The Effects of Sex Steroids on Energy Balance in Sheep

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All Amendments can be found at the back of this eThesis

Contents

Summary

Obesity develops during periods of prolonged positive energy balance, when energy intake exceeds that of energy expenditure. A greater understanding of the energy balance equation may provide new treatment strategies to combat obesity. The recent confirmation of function brown adipose tissue (BAT) in adult humans has renewed interest in adaptive thermogenesis – a component of energy expenditure whereby energy is dissipated via the production of heat. In BAT, adaptive thermogenesis can be induced by cold or dietary cues, and is mediated via the mitochondrial protein uncoupling protein 1 (UCP1). The thermogenic potential of skeletal muscle has also come into focus due to its similar embryonic origins to BAT, and its high expression of the UCP1 homologue UCP3 – which may also have uncoupling properties.

As evidenced by the sexual dimorphism of body composition observed over the lifespan of males and females, the sex hormones (testosterone and estrogen) are thought to play an important role in energy balance. This thesis sought to characterise the role of testosterone and estrogen on energy balance, with a specific focus on adaptive thermogenesis.

A model of postprandial temperature elevation in the sheep has previously been established by this laboratory. By restricting food intake to a daily "feeding window" temperature elevations during the postprandial period are entrained. They are measured by temperature recording devices (dataloggers) implanted in the peripheral tissue. Studies conducted in Chapter 3 of this thesis aimed to investigate the influence of regional blood flow on muscle temperature. Results confirm a disassociated of muscle temperature from regional blood flow in a number of paradigms including during entrained postprandial temperature elevation. In addition, intravenous infusion of high but not low doses of the β1/β2-adrenoreceptor agonist isoprenaline

increased muscle temperature in addition to blood flow. This effect was attributed to "spillover" activation of β3-adrenoreceptors, the primary peripheral receptor involved in the initiation of adaptive thermogenesis. Collectively, these studies strengthen the case for a role of skeletal muscle thermogenesis in energy balance.

Chapter 4 investigated the effects of chronic testosterone replacement in male and female castrate sheep. In males but not females testosterone decreased visceral and muscle temperature at baseline and during the postprandial period. This occurred without affecting the duration or amplitude of the postprandial temperature response. In males, testosterone decreased postprandial plasma glucose levels without changes to activation of skeletal muscle Akt or AMP- activated protein kinase (AMPK), key regulators of glucose uptake and fatty acid oxidation. In males, Expression of UCP1 and UCP3 mRNA was shown to be elevated in muscle taken during the postprandial period compared to baseline. This occurred independent of testosterone treatment. Elevations in UCP1 and UCP3 expression may be responsible for the temperature increases observed.

Chapter 5 investigated the effects of acute and chronic estrogen replacement on energy balance in castrated ewes. Acute intramuscular injection of estradiol-17β benzoate (50μg) increased skeletal muscle and visceral fat temperature within one hour, compared to vehicle control. Skeletal muscle blood flow was unaltered during this period. Gluteal fat had no such reaction to acute estradiol-17β benzoate administration. Chronic (7 days) estrogen replacement (3x3cm subcutaneous estradiol-17β benzoate implants) had no effect on tissue temperature compared to control (blank implants). Acute estradiol-17β benzoate replacement was shown to increase activation of Akt and AMPK in skeletal muscle during the postprandial period. This indicates increased fuel utilisation. Repeated intravenous bolus infusion of estrogen (25μg, 3 infusions 3 hours apart) induced temperature elevations in muscle with a greater duration and amplitude than a single intramuscular dose. Expression of UCP mRNA could not account for the estrogen-induced temperature elevations.

Collectively, the body of work presented in this thesis strengthens the notion that skeletal muscle is capable of thermogenesis. This work also presents evidence that estrogens and testosterone differentially regulate energy balance. Estrogens regulate energy expenditure acutely but not chronically in a tissue specific manor, increasing tissue temperature and fuel utilisation. Testosterone regulates energy expenditure in males but not females, decreasing tissue temperature. Understanding the actions of sex steroids on energy balance may lead to novel targets in combatting obesity.

Declaration

I hereby declare, that to the best of my knowledge, this thesis contains no material previously published or written by any other person, except where due reference is made in the text. In addition, no part of this thesis has been submitted for the award of any other degree or diploma at any university or equivalent institution. All experiments reported in this thesis complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and had approval from the Monash University Animal Welfare Committee.

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Publications

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Conference Presentations

Clarke S.D., Cowley M.A., Clarke I.J. and Henry B.A. Sexual divergence in the metabolic effects of testosterone. ENDO: 93rd Annual Meeting & Expo. Boston, U.S.A. (2011)

Clarke S.D., Clarke I.J., Cowley M.A. and Henry B.A. Testosterone but not α-melanocyte stimulating hormone (αMSH) modulates skeletal muscle temperature and glucose utilisation in male sheep. The 7th International Congress of Neuroendocrinology. Rouen, France. (2010)

Henry B.A., Cowley M.A., **Clarke S.D.**, Leury B. and Clarke I.J. Direct effects of α-melanocyte stimulating hormone on energy expenditure in skeletal muscle. The 7th International Congress of Neuroendocrinology. Rouen, France. (2010)

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Clarke S.D., Clarke I.J., and Henry B.A.. The effects of acute and chronic estrogen replacement on energy expenditure in the ovariectomised ewe. Endocrine Society of Australia Annual Scientific Meeting. Melbourne, Australia (2008).

Abbreviations, Symbols and Units of Measure

Abbreviations

18F	Fluorine-18
5α –DHT	5α-dihydro-testosterone
ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
ADR	Adrenergic receptor
AgRP	Agouti related protein
AICAR	5-Aminoimidazole-4-carboxamide ribotide
AMP	Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
AMPKK	AMPK kinase
approx.	Approximately
AR	Androgen receptor
ARC	Arcuate nucleus
ARKO	Androgen receptor knock-out
ATP	Adenosine triphosphate
AU	Arbitrary units
AVP	Arginine vasopressin
B2M	Beta-2 microglobulin
BAT	Brown adipose tissue
BMI	Body mass index
BMR	Basal metabolic rate
BSA	Bovine serum albumin

Ca2	Calcium ion
CAC	Citric acid cycle
cAMP	Cyclic adenosine
CAT [scan]	Computed axial tomography [scan]
CCK	Cholecystokinin
cDNA	Complimentary DNA
CoA	Coenzyme A
CPT1	Cytochrome oxidase subunit IV
CREB	cAMP response element-binding
CRH	Corticotropin releasing hormone
Ct	Threshold cycle
CVD	Cardiovascular disease
DEXA	Dual energy X-ray absorptiometry
DII	Type II thyroxine deiodinase
DMH	Dorsomedial hypothalamus
DNA	deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
Eg.	Example
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
et al.	*et alii* (and others)
ETC	electron transport chain
Etc	*et cetera* (and so forth)
FADH	Flavin adenine dinucleotide
FDG-PET	Fludeoxyglucose (18F)
FOXC2	Forkhead box protein C2
FSH	Follicle stimulating hormone
GDP	Guanosine diphosphate
GHS-R	Growth hormone secretagogue receptor
GLUT	Glucose transporter type

H^+	Proton
HK	Housekeeping
HSP	Heat shock protein
i.v.	Intra venous
LHA	Lateral hypothalamic area
MCH	Melanin-concentrating hormone
MCR	Melanocortin receptor
MDH1	Malate Dehydrogenase
mRNA	Messenger Ribonucleic acid
mTOR	Mammalian target of rapamycin
NA `	Noradrenaline
NADH	Nicotinamide adenine dinucleotide
NEAT	Non-exercise activity thermogenesis
NEFA	Non-esterified fatty acid
NPY	Neuropeptide Y
NRF-1	Nuclear respiratory factor 1
O2	Oxygen
OVX	Ovariectomised
p107	107 kDa retinoblastoma-associated protein
p38 MAPK	P38 mitogen-activated protein kinase
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PET	Positron emission tomography
PGC	Peroxisome proliferator-activated receptor gamma coactivator
Pi	Inorganic phosphate
PKA	Protein kinase A
POMC	Proopiomelanocortin
PPARγ	Peroxisome proliferator-activated receptor gamma
PRDM16	PR domain containing 16
PVN	Paraventricular nucleus

RIP140	Nuclear receptor-interacting protein 1
ROS	Reactive oxygen species
Rt-PCR	Real time Polymerase chain reaction
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error (of the) mean
Ser	Serine
T3	3,5,3'-triiodothyronine
Thr	Threonine
TMB	3,3',5,5'-Tetramethylbenzidine
UCP	Uncoupling protein
VMN	Ventromedial nucleus
WAT	White adipose tissue
Y1-Y5	NPY receptor (1 – 5)
YWHAZ	Tyrosine 3-monooxygenase
αERKO	Estrogen receptor alpha knock-out
α-MSH	Alpha melanocyte-stimulating hormone
βERKO	Estrogen receptor beta knock-out

Symbols

α	Alpha	~	Approximately
β	Beta	±	Plus or minus
γ	Gamma	Δ	Delta (change in)
%	Percent	P	P-value
$	Dollar	<	Less than

Units of Measure

µl	Microlitre	min	Minutes
C	Celsius	ml	Millilitre
G	Gauge	mm	Millimetre
h	Hours	ng	Nanogram
K	1,000 units	nl	Nanolitre
kcal	Kilocalorie	rpm	Revolutions per minute
kg	Kilogram	UI	International unit
L	Litre	V	Volt
M	Molar	W	Watts
m	Metre	Wks	Weeks
mg	Milligram		

CHAPTER 1
Review of the Literature

1.1 General Introduction

The prevalence of obesity in developed and developing nations has increased dramatically in the previous decades. This has come at great personal, social and economic cost. Presently, obesity prevention and treatment programs focus on controlling diet and encouraging exercise as the main tenants of body weight regulation. Empirical and anecdotal evidence suggests that these intervention methods yield only short term benefits at best and are wrought with non-compliance.

The regulation of body weight is a multifaceted process encompassing a wide range of physiological systems. Food intake and exercise are only two components of the equation. Energy expenditure is comprised of five major facets including basal metabolic rate, exercise, non-exercise activity thermogenesis (NEAT), shivering thermogenesis and adaptive thermogenesis. Recently, there has been an increasing emphasis on understanding the pathways and mechanisms involved in these forms of energy expenditure, particularly adaptive

thermogenesis. Greater understanding of these mechanisms may pave the way for new treatment strategies to combat obesity. The recent surge of interest in the role of adaptive thermogenesis in energy balance stems from a series of papers published in 2009 confirming the presence of functional brown adipose tissue (BAT) in adult humans. The thermogenic potential of skeletal muscle has also come into focus due to its similar embryonic origins with BAT.

Generally, sexual dimorphism of body composition is finalised after puberty, extending throughout adolescence and into adulthood. An android (central/visceral) distribution of fat is seen in males but can also develop in post-menopausal women (Trémollieres *et al.*, 1996). This type of fat distribution has associated health risks. Hormone replacement therapy in post-menopausal women can reverse the changes seen in this condition. A gynoid (subcutaneous/gluteal) adipose distribution is seen in premenopausal women and is relatively benign. While the sex steroids estrogens and testosterone can affect food intake, obesity associated with changes in sex steroids can develop independent of energy intake. This suggests an important role for energy expenditure in the aetiology of obesity in these cases. Associated changes in the levels of sex steroids (eg. menopause) may be a factor that exacerbates the obese condition. Little is known about how sex steroids regulate the different facets of energy expenditure. The regulatory roles of sex steroids on energy expenditure may occur at the level of the brain, or within peripheral tissues such as fat and muscle.

This thesis sought to investigate the effects of testosterone and estrogen on energy balance. This review of the literature encompasses the regulatory control of sex steroids on the many facets of the energy balance equation, with a particular focus on adaptive thermogenesis in BAT and skeletal muscle.

1.2 Obesity

1.2.1 Defining and Measuring Obesity

In its most simple definition, obesity is the accumulation of adipose tissue beyond normal or healthy levels. In actuality, obesity is a complex state of chronic dyshomeostasis incorporating interactions between central and peripheral factors and physiological systems such as inflammatory pathways and endocrine signalling.

Various methods are used to define what constitutes being 'overweight' or 'obese'. The simplest and most widely used method is the body mass index (BMI) - an index used to describe body weight in proportion to height. BMI is calculated via the following equation:

$$\text{BMI} = \text{Body Mass (kg)}/\text{Height (m)}^2$$

The normal or healthy range of BMI values is 20 – 24.9 kg/m^2, with the overweight range being 25 – 29.9 and obese range 30+ kg/m^2 (WHO, 2000). BMI is a quick and inexpensive diagnostic tool for the evaluation of an individual's weight in relation to height and as estimate of adiposity. In spite of its widespread use, the BMI has a number of shortcomings. In the context of large-scale population studies, BMI is often self-reported by participants. People tend to over-estimate their height and under-estimate their weight, leading to low estimates of BMI score (Spencer *et al.*, 2002). Also, BMI assumes variation in body mass between people of the same height is due to changes in adipose mass and it does not account for differences in lean mass (eg. muscle and bone). This can lead to inaccurate diagnosis for those with larger lean mass (Nawaz *et al.*, 2001; Garn *et al.*, 1986) and an over-estimation of the incidence of obesity. In addition, BMI does not take into account the distribution of adipose mass. Previous studies have suggested that an increase in subcutaneous adipose tissue is relatively benign and is not associated with the increased risk of the obesity-associated metabolic disorders including type II diabetes and cardiovascular disease (CVD; discussed below). In this regard, measurement of

waist circumference or waist to hip ratio may be a more accurate approximation of deleterious fat distribution. Indeed, waist circumference, an indicator of visceral adiposity, can be a more accurate predictor of health risks such as CVD (Huxley *et al.*, 2010).

Other techniques such as bioelectrical impedance analysis (Kushner and Schoeller, 1986; Houtkooper *et al.*, 1992) and anthropometric measurement of skin folds (Ball *et al.*, 2004) can also be used to assess body fat. Both require specialised equipment, and anthropometry requires a technician who has been trained to accurately measure and analyse the data.

The 'gold standard' for body fat measurement is dual-energy X-ray absorptiometry (DEXA); a sophisticated technique available in clinical and research settings to directly and accurately ascertain the distribution and abundance of adipose tissue within an individual (Haarbo *et al.*, 1991). In spite of its accuracy, the use of DEXA on humans is limited due to the potential negative effects of X-ray exposure. DEXA machines are also expensive, further limiting the use of the technique.

In summary, each method used for the measurement of body weight and obesity has relative advantages and disadvantages. Clinically, the most important component of obesity is visceral adiposity, due to the health risks associated with this particular fat depot. In this context, measures of adiposity such as waist circumference and dual energy X-ray absorptiometry (DEXA) analysis are more valuable than measures such as BMI.

1.2.2 Causes and Prevalence of Obesity

One popular theory poses that from an evolutionary standpoint the propensity to gain and store fat was an advantageous adaptation during early human history - in times of nutritional scarcity (Brown and Konner, 1987). The twentieth century saw dramatic changes in our standard of

living and our environment. The technological revolution, urbanisation and trends towards readily available, energy-dense food created a more obesogenic environment in many developed and developing countries (Townshend and Lake, 2009; Caballero, 2007; Ulijaszek, 2008). Consequently, from the latter half of the twentieth century until the present day, the proportion of overweight and obese people has increased markedly. The true extent and cost of these changes are only now being recognised.

In Australia, the prevalence of obesity doubled in the 20 years between 1983 and 2003 (Cameron *et al.*, 2003). According to the Australian Bureau of Statistics 2007-08 National Health Survey, 61% of Australians are categorised as overweight or obese (AustralianBureauofStatistics, 2009). A recent study of the prevalence of obesity in Australia highlighted that, not only is a larger proportion of the population overweight or obese, but there seems to be a rightwards skew in the BMI distribution pattern, meaning there is an increased prevalence of severely obese people (Walls *et al.*, 2010). If this trend continues, it is projected that by 2025, the proportion obese people will increase by 65% from current levels (Walls *et al.*, 2011). Sex differences in overweight and obesity rates exist. Recent estimates suggest that 48% of men and 30% of women in Australia are overweight, and an additional 19% of men and 22% of women are obese (Thorburn, 2005). Weather these sex differences are based on physiological, cultural or psycho-social reasons (or a mix of the three) is unknown. Obesity has become a major health issue within Australia, with the financial cost of obesity estimated to be $8.283 billion in 2008 (Economics, 2008).

Other developed, western countries such as the United States of America (Flegal *et al.*, 2010; Colditz, 1999), Canada (Bélanger-Ducharme and Tremblay, 2005), and Britain (Rennie and Jebb, 2005) report obesity rates similar to those in Australia. The obesity ‘epidemic’ was initially considered to be a problem of the developed world but as developing nations adopt a western-style of living, obesity rates in these countries are beginning to mirror those of developed countries (Prentice, 2006; Ewing *et al.*, 2008; Deckelbaum and Williams, 2001;

Uauy *et al.*, 2001). Childhood obesity is another, relatively more recent concern (Wang and Lobstein, 2006). It is a strong predictor of adulthood obesity (Eriksson *et al.*, 2003), as well as a precursor to increased morbidity and mortality rates later in life (Wang and Lobstein, 2006; Dietz, 1998). Clearly, obesity is an issue worthy of worldwide concern, affecting a growing number of people in developed and developing nations.

1.2.3 Metabolic Syndrome and Heath Consequences of Obesity

Initially popularised under the name syndrome X by endocrinologist Professor Gerald Reaven (Reaven, 1988), the metabolic syndrome was quickly adopted as a term to define a complex and perhaps synergistic amalgam of clinical outcomes seen to be related to obesity. Nebulous in its definition, the World Health Organisation (WHO) acted to create a unifying diagnostic tool for the metabolic syndrome. Although different sets of criteria have been suggested, it is generally agreed upon that a diagnosis of metabolic syndrome is given if a patient exhibits impaired glucose regulation/diabetes/insulin resistance and at least two of the following: chronic hypertension, dyslipidemia, central obesity or microalbuminuria (Alberti and Zimmet, 1998; Alberti *et al.*, 2009). It is of note that obesity is not a prerequisite for metabolic syndrome.

In Australia, 20-33% of the adult population is estimated to have metabolic syndrome, with the percentage depending on the diagnostic criteria used (Cameron *et al.*, 2007). It is estimated that approximately 65,000,000 citizens of the United States of America have metabolic syndrome (Ford *et al.*, 2004). In short, it is widespread throughout developed nations.

The use of the metabolic syndrome as a clinical endpoint rather than as a term used for a collection of distinct pathologies has recently come under fire (Kahn *et al.*, 2006). Even Prof. Reaven has warned of the phrase's misappropriation (Reaven, 2005). It is in doubt whether the

metabolic syndrome confers with it additional CVD risks above that of the pathologies that define it; in other words, weather it is more than the sum of its parts (Golden *et al.*, 2002).

Regardless of this, there is little doubt that obesity, and in particular visceral obesity is associated with a number of health risk factors. Cardiovascular disease risk factors such as high blood pressure and high circulating lipid levels, as well as the incidence of type II diabetes correlate to visceral adiposity regardless of total fat content (Despres *et al.*, 2008; Farajian *et al.*, 2008). The prevalence of stroke (Larsson *et al.*, 1984) and various forms of cancer (Calle and Thun, 2004) also increases with visceral adiposity.

The onset of obesity and metabolic syndrome may be related to energy expenditure. By understanding the mechanisms involved in how the body regulates energy expenditure, targets for the treatment of these conditions may be elucidated.

1.3 Energy Balance and the Regulation of Body Weight

1.3.1 Overview

Maintenance of body weight is achieved through a balance between energy intake and whole body energy expenditure. Energy intake is the sum total of energy derived from food consumed and energy expenditure is the sum total of energy utilised by the body.

Most individuals are able to maintain energy balance and experience only minor fluctuations in body weight. Chronic disruption of energy balance leads to changes in body weight. In particular, sustained periods during which food intake exceeds energy expenditure (positive energy balance) will result in weight gain and the development of obesity.

The regulation of food intake and energy expenditure is critical for the survival and wellbeing of an individual. It is maintained by a complex integration between neural and endocrine systems. The systems which regulate feeding and energy expenditure utilise many common pathways; whereby particular factors that reduce food intake (eg. Leptin) also increase energy expenditure (Woods *et al.,* 1998). As will be discussed, energy balance requires precise interplay between peripheral (blood borne) factors and neuropeptides/neurotransmitters in the brain. This interplay responds to changing metabolic states.

1.3.2 Regulation of Food Intake

To ensure equilibrium of the energy balance equation, food intake must be tightly regulated. This is achieved through the integration of metabolic, endocrine and neural parameters. At the level of the brain, the hypothalamus is thought to be a central hub involved in the regulation of food intake and metabolism (Spiegelman and Flier, 2001).

1.3.2.1 The Arcuate Nucleus

The arcuate nucleus (ARC) of the hypothalamus is comprised of the 'first order' neurons involved in energy balance. The 'first order' neurons receive peripheral signals related to energy balance, relaying information on energy status to 'second order' neurons. The ARC is well positioned to detect and respond to peripheral signals due to proximity to the blood brain barrier. At least some portions of the ARC are outside the blood-brain barrier, with fenestrations within the blood vessels allowing direct passage of blood borne factors into at least part of the nucleus (Norsted *et al.,* 2008). The ARC contains both anorexigenic orexigenic neuronal populations. This allows it to finely tune food intake.

The anorexigenic neurons of the ARC produce melanocortins, peptides derived from the proopiomelanocortin (POMC) precursor (Boston *et al.*, 1997). In the brain, the melanocortins (α, β, and γ melanocyte-stimulating hormones) act through melanocortin 4 receptors (MCR4) expressed by second order neurons to decrease food intake and increase energy expenditure (Mountjoy *et al.*, 1994; Woods *et al.*, 1998; Balthasar *et al.*, 2005). The POMC precursor can also produce endorphins, which have the opposite effects on energy balance (David McKay *et al.*, 1981; Giugliano and Lefebvre, 1991). The activity of these peptides is regulated by acetylation, whereby acetylation nullifies the action of endorphins and enhances the action of melanocortins (Tsujii and Bray, 1989).

The orexigenic neurons of the ARC produce neuropeptide Y (NPY) and agouti related protein (AgRP). AgRP is expressed exclusively in NPY-producing cells of the ARC (Broberger *et al.*, 1998; Boswell *et al.*, 2002). The stimulatory effects of NPY and AgRP on food intake are well documented (Clark *et al.*, 1984; Stanley and Leibowitz, 1984; Gropp *et al.*, 2005). NPY is a direct, positive driver of appetite and a negative driver of energy expenditure. At least five subtypes of the NPY receptor are expressed in mammalian species (Y1-Y5). In rodents, Y1, Y2 and Y5 are important for the regulation of energy balance (Pedrazzini *et al.*, 1998; Marsh *et al.*, 1998). In sheep, Y1 is thought to mediate the orexigenic effects of NPY (Clarke *et al.*, 2005). AgRP elicits its orexigenic effects by acting as an agonist of MCR4. Essentially, it blocks the anorexigenic effects of the melanocortins (as discussed above; Fong *et al.*, 1997; Mizuno *et al.*, 2003). Further interaction between NPY/AgRP and POMC neurons can be evidenced by their reaction to leptin stimulation, whereby NPY/AgRP neurons are able to directly modify POMC neuronal activity (Cowley *et al.*, 2001).

Neurons of the ARC are able to receive peripheral, blood-borne signals due to expression of different receptors types. For example, NYP/AgRP and POMC neurons express leptin (Hahn *et al.*, 1998; Ahima *et al.*, 2000; Iqbal *et al.*, 2001a) and insulin receptors (Choudhury *et al.*, 2005; Konner *et al.*, 2007). NPY/AgRP neurons also express the receptor for ghrelin, a peptide

released from the gut involved in the initiation of eating (Willesen *et al.*, 1999). ARC neurons can also sense and respond to glucose (Burdakov *et al.*, 2005; Lynch *et al.*, 1997; Fioramonti *et al.*, 2007) and fatty acids (Rossetti *et al.*, 2005). Of particular importance to this thesis, the actions of ARC neurons can be influenced by estrogens (Simerly *et al.*, 1990; Shughrue *et al.*, 1996) and androgens (Simerly *et al.*, 1990). Sex steroid receptors are not abundantly expressed in NPY/AgRP and POMC neurons of the ARC. Instead, the effects of sex steroids may be influenced by other neurons. For example, estrogen receptor expressing kisspeptin receptors are able to modulate energy balance in response to estrogens via NPY and POMC neurons (Backholer *et al.*, 2010).

1.3.2.2 The Lateral Hypothalamic Area.

The lateral hypothalamic area (LHA) is one region of the hypothalamus that is a 'second-order' target of ARC neurons. Based on lesioning studies, the LHA was originally defined as the feeding centre, as its ablation results in hypophagia and weight loss (Anand and Brobeck, 1951; Elmquist *et al.*, 1999). The regulatory role of the LHA on food intake was later explained by the discovery of two orexigenic neuropeptides expressed in this area: the orexins and melanin-concentrating hormone (MCH). These neuropeptides are found in a variety of species including rats (Kawano *et al.*, 2002; Bridget I, 1991) and sheep (Iqbal *et al.*, 2001b; Tillet *et al.*, 1996).

Orexins (subtypes A and B) are predominantly expressed in neurons of the LHA, the perifornical area in rats (Sakurai *et al.*, 1998), and the dorsomedial nucleus in sheep (Qi *et al.*, 2008). Dichotomous results have been found in relation to the effects of orexins on energy balance. Some rodent models have shown that orexins not only stimulate food intake (Elias *et al.*, 1998; Rodgers *et al.*, 2002) but have also been shown to increase metabolic rate (Lubkin and Stricker-Krongrad, 1998) and body temperature (Jászberényi *et al.*, 2002). Another model has shown the opposite effects (Verty *et al.*, 2010). In rats, strong reciprocal connection between

orexin neurons of the LHA and neurons of the ARC have been observed (Elias *et al.*, 1998), supporting the 'first order – second order' hypothesis. As discussed below, similar observations have been made in the sheep (Qi *et al.*, 2010).

MCH is expressed within neurons of the LHA and zona incerta (Bittencourt *et al.*, 1992). Both NPY/AgRP and α-MSH containing cells of the ARC project to MCH cells (Elias *et al.*, 1998). This further indicates a regulatory role of the ARC over the LHA. MCH potently stimulates food intake in a variety of species, as evidenced by studies whereby MCH was injected into the ARC, paraventricular nucleus and dosromedial nucleus (Kawano *et al.*, 2002; Qu *et al.*, 1996; Ludwig *et al.*, 2001; Abbott *et al.*, 2003). MCH receptors are expressed in a variety of brain regions, including those involved in regulation of food intake such as the ventromedial nucleus, dorsomedial nucleus and ARC. In mice, intracerebroventrical infusion of MCH results in reduced energy expenditure and body temperature (Glick *et al.*, 2009)

1.3.2.3 The Ventromedial Nucleus

The ventromedial nucleus (VMN) was initially termed the satiety centre of the brain; and was seen as complementary to the LHA (Beltt and Keesey, 1975). Electrophysiological studies have shown a relationship between these two areas whereby spontaneous firing of neurons in one centre is concurrent with decreased firing in the other (Oomura *et al.*, 1967; Roesch and Felix, 1984). Neurons of the VMN which regulate food intake express steroidogenic factor-1 (SF-1); nutritional factors such as leptin exert their effects through these neurons (Dhillon *et al.*, 2006). In the sheep, neurons of the VMN predominantly project to the preoptic area, bed nucleus of the stria terminalis and the anterior hypothalamic area (Pompolo *et al.*, 2001). Interconnectivity between the ARC and VMN has also been shown in sheep (Qi *et al.*, 2008) rodents (Luiten and Room, 1980; Elmquist, 2001; Bernardis and Bellinger, 1993) and humans (Elias *et al.*, 1998; Elias *et al.*, 1999). The VMN controls satiety by acting on a range of nuclei in the brain.

1.3.2.4 The Dorsomedial Hypothalamus

Stimulation of the dorsomedial hypothalamus (DMH) increases food intake (Larsson, 1954), while ablation of the nucleus via lesioning decreases food intake (Bellinger and Bernardis, 2002; Bernardis, 1975). The DMH is also critical for the regulation of food-entrainable circadian rhythms (Gooley *et al.*, 2006) and receives input from many hypothalamic nuclei including the LHA, and paraventricular nucleus. Additionally, at least in rodent species, the DMH receives a high number of both NPY (Broberger *et al.*, 1998) and α-MSH (Jacobowitz and O'Donohue, 1978) projections from the ARC. NPY is also expressed in neurons of the DMH as well as the ARC (Bi *et al.*, 2003). Its action in the DMH greatly increases food intake (Yang *et al.*, 2009). As mentioned above, orexin-expressing neurons are also found in the ovine DMH.

1.3.2.5 The Paraventricular Nucleus

The paraventricular nucleus (PVN) is an important regulator of the autonomic nervous system and behaviour (Luiten *et al.*, 1987). The PVN receives NPY/AgRP and POMC neuron projections from the ARC (Baker and Herkenham, 1995). NPY/AgRP neurons regulate the function of thyotropin-releasing hormone containing neurons of the PVN, influencing food intake and energy expenditure (Martin *et al.*, 2006). Accordingly, injection of orexigenic peptides such as NPY into the PVN results in hyperphagia (Kim *et al.*, 2000; Stanley *et al.*, 1985). Corticotropin releasing hormone (CRH), a potent anorexigenic hormone produced in parvocellular neurons of the PVN can inhibit these effects of NPY (Heinrichs *et al.*, 1993). Select neurons of the PVN also express arginine vasopressin (AVP) which can inhibit food intake and activate the hypothalamic-pituitary-axis (Martin *et al.*, 2006). Oxytocin neurons play an important function in the relay of signals from the ARC to the brain stem (Olson *et al.*, 1991;

Olson *et al.*, 1993). Oxytocin-releasing neurons of the PVN are thought to mediate the hypophagic response of leptin to the brainstem (Blevins *et al.*, 2004).

1.3.2.6 Summary of Neuronal Control of Food Intake

While the areas of the hypothalamus discussed above are important brain regions in the regulation of food intake, many other regions of the brain are also important. Another hypothalamic nuclei, the suprachiasmatic nucleus (SCN), is known as the 'body clock' and regulates circadian rythyms based predominantly on day/night exposure. It can also regulate food intake (Hillebrand *et al.*, 2002). Extra-hypothalamic areas, such as the brain stem also play roles in energy balance. The brain stem receives projections from various hypothalamic regions, and aides in the regulation of food intake and energy expenditure (Morrison *et al.;* Szelényi *et al.*, 1977; Williams *et al.*, 2003) be regulating sympathetic outflow to peripheral tissues.

Cholecystokinin (CCK) is a satiety signal released from the intestines in response to nutritional signals from chyme (Douglas *et al.*, 1990; Lewis and Williams, 1990). CCK injection into the brain can reduce meal size (Figlewicz *et al.*, 1986; Zhang *et al.*, 1986). CCK produces these effects via actions on the brainstem, which relays the signals to the hypothalamus (Schwartz, 2006; Fraser and Davison, 1992).

In summation, the neuronal control of energy balance is a tightly regulated system involving a number of brain regions and neuropeptides. Further complicating the picture, hypothalamic pathways involved in the regulation of food intake vary between species. In the ovine brain, the LHA does not appear to be a major 'second order' target of the ARC, though connections do exists between the LHA and VMN (Qi *et al.*, 2008). Instead, the ARC may have indirect effects on the LHA via the VMN, DMH, or PVN (Qi *et al.*, 2010). In the sheep, it has been shown that in the absence of the ARC, leptin can still assert effects via direct action on a number of nuclei

including the DMH, VMN and PVN (Qi *et al.*, 2010). These findings are contrary to the 'first order – second order' hypothesis, as the ARC is not needed for the anorectic effects of leptin to occur. Similar studies in rodents have not been carried out.

1.3.2.7 Peripheral Metabolic Factors Which Affect Food Intake

As indicated above, blood-borne metabolic factors regulate the activity of hypothalamic neurons. The following is a description of the main actions of the most salient metabolic factors which centrally drive change in food intake and energy expenditure.

Nutrient sensing by the hypothalamus allows food intake and energy expenditure to be directly affected by circulating levels of glucose and fatty acids. When administered into the hypothalamus long chain fatty acids decrease AgRP and NPY mRNA expression in the ARC, inhibiting food intake in the rat (Morgan *et al.*, 2004; Obici *et al.*, 2002). To a similar end, long chain fatty acids can excite POMC neurons of the ARC (Jo *et al.*, 2009). Malonyl-CoA, an intermediate in fatty acid synthesis plays a key role in nutrient signalling in the hypothalamus. Levels of malonyl-CoA inversely regulate food intake (Wolfgang and Lane, 2006). Malonyl-CoA levels can be modified by both fatty acids and glucose levels via the activation of 5' AMP-activated protein kinase (AMPK; Wolfgang *et al.*, 2007; Xue and Kahn, 2006). As discussed later, AMPK can sense cellular energy levels. As such, AMPK regulates food intake in response to acute changes in glucose levels in the hypothalamus (Minokoshi *et al.*, 2004).

In addition to direct actions of fatty acids and glucose, the hypothalamus is responsive to a number of metabolic signals originating in the periphery. One such regulator is the hormone leptin, which is primarily synthesised and released from white adipose tissue; the gene was first cloned in 1994 by Zhang *et al.* (Zhang *et al.*, 1994). A number of studies have demonstrated that circulating leptin levels are in proportion to total fat mass (Frederich *et al.*, 1995; Maffei *et al.*,

1995). Leptin acts to reduce food intake and increase energy expenditure via a number of hypothalamic areas including the ARC, VMN, DMH and PVN (Elias *et al.*, 2000; Henry *et al.*, 2008). Leptin resistance (the inability of the body to properly respond to leptin levels), has been linked to obesity (Myers Jr *et al.*, 2010). Leptin action can be modulated by estrogens, with increased hypothalamic leptin sensitivity in the presence of estrogens (Clegg *et al.*, 2006; Gao and Horvath, 2008). Clegg *et al.* (Clegg *et al.*, 2006). Administration of estrogen to male rats essentially 'feminises' their response to leptin. This exemplifies the important role sex steroids can play in the regulation of energy balance.

Insulin is secreted by the β-cells of the islets of Langerhans in the pancreas in response to elevated plasma glucose levels. This typically occurs as a result of food consumption. Plasma insulin levels correlate positively to levels of adiposity (Polonsky *et al.*, 1988). This may be because obese individuals become insulin-resistant with increased adiposity, leading to hyperinsulinemia. Insulin can act on peripheral tissues such as skeletal muscle to increase its rate of glucose uptake. It may also cross the blood brain barrier (Banks, 2006) and regulate energy balance. Accordingly, infusion of insulin into the brain results in decreased food intake and increased energy expenditure (Woods *et al.*, 1979; Porte and Woods, 1981). This is achieved via modulation of POMC and NPY/AgRP containing neurons in the ARC (Belgardt *et al.*, 2009). The central effects of insulin on energy balance may be sexually divergent, with male baboons experiencing accumulation of adipose tissue after treatment with insulin while females do not (Woods *et al.*, 1979).

In humans, plasma levels of ghrelin are elevated preceding a meal, decreasing quickly postprandially (Cummings *et al.*, 2001). In animal models, ghrelin treatment increases food intake and decreases fat utilisation (Nakazato *et al.*, 2001; Tschop *et al.*, 2000). The ghrelin receptor (GHS-R), is expressed by NPY/AgRP neurons of the ARC. Peripheral ghrelin administration induces activation of NPY/AgRP neurons of the ARC (Wang *et al.*, 2002).

Central administration of ghrelin also activates other hypothalamic areas such as the PVN and DMH and areas of the brainstem (Lawrence *et al.*, 2002).

1.3.3 Energy Expenditure

While regulation of food intake has been extensively studied, the importance of, and mechanisms underpinning energy expenditure are less well understood. This component of the energy balance equation is highly variable between individuals, with a strong genetic component (Goran, 1997; Ravussin and Bogardus, 2000). It may also serve as a realistic target for control of and/or prevention of obesity. Studies conducted on twins by Bouchard and Tremblay (Bouchard and Tremblay, 1997) suggest a strong genetic component of weight gain in humans. In this study, innate differences in weight gain between individuals was largely determined by differences in the rate of energy expenditure (Bouchard and Tremblay, 1997; Bouchard, 1997). Twins show low variation in their response to both positive (Bouchard *et al.*, 1990) and negative energy balance (Tremblay *et al.*, 1997). In contrast, highly variable weight change responses occur between non-related individuals. These studies emphasise the role of both genetics and energy expenditure in energy balance.

As mentioned, energy expenditure can be subdivided into five main categories: basal metabolic rate, shivering thermogenesis, exercise, NEAT and adaptive thermogenesis. The approximate relative contribution of these components towards daily energy can be seen in Figure 1.1, and will be discussed below in the context of physiological mechanisms and therapeutic potential in combating obesity.

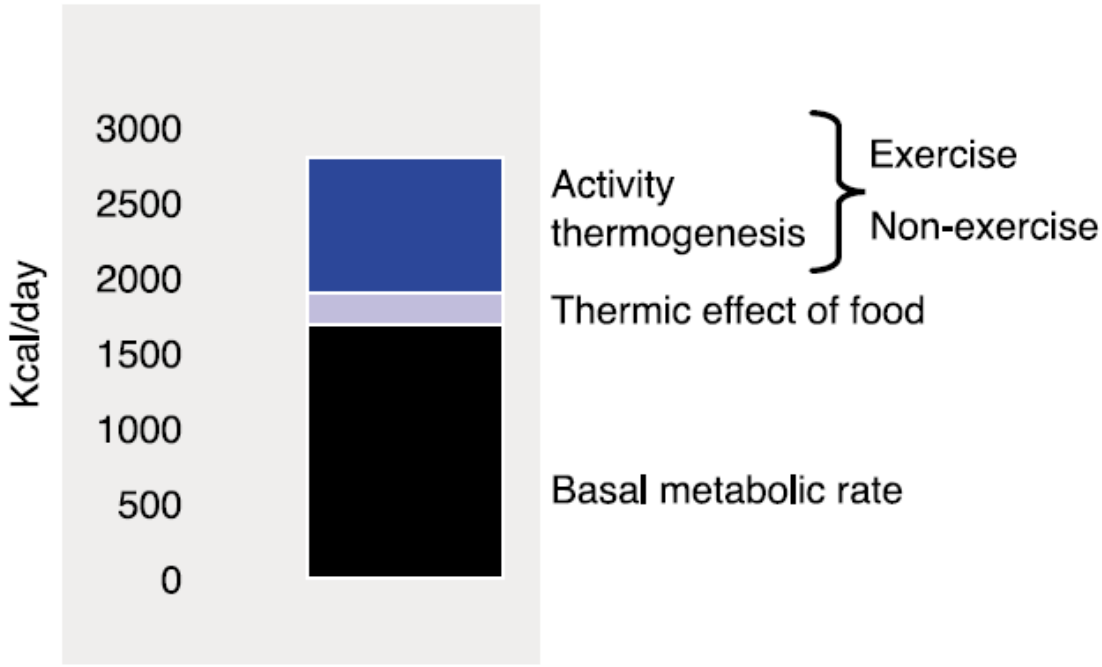

Figure 1.1: Relative contribution of different components of energy expenditure towards total daily energy expenditure. Activity thermogenesis comprises exercise, non-exercise activity thermogenesis (NEAT) and shivering. Diet-induced adaptive thermogenesis contributes to the thermic effect of food. Figure adapted from (Levine, 2002).

1.3.3.1 Basal Metabolic Rate

Basal metabolic rate (BMR) is the energy required to maintain all of the body's systems while at rest. Ideally, BMR is measured using direct or indirect calorimetry under the following conditions: 1. a resting state, 2. in the post-absorptive state, 3. in a thermoneutral environment and 4. free from emotional distress. Although BMR comprises approximately 60% of a sedentary individual's daily energy expenditure, it is highly dependent on an individual's mass, with three-quarters of it being predicted by lean body mass (Deriaz *et al.*, 1992; Ford, 1984). As such, a majority of studies show that BMR varies greatly between individuals but not between obese and lean individuals when expressed per unit body weight.

1.3.3.2 Shivering Thermogenesis

Shivering thermogenesis is common among many species, including humans. It involves involuntary skeletal muscle activity in response to cold exposure. The process of shivering thermogenesis is controlled by the central nervous system. Its primary function is to maintain core body temperature when an individual is exposed to hypothermic conditions. This is achieved by the induction of tremor-like vibrations in skeletal muscle, which increases metabolic respiration, resulting in the production of heat. Shivering thermogenesis can be measured via electromyography, which detects the electrical activity of the muscle. Shivering thermogenesis is a well characterised phenomenon (Hemingway, 1963) but has little bearing on whole body energy expenditure outside of the context of hypothermia.

1.3.3.3 Exercise

Exercise can be defined as physical activity undertaken for the means of maintaining or improving health. This wide ranging and loose definition can encompass activities such as

walking, running, sports and resistance training. Exercise, is often prescribed for the treatment of obesity or for the prevention of weight gain. Exercise utilises fuels in levels above that of BMR or NEAT, increasing energy expenditure (in a process referred to as exercise activity thermogenesis).

Exercise alone has been shown to result in only modest reductions in body weight (Garrow and Summerbell, 1995). In combination with improved diet and other lifestyle interventions, exercise can help in the maintenance of body weight in lean people, and weight loss in the obese, though weight regain often occurs (Wu *et al.*, 2009).

The majority of people in developed nations do not engage in prolonged exercise to any substantial extent. As such the contribution of exercise activity thermogenesis to total energy expenditure is often negligible. Some argue that even those who participate in moderate levels of exercise (~2hours/week) only contribute ~100 out of 2,000 – 2,500kcal to their daily energy expenditure (Levine *et al.*, 2006).

In theory, energy expenditure through exercise should be a robust method to combat obesity but a number of practical factors inhibit its effectiveness. To maintain the duration and/or intensity of exercise needed for substantial increases in energy expenditure an individual needs to have the motivation and ability to adhere to an exercise regime. Regimes of this nature can lead to injuries (especially in the untrained or obese), and can be time consuming and physically arduous; all of which can result in non-compliance. While modest losses in body weight can be achieved through the intervention of exercise, prolonged adherence is uncommon and weight-regain is a likely long-term outcome. Exercise has also been associated with a compensatory increase in food intake, essentially negating its effects (Church *et al.*, 2009). Exercise is often touted as a cure-all for obesity. However, in practice exercise as a health intervention is an unreliable form of energy expenditure to succeed in the long term.

1.3.3.4 Non-Exercise Activity Thermogenesis (NEAT)

NEAT constitutes energy utilised through activity but excluding volitional exercise. This includes activities such as walking, talking, fidgeting and energy utilised to maintain postural allocation. While activities that comprise NEAT may seem trivial, their net accumulation across time can contribute anywhere between 15 and 53% of daily energy expenditure in sedentary and highly active people, respectively (Levine *et al.*, 1999).

NEAT can be highly variable based on a number of factors such as an individual's occupation (Levine *et al.*, 2006) and daily level of activity (Levine *et al.*, 2000). As the proportion of energy spent through exercise is negligible in most populations, NEAT is seen as playing a more important role in energy balance and body weight determination.

Evidence to support the role of NEAT in the regulation of body weight is mostly indirect. It relies on self-reporting of daily activity by participants, or from epidemiological studies. A limited number of studies (outlined below) have shown a relationship between NEAT, energy balance and obesity. Individuals characterised as having high levels of NEAT are resistant to diet-induced obesity (Levine *et al.*, 1999). In this study, 16 non-obese participants were placed on a diet consisting of 1000 kilocalories above that required for weight maintenance for 8 weeks (Levine *et al.*, 1999). Approximately two thirds of the compensatory increases in daily energy expenditure observed were attributed to increases in NEAT (Levine *et al.*, 1999). There was a strong, inverse relationship between fat gain and an individual's NEAT (Levine *et al.*, 1999). NEAT has also been shown to decrease (per unit body weight) as the degree of obesity increases (Elbelt *et al.*, 2010). Yet in this case it may be argued that a reduction in NEAT is a consequence of obesity and not causally linked.

Mechanistically, little is known about the regulation of NEAT. Theories have been put forth that the brain processes external and internal cues of energy availability and metabolic parameters resulting in altered activity levels or altered efficiency of activity (Novak and Levine, 2007). Peptides related to hypothalamic control of energy balance such as the orexins, AgRP, and NPY, as well as the gut hormone ghrelin, have been implicated as mediators of NEAT. This indicates strong central regulation (Kotz *et al.*, 2008).

1.4 Adaptive Thermogenesis

1.4.1 Overview

An important component of energy expenditure is adaptive thermogenesis, the biochemical dissipation of energy through the production of heat. This type of thermogenesis occurs within specialised tissues and is regulated by the brain. Specifically, thermogenesis is controlled centrally via the sympathetic nervous system in response to cold and dietary stimuli. Sympathetic signals which promote thermogenesis are thought to originate in regions of the hypothalamus such as the POA, PVN, and VMN, and relayed via the brain stem to peripheral sites (Bamshad *et al.*, 1999). Figure 1.2 shows a schematic diagram of the above mentioned components involved in the initiation of adaptive thermogenesis in peripheral tissues.

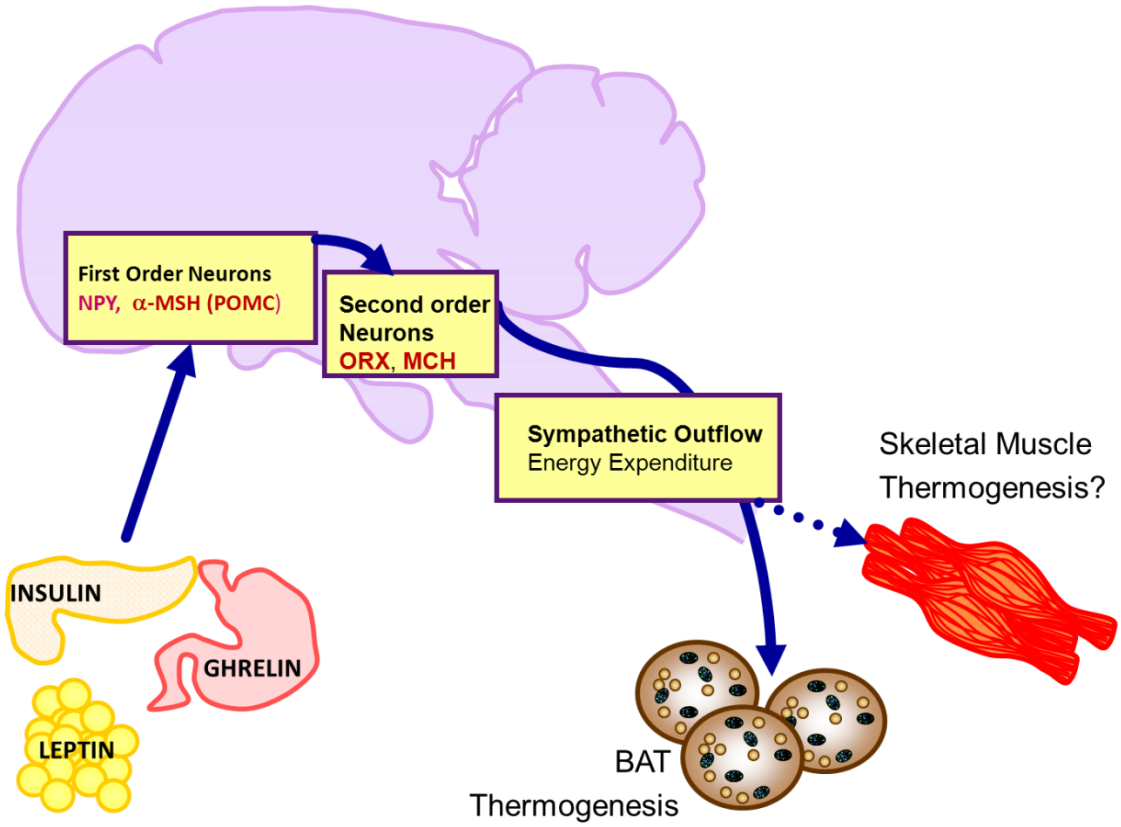

Figure 1.2: A schematic representation of the components believed to be involved in the elicitation of adative thermogenesis in peripheral tissue. Blood-borne nutritional signalling factos such as leptin are detected by the 'first order' neurons of the hypothalamus. The 'first order' neurons relay this information to the second order neurons. The sympathetic output through the brainstem innovates peripheral tissues, initiating the thermogenic response. Brown adipose tissue (BAT) thermogenesis is well characterised in rodents, and more recently in humans. The potential role of thermogenesis in skeletal muscle is controversial, and needs to be further investigated.

Classically, adaptive thermogenesis has been known to occur in specialised adipose tissue, termed BAT. As opposed to white adipose tissue (WAT), BAT is catabolic in nature, as evidenced by a number of morphological features. BAT cells are rich in uncoupling protein 1 (UCP1)-dense mitochondria. BAT is highly innervated by the sympathetic nervous system, and is highly vascularised allowing for ready uptake of plasma metabolites (Nnodim and Lever, 1988). In addition, brown adipocytes expresses β-adrenergic receptors (β-ADR; Nnodim and Lever, 1988). Histologically, BAT can be characterised by its multilocular morphology. This is due to the storage of multiple small lipid droplets within the cell. This contrasts with WAT cells, which have a monocular morphology due to the presence of a single large lipid droplet within the cytoplasm. Figure 1.3 depicts BAT's key morphological features in the human.

Historically, brown adipose tissue was thought to exist only in newborn and infant humans, when it is concentrated around internal organs. It was believed that as infants developed and gained the ability to produce heat through shivering thermogenesis, the presence and functional capacity of BAT was lost (Porth and Kaylor, 1978; Lean, 1989). Thus it was considered that adaptive thermogenesis occurred either in other tissues or that this process was not important in maintaining energy balance in adult humans. In 1989, data were then obtained to indicate the existence of BAT in adult humans (Lean, 1989). These data remained rooted in histological observations and gene/protein expression studies and were not conclusive in showing the presence of functional BAT. Around this time, a study from Scandinavia reported the presence of BAT-like cells in human adipose tissue from in neck region. BAT deposits were observed in subjects who worked outdoors, but not in those who worked indoors (Huttunen *et al.*, 1981). The identification of multilocular cells in these outdoor workers implied the presence of cold-activated brown adipocytes in adult humans (Huttunen *et al.*, 1981). Krief *et al.* also showed differential expression patterns of the β_3-ADR in different fat beds in adult humans. Receptor concentration was greater in retroperitoneal fat and lower in the subcutaneous fat bed. This pattern is analogous to BAT distribution in other species (Krief *et al.*, 1993). BAT was also shown to exist pathologically in some adults who have pheochromocytoma, a type of tumour

which can result in perpetual BAT stimulation due to over-production of noradrenaline (NA) (Breslau *et al.*, 1982; Iyer *et al.*, 2009; Kuji *et al.*, 2008). In spite of these data, the physiological significance of BAT in adult humans remained controversial until recently.

In large mammals, including humans and sheep, BAT is prevalent during the neonatal period (Porth and Kaylor, 1978; Lean, 1989) when it is essential for the maintenance of core body temperature. In human infants, BAT is predominantly found in the interscapular and retroperitoneal fat bed (Porth and Kaylor, 1978; Lean, 1989), while in lambs it is found primarily in the retroperitoneal fat bed (Alexander, 1978). Shortly after birth in these species, the amount of BAT is reduced and it was thought not to be present in the adults of non-rodent species. As discussed in detail later, this notion was dispelled in 2009 when a series of papers were published in the New England Journal of Medicine that unequivocally demonstrated that functional BAT exists in adult humans (Cypess *et al.*, 2009; van Marken Lichtenbelt, 2009; Virtanen *et al.*, 2009). As to whether the same is true for adult sheep remains controversial, since equivalent studies using similar techniques to the above mentioned human studies have yet to be conducted. Nonetheless, real time PCR has detected UCP1 mRNA in subcutaneous and visceral adipose depots of the sheep (Henry *et al.*, 2010) indicative of the presence of brown adipocytes.

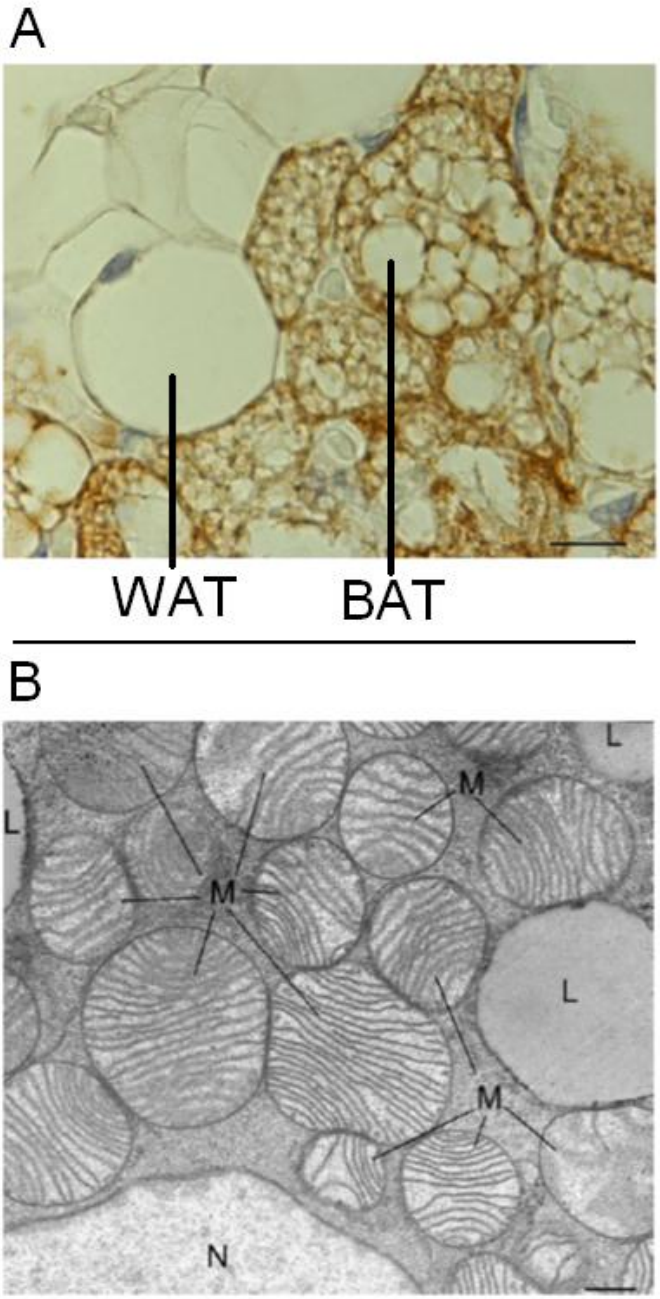

Figure 1.3: Histological sections showing the main characteristics of brown adipose tissue (BAT) in humans. Image A shows a light microscopy of an immunohistochemistry section of human-white adipose tissue (WAT) and BAT probed for uncoupling protein 1 (UCP1; bar =15 microns). UCP1 highlights the multilocular morphology of brown adipocytes in contrast to the monolocular morphology white adipocytes. Image B shows electron microscopy of a brown adipocyte (bar = 0.5 micron). Brown adipocytes are rich in mitochondria (M), and contain small lipid droplets (L). A portion of the nucleus is also visible (N). Figure adapted from (Cinti, 2006)

1.4.2 Molecular Control of Adaptive Thermogenesis

Energy is required for the maintenance life. Biological systems have evolved to use adenosine triphosphate (ATP) as an energy-intermediate. The production of ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) occurs within mitochondria. It involves the catabolism of metabolites such as non-esterified fatty acids (NEFA) and glucose, via a series of complex and highly regulated pathways involving the citric acid cycle (CAC) and the electron transport chain (ETC; see Figure 1.4).

In summary, NEFA and glucose are transported across the inner mitochondrial membrane, into the mitochondrial matrix. There they are metabolised for the production of Acetyl-CoA. Fatty acids undergo β-oxidation producing one NADH, FADH and Acetyl-CoA molecule during each round of oxidation, shortening the fatty acid backbone by two carbon atoms. Acetyl-CoA enters the CAC for the further production of NADH and FADH.

The proteins of the ETC utilise interaction between electron donors (NADH and FADH) and electron acceptors (O_2) to drive movement of protons (H^+) from the mitochondrial matrix across the mitochondrial inner membrane. This creates an electrochemical gradient across the membrane. The movement of these protons back along the electrochemical gradient across the mitochondrial inner membrane into the mitochondrial matrix via the enzyme ATP synthase creates a proton-motive force resulting in the production of ATP from ADP + Pi. This process is known as oxidative phosphorylation. The reactions involved in the production of ATP are stoichiometrically coupled in that ATP molecules are produced in proportion to the number of protons that cross the mitochondrial inner membrane via ATP synthase.

All cells utilise coupled respiration to derive energy (as ATP) from fuel sources. When stimulated by the sympathetic nervous system, brown adipocytes are able to uncouple oxidative phosphorylation from ATP synthesis. Thus, uncoupling protein 1 (UCP1) located on the mitochondrial inner membrane enables uncoupled respiration, whereby proton leakage across

the membrane into the matrix occurs without the production of ATP. This energy dissipates as heat. Low levels of proton leakage occurs at baseline (Nicholls, 1997), but the presence and activity of UCP1 increases proton flux and therefore heat production. It is thought that the thermogenic potential of BAT is derived primarily from the activity of UCP1 (Matthias *et al.*, 2000). As discussed in further detail later, homologues of UCP1 are expressed in other tissues. The thermogenic potential of these homologues will also be reviewed later.

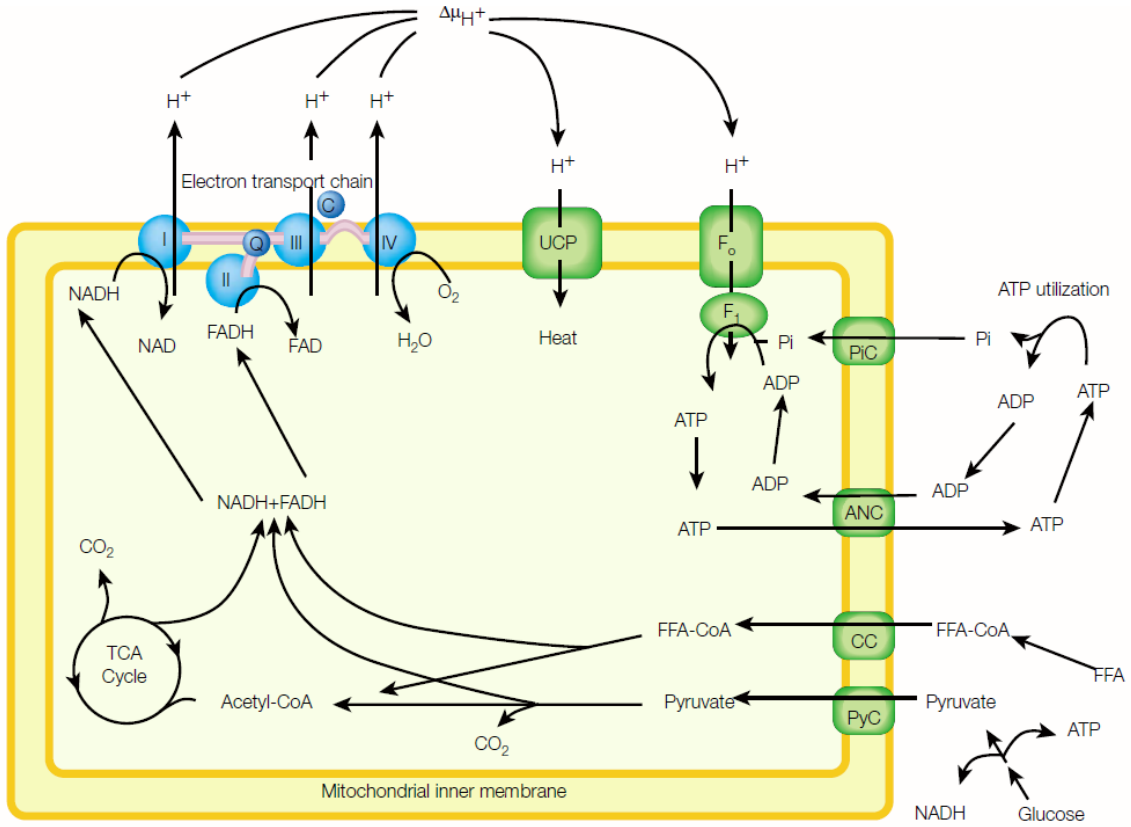

Figure 1.4: A schematic diagram of the processes involved in coupled and uncoupled respiration. In mitochondria, NADH and FADH, products of the oxidation of blood-borne fuel intermediates free fatty acids (FFA) and glucose, enter electron transport chain. The electron transport chain uses these substrates to produce a proton (H+) gradient across the mitochondrial inner membrane. During coupled respiration, H+ movement back across the mitochondrial inner membrane via ATP synthase (F_o and F_1) drives the production of ATP from ATP and Pi. During uncoupled respiration, uncoupling proteins located on the mitochondrial inner membrane allow the influx of H+ without the production of ATP; this potential energy is lost as heat. Figure adapted from (Lowell and Spiegelman, 2000)

1.4.3 Physiological Roles of Adaptive thermogenesis

Adaptive thermogenesis can be induced by cold and dietary stimuli. Cold-induced adaptive thermogenesis plays an important role in thermoregulation (especially in rodents, other small mammals, and infants). It prevents potentially critical reductions in body temperature during exposure to hypothermic conditions. The preference for thermoneutral environments (staying in-doors, adjusting clothing to ambient temperatures etc), and thus minimising the need for adaptive thermogenesis, has been proposed reason for increased obesity rates in humans (Johnson *et al.*, 2011; Keith *et al.*, 2006). Cold-induced thermogenesis provides a useful and robust model for studying adaptive thermogenesis, as it employs similar neural and molecular mechanisms as diet-induced thermogenesis.

In humans, the thermic effect of food is an important part of energy expenditure. The thermic effect of food has two components; 1) the obligatory component, which refers to the energy required to process and metabolise food for storage and use and 2) the facultative or adaptive component, which is the energy expended above that required to process food. The phenomenon of diet-induced adaptive thermogenesis accounts for approximately 15% of total daily energy expenditure in non-obese individuals (Schutz *et al.*, 1984; Levine, 2004).

Diet-induced adaptive thermogenesis may be an important factor that determines how energy is utilised and body weight is regulated (Rosenbaum *et al.*, 2008; Goele *et al.*, 2009). Lower levels of thermogenesis would allow a greater amount of energy from food to be converted to triglycerides, and stored in WAT. Unchecked, this could lead to obesity.

Given its role in the expenditure of energy, BAT is an important organ for body weight maintenance. This is exemplified by the removal of BAT in rodent species. Ablation of BAT in rodents (by DNA transgenic vector to drive brown fat specific expression of diphtheria toxin A-chain; which essentially kills brown adipocytes) leads to increased adiposity (Hamann *et al.*,

1996; Klaus *et al.*, 1998). The importance of BAT in determining whole body energy expenditure is well established in adult rodents. Its contribution to whole body energy expenditure in adult humans is a current area of interest in the scientific community.

1.4.3.1 Regulation of UCP1 Expression

The primary tenet of adaptive thermogenesis has UCP1 activity being regulated by sympathetic neuronal activation of the brown adipocyte via the membrane-bound g-protein coupled β_3-ADR (Lowell and Flier, 1997). Evidence also suggests a possible complementary role of β_1 and β_2 – ADR activation (Rohlfs *et al.*, 1995). Activation of β-ADR results in the production of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA). PKA is considered a keystone of several pathways involved in the initiation of adaptive thermogenesis. Beta-adrenergic stimulation of brown adipocytes further initiates a number of responses culminating in the induction of thermogenesis. These include 1) coordinated up-regulation of UCP1 expression and activity, 2) mitochondrial biogenesis and 3) lipolysis.

In rodents (Cassard-Doulcier *et al.*, 1993; Kozak *et al.*, 1994) and humans (Rim and Kozak, 2002), enhancer regions upstream of the UCP1 gene promote the expression of UCP1. This occurs exclusively in brown adipocytes, upon β-ADR stimulation. Transcription of the UCP1 gene is regulated at this enhancer site through the binding of a number of complexes including human thyroid receptor (Rabelo *et al.*, 1995; Rabelo *et al.*, 1996), retinoic acid receptor (Alvarez *et al.*, 2000; Alvarez *et al.*, 1995) and peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ is expressed in other cell types, but in BAT it is essential for the initiation of UCP1 gene translation (Sears *et al.*, 1996). PPARγ co-activator (PGC) and specifically PGC-1α is highly expressed in BAT (but not WAT), and other tissues such as skeletal muscle, It serves to bind co-activates PPARγ, along with the thyroid receptor and retinoic acid receptor. This leads to amplification of UCP1 gene translation.

PGC-1 can also bind to and co-activate the NRF system, enhancing transcription of genes involved in mitochondrial biogenesis. Adenovirus-mediated expression of PGC-1α in human WAT induces BAT-like protein expression profiles: UCP1 expression, and increased expression of mitochondrial proteins such as cytochrome c and COX-4 (Tiraby *et al.*, 2003). While important for the induction of UCP1-dependant thermogenesis, Uldry *et al.* have shown that PGC-1α is not essential for BAT differentiation from brown preadipocytes (Uldry *et al.*, 2006).

The induction of PGC-1 transcription can also be regulated by cAMP response element-binding (CREB), which has a dual effect on UCP-1 translation by also up-regulating type II thyroxine deiodinase (DII) expression, leading to formation of triiodothryronine (T3) - a ligand for the thyroid hormone receptor. All of which results in increased UCP1 expression. PKA-mediated activation of p38 MAPK has been shown to increase UCP1 expression as well as having independent, positive effects on PGC-1α gene transcription (Cao *et al.*, 2004) This further amplifies the β-adrenergic response (Collins *et al.*, 2004). A schematic representation of the above outlined processes can be viewed in Figure 1.5.

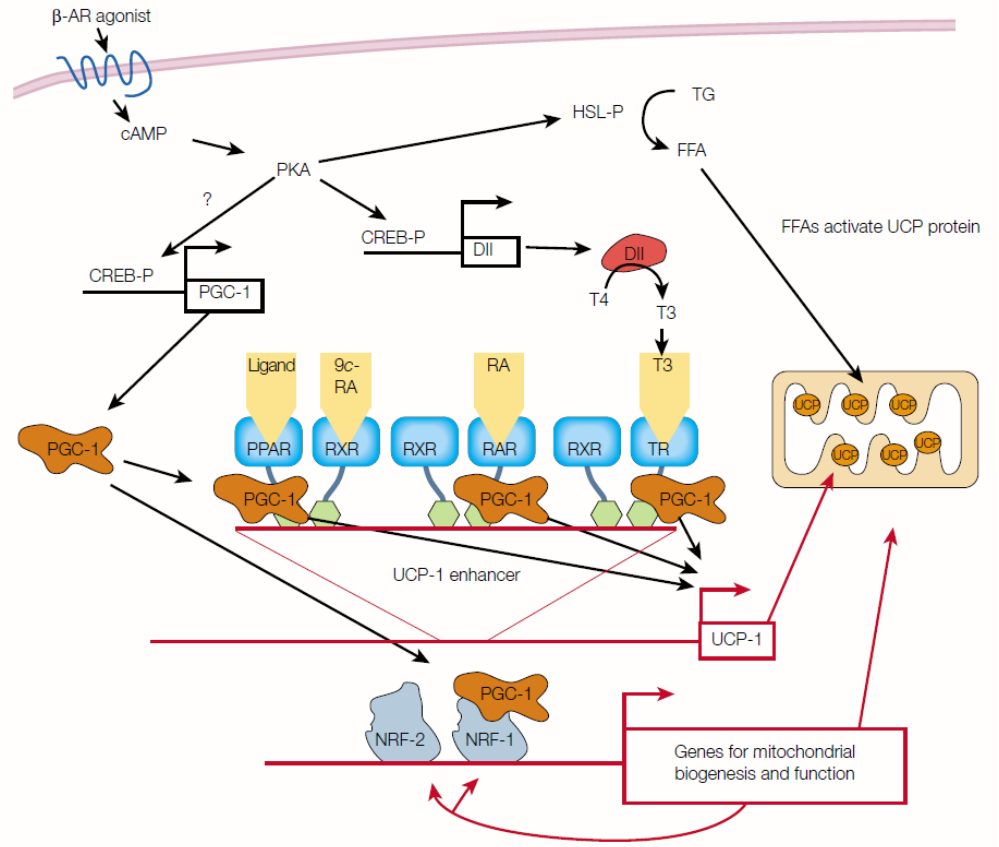

Figure 1.5: Schematic diagram of brown adipose tissue activation by adrenergic stimula Activation of the β3-ADR by the sympathetic nervous system results in the activation of pr kinase A (PKA). This leads to transcriptional activation of PGC-1 and DII expression. P(has several downstream effects including 1) co-activation of transcription factors assemble the UCP1 enhancer region, resulting in increased UCP1 expression, and 2) activatio mitochondrial biogenesis by the co-activation of transcription factor NRF-1. DII increase synthesis, which activates the thyroid receptor, promoting further UCP1 synthesis. Fi adapted from (Lowell and Spiegelman, 2000)

1.4.3.2 Ushering in the Human BAT Renaissance

As technology has progressed, so too has the availability of more advanced techniques to detect functional BAT in adult humans. The development of the medical imaging technique fluorodeoxyglucose positron emission tomography (FDG-PET) for the detection of tumours also led to the detection of active brown fat. FDG-PET involves the consumption of 2-[18F]-fluoro-2-deoxy-glucose - a glucose substrate taken up by the GLUT receptors within active tissues. This substrate has an 18F label which can be detected by PET. Due to its high metabolic activity, BAT consumes glucose at a much higher rate than most other peripheral tissues. Thus, islands or pockets of BAT may be detected in the supraclavicular and neck regions, as well as in paravertebral, mediastinal, para-aortic, and suprarenal localizations of humans. CAT scans are usually conducted in parallel to FDG-PET scans. They serve to confirm that the observed BAT has the same density as adipose tissue (van Marken Lichtenbelt, 2009; Cypess *et al.*, 2009; Nedergaard *et al.*, 2007). Tissue biopsies of the fat deposits detected by FDG-PET can also be confirmed histologically by the presence of UCP1, and PGC1 (Virtanen *et al.*, 2009). Additionally, administration of propranolol (a non-selective β-ADR blocker) in conjunction with FDG reduces BAT detection by PET due to decreased uptake of FGD uptake by BAT (Basu and Alavi, 2008). The reporting of functional BAT in adult humans (Cypess *et al.*, 2009; van Marken Lichtenbelt, 2009; Virtanen *et al.*, 2009) resulted in a paradigm shift, identifying this as a potential target for the combat of obesity.

Initially, the detection of BAT in adult humans via FDG-PET was achieved in retrospective studies. The prevalence of BAT detection was fairly low (~4-5%; Nedergaard *et al.*, 2010). Scans in these studies were predominantly from patients undergoing treatment for cancer, and were not controlled for the optimal detection of BAT. It is now apparent that in scans conducted during cold days (Ouellet *et al.*, 2010), or when participants are exposed to cold under experimental conditions (Saito *et al.*, 2009; Virtanen *et al.*, 2009; Yoneshiro *et al.*, 2010), the prevalence of BAT detection, and activity of BAT was greatly improved - being as high as 95%.

Au-young *et al.* reported a strong seasonal variation in BAT detection in humans, but attributed this effect to photoperiod rather than ambient temperature (Au-Yong *et al.*, 2009). The likelihood of BAT detection is also greatly enhanced when repeated scans are conducted (Lee *et al.*, 2010). More recently, Lee *et al.* (Lee *et al.*, 2011). have also demonstrated a high prevalence of people who express UCP1 and other genes associated with adaptive thermogenesis in supraclavicular adipose tissue, regardless of whether BAT is detected via FDG-PET (Lee *et al.*, 2011). Data suggest that BAT detection may also be influenced by age and sex: BAT detection correlates negatively with age and body weight/BMI (Gelfand *et al.*, 2005; Ouellet *et al.*, 2010; Döbert *et al.*, 2004; van Marken Lichtenbelt, 2009; Saito *et al.*, 2009) and is more readily detected in females than in males (Cypess *et al.*, 2009).

While initial studies indicated that such detection is possible in a minority of people at ambient temperatures, cold-induced adaptive thermogenesis has been shown in various studies (*vide supra*). The role of adaptive thermogenesis in the maintenance of body weight via diet-induced thermogenesis is, however, relatively poorly researched. Correlation between obesity and the presence/absence of BAT may be a key indicator that adaptive thermogenesis is involved in the control of body weight and that its dysfunction may result in the propensity to gain weight. The potential causative relationship between BAT presence and obesity warrants further investigation.

1.4.3.3 BAT in Sheep

Functional BAT can be detected in neonatal sheep during the first day and weeks of life. Through the use of guanosine diphosphate (GDP) binding (an indicator of BAT activity), Symonds *et al.* (Symonds *et al.*, 1992) were able to show that lambs exposed to cool prenatal conditions (mothers with shorn wool), had greater retroperitoneal BAT activity at birth than those exposed to warmer conditions (mothers with wool). At birth, cool-exposed lambs were

heavier with greater fat mass. Within 33 days of life, all lambs showed reduced GDP binding and increased lipid content within the retroperitoneal adipose tissue (Symonds *et al.*, 1992). Using a combination of GDP binding and Western and Northern blot analysis for UCP protein and mRNA expression, Clarke *et al.* (Clarke *et al.*, 1997) showed that BAT thermogenic activity peaked 4 days after birth, but was detectable at diminished capacity for 30 days. Similarly, Lomax *et al.* (Lomax *et al.*, 2007) were able to detect UCP1 mRNA and protein (by rt-PCR and western blot, respectively) in adipose tissue collected from neonates across a number of time points. UCP1 protein expression was greatest within the first neonatal day, detectable at day 7, but undetectable by day 21. (Lomax *et al.*, 2007). Casteilla *et al.* showed that UCP1 was detectable only in retroperitoneal fat at neonate day 1, and was undetectable by neonatal day 6 (Casteilla *et al.*, 1989). UCP1 protein has been shown to be expressed in 100% of retroperitoneal adipocytes on the first day of life, with levels declining to become undetectable by day 15 (Finn *et al.*, 1998).

Recently, Henry *et al.* have shown that retroperitoneal and subcutaneous fat depots in adult sheep express uncoupling protein mRNA. UCP1 and UCP2 mRNA were expressed at higher levels in the retroperitoneal fat than the subcutaneous fat. The distribution of UCP3 mRNA was similar in the two tissues (Henry *et al.*, 2010). This work also lent credence to the notion that BAT in sheep is capable of diet-induced thermogenic responses, as well as modulation of this response by central administration of leptin (Henry *et al.*, 2010; Henry *et al.*, 2008).

As more sensitive methods for the detection of UCP mRNA and protein are utilised (eg rt-PCR compared to northern blotting) the presence of BAT in sheep beyond the neonatal stages has been confirmed. The distribution and functionality of BAT in sheep seems to be analogous to that of humans. Both species have relatively high levels of active BAT at birth, which dissipates within the early neonatal period. In adulthood, both species have BAT interspersed within WAT deposits, which can be activated with both cold and dietary stimuli.

1.4.4 UCP1 Analogues and Their Functions

Four homologues of UCP1 have been identified. UCP2 and UCP3 are expressed in peripheral tissues such as fat and muscle. UCP2 is essentially ubiquitous in its distribution, while UCP3 is expressed predominantly in BAT and skeletal muscle (Brand and Esteves, 2005). UCP4 (Mao *et al.*, 1999) and UCP5 (Sanchis *et al.*, 1998) are expressed mainly in neuronal tissues and therefore are not involved in peripheral tissue adaptive thermogenesis. As discussed below, the exact roles of UCP2 and UCP3 are presently unclear, and their relative importance in uncoupled respiration is a matter of debate.

In early studies employing UCP1 knockout mice, it was observed that gene ablation had no effect on body weight (Enerback *et al.*, 1997). In these mice, UCP2 mRNA levels in BAT were 14-fold greater and UCP3 expression was slightly lower than wild types (Matthias *et al.*, 1999). This suggested a role of UCP2 in thermogenesis, but these mice were also cold sensitive, indicating a loss of cold-induced adaptive thermogenesis (Enerback *et al.*, 1997). Several theories have ascribed a role of UCP2 in cellular function; the most prominent of which is in the regulation of reactive oxygen species (ROS) production and glucose sensing in skeletal muscle and adipose tissue (Brand and Esteves, 2005; Bouillaud, 2009). A role in glucose-stimulated insulin secretion in pancreatic β-cells (Pi and Collins, 2010; Brand *et al.*, 2010) has also been proposed. Little evidence exists to suggest UCP2 is involved in thermogenesis.

There is continued controversy as to whether UCP3 is involved in regulating thermogenic processes. Gong *et al.* (Gong *et al.*, 1997) demonstrated that UCP3 expression in skeletal muscle was regulated positively by thyroid hormone, while in adipose tissue β-ADR activation upregulated UCP3 expression. Leptin treatment (intraperitoneal injection) of *ob/ob* (leptin lacking) mice show increased UCP3 in skeletal muscle and BAT, as well as increased UCP1 in BAT. This inferred a regulatory role of leptin on adaptive thermogenesis. In contrast, UCP2

levels remained relatively stable suggesting a greater likelihood of UCP3's involvement in thermogenesis.

UCP3 knockout mice exhibit higher coupled mitochondrial respiration in skeletal muscle - indicating an uncoupling role of UCP3 (Vidal-Puig *et al.*, 2000). In spite of this, UCP3 knockout mice have been shown to adapt to cold exposure - likely due to UCP1 expression. They also have similar bodyweights compared to wild-types (Vidal-Puig *et al.*, 2000). Mice engineered to overexpress human UCP3 in skeletal muscle have decreased fat mass in spite of being hyperphagic, indicating that UCP3 affects energy balance via energy expenditure rather than through altering intake (Clapham *et al.*, 2000). More recent studies show a naturally occurring mutation cause hamsters to not express UCP3 in BAT, conferring reduced cold-induced thermogenesis (Nau *et al.*, 2008). Elevated levels of UCP3 may also play a role in increased uncoupling in the skeletal muscle of UCP1 knockout mice (Monemdjou *et al.*, 2000) though this observation is inconsistent (Cadenas *et al.*, 1999). Proton leak and UCP3 expression have also been shown to correlate with weight loss success in humans on a set diet, highlighting a potential importance of this uncoupling agent in energy balance (Harper *et al.*, 2002).

Cold-induced UCP3 expression in skeletal muscle has been shown to peak (two to three fold increase) within 24 hours in rats, but declines to 50% of original expression within 6 days of continued cold exposure (Lin *et al.*, 1998). Another study saw no effect of cold exposure on UCP3 expression during cold exposure in rats, but did show elevation during prolonged fasting (Boss *et al.*, 1998). Zhou *et al.* (Zhou *et al.*, 2000) suggested that elevated NEFA levels as a result of fasting or acute exercise may up-regulate UCP3 expression. Consistent with this, infusion of NEFA to fed mice to mimic fasting levels resulted in elevated UCP3 expression (Weigle *et al.*, 1998). Adaptation of UCP3 expression to NEFA levels lends support to the notion that UCP3 is important for regulation of ROS production (Hoeks *et al.*, 2006; Nabben *et al.*, 2008) and thermogenesis.

Studies within this laboratory show that central infusion of leptin increases postprandial thermogenic output in ovine muscle, and gluteal and visceral fat (Henry *et al.*, 2008). In addition, leptin increased the expression of UCP2 and UCP3, but not UCP1 mRNA in muscle tissue (Henry *et al.*, 2011). This was concomitant with increases in both coupled and uncoupled respiration in skeletal muscle mitochondria from leptin treated animals - with a predominant effect on uncoupled respiration (Henry *et al.*, 2011). This model comprehensively combines molecular mechanisms with physiological outcomes. As UCP1 is predominantly expressed in BAT, and levels of UCP1 were not affected by central leptin treatment, thermogenic differences can be attributed to the muscle, and not BAT found surrounding muscle. These findings, in addition to the above mentioned studies, serve to highlight not only the potential of skeletal muscle as a key regulator of thermogenesis and energy expenditure, but also the importance of UCP3 in these functions.

1.4.5 The Thermogenic Potential of Skeletal Muscle

Adaptive thermogenesis in BAT is a well characterised process. However, the postprandial thermogenic potential of skeletal muscle is relatively unexplored. The latter is known to play a significant role in whole body energy expenditure, even at rest (Zurlo *et al.*, 1990). Skeletal muscle comprises approximately 30-40% of total body weight (Astrup *et al.*, 1985) and is thought to be responsible for 70-85% of glucose disposal in man (Yki-Jarvinen *et al.*, 1987). Muscle can contribute up to 50% of the whole body thermogenic response to adrenaline (Simonsen *et al.*, 1993). Together, this indicates a significant role of muscle in whole body energy expenditure which makes it a potential target for the manipulation of thermogenesis.

1.4.5.1 Embryology of Skeletal Muscle and BAT

It was originally believed that BAT shares a common embryonic lineage with white adipose tissue (Gesta *et al.*, 2007; Hansen and Kristiansen, 2006; Rosen and Spiegelman, 2000). This was due to morphological features and gene expression similarities between the two tissue types. Additionally, observations in non-rodent mammalian species that BAT was present in distinct depots during neonatal life but was apparently not present in adults, led to the notion that transdifferentiation occurred during the transition from infancy resulting in BAT converting to WAT. In spite of this, BAT and WAT have functionally distinct and contrasting functions in terms of lipid metabolism and storage. Recent studies demonstrate that true brown adipocytes are derived from a precursor common to myocytes rather than white adipocytes.

A number of transcriptional regulators act to either suppress (pBR, p107 and RIP140) or stimulate (FOXC2 and PRDM16) BAT development. Of these, PRDM16 has been shown to be essential in the formation of BAT from myoblasts, which are precursor cells for skeletal muscle (Seale *et al.*, 2008). It has also been demonstrated that BAT and skeletal muscle (but not WAT) share common progenitor cells - those expressing Myf5 (Kajimura *et al.*, 2009). The introduction of PRDM16 into Myf5 expressing myoblasts results in the formation of functional (UCP1 expressing) BAT. Those not exposed to PRDM16 form myocytes (Figure 1.6). Collectively, these data have led to the proposition that muscle and BAT have a common origin, consistent with the catabolic function in the two cell types.

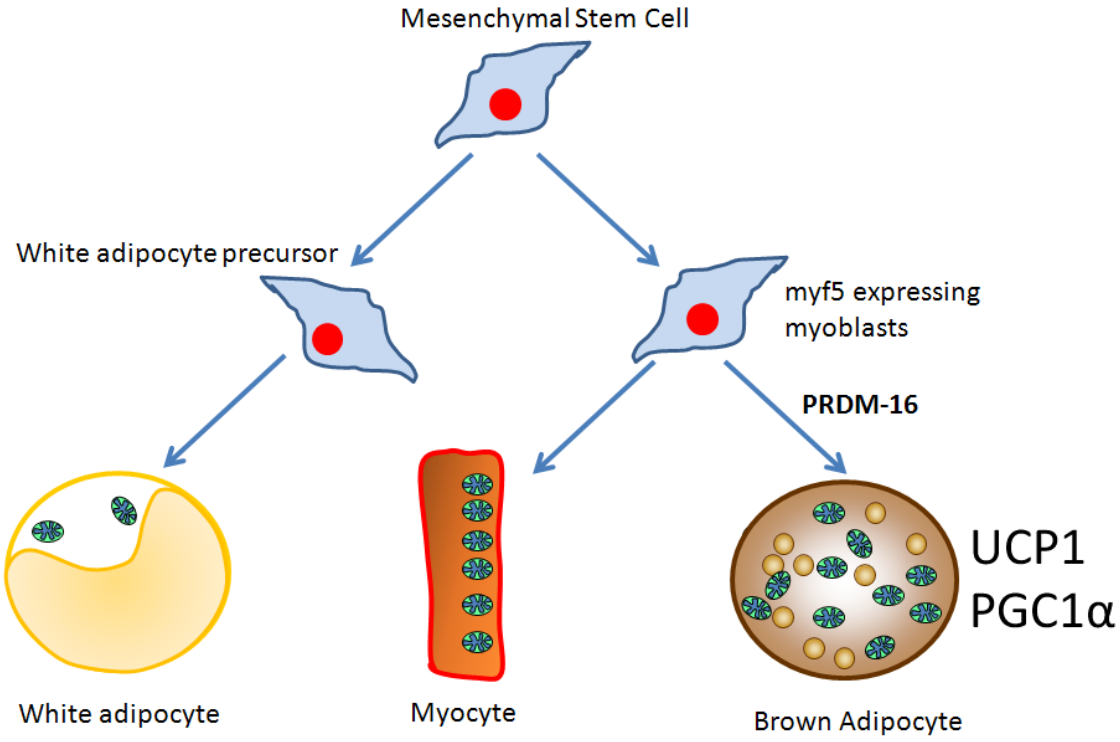

Figure 1.6: Skeletal muscle and brown adipose tissue share a common lineage through myf5 expressing cells derived from tripotent precursors in the dermomyotome (not pictured). PRDM-16 switches myf5 expressing myoblasts towards brown adipocyte differentiation. The shared lineage between brown adipocytes and myocytes is distinct and separate from that of white adipose tissue. Similarity of origin and catabolic function gives weight to skeletal muscle being capable, like brown adipose tissue, of adaptive thermogenesis. Figure adapted from (Petrovic *et al.*, 2010).

1.4.5.2 Evidence for the Thermogenic Potential of Skeletal Muscle

It has been proposed that skeletal muscle is a tissue that displays adaptive thermogenesis which can be induced by cold (Mollica *et al.*, 2005; Wijers *et al.*, 2008) or feeding (Kus *et al.*, 2008). Nevertheless, this is contested by some, who claim that BAT is the only true thermogenic tissue (Cannon and Nedergaard, 2010), Skeletal muscle (particularly Type I and Type IIa muscle fibres), like BAT, is rich in mitochondria. While UCP1 expression is a property unique to BAT, skeletal muscle has been shown to express the homologues, UCP2 and UCP3 (Schrauwen and Hesselink, 2002)**.** The uncoupling and thermogenic potential of these homologues remains to be elucidated, although there is evidence to suggest that UCP3 is involved in uncoupling oxidative phosphorylation (Gong *et al.*, 1997) and the regulation of energy expenditure (Costford *et al.*, 2008; Clapham *et al.*, 2000); see above.

The increase in NA release by sympathetic neurons, and the increase in NA uptake that occurs in skeletal muscle tissue during the postprandial period suggests a sympathetically mediated response (Vaz *et al.*, 1997). β_3-ADR stimulation in lean and obese mice causes up-regulation of UCP2 and UCP3 mRNA expression in skeletal muscle, in association with reduction in fat mass. This occurs without changes in UCP2 or UCP3 expression changes in BAT or WAT. (Nakamura *et al.*, 2001). Nagase *et al.* obtained similar results, although their study reported levels of total UCP mRNA only (Nagase *et al.*, 1996). It should be noted that β-ADR are localised to vessel walls within skeletal muscle, but little evidence exists to show that receptors can be found in myocytes themselves. This suggests that the adrenergic regulation of thermogenesis in skeletal muscle may occur due to some action within perivascular cells.

As discussed, Henry *et al.* (Henry *et al.*, 2008) have demonstrated postprandial temperature excursions in skeletal muscle, which are markedly up-regulated by central leptin infusion - indicative of neurally-mediated postprandial thermogenesis. One study has demonstrated that brown adipocytes are interspersed with WAT that surrounds muscle (Almind *et al.*, 2007), raising the possibility that thermogenic output of skeletal muscle may be due to the presence of

functional BAT. This seems unlikely, as the diet-induced elevation in skeletal muscle temperature caused by the central administration of leptin (Henry *et al.*, 2008) is associated with increased expression of UCP2 and UCP3 mRNA with no change in UCP1 mRNA (Henry *et al.*, 2011) If ectopic BAT were responsible for the temperature responses observed, UCP1 expression should be elevated. Analysis of isolated mitochondria from skeletal muscle samples during these conditions also showed a preference towards mitochondrial uncoupling, a hallmark of adaptive thermogenesis (Henry *et al.*, 2011). Manipulation of adaptive thermogenesis in skeletal muscle is a potential means of combating obesity. To realise this potential the underlying mechanisms and importance of this energy dissipation to daily energy expenditure needs to be investigated further.

1.5 Fuel Partitioning and Metabolic Pathways

As discussed earlier, whole body energy balance adapts in response to dynamic changes to metabolic status with respect to both energy intake and expenditure. As such, individual tissues and cells presumably possess mechanisms to detect and adapt to shifts between times of energy excess and scarcity. Indeed, fuel availability and metabolic demand govern fuel partitioning -the shunting of metabolic fuels to specific tissues, or into specific metabolic pathways. In the overfed state, fatty acids are transported to and stored in WAT. In a fasted state, fatty acids are transported to BAT and muscle, to be oxidised via the citric acid cycle, with ATP yielded via the ETC (Uauy and Díaz, 2005). By altering expression and activity of the proteins involved in fuel partitioning, energy expenditure and thermogenesis can be influenced (Uauy and Díaz, 2005; Towler and Hardie, 2007).

1.5.1 The Role of AMP-Activated Protein Kinase in Fuel Partitioning

ATP levels (in relation to that of AMP – a product of ATP hydrolysis) provide a valuable marker of the energy state of cells. The AMP to ATP ratio was first proposed to be a major determinant of fuel partitioning in the 1960s (Hathaway and Atkinson, 1963; Hathaway and Atkinson, 1965).

5' AMP-activated protein kinase (AMPK) was first identified and later named (Grahame Hardie *et al.*, 1989) for its ability to regulate synthesis of fatty acids (via inhibition of acetyl-CoA carboxylase; ACC; Carlson and Kim, 1973) and cholesterol (via activation of 3-hydroxy-3-methyl-glutaryl-CoA reductase; HMG-CoA reductase; Beg *et al.*, 1973). This occurs in response to fluctuating levels of AMP. AMPK is now regarded as a 'master switch' of cellular fuel partitioning. It is highly conserved across species (and yeast), and is widely distributed throughout many tissues types including liver, adipose, skeletal muscle and brain.

AMPK is a heterotrimer comprised of thee subunits: α, β and γ. For complete activation of AMPK two conditions must be met: 1) the AMP to ATP ratio needs to be relatively high, and 2) the α-subunit needs to be phosphorylated at Thr-172 by an upstream AMPK kinase (AMPKK) complex (Hawley *et al.*, 1996). As the AMP to ATP ratio increases, AMP is thought to act on the γ-subunit, resulting in a conformational change to the α-subunit, exposing its kinase domain (Cheung *et al.*, 2000). This allosteric change also allows the α-subunit to be phosphorylated, which greatly improves its activity (Stein *et al.*, 2000). AMP has also been shown to independently increase the actions of the AMPKK complex (Hawley *et al.*, 1995). The primary function of the β-subunit is to act as a scaffold for the α and γ subunits.

Activation of AMPK has the net effect of up-regulating energy consuming pathways such as glucose uptake (Kurth-Kraczek *et al.*, 1999) and fatty acid oxidation (Merrill *et al.*, 1997; Lee *et al.*, 2006), while down regulating energy storing pathways such as lipogenesis and protein synthesis. AMPK activation also upregulates mitochondrial biosynthesis.

AMPK activation is responsive to food consumption. In skeletal muscle of healthy men, ingestion of an amino acid and carbohydrate rich meal leads to a reduction in the phophorylation of AMPK and increased phophorylation of Akt and mTOR within one hour (Fujita *et al.,* 2007). Long-term feeding of a high fat di*et al*so decreases AMPK protein expression and activation (Liu *et al.,* 2006). In addition, hypothalamic AMPK activation has also been shown to increase food intake in rats (Andersson *et al.*, 2004; Xue and Kahn, 2006).

AMPK clearly plays an important role in fuel partitioning though its role in whole body energy balance is questionable. 5-Aminoimidazole-4-carboxamide ribotide (AICAR), is a kinase which phosphorylates AMPK. AICAR administration to mice induces increased fatty acid oxidation but with no change in whole body energy expenditure or adiposity (Hoehn *et al.*, 2010). AICAR-induced AMPK activation also increases skeletal muscle UCP3 expression in rats (Zhou *et al.*, 2000) In spite of this, the role of AMPK role in adaptive thermogenesis is also unclear. Recent studies in sheep suggest that leptin-induced adaptive thermogenesis can occur independent of AMPK activation (Laker *et al.,* 2011). Thus, AMPK activation may regulate thermogenesis in a species dependant manner, linked to thermogenesis in rodents but not in sheep.

1.5.2 The Role of Acetyl-CoA Carboxylase in Fuel Partitioning

One of the key downstream targets of AMPK is Acetyl-CoA Carboxylase (ACC). ACC regulates the carboxylation of acetyl-CoA into malonyl-CoA. AMPK phosphorylates ACC leading to inhibition of its actions. Malonyl-CoA is an essential, rate-limiting intermediate for fatty acid synthesis. It is also an inhibitor of fatty acid oxidation via its inhibition of carnitine palmitoyltransferase (CPT1; Wakil *et al.*, 1983).

Mammals express two isoforms of ACC encoded by different genes and with different tissue expression profiles and cellular localisation. ACC1 is localised in the cytosol of adipocytes and hepatocytes, and is thought to regulate fatty acid synthesis (Wakil and Abu-Elheiga, 2009). ACC2 is localised in the mitochondria of myocytes (skeletal and cardiac). Its primary function is the regulation of fatty acid oxidation (Abu-Elheiga *et al.*, 2001).

ACC2 knockout mice tend to display hyperphagia but maintain or lose body weight. This is explained by their elevated fatty acid oxidation rates. As a result, ACC2 knockout mice have lower levels of adiposity than wild types (Abu-Elheiga *et al.*, 2001; Choi *et al.*, 2007). ACC1 knockout mice are embryonically lethal, while heterozygous $ACC1^{+/-}$ mice develop similar to wild types, maintaining similar body weights and levels of malonyl-CoA (Abu-Elheiga *et al.*, 2001). In normal fed states, liver-specific ACC1 knockout mice have lower hepatic triglyceride stores compared to wild type (Mao *et al.*, 2006). In summary, ACC1 and ACC2 are important regulators of fatty acid synthesis and oxidation, and hence influence fuel partitioning.

1.5.3 The Role of Akt in Fuel Partitioning

Protein kinase B, also known as Akt is a serine/threonine protein kinase known for its role in a number of important cellular functions such as cell proliferation, apoptosis and glucose metabolism. Three isoforms of Akt are expressed in humans, Akt1, Akt2, Akt3. Expression of each isoform varies depending on tissue type. This variable pattern in expression is related to their different physiological functions (Yang *et al.*, 2003). Akt1 and Akt3 are thought to be responsible for growth and neuronal development, respectively. Akt2 is expressed in insulin-responsive tissues such as liver, adipose tissue and skeletal muscle (Yang *et al.*, 2003).

Akt2 deficient mice are severely insulin resistant; displaying hyperglycaemia, hyperinsulinaemia and glucose intolerance (Cho *et al.*, 2001). In one case, age-dependant

adipose tissue loss, and growth deficiency were observed in Akt2 deficient mice (Garofalo *et al.*, 2003). Constitutive expression of active Akt in adipose tissue has been shown to lead to continual glucose uptake in the absence of insulin (Kohn *et al.*, 1996). Akt2 is activated via the insulin/phosphatidylinositol 3-kinases (PI3K) signalling pathway, resulting in a variety of downstream effects. These include GLUT4 translocation to the cell membrane for glucose uptake in insulin sensitive tissues (Hill *et al.*, 1999; Hernandez *et al.*, 2001; Martin *et al.*, 1996); and inhibition of glycogen synthase kinase 3 (GSK3) resulting in increased levels of glycogen synthesis (Cross *et al.*, 1995). Clearly, Akt is an important regulator of glucose uptake, altering fuel partitioning in order to maintain glucose homeostasis.

1.6 Sexual Dimorphism and Body Composition

1.6.1 Sex Steroid Production and Function

The sex steroids testosterone, estrogens and progesterone are vital for an individual's proper development, maturation and for sexual reproduction. Testosterone is considered the 'male' sex hormone, while estrogens and progesterone are considered the 'female' sex hormones. Nevertheless, males produce estrogens and females produce testosterone. Each is synthesised at different rates, at different locations and have differing actions in the two sexes. The sex steroids have a number of physiological functions, primarily in sexual development. The sex steroids can also have both direct and centrally-mediated effects on food intake and metabolism; a simple schematic diagram of which can be seen in figure 1.7.

Any discussion on the effects of testosterone with respect to body energy dynamics must take account of biological processes that result in aromatisation of androgens to produce estrogens and 5α-reduction of androgens to produce 5α-dihydro-testosterone (5α -DHT). Specifically,

testosterone is readily converted to estradiol via aromatase which is expressed predominately in adipose tissue (Simpson *et al.*, 1994; Zhao *et al.*, 2009).

Testosterone is synthesised and released primarily from the testes in men and the ovaries in women, It acts at various tissues within the body including the brain, muscle and fat. It is found at much higher concentrations in males than females. Testosterone has two distinct roles, being essential to male reproduction and sexual behaviour as well as acting as an anabolic steroid. Anabolic steroids promote protein synthesis, increasing muscle mass and bone density, while reducing adipose accumulation.

In premenopausal women, estrogens are primarily secreted from the ovaries and are integral to the maintenance of reproduction. In males and post-menopausal females the primary site of estrogen production is the adipose tissue itself. Androgens and estrogens can act genomically via the relevant nuclear receptor or via non-genomic pathways which allow faster signalling than the former (D'Eon *et al.*, 2005). Estrogens and progesterone regulate the menstrual cycle in women by feedback action on the brain and the pituitary gland (Clarke, 1996) These steroids also play a role in a number of other body functions, including the regulation of adiposity.

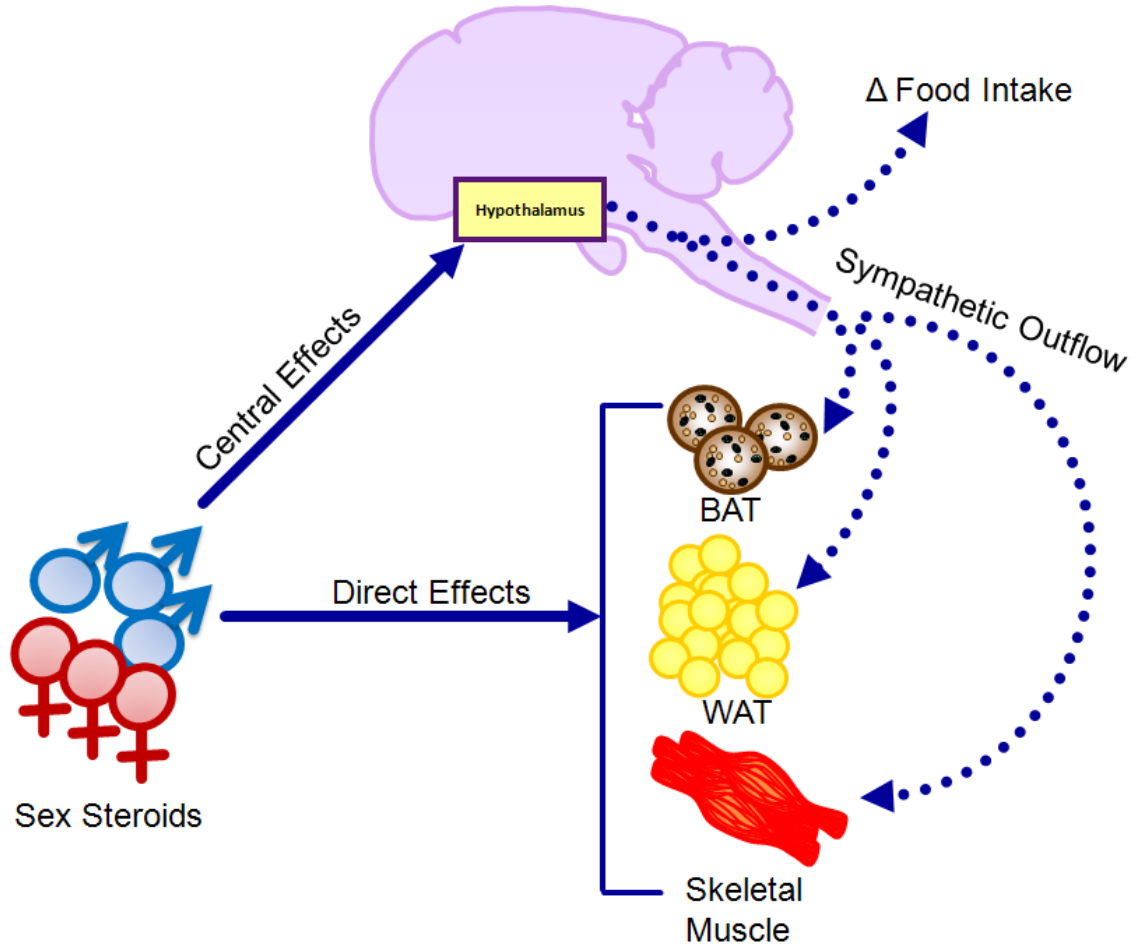

Figure 1.7: Schematic diagram of the direct and centrally mediated effects of sex steroids on energy expenditure. The sex steroids can act centrally via the hypothalamus to induce changes in food intake, as well as altered sympathetic outflow to peripheral tissues such as brown adipose tissue (BAT), white adipose tissue (WAT) and skeletal muscle. Sympathetic activation of peripheral tissue may have many effects such as change in metabolic rate and the induction of thermogenesis. Sex steroids may also act directly on peripheral tissue to alter metabolism and protein synthesis.

1.6.2 Sex Steroids and Body Composition

While some sex-based differences in body composition exist at birth (Koo *et al.*, 2000; Ay *et al.*, 2008; Rodríguez *et al.*, 2004), sexual dimorphism of body composition and adipose distribution manifests in a most notable way at puberty (Ogle *et al.*, 1995) and is maintained throughout adulthood until women reach menopause. Sexual dimorphism exists whereby an androgenous (male) hormonal profile is associated with a more central (visceral) distribution of adiposity (Blouin *et al.*). Conversely, pre-menopausal women tend to have a higher subcutaneous distribution of adipose tissue (in the gluteal/femoral region; Nindl *et al.*, 2002; Krotkiewski *et al.*, 1983). As the hormonal profile of women changes during menopause, declining levels of estrogens and increased (relative) concentrations of androgens are associated with the development of an android adipose distribution (Lee *et al.*, 2009; Lovejoy *et al.*, 2008; Lovejoy *et al.*, 1996). Additionally, hyper-androgenic women (e.g. those with polycystic ovarian syndrome and female-to-male transsexuals undergoing hormone replacement) display accumulation of visceral adipose tissue (Cascella *et al.*, 2008; Elbers *et al.*, 1997). Men also undergo age related changes in body composition due to decreasing testosterone and growth hormone levels and increasing cortisol levels (Isidori *et al.*, 2005; Morley *et al.*, 1997).

As previously discussed, central (visceral) adiposity is linked to increased risk factors for a number of medical conditions (Wajchenberg, 2000; Kannel *et al.*, 1991). Subcutaneous adiposity is regarded as relatively benign (Bjrntorp, 1996). This accounts for the higher prevalence of CVD in males compared to pre-menopausal women (Nedungadi and Clegg, 2009). There is also an increased risk of metabolic syndrome in male adolescents compared to females. This persists throughout adulthood (Syme *et al.*, 2008). As women enter menopause, and begin to display an androgenous distribution of adipose tissue, these risk factors come into line with their male counterparts but can be reversed under hormone replacement therapy (Stampfer *et al.*, 1991; Gambacciani *et al.*, 2001; Rosano *et al.*, 2007). The same can be said for

the risk of Type 2 diabetes in postmenopausal women (Lindheim *et al.*, 1994) which can be ameliorated by hormone replacement (Bonds *et al.*, 2006).

While sexual dimorphism of adipose distribution and CVD prevalence is well recognised in the clinical setting, the molecular and physiological mechanisms involved in these processes are not well understood.

1.6.3 Sex Steroid Receptors

Historically, although it was well established that sex steroids (or at least the presence of gonads) regulated body composition in humans, the mechanism for this was not elucidated until recent times. In particular, it was not known whether the underlying mechanisms were due to peripheral or central effects of steroids. Knowledge of the interplay between centrally mediated energy balance signals and peripheral tissues such as adipose tissue and skeletal muscle has been expanded in the past few decades. This has received impetus from the advancement of molecular biology techniques enabling the localisation of estrogen, androgen and progesterone receptor expression to specific tissue types and regions as well as the appreciation of membrane effects of estrogens (Mauvais-Jarvis, 2011).

Testosterone acts via the androgen receptor (AR), a transcription factor affecting the expression of a wide battery of genes. More recently, non-genomic actions of testosterone have been elucidated, whereby AR activation can lead to rapid intracellular calcium flux and activation of kinase-signalling cascades (Foradori *et al.*, 2008). Two basic subtypes of estrogen receptors (ER) are expressed in mammals, ERα and ERβ; these have both genomic and non-genomic actions (Levin, 2005).

Peripherally, estrogen (Mizutani *et al.*, 1994; Crandall *et al.*, 1998), testosterone (Pedersen *et al.*, 1996a) and progesterone (O'Brien *et al.*, 1998) receptors have been shown to be expressed in human adipose tissue. Of note, the levels and types of sex steroid receptors in adipose tissue differ in relation to sex and region. In male rats, the androgen receptors are more prevalent in visceral adipose tissue than in subcutaneous fat (Sjögren *et al.*, 1995). In a study of male and female humans, both androgen receptor number and binding was higher in visceral preadipocytes than in subcutaneous adipocytes (Joyner *et al.*, 2002). Another study found that the estrogen-binding capacity of visceral and subcutaneous fat deposits was equal in females, while in males, subcutaneous adipose tissue estrogen-binding capacity was twice that of visceral fat. (Pedersen *et al.*, 1996b). Although there is some contradictory literature, it seems that anatomical and molecular differences may provide a mechanistic explanation for the sex-based body composition disparities seen in humans.

Sex steroid receptors are also expressed by skeletal muscle cells. In humans, AR are expressed on many cell types (such as myosatellite cells the myocytes themselves) found in skeletal muscle, and expression is up-regulated by androgen action (Sinha-Hikim *et al.*, 2004). In rats, castration results in reduced AR expression in specific muscle groups (the bulbocavernosus but not levator ani muscle (Antonio *et al.*, 1999). A primary effect of androgens on skeletal muscle is protein synthesis, regulating skeletal muscle mass (Sheffield-Moore, 2000). Both ERα (Lemoine *et al.*, 2003) and ERβ (Glenmark *et al.*, 2003) are expressed in human skeletal muscle, with no sex differences in expression profiles (Wiik *et al.*, 2009). In mice, ERα positively regulates while ERβ suppresses expresseion of GLUT4-mediated glucose uptake by skeletal muscle (Barros *et al.*, 2006).

Widespread expression of ER and AR throughout many hypothalamic regions is relevant to the central control of energy balance. ER are located in various hypothalamic areas such as the medial preoptic area, PVN, the ARC and the VMN (Diano *et al.*, 1998). In general, estrogen delivery into the brain results in reduction in food intake (Butera and Czaja, 1984; Palmer and

Gray, 1986), which may be due to action at various loci. While POMC and NPY neurons of the ARC are often considered the 'first order' neurons in the control of food intake, in sheep only 10-20% of these neurons express ERα (Lehman and Karsch, 1993; Skinner and Herbison, 1997). In rodents 3% of POMC neurons express AR (Fodor and Delemarre-van de Waal, 2001) while in sheep approximately 20% of NPY do (Lehman and Karsch, 1993). As such, it is believed that the sex steroids act indirectly on POMC and NPY neurons to regulate energy balance. Neurons expressing kisspeptin (a peptide involved in hypothalamic control of puberty), also highly express ERα, and can directly act on POMC and NPY cells in response to leptin activation (Backholer *et al.*, 2010). These neurons may provide an indirect path for the actions of estrogens on POMC and NPY neurons.

Hypothalamic androgen receptor mRNA expression in sheep has been found to be similar between rams and ewes in most regions, though rams express the receptor in greater abundance in the rostral preoptic area, caudal preoptic area and rostral portion of the bed nucleus of the stria terminalis than ewes (Scott *et al.*, 2004). Ewes also have more estrogen receptor-positive cells in the hypothalamus and a greater abundance of receptors per cell in the ventromedial nucleus compared to rams; other minor differences can also be found (Scott *et al.*, 2000). In summary, the hypothalamic distribution of ERα, ERβ, and AR containing cells in farm animals is quite similar between the sexes, with the exceptions mentioned above (Scott *et al.*, 2000). Immunohistochemistry has revealed that the brains of male rats have more androgen receptors than females in areas such as the bed nucleus of the stria terminalis, posterior aspect, medial preoptic area, and dorsal and ventral aspects of the lateral septum (Lu *et al.*, 1998).

1.7 The effects of Testosterone on Energy Balance

1.7.1 Testosterone and Body Composition in Humans

As the primary sex steroid in males, testosterone modulates many systems. In relation to energy balance this was thought to be due to aromatisation and action via ER (Mauvais-Jarvis, 2011).

However, in mice, administration of the non-aromatizable androgen 5α -DHT to castrate females (McInnes *et al.*, 2006) and males (Moverare-Skrtic *et al.*, 2006) leads to an obese phenotype. These changes are not caused by altered food intake, suggestive of an effect via altered energy expenditure.

In humans, free testosterone concentrations and fat mass are inversely correlated in young males (Mauras *et al.*, 1998). As males age, plasma testosterone levels decrease (Bhasin *et al.*, 1996) which can, in part, explain the concurrent increase in adiposity (Shi and Clegg, 2009) The changes in total serum testosterone and adiposity are linear with age (Nielsen *et al.*, 2007). Site specific changes in adiposity in aging males show that fat gain is not uniform, such that there is an increase in the visceral:subcutaneous fat ratio (Wannamethee *et al.*, 2007; Yanase *et al.*, 2008). Additionally, cross-sectional and longitudinal studies have shown a correlation between declining testosterone levels and increasing leptin levels as males age, which may explain the reduced food intake of the elderly (Baumgartner *et al.*, 1999; Nicklas *et al.*, 1997; Morley, 2001). It should be noted that changes in cortisol and growth hormone may also be involved in altered body composition in aging men (Lamberts *et al.*, 1997).

Highlighting the potential anabolic effects of testosterone in women, year-long testosterone replacement in female-to-male transsexuals causes increased total body weight but reduced whole body adiposity, including visceral fat (Elbers *et al.*, 1999). In women, polycystic ovary syndrome (PCOS) is associated with visceral obesity, insulin resistance and hyperandrogenism, perhaps underlining deleterious effects of androgens in women (Zacur, 2001; Cascella *et al.*, 2008). It is clear that androgens play an important role in energy balance in male and females, but the mechanisms underpinning these actions remain less clear.

1.7.2 Testosterone and Food Intake

In rodent models, testosterone removal by orchidectomy reduces food intake and increases body weight (Gentry and Wade, 1976; Leshner and Collier, 1973; Mitchel and Keesey, 1974). These effects are reversed by testosterone replacement (Gentry and Wade, 1976; Chai *et al.*, 1999). Testosterone replacement to hyper-physiological levels initially reverses the effects of orchidectomy but, in the long term, a lowered food intake and body weight ensues, mainly through reduction in fat weight (Gentry and Wade, 1976; Kochakian and Endahl, 1959). On the other hand, there are some reports of increased body weight in response to testosterone replacement, being due to increased lean mass with no changes in fat mass (Bhasin *et al.*, 1997). A similar effect is seen in humans, whereby testosterone treatment of hypogonadal and normal men lowers fat mass and increases fat-free mass (Bhasin *et al.*, 1996; Bhasin *et al.*, 1997; Katznelson *et al.*, 1996).

In sheep, the effects of testosterone on energy balance are influenced by seasonal variation driven by photoperiod. Seasonal variation in NPY levels in the ARC, being highest during longer days, matches the time of peak food intake (Clarke *et al.*, 2003). Testosterone treatment of castrate rams during this period increases NPY expression (Dobbins *et al.*, 2004). Furthermore, differences in food intake exist between castrate and intact rams during short photoperiod exposure whereby food intake decreases during exposure, but in intact animals food intake increases following a decline in testicular activity (Anukulkitch *et al.*, 2007). This is due to the photorefractoriness in the intact animals but not in the castrates. These changes are linked to increased NPY and leptin receptor expression profiles, and decreased POMC expression (Anukulkitch *et al.*, 2007). Testosterone has clear effects on hypothalamic control of food intake, further modulated by photo-period signals in seasonal mammals.

1.7.3 Effects of AR Expression Manipulation

One way to investigate the role of androgens on energy balance is to manipulate expression of the AR *in vivo*. This can be achieved via a number of techniques to up-regulate, down-regulate or knock out AR in the whole body in a site or tissue specific manner. This is ideally performed in mice, in which transgenic technology is more advanced. The creation of whole body AR knockout (ARKO) has led to a number of discoveries in relation to androgen action. Male but not female ARKO mice display late-onset obesity (Lin *et al.*, 2005; Sato *et al.*, 2003), highlighting sexually divergent actions of androgens. Another hallmark of ARKO mice is skeletal muscle atrophy, which is observed in both sexes (MacLean *et al.*, 2008; Ophoff *et al.*, 2009). Interestingly, the creation of ARKO mice via deletion of different exons in the androgen receptor gene results in varying hormonal and metabolic profiles, although delayed onset obesity in males is always a feature. Knockout via deletion of exon 2 of AR causes leptin resistance and high leptin levels (Lin *et al.*, 2005), while exon 1 deletion causes reduced spontaneous activity and lowered oxygen consumption, while having no effect on insulin sensitivity (Fan *et al.*, 2005). Exon 1 deleted ARKO mice also exhibit high serum adiponectin levels (Fan *et al.*, 2005). Adiponectin is a hormone secreted by adipose tissue that aids in insulin sensitivity (Kadowaki *et al.*, 2006). These findings in ARKO mice are consistent with human studies in which testosterone has been shown to suppress adiponectin levels (Page *et al.*, 2005).

Tissue specific AR expression studies have provided insight into the importance of androgen action on skeletal muscle in whole body energy dynamics. Overexpression of AR receptor in the skeletal muscle of rats leads to increased muscle mass, decreased fat mass, greater activity of mitochondrial enzymes in skeletal muscle and increased whole body O_2 consumption (Fernando *et al.*, 2010). Unexpectedly and in contrast to the whole body ARKO studies discussed above, skeletal muscle specific ARKO mice have reduced fat mass (Ophoff *et al.*, 2009). These animals were studied at 16 weeks, while whole body ARKO mice generally develop obesity beyond 20 weeks which may explain the differences observed between these two genetic

models. Adipose specific ARKO mice do not exhibit late onset obesity, but are hyperleptinemic (Yu *et al.*, 2008). Taken together, it appears that androgen action via its receptor in both skeletal muscle and adipose tissue may play a more important role in regulating whole body energy expenditure though the underlying mechanism(s) remain to be elucidated.

1.7.4 Testosterone, Thermogenesis and Energy Expenditure

There is a sparse literature regarding the effects of androgens on energy expenditure. Dihydrotestosterone (DHT) treatment of ovariectomised rats leads to lower levels of phosphorylated AMPK and ACC in visceral fat. Since these enzymes are fundamental to the oxidation of fatty acids, this results in increased adiposity and body weight gain (McInnes *et al.*, 2006). In ARKO mice (discussed above), WAT exhibits transcriptional profiles indicative of inhibited lipolysis, through down-regulation of hormone sensitive lipase, a key lipolytic enzyme (Yanase *et al.*, 2008). Although there is no change in acyl-CoA carboxylase levels, a key fatty acid synthesis regulator, the net effect of this profile is to increase fat accumulation. The skeletal muscle of these mice also had increased expression of GLUT4, and responded to insulin; whether this is directly due to lack of androgen action on muscle and fat tissues is not known. It is possible that the loss of AR alters the expression of 'appetite regulating peptides' in the brain, which indirectly alters the function of muscle.

Androgens have been implicated in the regulation of core body temperature, with men experiencing over-night nadirs (approx. -0.5°C) in rectal temperatures, while women do not (Baker *et al.*, 1998). Rodent models have also shown that testosterone impacts on circadian rhythms in core body temperature, with adult rats castrated during the neonatal period-exhibiting an earlier rise in core body temperature during the light phase compared to intact control animals. In this study, female rats also display higher overall body temperatures than intact males (Zuloaga *et al.*, 2009), which may be due to androgen status, although this was not

defined. It should be noted that these studies may indicate steroid-independent effects of sex on body temperature, and not direct action of androgens *per se*.

In rodents, testosterone treatment decreases UCP1 mRNA expression in BAT, but also decreases the count and size of lipid droplets in WAT. In contrast, testosterone treatment accentuates noradrenaline-induced increases in UCP2 mRNA expression in BAT (Rodríguez *et al.*, 2002). Further to this, testosterone also down-regulates PGC-1, the main transcription factor responsible for UCP1 expression (Rodriguez-Cuenca *et al.*, 2007). The above studies were conducted *in vitro* on isolated cells, hence their relevance to *in vivo* models are unknown. *In vivo,* transcription profiling of male ARKO mice also show marked down-regulation of UCP1, further evidencing the effect androgen stimulation may have on UCP1 expression and adaptive thermogenesis (Yanase *et al.*, 2008). Data relating to androgen action on thermogenesis and energy expenditure do not yet form a comprehensive picture.

1.8 The effects of Estrogens on Energy Balance

1.8.1 Estrogens and Body Composition

As previously discussed, human menopause or ovariectomy (OVX) of animals is associated with reduced estrogen levels and increased accumulation of visceral adipose tissue (Lee *et al.*, 2009; Clegg *et al.*, 2006). In humans, these changes are usually associated with reduced energy expenditure (Poehlman *et al.*, 1995). Overall, removal of estrogens either via menopause, ovariectomy or genetic manipulation (knock out of the aromatase gene in mice; Jones *et al.*, 2000) results in an attenuation in lipolysis and an increase in triglyceride accumulation in a site specific manner.

1.8.2 Estrogens and Food Intake

In humans, a reduction in food intake is observed immediately prior to ovulation, which coincides with the rising levels of estrogens in the preovulatory period (Lyons *et al.*, 1989). Thus, in humans, food intake is greatest during the luteal phase of the estrous/menstrual cycle (Gong *et al.*, 1989; Dalvit, 1981; Dalvit-McPhillips, 1983) corresponding to low levels of estrogens.

In rodent models, estrogen has been shown to reduce the expression of NPY and AgRP (Titolo *et al.*, 2006). Lateral ventricular infusion of NYP acutely decreases food intake, an effect dampened by accompanying infusion of estrogens (Santollo and Eckel, 2008). It is unsure if these effects were due to receptor expression changes. Estrogen (mimicking the effects of leptin) also increases the amount of excitatory inputs to POMC cells, inducing catabolic effects (Gao *et al.*, 2007). Discrete ablation of ERα in the ventromedial nucleus of female rats and mice results in hyperphagia and a concomitant reduction in physical activity and diet-induced thermogenesis (Musatov *et al.*, 2007). Thus, estrogen not only regulates energy balance via modulating food intake, but also via energy expenditure pathways.

1.8.3 Effects of ER Expression Manipulation

Both male and female ERα knockout (αERKO) mice have been shown to have higher levels of adipose tissue, although there is no difference in food intake compared to wild-type animals (Heine *et al.*, 2000). This indicates a role for altered energy expenditure. These results were supported by another study showing that αERKO male mice became obese with altered lipoprotein profiles at maturity (Ohlsson *et al.*, 2000). On the other hand, Bryzgalova *et al.* (Bryzgalova *et al.*, 2006) observed obesity in female but not male αERKO mice, which was associated with hyperglycemia attributed to hepatic insulin resistance. In these studies ERβ knockout (βERKO) mice were shown to be of normal weight/adiposity (Bryzgalova *et al.*, 2006; Ohlsson *et al.*, 2000), indicating that this subtype of the estrogen receptor does not mediate

effects of estrogens on energy balance. One study has shown that ERα mediates the anorectic effects of estrogen on food intake, while ERβ does not (Roesch, 2006). These studies highlight the relative importance of ERα over ERβ in energy homeostasis in both males and females.

1.8.4 Estrogen, Thermogenesis and Energy Expenditure

Core body temperature fluctuations across the estrous cycle are thought to be regulated by progesterone, being higher when plasma progesterone levels are high (Baker and Driver, 2007; Coyne *et al.*, 2000; Lee, 1988). Though estrogen may have an effect on modulating the thermostatic effects of progesterone, leading to reduced core body temperature during the follicular phase (Stachenfeld *et al.*, 2000), when progesterone levels are low.

Studies in humans demonstrat that the incidence of active BAT is greater in females (7.5%) than in males (3.1%; Cypess *et al*, 2009), which may point to a role of estrogen plays a role in adaptive thermogenesis, at least in BAT. Effects of progesterone cannot be ruled out. As previously mentioned, gene silencing of ERα in the ventromedial nucleus leads to obesity, attributed to decreased energy expenditure, including decreased diet-induced thermogenesis (Musatov *et al.*, 2007). Ovariectomy, resulting in reduced levels of estrogens also leads to decreased UCP1 expression in BAT, and decreased UCP3 expression in skeletal muscle under mild energy restriction (Pedersen *et al.*, 2001). In contrast, administration of estrogen to *in vitro* brown adipocytes does not alter UCP1 mRNA levels (Rodríguez *et al.*, 2002). The same group has further demonstrated that cultured rat brown adipocytes exposed to 17-β-estradiol displayed lipid droplets larger in size and in greater number compared to unexposed cells, indicating reduced energy expenditure as a result of estrogen action.

The potential role of estrogens in adaptive thermogenesis is relatively unexplored, being an area of focus in this thesis. Further to this, recent studies have indicated that estrogen may play an

important role in regulating energy expenditure through altered fuel partitioning. Treating skeletal muscle cells with physiological levels of estrogen have been shown to rapidly stimulate phosphorylation of both Akt (Vasconsuelo *et al.*, 2008) and AMPK (D'Eon *et al.*, 2008; D'Eon *et al.*, 2005), the key regulators of glucose uptake and fatty acid oxidation. Interestingly, stimulation of skeletal muscle *ex vivo* has shown activation of Akt and AMPK within 10 mins of treatment, but no change in glucose uptake was observed (Rogers *et al.*, 2009), indicating that other mechanisms of glucose control may be involved.

1.9 Summary and Unifying Hypothesis

The discovery of functional BAT in adult humans has renewed interest in the role of adaptive thermogenesis in energy balance. Rodent models have been used extensively to investigate adaptive thermogenesis due to their conserved and well defined depots of BAT. It is apparent now, that BAT is present in human infants and is also found dispersed amongst WAT of adult humans. Similar observations have been made in sheep. Furthermore, recent studies indicate the potential thermogenic capacity of skeletal muscle, which may provide a novel target in the combat against obesity.

This laboratory has recently established a model in conscious sheep which allows the continued measure of adipose and skeletal muscle temperature in a site specific manner. With this model plasma and peripheral tissues (fat and muscle) may be sampled in a series, so that correlative data may be obtained to explain excursions in tissue temperatures. This model employs programmed feeding, whereby food intake is restricted to a set feeding time, to entrain a postprandial response. Previously this model has been used to ascertain postprandial temperature effects in these tissues as well as determining the effects of the appetite suppressing peptide, leptin, on energy expenditure.

In humans and in animal models, the actions of androgens and estrogens have profound effects on food intake, whole body energy expenditure and body composition. Work in this area has primarily focused on the regulatory role of sex steroids on appetite and the effects of sex steroid receptor knockout on energy homeostasis in mice. This thesis sought to expand on the knowledge of the effects of estrogen and testosterone on energy expenditure pathways, with the use of a large animal model. This was achieved by adapting the above mentioned sheep model of postprandial temperature excursion.

The unifying hypothesis tested within this thesis was that androgens and estrogens differentially regulate energy balance through tissue and site specific actions on adaptive thermogenesis and fuel partitioning. Three hypotheses were tested;

1. Postprandial temperature excursions in skeletal muscle are not explained by regional blood flow to the tissue.

2. Whole body androgen replacement in castrate ewes and rams affects thermogenesis and energy balance in a sex and tissue specific manner.

3. Chronic and acute estrogen replacement in castrate ewes affects energy balance in a time and tissue dependant manner.

CHAPTER 2
General Materials and Methods

2.1 Ethics and Animal Management

2.1.1 Ethics

All animal procedures were conducted in accordance with the Australian Prevention of Cruelty to Animals Act 1986 and the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation/Australian Animal Commission "Australian code of practice for the care and use of animals for scientific purposes". All procedures were approved by the School of Biomedical Sciences Animal Ethics Committee at Monash University, Australia

2.1.2 Animal Management

Adult Corriedale ewes (Chapters 3, 4 and 5) and Merino wethers (Chapter 4) were acclimated to single pen housing for at least 1 week prior to the onset of experimentation. Animals in Chapters 4 and 5 were kept at the Monash Large Animal Research Facility in Werribee, Victoria, Australia (latitude 38°S). These animals were exposed to natural light and ambient temperatures. Animals in Chapter 3 were housed in the Department of Physiology, Monash University, Clayton, Victoria, Australia. These animals were housed in an isolated room, exposed to a 12:12 (h) light/dark cycle (lights on at 0600h) and the ambient temperature of the room was maintained at 22°C. All animals were kept in pens designed to allow the animal to sit and stand but prevented further movement that may have influenced thermogenic output.

2.2 Surgery

All Surgical procedures were performed under sterile conditions on fasted animals by Mr. Bruce Doughton or Mr. Alex Satragno.

2.3.1 Anaesthesia

Animals were anaesthetised with an intravenous injection of Thiobarbitone sodium (10mg/kg; Lyppard, Keysborough, Vic., Australia). Animals were laid on their back and an endotracheal tube was introduced, prior to the animal being placed on an operating table and connected to an anaesthetic machine. Throughout surgery, anaesthesia was maintained with a 3-5% gaseous flurothane (Rhone Merieux Australia, West Footscray, VIC, Australia) in oxygen. Nitrous oxide was used to deepen anaesthesia when required. The mixture of gases was administered using an anaesthetic machine by an experienced operator.

2.3.2 Surgical Preparation

All animals were given a routine antibiotic injection of Terramycin i.m. (oxytetracycline hydrochloride 200 mg/ml, 1 ml/10 kg body weight; Pfizer Animal Health, West Ryde, NSW, Australia). In addition, ewes were administered the analgesic Rimadyl, i.v. (carprofen 50 mg/ml, 1 ml/12.5 kg body weight; Pfizer Animal Health, West Ryde, NSW, Australia).The animal was then prepared for surgery by clipping the wool over the operation site. The skin was then shaved and scrubbed with Betadine Surgical Scrub (7.5% w/v providone – iodine; Fauldings & Co. Ltd., Adelaide, SA, Australia) The site was then sprayed with 70% alcohol (Yarraville Distillery, Yarraville, Vic, Australia), and the animal was covered with surgical slit drape leaving the region of the operation exposed.

2.2.3 Post-Operative Care

Following suturing, the surgical site was sprayed with an antibiotic (Pinkeye aerosol, oxytetracycline hydrochloride 2.0mg/g; Pfizer Animal Heath, West Ryde, NSW, Australia). Following cessation of anaesthesia the animals were monitored for recovery, specifically by observation for adequate breathing, eye reflex, swallowing and chewing. The endotracheal tube was removed once the animal had regained consciousness and the swallowing reflex was restored. Animals were placed either in metabolic cages or in more spacious lambing pens (when available) and allowed to recover.

Animals were immediately fed and once they had achieved a stable standing position were allowed access to water. Animals were monitored for signs of distress, pain or infection during

the post-surgical period and for the duration of the experiments. Appropriate actions were taken to ease any pain and to treat any infected areas.

2.2.4 Surgical Techniques

2.2.4.1 Ovariectomy

All ewes (Chapters 3, 4 and 5) were ovariectomised bilaterally at least one month prior to experimentation. For this procedure, the animal was laid on its back and a mid-line laparotomy performed, the uterus and ovaries were then located and gently externalised. The ovarian blood vessels were ligated around the ovarian pedicle and the ovaries removed. The uterus was placed back into the abdomen, and the wound was closed with two series of sutures, first closing the abdominal wall and then the skin.

2.2.4.2 Datalogger Insertion

Dataloggers (SubCue, Calgary, Alberta, Canada) are implantable devices used for site-specific recording tissue temperatures. Dataloggers were placed in 3 sites, being the skeletal muscle of the hind limb, the visceral and subcutaneous fat. For each surgical procedure the animal is laid on its side. The dataloggers are sterilised overnight in 70% ethanol. Each datalogger comprises of a recording head (2cm diameter and 0.5cm deep), which is connected (10cm or 20cm) to a download point; the download point is tethered to allow externalisation, giving download access throughout the course of an experiment.

For implantation into skeletal muscle, a small incision (5cm) was made along the limb. Blunt dissection (with round ended surgical scissors) was used to separate the vastus lateralis and

bicep femoris muscles. This minimised damage to the muscle. The datalogger was implanted between the two muscles, with the recording side facing vastus lateralis and anchored in position with the use of chromic gut suture. The skin was closed with suture.

For implantation in the visceral fat, the retroperitoneal region was targeted. An incision was made below the rib cage by manual probing to identify the lower region of the spinal cord. An incision (10cm) was made and the abdominal cavity was accessed by blunt dissection through the abdominal wall. The retroperitoneal fat bed was located and a small pocket was made in the fat - again using blunt dissection. The datalogger was anchored within this pocket with chromic gut suture. Care was taken to ensure that the datalogger was implanted in the fat surrounding the kidney and not in proximity to the kidney itself to ensure accurate temperature recordings of the fat. The wound was closed with two sets of sutures, first to close the abdominal wall and then to close the skin.

For implantation in the subcutaneous fat, the datalogger was implanted using the same incision as for the skeletal muscle. The subcutaneous fat was separated from the underlying muscle using blunt dissection. The datalogger was anchored with chromic gut suture, ensuring that the recording side was facing the gluteal fat. The wound was sutured as outlined for the skeletal muscle surgery.

In each case, prior to closing the wound the download lead was exteriorsed and tagged for future use. Data could then be downloaded at any time.

2.2.4.3 Blood Flow Probe Insertion

Whole limb blood flow was measured using a trans-sonic flow probe that was surgically placed around the femoral artery. A transit time ultrasound flow probe (6SB, Transonics Systems Inc,

Ithaca, NY, USA) was placed around the femoral artery immediately distal to the caudal femoral artery branch to measure femoral artery blood flow. In order to determine whether whole limb flow reflected localised changes in tissue perfusion, two animals were additionally fitted with laser Doppler flowprobes (MSP300XP, Oxford Optronix, Milton Park, Oxford, UK) in order to measure capillary blood flow in muscle tissue adjacent to implanted dataloggers.

2.3 Model of Diet-Induced Thermogenesis

Sheep are continuous feeders and hence do not typically exhibit any circadian rhythms associated with meal feeding. Through temporal food restriction, however, or a set "meal feeding" paradigm meal, associated rhythms can be entrained. Previous studies have also demonstrated that fixed meal feeding can entrain a postprandial temperature response and a good example of this is the preprandial elevation in ghrelin secretion (Sugino *et al.*, 2002). For each of the experiments outlined in this thesis, food was made available between 1100-1600h daily (water was available *ad libitum*). Animals were subjected to this feeding window for at least 2 weeks prior to experimentation for entrainment, ensuring that a postprandial elevation in temperature occurred. Across each experiment, daily food intake was measured; animals were fed 2kg of lucerne chaff/day and refusals were weighed.

2.4 Peripheral Sex Steroid Replacement

Studies investigating the effects of sex steroids on energy expenditure compared control, gonadectomised conditions to those where whole body sex steroid replacement had been achieved. While whole body testosterone replacement (Chapter 4) was only administered

chronically; whole body estrogen placement (Chapter 4) was broadly categorised into two types: acute or chronic.

Acute estrogen replacement was achieved via intra-muscular (i.m.) injection of estrogen benzoate (1ml, 50µg/ml; Intervet International, VIC, Australia). Equal-volume injections of sterile peanut oil were given as control.

Chronic sex steroid replacement was achieved through the insertion of subcutaneous implants designed to defuse constant amounts of either testosterone (3 x 200 mg, Organon Pty. Ltd., Lane Cove, NSW Australia) or estrogen (3x3 cm). Blank Silastic tubes that were the same size as the estrogen implants were used for control conditions. For experiments incorporating a cross-over design (Chapter 5) implants were removed with an adequate washout period (4 weeks) to ensure removal of the sex steroids from the body.

2.5 Tissue Collection

2.5.1 Cannulation

Animals were cannulated with 12G indwelling venous cannulae (Teflon Dwellcath, Tuta Laboratories Australia Pty. Ltd. Lane Cove, NSW, Australia) on the day preceding the initiation of experiments. One external jugular vein was cannulated, the cannula connected to a manometer line (Portex Ltd., Hythe, Kent, UK) and closed with a 3-way Luer-lock tap (Baxter Travenol Laboratories Inc., Deerfield, Illinois, USA). Lines were kept patent with heparinised (100 or 50 units/ml; Fisons Pty, Ltd., Sydney, NSW, Australia) physiological (0.9% NaCl) saline.

2.5.2 Blood Sampling

Serial blood samples (6ml) were taken at regular intervals throughout the sampling periods (See Chapters 4 and 5 for specific time points). Prior to sampling the heparinised saline was removed from the line with a flushing syringe by drawing 4ml, a blood sample was then taken with a sample syringe and fresh heparinised saline used to re-fill the manometer line. Samples were place into tubes containing anti-coagulant lithium heparin (Sarsedt Australia, Mawson Lakes SA, Australia) and centrifuged for 10min at 3000g. The plasma was decanted into vials and stored at -20°C until assayed.

2.5.3 Skeletal Muscle and Adipose Tissue Collection

Muscle tissue was collected via a number of methods. In conscious animals, muscle tissue was collected by needle biopsy and under local anaesthesia s.c. (Lignocaine, 10ml; Astra Pharmaceuticals Pty Ltd, N. Ryd, NSW, Australia), while in unconscious animals tissue was collected via blunt dissection from animals under general anaesthesia. For the latter, the animals received 10 ml Thiopentone , by jugular venepuncture and the wool over the sample site of the back leg was quickly shaved. The skin was prepared as for surgery (see above) and a drape was placed over the biopsy site. An incision of 1.5cm was made with a scalpel, through the skin and down to the muscle. A slice of muscle (100mg) was taken, in the plane of the fibres. The muscle wound was closed with chromic 2 suture stitches and the skin was closed with 3 stitches. This procedure took less than 10 min and the animals were then allowed to recover from the anaesthetic in their home pen. Some muscle and all adipose tissue samples were collected immediately post-mortem from an overdose of Lethabarb (Virbarc, Regents Park, NSW, Australia). Tissue collection method is detailed in experimental chapters in the relevant sections.

2.6 Radioimmunoassays

2.6.1 Follicle Stimulating Hormone (FSH)

Plasma samples were assayed for follicle stimulating hormone (FSH) following the method of Bremner *et al.* (Bremner *et al.*, 1980). Assay specific details are given in the experimental chapters.

2.6.2 Testosterone

For measurement of testosterone we utilised a double extraction radioimmunoassay as outlined by Young *et al.* (Young *et al.*, 1989). Samples (100µl) were assayed in duplicate. Assay specific details are given in the experimental chapters.

2.7 Metabolic Characterisation

2.7.1 Glucose and Lactate

Blood glucose and lactate concentrations were measured in 25-ml samples of plasma using a YSI2300 STAT glucose/lactate analyzer (Yellow Springs Instrument Co., USA). The measurable range for blood glucose was 0–30mmol/l and for lactate was 0–16 mmol/l.

2.7.2 Non-Esterfied Fatty Acids (NEFA)

The plasma non-esterified fatty acid (NEFA) concentrations were determined by the enzymatic kit assay (Wako Diagnostics, Richmond, VA, USA) previously described by Sechen *et al.* (Sechen *et al.*, 1990). Assay sensitivity and the range will be provided in the relevant chapters.

2.7.3 Insulin

The plasma levels of insulin were measured in duplicate with an enzyme linked immuno-assay. Briefly, a 96-well plate was coated with anti-insulin antibody raised in guinea pigs (Antibodies Australia, Clayton, Vic. Australia) overnight at 4°C. The coating solution was decanted and blocking solution (1.5% BSA/ PBS) was added at room temperature for 2h. Once blocked, 10µl of standard or test samples were added in duplicate, with 90µl of biotinylated antibody (guinea pig anti-insulin 1:2500 dilution in 1%BSA/PBS/5mM EDTA) and incubated for 2h at room temperature. Finally, at room temperature horseradish peroxidase (100µl of 1:16 000in 0.1%BSA/PBS/0.05% Tween20) was added and incubated for 30min, followed by 100µl of chromogenic substrate reagent (TMB) for 45min and the colour reaction was stopped with 100µl of H_2SO_4. Assay sensitivity and the range will be provided in the relevant chapters.

2.8 Western Blot

2.8.1 Tissue Homogenisation

Frozen tissue samples were suspended in lysis buffer (0.1 M PBS) containing 50mM Tris, 1mM EDTA, 10% glycerol, 1% Triton X-100, a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA), and phosphatase inhibitors (1mM sodium vanadate, 0.05 M okadaic acid, 1

mM phenylmethylsulfonyl fluoride). Tissue was then homogenised in the lysis buffer by hand using a pestle, left on ice for 20min then homogenised again. The homogenate was centrifuged (12,000rpm) for 20min at 4°C and the supernatant was collected. After protein concentrations determined the protein preparations were stored at -80°C.

2.8.2 Protein Concentration Analysis

Protein concentration was determined with either a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, California, USA), or a bicinchoninic acid assay (BCA) kit (Pierce/ThermoFisher Scientific Australia Pty Ltd, Scoresby, Vic, Australia). Bovine serum albumin (BSA) samples of known protein concentration were used as protein assay standards. Assays were performed on 96-well plates and analysed using an absorbance microplate reader (Molecular Devices, Sunnyvale, California USA).

2.8.3 Western Blotting

Western blotting was performed to measure phosphorylation of AMP-activated protein kinase (AMPK) and Akt, as well as expression of uncoupling protein 3 (UCP3) in various tissues. Specific details about electrophoresis protocols and primary antibodies are given Table 2.1. The general methodology utilised in all wester blots is outlined below. All washes and blocking were performed at room temperature using a plate shaker, unless otherwise indicated.

Homogenised protein samples of known concentration were ran on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Gels consisted of two sections, the upper stacking gel, and the lower separating gel. Stacking gels were composed of 25% Tris/SDS

solution (Tris 0.5M, SDS 0.4%, [pH 6.8]), 0.3% Ammonium persulphate, 0.2% Tetramethylethylenediamine (TEMED) and 5% acryl/bis in Milli Q water. Separating gels were composed of 25% Tris/SDS solution (Tris 0.5M, SDS 0.4%, [pH 6.8]), 0.6% Ammonium persulphate and 0.07% TEMED in Milli Q water. The percentage of Acryl/bis used in the separating gels varied according to the protein examined. Details are given in Table 2.1.

Forty to sixty µg of protein were mixed with sample loading buffer (3:1; 0.0625 M Tris-HCl, [pH 6.8], containing 50 mM 1,4-dithiothreitol, 1% SDS, 10% glycerol, and 1% bromophenol blue), boiled for 4 min, and loaded onto the SDS-PAGE gel. Four µl of Full Range Rainbow Marker (GE Healthcare Australia, Rydalmere, NSW, Australia) were loaded into the first well on each gel and used to mark molecular weight. Electrophoresis was performed (separation buffer: 810ml Milli Q water, 90ml TG-SDS 10X; Astra Pharmaceuticals Pty Ltd, N. Ryd, NSW, Australia) until bands were adequately separated. Protein were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California, USA) in transfer buffer (720ml Milli Q water, 160ml Methanol, 80ml TG 10X ; Astra Pharmaceuticals Pty Ltd, N. Ryd, NSW, Australia).

After transfer, membranes were blocked using 5% non-fat milk powder in Tris-Buffered Saline and Tween 20 (TBST) for 60 min. Membranes were incubated with primary antibodies in 5% BSA in TBST over night at 4°C. A secondary antibody conjugated to horseradish peroxidise (Antibodies Australia, Clayton, Vic, Australia) was applied for 1 h at room temperature.

Detection of proteins was accomplished using an enhanced chemiluminescence reagent (ECL; GE Healthcare Australia, Rydalmere, NSW, Australia) for 4 min. Membranes were then exposed to film in a light-sealed cassette for the desired time. The films were scanned with a Canon scanner (Akimachi, Kunisaki-shi, Japan) and band density was determined using the ImageJ software program (Fujifilm Corp., Minatoku, Japan). The assessed band width was kept constant and density expressed relative to background.

To correct for sample loading variation, membranes were stripped of their bound primary antibody for re-probing using a dehybridisation buffer (0.2% SDS, 0.1 m NaCl, and 0.1 m glycine/HCl [pH 2]) for 1 h at room temperature and washed in TBST for 1 h. Membranes were then blocked with 5% non-fat milk in TBST and the primary antibody protocol continued. Membranes probed for phosphorylated proteins were stripped and re-probed for the total amount of the non-phosphorylated form of the same protein. Membranes probed for UCP3 were stripped and re-probed for β-Actin.

Table 2.1: Western blot protocol and antibody details sorted by protein of interest.

Protein	Gel Acryl/bis percentage	Electrophoresis	Transfer/Membranes	Primary Antibody Details
Phosphorylated AMP-activated protein kinase (pAMPK)	Acryl/bis: 7.5%	50V for 10 min 150V for 1.5 hours	1hour at 75V	Cell Signalling, USA
Total AMPK (AMPKtot)	Membranes probed for pAMPK were stripped and re-probed for AMPKtot			Cell Signalling, USA
Akt phosphorylated at Serine 473 (Akt Ser)	Acryl/bis: 7.5% or 12.5%	50V for 10 min 150V for 1.5 hours	1hour at 75V	Cell Signalling, USA
Akt phophorylated at Threonine 308 (Akt Thr)	Acryl/bis: 7.5% or 12.5%	50V for 10 min 150V for 1.5 hours	1hour at 75V	Cell Signalling, USA
Total Akt (Akt tot)	Membranes probed for Akt Ser or Akt Thr were stripped and re-probed for Akt tot.			Cell Signalling, USA
Uncoupling protein 3 (UCP3)	Acryl/bis: 12.5%	50V for 10 min, 100V for 2.5 hours.	1hour at 75V	Phoenix Pharmaceuticals, USA.
Beta actin (β-Actin)	Membranes probed for UCP3 were stripped and re-probed for β-Actin			Cell Signalling, USA

2.9 Real-Time Polymerase Chain Reaction (rt-PCR)

2.9.1 RNA Extraction

Total ribonucleic acid (RNA) was extracted from skeletal muscle and adipose tissue using a protocol adapted from the TRIzol technique established and revised by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987; Chomczynski and Sacchi, 2006). Tissue (300 – 500 μg) was placed in 2 ml TRIzol (Invitrogen, USA) and homogenised (using an electric tissue homogeniser/grinder) the supernatant was collected and centrifuged at 13,000 rpm for 10 min at 4°C. Homogenates were washed with 0.4 ml of chloroform-isoamyl alcohol and centrifuged at 13,000 rpm for 15 min at 4°C for the separation of RNA from DNA, proteins and lipids. The upper, aqueous phase (containing RNA), was recovered and rewashed with equal parts chloroform-isoamyl alcohol, before centrifugation at 13,000 rpm for 15 min at 4°C. The upper, RNA containing layer was collected again, precipitated in 0.5 ml isopropanol overnight at -20°C, with the addition of 2 μl glycogen (20 mg/ml) to aid in visualising the RNA pallet. The preparation was centrifuged at 13,000 rpm for 10 min at 4°C, to obtain an RNA pellet. The supernatant was discarded and the pellet washed with 500 μl of 75% Ethanol, before being re-centrifuged for 5 min at 4°C. Ethanol was removed from the tubes, which were then allowed to air dry. RNA was resuspended in RNase free water (Diethylpyrocarbonate; DEPC water) ready for concentration analysis.

RNA concentration and purity was analysed using a nanophotometer (IMPLEN, München, Bavaria, Germany), following the manufacturer's instructions. Aliquots of 0.5μg RNA in 10μl Tris/borate/EDTA (2μl; Sigma-Aldrich Australia, Sydney, NSW, Australia) were loaded onto 1% agarose gels containing ethidium bromide. Electrophoresis was conducted for approximately 30 min at 60V using a Submerged horizontal gel electrophoresis system (Bio-Rad Laboratories, Hercules, California, USA). Bands were visualised using fluorescence-detecting gel-doc imaging device (Syngene/Synoptics Group, Cambridge, Cambridgeshire, UK)

under an infrared light. The RNA preparation was treated with DNase (Ambion, Austin, TX, USA), as per the manufacturer's instructions and then re-analysed for RNA concentration.

2.9.2 cDNA Synthesis

DNase-treated RNA was transcribed into complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mulgrave, Vic, Australia), following the manufacturer's instructions; all reagents were provided in the kit. RNA (2µg) was diluted to 10µl in DEPC water and pipette-mixed with 10µl of 'RT Master Mix'. Thus, each aliquot of 'RT Master Mix' was comprised of 2.0µl 10x RT buffer, 0.8µl 25x dNTPs, 0.5µl RNasin, 1.0µl Multiscribe RT, 2.0µl 10x Random primers diluted in 7.4 DEPC water. The samples were incubated at room temperature for 10 minutes followed by 37°C for 120 min. The cDNA synthesis reaction was heat inactivated at 85°C for 5 sec and the resulting cDNA was stored at -20°C until assessed via rt-PCR.

2.9.3 Selection of Housekeeping Genes for rt-PCR

Improved accuracy of rt-PCR results can be achieved by employing multiple reference or housekeeping genes (Vandesompele *et al.*, 2002). For each gene of interest expressed in each tissue type we determined three optimal housekeeping genes from seven candidate genes. Candidate gene expression in our RNA samples were analysed by geNorm (Biogazelle, Zwijnaarde, Belgium) to obtain expression stability and efficiency values. The housekeeping genes that were selected for muscle and fat samples are given in Table 2.2.

2.9.4 Real-time Polymerase Chain Reaction

The level of expression of genes of interest and housekeeping genes was quantified by rt-PCR (Eppendorf Realplex4; Eppendorf South Pacific Pty. Ltd., North Ryde, NSW, Australia). Purified DNA of known concentrations was used for assay standards. Each sample was run in triplicate. Real-time PCR was performed in DEPC water, 1x Real MasterMix (Promega, Alexandria, NSW, Australia), assay forward and reverse primers (500nM) and 1xSYBR Green (agilent technologies australia Pty. Ltd.,Forrest Hill, Vic, Australia). Primer sequences for the genes of interest are given in Table 2.2. Real-time PRC was performed using the following sequence:

An initial denaturation step at 95°C for 10 min followed by cycles of:

1. Denaturation: 95°C for 15 sec
2. Annealing: at the appropriate temperature for the gene of interest, as indicated in table 1.2 for 30 sec
3. Extension: 72°C for 30 sec.

Double-stranded DNA was detected using the fluorescent dye SYBR Green, allowing for the quantification of the PCR products. Fluorescence was measured at the end of each cycle. After a set number of cycles, which was different for each gene of interest (see Table 2.2) a melt curve program was initiated which consisted of an initial denaturation step at 95°C for 15 sec, an incubation period for 15 seconds at 60°C was and then a ramp from 75°C to 95°C, increasing by 0.5°C increments over a period of 20 min. A final incubation period was conducted for 5 seconds at 95°C.

The level of fluorescence was plotted against the number of cycles, resulting in an amplification curve for each sample that was then analysed to provide the concentration of RNA. Each sample was analysed at the point at which the amplification plot crossed the 'threshold', which is set by the analysis software during the exponential replication phase. The threshold is set above the background fluorescence and below the plateau of replication. The cycle number at which each sample crosses the threshold is referred to as the cycle threshold (C_t). C_t Values of unknown samples were plotted against those of the standard curve to determine expression levels. Expression levels are normalised by dividing by the geometric mean of housekeeping gene expression.

2.10 Statistical Analysis

All data are presented as mean ± the standard error of the mean (SEM). Specific details about the types of statistical analysis utilised is presented in each of the experimental chapters. All figures were produced using GraphPad Prism version 5.04 (GraphPad Software Inc, USA). Statistical analysis was performed using either GraphPad Prism version 5 or SPSS version 18 (IBM, USA).

Table 2.2: rt-PCR genes of interest and housekeeping gene details.

Housekeep Genes	GenBank accession no	Sense sequence (5` to 3`)	Antisense sequence (3` to 5`)	Cycle details.
Tyrosine 3-monooxygenase (YWHAZ)	NM_174814	AGACGGAAGGTGCTGAGAAA	TCTCTGCTTGTGAAGCGTTG	40 cycles Annealing: 59.6°C
Beta-2 microglobulin (B2M)	AY549962	GCAAAGACCTCTACGCCAAC	TGATCTTGATCTTCATCGTGCT	40 cycles of: Annealing: 59.6°C
Beta Actin (β-Actin)	U39357	GCAAAGACCTCTACGCCAAC	TGATCTTGATCTTCATCGTGCT	40 cycles of: Annealing: 59.6°C
Malate Dehydrogenase (MDH1)	AF233351	CGTTGCAGAGCTGAGGATT	GGTGCACTGAGAGATCAAGG	40 cycles of: Annealing: 57°C
Peptidylproplyl isomerise A (Cyclophilin)	BC013915	GGTGACTTCACACGCCATAA	GGTGATCTTCTTGCTGGTCT	40 cycles of: Annealing: 61°C
Acetyl-CoA Carboxylase (Acetyl-CoA)	AJ001056	CATGCTTCAAAGATCCAGCA	TGGCGAGGCTACAGTGAAAT	40 cycles of: Annealing: 61°C
Cytochrome	NM_001129905	TCTCAGTGGCCAATGTCATC	GTTCCCGGAGGCAGTTATC	40 cycles of: Annealing: 61°C
Genes of Interest				
UCP1	AY371696	AGAGCCATCTCCACGGTCCCA	CCAAAGCCCCGTCAAG	50 cycles of: Annealing: 60°C
UCP2	NM_001033611	AAGGCCCACCTAATGACAGA	CCCAGGGCAGAGTTCATGT	40 cycles of: Annealing: 59.6°C
UCP3	NM_174210	TGACCTCCTCACCTTTCCAC	AAATCCGGGTAATGATGCTG	50 cycles of: Annealing: 61°C

CHAPTER 3
Characterising post prandial heat production and blood flow in skeletal muscle

3.1 Introduction

Confirmation of the presence of functional brown adipose tissue in adult humans (Huttunen *et al.*, 1981; Nedergaard *et al.*, 2007; Virtanen *et al.*, 2009; Zingaretti *et al.*, 2009) has produced a paradigm shift in obesity research. The manipulation of adaptive thermogenesis is now a valid prospective anti-obesity treatment (Wijers *et al.*, 2009). As discussed in Chapter 1.4, BAT expresses uncoupling protein 1 (UCP1), a mitochondrial protein which uncouples oxidative phosphorylation from ATP formation. This results in the loss of energy as heat. Although it is now known that functional BAT is present in adult humans, this tissue does not account for an individual's total thermogenic capacity, suggesting other tissues are involved (Astrup *et al.*, 1985; Simonsen, 1993). Skeletal muscle is a highly energy consumptive tissue and a major determinant of basal metabolic rate (Yki-Jarvinen *et al.*, 1987; Zurlo *et al.*, 1990). Due to its shared embryological lineage with BAT (Seale *et al.*, 2008; Kajimura *et al.*, 2009), it is a likely

candidate tissue for thermogenic potential. Skeletal muscle expresses the UCP1 homologues, UCP2 and UCP3 (Schrauwen and Hesselink, 2002). While the uncoupling and thermogenic potential of these homologues is debated, evidence exists to suggest that UCP3 may be involved in uncoupling oxidative phosphorylation (Gong *et al.*, 1997) and in regulating energy expenditure (Costford *et al.*, 2008; Clapham *et al.*, 2000).

Henry *et al.* (Henry *et al.*, 2008; Henry *et al.*, 2011) recently demonstrated postprandial temperature excursions in skeletal muscle of sheep. Central administration of leptin increases this response, indicative of neurally-mediated postprandial thermogenesis (Henry *et al.*, 2008). Furthermore, leptin-induced heat production in skeletal muscle is associated with a marked increase in UCP3 mRNA expression and a switch towards uncoupled respiration in isolated mitochondria (Henry *et al.*, 2011). These data suggest that leptin increases heat production in skeletal muscle via activation of thermogenic pathways. In spite of this, the precise mechanism which underpins postprandial heat production in skeletal muscle remains unknown. Increased heat production with meal feeding may be due to altered mitochondrial function, but may also be secondary to discrete changes in blood flow.

In skeletal muscle, blood flow is under the dual control of α1 and β2 adrenoceptors, which mediate vasoconstriction and vasodilation respectively (Gaballa *et al.*, 1998; Hagstrom-Toft *et al.*, 1998; Buckwalter *et al.*, 1998). During the postprandial period, blood flow can be modulated in a tissue and organ specific manner to reflect altered metabolic demand. In humans, postprandial increases in blood flow to the intestinal organs corresponds to the increased activity required for the distribution of substrates to peripheral tissues. Blood flow through the superior mesenteric artery has been reported to increase (58-250%) during the postprandial period; peaking approximately 1 hour of meal ingestion (Qamar and Read, 1988; Sieber *et al.*, 1991; Someya *et al.*, 2008). In humans, alterations in blood flow to skeletal muscle during the postprandial may be dependent on the macronutrient intake. Feeding participants a meal of mixed macronutrient content has been shown to increase blood flow to the forearm muscle

(Keske *et al.*, 2009), while a glucose load decreased blood flow (Bulow *et al.*, 1987). Given the role of blood flow in thermoregulation (Hodges and Johnson, 2009), for example the regulation of cutaeous blood flow in response to internal and external temperature (Charkoudian, 2003), it is possible that changes in blood flow around the time of feeding impacts on skeletal muscle temperature. Therefore, this chapter sought to characterise the potential relationship between blood flow and tissue temperature. In addition, I sought to determine the mitochondrial and cellular pathways involved in mediating postprandial heat production in the skeletal muscle of sheep.

3.2 Methods

3.2.1 Experiment 1: Effects of isoprenaline on blood flow and temperature in skeletal muscle.

Four ovariectomised Corriedale ewes (49.2 ±1.4kg) were housed in an isolated room, exposed to a 12:12 (h) light/dark cycle (lights on at 0700h), with the ambient temperature of the room maintained at 22°C. Dataloggers were surgically implanted into the hind limb to continually record skeletal muscle temperature (refer to section 2.2.4.2). A transit-time ultrasound flow probe (type 6SB, Transonic Systems Inc, Ithaca, NY, USA) was fitted to record femoral artery blood flow to the hind limb. Two animals were additionally fitted with Doppler flowprobes (MSP300XP, Oxford Optronix, Oxford, UK) to record microvascular perfusion (tissue blood flow) in the skeletal muscle tissue (refer to section 2.2.4.3). A jugular vein was cannulated for the purpose of i.v. infusion of various substances (refer to section 2.5.1). For the duration of the experiments, the animals were kept in metabolic cages designed to allow the animal to sit and stand but prevented further movement which may influence temperature readings.

To reduce confounding effects of feeding, treatments in experiment 1 and 2 were carried out in sheep in a fasted state, prior to being programmed to the feeding window. Isoprenaline (a non-specific β-adrenergic agonist) was administered as a bolus i.v. infusion at the following incremental doses: 0.1, 0.3, 1.0 and 3.0 μg/kg body weight. Blood flow was assessed 5 minutes prior to infusion and during a washout period of 30 minutes, allowing for blood flow to return to baseline levels.

3.2.2 Experiment 2: Effects of phenylephrine on blood flow and temperature in skeletal muscle.

On a separate day to Experiment 1, each animal was treated with phenylephrine (a non-specific α-adrenergic agonist) which was administered i.v. as a continuous infusion over 15 minutes at sequential doses of 1, 3, 6, 10 μg/kg body weight/min. Blood flow was assessed 5 minutes prior to, during and for 45 minutes after the infusion period. Sequential treatments were administered once blood flow had returned to baseline levels.

3.2.3 Experiment 3: Effects of programmed feeding on blood flow and temperature in skeletal muscle

In order to assess the relationship between postprandial changes in blood flow and temperature in skeletal muscle, measurements were taken in animals that had been subjected to the programme feeding regime (fed between 1100h and 1600h). Animals were maintained on this feeding regime for at least 1 week prior to experimentation. Temperature and blood flow recordings were taken between 1000h and 1600h.

3.2.4 Experiment 4: Effect of meal anticipation on blood flow and temperature in skeletal muscle

To establish a model of meal anticipation, animals were placed on the programme feeding schedule for 2 weeks. This laboratory has previously shown that the postprandial increase in temperature is dependent on food availability and this response is abolished in entrained animals that are fasted (Henry *et al.*, 2008). Meal anticipation, however, can be evoked in an entrained but fasted animal when co-housed with sheep that are fed at the standard feeding time (1100h) as established by Henry *et al.* (Henry *et al.*, 2010). Thus, the experimental animals are exposed to visual and olfactory cues associated with a meal, without the associated metabolic consequences of feeding. Blood flow and temperature were measured from 1000h to 1600h.

3.2.5 Experiment 5: Postprandial changes in cellular pathways underpinning thermogenesis and metabolism in skeletal muscle

To examine possible cellular mechanisms that underlie postprandial heat production muscle biopsies were collected at 3 select time points: 1) at baseline before undergoing programme feeding (baseline, 0900h); 2) during the preprandial period after being entrained by temporal feeding restriction (preprandial, 0900h); 3) during the peak of the postprandial temperature response (postprandial, 1200h). To do this, 6 ovariectomised ewes (body weight 51.3 ±0.9kg) were programme-fed for 2 weeks to entrain the postprandial response. Animals were anaesthetised and the pre- and post- meal entrainment biopsies were collected under surgical conditions. Tissue was frozen on dry ice.

The expression of UCP1, UCP2 and UCP3 mRNA was assessed via rt-PCR as described in section 2.9. Uncoupling protein mRNA expression was normalised to the geometric mean of the housekeeping genes expression levels (B2M, MDH1, YWHAZ and β-Actin; refer to Section 2.9).

Peroxisome proliferator-activated receptor gamma (PPARγ), glucose transporter 1 (GLUT1), GLUT4, heat shock protein 70 (HSP70) and HSP90 mRNA were analysed via rt-PCR. Each sample was normalised to the housekeeping gene (β-actin) by subtracting the housekeeping gene Ct value from the Ct value of the gene of interest to give ΔCt. Data was normalised to baseline by subtracting the average ΔCt value of samples taken at baseline before programme feeding from the ΔCt of each sample. This data was further transformed to obtain ΔΔCT expressing it in the following equation:

$$(\Delta\Delta CT) = 2\text{^}n;\ \text{where n equals the } \Delta Ct \text{ value normalised to baseline.}$$

In addition, UCP3 expression and AMPK phosphorylation (refer to Section 2.8 for general western blot protocol) was assessed via western blot. Experiment specific details are as follow:

Sixty micrograms of protein was mixed with sample loading buffer (3:1) and loaded into a SDS page gel. Electrophoresis was performed until bands were adequately separated (1.5 hours at 150V at room temperature for AMPK; 3.0 hours at 100V at room temperature for UCP3). Protein was transferred to a nitrocellulous membrane (1hour at 75V at room temperature). Membranes were incubated with primary antibodies raised against pAMPK, total AMPK protein, UCP3 and β-Actin. A secondary antibody conjugated to horseradish peroxidise (Antibodies Australia, Melbourne, Australia) was applied for 1 h at room temperature and detection was accomplished using enhanced chemiluminescence (ECL).

3.3.6 Data Analysis and Presentation

Experiments 1 and 2: Data are presented as 1min averages. For experiment 1, femoral artery blood flow and skeletal muscle temperature were analysed as the area under the curve of the first 5 minutes after isoprenaline infusion compared to area under the curve of the antecedent 5 minutes. For experiment 2, femoral artery blood flow and skeletal muscle temperatures were analysed as the area under the curve of the 5 minutes before infusion of phenylephrine compared to the area under the curve of the 5 minute period 25 – 30 mins post commencement of infusion. For both experiments 1 and 2, comparisons were performed using repeated measures ANOVA. Post hoc analysis was performed using Fisher's Least Significant Differences Test.

Experiments 3 and 4: Data are presented as 15min averages. Blood flow was continuously recorded every two seconds. Temperature data from experiments 3 and 4 were analysed by comparing average baseline (1000 – 1045h) recordings compared to the peak postprandial temperature. When expressed in 15 min averages, all animals experienced peak postprandial temperatures at 1130h. Blood flow measurements at these time points were also compared using paired t-tests.

Experiment 5: Real time PCR was used to measure levels of UCP1, 2 and 3 mRNA. For this, purified DNA of a known concentration was utilised to establish a standard curve and thus allowing detection of specific concentration. All data for UCP genes was corrected to the geometric mean of the three most stable housekeeping genes as determined by GeNorm analyses. Whereas, real time PCR for measurement of PPARγ, GLUT1, GLUT4, heat shock protein 70 (HSP70) and HSP90 were carried out without the use of a standard curve. In each case, the ΔΔCT was calculated with correction to the baseline samples (as above). All gene (real time PCR) and protein (Western Blot) data were analysed using a Friedman non-parametric test

and Dunn's post hoc test when appropriate. Data demonstrated unequal variance and therefore a nonparametric statistical test was utilised.

3.3 Results

3.3.1 Experiment 1: Effects of isoprenaline infusion on blood flow and temperature.

Isoprenaline increased ($P<0.05$) both femoral artery and tissue blood flow (Figures 3.1 and 3.2). Furthermore, changes in these variables were closely related. Increasing doses of isoprenaline elicited similar hyperaemic effects, with the peak increase in blood flow being equivalent across all doses. There was no significant effect of low dose isoprenaline treatment (0.1 and 0.3 µg/ kg body weight) on skeletal muscle temperature, but at high doses (1.0 and 3.0 µg/ kg body weight) increased skeletal muscle temperature (Figure 3.2).

3.3.2 Experiment 2: Effects of phenylephrine infusion on blood flow and temperature.

At low doses (1 and 3 µg/kg body weight/min) phenylephrine had no effect on blood flow. However, at high doses (6 and 10 µg/kg body weight/min) phenylephrine reduced ($P<0.05$) blood flow (Figure 3.3). There was no significant effect of infusion of phenylephrine on skeletal muscle temperature at any dose studied (Figure 3.3).

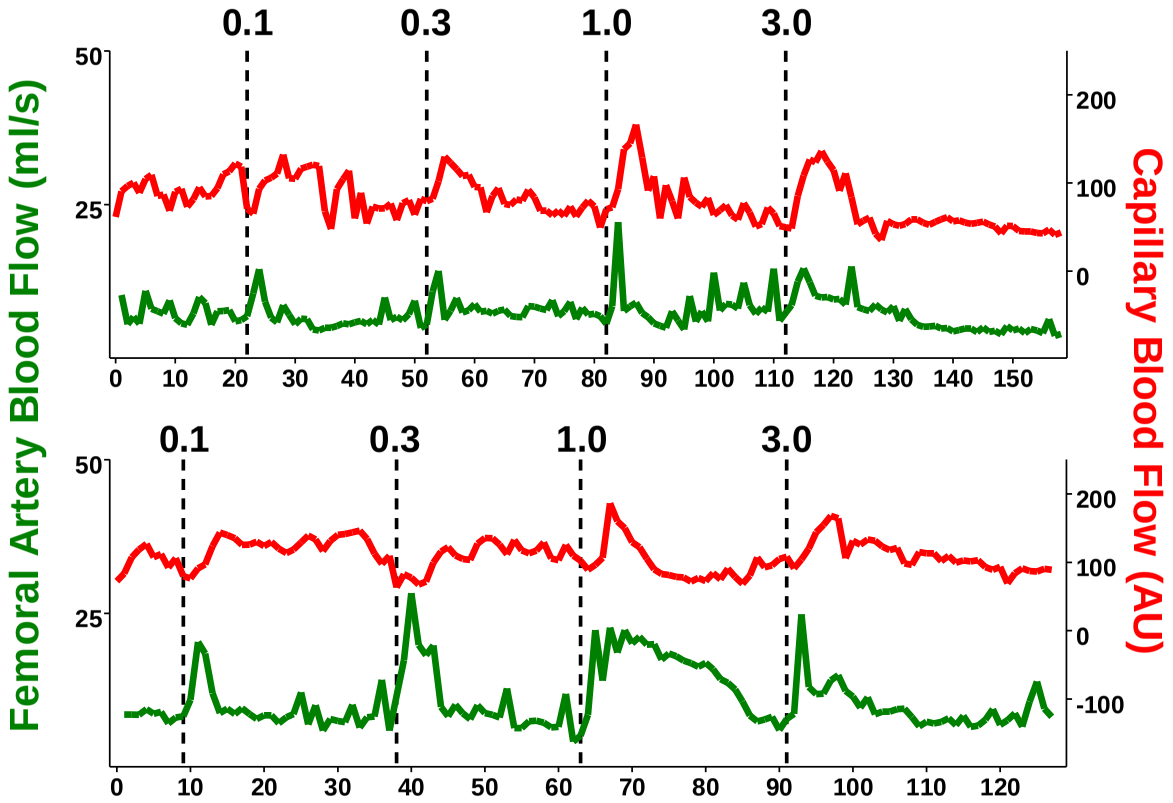

Figure 3.1: Representative data from two animals (Panels A and B) receiving bolus i.v. doses (0.1, 0.3, 1.0 and 1.0 µg/kg) of isoprenaline. Blood flow across the femoral artery (green) and muscle microvascular perfusion (red) increased in response to isoprenaline treatment. These data demonstrate that changes in muscle microvascular perfusion reflected changes in total limb blood flow as measured by femoral artery blood flow. AU= arbitrary units

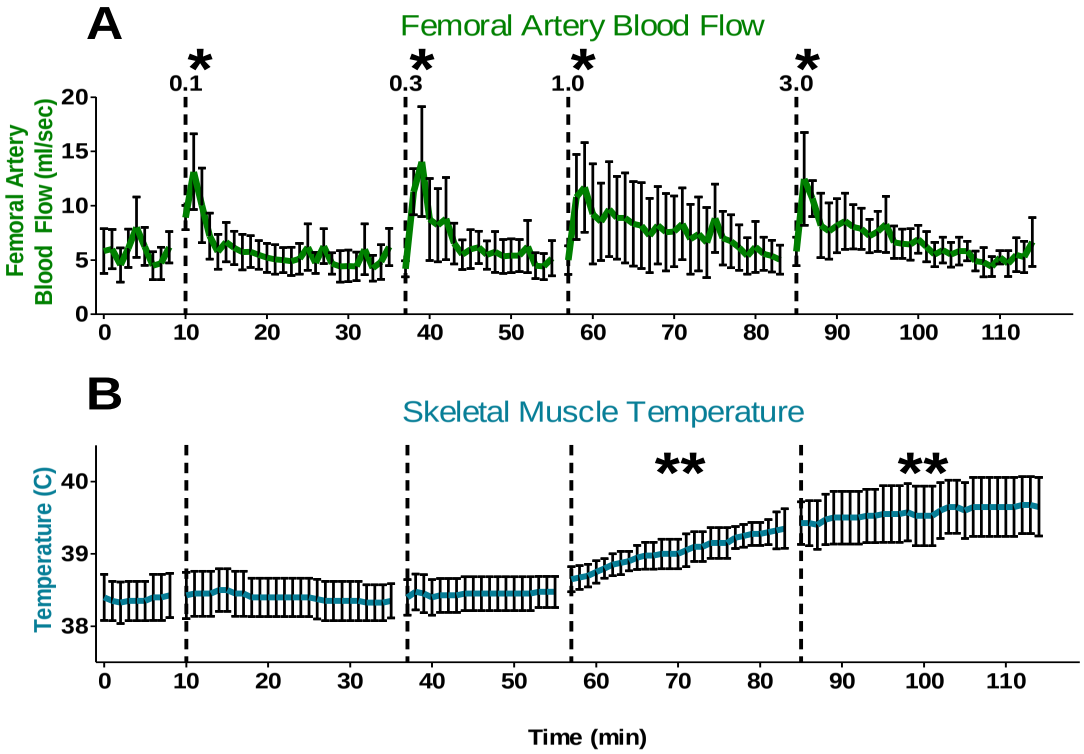

Figure 3.2: The effects of isoprenaline treatment on femoral artery blood flow (Panel A) and skeletal muscle temperature (Panel B). Femoral artery blood flow was increased ($P<0.05$) at all 4 doses of isoprenaline (0.1, 0.3, 1.0 and 3.0 µg/kg body weight). In contrast, isoprenaline treatment increased skeletal muscle temperature at high doses only (1.0 µg/kg body weight: $P<0.05$; 3.0 µg/kg body weight: $P<0.01$); there was no significant effect of isoprenaline on skeletal muscle temperature at low doses. All data are presented as the mean±SEM. *$P<0.05$; **$P<0.01$.

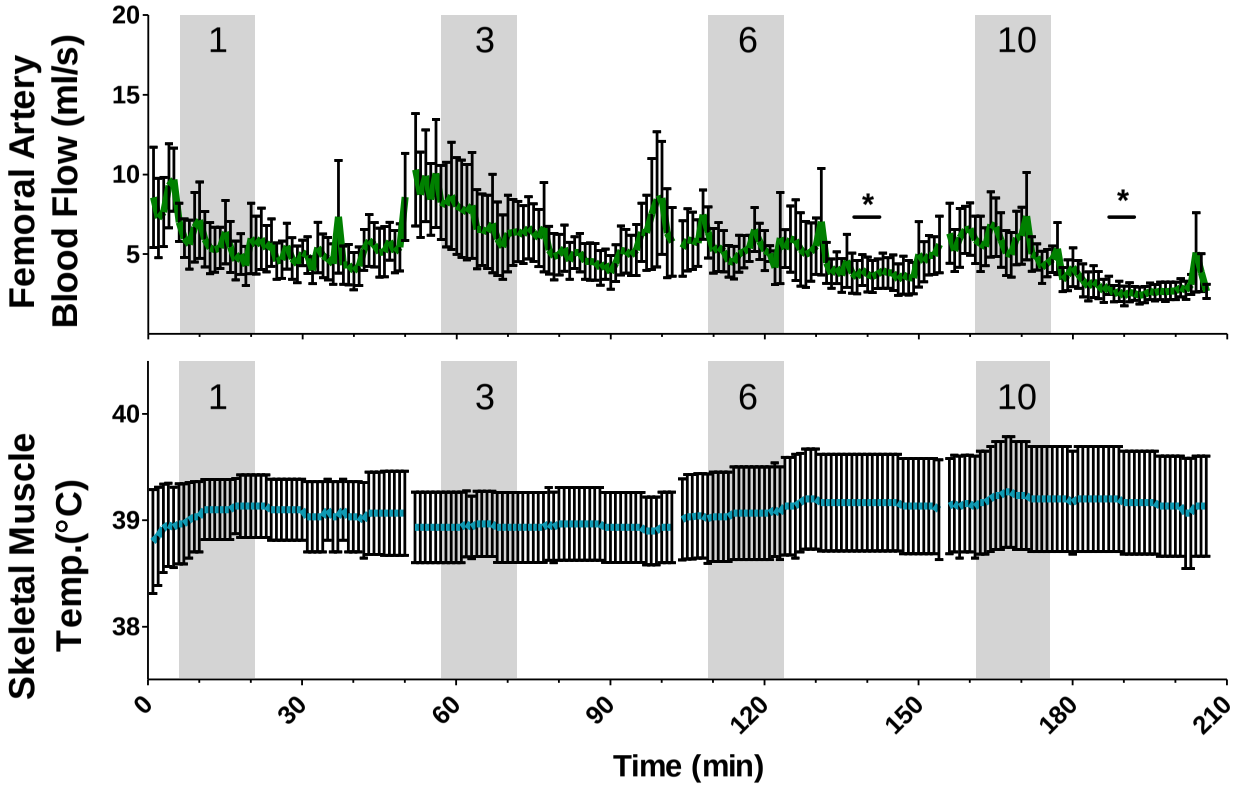

Figure 3.3: The effects of phenylephrine treatment on femoral artery blood flow (Panel A) and skeletal muscle temperature (Panel B). At lower doses (1.0 and 3.0 µg/kg body weight/min) infusion of phenyephrine did not impact on femoral artery blood flow. Higher doses (6 and 10 µg/kg body weight/ min) of phenyleprhine, however, reduced ($P<0.05$) blood flow to the hind limb. There was no effect of phenylephrine treatment on skeletal muscle temperature at any of the doses studied. All data are presented as the mean±SEM. *$P<0.05$.

3.3.3 Experiment 3: Effects of programmed feeding on blood flow and temperature in skeletal muscle

There was an increase in skeletal muscle temperature during the postprandial period. This effect was evident at the commencement of feeding. The mean temperature increase in skeletal muscle was 0.57±0.06°C ($P<0.01$; Figure 3.4). Despite the elevation in temperature, there were no associated changes in blood flow (Figure 3.4).

3.3.4 Experiment 4: Effects of meal anticipation on blood flow and temperature in skeletal muscle

Excursions in muscle temperature were elicited by the meal anticipation paradigm (Figure 3.5). Similar to the response seen in feeding animals, an increase ($P<0.05$) in mean skeletal muscle temperature was observed, but this was not associated with any change in blood flow (Figure 3.5).

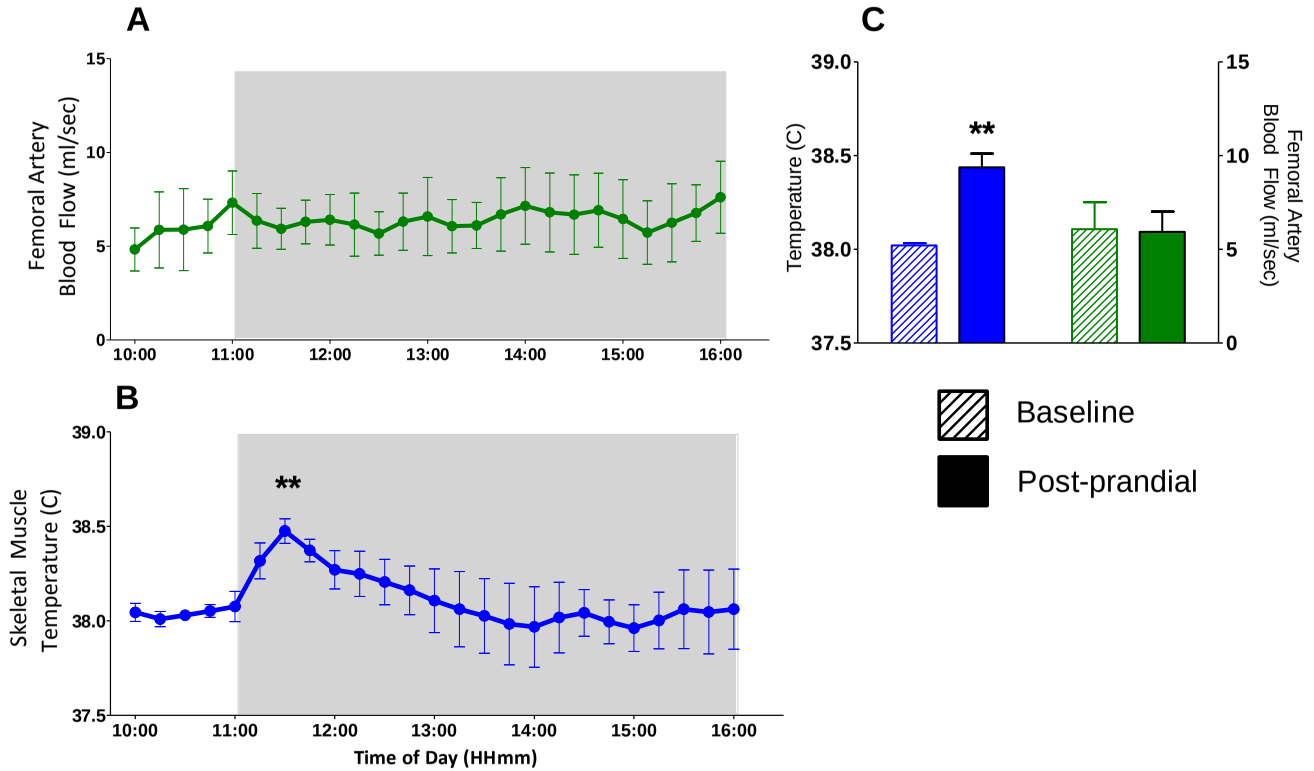

Figure 3.4: Hind limb blood flow (Panel A) and skeletal muscle temperature (Panel B) before (baseline) and during feeding (postprandial: grey area). Muscle temperature increased ($P<0.05$) following the commencement of the meal, but there was no associated change in femoral artery blood flow. The peak temperature (Panel C) response during the postprandial period demonstrated that on average temperature increased by 0.42 ±0.06°C. There was no detectable change in blood flow across this period. Baseline measurements are represented by the hatched bars and peak measurements by the solid bars. All data are presented as the mean ±SEM. *$p<0.05$

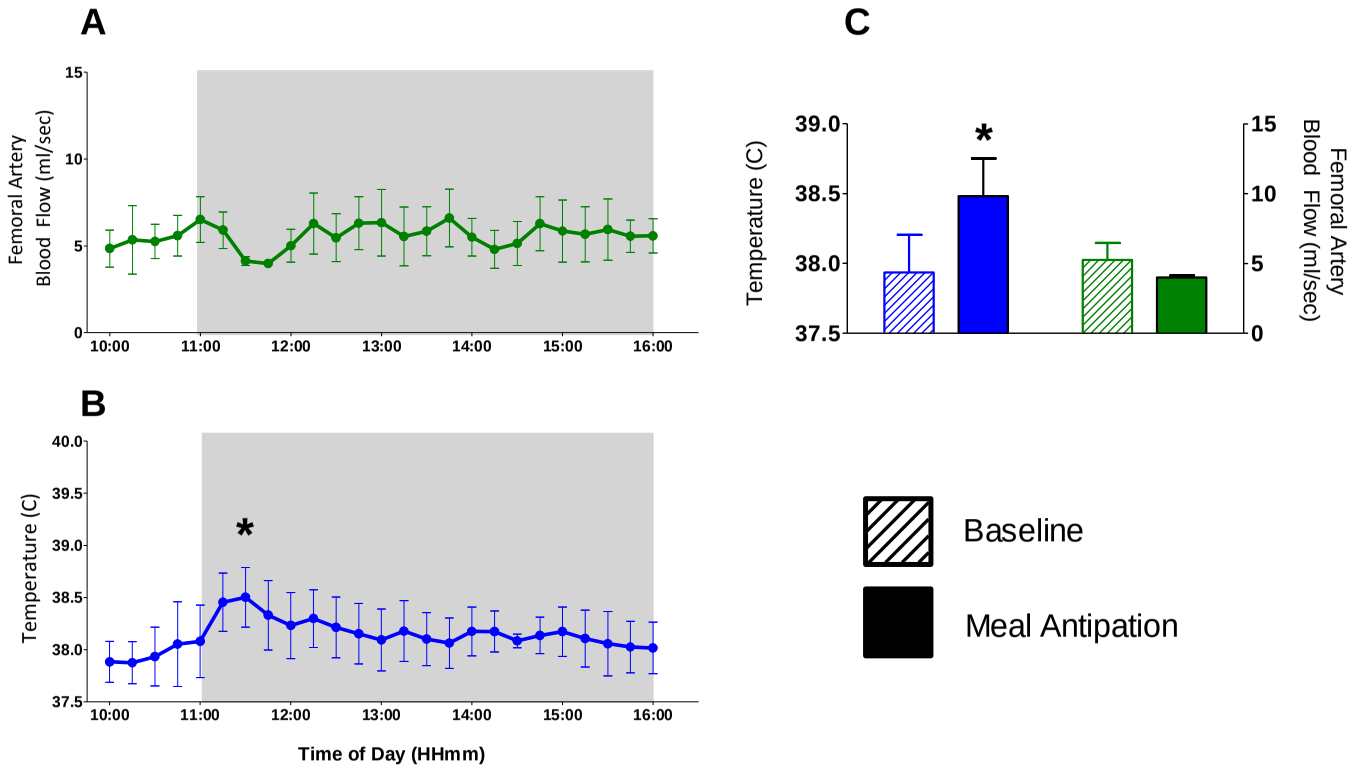

Figure 3.5: Hind limb blood flow (Panel A) and skeletal muscle temperature (Panel B) before (baseline) and during meal anticipation (grey area) Muscle temperature increased ($P<0.05$) during the meal anticipation period, yet no change in blood flow to the hind limb was observed. Analysis of the peak temperature response (Panel C, blue) revealed that the increase in temperature during meal anticipation was not associated with any change in blood flow (green). In Panel C, baseline measurements are represented by the hatched bars and peak postprandial measurements by the solid bars. All data are presented as the mean ±SEM.*$p<0.05$

3.3.5 Experiment 5: The effects of programmed feeding on molecular regulation of energy balance in skeletal muscle.

Entrainment of a postprandial temperature response via temporal feeding restriction elicited no change to skeletal muscle uncoupling protein mRNA expression as seen when comparing pre-programmed baseline to both pre- and post-feeding time points (Figure 3.6). Feeding did not result in changes to UCP1, UCP2 or UCP3 mRNA expression as evidenced by comparing preprandial and post-pranial time points (Figure 3.6). Additionally, no difference was observed in UCP3 protein content in skeletal muscle between the pre- and postprandial response time points. There was no effect of meal feeding on the phosphorylation of AMPK (Figure 3.8) as assessed in skeletal muscle during the pre- and postprandial time points. No differences were observed in PPARγ, GLUT1, GLUT4, heat shock protein 70 (HSP70) or HSP90 mRNA expression in skeletal muscle between baseline, the preprandial peroid and the postprandial peroid.

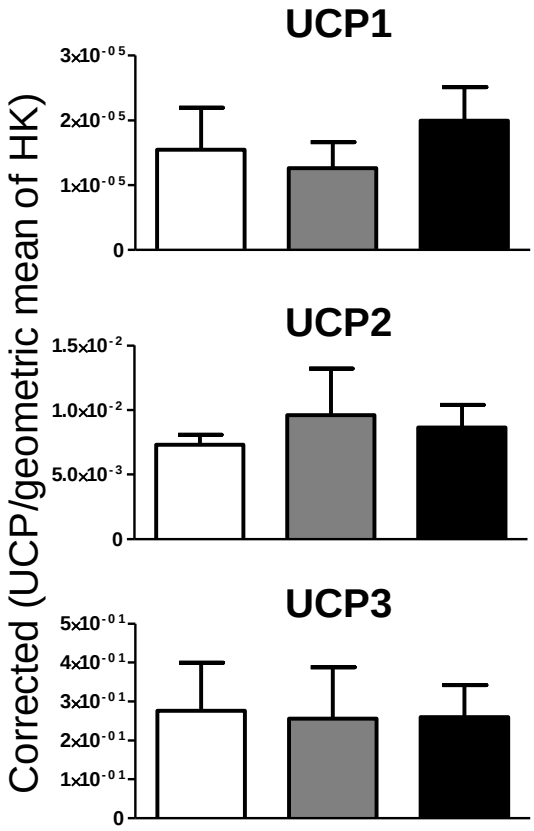

Figure 3.6: Uncoupling protein 1, 2 and 3 mRNA expression in skeletal muscle taken at three timepoints: 1) at baseline before commencing the programme feeding regime (baseline; white bars), 2) during the preprandial period (preprandial, grey bars), and 3) during the period of peak postprandial heat elevation (postprandial; black bars). There was no effect of the meal feeding regime on the expression of UCP 1, 2 or 3 mRNA.

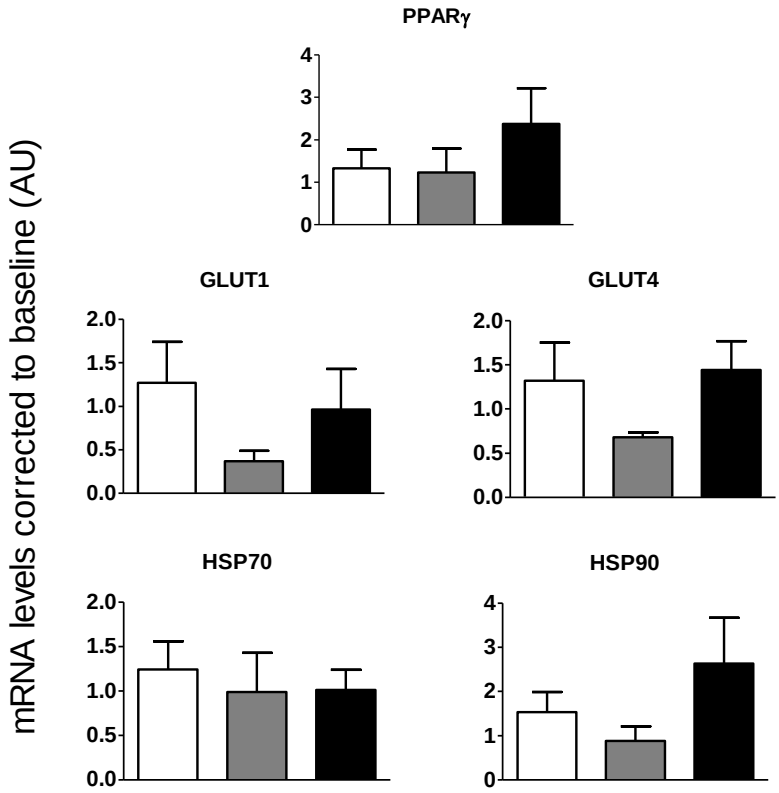

Figure 3.7: PPARγ, GLUT1, GLUT4, heat shock protein 70 (HSP70) or HSP90 mRNA expression in skeletal muscle taken under three conditions: 1) at baseline before commencing the programme feeding regime (baseline; white bars), 2) during the preprandial period (preprandial, grey bars), and 3) during the period of peak postprandial heat elevation (postprandial; black bars). The mRNA expression profiles of these proteins were not affected by the programmed feeding regime nor feeding itself.

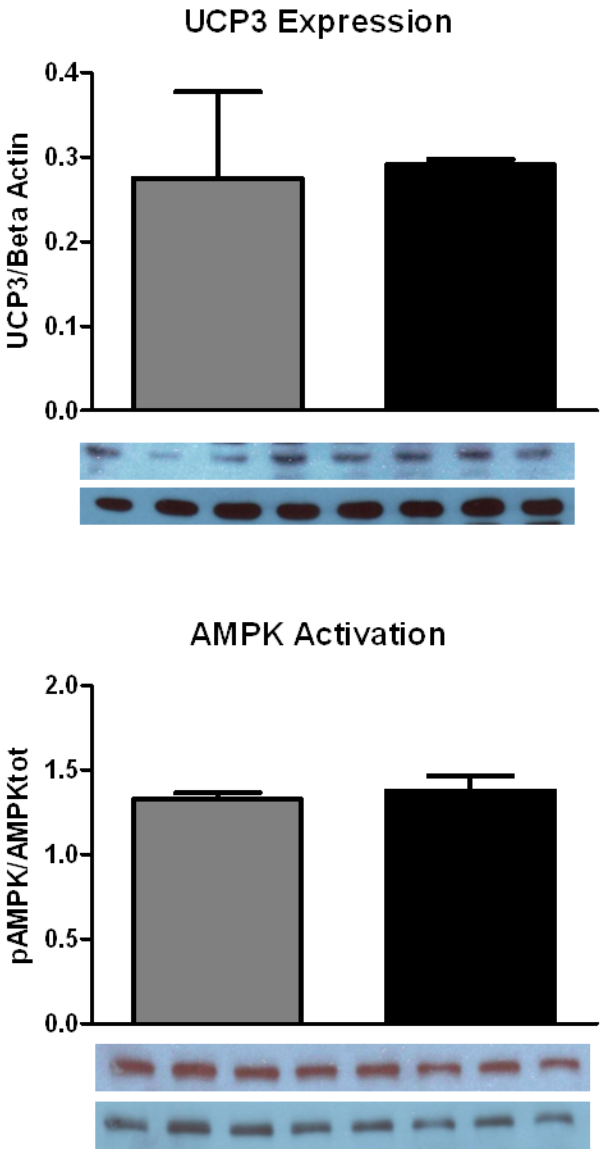

Figure 3.8: The effect of feeding state on uncoupling protein 3 (UCP3) protein expression and AMP-activated protein kinase (AMPK) phosphorylation in skeletal muscle. Neither UCP3 expression, nor AMPK phosphorylation was altered due to feeding (black bars) compared to the preprandial period (grey bars). UCP3 data are presented corrected to β-Actin expression. AMPK phosphorylation data are corrected to total AMPK protein expression.

3.4 Discussion:

Experiments detailed in this chapter characterise mechanisms that underpin postprandial heat production in the sheep model first established by Henry *et al.* (Henry *et al.*, 2008). These experiments comprehensively show disassociation between tissue temperature and acute changes in skeletal muscle blood flow. This was demonstrated utilising a variety of experimental paradigms including: models of postprandial and meal anticipatory heat production, as well as pharmacological intervention with α and β adrenergic agonist administration. These data support the notion that changes in skeletal muscle temperature are not brought about by changes in blood flow. Although postprandial temperature increases in skeletal muscle were observed, no significant changes in the expression of UCP1, UCP2 or UCP3 mRNA; or UCP3 protein were observed as a result of meal entrainment or feeding, itself.

Administration of increasing concentrations of isoprenaline induced acute elevations in both femoral artery blood flow and muscle microvascular perfusion. Femoral artery blood flow is regarded as a good indicator of blood flow through the skeletal muscle itself. Femoral artery blood flow (as detected by transit-time ultrasound), was therefore used exclusively in the remaining experiments (and subsequently in chapter 5) as a proxy for blood flow through skeletal muscle. This conclusion is supported by two studies showing strong relationships between total hindlimb flow and local muscle microvascular perfusion during hypoxia (Evans *et al.*, 2011a) and local infusion of vasoactive agents (Evans *et al.*, 2011b) in anesthetized rabbits. The current study differs from the aforementioned studies in that it was conducted in conscious animals.

Responses to administration of isoprenaline and phenylephrine observed in this chapter support the notion that blood flow and tissue temperature are disassociated. All four doses of isoprenaline acutely elevated blood flow to the hind limb. This was expected as β2-adrenergic

activation has known vasodilatory effects (Bachman *et al.*, 2002; van Baak, 2001; Blaak *et al.*, 1993). Low doses of isoprenaline had no impact on tissue temperature; showing clear dissociation between the two. However, high doses of isoprenaline significantly increased muscle temperature. Although isoprenaline has a high affinity for the β1/β2-adrenoreceptors, at higher doses it has been shown to bind β3-adrenoreceptors (Strosberg, 1996). β3-adrenoreceptor activation is a hallmark of the thermogenic response exhibited by BAT (Lowell and Flier, 1997). It is therefore proposed that in this study, higher doses of isoprenaline resulted in binding to β3-adrenoreceptors and activation of thermogenic pathways.

In contrast, high but not low doses of phenylephrine decreased blood flow to the hind limb. There was no concomitant effect of phenylephrine on tissue temperature at any of the doses. These findings are consistent with the known vasoconstrictive effects of α-adrenergic stimulation, and the notion that energy expenditure and thermogenesis is regulated independent of the α-adrenergic system (Bachman *et al.*, 2002; van Baak, 2001; Blaak *et al.*, 1993). Isoprenaline and phenylephrine administration served not only to validate the current model of simultaneous temperature and blood flow recording in the conscious sheep, but also demonstrate that alterations in blood flow does not in itself affect tissue temperature. Furthermore, it can be extrapolated that stimulation of β3-adrenoreceptors can induce temperature elevations in skeletal muscle.

Blood flow has consistently been shown to increase postprandially in the gut and adipose tissue (Qamar and Read, 1988; Sieber *et al.*, 1991; Someya *et al.*, 2008). The effects of feeding on blood flow to skeletal muscle have produced conflicting findings. In humans, blood flow to skeletal muscle during the postprandial period has been reported to both increase (Keske *et al.*, 2009) and decrease (Bulow *et al.*, 1987). In the canine model, blood flow to skeletal muscle is unchanged during feeding (Gallavan *et al.*, 1980). Data presented in this chapter are consistent with the canine model, possibly highlighting species differences.

Meal anticipation is a non-homeostatic phenomenon whereby tissue temperature elevations occur in a fasted subject when another animal is fed in close proximity. This is most likely in response to visual and olfactory stimuli. While the postprandial paradigm employed in this chapter measures tissue temperature and blood flow in response to altered metabolic demand of the tissue as well as putative thermogenesis, the meal anticipation paradigm established by Henry *et al.* (Henry *et al.*, 2010) only measures responses to putative thermogenesis. In rodents on prolonged temporal feeding restriction, there is a shift in circadian regulation. This results in anticipatory rises in BAT thermogenesis and core body temperature even when the animal is fasted (an absence of homeostatic and non-homeostatic stimuli; Gooley *et al.*, 2006; Boulos and Terman, 1980; Mistlberger, 1994). In contrast, sheep entrained to a feeding window will only exhibit temperature elevations when homeostatic (feeding) or non-homeostatic (meal anticipation) stimuli are provided (Henry *et al.*, 2010; Henry *et al.*, 2008). In this chapter, both the postprandial entrainment and meal anticipation models were able to elicit elevations in skeletal muscle temperature disassociated from any change in blood flow.

Mechanistically, BAT activation in mice via the β3-adrenoceptor agonist CL316,243 requires elevation of UCP1 expression to induce thermogenesis and energy expenditure (Inokuma *et al.*, 2006). In sheep at least, UCP3 is the dominant uncoupling protein in skeletal muscle. In this tissue, UCP3 mRNA is at least 100 fold more abundant than UCP1 mRNA (Clarke *et al.*, 2012; Henry *et al.* 2011). The literature concerning UCP2 and UCP3 expression in response to β3-adrenoceptor activation provides inconclusive results. In mice, chronic treatment with the β3 adrenoceptor agonist CL316,243 has been shown to either reduce (Yoshitomi, 1998) or increase (Nakamura *et al.*, 2001) the expression of UCP2 and UCP3 in skeletal muscle. These dichotomous findings may be due to differences in the methodology employed, with these studies quantifying changes in gene expression via Northern Blot and PCR respectively. In rats, β3-adrenoceptor agonist administration results in elevated expression of UCP3 in skeletal muscle (Emilsson *et al.*, 1998). Increased expression of UCPs in skeletal muscle is associated with increased GDP-binding (Yoshida *et al.*, 1998), a hallmark of mitochondrial uncoupling. To

assess molecular adaptations to the programme feeding regime (and to feeding, itself), mRNA expression of UCP1, UCP2 and UCP3 was measured. Surprisingly, programme feeding had no effect on UCP1, UCP2 or UCP3 expression in skeletal muscle. Additionally, UCP3 protein levels were consistent between the baseline and the postprandial period. In this body of work I was unable to link any molecular adaptations to the physiological outcome of postprandial temperature elevations. However, analysis of isolated mitochondria performed within this laboratory (unpublished) provides evidence that skeletal muscle is capable of adaptive thermogenesis. These data show that isolated muscle mitochondria taken from sheep after feeding elicit an increase in uncoupled respiration during compared to the preprandial period. Furthermore the increase in uncoupled respiration was supported by a decrease in the respiratory control ratio (RCR), deemed a precise measure of function in isolated mitochondria (Brand and Nicholls, 2011). The current work, however, demonstrates that this switch towards uncoupled respiration due to feeding is unlikely to be caused by altered expression of UCP 1, 2 or 3 mRNA.

If UCP expression is not driving the change in mitochondrial respiration observed during feeding, which other mechanisms can be attributed? Measurement of protein or gene expression does not provide an indication of UCP3 activity, which may drive the increase in uncoupled respiration. Therefore it remains to be elucidated if UCP3 drives this response, or if an as-yet undescribed mechanism is involved. Analysis of isolated mitochondria has also been used to show that central leptin treatment increases skeletal muscle temperature and induces a preference towards uncoupled respiration in muscle mitochondria in the model of ovine postprandial thermogenesis (Henry *et al.*, 2011). UCP2 and UCP3 but not UCP1 mRNA expression, as well as UCP3 protein is elevated during this time point (Henry *et al.*, 2011). Combined, these data suggest that skeletal muscle in sheep, may be capable of uncoupling protein-mediated adaptive thermogenesis which can be induced by feeding and further modulated by central treatment with leptin. Although the unpublished mitochondrial respiratory data demonstrates changes in mitochondrial function with feeding, it is also possible that other

subcellular pathways such as futile calcium cycling (Tseng *et al.* 2010) contribute to the postprandial increase in heat production in skeletal muscle.

In rodents, the induction of thermogenesis has been linked to AMPK activation (phosphorylation; Kus *et al.*, 2008; Lombardi *et al.*, 2008). Several studies have shown that central and peripheral administration of leptin activates AMPK in rodent skeletal muscle (Solinas *et al.*, 2004; Minokoshi and Kahn, 2003; Minokoshi *et al.*, 2002; Kus *et al.*, 2008; Roman *et al.*, 2010). The link between AMPK activation and thermogenesis has not been substantiated in the sheep. One study by this laboratory has shown that central leptin infusion increased thermogenesis without changes in AMPK activation (Laker *et al.*, 2011). Furthermore, this laboratory has demonstrated that direct infusion of AMPK activator 5-aminoimidazole-4-carboxyamide-ribonucleoside (AICAR) into the femoral artery phosphorylates AMPK (Laker *et al.*, 2011), but does not alter temperature in muscle tissue (Henry *et al.*, 2011). In this chapter I demonstrated that postprandial skeletal muscle temperature excursions were not associated with changes in AMPK activation. Collectively, these data suggest that AMPK activity is not associated with the induction of thermogenesis in sheep skeletal muscle. To further characterise the molecular mechanisms involved with skeletal muscle postprandial thermogenesis, I measured mRNA expression of PPARγ, GLUT1, GLUT4 and heat shock proteins HSP70 and HSP90. PPARγ in BAT is essential for UCP1 expression and hence thermogenesis (Sears *et al.*, 1996), in skeletal muscle it is important for insulin action (Hevener *et al.*, 2003; Park *et al.*, 1997). Similarly, GLUT1 and GLUT4 regulate glucose uptake into skeletal muscle. GLUT4 in particular is known to be responsive to insulin signalling (Douen *et al.*, 1990). It is therefore surprising that expression PPARγ, GLUT1 and GLUT4 were not increased during the post-period, a period of energy abundance. As a ruminant, sheep's glucodynamics differ from monogastric animals. Glucose is not derived from diet, but rather from gluconeogenesis in the liver. These differences may explain why PPARγ, GLUT1 and GLUT4 mRNA is not altered due to feeding. This may also explain why we see no change in AMPK phosphorylation due to feeding. HSP70 and HSP90 are expressed in response to a

variety of physiological stresses such as hyperthermia, exercise, oxidative stress and metabolic challenges. No differences were observed in their expression due to meal entrainment nor due to feeding itself.

In conclusion, I have demonstrated that there is a disassociation between regional blood flow and skeletal muscle temperature. This was demonstrated via a variety of paradigms, independently altering both blood flow and tissue temperature. Pharmacological use of adrenoceptor agonists to both decrease (high doses of phenylephrine) and increase (low doses of isoprenaline) blood flow to the hind limb did not cause a concomitant change in tissue temperature. Models of postprandial and meal anticipatory temperature excursions served to demonstrate that elevations in skeletal muscle temperature are not associated with altered blood flow. The induction of temperature elevations in response to high doses of isoprenaline is suggestive of a β-adrenergic mediated thermogenic response. These data, taken together with unpublished findings from this laboratory showing a preference towards mitochondrial uncoupling during the postprandial period compared to before feeding, support the notion that skeletal muscle is capable of adaptive thermogenesis. Mechanistically, I was unable to provide a link between skeletal muscle expression of UCP1, UCP2 and UCP3 and our model of postprandial thermogenesis. Despite this, our sheep model of postprandial temperature excursions provides a solid platform to explore the effects of the sex hormones testosterone and estrogen on energy balance. Investigation of these effects forms the basis of the proceeding chapters.

CHAPTER 4
The effects of testosterone on energy balance in the sheep

4.1 Introduction

Sexual dimorphism in energy balance and body weight regulation has been observed in many species including sheep (Clarke *et al.*, 2001) and rodents (Clegg *et al.*, 2003). Both the sex of the animal (Clarke *et al.*, 2001; Clegg *et al.*, 2003) and its sex steroidal profile (Clegg *et al.*, 2006) influence energy balance. As previously discussed, while the effects of estrogen have been well studied, the effects of androgens are less well explored.

Testosterone has been shown to act via the brain to reduce food intake and increase energy expenditure (Mauvais-Jarvis, 2011). However, the underlying mechanisms governing these actions remain to be elucidated. The ability of testosterone to be aromatised to estrogens complicates this picture. Weight gain observed in castrate male rats can be ameliorated by either estrogen or testosterone treatment (Mayes and Watson, 2004; Mauvais-Jarvis) but not by androgen 5α-dihydro-testosterone (5α -DHT), a non-aromatizable androgen. Furthermore, 5α-

DHT has been shown to exaggerate obesity in both male and female mice by up-regulating lipogenic pathways while down-regulating fatty acid oxidation (McInnes *et al.*, 2006; Moverare-Skrtic *et al.*, 2006). Thus, it is possible that the beneficial effects of testosterone are exerted via aromatization rather than via activation of androgen receptors.

Sexually divergent regulation of thermogenesis suggests a role of sex and sex steroids in heat production, with females being thought to have greater thermogenic capacity than males. This is evidenced by the greater amount of functional BAT found in human females (Cypess *et al.*, 2009; Cheng *et al.*, 2009). In addition, BAT taken from female rats is consumes more oxygen, related to increased UCP1 mRNA expression and increased sensitivity to β3 adrenoceptor activation (Rodriguez-Cuenca *et al.*, 2007). Sexual divergence has also been implicated in the regulation of core body temperature, with men experiencing over-night nadirs (approx. -0.5°C) in rectal temperatures, while women do not (Baker *et al.*, 1998). Rodent models have also shown that testosterone impacts on circadian rhythms in core body temperature; with neonate-castrate adult rats exhibiting an earlier rise in core body temperature during the light phase compared to intact controls (Zuloaga *et al.*, 2009). Female rats also displayed higher overall body temperatures than intact males (Zuloaga *et al.*, 2009).

In relation to androgen action on thermogenic pathways, *in vitro* studies indicate that testosterone may impair the function of BAT via reduction of PGC1α (Rodriguez-Cuenca *et al.*, 2007) and UCP1 mRNA levels (Rodriguez-Cuenca *et al.*, 2007). In another study, treatment of male rats with testosterone did not impact on BAT weight, mitochondrial mass or GDP-binding (Abelenda *et al.*, 1992). Inconsistencies between *in vitro* and *in vivo* models warrant further examination.

The melanocortin α-Melanocyte-Stimulating Hormone (αMSH) is a potent anorectic peptide. It acts centrally, mainly via melanocortin-4 receptor (MC4R), to reduce food intake. αMSH is also secreted by the pituitary gland, into the bloodstream (Engler *et al.*, 1988; Engler *et al.*, 1990).

Melanocortin receptors have been found in a number of peripheral tissues, including adipose (Boston and Cone, 1996) and skeletal muscle tissue (An *et al.*, 2007). In spite of this, potential peripheral actions of αMSH on energy expenditure remain to be elucidated. It has been shown that αMSH increases fatty acid oxidation in skeletal muscle cells *in vitro*, acting via the MC5R (An *et al.*, 2007) but further research is warrented. These pro-oxidative properties make αMSH a prime candidate for peripheral regulation of skeletal muscle thermogenesis.

This chapter addresses the effects of testosterone on thermogenic and metabolic pathways in male and female sheep. This was achieved by examination of the direct effects of testosterone on heat production in adipose tissue and skeletal muscle, as well as other metabolic pathways. Furthermore, the convergent effects of testosterone and αMSH on heat production in peripheral tissues of castrated rams were investigated.

4.2 Methods:

4.2.1 Animals

To examine sexual divergence in response to testosterone, castrated rams (44.1±1.1 kg body weight; n=5-6/group) and ovariectomized ewes (46.9 ±2.7kg body weight; n=4/group), which were housed in an isolated room, exposed to natural light and a temperature range of 17-22°C. Animals were held in pens designed to allow sitting and standing but prevented further movement which may impact on temperature recordings. Temporal food restriction was imposed 2 weeks prior to experimentation to entrain a postprandial temperature response as described in Chapter 2.3. Food refusals were weighed to determine daily food intake.

Temperature recordings were made using customized Dataloggers (SubCue, Calgary, Canada), which were surgically implanted to record tissue temperature of hind limb skeletal muscle and

retroperitoneal (visceral) fat as previously described (chapter 2.2.4.2). Dataloggers were programmed to record temperature at 15min intervals.

4.2.2 Experiment 1: Effects of testosterone on heat production in adipose tissue and muscle in male and female sheep.

Subcutaneous implants containing testosterone (3 x 200 mg, Organon, Lane Cove, NSW, Australia) were inserted within the ventral neck area of castrated male and female sheep and control animals received 3 blank silastic implants of similar size as previously described (chapter 2.4). These treatments began 2 weeks prior to experimentation. Two days prior to the onset of experiments, an external jugular vein was cannulated as previously described (Chapter 2.5.1) and closed with a three-way tap. The cannulae were kept patent with heparinized (100K IU) saline. On experimental days, blood samples (6ml) were collected at 30 min intervals from 0900 to 1600h. The samples were collected as previously described (chapter 2.5.2) and plasma was stored at -20°C until assayed for testosterone, glucose, lactate, insulin and non-esterified fatty acids (NEFA). Follicle stimulating hormone (FSH) was also assayed to determine the efficacy of testosterone treatment.

4.2.3 Experiment 2: Effects of testosterone treatment on uncoupling protein gene expression and energy signalling pathways in males

Tissues (retroperitoneal fat and skeletal muscle) were collected after 4 weeks of treatment to measure UCP1, 2 and 3 mRNA levels as well as the phosphorylation of AMP-activated protein kinase (AMPK) and Akt. Skeletal muscle was collected during the pre-prendial (baseline: 0900h) and postprandial periods (1200h), whereas adipose tissue was collected during the

preprandial period only (0900h). Muscle tissue was collected by needle biopsy and under local anaesthesia (Lignocaine, 10ml), and adipose tissue was collected post-mortem as previously described (Chapter 2.5.3). The tissues were snap-frozen in dry ice and stored at -80°C until analysis. UCP1, 2 and 3 mRNA in muscle and fat tissues was measured by real time PCR. Phosphorylated and non-phosphorylated forms of AMPK and Akt were measured by Western blot analysis in muscle tissues.

4.2.4 Experiment 3: Effects of peripheral α-MSH with or without testosterone replacement on heat production in adipose tissue and muscle in castrate males

To assess the effects of peripheral αMSH treatment on tissue temperature in castrate male sheep, animals were maintained on temporal feeding restriction as previously discussed. Two days prior to experimentation, both external jugular veins were cannulated; one cannula was used for blood sample collection, while the other was used for whole body, peripheral infusion treatments. Infusion treatments consisted of either αMSH (250μg/h) or saline from 1000 to 1600 h.

Blood samples (6ml) were taken and collected into heparinised tubes, centrifuged at 4°C and stored at -20°C until assayed. Samples were taken every 30min between 0900-1600h. Plasma was assayed for glucose, insulin, lactate and non-esterified fatty acids (NEFA) concentrations. Daily food intake was also monitored during the course of the experiment.

4.2.5 Western Blotting

Refer to Section 2.8 for general western blot protocol. Experiment specific details are as follow: Sixty micrograms of protein was mixed with sample loading buffer (3:1) boiled for 4 min and loaded into a 12.5% SDS page gel. Electrophoresis was performed until bands were adequately separated (1.5 hours at 150V at room temperature for). Protein was transferred to a nitrocellulous membrane (1hour at 75V at room temperature). Membranes were incubated with primary antibodies raised against pAMPK, total AMPK protein, pAktSer, pAktThr and total Akt (Akttot) over night at 4°C. A secondary antibody conjugated to horseradish peroxidise (Antibodies Australia, Melbourne, Australia) was applied for 1 h at room temperature and detection was accomplished using ECL. Densitometry was accomplished using the ImageJ software program (Fujifilm Corp., Minatoku, Tokyo, Japan).

4.2.6 Real time PCR

Real time-PCR for UCP1, UCP2 and UCP3 mRNA expression was performed. Uncoupling protein mRNA expression was normalised to the geometric mean of the housekeeping genes. In visceral fat, the housekeeping genes used were β2-microglobulin, β-actin and malate dehydrogenase-1. In skeletal muscle, the housekeeping genes used were β2-microgobulin, β-actin and cyclophilin. Refer to Chapter 2.9 for general rt-PCR protocols.

4.2.7 Assay of Metabolic Indicators

Blood glucose and lactate concentrations were measured in 25-ml samples of plasma using a YSI2300 STAT glucose/llactate analyzer (Yellow Springs Instrument Co., Yellow Springs,

OH). The measurable range for blood glucose was between 0–30mm and was 0–16 mm for lactate.

The plasma non-esterified fatty acids (NEFA) concentrations were determined by the enzymatic kit assay (Wako, Dallas, TX, US) previously described by Sechen *et al.* (Sechen *et al.*, 1990). The inter- and intra-assay CV were 5.8% and 4.7% respectively.

The plasma levels of insulin were measured in duplicate with an enzyme linked immuon-assay. Briefly, a 96-well plate was coated with anti-insulin antibody raised in guinea pigs (Antibodies Australia) overnight at 4°C. The coating solution was decanted and blocking solution (1.5% BSA/ PBS) was added at room temperature for 2h. Once blocked, 10µl of standard or test samples were added in duplicate with 90µl of biotinylated antibody (guinea pig anti-insulin 1:2500 dilution in 1%BSA/PBS/5mM EDTA) and incubated for 2h at room temperature. Finally, at room temperature horseradish peroxidase (100µl of 1:16 000 in 0.1%BSA/PBS/0.05% Tween20) was added and incubated for 30min, followed by 100µl of chromogenic substrate reagent (TMB) for 45min and the colour reaction was stopped with 100µl of H_2SO_4. The insulin ELISA was calculated to have a sensitivity of 0.2ng/ml and the following between assay co-efficient of variation of 16.7% at 2.9ng/ml, 17.9% at 1.8ng/ml, 11.7% at 3.3ng/ml and 13.9% at 1.2ng/ml.

4.2.8 Follicle Stimulating Hormone and Testosterone Assays

FSH assays were performed to determine whether the testosterone treatments were efficacious, with the expectation of lowered plasma levels. Assays were performed in duplicates of 100 µl, using the method of Bremner *et al.* (Bremner *et al.*, 1980). The standard was NIAMMD oFSH-RP-1 and the sensitivity of 2 assays was 0.06 ng/ml, and interassay CV of 6.9% at 2.8ng/ml and 3.1% at 11.5ng/ml. Plasma testosterone levels were assayed by the method of Young *et al.*

(Young *et al.*, 1989). Samples (100µl) were assayed in and the inter-assay CV of 9.1% at 241pg/100µl and 3.9% at 184pg/100µl.

4.2.9 Data Analysis and Presentation

Temperature and metabolite data were analysed using a repeated measures analyses of variance (ANOVA) incorporating time and treatment effects. Sexual dimorphism in temperature and metabolites were assessed using a repeated measures ANOVA incorporating gender and treatment. If a significant interaction was obtained, specific time-points were further analysed via the least significant difference post hoc test. To further characterise the feeding associated changes in temperature in males the amplitude and duration of the postprandial response was analysed. The amplitude was calculated as the difference between baseline (average temperature between 0900-1000h) and the peak temperature response across the feeding window. The duration was calculated as the time (min) for temperature to return to baseline levels for 4 consecutive (1 hour) time-points. Both the amplitude and duration were analysed using a single factor ANOVA. Data on FSH levels, testosterone levels, the expression of UCP1, 2 and 3 mRNA and the phosphorylation of AMPK and Akt were analysed using a single factor ANOVA.

4.3 Results

4.3.1 Experiment 1: Effects of testosterone on heat production in adipose tissue and muscle in male and female sheep.

Testosterone treatment increased ($P<0.001$) plasma levels of testosterone while reducing ($P<0.01$) plasma levels of FSH. These changes were similar in both male and female sheep (Table 5.1). Testosterone replacement had no effect on food intake in either males or females (Figure 5.1). Sexually divergent effects of testosterone treatment on temperature and plasma metabolites were observed.

Testosterone treatment reduced ($P<0.01$) skeletal muscle temperature in males (Figure 5.2; Panel A). However it had no effect on females (Figure 2, Panel B). Muscle temperature was lower ($P<0.01$) at all time points measured, including both during the pre- and postprandial period in testosterone-treated males (Figure 5.2, Panel A). To elucidate the effect of testosterone treatment in males on postprandial temperature responses, the amplitude and duration of the response was assessed. Testosterone did not alter either of these parameters (Figure 5.2, panels C and D) in spite of the net reduction in tissue temperature observed in these animals.

Sex differences were observed in skeletal muscle temperatures, whereby males had higher ($P<0.01$) temperatures than females, regardless of steroidal background. Castrate males had higher ($P<0.01$) visceral fat temperatures than females of either treatment group, though testosterone treatment in males eliminated these differences.

Sex differences were also observed in the preprandial levels of glucose and NEFA (Figure 5.3) whereby glucose levels were higher ($P<0.05$) in males, but NEFA levels were higher ($P<0.05$) in females. Plasma levels of lactate and insulin showed no sexual divergence. In females, plasma glucose levels increased ($P<0.05$) while NEFA levels decreased ($P<0.05$) during the postprandial period (Figure 5.3). However, in males, plasma metabolites did not change across

the feeding period (Figure 5.3). Testosterone treatment had no effect on plasma levels of insulin, lactate or NEFA in either males or females (Figure 5.3), though it reduced ($P<0.05$) postprandial glucose levels in males but not females (Figure 5.3).

Table 5.1 Plasma testosterone and follicle stimulating hormone levels in male and female castrate sheep with or without whole body testosterone replacement. Higher testosterone and lower FSH levels were measured in testosterone treated animals. Testosterone treatment induced greater testosterone levels in female sheep as compared to males.

	Males		Females			
	4Weeks		Baseline		4Weeks	
	Castrate	Testosterone	Castate	Testosterone	Castrate	Testosterone
Testosterone(pg/100ml)	4.1 (0.6)	141.2 (16.6)[a]	8.0 (4.4)	6.7 (3.3)	5.4 (3.4)	310.7 (65.2)[ac]
FSH (ng/ml)	11.4 (1.3)	4.6 (1.3)[b]	8.9 (1.1)	8.2(0.7)	8.6 (0.9)	1.7 (0.3)[b]

All data is shown as mean (SEM)

a difference between castrate and testosterone replacement animals of the same sex ($p < 0.001$).

b difference between castrate and testosterone replacement animals of the same sex ($p < 0.01$).

c difference between testosterone replacement animals of difference sex ($p<0.05$)

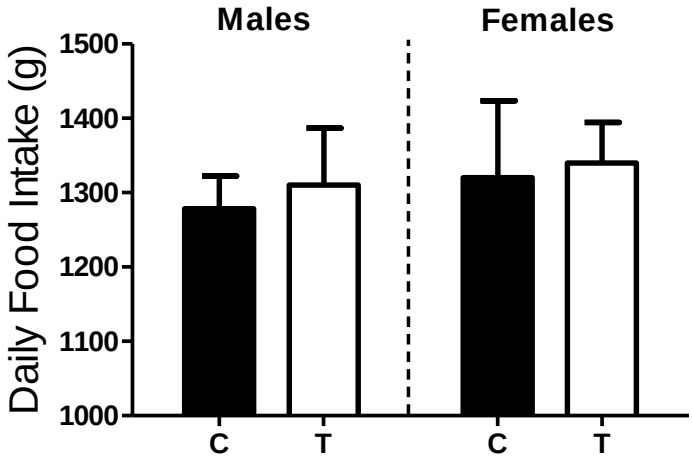

Figure 5.1. The effect of testosterone (T) treatment (3x 200mg s.c. implants for 2 weeks) on food intake in male and female castrate sheep. Control (C) animals received blank implants. Food intake was similar in castrate animals compared to testosterone-treated animals, irrespective of sex.

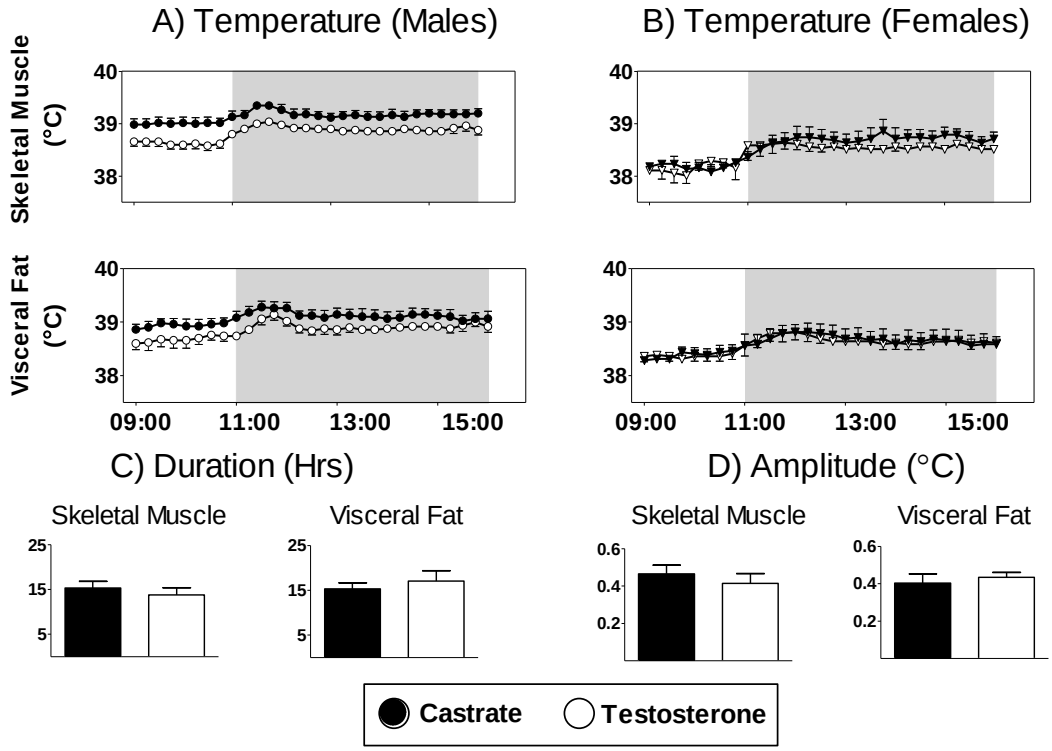

Figure 5.2. The effects of testosterone treatment on temperature in the visceral (retroperitoneal) fat and skeletal muscle of male (Panel A: circles) and female (Panel B: triangles) sheep. The feeding period (1100-1600h) is depicted by the grey box. Testosterone (open symbols) reduced ($P<0.001$ overall group effect) heat production in skeletal muscle compared to control (closed symbols), in male animals only. Males had higher skeletal muscle temperatures compared to females, regardless of steroidal background. Male visceral fat temperature was higher in control males compared to both female groups; testosterone treatment in the males eliminated this effect. There was no effect of testosterone treatment on heat production in either adipose tissue or skeletal muscle of the female sheep. To further analyse the effects of testosterone on heat production in males changes in the postprandial temperature profile were analysed. In response to feeding, there was no effect of testosterone treatment on the duration (Panel C) or the amplitude (Panel D) of the postprandial elevation in temperature in either the visceral fat or the skeletal muscle of either sex.

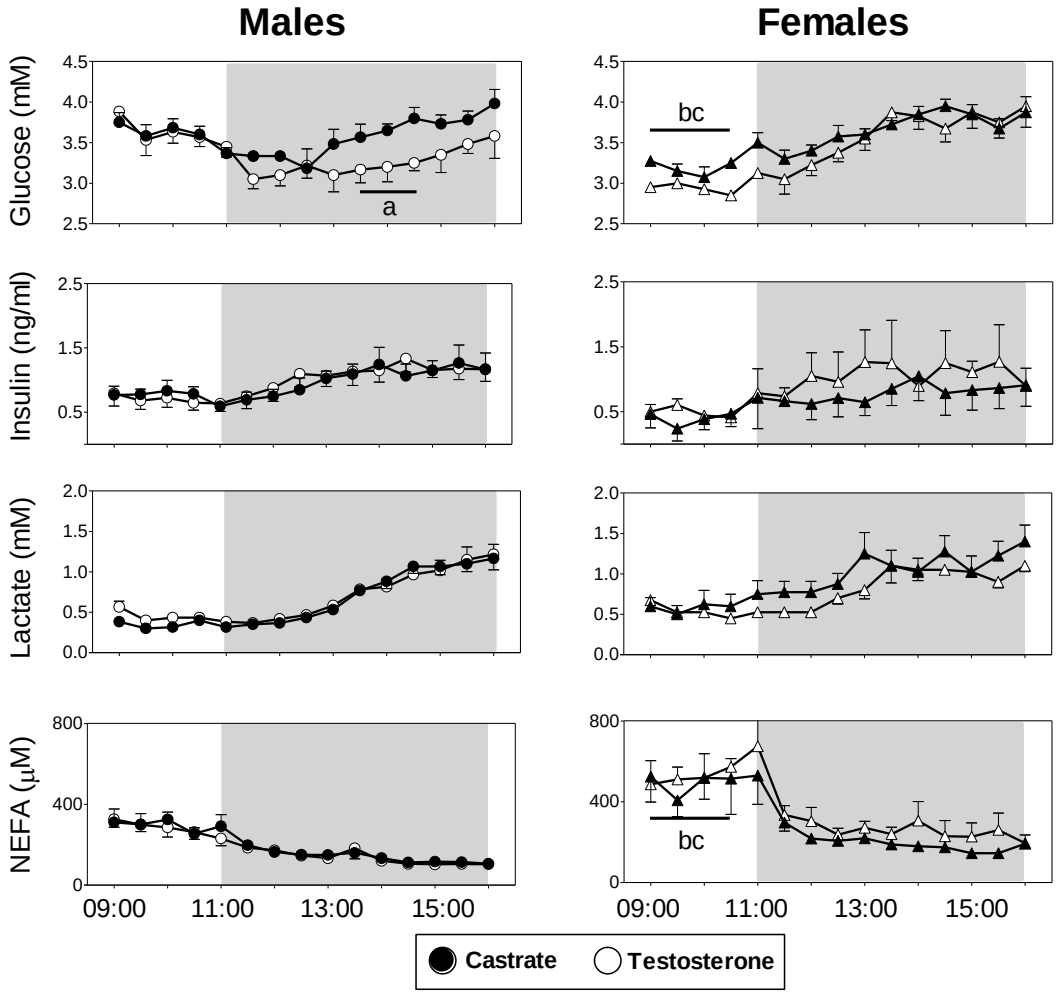

Figure 5.3. The effects of testosterone treatment on plasma levels of glucose, insulin, lactate and non-esterified fatty acids (NEFA) in male (circles: left panels) and female (triangles: right panels) sheep. Testosterone (open symbols) reduced ($P<0.05$) the plasma levels of glucose compared to control (black symbols), albeit in male sheep only. There was no effect of testosterone on the plasma levels of insulin, lactate and NEFA in males or females. During the preprandial period, plasma levels of glucose were lower ($P<0.05$) in females, whereas plasma levels of NEFA were higher ($P<0.05$) in females compared to males, irrespective of testosterone treatment. There were no sex differences in the levels of lactate or insulin. Feeding had little effect on plasma metabolite and insulin levels in the males, but there were distinct meal-associated patterns in the females. In females, plasma levels of glucose were higher, but NEFA levels were lower during the feeding window compared to the preprandial or baseline period. [a]$P<0.05$ testosterone compared to control; [b]$P<0.05$ female compared to males and [c]$P<0.05$ preprandial compared to postprandial.

4.3.2 Experiment 2: Effects of testosterone treatment on uncoupling protein gene expression and energy signalling pathways in males

To elucidate possible mechanisms involved in the temperature effects of testosterone replacement in male sheep observed in experiment 1, expression of UCP1, 2 and 3 mRNA was measured (Figure 5.4) in adipose tissue (preprandially) and skeletal muscle (pre and postprandially). Phosphorylation of AMPK and Akt (Figure 5.5) in muscle tissue was also measured. Levels of UCP1 and UCP3 mRNA were higher ($P<0.05$) in the postprandial period in skeletal muscle compared to the preprandial period, whereas there was no effect of feeding on UCP2 mRNA levels in muscle (Figure 5.4). Testosterone treatment did not alter the expression of UCP1, 2 or 3 mRNA in either adipose tissue or skeletal muscle of the male sheep (Figure 5.4) and did not alter the phosphorylation of either AMPK or Akt in skeletal muscle of male sheep (Figure 5.5).

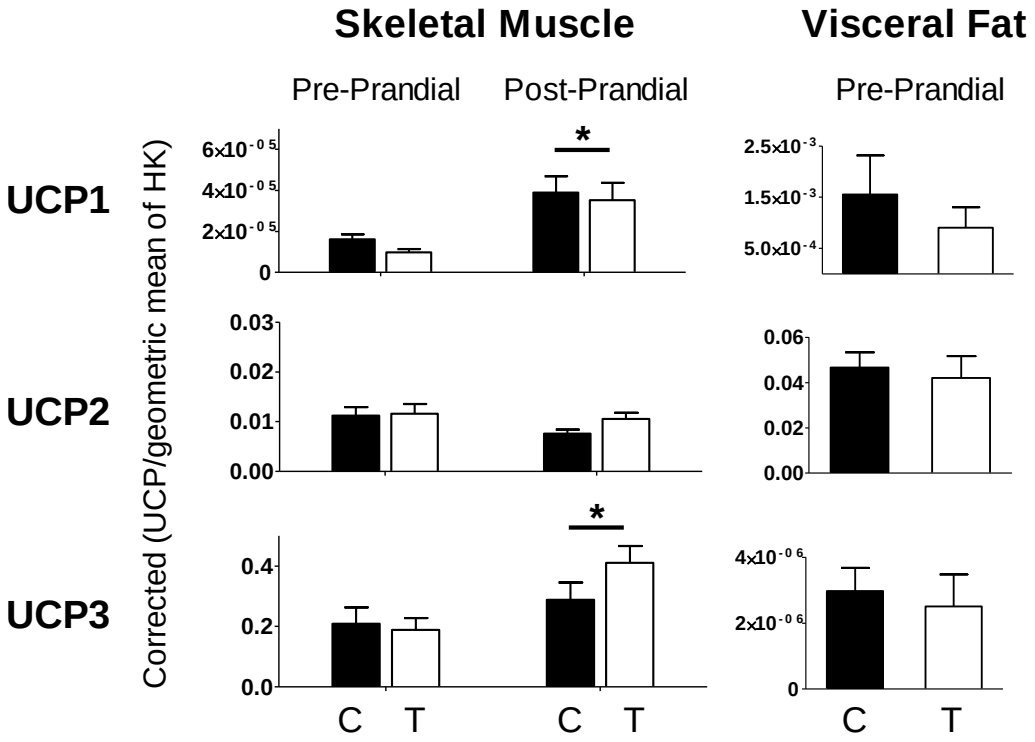

Figure 5.4: The effect of testosterone and feeding on the expression of UCP1, 2 and 3 mRNA in skeletal muscle and visceral fat in male sheep. Biopsies of muscle were taken during both the pre- and postprandial periods and used to measure UCP mRNA. There was no effect of testosterone treatment on expression of UCP1, 2 and 3 mRNA during the pre- or postprandial periods. On the other hand, the onset of feeding increased ($P<0.05$) the expression of UCP1 and UCP3 mRNA levels in skeletal muscle, irrespective of testosterone treatment. Expression of UCP mRNA levels was also measured in the visceral (retroperitoneal) fat bed, during the preprandial period only. There was no effect of testosterone treatment on the expression of UCP1, 2 or 3 mRNA in visceral fat. *$P<0.05$ compared to preprandial (baseline) levels.

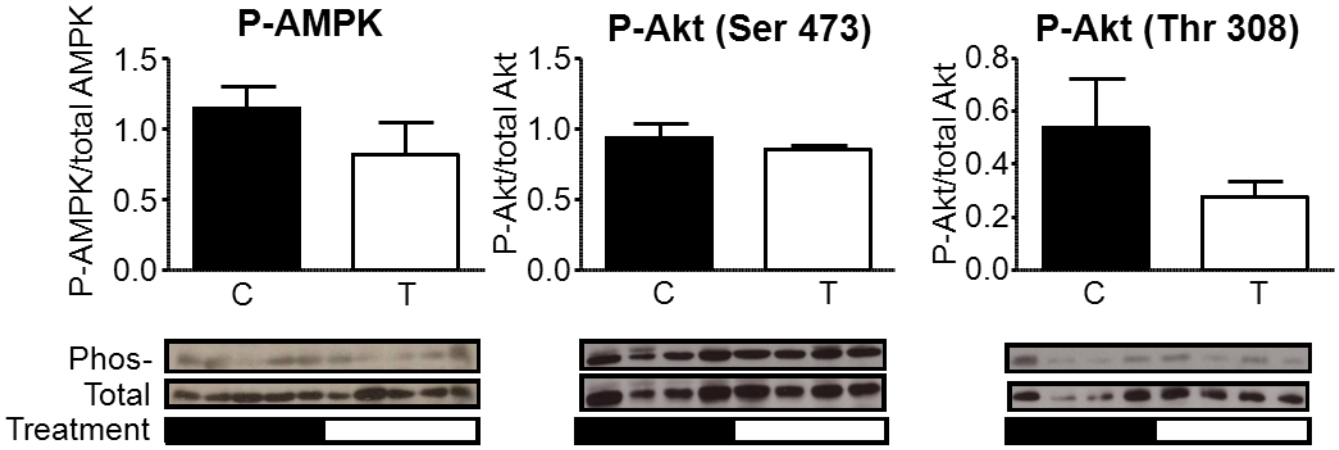

Figure 5.5: Effects of testosterone treatment on the phosphorylation of AMP-activate protein kinase (AMPK) and Akt in skeletal muscle of male sheep. There was no effect of testosterone on the phosphorylation of AMPK in Akt in male sheep. All data are presented as the phosphorylated protein corrected to total protein.

4.3.3 Experiment 3: Effects of peripheral alpha-melenocyte stimulating hormone on heat production in castate male sheep with or without testosterone replacement

Peripheral administration of α-MSH to castrate male sheep with and without testosterone replacement had no effect on tissue heat production in skeletal muscle or visceral fat before or during the feeding period (Figure 5A). Due to the effect of testosterone replacement on tissue temperature observed in experiment 1, temperature data was expressed as temperature change from a baseline period (average temperature between 08:00 and 08:45 h). Neither the amplitude nor duration of the postprandial response was affected by α-MSH treatment regardless of steroidal background. Food intake was not affected by α-MSH treatment in sheep with or without testosterone replacement, as compared to food intake from the preceding day (Figure 5B).

Treatment with α-MSH had no effect on plasma metabolites before or during the feeding period (figure 6). As with experiment 1, testosterone replacement reduced glucose levels during the postprandial period. Metabolite data presented in experiment 1 is taken from saline treated animals in this current experiment. These data are duplicated here for ready comparison between sheep treated with α-MSH or saline.

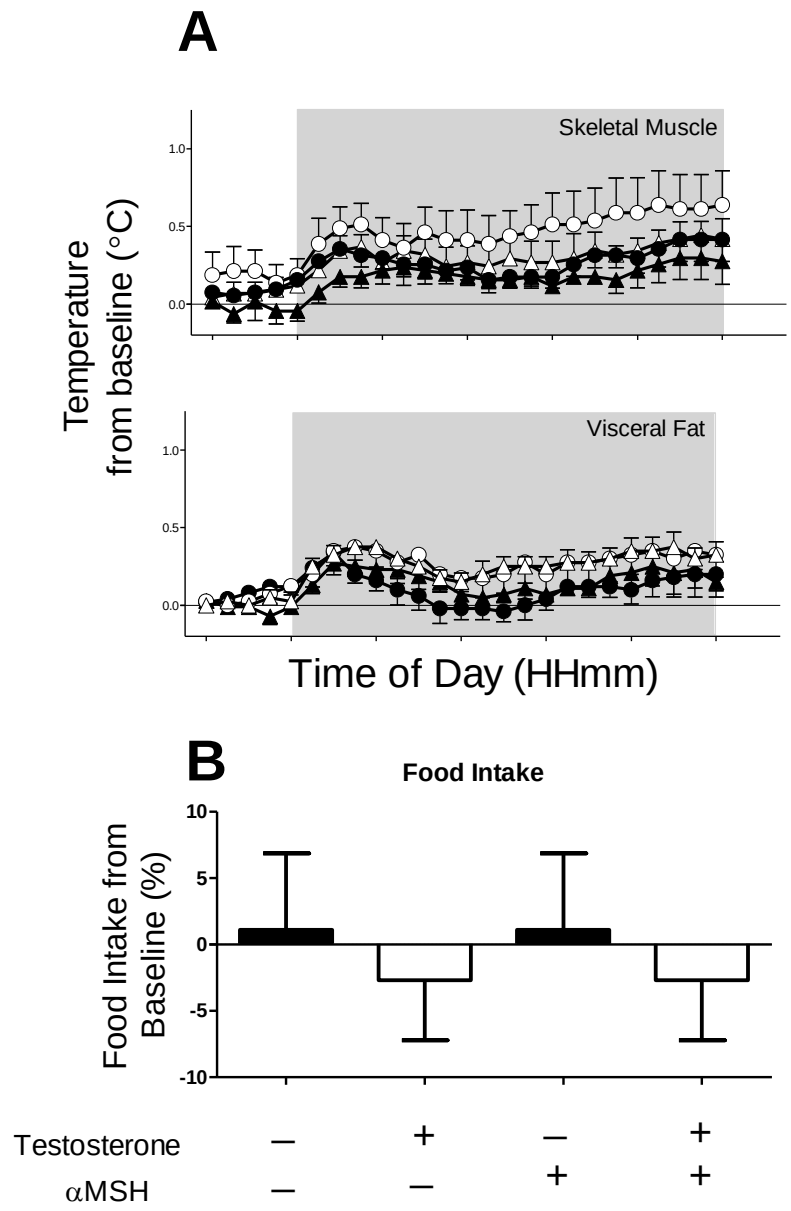

Figure 5.6: The effects of testosterone replacement and peripheral αMSH infusion on skeletal muscle (A, *upper panel*) and visceral fat (A, *lower panel*) temperature and postprandial (*gray box*) temperature responses in castrate rams. Temperature data is expressed as change from baseline (08:00 – 08:45 h average). Animals (n = 5 per group) were treated with testosterone implants (*open shapes*) or blank silastic tubing control implants (*closed shapes*), and infused with saline (*circles*) or αMSH (*triangles*). Neither steroidal background nor infusion type affected tissue temperature or the postprandial thermogenic response. Daily food intake (B) was not affected by infusion type irrespective of steroidal background.

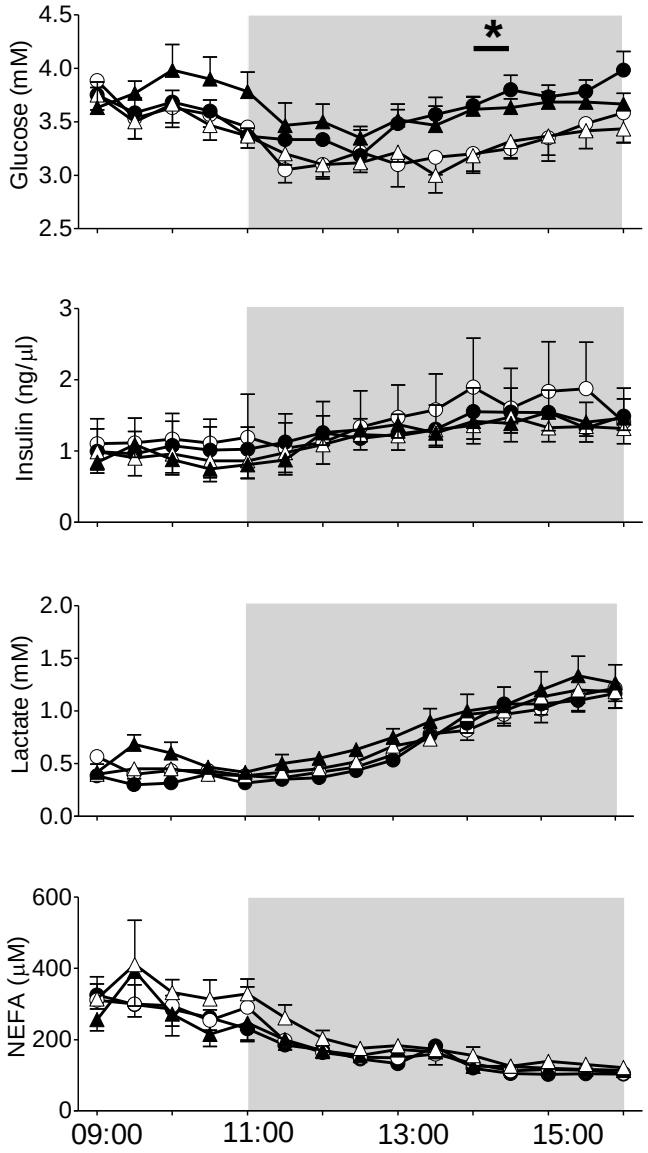

Figure 5.7: The effects of testosterone replacement and peripheral αMSH infusion on plasma metabolites before and during the feeding period in castrate rams. Animals (n = 5 per group) were treated with testosterone implants (*open shapes*) or blank silastic tubing control implants (*closed shapes*), and infused peripherally with saline (*circles*) or αMSH (*triangles*). Infusion type had no effect on any of the metabolites. Testosterone decreased ($p<0.05$) postprandial glucose levels regardless of infusion type.

4.4 Discussion

Within this chapter a sex-specific effect of testosterone was uncovered in relation to temperature output of skeletal muscle and visceral fat; this was observed in males but not female sheep. Both skeletal muscle and visceral adipose tissue temperature was reduced by whole body testosterone replacement in males. Reduced heat production in males due to testosterone was not associated with UCP1, UCP2 and UCP3 mRNA expression in these tissues. Sexual divergence in tissue temperature was also observed, whereby male skeletal muscle had higher temperatures than females, regardless of steroidal treatment. Castrate male visceral fat also had a higher temperature compared to females of both treatment groups, but testosterone replacement in males reversed this effect. In addition to temperature effects, during the postprandial period, plasma glucose levels were lower in males receiving testosterone. In females, testosterone had no effect on plasma glucose. The reduced glucose response to feeding in males was not associated with changes in insulin or the activation of AMPK or Akt.

Historically, direct effects of testosterone on energy expenditure were thought to be marginal. Testosterone was thought to primarily affect energy balance via its aromatization into estrogens. Our data are not consistent with this dogma, as testosterone treatment reduced heat production. In contrast, estrogen has previously been shown to up-regulate thermogenic pathways. Estrogen receptor-α deletion in the ventromedial nucleus (VMN) leads to hyperphagia and reduced energy expenditure (including reduced thermogenesis) resulting in an obese phenotype (Musatov *et al.*, 2007). Estrogen deficiency is also associated with decreased UCP1 in BAT which can be reversed by estrogen treatment (Pedersen *et al.*, 2001). Chapter 5 of this thesis examines the effects of acute and chronic estrogen replacement in female sheep on energy balance employing the same model used in this chapter. This data shows acute but not chronic effects of estrogen, whereby estrogen increased energy expenditure in females.

Sex steroids can influence core body temperature in both males and females. In general, females displayed higher overall body temperatures compared to intact males (Zuloaga *et al.*, 2009). Females experience fluctuations in body temperature across the menstrual cycle whereby progesterone exerts profound effects, specifically during the luteal phase when levels are elevated (Baker *et al.*, 2001). Estrogen and progesterone also increase markers of mitochondrial biogenesis in vitro in isolated brown adipocytes while testosterone has opposing effects (Rodríguez-Cuenca *et al.*, 2002; Rodriguez-Cuenca *et al.*, 2007). These findings support the sexual divergence in tissue temperature reported in this chapter. Females had lower skeletal muscle temperatures compared to males, as well as lower visceral fat temperatures compared to control males. This is most likely due to the females used in this chapter having been ovariectomised and would therefore have low levels of progesterone (Pope *et al.*, 1969).

Sex differences are implicated in the regulation of core body temperature, with men experiencing over-night nadirs (approx. -0.5°C) in rectal temperatures, while women do not (Baker *et al.*, 1998). Rodent models have also shown that testosterone impacts on circadian rhythms in core body temperature; with neonate-castrate adult rats exhibiting an earlier rise in core body temperature during the light phase compared to intact controls (Zuloaga *et al.*, 2009). Data presented in this chapter supports the notation that testosterone reduces core body temperature but exclusively in males. Females saw no temperature effects of testosterone replacement, although they experienced comparable elevations in plasma testosterone and decreases in follicle stimulating hormone compared to the male cohort. Sex differences in androgen receptor and estrogen receptor-α distribution may be key to the sexually dimorphic response to testosterone observed in this chapter. Androgen receptor mRNA is more greatly expressed in the rostral and caudal pre-optic area of male sheep compared to females (Scott *et al.*, 2004). These areas have been implicated as important centres for the regulation of thermogenic pathways (Nakamura and Morrison, 2008). Testosterone action on these regions may explain why testosterone replacement affected tissue temperature in males but not females.

To elucidate possible mechanisms involved in the reduced tissue temperature observed in testosterone treated males, thermogenic pathways were examined. These included postprandial responses and expression of UCP1, 2 and 3 mRNA. Postprandial elevations in temperature were observed in both tissue types and both sexes. Testosterone replacement had no effect on postprandial temperature elevation amplitude or duration. Consistent with this, testosterone replacement had no effect on preprandial or postprandial expression of UCP1, 2 or 3 mRNA in males. Nor were treatment differences observed in UCP1, 2 or 3 mRNA expression in adipose tissue taken during the preprandial period. Combined, data in relation to postprandial temperature responses and UCP1, 2 and 3 mRNA expression suggest that reduced tissue temperature observed in testosterone treated males is not related to altered adaptive thermogenic activity.

Humans and rodents are able to produce heat via thermogenesis due to the presence of functional BAT. BAT characterised by the expression of UCP1 (Zingaretti *et al.*, 2009; Golozoubova *et al.*, 2006; Lowell and Spiegelman, 2000). As discussed in chapter 1, in sheep, the picture is less clear. Adipose tissue expression of UCP1 mRNA in sheep has been demonstrated by Henry *et al.* (Henry *et al.*, 2010) in visceral fat(Henry *et al.*). Expression patterns are consistent with the modulation of thermogenesis in this tissue by leptin (Henry *et al.*, 2008). In contrast, Lomax *et al.* have shown ontogenic loss of BAT in sheep shortly after birth (Lomax *et al.*, 2007). This group was also unable to show the induction of UCP1 expression in the retroperitoneal fat bed by the β1/ β2 adrenergic agonist isoprenaline beyond 1 week of age in lambs (Lomax *et al.*, 2007). Data to support the presence of BAT in sheep beyond the neonatal period is scarce. Data presented in this chapter are consistent with earlier work by this lab, as decreased visceral fat temperatures in testosterone treated males were observed without any change in UCP1 expression. This may suggest that testosterone acts to decrease tissue temperature independent of thermogenic pathways. A potential role of functional BAT in adult sheep requires further investigation.

While the thermogenic potential of UCP3 is debated, Henry *et al.* (Henry *et al.*, 2011) has also demonstrated central administration of leptin induces skeletal muscle temperature elevations concomitant with increased UCP3 mRNA expression. In skeletal muscle from leptin treated animals there was a preferential switch towards uncoupled respiration in mitochondria, a biochemical hallmark of thermogenesis (Henry *et al.*, 2011). In addition rodent studies have highlighted the importance of UCP3 in energy balance. Genetic over-expression in skeletal muscle confers protective effects against diet-induced obesity (Tiraby *et al.*, 2007), and gene deletion increases susceptibility to obesity when placed on a high-fat diet (Costford *et al.*, 2008). There is mounting evidence to suggest a role of UCP3 skeletal muscle. In spite of this, data presented in this chapter reveals action of testosterone on tissue temperature in male sheep without associated changes in UCP3 mRNA in either adipose tissue or muscle. Coupled with data related to testosterone effects on UCP1 expression, it is unlikely that testosterone effects heat production via traditional thermogenic pathways.

In rodents, adaptive thermogenesis is not only associated with expression of the uncoupling proteins but also activation of AMPK. This has been observed in adipose tissue and skeletal muscle (Kus *et al.*, 2008; Lombardi *et al.*, 2008; Ahmadian *et al.*, 2011). AMPK activation is thought to be associated with adaptive thermogenesis as it results in up-regulation of fatty acid oxidation and glucose uptake, providing substrates for citric acid cycle and the electron transport chain. However, while characterised in rodent models, activation of AMPK in sheep has been shown not to be associated with altered thermogenesis (Laker *et al.*). Data presented in this chapter are consistent with this notion, as testosterone induced temperature decreases were not associated with change in AMPK activation in skeletal muscle.

Though testosterone had no effect on skeletal muscle AMPK or Akt activation, altered metabolic profiles were observed in males but not females. Testosterone replacement in males reduced postprandial levels of plasma glucose. No corresponding changes to insulin levels were observed. Conditions where females are exposed to hyper-androgenic hormonal profiles such as

polycystic ovarian syndrome, are associated with elevated glucose levels due to insulin resistance (Teede *et al.*, 2010; Cussons *et al.*, 2007). It is therefore surprising that no effect of testosterone treatment on glucose or insulin levels was detected in female sheep. Combined, these data suggest that the decreased postprandial glucose levels observed in testosterone treated males are not due to altered glucose utilisation by skeletal muscle. Sheep do not absorb glucose from their diet, they produce it via gluconeogenesis. Changes in plasma glucose observed during the postprandial period may be due to altered hepatic glucose production and release. This would be consistent with the lack of effect of testosterone on AMPK and Akt activation. Though, in humans at least, testosterone supplementation in elderly men has no effect on hepatic glucose kinetics (Basu *et al.*, 2007).

It appears, at least in sheep, that effects of testosterone on glucose homeostatis are sexually divergent, limited to males. In addition, the preprandial levels of glucose and NEFA were different between the male and female sheep, regardless of steroidal background. Female sheep had higher plasma levels of NEFA and lower plasma glucose levels during the preprandial period, compared to males. Possibly as a result, females exhibited distinct feeding associated rhythms in these two metabolites; glucose levels increased across the feeding period while NEFA levels decreased. No comparable rhythms were observed in males. No changes were observed in plasma lactate or insulin in males or females either due to feeding or testosterone treatment.

Peripheral administration of αMSH had no measurable effects on tissue temperature, plasma metabolites or food intake. αMSH is usually a potent anorectic peptide when administered centrally. In this study, αMSH had no effect on food intake, suggesting that the peptide did not cross the blood-brain barrier into the brain. In humans and other species, αMSH is found circulating in plasma, with levels showing little fluctuation (Catania *et al.*, 1998). Additionally, many of αMSH's receptor subtypes are expressed on peripheral tissues including adipose tissue and skeletal muscle (An *et al.*, 2007). In spite of this, the peripheral effects of αMSH have

scarcely been explored. In leptin deficient mice, peripheral administration of αMSH slow weight gain, reportedly due to altered adipose tissue metabolism (Hochgeschwender *et al.*, 2001). This treatment also improved thermoregulation during cold exposure (Hochgeschwender *et al.*, 2001) suggesting a role of αMSH in adaptive thermogenesis. Studies also suggest that α-MSH may help regulate leptin expression in adipocytes (Hoggard *et al.*, 2004; Norman *et al.*, 2003). As previously mentioned, α-MSH has also been shown to increase fatty acid oxidation in vitro (An *et al.*, 2007). It is therefore surprising that no effect of peripheral αMSH administration of skeletal muscle or adipose tissue temperate, nor in metabolite dynamics were oberved. Unpublished data from this laboratory shows that peripheral αMSH can increase skeletal muscle temperature during the postprandial period. These data were collected from a model similar to the one presented in this chapter. Instead of infusing αMSH throughout the periphery via the jugular vein, this model employed a hind-limb model whereby infusions of less concentration were infused directly into a vessel supplying the hind limb of castrate ewes. It is possible that experiments conducted in this chapter utilised a dose of αMSH which was either too low (and therefore had no effect on tissue metabolism) or that infusion to the whole periphery produced effects in several tissue types which essentially neutralised each other.

In conclusion, data presented in this chapter shows a profound and sexually divergent effect of testosterone on tissue temperature and glucose homeostasis in male but not female sheep. In males, testosterone lowered both visceral fat and skeletal muscle temperature and lowered plasma glucose levels during the postprandial period. These changes cannot be ascribed to changes in UCP1 2 or 3 mRNA expression or postprandial thermogenic output. These changes cannot be attributed to aromatization of testosterone to estrogen, and may be related to testosterone's action on the brain to regulate energy balance.

CHAPTER 5
The effects of estrogen on energy balance in the sheep

5.1 Introduction

Estrogen is known to be protective against weight gain in both males and females, whereby reduced levels of estrogen either through castration or with aging, is associated with increased body weight and adiposity (Brown and Clegg; Mauvais-Jarvis; Mayes and Watson, 2004; Lee *et al.*, 2009; Lovejoy *et al.*, 2008; Lovejoy *et al.*, 1996). In particular, estrogen is protective against abdominal (visceral) fat gain, and is paramount in determining sex differences in fat distribution. Males exhibit greater proportion of visceral adipose tissue compare to pre-menopausal females, who exhibit greater subcutaneous fat (Brown and Clegg, 2010; Shi and Clegg, 2009). Accumulation of visceral adiposity confers with it increased risk of a number of chronic obesity-associated metabolic complications such as cardiovascular disease (Wajchenberg, 2000; Kannel *et al.*, 1991); subcutaneous adiposity is regarded as being relatively benign (Bjrntorp, 1996). As such, pre-menopausal women have lower incidence of these obesity-associated adverse health risks compared to males. The importance of estrogen in

these health consequences is further evidenced by the increased risk of Type 2 diabetes in post-menopausal women (Lindheim *et al.*, 1994), which can be ameliorated by hormone replacement (Bonds *et al.*, 2006).

The beneficial effects of estrogen on energy balance are thought to be primarily derived from its action on the brain to reduce food intake (Butera and Czaja; Gao and Horvath, 2008). In most species, including humans, primates, sheep and rodents, fluctuations in food intake are evident across the menstrual cycle, corresponding to the secretion levels of estrogen from the ovaries (Lissner *et al.*, 1988; Buffenstein *et al.*, 1995). Preceding ovulation, a reduction in food intake is observed, coinciding with the rising levels of estrogen during the period of positive estrogen feedback (Lissner *et al.*, 1988; Buffenstein *et al.*, 1995). In rats, estrogen deficiency increases food intake and weight gain; estrogen replacement reverses these effects (Asarian and Geary, 2002). One mechanism whereby estrogen acts to reduce food intake is by sensitising the brain to leptin. This essentially enhances leptin's satiety effects in both male and female rats (Clegg *et al.*, 2006). While estrogen can have pronounced effects on food intake, a number of genetic studies that either deplete estrogen (aromatase knockout) or abolish estrogen action (deletion of the gene that encodes ERα), report the onset of obesity in the absence of hyperphagia (Heine *et al.*, 2000; Jones *et al.*, 2001; Jones *et al.*, 2000). These data suggest that estrogen regulates energy expenditure.

Previous studies in rodents have shown a reduction in physical activity as a precursor to obesity in both male and female aromatase knockout mice (Jones *et al.*, 2001; Jones *et al.*, 2000), and in female ERKOα mice (Ribas *et al.*, 2010). In the later study, activation of AMPK was lower in ERKOα mice, suggestive of decreased oxidative phosphorylation. (Ribas *et al.*). Altered thermogenesis has also been implicated in these phenotypes, though this aspect of energy expenditure remains relatively unexplored. Discrete ablation of ERα in the VMH and ARC of female rats and mice results in hyperphagia concomitant with a reduction in physical activity and diet-induced thermogenesis (Musatov *et al.*, 2007). In addition, deletion of ERα from SF-1

neurons of the VMN, reduces BAT thermogenesis without altering food intake, while deletion of ERα from pro-opiomelanocortin neurons (POMC) of the ARC increased food intake without associated effects on BAT thermogenesis (Xu *et al.*, 2011). This study highlights the dual roles of estrogen on energy intake and expenditure, mediated via the CNS.

In this chapter, an ovine model has been employed to investigate the acute and chronic effects of estrogen on food intake, energy expenditure and metabolic substrates in ovariectomized animals. It was hypothesised that estrogen regulates body weight via dual effects on energy intake and on energy expenditure. The primary focus of this work was to characterise the acute and chronic effects of estrogen on heat production in skeletal muscle and adipose tissue.

5.2 Methods:

5.2.1 Animals

Castrate ewes (n=6/group, 63.8+2.3kg bodyweight) were housed in an isolated room, exposed to natural light and a temperature range of 17-22°C. The animals were held in pens designed to allow sitting and standing but prevented further movement which might impact on temperature recordings. Temporal food restriction (between 1100-1600h daily) was imposed 2 weeks prior to experimentation to entrain a postprandial temperature response as previously described (Chapter 2.3). Food refusals were weighed to determine daily food intake. Temperature recordings were made using customized Dataloggers (SubCue, Calgary, Canada), surgically implanted to record tissue temperature of hind limb skeletal muscle and retroperitoneal (visceral) fat as previously described (Chapter 2.2.4.2). Dataloggers were programmed to record temperature at 15min intervals.

Prior to experimentation, one external jugular vein was cannulated, extended with a manometer line (Portex Ltd, Kent, UK) and closed with a three-way tap for the purpose of serial blood sampling. Cannulae were kept patent with heparinised saline (100K IU) until the day of experimentation as previously described (Chapter 2.5.1).

5.2.2 Experiment 1: Acute versus chronic estrogen replacement on heat production in muscle and fat

Tissue temperature was recorded under three conditions: baseline, acute treatment and chronic estrogen treatment. On each of these occasions skeletal muscle, visceral and gluteal adipose tissue temperature was recorded every 15min from 0800 to 1600h. Core body temperature was recorded hourly from 0900-1600 h with a rectal thermometer. In addition, blood samples (6ml) were collected and stored every 30min between 0900-1600 h, as previously described (Chapter 2.5.1). Samples were assayed for glucose, lactate, insulin and NEFA. Follicle stimulating hormone (FSH) was also assayed to determine the efficacy of estrogen treatment.

Baseline recordings were made on the day preceding acute treatment. For the acute treatment, animals received a single (i.m.) injection of either estradiol-17β benzoate (50μg, i.m.) or sterile peanut oil as control. Injections were performed at 0900 h. The initial blood sample and core body temperature recording (0900 h) were taken immediately prior to injection.

For chronic treatment, subcutaneous implants were inserted into the neck region of the animal as previously described (Chapter 2.4). Silastic implants contained either estradiol-17β benzoate (3x3 cm) or were blank (control). Acute and chronic treatments were performed in succession, with animals receiving acute and chronic treatments (exclusively estrogen or control, treatments were reversed when a cross-over design was implemented). Implants were inserted after 1600h

on the day of acute treatment. Chronic treatment was carried out for one week. On the seventh day, temperature recordings and blood samples were taken as described above (figure 5.1). A cross-over design was incorporated so each animal was treated with estrogen and control. A 2 week washout period was conducted between treatments during which animals were maintained on the feeding regime. Daily food intake was measured from baseline (the average of 3 days preceding acute treatment), through to day 7 of chronic treatment (Chapter 2.3).

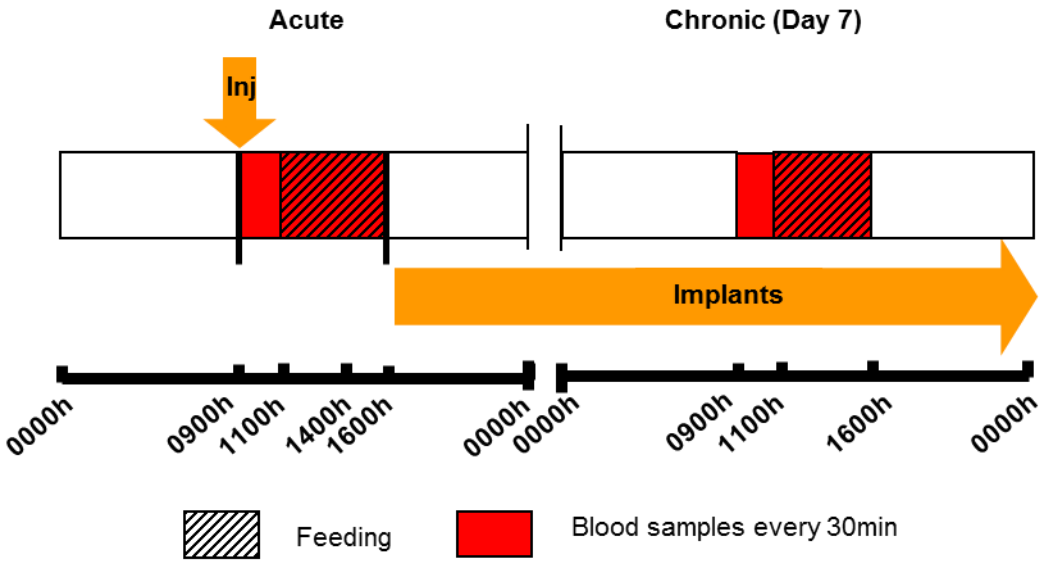

Figure 5.1: Timeline of experiment 5.1, highlighting acute and chronic treatments, blood sampling and feeding. Acute treatment was administered via i.m. injection at 0900h. Chronic treatment was administered via implants inserted after 1600h on the acute treatment day. Blood samples and temperature data were recorded on the 7th day of chronic treatment. After one round of acute and chronic treatment, a cross-over design was implemented whereby treatments were repeated but sheep who initially received estrogen then received control and *vice versa*.

5.2.3 Experiment 2: Repeated estrogen administration on postprandial temperature responses in muscle

To further elucidate the acute effects of estrogen administration on peripheral tissue temperature, animals (n=5/group; 51.7±2.8kg bodyweight) received serial injections (i.v.) of 25µg estradiol-17β benzoate. Control animals received vehicle (2.5% ethanol in sterile water). Injections were administered at 0900, 1200 and 1500h and skeletal muscle temperature recorded every 15min between 0800 and 2000h. A cross-over design was implemented allowing at least 2 days recovery between treatments.

5.2.4 Experiment 3: Acute estrogen treatment on metabolic pathways in muscle and fat

To investigate the molecular mechanisms underpinning the acute estrogen-induced temperature elevations observed Experiment 1, the acute treatment was repeated followed by tissue biopsies (n=4 in each group). Animals received an i.m. injection of 50µg estradiol-17β benzoate or vehicle at 0900h, fed at 1100 h and culled (20ml Lethabarb, i.v.) during the postprandial period (1400 h). Tissue samples from the gluteal muscle, subcutaneous gluteal fat and visceral fat were immediately collected, frozen in liquid nitrogen and stored at -80ºC until analysis.

Phosphorylation of AMPK and Akt were measured by Western Blotting and expression of UCP 1, 2 and 3 mRNA was measured by real time PCR.

5.2.5 Experiment 4: Relationship between estrogen-induced heat production and blood flow in skeletal muscle

In order to understand the relationship between estradiol-17β benzoate -induced skeletal muscle temperature increases and blood flow to this tissue, whole limb blood flow was measured using a transonic flow probe in conscious animals (n=4; 49.2±1.4). The transonic flow probe was surgically placed around the femoral artery as detailed in Chapter 2.2.4.3. The acute treatment detailed in Experiment 1 was repeated, whereby animals received an i.m. injection of 50µg estradiol-17β benzoate or vehicle at 0900h, and fed between 1100 and 1600 h. Skeletal muscle temperature was measured at 1min intervals and femoral artery blood flow was continuously recorded; both are presented as 15min averages.

5.2.6 Plasma Metabolites, Insulin and Follicle Stimulating Hormone (FSH)

Methodology for the measurement of glucose, lactate, plasma non-esterified fatty acids (NEFA), insulin and FSH can be found in chapters 2.6 and 2.7. Assay-specific details are as follows:

The measurable range for blood glucose was between 0–30mm and was 0–16 mm for lactate. For the measure of NEFA, the inter-assay co-efficient of variation (CV) was 3.6% at 117.2 µM and 0.4% at 300.8 µM. For the measure of insulin, the inter-assay CV was 1.3% at 0.98ng/ml and 4.3% at 4.26ng/ml and the sensitivity of the assay was 0.2ng/ml.

FSH assays were performed to determine whether the estradiol-17β benzoate treatments were efficacious in providing negative feedback to gonadotrophs in the anterior pituitary, with the expectation of lowered plasma levels. The inter-assay co-efficient of variation (CV) was 6.8% at

2.8ng/ml and 3.1% at 11.5ng/ml and the sensitivity of the assay was 0.06ng/ml. Plasma FSH data is presented as a daily mean.

5.2.7 Western Blotting

The general western blot protocol is provided in Section 2.8. Experiment-specific details are as follows:

Sixty micrograms of protein was mixed with sample loading buffer (3:1) boiled for 4 min and loaded into a 12.5% SDS page gel. Electrophoresis was performed until bands were adequately separated (150V at room temperature for1.5 hours). Protein was transferred to a nitrocellulose membrane (1hour at 75V at room temperature). Membranes were incubated with primary antibodies raised against pAMPK, total AMPK protein, pAktSer473, pAktThr308 and Akttot Over-night at 4°C. A secondary antibody conjugated to horseradish peroxidise (Antibodies Australia, Melbourne, Australia) was applied for 1 h at room temperature and detection was accomplished using ECL. Densitometry was accomplished using the ImageJ software program (Fujifilm Corp., Minatoku, Tokyo, Japan).

5.2.8 Real-Time Polymerase Chain Reaction (rtPCR)

The details of rtPCR methodology are provided in Chapter 2.9.. Experiment-specific details are as follows:

Skeletal muscle and visceral fat samples were analysed by rtPCR for UCP1, UCP2 and UCP3 mRNA expression. Uncoupling protein mRNA expression was normalised to the geometric

mean of the housekeeping genes. For visceral fat, the housekeeping genes included Tyrosine 3-monooxygenase (YWHAZ), β-actin and malate dehydrogenase-1 (MDH1). For skeletal muscle, the housekeeping genes included β2-microgobulin, β-actin and cyclophilin.

5.2.9 Data Analysis and Presentation

All data are presented as means ± SEM. Temperature data in experiment 4 are expressed as hourly averages corrected to baseline. FSH data is presented as the average concentration recorded across the treatment period.

Temperature data, plasma metabolites, FSH, insulin and food intake were analysed using a repeated measures analyses of variance (ANOVA). When a group x treatment effect was established, post-hoc comparisons were made using the least significance difference test. Effects of acute estrogen treatment on gene and protein levels were determined by a one-way ANOVA.

5.3 Results:

5.3.1 Experiment 1: Acute versus chronic estradiol-17β benzoate replacement on heat production in muscle and fat

Food intake was similar between the control and estradiol-17β benzoate -treated animals across the baseline period and after acute injection of EB (Figure 5.2A). Food intake, however, was decreased ($P<0.05$) between day 1 and day 4 in animals receiving chronic estradiol-17β benzoate treatment compared to control (Figure 5.2A). The reduction in food intake was transient and returned to control levels within 5 days of treatment (Figure 5.2A).

Plasma levels of FSH were similar between castrate and estradiol-17β benzoate -treated animals across the baseline period (Figure 5.2B). There was no effect of acute EB-injection of FSH levels, but chronic estrogen treatment reduced ($P<0.01$) FSH levels (Figure 5.2B).

Skeletal muscle, visceral fat, gluteal fat and core body temperatures were similar between control and treated animals during the baseline period (Figure 5.3). Injection of EB immediately increased ($P<0.05$) heat production in skeletal muscle and visceral fat compared to the control group (Figure 5.3). There was no effect of chronic treatment with estradiol-17β benzoate implants on skeletal muscle or visceral fat (Figure 5.3). There was no effect of either acute or chronic estradiol-17β benzoate treatment on heat production in gluteal fat or on core body temperature (Figure 5.3).

To characterise the metabolic state of the animals we measured plasma levels of glucose, lactate, insulin and NEFA. There were no effects of either acute or chronic estradiol-17β benzoate treatment on the plasma metabolites or insulin levels (Figure 5.4).

5.3.2 Experiment 2: Repeated estradiol-17β benzoate administration on postprandial temperature responses in muscle

Serial bolus i.v. injections of estradiol-17β increased skeletal muscle temperature compared to the vehicle-treated group. Unlike the effect of a single injection, repeated injections of -estradiol-17β elicited a sustained increase (group effect $P=0.001$) in skeletal muscle temperature (Figure 5.5), with a return to baseline within 5h after the last injection (Figure 4).

5.3.3 Experiment 3: Acute estradiol-17β benzoate treatment on metabolic pathways in muscle and fat

Acute estradiol-17β treatment had no effect on the expression of UCP1, UCP2 and UCP3 mRNA in skeletal muscle or visceral fat (Figure 5.6A).

Acute estradiol-17β treatment increased the phosphorylation of AMPK and Atk (phosphorylation of Thr308) in skeletal muscle during the postprandial period compared to animals who received vehicle (Figure 5.6B). There was no effect of estradiol-17β replacement on AMPK or Akt (Thr308; Ser473) phosphorylation in either visceral or gluteal fat.

5.3.4 Experiment 4: Relationship between estradiol-17β benzoate - induced heat production and blood flow in skeletal muscle

In accordance with Experiment 1, acute treatment estradiol-17β treatment replacement resulted in a rapid rise (group effect $P<0.05$) in heat production within one hour of i.m. injection (Figure 5.7A). This response was not observed when animals were treated with vehicle. Blood flow to the skeletal muscle was not altered due to either the estradiol-17β treatment injection or feeding (Figure 5.7B).

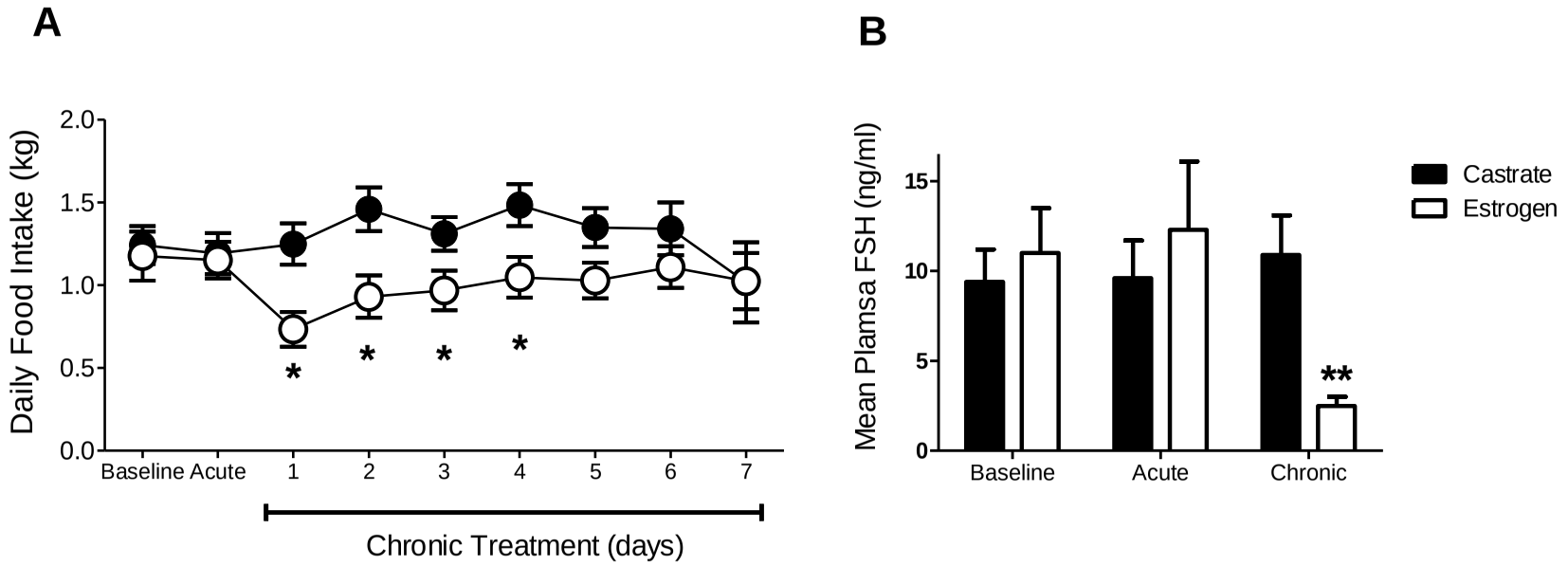

Figure 5.2: Estradiol-17β treatment replacement had a transient effect on food intake (A), decreasing ($P<0.05$) consumption on day 1 – 4 of chronic treatment. Food intake was similar between estradiol-17β treatment treated and castrate controls at baseline, after acute i.m. injection of EB, and at 7 days of chronic estradiol-17β treatment treatment: days in which temperature data was recorded. Mean plasma levels of FSH (B) were similar between castrate and estrogen-treated animals across the baseline period. Acute EB-injection had no effect on FSH levels, but 7 days of chronic estrogen treatment reduced ($P<0.01$) FSH levels.

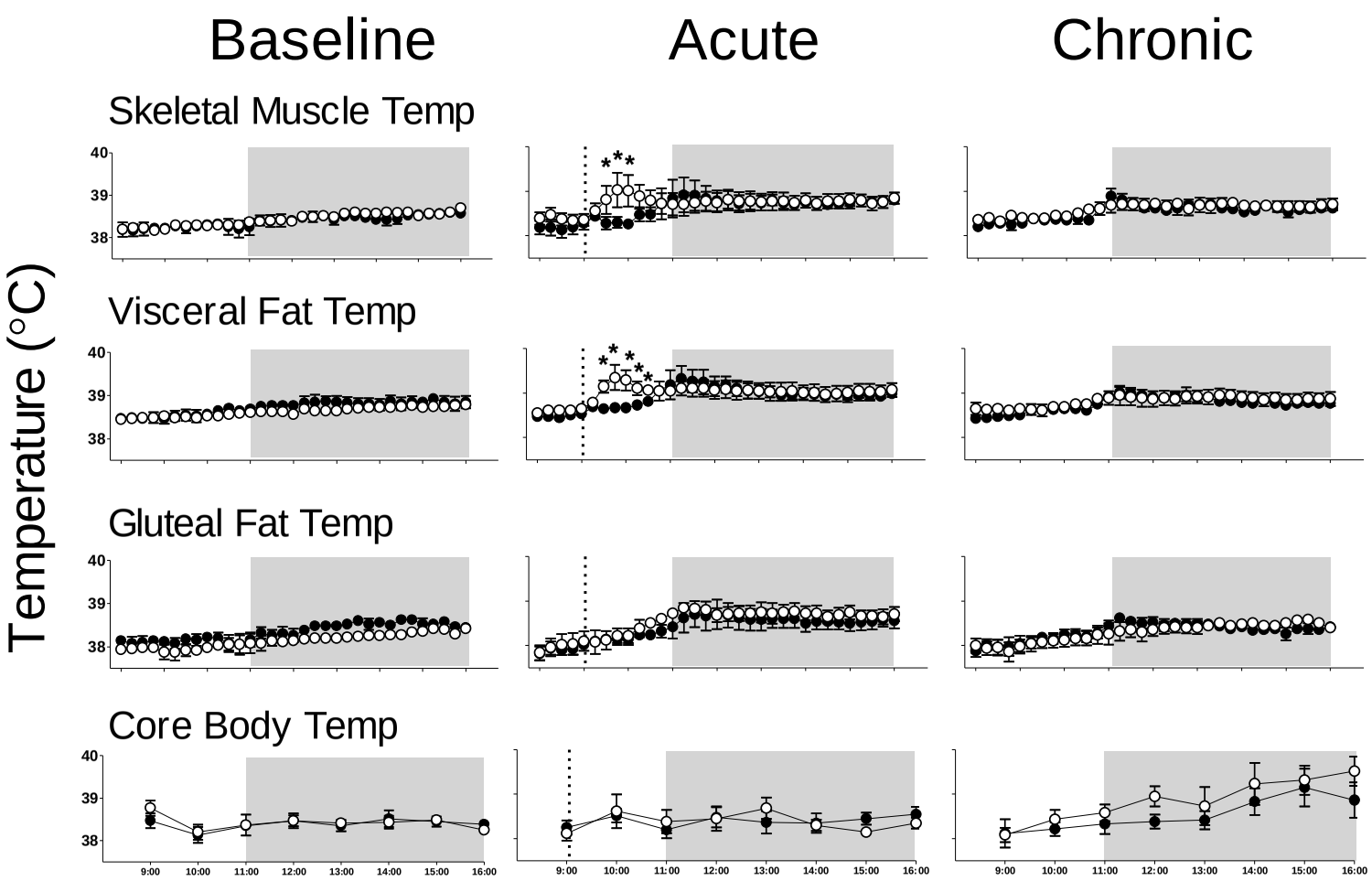

Figure 5.3: Figure legend on next page.

Figure 5.3: Effects of acute and chronic estradiol-17β benzoate replacement (○) on tissue and core temperature profiles in castrate control (control; ●) ewes before and during the postprandial period (grey box). Skeletal muscle, visceral fat, gluteal fat and core body temperatures were similar between control and treated animals during the baseline period. Intramuscular injection (50µg estradiol-17β treatment, 0900 h, dotted line) immediately increased ($P<0.05$) heat production in skeletal muscle and visceral fat compared to the vehicle control group. This acute effect of EB was not apparent in gluteal fat. Seven days of chronic treatment with estradiol-17β benzoate had no effect on skeletal muscle and visceral fat or gluteal fat temperature, being similar between treated and control animals. There was no acute or chronic treatment effect on core body temperature.

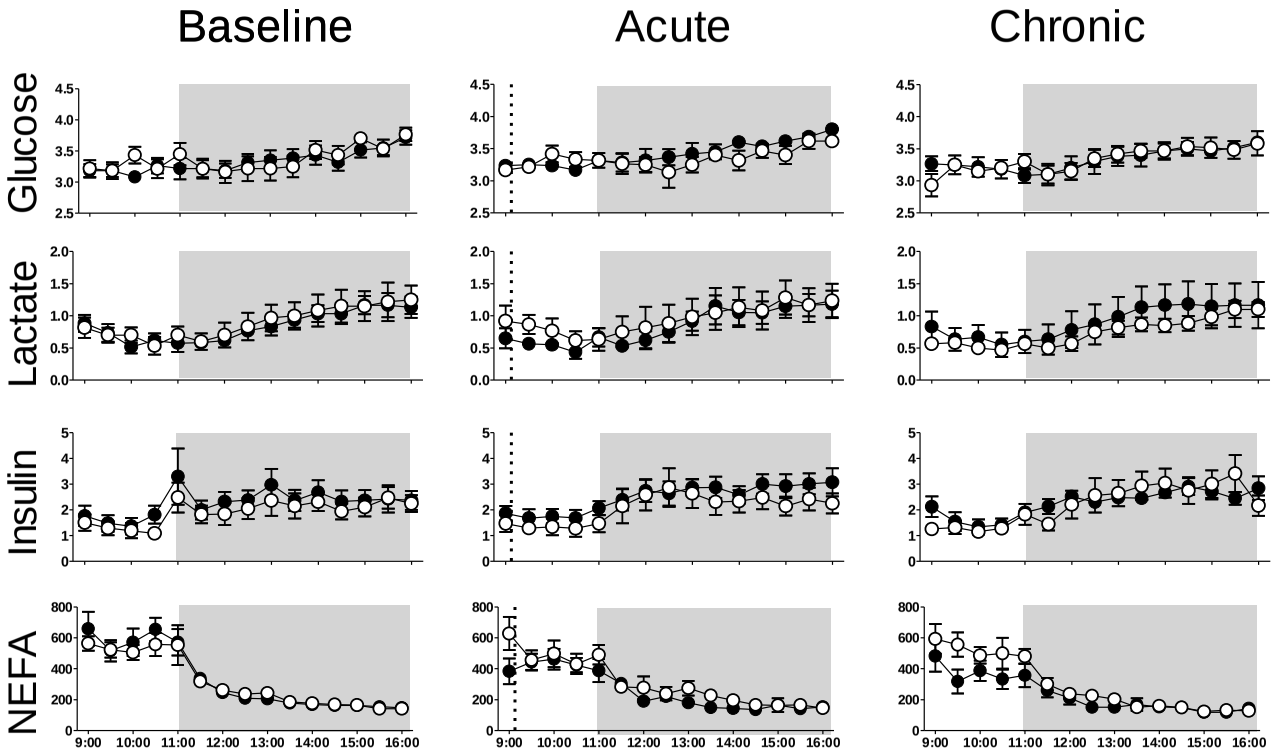

Figure 5.4:, Effects of acute and chronic estradiol-17β benzoate replacement (○) on plasma metabolite and insulin levels in castrate (control; ●) ewes, before and during the postprandial period (grey box). Metabolic profiles were measured at baseline, after acute i.m. injection (50µg estradiol-17β treatment or vehicle, 0900 h, dotted line), and after 7 days of chronic steroidal overlay (3x3cm estradiol-17β benzoate or blank s.c. implants). Estrogen replacement had no effect on plasma concentrations of glucose, lactate, insulin and non-esterified fatty acids (NEFA).

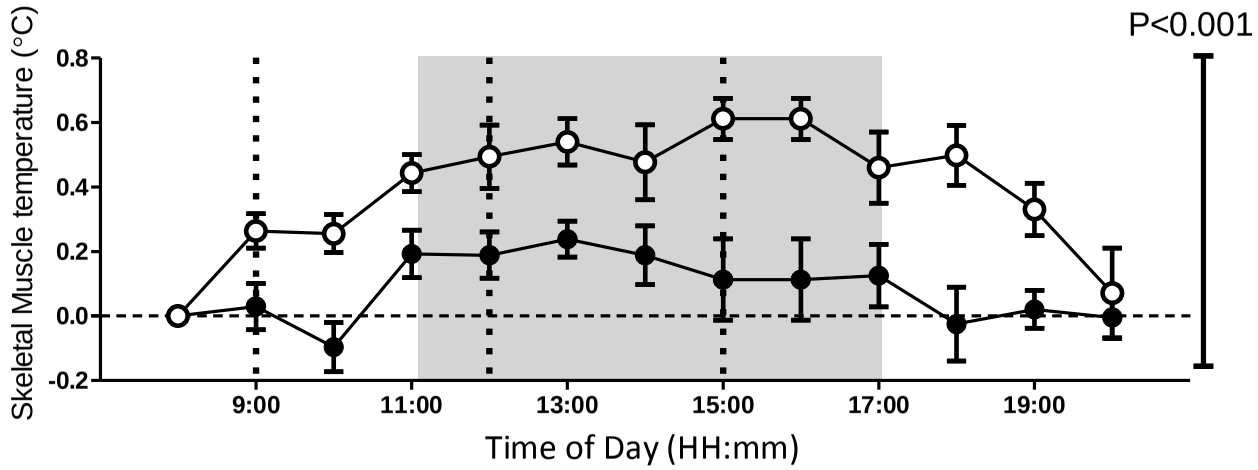

Figure 5.5: The effects of repeated estradiol-17β treatment infusion bolus (25ug, dotted lines) on skeletal muscle temperature in female castrate sheep. Skeletal muscle temperature is presented as hourly averages (±SEM) minus the 0800 h baseline temperature. Estradiol-17β treatment replacement (○) increased (group effect P<0.001) skeletal muscle temperature above that of animals receiving vehicle (●).

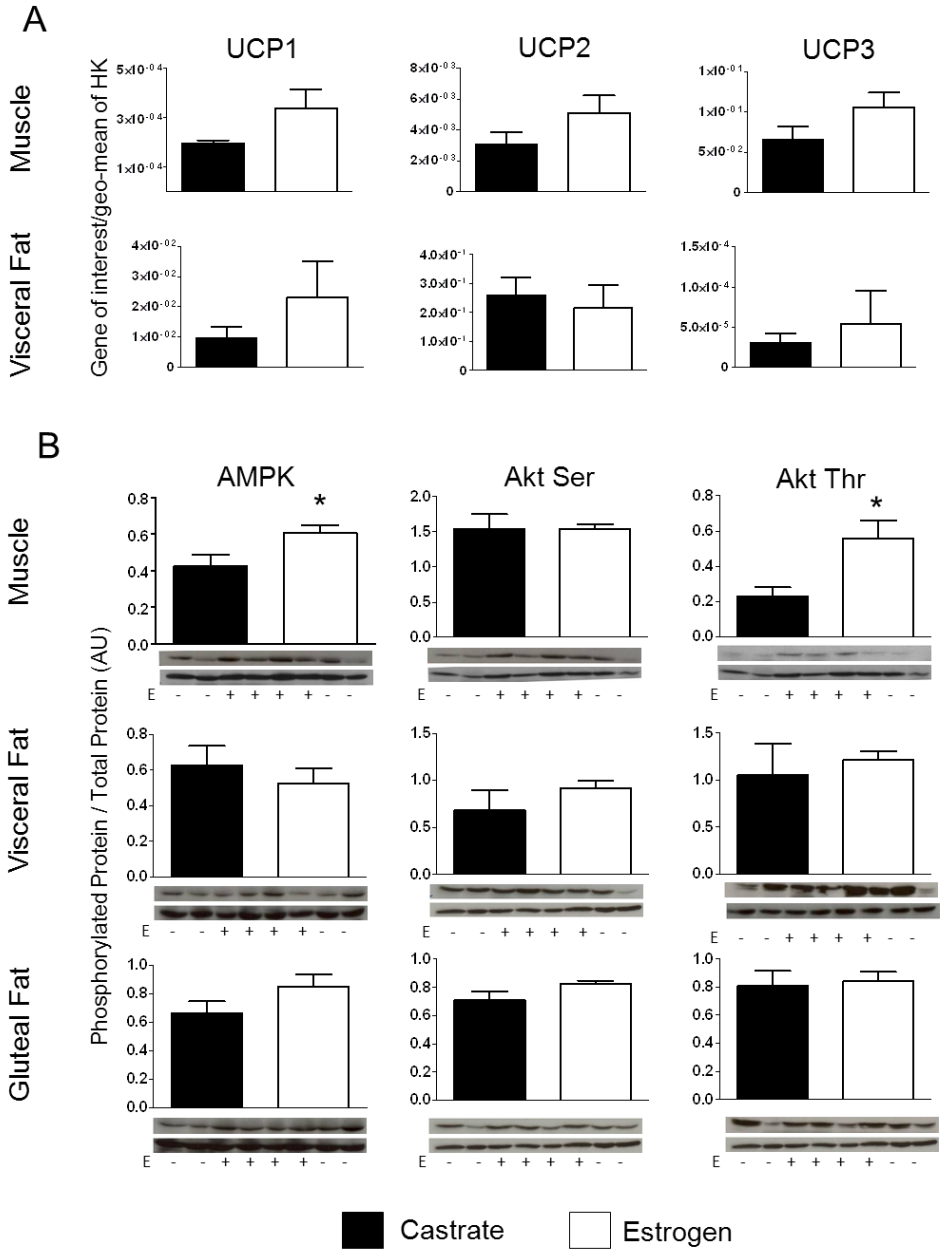

Figure 5.6: The effects of acute estradiol-17β treatment replacement (50µg i.m. injection; white bars) on metabolic pathways in peripheral tissues during the postprandial period in castrate (vehicle; black bars) ewes. Uncoupling protein 1, 2 and 3 A mRNA expression in skeletal muscle and visceral fat was not altered during the postprandial period under acute estradiol-17β treatment replacement as assessed via rtPCR (A). Acute estradiol-17β treatment replacement increased activation of p-AMPK ($p=0.05$) and p-Akt(Thr; $P<0.05$) but not p-Akt(Ser) in skeletal muscle (B).

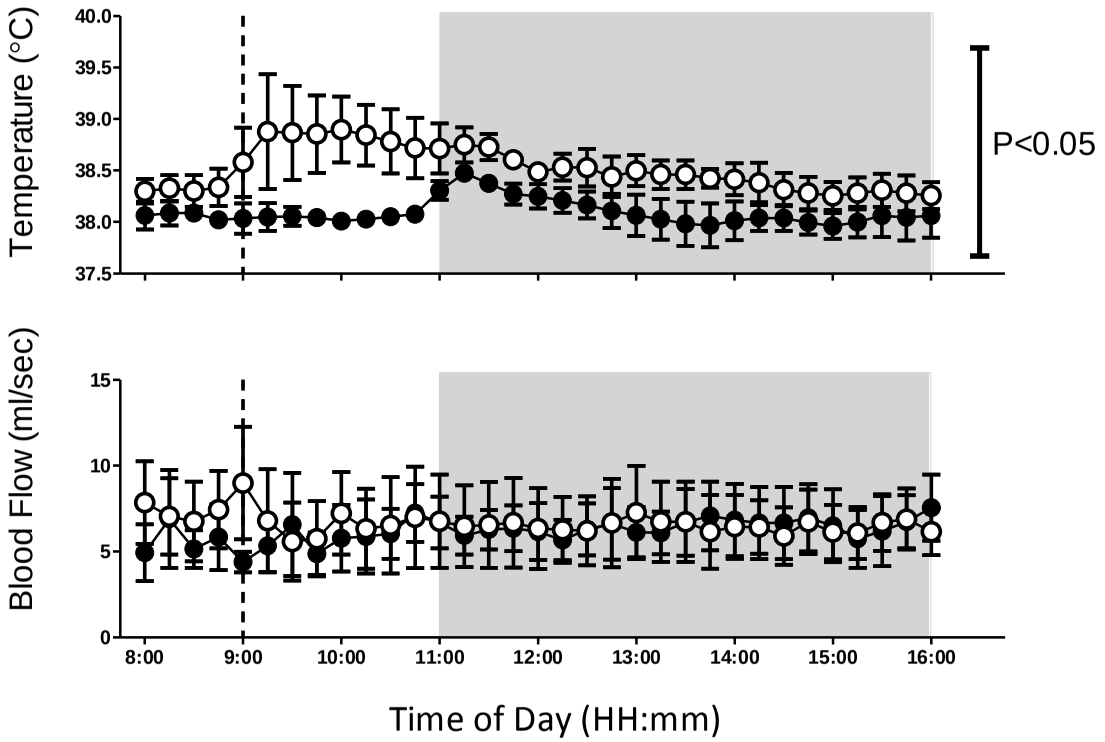

Figure 5.7: Acute estradiol-17β treatment replacement resulted in a rapid rise (group effect P<0.05) in heat production within one hour of i.m. injection (dotted line) in skeletal muscle. This response was not observed when animals were treated with vehicle. Blood flow to the skeletal muscle was not altered due to either the estradiol-17β treatment injection or feeding.

5.4 Discussion

Acute estradiol-17β treatment injection increased heat production in visceral fat and skeletal muscle of ovariectomised ewes. Interestingly, these effects appear to be tissue specific as this was not observed in gluteal fat. Additionally, repeated injection of estradiol-17β treatment (reflecting pulsatile or episodic estradiol-17β treatment release) caused a sustained and elevated temperature response in skeletal muscle. In contrast, these temperature effects were not achieved via continuous, chronic estradiol-17β benzoate delivery as administered via subcutaneous 17β-estradiol implants. This suggests that pulsatile release is essential to the thermogenic effect of estradiol-17β treatment. Furthermore, it was demonstrated that estrogen-induced heat production occurs within 30 min, and as such may be mediated via rapid cell signalling. The effects of estrogen on thermogenesis are rapid and episodic or pulsatile administration is essential in maintaining elevated heat production.

Traditionally, estrogen was thought to act through classical genomic signalling via its receptors. Recently, central effects of estrogen on metabolism have been linked to rapid, non-genonmic mechanisms. STX, a nonsteroidal compound, which targets a G-protein coupled estrogen receptor has been shown to reduce food intake as well as body temperature in ovariectomized hamsters (Qiu *et al.*, 2006; Roepke *et al.*, 2010). Site specific silencing of ERα gene within the ventromedial hypothalamus induces an obese phenotype characterised both hyperphagia and a reduction in thermogenesis (Musatov *et al.*, 2007). Furthermore, deletion of ERα from SF-1 neurons of the VMN, attenuates BAT thermogenesis, where deletion of ERα from pro-opiomelanocortin (POMC) neurons of the arcuate nucleus increased food intake without an associated effect on BAT thermogenesis (Xu *et al.*, 2011). Restoration of the non-genomic signalling pathways by genetic knock-in abolishes the metabolic phenotype of ERα knockout animals (Park *et al.*, 2011). Taken together, these studies begin to separate neuronal pathways associated with estrogen-mediated regulation of thermogenesis as being distinct from those which regulate food intake. Temperature data presented in this chapter are consistent with the

work by Wu *et al.* and Musatov *et al.* (Xu *et al.*, 2011; Musatov *et al.*, 2007), showing increased temperature in response to estrogen treatment. These data demonstrate an acute and rapid effect of estrogen treatment, but whether this effect is through classical genomic signalling or through non-classical signalling remains to be elucidated.

The rapid effects of estradiol-17β treatment on peripheral tissue temperature was site specific; resulting in increased heat production in skeletal muscle and visceral fat, but not in subcutaneous gluteal fat. As reviewed in chapter 1.6.3, in humans, the expression of ER α is similar in both visceral and subcutaneous fat depots. In contrast, an early study in sheep revealed greater estrogen binding within subcutaneous adipose tissue compared to visceral (Watson *et al.*, 1993). Therefore, site specific differences in response to acute estrogen treatment may be due to ERβ, although this has not been verified. Given that estrogen binding is greatest in subcutaneous fat, it was surprising that we did not find a change in subcutaneous fat temperature in response to estrogen treatment. Nonetheless, these data are consistent with previous work that suggested that subcutaneous fat has a lower thermogenic potential than retroperitoneal fat. Henry *et al.* (Henry *et al.*, 2008) reported that central infusion of leptin increased thermogenesis in skeletal muscle and visceral fat to a greater degree than in subcutaneous fat. This was substantiated by the additional finding that UCP1 mRNA expression is higher in visceral fat (Henry *et al.*, 2010). The lack of estrogen-induced temperature elevation in subcutaneous fat may therefore be due to the intrinsically low thermogenic potential of this tissue, even though estrogen receptors are expressed in this depot.

It is important to note that the effects of estradiol-17β benzoate on energy expenditure were independent of any effects on feeding or the reproductive axis. Acute estradiol-17β benzoate replacement increased tissue temperature in muscle and visceral fat before the onset of feeding. In addition, acute estradiol-17β benzoate treatment had no effect on food intake or plasma levels of FSH. Conversely, chronic estradiol-17β benzoate replacement suppressed FSH levels and had a transient effect on food intake, but had no effect on thermogenesis. These food intake data are

consistent with a number of studies showing transient reduction in food intake as a result of cyclic estrogen replacement in ovariectomised mice (Asarian and Geary, 2002; Geary and Asarian, 1999). Food intake data presented in this chapter confirm that changes in temperature are due to a direct effect of estrogen rather than a secondary change due to food intake.

Repeated injection of estradiol-17β benzoate caused a sustained increase in skeletal muscle thermogenesis. Thus, at least in sheep, the effect of estradiol-17β benzoate on heat production is amplified or accentuated through episodic delivery. This reflects the physiological pulsatile secretion of estrogens from the ovary. Core body temperature fluctuations across the estrous cycle are thought to be predominately linked to progesterone, whereby high progesterone levels correspond to high core body temperatures (Baker and Driver, 2007; Coyne *et al.*, 2000; Lee, 1988). Additionally, some studies indicate that estrogen may reduce tissue temperature when progesterone levels are low (Stachenfeld *et al.*, 2000). The current studies were performed in ovariectomised sheep, eliminating any effects of changing progesterone levels. Perhaps the present findings have unmasked a true thermogenic property of estrogen. In contrast to an earlier study (Stachenfeld *et al.*, 2000), these results suggest a dichotomous role of estrogen on thermogenesis in normally cycling animals compared to estrogen replacement in OVX animals. This may have implications for hormone replacement in post-menopausal women.

It was hypothesised that estradiol-17β benzoate -induced heat production in skeletal muscle and adipose tissue occurred via UCP-mediated thermogenesis. To elucidate this, UCP 1, 2 and 3 mRNA levels were measured under conditions of acute estrogen replacement. Estradiol-17β benzoate treatment had no effect on UCP1, UCP2 or UCP3 mRNA expression in either tissue. These tissue samples were obtained during the peak postprandial period – after tissue temperature had returned to baseline- and therefore these data are difficult to interpret. Experiment 4 (serial estradiol-17β benzoate treatment), has been repeated in a separate study with skeletal muscle biopsies taken at 1600h (the time of peak temperature divergence between estrogen-treated and control animals). Unpublished data show that estrogen treatment increased

UCP1 mRNA expression in 3 of 4 animals. Given the low abundance of UCP1 in skeletal muscle (Henry *et al.*, 2010), and the low level of brown adipocytes interspersed between muscle fibres (Almind *et al.*, 2007), these observed changes in UCP1 do not account for the large increase in tissue temperature during this time. Further study is needed to assess UCP expression and activation during estrogen-induced temperature excursions observed after single and repeated acute estrogen replacement.

To further characterise the peripheral effects of estrogen replacement on energy balance, activation of AMPK and Akt (key proteins involved in fuel partitioning) was measured in muscle and visceral fat. In rodents, activation of AMPK in both skeletal muscle and visceral fat (Kus *et al.*, 2008; Lombardi *et al.*, 2008; Ahmadian *et al.*) is associated with adaptive thermogenesis. As reviewed in Chapter 1.5, activated AMPK can exert dual effects on energy expenditure pathways, driving fatty acid oxidation via phosphorylation of acetyl-CoA carboxylase (ACCleading to inhibition of ACC activity), and increasing glucose uptake by up-regulating transcription factors leading to increased GLUT4 synthesis. Both of these pathways would provide substrates for the citric acid cycle and the electron transport chain. While it has been characterised in rodent models, activation of AMPK in sheep has been shown not to be associated with altered thermogenesis (Laker *et al.*, 2011; Henry *et al.*). In the present study, AMPK activation was shown to increase in the skeletal muscle (but not the adipose tissue) of estradiol-17β benzoate treated animals. This is consistent with the works of D'Eon *et al.* (D'Eon *et al.*, 2008; D'Eon *et al.*, 2005), providing evidence for the catabolic role of estrogens on skeletal muscle. Akt activation via phosphorylation of Thr308 was also elevated in muscle in response to estrogen treatment. In adipose tissue and skeletal muscle, phosphorylation of the Ser473 And Thr308 amino acids are differentially regulated by divergent up-stream pathways (Hresko and Mueckler, 2005; Kondapaka *et al.*, 2004; Weigert *et al.*, 2005). Despite this, phosphorylation of either (or both) amino acid results in the same physiological outcome. Activation of Akt in skeletal muscle further indicates increased glucose utilisation by the tissue due to acute estradiol-17β benzoate replacement. Rogers *et al.* have previously shown that

estrogen stimulation of *ex vivo* rat skeletal muscle results in rapid (<10min) activation of both Akt and AMPK, though interestingly, no increase in glucose uptake was observed in these preparations (Rogers *et al.*, 2009). This is consistent with our findings that estradiol-17β benzoate replacement had no effect on plasma metabolite concentrations.

The model established in chapter 3 to assess the relationship between skeletal muscle temperature blood flow to the tissue was adapted in the current study to investigate the effect of acute estradiol-17β benzoate administration on blood flow and tissue temperature. Results obtained from this study lead to the conclusion that the temperate effects of acute estradiol-17β benzoate replacement are independent of acute changes in blood flow, strengthening the case for temperature elevation intrinsic to the tissue.

We have observed a number of adaptations within energy expenditure pathways resulting from acute estradiol-17β benzoate replacement. These data support the notion that estrogens increases energy expenditure in a site specific manner, potentially underpinning differences in body composition observed in individuals pre and post menopause, and in males (reviewed in Chapter 1.6). Clearly, estrogens plays a role in regulating whole body energy expenditure, having major effects on skeletal muscle and visceral fat. The gradual changes in body composition between pre- and post-menopausal women results from prolonged changes in progesterone and estrogen levels. It was surprising therefore, that chronic estradiol-17β benzoate replacement did not alter thermogenesis at any of the tissue sites. Previous studies have shown a reduction in estrogen and progesterone in healthy women reduces resting energy expenditure (Day *et al.*, 2005) and sympathetic activity in skeletal muscle (Day *et al.*, 2011). Cyclic administration of synthetic progesterone (progestin) has also been shown to increase energy expenditure and decrease adipose accumulation in peri-menopausal women over a 12 month period. Collectively, these studies suggest a role of the 'female' sex hormones in regulating body weight via sympathetic-mediated energy expenditure pathways. Data presented

in this chapter further strengthen this paradigm, specifically highlighting the role of estradiol-17β benzoate in regulating heat production in peripheral tissues.

In conclusion, data presented in this chapter shows a profound effect of acute but not chronic estradiol-17β benzoate replacement on skeletal muscle and visceral fat heat production. Repeated, estradiol-17β benzoate injections produced a sustained increase in skeletal muscle heat production. In addition, acute estradiol-17β benzoate replacement was associated with increased activation of AMPK and Akt. In spite of this, no differences in plasma metabolites was observed. Estrogen-induced temperature elevations cannot be attributed to UCP1, 2 or 3-mediated uncoupling thermogenesis but may involve rapid non-genomic pathways. As such, further investigation is warranted to elucidate the molecular mechanisms underpinning the physiological outcomes observed in this chapter.

CHAPTER 6
General Discussion

6.1 Major Findings

The work presented in this thesis has advanced our understanding of how the sex steroids testosterone and estrogen regulate energy balance in the sheep. This thesis employed a recently developed model (Henry *et al.*, 2011; Henry *et al.*, 2008; Clarke *et al.*, 2012) whereby tissue temperature can be directly recorded, in the conscious animal, as a measure of energy expenditure. These recordings were performed concurrently with the collection of tissues as well measurements of food intake. Together, these techniques provided a large scope through which the governing roles of sex steroids on whole body energy balance were investigated using a novel approach. The experiments presented in chapter 3 show comprehensively that skeletal muscle temperature is independent of changes in blood flow to the tissue. This conclusion was based on the findings of four different paradigms each investigating the effects of altered blood flow due to pharmacological intervention or induction of muscle temperature by meal feeding or meal anticipation. The temperature elevations observed in response to high doses of isoprenaline suggest that β3-andrenergic stimulation can result in heat production, consistent

with the idea that skeletal muscle is capable of adaptive thermogenesis via sympathetic activation. The results presented in Chapter 4 reveal an important role of testosterone in regulating energy expenditure in male but not female sheep. Testosterone decreased overall tissue temperature and postprandial plasma glucose levels. Testosterone reduced baseline temperature but did not alter postprandial temperature excursions. Results presented in Chapter 5 show that acute but not chronic estrogen replacement increases muscle and visceral fat temperature while also increasing fuel utilisation in skeletal muscle. These results may directly relate to the changes in body composition observed in post-menopausal women where the lowering of estrogen and progesterone levels coincide with fat (specifically visceral) accumulation. In summation, the data presented in this thesis provide strong evidence that testosterone and estrogen can influence energy balance by regulating the production of heat. Furthermore, the data presented in this thesis demonstrate that skeletal muscle is thermogenic and sex steroids act to regulate thermogenic activity within this tissue. The exact mechanisms that underpin sex steroid regulation of thermogenesis at muscle and adipose tissue remains to be elucidated and will be discussed below.

6.2 Skeletal Muscle is a Thermogenic Tissue

For many years, the idea that adult humans expressed active brown adipose tissue was controversial. A series of publications in the New England Journal of Medicine (Cypess *et al.*, 2009; van Marken Lichtenbelt, 2009; Virtanen *et al.*, 2009), however, unequivocally demonstrate that functional brown fat exists in adult humans. Since these initial papers a number of studies have clearly demonstrated that the activity of brown fat in humans is increased by cold exposure (Ouellet *et al.*, 2010; Saito *et al.*, 2009; Virtanen *et al.*, 2009; Yoneshiro *et al.*, 2010), reduced in obesity (Saito *et al.*, 2009) and is also influenced by

photoperiod and seasonal variation (Au-Yong *et al.*, 2009). This discovery has renewed interest in the potential role of thermogenic pathways in the battle against obesity.

Although it is well known that BAT is thermogenic, a number of studies have demonstrated that this tissue does not account for total thermogenic capacity (Astrup *et al.*, 1985; Simonsen, 1993). Skeletal muscle is highly metabolic, and is thought to be responsible for up to 70-85% of glucose disposal in man (Yki-Jarvinen *et al.*, 1987). Indeed, recent advances (Seale *et al.*, 2008; Kajimura *et al.*, 2009) have indicated that BAT and skeletal muscle have similar embryonic lineage. These tissues also share phenotypic similarities such as a high abundance of mitochondria. Skeletal muscle also expresses UCP3, a homologue of UCP1, and recent data in sheep suggest that UCP3 has a similar function in muscle to that of UCP1 in BAT (Henry *et al.*, 2011).

As previously discussed, this laboratory has demonstrated postprandial temperature excursions in skeletal muscle, which can be up-regulated by central infusion of leptin (Henry *et al.*, 2011; Henry *et al.*, 2008). Furthermore, icv leptin treatment increases UCP3 expression and increases uncoupled respiration in mitochondria isolated from skeletal muscle. Work presented in this thesis furthers our understanding of skeletal muscle thermogenesis. Investigation into the relationship between blood flow and muscle temperature yielded a number of important findings. Primarily, the feeding-induced increase in skeletal muscle heat production was independent of changes in blood flow. Furthermore, the model of meal anticipation clearly shows skeletal muscle temperature elevation in response to visual and olfactory stimulation in lieu of gastrointestinal/metabolic cues. This strongly suggests a central component to the initiation of tissue temperature regulation. In addition, skeletal muscle temperature increased in response to isoprenaline only at high doses, suggesting that this effect may be mediated via β3-AR activation, pointing to a role for the sympathetic nervous system to control heat production in skeletal muscle.

In sum, these studies support a growing body of work that suggests that that skeletal muscle is capable of thermogenesis.

6.3 Sex and Sex Steroids in the Regulation of Energy Balance

Sexual dimorphism of body composition is seen in many species, including humans. These sex differences highlight a possible governing role of the sex steroids testosterone and estrogen in the regulation of energy balance. Additionally, sex hormone-reduced states such as menopause and gonadectomy provide further evidence for the importance of sex steroids in combatting weight gain and obesity. Work presented in this thesis provides strong evidence that both testosterone and estrogen are able to modify manipulating energy expenditure through altered temperature output of peripheral tissues.

I have shown that feeding-induced temperature elevations in rams (chapter 4) are associated with increased expression of UCP1 and UCP3 in skeletal muscle. However, in ewes (chapter 3) feeding increases uncoupled respiration in mitochondria isolated from skeletal muscle but there is no associated change in uncoupling protein expression (unpublished data). This difference is unlikely due to the time of sampling as this was similar between the two studies. On the other hand, this may reflect sexual dimorphism in the response of skeletal muscle to feeding and this merits further investigation. Other studies have set a precedent for sexual dimorphism in the control of thermogenesis. BAT mitochondria have greater oxidative and thermogenic capacities in female mice compared to males. This is associated with greater UCP1 expression (Justo *et al.*, 2005). In rats, weight loss after a cafeteria diet is greater in females, predominantly due to increased BAT thermogenic capacity (Rodriguez *et al.*, 2001). In addition, human BAT screening has shown that a greater percentage of females than males express functional BAT (Cheng *et al.*, 2009; Cypess *et al.*, 2009). While these studies suggest females have a greater

capacity for adaptive thermogenesis in visceral fat, whether these differences translate to skeletal muscle thermogenesis is unknown. In mice, feeding a cafeteria diet induces larger UCP3 mRNA elevations in skeletal muscle of males compared to females (Rodríguez *et al.*, 2003). However, in this study, UCP3 mRNA levels correlated with being overweight in males, but not in females (Rodríguez *et al.*, 2003). In rats, females show a greater capacity for storing fat in adipose tissue and for oxidizing fatty acids in muscle when fed a high fat diet compared to males (Priego *et al.*, 2008).The data presented in chapter 4 of this thesis demonstrated sexual dimorphism in the metabolic response to testosterone, whereby testosterone treatment reduced heat production in skeletal muscle of males but not females. In addition, testosterone decreased postprandial plasma glucose levels in males but not females. Collectively, these studies suggest sexual divergence in a range of metabolic parameters. This may account for the changes in UCP1 and UCP3 expression in response to feeding observed in males but not females reported in this thesis. Further investigation into the sex differences in uncoupling protein expression and activity was outside the scope of this thesis and constitutes a significant area of research worth pursuing.

Pathways involved in the central regulation of food intake by sex steroids are well characterised. Sexual dimorphism of androgen and estrogen receptor distribution within the brain – and specifically the hypothalamus - contribute towards variation in food intake between sexes in response to a number of stimuli (reviewed in chapter 1). Sex steroids have also been shown to modulate the effects of other signalling factors – such as the possible effects estrogen has on leptin action as reviewed in chapter 1.6 – 1.8. Neuronal pathways involved in sex steroid-mediated regulation of thermogenesis have not been fully characterised. Silencing of the ERα gene within the ventromedial hypothalamus leads to hyperphagia and a reduction in thermogenesis resulting in obesity (Musatov *et al.*, 2007a). This study indicates that neuronal control of food intake and thermogenesis is tightly linked. However Xu *et al.* (Xu *et al.*, 2011a), recently demonstrated that deletion of ERα from SF-1 neurons of the VMN, attenuated BAT thermogenesis without changes in food intake, while deletion of ERα from pro-

opiomelanocortin (POMC) neurons of the arcuate nucleus increased food intake without an associated effect on BAT thermogenesis. Further insight into this area would have large implications on the work presented in this thesis.

6.4 Cellular Mechanisms of Thermogenesis

As outlined above, the sex steroids estrogen and testosterone can profoundly regulate skeletal muscle and adipose tissue temperature. It was hypothesised that estrogen and testosterone-induced temperature responses in adipose tissue and skeletal muscle were derived from uncoupling protein-mediated adaptive thermogenesis. To elucidate these points, the expression of UCP1, UCP2 and UCP3 mRNA was measured as a possible index of thermogenic capacity.

Acute estrogen treatment increased both skeletal muscle and visceral fat temperature without any change to UCP1 UCP2 or UCP3 mRNA expression by these tissues. Unpublished data from this laboratory shows that repeated acute-estrogen treatment similar to that outlined in chapter 5 had no effect on UCP1, UCP2 or UCP3 mRNA expression in muscle, despite marked temperature elevations. Additionally, chronic testosterone treatment decreased muscle and visceral fat temperature in males without an effect on UCP1, UCP2 and UCP3 mRNA expression. Similarly, the feeding induced rise in muscle temperature did not correspond with increased UCP expression. Although mRNA expression does not account for changes in protein expression or protein activity, the current data indicate that thermogenesis may be driven by UCP-independent cellular mechanisms.

Uncoupling protein mRNA and UCP3 protein expression in response to feeding, and sex steroid treatment was not able to comprehensively account for temperature fluctuations that were observed. While this laboratory has been able to show that uncoupling protein expression is not

always indicative of mitochondrial uncoupled respiration we cannot rule out other cellular pathways. As reviewed by Tseng *et al.* (Tseng *et al.*, 2010), cellular mechanisms other than mitochondrial uncoupling can increase heat production and one such pathways is futile calcium cycling. In skeletal muscle, activation of the ryanodine 1 receptor can allow for calcium (Ca^{2+}) influx into the cytoplasm from outside of the cell or from the sarcoplasmic reticulum. Increased cytoplasmic Ca^{2+} levels can lead to thermogenesis via two pathways: the hydrolysis of ATP during muscle contraction and relaxation; and the action of active Ca^{2+} channels to pump Ca^{2+} back into the sarcoplasmic reticulum – creating a futile cycle. Ryanodine receptor-mediated thermogenesis is involved in malignant hyperthermia (Stowell, 2008). The involvement of ryanodine receptor-mediated calcium cycling is currently being investigated by this laboratory. Initial data from these studies show that feeding leads to an elevation in RYR1 (ryanodine receptor) mRNA expression and SERCA2a (intracellular Ca^{2+} pump found on the SR membrane) protein expression in muscle. Understanding the cellular mechanisms involved in sex steroid-induced temperature changes in muscle and fat constitutes a future direction for this project. Investigating UCP-mediated thermogenesis as well as other pathways such as ryanodine receptor-mediated calcium cycling, could lead to novel target areas to fight obesity.

6.5 Concluding remarks

In conclusion, the results generated from the experiments presented in this thesis demonstrate that the sex steroids testosterone and estrogen differentially regulate numerous facets of energy expenditure in the sheep. Testosterone and estrogens had effects on tissue temperature. These changes may be due to altered adaptive thermogenesis, though UCP mRNA expression did not reflect this. The mechanisms involved in the temperature effects of sex steroids on peripheral tissues remain to be elucidated, providing a future direction for this work. Data presented in this thesis not only promote the notion of sex steroidal regulation of energy expenditure but also

support the notion that skeletal muscle is capable of adaptive thermogenesis in a model of diet-induced thermogenesis. It is anticipated that further research in the field could yield therapeutic treatment options against obesity and weight gain, specifically obesity related to menopause, aging, and sex hormone suppression.

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AMENDMENTS

The following addendum is a collection of the responses to reviewer comments containing issues requiring additional consideration. Within this section, reviewer comments are highlighted in bold. Additional references are listed after this section.

The errata section is a collection of typographical errors listed by page (p), paragraph (para) and line (ln) numbers.

ADDENDUM

It may be helpful to comment in Figure legends [Figures 3.2 and 3.3] that the * refers to significant differences between AUC of 5min windows prior to and following drug treatment - rather than just showing * P<0.05. At the very least, it should state compared to pre-drug treatment.
A detailed description the statistical analysis performed throughout chapter 3 is presented in chapter 3.3.6 – including the above mentioned details.

The effect of meal anticipation on muscle temperature is of interest – has this been studied in humans?
To the best of my knowledge, similar research into the effects of meal anticipation on skeletal muscle temperature has not been conducted in humans.

The discussion concludes that higher doses of isoprenaline activated B3 adrenoceptors, accounting for the effects seen on temperature - this could be tested with a B3 agonist.
Our hypothesis that β3 adrenoceptor activation leads to increases in tissue temperature could be further supported by the use of selective β3 adrenoceptor agonists such as CL-316,243 and LY-368,842. These additional experiments fell outside of the scope of this thesis but could constitute an area for future research.

No changes in UCP mRNA expression were observed [in chapter 3]- what about timing of tissue collection- is it possible this contributed to the lack of significant effects (in later chapters too)?
It is possible that the timeframe of tissue collection employed in this and subsequent chapters may have contributed to the lack of significant effects. However, two studies by this lab (Clarke *et al.* 2012; Henry *et al.* 2011) have similarly been unable to find an association between UCP mRNA expression and function. In fact, the work by Henry et al. (2011) has shown a switch towards uncoupled respiration in spite of no change in UCP mRNA content in skeletal muscle.

Were the mitochondrial changes alluded to on page 103 found at the same time relative to feeding [as results presented in Chapter 3]?
As detailed by Henry *et al*. (2011), a change towards uncoupled respiration was observed in isolated skeletal muscle mitochondria during the post-prandial period. Chapter 3 of this thesis and the work presented in Henry *et al.* (2011) employ the same animal model and same time frames.

In Figure 4.2 [p118], the boxes in panels C and D require better definition; presumably one is castrate and one testosterone.
Boxes in Figure 4.2 are presented as: closed boxes – castrate; open boxes – testosterone. This follows from the colour coding used in panels A and B, and is indicated in the figure key presented at the bottom of the figure.

[Chapter 5] It was not clear to me whether supraphysiological levels were being achieved at the dose used - the text refers to treatment and replacement interchangeably. Were the blood E levels achieved different in the single 50 ug injections versus repeated 25 ug ones?

Animals were treated with 3 implants of the same dimensions. Based on work by Legan et al. (1977) it was expected this would achieve plasma levels of approximately 16pg/ml. This reflects plasma levels of estradiol-17β that circulate throughout the late follicular phase of ewes Thomas et al. (1988). Given the size of the sheep used in these experiments, injections of 25μg would produce plasma levels of 6.25 ng/ml, comparable to the production rate per min in the late follicular phase of the cycle (Campbell *et al.*, 1990). Thus both doses are comparable to the late follicular phase of the ewe whereby the continuous implant reflects the mean levels of estradiol-17β and the single injection represents the estradiol-17β production rate. The single 50ug injection was delivered i.m. while the repeated 25ug injections were delivered i.v. It was expected that the difference in dose delivery methods, and the size of the doses would results in approximately similar circulating levels of estrogen.

[Chapter 5] The discussion attempts to place, but I found it rather confusing. E.g. the 2 sentences at the start of the last paragraph seem contradictory - profound effect of acute but not chronic E replacement on muscle and fat heat production; Repeated E injections – sustained increase in muscle heat production… Obviously I had trouble discerning the subtleties of all the different treatment modalities - perhaps a summary table ?

The main findings of chapter 5 are highlighted in the below table. Three treatment groups were investigated: single, acute injection of estrogen benzoate; chronic treatment (one week) with subcutaneous EB implants; repeated injection of acute EB (3 injections, three hours apart). Temperature effects of estrogen on tissue temperature were observed as a result of both acute, and repeated acute treatments, but not due to chronic treatment.

Treatment	Food Intake	FSH	Tissue Temp.
Acute	No change	No Change	Increased in muscle and visceral fat
Chronic	Decreased on days 1-4 of treatment	Decreased	No change
Repeated (acute) inj.	No change (data not shown)	Not investigated	Increased in muscle. Visceral fat not investigated

How was non-specific binding assessed and was a single band detected at the correct molecular weight?

Molecular weight markers were included in all gels to verify the molecular weight of any bands detected on the film. Non-specific binding was not an issue as no non-specific binding occurred near the molecular weight of any bands of interest. The UCP3 antibody did, however, produce significant non-specific binding, but we were able to isolate the correct band due to its position in relation to the molecular weight markers.

Also when measuring mitochondrial proteins it is the norm to isolate the mitochondrial for such assessments but this does not seem to have been undertaken. Also why was UCP1 analysis not undertaken along the lines used for UCP2 and 3?

Whole-tissue homogenate was used in all western blot analysis. As such, UCP1 protein concentration was too low to be detected via western blot. UCP1 expression in muscle tissue is particularly low – due to its localised expression in ectopic brown adipocytes found amongst the muscle fibres. However, UCP1 mRNA was assessed, also from skeletal muscle whole tissue-homogenate, with UCP1 levels shown to increase in the postprandial period in males but not females.

Chapter Three - Experiment 1: It appears that the tissue was taken from anaesthetized animals, did they show the same temperature changes as fully conscious animals and would this procedure compromise the interpretation of the tissue measurements made?
Anaesthesia was given immediately prior to tissue collection after the postprandial thermogenic response had been established. These animals displayed a similar postprandial temperature response compared to fully conscious animals. Temperature recordings were typically conducted on the day preceding tissue collection so that tissue collection would not confound temperature recordings.

Chapter Four – Experiment 2: It appears here that muscle biopsies were taken under local anesthesia rather than general anesthesia as described in Chapter 3. Does this affect the interpretation of the results? In contrast adipose tissue was sampled at autopsy but a similar method was not used for skeletal muscle at this time, when it would be useful to validate the sampling technique.
During the time this thesis was completed, the tissue collection method was continually refined to increase efficiency of tissue collection and to decrease the level of distress felt by animals. No obvious differences were observed in tissue collected under the different sampling techniques.

Where is the mRNA data for females? Would it have been useful to include a group of intact males and females in order to establish that the changes in muscle temperature are thus similar to those seen in the hormone supplemented groups?
As females had no detectable response (temperature, metabolites) to testosterone treatment, mRNA was not measured. While it may have been useful to use intact males and females as a further control group, this would also have introduced unwanted variables. In particular, intact females would cycle – having fluctuating levels of estrogen – which may effect thermogenesis. Intact males would be considerably larger than wethers, which could lead to problems in comparing the two.

Chapter 5 – Experiment 3: Can you be certain that a local rise in tissue temperature equates to increased heat production? It may be a very localized change in blood flow that perhaps could be further examined using microspheres? Also how does the site of tissue sampling for gene expression analysis relate to the actual site in which changes in temperature were made?
These experiments were carried out in animals in which we had measured local perfusion to verify that local changes reflect femoral blood flow – see chapter 3. Tissue collection and temperature recording occurred in the same tissues types and, in the case of skeletal muscle, the same muscle, *vastus lateralis*.

ADDITIONAL REFERENCES:

Campbell, B. K., Mann, G. E., Mcneilly, A. S. & Baird, D. T. (1990). Pulsatile secretion of inhibin, oestradiol and androstenedione by the ovary of the sheep during the oestrous cycle. *J Endocrinol,* **126,** 385-93.

Legan, S. J., Karsch, F. J. & Foster, D. L. (1977). The endocrin control of seasonal reproductive function in the ewe: a marked change in response to the negative feedback action of estradiol on luteinizing hormone secretion. *Endocrinology,* **101,** 818-24.

Thomas, G. B., Martin, G. B., Ford, J. R., Moore, P. M., Campbell, B. K. & Lindsay, D. R. (1988). Secretion of LH, FSH and oestradiol-17 beta during the follicular phase of the oestrous cycle in the ewe. *Aust J Biol Sci,* **41,** 303-8.

ERRATA

p 7, para 1, ln 2: change “weather” to “whether”

p 12, para 2, ln 7: change “aides” to “aids”; ln 8: change “be” to “by”

p 23, para 1, ln 6: change “expresses” to “express”

p 33: Figure legend truncated due to formatting error. It should read as: “**Figure 1.5:** Schematic diagram of brown adipose tissue activation by adrenergic stimulation. Activation of the β3-ADR by the sympathetic nervous system results in the activation of protein kinase A (PKA). This leads to transcriptional activation of PGC-1 and DII expression. PGC-1 has several downstream effects including 1) co-activation of transcription factors assembled on the UCP1 enhancer region, resulting in increased UCP1 expression, and 2) activation of mitochondrial biogenesis by the co-activation of transcription factor NRF-1. DII increased T3 synthesis, which activates the thyroid receptor, promoting further UCP1 synthesis. Figure adapted from (Lowell and Spiegelman, 2000)”

p 38, para 3, ln 4: remove “during cold exposure in rats”

p 44, para 3, ln 1: change thee to three

p 60, para 3, ln 1: change demonstrat to demonstrate

p 73, para 1, ln 3: add “were” between “concentrations” and “determined”; **para 3, ln 4:** change “wester” to “western”

p 74, para 2, ln 4: change “protein” to “proteins”; ln 9: change “Ryd” to “Ryde”

p 77, para 1, ln 11: change “pallet” to “pellet”

p 83, para 3 ln 8: change “1 hour of” to “1 hour after”

p 84, para 1 ln 3: change “cutaeous” to “cutaneous”

p 95, figure key: change “meal antipation” to “meal anticipation”

p 107, para 2, ln 4: delete “is”

p 108, para 1, ln 5: change “warrented to “warranted”

p 111, para 3, ln 1: change “25-ml” to “25µl”

p 112 – 131: Figures throughout chapter 4 are incorrectly labelled as Figure 5.x etc. These should read Figure 4.x.

p 123, 4.3.3 Title: change “melanocyte” to “melanocyte”

p 131, para 1, ln 9: change “oberve” to “observe”

p 132, para 1, ln 3: Reference “Brown and Clegg” was published in 2010, “Mauvais-Jarvis *et al.*” in 2011.

p 133, para 2, ln 2: Refence “Butera and Czaja” was published in 1984.

p 134, para3, ln 1: change “+” to “±”

p 157, para 1, ln 13: change “andrenergic” to “adrenergic”

p 160, para 1, ln 6: remove “manipulating”

p 163, para 1, ln 3: change “pathways” to “pathway”