EQUINE OBESITY AND ITS ROLE IN INSULIN RESISTANCE, INFLAMMATION AND RISK FOR LAMINITIS

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Key words: exercise, horse, inflammation, insulin resistance, laminitis, obesity

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(ABSTRACT)

The present studies were conducted to determine the effects of obesity on insulin resistance, inflammatory state and risk for laminitis, and the effectiveness of exercise training to reduce obesity and insulin resistance in equids. Practical methods of assessing adiposity were developed and verified, including a condition scoring system for neck crest adiposity and morphometric measurements for generalized (girth:height) and localized (neck circumference:height) adiposity. Evaluation of 74 and 57 pony mares in March of two consecutive years resulted in the identification of predictive tests for incipient pasture-associated laminitis, including hyperinsulinemia (> 32 mU/L), hyperleptinemia (> 7.3 ng/mL), and generalized (body condition score ≥ 7) and localized (cresty neck score ≥ 4) obesity. Induction of obesity in 13 Arabian geldings by 4 months of overfeeding resulted in compensated insulin resistance (minimal model analysis) with hyperinsulinemia and hyperleptinemia. Although lipid concentrations (nonesterified fatty acids and triglyceride) decreased on a high concentrate diet, they did not differ before and after weight gain. The resulting obesity-induced insulin resistance was accompanied by an increase in chemokine (monocyte chemoattractant protein [MCP]-1, MCP-2, interleukin [IL]-8) but not inflammatory cytokine (tumor necrosis factor [TNF]α, IL-1β, IL-6) mRNA expression in subcutaneous adipose tissue. Additionally, there was a decrease in plasma TNFα protein concentration with weight gain. By using 12 of the obese, insulin resistant Arabian geldings (8 exercised, 4 control) it was demonstrated that 8 weeks of moderate intensity exercise training reduced adiposity (4% reduction in body weight, 35% reduction in fat mass) without affecting glucose and insulin dynamics or plasma hormone and lipid concentrations. Collectively, these studies demonstrate the impact obesity has on metabolism and risk for laminitis in equids, and that exercise training may provide an effective countermeasure for the reduction of obesity.

Key words: exercise, horse, inflammation, insulin resistance, laminitis, obesity

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INTRODUCTION

Obesity has become a major health concern in humans over the past few decades due to its increasing prevalence and association with risk factors for diseases that include type 2 diabetes mellitus and cardiovascular disease. Development of obesity is a causative factor for insulin resistance through several molecular mechanisms, including the creation of a low-grade inflammatory state. Additionally, central obesity in humans indicates a greater risk for insulin resistance and disease than generalized obesity alone.

With equids there is increasing concern about the metabolic effects of obesity as its association with insulin resistance, inflammation, and risk for disease has been revealed. Through analogies with human conditions, it has been hypothesized that equine obesity is linked to insulin resistance via development of a proinflammatory state. These metabolic disturbances contribute to mechanisms that induce laminitis, particularly the pasture-associated form of this disease. There is a lack of experimental evidence in horses demonstrating a causal role of obesity in insulin resistance or inflammation. Anecdotal evidence suggests that adipose tissue distributed along the crest of the neck, or the appearance of a “cresty neck”, is a high risk fat depot comparable to central obesity in humans.

Determination of specific consequences of obesity, such as insulin resistance or a proinflammatory state, and identification of their role in risk for laminitis would allow for the investigation of countermeasures to avoid these metabolic disturbances and prevent disease associated with obesity. Exercise training is often recommended as a beneficial management strategy because of its potential to reduce body weight and improve insulin sensitivity. However, there is minimal evidence to support its effectiveness in obese, insulin resistant horses or to create recommendations for exercise duration or intensity.

Given the influence of obesity on insulin resistance and risk for disease in humans and the lack of understanding regarding the role of obesity in equine health and disease, the following studies were designed to address this gap in knowledge. The objectives of these studies were to:

1) Develop and evaluate methods of measuring generalized and localized adiposity in horses and ponies;
2) Identify predictive tests for incipient pasture-associated laminitis in ponies;
3) Determine effects of diet-induced obesity on glucose and insulin dynamics and plasma hormone and lipid concentrations in horses;
4) Determine effects of obesity-induced insulin resistance on subcutaneous adipose tissue mRNA expression of inflammatory mediators and plasma TNFα concentrations in horses; and
5) Assess the effects of moderate intensity exercise training on adiposity, glucose and insulin dynamics, and plasma hormone and lipid concentrations in obese, insulin resistant horses.

LITERATURE REVIEW

Prevalence of equine obesity and related comorbid conditions

Equine obesity has been associated with comorbid conditions of insulin resistance and laminitis. Anecdotal observations by equine veterinarians have suggested that obesity is a growing problem in companion equid populations. However, the prevalence of obesity is poorly understood, as there are few epidemiological studies addressing this condition. A survey conducted by the USDA in 1998 estimated the prevalence of equine obesity as 5% (USDA, 2000). However, there were no defined measurement variables or criteria for obesity classification. Additionally, data were owner-reported, which may have lead to a significant bias of the results. To further address this problem, a recent cross-sectional study randomly sampled 300 mature horses (140 mares, 151 geldings and 9 stallions; 4 to 20 years of age) drawn from the Virginia-Maryland Regional College of Veterinary Medicine Equine Field Service practice. Contrary to the USDA survey, there were a reported 19% obese (BCS 8 or 9) and 32% overweight (BCS 6.5 – 7.5) in the study population (Thatcher et al., 2007).

The prevalence of insulin resistance in horses is unknown, as there is currently no standardized method of measurement or clinical criteria for classification of insulin resistance. However, prevalence of hyperinsulinemia, a nonspecific indicator of insulin resistance, was assessed in the cross-sectional study of 300 horses in Southwestern Virginia. Hyperinsulinemia, as defined by basal insulin concentration > 30 mU/L, was present in 10.0% (95% CI = 7.1% - 13.9%) of the horses evaluated (Geor et al., 2007).

Pasture-associated laminitis has major economic and welfare implications for the equine industry, accounting for an estimated 54% of cases of equine laminitis for which the initial cause is identifiable (USDA, 2000). According to a survey conducted by the USDA, apart from colic, laminitis is the most common cause for a horse or pony to be presented for veterinary treatment, as 13% of horse owners/operations reported problems with laminitis within the previous year, with ~5% of those affected with laminitis resulting in death or euthanasia (USDA, 2000). Similarly, in the United Kingdom, a

survey involving 113,000 horses estimated the prevalence of laminitis at 7.1%, with ~8,000 horses affected each year, of which ~600 were euthanized and ~1,300 left permanently unsound (Hinckley and Henderson, 1996).

Methods of assessing adiposity

The objective in assessing adiposity is to quantify fat mass (FM), either by direct measurement or indirect, more relative, measurements. Differentiation between FM and fat-free mass (FFM; e.g. bone and muscle) increases the accuracy of a method. In humans, the 'gold standard' for body composition assessment is hydrostatic weighing. This technique is based on Archimedes' principle and requires the subject to exhale completely and remain submerged underwater for ~10 seconds (Jensen, 1992). As it would be unreasonable to expect a horse to hold its breath underwater, this technique is not applicable to horses. Additionally, other accurate methods of body composition assessment used in humans and small animals, such as computed tomography and dual energy X-ray absorption (DEXA), are not applicable to horses because of their large size. Therefore, more accurate methods of assessing adiposity are often substituted for by less accurate, more practical methods.

Body condition scoring

The most common method for assessment of adiposity in horses is the rating of body condition (subcutaneous fat deposition) by use of a 9-point scale (Henneke et al., 1983). The scores are based on visual appraisal and palpable fat cover at six areas of the horse's body: neck, withers, back, tailhead, ribs, and behind the shoulder. The Henneke BCS system, originally developed for use in Quarter Horse broodmares, may be most appropriate for use in light breed horses (e.g. Quarter horses, Thoroughbreds and Arabians) that share similar body type and patterns of fat deposition. Although the Henneke system has been applied with modifications to other breeds, including warm-blooded horses (Kienzle and Schramme, 2004), its suitability for ponies has not been reported.

Although BCS is a subjective assessment, it is an accepted evaluation of adiposity in horses, and previous studies show that it correlates with subcutaneous fat thickness (Gentry et al., 2002; Henneke et al., 1983), leptin concentration (Buff et al., 2002), glucose tolerance (Frank et al., 2006), and insulin sensitivity (Vick et al., 2007). Obesity, as defined by a BCS above a certain cutpoint, is associated with an increased risk for laminitis (Treiber et al., 2006c), reduced insulin sensitivity (Hoffman et al., 2003), and altered metabolic and reproductive activity (Vick et al., 2006).

Morphometric measurements

In human medicine, morphometric measures such as body mass index (BMI) and waist to hip ratio are used for assessment of overall adiposity and regional (central) adiposity, respectively. Donaldson et al. (2004b) applied a BMI to horses (estimated weight (kg)/height $(m)^2$) and found a moderate correlation (r_s = 0.60, 95% confidence interval, 0.44 to 0.73) between this index and BCS. In the validation of the BCS system, condition scores were correlated with weight (r^2 = 0.50, $P < 0.01$), girth circumference (r^2 = 0.40, $P < 0.05$), weight:height (r^2 = 0.58, $P < 0.01$), and girth:height (r^2 = 0.51, $P <$ 0.01) measurements (Henneke et al., 1983). Overall, however, there has been little application of morphometric measurements for evaluation of adiposity in equids.

Ultrasonic measurement of subcutaneous fat depth

Percentage body fat has been calculated from subcutaneous fat thickness, with the assumption that fat depth at a specific body site (usually the rump) is proportional to generalized adiposity. By measuring ultrasound subcutaneous fat thickness on the rump and carcass composition in horses and ponies, linear regression was used to develop a predictive equation of percent body fat from rump fat thickness (Westervelt et al., 1976). The equation developed by Westervelt et al. (1976) of % body fat = 5.07*(fat depth, cm) + 6.22, was specific to a site of ultrasound fat depth over the rump 5 cm lateral from the midline at the center of the pelvic bone. Subsequently, a similar study evaluated five sites along the rump from the tailhead to the top of the croup, each site 10 cm from the midline (Kane et al., 1987). Fat depth at the site closest to the tailhead was most highly correlated

with fat mass, and a predictive equation was calculated as % body fat = 5.47*(fat depth, cm) + 2.47.

Subcutaneous fat thickness or body fat percentage calculated from fat depth are correlated to body condition. In the original validation of the BCS system, percent body fat calculated from ultrasonic fat thickness was correlated to BCS ($r^2 = 0.65$, $P < 0.001$) (Henneke et al., 1983). Repeated ultrasound measurement along the topline, from the withers to the tailhead, indicated that subcutaneous fat thickness at the tailhead had the highest correlation with BCS (Gentry et al., 2004).

Percent body fat calculated from subcutaneous fat thickness has been used to calculate FM and FFM based on body weight (Kearns et al., 2002b; Kearns et al., 2001; Kearns et al., 2006). Indirectly calculating FM and FFM with this method applies the assumption that subcutaneous fat thickness of the rump has a constant proportion to total FM, and that body weight differences in the absence of fat depth differences are attributable to differences in FFM. However, alternative fat depots in addition to subcutaneous fat, such as visceral or intramuscular adipose, also contribute to FM and are not accounted for in this calculation. Additionally, these studies used the ultrasound site specified by Westervelt et al. (1976), but the equation specified by Kane et al. (1987) which was developed for a different location.

Total body water

A more accurate method of measuring FM and FFM may be through the measurement of total body water (TBW), as these techniques account for multiple adipose depots. In horses, TBW has been assessed through the isotope dilution techniques, such as deuterium oxide dilutions (Andrews et al., 1997), and by bioelectrical impedance (Fielding et al., 2004; Forro et al., 2000). In dilution techniques, deuterium oxide is administered orally or intravenously. The principle behind this method is that isotope equilibrates into all body water spaces and does not equilibrate into lipid compartments, such as adipose tissue. Once equilibration occurs, the concentration of isotope in plasma is representative of all body water spaces, and total body water is calculated according to the amount of isotope administered and its final concentration in plasma. Fat mass and fat-free mass are then calculated based on a hydration constant of

0.73 for fat-free mass and zero for fat mass (Wang et al., 2000). Although this hydration constant has not been determined specifically in horses, in other mammals, including the mouse, rat, hamster, Rhesus monkey, baboon, goat, sheep, pig, cattle, gray seal and human, it ranges between 0.71 and 0.77, with an overall average of 0.73 (Wang et al., 1999).

Assessment of regional fat distribution

The BCS system and calculation of body composition by TBW are not useful to differentiate the differences in regional adiposity that may indicate increased risk for disease. In human subjects, abdominal (visceral) adiposity is more closely linked to risk for diabetes and cardiovascular disease than generalized obesity, and measurement of waist circumference is a better indicator of abdominal fat accumulation than is BMI (Murphy and Bloom, 2006).

In horses and ponies, adipose tissue deposited more heavily along the crest of the neck ("cresty neck"), has been suggested to be associated with altered metabolic states, including insulin resistance, and an increased risk for laminitis (Johnson, 2002; Treiber et al., 2006c). Measurements of neck circumference were correlated with glucose ($r = 0.71$; $P = 0.015$) and insulin ($r = 0.88$; $P < 0.001$) areas under the curve during combined glucose-insulin tests, indicating that horses with larger necks were more insulin resistant (Frank et al., 2006). However, the effects of neck crest adiposity were not independent of the effects of generalized adiposity in this study, as similar correlations were reported for BCS.

In addition to estimating generalized body adiposity, ultrasound assessment of regional distribution of subcutaneous adipose tissue at multiple body locations has been reported (Gentry et al., 2004; Westervelt et al., 1976). Ultrasound measurement over the shoulder, rib and rump after 0, 30, and 90 days of exercise indicated a decrease in shoulder and rump fat thickness, but no change in rib fat thickness in horses (Westervelt et al., 1976). When separate groups of exercised and control ponies were compared, the exercised ponies had less fat cover over the shoulder, rump and ribs. Repeated ultrasound measurements along the topline at 4 locations between the withers and rump demonstrated an increase in subcutaneous fat thickness with weight gain and a decrease

with weight loss at all locations (Gentry et al., 2004). Collectively, these studies indicate a potential usefulness of ultrasonic measurements at various body locations to track changes in subcutaneous fat thickness.

Insulin sensitivity

Insulin sensitivity is defined as the effectiveness of insulin to reduce blood glucose by promoting glucose uptake into muscle and adipose cells and by increasing hepatic glycogen storage and reducing hepatic glucose production (Trout et al., 2007). Insulin resistance is a condition in which there is a reduced level of insulin sensitivity. In this situation, the cells are less responsive to the effect of insulin, and therefore higher insulin concentrations are needed to maintain euglycemia in response to a glucose load.

Mechanisms of insulin-mediated glucose disposal

Insulin action mediates glucose uptake primarily into muscle, adipose and liver tissues. Skeletal muscle is responsible for the majority of insulin-mediated glucose disposal in the body, and glucose disposal in both skeletal muscle and adipose tissue is mediated by the insulin-dependent glucose transporter (GLUT) 4 (Musi and Goodyear, 2006). Insulin facilitates hepatic glucose uptake by promoting utilization and preventing production of glucose (Trout et al., 2007). This is accomplished by stimulating glycogen synthesis and glycolysis, and conversely inhibiting glycogenolysis and gluconeogenesis.

Glucose uptake in skeletal muscle and adipose cells is mediated by translocation of glucose transporters from intracellular vesicles into the plasma membrane. Once in the plasma membrane, glucose transporters facilitate glucose diffusion down a concentration gradient and into the cell. Signaling mechanisms resulting in the translocation and activation of glucose transporters can be either dependent or independent of insulin action (Kahn, 1994). Insulin-independent glucose uptake may occur through glucose transporters that are not reliant on insulin stimulation, such as GLUT1, whereas the primary insulin-dependent glucose transporter is GLUT4. However, during cellular energy deficit, such as during exercise, pathways utilizing AMP-activated protein kinase

(AMPK) may facilitate GLUT4 translocation and glucose uptake independent of insulin action (Musi and Goodyear, 2006).

Insulin-dependent glucose uptake into skeletal muscle and adipose tissue is initiated when an increase in glucose concentration stimulates the secretion of insulin from pancreatic β-cells into circulation, with subsequent stimulation of insulin signaling pathways in various tissues (Trout et al., 2007). Insulin induces an anabolic response in skeletal muscle and adipose tissue, stimulating glucose and free fatty acid uptake, inhibiting lipolysis, and stimulating *de novo* fatty acid synthesis (Laviola et al., 2006). Additionally, insulin regulates tissue growth and differentiation and may modulate gene expression of cytokines and tissue-specific transcription factors.

Insulin binding to the extracellular portion of insulin receptors located in the plasma membrane stimulates the second messenger systems involved in insulin signaling. Autophosphorylation and activation of the protein tyrosine kinase within the insulin receptor causes the recruitment of docking proteins, including insulin receptor substrate (IRS)-1 and Src homology collagen (Shc) (Youngren, 2007). These proteins initiate two divergent second messenger signaling pathways with separate biological functions. The stimulation of glucose transport and most other metabolic effects of insulin, such as inhibition of lipolysis, are regulated through the phosphatidylinositol 3-kinase (PI3K) pathway, which is activated by IRS-1. This pathway facilitates glucose uptake through the translocation of GLUT4-containing vesicles to the plasma membrane (Kahn and White, 1988). Conversely, cell proliferation and protein synthesis are more closely associated with mitogen activated protein kinase (MAPK) pathways, which are activated by Shc phosphorylation (Avruch, 1998).

While many of the mechanisms of insulin action described above have been studied using *in vitro* and *in vivo* rodent and human models, similar physiological mechanisms are assumed to occur in the horse. Indeed, in horses GLUT4 expression is related to insulin sensitivity and insulin signaling molecules have been identified and quantified (McCutcheon et al., 2002; McCutcheon et al., 2006; Stewart-Hunt et al., 2006). However, it is suspected that insulin signaling pathways may be differently regulated in the horse, as effects on insulin sensitivity that are often observed in humans

have not been consistently repeated in horses, such as an increase in insulin sensitivity immediately following exercise (Pratt et al., 2007).

Methods of measuring insulin sensitivity

Methods of measuring insulin sensitivity can be divided into nonspecific indications or quantitative measurements of insulin sensitivity (Kronfeld et al., 2005). Nonspecific indications of insulin sensitivity include basal glucose or insulin concentrations, glucose or insulin tolerance testing, and mathematical calculations utilizing basal glucose and/or insulin concentrations. Quantitative methods have the capacity to separate the function of pancreatic β-cells from peripheral actions of insulin, and include insulin suppression testing, euglycemic-hyperinsulinemic clamp (EHC) technique, and the minimal model of glucose and insulin dynamics.

Measurement of basal insulin concentration is a favorable method for clinical use, as it is easily obtained and inexpensive. As insulin sensitivity decreases, insulin secretion, and therefore insulin concentration, is expected to increase in order to compensate for the relative level of insulin resistance. Previous studies in horses have demonstrated inverse correlations between basal insulin concentration and insulin sensitivity as measured by the EHC technique ($r = -0.32$, $P < 0.05$) (Vick et al., 2007), minimal model ($r = -0.52$, $P < 0.001$) (Treiber et al., 2005b), or combined glucose-insulin testing ($r = 0.66$ and 0.83 for glucose and insulin areas under the curve, respectively, $P < 0.05$) (Frank et al., 2006). These studies indicate the potential usefulness of insulin concentration as an indicator of insulin sensitivity, however many factors influence its measurement, decreasing the accuracy of this method. Examples include dietary intake or stress.

The use of frequently sampled intravenous glucose tolerance tests (FSIGTT) with minimal model analysis provides advantages over other methods, as it is able to differentiate between glucose- and insulin-mediated glucose disposal (Bergman et al., 1979). Parameters calculated from the model include insulin sensitivity (SI), representing insulin-mediated glucose disposal; acute insulin response to glucose (AIRg), representing endogenous insulin response to a glucose bolus; glucose effectiveness (Sg), representing glucose-mediated glucose disposal; and disposition index (DI), representing

appropriateness of insulin response with relation to the degree of insulin sensitivity which is calculated as DI = SI x AIRg (Bergman et al., 1979; Hoffman et al., 2003).

An insulin-modified FSIGTT procedure was introduced to measure SI in insulin-dependent diabetes subjects (Finegood et al., 1990). During the modified procedure, insulin is injected 20 minutes after the glucose bolus in order to induce an artificial insulin peak to increase the test dynamics. Although particularly useful in subjects with little or no endogenous insulin response, the insulin-modified procedures are often routinely used in place of the regular procedures. Comparison of the regular and insulin-modified FSIGTT with minimal model analysis in humans demonstrated no difference ($P = 0.656$) between SI values and a 28% reduction ($P = 0.013$) in Sg values of the regular compared to the insulin-modified procedures (Pacini et al., 1998).

Analysis of the calculated parameters allows for the identification of compensated or uncompensated insulin resistance (Kronfeld et al., 2005). During compensated insulin resistance, a decrease in insulin sensitivity is matched by an increase in insulin release from the pancreas. During uncompensated insulin resistance, the decrease in insulin sensitivity cannot be completely compensated for by the increased insulin secretion from the pancreas, therefore DI decreases. Type 2 diabetes mellitus would be indicated by a decrease in SI accompanied by a decrease in AIRg and DI because of a failure of β-cells to secrete insulin in response to glucose.

Proxy measurements have been developed as surrogate tests for insulin sensitivity and insulin secretory responsiveness. These proxies include the reciprocal of the square root of insulin (RISQI) and modified insulin-to-glucose ratio (MIRG), calculated from basal insulin and glucose concentrations as estimates of insulin sensitivity and insulin secretory responsiveness, respectively (Treiber et al., 2005b). Minimal model parameters and proxy measurements were assessed in 46 healthy horses, including Thoroughbred geldings, pregnant Thoroughbred mares, Thoroughbred weanlings and Arabian geldings, and normal reference ranges and quintiles were determined.

Factors affecting the measurement of insulin sensitivity

A number of environmental or physiological factors may affect insulin sensitivity or the measurement of insulin sensitivity. Repeated measurement of insulin sensitivity

demonstrates inter-assay variability in measurements. For example, mean (range) within-horse coefficients of variation were 29 (4 – 89) % for basal insulin concentration, 5 (0 – 9)% for basal glucose concentration, 14 (7 – 20)% for the euglycemic hyperinsulinemic clamp, 24 (9 – 35)% or 33 (7 – 66)% for FSIGTT with minimal model analysis, and 15 (SD ± 6)% for the combined glucose-insulin test (Eiler et al., 2005; Frank et al., 2008b; Pratt et al., 2005). This variation may be partially explained by introduction of factors affecting the testing procedure or temporary changes in insulin sensitivity.

Depending on the method used for measurement of insulin sensitivity, certain factors may cause variations in measurement values without effectively changing actual insulin sensitivity. Slight deviations in execution of testing procedures, such as variations in administered doses, may introduce a source of error. For orally administered dose response tests, such as oral glucose tolerance tests, absorption rate and efficiency cause variation in the rate of glucose appearance into circulation. As oral glucose tolerance tests are affected by differences in absorption rate, these tests have been used to assess small intestinal malabsorption in horses (Mair et al., 1991). Additionally, if methods are performed in a non-fasting state, feed ingestion may increase glucose or insulin concentrations independent of the testing procedure. These factors introduce changes in the glucose appearance rate that cannot be accounted for in the measurements.

One of the major drawbacks to using basal insulin concentration or proxies as indicators of insulin sensitivity is that insulin concentration is heavily influenced by dietary consumption. Insulin concentration can vary dramatically with changes in dietary composition that occur seasonally and diurnally, as observed in grazing horses (McIntosh et al., 2007). Additionally, changes in insulin sensitivity as assessed by the EHC or minimal model have been observed without concurrent changes in basal insulin concentration, such as with decreased insulin sensitivity in response to a high nonstructural carbohydrate diet (Pratt et al., 2006; Treiber et al., 2005a).

Sedation may also influence measures of insulin sensitivity. Xylazine inhibits insulin secretion, resulting in hyperglycemia and hypoinsulinemia in horses (Thurmon et al., 1982). This would make the animal appear more insulin resistant by methods such as combined glucose insulin testing (Eiler et al., 2005), but more insulin sensitive by methods such as basal insulin concentration.

Many factors change insulin sensitivity either transiently or more continuously. Temporary changes in insulin sensitivity may be induced by factors including endogenous or exogenous changes in hormone concentrations or physical activity.

Cortisol exerts "anti-insulin" effects on the body by promoting glucose sparing rather than glucose uptake into skeletal muscle and adipose tissue. For instance, cortisol promotes gluconeogenesis in the liver and interferes with the insulin signaling pathway in peripheral tissue, resulting in decreased GLUT4 translocation and increased lipolysis (Andrews and Walker, 1999). Therefore, an increase in insulin production would be needed in order compensate for the reduction in insulin sensitivity to promote glucose uptake. An increase in cortisol concentration through a stress response or administration of glucocorticoids may produce temporary decreases in measured insulin sensitivity (Bailey et al., 2007; Eiler et al., 2005). Additionally, long-term effects of cortisol dysregulation during pituitary pars intermedia dysfunction (PPID), or Cushing's disease, may lead to a more permanent state of insulin resistance.

Progesterone may interfere with insulin signaling by decreasing the binding of insulin to insulin receptors. In both women and mares, insulin sensitivity is decreased during the luteal phase of the menstrual/estrus cycle, when progesterone concentration is elevated (Cubitt et al., 2007; Livingstone and Collison, 2002).

Seasonal changes in insulin sensitivity may occur through changes in secretion of hormones such as ACTH or melatonin. In ponies and horses, ACTH concentration was higher in September than January and May (Donaldson et al., 2005). An increase in ACTH stimulation may increase the effects of cortisol to decrease insulin sensitivity. Alternatively, melatonin may increase insulin sensitivity during times of short day length. Melatonin, which is secreted during periods of darkness, inhibits pancreatic β-cell responsiveness and decreases insulin concentrations in rats and humans (Peschke, 2008).

Defining insulin resistance

In human medicine, basal glucose concentrations or oral glucose tolerance tests are often assessed because of the concern for identifying glucose dysregulation, or the presence of diabetes mellitus. Diabetes is identified by a fasting glucose concentration ≥ 126 mg/dL or glucose concentration ≥ 200 mg/dL 2 h after an oral glucose challenge

(OGTT), whereas definitions of the metabolic syndrome identify "insulin resistance" as fasting glucose between 110 and 126 mg/dL or a 2 h post glucose challenge concentration of > 140 mg/dL (Grundy et al., 2004). However, these methods only address impaired fasting glucose or impaired glucose tolerance, and do not take into consideration the presence of compensated insulin resistance that may occur before glucose dysregulation.

In horses, no standardized testing procedure or criteria have been determined to define insulin resistance. Similar to measurement of adiposity, measurement of insulin sensitivity often compromises accuracy for practicality. Additionally, "insulin resistance" is a relative term, and choosing a cutoff value along a continuum of insulin sensitivity values at which a horse is denoted as "insulin resistant" or "insulin sensitive" is a theoretical exercise. By designating a horse as insulin resistant, it is assumed that the animal is in an abnormal physiological state or that it is at an increased risk for disease.

Abnormal test measurement may be identified by the use of reference intervals. Many commercial laboratories have reference ranges for insulin concentrations, usually identifying 20 or 30 mU/L as the upper limit for normal values. Reference ranges and quintiles for minimal model parameters, proxy measurements, and basal glucose and insulin concentrations have been developed in one study using 46 healthy horses (Treiber et al., 2005b). Relative insulin resistance may be considered as below the 95% reference interval ($SI < 0.16 \times 10^{-4}$ L/min/mU or $RISQI < 0.159$ $(mU/L)^{-1}$) or within the lowest quintile ($SI < 0.78 \times 10^{-4}$ L/min/mU or $RISQI < 0.295$ $(mU/L)^{-1}$). Using the combined glucose-insulin test, insulin resistance was identified as maintenance of glucose concentration above the pre-injection value for ≥ 45 minutes (Eiler et al., 2005; Frank et al., 2006). However, this cutoff was arbitrarily identified and was not based on reference intervals or classification of disease state.

Alternatively, insulin resistance may be defined based on an increased risk for disease. Included in the criteria identified for increased risk of pasture-associated laminitis was $RISQI < 0.32$ $(mU/L)^{-1}$ (Treiber et al., 2006c). This cutoff includes values greater than the lowest quintile identified in the previous study (Treiber et al., 2005b), indicating a difference between abnormal values and those that indicate risk for disease. Overall, the lack of a standardized method that is both practical and accurate, and the

implications of identifying a single cutoff value has limited the clinical identification of insulin resistance.

Obesity and related comorbid condition in humans

Obesity is the result of a prolonged positive energy balance, when excess energy is deposited as triglycerides in adipocytes. Adipose tissue is located in multiple regions of the body either subcutaneously or internally, surrounding visceral organs or within skeletal muscle tissue (Trayhurn, 2007). Classified as loose connective tissue, adipose is composed of not only adipocytes, but also other cell types, including fibroblasts, endothelial cells, and immune cells, which are referred to as the stromal-vascular fraction.

While an increase in fuel reserves through adipose tissue deposition is beneficial to provide energy during times of reduced feed availability, prolonged storage of excess amounts of lipid can have a negative impact on health. In humans, an increasing prevalence of obesity has become a major concern over the past few decades, with its incidence in the United States approaching one-third of all adults (Trayhurn and Wood, 2005). The rise in obesity is of considerable importance because of its influence on decreasing life expectancy and increasing risk for several diseases, including type 2 diabetes and cardiovascular disease.

Metabolic syndrome

The metabolic syndrome is an association of several risk factors, including central obesity, dyslipidemia, hyperglycemia and hypertension, that predicts an increased risk for cardiovascular disease or type 2 diabetes. With the introduction of the metabolic syndrome, or Syndrome "X", Reaven acknowledged the importance of obesity through its correlation with insulin resistance (Reaven, 1988). He further pointed out that "treatment" for syndrome X was weight maintenance and physical activity in order to reduce obesity. The importance of obesity as a risk factor for disease has subsequently led to its inclusion in all four definitions of clinical criteria for the metabolic syndrome by

the World Health Organization (WHO, 1999), National Cholesterol Education Program's Adult Treatment Panel III (ATPIII, 2001), American Association of Clinical Endocrinologists (AACE)(Grundy et al., 2004), and International Diabetes Federation (IDF, 2005) (Table A).

Characterization of the metabolic syndrome has lead to the necessity of defining obesity according to an increased risk for disease. However, each description of the metabolic syndrome uses a different definition of obesity, with no agreed upon definition. Methods of measuring adiposity were chosen for their practicality and include morphometric measurements of waist circumference, waist:hip ratio, or BMI. While two of the definitions include generalized obesity with BMI measurements (Grundy et al., 2004; WHO, 1999), three definitions realize the importance of central obesity over generalized obesity in disease risk (ATPIII, 2001; IDF, 2005; WHO, 1999).

Mechanisms of obesity-induced insulin resistance

Adipose tissue produces a variety of factors that may exhibit autocrine or paracrine signaling within adipose tissue, or may be released into circulation to mediate endocrine signaling to other insulin sensitive tissues, such as skeletal muscle or the liver. These factors include lipids and cytokines, which have been implicated in mediating the insulin resistance that accompanies obesity.

Circulating nonesterified fatty acids. Metabolic products of lipolysis, such as nonesterified fatty acids (NEFA), are released into circulation and may be used by peripheral tissue as a fuel source. However, NEFA may also be used as a signaling molecule and cause physiological changes unrelated to its use as an energy source. Plasma NEFA concentrations are often elevated during human obesity and type 2 diabetes as a result of increased lipolysis in adipose tissue due to its resistance to insulin action (Delarue and Magnan, 2007). Circulating NEFA then contribute to the insulin resistant state through effects on liver and peripheral tissues.

Table A: Clinical criteria for the metabolic syndrome in humans as defined by the World Health Organization (WHO), National Cholesterol Education Program's Adult Treatment Panel III (ATP III), American Association of Clinical Endocrinologists (AACE), and International Diabetes Federation (IDF)

	WHO, 1999	ATP III, 2001	AACE, 2003	IDF, 2005
Triglycerides	≥ 150 mg/dL	≥ 150 mg/dL	≥ 150 mg/dL	≥ 150 mg/dL
HDL cholesterol	Men < 35 mg/dL Women < 39 mg/dL	Men < 40 mg/dL Women < 50 mg/dL	Men < 40 mg/dL Women < 50 mg/dL	Men < 40 mg/dL Women < 50 mg/dL
Obesity	BMI > 30 kg/m^2 And/or waist:hip ratio: Men > 0.9 Women > 0.85	Waist circumference: Men > 102 cm Women > 88 cm	BMI ≥ 25 kg/m^2	Waist circumference: Men ≥ 94 cm Women ≥ 80 cm for Europids, other ethnicity specific values
Blood pressure	≥ 140 / ≥ 90 mm Hg or antihypertensive medication	≥ 130 / ≥ 85 mm Hg	≥ 130 / ≥ 85 mm Hg	≥ 130 / ≥ 85 mm Hg
Fasting glucose	Impaired	≥ 110 mg/dL	110-126 mg/dL	≥ 100 mg/dL
Insulin resistance/ impaired glucose tolerance	Type 2 diabetes, impaired fasting glucose, or impaired glucose tolerance		> 140 mg/dL, 2 h postglucose challenge	
Other	Urinary albumin excretion rate ≥ 20 μg/min or albumin:creatine ratio ≥ 30 mg/g		Family history of type 2 diabetes, hypertension, CVD, polycystic ovary syndrome, sedentary lifestyle, advancing age, high risk ethnic groups	
Diagnosis	Insulin resistance plus any other 2 factors	3 of the 5 factors are present	Clinical judgment based on risk factors	Central obesity plus any 2 factors

Fatty acids have been shown to interfere with hepatic insulin removal, leading to hyperinsulinemia (Svedberg et al., 1990). Additionally, increased NEFA delivery to the liver increases hepatic glucose production and very low density lipoprotein (VLDL) triglyceride secretion. Visceral adipose tissue may play a larger role than subcutaneous adipose tissue in NEFA-induced insulin resistance, as its secretions drain into the portal vein and directly increase NEFA delivery to the liver (Frayn, 2000). During euglycemic clamps in dogs, short-term elevation of portal NEFA caused peripheral hyperinsulinemia through decreased hepatic insulin clearance (Yoshii et al., 2006). In vitro studies of human adipose tissue demonstrate that visceral adipocytes have higher rates of lipolysis than subcutaneous adipocytes because of an increased sensitivity to catecholamine stimulation and higher resistance to insulin suppression of lipolysis (Ostman et al., 1979).

In peripheral tissue, adipocytes and macrophages, NEFA contribute to insulin resistance through stimulation of toll-like receptors (TLR). Using *in vitro* and *in vivo* rodent models, it has been demonstrated that activation of TLR4 by fatty acids initiates an inflammatory cytokine response (Shi et al., 2006), which may then contribute to insulin resistance as described below.

Intramuscular triglycerides. Triglycerides are stored intramuscularly either in myocytes or in adipocytes located between bundles of myocytes. Intramyocellular triglyceride (IMTG) content, as assessed by Oil Red O staining of muscle fibers, was increased in obesity and decreased by weight loss in humans (Goodpaster et al., 2000). Accumulation of IMTG in animals and humans during obesity has been linked to insulin resistance (Goodpaster and Kelley, 2002).

During human obesity, reduced capacity for fatty acid oxidation, rather than an increase in fatty acid uptake was responsible for IMTG accumulation (Kelley et al., 1999). Decreased oxidative capacity can result from both decreased levels of mitochondrial oxidative enzymes and decreased mitochondrial number. In obese women, markers of mitochondrial content, including citrate synthase, β-hydroxyacyl-CoA, and cytochrome c oxidase, were reduced compared to lean controls (Holloway et al., 2007). Although there was no impairment of isolated mitochondria to oxidize palmitate, whole muscle palmitate oxidation was decreased, indicating that decreased oxidative capacity

was a consequence of decreased mitochondrial number rather than mitochondrial dysfunction.

Accumulation of IMTG may only be a marker of other lipid metabolites that cause the alterations in the insulin signaling pathway (Goodpaster and Kelley, 2002). Reduced oxidative capacity results in the accumulation of not only IMTG, but also fatty acid intermediates, including diacylglycerol and ceramide. Both of these lipid metabolites interfere with insulin signaling to reduce GLUT4 translocation and glucose uptake. Diacylglycerol activates protein kinase C, which inhibits tyrosine kinase activity of the insulin receptor, as well as tyrosine phosphorylation of IRS-1 (Holland et al., 2007). Ceramide activates protein phosphatase 2A, which then dephosphorylates Akt/protein kinase B, resulting in its inactivation.

Inflammation. In humans, research demonstrates that inflammatory cytokines play a direct role in the development of obesity-associated insulin resistance. Obesity has been associated with activation of nuclear factor (NF)-κB pathways in adipocytes and increases in their target gene products, including proinflammatory cytokines (interleukin [IL]-6, IL-1β, tumor necrosis factor [TNF]α), anti-inflammatory cytokines (IL-10, IL-6), and chemokines that recruit immune cells to sites of inflammation (monocyte chemoattractant protein [MCP]-1, MCP-2, IL-8, plasminogen activator inhibitor [PAI]-1) (Shoelson et al., 2007).

An increase in adipose tissue is associated with adipocyte hypertrophy. As adipocyte size increases, their functional viability decreases because of an increased cell volume:cell surface ratio, as there is decreased ability for nutrient intake and waste removal in relation to cell size. In mice and humans, this leads to an increase in adipocyte cell death and the recruitment of immune cells, such as macrophages, to scavenge cell debris and free lipid droplets (Zeyda and Stulnig, 2007). “Crown-like” structures form as the dead adipocytes are surrounded by macrophages. Macrophage infiltration into adipose tissue enhances cytokine production and secretion, purportedly through a paracrine interaction between adipocytes and macrophages (Suganami et al., 2005). In human adipose tissue, although the non-adipocyte cells (including stromal-vascular and immune cells) in adipose tissue produce and release MCP-1, PAI-1 and IL-8 in a greater

abundance than adipocytes, there is significant secretion of these chemokines from adipocytes (Fain, 2006).

Secreted cytokines demonstrate autocrine or paracrine signaling within adipose tissue, or may be released into circulation to mediate endocrine signaling to other insulin sensitive tissues, such as skeletal muscle or the liver. Cytokines contribute to insulin resistance through activation of several serine-threonine kinase pathways, such as inhibitor of NF-κB kinase β (IKKβ) and c-Jun NH_2-terminal kinase (JNK)1, which interfere with insulin signaling through serine phosphorylation and subsequent inactivation of insulin receptor substrate (IRS)-1 (Chen, 2006).

Adipokines released exclusively by adipocytes, such as leptin and adiponectin, have also been shown to influence insulin sensitivity (Antuna-Puente et al., 2008). Although leptin displays insulin-sensitizing properties, obesity is often a leptin-resistant state where increased concentrations of leptin do not contribute to insulin sensitivity. Leptin promotes fatty acid oxidation and reduces ectopic fat accumulation in non-adipose tissue through the activation of AMP-activated kinase (AMPK), which results in increased insulin sensitivity (Kahn et al., 2005). Conversely, plasma adiponectin concentrations are inversely related to fat mass, and are reduced in obese, insulin-resistant, diabetic, or dyslipidemic subjects compared to healthy controls (Ouchi et al., 1999). Adiponectin exhibits insulin-sensitizing properties, partially mediated by activation of AMPK, which increases fatty acid oxidation in skeletal muscle and reduces the rate of glucose production in the liver (Kahn et al., 2005).

In humans, TNFα mRNA and protein expression in adipose tissue are positively correlated with body adiposity (Hotamisligil et al., 1995). TNFα has been implicated in causing insulin resistance by several mechanisms. It has been determined as a regulator of insulin sensitivity by inducing serine phosphorylation of insulin receptor substrate-1 (IRS-1), which then acts as an inhibitor of insulin receptor kinase activity and the downstream signaling of phosphatidylinositol 3-kinase (Hotamisligil et al., 1996). It also down-regulates IRS-1 and GLUT4 expression (Stephens et al., 1997). In addition, TNF-α stimulates lipolysis in adipocytes by autocrine signaling, thereby increasing circulating NEFA concentrations (Kahn and Flier, 2000).

As its name implies, plasminogen activator inhibitor 1 (PAI-1) is the most important endogenous inhibitor of tissue plasminogen activator and uro-plasminogen activator. As a result, it is one of the main determinants of fibrinolytic activity by inhibiting fibrin degradation. Thus, elevated circulating concentrations of PAI-1 increase the risk for thrombosis (Rondinone, 2006). Production and secretion of PAI-1 occurs in the liver, endothelial cells, thrombocytes, and adipose tissue. Overexpression of PAI-1 in adipose tissue occurs during obesity, and an increase in circulating concentrations during obesity has been suggested as a marker for type 2 diabetes (Schneider et al., 1993). Another important role of PAI-1 is its activity in cell migration and angiogenesis. The ability of preadipocytes to migrate is regulated by autocrine action of PAI-1, which may then contribute to adipogenesis (Crandall et al., 2000).

Obesity and related comorbid conditions in equids

An increasing amount of evidence suggests that obesity also has pathological consequences in horses. Obesity has been associated with insulin resistance (IR) in horses and ponies (Hoffman et al., 2003; Vick et al., 2007), and both obesity and IR have been associated with increased risk of laminitis, particularly the pasture-associated form of this disease (Treiber et al., 2006c).

As in humans, there is no agreed upon definition of obesity in horses. Body condition scoring (Henneke et al., 1983) is almost exclusively used for classifications of obesity, however different cutoff scores have been used depending on each investigator's interpretation of the scoring system. The original BCS system describes horses with a score of 5 as moderate, 6 as moderately fleshy, 7 as fleshy, 8 as fat, and 9 as extremely fat (Henneke et al., 1983). Studies utilizing the same BCS system have described horses as "obese" at BCS ≥ 7 (Buff et al., 2006; Frank et al., 2006; Hoffman et al., 2003) or ≥ 7.5 (Thatcher et al., 2007; Vick et al., 2006), and horses at a "high" BCS with scores ≥ 6 (Waller et al., 2006), ≥ 7.5 (Cartmill et al., 2003; Gentry et al., 2002), or ≥ 8 (Gentry et al., 2004). Additionally, obesity as defined by an increased risk for laminitis has been identified as BCS > 6 (Treiber et al., 2006c).

A metabolic syndrome in equids

As in human medicine, an analogous clinical syndrome in horses has been proposed to characterize the obesity-associated syndrome of mature-onset (usually between 8 – 18 years of age) laminitis in horses (Johnson, 2002). It was hypothesized that horses with the "equine metabolic syndrome" were afflicted with intra-abdominal obesity, insulin resistance (hyperinsulinemia), glucose intolerance, dyslipidemia, hypertension and insidious-onset laminitis. However, these characteristics were developed based on direct comparison to human data, and are unsubstantiated by epidemiological studies in horses. Additionally, the equine metabolic syndrome describes physiological abnormalities in the presence of chronic laminitis, whereas the use of factors to predict risk for laminitis may be more clinically relevant. Identification of susceptible ponies could facilitate the prediction of laminitic episodes and allow for implementation of countermeasures to avoid laminitis (Harris et al., 2006).

Increased risk for pasture-associated laminitis in apparently healthy ponies has been characterized by a set of risk factors known as the prelaminitic metabolic syndrome (PLMS) (Treiber et al., 2006c). These risk factors include insulin resistance (RISQI < 0.32 $[mU/L]^{-0.5}$), increased insulin secretory response (MIRG > 5.6 $mU_{insulin}^{2}/[10 \cdot L \cdot mg_{glucose}]$), hypertriglyceridemia (> 57.0 mg/dL), and obesity (body condition score > 6 on a scale of 1 to 9, with localized fat deposits on neck and tailhead). Ponies that were identified by the PLMS as surpassing three or more criteria were 10 times more likely to develop laminitis than those not identified by PLMS, according to subsequent occurrence of laminitis in the study group.

Individual variables, without their inclusion in a syndrome, have also been studied to determine differences between ponies with a history of laminitis ("predisposed" to laminitis) and control ponies. Studies have repeatedly determined ponies predisposed to laminitis to be more insulin resistant than control ponies by methods including oral glucose tolerance testing (Jeffcott et al., 1986), the minimal model (Bailey et al., 2007; Treiber et al., 2007b), and the proxy measurement RISQI (Bailey et al., 2008; Treiber et al., 2006c).

In humans, hypertension is a common feature of the metabolic syndrome. While hypertension was proposed as part of the equine metabolic syndrome, there is little

evidence to support its occurrence in laminitic ponies. When laminitis-prone ponies (n = 40) were compared to control ponies (n = 40), the laminitis-prone ponies had higher blood pressures in summer, but not winter (Bailey et al., 2008).

In the same study, laminitis-prone ponies had higher uric acid concentrations in summer, but not winter compared to control ponies (Bailey et al., 2008). In humans, uric acid concentration is an independent predictor of obesity, hyperinsulinemia and hypertension (Johnson et al., 2007b). High uric acid concentrations may result from high amounts of fructose consumption, as the metabolism of fructose leads to decreased tissue ATP content and subsequent production of lactic and uric acids. Uric acid reduces the concentration of endothelial nitric oxide to result in hypertension and increases in insulin and triglyceride concentrations (Johnson et al., 2007b).

Laminitis is a common sequela to pituitary pars intermedia dysfunction. Pituitary pars intermedia dysfunction, as defined by elevated ACTH concentration, was observed in 70% of horses with laminitis in a primary-care ambulatory setting (Donaldson et al., 2004a). However, concentrations of ACTH were similar ($P > 0.05$) between laminitis-prone and control ponies during both summer and winter evaluations (Bailey et al., 2008).

Regional adiposity in horses and ponies, especially adipose tissue deposited more heavily along the crest of the neck ("cresty neck"), has been suggested to be an indicator of insulin resistance and a risk factor for laminitis (Frank et al., 2006; Johnson, 2002; Treiber et al., 2006c). However, when laminitis-prone ponies and control ponies are matched for body condition, there is no difference between neck measurements of crest height, crest thickness or neck circumference between groups (Bailey et al., 2008). Additionally, there is currently no mechanistic link explaining the association of subcutaneous neck crest fat with insulin resistance or laminitis.

Association of obesity with insulin resistance and inflammation

Compared to lean horses, overweight or obese horses have lower insulin sensitivity and elevated blood hormone and lipid concentrations (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2006). Application of the minimal model to nonobese (BCS 5 – 5.9; n = 4), moderately obese (BCS 6 – 6.9; n = 3) and obese (BCS ≥ 7; n = 3)

Thoroughbred geldings, revealed that obese horses had 81% lower SI, 93% higher AIRg and 111% higher Sg than nonobese horses (Hoffman et al., 2003). Moderately obese horses had results between obese and nonobese horses. When euglycemic-hyperinsulinemic clamp procedures were applied to feed restricted (BCS 4 – 5; n = 6) and obese (BCS 7.5 – 9; n = 6) mares, the obese mares had ~60% lower glucose infusion rate than feed restricted mares (Vick et al., 2006). Additionally, obese mares had higher ($P <$ 0.05) baseline insulin and leptin concentrations and lower thyroxine concentrations than feed restricted mares throughout a 4 month period. In order to determine differences in hormone and lipid concentrations, lean horses (BCS 4 – 6; n = 5) were compared to obese, insulin resistant horses (BCS 7 – 9; n = 7) (Frank et al., 2006). As was the intention of the study design, obese horses had 68% higher glucose and 81% higher insulin areas under the curve during combined glucose-insulin testing. Additionally, obese horses had higher baseline insulin, glucose, leptin, NEFA, very-low-density lipoprotein (VLDL), VLDL-triglyceride, and high-density lipoprotein-cholesterol compared to lean horses.

Across a range of body conditions adiposity measures are associated with insulin sensitivity, circulating concentrations of insulin, leptin, adiponectin, TNFα, and blood mRNA expression of TNFα and IL-1 (Buff et al., 2002; Kearns et al., 2006; Vick et al., 2007). Leptin is positively correlated to body condition in Quarter horses (r = 0.64, $P <$ 0.001; BCS 3 – 8; n = 71) (Buff et al., 2002). In a group of mature Standardbred mares (n = 23) and weanling Quarter horse/Belgian fillies (n = 10), percent fat calculated from ultrasonic rump fat thickness (range 3 – 34% fat) was positively correlated to leptin concentration (r = 0.54, $P < 0.001$) and negatively correlated to adiponectin concentration (r = -0.65, $P < 0.001$) (Kearns et al., 2006). However, correlations were heavily dependent on differences in both % fat and adipokine concentrations between mature and weanling horses, therefore it is possible that effects observed in this study are due to differences in age or breed instead of fat mass. In a population of 60 mares, both BCS and % fat (ultrasonic assessment) were positively correlated with insulin concentration (r = 0.53, 0.47, respectively) and negatively correlated with insulin sensitivity assessed by EHC technique (r = -0.57, -0.64, respectively) (Vick et al., 2007). Additionally, adiposity measurements were positively correlated with inflammatory cytokines, including blood

mRNA expression of IL-1 and TNFα, and TNFα protein concentration, whereas insulin sensitivity was negatively associated with TNFα mRNA and protein expression.

Previous studies in horses utilized cross-sectional analysis with regard to comparing metabolic variables of horses at different levels of adiposity. From these associations it may be hypothesized that obesity causes insulin resistance through induction of a pro-inflammatory state and/or through alterations in lipid metabolism. However, in order to test this hypothesis, experimental evidence is necessary to determine cause and effect relationships.

Experimental evidence demonstrates that systemic inflammation causes insulin resistance in horses (Toth et al., 2008; Vick et al., 2008). Induction of systemic inflammation by intravenous administration of lipopolysaccharide (LPS) reduced the glucose infusion rate during a euglycemic-hyperinsulinemic clamp by ~80% at 24 h after LPS administration (Vick et al., 2008). In a similar study of LPS-induced systemic inflammation, minimal model analysis revealed a 69% reduction in SI accompanied by a 80% increase in AIRg at 24 hours after administration, demonstrating a compensated insulin resistance (Toth et al., 2008). Additionally, adipocytes may contribute to the inflammatory response, as TNFα, IL-1 and IL-6 were detected in preadipocytes or mature adipocytes stimulated with LPS. However, it is unknown whether a similar inflammatory state is induced during the development of obesity to contribute to an insulin resistant state.

Mechanisms linking obesity and insulin resistance with laminitis

Laminitis is a condition in which there is degradation of the laminar tissue between the hoof wall and pedal bone. Separation may occur between the secondary epidermal and dermal lamellae, specifically between the epidermal basal cells and their basement membrane (Pollitt, 1996). As there is a multitude of precipitating factors resulting in laminitis, including grain overload, enteritis, colitis, acute metritis, concussion, unilateral leg lameness, endocrinopathies and grazing "lush" pasture, there are multiple theories on the pathogenic mechanisms of laminitis (Colles and Jeffcott, 1977). Two predominating theories arise from proposed pathologic mechanisms, the enzymatic and vascular theories, which may not be mutually exclusive. Obesity-

associated insulin resistance and/or hyperinsulinemia may be implicated in the pathophysiology of both theories.

Although the mechanism by which laminitis may be induced by insulin resistance or hyperinsulinemia is unknown, substantial evidence supports a causal link. Low insulin sensitivity and/or high insulin concentrations have been associated with laminitic predisposition and risk for laminitis in previous studies (Coffman and Colles, 1983; Jeffcott et al., 1986; Treiber et al., 2007b; Treiber et al., 2006c). Additionally, experimental induction of marked hyperinsulinemia (> 1000 mU/L) while maintaining euglycemia induced laminitis in healthy ponies (Asplin et al., 2007b).

Enzymatic mechanisms. In the enzymatic theory, "trigger factors", such as bacterial proteases, activate matrix metalloproteinases (MMP). Epithelial cells are attached to their basement membrane by adhesion molecules, including integrins, which attach to collagen and laminin on the basement membrane. Matrix metalloproteinase (MMP) activation leads to collagen degradation, which may be part of the mechanism behind detachment of lamellar epithelial cells from the basement membrane (French and Pollitt, 2004).

Inflammation may contribute to the enzymatic induction of laminitis, as MMP production and secretion is stimulated by cytokines, including IL-1β, TNFα, and growth factor TGF-β (Bailey et al., 2004). Activation of MMP is then brought about by proteases, including trypsin, plasmin, cathepsin G, bacterial proteases (exotoxins), or by reactive oxygen species. Increased inflammatory cytokine mRNA expression, including IL-1β, IL-6 and IL-8, was observed in lamellar tissue during the development of laminitis induced by black walnut extract or oligofructose (Belknap et al., 2007; Loftus et al., 2007; Waguespack et al., 2004b).

Obesity may potentially contribute to insulin resistance through creation of a proinflammatory state (Vick et al., 2007). Therefore, it is possible that obesity may contribute to laminitis risk through an inflammatory response in addition to the effects of insulin resistance. An obesity-associated low-grade inflammatory response within lamellar tissue may sensitize this tissue to subsequent stressors and contribute to induction of laminitis.

Vascular mechanisms. In the vascular theory, reduced blood flow to the hoof results in ischemia, which is then followed by reperfusion injury (Hood et al., 1993). Ischemia followed by reperfusion may damage lamellar tissue in several ways, including generation of oxygen free-radicals, activation of MMPs or deprivation of nutrients. Although the trigger for changes in vascular function is uncertain, vasoactive amines produced in the cecum as a result of microbial fermentation of soluble carbohydrates have been implicated in contributing to vasoconstriction (Bailey and Elliott, 1998; Bailey et al., 2003; Elliott and Bailey, 2006).

Blood flow in the equine digit is measured indirectly, through methods such as hoof surface temperature, with the assumption that an increase in hoof temperature indicates an increase in blood flow (Hood et al., 2001; Pollitt and Davies, 1998). However, a large number of arteriovenous anastomoses (AVA) are associated with the digital circulation which may shunt blood flow away from the capillaries, and therefore lead to erroneous conclusions about blood flow measurements. Consequently, contradictory evidence has been collected either supporting or refuting the ischemia/reperfusion hypothesis (Bailey et al., 2004; Hood et al., 2001; Pollitt and Davies, 1998).

Lamellar tissue is dependent on glucose for energy, and *in vitro* studies with hoof lamellar explants demonstrate that glucose deprivation causes weakening of hemidesmisomes and collapse of the basal cell cytoskeleton (French and Pollitt, 2004). Therefore, a decrease in blood supply through vasoconstriction could affect glucose delivery to lamellar tissues and influence cellular function.

Insulin resistance has been proposed to induce laminitis by reducing glucose uptake into laminar cells. In one study, equine laminar keratinocytes, the mesenchymal cells responsible for synthesis and degradation of laminar tissue, were shown to express GLUT1 and GLUT4 (Mobasheri et al., 2004). Conversely, a subsequent study has shown that glucose uptake in lamellar tissue is insulin-independent, as demonstrated by the absence of GLUT4 (but a presence of GLUT1) and the inability of insulin to stimulate additional glucose uptake (Asplin et al., 2007a). Contradictory results between the two

studies may be due to differences in methodology, including subjects used (laminitic vs. healthy), measurement technique (immunohistochemistry vs. PCR), or site of tissue biopsy.

Alternatively, insulin resistance may contribute to the pathology of laminitis through its influence on vascular function. In rodent and human models, insulin stimulates nitric oxide (vasodilator) production through phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathways, and also stimulates endothelin-1 (vasoconstrictor) production and secretion through mitogen activated protein kinase (MAPK)-dependent signaling pathways (Muniyappa et al., 2007). During insulin resistance, there is an impairment of PI3K-dependent signaling pathways, while MAPK-dependent pathways are unaffected. This inhibits insulin from stimulating vasodilatory pathways, while continuing stimulation (often enhanced stimulation with hyperinsulinemia) of vasoconstrictive pathways.

Additionally, an obesity-associated low-grade inflammatory response may potentially mediate a vasoconstrictive state. Inflammatory cytokines, including TNF-α and IL-1β, activate JNK and IKKβ, which then activates activating protein (AP)-1 and NF-κB (Muniyappa et al., 2007). This inhibits insulin stimulation of eNOS, therefore reducing nitric oxide production and its effects on vasodilation.

Countermeasures for obesity and insulin resistance

While some treatments specifically aim to improve insulin sensitivity, theoretically the most effective method of improving obesity-associated insulin resistance is by removing the cause through a reduction in fat mass. Treatment of insulin resistance with insulin sensitizer agents, including metformin and levothyroxine, has been evaluated in horses (Durham et al., 2008; Frank et al., 2008a; Frank et al., 2008b; Vick et al., 2006). However, common recommendations for improving the health status of obese horses are to decrease energy intake and increase energy expenditure. Currently, there is a void of research-based evidence supporting the development of management strategies involving nutritional manipulation or exercise training for obese horses.

Nutrition

Nutritional management of obese horses includes restricting digestible energy intake to reduce the degree of obesity, and lowering the nonstructural carbohydrate (NSC) content of the diet to minimize the degree of insulin resistance.

In previous studies, adaptation to a high NSC diet decreased SI when compared to a high fiber and fat diet (Hoffman et al., 2003; Treiber et al., 2005a). In both Thoroughbred weanlings and mature geldings, horses adapted to a diet high in NSC had 36% or 38% lower SI, respectively, compared to horses adapted to a diet high in fat and fiber. Although no other minimal model parameters differed between groups in the weanlings, AIRg and DI were lower in high NSC-fed mature geldings. Additionally, in mature Standardbred horses, adaptation to a high NSC diet resulted in a reduction of metabolized glucose/unit insulin during the EHC and increased area under the insulin curve during an oral glucose tolerance test (Pratt et al., 2006). Horses adapted to a high-fat feed in the same study displayed no differences on the high-fat feed compared to forage cubes. However, adaptation to similar diets in exercise-trained Arabian geldings demonstrated no differences in minimal model parameters between high NSC or high fat and fiber fed horses at rest (Treiber et al., 2006a). Collectively, these results indicate modest, if any, decreases in SI with high NSC feeding.

In addition to potential effects on insulin sensitivity, ingestion of high-NSC feeds may aggravate an existing insulin resistant physiological state. The negative consequences of insulin resistance are often initiated by ingestion of feeds that induce a high insulinemic response. For example, ponies that have a predisposition for laminitis are more insulin resistant than control ponies, but laminitis most often occurs in the spring during intake of NSC-rich pasture (Bailey et al., 2008; Treiber et al., 2007b; Treiber et al., 2006c). Therefore, limiting exposure to feeds that cause a high insulinemic response may reduce the risks associated with insulin resistance.

Dietary restriction through decreasing caloric intake below maintenance requirements may increase insulin sensitivity by decreasing adiposity. Restricting feed intake decreases body weight and adiposity in horses, however limited evidence exists to determine the amount of feed restriction necessary to obtained a desired amount of

weight loss or change in metabolism (Gentry et al., 2004; Vick et al., 2006). In a study with obese pony mares (n = 5, BCS > 7), feed was provided in an amount that was 75% of ad libitum hay intake (2.9 ± 0.2 kg hay and 0.33 ± 0.02 kg oats) over a 6 week period to induce weight loss (Buff et al., 2006). Body weight decreased by ~10 kg and baseline insulin concentration decreased by ~75% over the 6 week time period. In a separate study involving obese ponies (n = 9, BCS 8 or 9), in order to achieve weight loss at a rate of 1% of body weight per week for 18 weeks, ponies were restricted to 70% of their DE requirements for 6 weeks, 50% for 8 weeks, and 35% for the remaining 4 weeks (Van Weyenberg et al., 2005). Areas under the glucose and insulin curves during an oral glucose tolerance test were lower after weight loss, indicating an improvement in insulin sensitivity.

Exercise

Exercise may temporarily improve insulin sensitivity in response to a single exercise session or may have more prolonged improvements in response to exercise training. Additionally, in humans exercise training without dietary restriction is an effective method of weight reduction (Ross et al., 2000b).

Acute exercise effects. In humans, an increase in insulin sensitivity is consistently observed during and after exercise, with possible mechanisms including increased expression or activity of proteins involved in signal transduction, increased glycogen synthase activity resulting from glycogen depletion, or increased muscle perfusion (Wojtaszewski et al., 2002). In addition to insulin-dependent pathways, insulin independent pathways, such as AMP-activated protein kinase (AMPK) facilitate glucose disposal.

In horses, there are conflicting results as to the effects of exercise on insulin sensitivity. By application of the FSIGTT during moderate intensity exercise, it has been demonstrated that SI, Sg, and DI increase during exercise, whereas AIRg decreases, indicating an overall increase in glucose disposal through both insulin dependent and independent pathways (Treiber et al., 2006a). However, in a separate study a decrease in insulin sensitivity was observed 0.5 h after a single bout of exercise, with no differences from pre-exercise values at 4 and 24 h after exercise (Pratt et al., 2007). Although there

was no difference in muscle GLUT4 content, there was a decrease in muscle glycogen and increase in glycogen synthase activity.

Exercise training effects. In humans, exercise training induces an increase of glucose transporter 4 (GLUT-4) content in skeletal muscle, and improvements in insulin sensitivity with training may be induced by increased expression or activity of proteins involved in signal transduction, including insulin receptor substrate 1 (IRS-1) and phosphatidylinositol 3-kinase (PI3-kinase) (Zierath, 2002).

Indirectly, insulin sensitivity may be increased through improvements in lipid metabolism. Moderate intensity exercise training with weight loss induces skeletal muscle mitochondrial biogenesis in obese humans (Menshikova et al., 2007). Consequently, improvements in lipid oxidation are observed with exercise (Holloway et al., 2006) and decreases in IMTG lipid droplet size with exercise and weight loss (He et al., 2004). These improvements in lipid oxidation may lead to decreases in lipid intermediates, such as diacylglycerol or ceramide, and therefore alleviating inhibition of the insulin signaling pathway.

In horses, short-term (7 days) exercise training has been shown to increase insulin sensitivity in lean and obese horses when measured 24 h after the last exercise session (Powell et al., 2002; Stewart-Hunt et al., 2006). Insulin sensitivity remained higher than pre-exercise values 5 days after the last exercise session when there was a decrease in body weight with exercise (Stewart-Hunt et al., 2006), but returned to pre-exercise values by 9 days after the last exercise session when there was no decrease in body weight with exercise training (Powell et al., 2002). Insulin sensitivity increased after 7 weeks of training when body weight remained constant, however this occurred only in horses adapted to a high NSC feed and not in horses adapted to a high fat feed (Pratt et al., 2006). In obese, hyperinsulinemic ponies insulin sensitivity improved after 6 wk training and remained higher after 2 wk detraining (Freestone et al., 1992). However, similar changes were observed in the control group, including decreased body weight and increased insulin sensitivity, therefore exercise exerted no additional improvements above controlled feed intake. Collectively, these studies indicate a sustained improvement in insulin sensitivity with exercise training in the presence of weight loss or when dietary factors contributed to the insulin resistance.

CHAPTER 1

Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies

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ABSTRACT: This study describes a scoring system for the assessment of apparent neck adiposity and evaluates morphometric measurements for assessing neck and overall adiposity. Twenty-one barren Thoroughbred mares (median [interquartile range] age 11 [8 – 14] years; body weight 568 [539 – 595] kg), 13 Arabian geldings (age 13 [11 – 14] years; body weight 455 [422 – 468] kg) and 75 Welsh, Dartmoor, or crossbred pony mares (44 pregnant, 32 barren; age 10 [6 – 20] years; body weight 345 [312 – 371] kg) were evaluated and basal blood samples analysed for insulin, glucose, leptin, and triglycerides. Body weight, height, length, girth and abdominal circumferences, neck length, neck crest height and neck circumference were measured, and body condition scores (BCS) and cresty neck scores (CNS) were rated. Girth:height ratio had the strongest associations with BCS ($r_s = 0.64$, $P < 0.001$ in horses; $r_s = 0.83$, $P < 0.001$ in ponies) and blood variables, such as leptin ($r_s = 0.39$, $P = 0.024$ in horses; $r_s = 0.68$, $P < 0.001$ in ponies). Crest height and neck circumference:height ratio had the strongest association with CNS ($r_s > 0.50$, $P < 0.01$) and blood variables, such as insulin ($r_s \geq 0.40$, $P < 0.05$). Cresty neck score is useful in the assessment of neck crest adiposity and has physiological relevance, as demonstrated by associations with blood variables. Girth:height is the most suitable morphometric for assessment of overall adiposity, and either crest height or neck circumference:height is a suitable morphometric for assessment of apparent neck adiposity.

Key words: adiposity, body condition, cresty neck, horse, morphometrics

Introduction

Anecdotal observations by equine veterinarians suggested that obesity is a growing problem in companion equid populations. Indeed, a recent cross-sectional study of 300 mature horses reported that 19% were obese (body condition score [BCS] ≥ 7.5, scale 1 – 9) and 32% were overweight (BCS 6.5 – 7) (Thatcher et al., 2007). Obesity has been associated with insulin resistance (IR) in horses and ponies (Hoffman et al., 2003; Jeffcott et al., 1986; Vick et al., 2007), and both obesity and IR have been associated with increased risk of laminitis, particularly the pasture-associated form of this disease (Treiber et al., 2006c). The most common method for assessment of adiposity in horses is the rating of body condition (subcutaneous fat deposition) by use of a 9-point scale (Henneke et al., 1983). The Henneke BCS system, originally developed for use in Quarter Horse broodmares, may be most appropriate for use in light breed horses (e.g. Quarter horses, Thoroughbreds and Arabians) that share similar body type and patterns of fat deposition. Although the Henneke system has been applied with modifications to other breeds, including warm-blooded horses (Kienzle and Schramme, 2004), its suitability for ponies has not been reported.

The BCS system is a valuable estimate of apparent adiposity (Henneke et al., 1983), however it is a subjective assessment. Therefore, use of more objective, yet easily obtained measurements of obesity would be helpful in the clinical assessment of adiposity. In human medicine, morphometric measures such as body mass index (BMI) and waist to hip ratio are used for assessment of overall adiposity and regional (central) adiposity, respectively. Donaldson et al. (2004) applied a BMI to horses (estimated weight (kg)/height $(m)^2$) and found a moderate correlation (r_s = 0.60, 95% confidence interval, 0.44 to 0.73) between this index and BCS. Overall, however, there has been little application of morphometric measurements for evaluation of adiposity in equids.

The BCS system is not useful to differentiate the differences in regional adiposity that may indicate increased risk for disease. In human subjects, abdominal (visceral) adiposity is more closely linked to risk for diabetes and cardiovascular disease than generalized obesity, and measurement of waist circumference is a better indicator of abdominal fat accumulation than is BMI (Lee et al., 2006; Murphy and Bloom, 2006).

Similarly, regional adiposity in horses and ponies, especially adipose tissue deposited more heavily along the crest of the neck ("cresty neck"), has been suggested to be associated with altered metabolic states, including insulin resistance, and an increased risk for laminitis (Johnson, 2002; Treiber et al., 2006c). A standardised scale or system for assessment of neck crest fat accumulation would be beneficial for further evaluating the relationship of regional adiposity with metabolism and disease risk in horses and ponies.

The objectives of the study reported here were to: (1) describe a scoring system for the assessment of apparent neck crest adiposity, in horses and ponies, and (2) evaluate morphometric measurements for assessment of neck and overall adiposity through associations with condition scores and blood variables. It was expected that after successful implementation of a scoring system for neck crest adiposity, chosen morphometric measurements would display relationships with scoring systems, and that these measurements of adiposity would be related to metabolic blood variables.

Materials and methods

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Subjects and blood sampling

Twenty-one barren Thoroughbred mares (age 11 [8 – 14] years; body weight 568 [539 – 595] kg), 13 Arabian geldings (age 13 [11 – 14] years; body weight 455 [422 – 468] kg) and 75 Welsh, Dartmoor, or crossbred pony mares (44 pregnant, 31 barren; age 10 [6 – 20] years; body weight 345 [312 – 371] kg) were evaluated and sampled. All horses and ponies were maintained on a routine vaccination and deworming program, had regular dental exams, and were healthy in appearance at the time of sampling. All horses and ponies were maintained on pasture and supplemented with mixed grass hay because the study occurred during the winter season when pasture was minimal. No concentrate feed had been fed for > 3 weeks prior to sampling. Horses and ponies had *ad libitum* access to fresh water and salt blocks. While at pasture and before daily hay feeding,

blood samples (20 mL) were collected between 0800 and 1000 by jugular venipuncture into 10 mL evacuated tubes containing sodium heparin or potassium EDTA as an anticoagulant. Plasma was separated by centrifugation (1500 × *g*) within 30 min of sample collection, separated into 1 mL aliquots, and then stored at -20 ^{o}C until analysis.

Plasma analyses

Each analysis was performed in a single assay run on separate aliquots of plasma. Analyses were performed 1 – 5 months after sample collection. Plasma glucose (in heparin) and triglyceride (in EDTA) concentrations were assayed enzymatically by use of commercial kits and an automated analyzer (CX5 Chemistry Analyzer, Beckman Instruments). Plasma insulin (in heparin) and leptin (in EDTA) concentrations were measured by use of commercial radioimmunoassays (Coat-A-Count Insulin, Diagnostic Products Corporation; Multi-species Leptin RIA, Linco Research) previously validated for use in equine plasma (Freestone et al., 1991; McManus and Fitzgerald, 2000). All analyses were performed in duplicate. Intra-assay coefficients of variation, calculated from duplicate analyses, were 0.7 % for glucose, 8.5 % for insulin, 2.8 % for triglycerides, and 6.3 % for leptin. Assay sensitivity was 5 mg/dL for glucose, 1.2 mU/L for insulin, 10 mg/dL for triglycerides, and 1 ng/mL for leptin. Range of linearity for each assay was 5 – 700 mg/dL for glucose, 5.4 – 460 mU/L for insulin, 10 – 1000 mg/dL for triglycerides, and 1 – 50 ng/mL for leptin.

Body measurements

Body weight (BW), height (at the wither), body length, girth and waist (abdominal) circumferences, neck length, neck crest height, and neck circumference (NC) at 0.25, 0.50, 0.75 of neck length were measured. Body length was measured from the point of the shoulder (intermediate tubercle of the humerus) to the point of the buttock (ischiatic tuberosity). Girth circumference was measured caudal to the elbow (olecranon tuber) and immediately behind the slope of the withers. Waist was taken as the abdominal circumference measured two-thirds the distance from the point of the shoulder to the point of the hip (tuber coxae). All neck measurements were taken while the neck was held in a relaxed position, at approximately a 45° angle. Neck length was

measured from the poll to the highest point of the withers. Crest height was measured at 0.50 of neck length from the dorsal midline of the neck to estimated differentiation between the crest (tissue apparent above the ligamentum nuchae) and neck musculature, identified by palpation and visual assessment. Intra-assay coefficients of variation, calculated from 3 repeated measurements measured by a single evaluator on 3 horses, were 1.1 % for girth, 0.4 % for waist, 3.0 % for neck length, 2.1 % for 0.25 NC, 2.4 % for 0.50 NC, 0.7 % for 0.75 NC, and 2.4 % for crest height.

Four evaluators graded BCS from 1 to 9 (Henneke et al., 1983) and neck crest adiposity as cresty neck score (CNS) from 0 to 5 (Table 1.1; Figure 1.1). Scores were rated to the nearest whole- or half-score increment and the median of the four scores for each horse or pony was used for data analysis. Intraclass correlation coefficients for the reliability of individual scores were 0.74 for BCS and 0.70 for CNS.

Statistical analyses

Two body mass indices were calculated as BW (kg)/[height (m)]^2 (BMI 1) (Donaldson et al., 2004b) and BW (kg)/[length (m) × height (m)] (BMI 2) (Hoenig and Ferguson, 2002). Ratios of girth:height, girth:length, girth:BW, waist:height, waist:length, waist:BW, waist:girth, NC:neck length, and NC:height were calculated. Mean NC was calculated as the average of 0.25 NC, 0.50 NC, and 0.75 NC. Results of the Shapiro-Wilk test revealed that not all variables were normally distributed within breed; therefore, associations between variables were quantified with Spearman rank correlation coefficients (r_s). Data were analyzed as two groups (horses and ponies) and differences in relationships of variables between groups were determined by comparison of regression lines if correlations were significant ($P < 0.05$) for both groups. Comparison of regression lines was analysed by an F test of the null hypothesis that one line fits both data sets (constraining both intercept and slope) with an alternate hypothesis that separate lines fit the data sets. Comparison of strength of associations was based on numerically higher r_s and lower P-values. Groups were compared by use of Kruskal-Wallis tests and data are reported as median (interquartile range). Increased likelihood of hyperinsulinemia (insulin concentration ≥ 30 mU/L) above a certain BCS or CNS was

assessed by odds ratios. Software programs (Intercooled Stata 9 and GraphPad Prism 4) were used for statistical computations.

Results

Body condition score, CNS, leptin, and triglyceride concentrations were lower in horses compared to ponies ($P < 0.05$), while insulin ($P = 0.12$) and glucose ($P = 0.80$) concentrations were similar between horses and ponies (Table 1.2). All blood variables were more strongly associated with BCS and CNS than morphometric measures, with similar relationships between horses and ponies for leptin and triglyceride ($P > 0.05$; Tables 1.3 & 1.4).

For the assessment of overall adiposity in horses, BCS had the strongest associations with girth:height and waist:height (Table 1.5). Leptin was associated with girth:height. There were no other significant associations of blood variables with morphometrics in horses (Table 1.3). In ponies, girth:height had the strongest association with BCS (Table 1.5; Figure 1.2) and blood variables (Table 1.3).

For the assessment of neck crest adiposity in horses, CNS was most strongly associated with 0.50 NC:height and mean NC:height (Table 1.6). Crest height was associated with insulin and glucose, whereas mean NC:height was associated with leptin, insulin, glucose, and TG (Table 1.4). In ponies, crest height had the strongest association with CNS (Table 1.6), and mean NC:height had the strongest association with blood variables (Table 1.4).

Regression lines of morphometrics with BCS, CNS, and blood variables were different between horses and ponies ($P < 0.01$; Tables 1.3, 1.4, 1.5, & 1.6; Figure 1.2). The only exceptions were for the regressions of girth:length or waist:length with BCS (Table 1.5).

To further evaluate the relationship of body condition with blood metabolic variables, horses and ponies classified as moderate ($4 < BCS < 7$), overweight ($7 \leq BCS < 8$), or obese ($BCS \geq 8$) were compared to classification of hyperinsulinemia (insulin concentration ≥ 30 mU/L). Nineteen percent of the moderately conditioned horses (5/27) were hyperinsulinemic, whereas 43% of the overweight or obese horses (3/7) were

hyperinsulinemic. Ten percent of the moderately conditioned ponies (3/30) were hyperinsulinemic, whereas 45% of the overweight ponies (13/29) and 63% of the obese ponies (10/16) were hyperinsulinemic.

The relationship of a moderate neck (CNS < 3) or a cresty neck (CNS ≥ 3) with hyperinsulinemia (insulin concentration ≥ 30 mU/L) was also evaluated. Twenty-one percent of horses with a moderate neck (6/29) were hyperinsulinemic, whereas 40% of horses with cresty necks (2/5) were hyperinsulinemic. Six percent of ponies with moderate necks (2/32) were hyperinsulinemic, whereas 56% of ponies with cresty necks (24/43) were hyperinsulinemic.

An overweight or obese horse (BCS ≥ 7) was not more likely ($P = 0.19$) to be hyperinsulinemic than a moderately conditioned horse. An overweight or obese pony was 9.4 times more likely ($P = 0.001$) to be hyperinsulinemic than a moderately conditioned pony. A horse with a cresty neck was not more likely ($P = 0.36$) to be hyperinsulinemic than a horse with a moderate neck, whereas a pony with a cresty neck was 18.9 times more likely ($P < 0.001$) to be hyperinsulinemic.

Discussion

This study is the first to describe a standardised scoring system for apparent neck crest adiposity in equids. Although CNS is related to overall adiposity, as indicated by an association with BCS, the purpose of CNS is to assess fat deposition along the crest of the neck independent of overall adiposity. Fulfillment of this objective is supported by association of CNS with morphometric measurements of neck dimensions. However, without a direct measure of adipose tissue for comparison, CNS only assesses apparent or visibly appreciable neck crest adiposity. Although CNS is associated with blood variables relevant to assessment of insulin resistance and other aspects of metabolism, the current study did not determine crest adiposity as a causative factor for altered metabolism nor could it fully describe associations with CNS independent of BCS.

Another limitation of the present study was the female bias, as all of the ponies and 62 % of the horses were mares. Some differences in blood variables between the Thoroughbred mares and Arabian geldings were observed (Appendix A), however it

could not be determined whether these were breed- or sex-dependent differences, and both groups were combined to provide enough power for statistical analyses. Further studies are necessary to determine specific differences in relationships of variables between mares, geldings, and stallions.

Body condition scoring is an accepted evaluation of apparent adiposity in horses, and previous studies have shown that it correlates with subcutaneous fat thickness (Gentry et al., 2004; Henneke et al., 1983), leptin concentration (Buff et al., 2002), glucose tolerance (Frank et al., 2006), and insulin sensitivity (Vick et al., 2007). Obesity, as defined by a BCS above a certain cutpoint, is associated with risk for laminitis (Treiber et al., 2006c), reduced insulin sensitivity (Hoffman et al., 2003), and altered metabolic and reproductive activity (Vick et al., 2006). The findings of the present study supported that BCS is a useful tool in the assessment of apparent adiposity and affirm that BCS is associated with blood variables relevant to assessment of insulin resistance and other aspects of metabolism. Although the BCS system used in the present study was originally developed on horses, it was successfully applied to both horses and ponies.

Morphometric measurements are more easily performed in the absence of trained evaluators and provide a more objective alternative for subjective scoring systems. The correlations of BCS with morphometric measurements, including BW, girth, BW:height, girth:height, and body mass index (estimated BW/height^2) have been demonstrated in previous studies (Donaldson et al., 2004b; Henneke et al., 1983). The present study compared these measurements, along with other morphometric measurements, and found that girth:height was the most suitable morphometric for assessment of overall body adiposity, as demonstrated by strong correlations with BCS and blood variables.

In ponies, but not horses, morphometric ratios utilising waist circumference had weaker associations with variables than morphometric ratios utilising girth circumference. This discrepancy may be attributed to the 58% of pony mares that were pregnant at the time of evaluation. Pregnant ponies had a greater ($P < 0.001$) waist:height than non-pregnant ponies (1.52 [1.48 – 1.57] and 1.40 [1.35 – 1.45], respectively), which indicated that waist should not be used during pregnancy, although it may be useful for adiposity measurement in non-pregnant equids.

Either crest height or NC:height was a suitable morphometric for assessment of neck adiposity, as demonstrated by its association with CNS and blood variables. Mean NC has previously been shown to be associated with glucose tolerance in horses (Frank et al., 2006). The present study demonstrated that correcting for body size by calculating the NC:height ratio is a more accurate assessment of neck adiposity than mean NC. Mean NC:height and 0.50 NC:height had similar ($P > 0.20$) associations with CNS and blood variables. As mean NC did not significantly improve associations with variables over 0.50 NC, the latter is recommended as a more practical measurement of neck crest adiposity.

Ideally, if morphometric ratios had completely adjusted for size, a single regression line for relationships between variables would fit both pony and horse data. However, relationships of morphometrics with BCS, CNS, and blood variables were different between horses and ponies, indicating that a morphometric measurement of a pony cannot be compared with the same morphometric measurement of a horse. The same value in a horse and pony would not signify a similarity in adiposity or have the same physiological relevance.

Using the populations of horses and ponies in the present study, it is possible to develop estimates of morphometric measurements that would indicate obesity and a "cresty neck". An equid may be considered overweight (equivalent to BCS ≥ 7) with a girth:height $\geq 1.26 \pm 0.01$ for horses or 1.33 ± 0.01 for ponies. An equid may be considered obese (equivalent to BCS ≥ 8) with a girth:height $\geq 1.29 \pm 0.01$ for horses or 1.38 ± 0.01 for ponies. An equid may be considered as having a cresty neck (equivalent to CNS ≥ 3) with a 0.50 NC:height $\geq 0.63 \pm 0.01$ for horses or 0.68 ± 0.01 for ponies. Future studies should evaluate the validity of these estimates in other horse and pony breeds.

As the link between obesity and health problems in horses and ponies is further elucidated, it is important to find convenient methods for the assessment of overall and regional adiposity. While body condition scoring is an accepted method for assessment of overall adiposity, cresty neck scoring would standardise the assessment of regional fat distribution on the crest of the neck. Morphometric measurements of girth:height for

overall adiposity, and crest height or 0.50 NC:height for neck crest adiposity are objective alternatives to subjective scoring systems.

Table 1.1: Cresty neck scoring system

Score	Description
0	No visual appearance of a crest (tissue apparent above the *ligamentum nuchae*). No palpable crest.
1	No visual appearance of a crest, but slight filling felt with palpation.
2	Noticeable appearance of a crest, but fat deposited fairly evenly from poll to withers. Crest easily cupped in one hand and bent from side to side.
3	Crest enlarged and thickened, so fat is deposited more heavily in middle of the neck than toward poll and withers, giving a mounded appearance. Crest fills cupped hand and begins losing side to side flexibility.
4	Crest grossly enlarged and thickened, and can no longer be cupped in one hand or easily bent from side to side. Crest may have wrinkles/creases perpendicular to topline.
5	Crest is so large it permanently droops to one side.

Table 1.2: Variable medians (25^{th} – 75^{th} percentile) for horses and ponies

Variable	Horses (n = 34)	Ponies (n = 75)	P[a]
Body condition score	6.0 (5.5 – 6.5)	7.0 (6.0 – 7.5)	< 0.001
Cresty neck score	2.0 (1.8 – 2.3)	3.0 (2.0 – 4.0)	0.001
Insulin, mU/L	9.6 (5.9 – 28.2)	16.0 (8.1 – 38.2)	0.12
Glucose, mg/dL	94.2 (89.6 – 99.2)	93.8 (89.1 – 100.0)	0.80
Triglyceride, mg/dL	23.9 (18.0 – 32.5)	47.2 (35.2 – 58.2)	< 0.001
Leptin, ng/mL	3.8 (2.4 – 5.4)	5.8 (3.4 – 8.1)	< 0.001

[a] P-value for Kruskal-Wallis test comparing horses and ponies

Table 1.3: Associations of blood variables with body condition score (BCS) or morphometric measurements of body adiposity

Correlation variables	Horse (n = 34)		Pony (n = 75)		
	r_s [a]	P [b]	r_s	P [b]	P [c]
BCS x Leptin	0.44	0.010	0.72	<0.001	0.090
Girth:Height x Leptin	0.39	0.024	0.68	<0.001	<0.001
Waist:Height x Leptin	0.32	0.066	0.44	<0.001	
BCS x Insulin	0.48	0.004	0.49	<0.001	0.001
Girth:Height x Insulin	0.31	0.075	0.41	<0.001	
Waist:Height x Insulin	0.16	0.35	0.06	0.59	
BCS x Glucose	0.46	0.007	0.21	0.071	
Girth:Height x Glucose	0.12	0.51	0.22	0.055	
Waist:Height x Glucose	0.09	0.60	-0.01	0.97	
BCS x Triglyceride	0.41	0.017	0.43	<0.001	0.21
Girth:Height x Triglyceride	0.17	0.33	0.44	<0.001	
Waist:Height x Triglyceride	0.07	0.68	0.35	0.002	

[a] Spearman rank correlation coefficient

[b] *P*-value for a test of the null hypothesis that the variables are independent

[c] *P*-value for a test of the null hypothesis that one line fits both pony and horse data sets. Tests were performed only if correlations were significant for both horses and ponies.

Table 1.4: Associations of blood variables with cresty neck score (CNS) or morphometric measurements of neck crest adiposity

Correlation variables	Horse (n = 34)		Pony (n = 75)		
	r_s [a]	P [b]	r_s	P [b]	P [c]
CNS x Leptin	0.48	0.005	0.62	<0.001	0.086
0.50 NC[d]:Height x Leptin	0.43	0.010	0.70	<0.001	<0.001
Mean NC[e]:Height x Leptin	0.53	0.001	0.74	<0.001	<0.001
Crest Height x Leptin	0.19	0.27	0.57	<0.001	
CNS x Insulin	0.50	0.002	0.59	<0.001	0.005
0.50 NC:Height x Insulin	0.40	0.021	0.50	<0.001	<0.001
Mean NC:Height x Insulin	0.49	0.003	0.51	<0.001	<0.001
Crest Height x Insulin	0.41	0.017	0.49	<0.001	<0.001
CNS x Glucose	0.45	0.007	0.27	0.020	0.001
0.50 NC:Height x Glucose	0.32	0.069	0.28	0.014	
Mean NC:Height x Glucose	0.42	0.013	0.28	0.016	<0.001
Crest Height x Glucose	0.39	0.023	0.23	0.046	<0.001
CNS x Triglyceride	0.43	0.012	0.43	<0.001	0.52
0.50 NC:Height x Triglyceride	0.38	0.029	0.41	<0.001	0.002
Mean NC:Height x Triglyceride	0.50	0.003	0.42	<0.001	0.001
Crest Height x Triglyceride	0.17	0.33	0.36	0.002	

[a] Spearman rank correlation coefficient

[b] P-value for a test of the null hypothesis that the variables are independent

[c] P-value for a test of the null hypothesis that one line fits both pony and horse data sets. Tests were performed only if correlations were significant for both horses and ponies.

[d] Neck circumference at 0.50 neck length

[e] Average of 0.25 NC, 0.50 NC, and 0.75 NC

Table 1.5: Associations of body condition score (BCS) with morphometric measurements of body adiposity

Morphometric measurement	Horse BCS (n=34)		Pony BCS (n=75)		
	r_s [a]	P [b]	r_s	P [b]	P [c]
BW [d]	0.09	0.61	0.05	0.67	
BMI 1[e]	0.35	0.044	0.43	<0.001	<0.001
BMI 2[f]	0.50	0.003	0.37	0.001	<0.001
Girth	0.21	0.22	0.20	0.091	
Waist	0.24	0.17	0.18	0.13	
Girth:Height	0.64	<0.001	0.83	<0.001	<0.001
Girth:Length	0.40	0.020	0.56	<0.001	0.86
Girth:BW	-0.01	0.96	0.05	0.69	
Waist:Height	0.68	<0.001	0.58	<0.001	<0.001
Waist:Length	0.51	0.002	0.45	<0.001	0.61
Waist:BW	0.06	0.72	0.07	0.57	
Waist:Girth	0.22	0.22	0.06	0.62	

[a] Spearman rank correlation coefficient
[b] *P*-value for a test of the null hypothesis that the variables are independent
[c] *P*-value for a test of the null hypothesis that one line fits both pony and horse data sets. Tests were performed only if correlations were significant for both horses and ponies.
[d] Bodyweight
[e] Body mass index 1 (BW (kg)/[height (m)]^2)
[f] Body mass index 2 (BW (kg)/[length (m) × height (m)])

Table 1.6: Associations of cresty neck score (CNS) with morphometric measurements of neck crest adiposity

Morphometric measurement	Horse CNS (n=34)		Pony CNS (n=75)		
	r_s [a]	P [b]	r_s	P [b]	P [c]
0.25 NC[d]	0.55	0.001	0.48	<0.001	<0.001
0.50 NC[e]	0.39	0.023	0.61	<0.001	<0.001
0.75 NC[f]	0.27	0.13	0.44	<0.001	
Mean NC[g]	0.40	0.019	0.56	<0.001	<0.001
0.50 NC:Neck length	0.43	0.011	0.65	<0.001	<0.001
Mean NC:Neck length	0.37	0.028	0.61	<0.001	<0.001
0.50 NC:Height	0.58	0.001	0.80	<0.001	<0.001
Mean NC:Height	0.65	<0.001	0.83	<0.001	<0.001
Crest Height	0.51	0.002	0.91	<0.001	<0.001

[a] Spearman rank correlation coefficient
[b] *P*-value for a test of the null hypothesis that the variables are independent
[c] *P*-value for a test of the null hypothesis that one line fits both pony and horse data sets. Tests were performed only if correlations were significant for both horses and ponies.
[d] Neck circumference at 0.25 neck length
[e] Neck circumference at 0.50 neck length
[f] Neck circumference at 0.75 neck length
[g] Average of 0.25 NC, 0.50 NC, and 0.75 NC

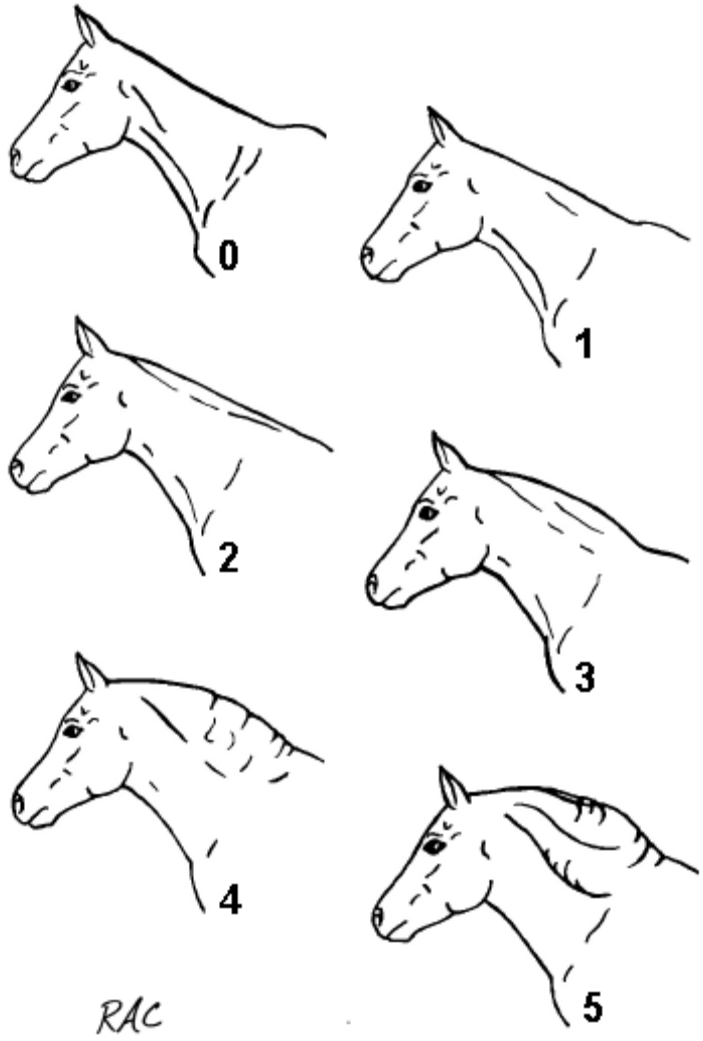

Figure 1.1: Illustrations of individual cresty neck scores.

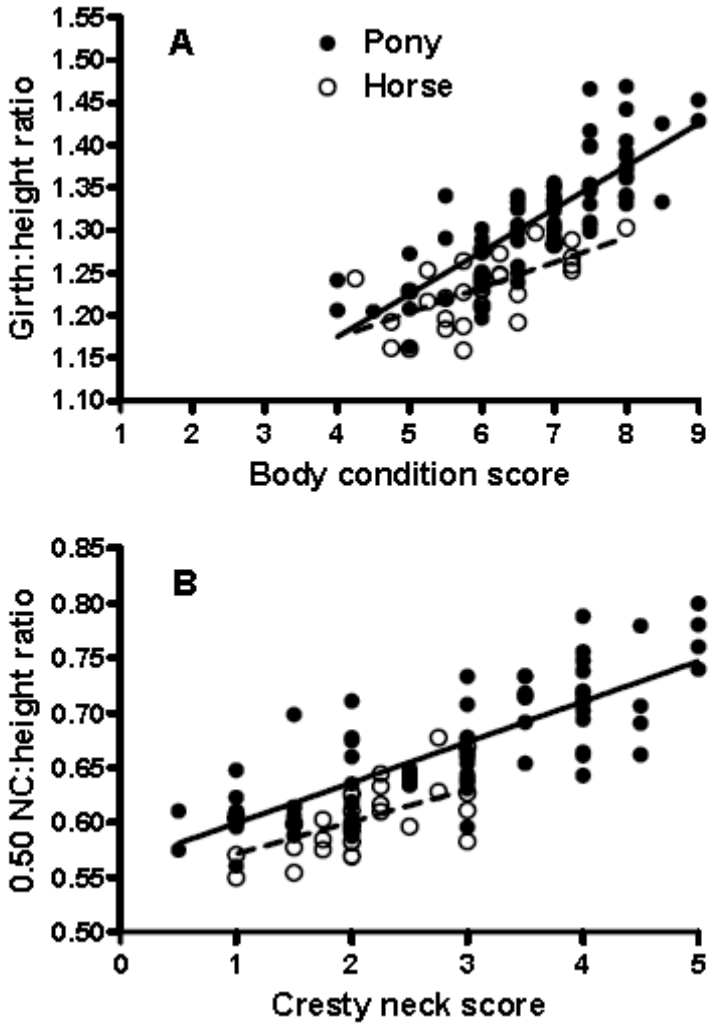

Figure 1.2: Linear relationship of girth:height and body condition score (A) in horses (y = 0.030x + 1.054; $P < 0.001$) and ponies (y = 0.050x + 0.975; $P < 0.001$). Linear relationship of neck circumference at half neck length (0.50 NC):height and cresty neck score (B) in horses (y = 0.030x + 0.541; $P < 0.001$) and ponies (y = 0.037x + 0.562; $P < 0.001$). Solid and dashed lines represent linear regression lines of ponies and horses, respectively.

CHAPTER 2

Prediction of incipient pasture-associated laminitis from hyperinsulinemia, hyperleptinemia, and generalized and localized obesity in a cohort of ponies

ABSTRACT: The ability to predict ponies at increased risk of laminitic episodes when exposed to high nonstructural carbohydrate pasture facilitates the implementation of management strategies to avoid disease. The objective of this study was to identify variables and clinically useful cutoff values with reproducible diagnostic accuracy for the prediction of ponies that subsequently developed laminitis when exposed to pasture high in nonstructural carbohydrate within 3 months of evaluation. A cohort of predominantly Welsh and Dartmoor ponies from a closed herd was evaluated in March 2006 (Evaluation 1; n = 74) and March 2007 (Evaluation 2; n = 57). Ponies were categorized as never laminitic (NL) or previously laminitic (PL) according to reported laminitic history and as clinically laminitic (CL) if laminitis was observed within 3 months following evaluation. Body condition score (BCS), cresty neck score (CNS), girth and neck circumferences, wither height, blood pressure, hoof surface temperature and plasma insulin, glucose, triglyceride, leptin, cortisol, ACTH, uric acid and TNFα concentrations were measured. Analysis of sensitivity, specificity, and receiver operating characteristic curves was used to evaluate the diagnostic accuracy for a variable to predict CL ponies. Reproducibility of predictors was evaluated during Evaluation 2. Variables with diagnostic accuracy for the prediction of CL ponies (n = 6 in Evaluation 1; n = 8 in Evaluation 2) included insulin, leptin, BCS, CNS, and neck circumference:height. Specific cutoff values of insulin > 32 mU/L, leptin > 7.3 ng/mL, BCS ≥ 7, CNS ≥ 4, and neck circumference:height ratio > 0.71 based on Evaluation 1 data had reproducible diagnostic accuracy for the prediction of laminitis in both evaluations. Combining tests did not result in higher diagnostic accuracy than the individual tests of insulin or leptin during both evaluations. Tests of insulin and leptin concentrations and measures of generalized (BCS) and localized (CNS or neck circumference:height ratio) obesity were beneficial in the prediction of laminitic episodes when ponies were exposed to high carbohydrate pasture. These results highlight the importance of monitoring and reducing insulin concentration as well as generalized

and regional obesity in ponies to improve metabolic health and therefore reduce laminitis risk.

Key words: cresty neck, insulin, laminitis, leptin, obesity, pony

Introduction

Pasture-associated laminitis has major economic and welfare implications for the equine industry, accounting for an estimated 54% of cases of equine laminitis for which the initial cause is identifiable (USDA, 2000). Physiologic or genetic factors may predispose some ponies more than others to recurrent, or pasture-associated, laminitis (Treiber et al., 2006c). Identification of susceptible ponies could facilitate the prediction of laminitic episodes and allow for implementation of countermeasures to avoid laminitis (Harris et al., 2006).

Increased risk for pasture-associated laminitis in apparently healthy ponies has been characterized by a set of risk factors known as the prelaminitic metabolic syndrome (PLMS) (Treiber et al., 2006c). These risk factors include insulin resistance (reciprocal of the square root of insulin [RISQI] < 0.32 $[mU/L]^{-0.5}$), increased insulin secretory response (modified insulin-to-glucose ratio [MIRG] > 5.6 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$), hypertriglyceridemia (> 57.0 mg/dL), and obesity (body condition score [BCS] > 6, scale 1 – 9, with localized fat deposits on neck and tailhead). Ponies identified by the PLMS as surpassing three or more criteria were 10 times more likely to develop laminitis than those not identified by PLMS, according to subsequent occurrence of laminitis in the study group.

Metabolic syndrome in humans relates factors of obesity, insulin resistance, hypertension, hypertriglyceridemia, and hyperglycemia to risk for type 2 diabetes and cardiovascular disease (ATPIII, 2001; WHO, 1999). Recent research has demonstrated a number of similar factors, including insulin resistance, hyperinsulinemia, obesity, hypertension, and increased uric acid concentrations in ponies with a history of laminitis (Bailey et al., 2008; Bailey et al., 2007; Treiber et al., 2007b; Treiber et al., 2006c). Additionally, laminitis is a common sequela to pituitary pars intermedia dysfunction. Pituitary pars intermedia dysfunction, as defined by elevated ACTH concentration, was observed in 70% of horses with laminitis in a primary-care ambulatory setting (Donaldson et al., 2004a).

Obesity has been associated with insulin resistance, hyperinsulinemia, altered lipid metabolism, and increased inflammatory cytokine expression (Frank et al., 2006;

Hoffman et al., 2003; Vick et al., 2007). Obesity may therefore be related to laminitis either directly or indirectly through its associated metabolic factors. In humans regional fat accumulation centrally (visceral adipose) is more closely linked to disease risk than generalized obesity (Murphy and Bloom, 2006). However, it has been proposed that in horses and ponies, adipose tissue distributed specifically on the crest of the neck ("cresty neck") could indicate or contribute to hyperinsulinemia, insulin resistance, or risk for laminitis (Frank et al., 2006; Johnson, 2002; Treiber et al., 2006c).

Considering these relationships with laminitic history, it was hypothesized that measurement of the above mentioned variables would provide useful tests for the prediction of laminitic episodes. The objective of the study reported here was to identify variables and clinically useful cutoff values with reproducible diagnostic accuracy for the prediction of ponies that subsequently developed laminitis when exposed to high carbohydrate pasture within 3 months of evaluation. Additionally, it was tested whether clustering variables into a syndrome, such as the PLMS, provided stronger diagnostic accuracy than assessing individual variables alone.

Materials and Methods

Animals and study design.

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. Following a cohort study design, a single herd of ponies was evaluated in March 2006 (Evaluation 1) and March 2007 (Evaluation 2). Eligibility criteria for inclusion in the study included being fully or partially descended from pony bloodlines, residing at the selected farm in northern Virginia, ≥ 3 years of age, female, and apparently healthy at the time of initial evaluation. Ponies < 3 years old were excluded based on a lack of laminitic episodes in this age range according to farm records, and males were excluded as mares were almost exclusively available for evaluation. For Evaluation 1, 74 ponies that fit the selected criteria were recruited and sampled on 3 consecutive days in March 2006 between 0800 and 1200 h. Ponies were used in Evaluation 2 if they had participated in Evaluation 1 and were apparently healthy at the

time of re-evaluation. For Evaluation 2, 57 ponies were evaluated on 2 consecutive days in March 2007.

Diagnosis of laminitis.

Ponies which were reported in farm records to have exhibited one or more episodes of idiopathic lameness characteristic of laminitis were categorized as previously laminitic (PL). Identification of previous laminitis episodes was performed by farm personnel knowledgeable in the identification of lameness due to laminitis as opposed to other causes of lameness. Unaffected ponies were categorized into a never laminitic (NL) control group. Following initial evaluations, episodes of acute pasture-associated laminitis were documented in spring and early summer (April to June, 2006 and 2007). Complete physical and lameness examinations were performed to confirm suspected episodes of laminitis. These ponies were subsequently allocated to a clinically laminitic (CL) group for each evaluation.

Sample collection and analysis.

During Evaluation 1, linear measurements were taken of wither height, girth and waist circumferences, and neck circumference (NC) at half of neck length with a tape measure. Waist circumference was measured as the abdominal circumference two-thirds the distance from the point of the shoulder (intermediate tubercle of the humerus) to the point of the hip (tuber coxae). Three experienced evaluators independently rated body condition score (BCS) on a scale of 1 to 9 (Henneke et al., 1983) and cresty neck score (CNS) of neck adiposity on a scale of 0-5 (Chapter 1). The median score for each pony was used for analysis. Systolic, diastolic and mean blood pressures were measured with an oscillometric device (Colin 8800, Colin Medical Instruments Corporation, San Antonio, TX) and pressure cuff applied to the base of the tail. Five consecutive measurements were taken from each pony, with the median value used for analysis. Hoof surface temperature was measured with a laser portable infrared thermometer (IR Man, MIKRON Instrument Company, Oakland, NJ) at the anterior midpoint of the hoof wall for each hoof. Ponies were removed from direct sunlight onto an asphalt barn aisle for ≥

5 min before hoof temperature measurement. Ambient temperature was recorded each day.

Basal blood samples were collected by jugular venipuncture between 0800 and 1200 h, before concentrate or cereal grains were fed. Samples were collected into evacuated tubes containing sodium heparin or potassium EDTA as anticoagulant. Plasma was separated by centrifugation within 30 min of sample collection, and stored at -20^{o}C until analysis. Plasma glucose, triglyceride, and uric acid concentrations were assayed enzymatically by commercial kits and an automated analyzer (CX5 Chemistry Analyzer, Beckman Coulter Inc, Fullerton, CA). Plasma insulin (Coat-A-Count Insulin, Diagnostic Products Corporation, Los Angeles, CA), leptin (Multi-species Leptin RIA, Linco Research Inc, St. Charles, MO), cortisol (Coat-A-Count Cortisol, Diagnostic Products Corporation, Los Angeles, CA), and ACTH (DiaSorin ACTH, DiaSorin, Stillwater, MN) concentrations were measured by commercial radioimmunoassay previously validated for use in equine plasma (Freestone et al., 1991; McFarlane et al., 2006; McManus and Fitzgerald, 2000). Plasma TNFα was measured by a commercially available equine-specific ELISA (Equine TNFα Screening Set, Endogen, Rockford, IL) previously validated in equine samples (Vick et al., 2007). Assays were performed in duplicate.

During Evaluation 2, BCS, CNS, wither height, girth circumference, NC, blood pressure, and plasma insulin, glucose, triglyceride and leptin concentrations were measured as described for Evaluation 1. Plasma ACTH concentrations were analyzed at a commercial laboratory (Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY) by an automated chemiluminescent enzyme immunoassay system (Immulite, Siemens Medical Solutions Diagnostics, Los Angeles, CA) previously validated in equine plasma (Perkins et al., 2002).

Statistical analyses.

Values for RISQI and MIRG were calculated as previously described (Treiber et al., 2005b). Results of the Shapiro-Wilk test revealed that not all variables were normally distributed within laminitis group, therefore data are reported as median (95% confidence intervals for the median) and laminitis groups (NL, PL and CL) were compared by Kruskal-Wallis tests with Dunn's multiple comparison post-test applied between groups.

Differences in medians between evaluations were assessed by Wilcoxon's matched pairs test. Values of $P < 0.05$ were considered significant.

For each variable, true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) were determined based on the ability of a test to distinguish CL ponies from all other ponies at each measured value or at a specified cutoff value. Sensitivity and specificity were calculated as TP/(TP + FN) and TN/(FP + TN), respectively (Gibson, 1990). Positive predictive value (PPV) was calculated as TP/(TP + FP) and negative predictive value (NPV) was calculated as TN/(TN + FN). To determine the diagnostic accuracy of a test, the true positive fraction (sensitivity) was plotted against the true negative fraction (1 – specificity) in receiver operating characteristic (ROC) plots and the area under the curve was calculated using the trapezoidal rule (Zweig and Campbell, 1993). Areas under the ROC curves were considered significant if there was rejection of the null hypothesis that the ROC area equals 0.50 by a one-tailed test ($\alpha = 0.05$). Continuous variables were used in global ROC (gROC) plots to determine the ability of the test to distinguish between CL and all other ponies over all decision thresholds (Figure 2.1A). Continuous variables with gROC > 0.50 ($P < 0.05$) or part of the original PLMS criteria were then transformed into binary variables (positive or negative) by categorization based on a cutoff value and local ROC (lROC) plots were generated (Figure 2.1B). An optimum cutoff value was chosen based on a maximum area under the lROC curve value, at which point there was a balanced minimum of false-positive and false-negative results. Likelihood ratios were calculated as the ratio of the true-positive rate to the false-positive rate (LR+) and the ratio of the false-negative rate to the true-negative rate (LR-) (Biggerstaff, 2000). Areas under the ROC curves and likelihood ratios are reported with 95% confidence intervals (Altman et al., 2000; Biggerstaff, 2000) and were compared using 95% confidence intervals for differences between variables. Intercooled Stata Version 9.2 (Stata-Corp, College Station, TX) was used for statistical computations.

Results

Evaluation 1.

Evaluated ponies were predominantly descended from Welsh (59%) or Dartmoor (17%) bloodlines or were cross-bred (21%) with horse bloodlines. According to farm manager-reported histories of laminitis, 42 of the 74 ponies presented for evaluation were categorized as PL (25 pregnant, 17 nonpregnant; age 7 – 34 years) and 32 were categorized as NL (18 pregnant, 14 nonpregnant; age 3 – 29 years). Six PL ponies developed pasture-associated laminitis during April or May 2006 (i.e. 2 to 3 months after evaluation) and were subsequently recategorized into a CL group (2 pregnant, 4 nonpregnant; age 7 – 22 years).

Median (95% confidence interval) values for measured test variables are reported in Table 2.1. Forefoot and hindfoot temperatures were similar ($P > 0.05$), therefore the median temperature of all 4 hooves for each pony was used for analysis. Hoof temperatures were different ($P < 0.05$) between days of evaluation, with higher hoof temperatures corresponding to a greater ambient temperature. Potential confounding factors of age and pregnancy status were evaluated in all variables. Girth:height ratio and triglyceride and uric acid concentrations were higher ($P < 0.05$) in pregnant than nonpregnant ponies. Hoof wall temperature and TNFα concentrations were positively associated ($P < 0.05$) with age.

Diagnostic accuracy of continuous variables to distinguish CL ponies from all other ponies was assessed by area under the gROC curve (Table 2.1). Tests of insulin, RISQI, leptin, ACTH, BCS, girth:height, CNS, NC:height, BCS, and blood pressure measurements had gROC areas greater ($P < 0.05$) than 0.50, indicating an ability to distinguish between ponies that did and did not develop clinical laminitis.

Predictions by individual tests were compared to combinations of tests, including 1) the original PLMS as previously defined (Treiber et al., 2006c), 2) a modified PLMS with the same variables as the original, but cutoff values specific for the prediction of CL in the present study (3 of 4 criteria: BCS ≥ 7 and CNS ≥ 4, TG > 94 mg/dL, RISQI < 0.17 $[mU/L]^{-0.5}$, MIRG > 11.3 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$), and 3) a new cluster of variables chosen for their superior ability to predict CL (3 of 4 criteria: BCS ≥ 7, CNS ≥ 4, basal insulin > 32 mU/L, leptin > 7.3 ng/mL) (Table 2.2). The new cluster had a higher lROC than the original PLMS, whereas the modified PLMS criteria lROC did not differ from

either the original PLMS or new cluster of variables. Combining tests did not result in higher lROC than the individual tests.

Evaluation 2.

Fifty-seven of the ponies that were evaluated in 2006 were re-evaluated in 2007. Reasons for withdrawal from the study between Evaluations 1 and 2 included being sold, located in inaccessible pastures, or deceased. Twenty-seven were NL (19 pregnant, 8 nonpregnant; age 4 – 21 years) and 30 were PL (11 pregnant, 19 nonpregnant; age 8 – 24 years). Eight PL ponies (3 pregnant, 5 nonpregnant; age 9 – 15 years) developed pasture-associated laminitis in April or May 2007. Two ponies were CL in both Evaluations 1 and 2. In the 57 ponies assessed during both evaluations, triglyceride, leptin, and ACTH concentrations decreased ($P < 0.001$), NC:height increased ($P < 0.037$) and mean blood pressure decreased ($P < 0.028$) from Evaluation 1 to 2, with no differences between evaluations in all other variables ($P > 0.05$).

Cutoff values established in Evaluation 1 were used in Evaluation 2 in order to examine the within herd accuracy of these cutoffs on the second evaluation's data. Median (95% confidence interval) values for variables are reported in Table 2.3. Variables with reproducible diagnostic accuracy (gROC greater ($P < 0.05$) than 0.50 in Evaluations 1 and 2) included insulin, RISQI, leptin, BCS, CNS, and NC:height (Table 2.3). Variables with reproducible diagnostic accuracy at specified cutoff values (lROC greater ($P < 0.05$) than 0.50 in Evaluations 1 and 2) included insulin, RISQI, MIRG, leptin, BCS, CNS, and NC:height (Table 2.4). Combining tests into the new cluster of variables had greater lROC than the original or modified PLMS. However, combining tests did not result in higher lROC than the individual tests of insulin, leptin, RISQI, or MIRG.

Discussion

In a closed herd of predominantly Welsh and Dartmoor ponies, variables with reproducible diagnostic accuracy for the prediction of laminitic episodes included basal insulin and leptin concentrations, RISQI, and measures of generalized (BCS) and

localized (CNS or NC:height ratio) obesity. Although the original PLMS criteria or combinations of new criteria predicted laminitis with reproducible diagnostic accuracy, their areas under the lROC curves were not higher than the individual tests from which they were produced.

Previously established PLMS criteria were determined on the basis of their ability to correctly differentiate historically PL from NL ponies, which was then applied for the prediction of CL cases (Treiber et al., 2006c). The present study has analyzed variables for their ability to differentiate CL ponies from ponies that did not develop laminitis. These risk factors identify ponies during times of low pasture nonstructural carbohydrate (NSC) levels (NSC < 10% of dry matter during March 2006 and 2007, Appendix B) that will be more likely to develop laminitis in the upcoming months when exposed to high NSC pasture (NSC > 15% of dry matter, Appendix B). This focuses on variables relevant to current risk of laminitis rather than previous conditions which may no longer apply. The small number of ponies that develop laminitis each year and the variability of measured variables create the necessity of using statistical analyses such as ROC plots and likelihood ratios.

The diagnostic accuracy of a test for the prediction of CL was assessed by evaluation of areas under the ROC curves (Figure 2.1). When a continuous variable is used in an ROC plot, a comprehensive picture is formed of the ability of the test to make the distinction being examined over all decision thresholds (Zweig and Campbell, 1993). This was referred to as global ROC (gROC) and provides information on a variable's relationship to laminitis independent of any specified cutoff value. When interpreting gROC, an AUC of 0.80, for example, would mean that a CL pony would have a test value greater than that of a pony that did not develop laminitis 80% of the time. When a continuous variable was transformed into a binary variable (positive or negative) by categorization based on a cutoff value, local ROC (lROC) plots were generated. Applying these cutoffs to the second year of data provides some degree of verification of the adequacy of values determined with the first year of data. Likelihood ratios correspond to the slopes of the ROC plot (Biggerstaff, 2000). The LR+ describes how probability of laminitis shifts with a positive test result, whereas LR- describes how the probability of laminitis shifts with a negative test result. A test with higher LR+ and lower LR- values

was preferred. Although likelihood ratios provide useful information on test performance at a specified cutoff value, it is necessary to interpret values with respect to sensitivity and specificity. For example, a test with high LR+ would indicate a high probability of laminitis with a positive test result, however in the presence of low sensitivity and a high specificity it would not identify many of the ponies that would develop laminitis due to an inappropriately high cutoff value.

Positive and negative predictive values were determined as the proportion of positive or negative tests that were true, respectively. For example, a PPV of 29% for the test of insulin concentration > 32 mU/L in Evaluation 1 indicates that 29% of the positive test results correctly predicted the occurrence of laminitis, whereas 71% of the ponies with positive test did not develop laminitis. Predictive values are dependent on the prevalence of a disease. When prevalence is low, even very sensitive and specific tests have relatively low PPV. Conversely, when prevalence is high, tests with low sensitivity and specificity may have relatively high PPV.

As components of the original PLMS, it was hypothesized that triglyceride concentration, RISQI, and MIRG would be useful for the prediction of incipient laminitis. Proxy measurements of RISQI and MIRG were developed as easily obtainable surrogates of minimal model parameters of insulin sensitivity and acute insulin response to glucose, respectively (Treiber et al., 2005b). As RISQI is inversely proportional to insulin concentrations, it had the same ability as basal insulin concentration for the prediction of CL ponies. Both MIRG and triglyceride concentration had low diagnostic accuracy for the prediction of laminitis, with gROC not significantly different than 0.50 for one or both evaluations. Although triglyceride concentration differentiated NL from PL ponies, it predicted CL ponies with low diagnostic accuracy. Additionally, MIRG has limited value when insulin concentrations are > 50 mU/L because its parabolic nature causes values to decline after insulin concentrations surpass 50 mU/L, and therefore restricts its utility of identifying laminitis risk in hyperinsulinemic ponies.

Although surface temperature of the hoof wall was greater in PL compared to NL ponies, this measurement was not an effective predictor of laminitis in CL ponies. The usefulness of this measurement is limited due to the effect of ambient temperature and the necessity of a controlled environment. Difference in hoof temperature between laminitis

groups may be a response to previous occurrences of laminitis, such as vascular remodeling or a continual inflammatory response from previous damage.

Results of the current study do not support blood pressure or uric acid concentrations as predictive measurements for the occurrence of laminitis. These variables were previously evaluated in ponies with a history of laminitis, and differences between NL and PL ponies were observed during summer but not winter (Bailey et al., 2008). Although seasonal differences in variables may be part of the phenotype of ponies with a history of laminitis (and suspected predisposition for laminitis), these variables were not predictive of laminitic episodes and did not differ between NL and PL groups in March in the present study.

Bailey et al. (2008) found that ACTH concentrations were similar between NL and PL ponies. In the present study, although ACTH concentration was higher in PL than NL ponies during Evaluation 1, it was not a useful predictor of laminitis nor did it differ between laminitis groups during Evaluation 2. Additionally, the concentrations in Evaluation 1 were higher than in Evaluation 2, perhaps due to annual variation or assay methodology.

Inclusion of CNS or NC:height in the PLMS obesity criterion complimented BCS, providing a standardized measurement of the localized fat deposits on the neck which were referred to in the original PLMS. However, the current study cannot support a role of CNS independent of BCS for the prediction of laminitis since all CL ponies displayed both generalized and localized obesity. In horses, generalized obesity has been linked to insulin resistance and inflammation (Hoffman et al., 2003; Vick et al., 2007), however researchers are only beginning to explore the possibility of a high-risk fat depot located along the crest of the neck (Bailey et al., 2008; Frank et al., 2006).

Laminitic episodes observed in the ponies in the present study were highly associated with the presence of obesity and obesity-related factors, including high BCS, high CNS, and elevated insulin and leptin concentrations. It is hypothesized that in this population, a chronic state of overnutrition and/or reduced physical activity contributed to the development of obesity (generalized and localized) which then contributed to an insulin resistant state with concurrent hyperinsulinemia (Hoffman et al., 2003; Vick et al., 2007). Therefore, it is suggested that the tests presented here are most applicable to cases

where an obesity-associated change in metabolism has increased the risk of laminitis. It is uncertain whether these tests will predict laminitis when obesity is not present. Additionally, the data in the present study were drawn from a closed herd of inbred ponies, so further studies are needed to determine the generality of results to wider populations (Carter et al., 2006).

Insulin concentration had reproducibly high diagnostic accuracy as a continuous variable and with a cutoff value > 32 mU/L in Evaluations 1 and 2. Low insulin sensitivity and/or high insulin concentrations have been associated with laminitic predisposition in previous studies (Bailey et al., 2008; Bailey et al., 2007; Coffman and Colles, 1983; Jeffcott et al., 1986; Treiber et al., 2007b; Treiber et al., 2006c), and experimental induction of continuous hyperinsulinemia (> 1000 mU/L) while maintaining euglycemia induced laminitis in healthy ponies (Asplin et al., 2007b). Although hyperinsulinemia effectively predicted laminitis in the current study, dependence on a single factor rather than a combination of multiple risk factors may limit sensitivity or generality. Insulin concentration can vary dramatically with changes in dietary composition that occur seasonally and diurnally, as observed in grazing horses (McIntosh et al., 2007). Additionally, a recent study by Bailey et al. (2007) demonstrated that basal insulin concentrations were not consistently different between NL and PL mixed-breed ponies, especially when fed a low glycemic diet. A more reliable identifier of the laminitis-predisposed phenotype was insulinemic response to a challenge of intravenous glucose, oral inulin, or intramuscular dexamethasone. Dynamic tests, or measuring insulin in response to a stimulus, could facilitate prediction of a pony's response to stressors that may induce laminitis, including changes in pasture carbohydrate levels. However, even dynamic tests of insulin sensitivity display inter-day variability, with coefficients of variation of 24 – 33 % reported for insulin-modified frequently sampled intravenous glucose tolerance tests (Frank et al., 2008b; Pratt et al., 2005).

Leptin concentration had high diagnostic accuracy as a continuous variable, with a cutoff value > 7.3 ng/mL in Evaluations 1 and 2. Leptin is primarily produced and secreted from adipose tissue at levels that are proportional to body condition (Buff et al., 2002), and its secretion is stimulated by insulin and inversely related to insulin sensitivity in obese horses (Cartmill et al., 2005; Cartmill et al., 2003; Frank et al., 2006).

Additionally, obesity is related to a state of relative insulin resistance (Hoffman et al., 2003; Vick et al., 2007), with adipose tissue deposited specifically along the crest of the neck associated with insulin concentration (Chapter 1) or glucose tolerance (Frank et al., 2006). Although previous studies have demonstrated that basal insulin concentration is a useful indicator of insulin sensitivity (Frank et al., 2006; Treiber et al., 2005b), measurement of variables which are associated with insulin sensitivity that do not fluctuate as acutely as insulin concentration, such as leptin, BCS or CNS, could compliment the prediction of laminitis.

Although a predisposition to develop laminitis may be genetically or physiologically determined, actual development of laminitis is dependent on interactions between the animal and its environment. Evaluations of the current study were performed in March, presumably before environmental stressors, such as changes in pasture composition, would affect metabolism. Although sampling at this time of year provides the greatest opportunity to implement countermeasures to avoid laminitis, repeating evaluation under various environmental conditions would provide a broader perspective of the factors contributing to laminitis.

In summary, using a cohort of ponies from a single inbred herd, tests of insulin and leptin concentrations as well as measures of generalized (BCS) and localized (CNS or NC:height ratio) obesity were beneficial in the prediction of laminitic episodes resulting from exposure to rapidly growing pasture within 3 months of evaluation. These results highlight the importance of monitoring and reducing insulin concentration and obesity in ponies in order to improve metabolic health and therefore reduce laminitis risk.

Table 2.1: Median values of variables for never laminitic (NL), previously laminitic (PL), and clinically laminitic (CL) groups of ponies measured in March 2006 during Evaluation 1. Area under the global receiver operating characteristic curve (gROC) was used as a measure of diagnostic accuracy for the prediction of laminitis. Values in ( ) are 95% confidence intervals.

	NL n = 32	PL n = 36	CL n = 6	gROC
Blood Variables				
Triglyceride, mg/dL	39[a] (33 – 47)	53[b] (48 – 58)	47[a,b] (31 – 122)	0.58 (0.28 – 0.88)
Insulin, mU/L	8.8[a] (7.1 – 14.6)	20.5[b] (10.1 – 36.9)	59.5[b] (35.3 – 333)	0.88* (0.80 – 0.96)
Glucose, mg/dL	91 (89 – 95)	96 (92 – 101)	93 (89 – 104)	0.53 (0.34 – 0.73)
RISQI, $[mU/L]^{-0.5}$	0.34[a] (0.26 – 0.38)	0.22[b] (0.16 – 0.32)	0.13[b] (0.05 – 0.17)	0.88[†]* (0.80 – 0.96)
MIRG, $mU_{insulin}^{2}/[10 \cdot L \cdot mg_{glucose}]$	4.9 (4.0 – 6.6)	5.1 (3.8 – 7.6)	11.4 (-315 – 12.8)	0.67 (0.28 – 1.0)
Leptin, ng/mL	4.9[a] (3.4 – 6.3)	5.8[a] (4.0 – 6.8)	11.3[b] (5.6 – 16.0)	0.87* (0.70 – 1.0)
Cortisol, μg/dL	5.6 (4.9 – 6.5)	5.6 (5.2 – 6.6)	4.4 (3.6 – 12.0)	0.66[†] (0.38 – 0.95)
ACTH, pg/mL	75.0[a] (57.2 – 87.1)	86.4[b] (80.7 – 97.6)	51.8[a] (20.5 – 100.4)	0.78[†]* (0.54 – 1.0)
Uric acid, mg/dL	0.5 (0.4 – 0.5)	0.5 (0.5 – 0.5)	0.45 (0.3 – 0.5)	0.63[†] (0.41 – 0.85)
TNF-α, pg/mL	373[a] (204 – 725)	1839[b] (1007 – 2527)	1341[ab] (493 – 2406)	0.59 (0.41 – 0.76)
Adiposity Measurements				
Body condition score	6.5[a] (6.0 - 7.0)	7.0[ab] (6.5 - 7.5)	7.5[b] (7.0 – 8.0)	0.75* (0.63 – 0.87)
Girth:height ratio	1.30 (1.26 - 1.34)	1.33 (1.29 – 1.35)	1.37 (1.31 – 1.47)	0.75* (0.56 – 0.94)
Waist:height ratio	1.45 (1.40 – 1.50)	1.48 (1.46 -1.53)	1.45 (1.39 – 1.68)	0.49 (0.25 – 0.73)
Cresty neck score	2.3[a] (2.0 – 3.0)	3.0[b] (3.0 – 4.0)	4.3[c] (3.5 – 5.0)	0.89* (0.80 – 0.99)
NC:height ratio	0.64[a] (0.61 – 0.66)	0.66[a] (0.64 – 0.71)	0.73[b] (0.71 – 0.80)	0.90* (0.81 – 0.98)
Blood Pressure				
Systolic, mmHg[‡]	119 (112 – 130)	121 (112 – 126)	111 (99 – 127)	0.71[†]* (0.50 – 0.92)
Diastolic, mmHg[‡]	60 (53 – 65)	59 (55 – 62)	53 (45 – 63)	0.72[†]* (0.50 – 0.95)
Mean, mmHg[‡]	84 (78 – 87)	81 (76 – 87)	74 (67 – 85)	0.72[†]* (0.52 – 0.91)
Hoof surface temperature, °C	14.6[a] (10.7 – 18.7)	21.4[b] (19.5 – 24.2)	21.8[ab] (14.4 – 24.2)	0.65 (0.47 – 0.83)

[a,b]Values in the same row with different letters indicate differences ($P < 0.05$) between laminitis groups within each variable

*Area under the gROC curve significantly greater ($P < 0.05$) than 0.50; gROC did not differ ($P > 0.05$) between variables greater than 0.50

[†]Inverse values were used for ROC calculations due to negative association with risk for laminitis

[‡]NL, n=30; PL, n=35; CL, n=5.

Table 2.2: Diagnostic statistics for the prediction of laminitis in ponies in Evaluation 1 (n = 74) using a defined cutoff value for each variable are sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), area under the local receiver operating characteristic curve (lROC), and log likelihood ratios for the positive (LR+) and negative (LR-) tests. Values in ( ) are 95% confidence intervals.

	Cutoff	Sensitivity, %	Specificity, %	PPV, %	NPV, %	lROC	LR+	LR-
Blood Variables								
Triglyceride, mg/dL	> 94	33[b] (10 – 70)	99[a] (92 – 100)	67 (21 – 94)	94 (86 – 98)	0.66[b] (0.54 – 0.77)	22.7[ab] (2.4 – 215)	0.7 (0.4 – 1.2)
Insulin, mU/L	> 32	100[a] (61 – 100)	78[b] (67 – 86)	29 (14 – 50)	100 (93 – 100)	0.89[a] (0.80 – 0.95)	4.5[bcd] (2.9 – 7.1)	0.0 (0.0 – 1.5)
RISQI, $[mU/L]^{-0.5}$	< 0.17	100[a] (61 – 100)	78[b] (67 – 86)	29 (14 – 50)	100 (93 – 100)	0.89[a] (0.80 – 0.95)	4.5[bcd] (2.9 – 7.1)	0.0 (0.0 – 1.5)
MIRG, $mU_{insulin}^{2}/[10 \cdot L \cdot mg_{glucose}]$	> 11.3	67[ab] (30 – 90)	97[a] (90 – 99)	67 (30 – 90)	97 (90 – 99)	0.82[ab] (0.72 – 0.90)	22.7[a] (5.2 – 99.4)	0.3 (0.1 – 1.1)
Leptin, ng/mL	> 7.3	83[ab] (44 – 97)	78[b] (67 – 86)	25 (11 – 47)	98 (90 – 100)	0.81[ab] (0.70 – 0.89)	3.8[bcd] (2.1 – 6.7)	0.2 (0.0 – 1.3)
ACTH, pg/mL	< 58	83[ab] (44 – 97)	79[b] (68 – 87)	26 (12 – 49)	98 (90 – 100)	0.83[ab] (0.71 – 0.90)	4.0[bcd] (2.2 – 7.3)	0.2 (0.0 – 1.3)
Adiposity Measurements								
Body condition score	≥ 7	100[a] (61 – 100)	44[c] (33 – 56)	14 (6 – 27)	100 (89 – 100)	0.72[b] (0.60 – 0.81)	1.8[e] (1.4 – 2.2)	0.0 (0.0 – 2.7)
Girth:height ratio	> 1.30	100[a] (61 – 100)	41[c] (30 – 53)	13 (6 – 26)	100 (88 – 100)	0.71[b] (0.59 – 0.80)	1.7[e] (1.4 – 2.1)	0.0 (0.0 – 2.9)
Cresty neck score	≥ 4	83[ab] (44 – 97)	78[b] (67 – 86)	25 (11 – 47)	98 (90 – 100)	0.81[ab] (0.70 – 0.89)	3.8[bcd] (2.1 – 6.7)	0.2 (0.0 – 1.3)
NC:height ratio	> 0.71	100[a] (61 – 100)	79[b] (68 – 87)	30 (15 – 52)	100 (93 – 100)	0.90[a] (0.80 – 0.95)	4.9[bc] (3.0 – 7.7)	0.0 (0.0 – 1.5)
Mean blood pressure, mmHg[#]	< 76	83[ab] (44 – 97)	69[b] (57 – 79)	19 (9 – 38)	98 (89 – 100)	0.76[ab] (0.64 – 0.85)	2.7[cde] (1.6 – 4.5)	0.2 (0.0 – 1.5)
Combined Criteria								
Original PLMS	Treiber et al., 2006[†]	67[ab] (30 – 90)	66[b] (54 – 76)	15 (6 – 32)	96 (86 – 99)	0.66[b] (0.54 – 0.77)	2.0[de] (1.0 – 3.8)	0.5 (0.2 – 1.6)
Modified PLMS	New cutoffs[‡]	67[ab] (30 – 90)	97[a] (90 – 99)	67 (30 – 90)	97 (90 – 99)	0.81[ab] (0.72 – 0.90)	22.7[a] (5.2 – 99.4)	0.3 (0.1 – 1.1)
New Cluster	New tests[§]	100[a] (61 – 100)	79[b] (68 – 87)	30 (15 – 52)	100 (93 – 100)	0.90[a] (0.80 – 0.95)	4.9[bc] (3.0 – 7.7)	0.0 (0.0 – 1.5)

[a-ef]Comparisons between variables within each diagnostic statistic. Values in the same column with different superscripts differ ($P < 0.05$) between variables, with "a" representing the variables with the greatest value for each statistic. Only lROC values greater ($P < 0.05$) than 0.50 were considered in comparisons.

[†]Surpass 3 of 4 criteria: BCS > 6, TG > 56 mg/dL, RISQI < 0.32, MIRG > 5.6

[‡]Surpass 3 of 4 criteria: BCS ≥ 7 and CNS ≥ 4, TG > 94 mg/dL, RISQI < 0.17, MIRG > 11.3

[§]Surpass 3 of 4 criteria: BCS ≥ 7, CNS ≥ 4, insulin > 32 mU/L, leptin > 7.3 ng/mL

[#]n = 70

Table 2.3: Median values of variables for never laminitic (NL), previously laminitic (PL), and clinically laminitic (CL) groups of ponies measured in March 2007 during Evaluation 2. Area under the global receiver operating characteristic curve (gROC) was used as a measure of diagnostic accuracy for the prediction of laminitis. Values in ( ) are 95% confidence intervals.

	NL n = 27	PL n = 22	CL n = 8	gROC
Blood Variables				
Triglyceride, mg/dL‡	25 (21 – 38)	44 (33 – 52)	48 (22 – 59)	0.67 (0.49 – 0.85)
Insulin, mU/L	9.8[a] (7.0 – 22.4)	25.4[ab] (14.5 – 30.7)	62.6[b] (32.8 – 73.4)	0.87* (0.78 – 0.96)
RISQI, $[mU/L]^{-0.5}$	0.32[a] (0.21 – 0.38)	0.20[ab] (0.18 – 0.26)	0.13[b] (0.12 – 0.17)	0.87†* (0.78 – 0.96)
MIRG, $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$	4.6[a] (3.6 – 8.0)	7.5[ab] (5.3 – 10.2)	10.8[b] (8.1 – 13.2)	0.87* (0.77 – 0.98)
Leptin, ng/mL#	3.7[a] (2.6 – 5.3)	4.6[ab] (2.3 – 6.7)	8.4[b] (4.0 – 11.7)	0.78* (0.59 – 0.96)
ACTH, pg/mL	19.7 (16.8 – 28.4)	28.5 (20.2 – 37.5)	23.7 (15.4 – 46.6)	0.54 (0.36 – 0.73)
Adiposity Measurements				
Body condition score	7.0[a] (6.5 – 7.5)	7.3[ab] (7.0 – 8.0)	8.0[b] (7.0 – 9.0)	0.80* (0.66 – 0.94)
Girth:height ratio	1.33 (1.28 – 1.36)	1.33 (1.27 – 1.37)	1.35 (1.28 – 1.42)	0.63 (0.44 – 0.82)
Cresty neck score	2.5[a] (2.0 – 3.0)	3.5[b] (3.0 – 4.0)	3.8[b] (3.0 – 4.5)	0.80* (0.67 – 0.93)
NC:height ratio	0.65[a] (0.64 – 0..69)	0.70[b] (0.65 – 0.74)	0.72[b] (0.67 – 0.81)	0.75* (0.59 – 0.92)
Mean blood pressure, mmHg§	74 (68 – 85)	77 (67 – 82)	82 (61 – 93)	0.67 (0.40 – 0.93)

[a,b]Values in the same row with different letters indicate differences ($P < 0.05$) between laminitis groups within each variable

*Area under the gROC curve significantly greater ($P < 0.05$) than 0.50; gROC did not differ ($P > 0.05$) between variables greater than 0.50

†Inverse values were used for ROC calculations due to negative associations with risk for laminitis

‡PL, n=21.

#NL, n=25; PL, n=21.

§NL, n=13; PL, n=10; CL, n=7

Table 2.4: Diagnostic statistics for the prediction of laminitis in ponies in Evaluation 2 (n = 57) using a defined cutoff value for each variable are sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), area under the local receiver operating characteristic curve (lROC), and log likelihood ratios for the positive (LR+) and negative (LR-) tests. Values in ( ) are 95% confidence intervals.

	Cutoff	Sensitivity, %	Specificity, %	PPV, %	NPV, %	lROC	LR+	LR-
Blood Variables								
Triglyceride, mg/dL[‡]	> 94	0[d] (0 – 32)	98[ab] (89 – 100)	0 (0 – 79)	86 (74 – 93)	0.49 (0.36 – 0.63)	0.0 (0.0 – 84.0)	1.0[c] (1.0 – 1.1)
Insulin, mU/L	> 32	100[a] (68 – 100)	80[de] (66 – 89)	44 (25 – 66)	100 (91– 100)	0.90[a] (0.78 – 0.96)	4.9[ab] (2.8 – 8.5)	0.0 (0.0 – 1.2)
RISQI, $[mU/L]^{-0.5}$	< 0.17	88[ab] (53 – 98)	84[cde] (71 – 91)	47 (25 – 70)	98 (88– 100)	0.86[a] (0.74 – 0.94)	5.4[ab] (2.7 – 10.6)	0.1[a] (0.0 – 0.9)
MIRG, $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$	> 11.3	50[bc] (22 – 78)	94[abc] (83 – 98)	57 (25 – 84)	92 (81 – 97)	0.72[ab] (0.58 – 0.83)	8.2[a] (2.2 – 29.9)	0.5[abc] (0.3 – 1.1)
Leptin, ng/mL[#]	> 7.3	63[abc] (31 – 86)	90[bcd] (78 – 96)	50 (24 – 76)	94 (83 – 98)	0.76[ab] (0.62 – 0.87)	6.1[ab] (2.3 – 16.5)	0.4[abc] (0.2 – 1.0)
ACTH, pg/mL	< 58	100[a] (68 – 100)	8[g] (3 – 19)	15 (8 – 27)	100 (51– 100)	0.54 (0.41 – 0.68)	1.1[d] (1.0 – 1.2)	0.0 (0.0 – 13.1)
Adiposity Measurements								
Body condition score	≥ 7	100[a] (68 – 100)	29[f] (18 – 42)	19 (10 – 33)	100 (78– 100)	0.64[b] (0.51 – 0.77)	1.4[c] (1.2 – 1.7)	0.0 (0.0 – 3.3)
Girth:height ratio	> 1.30	88[ab] (53 – 98)	37[f] (25 – 51)	18 (9 – 33)	95 (75 – 99)	0.62 (0.48 – 0.74)	1.4[cd] (1.0 – 1.9)	0.3[abc] (1.0 – 2.2)
Cresty neck score	≥ 4	50[bc] (22 – 78)	80[de] (66 – 89)	29 (12 – 55)	91 (78 – 96)	0.65[b] (0.45 – 0.84)	2.5[abc] (1.0 – 5.9)	0.6[abc] (0.3 – 1.3)
NC:height ratio	> 0.71	63[abc] (31 – 86)	67[e] (53 – 79)	24 (11 – 45)	92 (78 – 97)	0.65[b] (0.51 – 0.77)	1.9[bc] (1.0 – 3.7)	0.6[abc] (0.2 – 1.4)
Mean blood pressure, mmHg[#]	< 76	14[cd] (3 – 51)	52[f] (33 – 71)	5 (1 – 17)	67 (44 – 84)	0.33 (0.17 – 0.53)	0.3[e] (0.1 – 1.1)	3.1[e] (1.6 – 5.8)
Combined Criteria								
Original PLMS	Treiber et al., 2006[†]	100[a] (68 – 100)	43[f] (30 – 57)	22 (12 – 38)	100 (85– 100)	0.71[b] (0.58 – 0.83)	1.8[c] (1.4 – 2.2)	0.0 (0.0 – 2.2)
Modified PLMS	New cutoffs[‡]	25[cd] (7 – 59)	100[a] (92 – 100)	100 (34– 100)	88 (77 – 95)	0.63[b] (0.49 – 0.76)	∞ (1.1 - ∞)	0.8[bc] (0.5 – 1.1)
New Cluster	New tests[§]	86[ab] (49 – 97)	87[cd] (74 – 94)	50 (25 – 75)	98 (87– 100)	0.87[a] (0.75 – 0.95)	6.6[a] (2.9 – 14.7)	0.2[ab] (0.0 – 1.0)

[a-f]Comparisons between variables within each diagnostic statistic. Values in the same column with different superscripts differ ($P < 0.05$) between variables, with "a" representing the variables with the greatest value for each statistic. Only lROC values greater ($P < 0.05$) than 0.50 or LR greater ($P < 0.05$) than 0 were considered in comparisons.

[†]Surpass 3 of 4 criteria: BCS > 6, TG > 56 mg/dL, RISQI < 0.32, MIRG > 5.6

[‡]Surpass 3 of 4 criteria: BCS ≥ 7 and CNS ≥ 4, TG > 94 mg/dL, RISQI < 0.17, MIRG > 11.3

[§]Surpass 3 of 4 criteria: BCS ≥ 7, CNS ≥ 4, insulin > 32 mU/L, leptin > 7.3 ng/mL

[#]n = 30

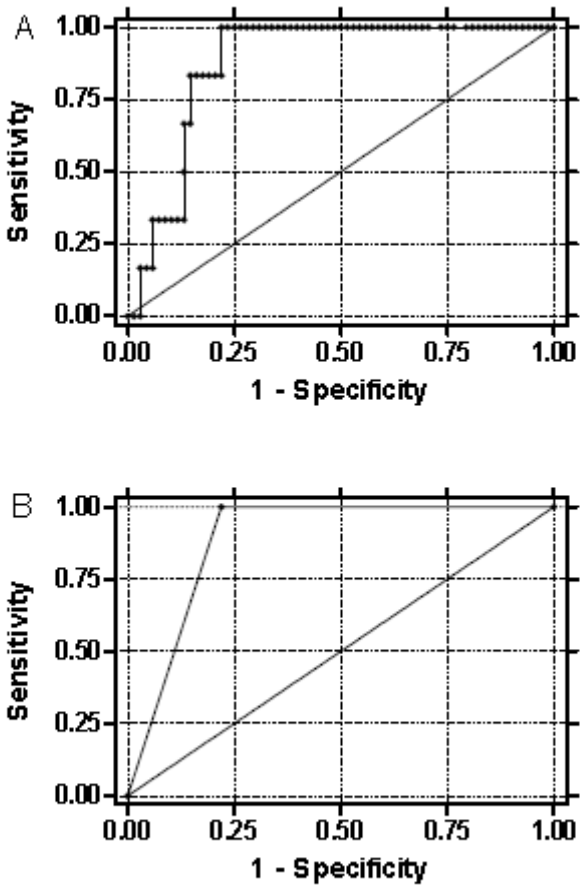

Figure 2.1: Example receiver operating characteristic (ROC) plots for the prediction of CL ponies in Evaluation 1 from insulin concentration among all insulin values in a global ROC plot (A) and at a specified cutoff value of > 32 mU/L in a local ROC plot (B).

CHAPTER 3

Effects of diet-induced obesity on glucose and insulin dynamics and plasma hormone and lipid concentrations in horses

ABSTRACT: The objective of this study was to determine the effects of diet-induced obesity on glucose and insulin dynamics and plasma hormone and lipid concentrations in horses. Thirteen adult Arabian geldings were used in a longitudinal study of 30 weeks duration. Horses initially at a moderate body condition (body condition score [BCS] 6 ± 1, scale 1 – 9) were fed 200% of their digestible energy requirements for maintenance to induce obesity. Frequently sampled intravenous glucose tolerance tests were performed before, during, and after weight gain. Glucose and insulin data were assessed by minimal model analysis. Adiposity and circulating concentrations of insulin, glucose, nonesterified fatty acids, triglycerides, and leptin were measured throughout the study. As a result of overfeeding, body weight increased ($P < 0.001$) by 20% from 440 ± 44 kg to 526 ± 53 kg and BCS increased ($P < 0.001$) from 6 ± 1 to 8 ± 1. Plasma glucose, triglyceride and nonesterified fatty acid concentrations were similar ($P > 0.05$) before and after weight gain, and leptin and insulin concentrations increased ($P < 0.001$) with the induction of obesity. Insulin sensitivity decreased ($P < 0.05$) to 19% of baseline values, accompanied by a 372% increase ($P < 0.05$) in the acute insulin response to glucose, producing a similar ($P > 0.05$) disposition index before and after weight gain. The results of this study demonstrated that diet-induced obesity in horses was associated with decreased insulin sensitivity that was effectively compensated for by an increase in insulin secretory response. Obesity resulted in hyperinsulinemia and hyperleptinemia with respect to baseline values, however no changes in lipid concentrations were apparent. Obesity should be prevented to avoid insulin resistance, hyperinsulinemia, and hyperleptinemia in horses.

Key words: horse, insulin resistance, leptin, lipid, minimal model, obesity

Introduction

Obesity is a growing health concern for equids because of its increasing prevalence and association with altered metabolic function and disease (Frank et al., 2006; Thatcher et al., 2007; Treiber et al., 2006c). Obesity has been associated with insulin resistance in horses and ponies (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007), and insulin resistance in the presence of obesity has been associated with altered reproductive activity in mares and an increased risk of and predisposition for pasture-associated laminitis (Treiber et al., 2007b; Treiber et al., 2006c; Vick et al., 2006).

Compared to lean horses, overweight or obese horses have lower insulin sensitivity and elevated circulating concentrations of insulin, glucose, leptin, and nonesterified fatty acids (NEFA) (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2006). Additionally, across a range of body conditions adiposity measures are associated with insulin sensitivity, circulating concentrations of insulin, leptin, tumor necrosis factor (TNF)α, and blood mRNA expression of TNFα and interleukin (IL)-1 (Buff et al., 2002; Carter et al., 2008; Vick et al., 2007). Previous studies in horses utilized cross-sectional analysis with regard to comparing metabolic variables of horses at different levels of adiposity. However, induction of obesity in a longitudinal study would permit intra-individual comparison of variables before and after weight gain. Such a study design would limit the influence of inter-individual variation and differentiate between metabolic abnormalities inherent to an individual horse from those induced by an increase in adiposity. Additionally, evaluating horses during a mean body condition change from moderate (body condition score [BCS] < 7, scale 1 – 9) to overweight (BCS 7 – 8) to obese (BCS ≥ 8) would be most applicable to determining metabolic abnormalities through developing obesity.

The present study was designed to test the hypothesis that diet-induced obesity in horses lowers insulin sensitivity and raises plasma concentrations of insulin, glucose, NEFA, triglyceride, and leptin. Specifically, the purpose of the study reported here was to identify changes in adiposity, minimal model parameters of glucose and insulin

dynamics, and basal concentrations of insulin, glucose, NEFA, triglyceride, and leptin as adiposity increases and obesity is achieved through overfeeding.

Materials and Methods

Horses

Thirteen Arabian or Arabian cross geldings ranging in age from 8 – 20 years from the Virginia Tech Middleburg Agricultural Research and Extension Center's research herd were evaluated during the study period (June 2006 to January 2007). Initial mean ± SD bodyweight was 448 ± 46 kg and horses were in moderate condition (mean BCS 6 ± 1, scale 1 – 9). Prior to study initiation, all horses were maintained on pasture as a single group for > 6 months. Horses were maintained as a single group on a drylot during the study. The experimental protocol was approved by the Virginia Tech Institutional Animal Care and Use Committee.

In order to exclude the possibility of pituitary pars intermediary dysfunction (PPID), dexamethasone suppression tests were performed according to the protocol of Donaldson et al. (2005). During Week 2, dexamethasone (Dexamethasone sodium phosphate injection, Vedco Inc., St. Joseph, MO) was administered intramuscularly (40 μg/kg body weight) at 1400 h. Blood samples for measurement of plasma cortisol were collected by jugular venipuncture into evacuated sodium heparin collection tubes immediately before and 19 h after dexamethasone administration.

Experimental design

In a longitudinal study lasting 30 weeks, all 13 horses were concurrently exposed to the protocol's dietary treatments and sampling procedures. During Period 1 (Weeks 0 to 3), horses were maintained at a moderate body condition on a forage diet consisting of mixed grass/legume hay (Figure 3.1). During Period 2 (Weeks 4 to 7), horses were maintained at a moderate body condition (mean BCS < 7) on a high concentrate (≥ 50% of diet by weight) and hay diet. During Periods 3 and 4 (Weeks 8 – 24), horses were fed approximately 200% of their digestible energy (DE) requirements for maintenance of a high concentrate diet to induce weight gain. During Period 5 (Weeks 25 – 30), horses

were maintained at an obese state (mean BCS ≥ 8) on a forage diet. The high concentrate diet consisted of approximately 60% of DE requirements from a concentrate feed (Grass Plus Performer, Buckeye Nutrition, Dalton, OH), 20% from chopped alfalfa forage, and 20% from a mixed grass/legume hay (Table 3.1). Horses were group-fed hay in the drylot and individually fed concentrate feed and chopped alfalfa forage in stalls three times daily at 0700, 1400, and 1900 h.

Basal blood samples were collected the first day of each week (Weeks 1 – 30) between 0700 and 0900. Body weight was measured the first day of each week and adiposity measurements were evaluated biweekly. Horses were divided into 3 groups and subjected to the frequently sampled intravenous glucose tolerance test (FSIGTT) procedure on the first, second, or third day of Weeks 3, 7, 16, 24, and 30, corresponding to the last week of each period.

As part of a companion study investigating changes in inflammatory cytokine expression during diet-induced obesity (Chapter 4), tissue biopsies were performed during the fourth or fifth day of Weeks 3, 7, 16, 24, and 30; 2 to 3 days after each horse underwent FSIGTT procedures. Muscle tissue samples were collected percutaneously from the middle gluteal muscle (*m. gluteus medius*) using a Bergstrom needle biopsy technique, and adipose tissue samples were collected from the subcutaneous adipose tissue of the nuchal crest through surgical incision. Horses were maintained in stalls during a 4 or 5 day recovery period after tissue biopsies were performed.

Measurements of adiposity

Girth and waist (abdominal) circumferences, height (at the wither), neck crest height, and neck circumference at 0.25, 0.50, 0.75 of neck length were measured. Girth circumference was measured caudal to the elbow (*olecranon tuber*) and immediately behind the slope of the withers. Waist was taken as the abdominal circumference measured two-thirds the distance from the point of the shoulder to the point of the hip (*tuber coxae*). All neck measurements were taken while the neck was held in a relaxed position, at approximately a 45° angle. Neck length was measured from the poll to the highest point of the withers. Crest height was measured at 0.50 of neck length from the

dorsal midline of the neck to estimated differentiation between the crest and neck musculature, identified by palpation and visual assessment.

Four evaluators independently graded body condition score (BCS) from 1 to 9 (Henneke et al., 1983) and neck crest adiposity as cresty neck score (CNS) from 0 to 5 (Chapter 1). Scores were rated to the nearest whole- or half-score increment and the mean of the four scores for each horse was used for data analysis. Intraclass correlation coefficients for the reliability of individual scores were 0.77 for BCS and 0.54 for CNS. Intraclass correlation coefficients for the reliability of the mean scores were 0.92 for BCS and 0.81 for CNS.

Ultrasound measurements of subcutaneous fat thickness were performed over the 1) rump, 5 cm lateral from the dorsal midline at the center of the pelvic bone (Westervelt et al., 1976); 2) shoulder, ¾ the distance from the dorsal midline to a point 1/3 the distance from the point of the shoulder to the point of the hip; 3) ribs, 2/3 the distance from the point of the shoulder to the point of hip between the 12^{th} and 13^{th} ribs; and 4) back, ¼ the distance from the dorsal midline to the point where rib measurement was taken. Intra- and inter-assay CV for ultrasound measurements were < 6%. Intra-assay CV for morphometric measurements were < 3%.

Basal blood sampling

On the first day of each week prior to daily feeding, blood samples were collected between 0700 and 0900 by jugular venipuncture into 10 mL evacuated tubes containing sodium heparin or potassium EDTA as an anticoagulant.

FSIGTT

On days of FSIGTT procedures, horses were removed from the drylot at 0700, catheters were inserted into a jugular vein, and horses were placed in stalls for the duration of the testing procedure. No feed or hay was offered prior to or during the testing procedure on FSIGTT days, however horses had continual access to water. Thirty minutes after catheter placement, the insulin-modified FSIGTT procedure was initiated as previously described (Hoffman et al., 2003). Briefly, a glucose bolus (50% [wt/vol] dextrose solution, Dextrose 50% solution, Vedco Inc., St. Joseph, MO) of 0.3 g/kg body

weight was administered rapidly (within 2 min) through the catheter, followed after 20 min by rapid administration (within 10 s) of an insulin (Humulin R, Eli Lilly & Co., Indianapolis, IN) bolus of 20 mIU/kg body weight. Blood samples were collected at -20 (between 0830 and 0900), -5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, 180, 210, and 240 min with respect to completion of glucose administration. Samples were immediately placed into 10 mL evacuated tubes containing sodium heparin and kept on ice until centrifugation.

Analysis of samples

Plasma was separated by 10 min of centrifugation (3000 × *g*) within 30 min of sample collection, separated into 1 mL aliquots, and stored at -80 ^{o}C until analysis. Plasma glucose and insulin (in heparin) concentrations were measured in weekly basal samples and in FSIGTT samples. Plasma triglyceride, NEFA, and leptin (in EDTA) concentrations were measured biweekly in basal blood samples. Plasma glucose, triglyceride, and NEFA concentrations were assayed enzymatically by use of commercial kits and an automated analyzer (CX5 Chemistry Analyzer, Beckman Coulter Inc., Fullerton, CA). Plasma insulin (Coat-A-Count Insulin, Diagnostic Products Corp, Los Angeles, CA), leptin (Multi-species Leptin RIA, Linco Research Inc., St. Charles, MO), and cortisol (Coat-A-Count Cortisol, Diagnostic Products Corp, Los Angeles, CA) concentrations were measured by use of commercial radioimmunoassays previously validated for use in equine plasma (Freestone et al., 1991; McManus and Fitzgerald, 2000). All analyses were performed in duplicate. Intra-assay coefficients of variation were 0.43%, 2.7%, 4.0%, 5.4%, 9.6%, and 3.9% for glucose, triglyceride, NEFA, insulin, leptin, and cortisol, respectively. Mean inter-assay coefficients of variation for analyses performed in this lab were 4.6 % for glucose and 6.4 % for insulin. All other analyses were performed in a single assay run for each analysis.

The minimal model of glucose and insulin dynamics was applied to glucose and insulin data for each FSIGTT by use of commercially available software (MinMod Millenium Version 5.15, Raymond Boston, University of Pennsylvania, Kennett Square, PA) and previously described methods (Boston et al., 2003; Hoffman et al., 2003). The

model was used to calculate values for insulin sensitivity (SI), glucose effectiveness (Sg), acute insulin response to glucose (AIRg), and disposition index (DI).

Statistical analyses

The Shapiro-Wilk test was used to test for normality of variables within each week or period. Grubbs test ($\alpha = 0.01$) was used to determine outliers within each week or period, which were subsequently removed from analyses. Five insulin, 1 glucose, 2 triglyceride, 1 NEFA, 2 SI, and 1 DI values were removed prior to analyses. A two-sample paired *t* test was used to compare cortisol measurements before and after dexamethasone administration. The effect of week on adiposity and basal blood variables was assessed by repeated measures ANOVA using the Huynh-Feldt ε correction factor to adjust for sphericity, with Fisher-Hayter pairwise comparisons made between weeks. Values during Weeks 1 to 3 (Period 1) were averaged, then compared to subsequent weeks by Fisher-Hayter pairwise comparisons. For minimal model parameters, the effect of period was assessed by repeated measures ANOVA using the Huynh-Feldt ε correction factor to adjust for sphericity, with Fisher-Hayter pairwise comparisons made between periods. Values of $P < 0.05$ were considered significant. Data are presented as mean ± SEM unless stated otherwise. Statistical analyses were conducted by use of a computer software program (Intercooled Stata Version 9.2, Stata Corp, College Station, TX).

Results

Horses

No horses were excluded based on the results of dexamethasone suppression testing to assess the possibility of PPID, as plasma cortisol decreased ($P < 0.001$) from resting concentrations of 4.1 ± 0.3 μg/dL to < 0.5 μg/dL in all horses 19 h after dexamethasone administration.

Three horses displayed mild signs of laminitis during the study, including bilateral forelimb lameness (Obel grade 1 – 2), prominent digital pulses, and increased hoof temperature. Phenylbutazone (2 g per day) was administered to these horses as needed for

pain relief. Clinical signs of laminitis began during Weeks 18, 20, and 22, and were resolved by Week 27. According to the Grubb's test for outliers within each week or period, these horses did not exhibit outlying values for any variables during the study, except a high ($P < 0.01$) value for triglyceride concentration during Week 14 and a high value for NEFA concentration during Week 5 for two separate horses, which were subsequently removed from data analysis.

Adiposity

All measurements of adiposity differed ($P < 0.001$) by week, increasing above ($P < 0.05$) Period 1 and 2 values (pre-weight gain) by the end of Period 3 (Table 3.2; Figure 3.2). The only exception was subcutaneous fat thickness over the rib, which did not differ by week ($P = 0.11$). Adiposity measurements were similar ($P > 0.05$) within the pre-weight gain period (Weeks 1 – 7) and within the post-weight gain period (Weeks 24 – 30). Therefore, measurements were pooled within pre- or post- weight gain periods to calculate the overall change in adiposity measurements (Table 3.2). Overall there was a 20% increase in body weight, 160% increase in rump subcutaneous fat thickness, almost 2 unit increase in BCS, and 1 unit increase in CNS.

Basal blood variables

Insulin concentration differed by week ($P < 0.001$), increasing above ($P < 0.05$) Period 1 concentrations during Period 4 and in Week 30 (Figure 3.3). Mean weekly concentrations of insulin ranged from 3.5 ± 0.3 (Week 2) to 61.7 ± 15.7 (Week 23) mU/L across the study. Glucose concentration differed by week ($P < 0.001$), however Period 1 concentrations were not different than subsequent weeks and no consistent changes were apparent between weeks (Figure 3.3). Mean weekly concentrations of glucose ranged from 88.1 ± 0.9 (Week 6) to 96.2 ± 1.0 (Week 30) mg/dL across the study. Leptin concentration differed by week ($P < 0.001$), increasing ($P < 0.01$) consistently from Period 1 concentrations during Weeks 10 to 24 (Figure 3.3). Additionally, leptin concentrations during Weeks 26 and 28 were lower ($P < 0.05$) than Week 24.

Triglyceride concentration differed by week ($P < 0.001$), with 5 of the 8 measured values during Periods 3 and 4 being lower ($P < 0.05$) than Period 1 concentrations

(Figure 3.4). Mean concentrations of triglyceride ranged from 19.4 ± 1.6 (Week 26) to 39.2 ± 3.5 (Week 28) mg/dL across the study. Concentrations of NEFA differed by week ($P < 0.001$; Figure 3.4). Concentrations were lower ($P < 0.01$) than Period 1 values during Periods 2, 3, and 4. Mean concentrations of NEFA ranged from 0.24 ± 0.02 (Week 28) to 0.34 ± 0.03 (Week 30) mEq/L during Periods 1 and 5, and from 0.05 ± 0.01 (Week 14) to 0.19 ± 0.02 (Week 24) mEq/L during Periods 2, 3 and 4.

Glucose and insulin dynamics

Mean SI was lower ($P < 0.05$) in Periods 4 and 5 compared to Periods 1 and 2, and increased from Period 1 to 2 (Figure 3.5). Mean AIRg increased across periods, with AIRg values for Periods 3, 4 and 5 higher ($P < 0.05$) than for Periods 1 and 2. Mean DI was higher ($P > 0.05$) during Periods 2 and 3 compared to Periods 1, 4, and 5. Mean Sg was higher in Period 2 than Period 1, but neither value differed from Periods 3, 4, or 5. Due to laboratory error, saline was injected in place of the insulin dose for all horses during Periods 2 and 3.

Discussion

In the present study, 16 weeks of overfeeding that resulted in 88 ± 11 kg of BW gain (20% increase) and obesity (BCS ≥ 7.5) was associated with decreased SI that was effectively compensated for by an increase in AIRg. Circulating insulin and leptin concentrations increased, whereas NEFA, triglyceride and glucose concentrations did not differ with the induction of obesity.

Insulin-mediated glucose disposal (SI) was 81% lower than Period 1 values after the induction of obesity. The decrease in SI was accompanied by a 372% increase in AIRg, representing the endogenous insulin secretion in response to the glucose dose. This resulted in a similar DI in Periods 1 and 5, indicating that as the degree of insulin resistance increased, there was a greater response of insulin secretion to compensate for the lack of insulin sensitivity. Additionally, Sg, or glucose-mediated glucose disposal, was not affected by weight gain. Collectively, these results indicate that changes in

glucose dynamics occurred through insulin-dependent rather than insulin-independent pathways.

As a result of laboratory error, insulin doses were not administered during Period 2 and 3 FSIGTT. As AIRg is calculated as the integral of insulin concentration above baseline from 0 to 10 min, values were not affected by inclusion or exclusion of the insulin dose at 20 min. In humans, when the regular and insulin-modified FSIGTT with minimal model analysis were compared, both tests produced similar SI values, but Sg was 28% lower during regular compared to insulin-modified tests (Pacini et al., 1998). However, in the present study values for SI, Sg, and DI were either higher than other periods or higher than expected for Periods 2 and 3, and therefore cannot be confidently compared to other time periods. All minimal model parameters increased between Periods 1 and 2 as horses adapted to a relatively high NSC diet. Although results are confounded by differences in FSIGTT procedures between periods, it is possible that glucose and insulin dynamics may have been affected by dietary adaptation.

As no comparable control group was assessed in the present study, it cannot be excluded that changes in variables were a result of factors other than an increase in adiposity. In previous studies, adaptation to a high nonstructural carbohydrate (NSC) diet decreased SI when compared to a high fiber and fat diet (Hoffman et al., 2003; Treiber et al., 2005a). In both Thoroughbred weanlings and mature geldings, horses adapted to a diet high in NSC had 36% or 38% lower SI, respectively, compared to horses adapted to a diet high in fat and fiber. Although no other minimal model parameters differed between groups in the weanlings, AIRg and DI were lower in high NSC-fed mature geldings. However, adaptation to similar diets in exercise-trained Arabian geldings (many of which were used in the present study) demonstrated no differences in minimal model parameters between high NSC or high fat and fiber fed horses at rest (Treiber et al., 2006a). Collectively, these results indicate modest, if any, decreases in SI with high NSC feeding compared to the 81% decrease in SI observed in the present study. Additionally, previous studies demonstrated a decrease or no change in AIRg with high NSC adaptation, whereas there was a 372% increase in AIRg in the present study. Therefore it is unlikely that changes in glucose and insulin dynamics in the present study were primarily a result of dietary adaptation.

Additionally, seasonal changes in insulin sensitivity may occur through changes in secretion of hormones such as ACTH. Although ACTH was not measured in the present study, a previous study demonstrated that in ponies and horses ACTH concentration was higher in September than January and May (Donaldson et al., 2005). An increase in ACTH stimulation may increase the effects of cortisol to decrease insulin sensitivity.

Nonetheless, the results from the present study are comparable to results from studies of diet-induced obesity in other species. Induction of weight gain by overfeeding has been demonstrated to decrease insulin sensitivity in humans, mice, dogs, and rabbits (Erdmann et al., 2008; Gayet et al., 2004; Gayet et al., 2007; Surwit et al., 1988; Zhao et al., 2008). Various mechanisms by which obesity may contribute to insulin resistance include an increase in inflammatory cytokines, accumulation of intramyocellular triglycerides, or increase in circulating NEFA. Adipose tissue in obesity has been shown to release increased levels of inflammatory cytokines, including TNFα and IL-6, which may then directly interfere with insulin signaling (Chen, 2006). Additionally, increases in fatty acids in circulation or within myocytes may directly interfere with insulin signaling pathways or indirectly affect insulin sensitivity through stimulation of inflammatory pathways (Chen, 2006; Delarue and Magnan, 2007).

Minimal model parameters during Periods 1 and 5 were compared to 95% reference intervals previously determined on 46 healthy horses, including Thoroughbred mares, geldings, weanlings, and the Arabian geldings used in the current study at a younger age (Treiber et al., 2005b). Before weight gain (Period 1), all values for minimal model parameters were within their respective reference intervals, except for one SI value which was above the reference range. After weight gain (Period 5), 4 SI values were below and 7 AIRg values were above their respective reference intervals, however these values were similar to values previously determined in insulin resistant ponies (Bailey et al., 2007; Treiber et al., 2007b).

As insulin clearance was not measured in this study, it cannot be excluded that changes in AIRg were partially attributable to decreased insulin clearance by the liver rather than an increase in insulin secretion from the pancreas. In a study of diet-induced weight gain in humans, changes in insulin concentrations after oral or intravenous glucose doses were fully attributable to decreased insulin clearance after weight gain

(Erdmann et al., 2008). Similarly, increases in basal insulin concentrations after weight gain were attributable to changes in both insulin secretion and clearance, with a greater contribution of reduced insulin clearance relative to hypersecretion (Erdmann et al., 2008). A decreased rate of insulin clearance would increase AIRg values, as circulating insulin concentrations would be increased during AIRg measurement.

Although insulin concentration increased with adiposity, it also decreased during Weeks 26 to 29, resulting in concentrations similar ($P > 0.05$) to those observed before weight gain. These results suggest an additive effect of obesity and the overfeeding of a high concentrate diet, as insulin concentration was highest when both were present (Period 4) but not different from baseline levels (Period 1) when horses were obese on hay (Period 5) or nonobese and overfed a high concentrate diet (Period 3). Previous studies have demonstrated decreased SI in healthy horses when adapted to a high nonstructural carbohydrate diet (Hoffman et al., 2003; Treiber et al., 2005a). Although hyperinsulinemia was not observed with dietary differences in these studies, hyperinsulinemia has been observed in insulin resistant ponies on a high compared to low nonstructural carbohydrate diet (Bailey et al., 2007). Collectively, these studies indicate that basal hyperinsulinemia results from an interaction of degree of insulin resistance and level of nonstructural carbohydrates in the diet.

In the presence of increased insulin concentrations, euglycemia was maintained throughout the study. In addition to similar DI values before (Period 1) and after (Period 5) weight gain, maintenance of euglycemia provides further support that the decrease in insulin sensitivity was effectively compensated for by an increase in insulin secretion. While in the present study relatively short-term obesity resulted in compensated insulin resistance, chronic obesity may eventually lead to uncompensated insulin resistance through β-cell exhaustion (Kasuga, 2006). The inability of the pancreas to secrete sufficient insulin to maintain euglycemia, despite persistently elevated circulating insulin levels, has been associated with clinical laminitis in ponies (Treiber et al., 2007a).

Obesity in the present study was induced by overfeeding a diet high in nonstructural carbohydrates over a relatively short period of time (16 weeks). In this model of obesity, although NEFA and triglyceride concentrations did not differ before and after weight gain, NEFA concentrations were lower during periods of high

concentrate feeding, and triglyceride concentrations were lower during 5 of the 8 sampling weeks during the high concentrate feeding. Both NEFA and triglyceride concentrations were similar ($P > 0.05$) when horses were fed a forage diet while in a moderate body condition (Weeks 1 and 3) or while obese (Weeks 28 and 30). Therefore, the effect of feed composition on lipid metabolism was greater than any effect of increased adiposity. Decreases in circulating lipids may result from decreased release into circulation and/or increased uptake and utilization by tissue. The high concentrate diet had higher starch levels and higher fat levels than the forage diet (Table 3.1). Although glycemic responses of the diets were not determined in this study, it is expected that consumption of the high concentrate diet resulted in a greater glycemic response than the forage diet, and therefore more fluctuation in glucose and insulin concentrations throughout the day. Insulin and glucose stimulate lipogenesis by increasing acetyl-CoA carboxylase expression, and insulin decreases lipolysis through inhibition of hormone-sensitive lipase (Bhathena, 2006). Alternatively, high-fat feeding to ponies reduced plasma triglyceride concentrations through increased fatty acid transport and oxidation in muscle and decreased fatty acid synthesis in the liver (Geelen et al., 2001).

Increases in circulating NEFA have been proposed as one possible mechanism of obesity-associated insulin resistance (Delarue and Magnan, 2007). When nonobese horses were compared to obese horses with insulin resistance, the obese horses had higher NEFA but similar triglyceride concentrations (Frank et al., 2006). In the present study an increase in NEFA concentration was not observed with weight gain or with decreases in insulin sensitivity, however it is still possible NEFA may affect the liver or peripheral tissue through increases in production and clearance that are not reflected in baseline concentrations. As the duration of the present study was relatively short, it cannot be excluded that NEFA concentrations would eventually increase in chronically obese horses or in cases where an increase in insulin secretion does not fully compensate for decreased insulin sensitivity. In humans, NEFA concentrations increase with insulin resistance because of the reduced ability of insulin to exert inhibitory effects on hormone-sensitive lipase (Delarue and Magnan, 2007).

Leptin concentrations after weight gain were comparable to previously reported concentrations in obese, insulin-resistant horses (Frank et al., 2006). Leptin is primarily

produced and secreted from adipose tissue at levels that are proportional to fat mass (Buff et al., 2002; Kearns et al., 2006), and its secretion is stimulated by insulin and inversely related to insulin sensitivity in obese horses (Cartmill et al., 2005; Cartmill et al., 2003; Frank et al., 2006). Leptin also signals satiety and energy status, and while overfeeding above maintenance requirements increases leptin concentrations, underfeeding below maintenance decreases leptin concentrations in horses (Weyenberg et al., 2007). In the present study, leptin concentration increased through Week 24, corresponding to increases in feed intake, adiposity, and insulin concentration. Additionally, leptin concentration decreased from Week 24 to 26, corresponding to decreases in energy intake and insulin concentration.

Three horses displayed mild signs of laminitis during the present study, however these cases were not confirmed radiographically and laminitis status was not evaluated prior to study initiation. Laminitic episodes were observed during weeks of the highest basal insulin concentrations, and dissipated in association with a significant decrease in insulin concentrations between Weeks 24 to 27. Low insulin sensitivity and/or high insulin concentrations have been associated with laminitic predisposition in ponies (Bailey et al., 2008; Bailey et al., 2007; Coffman and Colles, 1983; Jeffcott et al., 1986; Treiber et al., 2007b; Treiber et al., 2006c), and experimental induction of prolonged hyperinsulinemia (> 1000 mU/L) while maintaining euglycemia induced laminitis in healthy ponies (Asplin et al., 2007b). Although the three horses that developed laminitis did not display any outlying values for insulin concentration or minimal model parameters, individual susceptibility may be greater in these horses due to factors not measured, such as inflammatory mediators. Additionally, as speculated with experimental models of laminitis induced by carbohydrate overload, gastrointestinal disturbances such as changes in hindgut bacteria, the release of endotoxins, or increased intestinal mucosal permeability, may have been initiating factors of the laminitic episodes (Bailey et al., 2004).

By calculating the slope of the linear regression of mean body weight against BCS during Weeks 7 to 24, it was estimated that 46 kg (95% confidence interval 38 – 55 kg) were gained per one unit increase in BCS. With an estimated 15.0 Mcal/d intake above maintenance during Periods 3 and 4, it was calculated that 28 Mcal were needed

for each kg increase in body weight. These estimates are greater than previous reports of 16 – 20 kg body weight gain per one unit increase in BCS (from BCS 4 – 6) and 16 – 24 Mcal/kg gain (NRC, 2007). It is possible that at the higher levels of BCS, more gain is necessary to achieve an observable change in body condition.

Unexpectedly, subcutaneous fat thickness over the ribs did not change as other adiposity measurements increased. Previous reports of rib fat thickness used measurements near the 13th rib, 5 cm lateral from the spinous processes (Gentry et al., 2004; Westervelt et al., 1976). This represents an area of subcutaneous fat covering longissimus muscle groups. In the present study, subcutaneous fat thickness was measured two-thirds the distance from the point of the shoulder to point of the hip (*tuber coxae*), representing an area covering intercostal muscle groups. The latter location more accurately represents the area considered during body condition scoring and where the visual appearance of ribs is often observed in lean horses. Although qualitative assessment of this area may be related to overall body condition, quantitative assessment of subcutaneous fat thickness over the ribs was not useful to track increased adiposity.

In the present study, diet-induced obesity decreased insulin sensitivity while enhancing the acute insulin response to glucose and not altering glucose effectiveness in horses. Basal concentrations of insulin and leptin increased with adiposity, however triglyceride, NEFA and glucose were not affected by increased adiposity. Results suggest that avoiding obesity is important to minimize insulin resistance, hyperinsulinemia, and hyperleptinemia in horses. This avoidance may then reduce risk for laminitis and help maintain a healthy metabolic state.

Table 3.1: Estimated dietary intake per horse on a daily basis and calculated dietary composition during each period.

		Period				
		1	**2**	**3**	**4**	**5**
Feed intake, kg/d	Mixed grass/legume hay	8	1.6	2.7	3.2	12
	Chopped alfalfa forage	0	1.5	2.5	2.5	0
	Concentrate (sweet feed)	0	3.0	6.0	6.5	0
Nutrient intake, % of dry matter intake	Crude protein	12.3	16.4	16.3	16.2	16.0
	Acid detergent fiber	38.0	20.5	19.1	19.4	31.5
	Neutral detergent fiber	60.1	30.9	29.5	29.9	51.0
	Water-soluble carbohydrates	5.8	7.6	7.4	7.4	8.9
	Starch	1.8	25.2	27.3	27.1	2.0
	Ether extract	2.7	5.4	5.6	5.5	2.1
Digestible Energy (DE) intake	Mcal/d	15.5	15.8	29.5	32.1	23.6
	Percent of requirements*	104	110	197	193	133

*Percent of daily DE requirements that were fulfilled by estimated DE intake. Required DE = 33.3 kcal/kg BW (NRC, 2007), where BW is the mean group bodyweight for the first week of each period.

Table 3.2: Adiposity measurements during the final week of each period and overall change from before to after weight gain. Values are presented as mean ± SD.

Variable	Week 3 (Period 1)	Week 7 (Period 2)	Week 16 (Period 3)	Week 24 (Period 4)	Week 30 (Period 5)	Change*
Body weight, kg	440 ± 44[a]	435 ± 42[a]	494 ± 45[b]	528 ± 48[c]	526 ± 53[c]	88 ± 11
Body condition score	6.2 ± 1.0[a]	6.0 ± 1.0[a]	7.3 ± 0.9[b]	8.0 ± 0.7[c]	7.8 ± 0.8[c]	1.7 ± 0.5
Girth, cm	179 ± 7[a]	179 ± 7[a]	189 ± 7[b]	197 ± 7[c]	198 ± 10[c]	17 ± 3
Waist, cm	196 ± 8[a]	196 ± 8[a]	208 ± 7[b]	217 ± 8[c]	216 ± 9[c]	20 ± 4
Cresty neck score	2.2 ± 0.5[a]	2.1 ± 0.6[a]	2.9 ± 0.6[b]	3.2 ± 0.4[c]	3.1 ± 0.5[bc]	0.9 ± 0.4
Mean neck circumference, cm	91 ± 3[a]	91 ± 4[a]	96 ± 4[b]	97 ± 4[c]	98 ± 3[c]	6.2 ± 1.8
Crest Height, cm	9.8 ± 1.4[a]	9.7 ± 1.8[a]	12.7 ± 1.6[b]	14.0 ± 1.7[c]	14.7 ± 2.1[c]	4.2 ± 0.8
Rump, cm	1.74 ± 0.72[a]	1.66 ± 0.67[a]	2.70 ± 0.91[b]	3.41 ± 0.95[c]	3.24 ± 0.94[c]	1.59 ± 0.50
Back, cm	0.66 ± 0.22[a]	0.60 ± 0.22[a]	0.83 ± 0.29[b]	1.01 ± 0.39[c]	1.00 ± 0.33[c]	0.27 ± 0.08
Rib, cm	0.81 ± 0.15	0.78 ± 0.12	0.84 ± 0.11	0.81 ± 0.13	0.88 ± 0.20	0.02 ± 0.10
Shoulder, cm	0.78 ± 0.07[a]	0.78 ± 0.08[a]	0.95 ± 0.09[b]	0.98 ± 0.10[b]	0.97 ± 0.10[b]	0.19 ± 0.06
Body fat, %[†]	15.9 ± 4.5[a]	15.4 ± 4.2[a]	21.9 ± 5.6[b]	26.2 ± 5.9[c]	25.2 ± 5.8[c]	9.9 ± 3.1

*Difference between the mean of Weeks 1 – 7 (before weight gain) and Weeks 24 – 30 (after weight gain)
[†]Calculated as % fat = 6.22 + 5.07x, where x = subcutaneous rump fat thickness in cm (Westervelt 1976)
[a,b,c]Means in the same row with different superscripts differ ($P < 0.05$) according to Fisher-Hayter pairwise comparisons.

Week	0 to 3	4 to 7	8 to 16	17 to 24	25 to 30
Period	1	2	3	4	5
Energy balance	Maintenance		Weight gain		Maintenance
Dietary composition	100% hay	60% concentrate, 20% chopped alfalfa, 20% hay			100% hay

Figure 3.1: Experimental design. Basal blood sampling occurred weekly, adiposity measurements biweekly, and frequently sampled intravenous glucose tolerance tests during the final week of each period.

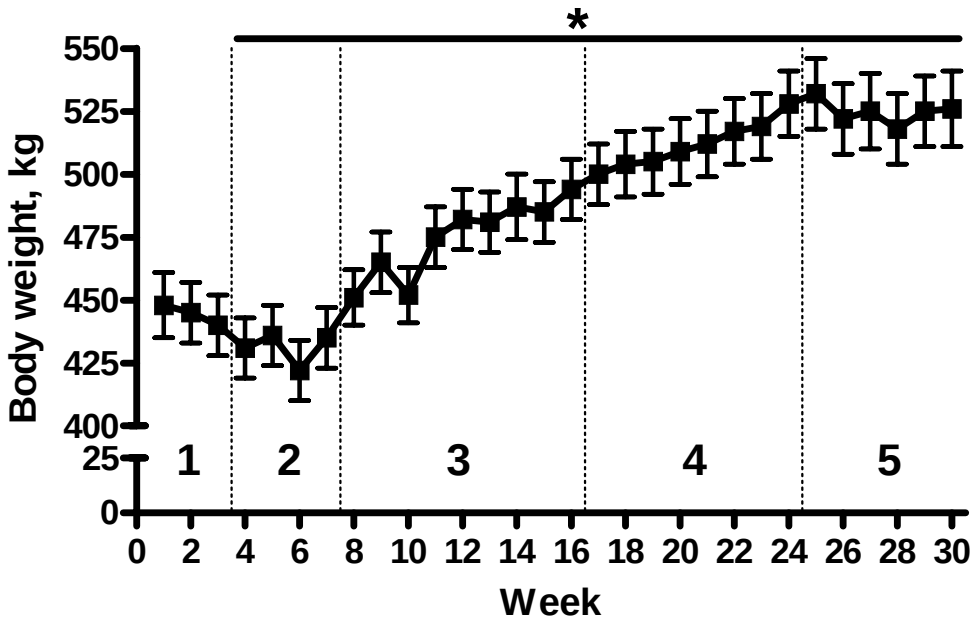

Figure 3.2: Body weight during Weeks 1 – 30. Body weight differed by week according to repeated measures ANOVA ($P < 0.001$). *Indicates a difference ($P < 0.05$) between the mean of Weeks 1-3 and each subsequent week using Fisher-Hayter pairwise comparisons. Dashed lines represent the different periods (1-5) of the study. Values are graphed as mean ± SEM.

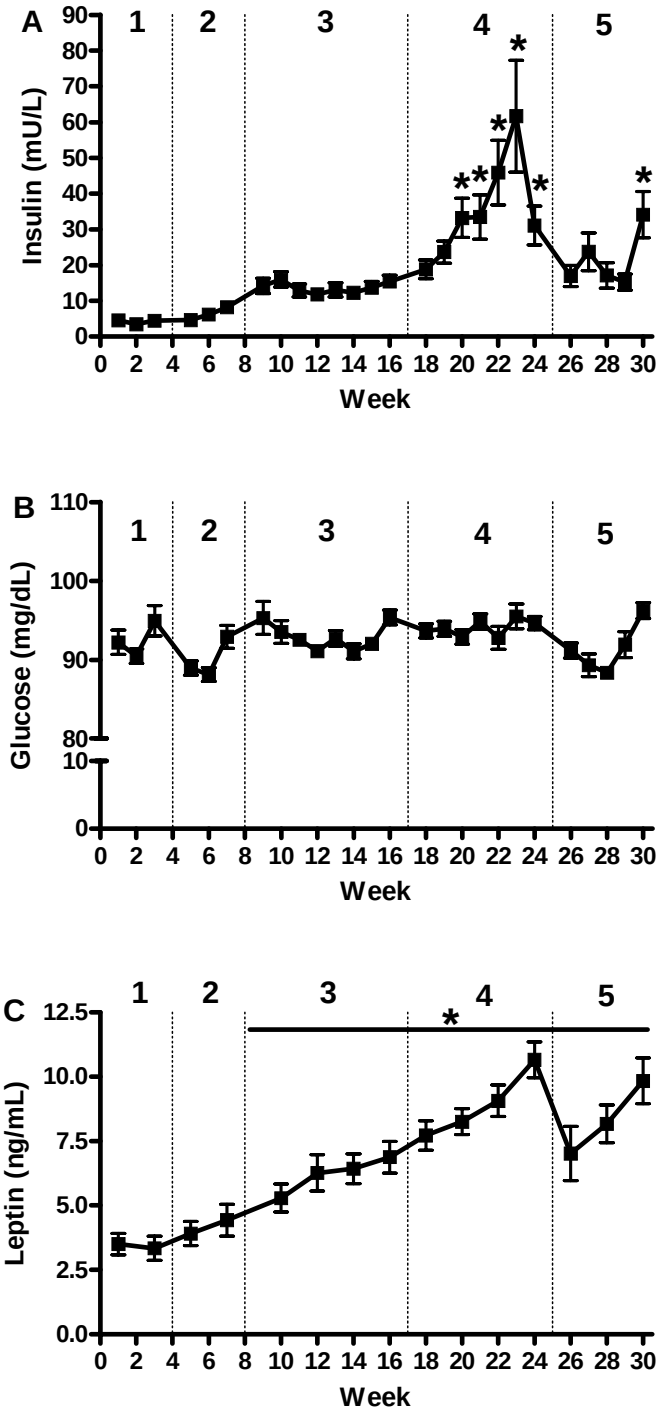

Figure 3.3: Basal concentrations of plasma insulin (A), glucose (B), and leptin (C) for Weeks 1 – 30. All variables differed by week according to repeated measures ANOVA ($P < 0.001$). *Indicates a difference ($P < 0.05$) between the mean of Weeks 1-3 and each subsequent week using Fisher-Hayter pairwise comparisons. Dashed lines represent the different periods (1-5) of the study. Values are graphed as mean ± SEM.

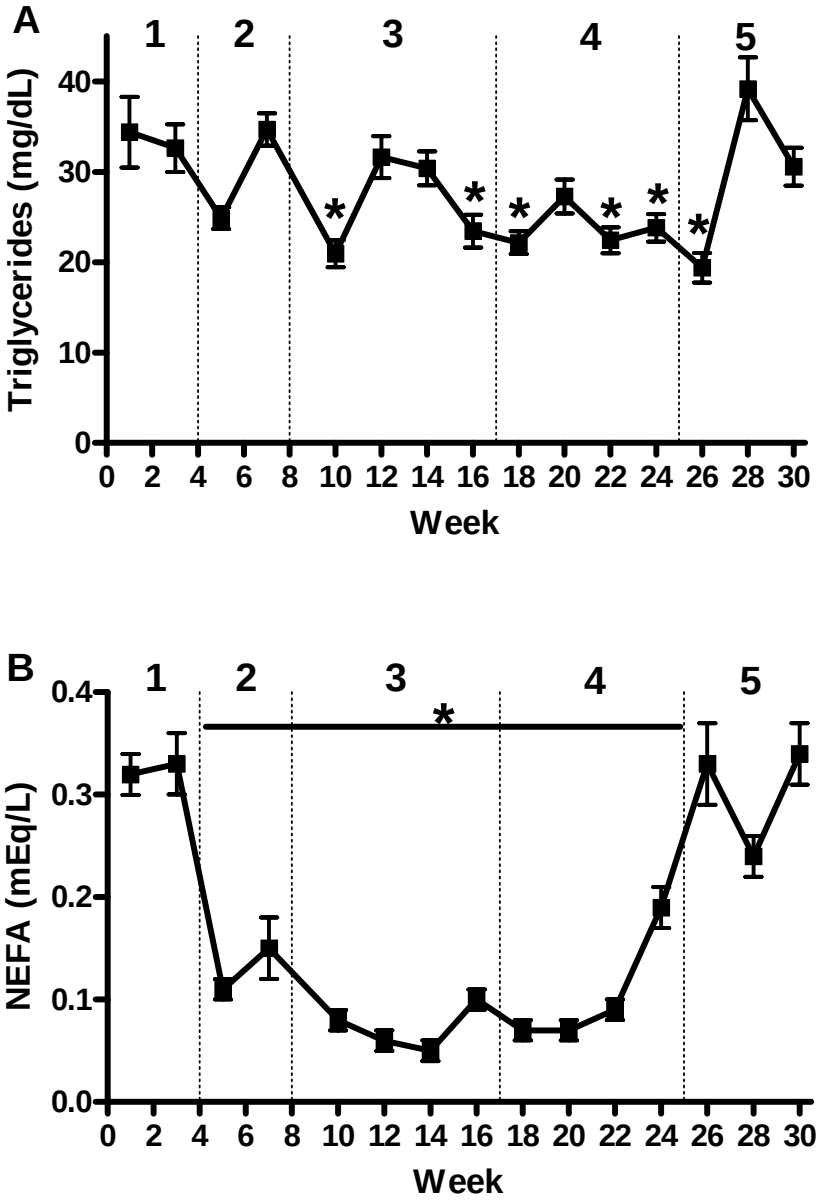

Figure 3.4: Basal concentrations of plasma triglycerides (A) and NEFA (B) for Weeks 1 – 30. All variables differed by week according to repeated measures ANOVA ($P < 0.001$). *Indicates a difference ($P < 0.05$) between the mean of Weeks 1-3 and each subsequent week using Fisher-Hayter pairwise comparisons. Dashed lines represent the different periods (1-5) of the study. Values are graphed as mean ± SEM.

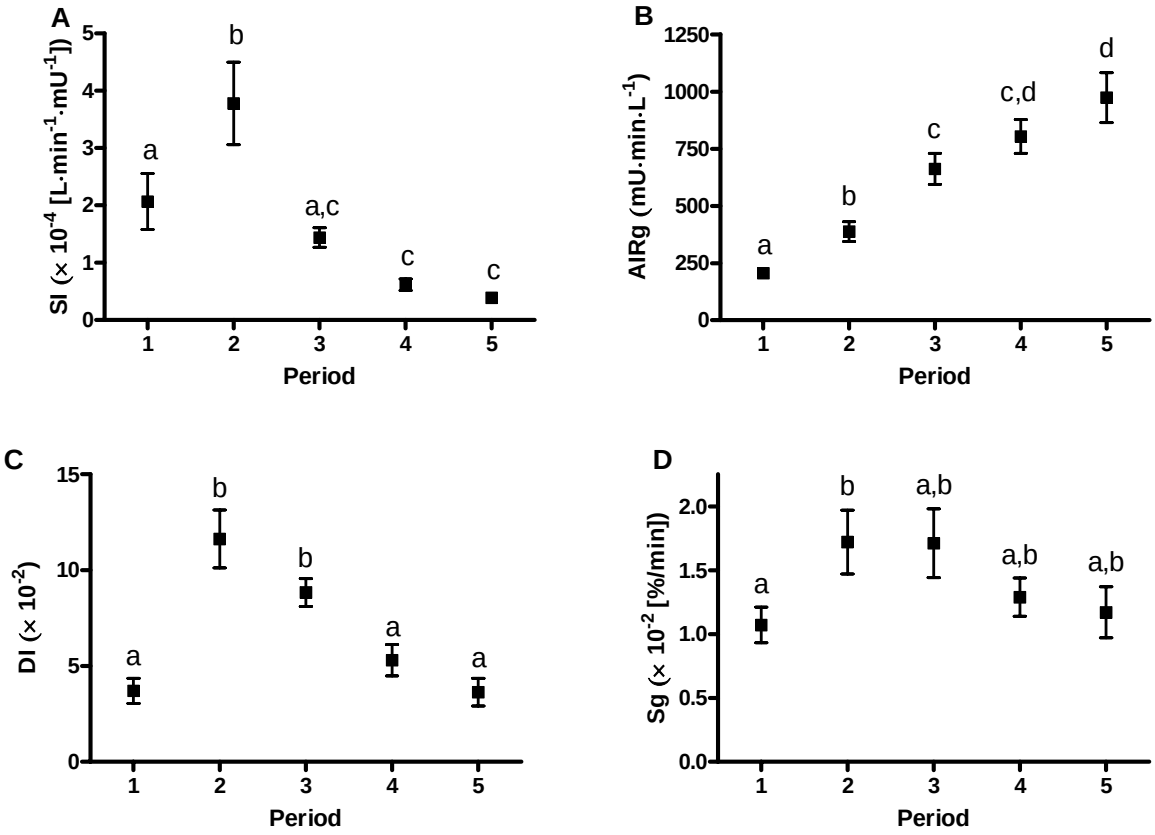

Figure 3.5: Minimal model parameters of SI (A), AIRg (B), DI (C), and Sg (D) calculated from glucose and insulin curves of FSIGT tests performed during the final week of each period. Parameters differed by week ($P < 0.05$) according to repeated measures ANOVA. Points with different letters indicate different ($P < 0.05$) means as identified by Fisher-Hayter pairwise comparisons. Values are graphed as mean ± SEM.

CHAPTER 4

Inflammatory response in subcutaneous adipose tissue during obesity-induced insulin resistance in horses

ABSTRACT: Increased production and secretion of inflammatory factors from adipose tissue is implicated as one of the causal mechanisms linking obesity and insulin resistance, however increased inflammation during the induction of obesity has not been demonstrated in horses. The objective of this study was to identify changes in subcutaneous adipose tissue mRNA expression of inflammatory mediators and plasma tumor necrosis factor (TNF)-α protein concentrations in horses documented to have an increase in adiposity and concurrent decrease in insulin sensitivity. Thirteen Arabian geldings underwent a 20% increase in body weight and a decrease in insulin sensitivity to 19% of baseline values after being fed twice their daily digestible energy requirements for 4 months. Subcutaneous adipose tissue biopsies from the nuchal crest were performed before, during, and after the induction of obesity. Total mRNA was isolated from adipose tissue followed by real time quantitative polymerase chain reaction for amplification of inflammatory genes, including proinflammatory cytokines (interleukin [IL]-6, IL-1β, TNFα), anti-inflammatory cytokines (IL-10, IL-6), chemokines (monocyte chemoattractant protein [MCP]-1, MCP-2, IL-8, plasminogen activator inhibitor [PAI]-1) and adipokines (leptin, adiponectin). Gene expression of TNFα, IL-1β, IL-6, IL-10 and adiponectin were not different before and after weight gain, whereas gene expression of MCP-1, MCP-2, IL-8, and PAI-1 increased with obesity. Additionally, plasma protein concentrations of TNFα decreased with obesity. Therefore, obesity-induced insulin resistance in horses was associated with no change in inflammatory cytokine gene expression but increased chemokine gene expression in subcutaneous adipose tissue. Results suggest that mechanisms other than a neck subcutaneous adipose tissue inflammatory response are the initiating causes of obesity-induced insulin resistance.

Key words: adipose, horse, inflammation, insulin resistance, obesity

Introduction

Obesity is of increasing concern in equid populations, as its presence is associated with altered metabolic function and risk for disease. Obesity has been associated with insulin resistance in horses and ponies (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007), and both obesity and insulin resistance have been associated with impaired reproductive function in mares and an increased risk of and a predisposition for development of pasture-associated laminitis (Bailey et al., 2008; Treiber et al., 2007b; Treiber et al., 2006b; Treiber et al., 2006c; Vick et al., 2006).

Multiple mechanisms have been suggested to connect obesity with insulin resistance, including an inflammatory response in the enlarged adipose tissue depots. Although it has been demonstrated that induction of a systemic inflammatory response via lipopolysaccharide infusion decreases insulin sensitivity in horses (Vick et al., 2008), it is unknown whether a similar inflammatory response occurs during obesity to induce insulin resistance. Across a range of body conditions, measurements of adiposity are related to insulin sensitivity and inflammatory cytokine expression, including blood mRNA expression and protein concentration of tumor necrosis factor (TNF)-α (Vick et al., 2007). Additionally, it has been demonstrated that cultured equine adipocytes and preadipocytes have the capacity to produce inflammatory cytokines, including TNFα, interleukin (IL)-6, and IL-1 (Vick et al., 2008).

In other species, including humans, studies show that inflammatory cytokines play a direct role in the development of obesity-associated insulin resistance. Obesity has been associated with activation of nuclear factor (NF)-κB pathways in adipocytes and increases in their target gene products, including proinflammatory cytokines (IL-6, IL-1β, TNFα), anti-inflammatory cytokines (IL-10, IL-6), and chemokines that recruit immune cells to site of inflammation (monocyte chemoattractant protein [MCP]-1, MCP-2, IL-8, plasminogen activator inhibitor [PAI]-1) (Shoelson et al., 2007). Macrophage infiltration of adipose tissue amplifies the production of proinflammatory cytokines, including IL-1β, IL-6, and TNFα (Fain, 2006). Secreted cytokines demonstrate autocrine or paracrine signaling within adipose tissue, or may be released into circulation to mediate endocrine signaling to other insulin sensitive tissues, such as skeletal muscle or the liver. Cytokines

contribute to insulin resistance through activation of several serine-threonine kinase pathways, such as inhibitor of NF-κB kinase β (IKKβ) and c-Jun NH_2-terminal kinase (JNK)1, which interfere with insulin signaling through serine phosphorylation and subsequent inactivation of insulin receptor substrate (IRS)-1 (Chen, 2006). Additionally, adipokines released exclusively by adipocytes, such as leptin and adiponectin, have also been shown to influence insulin sensitivity (Antuna-Puente et al., 2008).

While previous reports in horses have demonstrated associations between obesity, insulin resistance and inflammation, these studies were cross sectional with regard to comparing different levels of adiposity. A longitudinal study with the induction of obesity would permit intra-individual comparison of variables before and after weight gain and limit the influence of inter-individual variation. Such a study design would differentiate between metabolic abnormalities inherent to the horse from those induced by an increase in adiposity.

In a companion study of diet-induced obesity, thirteen Arabian geldings were documented to undergo a 20% increase in bodyweight and a decrease in insulin sensitivity to 19% of baseline values after being fed twice their daily digestible energy requirements for 4 months (Chapter 3). Utilizing this model of equine obesity, the present study was designed to test the hypothesis that obesity-induced insulin resistance in horses is associated with an inflammatory response in adipose tissue. Specifically, the objective of the study reported here was to identify changes in subcutaneous adipose tissue mRNA expression of inflammatory mediators, including proinflammatory cytokines (IL-6, IL-1β, TNFα), anti-inflammatory cytokines (IL-10, IL-6), chemokines (MCP-1, MCP-2, IL-8, PAI-1) and adipokines (leptin, adiponectin), and plasma TNFα concentrations in horses documented to have an increase in adiposity and concurrent decrease in insulin sensitivity.

Materials and methods

Horses

Thirteen Arabian or Arabian cross geldings (age 8 – 20) from the Virginia Tech Middleburg Agricultural Research and Extension Center's research herd were evaluated

during the study period (June 2006 to January 2007). Initial mean ± SD bodyweight was 448 ± 46 kg and horses were in moderate condition (body condition score 6.4 ± 0.8, scale 1 – 9). Horses were maintained as a single group on a drylot during the study. Prior to study initiation, all horses were maintained on pasture as a single group for > 6 months. The experimental protocol was approved by the Virginia Tech Institutional Animal Care and Use Committee.

Experimental design

In a longitudinal study lasting 30 weeks, all 13 horses were concurrently exposed to dietary treatments and sampling procedures. During Period 1 (Weeks 0 – 3), horses were maintained at a moderate body condition on a mixed grass/legume hay diet. During Period 2 (Weeks 4 – 7), horses were maintained at a moderate body condition on a high concentrate (≥ 50% of diet by weight) and forage diet. During Periods 3 and 4 (Weeks 8 – 24), horses were fed approximately 200% of their digestible energy requirements of a high concentrate diet to induce weight gain. During Period 5 (Weeks 25 – 30), horses were maintained at an obese state on mixed grass/legume hay. The high concentrate diet consisted of approximately 60% of digestible energy requirements from a concentrate feed (commercial sweet feed), 20% from chopped alfalfa forage, and 20% from a mixed grass/legume hay. Horses were group-fed hay in the drylot and individually fed concentrate feed and chopped alfalfa forage in stalls three times per day at 0700, 1400, and 1900 h.

As part of a companion study investigating the changes in glucose and insulin dynamics and plasma hormone and lipid concentrations during diet-induced obesity, blood samples were collected weekly and horses were subjected to a frequently sampled intravenous glucose tolerance test (FSIGTT) procedure on the first, second, or third day of Weeks 3, 7, 16, 24, and 30 (Chapter 3).

Blood sampling and analysis

Blood samples were collected between 0700 and 0900 prior to daily feeding on the first, second, or third day of the last week of each period, corresponding to Weeks 3, 7, 16, 24, and 30 of the study. Samples were collected by jugular venipuncture into 10

mL evacuated tubes containing sodium heparin as an anticoagulant. Plasma was separated by centrifugation (3000 × *g*) within 30 min of sample collection, separated into 1 mL aliquots, and stored at -80 ^{o}C until analysis. Plasma TNFα was measured via a commercially available equine-specific ELISA (Equine TNFα Screening Set, Endogen, Rockford, IL) previously validated in equine samples (Vick et al., 2007). Plasma samples were assayed in triplicate in a 1:4 dilution in reagent diluent, and samples with CV >10% were re-assayed.

Tissue collection

Adipose tissue biopsies were taken on the fourth or fifth day of the last week of each period, corresponding to Weeks 3, 7, 16, 24, and 30 of the study. For the collection of biopsy specimens, horses were sedated with xylazine hydrochloride (0.6 mg/kg bwt IV) and lidocaine was injected subcutaneously at the biopsy site as a local anesthetic. Subcutaneous adipose tissue samples (1 – 2 g) were collected from the nuchal crest approximately midway between the poll and withers through surgical incision. This adipose depot was chosen because of its association with metabolic variables, including measures of insulin sensitivity (Chapter 1) (Frank et al., 2006). Biopsies were performed on alternating sides of the neck between time periods, but > 5 cm from a previous biopsy site. Once adipose tissue was removed, it was cleaned of visible blood and connective tissue and immediately snap frozen in liquid nitrogen. Samples were then transferred to cryovials and stored at -80°C until analysis. Horses were maintained in stalls for 4 or 5 days and administered 2 g/d phenylbutazone per os for 3 days after tissue biopsies were performed.

RNA isolation and cDNA synthesis

Approximately 500 mg adipose tissue was disrupted with a rotor-stator homogenizer in 1 mL of a guanidine isothiocyanite-containing phenol reagent (TRIzol, Invitrogen Corp., Carlsbad, CA) per 100 mg tissue to achieve cell lysis. Chloroform was added to the lysate at a rate of 0.5 mL per 100 mg tissue and centrifuged at 3000 × *g* and 4°C for 20 min. The chloroform layer was then mixed with 0.5 mL isopropanol per 100 mg tissue and incubated at room temperature for 20 min. After a second 20 min

centrifugation at 3000 × *g* and 4°C, the supernatant was decanted and pellet resuspended in 1 mL 70% ethanol per 100 mg tissue. After a third 20 min centrifugation at 3000 × *g* and 4°C, the supernatant was decanted and pellet resuspended in 100 μL RNase-free water. Contaminants were removed from isolated total RNA by use of a commercial kit (RNeasy Mini Kit, Qiagen) used in accordance with the manufacturer's protocol for RNA cleanup. cDNA was made from total RNA via reverse transcription using a commercial kit (RETROscript, Ambion Inc., Austin, TX). The cDNA was used to perform a real-time quantitative polymerase chain reaction (RT-qPCR) procedure to determine mRNA expression of the different cytokines and chemokines.

RT-qPCR procedure

A commercial real-time thermocycler (LightCycler 480 System, Roche Molecular Biochemical, Indianapolis, IN) was used for amplification reactions and quantification with external standards was performed with the fluorescent format for SYBR Green I dye as previously described (Belknap et al., 2007; Waguespack et al., 2004a; Waguespack et al., 2004b). Primers for IL-1β, IL-6, IL-8, IL-10, PAI-1, MCP-1, MCP-2, TNFα, adiponectin, leptin, β-actin, GAPDH, β-2 microglobulin, and 28s rRNA were designed from equine-specific sequences using computer programs as previously described (Table 4.1) (Garton et al., 2002; Waguespack et al., 2004b). Amplified cDNA fragments were ligated into a linearized vector for use as standard templates for production of a standard curve for the RT-qPCR reactions (Waguespack et al., 2004b).

All PCR reactions were performed in glass capillaries in 20 μL volumes (5 μL sample DNA and 15 μL 1.33× PCR master mixture). The master mix included *Taq* polymerase (1 unit), uracil-N-glycosylase (0.2 units), SYBR Green stock solution (1:10,000 dilution), and PCR buffer. The PCR buffer (20 mM Tris-HCL, pH 8.4) contained 0.05% each of Tween 20 and a nonionic detergent. All primers were used at a concentration of 1 μM. For comparisons of Period 1 and 5, all amplification reactions from individual horses were performed in a single run. For comparisons among all time periods, amplification reactions for all time periods for 6 horses were performed in one run and for 7 horses in a second run, with the same groups of horses run together for all gene products. Standard curves were performed for each gene product and water included

as a negative control. Standards and targets were prepared in separate capillaries but always amplified during the same PCR run. Ten-fold serial dilutions of linearized plasmids containing the different gene-specific cDNA inserts were used at concentrations of 10^4 to 10^0 template molecules/reaction to create a standard curve. These curves were used for relative quantification of the target amplicon and the housekeeping genes in each cDNA sample for the normalization process. The amplification reactions were performed in duplicate from the individual cDNA samples.

Housekeeping genes and statistical analysis

RT-qPCR was performed on 4 potential housekeeping genes (β-actin, β_2 microglobulin, glyceraldehyde-3 phosphate dehydrogenase [GAPDH] and 28S rRNA) in samples from all horses at all 5 time periods. The resulting data for the 4 genes were assessed for normalization factors by use of a computer program (geNorm, Ghent University, Ghent, Belgium). The 2 housekeeping genes that changed < 2 fold between study periods (Appendix D) and received the best score from geNorm (β_2 microglobulin and 28S rRNA) were used to make normalization factors.

The amplification data obtained by RT-qPCR were divided by the normalization factors for each sample to attain mRNA expression values represented by cDNA copies per normalization factor. Fold changes in mRNA expression were determined by comparing normalized values between Periods 1 and 5. Normalized values were log transformed prior to statistical analysis. A 2-tailed paired *t* test was performed to determined differences in mRNA expression between Periods 1 and 5. For variables measured during all study periods, the effect of period was assessed by repeated measures ANOVA using the Huynh-Feldt ε correction factor to adjust for sphericity, with Fisher-Hayter pairwise comparisons made between periods. Of specific interest were the comparisons of Period 1 with Period 5 and of Period 2 with Period 4, representing comparisons between moderate and obese states while horses were on similar diets. Values of $P < 0.05$ were considered significant. Data are presented as mean ± SEM unless stated otherwise. Statistical analyses were conducted by use of a computer software program (Intercooled Stata Version 9.2, Stata-Corp, College Station, TX).

Results

RT-qPCR values (cDNA copies per normalization factor) were determined in samples from 12 horses during Periods 1 and 5 for MCP-1, IL-1β, IL-6, IL-8, IL-10, adiponectin, and in samples from 6 horses for leptin (Table 4.2). mRNA expression was higher in Period 5 compared to Period 1 for MCP-1 and IL-8, with a trend ($P = 0.076$) for higher expression of leptin.

RT-qPCR values were determined in samples from 13 horses during all time periods (n = 12 for Period 1) for PAI-1, MCP-2 and TNFα (Figure 4.1). Although PAI-1 mRNA expression was similar ($P > 0.05$) between Periods 1 and 5, mRNA expression increased by 5.1 ± 2.8 fold between Periods 2 and 4. MCP-2 mRNA expression increased by 2.7 ± 0.6 fold between Periods 1 and 5, and by 2.4 ± 0.3 fold between Periods 2 and 4. There was no effect of time period on TNFα mRNA expression ($P = 0.34$).

As β-actin and GAPDH were unsuitable as housekeeping genes because of variability between periods, these genes were normalized and values reported for 13 horses (n = 12 for Period 1) during all time periods (Figure 4.2). β-actin mRNA expression was higher in Periods 4 and 5 than Periods 1 and 2, increasing 1.9 ± 0.4 fold from Period 1 to 5 and 2.2 ± 0.3 fold from Period 2 to 4. GAPDH mRNA expression was higher in Period 5 than 1, increasing 1.9 ± 0.2 fold, and was higher in Period 4 than 2, increasing 2.1 ± 0.2 fold.

Plasma TNFα protein concentrations decreased to 71 ± 14% of pre-weight gain values from Periods 1 to 5, and to 55 ± 9% of pre-weight gain values from Periods 2 to 4 (Figure 4.3).

Discussion

In horses demonstrated to have developed obesity-induced insulin resistance, no changes were apparent in inflammatory cytokine mRNA expression in a subcutaneous adipose depot, whereas mRNA expression of chemokines known to recruit immune cells increased between 2- and 85-fold with the induction of obesity and insulin resistance.

In the present study, leptin gene expression exhibited a trend to increase with obesity, and it is expected that measurement in 12 horses would display a significant increase with obesity. Plasma leptin concentrations were measured as part of the companion study, with a 3-fold increase in concentration from 3.3 ± 0.5 ng/mL in Period 1 to 9.8 ± 0.9 ng/mL in Period 5. However, adiponectin gene expression did not differ before and after weight gain. The adipokines leptin and adiponectin are produced and released almost exclusively from adipocytes in other species (Fain, 2006). Previous studies in horses have demonstrated a proportional relationship of plasma leptin concentration with fat mass, whereas adiponectin concentration was inversely proportional to fat mass (Buff et al., 2002; Kearns et al., 2006).

In the present study, cytokine mRNA expression of IL-1β, IL-6, IL-10, and TNFα was not different in neck crest subcutaneous adipose tissue before and after induction of obesity and insulin resistance. Additionally, plasma TNFα protein concentrations decreased with weight gain. Previous studies in horses have reported that TNF protein and mRNA expression in blood is associated with measurements of both adiposity and insulin sensitivity (Vick et al., 2007). Additionally, it has been demonstrated that cultured equine adipocytes and preadipocytes have the capacity to produce TNFα, IL-6, and IL-1 in response to lipopolysaccharide stimulation (Vick et al., 2008).

In human and rodent studies, obesity is accompanied by increased mRNA expression of TNF in subcutaneous adipose tissue which is implicated as an initiating factor for a larger inflammatory response through activation of the NF-kB pathway and induction of insulin resistance by inhibiting intracellular signaling from the insulin receptor (Fain, 2006; Hotamisligil et al., 1995). Therefore, it was unexpected that in the present study mRNA expression in subcutaneous adipose tissue was unchanged and circulating protein concentrations of TNFα decreased with the induction of obesity. These results do not support a role of subcutaneous adipose expression of or circulating concentrations of TNFα in the initial stages of obesity-induced decreases in insulin sensitivity in horses.

Although the results of this study do not support a sufficient explanation for the observed decrease in plasma TNFα protein concentrations, it is possible that these changes are related to seasonal variation in inflammatory cytokine expression. In

humans, seasonal decreases in the response of TNFα to lipopolysaccharide stimulation occur in autumn, and may be influenced by photoperiod, temperature, or exposure to environmental antigens (Myrianthefs et al., 2003).

Although cytokine expression did not differ with weight gain, chemokine expression increased. Measured chemokines MCP-1, MCP-2, IL-8 and PAI-1, all of which have been implicated in the recruitment of immune cells to adipose tissue were increased after the induction of obesity. In other species, macrophage infiltration into adipose tissue enhances cytokine production and secretion, purportedly through a paracrine interaction between adipocytes and macrophages (Suganami et al., 2005). It is possible that in the short-term model of equine diet-induced obesity of the present study macrophage infiltration had not yet occurred, and therefore cytokine production was limited.

In human adipose tissue, although the non-adipocyte cells (including stromal-vascular and immune cells) in adipose tissue produce and release MCP-1, PAI-1 and IL-8 in a greater abundance than adipocytes, there is significant secretion of these chemokines from adipocytes, and in greater amounts than leptin (Fain, 2006). Additionally, during the early stages of obesity in mice MCP-1 production is increased before macrophage infiltration into adipose tissue (Ito et al., 2007). Therefore, it is possible that non-immune cells, including adipocytes or stromal-vascular cells, may be responsible for the increase in chemokine expression in the early stages of obesity.

In humans, euglycemic hyperinsulinemia increased MCP-1 mRNA expression and protein content in subcutaneous adipose tissue, and this response was exaggerated in insulin resistant compared to insulin sensitive subjects (Westerbacka et al., 2008). Similarly, PAI-1 continues to respond to insulin stimulation during insulin resistant states (Samad et al., 2000). Taking these studies into consideration, it is possible that the increase in cytokine expression was induced by the hyperinsulinemia resulting from obesity, or even the hyperinsulinemia induced by the FSIGTT with a stronger response after obesity due to the insulin resistance. In this respect, an inflammatory response would be secondary to the insulin resistance or hyperinsulinemia rather than a stimulatory cause of this metabolic alteration.

Although many of the cytokines and chemokines measured in the present study are regulated via the NF-κB pathway, differential expression between cytokines and chemokines was observed in subcutaneous adipose tissue. It has been demonstrated that chemokine expression of MCP-1 and IL-8 in adipose tissue is regulated by mitogen-activated protein kinase (MAPK)-dependent pathways in addition to NF-κB pathways (Fain et al., 2005; Ito et al., 2007). Although MAPK-dependent pathways are stimulated by insulin, they are not down-regulated during insulin resistance, such as with phosphatidylinositol 3-kinase (PI3K)-dependent pathways, an occurrence well described for cardiovascular actions of insulin (Muniyappa et al., 2007). It is possible that chemokines are similarly regulated by MAPK-dependent pathways, therefore responding to insulin stimulation during insulin resistance.

Although cytokine expression did not increase in the present study, there are alternative explanations that may still implicate a pro-inflammatory state in the induction of insulin resistance during the early stages of obesity. First, chemokines may directly affect the insulin signaling pathway independent of their effect on macrophage infiltration and cytokine production. Addition of MCP-1 to differentiated adipocytes *in vitro* decreased insulin-stimulated glucose uptake and glucose transporter (GLUT)-4 mRNA expression (Sartipy and Loskutoff, 2003). Second, it is possible that the inflammatory response responsible for inducing insulin resistance occurs in another fat depot besides neck subcutaneous fat, such as visceral adipose. In obese humans, visceral adipose tissue releases greater amounts of cytokines than abdominal subcutaneous fat (Fain, 2006). A further possibility is that a pro-inflammatory response in the muscle tissue is more important than in the adipose tissue. Similar to adipose tissue, muscle tissue produces and secretes cytokines and may display macrophage infiltration. In humans, insulin resistant states, such as type 2 diabetes mellitus, are associated with increased inflammatory cytokine expression in skeletal muscle (Wei et al., 2008). As skeletal muscle is responsible for the majority of insulin-mediated glucose disposal, autocrine or paracrine cytokine action may have more of an impact on insulin sensitivity in this tissue. Finally, it is possible that inflammation is not the initiating factor for insulin resistance during obesity, but rather plays a role in maintaining and exacerbating established insulin resistance.

Alternative mechanisms of obesity-induced insulin resistance, such as intramyocellular triglyceride accumulation, may be more important in the horse. Research in humans links mitochondrial dysfunction with intramyocellular triglyceride accumulation in obesity and insulin resistance (Goodpaster and Wolf, 2004). Intramyocellular triglyceride content, as assessed by Oil Red O staining of muscle fibers, was increased in obesity and decreased by weight loss in humans (Goodpaster et al., 2000). Accumulation of triglycerides and fatty acid metabolites, including diacylglycerol and ceramide, within myocytes results in defects of the insulin signaling cascade.

Considering the longitudinal design of the present study, it is possible that other factors besides obesity could have contributed to the changes in glucose and insulin dynamics, therefore not creating a representative obesity-induced insulin resistance. As no comparable control group was assessed in the present study, it cannot be excluded that changes in variables were a result of factors other than an increase in adiposity. In previous studies, adaptation to a high nonstructural carbohydrate diet decreased insulin sensitivity when compared to a high fiber and fat diet (Hoffman et al., 2003; Treiber et al., 2005a). Additionally, photoperiod may exert a seasonal effect on insulin sensitivity through a pineal-hypothalamic-adipocyte pathway (Scott and Grant, 2006). Additional factors may have influenced an inflammatory response independent of changes in obesity or insulin sensitivity. The biopsy procedure may elicit up- or down-regulation of genes during the time prior to snap freezing. Similarly, although biopsies were performed > 5 cm apart, repeated biopsies may change cytokine expression *in vivo*.

Prior to normalization, GAPDH and β-actin differed > 2-fold between time periods (Appendix D). Therefore these genes were excluded as housekeeping genes. These genes have previously been demonstrated to increase with insulin stimulation. Studies in cultured human adipocytes demonstrate a threshold cycle difference of 0.5 with RT-qPCR, representing an approximate 40% increase in these genes between insulin-stimulated and control adipocytes (Gorzelniak et al., 2001). Transcription of GAPDH is regulated by multiple insulin-responsive elements (Alexander-Bridges et al., 1992). Both GAPDH and β-actin facilitate glucose metabolism, as GAPDH is facilitates glycolysis and the translocation of GLUT4 in adipocytes requires insulin stimulation of intracellular actin remodeling (Kanzaki, 2006).

In the present study, obesity-induced insulin resistance in horses was associated with no change in inflammatory cytokine gene expression but increased chemokine gene expression in subcutaneous adipose tissue. Results suggest that alternative mechanisms besides a subcutaneous adipose tissue inflammatory response are initiating causes of obesity-induced insulin resistance.

Table 4.1: Primer sequences for the genes used in real-time quantitative polymerase chain reactions.

Gene	Upper primer	Lower primer
28S rRNA	TTGGGCAGGGCGAAGCAGAGG	TGAGATCGTTTCGGCCCCAAGACC
Adiponectin	CCAAGGCAGGAAAGGAGAACC	GGAATTTGCCAGTGGTGCCATCATAGT
β_2 microglogulin	CAGGTTTACTCACGTCACCC	CTGGTTAGAGGTCTCGATCCC
β-actin	GGGAAATCGTGCGTGACAT	AGCACTGTGTTGGCGT
GAPDH	ATGGGGGCATCAGCAGAAGGAGCAGAGAT	CAGGAGCGAGATCCCGCCAACATCAAAT
IL-1β	CCAGACGCGGCCGGGACATAAC	GGGAAGGCAGCTGGGCATTGATT
IL-6	CACCGTCACTCCAGTTGCCTT	GGATGTACTTAATGTGCTGTTTGGTTTTGTCTG
IL-8	TCTTGGCCGTCTTCCTGCTTTCT	TGGCCCACTCTCAATCGCTCTC
IL-10	AAAGGTGTCTACAAAGCCATGAG	AGGGTTTTCCAAGGAGCTG
Leptin	TCCAGGATGACACCAAAACC	GGAGAAGGCCAGCAGATG
MCP-1	AGTCACCTGCTGCTATACATTCACC	AGGCTTTGGAGTTTGGGCTTTCT
MCP-2	GCCCTCCTGTGCCTGCTGCTC	ACTGGCTGCTGGTGATTCTTGTGTAG
PAI-1	GGTCCGCTTCCTACAATCCCGAGTCC	CCAGCACCGAGGCCACCCCGTAAG
TNFα	AGCCACGGGTTGCCTTATTTTA	ATGTGTATCCGGTGGGTGTGAA

Table 4.2: Changes in subcutaneous adipose tissue mRNA expression* of cytokines before (Period 1) and after (Period 5) induction of obesity in 12 horses.

Variable	Period 1	Period 5	*P* value	Fold Change
MCP-1	25.7 ± 4.5	353 ± 193	0.047	17.9 ± 10.2
IL-1β	350 ± 98	203 ± 38	0.26	NS
IL-6	202 ± 66	135 ± 25	0.81	NS
IL-8	0.90 ± 0.34	15.0 ± 7.9	0.003	84.5 ± 36.5
IL-10	901 ± 304	706 ± 216	0.89	NS
Adiponectin	59550 ± 9485	77217 ± 12427	0.25	NS
Leptin†	1.45 ± 0.49	3.67 ± 1.12	0.076	3.6 ± 1.5

*Values expressed as mean ± SEM of cDNA copies per normalization factor.
NS = nonsignificant difference between Periods 1 and 5.
†n = 6

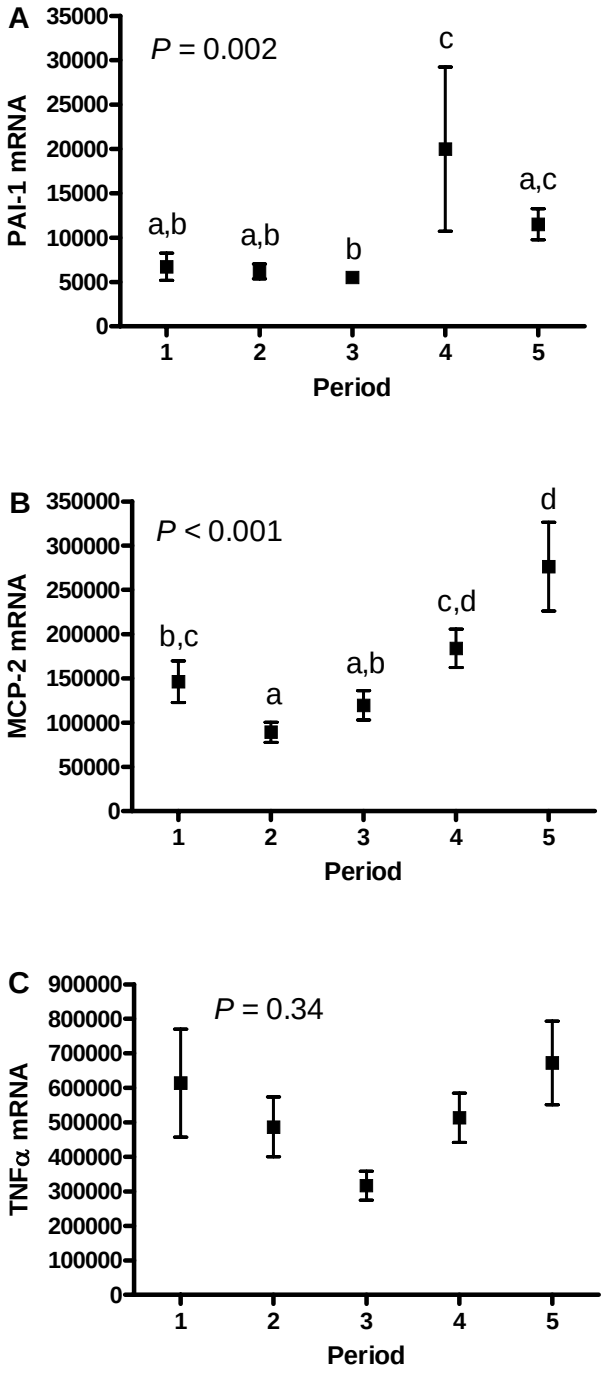

Figure 4.1: Cytokine mRNA expression of PAI-1 (A), MCP-2 (B), and TNFα (C) in subcutaneous adipose tissue before (Periods 1 and 2), during (Period 2), and after (Periods 4 and 5) the induction of obesity in 13 horses. Values represent mean ± SEM cDNA copies per normalization factor. *P* values indicate significance of repeated measures ANOVA of log-transformed data; means with different letters differ ($P < 0.05$) according to Fisher-Hayter pairwise comparisons.

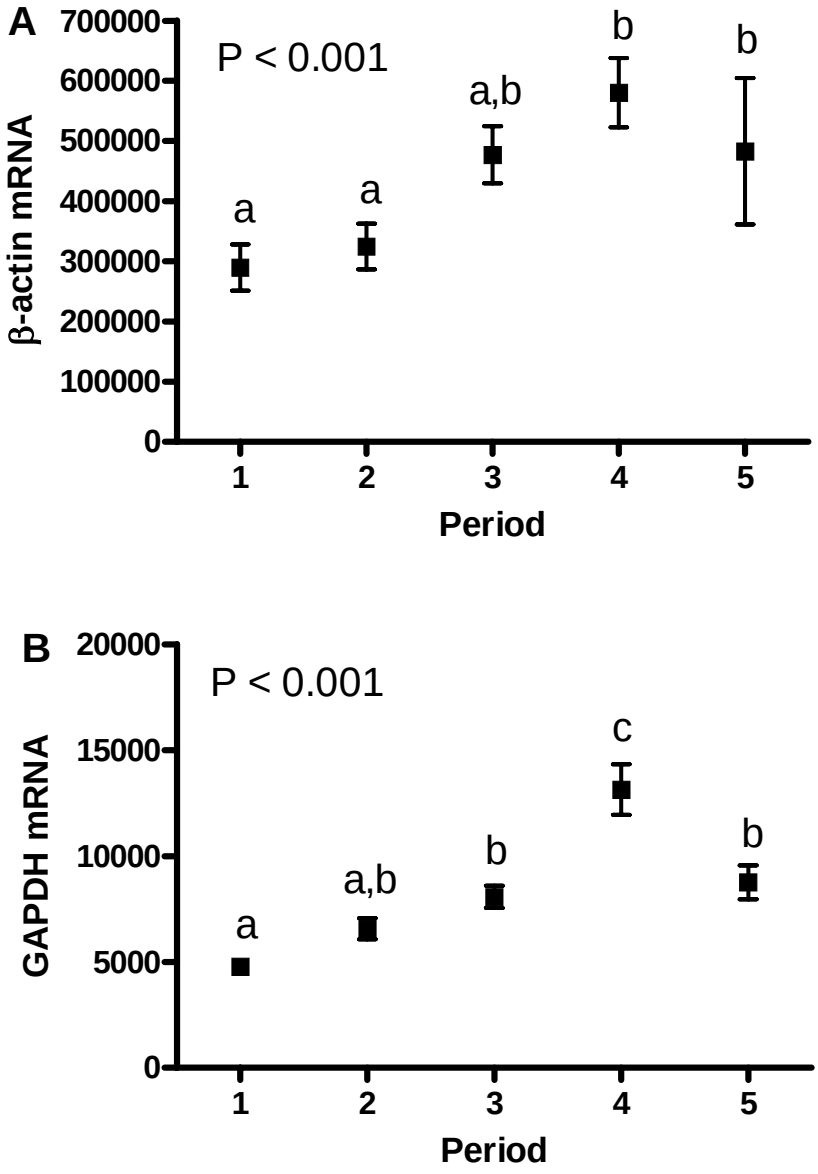

Figure 4.2: β-actin (A) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; B) mRNA expression in subcutaneous adipose tissue before (Periods 1 and 2), during (Period 2), and after (Periods 4 and 5) the induction of obesity in 13 horses. Values represent mean ± SEM cDNA copies per normalization factor. *P* values indicate significance of repeated measures ANOVA of log-transformed data; means with different letters differ ($P < 0.05$) according to Fisher-Hayter pairwise comparisons.

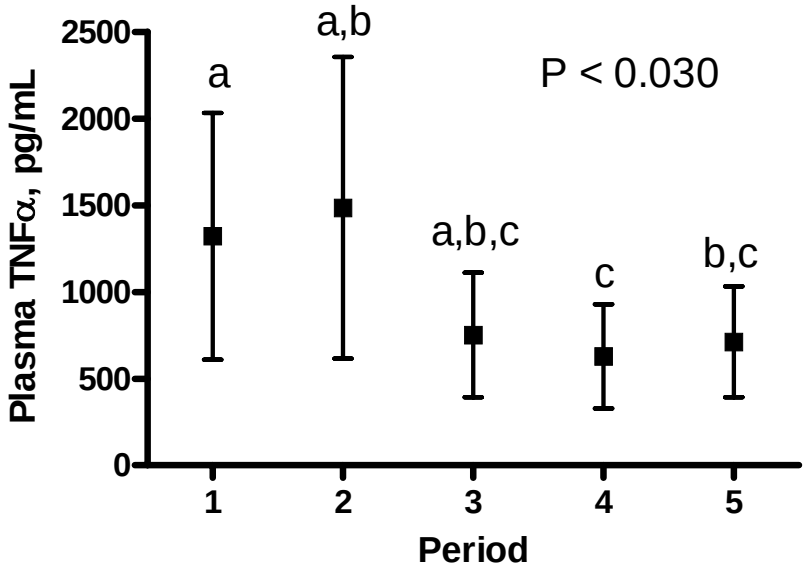

Figure 4.3: Mean ± SEM plasma protein concentrations of TNFα before (Periods 1 and 2), during (Period 2), and after (Periods 4 and 5) the induction of obesity in 13 horses. *P* values indicate significance of repeated measures ANOVA of log-transformed data; means with different letters differ ($P < 0.05$) according to Fisher-Hayter pairwise comparisons.

CHAPTER 5

Effect of exercise training on adiposity, glucose and insulin dynamics, and plasma hormone and lipid concentrations in obese, insulin resistant horses

ABSTRACT: Increasing physical activity is a common recommendation to reduce adiposity and improve insulin sensitivity in obese horses. The present study evaluates the influence of exercise training without dietary restriction on adiposity and basal insulin and glucose concentrations in obese horses. Twelve overweight or obese (body condition score ≥ 7, scale 1 – 9) Arabian geldings (age 9 – 21 years; body weight 433 – 638 kg) were used in the study. Four horses remained sedentary (CON) and 8 horses (EX) were exercised 4 times per week for 4 wk at low intensity (30 min trot), then an additional 4 wk at higher intensity (10 min trot, 20 min canter, 3° incline) exercise, followed by 2 wk detraining. Prior to and after each training period, frequently sampled intravenous glucose tolerance tests with minimal model analysis were performed and adiposity was assessed by body condition scores, morphometric measurements, subcutaneous fat thickness (ultrasonic assessment), and total body water (deuterium oxide dilution). Plasma insulin, glucose, triglycerides, nonesterified fatty acids, and leptin were analyzed in baseline blood samples. Four weeks of low intensity exercise training was associated with a 2% (10 ± 4 kg) reduction in body weight and 21% (20 ± 7 kg) reduction in fat mass, and a further 4 wk of higher intensity exercise training reduced body weight a total of 4% (20 ± 8 kg) and fat mass a total of 35% (32 ± 9 kg) compared to pre-exercise values ($P < 0.05$). However there were no changes ($P > 0.05$) in subcutaneous fat thickness over the rump, shoulder, back, or ribs with exercise training. Additionally, metabolic changes resulting from exercise training were not observed, as plasma hormone and lipid concentrations and minimal model parameters changed similarly ($P > 0.05$) over time in CON and EX groups. The results of this study demonstrate a decrease in fat mass with exercise training independent of changes in subcutaneous fat thickness and without changes in metabolic variables, including insulin sensitivity.

Key words: exercise, horse, insulin resistance, lipid, obesity

Introduction

Obesity is of increasing concern in equid populations, as its presence is associated with altered metabolic function and risk for disease. Obesity has been associated with insulin resistance in horses and ponies (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007), and both obesity and insulin resistance have been associated with impaired reproductive function in mares and an increased risk of and a predisposition for developing pasture-associated laminitis (Bailey et al., 2008; Treiber et al., 2007b; Treiber et al., 2006b; Treiber et al., 2006c; Vick et al., 2006). Therefore, both obesity and insulin resistance are significant risk factors for disease in horses. Developing management strategies that mitigate the extent of obesity and insulin resistance is essential to improving the health of obese horses.

In humans, regular exercise is recommended for weight reduction and management of metabolic disease (Pate et al., 1995). Research has helped elucidate the amount and intensity of exercise necessary for desired effects (Houmard et al., 2004; Johnson et al., 2007a). Additionally, research in humans and rodents is exposing the molecular mechanisms of increased insulin sensitivity resulting from exercise training. Exercise training induces an increase of glucose transporter 4 (GLUT-4) content in skeletal muscle, and improvements in insulin sensitivity with training may be induced by increased expression or activity of proteins involved in signal transduction, including insulin receptor substrate 1 (IRS-1) and phosphatidylinositol 3-kinase (PI3-kinase) (Zierath, 2002).

Previous studies in horses support a role of short-term (7 days) exercise training for increasing insulin sensitivity, although sustained effects lasting > 1 day after the last exercise session are inconsistent (Powell et al., 2002; Stewart-Hunt et al., 2006). Additionally, in obese, insulin resistant ponies, decreased adiposity through controlled feed intake increases insulin sensitivity to a similar degree as exercise training alone (Freestone et al., 1992). However, it is unknown whether longer-term exercise training without feed restriction would induce sustained increases in insulin sensitivity or reduce adiposity in obese, insulin resistant horses. It is also of interest to determine whether

alterations in obesity and metabolism are dependent on intensity level of exercise training.

The present study was designed to test the hypothesis that exercise mitigates risk factors for metabolic disease and laminitis, that higher intensity exercise is more effective at altering these risk factors, and that these alterations will be present up to 2 weeks after the cessation of exercise training. The specific objectives of this study were to identify changes in measurements of adiposity, minimal model parameters of glucose and insulin dynamics, and circulating concentrations of insulin, glucose, NEFA, triglyceride, and leptin in response to low intensity and higher intensity exercise training compared to a sedentary control group and to determine whether these changes persist for a 2 week sedentary, detraining period.

Materials and methods

Horses

Twelve Arabian or Arabian cross geldings (age 9 – 21 years) from the Virginia Tech Middleburg Agricultural Research and Extension Center's research herd were evaluated during the study period (August 2007 to November 2007). Horses were previously used as a model of diet-induced obesity in a study concluding 6 months prior to the initiation of the present study, and therefore horses had been maintained at a high body condition (body condition score [BCS] ≥ 7, scale 1 – 9) for > 7 months. At study initiation, horses were either overweight ($7 \leq$ BCS < 8; n = 4) or obese (BCS ≥ 8; n = 7) and insulin resistant (insulin sensitivity [SI] $\leq 1.2 \times 10^{-4}$ L/min/mU), with SI values in the lowest (n = 7) or second to lowest (n = 4) reference quintiles for normal horses (Treiber et al., 2005b). One horse was insulin resistant but not overweight or obese, and one was overweight but not insulin resistant. Horses were divided into two groups (n = 4 and n = 8) to achieve similar mean values between groups for body weight, BCS, cresty neck score (CNS), and previously obtained values of SI.

Horses were maintained as two separate groups on a divided drylot beginning 2 weeks prior to and for the duration of the study. A mixed alfalfa/grass hay was group-fed at 2.4% of bodyweight (12 kg/horse/day). Before study initiation, feeding requirements

were determined by adjusting hay intake until a stable body weight was maintained for each group with minimal hay wastage. Each horse also received 1 kg/d of a concentrate feed to supplement vitamins and minerals and was provided access to fresh water and a salt block at all times. The overall nutrient profile of the diet was 18% crude protein, 4% crude fat, 10% nonstructural carbohydrate (NSC), 30% acid detergent fiber, and 43% neutral detergent fiber on a dry matter basis (Appendix E). Total digestible energy (DE) intake was estimated at 23 Mcal/d, accounting for an estimated 15% hay wastage. The experimental protocol was approved by the Virginia Tech Institutional Animal Care and Use Committee.

Experimental design

In a longitudinal study lasting 14 weeks, the two groups of horses were allocated to either an exercise training protocol (EX, n = 8) or remained sedentary (CON, n = 4). Horses in the EX group were exercised 4 wk at low intensity followed by 4 wk at higher intensity exercise followed by 2 weeks of no structured exercise (detraining). Low intensity exercise consisted of 10 min walking (1.1 m/s) and 30 min trotting (2.5 m/s) on an automated horse exerciser (Odyssey Horse Exerciser, Odyssey Performance Limited, Rockwood, Ontario, Canada) 4 times per week. Higher intensity exercise consisted of 2 days per week of 10 min walking (1.1 m/s) and 30 min trotting (3.0 m/s) on an automated horse exerciser and 2 days per week of 10 min walking (1.3 m/s), 10 min trotting (3.7 ± 0.3 m/s, 3° incline) at a target heart rate of 130 bpm, and 20 min cantering (6.2 ± 0.8 m/s, 3° incline) at a target heart rate of 160 bpm on a treadmill (Equi Gym Products LLC, Paris, Kentucky, USA). Heart rate was monitored by a commercial digital heart rate monitor (Polar Pacer, Polar CIC Inc, Port Washinton, New York, USA).

Weekly exercise amounts during the low intensity training totaled 2 h 40 min of 25% walking and 75% trotting. This exercise regimen was chosen to represent a training program with potential to have a high compliance if recommended for use in obese equids. Each exercise session was estimated to have an average energy expenditure of 2.3 Mcal for a 500 kg horse working at a heart rate of 100 bpm for 30 min and 80 bpm for 10 min (NRC, 2007).

During the higher intensity exercise training, horses were exercised the same amount of time (2 h 40 min per week), except 2 of the sessions were performed on the treadmill and included 25% walking, 25% trotting at a target heart rate of 130 bpm, and 50% cantering at a target heart rate of 160 bpm. This exercise regimen was chosen to be comparable to current recommendations for obese humans (Johnson et al., 2007a). Each of the treadmill sessions was estimated to have an average energy expenditure of 4.8 Mcal for a 500 kg horse working at a heart rate of 160 bpm for 20 min, 130 bpm for 10 min, and 80 bpm for 10 min (NRC, 2007).

Testing procedures consisted of measurements of adiposity, frequently sampled IV glucose tolerance tests (FSIGTT), and deuterium oxide dilutions. Testing procedures were performed over a 4 day period during the weeks prior to low intensity exercise, between low and higher intensity exercise, between higher intensity exercise and detraining, and after detraining. Deuterium oxide dilutions were not performed after detraining. For testing procedures, horses were divided into 3 groups (n = 1 or 2 CON and 2 or 3 EX) and each group underwent tests on consecutive days. On day one of testing procedures, catheters were inserted into a jugular vein and the deuterium oxide infusion protocol was performed, beginning between 1000 and 1200 h. On day two of testing procedures, FSIGTT were performed in the morning, starting between 0800 and 0830 h, followed by measurements of adiposity. FSIGTT were performed on horses in the EX group approximately 48 h after their last exercise session.

Morphometrics, condition scores, and subcutaneous fat thickness

Bodyweight, girth and waist (abdominal) circumferences, height (at the wither), neck crest height, and neck circumference (NC) at 0.25, 0.50, 0.75 of neck length were measured. Girth circumference was measured caudal to the elbow (oleccranon tuber) and immediately behind the slope of the withers. Waist was taken as the abdominal circumference measured two-thirds the distance from the point of the shoulder to the point of the hip (tuber coxae). All neck measurements were taken while the neck was held in a relaxed position, at approximately a 45° angle. Neck length was measured from the poll to the highest point of the withers. Crest height was measured at 0.50 of neck

length from the dorsal midline of the neck to estimated differentiation between the crest and neck musculature, identified by palpation and visual assessment.

Three evaluators graded BCS from 1 to 9 (Henneke et al., 1983) and neck crest adiposity as CNS from 0 to 5 (Chapter 1). The mean of the three scores for each horse was used for data analysis. Intraclass correlation coefficients for the reliability of individual scores were 0.83 for BCS and 0.80 for CNS. Intraclass correlation coefficients for the reliability of the mean scores were 0.93 for BCS and 0.92 for CNS.

Ultrasound measurements of rump subcutaneous fat thickness were performed over the rump 5 cm lateral from the dorsal midline at the center of the pelvic bone (Westervelt et al., 1976). Shoulder subcutaneous fat thickness was measured ¾ the distance from the dorsal midline to a point 1/3 the distance from the point of the shoulder to the point of the hip. Back subcutaneous fat thickness was measured ¼ the distance from the dorsal midline to the point where rib measurement was taken. Rib subcutaneous fat thickness was measured 2/3 the distance from the point of the shoulder to the point of hip, between the 12^{th} and 13^{th} ribs. Intra- and inter-assay CV for ultrasound measurements were < 6%. Intra-assay CV for morphometric measurements were < 3%.

Deuterium oxide dilution

After catheter placement into a jugular vein, baseline blood samples were drawn and placed into 10 mL evacuated tubes containing sodium heparin as an anticoagulant and kept on ice until centrifugation. Deuterium oxide (D_2O) solution (Cambridge Isotope Laboratories Inc, Andover, MA) was then infused at a rate of 0.2 g/kg body weight as rapidly as possible (< 2 min). Actual weight of infused deuterium oxide solution was calculated from syringe weights before and after infusion. Blood samples were taken 180 min after completion of deuterium oxide infusion.

FSIGTT

On the days of FSIGTT procedures, horses were removed from the drylot at 0700 and placed in stalls for the duration of the testing procedure. No feed or hay was offered prior to or during the testing procedure on FSIGTT days, however horses had continual access to water. Extension sets were attached to catheters already in place from the

previous day, and the insulin modified FSIGTT procedure was initiated as previously described (Hoffman et al., 2003). Briefly, a glucose bolus (50% [wt/vol] dextrose solution, Dextrose 50% solution, Vedco Inc., St. Joseph, MO) of 0.3 g/kg body weight was administered rapidly (within 2 min) through the catheter, followed after 20 min by rapid administration (within 10 s) of an insulin (Humulin R, Eli Lilly & Co., Indianapolis, IN) bolus of 20 mIU/kg BW. Blood samples were collected at -20 (between 0800 and 0830), -5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, 180, 210, and 240 min with respect to completion of glucose administration. Samples were immediately placed into 10 mL evacuated tubes containing sodium heparin as an anticoagulant and kept on ice until centrifugation. Additional blood was collected during the -5 min sampling time into tubes containing potassium EDTA as an anticoagulant.

Analysis of samples

Plasma was separated by centrifugation (3000 × *g*) within 30 min of sample collection, separated into 1 mL aliquots, and stored at -80 ^{o}C until analysis. Plasma glucose and insulin (in heparin) concentrations were measured in all FSIGTT samples. Plasma triglyceride, NEFA, and leptin (in EDTA) concentrations were measured in the -5 min blood samples. Plasma glucose, triglyceride, and NEFA concentrations were assayed enzymatically by use of commercial kits and an automated analyzer (CX5 Chemistry Analyzer, Beckman Coulter Inc., Fullerton, CA). Plasma insulin (Coat-A-Count Insulin, Diagnostic Products Corp, Los Angeles, CA), leptin (Multi-species Leptin RIA, Linco Research Inc., St. Charles, MO) concentrations were measured by use of commercial radioimmunoassays previously validated for use in equine plasma (Freestone et al., 1991; McManus and Fitzgerald, 2000). All analyses were performed in duplicate. Intra-assay coefficients of variation were 0.9%, 3.0%, 3.8%, 5.2%, and 5.6% for glucose, triglyceride, NEFA, insulin, and leptin, respectively. Inter-assay coefficients of variation were 1.1% and 6.4% for glucose and insulin. All other analyses were performed in a single assay for each analysis.

Deuterium oxide analysis was performed in a commercial laboratory (Metabolic Solutions Inc, Nashua, NH) as previously described (Andrews et al., 1997). Briefly, the

D_2O concentration of plasma was measured by zinc reduction at 490°C to produce deuterium gas that was measured with an isotope ratio mass spectrometer. Data were determined as delta D/mL (δ) relative to Vienna standard mean ocean water (VSMOW).

The total body water (TBW) content (mol) was calculated using the following equation:

$$\text{TBW (mol)} = (WA/18.02a) \times [(\delta_{dose} - \delta_{dil})/(\delta_{post} - \delta_{pre})] / 1.04$$

where W is the weight (g) of the dilution water, A is the weight (g) of the D_2O dose administered, a is the weight (g) of the dose used for analysis, δ_{pre} and δ_{post} are the delta deuterium values determined for the predose (0 min) and postdose (180 min) samples, δ_{dose} is the measured deuterium content of the diluted dose, and δ_{dil} is the measured deuterium content of dilution water. To correct for the nonexchange of deuterium in the body with acidic amino acids and other binding sites, TBW (mol) was adjusted by 4% by dividing by 1.04 (Forro et al., 2000).

TBW (mol) was converted into kilograms using the following equation:

$$\text{TBW (kg)} = \text{TBW (mol)} \times 18.02 \text{ (g/mol)} / 1{,}000 \text{ g/kg}$$

Fat-free mass (FFM; kg) was calculated as TBW (kg) / 0.73, using a hydration factor of 73% for FFM (Wang et al., 2000). Fat mass (FM; kg) was calculated as body weight (kg) – FFM (kg).

The minimal model of glucose and insulin dynamics was applied to glucose and insulin curves for each FSIGTT by use of commercially available software (MinMod Millenium Version 5.15, Raymond Boston, University of Pennsylvania, Kennett Square, PA) and previously described methods (Boston et al., 2003; Hoffman et al., 2003). The model was used to calculate values for insulin sensitivity (SI), glucose effectiveness (Sg), acute insulin response to glucose (AIRg), and disposition index (DI).

Statistical analysis

The Shapiro-Wilk test was used to test for normality of variables within each group. Grubbs test ($\alpha = 0.01$) was used to determine outliers, which were subsequently removed from analysis. Data points removed from analysis included two high insulin concentrations (1 CON, 1 EX during detraining) and one high glucose concentration (EX during detraining). The effect of group and time point on variables was assessed by repeated measures ANOVA with horse nested within group, and using the Huynh-Feldt ε correction factor to adjust for sphericity. When a significant value for the *F* ratio was obtained, Fisher-Hayter pairwise comparisons were made between groups or time points. Values of $P < 0.05$ were considered significant. Values are reported as mean ± SD unless stated otherwise. Statistical analyses were conducted by use of a computer software program (Intercooled Stata Version 9.2, Stata Corp, College Station, TX).

Results

Morphometrics, condition scores, and subcutaneous fat thickness

There was no effect of exercise group on any measurements of morphometrics, condition scores, or subcutaneous fat thickness ($P > 0.05$). There was no effect of group, time point, or their interaction on measurements of crest height, CNS, or shoulder fat thickness ($P > 0.05$). Only an effect of time point was present in measurements of girth ($P = 0.006$), mean NC ($P = 0.001$), back fat thickness ($P = 0.002$), and rib fat thickness ($P = 0.026$), indicating that they changed similarly in both groups during the study. There were group×time point effects for body weight ($P < 0.001$), waist circumference ($P = 0.036$) and BCS ($P = 0.013$), indicating a decrease in adiposity in EX compared to CON (Table 5.1). In the EX group, body weight decreased by 10 ± 4 kg (2.0%) after low intensity exercise, by 20 ± 8 kg (4.0%) after higher intensity exercise, and remained 12 ± 6 kg (2.4%) lower after detraining compared to pre-exercise measurements. A group×time point effect ($P = 0.003$) indicated an increase in rump fat thickness in CON throughout the study compared to baseline (pre-exercise) measurements.

Total body water

There was no effect of exercise group, time point, or their interaction on TBW or FFM ($P > 0.05$). There was a group×time point effect ($P < 0.001$) on FM, which decreased ($P < 0.05$) in EX horses by 20 ± 7 kg (21%) after low intensity exercise and 32 ± 9 kg (35%) after high intensity exercise compared to pre-exercise values (Table 5.2). Similarly, percent body fat calculated by FM and body weight decreased ($P < 0.001$) with exercise training.

FSIGTT

Baseline circulating concentrations of insulin, glucose, NEFA, TG and leptin did not differ between groups ($P > 0.05$), with no group×time point interactions ($P > 0.05$). However, there was an effect of time point on insulin (P = 0.013), glucose ($P = 0.003$), NEFA ($P = 0.004$) and triglyceride ($P = 0.001$) concentrations, indicating that they changed similarly in both groups during the study (Figures 5.1 and 5.2). Compared to pre-exercise values, insulin concentration increased during detraining, glucose concentration decreased after low intensity exercise, NEFA concentration decreased after detraining, and triglyceride concentration decreased after higher intensity exercise and detraining ($P < 0.05$).

A time point effect was detected for AIRg ($P = 0.001$), with values of both groups measured after higher intensity exercise and detraining higher ($P < 0.05$) than pre-exercise values (Table 5.3). No other group, time point or group×time point effects were observed for AIRg, SI, Sg or DI ($P > 0.05$).

Discussion

Four weeks of low intensity exercise training was associated with a 2% reduction in body weight and 21% reduction in FM, and a further 4 wk of higher intensity exercise training reduced body weight a total of 4% and FM a total of 35% compared to pre-exercise values. However there were no changes in subcutaneous fat thickness over the rump, shoulder, back, or ribs with exercise training. Additionally, metabolic changes resulting from exercise training were not observed, as baseline blood variable

concentrations and minimal model parameters changed similarly between CON and EX over time.

As metabolic variables did not differ between EX and CON throughout the study, there was no benefit of higher intensity exercise training or effect of detraining for these variables. There was also no benefit of higher intensity exercise training on reducing adiposity, as body weight and FM reduction was not enhanced with the inclusion of higher intensity exercise. Although FM was not measured during the detraining time point, half of the body weight that was lost during exercise training was re-gained during 2 wk without structured exercise.

Previous studies in horses indicate a sustained improvement in insulin sensitivity with exercise training in the presence of weight loss or when dietary factors, such as a high NSC diet, contributed to the insulin resistance. Short-term (7 day) exercise training has been shown to increase insulin sensitivity in lean and obese horses when measured 24 h after the last exercise session (Powell et al., 2002; Stewart-Hunt et al., 2006). Insulin sensitivity remained higher than pre-exercise values 5 d after the last exercise session when there was a decrease in body weight with exercise (Stewart-Hunt et al., 2006), but returned to pre-exercise values by 9 d after the last exercise session when there was no decrease in body weight with exercise training (Powell et al., 2002). Insulin sensitivity increased after 7 wk training when body weight remained constant, however this occurred only in horses adapted to a high NSC feed and not in horses adapted to a high fat feed (Pratt et al., 2006). In obese, hyperinsulinemic ponies insulin sensitivity improved after 6 wk training, and remained higher after 2 wk detraining (Freestone et al., 1992). However, similar changes were observed in the control group, including decreased body weight and increased insulin sensitivity, therefore exercise exerted no additional improvements above controlled feed intake.

Previous studies have determined exercise training effects by measuring insulin sensitivity in less than 48 h with respect to completion of the last exercise session (Powell et al., 2002; Stewart-Hunt et al., 2006). Results may be confounded by the effects of acute exercise, or a single exercise session, on insulin sensitivity. Insulin sensitivity increases dramatically during moderate intensity exercise in horses (Treiber et al., 2006a). Although short-term increases in insulin sensitivity within 24 h after exercise

have not been demonstrated in horses (Pratt et al., 2007), there is substantial data in humans suggesting an increase in insulin sensitivity as long as 2 days after an exercise bout (Wojtaszewski et al., 2002). Therefore, the present study assessed insulin sensitivity 48 h after the last exercise session in order to determine the effects of exercise training independent of the effects of an exercise session.

Studies in humans and rodents indicate a sustained increase in skeletal muscle insulin sensitivity with exercise training through molecular mechanisms of increased GLUT4 or insulin signaling protein expression (Zierath, 2002). However, in studies performed on obese subjects, exercise training without weight loss did not increase insulin sensitivity when tests were performed at least 2 days after the last exercise session (Dekker et al., 2007; Ross et al., 2000a; Ross et al., 2004). Insulin sensitivity increases in the presence of weight loss, whether it is diet or exercise induced (Goodpaster et al., 2003; Kim et al., 2003; Ross et al., 2000a). Additionally, when similar amounts of weight loss are induced by either diet or exercise, the increase in insulin sensitivity is also similar, indicating that exercise does not enhance insulin sensitivity above the effects of weight loss (Ross et al., 2000a).

The results of the present study contrast previous findings in both horses and humans demonstrating that exercise-induced weight loss improves insulin sensitivity. In the present study, although there was a decrease in body weight and FM, there was no associated improvement in insulin sensitivity. A more pronounced decrease in FM may be necessary to have an effect on SI during obesity/overweight in horses.

Although significant changes occurred in metabolic variables between periods, these changes were similar between CON and EX groups. Overall, there was an increase in AIRg and insulin and triglyceride concentrations and decrease in NEFA concentrations as the study progressed. Collectively, these data indicate either an increase in insulin secretion or decrease in clearance which is elevating insulin concentrations and subsequently increasing the availability of insulin to inhibit lipolysis. As there were no changes in dietary intake or composition, the most likely explanation for changes in metabolic variables is an influence of other environmental factors, such as weather or day length. Melatonin, which is secreted during periods of darkness, inhibits pancreatic β-cell responsiveness and decrease insulin concentrations (Peschke, 2008). However, decreases

in insulin secretion would have been expected as there was a decrease in day length with the change in season from summer (August) to autumn (November) in the present study.

In EX horses, waist (abdominal) circumference decreased after higher intensity exercise and FM decreased after both low and higher intensity exercise training. However, there were no changes in subcutaneous fat thickness at the four measured body sites, suggesting a decrease in adipose depots other than subcutaneous adipose. Although not directly measured in the present study, changes in visceral or intramuscular adipose depots may decrease with exercise training. In humans, visceral adipose tissue decreases to a greater degree with weight loss than total FM (Hallgreen and Hall, 2008). Visceral adipocytes are hypothesized to have a higher fat turnover rate than subcutaneous adipocytes, as *in vitro* studies have demonstrated visceral adipocytes to be more lipolytically active (Mauriege et al., 1995). Additionally, fat stored in or around muscle fibers could decrease with exercise. Although endurance trained athletes have increased intramyocellular triglyceride content associated with increased oxidative capacity, obese individuals have increased intramyocellular triglyceride content associated with decreased oxidative capacity (van Loon and Goodpaster, 2006). Intramyocellular triglycerides are an important substrate source during exercise for endurance trained human athletes (Stellingwerff et al., 2007), and decrease in obese subjects with weight loss (Goodpaster et al., 2000).

Interestingly, in the CON group a slight increase in body weight (13 kg) corresponded to an increase in rump subcutaneous fat rather than an increase in total fat mass, perhaps indicating differential regulation of fat deposition and fat utilization in the different adipose depots.

In the present study, TBW ranged from 59 ± 2% of body weight in both groups at the pre-exercise sampling, to 64 ± 2% in EX after higher intensity exercise, which is within the range of 56 – 68% observed in previous studies of TBW measurements in horses (Forro et al., 2000). Body condition was not indicated, but calculation of % body fat from TBW provides a range from 7 – 23% FM, with 19 ± 3% FM in horses at the initiation of the present study toward the upper end of this range. Additionally, results are within similar ranges of adiposity obtained by chemical analysis of carcass composition (Kane et al., 1987; Kearns et al., 2002a; Westervelt et al., 1976). A number of

assumptions are made with the measurement of TBW, and derivation of FM and FFM, by the deuterium oxide dilution method. When calculating TBW, it is assumed there is equilibration of deuterium within all hydrated compartments at the time of the post-infusion sample, including intracellular and extracellular fluid compartments. In the present study, a post-infusion sample was collected at 180 min based on appearance of a plateau phase identified during a more intensive sampling protocol for preliminary studies (Appendix E) and based on equilibration times observed in previous studies (Andrews et al., 1997; Fielding et al., 2004; Forro et al., 2000).

In the present study it cannot be excluded that changes in hydration status or gastrointestinal content partially contributed to the observed changes in FM. When calculating FFM from TBW, it is assumed that fat-free mass is 73% water content (Wang et al., 2000), water does not equilibrate into fat mass, and hydration status is similar during each infusion (pre-exercise, post-low intensity, post-higher intensity exercise). Deuterium oxide dilution protocols were performed in horses at least 24 h after the last exercise session to minimize effects of exercise on hydration, as it has been previously demonstrated that TBW re-equilibrates to pre-exercise values 24 h after an exercise session (McKeen and Lindinger, 2004). Additionally, differences in transcellular fluid, including intraluminal gastrointestinal water and urinary excretion, and timing of urination and defecation with respect to body weight determination and D_2O infusion could potentially influence calculations of TBW, FFM, and FM. In rabbits, intraluminal gastrointestinal water accounted for 12% of measured TBW and 0.1 – 0.6% of injected D_2O was excreted in urine (Gotch et al., 1957).

Over the entire training period (low and higher intensity exercise) it was estimated that horses utilized an excess 100 Mcal through structured exercise. With an estimated 1.6 Mcal energy expenditure necessary per 1 kg body weight loss (Hall, 2008), it was expected that horses would lose 13 kg throughout the training periods. Therefore, the modest loss of 20 kg observed after higher intensity exercise was actually more than, and the 12 kg loss maintained after detraining was similar to what was expected.

Considering the management effort necessary for modest decreases in body weight, it may be discouraging for horse owners to implement an exercise training program to reduce obesity in their horses. However, exercise training in combination with

dietary restriction would be expected to have an additive effect on weight reduction. Additionally, exercise may have beneficial effects that dietary restriction alone would not provide. In humans, exercise training with weight reduction has been shown to maintain or increase FFM (primarily muscle mass), whereas dietary restriction alone may decrease FFM with weight reduction (Garrow and Summerbell, 1995; Hunter et al., 2008). Similar occurrences in horses are supported by the present study, as EX horses had a nonsignificant 13 kg increase in FFM after exercise training. Additionally, in humans maintenance of weight loss is more likely to succeed if exercise is part of the daily routine (Tate et al., 2007).

In summary, 8 wk of low to moderate intensity exercise training modestly decreased adiposity (body weight and fat mass) without concurrent changes in glucose and insulin dynamics or basal metabolic blood variables. Results suggest that moderate exercise training without concurrent dietary restriction does not mitigate insulin resistance in the presence of obesity/overweight, and that a more pronounced reduction in adiposity may be necessary for a sustained metabolic effect.

Table 5.1: Mean ± SD values for adiposity measurements in 4 control (CON) and 8 exercised (EX) horses. If no group or group×time point effect was observed, groups were combined (ALL). Measurements were performed with respect to the exercise protocol before exercise training, after 4 weeks of low intensity exercise, after 4 weeks of higher intensity exercise, and after 2 weeks of detraining.

		Time point			
Variable	**Group**	**Pre-exercise**	**Low intensity**	**Higher intensity**	**Detraining**
Bodyweight, kg	CON	513 ± 90[a]	518 ± 94[a,b]	520 ± 91[a,b]	526 ± 94[b]
	EX	503 ± 29[c]	492 ± 26[d]	483 ± 28[e]	491 ± 29[d]
Body condition score	CON	8.0 ± 1.2[a]	8.0 ± 1.3[a]	8.0 ± 1.1[a]	8.1 ± 1.2[a]
	EX	7.9 ± 0.5[a]	7.4 ± 0.7[b]	7.3 ± 0.6[b]	7.5 ± 0.7[b]
Waist, cm	CON	213 ± 15[a,c]	217 ± 13[c]	214 ± 14[a,c]	215 ± 14[a,c]
	EX	211 ± 3[a]	212 ± 5[a]	207 ± 5[b]	212 ± 5[a]
Girth, cm	ALL	190 ± 8[a]	191 ± 9[a]	190 ± 8[a]	193 ± 9[b]
Cresty neck score	ALL	3.2 ± 0.6	3.0 ± 0.7	2.9 ± 0.7	2.9 ± 0.6
Mean neck circumference, cm	ALL	96.4 ± 4.8[a,c]	95.1 ± 4.9[b]	95.8 ± 4.7[a,b]	97.3 ± 4.9[c]
Crest Height, cm	ALL	14.2 ± 2.5	13.3 ± 2.5	13.6 ± 1.9	13.9 ± 2.3
Rump, cm	CON	2.44 ± 1.04[a]	2.88 ± 1.24[b]	2.95 ± 1.18[b]	3.07 ± 1.19[b]
	EX	2.57 ± 0.70[a]	2.45 ± 0.66[a]	2.49 ± 0.66[a]	2.52 ± 0.65[a]
Back, cm	ALL	0.87 ± 0.29[a,b]	0.80 ± 0.30[a]	0.93 ± 0.32[b]	0.96 ± 0.34[b]
Rib, cm	ALL	0.89 ± 0.17[a,b]	0.86 ± 0.14[a]	0.95 ± 0.15[b]	0.96 ± 0.15[b]
Shoulder, cm	ALL	0.87 ± 0.11	0.90 ± 0.10	0.91 ± 0.10	0.93 ± 0.10
Body fat, %[†]	CON	18.6 ± 5.3[a]	20.8 ± 6.3[b]	21.2 ± 6.0[b]	21.8 ± 6.0[b]
	EX	19.3 ± 3.5[a]	18.6 ± 3.3[a]	18.9 ± 3.4[a]	19.0 ± 3.3[a]

[a-e]Within a variable, means with different superscripts differ ($P < 0.05$) according to repeated measures ANOVA with Fisher-Hayter pairwise comparisons

[†]Calculated as % fat = 6.22 + 5.07x, where x = subcutaneous rump fat thickness in cm (Westervelt 1976)

Table 5.2: Mean ± SD values for total body water (TBW), fat-free mass (FFM), fat mass (FM) and percent body fat obtained from deuterium oxide infusions in 4 control (CON) and 8 exercised (EX) horses. Deuterium oxide infusions were performed with respect to the exercise protocol for EX horses before exercise training, after 4 weeks of low intensity exercise, and after 4 weeks of higher intensity exercise.

		Time point		
Variable	**Group**	**Pre-exercise**	**Low intensity**	**Higher intensity**
TBW, kg	CON	298 ± 42	302 ± 46	300 ± 39
	EX	298 ± 21	305 ± 21	307 ± 23
FFM, kg	CON	409 ± 58	413 ± 62	411 ± 54
	EX	408 ± 29	418 ± 29	421 ± 32
FM, kg	CON	104 ± 36[a]	105 ± 34[a]	109 ± 38[a]
	EX	95 ± 8[a]	75 ± 6[b]	62 ± 9[c]
Body fat, %[†]	CON	19.9 ± 4.3[a]	19.9 ± 3.6[a]	20.6 ± 3.8[a]
	EX	18.9 ± 1.8[a]	15.2 ± 1.6[b]	13.0 ± 2.3[c]

[a-c]For variables with a significant ($P < 0.05$) group×time point effect, mean values with different superscripts differ, as determined by repeated measures ANOVA with Fisher-Hayter pairwise comparisons.
[†]Calculated as FM percentage of body weight

Table 5.3: Mean ± SD values for minimal model parameters obtained from FSIGTT glucose and insulin curves in 4 control (CON) and 8 exercised (EX) horses. FSIGTT were performed with respect to the exercise protocol for EX horses before exercise training, after 4 weeks of low intensity exercise, after 4 weeks of higher intensity exercise, and after 2 weeks of detraining.

		Time point			
Variable	**Group**	**Pre-exercise**	**Low intensity**	**Higher intensity**	**Detraining**
SI ($\times 10^{-4}$ L/min/mU)	CON	0.52 ± 0.48	0.98 ± 1.26	0.37 ± 0.20	0.42 ± 0.18
	EX	0.93 ± 0.87	1.49 ± 1.58	0.89 ± 0.93	1.46 ± 1.72
Sg (%/min)	CON	1.06 ± 0.14	1.20 ± 0.29	0.90 ± 0.72	1.36 ± 0.47
	EX	0.92 ± 0.64	0.97 ± 0.64	0.72 ± 0.86	1.20 ± 0.84
AIRg ([mU×min]/L	CON	560 ± 191[a]	720 ± 266[a,b]	862 ± 406[b,c]	1215 ± 602[c]
	EX	424 ± 169[a]	591 ± 204[a,b]	786 ± 302[b,c]	865 ± 602[c]
DI ($\times 10^{-2}$)	CON	2.40 ± 1.70	5.34 ± 5.01	2.89 ± 1.28	4.36 ± 1.39
	EX	3.59 ± 2.68	7.92 ± 5.87	5.63 ± 2.94	7.47 ± 7.52

[a-c]For variables with a significant ($P < 0.05$) time point effect, mean values with different superscripts differ, as determined by repeated measures ANOVA with Fisher-Hayter pairwise comparisons. A time point effect was detected for AIRg ($P = 0.001$), with no other treatment, time or treatment-time effects observed for variables.

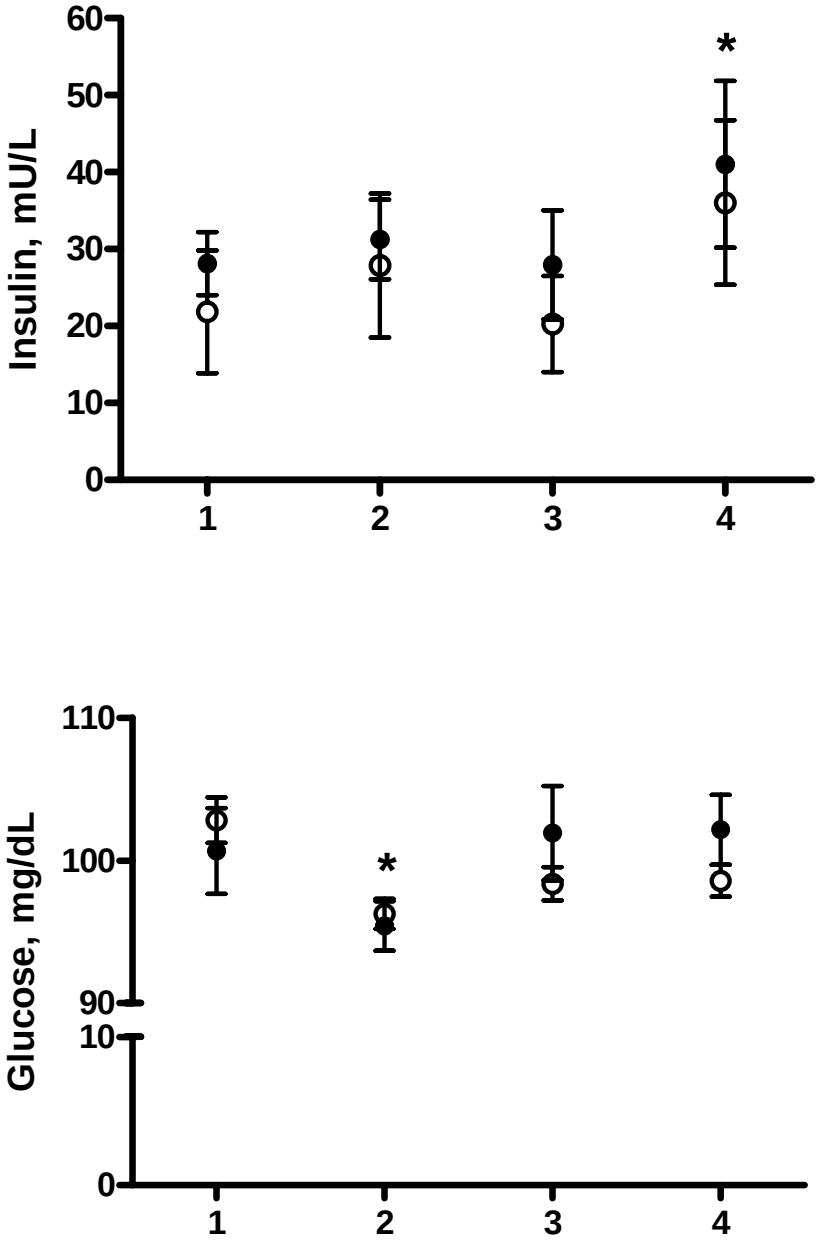

Figure 5.1: Mean ± SEM baseline insulin and glucose concentrations in control (open circles) and exercised horses (filled circles) at 4 time points representing (1) pre-exercise, (2) after low intensity exercise, (3) after higher intensity exercise, and (4) after detraining. *Value for both the control and exercised groups different ($P < 0.05$) than pre-exercise value.

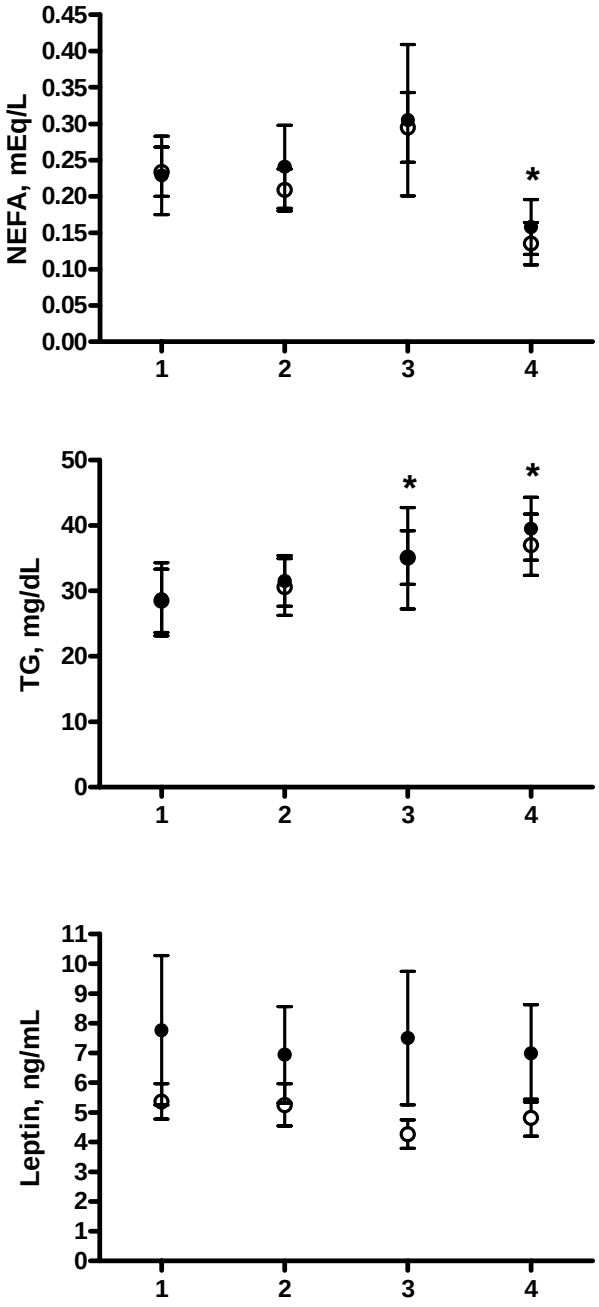

Figure 5.2: Mean ± SEM baseline nonesterified fatty acid (NEFA), triglyceride (TG), and leptin concentrations in control (open circles) and exercised horses (filled circles) at 4 time points representing (1) pre-exercise, (2) after low intensity exercise, (3) after higher intensity exercise, and (4) after detraining. *Value for both the control and exercised groups different ($P < 0.05$) than pre-exercise value.

GENERAL SUMMARY

The main findings of the present studies are as follows:

1) Practical methods of assessing adiposity were developed in horses and ponies and verified, including a condition scoring system for neck crest adiposity (CNS) and morphometric measurements for generalized (girth:height) and localized (neck circumference:height) adiposity.
2) Predictive tests for incipient pasture-associated laminitis in a cohort of ponies were identified, including hyperinsulinemia, hyperleptinemia, and generalized and localized (neck) obesity.
3) Diet-induced obesity in healthy Arabian geldings resulted in a compensated reduction in insulin sensitivity with hyperinsulinemia and hyperleptinemia. Although lipid concentrations (NEFA and triglyceride) decreased on a high concentrate diet, they did not differ before and after weight gain.
4) Obesity-induced insulin resistance in Arabian geldings was accompanied by an increase in chemokine but not inflammatory cytokine mRNA expression in subcutaneous adipose tissue. Additionally, there was a decrease in plasma TNFα protein concentration with weight gain.
5) Moderate intensity exercise training for 8 weeks in obese, insulin resistant Arabian geldings modestly reduced adiposity without affecting glucose and insulin dynamics or plasma hormone and lipid concentrations.

Collectively, these studies demonstrate the impact of obesity on metabolism and risk for laminitis in equids, and that exercise training may provide an effective countermeasure for modest reductions of obesity.

Implementation of the CNS system or neck circumference:height ratio in clinical practice would compliment BCS by providing a standardized assessment of a potentially high risk fat depot. Additionally, inclusion of neck adiposity measurements in obesity related studies will help elucidate the influence of this depot on metabolism and disease.

Hyperinsulinemia, hyperleptinemia, and generalized and localized (neck) obesity were consistently related to altered metabolic states, as all factors were epidemiologically

associated with risk for laminitis and experimentally related to compensated insulin resistance, where a decrease in insulin sensitivity is compensated for by an increase in insulin secretion. However, disturbances in lipid metabolism did not appear to play a role, as triglyceride concentration was not related to risk for laminitis and NEFA and triglyceride concentrations did not change with obesity or insulin resistance.

While measurement of BCS, CNS, and insulin and leptin concentrations would be beneficial to evaluate metabolic status and risk for laminitis, the necessity of identifying cutoff values to classify abnormal values presents a number of problems. Heavily relying on a single cutoff value to determine management strategies may be detrimental, as the efficacy of these values may vary depending on breed, season, assay methods, individual variability, or subsequent exposure to environmental factors. Therefore, cutoff values should only be used as a tool in the overall assessment and integration of physical, physiological, and environmental factors that are considered when developing management strategies for horses or ponies with a suspected risk of laminitis.

A role for inflammation in the link between obesity and insulin resistance or the induction of pasture-associated laminitis is not supported by the present studies. Plasma TNFα concentrations were not elevated during obesity-induced insulin resistance and were not predictive of incipient laminitis. Although chemokine mRNA expression was increased in subcutaneous adipose tissue after weight gain, inflammatory cytokine expression did not differ before and after weight gain. Of particular interest is the lack of change in TNFα, IL-1β and IL-6, which are the focal cytokines implicated in the induction of insulin resistance during obesity in humans (Gil et al., 2007). As there was insufficient evidence supporting a pro-inflammatory state during obesity and insulin resistance, these measurements were not included in the exercise training study.

The lack of a role of inflammation was unexpected, as previous associations between adiposity, insulin sensitivity, and blood inflammatory cytokine mRNA expression have been reported in horses (Vick et al., 2007). Additionally, plasma TNFα concentrations measured in the laminitis study were significantly greater in previously laminitic ponies than control ponies, even though TNFα was not a suitable predictor of incipient laminitis. These findings indicate a discrepancy between inflammatory state during chronic obesity or insulin resistance as opposed to the early stages of weight gain

or prior to incipient laminitis. In order to address this discrepancy, it is necessary for future studies to investigate the expression of a comprehensive selection of inflammatory cytokines in alternative adipose depots (e.g. visceral adipose), in alternative tissue types (e.g. skeletal muscle), and during chronic obesity. Additionally, determining the presence or absence of macrophage infiltration in adipose tissue during obesity would aid in the interpretation of the observed increase in chemokine but not cytokine expression in the present study.

Current recommendations to reduce obesity and insulin resistance include decreasing caloric intake and increasing physical activity. While the present study indicates that adiposity may be modestly reduced with as little as 4 weekly sessions of 30 minutes trotting, insulin sensitivity was not improved after 8 weeks of moderate intensity exercise training without dietary restriction. These results highlight the importance of providing evidence-based recommendations, as insulin resistance may not be reduced in obese horses by exercise alone. It is possible that a more intense exercise routine or the reduction of adiposity to a lean/moderate condition is necessary for the improvement of insulin sensitivity in obese horses. In order to develop effective recommendations, it is necessary for future research to investigate the effects of various levels of dietary restriction in addition to exercise training on reducing adiposity and insulin resistance.

APPENDICES

Appendix A: Supplementary material for Chapter 1

Additional measurements performed on 34 horses (21 Thoroughbreds and 13 Arabians)

In addition to the blood samples collected and analyzed in Chapter 1, collection of blood samples were collected again one week after the initial sampling time point for horses only (n = 34). Plasma samples from both weeks were analyzed in the same assay. Reproducibility of baseline concentrations was assessed by inter-week coefficients of variation and concordance correlation coefficients (Table A.1).

Repeatability of morphometric measurements was assessed by 3 repeated measurements on 3 separate horses by a single evaluator (RAC) within a one hour time period (Table A.2).

Ultrasound measurements of subcutaneous fat thickness were performed over the rump, back, rib, and shoulder as described in Chapter 2. Measurement variability was assessed in 3 horses by measuring each site on the left and right side in triplicate within a one hour period (intra-assay variability) and repeated once each day for two more consecutive days (iner-assay variability) by a single evaluator (RAC) (Table A.3). Correlations of condition scores, morphometric measurements and blood variables are presented in Table A.4.

Measurement differences between Thoroughbreds and Arabians

Although Thoroughbreds and Arabians were grouped together in a horse category in order to provide enough power for statistical analysis, there were differences between breeds for some variables. Median values for all measured variables and differences between breeds are reported in Table A.5.

Correlations of morphometrics with blood variables

Selected morphometric measurements that had strong correlations with condition scores were chosen to determine correlations with blood variables in Chapter 1. Correlations of additional morphometric measurements with leptin (Table A.6), insulin (Table A.7), glucose (Table A.8), and triglyceride (Table A.9) concentrations are reported.

Statistical analyses for Chapter 1

STATA code- Italicized words were changed depending on variable or group tested

Shapiro-Wilk test for normality within breed ($\alpha = 0.05$):

· swilk *varlist* if breed = = *1*

Spearman rank correlation coefficient within breed:

· spearman *varlist* if breed = = *1*

Median and interquartile ranges within each breed:

· tabstat *varlist* , by (breed) s(median p25 p75)

Kruskal-Wallis test for nonparametric differences between groups:

· kwallis *varlist* , by (breed)

Odds ratios for increased likelihood of hyperinsulinemia:

· logistic hi overweight if breed = = *1*

· logistic hi cresty if breed = = *1*

where hi = binomial variable of hyperinsulinemia (insulin concentration > 30 mU/L), overweight = binomial variable of BCS ≥ 7, and cresty = binomial variable of CNS ≥ 3

Comparisons of linear regression lines were performed in GraphPad Prism 4. An example of the comparison between ponies and horses for the regression analysis of girth:height vs. body condition score is presented in Table A.10.

Table A.1: Inter-week variability of hormone/metabolite measurements in 34 horses.

	Intra-assay CV	Inter-week CV	Concordance correlation coefficient
Insulin	8.5	39.9	0.36
Glucose	0.7	3.6	0.47
TG	2.8	22.4	0.62
NEFA	4.7	40.4	0.13
Leptin	6.3	9.9	0.93
Cortisol	4.5	17.6	0.20

Table A.2: Morphometric measurement variability of 3 repeated measurements in 3 horses.

Morphometric Measurement	Intra-assay CV, %
Girth	1.1
Waist	0.4
Neck Length	3.0
0.25 Neck circ.	2.1
0.50 Neck circ.	2.4
0.75 Neck circ.	0.7
Crest Height	2.4

Table A.3: Ultrasound measurement variability of 3 measurements within one day (intra-assay) or across 3 days (inter-assay) in 3 horses.

	Intra-assay CV, %	Inter-assay CV, %
Rump	4.1	3.6
Tailhead	2.1	3.0
Back	5.1	5.3
Rib	3.5	3.4
Shoulder	2.3	2.7

Table A.4: Correlations of ultrasound subcutaneous fat thickness of the rump, back, rib, and shoulder measured in 34 horses (21 Thoroughbreds and 13 Arabians) with condition scores, morphometric measurements, and blood variables.

	Rump		Back		Rib		Shoulder	
Variable	r_s	*P*-value	r_s	*P*-value	r_s	*P*-value	r_s	*P*-value
BCS	0.68	< 0.001	0.53	0.001	0.52	0.002	0.14	0.42
Body weight	0.22	0.21	0.33	0.054	0.25	0.15	-0.05	0.80
Girth	0.40	0.020	0.50	0.003	0.30	0.087	-0.13	0.46
Waist	0.38	0.029	0.44	0.008	0.34	0.053	-0.08	0.65
BMI 1	0.38	0.029	0.38	0.026	0.34	0.047	0.02	0.89
BMI 2	0.39	0.023	0.47	0.005	0.53	0.001	0.02	0.90
Girth:Height	0.54	0.001	0.49	0.003	0.37	0.032	-0.13	0.45
Waist:Height	0.55	0.001	0.48	0.004	0.45	0.007	-0.09	0.62
Girth:Length	0.23	0.20	0.24	0.17	0.27	0.12	-0.12	0.49
Waist:Length	0.26	0.14	0.24	0.17	0.33	0.060	-0.05	0.80
CNS	0.68	< 0.001	0.54	0.001	0.34	0.051	0.16	0.37
0.50 NC:Height	0.40	0.020	0.31	0.077	0.28	0.10	-0.02	0.90
Mean NC:Height	0.45	0.008	0.28	0.10	0.31	0.071	0.07	0.69
Crest Height	0.35	0.040	0.30	0.082	0.27	0.12	0.03	0.87
0.25 NC	0.51	0.002	0.43	0.012	0.56	0.001	-0.09	0.63
0.50 NC	0.45	0.008	0.47	0.005	0.29	0.10	-0.13	0.48
0.75 NC	0.41	0.016	0.44	0.009	0.32	0.064	0.03	0.86
0.50 NC:Length	0.42	0.015	0.35	0.040	0.30	0.086	-0.10	0.58
Mean NC:Length	0.40	0.019	0.34	0.052	0.28	0.10	-0.07	0.69
Insulin	0.26	0.13	0.15	0.40	0.36	0.038	0.05	0.76
Glucose	0.08	0.66	-0.06	0.72	0.18	0.32	0.15	0.40
Triglyceride	0.14	0.42	-0.05	0.78	0.26	0.13	-0.01	0.96
Leptin	0.35	0.041	0.27	0.13	0.33	0.056	0.11	0.55

Table A.5: Median (interquartile range) of variables measured in Chapter 1 for groups of Thoroughbreds, Arabians, and ponies.

Variable	Thoroughbred (n = 21)	Arabian (n = 13)	Pony (n = 75)
BCS	5.8 (5.3 - 6.3)[a]	6.3 (6 - 6.5)[a]	7 (6 - 7.5)[b]
Body weight, kg	568 (539 - 595)[a]	455 (422 - 468)[b]	345 (312 - 371)[c]
Height, cm	162 (159 - 163)[a]	151 (149 - 152)[b]	128 (123 - 136)[c]
Length, cm	181 (175 - 182)[a]	157 (155 - 161)[b]	146 (141 - 152)[c]
Girth, cm	197 (194 - 203)[a]	186 (181 - 188)[b]	170 (164 - 179)[c]
Waist, cm	216 (210 - 223)[a]	205 (196 - 207)[b]	190 (177 - 201)[c]
BMI 1, kg/m^2	223 (209 - 227)[a]	197 (192 - 207)[b]	209 (192 - 224)[b]
BMI 2, kg/m^2	198 (191 - 204)[a]	191 (182 - 196)[b]	184 (169 - 196)[b]
Girth:Height	1.23 (1.21 - 1.26)[a]	1.25 (1.21 - 1.25)[a]	1.32 (1.27 - 1.35)[b]
Waist:Height	1.36 (1.32 - 1.38)[a]	1.36 (1.34 - 1.38)[a]	1.48 (1.40 - 1.54)[b]
Girth:Length	1.11 (1.07 - 1.13)[a]	1.16 (1.15 - 1.18)[b]	1.15 (1.12 - 1.20)[b]
Waist:Length	1.21 (1.19 - 1.26)[a]	1.27 (1.26 - 1.30)[b]	1.29 (1.23 - 1.34)[b]
CNS	2 (1.5 - 2)[a]	2 (2 - 2.5)[a,b]	3 (2 - 4)[b]
Crest Height, cm	6 (5 - 7)[a]	6 (5 - 8)[a]	10 (8 - 13)[b]
0.25 NC, cm	76 (74 - 77)[a]	77 (75 - 79)[a]	70 (67 - 71)[b]
0.50 NC, cm	95 (92 - 98)[a]	91 (89 - 95)[a]	86 (83 - 90)[b]
0.75 NC, cm	112 (110 - 116)[a]	107 (104 - 111)[b]	100 (96 - 104)[c]
0.50 NC:Height	0.60 (0.58 - 0.61)[a]	0.60 (0.60 - 0.63)[a]	0.66 (0.62 - 0.71)[b]
Mean NC:Height	0.59 (0.58 - 0.60)[a]	0.61 (0.60 - 0.63)[b]	0.65 (0.62 - 0.69)[c]
0.50 NC:Length	1.03 (1.03 - 1.07)[a]	1.13 (1.04 - 1.17)[b]	1.18 (1.13 - 1.26)[c]
Mean NC:Length	1.04 (1.02 - 1.06)[a]	1.12 (1.06 - 1.15)[b]	1.17 (1.12 - 1.23)[c]
Rump, cm	1.77 (1.33 - 2.54)[a]	1.73 (1.47 - 2.35)[a]	
Back, cm	0.59 (0.52 - 0.74)[a]	0.52 (0.45 - 0.64)[a]	
Rib, cm	0.75 (0.70 - 0.80)[a]	0.75 (0.71 - 0.83)[a]	
Shoulder, cm	0.79 (0.73 - 0.86)[a]	0.84 (0.73 - 0.89)[a]	
Insulin, mIU/L	7.7 (4.5 - 9.9)[a]	34.7 (24.2 - 72.4)[b]	16.0 (8.1 – 38.2)[b]
Glucose, mg/dL	90.7 (88.5 - 93.3)[a]	101.5 (98.7 - 104.6)[b]	93.8 (89.1 – 100.0)[c]
Triglyceride, mg/dL	18.4 (17.4 - 23.4)[a]	32.6 (28.4 - 36.3)[b]	47.2 (35.2 – 58.2)[c]
Leptin, ng/mL	2.5 (2.2 - 3.8)[a]	5.4 (4.5 - 5.5)[b]	5.8 (3.4 – 8.1)[b]

[a-c]Medians with different superscripts differ ($P < 0.05$) between groups by Kruskal-Wallis test.

Table A.6: Correlations of leptin concentration and adiposity measurements in horses and ponies.

	Horse (n=34)		Pony (n=75)	
	rs	P-value	rs	P-value
BCS	0.38	0.027	0.74	<0.001
Body weight	-0.18	0.3	-0.05	0.68
Girth	-0.08	0.66	0.04	0.72
Waist	-0.10	0.58	0.04	0.71
BMI 1	-0.02	0.91	0.28	0.017
BMI 2	0.15	0.39	0.23	0.048
Girth:Height	0.39	0.024	0.68	<0.001
Waist:Height	0.32	0.066	0.44	<0.001
Girth:Length	0.55	0.001	0.42	<0.001
Waist:Length	0.51	0.002	0.31	0.001
CNS	0.50	0.003	0.63	<0.001
Crest Height	0.19	0.27	0.57	<0.001
0.25 NC	0.36	0.34	0.37	<0.001
0.50 NC	0.15	0.40	0.46	<0.001
0.75 NC	0.01	0.95	0.30	0.009
0.50 NC:Height	0.43	0.010	0.70	<0.001
Mean NC:Height	0.53	0.001	0.74	<0.001

Table A.7: Correlations of insulin concentration and adiposity measurements in horses and ponies.

	Horse (n=34)		Pony (n=75)	
	rs	P-value	rs	P-value
BCS	0.43	0.011	0.51	<0.001
Body weight	-0.39	0.021	-0.25	0.028
Girth	-0.22	0.21	-0.09	0.43
Waist	-0.33	0.060	-0.26	0.02
BMI 1	-0.25	0.15	-0.06	0.61
BMI 2	0.01	0.96	-0.11	0.35
Girth:Height	0.31	0.075	0.41	<0.001
Waist:Height	0.16	0.35	0.06	0.59
Girth:Length	0.67	<0.001	0.27	0.020
Waist:Length	0.52	0.002	-0.04	0.74
CNS	0.49	0.003	0.58	<0.001
Crest Height	0.41	0.017	0.49	<0.001
0.25 NC	0.36	0.037	0.12	0.29
0.50 NC	-0.01	0.95	0.27	0.017
0.75 NC	-0.22	0.21	0.16	0.18
0.50 NC:Height	0.40	0.021	0.50	<0.001
Mean NC:Height	0.49	0.003	0.51	<0.001

Table A.8: Correlations of glucose concentration and adiposity measurements in horses and ponies.

	Horse (n=34)		Pony (n=75)	
	rs	P-value	rs	P-value
BCS	0.39	0.023	0.21	0.066
Body weight	-0.48	0.004	-0.21	0.077
Girth	-0.45	0.008	-0.08	0.48
Waist	-0.44	0.009	-0.22	0.064
BMI 1	-0.29	0.094	-0.06	0.63
BMI 2	-0.08	0.65	-0.11	0.34
Girth:Height	0.12	0.51	0.22	0.055
Waist:Height	0.09	0.60	-0.01	0.97
Girth:Length	0.48	0.004	0.10	0.4
Waist:Length	0.52	0.002	-0.11	0.35
CNS	0.42	0.014	0.27	0.021
Crest Height	0.39	0.023	0.23	0.046
0.25 NC	0.24	0.17	-0.01	0.96
0.50 NC	-0.14	0.44	0.16	0.18
0.75 NC	-0.37	0.029	0.07	0.58
0.50 NC:Height	0.32	0.069	0.28	0.014
Mean NC:Height	0.42	0.013	0.28	0.016

Table A.9: Correlations of triglyceride concentration and adiposity measurements in horses and ponies.

	Horse (n=34)		Pony (n=75)	
	rs	P-value	rs	P-value
BCS	0.35	0.043	0.46	<0.001
Body weight	-0.39	0.022	-0.07	0.56
Girth	-0.34	0.050	0.001	0.99
Waist	-0.34	0.052	0.08	0.52
BMI 1	-0.28	0.11	0.28	0.016
BMI 2	-0.05	0.77	0.24	0.035
Girth:Height	0.17	0.33	0.44	<0.001
Waist:Height	0.07	0.68	0.35	0.002
Girth:Length	0.59	0.001	0.34	0.003
Waist:Length	0.50	0.003	0.32	0.005
CNS	0.42	0.014	0.47	<0.001
Crest Height	0.17	0.33	0.36	0.002
0.25 NC	0.34	0.047	0.24	0.041
0.50 NC	-0.01	0.96	0.20	0.080
0.75 NC	-0.22	0.22	0.07	0.570
0.50 NC:Height	0.38	0.029	0.41	<0.001
Mean NC:Height	0.50	0.003	0.42	<0.001

Table A.10: Example of GraphPad Prism output for the comparison of linear regression lines between horses and ponies. Comparisons of linear regression lines of girth:height v. body condition score for ponies or horses is shown.

	Girth:Height(Pony)	Girth:Height(Horse)	Global (shared)
Comparison of Fits			
Null hypothesis			One curve for all data sets
Alternative hypothesis			Different curve for each data set
P value			P<0.0001
Conclusion (alpha = 0.05)			Reject null hypothesis
Preferred model			Different curve for each data set
F (DFn, DFd)			23.28 (2,106)
Different curve for each data set			
Best-fit values			
INTERCEPT	0.9614	1.039	
SLOPE	0.05236	0.03182	
Std. Error			
INTERCEPT	0.02962	0.03975	
SLOPE	0.004297	0.006467	
95% Confidence Intervals			
INTERCEPT	0.9023 to 1.021	0.9582 to 1.120	
SLOPE	0.04379 to 0.06093	0.01864 to 0.04500	
Goodness of Fit			
Degrees of Freedom	74	32	
R^2	0.6674	0.4307	
Absolute Sum of Squares	0.1101	0.02804	
Sy.x	0.03857	0.0296	
One curve for all data sets			
Best-fit values			
INTERCEPT	0.9252	0.9252	0.9252
SLOPE	0.05563	0.05563	0.05563
Std. Error			
INTERCEPT	0.02685	0.02685	0.02685
SLOPE	0.004024	0.004024	0.004024
95% Confidence Intervals			
INTERCEPT	0.8719 to 0.9784	0.8719 to 0.9784	0.8719 to 0.9784
SLOPE	0.04765 to 0.06362	0.04765 to 0.06362	0.04765 to 0.06362
Goodness of Fit			
Degrees of Freedom			108
R^2	0.6201	-0.4827	0.639
Absolute Sum of Squares	0.1258	0.07302	0.1988
Sy.x			0.0429
Constraints			
INTERCEPT	INTERCEPT is shared	INTERCEPT is shared	
SLOPE	SLOPE is shared	SLOPE is shared	

Appendix B: Supplementary material for Chapter 2

Pasture composition

Pasture sampling and analysis was performed during March 2006, March 2007 and May 2007 (Table B.1). During March 2006, 6 pastures were sampled on which a majority of the evaluated ponies were maintained. For each pasture, grass was collected at 10 – 20 different locations to form one composite, representative sample. Samples were dried in a drying oven and sent to Dairy One Forage Laboratory (Ithaca, NY) for analysis.

In March and May 2007, two pastures were sampled on which a majority of the evaluated ponies were maintained. A battery-powered reel lawnmower was used to collect the samples in 2007. After strips from all areas of the pasture were mowed, pieces of dirt, manure and other debris were removed from the collected clippings within 5 min. For each month, pasture samples were sampled at 2pm on 3 or 4 consecutive days on the week prior to sampling the ponies. Each day the pasture clippings were collected in cloth bags and immediately placed in liquid nitrogen. Samples were stored at -80°C until analysis. For analysis, grab samples of each bag were weighed and oven dried, then sent to Dairy One Forage Laboratory (Ithaca, NY).

Statistical analyses for Chapter 2

STATA code- Italicized words were changed depending on variable or group tested

Median and 95% confidence intervals within each laminitis group:

· centile *varlist* if laminitis = = *0*, cci

Kruskal-Wallis test for nonparametric comparisons between laminitis groups:

· kwallis *varlist* , by (laminitis)

Wilcoxon's matched pairs test for nonparametric comparison between years/evaluations:

· signrank *var1 = = var2*

where var1 and var2 are the same variable measured in two separate years

Area under the global receiver operating characteristic (ROC) curves:
· roctab *depvar indepvar*, detail
where depvar is the binomial variable for occurrence of laminitis within the year of evaluation and indepvar is a continuous predictor variable

Area under the local ROC curves and likelihood ratios:
· roctab *depvar indepvar*, detail binomial
where depvar is the binomial variable for occurrence of laminitis within the year of evaluation and indepvar is a binary predictor variable

Comparison of ROC curves:
· roccomp *depvar indepvars*

Table B.1: Nutrient composition of pasture during Evaluations 1 and 2.

Nutrient	March 2006[a]	March 2007[b]	May 2007[c]
% Crude protein	16.8 ± 4.1	9.8 ± 2.7	21.9 ± 3.4
% Acid detergent fiber	33.8 ± 4.9	20.6 ± 7.2	18.6 ± 2.2
% Neutral detergent fiber	58.9 ± 8.1	39.2 ± 13.6	39.6 ± 1.9
% Nonstructural carbohydrate	9.9 ± 3.1	1.9 ± 0.9	18.6 ± 7.8
% Starch	1.2 ± 0.3	0.46 ± 0.30	3.2 ± 5.3
% Water soluble carbohydrate	8.8 ± 2.9	1.4 ± 1.0	15.4 ± 4.7
% Ethanol soluble carbohydrate		2.0 ± 1.2	11.5 ± 4.0
% Crude fat	2.4 ± 0.6	1.3 ± 0.5	3.7 ± 0.7
% Ash	10.4 ± 2.3	44.5 ± 16.1	20.0 ± 8.9
% Ca	0.87 ± 0.47	0.59 ± 0.15	0.54 ± 0.20
% P	0.23 ± 0.05	0.14 ± 0.04	0.31 ± 0.05
% Mg	0.17 ± 0.02	0.12 ± 0.02	0.18 ± 0.02
% K	1.6 ± 0.8	0.36 ± 0.16	2.3 ± 0.7
% Na	0.006 ± 0.002	0.008 ± 0.002	0.008 ± 0.004
ppm Fe	720 ± 368	8833 ± 2782	2515 ± 1948
ppm Zn	22 ± 5	45.3 ± 6.7	30.1 ± 3.9
ppm Cu	6.2 ± 2.5	15.8 ± 1.8	11.0 ± 2.8
ppm Mn	129 ± 55	504 ± 96	218 ± 115
ppm Mo	0.58 ± 0.15	0.34 ± 0.11	0.52 ± 0.13
Digestible energy (Mcal/kg)	2.34 ± 0.27	2.79 ± 0.47	3.28 ± 0.06

[a]Values represent the mean ± SD of 6 pastures from which one representative sample of each was analyzed.
[b]Values represent the mean ± SD of 2 pastures from which 4 replicate samples of each were analyzed.
[c]Values represent the mean ± SD of 2 pastures (same pastures as March 2007) from which 3 replicate samples of each were analyzed.

Appendix C: Supplementary material for Chapter 3

Additional data for adiposity measurements

Presentation of data for mean neck circumference (NC) and girth circumference was chosen instead of NC:height or girth:height ratios, as intra-horse comparisons were made in this longitudinal study and it was expected that the height of each horse would not change. However, ratios were calculated, and are presented with measurements of 0.25 NC, 0.50 NC, and 0.75 NC in Table C.1. In tables 3.2 and C.1, changes in adiposity measurements before and after weight gain are presented as the difference between the mean of Weeks 1 – 7 (before weight gain) and Weeks 24 – 30 (after weight gain). These changes are presented as a percentage of before weight gain values, along with the mean before and after weight gain values in Table C.2.

Adiposity was assessed once every two weeks during the study, however for simplicity only data for the last week of each period is presented in Chapter 3. Mean values for all measurement time points are presented in Figures C.1, C.2 and C.3.

Tabular presentation of blood variable data

To supplement the graphical presentation of blood variables in Chapter 3, mean ± SEM values are presented in Table C.3 and C.4. Included in Table C.3 are the glucose and insulin concentrations for Weeks 4, 8, 17, and 25, which were omitted from the graphs. Horses were in stalls for biopsy recovery during these sampling time points, and therefore the observed increase in insulin concentration during these weeks may have occurred because of a stress response.

Additional presentation minimal model data

Mean values of glucose and insulin concentrations during the frequently sampled intravenous glucose tolerance tests (FSIGTT) are presented graphically. Values for Periods 1 and 5 are presented in Figure C.4 and values for all periods are presented in Figure C.5.

To supplement the graphical presentation of minimal model parameters in Chapter 3, mean ± SEM values are presented in Table C.5.

Dexamethasone suppression test

The dexamethasone suppression test has been suggested as potentially useful as a dynamic test for altered insulin sensitivity or β-cell function (Bailey et al., 2007). Therefore, in addition to cortisol, insulin concentration was measured during the dexamethasone suppression. Insulin concentration increased ($P < 0.001$) in all horses (paired *t* test) by 8 ± 3-fold 19 hours after dexamethasone administration (Figure C.6). The change in insulin concentration during the dexamethasone suppression test (during Week 2) was compared to SI and AIRg calculated from the FSIGTT during Week 3 (Period 1) (Figure C.7). Relationships were not linear, therefore spearman correlation coefficients were used for analysis. Change in insulin concentration was not significantly related to SI ($r_s = -0.36$, $P = 0.23$), but was positively correlated with AIRg ($r_s = 0.58$, $P = 0.037$).

Statistical analyses for Chapter 3

STATA code- Italicized words were changed depending on variable or time interval (week or period) tested

Shapiro-Wilk test for normality within week/period (α = 0.05):

· swilk *varlist* if *week = = 1*

Grubb's test for outliers within week/period (α = 0.01):

· grubbs *varlist* if *week = = 1*, level (99)

· list *grubbs_varlist* horse if *week = = 1*

Mean, standard deviation and standard error of the mean within each week/period:

· tabstat *varlist* , by (*week*) s(mean sd sem)

Two sample paired *t* test:

· ttest *var1 = = var2*

Repeated measures ANOVA with Fisher-Hayter pairwise comparisons (α = 0.05):

· anova *depvar* horse *week*, repeated (*week*)

· fhcomp *week*

Statistical outliers

Outlying values were determined by Grubb's test ($\alpha = 0.01$) within each week or period. These values were removed from the data set prior to statistical analysis. A complete list of outliers for Chapter 3 analysis is presented in Table C.6.

Table C.1: Additional morphometric measurements measured during the final week of each period and overall change from before to after weight gain. Values are presented as mean ± SD.

Variable	Week 3	Week 7	Week 16	Week 24	Week 30	Change*
0.25 NC, cm	75 ± 3[a]	75 ± 3[a]	78 ± 3[b]	80 ± 2[c]	80 ± 3[c]	5 ± 2
0.50 NC, cm	91 ± 4[a]	91 ± 5[a]	96 ± 5[b]	98 ± 5[b]	98 ± 4[b]	6 ± 2
0.75 NC, cm	108 ± 4[a]	107 ± 4[a]	112 ± 5[b]	114 ± 4[c]	115 ± 4[c]	7 ± 2
0.50 NC:Height	0.60 ± 0.02[a]	0.61 ± 0.03[a]	0.64 ± 0.03[b]	0.65 ± 0.03[b]	0.65 ± 0.02[b]	0.05 ± 0.01
Girth:Height	1.19 ± 0.04[a]	1.19 ± 0.04[a]	1.26 ± 0.04[b]	1.31 ± 0.04[c]	1.32 ± 0.06[c]	0.11 ± 0.02

*Difference between the mean of Weeks 1 – 7 (before weight gain) and Weeks 24 – 30 (after weight gain)

[a,b,c]Means in the same row with different superscripts differ ($P < 0.05$) according to Fisher-Hayter pairwise comparisons.

Table C.2: Overall differences in adiposity measurements before (Weeks 1 – 7) and after (Weeks 24 – 30) weight gain in 13 horses. Values are reported as mean ± SD.

Variable	Before weight gain[a]	After weight gain[b]	Change[c]	Percent change[d]	*t* test[e]
Body weight	437 ± 43	525 ± 50	88 ± 11	20 ± 2	< 0.001
BCS	6.1 ± 1.0	7.9 ± 0.7	1.7 ± 0.5	29 ± 11	< 0.001
Girth, cm	181 ± 7	198 ± 8	17 ± 3	9.6 ± 1.5	< 0.001
Girth:Height	1.20 ± 0.04	1.31 ± 0.05	0.11 ± 0.02	9.6 ± 1.6	< 0.001
Waist, cm	198 ± 8	218 ± 9	20 ± 4	10 ± 2	< 0.001
CNS	2.1 ± 0.6	3.1 ± 0.5	0.9 ± 0.4	48 ± 25	< 0.001
Mean NC, cm	92 ± 4	98 ± 4	6 ± 2	6.7 ± 2.1	< 0.001
0.25 NC, cm	75 ± 3	80 ± 3	5 ± 2	6.7 ± 2.4	< 0.001
0.50 NC, cm	92 ± 4	98 ± 4	6 ± 2	7.0 ± 2.7	< 0.001
0.75 NC, cm	108 ± 4	115 ± 4	7 ± 2	6.5 ± 1.9	< 0.001
0.50 NC:Height	0.61 ± 0.03	0.65 ± 0.02	0.05 ± 0.01	7.0 ± 2.7	< 0.001
Crest Height, cm	9.8 ± 1.5	14.1 ± 1.5	4.2 ± 0.8	44 ± 12	< 0.001
Rump, cm	1.70 ± 0.69	3.29 ± 0.94	1.59 ± 0.50	160 ± 47	< 0.001
Back, cm	0.63 ± 0.13	0.91 ± 0.18	0.27 ± 0.08	44 ± 12	< 0.001
Rib, cm	0.81 ± 0.14	0.83 ± 0.12	0.02 ± 0.10	3.1 ± 11.5	0.56
Shoulder, cm	0.79 ± 0.06	0.99 ± 0.10	0.19 ± 0.06	24 ± 7	< 0.001
% Fat	15.6 ± 4.3	25.5 ± 5.8	9.9 ± 3.1	66 ± 24	< 0.001

[a]Within each horse, the mean of Weeks 1 – 7 were calculated then averaged over all horses

[b]Within each horse, the mean of Weeks 24 – 30 were calculated then averaged over all horses

[c]Within each horse, the before mean before weight gain value was subtracted from the mean after weight gain value, then averaged over all horses

[d]Percentage change from before weight gain was calculated for each horse then averaged over all horses

[e]Paired *t* test comparing before and after weight gain values

Table C.3: Insulin and glucose concentrations during all sampling time points. Values are presented as mean ± SEM.

Week	Insulin, mU/L	Glucose, mg/dL
1	4.57 ± 0.5	92.21 ± 1.55
2	3.45 ± 0.27	90.46 ± 0.92
3	4.48 ± 0.54	94.93 ± 1.93
4	3.44 ± 0.37	93.27 ± 1.31
5	4.63 ± 0.52	88.95 ± 0.9
6	6.2 ± 0.57	88.11 ± 0.89
7	10.62 ± 2.59	92.9 ± 1.46
8	32.01 ± 5.5	95.21 ± 2.09
9	17.19 ± 3.58	95.3 ± 2.1
10	15.97 ± 2.11	93.52 ± 1.42
11	12.89 ± 1.82	92.52 ± 0.7
12	11.82 ± 1.39	91.12 ± 0.73
13	13.06 ± 2	92.71 ± 0.98
14	14.53 ± 2.68	91.07 ± 0.95
15	13.7 ± 1.76	92.03 ± 0.79
16	15.45 ± 1.78	95.37 ± 0.94
17	36.63 ± 8.32	93.29 ± 1.07
18	18.79 ± 2.63	93.67 ± 0.89
19	23.65 ± 3.1	93.92 ± 0.96
20	33.2 ± 5.5	92.91 ± 0.93
21	33.45 ± 6.19	94.83 ± 0.98
22	45.88 ± 9.03	92.78 ± 1.45
23	61.66 ± 15.65	95.5 ± 1.57
24	31.08 ± 5.47	94.64 ± 0.85
25	52.53 ± 11.69	94.16 ± 1.66
26	16.96 ± 2.97	91.17 ± 0.96
27	32.34 ± 9.9	89.29 ± 1.43
28	17.11 ± 3.57	88.36 ± 0.58
29	19.82 ± 5.03	91.92 ± 1.63
30	34.1 ± 6.49	96.22 ± 1.01

Table C.4: Triglyceride, nonesterified fatty acid (NEFA) and leptin concentrations during all sampling time points. Values are presented as mean ± SEM.

Week	Triglyceride, mg/dL	NEFA, mEq/L	Leptin, ng/mL
1	34.39 ± 3.91	0.32 ± 0.02	3.5 ± 0.42
3	32.63 ± 2.64	0.33 ± 0.03	3.34 ± 0.47
5	24.89 ± 1.21	0.12 ± 0.01	3.91 ± 0.47
7	34.69 ± 1.79	0.15 ± 0.03	4.43 ± 0.62
10	20.93 ± 1.5	0.08 ± 0.01	5.29 ± 0.55
12	31.62 ± 2.33	0.06 ± 0.01	6.27 ± 0.7
14	30.39 ± 1.88	0.05 ± 0.01	6.43 ± 0.58
16	23.44 ± 1.81	0.1 ± 0.01	6.88 ± 0.61
18	22.15 ± 1.26	0.07 ± 0.01	7.72 ± 0.57
20	27.28 ± 1.88	0.07 ± 0.01	8.26 ± 0.51
22	22.43 ± 1.44	0.09 ± 0.01	9.07 ± 0.61
24	23.82 ± 1.51	0.19 ± 0.02	10.66 ± 0.7
26	19.39 ± 1.64	0.33 ± 0.04	7.02 ± 1.06
28	39.18 ± 3.46	0.24 ± 0.02	8.17 ± 0.73
30	30.57 ± 2.1	0.34 ± 0.03	9.84 ± 0.89

Table C.5: Minimal model parameters during all study periods. Values are presented as mean ± SEM.

Parameter	Period 1	Period 2	Period 3	Period 4	Period 5
SI ($\times 10^{-4}$ L/min/mU)	2.07 ± 0.49	3.78 ± 0.72	1.44 ± 0.17	0.62 ± 0.10	0.39 ± 0.07
Sg (%/min)	1.07 ± 0.14	1.72 ± 0.25	1.71 ± 0.27	1.29 ± 0.15	1.17 ± 0.20
AIRg ([mU×min]/L	206 ± 24	388 ± 44	662 ± 68	804 ± 74	973 ± 109
DI ($\times 10^{-2}$)	3.70 ± 0.66	11.63 ± 1.51	8.84 ± 0.73	5.30 ± 0.82	3.63 ± 0.72

Table C.6: Statistical outliers for Chapter 3.

Variable	Value	Time point	Horse
Insulin, mU/L	39.2	Week 7	244
Insulin, mU/L	53.2	Week 9	230
Insulin, mU/L	41.4	Week 14	230
Insulin, mU/L	135.9	Week 27	232
Insulin, mU/L	74.70128	Week 29	248
Glucose, mg/dL	124.7225	Week 7	244
Triglyceride, mg/dL	90.86	Week 14	233
Triglyceride, mg/dL	76.72	Week 26	251
NEFA, mEq/L	0.25	Week 5	229
SI ($\times 10^{-4}$ L/min/mU)	4.9117	Period 3	250
SI ($\times 10^{-4}$ L/min/mU)	2.3216	Period 4	250
DI ($\times 10^{-2}$)	2117	Period 3	230

Table C.7: Nutrient composition of individual feeds and hays fed during the weight gain study. Analysis was performed on a representative composite sample from 3 bags of feed or 10 bales of hay.

	Alfalfa/timothy hay	Alfalfa/orchard grass hay 1	Chopped alfalfa	Sweet feed	Alfalfa/orchard grass hay 2
Period 1, kg/d/horse	8	0	0	0	0
Period 2, kg/d/horse	0	1.6	1.5	3	0
Period 3, kg/d/horse	0	2.7	2.5	6	0
Period 4, kg/d/horse	0	3.2	2.5	6.5	0
Period 5, kg/d/horse	0	0	0	0	12
Dry matter, %	89.8	88.6	86.0	88.5	88.5
Crude protein, %	12.3	15.3	19.4	15.5	16
Acid detergent fiber, %	38	39.8	32.6	4.4	31.5
Neutral detergent fiber, %	60.1	54.8	38.2	14.5	50.9
Nonstructural carbohydrate, %	7.5	6.8	14.4	55.7	10.8
Starch, %	1.8	0.3	3.0	49.3	2.0
Water-soluble carbohydrate, %	5.8	6.5	11.3	6.3	8.9
Crude fat, %	2.7	2.6	3.9	7.6	2.1
Ash, %	8.4	9.4	10.4	6.0	10.1
Ca, %	0.87	0.88	1.17	0.78	1.16
P, %	0.25	0.25	0.29	0.62	0.24
Mg, %	0.18	0.27	0.33	0.28	0.27
K, %	2.23	2.79	2.67	0.88	2.20
Na, %	0.013	0.017	0.053	0.202	0.034
Fe, ppm	82	97	307	315	184
Zn, ppm	28	22	26	138	21
Cu, ppm	11	8	10	57	6
Mn, ppm	44	69	41	82	63
Mo, ppm	1.4	1.9	2.5	1.6	1.2
Cl, %	0.85	0.88	0.56	0.50	0.52
Digestible energy, Mcal/kg	2.2	2.2	2.4	3.6	2.2

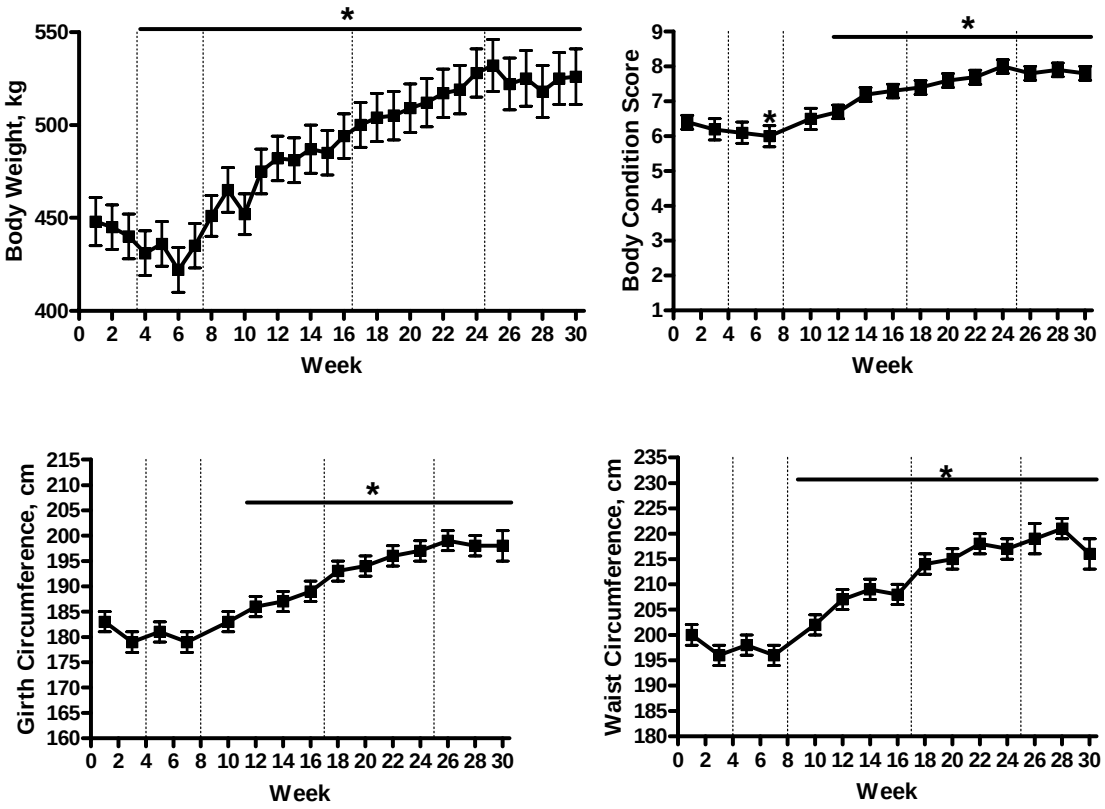

Figure C.1: Generalized adiposity as assessed by body weight, body condition score, girth and waist circumferences during all evaluation time points. Data are presented as mean ± SEM. Dashed lined indicated different study periods. *$P < 0.05$ compared to Period 1 (Weeks 1 – 3) according to Fisher-Hayter pairwise comparisons.

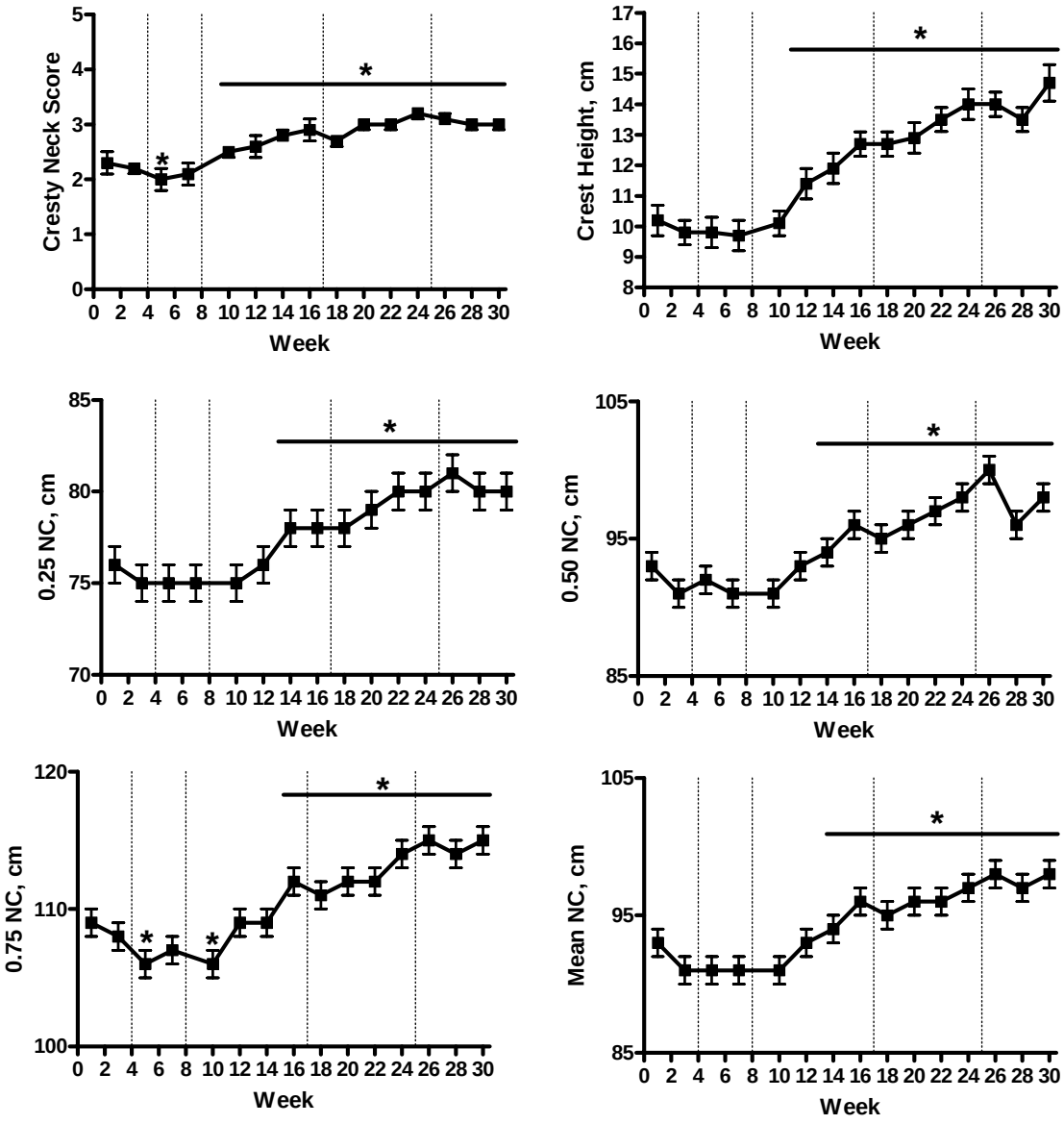

Figure C.2: Neck adiposity as assessed by cresty neck score, crest height, 0.25 neck circumference (NC), 0.50 NC, 0.75 NC, and mean NC during all evaluation time points. Data are presented as mean ± SEM. Dashed lined indicated different study periods. *$P <$ 0.05 compared to Period 1 (Weeks 1 – 3) according to Fisher-Hayter pairwise comparisons.

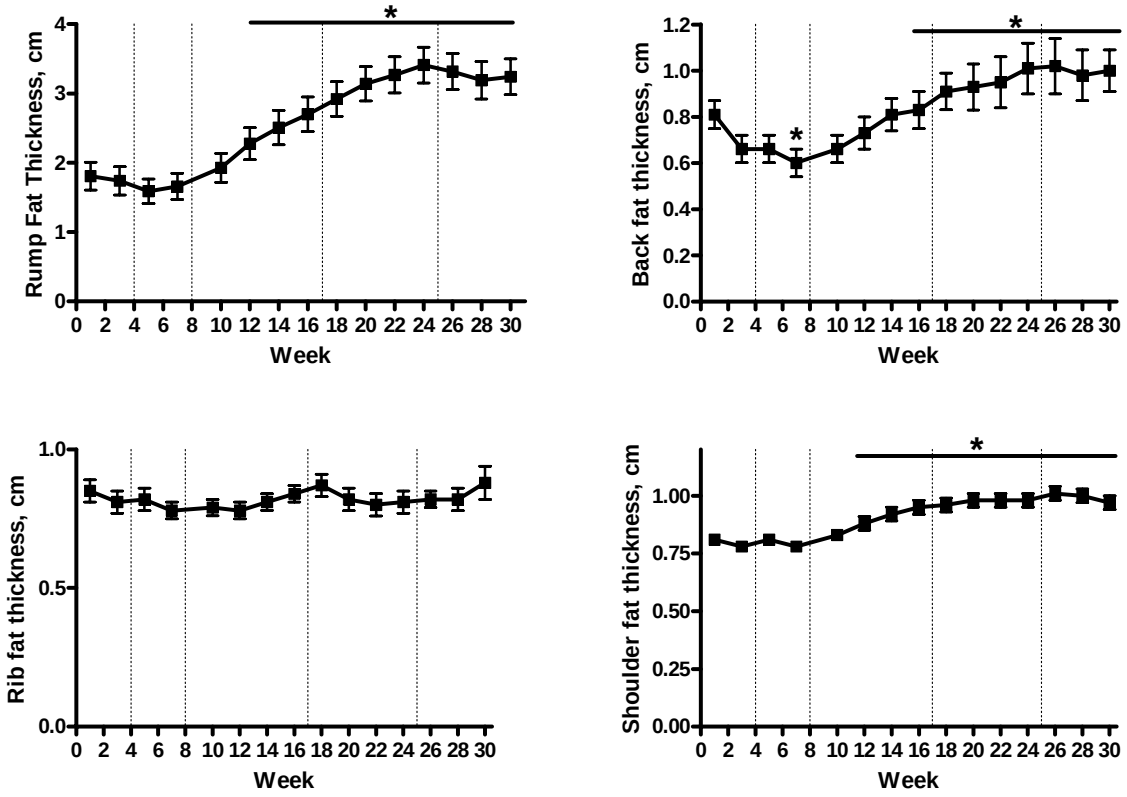

Figure C.3: Subcutaneous fat thickness assessed by ultrasound over the rump, back, rib, and shoulder during all evaluation time points. Data are presented as mean ± SEM. Dashed lined indicated different study periods. **P* < 0.05 compared to Period 1 (Weeks 1 – 3) according to Fisher-Hayter pairwise comparisons.

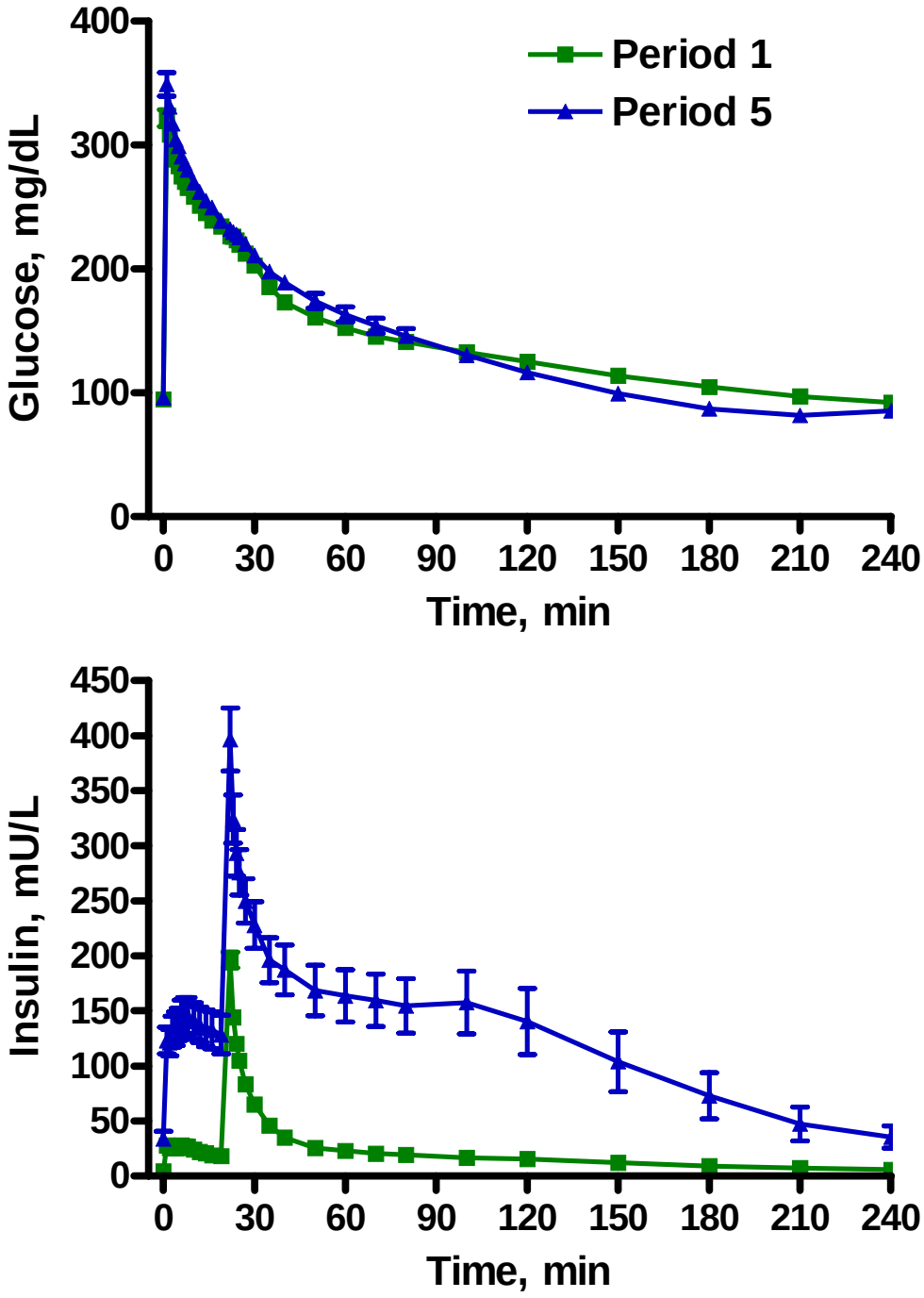

Figure C.4: Glucose and insulin concentrations during the frequently sampled intravenous glucose tolerance tests during Periods 1 and 5.

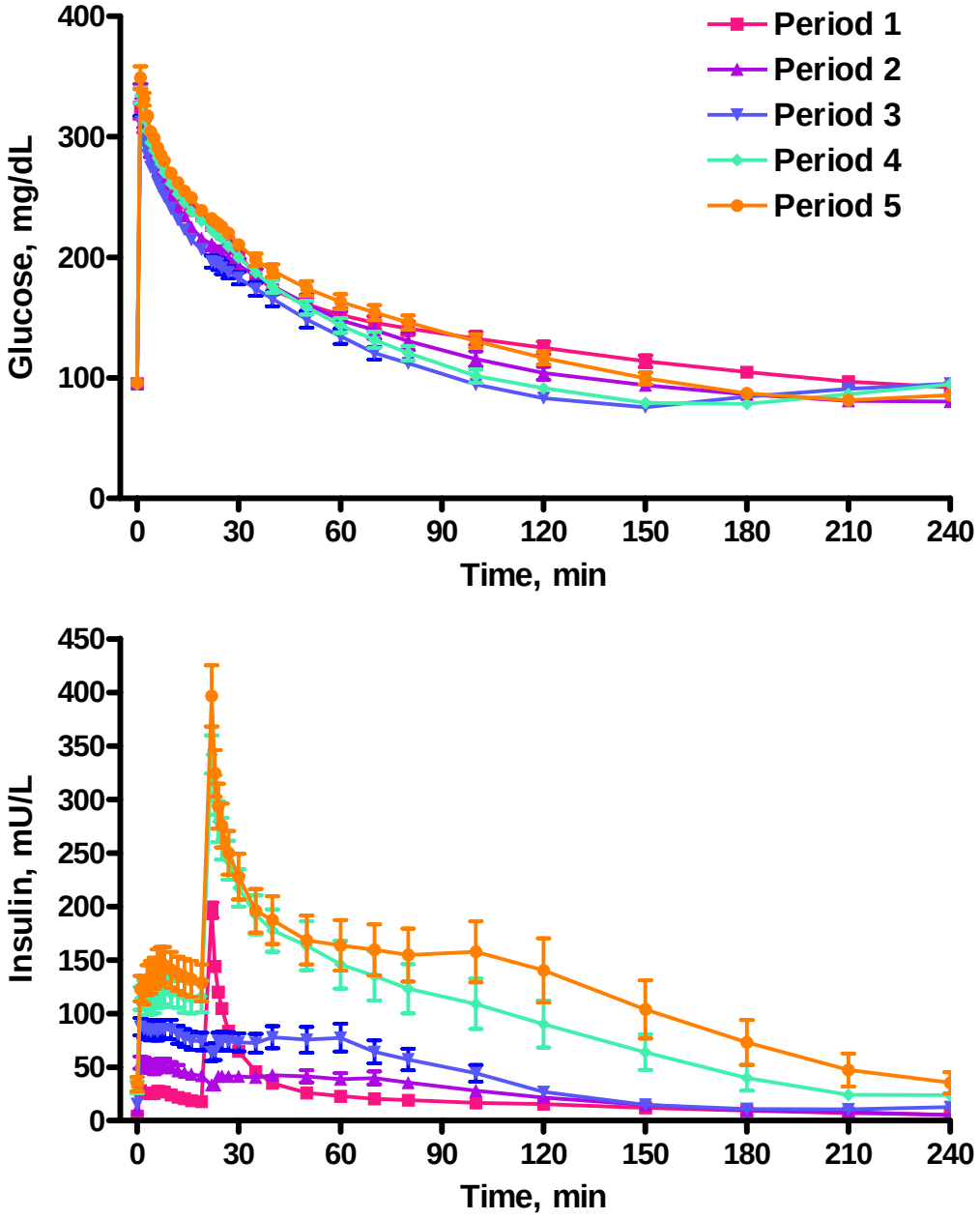

Figure C.5: Glucose and insulin concentrations during the frequently sampled intravenous glucose tolerance tests during all time periods.

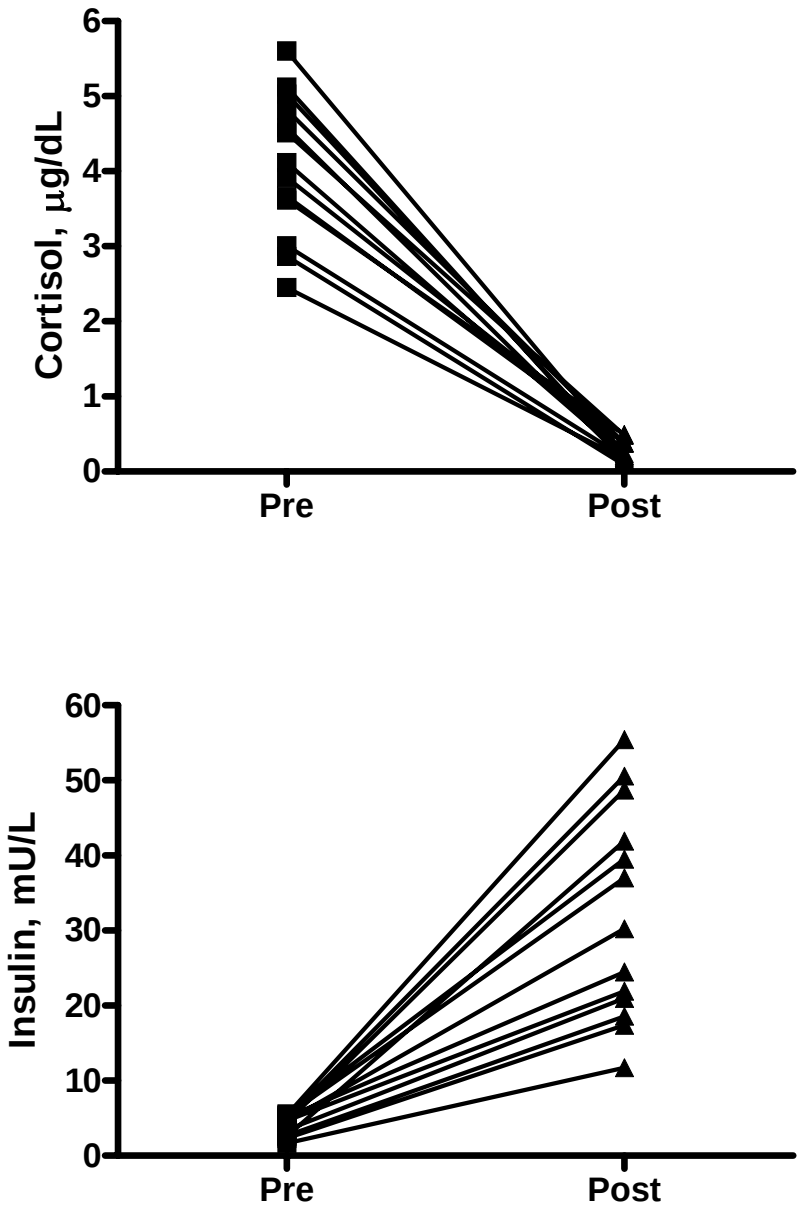

Figure C.6: Cortisol and insulin concentrations before (pre) and 19 hours after (post) dexamethasone administration.

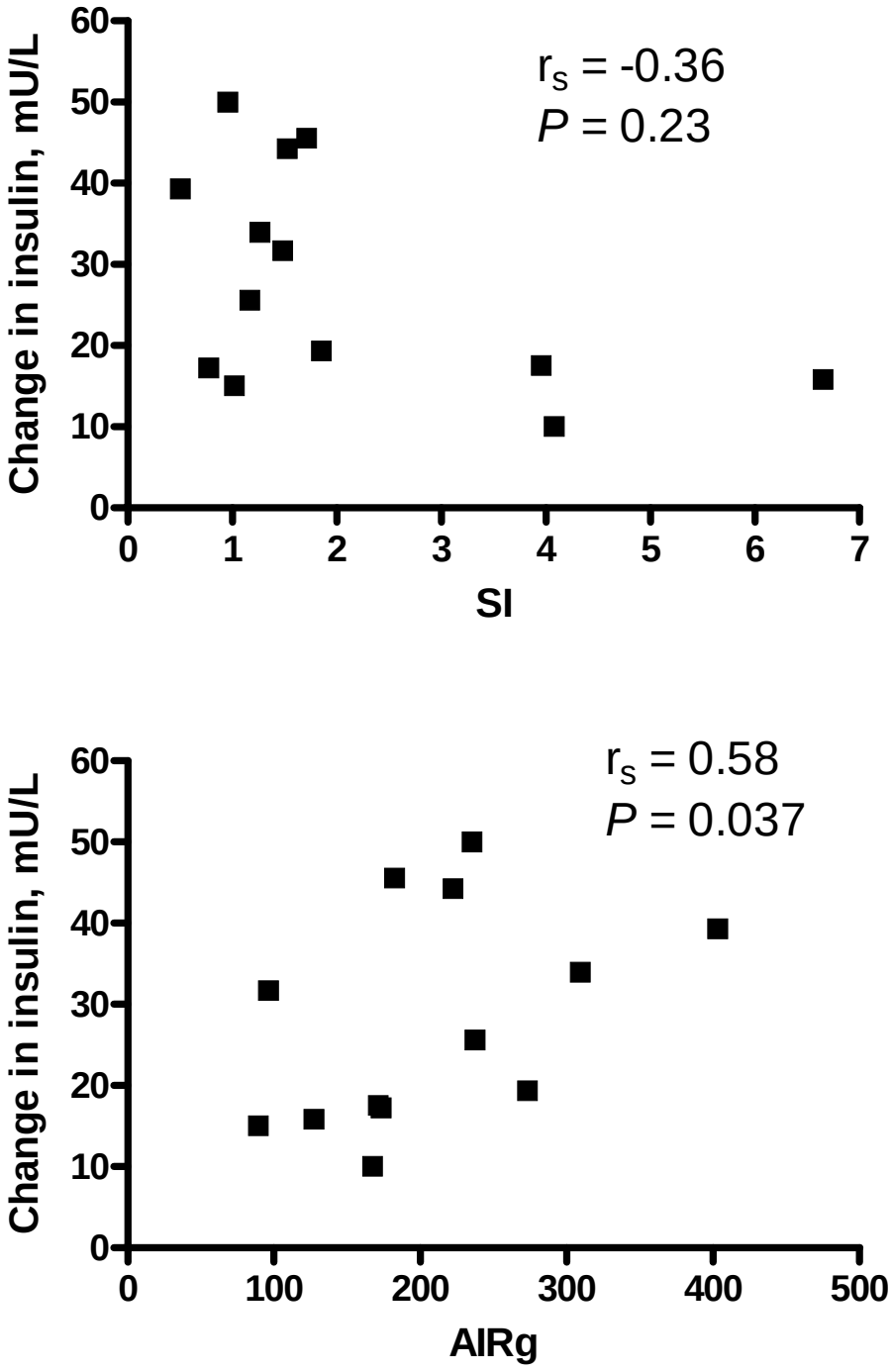

Figure C.7: Correlations of the change in insulin concentration during the dexamethasone suppression test (Week 2) and SI or AIRg (Week 3).

Appendix D: Supplementary material for Chapter 4

Protocol for total RNA extraction from adipose tissue

(Provided by Nick Frank and Sarah Elliot, University of Tennessee)

1. Add 1 mL Trizol per 100 mg of tissue. The tissue is always kept stored at -80 until used for RNA extraction.
2. Homogenize at maximum speed for 30 sec.
3. Keep at room temperature for 5 min.
4. Add 0.5 mL chloroform per 100 mg of tissue (half the volume of Trizol added in Step 1).
5. Vortex for 15 sec.
6. Keep at room temperature for 5 min.
7. Centrifuge 3000 rpm, 4 degrees, 20 min.
8. Collect the top layer, be careful not to get bits of bottom layer.
9. Repeat centrifugation if the layer is dirty, (try to avoid having to do this, it decreases yield).
10. Add 0.5 mL 100% isopropyl alcohol per 100 mg of tissue (same volume as that of chloroform added in Step 4).
11. Vortex for 15 sec.
12. Keep at room temperature for 20 min.
13. Centrifuge 3000 rpm, 4 degrees, 20 min.
14. Decant the supernatant from the pellet.
15. Add 1 mL 70% ethanol per 100 mg of tissue (same volume as Trizol added in Step 1).
16. Vortex to disperse the pellet.
17. Centrifuge 3000 rpm, 4 degrees, 20 min.
18. Decant the supernatant from the pellet, dab lip of tube on Kimwipe to get all ethanol before turning upright.
19. Dry the pellet at room temperature for 10 min.
20. Add 100 uL DEPC water to pellet to reconstitute.

21. Start on Step 1 with the RNeasy Mini Kit from Qiagen. I use the RNA Cleanup Protocol on page 80 of the handbook.
22. Quantitate using the Biophotometer.
23. Store at -80°C.

Protocol for reverse transcription

(Provided by Jim Belknap and Cailing Yin, The Ohio State University)

1. In thermowell tubes add volume of sample equivalent to 1 μg total RNA, 2 μL random decamers, and enough nuclease-free water to make 12 μL total volume.
2. Mix, spin, and heat at 85°C for 3 min. in thermocycler.
3. Spin, replace on ice.
4. Add 2 μL 10× RT buffer, 4 μL dNTP mix, 1 μL RNase inhibitor, and 1 μL MMLV-RT reverse transcriptase to make a total volume of 20 μL.
5. Mix, spin.
6. Incubate in thermocycler at 42°C for 1 hour and 92°C for 10 min.
7. Store at -20°C.

Protocol for polymerase chain reaction

(Provided by Jim Belknap and Cailing Yin, The Ohio State University)

1. Prepare master mix for the number of samples and standards to be analyzed. 10% excess is calculated into the recipe. Prepare at room temperature. Platinum Taq and uracil glycosylase should remain in the freezer box until use.

   Per reaction use:

   6.8 μL dH_2O

   4.4 μL 5X SYBR PCR stock

   4.4 μL 5 μM primer mix

   0.44 μL Nucleotide Mix Plus

   0.22 μL Platinum Taq polymerase

   0.22 μL uracil N-glycosylase
2. Pipette master mix into capillary tubes in this order:15 μL master mix into negative control, then 5 μL dH_2O, cover with cap; 15 μL master mix into all

remaining tubes, then 5 µL of unknown samples, cover with cap; 5 µL of known standard template concentrations, cover with cap.

3. Run PCR reactions with a program following these general guidelines, altering the annealing temperature and elongation step time according to the specific target:

UNG Activation	1 cycle	50°C	2 min
Denaturation	1 cycle	95°C	5 min
Thermal Cycling	40 cycles	95°C	0 s
		X°C	5 s (target-specific annealing temp)
		72°C	X s amplicon bp/25
		X°C	10 s determine the best fluorescent temp based on the melt curve analysis
Melt Curve	1 cycle	95°C	0 s
		65°C	10 s, then continuous temp increase of 0.1 per second to 95°C
		95°C	0 s
Cooling	1 cycle	38°C	0 s

Housekeeping genes

Four housekeeping genes were measured in order to find two that did not differ >2-fold in concentration between Periods. Non-normalized concentrations of potential housekeeping genes (β-actin, β_2 microglobulin, glyceraldehyde-3 phosphate dehydrogenase [GAPDH] and 28S rRNA) are reported in Table D.1.

Preliminary measurements of MCP-2, PAI-1 and TNFα

Prior to running PCR for MCP-2, PAI-1 and TNFα genes in all 5 periods, they were first analyzed in Periods 1 and 5 samples. Preliminary data demonstrated an ~5-fold increase in PAI-1 and MCP-2 mRNA expression, and no difference in TNFα mRNA expression (Table D.2). Because of the large increase in PAI-1 and MCP-2 genes between Periods 1 and 5, further analysis was performed to determine at what time point

a significant change in gene expression occurs. Gene expression of TNFα was performed during all time periods to compare with plasma protein concentrations of TNFα and because of its strong association with obesity previously determined in both humans and horses (Gil et al., 2007; Vick et al., 2007).

Statistical analyses for Chapter 4

STATA code- Italicized words were changed depending on variable or time interval (week or period) tested

Mean, standard deviation and standard error of the mean within each period:

· tabstat *varlist* , by (period) s(mean sd sem)

Two sample paired *t* test:

· ttest *var1 = = var2*

Repeated measures ANOVA with Fisher-Hayter pairwise comparisons ($\alpha = 0.05$):

· anova *depvar* horse period, repeated (period)

· fhcomp period

Table D.1: Mean ± SD cDNA copies/μL (non-normalized) of potential housekeeping genes during each time period.

Housekeeping gene	Period 1	Period 2	Period 3	Period 4	Period 5	Maximum fold change*
β-actin	$4.1 \pm 2.8 \times 10^5$	$4.8 \pm 3.5 \times 10^5$	$9.3 \pm 4.0 \times 10^5$	$10.8 \pm 4.5 \times 10^5$	$7.3 \pm 4.0 \times 10^5$	2.6
GAPDH	$4.0 \pm 1.0 \times 10^3$	$5.4 \pm 2.9 \times 10^3$	$10.1 \pm 2.8 \times 10^3$	$15.3 \pm 4.5 \times 10^3$	$9.3 \pm 2.9 \times 10^3$	3.8
28S rRNA	$8.7 \pm 2.5 \times 10^6$	$9.3 \pm 2.6 \times 10^6$	$10.7 \pm 3.5 \times 10^6$	$10.3 \pm 3.4 \times 10^6$	$10.6 \pm 2.8 \times 10^6$	1.2
β_2 microglobulin	$3.2 \pm 1.3 \times 10^5$	$3.1 \pm 2.3 \times 10^5$	$5.9 \pm 1.8 \times 10^5$	$5.5 \pm 1.8 \times 10^5$	$4.5 \pm 1.8 \times 10^5$	1.9

*Maximum change in mean concentration between periods.

Table D.2: Changes in subcutaneous adipose tissue mRNA expression* of cytokines before (Period 1) and after (Period 5) induction of obesity in 12 horses.

Variable	Period 1	Period 5	*P* value	Fold Change
PAI-1	2338 ± 863	8113 ± 1661	< 0.001	5.6 ± 1.2
MCP-2	14156 ± 3378	35963 ± 7999	0.007	5.5 ± 1.8
TNFα[†]	3041 ± 535	4477 ± 1094	0.21	NS

*Values expressed as mean ± SEM of cDNA copies per normalization factor.
NS = nonsignificant difference between Periods 1 and 5.
[†]n = 6

Appendix E: Supplementary material for Chapter 5

Additional presentation of adiposity and minimal model data

In Chapter 5, adiposity measurements with no significant group or group×time point effect were reported as a single value for both groups. Adiposity measurements are reported separately for each group in Table E.1.

Mean values of glucose and insulin concentrations during the frequently sampled intravenous glucose tolerance tests (FSIGTT) are presented graphically. Values for all time periods are presented in Figure E.1.

Preliminary deuterium oxide analysis

During the pre-exercise deuterium oxide dilutions, blood samples were collected and analyzed at 90 min and 180 min for 4 horses. Total body water calculated from the 90 min or 180 min samples were not different ($P = 0.068$), demonstrating a plateau in deuterium concentration by 180 min (Table E.2).

Statistical analyses for Chapter 5

STATA code- Italicized words were changed depending on variable or time interval (week or period) tested

Shapiro-Wilk test for normality ($\alpha = 0.05$):

· swilk *varlist*

Grubb's test for outliers ($\alpha = 0.01$):

· grubbs *varlist*, level (99)

· list *grubbs_varlist* horse period

Mean, standard deviation and standard error of the mean within each group or time period:

· tabstat *varlist* if group = = *1*, by (period) s(mean sd sem)

Repeated measures ANOVA with horse nested within group:

· anova *depvar* group / horse|group period period*group, repeated (period)

Fisher-Hayter pairwise comparisons (α = 0.05) between periods across all groups:

· fhcomp period

Fisher-Hayter pairwise comparisons (α = 0.05) between periods within each group and between groups within each period:

· egen z = group (group period)

· fhcomp z

Statistical outliers

Outlying values were determined by Grubb's test (α = 0.01) within each group. These values were removed from the data set prior to statistical analysis. A complete list of outliers for Chapter 5 analysis is presented in Table E.3.

Table E.1: Mean ± SD values for adiposity measurements in 4 control (CON) and 8 exercised (EX) horses. Measurements were performed with respect to the exercise protocol before exercise training, after 4 weeks of low intensity exercise, after 4 weeks of higher intensity exercise, and after 2 weeks of detraining.

		Time point			
Variable	**Group**	**Pre-exercise**	**Low intensity**	**Higher intensity**	**Detraining**
Bodyweight, kg	CON	513 ± 90[a]	518 ± 94[a,b]	520 ± 91[a,b]	526 ± 94[b]
	EX	503 ± 29[c]	492 ± 26[d]	483 ± 28[e]	491 ± 29[d]
Body condition score	CON	8.0 ± 1.2[a]	8.0 ± 1.3[a]	8.0 ± 1.1[a]	8.1 ± 1.2[a]
	EX	7.9 ± 0.5[a]	7.4 ± 0.7[b]	7.3 ± 0.6[b]	7.5 ± 0.7[b]
Waist, cm	CON	213 ± 15[a,c]	217 ± 13[c]	214 ± 14[a,c]	215 ± 14[a,c]
	EX	211 ± 3[a]	212 ± 5[a]	207 ± 5[b]	212 ± 5[a]
Girth, cm	CON	193 ± 13[a]	196 ± 15[a]	194 ± 13[a]	197 ± 15[a]
	EX	189 ± 5[a,b]	189 ± 5[b]	188 ± 5[b]	192 ± 6[b]
Cresty neck score	CON	3.3 ± 0.8[a]	3.3 ± 0.9[a]	3.3 ± 0.8[a]	3.3 ± 0.7[a]
	EX	3.1 ± 0.5[a,b]	2.8 ± 0.7[b]	2.7 ± 0.5[c]	2.8 ± 0.6[c]
Mean neck circumference, cm	CON	97 ± 7[a,b]	96 ± 8[a]	98 ± 7[a,b]	100 ± 7[b]
	EX	96 ± 4[a]	94 ± 3[a]	95 ± 4[a]	96 ± 4[a]
Crest Height, cm	CON	15 ± 3[a]	14 ± 4[a]	15 ± 2[a]	16 ± 3[a]
	EX	14 ± 2[a,b]	13 ± 2[a,b]	13 ± 2[a,b]	13 ± 2[b]
Rump, cm	CON	2.44 ± 1.04[a]	2.88 ± 1.24[b]	2.95 ± 1.18[b]	3.07 ± 1.19[b]
	EX	2.57 ± 0.70[a]	2.45 ± 0.66[a]	2.49 ± 0.66[a]	2.52 ± 0.65[a]
Back, cm	CON	0.88 ± 0.35[a,b]	0.85 ± 0.25[a,b]	0.99 ± 0.32[a,b]	0.97 ± 0.36[a,b]
	EX	0.87 ± 0.29[a,b]	0.77 ± 0.33[a]	0.90 ± 0.34[a,b]	0.95 ± 0.35[b]
Rib, cm	CON	0.80 ± 0.09[a]	0.82 ± 0.11[a,b]	0.89 ± 0.12[a,b]	0.90 ± 0.07[a,b]
	EX	0.94 ± 0.19[b]	0.89 ± 0.16[a,b]	0.98 ± 0.16[b]	0.99 ± 0.17[b]
Shoulder, cm	CON	0.91 ± 0.15[a,b]	0.94 ± 0.11[a,b]	0.95 ± 0.11[a,b]	0.97 ± 0.13[b]
	EX	0.86 ± 0.10[a]	0.87 ± 0.09[a,b]	0.90 ± 0.10[a,b]	0.91 ± 0.08[a,b]
Body fat, %[†]	CON	18.6 ± 5.3[a]	20.8 ± 6.3[b]	21.2 ± 6.0[b]	21.8 ± 6.0[b]
	EX	19.3 ± 3.5[a]	18.6 ± 3.3[a]	18.9 ± 3.4[a]	19.0 ± 3.3[a]

[a-e]Within a variable, means with different superscripts differ ($P < 0.05$) according to repeated measures ANOVA with Fisher-Hayter pairwise comparisons

[†]Calculated as % fat = 6.22 + 5.07x, where x = subcutaneous rump fat thickness in cm (Westervelt 1976)

Table E.2: Total body water (kg) of 4 horses calculated from deuterium concentration in plasma collected 90 or 180 minutes after infusion of deuterium oxide.

Horse	90 min	180 min
234	285.6	290.5
241	291.5	296
248	352.8	360.5
251	306.8	307
Mean ± SD	309 ± 30	314 ± 32*

*P = 0.068 compared to 90 min sample (paired t test)

Table E.3: Statistical outliers for Chapter 5.

Variable	Value	Period	Group	Horse
Insulin, mU/L	211.2	4	Exercise	248
Insulin, mU/L	105.8	4	Control	251
Glucose, mg/dL	131.4	4	Exercise	248

Table E.4: Nutrient composition of individual feeds and hays fed during the exercise study. Analysis was performed on one representative composite sample from 3 bags of concentrate feed and on three representative samples of hay, each sample a composite of 10 bales .

Nutrient	Concentrate feed	Alfalfa/orchard grass hay
Dry matter, %	89.7	90.8 ± 0.2
Crude protein, %	18.6	17.8 ± 1.0
Acid detergent fiber, %	17.4	31.3 ± 1.8
Neutral detergent fiber, %	33.6	43.7 ± 3.4
Nonstructural carbohydrate, %	21.4	9.1 ± 0.4
Starch, %	15.2	2.1 ± 0.2
Water-soluble carbohydrate, %	6.2	7.0 ± 0.4
Ethanol-soluble carbohydrate, %	8.6	8.3 ± 0.7
Crude fat, %	7.5	3.4 ± 0.2
Ash, %	9.9	9.5 ± 0.4
Ca, %	1.23	1.05 ± 0.13
P, %	0.74	0.23 ± 0.02
Mg, %	0.35	0.24 ± 0.01
K, %	1.01	2.65 ± 0.08
Na, %	0.211	0.021 ± 0.006
Fe, ppm	947	174 ± 21
Zn, ppm	335	24 ± 3
Cu, ppm	133	9 ± 1
Mn, ppm	158	68 ± 11
Mo, ppm	1.4	0.8 ± 0.1
Digestible energy, Mcal/kg	2.85	2.34 ± 0.03

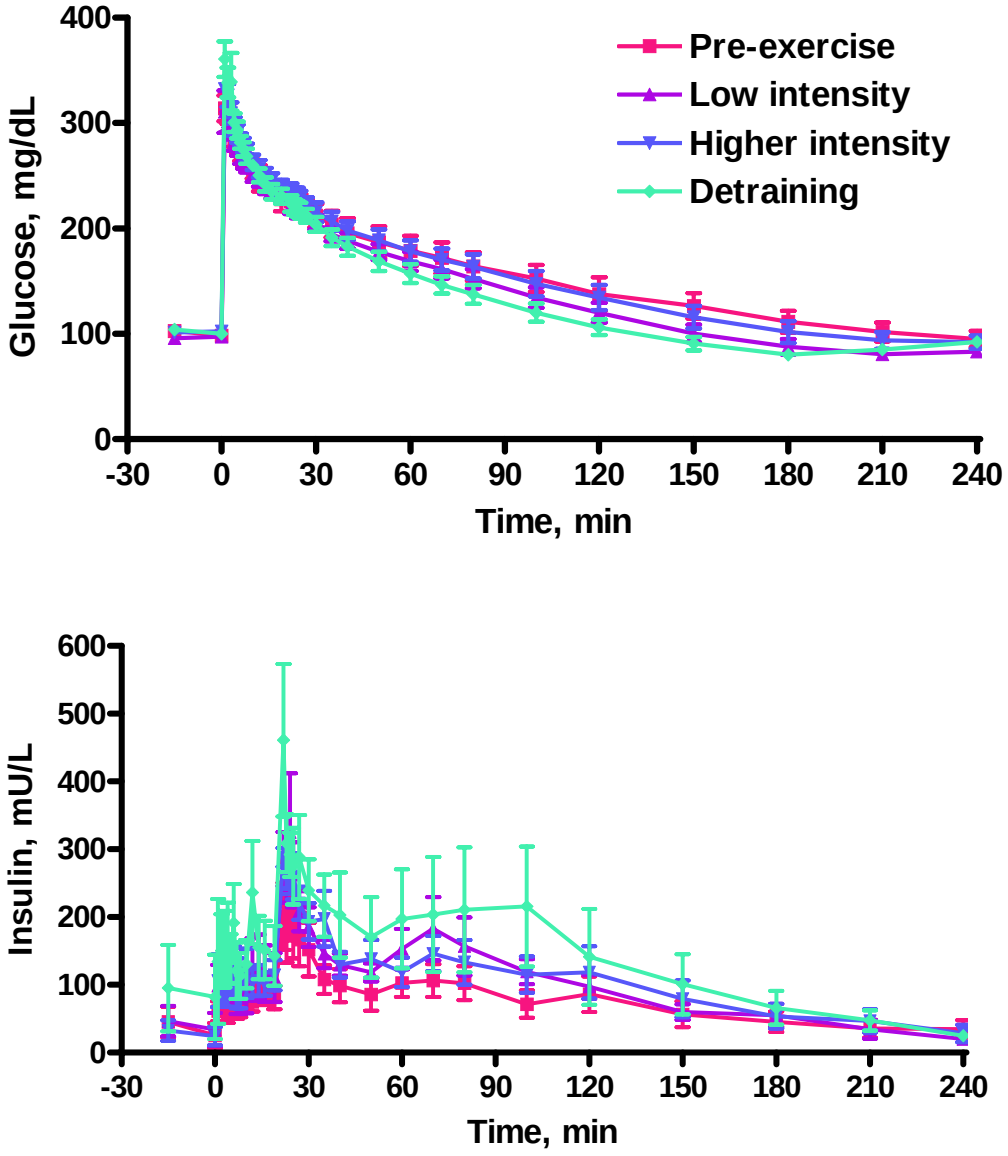

Figure E.1: Glucose and insulin concentrations during the frequently sampled intravenous glucose tolerance tests in control horses during all time points.

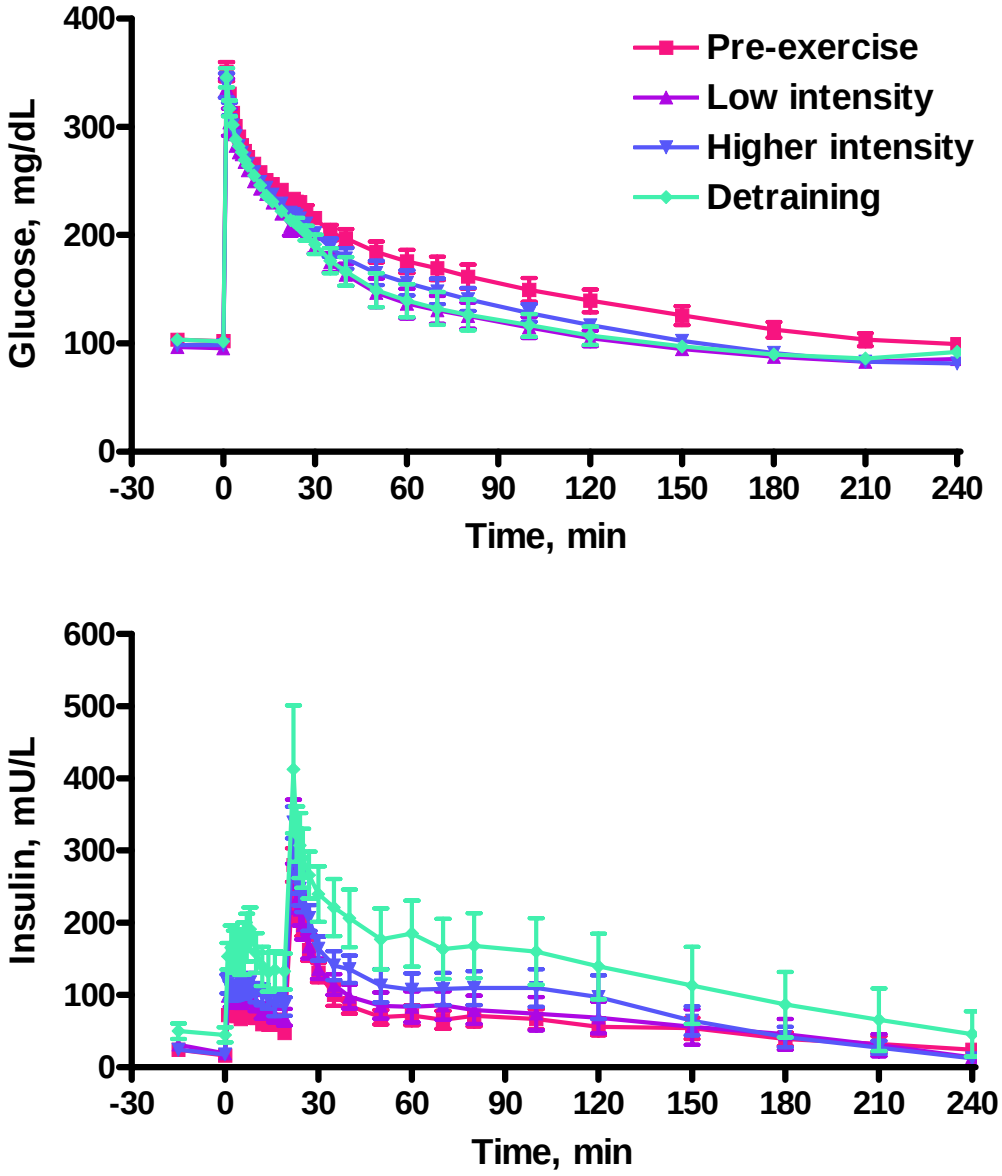

Figure E.2: Glucose and insulin concentrations during the frequently sampled intravenous glucose tolerance tests in exercised horses during all time points.

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