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

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

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## 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. Eighteen obese (body condition score>7) Shetland ponies were studied over a 23.5 week trial, divided into 3 periods. First a 4 week adaptation period in which each animal was fed 100% of their maintenance energy requirements needed to maintain stable obese body weight (MERob). Then a 16.5 weeks weight loss period in which ponies were assigned to receive either 100% (control group, CONTROL), 80% (slow weight loss group, SLOW) or 60% (rapid weight loss group, RAPID) of their MERob. During the 3 week end phase period all animals were again fed 100% of their MERob. Relative weight loss was higher in RAPID ($P<0.001$) compared to SLOW. No linear relationship was found as a doubling in caloric restriction was accompanied with a tripling in weight loss. Relative weight gain afterwards in the end phase period was higher in RAPID ($P<0.001$) compared to SLOW and CONTROL. During the weight loss period, triacylglycerol and non-esterified fatty acids levels were highest in RAPID, as were α-tocopherol and ferric reducing ability of plasma. After 8 weeks of weight loss, advanced oxidation protein products were higher in RAPID compared to SLOW and CONTROL ($P<0.001$). 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 post restriction.

## Introduction

With a prevalence of 19 to 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]. Whilst 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 where increased physical activity may be contra-indicated[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 caloric restriction (food intake restricted to 1.25% as dry matter intake (DMI) of actual body mass (BM)) with appropriate levels of weight loss, others may require more marked levels of reduction (1.00% of BM as daily DMI)[9]. However, it is well known that too severe caloric restriction in obese equidae may lead to hyperlipemia[10]. In dogs, the degree of caloric restriction also affected long term body weight

change. A higher caloric restriction resulted initially in a greater level of weight loss, but when returned to a 'normal' diet, the 'rebound' weight gain was higher[11]. This rebound weight gain was significantly correlated with the amount of lost body weight and the caloric restriction level[11]. This effect was also seen in human patients[12].

In obese humans, oxidative stress is related to chronic disease (e.g. hypertension, diabetes, metabolic syndrome, polycystic ovarian syndrome, liver disease)[13]. Human obesity increases the level of oxidative stress[14] as indicated by increased lipid peroxidation[15,16] and decreased systemic antioxidants[13,16-19]. Moreover, the altered oxidant-antioxidant status in obese children was reversible by a dietary restriction-weight loss program[19,20]. Restricted caloric intake reduced oxidative damage to proteins, lipids, and DNA in rodents[21]. It also reduced serum advanced glycation end products formation in healthy and overweight or obese human adults[22]. In horses, oxidative stress has been associated with several disorders such as recurrent airway obstruction[23,24], joint disease[25], neurological disorders[26] and perfusion related disorders[27]. Laminitis can be classified under this last condition. Equine digital laminea have relatively limited SOD capacity, which can make this tissue more susceptible to damage by reactive oxygen species, such as superoxide anion[28]. Insulin induced laminitis on the other hand has been associated with the accumulation of advanced glycation end products in the lamellar tissues of horses[29]. Insulin resistance, together with general obesity, regional accumulations of fat tissue and hyperleptineamia are features of equine metabolic syndrome (EMS)[4]. Higher plasma advanced glycation end product concentrations, more specific pentosidine, were found in ponies exhibiting clinical features of EMS and with a recent history of or current episode of laminitis compared with ponies with no recent history of laminitis[30]. Lower antioxidant capacity, by means of by means of decreased erythrocyte glutathione peroxidase activities, has also been described in obese horses[31].

In equidae, little is known about the effect of weight reduction programmes on the oxidant-antioxidant status. Therefore, the aim of the present study was to test the effect of different levels of energy restriction on weight loss and subsequent rebound weight gain, and oxidant/antioxidant balance. It was hypothesised that greater energy restriction would result in more weight loss, accompanied with an improved oxidant/antioxidant balance (increased antioxidant defence and lowered 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*

Eighteen obese (body condition score, BCS ≥ 7/9)[32] Shetland geldings, aged 9.3 ± 3.9 years (Table 1) were studied over a 23.5 week period (August to January). Only animals in good health and dental status were recruited. No prior history of clinical lameness and laminitis was reported for the ponies involved in the present study. No evidence of hyperinsulinemia was noticed at the start and throughout the study. The aim of the presents study was not to evaluate the effect of weight loss on glucose and insulin dynamics, therefore, no glucose tolerance tests were implemented. Routine foot care, vaccination and anthelmintic treatments were undertaken before and, if necessary, during the study. Ponies were housed individually during feeding times in 9 indoor boxes of $9m^2$ or in 9 stalls with an indoor and outdoor unit with a combined area of $13.83m^2$. During the rest of the day, ponies were group-housed in a large barn (inner part: $285m^2$, outer part: $275.5m^2$). 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 seen.

*Study design*

For a month before the start of the adaptation period, the ponies were given *ad libitum* access to the same low energy hay (Table 2) as would be used during the trial. The ponies also received the same vitamin, protein and mineral supplement (Spillers Gro'N Win®, MARS Horsecare) (Table 3) as fed during the trial. The trial itself was divided into 3 periods: an adaptation period of 4 weeks, a weight loss period of 16.5 weeks, and an end phase period of 3 weeks (Table 3).

During the adaption period, the maintenance energy requirements to maintain 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.[33], based on their actual obese body weight (BW). Ponies also received the protein/vitamin/mineral balancer at approximately 1.32 g/kg ideal BW/day. This amount of balancer is similar to 12.5% of their maintenance digestible energy requirements for (estimated) ideal body weight (according to NRC recommendations)[34] and corrects for all possible vitamin and protein insufficiencies from the hay diet. During the adaption period, BW was measured 3 times a week and based on these measurements, changes in the amount of hay fed were made to maintain a stable obese body weight. At the end of the last week of the adaption period individual MERob (100% iMERob) could be determined for each animal.

In the following weight loss period, ponies were divided into 3 groups stratified for balanced distribution according to age and BCS. Age was taken into account as plasma protein glycation,

more specific pentosidine, increase in horses during aging[35]. The control group (CONTROL) received 100% of their iMERob during this entire period. The 'slow' weight loss group (SLOW) was offered 80% of their iMERob. The 'rapid' weight loss group (RAPID) was restricted to 60% of their iMERob.

During the end phase period, all animals were again fed 100% of their original iMERob determined at the end of the adaptation period for another 3 weeks.

Throughout the study, daily hay rations were equally divided between 2 meals (09:00 am and 03:00 pm) and offered from small holed haylage nets in order to maximise the time spent foraging. The balancer was only offered during the morning feeding.

*Determination of body mass*

During the adaptation period and the end phase period, ponies were weighed (± 0.1 kg) 3 times a week between 08.00 and 09.00 am. During the weight loss period, ponies were weighed between 08.00 and 09.00 am on the first day of each week. All measurements were carried out on a calibrated scale (error ± 0.01kg) (Bascules Robbe, serial number 137, Torhout, Belgium).

*Blood sampling*

Blood sampling was undertaken in the early morning prior to feeding. During the weight loss period, blood samples were drawn every week on Monday into Vacuette® tubes (Z Serum Clot Activator, 4 ml) in order to monitor triacylglycerol (TAG) and non-esterified fatty acids (NEFA). In weeks 1, 5, 9, 13, 17, 21.5, and 24.5, blood samples were also taken for the analysis of glucose (Vacuette® tube, FX Sodium Fluoride/Potassium oxalate, 2 ml), and insulin, ferric reducing ability of plasma (FRAP), thiobarbituric reactive substances (TBARS), superoxide dismutase (SOD), α-tocopherol, and leptin (Vacuette® tube, Z Serum Clot Activator, 9 ml). Every 8 weeks, heparin plasma (Vacuette® tube, LH Lithium Heparin, 9 ml) was collected for subsequent analysis of protein, advanced glycation end products (pentosidine and carboxymethyllysine), and advanced oxidation protein products (AOPPs). The blood samples were stored at 4°C until centrifugation at 3000 x **g** for 10 minutes. Subsequently, plasma and serum samples were stored at -20°C until analysis.

*Plasma glucose and insulin concentrations*

Fasting plasma glucose concentration was measured by enzymatic colorimetric assay method (REF 3L82-21 and 3L82-41) using an Abbott Architect C16000 autoanalyzer (Abbott Diagnostic Laboratories, Chicago, IL, USA) with the hexokinase-G6PDH method[36,37].

Serum insulin concentrations were measured immunoradiometrically[33,37] (insulin IRMA Ref 5251, Diasource Europe S.A., Nivelles, Belgium). An implementation validation procedure has previously briefly been described by Bruynsteen et al.[37].

*Markers of lipid metabolism*

Serum TAG was measured enzymatically (REF 7D74 304706/R02) using an Abbott Architect C16000 autoanalyzer (Abbott Diagnostic Laboratories, Chicago, IL, USA). Serum NEFA concentrations were measured by the Randox NEFA kit (REF FA 115, Randox Laboratories Limited, United Kingdom) modified for use in the Daytona System.

*Markers of antioxidant status*

Analysis of FRAP was determined by spectrophotometrical analysis (Monarch Chemistry System, Instrumentation Laboratories, Zaventem, Belgium) as described by Benzie and Strain[38] and previously validated in horses by Balogh et al.[39]. In this assay, antioxidant activity was measured in terms of reduction of 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 $Fe^{2+}$ measured per L of serum after reaction (µmol/l). The SOD concentration was measured with a commercially available assay kit (REF 19160 SOD determination kit, Sigma-Aldrich) which is based on the colorimetric reaction between water-soluble tetrazolium salt (WST) and superoxide anion. Absorbance was read at 450 nm with the Victor 3 Plate reader (Perkin Elmer). Measurement of α-tocopherol was performed by reversed phase HPLC and spectrophotometrical UV detection. The RT-HPLC analytical column was a Prontosil 120-3-C18 (particle size 3 µm and length 100 mm; Thermo Fisher Scientific Inc., West Palm Beach, Florida, USA) and UV detection was determined at an absorbance of 295 nm (UV2000, Thermo Fisher Scientific).

*Markers of oxidant status*

The TBARS concentration was measured spectrophotometrically (Monarch Chemistry System, Instrumentation Laboratories, Zaventem, Belgium) as described by Lin et al.[40] and reported as concentration of malondialdehyde (MDA) measured per ml of serum after reaction (nmol/ml). Plasma protein content (protein) was determined using the BCA protein assay kit according to the manufacturer's instruction (Pierce® BCA Protein Assay Kit, Pierce Biotechnology). Pentosidine (PENT) was determined with high performance liquid chromatography (HPLC) detection according to Valle et al.[30] with slight modifications. Chromatography was performed using a Waters system (Waters S.P.A., Milan, Italy) equipped with a Bio-Tek SFM25 fluorimeter detector (Kontron

Instruments, Milan, Italy). Briefly, protein content, after delipidation with hexane and precipitation with trichloroacetic acid, was hydrolyzed with 6 mol/l hydrochloric acid for 18 h at 110 °C in borosilicate screw-capped tubes, dried in a Speed-Vac concentrator and then reconstituted in HPLC-grade water containing 0.01 mol/l heptafluorobutyric acid (HFBA), filtered through a 0.45-µm pore diameter Ultrafree MC (Millipore, Milano, Italia) and injected into a Aeris peptide 3.6u XB-C18 Reverse-Phase column (25 cm × 0.46 cm, 3.6 µm) with a curvilinear gradient program of 20%-40% phase B (methanol) in 30 min, while solvent A was $H_2O$. Both water and methanol contained 0.01 mol/l HFBA. The PENT peaks were monitored using a fluorescent detector at $\lambda_{ex}$ 335 nm and $\lambda_{em}$ 385 nm wavelengths. A PENT synthetic standard (prepared as described by Grandhee and Monnier, 1991)[41] was injected at the start of each run to determine the PENT concentration in the sample by peak area comparison. The amount of PENT was expressed as pmol per mg of plasma protein content. Inter and intra-assay CV was 4.8 and 5.4% respectively. Carboxymethyllysine (CML) was evaluated by ELISA methods (EIAab, Wuhan, China) according to the manufacturer's instructions. The detection range of CML ELISA kit was 0.78-58 ng/ml, so the plasma samples were diluted 1:50. Absorbance was read at 450 nm. Results were expressed as pg/mg protein. Inter and intra-assay CV was 10.7 and 9.8% respectively. Determination of AOPP was based on spectrophotometric detection according to Witko-Sarsat (1996)[42]. The AOPP levels were measured by spectrophotometry on a microplate reader and were calibrated with chloramine-T (CT) solutions, which in presence of potassium iodide absorb at λ 340 nm. In test wells, 200 µl of HSA preparation (diluted 1/10 in PBS) was placed on a 96-well microtiter plate, and 20 µl of acetic acid was added. In standard wells, 10 µl of 1.16 M potassium iodide was added to 200 µl of CT solution (0–100 µM) followed by 20 µl of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 200 µl of PBS, 10 µl of potassium iodide, and 20 µl of acetic acid. The AOPP concentrations were expressed as µmol/l of CT equivalents. The inter and intra-assay CV was 7.2 and 6.9% respectively.

*Serum leptin concentrations*

Leptin was measured using a multispecies RIA kit (Millipore, St. Charles, Missouri, USA) validated for the use in horses[43]. The antibody used was guinea pig anti-human leptin. In absence of a purified leptin preparation, results are reported as human equivalents of immunoreactive leptin (ir-leptin).

*Statistical analysis*

The statistical analysis was based on a linear mixed model with pony as random effect and treatment, time and their interaction as categorical fixed effects. A separate analysis was done for

the weight loss period and the end phase period. Because there was a large spread in initial body weight at the start of the weight loss period (100 – 243.3kg), changes in body weight were expressed as relative difference (% change) with the baseline value (i.e., week 5 for weight loss period and week 21.5 for end phase period) were used as 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 equal to 5%.

## Results

*Feed intake*

Daily hay intake throughout the study is described in table 3. All ponies ate all of the balancer throughout the entire study. The average DMI at the end of the adaptation period in the CONTROL, SLOW, and RAPID group respectively was 1.86 ± 0.18, 1.96 ± 0.15, and 1.95 ± 0.27 % of the obese BW at the end of the adaptation period. The DMI intake during the weight loss period in CONTROL, SLOW, and RAPID was 1.86 ± 0.18, 1.57 ± 0.12, and 1.17% ± 0.16 of the BW at the end of the adaptation period. During the end phase period, ponies received the same DMI as in the adaptation period. Energy intake is described in table 3.

*Relative weight changes*

During the entire weight loss period (w5-21), CONTROL lost an average of 0.42 ± 0.45%, SLOW 3.59 ± 0.63%, and RAPID 10.81 ± 0.77% of their initial body weight (Figure 1). A more rapid weight loss was seen in the group with the highest energy restriction (RAPID, $P<0.001$), but the relationship between caloric restriction and percentage weight loss was not proportional as a doubling in caloric restriction (20% compared to 40%) was accompanied with a tripling in weight loss (3.59 compared to 10.81%).

At the end of the end phase period, CONTROL, SLOW, and RAPID had respectively regained 1.11 ± 1.64, 1.41 ± 1.04, and 3.40 ± 0.94% of their BW at the start of the end phase period (w21.5). During the end phase period, a treatment effect was found between CONTROL and RAPID ($P<0.001$), and SLOW and RAPID ($P<0.001$) meaning that RAPID gained significantly more weight during the send phase period than SLOW and CONTROL.

*Plasma glucose and insulin concentrations*

Throughout the weight loss period, glucose values changed over time independently of treatment (P<0.001) (data not shown). No significant effects were found for insulin during the weight loss period and/or end phase period.

*Markers of lipid metabolism*

Higher and more rapid maximum TAG values were reached in RAPID (P=0.001) during the first part of the weight loss period (Figure 2). At the end of the end phase period, TAG values were decreased more rapidly in RAPID compared to SLOW and CONTROL (P<0.001).

The NEFA concentrations changed during the weight loss period (P<0.001), with higher values in RAPID compared to SLOW and CONTROL (P=0.021). At the end of the end phase period, the NEFA concentrations were decreased in RAPID, whereas in CONTROL and SLOW values respectively increased and remained stable (P=0.014) (data not shown).

*Antioxidant status*

The FRAP concentrations changed during the weight loss period (P<0.001), with the highest values in RAPID (P<0.001) (Figure 3). Comparing the end of the end phase period with the beginning, FRAP concentrations increased in SLOW and CONTROL, while they remained constant in RAPID (P<0.001).

During the weight loss period, SOD values changed (P=0.036) with lower values in RAPID after 8 weeks of weight loss (P=0.003). The SOD concentrations increased in the 3 groups during the end phase period (P=0.003) (data not shown).

The α-tocopherol values changed over time during the weight loss period with an increase in RAPID compared to more stable values in SLOW and slightly decreasing values in CONTROL (P=0.004) (Figure 4). Rapidly decreasing values were noticed in CONTROL and RAPID during the end phase period, whereas α-tocopherol values only slightly decreased in SLOW (P<0.001).

*Oxidant status*

During the weight loss and end phase period, TBARS values changed over time independently of treatment (P<0.001 and P=0.042 respectively) (data not shown).

Throughout the weight loss period, AOPP values changed differently over time between the 3 treatment groups, with an increase in RAPID in the first part compared to stable or decreasing values in SLOW and CONTROL respectively (P=0.015) (figure 5).

No significant effects were found for PENT and CML.

*Serum leptin concentration*

Serum leptin concentrations changed during the weight loss period (P=0.002) and between the treatments (P=0.001) with the lowest values in RAPID (Figure 6). At the end of the end phase period, most rapidly decreasing leptin values were found in CONTROL compared to RAPID and SLOW (P<0.001).

## Discussion

To the authors' knowledge, this is the first study describing the effect of different levels of energy restriction on weight loss and oxidative stress in ponies/equidae. In the present study, the higher calorie restriction (RAPID) was associated with the greatest weight loss, as has also been described previously in dogs[11]. A doubling of the percentage of caloric restriction was associated with a tripling in percentage of weight loss (3.59% in SLOW compared to 10.81% in RAPID). The highest energy restricted group (RAPID), however, also showed the greatest weight gain when they received again their 100% iMERob, a similar finding to that described in dogs[11] and humans[12]. Increased metabolic efficiency, as has been described in obesity-prone rats after weight loss[44], could be a possible explanation, although specific parameters (thyroid hormones, metabolic rate, individual total and resting expenditure) to confirm this were not measured in the present study. In obesity-prone rats, metabolic efficiency remained elevated and contributed to a lower energy expenditure, a greater energy imbalance, and a high rate of weight regain early in relapse[44]. MacLean and colleagues[44] estimated that adjustments in appetite and resting energy expenditure significantly contributed to the energy imbalance when rats were given free access to a low-fat diet.

Mean weekly weight loss in RAPID corresponded to 0.68%, which is comparable to the losses described in the weight loss trial of Dugdale and co-workers[8] who observed weekly weight losses of 0.7% by feeding 1% of actual BW as DMI daily in obese pony mares. In that same study, BW decreased by 4.3% during the first week of the weight loss period, which was attributed to decreased gut fill and possibly depletion of glycogen reserves associated with the transition from *ad libitum* food intake (positive energy balance) to restricted food intake (negative energy balance). This phenomenon was not seen in the present study where BW loss of RAPID in the first week only reached 0.87%. This could possibly be attributed to the feeding strategy in the adaptation period in this study, in which the ponies were fed at their maintenance energy requirements to maintain stable obese body weight instead of *ad libitum* food intake in the trial of Dugdale and co-workers[8].

Leptin levels at the end of the weight loss period were lower compared to the start of the weight loss period, which is in accordance with findings in other weight loss studies in ponies[33,45]. In the present study, refeeding the animals to 100% iMERob resulted in further lowering of the leptin levels in the energy restricted groups, a rather unexpected finding as higher leptin levels would be expected because of the higher energy intake and subsequent satiety effect of this hormone[46]. No clear explanation could be found for this.

When the horse's body is in a state of negative energy balance, it changes to a more catabolic state. In an attempt to maintain normoglycemia, there is a shift toward use of fatty acids as primary energy source[10]. Given the higher predisposition of hyperlipemia in Shetland ponies with obesity as a fortifying factor[47,48], this was obviously a potential concern. However, a previous study with Shetland pony geldings with even more severe (but introduced very gradually) caloric restriction at 35% of maintenance energy requirements had reported no adverse health effects[33]. In the present study, statistically higher serum TAG and NEFA levels were found in SLOW and RAPID compared to CONTROL, with the highest levels in RAPID. Triglyceride concentrations, however, never exceeded the upper limit of the normal range (< 500 mg/dl)[10] and none of the ponies showed any adverse clinical signs. During the first weeks of the weight loss period, accumulation of TAG in the serum was positively associated with the extent of the weight loss. After 4 weeks of caloric restriction, even though weight loss was still occurring, TAG concentrations gradually returned to baseline levels.

Together with the increase in percentage weight loss, and TAG and NEFA concentration in the blood, an increase in plasma antioxidant capacity (as indicated by α-tocopherol and FRAP) was seen during the first 4 to 8 weeks of the study. The increase in α-tocopherol (as part of the vitamin E) could also be related to the fact that this liposoluble vitamin is released from the fat deposit as this is one of the major storage sites of vitamin E[43]. By the end of the weight loss period, these values had returned to baseline levels, suggesting perhaps that the stimulus for increased antioxidant demand had been resolved (lowered blood TAG and NEFA concentrations). The pro-oxidant marker TBARS was lower at the end of the weight loss period compared to the start, indicating that, as in humans[13], 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[50]. However, its use has been criticised due to low specificity[50] and sensitivity[51]. In order to have a good understanding of the oxidant/antioxidant status, multiple measures of oxidative damage should be evaluated[27,50]. Therefore, in the present study, oxidative damage to proteins was also evaluated. In

humans, lipids as well as carbohydrates are important contributors to chemical modification of proteins, leading to lipoxidation products[52]. Chemical modification of amino acids in proteins during lipid peroxidation results in the formation of lipoxidation products like AOPP. The higher increase in AOPP in RAPID after 8 weeks of weight loss compared to SLOW could therefore be related to the higher TAG and NEFA values in RAPID, which are prone to oxidation. No significant effects were found for CML and PENT, which could be due to the tight glycemic control in the ponies and the fact that TAG and NEFA levels were not high enough to form these glycoxidation and lipoxidation markers[53].

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 percentage of weight loss. A doubling in percentage of energy restriction was associated with a tripling in % of weight loss. Following the weight loss period, the more extensive weight loss was associated with more rapid and greater weight regain when ponies were fed again at 100% of their MERob. Based on the results of the present study, it can be recommended in practice that if the obese equids are fed a more severe calorie restricted diet in order to achieve weight loss within a reasonable time period, it is even more important that once the animal's ideal weight is reached, monitoring continues in order to avoid rapid weight gain rebound effect.

Finally, energy restriction and consequently weight loss can affect the oxidant/antioxidant balance, although significant effects were only seen in this study with the highest level of calorie restriction. However, these rather small effects are thought not to be of any biological significance and further research into the effect of weight loss on the oxidant/antioxidant balance is warranted.

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**Conflict of Interest**

None.

**Authorship**

L.B. was responsible for the study design, study performance, data analysis and manuscript drafting. M.H. and G.P.J.J., supervisor and co-supervisor of L.B. respectively, contributed to the development of the study design, data analysis and manuscript drafting. P.A.H. contributed to the study design and manuscript drafting. L.D. was responsible for data analysis and manuscript drafting. E.V. was responsible for advanced glycation end products analysis and manuscript drafting. P O. was responsible for advanced glycation end products analysis. K.V. collaborated in the study performance and contributed to the manuscript drafting.

**REFERENCE LIST**

1. Thatcher C, Pleasant R, Geor R *et al.* (2007) Prevalence of obesity in mature horses: an equine body condition study. *The American Academy of Veterinary Nutrition 7th Annual Clinical Nutrition and Research Symposium,* Seattle, pp. 6.
2. Wyse CA, McNie KA, Tannahil VJ *et al.* (2008) Prevalence of obesity in riding horses in Scotland. *Vet Rec* **162**, 590-591.
3. Quinn RW, Burk AO, Suagee JK *et al.* (2006) Equine obesity: implications for health and strategies for control. *Proceedings of the 4th Mid-Atlantic Nutrition conference,* March 29-30, 2006 Timonium, Maryland, pp. 23-36.
4. Geor RJ (2008) Metabolic predisposition to laminitis in horses and ponies: obesity, insulin resistance and metabolic syndromes. *J Equine Vet Sci* **28**, 753-759.
5. Frank N, Geor RJ, Bailey SR *et al.* (2010). Equine Metabolic syndrome. *J Vet Intern Med* **24**, 467–475.
6. King C & Mansmann RA (2004). Preventing laminitis in horses: Dietary strategies for horse owners. *Clin Tech Equine Pract* **3**, 96-102.
7. Carter RA, McCutcheon J, Valle E *et al.* (2010) Effects of exercise training on adiposity, insulin sensitivity, and plasma hormone and lipid concentrations in overweight or obese, insulin-resistant horses. *Am J Vet Res* **71**, 314-321.

8. Dugdale AHA, Curtis GC, Cripps P *et al.* (2010) Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares. *Equine Vet J* **42**, 600-610.
9. Argo CMcG, Curtis GC, Grove-White D *et al.* (2012) Weight loss resistance: A further consideration for the nutritional management of obese *Equidae; Vet J* **194**, 179-188.
10. McKenzie III HC (2011). Equine Hyperlipidemias. *Vet Clin North Am Equine Pract* **27**, 59–72.
11. Laflamme DP & Kulman G (1995) The effect of weight loss regimen on subsequent weight loss maintenance in dogs. *Nutr Res* **15**, 1019-1028.
12. Weiss EC, Galuska DA, Khan LK *et al.* (2007) Weight regain in U.S. adults who experienced substantial weight loss, 1999-2002. *Am J Prev Med* **33**, 34-40.
13. Vincent HK, Innes KE & Vincent KR (2007) Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity. *Diabetes Metab.* **9**, 813-839.
14. Suzuki K, Ito Y, Ochiai J *et al.* (2003) Relationship between obesity and serum markers of oxidative stress and inflammation in Japanese. *Asian Pac J Allergy* **4**, 259-266.
15. Dandona P, Mohanty P, Ghanim H *et al.* (2001) The suppressive effect of dietary restriction and weight loss in the obese on the generation of reactive oxygen species by leukocytes, lipid peroxidation, and protein carbonylation. *J Clin Endocr Metab* **86**, 355-362.
16. Roberts CK & Sindhu KK (2009) Oxidative stress and metabolic syndrome. *Life Sci* **84**, 705-712.
17. Ozata M, Mergen M, Oktenli C *et al.* (2002) Increased oxidative stress and hypozincemia in male obesity. *Clin Chem* **35**, 627-631.
18. Reitman A, Friedrich I, Ben-Amotz A *et al.* (2002). Low plasma antioxidants and normal plasma B vitamins and homocysteine in patients with severe obesity. *Israel Med Assoc J* **4**, 590-593.
19. Mohn A, Catino M, Capanna R *et al.* (2005) Increased oxidative stress in prepuberal severely obese children: effect of a dietary restriction-weight loss program. *J Clin Endocr Metab* **90**, 2653-2658.
20. Kelishidaki R, Hashemi M, Mohammadifard N *et al.* (2008) Association of changes in oxidative and proinflammatory states with changes in vascular function after a lifestyle modification trial among obese children. *Clin Chem* **54**, 147-153.
21. Gredilla R & Barja G (2005) Minireview: the role of oxidative stress in relation to caloric restriction and longevity. *Endocrinology* **146**, 3713-1717.

22. Gugliucci A., Kotani K., Taing J *et al.* (2009) Short-term low calorie diet intervention reduces serum advanced glycation end products in healthy overweight or obese adults. *Ann Nutr Metab* **54**,197–201.
23. Kirschvink N, Smith N, Fiévez *et al.*(2002) Effect of chronic airway inflammation and exercise on pulmonary antioxidant status of healthy and heaves-affected horses. *Equine Vet J* **34**, 705-712.
24. Deaton CM, Marlin DJ, Smith NC *et al.* (2004) Pulmonary epithelial lining fluid and plasma ascorbic acid concentrations in horses affected by recurrent airway obstruction. *Am J Vet Res* **65**,80-87.
25. Dimock AN, Siciliano PD & McIlwraith (2000) Evidence supporting an increased presence of reactive oxygen species in the diseased equine joint. *Equine Vet J* **32**, 439-443.
26. Divers TJ, Cummings JE, de Lahunta A *et al.* (2006) Evaluation of the risk of motor neuron disease in horses fed a diet low in vitamin E and high in copper and iron. *Am J Vet Res* **67**, 120-126.
27. Kischvink N, de Moffarts B & Lekeux P (2008) The oxidant-antioxidant equilibrium in horses. *Vet J* **177**, 178-191.
28. Loftus JP, Belknap JK, Stankiewicz KM *et al., (*2007) Laminar xanthine oxidase, superoxidase dismutase and catalase activities in the prodromal stage of black-walnut induced equine laminitis. *Equine Vet J* **39**, 48-53.
29. de Laat MA, Kyaw-Tanner MT, Sillence *et al.* (2012) Advanced glycation endproducts in horses with insulin-induced laminitis. *Vet Immunol Immunop* **145**, 395-401.
30. Valle E, Storace D, Sanguinetti R *et al.* (2012) Association of the glycoxidative stress marker pentosidine with equine laminitis. *Vet J*, **3**, 445-450.
31. Pleasant RS, Suagee JK, & Thatcher CD (2013) Adiposity, plasma insulin, leptin, lipids, and oxidative stress in mature light breed horses. *J Vet Intern Med* **27**:576–582.
32. Henneke DR, Potter GD, Kreider JL *et al.* (1983). Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet J* **15**, 371-372.
33. Van Weyenberg S, Hesta M, Buyse J *et al.* (2008) The effect of weight loss by energy restriction on metabolic profile and glucose tolerance in ponies. *J Anim Physiol Nutr* **92**, 538-545.
34. National Research Council, 2007, *Nutrient Requirements of Horses, sixth ed. National Academies Press*, Washington DC.

35. Valentini S, Valle E & Bergero D (2007) Plasma protein glycoxidation and aging in horses. *Proceedings of the 11th European Society of Veterinary and Comparative Nutrition Congress*, November 1st – 3rd, Leipzig, Germany.
36. Moreaux S, Nichols J, Bowman J *et al.* (2011) Psyllium Lowers Blood Glucose and Insulin Concentrations in Horses. *J Equine Vet Sci* **31**, 160-165.
37. Bruynsteen L, Erkens T, Peelman LJ *et al.* (2013) Expression of inflammation-related genes is associated with adipose tissue location in horses. *BMC Vet Res* **9**: 240.
38. Benzie IFF & Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* **239**, 70-76.
39. Balogh N, Gaál T, Ribiczeyné PS *et al.* (2001) Biochemical and antioxidant changes in plasma and erythrocytes of pentathlon horses Before and after exercise. *Vet Clin Path* **30**, 214-218.
40. Lin H, Decuypere E & Buyse J (2004) Oxidative stress induced by corticosterone administration. *Comp Biochem and Phys B* **139**, 737 – 744.
41. Grandhee SK & Monnier VM (1991) Mechanism of formation of the Maillard protein cross-link pentosidine. Glucose, fructose, and ascorbate as pentosidine precursors. *J Biol Chem* 266, 11649-11653.
42. Witko-Sarsat V, Friedlander M, Capaillère-Blandin C *et al.* (1996). Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Journal* **49**, 1304-1313.
43. McManus C & Fitzgerald B (2000) Effects of a single day of feed restriction on changes in serum leptin, gonadotropins, prolactin, and metabolites in aged and young mares. *Domest Anim Endocrin* **19**, 1–13.
44. MacLean PS, Higgins JA, Johnson GC *et al.* (2004) Enhanced metabolic efficiency contributes to weight gain after weight loss in obesity-prone rats. *Am J Physiol Regul Integr Comp Physiol* **287,** R1306-R1315.
45. Ungru J, Blüher M, Coenen M *et al.* (2012) Effects of body weight reduction on blood adipokines and subcutaneous adipose tissue adipokine mRNA expression profiles in obese ponies. *Vet Rec* doi: 10.1136/vr.100911.
46. Radin MJ, Sharkey LC & Holycross BJ (2009) Adipokines: a review of biological and analytical principles and an update in dogs, cats, and horses. *Vet Clin Pathol* **38**, 136-156.
47. Watson TDG, Burns L, Love S *et al.* (1992) Plasma lipids, lipoproteins and post-heparin lipases in ponies with hyperlipaemia. *Equine Vet J* **24**, 341-346.

48. Hughes KJ, Hodgson DR & Dart AJ (2004) Equine hyperlipaemia: a review. *Aust Vet J* **82**, 136-142.
49. Ball GFM (1998). *Bioavailability and Analysis of Vitamins in Foods*. New York: Chapman and Hall.
50. Lykkesfeldt J & Svendsen O (2007) Oxidants and antioxidants in disease: oxidative stress in farm animals. *Vet J* **173**, 502-511.
51. McMichael MA (2007) Oxidative stress, antioxidants, and assessment of oxidative stress in dogs and cats. *J Am Vet Med Ass* **231**, 714-720.
52. Bengmark S (2007) Advanced glycation and lipoxidation end products-amplifiers of inflammation: the role of food. *JPen- Parenter Enter* **31**, 430-440.
53. Requena JR, Fu M, Ahmed MU *et al.* (1996) Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. *Neprhol Dial Transplant* **11** (Suppl 5), 48-53.

## TABLES

Table 1**:** Phenotypic summary of each study group at the beginning of the weight loss period. Data describing age (years), body weight (kg), % overweight based on estimated ideal body weight and body conditions score (BCS) are presented. Data are provided as means ± SE.

| Group | Age (years) | | BW (kg) | | % overweight based on estimated ideal BW | | BCS (1-9) | |
|---|---|---|---|---|---|---|---|---|
| | mean | SE | mean | SE | mean | SE | mean | SE |
| CONTROL | 9.7 | 1.9 | 140.8 | 39.8 | 26.4 | 3.6 | 8.3 | 0.3 |
| SLOW | 9.0 | 1.5 | 170.3 | 38.0 | 28.0 | 4.0 | 8.2 | 0.3 |
| RAPID | 9.3 | 1.7 | 154.1 | 25.6 | 27.4 | 6.4 | 8.3 | 0.3 |

Table 2: Analysed (hay) and labelled (supplement) nutrient composition of the hay and supplement (Spillers Gro'n Win®, MARS Horsecare) on dry matter basis.

| Nutrient | Hay | Supplement | |
|---|---|---|---|
| Dry matter | 885 | 888.1 | g/kg |
| Crude protein | 89.3 | 320 | g/kg |
| Crude ash | 63.3 | 150 | g/kg |
| Crude fibre | 371.8 | 50 | g/kg |
| Crude fat | 14.7 | NI | g/kg |
| Starch | 12.4 | 50 | g/kg |
| alpha-tocopherol | 17.5 | 1500 | mg/kg |
| DE | 8.1 | 13.5 | MJ/kg |

NI, not indicated on label; DE, digestible energy

Table 3: Energy intake during adaptation period, weight loss period, and end phase period. Individual maintenance requirements to maintain stable obese body weight were determined during the adaptation period. During the weight loss period, ponies were divided into 3 treatment groups (n=6), were they received 100% of individual maintenance requirements to maintain stable obese body weight (control group, CONTROL), 80% of individual maintenance requirements to maintain stable obese body weight (slow weight loss group, SLOW) or 60% of individual maintenance requirements to maintain stable obese body weight (rapid weight loss group, RAPID). Energy intake was calculated as digestible energy (DE). Daily dry matter intake (DMI) as % of body weight (BW) is also given in the table.

| Group | Adaptation period and end phase period | | | | | Weight loss period | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy intake (% of iMERob) | Energy intake (MJ/kg BW) | | % of offered hay consumed | | Energy intake (% of iMERob) | Energy intake (MJ/kg BW) | | % of offered hay consumed | |
| | Mean | Mean | SE | Mean | SE | Mean | Mean | SE | Mean | SE |
| 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 |

iMERob, individual maintenance energy requirements to maintain stable obese body weight; CONTROL, control group; SLOW, slow weight loss group; RAPID, rapid weight loss group.

## FIGURES

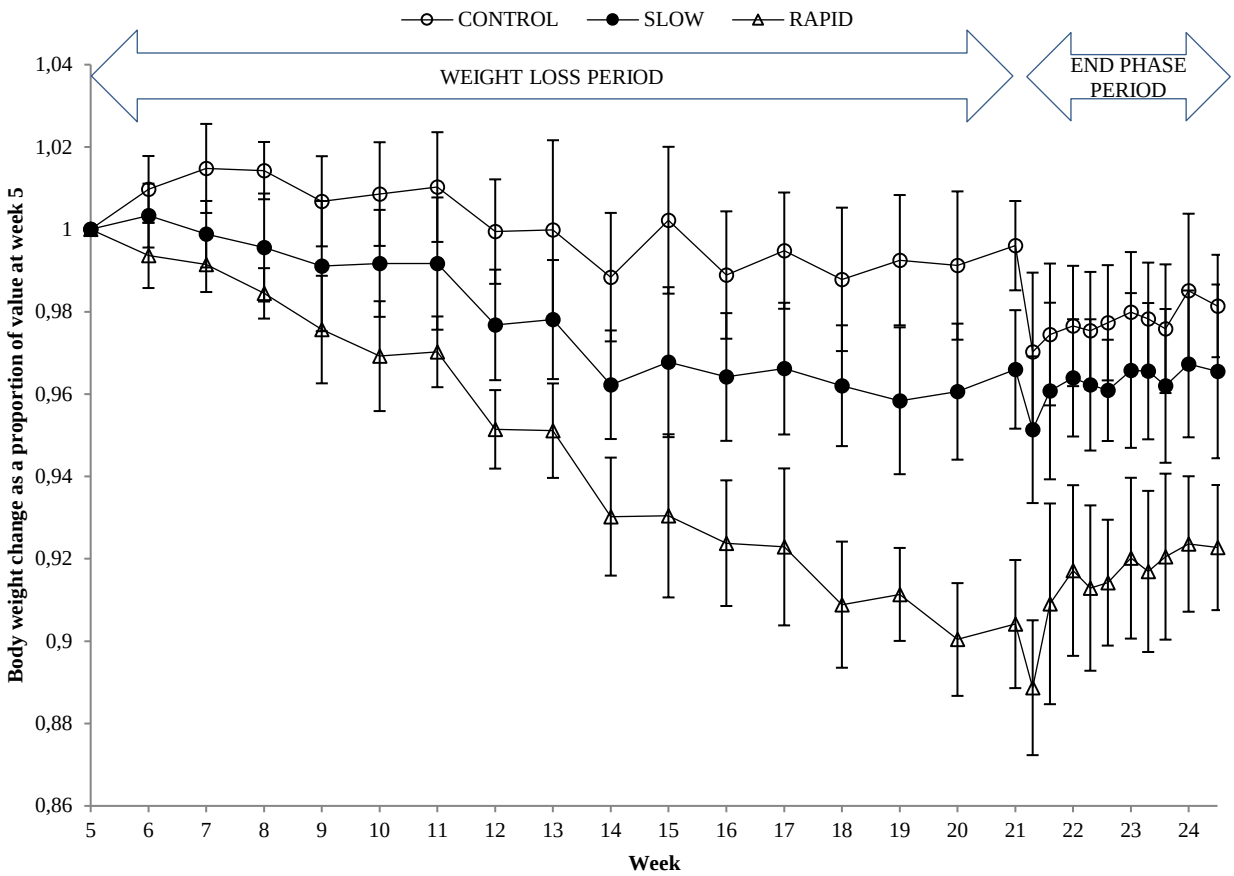


Figure 1: Body weight change (%) during the weight loss period and end phase period as a proportion of the value at week 5 (beginning of weight loss period) in the control group (CONTOL,○), slow weight loss group (SLOW,●) and rapid weight loss group (RAPID,Δ). Data are given as means ± SEM. Abbreviations: CONTROL, control group; SLOW, slow weight loss group; RAPID, rapid weight loss group.

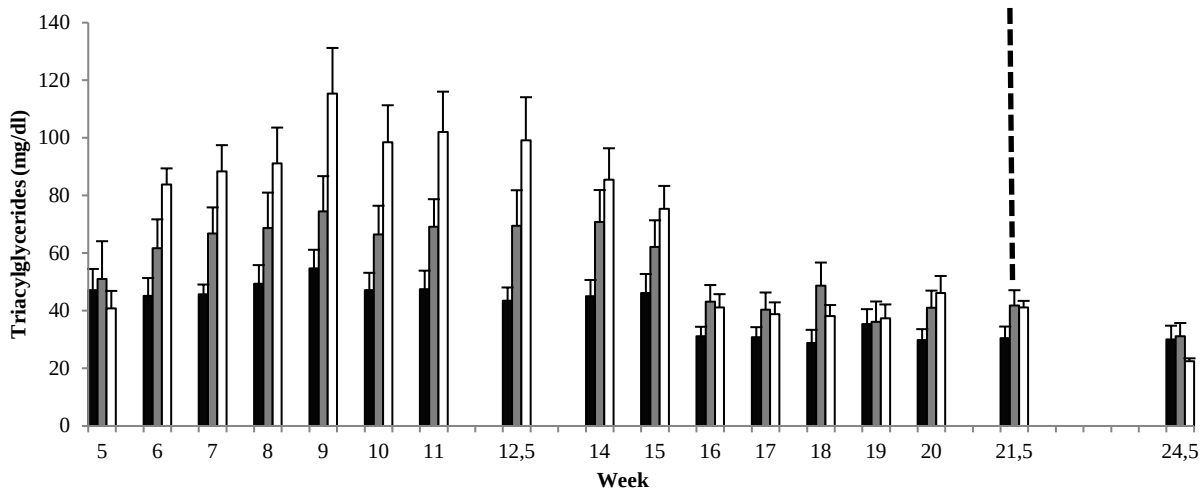


Figure 2: Serum TAG concentration during the weight loss period and end phase period in the CONTROL (black bar), SLOW (grey bar) and RAPID (white bar) group. Data are given as means ±

SEM. The vertical dotted line on the graphs separates the weight loss period from the end phase period. Abbreviations: CONTROL, control group; SLOW, slow weight loss group; RAPID, rapid weight loss group.

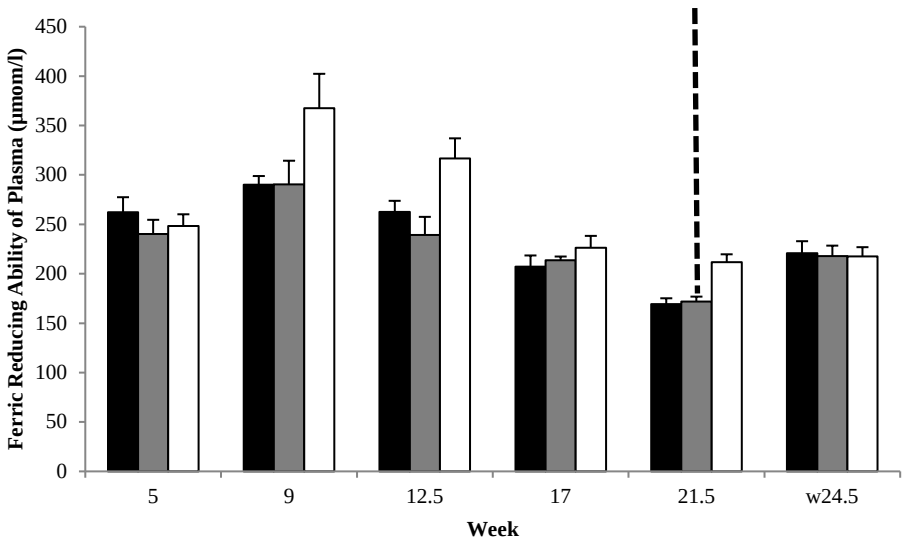


Figure 3: Serum FRAP concentration during the weight loss period and end phase period in the CONTROL (black bar), SLOW (grey bar) and RAPID (white bar) group. Data are given as means ± SEM. The vertical dotted line on the graphs separates the weight loss period from the end phase period. Abbreviations: CONTROL, control group; SLOW, slow weight loss group; RAPID, rapid weight loss group.

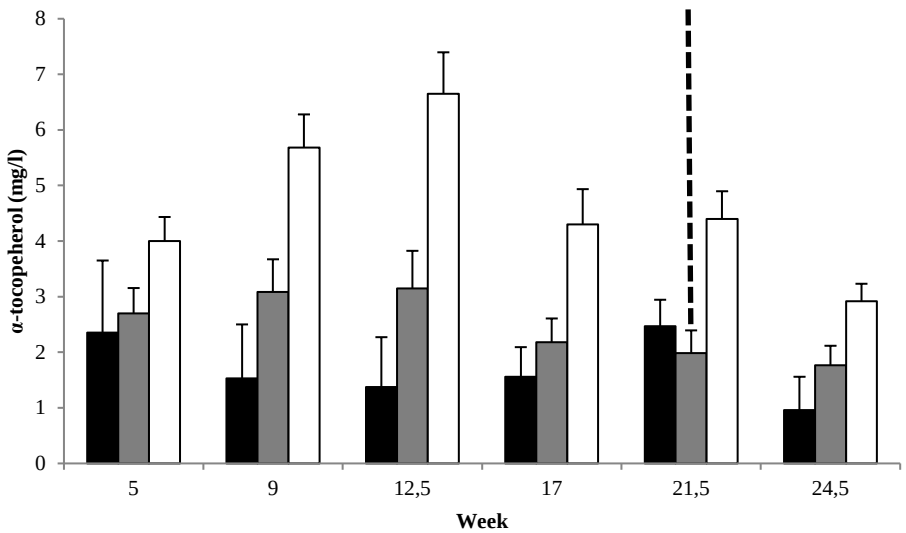


Figure 4: Serum α-tocopherol concentration during the weight loss period and end phase period in the CONTROL (black bar), SLOW (grey bar) and RAPID (white bar) group. Data are given as means ± SEM. The vertical dotted line on the graphs separates the weight loss period from the end phase period. Abbreviations: CONTROL, control group; SLOW, slow weight loss group; RAPID, rapid weight loss group.

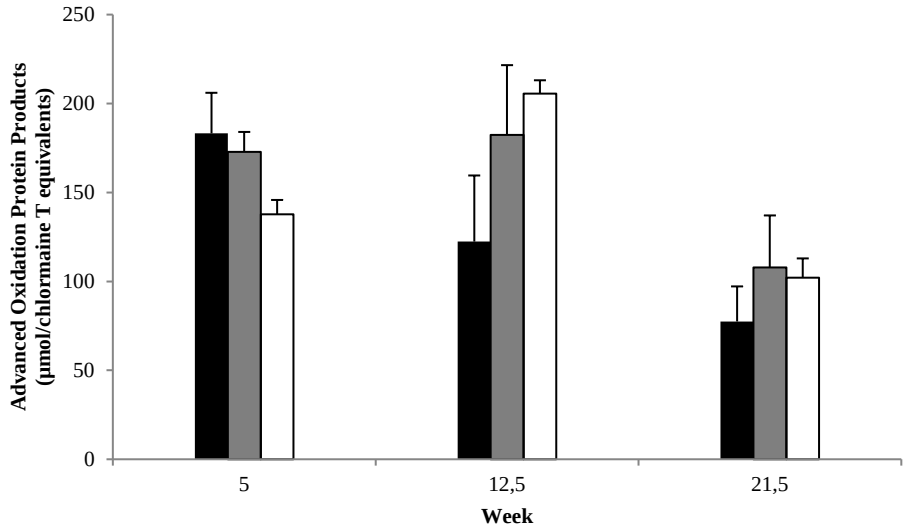


Figure 5: Plasma AOPP concentration during the weight loss period in the CONTROL (black bar), SLOW (grey bar) and RAPID (white bar) group. Data are given as means ± SEM. Abbreviations: AOPP, advanced oxidation protein products; CONTROL, control group; SLOW, slow weight loss group; RAPID, rapid weight loss group.

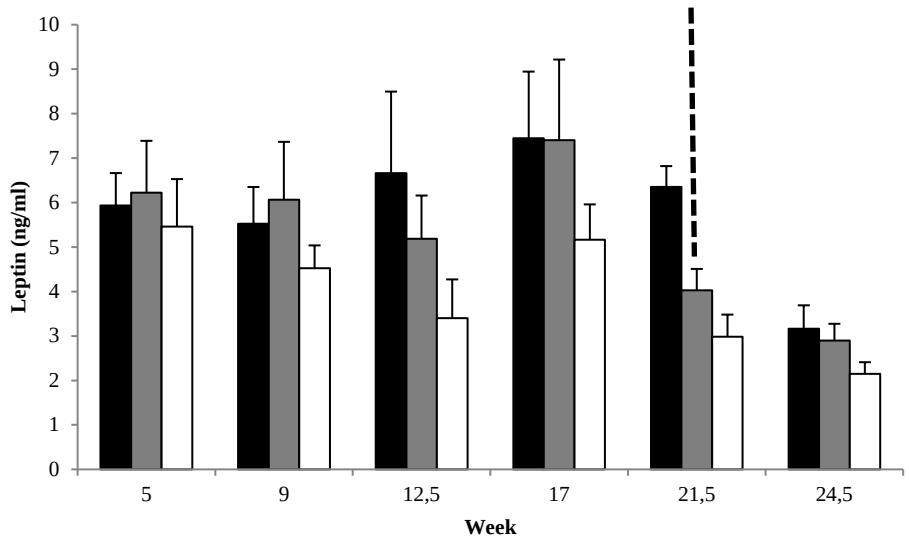


Figure 6: Serum leptin concentration during the weight loss period in the CONTROL (black bar), SLOW (grey bar) and RAPID (white bar) group. Data are given as means ± SEM. The vertical dotted line on the graphs separates the weight loss period from the end phase period. Abbreviations: CONTROL, control group; SLOW, slow weight loss group; RAPID, rapid weight loss group.