Aspects of obesity and energy restriction in equines

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TABLE OF CONTENTS

List of abbreviations

%mDE: percentage of DEI relative to estimated maintenance energy requirements

ACTB: actin beta

ADIPOQ: adiponectin

AGE: advanced glycation end products

AMPK: adenosine monophosphate-activated protein kinase

AOPP: advanced oxidation protein products

AP: adaptation period

APC: antigen presenting cells

APES: 3-aminopropyltriethoxysilane

AT: adipose tissue

BCA: bicinchoninic acid

BCS: Body Condition Score

BM: body mass

BMI: Body Mass Index

BW: body weight

CCL5: chemokine ligand 5

cDNA: complementary DNA

CLS: crown-like structure

CML: carboxymethyllysine

CNS: Cresty Neck Score

CONTROL: control group

CRP: C-reactive protein

CT: chloramine-T

Ct: transcription value

DEI: digestible energy intake

DMI: dry matter intake

EMS: Equine Metabolic Syndrome

FRAP: ferric reducing ability of plasma

FUP: follow up period

G6PDH: glucose-6-phosphate dehydrogenase

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GE: gross energy

GLUT: glucose transporter

HE: haematoxylin-eosin

HFBA: heptafluorobutyric acid

HIF-1α: hypoxia-induced factor 1α

HLA-DR: human leukocyte antigen-DR

HPF: high power field

HPLC: high performance liquid chromatography

HPRT1: hypoxanthine phosphoribosyltransferase 1

HSA: human serum albumin

IL: interleukin

iMERob: individual maintenance energy requirements to maintain stable obese body weight

IR: insulin resistance

IRMA: immunoradiometric assay

L: Loin

LH: lithium heparin

M: mesenteric

MCP: monocyte chemotactic protein

MDA: malondialdehyde

ME: metabolizable energy

MERob: maintenance energy requirements to maintain stable obese body weight

MHC II: major histocompatibility complex II

MMP: matrix metalloproteinase

MOD: moderate energy restricted group

mRNA: messenger ribonucleic acid

N ¼: Neck ¼

NAPDH: nicotinamide adenine dinucleotide phosphate

NE: net energy

NEFA: non esterified fatty acids

NF: normalization factor

NO: nitric oxide

NRC: National Research Council

PBS: phosphate buffered saline

PENT: pentosidine

PPAR$_\gamma$: peroxisome proliferator-activated receptor gamma

RK: right kidney

ROS: reactive oxygen species

RP: retroperitoneal

RPL 32: ribosomal protein L32

RQI: RNA quality indicator

RT-qPCR: reverse transcriptase quantitative polymerase chain reaction

SAA: serum amyloid A

SC: subcutaneous

SDHA: succinate dehydrogenase complex

SEV: severe energy restricted group

SOD: superoxide dismutase

TAG: triacylglycerol

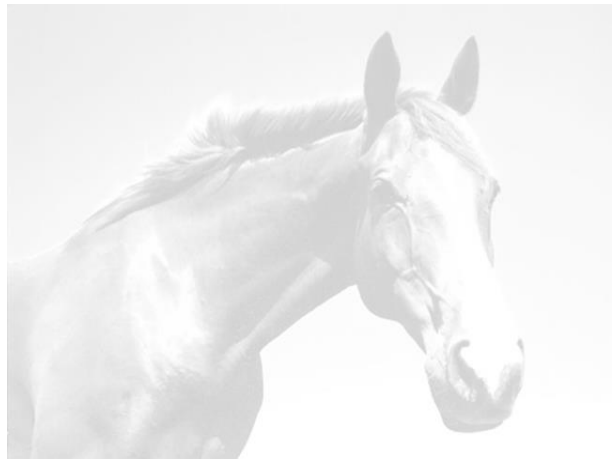TBARS: thiobarbituric reactive substances

TH: tail head

TNF-α: tumor necrosis factor alpha

TUBA4A: tubulin 4A

WLP: weight loss period

WST: water-soluble tetrazolium salt

β-GUS: beta-glucuronidase

CHAPTER 1
GENERAL INTRODUCTION

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1.1 Definition of obesity

Obesity can be defined as an **expanded mass of adipose tissue** within the body (Johnson et al., 2006), which can be either due to adipocyte hypertrophy (increasing size of individual cells) or adipocyte hyperplasia (recruiting new adipocytes from the resident pool of progenitors) (Forsythe et al., 2008; Rosen and Spiegelman, 2014). Obesity is considered to be a **pathological condition** in which the accumulation of body fat is so increased that health can be compromised. In horses, obesity (general and regional) has been considered for a long time as one of the principal components of the **Equine Metabolic Syndrome** (EMS), however, recent work suggests that insulin dysregulation (excessive insulin response to sugars, insulin resistance (IR), or fasting hyperinsulinemia) is the key component of EMS (Geor, 2013; Frank and Tadros, 2014). This syndrome is also associated with other physiological alterations, such as hypertriglyceridemia and hyperleptinemia (Frank, 2006; Frank, 2009).

1.2 Determination of equine body mass and composition

To estimate body condition and body composition in horses, multiple techniques have been described. Before discussing these, it is important to note that there appears to be breed-related differences in fat and muscle distribution between horses and ponies depending on their genetic heritage (Kearns et al., 2002; Robin et al., 2014). A clear example is the comparison between the active sport horse type Thoroughbred and the inactive 'native' breed type/companion animal Shetland pony.

Over the years, various methods have been described to determine body composition in horses. The first method described, the gold standard, is **carcase dissection** (Westervelt et al., 1976; Martin-Rosset et al., 2008; Dugdale et al., 2011a). This method has also been used to compare the calculated values after ultrasound measurement (Westervelt et al., 1976) or deuterium oxide dilution technique (Dugdale et al., 2011b). This is not a practical technique as the horses have to be sacrificed.

The most frequently used, although subjective, method in the horse for estimating body condition is the **Body Condition Score** (BCS). Over decades, multiple scoring methods have been developed, with these two being currently the most frequently used: the Henneke et al. (1983) system, developed in Quarter Horse mares and ranging from 1 to 9 (1=emaciated,

5=normal, 9=obese), and the Carroll and Huntington (1988) system ranging from 0 to 5 (0= very poor, 5= very fat). In the Henneke et al. system (1983), fat deposits in specific areas of the body, including the crest of the neck, on the withers, behind the shoulder, over the ribs, along the loin, and around the tail head are palpated and evaluated to create a numerical score (Figure 1.1). In the Carroll and Huntington system (1988), deposition of body fat at the level of the neck, back and ribs, and pelvis area are assessed individually and then combined to give an overall condition score.
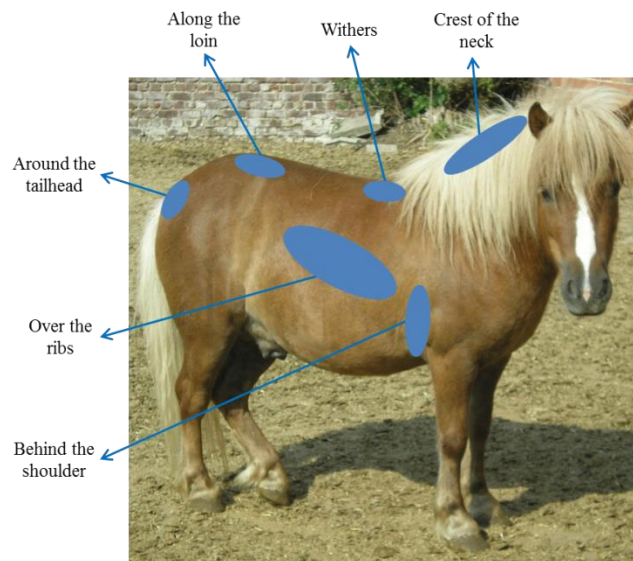
Figure 1.1: The fat deposition at six areas used to estimate the body condition score in equines according to the Henneke et al. (1983) system.

According to the body condition scoring system developed by Henneke et al. (1983), horses with a BCS of 8 (fat) or 9 (extremely fat) can be defined as obese. Animals with a BCS of 7 are considered to be overweight. Care should be taken when using body condition scoring systems, as these methods were originally developed by the food industry for the evaluation of superficial 'flesh' in meat producing animals (e.g. cows) (Jefferies, 1961), and were not intended for the evaluation of fat alone. Body condition scoring is a subjective measure of adiposity. Therefore, the reliability and repeatability of this measure is dependent on the within and between variation of the scorers. In a study performed in 56 horses, with the BCS estimated by 5 experienced individuals on 2 different time points, the scorers' ability to detect changes in adiposity over time was relatively poor. The coefficient of determination was less than 10% for most of the scorers (3 out of 5) (Mottet et al., 2009).

In free-living equines, body condition can vary seasonally. During summer months, when high quality forages are available, an increase in appetite and metabolic rate results in fat deposition (Arnold et al., 2006). During winter months, decreased appetite and lowered metabolic rate (hypometabolism) can adapt equines through an increased metabolic efficiency to survive under these seasonal harsh circumstances (McGregor-Argo, 2009; Dugdale et al., 2011c; Brinkmann et al., 2012). Endogenous circannual mechanisms to suppress winter weight gain, however, are insufficient to prevent the development of obesity in ad libitum fed ponies (Dugdale et al., 2011c). Therefore, in pony breeds and obesity-prone horse breeds, body condition score will be high throughout the entire year when there is a year-round intake of high quality forages.

An exponential relationship exists between BCS and body fat percentage in equines (Martin-Rosset et al., 2008; Dugdale et al., 2012). As a result of this nonlinear relationship, a reduction in the sensitivity of BCS is suggested when the BCS exceeds 7/9 (Dugdale et al., 2012). This implicates that it's impossible to estimate the exact percentage of body fat in equines with a BCS of 7 or higher, but it can be supposed that horses exceeding a BCS of 7 presumably will have a higher percentage of body fat compared with horses with a BCS lower than 7.

Becvarova and Pleasant (2012) developed an equation to predict optimal body weight in light breed horses based on BCS. This formula can be used to estimate the amount of weight loss to reach optimal body weight. Depending on the type of horse, each incremental change in BCS represents 18 to 27 kg:

$$\text{Predicted optimal body weight (kg)} =$$
$$\text{starting body weight (kg)} - ([\text{starting BCS} - \text{desired BCS}] \times 22.5\text{kg})$$

This equation has not been further used in scientific research, due to low accuracy, although it can be a starting point in clinical settings for the calculation of weight loss rations in overweight and obese horses. This equation is not suitable for use in ponies.

Regional condition scores have also been described. The **Cresty Neck Score** (CNS) was developed by Carter and co-workers (2009a). This is a regional scoring system with values between 0 (no visual appearance of a crest) and 5 (crest is so large that it permanently drops

to one side). Nuchal fat, however, is a poor predictor of total body white adipose tissue in mature Welsh Mountain pony mares according to Dugdale et al. (2011a).

Ultrasound measurement at different parts of the body is a non-invasive method which has been used to evaluate the thickness of subcutaneous fat deposition, but also for the estimation of total body fat as described in 1976 by Westervelt and colleagues. They carried out four different experiments using ultrasound measurements to predict total body fat and to evaluate the influence of exercise and energy intake. The following equations for the determination between rump fat (x, cm) and extractable fat (y,%), respectively in horses and ponies, y = 8.64 + 4.70x and y = 3.83 + 5.58x, were developed. Several other authors have used ultrasound measurements for monitoring subcutaneous fat depth during weight loss trials (Gordon et al., 2009; Dugdale et al., 2010; Dugdale et al., 2011c; Argo et al., 2012). Weight loss, however, is not necessarily accompanied with a decrease in fat depth measurements, as was evidenced in a study of Argo and co-workers (2012). Although there was a weight loss of 7.2% and a fat mass loss of 19.3%, rib and rump fat depth increased. Environmental conditions, such as cold temperatures, probably were responsible for a re-sequestering of fat in those subcutaneous areas. This indicates that ultrasound measurements can be a useful additional tool in monitoring weight loss, but they have to be combined and evaluated with other measurements to completely understand what happens in the equine body during weight loss.

Morphometric measurements are a good addition for the determination of equine body fat distribution as it can give an estimation of regional adiposity. Rump width, belly girth, heart girth, and neck circumference at mid-crest are measurements used in the evaluation of regional fat deposition in horses and ponies (Frank et al., 2006; Carter et al., 2009a; Dugdale et al., 2010; Argo et al., 2012). When using belly girth, one must keep in mind that this measurement can be an indication of gut fill and/or fat deposition in the abdominal area. When belly girth decreases during weight loss, this can be an indication of a decreased gut fill and/or a decrease in abdominal fat. Evaluating differences in neck circumference in smaller pony breeds can be difficult as angle of head position and degree of muscle tension can have a big influence on the measurement outcome, even when the measuring points are clearly defined.

Combined use of ultrasound and morphometric measurements can give an estimation of adipose tissue distribution in equines, however, inter-individual variation in body

conformation is high. Therefore, the ideal equation using ultrasound and morphometric indices to predict body mass and body fat percentage in every type of equine has not been developed yet, and probably, will not ever be formulated. However, in the past, there have been some efforts to predict body weight based on morphometric measurements in different types of equines.

Caroll and Huntington (1988) developed an equation to predict body weight based on the heart girth (measured immediately posterior of elbow following respiratory expiration) and length of the horse (from point of the shoulder to the tuber ischii):

$$\text{Weight (kg)} = \frac{[\text{girth}^2 \text{ x length (cm)}]}{Y}$$

With Y = 11900

A similar equation was developed for predicting live weight in adult Moroccan working donkeys (Pearson and Ouassat, 1996):

$$\text{Live weight (kg)} = \frac{[\text{heart girth (cm)}^{2.12} \text{ x length (cm)}^{0.688}]}{380}$$

Measurements of body condition. More recently, an objective determination of body composition has been validated in mature Welsh Mountain pony mares (Dugdale et al., 2011b). By using the **deuterium oxide dilution technique**, body lean mass and fat mass percentages can be calculated. In the validation study, total body water was only slightly overestimated (0.79%), and total body lipid slightly underestimated (1.78%), indicating that this is a reliable method for the estimation of body composition in mature Welsh Mountain pony mares. A formula has been developed to calculate percentage body fat from the percentage total body water (Dugdale et al., 2011b):

$$\text{percentage body fat} = 100 - (\text{percentage total body water}/0.732)$$

The value '0.732' is the hydration factor for lean tissues as suggested by Pace and Tathburn (1945). This hydration factor is applicable for normally hydrated animals. In dehydrated animals, however, total body water decreases, and thus increases the percentage total body fat in dehydrated animals. This can give false results of animals having a high percentage of body fat, whereas in reality, they were low in total body water. Variations in lean tissue hydration will bias total body fas mass values calculated from total body water,

giving problems to estimate total body fat mass in animals at the extremes of the BCS scale. Dugdale and colleagues (2011b) reported even a negative total body fat mass value in the leanest animal (BCS = 1.25) involved in their validation trial. Animals with BCS > 7/9 were not included in their validation study. The deuterium oxide dilution technique is very useful in the follow-up of the composition of the weight loss during weight loss studies in normally hydrated animals, although, no information is available about the region of adipose tissue loss.

Other techniques to measure equine body composition such as **dual X-ray** (Grier, 1996) and **bioelectrical impedance analysis** are also described but are rarely used in practice (Forro et al., 2000; Gee et al., 2003; Van der Aa Kuhle et al., 2008).

Although the use of the above described methods is widespread, both in the field and in equine scientific research, most of them are not validated, except for the deuterium oxide dilution technique that was only validated in mature Welsh Mountain pony mares (Dugdale et al, 2011b).

1.3 Prevalence

Being overweight or obese is a **growing problem** in today's society. These conditions are prevalent among **both humans and animals**. Global burden of people suffering due to overnutrition exceeds that due to undernutrition (Rosen and Spiegelman, 2014). In the United States, results from the 2009–2010 National Health and Nutrition Examination Survey (NHANES) indicate that 36% of adults and 17% of youth are obese (Ogden et al., 2012). If no remedial actions are taken, it has been predicted that more than 44% of people in the United States could be obese by the year 2030 (Lavizzo-Mourey and Levi, 2002).

In companion animals, 25 to 40% of adult cats are overweight or obese (Laflamme, 2006). In Western countries, obesity has been described as the most common nutritional disease in dogs, with a reported obesity prevalence of up to 44% (Zoran, 2010).

Equine obesity has been widely reported. Thatcher and co-workers evaluated a subpopulation of 300 mature Light Breed horses in Virginia (Thatcher et al., 2007). Thirty two % of the horses were overweight (BCS 6.5-7/9; BCS according to Henneke et al., (1983) and 19% were obese (BCS 7.5-9/9). In a study of 319 pleasure riding horses in Scotland (Wyse et al., 2008), 45% of the horses were classified as obese (BCS 5-6/6 according to Webb and Weaver (1979)). In other UK and US surveys in mixed breed ponies and horses

performing exercise at different intensity levels (from no exercise to competition), combined overweight and obesity percentages varied between 41.4 and 62% (Pratt-Phillips et al., 2010; Harker et al., 2011; Robin et al., 2014) indicating the importance of overweight and obesity in the modern horse industry, especially in leisure horses. Equine athletes performing exercise at high intensity levels, such as racehorses, will probably not suffer from this disorder.

There are several potential reasons for the increased incidence of human and equine obesity as obesity is the result of both genetic (genetic susceptibility) and environmental factors (increased availability of high-energy feeds, and a reduced need for physical activity in modern society) (Friedman and Halaas, 1998; Kopelman, 2000; Johnson et al., 2013).

1.3.1 Genetic factors

Several genetic causes of weight gain have been described in humans (such as alterations in leptin receptor, presence/absence of fat mass and obesity associated gene, gene variant of insulin-induced gene 2) (Bessesen, 2008). Ponies and some horse breeds, such as Morgan, Paso Fino, Arabian, Andalusian horse, and Quarter Horse are genetically predisposed to the development of EMS. In a study of Giles and colleagues (2014) in outdoor living (at least six hours a day) domestic horses and ponies, breed was the risk factor most strongly associated with obesity. Genetically EMS-prone horses are often referred to as "**easy keepers**" (Frank, 2009) or "good doers" (Frank et al., 2010). As obesity is an important component of EMS, these equines probably will have an increased predisposition to the development of overweight and obesity. However, environmental, nutritional, and gut microbiome influences always need to be taken into account, as it is difficult to divide the influence of these factors from the genetic load of an equid on the final development of overweight and obesity.

Breed-related differences in lipid profiles, glucose and insulin dynamics have been shown in equines (Robie et al., 1975; Rijnen et al., 2003; Annandale et al., 2004; Firshman et al., 2008; Bamford et al., 2013). Insulin resistance seems to be a biological adapation that allows animals to store fat in preparation for hibernation, or possible forage shortage especially in herbivorous species depending on grassland availability (Johnson et al., 2013). Native pony and horse breeds originating from temperate latitudes seem to be adapted to survive under these harsh circumstances (Dugdale et al., 2011c). This could be attributed to an increased metabolic efficiency, or "**thrifty genes**", which was described in humans with diabetes mellitus being exeptionally efficient in the intake and/or utilization of food (Neel, 1962).

Under 'modern' circumstances, with a year-round access to highly fermentable forages and concentrates, a shelter and minimal work, these equines are predisposed to become obese.

1.3.2 Environmental factors

In humans, the range of body mass index (BMI) of a population varies significantly according to the stage of economic transition and associated industrialization of a country (Kopelman, 2000). It is not known if this also accounts for the incidence of overweight and obesity in horses. Johnson (2009) stated that attractive advertising and free advice provided by the influential equine food industry can make horse owners to buy energy-dense rations for their herd. Nowadays, the management of horses is in contrast to the natural/feral conditions where equines spent 12 to 16h (Prache et al., 1998) or even up to 20h a day (Ödberg and Francis-Smith, 1976) foraging and eating. Many horses are currently confined to stables the entire day with little to no exercise, or are put on lush pastures with grassland species genetically improved to suit the needs of the food animal industry (Johnson et al., 2009). Giles and co-workers, however, found that supplementary feed in equines living outdoors for ≥ 6h per day had limited to no effect on obesity levels (Giles et al., 2014).

1.4 Reasons for increased incidence of overweight and obesity in equines

An important issue in the problem of overweight and obesity discussion is the **underrecognition** of animals being overweight or obese by both veterinary clinicians and horse owners (Johnson et al., 2009). In a study of 319 pleasure riding horses in Scotland, the horse's body condition was generally underestimated by the owners. Only 50% of the owners of fat horses estimated the BCS of their horse correctly (Wyse et al., 2008). Many horse owners tend to prefer their horse to be in a 'healthy fleshy' condition, not knowing the impact of the extra weight.

Another important cause of the increased overweight and obesity prevalence in the modern horse industry of more developed industrial countries is the high numbers of horses kept for **recreation purposes** and as **pet animals** (e.g. Shetland pony as 'grassland manager'), with many of them undertaking little to no physical exercise. Unfortunately, many horse owners know little about the physical and nutritional needs of horses.

This leads to a third potential reason for the increased incidence of overweight and obesity, which is the use of **inappropriate rations**. Equines, particularly the pleasure/leisure horse and ponies, nowadays are often fed rations that are largely different from their natural

nutritional availabilities. Firstly, a broad spectrum of concentrates and cereals could stimulate uneducated horse owners to purchase energy-rich rations, which are too energy-dense for leisure horses and ponies performing little to no exercise. Secondly, forage sources, which are supposed to be the major part of ration, are often genetically improved to suit the needs of production animals (e.g. high yielding cows) (Johnson et al., 2009), making them less appropiate for the inactive horse. Under natural circumstances and in the wild, horses have to walk large distances to find food (Prache et al., 1998). Many horses nowadays are confined to stables sometimes for the entire day, receiving once or twice a day their meal, other horses are kept on lush pastures the entire day, having the opportunity to eat large amounts of grass, and so exceed their required daily energy intake. Every horse is different and needs individual feeding advice (Harris, 2011), paying attention to the following parameters, such as age, health and temperament of the horse, the amount and type of work the horse is subjected to, environmental conditions, metabolic and digestive efficiencies. One should keep in mind that obesity in animals in many cases is a consequence of management decisions made by the animal owners.

Some pony and horse breeds are **genetically predisposed** to the development of EMS and are often referred to as 'easy keepers' (Frank, 2009). These equines require less calories to maintain body condition. Increased attention is required when placing these horses on lush pastures. Insulin sensitivity differences have been identified between Quarter Horses and Belgian horses (Annandale et al., 2004; Firshman et al., 2008), Warmblood horses and ponies (Rijnen et al., 2003), and mixed-breed ponies compared to Standardbreds (Bamford et al., 2014)

Dugdale and co-workers (2011c) have evidenced that under domestic conditions, with an ad libitum access to a diet of constant quality, **seasonal physiological mechanisms** may be **insufficient** to prevent the excessive accumulation of body fat in at least Welsh Mountain pony mares, and probably by extension for a lot of (inactive) pleasure/leisure equines.

Horse temperament and hierarchial place also seems to influence the incidence of obesity. In a study of Giles and co-workers (2014), more dominant, more aggressive, and more interactive individuals generally had a higher BCS. Additionally, a greater proportion of the dominant individuals fell into the obese category (BCS $\geq$ 7).

1.5 Functions of body fat

Originally, the main function of adipose tissue was believed to be the **storage of excess energy** as triglycerides, and its release according to need in the form of fatty acids (Fisher-Posovszky et al., 2007). Indeed, adipose tissue is an important regulator of energy balance and nutritional homeostasis (Rosen and Spiegelman, 2014).

Aside from the energy storage site, adipose tissue can act as a **thermal insulator** (Trayhurn, 2007) and has important **mechanical properties**. It protects delicate organs from trauma, such as the eyes and kidneys in humans and animals. Adipose tissue also cushions body parts exposed to high levels of mechanical stress, such as the heel in humans (Rosen and Spiegelman, 2014), toe pads in cats and dogs (Budras et al., 2007), and the digital cushion within the hoof in equines (Budras et al., 2008).

Nowadays, with the increased knowledge of adipose tissue composition, adipose tissue can be recognized as the largest **endocrine organ** in the body (See 1.7), certainly in overweight and obese humans (Trayhurn, 2007). Adipose tissue interacts with other organs through the production and release of adipokines (Trujillo and Scherer, 2006).

1.6 Composition of adipose tissue

Human adipose tissue can be divided into brown and white adipose tissue (Hahn and Novak, 1975). As mentioned before, white adipose tissue is more than just an energy storage site and is now recognized as a highly active metabolic and endocrine organ (Kershaw and Flier, 2004; Fantuzzi, 2005) comprising **different cell types**, such as adipocytes, pre-adipocytes, endothelial cells, fibroblasts, mesenchymal cells, and immune cells (macrophages, lymphocytes, natural killer cells, mast cells) (Maury and Brichard, 2010) (Figure 1.2). Resident macrophages are present as part of the innate immune system (Ozinsky et al., 2000).
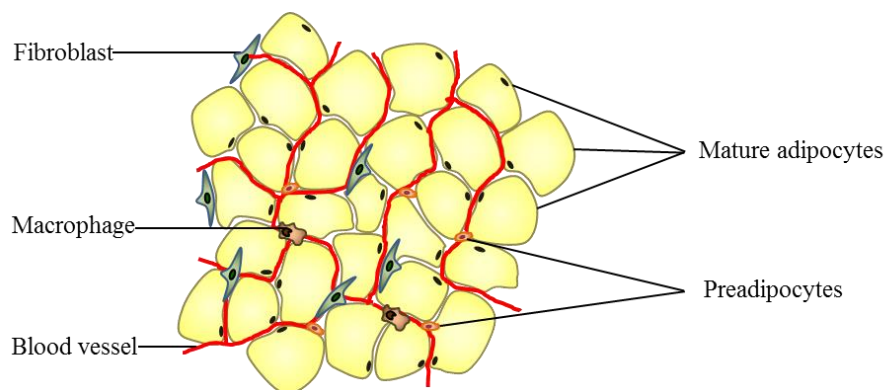

Figure 1.2: Composition of human adipose tissue. (own picture)

The variety of cells are capable of secreting proteins (also known as **adipo(cyto)kines**) involved in the regulation of energy, as well as neuroendocrine, autonomic, and immune metabolism (Radin et al., 2009) (Figure 1.3). Mature, lipid-laden adipocytes are believed to make up only 20-40% of the cellular content of a fat pad in humans, although they account for over 90% of fat pad volume (Rosen and Spiegelman, 2014).

To the author's knowledge, research into the composition of equine adipose tissue is limited to equine adipose tissue-derived mesenchymal stem cells, which are used in stem cell therapy for orthopaedic and musculoskeletal diseases (Frisbie et al., 2010; Watts, 2014). Information on the different cell types residing in equine adipose tissue is lacking. Therefore, further research in this area could give more information about the adipose tissue composition in equines and if the development of obesity in this species will also be associated with an increase in adipokine production.
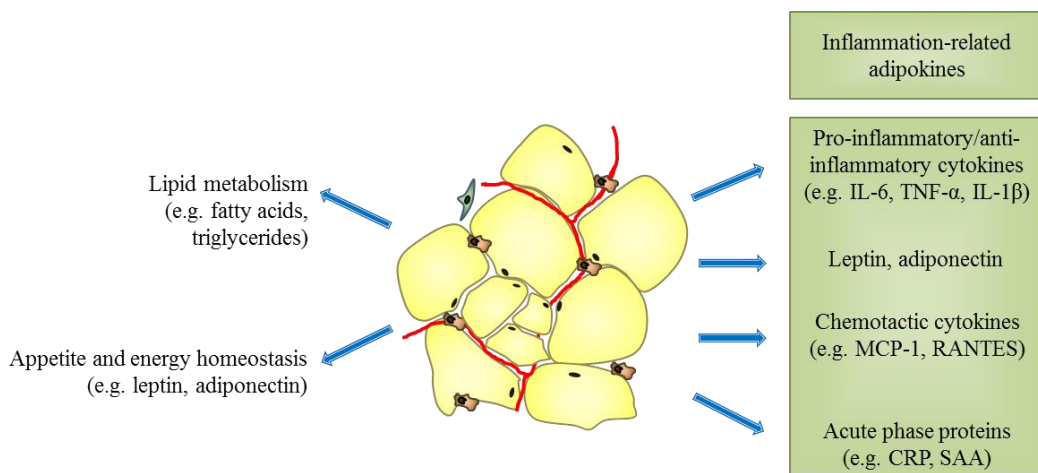
Figure 1.3: Adipo(cyto)kines expressed and secreted by white adipose tissue (based on studies in humans and other species) (Abbreviations: IL-6, interleukin-6; TNF-α, tumor necrosis factor α; MCP-1, monocyte chemotactic protein 1; CCL5, chemokine ligand 5; CRP, C-reactive protein; SAA, serum amyloid A). (own picture)

Leptin and adiponectin are adipokines expressed predominantly or exclusively by adipose tissue. **Leptin** is a 167 amino acid protein that is encoded by the *ob* gene and is generally known as the 'satiety hormone' (Heini et al., 1998). The leptin receptor (Ob-R) is expressed in the satiety centre of the hypothalamus, but splice variants are located throughout the entire body. This explains the involvement of leptin in the regulation of multiple physiological processes in cats, dogs and horses (Radin et al., 2009). Binding of leptin on its receptor results in suppression of appetite and increased energy expenditure in humans (Houseknecht and Portocerraro, 1998). In the absence of leptin or in leptin resistant state, appetite control is lost

(Friedman, 2009). Fasting generally leads to fall in serum leptin levels in humans (Boden et al., 1996). Leptin concentrations in equines decrease after periods of feed restriction (Van Weyenberg et al., 2008) and increase after meal ingestion (Cartmill et al., 2005). It is suggested that adipose tissue leptin production is driven by insulin as insulin infusion increases plasma leptin concentrations in horses (Cartmill et al., 2005). In humans, there is a strong positive correlation between serum leptin and amount of body fat (Caro et al., 1996). In horses, however, leptin levels are not always positively associated with BCS. Several authors found varying leptin levels in horses with similar BCS (Gentry et al., 2002; Cartmill et al., 2003; Van Weyenberg et al., 2008).

In humans, leptin has pro-inflammatory properties, such as controlling tumor necrosis factor alpha (TNF-α) production, macrophage activation, triggering of nitric oxide-synthase synthesis and oxygen-derivative molecular species production and monocyte chemotactic protein-1 (MCP-1) expression (Pérez de Heredia et al., 2012). Furthermore, pro-inflammatory cytokines TNF-α and interleukin-6 (IL-6) are capable of stimulating adipocyte leptin production, and thereby sustaining the pro-inflammatory activity.

Adiponectin is an anti-inflammatory cytokine with decreased blood levels in obesity (Fantuzzi, 2005). This adipokine has insulin sensitizing and vascular-protective effects. The increase in insulin sensitivity could be explained by the stimulation of adenosine monophosphate-activated protein kinase (AMPK), which increases glucose uptake by promoting translocation of the glucose-transporter 4 (GLUT-4) to the cell surface (Radin et al., 2009). In humans, adiponectin could also reduce inflammatory response induced by TNF-α and IL-6. *In vitro* human studies have indicated a decreased TNF-α production and macrophage activity in macrophages treated with adiponectin (Suganami et al., 2005). Adiponectin expression in human adipocytes is reduced by TNF-α and IL-6 (Bruun et al., 2003), which could explain the lowered blood adiponectin levels in obese individuals. Blood adiponectin levels in horses are negatively correlated with fat mass, percent body fat, BCS, serum insulin activity, and leptin levels (Kearns et al., 2006; Gordon et al., 2007; Wooldridge et al., 2012). Lowered plasma adiponectin levels were demonstrated in previously laminitic animals (Wray et al., 2013).

There are also a lot of other adipokines expressed as well in adipose tissue as in a variety of different tissues. A detailed list of all cytokines expressed in and released from the different tissues is beyond the scope of this introduction. Therefore, only the most important ones will

be mentioned. **Resistin** is a pro-inflammatory cytokine which stimulates pro-inflammatory cytokine secretion by macrophages (Radin et al., 2009). **Interleukin-1, IL-6, TNF-α, interferon-γ, chemokine ligand 5 (CCL5)** and **MCP-1** are cytokines with a pro-inflammatory profile. **Interleukin-10** on the other hand has anti-inflammatory properties (Kershaw and Flier, 2004; Fantuzzi, 2005, Radin et al., 2009).

Macrophages and pro-inflammatory cytokines are essential for adipose tissue remodelling and adipocyte differentiation. Elevation of pro-inflammatory cytokines in humans increases energy expenditure and therefore acts to prevent obesity (Ye and McGuinness, 2013). Interleukin-1 and IL-6 reduce food intake and prevent hyperphagia in mice and rats (Wallenius et al., 2002; Anforth et al., 1998). Inflammatory cytokines may serve as an anti-obesity signal by modifying both energy intake and energy expenditure. They function as an important link between the peripheral tissues and the central nervous system in the control of energy balance in humans and rodents (Ye and Keller, 2010). When there is a disruption in this delicate relation, however, energy expenditure will be interrupted and fat will accumulate in the body causing deleterious effects such as inflammation and oxidative stress. These are results from research in humans and laboratory animals. Therefore, this information cannot simply be extrapolated to equines.

1.7 Negative effects of obesity

1.7.1 Expanding adipose tissue

Adipose tissue increase in human obesity is associated with **adipocyte hyperplasia** (recruiting new adipocytes from the resident pool of progenitors) and **adipocyte hypertrophy** (increasing size of individual cells) (Rosen and Spiegelman, 2014) (Figure 1.4). Enlarged adipocytes become hypoxic when they outgrow their blood supply (increased cell volume and a lowered blood supply per adipocyte). This eventually leads to cellular stress (Goossens, 2008), adipocyte cell death, and recruitment of immune cells, such as macrophages, to clean up the debris of that adipocyte. Cinti and colleagues (2005) demonstrated that > 90% of all macrophages in white adipose tissue of obese mice and humans were localized around dead adipocytes, forming **crown-like structures** (CLS).

Obese human adipocytes activate the oxygen-sensitive transcription factor hypoxia-induced factor 1α (HIF-1α) (Krishman et al., 2012). This leads to metabolic dysfunction, such as reduction of adiponectin (Jiang et al., 2013) and promotion of inflammation in mice

(Halberg et al., 2009). Hypoxic adipocytes also release chemokines, such as monocyte chemotactic proteins (MCP), that attracts new macrophages to expanding human and mice adipose tissue (Cinti et al., 2005; Kamei et al., 2006; Strissel et al., 2007; Murano et al., 2008), and CCL5 that attracts various leukocytes (Skurk et al., 2009).
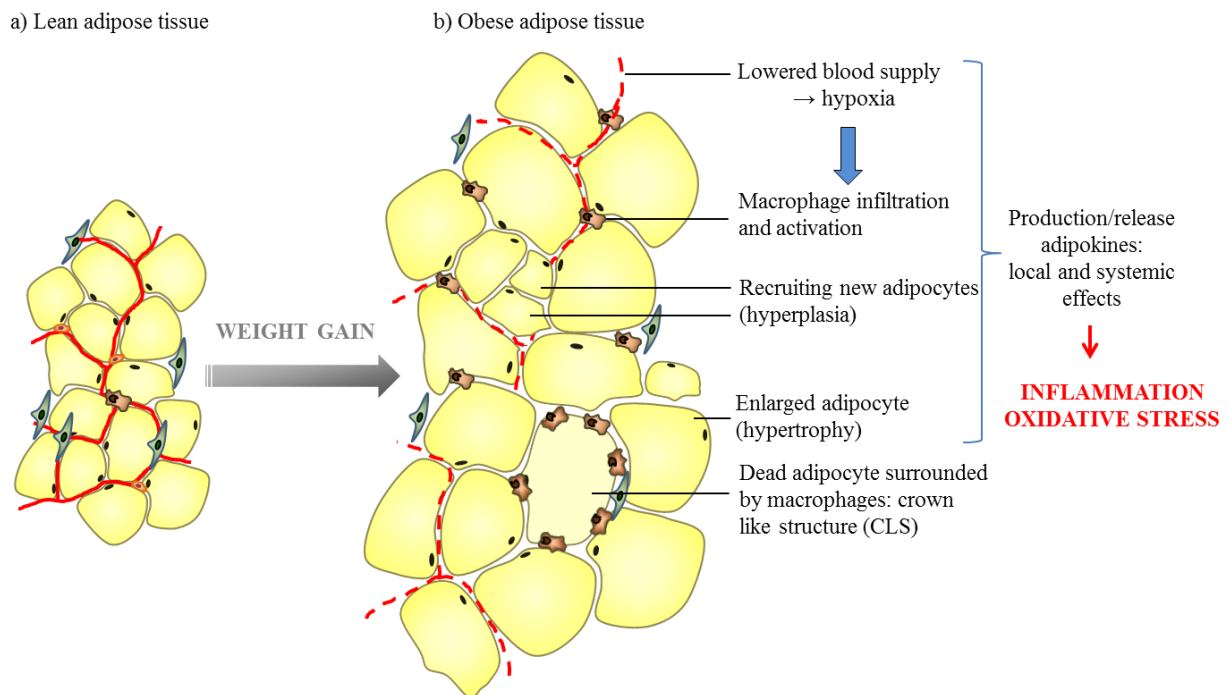
Figure 1.4: Adipose tissue in the lean and obese state (situation in humans and laboratory animals). (own picture)

Phenotypically, two types of macrophages exist: M1 or classically activated and M2 or alternatively activated macrophages. **M1 macrophages** tend to have a more pro-inflammatory phenotype by expressing TNF-α, IL-6 and IL-1β upon lipopolysaccharide or interferon gamma stimulation, and generate reactive oxygen species (ROS) (nitric oxide (NO) and reactive oxygen intermediates) (Mantovani et al., 2004). **M2 macrophages** are responsible for tissue remodelling, wound healing, secreting anti-inflammatory cytokines (IL-10 and IL-1 receptor antagonist) (Lumeng et al., 2007a), and expression of peroxisome proliferator-activated receptor gamma (PPARγ) (Stienstra et al., 2008). They also participate in the blockade of inflammatory responses (Gordon and Taylor, 2005). In lean mice, M2 macrophages dominate the adipose tissue resident population. Diet induced obesity leads to a shift in the activation state of adipose tissue macrophages from a M2-polarized stage in lean

animals that may protect adipocytes from inflammation to a M1 pro-inflammatory state that contributes to insulin resistance (Lumeng et al., 2007a; Oh et al., 2012). There is a major shift in the M1/M2 ratio favouring a pro-inflammatory state under conditions of adipose tissue hypoxia or when influenced by recruited pro-inflammatory macrophages (Lumeng et al., 2007a; Lumeng et al., 2007b). Due to the imbalance in cytokine production in favour of the pro-inflammatory cytokines in obese humans, obesity is considered as a **state of chronic inflammation** (Dandona et al., 2004).

The infiltrated immune cells enhance cytokine production and secretion through a paracrine interaction between adipocytes and macrophages, as evidenced by *in vitro* research in mice adipocytes (Suganami et al., 2005). A study by Fain and colleagues (2004) in obese women revealed that over 90% of the adipokine release by AT, except for adiponectin and leptin, could be attributed to non-fat cells. Hotamisligil and co-workers (1993) first demonstrated that adipocytes constitutively express the pro-inflammatory cytokine (TNF-α) and that its expression is increased in obese mice and rats. In obese humans, TNF receptor concentration is also increased (Mantzoros et al., 1997). Therefore, an important consequence of increased fat mass is a dysregulation of production and secretion of adipokines that influence glycaemic control, inflammation, and cardiovascular function. In human medicine, increased circulating levels of specific inflammatory proteins have been useful to predict future metabolic disease (e.g. elevated levels of C-reactive protein have been associated with increased cardiovascular risk) (Clearfield, 2005). A similar protein to predict the risk for metabolic disease in horses would be of great value, but has not been detected yet (Suagee et al., 2012).

Equine obesity has been suggested to be associated with inflammation by some researchers (Vick et al., 2007; Adams et al., 2009; Treiber et al., 2009; Suagee et al., 2011; Suagee et al., 2013; Tadros et al., 2013) but not by others (Holbrook et al., 2012; Banse, 2013; Suagee et al., 2013). In two studies, obesity was characterized by increased systemic concentrations of proinflammatory TNF proteins, leukocyte TNF mRNA and interleukin-1β (IL-1β) (Vick et al., 2007), and higher acute phase protein amyloid A plasma concentrations (Suagee et al., 2013). Increased levels of TNF-α protein have been found in fat old horses (fat % 22.7, BCS 7.25) (Table 1.1), which reduced significantly by reducing body mass and fat (fat % 17.3, BCS 5) (Adams et al., 2009). Elevated serum TNF levels were also found in obese, insulin resistant pony mares compared with their lean and insulin-resistant counterparts (Treiber et al., 2009). In slight overweight horses (BCS 6.3), higher plasma TNF and IL-6

concentrations were found after acute hyperinsulinemia induced by exogenous insulin infusion compared to horses infused with saline solution, suggesting insulin to play an important role in the production of inflammatory cytokines. This is also one of the mechanisms to explain the increased risk for laminitis in horses with hyperinsulemia and/or insulin resistance (Suagee et al., 2011). Altered inflammatory response has been found in horses with EMS as a prolonged cytokine (IL-6, IL-8, IL-10, and TNF-α) gene expression was seen after lipopolysaccharide infusion (Tadros et al., 2013).

In contrast, Holbrook and co-workers (2012) suggested that cytokine-mediated inflammation in horses did not increase in direct response to obesity or insulin resistance, quite different as what is seen in humans, as the peripheral bood cells of obese hyperinsulinemic horses showed decreased endogenous pro-inflammatory cytokine gene expression (IL-1 and IL-6) and comparable cytokine response following immune stimulation as the control horses with significantly lower body mass index and fasting insulin concentrations. Banse (2013) also found no increased systemic inflammation (by means of serum amyloid A and TNF-α) in obesity. In an other study performed in 110 horses of varying body condition score (2 to 9/9), no correlations were found between TNF, IL-1β, and IL-6 concentrations and BCS (Suagee et al., 2013). Age and gender related differences in inflammatory cytokine profile in horses, on the other hand, were prevelant as higher plasma TNF and IL-6 concentrations were found in females compared to males. Interleukin-6 concentrations were positively correlated with age (Suagee et al., 2013). Further research into the specific impact of equine obesity on inflammation and vice versa would give further information on their possible relationship. One must also consider here the important role of insulin, insulin resistance, and age as possible confounding factors.

Obesity in humans has also been associated with increased markers of **oxidative stress** (Couillard et al., 2005; Pou et al., 2007; Vincent et al., 2007). Oxidative stress can be described as a disturbance in prooxidant/antioxidant balance in favour of the former, leading to potential damage (Sies, 1997) (Figure 1.5), or as a serious imbalance between the production of reactive species and antioxidant defence (Halliwell and Whiteman, 2004). Oxidants are usually generated by metabolic enzymes, inflammatory cells, and mitochondrial electron leakage (Kirschvink et al., 2008). In a state of overnutrition, excess glucose (by Krebs cycle) and fatty acids (by beta-oxidation and Krebs cycle) can finally generate ROS (Brownlee, 2005). In adipose tissue of obese mice, overexpression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunints, an enzyme that converts molecular

oxygen to its superoxide radical, has been evidenced. This potentially contributes to the increased levels of oxidative stress in adipose tissue (Furakawa et al., 2004).

Human overweight and obese juveniles tend to have higher blood levels of advanced oxidation protein products (AOPP), a biomarker of oxidative stress, compared to lean controls (Krystek-Korpacka et al., 2008). Body mass reduction lowers oxidative pressure by means of lowered AOPP (Krystek-Korpacka et al., 2008), advanced glycation end products (AGE) (Gugliucci et al., 2009), and thiobarbituric reactive substances (TBARS)/malondialdehyde (MDA) (Dandona et al., 2001; Kelishidaki et al., 2008).
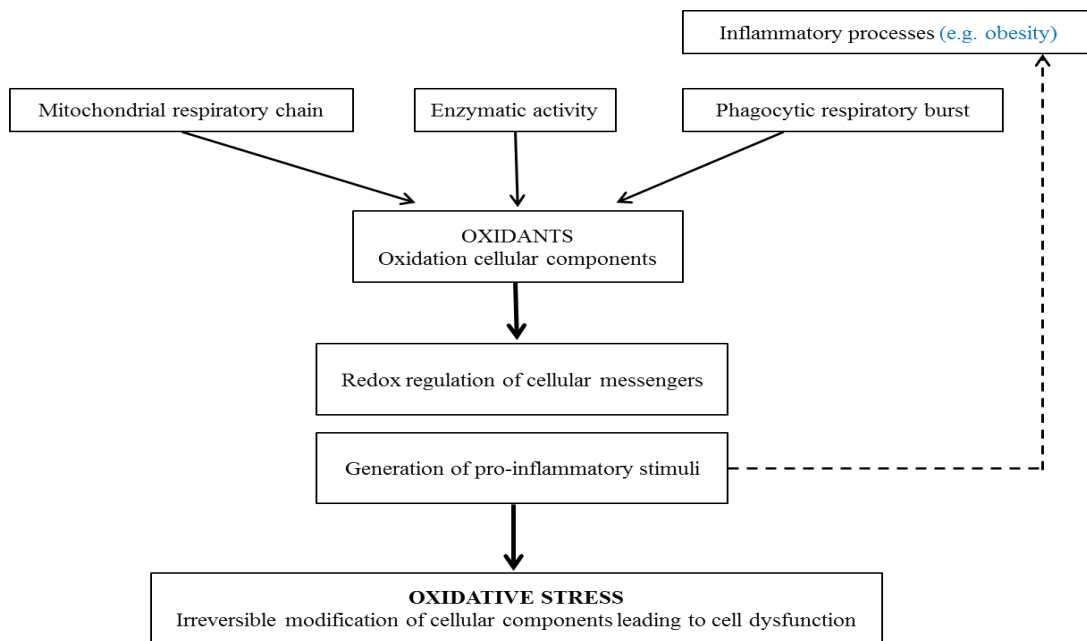
Figure 1.5: Oxidative stress mechamism and the potential influence of obesity (Adapted from Kirschvink et al., 2008).

In equines, little information is available about the relationship between oxidative stress and obesity. Pleasant and co-workers (2013) found lowered antioxidant capacity, by means of decreased erythrocyte glutathione peroxidase activity, in obese light breed horses compared to optimal condition light breed horses. No information, however, was available on the diet of these horses. There was also a lowered antioxidant status in old horses (17 to 20 years) compared with younger horses (4 to 8 years), which further indicates the importance of age in oxidant/antioxidant balance next to its importance in the inflammatory profile in equines (Suagee et al., 2013). Holbrook and co-workers (2002) found significantly higher oxidative burst activity or increased neutrophil oxidative stress in obese hyperinsulinemic horses compared to normal weight normoinsulinemic horses. This could indicate that insulin may be

an important regulator of equine neutrophil function (upregulating oxidative burst activity), as has already been evidenced in humans (Walrand et al., 2004) and laboratory animals (Okouchi et al., 2002). Banse (2013) on the other hand found an increased anti-oxidant capacity in obese (BCS≥7) light breed horses, together with increased skeletal muscle oxidative stress (lipid peroxidation). To the author's knowledge, no information is available on the effect of weight loss on oxidative stress parameters in equines. More research in this field, with the inclusion of multiple oxidative stress related parameters, in equines during weight loss would give more valuable information about the oxidant/antioxidant balance in equines. So far, there is no consensus about the effect of obesity on oxidative stress in equines.

Oxidative stress has, however, been associated with several other disorders in the equines, such as recurrent airway obstruction, joint disease, neurological diseases, and perfusion related disorders (Kirschvink et al., 2002; Deaton et al., 2004; Dimock et al., 2000; Divers et al., 2006, Kirschvink et al., 2008). Disturbed perfusion at the level of the hoof perhaps also plays a role in the development of laminitis. Equine digital laminae have relatively limited superoxide dismutase (anti-oxidant) capacity, making this tissue more susceptible to oxidative damage (Loftus et al., 2007). Laminitis in horses has been associated with increased levels of advanced glycation products in the lamellar tissues (de Laat et al., 2012) and in the plasma (Valle et al., 2013). Increased advanced glycation product formation has been associated with increased oxidative pressure in humans and vice versa (Dalle-Donne et al., 2003). Given this positive relationship, increased systemic and/or AGE products in the laminitic horse could be a sign of increased oxidative stress.

1.7.2 Adipose tissue location

Adipose tissue is distributed throughout the entire body in different regions. Adipocytes from different anatomic locations may vary in their biology due to local influences on differentiation and gene expression, and may be considered "**mini-organs**" (Trujillo and Scherer, 2006). The distribution of adipose tissue influences the pathologic sequelae characterizing obesity, e.g. increased intra-abdominal adipose tissue is associated with increased diabetes risk and vascular disease in humans (Hajer et al., 2008). Because the venous drainage from omental tissue flows directly into the liver, the metabolic impact of interleukin-6 release from omental adipose tissue may be of particular importance. Interleukin-6 increases hepatic triglyceride secretion, and may, therefore, contribute to the

hypertriglyceridemia associated with visceral obesity in rats (Nonogaki et al., 1995). Montague and co-workers (1997) have shown that leptin mRNA was greater in subcutaneous than in omental human adipocytes. Subcutaneous fat depot is the major source of leptin in women (Van Harmelen et al., 1998).

In overweight horses, it has been shown that expression of glucose transporter was influenced by adipose tissue location in insulin sensitive and insulin resistant horses (Waller et al., 2011). It has also been suggested, although not scientifically proven, in equines that adipose tissue distributed specifically on the crest of the neck could indicate or contribute to hyperinsulinemia, IR, and/or an increased risk for laminitis (Carter et al., 2009a; Frank et al., 2010). Therefore, equine clinical interest in nuchal adipose tissue is increased (Carter et al., 2009a; Burns et al., 2010). Burns and co-workers found higher mRNA concentrations of the pro-inflammatory cytokine IL-1β and IL-6 mRNA expression in the nuchal ligament adipose tissue compared to the other adipose tissue depots sampled in that study. Limited research has been performed in this area, in particular in respect to the presence of macrophages and other immune cells in the equine adipose tissue. Further research in this area would give more information about the importance of these immune cells in the development of inflammation and other related disorders in equine obesity.

1.7.3 Gender differences

In humans, there are gender differences in adipose tissue distribution. Women tend to have higher percentages of body fat than men and tend to store adipose tissue preferably in the gluteal-femoral region, in contrast to the classic male pattern of obesity in which adipose tissue deposition is concentrated in the visceral and abdominal depots (Blaak, 2001). The existence of similar gender differences in horses are not described yet, however, stallions generally have a more pronounced crest compared to geldings and mares, partially depending on their training status.

1.7.4 Obesity associated conditions in horses

The most important co-morbidities of equine obesity are further summerized in Table 1.1. General and/or regional obesity together with insulin dysregulation, hypertriglyceridemia, and hyperleptineamia are the major features of the **Equine Metabolic Syndrome (EMS)** (Johnson, 2002; Geor, 2008; Frank and Tadros, 2014). The EMS clusters physiological alterations which are important for their association with the predisposition to laminitis.

Equine obesity has been suggested to be a predisposing factor for **laminitis** (Alford et al., 2001). Belknap and co-workers (2007) found significantly increased lamellar expression of the cytokines IL-1β, IL-6, and IL-8 in the developmental stages of laminitis. Obesity and the associated increase in adipokine activity may predispose equines to laminitis by increased laminar matrix metalloproteinase (MMP) expression (Clutterbuck et al., 2010). These molecules can cause lamellar basement membrane destruction and are therefore important in the development of laminitis. Equine obesity is often negatively associated with insulin sensitivity (Vick et al., 2007) and associated with an increased risk of hyperinsulinemia (Muno et al., 2009). **Insulin resistance** has been suggested as a predisposing factor for laminitis as ponies prone to laminitis have an insulin resistant phenotype (Treiber et al., 2006). Insulin enables vasodilation (Cleland et al., 2000), so insulin resistance may contribute to laminitis via restricting blood flow to the hoof. Insulin resistance may also play a role in the development of laminitis through promoting MMP activity (Asplin et al., 2007). More recently, it has been suggested that insulin resistance may play a role in the development of laminitis through stimulation of insulin-like growth factor 1 receptor (Burns et al., 2013). Prolonged hyperinsulinemia can induce laminitis in clinically normal ponies (Asplin et al., 2007) and therefore further emphasizes the association between insulin and laminitis. A relationship between obesity and insulin levels could be demonstrated in a study performed in 13 Arabian or Arabian cross geldings, in which diet-induced weight gain resulted in hyperinsulinemia and hyperleptinemia (Carter et al., 2009c), however, the potential for variable effects on glucose and insulin dynamics according to the nature of the diet cannot be ignored.

Table 1.1: Suggested obesity associated conditions in horses (with IR as possible contributing factor) (non-exhaustive list)

Disease/condition associations	Reference
Insulin resistance/Insulin dysregulation	Jeffcott et al., 1986; Freestone et al., 1992; Powell et al., 2002; Hoffman et al., 2003; Frank et al., 2006; Vick et al., 2007; Frank and Tadros, 2014.
Abnormal reproductive performance (e.g. extended interval between successive ovulations, persistent elevated blood levels of progesterone)	Henneke et al., 1984; Nagy et al., 1998; Fitzgerald et al., 2003; Sessions et al., 2004; Vick et al., 2006.
Age-related dysregulation of inflammatory cytokine production	Adams et al., 2009.
Benign (pedunculated) lipomas in mesenteric adipose tissue	Garcia-Seco et al., 2005.
Developmental orthopaedic disease (osteochondrosis dissecans)	Ralston, 1996; Harris et al., 2005; Lawrence and Pagan, 2005; Secombe and Lester, 2012.
Exercise intolerance (reduced athleticism)	Lawrence et al., 1992; Garlinghouse and Burrill, 1999; Kearns et al., 2002.
Hyperleptinemia	Cartmill et al., 2003; Frank et al., 2006.
Hyperlipemia	Jeffcot and Field, 1985; Watson et al., 1992; Reid and Mohammed, 1996.
Impaired thermoregulation (increased heat stress during hot weather)	Webb et al., 1989; Cymbaluk and Christison, 1990.
Laminitis	Coffman and Colles, 1983; Field and Jeffcott, 1989; Johnson, 2002; Frank, 2006; Treiber et al., 2006.
Lowered antioxidant capacity	Pleasant et al., 2013.
Pro-inflammatory state	Fontaine et al., 2001; Vick et al., 2007; Treiber et al., 2009.
Lowered blood adiponectin	Kearns et al., 2006; Gordon et al., 2007; Wooldridge et al., 2012.

1.8 Intervention strategies

Weight loss in both humans and animals can be achieved by different strategies (Figure 1.6), with dietary energy restriction and exercise being the most important ones in equine overweight and obesity (Table 1.2).

1.8.1 Dietary energy restriction

Energy accumulation can lead to elevated blood glucose and fatty acid levels. Their metabolites (e.g. diacylglycerol and reactive oxygen species) may activate inflammatory signalling pathways and transcription factors, such as IkBα kinase beta, nuclear factor kappa beta, and c-Jun N-terminal kinase 1. In case of overstimulation of these pathways, inflammatory cytokine expression will increase and a vicious cycle can be initiated. These events can be prevented by energy restriction in laboratory animals and humans (Ye and Keller, 2010). Suagee and co-workers (2011) demonstrated that geldings consuming a high glycaemic diet have increased pro-inflammatory serum TNF levels compared to geldings on a low glycaemic diet, suggesting that a high glycaemic diet might promote inflammation independent of obesity. Therefore, a specific diet should be designed for obese equines and equines with EMS to reduce the inflammatory impact of the diet. This implies that energy dense sweet feeds (commercial sweet feeds) need to be eliminated from the diet.
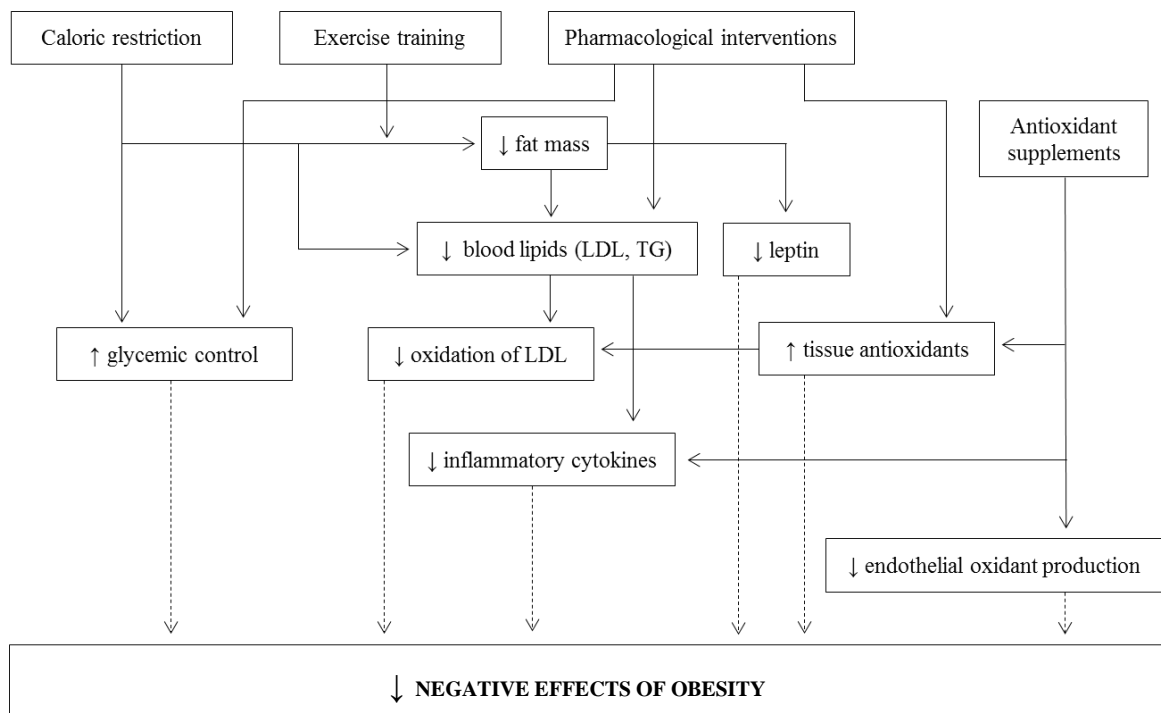

Figure 1.6: Possible intervention strategies to reduce negative effects of obesity. (Adapted from Vincent et al., 2007)

Table 1.2: Overview of weight loss trials in equines

Reference	number of animals	energy restriction	exercise	daily energy intake during weight loss period
Sticker et al., 1995	16	+	-	16.14 DE Mcal → 9.24 DE Mcal
Buff et al., 2006	15	+	-	restricted to 75% of ad lib hay consumption + oats mixture; energy content not known
Van Weyenberg et al., 2008	9	+	-	409 kJ NE/kg0.75 → 119 kJ NE/kg0.75
Gordon et al., 2009	23	+	+	control group: hay at 1% BM*1.96 DE Mcal/kg + supp at unknown% BM*3.48 DE Mcal/kg / diet: hay at 1% BM*1.96 DE Mcal/kg + supp at 0.5% BM* 1.98 DE Mcal/kg / diet + exercise: hay at 1% BM*1.96 DE Mcal/kg + supp at 0.5 or 0.3% BM* 1.98 DE Mcal/kg
Dugdale et al., 2010	5	+	-	1% BM as DMI*8.5 MJ/kg DM
Carter et al., 2010	12	-	+	23Mcal
Argo et al., 2012	16	+	-	1.25% BM as DMI: 0.115 DE MJ/kg BM / extension study: 1% BM as DMI
Ungru et al., 2012	15	+	+	8.4 or 7 MJ DE/100kg BM
Brinkmann et al., 2013	10	+	-	1st restriction: 12.5 ME MJ/100kg BM + supplement / 2nd restriction: 11 ME MJ/100kg BM + supplement
McGowan et al., 2013	12	+	-	1.25% of BM as DM hay + 25g/100kg supplement (with or without sc-FOS)
Schmengler et al., 2013	16	+	+	same study as Ungru et al. (2012) + supplement (L-carnitine/silicic acid at 2.6g/100 kg BM)
Potter et al., 2013	18	+	+	1.25% BM as DMI*2.0Mcal/kg DM
Chapter 5 and 6	18	+	-	control group: 0.17 DE MJ/kg BM (100% of iMERob) / moderate energy restricted: 0.14 DE MJ/kg BM (80% of iMERob) / severe energy restricted: 0.10 DE MJ/kg BM (% of MERob)

Abbreviations: DE, digestible energy; NE, net energy; BM, body mass; supp, supplement; DMI, daily dry matter intake; ME, metabolizable energy; iMERob, individual maintenance energy requirements to maintain stable obese body weight.

Pasture turnout has to be re-evaluated in equines on a weight loss diet. Restricted grazing could be a possible solution to reduce the energy intake from grass, although care should be taken as ponies can ingest between one half and two-thirds of their advised daily total DMI during a 3 hours pasture turnout if they are habituated to this procedure (Longland et al., 2011). Grazing muzzles appear to be an effective means of restricting pasture intake by ponies as there was an average reduction of 83 % in DMI between the muzzled ponies and the ponies without muzzles (Longland et al., 2011). One must also keep in mind that there has been a general underestimation of the DMI intake in ponies on pasture (2-2.5% of live weight) for a long time. In a study performed by Longland and co-workers (2011), estimated voluntary DMI ranged between 2.9 to 4.9 % of live weight in mature pony mares at pasture for 6 weeks. Also in horses, similar DMI intakes have been reported (Smith et al., 2007). Therefore, to have an exact estimation of energy intake in horses on a weight loss diet, it is recommended to restrict all pasture turnout.

Forage is preferably a late-cut mature hay which tends to have a lower energy content compared to early-cut hay with a high leaf to stem ratio (Geor and Harris, 2013). Straw (without cereal heads) can be gradually introduced in the diet to further decrease the energy content of the diet, although the risk for impaction is prevalent. To mimic the natural eating behaviour of horses, the forage is preferably given in multiple feedings per day or in haylage nets to increase the time spent eating. To meet the vitamin, mineral and protein requirements, a supplement or ration balancer should be provided.

In some cases, such as very painful laminitic obese horses, dietary energy restriction is the only option at the start of the treatment (King and Mansmann 2004). Several energy restriction programmes have been described in horses (Table 1.2), with (McGowan et al., 2013; Schmengler et al., 2013) or without the addition of a dietary supplement (short chain fructo-oligosaccharides), either based on net energy requirements starting from ideal body weight (Van Weyenberg et al., 2008), or on percentage dry matter intake (BMI) of actual body mass (BM) (Dugdale et al., 2010; Argo et al., 2012; Ungru et al., 2012; Potter et al., 2013). To obtain a weekly weight loss of ~ 1% of BM, severe energy restriction is sometimes required in native breeds, such as Shetland ponies (Van Weyenberg et al., 2008) and Welsh Mountain ponies (Dugdale et al., 2010). To maintain a 1% weekly weight loss in Shetland pony geldings, energy intake had to be decreased from 70% to 35% of maintenance net energy requirements (Van Weyenberg et al., 2008). In Welsh Mountain pony mares, energy intake had to be restricted to 67% of estimated maintenance digestible energy requirements or

a DMI of 1% of actual body mass (Dugdale et al., 2010). Argo and co-workers (2012) also had to decrease the DMI from 1.25% to 1% of DMI in some weight loss resistant ponies to achieve (further) weight loss in those ponies. In a study performed by Potter et al. (2013), breed-related differences in responsiveness to energy restriction was described. Obese Standardbred horses lost significant amounts of weight when maintained on ad-lib hay, whereas mixed pony-breeds and Andalusian-cross horses were relatively resistant to body mass loss when hay was reduced to 1.25% of body weight. Weight loss through energy restriction in Welsh Mountain ponies of 7.2 and 10.1% was accompanied with decreases in fat mass of respectively 19.3 and 10% (Argo et al., 2012; Dugdale et al., 2010), which indicates a positive influence on body mass composition.

Energy restriction is necessary to treat obesity, but it can be a stressful situation. When dietary energy restriction is used as only method to achieve weight loss, care should be taken not to restrict feed intake too severely as this may trigger life-threatening hyperlipemia (Hughes et al., 2004). Too severe restriction of feed intake can also lead to the development of undesirable and/or stereotypic behaviours such as crib biting (Cooper and Albentosa, 2005), ingestion of wood shavings bedding (Curtis et al., 2011), and/or gastric ulcer formation (Murray and Eichorn, 1996). Therefore, low energy forages with a high fibre content (more mature late seed-cut hay) presented in haylage nets should be used to maximise the satiety effect and to increase the time spent eating (Harris, 2011).

Energy restriction will induce weight loss, but the most ideal speed of weight loss has not been determined yet in horses. Further research is wanted in that field, as too fast weight loss can induce hyperlipidemia in horses due to the high triacylglycerol concentrations in the blood (Hughes et al., 2004). Therefore, it is important to monitor the speed and extent of weight loss, if necessary in combination with blood parameters. Weight loss resistant animals will require a more severe energy restriction compared to weight loss sensitive animals (Argo et al., 2012), so this first group certainly needs a regular follow-up, so energy levels can be adjusted as soon as possible to ensure weight loss.

1.8.2 Exercise

Human data suggest that exercise alone is generally less effective than caloric restriction in achieving and maintaining weight loss (Miller et al., 1997; Catenacci and Wyatt, 2007; Moinuddin et al., 2012). However, substantial weight loss similar to that achievable by diet is seen if efforts are made to assure an equal energy deficit, e.g. high levels of physical activity

which will consume a lot of energy such as military training and mountain climbing (Donnelly et al., 2009). Exercise in humans has a lot of beneficial and protective effects on body composition (increased lean body mass), improved insulin sensitivity, cardiovascular fitness, and preventing weight regain after weight loss (Hawley, 2004; Donnelly et al., 2009; Moinuddin et al., 2012). In horses fed a diet high in concentrates but moderate in soluble carbohydrates, moderate exercise performed at least 5 days a week improved insulin sensitivity, even in insulin-sensitive horses (Turner et al., 2011). Regular low intensity exercise appears to have an anti-inflammatory effect (decreased serum amyloid A and haptoglobulin concentrations) in native breed ponies, which is possibly greater in previously laminitic nonobese ponies compared to normal nonobese ponies (Menzies-Gow et al., 2014).

Equine studies on the effect of exercise as the single intervention for weight loss are limited. In a study performed in 12 overweight to obese Arabian and Arabian cross geldings, including a control group of 4 horses and an exercise group of 8 horses, moderate and high intensity exercise decreased body mass (minus 4%), abdominal circumference and fat mass (minus 34%), but there were only minimal changes in insulin and glucose dynamics (Carter et al., 2010). Also no effect of intensity (low intensity exercise (estimated mean energy expenditure of 2.3 Mcal for a 500 kg horse) compared to high intensity exercise (estimated mean energy expenditure of 4.8 Mcal for a 500 kg horse) for times a week) was found on most of the morphometric measurements (girth and neck circumferences), BCS and subcutaneous fat thickness at the level of the trunk, back, rib, and shoulder. Fat mass and body fat percentage decreased significantly more in the exercise group compared to the control group. Also higher intensity exercise was associated with more significant body fat loss and percentage body fat loss compared to lower exercise (Carter et al., 2010).

1.8.3 Combination

In humans, most recommendations for weight loss include both physical activity and energy restriction (Donnelly et al., 2009). The amount of physical activity and energy restriction can be very different, with a higher expected weight loss when a greater energy deficit is present.

In a weight loss trial performed in 23 Quarter Horse and Thoroughbred geldings and mares, energy restriction (moderate quality hay fed at 1% of body weight and a low calorie mixture fed at 0.5% of body weight) combined with exercise (3 days per week a walk and trot exercise with increasing intensity towards the end of the trial) was significantly more

successful in achieving weight loss compared to energy restriction alone (Gordon et al., 2009). It has to be mentioned, however, that the amount of mixture, in addition to hay, provided to the combined energy restriction/exercise group was further restricted from 0.5% to 0.3% of body weight after 6 weeks of weight loss, whereas in the energy restricted group, this amount was maintained at 0.5% of body weight for the entire 12 weeks of weight loss. This could have confounded the results. In a study performed in 12 obese animals (4 Standardbreds, 4 mixed breed ponies, and 4 Andalusian-cross horses), light to moderate exercise in addition to caloric restriction did not appear to be sufficient to further reduce the weight, BCS, or percentage of fat compared to caloric restriction alone (Potter et al., 2013). Potentially, the intensity of exercise (5 minutes walk, 15 minutes working trot, and another 5 minutes of walk), was not high enough to elicit any effects. At the start of the trial, different mean body condition scores were present in the 3 groups, which possibly also could have an influence on the extent of weight loss through energy restriction and/or exercise.

When possible, the combination of these two weight loss strategies should be encouraged, as the addition of exercise also prevents boredom in energy restricted horses. As described by Argo and co-workers (2012), the superimposition of controlled-exercise on less severe levels of energy restriction may be more '**welfare-protective**' than a progressive increase in the severity of feed restriction.

A second important advantage of the combination of physical activity and energy restriction to achieve weight loss that has been described in humans is the maintenance or even increase in lean body mass and thus the block of the adaptive suppression of resting metabolism (Redman et al., 2009; Dugdale et al., 2010). This can be of high importance for weight-loss maintenance (Catenacci and Wyatt, 2007) once ideal body weight is reached.

1.9 Conclusion

From this introduction, it is clear that obesity and its related disorders are very important issues that receive a lot of attention in human and equine medicine. There are similarities between humans and horses in the specific mechanisms by which obesity may cause deleterious effects in the body, but there are also important differences. Visceral adipose tissue being the most harmful adipose tissue depot in humans, it is suggested that the equivalent in equines could be the nuchal adipose tissue although this has yet to be proven. Further research into the composition of equine adipose tissue and the impact of different

adipose tissue locations in the equine body would give some more insights into the specific role of adipose tissue in total body inflammation in equines.

A recommendation for the treatment of obesity is given in the ACVIM consensus statement (Frank et al., 2010). Ideally, the combination of physical exercise and energy reduction should be recommended, but in practice, this is not always possible. Energy restriction is often the only weight loss strategy that can be chosen, e.g. painful laminitic animals. Therefore, more research into different degrees of energy restriction could give a clearer view what happens in the equine body depending on the rate of weight loss. It could also provide additional information about which adipose tissue depot will decrease depending on the rate of weight loss.

1.10   References

Adams AA, Katepalli MP, Kohler K, Reedy SE, Stilz JP, Vick MM, Fitzgerald BP, Lawrence LM, Horohov DW (2009) Effect of body condition, body weight and adiposity on inflammatory cytokine responses in old horses. Vet. Immunol. Immunopathol. 127, 286–294.

Annandale EJ, Valberg SJ, Mickelson JR, Seaquist ER (2004) Insulin sensitivity and skeletal muscle glucose transport in horses with equine polysaccharide storage myopathy. Neuromuscular disorders 14, 666-674.

Alford P, Geller S, Richrdson B, Slater M, Honnas C, Foreman J, Chaffin M (2001) A multicenter, matched case-control tudy of risk factors for equine laminitis. Preventive veterinary medicine, 49, 209-222.

Anforth HR, Bluthe RM, Bristow A, Hopkins S, Lenczowski MJP, Luheshi G, Wollman EE (1998) Biological activity and brain actions of recombinant rat interleukin-1alpha and interleukin-1beta. European cytokine network 9, 279-88.

Argo CMcG, Curtis GC, Grove-White D, Dugdale AHA, Barfoot CF, Harris PA (2012) Weight loss resistance: A further consideration for the nutritional management of obese Equidae. Vet. J. 194, 179-188.

Arnold W, Ruf T, Kuntz R (2006) Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (Equus ferus przewalskii) II. Energy expenditure. J. Exp. Biol. 209, 4566-4573.

Asplin KE, Sillence MN, Pollitt CC, McGowan CM (2007) Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. The Vet. J. 174, 530-535.

Bamford NJ, Potter SJ, Harris PA, Bailey SR (2013) Breed differences in insulin sensitivity and insulinemic responses to oral glucose in horses and ponies of moderate body condition score. Domest. Anim. Endocrin. 47, 101-107.

Banse H (2013) Mechanisms of equine insulin resistance. Dissertation thesis Oklahoma State University.

Becvarova I, Pleasant RS (2012) Managing obesity in pasture-based horses. Compend. Contin. Educ. Vet. 34, E1-E4.

Belknap JK, Giguere S, Pettigrew A, Cochran AM, Eps A, Pollitt CC (2007) Lamellar pro-inflammatory cytokine expression patterns in laminitis at the developmental stage and at the onset of lameness: innate vs. adaptive immune response. Equine Vet. J. 39, 42-47.

Bessesen DH (2008) Update on obesity. J. Clin. Endocrinol. Metab. 93, 2027-2034.

Blaak E (2001) Gender differences in fat metabolism. Curr. Opin. Clin. Nutr. Metab. Care 4, 499-502.

Boden G, Chen X, Mozzoli M, Ryan I (1996) Effect of fasting on serum leptin in normal human subjects. The Journal of Clinical Endocrinology & Metabolism 81, 3419-3423.

Brinkmann L, Gerken M, Riek A (2012) Adaptation strategies to seasonal changes in environmental conditions of a domesticated horse breed, the Shetland pony (Equus ferus caballus). J. Exp. Biol. 215, 1061-1068.

Brownlee M (2005) The pathobiology of diabetic complications a unifying mechanism. Diabetes 54, 1615-1625.

Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A, Richelsen B (2003) Regulation of adiponectin by adipose tissue-derived cytokines: *in vivo* and *in vitro* investigations in humans. Am. J. Physiol. Endocrinol. Metab. 285, E527-E533.

Budras KD, McCarthy PH, Fricke W, Richter R (2007) Anatomy of the dog (5th edn), Schlütersche Verlagsgesellschaft mbH & Co, Hannover, Germany.

Budras KD, Sack WO, Rock S (2008) Anatomy of the horse (5th edn). Schlütersche Verlagsgesellschaft mbH & Co, Hannover, Germany.

Burns TA, Geor RJ, Mudge MC, McMutcheon LJ, Hinchcliff KW, Belknap JK (2010) Proinflammatory Cytokine and Chemokine Gene Expression Profiles in Subcutaneous and Visceral Adipose Tissue Depots of Insulin-Resistant and Insulin-Sensitive Light Breed Horses. J. Vet. Inter. Med.24, 932-939.

Burns TA, Watts MR, Weber PS, McCutcheon LJ, Geor RJ, Belknap JK (2013) Distribution of insulin receptor and insulin-like growth factor-1 receptor in the digital laminae of mixed-breed ponies: An immunohistochemical study. Equine Vet. J. 45, 326-332.

Caro JF, Sinha MK, Kolaczynski JW, Zhang PL, Considine RV (1996) Leptin: the tale of an obesity gene. Diabetes 45, 1455–1462.

Carroll CL, Huntington PJ (1988) Body condition scoring and weight estimation. Equine Vet. J. 20, 41-45.

Carter RA, Geor RJ, Stanair WB, Cubitt TA, Harris PA (2009a) Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies. Vet. J. 179, 204-210.

Carter RA, Treiber KH, Geor RJ, Douglass L, Harris PA (2009b) Prediction of incipient pasture-associated laminitis from hyperinsulineamia and generalized and localized obesity in cohort of ponies. Equine Vet. J. 41, 171–178.

Carter RA, McCutcheon J, George LA, Smith TL, Frank N, Geor RJ (2009c) Effects of diet-induced weight gain on insulin sensitivity and plasma hormone and lipid concentrations in horses. Am. J. Vet. Res. 70, 1250-1258.

Carter RA, McCutcheon J, Valle E, Meilahn EN, Geor RJ (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.

Cartmill JA, Thompson DL, Storer WA, Gentry LR, Huff NK (2003) Endocrine responses in mares and geldings with high body condition score grouped by high vs. low resting leptin concentrations. J. Anim. Sci. 81, 2311-2321.

Cartmill JA, Thompson DL, Storer WA, Crowley JC, Huff N, Waller CA (2005) Effect of dexamethasone, feeding time, and insulin infusion on leptin concentrations in stallions. J. Anim. Sci. 83, 1875-1881.

Catenacci VA, Wyatt HR (2007) The role of physical activity in producing and maintaining weight loss. Nat. Clin. Pract. Endocrinol. Metab., 3:518-29.

Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin, M.S. (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J. Lipid Res. 46, 2347-2355.

Clearfield MB (2005) C-reactive protein: a new risk assessment tool for cardiovascular disease. JAOA: Journal of the American Osteopathic Association 105, 409-416.

Cleland SJ, Petrie JR, Small M, Elliott HL, Connell JM (2000) Insulin action is associated with endothelial function in hypertension and type 2 diabetes. Hypertension 35, 507-511.

Clutterbuck A.L, Harris P, Allaway D, Mobasheri A (2010). Matrix metalloproteinases in inflammatory pathologies of the horse. Vet. J. 18, 27-38.

Coffman JR, Coles CM (1983) Insulin intolerance in laminitic ponies. Can. J. Comp. Med. 47,347-351.

Cooper JJ, Albentosa MJ (2005) Behavioural adaptation in the domestic horse: potential role of apparently abnormal responses including stereotypic behaviour. Livest. Prod. Sci. 92, 177-182.

Couillard C, Ruel G, Archer WR, Pomerleau S, Bergeron J, Couture P, Lamarche B, Bergeron N (2005) Circulating levels of oxidative stress markers and endothelial adhesion molecules in men with abdominal obesity. J. Clin. Endocrinol. Metab. 90, 6454-6459.

Curtis GC, Barfoot CF, Dugdale AHA, Harris PA, Argo McG (2011) Voluntary ingestion of wood shavings by obese horses under dietary restriction. Brit. J. Nutr. 106, S178-S182.

Cymbaluk, NF, Christison GI (1990) Environmental effects on thermoregulation and nutrition of horses. Vet. Clin. N. Am. Eq. Prac. 6, 355-372.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. Clinica Chimica Acta 329, 23-38.

Dandona P, Mohanty P, Ghanim H, Aljada A, Browne R,l Hamouda W, Prabhala A, Afzal A, Garg R (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.

Dandona P, Aljada A, Bandyopadhyay A (2004) Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol. 25, 4–7.

de Laat MA, McGowan CM, Sillence MN, Pollitt CC (2010) Equine laminitis: Induced by 48h hyperinsulinemia in Standardbred horses. Equine Vet. J. 42, 129-135.

de Laat MA, Kyaw-Tanner MT, Sillence, McGowan CM, Pollitt CC (2012) Advanced glycation endproducts in horses with insulin-induced laminitis. Vet. Immunol. Immunop. 145, 395-401.

Deaton CM, Marlin DJ, Smith NC, Harris PA, Roberts CA, Schroter RC, Kelly FJ (2004) Pulmonary epithelial lining fluid and plasma ascorbic acid concentrations in horses affected by recurrent airway obstruction. Am. J. Vet. Res. 65,80-87.

de Heredia FP, Gómez-Martínez S, Marcos A (2012) Obesity, inflammation and the immune system. Proceedings of the Nutrition Society 71, 332-338.

Dimock AN, Siciliano PD, McIlwraith CW (2000) Evidence supporting an increased presence of reactive oxygen species in the diseased equine joint. Equine. Vet. J. 32, 439-443.

Divers TJ, Cummings JE, de Lahunta A, Hintz HF, Mohammed HO (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.

Donnelly JE, Blair SN, Jakicic JM, Manore MM, Rankin JW, Smith BK (2009) Appropriate physical activity intervention strategies for weight loss and prevention of weight regain for adults. Med. Sci. Sport Exer. 41, 459-471.

Dugdale AHA, Curtis GC, Cripps P, Harris PA, Argo CMcG (2010) Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares. Equine Vet. J. 42, 600-610.

Dugdale AHA, Curtis GC, Harris PA, Argo CMc (2011a) Assessment of body fat in the pony: Part I. Relationships between the anatomical distribution of adipose tissue, body composition and body condition. Equine Vet. J. 43, 552-561.

Dugdale AHA, Curtis GC, Milne E, Harris PA, Argo C.McG (2011b) Assessment of body fat in the pony: Part II. Validation of the deuterium oxide dilution technique for the measurement of body fat. Equine Vet. J. 43, 562-570.

Dugdale AHA, Curtis GC, Cripps PJ, Harris PA, Argo CMcG (2011c) Effects of season and body condition on appetite, body mass and body composition in ad libitum fed pony mares. Vet. J. 190, 329-337.

Dugdale AHA, Grove-White D, Curtis GC, Harris PA, Argo CMcG (2012) Body condition scoring as a predictor of body fat in horses and ponies. Vet. J. 194, 173-178.

Fain JN, Madan AK, Hiler MJ, Cheema P, Babouth SW (2004) Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinol. 145, 2273–2282.

Fantuzzi G (2005) Adipose tissue, adipokines, and inflammation. J. Allergy Clin. Immunol. 115, 911–919.

Field JR, Jeffcott LB (1989) Equine laminitis-another hypothesis for pathogenesis. Med. Hypotheses 30, 203-210.

Firshman AM, Valberg SJ, Baird JD, Hunt, L DiMauro S (2008) Insulin sensitivity in Belgian horses with polysaccharide storage myopathy.American journal of veterinary research 69, 818-823.

Fischer-Posovszky P, Wabitsch M, Hochberg Z (2007) Endocrinology of adipose tissue – an update. Horm. Metab. Res. 39, 314-321.

Fitzgerald BP, Reedy SE, Sessions DR, Vick MM, Murphy BA (2003) Obesity disrupts the duration of the estrous cycle in the mare. J. Anim. Sci. 81 (Suppl. 1), 102.

Fontaine GL, Belknap JK, Douglas A, Moore JN, Kroll DL (2001) Expression of interleukin-1β in the digital lamina of horses in the prodromal stage of experimentally induced laminitis. Am. J. Vet. Res. 62, 714-720.

Forro M, Cieslar S, Ecker GL, Walzak A, Hahn J, Lindinger MI (2000) Total body water and ECFV measured using bioelectrical impedance analysis and indicator dilution in horses. Journal of Applied Physiology 89, 663-671.

Forsythe LK, Wallace JMW, Livingstone MBE (2008) Obesity and inflammation: the effects of weight loss. Nutr. Res. Rev. 21, 117-133.

Frank N, Elliott SB, Brandt LE, Keisler DH (2006) Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. J. Am. Vet. Med. Assoc. 228, 1383-1390.

Frank N (2009) Equine Metabolic Syndrome. J. Eq. Vet. Sci. 29, 259-267.

Frank N, Geor RJ, Bailey SR, Durham AE, Johnson PJ (2010) Equine metabolic syndrome. J. vet. Internal. Med. 24, 467-475.

Frank N, Tadros EM (2014) Insulin dysregulation. Equine Vet. J. 46, 103-112.

Freestone JF, Beadle R, Shoemaker K, Bessin RT, Wolfsheimer KJ, Church C (1992) Improved insulin sensitivity in hyperinsulinemic ponies through physical conditioning and controlled feed intake. Equine Vet. J. 24, 187-190.

Friedman JM, Halaas JL (1998) Leptin and the regulation of body weight in mammals. Nature 395, 763-770.

Friedman JM (2009) Leptin at 14y of age: an ongoing story. Am. J. Clin. Nutr. 89, 973S-979S.

Frisbie DD, Smith RKW (2010) Clinical update on the use of mesenchymal stem cells in equine orthopaedics. Equine Vet. J. 42, 86-89.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Shimomura I (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. Journal of Clinical Investigation 114, 1752-1761.

Garcia-Séco E, Wilson DA, Kramer J; Keegan KG, Branson KR, Johnson PJ, Tyler JW (2005) Prevalence and risk factors associated with outcome of surgical removal of pedunculated lipomas in horses: 102 cases (1987-2002). J. Am. Vet. Assoc. 226, 1529-1537.

Garlinghouse SE, Burrill MJ (1999) Relationship of body condition score to completion rate during 160 km endurance races. Equine Vet. J. Suppl. 30,591-595.

Gee EK, Fennessy PF, Morel PCH, Grace ND, Firth EC, Mogg TD (2003) Chemical body composition of 20 Thoroughbred foals at 160 days of age, and preliminary investigation of techniques used to predict body fatness, New Zeal. Vet. J. 51, 125-131.

Gentry LR, Thompson DL, Gentry GT, Davis KA, Godke RA, Cartmill JA (2002) The relationship between body condition, leptin, and reproductive and hormonal characteristics of mares during the seasonal anovulatory period. Journal of animal science 80, 2695-2703.

Geor RJ (2008) Metabolic predisposition to laminitis in horses and ponies: obesity, insulin resistance and metabolic syndromes. J. Eq. Vet. Sci. 28, 753-759.

Geor RJ, Harris PA (2013) Obesity In Geor RJ, Harris PA, Coenen M (eds) (2013) Equine Clinical and Applied Nutrition, 487-502. Elsevier

Geor (2013) Equine metabolic syndrome – new insights in pathophysiology. AAEP proceedings, 123-125.

Giles SL, Rands SA, Nicol CJ, Harris PA (2014) Obesity prevalence and associated risk factors in outdoor living domestic horses and ponies. PeerJ 2:e299; DOI 10.7717/peerj.299.

Goossens GH (2008) The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol. Behav. 94, 206-218.

Gordon S, Taylor PR (2005) Monocyte and macrophage heterogeneity. Nature Reviews Immunology 5, 953-964.

Gordon ME, McKeever KH, Betros CL, Manso Filho HC (2007) Exercise-induced alterations in plasma concentrations of ghrelin, adiponectin, leptin, glucose, insulin, and cortisol in horses. Vet. J. 173, 532-540.

Gordon ME, McKeever KH, Betros CL, Manso Filho HC (2007) Plasma leptin, ghrelin and adiponectin concentrations in young fit racehorses versus mature unfit standardbreds. The Vet. J. 173, 91-100.

Gordon ME, Jerina ML, Raub RH, Davison KA, Young JK and Williamson KK (2009) The effects of dietary manipulation and exercise on weight loss and related indices of health in horses. Comparative Exercise Physiology 6, 33-42.

Grier SJ, Turner AS, Alvis MR (1996) The use of dual-energy X-ray absorptiometry in animals. Invest. Radiol. 31, 50-62.

Gugliucci A, Kotani K, Taing J, Matsuoka Y, Sano Y, Yoshimura M, Egawa K, Horikawa C, Kitagawa Y, Kiso Y, Kimura S, Sakane N (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.

Hahn P, Novak M (1975) Development of brown and white adipose tissue. J. Lipid. Res. 16, 79–91.

Hajer GR, van Haeften TW, Visseren, FL (2008). Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur. Heart J. 29, 2959-2971.

Halberg, N, Khan, T, Trujillo, ME, Wernstedt-Asterholm, I, Attie, AD, Sherwani, S, Wang, ZV, Landskroner-Eiger, S, Dineen, S, Magalang, UJ, Brekken, RA, Scherer, PE (2009) Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. Mol. Cell. Biol. 29, 4467-4483.

Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? Brit. J. Pharmacol. 142, 231-255.

Harker IJ, Harris PA, Barfoot CF (2011) The body condition score of leisure horses competing at an unaffiliated championship in the UK. J. Eq. Vet. Sci. 31, 253-254.

Harris PA, Staniar W, Ellis AD (2005) Effect of exercise and diet on the incidence of DOD. The growing horse: nutrition and prevention of growt disorders. In: Juliand, V. and W. Martin-Rosset (eds.) Nutrition of the performance horse: which system in Europe for evaluating the nutritional requirements, 273-290.

Harris P (2011) How can we practically manage obese horses and ponies? In; Lindner, A. (ed.) Applied equine nutrition and training. Wageningen Academic Publishers, Wageningen, the Netherlands, 71-90.

Hawley JA (2004) Exercise as a therapeutic intervention for the prevention and treatment of insulin resistance. Diabetes Metab. Res. Rev. 20, 383-393.

Heini AF, Lara-Castro C, Kirk KA, Considine RV, Caro JF, Weinsier RL (1998) Association of leptin and hunger-satiety ratings in obese women. Int. J. Obesity 22, 1084-1087.

Henneke DR, Potter GD, Kreider JL (1984) Body condition during pregnancy and lactation and reproductive efficiency of mares. Theriogenology 21, 897-909.

Henneke DR, Potter GD, Kreider JL, Yeates BF (1983). Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15, 371-372.

Hoffman RM, Boston RC, Stefanovski D, Kronfeld DS, Harris PA (2003) Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. J. Anim. Sci. 81, 2333-2342.

Holbrook TC, Tipton T, McFarlane D (2012) Neutrophil and cytokine dysregulation in hyperinsulinemic obese horses. Veterinary immunology and immunopathology, 145, 283-289.

Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259, 87-91.

Houseknecht KL, Portocarrero CP (1998) Leptin and its receptors: regulators of whole-body energy homeostasis. Domest. Anim. Endocrin. 15, 457-475.

Hughes KJ, Hodgson DR, Dart AJ (2004) Equine hyperlipaemia: a review. Aust. Vet. J. 82, 136-142.

Jeffcot LB, Field JR (1985) Current concepts of hyperlipaemia in horses and ponies. Vet. Rec. 116, 461-466.

Jeffcott LB, Field JR, McLean JG, O'Dea K (1986) Glucose tolerance and insulin sensitivity in ponies and Standardbred horses. Equine Vet. J. 18, 97-101.

Jefferies BC (1961) Body condition scoring and its use in management. Tasmanian J. Agric., 39, 19-21.

Jiang C, Kim JH, Li F, Qu A, Gavrilova O, Shah YM, Gonzalez FJ (2013) Hypoxia-inducible factor 1α regulates a SOCS3-STAT3-adiponectin signal transduction pathway in adipocytes. J. Biol. Chem. 288, 3844-3857.

Johnson PJ (2002) The equine metabolic syndrome peripheral Cushing's syndrome. Vet. Clin. North. Am. Equine Pract. 18, 271-293.

Johnson PJ, Ganjam VK, Messer NT, Turk JR, Keisler DH, Buff PR, Wiedmeyer CE (2006) The obesity paradigm: an introduction to the emerging discipline of adipobiology. Proceedings of the 52nd Annual convention of the American Association of Equine Practitioners, Dec 2-6, San Antonio, TX, p. 41-50.

Johnson PJ, Wiedmeyer CE, Messer NT, Ganjam VK (2009) Medical implications of obesity in horses – Lessons for human obesity. J. Diabetes Sci. Technol. 3, 163-174.

Johnson RJ, Stenvinkel P, Martin SL, Jani A, Sánchez-Lozada LG, Hill JO, Lanaspa MA (2013) Redefining metabolic syndrome as a fat storage condition based on studies of comparative physiology. Obesity 21, 659-664.

Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, Ohtsuka-Kowatari N, Kumagai K, Sakamoto K, Kobayashi M, Yamauchi T, Ueki K, Oishi Y, Nishimura S, Manabe I, Hashimoto H, Ohnishi Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Nagai R, Kadowaki T (2006) Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. J. Biol. Chem. 281, 26602–26614.

Kearns CF, McKeever KH, John-Alder H, Abe T, Brechue WF (2002) Relationship between body composition, blood volume and maximal oxygen uptake. Equine Vet. J. Suppl. 34, 485-490.

Kearns CF, McKeever KH, Roegner V, Brady SM, Malinowski, K (2006) Adiponectin and leptin are related to fat mass in horses. Veterinary J., 172, 460-465.

Kelishidaki R, Hashemi M, Mohammadifard N, Asgary S, Khavarian N (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.

Kershaw EE, Flier JS (2004) Adipose tissue as an endocrine organ. J. Clin. Endocr. Metab. 89, 2548–2556.

King CK, Mansmann RA (2004) Preventing laminitis in horses: Dietary strategies for horse owners. Clin. Tech. in Equine Pract. 3, 96-102.

Kirschvink N, Smith N, Fiévez L, Bougnet V, Art T, Degand G, Marlin D, Roberts C, Génicot B, Lindsey P, Lekeux P (2002) Effect of chronic airway inflammation and exercise on pulmonary antioxidant status of healthy and heaves-affected horses. Equine Vet. J. 34, 705-712.

Kischvink N, de Moffarts B, Lekeux P (2008) The oxidant-antioxidant equilibrium in horses. Vet. J. 177, 178-191.

Kopelman PG (2000) Obesity as a medical problem. Nature 404, 635-643.

Krzystek-Korpacka M, Patryn E, Boehm D, Berdowska I, Zielinski B, Noczynska A (2008) Advanced oxidation protein products (AOPPs) in juvenile overweight and obesity prior to and following weight reduction. Clin. Biochem. 41, 943-949.

Laflamme DP (2006) Understanding and managing obesity in dogs and cats. Vet. Clin. N. Am. Small Anim. Pract. 36, 1283-1295.

Lavizzo-Mourey R, Levi J (2002) F as in Fat: how obesity threatens America's future. Trust for America's health. Issue report, September 2012.

Lawrence L, Jackson S, Kline K, Moser L, Powell D, Biel M (1992) Observations on body weight and condition of horses in a 150-mile endurance race. J. Eq. Vet. Sci. 12, 320-324.

Lawrence LA, Pagan JD (2005) Nutritional management of developmental orthopaedic disease in the equine. In: Conference Sponsors, p. 177.

Lewis GF, Carpentier A, Adeli K, Giacca A (2002) Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. Endocr. Rev. 23, 201–229.

Loftus JP, Belknap JK, Stankiewicz KM, Black SJ (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.

Longland AC, Ince J, Newbold JC, Barfoot C, Harris PA (2011a) Pasture intake in ponies with and without a grazing muzzle over time. Proceedings of the 15th ESVCN Congress 2011, Zaragoza, Spain.

Longland AC, Barfoot C, Harris PA (2011b) The effect of wearing a grazing muzzle vs not wearing a grazing muzzle on pasture dry matter intake by ponies. J. Equine Vet. Sci. 31, 282-283.

Longland AC, Ince J, Harris PA (2011c) Estimation of pasture intake by ponies from liveweight change during six weeks at pasture. J. Equine Vet. Sci.31, 275-276.

Lumeng CN, Bodzin JL, Saltiel AR (2007a) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J. Clin. Invest., 117, 175–184.

Lumeng CN, Deyoung SM, Bodzin JL, Saltiel AR (2007b) Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes, 56, 16–23.

Mantovani A, Sicab A, Sozzanib S, Allavenab P, Veccheb A, Lacatia M (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25, 677-686.

Mantzoros CS, Moschos S, Avramopoulos I, Kaklamani V, Liolios A, Doulgerakis DE, Griveas I, Katsilambros N, Flier JS (1997) Leptin concentrations in relation to body mass index and the tumor necrosis factor-α system in humans. J. Clin. Endocrinol. Metab. 82, 3408-3413.

Martin-Rosset W, Vernet J, Dubroeucq H, Arnaud G, Picard A, Vermorel M (2008) Variation of fatness and energy content of the body with body condition score in sport horses and its prediction. In: Nutrition of the Exercising Horse, Eds: M.T. Saastamoinen and W. Martin-Rosset, European Association for Animal Production Publication Number 125. Wageningen Academic Publishers, Wageningen. pp 167-176.

Maury E, Brichard SM (2010) Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol. Cell. Endocrinol. 314, 1–16.

McGowan CM, Dugdale AH, Pinchbeck GL, Argo CMcG (2013) Dietary restriction in combination with a nutraceutical supplement for the management of equine metabolic syndrome in horses. Vet. J. 196, 153-159.
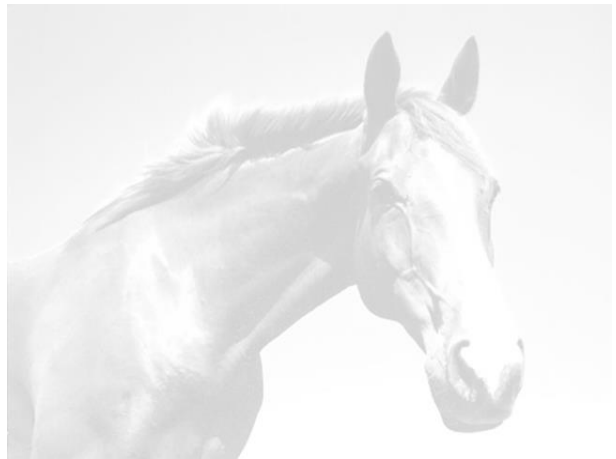
McGregor-Argo C (2009) Appraising the portly pony: Body condition and adiposity. Vet. J. 179, 158-160.

Menzies-Gow, N. J., Wray, H., Bailey, S. R., Harris, P. A., & Elliott, J. (2014) The effect of exercise on plasma concentrations of inflammatory markers in normal and previously laminitic ponies. Equine Vet. J., 46, 317-321.

Miller WC, Koceja DM, Hamilton EJ (1997) A meta-analysis of the past 25 years of weight loss research using diet, exercise or diet plus exercise intervention. Int. J. Obes. Relat. Metab. Disord. 21, 941-947.

Moinuddin IK, Collins EG, Kramer HJ, Leehey DJ (2012) Exercise in the management of obesity. J. Obes. Weigh. Los. Ther 2, 117.

Montague CT, Prins JB, Sanders L, Digby JE, O'Rahilly S (1997) Depot- and sex-specific differences in human leptin mRNA expression: implications for the control of regional fat distribution. Diabetes 46, 342–347.

Mottet, R, Onan G, Hiney K. (2009) Revisiting the Henneke body condition scoring system: 25 years later. J. Equine Vet. Sci. *29*, 417-418.

Muno JD (2009) Prevalence, risk factors and seasonality of plasma insulin concentrations in normal horses in central Ohio, Doctoral dissertation, The Ohio State University.

Murray MJ, Eichorn ES (1996) Effects of intermittent feed deprivation, intermittent feed deprivation with ranitidine administration, and stall confinement with ad libitum access to hay on gastric ulceration in horses. Am. J. Vet. Res. 57, 1599-603.

Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M, Cinti S (2008) Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J. Lipid Res. 49, 1562-1568.

Nagy P, Huszenicza G, Juhász J, Kulcsár M, Solti L, Reiczigel J, Abaváry K (1998) Factors influencing ovarian activity and sexual behaviour of postpartum mares under farm conditions. Theriogenology 50, 1109-1119.

Neel JV (1962) Diabetes mellitus: a thrifty genotype rendered detrimental by progress. Am. J. Human Gen. 14, 353-362.

Nonogaki K, Fuller GM, Fuentes NL, Moser AH, Staprans I, Grunfeld C (1995) Interleukin-6 stimulates hepatic triglyceride secretion in rats. Endocrinology 136, 2143-2149.

Ödberg FO, Francis-Smith K (1976) A Study on Eliminative and Grazing Behaviour — The Use of the Field by Captive Horses. Equine Vet. J. 8, 147-149.

Oh J, Riek AE, Weng S, Petty M, Kim D, Colonna M, Cella M, Bernal-Mizrachi C (2012) Endoplasmic reticulum stress controls M2 macrophage differentiation and foam cell formation. J. Biol. Chem. 287, 11629-11641.

Ogden C, Carroll M, Kit B, Flegal K (2012) Prevalence of obesity in the United States, 2009–2010. NCHS Data Brief No. 82.

Okouchi M, Okayama N, Imai S, Omi H, Shimizu M, Fukutomi T, Itoh M (2002) High insulin enhances neutrophil transendothelial migration through increasing surface expression of platelet endothelial cell adhesion molecule-1 via activation of mitogen activated protein kinase. Diabetologia 45, 1449-1456.

Ozinsky A, Underhill DM, Fontenot JD, Haijar AM, Smith KD, Wilson CB, Schroeder L, Aderem A (2000) The repertoire for pattern recognition of pathogens by the innate system is defined by cooperation between Toll-like receptors. Proc. Natl. Acad. Sci. USA, 97, 13766–13771.

Pace N, Rathbun EN (1945) Studies on body composition III. The body water and chemically combined nitrogen content in relation to fat content. J. Biol. Chem.158, 685-691.

Pearson RA, Ouassat M (1996) Esimation of the liveweight and body condition of working donkeys in Morocco. Vet. Rec. 138, 229-233.

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.

Potter, S. J., Bamford, N. J., Harris, P. A., & Bailey, S. R. (2013) Comparison of weight loss, with or without dietary restriction and exercise, in Standardbreds, Andalusians and mixed breed ponies. J. Equine Vet. Sci., 33(5), 339-339.

Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG, Keany Jr JF, Meigs JB, Lipinska I, Kathiresan S, Murabito JM, O'Donnell CJ, Benjamin EJ, Fox CS (2007) Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham heart study. Circulation 116, 1234-1241.

Powell DM, Reedy SE, Sessions DR, Fitzgerald BP (2002) Effect of short-term exercise training on insulin sensitivity in obese and lean horses. Equine Vet. J. 34, 81-84.

Prache S, Gordon IJ, Rook AJ (1998) Foraging behaviour and diet selection in domestic herbivores. Ann. Zootech. 47, 335-345.

Pratt-Phillips SE, Owens KM, Dowler LE, Cloninger MT (2010) Assessment of resting insulin and leptin concentrations and their association with managerial and innate factors in horses. J. Eq. Vet. Sci. 30, 127-133.

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.

Ralston SL (1996) Hyperglycemia/hyperinsulinemia after feeding a meal of grain to young horses with osteochondrosis dessicans (OCD) lesions. Pferdeheilkunde 12, 320-322.

Redman LM, Heilbronn LK, Martin CK, de Jonge L, Williamson DA, Delany JP, Ravussin E (2009) Metabolic and behavioral compensations in response to caloric restriction: Implications for the maintenance of weight loss. PLoS ONE, 4 (2009), p. e4377.

Reid SW, Mohammed HO (1996) Several analysis approach to risk factors associated with hyperlipemia in donkeys. J. Am. Vet. Med. Assoc. 209, 1449-1452.

Rijnen, K. E., & van der Kolk, J. H. (2003) Determination of reference range values indicative of glucose metabolism and insulin resistance by use of glucose clamp techniques in horses and ponies. American journal of veterinary research, 64(10), 1260-1264.

Robie SM, Janson CH, Smith SC, O'Connor Jr JT (1975) Equine serum lipids: serum lipids and glucose in Morgan and Thoroughbred horses and Shetland ponies. American journal of veterinary research 36, 1705-1708.

Robin CA, Ireland JL, Wylie CE, Collins SN, Verheyen KLP, Newton JR (2014) Prevalence and risk factors for equine obesity en Great Britain based on owner-reported body condition scores. Equine vet. J. DOI: 10.1111/evj.12275.

Rosen E and Spiegelman B (2014) What we talk about when we talk about fat. Cell 156, 20-44.

Schmengler U, Ungru J, Boston R, Coenen M, Vervuert I (2013) Effects of L-carnitine supplementation on body weight losses and metabolic profile in obese and insulin-resistant ponies during a 14-week body weight reduction programme. Livest. Sci. 155, 301-307.

Sessions DR, Reedy SE, Vick MM, Murphy BA, Fitzgerald BP (2004) Development of a model for introducing transient insulin resistance in the mare: Preliminary implications regarding the estrous cycle. J. Anim. Sci. 82, 2321-2328.

Sies H (1997) Oxidative stress: oxidants and antioxidants. Exp. Physiol. 82, 291-295.

Skurk T, Alberti-Huber C, Herder C, Hauner H (2007) Relationship between adipocyte size and adipokine expression and secretion. J. Clin. Endocrinol. Metab. 92,1023-1033.

Smith DG, Mayes RW, Hollands T, Cuddeford D, Yule HH, Malo Ladrero CM, Gillen E (2007) Validating the alkane pair technique to estimate dry matter intake in equids. The Journal of Agricultural Science 145, 273-281.

Stienstra R, Duval C, Keshtkar S, van der Laak J, Kersten S, Müller M (2008) Peroxisome proliferator-activated receptor γ activation promotes infiltration of alternatively activated macrophages into adipose tissue. Journal of Biological Chemistry 283, 22620-22627.

Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, DeFuria J, Jick Z, Greenberg AS, Obin, MS (2007) Adipocyte death, adipose tissue remodeling, and obesity complications. Diabetes 56, 2910-2918.

Suagee JK, Burk AO, Quinn RW, Petersen ED, Hartsock TG, Douglass LW (2008) Effect of diet and weight gain on body condition scoring in Thoroughbred geldings. J. Equine Vet. Sci. 28, 156-166.

Suagee JK, Corl BA, Crisman MV, Hulver MW, McCutcheon LJ, Geor RJ (2011) Effects of acute hyperinsulinemia on inflammatory proteins in horses. Vet. Immunol. Immunopathol. 142, 141-146.

Suagee JK, Corl BA, Geor RJ (2012) A Potential Role for Pro-Inflammatory Cytokines in the Development of Insulin Resistance in Horses. Animals 2, 243-260.

Suagee JK, Corl BA, Crisman MV, Pleasant RS, Thatcher CD, Geor RJ (2013) Relationships between Body Condition Score and Plasma Inflammatory Cytokines, Insulin, and Lipids in a Mixed Population of Light-Breed Horses. Journal of Veterinary Internal Medicine 27, 157-163.

Secombe CJ, Lester GD (2012).The role of diet in the prevention and management of several equine diseases. Animal feed science and technology 173, 86-101.

Suganami T, Nishida J, Ogawa Y (2005) A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor α. Arterioscler. Thromb. Vasc. Biol. 25, 2062-2068.

Tadros EM, Frank N, Donnell RL (2013) Effects of equine metabolic syndrome on inflammatory responses of horses to intravenous lipopolysaccharide infusion. American journal of veterinary research, 74, 1010-1019.

Thatcher C, Pleasant R, Geor R, Elvinger F, Negrin KA, Franklin J, Gay L, Were SR (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.

Trayhurn (2007) Adipocyte biology. Obesity reviews 8, 41-44.

Treiber KH, Kronfeld DS, Hess TM, Byrd BM, SPLAN RK, Stanair WB (2006) Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies. J. Am. Vet. Med. Ass. 228, 1538-1545.

Treiber K, Carter R, Gay L, Williams C, Geor R (2009) Inflammatory and redox status of ponies with a history of pasture-associated laminitis. Vet. Immunol. Immunopath. 129, 216-220.

Trujillo ME and Scherer PE (2006) Adipose tissue-derived factors: Impact on health and disease. Endocr. Rev 27, 762-778.

Turner SP, Hess TM, Treiber K, Mello EB, Souza BG, Almeida, FQ (2011) Comparison of insulin sensitivity of horses adapted to different exercise intensities. J. Equine Vet. Sci. 31, 645-649.

Ungru J, Blüher M, Coenen M, Raila J, Boston R, Vervuert I (2012) Effects of body weight reduction on blood adipokines and subcutaneous adipose tissue adipokine mRNA expression profiles in obese ponies. Vet. Rec. 171:528 doi 10.1136/vr.100911.

Valle E, Storace D, Sanguinetti R, Carter R, Odetti P, Geor R, Bergero D (2013) Association of the glycoxidative stress marker pentosidine with equine laminitis. Vet. J., 3, 445-450.

Van der Aa Kuhle K, Cawdell-Smith AJ, Ward LC, Bryden WL (2008) Prediction of equine body composition with bioelectrical impedance spectroscopy. In: Proceedings of Nutrition Society Australia, Adelaide, Australia.

Van Weyenberg S, Hesta M, Buyse J, Janssens GPJ (2008) The effect of weight loss by energy restriction on metabolic profile and glucose tolerance in ponies. J. Anim. Physiol. Nutr. 92, 538-545.

Vick MM, Sessions DR, Murphy BA, Kennedy EL, Reedy SE, Fitzgerald BP (2006) Obesity is associated with altered metabolic and reproductive activity in the mare: Effects of metformin on insulin sensitivity and reproductive cyclicity. Reprod. Fertil. Dev.18, 609–617.

Vick MM, Adams AA, Murphy BA, Sessions DR, Horohov DW, Cook RF, Shelton BJ, Fitzgerald BP (2007) Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85, 1144–1155.

Vincent HK, Innes KE, Vincent KR (2007) Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity. Diabetes Obes. Metab. 6, 813-839.

Wallenius V, Wallenius K, Ahrén B, Rudling M, Carlsten H, Dickson SL, Jansson JO (2002) Interleukin-6-deficient mice develop mature-onset obesity. Nature medicine 8, 75-79.

Waller AP, Kohler K, Burns TA, Mudge MC, Belknap JK, Lacombe VA (2011) Naturally occurring compensated insulin resistance selectively alters glucose transporters in visceral and subcutaneous adipose tissues without change in AS160 activation. Biochim. Biophys. Acta 1812, 1098-1103.

Walrand S, Guillet C, Boirie Y, Vasson MP (2004) *In vivo* evidences that insulin regulates human polymorphonuclear neutrophil functions. Journal of leukocyte biology 76, 1104-1110.

Watson TDG, Burns L, Love S, Packard CJ, Shepard J (1992) Plasma lipids, lipoproteins and post-heparin lipases in ponies with hyperlipaemia. Equine Vet. J. 24, 341-346.

Watts AE (2014) Use of stem cells in equine musculoskeletal disorders. Equine Vet. Educ. 26, 492-498.

Webb AI, Weaver BMQ (1979) Body composition of the horse. Equine Vet. J. 11, 39-47.

Webb SP, Potter GD, Evans JW, Webb GW (1989) Influence of body fat content on digestible energy requirements of exercising horses in temperate and hot environments. P 279-284. In: Proc. 11th Eq. nutr. Physiol. Symp., Stillwater, OK.

Westervelt RG, Stouffer JR, Hintz HF, Schryver HF (1976) Estimating fatness in horses and ponies. J. Anim. Sci. 43, 781-785.

Wooldridge AA, Edwards HG, Plaisance EP, Applegate R, Taylor DR, Taintor J, Judd RL (2012) Evaluation of high–molecular weight adiponectin in horses. American journal of veterinary research 73, 1230-1240.

Wray H, Elliott J, Bailey SR, Harris PA, Menzies-Gow NJ (2013) Plasma concentrations of inflammatory markers in previously laminitic ponies. Equine Vet. J., 45, 546-551.

Wyse CA, McNie KA, Tannahil VJ, Murray JK, Love S (2008) Prevalence of obesity in riding horses in Scotland. Vet. Rec. 162, 590-591.

Ye J, Keller JN (2010) Regulation of energy metabolism by inflammation: a feedback response in obesity and calorie restriction. Aging 2, 361.

Ye J, McGuinness OP (2013) Inflammation during obesity is not all bad: evidence from animal and human studies. American Journal of Physiology-Endocrinology and Metabolism 304, E466-E477.

Zoran DL (2010) Obesity in dogs and cats. Vet. Clin. Small Anim. 40, 221–239.
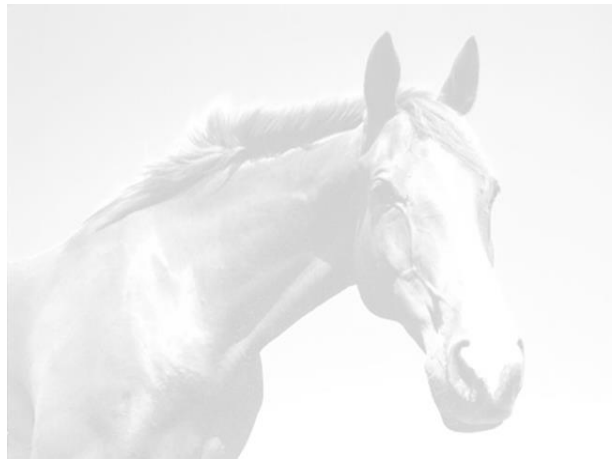
CHAPTER 2
SCIENTIFIC AIMS

CHAPTER 2: SCIENTIFIC AIMS

Equine obesity is a growing concern in the modern horse industry, especially in leisure horses, due to the development of obesity-related disorders, such as insulin resistance, hyperlipemia, hyperleptinemia, and a pro-inflammatory state (Frank et al., 2006; Vick et al., 2007; Frank and Tadros, 2014) . In humans, increased pro-inflammatory cytokine expression and protein levels are seen in obese compared to lean individuals (Fantuzzi, 2005). Inflammatory cytokines expression in humans is adipose tissue depot-dependent, with the visceral adipose tissue depot being the most harmful (Hajer et al., 2008). In equines, higher expression of pro-inflammatory cytokines IL-1β and IL-6 were found in the nuchal adipose tissue region compared to visceral and subcutaneous adipose tissue (Burns et al., 2010).We hypothesised that the equine inflammatory and oxidative response to changes in overweight depends on the changes in the different adipose tissues. To test this hypothesis, the first aim is to determine the effect of specific adipose tissue location on mRNA expression of inflammation-related genes in different equine adipose tissue depots. In addition, the effect of adipose tissue location on adipocyte area and presence of antigen presenting cells will be determined (**Chapter 3 and 4**).

An effective approach to counteract the negative effects of being either overweight or obese is to lose weight. Adipose tissue deposition and volume in the various locations is influenced by weight loss, however weight loss is not necessarily accompanied with a decrease in fat depth at all locations (Argo et al., 2012). In dogs, level of energy restriction was positively associated with amount of weight loss (Laflamme and Kuhlman, 1995). Weight rebound afterwards was also positively associated with the level of energy restriction. In equines, however, the effect of different levels of energy restriction on weight loss and subsequent changes in adipose tissue volume has not been investigated yet. Therefore, we want to test the effect of 2 levels of energy restriction on body mass and subsequent rebound weight gain. In addition, morphometric parameters will be evaluated (**Chapter 5**).

Energy restriction can be a stressful situation in equines, as under natural circumstances, they spend the greatest part of the day foraging and eating (Prache et al., 1998), with the potential development of stereotypic, unwanted behaviour and/or gastric ulceration (Murray and Eichorn, 1996, Cooper et al., 2005; Curtis et al., 2011). Therefore, behaviour and gastric health as features of animal welfare will also be evaluated (**Chapter 5**).

Human obesity increases the level of oxidative stress as indicated by decreased systemic antioxidants and increased oxidants (Vincent et al., 2007). Weight loss can reverse this altered status (Vincent et al., 2007). In equines, little is known about the effect of weight reduction programmes on the oxidant/antioxidant balance. We hypothesised that energy restriction will be accompanied with an improved oxidant/antioxidant balance (decreased oxidants and increased antioxidants) and that improvement will we associated with amount of weight loss. Therefore, we want to test if the level of energy restriction influences oxidative profile (**Chapter 6**).

CHAPTER 3

REFERENCE GENE EXPRESSION IN EQUINE ADIPOSE TISSUE

CHAPTER 3: DETERMINATION OF REFERENCE GENES IN EQUINE ADIPOSE TISSUE

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Extended version of materials and methods for the determination of reference genes used in Chapter 4.

3.1 Abstract

Background

The use of reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) to reveal differences in mRNA expression between equine adipose tissue depots may be a useful methodology for investigating clinical risk associated with regional obesity. However, for a correct interpretation of the qPCR results, expression data have to be normalized. This can be achieved by the use of 1 or multiple validated reference genes. Little information is available on the expression stability of reference genes in equine adipose tissue. Therefore, the goal of the present study was to establish a set of reference genes which can be used for normalization of mRNA expression data of genes expressed in different equine adipose tissue depots.

Results

In this study, the expression stability of 6 candidate reference genes (ACTB, HPRT1, TUBA4A, RPL32, GAPDH and SDHA) was investigated in 8 different adipose tissue depots (3 samples in the neck region, 3 abdominal samples and 2 samples from subcutaneous adipose tissue). HPRT1, RPL 32 and GAPDH were identified by using GeNorm analysis to be most stable in equine adipose tissue depots.

Conclusions

The results of this study provide a set of references genes (HPRT1, RPL32 and GAPDH) that can be recommended for accurate normalization of RT-qPCR data in equine adipose tissue depots. Depending on the adipose tissue depot, a different set of reference genes should be used.

3.2 Background

Nowadays, white adipose tissue is considered to be an important metabolic and endocrine organ (Fantuzzi, 2005). It is made up of several cell types, such as adipocytes, pre-adipocytes, endothelial cells, fibroblasts and macrophages (Maury and Brichard, 2010), expressing and secreting several adipokines that can exert its effects locally and or systemically (Radin et al., 2009). As a consequence of the adipokine-secreting ability of adipose tissue, inflammatory markers in the circulation can rise under certain circumstances, for example when excessive amounts of adipose tissue are deposited, as in obesity, leading to a state of low-grade inflammation (Dandona et al., 2004; Fantuzzi, 2005; Maury and Brichard, 2010).

Persons with a higher accumulation of visceral fat are at a higher risk for the development of obesity-related metabolic alterations (Després and Lemieux, 2006). In equines, adipose tissue distributed specifically on the crest of the neck could be associated with hyperinsulinemia, insulin resistance and/or an increased risk for laminitis (Carter et al., 2009; Frank et al., 2010), however, no causal link has been proven. Increasing equine clinical interest is being focused on adipose tissue in this region (Burns et al., 2010). To study depot-related variation in mRNA expression in adipose tissue, a very sensitive and specific technique is required.

When determining gene expression levels in fat mass depots, RT-qPCR is a very useful technique because of the low yield of mRNA isolated from adipose tissue (Gorzelniak et al., 2001). Real-time RT-qPCR is a potentially very reliable technique enabling comparison of mRNA transcription levels between tissue sites due to its high sensitivity and reproducibility (Bustin et al., 2005). Before comparing mRNA expression profiles across samples, correction for variables such as quality and quantification of the starting material and enzymatic efficiencies must be carried out (Bustin et al., 2002 and 2004; Fleige and Pfaffl, 2006). Consequently, the need for accurate data normalization is crucial (Thellin et al., 1999). In human adipose tissue, obesity and type 2 diabetes can exert a detectable influence on reference gene expression in subcutaneous and visceral fat depots (Mehta et al. 2010). This demonstrates that the expression level of reference genes is influenced by body region and health state of the experimental subject. Hence, reference genes need to be validated for each adipose tissue depot and every experimental setup. The normalization corrects for differences in amount and quality of starting material, and differences in mRNA isolation and cDNA

synthesis, as the reference genes are exposed to the same preparation steps as the genes of interest (Nygard et al., 2007).

Up till now, only a few gene expression studies in adipose tissue using RT-qPCR have been performed in equines (Vick et al., 2008; Burns et al., 2010; Packer et al., 2011; Ungru et al., 2012; Wearn et al., 2012; Morrison et al., 2014) (Table 1). In the majority of these studies, only 1 (Vick et al., 2008; Packer et al., 2011; Ungru et al., 2012; Wearn et al., 2012) or 2 (Burns et al., 2010; Morrison et al., 2014) reference genes have been used for normalisation (Table 3.1).

Table 3.1: Overview of reference gene determination in equine adipose tissue

Reference	Considered reference genes	Most stable reference genes	Adipose tissue depot	Animals	Gender
Vick et al., 2008	β-GUS	β-GUS	tail head	mixed light breed horse	female
Burns et al., 2010	ACTB, β-microglobulin	ACTB, β-microglobulin	omental, retroperitoneal, mesocolonic, nuchal, tail head	light breed horse	female
Packer et al., 2011	ACTB, SDHA	ACTB, SDHA	retroperitoneal	mixed breed ponies and horses	male and female
Ungru et al., 2012	18S	18S	tail head	Shetland ponies and mixed breed ponies	male and female
Wearn et al., 2012	ACTB	ACTB	nuchal	Thoroughbred	female
Morrison et al., 2014	GAPDH, ACTB, HPRT1, RPL32	RPL32, HPRT1	perirenal, ventro-abdominal, epicardial, mesenteric, nuchal, -tail head	Thoroughbred	male and female
	GAPDH, ACTB, HPRT1, RPL32	HPRT1, ACTB	perirenal (combined with muscle tissue and visceral organs)	Thoroughbred	male and female

Abbreviations: β-GUS: β-glucuronidase; ACTB, β-actin; SDHA, succinate dehydrogenase complex subunit A; HPRT1, hypoxanthine phosphoribosyltransferase 1; RPL32, ribosomal protein L32; TUBA4, tubulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

The aim of this study was to determine out of a set of candidate reference genes the most stable ones that can be used for the normalization of real-time RT-qPCR mRNA expression data originating from different adipose tissue regions in the horse.

3.3 Methods

3.3.1 Sample collection

The 12 equines sampled in this study were euthanized for non-research purposes at a local abattoir. More details on the equines involved in the present study are available in Chapter 4. Adipose tissue samples were collected from different places in the body within 15 minutes of euthanasia, so good quality RNA could be expected (Morrison et al., 2014): 3 samples in the neck region, 3 abdominal samples and 3 samples from subcutaneous adipose tissue. In the neck region, the samples were taken at ¼, ½ and ¾ of the distance between the poll and the withers from a clieved carcase. The abdominal adipose tissue samples were taken from the inside of a clieved carcase at the level of the right kidney from the fat surrounding the right kidney, retroperitoneally ± 10 cm lateral to the linea alba at the level of the last rib and at the level of the mesenterium (sample collected from fat from the suspensory ligament of the small intestine). The subcutaneous adipose tissue samples were taken at the level of the withers (± 5cm next to the highest point of the withers), the back at the level of the loin (at the level of the sacrum), and around the tail head (± 5cm lateral from the tail head basis). All the samples were taken in duplicate. Adipose tissue samples collected at the level of the withers were rather small, and sometimes included small fractions of tendon tissue. Therefore, these samples were excluded from further analysis. The samples (thickness 0.4 – 0.5 cm) were immediately submerged in RNAlater (Sigma-Aldrich, Ambion Inc., Austin, Texas) for RNA preservation and stored at 4°C for 24 hours and then stored at -20°C until RNA extraction.

3.3.2 Selection of candidate reference genes

Six reference genes were selected based on previous gene expression studies in the horse (Bogaert et al., 2006; Capelli et al., 2008; Smits et al., 2009), and bovine (Hosseini et al., 2010) and human adipose tissue (Gabrielsson et al., 2005; Hurtado del Pozo et al., 2010; Ferguson et al., 2010; Mehta et al., 2010). The NCBI database was searched for available equine primer sequences of reference genes necessary for gene expression normalization and of the genes of interest. Up to date, several gene expression studies using reverse-transcription quantitative real-time PCR (RT-qPCR) have been performed in horses. Waguespack et al.

(2004) compared 4 reference genes (ACTB, B2M, GAPDH and TBP) in the lamellae of the hoof in healthy horses and horses with black walnut-induced laminitis. Bogaert et al. (2006) selected a set of reliable reference genes in normal equine skin (TUBA1, ACTB and UBB) and in equine sarcoids (B2M, ACTB and UBB). When normal skin and equine sarcoids are compared, the use of the geometric mean of UBB, ACTB and B2M can be recommended as a reliable normalization factor. Smits et al. (2009) selected reference genes for RT-qPCR in equine in vivo and fresh and frozen-thawed vitro blastocysts. Based on the results of this study, the geometric mean of UBC, ACTB, RPL32 and GAPDH is to be recommended for accurate normalization of RT-qPCR data in equine in vivo and in vitro blastocysts. However, it has been extensively proven that it is necessary to evaluate reference gene expression stability in every experimental setup and every tissue type before they can be used for gene expression normalization (Bustin et al., 2009). More information on the function of the selected refences genes is presented in Table 3.2.

Table 3.2: Function of selected reference genes

	Gene name	Function
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	glycolytic enzyme
TUBA4A	Tubulin	member of tubulin family: structural protein
ACTB	actin beta	cytoskeletal structural protein
HPRT1	hypoxanthine phosphoribosyltransferase 1	nucleotide metabolism: generation of purine nucleotides from intermediates
RPL32	ribosomal protein L32	structural constituent of ribosome
SDHA	succinate dehydrogenase complex	involved in Krebs cycle as electron carrier

3.3.3 RNA isolation and cDNA synthesis

Total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen) and the TissueRuptor (Qiagen) for complete sample disruption/homogenization, as described in the manufacturer's protocol. An on-column DNase digestion (RNase-Free DNase Set, Qiagen) was included and was empirically verified by a minus reverse transcription (RT) control reaction. RNA quantity and purity (OD 260/280 ratio 1.9-2.1) were measured with the ND-1000 spectrophotometer (NanoDrop). RNA quality was verified on an agarose gel and was

assessed with the Experion RNA StdSens Analysis Kit (Bio-Rad) on an Experion Automated Electrophoresis System (Bio-Rad). The RNA quality indicator (RQI) for the adipose tissues ranged between 7-8.5. Subsequently, the iScript cDNA Synthesis Kit (Bio-Rad) was used to convert approximately 0.6 µg of total RNA into cDNA, which was verified by a control PCR. This cDNA was diluted 8 times to perform the qPCR analysis.

3.3.4 Real-time quantitative PCR

All PCR reactions were performed in a 15µl reaction volume on an iCycler iQ Real-Time PCR Detection System (Bio-Rad) using 7.5 µl of Kapa SYBR Fast Bio-Rad qPCR Master Mix (Sopachem) supplemented with 2.5 µl of diluted cDNA. The addition of RNAse free water and primer concentration varied according to the primer used. qPCR measurements for all samples were performed in duplicate and every run included a no-template control.

The PCR program started with an initial denaturation at 95°C for 3 minutes to activate the Taq polymerase, followed by 40 cycles of denaturation at 95°C for 10 seconds and a combined primer annealing/extension at the primer specific annealing temperature (Ta. see table 3.1) for 30 seconds during which fluorescence was measured. A melting curve was constructed to verify the presence of a single gene-specific amplicon and the absence of any primer dimers by heating the samples from 70 to 95°C in 0.5°C increments with a dwell time at each temperature of 10 seconds while continuously monitoring the fluorescence. The efficiency of each qPCR run was calculated from a relative standard curve based on a 5-point 5-fold cDNA dilution series. The pooled cDNA was obtained from adipose tissue in the neck, loin and tail head region and liver, using the same RNA extraction and cDNA synthesis protocols as described above.

3.3.5 Determination of reference gene expression stability

The geNorm algorithm was used to determine reference gene stability and their optimal number to be used for accurate normalization (Vandesompele et al., 2002). Reference gene expression stability was evaluated with the M value. The most stable control genes (lowest variation in mRNA expression) have the lowest M value. The raw gene expression data from the genes of interest were then normalised using the geometric mean of the best performing reference genes.

Table 3.3: Information on the primers used in the present study

Gene	Primer sequence (5'→3')	Annealing temperature (°C)	Ct range	Efficiency (E%)
HPRT1	GGCAAAACAATGCAAACCTT	62	19,7 - 24,6	94,5
	CAAGGGCATATCCTACGACAA			
RPL32	AGCCATCTACTCGGCGTCA	64	16,9 - 21,6	94
	TCCAATGCCTCTGGGTTTC			
GAPDH	CAGAACATCATCCCTGCTTC	59	15 - 21,1	95
	ATGCCTGCTTCACCACCTTC			
ACTB	CCAGCACGATGAAGATCAAG	62	16,4 - 20,1	93
	GTGGACAATGAGGCCAGAAT			
SDHA	TCCATCGCATAAGAGCAAAG	61	20,4 - 26,8	98
	GGTGGAACTGAACGAACTCC			
TUBA4	GCCCTACAACTCCATCCTGA	62	21,8 - 27,4	102,5
	ATGGCTTCATTGTCCACCA			

3.4 Results and discussion

Adipose tissue samples collected from 8 different adipose tissue depots in 12 healthy equines were used for the quantification of reference genes. To determine the expression stability of the six selected references genes, mRNA expression levels of the candidate reference genes were measured.

It should be mentioned that adipose tissue is made up of multiple cell types. The aim of the study was not to examine the individual cell populations, but to consider adipose tissue as a whole.

A RT-qPCR assay, based on SYBR® Green detection, was designed for the transcription profiling of the 6 selected genes (ACTB, HPRT1, TUBA4A, RPL32, GAPDH and SDHA). Melting curve analysis was used to confirm the specificity of the amplifications. The efficiency of each qPCR run was calculated from a relative standard curve based on a 5-point 5-fold cDNA dilution series, and ranged between 93 and 102.5%. Linear correlation coefficients varied between 0.996 and 0.999. The qPCR data from all genes were converted to raw data as described in Erkens et al. (2006).

Transcription levels (Ct value: the fractional PCR cycle at which the fluorescent signal significantly rises above the background signal (Erkens et al., 2006)) across all adipose tissue studied were almost similar for ACTB, GAPDH and RPL 32, which had lower Ct values than HPRT1, SDHA and TUBA4A. All the candidate reference genes had Ct values between 15 and 26.8 (Figure 3.1).

One sample from the neck region, 2 samples from the subcutaneous region and 4 from the abdominal region showed consistently higher Ct values compared to the other samples. Amplification problems could be the cause as the RNA quantity and quality was comparable to the other samples of the same region (Experion analysis). Those 7 adipose tissue samples were excluded from further analysis.

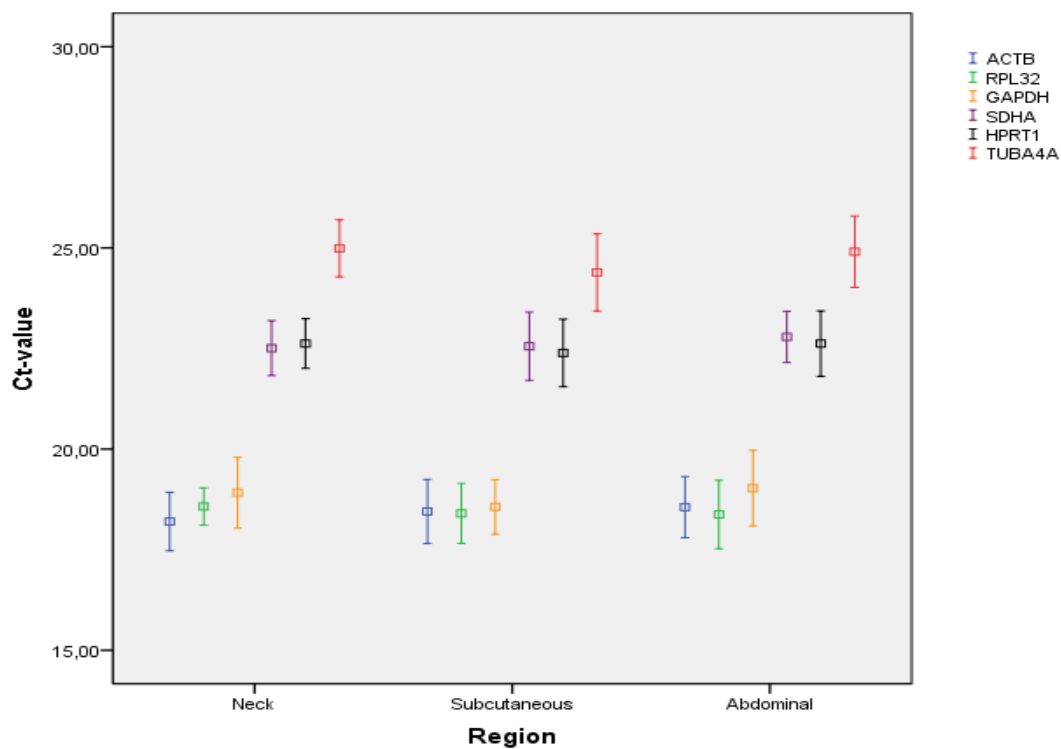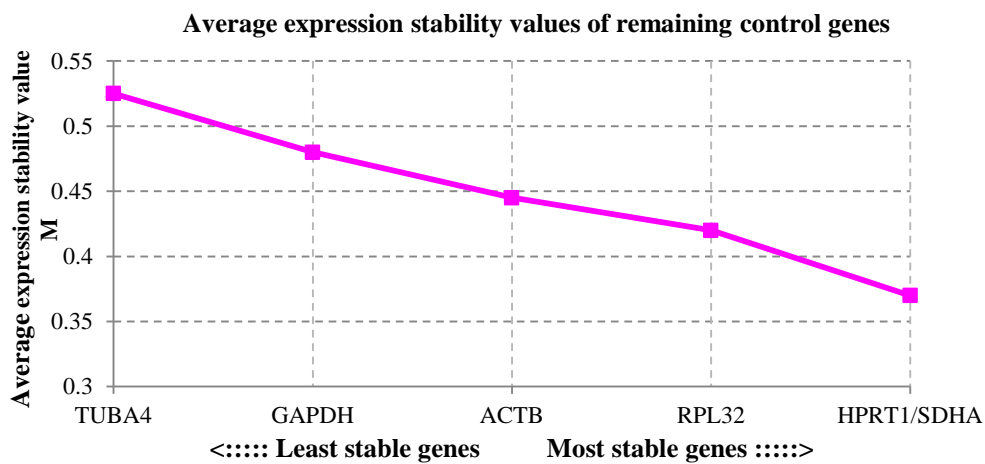Figure 3.1 - Ct-value range of the reference genes. Average expression levels of the 6 candidate reference genes in the 3 adipose tissue regions. Squares represent mean Ct values, bars indicate the standard variation.

3.3.1 GeNorm analysis

By using the geNorm algorithm, candidate reference genes can be classified on the basis of their expression stability (M), from least stable to most stable (Figure 3.2). The

classification of the 6 reference genes consistent with their M value was not equal for the different adipose tissue regions and the combination of all adipose tissue regions. For neck adipose tissue, HPRT1, SDHA and RPL32 were found to be most stable (Figure 3.2A). For the abdominal adipose tissue HPRT1, GAPDH and SDHA were the 3 most stable genes (Figure 3.2B). In the subcutaneous region HPRT1, RPL32 and ACTB were found to be most stable (Figure 3.2C). HPRT1, RPL32 and GAPDH were the 3 most stable reference genes when the 3 adipose tissue regions were combined (Figure 3.2D).

A)

B)

C)

Average expression stability values of remaning control genes

D)

Average expression stability values of remaining control genes
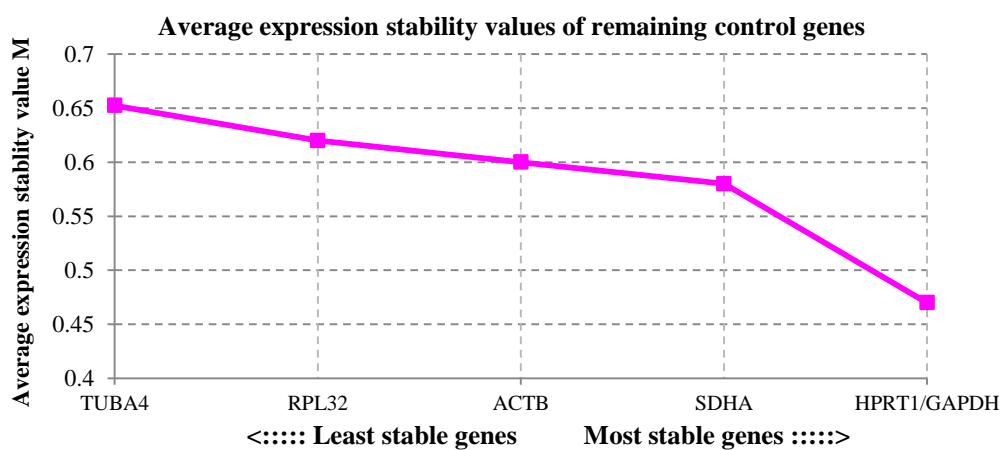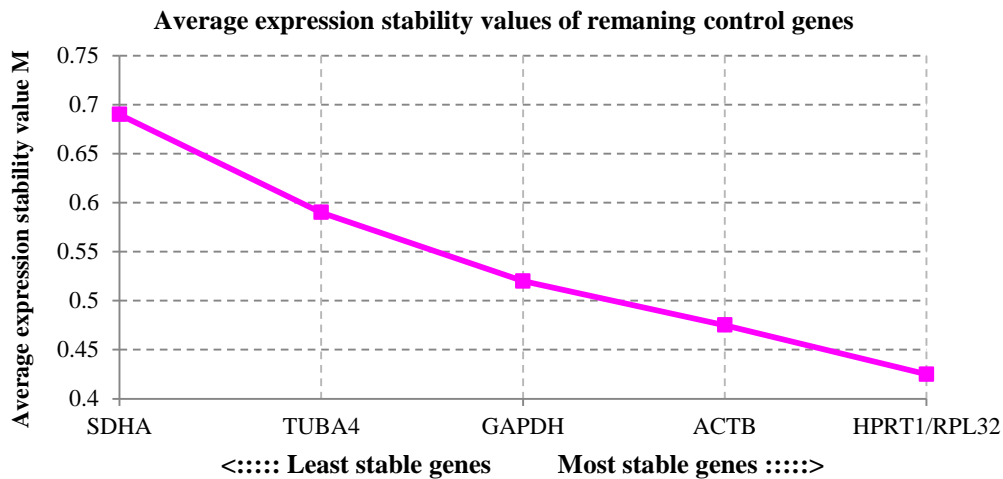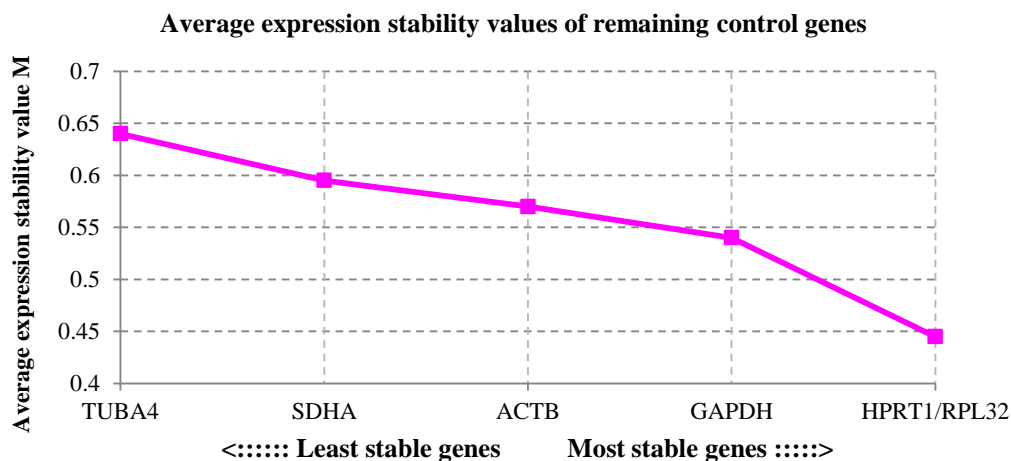
Figure 3.2 - Average reference gene mRNA expression stability values in equine adipose tissue. The average stability values (M) of the control genes were analysed by the geNorm program. (A) In the neck region HPRT1, SDHA and RPL32 were found to be most stable. (B) In the abdominal region, HPRT1, GAPDH and SDHA were found to be most stable. (C) In the subcutaneous region HPRT1, RPL32 and ACTB were found to be most stable. (D) HPRT1, RPL32 and GAPDH were found to be the most stable reference genes when the 3 adipose tissue regions were combined; A: Neck region – B: Abdominal region – C: Subcutaneous region – D: Combination of the 3 adipose tissue regions.

The optimal number of reference genes for accurate normalization depends on the adipose tissue region (Figure 3.3). In the neck region, the combination of 2 reference genes (V2/3= 0.136) is sufficient for adequate normalization. In the abdominal and subcutaneous region, theoretically the inclusion of a fourth gene does contribute (V3/4 = 0.130 and 0.122)

to the stability. In practice, however, if the V2/3 value is not that high, only 3 reference genes are used due to practical reasons.

A)

B)

C)
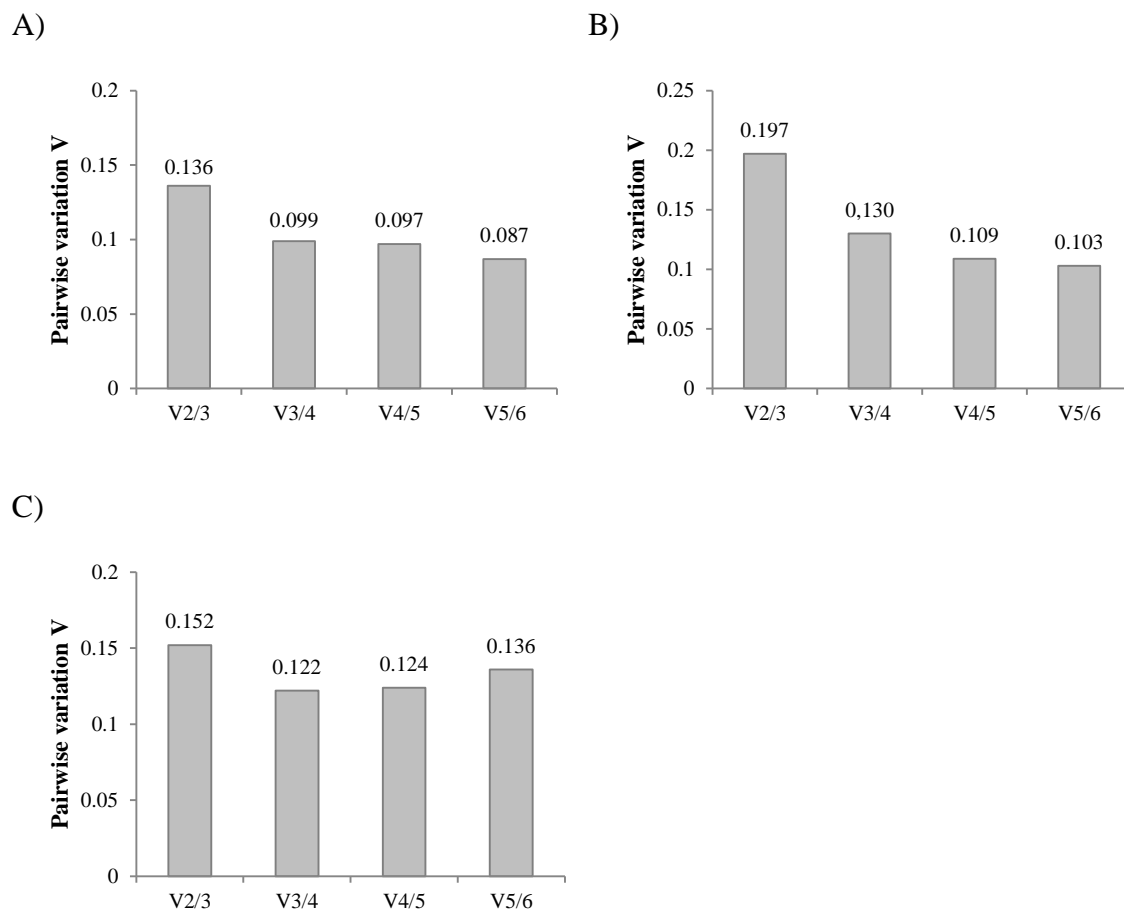
Figure 3.3 - Determination of the optimal number of control genes for normalization in the 3 adipose tissue regions. (A) The combination of 2 genes is sufficient for adequate normalization: V2/3 <0.15 (Vandesompele et al., 2002) (B,C) The inclusion of a fourth gene does contribute to the stability (V3/4 < 0.15), however, from a practical point of view, only 3 reference genes are used. The combination of 3 genes is sufficient for adequate normalization: V2/3 >0.15>V3/4 ; A: Neck region – B: Abdominal region – C: Subcutaneous region.

When mRNA expression of a gene is examined between the different adipose tissue regions, an adequate set of reference genes should be used. When the 3 regions are combined, theoretically the inclusion of a fourth gene does contribute (V3/4 < 0.15) to the stability. In practice, however, only 3 reference genes are used (Figure 3.4).
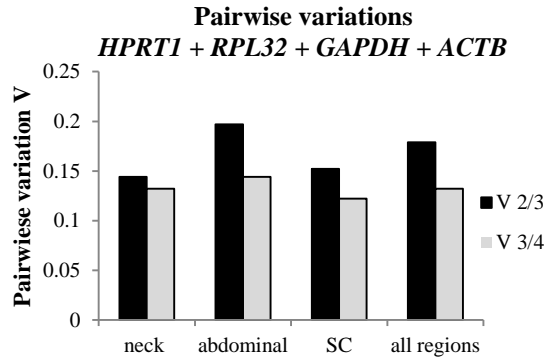
Figure 3.4 - Determination of the optimal number of control genes for normalization. Pairwise variation analysis between the normalization factors NF_n and NF_{n+1}, to determine the optimal number for normalization. The use of 3 reference genes to compare the gene expression between the 3 adipose tissue regions (all regions) or gene expression in one region (neck, abdominal, subcutaneous (SC)) is sufficient. The inclusion of a fourth gene does contribute to the stability (V3/4 < 0.15), however, from a practical point of view, only 3 reference genes are used.

The results of this study provide a set of references genes (HPRT1, RPL32 and GAPDH) that can be recommended for accurate normalization of RT-qPCR data for equine adipose tissue depots. These results are in accordance with findings in human visceral adipose tissue in which ACTB was recommended as stable reference gene for gene expression studies of human visceral adipose tissue (Mehta et al., 2010). Using microarray analysis, Hurtado del Pozo and colleagues (2010) found that when combining human subcutaneous and omental adipose tissue, HPRT1 was the control gene with the most stable expression. This agrees with the finding in our study that categorized HPRT1 as the most stable reference gene. In bovine adipose tissue, the expression of GAPDH in subcutaneous and retroperitoneal adipose tissue explants is fairly stable (Hosseini et al., 2010), which is in accordance with the results of Gorzelniak and colleagues (2001) who indicated that GAPDH was a well suited housekeeping gene for gene expression studies in human adipose tissue. Catalán and co-workers (2007), however, found that GAPDH showed the greatest variability in expression levels in omental and subcutaneous adipose tissue In the present study, GAPDH is recommended to use as a reference gene for the calculation of the normalisation factor when equine adipose tissue regions are combined.

3.5 Conclusion

Normalization with adequate reference genes is a critical step in the interpretation of RT-qPCR data. Not only between different tissues, but also for a single tissue originating from different sites within the body. This study demonstrates that the expression stability of

reference genes varies between adipose tissue regions in the horse. Extra attention to the choice of reference genes should be made if a comparison between gene expression in different adipose tissue regions is made. As for normalization of mRNA expression in neck adipose tissue 2 reference genes (HPRT1 and SDHA) are sufficient, 3 reference genes (HPRT1, GAPDH, SDHA and HPRT, RPL32, ACTB) are respectively needed for an accurate normalization of mRNA expression in intra-abdominal and subcutaneous adipose tissue samples. Conclusions regarding gene expression across different adipose tissue regions should be based on data normalized with a set of 3 reference genes (HPRT1, RPL32 and GAPDH).
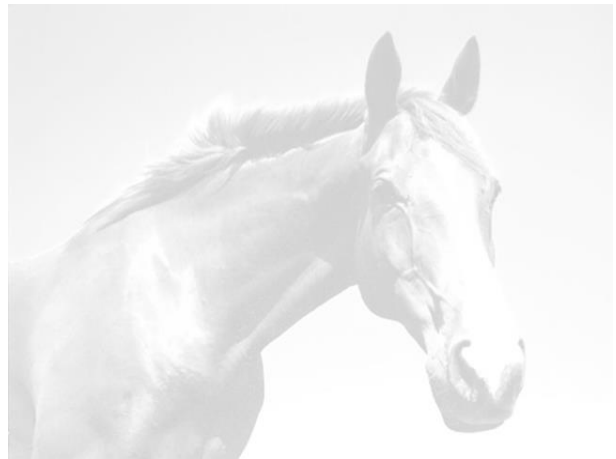
3.6 Acknowledgements

3.7 References

Antuna-Puente B, Feve B, Fellahi S, Bastard JP (2008) Adipokines: The missing link between insulin resistance and obesity. Diabetics & Metabolism 34, 2-11.

Bogaert L, Van Poucke M, De Baere C, Peelman L, Gasthuys F, Martens A (2006) Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. BMC Biotechnol. 6, 24.

Burns TA, Geor RJ, Mudge MC, McMutcheon LJ, Hinchcliff KW, Belknap JK (2010) Proinflammatory Cytokine and Chemokine Gene Expression Profiles in Subcutaneous and Visceral Adipose Tissue Depots of Insulin-Resistant and Insulin-Sensitive Light Breed Horses. J. Vet. Inter. Med.24, 932-939.

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol.29, 23-39.

Bustin SA, Nolan T (2004) Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction.. J. Biomol. Tech. 15: 155-166.

Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR – a perspective. J. Mol. Endocrinol. 34, 597-601.

Capelli K, Felicetti M, Capomaccio S, Spinsanti G, Silvestrelli M, Supplize AV (2008) Exercise induced stress in horses: Selection of the most stable reference genes for quantitative RT-PCR normalization. BMC Mol. Biol. 9, 49.

Carter RA, Treiber KH, Geor RJ, Douglass L, Harris PA (2009) Prediction of incipient pasture-associated laminitis from hyperinsulineamia and generalized and localized obesity in cohort of ponies. Equine Vet .J. 41, 171-178.

Catalán V, Gómez-Ambrosi J, Rotellar F, Silva C, Rodríguez A, Salvador J, Gil MJ, Cienfuegos JA, Frühbeck G (2007) Validation of Endogenous Control Genes in Human Adipose Tissue: Relevance to Obesity and Obesity-associated Type 2 Diabetes Mellitus. Horm. Metab. Res. 39, 495-500.

Dandona P, Aljada A, Bandyopadhyay A (2004) Inflammation: the link between insulin resistance, obesity and diabetes. TRENDS in Immunology 25, 4-7.

Deprés JP, Lemieux I (2006) Abdominal obesity and metabolic syndrome. Nature 444: 881-887.

Erkens T, Van Poucke M, Vandesompele J, Goossens K, Van Zeveren A, Peelman LJ (2006) Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and *longissimus dorsi* muscle, and evaluation with *PPARGCIA*. BMC Biotechnol. 6, 41.

Fantuzzi G: (2005) Adipose tissue, adipokines, and inflammation. J. Allergy Clin. Immunol. 115: 911-919.

Ferguson BS, Nam H, Hopkins RG, Morrison RF (2010) Impact of Reference Gene Selection for Target Gene Normalization on Experimental Outcome Using Real-Time qRT-PCR in Adipocytes. PLoS ONE 5, e15208.

Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol. Aspects Med. 27, 126-139.

Frank N, Geor RJ, Bailey SR, Durham AE, Johnson PJ (2010) Equine Metabolic Syndrome. J. Vet. Intern. Med. 24, 467-475.

Gabrielsson BG, Olofsson LE, Sjögren A, Jernås M, Elander A, Lönn M, Rudemo M, Carlsson LMS (2005) Evaluation of Reference Genes for Studies of Gene Expression in Human Adipose Tissue. Obesity Research 13, 649-652.

Gorzelniak K, Janke J, Engeli S, Sharma AM (2001) Validation of Endogenous Controls for Gene Expression Studies in Human Adipocytes and Preadipocytes. Horm. Metab. Res. 33, 625-627.

Hosseini A, Sauerwein H, Mielenz M (2010) Putative reference genes for gene expression studies in proprionate and β-hydroxybutyrate treated bovine adipose tissue explants. J. Anim .Physiol. An. N. 94, e178-e184.

Hurtado del Pozo C, Calvo RM, Vesperinas-García G, Gómez-Ambrosi J, Frühbeck G, Corripio-Sánchez R, Rubio MA, Obregon MJ (2010) *IPO8* and *FBXL10*: New Reference Genes for Gene Expression Studies in Human Adipose Tissue. Obesity 18, 897-903.

Maury E, Brichard SM (2010) Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol. Cell. Endocrinol. 314, 1-16.

Mehta R, Birerdinc A, Hossain N, Afendy A, Chandoke V, Younossi Z, Baranova A (2010) Validation of endogenous reference genes for qRT-PCR analysis of human visceral adipose samples. BMC Mol. Biol. 11, 39.

Morrison PK, Bing C, Harris PA, Maltin CA, Grove-White D, Argo CM (2014). Post-Mortem Stability of RNA in Skeletal Muscle and Adipose Tissue and the Tissue-Specific Expression of Myostatin, Perilipin and Associated Factors in the Horse. PloS one, 9, e100810.

Nygard AB, Jørgensen CB, Cicera S, Fredholm M (2007) Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC Mol. Biol. 8, 67.

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.

Smits K, Goossens K, Van Soom A, Govaere J, Hoogewijs M, Vanhaesebrouck E, Galli C, Colleoni S, Vandesompele J, Peelman L (2009) Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen-thawed *in vitro* blastocysts. BMC Res. Notes 2, 246.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999) Housekeeping genes as internal standards: use and limits: J. Biotechnol. 75, 291-295.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, 0034.I-0034.I.

CHAPTER 4

INFLAMMATION-RELATED GENE EXPRESSION IN DIFFERENT EQUINE ADIPOSE TISSUE DEPOTS

CHAPTER 4: EXPRESSION OF INFLAMMATION-RELATED GENES IS ASSOCIATED WITH ADIPOSE TISSUE LOCATION IN HORSES

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4.1 Abstract

Background

In humans, adipose tissue (AT) originating from different depots shows varying gene expression profiles. In horses, the risk of certain metabolic disorders may also be influenced by the impact of specific AT depots. Macrophage infiltration in human and rat AT is considered to be a source of inflammatory changes. In horses, this relationship has not been extensively studied yet. The main objectives of this study were to compare mRNA expression of inflammation-related genes, as well as adipocyte morphology and number between different equine AT depots; and in addition, to investigate the presence of antigen presenting cells in equine AT and any potential relationship with adipokine mRNA expression.

Results

In this study, the mRNA expression of inflammation-related genes (leptin, chemokine ligand 5, interleukin-1β, interleukin-6, interleukin-10, adiponectin, matrix metalloproteinase 2, and superoxide dismutase 2) and candidate reference gene stability was investigated in 8 different AT depots collected from the nuchal, abdominal (mesenteric, retroperitoneal, and peri-renal) and subcutaneous (tail head and loin) AT region. Highest leptin expression was found in the nuchal adipose tissue, whereas highest CCL5, IL-1β and IL-10 were found in the abdominal adipose tissue. Subcutaneous adipose tissue showed the highest expression of MMP2 and the highest amount of antigen presenting cells per adipocyte.

Conclusions

Adipose tissue location was associated with differences in mRNA expression of inflammation-related genes. This depot-specific difference in mRNA expression suggests that the overall inflammatory status of equines could be partially determined by the relative proportion of the different AT depots.

4.2 Background

Adipose tissue (AT) can be divided into brown and white AT (Hahn and Novak, 1975). The latter is now recognized as being more than an energy storage site. It is accepted as a highly active metabolic and endocrine organ (Fantuzzi, 2005; Kershaw and Flier, 2004) comprising different cell types (adipocytes, pre-adipocytes, endothelial cells, fibroblasts and macrophages) (Maury and Brichard, 2010) that actively secrete proteins involved in the regulation of energy, as well as neuroendocrine, autonomic, and immune functions (Radin et al., 2009). These different cell types may contribute to the secretion of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), chemokine ligand 5 (CCL5), and anti-inflammatory cytokine interleukin-10 (IL-10), as well as hormones such as resistin, leptin, and adiponectin that are involved in the inflammatory response and insulin sensitivity (Juge-Aubry et al., 2005; Radin et al., 2009; Antuna-Puente et al., 2010).

The AT also secretes matrix metalloproteinases (MMPs, e.g. MMP-2 or gelatinase A, MMP-9 or gelatinase B (Demeulemeester et al., 2005), and MMP-1,3,7,… (Halberg et al., 2008)) which have a functional role in the development of the AT (Lijnen et al., 2002) and are important for the extracellular matrix remodelling, which occurs during obesity-mediated AT formation, at least in mice (Chavey et al., 2008).

A study by Fain and colleagues (Fain et al., 2004) in obese women revealed that over 90% of the adipokine release by AT, except for adiponectin and leptin, could be attributed to non-fat cells. When excessive amounts of AT are deposited, inflammatory markers in the circulation can rise as result of the adipokine secreting ability of AT. Cinti and colleagues (Cinti et al., 2005) demonstrated that > 90% of all macrophages in white AT of obese mice and humans were localized around dead adipocytes, forming crown-like structures (CLS). Vick and colleagues (Vick et al., 2007) first demonstrated an association between obesity and increased inflammatory markers (TNF-α and IL-1) in horses, although age was also an important and possible confounding factor. Currently, there is some controversy whether obesity in horses is or is not associated with low grade inflammation (Vick et al., 2007; Vick et al., 2008; Adams et al., 2009) and there is no evidence whether CLS do or do not form in the obese horse.

Adipocyte size is positively correlated with frequency of adipocyte death, macrophage numbers, as well as CLS in visceral and subcutaneous (SC) depots in mice (Cinti et al., 2005;

Murano et al., 2008), and leptin mRNA expression in humans and cattle (Van Harmelen et al., 1998; Yang et al., 2008). To the author's knowledge, size of adipocytes originating from different horse AT regions has not been previously reported.

The specific site of AT deposition is clinically very important. Humans with a higher accumulation of visceral fat are at a higher risk for the development of obesity-related metabolic disorders (Deprés and Lemieux, 2006). Similarly in horses, it has recently been demonstrated that expression of glucose transporters was influenced by AT location in insulin sensitive and insulin resistant individuals (Waller et al., 2011). It has also been suggested in equidae that AT distributed specifically on the crest of the neck could indicate or contribute to hyperinsulinemia, insulin resistance (IR), and/or an increased risk for laminitis (Carter et al., 2009; Frank et al., 2010) although no causal link has been proven. Therefore, clinical interest on AT in this region is increased in horses (Carter et al., 2009; Burns et al., 2010). Burns and co-workers (Burns et al., 2010) found no differences in pro-inflammatory cytokine IL-1β and IL-6 mRNA expression between insulin resistant and insulin sensitive horses. Higher mRNA concentrations of these two cytokines, however, were found in the nuchal ligament AT compared to the other AT depots sampled in that study.

Our hypothesis was that mRNA expression of inflammation-related genes varied across AT depots. Therefore, the first aim of this study was to compare adipocyte size and mRNA expression between different equine AT depots with special interest in the nuchal AT region. The second aim was to investigate the presence of antigen presenting cells in equine AT and any potential relationship with adipokine mRNA expression.

4.3 Methods

4.3.1 Study animals

Twelve equines, due to be euthanized for non-research purposes, were selected at a local abattoir. Equines were chosen so that : 1) they were all geldings to exclude potential gender-related gene expression (Roth et al., 2002); 2) they represented a variety of different breeds presented at the abattoir (Table 4.1); 3) they showed no obvious lameness or overt laminitic rings; 4) they all had healthy appearance; 5) they ranged in their nutritional status (as scored visually by 2 experienced veterinarians) from normal to obese (Table 4.1); and 6) they ranged in age from 1-25 years (Table 4.1). All animals were sampled at the same day in April

2010. Equines were euthanized according to the procedure Royal Decree of January 16, 1988 concerning the protection of animals at killing.

Table 4.1: Information on the equines involved in this study

Equine	Breed	Age (years)	Nutritional status
1	German riding horse	16	overweight to obese
2	Dutch riding horse	20	normal
3	Belgian riding horse	1	normal
4	French riding pony, breed Haflinger	17	obese
5	Selle Français	10	normal
6	French Thouroughbred	12	normal
7	Royal Dutch Sport Horse (KWPN)	11	normal
8	Belgian trotter	3	normal
9	Belgian Warmblood (BWP)	21	overweight to obese
10	Dutch riding horse	25	overweight to obese
11	Dutch riding horse	11	overweight to obese
12	French trotter	15	overweight to obese

4.3.2 Sample collection

Blood samples were taken immediately post mortem from the vena jugularis for the analysis of glucose (Vacuette® tube, FX Sodium Fluoride/Potassium oxalate, 2 ml), as well as insulin and leptin (Vacuette® tube, Z Serum Clot Activator, 9 ml). Within 15 minutes after euthanasia, AT samples were collected from the different sites: three samples in the nuchal region taken at ¼, ½, and ¾ of the distance between the poll and the withers; three abdominal samples taken at the right kidney, retroperitoneally 10 cm lateral to the linea alba, and at the mesenterium; and two samples from SC AT taken at the level of the loin and around the tail head. All samples were taken in duplicate. Gene expression samples (thickness $0.4 - 0.5$ cm) were immediately submerged in RNAlater (Sigma-Aldrich, AMBION, Inc., Austin, Texas, USA) for RNA preservation and stored at 4°C for 24 hours and then stored at -20°C until RNA extraction. Histology AT samples were stored in formalin until further processing.

4.3.3 Blood sample analysis

Plasma glucose analysis was performed using a spectrophotometric method based on glucose hexokinase (Moreaux et al., 2011) (Architect C16000; Abbott, Abbott Laboratories, Abbott Park, Illinois, USA). Serum insulin concentrations were measured with an immunoradiometric assay test kit (Van Weyenberg et al., 2008) (insulin IRMA Ref 5251, Diasource Europe S.A., Nivelles, Belgium). An implementation validation has been carried out before use in horses. A dilution curve has been designed (100 - 80 – 60 – 40 – 20 – 0 % sample). Theoretical and measured values were compared to evaluate possible matrix-influences. Inter-assay variance was < 4 %, intra-assay variance in the high sample % was 9.2 %, in the low sample % 1.9 %. Leptin was measured using a multispecies RIA kit (Merck Millipore., Billerica, MA 01821, USA), previously validated for use in equine plasma (McMannus and Fitzgerald, 2000).

4.3.4 RNA isolation and cDNA synthesis, quantitative real-time PCR and determination of candidate reference gene expression stability

This was described in Chapter 3.

4.3.5 Primers for the genes of interest

Primers for the genes of interest CCL5, IL-10, IL-1β, IL-6, and SOD2 were used from Figueiredo et al. (2009), and for MMP2 from Loftus et al. (2006). For ADIPOQ and LEP, primers were designed using Primer3 (Rozen and Skaletsky, 1999)**,** while taking primer specificity (Blast, (Altschul et al., 1990)) and possible secondary structures (Mfold, (Zuker, 2003)) into account. As for the candidate reference genes, all primer amplicons were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystems 3730xl DNA Analyser. In addition, gel electrophoresis was performed to check the formation of 1 amplicon of the expected size, and to control the absence of primer dimers.

4.3.6 Histology

Adipose tissue samples were fixated by immersion in 4% paraformaldehyde, embedded in paraffin and sectioned. Two five µm thick serial sections were obtained, the first stained by haematoxylin and eosin (HE) to assess morphology (adipocyte area) and the rest processed for immunohistochemistry (see below). Adipocyte numbers were counted in 10

high power fields (HPF) and the average number of adipocytes was calculated per HPF. The surface of 1 HPF ($\pi r^2 = \pi*250 \ \mu m^2 = 196250 \ \mu m^2$) was divided through the numbers of adipocytes/HPF to calculate the mean surface area per adipocyte.

4.3.7 Immunohistochemistry

The presence of antigen presenting cells (APC) was evaluated by the use of Monoclonal Mouse Anti-Human HLA-DR antigen, alpha-chain clone TAL.1B5 (Code No. M0746; DakoCytomation, DakoCytomation, DK-2600, Glostrup, Denmark). This stain colours the major histocompatibility II (MHC II) molecules that are expressed on cells that serve as APC for CD4+, such as macrophages, monocytes, dendritic cells, and B cells (Ting and Trowsdale, 2002, Al-Daccak et al., 2004). Five μm-thick paraffin-embedded sections mounted on coated slides (APES, 3-aminopropyltriethoxysilane) were deparaffinised in xylene and with ethanol. Subsequently, the slides were pre-treated according to the microwave pressure cooker protocol for antigen retrieval (Citrate Buffer 10x, pH 6.0, Klinipath CBB 999, Klinipath BV, 6920 AD, Duiven, Netherlands). The immunohistochemistry was performed in an automated immunostainer (Dako, Glostrup, Denmark; S/N S38-7410-01) according to the manufactures protocol. For visualization the Envision+/HRP mouse (DAB) kit (Dako Ref K4007, DakoCytomation, DK-2600, Glostrup, Denmark) was used. Antibody diluent (Dako Ref S302283) was used to block hydrophobic interactions. Sections were counterstained with Mayer's hemalum solution (Klinipath). A positive control (thoracic mass, high grade sarcoma) was included in each run to ensure specificity. In negative controls, the primary antibody was replaced by a nonsense antibody of similar isotype (Monoclonal Mouse Anti-Human Cytokeratin Clones AE1/AE3). A second type of negative control was carried out by using central nervous system parenchyma with the original primary antibody (Monoclonal Mouse Anti-Human HLA-DR antigen (alpha-chain clone TAL.1B5), as within the normal central nervous system parenchyma, MHC expression is minimal or absent (Aloisi, 2001). To calculate APC/adipocyte, number of APC/10 HPF was divided through the number of adipocytes/10 HPF.

4.3.8 Statistical analysis

Data are reported as means ± SD and significance was set at $P < 0.05$. Statistical analyses were performed using IBM SPSS Statistics 20. Gene expression data were analysed with a general linear model by means of repeated measures with depot as within variable and animal as between variable, followed by a Bonferroni post hoc test when a significant difference

between depots was detected. Histology data were analysed with a general linear model, univariate analysis and Tukey post hoc test. Correlation analysis (Pearson's correlation test) was performed to identify relationships between blood parameters, histology findings and mRNA expression of cytokines.

4.4 Results and discussion

4.4.1 Animals

A variety of different breeds was chosen for this study (Table 4.1). Average age was 14 ± 7 years.

4.4.2 Blood analysis

Average glucose, insulin and leptin levels were 106 ± 16 mg/dl, 5.9 ± 0.9 mU/l, and 3.3 ± 1.4 ng/ml respectively. Glucose values can be increased in slaughterhouse animals due to increased stress from transport to the slaughterhouse, during lairage and stunning. The effect of these procedures on insulin and leptin levels are not known. The normal rations of the horses and the time of fasting were not known for the equine involved in the present study. Therefore, no conclusions on glucose and insulin dynamics were drawn.

4.4.3 Candidate reference gene selection and geNorm analysis

The efficiency of each RT-qPCR run was calculated from a relative standard curve based on a 5-point 5-fold cDNA dilution series, and ranged between 93 and 102.5 %. Linear correlation coefficients varied between 0.996 and 0.999.

One sample from the neck region, two from the SC region, and four from the abdominal region showed consistently higher transcription levels (Ct value: the fractional PCR cycle at which the fluorescent signal significantly rises above the background signal (Erkens et al., 2006) compared to the other samples. Amplification problems were considered to be the cause as the RNA quantity and quality was comparable to the other samples of the same region (Experion analysis). These 7 AT samples were therefore excluded from further analysis. Transcription levels across all AT studied were almost similar for ACTB, GAPDH, and RPL32, which had lower Ct values than HPRT1, SDHA, and TUBA4A. The raw gene expression data from the genes of interest were normalised using the geometric mean of the most stable candidate reference genes GAPDH, HPRT1, and RPL32.

4.4.4 Depot-specific mRNA expression

The present study investigated the AT depot related mRNA expression of inflammation-related genes in equines of different breeds, different ages, and with varying body condition or nutritional status. Adipokine expression was primarily studied at the transcription level, which does not necessarily reflect the protein level and/or its activity. It should be mentioned that AT is made up of multiple cell types (Maury and Brichard, 2010). The aim of mRNA expression was not to examine the individual cell populations, but to consider AT as a whole (Fain et al., 2004).

Leptin mRNA expression was significantly higher in the three neck samples compared to the mesenteric AT samples (Neck (N) ¼, N ½, N ¾: P = 0.034, 0.008, and 0.015 respectively). In contrast, CCL5 and IL-10 showed significantly lower mRNA expression in the nuchal AT compared to the mesenteric AT (N ¼, N ½, N ¾; P = 0.009, 0.009, 0.019 for CCL5, and 0.032, 0.008, 0.009 for IL-10 respectively). A significant lower expression of adiponectin mRNA was found in the tail head AT region compared to the nuchal AT (N ¼, N ½, N ¾: P = 0.010, 0.014, and 0.004 respectively), retroperitoneal (P = 0.003), and peri-renal AT region (P = 0.009). Pro-inflammatory cytokine IL-1β mRNA expression was significantly lower in the loin AT compared to the mesenteric and peri-renal AT (P = 0.004). A trend for lower mRNA expression was found in the retroperitoneal AT (P = 0.074). The MMP2 mRNA expression was significantly lower in the peri-renal region compared to AT originating from N ¼ (P = 0.019), N ½ (P = 0.006), tail head (P = 0.004) and loin region (P = 0.008). Mesenteric AT had a significantly lower MMP2 mRNA expression compared to N ½ (P < 0.001), tail head (P = 0.004) and loin AT (P = 0.001). Retroperitoneal AT had a significantly lower MMP2 mRNA expression compared to the loin AT (P = 0.014). Interleukin-6 tended to have a higher mRNA expression in the N ½ AT compared to mesenteric AT (P = 0.073). No significant AT depot effect was found for superoxide dismutase (SOD) 2.

A correlation was found between plasma leptin and insulin concentrations (P = 0.035; r = 0.610). There was also a correlation between IL-6 and IL-1β mRNA expression in the nuchal AT region (N ¼, N ½, and N ¾: P = 0.004, 0.001, 0.003; r = 0.756, 0.827, 0.782 respectively) and the tail head region (P = 0.007; r = 0.734). Higher leptin mRNA expression in the nuchal AT region compared to the mesenteric AT suggests that nuchal AT may contribute proportionally more to the overall leptin concentration in equines. The strong correlation between leptin concentration and degree of IR (Van Weyenberg et al., 2008)

supports the hypothesis that enlarged nuchal AT indeed is an important risk factor for IR (Carter et al., 2009; Frank et al., 2010). In a study of Liburt and co-workers, decreased IL-6 mRNA in nuchal AT was associated with increased insulin sensitivity (Liburt et al., 2011). Higher nuchal AT IL-6 mRNA expression together with a significant correlation between the expression of IL-6 and IL-1β in the nuchal AT depot, could indicate that in equines, the nuchal AT depot is an important contributor for gene expression of these pro-inflammatory markers whereas in humans, the visceral AT is responsible for this (Fried et al., 1998; Park et al., 2005). If such expression leads to increased protein formation, then an increase in the size of nuchal AT depot could potentially contribute more to the total body inflammatory status. In humans, elevated inflammatory cytokines such as TNF-α, IL-6, and IL-1 play important roles in the development of obesity-associated IR (Kerschaw and Flier, 2004; Chavey et al., 2008; Tilg and Moschen, 2008). If this is also the case in equines, it would further confirm the link between a high cresty neck score and IR.

Lower adiponectin mRNA expression in the SC region compared to the abdominal and nuchal region suggests that abdominal AT and nuchal AT may be more important for circulating adiponectin concentrations. Differences between gene expression in the different AT depots and the protein levels in the blood can be caused by differences at the translation level, which can be influenced by cytokines. Bruun and colleagues (Bruun et al., 2003) showed that TNF-α and IL-6 significantly decreased the human adiponectin mRNA levels *in vitro* suggesting that endogenous cytokines may affect adiponectin. In the present study, however, no correlations between adiponectin gene expression and cytokine mRNA expression were found.

Chemotactic cytokine CCL5 mediates chemotaxis of different leukocytes, depending on the tissue protein levels. High levels of CCL5 can trigger cytokine release. In humans, CCL5 production is upregulated by inflammatory cytokines, such as IL-1 (Thalmann and Meier, 2007). In the present study, CCL5, IL-10, and IL-1β mRNA expression was higher in the abdominal region, although no correlations between these cytokines were found. This could indicate that in equines, other cytokines regulate CCL5 production. Higher mRNA expression of CCL5, IL-10, and IL-1β in the abdominal AT could suggest that this AT depot may be more important for the circulating levels of these cytokines, although high mRNA expression does not necessarily leads to increased protein levels in the blood. This is in contrast with the findings from Burns and co-workers (Burns et al., 2010) who found higher IL-1β mRNA expression in the nuchal ligament AT compared with the other depots sampled

in that study. It has been suggested that different reference genes should be tested in each study setup to find the most suitable one not influenced by the experimental treatment (Overbergh et al., 2003). As candidate reference gene selection was different in the present study (HPRT1, RPL32, and GAPDH) and the Burns' study (β-actin and β_2-microglobulin), it may mean that results from both studies cannot be simply compared.

In mice, many MMPs are expressed by AT and stromal vascular cells in a depot-specific manner (Maquoi et al., 2002). Higher MMP2 mRNA expression in the SC AT suggests that in equines, this AT depot is more stimulated to differentiate pre-adipocytes into adipocytes (Lijnen et al., 2002; Croissandeau et al., 2002) and extracellular matrix remodelling (Cavey et al., 2008).

In conclusion, different mRNA expression of inflammation-related genes in different AT depots suggests that AT depots may be a driving force for total body inflammation if increased mRNA expression levels lead to increased circulating protein levels. It is possible that if more fat is deposited in an AT depot with high mRNA expression levels of pro-inflammatory cytokines, such as nuchal AT with high leptin and pro-inflammatory IL-6 mRNA expression, this may contribute to a greater overall inflammation in that individual equine than if the fat had been deposited in an AT depot with a low mRNA expression of pro-inflammatory cytokines. Further research into the final translation of mRNA expression of inflammation-related genes into adipokines will be necessary to correctly evaluate the impact of fat deposition at specific places in the equine body.

4.4.5 Histology and immunohistochemistry

To the author's knowledge, adipocyte size and area in cross-section in different AT regions in the equine body have not been previously reported.

Adipocyte area and number of antigen presenting cells (APC)/adipocyte could not be determined in 10 samples from different AT depots in 6 different equines due to technical cutting and staining difficulties (N ¼ AT for equine number 4; N ¾ AT for equine number 8; tail head AT for equine number 3; mesenteric AT for equine number 3,4,7,8 and right kidney AT for equine number 2,7,9). Negative controls using isotype-matched nonsense antibody showed no staining.

Average adipocyte diameter was 70 ± 7 μm, with the largest average adipocyte diameter being found in peri-renal AT (82 ± 14 μm). Average adipocyte area in cross-section

was 3980 ± 1355 μm^2. Peri-renal adipocyte area (5370 ± 1919 μm^2) was significantly higher compared to N ½ (3116 ± 556 μm^2; P < 0.001), N ¼ (3195 ± 831 μm^2, P = 0.003) and tail head adipocyte area (3537 ± 1375 μm^2, P = 0.022) (Figure 4.1). Retroperitoneal adipocyte area (4795 ± 1610 μm^2) was significantly higher than N ½ (3116 ± 556 μm^2; P = 0.020) adipocyte area. A significant lower number of APC/adipocyte was found in the N ½ AT compared to the loin AT (P = 0.024) (Figure 4.2).

Capping structures (dead adipocytes engulfed by APC) (Figure 4.3), similar to CLS (dead adipocytes surrounded by macrophages (Murano et al., 2008)) in mice), were found in 6 equines in 4 different AT depots (N ¼, N ½, loin, and peri-renal). In 2 equines (number 3 and 4) CLS were found in multiple AT depots.
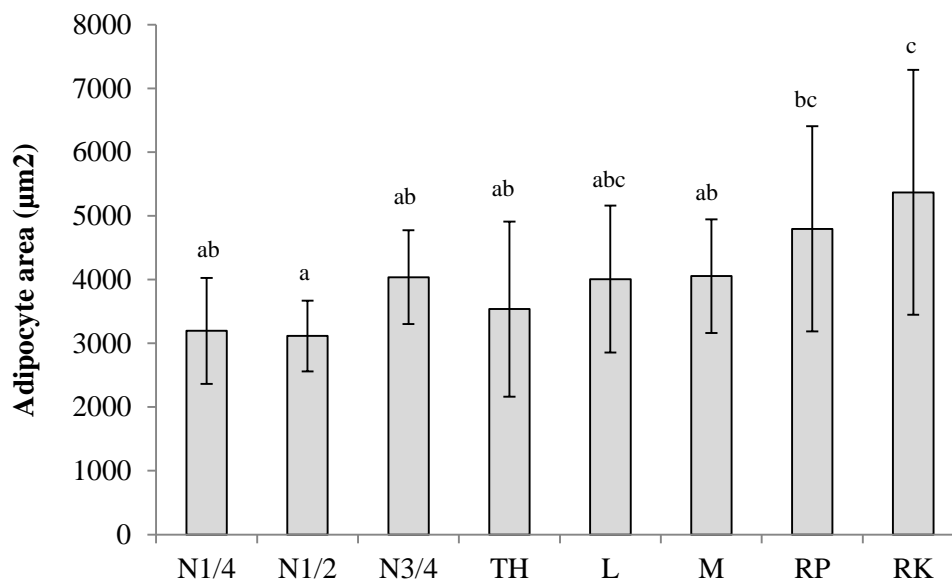
Figure 4.1 - Mean adipocyte area of equine adipocytes. Data are reported as means ± SD. Peri-renal adipocyte area is higher compared to N ½ (P<0.001), N ¼ (P=0.003) and loin adipocyte area (P=0.022). Retroperitoneal adipocyte area is higher than N ½ (P=0.020) adipocyte area. Superscripts (abc) indicate differences between adipose tissue location (P<0.05). Missing values and outliers were excluded from this analysis (N3/4 8; N1/4 4; TH 3; M 3,4,7,8; RK 2,7,9; L 11, RP 3). Abbreviations: N ¼, neck ¼; TH, tail head; L, loin; M, mesenteric; RP, retroperitoneal; RK, right kidney.

Clear differences in adipocyte area between AT depots were found, which is in accordance with the findings in other species (O'Connell et al., 2010; Kabir et al., 2011; Van de Velde et al., 2013). Differences in adipocyte area can influence mRNA expression (Skurk et al., 2007; Huber et al., 2008; Drolet et al., 2009). Significant correlations between mRNA expression of inflammation-related genes and adipocyte area were also found in the present study between multiple AT depots (N ¼, N ¾, tail head, mesenteric, retroperitoneal, and

perirenal) and multiple genes (adiponectin, CCL5, SOD, IL-10, leptin, and MMP2), although no clear pattern could be determined.

In the subcutaneous region, APC/adipocyte was high compared to the nuchal and abdominal region. In humans, this is related to high adipocyte death and CLS formation (Cinti et al., 2005), but in the current study, this relationship was not found. Macrophage inflammation in AT is also correlated with inflammation in humans (Weisberg et al., 2003; Xu et al., 2003).
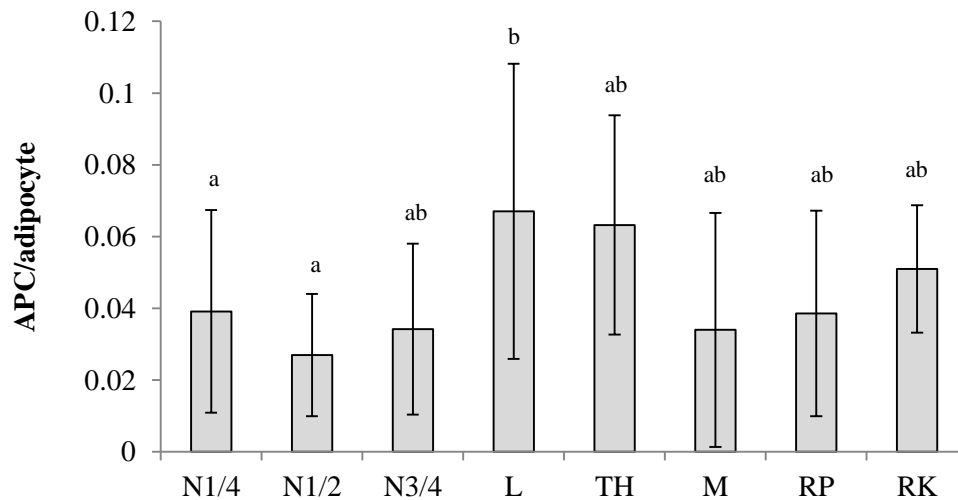
Figure 4.2 - Antigen presenting cells (APC) per adipocyte in equine adipose tissue. Major Histocompatibility Complex II (MHC II) stain was performed to enable the identification of antigen presenting cells/HPF (APC/HPF) in the different AT depots. Data are reported as means ± SD. A lower amount of APC/adipocyte was found in the N ½ AT compared to the loin AT (0.024). Superscripts (ab) indicate differences between adipose tissue location (P<0.05). Missing values and outliers were excluded from this analysis (N3/4 8; N1/4 4; TH 3; M 3,4,7,8; RK 2,7,9; L 11, RP 3). Abbreviations: N ¼, neck ¼; L, loin; TH, tail head; M, mesenteric; RP, retroperitoneal; RK, right kidney.

This is the first study describing capping structures (adipocytes surrounded by APC) in equines (Figure 4.3), probably similar to CLS in mice and humans (Cinti et al., 2005; Murano et al., 2008).
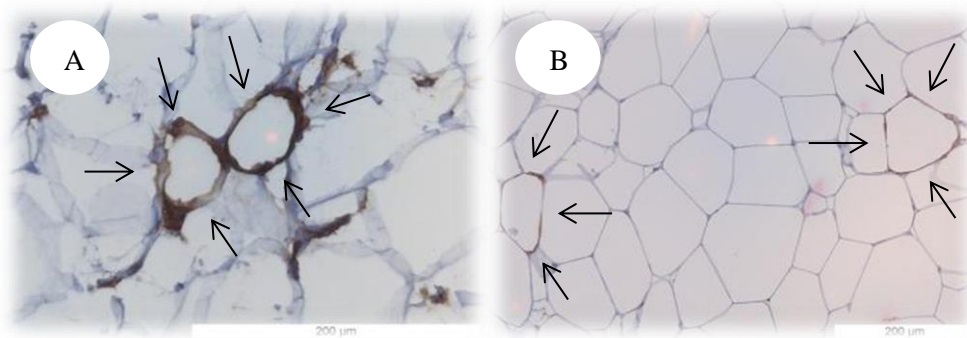
Figure 4.3 - Capping structures in equine adipose tissue. Major Histocompatibility Complex II (MHC II) stain was performed to enable the identification of antigen presenting cells in the different AT depots. Capping structures in the loin adipose tissue (a) and Neck ¼ (b) are indicated with black arrows.

One limitation of the present study was the staining method used, as this labelled MHC II molecules, which are not exclusively expressed on macrophages, but also on other APC such as monocytes, B-cells, and dendritic cells (Ting and Trowsdale, 2002; Al-Daccak et al., 2004).

One important item to address is the accessibility of the different adipose tissue locations samples in the present study. Practically, it is very difficult or even impossible to sample the adipose tissue at the level of the mesenterium or the kidney in the standing animal. Samples could be taken when horses are under general anesthesia and in dorsal recumbency, however, this is not practical.

4.5 Conclusions

Despite the fact that mRNA levels of inflammation-related genes were studied instead of protein levels, still interesting conclusions concerning the deposition of fat in various depots in equines could be drawn. The inflammatory profile in AT clearly varies with its location in the equine body in equines of different breeds varying in age and nutritional status, suggesting that the total inflammatory status of equines may be at least partly a reflection of the relative contribution of each AT. The factors driving the interindividual differences in AT distribution thus warrant further investigation.

4.6 Authors' contributions

LB was the primary author of the manuscript, responsible for the study design and performed most of the study procedures. TE participated in the study procedures and provided real-time instrument procedures. LJP, RD, GPJJ, PAH, and MH participated in the design of

the project, helped to draft the manuscript and supervised the study. All authors read and approved the final manuscript.
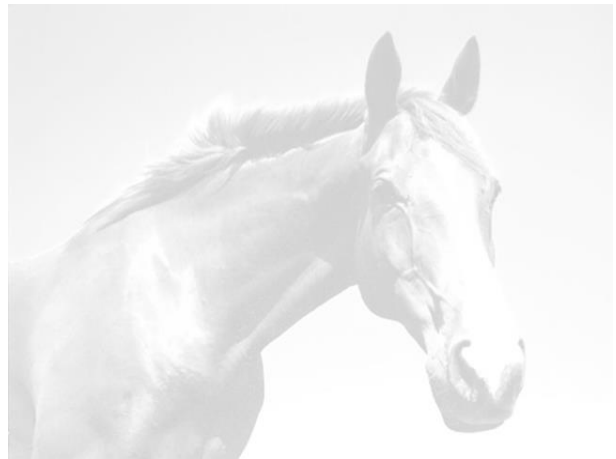
4.7 Acknowledgements

4.8 References

Adams AA, Katepalli MP, Kohler K, Reedy SE, Stilz JP, Vick MM, Fitzgerald BP, Lawrence LM, Horohov DW (2009) Effect of body condition, body weight and adiposity on inflammatory cytokine responses in old horses. Vet. Immunol. Immunopathol. 127, 286-294

Al-Daccak R, Mooney N, Charron D (2004) MHC II class signaling in antigen-presenting cells. Curr. Opin. Immunol. 16, 108-113.

Aloisi F (2001) Immune function of microglia. Glia 36: 165-179.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403-410. (http://www.ncbi.nlm.nih.gov/blast)

Bogaert L, Van Poucke M, De Baere C, Peelman L, Gasthuys F, Martens A (2006) Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. BMC Biotechnol. 6, 24.

Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A, Richelsen B (2003) Regulation of adiponectin by adipose tissue-derived cytokines: *in vivo* and *in vitro* investigations in humans. Am. J. Physiol. Endocrinol. Metab. 285, E527-E533.

Burns TA, Geor RJ, Mudge MC, McCutcheon LJ, Hinchcliff KW, Belknap JK (2010) Proinflammatory cytokine and chemokine gene expression profiles in subcutaneous and visceral adipose tissue depots of insulin-resistant and insulin-sensitive light breed horses. J. Vet. Intern. Med. 24, 932-939.

Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR – a perspective. J. Mol. Endocrinol. 34, 597-601.

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29, 23-39.

Capelli K, Felicetti M, Capomaccio S, Spinsanti G, Silvestrelli M, Supplize AV (2008) Exercise induced stress in horses: Selection of the most stable reference genes for quantitative RT-PCR normalization. BMC Mol. Biol. 9, 49.

Carter RA, Treiber KH, Geor RJ, Douglass L, Harris PA: Prediction of incipient pasture-associated laminitis from hyperinsulineamia and generalized and localized obesity in cohort of ponies. Equine Vet. J. 41, 171-178.

Chavey C, Mari B, Monthoue MN, Bonnafous S, Anglard P, Van Obberghen E, Tartare-Deckert S (2008) Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. J. Biol. Chem. 278, 11888-11896.

Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J. Lipid Res. 46, 2347-2355.

Croissandeau G, Chrétien M, Mbikay M (2002) Involvement of matrix metalloproteinases in the adipose conversion of 3T3-L1 preadipocytes. Biochem. J. 364, 739-746.

Demeulemeester D, Collen D, Lijnen HR (2005) Effect of matrix metalloproteinase inhibition on adipose tissue development. Biochem. Bioph. Res. Co. 329, 105-110.

Després JP, Lemieux I (2006) Abdominal obesity and metabolic syndrome. Nature 444: 881-887.

Drolet R, Bélanger C, Fortier M, Huot C, Mailloux J, Légaré D, Tchernof A (2009) Fat Depot-specific Impact of Visceral Obesity on Adipocyte Adiponectin Release in Women. Obesity 17, 424-430.

Erkens T, Van Poucke M, Vandesompele J, Goossens K, Van Zeveren A, Peelman LJ (2006) Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and *longissimus dorsi* muscle, and evaluation with *PPARGCIA*. *BMC* Biotechnol. 6, 41.

Fain JN, Madan AK, Hiler MJ, Cheema P, Babouth SW (2004) Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 145, 2273–2282.

Fantuzzi G (2005) Adipose tissue, adipokines, and inflammation. J. Allergy Clin. Immunol. 115, 911-919.

Ferguson BS, Nam H, Hopkins RG, Morrison RF (2010) Impact of Reference Gene Selection for Target Gene Normalization on Experimental Outcome Using Real-Time qRT-PCR in Adipocytes. PLoS ONE 5, e15208.

Figueiredo MD, Salter CE, Andrietti ALP, Vandenplas ML, Hurley DJ, Moore JN (2009) Validation of a reliable set of primer pairs for measuring gene expression by real-time quantitative RT-PCR in equine leucocytes. Vet. Immunol. Immunopathol. 131, 65-72.

Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol. Aspects Med. 27, 126-139.

Frank N, Geor RJ, Bailey SR, Durham AE, Johnson PJ (2010) Equine Metabolic Syndrome. J Vet. Intern. Med. 24, 467-475.

Fried SK, Bunkin DA, Greenberg AS (1998) Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J. Clin. Endocrinol. Metab. 83, 847-850.

Gabrielsson BG, Olofsson LE, Sjögren A, Jernås M, Elander A, Lönn M, Rudemo M, Carlsson LMS (2005) Evaluation of Reference Genes for Studies of Gene Expression in Human Adipose Tissue. Obes. Res. 13, 649-652.

Gorzelniak K, Janke J, Engeli S, Sharma AM (2001) Validation of Endogenous Controls for Gene Expression Studies in Human Adipocytes and Preadipocytes. Horm. Metab. Res. 33, 625-627.

Hahn P, Novak M (1975) Development of brown and white adipose tissue. J. Lipid Res. 16, 79-91.

Halberg N, Wernstedt-Asterholm I, Scherer PE (2008) The Adipocyte as an Endocrine Cell. Endocrinol. Metab. Clin. N. Am. 37, 753-768.

Hosseini A, Sauerwein H, Mielenz M (2010) Putative reference genes for gene expression studies in proprionate and β-hydroxybutyrate treated bovine adipose tissue explants. J. Anim. Physiol. An. N. 94, e178-e184.

Huber J, Kiefer FW, Zeyda M, Ludvik B, Silberhumer GR, Prager G, Zlabinger GJ, Stulnig TM (2008) CC chemokines and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity. J. Clin. Endocrinol. Metab. 93, 3215-3221.

Hurtado del Pozo C, Calvo RM, Vesperinas-García G, Gómez-Ambrosi J, Frühbeck G, Corripio-Sánchez R, Rubio MA, Obregon MJ (2010) *IPO8* and *FBXL10*: New Reference Genes for Gene Expression Studies in Human Adipose Tissue. Obesity 18, 897-903.

Juge-Aubry CE, Henrichot E, Meier CA (2005) Adipose tissue: a regulator of inflammation. Best Pract. Res. Cl. En. 19, 547-566.

Kabir M, Stefanovski D, Hsu IR, Iyer M, Woolcott OO, Zheng D, Catalano KJ, Chiu JD, Kim SP, Harrison LN, Ionut V, Lottati M, Bergman RN, Richey JM (2011) Large size cells in the visceral adipose depot predict insulin resistance in the canine model. Obesity 19, 2121–2129.

Kershaw EE, Flier JS (2004) Adipose Tissue as an Endocrine Organ. J. Clin. Endocr. Metab. 89, 2548-2556

Liburt NR, Fugaro MN, Wunderlich EK, Zambito JL, Horhov DW, Betancourt A, Boston RC, McKeever KH, Geor RJ (2011) The effect of exercise training on insulin sensitivity and fat and muscle tissue cytokine profiles of old and young Standardbred mares [abstract]. J. Equine Vet. Sci. 31, 237-238.

Lijnen HR, Maquoi E, Hansen LB, Van Hoef B, Frederix L, Collen D (2002) Matrix metalloproteinase inhibition impairs adipose tissue development in mice. Arterioscler. Thromb. Vasc. Biol. 22, 374-379.

Loftus JP, Belknap JK, Black SJ (2006) Matrix metalloproteinase-9 in laminae of black walnut extract treated horses correlates with neutrophil abundance. Vet. Immunol. Immunopathol. 133, 267-276.

Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR (2002) Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. Diabetes 51, 1093-1101.

Maury E, Brichard SM (2010) Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol. Cell. Endocrinol. 314, 1-16.

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.

Mehta R, Birerdinc A, Hossain N, Afendy A, Chandoke V, Younossi Z, Baranova A (2010) Validation of endogenous reference genes for qRT-PCR analysis of human visceral adipose samples. BMC Mol. Biol. 11, 39.

Moreaux S, Nichols J, Bowman J, Hatfield P (2011) Psyllium Lowers Blood Glucose and Insulin Concentrations in Horses. J. Equine Vet. Sci. 31, 160-165.

Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M, Cinti S (2008) Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J. Lipid Res. 49, 1562-1568.

O'Connell J, Lynch L, Cawood TJ, Kwasnik A, Nolan N, Geoghegan J, McCormick A, O'Farelly C, O'Shea D (2010) The Relationship of Omental and Subcutaneous Adipocyte Size to Metabolic Disease in Severe Obesity. PLoS ONE 5 4, e9997.

Overbergh L, Giulietti A, Valckx D, Decallonne B, Bouillon R, Mathieu C (2003) The Use of Real-Time Reverse Transcriptase PCR for the Quantification of Cytokine Gene Expression. J. Biomol. Tech. 14, 33-43.

Park HS, Park JY, Yu R (2005) Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-α and IL-6. Diabetes Res. Clin. Pr. 69, 29-35.

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.

Roth SM, Ferrell RE, Peters DG, Metter EJ, Hurley BF, Rogers MA (2002) Influence of age, sex, and strength training on human muscle gene expression determined by microarray. Physiol. Genomics 10, 181-190.

Rozen S, Skaletsky HJ (1999) Primer3 on the WWW for general uses and for biologist programmers. Bioinformatics Methods and Protocols: Methods in Molecular Biology 132, 365-386. (http://primer3.wi.mit.edu/).

Skurk T, Alberti-Huber C, Herder C, Hauner H (2007) Relationship between adipocyte size and adipokine expression and secretion. J. Clin. Endocrinol. Metab. 92, 1023-1033.

Smits K, Goossens K, Van Soom A, Govaere J, Hoogewijs M, Vanhaesebrouck E, Galli C, Colleoni S, Vandesompele J, Peelman L (2009) Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen-thawed *in vitro* blastocysts. BMC Res. Notes 2, 246.

Thalmann S, Meier CA (2007) Local adipose tissue depots as cardiovascular risk factors. Cardiovasc. Res. 75, 690-701.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999) Housekeeping genes as internal standards: use and limits: J.Biotechnol. 75, 291-295.

Tilg H, Moschen R (2008) Role of adiponectin and PBEF/visfatin as regulators of inflammation: involvement in obesity-associated diseases. Clin. Sci. 114, 275-288.

Ting JP-Y, Trowsdale J (2002) Genetic Control of MHC Class II Expression. Cell 109, S21-S33.

Van de Velde H, Janssens GPJ, de Rooster H, Polis I, Peters I, Ducatelle R, Nguyen P, Buyse J, Rochus K, Xu J, Verbrugghe A, Hesta M (2013) The cat as a model for human obesity: insights into depot-specific inflammation associated with feline obesity. Brit. J. Nutr. 110, 1326-1335.

Van Harmelen V, Reynisdottir S, Eriksson P, Thörne A, Hoffstedt J, Lönnqvest F, Arner P (1998) Leptin secretion from subcutaneous and visceral adipose tissue in women. Diabetes 47, 913-917.

Van Weyenberg S, Hesta M, Buyse J, Janssens GPJ (2008) The effect of weight loss by energy restriction on metabolic profile and glucose tolerance in ponies. J. Anim. Physiol. An. N. 92, 538-545.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome. Biol. 3, 0034.I-0034.II.

Vick MM, Adams AA, Murphy BA, Sessions DR, Horohov DW, Cook RF, Shelton BJ, Fitzgerald BP (2007) Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85, 1144-1155.

Vick MM, Murphy BA, Sessions DR, Reedy SE, Kennedy EL, Horohov DW, Frank Cook R, Fitzgerald BP (2008) Effects of systemic inflammation on insulin sensitivity in horses and inflammatory cytokine expression in adipose tissue. Am. J. Vet. Res. 69, 130-139.

Waller AP, Kohler K, Burns TA, Mudge MC, Belknap JK, Lacombe VA (2011) Naturally occurring compensated insulin resistance selectively alters glucose transporters in visceral and subcutaneous adipose tissues without change in AS160 activation. Biochimica et Biophysica Acta 1812, 1098-1103.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW (2003) Obesity is associated with macrophage accumulation in adipose tissue. J. Clin. Invest. 112, 1796-1808.

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J. Clin. Invest. 112, 1821-1830.

Yang SH, Matsui T, Kawachi H, Yamada T, Nakanishi N, Yano H (2008) Fat depot-specific differences in leptin mRNA expression and its relation to adicyte size in steers. Anim. Sci. J. 74, 17-21.

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31: 34.

CHAPTER 5

ENERGY RESTRICTION EFFECT ON METABOLIC, MORPHOMETRIC AND WELFARE PARAMETERS

CHAPTER 5: HIGHER ENERGY RESTRICTION RATE IN OBESE SHETLAND PONIES IS ASSOCIATED WITH A FASTER DECREASE IN BCS AND GIRTH MEASUREMENTS

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5.1 Abstract

Due to high prevalence of obesity, especially in the leisure horse sector, effective and safe weight loss strategies are required.

The present study evaluated the effect of two different energy restriction rates on physical, morphometric and welfare parameters in 18 obese (BCS7-9/9) Shetland geldings. The trial was divided into three periods: 1) a 4-week adaptation period, during which the maintenance energy intakes to maintain stable obese body weight were determined (100% MERob), 2) a 16.5-week weight loss period during which the ponies were randomly divided into three groups (n=6/group): control group (CONTROL), moderate energy restricted (MOD), and severe energy restricted group (SEV) that were respectively fed at 100, 80 and 60% of their individual MERob, and 3) a 3-week follow up period in which the ponies were again fed at their individual 100% MERob.

Between the beginning and the end of the weight loss period, significant pairwise differences between the three treatment groups were seen for body weight, BCS, heart girth, belly girth, and relative ultrasound fat depth at the level of loin and ribs (P<0.05). The higher energy restriction was associated with a higher decrease in BCS, tail head, and heart plus belly girth, but not with the development of gastric ulcers or stereotypic behaviours.

5.2 Introduction

Leisure horse obesity is a growing problem, with overweight and obesity prevalence up to 62% in some populations (Harker et al., 2011; Robin et al., 2014). Equine obesity has been associated with conditions, such as insulin resistance and an increased risk for laminitis (Quinn et al., 2006; Geor, 2008).

Reduced energy intake (Van Weyenberg et al., 2008; Argo et al., 2012) and increased physical activity (Carter et al., 2010) are two successful weight loss strategies in horses. In dogs and humans, the rate and extent of body weight loss can be affected by the degree of caloric restriction (Laflamme et al., 1995; Sweeney et al., 1993).

In horses, adipose tissue deposited at the crest of the neck has been suggested to be associated with metabolic disturbances, such as insulin resistance (Frank et al., 2006) and increased laminitis risk (Carter et al., 2009, Johnson et al., 2002, Treiber et al., 2006). During weight loss, the individual adipose tissue depots may vary in the extent of their reduction and the timing. In the previous weight loss study of Dugdale and co-workers (2010) in Welsh Mountain ponies, there was no change in body condition score (BCS) despite a significant overall loss in weight. The weight loss most likely resulted from the preferential use of intra-abdominal adipose tissue and/or the loss of muscle mass. Neither of these are evaluated by the body condition scoring system (Henneke et al, 1983). Prediction of body fat mass by the body condition scoring system (BCS) is more subjective and less accurate in obese ponies and horses (Dugdale et al., 2012) and is not thought to be sufficiently accurate for monitoring changes in regional adiposity. Therefore, the use of regional morphometric measurements and (real-time) ultrasonography has been suggested (Dugdale et al., 2010; Gobesso et al., 2012). However, changes in certain adipose tissue depots may not mirror changes in body weight or total body fat content, especially under certain climatic condition due to body fat reorganisation (Argo et al., 2012).

Energy restriction can be a stressful situation for horses, as they spend 12 to 20 h a day foraging and eating under natural circumstances (Prache et al., 1998; Ödberg and Francis-Smith, 1976). Therefore, in energy restricted horses, care should be taken to try and enable them to maintain as much of their natural eating behaviour as possible to help prevent the development of stereotypic behaviour such as crib biting or wood chewing (Cooper et al., 2005; Curtis et al., 2011) and/or gastric ulcer formation (Murray and Eichorn, 1996). Management practices, including the use of low energy, high fibre feeds/forages are therefore often recommended in order to try to increase the time spent foraging even when energy

intakes are restricted (Geor and Harris, 2009). However, the effect of weight loss protocols on gastric ulcer development has not been reported.

To the authors' knowledge, the effect of different levels of energy restriction in equids has not been studied to date. Therefore, the aim of the present study was to investigate the effect of two levels of energy restriction on various morphometric and animal welfare parameters.

5.3 Materials and methods

5.3.1 Animals and husbandry

Eighteen obese (body condition score, BCS $\geq$ 7/9) (Henneke et al., 1983) Shetland geldings, aged 9.3 $\pm$ 3.9 years (Table 5.1) 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. 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.

5.3.2 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 as would be used during the trial. The ponies also received the same vitamin, protein and mineral supplement (Spillers Gro'N Win®, MARS Horsecare) 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 a follow up period of 3 weeks.

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. (2008), based on their ideal 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) (National Reasearch Council, 2007) and corrected for all possible vitamin and protein insufficiencies from the hay diet. During the adaptation 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. If body weight changed with more than 1%, amount of offered hay immediately was increased or decreased in case of weight loss or weight gain. When weight was stable, no adjustments were made to the amount of hay. At the end of the last week of the adaptation period, when BW was stable for at least 3-5 least days, the energy intake from this amount of hay together with the fixed amount of balancer was considered to be the individual maintenance energy requirements to maintain stable obese body weight (iMERob).

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, increases in horses during aging (Valentini et al., 2007). The control group (CONTROL) received 100% of their iMERob during this entire period. The moderate energy restricted group (MOD) was offered 80% of their iMERob. The severe energy restricted group (SEV) was restricted to 60% of their iMERob.

During the follow up 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 two 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.

5.3.3 Determination of body mass

The ponies were weighed ($\pm$ 0.1 kg) 3 times a week during the adaptation period and follow up period. During the weight loss period, ponies were weighed weekly.

5.3.4 Morphometric and ultrasound measurements

Belly girth circumference at the level of the umbilicus was performed every 2 to 4 weeks. Cresty neck score (CNS), BCS, neck circumference at mid-crest and heart girth was performed every 4 weeks as described by Dugdale and colleagues (2010).

Every four weeks during the weight loss period, and at the end of the follow up period, the depths of 4 superficially accessible fat depots (tail head, loin, neck, ribs) were recorded by transcutaneous ultrasonography with a variable frequency linear array transducer (7,5-12,0

MHz) (Esaote Pie Medical MyLab30VetGold, LA523). Measurements at each site were carried out at the left side of the animal and repeated in triplicate and mean values were calculated. Neck fat depth was measured in the half of the distance between the poll and the withers with probe centered 7cm lateral from the top of the crest. Rib fat dept was measured with the probe centered lateral to the dorsal midline in the 14th intercostal space, at a distance of 1/6th of the abdominal circumference at the level of the umbilicus. To measure loin fat depth, the probe was centered 10 cm lateral to the dorsal midline at the level of the hair whorl in the flank of the pony (between the last rib and the massive muscles of the thigh). Finally, to measure fat depth at the level of the tail head, the probe was positioned 10 cm lateral to the vertebral column, 6 to 8 cm cranial of the first tail hairs, depending on the size of the animals (6 cm in the smallest animals, 8 cm in the largest animals).

Neck circumference at mid-crest and neck fat depth determinations were not repeatable in our trial despite accurate localization of the measurement point and clipping of the manes. Differences of angle of the head position, degree of muscle tension, and the small size of the animals led to too high coefficient of variation between intra-animal measurements. Therefore, results and further interpretations are not shown.

5.3.5 Gastroscopy

An endoscopic gastroscopy was performed as a routine clinical procedure to evaluate the gastric health at the start of the weight loss period, after 8 weeks of weight loss, and at the end of the weight loss period. Gastroscopic examination was carried out using a 1.75m long fiberoptic endoscope with a 9.5 mm outer diameter. Fasted animals were sedated with Domosedan (Pfizer A.H.) (0,1 ml/100kg BW). The visualization of the stomach was performed by an experienced endoscopist, blinded to treatment group assignments. To provide good visualisation of the squamous mucosa, margo plicatus, and glandular mucosa, the stomach was inflated with air and the mucosa rinsed with tap water. For each horse, a gastric ulcer score was given based on the worst gastric ulcer visualised according to the Practitioner's simplified scoring system adapted from Andrews et al. (1999).

Table 5.1: Phenotypic summary, daily digestible energy intake (DEI), and daily dry matter intake (DMI) of the individual animals involved in the 3 treatment groups. Age, body weight (BW), and body condition score (BCS) at onset of the weight loss period are given. Group summaries are given as means ± SEM

Treatment group		BM*	Age	BCS	DEI (MJ/day) Fed		DEI /kg BM*		DMI (% of BM°)			
		(kg)	(years)		AP/FUP	WLP	AP/FUP	WLP	end AP	start WLP	end WLP	start FUP
CONTROL	1	100.0	5	8.5	17.8	17.8	0.18	0.18	2.01	2.01	2.02	2.11
	2	108.8	13	7	20.1	20.1	0.18	0.18	2.09	2.09	2.06	2.09
	3	110.9	3	8	17.3	17.3	0.16	0.16	1.75	1.75	1.79	1.82
	8	152.0	11	8	26.3	26.3	0.17	0.17	1.95	1.95	1.96	2.05
	15	187.8	12	9	26.8	26.8	0.14	0.14	1.60	1.60	1.60	1.63
	17	185.5	14	9	29.4	29.4	0.16	0.16	1.79	1.79	1.81	1.83
	mean	**140.8**	**9.7**	**8.3**	**22.9**	**22.9**	**0.17**	**0.17**	**1.86**	**1.86**	**1.87**	**1.92**
	SEM	16.2	1.9	0.3	2.1	2.1	0.01	0.01	0.07	0.07	0.07	0.08
MOD	6	152.9	8.0	7	27.1	21.7	0.18	0.14	2.00	1.59	1.61	2.06
	7	149.6	5.0	7.5	26.4	21.1	0.18	0.14	1.99	1.58	1.61	2.07
	10	137.5	11.0	8.5	25.3	20.1	0.18	0.15	2.08	1.65	1.72	2.19
	12	175.5	9.0	8	30.3	24.2	0.17	0.14	1.95	1.54	1.60	2.03
	14	162.8	6.0	9	24.2	19.4	0.15	0.12	1.67	1.33	1.39	1.82
	18	243.3	15.0	9	44.4	35.4	0.18	0.15	2.07	1.64	1.72	2.18
	mean	**170.3**	**9.0**	**8.2**	**29.6**	**23.6**	**0.17**	**0.14**	**1.96**	**1.55**	**1.61**	**2.06**
	SEM	15.5	1.5	0.3	3.1	2.4	0.01	0.00	0.06	0.05	0.05	0.05
SEV	4	140.0	8.0	7.5	24.9	14.8	0.18	0.11	2.01	1.16	1.26	2.25
	5	118.0	16.0	8.5	23.0	13.7	0.20	0.12	2.21	1.30	1.43	2.53
	9	180.5	5.0	7	36.1	21.3	0.20	0.12	2.26	1.32	1.43	2.48
	11	137.0	11.0	9	22.4	13.3	0.16	0.10	1.85	1.07	1.19	2.05
	13	176.5	5.0	8.5	29.2	17.2	0.17	0.10	1.86	1.08	1.21	2.13
	16	172.8	11.0	9	23.4	13.8	0.14	0.08	1.52	0.88	0.99	1.74
	mean	**154.1**	**9.3**	**8.3**	**26.5**	**15.69**	**0.17**	**0.10**	**1.95**	**1.13**	**1.25**	**2.19**
	SEM	10.6	1.7	0.3	2.2	1.27	0.01	0.01	0.11	0.07	0.07	0.12

Abbreviations: CONTROL, control group: MOD, moderate energy restricted group; SEV, severe energy restricted group; AP, adaptation period; WLP, weight loss period; FUP, follow up period; %mDE, percentage of DEI relative to estimated maintenance requirements (NRC, 2007); *: based on BM at the start of the weight loss period; °: based on actual BM.

5.3.6 Behaviour

Behaviour was evaluated every two weeks via direct observations, resulting in a total of 12 observation days (adaptation period: 2, weight loss period: 8, follow up period: 2). Observations were carried out for 30 min in the morning (08:15 to 08:45) and for 2 hours in the afternoon (13:30 to 14:30; 14:45 to 15:45) prior to feeding times. Scan sampling (Altmann, 1974) was performed using instantaneous recording (5-min intervals), resulting in information for several behavioural categories (Table 5.2). In addition, all occurrences sampling (Altmann, 1974) using one-zero recording (5-min intervals) was carried out for the four behavioural categories (Table 5.2).

Prior to each observation session, the animals were habituated to the presence of the observer for 15-30 minutes. Data collection was performed by two observers (observation day 1 through 10 by observer 1, observation day 10 through 12 by observer 2). The first observer was blinded to the distribution of the animals across treatments, but could not finish the observations for logistical reasons. The second observer, the trial leader and therefore not blinded to the treatments, trained alongside the first observer and Cohen's kappa was calculated to determine the agreement between observations on observation day 10, in order to validate the results for the last two observation days.

Table 5.2: Behavioural parameters. Scan sampling was performed using instantaneous recording (5-min intervals), resulting in information for several behavioural categories. All occurrences sampling using one-zero recording (5-min intervals) was carried out for 4 behavioural categories.

Behavioural category	Examples	Main effect	Post hoc	P-value
Scan sampling				
Inactive	Resting standing, resting recumbent, sleeping	Time	AP>WLP1, WLP2, FUP	<0.0001, <0.0001, <0.0001
			FUP>WLP1, WLP2	<0.0001, 0.0032
			AP>WLP2, FUP	0.0010, 0.0002
Immobile active	Standing alert, standing active	Time	WLP1>WLP2, FUP	0.0226, 0.0049
		Treatment	SEV>MOD	0.0102
Locomotion	Walk, trot, canter	Time	AP>WLP1, WLP2, FUP	0.0002, <0.0001, 0.0002
		Treatment	MOD>CONTROL, SEV	0.0329, 0.0262
Solitary behaviour	Solitary play, foraging, exploration	Time	AP>WLP1, WLP2, FUP	<0.0001, <0.0001, <0.0001
Eliminative behaviour	urinating, defecating	Time	WLP2>FUP	0.0077
Ingestive behaviour	Eating and drinking	NS		
All occurrences				
Affiliative	Allogrooming, social play, following, other affiliative	Time	AP<WLP2	0.0024
			FUP<WLP1, WLP2	0.0002, <0.0001
Offensive	Display, offensive aggression	Time	WLP2>AP, WLP1	0.0208, 0.0068
			AP<WLP1, WLP2	<0.0001, <0.0001
Coprophagy		Time	FUP<WLP1, WLP2	0.0180, 0.0285
Defensive	Defensive aggression, avoid	NS		0.0028, 0.0068

Abbreviations: AP, adaptation period; WLP1, weight loss period 1; WLP2, weight loss period 2; FUP, follow up period; CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group.

5.3.7 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 follow up period. For body weight, heart and belly girth, tail head, loin, and rib fat depth, the relative difference (% change) from the baseline value, i.e., week 5 for weight loss period and week 21.5 for follow up period, was used as the response variable. Absolute values were used for all 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%. A statistical trend was defined when $0.05 < P < 0.10$. Data are reported as mean ± SEM.

Proportions for each behavioural category were calculated per period as the total number of time this category was recorded divided by the total number of measurements (sample points in case of instantaneous recording or intervals for zero-one recording). Due to the extended duration of weight loss period compared to the adaptation period and follow up period, for the behavioural analysis, weight loss period was divided into weight loss period 1 (WLP1) (observation days 3 to 6) and weight loss period 2 (WLP2) (observation days 7 to 10), resulting in four periods.

The linear mixed model used included pony as random effect and treatment, period, and their interaction as fixed effects. When a significant effect was found, a multi comparisons post-hoc testing using Bonferroni adjustment was carried out to examine pair-wise differences.

5.4 Results

5.4.1 Feed intake & relative weight changes

Daily digestible energy intake (DEI) and daily dry matter intake (DMI) are described in table 5.1.

During the entire weight loss period (w5-21), CONTROL lost an average of 0.42 ± 0.45%, MOD 3.59 ± 0.63%, and SEV 10.81 ± 0.77% of their initial body weight. A more rapid weight loss was seen in the group with the highest energy restriction (SEV, 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%) (Figure 5.1; Table 5.3).

Table 5.3: Weight change as a proportion of the value at week 5 (start weight loss period)for each group throughout the WLP.

	Body weight change as a proportional value at week 5 (%)							
	after 4 weeks		after 8 weeks		after 12 weeks		after 16 weeks	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
CONTROL	-0.69*	0.45	0.03	0.90	0.54	0.59	0.42	0.45
MOD	0.94	0.68	2.32	0.63	3.58	0.71	3.59	0.63
SEV	2.74	1.47	5.52	1.36	8.70	2.27	10.81	1.88

Abbreviations: CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group; *, '-' value means weight gain.
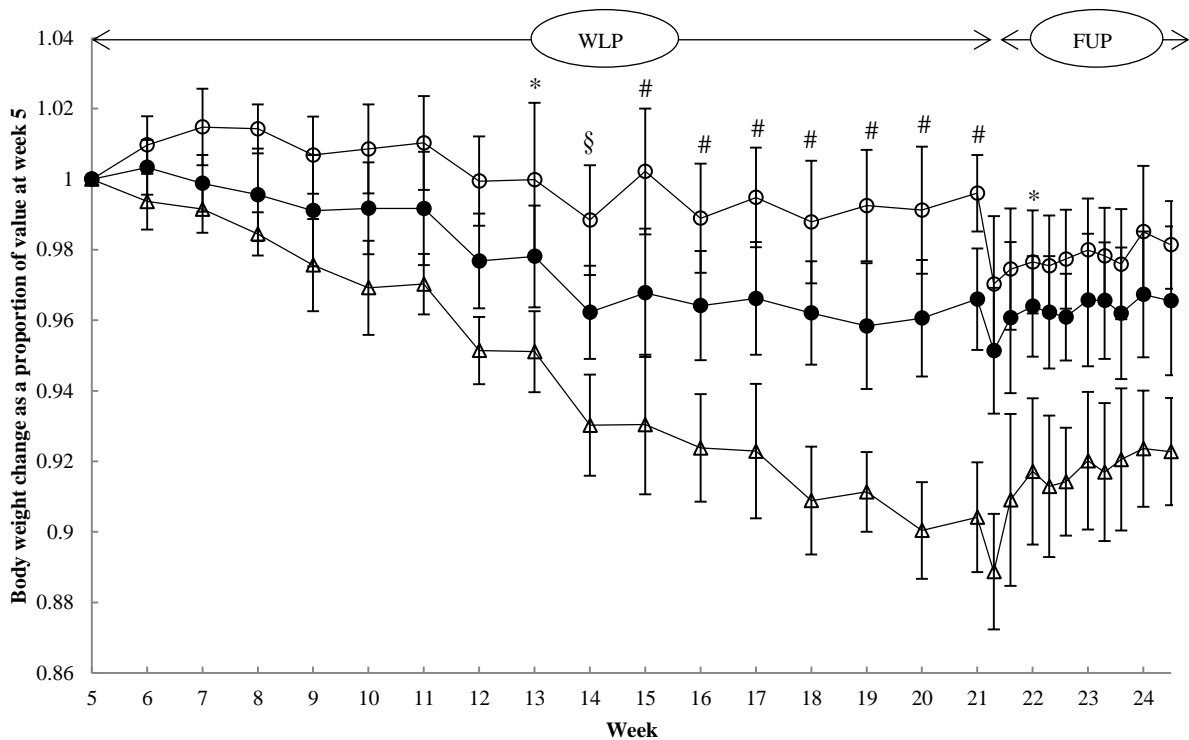

Figure 5.1: Body weight change (%) during the WLP and FUP as a proportion of the value at week 5 (beginning of WLP) in the control(○), moderate (●) and severe energy restricted (Δ) group. Data are given as means ± SEM. Significant differences between 2 or 3 groups at individual time points are indicated with symbols (*,γ, §) and differ from zero at a comparisonwise level of 0.05/g (g=16 during the WLP; g=9 during the EPP; g=number of comparisons; *: significant difference between control group and rapid weight loss group; §: significant difference between control group and the 2 weight loss groups; #: significant difference between the 3 treatment groups). Abbreviations: WLP, weight loss period; FUP, follow up period.

At the end of the follow up period, CONTROL, MOD, and SEV had respectively regained 1.11 ± 1.64, 1.41 ± 1.04, and 3.40 ± 0.94% of their BW at the start of the follow up period (w21.5). During the follow up period, a treatment effect was found between CONTROL and SEV (P<0.001), and MOD and SEV (P<0.001) meaning that SEV gained significantly more weight during the follow up period than MOD and CONTROL. When results from w24.5 (end of FUP) and w22.5 (one week after refeeding at 100% of iMERob determined in the adaptation period) were further evaluated, the difference in weight gain between the different treatment groups is negligible (relative weight gain of respectively 0.56, 0.26 and 0.68% in CONTROL, MOD and SEV). A possible explanation for the highest weight gain in the highest energy restricted group probably is the increase in gut fill compared to the weight loss period.

5.4.2 Changes in body condition score

At the end of the weight loss period, BCS was decreased more in SEV compared to MOD and CONTROL (P=0.016 and 0.006 respectively). A higher increase in BCS was found in SEV compared CONTROL by the end of the follow up period (Figure 5.2). The cresty neck score was numerically decreased in the two weight loss groups during the weight loss period, although no significant effects were found (data not shown).

5.4.3 Morphometric measurements

At the end of the weight loss period, loin and rib adipose tissue fat depth (Figure 5.3), and belly and heart girth (Figure 5.4) were lower in the two energy restricted groups compared to the control group (P=0.0482, 0.0235, 0.009 and 0.009 respectively). Only a time effect was found for tail head fat depth during the weight loss period (P<0.001) with decreasing fat depth in all 3 groups. At the end of the weight loss period, there was a trend (P=0.097) that tail head fat depth was decreased more in SEV compared to CONTROL.

During the follow up period, a time effect was found for loin adipose tissue fat depth (P=0.026). At the end of the follow up period, belly and heart girth were increased in the two energy restricted groups (P=0.007 and 0.003 respectively).
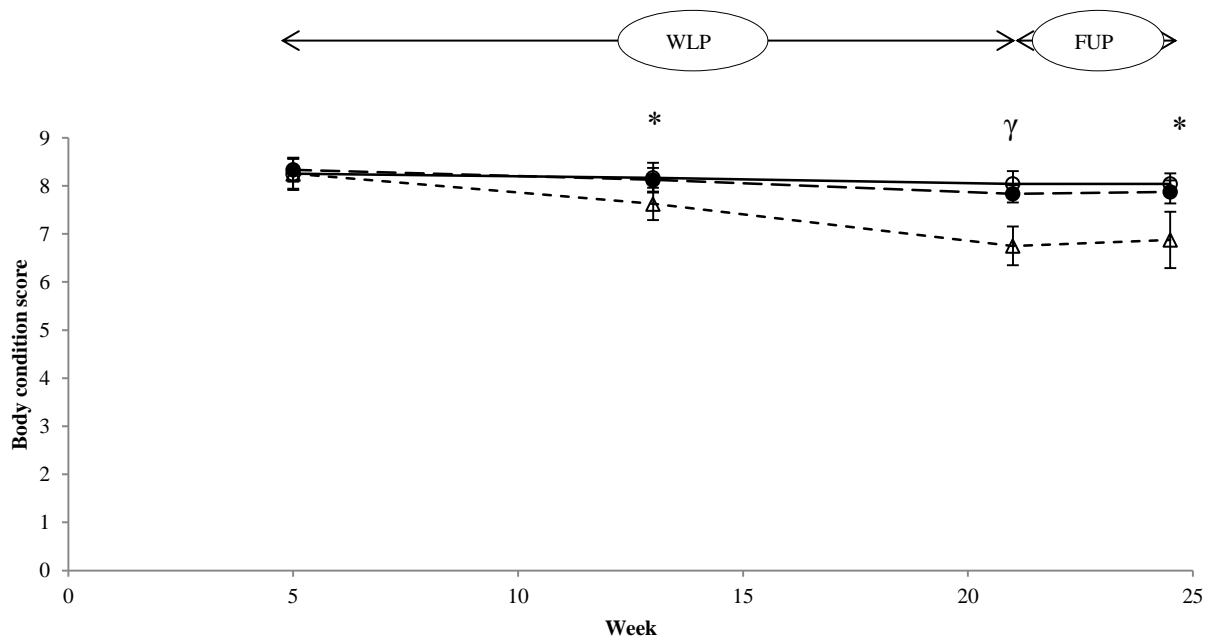
Figure 5.2: Body condition score during the WLP and FUP in the control (○), moderate (●), and severe energy restricted (Δ) group. Data are given as means ± SEM. Significant pairwise differences between 2 or 3 groups at individual time points are indicated with symbols (*,γ) and differ from zero at a comparisonwise significance level of 0.05/g (g=2 in WLP and 1 in FUP; g=number of comparisons; *: significant pairwise difference between control group and severe energy restricted group; γ: significant pairwise difference between the severe energy restricted group and the other 2 treatment groups). Abbreviations: WLP, weight loss period; FUP, follow up period.

5.4.4 Gastroscopy

At the start of the trial, a few non clinically significant gastric lesions were found in the 3 groups (lesion score ≤ 1). After 8 weeks, no lesions were found. By the end of the weight loss period, again a few non clinically significant lesions were seen, all scored ≤ 1. No significant treatment effect and/or time effect was found for gastric ulcer score (data not shown).

(A)

(B)

(C)
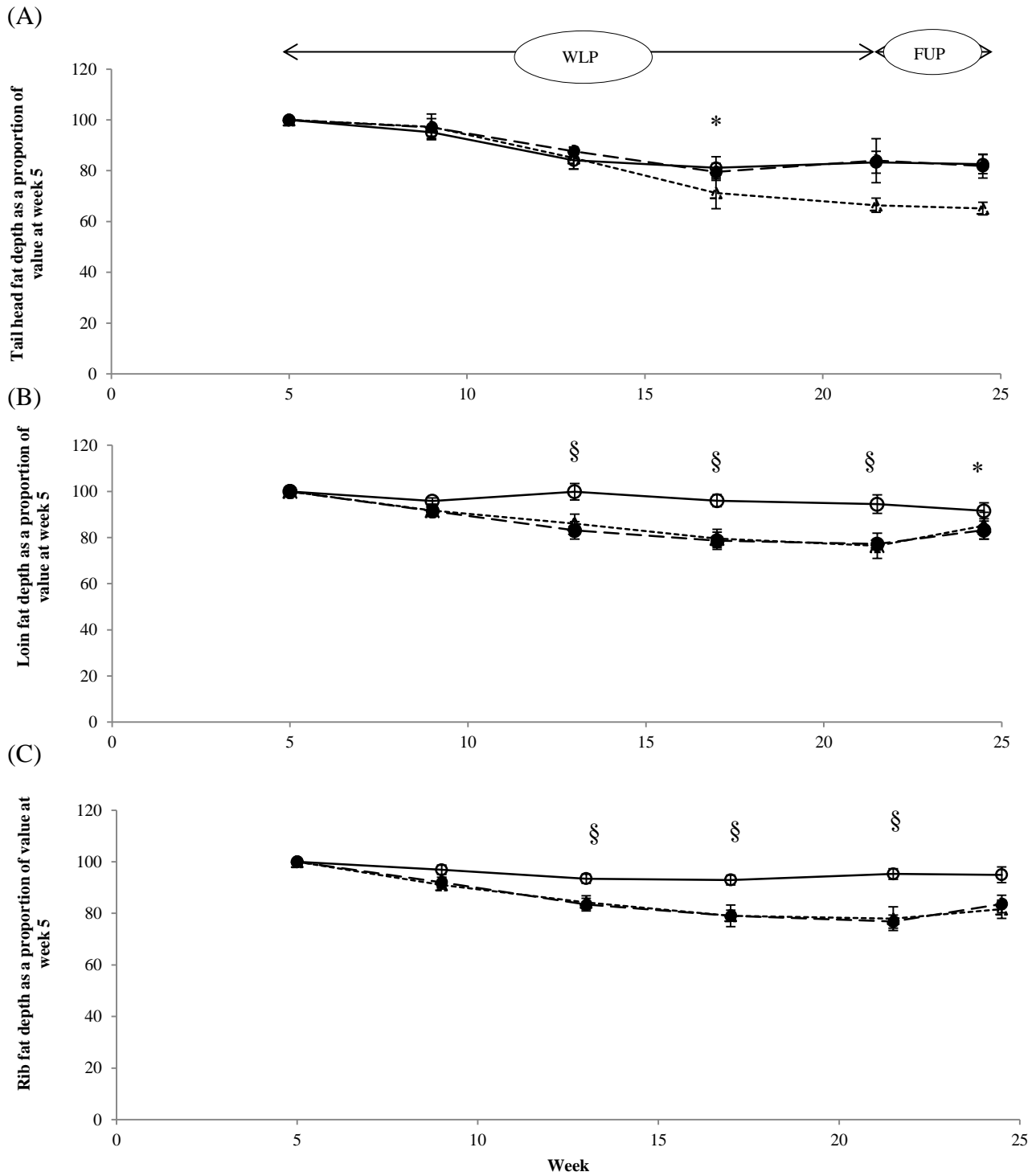
Figure 5.3: Fat depth change (%) during the WLP and FUP at the level of the tail head (A), loin (B), and ribs (C) as a proportion of the value at week 5 (beginning WLP) in the control (○), moderate (●), and severe (Δ) energy restricted group. Data are given as means ± SEM. Significant pairwise differences between 2 or 3 groups at individual time points are indicated with symbols (§,*) and differ from zero at a comparisonwise significance level of 0.05/g (g=4 in WLP and 1 in FUP; g=number of comparisons); §: significant pairwise difference between control group and the 2 weight loss groups; *: significant pairwise difference between control group and severe energy restricted group.
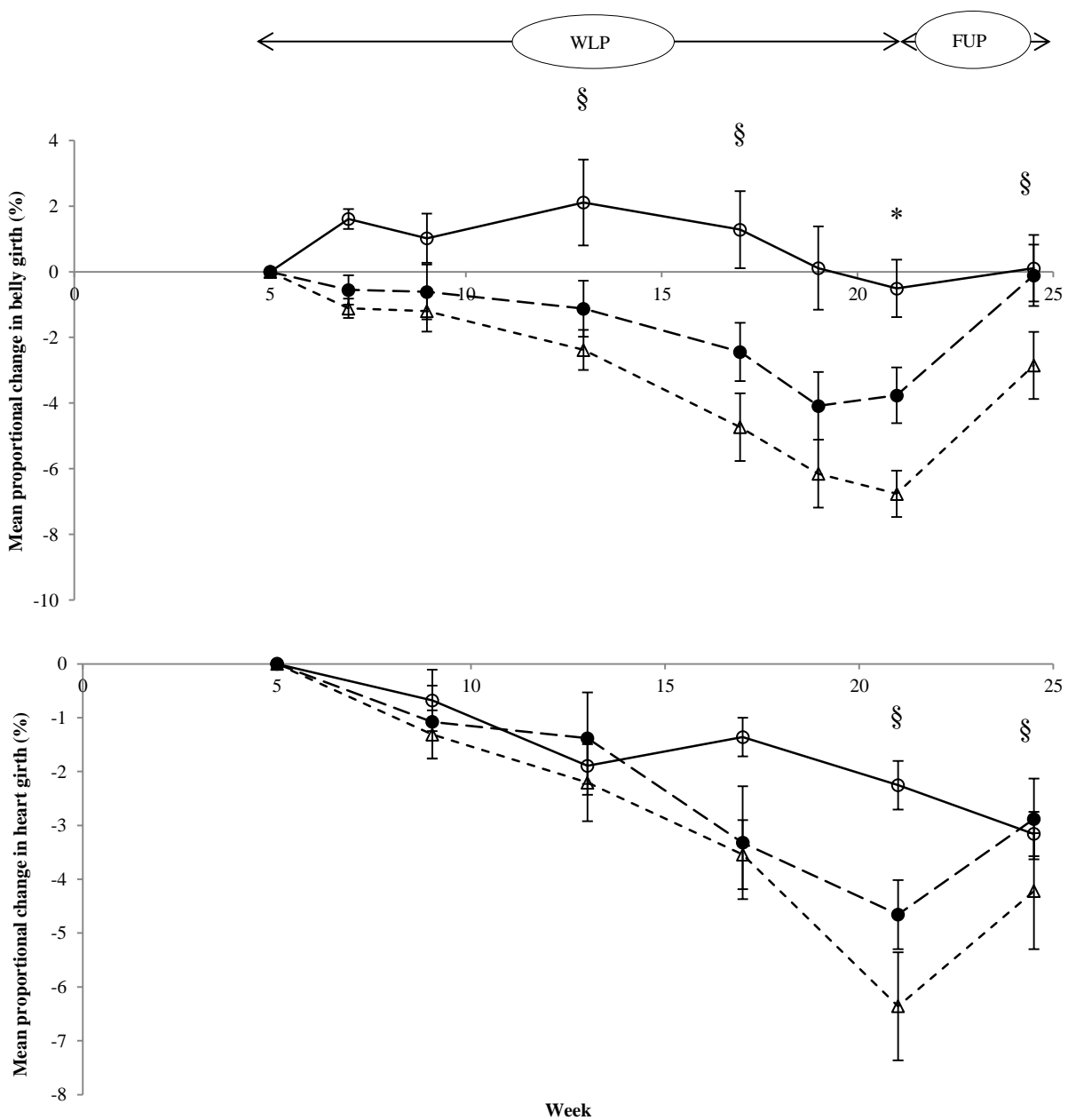
Figure 5.4: Belly girth (A) and heart girth (B) change (%) during the WLP and FUP as a proportion of the value at week 5 (beginning WLP) in the control (○), moderate (●), and severe energy restricted (Δ) group. Data are given as means ± SEM. Significant pairwise differences between 2 or 3 groups at individual time points are indicated with symbols (*,§) and differ from zero at a comparisonwise significance level of 0.05/g (g=6 in WLP and 1 in FUP for belly girth; g=4 in WLP and 1 in FUP for heart girth ; g=number of comparisons; *: significant pairwise difference between control group and severe energy restricted group; §: significant pairwise difference between control group and the 2 weight loss groups). Abbreviations: WLP, weight loss period; FUP, follow up period.

5.4.5.Behaviour analysis

For all occurrences observations, data were not analysed for 'performing stereotypies' and 'chewing fence', due to insufficient prevalence of these behaviours.

No significant interactions between time and period were found for any of the analysed behavioural parameters. Significant time or treatment effects are shown in Table 5.2.

A significant time effect was found for coprophagy (P<0.0001), with higher incidence in the 2 parts of the weight loss period compared to the adaptation period (P<0.0001 and P<0.0001) and follow up period (P=0.0180) and P=0.0285). There was a trend for a higher incidence of coprophagy in the follow up period compared to the adaptation period (P=0.0784). Although not statistically significant, in SEV there was a higher increase in the incidence of coprophagy in the weight loss period compared to the adaptation period (6.3 times higher incidence) compared to the MOD and CONTROL (2.2 and 3.8 higher increase respectively). Compared to the weight loss period, the incidence of coprophagy decreased in the follow up period in all 3 groups, but the largest decrease was seen in SEV (2.9 compared to 1.88 and 1.5 in MOD and CONTROL respectively).

No significant changes were seen for defensive and ingestive behaviour.

5.5 Discussion

Different levels of energy restriction clearly affected BCS, with the largest decrease in the severe energy restricted group, including a greater decrease in BCS per unit of energy restriction in this group. This is in accordance with other weight loss trials (Van Weyenberg et al. 2008; Gordon et al., 2009), although in one study considerable weight loss percentages (mean 11.4 ± 1.9%) in a group of 5 overweight/obese Welsh Mountain pony mares were accompanied by only minor decreases in BCS (average 0.3 ± 0.14 BCS points). Fat loss, however, accounted for 45 ± 19% of the body mass lost (Dugdale et al., 2010). Weekly weight losses from week 2 onwards in that trial (0.7 ± 0.1% of BM at end of week 1) were comparable with the weight losses in the severe energy restricted group of the present trial (0.7 ± 0.01%). Comparable weekly weight loss was achieved with a daily DMI of 1% of actual BM in that trial and a restriction of 1.13% daily DMI in the present study. Possible confounding factors can be breed (Welsh Mountain ponies compared with Shetland ponies), gender (mares compared to geldings), type of diet (complete chaff based diet compared with hay and vitamin/protein/mineral supplement), and actual DE intake.

During the weight loss period, CNS decreased in the two weight loss groups, but no significant differences between these two groups were found, indicating that a significant

decrease in BCS is not necessarily accompanied by a significant decrease in CNS. This might suggest that depending on the rate of weight loss, different adipose tissues are utilised. The same effect was seen for the subcutaneous adipose tissue depots at the level of the loin, ribs, and tail head. A higher rate of weight loss clearly decreased fat depth in all three AT depots, whereas the lower weight loss rate only decreased fat depth at the level of the loin and ribs relative to the control group.

Girth circumference progressively decreased throughout the weight loss period in the two weight loss groups. The reason for decreased belly circumference is suggested to be twofold: decreased gut fill and/or decreased intra-abdominal fat tissue (Argo et al., 2012).

In equines, it is not known which depot favourably will be mobilised during weight loss. Environmental temperature seems to have an important influence on subcutaneous adipose tissue (re)distribution in equines undergoing a weight loss treatment, as a re-sequestering of fat into the subcutaneous adipose tissue region was seen in a weight loss trial performed in cold ambient temperatures (Argo et al., 2012).

The decrease in heart girth found in all three groups during the weight loss period can either be the effect of fat tissue loss in that depot in proportion of general weight loss, or a consequence of a redistribution of fat from the heart area to other adipose tissue depots in the transition from summer to winter, which could explain the fact that a decrease in heart girth also occurred in the control group. As the number of fat depth measurements in the present study was limited (3 regions: rib, loin and tail head), this suggestion of redistribution could not be evidenced. Future research with fat depth measurements on a regular basis (monthly) in animals with a stable body weight throughout an entire year, could give more information if fat redistribution is an adaptive response to seasonal temperature changes in equines.

According to Ellis (2010), foraging and coprophagy can be seen as a common part of the feed intake behaviour in equids as it is also seen in wild and free-ranging horses. The incidence of coprophagy in the severe energy restricted group was higher at the beginning of the weight loss period compared to the adaptation period, which could be an indication of an increased motivation for foraging due to a decrease in fibre intake. As horses rarely fast for more than 3-5 hours when forage is freely available (Ralston et al., 1984), coprophagy can reflect the motivation to feed outside of mealtimes (Hothersall and Nicol, 2009). It may represent attempts to ingest fibre as coprophagy often decreases by greater provision of roughage (Hothersall and Nicol, 2009). However, all ponies in the present study showed coprophagic behaviour, suggesting that for our test population, also other factors than

forage/fibre restriction were responsible for this phenomenon. Copying behaviour, as has been described for other 'unwanted' behaviours in horses (Lindberg 1999, Albright 2009), could have played a role. Ingesting food (in the present case faeces) could be a strategy to reduce gastric acidity during times when no forage is available, as horses only produce saliva when biting or chewing (Alexander, 1966). However, coprophagy in this study was already present in 11 of the 18 animals (4 from CONTROL, 4 from MOD, and 3 from SEV) during the adaptation period (when not restricted), suggesting that restriction was not responsible for the initiation in most of the animals. The presence of this coprophagic behaviour could, however, partly explain why no gastric ulcers were found in these ponies, even those in the severe energy restricted group that had no access to forage for about 12 to 15 hours (depending on feed consumption rate). Another factor that may have reduced the risk of ulcer formation may have been how they were managed. As horses are social animals (Goodwin, 2007; Krueger and Heinze 2008), and the ponies in the present study were composed of the same members for several months, we could assume that the possibility to have interactions with the other ponies could lower their stress levels and could be another reason why they did not develop gastric ulcers, as outside of the feeding moments, they were group housed. Another consequence of this housing system is that any behavioural change, is not necessarily solely due to the energy restriction regimen as inter-individual relationships and/or temperament of the individual pony could potentially be more important than the energy restriction regimen. Therefore, it is inappropriate to make further conclusions concerning behaviour as the triggering factors cannot be unambiguously identified.

5.6 Conclusions

The level of energy restriction influenced the morphometric profile. The more severe level of energy restriction in Shetland geldings resulted in significant reduction of general obesity (BCS). Animal welfare was apparently not compromised by any of the two energy restriction regimens.

5.7 Acknowledgements

5.8 References

Albright JD, Mohammed HO, Heleski CR, Wickens CL, Houpt KA (2009) Crib-biting in US horses: Breed dispositions and owner perception of aetiology. Equine Vet. J. 41, 455-458.

Alexander F (1966) A study of parotid salivation in the horse. J. Physiol. 184, 646–656.

Andrews FM, Sifferman RL, Bernard W, Hughes FE, Holsteo JE, Daurio CP, Alva R., Cox JL (1999) Efficacy of omeprazole paste in the treatment and prevention of gastric ulcers in horses. Equine Vet. J. Suppl 29, 81-86.

Argo C.McG, Curtis GC, Grove-White D, Dugdale AHA, Barfoot CF, Harris PA (2012) Weight loss resistance: A further consideration for the nutritional management of obese *Equidae*. Vet. J. 194, 179-188..

Carter RA, Treiber KH, Geor RJ, Douglass L, Harris PA (2009) Prediction of incipient pasture-associated laminitis from hyperinsulineamia and generalized and localized obesity in cohort of ponies. Equine Vet. J. 41, 171-178.

Carter RA, McCutcheon LJ, Valle E, Meilahn EN, Geor RJ (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.

Cooper JJ, Albentosa MJ (2005) Behavioural adaptation in the domestic horse: potential role of apparently abnormal responses including stereotypic behaviour. Livest. Prod. Sci. 92, 177-182.

Curtis GC, Barfoot CF, Dugdale AHA, Harris PA, Argo McG (2011) Voluntary ingestion of wood shavings by obese horses under dietary restriction. Br. J. Nutr. 106, S178-S182.

Davidson N, Harris P (2002) Nutrition and welfare. Waran N. (Ed.), The Welfare of Horses, Kluwer Academic Press, Amsterdam, pp. 45–76.

Dugdale AHA, Curtis GC, Cripps P, Harris PA, Argo CMcG (2010) Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares. Equine Vet. J. 42, 600-610.

Dugdale AHA, Grove-White D, Curtis GC, Harris PA, Argo CMcG (2012) Body condition scoring as a predictor of body fat in horses and ponies. Vet. J. 194, 173-178.

Ellis AD (2010) Biological basis of behaviour and feed intake. In: Ellis, AD, A.C. Longland and M. Coenen (eds.) The impact of nutrition on the health and welfare of horses. EAAP Publication No. 128. Wageningen Academic Publishers, Wageningen, the Netherlands, 53-74.

Frank N, Elliott SB, Brandt LE, Keisler DH (2006) Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. J. Am. Vet. Med. Assoc. 228, 1383-1390.

Geor RJ (2008) Metabolic predisposition to laminitis in horses and ponies: obesity, insulin resistance and metabolic syndromes. J. equine vet. Sci. 28, 753-759.

Geor RJ, Harris PA (2009) Dietary management of obesity and insulin resistance: countering the risk for laminitis. Vet. Clin. N. Am.-Equine 25, 51-65.

Gobesso AAO, Françoso R, Toledo RAD, Centini TN, Gonzaga IVF, Gil PCN, Baldi F (2012) Evaluation of body condition score in horses by ultrasonography. In: Saastamoinen, M., Fradinho, M.J., Santos, A.S., Miraglia, N. (Eds.), Forages and Grazing in Horse Nutrition. EAAP Scientific Series, Publication 132. Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 387–390.

Goodwin D (2002) Horse behaviour: Evolution, Domestication and Feralisation. Waran N. (Ed.), The Welfare of Horses, Kluwer Academic Press, Amsterdam, pp. 1–18.

Gordon ME, Jerina ML, Raub RH, Davison KA, Young JK, Williamson KK (2009) The effects of dietary manipulation and exercise on weight loss and related indices of health in horses. Comp. Exerc. Physiol. 6, 33-42.

Harker IJ, Harris PA, Barfoot CF (2011) The body condition score of leisure horses competing at an unaffiliated championship in the UK. J. Eq. Vet. Sci. 31, 253-254.

Henneke DR, Potter GD, Kreider JL, Yeates BF (1983) Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15, 371-372.

Hothersall B, Nicol C (2009) Role of diet and feeding in normal and stereotypic behaviors in horses. Vet. Clin. N. Am-Equine 25, 167-Alexander F. (1966) A study of parotid salivation in the horse. J. Physiol. 184, 646-656.

Johnson PJ (2002) The equine metabolic syndrome peripheral Cushing's syndrome. Vet. Clin. North. Am. Equine Pract. 18, 271-293.

Krueger K, Heinze J (2008) Horse sense: social status of horses (*Equus caballus*) affects their likelihood of copying other horses' behavior. Anim. Cogn. 11, 431-439.

Laflamme DP, Kulman G (1995) The effect of weight loss regimen on subsequent weight loss maintenance in dogs. Nutr. Res. 15, 1019-1028.

Lindberg AC, Kelland A, Nicol CJ (1999) Effects of observational learning on acquisition of an operant response in horses. Appl. Anim. Behav. Sci. 61, 187-199.

Murray MJ, Eichorn ES (1996) Effects of intermittent feed deprivation, intermittent feed deprivation with ranitidine administration, and stall confinement with ad libitum access to hay on gastric ulceration in horses. Am. J. Vet. Res. 57, 1599-603.

Ödberg FO, Francis-Smith K (1976) A Study on Eliminative and Grazing Behaviour — The Use of the Field by Captive Horses. Equine Vet. J. 8, 147-149.

Prache S, Gordon IJ, Rook AJ (1998) Foraging behaviour and diet selection in domestic herbivores. Ann. Zootech. 47, 335-345.

Quinn RW, Burk AZO, Suagee JK, Hartsock TG (2006) Equine obesity: implications for health and strategies for control. Proceedings of the 4th MID-ATLANTIC NUTRITION CONFERENCE March 29-30, 2006 Timonium, Maryland, p23-36.

Ralston SL, Baile CA (1983) Factors in the control of feed intake of horses and ponies. Neurosci. Biohav. R. 7, 465-470.

Robin CA, Ireland JL, Wylie CE, Collins SN, Verheyen KLP, Newton JR (2014) Prevalence and risk factors for equine obesity en Great Britain based on owner-reported body condition scores. Equine Vet. J. DOI: 10.1111/evj.12275.

Sweeney ME, Hill JO, Heller PA, Baney R, DiGirolamo M (1993) Severe vs moderate energy restriction with and without exercise in the treatment of obesity: efficiency of weight loss. Am. J. Clin. Nutr. 57, 127-134.

Treiber K, Kronfeld D, Hess T, Byrd B, Splan R, Stanair W (2006) Evaluation of genetic and metabolic predispositions and nutritional risk factor for pasture-associated laminitis in ponies. J. Am. Vet. Med. Assoc. 228, 1538-1545.

Van Weyenberg S, Hesta M, Buyse J, Janssens GPJ (2008) The effect of weight loss by energy restriction on metabolic profile and glucose tolerance in ponies. J. Anim. Physiol. Anim. Nutr. 92, 538-545.

CHAPTER 6

OXIDATIVE STRESS CHANGES IN ENERGY RESTRICTED PONIES

CHAPTER 6: CHANGES IN OXIDATIVE STRESS IN RESPONSE TO DIFFERENT LEVELS OF ENERGY RESTRICTION IN OBESE PONIES

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6.1 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≥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 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% (moderate energy restricted group, MOD) or 60% (severe energy restricted group, SEV) of their MERob. During the 3-week follow up period, all ponies were again fed 100% of their MERob. Relative weight loss was higher in SEV ($P<0.001$) compared with MOD. 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 follow up period was higher in SEV ($P<0.001$) compared with MOD and CONTROL. During the weight loss period, triacylglycerol and non-esterified fatty acids levels were highest in SEV, as were α-tocopherol and ferric reducing ability of plasma. After 8 weeks of weight loss, advanced oxidation protein products were higher in SEV compared to MOD 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.

6.2 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 (Thatcher et al., 2008; Wyse et al., 2008). Obesity is associated in particular with an increased risk of insulin resistance as well as laminitis (Quinn et al., 2006; Geor et al., 2008; Frank et al., 2010). 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 (King and Mansmann, 2004). Recently, several equine studies have been published looking at the efficacy of weight loss programmes with and without exercise (Carter et al., 2010; Dugdale et al., 2010; Argo et al., 2012).

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 (low calorie 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) (Argo et al., 2012). However, it is well known that too severe caloric restriction in obese equidae may lead to hyperlipemia (McKenzie, 2011). 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 (Laflamme and Kuhlman, 1995). This rebound weight gain was significantly correlated with the amount of lost body weight and the caloric restriction level (Laflamme and Kuhlman, 1995). This effect was also seen in human patients (Weiss et al., 2007).

In obese humans, oxidative stress is related to chronic disease (e.g. hypertension, diabetes, metabolic syndrome, polycystic ovarian syndrome, liver disease) (Vincent et al., 2007). Human obesity increases the level of oxidative stress (Suzuki et al., 2003) as indicated by increased lipid peroxidation (Dandona et al., 2001; Roberts et al., 2009) and decreased systemic antioxidants (Vincent et al., 2007; Roberts et al., 2009; Ozata et al., 2002; Reitman et al., 2002; Mohn et al., 2005). Moreover, the altered oxidant-antioxidant status in obese children was reversible by a dietary restriction-weight loss program (Mohn et al., 2005; Kelishidaki et al., 2008). Restricted caloric intake reduced oxidative damage to proteins, lipids, and DNA in rodents (Gredilla et al., 2005). It also reduced serum advanced glycation

end products formation in healthy and overweight or obese human adults (Gugliucci et al., 2009). In horses, oxidative stress has been associated with several disorders such as recurrent airway obstruction (Kirschvink et al., 2002; Deaton et al., 2004), joint disease (Dimock et al., 2000), neurological disorders (Divers et al., 2006), and perfusion related disorders (Kirschvink et al., 2008). Laminitis can be classified under this last condition. Equine digital laminae have relatively limited SOD capacity, which may make this tissue more susceptible to damage by reactive oxygen species, such as superoxide anion (Loftus et al., 2007). Insulin induced laminitis however has been associated with the accumulation of advanced glycation end products in the lamellar tissues of horses (de Laat et al., 2012). Insulin resistance, together with general obesity, regional accumulations of fat tissue and hyperleptinemia are features of equine metabolic syndrome (EMS) (Geor et al., 2008). 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 laminitis or a current episode of laminitis compared with ponies with no recent history of laminitis (Valle et al., 2012). Lower antioxidant capacity, as measured by decreased erythrocyte glutathione peroxidase activities, has also been described in obese horses (Pleasant et al., 2013).

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, as well as 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 (severe energy restricted) would be accompanied by a greater weight gain when the ponies were fed again at maintenance energy levels.

6.3 Materials and methods

6.3.1 Animals and study design

The study protocol has been described in Chapter 5. Briefly, 18 obese Shetland ponies (BCS ≥ 7/9; Henneke et al., 1983) with an average age of 9.3 years (± 3.9 year) (figure 6.1) were studied over a 23.5 week period (August to January). Average body condition score at the start of the study (BCS) was 8 ± 1 (scale 1 = emaciated to 9 = obese) (Table 5.1). In accordance with the range reported by Treiber et al. (2005) (1.22-40.40mU/l), baselines of insulin values at the start of the adaptation period and weight loss period were normal (range

5.5-39.6 mU/l). 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. Ponies were individually housed during feeding times for 4 hours before noon (9 am to 1 pm) and 5 hours in the afternoon (3 pm to 8 pm). During the rest of the day, ponies were group housed. On the floor of the loose barn, rubber mats were placed as bedding material. Water was freely available under all circumstances. Environment temperature was measured daily, minimum and maximum temperatures were 1.8 and 27.6°C respectively. The study design was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011/098).

Table 6.1: Phenotypic summary of each study group of obese ponies 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
MOD	9.0	1.5	170.3	38.0	28.0	4.0	8.2	0.3
SEV	9.3	1.7	154.1	25.6	27.4	6.4	8.3	0.3

Abbreviations: CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group.

The study was divided into three periods: 1) an adaptation period of 4 weeks in which individual maintenance energy intakes required to maintain stable obese body weight (iMERob) were determined , 2) a weight loss period of 16.5 weeks in which ponies were divided into a control group (CONTROL: receiving 100% of iMERob), a moderate energy restricted group (MOD: receiving 80 % of iMERob), and a severe energy restricted group (SEV: receiving 60% of iMERob); and 3) a follow up period of 3 weeks in which all ponies were again fed at 100% of iMERob determined in the adaptation period (Table 6.2).

Diet consisted of a low energy/high fibre hay (DE 8.1 MJ/kg and crude fibre 371.8 g/kg on a dry matter basis) offered twice a day in haylage nets. A vitamin, protein, and mineral balancer at approximately 1.32 g/kg ideal body weight (BW)/d (Spillers Gro'N Win®, MARS Horsecare) was given with the morning feeding (Table 6.3).

Table 6.2: Energy intake during adaptation period, weight loss period, and follow up period in obese ponies. Individual maintenance requirements to maintain stable obese body weight were determined during the adaptation period. 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	Energy intake (% of iMERob)	Energy intake (MJ/kg BW)		% of offered hay consumed	
	Adaptation period and follow up period				
	Mean	Mean	SE	Mean	SE
CONTROL	100%	0.17	0.01	97.50	1.45
MOD	100%	0.17	0.01	99.84	0.21
SEV	100%	0.17	0.01	99.98	0.06
	Weight loss period				
	Mean	Mean	SE	Mean	SE
CONTROL	100%	0.17	0.01	99.63	0.31
MOD	80%	0.14	0.00	99.21	1.42
SEV	60%	0.10	0.01	98.80	1.7

Abbreviations: iMERob, individual maintenance energy requirements to maintain stable obese body weight; CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group.

Table 6.3: 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	360.3	g/kg
Crude ash	63.3	168.9	g/kg
Crude fibre	371.8	56.3	g/kg
Crude fat	14.7	NI	g/kg
Starch	12.4	56.3	g/kg
alpha-tocopherol	17.5	1688.9	mg/kg
DE	8.1	13.5	MJ/kg

Abbreviations: NI, not indicated on label; DE, digestible energy.

6.3.2 Determination of body mass

As described in Chapter 5, ponies were weighed ($\pm$ 0.1 kg) 3 times a week during the adaptation period and follow up period. During weight loss period, ponies were weighed weekly.

6.3.3 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.

6.3.4 Measurements of plasma glucose and serum 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 (Moreaux et al., 2011; Bruynsteen et al., 2013).

Serum insulin concentrations were measured immunoradiometrically (Van Weyenberg et al., 2008; Bruynsteen et al., 2013) (insulin IRMA Ref 5251, Diasource Europe S.A., Nivelles, Belgium). An implementation validation procedure has previously been described briefly by Bruynsteen et al. (2013).

6.3.5 Measurements 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.

6.3.6 Measurements of antioxidant status markers

Analysis of FRAP was determined by spectrophotometrical analysis (Monarch Chemistry System, Instrumentation Laboratories, Zaventem, Belgium) as described by Benzie and Strain (1996) and previously validated in horses by Balogh et al. (2001). 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 litre 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).

6.3.7 Measurements of oxidant status markers

The TBARS concentration was measured spectrophotometrically (Monarch Chemistry System, Instrumentation Laboratories, Zaventem, Belgium) as described by Lin et al. (2004) 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. (2013) 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 λ_{ex} 335 nm and λ_{em} 385 nm wavelength. A PENT synthetic standard (prepared as described by Grandhee and Monnier, 1991) 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). 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.

6.3.8 Measurements of serum leptin concentrations

Leptin was measured using a multispecies RIA kit (Millipore, St. Charles, Missouri, USA) validated for the use in horses (McMannus and Fitzgerald, 2000). 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).

6.3.9 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 follow up 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 follow up 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%. Data are reported as means ± SEM.

6.4 Results

6.4.1 Feed intake

Daily hay intake throughout the study is described in table 6.3. All ponies ate all of the balancer throughout the entire study. The average DMI at the end of the adaptation period in the CONTROL, MOD, and SEV 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 during the weight loss period in CONTROL, MOD, and SEV was 1.86 ± 0.18, 1.57 ± 0.12, and $1.17\% \pm 0.16$ of the BW at the end of the adaptation period. During the follow up period, ponies received the same DMI as in the adaptation period. Energy intake is described in table 6.3.

6.4.2 Relative weight changes

As mentioned in Chapter 5, greater weekly weight loss ($P<0.001$) was seen in the group with the severe energy restriction (SEV; 0.68%) compared to MOD (0.22%) and CONTROL (0.03%).

Weight regain during the follow up period was higher in SEV ($3.40 \pm 0.94\%$) compared to MOD ($1.41 \pm 1.04\%$) and CONTROL ($1.11 \pm 1.64\%$) ($P<0.001$).

6.4.3 Plasma glucose and serum insulin concentrations

Throughout the weight loss period, glucose values (range 65-91 mg/dl) changed over time independently of treatment ($P<0.001$). No significant effects were found for insulin during the weight loss period and/or follow up period. Insulin means at all time points were <30mU/l, although at the end of the weight loss period there were 2 ponies with insulin values >30mU/l, 75.6 mU/l (CONTROL) and 75.2 mU/l (MOD) respectively. At the end of the follow up period, another 4 ponies (2 CONTROL, 1 MOD and 1 SEV) had values >30mU/l (range 40.9-73.3 mU/l).

6.4.5 Markers of lipid metabolism

Higher and more rapid maximum TAG values were reached in SEV ($P=0.001$) during the first part of the weight loss period (Figure 6.1). At the end of the follow up period, TAG values were decreased more rapidly in SEV compared to MOD and CONTROL ($P<0.001$).

The NEFA concentrations changed during the weight loss period ($P<0.001$), with higher values in SEV compared to MOD and CONTROL ($P=0.021$). At the end of the follow

up period, the NEFA concentrations were decreased in SEV, whereas in CONTROL and MOD values respectively increased and remained stable (P=0.014) (data not shown).
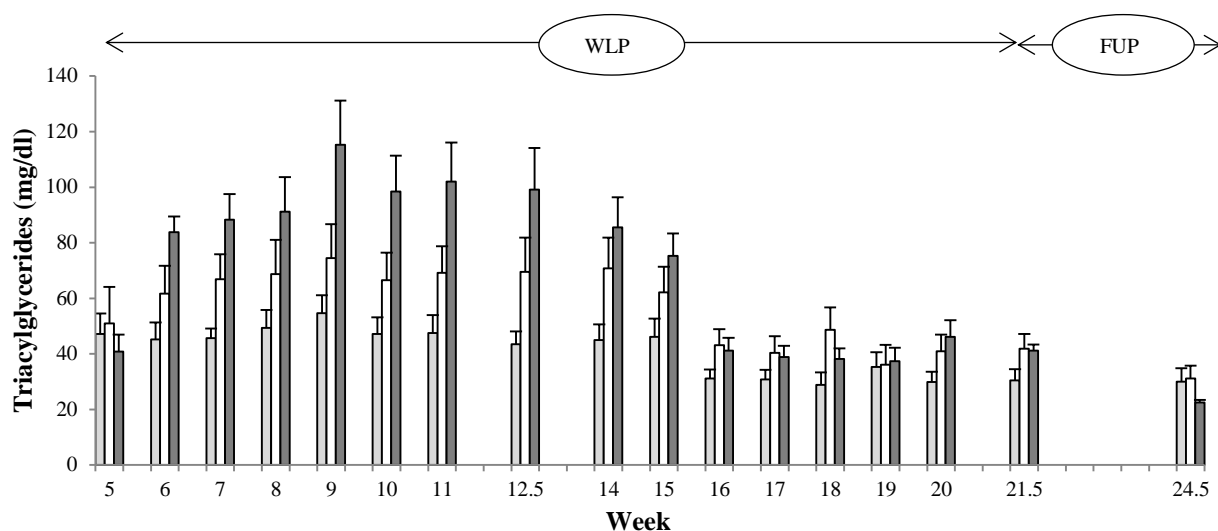

Figure 6.1: Serum triacylglycerol (TAG) concentration in obese ponies during the weight loss period and follow up period in the CONTROL (light grey bar), MOD (white bar) and SEV (dark grey bar) group. Data are given as means ± SEM. During the weight loss period, and follow up period, a significant interaction between time and treatment was seen (P=0.001 and P<0.001 respectively). Abbreviations: CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group; WLP, weight loss period; FUP, follow up period.
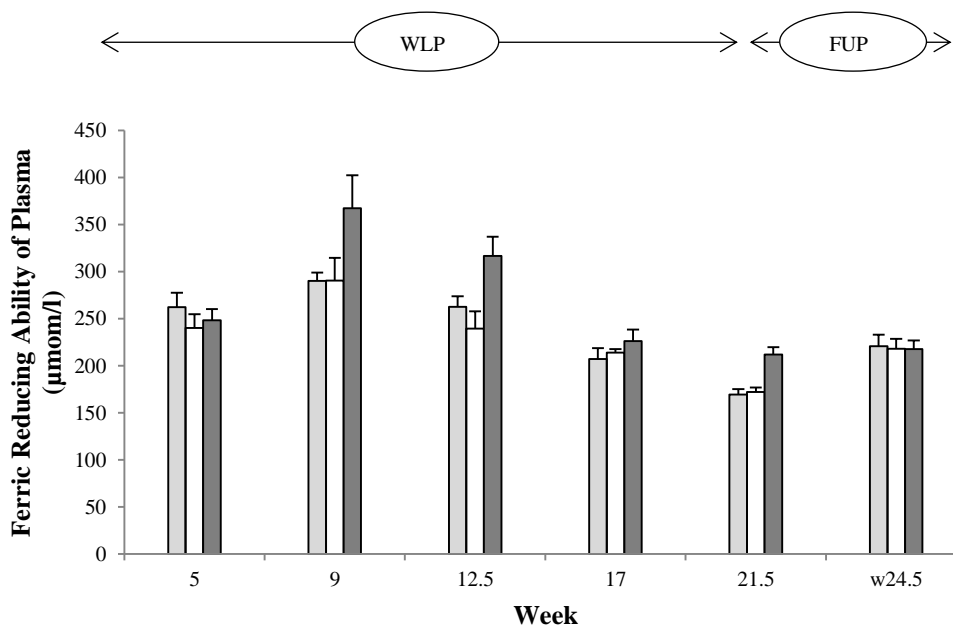
Figure 6.2: Serum ferric reducing ability of plasma (FRAP) concentration in obese ponies during the weight loss period and follow up period in the CONTROL (light grey bar), MOD (white bar) and SEV (dark grey bar) group. Data are given as means ± SEM. During the weight loss period, a significant time (P<0.001) and treatment (P<0.001) effect was seen. During the follow up period, a significant interaction between time and treatment was seen (P<0.001). Abbreviations: CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group; WLP, weight loss period; FUP, follow up period.

6.4.6 Antioxidant status

The FRAP concentrations changed during the weight loss period (P<0.001), with the highest values in SEV (P<0.001) (Figure 6.2). Comparing the end of the follow up period with the beginning, FRAP concentrations increased in MOD and CONTROL, whereas they remained constant in SEV (P<0.001).

During the weight loss period, SOD values changed (P=0.036) with lower values in SEV after 8 weeks of weight loss (P=0.003). The SOD concentrations increased in the three groups during the follow up period (P=0.003) (data not shown).

The α-tocopherol values changed over time during the weight loss period with an increase in SEV compared to more stable values in MOD and slightly decreasing values in CONTROL (P=0.004) (Figure 6.3). Rapidly decreasing values were noticed in CONTROL and SEV during the follow up period, whereas α-tocopherol values only slightly decreased in MOD (P<0.001).

6.4.7 Oxidant status

During the weight loss and follow up 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 three treatment groups, with an increase in SEV after 8 weeks of weight loss compared to stable or decreasing values in MOD and CONTROL respectively (P=0.015) (figure 6.4).

No significant effects were found for PENT and CML.

6.4.8 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 SEV (Figure 6.5). At the end of the follow up period, most rapidly decreasing leptin values were found in CONTROL compared to SEV and MOD (P<0.001).

Figure 6.3: Serum α-tocopherol concentration in obese ponies during the weight loss period and follow up period in the CONTROL (light grey bar), MOD (white bar) and SEV (dark grey bar) group. Data are given as means ± SEM. During the weight loss period and follow up period, a significant interaction between time and treatment was seen (P=0.004 and P<0.001 respectively). Abbreviations: CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group; WLP, weight loss period; FUP, follow up period.
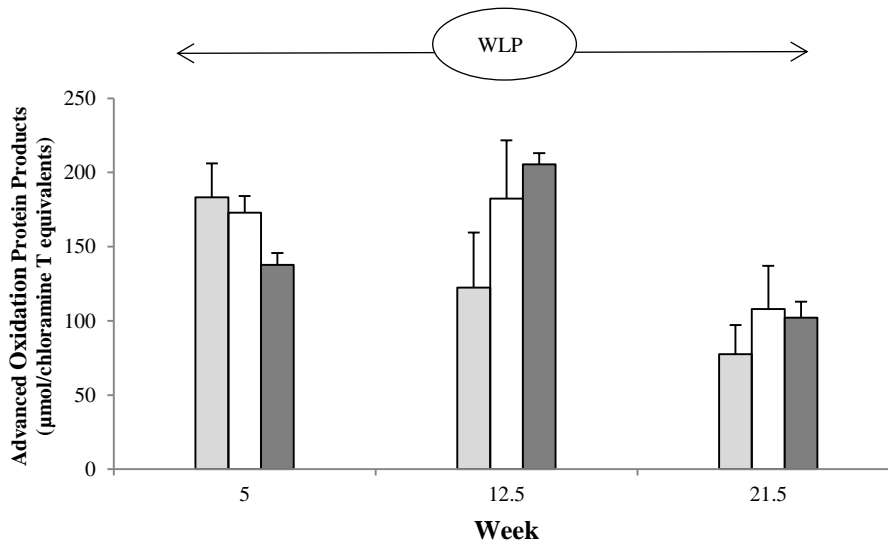
Figure 6.4: Plasma AOPP concentration in obese ponies during the weight loss period in the CONTROL (light grey bar), MOD (white bar) and SEV (dark grey bar) group. Data are given as means ± SEM. A significant interaction between time and treatment was seen (P=0.015). Abbreviations: AOPP, advanced oxidation protein products; CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group; WLP, weight loss period.
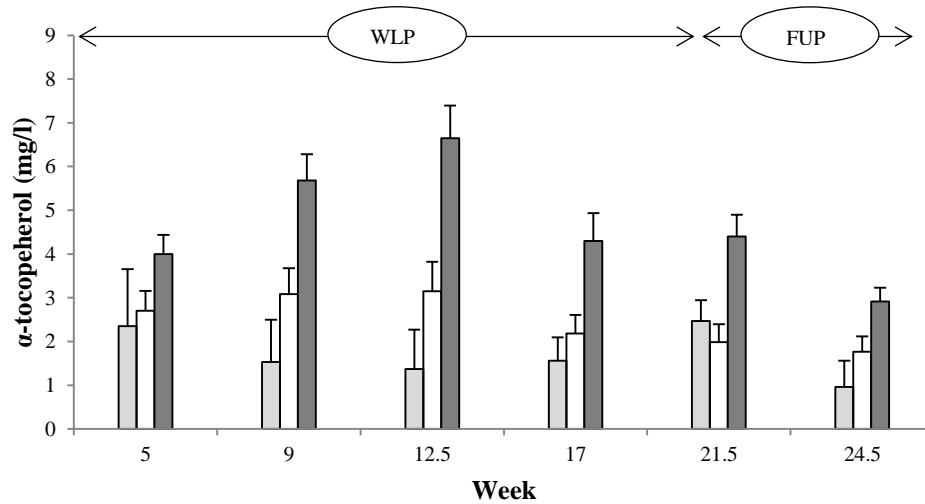
Figure 6.5: Serum leptin concentration in obese ponies during the weight loss period in the CONTROL (light grey bar), MOD (white bar) and SEV (dark grey bar) group. Data are given as means ± SEM. During the weight loss period, a significant time ($P=0.002$) and treatment ($P=0.001$) effect was seen. During the follow up period, a significant interaction between time and treatment was seen ($P<0.001$) Abbreviations: CONTROL, control group; MOD, moderate energy restricted group; SEV, severe energy restricted group; WLP, weight loss period; FUP, follow up period.
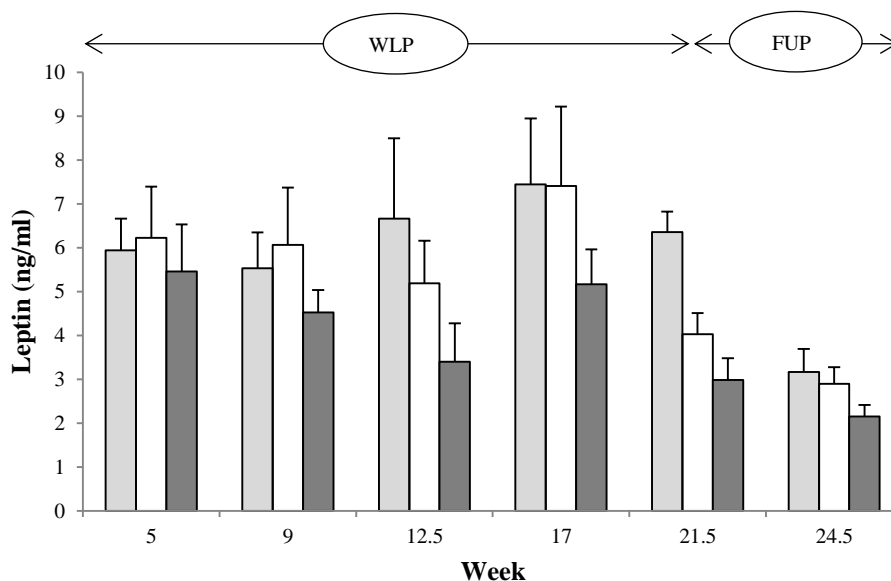
6.5 Discussion

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 (SEV) was associated with the greatest weight loss, as has also been described previously in dogs (Laflamme and Kuhlman, 1995). A doubling of the percentage of caloric restriction was associated with a tripling in percentage of weight loss (3.59% in MOD compared to 10.81% in SEV). The highest energy restricted group (SEV), however, also showed the greatest weight gain when they received again their 100% iMERob, a similar finding to that described in dogs (Laflamme and Kuhlman, 1995) and humans (Weiss et al., 2007). Increased metabolic efficiency, as has been described in obesity-prone rats after weight loss (McLean et al., 2004), 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 (McLean et al., 2004). MacLean and colleagues (2004) 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 SEV corresponded to 0.68%, which is comparable to the losses described in the weight loss trial of Dugdale and co-workers (2010) 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 SEV 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 (2010).

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 (Van Weyenberg et al., 2008; Ungru et al., 2012). 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 (Radin et al., 2009). 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 (McKenzie, 2011). Given the higher predisposition of hyperlipemia in Shetland ponies with obesity as a fortifying factor (Watson et al., 1992; Hughes et al., 2004), this was obviously a potential concern. However, a previous study in 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 (Van Weyenberg et al., 2008). In the present study, statistically higher serum TAG and NEFA levels were found in MOD and SEV compared to CONTROL, with the highest levels in SEV. Triglyceride concentrations, however, never exceeded the upper limit of the normal range ($<$ 500 mg/dl) (McKenzie, 2011) 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 (Ball, 1998). 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 (Vincent et al., 2007), 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 (Lykkesfeldt and Svendsen, 2007). However, its use has been criticised due to low specificity (Lykkesfeldt and Svendsen, 2007) and sensitivity (McMichael, 2007). In order to have a good understanding of the oxidant/antioxidant status, multiple measures of oxidative damage should be evaluated (Kirschvink et al., 2008; McMichael, 2007). 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 (Bengmark, 2007). 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 SEV after 8 weeks of weight loss compared to MOD could therefore be related to the higher TAG and NEFA values in SEV, 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 (Requena et al., 1996).

Since the diet was the main focus of the present study and not the effect of weight loss on glucose and insulin dynamics, no glucose tolerance tests were implemented. We should nevertheless acknowledge that insulin resistance and hyperinsulinemia (Paolisso and Giugliano, 1996; Ceriello, 2000; Kyselová et al., 2002) are associated with oxidative stress and therefore could be a confounding factor. However, no significant correlations were found between plasma insulin levels and TBARS, AOPP, PENT, SOD, FRAP, and α-tocopherol.

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 equines 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.

6.6 Acknowledgements

manuscript drafting. PO was responsible for advanced glycation end products analysis. JB supervised the plasma analysis of SOD, TBARS, FRAP, and leptin. KV collaborated in the study performance and contributed to the manuscript drafting.

The authors declare no conflict of interest.

6.7 References

Argo CMcG, Curtis GC, Grove-White D, Dugdale AHA, Barfoot CF, Harris PA (2012) Weight loss resistance: A further consideration for the nutritional management of obese Equidae. Vet. J. 194, 179-188.

Ball GFM (1998). Bioavailability and Analysis of Vitamins in Foods. New York: Chapman and Hall.

Balogh N, Gaál T, Ribiczeyné PS, Petri Ả (2001) Biochemical and antioxidant changes in plasma and erythrocytes of pentathlon horses Before and after exercise. Vet. Clin. Path. 30, 214-218.

Bengmark S (2007) Advanced glycation and lipoxidation end products-amplifiers of inflammation: the role of food. JPen- Parenter. Enter. 31, 430-440.

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.

Bruynsteen L, Erkens T, Peelman LJ, Ducatelle R, Janssens GPJ, Harris PA, Hesta M (2013) Expression of inflammation-related genes is associated with adipose tissue location in horses. BMC Vet. Res. 9: 240.

Carter RA, McCutcheon J, Valle E, Meilahn EN, Geor RJ (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.

Ceriello A (2000) Oxidative stress and regulation. Metabolism 49, 27-29.

Dandona P, Mohanty P, Ghanim H, Aljada A, Browne R, Hamouda W, Prabhala A, Afzal A, Garg R (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.

de Laat MA, Kyaw-Tanner MT, Sillence, McGowan CM, Pollitt CC (2012) Advanced glycation endproducts in horses with insulin-induced laminitis. Vet. Immunol. Immunop. 145, 395-401.

Deaton CM, Marlin DJ, Smith NC, Harris PA, Roberts CA, Schroter RC, Kelly FJ (2004) Pulmonary epithelial lining fluid and plasma ascorbic acid concentrations in horses affected by recurrent airway obstruction. Am. J. Vet. Res. 65,80-87.

Dimock AN, Siciliano PD, McIlwraith CW (2000) Evidence supporting an increased presence of reactive oxygen species in the diseased equine joint. Equine. Vet. J. 32, 439-443.

Divers TJ, Cummings JE, de Lahunta A, Hintz HF, Mohammed HO (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.

Dugdale AHA, Curtis GC, Cripps P, Harris PA, Argo CMcG (2010) Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares. Equine Vet. J. 42, 600-610.

Frank N, Geor RJ, Bailey SR, Durham AE, Johnson PJ (2010) Equine metabolic syndrome. J. vet. Internal. Med. 24, 467-475.

Geor RJ (2008) Metabolic predisposition to laminitis in horses and ponies: obesity, insulin resistance and metabolic syndromes. J. Equine. Vet. Sci. 28, 753-759.

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.

Gredilla R, Barja G (2005) Minireview: the role of oxidative stress in relation to caloric restriction and longevity. Endocrinology 146, 3713-1717.

Gugliucci A, Kotani K, Taing J, Matsuoka Y, Sano Y, Yoshimura M, Egawa K, Horikawa C, Kitagawa Y, Kiso Y, Kimura S, Sakane N (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.

Henneke DR, Potter GD, Kreider JL, Yeates BF (1983). Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15, 371-372.

Hughes KJ, Hodgson DR, Dart AJ (2004) Equine hyperlipaemia: a review. Aust. Vet. J. 82, 136-142.

Kelishidaki R, Hashemi M, Mohammadifard N, Asgary S, Khavarian N (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.

King C, Mansmann RA (2004). Preventing laminitis in horses: Dietary strategies for horse owners. Clin. Tech. Equine Pract. 3, 96-102.

Kirschvink N, Smith N, Fiévez L, Bougnet V, Art T, Degand G, Marlin D, Roberts C, Génicot B, Lindsey P, Lekeux P (2002) Effect of chronic airway inflammation and exercise on pulmonary antioxidant status of healthy and heaves-affected horses. Equine Vet. J. 34, 705-712.

Kischvink N, de Moffarts B, Lekeux P (2008) The oxidant-antioxidant equilibrium in horses. Vet. J. 177, 178-191.

Kyselová P, Žourek M, Rušavý Z, Trefil L, Racek J (2002) Hyperinsulinemia and oxidative stress. Physiol. Res. 51, 591-595.

Laflamme DP, Kulman G (1995) The effect of weight loss regimen on subsequent weight loss maintenance in dogs. Nutr. Res. 15, 1019-1028.

Lin H, Decuypere E, Buyse J (2004) Oxidative stress induced by corticosterone administration. Comp. Biochem. and Phys. B. 139, 737 – 744.

Loftus JP, Belknap JK, Stankiewicz KM, Black SJ (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.

Lykkesfeldt J, Svendsen O (2007) Oxidants and antioxidants in disease: oxidative stress in farm animals. Vet. J. 173, 502-511.

MacLean PS, Higgins JA, Johnson GC, Fleming-Elder BK, Donahoo WT, Melanson EL, Hill JO (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.

McKenzie III HC (2011). Equine Hyperlipidemias. Vet. Clin. North. Am. Equine Pract. 27, 59–72.

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.

McMichael MA (2007) Oxidative stress, antioxidants, and assessment of oxidative stress in dogs and cats. J. Am. Vet. Med. Ass. 231, 714-720.

Mohn A, Catino M, Capanna R, Giannini C, Marcovecchio M, Chiarelli F (2005) Increased oxidative stress in prepuberal severely obese children: effect of a dietary restriction-weight loss program. J. Clin. Endocr. Metab. 90, 2653-2658.

Moreaux S, Nichols J, Bowman J, Hatfield PG (2011) Psyllium Lowers Blood Glucose and Insulin Concentrations in Horses. J. Equine Vet. Sci. 31, 160-165.

National Research Council, 2007, Nutrient Requirements of Horses, sixth ed. National Academies Press, Washington DC.

Ozata M, Mergen M, Oktenli C, Aydin A, Sanisoglu SY, Bolu E, Yilmaz MI, Sayal A, Isimer A, Ozdemir IC (2002) Increased oxidative stress and hypozincemia in male obesity. Clin. Chem. 35, 627-631.

Paolisso G, Giugliano D (1996) Oxidative stress and insulin action: is there a relationship? Diabetologia 39, 357-363.

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.

Quinn RW, Burk AO, Suagee JK, Hartsock TG (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.

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.

Reitman A, Friedrich I, Ben-Amotz A, Levy Y (2002). Low plasma antioxidants and normal plasma B vitamins and homocysteine in patients with severe obesity. Israel Med. Assoc. J. 4, 590-593.

Requena JR, Fu M, Ahmed MU, Jenkins AJ, Lyons TJ, Thorpe SR (1996) Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. Neprhol. Dial. Transplant. 11 (Suppl 5), 48-53.

Roberts CK, Sindhu KK (2009) Oxidative stress and metabolic syndrome. Life Sci. 84, 705-712.

Suzuki K, Ito Y, Ochiai J, Kusuhara Y, Hashimoto S, Tokudome S, Kojima M, Wakai K, Toyoshima, Koji Tamakoshi H, Watanabe Y, Hayakawa N, Maruta M, Watanabe M, Kato K, Ohta Y, Tamakoshi

A (2003) Relationship between obesity and serum markers of oxidative stress and inflammation in Japanese. Asian Pac. J. Allergy 4, 259-266.

Thatcher C, Pleasant R, Geor R, Elvinger F, Negrin KA, Franklin J, Gay L, Were SR (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.

Treiber KH, Kronfeld DS, Hess TM, Boston RC, Harris PA (2005) Use of proxies and reference quintiles obtained from minimal model analysis for determination of insulin sensitivity and pancreatic beta cell responsiveness in horses. Am. J. Vet. Res. 66, 2114-2121.

Ungru J, Blüher M, Coenen M, Raila J, Boston R, Vervuert I (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.

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.

Valle E, Storace D, Sanguinetti R, Carter R, Odetti P, Geor R, Bergero D (2013) Association of the glycoxidative stress marker pentosidine with equine laminitis. Vet. J., 3, 445-450.

Van Weyenberg S, Hesta M, Buyse J, Janssens GPJ (2008) The effect of weight loss by energy restriction on metabolic profile and glucose tolerance in ponies. J. Anim. Physiol. Nutr. 92, 538-545.

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.

Watson TDG, Burns L, Love S, Packard CJ, Shepherd J (1992) Plasma lipids, lipoproteins and post-heparin lipases in ponies with hyperlipaemia. Equine Vet. J. 24, 341-346.

Weiss EC, Galuska DA, Kettel Khan L, Gillespie C, Serdula MK (2007) Weight regain in U.S. adults who experienced substantial weight loss, 1999-2002. Am. J. Prev. Med. 33, 34-40.

Witko-Sarsat V, Friedlander M, Capaillère-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, Jungers P, Descamps-Latscha B (1996). Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Journal 49, 1304-1313.

Wyse CA, McNie KA, Tannahil VJ, Murray JK, Love S (2008) Prevalence of obesity in riding horses in Scotland. Vet. Rec. 162, 590-591.

Addendum: mean and SEM values of parameters measured in Chapter 5 and 6

		start WLP		end WLP		end FUP	
		mean (mm)	SEM	mean (mm)	SEM	mean (mm)	SEM
loin fat depth	CONTROL	9.28	0.77	8.80	0.87	8.54	0.84
	MOD	12.94	1.09	9.94	0.78	10.77	1.05
	SEV	11.95	1.26	8.98	0.79	9.91	0.62
rib fat depth	CONTROL	12.19	0.45	11.61	0.46	11.55	0.45
	MOD	15.30	1.62	11.63	1.04	12.61	1.03
	SEV	13.01	1.02	10.06	0.81	10.49	0.62
tail head fat depth	CONTROL	16.11	1.94	13.35	1.55	13.06	1.28
	MOD	15.18	1.91	11.49	1.54	12.37	1.58
	SEV	16.22	2.43	10.61	1.29	10.44	1.31
		mean (cm)	SEM	mean (cm)	SEM	mean (cm)	SEM
belly girth	CONTROL	144.50	6.10	143.75	6.10	144.75	6.67
	MOD	154.67	4.46	148.83	4.46	154.50	4.75
	SEV	148.50	3.52	138.42	3.12	144.67	3.12
heart girth	CONTROL	124.25	5.21	121.50	4.99	120.50	5.29
	MOD	131.42	4.55	125.50	3.79	127.67	3.88
	SEV	127.08	3.01	119.50	2.71	121.92	2.41
		mean (μM)	SEM	mean (μM)	SEM	mean (μM)	SEM
FRAP	CONTROL	262.25	15.21	169.41	5.77	220.79	12.15
	MOD	240.01	14.51	172.03	4.82	217.86	10.60
	SEV	248.21	11.97	211.82	7.86	217.46	9.44
		mean (mg/l)	SEM	mean (mg/l)	SEM	mean (mg/l)	SEM
α-tocopherol	CONTROL	2.35	1.30	2.47	0.48	0.96	0.60
	MOD	2.70	0.46	1.98	0.41	1.77	0.35
	SEV	4.00	0.43	4.40	0.50	2.92	0.32

		mean (U/ml)	SEM	mean (U/ml)	SEM	mean (U/ml)	SEM
SOD	CONTROL	9.70	0.65	8.04	0.59	10.18	1.96
	MOD	9.41	0.63	7.73	0.25	13.16	2.54
	SEV	10.43	1.32	7.32	0.21	11.15	1.75

		mean (mg/dl)	SEM	mean (mg/dl)	SEM	mean (mg/dl)	SEM
TG	CONTROL	47.17	7.34	30.50	4.01	30.00	4.81
	MOD	51.00	13.13	41.83	5.30	31.17	4.57
	SEV	40.83	6.05	41.17	2.25	22.50	0.99

		mean (mmol/l)	SEM	mean (mmol/l)	SEM	mean (mmol/l)	SEM
NEFA	CONTROL	0.36	0.06	0.16	0.04	0.31	0.06
	MOD	0.44	0.07	0.22	0.04	0.22	0.05
	SEV	0.57	0.04	0.55	0.03	0.33	0.08

		mean (ng/ml)	SEM	mean (ng/ml)	SEM	mean (ng/ml)	SEM
leptin	CONTROL	5.94	0.73	6.35	0.47	3.16	0.53
	MOD	6.22	1.17	4.03	0.48	2.88	0.38
	SEV	5.46	1.07	2.99	0.50	2.15	0.26

		mean (nmol/ml)	SEM	mean (nmol/ml)	SEM	mean (nmol/ml)	SEM
TBARS	CONTROL	1.51	0.10	0.94	0.12	1.25	0.11
	MOD	1.59	0.10	1.03	0.09	1.12	0.06
	SEV	1.60	0.20	0.98	0.08	0.99	0.03

		mean (µmol/chloramine T equivalents)	SEM	mean	SEM
AOPP	CONTROL	183.23	22.81	77.50	8.16
	MOD	172.85	37.18	107.92	7.50
	SEV	137.63	19.70	102.10	10.86

CHAPTER 7
GENERAL DISCUSSION

CHAPTER 7: GENERAL DISCUSSION

7.1 Brief overview of the results

Stable reference genes (HPRT1, RPL32 and GAPDH) were determined in different equine adipose tissue regions (**Chapter 3**). HPRT1, RPL32 and GAPDH were used for accurate normalization of RT-qPCR data to determine differences in mRNA expression between adipose tissue originating from the nuchal, subcutaneous, and abdominal region (**Chapter 4**). Differences in mRNA expression were found between the different adipose tissue depots, with higher pro-inflammatory cytokine mRNA expression of CCL-5 and IL-1β in the abdominal region, and leptin and IL-6 in the nuchal region. Higher mRNA expression of the anti-inflammatory cytokine IL-10 was found in the abdominal region. Depot-specific differences in adipocyte area, density of antigen presenting cells, and the presence of capping structures in equine adipose tissue were discovered for the first time.

A more severe energy restriction in obese Shetland ponies resulted in a relatively higher decrease in body weight, BCS, belly and heart girth, and tail head fat depth compared to a lower level of energy restriction. However, relative weight gain afterwards was positively correlated with energy restriction level. Energy restriction level did not affect behavioural parameters and gastric ulcer formation (**Chapter 5**). Early weight loss was accompanied with increasing NEFA, TAG, FRAP, α-tocopherol, and AOPP levels that were more pronounced in the highest energy restricted group. Towards the end of the weight loss period, these values returned to baseline levels (**Chapter 6**).

7.2 Introduction

Equine obesity has become a problem, especially in leisure horses (Harker et al., 2011; Robin et al., 2014). It has been associated with conditions such as insulin resistance (Frank et al., 2006; Vick et al., 2007), laminitis (Johnson, 2002; Treiber et al., 2006), abnormal lipid metabolism (Jeffcot and Field, 1985; Watson et al, 1992), and many more (see **Chapter** 1).

Adipose tissue is distributed throughout the entire body in different regions. From human research and research in rodents (Trujillo and Scherer, 2006), it is known that adipocytes from different anatomic location may vary in their biology due to local influences on differentiation and gene expression, and may be considered as "mini-organs". In humans, increased intra-abdominal adipose tissue has been associated with increased diabetes risk and vascular disease (Hajer et al., 2008). In horses this has not been extensively investigated and, therefore, the main objective of the present thesis was to gain further insight into the importance of adipose tissue depots in the equine body. What is the impact of adipose tissue depot on inflammatory profile and what is the impact of weight loss on the individual adipose tissue depots?

Answers to these research questions were searched by performing an observational (sampling of different adipose tissues at one moment) and a longitudinal (effect of weight loss on external indicators of changes in accessible key adipose tissue depots) study.

7.3 Equine adipose tissue: is there one specific depot that can be classified as the most harmful?

In the small test population of equines used in the present PhD thesis with varying age (range 1-25 years), nutritional status (lean to obese), and genetic background (1 pony and 11 horses of different breeds), depot-specific differences in gene expression levels of inflammation-related genes were found, as well as differences in adipocyte size and presence of antigen presenting cells.

7.3.1 Relation adipose tissue depot and gene expression

Whereas in an earlier study in insulin sensitive and insulin resistant light breeds horses (Burns et al., 2010) the nuchal adipose tissue depot was indicated as being the most pro-inflammatory (highest mRNA expression of the inflammatory cytokines IL-6 and IL-1β), this result was not found in our study. We discovered that also the abdominal adipose tissue depot potentially had a pro-inflammatory profile (highest expression of pro-inflammatory cytokine

IL-1β and chemotactic cytokine CCL5). The inconsistency between gene expression results in the present study and the study of Burns and co-workers could be due to several reasons. Briefly, a first reason could be the study population we used. It consisted of horses and 1 pony of different breeds, including at least 1 equine (Halflinger pony) that is predisposed to become readily obese, and 3 horses that have a phenotype that could be classified as being more resistant to develop obesity (French Thoroughbred and trotters). It is possible that horses classified as easy keepers have different gene expression levels of inflammation-related genes compared to horses that are bred to perform exercise at high intensity levels. No history of the horses was available, meaning that the fitness level of the horses was not known. To the author's knowledge, the impact of fitness level on gene expression of inflammation-related genes has not been previously investigated in equines. A second reason for a different outcome between the 2 studies could be different reference genes used for normalization of the data (geometric mean of β-actin and β$_2$-globulin compared to geometric mean of HPRT1, RPL32 and GAPDH). A third reason could be the gender of the investigated animals. In our study only geldings were used, as compared to the study of Burns and co-workers, only using mares. In humans, gender specific gene expression has previously been described (Roth et al., 2002), indicating that this possibly could be a reason for differences between the 2 trials.

The different outcome, however, does not indicate that nuchal adipose tissue is not important, because when considering

1. the high leptin gene expression in nuchal adipose tissue (**Chapter 4**),

2. the strong correlation between leptin and IR (Van Weyenberg et al., 2008),

3. the positive correlation between IL-6 and IL-1β gene expression in the nuchal adipose tissue region (**Chapter 4**), and

4. the increased local adipose tissue deposition at the level of the crest in a lot of obese and laminitis prone equines (Carter et al., 2009),

nuchal adipose tissue could still be an important contributor to a pro-inflammatory state in obese equines, but more work is needed to confirm this.

On the other hand, the importance of the adipose tissue in the abdominal region cannot be ignored, as in our test population, higher expression of the pro-inflammatory CCL5 and IL-1β, and anti-inflammatory IL-10 was shown. This implicates that, as in humans, this adipose tissue depot could also play an important role in the local and general inflammatory load in the equine body. Therefore, this depot certainly is important in follow-up during weight loss.

If this specific adipose tissue decreases during weight loss in equines, this could have a positive influence on the local and general inflammatory profile in equines.

Before making too early conclusions about the importance of each adipose tissue depot in equines, some limitations of the first trial have to be recognized.

1. To investigate the effect of obesity on gene expression, clearly lean and obese equines should be sampled. It was the intention of the first trial to select lean and obese horses, but due to practical limitations (slaughterhouse horses that could only be inspected for a limited time (<1 minute before slaughtering)), no proper estimation of body condition could be made. It was not possible to palpate the horses to estimate the BCS. Therefore, horses were only classified as 'normal' and 'overweight to obese'. This implies that horses that looked normal on quick visual examination could have been overweight or vice versa. Therefore, from the results of our study, we cannot conclude that there is or is not a higher expression of inflammation-related genes in obese horses compared to lean horses.

2. Another limitation of the present study was the lack of history of our test population. No information was available about the diet of the horses and the time the horses were fasted. Some horses were already present at the slaughterhouse the evening before slaughtering, whereas others were only presented the day itself. Although glucose values were only slightly increased, the influence of transport, fast, and lairage time could not be excluded.

7.3.2 Adipose tissue depot size and gene expression

Next to the important influence of adipose tissue deposition, the size of each adipose tissue depot must also be taken into consideration when evaluating the inflammatory profile in the equine body. Adipose tissue depots that only express pro-inflammatory cytokines at a low level can become very important in the overall inflammatory load if this depot has the ability to enlarge extremely during the onset of obesity. On the other hand, if the volume of an adipose tissue depot with high mRNA expression of pro-inflammatory cytokines significantly reduces during weight loss, this could also contribute to a decreased overall inflammatory load. Therefore, not only the adipose tissue depot-specific inflammatory gene expression should be evaluated, but also its contribution to the total gene expression of all adipose tissue depots in the body.

Dugdale and colleagues (2011a) evaluated body fat content in seven mature Welsh Mountain pony mares. Depending on body condition score (1.25-7/9), 1.77 to 29.88% of

recovered empty body mass (live body weight minus digesta and unaccounted mass (water lost or gained by evaporation and condensation during dissection)) was white adipose tissue, originating from 4 regional adipose tissue depots (internal carcase (body wall associated), internal carcase (organ associated), external carcase and head/lower limb). Except for the pony with the lowest BCS, the largest white adipose tissue depot in ponies was at the level of the external carcase, including subcutaneous, udder-associated, nuchal and inter-muscular white adipose tissue. Irrespective of BCS, the inter-muscular tissue had the greatest contribution to that region. In the same trial, an exponential relationship between BCS and total body white adipose tissue/lipid was found. As obese ponies (BCS > 7/9) were not included, no information was available on the exact fat distribution in those obese animals. It could be suggested, however, that in obese equines with a BCS > 7/9 huge amounts of adipose tissue will be deposited. It yet has to be determined in which adipose tissue depot the extra fat will be deposited. Therefore, body composition and conformation analyses in equines with different genetic backgrounds and varying BCS, coupled with gene expression analysis under standardized conditions would give very valuable information regarding the 'gene expression load' of equine adipose tissue.

7.2.3 Gene expression and blood protein levels

When discussing mRNA expression, one very important remark has to be made. Cytokine mRNA expression may not always be reflective of protein content due to post-transcriptional regulation (Anderson, 2008). As example, in **Chapter 4**, there was no correlation between leptin mRNA gene expression in adipose tissue and blood leptin levels. To have a good insight in the final conversion to the active protein and its influence on total body, gene expression analysis and protein analysis should always be combined.

It is, however, still not clear whether obesity in equines is associated with inflammation, as some authors found a systemic increase in some pro-inflammatory cytokines (TNF, leukocyte mRNA, IL-1β, amyloid A, IL-6) in obese equines (Vick et al., 2007; Adams et al., 2009; Treiber et al., 2009; Suagee et al., 2013), whereas other authors found no positive relation between obesity and systemic pro-inflammatory cytokines (Banse, 2013; Suagee et al., 2013) or even a decrease in pro-inflammatory cytokine gene expression (IL-1 and IL-6) (Holbrook et al., 2012).

7.2.4 Relation adipose tissue depot, adipocyte size and antigen presenting cells

The study presented in **Chapter 4** was the first to investigate the impact of adipose tissue depot on adipocyte size and presence of antigen presenting cells. Depot-specific differences in adipocyte size were identified in humans (O'Connell et al., 2010), dogs (Kabir et al., 2011), and cats (Van de Velde et al., 2013). In mice, adipocyte size was positively correlated with frequency of adipocyte death, macrophage, and CLS numbers in visceral and subcutaneous adipose tissue depots (Cinti et al., 2005; Murano et al., 2008). In humans and cattle, mRNA expression of leptin was positively correlated with adipocyte size (Van Harmelen et al., 1998; Yang et al., 2008). Also in humans, secretion of IL-6, IL-8, TNF-α, monocyte chemoattractant protein-1 was positively correlated with subcutaneous adipocyte size (Skurk et al., 2007). In that same trial, there was a decrease of IL-10 secretion with increasing adipocyte size. In equines, however, no information is available on differences in adipocyte size and presence of immune cells between adipose tissue depots. **Chapter 4** showed that adipocyte size differed between regions (nuchal, visceral and subcutaneous) in a mixed breed, normal to obese gelding group. Differences in adipocyte area can influence mRNA expression in humans (Skurk et al., 2007; Huber et al., 2008; Drolet et al., 2009), which was also seen in **Chapter 4**, as positive correlations were found between mRNA expression of the evaluated inflammation related genes and adipocyte area in multiple adipose tissue depots. Unfortunately, no clear pattern could be determined in our very small and diverse study population. In obese cats adipocyte size is larger compared to lean individuals (Van de Velde et al., 2013), indicating the importance of further research comparing obese, overweight and lean equines.

During the present PhD thesis, an attempt was made to examine the presence of immune cells in equine adipose tissue. Using Monoclonal Mouse Anti-Human HLA-DR antigen, alpha-chain clone TAL.1B5 (Code No. M0746; DakoCytomation, DK-2600, Glostrup, Denmark), major histocompatibility II (MHC II) molecules can be visualised. A limitation of this staining method is the staining of not only macrophages, but also other antigen presenting cells, such as monocytes, B-cells and dendritic cells (Ting and Trowsdale, 2002; Al-Daccak et al., 2004). Therefore, we must be careful to make comparisons with trials in other species using a macrophage specific staining method.

The presence of antigen presenting cells differed between adipose tissue regions (**Chapter 4**), with the highest concentration in the subcutaneous adipose tissue depot. The presence of capping structures (adipocytes surrounded by antigen presenting cells, similar to crown like

structures in mice (Murano et al., 2008)) was demonstrated for the first time in equine adipose tissue. Their presence was demonstrated in 6 of the 12 equines involved in that trial, in 4 of the 8 adipose tissue depots examined (N ¼, N ½, loin, and perirenal adipose tissue), in both equines with a normal and an overweight to obese status. Their presence could not be related to increased gene expression of pro-inflammatory cytokines. As mentioned before, it would have added value to further investigate if there are differences in the number of antigen presenting cells in adipose tissue originating from horses with different body condition scores.

Take home messages from this first study are:

1. Different adipose tissue depots in the equine body have different patterns of inflammation-related gene expression, different adipocyte size and different amounts of antigen presenting cells.
2. In contrast to humans, in a mixed population of equines with varying nutritional status, age and breed, there is not one adipose tissue depot that could be referred to as most harmful. Neck adipose tissue and abdominal adipose tissue had a higher expression of inflammatory cytokines compared to subcutaneous adipose tissue.

7.2.5 Future research

To gain more insight in the importance of each adipose tissue depot and the influence of obesity, breed and/or age, suggestions for future research in this area are listed below.

1. It is advised to use a more controlled study population for equine scientific research to remove background noise in statistics.
2. To investigate the influence of obesity on gene expression of inflammation-related genes, adipocyte size and presence of antigen presenting cells, a population of lean and obese horses should be used. Next to an accurate of estimation of BCS, additional information on local adipose tissue deposition (morphometric measurements, fat depth measurements) and general obesity (body composition analysis) should be carried out. With this information, it would be possible to make further conclusions if the differences in gene expression, adipocyte size and presence of antigen presenting cells between adipose tissue depots in obese horses are comparable to differences between adipose tissue depots in lean horses.
3. To further elucidate the impact of adipose tissue deposited in the nuchal area, gene expression of inflammation-related horses with an extremely high deposition of nuchal

fat (high CNS) should be compared with horses a normal deposition of adipose tissue in that area.

4. To have more insight in the gender-specific and age-related gene expression of inflammation-related genes, male and female equines originating from different age categories should be included in future research studies. Suagee and co-workers (2013) found higher plasma TNF and IL-6 concentrations in females compared to males. Interleukin-6 concentrations were positively correlated with age.

5. As the influence of time of fast, diet and fitness level on gene expression of inflammation-related genes is not known, a test population with a known feeding and physical exercise history should be used.

6. Gene expression analysis and protein analysis should be combined to evaluate its influence on total body inflammatory profile. Systemic levels of inflammatory cytokines can be influenced by gene expression and translation in different tissues and cells.

7. Future research into differences in gene expression between animals with a different genetic background (ponies vs horses, easy keepers vs hard keepers) would also provide valuable information on the inflammation sensitivity of different equines.

8. Equines with different insulin sensitivity levels and/or hyperinsulinemia also warrant further research. Tadros and co-workers (2013) stated that EMS affects the inflammatory response to endotoxin by prolonging cytokine expression in circulating leukocytes. During compensated IR, a pro-inflammatory state (increased expression of Toll-like receptor 4 and suppressor of cytokine signalling 3) was seen in skeletal muscle and visceral adipose tissue, but not in subcutaneous adipose tissue (Waller et al., 2011).

9. Next to the importance of inflammatory profile of each individual adipose tissue depot, it would also be interesting to investigate if there are differences in metabolic activity between adipose tissue depots. This would give further information on the response of that specific adipose tissue depot to weight loss or weight gain. In humans, adipose tissue lipolytic activity differs between regions. A higher basal lipolysis was seen in adipocytes from the omental and mesenteric adipose tissue depot compared to adipocytes originating from the subcutaneous region (Arner, 2005).

These suggestions for future research could give further information if one or more adipose tissues depots need more specific attention in obese horses. Next to the knowledge of

the influence of different adipose tissue depots on the inflammatory profile in the equine body, it would also be very interesting to know what happens in the equine body during weight loss, how adipose tissue will respond to weight loss and the influence it will have on total body inflammatory profile. Therefore, a weight loss trial was performed during the PhD thesis to further examine this.

7.4 Weight loss in equines: do all adipose tissue depots react the same?

To investigate the effect of weight loss on the different adipose tissue depots in the equine body, different rates of weight loss should be implemented. In 18 Shetland gelding ponies, this was achieved by using 2 different levels of energy restriction (**Chapter 5**). A control group was included to recognize potentially seasonal influences on differences in adipose tissue deposition as this has been suggested in earlier weight loss trials in ponies (Argo et al., 2012).

Differences in energy restriction clearly influenced the rate of weight loss, although not linearly. An energy intake of 80% of MERob was accompanied with an average weekly weight loss of 0.23 %, whereas a more severe energy restriction (energy intake at 60% of MERob) was accompanied with an average weight loss of 0.68 %. A doubling in caloric restriction was accompanied with a tripling in weight loss.

7.4.1 Effect on subcutaneous fat depth

At the end of the weight loss period, subcutaneous fat depth decreased in all depots (loin, rib, tail head) in the 3 groups (2 energy restricted groups and the control group), indicating that there probably is a seasonal influence on subcutaneous adipose tissue distribution, as relative body weight loss at the end of the weight loss period was negligible in the control group (minus 0.5%). Although no further weight loss was seen in the moderate energy restricted group between 9 and 16 weeks of weight loss, there was a mild further reduction in subcutaneous fat depth at the level of the loin and ribs.

A moderate level of energy restriction (80% of MERob) and subsequent weight loss (3.59% after 16 weeks), did not result in a greater decrease in fat mass at the level of the tail head compared to the control group (fed to maintain body weight). More severe energy restriction (60% of iMERob) and subsequent higher weight loss (10.81%), however, was accompanied with a greater decrease in tail head fat depth (although not significantly) and BCS, indicating that tail head fat depth could be identified as a more important adipose tissue

depot to evaluate BCS changes during weight loss compared to other subcutaneous adipose tissue depots. In previous trials, this adipose tissue depot has also been found to have the highest correlation with BCS (Gentry et al., 2004). The impact of a decrease in tail head fat depth on inflammatory load is expected to be minimal, however, as gene expression of pro-inflammatory genes in this specific adipose tissue depot is low compared adipose tissue originating from the nuchal and abdominal region (**Chapter 4**).

Rate of weight loss did not affect decrease in subcutaneous rib and loin fat depth. Evaluation of subcutaneous fat depth at the level of rump and ribs in other trials also showed that these subcutaneous measurements are not the best indicators of weight loss. Despite the fact that a considerable amount of weight loss was achieved (average of 7.2% BM loss or a weekly 0.45% loss) in a trial performed by Argo and co-workers (2012), proportional rump fat depth and rib fat depth were increased towards the end of the study. As this study was performed in cold temperatures (as low as -10°C), they suggested that a re-sequestering of adipose tissue in the subcutis probably has taken place. No control group was included in that trial, and hence no information was available if this would also have occurred in non-energy restricted ponies, when no lipids mobilized due to weight loss are circulating in the body.

During the last 4 weeks of the weight loss period, the decrease in fat depth in the subcutaneous adipose areas was low compared to the first 12 weeks of the weight loss period, with almost a stabilisation in the loin and rib area. This is in contrast to the decrease in belly girth.

7.4.2 Effect on belly circumference

The decrease in belly girth was positively associated with energy restriction level. Whereas the decrease in fat depth in the subcutaneous area was low during the last 4 weeks of the weight loss trial, there was a further decrease in belly girth in the 2 energy restricted groups. The impact of a decrease in abdominal adipose tissue on inflammatory load is expected to be important, as this adipose tissue depot shows a higher expression of pro-inflammatory cytokines compared to subcutaneous adipose tissue. It has to be mentioned, however, that a decrease in belly girth can be due to a decrease in gut fill and/or a decrease in abdominal fat tissue (Argo et al., 2012). One can expect that at the start of the weight loss trial the decrease in gut fill will be the most important, whereas during the rest of the trial, the change in abdominal adipose tissue will also be important. Until recently, attention has mainly been focussed on the more visible adipose tissue depots (neck adipose tissue, subcutaneous

adipose tissue) during weight loss, however, the more invisible adipose tissue depots (intra-abdominal adipose tissue such as mesenterial, omental, and retroperitoneal) are also very important, as sometimes this is one of the only adipose tissue depots that decreases during weight loss. In a study performed by Dugdale and colleagues (2010), an average body mass loss of 11.4% was accompanied with only minor changes in BCS (average minus 0.25). There was, however, a significant decrease in heart and belly girth. In all equine weight loss trials measuring belly girth, a positive relation between weight loss and belly girth was found (Dugdale et al., 2010; Carter et al. 2010; Argo et al.; 2012, **Chapter 5**). In humans, higher basal lipolysis of omental and mesenterial adipocytes compared to adipocytes from the subcutaneous region has been detected (Arner, 2005). Differences in lipogenesis between various adipose tissue depots were seen. If this is also the case in equines, has to be further elucidated. One has to be prudent, however, to extrapolate data from human to equine research as the function of specific adipose tissue depots can differ between humans and equines. For example, subcutaneous adipose tissue in equines has an important thermoregulatory function in addition to the storage of TAG, whereas in humans, the thermoregulatory function of subcutaneous adipose tissue is probably less important.

When evaluating different previous weight loss trials in equines, very different outcomes on local adipose tissue depots were observed (Table 7.1). This potentially could not only be attributed to the level of energy restriction (Dugdale et al., 2010; Argo et al., 2012, **Chapter 5 and 6**), but also to breed (Welsh Mountain ponies vs Shetland ponies), gender (mares vs geldings), and weight loss intervention strategy (energy restriction (Dugdale et al., 2010; Argo et al., 2012, **Chapter 5** and **6**), exercise (Carter et al., 2010) or combination of both (Gordon et al., 2009)).

Table 7.1: Overview of monitoring weight loss using morphometric and ultrasound measurements.

Monitoring tool	Weight loss intervention		
	Energy restriction	Exercise	Combination
Belly girth/abdominal circumference	↓	↓	NA
Heart girth	↓	NA	NA
Tail head fat depth	↓ or =	NA	NA
Rib fat depth	↓ or = or ↑	=	NA
Retroperitoneal/belly fat depth	↓	NA	NA
Rump/loin fat depth	↓ or = or ↑	=	↓

Abbreviations: NA, not available. References: Dugdale et al., 2010; Argo et al., 2012; **Chapter 5**; Carter et al., 2010; Gordon et al., 2009.

7.4.3 Effect on adipose tissue in the nuchal area

In our weight loss trial, body weight change was accompanied with only minimal changes in CNS. Changes in neck circumference and CNS were only evaluated in a limited number of weight loss trials (Dugdale et al., 2010; Carter et al., 2010), with minimal to no influence of weight loss on these measurements. Experience from the field also indicates that adipose tissue distributed at the crest of the neck can be very resistant to weight loss. The mechanism for this resistance has not been elucidated yet. To gain further insights, equines with high CNS should be evaluated during weight loss. As adipose tissue distributed at the crest of the neck potentially has a pro-inflammatory profile (Burns et al, 2010; **Chapter 4**), its influence on total body inflammation could be high and deserver further attention.

7.4.4 Effect of weight loss strategy on body composition: energy restriction vs exercise

During our weight loss trial, main attention was focussed on changes in adipose tissue depots. During weight loss, however, lean tissue can also decrease. As fat mass is the tissue responsible for the deleterious effects of obesity, the amount of adipose tissue loss during weight loss has to be maximised, preserving lean body mass. Lean body mass is considered to be the main factor that accounts for the magnitude of resting metabolism (Stiegler and Cunliffe, 2006). When lean body mass is lost during weight loss, resting metabolic rate can also decrease (Redman et al., 2009), which can potentially contribute to a more difficult weight maintenance or even weight regain when individuals terminate the weight loss program (Catenacci and Wyatt, 2007). In humans, exercise training promotes changes in body composition and sustaining lean body mass (Tsai et al., 2003).

As described before (**Chapter 1**), body composition analysis by means of deuterium oxide dilution technique gives valuable information about the percentage body lean and fat mass (**Chapter 1**). This validated technique in Welsh Mountain pony mares (Dugdale et al., 2011b) has been used in previous weight loss trials (Table 7.2).

Table 7.2: Overview of body composition determination using the deuterium oxide dilution technique in equines in previous weight loss trials.

Reference	Weekly weight loss (%)	BCS		fat %		% fat loss	ER	Exercise
		start	end	start	end			
Dugdale et al., 2010	0.84	7	6.7	20.1	18	10	1% BM as DMI	
Carter et al., 2010	0.50	7.9	7.5	18.1	13	31		x
Argo et al., 2012	0.45	7.9	6.4	24.4	19.7	19.3	1.25% BM as DMI	

Abbreviations: BCS, body condition score; ER, energy restriction; BM, body mass; DMI, dry matter intake.

As presented in Table 7.2, a different pattern of body fat mass loss is seen between weight loss through increased exercise (34% of fat loss after 8 weeks; Carter et al., 2010) and weight loss achieved by energy restriction (10 to 19.3% of fat loss after 12 and 16 weeks respectively; Dugdale et al., 2010; Argo et al., 2012). When obese equines are subjected to a weight loss diet, we need to aim for fat mass loss over lean mass loss, which could be achieved by increased exercise. As there is only one trial examining the effect of exercise as only strategy for weight loss (Carter et al., 2010), these results have to be interpreted with care. However, it is a very interesting starting point for further research as 4% body mass loss was associated with 34% of fat mass loss, indicating an improvement in the lean-to-fat ratio. Glucose and insulin dynamics were not influenced by exercise, whereas there has been an improvement in these dynamics in weight loss studies through energy restriction (Van Weyenberg et al., 2008; Argo et al., 2012). Therefore, the combination of energy restriction and (increased) exercise should be further investigated to see if this weight loss strategy can influence glucose/insulin dynamics as well as fat mass loss in equines.

7.4.5 Breed-related response to energy restriction

It is difficult to extrapolate the results of a weight loss trial in Shetland ponies to other breeds, as breed-related differences in response to energy restriction have been described. Obese Standardbred horses lost a significant amount of weight and body condition when maintained on ad-lib hay, whereas mixed pony-breeds and Andalusian-cross horses had to be restricted to a DMI of 1.25% of live body weight to achieve changes in BCS (Potter et al., 2013). In a study performed by Argo and co-workers (2012) in a mixed group of horse and pony mares, 4 cob breeds were diagnosed as weight loss resistant, as in a 16 week energy restriction trial, their weight loss decreased or even reversed during the last for 4 weeks. When these animals were further restricted in daily DMI from 1.25 to 1% of actual body mass, rates of weight loss were almost 3-fold greater compared with the first 12 weeks of that trial.

7.4.6 Possible limitations of the present study

For a correct assessment of the influence of weight loss rate on adipose tissue distribution, these depots should be evaluated when equal amounts of weight are lost. In our trial, however, morphometric measurements were carried out every 4 weeks. It would have given extra information if measurements would be performed when a certain percentage of weight was lost. This implicates that equines who are submitted to a slower weight loss rate need to be

followed up for a longer time compared to the equines submitted to a higher weight loss rate. In such a trial, influence of season could be an important environmental factor biasing the results.

The use of Shetland ponies, which can be classified as an easy keeper, in a weight loss trial can give valuable information about the response of these types of equines on a certain amount of energy restriction. The size of the animals, however, can be disadvantageous when morphometric measurements have to be evaluated. Small morphometrical measurement errors will bias the outcome more compared to larger breeds.

Different levels of energy restriction and subsequent different weight loss rates resulted in changes in adipose tissue depots, however, no significant differences in adipose tissue changes were found between the 2 energy restriction regimens. This could probably be due to the fact that energy restriction, and thus weight loss rate, was not severe enough. Future trials using more severe energy restriction levels could give more valuable information about the impact of weight loss on the different adipose tissue depots in the equine body. In contrast to other trials (Dugdale et al., 2010; Argo et al., 2012) DMI was not adjusted weekly. This could be responsible for the stabilization in weight loss towards the end of the weight loss period in the 2 energy restricted groups.

Attempts were made to determine body composition in **Chapter 5**. Unfortunately, due to unidentified reasons (deuterium oxide administration, preparation of deuterium oxide solution, calculation problems, storage and transport problems, analysis problems, …), unreliable results were obtained. Results from body composition analysis would have been interesting to see if different levels of energy restriction are accompanied with different patterns of fat mass loss and lean body mass loss. Performing body composition analysis during a weight loss trial could give further information in what is happening during early weight loss and weight loss later on.

7.5 Influence of weight loss on inflammatory profile and oxidative stress

7.5.1 Influence on inflammatory profile

It would have been an added value if the effect of weight loss on inflammatory profile was determined during the weight loss trial. However, cytokine analysis (by means ELISA) was carried out, but no reliable results could be obtained. Therefore, the effect of weight loss on inflammatory profile could not be evaluated. Gene expression of inflammation-related genes

in the different adipose tissue depots was not evaluated in the weight loss trial, partially due to the fact that there was not 1 single adipose tissue depot detected in the first trial that could be referred to as most pro-inflammatory, but also due to welfare reasons, as a lot of other examinations were carried in our test population, such as gastroscopy and deuterium analysis.

Because of this, we shifted our focus to another important obesity-associated disorder, oxidative stress, as in humans, obesity has been associated with increased markers of oxidative stress (Vincent et al., 2007).

7.5.2 Influence of weight loss on oxidative stress in equines

Oxidative stress in equines has been related to several disorders, such as joint disease (Dimock et al., 2000), neurological disorders (Divers et al., 2006), recurrent airway obstruction (Kirschvink et al., 2002; Deaton et al., 2004), and perfusion-related disorders (Kirschvink et al., 2008). Laminitis, which can develop as a consequence of differences in regional perfusion, or experimental induction of hyperinsulinemia, has been associated with increased concentrations of advanced glycation end products (de Laat et al., 2012; Valle et al., 2013). In a study performed in normal to obese pony mares with or without history of laminitis, no differences in antioxidant function (glutathione, glutathione peroxidase, superoxide dismutase) or increased oxidative pressure (malondialdehyde, apoptosis, 3-nitrotyrosine) were noted between the two groups (Treiber et al., 2009). Another study also found no difference in oxidative markers (including glutathione peroxidase and MDA) between ponies with equine Cushing's disease and control ponies with different propensities for laminitis (Keen et al., 2004). These findings suggest that disrupted redox status may not be a component in equine laminitis, or that current markers for redox status are insufficient.

On the other hand, lowered antioxidant capacity (decreased erythrocyte glutathione peroxidase activity) has been reported in obese horses and aged horses (Pleasant et al., 2013), however, no individual dietary information was available which makes interpretation of the data difficult.

To the best of the author's knowledge, the effect of weight loss and different levels of energy restriction on oxidative stress related parameters has not been reported previously in equines (**Chapter 6**).

In general, only limited effects of weight loss on oxidative stress parameters between the start and end of the weight loss period were noted. On the other hand, the evolution of the

parameters during weight loss was very interesting. Independent of weight loss regimen, increasing triglycerides, antioxidant capacity (FRAP and α-tocopherol levels) and pro-oxidant levels (AOPP) were found during the first 4 to 8 weeks of weight loss, followed by a decrease towards the end of the weight loss period to baseline values. The changes in those parameters were associated with level of energy restriction. The parallelism between the pro- and anti-oxidant markers indicates that the ponies tried to cope with increasing oxidative stress by increasing the anti-oxidant capacity, although this was not fully successful as there was a temporary increase in oxidative stress (increased AOPP).

7.5.3 Parameters influencing the outcome and possible limitations of the study

Ponies in the present study received a nutritionally balanced diet one month before and throughout the entire trial, with the inclusion of a vitamin E enriched ration balancer. This could be a possible explanation for the limited effects of weight loss on oxidative stress, especially at the level of the α-tocopherol concentrations. We should mention that only a limited number of oxidant markers were studied throughout the trial, and that more accurate pro-oxidant markers, such as isoprostane, could have given additional information (Kirschvink et al., 2002; Lykkesfeldt et al., 2007).

Important to mention is that at the end of the trial, ponies were still overweight to obese (average BCS of 7.83 ± 0.58 and 6.75 ± 1.43 respectively in the moderately and severely energy restricted group), meaning that there may not have been sufficient weight loss to induce significant changes in oxidative stress related parameters. On the other hand, it is possible that too severe energy restriction levels and subsequent weight loss can lead to deleterious effects. Next to the known risk of hyperlipemia (Hughes et al., 2004), increased NEFA and TAG concentrations due to too high fat metabolism could induce high levels of oxidative stress as these molecules are prone to oxidation. It is known in humans that lipids also are important contributors to chemical modification of proteins and can result in the formation of lipoxidation products (Bengmark, 2007). Increasing circulating free fatty acids may also be pro-inflammatory via signalling through the Toll like receptors, which can potentiate expression and secretion of pro-inflammatory cytokines. This can potentially increase the pro-inflammatory state in obese equines or during too severe weight loss (De Luca and Olefsky, 2008). Future weight loss trials investigating the effect of higher rates of weight loss compared to our trial could give more information on the possible negative effects of severe weight loss on oxidative stress and pro-inflammatory profile in equines.

Another limitation of the weight loss trial discussed in the present PhD thesis could be the fact that only the changes in oxidative stress markers were evaluated in **Chapter 6.** The baseline values of those markers in healthy normal weight Shetland ponies were not available. So although obese, it is not clear if our ponies suffered from oxidative stress at the start of the trial. Hence, observed changes could be within a normal range for the parameters evaluated, and therefore being of low biological significance. Taking into account the 1) high variance between blood values of oxidant and antioxidant markers between and within the groups, and 2) lack of difference in oxidant and antioxidant markers between begin and end of severe energy restriction, it could be suggested that the importance of oxidative stress in our test population of obese Shetland ponies is rather low.

Unfortunately, no inflammation-related parameters (cytokines) were measured during this weight loss trial. Therefore, it was not possible to examine if there were possible relationships between levels of oxidative stress and inflammatory profile, and the impact of different rates of weight loss on these relationships.

7.5.4 Future research

To gain more insight in the potential relationship between obesity and inflammation/oxidative stress in equines, the following trials are suggested.

1. Both oxidative stress related parameters and inflammatory parameters should be evaluated during weight loss trials to know the impact of oxidative stress and inflammation in equines. So far, their influence on obesity-related disorders is questionable.

2. Not only weight loss trials could give further information, a lot of information could also be gained from weight gain trials, if possible with different rates of weight gain, and with different rations. Starting from lean healthy animals gives information on baseline values of the parameters that want to be investigated. A weight gain trial could give further information on adipose tissue deposition during the onset of obesity, and its influence of oxidative and inflammatory profile. Ideally, weight gain and weight loss trials should be combined with gene expression analysis to see if differences will occur.

7.6 General conclusion

The results of this thesis show that equine adipose tissue depots have a unique behaviour as differences in gene expression of inflammation-related genes between adipose tissue depots were found in a group of normal to obese ponies and horses. In contrast to humans, there was not one particular adipose tissue depot that could be referred to as the most harmful, as there was an increased mRNA expression of pro-inflammatory cytokines in both abdominal and nuchal adipose tissue. Depot-related differences in mRNA expression could not be related to adipocyte size or presence of antigen presenting cells.

Weight loss influences adipose tissue depot magnitude in Shetland ponies, although seasonal influences on adipose tissue distribution in ponies without significant weight loss cannot be ignored. Level of energy restriction influenced morphometric and metabolic parameters in obese Shetland geldings. After 16 weeks of weight loss, greatest decreases in body weight, BCS, heart and belly circumference, and tail head fat depth were detected in the most severe energy restricted group. Following the weight loss period, weight gain was positively associated with level of energy restriction when ponies were again fed at their maintenance energy levels to maintain stable obese body weight.

The small effects of energy restriction on oxidative stress related parameters in the present study were thought not to be of any biological significance.

This thesis identified weight loss strategy as an important factor in healthy weight management in equines, with possible implications for the oxidative and inflammatory status in these animals.

7.7 References

Adams AA, Katepalli MP, Kohler K, Reedy SE, Stilz JP, Vick MM, Fitzgerald BP, Lawrence LM, Horohov DW (2009) Effect of body condition, body weight and adiposity on inflammatory cytokine responses in old horses. Vet. Immunol. Immunopathol. 127, 286–294.

Al-Daccak R, Mooney N, Charron D (2004) MHC II class signaling in antigen-presenting cells. Curr. Opin. Immunol. 16, 108-113.

Anderson (2008) Past-transcriptional regulons coordinate the initiation and resolution of inflammation. Nat. Rev. Immunol. 10, 24-35.

Argo CMcG, Curtis GC, Grove-White D, Dugdale AHA, Barfoot CF, Harris PA (2012) Weight loss resistance: A further consideration for the nutritional management of obese Equidae. Vet. J. 194, 179-188.

Arner P (2005) Human fat cell lipolysis: biochemistry, regulation and clinical role. Best Pract. Res. Clin. Endocrinol. Metab. 19, 471–482.

Banse H (2013) Mechanisms of equine insulin resistance. Dissertation thesis Oklahoma State University.

Bengmark S (2007) Advanced glycation and lipoxidation end products-amplifiers of inflammation: the role of food. JPen- Parenter. Enter. 31, 430-44.

Burns TA, Geor RJ, Mudge MC, McMutcheon LJ, Hinchcliff KW, Belknap JK (2010) Proinflammatory Cytokine and Chemokine Gene Expression Profiles in Subcutaneous and Visceral Adipose Tissue Depots of Insulin-Resistant and Insulin-Sensitive Light Breed Horses. J. Vet. Inter. Med.24, 932-939.

Carter RA, Geor RJ, Stanair WB, Cubitt TA, Harris PA (2009) Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies. Vet. J. 179, 204-210.

Carter RA, McCutcheon LJ, Valle E, Meilahn EN, Geor RJ (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.

Catenacci VA, Wyatt HR (2007) The role of physical activity in producing and maintaining weight loss. Nat. Clin. Pract. Endocrinol. Metab., 3:518-29.

Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin, M.S. (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J. Lipid Res. 46, 2347-2355.

de Laat MA, Kyaw-Tanner MT, Sillence, McGowan CM, Pollitt CC (2012) Advanced glycation endproducts in horses with insulin-induced laminitis. Vet. Immunol. Immunop. 145, 395-401.

de Luca C., Olefsky JM (2008) Inflammation and insulin resistance. FEBS letters 582, 97-105.

Deaton CM, Marlin DJ, Smith NC, Harris PA, Roberts CA, Schroter RC, Kelly FJ (2004) Pulmonary epithelial lining fluid and plasma ascorbic acid concentrations in horses affected by recurrent airway obstruction. Am. J. Vet. Res. 65,80-87.

Dimock AN, Siciliano PD, McIlwraith CW (2000) Evidence supporting an increased presence of reactive oxygen species in the diseased equine joint. Equine. Vet. J. 32, 439-443.

Divers TJ, Cummings JE, de Lahunta A, Hintz HF, Mohammed HO (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.

Drolet R, Bélanger C, Fortier M, Huot C, Mailloux J, Légaré D, Tchernof A (2009) Fat Depot-specific Impact of Visceral Obesity on Adipocyte Adiponectin Release in Women. Obesity 17, 424-430.

Dugdale A.H.A, Curtis G.C, Cripps P., Harris P.A. and Argo C. McG. (2010) Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares. Equine Vet. J. 42, 600-610.

Dugdale AHA, Curtis GC, Harris PA, Argo CMc (2011a) Assessment of body fat in the pony: Part I. Relationships between the anatomical distribution of adipose tissue, body composition and body condition. Equine Vet. J. 43, 552-561.

Dugdale AHA, Curtis GC, Milne E, Harris PA, Argo C.McG (2011b) Assessment of body fat in the pony: Part II. Validation of the deuterium oxide dilution technique for the measurement of body fat. Equine Vet. J. 43, 562-570.

Frank N, Elliott SB, Brandt LE, Keisler DH (2006) Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. J. Am. Vet. Med. Assoc. 228, 1383-1390.

Gentry LR, Thompson JDL, Gentry JrGT, Del Vecchio RP, Davis KA, Del Vecchio PM (2004) The relationship between body condition score and ultrasonic fat measurements in mares of high versus low body condition. J. Equine Vet. Sci. 24, 198-203.

Gordon ME, Jerina ML, Raub RH, Davison KA, Young JK and Williamson KK (2009) The effects of dietary manipulation and exercise on weight loss and related indices of health in horses. Comparative Exercise Physiology 6, 33-42.

Hajer GR, van Haeften TW, Visseren, FL (2008) Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur. Heart J. 29, 2959-2971.

Harker IJ, Harris PA, Barfoot CF (2011) The body condition score of leisure horses competing at an unaffiliated championship in the UK. J. Eq. Vet. Sci. 31, 253-254.

Holbrook TC, Tipton T, McFarlane D (2012) Neutrophil and cytokine dysregulation in hyperinsulinemic obese horses. Veterinary immunology and immunopathology, 145, 283-289.

Huber J, Kiefer FW, Zeyda M, Ludvik B, Silberhumer GR, Prager G, Zlabinger GJ, Stulnig TM (2008) CC chemokines and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity. J. Clin. Endocrinol. Metab. 93, 3215-3221.

Hughes KJ, Hodgson DR, Dart AJ (2004) Equine hyperlipaemia: a review. Aust. Vet. J. 82, 136-142.

Jeffcot LB, Field JR (1985) Current concepts of hyperlipaemia in horses and ponies. Vet. Rec. 116, 461-466.

Kabir M, Stefanovski D, Hsu IR, Iyer M, Woolcott OO, Zheng D, Catalano KJ, Chiu JD, Kim SP, Harrison LN, Ionut V, Lottati M, Bergman RN, Richey JM (2011) Large size cells in the visceral adipose depot predict insulin resistance in the canine model. Obesity 19, 2121–2129.

Keen JA, McLaren M, Chandler KJ, McGorum BC (2004) Biochemical indices of vascular function, glucose metabolism and oxidative stress in horses with equine Cushing's disease. Equine Vet. J. 36, 226-229.

Kirschvink N, Smith N, Fiévez L, Bougnet V, Art T, Degand G, Marlin D, Roberts C, Génicot B, Lindsey P, Lekeux P (2002) Effect of chronic airway inflammation and exercise on pulmonary antioxidant status of healthy and heaves-affected horses. Equine Vet. J. 34, 705-712.

Kischvink N, de Moffarts B, Lekeux P (2008) The oxidant-antioxidant equilibrium in horses. Vet. J. 177, 178-191.

Lykkesfeldt J, Svendsen O (2007) Oxidants and antioxidants in disease: oxidative stress in farm animals. Vet. J. 173, 502-511.

Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M, Cinti S (2008) Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J. Lipid Res. 49, 1562-1568.

O'Connell J, Lynch L, Cawood TJ, Kwasnik A, Nolan N, Geoghegan J, McCormick A, O'Farelly C, O'Shea D (2010) The Relationship of Omental and Subcutaneous Adipocyte Size to Metabolic Disease in Severe Obesity. PLoS ONE 5 4, e9997.

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.

Potter, S. J., Bamford, N. J., Harris, P. A., & Bailey, S. R. (2013) Comparison of weight loss, with or without dietary restriction and exercise, in Standardbreds, Andalusians and mixed breed ponies. J. Equine Vet. Sci., 33(5), 339-339.

Redman LM, Heilbronn LK, Martin CK, de Jonge L, Williamson DA, Delany JP, Ravussin E (2009) Metabolic and behavioral compensations in response to caloric restriction: Implications for the maintenance of weight loss. PLoS ONE, 4 (2009), p. e4377.

Robin CA, Ireland JL, Wylie CE, Collins SN, Verheyen KLP, Newton JR (2014) Prevalence and risk factors for equine obesity en Great Britain based on owner-reported body condition scores. Equine vet. J. DOI: 10.1111/evj.12275.

Suagee JK, Corl BA, Crisman MV, Pleasant RS, Thatcher CD, Geor RJ (2013) Relationships between Body Condition Score and Plasma Inflammatory Cytokines, Insulin, and Lipids in a Mixed Population of Light-Breed Horses. Journal of Veterinary Internal Medicine 27, 157-163.

Skurk T, Alberti-Huber C, Herder C, Hauner H (2007) Relationship between adipocyte size and adipokine expression and secretion. J. Clin. Endocrinol. Metab. 92, 1023-1033.

Stiegler P, Cunliffe A (2006) The role of diet and exercise for the maintenance of fat-free mass and resting metabolic rate during weight loss. Sports Med. 36, 239-262.

Tadros EM, Frank N, Donnell RL (2013) Effects of equine metabolic syndrome on inflammatory responses of horses to intravenous lipopolysaccharide infusion. American journal of veterinary research, 74, 1010-1019.

Ting JP-Y, Trowsdale J (2002) Genetic Control of MHC Class II Expression. Cell 109, S21-S33.

Treiber K, Carter R, Gay L, Williams C, Geor R (2009) Inflammatory and redox status of ponies with a history of pasture-associated laminitis. Vet. Immunol. Immunopath. 129, 216-220.

Trujillo ME and Scherer PE (2006) Adipose tissue-derived factors: Impact on health and disease. Endocr. Rev 27, 762-778.

Tsai AC, Sandretto A, Chung YC (2003) Dieting is more effective in reducing weight but exercise is more effective in reducing fat during the early phase of a weight-reducing program in healthy humans. J. Nutr. Biochem. 14, 541-549.

Valle E, Storace D, Sanguinetti R, Carter R, Odetti P, Geor R, Bergero D (2013) Association of the glycoxidative stress marker pentosidine with equine laminitis. Vet. J., 3, 445-450.

Van de Velde H, Janssens GPJ, de Rooster H, Polis I, Peters I, Ducatelle R, Nguyen P, Buyse J, Rochus K, Xu J, Verbrugghe A, Hesta M (2013) The cat as a model for human obesity: insights into depot-specific inflammation associated with feline obesity. Brit. J. Nutr. 110, 1326-1335.

Van Harmelen V, Reynisdottir S, Eriksson P, Thörne A, Hoffstedt J, Lönnqvist F, Arner P (1998) Leptin secretion from subcutaneous and visceral adipose tissue in women. Diabetes 47, 913–917.

Van Weyenberg S, Hesta M, Buyse J, Janssens GPJ (2008) The effect of weight loss by energy restriction on metabolic profile and glucose tolerance in ponies. J. Anim. Physiol. An. N. 92, 538-545.

Vick MM, Adams AA, Murphy BA, Sessions DR, Horohov DW, Cook RF, Shelton BJ, Fitzgerald BP (2007) Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85, 1144-1155.

Vick MM, Murphy BA, Sessions DR, Reedy SE, Kennedy EL, Horohov DW, Frank Cook R, Fitzgerald BP (2008) Effects of systemic inflammation on insulin sensitivity in horses and inflammatory cytokine expression in adipose tissue. Am. J. Vet. Res. 69, 130-139.

Waller AP, Kohler K, Burns TA, Mudge MC, Belknap JK, Lacombe VA (2011) Naturally occurring compensated insulin resistance selectively alters glucose transporters in visceral and subcutaneous adipose tissues without change in AS160 activation. Biochim. Biophys. Acta 1812, 1098-1103.

Watson TDG, Burns L, Love S, Packard CJ, Shepard J (1992) Plasma lipids, lipoproteins and post-heparin lipases in ponies with hyperlipaemia. Equine Vet. J. 24, 341-346.

Yang SH, Matsui T, Kawachi H, Yamada T, Nakanishi N, Yano H (2008) Fat depot-specific differences in leptin mRNA expression and its relation to adicyte size in steers. Anim. Sci. J. 74, 17-21.

SUMMARY

Summary

Overweight and obesity are a growing problem in the modern horse industry, especially in leisure horses and ponies. Increased interest in the influence of adipose tissue region on inflammatory profile and effective strategies to treat equine obesity is present in current equine research. This thesis aimed to give further insights into these two topics.

Chapter 1 first gives a definition of obesity. Then, a general overview to determine and evaluate the nutritional status, adipose tissue distribution, and body composition in equines is presented. Furthermore, multiple reasons for the increased incidence of equine overweight and obesity are given. The positive effects of adipose tissue, the composition of adipose tissue in the lean and obese state, and the impact of obesity in humans and horses are described. Finally, the most important weight loss strategies in equines described in literature are discussed. From this overview, multiple research questions could be formulated (**Chapter 2**), to further examine the importance of adipose tissue distribution in equines and to gain insight in what is happening during weight loss in the equine body.

The first aim of the present PhD thesis was to evaluate the effect of adipose tissue distribution in the equine body on the expression of inflammation-related genes, adipocyte size, and presence of antigen presenting cells. In humans, harmful effects of obesity have been mainly attributed to visceral adipose tissue. In equines, special interest has been focussed on adipose tissue distributed at the crest of the neck as increased deposition of adipose tissue in that region could indicate or contribute to hyperinsulinemia, insulin resistance and/or an increased risk for laminitis.

To evaluate differences in gene expression of inflammation-related genes in the different adipose tissue depots, reverse transcriptase real time PCR was used. An accurate normalization is crucial to obtain a correct interpretation of the gene expression data. The use of reference genes as internal control is most common. Therefore, a set of reliable reference genes was determined (**Chapter 3**). It was evidenced that even in one tissue (in this case adipose tissue) originating from different locations in the equine body (neck region, subcutaneous region, and abdominal region) differences in reference gene stability were present. To overcome this location dependent stability of the references genes, most stable reference gens (HPRT1, RPL32, and GAPDH) were determined in an adipose tissue sample in which all the depots were combined.
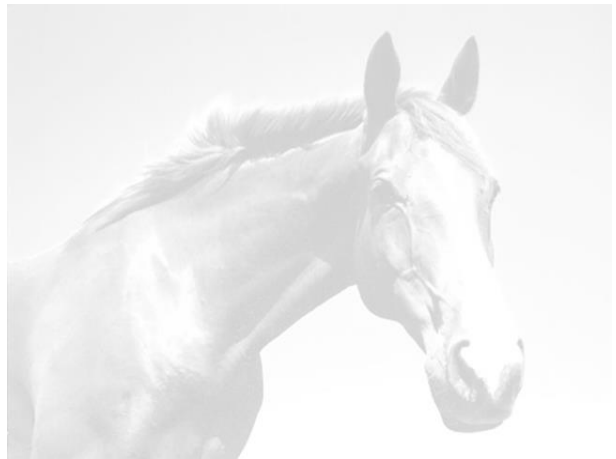
This set of references genes was used for normalization of the results in **Chapter 4**. Differences in gene expression of inflammation-related genes in adipose tissue originating from different location in the equine body were evaluated. Therefore, 12 ponies and horses with a normal to obese body condition, euthanized for non-research purposes, were sampled. Adipose tissue samples from the neck, subcutaneous, and abdominal region were collected and further analysed. Within each region, 2 or 3 samples were taken. As in humans, depot related differences in inflammation-related genes were present. The higher pro-inflammatory profile of the nuchal adipose tissue region as described in another equine trial could not be confirmed in our trial. An increased expression of both pro- and inflammatory genes was observed in the abdominal region. We could not conclude that, as opposed to humans, one adipose tissue depot clearly is the most harmful. Regional differences in adipocyte size and presence of antigen presenting cells were also observed. Further research in a larger population of normal and obese equines with the inclusion of more inflammation-related genes could provide more information about the regional inflammatory profile in equines with different body condition scores.

The second aim of this thesis was to investigate the effect of different levels of energy restriction on morphometric, behavioural, metabolic, and oxidative stress related parameters. Therefore, 18 obese Shetland geldings were subjected to a 16-week weight loss trial and a 3-week follow up period. More severe energy restriction was accompanied by a greater decrease in body condition score, relative body weight, belly girth, and heart girth (**Chapter 5**). At the end of the follow up period, when all ponies were again fed 100% of their maintenance energy requirements to maintain stable obese body weight, relative weight gain was highest in the severe energy restricted group. No effect of level of energy restriction on gastric ulcer development was noted. Coprophagy was present in all treatment groups, including the control group throughout the entire trial.

In the same trial, effect of level of energy restriction oxidative profile, lipid metabolism, glucose, insulin, and leptin was studied (**Chapter 6**). No effect energy restriction regimen was found for glucose and insulin. The highest increase in lipid markers (triacylglycerol and non-esterified fatty acids) was found in the most energy restricted group after 4 to 8 weeks of weight loss. By the end of the weight loss period, these values returned to baseline values. Prooxidant (advanced oxidation protein products) and antioxidant (ferric reducing ability of plasma and α-tocopherol) also increased during the first 4 to 8 weeks of weight loss and returned to baseline values by the end of the weight loss period. We could

conclude that the rate of weight loss influenced blood pro- and antioxidant markers during the weight loss process, but it did not result in significantly decreased values at the end of the weight loss period in Shetland ponies. We should mention, however, that ponies were still overweight at that time. Longer term weight loss trials in which equines are energy restricted until ideal body weight and body condition score is reached can give very valuable information into the changes that occur in the equine body during the weight loss process. Further research in more breeds and more (sensitive) oxidative stress related parameters could give further information about the influence of weight loss on oxidative profile in equines.

From the results of the present study, we can conclude that regional differences in gene expression, adipocyte size and presence of antigen presenting cells occur in ponies and horses. Rate of energy restriction and rate of weight loss influence morphometric and oxidative stress related parameters in the transition from the obese to the overweight state. Weight loss trials in which equines reach ideal body weight are essential to gain further insights in the events occurring in the equine body during weight loss.

SAMENVATTING

Samenvatting

Overgewicht en obesitas zijn een steeds groter wordend probleem in huidige paardenhouderij, niet in het minst bij de gezelschapspaarden/pony's en paarden gebruikt voor recreatieve doeleinden. Bij de mens worden overgewicht en obesitas geassocieerd met een chronische ontsteking, die op haar beurt dan weer bijdraagt tot de ontwikkeling van onder andere cardiovasculaire problemen. Bij het paard er tot nu toe nog niet zoveel gekend over de relatie tussen overgewicht en obesitas enerzijds en een verhoogde chronische ontstekingstoestand anderzijds.

In **Hoofdstuk 1** wordt een algemeen overzicht gegeven van wat obesitas precies is, en hoe we de voedingstoestand, de vetverdeling en de lichaamssamenstelling bij paardachtigen kunnen beoordelen. Verder worden verschillende redenen aangehaald waarom overgewicht en obesitas een toenemend probleem zijn, maar wordt ook aangetoond dat vetweefsel essentieel is in het lichaam. Een overzicht van de samenstelling van vetweefsel en de negatieve effecten van overgewicht en obesitas bij zowel de mens als paardachtigen worden er verder behandeld. Er wordt afgesloten met de belangrijkste vermageringsstrategieën bij paardachtigen zoals deze beschreven zijn in de literatuur.

Het eerste doel van deze thesis was nagaan wat de negatieve effecten waren van overgewicht en obesitas bij paardachtigen, met specifieke aandacht voor het belang van de verschillende vetdepots in het paardenlichaam. Waar bij de mens vooral het intra-abdominaal vetdepot verantwoordelijk is voor de negatieve effecten geassocieerd met obesitas, werd dit bij het paard grotendeels toegewezen aan het vetdepot ter hoogte van de nek.

Om verschillen in genexpressie van ontstekingsgerelateerde cytokines in vetdepots verspreid over het gehele paardenlichaam te kunnen bestuderen werd gebruik gemaakt van reverse-transcriptase real time PCR. Om deze verschillen correct te kunnen interpreteren, is het van groot belang deze resultaten op een juiste manier te normaliseren. Dit kan met behulp van stabiele referentiegenen. Daarom werd in **Hoofdstuk 3** uit een set beschikbare referentiegenen de meest stabiele referentiegenen gedetermineerd. Er werd aangetoond dat er regionale verschillen (vetweefsel afkomstig van de nekregio, subcutaan vet en abdominaal vet) waren in stabiliteit van de referentiegenen, en daarom werden de meest stabiele referentiegenen (*HPRT1, RPL32* en

GAPDH) bepaald in vetweefsel waarbij alle depots gecombineerd werden. *HPRT1, RPL32* en *GAPDH* werden aangewend voor de normalisatie van de resultaten uit **Hoofdstuk 4**. Daar werd nagegaan of er regionale verschillen waren in genexpressie van ontstekingsgerelateerde genen tussen vetweefsel afkomstig van de nekregio, subcutaan vet en abdominaal vet. Als testpopulatie werden 12 paarden en pony's van verschillende rassen met een normale tot obese lichaamsconditie bemonsterd. Per regio werden 2 of 3 vetstalen verzameld. We concludeerden dat er, net zoals bij de mens en andere diersoorten, duidelijke regionale verschillen in genexpressie aanwezig waren. Een hoger pro-inflammatoir profiel van vetweefsel afkomstig uit nekregio kon in deze studie niet volledig bevestigd worden. Ook in het buikvet werd een verhoogde expressie van zowel pro- als anti-inflammatoire genen waargenomen. Regionale verschillen in vetcelgrootte en aantal antigeen presenterende cellen werden eveneens aangetoond. We kunnen concluderen dat er uit de resultaten van Hoofdstuk 4 niet 1 vetregio als duidelijk pro-inflammatoir kan aangeduid worden. Verder onderzoek in een grotere populatie normale en obese paardachtigen kan meer informatie verschaffen over het regionale inflammatoire profiel bij paarden met verschillende lichaamsconditiescores.
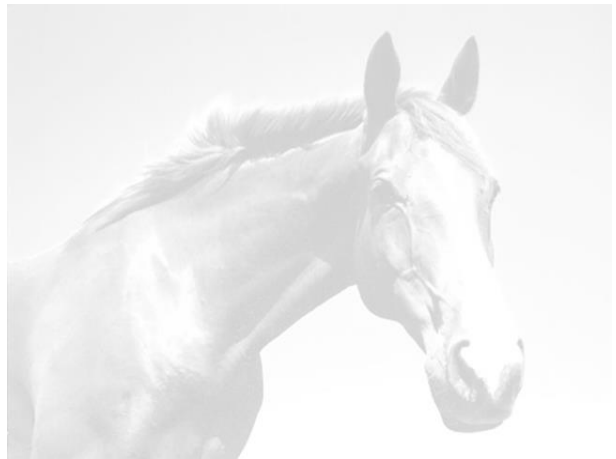
Het tweede doel van deze thesis was nagaan welk effect energiebeperking had op de verschillende vetdepots, en of er verschillen waren tussen matige en strengere energiebeperking (**Hoofdstuk 5**). Strengere energiebeperking ging gepaard met een procentueel groter gewichtsverlies, grotere afname van buikomtrek en hartomtrek, evenals een trend voor een grotere afname van de subcutane vetdikte ter hoogte van de staart. Ook de daling in lichaamsconditiescore was hoger in de strenger energiebeperkte groep. Geen verschillen werden waargenomen in subcutane vetdikte ter hoogte van rug en de ribben. Wanneer de dieren na 16 weken energiebeperking opnieuw gevoederd werden naar 100% van hun energiebehoefte om obees lichaamsgewicht te behouden, was de relatieve gewichtstoename het grootst in sterkt beperkte groep. De mate van energiebeperking had geen invloed op de ontwikkeling van maagzweren of agressief gedrag. Coprofagie kwam voor in alle groepen, zowel in de controle groep die niet in energie beperkt was, als de 2 vermageringsgroepen. Hoewel niet significant, was er toch een grotere toename in incidentie van coprofagie in de strenge energiebeperkte groep tijdens de vermageringsperiode. Wanneer deze dieren opnieuw gevoederd werden naar 100% van hun energiebehoefte om obees lichaamsgewicht te behouden was er een

opnieuw een sterkere afname in incidentie van coprofagie ten opzichte van de controlegroep en de minder energiebeperkte vermageringsgroep.

Ook werd er in deze studie nagegaan wat het effect was van snelheid van vermageren op metabole parameters, zoals insuline, glucose, vetmetabolisme parameters en oxidatieve stress gerelateerde parameters (**Hoofdstuk 6**). Snelheid van vermageren had geen invloed op glucose en insuline. In de meest energiebeperkte groep was er een sterkere stijging in triglyceriden en vrije vetzuren, dat veroorzaakt kan worden door de grotere vetafbraak in de deze groep. Tegen het einde van de vermageringsperiode waren deze parameters terug op hun beginwaarden. De triglyceridegehaltes in het bloed hebben nooit de kritische waarde van 500mg/dl overschreden, dus het gevaar voor hyperlipemie was gering. Ook werden de dieren klinisch geëvalueerd op tekenen van hyperlipemie. Zowel pro- (advanced oxidation protein products) als anti-oxidante parameters (ferric reducing ability of plasma en α-tocopherol) vertoonden tijdens de eerste weken van de vermageringsperiode een stijging, die sterker was in de strengst beperkte groep. Naar het einde van vermageringsperiode daalden al deze parameters terug naar hun beginwaarden. Algemeen gezien was er een lichte daling in leptine gehaltes in het bloed tussen het begin en het einde van de vermageringsperiode in de 2 vermageringsgroepen, terwijl het in de controlegroep op een constant niveau bleef. Uit de resultaten van deze proef kunnen we besluiten dat vermageren en snelheid van vermageren een invloed hadden op anti- en pro-oxidatieve parameters tijdens het verloop van het vermageringsproces, maar niet resulteerden in gedaalde gehaltes op het einde van de vermagering in een groep Shetland pony's die nog steeds overgewicht hadden op het einde van de vermageringsperiode. Verder onderzoek in meerdere rassen, en meerdere (gevoeligere) oxidatieve stress gerelateerde parameters kunnen verder informatie verschaffen over de invloed van vermageren op oxidatieve stress. In de studie beschreven in hoofdstuk 5 en 6 hadden de dieren op het einde van de vermageringsperiode nog steeds overgewicht, dus een langere termijn vermageringsstudie waarbij dieren opgevolgd worden tot ze op ideaal lichaamsgewicht zijn kan verdere nuttige informatie verschaffen in verband met invloed van vermageren op zowel morfologische als metabole parameters.

De onderzoeken uitgevoerd in deze thesis maken duidelijk dat er ook bij paardachtigen met een normale tot obese lichaamsconditie regionale verschillen zijn inflammatoir profiel, vetcelgrootte en aantal antigeenpresenterende cellen tussen de verschillende vetdepots. Snelheid van vermageren heeft invloed op bepaalde

morfometrische en oxidatieve stress gerelateerde parameters, maar langere termijn studies waarbij paarden opgevolgd worden tot op ideaal gewicht zijn essentieel om een nog diepere kennis te verschaffen in verband met de invloed van gewichtsverlies op voorgenoemde parameters.

CURRICULUM VITAE

Curriculum vitae

Lien Bruynsteen werd geboren op 19 augustus 1983 te Torhout. Na het behalen van het diploma hoger secundair onderwijs aan het Sint-Jozefscollege te Torhout in de richting wiskunde-wetenschappen, begon zij in 2001 met de studie Diergeneeskunde aan de Universiteit Gent. Zij behaalde haar diploma van Dierenarts in 2007 met onderscheiding, optie Paard.

Na een kortstondige tewerkstelling als leerkracht secundair onderwijs aan het Immaculata Instituut te Brugge, begon zij als zelfstandig dierenarts bij DAP De Geest Jan in Watou. Zij werkte daar gedurende 14 maand alvorens in juli 2009 te starten aan het Laboratorium voor Diervoeding, Vakgroep Voeding, Genetica en Ethologie. De eerste 3 maand als onbezoldigd medewerker, nadien als doctoraatsbursaal met een facultaire onderzoeksbeurs. Van februari 2010 tot eind december werkte ze als Dehousse bursaal en in januari 2011 behaalde zij een IWT specialisatiebeurs. Gedurende meer dan 5 jaar deed zij onderzoek naar overgewicht en obesitas bij het paard, de invloed van plaats van vetopslag in het paardenlichaam en de invloed van vermageren op verschillende metabole parameters bij het paard. Daarnaast was zij promotor en medepromotor van meerdere hogeschoolstudenten en studenten diergeneeskunde. Gedurende haar doctoraatsopleiding hielp ze mee met het praktisch werk bij verschillende collega's en verschillende diersoorten.

Lien Bruynsteen is auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Tevens was zij spreker op meerdere nationale en internationale congressen.
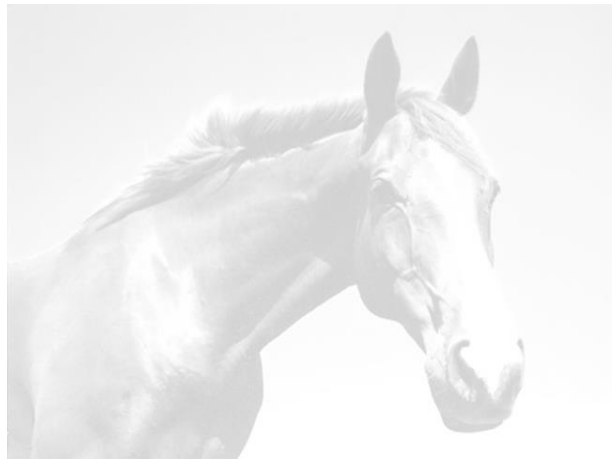
BIBLIOGRAPHY

Bibliography

1. Publications in international peer-reviewed journals

Bruynsteen L., Erkens T., Peelman L., Ducatelle R., Janssens G., Harris P., Hesta M. (2013) Expression of inflammation-related genes is associated with adipose tissue location in horses. BMC Veterinary Research 9; 240.

Bruynsteen L., Janssens G., Harris P., Duchateau L., Valle E., Odetti P., Vandevelde K., Buyse J., Hesta M. (2014) Changes in oxidative stress in response to different levels of energy restriction in obese ponies. British Journal of Nutrition, 112, 1402-1411.

Bruynsteen L., Moons C., Janssens G., Harris P., Vandevelde K., Lefère L., Duchateau L., Hesta M. (2014) Level of energy restriction alters Body Condition Score and morphometric profile in obese Shetland ponies (submitted to the Veterinary Journal, revisions).

2. Abstracts presented at national and international conferences

Bruynsteen L., Janssens G.P.J., Harris P., Duchateau L., Valle E., Vandevelde K., Hesta M. (2013) Changes in physical and metabolic parameters in function of weight loss rate in obese ponies. In: *Proceedings of the 38th Animal Nutrition Research Forum,* pp. 7.

Valle E., Bruynsteen L., Odetti P., Borghi R., Pacini D., Traverso N., Hesta M. (2013) Effect of different degrees of caloric restriction on AOPPs and AGEs formation in healthy obese ponies. In: *Proceedings of the 6th European Equine Health and Nutrition Congress,* pp. 173.

Bruynsteen L., Vandevelde K., Harris P., Janssens G.P.J., Hesta M. (2012) Changes in oxidative stress as a function of weight loss rate in obese ponies. In: *Proceedings of 16th congress of the European Society of Veterinary and Comparative Nutrition,* p.22

Bruynsteen L., Vandevelde K., Harris P., Janssens G.P.J., Hesta M (2012) The effect of different levels of energy restriction on physical, metabolic, morphometric and animal welfare parameters. In: *Proceedings of 16th congress of the European Society of Veterinary and Comparative Nutrition,* p.87.

Bruynsteen L., Erkens T., Peelman L., Janssens G., Harris P.A., Hesta M. (2011) Determination of depot-specific mRNA expression by equine adipocytes. In: *Proceedings of the 5th European Equine Health and Nutrition Congress,* pp. 149.

Bruynsteen L., Erkens T., Peelman L., Janssens G., Harris P.A., Hesta M. (2011) Depot-specific mRNA expression by equine adipocytes. *Proceedings of 15th congress of the European Society of Veterinary and Comparative Nutrition,* p.63.

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Dankwoord

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Liefs…

Lien