice and Sprague-Dawley rats, even though the percentage of fat given in the HF diet group was similar (LDLr KO mice given 60% kcal fat, Sprague-Dawley rats given 55% kcal fat), the source of fat was different. HFD given to the LDLr KO mice was mainly made from lard while the HFD given to Sprague-Dawley rats was mainly made from full-cream milk powder and corn oil.

The effects of SCE were further investigated *in vitro* using cultured rat epididymal and human omental adipose tissue explants. We hypothesized that application of SCE would result in an enhancement of lipolysis in rat and human adipose tissue explants. This hypothesis was not met as SCE at three different concentrations (0.13%, 0.013% and 0.0013%) did not appear to have any direct effect on lipolysis in either rat or human adipose tissue explants. These concentrations of SCE when converted to Gallic Acid Equivalents represent the addition of 1000μM, 100μM and 10μM equivalents of phenolics, respectively. A review from 97 polyphenol bioavailability studies (Manach *et al.*, 2005) concluded that the plasma concentrations of total polyphenol metabolites ranged from 0 to 4 μM with an intake of 50 mg aglycone equivalents, and the relative urinary excretion ranged from 0.3% to 43% of the ingested dose, depending on the polyphenol. Thus the lack of effect in the explants studies is unlikely to be due to the concentrations used as these were relatively high. Another possible explanation is that the types of polyphenols available in the SCE used in the *in vitro* study might not be similar to the types of polyphenols available in the SCE that was being absorbed and utilized in the *in vivo* study. As discussed in the introduction dietary polyphenols can be subject to metabolism by the gut microfloraa and also once absorbed into the body. Further studies on the bioavailability of constituents within the SCE *in vivo* should be done to confirm this.

Another possibility is that the increase in plasma glycerol concentrations and lipid oxidation with SCE treatment found in the *in vivo* experiment may have been due to an indirect effect of SCE on lipolysis through the activation of PPARα in the liver instead of acting directly as a β-adrenoreceptor agonist. The increase of plasma lipid in the *in vivo* study could be the result of the reduced capability of the liver to convert the lipids into bile acids which could then be removed out of the capillary system into the fecal excretion. All these actions are related to specific interaction of polyphenols with enzymes and receptors in various organs in the body and thus may be the reason of the different effects of SCE found in the *in vivo* and the *in vitro* system.

Since polyphenols were still the main interest in this study and we had already developed the rat adipose tissue explants system, the effects of individual polyphenols on lipolysis were then studied in rat and human adipose tissue

explants (the latter successfully developed from the rat adipose tissue explants method) results were then compared with the results from a previous pig adipose tissue explant study (Headland, 2007). The hypothesis was that effects of individual polyphenols on rat and human adipose tissue explants are similar to that found in the pig adipose tissue explants and other studies using cultured adipocytes and cell lines. However, this hypothesis was not supported by the findings. Consistency of the effects on lipolysis was found to depend on the polyphenol being studied and the length of incubation. When comparisons were made between the lipolytic effects of individual polyphenols on the rat, human and pig adipose tissue explants only EGCG was found to have consistent effects at similar concentrations and incubation times, whereas Phloridzin and Resveratrol showed different effects in different species. The consistency of EGCG in inhibiting lipolysis in the adipose tissue explants of all three species could be due to the fact that a toxic concentration had been used. EGCG Cmax value was found to range from 25 to 126nM in human plasma (Stalmach *et al.*, 2008) and the concentration that was used in the adipose tissue explants was 10 fold higher. However, if these EGCG concentrations are toxic, then this must be specific to EGCG since at similar concentrations there was no effect of Phloridzin, and Resveratrol increased lipolysis in some experiments. When the molecule structures of IP (lipolysis stimulator) and Adenosine (lipolysis inhibitor) was compared with the molecule structures of all the individual polyphenols (Figure 5.9 and 5.10 in Chapter 5), there seems to be no relation between molecular structures and their effects as either a lipolysis stimulant or inhibitor.

The results from the *in vitro* study of individual polyphenols showed variable responses in adipose tissue across different species although the differential effect is not really clear. For future work, it is suggested that the effect of the individual polyphenols on adipose cell apoptosis induction and lipogenesis inhibition were also studied because these mechanisms could be the factors that were affecting the response of lipolysis in the rat, human and pig adipose tissue explants. When planning future work, we have to note that the age of the rats, pigs and humans and also the state of health (normal rats and pigs, obese humans) of the species used in this study were not the same and this might affect the composition of adipocytes (pre-adipocytes, adipocytes and mature cells) in the adipose tissues, resulting in the different effects on lipolysis found.

Additionally, when the effects of polyphenols on lipolysis-in rat adipose tissue explants was compared to similar treatments carried out on isolated adipocytes and cultured adipocyte cell lines, the rat adipose tissue explants response was found to be very similar to the response found in the isolated adipocytes but not to cultured adipocyte cell lines. It is not clear whether this is due to the higher proportion of mature adipocytes found in adipose tissue explants and the isolated adipocytes compared to the cultured adipocyte cell lines.

Since the response to treatment with individual polyphenols was found to be different between human, rat and pig fat explants, the hypothesis that basal and stimulated lipolysis in the rat, human and pig adipose tissue explants are different was tested. This hypothesis was proven to be true as it was found that across species, basal lipolysis was found to be higher in pig perirenal adipose tissue explants (Headland, 2007) than in human adipose tissue explants, but lower than in rat adipose tissue explants. IP stimulated lipolysis was also higher than in the human system, in pig perirenal adipose tissue explants, but not in the pig subcutaneous adipose tissue explants, where IP stimulated lipolysis was similar to that in human omental adipose tissue. However, there was similarity across species in the response to ADA. The presence of adenosine does have an effect on the lipolysis rate in rat, human and pig adipose tissue explants, since the addition of ADA (to metabolise/remove adenosine) increased basal lipolysis in all three cases. Adenosine is known as a major regulator of adenyl cyclase which leads to lipolysis in the adipose tissue (Chaves *et al.*, 2011). Recently, it was shown that removal of endogenous adenosine with adenosine deaminase enhanced lipolysis in adenosine α_1 receptor (+/+) but not adenosine α_1 receptor (-/-) adipocytes (Johansson *et al.*, 2008) and that incubation of rat isolated adipocytes with an adenosine α_1 receptor antagonist induces an increase in lipolytic activity (Szkudelski *et al.,* 2009). These findings provide strong evidence that the antilipolytic effect of adenosine involves impairment of PKA activation.

The rates of lipolysis in adipose tissue explants appears to differ between rat, human and pig, but other variables such as age, cell size, BMI and specific adipose tissue depot are also likely to be important contributory factors in these apparent differences. It is thus not clear that either animal species is a "better" model for humans.

In summary, SCE was found to have an effect on lipolysis in the obese LDLr KO mice but the mechanism for this effect is still unclear. In the future, the study could be extended to investigate changes in the expression of genes related in energy expenditure and lipolysis such as peroxisome proliferator alpha (PPARα) and carnitine palmitoyltransferase (CPT1) to obtain more clues to the mechanism. Activation of PPARα leads to changes in transcription of a large number of genes that regulate lipoprotein metabolism including LPL and changes in transcription of LPL are thought to lead to enhanced lipolysis of VLDL triglycerides resulting in reduced plasma triglyceride levels (Shah *et al.*, 2010). Fatty acid degradation occurs in the mitochondria through the process of β-oxidation and CPT-1 is the rate-limiting step for the entry of fatty acids into the mitochondria (McGarry *et al.*, 1983; Mc Garry *et al.*, 1997; Eaton *et al.*, 2001).

Future *in vivo* work should also consider calculating the composition of adipose tissue in the mice using a more sensitive tool such as the Dual Energy X-ray Absorptiometry (DXA) as the effect of SCE on LDLr KO mice adipose tissue weight might be too small to be detect using conventional methods whilst the CLAMS was able to detect very small changes in RER and heat production in the treated mice.

The bioavailability of the polyphenols in the SCE should also be investigated as it could be different across species. It would be more interesting if humans would be included in this species comparison study as the final application of the SCE would be in the human. However, the treatment of humans with a chloroform-methanol extract might not be approved as chloroform is considered a toxic material. In this case, the polyphenol contents of SCE should be analyzed and identified. Other non-toxic methods of extraction can then be applied to extract the identified polyphenols. These polyphenols can then be used for human study although it may be subjected to an *in vitro* study first.

Since most of the polyhenols studied in the rat and human adipose tissue explants had inconsistent effects on lipolysis, except for EGCG, studies on the metabolized forms of polyphenols might be carried out to compare with the results for the individual polyphenols. Further studies should also measure the effect of individual polyphenols on adipocyte cell apoptosis.

Chapter 9 - References

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Appendix A

Calculation of Heat from the CLAMS

Oxymax will calculate heat with one of two methods. Both methods calculate the heat before the application of any normalization or correction and, thus, reflect the exact heat of the subject. The method termed Internal will derive a calorific value (CV) based on the observed respiratory exchange ratio. This calorific value is then used with the observed oxygen consumption (VO_2) to calculate heat. The equations are:

Heat = CV × VO_2
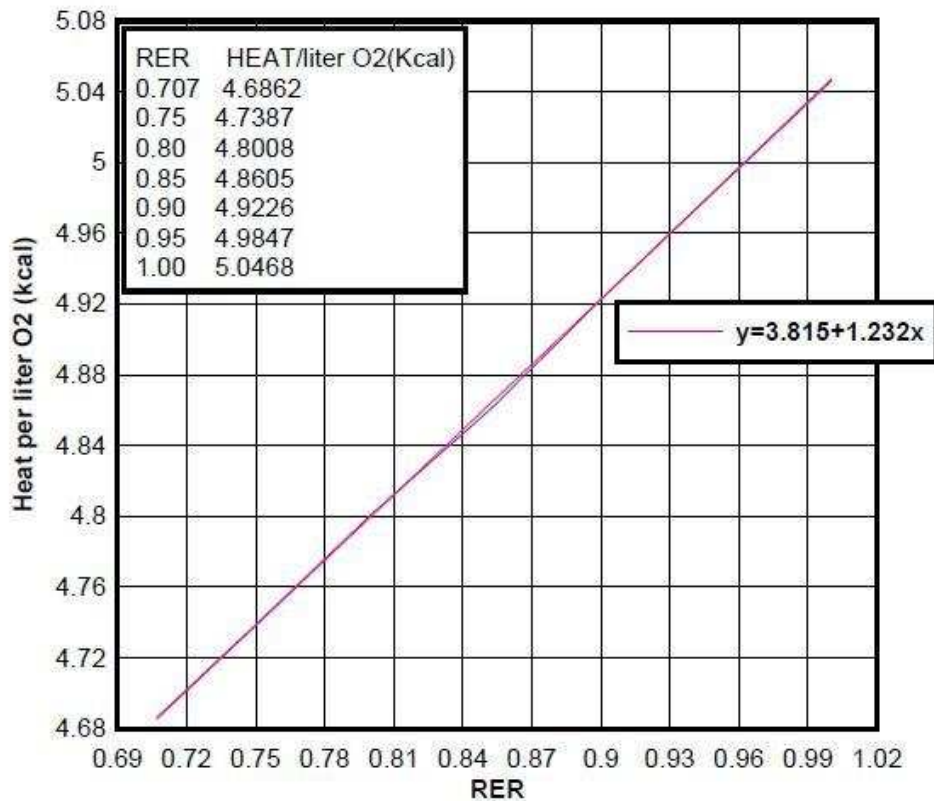
CV = 3.815 + 1.232 × RER

The constants are derived from the following data:

Analysis of the oxidation of mixtures of carbohydrate and fat
(Converted from the table of Lusk, 1928)

RER	% of total O2 consumed by:		% of total heat produced by:		Heat per liter of O2	
	Carbohydrate	fat	Carbohydrate	fat	(kJ)	(kcal)
0.707	0	100.0	0	100.0	19.62	4.6862
0.75	14.7	85.3	15.6	84.4	19.84	4.7387
0.80	31.7	68.3	33.4	66.6	20.10	4.8008
0.85	48.8	51.2	50.7	49.3	20.35	4.8605
0.90	65.9	34.1	67.5	32.5	20.61	4.9226
0.95	82.9	17.1	84.0	16.0	20.87	4.9847
1.00	100.0	0	100.0	0	21.13	5.0468

HEAT per liter of O2 vs. RER
Analysis of the oxidation of mixtures of carbohydrate and fat.
(Converted from the table of Lusk,1928)

RER	HEAT/liter O2(Kcal)
0.707	4.6862
0.75	4.7387
0.80	4.8008
0.85	4.8605
0.90	4.9226
0.95	4.9847
1.00	5.0468

y=3.815+1.232x

(Y-axis: Heat per liter O2 (kcal); X-axis: RER)

A least squares linear fit to the data provides the formula Heat (kcal/liter of O2) = 3.815 + 1.232*RER.

Multiplication by VO2 in liters/unit time yields a heat value in kcal/unit time.

Example calculation: assume VO2 = 4000 ml/hr/kg for a 25 gram mouse and RER = 0.85.

Heat (kcal/hr) = (3.815 + 1.232 * 0.85) * 4000 ml/hr/kg * 1L/1000 ml * 1kg/1000g * 25 g = 0.486 kcal/hr

The other method, termed User Defined, allows the user to enter calorific values (CVx) relating to the oxygen consumption (VO2) the carbon dioxide production (VCO2), and the methane production (VCH4).

Note that the methane term drops out if a methane sensor is not present. Each of the calorific values is entered in the form Kcal/Liter of the respective gas. The equation used is:

Heat = CV1× VO2 +CV2 × VCO2 +CV3 × VCH4

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