Disparate effects of feeding on core body and adipose tissue temperatures in animals selectively bred for Nervous or Calm temperament

Belinda A. Henry,1 Dominique Blache,2 Alexandra Rao,1 Iain J. Clarke,1 and Shane K. Maloney3

1Department of Physiology, Monash University, Wellington Road, Melbourne, Victoria, Australia; 2School of Animal Biology and 3Department of Physiology, School of Biomedical, Biomolecular and Chemical Science, The University of Western Australia, Crawley, Western Australia, Australia

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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 299: R907–R917, 2010. First published June 23, 2010; doi:10.1152/ajpregu.00809.2009.—In addition to homeostatic regulation of body mass, nonhomeostatic factors impact on energy balance. Herein we describe effects of temperament on adipose and core body temperatures in sheep. Animals were genetically selected for Nervous or Calm traits. We characterized the effects of 1) high- and low-energy intake and maintenance feeding, 2) meal anticipation, and 3) adrenocorticotropic challenge on core body and adipose temperatures. Temperature measurements (5 min) were made using a thermistor inserted into the carotid artery (core body) and a probe in the retroperi toneal fat. An imposed feeding window was used to establish postprandial elevations in temperature. Fat tissue was taken from retroperitoneal and subcutaneous regions for real-time PCR analyses. We demonstrate that innate differences in temperament impact on adipose and core body temperatures in response to various dietary and evocative stimuli. In response to homeostatic cues (low-energy intake and maintenance feeding) core body temperature tended to be higher in Calm compared with Nervous animals. In contrast, in response to nonhomeostatic cues, Nervous animals had higher anticipatory thermogenic responses than Calm animals. Expression of uncoupling protein (UCP)-1 and -2 mRNA were higher in retroperi toneal tissue than in subcutaneous tissue, but UCP3 and leptin mRNA levels were similar at both sites; expression of these genes was similar in Nervous and Calm animals. There were no differences in stress responsiveness. We conclude that temperament differentially influences adipose thermogenesis and the regulation of core body temperature in responses to both homeostatic and non homeostatic stimuli.

Brown adipose tissue; energy expenditure; nonexercise activity thermogenesis; stress responsiveness

IN HUMANS, INHERENT DIFFERENCES in temperament are considered important determinants for the propensity to gain weight and develop obesity. In 1999, studies began to define the role of nonexercise activity thermogenesis (NEAT) in the determination of susceptibility of individuals to become obese (18). NEAT is characterized as the energy expended through daily activities that do not involve voluntary exercise, such as fidgeting, talking, walking, and postural allocation (18, 19). Subjects that display high levels of NEAT (or have a high-activity temperament) are less likely to become obese than those that display low levels (2, 17–19). In addition to NEAT, various aspects of temperament, including low inhibitory control (3), novelty seeking (28), and calmness (35) have all been associated with altered energy balance and adiposity. More specifically, girls with low inhibitory control are more likely to display increased weight gain and to be overweight by age 15 yr (3), whereas calm infants have been shown to have reduced adiposity at childhood (2–3.5 yr of age) compared with infants that are easily distressed (35). Furthermore, temperament and personality traits have been shown to differ between obese and lean subjects; obese subjects score higher on novelty seeking, but score lower in persistence and self-directedness (28). Indeed, obese subjects that score high in novelty seeking are less likely to be successful in weight loss through lifestyle interventions (28). The full spectrum of effects of temperament on energy balance, however, remains to be elucidated.

Energy balance is determined by the difference between energy intake and energy expenditure and the latter has three major components, being basal metabolic rate, physical activity/exercise and thermogenesis. Adaptive thermogenesis is altered by the brain in response to both dietary and cold stimuli and is thought to contribute to ~15% of total daily energy expenditure (21). There has been a recent surge of interest in understanding the role of thermogenesis in weight regulation and the maintenance of energy balance, following a series of papers that clearly and irrefutably demonstrate that functional brown adipose tissue exists in adult humans (9, 25, 27, 32, 33, 36). These studies demonstrated islands or pockets of brown fat cells dispersed among white adipose tissue, which are activated in response to cold exposure (27, 32, 33). Adipose tissue within the neck, supraclavicular, para-aortic, paravertebral, and suprarenal regions contains concentrated deposits of brown adipocytes (9, 25, 27, 32, 33, 36). In these brown fat deposits, thermogenic activity is inversely associated with body mass index (9, 27). Sheep represent a novel model to investigate changes in adipose thermogenesis because brown fat cells are also interspersed among white adipose tissue in this species (20). Indeed, we have demonstrated temperature excursions in adipose tissue that are consistent with postprandial or diet-induced thermogenesis, and this response is markedly upregulated by leptin action in the brain (15). These observations provide strong support for the notion that thermogenesis occurs in diffuse fat beds in nonrodent species.

It is not known whether genetic or inherent differences in temperament impact on thermogenic function. To investigate this, we have employed a unique ovine genetic model (24) to determine whether innate differences in temperament impact on postprandial heat production in adipose tissue. These studies demonstrate that sheep selectively bred for either Nervous or Calm behavioral traits display inherent differences in adipose tissue thermogenesis and thermoregulation. We propose that temperament may be an important factor in determining innate differences.

Address for reprint requests and other correspondence: B. A. Henry, Dept. of Physiology, Monash Univ., Wellington Rd., Melbourne 3800, Victoria, Australia (e-mail: belinda.henry@med.monash.edu.au).
in energy expenditure and that these will be reflected in differences in core body and adipose tissue temperatures.

MATERIALS AND METHODS

Ethics. The experimental work was approved by the animal ethics committee of the University of Western Australia (AEC 100/594). During experiments, the animals were housed in individual pens at the Large Animal Facility at the University of Western Australia, Crawley, Australia. The sheep were exposed to a constant 22–23°C and a 12:12-h light-dark cycle with lights on at 0730 h.

Animals. In animals, temperament models are based on emotional reactivity, and this is determined by tests that largely assess behavioral responses to fear and anxiety (4, 5, 10). Genetically divergent lines of Merino sheep have been created by selective breeding for Nervous and Calm temperament (24). Temperament selection has been based on a method developed at The University of Western Australia and is a combination of two objective behavioral tests: the arena test and the box test (24). Briefly, the arena test is a motivational choice test that measures the approach/avoidance behavior of the animal to humans (24). Although domestication of species is associated with reduced fear to humans, such behaviors are still observed in farm species and therefore can be used to index “fearlessness” or temperament (10). The box test is similar to isolation tests used for sheep and cattle (8, 14) and provides an objective measure of the degree of anxiety associated with isolation. Animals that display heightened anxiety within the box test and greater fear to humans have been selected as Nervous, whereas those that show attenuated anxiety and fear have been selected as Calm. A genetic selection index was calculated using both tests and was used to selectively breed two divergent lines of sheep (Nervous or Calm). In these animals, temperament is moderately heritable (∼0.4) so it responds favorably to selection (24). Animals used in the present study were selected from a flock that has been continuously selected and bred for either Nervous or Calm temperament for 15 generations. We studied female sheep, which were ovariectomized at least 2 wk prior to experimentation, to remove cyclic influences of ovarian steroids. All experiments used virgin female animals of similar age (3–5 yr) that were randomly selected from the flock.

At the commencement of experimentation, Nervous and Calm sheep had similar body mass (Nervous: 54.3 ± 1.7 kg, Calm: 55.1 ± 2 kg).

Surgery. Measurements of core body temperature were made using ruggedized glass-coated bead thermistors (bead diameter 0.3 mm, model ABOE3-BR11KA103N; Thermometrics, Edison, NJ) in a blind-ended, thin-walled polytetrafluoroethylene tube (OD, 1.35 mm; ID, 0.97 mm; Straight Aortic Flush 4F Catheter; Cordis, Bridgewater, NJ) placed into the left carotid artery (11). The thermistor was connected to insulated extension leads attached to data loggers (Stowaway XT; Onset Computer, Pocasset, MA) implanted in the neck. Retropertioneal adipose tissue temperature (used as an index of thermogenic output) was measured using loggers with inbuilt temperature sensors (12-mm diameter × 6-mm width, iButton; Maxim Integrated Products, Sunnyvale, CA).

With the sheep under isoflurane anesthesia, an incision was made along the neck, the left carotid artery isolated, and the catheter inserted. The thermistor was positioned midway along the length of the neck and advanced 100 mm toward the heart. The base of the thermistor probe was embedded in the fat and not in close proximity to the kidney. All recording devices were calibrated against a certified mercury in glass thermometer (National Association of Testing Authorities, Sydney, Australia) between 30 and 42°C and programmed to record temperature every 5 min. At the end of experimentation, the animals were again anesthetized, the thermistors and data loggers were retrieved and recalibrated, and the data was downloaded.

Postprandial thermogenesis. To investigate the effects of temperament on postprandial thermogenesis and core body temperature, we employed a model of temporal feed restriction to entrain the response, as previously described (15), wherein the animals were exposed to scheduled maintenance feeding for 4.5 wk prior to experimentation. Sheep are normally continuous feeders and therefore do not display postprandial responses, but we have demonstrated that a postprandial response can be entrained by imposing a daily meal period (15). In the present studies, the following feeding windows were employed: experiment 1, 0900–1600 h; experiment 2, 1200–1600 h; and experiment 3, 0930–1600 h. During this period, the animals were offered a standard maintenance diet of 800 g/day of lucerne chaff and 100 g/day of lupin grain, unless the energy intake was altered (see below).

Experiment 1. Effect of temperament on temperatures in animals fed in excess to, or less than, maintenance requirements. Prior to experimentation, the 12 sheep (6 Calm and 6 Nervous) were brought into the Large Animal Facility in Crawley and acclimated for 2 wk. Surgeries for implantation of recording devices and ovarioectomy were performed during the third week, and then at least 10 days recovery was allowed before experimentation began. Throughout the aclimatization, the feeding window was 0900–1600 h. The animals were fed a maintenance diet of lucerne chaff; individual maintenance requirements were calculated as predicted by the daily energy requirement (MJ); this was based on body mass and predicted growth rate. This feeding regimen entrained the postprandial response in both adipose tissue and core body temperatures.

To examine the effects of high- and low-energy intake on thermoregulation and thermogenesis, for 12 days the sheep were fed either 1.5 or 0.7 times the maintenance energy requirements. To improve the palatability of the excess energy, the high-energy diet was achieved mainly by supplementation with lupin grain, not simply a proportional increase in chaff and lupin. Thus, animals on the high-energy diet were offered 1,050 g/day of lucerne chaff supplemented with 200 g/day of lupin grain, where those on the low-energy diet were offered 550 g/day of lucerne chaff and 60 g/day of lupin grain. Animals were maintained on either the high (n = 3/temperament) or the low (n = 3/temperament) energy intake diet for 12 days, after which they were returned to maintenance feeding for 12 days. The high- and low-intake diets were then reintroduced using a cross-over design so that group size was n = 6/group. Food intake and body mass were measured before, during, and after dietary manipulation. Food intake was calculated each day by measuring the residual food at 1600 h; intake was then converted into the energy consumed (MJ).

Experiment 2. Effect of temperament on temperatures with fasting, meal anticipation, and refeeding. We examined the effects of fasting, meal anticipatory behavior, and refeeding on postprandial temperatures in Nervous and Calm sheep. Baseline measures of postprandial temperatures in both adipose and core body temperature were established by programmed feeding for 1 wk, during which a maintenance diet of lucerne chaff was offered between 1200 and 1600 h. On day 1 of the experiment, the animals were fed the maintenance diet at the usual time (n = 6/group). The following day, all of the animals were fasted (n = 6/group), where food was not offered at the scheduled time of 1200 h. To elicit meal anticipatory behavior (day 3), the fasted animals were exposed to a “friend-feeding” paradigm, where half of the animals were fed and the remaining animals remained fasted (n = 3/group). The fasted animals were in pens adjoining the fed animals, separated by wire mesh pen walls, and thus were in visual contact with the feeding animals. Finally, on the last day of the experiment (day 4) all of the animals were fed to measure the refeeding response. This 4-day protocol was repeated in a cross-over design incorporating a 3-day recovery period.

Experiment 3. Effect of temperament on the cortisol response after an ACTH-challenge: implications for site-specific temperature regulation. The animals received an ACTH challenge to determine whether 1) cortisol responsiveness differed in Nervous and Calm sheep and 2) whether increased cortisol secretion impacted on core
body and adipose temperatures. The animals were injected intramuscularly with either 0.5 mg Synacthen Depot (1 mg/ml ACTH) or an equivalent volume of saline as control at 0900 h and a cross-over design was incorporated with 1-day recovery between injections. On the day prior to the first injection, indwelling cannulas were inserted into the jugular vein for blood sampling. Blood samples (5 ml) were collected every 10 min for 3 h (0800–1100 h) and then every 20 min for 5 h (1100–1600 h); plasma was harvested and used to characterize cortisol levels. Across this experiment, the animals were allowed access to food between 0930 and 1600 h.

Plasma cortisol concentrations were measured in duplicate after extraction with 2 ml of dichloromethane using a radioimmunoassay based on separation with dextran-coated charcoal (1). The samples were assayed as duplicate 50 μl aliquots of plasma and the limit of detection was 0.2 ng/ml. The assay included six replicates of two control samples containing 2.6 and 4.7 ng/ml, which were used to estimate the intra-assay coefficients of variation as 7.5 and 10%. To characterize cortisol responsiveness, we analyzed the mean cortisol concentration and the amplitude of response after ACTH challenge.

Experiment 4. Expression of genes for leptin and uncoupling proteins in adipose tissue of nervous and calm sheep. Nervous and Calm animals (n = 5 Nervous, n = 6 Calm) were killed, and subcutaneous and retroperitoneal adipose samples were taken and snap frozen in liquid nitrogen, with subsequent storage at −80°C. RNA was extracted from the fat samples using the Trizol method. Quality of RNA was determined by the visualization of 18- and 28S bands. Uncoupling proteins (UPC)-1, -2, and 3, and leptin mRNA expression were analyzed using real-time PCR (Realplex4, Eppendorf). In each case, detection was 0.2 ng/ml. The assay included six replicates of two control samples containing 2.6 and 4.7 ng/ml, which were used to estimate the intra-assay coefficients of variation as 7.5 and 10%. To characterize cortisol responsiveness, we analyzed the mean cortisol concentration and the amplitude of response after ACTH challenge.

Table 1. Food intake and body mass loss and gain in Nervous and Calm sheep during maintenance feeding as well as high- and low-energy intake

<table>
<thead>
<tr>
<th></th>
<th>Nervous Animals</th>
<th>Calm Animals</th>
<th>Statistical Significance</th>
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<tbody>
<tr>
<td>Food Intake, MJ/day</td>
<td></td>
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<tr>
<td>Maintenance feeding</td>
<td>8.7 ± 0.2</td>
<td>7.8 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>High-energy diet</td>
<td>9.44 ± 0.52</td>
<td>8.93 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Low-energy diet</td>
<td>4.77 ± 0.1</td>
<td>4.14 ± 0.43</td>
<td>P &lt; 0.05*</td>
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<tr>
<td>% Change in Body Mass</td>
<td></td>
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<tr>
<td>Initial maintenance feeding from pasture</td>
<td>−5.25 ± 1.3</td>
<td>−11.66 ± 1.38</td>
<td>P &lt; 0.05†</td>
</tr>
<tr>
<td>High-energy diet</td>
<td>3.01 ± 1.16</td>
<td>2.97 ± 0.76</td>
<td>NS</td>
</tr>
<tr>
<td>Maintenance feeding after high-energy diet</td>
<td>0.24 ± 0.73</td>
<td>1.14 ± 0.82</td>
<td>NS</td>
</tr>
<tr>
<td>Low-energy diet</td>
<td>−5.24 ± 1.04</td>
<td>−3.84 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Maintenance feeding after low-energy diet</td>
<td>4.27 ± 0.44</td>
<td>4.81 ± 1.24</td>
<td>NS</td>
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Values are means ± SE. *Compared with the high-energy diet; †effect of temperament. NS, not significant.

Fig. 1. Effects of high-energy intake on core body temperature (CBT) in Nervous (○) and Calm (□) sheep. There was little effect of temperament on the postprandial elevation in CBT (top), albeit CBT was higher (*P < 0.05, effect of temperament) in Calm animals on days 6 and 12, but this only occurred at discrete time points during the postprandial period. There was no effect of temperament on the mean preprandial temperature (middle) or the amplitude of response (bottom) in Nervous or Calm animals on high-energy intake.
purified DNA of known concentration was used as the assay standard. In the initial optimization of each primer set, PCR products were separated by agarose gel electrophoresis, purified, and sequenced to confirm identity. The levels of expression of each mRNA and the estimated concentrations were determined relative to the standard preparation (determined by spectrophotometer) using Realplex4 computer software. We used similar amounts of RNA for each amplification, but to correct for minor differences in the total amount of RNA used between samples, we calculated the ratio of each mRNA to the geometric mean of the three most stable reference genes (determined by geNorm analysis from a panel of 7 possible reference genes). In this case, we used β-actin, malate dehydrogenase-1, and β-2-microglobulin as housekeeping genes.

For each sample, 1 µg of total RNA was treated with DNA-free from Ambion according to the manufacturer’s instructions. First-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems.

For PCR, 4 µl of each DNA standard and sample cDNAs (diluted 1:2) were added to individual wells. A master mix was prepared by using Brilliant II SYBR Green Master Mix from Stratagene, sterile water, and primers (sense and antisense) for a final volume of 20 µl. Based on available sequence data of the genes of interest, the primers used were: UCP1 (GenBank accession no. AY371696), sense 5′-AGAGCCATCTCCAGGTCCCA-3′, antisense 5′-CCAAAGCCTCCCGTCAAG-3′; UCP2 (GenBank accession no. NM_001033611), sense 5′-AAGGCCACCTAATGACAGA-3′, antisense 5′-CCCAGGGCAGAGTTCATGT-3′; UCP3 (GenBank accession no. NM_174210), sense 5′-TGACCTCCTACCGTCTTC-3′, antisense 5′-AAATCCGGGTAATGATGCTG-3′; and leptin (GenBank accession no. OAU84247), sense 5′-GGGTCACTGGTTTGGACTTC-3′, antisense 5′-GCGAGGATCTGGTGTGAG-3′.

Data and statistical analyses. Postprandial thermogenesis was measured by averaging data into 1-h blocks. These data were then used to characterize the temperature responses in the preprandial (baseline) and postprandial periods. In experiments 1 and -2, further analyses were undertaken to calculate the amplitude of the postprandial rise in adipose and core body temperatures. In experiment 1 the amplitude was calculated by subtracting the temperature at 1000 h (hour after feeding) from the mean preprandial temperature. In experiment 2, the amplitude was calculated by determining the difference between the hourly averages from 1100 h (the hour preceding feeding) and 1300 h (the hour after feeding). In addition, in experiment 1 the mean preprandial temperature was calculated (average temperature between 0400 and 0700 h). Data were checked for homogeneity of variance using Levene’s test and then analyzed by repeated-measures ANOVA. When a significant interaction between temperament and time was detected, analyses of specific time points were performed using a single-factor ANOVA. For analyses of gene expression, we performed repeated-measures ANOVA with the specific adipose depots as the repeated factor within animals.

RESULTS

Experiment 1. the effect of temperament on site-specific temperature regulation in animals fed in excess to, or less than, maintenance requirements. During the initial housing familiarization and maintenance feeding period, food intake was similar in Nervous and Calm animals (Table 1). Total daily energy intake (Table 1) was greater \( P < 0.05 \), effect of diet) when the animals were offered the high-intake diet compared with when the animals were offered the low-intake diet, regardless of

![Fig. 2. Effects of high-energy intake on retroperitoneal fat temperature in Nervous (●) and Calm (○) sheep. There was little effect of temperament on the postprandial elevation in adipose temperature (Ad T) (top). There was a trend \( P = 0.066, \) effect of temperament) toward a reduction in Ad T in Nervous compared with Calm animals on day 12. The mean preprandial temperature (middle) and the amplitude of the postprandial response (bottom) were similar in the Nervous and Calm groups across the experimental period.](http://ajpregu.physiology.org/)
temperament. In spite of similar levels of food intake, however, the %change in body mass (mass gain/initial body mass × 100) differed between the Nervous and Calm animals (Table 1). The Calm animals initially displayed a greater (P < 0.05, effect of time) loss in body mass when placed on the maintenance diet (Table 1) compared with Nervous sheep. Subsequently there was no effect of temperament on mass gain or loss during the times of high or low intakes (Table 1).

There was no effect of temperament on either core body or adipose tissue temperatures in animals on high intake (Figs. 1 and 2), albeit there was a trend (P = 0.066) toward a reduction in the postprandial elevation in adipose tissue temperature in Nervous animals on day 12 (Fig. 2). The mean preprandial core body (Fig. 1) and adipose temperatures (Fig. 2) were similar in Nervous and Calm sheep on high intake. Furthermore, there was no effect of temperament on the amplitude of the postprandial response in animals on the high diet.

In contrast, temperament significantly impacted on core body temperature when animals were on low feed intake (Fig. 3). The effect of low-energy intake on core body temperature was greater in the Nervous animals compared with the Calm group. On days 1–6, core body temperatures were typically lower (P < 0.05) in Nervous compared with the Calm animals (Fig. 3). The mean preprandial core body temperature was lower (aa P < 0.01; effect of temperament) in Nervous compared with Calm animals on days 1, 3, and 6. Furthermore, the mean preprandial core body temperature was reduced (b P < 0.05; effect of time) on days 6 and 12 compared with day 1 in Nervous animals, but this effect did not manifest in Calm animals until day 12. In Calm animals only, the amplitude of the postprandial response was increased (c P < 0.05; effect of time) on day 12 compared with day 1 (due to a reduction in core body temperature during the preprandial period on this day) (Figs. 1 and 2). There was no effect of low-energy intake on the amplitude of the postprandial response in core body temperature in the Nervous group. With respect to adipose tissue temperature, there was little effect of low-energy intake in both the Nervous and Calm animals (Fig. 4). On day 12, adipose temperature was lower (d P < 0.05; effect of temperament) in Calm animals during the preprandial period compared with Nervous animals (Fig. 4). Thus when animals were on low-energy intake, changes in adipose temperature were consistent with changes in core body temperature in the Nervous animals. In Calm sheep, reduced core body temperature was not associated with any change in adipose temperature.

There was no effect of low-energy intake on the amplitude of the postprandial response in adipose tissue in either the Nervous or Calm groups (Fig. 4).

Experiment 2. the effect of temperament on thermogenesis with fasting, meal anticipation, and refeeding.

During maintenance feeding, Calm animals displayed an extended (a P < 0.05, effect of temperament) postprandial elevation in core body temperature (Fig. 3). During this period (days 1–6), the mean preprandial temperature (middle) was lower (aa P < 0.01) in the Nervous animals compared with the Calm. In Nervous animals, feeding the low-energy diet reduced (b P < 0.05, effect of time) the mean preprandial temperature on days 6 and 12 compared with day 1, whereas in Calm animals the low-energy diet reduced (c P < 0.05, effect of time) the mean preprandial temperature on day 12 alone. There was no effect of diet on the amplitude of the postprandial response in Nervous animals. In Calm animals, however, the amplitude of the postprandial response increased (d P < 0.05, effect of time) on day 12 compared with day 1 of dietary manipulation. This effect was primarily due to a reduction in preprandial temperature on this day.

Fig. 3. Effects of temperament on CBT were unmasked when animals were fed a low-energy diet. CBT was lower (P < 0.05) in Nervous (●) compared with Calm (○) sheep on days 1–6 (top). During this period (days 1–6), the mean preprandial temperature (middle) was lower (aa P < 0.01) in the Nervous animals compared with the Calm. In Nervous animals, feeding the low-energy diet reduced (b P < 0.05, effect of time) the mean preprandial temperature on days 6 and 12 compared with day 1, whereas in Calm animals the low-energy diet reduced (c P < 0.05, effect of time) the mean preprandial temperature on day 12 alone. There was no effect of diet on the amplitude of the postprandial response (bottom) in Nervous animals. In Calm animals, however, the amplitude of the postprandial response increased (d P < 0.05, effect of time) on day 12 compared with day 1 of dietary manipulation. This effect was primarily due to a reduction in preprandial temperature on this day.
perature compared with the Nervous animals (Fig. 5A). The exacerbated response in Calm animals, however, was not evident with respect to the retroperitoneal fat temperature. In both the Nervous and Calm animals, fasting reduced (bP/H110210.05, effect of fasting) core body and adipose temperatures during the preprandial periods, as evident on the day of meal anticipation and refeeding (Fig. 5, C and D). While there remained a small increase in core and adipose temperatures prior to the usual feeding time on the day of fasting (Fig. 5B), temperatures were lower (P < 0.05, effect of fasting) in the afternoon (after 1200 h or during the period generally associated with feeding) in both the Nervous and Calm groups. Regarding the meal anticipation paradigm (Fig. 5C), there was a reduction in temperature in both core body and adipose tissues lower (bP < 0.05, effect of time compared with maintenance feeding) during the postprandial period in both groups compared with the same times when food was received. The Nervous and Calm animals exhibited similar responses to refeeding (Fig. 5D).

The amplitude of the postprandial thermogenic response in adipose tissue was similar in Nervous and Calm sheep during maintenance feeding (Fig. 6A), fasting (Fig. 6B), and refeeding (Fig. 6D), but the amplitude of the response was greater (bP < 0.001, effect of temperament) in the Nervous animals than in the Calm animals during meal anticipation (Fig. 6C). In contrast, the amplitude of the postprandial response in core body temperature was similar in Nervous and Calm sheep across all four paradigms. Thus, during meal anticipation there was a divergent effect of temperament on core body and adipose temperature responses.

Experiment 3. the effect of temperament on responsiveness after ACTH challenge. The mean plasma concentration of cortisol and the amplitude of the cortisol response to ACTH were similar in the Nervous and Calm animals (Table 2). Core body and fat temperatures increased after the injection of either saline or ACTH (Fig. 7) but there was no difference between the saline control and the ACTH-challenge in either adipose temperature or core body temperature in either group of sheep (Fig. 7).

Experiment 4. the expression of leptin and uncoupling proteins in adipose tissue of nervous and calm sheep. The expression of UCP1, UCP2, UCP3, and leptin mRNA was analyzed in both subcutaneous and retroperitoneal fat. The expression of UCP1 and UCP2 mRNA was higher (P < 0.05) in the retroperitoneal fat compared with the subcutaneous fat depot, whereas expression of UCP3 and leptin was similar in the two depots (Fig. 8). There was no effect of temperament on the expression of these genes (Fig. 8).

DISCUSSION

This study demonstrates that selection for temperament impacts on the regulation of adipose tissue and core body temperatures in sheep. We investigated the effects of both homeostatic (maintenance feeding, high- and low-energy diet) and nonhomeostatic (meal anticipation) cues on temperature.
Under homeostatic (experiment 1: low-energy intake, and experiment 2: maintenance feeding) conditions, core body temperature was generally higher in Calm animals than in Nervous animals. The marked reduction in core body temperature in Nervous animals during low-energy intake, however, was dissociated from any change in adipose tissue temperature. This discrepancy strongly suggests that changes in adipose thermogenesis are not a major determinant of core body temperature, at least in animals selectively bred for Nervous temperament exposed to homeostatic cues. This further indicates that the generation of heat by other tissues (such as skeletal muscle) may contribute more substantially to core body temperature or that other processes are involved. In contrast, under nonhomeostatic (meal anticipation) conditions, Nervous animals displayed an exacerbated increase in adipose tissue heat production compared with the Calm group, indicative of enhanced thermogenic potential in the Nervous animals. Thus, these studies suggest that under homeostatic conditions, Calm animals display greater rates of energy expenditure as suggested by higher core body temperature. Whereas, under nonhomeo-

**Fig. 5.** The effect of fasting, meal anticipation, and refeeding on CBTs and retroperitoneal fat temperatures in Nervous (●) and Calm (○) sheep. A: maintenance feeding resulted in an increase in both CBTs and Ad Ts in Nervous and Calm sheep; the duration of the increase in CBT was greater in Calm animals. B: fasting for 24 h abolished all postprandial temperature responses in both Nervous and Calm sheep. C: preprandial temperatures (both CBT and Ad T) were lower on the days of meal anticipation and refeeding since this was after 24 h without food. Meal anticipation increased both CBT and adipose thermogenesis in the Nervous and Calm sheep. D: finally, refeeding restored the postprandial response in CBT and adipose thermogenesis and these responses were similar in Nervous and Calm animals. *P < 0.05 effect of temperament, †P < 0.05 effect of diet in both Calm and Nervous animals.

**Fig. 6.** The effect of maintenance feeding (A), fasting (B), meal anticipation (C), and refeeding (D) on the amplitude of the postprandial response in Ad Ts and CBTs in Nervous (black bar) and Calm (white bar) animals. Nervous and Calm animals exhibited a similar increase in CBT and Ad T after maintenance and refeeding. Likewise, there was a similar reduction in response in both CBTs and Ad Ts in fasted animals. In contrast, the response to meal anticipation differed in Nervous and Calm sheep. The amplitude of response in retroperitoneal fat was greater (aaaP < 0.001, effect of temperament) in Nervous compared with Calm animals, although there was no difference in the amplitude of response in CBT.

**EFFECT OF TEMPERAMENT ON THERMOGENESIS IN FAT**

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static conditions, Nervous animals display enhanced adipose thermogenesis compared with the Calm group.

In humans and cattle, innate differences in temperament are known to impact on energy balance and the predisposition to obesity. In humans, temperament traits differ between obese and lean subjects with obese subjects showing greater tendency toward novelty-seeking behaviors but a reduction in self-directedness and persistence (28). Furthermore, obese subjects who display higher scores on novelty-seeking tests are generally less likely to succeed in weight loss programs (28). In animal models, temperament is deemed an objective measure of the levels of the fear and anxiety an animal experiences in the face of either an actual (fear) or perceived (anxiety) threat (5, 10). Thus, animals that are characterized as Calm, generally display reduced fear and anxiety in behavioral paradigms, whereas animals that are Nervous display increased levels of fear and anxiety (4). Previous studies in cattle have demonstrated that animals with a Calm temperament have greater daily weight gain when held in a feedlot compared with those with excitable temperament (34). Cattle with a Calm temperament are thought to have a higher feed efficiency and therefore eat less than predicted based on growth requirements (26), leading to the suggestion that calm animals have lower levels of energy expenditure. In contrast, infants that display a Calm temperament have lower levels of adiposity in childhood than infants that are easily distressed (35). Interestingly, in the current study we report that under homeostatic conditions, such as maintenance feeding or low-energy intake, core body temperatures were higher in the Calm compared with Nervous animals, suggesting that the former either have higher levels of heat production or reduced levels of heat loss than Nervous sheep. One possibility is that the Calm animals have greater fat deposition, which provides increased insulation against heat loss, and indeed, this could play a role in the maintenance of higher core temperature in the postprandial period in the Calm animals during maintenance feeding. On the other hand, consistent with the notion that heat production was the cause of the elevated core temperature was the observation that Calm animals lost more body mass than the Nervous animals when initially placed on the maintenance diet. It is possible that this reduction in body mass resulted from altered dietary intake in that Calm animals may have a tendency to over eat when on an ad libitum diet on pasture. Given that Calm and Nervous animals ate similar amounts during this period of weight loss, another explanation for the greater reduction in body mass was increased energy expenditure. To further delineate whether Calm animals have greater propensity to over eat when excess food is available or whether these animals display increased rates of energy expenditure, requires further investigation. The current data, however, demonstrate that Calm animals have increased core body temperatures under various feeding paradigms, including low-energy intake and maintenance feeding indicative of increased rates of energy expenditure. It must be noted, however, that body mass loss and gain did not differ

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<th>Table 2. Effect of ACTH-treatment on cortisol secretion in Nervous and Calm sheep</th>
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Values are means ± SE in ng/ml. *Compared with saline treatment.

Fig. 7. The effect of ACTH-challenge on CBTs and Ad Ts in Nervous and Calm sheep. On different days, the animals were given either an intramuscular injection of the ACTH analog [0.5 mg Synacthen Depot (●)], or an equivolume injection of saline (○). All injections were performed at 0900 h. There was no effect of ACTH treatment or the elevated cortisol levels on the postinjection or postprandial increases in CBTs or Ad Ts in Nervous or Calm sheep.
between the two genotypes when they were given high- and low-energy diets. This similarity between the genotypes may be due to the short time frame of feeding (12 days) that we employed. These studies demonstrate that in a sufficient time frame (4–5 wk) on a set dietary regimen, Calm animals display higher levels of mass loss when intake is reduced from ad libitum to maintenance requirements or from a continuous-grazing diet to a meal-feeding regimen, which appears to relate to inherently higher levels of energy expenditure. Thus, under homeostatic conditions, Calm animals appear to be more susceptible to weight loss by virtue of increased energy expenditure.

A common feature of these studies, across a number of the experimental paradigms (maintenance feeding, low-energy intake, and meal anticipation), was the lack of association between core body temperature and adipose temperature. This observation was particularly evident in the Nervous animals on a low-energy diet, wherein core body temperature was markedly reduced without an associated change in adipose temperature. This dissociation was not reflected in the Calm animals under the same paradigm; the low-energy diet reduced core body and adipose temperatures during the preprandial period on day 12. Thus suggesting that a reduction in adipose thermogenesis in Calm animals may be involved in the adaptive response to low-energy feeding. A likely candidate for this is skeletal muscle, which displays robust thermogenic potential in sheep (15). Ongoing studies are investigating the effects of temperament on thermogenic activity in muscle.

In addition to studying the effects of programmed feeding and the effects of altering levels of dietary intake, we investigated the effects of fasting, meal anticipation, and refeeding on body temperatures. Meal anticipation is considered a nonhomeostatic paradigm, which is particularly pertinent to the investigation of temperature regulation and thermogenesis in Nervous and Calm sheep since temperament is also considered a nonhomeostatic effector of energy balance. Studies in rodents have demonstrated that temporal food restriction resets various circadian and biological rhythms in what is referred to as the food entrainable oscillator (6, 12, 22), which program a number of anticipatory behaviors and events that occur around meal time, including an anticipatory elevation in core body temperature (6, 12, 22). This anticipatory increase in temperature, once programmed, exists even in the fasted state (22). Sheep do not typically exhibit this anticipatory response, since increased temperature is dependent on food availability (15). In sheep, however, an anticipatory response can be elicited in fasted animals that have previously been exposed to programmed feeding to entrain the postprandial response, and that are then housed with a feeding animal. We have referred to this phenomenon as meal anticipation. The present study demonstrated that while the temperature responses to fasting and refeeding

Fig. 8. Expression patterns of uncoupling protein (UCP)-1, -2, and -3 and leptin mRNA in retroperitoneal and subcutaneous fat in Nervous (black bars) and Calm (white bars) sheep. Expression of UCP1 and UCP2 mRNA were higher (*P < 0.05; ***P < 0.001) in the retroperitoneal fat bed than subcutaneous fat, whereas expression of UCP3 and leptin mRNA were similar at both sites. There was no effect of temperament on the expression of any of the 4 genes studied.
were similar in the Nervous and Calm sheep, temperament impacted on the response to meal anticipation. Adipose tissue temperature increased more in response to meal anticipation in the Nervous than the Calm sheep, while the amplitude of response in core body temperature was similar, suggesting that adipose thermogenesis was greater in Nervous compared with Calm sheep. These data suggest that with respect to nonhomeostatic cues, such as meal anticipation, effects on energy expenditure and thermogenesis and adipose metabolism may be greater in the Nervous animals.

To determine a possible mechanism behind disparate temperature regulation in Nervous and Calm sheep we investigated the role of the hypothalmo-pituitary-adrenal axis. In humans, aberrations in the responsiveness of the hypothalmo-pituitary-adrenal axis have been linked to innate differences in temperament (29, 30). However, we did not find any differences in cortisol responsiveness to ACTH challenge between the Nervous and Calm animals. Furthermore, the subsequent increase in plasma levels of cortisol did not impact on either adipose thermogenesis or core body temperature in either group. ACTH challenge was performed using Synacthen Depot, a long-acting synthetic ACTH analog. It is possible that this may have compounded the data and that small differences in the Nervous and Calm sheep were not revealed. Nonetheless, the data demonstrate that altered cortisol responsiveness is most likely not responsible for differences in core body temperature or adipose thermogenesis in the Nervous and Calm sheep.

Expression of UCP1 and UCP2 mRNA levels were higher in the retroperitoneal fat than in subcutaneous fat, whereas levels of UCP3 and leptin mRNA were similar in both depots. Furthermore, there was no effect of temperament on expression of any of the genes analyzed, albeit there was a trend toward elevation of UCP1 mRNA in the retroperitoneal fat of Nervous animals. Leptin mRNA expression was similar in Nervous and Calm animals and across adipose depots. As to whether leptin is differentially expressed in different fat beds in the sheep remains unknown, since previous studies have reported higher expression levels in subcutaneous fat (7) or similar levels across different depots (23). With respect to temperament, previous studies have demonstrated that plasma levels of leptin are similar in Nervous and Calm sheep, albeit Nervous animals display an exacerbated increase in leptin secretion in response to LPS challenge (4). Given that baseline secretion of leptin is similar between Nervous and Calm sheep, the lack of effect of temperament on leptin mRNA levels is not surprising.

**Perspectives and Significance**

In conclusion, we have demonstrated that inherent differences in temperament impact on body temperature and thermogenesis in Nervous and Calm sheep. Nervous animals are more susceptible to the effects of low-energy intake than Calm animals, with the Nervous animals displaying lower core body temperature than Calm sheep. This effect in Nervous animals, however, is not driven by changes in adipose tissue thermogenesis, since temperature in the retroperitoneal fat bed was not influenced by low-energy intake. Thus, in Nervous animals on a low-energy diet, either heat production in other tissues, such as skeletal muscle, or differences in heat loss are likely to be important in determining core body temperature. On the other hand, in a nonhomeostatic paradigm (meal anticipation) Nervous animals display greater postprandial heat production in the retroperitoneal fat bed, indicative of enhanced putative thermogenesis. Overall, divergence in thermogenic capacity of Nervous and Calm sheep appears to be determined by the experimental paradigm. Calm animals display greater core body temperatures under homeostatic (low-energy intake and maintenance feeding) conditions, whereas Nervous animals display enhanced adipose thermogenesis in nonhomeostatic (meal anticipation) paradigms.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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