Cortisol Responsivity to ACTH Marks Innate Predisposition to Obesity, Changes in Energy Homeostasis in response to Stress & Coping Behaviours.

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Summary

Overcoming the innate determinants of body weight in the overweight and obese person is not easy in today's obesogenic environment. Despite strong public awareness of what a healthy lifestyle entails, the obesity epidemic continues to worsen. The difficulty lies in an individual not being able to sustain the effort required to maintain a lower body weight leading to a return to a higher natural setpoint.

In this Thesis, I present a novel way of viewing this growing epidemic by evaluating stress responsiveness as a way to mark the innate determinants of weight gain. Stress plays an instrumental part in regulating our desire for food and sex, or in physiological terms, energy homeostasis and reproduction. Stress responsiveness can be determined by measuring the stress hormone, cortisol. In the animal model used in the present studies, I have identified high stress responders (HR) and low stress responders (LR) from a population of outbred sheep by injecting Synacthen (synthetic adrenocorticotropin –ACTH) and selecting those animals at either extreme in terms of cortisol response.

In the first study, it was shown that HR have a greater tendency to become obese when exposed to a high energy diet compared with LR and this was found to be due to a greater positive energy balance in

HR. This greater positive energy balance was not a result of increased food intake but rather a lower thermogenic output from muscle. The characterstics of these animals in terms of leptin sensitivity, insulin sensitivity were also detailed showing no difference between HR and LR groups. Finally, the study included measures of muscle thermogenesis in terms of mitochondrial respiration and futile calcium cycling, demonstrating that though increased futile calcium cycling and mitochondrial respiration can explain the mechanism of postprandial thermogenesis, neither of the mechanisms adequately explained the difference observed between HR and LR groups.

In the second study, it was shown that energy homeostasis can be marked by cortisol responsiveness to stress in HR and LR. In other words, the greater the difference in cortisol response to particular stressors, the greater the difference in the metabolic response to stress in the HR and LR in terms of food intake and thermogenic output. Three stressors were used to demonstrate this relationship, namely metabolic stress in the form of insulin induced hypoglycaemia, psychosocial stress in the form of a barking dog and immune stress in the form of lipopolysaccharide (LPS) infusion.

Of the three stressors, the greatest divergence in cortisol response between HR and LR was seen with LPS immune stress where LPS treatment reduced ($P<0.01$) food intake in both groups, but LR showed a greater ($P<0.05$) reduction in food intake and a more substantial ($P<0.05$) rise in muscle temperature. Introduction of the barking dog demonstrated differences in peak cortisol and reduced ($P<0.05$) food intake in LR only; there was no significant difference in

thermogenic output. In metabolic stress, no difference was seen in cortisol response between HR and LR and there was also no difference in food intake and thermogenic output between the two groups. Thus, LR animals typically displayed a greater catabolic response to stress than HR.

Finally, I characterized behavioural differences in HR and LR animals. Cortisol responsiveness was also shown to mark an innate difference in coping styles to stress. By using three different behavioural tests, it was shown that LR have greater activity as well as more initiative and less fearfulness in response to stress than HR. Thus, LR may be considered to have a proactive coping style, whereas HR exhibit a relatively reactive series of coping strategies.

In conclusion, cortisol responsiveness can predict the susceptibility for obesity in sheep fed a high energy diet, predict changes to energy balance in relation to stressors and also predict coping behavioural strategies. The teleological implication and potential of these findings is to provide novel insight into mechanisms underlying propensity for obesity and there is an indication that Synacthen testing may be useful as a predictor of subsequent metabolic outcome.

Declaration

I hereby declare that to the best of my knowledge, this thesis contains no material 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 Monash University Animal Welfare Committee.

Tao-kwang Kevin Lee

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Abbreviations, Units of Measure and Symbols

ADP	Adenosine diphosphate
AgRP	Agouti related protein
AICAR	5-Aminoimidazole-4-carboxamide ribotide
AMP	Adenosine monophosphate
ARC	Arcuate nucleus
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
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine
CT	Computed axial tomography [scan]
CCK	Cholecystokinin
cDNA	Complimentary DNA
CoA	Coenzyme A
CRH	Corticotropin releasing hormone
CVD	Cardiovascular disease
DEXA	Dual energy X-ray absorptiometry
DMH	Dorsomedial hypothalamus
DNA	deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDGPET	Fludeoxyglucose
FSH	Follicle stimulating hormone
GDP	Guanosine diphosphate
GLUT	Glucose transporter type
GR	Glucocorticoid Receptor
H^+	Proton
HK	Housekeeping
HPA	Hypothalamic-Pituitary-Adrenal
IL	Interleukin
i.v.	Intravenous
LHA	Lateral hypothalamic area
LPS	Lipopolysaccharide
MCH	Melanin-concentrating hormone
MCR	Melanocortin receptor
MDH1	Malate Dehydrogenase
MR	Mineralocorticoid Receptor
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
O_2	Oxygen
OVX	Ovariectomised
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction

PET	Positron emission tomography
P_i	Inorganic phosphate
PKA	Protein kinase A
POMC	Proopiomelanocortin
PPARγ	Peroxisome proliferator-activated receptor gamma
PVN	Paraventricular nucleus
PSS	Porcine Stress Syndrome
ROS	Reactive oxygen species
RT-PCR	Real time Polymerase chain reaction
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate
SEM	Standard error (of the) mean
SERCA	Sarcoendoplasmic Reticulum Calcium ATPase
SR	Sarcoplasmic Reticulum
T_3	3,5,3'-triiodothyronine
UCP	Uncoupling protein
VMN	Ventromedial nucleus
WAT	White adipose tissue
α-MSH	Alpha melanocyte stimulating hormone
11βHSD	11-beta-hydroxysteroid dehydrogenase

Sympbols

α	Alpha
β	Beta
γ	Gamma
%	Percent
±	Plus or minus
Δ	Delta (change in)
P	P-value

Units of Measure

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

Publications Arising from PhD

Clarke S. D., Lee K., Andrews Z. B., Fahri F., Evans R.G., Clarke I. J. and Henry B.A. (2012) Postprandial Heat Production in Skeletal Muscle is Associated with Altered Mitchondrial Function and Futile Calcium Cycling. American Journal of Physiology 303(10):R1071-9.

T. Kevin Lee, Iain J. Clarke, Justin St John, I. Ross Young, Brian L. Leury, Alexandra Rao, Zane B. Andrews, and Belinda A. Henry. (2014) High Cortisol Responses Identify Propensity for Obesity that is Linked to Thermogenesis in Skeletal Muscle. FASEB Journal, 13: 238345.

T. Kevin Lee, Iain J. Clarke, Gavin Lambert, Robert Bischof, Caroline Lee and Belinda A Henry (2014) Stress-induced Behavioural and Metabolic Adaptations Lead to an Obesity-prone Phenotype in Individuals with Elevated Cortisol Responses. Psychoneuroendocrinology, 47: 166-177.

Conference Presentations

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Lee, T. K., Clarke, I. J., Henry, B. A. Skeletal Muscle Thermogenesis Explains the Propensity to Obesity in High Cortisol Responders. Poster Presentation at Victorian Obesity Consortium (2012), Melbourne, Australia.

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Awards

Winner of Endocrine Society of Australia Novartis Young Scientist of the Year Award, 2013.

Winner of Research Establishment Grant by Diabetes Australia and Australasian Royal College of Physicians. 2013.

Chapter 1:
Introduction

The Obesity Epidemic

The incidence of obesity is rapidly escalating and projection analyses from the AusDiab study indicate that the number of obese subjects is likely to exceed 65% within the adult population of Australia by 2025[1]. Obesity is a strong predictor of mortality both directly and indirectly through its association with metabolic syndrome as well as its links to certain cancers and infections like cellulitis and pneumonia[2]. Whilst there has been considerable emphasis placed upon population approaches to tackle the issue, which have addressed the aetiological contributors including lifestyle and affluence, it is quite clear that not all who live in affluent and calorically plentiful first world countries succumb to the epidemic and even those who do succumb do so to varying extent.

Animal and twin studies show that around 40% of variablility in human body weight may be due to genetic factors[3]. It is the hope that the elucidation of these genetic factors will allow us to treat obesity in a better way and to fully understand one of the most fundamental of biological processes for survival, namely food intake and energy expenditure. A complete understanding of the etiology of obesity, from a molecular standpoint, is very complex. This involves a thorough knowledge of the neuroendocrinology of appetite as well as the biochemistry of metabolism. The role that genetics and epigenetics play in the determinants of a natural set point in these various parameters and body weight, has become critical if we are to combat the problem.

Obesity leads to sequalae synonymous with the metabolic syndrome, which shares many of its characteristics with hypercortisolemic conditions such as Cushing's syndrome[4]. In both conditions, elevated circulating levels of cortisol are displayed, with associated abdominal adiposity, hypercholesterolaemia, hypertension, hyperuricaemia and hyperglycaemia[5]. Epidemiological data indicates a role of psychosocial stress in weight gain[6] and emerging data have revealed how

hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is linked to patients with obesity and metabolic syndrome. Obesity in animals such as sheep[7] and in humans[8] is similarly associated with increased stress-responsiveness or exacerbated secretion of cortisol in response to a stressor. Remarkably, this is the case, even though the sheep is a ruminant. To date, however, whether increased stress-responsiveness results from the obese phenotype or whether stress-responsiveness can be causally linked to the manifestation of obesity remains a topic of debate.

The Hypothalamo-Pituitary Adrenal Axis (HPA)

The HPA axis is driven by the production of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) in the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN). The combined action of CRH and AVP stimulate the pituitary corticotrophs to release of peptides derived from pro-opiomelanocortin (POMC) like the opioid peptide β-endorphin, α-melanocyte stimulating hormone and adrenocorticotropin (ACTH). ACTH is released into the circulation and acts at the melanocortin 2 receptors in the adrenal gland to stimulate glucocorticoid secretion. In sheep and humans the predominant glucocorticoid is cortisol whereas, in rodents, it is corticosterone[9].

Cortisol, released by the adrenal glands, acts via the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Cortisol can, however, be rendered inactive by conversion into cortisone by peripheral tissues through the action of the enzyme 11beta-hydroxysteroid dehydrogenase type II (11βHSD type 2). This enzyme is predominantly found in the kidney and prevents the weak mineralocorticoid receptor agonism, which can lead to a hypertensive state as seen in ectopic ACTH syndrome. The inactive form of glucocorticoid, cortisone, can also be converted back to cortisol by 11βHSD type I, found predominantly in the liver but also in adipose tissue[10]; an increase in active glucocorticoid correlates with increased 11βHSD type I activity in adipose tissue [11].

As mentioned earlier, glucocorticoids act via binding to two types of intracellular nuclear receptors, the GR and the MR[12]. Upon binding, the receptors translocate to the nucleus where they act as transcription factors for specific target genes. Therefore, glucocorticoid responsiveness can be regulated by GR coactivators and corepressors[13], GR polymorphisms[14], splice variants and isoforms[15, 16], as well as the regulation of glucocorticoid retrograde transport to the nucleus[17] and most recently, micro-RNAs that involve non-coding RNA stabilizing and influencing translation of mRNA, specifically microRNA 18 and 124a which down regulate the GR[18].

As with all endocrine systems, the HPA axis is a closed loop. Thus, negative feedback by cortisol regulates CRH, AVP and ACTH production and secretion. The secretion of these hormones is pulsatile at all levels, with CRH/AVP pulses secreted into the hypophysial portal system driving pulsatile secretion of ACTH from the pituitary corticotropes[19]. The pulsatile secretion of the hypothalamic hormones is modulated by a wide range of neural factors as evidenced by studies quantifying the effects of intracerebroventricular adminstration of norepinephrine or the hunger hormone, neuropeptide Y, both of which have been shown to elicit acute and sustained increases in mean plama ACTH and cortisol[19]. This effect was not seen in cultured anterior pituitary cells exposed to NPY or norepinephrine, suggesting that upregulation of the HPA axis by these factors is mediated via central pathways.

As in all hormonal axes, the HPA axis is also subject to diurnal variation. In humans, HPA activity peaks in the early hours of the morning around 30 minutes after awakening followed by a trough in the late evening. Changes in this diurnal variation have been demonstrated in large cohort studies like the Whitehall II study when sleep disturbances are encountered[20] and also attributable to the development of Type 2 Diabetes[21]. Interestingly, cortisone, the inactive form of cortisol, circulates at 60nmol/L, showing little diurnal variation[22]. In addition to circadian rhythms, there is also a minute to minute fluctuation in plasma cortisol

concentrations that can be characterized using frequent blood sampling regimens. If blood is sampled every 10 minutes, pulses of cortisol (approximately 19 per day) varying in amplitude and duration are seen in humans (Figure 1)[23].

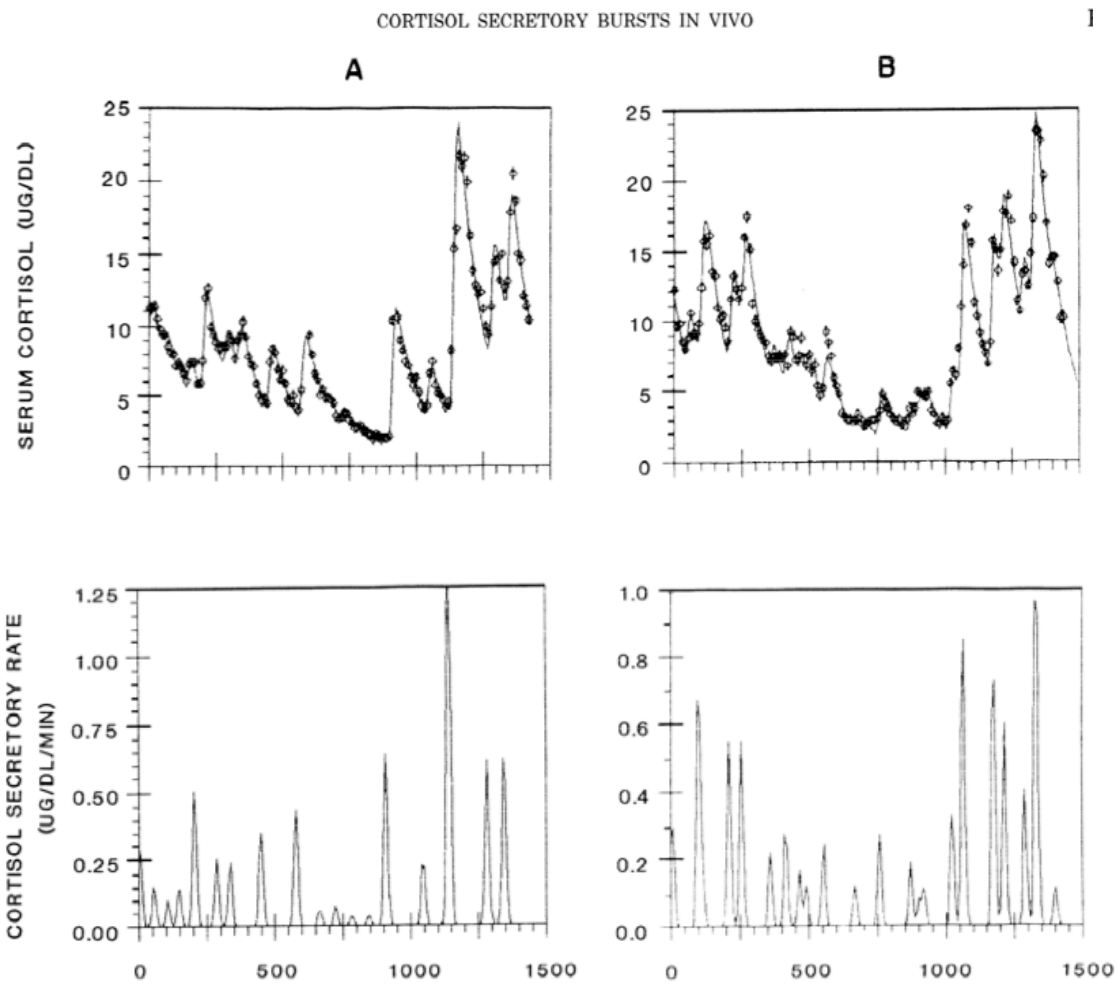

Figure 1 Circadian patterns of cortisol secretion. The upper two panels show the circadian pattern of serum cortisol concentrations. The lower panels demonstrate secretory bursts of cortisol production after deconvolution analyses revealing the ultradian pattern (Windle et al., 1998)[24].

The HPA axis is also regulated at all levels by hormones from other endocrine systems. The physiological changes that occur may be secondary to circadian clock disruptions of a number of hormones, not just cortisol alone, and has strong implication for obesity as reviewed by Froy[25]. Of particular interest are two of the most pertinent metabolic regulators, leptin and insulin.

Insulin is an anabolic hormone secreted from pancreatic beta islet cells. Glucocorticoids stimulate the secretion of insulin and thus promote peripheral tissue glucose utilization. Insulin also acts centrally to reduce food intake, specifically targeting the neuropeptide Y (NPY) cells of the hypothalamic arcuate nucleus (ARC) [26]. In rats, insulin promotes a preference for a fatty macronutrient selection over standard chow[27]. La Fleur et al. studied rats in which diabetes was induced by streptozocin treatment and found a dose-dependent relationship of fatty macronutrient selection over standard chow with insulin re-administration[28]. The mechanism by which this occurs is not yet known, but there is a growing awareness of how orexigenic and anorexigenic factors are able to modulate sweet or fatty taste sensitivity by acting directly on taste receptors and taste neurons[29] which will not be reviewed here.

Leptin is produced by white adipocytes and is secreted into the bloodstream to act on the brain to reduce food intake and increase thermogenesis via activation of the sympathetic nervous system[30]. Leptin can also regulate the the HPA axis, although studies that address this interaction are somewhat conflicted. In mice, Heiman et al. [31] found that intraperitoneal injection of leptin led to inhibition of the HPA axis in animals exposed to restraint stress, whereas Van Dijk et al.[32] reported a stimulation of the HPA axis when leptin was given intracerebroventricularly. It may be that the basal or stress state of the organism is important in understanding the relationship between leptin and the HPA axis. Studies of leptin deficient humans show that after 6 months of leptin replacement, higher 24h mean cortisols levels occur, which is associated with fewer pulses but greater peak height of individual secretory episodes of cortisol secretion; thus treatment with leptin restores the ultradian pattern of cortisol secretion[33]. The relationship between leptin and the HPA axis is further complicated, as there is a reciprocal relationship

where cortisol is also able to regulate leptin production. Laferrere et al. proposed that cortisol actually drives the diurnal pattern of leptin secretion, which peaks late in the evening[34]. Thus, treatment with metyrapone, an inhibitor of cortisol production, blunted the meal-entrained rise in serum leptin levels. Accordingly, it might be considered that there is a 'chicken-and-egg' relationship between the leptin system and the HPA axis, such that one modulates the other and vice versa.

A similar reciprocal relationship is also seen between the HPA axis and the hypothalamic-pituitary-gonadal (HPG) axis with stress-related hormones generally suppress pulsatile release of GnRH from the hypothalamus and pituitary LH secretion with lesser direct effect on the gonads [35]. On the other hand, circulating gonadal steroids have mixed effects on the HPA axis, with differential effects of male or female steroids. In ewes, an enhanced cortisol response to synthetic ACTH is seen in gonadally intact females while suppression of the HPA axis is seen in gonadally intact males[36]. Interestingly, oxytocin, another hormone related to reproduction that is produced in the cells of the PVN, is also able to modulate HPA activity by attenuating response of cortisol to ACTH and also reducing anxiety behaviour[37].

Stress Responsiveness and Obesity

The HPA axis is responsive to a variety of stressors, that can be metabolic, psychological or physical[38] and often, the communication between stressor and the HPA axis is bi-directional. An example of this can be found in the response to an immune challenge, such as administration of bacterial endotoxin. This elicits the release of pro-inflammatory cytokines that subsequently activate the HPA axis[39]. The HPA axis then dampens the immune response by increasing glucocorticoid secretion from the adrenal gland to effect an anti-inflammatory response. Accordingly, it is possible to identify high and low cortisol responders based on immune challenge with lipopolysaccharide (LPS)[40]. The 'set-point' of the HPA axis is maintained throughout life - high responders (HR) and low responders (LR)

displayed the disparate stress responses across their lifespan in rodents[41]. The mechanism by which this was mediated did not appear to be related to cytokine levels, since plasma concentrations of IL-1 and IL-6 measured four hours after stimulating the HPA axis were not different between the groups. This disparity was substantiated by microarray analyses of the two groups (LR and HR) of hepatic tissues from the sheep after they had been treated with LPS. The authors speculated and showed in a subsequent paper that differences in genetic and epigenetic influences contribute to HPA responsiveness[42] which led them to conclude that the level of cortisol response to LPS was maintained throughout life and that the same groupings of responsiveness, be that HR or LR, was observable with CRH, AVP or ACTH challenge.

Another way to examine the function of the HPA axis is to employ the dexamethasone suppression test. Dexamethasone is a synthetic glucocorticoid that preferentially binds to the Type II glucocorticoid receptors (GR)[43]. In a clinical setting, dexamethasone suppression testing (DST) is commonly used in the diagnosis of over active HPA function as in disease states like Cushing's Disease and variable suppressability of cortisol levels with administration of 0.25mg dexamethasone has been found to be associated with certain polymorphisms of GR. Interestingly, polymorphisms in GR that associated with greater glucocorticoid sensitivity were linked to increased BMI[44]. In fact, a meta-analysis indicates that non-suppression of cortisol levels with serial measurements the day following an overnight dose of 1mg dexamethasone is predictive of suicidality[45]. Jokinen and Nordstrom showed that there was an excellent negative predictive value for elderly mood-disordered inpatients of 100% and a positive predictive value of 71% if a 1600h cortisol measurement of less than 8.7mg/dl cutoff was used[46]. In other words, those that had a cortisol value below 8.7mg/dl had a 100% non-suicide rate and those that had a value above 8.7mg/dl had a 71% likelihood of commiting suicide. As for the cortisol stimulation tests, higher non-suppression of the dexamethasone suppression test is seen in women than men[47] meaning that the evaluation of the HPA axis whether it be by stimulation testing or suppression testing is consistent with sexual dimorphism in the control of the HPA axis.

In terms of understanding the role of the HPA axis and stress, the dexamethasone suppression test is not as well studied as the stimulation test. The degree of cortisol responsiveness to stress has been well characterized and is beyond the scope of the introduction here, but this has been shown to be influenced by many factors including gender[48], seasonality (eg sheep[36]), lactation[49], pregnancy[50] as well as the stage of the estrous cycle[51]. In humans, chronic use of ecstasy or 3,4-methylenedioxymethamphetamine has been shown to attenuate stress reactivity in terms of the cortisol response[52] as well as lower baseline cortisol levels[53] compared to controls[54]. This demonstrates that many factors need to be taken into account in terms of the heterogeneity of subjects when studying the HPA axis and stress responsiveness.

Another major determinant of the stress response is the degree of adiposity[55]. ACTH and cortisol levels that are seen in response to isolation restraint stress are higher in obese sheep than in lean calorie restricted sheep[7]. Similar results are obtained in humans, with overweight individuals exhibiting an exaggerated cortisol response (compared to lean individuals) in response to a mental challenge[56] or a physical stressor such as cold exposure[57]. On the other hand, it has been suggested that chronic stress can lead to increased adiposity. The proponents of stress-induced obesity have postulated several mechanisms and recently a number of theories have been developed, the most prominent being the 'comfort food' hypothesis. This states that intake of foods high in fat and sugar is a maladaptive response to stress; increased fat and sugar intake has been shown to blunt the neuroendocrine stress response[58]. In humans, stress or increased levels of glucocorticoids is associated with higher calorie intake with the exception of a small number of subjects (15%) who display reduced food intake in response to stress[6]. One issue with this hypothesis is that species differences exist. In rodents, for example, stress or exogenous administration of corticosterone has been shown to reduce food intake with a subsequent reduction in body weight[59]. Generally, however, it is important to note that glucocorticoid-treated rodents exhibit increased levels of adiposity compared to pair-fed controls, suggesting that factors other than food intake are important in glucocorticoid-mediated changes in

adiposity. In contrast to rodents, chronic elevation in cortisol in sheep leads to an increase in food intake consistent with observations in humans[60].

Another clue that the HPA axis is important in the etiology of obesity is the observed difference in response to ACTH challenge found in women with abdominal obesity as opposed to peripheral obesity[61]. Epidemiological studies have shown that fat distribution in the abdominal region increases the risk factors for cardiovascular and cerebrovascular disease as well as diabetes[62]. In these studies, the women had similar body mass index, but the waist-to-hip ratio was different. Baseline cortisol levels were similar between the two groups, but those with abdominal obesity (increase waist-to-hip ratio) showed an exacerbated cortisol response to ACTH. In another study, Duclos et al.[63] made similar findings, showing that patients with increased abdominal obesity exhibited attenuated suppression of cortisol when given dexamethasone after taking into account the effects of BMI.. However, very few studies to date have attempted to determine the direct causality between obesity and HPA axis over-activity. In otherwords, it remains unknown as to whether the increased HPA axis response causes the obese phenotype to emerge or the obese phenotype leads to increased HPA axis response. To address this would require prospective data partitioning HPA axis differences and observing for phenotypic changes over time.

The earliest human trials that correlated stress responsivity to eating behavior were those of Epel et al.[64] Here it was shown that subjects with high cortisol response to psychological stress consumed more calories and ate significantly more sweet food after the stressful episode. This study included fifty-nine healthy premenopausal women; to control for the effect of the menstrual cycle (ovarian steroids) on appetite women were selected to be within the first five days of the follicular stage of their menstrual cycle. Cortisol reactivity was assessed by median split of AUC, categorizing women as high reactors, above the median or low cortisol reactors, below the median after psychological stress (Trier social stress test). Interestingly, food intake was similar between both groups on non-stress (control) days but diverged only after stress where high cortisol responders ate more compared to the control period and low cortisol

responders ate less after the Trier social stress test. Thus, low cortisol responders tended to reduce food intake in response to stress compared to the high cortisol responders. To test whether this relationship was maintained during daily life stress outside the laboratory, Newman et al.[65] correlated a daily hassles questionaire and snack intake diary to the cortisol responsiveness. The authors found a consistent association with increased snack intake in high cortisol responders than in low cortisol responders. More recently, George et al.[66] demonstrated that CRH stimulation could increase food intake, relative to placebo treatment, and that the higher the peak cortisol response to CRH, the greater the caloric consumption. In this study, fourteen non-obese subjects (8 females, 6 males) were stimulated with CRH at 1500h, followed by blood sampling (for cortisol measurements) and food was offered at 1730h. Once again, subjects were blinded to the fact that intake was monitored and a variety of snacks were offered, including salty and sweet high fat snacks as well as salty and sweet low fat snacks. These studies attempted to show prospectively that high stress reactivity leads to increased caloric intake but they lacked morphometric data with no follow up on weight or adiposity.

The only study that has included morphometric data is that of Roberts et al. [67]. In this case, 71 healthy women volunteers enrolled in a university based nurse practitioner program. Salivary cotisol measurements were taken at 2-hourly intervals from 8am to 8pm prior to and at week 12 of the study semester. Those individuals with a greater increase in salivary cortisol concentration after the end of year examination period had a greater BMI increase over the study period. In terms of body weight, those with the greatest increase in cortisol levels averaged a weight gain of 5 pounds greater than those who did not display an increase in cortisol at the end of follow-up. The initial BMI and initial cortisol level (as measured by area under the curve-AUC) was similar in the two groups. Aside from being a longer term prospective longitudinal study, one of the strengths of this study was that the stressor used was in the context of an exam and therefore has high societal validity. Nevertheless, it is uncertain whether the increase in cortisol levels was a direct result of weight gain over the 12 week period or whether there was an intrinsically higher cortisol responsiveness in the group that gained weight. Ideally, the study would have included a stressor at the outset of the investigation, allowing the ranking of the individuals in terms of innate differences in cortisol responses. This could have then been correlated with morphometric assessment at the end of the study with another stress responsiveness assessment. This study, like all

previous human studies detailing the effects of stress on body weight, examined food intake but did not quantify energy expenditure, which is a vital half of the energy balance equation.

Recent work in sheep has attempted to address the causal relationship further between stress responsiveness to ACTH (synacthen) and obesity. Knott et al.[68] measured feeding behaviour in 52 cross-bred rams and studied the relationship between cortisol response after an ACTH challenge to residual food intake (RFI), feed efficiency and adiposity. Residual food intake, or RFI, is a measure of how efficiently an animal utilizes feed for growth and is the difference between an animal's actual intake and its expected intake based on its weight and growth rate over a specified period of time. If an animal eats more than the amount predicted for maintenance, it is classified as having a high RFI, which is a low feed efficiency. Knott et al. found that the rams with high cortisol responses had high RFI. Using dual energy x-ray absorptiometry (DXA) before and after a 62 day period of feeding, the higher cortisol response was also found to be positively correlated with adiposity and negatively correlated with lean mass gain. The fatter animals were less 'feed–efficient' and these were the animals that were characterized as high cortisol responders. In a subsequent paper, Knott et al.[69] examined the role of stress in the same rams. The animals were challenged by insulin-induced hypoglycaemia and those with high cortisol response to synacthen also had a high cortisol response to the metabolic stressor[68]. In short, the high responders were consistently high responders, whether in terms of response to Synacthen or response to stress and they also consumed food beyond the predicted requirement. This indicated that these animals were prone to overeating and that inherent predisposition toward high cortisol response to synacthen may mark weight gain across time in terms of adiposity. As with the human studies discussed above, there were no attempts to quantify energy expenditure in these animals.

The question can be posed as to whether cortisol responsiveness is directly causative of adiposity or merely a marker of adiposity. The answer is likely to be that both pertain, with the former being useful in a predictive sense, however,

abberations in cortisol responses are likely to be exacerbated by obesity. Cortisol responsiveness marks the hormonal milieu of an organism, a composite of the many hormones and possible non-hormonal factors that influence how much cortisol is secreted when the adrenal glands are stimulated with ACTH. The adrenal glands show a greater cortisol secretion with the presence of noradrenaline or adrenaline, leptin, cytokines, whereas responsiveness is attenuated in the presence of hormones like testosterone, prolactin and oxytocin[70]. Although it was mentioned earlier that the phenotype of Cushing's syndrome and obesity was similar to that of individuals with high cortisol responsiveness, the overall direct contribution of a functional hypercortisolaemia to obesity is likely to be only one factor in the metabolic equation. Perturbations in the levels of other hormones are also known to contribute to the development of obesity and metabolic syndrome[71, 72].

In summary, cortisol responsiveness to a defined hormonal stimulus (Synacthen) or to stress has been proposed as a marker of the susceptibility to become obese as well as a marker of the obese state. To date, however, there is no study that has directly addressed this. Furthermore, to my knowledge, there has been no published information that relates stress responsiveness to energy expenditure in either animal models or humans[64, 65, 68]. Furthermore, studies to date consistently correlate increased stress responsiveness with increased caloric intake, with no prospective studies showing increased adiposity in humans [27, 65, 66, 73].

Energy Expenditure

The metabolic state of an individual is dictated by the balance between the energy consumed and the energy expended. When the latter exceeds the former, obesity ensues. Thus weight gain results when energy expenditure is inappropriately low for the caloric intake of an individual. There has been significant focus on ways to control energy consumption as a means of controlling the obesity epidemic, but the role of energy expenditure has been less well studied.

Total daily energy expenditure consists of basal metabolic rate (BMR), adaptive thermogenesis which includes (1) thermic effect of food (TEF) or food induced thermogenesis and (2) cold induced thermogenesis and finally the energy expenditure of activity (EEA). In humans and rodents, the BMR is thought to account for over half of energy expenditure, with 15 - 20% from adaptive thermogenesis and the remainder by the EEA[74]. EEA can be broken down in humans to non-exercise thermogenesis (NEAT) and exercise activity thermogenesis (EAT). EAT is more relevant to humans as it implies a degree of consciousness and volition, as opposed to the spontaneous nature of non-exercise activity, sometimes termed spontaneous physical activity or SPA (Figure 2)[75].

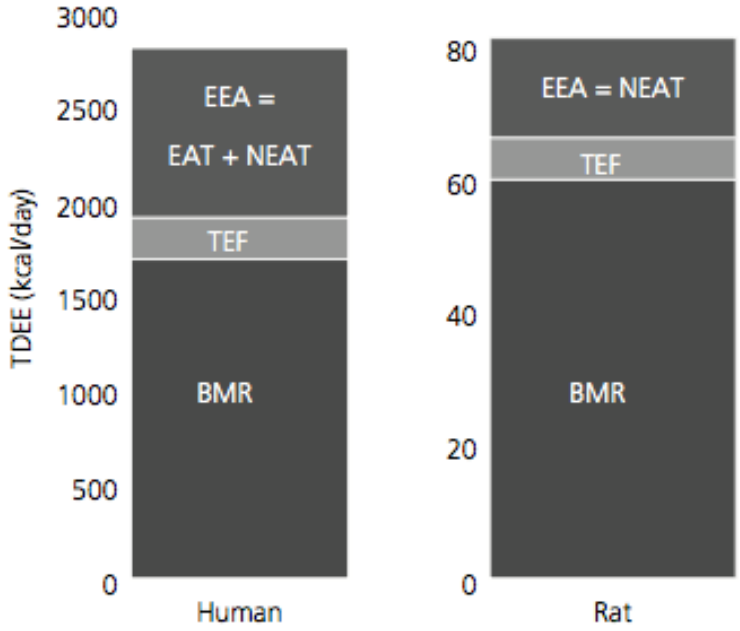

Figure 2 Comparison of total daily energy expenditure (TDEE) in the human and the rat, TDEE being the sum of basal metabolic rate (BMR), adaptive thermogenesis represented in this diagram as thermogenic effect of food (TEF) and energy expenditure of activity (EEA) which is comprised of exercise (EAT) and non-exercise activity thermogenesis (NEAT). (Adapted from Novak & Levine, 2007)[75].

There is some controversy regarding the extent to which each component of energy expenditure contributes to the pathogenesis of obesity. Obesity research to date shows that there is reduction in all three components of expenditure in

individuals who are obese compared to lean individuals[74, 76, 77] and controversy as to the cause or effect of this reduction is unresolved[78, 79]. The most variable component of total daily energy expenditure is NEAT and innate differences in NEAT have been shown to correlate to weight gain in response to over-feeding which Levine et al. estimate to be 100 to 800kcal/day.

Humans show considerable inter-individual variation in susceptibility to weight gain in response to overeating. The physiological basis of this variation was investigated by measuring changes in energy storage and expenditure in non-obese volunteers who were fed 1000 kilocalories per day in excess of weight-maintenance requirements for 8 weeks[80]. This resulted in an increase in total energy expenditure with two-thirds of this increase attributed to NEAT. In short, changes in NEAT predicted resistance to fat gain with overfeeding suggesting that, as humans overeat, activation of NEAT dissipates excess energy to preserve leanness. In other words, failure to activate NEAT may result in fat gain.

A smaller but still significant contributor to total energy expenditure is adaptive thermogenesis, thermogenesis induced in response to either cold exposure or diet[74]. Thermogenesis in this category can be increased by a drop in ambient temperature or by feeding in order to protect the organism from cold exposure or to regulate energy balance after changes in diet. Historically, adaptive thermogenesis is well known to occur in specialized tissues, such as brown adipose tissue (BAT). In BAT, thermogenesis is defined as the dissipation of energy via the production of heat and occurs through the process of 'uncoupling'. Typically, within the mitochondria of brown adipocytes, ATP is synthesized via the process of oxidative phosphorylation. Activation of uncoupling proteins (UCP), and in particular UCP1 in brown adipocytes leads to uncoupled respiration, by causing a proton leak across the inner mitochondrial membrane. This leak, 'steals' protons away from ATPsynthase, diverting energy from the production of ATP to heat production. This process will be discussed in detail below. In terms of feeding, this process is believed to serve the process of acquiring enough substrate to sustain protein biosynthesis whilst fending off weight gain during this pursuit. If a diet is low in protein, then food intake needs be increased and adaptive thermogenesis 'wastes' excess calories for the purposes of further food ingestion. This

is apparent in the fact that the thermic effect of food is the greatest during ingestion of diets that are low in protein[81, 82].

The process of adaptive thermogenesis, be it to cold or food, is regulated by the brain through sympathetic outflow. The hypothalamus is essential in the control of adaptive thermogenesis as shown by injecting the polysynaptic tracers pseudorabies virus[83] and herpes simplex virus[84] into BAT and then retrogradely tracing the innervation from the hypothalamus to BAT. The characterzation of different nuclei projecting to different depots of fat have been characterized but is beyond the scope of this thesis and have been examined extensively in the literature[85 86 87] but it must be recognized that these hypothalamic nuclei and the associated neuropeptides (e.g. neuropeptide Y (NPY), α melanocyte stimulating hormone (αMSH), orexin and melanin concentrating hormone (MCH)) regulate not only food intake but also energy expenditure. Indeed, dogma dictates that factors that increase food intake typically inhibit thermogenesis and vice versa[88].

Caloric intake and energy expenditure constitute opposite arms of the energy equation, so it is logical that the factors that regulate one also regulate the other. Thus, in addition to the neuropeptides mentioned above, circulating factors such as leptin, cholecystokinin and ghrelin exert dual effects on food intake and energy expenditure[89]. Leptin, for example, not only reduces food intake in ob/ob mice but also increases spontaneous physical activity, energy expenditure and body temperature to the level of non-obese wild-type controls[90, 91]. The energy expenditure induced by leptin is due to action on the brain, as demonstrated in transgenic mice lacking leptin-receptor-STAT3 signaling[92]. Normalization of reduced spontaneous physical activity is demonstrated in the transgenic mice lacking leptin-receptor-STAT3 upon restoration of the inducible transgene for leptin-receptor-STAT3 in the ARC. In humans, however, there are no studies in which an infusion of leptin has been performed to measure spontaneous physical activity or NEAT. In a human study by Levin et al[93] however, they showed a positive correlation of leptin levels with weight gain by overfeeding and this correlation was stronger than the correlation of leptin in the same individuals with changes in NEAT potentially highlighting a role of leptin resistance which is a topic reviewed extensively elsewhere[94].

There is good evidence that leptin is a factor that regulates adaptive thermogenesis in brown fat, however, the evidence for this in relation to the thermic effect of food comes predominantly from rodent models that have shown that during starvation, leptin secretion and consequently sympathetic activation to brown fat declines[95, 96]. The molecule responsible for brown fat thermogenesis mentioned earlier are the uncoupling proteins capapble of dissipating the mitochondrial transmembrane proton gradient and generating heat. This process is shown to be mediated centrally by leptin via sympathetic activation[97] and indeed in rodents, central administration of a leptin agntagonist has been shown to inhibit diet-induced thermogenesis [98].

The evidence of BAT thermogenesis being significant in humans in the pathogenesis of obesity is beginning to gain traction since the detection of brown adipose tissue in adults using PET-CT imaging techniques combined with tissue bopsies and histology[99]. Cold exposure activates BAT in essentially all lean individuals, but this also highlights the discrepant brown fat activation between obese and lean individuals[100]. Obese individuals consistently show reduced BAT activity. Unlike rodents where brown adipocytes are located in the interscapular fat pad, the brown adipocytes in humans have been shown to be dispersed throughout white adipose tissue within the neck, supraclavicular, paraarotic, paravertebral and suprarenal regions[101]. In terms of adaptive thermogenesis in humans and animals, focus has primarily been in relation to cold induced thermogenesis and the role of BAT not the thermic effect of food nor the role of other tissues like skeletal muscle in adaptive thermogenesis (which will be discussed in detail below).

Endocrine Control of Energy Balance

Evidence points toward a prominent role of lowered physical activity and NEAT with increasing adiposity. Dysregulation of energy balance is not merely under voluntary control, but is also subject to prominent biological, involuntary regulation. In general, neuropeptides within the brain and hormones that regulate energy expenditure also have a role in appetite regulation. Hence, for the majority

of these signaling molecules, those that increase food intake generally reduce energy expenditure and vice versa. Ghrelin, synthesized by the stomach, promotes hunger and reduces spontaneous locomotor activity in rats[102] while leptin, synthesized by white adipose tissue, promotes satiety and increases adaptive thermogenesis along with NEAT[92] . In terms of mechanism, leptin has been shown to stimulate release of anorexigenic αMSH within the hypothalamus and also promotes acetylation of this peptide, enhancing function[103] as well as inhibiting orexigenic neuropeptides, NPY and AgRP release from the arcuate nucleus[104]. Leptin also lowers caloric intake by inhibiting sweet taste sensitivity without affecting responses to sour, salty and bitter substances in lean mice. [29] There are many other pathways to increasing caloric consumption other than those operating through appetite centres in the hypothalamus, such as reward pathways and hedonic circuits in the orbitofrontal cortex and striatum which were reviewed recently by Weltens et al.[105]

There are a host of other signals that determine appetite drive and these sometimes perturb the energy balance equation. One of the first hormones found to influence appetite and energy expenditure was cholecystokinin (CCK). CCK is synthesized throughout the gastrointestinal tract and acts to reduce appetite via vagal input to brain stem nuclei and to lower energy expenditure by reducing locomotion in rats[106]. CCK also plays a role in the successful passage of food through the gastrointestinal tract and once intake exceeds the processing ability of the duodenum, foraging and consumption is inhibited by CCK. An exception to the rule that hypothalamic peptides have reciprocal functions is found in the case of orexin A and B. Unlike the factors mentioned above, orexin is important to the sleep-wake cycle and has been shown to consistently increase both locomotion and appetite[107]. This may reflect the importance of circadian information in regulating feeding behaviour[108].

To further illustrate the diverse patterns of peripheral signaling, hormones such as thyroid hormone[109] increase both appetite and NEAT. This complexity of signaling from the periphery is integrated at the level of the hypothalamus and the sum of activity of orexigenic and anorexigenic neuropeptides and neurotransmitters

determines whether an organism is in a state of positive or negative energy balance.

Importantly, this funneling of information occurs in the hypothalamus within several key nuclei. The arcuate nucleus appears to be a key site where peripheral and central signals converge for appetite regulation[89]. Two key cell types are found in this nucleus[110]. In one, the orexigenic peptide neuropeptide Y (NPY) is produced, but the same cells also produce the anorexigenic peptide, agouti-related protein (AgRP). In another set of neurons, pro-opiomelanocortin (POMC) is expressed and this encodes for the melanocortins that are anorexigenic as well as β-endorphin, which is orexigenic. These cells may also express cocaine and amphetamine regulated transcript (CART)[111]. These central signals that regulate appetite have inverse effects with regard to NEAT. Those central signals that increase appetite like NPY, AgRP, melanin-concentrating hormone and galanin-like peptide generally reduce NEAT whereas melanocortins , CRH, neuromedin and CART lower appetite and increase NEAT[89].

Mechanisms that control appetite and NEAT are also important with respect to anxiety and stress response. For example, the ability of CCK to reduce locomotion is only apparent when rats are in a novel and a stressed state[112]. In a habituated environment, the effect of CCK is reduced. Wunderlich et al. [113] found that the effect of CCK can be attenuated by an antagonist only in rats that have been pre-stressed. This dependence on the stress response for CCK function to affect activity is thought to be mediated by different receptor subtypes in different regions of the hypothalamus. Thus, CCK-B receptors in the amygdala mediate anxiety-related behaviours, whereas CCK-A receptors in the nucleus accumbens in the basal forebrain rostral to the preoptic area of the hypothalamus regulate reward related behaviors[114]. Other NEAT-inducing factors, such as oestradiol and galanin, have been shown to influence fear and anxiety related behaviors, which is interesting because this suggests a link between weight regulation, the reproductive axis and the stress response[110]. It is assumed that brain mechanisms controlling the behavioral and physiological response to stress have been co-opted to alter NEAT in the service of energy balance.

Cellular Mechanisms of Adaptive Thermogenesis

Upon activation of the sympathetic nervous system by leptin, noradrenaline is released from the nerve endings of postganglionic fibres to act primarly at β_3 adrenoreceptors on the brown adipocytes[74], fundamental to the activation of BAT thermogenesis. This in turn increases the expression and function of UCP1 in mitochondria and causes the disassociation of the innermembrane proton gradient resulting in the generation of heat. In addition to UCP1, there are a number of UCP homologues including UCP2 and UCP3. The role of UCPs in adaptive thermogenesis is reviewed here with discussion of the role of various homologues of UCP1. An increasing body of work has examined the role of muscle tissue in adaptive thermogenesis and possible molecular mechanisms involved in thermogenesis in this tissue will also be discussed.

Uncoupling Proteins

The archetypal UCP is UCP-1. It is found primarily in brown adipocytes and is known to be positively regulated by β-adrenergic stimulation, cold exposure and fatty acids[74]. Homologues of UCP-1, namely UCP-2 and UCP-3, have also been identified, but their role in mediating adaptive thermogenesis has been contentious and this is summarized in Table 1.

Table 1 Summary of Uncoupling Proteins 1, 2 and 3 in terms of their site location, function and regulation (Adapted from Krauss et al., 2005 and Azzu et al., 2010)[115, 116].

Uncouping Protein	**Location**	**Function**	**Regulation**
UCP1	Brown Adipose Tissue	-Mediates thermogenesis in response to cold and	POSITIVE - sympathetic nervous system - Thyroid hormone - increase transport and

		dietary stimuli.	oxidation of fuel substrate like free fatty acids (FFA) NEGATIVE - Purine nucleotides, ATP and GTP
UCP2	Ubiquitous, including pancreatic beta cells, spleen, lung, stomach	Protection against reactive oxygen species (ROS)	Not known.
UCP3	-Predominantly in skeletal muscle -Smaller amounts in heart tissue and brown adipose tissue	- Mediates thermogenesis in food exposure and exercise induced thermogenesis in large mammal models but not in rodent models.	POSITIVE -Expression increased with FFA and carbohydrates -States of fasting and exercise - Centrally administered leptin[117]. NEGATIVE -Denervation to skeletal muscle

Among the factors that lead to the stimulation of UCP-1 in brown adipocytes, noradrenaline via its action on β_3 adrenoceptor is perhaps the most important and is widely studied. Lipolysis and the release of fatty acids provides a thermogenic substrate required to generate acetyl CoA moieties, so that the electron transport chain can generate a proton gradient. Another stimulus of UCP-1 mediated uncoupled respiration is cold exposure and its role in adaptive thermogenesis has recently assumed importance because of the link to the etiology of obesity[118, 119]. Mice with deletion of the UCP-1 gene, for example, have lower body temperature during cold exposure than normal mice[120]. The effects of UCP-1 deficiency on thermogenesis in these mice was assessed by exposure to 5°C and measuring the time it took to lose 10°C of their body heat. In the experiment by Enerback et al.[120], a higher proportion of UCP-1 knockout

animals were considered to be cold sensitive after 24h of cold exposure. In the animals with a deficiency of UCP-1, no obese phenotype was apparent on either a standard or high fat diet because animals were housed at normal laboratory temperatures however the animal house temperatures are not thermoneutral and this leads to the comfounding the true phenotypes. For mice, Cannon and Nedergaard noted that normal animal housed at laboratory temperatures of 22 degrees celsius are markedly below thermoneutrality which for mice is 30 degrees celsius, and the mice therefore have a metabolic rate and food consumption about 1.5 times higher than their intrinsic requirements in most studies, which confound the effects of UCP1 deletion on body weight and thermogenesis[121]. Nonetheless, in the UCP-1 knockouts mentioned above by Enerback et al.[120] which were housed unknowlingly below thermoneutrality, demonstrated UCP-2 expression increasing 5-fold, suggesting a role of other UCP homologues in adaptive thermogenesis.

UCP-2 has approximately 66% sequence homology to UCP-1, but UCP-2 is not specifically localised to BAT. In fact, it is ubiquitously expressed, with significant levels found in white adipose tissue, muscle, pancreatic beta islet cells, immune cells, spleen lung and stomach cells[122]. Several theories have been advanced regarding the role of UCP-2, including the following. In the process of oxidative phosphorylation, mitochondria generate reactive oxygen species (ROS), such as superoxides at complex I. This process is highly sensitive to the transmembrane proton motive force. Uncoupling of the proton motive force would decrease mitochondrial-derived ROS, reducing damage to cell constituents as is shown in studies that acutely overexpress UCP-2 in cell types like beta-islets and neurons[123, 124]. The transcription of UCP-2 itself is highly inducible under conditions of oxidative stress, for example by exposure to agents such as lipopolysaccharide[125] and TNFα[126] as well as a high fat diet challenge[127]. Most recently, Pi et al.[128] have shown that UCP-2 knockout mice have impaired glucose-stimulated insulin secretion in the presence of oxidative stress. Thus, although initial studies suggested that UCP-2 may be thermogenic due to it's ability to uncouple oxidative phosphorylation in yeast cells, this notion has now been dispelled and it's primary function appears to be the buffering of reactive oxygen species.

UCP-3 is another uncoupling protein and this has long been known to lead to increased thermogenesis during exercise[129] as well as thermogenesis induced by the drug ecstacy[130]. Some studies have also suggested a role for UCP-3 in the process of adaptive thermogenesis. It has been observed that mice lacking UCP-1 can survive in a cold environment as long as they are exposed to a gradual adaption[131]. UCP-3 in muscle is a suggested candidate for this ability to adapt to cold. UCP-3 is largely localized to skeletal muscle, less in BAT and at low levels in cardiac tissue[132]. Its role in adaptive thermogenesis has been shown to be significant in larger mammals like sheep that have a comparatively lower BAT content relative to rodents[133]. One of the factors that has led to an under-appreciation of UCP-3 and adaptive thermogenesis in humans is that inferences have largely been made from rodent models alone, which have focused on the role of BAT. There are critical differences between large and small mammals in this respect only anatomically but also biochemically. For example, the increase in the energy-sensing enzyme AMP kinase (AMPK) in promoting skeletal muscle glucose uptake and fat oxidation in rodents was not seen in sheep receiving a central infusion of leptin.[134]. Also, AICAR, an analog of AMP that is capable of stimulating AMPK when infused directly into the artery of rodent models to induce heat production does not lead to heat production in skeletal muscle of large mammals like sheep despite AMPK activation[117].

Another important difference when looking at metabolism between large mammals and rodents is that large mammals like humans are not nocturnal and would therefore display hormonal patterns that are different to those in rodents. For example, under fasted conditions, both sheep[135] and humans[136] exhibit an increase in growth hormone secretion, which is a hormone that promotes leaness, whereas rodents exhibit a decrease in the levels of the same[137]. Another factor which has led to an under appreciation of UCP-3 and its role in postprandial thermogenesis is the observation that fasting acutely increases UCP-3 in rodents, which is not consistent with the preservation of energy during negative energy balance[138]. However, physiological changes in the fed state are not just simply the opposite of the fasted state and postprandial thermogenesis should be studied in fed animals rather than inferred. A recent study by Henry et al. [117] has shown that

central infusion of leptin, in meal-fed sheep results in increased post-prandial heat production and that this increase coincides with increased uncoupled respiration in isolated mitochondria as well as an increase in UCP-3 mRNA and UCP-3 protein expression in skeletal muscle. This provides associative evidence to suggest that elevated UCP3 may drive the leptin-induced increase in post-prandial thermogenesis.

A direct role and capacity for muscle to be a major thermogenic organ related to adipose weight gain is also strongly suggested through several other lines of enquiry. Firstly, mice treated with ecstacy (3,4-methylenediooxy-methamphetamine) display rapid elevation in rectal and muscle temperature whereas UCP3 knockouts do not[139]. With the caveat that ecstasy causes a supraphysiological release of noradrenaline, the role of UCP-3 in non-shivering thermogenesis in skeletal muscle is clear when normal mice given increasing doses of ecstasy developed rhabdomyolysis with muscle breakdown and renal impairment, as seen in humans, whereas the UCP-3 knockouts were immune.

Arguably the adminstration of ecstasy may not necessarily be applicable to physiological processes, but the recent work in sheep does strongly suggest that skeletal muscle is thermogenic. A meal entrained rise in temperature was measured using dataloggers embedded in skeletal muscle by Clarke et al.[140] soon after commencing feed in sheep skeletal muscle heat production increased, but this was not related to a change in blood flow to the tissue. Subsequent studies by Clarke et al. [141] also demonstrated that estrogen administered in pulses, but not continuous infusion was able to increase thermogenesis in muscle. It has been suggested that the thermogenic potential of skeletal muscle is derived from brown adipocytes interspersed within the muscle tissue[142], but in the study by Henry et al., it was demonstrated that thermogenesis in muscle occurred without a concomitant increase in in UCP-1, thus suggesting that the source of heat production may be the myocyte itself.

A second and important line of evidence that UCP-3 can influence energy expenditure is derived from UCP-3 knockout studies where observations over a

sufficiently long duration becomes critical. Uncertainty regarding the role of UCP-3 and its impact on weight regulation was raised with the observation that UCP-3 knockout mice were not overweight compared with wild type controls on a normal chow diet or a high fat diet and that body temperature was maintained even when the knockout animals were exposed to cold[143, 144]. Gong et al.[143] for example found that the baseline metabolic rate, as determined by indirect calorimetry, was not statistically different between UCP-3 knockouts and wildtype animals on a normal diet. Vidal-Puig et al.[144] showed that UCP-3 knockout mice have reduced uncoupled respiration but no change in body weight after 3 months on a high fat diet. Recently, however, Costford et al.[145] have shown that exposing the UCP-3 knockouts to a long-term (8 month) high-fat (45%) feeding regimen did enhanced accumulation of epididymal white adipose. This fat accumulation in the UCP-3 knockouts was 20% higher than in wild-type controls and the difference was not observed at 4 month on a high fat diet, which is when previous studies ended their morphometric measurements. This highlights the need to monitor changes in weight over a long period of time, which was not done by either Gong et al nor Vidal-Puig et al.

It is also evident that UCP-3 plays a significant role in the development of obesity in humans. Harper et al. [146] recruited over 300 overweight women who had completed a weight management clinic program and were ranked according to percent body weight loss during the first 6 weeks of a 900kcal meal replacement protocol. The highest and lowest quintiles of weight loss were defined as diet-responsive and diet-resistant, respectively. After achieving a stable body weight for 10 weeks, muscle biopsies and blood samples were taken and it was found that, despite having similar baseline weight and age, the diet-responsive group had significantly greater uncoupled respiration (51%) which was associated with higher UCP-3 expression (25%). In particular, these authors identified a negative correlation between the change in UCP-3 protein levels and BMI, suggesting that UCP3 abundance facilitates weight loss. In terms of cause and effect, it would have been more informative had a biopsy been done before weight loss as well, but the authors argued that there were no statistical differences between the diet-responsive and diet-resistant groups when the muscle biopsy was done, which

suggested that the higher UCP-3 levels were a function of absolute weight. By measuring UCP-3 levels prior to weight loss, however, Schrauwen et al.[147] showed that absolute UCP-3 levels, in terms of both mRNA and protein, were not only lower at the end of an intentional weight loss period over 10 weeks, but more importantly, greater weight loss was associated with smaller decrements of UCP-3. This study, which was performed in obese subjects with Type II diabetes, demonstrates a negative correlation between changes in UCP-3 and weight loss. Overall, this suggests that the body has a capacity to alter energy expenditure and those individuals with greater weight loss maintain higher UCP-3 levels.

Aside from diet-induced thermogenesis, muscle is also capable of cold-induced thermogenesis, which is a response similar to that seen in BAT, not only in form but function as well. Wijers et al.[148] showed that exposure of 10 healthy lean male subjects to a mildly cold environment of 16°C (above the threshold required for shivering), was able to induce thermogenesis in skeletal muscle. Measurements were made during cold exposure over 84 hours with subjects living in a respiratory chamber, to allow calculation of energy expended based on oxygen consumption, carbon dioxide production and urine nitrogen excretion. Muscle biopsies were obtained at the end of the period to measure mitochondrial respiratory function. Importantly, they found that state 4 uncoupled respiration was increased as a result of cold exposure, lending credence to the role of muscle in cold induced thermogenesis. This would be due to dissipation of the proton gradient in mitochondria by UCP-3. Unfortunately, neither UCP-3 mRNA nor protein levels were measured. By contrast, Shabalina et al.[149] performed similar studies in UCP-1 knockout mice and were able to demonstrate that cold exposure did induce increased expression of UCP-3 protein. The mitochondrial analyses that were performed in this study, however, did not show increased uncoupling as was observed the human study by Wijers et al.[148] Interestingly, Wijers et al. administered a non-selective adrenoceptor blocker to their human subjects which did not impair cold-induced thermogenesis but was able to stop state 4 respiration in muscle. The authors hypothesized that propranolol, being more selective for β_1 and β_2 adrenoceptors which are found in muscle, down-regulated thermogenesis in the muscle and allowed a switch to BAT thermogenesis, normally induced via β_3

adrenoceptors. This receptor subtype is 100-1000 times less sensitive to propranolol. It could not be proven, however, since measurement of BAT activity in the presence of propanolol is unreliable[150, 151]

Perhaps it should come as no surprise that muscle and BAT have similar roles and characteristics, given that recent studies indicate brown adipocytes are derived from a myogenic lineage, which is distinct from the white adipocyte lineage[152, 153]. Using microarray analysis, Timmons et al. [152] revealed that brown pre-adipocytes share the same transcriptional signature as myocytes, while Seale et al.[153] showed that PRDM16 functions, in part, as a switch for precursor cells to differentiate into either brown adipocytes or myocytes. More recently, this common precursor has been identified in cells that express Myf5[148]. It is not too much of an extension to hypothesize that the role of adaptive thermogenesis can be supported by either brown adipose tissue and or muscle. In fact, the recent discovery of a myokine called irisin indicates that muscle regulates brown-like cell differentiation in subcutaneous adipocytes[154]. The function of this myokine is most likely induced by PGC1-α, acting via PPARγ signaling pathways that are activated through exercise. It has been shown that neutralization of irisin with antibodies ameliorates the effect of this factor to induce UCP-1 expression, while overexpression of the myokine with an adenoviral vector is able to induce weight loss in high fat diet fed mice as well as improve insulin sensitivity[154]. This work has primarily been carried out in rodents, and the functional role of irisin in humans is less clear. Various clinical studies have failed to show an increase in irisin secretion post-exercise[155, 156], albeit a recent study has evoked a role for irisin in cold-induced thermogenesis in humans[156].

Regulation of thermogenic tissue is also seen in BAT by the inflammatory process linking the immune system with energy homeostasis. Nguyen et al. [157] have reported that BAT activation during cold exposure is subject to regulation through macrophage stimulation. Classically, macrophages are activated by IFNγ secreted by T cells but an alternative activation of macrophages has been demonstrated with IL-4 and IL-13. This means of macrophage activation is known as 'alternative activation' of macrophages[158]. By comparing wild type mice to IL-4$^{-/-}$/IL-13$^{-/-}$ and

Stat $6^{-/-}$ knockout mice which lack the ability to produce IL-4 and IL-13, it was demonstrated that the knockouts lacked the ability to respond to cold (4°C)[157] and that this was due to reduced thermogenic capacity of BAT. It was further demonstrated that IL-4 was mandatory for stimulation of macrophage secretion of catecholamines that, in turn, switched on BAT thermogenesis[157]. Given that glucocorticoids are known to interact with IL-4 in a synergistic fashion with the combination of stimuli inducing special features in relation to receptor expression[159] and signaling[160], this is a potential mechanism by which the cortisol response to synacthen in sheep might be linked to thermogenesis. The role of muscle in relation to stimulation by cytokines in adaptive thermogenesis would be a logical extension of this work.

Calcium Cycling Thermogenesis

Aside from uncoupling proteins, heat can also be generated via alternative pathways within skeletal muscle tissue. Muscle can generate heat via other uncoupled processes such as leakage of ions through channels along calcium chemical gradients known as futile calcium cycling thermogenesis. [161]. In this process, calcium fluxes into the cytoplasm of myocytes, be it from either outside the cell via the opening of neutrotransmitter-mediated Na^{2+} channels or via the calcium storing organelle, the sarcoplasmic reticulum (SR) via a receptor known as ryanodine receptor (RyR). The calcium is then pumped back into the SR via the Sarco Endocplasmic Resticulum Calcium ATPase or SERCA. The hydrolysis of ATP by SERCA is needed in order to pump the calcium against the concentration gradient back into the SR, a process that releases part of the energy from the hydrolysis of ATP as heat energy. Links between a stress response and mutations in the RyR leading to thermogenesis has been examined in porcine models in relation to the malignant hyperthermia that results in a small but appreciable number (0.7%) of pigs suffering from porcine stress syndrome (PSS). Upon slaughter, pigs with this condition exhibit a rapid decrease in muscle pH and elevation in muscle temperature resulting in denaturation of muscle proteins. The meat from these pigs has a pale, soft, exudative appearance and reduces the

commercial value.[162] PSS results from a single nucleotide substitution in the gene encoding the skeletal muscle ryanodine receptor. The presence of this mutation becomes evident during the stress of slaughter. Accordingly, researchers have examined the role of the HPA axis in this thermogenic process. Lower basal levels of cortisol and lack of diurnal variation in cortisol levels have been identified in heterozygotes for this mutation (compared with wild type) and these heterozygotes are also leaner have higher muscle temperature at the time of slaughter[163]. Interestingly, however, the authors did not report increased HPA response to the stressor which consisted of restraint with a nose snare for 5 minutes. To date, no significant stressor has been applied to pigs that have PSS nor has a synacthen challenge been administered.

These findings have direct correlates to humans. This is amply demonstrated in individuals who suffer from the potentially fatal pharmacogenetic disorder of malignant hyperthermia (MH), secondary to the response to volatile anaesthetic agents (eg isoflurane, halothane and sevofluorane) and depolarizing muscle relaxants (eg succinlcholine). This occurs in up to 1 in 12,000 individuals[164]. During an episode of MH there is a rapid and sustained elevation of body temperature by as much as one degree per 5 minutes and core body temperature can exceed 43°C. In addition, tachycardia, lactic acidosis and elevated arterial CO_2 occurs, indicating increased aerobic and anaerobic metabolism along with muscle contracture and breakdown. There are reports of physical or emotional stress, anxiety and or sudden changes in environment that can initiate MH in some patients[165] with a proposed human stress syndrome based on observations of a number of families with a history of MH[166]. Though the mechanism of how the HPA axis activates the calcium cycling process for heat production is not fully understood, these observations imply signaling pathways exists linking stress and thermogenesis via the calcium cycling pathway.

These signaling pathways is likely to involve proteins like sarcolipin and phospholamban that can bind to different conformations of SERCA, changing the extent to which heat production is regulated[167]. The regulation of these proteins is not reviewed here but importance of these proteins should not be

underestimated in terms of adaptive thermogenesis given that sarcolipin null mice have been demonstrated to be prone to diet induced obesity and that sarcolipin can compensate for reduced BAT function on cold exposure[168]. Nonetheless, the most important feature of heat production relates to intracytoplasmic calcium levels and the rise in calcium required for myocyte contraction is provided by the activation of the RyR to which mutations can affect heat production in calcium cycling.[169] Calcium is released from the SR via the RyR and pumped back into the SR through calcium ATPase in order to maintain a low level of cytosolic calcium so it serves to reason that the mutations of the RyR that allow greater flux of calcium are also more likely to induce thermogenesis as this would then induce SERCA to hydrolyse more ATP to pump calcium against a concentration gradient into the SR and generate heat. In summary, the higher the rate of calcium cycling, the greater the rate of ATP hydrolysis by SERCA and the larger the amount of heat released. One would also expect that, in order to maintain the cellular level of ATP used, mitochondrial respiration rate would increase as well.

SERCA and RyR

SERCA is a 110kDa transmembrane protein that is found in all living organisms from yeast to mammals. In vertebrates, there are 3 distinct forms of the receptor encoded by 3 different genes that encode SERCA 1, 2 and 3. Alternate splicing creates 10 isoforms, of which two are potentially involved in skeletal muscle thermogenesis (see Table 2). SERCA 1 is expressed in fast twitch skeletal muscle as well as BAT with SERCA1a being seen in adults and 1b in fetal tissues. SERCA2a is expressed in slow twitch muscle and 2b is expressed in all tissues at low levels, including muscle and non-muscular tissues. Regulation of SERCA1a and 2a is also similar to that of UCP-1 in BAT, suggesting a potential role in adaptive thermogenesis. Beta adrenergic stimulation increases uncoupling of the electrochemical gradient in BAT to generate heat and also inhibits phosphorylation of phospholamban. This latter moiety is a small molecular weight inhibitor of SERCA, which in essence causes disinhibition of SERCA[170]. As with UCP-1 in BAT, thyroid hormone is able to increase SERCA levels in a fibre specific way by

upregulating gene transcription of SERCA[171]. There is differential upregulation dependent on muscle fibre type and thyroid status.

Table 2 Summary of SERCA subtypes, location and details of fibre types in which the various subtypes are expressed (adapted from Perisamy and Kalyanasundaram)[172].

SERCA Type	Location	Details
I_a	Muscle Brown adipose tissue	I_a is found in adult muscle and I_b is found in foetal muscle.
I_b	Muscle Brown adipose tissue	
II_a	Heart Muscle	II_a is found in slow twitch muscle and foetal fast twitch fibres
II_b	Ubiquitous (SMC)	
III	Specialized cell types like epithelial cells and endothelial cells.	

There is evidence that a number of factors modulate energy production from SERCA via ATP hydrolysis, allowing SERCA function to switch between performing calcium transport and generating heat. This is done via changes to the conformation of SERCA's tertiary structure exposing the calcium binding site. If the calcium binding site is exposed on the external side of the calcium containing vesicle, it has high affinity for calcium (Ka = 10^{-6}M at pH 7) compared with a conformation where SERCA's calcium binding site faces the vesicle lumen (Ka = 10^{-3}M at pH 7)[173, 174]. In other words, the ATPase activity can be coupled to calcium transport, releasing only a little heat or if completely uncoupled, generate a lot

more heat. The heat release in coupled ATPase activity varies between -10 and -12 kcal/mol ATP compared with -20 to -24 kcal/mol ATP in uncoupled ATPase activity[175]. More recently, it has been demonstrated that there is crosstalk between RyR and SERCA which regulates the amount of heat released during ATP hydrolysis. Work by Arruda et al.[176] examined ATPase activity fractions of SR including both RyR and SERCA and calculated calometric enthalpy. They showed that this can change from -12 to -15 kcal/mol to -20 to -22 kcal/mol ATP upon addition of an inhibitor of calcium transport in the RyR of 5μM ruthenium red and 5mM $MgCl_2$. This increase in heat released per mol of ATP is a result of less ATP being hydrolysed by SERCA when the RyR is inhibited with a compensatory increase in energy released per ATP molecule, in effect, maintaining thermoneutrality *in vitro*. The authors of this work speculated that when more calcium leaks through the RyR *in vivo*, more ATP is cleaved to maintain the cytosolic calcium concentration within the physiological range. Interestingly, the authors also speculated that during calcium cycling, heat would be produced not just from cleavage of ATP but also from the activation of mitochondrial respiration needed to maintain intracellular ATP concentration.

The RyR also plays a in the calcium signaling and influences the metabolic processes of the mitochondria. The RyR found in the mitochondria (mRyR), is of the same subtype as that in skeletal muscle (RyR1) and also sensitive to dantrolene, as opposed to the cardiac subtype (RyR2), which is not. This has been comfirmed by the lack of detection of mRyR in hearts of newborn RyR1 knockout mice[177]. Along with other transporters, mRyR is able to increase the calcium permeability of the mitochondrial inner membrane ultimately leading to activation of ATP synthase[178]. There are other regulators of RyR such as the dihydropyridine receptors (DHPR)[179] which will be mentioned briefly. These are voltage-gated calcium channels that help activate RyR through an indirect mechanism in cardiac muscle (known as calcium-induced calcium release - CICR) or a direct physical interaction with the RyR in skeletal muscle. In skeletal muscle, changes in the confirmation of DHPR following membrane depolarization, induces the opening of RyR to release calcium needed for excitation contraction coupling. Other

moderators of RyR function include calstabin 1 and calstabin 2 which maintain stability of RyR in muscle[180], calmodulin which activates the receptor when it is calcium free, but inhibits RyR if calcium bound[181, 182]. Other mediators are [169] adenine nucleotides, cyclic ADP-ribose, kinases like PKA, CamK, phosphatases like PP1 and PP2A, phosphodiesterase PDE4DE and the muscle A-kinase anchoring protein[183]. This implies complex regulation of RyR beyond simple expression level of the receptor. To date, there have been no studies on the role that calcium plays in relation to diet or postprandial thermogenesis. Given the recent interest in BAT and thermogenesis, considerable effort has been directed towards examination of adaptive thermogenesis in BAT in terms of calcium cycling via SERCA and RyR. Until now, the heat generated in rodent models has been thought to derive from UCP-1 activity and the uncoupling of the proton gradient in mitochondria in BAT. Recent evidence, however, has shown the existence of SERCA in BAT isolated from the endoplasmic reticulum[184]. The isoform present in BAT, SERCA 1, is also found in skeletal muscle but there are several kinetic properties that are different in the two tissues. There appears to be a different calcium binding affinity and a higher degree of ATPase uncoupling in BAT than in muscle. The role of SERCA in BAT during adaptive thermogenesis is less well defined. Aydin et al.[185] have shown that UCP-1 KO mice exhibit severe RyR hyperphosphorylation and calstabin 1 depletion during cold exposure leading to markedly reduced SR calcium release and decreased force of muscle contraction. Unequivocal evidence of the role of skeletal muscle in adaptive thermogenesis has been reported recently, with the generation of sarcolipin knockout mice[186].

As mentioned earlier, sarcolipin is a modulator of SERCA and has the ability to promote uncoupling of SERCA leading to increased ATP hydrolysis and heat production. Placed on a high fat diet for 22 weeks, the sarcolipin knockout animals showed a gain of almost twice the amount of white adipose tissue compared to wild type mice. The wildtype mice showed upregulation of sarcolipin within the soleus muscle to a level 3-5 fold higher than in the knockout animals. Interestingly, the SERCA1a and SERCA2a protein expression was not altered in sarcolipin knockout animals compare to the wildtype controls. The study also showed that skeletal muscle thermogenesis has a strong role to play in maintaining

core body temperature through the model of interscapular BAT surgical ablation rodent model.

The complex regulation of adaptive thermogenesis involving BAT and muscle, which involves SERCA, RyR, DHPR and sacrolipin suggests a role of this process for the support and enhancement of survival of the organism, as opposed to it being a byproduct of more important physiological processes. Good argument has been put forward in support of its role of adaptive thermogenesis to cold by allowing animals to survive and be active during the night or maintaining core temperature during hybernation as well as allowing survival during the cold stress of birth. The role of postprandial thermogenesis is arguably less clear but many believe that it is able to promote our survival on diets low in essential macronutrients, especially protein. Adding fat or carbohydrate to diets has been shown to stimulate the thermic effect of food in BAT and the significance of this can be understood by remembering that food serves two purposes. Firstly, food provides calories to meet energy demands and secondly, it provides amino acids for protein synthesis. If the diet is low in protein, then food intake must be increased to obtain enough protein to sustain protein biosynthesis. This would lead to obesity if the organism lacked the capacity to use up excess calories. The dilution of protein leading to increased adaptive thermogenesis is called the protein-dilution hypothesis. If this hypothesis is correct, then populations that have low protein in their diets should have greater selective pressure favouring higher temperature excursions in relation to feeding compared with populations that have higher protein diets. To date, no such human data has been gathered.

Behavioral Responses and the HPA Axis

Although thermogenesis is an example of an adaptive homeostatic mechanism that can determine and predict one's susceptible toward obesity, it is also suggested that behavioural phenotypes are linked to the development of increasing obesity in humans. To date, there have been no weight-loss studies that take the personality

trait or coping behaviour into account when devising a management plan for weight loss and this could be a reason why many interventions fail. It may also account for heterogeneity of weight maintenance outcomes with the vast majority leaning toward weight re-gain beyond the 3 year period.

Coping behaviour to stress in humans is determined by a combination of innate mechanisms and learned strategies. Interestingly, there are broad categories of coping strategies in animal models that have been correlated with physiological responses of the HPA axis, bridging a mind body divide that was traditionally separate since the days of Descarte[187].

The relationship between the HPA axis and its association with stress-induced behaviour has been studied through selection of animals with different behavioural traits and the elucidation of the hormonal differences in the same. Often, individuals with identified behavioural traits have been back-crossed through a number of generations and the function of the HPA has segregated with behavior[41, 188]. For example Beausoliel et al.[189] selected sheep with either 'More Active' (MA) or 'Less Active' (LA) response to social isolation. Here, it was found that MA sheep, which had greater locomotion and bleating in response to stress, also had a lower cortisol concentrations post-stress than LA sheep. These differences in behavioral performance, followed by discovery of differences in neuroendocrine responses, were also found in mice selected on time it took or latency to attack an intruder[188]. Lines of wild house mice selected on the basis of attack latency in response to territorial infringement were found to correlate with corticosterone fluctuations during the light and dark phase as well as corticosterone responses to stressors, whereby short-attack latency mice, which were more aggressive than long-attack latency mice, demonstrated lower corticosterone increments following the stress of a forced swim test. The mechanism would seem to be centrally mediated as it was observed that short attack latency mice had correspondingly lower CRH mRNA expression in the hypothalamus and lower percent change from basal ACTH secondary to the stressor. Thus short-attack latency mice are akin to low cortisol responders, which is a relationship observed not only in mice. Coping strategy has been associated

with HPA activity in a number of other species including rats[190], chicks[191] and pigs[192]. The explanation for this may lie in the differential effect of cortisol on different parts of the brain.

The amygdala is part of the limbic system and produces many peptides implicated in stress and anxiety regulation, such as CRH[193]. In contrast to the PVN in the hypothalamus, it has been shown by immunohistochemistry that injection of corticosterone stimulates CRH release in the amygdala, whereas the same causes a reduction of CRH mRNA levels in the PVN. [194]. This suggests that stress responses not only can promote physiological adapation to challenges, but may also lead to psychosocial changes that need to be coordinated to promote survival.

Exposure to aggression has long been known to be a powerful stressor that has been shown to activate the HPA axis as well as sympathoadrenomedullar system[195]. Aggressive behavior is, in turn, affected by the functional properties of the HPA axis and of the SAM system. Baseline levels of glucocorticoids, for example, are inversely related with aggressiveness in various species, including pigs[196]. Thus, Murani et al. [197] evaluated aggression by introducing an aggressive animal to a resident pig and scoring the number of skin lesions on the resident animal; the number of lesions was correlated with aggression. They found that the animals that were heterozygous for a mutated ryanodine receptor (a mutation that leads to malignant hyperthermia) showed more aggressive behaviour. Their work was based on the earlier studies of Weaver et al.[198] showing that changes to carcass meat quality (suggestive of increased temperatures) were found in pigs that were heterozygous for the mutation in the RYR. They also showed that the heterozygotes had lesser adiposity, with lower carcass fat depth and increased lean mass. These animals also had lower basal plasma ACTH and cortisol levels but stressor testing by snare restraint for 5 minutes did not reveal differences in cortisol response. The lack of a difference in HPA responsiveness may be due to type of stressor and magnitude of the stressor used, as others have shown differences in stressor-induced activity of the HPA axis in pigs with porcine stress syndrome[199]. This study also demonstrated that pigs with higher cortisol response to stress had a higher emotional response when loading for

transportation. In combination, the above studies suggest that a less 'active' HPA axis correlates with leaner mass, higher muscle temperatures on slaughter and a greater tendency toward aggressive behaviour. It is also possible that cortisol may have a direct effect on aggressiveness, because aggressive behaviour can be induced in rats with an acute increase in glucocorticoids via a fast feed-forward mechanism[200].

Given the universality of the correlation between neuroendocrine response and behavioural response to stress, it is an interesting question as to whether this is heritable. Touma et al.[41] addressed this question in mice that were high cortisol responders; these animals had a hyperactive, restless and agitated phenotype, not unlike the behavior seen in humans with melancholic depression. High (HR) and low cortisol responders (LR), which were identified by restraint stress test and behaviorally phenotyped, were then back-crossed through 3 generations. Progeny of the high and low cortisol responders were retested and it was shown that cortisol responsive status and the behavior of these mice was indeed heritable. The animals were examined in a series of well-characterized tests to determine their emotionality and coping style. These tests included examining the exploratory drive of the animals by using an elevated platform test, examining aggressiveness by measuring the duration of attack on a intruder to a resident rodent and depression like behavior in a forced swim test and/or tail suspension test. Higher exploratory drive was displayed by the HR animals, which also exhibited a longer attack latency[41]. Depression-like behavior was more prominent in the LR animals. The results of these studies suggested that HR exhibited more exploratory, agitated phenotype but were less likely to demonstrate aggression toward a threat, the combination of which would more likely lead to flight rather than fight response. A series of other tests were performed including open field testing that examines locomotor activity as well as an elevated plus maze test to examine anxiety related behavior. These tests, however, did not reveal a difference in behavior between the HR and LR mice, which highlights the need to explore animal behavior with multiple tests in order to draw meaningful conclusions.

In summary, the relationship between behaviour and cortisol responsiveness has been demonstrated in rodent species[201-203] and hens[191] and it has been shown consistently that high cortisol responsiveness is associated with a reactive behaviour in response to stress while low cortisol responsiveness is associated with a proactive behaviour in response to stress[204]. The reactive group tend to be less aggressive, less territorial but more adaptive and flexible than the proactive, high cortisol response group[204]. Adrenalectomy and subsequent replacement of corticosteroid has been used to show a causal physiological to behavioural relationship in terms of the importance of cortisol or corticosterone effecting a reactive behavior. Antagonists of the mineralocorticoid or glucocorticoid receptor have also been administered to the brain, yielding a surprising importance of the mineralocorticoid receptor in the promulgation of the corticosteroid effect[205, 206].

Coping Sytle in Humans

The study of behaviour and HPA axis has also been studied in humans, especially in relation to sport. Psychophysiological stress in tennis players across a tournament was studied by Filaire et al.[207]. Here, Competitive State Anxiety Inventory Scales (CSAI-2) were correlated with salivary cortisol concentration and individuals who won their matches had a lower CSAI-2 scores as well as lower pre- and post-tournament cortisol levels. In other words, the higher the cortisol level difference between pre and post tournament stress, the likelier those individuals had higher anxiety levels. It should be noted though that this is just a correlation and not causation as it is not clear whether anxiety causes a higher HPA response or a higher HPA response leads to greater anxiety as a phenotype.

There have been other studies correlating personality with HPA axis function, but these human studies have not been able to elucidate the nature of this relationship. The relationship between personality trait and HPA function in most studies leans toward a causal relationship, whereby personality traits like neuroticism and introversion render the individual more susceptible to stress and activation of the

HPA axis[208]. Studies by Jokinen et al.[46, 209] employed a dexamethasone suppression test, instead of a synacthen challenge, to evaluate the activity of the HPA axis and found that those who had attempted suicide were 3 times more likely not to suppress their HPA function. In other words, non-suppression of HPA function could predict an increased risk of attempting suicide. A likely explanation for this phenomenon is that those who attempt suicide are further down the depressive scale and so the HPA axis is merely reflecting the psychological stress in which the individual is experiencing instead of being a marker of a personality trait.

As in animal studies, the extent to which stress-responsiveness marks behavioural differences in the general population is unknown. There has been no systematic examination of whether the clustering of coping styles to stress is that seen in animals, such as the different pig breeds or selected lines of sheep (mentioned above) may also pertain to humans. A psychological assessment of over a hundred morbidly obese women led to the emergence of two personality subtypes. [210]. One was a resilient subtype with a 'normal' personality profile while another was an emotionally dysregulated subtype characterized by low extraversion and high neuroticism. The latter had an avoidant trait that is similar to that in the LA sheep studied by Beausoliel et al.[189] This may indicate that the two groups may need to be managed differently with regard to compliance to therapy for weight reduction.

Conclusion

The obesity epidemic thus far tells us that the innate determinants of body weight are formidable and clearly require more than just telling patients to eat well and exercise. The aetiology is multifactorial, with innate and environmental factors, with innate factors being polygenic and under the influence of epigenetic change. Stress responsiveness is an innate feature, one of several factors that have not been well characterized in terms of how it interacts in a social environment that is present today.

Stress plays an instrumental part in regulating our desire for food and sex, or in physiological terms, energy homeostasis and reproduction. Stress responsiveness can be determined by measuring the stress hormone, cortisol. As reviewed above, there are individuals that tend to lose weight in relation to various forms of stressors while a majority tend to gain weight or demonstrate positive energy balance in relation to the same stressors and those that gain tend to be individuals that have a greater cortisol response.

In the animal model used in the present studies, high stress responders (HR) and low stress responders (LR) are identified from populations of outbred sheep and the aims were to determine if HR have a greater tendency to become obese when exposed to a high energy diet compared with LR. Having demonstrated this, the mechanism in terms of energy balance was investigated, in order to gain information on the biological mechanism for this difference.

The second aim was to determine if energy homeostasis changes in relation to stress was differentin LR and HR. In particular, the question was asked as to whether differences in food intake and energy expenditure between HR and LR would lead to a more positive energy balance in HR than the LR, when subjected to metabolic stress by insulin induced hypoglycaemia, psychosocial stress in the form of a barking dog and immune stress in the form of lipopolysaccharide (LPS) infusion.

The final aim was to characterize the behavioural differences in HR and LR animals and it was hypothesized that the innate coping styles are different in HR and LR. It was further hypothesize that LR demonstrate a more proactive coping style whereas HR exhibit a relatively reactive series of coping strategies.

Chapter 2
General Materials and Methods

This chapter details the materials used and methods employed which are common to Chapters 3, 4 and 5. Methodology specific to individual chapters will be found within the respective chapters within the methods section of the published manuscripts.

Animal Ethics

Experiments and procedures on all animal 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

Animal Management

Adult Corriedale ewes were acclimated to single pen housing for experiments set out in Chapter 3, 4 and 5 where no significant movement aside from standing and sitting were possible. Animals were held in single pens that allowed visual contact with their flockmates unless otherwise stipulated. In all experiments, animals were kept at the Monash Large Animal Research Facility in Werribee, Victoria, Australia (latitude 38°S) with exposure to natural variations in light and ambient temperatures.

Animal Surgery

All surgical procedures were performed under sterile conditions on fasted animals by Mr. Bruce Doughton, Dr Ross Young and Mr. Alex Satragno. Anaesthesia prior to surgery was performed with an intravenous injection of Thiobarbitone sodium (10mg/kg; Lyppard, Keysborough, Vic., Australia). Once anaesthesia had taken effect, animals were laid on their back and an endotracheal tube was introduced. The animals was then placed on an operating

table and connected to an anaesthetic gas in order to maintain anaesthesia throughout surgery, this consisted of a 3-5% gaseous fluothane (Rhone Merieux Australia, West Footscray, VIC, Australia) in oxygen. Nitrous oxide was used to deepen anaesthesia when required.

The animals were prepared for surgery subsequently 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) and sprayed with 70% alcohol (Yarraville Distillery, Yarraville, Vic, Australia). A surgical slit drape was used, but the region of incision remained exposed.

Datalogger Insertion

Dataloggers (SubCue, Calgary, Alberta, Canada) are implantable devices used for site-specific recordings of tissue temperatures. Dataloggers were implanted intoskeletal muscle of the hind limb and retroperitoneal fat. The dataloggers are sterilised overnight in 70% ethanol the recording head (2cm diameter and 0.5cm deep) embedded in the tissue and the download lead (10cm long) exteriorized. This allowed collection of data from the dataloggers at any time, by connection to a computer with the relevant program (supplied by the manafacturers).

For implantation into skeletal muscle, a small incision (5cm) was made along the limb followed by blunt dissection (with round ended surgical scissors) to separate the vastus lateralis and bicep femoris muscle minimizing 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 retroperitoneal region, 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 and a small pocket was made in the fat – where 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.

Following suturing, the surgical sites were sprayed with an antibiotic (Pinkeye aerosol, oxytetracycline hydrochloride 2.0mg/g; Pfizer Animal Heath, West Ryde, NSW, Australia). All animals were given a routine intramuscular antibiotic injection of Terramycin (oxytetracycline hydrochloride 200 mg/ml, 1 ml/10 kg body weight; Pfizer Animal Health, 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.

After surgery the animals were immediately offered food 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.

Cannulation and Blood Sampling

Jugular vein cannulations were carried out using 12G indwelling venous cannulae (Teflon Dwellcath, Tuta Laboratories Australia Pty. Ltd., Lane Cove, NSW, Australia). In each case, the cannula was inserted at least one day prior to the onset of experimentation where blood sampling was necessary. The external jugular vein was cannulated and connected to a manometer line (Portex Ltd., Hythe, Kent, UK) followed by closure 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) saline.

Serial blood samples (6ml) were taken at regular intervals throughout the sampling periods (See Chapters 3, 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. Unless otherwise stated in Chapter 4 and 5, samples were placed into tubes containing anti-coagulant lithium heparin (Sarsedt Australia, Mawson Lakes, SA, Australia) and centrifuged for 10min at 3000g after which the plasma was decanted into vials and stored at -20°C until assayed.

Selection of Animals

We used an outbred populations (n=100) of sheep to identify HRs and LRs. We challenged animals with synthetic ACTH (Synacthen) at 0.2μg/kg body weight; (Novartis Pharmaceuticals, North Ryde, NSW, Australia). During the breeding season, ovarian cycles were first synchronized by i.m. injection of 125μg prostoglandin (Cloprostenol (Estrumate); Pitman-Moore, Sydney, NSW, Australia), and the ACTH challenge was conducted 1 week later, during the luteal phase of the estrous cycle. Blood samples (6mL) were collected by venepuncture at -30, 0, 30, 60 and 90 minutes relative to injection and plasma was stored at -20 degrees celsius until assayed for cortisol.

The animals underwent an initial screening and were characterized as either HRs or LRs on the basis of cortisol response, as determined by the AUC. The selected animals classified as HRs or LRs were subjected to a second Synacthen challenge to confirm the initial results.

Chapter 3

High Cortisol Responses Identify Propensity for Obesity that is Linked to Thermogenesis in Skeletal Muscle

Declaration for Thesis Chapter 3

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution
Design & carrying out of experiments, coordination of groups involved, laboratory analysis of samples and statistical analysis of data. Writing up paper and creation of graphs for publication. Extent of Contribution: 80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Justin St John	Mitochondrial Gene Sequencing
Ross Young	Surgery of animals for insertion of ICV drains and dataloggers
Alex Rao	Guidance with PCR
Zane Andrews	Mitochondrial Respiratory Measurements

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	

Main Supervisor's Signature	

Date:

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

High cortisol responses identify propensity for obesity that is linked to thermogenesis in skeletal muscle

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ABSTRACT **Subjects characterized as cortisol high responders (HRs) consume more calories after stress, but it is unknown whether cortisol responsiveness predicts a propensity for obesity. Female sheep with either high or low cortisol responses to adrenocorticotropin (ACTH) were identified. Body composition was similar in HRs and cortisol low responders (LRs), but the HRs had greater ($P<0.01$) adiposity than did the LRs (40.5±0.7 *vs.* 35.8±1.4%) after high-energy feeding, despite comparable food intake. Postprandial thermogenesis in muscle temperature was 0.8 ± 0.08°C higher in the LRs than in the HRs ($P<0.01$), whereas feeding-induced changes in fat temperature were similar. Leptin and insulin sensitivity were similar in the HRs and LRs. Feeding lowered ($P<0.001$) the respiratory control ratio in muscle (HRs 9.2±0.8–5.2±1.2; LRs 8.4±0.5–5.2±0.7), indicative of increased uncoupled respiration. Also in muscle, the feeding-induced increases in uncoupling protein (UCP)-3 (fold increase: HRs, 2.4; LRs, 2.0), ryanodine 1 receptor (RyR1; fold increase: HRs 3.1; LRs 2.1), and sarcoendoplasmic reticulum Ca^{2+}-dependent ATPase (fold increase: HRs 1.5; LRs 1.6) were equivalent in the HRs and LRs. Sequencing of mitochondrial DNA revealed no haplotypic differences between the 2 groups. We conclude that predisposition to obesity can be predicted by cortisol responsiveness to an ACTH challenge and that the response is due to innate differences in muscle thermogenesis.—Lee, T. K., Clarke, I. J., St. John, J., Young, I. R., Leury, B. L., Rao, A., Andrews, Z. B., Henry, B. A. High cortisol responses identify propensity to obesity that is linked to thermogenesis in skeletal muscle. *FASEB J.* 28, 35–44 (2014). www.fasebj.org**

Key Words: stress · energy expenditure · hypothalamic-pituitary-adrenal axis · postprandial

THE EPIDEMIC OF OBESITY and type 2 diabetes in the world is growing unimpeded, with most current strategies for prevention and treatment being ineffective. Currently, the only effective treatment for morbid obesity is surgical intervention. Efforts to curtail food intake by pharmacological means have failed, but relatively little attention has been paid to the other arm of the energy equation—energy expenditure. Targeting energy expenditure for therapeutic intervention may provide a solution to the escalating problem. In this study, we identified a means by which propensity to become obese can be predicted—in essence, the cortisol response of sheep to a Synacthen [adrenocorticotropin (ACTH)] challenge. The method identifies high responders (HRs) and low responders (LRs), with the former showing a relatively greater gain in adiposity on a high-energy diet. This predictor of the propensity to become obese may be a useful tool in managing obesity.

A relationship between the set point of the stress axis and the tendency to become obese has been suggested in various studies. A classic example is that of Cushing's syndrome, in which elevated cortisol levels are associated with an obese phenotype. In obese, non-Cushing's patients, a high cortisol response to either corticotropin-releasing hormone (CRH) or ACTH (1, 2) is also associated with elevated visceral adiposity. Various rodent models of obesity, such as ob/ob mice and fatty Zucker rats, have elevated glucocorticoid levels (3–5), and the obese phenotype is attenuated by adrenalectomy (5). Although glucocorticoid levels are associated with obesity, it is not known whether individuals with a relatively higher activity within the hypothalamic-pituitary-adrenal (HPA) axis have a greater propensity to become obese. An important question, therefore, is

Abbreviations: aCSF, artificial cerebral spinal fluid; ACTH, adrenocorticotropin; AUC, area under the curve; CRH, corticotropin-releasing hormone; CV, coefficient of variation; ECL, enhanced chemiluminescence; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; GTT, glucose tolerance test; HPA, hypothalamic-pituitary-adrenal; HR, high responder; HRP, horseradish peroxidase; i.c.v., intracerebroventricular; LR, low responder; NEFA, nonesterified fatty acid; RCR, respiratory control ratio; RyR1, ryanodine 1 receptor; SERCA, sarcoendoplasmic reticulum Ca^{2+}-dependent ATPase; UCP, uncoupling protein

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whether up-regulation of the HPA axis is a cause or a consequence of obesity.

In humans, prednisolone treatment increases food intake in postmenopausal women (6), and, in female sheep, chronic elevation in cortisol increases food intake (7). Clinical studies have shown that most subjects increase food intake in response to stress, but there is a small subpopulation (~15%) in which food intake is reduced (8). Furthermore, cortisol and stress responsiveness may predict the metabolic effects of glucocorticoids. Thus, subjects characterized as HRs tend to eat more in response to stress or after corticotropin-releasing factor (CRF) challenge than do those classified as LRs (8–10). Furthermore, male sheep with high responsiveness to ACTH eat beyond their predicted requirement, whereas those with low responsiveness do not (11).These findings indicate that stress and innate differences in cortisol responsiveness may have an effect on predictive adiposity; however, this possibility has not been directly addressed.

We sought to determine whether differences in cortisol responsiveness predict the propensity for obesity and to identify the possible mechanisms, particularly with regard to energy expenditure. Energy expenditure comprises basal metabolism, physical activity, and thermogenesis. Dissipation of energy through the production of heat (thermogenesis) is well characterized in brown adipose tissue (12, 13), but we, and others, have shown that skeletal muscle also exhibits thermogenic properties (14–20). In sheep, postprandial muscle thermogenesis coincides with an increase in the expression of *ryanodine 1 receptor (RyR1)* and sarcoplasmic Ca^{2+}-dependent ATPase (SERCA)-2a, as well as altered mitochondrial function, which is indicative of adaptive thermogenesis (21). It is not known whether innate differences in skeletal muscle thermogenesis alter the predisposition to obesity. The present studies were undertaken in animals derived from an outbred population, in which we identified ewes at the extreme ends (high and low) of the cortisol response to ACTH. High cortisol responsiveness, in the presence of lowered thermogenesis in skeletal muscle, predicted the propensity to become obese.

METHODS AND MATERIALS

Selection of HRs and LRs

We used an outbred population (n=100) of sheep to identify HRs and LRs. We challenged animals with synthetic ACTH (Synacthen, 0.2 μg/kg body weight; Novartis Pharmaceuticals, North Ryde, NSW, Australia). During the breeding season, ovarian cycles were first synchronized by i.m. injection of 125 μg prostaglandin [cloprostenol (Estrumate); Pitman-Moore, Sydney, NSW, Australia], and the ACTH challenge was conducted 1 wk later, during the luteal phase of the estrous cycle. Blood samples (6 ml) were collected by venipuncture at −30, 0, 30, 60, and 90 min from injection, and the plasma was stored at −20°C until assayed for cortisol.

The animals underwent an initial screening and were characterized as either HRs or LRs on the basis of cortisol response, as determined by the area under the curve (AUC). The selected animals classified as HRs or LRs were subjected to a second Synacthen challenge, to confirm the initial results.

Cortisol levels were determined by the radioimmunoassay method of Bocking *et al.* (22). Assay sensitivity was 0.4 ± 0.02 ng/ml, the intra-assay coefficient of variation (CV) was 8%, and the interassay CV was 10%.

Experiment 1: effects of a high-energy diet on body weight and body composition in LRs and HRs

Subsets of selected animals were subjected to a high-energy diet (n=5). The animals were held in pasture and fed a supplemented diet of lupin grain and oats for 16 wk (23, 24). This diet causes an increase in adiposity (23, 24). Body weight was monitored weekly, and body composition was determined by dual energy X-ray absorptiometry (DXA) before the start of the high-energy diet and after 8 and 16 wk of dietary manipulation.

Food intake was measured at 2 time points. To account for seasonal changes in feeding (25–27), food intake was measured in January (when voluntary food intake is high) and June (when food intake is at the nadir). Animals were housed in single pens and offered lucerne chaff (2 kg/d) or a high-energy diet (1 kg/d), and refusals were measured; food intake was monitored for 4 d.

Experiment 2: effects of meal feeding and central leptin infusion on thermogenesis in LRs and HRs

This work was carried out in HRs and LR animals (n=5) fed a normal diet, when body weight and lean body mass were similar. Silastic cannulae were implanted into the lateral ventricles for infusion of either artificial cerebrospinal fluid (aCSF; 150 mM NaCl, 1.2 mM CaCl, 1 mM MgCl, and 2.8 mM KCl) or recombinant human leptin (15). Intracerebroventricular (i.c.v.) infusion lines were connected to a microinfusion pump (Graseby Medical Ltd., Gold Coast, QLD, Australia), and infusions (110 μl/h) were performed between 09:00 and 15:00. A crossover design was used, wherein all animals received both vehicle and leptin treatments, with 1 wk between treatments.

Leptin was synthesized and purified, and efficacy was tested in a cell proliferation bioassay (28). Temperature recordings were made as an index of thermogenic output (14, 15, 29). Dataloggers (SubCue, Calgary, AB, Canada) were surgically implanted into the retroperitoneal fat and skeletal muscle of the hind limb (vastus lateralis) and were set to record temperature at 15-min intervals.

The experiment was conducted during the late breeding season, and ovarian cyclicity was synchronized by an injection of cloprostenol (125 μg i.m.) 1 wk before the temperature recordings. At 2 wk before the recordings, the animals were brought into a shed and placed in single pens with natural light and ambient temperature. A programmed meal-feeding regimen (access to food between 11:00 and 16:00 daily) was applied, commencing 2 wk before the experiment. This feeding regimen entrains a postprandial elevation in peripheral heat production in fat and muscle (14, 15, 29, 30). Food intake was measured. Serial blood samples (6 ml) were collected at 30-min intervals across the feeding period, *via* an indwelling cannula inserted into a jugular vein, extended by a manometer line, and closed with a 3-way tap. Samples were collected into heparinized tubes and centrifuged, and the plasma was harvested. Plasma samples were stored at −20°C until assayed for metabolites and insulin.

On the day of experimentation, the animals received an infusion of either leptin (10 μg/h) or aCSF (110 μl/h) between

0900 and 1500. Blood samples were collected every 30 min between 08:00 and 16:00 and used to measure glucose, lactate, insulin, and nonesterified fatty acids (NEFAs). Skeletal muscle and retroperitoneal fat temperatures were recorded across the experimental period. Insulin was measured with a validated kit (Linco, St. Louis, MO, USA; ref. 29). Assay sensitivity was 0.1 ng/ml, intra-assay CV was 9%, and interassay CV was 7%. Plasma glucose and lactate concentrations were measured with a YSI2300 STAT glucose/lactate analyzer (Yellow Springs Instrument Co., Yellow Springs, OH, USA). Plasma NEFAs were analyzed with an enzymatic kit assay (Wako, Dallas, TX, USA; ref. 31), with inter- and intra-assay CVs of 10 and 7%, respectively.

In addition to characterizing the effects of leptin, insulin sensitivity was assessed by a glucose tolerance test (GTT). Intravenous GTTs were performed in unfed (24 h) sheep during the nonbreeding season and analyzed with minimal model (MINMOD) computer software (32). MINMOD was developed by Bergman *et al.* (33) and is used to assess parameters of glucose and insulin metabolism. The animals received a bolus injection of glucose (0.25 g/kg body weight). Blood samples were collected at −10, −5, 0, 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min relative to injection and were used to measure glucose and insulin levels.

Experiment 3: measurement of mitochondrial function and futile calcium cycling in skeletal muscle

Real-time PCR

Animals (n=4–7/group) were program fed for 2 wk before the experiment, and biopsies of skeletal muscle were collected before (10:00) and during (14:00) the feeding window. Expression of *uncoupling protein (UCP)-1, UCP-2*, and *UCP-3*, as well the futile calcium cycling genes *RyR*, *SERCA1a*, and *SERCA2a*, were quantified by real-time PCR (14). The sequences for the primers (not previously described) were RyR: forward 5′-GGGATATGGGTGACACGAC-3′, reverse 3′-TCTCAGCATCAGCTTTCTCC-5′; SERCA1: forward 5′-ATCCGCCAGCTAATGAAGAA-3′, reverse 3′-GAGACGCTGGAATCCGAGTA-5′; and SERCA2a: forward 5′-GTACACCAAACAAACCAAGTCG-3′, reverse 3′-TCTGTGAAGCTGTGCCGG-5′.

The quantified RNA was then normalized against the geometric mean of the 3 most stable reference genes determined by geNorm analysis (http://medgen.ugent.be/~jvdesomp/genorm/) from a panel of 7 possible reference genes. We used β-*actin*, *cyclophilin*, and *malate dehydrogenase I*.

Western blot analysis

Protein levels of SERCA1 and SERCA2a were quantified. In brief, 60 μg of protein was loaded onto a precast gel (Mini-Proteans; Bio-Rad, Hercules, CA, USA), and the gel was run at 150 V for 1 h. Following transfer to a nitrocellulose membrane, the reaction was blocked overnight in skim milk. SERCA1 and SERCA2a levels were measured by using the mouse monoclonal primary antibodies anti-SERCA1 (clone VE121GI, 1:2500; Sigma-Aldrich, Castle Hill, NSW, Australia) and anti-SERCA2 (clone 2A7-A1, 1:1000; Sigma-Aldrich), and the membranes were probed at 4°C overnight. The secondary antibody was goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (Antibodies Australia, Melbourne, VIC, Australia), used at a dilution of 1:2000 and applied for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence (ECL; Amersham, Amersham, UK) with 30 s exposure. The membrane was then stripped and reblocked, followed by incubation in rabbit polyclonal total actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:2000 for 2 h and then in goat anti-rabbit antibody HRP at a dilution of 1:2000 (Antibodies Australia) for 1 h at room temperature. Detection was by ECL, and the X-ray films were exposed for 1 min.

Mitochondrial respiration

Respiratory capacity was measured in isolated mitochondria (14) with a Clark electrode (Hansatech Instruments, King's Lynn, UK). Respiration was measured at 37°C in 250 μg of mitochondrial protein. Mitochondria were stimulated with 5 mM pyruvate and 2.5 mM malate, to determine substrate-driven respiration. Coupled or state 3 respiration was assessed on the addition of 150 μM ADP. To characterize uncoupled or state 4 respiration, ATP synthase was blocked by oligomycin (1 μM), and maximum respiratory capacity was determined by the addition of 1 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). The respiratory control ratio (RCR) was calculated as a measure of mitochondrial uncoupling, by determining the ratio of state 3 to state 4 respiration.

Mitochondrial DNA sequencing

DNA was obtained from lymphocytes according to a cell culture DNA isolation protocol, with the Puregene DNA isolation kit (Qiagen, Chadstone, VIC, Australia). PCR amplification of 895 bp of the D-loop region of the ovine mtDNA D-loop region (15,532–16,427; GenBank accession no. NC001941) was performed in 50 μl reactions. The reaction consisted of 200 ng of total DNA, 1× PCR buffer (BioLine, Alexandria, NSW, Australia), 1.5 mM $MgCl_2$ (BioLine), 200 μM dNTPs (BioLine), 0.5 μM of the forward (15,532–15,550: CTTCCCACTCCACAAGCC) and reverse (16,399–16,427: CATTAATTATATTATGCCCATGCTTACC) primers of the ovine mtDNA genome (GenBank accession no. NC001941), and 2.5 U BioTaq DNA polymerase (BioLine). Reaction conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 56°C for 30 s, and 72°C for 60 s and then 72°C for 3 min. The reactions were run in a PTC-200 DNA engine (MJ Research, Waltham, MA, USA). The PCR products were resolved on 2% agarose gels (BioLine) at 100 V for 1 h against a 100-bp DNA ladder (Hyperladder; BioLine), excised from the agarose gels, and purified for DNA sequencing with the QIAquick gel extraction kit (Qiagen), as described in the manufacturer's protocol,. The purified mtDNA was then sequenced according to the automated direct-sequencing protocol (34), with a GeneAmp Veriti 96-well Thermal Cycler and the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), with both the respective forward and reverse primers. Electrophoresis of cycle-sequencing products was performed on a 16-capillary 3130xl Genetic Analyzer (Applied Biosystems). Sequences were aligned and phylograms produced with ClustalW2 [European Microbiology Laboratory-European Bioinformatics Institute (EMBL-EBI), Hinxton, UK; http://www.ebi.ac.uk/Tools/msa/clustalw2/].

Statistical analyses

All data were analyzed by repeated-measures ANOVA, and *post hoc* analyses were carried out using a least significant difference test, where appropriate.

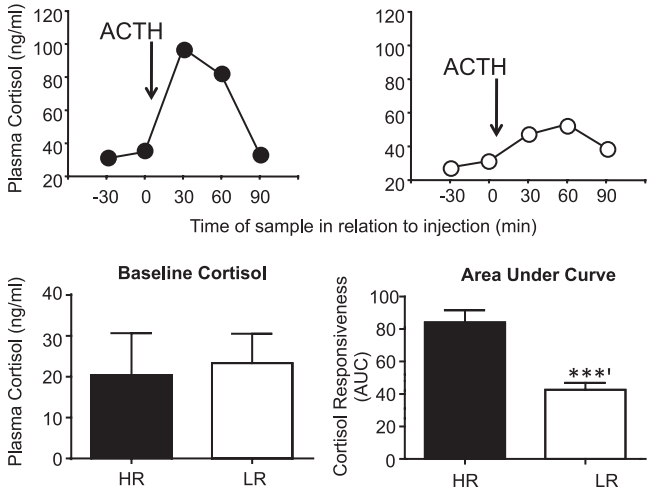

Figure 1. Cortisol profiles in HRs (solid circles and bars) and LRs (open circles and bars) using a Synacthen challenge. Top panels: representative profiles of an HR and an LR animal. Bottom panels: baseline cortisol levels were similar in HRs and LRs. Approximately 10% of animals segregated as either LRs or HRs based on measurement of the area under the cortisol concentration × time curve (n=10/group). ***$P < 0.001$.

RESULTS

Selection of HRs and LRs

From 100 sheep, 10 HRs and 10 LRs were identified by Synacthen challenge (**Fig. 1**). Before injection of Synacthen, plasma cortisol levels were equivalent in HRs and LRs. Cortisol responsiveness, as determined by the AUC, was greater (P<0.001) in HRs compared to LRs.

Experiment 1: a high-energy diet unmasks predisposition to obesity in HRs

At baseline, without dietary intervention, body composition was similar in HRs and LRs (**Fig. 2*A***). High-energy feeding unmasked differences in the propensity for obesity between the two groups, such that HRs had a greater gain in percentage of adiposity than LRs. After 16 wk of high-energy feeding, the percentage of adiposity was 4.6 ± 1.4% greater (P<0.01) in HRs than LRs (Fig. 2*B*). To further analyze the effects of the high-energy diet on body composition, we performed a correlation analysis to determine the relationship between cortisol responsiveness and percentage of adiposity. At baseline, there was no significant relationship between the AUC of cortisol response and the degree of adiposity (Fig. 2*C*). During high-energy feeding, cortisol responsiveness and percentage of adiposity at both 8 and 16 wk of dietary manipulation showed a strong correlation (P<0.01). Thus, HRs exhibited an increased propensity for obesity that was unmasked by the high-energy diet.

Experiment 2: increased susceptibility to obesity is underpinned by a reduction in thermogenesis

To determine the physiological mechanisms that underpin the differing susceptibility to obesity in HRs and LRs, we measured food intake and thermogenic out-

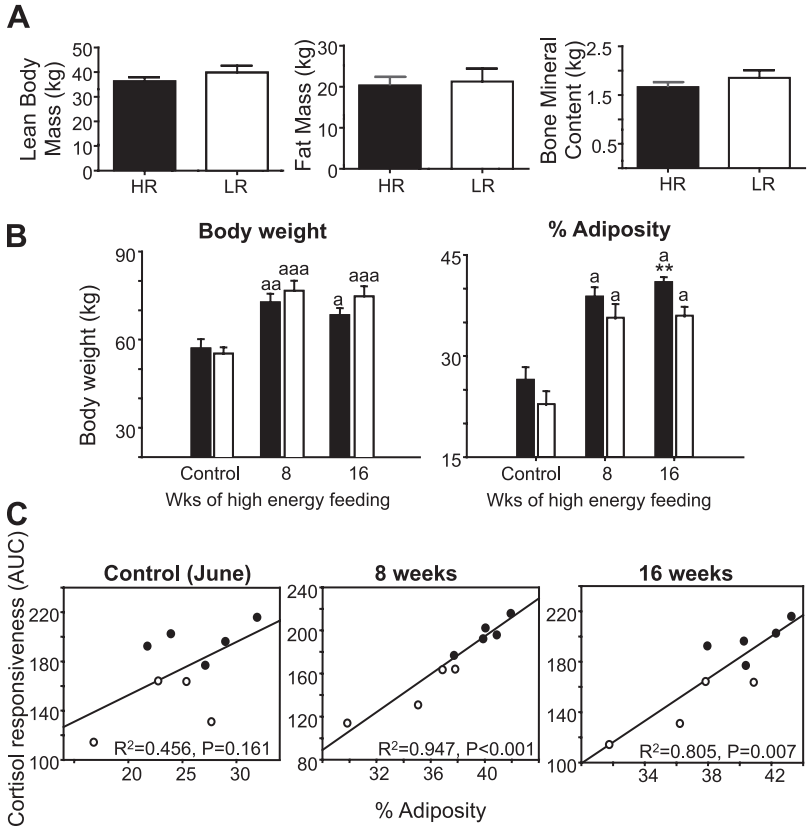

Figure 2. Body composition in HRs and LRs before (*A*) and during (*B*) consumption of a high-energy diet. *A*) Before dietary manipulation, body composition was similar in the 2 groups. *B*) Body weight and percentage of adiposity increased in HR and LR animals after 8–16 wk of high-energy feeding. At 16 wk, percentage of adiposity was greater in HRs (solid bars) than LRs (open bars). *C*) Feeding of a high-energy diet (solid circles, HR group; open circles, LR group) revealed a correlation between cortisol responsiveness and percentage of adiposity (n=4–5/group). $^{a}P < 0.05$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$ *vs.* control; **$P < 0.01$ *vs.* LRs.

put. Food intake was measured at 2 times of the year, since sheep are known to exhibit a circannual feeding pattern and response to leptin (26, 27, 35). Intake of either chaff or high-energy pellets was similar in HRs and LRs, but the LRs exhibited a small increase ($P<0.05$) in lucerne chaff intake in January. Also, in LRs, intake of high-energy pellets was higher ($P<0.05$) in June (**Fig. 3*A***).

Temperature profiling in skeletal muscle and retroperitoneal adipose tissue was undertaken to assess thermogenesis in HRs and LRs, during programmed feeding with a normal diet (Lucerne chaff) and when body weights and body composition were similar. Consistent with increased adiposity on the high-energy diet, the feeding-induced thermogenic responses in the muscle were lower ($P<0.01$) in HRs than LRs (Fig. 3*B*, top panels). This difference was enhanced during i.c.v. infusion of leptin ($P<0.01$; Fig. 3*B*, bottom panels). Leptin treatment increased ($P<0.01$) the temperature (treatment×time interaction) in adipose tissue, but the effect was similar in HRs and LRs (Fig. 3*B*, right panels). As expected, leptin reduced ($P<0.001$) food intake, but the effect was equivalent in both groups (**Fig. 4*A***).

We also measured plasma metabolites and insulin concentrations in HRs and LRs before and after feeding. Levels of glucose, lactate, and NEFAs were similar in both groups. Plasma concentrations of insulin were higher ($P<0.05$) in LRs. There was no effect of i.c.v. leptin treatment on plasma metabolites or insulin levels (Fig. 4*B–E*). To determine whether the elevated levels of insulin conferred differences in insulin sensitivity and glucose metabolism, we used MINMOD to conduct a GTT (**Fig. 5** and **Table 1**). The GTT was conducted when animals were at a similar body weight, had similar body composition, and were consuming a normal diet. Basal levels of insulin and glucose were similar in HRs and LRs, as were insulin sensitivity, glucose effectiveness, acute insulin response to glucose, and indices of β-cell function and insulin resistance. Insulin sensitivity and glucose metabolism were similar in HRs and LRs when body weights and percentage of adiposity were similar between the groups.

Experiment 3: no difference in mitochondrial function and futile calcium cycling in cortisol HRs and LRs

To characterize the cellular mechanisms that are responsible for divergent muscle thermogenesis in the HRs and LRs, we measured mitochondrial function (mitochondrial respiratory capacity and expression of *UCP-1, UCP-2*, and *UCP-3*) and indices of calcium cycling (expression of *RyR1, SERCA1*, and *SERCA2a*), before and during the feeding window. We aimed to determine whether there are functional disparities that explain the differences in propensity to become obese on a high-energy diet. In isolated mitochondria, feeding did not affect substrate-driven respiration (**Fig. 6*A***), but reduced both state 3 respiration ($P<0.05$) and total respiratory capacity ($P<0.001$) (Fig. 6*B*, *D*) in both HR and LR animals. In addition, state 4 respiration increased ($P<0.05$) during feeding, indicating a switch toward uncoupled respiration, and there was an attendant reduction ($P<0.01$) in RCR (Fig. 6*C*, *E*). Feeding-induced changes in mitochondrial function were similar in HRs and LRs.

Expression of *UCP-1, UCP-2*, and *UCP-3* in skeletal muscle was similar in HRs and LRs. There was no effect of feeding on the levels of *UCP-1* or *UCP-2* mRNA, but *UCP-3* expression increased ($P<0.05$) during the feeding window (**Fig. 7**). Analysis of the mitochondrial

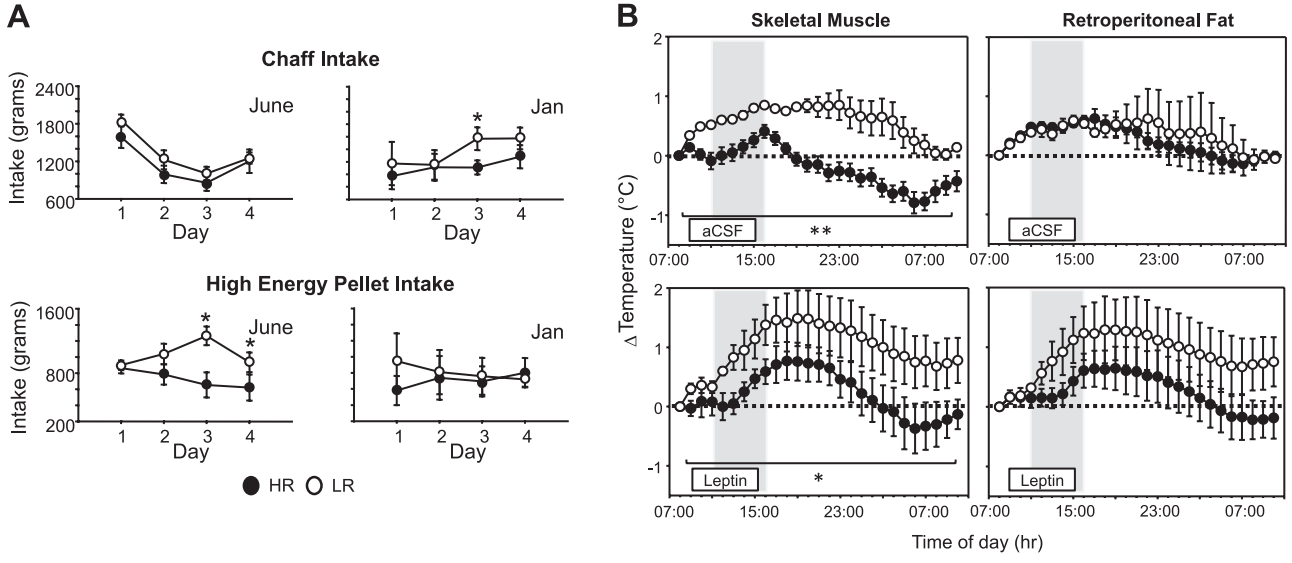

Figure 3. *A*) Food intake (left panels) and thermogenesis (right panels) in HRs and LRs. Food intake was measured at 2 different times of the year, because of the circannual rhythm (27, 46). Food intake was similar in LRs and HRs. *B*) Longitudinal temperature recordings provided an index of thermogenic output in skeletal muscle and retroperitoneal adipose tissue. Skeletal muscle temperature was higher in LRs than HRs in response to both feeding (aCSF infusion) and i.c.v. leptin infusion (overall group effect). Retroperitoneal fat temperature was similar in HRs and LRs ($n=4$/group). $^*P < 0.05$, $^{**}P < 0.01$ for HRs *vs.* LRs.

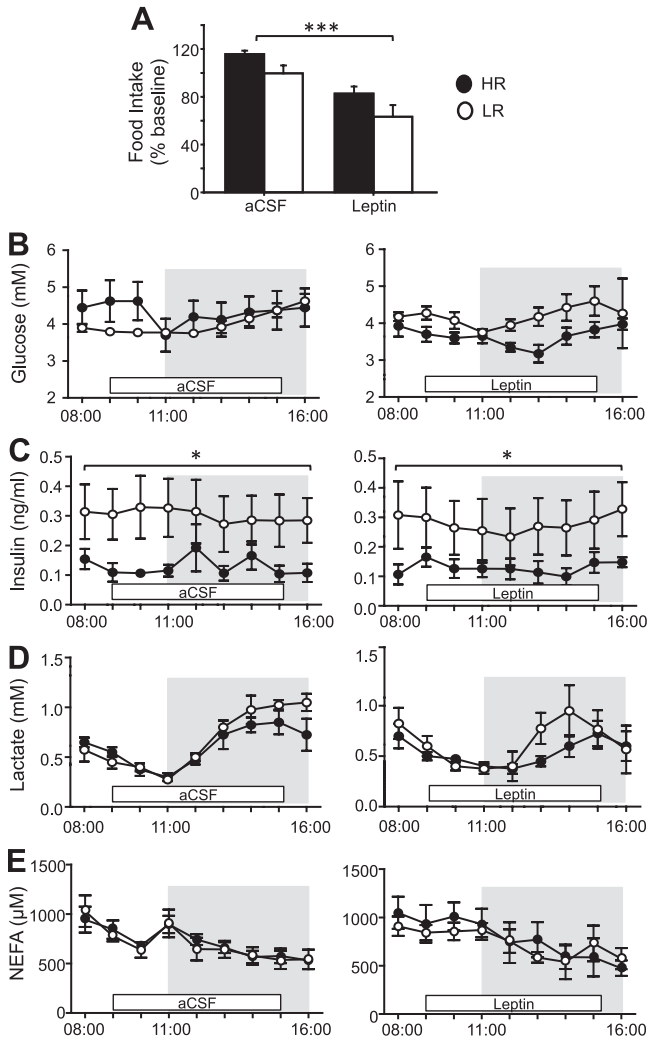

Figure 4. Effect of i.c.v. leptin infusion on food intake and plasma metabolites in HRs and LRs. *A*) Food intake was similar in both groups. *B–E*) Plasma levels of glucose (*B*), lactate (*D*), and NEFAs (*E*) were similar in HR and LR animals before and during leptin treatment; plasma insulin levels (*C*) were higher in LRs, irrespective of treatment (overall group effect; $n=4$/group). $^*P < 0.05$ for HRs *vs.* LRs; $^{***}P < 0.001$ for leptin *vs.* control.

genome by DNA sequencing demonstrated that there was no haplotypic variation or clustering between the HRs and LRs, based on the genetic distance across the 2 groups, which suggests that innate differences in mitochondrial function do not determine the thermogenic phenotype within skeletal muscle. Accordingly, we measured the expression of *RyR1, SERCA1*, and *SERCA2a* in skeletal muscle as an index of calcium cycling. Feeding increased *RyR1* ($P<0.001$) and *SERCA1* ($P<0.05$) mRNA levels in HRs and LRs without an associated effect on *SERCA2a* mRNA levels (**Fig. 8**). In contrast, feeding did not alter SERCA1 protein levels, but increased ($P<0.05$) SERCA2a protein. As with mitochondrial respiration, up-regulation of the indices of calcium cycling during feeding was similar in HRs and LRs. Thus, feeding altered mitochondrial function as well as the expression of factors that mediate calcium cycling in muscle, but these changes do not account for the differences in muscle thermogenesis in HR and LR animals.

DISCUSSION

In our study, the 10% extremes in an outbred population of animals were identified as cortisol HRs and LRs after ACTH challenge, and HR animals had an increased propensity to preferentially gain adipose tissue on an obesogenic diet. Innate differences in cortisol responsiveness have been shown in several species (8, 11, 36, 37), but the current study is the first to indicate a direct link between cortisol responsiveness in the propensity to become obese. Without dietary manipulation, HR and LR animals have similar body composition, but, when fed a high-energy diet, HR animals become relatively more obese. HR animals preferentially gained adipose tissue, whereas LR animals gained lean body mass on the high-energy diet. Food intake was essentially similar in HRs and LRs, suggesting that the observed differences in those on a high-energy diet were due to innate differences in energy expenditure. We found that differences in susceptibility to obesity were underpinned by innate differences (measured on a normal diet) in muscle thermogenesis. This difference was confined to skeletal muscle, which represents 40% of body mass. Thus, in the current study, cortisol responses to ACTH predicted the propensity to become obese, and the effect was explained by differences in muscle thermogenesis.

Studies in humans have shown that altered thermogenic function in skeletal muscle determines the ability to successfully lose and maintain weight loss in obese individuals. Harper *et al.* (20) linked reduced expres-

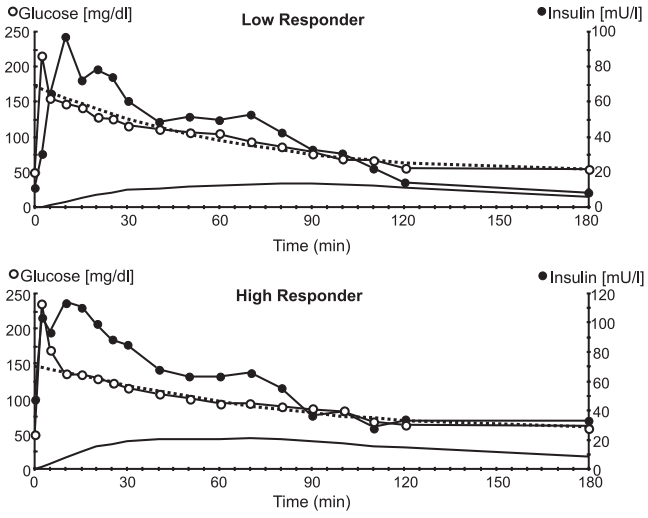

Figure 5. Plasma insulin and glucose responses to a glucose challenge in HRs and LRs. Analyses were performed with MINMOD software (millennium edition). Representative insulin and glucose profiles are shown ($n=5$/group). There were no significant differences in any of the metabolic parameters assessed during the GTT (Table 1).

TABLE 1. *Plasma insulin and glucose responses to a glucose challenge in HRs and LRs*

Parameter	HRs	LRs	*P*
Basal glucose (mg/dl)	53.2 ± 1.2	55.5 ± 0.3	0.4
Basal insulin (mU/L)	16.7 ± 4.5	24 ± 8.4	0.4
S_i (mU/L/min)	0.7 ± 0.1	2.4 ± 1	0.09
S_g (min^{-1})	0.01 ± 0.002	0.02 ± 0.001	0.1
AIR_g (mU/L/min)	511.3 ± 73	734.7 ± 147.2	0.3
β-cell function (mU/mM)	−1119.4 ± 363.8	−1778.6 ± 1358.3	0.7
HOMA-IR (mU/mM/min^2)	3.3 ± 1.1	2.4 ± 0.7	0.5

Analyses were performed with MINMOD software (millennium edition). There were no significant differences in any of the metabolic parameters assessed ($P \geq 0.05$; $n=5$/group). S_i, insulin sensitivity; S_g, glucose effectiveness; AIR_g, acute insulin response to glucose; HOMA-IR, homeostasis model of assessment-insulin resistance.

sion of UCP-3 in muscle to a reduction in mitochondrial proton leak and impaired ability to lose weight. In our study, lowered muscle thermogenesis was evident before weight gain in HRs and was therefore likely to be an important determinant of susceptibility to obesity. To define the mechanism that underpins differences between LRs and HRs in skeletal muscle thermogenesis, we measured mitochondrial function and indices of calcium cycling. Consistent with our previous work, we showed that postprandial thermogenesis in skeletal muscle was associated with increased *UCP-3* mRNA levels (29) and an increase in uncoupled mitochondrial respiration (21). Changes in *UCP-3* expression and mitochondrial respiration, however, were equivalent in HRs and LRs. To further investigate a possible link between mitochondrial function and muscle thermogenesis in HR and LR animals, we sequenced the mitochondrial genome. Mitochondrial DNA encodes key genes of the electron transport chain. Haplotypic variation in mitochondria DNA sequences suggest variation in the generation of ATP and have been linked to innate differences in reproductive function as well as cold tolerance (38). There was no apparent haplotypic segregation between the HR and LR groups, which suggests that the differences in thermogenic capacity between HR and LR animals were not determined by maternal inheritance and further substantiates the notion that the divergence in thermogenesis in LRs and HRs was not due to altered mitochondrial capacity or function. It seems, therefore, that the disparity was the result of an innate difference in muscle thermogenesis, especially in response to feeding cues.

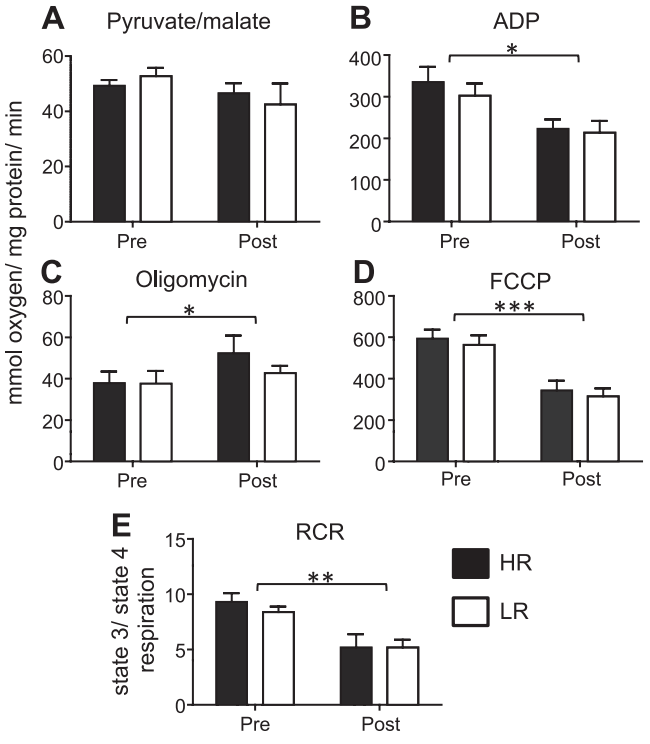

Figure 6. Mitochondrial respiration was measured in isolated skeletal muscle mitochondria taken before the feeding window (pre) and during the feeding window (post). There was no effect of feeding on substrate-driven (pyruvate and malate) respiration (*A*). Coupled respiration (ADP, *B*) and total respiratory capacity (FCCP, *D*) were reduced after feeding. Uncoupled respiration (oligomycin, *C*) increased after feeding in both the HRs and LRs and produced an overall reduction in the RCR (*E*). Thus, during feeding, mitochondria displayed increased uncoupled respiration. Although there were effects of feeding on respiratory function, there were no apparent differences between HRs and LRs ($n=5$/group). $^*P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$.

In addition to mitochondrial function, futile calcium cycling has been linked to muscle thermogenesis and energy expenditure. Calcium cycling occurs across the sarcoplasmic reticulum (SR), whereby it is expelled *via* the RyR1. Increased cytosolic calcium concentrations activate SERCA1 and SERCA2a in skeletal muscles, and they propel the calcium back into the SR through the hydrolysis of ATP. A recent study has shown that genetic deletion of sarcolipin (an endogenous activator of SERCA) impairs cold tolerance and predisposes animals to diet-induced obesity (19). In our study, postprandial thermogenesis coincided with increased *RyR1* and SERCA levels in skeletal muscle (21). Activating mutations in RyR1 cause malignant hyperthermia (18, 39) and have been linked to stress responsiveness and aggressive behavior in pigs (40). Mutations that lead to malignant hyperthermia are also linked to lower cortisol levels (40), which is consistent with our present data indicating that LRs have relatively higher muscle thermogenesis. Despite this, postprandial levels of *RyR1* and SERCA were similar in HRs

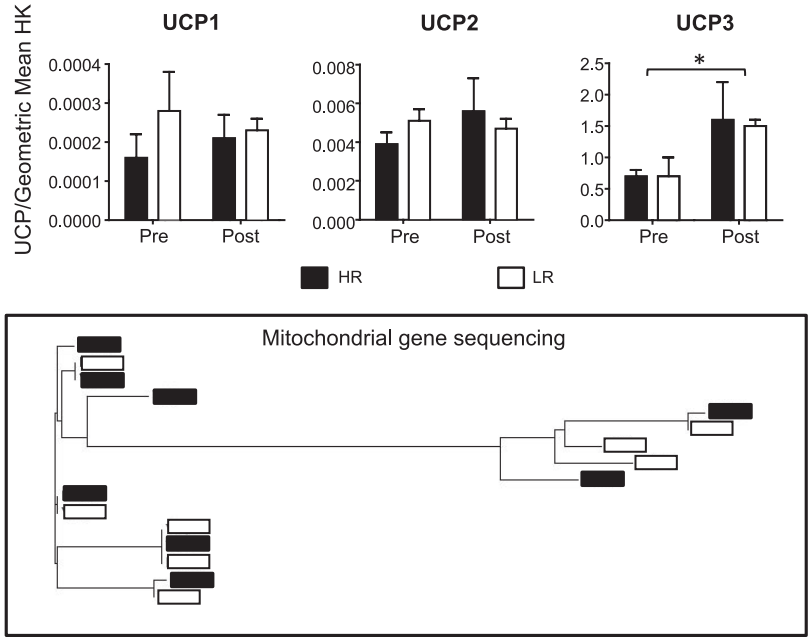

Figure 7. Top panel: expression of *UCP-1*, *UCP-2* and *UCP-3* mRNA in skeletal muscle. Feeding increased levels of *UCP-3* mRNA in muscle, but the effect was equivalent in HRs and LRs. There was no effect of feeding, nor were there any differences between HRs and LRs in the expression of *UCP-1* and *UCP-2*. HK, housekeeping genes. Bottom panel: mitochondrial DNA was sequenced to profile mitochondrial gene differences. Phylogenetic tree indicates no haplotypic segregation based on genetic differences between HRs and LRs (n=6–8/group). *$P < 0.05$.

and LRs. Thus, although postprandial thermogenesis in skeletal muscle is associated with up-regulation of markers of futile calcium cycling, it does not appear to account for differences in the propensity to obesity in HR and LR animals.

We assessed leptin and insulin sensitivity to determine whether altered responsiveness to either hormone could account for differences in muscle heat production in LRs and HRs. In earlier studies, we showed that central leptin treatment increases postprandial thermogenesis in adipose tissue and skeletal muscle (14, 15), and this finding was replicated in the current study. It is notable, however, that the muscle thermogenic response to leptin was greater in LRs compared to HRs. Despite this, the satiety response to leptin was also similar in HRs and LRs, further indicating that the fundamental set-point difference is not due to this arm of the energy equation. In contrast, plasma insulin levels were higher in LRs than in HRs, and there was also a tendency toward increased plasma glucose levels in LR animals treated with leptin. This disparity, however, does not translate into differences in insulin sensitivity, as demonstrated by the GTT. Of note, at the time of the GTT, basal levels of insulin in HRs and LRs converged as a result of food withdrawal. Overall, differences in muscle heat production were not due to inherent differences in either leptin or insulin sensitivity. We propose that the variance in muscle heat production may be caused by differences

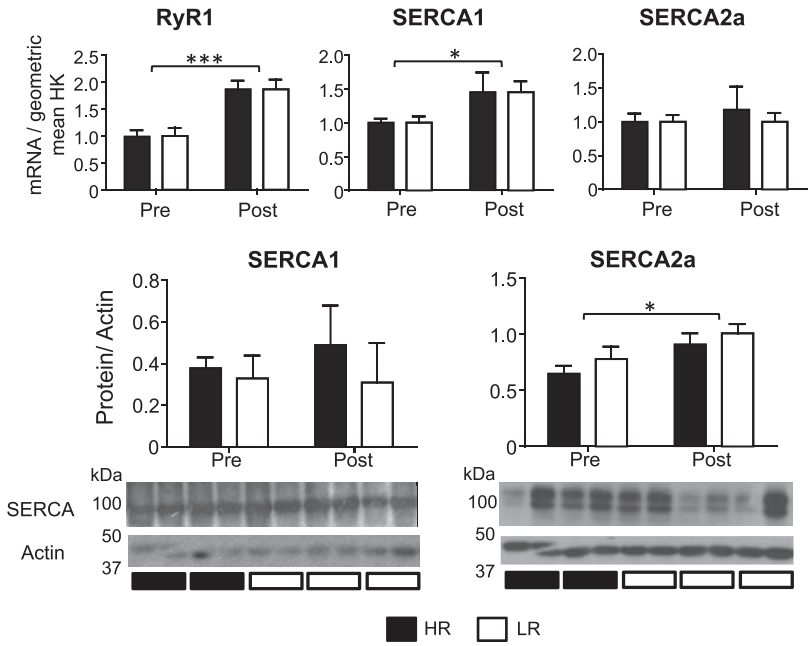

Figure 8. Quantification of components of calcium cycling. Expression of *RyR1, SERCA1*, and *SERCA2a* mRNA (top panel) and SERCA protein (bottom panel). Feeding increased *RyR1* mRNA and *SERCA1* expression without a corresponding effect on *SERCA2a* expression. SERCA2a protein levels (Western blots) increased during feeding. There were no differences in the expression of markers of calcium cycling in HRs and LRs (n=4–6/group). HK, housekeeping genes. *$P < 0.05$, ***$P < 0.001$.

in the metabolic function of muscle, and we are pursuing this line of inquiry.

I.c.v. infusion of leptin increased heat production in both skeletal muscle and retroperitoneal fat. Heat production in the latter was equivalent in HRs and LRs, whereas muscle temperature was higher in the LR group. In sheep, brown adipocytes are interspersed among white adipocytes in the retroperitoneal fat, and a recent study in lambs also indicated that the sternal and clavicular adipose depots are enriched in brown adipocytes, expressing high levels of UCP-1 mRNA (41). To date, however, there are no equivalent data on these fat beds in adult sheep. We focused on the retroperitoneal fat bed, because our work in adults has shown greater expression of UCP-1 in this fat bed than in subcutaneous fat (30). Furthermore, the thermogenic effect of both leptin and estrogen is greater in retroperitoneal fat than in subcutaneous adipose tissue (15, 42). Thus, our previous work suggests that, at least in adult sheep, the retroperitoneal fat depot contributes to total body adaptive thermogenesis. Nonetheless, in the current study, the fundamental difference in adaptive thermogenesis in the LRs and HRs occurred in muscle and not in retroperitoneal fat.

In summary, cortisol responsiveness to an ACTH challenge is a simple test that predicts the propensity for obesity. HR animals have increased susceptibility to gain adipose tissue when fed a high-energy diet, and this is due to reduced skeletal muscle thermogenesis. It is unequivocal that most obese subjects resist weight loss and that long-term maintenance of weight loss is problematic (43–45). We propose that cortisol responsiveness is a marker for predisposition to obesity and provides a means to identify individuals that exhibit reduced thermogenesis and consequent energy expenditure. This innate property of thermogenesis affects susceptibility to weight gain. FJ

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Chapter 4

Stress-Induced Behavioural and Metabolic Adaptations Lead to an Obesity-prone Phenotype in Ewes with Elevated Cortisol Responses.

Declaration for Thesis Chapter 4

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution
Design & carrying out of experiments, coordination of groups involved, laboratory analysis of samples and statistical analysis of data. Writing up paper and creation of graphs for publication. Extent of Contribution: 90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Caroline Lee	Supervise design of animal behaviour experiments
Robert Bischof	Analyse samples for cytokines
Gavin Lambert	Analyse samples for catecholamines

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	

Main Supervisor's Signature	

Date:

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

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Stress-induced behavioral and metabolic adaptations lead to an obesity-prone phenotype in ewes with elevated cortisol responses

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KEYWORDS
Hypothalamo-pituitary-adrenal axis;
Lipopolysaccharide;
Thermogenesis;
Energy balance

Summary The underlying cause of predisposition to obesity is complex but one marker is cortisol responsiveness. Selection of sheep for high (HR) or low (LR) cortisol responses to adrenocorticotropin shows that HR are more likely to become obese. Increased propensity to obesity is associated with reduced skeletal muscle thermogenesis. We sought to determine whether metabolic or behavioral responses to stress also contribute to altered propensity to obesity in LR and HR. Animals ($n = 5—10$/group) were exposed to 3 stressors and we measured food intake and thermogenesis (recorded with dataloggers implanted into muscle). Stressors were hypoglycaemia (0.125 units/kg insulin, IV), a barking dog and immune challenge (200 ng/kg lipopolysaccharide — LPS, IV). LR animals showed a greater catabolic state in response to both immune and psychosocial stressors. LPS reduced ($P < 0.01$) food intake in both groups but LR showed a greater ($P < 0.05$) reduction in food intake and a more substantial ($P < 0.05$) rise in muscle temperature. Introduction of the barking dog reduced ($P < 0.05$) food intake in LR only. These metabolic differences coincided with differences in cortisol responsiveness, where HR animals had increased ($P < 0.05$) cortisol in response to both immune and psychosocial stressors. We also

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assessed behavior in the following paradigms: 1, isolation in the open field test; 2, response to a human intruder; and 3, food competition. LR had greater ($P < 0.05$) activity, reduced fearfulness and displayed a proactive coping style of behavior. Thus we demonstrate that high cortisol responsiveness identifies animals with stress-induced metabolic and behavioral traits that may contribute to susceptibility to obesity.
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1. Introduction

There is a strong nexus between the hypothalamo-pituitary-adrenal (HPA) axis and energy homeostasis. Hallmark studies in rats by Dallman and colleagues demonstrate that stress increases the preference for palatable foods that are high in fat and sugar (Pecoraro et al., 2004; Dallman et al., 2005; Dallman, 2010; Tomiyama et al., 2011). This led to the 'comfort' food hypothesis, stating that increased consumption of foods high in fat and sugar leads to feedback on the HPA axis to dampen the stress-induced elevations in circulating corticosteroid levels (la Fleur et al., 2005).

In addition to the availability of palatable foods, effects of stress on feeding are influenced by the type of stressors as well as the severity and duration of the stressor (Valles et al., 2000; Solomon et al., 2007; Calvez et al., 2011). In humans, stress has been shown to increase food intake in the majority of subjects and reduces intake in only a small sub-population of around 10—15% (Epel et al., 2001). Interestingly, the effect of stress on food intake may be primarily determined by individual variation in cortisol responsiveness. In this regard, we refer to variation in the rise of plasma cortisol as a difference in cortisol responsiveness. Thus in any given population, high or low cortisol responder individuals can be identified (Epel et al., 2001; Touma et al., 2008; Tomiyama et al., 2011; Lee et al., 2014). Cortisol responsiveness is a strong determinant of the feeding response to stress, whereby subjects characterized as high cortisol responders (HR) tend to eat more in response to stress than do low cortisol responders (LR) (Epel et al., 2001; Adam and Epel, 2007; Tomiyama et al., 2011).

We have developed a model of cortisol responsiveness using the sheep (Lee et al., 2014), where we demonstrate that high cortisol responsiveness confers a predisposition to obesity. This increased propensity to weight gain was primarily associated with a reduction in thermogenesis in skeletal muscle, without an attendant change in food intake. In addition to the predisposition to obesity, studying animals of differing cortisol response to stress may have important ramifications for animal welfare and livestock practices.

In addition to the disparate thermogenic feature, we predict that differing propensity to obesity in LR and HR may also be associated with factors such as altered metabolic responses to stress and/or innate differences in temperament and behavior. Indeed, obesity is associated with differences in temperament in humans, which manifests as low inhibitory control (Anzman and Birch, 2009) and impulsiveness (Sullivan et al., 2007). Furthermore, during infancy a calm temperament has been associated with increased adiposity (Wells et al., 1997). In sheep, adipose tissue thermogenesis is lower in animals selected for a calm temperament than in those selected for a nervous phenotype (Henry et al., 2010). It has also been hypothesized that innate differences in coping strategy (e.g. reactive compared to proactive) may impact on energy expenditure and metabolism (Garland et al., 2011). Accordingly, the current study sought to determine whether ewes characterized as either HR or LR display differences in behavioral and metabolic responses to stress, which align with altered propensity to obesity. We tested the hypothesis that LR animals generate greater negative energy balance in response to stress. We also tested the hypothesis that LR animals show behavioral characteristics associated with an aggressive and fearless phenotype. We propose that increased propensity to obesity in HR will be underpinned by an innate reduction in energy expenditure as well as distinct behavioral and metabolic coping strategies in response to stress.

2. Materials and methods

2.1. Selection of low and high cortisol responding sheep

This work was performed in Corriedale ewes (age 3—5 years) and was approved by the Monash Animal Research Platform animal ethics committee. Prior to the selection of low (LR) and high (HR) cortisol responders and prior to each experiment, reproductive status was standardized by synchronizing the estrous cycles of the ewes with an IM injection of 125 μg Cloprostenol, which causes demise of the corpus luteum and initiation of a new estrous cycle (Challis et al., 1976). Experiments were carried out 7 days later in the mid-luteal phase of the estrous cycle. Animals ($n = 100$) were given an IV injection of synthetic ACTH (Synacthen, Novartis, North Ryde, NSW, Australia) to identify HR and LR animals as described previously (Lee et al., 2014). The synacthen challenge was performed at 1300 h, prior to which the animals were habituated to the experimental facility. Accordingly, these tests were carried out under non-stressful conditions. In brief, outbred ewes received indwelling jugular venous cannulae 24 h prior to sampling and these were kept patent with heparinized saline (100 KIU/1 L). Injection of synacthen (0.2 μg/kg body weight) and blood samples were collected at −30, 0, 30, 60 and 90 min relative to injection, centrifuged immediately at 3000 rpm at 4 °C and the plasma was harvested. The samples were stored at −20 °C until assayed for cortisol (see below). After the initial challenge, 20% of the animals identified with extreme high or low responses and the synacthen test was repeated 2 weeks later to verify the results. After the second challenge 10% of animals were selected as either a LR or HR ($n = 10$/group).

Plasma cortisol concentrations were measured by radioimmunoassay as described previously (Bocking et al.,

1986). For the selection of LR and HR cortisol responsiveness was determined by calculating the area under the curve (AUC). Across all of the assays, the average sensitivity was 0.3 ng/ml, the intra-assay coefficient of variation was 4.2% and the inter-assay coefficient of variation was 3.2% at 12 ng/ml and 4.5% at 80 ng/ml.

2.2. Experiment 1: Episodic profiling of cortisol secretion in HR and LR

There is some evidence in pregnant and fetal sheep to show that like humans, sheep display diurnal variation in cortisol secretion and this is exacerbated by meal feeding (Simonetta et al., 1991). To measure basal cortisol secretion, blood samples were collected at 10 min intervals for 6 h (09:00—15:00 h) and cortisol levels were measured as above. To characterize the secretory profile we performed deconvolution analysis and measured the number of pulses, mode (time taken from the onset of a pulse to reach the peak; min), basal secretion (ng/ml), pulsatile secretion (ng/ml), total secretion (ng/ml), pulse mass (ng/ml) and approximate entropy (ApEn) (Veldhuis et al., 1989, 1990). In addition, the AUC was assessed across the first hour of sampling (09:00—10:00 h) and for the total baseline period (09:00—15:00 h).

2.3. Experiment 2: Effect of stress on food intake, thermogenesis and plasma cortisol levels in LR and HR

Animals were subjected to 3 different stressors to quantify effects on food intake, thermogenesis and plasma cortisol levels. The animals were given a metabolic stress (insulin), a psychosocial challenge (barking dog) and an immune challenge with lipopolysaccharide, in a sequential manner. These were applied with intervening intervals of 3—4 days.

2.3.1. Part A: Insulin-induced hypoglycaemia

Two weeks prior to experimentation dataloggers (SubCue, Calgary, Canada) were implanted into the skeletal muscle (vastus lateralis) of the hind limb as previously described (Henry et al., 2008, 2011; Clarke et al., 2012), to monitor tissue temperature. Dataloggers were set to record temperature at 15 min intervals. In addition to characterizing thermogenesis, food intake was measured across the experimental period. Baseline food intake was established across the week preceding the onset of experiments. Animals were fed 2 kg of lucerne chaff at 09:00 h and refusals were weighed to determine daily intake.

Hypoglycaemia was induced by an IV bolus injection of insulin (Actrapid, Novo Nordisk Pharmaceuticals, Baulkham Hills, NSW). Blood samples (7 ml) were collected between 09:00 and 15:00 h and insulin (0.125 U/kg body weight) was injected at 12:00 h. Sampling then continued for 3 h. This dose was established in earlier work (Knott et al., 2010). Cortisol concentrations were measured in all plasma samples. Selected samples (−30, −20, −10, 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180 min relative to insulin injection) were used to measure plasma glucose concentrations, using a YSI2300 STAT glucose/lactate analyzer (Yellow Springs Instrument Co., USA). The measurable range for blood glucose was 0—30 mmol/l.

2.3.2. Part B: Psychosocial stress

To induce psychosocial stress, animals were exposed to a barking dog for 5 min at 12:00 h. The dog was guided to move from pen to pen whilst continuously barking, but direct physical contact was prevented (Pierce et al., 2008). Food intake, muscle thermogenesis and plasma cortisol concentrations were measured as outlined in Experiment 2a. Additional blood samples were collected at −10, 0, 10, 30, 50 and 70 min into tubes containing an inhibitor mix (30 mg/mL reduced glutathione and 95 mg/mL EGTA) for subsequent measurement of catecholamines. Plasma levels of adrenaline, noradrenaline and dihydroxyphenylglycol (the deaminated metabolite of adrenaline and noradrenaline) were measured by high performance liquid chromatography and coulorimetric detection as previously described (Lambert and Jonsdottir, 1998; Tilbrook et al., 2008).

2.3.3. Part C: Immune challenge

This experiment was carried out following the protocol outlined for experiment 2 except that the animals were given IV injections of lipopolysaccharide (LPS; 200 ng/kg body weight, *E. coli* 0127:B8, Sigma—Aldrich, St Louis, MO, USA) at 12:00 h. This dose was based on earlier studies (Briard et al., 2000; Elsasser et al., 2004). Food intake, muscle thermogenesis and plasma cortisol concentrations were measured. Hourly blood samples were taken (09:00—15:00 h) to measure plasma levels of tumor necrosis factor α (TNFα), interleukin (IL)-4, -6, -10 and -12, using ovine-specific enzyme-linked immunosorbent assays (ELISA) as described previously (Hope et al., 2002, 2005; Kwong et al., 2002; Rahman et al., 2004; Abeynaike et al., 2010).

2.3.4. Part D: Statistical analyses

The AUC of cortisol response as well as deconvolution data were analyzed using Student's unpaired *t*-test. Changes in glucose, cortisol (Experiments 2—4), cytokine, catecholamine levels and temperature were analyzed by repeated measures ANOVA and *post-hoc* comparisons were made using the Bonferroni test. Data are presented as means $\pm$ SEM and $P < 0.05$ was considered significant.

2.4. Experiment 3: Behavioral phenotype of LR and HR

All behavioral tests were carried out in the non-breeding season, obviating confounding effects of changes in ovarian steroids. In each experiment, except for the food competition test, animals were fed *ad libitum* prior to the onset of study. Behavior was recorded by video camera and was subsequently analyzed in a blind fashion.

2.4.1. Part A: Open field test

This was carried out to characterize behavioral responses to isolation. The test was conducted in an area of 5 m $\times$ 3 m divided into 6 equal-sized regions. The enclosure was surrounded by a 1.5 m enclosed fence. Single animals were exposed to the area for 5 min during which time behavior was

recorded. Recordings were subsequently used to measure the following indices:

Locomotor activity: the number of times the animal crossed from one of the 6 regions to another.
Vocalization: number of bleats.
Tunneling: number of attempts to bury their head under the arena entry/exit gate or fence.
Scratching: number of times the animals investigated the entry/exit gate by scratching or knocking.

The summation of these active behaviors, except locomotor activity, provided the total activity score. The data were analyzed using the non-parametric test, Mann—Whitney *U*-test.

2.4.2. Part B: Arena test

The arena test was used to characterize the response to a conflict between attractiveness to flock-mates and avoidance due to fearfulness of a human. The animals entered an arena (5 m × 3 m) that was enclosed by 3 walls. At the open end of the arena 2 flock mates were located in visible and auditory contact with the test animal. The human stood between the test subject and its conspecifics. The arena was equally divided into 3 areas, with zone 1 being the closest to the human observer and zone 3 being the furthest. Behavior of the test animal was recorded for 5 min. We quantified the amount of time each animal spent in zone 1, 2 or 3. Spending time in zone 1 facing the human intruder was deemed to indicate less fearfulness and greater motivation to be with flock-mates than standing further away in zone 2 or zone 3. All data were analyzed using a Mann—Whitney *U*-test.

2.4.3. Part C: Food competition test

The food competition test was used to examine the competitiveness of an animal in getting to a food source. Prior to the food competition test all animals were fasted for a minimum of 12 h. We used the test to mark one aspect of a proactive coping style which is to demonstrate initiative, as opposed to a reactive coping style which tends to be more passive. The test was carried out by placing either a HR or LR ewe in a pen next to a randomly selected control animal. At $t = 0$, the pen doors were opened and both sheep were allowed to move down a corridor measuring 13 m in length. A trough of food (chaff and lupin grain) was placed at the end of the corridor, which was 0.8 m wide. Animals were trained to learn the feed source location for 2 days prior to testing; animals were exposed to the test area on 2 occasions each day. The time latency for each test animal to reach the food and begin eating was measured and analyzed relative to the latency for the control. Results were analyzed using an unpaired Student's *t*-test.

3. Results

3.1. Selection of high and low cortisol responding sheep

Cortisol responses to ACTH were greater ($P < 0.01$) in HR than in LR as assessed by AUC (Fig. 1A). Of 100 outbred animals, 10% were selected as HR and LR ($n = 10$/group). Our previous data demonstrates that, on a normal diet, body weights and body composition are similar in LR and HR (Lee et al., 2014) and this was replicated in the current study. At the time of experimentation LR were 66.4 ± 4 kg and HR were 64.4 ± 4.8 kg.

3.2. Experiment 1: Episodic profiling of cortisol secretion in LR and HR

At the time of experimentation food intake was similar in LR (1.4 ± 0.2 kg/day) and HR (1.6 ± 0.2 kg/day). Deconvolution analysis of the cortisol data showed that the number of pulses, basal secretion, pulsatile secretion, total secretion and pulse mass were similar in HR and LR (Fig. 1). There were no significant differences in the approximate entropy value, but the mode was lower ($P < 0.05$) in HR, suggesting a shorter latency between the onset of a pulse and the peak of the pulse episode (Fig. 1). In addition, the AUC in the first hour of sampling (09:00—10:00 h) was greater ($P < 0.01$) in HR than in LR, but AUC was similar in LR and HR across the entire sampling period (09:00—15:00 h) (Fig. 1) Thus, in the non-stressed state, HR had higher morning cortisol levels than LR (Fig. 1B and C).

3.3. Experiment 2a: Effect of insulin-induced hypoglycaemia on food intake, thermogenesis and plasma cortisol levels in LR and HR

Injection of insulin induced an equivalent degree of hypoglycaemia in LR and HR animals (Fig. 2D). In response to this stressor, plasma levels of cortisol increased ($P < 0.0001$) and skeletal muscle heat production increased ($P < 0.0001$) to a similar extent in LR and HR (Fig. 2A and B). There was no effect of insulin-induced hypoglycaemia on food intake in either group (Fig. 2C).

3.4. Experiment 2b: Effect of psychosocial stress on food intake, thermogenesis, plasma cortisol and catecholamine levels in LR and HR

Exposure to a barking dog increased ($P < 0.05$) plasma cortisol levels in LR and HR with the response being greater ($P < 0.05$) in HR (Fig. 3A). In spite of this, skeletal muscle temperature was similar with little overall change in LR and HR across the experimental period (Fig. 3B). The psychosocial stress reduced ($P < 0.05$) food intake in LR only (Fig. 3C). Baseline catecholamine levels were similar in LR and HR (data not shown). The catecholamine response to barking dog was quantified by assessing the AUC for adrenaline, noradrenaline and DHPG (Fig. 3D—F). The AUC for noradrenaline and DHPG were similar in LR and HR, but the adrenaline response was greater ($P < 0.05$) in LR, demonstrating an inverse relationship between plasma levels in cortisol and adrenaline in LR and HR animals in response to psychosocial stress.

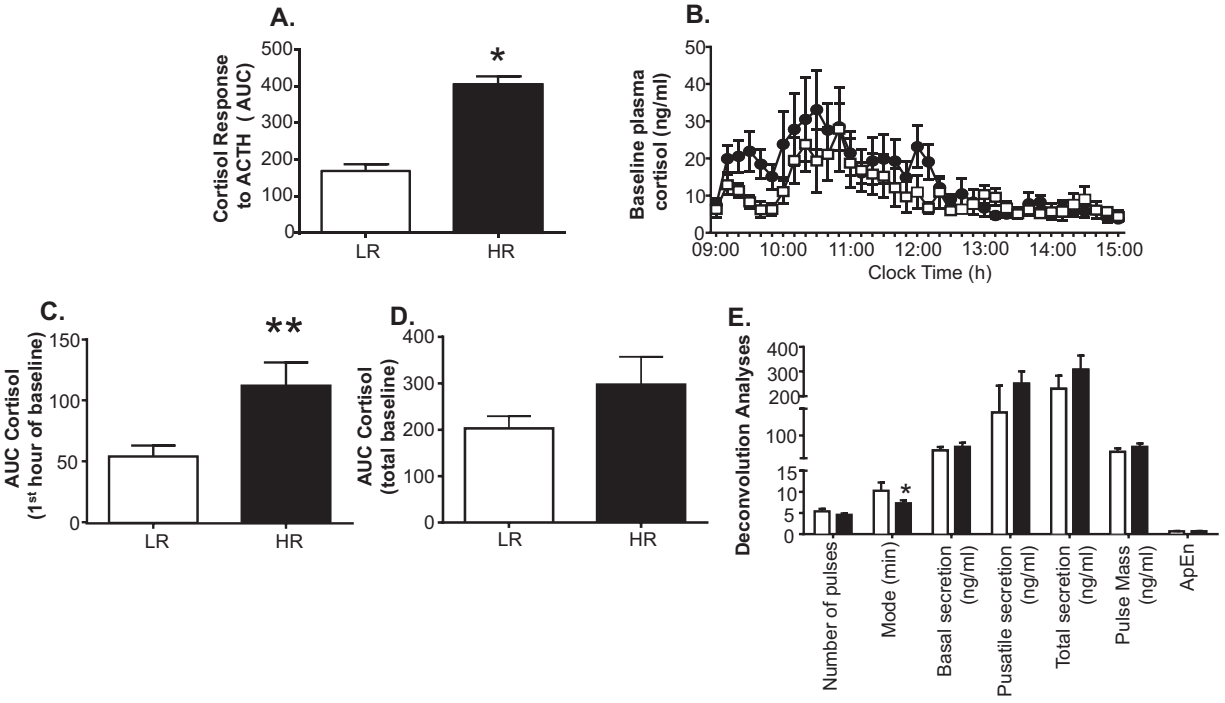

Figure 1 Cortisol secretory profiles in animals selected for low (LR) and high (HR) cortisol responses after synacthen challenge. The cortisol response to adrenocorticotropin (ACTH) challenge was greater in HR compared to LR (Panel A). Characterization of baseline cortisol levels in LR (white symbols) and HR (black symbols) is shown in panels B—E. Plasma levels of cortisol were elevated during the first hour of sampling (09:00—10:00 h), but there was little difference in concentrations across the remaining sampling period (Panels B—D). The area under the curve during the first hour was greater in HR (AUC: 09:00—10:00 h, Panel C), but this difference was not significant across the entire sampling period (09:00—15:00 h, Panel D). Deconvolution analysis of the baseline cortisol data is presented in Panel E. Secretory parameters including the number of pulses, basal secretion, pulsatile secretion, total secretion, pulse mass and the approximate entropy score were similar in LR and HR, whereas the mode was higher ($P < 0.05$) in LR, which is consistent with an elevation in cortisol in HR compared to LR. All data are presented as the mean ± SEM, $n = 7—10$/group. *$P < 0.05$, **$P < 0.01$, LR compared to HR.

3.5. Experiment 2c: Effect of immune challenge on food intake, thermogenesis, plasma cortisol and inflammatory cytokine levels in LR and HR

Injection of LPS increased ($P < 0.01$) plasma cortisol levels in LR and HR with a greater ($P < 0.05$) effect in HR than in LR (Fig. 4A). This divergence in cortisol response was concomitant with different metabolic responses. Skeletal muscle thermogenesis was increased after LPS injection in both groups, but LR had higher muscle temperature responses than HR (Fig. 4B). Similarly, LPS treatment reduced food intake in both groups ($P < 0.05$), but this effect was greater ($P < 0.05$) in LR than in HR (Fig. 4C). Treatment with LPS increased ($P < 0.0001$) the secretion of TNFα, IL6 and IL10, to a similar degree in LR and HR. The increase in IL6 levels was sustained over the experimental period, whilst IL10 and TNFα levels peaked within 2 h of LPS treatment. There was no effect of LPS treatment on the plasma levels of IL4 or IL12 in either group (Fig. 4D—H).

3.6. Experiment 3a: Behavioral responses in the open field test

There was little difference in the behavioral response to isolation in LR and HR animals subjected to the open field test (Fig. 5). The number of bleats, gate knocking and tunneling was similar in LR and HR. On the other hand, LR animals showed greater levels of activity such that the total activity ($P < 0.05$) and locomotor activity ($P < 0.05$) was higher in LR compared to HR.

3.7. Experiment 3b: Behavioral response to the arena test

LR animals spent a greater ($P < 0.05$) amount of time in zone 1 than did HR (Fig. 5B). Time spent in zones 2 and 3 was similar in LR and HR.

3.8. Experiment 3c: Food competition test

LR animals had a lower latency ($P < 0.05$) to initiate feeding compared to the control group (Fig. 5C).

4. Discussion

We have characterized cortisol secretion, metabolic indices and behavior in response to stress in animals selected for either high or low cortisol response to ACTH. In the non-stressed state, HR animals had a higher morning plasma

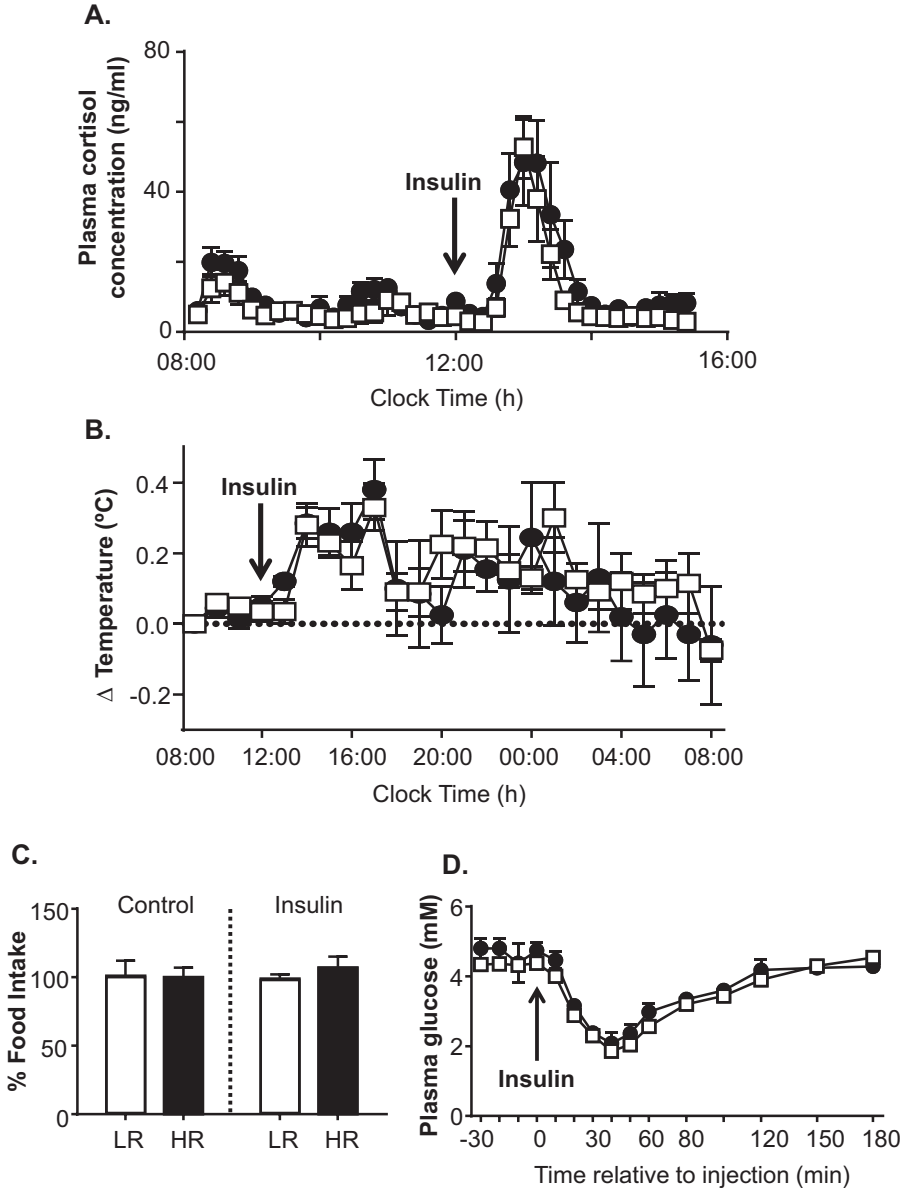

Figure 2 Effects of insulin-induced hypoglycaemia on plasma levels of cortisol and glucose, temperature in skeletal muscle and food intake in ewes selected for low (LR: white symbols) and high (HR: black symbols) cortisol responsiveness. Insulin-induced hypoglycaemia was induced by a single injection of actrapid at 12:00 h, as dictated by the arrow. Hypoglycaemia increased plasma cortisol levels (Panel A) as well as skeletal muscle temperature (Panel B) ($P < 0.0001$ effect of time) in both LR and HR. There was no significant effect of insulin-induced hypoglycaemia on food intake in either group (Panel C), nor were there any differences in plasma cortisol levels or muscle temperature in LR and HR. Injection of actrapid caused an equivalent hypoglycaemia in LR and HR (Panel D). All data are presented as the mean ± SEM, $n = 5$/group.

cortisol level. In response to both immune and psychosocial stressors, secretion of cortisol was greater in HR than LR. Notably, this divergence in cortisol secretion was associated with differences in food intake, such that LR animals showed a greater reduction in food intake in response to stress than HR. In response to LPS, muscle temperature was increased to a greater degree in LR, suggesting that they develop an enhanced catabolic state in response to stress compared to HR. In addition to the metabolic differences in response to stress, LR and HR animals displayed diverse and disparate behavioral phenotypes. LR animals showed increased physical activity and relative fearlessness, which is indicative of a proactive coping style, whereas HR animals displayed a cohesive set of reactive behaviors. This suggests that differences in the propensity to obesity in HR and LR may be due to a complex interplay between the control of food intake, energy expenditure, stress responsiveness and behavioral temperament. Overall, HR animals have increased propensity to obesity, which is driven by reduced energy expenditure (thermogenesis and physical activity), but also HR have a tendency to have a diminished reduction in food intake when compared to LR.

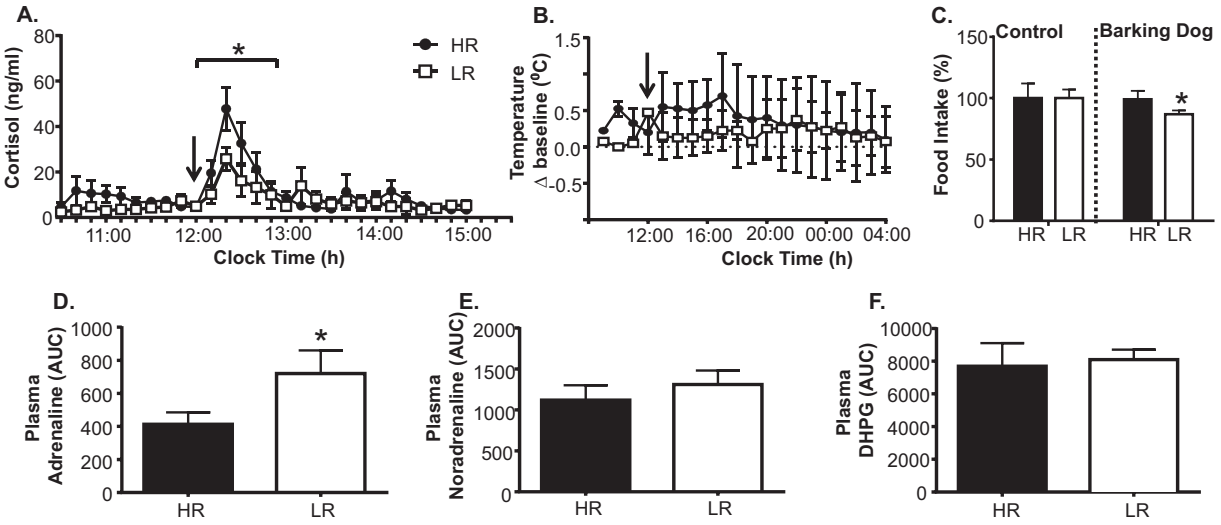

Figure 3 Effect of psychosocial stress on plasma levels of cortisol and catecholamines as well as metabolic indices in low (LR: white symbols) and high (HR: black symbols) cortisol responding animals. Plasma cortisol concentration was increased in LR and HR in response to barking dog stress, but this effect was greater in HR (Panel A). There was little effect of psychosocial stress on skeletal muscle temperature in either LR or HR (Panel B), whereas food intake was decreased in response to stress in LR only (Panel C). With regards to plasma catecholamine levels, the adrenaline response to stress as determined by measuring the area under the curve (AUC) was greater in LR compared to HR. Plasma, noradrenaline and its metabolite, dihydroxyphenylglycol (DHPG) was similar in LR and HR. The arrow depicts the onset of the barking dog stress. All data are presented as the mean ± SEM, $n = 5$/group. *$P < 0.05$ LR compared to HR.

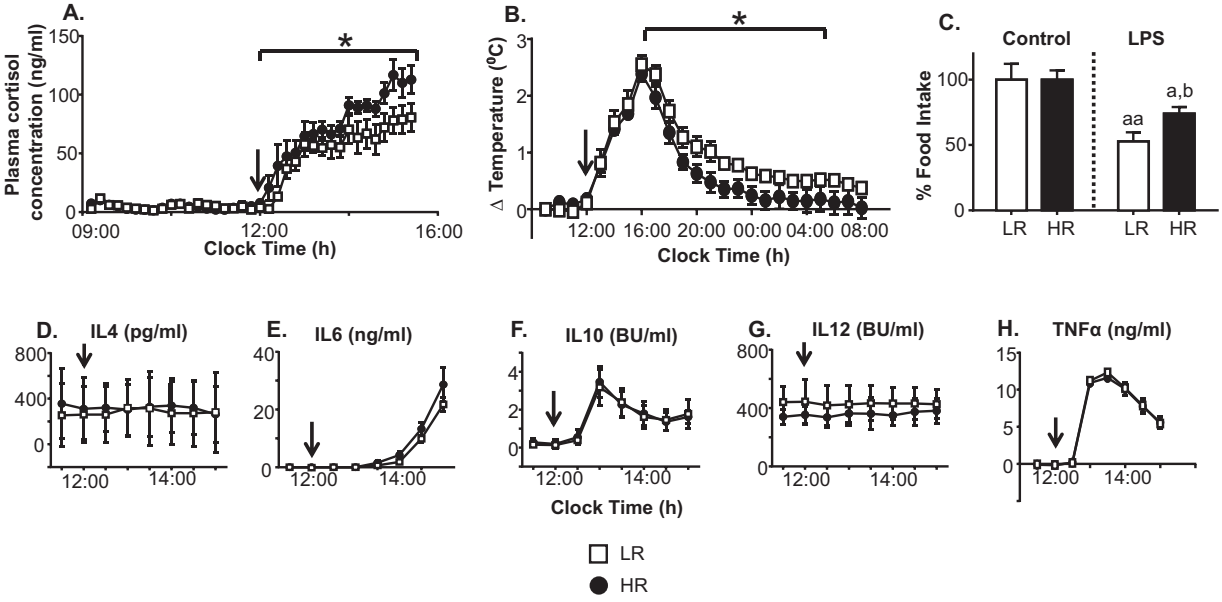

Figure 4 Effects of lipopolysaccharide (LPS) treatment on plasma levels of cortisol and cytokines, temperature in skeletal muscle and food intake in ewes selected for low (LR: white symbols) and high (HR: black symbols) cortisol responsiveness. Injection of LPS (as shown by the arrow at 12:00 h) increased plasma cortisol in both LR and HR ($P < 0.0001$ effect of time), however the increase in cortisol was greater in HR (Panel A). LPS treatment also increased skeletal muscle heat production in both groups ($P < 0.0001$, effect of time) but the temperature was higher ($P < 0.05$) in LR (Panel B). LPS treatment also reduced food intake in both LR ($P < 0.01$) and HR ($P < 0.05$) compared to control (baseline) food intake (Panel C). The reduction in food intake, however, was greater ($P < 0.05$) in LR compared to HR. In addition we measured plasma levels of interleukin 4 (IL4, Panel D), interleukin 6 (IL6, Panel E), interleukin 10 (IL10, Panel F), interleukin 12 (IL12, Panel G) and tumor necrosis factor α (TNFα, Panel H). Plasma cytokine levels were similar in LR and HR before and after injection of LPS. Injection of LPS increased plasma levels of IL6, IL10 and TNFα ($P < 0.0001$ effect of time), but there was no effect on plasma levels of IL4 or IL12. All data are presented as the mean ± SEM, $n = 5$/group. *$P < 0.05$, LR compared to HR; $^{a}P < 0.05$, $^{aa}P < 0.01$, compared to control; $^{b}P < 0.05$, LR compared to HR.

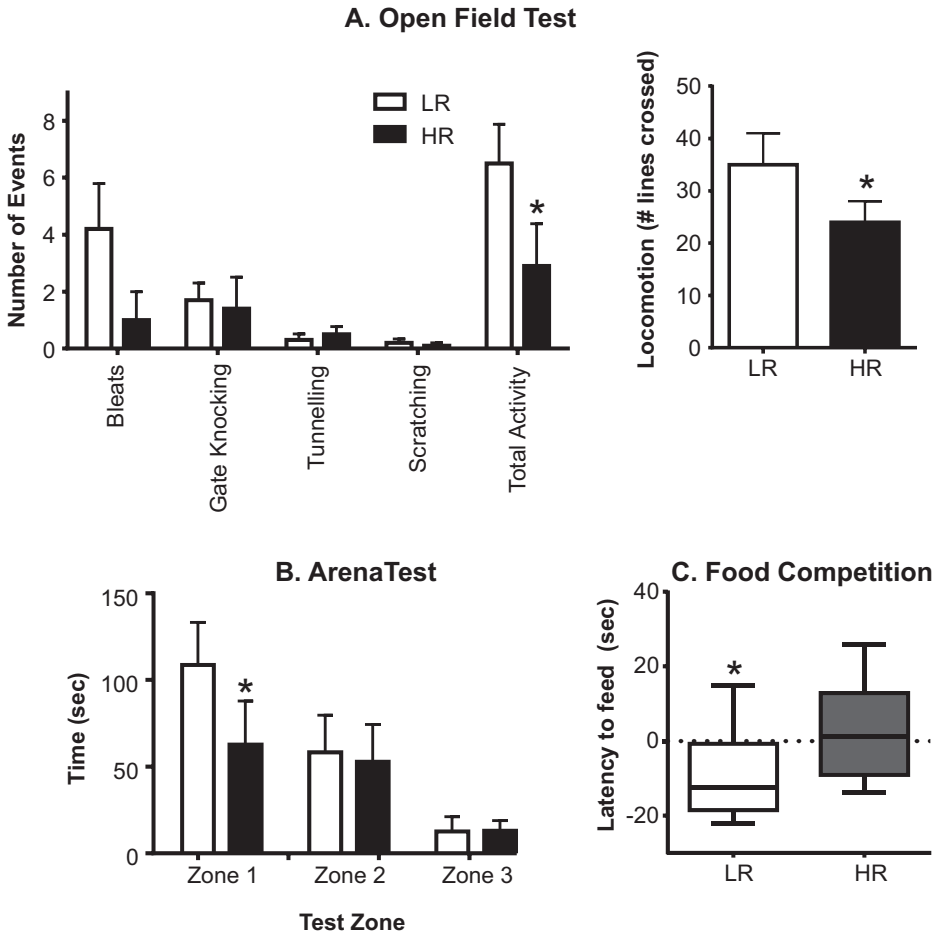

Figure 5 Behavioral differences in animals selected for either low (LR: white bars) or high (HR: black/gray bars) cortisol response to adrenocorticotropin. Behavioral responses were characterized in the open field test (Panel A), arena test (Panel B) and a food competition test (Panel C). LR animals had increased locomotion and total physical activity in response to isolation in the open field test. Furthermore, LR animals displayed reduced fearfulness in the arena test as demonstrated by a greater amount of time spent in zone 1, closest to the human. Finally, the LR animals showed a proactive coping style which was associated with the aforementioned behaviors as well as a reduced latency to feed when compared to control animals in the food competition test. All data are presented as the mean ± SEM, $n = 5$/group. *$P < 0.05$ LR compared to HR.

Reports of the effects of stress and elevated glucocorticoid concentrations on food intake have been dichotomous with a number of studies reporting conflicting findings. The comfort food hypothesis dictates that stress increases intake of foods high in fat and sugar (Pecoraro et al., 2004; Dallman et al., 2005). Increased intake of fat and sugar constitutes a negative feedback pathway, which dampens the HPA axis. Further to this, clinical studies have shown cortisol responsiveness to be an important determinant of the effect of stress on food intake, whereby individuals characterized as high cortisol responders tend to eat more after a stressful episode than those characterized as low cortisol responders (Epel et al., 2001; Tomiyama et al., 2011; Groesz et al., 2012; Tomiyama et al., 2012). Indeed recent work has shown that consumption of ''comfort foods'' is also associated with cortisol responses in that high cortisol responding subjects are more likely to consume high fat/high sugar foods after a stressful episode (Laugero et al., 2002; Pecoraro et al., 2004; la Fleur et al., 2005; Tomiyama et al., 2011). Importantly, with respect to the present work, sheep are ruminants and do not derive either fat or sugar from their diet. Our sheep are maintained on a homogeneous diet of lucerne chaff, deriving volatile fatty acids from their food. Thus, the nexus between the HPA axis, cortisol responsiveness and metabolic function extends to animals that are not mono-gastric.

The current study demonstrates that the effect of stress on food intake is determined not only by cortisol responsiveness but also by the type of stressor. We found that although an immune challenge (LPS) and psychosocial stress (barking dog) reduce food intake, there was no effect of a metabolic insult (insulin-induced hypoglycaemia) on feeding. Again, this may be a particular characteristic of the ruminant animal or may result due to the heterogeneous responses to different stressors (see below). Nonetheless, we demonstrate that LR animals are more affected by stress in terms of food intake whereby LPS reduced intake in both LR and HR animals, but food intake was lower in LR.

Likewise, psychosocial stress reduced food intake in LR only. This could contribute to the maintenance of a lean phenotype in LR, consistent with our earlier work (Lee et al., 2014).

Our previous work also demonstrated that LR and HR exhibit innate differences in the propensity to obesity (Lee et al., 2014) when fed a high energy diet and that this is related to their inherent differences in thermogenesis in skeletal muscle. HR animals have a lower thermogenic output from skeletal muscle in response to feeding and to the central infusion of leptin (Lee et al., 2014). Interestingly, in the non-stressed state, food intake is similar in LR and HR, which is concordant with results in humans, where patients characterized as having high and low cortisol responses had similar food intake at baseline, but food intake diverged after stress; high cortisol responders eat more than low cortisol responders (Epel et al., 2001; Tomiyama et al., 2011). The present work shows that, after immune challenge, HR and LR show differences in food intake and skeletal muscle heat production, which reflects a greater negative energy balance in LR. Injection of LPS reduced food intake in HR and LR, but food intake was lower in the latter. In concert with this greater reduction in food intake, skeletal muscle heat production was elevated in LR, and we propose that this would lead to greater negative energy balance. Despite metabolic differences, circulating levels of cytokines were similar in LR and HR, suggesting that cortisol responsiveness did not impact on the immune response to LPS. LPS treatment increased the circulating levels of some pro-inflammatory (TNFα and IL6) and anti-inflammatory (IL10) cytokines. On the other hand, plasma levels of IL4 and IL12 were not altered by LPS injection. Thus, our cytokine data suggest that the different metabolic responses to LPS challenge in HR and LR are not driven by any immediate differences in immune function or altered sickness predisposition. Nonetheless in response to immune challenge we predict that LR animals are more likely to lose weight due to an enhanced 'catabolic' state. On the other hand, HR animals appear to protect their body weight and this aligns with the previously observed increased propensity to obesity (Lee et al., 2014). This supports the notion that cortisol responsiveness is a marker for innate differences in metabolic set-point and altered susceptibility to obesity (Knott et al., 2008; Block et al., 2009; Lee et al., 2014).

In addition to immune challenge, we demonstrated divergence in cortisol response, food intake and plasma adrenaline levels after psychosocial stress in LR and HR. The LR animals displayed an attenuated increase in cortisol, but an inverse adrenaline response, whereby the increase in plasma adrenaline is heightened in LR compared to HR. Stress increased noradrenaline and the metabolite DHPG to an equivalent degree in LR and HR. The difference in cortisol secretion in response to a barking dog corresponded to differences in food intake, as outlined above, in that psychosocial stress reduced food intake in LR only. Interestingly, we demonstrated an inverse relationship between the catecholamine, adrenaline, and cortisol responses to stress, which is discussed in detail below.

In contrast to psychosocial and immune challenges, the cortisol responses to insulin-induced hypoglycaemia did not differ between HR and LR. This metabolic challenge stimulated the HPA axis, but the effect was equivalent in HR and LR animals. Earlier work in rodents clearly demonstrate heterogeneity in the neuroendocrine responses to various stressors and furthermore show that even within a stress the intensity of that insult determines the endocrine responses (Pacak et al., 1998). Earlier work in sheep has utilized higher doses of insulin to induce hypoglycaemia, for example the study of Saifullizam and colleagues utilized a dose of 4 IU/kg (Saifullizam et al., 2010). The current dose, however was based on previous work in rams (both studies used 0.125 IU/kg) and in this sex, the cortisol response to insulin-induced hypoglycaemia differed in LR and HR (Knott et al., 2010). It should be noted, however, that the initial selection of rams was carried out with a 10 fold higher dose of ACTH (Knott et al., 2008, 2010) than we used for the selection of ewes in the current study. Furthermore, work in sheep has also shown that males are more susceptible to the effects of insulin-induced hypoglycaemia than females, irrespective of gonadal steroids (Turner et al., 2002). This previous study demonstrated that an insulin challenge increases cortisol levels to a greater degree in males than in females (Turner et al., 2002). Such sexual dimorphism may explain why there are differences between the two studies in different sexes. Although Knott and colleagues (Knott et al., 2010) measured cortisol responsiveness it would be interesting to know whether the observed differences in cortisol levels correlated to acute change in food intake or thermogenic output in the rams. Nonetheless, the current study demonstrates that in female sheep insulin-induced hypoglycaemia stimulates cortisol secretion and increases muscle temperature, without an associated effect on food intake.

Further to the metabolic sequelae in response to stress, we demonstrated divergence in behavior, temperament and coping strategy in LR and HR. In obese patients, emotional eating is positively associated with impulsiveness and depression, whereas restrained eating correlates with openness, conscientiousness and extraverted personality traits (Elfhag and Morey, 2008). Moreover, successful weight loss is less likely in obese subjects that exhibit increased novelty seeking behavior (Sullivan et al., 2007), which suggests a lack of impulse control. We therefore sought to identify possible behavioral correlates in LR and HR that may contribute to altered propensity to obesity. Indeed, in humans and a number of animal models, differences in cortisol responsiveness have been shown to associate with differences in behavior, temperament and coping strategies. In humans, low cortisol response to stress is associated with higher neuroticism in women, low extraversion in men and low openness in both sexes (Oswald et al., 2006). It is possible, therefore, that differing set-point of the HPA axis is associated with personality traits that are also evident in obese subjects.

Various animal models have demonstrated that low cortisol responses are associated with increased aggression (Touma et al., 2008; Murani et al., 2010; Terenina et al., 2013). In pigs, recent studies have identified a number of single nucleotide polymorphisms that are associated with both HPA axis activity and aggression. Indeed, these molecular variants are largely found in genes that govern the HPA axis, catecholaminergic and serotonergic systems (Terenina et al., 2013). In the current study we link low cortisol

response to reduced fearfulness, whereby LR animals spent a greater amount of time in zone 1 (closest to the human) compared to HR. In addition to aggression, innate differences in cortisol responsiveness are correlated to differences in coping strategy. A coping strategy refers to a cohesive set of behaviors and physiological responses to stress. In this regard, LR animals displayed a proactive coping strategy, because they have a low cortisol response, increased physical activity, reduced fearlessness and a reduced latency in the food competition test. Innate differences in the HPA axis have been associated with proactive/reactive coping strategies in rodents (Koolhaas et al., 1999, 2010), pigs (Ruis et al., 2000) and chickens (Korte et al., 1997), so that low cortisol responsiveness may be considered a neuroendocrine hallmark of the proactive phenotype. On the other hand, the proactive coping style is associated with elevation in catecholamine levels, especially noradrenaline (Korte et al., 1997; Koolhaas et al., 1999). We show that LR have a greater elevation in adrenaline levels in response to a barking dog than HR, but the increase in noradrenaline levels is similar. Further work is required to determine whether the neuroendocrine differences in our model are causally linked to behavioral variation in LR and HR.

As to whether a proactive coping strategy leads to altered susceptibility to obesity or to weight loss remains unknown, but it has been hypothesized that animals of a proactive nature are more likely to expend energy — with the proactive behaviors such as aggression and physical activity being more likely to expend energy than the reactive behaviors such as freezing (Garland et al., 2011). We have demonstrated that LR increase physical activity in response to stress (as shown by isolation in the open field), which may contribute to their relative resistance to weight gain. Directly addressing changes in energy expenditure is difficult, however, whilst simultaneously performing behavioral studies. Nonetheless, we demonstrate that two components of energy expenditure are altered in LR animals compared to HR; LR have increased physical activity and increased thermogenesis and thus this is likely to impact on total energy expenditure.

In conclusion, we demonstrated that cortisol responses to ACTH can be used to predict the metabolic and behavioral responses to stress. We show that animals characterized as LR have reduced cortisol secretion in response to LPS and psychosocial stressors that coincide with a greater reduction in food intake. Furthermore, immune challenge causes a greater increase in skeletal muscle thermogenesis in LR than HR. Despite the metabolic differences, HR and LR had similar levels of circulating cytokines, suggesting that immune function is similar in the two groups. In addition to the metabolic response to stress, we demonstrate that LR animals adopt a proactive coping strategy, which may also contribute to an innate increase in energy expenditure. Thus, we demonstrate that in response to stress animals characterized as LR are more likely to enter negative energy balance and are therefore relatively protected against diet-induced obesity. On the other hand, HR are more likely to defend their body weight under stressful conditions and are therefore more susceptible to diet-induced obesity. Behavioral testing may identify individuals that are susceptible to obesity.

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Conflict of interest statement

Dr Kevin Lee delivers lectures sponsored by Novo Nordisk and Novartis. The authors have nothing else to disclose.

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Chapter 5

Other Papers

Declaration for Thesis Chapter 5

Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution
Analyse samples for SERCA by Western Blot, graphing and analysis. Extent of Contribution: 5%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Scott Clarke	PhD student who designed and carried out experiment. Extent of contribution: 90%.
Zane Andrews	Supervise mitochondrial respiratory measurements
Robert Bischof	Analysis
Fahri Fahri	Analysis
Roger Evans	Help with measurement of femoral artery blood flow.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	

Main Supervisor's Signature	

Date:

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

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Postprandial heat production in skeletal muscle is associated with altered mitochondrial function and altered futile calcium cycling

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Clarke SD, Lee K, Andrews ZB, Bischof R, Fahri F, Evans RG, Clarke IJ, Henry BA. Postprandial heat production in skeletal muscle is associated with altered mitochondrial function and altered futile calcium cycling. *Am J Physiol Regul Integr Comp Physiol* 303: R1071–R1079, 2012. First published September 12, 2012; doi:10.1152/ajpregu.00036.2012.—This study aimed to determine whether postprandial temperature excursions in skeletal muscle are consistent with thermogenesis or altered blood flow. Temperature probes were implanted into the vastus lateralis muscle of ovariectomized ewes, and blood flow was assessed using laser-Doppler flowmetry (tissue flow) and transit-time ultrasound flowmetry (femoral artery flow). The animals were program-fed between 1100 and 1600, and temperature and blood flow were measured during intravenous administration of either isoprenaline or phenylephrine and during feeding and meal anticipation. In addition, muscle biopsies were collected prefeeding and postfeeding to measure uncoupling protein (UCP) expression and mitochondrial function, as well as indices of calcium cycling (ryanodine 1 receptor: RyR1 and sarcoendoplasmic calcium-dependent ATPases SERCA1/ SERCA2a). Isoprenaline increased femoral artery blood flow, whereas phenylephrine reduced blood flow. At high doses only, isoprenaline treatment increased heat production in muscle. Phenylephrine treatment did not alter muscle temperature. Meal anticipation was evoked in fasted animals (previously program-fed) that were housed beside animals that were fed. Increases in muscle temperature were elicited by feeding and meal anticipation, without changes in blood flow during either paradigm. Analyses of respiration in isolated mitochondria indicated that the postprandial increase in heat production was associated with an increase in state 4 respiration, without increased UCP1, UCP2, or UCP3 expression. Feeding increased the expression of RyR1 and SERCA2a. We conclude that excursions in muscle temperature may occur independent of blood flow, suggesting that postprandial heat production is driven by altered mitochondrial function and changes in calcium cycling.

thermogenesis; uncoupling protein; calcium cycling; mitochondria

BODY WEIGHT IS DETERMINED by energy intake and energy expenditure. An important component of the latter is adaptive thermogenesis, a centrally mediated response to cold and dietary stimuli. Adaptive thermogenesis comprises ~15% of total daily expenditure in nonobese individuals (43) and has been extensively studied in brown adipose tissue (BAT). Activation of sympathetic drive to BAT increases the activity of uncoupling protein 1 (UCP1), promoting the loss of energy through the futile production of heat. Recent studies have demonstrated the unequivocal presence of functional BAT in adult humans (27, 40, 56, 60) and manipulation of thermogenesis is a prospective antiobesity treatment (58). Nonetheless, various studies have demonstrated that BAT does not account for the total thermogenic capacity of an individual, and other tissues, such as skeletal muscle, may also be involved (3, 48).

We have characterized postprandial temperature excursions in skeletal muscle of sheep (13), which is augmented by central leptin infusion indicative of neurally mediated postprandial thermogenesis (25). Furthermore, leptin-induced heat production in skeletal muscle is associated with elevated levels of UCP3 and an increase in uncoupled respiration (23), suggesting that these temperature excursions represent thermogenesis. Despite this, the mechanisms that underpin postprandial thermogenesis in muscle remain to be elucidated. In this regard, increased heat production with meal feeding may be caused by altered mitochondrial function but may also be secondary to alternative cellular pathways, such as futile calcium cycling and/or increased muscle blood flow.

Futile calcium cycling occurs across the sarcoendoplasmic reticular (SR) membrane, whereby activation of the ryanodine 1 receptor (RyR1) pumps calcium across the SR membrane leading to an increase in cytosolic calcium levels (15). Indeed, activating mutations in RyR1 can lead to malignant hyperthermia (32, 34). An increase in cytosolic calcium levels activates the sarcoendoplasmic calcium-dependent ATPases (SERCA), which propel calcium back into the SR and restores intracellular calcium homeostasis. This calcium pump is dependent upon the hydrolysis of ATP to ADP, which results in the production of heat. Thus, activation of the RyR1/SERCA system is thought to constitute a futile form of energy expenditure, resulting in the release of energy through heat. Skeletal muscle expresses the SERCA1 and SERCA2a isoforms, and it is the latter that is thought to be associated with heat production (2, 15, 59). It is possible, therefore, that postprandial heat production in skeletal muscle is driven by changes in calcium cycling.

On the other hand, α_1 and β_2 adrenoceptors control blood flow in skeletal muscle, by mediating vasoconstriction and vasodilation, respectively (9, 18, 21). During the postprandial period, blood flow to specific tissues and organs can be modulated in response to altered metabolic demand. In humans, postprandial elevation of blood flow to the intestinal organs corresponds to increased gut activity and the distribution of substrates to peripheral tissues. For example, an increase in postprandial blood flow of 58–250% has been recorded from the superior mesenteric artery, peaking within 1 h of meal initiation (41, 45, 50). Other studies have suggested that blood flow to skeletal muscle is lowered after a glucose load in humans (10) or not altered following feeding in dogs (19). In contrast, feeding subjects a meal of mixed macronutrient con-

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tent increased blood flow to the forearm muscle (29). We have consistently shown increased temperature in skeletal muscle of sheep at feeding, and the present study sought to determine the role of mitochondria and thermogenic pathways, as well as changes in blood flow that are associated with this elevation in heat production.

METHODS

Animals. All experimentation was approved by the Ethics Committee of Monash University School of Biomedical Sciences. Corriedale ewes were ovariectomized at least 1 mo prior to experimentation to avoid the confounding effects of changing ovarian steroid levels. The sheep were housed in an isolated room, exposed to a 12:12-h light-dark cycle (lights on at 0700), and the ambient temperature of the room was maintained at 22°C. During recordings, the animals were kept in pens designed to allow the animal to sit and stand but prevent further movement that might influence hind limb temperature and blood flow.

Postprandial thermogenesis. To establish a postprandial thermogenic response, the sheep were program-fed as previously described (23, 25), with lucerne chaff given between 1100 and 1600 daily. Animals in *experiments 2–5* were program fed for at least 2 wk prior to experimentation. Water was available ad libitum. Studies were carried out after a postprandial response had been confirmed.

In each experiment (*1–5*), temperature recordings were made using customized dataloggers (SubCue, Calgary, Canada) with leads of either 10 cm or 20 cm (25). The dataloggers were implanted so that the recording side faced the vastus lateralis muscle and were programmed to record temperature at 1-min intervals. Dataloggers and other equipment (see below) were implanted at a single operation under anesthesia induced by intravenous injection of 10 mg/kg thiobarbital sodium, (Lyppard, VIC, Australia) and maintained by inhalation of 3–5% vol/vol halothane. Surgery was performed 1 wk prior to experimental recordings.

Blood flow. For *experiments 1–3*, whole limb blood flow ($n = 3$/group) was measured using a transit-time ultrasound flow probe (type 6SB; Transonic Systems, Ithaca, NY) placed around the femoral artery immediately distal to the caudal femoral artery branch. To determine whether whole limb flow reflected localized changes in tissue perfusion, two animals were additionally fitted with laser-Doppler flow probes (MSP300XP, Oxford Optronix, Oxford, UK) to measure microvascular perfusion in the muscle tissue adjacent to the datalogger.

Experiment 1: effects of isoprenaline and phenylephrine on blood flow and temperature in skeletal muscle. Four ovariectomized ewes with a mean body weight of 49.2 ± 1.4 kg had a polyvinyl cannula (I.D. 1.5 mm; O.D. 2.7 mm; Dural Plastic, Sydney, Australia) surgically implanted into one jugular vein. This was performed at the same time that the datalogger and flow probes were fitted. The cannula was inserted 10 cm toward the heart, and patency was maintained with heparinized (100 units/ml) physiological saline. To reduce confounding effects of feeding or meal anticipation, treatment was carried out in sheep in a fasted state, prior to being program-fed. Isoprenaline (a combined β1/β2 adrenergic agonist) was administered as a bolus with incremental doses of 0.1, 0.3, 1.0, and 3.0 μg/kg body wt and phenylephrine (a α_1-adrenergic agonist) given as a continuous infusion over 15 min at sequential doses of 1, 3, 6, and 10 μg·kg body $wt^{-1}\cdot min^{-1}$. A washout period of 30 min was allowed between each dose to ensure that blood flow returned to baseline levels. Femoral artery blood flow and skeletal muscle temperature were analyzed as the area under the curve of the first 5 min after isoprenaline treatment compared with area under the curve of the antecedent 5 min. To assess the effects of phenylephrine, the area under the curve for the 5 min before infusion was compared with the area under the curve between 25 and 30 min after the commencement of infusion. Comparisons were performed using repeated-measures ANOVA, and post hoc analyses were performed using Fisher's least significant difference test.

Experiment 2: effects of programmed feeding on blood flow and temperature in skeletal muscle. To assess the relationship between postprandial changes in blood flow and temperature in skeletal muscle, these parameters were measured between 1000 and 1600 in program-fed animals (fed between 1100 and 1600). To characterize changes in blood flow, 15-min averages were calculated across the baseline (1000–1045) and feeding periods (1100–1600). The peak temperature response (1130) was compared with baseline using a paired *t*-test. To determine whether changes in temperature coincided with changes in blood flow, we compared blood flow at the time points corresponding to the peak temperature response.

Experiment 3: effects of meal anticipation on blood flow and temperature in skeletal muscle. To establish a model of meal anticipation, animals were placed on the programmed-feeding schedule for 2 wk. We have previously shown that the postprandial temperature response in a meal-entrained animal is dependent on food availability and is, therefore, abolished with fasting (24, 25). Meal anticipation, however, can be evoked in fasted sheep when cohoused with flockmates that are fed at a standard feeding time (1100) (24). In the present experiment, sheep were exposed to visual and olfactory cues associated with the feeding of an adjacent animal, but without the associated metabolic consequences of feeding. Skeletal muscle blood flow and temperature were measured between 1000 to 1600. Data were analyzed as for *experiment 2*.

Experiment 4. cellular or molecular pathways associated with postprandial heat production:uncoupling proteins, AMP-activated protein kinase, and calcium-cycling pathways. To characterize possible cellular or molecular pathways that underpin postprandial heat production in skeletal muscle, we measured changes in mRNA expression of UCP1, UCP2, and UCP3, as well as UCP2 and UCP3 protein and AMPK phosphorylation. Four to six ovariectomized ewes (51.3 ± 0.9 kg body wt) were program-fed (as above) for 2 wk prior to tissue collection to entrain postprandial elevation in heat production. Biopsies were taken from skeletal muscle during the preprandial period (0900) and postprandially (1200). Muscle tissue was collected under general anesthesia, whereby animals were briefly anesthetized with pentobarbital sodium, and a small biopsy of muscle was collected. Samples (~400 mg) were rapidly frozen on dry ice and stored at −80°C for subsequent gene and protein analyses. All gene and protein analyses were carried out on whole muscle homogenate.

UCP1, UCP2, and UCP3 mRNA levels were measured using real-time PCR, as previously described (23). In addition, UCP3 protein levels and the level of AMPK phosphorylation were measured by Western blot analysis, as described previously (23, 31). Western blot analysis used the following antibodies: UCP3 (1:1,000; Abcam, Cambridge, MA), phosphorylated-AMPK (p-Thr172-AMPK) (1:1000; Millipore, Billerica, MA), and anti-AMPK (1:1,000; Cell Signaling Technology, Beverly, MA). For gene expression, UCPs were corrected to the geometric mean of the housekeeping genes, including β-actin, cyclophilin, and malate dehydrogenase. For Western blot analysis, p-AMPK was corrected to total AMPK, and UCP3 was corrected to β-actin. In addition, we measured UCP2, SERCA1, and SERCA2a by Western blot analysis following standard methodology. In brief, for UCP2 measurements, 40 μg of protein was loaded to each lane of a precast gel and the gel run at 150 V for 1 h. Following transfer to nitrocellulose membrane, this was blocked overnight in skim milk. The membrane was subsequently incubated with goat polyclonal UCP2 antibody (ab77363; Abcam, Cambridge, MA) at 1:5,000 for overnight at 4°C, followed by incubation in secondary HRP anti-goat antibody at 1:4,000 (Antibodies Australia, Melbourne, Australia) for 1 h at room temperature. The membrane was then stripped and reblocked, followed by incubation in rabbit polyclonal total actin antibody 1:2,000 for 2 h and then in HRP anti-rabbit antibody at 1:2,000 (Antibodies Australia, Melbourne, Australia) for 1 h at room temperature. Detection was by chemiluminescence (ECL),

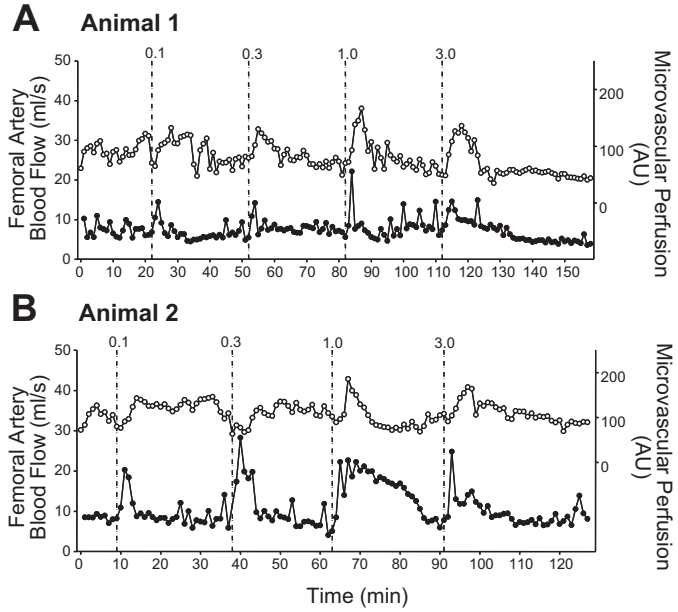

Fig. 1. Representative data from two animals (*A* and *B*) receiving bolus intravenous doses (0.1, 0.3, 1.0, and 1.0 μg/kg) of isoprenaline. Blood flow in the femoral artery (solid circles) and microvascular perfusion assessed by laser Doppler flowmetry (open circles) increased in response to isoprenaline treatment. These data demonstrate that changes in capillary blood flow reflected changes in total limb blood flow as measured by femoral artery blood flow. AU, arbitrary units.

and X-ray films were exposed for 1 min each. Western blot analysis for SERCA1 and SERCA2a followed a similar protocol with the following changes incorporated. Twenty-five micrograms of protein was loaded on the precast gel. SERCA protein expression was measured using mouse-derived primary antibodies, anti-SERCA1 (clone VE121GI; Sigma, St. Louis, MO), and anti-SERCA2 (clone 2A7-A1; Sigma) made up to the dilution of 1:2,500 and 1:1,000, respectively, in 5% nonfat dried milk in Tris-buffered saline with 1% Tween (TBST). Membranes were probed overnight at 4°C. The following day, a secondary antibody (anti-mouse IgG HRP conjugates, goat: diluted to 1:2,000, Antibodies Australia) was applied and incubated for an hour at room temperature. Bands were visualized by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, UK) with 30-s exposure to film. In each case, membranes were then stripped and reprobed with a monoclonal anti-β actin antibody (1: 2,000 in TBST, sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA) purified mouse immunoglobulin for 2 h at room temperature. This was followed by a secondary antibody (anti-mouse IgG HRP conjugates; goat, diluted to 1:2,000; Antibodies Australia) and ECL incubation and exposure to X-ray film for 30 s.

To further assess the calcium cycling pathway, we measured RyR1 mRNA levels using real-time PCR (Realplex; Eppendorf, Hamburg, Germany). RNA was extracted using the TRIzol method, and the quality of RNA was determined by the visualization of the 18S and 28S bands. A master mix for PCR was prepared, consisting of Brilliant II SYBR Green Master Mix (Stratagene, La Jolla, CA), sterile water and primers (sense and anti-sense) to a final volume of 20 μl. The sequence of the primers for RyR was sense 5′-GGG ATA TGG GTG ACA CGA C-3′ and antisense 3′-TCT CAG CAT CAG CTT TCT CC-5′. The quantified RNA was then normalized against the geometric mean of three most stable reference genes (β-actin, cyclophilin, and malate dehydrogenase I) determined by geNorm analysis from a panel of seven possible reference genes.

Equal variance and homogeneity were determined using the Levene's test of equal variance. Gene and protein data were shown to be of unequal variance, and therefore, all data were analyzed using a nonparametric test, the Wilcoxon sign rank test.

Experiment 5. effect of feeding on mitochondrial respiration in skeletal muscle. Ovariectomized ewes ($n = 6$/ group, body wt 63.2 ± 1.8 kg) were program-fed (as above) for 10 days prior to experimentation. Biopsies of skeletal muscle were taken under a light general anesthesia, as previously described (23), prior to feeding (1000) and during the feeding window (1200). Mitochondria were isolated immediately for respiration studies, as previously described (23). Mitochondrial respiration was assessed using a Clark-type electrode (Hansatech Instruments, Norfolk, UK) at 37°C with 250 μg of mitochondrial protein stimulated with pyruvate and malate (5 mM and 2.5 mM) as oxidative substrates in respiration buffer (mannitol 230 mM, sucrose 70 mM, $MgCl_2$ 2 mM, K_2HPO_4 5 mM, 0.1% BSA). State 3 (coupled) respiration was assessed by the addition of ADP (150 μM), which was subsequently inhibited by the ATP synthase inhibitor, oligomycin (1 μM) as an indicator of state 4 respiration. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 1 μM) was added as a final step, to measure maximal respiratory capacity. The respiratory control ratio (RCR) is a measure of mitochondrial uncoupling and was calculated as state 3 respiration:state 4 respiration. Preprandial and postprandial differences in respiration were determined using a one-way ANOVA.

RESULTS

Experiment 1: effects of isoprenaline and phenylephrine infusion on blood flow and temperature. Isoprenaline increased ($P < 0.05$) both femoral artery blood flow and muscle microvascular perfusion (Figs. 1 and 2). Furthermore, changes in these variables were closely related (Fig. 1). The hyperemic effects of isoprenaline were not dose-related, with the peak increase in blood flow being equivalent at both low (0.1 and 0.3 μg/ kg body wt) and high (1.0 and 3.0 μg/kg body wt) doses. There was no significant effect of low-dose isoprenaline treatment on skeletal muscle temperature, but at high doses, isoprenaline increased skeletal muscle temperature (Fig. 2).

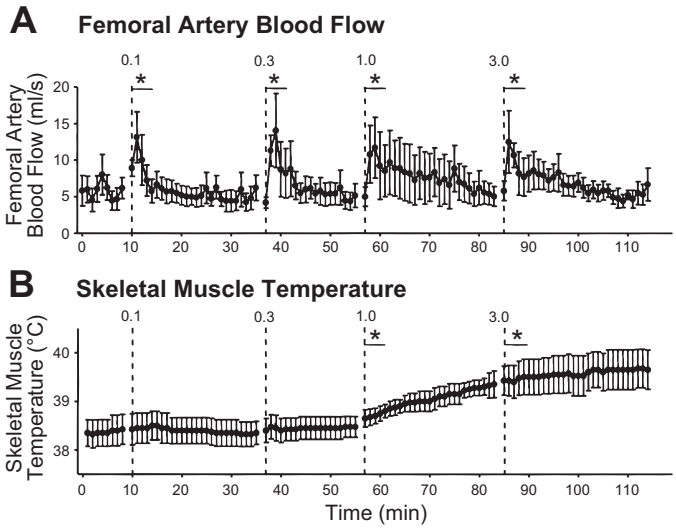

Fig. 2. The effects of isoprenaline treatment on femoral artery blood flow (*A*) and skeletal muscle temperature (*B*). Femoral artery blood flow was increased ($P < 0.05$) at all four doses of isoprenaline (0.1, 0.3, 1.0, and 3.0 μg/kg body wt). In contrast, isoprenaline treatment increased skeletal muscle temperature at high doses only (1.0 μg/kg body wt: $P < 0.05$; 3.0 μg/kg body wt: $P < 0.01$); there was no significant effect of isoprenaline on skeletal muscle temperature at low doses. All data are presented as the means ± SE; $n = 4$/group. *$P < 0.05$.

There was no significant effect of low-dose phenylephrine on blood flow (1 and 3 μg·kg body wt^{-1}·min^{-1}), but high doses (6 and 10 μg·kg body wt^{-1}·min^{-1}) reduced ($P < 0.05$) blood flow (Fig. 3). There was no significant effect of infusion of phenylephrine on skeletal muscle temperature at any dose studied (Fig. 3).

Experiment 2: effects of programmed feeding on blood flow and temperature in skeletal muscle. Muscle temperature increased ($P < 0.01$) at the commencement of feeding (Fig. 4). The mean temperature increase in skeletal muscle was 0.57 ± 0.06°C ($P < 0.01$) (Fig. 4). Despite the elevation in temperature, there were no associated changes in blood flow across the feeding window (Fig. 4).

Experiment 3: effects of meal anticipation on blood flow and temperature in skeletal muscle. Excursions in muscle temperature were elicited by the meal anticipation paradigm (Fig. 5). Similar to the response seen in feeding animals, an increase ($P < 0.05$) in skeletal muscle temperature was observed, and this was not associated with any significant change in blood flow (Fig. 5).

Experiment 4: molecular pathways associated with postprandial heat production. Expression of UCP1, UCP2, and UCP3 mRNA was similar in skeletal muscle prior to and following a meal (Fig. 6), and there was no significant effect of feeding on either UCP2 or UCP3 protein levels (Fig. 6). Further, feeding did not significantly alter the level of phosphorylation of AMPK in skeletal muscle (Fig. 6).

To further investigate cellular/molecular factors that might underpin postprandial heat production in skeletal muscle, we examined the effect of feeding on the expression of RyR1 mRNA, as well as SERCA 1 and SERCA2a protein. Feeding increased levels of RyR1 mRNA and SERCA2a protein. In contrast, SERCA1 protein levels in skeletal muscle were similar before and during feeding (Fig. 6).

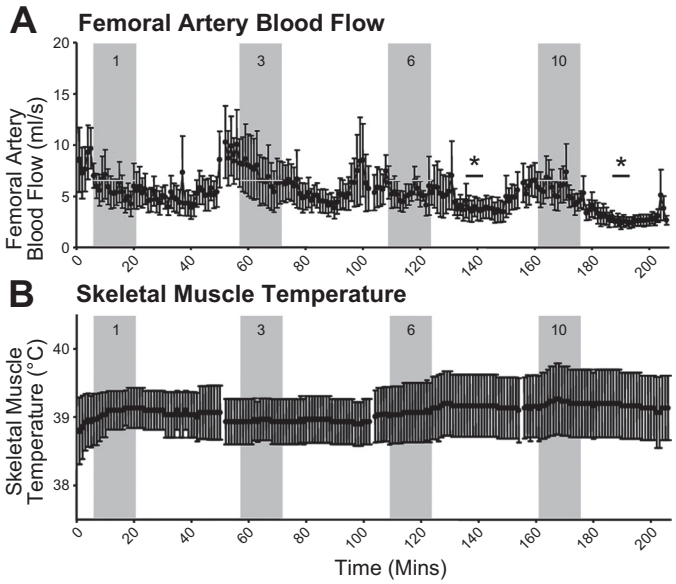

Fig. 3. The effects of phenylephrine treatment on femoral artery blood flow (*A*) and skeletal muscle temperature (*B*). At lower doses (1.0 and 3.0 μg·kg body wt^{-1}·min^{-1}) infusion of phenylephrine did not impact on femoral artery blood flow. Higher doses (6 and 10 μg·kg body wt^{-1}·min^{-1}) of phenyleprhine, however, reduced ($P < 0.05$) blood flow to the hind limb. There was no appreciable effect of phenylephrine treatment on skeletal muscle temperature at any of the doses studied. All data are presented as the mean ± SE; n = 4/group. Shaded areas represent periods of infusion, with the infused dose shown at the top of each area. *$P < 0.05$.

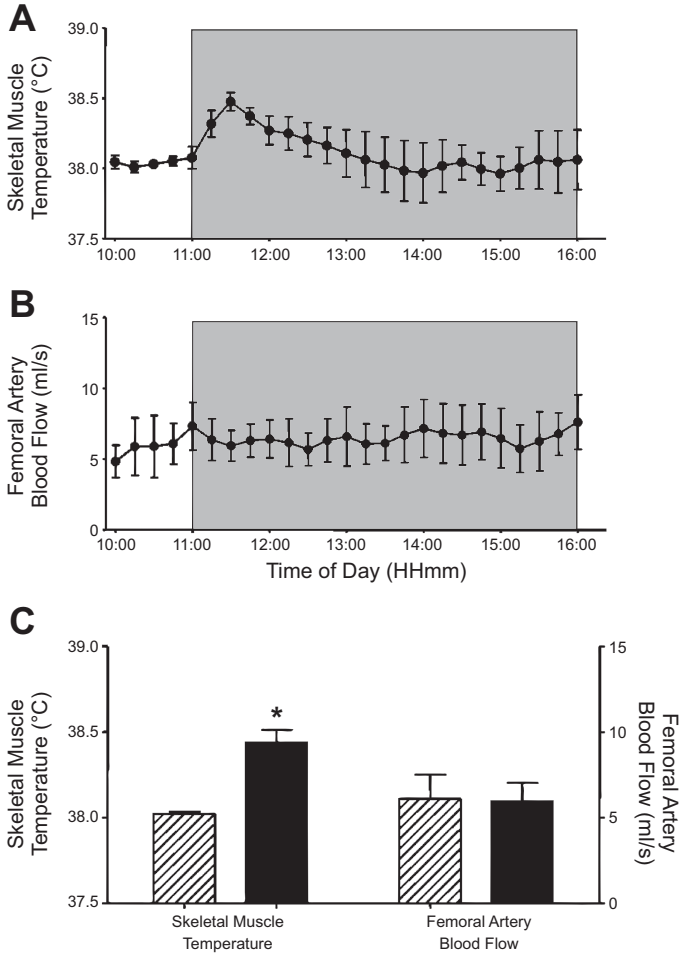

Fig. 4. Skeletal muscle temperature (*A*) and hind limb blood flow (*B*) at baseline before (baseline) and postprandial (PP): gray area. Muscle temperature increased ($P < 0.05$) following the commencement of meal time, yet no change in blood flow to the hind limb was observed. The peak temperature (*C*) response (at 1130) during the postprandial period demonstrated that on average temperature increased by ~0.5°C. There was no detectable change in blood flow across this period. Baseline measurements are represented by the hatched bar and peak measurements by the black bar. All data are presented as the mean ± SE; n = 4/group. *$P < 0.05$.

Experiment 5: effects of feeding on mitochondrial respiration in skeletal muscle. There was no significant effect of feeding on substrate (malate and pyruvate)-driven or state 3 (ADP) respiration (Fig. 7), but state 4 respiration (oligomycin) was increased ($P < 0.05$), indicating an increase in uncoupled respiration. This was consistent with the observed decrease ($P < 0.05$) in the RCR during the feeding window (Fig. 7). Total respiration capacity (FCCP) was lower ($P < 0.05$) in the postprandial compared with the preprandial period.

DISCUSSION

The presented data indicate a lack of association between tissue temperature and acute changes in blood flow in skeletal muscle. This conclusion was demonstrated in four different experimental paradigms, including the administration of α- and

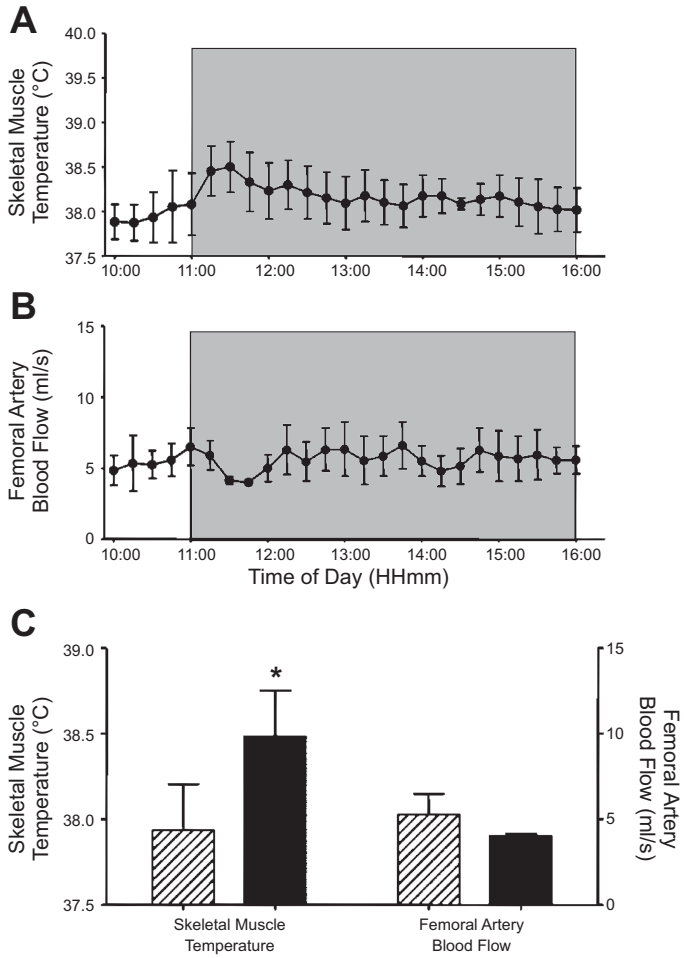

Fig. 5. Skeletal muscle temperature (*A*) and hind limb blood flow (*B*) at baseline before (baseline) and meal anticipation (gray area). Muscle temperature increased ($P < 0.05$) during the meal anticipation period, yet no change in blood flow to the hind limb was observed. Analysis of the peak temperature response (*C*) revealed that the increase in temperature during meal anticipation was not associated with any change in blood flow. There was no detectable change in blood flow across this period. Baseline measurements are represented by the hatched bar and peak measurements by the black bar. All data are presented as the means ± SE; $n = 4$/group. *$P < 0.05$.

β-adrenergic agonists, programmable postprandial responses and meal anticipation. None of these studies showed an association between blood flow and temperature. On the other hand, we demonstrate that the increase in temperature at the onset of feeding is concomitant with a switch toward state 4 respiration in mitochondria isolated from skeletal muscle, which suggests an increase in thermogenesis. Despite this, we did not demonstrate changes in the expression of UCP1, UCP2, or UCP3 mRNA or UCP2 and UCP3 protein. We did, however, demonstrate that feeding increases expression of RyR1 and SERCA2a, which suggests that altered calcium cycling may be the primary cellular driver underpinning postprandial thermogenesis in skeletal muscle. These data offer strong support for the notion that thermogenesis drives postprandial changes in skeletal muscle temperature independent of changes in blood flow.

There has been a recent surge of interest in the mechanisms of adaptive or putative thermogenesis, with particular focus on the potential for the development of novel antiobesity agents (58). To investigate this, we developed a model of meal entrainment where a postprandial elevation in temperature is evident in skeletal muscle after 1–2 wk of temporal food restriction (25). The present data suggest that changes in cellular function, specifically altered calcium cycling, or altered mitochondrial function, contribute to the postprandial elevation in temperature in skeletal muscle. We demonstrate that feeding induced the greatest change in factors that mediate calcium cycling. The expression of RyR1 mRNA and SERCA2a protein was increased after the onset of feeding, suggesting that upregulation of futile calcium cycling is a significant determinant of postprandial thermogenesis in skeletal muscle. Indeed, activating mutations of the RyR1 is considered the principal cause of malignant hyperthermia (32, 34). In vitro work using a perfused hind limb model showed that administration of triiodothyrodine (T3) increased expression of SERCA1 (and a corresponding decrease in SERCA2a) in rat skeletal muscle, leading the authors to hypothesize that the metabolic effects of T3 at muscle are, at least in part, mediated via SERCA1 (46, 47). This contrasts with our current data that show increased expression of SERCA2a, but not SERCA1, in response to feeding. Earlier work in rodents has also linked upregulation of SERCA2a to the induction of muscle thermogenesis in response to leptin (53). Accordingly, the current data suggest that increased expression of RyR1 and SERCA2a drive postprandial thermogenesis in skeletal muscle of sheep.

In addition to changes in calcium cycling, we demonstrate that feeding increased state 4 respiration in mitochondria isolated from skeletal muscle. In spite of this increase, there was no associated change in UCPs. The expression of UCP1, UCP2, and UCP3 mRNA or UCP2/UCP3 protein were similar in preprandial and postprandial periods. Previous studies in both humans and rodents have shown altered state 4 respiration in skeletal muscle mitochondria, without associated changes in UCP3 expression (11, 44, 57). It is important to note, however, that changes in UCP3 activity (which are not detected at the protein or gene level) may account for increased state 4 respiration and, therefore, contribute to postprandial thermogenesis. Indeed, UCP3 is essential for 3,4-methylenedioxymethamphetamine (MDMA or ecstasy)-induced hyperthermia (35), and this effect can be attenuated using β3-adrenoceptor antagonists (51). In addition to changes in UCP3, altered state 4 respiration in skeletal muscle mitochondria, may be driven by the adenine nucleotide translocase (ANT) (22); this warrants future investigation. Nonetheless, the current study demonstrates that postprandial thermogenesis in skeletal muscle occurs in association with altered mitochondrial function and possibly altered calcium cycling.

The lack of a feeding effect on the expression of UCP1 and UCP3 in skeletal muscle contrasts our previous work in male sheep, in which feeding increased expression of UCP1 and UCP3 mRNA (13). It is important to note, that the expression of UCP1 within skeletal muscle samples is likely to be due to the presence of brown adipocytes and not due to UCP1 expression within the myocytes. Indeed, previous work in mice has shown that brown adipocytes interspersed within skeletal muscle contribute to thermogenic output (1). As to whether this difference in the effect of feeding reflects sexual dimorphism in the response of skeletal muscle to feeding merits further investigation. Our earlier work has demonstrated sexual dimorphism in the metabolic response to testosterone, whereby

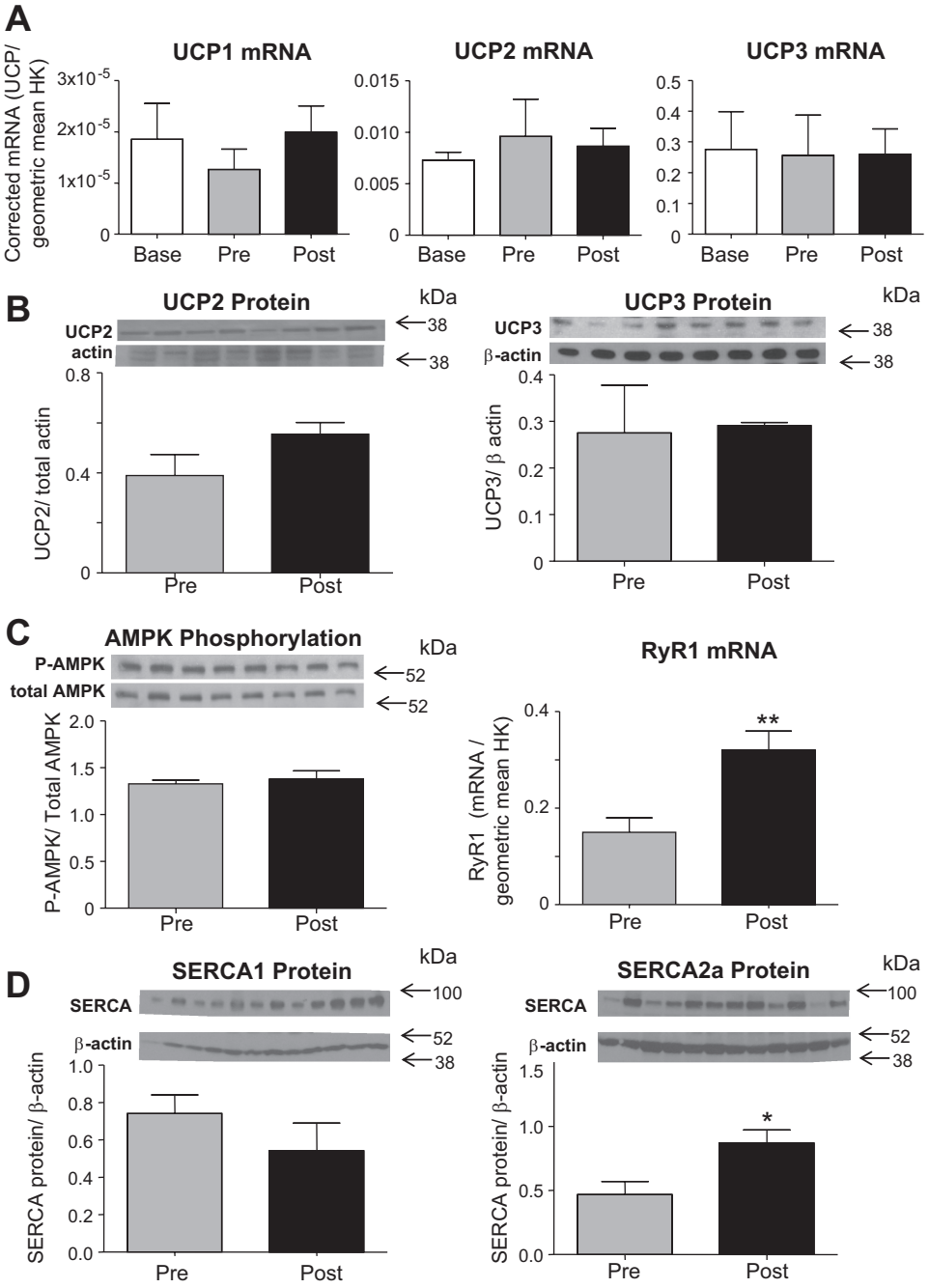

Fig. 6. Effects of meal feeding on the expression of uncoupling proteins (UCP), ryanodine 1 receptor (RyR1), sarcoplasmic calcium-dependent ATPase (SERCA) 1 and 2a and the phosphorylation of AMP-activated protein kinase (AMPK). Biopsies were taken prior to the initiation of meal feeding (base) and after 2 wk of program feeding (1100–1600) (Pre: preprandial; Post postprandial). For each Western blot, preprandial and postprandial samples were run in alternating lanes. The expression of UCP 1, 2, and 3 mRNA (*A*) was similar before and after feeding. There was no effect of meal feeding on the level of UCP2 or UCP3 protein or the phosphorylation of AMPK (*B* and *C*). On the other hand, expression of RyR1 mRNA (*C*) and SERCA 2a (*D*) was increased across the postprandial period compared with the preprandial period. Expression of SERCA1 was not influenced by feeding. All data are presented as the means ± SE; $n = 6$/group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with baseline.

testosterone treatment reduces heat production in skeletal muscle of males but not females (13). Furthermore, we have demonstrated that central infusion of leptin markedly increases postprandial heat production in skeletal muscle and that this is concomitant with an increase in UCP3 expression and an increase in state 4 respiration (23, 25). The current study demonstrates an additional change in total respiratory capacity; we demonstrate that feeding resulted in a reduction in total respiratory capacity in mitochondria isolated from skeletal muscle, which was an unexpected finding. Whereas there are no studies that provide a precedent, previous studies in humans have demonstrated reduced respiratory capacity in mitochondria isolated from skeletal muscle of either obese or type 2 diabetic patients (5, 39), but such changes are thought to be a consequence of the diabetic state (26).

In addition to measuring expression of genes/proteins for the uncoupling proteins, RyR1 and the SERCAs, we characterized AMPK activation (phosphorylation), since this has been linked to the induction of thermogenesis in rodents (30, 33). Several studies have shown that central and peripheral administration of leptin activates AMPK in skeletal muscle (28, 36, 37, 42, 49), which is contrary to our findings in sheep (31). Furthermore, we have demonstrated that direct infusion of AICAR into the femoral artery phosphorylates AMPK (31), but this does not result in altered temperature in muscle tissue (23). Collectively, these data suggest that AMPK activity is not

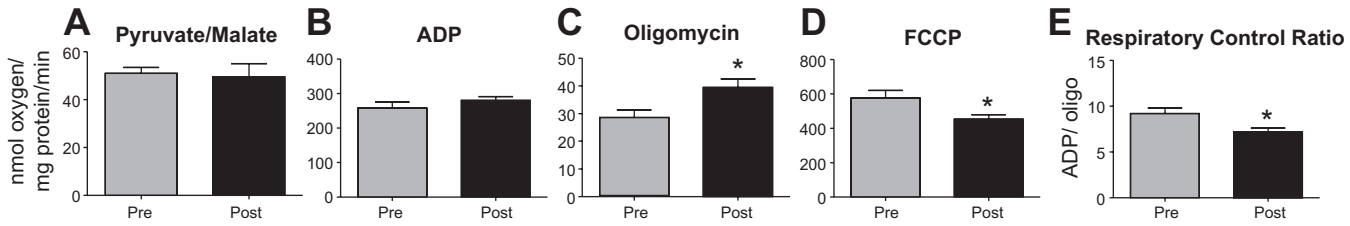

Fig. 7. Postprandial changes in the respiration rates of mitochondria isolated from skeletal muscle. Animals were program fed for 2 wk, and then biopsies of muscle were collected before (Pre: preprandial) and after (Post: postprandial) the onset of feeding. There was no effect of meal feeding on substrate-driven (*A*) or state 3 respiration (*B*). On the other hand, state 4 respiration was increased across the postprandial period compared with the preprandial period (*C*), whereas total respiratory capacity (*D*) was lower after meal feeding. The respiratory control ratio was lower during the postprandial period, consistent with a feeding-induced switch to state 4 respiration in isolated mitochondria (*E*). All data are presented as the means ± SE; $n = 6$/group. *$P < 0.05$ compared with the preprandial period. FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone.

associated with the induction of thermogenesis in sheep skeletal muscle, and the current data are consistent with this.

Consistent with the notion that cellular function drives postprandial heat production, our consistent finding is that postprandial elevation in temperature is not related to changes in blood flow. On the other hand, blood flow to tissues, such as the gut and adipose tissue, have been shown to increase during feeding (41, 45, 50), whereas effects on blood flow to skeletal muscle have produced disparate findings. Previous studies in humans have demonstrated that forearm blood flow can increase during a meal (29), but other data show reduced blood flow after a glucose load (10). Blood flow to the upper gastrointestinal organs, but not skeletal muscle, increases during feeding in dogs (19). This latter observation supports our assertion that feeding has little impact on skeletal muscle blood flow. Increased blood flow to the gastrointestinal organs and adipose tissue after feeding likely reflects the change in metabolic demand driven by increased activity for digestion and/or the distribution of metabolic substrates. Whereas there is no association between blood flow and postprandial elevation of temperature in muscle, a range of other factors influence the latter; these include central action of leptin via the sympathetic nervous system (25) and sex steroids, such as estradiol-17β, and testosterone (13) (Clarke SD, Clarke IJ, Rao A, Evans RG, Henry BA, unpublished data). We have also documented a role for circulating α-melanocyte-stimulating hormone (unpublished data). Many other factors are also likely to impact on this process, such as photoperiod (6), ambient temperature (55), thyroid, and stress hormones (12), for example.

Our meal anticipation studies are important, in distinguishing changes in temperature that are driven by altered metabolic function of skeletal muscle due to substrate utilization from those that may be attributable to putative thermogenesis (see above). Meal anticipation is a nonhomeostatic cue that causes heat production in muscle, most likely due to visual and olfactory stimuli that are provided to a fasted subject when another animal is fed (24). Unlike the situation in rodents, where prolonged temporal food restriction causes a shift in circadian regulation resulting in an anticipatory rise in core body and BAT temperature that persists in the fasted state (8, 20, 38), we have previously shown sheep that have been entrained to a feeding window do not exhibit excursions in heat production when fasted unless a homeostatic (feeding) or nonhomeostatic (meal anticipation) cue is provided (24, 25). Increased skeletal muscle temperature that occurs with meal anticipation is not associated with a change in blood flow. In two paradigms, postprandial entrainment and meal anticipation, we demonstrate an increase in temperature in skeletal muscle that is not driven by altered blood flow.

To further characterize the relationship between changes in blood flow and the regulation of temperature in skeletal muscle, we administered the β_1/β_2-adrenoreceptor agonist isoprenaline and the α_1-adrenoreceptor agonist phenylephrine. Blood flow changes in skeletal muscle were detected using both the transit-time ultrasound and laser Doppler flow probes, albeit with a slight time-delay in the Doppler recordings. Given that the transonic flow probes measured blood flow to the entire hind limb, via the femoral artery, while the laser-Doppler flow probe measured microvascular perfusion in muscle tissue, it can be concluded that changes in femoral blood flow are an accurate indicator of changes in blood flow through the muscle tissue itself. This conclusion is also supported by the finding of strong relationships between total hindlimb flow and local muscle microvascular perfusion during hypoxia (16) and local infusion of vasoactive agents (17) in anesthetized rabbits. Importantly, the responses to phenylephrine and isoprenaline observed in the current study support our assertion that blood flow and muscle temperature are dissociated, further supporting the case for thermogenesis in muscle. High doses of phenylephrine caused a small reduction in blood flow during the postinfusion period, but this had no apparent effect on skeletal muscle temperature. These observations are consistent with the notion that the α-adrenergic system has little impact on energy expenditure, thermogenesis, and heat production in peripheral tissues (4, 7, 54). In contrast, isoprenaline increased blood flow at all doses studied, consistent with the known vasodilator role of the β_2-adrenoreceptors (18, 21). On the other hand, muscle temperature was increased by isoprenaline treatment at the higher doses, only. This is not surprising, since isoprenaline exhibits preferential agonist activity at β_1/β_2-adrenoceptors, but can activate β_3 adrenoceptor at higher concentrations (52).

In conclusion, we demonstrate dissociation between blood flow and skeletal muscle temperature in a number of paradigms. We have demonstrated that pharmacologically reduced (phenylephrine) or increased (isoprenaline low doses) blood flow has little or no impact on muscle temperature. Furthermore, we have demonstrated that during both postprandial periods and during meal anticipation, temperature in skeletal muscle is increased without associated changes in blood flow. We also demonstrate that postprandial heat production is associated with a switch to state 4 respiration in isolated mito-

chondria. These data support the notion that postprandial increases in temperature are not due to changes in blood flow, but are due to altered cellular function and/or metabolism.

Perspectives and Significance

Herein, we have demonstrated that postprandial thermogenesis in skeletal muscle of sheep is due to altered calcium cycling and altered mitochondrial function. Feeding increases state 4 respiration in mitochondria isolated from skeletal muscle. In addition, the expression of RyR1 and SERCA2a, indices of futile calcium cycling, is increased after the onset of feeding. Changes in thermogenesis in muscle are not driven by altered blood flow. This is an important observation as it demonstrates that cellular mechanisms underpin thermogenesis in skeletal muscle. Given that skeletal muscle constitutes a significant proportion of total body mass (30–40%), even small changes in thermogenesis are likely to significantly impact on total energy expenditure. Understanding the cellular mechanisms that are responsible for thermogenesis in muscle will provide new targets for the development of novel therapies to control body weight.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.D.C., K.L., Z.B.A., R.B., F.F., R.G.E., and I.J.C. performed experiments; S.D.C., K.L., Z.B.A., and B.A.H. analyzed data; S.D.C. prepared figures; K.L., Z.B.A., R.G.E., I.J.C., and B.A.H. edited and revised manuscript; Z.B.A., R.G.E., I.J.C., and B.A.H. interpreted results of experiments; R.G.E., I.J.C., and B.A.H. conception and design of research; I.J.C. and B.A.H. approved final version of manuscript.

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Chapter 6

Discussion

Discussion of Results

In this body of work, I have demonstrated that synacthen testing, which was used to stimulate the secretion of cortisol, is able to consistently identify animals with different cortisol response set points. These set points are known to have genetic and epigenetic origins and are shown to be consistent upon re-testing in selection of HR and LR animals. It was further shown that animals with a high set point in cortisol response to synacthen, or High Responders (HR), have a greater propensity to gain adiposity when given a high energy diet compared with animals with a low cortisol response to synacthen (Low Responders -LR).

HR animals preferentially gained more adipose tissue compared with LR whereas LR animals gained more lean body mass relative to HR. Food intake was similar in HR and LR during this period of increased weight gain, prompting the postulation that innate differences in energy expenditure account for these differences in our animal model. This was indeed the case when post-prandial rates of energy expenditure were measured by insertion of dataloggers into skeletal muscle and brown fat depots. This innate differences in energy expenditure between HR and LR were confined to thermogenesis in skeletal muscle which represents 40% of body mass.

The mechanism by which skeletal muscle thermogenesis leads to differences in propensity for weight gain between HR and LR animals was demonstrated and explored in Chapter 2. The potential molecular mechanisms for muscle thermogenesis were explored by quantifying levels of SERCA and UCP-3 expression pre- and post-prandially, but any differences were not significantly discrepant between HR and LR to account for the differences in datalogger recorded temperature excursions. Subtle differences in the regulation beyond the mRNA and protein expression of SERCA and uncoupling proteins may be at play, but this is beyond the detection limits of our methodology and perhaps functional studies would be the next step. However, given the polygenic and

complex nature of weight gain, it is unlikely to be a simple differences in SERCA or UCP-1 expression alone but more likely a combination of these mechanisms with other yet to be discovered modes of thermogenesis as outlined in the Introduction chapter as well as differences in substrate availability and processing that will give us a more complete view. Similarly, one can predict the same complexity of mechanism to account for differences between HR and LR when it comes to understanding why there are differences in food intake and thermogenesis in response to different forms of stress as well as the neuroendocrine mechanisms underlying the proactive versus reactive coping styles which is discussed below. It has also been postulated that subtle differences in ghrelin and leptin signaling, melanocortin receptor expression and neural network differences may culminate in the observed phenotype.

Regardless of mechanism, the innate differences in thermogenesis and cortisol responsiveness displayed in this animal model demonstrate a correlation between programmed factors (genetic/epigenetic makeup) and weight gain. Though there have been no prospective human studies that follow cortisol responsiveness and weight gain, human studies have shown that altered thermogenic function in skeletal muscle can detemine the ability to successfully lose weight in obese individuals[146]. This suggests that innate determinants of weight gain and innate determinants of thermogenesis are most likely linked to innate determinants of cortisol responsiveness in humans.

Innate Determinants of Weight Regulation and Cortisol Responsiveness

Despite the most intense efforts to combat the obesity epidemic, longterm weight loss is challenging for the vast majority of people who try to lose and maintain a lowered body weight that has been achieved through strict dieting and exercise alone[211, 212]. This has been shown in meta-analyses and large

multicentre trials where less than 5% of subjects maintain weight reduction after two or more years.

The most recent lifestyle intervention trial, Look AHEAD, showed that an intensive lifestyle interventional approach for obese individuals over 100kg, involving a multidisciplinary team of dietitians, exercise physiologists, psychologists, doctors and diabetes educators achieved a 3% relative reduction of weight over a 10 year follow up period in over five thousand patients across the finest institutions in the United States[213]. They instigated very intensive education, support and follow up processes consisting of 3 group sessions and 1 individual session per month for the first six months. This tapered off to three sessions per month for the next six months and in Years 2 to 4, the intervention was delivered more on an individual basis with at least one person in contact each month and additional phone or email contact. By contrast, the Swedish Obesity Study that examined the effectiveness of bariatric surgery was able to achieve a 20-25% weight reduction from individuals over 110kg over 10 years of follow up without such an overwhelming effort expanded[214]. This suggests that the 'hard-wired' mechanisms that regulate weight are not amenable to conscious control alone and that something as drastic as gastric bypass surgery is needed for those that are obese. Considering the prevalence of the obesity epidemic however, gastric bypass surgery is not a population approach that can be implemented universally.

Aside from the difficulty in overriding innate determinants of weight, we also know that these determinants are not the same for everyone. In other words, not everyone who lives in the same calorically rich, obesogenic environment become overweight or obese. It is estimated that the heritability or the proportion of phenotypic variation attributable to genetic differences among individuals of BMI range from 50% to 80% and that twin studies suggest that genetic differences account for the majority of variation in body fatness within the population[215]. Furthermore, there is an increasing genetic variance observed during the obesity epidemic predisposing those in the obese range to produce offspring that are even more obese[216, 217]. Numerous studies have shown

associations and linkage of individual genes to obesity and many different loci have been identified but the overall contribution of individual genes is small and unlikely to be predictive of a polygenic trait or condition like obesity[217]. Furthermore, there is information concealed within the interaction of these loci that is not easily attained even from microarray analysis. This makes the role of synacthen testing so valuable in predicting obesity, allowing us to bypass the complexity of the origins of one trait, i.e. obesity, by using another trait that is linked to the phenotype, i.e. stress responsiveness.

Synacthen responsiveness was shown to correlate with stress responsiveness in the second paper (Chapter 4). This trait is, like obesity, equally complex in origin having similar gene-environment interactions as determinants of set point[42, 218] although further investigation of the underlying mechanisms was beyond the scope of this thesis. HR and LR animals showed divergence of responses to stress similar to their synacthen responsiveness, with HR being more likely to have a higher cortisol response to stress than LR. Consistent with the propensity to becoming obese, HR also had a greater energy deficit than LR in response to acute stress. Metabolic stress (insulin induced hypoglycaemia), psychosocial stress (barking dog) and immune stress (LPS) stimulated cortisol secretion in HR and LR, although the degree of difference in cortisol responsiveness differed in HR and LR depending on the type of stressor. More importantly, however, the differing cortisol responses to stress predicted whether there was a difference in energy homeostasis between the groups. Where there was no difference in response to hypoglycaemic stress between LR and HR for example, there was no difference in food intake or thermogenesis between the two groups. When there was a significant difference in peak cortisol alone (as observed in response to a barking dog), there was a reduction in food intake in LR only, with no reduction in food intake in HR. Since both HR and LR had similar thermogenic output, it can be inferred that psychosocial barking dog stress resulted in a greater negative energy balance in LR than HR. Finally, the cortisol response showed the greatest divergence between HR and LR in response to immune challenge, whereby the entire period of cortisol measured post-LPS stress was greater in HR. With this immune stress, the

differences in energy homeostasis was the greatest of all 3 stressors examined, in terms of both food intake and thermogenesis. Accordingly, it has been shown that with different forms of stress, cortisol responsiveness is able to predict differences in energy homeostasis whereby the greater the cortisol response, the greater the shift towards positive energy balance.

The implication for LR being more likely to have a greater negative energy balance and a leaner body morphometry is suggestive of a physiological system which is consistently less predisposed to store energy as fat than their HR counterparts. The mobilization of energy or fuel to meet the demands of stress has been well studied in immune stress, given that one of the best recognized energetic cost of innate immunity is fever. LR maintained higher temperatures over 18 hours after LPS administration and demonstrated a trend for increased IL-6 levels over the 3 hours of measurement post–LPS challenge suggesting a more robust response than that of HR. Others in the field have shown a more robust immune response in rainbow trout selected for cortisol responsiveness to stress, whereby HR trout have a lower vibrio antibody titre after being vaccinated for Vibrio Anguillarum compared with low cortisol responder trout[219].

Vaccination results in an increase in metabolic rate by 20 to 30% for the purposes of antibody formation and entails significant energetic costs which the LR phenotype is presumably better able to mobilize fuel[220]. In this study, the authors found that HR trout had a greater mortality compared with the low responders. This greater ability to mobilize energy during stress in trout corresponds and is consistent with the sheep model studied by Knott et al. [69]. who demonstrated that LR rams had a higher feed efficiency than HR rams. This is associated greater lean mass gain rather than adiposity which corresponds with the LR in our study.

Interestingly, 10% of humans usually lose weight in response to chronic stress whereas the vast majority are more likely to exhibit increased food intake after a stressful episode[67]. It is not known whether this subpopulation of individuals whom lose weight in response to stress correspond to LR biology and neither is it known whether the impaired immune function commonly observed in the

obese person is secondary to obesity or innate as part of the makeup of the individual prone to becoming obese.

In summary, cortisol responsiveness to an ACTH challenge is a simple test that predicts the propensity for obesity with LR and HR phenotypes being discernably different and divergent to a variety of stressors. LR animals are more likely to enter negative energy balance and therefore relatively protected against diet induced obesity. On the other hand, HR are more likely to defend their obdy weight under stressful conditions are are therefore more susceptible to diet induced obesity.

Physiology and Behaviour are Inextricably Linked

It is astounding that an incidental observation made whilst working with the HR and LR ewes led to the examination of cortisol responsiveness and its ability to predict innate coping behavioural responses to stress. This was the difference in behavioural responses to psychosocial stress. Casual observation made by the animal technicians was that LR animals were more recalcitrant than HR when being herded or handled e.g. for surgery. This prompted exploration of the literature and formal examination of behavioural responses of animals to stress. To date, behaviour can broadly be categoried into proactive versus reactive coping styles[204]. In some of these studies, cortisol was measured after the stressor and where it was measured, the proactive animals had a lower cortisol response compared with animals with a reactive coping style. Accordingly, it was decided to examine whether our HR and LR animal had distinct reactive and proactive coping styles, respectively.

In various behavioural stress paradigms, it was shown that the LR had greater activity in terms of locomotion, bleating and gate knocking in the open field test. Furthermore, LR were less fearfulof a human intruder in the arena test and less likely to freeze; they also showed more initiative in the food competition test. In

measuring hormonal responses to psychosocial stress, an inverse relationship between the sympathoadrenal system and cortisol responsiveness was demonstrated. Taken together, the hormonal and behavioural responses indicated that the selection of HR and LR animals from synacthen testing yields the same groupings of animals from the selection of proactive and reactive animals from psychosocial stress.

This bidirectionality of selection indicates that physiology and behaviour are inextricably linked in terms of a response to stress. HR have are predisposed to greater positive energy balance compared to LR as well as having a more reactive behavioural response. This reactive response is characterized by more freezing and less activity which is in keeping with an energetically more conservative biology than the LR. The extent to which behaviour and physiological characteristics are causally related is beyond the scope of this thesis, but there is evidence to suggest that the hormonal differences are likely to mediate the behavioural differences seen in HR and LR.

The work on sheep described herein suggest that synacthen responsiveness is able to elucidate intrinsic differences in HR and LR energy homeostasis and an organism's ability to mobilize energy to meet psychosocial threats or immune challenges. The physiological-behavioural integration and the phenotypic variation of this integration observed within the group have been reported in different animal species and kingdoms from fish to birds to small and large mammals. The robustness of the relationships as delineated by synacthen testing would, according to the theory of natural selection, imply that it is an important characteristic necessary to promote survival of the group, thus allowing successful gene transmission. It is thought that HR animals, for example, are able to adapt better than LR during times of drought and famine. They would have a biology predisposed toward greater positive energy balance as well as behaviour that allows them to conserve and withdraw to a more quiescent phenotype in times of starvation induced stress compared with their LR counterparts.

LR, on the other hand, might be more robust in times of stable and a calorically plentiful environment with a more proactive nature tending to be better at mobilizing energy stores for activity, immune challenges[196] and behaviour[221] which are necessary for a more dominant place in the social hierarchy like showing initiation, assertiveness and less fearfulness. Koolhaas et al.[222] have postulated that HR are more flexible and able to react to a changing environment, whereas LR are intrinsically driven, rigid and routine forming and consequently more adept to stable environments. If this is so, then the importance of stress in understanding weight gain as well as the uniform management strategy for obesity will be inadequate in managing the epidemic we are facing.

Future Direction and Therapeutic Implication for Weight Management

In obese patients contemplating bariatric surgery, psychological assessment revealed two personality subtypes[210]. One is an active problem solving while another is characterized by dysregulated high avoidant and low extraversion coping behaviour. Cluster analyses identifies these distinct groupings and the implication is that different subgroups may warrant fine tuning of our weight management strategies like psychological support, cognitive behavioural therapy and mindfulness based techniques. What is not known is whether these groupings correspond to our HR and LR subtypes in sheep and if so, whether they also have differences in thermogenesis and whether motivation to sustain lifestyle changes differ between the groups. If so, treatment that suits the individual best can be achieved by designing a comprehensive plan for weight management if synacthen testing helps us identify HR and LR subtypes within the obese. Without refining or improving understanding of obesity as a multifactorial disease and through treating everyone the same, it is likely that the efficacy of the interventions we employ is reduced, whether these be food

intake reducing therapeutics, thermogenic compounds, stress reduction techniques or lifestyle interventions[223].

The role in which cognition plays in proactive and reactive coping strategies is not as straight forward in humans as it is in animals. It would be interesting to understand the extent to which innate coping strategies influence our thinking faculties when faced with adversity. It may be that those humans who display LR phenotype will be able to act proactively when they start gaining a few kilos while those that have HR coping styles maybe add on the pounds not only through metabolic predisposition but also behaviourally predisposition as well. Our findings in relation to innate coping strategies add to the debate of freewill versus determinism, whereby those that believe that they are acting freely to be proactive to lose weight after gaining a few pounds maybe innately led to think so.

Unlike animals, humans also have the ability for metacognition, the capacity to think about our style and pattern of thinking, recognizing these patterns if dysfunctional and growing beyond our innate tendencies. This varies from individual to individual, however, and the role of mindfulness in developing metacognition is a promising methodology that could be used to test effectiveness in weight regulation. To date, no randomized control trial has studied weight management using mindfulness techniques, but an RCT investigated showed improved immune cell telomerase activity and well-being also demonstrated an incidental but significant weight loss in individuals engaged with mindfulness[224]. This program was not coupled with exercise and dietetics and the follow up period was merely 3 months. Accordingly, some caution is necessary when evaluating the effectiveness of mindfulness because we know from many large non-mindfulness based multi-interventional RCTs, the dominance of biology in long term weight maintenance is not to be underestimated.

In spite of the limitations indicated above, the research presented in this thesis may lead to significant change in practice in the clinic, through awareness that

many patients who struggle to lose weight are not merely lacking the will to implement healthy lifestyle measures. Built into the biology that predisposes to obesity could be behavioural coping strategies that are inadequate to implement life changing decisions in our stress-ridden society. The judgementalism that plagues this area of care when it comes to weight loss is not only unnecessary but not supported by the science as outlined in this thesis. This prejudice exists within the lay community but may also be prevalent amongst health professionals. Without looking more deeply into the extent to which biology dictates weight and behaviour, a HR phenotype can be easily mistaken by an LR person as someone that is not applying their will power to control his or her weight. As long as such judgementalism exists in the community, compassion and funding for obesity research will be lacking from philatropists and funding bodies and the epidemic will continue to grow.

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