Changes in oxidative stress in response to different levels of energy restriction in obese ponies

Lien Bruynsteen1*, Geert P. J. Janssens1, Patricia A. Harris2, Luc Duchateau3, Emanuela Valle4, Patrizio Odetti5, Kimberley Vandevelde1, Johan Buyse6 and Myriam Hesta1
1Laboratory of Animal Nutrition, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820 Merelbeke, Belgium
2Equine Studies Group, WALTHAM Centre for Pet Nutrition, Freeby Lane, Waltham-on-the-Wolds, Melton Mowbray, Leicestershire LE14 4RT, UK
3Department of Comparative Physiology and Biometry, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
4Department of Veterinary Science, University of Turin, Torino, Italy
5Division of Geriatrics, Department of Internal Medicine and Medical Specialities, University of Genova, Genova, Italy
6Laboratory of Livestock Physiology, Immunology and Genetics of Domestic Animals, Department of Biosystems, K.U. Leuven, Heverlee, Belgium

(Submitted 10 October 2013 – Final revision received 28 May 2014 – Accepted 13 June 2014 – First published online 2 September 2014)

Abstract
The present study evaluated the effect of different levels of energy restriction on metabolic parameters in obese ponies. Relative weight changes, markers of lipid metabolism and oxidant/antioxidant balance were monitored. A total of eighteen obese (body condition score $\geq 7/9$) Shetland ponies were studied over a 23·5-week trial, which was divided into three periods. The first period involved a 4-week adaptation period in which each animal was fed 100 % of their maintenance energy requirements needed to maintain a stable obese body weight (MERob). This was followed by a 16·5-week weight-loss period in which ponies were assigned to receive either 100 % (control group, CONTROL), 80 % (slow weight-loss (SLOW) group) or 60 % (rapid weight-loss (RAPID) group) of their MERob. During the 3-week end-phase period, all ponies were again fed 100 % of their MERob. Relative weight loss was higher in the RAPID group ($P < 0·001$) compared with the SLOW group. No linear relationship was found as a doubling of the percentage of energy restriction was accompanied by a tripling of the percentage of weight loss. Relative weight gain afterwards in the end-phase period was higher in the RAPID group ($P < 0·001$) compared with the SLOW and CONTROL groups. During the weight-loss period, TAG and NEFA concentrations were highest in the RAPID group, as were $\alpha$-tocopherol and ferric-reducing ability of plasma concentrations. After 8 weeks of weight loss, the concentrations of advanced oxidation protein products were higher in the RAPID group compared with the SLOW and CONTROL groups ($P < 0·001$). In conclusion, the level of energy restriction influences the extent of changes in oxidant/antioxidant balance. Practically, more severe energy restriction regimens may be associated with a greater regain of weight after the restriction period.

Key words: Ponies: Energy restriction: Weight-loss rate: Oxidative stress

With a prevalence of 19–45 %, overweight and obese horses have become a major welfare problem in modern horse management in developed countries (1,2). Obesity is associated in particular with an increased risk of insulin resistance as well as laminitis (3–5). While preventing animals from becoming obese is the preferred route, given the current scale of this problem, effective safe weight-loss protocols are required especially for the laminitic pony for which increased physical activity may be contraindicated (6). Recently, several equine studies have been published looking at the efficacy of weight-loss programmes with and without exercise (7–9). Most recently, the concept of weight-loss resistance in the horse has been highlighted with the suggestion that whereas some animals may respond to moderate energy restriction (low-energy food intake restricted to 1·25 % of the actual body weight as DM intake (DMI)) with appropriate levels of activity, others may not (10).
In the present study, the aim was to evaluate the effect of different levels of energy restriction on weight loss and subsequent rebound weight gain, as well as oxidative–antioxidant balance. It was hypothesised that greater energy restriction would result in more weight loss, accompanied by an improved oxidant–antioxidant balance (increased antioxidant defence and decreased levels of oxidant markers). A second hypothesis was that a more rapid weight loss would be accompanied by a greater weight gain when the ponies were fed again at maintenance energy levels.

### Materials and methods

#### Animals and husbandry

A total of eighteen obese (body condition score ≥7/9) Shetland geldings, aged 9.3 (SEM 3.9) years (Table 1), were studied over a 23.5-week period (August to January). Only the ponies in good health and dental status were selected. No prior history of clinical lameness and laminitis was reported for the ponies included in the present study. In accordance with the range reported by Treiber et al. (33) (7.32–242.40 pmol/l; 1.22–40.40 mU/l), baseline insulin concentrations at the start of the adaptation period and the weight-loss period were normal (range 33.00–237.60 pmol/l; 5.5–39.60 mU/l). As the aim of the present study was not to evaluate the effect of weight loss on glucose and insulin dynamics, glucose tolerance tests were not implemented. Routine foot care, vaccination and anthelmintic treatments were undertaken before and, if necessary, during the study. The ponies were housed individually during feeding times in nine indoor boxes of 9 m² or in nine stalls with an area of 13.83 m². During the rest of the day, the ponies were group-housed in a large barn (inner part 285 m²; outer part 275.5 m²). On the floor of the barn, rubber mats were placed as bedding material. Water was freely available at all times. The study design was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011/098). All ponies remained healthy and no clinical abnormalities were observed.

#### Study design

Before the start of the adaptation period, the ponies were given *ad libitum* access to the same low-energy hay (Table 2) as that fed during the trial, for 1 month. The ponies also

### Table 1. Phenotypic summary of each study group of obese ponies at the beginning of the weight-loss period

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age (years) Mean SEM</th>
<th>BW (kg) Mean SEM</th>
<th>Percentage of overweight based on estimated ideal BW Mean SEM</th>
<th>BCS (1–9) Mean SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>9.7 1.9</td>
<td>140.8 39.8</td>
<td>26.4 3.6</td>
<td>8.3 0.3</td>
</tr>
<tr>
<td>SLOW</td>
<td>9.0 1.5</td>
<td>170.3 38.0</td>
<td>28.0 4.0</td>
<td>8.2 0.3</td>
</tr>
<tr>
<td>RAPID</td>
<td>9.3 1.7</td>
<td>154.1 25.6</td>
<td>27.4 6.4</td>
<td>8.3 0.3</td>
</tr>
</tbody>
</table>

BW, body weight; BCS, body condition score; SLOW, slow weight loss; RAPID, rapid weight loss.
Table 2. Analysed (hay) and labelled (supplement) nutrient compositions of the hay and supplement
(Spillers Gro ‘N Win®; MARS Horsecare) on a DM basis

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Hay</th>
<th>Supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (g/kg)</td>
<td>885</td>
<td>888-1</td>
</tr>
<tr>
<td>Crude protein (g/kg)</td>
<td>89.3</td>
<td>360-30</td>
</tr>
<tr>
<td>Crude ash (g/kg)</td>
<td>63.3</td>
<td>168-9</td>
</tr>
<tr>
<td>Crude fibre (g/kg)</td>
<td>371-8</td>
<td>56-3</td>
</tr>
<tr>
<td>Crude fat (g/kg)</td>
<td>14.7</td>
<td>Nl</td>
</tr>
<tr>
<td>Starch (g/kg)</td>
<td>12.4</td>
<td>56-3</td>
</tr>
<tr>
<td>α-Tocopherol (mg/kg)</td>
<td>17.5</td>
<td>1698-9</td>
</tr>
<tr>
<td>DE (MJ/kg)</td>
<td>8.1</td>
<td>13-5</td>
</tr>
</tbody>
</table>

NI, not indicated on the label; DE, digestible energy.

received the same vitamin, protein and mineral supplement (Spillers Gro ‘N Win®; MARS Horsecare; Table 2) as that fed during the trial. The trial itself was divided into three periods: an adaptation period of 4 weeks; a weight-loss period of 16-5 weeks; an end-phase period of 3 weeks (Table 3).

During the adaptation period, the maintenance energy requirements to maintain a stable obese body weight (MERob) were determined for each pony individually. Initially, the low-energy hay was fed to provide 121% of maintenance net energy requirements as described by Van Weyenberg et al.135 based on their actual obese BW. The ponies also received a protein–vitamin–mineral balancer at an amount of approximately 1-32 g/kg ideal BW daily. This amount of the balancer is similar to 12·5% of their main-

Table 3. Energy intake during the adaptation period, weight-loss period and end-phase period in obese ponies
(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Adaptation period and end-phase period</th>
<th>Weight-loss period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage of offered hay consumed</td>
</tr>
<tr>
<td></td>
<td>Energy intake (%) of MERob</td>
</tr>
</tbody>
</table>

Groups | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
---|------|-----|------|-----|------|-----|------|-----|------|-----|
CONTROL | 100 | 0·17 | 0·01 | 97·50 | 1·45 | 100 | 0·17 | 0·01 | 99·63 | 0·31 |
SLOW | 100 | 0·17 | 0·01 | 99·84 | 0·21 | 80 | 0·14 | 0·00 | 99·21 | 1·42 |
RAPID | 100 | 0·17 | 0·01 | 99·98 | 0·06 | 60 | 0·10 | 0·01 | 98·80 | 1·7  |

MERob, individual maintenance energy requirements to maintain a stable obese body weight; BW, body weight; SLOW, slow weight loss; RAPID, rapid weight loss.

† BW at the end of the adaptation period.
products (PENT and carboxymethyllysine (CML)) and advanced oxidation protein products (AOPP). The blood samples were stored at 4°C until centrifugation at 3000 g for 10 min. Subsequently, plasma and serum samples were stored at −20°C until analysis.

**Measurements of plasma glucose and serum insulin concentrations**

Fasting plasma glucose concentrations were measured by the enzymatic colorimetric assay method (REF 3L82-21 and 3L82-41) using an Abbott Architect C16000 autoanalyzer (Abbott Diagnostic Laboratories) with the hexokinase–glucose-6-phosphate dehydrogenase method. Serum insulin concentrations were measured immunoradiometrically (REF 34,38) (insulin immunoradiometric assay (IRMA) reference no. 5251; DIAsource Europe S.A.). An implementation and validation procedure has previously been described briefly by Bruynsteen et al. (REF 38).

**Measurements of lipid metabolism markers**

Serum TAG concentrations were measured enzymatically (REF 7D74 304706/R02) using an Abbott Architect C16000 autoanalyzer (Abbott Diagnostic Laboratories). Serum NEFA concentrations were measured by the Random NEFA kit (REF FA 115; Randox Laboratories Limited) with modifications for use in the Daytona System (Randox Laboratories Limited).

**Measurements of antioxidant status markers**

FRAP was determined by spectrophotometric analysis (Monarch Chemistry System; Instrumentation Laboratories), as described by Benzie & Strain (REF 39) and previously validated in horses by Balogh et al. (REF 40). In this assay, antioxidant activity was measured via the reduction of the ferric tripyridyl triazine complex to the ferrous form at low pH, which was monitored by measuring the change in absorption at 593 nm. Results are reported as the concentration of Fe²⁺ measured per litre of serum (mol/l). SOD concentrations were determined by the ELISA method (EIAab), according to the manufacturer’s instructions. The detection range of the SOD ELISA kit was 0.78–58 ng/ml; therefore, plasma samples were diluted at 1:50. Absorbance was read at 450 nm. Results are expressed as pg/mg protein. Inter- and intra-assay CV were 10.7 and 9.8%, respectively. AOPP concentrations were determined by the ELISA method (EIAab), according to the manufacturer’s instructions. The detection range of the AOPP ELISA kit was 7.8–58 ng/ml; therefore, plasma samples were diluted at 1:50. Absorbance was read at 450 nm. Results are expressed as pg/mg protein. Inter- and intra-assay CV were 10.7 and 9.8%, respectively. OPO concentrations were measured spectrophotometrically, as described previously by Witko-Sarsat et al. (REF 41), on a microplate reader and calibrated with chloramidine-T solutions that absorb at λ = 340 nm in the presence of potassium iodide. Human serum albumin preparation (200 µl, diluted at 1:10 in PBS) was added to the test wells of a ninety-six-well microtitre plate, followed by the addition of acetic acid (20 µl). Potassium iodide (10 µl, 1-16 mol/l) was added to the standard wells containing chloramidine-T solution (200 µl, 0–100 µmol/l), followed by the addition of acetic acid (20 µl). The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 200 µl PBS, 10 µl potassium iodide and 20 µl acetic acid. AOPP concentrations are expressed as µmol/l of chloramidine-T equivalents. The inter- and intra-assay CV were 7.2 and 6.9%, respectively.

**Measurements of serum leptin concentrations**

Leptin concentrations were measured using a multispecies RIA kit (Millipore), validated for the use in horses (REF 42). The antibody used was guinea pig anti-human leptin. In the absence of purified equine leptin, results are reported as human equivalents of immunoreactive leptin.
**Statistical analysis**

Statistical analysis was based on a linear mixed model with pony as the random effect and treatment, time and their interaction as (categorical) fixed effects. A separate analysis was carried out for the weight-loss period and the end-phase period. Because there was a large difference in initial BW at the start of the weight-loss period (100–243·3 kg), changes in BW are expressed as relative differences (% change) from the baseline value (i.e. week 5 for the weight-loss period and week 21·5 for the end-phase period), which were used as the response variable. Absolute values were used for the other measured parameters. After the overall analysis, the three treatment groups were compared pairwise at each time point using Bonferroni’s adjustment technique for multiple comparisons. The global significance level was set at 5%.

**Results**

**Feed intake**

The daily hay intake throughout the study is described in Table 3. All ponies ate all of the supplement throughout the study. The average DMI was 1·86 (SEM 0·18), 1·96 (SEM 0·15) and 1·95 (SEM 0·27) % of obese BW in the CONTROL, SLOW and RAPID groups, respectively, at the end of the adaptation period. DMI was 1·86 (SEM 0·18), 1·57 (SEM 0·12) and 1·17 (SEM 0·16) % of BW in the CONTROL, SLOW and RAPID groups, respectively, at the start of the weight loss period. During the end-phase period, the ponies received the same DMI as that in the adaptation period. Energy intake from hay is described in Table 3.

**Relative weight changes**

During the entire weight-loss period (weeks 5–21), the CONTROL, SLOW and RAPID groups lost an average of 0·42 (SEM 0·45), 3·59 (SEM 0·63) and 10·81 (SEM 0·77) % of their initial BW, respectively (Fig. 1). A more rapid weight loss was observed in the group with the highest energy restriction (RAPID group, \( P<0·001 \)); however, the relationship between energy restriction and the percentage of weight loss was not proportional as a doubling of the percentage of energy restriction (20 % v. 40 %) was accompanied by a tripling of the percentage of weight loss (3·59 % v. 10·81 %).

At the end of the end-phase period, the CONTROL, SLOW and RAPID groups had, respectively, regained 1·11 (SEM 1·64), 1·41 (SEM 1·04) and 3·40 (SEM 0·94) % of their BW at the start of the end-phase period (week 21·5). During the end-phase period, a treatment effect was found between the CONTROL and RAPID groups (\( P<0·001 \)), and between the SLOW and RAPID groups (\( P<0·001 \)), meaning that the RAPID group gained significantly more weight during the end-phase period than the SLOW and CONTROL groups.

**Plasma glucose and insulin concentrations**

Throughout the weight-loss period, glucose concentrations (range 3·61–5·05 mmol/l; 65–91 mg/l) changed over time independently of the treatment (\( P<0·001 \)). No significant changes were found in the concentration of insulin during the weight-loss period and/or the end-phase period. Mean insulin concentrations at all time points were \( 180 \) pmol/l \( (\pm 30 \text{ mU/l}) \), although at the end of the weight-loss period, two ponies had higher insulin concentrations of 453·6 pmol/l \( (\pm 75·6 \text{ mU/l}) \) (CONTROL group) and 451·2 pmol/l \( (\pm 75·2 \text{ mU/l}) \) (SLOW group). At the end of the end-phase period, another four ponies (CONTROL group, \( n = 2 \); SLOW group, \( n = 1 \); RAPID group, \( n = 1 \)) had insulin concentrations \( >30 \text{ mU/l} \) (range 245·4–439·8 pmol/l; 40·9–73·3 mU/l).

![Fig. 1. Body-weight changes (%) in obese ponies from the control group (○), slow weight-loss group (●) and rapid weight-loss group (△) during the weight-loss period (WLP) and end-phase period (EPP) as a proportion of the value at week 5 (beginning of the WLP). Values are means, with their standard errors represented by vertical bars. During the WLP, a significant interaction between time and treatment was observed (\( P<0·001 \)). During the EPP, a group (\( P<0·001 \)) and week (\( P<0·001 \)) effect was observed.](https://www.cambridge.org/core/appendices/1406/33a075f59e39e388284c5c65b407412a)
Markers of lipid metabolism

Higher and more rapid maximum TAG concentrations were reached in the RAPID group (P=0·001) during the first part of the weight-loss period (Fig. 2). At the end of the end-phase period, TAG concentrations decreased more rapidly in the RAPID group compared with the SLOW and CONTROL groups (P<0·001).

NEFA concentrations changed during the weight-loss period (P<0·001), with higher concentrations found in the RAPID group compared with the SLOW and CONTROL groups (P=0·021). At the end of the end-phase period, NEFA concentrations decreased in the RAPID group, whereas the concentrations increased in the CONTROL group and remained stable in the SLOW group (P=0·014; data not shown).

Antioxidant status

FRAP concentrations changed during the weight-loss period (P<0·001), with the highest concentrations observed in the RAPID group (P<0·001) (Fig. 3). When comparing the end and the beginning of the end-phase period, FRAP concentrations increased in the SLOW and CONTROL groups, but remained constant in the RAPID group (P=0·001).

During the weight-loss period, SOD concentrations changed (P=0·036), with lower concentrations being observed in the RAPID group after 8 weeks of weight loss (P=0·003). SOD concentrations increased in the three groups during the end-phase period (P=0·003; data not shown).

α-Tocopherol concentrations changed over time during the weight-loss period, with the concentrations increasing in the RAPID group but being more stable in the SLOW group and slightly decreasing in the CONTROL group (P=0·004; Fig. 4). The concentrations of α-tocopherol rapidly decreased in the CONTROL and RAPID groups during the end-phase period; however, a slight decrease in concentration was observed in the SLOW group (P<0·001).

Oxidant status

During the weight-loss period and the end-phase period, TBARS concentrations changed over time independently of the treatment (P<0·001 and P=0·042, respectively; data not shown).

Throughout the weight-loss period, AOPP concentrations changed differently over time between the three treatment groups, with concentrations increasing in the RAPID group in the first part but remaining stable in the SLOW group and decreasing in the CONTROL group, respectively (P=0·015; Fig. 5).

No significant changes were found in the concentrations of PENT and CML.

Serum leptin concentrations

Serum leptin concentrations changed during the weight-loss period (P=0·002) and between the treatments (P=0·001) with the lowest concentrations being observed in the RAPID group (Fig. 6). At the end of the end-phase period, leptin concentrations decreased most rapidly in the CONTROL group compared with the RAPID and SLOW groups (P<0·001).
The feeding strategy during the adaptation period in the present study, in which the ponies were fed at their MERob instead of *ad libitum* food intake followed in the trial of Dugdale *et al.*

Leptin concentrations were lower at the end than at the beginning of the weight-loss period, which is in accordance with the findings reported in other weight-loss studies in ponies. In the present study, refeeding the ponies to 100% iMERob resulted in further lowering of leptin concentrations in the energy-restricted groups, which is a rather unexpected finding as higher leptin concentrations would be expected because of the higher energy intake and the subsequent satiety effect of this hormone. No clear explanation could be found for this finding.

When the body of the horse is in a state of negative energy balance, it changes to a more catabolic state. In an attempt to maintain normoglycaemia, there is a shift towards the use of fatty acids as the primary energy source. Given the higher predisposition of hyperlipaemia in Shetland ponies with obesity as a fortifying factor, this was obviously a potential concern. However, a previous study in Shetland geldings with even more severe energy restriction (but introduced very gradually) at 35% of maintenance energy requirements had reported no adverse health effects. In the present study, significantly higher serum TAG and NEFA concentrations were found in the SLOW and RAPID groups compared with the CONTROL group, with the highest concentrations being found in the RAPID group. However, TAG concentrations never exceeded the upper limit of the normal range (< 5000 mg/l), and none of the ponies showed any adverse clinical signs. During the 1st weeks of the weight-loss period, accumulation of TAG concentrations in the serum was positively associated with the extent of weight loss. After 4 weeks of energy restriction, even though weight loss was still continuing, TAG concentrations gradually returned to the baseline levels.

Together with the increase in the percentage of weight loss, TAG and NEFA concentrations in the blood, an increase in plasma antioxidant capacity (as indicated by the concentrations of α-tocopherol and FRAP) was observed during the first 4 to
Energy restriction rate and oxidative stress

8 weeks of the study. The increase in α-tocopherol concentrations (as part of vitamin E) could also be attributed to the release of this liposoluble vitamin from the fat deposit, which is one of the major storage sites of vitamin E (50). At the end of the weight-loss period, these concentrations returned to the baseline levels, perhaps suggesting that the stimulus for increased antioxidant demand had been resolved (decreased blood TAG and NEFA concentrations). The concentration of the pro-oxidant marker TBARS was lower at the end than at the start of the weight-loss period, indicating that, as reported in human subjects (51), dietary interventions resulting in even limited weight loss may help the oxidant–antioxidant equilibrium. The parameter TBARS has been broadly used for the measurement of lipid peroxidation, as it is one of the better predictors of oxidative damage (51). However, the use of this parameter has been criticised due to its low specificity (51) and sensitivity (52). To have a good understanding of the oxidant–antioxidant balance is warranted.

In conclusion, different levels of energy restriction will influence the extent of any weight loss, although there was no apparent linear relationship between the extent of energy restriction and the percentage of weight loss. A doubling of the percentage of energy restriction was associated with a tripling of the percentage of weight loss. Following the weight-loss period, more extensive weight loss was associated with more rapid and greater weight regain when ponies were fed again at 100% of their MERob. On the basis of the present results, it can be recommended in practice that if the obese equid is fed with a more severe energy-restricted diet in order to achieve weight loss within a reasonable time period, it is even more important that once the ideal weight of the animal is reached, monitoring should continue in order to avoid the rapid rebound effect of weight gain.

Finally, energy restriction and consequently weight loss can affect the oxidant–antioxidant balance, although significant effects were only observed in the present study with the highest level of energy restriction. However, these rather small effects are unlikely to be biologically significant, and further research into the effect of weight loss on the oxidant–antioxidant balance is warranted.

Acknowledgements

The authors gratefully acknowledge Ségolène Levéillée Nizerolle, An Cools, Kristel Rochus, Hannelore Van de Velde, Sanne Ott, Annelies De Spiegeleer, Galena Quist-Rybachuk, Christel Moons, Adronie Verbrugghe and Ruben Decaluwe for helping with the feeding practices and blood sampling of the ponies, and Sarah Van Beirs, Laura Stautus, Steven Galle and Ellen Van de Maelle for their excellent care of the ponies. The authors also thank Herman De Rycke, Roberta Borghi, Nicola Traverso, Daniel Vermeulen and Inge Vaessen for their assistance in the analyses.

The present study was part of the postgraduate study of L. B. and funded by the Institute for Promotion of Innovation through Science and Technology in Flanders (IWT, grant no. 101572). The study was also funded by the 2011 WALTHAM-Buckeye Equine Research Grant. Professor Pat Harris, who is affiliated with WALTHAM, was involved in the study design and drafting of the manuscript.

The authors’ contributions are as follows: L. B. was responsible for the study design, study performance, data analysis and drafting of the manuscript; M. H. and G. P. J. J., who were the supervisor and co-supervisor of L. B. respectively, contributed to the development of the study design, data analysis and drafting of the manuscript; P. A. H. contributed to the study design and drafting of the manuscript; L. D. was responsible for the data analysis and drafting of the manuscript; E. V. was responsible for the analysis of the advanced glycation end products and drafting of the manuscript;

References


