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Ontogeny and thermogenic role for sternal fat in female sheep.

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Précis

We demonstrate in a large animal that brown adipose tissue mitochondrial uncoupling protein is highly abundant in sternal adipose tissue and could contribute to the thermogenic response to feeding.

Abstract

Brown adipose tissue acting through a unique uncoupling protein (UCP1) has a critical role in preventing hypothermia in new-born sheep but is then considered to rapidly disappear during postnatal life. The extent to which the anatomical location of fat influences postnatal development and thermogenic function, particularly following feeding, in adulthood, are not known and were both examined in our study. Changes in gene expression of functionally important pathways (i.e. thermogenesis, development, adipogenesis and metabolism) were compared between sternal and retroperitoneal fat depots together with a representative skeletal muscle over the first month of postnatal life, coincident with the loss of brown fat and accumulation of white fat. In adult sheep, implanted temperature probes were used to characterise the thermogenic response of fat and muscle to feeding and the effects of reduced or increased adiposity. UCP1 was more abundant within sternal than retroperitoneal fat and was only retained in the sternal depot of adults. Distinct differences in the abundance of gene pathway markers were apparent between tissues, with sternal fat exhibiting some similarities with muscle that were not apparent in the retroperitoneal depot. In adults, the post-prandial rise in temperature was greater and more prolonged in sternal than retroperitoneal fat and muscle, a difference that was maintained with altered adiposity. In conclusion, sternal adipose tissue retains UCP1 into adulthood when it shows a greater thermogenic response to feeding than muscle and retroperitoneal fat. Sternal fat may be more amenable to targeted interventions that promote thermogenesis in large mammals.

Introduction

In the majority of large mammals studied to date birth is a critical period for the rapid recruitment of non-shivering thermogenesis in brown adipose tissue (BAT) and this coincides with maximal appearance of uncoupling protein (UCP1) (1,2). This is followed by a transformation of fat from a brown to white appearance although the rate and magnitude of this process could vary between depots (3). For example, in humans the supraclavicular (or neck) depot retains UCP1 into adulthood (4), whereas the peri-adrenal depot does not (5). Consequently, in adults the supraclavicular depot has the capacity to exhibit a significant thermogenic response to both cold exposure (6) and diet (7). The extent to which the retention of UCP1 through the life cycle, and thus associated thermogenic potential, are determined by a fat depots early development and/or anatomical location are currently unknown.

Studies in rodents initially suggested that brown and white adipocytes arise from different lineages, and that brown adipocytes may originate from the same precursor as skeletal muscle (8). This relationship now appears to be more complex and depot specific as white adipocytes can have diverse and mixed origins (9,10) potentially overlapping with brown adipocytes (11). Additional populations of adipocytes have also been identified, being beige or 'brite' (i.e. 'brown-in-white'), which are characterised as being small populations of UCP1-expressing cells surrounded by large numbers of white adipocytes (12,13). The thermogenic

relevance of these cells remains to be established, as their UCP1 content is only 10% of that of classical BAT (14). To date, the majority of studies investigating beige fat have been confined to adult rodents in which "almost everything" examined to date is able to 'brown' white adipose tissue (15). Furthermore, a diverse range of molecular markers for beige adipocytes have been suggested, but their applicability across species (16), as well as the optimal conditions in which these classifications are defined (17), are now being questioned.

Our study therefore had two aims, the first was to compare the gene expression profiles for the primary thermogenic, metabolic and functional markers of brown, beige and white adipose tissue in retroperitoneal (the most abundant depot in the fetal sheep and a "classic" adipose tissue depot (18)), sternal (or neck) fat, and in hind limb muscle, a representative skeletal muscle. Sheep, like many large mammals do not possess interscapular BAT (19) that is present in rodents. The current comparison, undertaken in young sheep, spanned the period from birth to one month of postnatal life, examining three important time points that in retroperitoneal fat are coincident with the peak abundance of UCP1 after birth (i.e. 1 day of age), the age at which UCP1 has declined to basal amounts prior to the onset of rapid growth (i.e. 7 days of age) and subsequent loss of UCP1 (i.e. 28 days of age (1)). We also conducted detailed molecular analyses to establish whether each depot could have a different developmental origin and if adipose tissue in the sternal depot is more similar to skeletal muscle than "classical" (i.e. retroperitoneal) adipose tissue. The second aim was to examine

whether the sternal depot was able to respond to the thermogenic stimulus of feeding in adulthood (20) and if this was comparable to responses in skeletal muscle rather than those in a “classic” adipose tissue. In addition, we examined whether diet-induced obesity or low body weight modulated this response and thus whether sternal fat could be a potential target for promoting thermogenesis.

Methods

Animal experimentation

All animal work was approved by the relevant Animal Ethics Committees at The University of Nottingham or Monash University. In order to avoid any confounding effects of sex and/or a disproportionate increase in muscle mass following overfeeding (as seen in males (21)) only females were studied.

Study 1. The effects of postnatal age on sternal adipose tissue development.

Ten triplet bearing sheep of mixed breed that all gave birth naturally to appropriately grown offspring at term (over a 4 week period) were entered into the study. Triplets were chosen for the study as this meant there would be no confounding maternal influences between each sampling date. One lamb from each mother was therefore randomly selected at either 1, 7 or 28 days of age, blood sampled from the jugular vein and then euthanized by injection of sodium pentobarbital (0.5 ml/kg). The sternal and retroperitoneal adipose tissues depots were dissected and weighed, together with a representative sample from the hind limb muscle (vastus lateralis). Samples were immediately placed in liquid nitrogen and then stored at −80°C until analysed.

Study 2. The effects of altered body weight and fat mass on the temperature response to feeding in sternal adipose tissue.

Manipulation of adult body weight. Fifteen adult female Corriedale sheep aged between 3-5 years, of normal body weight (~55kg), were all ovariectomized (in order to avoid any confounding effects of the reproductive cycle on tissue temperature) and then randomly divided into three different body weight groups. Five animals were made lean (32.5±1.5 kg) by feeding a restricted diet of ~500 g of lucerne chaff per day and five became obese (79.9±3.7 kg) after receiving a supplemented diet of lucerne hay *ad libitum* (*ad lib*) and ~300 g daily supplement of high energy food (i.e. oats and lupin grain) (22,23). The remaining five "control" animals (52.6±1.1 kg) were maintained on pasture. Differential body weights were then maintained for 1 year prior to experimentation. To assess the temperature response to feeding, the diets were standardised across all groups and the high energy supplementation of the obese group stopped. Visceral adiposity was determined at the time of euthanasia (Lean: 0.04±0.01 kg; Control: 1.58±0.18 kg; Obese: 4.11±0.19 kg).

Profiling post-prandial changes in temperature and metabolites

For tissue-specific temperature recordings, customised Dataloggers with 10 cm or 20 cm (SubCue, Calgary, Canada) download leads were inserted into either the skeletal muscle of the hind limb (vastus lateralis), sternal (midline) and retroperitoneal fat and set to record temperature at 15 minute intervals as previously published (20,24). After surgery, the

animals were housed indoors to enable precisely timed ‘meal’ feeding and exposed to natural variations in photoperiod and ambient temperature. To entrain post-prandial thermogenesis, animals were placed on a temporal program-feeding regime, in which they had access to food at set ‘meal’ times between 11.00 h and 16.00 h each day (20,24,25). Animals were “program-fed” for 2 weeks prior to the onset of experimentation. To characterise changes in plasma metabolites and insulin with feeding, blood samples (6 ml) were collected into heparinised tubes at 30 min intervals between 10.00 h and 16.00 h. The samples were centrifuged to obtain plasma, which was stored at −20°C until assayed.

Food intake was recorded after offering the obese and standard animals 2 kg of lucerne chaff and monitoring any refusals, whilst the lean animals received and consumed 500 g of chaff per day. Food intake, once corrected to body weight, was similar in the obese and lean animals, whereas the control group ate slightly more ($P<0.01$) than the other two groups (data not shown).

Temperature data were downloaded and the diurnal thermogenic pattern and the post-prandial response were then analysed in each tissue. After 3 weeks of program feeding, all animals were euthanized as described above, between 08.30-10.30 h. Representative fat and muscle samples were collected and stored as described above for Study 1. At this time it was

confirmed that each temperature probe was still located within the same anatomical position as at surgery.

Laboratory analysis

Gene expression: Total RNA was extracted and, following confirmation of RNA integrity, gene expression was determined using real time PCR (1). The specificity of each ovine primer was confirmed by classical PCR with ovine cDNA from suitable tissue samples, negative controls and ovine genomic DNA and analysing the products by agarose gel electrophoresis. The primers were only used if there was a clear single band on the gel corresponding to the expected amplicon size, negative control lanes were clear, and any products from amplification of genomic DNA could be easily distinguished from the target. The PCR products of the selected primers were analysed using high-sensitivity Sanger dideoxy sequencing, and the returned sequences were verified by alignment with the predicted on-line sequence to ensure that they were specific to the intended target. A summary of the primers used are given in Table 1. The relative amount of mRNA was calculated relative to the geometric mean of the most stable reference genes as determined by geNorm and/or NormFinder analysis. For the postnatal tissues, housekeeping genes included *IPO8, KDM2B, RPLP0* and *TBP* and cyclophilin, β actin, β-2-microglobulin and malate dehydrogenase 1 were used for the adult tissue.

Histology: Tissue sections were prepared as previously published (1) and stained using haematoxylin and eosin and for UCP1. The number of adipocytes was counted in randomly positioned grids using a counting frame area of 62 500μm^2. The Schaffer method was used and a total of approximately 120 adipocytes were examined with the coefficient of variation <2 %. Adipocyte size was measured using the nucleator method (26,27), and cell area calculated by using orthogonal lines originating from the midpoint of the cell, which was taken as the centre of the lipid droplet within complete adipocytes. For UCP1 immunohistochemistry, adjacent sections were collected. Sections were deparaffinised, and endogenous peroxide activity blocked with 0.3 % hydrogen peroxide in methanol. Sections were then washed and blocking serum (normal goat serum in 0.1 M phosphate-buffered saline) added, re-washed and then incubated with primary antibody (1:100 rabbit anti-UCP1) for 24 h at room temperature. Slides were then washed and incubated for 1 hour with secondary antibody (1:200 biotinylated anti-rabbit antibody, Antibodies Australia, Melbourne). Immunostaining was revealed using 3,3′-diaminobenzidine colour reagent. Rat brown adipose tissue was used as a positive control (primary antibody 1:1000) and staining without primary antibody was used to determine staining specificity.

Mitochondrial content and immunoblotting: The relative abundance of UCP1 and total mitochondrial protein content were determined in the postnatal samples as previously

described (28). In the adult samples the relative abundance of UCP1, UCP3, SERCA1 and SERCA2a were determined using antibodies as previously published (20,29,30) and summarised in Table 2. All data was corrected against the density of staining for total protein. Each antibody gave a signal at the correct molecular weight (see Supplementary Figure 1) and the specificity of binding for each anti-body was confirmed using non-immune rabbit serum.

Plasma metabolite and hormone analysis: Plasma glucose and lactate were analysed using an auto-analyser (YSI, Inc., Yellow Springs, OH), NEFA enzymatically (31). Plasma insulin (32) and irisin (33) by ELISA (Kit no. EK-067-29; Phoenix Pharmaceuticals, Inc., 330 Beach Road, Burlingame, CA 94010, USA) in single assays.

Statistical analyses

Differences in gene expression and protein abundance between depots with age and/or different body weights were analysed using Kruskal-Wallis non-parametric tests with Bonferroni correction for multiple analyses. In Study 2, longitudinal data for temperature and plasma analysis were analysed by repeated measures ANOVA. Differences in the temperature response to feeding, and in adipocyte size, were analysed by one-way ANOVA using Fishers least significant differences for post-hoc analyses.

Results

Changes in UCP1 and gene expression profile between depots during early development

As expected, the abundance of the *UCP1* gene and a key regulator of BAT function, *DIO2* were highest in both fat depots examined at 1 day of age and then declined (Figure 1). This adaptation in gene expression occurred as white adipose tissue mass increased substantially, with growth up to 28 days of age being greater in terms of relative body weight in the retroperitoneal depot (sternal 4.7±0.5; retroperitoneal 9.6±1.2 g/kg; $P<0.05$). At 1 day of age the total mitochondrial protein content was greater in sternal than retroperitoneal fat, which resulted in the total amount of UCP1 protein, being higher in the sternal depot (sternal 1.4±0.5; retroperitoneal 0.3±0.1 arbitrary units (au) per depot; $P<0.05$). The rate of decline in UCP1 was, however greater between 1 and 7 days of age in the sternal depot (i.e. 7 days: sternal 0.7±0.1; retroperitoneal 0.5±0.1 au per depot). UCP1 protein was undetectable in muscle at any time point.

Very low amounts of *UCP1* and *DIO2* mRNA were detected in skeletal muscle (Figure 1). As each fat depot lost UCP1, there was a parallel decrease in gene expression for *PDK4*, (that is present in murine BAT (34)), which was also apparent in muscle. In contrast, the gene that encodes for irisin, i.e. *FNDC5*, was highly abundant in all three tissues examined, with a clear peak in muscle at 7 days of age. Whilst there were no changes in *FNDC5* expression with age in retroperitoneal adipose tissue, in sternal fat it decreased. No change in plasma irisin

concentration was observed with age (7 days: 117±7; 28 days 129±11 ng/ml). The lipid droplet protein *CIDEA* exhibited high transcript expression in both fat depots and did not change with age, whereas expression was very low in muscle. RIP140 was equally abundant in both fat and muscle, and while it showed a clear rise at 28 days in both fat depots, there were no significant age-related changes in muscle.

In order to establish a clearer overview of the differences in relative gene expression of a range of markers previously considered to be indicative of brown, beige, and white adipose tissue or skeletal muscle, a more exhaustive analysis was undertaken on the samples obtained at 7 days of age (Figure 2). This demonstrated that other BAT related-genes (e.g. Eva1) were highly expressed in both fat depots, as were those genes primarily involved in either lipid metabolism (i.e. *FABP4*) or adipogenesis (i.e. *PPARγ*, *CEBPα* and *β*), whereas *ADIRF* was more abundant in sternal than retroperitoneal fat. None of these genes were present in muscle. Some other genes considered to potentially regulate adipose development, such as *HOXC8* and *HOXC9*, and the "white fat marker" gene *TCF21* were also highly expressed in retroperitoneal adipose tissue, but not in muscle or, more surprisingly, in sternal adipose tissue. Messenger RNA for *En-1* was highly abundant in sternal fat, but not in the other two tissues. In contrast, *SHOX2* mRNA was abundant in sternal fat and muscle, but was barely detectable in the retroperitoneal depot. Specific muscle marker genes *CPT1b* and *Tbx15*, which have been reported to be expressed during the differentiation or induction of BAT

(35,36), were highly expressed in muscle, but minimally expressed in fat. Finally, *ACSM5* mRNA was more abundant in muscle than in retroperitoneal fat, but hardly detectable in sternal fat.

To further understand the development of sternal fat the expression of additional genes were examined (Table 3), and divided into four categories: developmental genes previously considered to be markers of brown or beige fat; and those which regulate thermogenesis, metabolism or adipogenesis. For thermogenic genes, we observed a reduction in *PRLR* and *PGC1α* expression between 7 and 28 days, whereas *ATF2* expression increased. The expression of the beige/white marker gene *HOXC9* and the "classical" BAT marker gene *LHX8* both rose substantially at each age, whereas that of the BAT fate-determining gene *PRDM16* showed a small decrease by 28 days, whilst the beige marker gene *SHOX2* was unchanged. Surprisingly, changes in the expression profiles of adipogenic genes varied. The mRNA abundance of *PPARγ* and *NR3C1* both increased, whereas that of CEBPα transiently increased at 7 days of age and that of *SREBF1* declined. A majority of other metabolic genes also showed increased expression with age, i.e. adiponectin, leptin and *GPR120*. However, mRNA abundance for *FABP4* and the *INSR* were unchanged.

Differences in tissue temperature and metabolites in response to feeding and altered fat mass in adults

In adult sheep, the temperature of retroperitoneal fat was consistently higher than in sternal fat and skeletal muscle (Figure 3), which might reflect its deep-body location. Nevertheless, retroperitoneal fat and skeletal muscle displayed comparable temperature responses to feeding. The greatest feeding-induced temperature rise was in sternal fat (Figure 4). There was no effect of increased fat mass on the temperature of the three tissues studied (Figures 3-4). Whereas low body weight/adiposity was associated with reduced temperature of both adipose tissue and skeletal muscle, this effect was less pronounced in retroperitoneal fat (Figure 3). Plasma glucose and insulin were lower in the lean animals, but there was little effect of altered adiposity on plasma NEFA and lactate (Figure 5).

Differences in gene profile with altered fat mass in adults

The abundance of *UCP1* mRNA was very low in all adult tissues and *UCP1* was only consistently detectable by immunohistochemistry in sternal fat (Figure 6). *UCP3* was highly abundant in skeletal muscle, but was very low in sternal and retroperitoneal fat; being expressed 100-fold more in muscle than fat (i.e. skeletal muscle 5±1; adipose tissue 0.05±0.1). Gene expression for *UCP3* was increased in sternal adipose tissue in lean compared to obese animals (Lean 18±4; Obese 0.3±0.2 au; $P<0.05$), whereas protein abundance was reduced (Table 4). In contrast, neither *UCP1* mRNA nor protein, were altered

by changes in adiposity in either fat depot (data not shown). *UCP2* mRNA was lower in sternal and retroperitoneal fat of lean compared to control animals (e.g. retroperitoneal: Lean 1.0±0.3; Control 3.3±0.5 au; $P<0.05$). There were also no effects of body weight on *UCP3*, *RyR1* or *SERCA2a* in skeletal muscle. Expression of *SERCA1* mRNA was lower in skeletal muscle of lean animals, but protein concentrations were again unaffected by fat mass. Finally, as expected, adipocyte cell size was influenced by altered body weight and adiposity. In retroperitoneal fat, adipocyte size changed in proportion to increased body weight. On the other hand, adipocyte size was decreased in sternal fat of lean animals but there was no effect of obesity on adipocyte size in this depot (Figure 6).

Discussion

We have shown that during postnatal development in sheep, the sternal and retroperitoneal fat depots exhibit contrasting gene expression profiles that could be indicative of divergent prenatal origins. These differences potentially contribute to the enhanced temperature responses seen in sternal compared with retroperitoneal fat and skeletal muscle following feeding in adulthood. Although the abundance of UCP1 within the sternal depot declines with increased fat mass both during postnatal and adult life, it does not appear to compromise the ability of this depot to increase its temperature in response to feeding in adulthood. Furthermore, adipocyte cell size in the sternal fat depot appears unresponsive to increased adiposity, suggesting that it is likely to serve a function other than storing surplus lipid during nutrient excess and obesity.

Divergent patterns of development between fat and muscle in early life

It is becoming apparent that identifying functional markers in BAT and/or beige adipocytes is a complex process that is influenced by depot and whether in vivo or in vitro methodologies are used (17,37,38). As we have shown previously in ovine retroperitoneal fat, there are at least three distinct phases of postnatal development (1). The major functionally related changes are seen between 1 and 28 days of postnatal age, coincident with the loss of UCP1 and the transition of brown to white adipose tissue (1). In the present study, we confirm that this critical stage of development extends to both sternal fat and muscle and is coincident

with rapid growth and functional changes within each tissue (39,40). These findings are in accord with that recently described within epicardial fat during development of humans that have undergone heart surgery (41). Consideration of each gene or group of genes examined, the accepted function of each, and known developmental ontogeny in other species is given below, thereby providing new insights into the pronounced differences in the molecular signatures of fat and muscle.

The most notable characteristic of sternal, compared with both retroperitoneal fat and muscle, was the very high abundance of *En-1* mRNA. Lineage tracing studies in mice indicate cells showing early expression of En-1 during development give rise to dermis and epaxial muscle, but not other muscles, and interscapular BAT "bundles" (42), which is in accord with our findings postnatally. Anatomical location during early development is determined along three axes: anterior-posterior, proximal-distal, and dorsal-ventral (43), but the primary regulators remain to be fully elucidated, with a variety of gene families such as Wnt (44,45), HOX (46) and Pax playing roles. HOX genes are important regulators of development (47,48), for which expression of specific combinations of the paralogous HOX gene sets specify a particular anatomical location along the anterior-posterior axis. Our data show that *HOXC8* and *HOXC9* are highly expressed in the more posteriorly located retroperitoneal depot, but not in the more anterior sternal depot, and that this pattern of gene expression

persists to 28 days. In mice, *HOXC8* and *HOXC9* gene expression is higher in retroperitoneal than in interscapular adipose tissue (49).

TCF21 was originally proposed as a marker for white pre-adipocytes (50), but its gene expression differs between fat depots (51) (52). Therefore, our finding that *TCF21* mRNA was only abundant in retroperitoneal fat support the hypothesis that tissue-specific patterns of *TCF21* gene expression are indicative of fundamental differences between depots that are dependent on anatomical location. Further indirect evidence of depot-specific rates of development comes from examining *SHOX2*. Its pattern of gene expression was opposite to the *HOXC* genes measured, being expressed in both muscle and sternal fat, which is anteriorly located to the retroperitoneal depot, where there is little, if any, detectable expression. SHOX2 is able to interact with CEBPα to modulate ADRB3 and, by extension, regulate lipolysis in adipose tissue (53). Additionally, in mice, ablation of *SHOX2* promotes lipolysis (53). The low expression of this gene within retroperitoneal adipose tissue could therefore be indicative of a more rapid mobilisation of NEFA, as well as capacity for greater growth through adulthood compared with sternal fat. Differences in metabolic capacity, with respect to medium chain fatty acid synthesis between tissues, could also explain the much higher mRNA abundance of *ACSM5* in retroperitoneal compared with sternal adipose tissue. ACSM5 is also considered to be a characteristic of white fat rather than BAT (34), and its paucity reflects the retention of UCP1 within sternal fat. Our finding of greater gene

expression for ACSM5 within muscle contrasts with findings in adult rodents (54), and could be indicative of the significant changes seen within muscle during early postnatal development. In sheep, this is coincident with the recruitment of shivering thermogenesis (40), that could be accompanied with the increased utilisation of intramuscular fat as suggested in pigs (55). Gene expression for *FNDC5* also peaked at 7 days in muscle, coincident with maximal recruitment of shivering thermogenesis as UCP1 declined (40). There was no parallel change in plasma concentrations of irisin at this stage. This was not entirely unexpected given the current controversy regarding the measurement of irisin (56) and its potential functionality or existence (57).

Some changes in gene expression with age were similar in both muscle and fat depots (e.g. *PDK4*) that could reflect the overall decline in basal metabolic rate (40), loss of UCP1 and pronounced fat deposition up to one month of age (39). At the same time, there is a transition from lipid to glucose metabolism (58) that would be facilitated by a decline in PDK4 activity (59), whilst the increase in leptin, adiponectin, *RIP140* and *GPR120* gene expression are indicative of increased adiposity. In adults, however, fat mass and plasma adiponectin and its gene expression are normally negatively correlated (60). A different type of relationship during early life, coincident with the rapid growth of fat, is not unexpected, as has been seen for plasma leptin and the loss of its positive correlation with fat mass (61). At the same time, both *LHX8* and *ATF2* gene expression increased with age and fat mass, which was not

expected given their putative “BAT identity marker roles”, as described by others in ovine retroperitoneal adipose tissue with development (62). As suggested by rodent studies, both ATF2 and GRP120 may, therefore, have a greater role in stimulating adipogenesis rather than thermogenesis (63,64), whilst raised RIP140 would facilitate the loss of UCP1 (65). In summary, sternal fat development shares some characteristics with skeletal muscle that may also impact on the retention of UCP1 and its thermogenic capacity in adulthood.

Functional consequences of UCP1 in sternal fat

The contribution of BAT to diet-induced thermogenesis in rodents remains contentious (66), although it does appear to have a role in both young sheep (58), children (67) and adults (7). There is good evidence from developmental studies in both rodents and young sheep that muscle is recruited to generate heat when UCP1 is absent (68) and/or nonshivering thermogenesis is compromised (40). In adult sheep temperature excursions in skeletal muscle in response to feeding and central infusion of leptin are consistent with increased thermogenesis (24,29). The basal temperature of muscle also appears to be more sensitive to total fat mass than that of either fat depot studied. This could reflect both its anatomical position and/or the impact of an increase in the surrounding fat and its insulating properties. It should also be noted that in large mammals, such as sheep, the thermogenic response to feeding is an entrained response (20) whereas, in rodents, there is an influence of circadian rhythm (69). Furthermore, rodents are normally active in the dark phase and the sensitivity of

UCP1 to further stimulation is modulated by these diurnal activity patterns (70). These are dependent, in part, on both light exposure and activity of the sympathetic nervous system (71). A range of other factors can have a critical role in determining the thermogenic role of muscle in rodents, including sarcolipin (72) and UCP3 (73). In adult sheep gene expression of UCP3 is increased in skeletal muscle after central infusion of leptin and is associated with increased heat production and a switch towards uncoupled respiration in isolated mitochondria (24). Additionally, increased expression of *RyR1* mRNA and SERCA2a protein in skeletal muscle coincides with dietary induced thermogenesis (29). We, however, found no difference in protein abundance for either SERCA1 or 2a or UCP3 in muscle of animals of differing body weights.

In contrast to the acute effects of feeding, prolonged food restriction caused a marked decrease in temperature in skeletal muscle and sternal adipose tissue, an effect attenuated in the retroperitoneal fat. Consistent with the decreased temperature in muscle, *UCP3* gene expression declined markedly in skeletal muscle of lean animals, but there was no associated change in protein. On the other hand, altered adiposity had no effect on *UCP1* gene or protein abundance in sternal fat, but *UCP3* mRNA was reduced in the lean group. Due to the much larger mass of muscle than BAT, its contribution to metabolic homeostasis is appreciably greater in sheep and humans (74), especially when plasma glucose concentrations are raised. Notably, glucose concentrations were considerably lower in the lean group in the current

study. The reduced temperature in both skeletal muscle and sternal adipose tissue of lean animals is indicative of homeostatic reduction in thermogenesis in response to chronic food restriction and weight loss. This may be a mechanism to reduce energy expenditure in an effort to maintain body weight in states of negative energy balance and/or in the lean condition and may be mediated by lower thyroid hormone secretion (75).

In summary, sternal and retroperitoneal fat have distinct developmental profiles which are different to that seen in muscle. The different developmental profiles are not only associated with early adipose growth but also on thermogenesis in these tissues later in life. The extent to which sternal fat expansion, and particularly UCP1 abundance, can be modulated in early life may inform new strategies to manipulate energy balance, especially following feeding or in response to chronic food restriction during adulthood.

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References

1. Pope M, Budge H, Symonds ME. The developmental transition of ovine adipose tissue through early life. Acta Physiol Scan 2014; 210:20-30

2. Lean MEJ. Brown adipose tissue in humans. Proc Nutr Soc 1989; 48:243-256

3. Symonds ME, Pope M, Sharkey D, Budge H. Adipose tissue and fetal programming. Diabetologia 2012; 55:1597-1606

4. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S, Nuutila P. Functional brown adipose tissue in healthy adults. N Engl J Med 2009; 360:1518-1525

5. Vergnes L, Davies GR, Lin JY, Yeh MW, Livhits MJ, Harari A, Symonds ME, Sacks HS, Reue K. Adipocyte Browning and Higher Mitochondrial Function in Periadrenal But Not SC Fat in Pheochromocytoma. J Clin Endocrinol Metab 2016; 101:4440-4448

6. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, Schrauwen P, Teule GJ. Cold-activated brown adipose tissue in healthy men. N Engl J Med 2009; 360:1500-1508

7. Scotney H, Symonds ME, Law J, Budge H, Sharkey D, Manolopoulos KN. Glucocorticoids modulate human brown adipose tissue thermogenesis in vivo. Metabolism 2017:(In press)

8. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scime A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, Spiegelman BM. PRDM16 controls a brown fat/skeletal muscle switch. Nature 2008; 454:961-967

9. Shan T, Liang X, Bi P, Zhang P, Liu W, Kuang S. Distinct populations of adipogenic and myogenic Myf5-lineage progenitors in white adipose tissues. J Lipid Res 2013; 54:2214-2224

10. Sanchez-Gurmaches J, Hung CM, Sparks CA, Tang Y, Li H, Guertin DA. PTEN loss in the Myf5 lineage redistributes body fat and reveals subsets of white adipocytes that arise from Myf5 precursors. Cell Metab 2012; 16:348-362

11. Sanchez-Gurmaches J, Guertin DA. Adipocytes arise from multiple lineages that are heterogeneously and dynamically distributed. Nat Comm 2014; 5:4099

12. Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. Chronic peroxisome proliferator-activated receptor gamma activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. J Biol Chem 2010; 285:7153-7164

13. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. Nat Med 2013; 19:1252-1263

14. Nedergaard J, Cannon B. UCP1 mRNA does not produce heat. Biochem Biophys Acta 2013; 1831:943-949

15. Nedergaard J, Cannon B. The browning of white adipose tissue: some burning issues. Cell Metab 2014; 20:396-407
16. Scheele C, Larsen TJ, Nielsen S. Novel nuances of human brown fat. Adipocyte 2014; 3:54-57
17. de Jong JM, Larsson O, Cannon B, Nedergaard J. A stringent validation of mouse adipose tissue identity markers. Am J Physiol Endocrinol Metab 2015; 308:E1085-1105
18. Alexander G. Quantitative development of adipose tissue in foetal sheep. Aust J Biol Sci 1978; 31:489-503
19. Alexander G, Bell AW. Quantity and calculated oxygen consumption during summit metabolism of brown adipose tissue in newborn lambs. Biol Neonate 1975; 26:214-220
20. Henry BA, Dunshea FR, Gould M, Clarke IJ. Profiling postprandial thermogenesis in muscle and fat of sheep and the central effect of leptin administration. Endocrinology 2008; 149:2019-2026
21. Bloor ID, Sebert SP, Saroha V, Gardner DS, Keisler DH, Budge H, Symonds ME, Mahajan RP. Sex differences in metabolic and adipose tissue responses to juvenile-onset obesity in sheep. Endocrinology 2013; 154:3622-3631
22. Henry BA, Tilbrook AJ, Dunshea FR, Rao A, Blache D, Martin GB, Clarke IJ. Long-term alterations in adiposity affect the expression of melanin-concentrating hormone and enkephalin but not proopiomelanocortin in the hypothalamus of ovariectomized ewes. Endocrinology 2000; 141:1506-1514
23. Kurose Y, Iqbal J, Rao A, Murata Y, Hasegawa Y, Terashima Y, Kojima M, Kangawa K, Clarke IJ. Changes in expression of the genes for the leptin receptor and the growth hormone-releasing peptide/ghrelin receptor in the hypothalamic arcuate nucleus with long-term manipulation of adiposity by dietary means. J Neuroendocrinol 2005; 17:331-340
24. Henry BA, Andrews ZB, Rao A, Clarke IJ. Central leptin activates mitochondrial function and increases heat production in skeletal muscle. Endocrinology 2011; 152:2609-2618
25. Henry BA, Blache D, Rao A, Clarke IJ, Maloney SK. Disparate effects of feeding on core body and adipose tissue temperatures in animals selectively bred for Nervous or Calm temperament. Am J Physiol Regul Integr Comp Physiol 2010; 299:R907-917
26. Møller A, Strange P, Gundersen HJG. Efficient estimation of cell volume and number using the nucleator and the disector. Journal of Microscopy 1990; 159:61-71
27. Horvath TL, Erion DM, Elsworth JD, Roth RH, Shulman GI, Andrews ZB. GPA protects the nigrostriatal dopamine system by enhancing mitochondrial function. Neurobiology of disease 2011; 43:152-162

28. Symonds ME, Bryant MJ, Clarke L, Darby CJ, Lomax MA. Effect of maternal cold exposure on brown adipose tissue and thermogenesis in the neonatal lamb. J Physiol 1992; 455:487-502

29. 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 2012; 303:R1071-R1079

30. Lee TK, Clarke IJ, St John J, Young IR, Leury BL, Rao A, Andrews ZB, Henry BA. High cortisol responses identify propensity for obesity that is linked to thermogenesis in skeletal muscle. FASEB J 2014; 28:35-44

31. Sechen SJ, Dunshea FR, Bauman DE. Somatotropin in lactating cows: effect on response to epinephrine and insulin. Am J Physiol 1990; 258:E582-588

32. Clarke SD, Clarke IJ, Rao A, Cowley MA, Henry BA. Sex differences in the metabolic effects of testosterone in sheep. Endocrinology 2012; 153:123-131

33. Nygaard H, Slettalokken G, Vegge G, Hollan I, Whist JE, Strand T, Ronnestad BR, Ellefsen S. Irisin in blood increases transiently after single sessions of intense endurance exercise and heavy strength training. PLoS One 2015; 10:e0121367

34. Forner F, Kumar C, Luber CA, Fromme T, Klingenspor M, Mann M. Proteome differences between brown and white fat mitochondria reveal specialized metabolic functions. Cell Metab 2009; 10:324-335

35. Gburcik V, Cawthorn WP, Nedergaard J, Timmons JA, Cannon B. An essential role for Tbx15 in the differentiation of brown and "brite" but not white adipocytes. Am J Physiol Endocrinol Metab 2012; 303:E1053-1060

36. Christian M. Transcriptional fingerprinting of "browning" white fat identifies NRG4 as a novel adipokine. Adipocyte 2015; 4:50-54

37. Xue R, Lynes MD, Dreyfuss JM, Shamsi F, Schulz TJ, Zhang H, Huang TL, Townsend KL, Li Y, Takahashi H, Weiner LS, White AP, Lynes MS, Rubin LL, Goodyear LJ, Cypess AM, Tseng YH. Clonal analyses and gene profiling identify genetic biomarkers of the thermogenic potential of human brown and white preadipocytes. Nat Med 2015; 21:760-768

38. Shinoda K, Luijten IH, Hasegawa Y, Hong H, Sonne SB, Kim M, Xue R, Chondronikola M, Cypess AM, Tseng YH, Nedergaard J, Sidossis LS, Kajimura S. Genetic and functional characterization of clonally derived adult human brown adipocytes. Nat Med 2015; 21:389-394

39. Clarke L, Buss DS, Juniper DS, Lomax MA, Symonds ME. Adipose tissue development during early postnatal life in ewe-reared lambs. Exp Phys 1997; 82:1015-1017

40. Symonds ME, Andrews DC, Johnson PJ. The control of thermoregulation in the developing lamb during slow wave sleep. J Dev Physiol 1989; 11:289-298

41. Ojha S, Fainberg HP, Wilson V, Pelella G, Castellanos M, May ST, Lotto AA, Sacks H, Symonds ME, Budge H. Gene pathway development in human epicardial adipose tissue during early life. JCI Insight 2016; 1:e87460
42. Atit R, Sgaier SK, Mohamed OA, Taketo MM, Dufort D, Joyner AL, Niswander L, Conlon RA. Beta-catenin activation is necessary and sufficient to specify the dorsal dermal fate in the mouse. Dev Biol 2006; 296:164-176
43. Loomis CA, Harris E, Michaud J, Wurst W, Hanks M, Joyner AL. The mouse Engrailed-1 gene and ventral limb patterning. Nature 1996; 382:360-363
44. Moldes M, Zuo Y, Morrison RF, Silva D, Park BH, Liu J, Farmer SR. Peroxisome-proliferator-activated receptor gamma suppresses Wnt/beta-catenin signalling during adipogenesis. Biochem J 2003; 376:607-613
45. Christodoulides C, Lagathu C, Sethi JK, Vidal-Puig A. Adipogenesis and WNT signalling. Trends in endocrinology and metabolism: TEM 2009; 20:16-24
46. Cantile M, Procino A, D'Armiento M, Cindolo L, Cillo C. HOX gene network is involved in the transcriptional regulation of in vivo human adipogenesis. J Cell Physiol 2003; 194:225-236
47. Hunt P, Krumlauf R. Deciphering the Hox code: clues to patterning branchial regions of the head. Cell 1991; 66:1075-1078
48. Wellik DM. Hox patterning of the vertebrate axial skeleton. Developmental dynamics : an official publication of the American Association of Anatomists 2007; 236:2454-2463
49. Yamamoto Y, Gesta S, Lee KY, Tran TT, Saadatirad P, Kahn CR. Adipose depots possess unique developmental gene signatures. Obesity (Silver Spring) 2010; 18:872-878
50. Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, Hamilton DL, Gimeno RE, Wahlestedt C, Baar K, Nedergaard J, Cannon B. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. Proceedings of the National Academy of Sciences of the United States of America 2007; 104:4401-4406
51. Macotela Y, Emanuelli B, Mori MA, Gesta S, Schulz TJ, Tseng YH, Kahn CR. Intrinsic differences in adipocyte precursor cells from different white fat depots. Diabetes 2012; 61:1691-1699
52. Sacks HS, Fain JN, Bahouth SW, Ojha S, Frontini A, Budge H, Cinti S, Symonds ME. Adult epicardial fat exhibits beige features. J Clin Endocrinol Metab 2013; 98:E1448-1455
53. Lee KY, Yamamoto Y, Boucher J, Winnay JN, Gesta S, Cobb J, Bluher M, Kahn CR. Shox2 is a molecular determinant of depot-specific adipocyte function. PNAS, USA 2013; 110:11409-11414
54. Ellis JM, Bowman CE, Wolfgang MJ. Metabolic and tissue-specific regulation of acyl-CoA metabolism. PLoS One 2015; 10:e0116587

55. Ramayo-Caldas Y, Ballester M, Fortes MR, Esteve-Codina A, Castello A, Noguera JL, Fernandez AI, Perez-Enciso M, Reverter A, Folch JM. From SNP co-association to RNA co-expression: novel insights into gene networks for intramuscular fatty acid composition in porcine. BMC Genomics 2014; 15:232

56. Elsen M, Raschke S, Eckel J. Browning of white fat: does irisin play a role in humans? J Endocrinol 2014; 222:R25-R38

57. Albrecht E, Norheim F, Thiede B, Holen T, Ohashi T, Schering L, Lee S, Brenmoehl J, Thomas S, Drevon CA, Erickson HP, Maak S. Irisin - a myth rather than an exercise-inducible myokine. Scientific reports 2015; 5:8889

58. Symonds ME, Andrews DC, Johnson PJ. The endocrine and metabolic response to feeding in the developing lamb. J Endocrinol 1989; 123:295-302

59. Barquissau V, Beuzelin D, Pisani DF, Beranger GE, Mairal A, Montagner A, Roussel B, Tavernier G, Marques MA, Moro C, Guillou H, Amri EZ, Langin D. White-to-brite conversion in human adipocytes promotes metabolic reprogramming towards fatty acid anabolic and catabolic pathways. Molecular metabolism 2016; 5:352-365

60. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol 2000; 20:1595-1599

61. Bispham J, Budge H, Mostyn A, Dandrea J, Clarke L, Keisler DH, Symonds ME, Stephenson T. Ambient temperature, maternal dexamethasone, and postnatal ontogeny of leptin in the neonatal lamb. Pediatr Res 2002; 52:85-90

62. Basse AL, Dixen K, Yadav R, Tygesen MP, Qvortrup K, Kristiansen K, Quistorff B, Gupta R, Wang J, Hansen JB. Global gene expression profiling of brown to white adipose tissue transformation in sheep reveals novel transcriptional components linked to adipose remodeling. BMC Genomics 2015; 16:215

63. Cao W, Daniel KW, Robidoux J, Puigserver P, Medvedev AV, Bai X, Floering LM, Spiegelman BM, Collins S. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. Mol Cell Biol 2004; 24:3057-3067

64. Rosell M, Kaforou M, Frontini A, Okolo A, Chan YW, Nikolopoulou E, Millership S, Fenech ME, MacIntyre D, Turner JO, Moore JD, Blackburn E, Gullick WJ, Cinti S, Montana G, Parker MG, Christian M. Brown and white adipose tissues: intrinsic differences in gene expression and response to cold exposure in mice. Am J Physiol Endocrinol Metab 2014; 306:E945-964

65. Kiskinis E, Chatzeli L, Curry E, Kaforou M, Frontini A, Cinti S, Montana G, Parker MG, Christian M. RIP140 represses the "brown-in-white" adipocyte program

including a futile cycle of triacylglycerol breakdown and synthesis. Mol Endocrinol 2014; 28:344-356

66. Symonds ME, Pope M, Budge H. The ontogeny of brown adipose tissue. Ann Rev Nutr 2015; 35:295-320

67. Symonds ME. Brown adipose tissue growth and development. Scientifica 2013; 2013:14. http://dx.doi.org/10.1155/2013/305763

68. Rowland LA, Bal NC, Kozak LP, Periasamy M. Uncoupling Protein 1 and Sarcolipin Are Required to Maintain Optimal Thermogenesis, and Loss of Both Systems Compromises Survival of Mice under Cold Stress. J Biol Chem 2015; 290:12282-12289

69. Blessing W, Mohammed M, Ootsuka Y. Heating and eating: brown adipose tissue thermogenesis precedes food ingestion as part of the ultradian basic rest-activity cycle in rats. Physiol Behav 2012; 105:966-974

70. Ootsuka Y, de Menezes RC, Zaretsky DV, Alimoradian A, Hunt J, Stefanidis A, Oldfield BJ, Blessing WW. Brown adipose tissue thermogenesis heats brain and body as part of the brain-coordinated ultradian basic rest-activity cycle. Neuroscience 2009; 164:849-861

71. Kooijman S, van den Berg R, Ramkisoensing A, Boon MR, Kuipers EN, Loef M, Zonneveld TC, Lucassen EA, Sips HC, Chatzispyrou IA, Houtkooper RH, Meijer JH, Coomans CP, Biermasz NR, Rensen PC. Prolonged daily light exposure increases body fat mass through attenuation of brown adipose tissue activity. Proc Natl Acad Sci U S A 2015; 112:6748-6753

72. Bal NC, Maurya SK, Sopariwala DH, Sahoo SK, Gupta SC, Shaikh SA, Pant M, Rowland LA, Goonasekera SA, Molkentin JD, Periasamy M. Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. Nat Med 2012; 18:1575-1579

73. Clapham JC, Arch JR, Chapman H, Haynes A, Lister C, Moore GB, Piercy V, Carter SA, Lehner I, Smith SA, Beeley LJ, Godden RJ, Herrity N, Skehel M, Changani KK, Hockings PD, Reid DG, Squires SM, Hatcher J, Trail B, Latcham J, Rastan S, Harper AJ, Cadenas S, Buckingham JA, Brand MD, Abuin A. Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. Nature 2000; 406:415-418

74. Blondin DP, Labbe SM, Phoenix S, Guerin B, Turcotte EE, Richard D, Carpentier AC, Haman F. Contributions of white and brown adipose tissues and skeletal muscles to acute cold-induced metabolic responses in healthy men. J Physiol 2015; 593:701-714

75. Symonds ME, Clarke L. Influence of thyroid hormones and temperature on adipose tissue development and lung maturation. Proc Nutr Soc 1996; 55:567-575

Figure Legends

Figure 1. Summary of the changes in gene expression for putative markers of brown, beige or white adipose tissue or skeletal muscle in the sternal and retroperitoneal fat depots and hind limb muscle over the first 28 days of life in young sheep. Values are means with their standard errors, n=4-6 per age group and significant differences between age groups are indicated by * $P<0.05$.

Figure 2. Summary heat map comparison of qPCR gene expression between sternal and retroperitoneal adipose tissue and skeletal muscle from six individual sheep sampled at 7 days of age. All normalised data were made relative to the highest expressing sample and are given in arbitrary units (au) between 0-1. Each column contains data from an individual animal for the three tissues examined and each row contains data for a specific gene. Red squares represent the highest expression (1) and blue squares the lowest (0).

Figure 3. Summary of the effect of altered body weight and fat mass on changes in the temperature of sternal and retroperitoneal (RP) adipose tissue and skeletal muscle as measured continuously over a 24 h period. Values are means with standard errors of the mean and n=4-5 per age group. Grey box indicates the time at which food was available. Obese animals are shown in white squares, control in black triangles and lean in black circles. ** $P<0.01$ lean compared to obese and control, [a] $P<0.05$ lean compared to obese.

Figure 4. Summary of the effect of altered body weight and fat mass on changes in the post-prandial rise in tissue temperature of sternal and retroperitoneal (RP) adipose tissue and skeletal muscle as measured after feeding. This was calculated as the amplitude of the temperature change that occurred within the feeding window (11.00 h – 16.00 h). Values are means with standard errors of the mean and n=4-5 per group and significant differences between depot for each body weight group of sheep indicted by * $P<0.05$; ** $P<0.01$ sternal compared to muscle and RP fat.

Figure 5. Summary of the effect of increased body weight and fat mass on changes in plasma metabolites and insulin with feeding (represented by shaded region). Values are means with their standard errors and n=4-5 per group and significant differences between depot for each body weight group of sheep indicted by ** $P<0.01$ *** $P<0.001$ lean compared to control and obese. NEFA, non-esterified fatty acids.

Figure 6. The effects of body weight on adipocyte size and histological appearance in sternal and retroperitoneal (RP) fat of adult sheep. Panel A: Mean adipocyte cell size with sternal represented by closed boxes and RP by open boxes. Panels B-G: representative photomicrographs of haematoxylin and eosin stained sections; Panel H: example of uncoupling protein (UCP1) immunostaining from sternal adipose tissue. Rat brown adipose tissue was used as the positive control (Panel I) and staining specificity was determined with

rat brown adipose tissue in the absence of primary antibody (Panel J). Open bars: sternal fat; closed bars: retroperitoneal fat. Values are means with their standard errors and n=4-5 per group and significant differences between depot for each body weight group of sheep indicted by *P<0.05 compared to sternal fat, [a]P<0.01 compared to obese animals (within a fat depot). Scale bar represents 50µm.

Table 1. Summary of specific ovine sequence of forward and reverse oligonucleotides primers used for real time PCR.

Gene	Accession number	Forward primer	Reverse primer	Amplicon length (bp)
ACSM5	XM_015469322.1	CCACCATATGATGTGCAGGT	TGTCTTCTCAGGGTTGTCCA	138
ADIPOQ	NM_174742.2	ATCAAACTCTGGAACCTCCTATCTAC	TTGCATTGCAGGCTCAAG	232
ADIRF	NM_001114513.2	CCACAGAAGCAGGGCAGA	AAACCCGAGAAAGCCTCA	100
ATF2	XM_004004570.1	TCCCACTTGTTCGACCAGTCA	TTGACAGTATCGCCGTTGGT	151
C/EBPα	XM_004015623.1	CTGGAGCTGACCAGTGACA	GGGCAGCTGACGGAAGAT	96
C/EBPβ	NM_176788.1	ACGACTTCCTCTCCGACCTC	CCCAGACTCACGTAGCCGTA	85
CIDEA	NM_001083449.1	AAGGCCACCATGTACGAGAT	GGTGCCCATGTGGATAAGACA	138
CPT1b	NM_001034349.2	TGATCACGTATCGCCGTAAA	GAGCACATCTGTGTCCTTCC	137
DIO2	NM_001010992.3	AGCCGCTCCAAGTCCACTC	TTCCACTGGTGTCACCTCCT	175
En-1	XM_003581845.4	AACCCGGCCATACTGCTAAT	TTCTTCTTCAGCTTCCTGGTG	152
Eva-1	XM_004016067.3	GGAATTTCCGTCCTCGAGAT	AGGATGGAGACGTCATACCG	139
FABP4	NM_174314.2	TGAAATCACTCCAGATGACAGG	TGGTGGTTGATTTTCCATCC	98
GPR120	XM_002698388.1	CCTGGGACGTGTCATTTGCTA	CTGGTGGCTCTCCGAGTAGG	140
HOXC8	XM_002704245.5	TGTAAATCCTCCGCCAACAC	TGATACCGGCTGTAAGTTTGC	140
HOXC9	XM_002704244.2	GACCTGGACCCCAGCAAC	GCTCGGTGAGGTTGAGAAC	175
INSR	XM_002688832.3	CTGCACCATCATCAACGGAA	CGTAACTTCCGGAAGAAGGA	162
LEP	NM_173928.2	CCAGGATGACACCAAAACC	TGGACAAACTCAGGAGAGG	140
LHX8	XM_004003563.1	AGAGCACGCCACAAGAAACA	AGGGCTGGAGTCCAAGAGTT	199
NR3C1	NM_001206634.1	ACTGCCCCAAGTGAAAACAGA	ATGAACAGAAATGGCAGACATTTTATT	151
PGC1α	NM_177945.3	GATTGGCGTCATTCAGGAGC	CCAGAGCAGCACACTCGAT	84
PPARγ	NM_181024.2	GACCCGATGGTTGCAGATTA	TGAGGGAGTTGGAAGGCTCT	145

PPARγ	NM_181024.2	GACCCGATGGTTGCAGATTA	TGAGGGAGTTGGAAGGCTCT	145
PRDM16	XM_003583245.1	TGGCAGCTGGCTCAAGTACA	CGGAACGTGGGCTCCTCATC	198
PRLR	NM_174155.3	CTCCACCCACCATGACTGAT	CAGCGAATCTGCACAAGGTA	169
RIP140	XM_002684642.2	CGAGGACTTGAAACCAGAGC	TCTTAGGGACCATGCAAAGG	179
RyR1	NM_001206777	GGGATATGGGTGACACGAC	TCTCAGCATCAGCTTTCTCC	158
Serca 1a	XM_004020863.3	GCTGCTGTGGGCAATAAGAT	GCCAGTACCCCACTCTTTGA	150
Serca 2a	XM_012097784.2	CAGGTGTACCCACATTCGAG	TTCCCGAATGACAGACATGA	85
SHOX2	NM_001205527.1	CGCCTTTATGCGTGAAGAAC	TTGGCTGGCAGCTCCTAT	142
SREBF1	XM_004013336.1	AGGGGGACAAGGAGTTCTCA	CTCCGGCCATATCCGAACAG	72
Tbx15	NM_001079775.1	AATGGACATTGTACCTGTGGAC	TGACCACCTGTCTCATCCAA	158
TCF21	XM_014480981.1	ATCCTGGCCAACGACAAGTA	TCAGGTCACTCTCGGGTTTC	94
UCP1	XM_003587124.1	GGGCTTTGGAAAGGGACTACT	CAGGGCACATCGTCTGCTAAT	128
UCP2	NM_001033611	AAGGCCCACCTAATGACAGA	CCCAGGGCAGAGTTCATGT	128
UCP3	NM_001308581.1	ACCTGCTCACCGACAACTTC	CATATACCGCGTCTTCACCA	107
β-2-microglobulin	AY549962	CCAGAAGATGGAAAGCCAAA	CAGGTCTGACTGCTCCGATT	117
β-Actin	U39357	GCAAAGACCTCTACGCCAAC	TGATCTTGATCTTCATCGTGCT	120
Cyclophilin	JX534530	GCATACAGGTCCTGGCATCT	CATGCCCTCTTTCACTTTGC	136
IPO8	NM_001206120.1	GCCCTTGCTCTTCAGTCATT	GTGCAACAGCTCCTGCATAA	93
KDM2B	XM_004017579.1	CGGTCCTACCTCACTCAGGA	CCGTCTATGCTGGGCTTTCT	74
Malate dehydrogenase 1	AF233351	CGTTGCAGAGCTGAAGGATT	GGTGCACTGAGAGATCAAGG	100
RPLP0	NM_001012682.1	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	227
TBP	NM_001075742.1	CTTGGACTTCAAGATTCAGAACA	CCAGGAAATAACTCTGGCTCA	120
YWHAZ	NM_174814.2	CCGGACACAGAACATCCAGTC	TCAGCTCCTTGCTCAGTTACAG	125

Those listed at the bottom of Table that are shaded are the house-keeping genes.

Table 3. Summary of the changes in gene expression of putative markers of a) thermogenic, b) developmental, c) adipogenic and d) metabolic pathways in sternal adipose tissue over the first month of postnatal life in sheep.

Animal age	1 day	7 days	28 days
Thermogenesis			
PRLR	400 ± 80 [c]	390 ± 50 [e]	100 ± 10 [c, e]
PGC1α	11 270 ± 1 970 [c]	10 800 ± 1 540 [d]	2 220 ± 650 [c, d]
ATF2	4 770 ± 280 [a]	4 300 ± 180 [e]	7 090 ± 510 [a, e]
CIDEA	304 ± 56	400 ± 30	310 ± 70
Development			
HOXC9	65 ± 10 [c]	110 ± 10	190 ± 30 [c]
LHX8	10 ± 1 [a, b]	120 ± 40 [a]	210 ± 70 [b]
PRDM16	120 ± 40 [a]	80 ± 10	60 ± 10 [a]
SHOX2	1 190 ± 420	990 ± 90	1 420 ± 210
Adipogenesis			
PPARγ	21 810 ± 4 340 [c]	34 670 ± 1 890	46 480 ± 6 110 [c]
C/EBPα	12 300 ± 3 070 [c]	27 130 ± 1 920 [c, d]	12 820 ± 1 180 [d]
NR3C1	17 820 ± 2 590 [a]	15 870 ± 680 [e]	26 450 ± 910 [a, e]
SREBF1	8 850 ± 2 860 [c]	4 440 ± 270 [a]	3 350 ± 260 [a, c]
Metabolism			
LEP	110 ± 40 [a, e]	1 970 ± 470 [a]	6 100 ± 1 200 [e]
ADIPOQ (x10^3)	130 ± 30 [a, c]	370 ± 30 [a]	530 ± 80 [c]
FABP4 (x10^3)	1 450 ± 390	1 730 ± 110	2 090 ± 300
GPR120	10 ± 5 [e]	60 ± 20 [c]	560 ± 150 [c, e]
INSR	5 150 ± 1 960	7 240 ± 860	5 290 ± 580

Values are mean copy number with their standard errors, n=5-6 per group

Significant differences with age indicated by similar superscripts: [a, b] $p<0.05$; [c, d] $p<0.01$; [e] $p<0.001$

Table 4. Summary of the effects of altered adult body weight on the protein abundance of uncoupling protein (UCP) 1 and 3 in fat and skeletal muscle and of potential thermogenic proteins (i.e. sarcoplasmic reticulum calcium-transporting ATPases (SERCA)) in muscle.

	Lean	Control	Obese
Skeletal muscle			
UCP3	0.63±0.30	1.0±0.26	1.00±0.25
SERCA1	1.26±0.16	1.0±0.14	1.06±0.17
SERCA2a	1.18±0.11	1.0±0.08	1.06±0.10
Sternal adipose tissue			
UCP1	1.22±0.16	1.0±0.18	0.99±0.20
UCP3	0.68±0.17[a]	1.0±0.08	1.35±0.10[c]
Retroperitoneal adipose tissue			
UCP1	1.05±0.16	1.0±0.09	1.04±0.10
UCP3	0.73±0.08	1.0±0.16	1.02±0.10

All results expressed in arbitrary units, relative to controls and corrected against the density of staining for total protein.

Significant differences between body weight groups indicated by different superscripts a vs b $P<0.05$; a vs c $P<0.01$

Figure 1

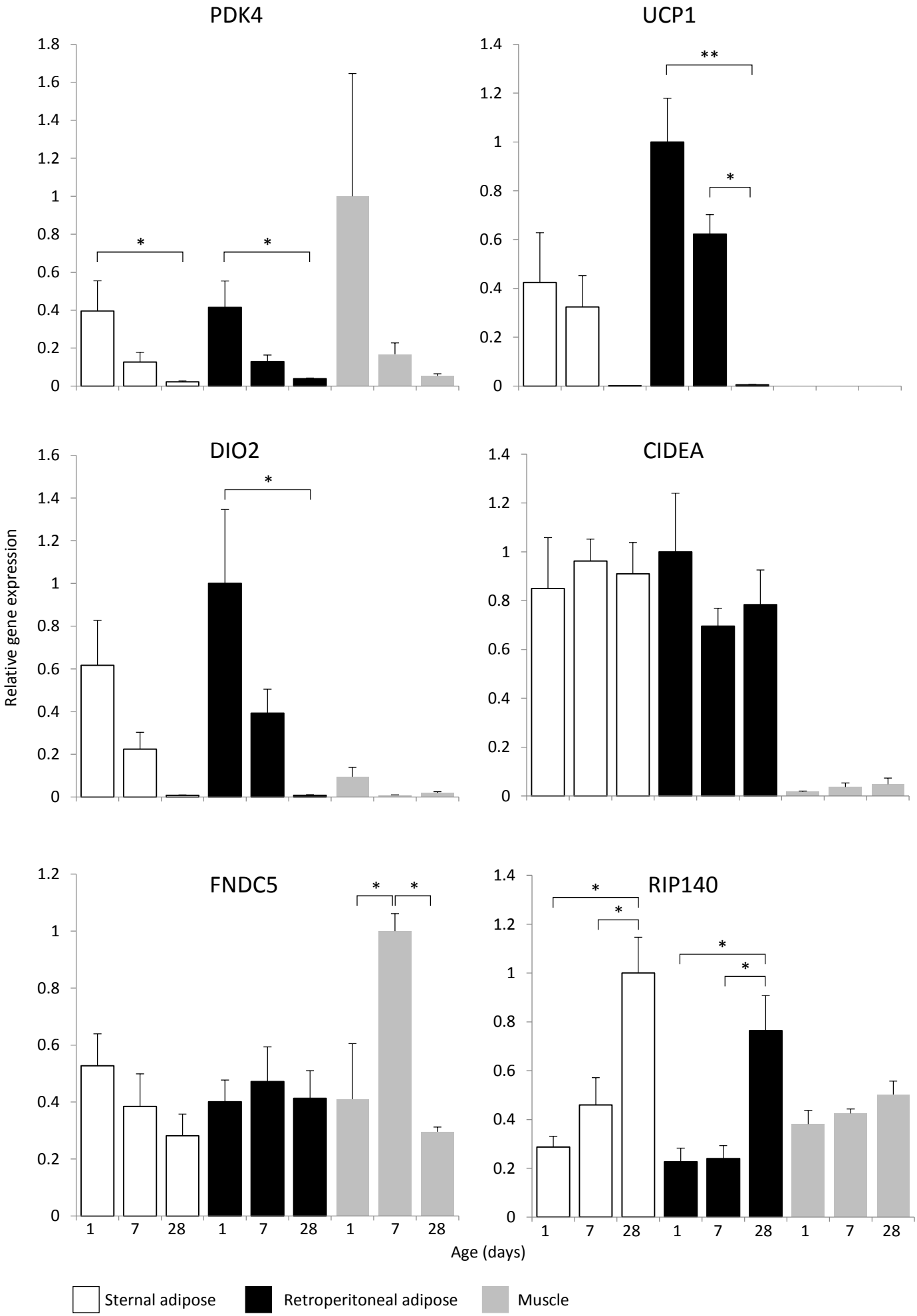


Figure 2

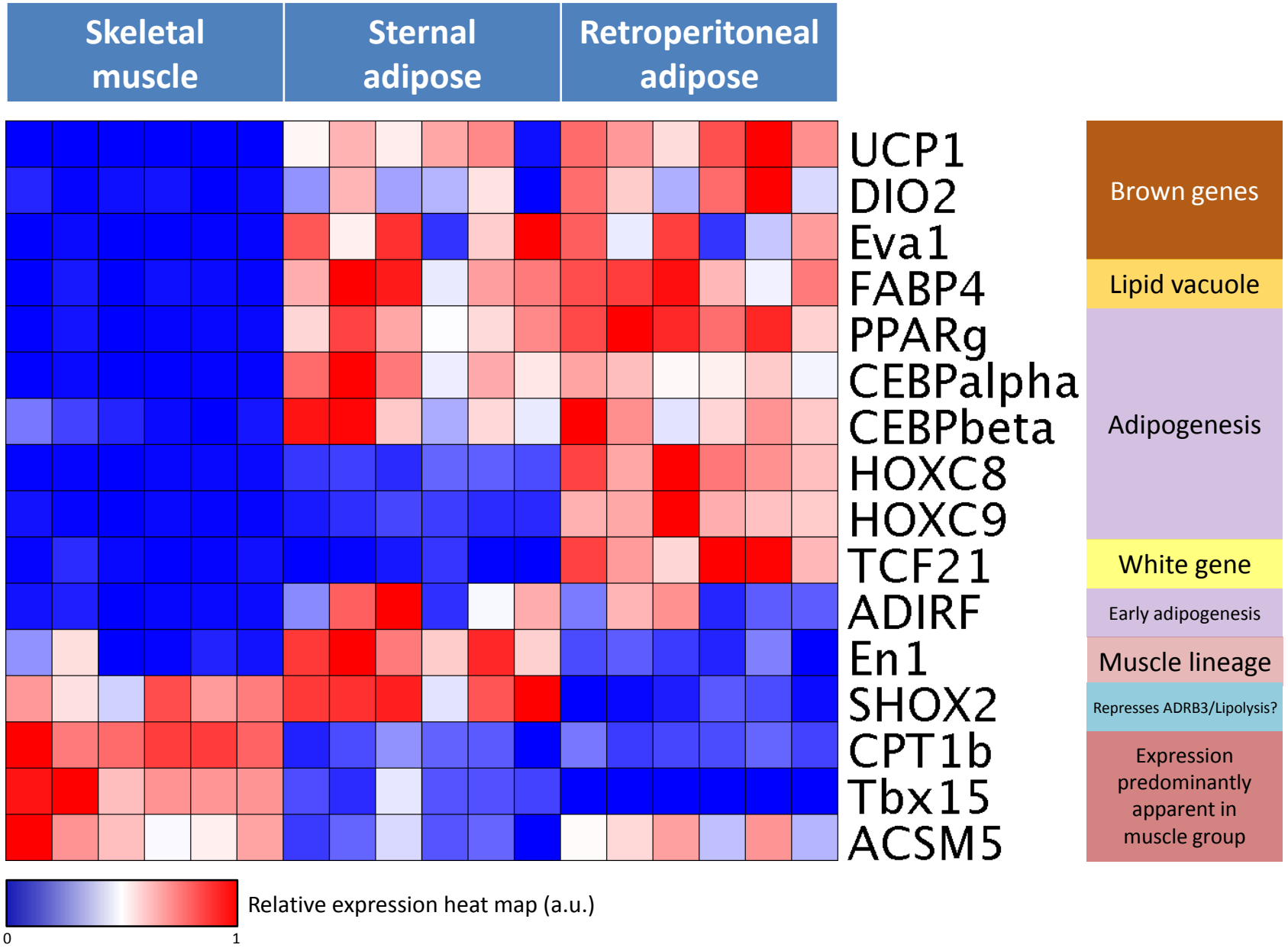


Figure 3

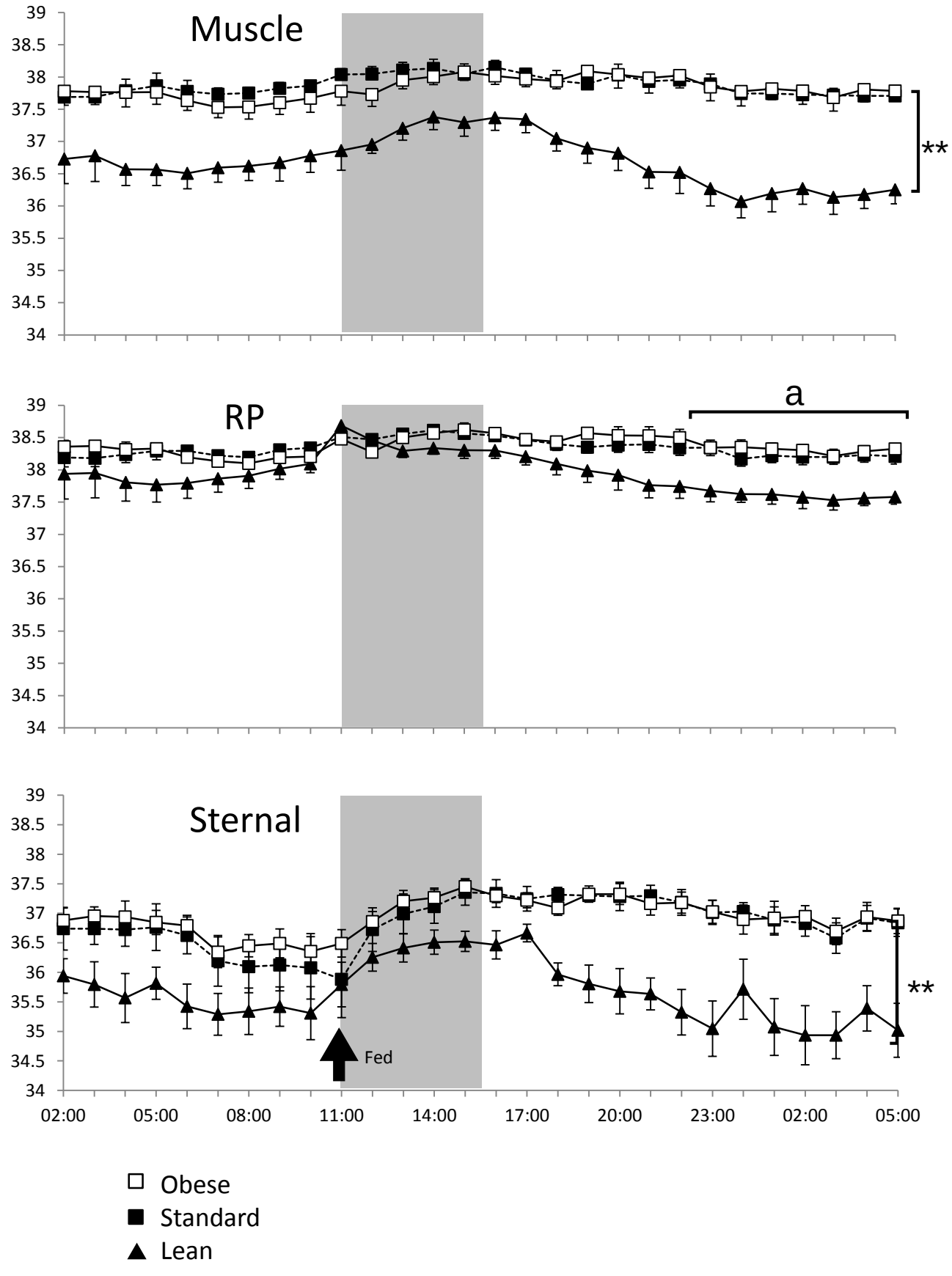


Figure 4

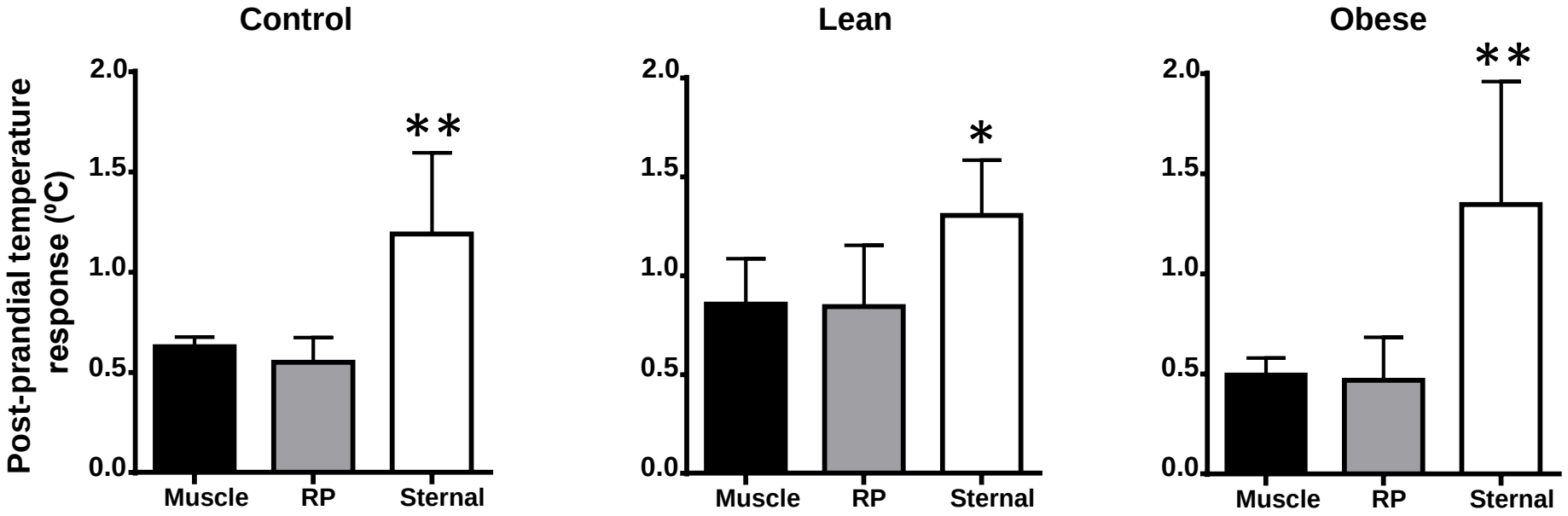


Figure 5

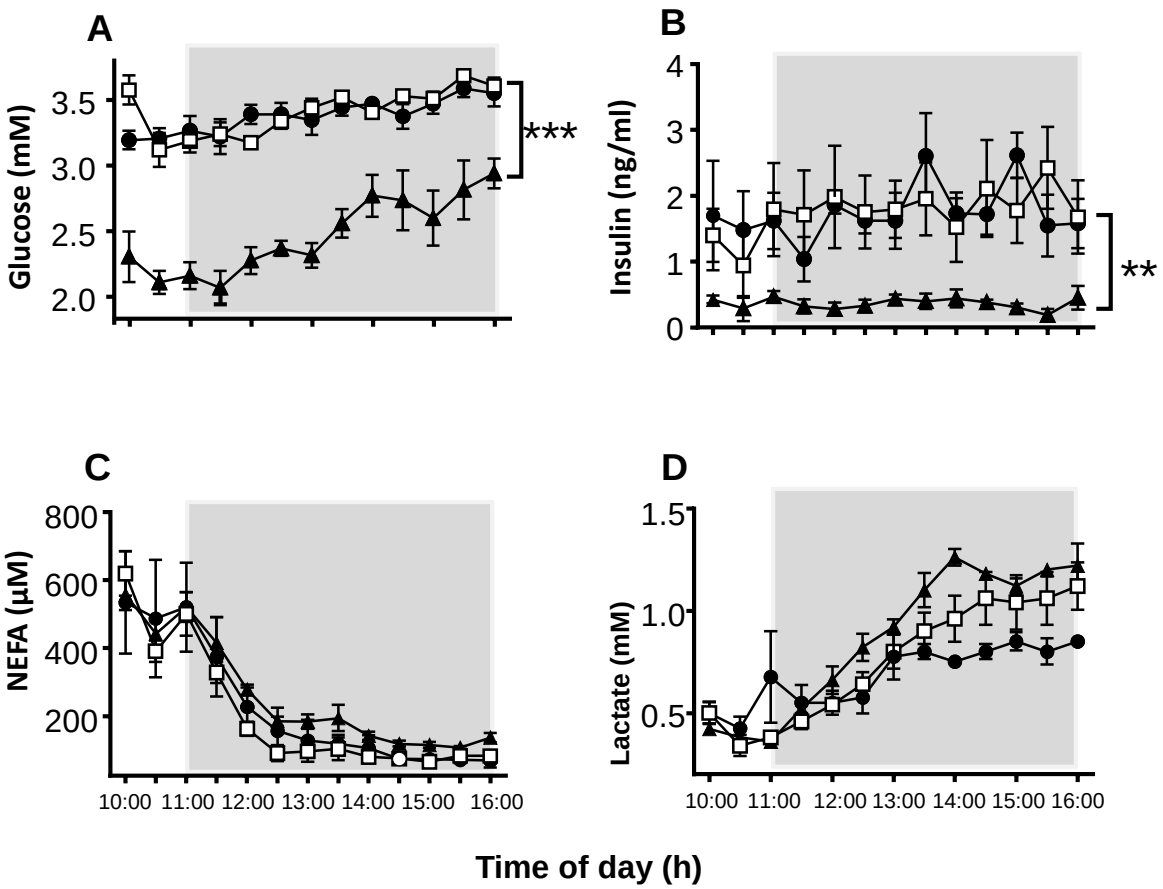
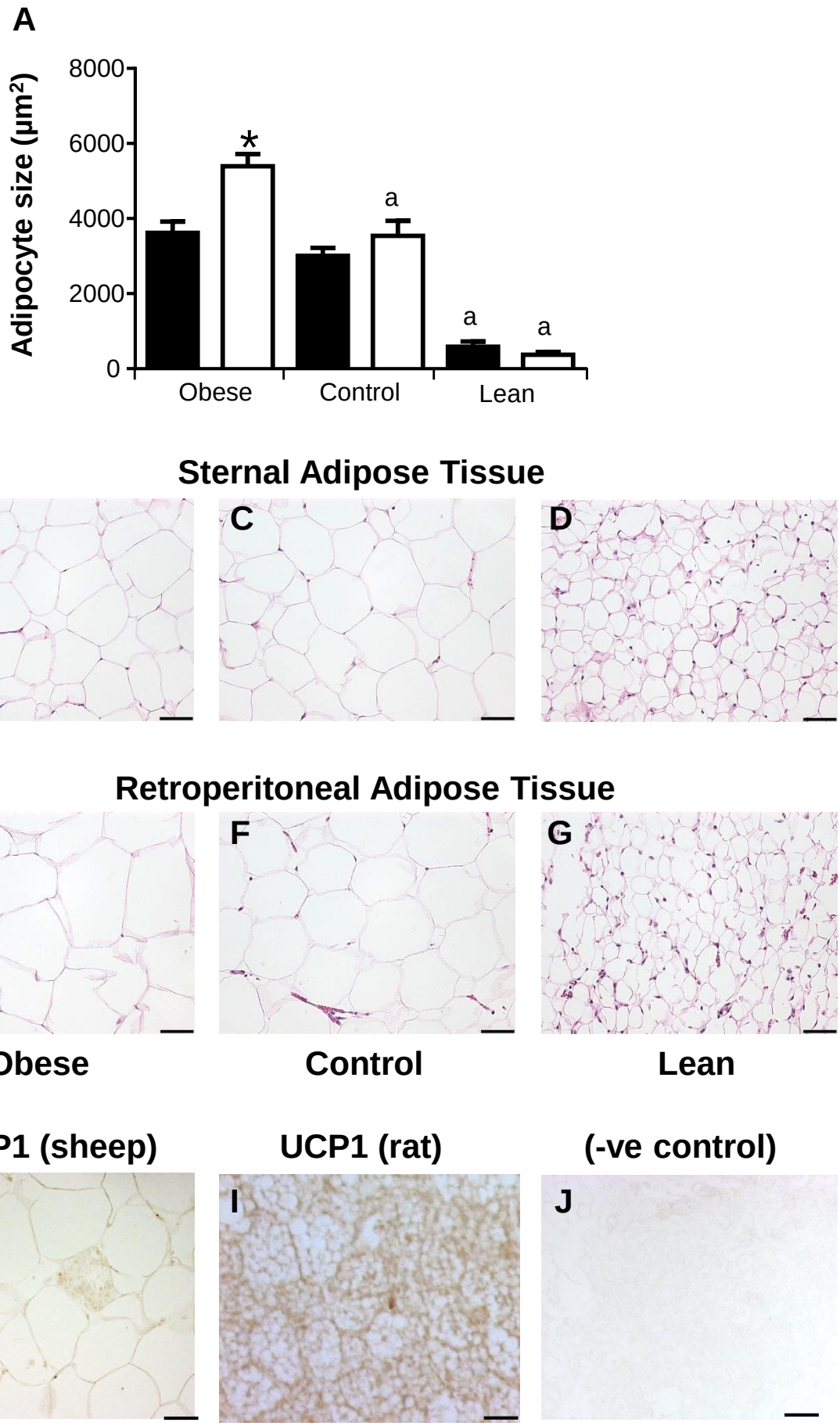


Figure 6



Antibody Table

Peptide/ Protein Target	Antigen Sequence (If Known)	Name of antibody	Manufacturer, Catalog No., o	Species Raise
UCP1		anti-UCP1	Antibodies Australia	Rabbit
UCP3	RALMKVQVLRESPF	Ab34677	Abcam	Rabbit
SERCA1		CaF2-5D2	Developmental Studies	Mouse
SERCA2a		CaS/C1	Developmental Studies	Mouse

d in Monoclon	Dilution used
	0.111111
	0.736111
	0.736111
	3.513889