ABSTRACT

Title of Document:	Effects of Diet and Weight Gain on Subcutaneous Body Fat Accretion Patterns and Adipocytokine Production in Thoroughbred Geldings
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Fifteen Thoroughbred geldings on an eight month weight gain study were used to evaluate 1) the effects of weight gain and diet on body area (neck, withers, shoulders, ribs, loin, tailhead) condition scores, and 2) the effects of weight gain, diet, and exercise on serum concentrations of tumor necrosis factor-α. The body condition scoring system developed in Quarter horse was slightly modified for use Thoroughbred geldings and involved developing prediction equations that utilized a smaller subset of the body areas. Horses at a BCS of 4 were found to be at a higher risk of inflammation that horses at a BCS of 7. The degree of inflammation was similar between horses fed either a high starch and sugar diet or high fiber and fat diet, indicated by similar TNF concentrations. Restricting daily exercise for two weeks was associated with increased inflammation.

EFFECTS OF DIET AND WEIGHT GAIN ON SUBCUTANEOUS BODY FAT ACCRETION PATTERNS AND ADIPOCYTOKINE PRODUCTION IN THOROUGHBRED GELDINGS

By

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Chapter 1: Introduction

Body condition scoring (BCS) is a subjective method of estimating subcutaneous fat cover in many livestock species and is one of the most widely used indices of energy balance in equine feeding management. The most commonly used equine BCS method was developed by Henneke et al. (1983) in pre-parturient Quarter horse mares. Fat cover of six discrete body areas; neck, withers, shoulders, ribs, loin, and tailhead was scored on a scale of 1 (thin) to 9 (fat) by physical palpation and visual assessment and then averaged for a mean BCS. The Henneke system has since been applied to all breeds, ages, and genders of horses, without determining if the system accurately describes body area fat accretion in these different classes of horses, if all classes of horses accrue subcutaneous fat in the same locations or if different types of diets have effects on body area fat accumulation.

Body condition scoring is an inexpensive and easily applied method for determining temporal changes in nutritional status. However, a valid BCS system is required to accurately assess these changes. Body condition scoring is an important management tool that can be used to prevent obesity. To date, obesity in horses has been linked with the development of life threatening metabolic conditions such as laminitis (Jeffcott et al., 1986; Fontaine et al., 2001) and hyperlipemia (Jeffcott et al., 1985; Hughes, 2004). Obesity in horses is often the result of a lack of knowledge on the owners' part about the serious risks associated with unhealthy levels of fat in their animals. Weight gain is positively correlated with increasing systemic concentrations of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF) in humans

(Hotamisligil et al., 1995) and horses (Vick et al., 2007), which are produced by the accumulating adipose tissue. In humans, an increase in TNF results in a low grade inflammation (Sethi et al., 1999) that precludes development of the metabolic diseases of obesity (Permana et al., 2006; Skurk et al., 2004). Since the obese equine also appears to be at risk for metabolic diseases (Johnson et al., 2002; Kronfeld et al., 2006; Treiber et al., 2006), a similar mechanism of action is probable (Vick et al., 2007).

Based on the importance of understanding both how horses accrue body fat and the link between excessive body fat and disease, the objectives of these studies were to:

1) Determine the accuracy and precision of the Henneke BCS system for Thoroughbred geldings
2) Assess the effect of dietary energy source on the distribution of subcutaneous body area fat accretion
3) Determine if a smaller subset of body areas could be used to accurately predict the overall mean BCS for the Henneke BCS system in Thoroughbred geldings
4) Determine if BCS gain is associated with serum concentrations of TNF
5) Assess the effects of dietary energy source on serum TNF concentrations
6) Determine whether cessation of daily exercise in horses fed to gain weight would influence their inflammatory state.

Chapter 2: Review of Literature

The ability to quantify stored body fat is important for nutritional, reproductive, and health management of livestock animals. The most common method reported in the literature for assessing body fat in horses is body condition scoring, a qualitative assessment of subcutaneous fat cover. Other techniques that are used, but are less common, include ultrasonography and body mass index. Managing horses properly involves the ability to maintain them at a healthy body condition and weight (Johnson, 2002; Treiber et al., 2006), and this in turn requires tools that are easily applied to large animals under field conditions.

The most common cause of unhealthy levels of fatness in horses is the feeding of too much grain in comparison to the level of physical activity a horse exerts (Johnson, 2002). This is often compounded with a lack of knowledge on the manager's part about how to assess body fat. Overfeeding can come in the form of concentrates, forages or lush pasture and because horses are often maintained in large group-feeding situations, horses that are prone to weight gain may need special attention during feeding to avoid obesity (Johnson, 2002). Maintaining horses at a healthy condition is important because there are several metabolic disorders associated with obesity in horses (Jeffcott et al., 1986; Hughes, 2004; Kronfeld et al., 2006; Treiber et al., 2006). Therefore, using a system such as body condition scoring to manage feed and exercise needs may aid in the prevention of metabolic disorders and the costly veterinary care required to treat these disorders.

METHODS FOR ASSESSING BODY COMPOSITION IN THE HORSE

Body Condition Scoring

Body condition scoring (BCS) systems estimate subcutaneous fat cover by dividing the continuous scale of fat deposition into categories based on specific characteristics (Burkholder, 2000; Evans, 1978). This is done through both visual assessments and physical palpations. Most systems use low scores to indicate an animal with minimal fat cover and easily visible bony structures, mid range scores to indicate an animal with bony structures that can be easily felt but not seen, and high range scores to indicate an animal whose bony structures are difficult to feel (Burkholder, 2000). Generally, mid range scores are the most desired, with athletes slightly lower and breeding animals slightly higher (Kronfeld, 1997).

Benefits and Limitations. Body condition scoring is the most often used method for determining fat cover in research publications, most likely due to ease of use, little expense, and no required equipment. Estimating an animal's fat cover is necessary for monitoring health and nutrition status as well as enabling the implementation of suitable feed and exercise management strategies that will bring about the optimal condition of that particular animal (Kronfeld, 1997). Within the equine species, healthy body weights range from 100 kg in Miniature Horses to 1000 kg in draft horse breeds, therefore, body weight (BW) alone is not sufficient for determining adequate nutritional status and health. In comparison, BCS are independent of frame size, allowing for a better assessment of health than BW alone (Burkholder, 2000). Body condition scoring is a good method of estimating stored body fat because it uses the whole body, and may therefore be more accurate than two-measurement

indicators such as BMI. However the benefit of the BMI measurement is that it is an indicator of visceral fat, whereas BCS only takes into account subcutaneous fat.

Body condition scoring systems are highly subjective when not accompanied by an unambiguous protocol, including both specific characteristics of each score and instructions for use (Evans, 1978). Therefore, in order to be valid, a BCS system should demonstrate three qualifications: repeatability, reproducibility, and predictability (Burkholder, 2000). Repeatability is a test of the system's precision; such that one assessor repeatedly assigns the same score to a given animal that is maintaining condition. Reproducibility is a test of between-assessor variability, such that two or more assessors assign the same score to one animal, while predictability is the ability of a BCS system to accurately reflect the fat cover of an animal. The main limitation of a BCS system is its subjective nature, thus valid results require user knowledge and familiarity of the judge with each system. Subjectivity is reduced and reliability of scores increased when the assignment of scores is conducted according to specific protocols (Burkholder, 2000).

Leighton-Hardman. The first published BCS system for horses was that of Leighton-Hardman (1980), which defines condition as the amount of subcutaneous fatty tissue present in a given animal (Table 1). This system identifies three areas of fat accumulation: the pelvic area as viewed by standing directly behind the horse, backbone and ribs, and neck area immediately cranial to the withers. The scale is from 0 (thin) to 5 (fat) with half point demarcations, resulting in an 11 point scale, although only whole scores are defined. Basic user instructions include visuals and brief written descriptions of a horse at each whole point condition score. The

assessment is conducted on the pelvic region first, and the score given can be adjusted by a half score if the score given to the back/ribs and neck area differs from the pelvic score by one point or more. Therefore the pelvic region score receives the greatest weight when calculating the final score.

The drawbacks of this system include that it utilizes only 6 whole scores, which may not be specific enough for research purposes. Additionally, there is no validation or explanation for using an adjusted pelvis score, which gives the pelvic score more weight than the other body areas being scored.

Table 1. Body condition scores as described by Leighton-Hardman (1980)

	Body Areas		
Score	Pelvis	Back and Ribs	Neck
0: Very Poor	Deep cavity under tail and either side of croup. Skin drawn tightly over pelvis with no tissue detectable beneath.	Individual processes sharp to touch and very clearly defined. Skin drawn tightly over clearly defined ribs.	Ewe neck, very narrow and slack at base.
1: Poor	Croup and pelvis well defined. No fatty tissue present but skin is supple. Deep depression under tail.	Individual processes of the backbone sharp to touch and clearly defined. Ribs clearly visible.	Ewe neck, narrow and slack at base.
2: Moderate	Croup well defined but some fatty tissue felt under the skin. Pelvis felt easy. Slight cavity under the tail.	Backbone is covered. Individual processes not apparent but can easily be felt with pressure. Ribs just visible.	Narrow but firm.
3: Good	Fatty tissue covering whole area giving a rounded appearance without any 'gutter'. Skin appears smooth but pelvis easily felt	Vertebrae well covered but without a 'gutter'. Backbone easily felt with pressure. Ribs just covered	Firm, but with no crest except for stallions.
4: Fat	Pelvis buried in soft fatty tissue. 'Gutter' to root of tail. Pelvis felt only with firm pressure.	Gutter' along length of backbone. Ribs well covered by fatty tissue. Bone structure felt only with firm pressure.	Wide and firm, with folds of fatty tissue present. Slight crest even in mares.
5: Grossly Fat	Pelvis buried in fatty tissue. Deep 'gutter' to root of tail. Skin distended. No part of pelvis felt even with firm pressure	Deep 'gutter' along backbone. Ribs buried in fatty tissue. Bone structure cannot be felt. Back more like a table.	Very wide and firm, with folds of fatty tissue present. Marked crest even in mares.

Carroll and Huntington. Carroll and Huntington (1988) adapted the Leighton-Hardman (1980) system by adding detailed descriptions of each score (Table 2). In addition, they suggest a more concrete overall condition score calculation, where pelvic score is adjusted by 0.5 if it differs by 1 or more points from the back or neck scores. The primary goal of their work was to develop nomograms for BW prediction from BCS and height, and so the 0-5 scale was chosen for its simplicity when compared to a 1-9 scale (Henneke et al., 1983). They used the same score descriptions described by Leighton-Hardman.

An evaluation of the 0-5 scale was carried out, which included condition scoring and BW measurement of 30 horses by one of the authors and several other horsemen, unfamiliar to condition scoring. The number of other horsemen used to score the horses was not reported. Condition score results between the one author and the other horsemen were the same in 65% of the horses and the maximum difference was 0.5 scores. The authors do not report statistical agreement of scores, or the correlation coefficient in their paper, which is a drawback to their study. There is also no defined requirement in the literature for either agreement between judge scores or maximum allowable difference**;** therefore although the authors claim that their system had good correlation, there is no way of determining if it is indeed biologically acceptable. A second evaluation included condition scoring, body weight (BW), and height measurements from 20 non-pregnant Thoroughbred (TB) mares. Body weight was calculated from the nomograms and compared to the measured BW of horses in the same condition and height categories from the sample of 30 horses previously measured. There were no significant differences present

between calculated and measured BW, while score had a correlation coefficient of 0.704 with BW.

Table 2. Body condition system for horses as described by Carroll and Huntington (1988)

Score	Neck	Back and Ribs	Pelvis
0: Very Poor	Marked 'ewe' neck. Narrow and slack at base.	Skin tight over ribs. Spinous processes sharp and easily seen.	Angular pelvis- skin tight. Deep cavity under tail and either side of croup.
1: Poor	'Ewe' neck. Narrow and slack at base.	Ribs easily visible. Skin sunken either side of backbone. Spinous processes well defined.	Rump sunken, but skin supple. Pelvis and croup well defined. Deep depression under tail.
2: Moderate	Narrow but firm.	Ribs just visible. Backbone well covered. Spinous processes felt.	Rump is flat on either side of backbone. Croup is well defined, some fat. Slight cavity under tail.
3: Good	No crest (except in stallions).	Ribs just covered- easily felt. No 'gutter' along back. Spinous processes covered, but can be felt.	Pelvis covered by fat and rounded. Pelvis easily felt.
4: Fat	Slight crest. Wide and firm.	Ribs well covered- need firm pressure to feel. 'Gutter' along backbone.	Gutter' to root of tail. Pelvis covered by soft fat- felt only with firm pressure.
5: Very Fat	Marked crest. Very wide and firm. Folds of fat.	Ribs buried- cannot feel. Deep 'gutter'. Back broad and flat.	Deep 'gutter' to root of tail. Skin distended. Pelvis buried- cannot be felt.

Rudman and Keiper. Rudman and Keiper (1991) further modified the 0-5 scale through the development of a visual system to monitor the body condition of feral ponies on Assateague Island, MD (Figure 1). A visual BCS system is much more applicable to feral horse populations since it is usually not possible to physically palpate them. It may also be applicable to large ranching or farming operations that keep more animals than is possible to physically palpate. The authors utilized the same three body areas**:** the pelvic area, back/ ribs, and neck. They developed a pictograph describing the fat accretion characteristics of each score, which are based

on the prominence or rounding of the bone structures- ribs, vertebrae, point of hip, and whether the neck is "ewe" or "cresty". A major flaw of the system includes the fact that differences in conformation, muscle tone, and condition all change the appearance of an animal and the rounding and appearance of bone structures. Without physical palpation it may be hard to tell whether a particular animal's shape is influenced by conformation, muscle tone, or condition.

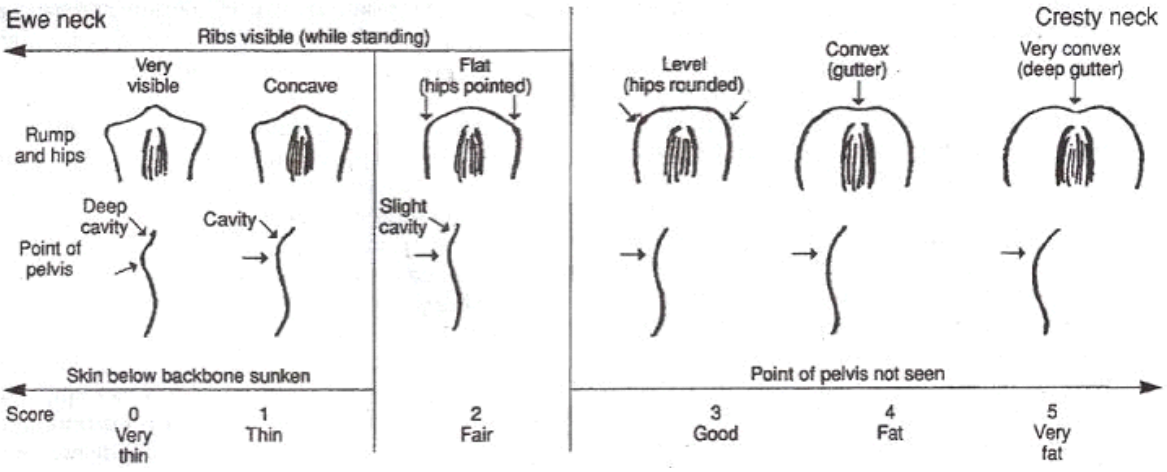

Figure 1. Body condition scores as defined by Rudman and Keiper (1991)

Henneke. The most common BCS system used today in research and management was developed in pre-parturient Quarter Horse mares (Henneke et al., 1983) as a way to accurately compare stored body fat by assigning a subjective score of fat accretion at six different body areas (Table 3). Sites of subcutaneous fat storage (Figure 2) specific to horses were determined by single time point observations of several mature horses, of mixed body condition and gender. A preliminary system was developed by Henneke (1981) and tested on 20 horses of Quarter Horse breeding by faculty, staff, and graduate students of the Texas A&M Animal Science Department. After refinement, the system was distributed to four Texas horse producers for

evaluation. Henneke identified six specific body areas of subcutaneous fat deposition as the neck, withers, behind the shoulders, ribs, lumbar spinous processes, and the tailhead. This condition scoring system defines a range of 1 (thin) to 9 (obese), with measurements in 0.5 units.

Body condition scores were compared by Henneke et al. (1983) to percent body fat by ultrasonic scans of rump fat thickness based on the methods of Westervelt et al. (1976). Correlations were run between BCS, BW, height, heart-girth, BW/height, heart-girth/height, and percent body fat. Body condition score was highly correlated to percent body fat, while there were no significant correlations between BW, height, or heart-girth measurements and percent body fat. Body weight/height and heart-girth/height were also significantly correlated to percent body fat. These results indicate that when used alone, single measurements of body size do not provide adequate estimates of body fat; however, ratios of these measurements can be used for this purpose.

Different breeds of horses were bred for different purposes- racing, pulling, pleasure riding, etc. These purposes may have had effects on breed specific inherent levels of muscling and bone structure, and these differences may affect the assignment of a BCS. The Henneke BCS system was primarily developed for use in reproductively active Quarter Horse mares, whose conformation may not only be affected by breed specific muscling but also by the weight of the fetus (Henneke et al., 1983). Because conformational differences between breeds may have significant impacts on scores, Henneke (1981) suggests that instead of averaging body area scores to calculate the final score, assessors should weight body areas differently

depending on these conformational differences. However, no weighting factors were suggested. Throughout the rest of this paper, BCS will refer to that determined by using the Henneke method, unless otherwise specified.

The benefits of using the Henneke BCS system include that it offers more categories for classifying animals, as well as identifying more body areas that accrue body fat than the other BCS systems. The Henneke system is the most often used BCS system in equine research because it offers the most flexibility when assigning scores, and it also has more comprehensive descriptions than the other BCS systems.

Table 3. Body condition scores as described by Henneke (1983)

Score	Description
1: Poor	Animal extremely emaciated. Spinous processes, ribs, tailhead, tuber coxae and ischii projecting prominently. Bone structure of withers, shoulders, and neck easily noticeable. No fatty tissue can be felt.
2: Very Thin	Animal emaciated. Slight fat covering over base of spinous processes, transverse processes of lumbar vertebrae feel rounded. Spinous processes, ribs, tailhead, tuber coxae, and ischii prominent. Withers, shoulders, neck structures faintly discernible.
3: Thin	Fat buildup about halfway on spinous processes, transverse processes cannot be felt. Slight fat cover over ribs. Spinous processes and ribs easily felt. Tailhead prominent but individual vertebrae cannot be visually identified. Tuber coxae appear rounded, but easily discernible. Tuber ischii not distinguishable. Withers, shoulders, and neck accentuated.
4: Moderately Thin	Negative crease along back. Faint outline of ribs discernible. Tailhead prominence depends on conformation, fat can be felt around it. Tuber coxae not discernible. Withers, shoulders, and neck not obviously thin.
5: Moderate	Back level. Ribs cannot be visually distinguished but can be easily felt. Fat around tailhead beginning to feel spongy. Withers appear rounded over the spinous processes. Shoulders and neck blend smoothly into body.

Table 3 continued. Body condition scores as described by Henneke (1983)

Score	Description
6: Moderately Fleshy	May have slight positive crease down back. Fat over ribs feel spongy. Fat around tailhead feels soft. Fat beginning to be deposited along the side of the withers, behind the shoulders and along the sides of the neck.
7: Fleshy	May have a crease down the back. Individual ribs can be felt, but noticeable filling between the ribs with fat. Fat around tailhead is soft. Fat deposited along withers, behind shoulders, and along neck.
8: Fat	Crease down back. Difficult to feel ribs. Fat around tailhead very soft. Area behind shoulder filled with fat. Noticeable thickening of neck. Fat deposited along inner thighs.
9: Extremely fat	Obvious crease down back. Patchy fat appearing over ribs. Bulging fat around tailhead, along withers, behind shoulders and along neck. Fat along inner things may rub together. Flank filled with fat.

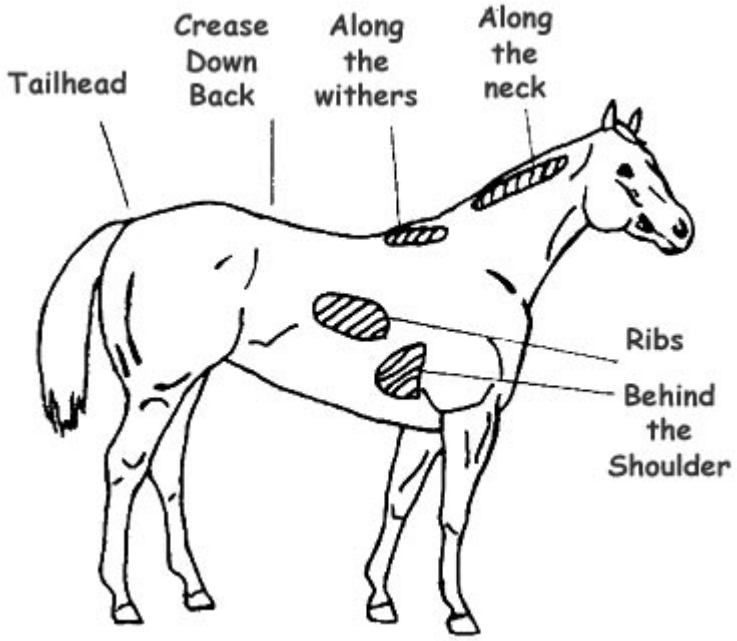

Figure 2. Body areas described by Henneke et al. (1983).

Body Mass Index

Body mass index (BMI) is rarely used as a measure of body fat in equine research; however, it is frequently used in human nutrition consultations. Donaldson et al. (2004) first used BMI (BW [kg]/ height at withers [m^2]) as an index of health in

horses, to correlate body fat to α- melanocyte stimulating hormone, an appetite control and energy balance hormone. Body mass index is an easily obtained and objective measure of body fat that is possibly more reliable than BCS, due to the subjective nature of body condition scoring (Donaldson et al., 2004). Body mass index was found to be a reasonable measure of body fat ($r^2 = 0.60$, $p < 0.001$). Healthy BMIs in horses ranged from 175 to 250, but because BW was calculated from a formula (Henneke et al., 1983) and not actual BW, BMI calculations based on actual BW might be slightly different. A drawback of using BMI is that BW may be influenced by more than just body fat, and therefore large ranges of BMI may be healthy.

Ultrasound Measurements

Ultrasonic measurements of fat cover are frequently used in equine nutrition and physiology research. The original work was done by Westervelt et al. (1976) on 8 horses and 11 ponies evaluated for fat thickness at the shoulder (directly posterior to scapula), rib (5 cm lateral from the spinous processes between the 12^{th} and 13^{th} ribs), and rump (5 cm lateral from the midline at the center of the pelvic bone) prior to slaughter. Carcass analysis was performed to determine total body fat by ether extract on homogenized carcass samples. Total body fat for horses was accurately predicted by rump fat measurements ($r^2 = 0.93$, $p < 0.01$), by the equation $Y = 8.64 + 4.70X$; where X = cm of rump fat and Y = percent fat. The equation for ponies was determined to be $Y = 3.83 + 5.58X$. Body condition scores correlated well to percent body fat ($r^2 = 0.65$).

A similar study by Kane et al. (1989) looked at site differences along the rump. Five sites were located 10 cm off the midline, at 5 cm intervals starting 6 cm from the tailhead. Site 1 was closest to the tailhead while site 5 was almost at the top of the croup. Ultrasound measurements were made to determine fat thickness at these 5 sites, and then horses were slaughtered and actual fat thickness determined. Sites 1-4 showed good correlation between ultrasound-derived fat thickness and actual body fat percentage, with sites 2 and 3 showing the strongest correlations ($r^2 = 0.98$ and 0.97, respectively). Derived equations varied greatly between sites, however, equations predicted from actual fat ($Y = 5.01 + 5.7X$) versus ultrasound measurements ($Y = 2.89 + 5.47X$) were most similar at site 2.

Gentry et al. (2004) found correlation coefficients of 0.87, 0.84, 0.82 and 0.86 between BCS and back fat thickness at the tailhead, rump, 13th rib, and withers respectively. In this study, light-breed mares at a starting BCS of 7 were fed for either BW gain to a BCS of 8.5, or BW loss to a BCS of 3.5, over a 2 mo period, and maintained there for 7 mos. Over the study period, there was little change in back fat thickness over the rump area as compared to the other three body areas, indicating that rump (correlates to site 5) might not be an accurate indicator of BCS in the horse. The tailhead area was the best indicator of BCS, as the tailhead averaged the most changes in fat cover, followed by fat cover of both the 13th rib and the withers.

Relationship between BCS, BW, and Body Fat

Body condition has been correlated to percent body fat in several studies, as well as BW gain required to achieve an increase of one BCS. Mature horses consuming an average 2.8% of their BW (24 Mcal of DE above daily maintenance)

achieved an average daily gain of 1 kg (Heusner, 1993). Horses increased their BCS from a 4 to a 6, with an increase of one BCS correlating to an increase of 16-20 kg BW. These horses averaged a gain of at least one BCS over a three week period. Graham-Thiers et al. (1999) demonstrated that a daily intake of 7 Mcal above DE resulted in an average daily gain of 0.3 kg, thus suggesting a requirement of 23 Mcal DE above maintenance for a one kg increase in BW. Taken together, these data support a requirement of at least 23 Mcal above daily DE for each one kg increase in BW, and within the BCS range of 4 to 6 an increase in one BCS requires a BW gain of 20 kg. This BW gain of 20 kg can be achieved with an average daily gain of one kg, and would require 20 days of a horse being fed 23 Mcal above daily DE.

There are broad ranges of percent body fat for each BCS that were identified across several breeds. Hines et al. (1987) estimated Quarter Horse mares at an average BCS of 3.4, to have 11-13% body fat. Estimations of percent body fat in BCS 4-5 animals come from Standardbreds (Kearns et al., 2002), Arabians (Lawrence et al., 1992), TB broodmares (Vick et al., 2006) and various breeds of mares (Powell et al., 2002), which was found to range between 7-11%. Body fat percentages for horses in moderate to moderately fleshy condition (BCS 5-7) have been identified in Quarter Horse broodmares (Henneke, 1981; Kubiak et al., 1991) and Standardbred mares (Kearns et al., 2001) and ranged from 12% to 24%. Hines et al. (1987) estimated Quarter Horse brood mares at a BCS of 7 to have 16-18% body fat, while TB mares at a BCS of 8 averaged 16-18% body fat (Vick et al., 2006), and Quarter Horses at a BCS of 9 averaged 16-17% body fat. However, Powell et al. (2002) estimated mares of various breeds at a BCS of 9 to have 30- 35% body fat. The

differences in ranges may be explained two ways**:** 1) BCS estimates subcutaneous fat only and not intra-abdominal fat, therefore breed specific propensity to lay down fat in these two distinct areas may affect the range of percent body fat associated with that BCS, and 2) breeds may have different propensities to lay down rump fat, making the equations less accurate. To summarize, horses with a BCS of 3-4 have a percent body fat of 11-13%; a BCS of 4-5 correlates to a percent body fat of 7-11%; BCS 5-7 correlates to a percent body fat of 12-24% body fat; and horses of BCS 7-9 have a percent body fat of 16-35%.

THE RELATIONSHIP BETWEEN OBESITY AND METABOLIC DISORDERS

An association between obesity and the onset of metabolic disorders was first described in humans as the prevalence of hypertension, hyperglycemia, and gout in obese patients (Studien, 1923; Vague, 1947). More recently, this clustering of metabolic diseases with obesity has been termed the 'Metabolic Syndrome (MS)' by the World Health Organization. Metabolic diseases of the MS include insulin resistance (a metabolic precursor to diabetes) as a central component, diabetes type II, hypertension, microalbuminuria, dyslipidemia, and abdominal obesity (Balkau and Charles, 1999). One of the theories relating obesity and metabolic disturbances is that adipose tissue is a metabolically active tissue that produces several hormones and immune system-associated proteins, or adipokines (Eckel, 2005; Despres, 2003; Duncan, 2001; Pickup, 2004). Of these adipokines, the pro-inflammatory cytokines (PIC) are also active innate immune system components with a multitude of functions in the response of an animal to an infectious event (Razonable, 2006). These

functions include the production of the inflammatory response: including both fever (Saigusa, 1990; Kawasaki et al., 1989) and cachexia (Tracey et al., 1988), which are physiological responses that promote the recovery of an animal from an infection (Hermann et al., 2005; Tracey et al., 1988). During cachexia, energy intake decreases and therefore, one important function of the PIC is to increase energy availability through disrupting normal lipid and glucose metabolism (Delano and Moldawer, 2006; Plata-Salaman, 1998). Because the sickness response is transient, normal metabolism is restored once the animal recovers from infection.

In obesity, the continual up-regulation of PIC is thought to contribute to the development of both dyslipidemia (Hardardottir et al., 1994) and insulin resistance (Hotamisligil, 1999). Through their finding that the onset of diabetes type II was consistent with activation of the innate immune system, Pickup et al. (1997) were the first to uncover a link between the development of metabolic diseases of obesity and inflammatory markers, including both pro-inflammatory cytokines (PIC) and acute phase proteins. Later studies corroborated this evidence through the repeated association of the onset of type II diabetes, insulin resistance (a metabolic precursor to diabetes type II), or dyslipidemia, or both with increased circulating levels of PIC (Bonora et al., 2003; Chan et al., 2002; Schmidt et al, 1999; Pickup and Crook, 1998; Pickup, 2004). One of the PIC associated with increasing adiposity is tumor necrosis factor-α (TNF), which will be discussed in detail later in this review. The metabolic diseases of obesity are therefore theorized to result from a chronic low-grade systemic inflammation (Das et al., 2001; Dunkin and Schmidt, 2001; Engstrom et al., 2003;

Festa et al., 2000; Kershaw and Flier, 2004; Pickup et al., 2004; Trayhurn and Wood., 2004; Vettor, 2005; Yudkin et al., 1999).

The progression of BW gain and onset of obesity in the horse have also been linked to several metabolic disorders including insulin resistance (Freestone et al., 1991a; Hoffman et al., 2003), laminitis (Fontaine et al., 2001; Jeffcott et al., 1986), and disrupted lipid metabolism such as hypertriglyceridemia, hyperlipidemia, or hyperlipemia (Jeffcott and Field, 1985a; Hughes et al., 2004; Kronfeld et al., 2006). Obesity in the equid has not been completely defined; however according to the Henneke BCS system, an 8 is considered fat, and 9 as extremely fat. Obesity may be better defined after the mechanisms relating obesity to metabolic disorders in the horse are more completely understood.

The Production of TNF in Adipose Tissue

Abdominal adipose tissue is composed of stromal vascular cells (preadipocytes, fibroblasts, and non-differentiated mesenchymal cells), differentiated adipocytes, and infiltrated macrophages, all of which have the ability to produce TNF (Cousin et al., 1999; Xu et al., 2003). The accumulation of adipose tissue in obesity parallels an increased infiltration of macrophages (Weisberg et al., 2003; Wellen, 2003; Xu et al., 2003). These macrophages are an important source of TNF production in adipose tissue through molecular signaling between adipocytes and macrophages (Weisberg et al., 2003; Xu et al., 2003). Adipocytes produce two chemokines that affect macrophage infiltration into adipose tissue: macrophage migration inhibitory factor (MIF) and monocyte chemoattractant protein-1 (MCP-1) (Do et al., 2006). Monocytes that are retained in the adipose tissue are able to

differentiate into macrophages that have enhanced inflammatory activity (Verhoeckx et al., 2004).

Macrophage migration inhibitory factor is a pleitropic hormone with inflammatory effects (Hirokawa et al., 1998) that includes its ability to prolong the lifespan of macrophages by protecting them from apoptosis (Bernhagen et al., 1998), counteract glucocorticoid- induced suppression of the immune system (Calandra et al., 1995; Calandra and Bucala, 1997), and up-regulate TNF expression in immune cells (Roger et al., 2001). Macrophage migration inhibitory factor deficient cells have down-regulated TNF receptor expression, and are hypo-responsive to TNF induced activity (Toh et al., 2006). Also, MIF levels are up-regulated in human patients with type 2 diabetes (Yabunaka et al., 2000). Skurk et al. (2005) showed *in vitro* that preadipocytes undergoing differentiation to mature adipocytes secrete MIF, and serum concentrations of MIF correlate to BMI in humans. Additionally, adipocytes may have different immunological properties than innate immune cells with regard to MIF expression, and may in fact constitutively secrete MIF (Calandra et al., 1994, 1998; Martiney et al., 2000).

Monocyte chemoattractant protein-1 is a hormone that attracts monocytes from the blood stream into the adipose tissue (Kanda et al., 2006). Serum concentrations of MCP-1 are significantly correlated to BMI, waist circumference, and other inflammatory markers (Kim et al., 2006), and it is up-regulated in the white adipose tissue of obese mice (Sartipy and Loskutoff, 2003; Takahashi et al., 2003), and humans (Christiansen et al., 2005). Both macrophage number and MCP-1 expression decrease after gastric bypass surgery- induced drastic weight loss in

morbidly obese patients (Cancello et al., 2005). Chen et al. (2005) found that diet-induced obese mice had elevated adipose tissue expression and plasma levels of MCP-1.

The major source of MCP-1 in adipose tissue has not been determined, but co-culturing macrophages with adipocytes results in a significant upregulation of both MCP-1 and TNF. The upregulation of MCP-1 does not require direct contact between cell types, as media conditioned from macrophages could induce the MCP-1 increase from adipocytes (Suganami et al., 2005). Suganami et al. (2005) did not find that undifferentiated adipocytes produced MCP-1, while Wang et al. (2004) did, however production increased upon differentiation. In opposition to this Gerhardt et al. (2001) found that MCP-1 release was higher in undifferentiated adipocytes than in mature adipocytes. However, the majority of *in vitro* MCP-1 release may actually be due to the non-adipocyte cells in adipose tissue because endogenous TNF promotes an increase in MCP-1 levels from non-fat cells during incubation (Fain et al., 2005). It is probable that all cell types in adipose tissue produce MCP-1, and differences in experimental results may be due to *in vitro* conditions that are unable to exactly simulate *in vivo* conditions.

Increases in MCP-1 protein levels precede increases in TNF expression in adipose tissue, suggesting that MCP-1 has a role in TNF expression (Chen et al., 2005). However, TNF exposure also stimulates MCP-1 release (Fain, 2006; Bruun et al., 2005; Gerhardt et al., 2001). The drug etanercept (Enbrel®, Immunex Corp., Seattle WA), a TNF inhibitor does not reduce MCP-1 production, suggesting that if TNF is involved in MCP-1 release, it is at least not required for it (Fain and Madden,

2006). From this evidence it is plausible that recruitment of macrophages through MCP-1 production also results in increased TNF production from macrophages and adipocytes, and that this increased TNF then has a feed forward effect on MCP-1 production and further macrophage recruitment- a feed forward cycle of obesity.

Tumor necrosis factor-α production is stimulated by bacterial products such as LPS (Chung et al., 2006; Fain, 2006; Flad et al., 1989; Islam et al., 2004) , viruses, TNF (Wang et al., 2005) and free fatty acids (Baldeweg et al., 2000). The mechanisms of production and release of TNF in obese adipose tissue are incompletely understood, but are most likely due to more than one factor. It is down-regulated by drugs such as cyclooxygenase-2 inhibitors (Fain, 2006), phosphodiesterase inhibitors (Morjaria, et al., 2006), glucocorticoids (Waage, 1987), and thiazolidinediones (Murase et al., 1998). Lipopolysaccharide (LPS) is a known inducer of TNF production and release, from most cell types, and induces a concentration dependent stimulation of the synthesis of TNF in hepatocytes (Thirunavukkarasu et al., 2006). In adipocytes, pretreatment with inhibitors to proteins in the LPS-stimulated TNF signal transduction cascade does not affect basal release levels of TNF, suggesting that basal TNF release is constitutive and independent of the LPS-stimulated pathway (Nakajima et al., 2004; Thirunavukkarasu et al., 2006). Kern et al. (1995) also found that adipocyte TNF was not stimulated by LPS. Therefore, adipocyte TNF expression and release may be regulated differently from the mechanisms that regulate macrophage TNF expression and release.

The major producer of TNF may not be adipocytes, but instead a different cell type in adipose tissue. Sewter et al. (1999) found that stimulation of adipose tissue with LPS resulted in a 1000% increase in TNF release, whereas release from isolated adipocytes only increased 150%, suggesting that the major TNF producer in adipose tissue is not adipocytes. Chung et al. (2005) and Creeley et al. (2006) also found that TNF expression increased in adipose tissue following LPS exposure, and that this occurred primarily in the non-adipocyte fraction. LPS is often used as a TNF stimulator to measure tissue TNF production, however, LPS is present only during states of acute infection. Therefore, the increases of TNF seen in obesity may be regulated by a pathway that is independent of LPS stimulation.

Without LPS stimulation, Fain et al. (2004) found that TNF release from obese human adipose tissue was primarily attributed to the non-fat cells. It is also possible that that the non-adipocyte fraction of adipose tissue is responsible for producing a necessary stimulatory factor that is absent when the cell types are separated. A time dependent study showed that TNF release from isolated adipocytes decreased across time, while the non-fat portion of adipose tissue had no change in TNF release (Fain, 2006). These findings were later reinforced through demonstrating that TNF release by adipose tissue without lipid was the same as adipose tissue with lipid and both were significantly greater than isolated adipocytes (Fain, 2006). Xu et al. (2003) also noted that the majority of TNF mRNA was lowest in adipocyte fractions of adipose tissue. In opposition to these results, Hotamisligil (1993) found that when adipocytes were separated from the rest of the adipose tissue, the majority of TNF mRNA fractionated with adipocytes and not the stromal vascular

fraction. Overall, these results indicate that the non-adipocyte fraction of adipose tissue is most likely the main producer of TNF, and that while adipocytes may produce some constitutive TNF, up-regulation of TNF most likely requires a signal from the non-adipose fraction (Fain et al., 2004b).

Effects of Body Fat on TNF

In horses, circulating concentrations of TNF have been positively correlated to BCS (Vick et al., 2007); however most of the evidence for the effects of body fat on TNF come from either rodent or human studies. In humans, adipose tissue TNF mRNA expression correlates with degree of adiposity (Bullo et al., 2003; Hotamisligil et al., 1995; Hube and Hauner, 1999; Kern et al., 1995), while BW loss correlates with decreasing TNF concentrations (Dandona et al., 1998; Samuelsson et al., 2003; Ziccardi et al., 2002). In rodents, adipose tissue TNF mRNA levels are five to ten fold higher in obese vs. lean animals (Hotamisligil et al., 1993). There are a few conflicting results however, as even when adipose tissue mRNA levels of TNF are markedly increased, a few authors have not found circulating protein levels to increase (Kellerer et al., 1996; Hauner et al., 1998a; Pfeiffer et al., 1997) or adipose tissue TNF concentration increased while circulating concentrations did not (Hotamisligil et al., 1995). Interestingly, patients with BMI > 45 (40 to 45% body fat), had decreased adipose tissue TNF mRNA (Kern, 1995).

In addition to changes in TNF mRNA and protein levels, changes in receptor levels have been noted in obesity. However, serum concentrations of the two types of receptors show dichotomous results; the 75 kd receptor may increase as much as six fold in obese vs. lean subjects (Hotamisligil et al., 1996), while no differences were

found in the concentrations of the 55kd receptor. In opposition to this, Hauner et al. (1998b) found that both receptor subtypes were increased 30-40% in obesity. These findings are not completely understood, though Hube and Hauner (1999) hypothesized that receptor up-regulation in obesity may be due to 1) a generally up regulated TNF system, 2) obesity induced receptor resistance, or 3) that soluble receptors serve to neutralize circulating TNF.

Obesity Associated Metabolic Disorders in the Horse

Insulin Resistance. Insulin resistance has been associated with obesity in the horse (Hoffman et al., 2002; Vick et al., 2007), although it is more prominent among pony breeds than horse breeds (Jeffcott and Field, 1986). Horses in a BCS range of 4-6 were not found to be insulin resistant (Nadeau et al., 2006). Insulin resistance is improved with exercise, as 7 days of light training improved insulin sensitivity in obese mares (Powell et al, 2002). Freestone et al. (1991b) noted that obese ponies (BCS 8-9) conditioned through exercise for 6 weeks, had decreased plasma insulin concentrations. However, ponies that lost BW through diet alone also had improved insulin sensitivity.

Insulin resistance in humans has been associated with abdominal adipose mass and high BMI (Abate et al., 1996; Farin et al., 2006; Festa et al., 2001; Janowska et al., 2006; Karter et al., 1996; Mazzali et al., 2006; Salmenniemi et al., 2004; Snijder et al., 2004; Weiss et al., 2004). Following a 17% reduction in weight, obese subjects had significantly decreased serum insulin levels, indicating decreased insulin resistance (Hotamisligil et al., 1995). However, Xydakis et al. (2004) showed that after 7% weight loss (BMI decreased from 39 to 36), obese subjects did not have

decreased TNF levels, but did have increased insulin sensitivity. The lack of change in TNF may be due to the only slight decrease in BMI, while the concurrent increase in insulin sensitivity indicates that there are multiple factors involved in the regulation of insulin resistance in obesity.

The overproduction of TNF in adipose tissue of obese rodents and obese people (Hotamisligil et al., 1995) has been implicated in the development of obesity related insulin resistance (Hotamisligil et al., 1993; Hotamisligil et al., 1995). In humans, infusing recombinant human TNF reduces whole body glucose uptake (Krogh-Madsen et al., 2006; Plomgard et al., 2005), and patients with the highest expression levels of adipose tissue TNF mRNA levels were also the patients with the most cases of diabetes, dyslipidemia, and hypertension (Bullo et al., 2003). You et al. (2005) demonstrated that hyperinsulinemia, a metabolic precursor to diabetes type II, was positively associated with adipose tissue TNF gene expression in women. Additionally, the number of metabolic syndrome components observed in obese subjects was negatively associated with circulating concentrations of an alternatively spliced TNF receptor, known to antagonize TNF action (Fernandez-Real et al., 2006). Thus, Hotamisligil (1999) hypothesized that TNF may directly result in obesity induced insulin resistance.

In vitro TNF results in the loss of insulin's stimulatory effect on glucose uptake into adipocytes (Hauner et al., 1995). Tumor necrosis factor-α (TNF) may induce insulin resistance through increasing serine phosphorylation on insulin receptor substrate-1(IRS-1). Increased serine phosphorylation decreases electrophoretic mobility, thereby interfering with tyrosine phosphorylation of IRS-1

and impairing insulin action (Feinstein et al., 1993; Kanety et al., 1995). Administration of a soluble TNF receptor resulted in marked increases in IRS-1 phosphorylation in the muscle and adipose tissue of obese Zucker rats (Hotamisligil et al., 1994). Tumor necrosis factor-α also acts to decrease insulin receptor, IRS-1, and GLUT4 mRNA expression and protein levels, as well as GLUT4 plasma membrane translocation (Hauner et al., 1995; Lumeng et al., 2006; Stephens et al., 1997) . Thiazolidinediones have a counteractive effect on TNF induced insulin resistance (Hernandez et al., 2004; Osei et al., 2005) . However, while Uysal et al. (1997) noted that TNF deficient ob/ob mice had improved insulin sensitivity over obese controls, other studies suggest that a TNF knockout is not sufficient to prevent obesity associated insulin resistance in rodent models of obesity (Schreyver, 1998; Ventre, 1997). In horses, TNF concentration is positively correlated with the degree of insulin resistance (Vick et al., 2007).

There is significant evidence for the involvement of TNF in the induction of insulin resistance of obese individuals, however there may be other mechanisms involved in this disorder. Insulin resistance is considered to be the central component of the human metabolic syndrome and therefore it is also possible that insulin resistance is also the central component of equine obesity associated metabolic disorders.

Laminitis. Laminitis is a metabolic disease characterized by inflammation of the laminar structures (epidermal-dermal junction) which attach the third phalanx (P3) to the inner hoof wall. Healthy laminae are a metabolically active tissue requiring constant energy sources in order to continually maintain attachment of P3 against the

forces of locomotion and BW of the horse. The equine hoof contains two extensive vascular beds that are under hypothalamic control (Hales and Molyneux, 1985; Pollitt, 1991): the arteriovenous anastomoses, the shunts that allow blood to bypass the capillaries at a faster rate and prevent the horse from developing frostbite (Pollitt, 1991), and the capillary bed- the microvasculature of the laminar structures (Field and Jeffcott, 1989; Hood et al., 1993; Pollitt and Davies, 1998). In severe cases, P3 can completely detach from the hoof wall and rotate through the bottom of the sole.

Obesity is a risk factor for the development of laminitis that may allow certain triggers to have more of an effect than in lean animals (Alford et al., 2002; Budras et al., 2001). Documented laminitic triggers include the ingestion of large amounts of grain (carbohydrate overload) (Garner, et al., 1975), lush grass (Longland et al., 1999), black walnut shavings (Eaton, et al., 1995; Minnick et al., 1975), endometritis (Broome et al., 1992), severe infections (Miller et al., 1983), cortisol dysfunction (French, 2000; Johnson, 2004) and obesity (Kronfeld et al., 2006). Laminitis occurs in both a chronic, long term form and an acute form (Cripps and Eustace, 1999).

The most common method of experimentally inducing laminitis is through carbohydrate overload. Excess carbohydrates are fermented in the hindgut, leading to pH changes that cause death of bacteria and the release of lipopolysaccharide (LPS) across the intestinal mucosa into the circulation (Field and Jeffcott, 1989). Following carbohydrate overload there is high capillary pressure, which may enhance capillary filtration and interstitial fluid accumulation, allowing a compartment syndrome to develop (Allen et al., 1990). Following carbohydrate overload, total vascular resistance in the hoof increased to 3.5 times normal, while digital blood flow was

reduced to one half. The disproportionate change in pressure and flow suggests the use of the arteriovenous anastomoses to shunt blood through the digit. Peroni et al. (2005b) described a dysfunction to venoconstriction and relaxation during the development of laminitis that was restricted to laminar veins, with laminar arteries not affected. Constriction of the veins would increase capillary pressure, forcing extra blood to flow through the arteriovenous anastomoses. It is possible that the vasculature of the equine hoof is biologically predisposed to venoconstriction, explaining why laminitis can result from such a variety of conditions (Peroni, 2005a).

Lipopolysaccharide, when released into the blood stream, may be a factor in the onset of laminitis; however, experimentally induced endotoxemia does not cause laminitis (Pollitt, 1999). Lipopolysaccharide alone has little effect on constriction and relaxation of the vascular system; therefore, it is most likely that the effects of LPS are mediated through the induction of vasoconstrictors. These may include TNF, as Menzies-Gow et al. (2004) found a 30-fold increase in TNF concentrations at 60 minutes after administration of LPS. Bueno et al. (1999) and Seethanathan et al. (1990) also found increased TNF activity after LPS administration. The PIC, interleukin-1β, had upregulated mRNA levels in the perivascular cells of capillaries in horses administered black walnut extract (Fontaine et al., 2001), supporting the idea of inflammatory changes in the hooves of horses with laminitis. In opposition to this, Rodgerson et al. (2001) did not find TNF mRNA to be upregulated in smooth muscle cells obtained from digital arteries exposed to LPS *in vitro*. Because it appears that most of the vascular changes occur in the venous side of the capillary bed, it would be more relevant to study inflammatory changes there.

During the development of laminitis, the enzymatic remodeling process of the hoof may be disrupted (Pollitt, 1999). In the normal healthy hoof, metalloproteinase-2 (MMP2) and metalloproteinase-9 (MMP9) act catabolically to facilitate the constant downward growth of the hoof wall and are regulated by controlled release of specific MMP inhibitors. Increased MMP production has been noted in lamellar tissues affected by laminitis (Johnson et al., 1998), and in other species, MMP production increases upon exposure to PIC (Pollitt, 1999). Although it is unknown as to whether MMP upregulation directly causes disruption of the lamellar attachment, tissues cultured from laminitic hooves show this MMP upregulation preceding detachment of P3 (Pollitt, 1999). *In vitro* both gram positive and gram negative bacteria induce dose dependent activation of MMP2 and MMP9, as well as causing separation of lamellar tissues in hoof explants. This is most likely through the production of exotoxins and endotoxins (Mungall et al., 2000).

Pro-inflammatory cytokines may also control the endothelins, which are implicated in laminitis induction (Russell and Davenport, 1999; Battistini et al., 1993). Endothelin-1 expression is increased in the hoof connective tissue of laminitic horses (Katwa et al., 1999) and plasma concentrations are increased in horses with experimentally induced laminitis (Holm, 2002). However, Menzies-Gow et al. (2004) did not find increased endothelin-1 concentrations after experimental LPS administration. Endothelin-1 induces a concentration-dependent contraction in both equine arterial veins and arteries, with veins being more sensitive, suggesting that endothelins can selectively induce digital venoconstriction (Katz et al., 2003). Normal vascular flow is maintained through interactions with nitric oxide, a

vasodilator (Fleming and Busse, 1999; Russell and Davenport, 1999). Vascular endothelial cells produce and continually release endothelin-1, in low concentrations.

Two endothelin receptors, endothelin receptor A (ETA) and endothelin receptor B (ETB) are differentially expressed throughout the capillary beds of the hoof. Endothelin receptor A and ETB present on the vascular smooth muscle cells mediate vasoconstriction, while ETB present on the vascular endothelial cells mediate vasodilation through nitric oxide pathways. High levels of these receptors may be induced by several factors, including activated platelets, endotoxin, thrombin, PIC, hormones, hypoxia, and ischemia (Russell and Davenport, 1999; Battistini et al., 1993). However, the specific interactions between PIC and endothelins, and their effects on the vascular system are unknown. While it is possible that increases in systemic TNF occurring during obesity may play a role in the development of chronic laminitis through inducing vascular changes or disrupting enzymatic remodeling, it is also possible that TNF action is restricted to the acute form of laminitis seen after exposure to endotoxin.

Another possible explanation for the relationship of laminitis and obesity is that of insulin resistance, as laminitis has been directly linked to insulin resistance in high BCS ponies. Kronfeld et al. (2006) found that previously laminitic ponies had compensated insulin resistance- a decreased insulin sensitivity with a greater pancreatic secretion of insulin. When a portion of these ponies developed clinical laminitis, insulin levels significantly increased. In two of the cases, insulin secretion began to fail, indicating a diabetic like condition. Coffman and Colles (1983) found that ponies affected with chronic laminitis were significantly less sensitive to insulin

than normal ponies. These results were also noted by Jeffcott et al. (1986), when comparing laminitic ponies to normal controls. The link between insulin resistance and laminitis may also be due to decreased glucose uptake by the lamellar tissues (French and Pollitt, 2004). Explants of horse hooves maintained in glucose solution did not undergo lamellar separation, whereas those maintained in saline underwent a lamellar separation characteristic of laminitis (Pass et al., 1998). This provides evidence for the hypothesis that insulin resistance leading to decreased glucose uptake is a risk factor for the development of laminitis through the reduction of energy substrates available to the metabolically active laminae.

Hyperlipemia/Hyperlipidemia. During normal metabolism, fatty acids are released from the adipose tissue by the enzyme hormone sensitive lipase (HSL). Hormone sensitive lipase activity is inhibited by insulin and glucose, but increased by glucagon, thus releasing fatty acids during fasting, when glycogen stores are low (Watson et al., 1992a). Fatty acids are primarily taken up by the liver, where they are either oxidized to acetyl CoA, used for gluconeogenesis, or esterified to triglycerides and stored, or released as very low density lipoproteins (VLDL) (Jeffcott and Field, 1985; Naylor, 1982; Watson and Love, 1994). In circulation, fatty acids are released from VLDL's via the enzyme lipoprotein lipase (LPL) located in the capillary endothelium, and used for energy by adipocytes, skeletal, and cardiac cells (Watson and Love, 1994; Mogg and Palmer, 1995) .

In horses, hyperlipidemia is characterized by circulating triglycerides (TGL) levels of 1-5 mmol/liter, while hyperlipemia is characterized by circulating TGL greater than 5 mmol/ liter, giving the plasma a cloudy appearance (Naylor, 1982).

Hyperlipemia is marked by hepatic lipidosis, fat infiltration of body tissues, organ dysfunction, and a high mortality rate (Naylor, 1980; Watson et al., 1992a; Schotman and Wagenaar, 1968). Schotman and Wagenaar (1968) found that as plasma total lipids increased, the percent of Shetland ponies that survived hyperlipemia decreased, demonstrating the lethality of this condition.

Hyperlipemia most often occurs as a result of a disease or metabolic process that causes marked negative energy balance, such as food deprivation or the metabolic demands of pregnancy and lactation (Frank et al., 2002; Schotman and Wagenaar, 1968; Seifi et al., 2002). Eighty-three to 100% of miniature horse cases (Mogg and Palmer, 1995), 30% of pony breeds cases (Gay, 1978; Watson, 1992), and 100% of horse cases of hyperlipemia or hyperlipidemia are caused by a primary disease or metabolic problem. Hyperlipemia is most prevalent among pony breeds and donkeys (Jeffcott, 1985; Watson and Love, 1992; Gilbert, 1986), with horse breeds rarely effected, although horses may develop hyperlipidemia. Ponies have a greater propensity to release free fatty acids from adipocytes upon norepinephrine stimulation than do horses; however plasma clearance rates do not differ between sub-species (Breidenbach et al., 1999).

Hyperlipemia may also occur in insulin resistant animals (Jeffcott and Field, 1985). Insulin signaling is important for regulating the activity of HSL through inhibition, and LPL through activation. In the case of insulin resistance, HSL and LPL may not be regulated properly, leading to over release of triglycerides (TGL) from the liver, and TGL accumulation in the blood (Watson and Love, 1992; Breidenbach et al., 1999). Hyperlipemia may also result from inadequate clearance

of VLDL from the blood (Freestone, 1991b); however, others report that LPL activity was not decreased in hyperlipemia (Watson and Love, 1992). Because ponies have a propensity to develop insulin resistance, they also have a greater risk of hyperlipemia (Jeffcott and Field, 1985).

Obesity tends to be a predisposing factor (Watson and Love, 1992; Jeffcott and Field, 1985; Mogg, 1995, Jeffcott, 1986), and there is incidental evidence that a positive correlation exists between body condition score and hyperlipemia risk (Forhead, 1994; Freestone, 1992; Reid and Mohammed, 1996). Reid and Mohammed (1996) found that overweight donkeys were at a higher risk for developing hyperlipemia, and when Watson et al. (1990) compared donkeys in thin, ideal, and obese body condition, they found that plasma VLDL concentration correlated significantly with condition. Obese animals have a greater store of fat to mobilize, and insulin resistance may promote lipid enzyme regulatory problems. In a study on 31 donkeys with naturally occurring hyperlipidemia/ hyperlipemia, Forhead et al. (1994) found a positive correlation between plasma insulin and TGL concentrations, and greater insulin concentrations in greater body condition score animals. While Frank et al. (2006) found that obese insulin resistant horses had higher resting concentrations of VLDL, TGL, free fatty acids, and HDL- cholesterol. Additionally, 4 of 7 of these horses had chronic laminitis.

Tumor necrosis factor- α may also be involved in the onset of hyperlipemia. Kawakami et al. (1981) first noticed that exudate from LPS treated cells caused LPL suppression *in vitro*, and that the mediator of this effect was a heat labile protein. Pekala et al. (1983) showed that the exudate from LPS treated cells suppressed the

activities of acetyl CoA carboxylase and fatty acid synthetase in differentiating preadipocytes. Later studies showed that this protein was TNF and also that TNF may mediate some of the metabolic changes that produce hyperlipidemia through its effects on lipid metabolism (Grunfeld and Feingold, 1991). Circulating free fatty acids and glycerol levels increase following TNF administration in both rodents and humans, indicating that TNF has a role in inhibiting the anabolic activities of adipose tissue (Grunfeld et al., 1988; Feingold et al., 1987; Starnes et al., 1988). Patton et al. (1986) found that following *in vitro* TNF exposure, adipocytes had decreased lipid storage and synthesis, as well as stimulated release of fatty acids from adipocytes.

Within adipose tissue the functions of TNF include the suppression of several enzymes lipoprotein lipase at both the protein and gene levels (Ogawa et al., 1989; Kawakami et al., 1987; Zechner et al., 1988), decrease the synthesis of LPL (Kawakami et al., 1982; Pekala et al., 1983; Patton et al., 1986; Hauner et al., 1995), inhibition of both transcription factor C/EBP-α and the nuclear receptor PPAR γ (two masters of adipose cell differentiation), fatty acid binding protein 2, and metabolic enzymes such as fatty acid synthetase, acetyl CoA carboxylase, and GDPH, (Ogawa et al., 1989; Pekala et al., 1984; Torti et al., 1985). Tumor necrosis factor-α may stimulate adipocytes to mobilize lipids through activation of hormone-sensitive lipase (HSL) (Ogawa et al., 1989), although Green et al. (1994) found no effect of TNF on HSL activity. Tumor necrosis factor-α has also been associated with increased lipolysis of adipose tissue (Feingold, 1992; Green, 1994), inhibition of fat cell recruitment, and possibly induction of fat cell apoptosis (Coppack et al., 2001; Hube and Hauner, 199; Prins et al., 1997; Wellen, 2003). It also acts to stimulate hepatic

triglyceride synthesis (Feingold and Grunfeld, 1987; Feingold et al., 1989; Chajek-Shaul et al., 1989) in rodents.

Tumor necrosis factor- α administered to healthy rats resulted in increased hepatic weight, plasma TGL concentrations, hepatic TGL content, and fatty acid synthesis in the liver (Feingold, 1987). However, the ability of TNF to induce hepatic fatty acid synthesis was limited to the liver, as no changes were seen in other tissues. On an immediate basis, TNF increases fatty acid synthesis by increasing intracellular levels of citrate, an allosteric activator of acetyl CoA carboxylase (Grunfeld et al., 1988). Long term, TNF increases levels of acetyl CoA carboxylase and fatty acid synthetase, and decreases levels of hepatic fatty acyl CoA. Additionally, in obese adult men, LPL activity was inversely proportional to TNF expression, and with BW loss, LPL activity increased to 411% of initial levels (Kern, 1995). During the sickness response, the normal function of TNF is to increase circulating levels of fatty acids for energy, and it is possible that this function is extrapolated to the disruption of lipid metabolism found in the metabolic syndrome.

Dietary Effects on TNF and Metabolic Syndrome Components

In both rodents and humans, high fat diets may result in increased TNF expression and release as shown by Borst and Conover (2005). Rats fed a high fat diet showed significantly higher mRNA levels of the 17 Kd form of TNF in both red gastrocnemius muscle (2 fold elevation) and in visceral fat (54% increase) when compared to rats on a control diet. However, serum levels were undetectable in both groups. Rats fed to obesity on a 45% fat diet (primarily consisting of corn oil) had

significantly greater TNF bioactivity in the retro-peritoneal fat pad, than rats fed a control 12% fat diet (Morin et al., 1997).

Type of dietary fat may have effects on TNF concentrations, but the results of several studies are conflicting. Obese men fed fish oil caplets did not experience any changes in TNF plasma concentrations (Jellema et al., 2004). This result is consistent with those found in rodents with sucrose-induced metabolic syndrome, in which fish oil administration had no effect on TNF concentration (Alexander et al., 2004). However, in these rats, the symptoms of the metabolic syndrome were alleviated by the fish oil diet. Opposing results were seen by Muurling et al. (2003), in which mice with preexisting insulin resistance were fed fish oil. After 10 weeks of 3g/day of fish oil, the insulin resistant mice had lower TNF protein in their white adipose tissue, but there was no improvement in insulin sensitivity. Mice on the same study that were diet restricted to 75% of ad libitum intake, but not fed fish oil, had improved insulin sensitivity as well as decreased TNF protein levels. Additionally, when mice on two different fat diets were injected with LPS, the mice fed a fish oil diet had significantly lower TNF responses than mice fed a low fat diet (Sadeghi et al., 1999). These results suggest that fish oil has the ability to diminish the production of TNF *in vivo*. In horses, α-linolenic acid supplementation reduces LPS-induced TNF production by macrophages *in vitro* (Morris, 1991).

TNF concentrations in humans are increased by diets that are high in hydrogenated fats when compared to the polyunsaturated fat found in soybean oil, although saturated fat (butter) had no effect on TNF (Han et al., 2002). Interestingly, baboons fed a high fat diet (41% fat, ratio of polyunsaturated/saturated fats = 0.33), in

comparison to a low fat diet (7% fat, ratio of polyunsaturated/saturated fats = 2.2), had increased TNF plasma concentrations at 3 week dietary challenge. By week 7 though, their TNF values had fallen below baseline (Shi et al., 2005). It is possible that the initial increase was due to an acute stress response to the dietary change. Contrary to these results, diets high in starch and sugar, and low in fat and fiber, have been shown to decrease insulin sensitivity in the horse (Hoffman et al., 2003; Treiber et al., 2005). The dichotomy of results may be due to the relatively low percent of fat in horse feeds as compared to the diets of humans or rodents. In this case insulin resistance may be due to the high sugar content of the feeds as well as meal feeding, which contrasts to the evolutionary development of the horse as a continual grazer. To date no studies have been conducted on the effects of high fat diets on TNF production in the horse, and therefore, the interaction and effects are unknown.

SUMMARY

Obesity in horses is a condition sometimes accompanied by the development of metabolic disorders. Not only are these metabolic disorders potentially fatal, but they are also expensive to treat and in the case of laminitis, extremely painful to the animal. The mechanisms by which obese animals develop metabolic disorders are not completely understood, but it is possible that obesity induced up-regulation of TNF is involved. While a complete understanding of these mechanisms is required in order to develop adequate treatment strategies, the prevention of disorders that result from obesity, through the prevention of obesity, is also an important management tactic. This includes the ability to monitor equine condition on a continual basis in order to adjust feed and exercise requirements. Body condition scoring is a no-

expense, easily applied system for measuring adiposity, and therein lays its usefulness for the prevention of obesity.

Chapter 3: Manuscript 1

Effects of diet and weight gain on body condition scoring in Thoroughbred geldings

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ABSTRACT

Subcutaneous fat deposition is an indicator of energy balance in horses, and is therefore useful in making feeding management decisions. The most commonly used body condition scoring (BCS) system was developed in light breed mares and assigns a subjective score of fat accretion at six different body areas. While the system is useful, its drawbacks include that it may not be applicable to all horse breeds or genders and that the written definitions of fat accretion are non specific and often difficult to use. The main objective of this study was to examine the usefulness of the Henneke BCS system for Thoroughbred (TB) geldings through examining effects of weight gain and diet on body area fat accretion in these horses. A second goal of this study was to use the information gained to modify the Henneke BCS system, such that it is specific to TB geldings. In this study, fifteen mature TB geldings were fed for weight gain over a nine month period on either a high in starch and sugar (HS) diet or a high in fat and fiber (HF) diet, and were independently assessed for body

area BCS on a monthly basis by two experienced judges using the Henneke BCS system. Throughout the study body area fat accretion characteristics were recorded. Differences between judge scores ranged from 0.10 to 0.81 across all time periods and body areas. Neither judge scored consistently higher than the other. Across the study period, neck scores averaged greater than those of withers and during months two through eight, neck scores averaged greater than loin scores. Out of 62 possible combinations of body areas, 37 were able to predict the mean BCS with a 95% prediction interval of ≤ 0.5 scores, including six 5-body area equations, eleven 4-body area equations, and seventeen 3-body area equations. The results of this study suggest that a more accurate BCS system for Thoroughbreds should be developed and that a fewer subset of body areas may be used to predict BCS.

Keywords: body condition score, body fat, horse, weight gain

INTRODUCTION

Body condition scoring is a subjective method of estimating subcutaneous fat cover in many livestock species, and is one of the most widely used indices of energy balance in equine feeding management. Body condition scores (BCS) are often used as either a statistical blocking factor or response factor in nutrition, physiology, and reproductive studies. Therefore, the ability to assess temporal changes in fat deposition, hence energy balance, is useful not only to researchers, but also to veterinarians and horse owners. The Henneke (1983) BCS system was developed in pre-parturient Quarter horse mares with fat cover scored on a scale of 1 (thin) to 9 (fat) by physical palpation and visual assessment of six discrete body areas; neck, withers, shoulders, ribs, loin, and tailhead. Henneke's system is the most widely used

system and has been applied to all breeds, ages, and genders of horses. This has been done without determining if the system accurately describes body area fat accretion in these different classes of horses, or if all classes of horses accrue subcutaneous fat in the same locations.

Diet may also affect body area adipose gain as a result of the possible differences in efficiency of fat storage when the proportion of fat to carbohydrate changes. High fat diets have been used to induce obesity in rodents (Borst et al., 2005; West and York, 1998), however, in horses it is unknown whether a high fat diet will increase either overall BCS gain or individual body area rates of gain.

The main objective of this study was to evaluate the usefulness of the Henneke BCS system for Thoroughbred (TB) geldings, and also to determine whether modifications to the system are needed for that specific population of horses. Two additional goals of the study were to determine if 1) dietary energy source affected fat accretion rates, and 2) if a smaller subset of body areas could be used to accurately predict the overall mean BCS using the Henneke BCS system.

MATERIALS AND METHODS

The study was conducted over a period of 10 months, from July 2005 to May 2006 at the University of Maryland's Equine Research Unit, in Ellicott City, MD and was approved by the University of Maryland's Institutional Animal Care and Use Committee. It was conducted in accordance with a companion study investigating the effect of diet and weight gain on insulin sensitivity (Quinn, 2007).

Horses

Fifteen mature Thoroughbred geldings (initial BW range 466 to 596 kg, initial BCS range 3.8 to 5.1; initial age range 5 to 15 y) were used in this study. All horses received routine veterinary health care including vaccinations- Eastern, Western, and Venezuelan encephalitis, Rhinopneumonitis, Influenza, Tetanus (Prestige V + VEE, Intervet, Millsboro, DE) Potomac horse fever and West Nile (Potomac Guard and West Nile Innovator, Fort Dodge Animal Health, Overland Park, KS), dental care, and a broad spectrum dewormer (Zimecterin Gold Ivermectin/Praziquantel, Merial, Duluth, GA) before the start of the study and throughout the study as needed. Horses were housed in individual (3.6 m^2) box stalls with rubber mats and wood pellet litter (Woody Pet Products, Surrey, BC, Canada). Horses were weighed on a livestock platform scale (Central Carolina Scale, Sanford, NC) prior to the start of the study, and then weekly thereafter. Horses were housed in the barn for three weeks prior to the start of the study to allow for acclimation the experimental conditions.

Diets

During the three week acclimation period, horses received mixed grass/alfalfa hay and a commercially available concentrate (Legends 10, Southern States Cooperative, Richmond, VA) fed to meet their daily DE requirement for maintenance (NRC, 1989). After the acclimation period horses were randomly assigned to one of two experimental concentrates designed to be isonitrogenous and isoenergetic, but differing in energy source, either high in starch and sugar (HS; n = 9) or fiber and fat (HF; n = 6) (Table 1). Horses were transitioned onto the treatment feeds over a period of 14 d. Horses were fed a mixed grass/legume hay and either the HS or HF

concentrate at a ratio of 60% DE from hay and 40% DE from concentrate. Hay was fed in a wooden box mounted on the floor in the corner and grain was fed from canvas feed bags (Country Supply, Louisiana, MO). Total daily intake of hay and concentrate was split into two meals fed at 0800 and 1900. Hay was offered first, with concentrate offered immediately following. Refusals of concentrate were collected and weighed after each feeding. Refusals of hay were collected and weighed on a daily basis, before the 0800 feeding. Intake of hay and concentrate was recorded daily.

Hay samples were obtained by random core sampling from no less than 25% of the hay used in the study (n = 14). Concentrate samples were obtained by random grab sampling (n = 8) on a monthly basis. Hay and concentrate were submitted for nutrient analysis to a commercial laboratory (Table 1) (Dairy One, Ithaca, NY). Access to fresh clean water and salt blocks containing only sodium chloride were available at all times.

Weight Gain

Horses were fed at maintenance for four weeks to allow for acclimation to the treatment diets, and then feeds were increased to weight gain intake levels over a period of one week. Horses were fed for a targeted weight gain of 23 kg per three weeks, which was previously shown to result in a gain of one BCS score (Heusner, 1993). In order to achieve the targeted weight gain, hay and concentrate was fed to provide each horse with an additional 20 Mcal DE above their daily DE requirement for maintenance. Horses were fed for weight gain over a period of eight months, August, 2005 to April, 2006. Five months after the start of the study, an additional

seven Mcal DE was offered daily to each horse via concentrate at 1400 h in order to encourage an increase in ADG.

Exercise

Horses were exercised at a walk (1.5m/s) for 35 min twice daily on a six horse automated equine exerciser (Priefert, Mt. Pleasant, TX). Horses were permanently assigned to one of three exercise groups, which were exercised in a random order, between 0900-1100 and 1630-1830. The companion study included a two week minimal exercise period following the weight gain period of the study; however data from that period was not used in this study.

Body Condition Scoring

Body condition scores were assessed monthly by two independent judges using a BCS system with a nine point scale (Henneke et al., 1983). Judges were selected based on their equine expertise and knowledge of the BCS system. Each judge conducted their body condition scoring independently of each other, but scores were obtained within 24 h of each other. On three occasions, scores could not be obtained by both judges, and in this case, body condition scoring by both judges was conducted the following week. Each of the six body areas (neck, withers, shoulders, ribs, loin, and tailhead) were assessed using both physical palpation (e.g., gently pressing or pinching fat along the designated body area) and visual appraisal in order to determine the amount of fat present. Body areas were scored, and an average of the six was used as a mean BCS for each horse at each assessment period. Additionally, observations of fat accretion characteristics were recorded throughout

the study, which included both visual and physical observations of fat fill, tone, and feel, to be used for describing subcutaneous fat accumulation in TB geldings.

Statistical Analysis

Monthly body weight data were analyzed by repeated measures ANOVA using the mixed models procedure of SAS (v. 9.1 SAS Institute Inc., Cary, NC) in order to determine the effects of month and diet. Monthly BCS data were analyzed by repeated measures ANOVA in order to determine the effects of month and diet. Monthly DE intakes were analyzed at each month by ANOVA in order to determine the effects of diet.

Judge precision. In order to analyze the precision between the two judges, data were analyzed using aggregate analysis according to methods adapted from those described previously (Bland and Altman, 1986), where the differences between judges' scores (DIFF) of each body area served as the dependent variable. The effects of diet, body part, and month on DIFF were analyzed by repeated measures ANOVA. Significance was established from least squares means, which tested the mean DIFF as being significantly different from zero. A α level of $P < 0.05$ was used for the determination of significance.

Agreement of body area scores. The effects of time and diet on body area scores were determined using repeated measures ANOVA. A α level of $P < 0.05$ was used for the determination of significance.

Prediction of BCS. To determine whether a smaller subset of body areas could accurately predict the overall BCS, prediction equations were generated. For this analysis, judge scores were averaged. All possible combinations of body areas were

run individually through a mixed model ANOVA, with mean BCS as the dependent variable and the specific combination of body areas to be tested as the predictor variables. Influence diagnostics were used to remove one horse at a time from each equation through the use of influence statistics. The predicted residual sums of squares (PRESS) statistic represents the observed (actual mean BCS) minus predicted (group mean BCS) when one observation is deleted, which in this case is the mean BCS for an individual horse. The PRESS statistics were summed (sPRESS) and used to determine the prediction r^2 using the equation [(Total sums of squares- Month sums of squares) – (sPRESS] / [Total sums of squares - month sums of squares]. Prediction intervals were calculated from the confidence intervals by the equation (√ (sPRESS/ horse sums of squares))*confidence interval. Thus prediction equations were developed on all horses minus one, and then asked to predict the one horse that was deleted. Weighted coefficients for each prediction equation were determined using estimate statements.

RESULTS

Average monthly digestible energy (DE) intakes were similar between dietary groups and shown in Table 2. Average BW prior to the initiation of and at the end of weight gain was 519 ± 12 kg and 608 ± 12 kg, respectively. Weight gain over the course of the study resulted in an average increase in BCS from 4.3 ± 0.1 to 6.9 ± 0.1. There was no main effect of diet on either mean BW or BCS, therefore monthly means were averaged across diets and presented in Figure 1.

Judge precision was not different between dietary groups, however there was an interaction between time and body area ($P < 0.001$) for judge precision (Table 3).

Judges scored differently at 52% of the assessments with differences ranging from 0.28 to 0.81 scores ($P < 0.05$). Neither judge scored consistently either higher or lower than the other.

Figure 2 shows the interaction between body area and diet ($P = 0.015$) for body area scores. Horses on the HF diet had higher scores at the neck ($P = 0.030$) and withers ($P = 0.060$). Figure 3 shows the interaction between time and body area ($P < 0.001$) for body area scores. Neck scored higher than withers and loin from Sep through Apr, and neck also scored higher than loin from Oct through Apr.

There were 62 possible combinations of body areas that were evaluated for their ability to predict the mean BCS (Table 4). Of these, 37 equations had a 95% prediction interval of ≤ 0.50 scores, which ranged from 0.16 to 0.50. Characteristics of fat accretion observed during this study are described for each body area (Table 4).

DISCUSSION

The Henneke BCS system was developed on pre-parturient Quarter Horse mares, and has since been extrapolated to all breeds and genders without determining its suitability for these other classes of horses, such as its use in ponies (Freestone et al., 1992) and draft horses (Potter et al., 1987). Body condition scoring systems need to demonstrate three specific qualifications in order to be valid: repeatability, reproducibility, and predictability (Burkholder, 2000; Evans, 1987). Repeatability is one test of a BCS system's precision, such that one judge repeatedly assigns the same score to one animal. Reproducibility is another test of a BCS system's precision, such that one or more judges assign the same score to the same animal, and is the focus of this analysis. Lastly, predictability is a test of a BCS system's ability to

accurately reflect the fat cover of an animal. The predictability of the Henneke BCS system was determined by Henneke et al. (1983) using body fat equations developed by Westervelt et al. (1976).

The main objective of this study was to examine the usefulness of the Henneke BCS system for TB geldings as part of a companion study investigating the effects of weight gain on insulin sensitivity. Precision of the Henneke BCS system was analyzed by comparing two judge's scores to each other. Body condition scores are defined at the whole-score level, yet half scores may be used, and because of this, it was our goal to determine if the two judges were able to agree with each other within a half score or 0.5. The fact that two judges scored differently from each other more than half of the time indicates the subjective nature of the BCS scoring system. Reasons that the judges may not have been able to more consistently assign the same score to body areas include 1) the descriptions of fat accretion within each body area score may be too ambiguous too allow for objective interpretation, and 2) because the system relies on visual and physical appraisal, there is an inherent degree of user bias involved in the assigning of scores. Both factors are inevitably involved in the error associated with BCS. The purpose of a BCS system is to describe an amount of subcutaneous fat by assigning a discrete number, but inherently a human's ability to accurately quantify fat accretion is limited. This has impacts on both the ability to develop a system with explicit BCS descriptions, as well as the ability of a user to interpret BCS descriptions in an unambiguous manner.

While score disagreement could be expected at all body areas, the unequal distribution of judge disagreement suggests that the score descriptions given to those

body areas most disagreed upon are not specific enough to allow for score differentiation. It is also possible that there are extraneous factors specific to those body areas, such as TB conformation, that affected unbiased score assignment in this BCS range. That the two judges used in this study were not consistently able to assign the same score to all body areas, even with their substantial prior experience with body condition scoring suggests that between the score range of a four to seven, the score characteristics of these body areas require refinement for use in for TB geldings.

Assessing the usefulness of the Henneke BCS system for TB geldings included determining whether body area scores need adjustment in relation to each other. This was done with the goal of determining if the TB geldings on this study were being assigned the same scores to each body area during each assessment. There were several differences in scores assigned to body areas during all months of the study. Neck scores were consistently higher than withers and were also higher than loin scores during seven of the eight months. Although differences were expected, the consistency of the neck scoring being greater than withers and loin may indicate that TB's put fat down on their neck earlier during weight gain than the loin or withers. It could also indicate that this area is easier to score through allowing judges to more readily differentiate when a horse had achieved a higher score at the neck than at the other two body areas. Neck also scored greater than shoulders, ribs, or tailhead, albeit inconsistently throughout the study, but only at three time points was the neck greater than all three. Additionally at most of the monthly assessments the body areas of shoulders, ribs and tailhead did not score consistently different from

each other, suggesting that the Henneke system descriptions of these three body areas for those levels of fat are adequate. The overall results of this analysis suggest that between the BCS range of four to seven, the fat accretion characteristics of each score need a minor adjustment in order to be specific to the way that TB geldings lay down fat. This includes adjusting the neck score such that scores assigned to the neck are more likely to reflect the scores given to the other body areas. For these reasons we adjusted the neck score and developed more specific descriptions of both the anatomical locations of fat accretion and the characteristics of fat accretion scores for each body area between a BCS of four and seven. We suggest that these be tested on a separate population of TB geldings and used in the future.

The second goal of this study was to determine if dietary energy source might effect fat accretion at different body areas. The diets were designed to be isoenergetic, but differ in percent fat, ADF, NDF and NSC. Concentrates were formulated based on theoretical DE values of feed stuffs as calculated from Harris and Kronfeld (2003). The "by difference method" of determining DE values of feeds may underestimate the actual DE value of high fiber feeds due to the energy available from the fermentation products. In this study horses on the HF diet scored higher at the neck and withers than horses on the HS diet. However, while this may suggest that dietary energy source had an effect on body area fat accretion, the effect was not great enough to cause a main effect on the mean BCS. Previous studies also reported no difference in mean BCS for horses on either a high in fat and fiber diet or a high in starch and sugar diet (Hoffman et al., 2003; Williams et al., 2001). This may mean that both the withers and the neck areas of TB geldings accrue fat at a greater rate on

high fat and fiber diets than high starch and sugar diets, and may have implications for feeding strategies.

The third goal of this study was to identify one or more equations consisting of a smaller subset of body areas that would accurately predict the mean BCS instead of using all 6 body areas identified by Henneke et al. (1983). There are several benefits to using fewer body areas, including less time required, less education and practice required for inexperienced users to become proficient, and the ability to exclude body areas that are confounded by conformation. The use of prediction equations allows for the equations developed on these study horses to be extrapolated to use in other TB geldings. Additionally, weighted coefficients for each body area in each equation were reported, and allowed for determination of body areas that continually carried lesser or more weight than other body areas. Within the equations that used five body areas, the loin was weighted less, on average than all other body areas, followed by the neck and then the withers, while shoulders were consistently weighted higher than all other body areas.

The analyses used in this study helped us to determine that of the six body areas, withers and loin tended to consistently score lower than the others. These may be in part due to conformational differences between breeds, as well as within a breed (Weller et al., 2006). Many TB tend to have prominent, well defined withers, however there is a great deal of variation in the population (Anderson, 2004). It is possible that differences in wither conformation complicate the process of qualifying the amount of fat present. Secondly, the fit of equipment such as the saddle places significant pressure on the 4th vertebrae, resulting in greater vertical motion than the

un-saddled horse (Winkelmayr et al., 2006) and this may have long term effects on wither conformation. Another factor is that of fitness level, as conditioning horses to carry weight increases wither height (O'Connor et al., 2002) and this may also have a confounding effect on score. Therefore the withers score may confound mean BCS results when comparing horses to each other when horses significantly differ in their wither conformation. Interestingly, the body area with the lowest precision was the withers, further evidence for the possible confounding tendency of this body area.

The loin area in the TB breed may also be difficult to accurately score based on conformational and muscular differences. The extent to which Thoroughbreds and Quarter Horses are asked to perform, and the level of athletic difficulty may change the musculature across the back (Eto et al., 2003; Yamano et al., 2002). This in turn may change the appearance of fat deposition across the loin area, based on the muscles filling in beneath the subcutaneous fat (Graham -Thiers and Kronfeld, 2005). Gentry et al. (2002) also found that the loin area was least correlated to percent body fat gain in mares fed for weight gain. During that study, fat thickness across the loin showed little change even as mares gained condition from a BCS of six to nine.

This study shows that an accurate BCS can be derived using several combinations of fewer body areas. Although the goal of this research was to determine a best fit equation, the presence of multiple equations meeting the defined criteria made this difficult. The authors support the removal of the withers area from the BCS equation due to the possible effect of conformational differences on score assignment, as supported by the fact that it was the least agreed on body area. However, there is no clear delineation for choosing a best fit equation using the

remaining five body areas. Possible recommendations include using the shoulders, ribs and tailhead areas, as they tended to agree most often with each other as well as receiving the highest weight coefficients within the equations utilizing five body areas. One of the goals of this analysis was to identify the equation with the fewest body areas and lowest prediction interval; however, whereas many of the weighted coefficients for the four body area equations were relatively equal, very few of the three body area equations have near equal weighted coefficients. Therefore it is difficult to recommend one equation over the others. Ideally, a few selected equations, such as those including shoulders, ribs, and tailhead should be tested using a separate population of horses

IMPLICATIONS

Although horses accrue subcutaneous fat at six different areas of their body, fewer body areas may be used to accurately predict mean BCS. More specific descriptions of fat accretion characteristics have been reported and require testing to delineate their usefulness for more accurately predicting BCS in TB's.

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TABLES

Table 1. Nutrient composition of a mixed grass/alfalfa hay (Hay) and either a concentrate high in starch and sugar (HS) or high in fiber and fat (HF) fed to horses[1, 2]

Nutrient	Hay	Hay SEM	HS	HF	Pooled SEM[3]	*P*-value[4]
CP, %	14.4	1.0	10.0	10.1	0.6	0.965
ADF, %	34.2	0.4	8.0	21.7	1.3	<0.001
NDF, %	53.9	0.1	17.0	32.8	1.3	<0.001
NSC[5], %	14.2	0.1	56.7	20.1	1.4	<0.001
Fat, %	3.1	0.8	4.3	17.2	0.9	<0.001
Ash, %	7.0	0.02	4.6	7.4	0.5	0.001
Ca, %	0.8	1.9	0.7	1.2	0.1	0.010
P, %	0.3	1.5	0.3	0.3	0.03	0.041
DE, Mcal/kg	1.0	0.02	3.6	2.9	0.03	<0.001
DE, Mcal/kg[6]			3.6	3.5		

[1] DM basis as analyzed by Dairy One (Ithaca, NY)

[2] n = 15 for Hay; n = 9 for HS; n = 6 for HF

[3] SEM pooled for HS and HF concentrates

[4] Significance of differences between HS and HF concentrates

[5] Non-structural carbohydrates

[6] Theoretical DE value calculated based on the feed formulation, as proposed by Harris and Kronfeld (2003)

Table 2. Digestible energy (DE) intakes of horses fed to gain weight over eight months on a mixed grass/alfalfa hay and either a concentrate high in starch and sugar (HS) or high in fat and fiber (HF) [1, 2]

Month	HS (n=8)[3]	HF (n=6)	*P*-value
	--- Mcal/d---		
Aug	18.0 ± 0.5	18.2 ± 0.5	0.735
Sep	35.8 ± 0.5	34.8 ± 0.5	0.946
Oct	33.4 ± 1.0	32.1 ± 1.1	0.414
Nov	35.1 ± 1.7	32.7 ± 1.8	0.349
Dec	39.2 ± 2.0	37.5 ± 2.2	0.576
Jan	33.0 ± 1.6	28.3 ± 1.7	0.066
Feb	34.6 ± 1.9	31.5 ± 2.0	0.286
Mar	36.5 ± 2.7	32.7 ± 2.9	0.347
Apr	36.0 ± 2.1	30.5 ± 2.2	0.091

[1] DE calculated based on formulations proposed by Harris and Kronfeld (2003)

[2] DE intakes are represented as means ± SEM

[3] One horse removed from analysis due to an inability to collect accurate refusal data

Table 3. Precision of body condition scores (BCS) assigned to six body areas by two judges over the 8 mo study [1]

Month	Body Area	Precision[2]	SE	*P* - value
Aug	Neck	-0.18	0.13	0.156
	Withers	0.00	0.13	1.000
	Shoulders	-0.37	0.13	0.003
	Ribs	-0.26	0.13	0.039
	Loin	-0.17	0.13	0.191
	Tailhead	-0.13	0.13	0.326
Sep	Neck	0.08	0.13	0.513
	Withers	0.38	0.13	0.003
	Shoulders	0.12	0.13	0.326
	Ribs	0.18	0.13	0.156
	Loin	0.06	0.13	0.663
	Tailhead	0.04	0.13	0.743
Oct	Neck	0.21	0.13	0.102
	Withers	0.85	0.13	<0.001
	Shoulders	0.47	0.13	<0.001
	Ribs	0.57	0.13	<0.001
	Loin	0.31	0.13	0.017
	Tailhead	0.38	0.13	0.003
Nov	Neck	-0.47	0.13	<0.001
	Withers	0.28	0.13	0.029
	Shoulders	-0.07	0.13	0.585
	Ribs	-0.08	0.13	0.513
	Loin	0.06	0.13	0.663
	Tailhead	-0.01	0.13	0.913

[1] Scores assigned using the method of Henneke et al. (1983)

[2] Precision calculated as score of Judge 1 – score of Judge 2

Table 3 continued. Precision of body condition scores (BCS) assigned to six body areas by two judges over the 8 mo study [1]

Month	Body Area	Precision[2]	SE	*P* - value
Dec	Neck	0.13	0.13	0.039
	Withers	0.24	0.13	0.064
	Shoulders	0.07	0.13	0.585
	Ribs	0.14	0.13	0.275
	Loin	0.13	0.13	0.326
	Tailhead	0.06	0.13	0.663
Jan	Neck	-0.29	0.13	0.022
	Withers	0.56	0.13	<0.001
	Shoulders	0.17	0.13	0.191
	Ribs	-0.19	0.13	0.127
	Loin	0.15	0.13	0.230
	Tailhead	0.00	0.13	1.000
Feb	Neck	-0.01	0.13	0.913
	Withers	0.56	0.13	0.050
	Shoulders	0.06	0.13	0.662
	Ribs	0.18	0.13	0.156
	Loin	0.32	0.13	0.012
	Tailhead	0.17	0.13	0.191
Mar	Neck	-0.38	0.13	0.003
	Withers	-0.32	0.13	0.012
	Shoulders	-0.31	0.13	0.017
	Ribs	-0.81	0.13	<0.001
	Loin	-0.31	0.13	0.017
	Tailhead	-0.51	0.13	<0.001
Apr	Neck	-0.49	0.13	<0.001
	Withers	-0.07	0.13	0.585
	Shoulders	-0.43	0.13	<0.001
	Ribs	-0.57	0.13	<0.001
	Loin	0.54	0.13	<0.001
	Tailhead	-0.18	0.13	0.156

[1] Scores assigned using the method of Henneke et al. (1983)

[2] Precision calculated as score of Judge 1 – score of Judge 2

Table 4. Weighted body condition score prediction equations, 95% prediction intervals, and prediction r^2 for all possible combinations of body areas[1]

Body Areas	Weighted Equations	95% Prediction Interval	Prediction r^2
NWRLT	0.20*N + 0.21*W + 0.23*R + 0.18*L + 0.18*T	0.16	0.99
NWSLT	0.17*N + 0.19*W 0.24*S + 0.21*L + 0.19*T	0.17	0.99
NWSRL	0.20*N + 0.17*W + 0.19*S + 0.21*R + 0.23*L	0.19	0.98
NSRLT	0.21*N + 0.25*S + 0.21*R + 0.16*L + 0.17*T	0.23	0.98
NWSRT	0.15*N + 0.17*W + 0.18*S + 0.25*R + 0.26*T	0.24	0.98
WSRLT	0.23*W + 0.24*S + 0.16*R + 0.14*L + 0.22*T	0.30	0.97
NWRL	0.24*N + 0.22*W + 0.29*R + 0.25*L	0.27	0.98
NWRT	0.18*N + 0.21*W + 0.33*R + 0.28*T	0.29	0.95
NWSL	0.20*N + 0.20W + 0.30*S + 0.30*L	0.30	0.97
NSRL	0.24*N + 0.28*S + 0.25*R + 0.23*L	0.30	0.97
NWLT	0.22*N + 0.29*W + 0.25*L + 0.24*T	0.30	0.97
NSLT	0.21*N + 0.37*S + 0.21*L + 0.21*T	0.31	0.97
NSRT	0.19*N + 0.27*S + 0.28*R + 0.26*T	0.31	0.97
WSLT	.025*W + 0.31*S + 0.19*L + 0.25*T	0.32	0.97
WSRT	0.21*W + 0.25*S + 0.24*R + 0.30*T	0.32	0.97
NRLT	0.28*N + 0.35*R + 0.17*L + 0.20*T	0.35	0.97
WRLT	0.31*W + 0.27*R + 0.15*L + 0.27*T	0.36	0.97
NWST	0.14*N + 0.19*W + 0.32*S + 0.35*T	0.36	0.97
WSRL	0.24*W + 0.30*S + 0.22*R + 0.24*L	0.40	0.97
SRLT	0.40*S + 0.22*R + 0.13*L + 0.25*T	0.41	0.96
NWSR	0.21*N + 0.15*W + 0.26*S + 0.38*R	0.42	0.96
NSL	0.26*N + 0.43*S + 0.31*L	0.39	0.97
WRT	0.30*W + 0.35*R + 0.35*T	0.40	0.97
WST	0.24*W + 0.38*S + 0.38*T	0.41	0.97
NRL	0.34*N + 0.40*R + 0.26*L	0.41	0.96
NRT	0.27*N + 0.43*R + 0.30*T	0.42	0.96
SRT	0.40*S + 0.28*R + 0.32*T	0.43	0.96
NWL	0.28*N + 0.33*W + 0.39*L	0.43	0.96
NST	0.19*N + 0.45*S + 0.36*T	0.45	0.96
NSR	0.25*N + 0.33*S + 0.42*R	0.46	0.96
WSL	0.28*W + 0.41*S + 0.31*L	0.46	0.96

[1] Where N=neck, W=withers, S=shoulders, R=ribs, L=loin, and T=tailhead

Table 4 continued. Weighted body condition score prediction equations, 95% prediction intervals, and prediction r^2 for all possible combinations of body areas

Body Areas	Weighted Equations	95% Prediction Interval	Prediction r^2
NWR	0.26*N + 0.23*W + 0.51*R	0.48	0.96
WLT	0.41*W + 0.24*L + 0.35*T	0.48	0.96
SLT	0.52*S + 0.18*L + 0.30*T	0.48	0.86
NWT	0.20*N + 0.34*W + 0.46*T	0.48	0.85
WRL	0.36* W + 0.37*R + 0.27*L	0.49	0.96
WSR	0.23*W + 0.37*S + 0.40*R	0.50	0.96
SRL	0.48*S + 0.29*R + 0.23*L	0.52	0.84
NLT	0.37*N + 0.31*L + 0.32*T	0.55	0.81
RLT	0.50*R + 0.12*L + 0.38*T	0.61	0.76
NWS	0.23*N + 0.22*W + 0.55*S	0.62	0.76
ST	0.58*S + 0.42*T	0.52	0.83
WT	0.42*W + 0.58*T	0.56	0.80
NR	0.38*N + 0.62*R	0.57	0.80
SR	0.54*S + 0.46*R	0.59	0.78
SL	0.68*S + 0.32*L	0.60	0.77
RT	0.55*R + 0.45*T	0.60	0.77
WR	0.38*W + 0.62*R	0.61	0.77
WL	0.53*W + 0.47*L	0.67	0.72
NL	0.49*N + 0.51*L	0.67	0.72
WS	0.30*W + 0.70*S	0.68	0.71
NS	0.29*N + 0.71*S	0.69	0.71
NT	0.39*N + 0.61*T	0.72	0.67
RL	0.71*R + 0.29*L	0.77	0.63
NW	0.44*N + 0.56*W	0.90	0.50
LT	0.34*L + 0.66*T	1.02	0.49
S	S	0.79	0.61
R	R	0.82	0.57
W	W	0.89	0.50
L	L	0.99	0.38
N	N	1.27	0.00
T	T	1.35	-0.14

[1] Where N=neck, W=withers, S=shoulders, R=ribs, L=loin, and T=tailhead

Table 5. Characteristics of individual body condition scores[1]

	Characteristics[2]					
BCS	Neck Area	Wither Area	Shoulder Area	Rib Area	Loin Area	Tailhead Area
Anatomical Description[3]	From the poll to the 3rd vertebrae	From the top of the shoulder blade to the top of the spinous processes of the 3rd, 4th, and 5th vertebrae	Between scapula / triceps muscle and barrel	Includes 6th through 12th ribs	Lumbar vertebrae	Caudal sacral vertebrae to tuber ischii
4	Small amount of fat deposited on crest of neck with slight depression in front of withers, otherwise flat between poll and withers.	Wither prominence depends on conformation. Shape of spinous processes visible. Fat begins to accumulate around transverse processes.	Point of shoulder easily identified. Top of scapular spine may be visible. Some fat felt but area is concave in appearance.	Central ribs visible (ribs 6-12) with others easily felt.	Some filling around sides but tops of spinous processes easily visible.	Tailhead prominence depends on conformation. Spinous processes of sacral vertebrae visible with little fat fill on sides. When viewed from side, some fat accumulation but concave in appearance.
5	Even deposition of fat along crest of neck, creating a smooth nearly flat line between the poll and the withers.	Fat accumulating from top of shoulder blade to point of withers lending a nearly flat appearance.	Fat fill in shoulder area creates smooth transition from shoulder blade to barrel though slightly concave. Can no longer identify scapular ridge.	Ribs cannot be visually distinguished, but can be easily felt.	Fat fill along spinous processes makes loin area level.	Fat filled along either side of tailhead, spinous processes of sacral vertebrae no longer visible. Fat along tailhead results in flat appearance when viewed from side.

[1] Adapted from those of Henneke et al. (1983)
[2] Descriptions from Henneke et al. (1983) in italics
[3] Adapted from Henneke et al. (1983)

Table 5 continued. Characteristics of individual body condition scores[1]

	Characteristics[2]					
BCS	Neck Area	Wither Area	Shoulder Area	Rib Area	Loin Area	Tailhead Area
6	Fat cover on crest of the neck slightly increases height of neck (e.g. a "cresty" neck beginning to develop).	Fat fill results in flat appearance of withers.	Fat fill appears convex and increasing in size ventrally.	Fat laid down in between ribs making them difficult to distinguish from each other. Can be felt with direct pressure.	Fat beginning to accumulate above spinous processes creating a slight depression..	Fat fill slightly convex in appearance.
7	Obvious crest with fat fill increasing the width of neck. Fat fill along crest filled in cranially and caudally. Fat laid down in front of shoulder, at point where neck and body meet.	Fat fill convex in appearance.	Fat fill causes obvious convexity, and has increases in size ventrally to encompass the area just behind the point of the elbow.	Noticeable filling between and on top of ribs. Individual ribs can be felt but difficult, even with direct pressure.	Fat accumulated above spinous processes creating an obvious depression on the loin area.	Fat fill above level of bony processes of tailhead.

[1]Adapted from those of Henneke et al. (1983)
[2] Descriptions from Henneke et al. (1983) in italics
[3] Adapted from Henneke et al. (1983)

FIGURES

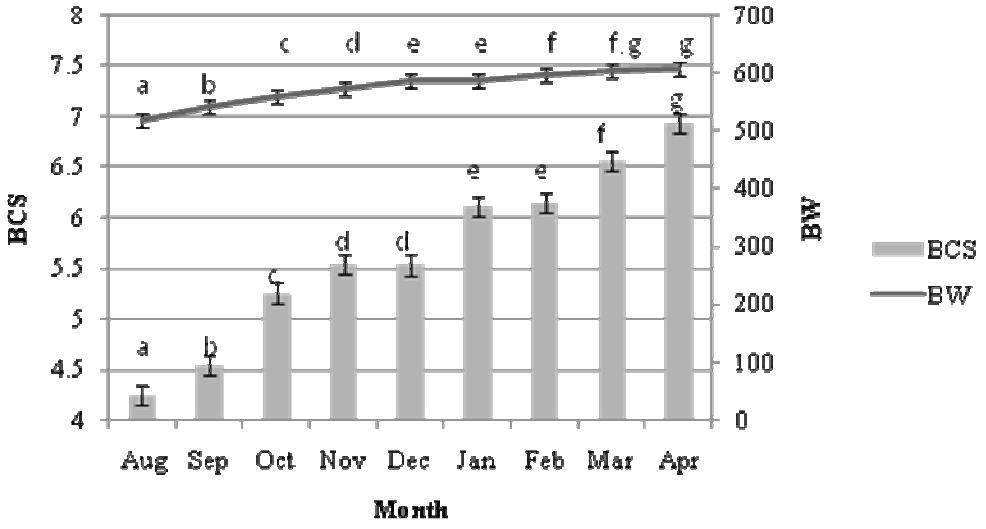

Figure 1. Body condition scores (BCS) and body weight (BW) of horses fed for weight gain on a mixed grass/alfalfa hay and either a concentrate high in starch and sugar or high in fat and fiber. Monthly means are averaged across diets and represented as mean ± SE. ^{a,b,c,d,e,f,g} Means with unlike superscripts within either BCS or BW differ ($P < 0.05$).

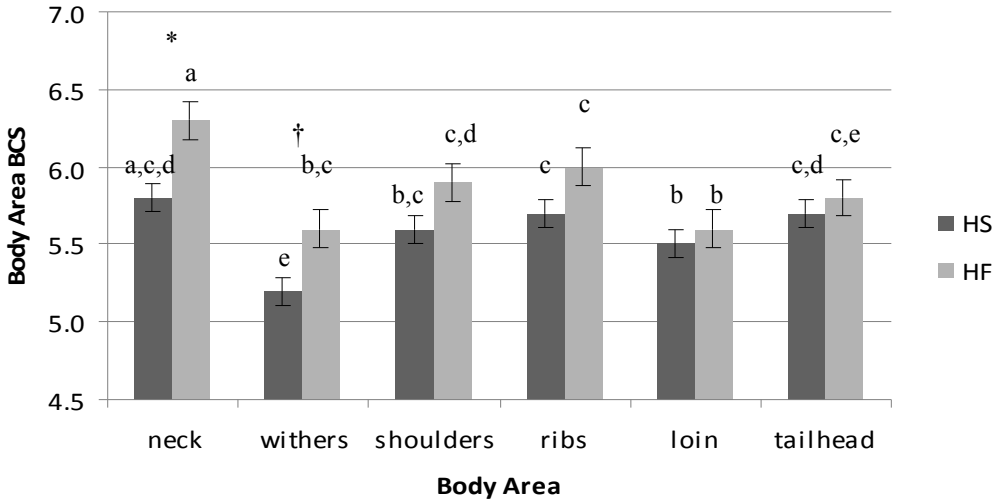

Figure 2. Differences in body area body condition score (BCS) for horses fed mixed grass/alfalfa hay and either a concentrate high in starch and sugar (HS) or high in fat and fiber (HF). Data averaged for two judges and represented as the mean ± SE. [a,b,c,d,e] Means with unlike superscripts differ ($P < 0.05$). *Dietary groups differ ($P < 0.05$). † Dietary groups differ ($P < 0.1$).

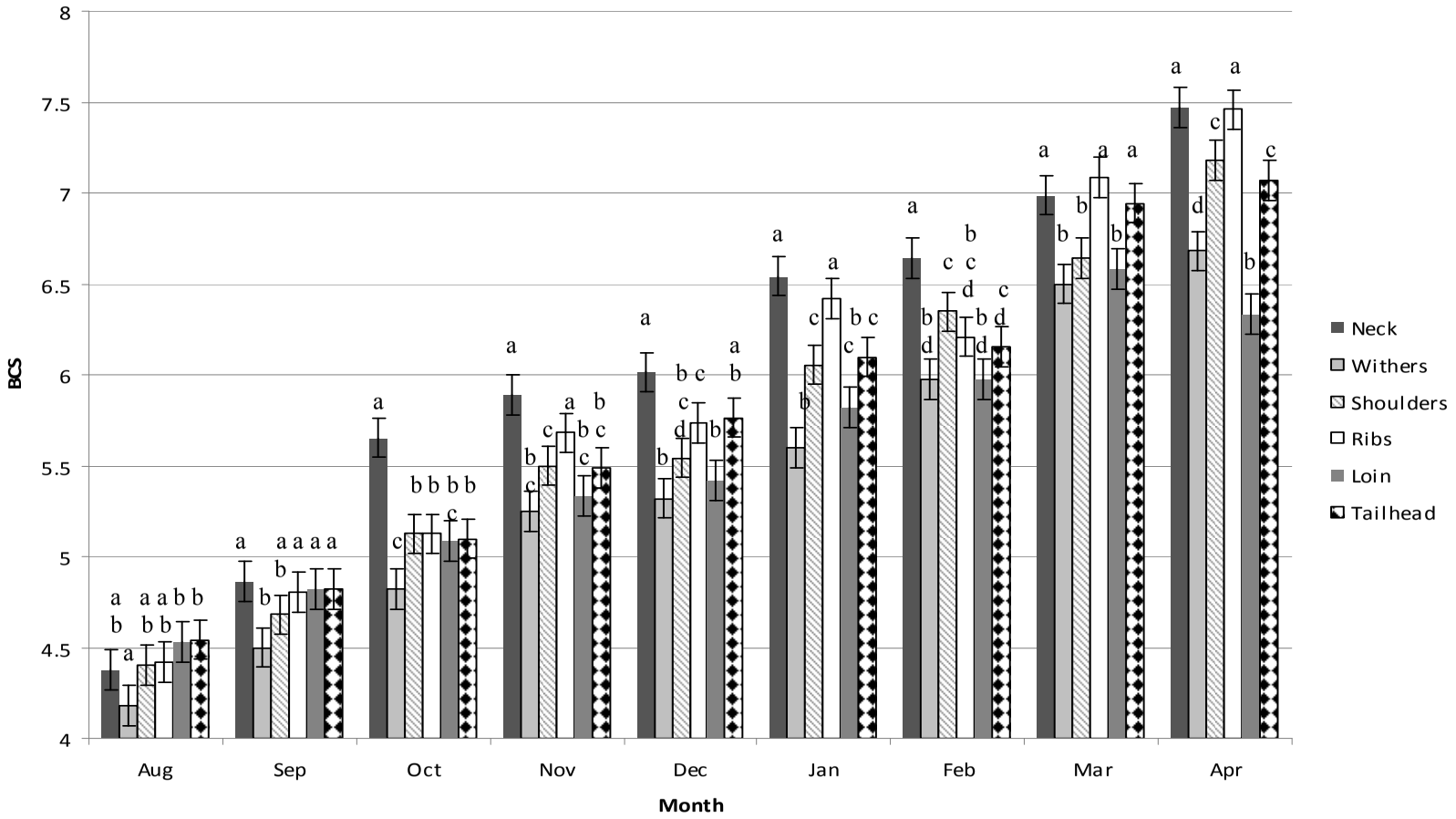

Figure 3. Differences in body area body condition score (BCS) at each month. Data averaged for two judges and represented as mean ± SE. [a,b,c,d] Means with unlike superscripts differ ($P < 0.05$).

Chapter 4: Manuscript II

Effects of diet, weight gain, and exercise on serum TNF-α concentration in Thoroughbred geldings[1]

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ABSTRACT

Obesity in horses is associated with an increased risk for the development of metabolic disorders such as insulin resistance, hyperlipemia, and laminitis. As yet there is no defined upper "safe" limit of adiposity; however in other species increasing adiposity has been associated with an increased level of systemic inflammation, as indicated by circulating concentrations of tumor necrosis factor- α. This increase in inflammation may preclude the development of the metabolic diseases of obesity seen in humans. The objective of this study was to assess whether the level of fatness, type of diet, and/or a cessation of exercise would affect the inflammatory state of the horse as measured by serum TNF concentrations. Fifteen mature Thoroughbred (TB) geldings with an average initial body condition score (BCS) of 4.4 were fed for weight gain over eight months on a diet of hay plus a concentrate that was either high in starch and sugar (HS) or high in fat and fiber (HF). Horses were assessed monthly for BCS and blood samples were taken for

determination of serum TNF. For two weeks following the weight gain period of the study, normal daily exercise of 1.5 m/s for 35 min twice daily was restricted. There was no effect of diet on serum TNF concentrations. Horses at a BCS of four had higher TNF concentrations than horses at a BCS of seven; however, TNF was not different in horses at a BCS of five, six, or seven. There was a trend for an increase of TNF ($P = 0.075$) when horses were not exercised. Horses at a BCS less than five are at a higher risk for inflammation than those between a five and seven.

Keywords: diet, condition, fat, horse, TNF, weight gain

INTRODUCTION

Obesity is the result of long term positive caloric balance and in horses is often the result of a lack of knowledge on the owners' part about proper feed types and amounts, and exercise management (Johnson, 2002). As yet, there is no defined upper "safe" limit of adiposity in horses; however it is imperative that this be determined because obese animals are at a greater risk for the development of life threatening metabolic conditions such as laminitis (Jeffcott et al., 1986; Fontaine et al., 2001) and hyperlipemia (Jeffcott et al., 1985; Hughes, 2004). Adiposity has been positively correlated with increasing systemic concentrations of pro-inflammatory cytokines such as TNF in humans (Hotamisligil et al., 1995) and horses (Vick et al., 2007) which are produced by the accumulating adipose tissue. In humans, an increase in TNF has been associated with low grade inflammation (Sethi et al., 1999) that precludes development of the metabolic diseases of obesity, such as diabetes type II and dyslipidemia (Permana et al., 2006; Skurk et al., 2004). Since the obese equine

also appears to be at risk for metabolic conditions (Johnson et al., 2002; Kronfeld et al., 2006; Treiber et al., 2006), a similar mechanism of TNF action may exist.

Diet and exercise may also affect the relationship between obesity and TNF secretion from adipose tissue. The HF diet was supplemented with corn oil, a feed component that is commonly added to horse feeds to aid in weight gain or to increase the caloric density of feeds for equine athletes. Corn oil has a high ratio of n-6: n-3 fatty acids, which has been found to increase inflammation in obese rats (Morin et al, 1997; Alexander et al., 2004). Alternatively, regular exercise decreased the risk of metabolic diseases in obese humans (Ziccardi et al., 2006) and horses (28-34% body fat, Powell et al., 2002), but it was not known whether it occurred as a result of a change in inflammatory status or another mechanism.

The objectives of this study were to assess levels of inflammation in Thoroughbred geldings as they gained weight and when fed diets differing in energy source. Another objective of this study was to evaluate whether a short term restriction in daily exercise would influence inflammatory state in fleshy horses.

MATERIALS AND METHODS

The study was conducted over a period of 10 months, from July 2005 to May 2006 at the University of Maryland's Equine Research Unit, in Ellicott City, MD and was approved by the University of Maryland's Institutional Animal Care and Use Committee. It was conducted in accordance with a companion study investigating the effect of diet and weight gain on insulin sensitivity (Quinn, 2007).

Horses

Fifteen mature Thoroughbred geldings (initial BW range 466 to 596 kg, initial BCS range 3.8 to 5.1; initial age range 5 to 15 y) were used in this study. All horses received routine veterinary health care including vaccinations- Eastern, Western, and Venezuelan encephalitis, Rhinopneumonitis, Influenza, Tetanus (Prestige V + VEE, Intervet, Millsboro, DE) Potomac horse fever and West Nile (Potomac Guard and West Nile Innovator, Fort Dodge Animal Health, Overland Park, KS), dental care, and a broad spectrum dewormer (Zimecterin Gold Ivermectin/Praziquantel, Merial, Duluth, GA) before the start of the study and throughout the study as needed. Horses were housed in individual (3.6 m^2) box stalls with rubber mats and wood pellet litter (Woody Pet Products, Surrey, BC, Canada). Horses were weighed on a livestock platform scale (Central Carolina Scale, Sanford, NC) prior to the start of the study, and then weekly thereafter. Horses were housed in the barn for three weeks prior to the start of the study to allow for acclimation the experimental conditions.

Diets

During the three week acclimation period, horses received mixed grass/alfalfa hay and a commercially available concentrate (Legends 10, Southern States Cooperative, Richmond, VA) fed to meet their daily DE requirement for maintenance (NRC, 1989). After the acclimation period horses were randomly assigned to one of two experimental concentrates designed to be isonitrogenous and isoenergetic, but differing in energy source, either high in starch and sugar (HS; n = 9) or fiber and fat (HF; n = 6) (Table 1). Horses were transitioned onto the treatment feeds over a period of 14 d. Horses were fed a mixed grass/legume hay and either the HS or HF

concentrate at a ratio of 60% DE from hay and 40% DE from concentrate. Hay was fed in a wooden box mounted on the floor in the corner and grain was fed from canvas feed bags (Country Supply, Louisiana, MO). Total daily intake of hay and concentrate was split into two meals fed at 0800 and 1900. Hay was offered first, with concentrate offered immediately following. Refusals of concentrate were collected and weighed after each feeding. Refusals of hay were collected and weighed on a daily basis, before the 0800 feeding. Intake of hay and concentrate was recorded daily.

Hay samples were obtained by random core sampling from no less than 25% of the hay used in the study (n = 14). Concentrate samples were obtained by random grab sampling (n = 8) on a monthly basis. Hay and concentrate were submitted for nutrient analysis to a commercial laboratory (Table 1) (Dairy One, Ithaca, NY). Access to fresh clean water and salt blocks containing only sodium chloride were available at all times.

Weight Gain

Horses were fed at maintenance for four weeks to allow for acclimation to the treatment diets, and then feeds were increased to weight gain intake levels over a period of one week. Horses were fed for a targeted weight gain of 23 kg per three weeks, which was previously shown to result in a gain of one BCS score (Heusner, 1993). In order to achieve the targeted weight gain, hay and concentrate was fed to provide each horse with an additional 20 Mcal DE above their daily DE requirement for maintenance. Horses were fed for weight gain over a period of eight months, August, 2005 to April, 2006. Five months after the start of the study, an additional

seven Mcal DE was offered daily to each horse via concentrate at 1400 h in order to encourage an increase in ADG.

Exercise

Horses were exercised at a walk (1.5m/s) for 35 min twice daily on a six horse automated equine exerciser (Priefert, Mt. Pleasant, TX). Horses were permanently assigned to one of three exercise groups, which were exercised in a random order, between 0900-1100 and 1630-1830. A two week minimal exercise (MinEx) period occurred following the weight gain period of the study.

Data Collection

Horses were assessed for BCS (Henneke et al., 1983) at the end of the acclimation period, at the start of weight gain, and monthly thereafter. Judges were selected based on knowledge of condition scoring and conducted their surveys independently of each other. Body area condition scores as well as an overall score were determined for each horse at each time period.

Blood samples were collected from each horse when they achieved a gain of one BCS, and before and after the minimal exercise period. A total of 10ml blood was collected via jugular venipuncture into 10 ml uncoated Vacutainer tubes (Vacutainer, Becton Dickenson, Franklin Lakes, NJ), between 0700 and 0800, on days corresponding to BCS assessments. Blood samples were allowed to clot for two hours and were then centrifuged at 15*g for 15 min at 4°C. Serum was then divided into 1 ml aliquots and frozen at -20°C until further analysis.

Sample Analysis

Serum samples were analyzed for TNF concentrations using an enzyme linked immunosorbent assay (Equine TNFα Screening Set, Pierce, Rockford, IL) consisting of recombinant equine TNF, coating antibody, detection antibody, streptavidin HRP, substrate solution (3, 3', 5, 5'- tetramethyl benzidine) and stop solution (0.18 M sulfuric acid). Nunc-Immuno 96 MicroWell flat bottom plates coated with Maxisorb (Nalge Nunc International, Rochester, NY) were coated with 100 uL of 1.0 ug/ml coating antibody prepared in 0.2 M sodium carbonate-bicarbonate buffer (BupH Carbonate/Bicarbonate Buffer, Pierce, Rockford, IL) and allowed to incubate for 18 hr. After incubation, plates were blocked with a blocking buffer (BB) consisting of 4% BSA (Albumin, Bovine Serum, Fraction V, RIA and ELISA grade, Calbiochem, La Jolla, CA), and 5% sucrose (Sucrose, Fisher Scientific, Fair Lawn, NJ), in PBS solution (BupH Modified Dulbecco's Phosphate Buffered Saline Packs, Pierce, Rockford, IL), for 1 hr. After blocking, the BB was aspirated and wells were rinsed 3 times with 300 uL each of a wash buffer (WB) consisting of 0.05% Tween® 20 (Tween® 20 Enzyme Grade, Fisher Scientific, Fair Lawn, NJ) in PBS. After washing, 100 uL of sample or standard was added and incubated for one hr. Then the wash step was repeated and 100 uL of 0.5 ug/ml detection antibody prepared in a reagent Diluents (RD) consisting of 4% BSA in PBS was added. Detection antibody was incubated for 1 hr and then the wash step repeated a third time. Next, 100 uL of streptavidin HRP (diluted 1:400 in RD), was added and incubated for 30 minutes. The wash step was repeated a fourth time and 100 uL of substrate solution was added and incubated for 20 minutes. At this point 100 uL of stop solution was added and

the optical density (OD) values of each well were read in a plate reader (Tecan Sunrise, Phenix Research Products, Hayward, CA) at 450 and 550 nm. The 550 nm reading was used as a correction factor and subtracted from the 450 nm reading for a final value. Serum samples were assayed in duplicate at a 1:4 dilution in RD, and all samples with CV's greater than 10% were re-assayed.

Prior to running samples, the range of the standard curve, parallelism, and recovery were determined using pooled serum sample standards. Sample standards consisted of pooled serum collected from the geldings at three BCS ranges 1) 3.5 to 4.5, 2) 5 to 6, and 3) 6.5 to 7.5. The standard curve was prepared from recombinant equine TNF in RD at concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.68, 7.81, 3.91, 1.95, and 0.98 pg/ml, and assayed in triplicate. The point at which the optical density (OD) values no longer declined with decreasing concentration was determined to be the limit of quantitation, and the lowest point on the standard curve for all future assays.

One ml of each pooled sample was diluted 1:2, 1:4, 1:8, 1:16, and 1:32 in RD. All serum samples were vortexed 30s before diluting. Samples were assayed neat, and at the above dilutions, in triplicate. Parallelism was determined as the range of dilutions where the slopes of the pooled standards paralleled that of the standard curve. Regions determined to be parallel were used to determine recovery. Recovery was determined by diluting serum standards 1:4 in RD and then spiking with 1000 pg/ml recombinant equine TNF. Spiked samples were diluted parallel to the entire range of the standard curve. In addition, a non-spiked sample was assayed at a 1:4 dilution in order to determine the endogenous TNF of the serum standard. Each

sample was vortexed 30 s before diluting. Recovery was determined as measured/predicted x 100%, where measured was the concentration determined from the OD value and predicted was the calculated concentration. To assess effects of incubation time on TNF serum concentrations, samples from three horses were incubated for 15 m, 30 m, one hour and two hours, prior to centrifugation and storage of serum.

The range of the standard curve was determined to be from 7.8 to 1000 pg/ml. Parallelism was confirmed between neat and the 1:4 dilutions, and recovery of spiked 1:4 dilutions averaged 104%. Results of spiking and recovery assay are represented in Table 3. There was no difference in serum concentrations of TNF between samples that were incubated 15 m, 30 m, one hour, or two hours prior to centrifugation and storage of serum (data not shown). Because there were no differences, the two hour incubation time was chosen.

Statistical Analysis

Body condition score data were analyzed by ANOVA with repeated measures using the mixed models procedure of SAS (SAS Institute Inc, Cary, NC), to determine the effects of time and diet on BCS. Body weight data were analyzed by ANOVA with repeated measures, to determine the effects of time and diet on BW gain. Digestible energy intakes between treatment groups within each month were analyzed by ANOVA. Data for serum TNF was non-normal skewed right, and was therefore $\log_{10}$ transformed. The effects of diet and BCS on serum TNF were determined by repeated measures ANOVA. Data were blocked by plate number.

Effects of incubation time on serum TNF concentrations were determined by ANOVA with repeated measures. For all analyses, significance was set at $P \leq 0.05$.

RESULTS

Average monthly digestible energy (DE) intakes were similar between dietary groups and shown in Table 2. There was no effect of diet on either BW or BCS therefore means were combined and averaged across diets (Figure 1). Body weight at the initiation of and at the end of weight gain was 519 ± 12 kg and 608 ± 12 kg, respectively. Body condition score at the initiation of and at the end of weight gain was 4.3 ± 0.1 and 6.9 ± 0.1, respectively. Removing exercise from the horse's daily routine did not result in a change in BW or BCS (Figure 1).

There was no main effect of diet on serum TNF concentrations, therefore means were combined and averaged across diets (Figure 2). The mean TNF concentration for all horses over the entire study was 388.5 pg/mL (range 16.1 – 1871.3 pg/mL). There was a trend ($P = 0.099$) for the effect of BCS on serum TNF (Figure 2). There was also a trend ($P = 0.075$) for an increase in TNF concentrations after two weeks of minimal exercise (Figure 3).

DISCUSSION

The association between weight gain and metabolic disorders was first identified in humans over 80 years ago (Carswell, 1923). One of the theories relating obesity with the development of metabolic disturbances is that adipose tissue is a metabolically active tissue that produces and secretes several hormones and inflammatory proteins (Eckel, 2005; Despres, 2003; Duncan, 2001; Pickup, 2004). In humans, the onset of obesity leads to increased release of the inflammatory proteins

which in turn leads to the development of a low-grade systemic inflammation. This systemic inflammation precludes the development of some metabolic diseases, such as insulin resistance and dyslipidemia (Permana et al., 2006; Skurk et al., 2004), making an increase in inflammation a predictor for the development of these metabolic disorders. Similarly, metabolic disorders in horses and ponies have also been associated with adiposity (Kronfeld, 2006; Treiber et al., 2006), and recent research has come to focus on the role of inflammation in the development of these equine diseases (Vick et al., 2007).

Accumulation of adipose tissue in obesity parallels an increased infiltration of macrophages (Weisberg et al., 2003; Wellen, 2003; Xu et al., 2003). These macrophages are an important source of inflammatory protein production in adipose tissue through molecular signaling between adipocytes and macrophages (Weisberg et al., 2003; Xu et al., 2003). Adipocytes produce two chemokines that affect macrophage infiltration into adipose tissue: macrophage migration inhibitory factor and monocyte chemoattractant protein-1 (Do et al., 2006). The latter is involved with attracting and retaining macrophages in the adipose tissue and is significantly correlated to BMI, waist circumference, and other inflammatory markers in humans (Kim et al., 2006).

Inflammation is indirectly measured by determining levels of different markers such as the pro-inflammatory cytokines and acute phase proteins (Salmenniemi et al., 2004; Schmidt et al., 1999). The normal functions of the pro-inflammatory cytokines are to initiate fever and increase energy availability through disruption of normal glucose and lipid metabolism during the sickness response

(Delano and Moldawer, 2006; Kawasaki et al., 1989; Saigusa, 1990). In the case of sickness, the disruption of lipid and glucose metabolism is beneficial to fuel the increased metabolic rate and decreased food intake (Plata-Salaman, 1998). Recovery from the infection restores normal metabolism (Tracey et al., 1988). In the case of obesity, the continual upregulation of inflammation results in long term disrupted metabolism and the development of the metabolic diseases (Hardardottir et al., 1994; Hotamisligil, 1999).

The pro-inflammatory cytokine, TNF has been used as a marker of inflammation and correlates directly with fat mass in humans (Dandonna et al., 1998; Samuelsson et al., 2003; and Ziccardi et al., 2002) and rodents (Hotamisligil et al., 1993). Remarkably, when injected intravenously into human subjects, TNF has initiated the development of insulin resistance (Krogh-Madsen et al., 2006; Plomgard et al., 2005). Injection of TNF has also been associated with hypertriglyceridemia in dairy cows (Kushibiki et al., 2002). We chose to study TNF as a marker of inflammation in horses because it has been extensively studied in other species and has not only been shown to increase with increasing adiposity but also to induce metabolic disruptions in those species.

The first objective of this study was to assess inflammation in Thoroughbred geldings as they were fed for weight gain, as determined by serum concentrations of TNF. We found that TNF concentrations did not increase when horses gained weight from BCS four to seven, in contrast to Vick et al. (2007). Vick et al. (2007) performed a single assessment of mixed light breed mares between a BCS four to nine that were maintained on pasture, whereas the geldings in this study were

between a BCS of four to seven and were maintained in stalls. Rather, a higher TNF concentration was found when horses were at a lower BCS of four in our study. The higher TNF concentrations seen in the horses of BCS four could have been due to a negative energy balance, as increased concentrations of TNF have been shown to increase in human subjects carrying less than 80% of their ideal body weight (Allende et al., 1998). The increased cytokine production in low body condition could be a metabolic adaptation to increase energy stores. Tumor necrosis factor-α has catabolic effects on energy stores and may be increased in animals of low body condition in order to increase energy availability (Tracey et al., 1987). The fact that there was no increase in TNF concentrations between a BCS of five, six, and seven may indicate that horses within this BCS range are at a healthy level of adiposity.

The levels of TNF reported in this study were lower than those reported by Vick et al. (2007). That study reported values ranging from 156 – 5000 pg/mL, whereas our concentrations were between 16.1 – 1833.8 pg/mL. The higher TNF observed by Vick et al. (2007) is likely due to the higher BCS of the horses on their study, which included horses from a BCS four to nine. In comparison, concentrations of TNF have been reported in the range of 3-18 pg/mL in humans (Dandona et al., 1998; Samuelsson et al., 2004; Ziccardi et al., 2002), 150- 220 pg/mL in sheep (Daniel et al., 2003), and an average of 125 pg/mL in healthy steers (Kahl and Elsasser, 2006). Normal levels of circulating levels of TNF in healthy animals may be species specific with concentrations in horses being higher than those found in other species. Whether horses truly have a higher degree of inflammation in association with those higher TNF concentrations is uncertain.

The second goal of this study was to determine the dietary effects of feeding a diet high in starch and sugar vs. a diet high in fat and fiber on inflammation. High fat diets have previously been shown to induce an inflammatory state in rodents (De Souza et al., 2005). This may be due to the increased availability of free fatty acids when consuming a high fat diet, as free fatty acids may induce TNF release from both adipocytes and macrophages (Suganami et al., 2005; 2007). One possible reason for not seeing a dietary effect is that the 60:40 ratio of hay to grain reduced the overall percent fat in the diet (3.6% on the HS diet and 8.7% on the HF diet) to a level lower than that needed to effect a significant change in inflammation.

The third goal of this study was to determine if the restriction of exercise would have an effect on inflammation in overweight horses, because horses that are diagnosed with metabolic disorders are often prescribed stall rest or are otherwise unable to exercise due to severe pain (Pollit, 1999). The trend for increased TNF following exercise restriction seen in this study suggests that exercise may have a modulating effect on inflammation. In humans, exercise often reduces inflammation (Kondo et al., 2005; Niessner et al., 2006) and this effect is possibly mediated through the effect of exercise increasing oxygen uptake. However, the relationship between inflammation and oxidative status is not completely known. In this study, the lack of a significant effect may due to the length of the exercise restriction being too short to have a significant influence on inflammatory status.

Although the inter-assay CV for the TNF assay was at 5.5%, the intra-assay CV was higher than desired at 18.3%. The large between-assay variability may be due to inconsistencies with the assay, such as antibody stability, slight differences in

concentrations of diluted antibodies, recombinant TNF, or streptavidin HRP, as well as differences in the specificity of recombinant TNF between different vials of recombinant TNF. A fresh standard of recombinant TNF was prepared for each plate unless plates were run on the same day, therefore standard degradation should not have been a source of variability, although there could have been slight differences between vials of recombinant TNF. Secondly, preparations of carbonate-bicarbonate and RD were made up in 100 mL aliquots; therefore slight differences in concentrations may also have played a role, although buffers and reagents were made using the same balance, pipettes, and graduated cylinders to ensure as little variation as possible. In future experiments buffers should be made up in a larger quantity or in the total volume needed to complete the entire experiment to ensure the least buffer variability. Another possible source of variability is the differences in serum clotting time between the collection of the first and last serum samples. Although samples were taken in a random order each collection time and centrifuged within two hours, there may have been differences in serum clot time between months depending on the number of staff available to collect blood samples. The effect of serum clotting time on TNF concentrations was evaluated using only three horses, and should be repeated on a different population of horses to ensure repeatability. Another possible weakness of the assay is evidenced from the 104% average recovery, suggesting that there may be factors in the serum that the antibodies are measuring besides TNF.

IMPLICATIONS

Concentrations of TNF have been reported for clinically healthy TB geldings at a BCS between four and seven, which are useful to be used as reference ranges for

future studies. Horses at a BCS of four and horses with minimal exercise may be at a higher risk of inflammation, and these relationships should be taken into consideration for future research. Horses consuming a total dietary fat up to %8 are not at risk for increased inflammation.

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TABLES

Table 1. Nutrient composition of a mixed grass/alfalfa hay (Hay) and either a concentrate high in starch and sugar (HS) or high in fiber and fat (HF) fed to horses[1, 2]

Nutrient	Hay	Hay SEM	HS	HF	Pooled SEM[3]	*P*-value[4]
CP, %	14.4	1.0	10.0	10.1	0.6	0.965
ADF, %	34.2	0.4	8.0	21.7	1.3	<0.001
NDF, %	53.9	0.1	17.0	32.8	1.3	<0.001
NSC[5], %	14.2	0.1	56.7	20.1	1.4	<0.001
Fat, %	3.1	0.8	4.3	17.2	0.9	<0.001
Ash, %	7.0	0.02	4.6	7.4	0.5	0.001
Ca, %	0.8	1.9	0.7	1.2	0.1	0.010
P, %	0.3	1.5	0.3	0.3	0.03	0.041
DE, Mcal/kg	1.0	0.02	3.6	2.9	0.03	<0.001
DE, Mcal/kg[6]			3.6	3.5		

[1] DM basis as analyzed by Dairy One (Ithaca, NY)

[2] n = 15 for Hay; n = 9 for HS; n = 6 for HF

[3] SEM pooled for HS and HF concentrates

[4] Significance of differences between HS and HF concentrates

[5] Non-structural carbohydrates

[6] Theoretical DE value calculated based on the feed formulation, as proposed by Harris and Kronfeld (2003)

Table 2. Digestible energy (DE) intakes of horses fed for weight gain over an eight month period on a mixed grass/alfalfa hay and either a high in starch and sugar (HS) concentrate or a high in fat and fiber (HF) concentrate and then exercise restricted for two weeks (MinEx)

	Concentrate		
Month	HS	HF	*P*-value
	--- Mcal/d---		
Aug	18.0 ± 0.5	18.2 ± 0.5	0.735
Sep	35.8 ± 0.5	34.8 ± 0.5	0.946
Oct	33.4 ± 1.0	32.1 ± 1.1	0.414
Nov	35.1 ± 1.7	32.7 ± 1.8	0.349
Dec	39.2 ± 2.0	37.5 ± 2.2	0.576
Jan	33.0 ± 1.6	28.3 ± 1.7	0.066
Fen	34.6 ± 1.9	31.5 ± 2.0	0.286
Mar	36.5 ± 2.7	32.7 ± 2.9	0.347
Apr	36.0 ± 2.1	30.5 ± 2.2	0.091
MinEx	29.1 ± 2.1	27.1 ± 2.4	0.534

Table 3. Recovery of tumor necrosis factor-α (TNF) from serum of horses from varied body condition scores (BCS) after spiking with recombinant equine TNF and dilutional parallelism of an ELISA specific for equine TNF (Endogen, Pierce, Rockford, IL) [1]

		BCS 3.5-4.5			BCS 5-6			BCS 6.5-7.5		
Dilution	Spike[2], pg/mL	O, pg/ml	E, pg/ml	R, %	O, pg/ml	E, pg/ml	R, %	O, pg/ml	E, pg/ml	R, %
1:4	0	1.2	-	-	27.6	-	-	71.6	-	-
1:4	1000.0	848.8	1001.2	84.8	884.5	1027.6	86.1	919.1	1071.6	85.8
1:8	500.0	481.6	501.3	96.1	547.0	529.1	103.4	625.7	575.6	108.7
1:16	250.0	255.7	251.3	101.8	302.5	279.9	108.1	372.5	327.6	113.7
1:32	125.0	129.3	126.3	102.4	181.4	156.0	116.3	230.0	203.6	113.0
1:64	62.5	66.4	63.9	103.9	99.5	93.0	107.0	160.2	141.6	113.1
1:128	31.3	35.7	32.6	109.5	64.5	61.8	104.4	121.8	110.5	110.2
1:256	15.6	20.0	17.0	117.6	50.2	46.2	108.7	104.5	95.1	109.9

[1] O=observed concentrations; E=expected concentrations; R=percent recovery

[2] Spiked with recombinant TNF (Endogen, Pierce, Rockford, IL)

FIGURES

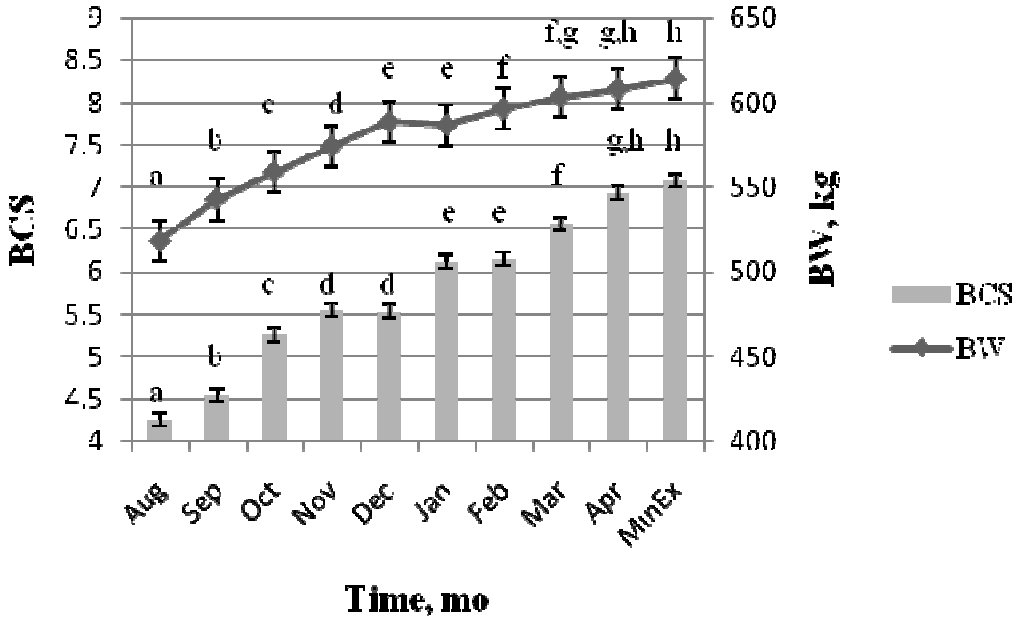

Figure 1. Body condition scores (BCS) and body weight (BW) of horses fed for weight gain on a mixed grass/alfalfa hay and either a concentrate high in starch and sugar or high in fat and fiber. A two week minimal exercise period (MinEx) followed the weight gain period. Monthly means are averaged across diets and represented as mean ± SE. $^{a,b,c,d,\ e,\ f,\ g}$ Means with unlike superscripts within either BCS or BW differ ($P < 0.05$).

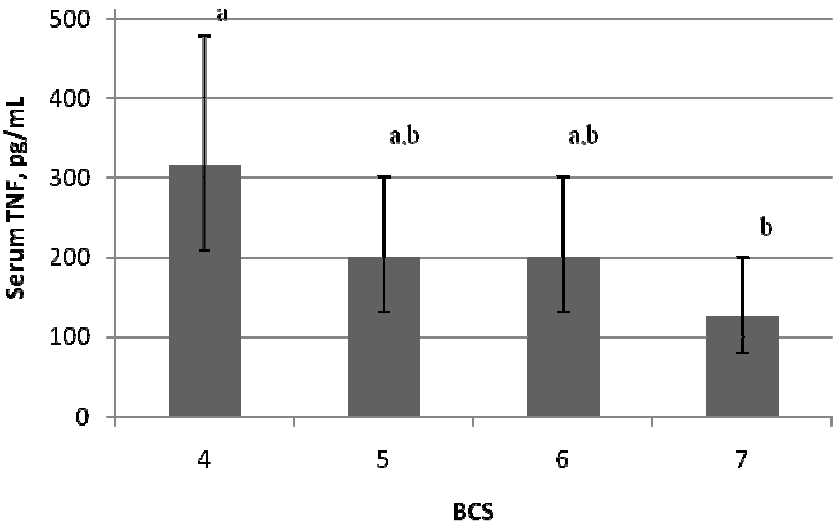

Figure 2. Effects of body condition score (BCS) on serum tumor necrosis factor-α (TNF) concentrations of horses fed for weight gain on a mixed grass/alfalfa hay and either a high in starch and sugar or a high in fat and fiber diet. Geometric means are averaged across diets and represented as mean ± SE. [a, b] Means with unlike superscripts differ ($P < 0.05$).

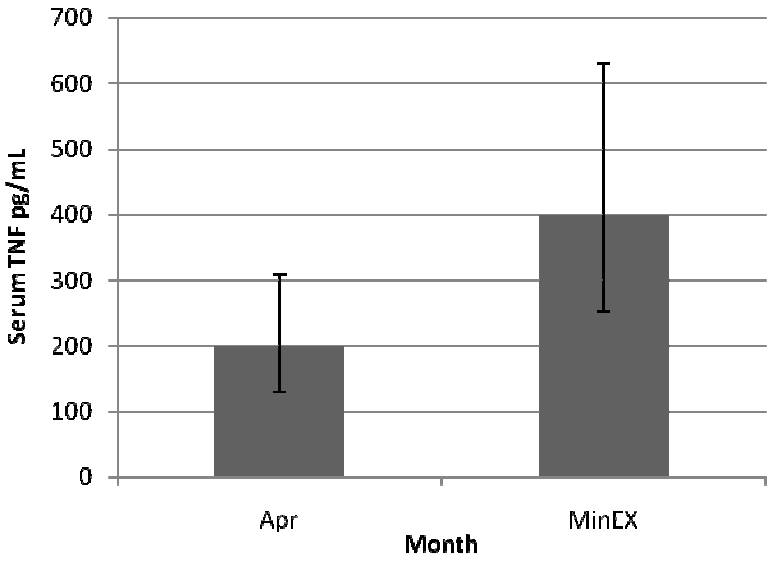

Figure 3. The effects of exercise restriction on serum tumor necrosis factor-α (TNF) concentrations of horses fed for weight gain on a mixed grass/alfalfa hay and either a high in starch and sugar or a high in fat and fiber diet. Geometric means are averaged across diets and represented as mean ± SE. There was a trend for increased TNF after exercise restriction ($P = 0.075$).

Chapter 5: General Summary

The main objectives of these studies were to assess the effects of weight gain and diet on body condition scoring in Thoroughbred (TB) geldings as well as to assess the effects of weight gain, diet and exercise on inflammation in the horse as indicated by serum concentrations of the pro-inflammatory cytokine, tumor necrosis factor-α (TNF). For these studies, fifteen TB geldings were fed for weight gain on hay plus either a high in starch and sugar (HS) concentrate or a high in fat and fiber (HF) concentrate over an eight month period. Monthly body area (neck, withers, shoulders, ribs, loin, and tailhead) body condition scores (BCS) were recorded by two judges, and blood was analyzed for serum TNF concentrations. The BCS method used was that of Henneke et al. (1983).

We found no dietary effect on body area body condition score, but there were significant differences between body area scores within months. Neck scored consistently higher than both withers and loin, while shoulders, ribs, and tailhead agreed during most monthly assessments. Additionally, the agreement between the two judge's scores was not consistent across either time periods or body areas, indicating the subjective nature of body condition scoring. The withers and loin were two areas that may be most influenced by conformation and should therefore be removed from BCS scoring. Several equations able to accurately predict the mean while utilizing a smaller subset of body areas were identified, however

recommendation of one above all others will require assessment on a different population of horses.

In the second study, the eight month weight gain period was followed by two weeks of no exercise. There was no dietary effect on serum TNF concentrations, while horses in low body condition had higher TNF concentrations than horses in high body condition. Restricting exercise tended to increase TNF concentrations. These findings imply that horses in low body condition as well as those with minimal exercise are at an increased risk for inflammation.

Chapter 6: Vita

Jessica K. Suagee was born on March 8, 1983 in Sylva, North Carolina to Mr. and Mrs. Dean B. Suagee. She graduated from the Science and Technology Program at Eleanor Roosevelt High School in Greenbelt, Maryland in 2001. She went on to pursue a Bachelor of Science degree in Nutritional Science from the University of Maryland, and graduated in 2005 with a citation from the University Honors Program. Jessica was employed as a research assistant at the Food and Drug Administration in Laurel, Maryland following graduation.

In August of 2005, Jessica returned to the University of Maryland to pursue a Master of Science degree in Animal Science with an emphasis on equine nutrition. This was done under the direction of Dr. Amy Burk. Jessica was recently admitted to the graduate program in Animal and Poultry Science at Virginia Polytechnic Institute and State University to pursue a PhD under the direction of Dr. Ray Geor. She was also awarded a John Lee Pratt Fellowship in Animal Nutrition.

Jessica K. Suagee

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