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# Ultrasonographic measurements of localized fat accumulation in Shetland pony mares fed a normal *v.* a high energy diet for 2 years

E. W. Siegers[1†], M. de Ruijter-Villani[1], D. A. van Doorn[1,2], T. A. E. Stout[1] and E. Roelfsema[1]

[1] *Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 112, 3584 CM, Utrecht, The Netherlands;* [2] *Division of Nutrition, Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL, The Netherlands*

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*Health risks associated with obesity are more likely a factor of the localization of fat excess, rather than of elevated BW* per se. *The aim of this randomized controlled clinical trial was to determine the effect of a long-term high energy diet on BW, fat accumulation and localization. Eight Shetland pony mares, 3 to 7 years old, were randomly divided into a control and a high energy (HE) diet group fed either maintenance or double maintenance energy requirements (200% net energy (NE)) for two consecutive summers, with a low energy diet in the winter in between. Body condition score (BCS) did not differ between the groups at the onset of the study (control 5.6 ± 0.75* v. *HE 6.3 ± 0.5). From 12 weeks after starting the diet, ultrasonography of five different locations (retroperitoneal, axillary, withers, intercostal and rump) for adipose deposition, BCS and BW were measured monthly during the period that ponies received different diets. Statistical analysis was performed using a linear mixed-effects model with* post hoc *Bonferroni testing.* P *values <0.05 were considered significant. At week 12 after the onset of the diet, fat thickness in the HE group was significantly greater than in the control group. During the monitoring period, the HE group showed a significant increase in mean (± SE) BW (+52%, 265 ± 13.94 kg) and BCS (+70%; to 9.0 ± 0.4), while the control group was unchanged (BW 160 ± 13.98 kg; BCS 3.8 ± 0.4). At all locations, the fat depth in the HE group increased significantly, with the highest increase noted for retroperitoneal deposits. The conclusions were that a 200% NE diet induced subcutaneous and retroperitoneal fat accumulation, with the greatest increase in intra-abdominal deposits. The moderate increase of the subcutaneous fat depth followed by a plateau phase suggests the existence of a limit of adipose tissue expandability, as in man.*

**Keywords:** adipose tissue, obesity, horse, equine metabolic syndrome, intra-abdominal fat

## Implications

In horses and ponies, obesity is common and is associated with several health risks. In man the location of the fat excess has a greater association with health risk than elevated BW *per se*. This study showed the existence of a limit in fat tissue expandability of subcutaneous fat tissue in Shetland ponies, which contributes to a better understanding of fat tissue dynamics. It is not yet known if the greater increase in abdominal fat in Shetland ponies has similar negative effects as those described for man and dogs.

## Introduction

In parallel with the human obesity 'epidemic', obesity is common in horses (Giles *et al.*, 2014; Visser *et al.*, 2014). Incidences of obesity of up to 45% have been reported among riding horses in the United Kingdom and the Netherlands (Wyse *et al.*, 2008; Stephenson *et al.*, 2011; Visser *et al.*, 2014). Obesity is defined as the accumulation of excessive adipose tissue (Robin *et al.*, 2014). Feeding a diet that contains more energy than physiologically required results in fat accumulation and weight gain (Carter *et al.*, 2009; Lindåse *et al.*, 2016). Breed differences in the predilection to developing obesity have been documented, underpinning the suggestion that there is a genetic basis to obesity (Bamford *et al.*, 2014). In this respect, ponies and certain breeds of horses show a greater tendency to becoming obese and undergoing changes in insulin sensitivity than Standardbreds (Frank *et al.*, 2010; Bamford *et al.*, 2014). This makes them more susceptible to the associated negative health effects of obesity including exercise intolerance, insulin dysregulation, laminitis, osteoarthritis and reduced fertility (Wyse *et al.*, 2008; Johnson *et al.*, 2009; Giles *et al.*, 2014).

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[†] E-mail: e.w.siegers@uu.nl

Ectopic fat depots of white adipose tissue are thought to be major drivers of insulin dysregulation and associated metabolic complications, and to trigger higher expression of inflammation-related genes (Tchkonia *et al.*, 2013; Gustafson and Smith, 2015; Wensveen *et al.*, 2015). Dysregulation of secretion of these metabolically active substances occurs in obese animals, leading to a state of low-grade inflammation (Frank *et al.*, 2010). The distribution of adipose tissue appears to influence the severity and likelihood of the pathological consequences of obesity (Bruynsteen *et al.*, 2013). Therefore it is important to understand the predilection sites for fat accumulation in breeds predisposed to the negative health consequences of obesity. The objective of this study was to monitor BW gain, increase in body condition scores (BCS) and accumulation and relative distribution of fat deposition in Shetland ponies fed either a control diet at maintenance (100% net energy (NE)) or a high energy (200% NE) diet over a prolonged period.

## Material and methods

### Animals and husbandry

The study was executed between February 2014 and October 2015. The population consisted of eight Shetland pony mares (age 3 to 7 years, mean 4.7 years), mean body weight 164 (±31) kg, BCS 5.9 (±0.7) out of nine at the start of the study. Only mares were used in this study, which was part of a larger study examining effects of obesity on reproductive parameters. Routine foot care, vaccination and anthelmintic treatments were performed when necessary and rectal temperature and heart rate were monitored daily to assess general health. The ponies were randomly divided among the control ($n=4$) and the high energy (HE) group ($n=4$); one pony from the HE group was exchanged with a pony from the control group directly after the start of the experiment because she was not able to eat the amount of feed provided. No medical abnormalities were found in this pony.

### Study design

All ponies were allowed an acclimatization period of at least 2 weeks before starting on the experimental diet. Measurements of BW and BCS were performed before the start of the experiment and continued on a monthly basis. For practical reasons, the ultrasonographic fat measurements were initiated 12 weeks after the start of the diet in 2014 and performed monthly from June 2014 to October 2014 (period 1 to 5). In 2015, ultrasound measurements were performed monthly during the entire period of high energy feeding from March 2015 to October 2015 (period 6 to 13) (Figure 1). Basal blood plasma glucose and insulin concentrations were analyzed before and after the feeding periods in 2014 and 2015.

### Diet

All ponies were fed individually according to BW on a NE basis, and received a ration consisting of a semi-purified concentrate (Table 1), grass hay and a mineral and vitamin supplement. Ponies in the control group were fed an energy level equating to maintenance according to NE requirements set by the Centraal Veevoederbureau (CVB, 2004) ($\approx 0.348\ \mathrm{MJ\ NE} \times \mathrm{BW}^{0.75}$). The ponies in the control group received 85% of NE intake in the form of hay. The remaining 15% was provided as concentrates. Ponies in the HE group received 200% of their NE requirements to induce weight gain, as previously described by Carter *et al.* (2009). Energy intake was adapted to weight gain throughout the whole experiment, to maintain a 200% NE intake. The diet for ponies in the HE group was designed such that 42.5% of NE intake originated from hay, and 57.5% from the semi-purified concentrate. All ponies received >1 kg dry matter (DM) forage per 100 kg BW per day. The NE content of the hay was analyzed (BLGG AgroXpertus; Wageningen, the Netherlands) and hay intake was adapted accordingly to maintain a similar energy partitioning during the study. The concentrate and hay was fed in multiple meals at 0800, 1300 and 1700 h. All ponies received (0800 h) a fixed amount of a supplement (Pavo Vital Complete; Pavo, Boxmeer, the Netherlands) (30 g/day) to ensure adequate provision of minerals, trace elements and vitamins (NRC, 2007). The ponies had free access to water and a salt lick (KNZ®; Hengelo, the Netherlands). During the 1st year (2014), the control ponies were housed in a group but fed individually, and bedded on wood shavings. The HE ponies were housed and fed individually. In between the experimental periods of 2014 and 2015 (winter 2014

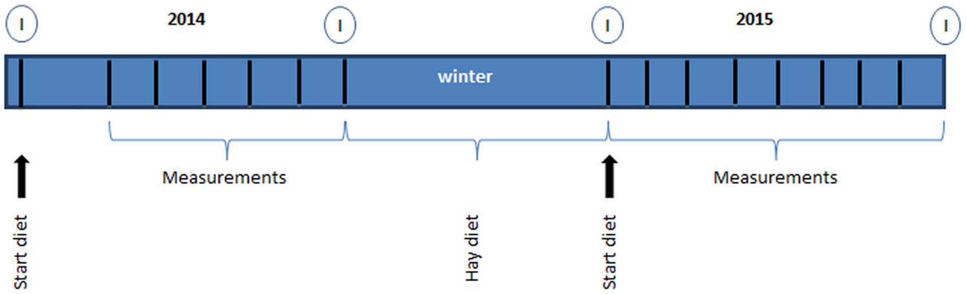

**Figure 1** Design of the study to examine the effects of feeding Shetland pony mares a normal ($n=4$) or double maintenance (high energy (HE); $n=4$) diet on parameters of obesity. Arrow: start diet, body condition score (BCS) and BW measurements. Measurements: weekly BW, monthly BCS and ultrasound measurements. Hay diet: winter period no measurements, only hay diet for both HE and control group. I = samples for glucose and insulin analysis.

**Table 1** *Calculated ingredient and macronutrient composition of the semi-purified diet[1] and hay fed to Shetland pony mares*

| Item | Concentrate composition (g/kg) (as-fed) | Hay[2] |
|---|---|---|
| Molasses (cane) | 20 | – |
| Wheat | 250 | – |
| Barley | 216 | |
| Linseed (crushed) | 184 | – |
| Soy hulls | 134 | – |
| Corn | 70 | |
| Wheat semolina | 50 | |
| Vegetable oil (Soy) | 35 | – |
| Salt | 16.16 | – |
| Mono calcium phosphate | 3.23 | – |
| Limestone | 0.95 | – |
| Vitamin E | 14 | – |
| Magnesium oxide | 5.87 | |
| Macronutrient composition | | |
| DM (g/kg) | 890 | 902 |
| Ash (g/kg DM) | 66 | 62 |
| CP (g/kg DM) | 117 | 70 |
| Crude fat (g/kg DM) | 127 | 16 |
| Starch (g/kg DM) | 330 | – |
| Sugars (g/kg DM) | 31 | 92 |
| Crude fiber (g/kg DM) | 86.1 | 318 |

[1] PAVO, Heijen, the Netherlands.

[2] Analysis provided by BLGG, AgroXpertus, Wageningen, the Netherlands.

**Table 2** *Specific anatomical locations used for the transcutaneous ultrasonographic measurement of adipose tissue thickness in Shetland pony mares fed a normal or a double maintenance energy requirement (high energy (HE)) diet*

| Fat deposit | Anatomical location |
|---|---|
| MP1 | Probe positioned parallel and immediately lateral to ventral midline, just caudal to the xiphisternum, fat depth between the peritoneum and ventral muscle layer was measured |
| MP2 | Lateral thoracic vein identified at point of emergence between deep pectoral and latissimus dorsi muscles. With lateral thoracic vein in cross section, fat depth immediately adjacent to vein was measured |
| MP3 | Probe was positioned perpendicular to the dorsal midline, just cranial to the highest point of the withers. Measurement was made 2.5 cm cranial to the scapula |
| MP4 | Probe centered 20 cm lateral to the dorsal midline in the 14th intercostal space, fat thickness between the skin and muscle layers was measured |
| MP5 | Probe centered equidistant between point of hip (tuber coxae) and center of tail head |

MP1 = retroperitoneal; MP2 = axillary; MP3 = withers; MP4 = intercostal; MP5 = rump.
All measurements were recorded on the left side of the pony, with the animal standing squarely.

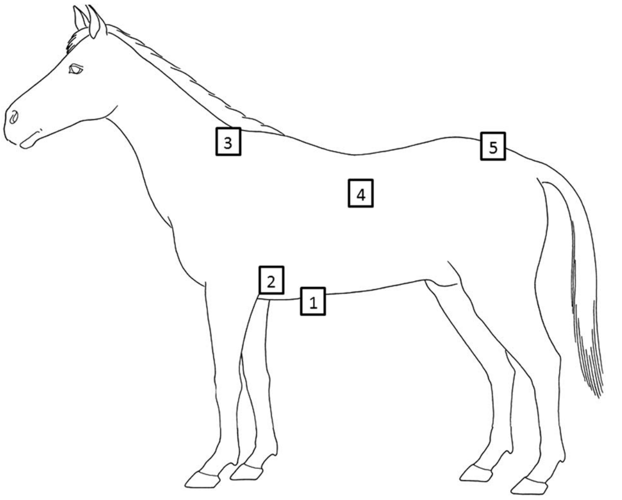

**Figure 2** Schematic view of anatomic locations used to asses adipose tissue thickness in Shetland pony mares fed a normal ($n = 4$) or double maintenance (high energy (HE); $n = 4$) diet by transcutaneous ultrasound. 1 = retroperitoneal; 2 = axillary; 3 = withers; 4 = intercostal; 5 = rump.

to 2015), all ponies were housed together in a group on straw, with a maintenance hay-only diet of at least 100% NE requirements. In the 2nd year (2015), all ponies were housed and fed individually and bedded on shavings. Groups were allowed access to a sand paddock every other day for social contact and limited exercise during the experimental periods.

### *Physical measurements: body weight, body condition scores and ultrasonographic fat measurements*

The BW of each pony was measured monthly (mean of three measurements) using a weighing scale (Epelsa BCN100M; Grupo Epelsa, Madrid, Spain). All BCS and fat thickness estimations were performed by the same observer throughout the study, who was blinded to the diet the ponies received. The BCS of each animal was assessed by visual appraisal and palpation and scored on a nine-point scale in accordance with criteria described by Henneke *et al.* (1983). The superficial fat deposits were measured to the nearest 0.01 mm by transcutaneous B-mode ultrasonography using a Logiq 7 Pro ultrasound machine equipped with a 10 MHz linear array probe (GE Medical Systems; Milwaukee, WI, USA). Five locations named measurement point 1 to 5 (MP1 to MP5; retroperitoneal, axillary, withers, intercostal and rump), were examined on each pony (Table 2 and Figure 2) as described by Dugdale *et al.* (2011a). All locations except for MP1 are referred to as subcutaneous fat tissue, MP1 is referred to as retroperitoneal fat tissue. All measurements were performed on the left side with the pony standing squarely, and repeated three times. Before the measurements the locations were clipped and cleaned using a chlorohexidine solution (Hibiscrub; BMC Ltd., Nottingham, UK) and 70% alcohol solution. Ultrasound imaging was performed using transmission gel (Chemolan; Chemodis, Alkmaar, the Netherlands). Images were saved and fat thickness was measured (GE Truscan architecture; Wauwatosa, WI, USA); the mean of the three measurements per location was used for statistical analysis.

### *Blood glucose and insulin measurements*

After fasting for 8 h whole blood samples were recovered between 0600 and 0800 h into 4 ml sodium-fluoride (glucose) and sodium-heparin (insulin) tubes. Glucose analysis was performed directly. The heparinized tubes were immediately centrifuged at 3000 × **g** for 10 min, the plasma was frozen at −20°C until analysis. In 2014, insulin concentrations were measured using a commercial radioimmunoassay (RIA Coat a Count; Siemens Diagnostic Products Corp, Los Angeles, CA, USA) validated for horses (Kolk van der *et al.*, 1995). In 2015, the RIA kit was no longer available and the plasma insulin concentrations were determined using a new commercially available solid-phase, enzyme-labeled chemiluminescent immunometric assay (CLIA) designed for human insulin (Immulite 2000 Insulin; Siemens Diagnostic Products Corp, Los Angeles, CA, USA). This CLIA was validated using equine plasma samples held in our laboratory and compared with the previously used RIA (CAC insulin). Linearity plots were constructed by comparing the CAC insulin concentrations with the CLIA insulin. The relationship between the two assays was determined by linear regression to be: $\text{CLIA} = 0.76 \times \text{CAC}$; $R^2 = 0.98$. All the insulin concentrations from 2014 were recalculated using this formula to allow comparison with the results from 2015. Hyperinsulinemia was defined as basal plasma insulin $\geqslant 20\ \mu\text{IU/ml}$, in accordance with the American College of Veterinary Internal Medicine consensus statement (Frank *et al.*, 2010).

### *Statistical analysis*

Statistical analyses were performed using SPSS software (SPSS 22-24; Quarry bay, Hong Kong). For all data, normal distribution of the residuals was confirmed using a Shapiro–Wilk test or by visual inspection of the Q-Q plot. MP5 and insulin data needed log transformation in order to achieve a normal distribution, left-censoring for observations below the detection limit was used for the insulin data. For the normally distributed data, a linear mixed-effects model followed by a *post hoc* Bonferroni test was used; with period and group as fixed effects and pony as random effect. For the non-normally distributed MP3, a non-parametric Mann–Whitney *U*-test was used to compare between groups, and a Wilcoxon-Signed Rank test was used to compare between measurement periods. For the insulin data a likelihood ratio test was used to test the effect of the diet. In the text, all results except for MP3, are presented as mean ± SE of the residuals. MP3 is presented as median (interquartile range (IQR)). *P* values <0.05 were considered to indicate statistically significant differences. Spearman's rank correlation coefficient was used to investigate correlations between BW, BCS and subcutaneous and retroperitoneal fat depth.

## Results

During the course of the study, one pony in the control group developed mild colic once; one pony in the control group was not always able to finish the concentrate. None of the ponies developed any signs of laminitis or any other significant clinical symptoms. There was no significant difference in BW between the control (160 ± 1.1 kg) and the HE (168 ± 1.1 kg) group at the onset of the study ($P = 0.24$). The HE group exhibited a significant increase in BW over time in all periods, with the exception of the winter period where they showed a significant decrease in BW ($P < 0.0001$). From period 4 (week 24), the HE ponies were significantly heavier than the control ponies ($P < 0.05$) (Figure 3a). After the winter period there was again no difference in BW between the control and the HE group, although the latter were still considerably heavier than their initial BWs. After 28 weeks (period 5) of the HE diet in 2014, the respective ponies showed a mean increase in BW of 30% ($P = 0.001$). In 2015, the mean increase in BW of the HE group after 34 weeks of receiving the diet (period 13) was 28% ($P < 0.0001$). The overall mean increase in BW from period 1 to 13 in the HE ponies was 52%. The control group showed no significant changes in BW over the entire monitoring period (Figure 3a).

The BCS at the start of the study was not significantly different between the two groups (5.6 ± 0.02 for the control group *v.* 6.3 ± 0.02 for the HE group). In 2015, the BCS of the control group differed slightly in several periods (Figure 3b). The HE group showed an increase in BCS from period 1 (5.3 ± 0.02) to period 5 (8.8 ± 0.02; $P < 0.001$), with a drop in the winter period from 8.8 ± 0.02 to 7.3 ± 0.02 ($P < 0.001$). In 2015, the increase in BCS resumed with the start of the HE diet and progressed to 9.0 ± 0.02 by period 13 ($P < 0.001$) in all HE-fed ponies. The HE group showed a mean increase in BCS of 67% in 2014 ($P < 0.001$) and 24% in 2015 ($P < 0.002$). The overall mean increase from period 1 to period 13 was 70% ($P < 0.001$) (Figure 3b).

In period 1 (12 weeks on the diet), the HE group had significantly thicker fat layers at all locations compared with the control group, with the exception of the axillary fat deposit (MP2; Table 3). The control group showed some fluctuations in fat depth over time (Table 3 and Figure 4).

For the HE group, the fat depth increased significantly over time at all locations during 2014 (Table 3 and Figure 4). After the winter hay-only period, the HE group showed a slight decrease in fat depth that only reached statistical significance for MP2 and MP3. The retroperitoneal adipose tissue (RAT, MP1) showed a gradual increase in depth over the entire feeding period, and was eight times greater than in the control group by the end of 2015, when the subcutaneous deposits (SAT) were only 1.6 to 2.2 times greater in the HE group. In 2014, fat thickness at MP1, MP2, MP3 and MP4 stabilized during period 3 to 5 in the HE group. A similar plateau was seen during the 2nd year, data from the HE group for MP3 and MP4 in period 13 were missing due to difficulty in obtaining representative ultrasound images. For MP5 only data from periods 1 to 3 in 2014 was available for analysis. Nevertheless, the HE group showed a significant increase in fat depth at MP5 over time. A strong positive correlation ($\rho = 0.81$) was found between BCS and BW. Correlations ranging from 0.84 to 0.91 were found between BCS and the various ultrasonographic measurements locations of fat depth. The correlations

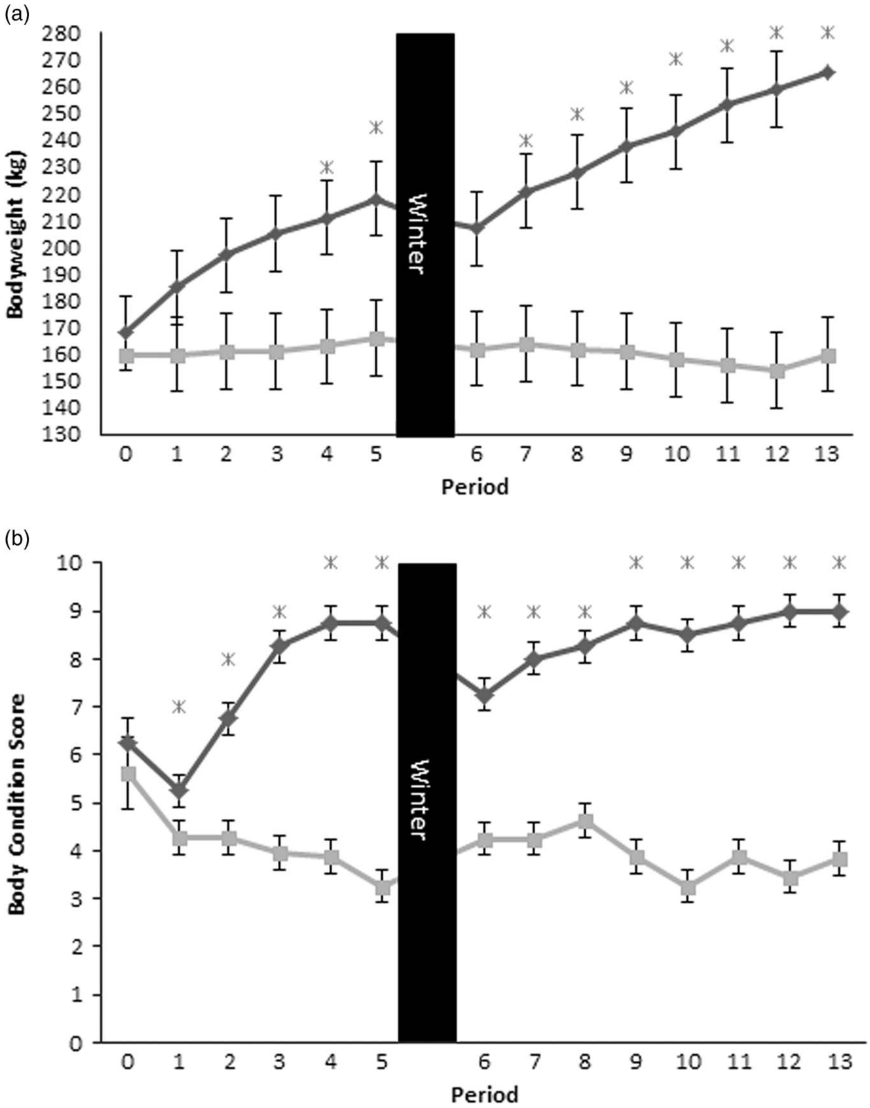

**Figure 3** (a) Monthly mean ± SE BW measurements and (b) monthly mean body condition scores in normal or double maintenance fed Shetland pony mares ($n = 4$ per group) during a 2-year feeding trial. Period 0 was at the start point of the diet, periods 1 to 5 were in year 1, and periods 6 to 13 in year 2. ■ = Control group; ◆ = High Energy group; * = test group significantly different from control group, $P < 0.05$; winter = period with hay-only diet in both groups.

between BW and measurement locations ranged from 0.73 to 0.76.

Two basal glucose values at the start of 2014 in the HE group were below the normal reference range of 3.9 to 5.6 mmol/l, all other values were within the reference range. No changes were observed in glucose concentrations over time in the control group. In the HE group, there was a significant increase in mean glucose concentration between the start and the end of 2015, starting at 4.3 ± 0.02 mmol/l and increasing to 4.9 ± 0.02 mmol/l ($P = 0.02$). Mean basal insulin levels in the control group before (2.1 ± 0.23 μIU/ml) and at the end (all ponies below detection limit of 2.0 μIU/ml) of the experiment did not differ. In the HE group, insulin levels before the start of the diet were below the level of detection in both years (<2.0 μIU/ml), and had increased after the dietary period (3.8 μIU/ml ± 0.23 in 2014, $P = 0.04$; 3.5 μIU/ml ± 0.23 in 2015, $P = 0.04$) with a likelihood ratio of 4.14.

## Discussion

In this study, ultrasonography was used to measure changes in subcutaneous and retroperitoneal fat depth in Shetland pony mares on a high energy diet equating to 200% of maintenance energy compared with Shetland pony mares on a maintenance energy diet. The excess dietary energy was shown to be stored in the form of subcutaneous and retroperitoneal fat. Across the monitoring period, retroperitoneal fat depth increased by a factor of 8 compared with more modest increases in subcutaneous fat thickness (1.6 to 2.2 times) within which the accumulation of axillary fat appeared to be slower than at other locations. Carter *et al.*, found a similar 1.6 times increase in subcutaneous fat thickness in horses fed 200% maintenance energy diet for 16 weeks, although different measurement locations were used (Carter *et al.*, 2009). The increase in BW in the HE group in the present study is comparable

**Table 3** *Mean fat depth (cm) ± SE at the different time points in Shetland pony mares fed a normal (*n = 4*) or double maintenance (high energy (HE):* n = 4*) diet, comparing the HE and control group at the different time points (period)*

| | Control | SE | HE | SE | *P* |
|---|---|---|---|---|---|
| 2014 | | | | | |
| MP1 | | | | | |
| Period 1 | 0.38 | 0.02 | 1.69 | 0.02 | 0.000 |
| Period 2 | 0.59 | 0.02 | 1.97 | 0.02 | 0.000 |
| Period 3 | 0.66 | 0.02 | 2.63 | 0.02 | 0.000 |
| Period 4 | 0.55 | 0.02 | 2.94 | 0.02 | 0.000 |
| Period 5 | 0.53 | 0.02 | 3.11 | 0.02 | 0.000 |
| MP2 | | | | | |
| Period 1 | 1.09 | 0.01 | 1.67 | 0.01 | 0.072 |
| Period 2 | 0.91 | 0.01 | 1.24 | 0.01 | 0.289 |
| Period 3 | 0.83 | 0.01 | 2.29 | 0.01 | 0.000 |
| Period 4 | 1.03 | 0.01 | 2.27 | 0.01 | 0.001 |
| Period 5 | 0.49 | 0.01 | 2.29 | 0.01 | 0.000 |
| MP3 | | | | | |
| Period 1 | 0.30 | 0.23 (IQR) | 1.43 | 0.67 (IQR) | 0.000 |
| Period 2 | 0.36 | 0.28 (IQR) | 1.31 | 1.28 (IQR) | 0.000 |
| Period 3 | 0.45 | 0.29 (IQR) | 1.87 | 1.38 (IQR) | 0.000 |
| Period 4 | 0.48 | 0.33 (IQR) | 3.33 | 1.90 (IQR) | 0.000 |
| Period 5 | 0.49 | 0.34 (IQR) | 2.00 | 0.99 (IQR) | 0.000 |
| MP4 | | | | | |
| Period 1 | 0.49 | 0.01 | 1.42 | 0.01 | 0.009 |
| Period 2 | 0.99 | 0.01 | 2.22 | 0.01 | 0.001 |
| Period 3 | 1.20 | 0.01 | 2.83 | 0.01 | 0.000 |
| Period 4 | 0.94 | 0.01 | 3.17 | 0.01 | 0.000 |
| Period 5 | 0.61 | 0.01 | 3.17 | 0.01 | 0.000 |
| MP5 | | | | | |
| Period 1 | 0.39 | 0.01 | 0.86 | 0.01 | 0.031 |
| Period 2 | 0.29 | 0.01 | 1.26 | 0.01 | 0.000 |
| Period 3 | 0.39 | 0.01 | 1.69 | 0.01 | 0.000 |
| Period 4 | 0.22 | 0.01 | N.A. | | |
| Period 5 | 0.15 | 0.01 | N.A. | | |
| 2015 | | | | | |
| MP1 | | | | | |
| Period 6 | 0.51 | 0.02 | 2.87 | 0.02 | 0.000 |
| Period 7 | 0.48 | 0.02 | 3.64 | 0.02 | 0.000 |
| Period 8 | 0.37 | 0.02 | 3.50 | 0.02 | 0.000 |
| Period 9 | 0.42 | 0.02 | 3.78 | 0.02 | 0.000 |
| Period 10 | 0.16 | 0.02 | 4.42 | 0.02 | 0.000 |
| Period 11 | 0.32 | 0.02 | 4.33 | 0.02 | 0.000 |
| Period 12 | 0.27 | 0.02 | 4.13 | 0.02 | 0.000 |
| Period 13 | 0.55 | 0.02 | 4.49 | 0.02 | 0.000 |
| MP2 | | | | | |
| Period 6 | 0.42 | 0.01 | 1.51 | 0.01 | 0.002 |
| Period 7 | 0.35 | 0.01 | 1.45 | 0.01 | 0.002 |
| Period 8 | 0.59 | 0.01 | 2.08 | 0.01 | 0.000 |
| Period 9 | 0.35 | 0.01 | 1.88 | 0.01 | 0.000 |
| Period 10 | 0.30 | 0.01 | 2.47 | 0.01 | 0.000 |
| Period 11 | 0.42 | 0.01 | 2.92 | 0.01 | 0.000 |
| Period 12 | 0.28 | 0.01 | 2.78 | 0.01 | 0.000 |
| Period 13 | 0.66 | 0.01 | 2.84 | 0.01 | 0.000 |
| MP3 | | | | | |
| Period 6 | 0.20 | 0.07 (IQR) | 1.24 | 1.27 (IQR) | 0.000 |
| Period 7 | 0.30 | 0.18 (IQR) | 2.41 | 2.22 (IQR) | 0.001 |
| Period 8 | 0.32 | 0.15 (IQR) | 2.74 | 1.77 (IQR) | 0.000 |
| Period 9 | 0.33 | 0.17 (IQR) | 2.28 | 1.23 (IQR) | 0.000 |
| Period 10 | 0.22 | 0.17 (IQR) | 2.38 | 1.31 (IQR) | 0.000 |
| Period 11 | 0.39 | 0.26 (IQR) | 2.47 | 0.58 (IQR) | 0.000 |
| Period 12 | 0.32 | 0.14 (IQR) | 3.14 | 1.35 (IQR) | 0.003 |
| Period 13 | 0.41 | 0.05 (IQR) | N.A. | | |
| MP4 | | | | | |
| Period 6 | 0.47 | 0.01 | 2.52 | 0.01 | 0.000 |
| Period 7 | 0.73 | 0.01 | 2.45 | 0.01 | 0.000 |
| Period 8 | 0.38 | 0.01 | 3.04 | 0.01 | 0.000 |
| Period 9 | 0.46 | 0.01 | 3.25 | 0.01 | 0.000 |
| Period 10 | 0.42 | 0.01 | 2.89 | 0.01 | 0.000 |
| Period 11 | 0.45 | 0.01 | 2.45 | 0.01 | 0.000 |
| Period 12 | 0.14 | 0.01 | 2.86 | 0.01 | 0.000 |
| Period 13 | 0.73 | 0.01 | N.A. | | |
| MP5 | | | | | |
| Period 6 | 0.42 | 0.01 | 1.54 | 0.01 | 0.000 |
| Period 7 | N.A. | | N.A. | | |
| Period 8 | N.A. | | N.A. | | |
| Period 9 | N.A. | | N.A. | | |
| Period 10 | N.A. | | N.A. | | |
| Period 11 | 0.33 | 0.01 | N.A. | | |
| Period 12 | N.A. | | N.A. | | |
| Period 13 | N.A. | | N.A. | | |

MP1 = retroperitoneal; MP2 = axillary; MP3 = withers; MP4 = intercostal; MP5 = rump.

For MP3 median (IQR) (cm) noted. $P < 0.05$ indicating significant difference between control and HE group.

with the BW increase found by Carter *et al.* (2009) of 20% over 24 weeks.

In the winter period the HE group showed significant decreases in BW, BCS and withers and axillary fat thickness which reduced to pre-study levels, while retroperitoneal fat (MP 1) did not decrease notably. This finding suggests that the retroperitoneal fat depot not only increases the most, but during a period of relatively lower energy supply it reduces less compared with the subcutaneously located fat deposits. The decrease in BCS despite a constant BW observed in the control group during 2015 might be attributed to growth of the young ponies.

Dugdale *et al.* (2010) evaluated five overweight pony mares during a 12-week period of weight loss and found decreases in BW and subcutaneous fat depots (gluteal region and 12th intercostal space) but no decrease in BCS which is in contrast with the present study where the sonographic reduction in fat thickness was reflected by decreased BCS in the HE group. The difference with the present study might be explained by a longer period of low energy feeding and, possibly, season (Dugdale *et al.*, 2011a). Strong correlations between BCS and ultrasound fat thickness have been reported previously in horses ($\rho > 0.8$) and donkeys ($\rho = 0.7$ to 0.9) and showed similarity to our findings ($\rho = 0.8$ to 0.9) in Shetland ponies (Gentry *et al.*, 2004; Quaresma *et al.*, 2013). A relative stability of rump fat has previously been reported in studies that monitored weight loss in horses and

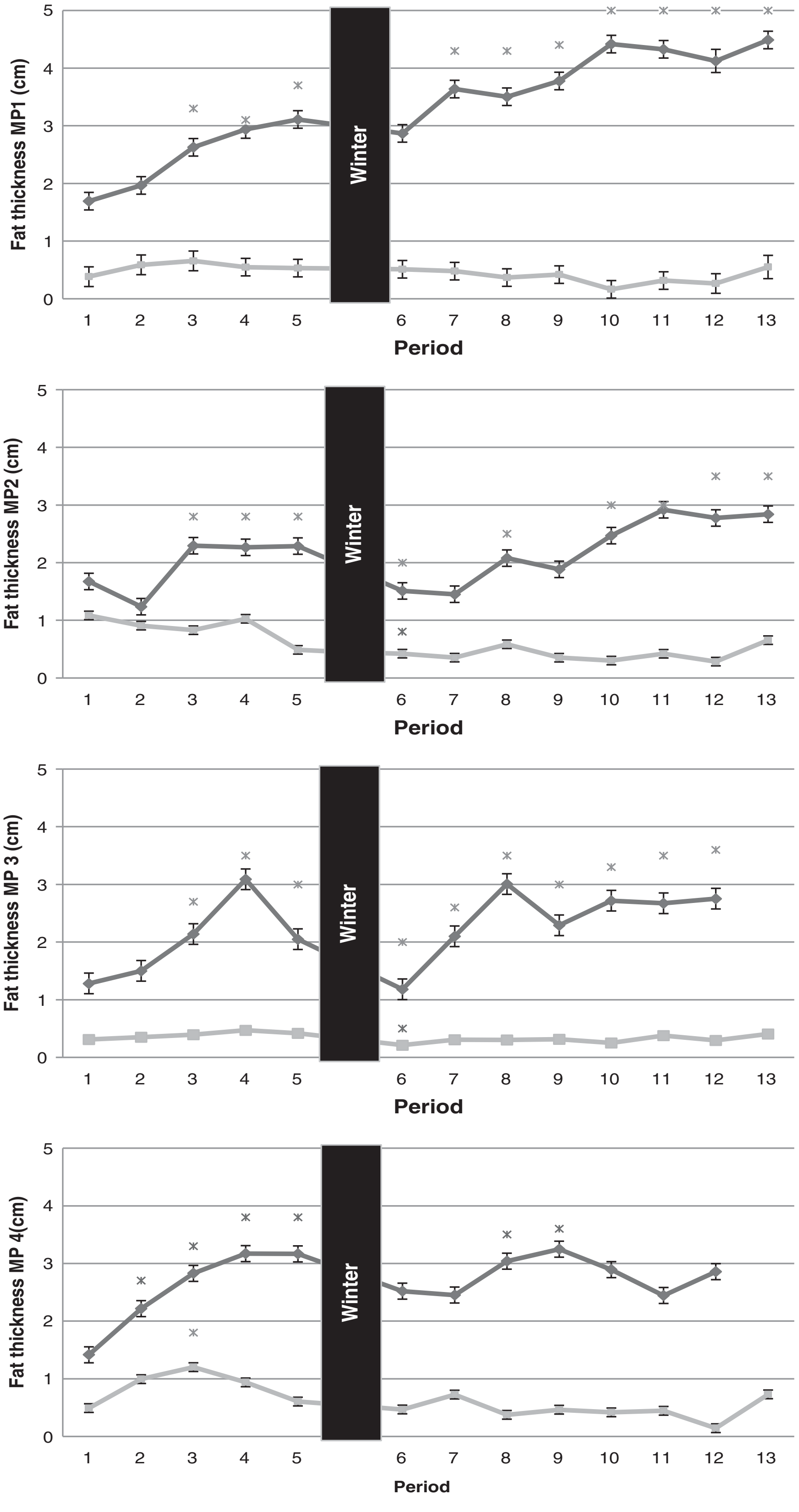

**Figure 4** Monthly mean ± SE (MP1, 2 and 4) or median (MP3) transcutaneous ultrasonographic adipose tissue thickness measurements in normal or double maintenance fed Shetland pony mares ($n = 4$ per group) during a 2-year feeding trial on four different locations. Period 0 was at the start point of the diet, periods 1 to 5 were in year 1, and periods 6 to 13 in year 2. Locations: MP1 = retroperitoneal; MP2 = axillary; MP3 = withers; MP4 = intercostal; MP5 (rump) was not shown in this graph ■ = Control group; ◆ = High Energy group; * = significantly different from start of diet, $P < 0.05$; winter = period with hay-only diet in both groups.

ponies (Gentry *et al*., 2004; Dugdale *et al*., 2011a and 2012). In the present study, we observed an increase in rump fat over time in the HE compared with the control group, and a strong correlation between rump fat thickness and BW and BCS in the early stage of the study period. In the later stages of the study the image quality at the rump was poor, breed and local thickening of the skin are assumed to be contributory factors to these difficulties. This was not reported in other studies; however, other studies collected fat thickness results only once or over a shorter period of time (Gentry *et al*., 2004; Dugdale *et al*., 2011a).

In man, the adipose tissue expandability theory states that SAT has limited expandability (Gustafson and Smith, 2015). When the SAT reaches its limits, further accumulation will occur in visceral adipose tissue or as ectopic fat depots, which are known to have more negative effects on metabolic parameters than SAT (Frank *et al*., 2010; Huang *et al*., 2011; Alligier *et al*., 2013; Gustafson and Smith, 2015; Moreno-Indias and Tinahones, 2015; Wensveen *et al*., 2015). Differences have been described in the expandability of fat depots per location, sex and ethnicity (Tchkonia *et al*., 2013; Gustafson and Smith, 2015). Men accumulate their excess fat intra-abdominally whereas women are more prone to excess fat storage in subcutaneous fat depots, like on the legs. This is considered the safest location, in terms of health repercussions, for fat accumulation in man (Tchkonia *et al*., 2013; Gustafson and Smith, 2015). Dogs with abdominal obesity have been shown to have a higher risk of heart disease (Thenchaisri *et al*., 2014), suggesting a similar negative effect of visceral adipose tissue as in man. In our study, the predominant site of fat deposition in overfed Shetland pony mares was retroperitoneal fat; it is yet not known if this has similar negative effects as described for man and dogs. The moderate increase of the subcutaneous fat depth in the different subcutaneous regions followed by the plateau phase seems to prove the existence of a limit of adipose tissue expandability in Shetland ponies, just as in man.

Ultrasound is a noninvasive technique for accurately and reliably measuring fat deposition at a specific body site or estimating total fat accumulation (Gee *et al*., 2003; Dugdale *et al*., 2011b; Quaresma *et al*., 2013). It is useful in a clinical setting due to its low cost and relative simplicity (Quaresma *et al*., 2013; Giménez *et al*., 2015). In this study there were significant differences in the control group in fat thickness over time, especially in the 1st year of measurements at the intercostal space (MP4). Although the measurements were executed by a single operator it is probable that regular use of the technique improved its accuracy. In the HE group the increase in SAT stabilized whereas BW and BCS continued to increase. This could be because of limitations in the expandability of the subcutaneous fat layers. It might also be partly due to increased pressure of the US probe as, with increasing fat tissue depth, the quality of the image decreases. The observer performing the ultrasound measurements was blinded to which diet each pony received, however it soon became clear which pony received which diet.

No hyperinsulinemia or hyperglycemia was found, based on basal glucose and insulin samples before and after the feeding period. Nevertheless, significant increases in glucose and insulin concentrations were found for the HE group in the 2nd year, indicating changes in glucose metabolism presumably associated with the increased fat deposits and BW. This finding is in agreement with the results of Frank *et al.* who found that basal insulin and glucose concentrations were not very sensitive markers of early insulin dysregulation in horses and changed only at advanced stages of insulin dysregulation (Frank *et al*., 2010). The ponies used in this study were all very young (3 to 7 years). According to Frank *et al.* (2010) horses with equine metabolis syndrome often present clinical signs between 5 and 15 years of age. This could be a factor contributing to the relatively low insulin values found in these long-term overfed/obese ponies.

Although the number of horses used in the present study was limited to eight, the large differences between the two groups made the data powerful enough to evaluate the different parameters. Further research is necessary to evaluate which adipose tissue has the most important negative impacts on health. In conclusion, this study showed that BW, BCS and subcutaneous and retroperitoneal fat measurements are correlated both in normal weight and in obese ponies and increase during a high energy diet. Increases in adipose tissue occur in both retroperitoneal and subcutaneous deposits, with the largest increase occurring in the retroperitoneal area. The moderate increase of the subcutaneous fat depth in the different subcutaneous regions followed by the plateau phase seems to prove the existence of a limit to adipose tissue expandability in Shetland ponies, as reported in man.

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