THESIS

DIETARY INTAKE IN A GROUP OF OLD MARES FED A SUPPLEMENT

CONTAINING LONG CHAIN 18:3 (N-3) FATTY ACID AND CHROMIUM

Submitted by

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ABSTRACT

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Introduction: Differences in dietary maintenance requirements for old horses compared to adult horses is unknown (NRC, 2007). Older horses are prone to developing decreased insulin sensitivity due to an increase in inflammation, disease, fat accumulation, and a decrease in physical activity (Adams et al., 2009). Studies show a relationship between obesity, inflammation, and insulin resistance (IR) in horses (Vick et al., a,b). An increased inflammatory status in older horses may cause of pituitary pars intermedia dysfunction (PPID); which, predisposes horses to laminitis and insulin resistance (McFarlane & Holbrook, 2008). Polyunsaturated fatty acids (PUFA), such as n-3 α-linolenic acid (ALA), are absorbed and incorporated into cell membranes. In rat and human studies, PUFAs change fatty acid composition of phospholipids surrounding insulin receptors found in muscle (Luo et al., 1996; Rasic-Milutinovic et al., 2007) and reduce inflammation when incorporated into white blood cells (Calder, 2008). Chromium has been found to be beneficial in diabetic experimental animals and also in conditions resulting from insulin sensitivity and defects in glucose transportation (Liu et al., 2010). The objective of this study was: to investigate the dietary intake in a group of old mares while testing the effects of a fatty acid supplement containing α-linolenic acid and chromium yeast on insulin sensitivity and inflammatory markers.

Materials and Methods: Dietary intake for thirty-two old (mean ± SE, 21.9 ± 0.65; 16-26 yr), nonpregnant, idle mares was tracked between February to July of 2010 and

was compared to the 2007 National Research Council predicted values for idle maintenance horses. Fourteen of the 32 mares were supplemented between July 2009 and July 2010 with 190 g/d of Equine Platinum Plus Metabolic Support (EPPMS): a fatty acid formula from Platinum Performance™ containing ALA with an addition of Chromium Yeast and other minerals and vitamins. Baseline blood samples were taken on those 14 mares, weight was taken using a calibrated electronic livestock scale (Cardinal Scale Manufacturing Company; Webb City, MO). Mares were classified into non-metabolic or insulin resistant using basal proxies (Treiber et al., 2005). Monthly assessment of dietary intake, BW, BCS and neck condition score (NCS) began in February. Twenty-two mares participated on an exercise schedule 3 to 5 d/wk; which included: walking on an automated walker panel system (Priefert® 6-Horse Panel Walker; Priefert®, Mount Pleasant, TX), between 2-3 mph, for 30-40 min. Dietray intake predicted requirements were adjusted for those walked versus idle, voluntary activity by other mares. Dental evaluation and care was given prior to the start of the study. Mares were maintained in stalls with access to ad libitum water and a salt block. Dietary intake was assessed weekly and changes were averaged. A mix diet of alfalfa and grass hay in addition to commercial grain concentrates were weighed, fed twice a day, and any refusals were recorded. Caloric intake per kilogram of body weight was calculated by dividing the total calorie intake by the body weight (kg). Forage was nutritionally analyzed at a commercial lab and grain nutrient content was provided by feed companies. Supplemented mares were re-sampled May 2010 and proxies for insulin sensitivity were analyzed and compared. Non-supplemented old mares which arrived to the Equine Reproductive Lab in April were utilized for a further evaluation of the supplement effect when compared to those

mares which were supplemented in July 2009. Non-supplemented mares had baseline blood samples taken in May 2010 and all mares were re-sampled in July 2010. Mares were blocked by supplemented versus baseline, supplemented versus non-supplemented, and overall group dietary intake. The six months of predicted vs. actual nutrient intake values were analyzed by ANOVA and significant differences were compared by the least square means analysis.

Results & Discussion: Overall, actual caloric intake was 13.8% higher than predicted caloric intake ($P < 0.001$); more specifically, in the month of June, actual was higher than predicted by 21.6%. In addition, actual intake of CP, calcium (Ca), phosphorus (P), magnesium (Mg), zinc (Zn), and copper was higher in June than predicted intake of those same nutrients ($P < 0.001$) in all other months. An increase in WSC ($P < 0.028$) occurred with the increase in DMI from May (8.4%) to June (9.5%). Dietary caloric intake increased by an average of 20% from May to July and BCS increased ($P < 0.05$) from 5.8 to 6.4 (out of a 1-9 possible range); yet NCS ($P = 0.71$) and weight ($P = 0.99$) did not vary even though most of the time the mares were fed on average, 13.8% above DE requirements. An increase in BCS by 0.6 may be due to the even greater increase (21.6%) in calories during the month of June. Additionally, those supplemented with EPPMS increased in insulin sensitivity and pancreatic-beta cell function from July 2009 to May 2010 ($P < 0.001$); yet weight trended to increase ($P = 0.07$). Caloric intake increased between sampling from 1.35% BW to 1.47% BW. Inflammatory cytokines COX-2 ($P < 0.05$) and IL-1 ($P < 0.05$) increased. TNF-alpha trended higher ($P = 0.07$) after supplementation and IL-10 was lower for those walked ($P < 0.05$). No effect was seen for cortisol or exercise compared with any other parameters.

Vitamins, minerals, and amino acids were present in the supplement, but not measured and resultsare inconclusive for insulin sensitivity. In comparison with the short-run study of those supplemented versus non-supplemented, no change in insulin sensitivity was found for those supplemented. Dietary intake increased from May to July by 6 kcal/kg of BW. Weight increased ($P < 0.05$) amongst both groups of walked or idle and weight trended to increase ($P < 0.06$) between supplemented versus non-supplemnted. An increase in BCS ($P < 0.003$) was found between May (5.6 ± 0.22) to July (6.25 ± 0.22).

Conclusion: Although previous research has indicated that nutrient intake is similar between old and young horses, in the current study mares did not increase in body weight or condition when fed 10% above energy requirements. A long-term controlled study measuring dietary nutrient inputs and outputs as well as left-over feed coupled with more frequent body weighing for tracking body weight fluctuations is needed to confirm current results. Additionally, those fed EPPMS from July 2009 to May 2010 increased in insulin sensitivity and pancreatic-beta cell function; however, when BCS and weight increased due to dietary calorie increase occurred in the short-run study compared to those non-supplemented, no effect was found on insulin sensitivity. In terms of improvement, a more controlled study including IR old horses with simiar BCS given a consistent diet would be needed to evaluate the effects of a fatty acid supplement.

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DEDICATION

This thesis is dedicated to my loving husband, Landon; family, friends, and beloved pets.

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CHAPTER I: INTRODUCTION

The percentage of horses ages 20 years or older has increased from 5.6 % in 1998 to 7.6 % in 2005 (USDA, 2006b). Further advancement in the care and consideration for old horse nutrition is important for improving well being and health for senior companions. Furthermore, old horses are less likely to be euthanized if they can survive at a comfortable and pain-free level (Traub-Dargatz and Long, 2006). In a survey of pet owners, 61.1% of horse-owning households had at least one veterinary visit in 2006, an increase of 11.9% from 2001 (Anon, 2007), and the number of horse owners maintaining older horses has increased (Schott, 2002).

A decrease in immune function may increase infectious morbidity in aged populations. This age-related immune decrease, termed immunosenescence, has been evaluated in horses, humans, dogs, and rodents (Thoman and Weigle, 1989; Greeley, 1996; Miller, 1996; Horohov et al., 1999, 2002).

Advanced age is also associated with a low-grade, chronic inflammatory state termed "inflamm-aging" (Franceschi et al., 2000). Aging in itself is not a disease, but age-associated inflammation can result in physiological changes that may predispose older horses to the most common endocrinopathy found in old equids: equine Cushing's disease (van der Kolk et al., 1993; McFarlane et al., 1998; McCue, 2002; Paradis, 2002; Schott, 2002; Brosnahan and Paradis, 2003a; van der Kolk et al., 2004; Holland, 2006). Equine Cushing's disease predisposes horses to chronic laminitis, hirsutism, muscle atrophy, polydipsia, polyuria, infertility, chronic infections, and insulin resistance

(McFarlane & Holdbrook, 2008; Messer, 2010). Failing dentition such as diastema, periodontal disease, wave mouth, shear mouth, hooks, ramps, and incisor malalignments are common in old horses, and can contribute towards esophageal choke, halitosis, feed-packed cheeks, impaction colic and diarrhea, and weight and body condition losses (Knottenbelt, 2003; Ralston, 2005; Baker and Chandler, 2006). Reduced fertility has been documented with advanced age in the mare, with decreased pregnancy and increased pregnancy loss rates (Ginther, 1992; Vick, 2006).

In old horses, there is a decrease in insulin sensitivity (Vick et al., 2008). Insulin resistance can be associated with obesity (Masuzaki & Paterson 2001; Johnson, 2002; Lyon et al., 2003; Vick et al., 2007), laminitis (Johnson, 2002; Treiber et al., 2006c; Asplin et al., 2007; Geor et al., 2008), equine metabolic syndrome (Bailey et al., 2008; Carter et al., 2009b; Frank et al., 2010b) , type 2 diabetes (Anon, 1999, 2001) and aberrant reproductive cycles (Gentry et al., 2002; Sessions et al., 2003). Data suggests that an interrelationship exists among obesity, inflammatory cytokines, and decreased insulin sensitivity in the old horse (Vick et al., 2007).

Knowledge of age-related changes in digestion, absorption, and metabolism is necessary to define the nutrient requirements in old horses. Although many aged horses are able to maintain good to excellent body condition and health on normal maintenance rations (Ralston and Breuer, 1996a; Brosnahan and Paradis, 2003a; Elzinga et al., 2011), weight loss in geriatric horses is not uncommon due to failing dentition, decreased digestion or absorption of nutrients, and equine Cushing's disease, so special care considerations may be warranted (Ralston, 2006). Whether an age-related increase or decrease in maintenance energy requirement occurs in horses is unknown (NRC, 2007),

therefore, guidance from the National Research Council (2007) accounts for aged horses by including their requirements with average maintenance guidelines for adult horses. Since older horses can still be used as athletes, the energy requirements for maintenance horse guidelines may not apply to the geriatric athletes or those with physical activity (NRC, 2007). The crude protein, macro- and micro- minerals requirements have not yet been established for the older horse (NRC, 2007); although, horses with reduced renal or hepatic function may be affected adversely to excess dietary protein, calcium, and fat (Ralston, 2006). Surveying body condition and weight in relation to nutritional management in old horses would be advantageous to equine managers and professionals to understand if the horse is meeting the nutritional requirements, and potentially furthering the health and wellness of their older horses.

One nutritional intervention to improve inflammatory status in horses while increasing insulin sensitivity and possibly preventing insulin resistance with the occurrence of laminitis, may be accomplished with supplementation of long chain highly unsaturated omega-3 fatty acids (LCHUFA), or polyunsaturated fatty acids (PUFA), such as n-3 α-linolenic acid (ALA) (Figure 2.1). In addition, the supplementation of chromium shows benefits for diabetic experimental animals and in conditions resulting from insulin resistance and defects in glucose transportation (Liu et al., 2010) by increasing cellular uptake of glucose and stimulated insulin metabolism (Mertz, 1993; Lukaski, 1999; Cefalu et al., 2002). However, the mechanism for chromium in the cellular-level is unknown. Chromium supplementation for increasing insulin sensitivity in old horses is unknown and further investigation is needed.

CHAPTER II: REVIEW OF LITERATURE

Old Age

Defining Age:

No exact chronologic threshold has been established for defining old age in horses, but several investigators have used 20 years of age (Ralston et al., 1989; Malinowski et al., 1997; Brosnahan and Paradis, 2003b). Some researchers have tried to define the age of the old horse using an equivalent to the average old age in human beings of 71 years of age, which is translated to 25 years of age for the horse (Mauderly and Hahn, 1982). When using these figures, a 20-year-old horse is equivalent to a 57-year old person and a 30-year old horse is equivalent to an 85-year old person. However, the functional age of the horse must be taken into consideration and the functional age always takes into account the horse's use (Bertone, 2006). For example, old broodmares may be defined in terms of reproductive capability, which may be any mare over 16 years old (Bertone, 2006). However, 16 years old may be the prime age of a hunter, polo, or dressage horse's career.

Another item of consideration is the demographic age and this is widely used by national surveys. Demographic age relates to the survivorship relative to a population; for instance, horses are considered 'mature' when 100% to 75% of a population is still alive; 'old' when there is only 75% to 25% survivorship; and 'very old' when the survivorship is less than 25% (Timras, 1988, 2007). As defined, the age at which one becomes demographically 'old' is when there is only 25 percent survivorship at or above that

specific age (Grundy, 2003). In real terms, the demographic age has almost no relevance to the life of a horse, unless one looks at the demographic age of broodmares, for example, and defines age by functionality. Thus, an old broodmare may be defined as a mare that surpasses the point at which only 25 percent of mares are expected to be fertile.

Even though 20 years of age has been the estimate of the threshold of old age, the degree of variation of this estimation is unknown; however, by surveying owners of when they noticed aging changes in their horses, the results were for horses approximately 22 years of age (Brosnahan and Paradis; 2003b). Thus, the combination of chronological age and physical signs of aging may be the most effective means of establishing the "old age" threshold for individual horses (NRC, 2007). Physical signs of aging may include a chronically low body condition score, loss of muscle mass over the top line, hollowing out of the grooves above the eyes, graying of the coat, and dental disease (Ralston et al., 1989; Ralston and Breuer, 1996; Paradis, 2002).

Demographical Data for Aged Equine:

The population percentage of equine 20 years of age or older increased from 5.6 percent in 1998 to 7.6 percent in 2005 (USDA, 2006b). Also, the percentage of geriatric horses (> 20 years) admitted to Tufts University School of Veterinary Medicine Large Animal Hospital has increased from 2.2 to 12.5% from 1989 to 1999 (Brosnahan and Paradis, 2003a). In a survey of pet owners, 61.1% of horse-owning households had at least one veterinary visit in 2006, an increase of 11.9% from 2001 (Anon, 2007), and the number of horse owners maintaining older horses has increased (Schott, 2002). A 2009 postal survey completed by horse owners in England and North Wailes, reported that 29% of horses and ponies were aged $\geq$ 15 years, with a decline in numbers within the

population after the age of 15 years (Ireland et al., 2011a). Compared to data from the National Animal Health Monitoring System, surveying properties with greater than five horses in the USA (Anon, 2005), the percentage of older animals was lower in the USA, as 7.6% of the population was aged ≥ 20 years and only 0.7% ≥ 30 years of age, while in the United Kingdom (Ireland et al., 2011a), 11% were aged 20 to 30 years of age and 2% were > 30 years of age. Pony breeds became over-represented in the very old age category (> 30 years), accounting for 70.8% of equine (Ireland et al., 2011a). In addition, more than one-quarter of horses were still in competition (25.9%) and the age of these horses (median = 18.0 years of age) was significantly lower than horses not competing (median = 21.0 years of age) ($P < 0.001$). The most common disciplines were showing, dressage, and show jumping, predominantly at local show/riding club level competitions, although 7.1% were reported to compete at a national or international level (Ireland et al., 2011a). These data suggest there could be over 300,000 geriatric horses and ponies in the UK.

The National Animal Health Monitoring Systems (NAHMS) Equine 1998 and 2005 Studies showed an increase in equids aged 20 years and older (USDA, 2006b). According to the 1998 NAHMS, equids of 20 years of age and older were represented at 5.6% and rose to 7.6% (SE = 0.4) in the 2005 (USDA, 2006b). The leading cause of death (including euthanasia) amongst equids was grouped into a category including those 6 months of age and older in 1998 and 2005. "Old age" was the leading cause of death at 24.8% (SE = 5.8%) in 1998 and 30.4% in 2005 (SE = 2.4%). Second to old age was colic at 22% (SE= 5.5%) in 1998 and injury, wounds, and trauma at 16% (SE = 1.7%) in 2005. Ranked third in 1998 at 18% (SE = 3.6%) was 'other known causes', including: cancer,

heart disease, poisoning, lightning strike, liver disease, and birth defects. Yet in 2005, colic was ranked third at 15.2% (S = 1.8%), although much lower than reported cases in 1998, where incidence of colic was reported at 22% (5.5%). The death rate for equids 20 years to less than 30 years was 6.7% (SE = 0.7%), while the 30 years and older population ranked highest at 45.7% (SE = 6.7%).

In several demographic studies completed in the United States, pony breeds make up a large demographic of the very old population (Williams, 2000; Brosnahan and Paradis, 2003a,b), although reasons for this are unknown. Ponies made almost one half of the very old population (≥ 30 years) in a clinical study (Brosnahan and Paradis, 2003b) and 33% of the equids in a study in which data obtained at necropsy from 817 equids were analyzed (Williams, 2000). Older horses are less likely to be euthanized if able to survive at a comfortable and pain-free level (Traub-Dargatz et al., 2006). Certainly, the care of older horses has been the focus of many recent articles for horse owners, managers, and veterinarians; highlighting the points of unique health and nutritional needs as resulted in an ever-growing market for products that address these needs (Bertone, 2006). Nutritional products have been specifically designed for senior horses, advancement in equine dental equipment has improved the dental health in older horses, and diagnostic testing and drugs to treat PPID have been developed, which helps regulate the horse's endocrine function and prevent laminitis.

According to the Guinness Book of World Records, the oldest horse on record was "Old Billy," an English draft horse who lived for 62 years (Scott, 1996). In 1994, a nation-wide search conducted by Purina Mills entitled "Americas Oldest Horses," evaluated over 2,000 equine owner's submitted information about their senior horses.

The winner was Theodore Edward (Teddy) Bear, a Shetland pony that was 52 years old in 1994. According to the Jockey Club's records, published in the Equine Geriatric Medicine and Surgery book (Bertone, 2006), the oldest thoroughbred mare to have a foal was "Betsy Ross," who was 30 years old in 1937 when she had her last foal; and the oldest Thoroughbred stallion to sire a foal was McGee, who was 31 years old when he sired his last foal crop in 1931.

Diseases in Old Horses

Common diseases that affect older horses include osteoarthritis (Brommer et al., 2003), degenerative arthritis (Bertone, 2006), laminitis (Alfort, 2001), recurrent airway obstruction (RAO) (Hotchkiss et al., 2007), left-sided valvular regurgitation, ophthalmic lesions, and neoplasia (Williams, 2000) age-associated inflammation can result in physiological changes that may predispose older horses to the most common endocrinopathy: equine Cushing's disease (van der Kolk et al., 1993; McFarlane et al., 1998; McCue, 2002; Paradis, 2002; Schott, 2002; Brosnahan and Paradis, 2003a; van der Kolk et al., 2004; Bertone, 2006); which predisposes horses to chronic laminitis, hirsutism, muscle atrophy, polydipsia, polyuria, infertility, chronic infections, and insulin resistance (McFarlane & Holdbrook, 2008; Messer, 2010). Failing dentition is common in old horses, and can contribute towards esophageal choke, halitosis, feed-packed cheeks, impaction colic and diarrhea, weight and body condition losses (Knottenbelt, 2003; Bertone, 2006). Reduced fertility has been documented with advanced age in the mare, with decreased pregnancy and increased pregnancy loss rates (Ginther, 1992; Vick, 2006).

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Inflammation, Immune Function, & Obesity

Inflammatory Markers:

Local and systemic inflammation is an immune response to the presence of micro-organisms or to injury which allows repair to damaged or infected tissue and a return to homeostatic conditions through a balance of local innate immune mechanisms and brain-derived immuno-regulatory output via the autonomic nervous system (Higgins and Lees, 1984). The immune system responds to infections and injury by inducing acute inflammation (Calder, 2001, 2006). The inflammatory response is a natural and immediate response noted by the presence of redness, swelling, heat and pain due to increased blood flow and vessel wall permeability of the surrounding vasculature (Calder, 2006; Parham, 2009). The inflammatory process allows for immune cells and large molecules involved in the immune response (i.e. chemokines, cytokines) to enter the site of infection or injury from the blood stream (Calder, 2006). In general, the inflammatory response is known for the local accumulation of fluid, plasma proteins, and white blood cells that is initiated by physical injury, infection, or a local immune response (Parham,

2009). The cells that invade tissues undergoing inflammatory responses are often called inflammatory cells or an inflammatory filtrate. The local response to pathogens involves the production and release of inflammatory cytokines, which are released at the site of inflammation (Pedersen et al., 1998; Parham, 2009). Those cytokines that promote inflammation are known as inflammatory cytokines (Parham, 2009).

The immune system cells are principally the white blood cells or leukocytes, and the tissue cells related to them. Leukocytes derive from a common progenitor called the pluripotent hematopoietic stem cell, which also gives rise to red blood cells (erythrocytes) and megakaryocytes, the course of platelets. All these cells together with their precursor cells are collectively called hematopoietic cells. Hematopoietic stem cells can commit to one of three cell lineages: the erythroid, myeloid, and the lymphoid.

One group of myeloid cells consists of the granulocytes (neutrophils, eosinophils and basophils), which have prominent cytoplasmic granules containing reactive substances that kill microorganisms (pathogen) and enhance inflammation. The most abundant of the granulocytes, and of all white blood cells, is the neutrophil, which is specialized in the capture, engulfment, and killing of microorganisms. Cells with this function are called phagocytes, of which, neutrophils are the most abundant and most lethal. Neutrophils are effector cells from innate immunity that are rapidly mobilized to enter sites of infection and can work in the anaerobic conditions that often prevail in damaged tissue. They are short-lived and die at the site of infection, forming pus.

The second group of myeloid cells consists of monocytes, dendritic cells, and macrophages. Monocytes are leukocytes that circulate in the blood and are bigger than granulocytes. Dendritic cells are resident in the body's tissues and act as cellular

messengers that are sent to call up an adaptive immune response (white blood cells called lymphocytes increase the power and focus of the immune system and contribute to defense against pathogen). Also, dendritic cells that reside in the infected tissue will leave the tissue with a cargo of intact and degraded pathogens and take it to one of several lymphoid organs that specialize in making adaptive immune responses. Other immune cells apart from the memory (acquired) immune response known as lymphocytes (B and T cells) will arrive later at the site of inflammation if reinforcement from the adaptive immune system is needed (Calder, 2006).

When a pathogen invades tissue, the first effector cells of the immune system it encounters are the resident macrophages. Macrophages are the mature forms of circulating monocytes that have left the blood and taken up residence in the tissues. They are prevalent in the connective tissues, the linings of the gastrointestinal and respiratory tracts, the alveoli of the lungs, and in the liver, where they are known as Kupffer cells. Macrophages are long-lived phagocytic cells that participate in both innate and adaptive immunity. They are the general scavenger cells of the body, phagocytosing and disposing of dead cells and cell debris as well as invading microorganisms. Macrophages carry a variety of receptors for communication within and around the cell, some of which are sent to the interior of the cell when pathogens are detected (Parham, 2009). Macrophages respond to pathogens by using different receptors to stimulate phagocytosis and to make and secrete small biologically active proteins, called cytokines (Parham, 2009). Cytokines can be considered markers of inflammation (Cannon, 2000) and can recruit other immune-system cells into the infected tissue, where they work together with macrophages to limit the spread of infection. These cytokines are not initially present as

part of the macrophage's fixed defenses of innate immunity, but their synthesis is induced by the presence of pathogens (Parham, 2009).

The last type of myeloid cell is the mast cell, which is resident in all connective tissues and is a major contributor to inflammation, yet its blood-borne progenitor is not yet known (Parham, 2009). Mast cells are posted throughout the body's tissues, particularly in the connective tissues underlying the mucosa of the gastrointestinal and respiratory tracts and along the blood vessels, especially those in the dermis of the skin (Parham, 2009). The cytoplasm of the mast cell is filled with large granules containing histamine and other molecules that contribute to inflammation (Parham, 2009). Mast cells become activated to release their granules when antigen binds to the IgE molecules on the surface of the mast cell (Parham, 2009). Large granules containing histamine and other molecules that contribute to inflammation are secreted into the tissues by activated mast cells, basophils, and eosinophils increase the permeability of the local blood vessels, enabling other cells and molecules of the immune system to move out of the bloodstream and into the tissues. This causes a local accumulation of fluid, swelling, reddening, and pain (Parham, 2009).

The Release of Inflammatory Cytokines:

Macrophages express many receptors that work in concert with complement receptors to phagocytose bacteria and other pathogens, many of these microbial ligands for the receptors of innate immunity are carbohydrates and lipids. One example of bacterial pathogen is called bacterial lipopolysaccharide (LPS), which is a major component of Gram-negative bacteria. The complement receptors, CR3 and CR4 on macrophages, recognize several microbial products such as LPS. The binding of these

macrophage receptors to their microbial ligands initiates a process of engulfment called receptor-mediated endocytosis, in which the receptor-bound pathogen is surrounded by the macrophage membrane and internalized into a membrane-bound vesicle called an endosome or phagosome. Phagosomes then fuse to the cellular organelles called lysosomes to form phagolysosomes, vesicles that are loaded with degradative enzymes and toxic substances that destroy the pathogen.

In addition to phagocytic receptors, the macrophage carries another class of receptors whose job is not to promote phagocytosis but to send signals via cytokines into the interior of the cell when the pathogens are detected. The Toll-like receptors (TLRs) are a family of signaling receptors, each of which is specific for a different set of microbial products. Toll-like receptors are transmembrane proteins composed of an extracellular domain that recognizes the pathogen and a cytoplasmic signaling domain that conveys that information to the inside of the cell. Macrophages express TLR4, which has specificity for LPS, and sends signals to the macrophage's nucleus that change the pattern of gene expression. The genes for cytokines that induce innate immune responses and inflammation at the site of infection are switched on and are known as inflammatory cytokines. The stimulation of TLRs by microbial ligands at an early phase of infection is not only essential for the innate immune response but also provides the conditions necessary for the adaptive immune response, should it be needed.

Intracellular reactions that lead to the macrophage's secreting inflammatory cytokines occurs initially in the cytoplasm and leads to the activation of the transcription factor nuclear factor ƙB (NFƙB), where its released from the cytoplasm and into the nucleus. The second part of the pathway takes place in the nucleus, where NFƙB initiates

the transcription of genes encoding inflammatory cytokines. Extracellular recognition of LPS causes the Toll-interleukin receptor (TIR) of TLR4 inside the cell to bind to a similar TIR domain in the protein MyD88. MyD88 is an example of an adaptor, which is a protein that acts as a bridge to bring other signaling proteins together. MyD88 binds the protein kinase IRAK4, which then binds and phosphorylates the adaptor TRAF6, which leads via a kinase cascade to the activation of inhibitor of ƙB kinase (IƙB). This phosphorylates IƙB, causing its dissociation from the complex with NFƙB and its eventual destruction. Once released from its' inhibitor, NFƙB moves into the nucleus, where it directs the activation of genes for cytokines, adhesion molecules and other proteins that expand and intensify the macrophage's effector functions. Cytokines are synthesized from cytokine mRNA in the cytoplasm and secreted via the endoplasmic reticulum (ER). The infiltrating cells cause a state of inflammation to develop within the tissue.

Changes that contribute to inflammation occur within the local blood capillaries that lead to an increase in their diameter (a process called dilation), reduction in the rate of blood flow, and increased permeability of the blood vessel wall. The increased blood supply to the region causes the local redness and heat associated with inflammation. Also, the increased permeability of blood vessels allows the movement of fluid, plasma proteins, and white blood cells from the blood capillaries into the adjoining connective tissues, causing the swelling and pain.

Inflammatory Cytokines:

Cytokines are small hormone-like proteins with a molecular mass of about 25kDa and mediate inflammatory responses by autocrine, paracrine, and endocrine effects and

can serve as protein chemical messengers (Calder, 2001). Strenuous exercise, energy crisis, stress hormones and oxidative stress are examples of physiological stimuli that modulate cytokine production (Cannon, 2000). When the immune system lacks regulation and resolution of the inflammatory response, the system enters a chronic state of inflammation leading to damage of host tissue and disease (Calder, 2006).

Prominent cytokines produced by activated macrophages are interleukin-1 (IL-1), interleukin-6 (IL-6), CXCL8 (previously called interleukin-8), interleukin-12 (IL-12), and Tumor necrosis factor-alpha (TNF- α) (Parham, 2009). These inflammatory cytokines have powerful effects that can be localized to the infected tissue or can be manifested systemically throughout the body. High levels of TNFα, IL-1 and IL-6 influence systemic effects of inflammation by mediating fever and weight loss (Calder, 2001), by acting on temperature-controlling sites in the hypothalamus, and on muscle and fat cells, altering energy mobilization to generate heat (Parham, 2009). A further systemic effect of IL-1, IL-6, and TNF-α is to change the spectrum of soluble plasma proteins secreted by hepatocytes in the liver, which acts on hepatocytes to induce synthesis of acute-phase proteins. They are also released by adipose tissue because of residing macrophages that clean up dead adipocytes and muscle stores, where protein and energy mobilization generates an increased body temperature (Parham, 2009). Increased levels of IL-1, IL-6, and TNFα are particularly destructive and implicated in inflammatory diseases such as rheumatoid arthritis, neurodegenerative diseases of aging in humans and septic/systemic inflammatory response syndrome (Calder, 2003a,b; 2006; Feldmann et al., 1996; Fetterman and Zdanowicz, 2009; Stulnig and Waldhausl, 2004). They are also involved in stimulating multiple cartilage degradation and the development

of arthritis (Shinmei et al., 1989). IL-1 beta (IL-1β) up-regulates a variety of genes, including the expression of itself and IL-6 (Moldoveanu et al., 2001).

IL-6 is considered a multifunctional cytokine and is released by skeletal muscle and adipose tissue (Ostrowski et al., 1998; Febbraio and Pedersen, 2002; Helge et al., 2003). IL-6 is identified in various immune system processes throughout the body (Pedersen et al., 2003), including modulation of stress proteins (Febbraio et al., 2002), autoimmune diseases (Ishihara and Hirano, 2002), lipid metabolism (Mohamed-Ali et al., 1997), and the regulation of glucose homeostasis (Helge et al., 2003). IL-6 functions as both a pro-inflammatory and an anti-inflammatory cytokine (Moldoveanu et al., 2001). Prostaglandins and IL-6 up-regulate the production and secretion of IL-10 which in turn, inhibits TNF-α, IL-1β, and IFN- ɣ production; thus, IL-6 can be considered an inflammatory response cytokine since it does not induce an inflammatory response (Petersen and Pedersen, 2005).

In order to heal damaged tissue, IL-8 or CXCL8 chemoattractant cytokines or chemokines promote the influx of lymphocytes, neutrophils, monocytes and other cells into the tissue, which helps clear any antigens (Pedersen, et al., 1998). Specifically, the principal function of CXCL8 is to recruit neutrophils from the blood into infected areas. Circulating neutrophils express two chemokine receptors, CXCR1 and CXCR2, which will bind to CXCL8 emanating from an infected tissue (Parham, 2009).

The effect of the pro-inflammatory cytokines such as IL-1, IL-6, and chemokines, including IL-8 and macrophage inflammatory protein (MIP)-α from LPS-activated human monocytes is opposed by the production of anti-inflammatory cytokine, IL-10, which studies have shown inhibits the synthesis of pro-inflammatory cytokines (Moore et

al., 1993; Goldman et al., 1997; Calder, 2006). With the addition of IL-10 to LPS-stimulated human mononuclear cells and neutrophils, a suppression of cytokine synthesis is observed, mainly by the inhibition of transcription of genes (Wang et al., 1994a,b). In addition, the ability of IL-10 to decrease mRNA stability has also been shown for several cytokines, including IL-6, IL-8, and IL-10 itself (Wang et al., 1994a,b; Brown et al., 1996; Takeshita et al., 1996).

A class of lymphocytes called natural killer (NK) lymphocytes are activated by the cytokine interleukin-12 (IL-12) and are lymphocytes of innate immunity that specialize in defense against viral infections. The cytokines IL-1 and TNF-α facilitate entry of neutrophils, NK cells, and other effector cells into infected areas by inducing changes in the endothelial cell walls of the local blood vessels. The TNF-α released by macrophages as a result of TLR stimulation can have both beneficial and harmful consequences (Parham, 2009). For example, vascular endothelial cells make platelet-activating factor, which triggers blood-clotting and blockage of the local blood vessels in response to TNF-α. This restriction prevents plasma leakage from the blood and prevents pathogens from entering the blood and disseminating infection throughout the body. If an infection spreads throughout the body, otherwise known as systemic infection, bacterial endotoxins such as LPS provoke widespread production of TNF-α, which then acts in ways that can become catastrophic. Specifically, when an infection develops in the blood, the systemic release of TNF-α and the effect it has on the venules in all tissues can simultaneously induce a state of shock that can lead to organ failure and death (Parham, 2009).

TNF- α may also play a role in muscle damage (Kimura et al., 2001), muscle proteolysis (Nawabi et al., 1990), impaired skeletal muscle glucose uptake (Steensberg et al., 2002), multiple sclerosis (Malamud et al., 2003), endotoxemia, sarcopenia or loss of muscle mass associated with aging (Petersen and Pedersen, 2005), insulin resistance, obesity, and diabetes (Saghizadeh et al., 1996; Ferrier et al., 2004; Keller et al., 2004; Petersen and Pedersen, 2005). In human studies, TNF-α has been implicated in cardiomyopathy and heart failure (Bristow, 1998) and bone loss associated with postmenopausal osteoporosis with diminished estrogens, where an array of systems limit bone formation by osteoblasts and enhance bone resorption by osteoclasts (Hamerman, 2007).

The cytokine IFN-ɣ is identified as one of the major pro-inflammatory cytokines (Mosmann and Sad, 1996) and is produced primarily by NK cells in the early stage of infection, which activates macrophages to secrete cytokines that help activate T cells, thus initiating the adaptive immune response (Parham, 2009) . IFN-ɣ plays a role in the inflammatory response (Ijzermans and Marquet, 1989) by not only activating macrophages and T cytotoxic cells, but also NK cells, nitric oxide synthesis (Elenkov, 2004), B lymphocytes and antibody production, as well as antiviral activity (Ijzermans and Marquet, 1989). With the arrival of effector T cells, NK-cell functions are turned off by IL-10, an inhibitory cytokine made by cytotoxic T cells (Parham, 2009). In addition, IFN-ɣ has been noted for its relation to the ageing process (Caruso et al., 1996; Venjatraman and Fernandes, 1997; Drela et al., 2004) and in response to exercise (Baum et al., 1997; LaManca et al., 1999; Kimura et al, 2001).

In a horse study that tested the effects of laminar inflammatory gene expression in the carbohydrate overload model in laminitis, researchers found increased mRNA concentrations ($P < 0.05$) for IL-1β, IL-6, IL-12p35, COX-2, E-selectin and ICAM-1 in laminae from horses with Obel Grade 1 lameness when compared to the control horses (Leise et al., 2011). Yet, no differences between the groups were found for IL-2, IL-4, IL-10, TNF-α, IFN-γ or COX-1 (Leise et al., 2011).

Inflammation and Aging:

Inflammation is associated with, or predicative of, virtually all chronic diseases of older age, including heart failure, type 2 diabetes, cancer, frailty, cognitive decline and dementia, and osteoporosis (Tracy, 2003). Elderly people that were experiencing "stressful" events showed elevated levels of circulating cytokines, especially IL-6, and displayed a variety of symptoms, including fatigue, depression, frailty, anorexia, weight loss, failure to thrive, and muscle weakness (Cohen et al., 1997). Another study with "healthy" elderly people, ages 70-79 years, levels of IL-6 and TNF-α were in general associated with lower muscle mass and muscle strength (Visser et al., 2002). Age-related functional disability and declines are poorly understood but may involve both low insulin-like growth factor 1 (IGF-1) and high IL-6 levels (Cappola et al., 2003). Cytokine elevation may account for "tiredness" in daily activities and be an indicator for later disability, mortality, and increased use of social and health services (Avlund, 2004).

Stress is defined as any actual or perceived threat to well-being, which then induces hypothalamic-pituitary-adrenal (HPA) activity (Jacobson, 2005). Afferent inputs to the hypothalamus and transduced into an endocrine signal by specific combination of hypothalamic ACTH-releasing factors, such as hypercortisolism (Khosla, 2002). Thus,

stress in this way enhances adrenal glucocorticoid secretion (Syed and Weaver, 2005).

People can have chronic stress associated with a variety of factors, such as physical,

financial, and psychological, which can then lead to cellular disturbances considered

"pro-aging." Stress is able to promote harmful health events that share cytokine

production and release at the cellular level, activating the sympathetic and central

nervous systems, resulting in a variety of immune, physiologic, neuroendocrine, and

behavioral responses (Sternberg, 1997). In horses, an increase in cytokine expression is

known to be associated with an increase in stress and aging, which may also decrease

immune function (Horohov et al., 1999, 2002; Holbrook et al., 2010, 2012).

Cellular-based stress exists. Inflammation, stress, and diabetes impose metabolic

overload with endoplasmic reticulum "stress," leading to the activation of signaling

pathways; and there is mitochondrial "stress," with the production of reactive oxygen

species (ROS), organelle damage, and inflammation (Wellen and Hotamisigil, 2005).

Prominent diseases associated with inflammation include diabetes and metabolic

syndrome. Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 in humans and

rodents are secreted from adipocytes and macrophages (Hotamisligil et al., 1993;

Weisber et al., 2003; Kershaw and Flier, 2004; Tilg and Moschen, 2006); and thus,

studies have focused on the role of fat tissue (Pedersen et al., 2003).

As previously mentioned, inflammation increases with age, and is termed

"inflamm-aging." This process accounts for an increase in pro-inflammatory leukocytes,

acute phase proteins and cytokines and a decrease in anti-inflammatory hormones

(Gruver et al., 2007). In old horses there is an increased frequency of IFNɣ and TNF-α

positive lymphocytes and monocytes after stimulation with phorbol 12-myristate 13-

acetate (PMA) and ionomycin (Adams et al., 2008). In the brain of elderly people, markers of neuro-inflammation accumulate, including activated microglia and pro-inflammatory cytokines (Conde and Streit, 2006), suggesting that with age a deterioration of the regulatory process of inflammation occurs (McFarlane and Holbrook, 2008).

An increased inflammatory status in older horses is also thought to be a possible cause of PPID (McFarlane & Holdbrook, 2008; Messer, 2010). For instance, in PPID-affected horses, the periventricular neurons terminate in the pituitary gland, outside the blood-brain-barrier; therefore, the systemic inflammatory state of a horse may influence neurodegeneration of the dopaminergic periventricular neurons (McFarlane and Holbrook, 2008). An increase in the expression of pro-inflammatory cytokines IL-6 and IFN-ɣ was found in healthy aged horses (≥ 16 years of age), as well as an increase in cytokine IL-8 in both healthy and aged horses with equine Cushing's disease (McFarlane and Holbrook, 2008).

The most commonly diagnosed respiratory tract disease was heaves and was recorded for 6% (30/467) of old horses (≥ 20 years) included in the clinical study at Tufts University School of Veterinary Medicine's Large Animal Hospital (Brosnahan and Paradis, 2003). Both pneumonia and laryngeal disease each were diagnosed in 2% (10/467) of the horses. In most instances, pneumonia developed as a result of aspiration during an episode of esophageal obstruction (Brosnahan and Paradis, 2003).

Immune Function and Aging:

A number of model systems have been employed to investigate age-associated changes in immune function. The purpose for Adams and others (2008) was to characterize senescent T cells and to investigate the "inflamm-aging" phenomenon both

in vitro and in vivo using the old horse as a model. Old horses have an increased

frequency of CD8-IFNɣ+ T cells and TNF-α producing cells. Also, old horses have

elevated levels of IL-1beta, IL-15, IL-18 and TNF-α gene expression in peripheral blood

and significant levels of TNF-α protein in serum; all characteristics of 'inflamm-aging''

(Adams et al., 2008). In addition, there are hematologic differences between younger and

older horses. The total lymphocytes, such as B and T cells and CD4 and CD8 counts were

decreased in older horses (McFarlane et al., 1998) and hemoglobin was increased

(McFarlane et al., 1998; Ralston et al., 1988). A non-specific indicator of inflammation

was an increase in the CD4:CD8 ratio (McFarlane et al., 2001). Immunoglobulin levels of

IgG, IgG (T), IgM, or IgA do not seem different (Ralston et al., 1988). Yet there was a

decrease gross response to vaccination with equine influenza (Ralston et al., 1988).

Inflammation Linked to Obesity and Insulin Resistance:

Inflammation in the old horse may be a key link between obesity and laminitis in

horses which are insulin resistant. Insulin resistance develops following systemic

inflammation in horses and adipose tissue may contribute to this inflammatory response

(Vick et al., 2008). Effects of adiposity in old horses were evaluated and a positive

correlation between increasing weight and fat, and inflammatory cytokine production was

found (Adams et al., 2008). In general, fat horses have an increase in the percent TNF-α

positive lymphocytes and monocytes following stimulation with PMA and ionomycin

when compared to thin horses (Adams et al., 2008). Decreasing body fat over time

significantly reduced the percentage of IFNɣ and TNF-α (Adams et al., 2008). These

findings demonstrate that age-related obesity potentially plays a role in the dysregulation

of inflammatory cytokine production by the immune system with increased age or "inflamm-aging" in the horse (Adams et al 2008).

In human studies evidence shows that elevated inflammatory cytokines such as TNF-α, IL-1, and IL-6 play direct roles in development of obesity-associated insulin resistance. In a horse study that investigated relationships among inflammatory cytokines, obesity, and insulin sensitivity, age was recorded along with body condition score (BCS) and the percent body fat (% FAT). These factors acted as measures of obesity in 60 mares (Vick et al., 2007) and insulin sensitivity decreased as BCS and % FAT increased ($P < 0.001$) (Vick et al., 2007). Additionally, increased IL-1 ($P < 0.05$) and TNF-α ($P < 0.01$) were associated with decreased insulin sensitivity. However, increased TNF-α ($P < 0.001$) was associated with decreased insulin sensitivity only in mares 20 years of age and older. Increased BCS and % FAT were associated with increased expression of TNF-α ($P = 0.053$) and IL-1 ($P < 0.05$). Surprisingly, increased BCS and % FAT were associated with decreased IL-6 expression ($P = 0.05$) in mares less than 20 years of age. Finally, BCS and % FAT ($P < 0.001$) with TNF-α mRNA ($P = 0.07$) and protein ($P < 0.05$) are inversely associated with insulin sensitivity independently of one another. Combined, these results provide the first evidence associating obesity and age with increased inflammatory factors in the horse (Vick et al., 2007).

Increased oxidative burst activity of neutrophils in hyperinsulinemic horses may predispose horses with metabolic syndrome to laminitis (Holbrook et al., 2012). In contrast with other findings, Holbrook and others (2012) showed peripheral blood cells of obese hyperinsulinemic horses had decreased endogenous pro-inflammatory cytokine gene expression (IL-1 and IL-6) and similar cytokine response following immune

stimulation compared to that of control horses, which suggests that unlike in people, cytokine-mediated inflammation does not increase in direct response to obesity or insulin resistance in horses.

Overweight and obese horses may struggle with multiple endocrine diseases, because of decreasing insulin sensitivity due to an increase of fat accumulation and decreasing physical activity (Adams et al., 2009). Obesity is considered a chronic mild inflammatory state (Rajala and Scherer, 2003; Xu, et al., 2003; Dandona et al., 2004) and leads to elevated circulating levels of free fatty acids (FFAs) in the skeletal muscle and liver, which can then decrease insulin sensitivity. In older horses, TNF-α was negatively related to insulin sensitivity (Vick et al., 2007); thus, FFAs represent an important link between the development of obesity and the development of insulin resistance. The liver uses little FFAs for energy, and the pathway for ketone body formation is not well developed in horses, with the result that large amounts of triglycerides are produced. Excessive fat mobilization may overwhelm the capacity of the liver to process triglycerides into VLDL, leading to triglyceride accumulation and hepatic lipidosis.

Increasing triglyceride concentrations can interfere with numerous normal physiologic functions, particularly in regard to reducing insulin sensitivity. This interference can result in the exacerbation of hyperlipidemia by impairing the ability of the body to limit fat mobilization, leading to worsening of lipid accumulation and severe complications, including renal and hepatic lipidosis and even death. Insulin-resistant individuals are at risk for hyperlipidemia, with the most commonly affected animals being ponies, miniature horses, and donkeys (Hughes et al., 2004). The true incidence of hyperlipidemias in large-breed horses is not known, but these conditions seem to be

increasingly encountered in the clinical setting, perhaps in relation to the increasing

degree of obesity in the equine population.

White adipose tissue is composed of adipocytes, fibroblasts, endothelial cells and

macrophages (Weisberg et al., 2003; Berg and Scherer, 2005). When adipose tissues

reach their capacity for fat storage, they can become stressed and release cytokines,

causing a pro-inflammatory state. In obesity, there is a progressive dysregulation of

adipose function with increased recruitment of monocytes in response to chemokines,

such as monocyte chemoattractant protein-1 (MCP-1) and PAI-1. This increases the

number of macrophages within adipose tissues and greatly amplifies the production and

secretion of pro-inflammatory adipokines, many of which decrease insulin sensitivity,

induce oxidant stress, and impair microvascular function, which may lower the horse's

threshold for laminitis (Firshman and Valberg, 2007).

The tendency to develop intra-abdominal, omental-type obesity has been well

documented in Cushing's syndrome in both human and equine patients. However, for

horses (and humans) affected with obesity, the circulating levels of cortisol are often

unremarkable and specific tests for Cushing's syndrome (associated with pars intermedia

dysfunction) yield normal results (Johnson, 2002).

Adipocytes are capable of secreting endocrine signals (adipokines) that cause

insulin resistance (Lyon et al., 2003). Omental adipocytes contain an enzyme (11-Beta,

hydroxysteroid DH; 11-Beta, HSD) capable of converting inactive cortisone to active

cortisol; which can play a role in the pathogenesis of insulin resistance (Masuzaki &

Paterson, 2001). Cortisol opposes insulin's effect in regulating carbohydrate metabolism

in vivo which then means an impaired glucose uptake in peripheral tissues and excess hepatic glucose production.

Obesity-Related Hormones and Reproduction

Leptin:

Leptin is primarily an adipose tissue-derived protein product of the obesity gene and an important regulator of energy metabolism. Leptin acts in the hypothalamus to suppress appetite in normal individuals and thereby regulate the accumulation of adipose tissue in the body. Leptin resistance may contribute to the development of obesity and associated disorders (Chu et al., 2001). Also, elevated levels of plasma resistin (adipocyte hormone) were found in obese individuals (Firshman and Valberg, 2007). Data regarding environmental pollutants and the association between them and insulin insensitivity in horses is currently lacking. However, deficiencies in magnesium, chromium and vanadium, as well as low serum levels of high density lipoprotein, and hypophophatemia have all been associated with development of insulin insensitivity, and may play a role in the development of obesity in horses (Johnson, 2002).

An increase in insulin prompted an increase in leptin in both humans and rats (Cusin et al., 1995). A study found that horses of a similar high body condition fell into 2 distinct groups based on leptin levels: hyperleptinemic or normal. The hyperleptinemic horses had metabolic profiles similar to that of human Type II diabetes. These horses were characterized by high glucose and insulin and exaggerated insulin response to glucose, they had exaggerated glucose and insulin responses to dexamethasone treatment.

Leptin and Reproductive Function in Mares:

Leptin provides information to the brain on energy status and may serve as a signal to the reproductive axis indicating a nutritional status adequate for the onset of cyclicity (Barash et al., 1996). Leptin has an apparent association with reproductive activity in mature mares. Higher amounts of body fat are associated with high circulating concentrations of leptin during the summer and autumn months when mares were reproductively active (Fitzgerald and McManus, 2000). In pony mares, circulating leptin concentrations were dependent on age, gender and body condition score. Concentrations of leptin are higher in horses between 5 and 12 years compared to horses younger than 5 years and higher in stallions, but not different between geldings and mares. Obese pony mares tend to have greater serum concentrations of leptin than thin pony mares (Buff et al., 2002). Fitzgerald and McManus (2000) found that mature, fat mares were more likely to have estrous cycles during the winter than young, lean mares. Other results show that mares with a high body condition score fail to exhibit reproductive quiescence, but those that lose body condition exhibit anestrus (Gentry et al., 2002). Therefore, the occurrence of seasonal anestrus is determined, in part, by metabolic signals.

In a study relating obesity to reproduction, a high degree of body fat produced by overfeeding during gestation did not adversely affect postpartum interval to ovulation, expression or intensity of estrus, and fertility (Kubiack et al., 1989). The insulin-leptin interactions in grazing mares and their impact on aspects of reproductive function were evaluated (Cubitt, 2007). In an initial study, 3 groups of 9 mares adapted to diets of either fat and fiber, sugar and starch, or pasture alone; while undergoing two frequent intravenous glucose tolerance tests: once during the luteal phase and another during the

follicular phase of the estrous cycle. Diet affected insulin sensitivity. Not surprisingly, progesterone was higher in the luteal phase when compared to the follicular phase; however, insulin sensitivity was lower in the luteal phase than the follicular phase of the estrous cycle (Cubitt, 2007).

In another study by Cubitt (2007), 15 mares that had adapted to fat and fiber, sugar and starch, or pasture forage were utilized. The mares had all accessible follicles ablated, and the follicular fluid was collected during the luteal and follicular phase of the estrous cycle. Insulin concentrations were 52% higher in large (> 25mm) follicles than either medium (16-25mm) or small (≤ 15mm) follicles irrespective of estrous cycle phase. A positive correlation was observed between follicular fluid (FFL) leptin and plasma leptin. A similar relationship was observed between FFL insulin and plasma insulin. Plasma insulin and leptin were positively associated along with FFL insulin and FFL leptin.

In both non-diabetic and diabetic women insulin sensitivity was lower during the luteal phase when compared to the follicular phase of the menstrual cycle (Pulido and Salazar, 1999). In bovine oocytes *in vitro,* diets high in sugar and starch increased basal insulin concentrations and reduced oocyte quality and embryo development when at elevated concentrations (Adamiak et al., 2006).

Adiponectin:

Adiponectin, has been shown to be inversely proportional to adiposity in humans (Arita et al., 1999; Weyer et al., 2001; Yang et al., 2001) and decreased in diabetes mellitus (Hotta et al., 2000) and insulin resistance (Hotta et al., 2001; Weyer et al., 2001). Plasma adiponectin and leptin concentrations were correlated to measures of adiposity in

horses as has been shown in humans (Arita et al., 1999; Considine et al., 1996; Takahashi et al., 1996; Weyer et al., 2001; Yang et al., 2001). Adiponectin gene expression is inhibited by increased insulin, TNF-α and dexamethasone concentration in adipocytes (Fasshauer et al., 2002). Adiponectin suppresses the TNF-α signaling in endothelial cells (Ouchi et al., 1999) and may therefore be able to protect adipocytes from the development of insulin resistance (Hotta et al., 2001). Since TNF-α increases with obesity (Hotamisligil et al., 1993), it may act to suppress adiponectin production. In addition, it was observed that plasma adiponectin concentrations were inversely correlated to plasma leptin concentrations and this has been previously described in humans (Matsubara et al., 2000). The significance of this finding is that leptin and adiponectin may work in concert during the development of obesity and insulin resistance. Adiposity may serve as a controller of energy homeostasis and therefore may affect nutrient utilization and fuel selection (Martinez, 2000). Conversely, adiponectin mRNA expression decreases with adiposity (Hotta et al., 2001).

Insulin

Definition, Function, and Biological Importance:

Within the pancreas, the islets of Langerhans are exocrine tissue, which make up small clumps of endocrine cells and synthesize hormones - insulin, glucagon, and somatostatin (Parham, 2009). The pancreas contains about half a million islets, each consisting of a few hundred cells. Each islet cell is programmed to make a single hormone: α-cells make glucagon, β-cells make insulin, and δ-cells make somatostatin (Parham, 2009). Insulin is a protein hormone that is essential to regulating the homeostasis and efficiency of cellular uptake of carbohydrates and influences lipid and

amino acid metabolism in the body. Insulin is secreted from the pancreas in response to the increased blood glucose levels arising after a meal. By binding to surface receptors, insulin stimulates the body's cells to take up glucose and incorporate it into carbohydrates and fats. After a meal, a glucose bolus injection, or glycogen metabolism, blood glucose concentrations rise, and insulin is released to signal cells to allow glucose to enter and be utilized or stored. The process starts by glucose entering the beta cells in the pancreas through the glucose transporter GLUT-2. Glucose enters glycolysis and the TCA cycle which produces adenosine triphosphate (ATP). Adenosine triphosphate controlled potassium channels close and the cell membrane depolarizes, which opens voltage-gated calcium channels, allowing calcium to enter the cell. This increased level of calcium activates phospholipase C, which cleaves the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP3) and diacylglycerol. Inositol 1,4,5-triphosphate (IP3) binds to receptor proteins in the endoplasmic reticulum (ER), which allows the release of calcium from the ER through IP3 gated channels, and in turn increases the calcium concentration of the cell even further. Substantially increased amounts of calcium in the cells causes the release of previously synthesized insulin, which has been stored in secretory vesicles. Insulin is released through circulation to its target organs (skeletal muscle and adipose tissue) where it then binds to its receptor on the cell surface and triggers a cascade of events. Glucose transporter, GLUT-4, translocates from intracellular lipid environment to plasma membrane, which allows glucose to enter the cell and either be stored as glycogen or lipid for energy use at a later time (Kanzaki, 2006).

Insulin Sensitivity and Insulin Resistance:

In old horses, there is a decrease in insulin sensitivity (Vick et al., 2008), and comparatively, ponies have lower insulin sensitivity than horses (Jeffcott et al., 1986; Rijnen and van der Kolk, 2004; Pratt et al., 2009; Borer et al., 2011). Insulin resistance (IR) is a condition in which an increased production of insulin is required in order to maintain circulating blood glucose levels within normal limits. Several factors can lead to a decreased sensitivity to insulin, such as diet, age, breed/genetics and obesity (Jeffcott et al., 1986; Treiber et al., 2006c; Vick et al., 2007). Changes in insulin sensitivity are associated with certain disease, including some forms of exertional rhabdomyolysis, osteochondrosis, hyperadrenocorticism, and related syndromes, such as hyperlipidemia and laminitis (Garcia and Beech, 1986; Jeffcott et al., 1986; Ralston, 1996; De La Corte et al., 1999; Pagan et al., 2001; Annandale, 2004; de Laat et al., 2012). Chronic IR may also result from genetic determinants, high starch and sugar diets, lack of exercise, obesity, equine Cushing's disease, and pregnancy (Ralston, 2002; Hoffman et al., 2003; Treiber et al., 2005, 2006a,b; Pratt et al., 2006). Studies of human patients suffering from IR have generally found the most favorable outcomes following control of predisposing factors such as diet, obesity, and lack of aerobic fitness, via lifestyle changes (Knowler et al., 2002).

A decreased sensitivity to insulin and documented insulin resistance (IR) is the most important predisposing factor for pasture-associated laminitis in ponies (Stulnig and Waldhausl, 2004; Treiber et al., 2006c), as diagnosis of insulin resistance may help identify laminitis-prone animals and allow the application of preventive countermeasures.

However, not all laminitis-prone animals are insulin resistant and not all insulin-resistant animals are prone to laminitis.

Measures to Assess Insulin Sensitivity:

Insulin sensitivity or IR can be assessed by dynamic evaluation of glucose and insulin responses or by simple analysis of steady-state/resting blood glucose and insulin concentrations. There are advantages and disadvantages of both approaches and diagnosis of insulin resistance in equines is difficult, with no general agreement over the best method to use. Currently, the gold-standard method of determining IR is by a specific and quantitative method for assessment of both insulin sensitivity and β-cell response, the Minimal Model (MINMOD) analysis of a frequently sampled intravenous glucose tolerance test (FSIGT) (Monzillo and Hamdy, 2003; Kronfeld et al., 2005) and the euglycemic hyperinsulinemic clamp (HEC), which provide direct and specific measurement of insulin-mediated glucose disposal (Firshman et al., 2007; Muniyappa et al., 2008). Minimal Model analysis has been used primarily to elucidate etiologies of diabetes in humans and other species (Bergman, 1989), and has effectively estimated insulin sensitivity in horses (Hoffman et al., 2003), while being a simpler means of investigating suspected T2DM in horses (Wallace and Mattews, 2002; Kronfeld et al., 2005; Treiber et al., 2006a). The procedure involves administration of a glucose bolus through a catheter defining minute 0 of the test. Samples are then taken at minutes 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 19 and at minute 20 insulin are administered. Blood is sampled at minutes 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 post glucose injection. Glucose and insulin curves are applied to the minimal model using computer software. While administering the HEC technique, a steady concentration of

insulin are given intravenously during which the rate of glucose infusion required to maintain euglycaemia during the clamp which serves as a measure of insulin sensitivity of muscle and adipose tissues (Firshman and Valberg, 2007). These techniques are laborious and impractical in clinical settings; however, they are probably more sensitive for detecting IR when compared with static resting measurements.

The simplest approach in clinical practice is to measure basal insulin and glucose concentrations for screening evaluation of IR. Basal plasma concentrations of insulin and glucose have been used as surrogates and proxies in human studies (Parra et al., 1994; Legro et al., 1998; Fukushima et al., 1999; Katz et al., 2000; Vuguin et al., 2001; Laaksonen et al., 2002; Uwaifo et al., 2002; Gungor et al., 2004). The combined use of proxies and reference quintiles facilitates the diagnosis and characterization of clinical cases (Treiber et al., 2005). In an equine study, basal plasma concentrations of glucose (mg/dL) and insulin (mU/L) were determined from the mean value of 2 or 3 baseline samples taken from each horse prior to conducting FSIGT for the minimal model (Treiber et al., 2005). Horses were kept in stalls with free access to grass hay and water but no concentrate. Blood samples were collected between 8:00am and 9:00am and glucose concentrations were determined by use of an enzymatic assay. Plasma insulin concentrations were determined by use of a radioimmunoassay previously validated for equine insulin (Freestone et al., 1991). Minimal model results for 46 horses were compared by equivalence testing with proxies for screening insulin sensitivity and pancreatic beta-cell responsiveness in humans with two new proxies for screening in horses, including the reciprocal of the square root of insulin (RISQI) and the modified insulin-to-glucose ratio (MIRG). Combined use of RISQI and MIRG, which represent

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insulin sensitivity and insulin response, will enable assessment of compensatory insulin secretion in apparently healthy horses and insulin signaling failure in hyperglycemic horses (Treiber et al., 2005). Together, RISQI and MIRG identify apparently healthy individuals that are compensating for low insulin sensitivity with increased beta-cell activity. The combination also allows for assessment of the ability of an individual to tolerate increases in plasma glucose that might be encountered following meals of concentrated feeds, when grazing rich pasture, or during veterinary treatment (Treiber et al., 2005).

Proxies are less accurate than the specific quantitative parameters they predict. However, proxies identify well-studied properties of IR and regulation with statistically determined power and are therefore superior to nonspecific indications, such as basal hyperinsulinemia, glucose intolerance, or analogies to diseases in other species (Kronfeld et al., 2005). Elevated basal plasma glucose concentrations is more likely to be a sign of current disease, such as hyperlipidemia, which is common in ponies (Jeffcott et al., 1986), or possibly type 2 diabetes mellitus, which is infrequently observed in horses (Tasker et al., 1966; Baker and Ritchie, 1974; Muylle et al., 1986; Durham et al., 2009). In humans, high insulin secretion is generally preempted by beta-cell failure, where insulin secretion reaches a limit; at this point, hyperglycemia and type 2 diabetes mellitus ensue (Bland, 1986).

When measuring resting insulin and glucose concentrations, standardization of sampling and analytic procedures is critical for reliable interpretation of results, because a large number of animal and environmental factors can affect these measurements. Basal insulin concentration is affected by individual, diurnal and seasonal variation (Firshman

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and Valberg, 2007; Pratt et al., 2009; Frank et al., 2010a; Borer et al., 2011). Cortisol and

epinephrine are released as a result of pain or stress can lower tissue insulin sensitivity

and raise resting glucose and insulin concentrations (Geor et al., 2000; Tiley et al., 2007).

In addition, horses suffering from laminitis are likely to have higher insulin

concentrations, so testing should be delayed until after the pain and stress of this

condition has subsided. Hyperinsulinemia (insulin value >20 μU/mL) in the absence of

confounding factors such as stress, pain, and a recent feeding provides evidence of

insulin resistance in horses and ponies (Frank et al., 2010b). Approximately 60% of the

insulin secreted by the pancreas is extracted from the portal blood by the liver in healthy

humans, so hyperinsulinemia can develop as a result of reduced insulin clearance and/or

increased pancreatic secretion (Frank et al., 2010b). Hyperglycemia occurs in a smaller

number of animals (Reeves et al., 2001; McGowan et al., 2004; Walsh et al., 2009).

For insulin resistant equine maintained at pasture, removal from pasture to a dry

lot or stall is recommended, especially during periods of active forage growth when the

high-sugar content of pasture forage can affect resting blood glucose and insulin

concentrations. However, equine have evolved to graze virtually continuously, and it is

unclear whether fasting would significantly alter measures of insulin sensitivity and

secretion, evaluated using either proxy estimates or more quantitative measurements

(Treiber et al., 2005). Previous studies evaluating RISQI and MIRG in equine have not

eliminated hay, but did fast animals from grain before sampling (Treiber et al., 2005;

Treiber et al., 2006c; Baily et al., 2008; Pratt et al., 2009). In rodents, a physiological

fasting state isn't present, and the imposition of a period of fasting before blood sampling

causes depletion of hepatic glycogen content and increases in insulin sensitivity (Muniyappa et al., 2008).

Endocrine Disorders: Equine Cushing's Disease, Equine Metabolic Syndrome, Diabetes Mellitus

Equine Metabolic Syndrome:

Equine metabolic syndrome (EMS) was adopted as the name for this disease because of its similarities with the metabolic syndrome in humans, which is a collection of risk factors assessed to predict the occurrence of coronary artery disease and type 2 diabetes mellitus in people (Fulop et al., 2006). Equine metabolic syndrome is diagnosed based on clinical signs, by ruling out other diseases, such as hypothyroidism and Pituitary Pars Intermedia Dysfunction (PPID), and by identifying insulin resistance via blood samples. Horses with EMS are typically between the ages of 5 and 15 years of age when veterinary or farrier services are first sought out because of laminitis (Frank et al., 2010b).

People affected by metabolic syndrome have an interconnection with atherosclerosis or a thickening of arterial walls and cardiovascular risk (Haffner et al., 2006). Furthermore, an inflammatory underpinning which predisposes people with metabolic syndrome is insulin resistance with impaired glucose tolerance and overt type 2 diabetes, dyslipidemia, hypercoagulability, and hypertension (Haffner et al., 2006). Pre-diabetic people have lower HDL; higher plasma triglycerides, fasting glucose, and insulin levels; and higher systolic blood pressure (Hamerman, 2007).

Equine Metabolic Syndrome Phenotypes:

The consensus statements of the American College of Veterinary Internal Medicine proposed that the EMS phenotype for the majority of affected equids should include an increase regional adiposity (Figure 2.2) or general obesity, insulin resistance characterized by hyperinsulinemia or abnormal glycemic and insulinemic responses to oral or intravenous glucose and/or insulin changes, and a predisposition toward laminitis. Other components of EMS phenotype include hypertriglyceridemia or dyslipidemia (Frank et al., 2006; Treiber et al., 2006c; Carter et al., 2009b), hyperleptinemia (Cartmill et al., 2002), arterial hypertension (Cartmill et al., 2002; Houskneicht et al., 2003; Frank et al., 2006; Treiber et al., 2006c; Bailey et al., 2008; Carter et al., 2009b), altered reproductive cycling in mares (Gentry et al., 2002), and an increase in systemic markers of inflammation in association with obesity (Vick et al., 2007).

Expanded subcutaneous adipose tissue surrounding the nuchal ligament in the neck (over the top of the neck) gives the neck a "cresty" appearance. Additionally, fat pads close to the tail head or accumulation of fat behind the shoulder or in the prepuce or mammary gland region. Geldings affected by EMS often develop a "swollen sheath" due to enhanced subcutaneous adiposity. Further, most horses also show ample amounts of adipose tissue in the omentum at necropsy (Johnson, 2002).

While obesity is observed in most cases of equids affected by EMS, some have a leaner overall body condition (Figure 2.3) and regional adiposity (Figure 2.2), and some others are normal in appearance. Still, obesity-related insulin resistance is a well-established phenomenon in horses (Hoffman et al., 2003) and is associated with several disorders, including altered reproductive function and laminitis (Treiber et al., 2006c;

Vick et al., 2006). Typically, equids labeled "easy-keepers" by their owners tend to be less insulin sensitive (may even be insulin resistant) and can maintain a "healthy appearance" (body condition score of at least five) with small portion sizes. "Easy keepers" are at risk of insulin resistance and EMS, as insulin resistance is another phenotypic attribute of EMS. Insulin resistance is characterized by hyperinsulinemia or abnormal glycemic and insulinemic responses to oral or intravenous glucose and/or insulin challenges. Additionally, horses affected with EMS are predisposed to laminitis, including clinical or subclinical laminitis that has developed in the absence of recognized causes such as grain overload, colic, colitis, or retained placenta (Frank et al., 2010b).

History:

Contributing factors for obesity should be assessed from the history, including the quantity of feed provided, size and quality of the pasture, and amount of exercise. Previous episodes of laminitis may be associated with changes in the abundance or composition of pasture grass, or alterations in grain feeding. Familial patterns have been recognized for EMS (Treiber et al., 2006c), so relevant information about the horse's lineage should be collected for future reference.

Laminitis:

Laminitis can be experimentally induced in healthy, non-obese ponies by infusing supraphysiological amounts of insulin intravenously over 2-3 days (Asplin et al., 2007; de Laat et al., 2012). This suggests that insulin itself can trigger events leading to laminar failure. Insulin resistance (IR) in horses has been linked to the development of laminitis, osteochondrosis, and equine metabolic syndrome (Coffman and Colles, 1983;

Ralston et al., 1996; Treiber et al., 2006c; Asplin et al., 2007; Carter et al., 2009b; Frank, 2010b).

Potential mechanisms relating obesity, hyperinsulinemia, and IR to laminitis are largely extrapolated from studies in other species and include endothelial cell dysfunction within blood vessels of the foot (Jansson, 2007), digital vasoconstriction (Sarafidis and Bakris, 2007), impaired glucose uptake by epidermal laminar cells (French and Pollitt, 2004), altered epidermal cell function or mitosis (Nourian et al., 2007) and matrix metalloproteinase activation by glucose deprivation or reactive oxygen species (French and Pollitt, 2004). Yet, glucose uptake mechanisms in the hoof are not well understood. In contrast to previous theories about glucose starvation as a possible mechanism of laminitis, Asplin and others (2011) showed that glucose uptake was not affected by insulin. In addition, hoof lamellae rely on a GLUT1-mediated glucose transport system, and it is unlikely that GLUT4 proteins play a substantial role in this tissue (Asplin et al., 2011).

Insulin may participate in the regulation of peripheral vascular responses and some studies have shown that the vascular endothelium is responsive to insulin, with insulin stimulating both vasodilatory and vasoconstrictive pathways (Geor, et al., 2008). This is thought to underlie hypertension, which is observed in humans with metabolic syndrome. Hypertension was also observed in insulin resistant, laminitis-prone ponies at summer pasture, which suggests that vascular endothelial dysfunction is a component of the metabolic syndrome phenotype in equids (Bailey et al., 2008).

Another theory may be the effect non-structural carbohydrates (NSC) have on inducing laminitis. As dietary intake of NSC increases, milder intestinal events may

induce inflammatory and vascular responses that combine with exacerbated insulin resistance and hyperinsulinemia to produce the onset of laminitis. Insulin resistance may decrease the amount of glucose getting into the hoof tissue cells, which could starve them and hamper their function, leading to laminitis. A study evaluating insulin resistance and laminitis in ponies concluded pre-laminitic metabolic syndrome in apparently healthy ponies is comparable to metabolic syndrome in humans (Treiber et al., 2006c). Welsh-crossed ponies (n = 160) were divided into groups based on age, sex, and reproductive maturity. They grazed on lush summer pasture between the months of May and September; some with a history of laminitis and others with none. The risk factors include a high body condition score, insulin resistance, and record of having laminitis. Further recommendations were made regarding special management for the ponies in the future; such as avoiding high starch intake, which can exacerbate insulin resistance and lead to chronic laminitis.

Increased fructosamine in laminitic horses may represent abnormal glycemic control and may become a clinically useful marker of abnormal glucose homeostasis in laminitic horses (Knowles et al., 2011). Laminitic horses have higher mean fructosamine than normal horses ($P < 0.001$) and 13/30 laminitic horses had fasting hyperinsulinemia. Additionally, Knowles and others (2011) showed statistically significant univariable correlations between fructosamine, glucose, insulin, and the proxies RISQI and MIRG (Treieber et al., 2005).

Diagnosis:

EMS can be diagnosed by obtaining a complete history, performing a physical examination, taking radiographs of the feet, and conducting laboratory tests. Blood

samples which test insulin sensitivity can aid in diagnosing insulin resistance (*see measuring insulin sensitivity*). Physical examination should include assessment of the horse for evidence of regional adiposity (Figure 2.2), including adipose tissue expansion within the neck crest (Figure 2.4), and body condition scoring (Figure 2.3).

Physical Measurements of Fat Indication and Body Condition:

Body weight should be measured with a scale or by using a weight tape. Body condition score (Figure 2.3; Henneke et al., 1983) is a physical measurement that can be used to assess body fat (Figure 2.2). The body condition score (BCS) ranges from 1 to 9, with 1 being emaciated and nine being extremely obese (Figure 2.3; Henneke et al., 1983). A considerable challenge for the older horse is that of maintaining body condition. Typically, diet and dental efficiency are of the utmost concern when determining quality of care. It is important to keep the BCS of an aged horse as close to the ideal of five as possible. Too fat (scores of 7, 8, and 9) leads to a higher risk of insulin resistance, equine metabolic syndrome and laminitis. Too thin (scores of 2, 3, and 4) makes an aged horse more susceptible to developing infections or to becoming anemic due to under-nutrition (Bertone, 2006). Mares with a BCS < 5 had a significantly longer interval to first ovulation from winter anestrous to spring compared to mares with a BCS < 5 (Henneke et al., 1984).

Regional Adiposity:

The mechanisms underlying generalized obesity or regional adiposity in EMS are unknown but chronic over-feeding in association with limited physical activity appears to be a contributing factor (Frank et al., 2010b). It has been suggested that horses and ponies evolved to adapted and survive in nutritionally sparse environments; and are especially

predisposed to obesity and insulin resistance, under modern management conditions in which plentiful feed is available year round (Frank et al., 2010b). Seasonal changes on insulin sensitivity may not only occur in spring when pasture is sugar-rich, but in the winter; which reflects alterations in decreased food availability, decreased physical activity, and a change in body condition. For instance, seasonality affected resting serum insulin concentrations in a study of insulin sensitivity in obese mares; and higher concentrations were detected in December, compared with September, October, and November (Vick et al., 2006). One reason for these changes, including the increase of laminitis seen especially in obese ponies, is the increase in cortisol levels, which can exacerbate insulin insensitivity by increasing stress. In hyperlypemia, triglycerides are mobilized in the blood from the utiilization of lipids from fat storage used in energy metabolism to maintain body condition, due to a negative energy balance (Jeffcott and Field, 1985).

In people, abdominal adiposity is more closely linked to risk for diabetes and cardiovascular disease than generalized obesity. Similarly, regional adiposity in horses and ponies, especially adipose tissue deposited more heavily along the crest of the neck (Figure 2.2 and 2.4), has been suggested to be associated with altered metabolic states, including reduced insulin sensitivity (Hoffman et al., 2003; Vick et al., 2007), an increased risk for laminitis (Johnson, 2002; Treiber et al., 2006c), and altered reproductive activity (Vick et al., 2006).

A cresty neck score (CNS) (Figure 2.4; Carter et al., 2009a) has been developed to assess the expansion of adipose tissues within the neck region and scores range from 0 to 5. Scores ≥ 3 are often detected in horses or ponies with EMS (Frank et al., 2010b).

The description for a score of 3 is "Crest enlarged and thickened, so fat is deposited more heavily in middle of the neck than toward poll and withers, giving a mounded appearance. Crest fills cupped hand and begins losing side-to-side flexibility." CNS has been shown to be useful in the assessment of adiposity in horses and has been associated with insulin, leptin, and triglycerides (Carter et al., 2008).

Ponies with a "cresty neck" are 18.9 times more likely to be hyperinsulinemic (Carter et al., 2009a) than horses with the same score of neck condition. Data reported in another study suggests that the nuchal ligament depot has unique biological behavior in the horse and is more likely to adopt an inflammatory phenotype than other depots examined, such as the tail head and mesenteric adipose tissue (Burns et al., 2010). Inflammatory cytokines IL-1 beta and IL-6 were higher in nuchal ligament adipose tissue than in other depots (Burns et al., 2010). These findings suggest that visceral fat may not contribute to the pathogenesis of obesity-related disorders in the horse as in other species (de Koning et al., 2007; Lee et al., 2007). In a study in which body condition and neck adiposity were assessed in horses and ponies, researchers found that overweight or obese horses (BCS ≥ 7) were not more likely ($P = 0.19$) to be hyperinsulinemic than a moderately conditioned horse ($4 ≥ BCS ≤ 7$), but an overweight or obese pony was 9.4 times more likely ($P < 0.001$) to be hyperinsulinemic than a moderately conditioned pony.

Managing Equine Metabolic Syndrome:

Goals for managing EMS are (1) reduce body fat mass in obese animals to improve insulin sensitivity, (2) avoid feeds that will exacerbate IR *(See: Feeding Insulin-Resistant Horses and Ponies)*, (3) lower the risk of laminitis by improving insulin

sensitivity through weight loss, diet, and exercise, and (4) avoid sudden changes in bacterial flora that might trigger laminitis. Changes in lifestyle and diet are designed to alleviate risk factors for laminitis in EMS and compliance of the owner or manager us critical to the success of the management changes.

Regular physical exercise is an effective therapeutic intervention to improve insulin sensitivity in obese insulin-resistant people (Houmard et al., 2004; Bajpeyi et al., 2009). Improvements in insulin sensitivity associated with physical activity can occur in the absence of weight loss or change in fat distribution; thus, an increase in physical activity is recommended for equids with EMS in order to promote weight loss and improve insulin sensitivity. Weight loss should be induced in obese horses by restricting the total number of calories consumed and by increasing the individual's level of physical activity. A general recommendation is to start with 2-3 exercise sessions per week (riding and or lounging), 20-30 minutes per session. Gradual increases in intensity and duration of exercise to five sessions per week may be beneficial. For those individuals suffering from chronic laminitis, it is important to permanently restrict access to pasture and house them in dirt paddocks so they are able to exercise once hoof structures have stabilized. In addition to exercise, restricting dry matter intake (DMI) to 1% of body mass resulted in a rate of weight loss of 1% of outset body mass weekly in mature, overweight/obese pony mares (Dugdale et al., 2010).

The use of pharmacologic agents for medical therapy of IR is indicated in refractory or severe cases and includes: levothyroxine sodium, which can induce weight loss and improve insulin sensitivity (Frank et al., 2005; 2008a,b) and metformin (a biguanide) *(See: Diabetes Mellitus: Treatment).* In addition to medical therapy,

supplements and nutraceuticals such as chromium, magnesium, cinnamon, and chasteberry are commonly recommended for the management of EMS *(See: Feeding Insulin-Resistant Horses).* However, there seems to be insufficient scientific evidence to support the use of these supplements at this time.

Feeding Insulin-Resistant Horses and Ponies

When considerations regarding the diet are concerned, the primary goal is to lower the glycemic and insulinemic response to the meal, which is the degree to which blood glucose and insulin concentrations rise in response to the feed. Key considerations are the quantity and composition of the ration. Avoidance of feeds rich in non-structural carbohydrates (NSCs) (starches, sugars, or fructans) that may increase risk for laminitis by exacerbation of IR and hyperinsulinemia or by disturbances to the hindgut microbial community that may trigger events leading to laminitis. Knowledge of the carbohydrate composition of feedstuffs is required in order to adequately manage the diet. Furthermore, removal from pasture is necessary for adequate control of dietary intake. Pasture-associated laminitis is triggered by gastrointestinal disturbances arising from alterations in bacterial flora that occur after consumption of pasture grass that is rich in fermentable carbohydrate (Treiber et al., 2006a,c; van Eps and Politt, 2006). Insulin sensitivity therefore seems to affect the threshold for laminitis, because IR horses and ponies are more susceptible to the disease, whereas equine with normal insulin sensitivity are less likely to develop the condition. For those individuals suffering from recurrent laminitis, it is important to permanently restrict access to pasture. As forage only diets may not provide adequate protein, minerals, or vitamins, supplementing with a low-calorie commercial ration balancer product (0.5-1.0 kg total per day) that contains

sources of high-quality protein and a mixture of vitamins and minerals to balance the low vitamin E, vitamin A, copper, zinc, selenium, and other minerals is recommended.

In horses that are being overfed, removal of all concentrates from the diet is sometimes sufficient to induce weight loss (Frank et al., 2010b). An obese horse should be placed on a diet consisting of hay fed in an amount equivalent to 1.5% of ideal body weight (1.5 lb. hay/100 lb. BW). Hay should be weighed on scales to ensure that correct amounts are fed. If an obese horse or pony fails to lose weight after hay has been fed at an amount equivalent to 1.5% of ideal body weight for 30 days, this amount should be lowered to 1%, but no less than 1%, as severe calorie restriction may lead to worsening IR and hyperlipemia (Frank et al., 2010b). In addition, obese horses and ponies should be supplemented with a mineral/vitamin supplement, while access to pasture grass should be limited or eliminated. Strategies for limiting grass consumption include short (< 1 hour) turnout periods (or hand-grazing), confinement in a small paddock, round pen, or area enclosed with electric fence, or use of a grazing muzzle (Frank et al., 2010b). Horses and ponies with EMS can have rapid rates of grass intake, so more than 1 to 2 hours of grazing may be excessive for these animals (Longland and Byrd, 2006).

Feed Restrictions of Non-Structural Carbohydrates:

Dietary management of EMS involves reducing the amount of energy provided in the diet to induce weight loss if obesity is presented, and lower the non-structural carbohydrate (NSC) content of the diet to reduce glycemic and insulinemic responses to meals. In general, rations for obese insulin-resistant horses should be high in fiber and low in NSCs. Simple sugars, starches, and fructans are NSC, whereas cellulose and hemicelluloses are structural carbohydrates (Longland and Byrd, 2006). Non-structural

carbohydrates includes both hydrolysable carbohydrates (CHO-H) and nonhydrolyzable but rapidly fermentable carbohydrates (CHO-F_R). Both fractions are fermented rapidly in the rumen, but, in the horse, CHO-H is digested mainly in the small intestine and is fermented in the hindgut if starch intake exceeds 0.4% of body weight per feeding (Potter et al., 1992). Seasonal variation in pasture NSC occurs and may increase the risk of certain diseases, such as laminitis and colic (Hoffman et al., 2000). Recommendations include that non-structural carbohydrates (NSC) be calculated by adding starch and water-soluble carbohydrates (WSC) percentages together, and this value should ideally fall below 10%. Hay with low NSC content (< 12%) should be selected and it can be soaked in cold water for 60 minutes to lower the soluble carbohydrate content if the amount of NSC exceeds 10% (Cottrell et al., 2005; Longland et al., 2011), but remember to discard the water after soaking. However, although soaking hay results in reductions of WSC content, the extent of the losses may be highly variable between types of hays used, and not related to their initial WSC content (Longland et al. 2011).

Insulin-resistant horses with a thinner overall body condition are challenging to manage from a dietary standpoint because hay alone may not meet energy requirements. Insulin-resistant ponies exhibit an insulin response to dietary fructans (Bailey et al., 2007). Utilizing commercial low-NSC feeds are useful in which digestible fibers (beet pulp or soya hulls) and/or vegetable oils are included in place of starch-rich ingredients. The energy density of the ration can be increased by feeding soaked beet pulp shreds (molasses-free) or vegetable oil.

Effects of Fatty Acid Supplementation on Insulin Sensitivity:

One nutritional intervention to improve inflammation status, increase insulin sensitivity, and possibly prevent insulin resistance, and consequently, laminitis, may be accomplished via supplementation of long chain highly unsaturated omega-3 fatty acids (LCHUFA), or polyunsaturated fatty acids (PUFA), such as n-3 α-linolenic acid (ALA). Conversion of ALA can produce eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 2.1) which are absorbed and incorporated into cell membranes (Cook, 1985). In a horse study done by Rexford and others (2012), those who were considered IR (< 0.78 RISQI) and supplemented with PUFAs showed a main time effect ($P < 0.008$) and a trend towards a treatment effect ($P < 0.09$). Horses classified in the lowest quintile for insulin sensitivity (< 0.29 RISQI) showed a trend for a treatment effect, whereas horses supplemented with flaxseed (149.8 mg/kg of BW) and fish oil (142.4 mg/kg of BW) had higher insulin sensitivity compared to the control group (Rexford, 2012). In rodent and human studies PUFAs change fatty acid composition of phospholipids surrounding insulin receptors found in muscle (Luo et al., 1996; Rasic-Milutinovic et al., 2007) and reduce inflammation when incorporated into white blood cells (Calder, 2008).

Polyunsaturated fatty acids (PUFA) comprise two classes of fatty acids: n-3 fatty acids, which include α-linolenic (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and n-6 fatty acids, which include arachidonic acid (ARA) and linoleic acid (LA) (Figure 2.1). When PUFAs (mainly omega-3 fatty acids) are absorbed they are incorporated into cell membranes affecting membrane characteristics, changing fatty acid composition of phospholipids surrounding insulin receptors in muscle in rats (Luo et al., 1996). Furthermore, n-3 PUFAs have a protective effect in rodents in

vivo against a high fat diet that induces insulin resistance (Kraegen et al., 1991). In addition, n-3 PUFAs, specifically eicopentaenoic (EPA) and docosaenoic acid (DHA) may reduce the rate at which insulin progresses to Type 2 diabetes mellitus in people (Kesavulu et al., 2002).

Fish oil has been shown to have many beneficial effects in other species including improvement of insulin sensitivity. Feeding essential fatty acids may reduce the risk of certain metabolic and digestive disorders that have been linked to feeding grain-based concentrates (Kronfeld, 2003). In horses, a study has shown that supplementation with flaxseed oil leads to plasma concentrations with increased linoleic (C18:2 n-6), linolenic (C18:3 n-3), EPA, but not DHA acids (Hansen et al., 2002). Flaxseed oil has omega 3 and omega 6 fatty acids in the proportion of 2:1. One possible reason for not finding significant increases in DHA could occur due to the competition between omega-6 and EPA at the conversion step to DHA. Supplementation of EPA and DHA has been shown to be effective in the incorporation to cell membranes (King et al., 2007; Wilson et al., 2007). Other anti-inflammatory effects of n-3 LCHUFA from plant and animal sources include reduced cytokine production *in vitro* (De Caterina et al., 1994) and *in vivo* (Grimm et al., 1994; McCann et al., 2000). Additionally, a trend was observed for improved insulin sensitivity in horses that were supplied with a fish oil supplement containing EPA and DHA, and in horses supplied with flaxseed meal in addition to hay (Rexford et al., 2010).

Chromium:

Chromium (Cr), in theory, is an essential trace element that is involved in the metabolism of carbohydrates, lipids and proteins by amplifying the activity of insulin

(Mertz, 1993; Lukaski, 1999). The hypothesized theory is that Cr forms a complex between insulin and insulin receptors that facilitates the insulin-tissue interaction (Mertz, 1993). The proposed mode of action of Cr is that increases in blood insulin levels stimulate its uptake by insulin-dependent cells, resulting in the binding of Cr to a low-molecular-weight, chromium-binding substance, chromodulin. The binding of four Cr ions to chromodulin enables the link to the insulin-stimulated, insulin receptor, and thereby amplifies insulin signaling (Davis et al., 1996; Davis and Vincent, 1997a,b; Vincent, 2000). Specifically, enhanced tyrosine kinase activity or reduced tyrosine phosphatase activity, which prolongs the effects of insulin binding to its receptor can thereby increases insulin sensitivity, in theory (Vincent 2000; Hummel et al., 2007).

In Type 2 diabetic and obese humans and rats, Cr supplementation has been shown to increase the cellular uptake of glucose, evidenced by lower fasting plasma concentrations, and stimulating insulin metabolism by increasing insulin sensitivity (Mertz, 1993; Lukaski, 1999; Vincent 2000; Cefalu et al., 2002; Hummel et al., 2007), whereas in healthy humans, Cr supplements did not benefit glucose metabolism (Mertz, 1993; Lukaski, 1999). Other functions of Cr relate to its effects on growth, lipid metabolism, immune response and interactions with nucleic acids (McDowell, 2003).

Chromium deficiency is evidenced by impaired glucose tolerance, a fasting hyperglycemia, elevated circulating insulin levels and high blood cholesterol and triglyceride concentrations (Anderson, 1994). There is little retention of Cr and that is absorbed and Cr is excreted primarily via the kidneys (McDowell, 2003). Circulating Cr does not reflect tissue Cr concentrations (Anderson, 1994). There is no adequate tool to assess Cr status and, currently, the best method to diagnose Cr deficiency is based on the

improvement in glucose tolerance following Cr supplementation (Mertz, 1993); as the effects of different Cr supplements on post-prandial serum Cr levels have not been established in horses.

Chromium failed to alter glucose or insulin dynamics in horses (Cartmill et al., 1995; Vervuert et al., 2006; Uyanik et al., 2008; Chameroy et al., 2011). Although, other studies have shown that chromium significantly alters glucose and insulin concentrations in horses (Pagan et al., 1995; Ralston et al., 1997; Ott and Kivipelto, 1999). Quarter horse and Thoroughbred yearlings that received chromium at the highest dosage (175,350 μg/kg BW) had faster glucose clearance rates than animals in the control group. Pagan and others (1995) used exercising Thoroughbreds and results showed a positive effect of supplementing Cr-enriched yeast (5mg Cr per horse over a 14-day period) to mature horses had some beneficial effect on glucose metabolism. Significantly lower levels of insulin, cortisol, and glucose during exercise were measured. Lower plasma glucose concentrations were also detected one hour post-feeding in the same horses. Aged mares (> 20 years) that were supplemented with 0.02 mg/kg BW chromium-L-methionine for four weeks had a lower peak insulin response to a concentrate meal after four weeks of supplementation (Ralston et al., 1997). Five trained Standardbred horses with a mean body weight of 412 ± 46kg, a body condition score of 4 (Henneke et al., 1983) and a mean age of 3 ± 2.2 years were individually stalled and fed a constant basal diet formulated to meet or exceed energy and nutrient requirements according to GEH (1989) recommendations for the performance horse. Each horse completed an identical 3-month training program on a high speed treadmill before the experiment began. Exercise tests were started 3 hours after the morning feed. The results showed there was no close

relationship between serum insulin and plasma glucose values ($P < 0.05$), and glucose

and insulin concentrations were not affected by Cr supplementation in healthy horses 2

hours after a meal. However, slightly lower serum cortisol concentrations (non-

significant) were measured in horses fed 8.3 mg Cr at rest than for those fed the control

or 4.15 mg Cr. There was a strong relationship between serum cortisol and plasma

glucose ($P < 0.001$). Although Cr supplementation in healthy trained horses elicited no

beneficial effects on glucose and insulin metabolism during both rest and exercise

(Vervuert et al., 2005), Cr-supplementation may have beneficial effects in obese diabetic

horses, which requires further investigation. A study done by Chameroy et al. (2011)

concluded no beneficial effects of chromium supplementation on morphometric

measurements, blood parameters, or insulin sensitivity in obese laminitic horses.

In a study done by Liu and others (2010), after 15 weeks of treatment with high –

chromium yeast (0-1,000 mug Cr/kg body mass) of diabetic mice, the effect on blood

lipids and blood glycosylated hemoglobin (GHb) of diabetes were not consistent. High-

chromium yeast results in a lowering ($P < 0.05$) of GHb and triglyceride, lowering ($P <$

0.01) of total cholesterol, and restoration ($P < 0.01$) of insulin; these results are in stark

contrast to those of diabetic mice of administration of normal yeast, which have no effect

on these parameters and serve as control group. The histopathological analysis of

pancreas islet shows that high-chromium yeast could profoundly protect the impaired

pancreatic islet and beta-cells from inflammatory infiltration and fibrosis (Liu et al.,

2010).

A study done by Lydic and others (2006) used Trivalent chromium (1000

microg), as chromium picolinate, and their results showed that without change in diet or

activity level, a 38% mean improvement in glucose disposal rate in five obese women with polycystic ovary syndrome who were tested with a HEC technique. This suggests that chromium picolinate, an over-the-counter dietary product, may be useful as an insulin sensitizer in the treatment of polycystic ovary syndrome (Lydic et al., 2006).

Finally, in women with polycystic ovary syndrome, chromium picolinate (200 microg/d) improved glucose tolerance compared with placebo, but does not improve ovulatory frequency or hormonal parameters (Lucidi et al., 2005). This pilot study done by Lucidi and others (2005) indicates that future studies in the polycystic ovary syndrome population should examine higher dosages or longer durations of treatment.

Magnesium:

This mineral is thought to elicit positive effects on insulin sensitivity by enhancing intracellular signaling and increasing tyrosine kinase activity via calcium channel blockade (Paolisso and Barbagallo 1997; Takaya et al., 2004). Magnesium deficiency has been associated with IR and metabolic syndrome in rodents and people (Volpe et al., 2001). Magnesium supplementation has been associated with improved homeostatic model assessment-IR scores in people, indicating improved insulin sensitivity (Volpe et al., 2001; Guerrero-Romero et al., 2004). There is little information available regarding magnesium supplementation on insulin sensitivity in horses. One study which used chromium and magnesium supplementation on laminitic, obese (BCS $\geq$ 7) horses (ages 8 to 20 years) found no effects on morphometric measurements, blood variables or insulin sensitivity (Chameroy et al., 2011). No product information was given at the request of the manufacturer, so results do not reflect any known amount of chromium or magnesium given. Different results have been reported for humans, as

magnesium supplementation increased insulin sensitivity in type 2 diabetic patients with low serum magnesium concentrations ($\leq$ 0.74 mmol/l) prior to treatment. However, in overweight nondiabetic adults, there was no improvement in insulin sensitivity after receiving a magnesium oxide supplement for 12 weeks (Lee et al., 2009). These differences may indicate that supplements have a greater effect on insulin sensitivity when subjects suffer from magnesium deficiency, and thus, magnesium status would need to be measured in individuals prior to the start of a study.

Diabetes Mellitus:

Diabetes Mellitus (DM) is reported rarely in horses (King et al., 1962; Loeb et al., 1966; Jeffrey, 1968; Riggs, 1972; Baker and Ritchie, 1974; Bulgin and Anderson, 1983; McCoy, 1986; Muylle et al., 1986; Ruoff et al., 1986; Johnson et al., 2005; Durham et al., 2009; Giri et al., 2011). Primary pancreatic disease leading to suspected type 1 DM (T1DM) in horses has been reported rarely (Jeffrey, 1968; Riggs, 1972; Bulgin and Anderson, 1983; Giri et al., 2011). Although insulin resistance is a common problem in equids, most affected horses do not develop overt DM. Possibly, the lower risk for DM in horses, compared with humans, is a result of their shorter longevity or the fact that equine rations typically contain little fat (Johnson et al., 2005).

Development of DM Type 2 diabetes mellitus is considered to be the more common form of DM, yet still rarely reported, and is seen most frequently in elderly horses affected from weight loss, polydipsia and polyuria, in association with equine Cushing's disease (King et al., 1962; Loeb et al., 1966; Baker and Ritchie, 1974), granulosa cell tumors (McCoy 1986), chronic pancreatitis (Jeffrey, 1968), pregnancy, and as a condition of unknown etiology (Muylle et al., 1986; Ruoff et al., 1986; Johnson et

al., 2005). Of 14 reported cases of equine DM, where age and gender were stated, the mean age was 19 years (range 7-30 years) with 5 geldings and 9 mares affected (Durham et al., 2009).

An 18-year-old Spanish Mustang mare was referred to the University of Missouri Veterinary Medical Teaching Hospital for evaluation of progressive weight loss, persistent hyperglycemia, and intermittent diarrhea (Johnson et al., 2005). Abnormalities identified on physical examination included poor body condition score (BCS of 3 on a scale from 1 to 9) (Figure 2.3; Henneke et al., 1983), fecal staining and urine scalding of the hindquarters, and excessive hoof growth affecting all four feet, although laminitis was not evident (Johnson et al., 2005). Biochemical abnormalities included hyperfibrinogenemia (0.7 g/dL; reference range: 72 to 114 mg/dL), hyperglycemia (359 mg/dL; reference range: 72 to 114 mg/dL), hyperproteinemia (8.0 g/dL; reference range: 4.9 to 6.9 g/dL), hyperglobulinemia (4.9 g/dL; reference range: 3.2 to 4.0 g/dL), hypertriglyceridemia (103 mg/dL; reference range: 4 to 50 mg/dL), high alkaline phosphatase activity (379 U/L; reference range: 109 to 315 U/L), and high ɣ-glutamyltransferase activity (70 U/L; reference range: 12 to 45 U/L). Plasma concentration of C-peptide was substantially lower (0.18 pmol/mL) than control horses (0.64 ± 0.17 pmol/mL) and fell into the lower reference limit for healthy humans (reference range: 0.22 to 1.08 pmol/mL). Researchers report that it is possible that the horse may have had insulin resistance for some time and that DM developed as a consequence of heightened β-cell stimulation over time (pancreatic β-cell exhaustion), which may have been exacerbated by a high glycemic ration from the owners (Johnson et al., 2005).

Diabetes Mellitus is the fifth-leading cause of death in the United States, as well as the leading cause of blindness among working-age adults, end-stage renal disease, and non-traumatic loss of a limb. Medical costs for diabetic patients amounts $90-130 billion in the United States (Perlin and Pogach, 2006; Petersen and Shulman, 2006). Diabetes is defined as chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Anon, 1999, 2003, 2006).

Type 1 Diabetes Mellitus:

Type 1 diabetes, also called insulin-dependent diabetes mellitus (IDDM or type 1) or juvenile-onset diabetes in humans, is caused by selective autoimmune destruction of the insulin-producing cells of the pancreas. Antibody and T-cells responses are made against insulin, glutamic acid decarboxylase, and other specialized proteins of the pancreatic β cell, which make about two-thirds of the islet cells; as they die the architecture of the islet degenerates (Parham, 2009). Disease commences when there are insufficient β cells to provide the insulin necessary to control the level of blood glucose. In humans, the usual treatment for patients with IDDM is daily injections with insulin purified from the pancreas of pigs or cattle, or recombinant human insulin produced in the laboratory from the cloned insulin gene is prescribed to patients who make antibodies against animal insulin.

Insulin-dependent diabetes mellitus is successfully managed in several species, most notably humans, dogs, and cats. Of the many challenges in the treatment of IDDM in horses, among the most significant is the lack of clinical and reported experience with long-term administration of insulin, the absence of monitoring tools, and little experience regarding dietary and exercise management (Giri et al., 2011).

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A 5-year-old Thoroughbred-Percheron cross mare was presented to the University of California's Veterinary Medical Teaching Hospital, for a six week history of weight loss despite polyphagia, as well as polydipsia and polyuria (Giri et al., 2011). Biochemical assessment of blood revealed marked hyperglycemia (16.0 mmol/L, reference range: 4.4 to 5.9 mmol/L), increased liver enzymes (AST 940 IU/L, reference range: 168 to 494 IU/L; GGT 45 U/L, reference range: 8 to 22 IU/L), increased anion gap (AG 21, reference range: 9 to 17); hypochloremia (84 mEq/L, reference range: 91 to 104 mEq/L), and a mild anemia (hematocrit = 27.3%, reference range: 30% to 44%). The mare's serum was grossly lipemic and the triglyceride concentration was markedly increased (34.1 mmol/L, reference interval: 0.02 to 0.46 mmol/L). The endogenous insulin concentration was below the level of detection (9 pmol/L, reference interval: 29 to 179 pmol/L). The fructosamine concentration was increased (559 mmol/L, reference interval: 316 to 402 mmol/L, based on concentrations found in 3 clinically normal age-matched horses), as was glycosylated hemoglobin (Hb A1c) (24.3%, reference interval: 2.5% to 5.0%). A urinalysis revealed marked glucosuria, mild to moderate ketonuria, and trace hematuria. The urine specific gravity was 1.035, although this was likely increased due to the presence of glucose and ketones. Although multiple experimental treatments were used over an 18-month span, the mare died from terminal electrolyte abnormalities consistent with Addison's disease (defined in humans) and had IDDM, which has not been previously described in horses (Giri et al., 2011).

Type 2 Diabetes Mellitus:

Type 2 diabetes mellitus (T2DM) also called noninsulin-dependent diabetes mellitus (NIDDM) is a relative insulin deficiency and is a complex metabolic disorder resulting from a gradual onset of both insulin resistance (IR) and pancreatic β-cell dysfunction (Anon, 1999; Kahn, 2000, 2003). Although rare, pancreatic β-cell failure may contribute to the development of T2DM in horses (Johnson et al., 2005; Durham et al., 2009). Typically, in people, higher levels of insulin are found in adults with T2DM (Yalow and Berson, 1960). Hyperinsulinemia enhances hepatic gluconeogenesis and impaired insulin-stimulating glucose uptake into skeletal muscle and fat. Furthermore, elevated levels of free fatty acids (FFAs) associated with obesity increases fat accumulation in insulin target tissues, especially in muscle.

Pathophysiology:

The pathophysiological progression of human T2DM ranges from: stage 1, compensated IR with normoglycemia, normal β cell function and hyperinsulinemia ('pre-diabetes'); to stage 2, uncompensated IR with mild hyperglycemia, mild β cell dysfunction and normal to hyperinsulinemia; to stage 3, uncompensated IR with marked hyperglycemia, severe β cell dysfunction and normal to low serum insulin (Martin et al., 1992; Weyer et al., 2001; Kahn 2000, 2003). Serum insulin concentrations may therefore be low, normal or high in subjects with T2DM, but even in cases with hyperinsulinemia, the coexisting hyperglycemia indicates that the insulin concentration is nevertheless insufficient (Kahn, 2001). One factor that may influence an increase in recognition of diabetes mellitus in horses and ponies is that the equine demographic analyses reveal an increasing population of elderly horses presenting for veterinary attention (Anon, 2007).

Diagnosis:

Confirmation of insulin resistance (IR) is required for the diagnosis of T2DM and further diagnostic grading of T2DM can be made on the basis of severity of β-cell dysfunction. Specific and quantitative tests of insulin sensitivity and β-cell function include the hyperinsulinemic-euglycemic clamp (HEC) and hyperglycemic clamp techniques (Wallace and Matthews, 2002; Kronfeld et al., 2005); the minimal model of glucose-insulin dynamics (MINMOD) (Wallace and Matthews, 2002; Kronfeld et al., 2005; Treiber et al., 2006a); and basal plasma insulin (mU/L) and glucose (mg/dL) concentrations using the basal proxies and reference quintiles obtained from minimal model analysis for determination of insulin sensitivity and pancreatic β-cell responsiveness by RISQI and MIRG equations (Treiber et al., 2005). In addition, endogenous insulin secretion may be assessed by measuring concentration of C-peptide, which is co-secreted with insulin in a 1:1 molar ration but which, unlike insulin, experiences little first-pass clearance by the liver (Sjoquist et al., 1998; Wahren et al., 2000; Torn, 2003). Measurement of C-peptide concentration under standard conditions is clinically validated for the assessment of β-cell function (Wahren et al., 2000; Torn, 2003).

If persistent hyperglycemia is detected, a diagnosis of DM should be considered and a diagnosis of T2DM should be considered only when hyperglycemia cannot be attributed to other causes such as stress, recent feeding, administration of α-2 agonist drugs, or inflammatory processes (Frank et al., 2010b).

Treatment:

Literature on the treatment for DM in horses is limited and research has variable results on the medications used, as the dosage and type is experimental at this point. Many medical options exist in the management of human patients with T2DM (Donner, 2006; Aronne, 2007). Pharmacological treatment with insulin-sensitizing agents, insulin secretagogues and exogenous insulin are all likely to be of limited benefit in the presence of low levels of endogenous insulin, severe β-cell dysfunction and marked IR (Durham et al., 2009). A pony with suspected T1DM was successfully managed using protamine zinc insulin at a dose of 1.0 iu/kg body weight daily, although hypoglycemic episodes were encountered (Jeffrey, 1968). Yet, insulin therapy in cases of T2DM in horses has been unsuccessful even with doses as high as 6.6 iu/kg body weight protamine zinc insulin daily (King et al., 1962; Loeb et al., 1966; Baker and Ritchie, 1974). Also, it should be considered that maintenance of high serum concentrations of insulin probably required for an effective response in the face of IR may be harmful (Asplin et al., 2007) and cost prohibitive in equine cases.

Medical treatment with the anti-hyperglycemic drug metformin (dimethylbiguanide), a frequently prescribed insulin-sensitizing drug in human patients (Nathan et al., 2008) and enhances insulin sensitivity by increasing peripheral glucose uptake. It also decreases blood glucose concentrations by inhibiting hepatic glucose production and intestinal absorption of glucose (Saenz et al., 2005). Metformin promotes weight loss and reduces lipid levels; adverse effects are rare (Salpeter et al., 2008).

Several reports describe the effects of metformin use in horses and ponies with mixed results (Johnson et al., 2005; Vick et al., 2006; Durham et al., 2008, 2009; Firshman et al., 2009; Hustace et al., 2009). A single dose of metformin (1.9 mg/kg bodyweight, orally) administered with Glibenclamide (an insulin secretagogue) to a hyperglycemic horse reduced plasma glucose concentrations to values within the reference interval (Johnson et al., 2005). Glyburide apparently lowers blood glucose concentrations by stimulating the release of insulin from the pancreas, an effect dependent on functioning β cells in the pancreatic islets (Dailey, 2003; Giannarelli et al., 2003). Since the mare had few β cells seen in the pancreatic islets, it seems likely that the decrease in glucose concentration was a result of metformin's action to improve the effectiveness of insulin at its receptor targets (Johnson et al., 2005).

In another case, metformin (2.8 mg/kg bodyweight, orally every 12 hours) administered to 14 obese mares for 30 days enhanced insulin sensitivity, measured with the hyperinsulinemic-euglycemic clamp (Vick et al., 2006). However, in the same study, metformin became ineffective when given for longer or at an increased dose (Vick et al., 2006). Yet, when metformin and glibenclamide were used as a therapy treatment for an aged 24 year old Warmblood mare that was diagnosed T2DM and hyperglycemic, along with a strict cereal-free diet, conditions improved (Durham et al., 2009). Results 11 months after therapy revealed that the mare was clinically normal, with plasma glucose at 5.8 mmol/l and serum insulin concentration at 2.8 miu/l (Durham et al., 2009).

Another case study used metformin and after five months of treatment and a dosage change, the 27 year old Hanovarian-cross gelding was reported to be bright and active, while exhibiting a good appetite (Durham et al., 2009). However, metformin

shows no significant change in insulin sensitivity in insulin resistant horses and ponies, but may be effective in obese ponies losing weight or with hyperglycemia (Tinworth et al., 2011). In addition, the lack of apparent long-term efficacy of metformin is attributable to its low bioavailability in the horse/pony. The bioavailability of metformin in horses after oral administration was reportedly $3.9 - 7.1\%$, depending on the feeding status (Hustace et al., 2009). In contrast, bioavailability in humans is $40 - 60\%$ (Scheen, 1996).

Concerns with treating horses with glibenclamide (glyburide) exist because of its potential to induce hypoglycemia in association with increased insulin secretion (Durham et al., 2008; Phillipe and Raccah, 2009). Until further research data are gathered on the use of metformin and glibenclamide in horse, caution must be taken when treating horses suspected with DM.

Equine Cushing's Disease:

Equine Cushing's disease, also known as pituitary pars intermedia dysfunction (PPID) is the most common endocrinopathy that occurs in horses 15 years and older and affects $15 - 30\%$ of aged equids (Schott, 2002; Brosnahan and Paradis, 2003; van der Kolk, et al., 2004; Ireland et al., 2011a,b). Pituitary pars intermedia dysfunction is a chronic progressive disease of the pars intermedia lobe of the anterior pituitary gland. Although all breeds of horses are subject to developing PPID, Morgan horses and ponies seem to be at greater risk (Brosnahan and Paradis, 2003; van der Kolk, et al., 2004).

Clinical Observations:

Hyperhidrosis or an increase in sweat on the neck and shoulder is usually visible on PPID-affected equids (Loeb, 1966; Hillyer et al., 1992), as well as hirsutism, or a long

and curly hair coat that fails to shed (van der Kolk, 1998). An increase in weight loss, lethargy, and protein catabolism is prevalent (Schott, 2002). Although affected horses appear thin overall because of loss of skeletal muscle mass, they also acquire regional deposits of body fat in abnormal locations such as over the top of the neck (Figure 2.4), over and behind the supra-orbital fossae (Hillyer et al., 1992; van der Kolk, 1998), over the tail head (Figure 2.2), and the sheath of a male (Messer and Johnson, 2007). Chronic laminitis and abnormal distribution of fat were the most frequent clinical expressions of PPID (Donaldson et al., 2004), even in the absence of hirsutism. In addition, PPID may also develop in overtly obese horses in which hirsutism is not present (Schott, 2002; Messer and Johnson, 2007). Horses with PPID are often predisposed to life-threatening complications such as laminitis, secondary infections, pseudo-lactation, and insulin resistance.

Horses affected by PPID tend to have delayed wound healing and secondary infections, such as urinary, sinusitis, and conjunctivitis, as their inflammatory status increases with age (van der Kolk et al., 1993; Hillyer et al., 1998; McFarlane and Holbrook, 2008). Mares may have persistent lactation and infertility; however, they can have normal estrous cycles and carry a foal to term (van der Kolk, 1998). Although rare, corneal ulcers, blindness, seizures, and ataxia can be some physical nervous system problems related with horses diagnosed PPID, due to a compression of adjacent tissue by the PPID tumor (van der Kolk et al., 1993).

Physiology:

The anterior lobe of the pituitary consists of three parts: pars distalis, pars intermedia, and pars tuberalis. The main secretory products of the corticotropes, located

in the pars distalis, are adrenocorticotropic hormone (ACTH) and β-endorphin-related

peptides, whereas the main secretory products of melanotropes, located in the pars

intermedia, are melanocyte-stimulating hormone and β-endorphin-related peptides

(Messer, 2006). Both corticotropes and melanotropes synthesize the same precursor

hormone, pro-opiomelanocortin (POMC), but cleave into different hormones. Pro-

opiomelanocortin is a single large pre-pro-hormone that has multiple hormones referred

to as POMC peptides derived from it. Such POMC peptides are alpha-melanocyte

stimulating hormones (α-MSH), beta-endorphin (β-END), adrenocorticotropin (ACTH)

and corticotropin-like intermediate peptide (CLIP) (Schott, 2002; Messer and Johnson,

2007; McFarlane et al., 2006; Millington et al. 1988).

In normal horses, melanotropes in the pars intermedia secrete relatively small

quantities of the POMC peptides and is under tonic inhibitory influence of dopaminergic

nerves originating in the periventricular nuclei (cell bodies) of the hypothalamus

(McFarlane et al., 2006). Usually, adrenocortical steriodogenesis is maintained by

corticotrope secretion of ACTH and corticotropes are inhibited via negative feedback of

glucocorticoids (Messer, 2006). Frequent pulses of ACTH are released from the pituitary

in a pulsatile manner consisting of at least 2 to 4 pulses per hour (Redekopp et al., 1986),

causing oscillations of ACTH and cortisol in the peripheral blood (Cudd et al., 1995) and

characterized as ultradian rhythms. The peaks in plasma ACTH occur approximately

every 11 - 17 minutes, but are not equal in all horses and can significantly affect a single

plasma ACTH measurement (Cudd et al., 1995). However, ACTH concentration in

jugular vein plasma is 50-times lower than that in pituitary venous effluent blood.

Therefore, most of the pituitary spikes are not detectable in jugular blood (Redekopp et

al., 1986). Due to the dilution effect, ACTH in jugular blood follows a diurnal rhythm with minor hourly fluctuations; therefore, a single ACTH measurement is a good reflection of pituitary secretory activity. Furthermore, ACTH levels are similar in young and old healthy horses (McFarlane et al., 1998).

Pathophysiology:

The endocrine cells of the pars intermedia (melanotropes) remain active in horses with PPID, secreting relatively large quantities of POMC-derived peptides into the peripheral circulation. Horses with PPID may have as much as a 40 to 50-fold increases in plasma concentration of pars intermedia POMC-derived peptides (Orth, et al., 1982). Clinical signs result from effects of increased POMC-peptide secretion, physical enlargement of the pituitary glands because of expansion of the pars intermedia and stimulated secretion of cortisol by the adrenal glands (hypercortisolism). However, upon evaluation of post-mortem examinations, only approximately 20 % of PPID-affected horses are identified with adrenocortical hyperplasia, although excess secretion of cortisol results from increased pars intermedia-derived ACTH, the steriodogenic properties of ACTH are significantly enhanced by other POMC peptides (Dybdal, et al., 1994).

High plasma insulin concentrations and insulin resistance can be seen in ponies and horses with equine Cushing's disease (Garcia and Beech, 1986; van der Kolk et al., 1995; McGowan et al., 2004), because of excessive secretion of endogenous glucocorticoids and POMC-derived peptides (Garcia and Beech, 1986). A seasonal increase in POMC-derived peptides to may metabolically prepare horses and ponies for a decrease in accessible food observed in the wild in winter (McFarlane et al., 2004).

Pituitary pars intermedia dysfunction occurs as a result of hyperplasia and hypertrophy of the melanotrope cell population in the pars intermedia of the pituitary gland secondary to loss of dopaminergic inhibition caused by neurodegeneration of the dopaminergic periventricular neurons (McFarlane et al., 2005). A decrease in the number of pars intermedia periventricular dopaminergic nerve terminals and associated hypothalamic cell bodies in horses with PPID compared with age-matched controls (McFarlane et al., 2005) suggests PPID is primarily a dopaminergic neurodegenerative disease rather than the consequence of a spontaneously forming pituitary adenoma (McFarlane and Holbrook, 2008). The resulting changes to the pars intermedia have been referred to as pituitary hypertrophy, pituitary hyperplasia, or pituitary adenoma (Schott, 2002).

Baseline plasma ACTH concentration is increased in horses and ponies with pituitary dysfunction, compared with that in clinically normal horses (Couteli et al., 1996). Excessive secretion of pars intermedia derived POMC peptides and hyperplasia of melanotropes in the pars intermedia of PPID affected-horses can result from the destruction of inhibitory dopaminergic nerves originating in the hypothalamus as a consequence of oxidative stress (McFarlane et al., 2005). Loss of dopamine inhibition of the endocrine cells of the pars intermedia (melanotropes) is critical in the pathology of PPID. Dopamine and dopamine metabolite concentrations in the pars intermedia of PPID horses were shown to decrease eight-fold compared with age-matched controls (Millington et al., 1988).

The pathogenesis of dopaminergic neurodegeneration in other species is unknown, but several factors are suggested to play a role in humans and rodents; such as oxidative stress, mitochondrial impairment, inflammation, and neuronal accumulation of

misfolded alpha-synuclein protein in humans and rodents (Dauer and Przedborski, 2003). In people with the dopaminergic neurodegenerative disease, Parkinson's disease (PD), activated microglia are found adjacent to degenerating neurons in the substantia nigra (Dauer and Przedborski, 2003), and these cells secrete inflammatory cytokines and produce free radicals (McGreer et al., 1988), highlighting the role of inflammation with the development of dopaminergic neurodegeneration. In addition, depletion of microglia or antagonism of the inflammatory response using genetic or pharmacologic manipulation prevents dopaminergic neurodegeneration after neurotoxin exposure (Bronstein et al., 1995; Gao et al., 2002; Barcia et al., 2004; Kurkowska-Jastrzebska et al., 2004).

Diagnoses:

Pituitary pars intermedia dysfunction is being more commonly diagnosed because of an increase in the awareness of the disease, availability of convenient diagnostic tests, and the growing population of geriatric horses (Donaldson et al., 2004; NAHMS, 2005). Diagnosing PPID can be challenging in less severe cases and is much easier to find in more advanced cases; hence more cases are found in older horses (Brosnahan and Paradis, 2003a,b). Also, it is unknown when this dysfunction generally occurs in equine, except that the reported cases seem to be in horses around 20 years of age and older (Brosnahan and Paradis, 2003a,b; Donaldson et al., 2004). Diagnosis is usually based on clinical signs and endocrine tests and early detection is important for long-term care and management of affected horses to minimize the complications that are often associated with the disease (Schott, 2002; Messer and Johnson, 2007).

Dexamethasone-Suppression Test:

The "gold standard" test for PPID in 1999 was the overnight dexamethasone suppression test (DST) (Messer et al., 2008). Suppression of endogenous cortisol concentration via administration of dexamethasone was long regarded as the optimal test for PPID, but results can be inconsistent and even variable within the same horse with PPID (Dybdal et al., 1994; Schott et al., 2001). In addition, the use of dexamethasone has a slight risk of inducing laminitis (Eyre et al., 1979), and often, people are reluctant to perform DST, especially in horses with a history of chronic laminitis (Couetil et al., 1996).

A study of 52 horses with clinical and pathologic evidence of PPID, showed all affected horses had plasma cortisol levels of > 1μg/dl 19 hours after the administration of 40 μg/kg of dexamethasone, whereas all unaffected, normal, healthy horses had plasma cortisol levels < 1μg/dl 19 hours after administration of 40 μg/kg dexamethasone (Dybdal et al., 1994). Based on these criteria, the test was 100% sensitive and 100% specific in the population of horses included in the study (Dybdal et al., 1994; van der Kolk et al., 1995). However, in another study, seven horses were all diagnosed with PPID at the beginning of the study based on the DST; in later evaluations 30 and 60 days after the initial DST, they were found to have variable DST results (Meisner et al., 2003). Five of the seven horses had normal DST results 60 days after all seven horses had showed abnormal DST results (Meisner et al., 2003). Also, this study was done before the time it was discovered that horses experience a normal physiologic increase in hypothalamic-pituitary-adrenal axis activity in the fall of the year. Thus, knowledge of the normal increase in POMC-derived peptides in the fall would have influenced the results of the study.

Thyrotropin-Releasing Hormone:

A study using ACTH response to thyrotropin-releasing hormone (TRH) administration was evaluated by itself and compared with DST results in 29 horses having variable involvement of the pars intermedia and ranged from normal to abnormal (Beech et al., 2007). Even though ACTH levels increased for all horses, the magnitude and duration of the increase was significantly greater in horses with PPID (Beech et al., 2007). The ACTH baseline concentration and response to TRH did not correlate with the DST results (Beech et al., 2007). Furthermore, DST results were abnormal only in clinically abnormal horses or in horses that were clinically normal but afflicted with pars intermedia hyperplasia as determined by post-mortem examination histopathologic assessment.

A study looking at the response of pituitary melanotropes to TRH both in situ and in explants from the pars intermedia from normal horses and horses with PPID concluded that TRH administration is unlikely to be useful in the diagnosis of PPID (McFarlane et al., 2006). All horses in that study had an increase in cortisol after TRH administration; however, unlike the first study reported by Beech and others (2007), normal horses had an increase in cortisol levels. Of the 16 normal horses, ten had a cortisol increase > 30% and of the ten, 7 had an increase > 50%, which would have meant that they would have been falsely diagnosed with PPID (McFarlane et al., 2006; Messer et al., 2008).

Thyrotropin-releasing hormone directly stimulates the par intermedia in normal and PPID-affected horses to release POMC derived peptides. A combined dexamethasone (DEX) suppression/thyrotropin-releasing hormone (TRH) test (DEX/TRH test) has been developed to evaluate horses for presence of a pars intermedia

pituitary adenoma (PIPA). A DEX/TRH test on 42 horses followed by post-mortem examination found that 17 of the 42 horses (40%) had PPID (Frank et al., 2006). The DEX/TRH test had a specificity of 88% and a sensitivity of 76% and was more sensitive than either of its components, but it was not specific as the results of the TRH component alone (92%) (Frank et. al, 2006).

Although ACTH response to TRH stimulation is affected by season, seasonal effects did not change the classification of individual horses as clinically normal or as having PPID; the TRH test is more cumbersome than use of basal samples (Beech et al., 2007).

Alpha-Melanocyte Stimulating Hormone:

It has been suggested that measuring α-MSH concentration may be superior to measuring plasma ACTH concentration to detect PPID because the former is primarily a product of the pars intermedia and ACTH is secreted primarily from the pars distalis (Orth et al., 1982; McFarlane et al., 2006). Alpha-melanocyte stimulating hormone (α-MSH) was released in response to TRH in healthy normal horses and those with PPID from the equine pars intermedia explants in vitro, but ACTH is not released (MacFarlane et al., 2006). Another study investigated effects of sample handling, storage, and collection time and season on plasma α-MSH concentration in healthy equids (McFarlane et al., 2004). They concluded that seasonal variation in plasma α-MSH concentrations must be considered when evaluating PPID (McFarlane et al., 2004). More recently, a study researching the evaluation of plasma ACTH, α-MSH, and insulin concentrations during various photoperiods in clinically normal horses and ponies and those with PPID proved that the plasma α-MSH and ACTH concentrations increased as daylight decreased

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from summer solstice (maximum daylight hours) to 12 hours of daylight (Beech et al., 2009).

Using alpha-melanocyte stimulating hormone (α-MSH) as a marker of a pars intermedia response and ACTH as a marker of a pars distalis response, an increase in plasma concentration of α-MSH and a significant increase in ACTH after TRH treatment was seen in normal horses (McFarlane et al., 2004, 2006). Both baseline and post-TRH and α-MSH concentrations were significantly greater in PPID horses compared with normal horse (McFarlane et al., 2006).

Domperidone-Challenge Test:

Domperidone is an orally administered dopamine (D2) receptor antagonist, a synthetic benzimidazole, and is commonly used for the management of endophyte-infected fescue agalactia in pregnant mares (Messer and Johnson, 2007). In the pars intermedia, domperidone releases melanotrophs to express POMC-derived peptides. In PPID-affected horses, treatment with domperidone caused a significant elevation of the plasma ACTH concentration (Sojka et al., 2006; Miller et al., 2008), although no effect was seen in normal horses. The result is due to the hypertrophied melanotropes in the pars intermedia of PPID-affected horses constantly being under some degree of dopaminergic inhibition, which leads to an elevation in the plasma ACTH concentration (Sojka et al., 2006; Miller et al., 2008). In most of the horses older than 20 years a large increase in pars intermedia area and an increase in ACTH concentration after domperidone which exceeded the laboratory reference range was seen (Miller et al., 2008). In addition, most old horses presented pituitary adenomatous hyperplasia,

microadenomas, or adenoma (grade 3, 4, or 5), which is considered the gold standard for diagnosing PPID (McFarlane et al., 2005).

Further work is needed to evaluate any seasonal effect on ACTH response to domperidone, to determine the effect of repeat-testing, and to compare the ACTH response to domperidone-treated horses with that in vehicle-treated control horses (Miller et al., 2008). Domperidone is commercially available for use in horses and can be administered orally in paste formation with completion of blood collection for the ACTH assay in 8 hours (Miller et al., 2008). In addition, the test should be initiated at a standard time to avoid diurnal variation in plasma ACTH concentration; plasma should be promptly harvested and frozen for accurate results (Miller et al., 2008).

Endogenous Adrenocorticotropic Hormone Test:

The endogenous ACTH test is nearly as sensitive as the DST, without the risk of causing laminitis (Donaldson et al., 2002; Perkins et al., 2002); however, both tests can be effected by seasonal variation, specifically seen in the fall months of August to October (Place et al., 2010). Plasma ACTH is under an ultradian rhythm and levels can change more than 50% during a peak or a low, horses that have a clinical suspicion of PPID and test normal values would benefit from a repeat sample (Cudd et al., 1995; Perkins et al., 2002). Additionally, plasma ACTH level can be a useful screening test for PPID for the ambulatory equine practitioner due to practicality, although the sample needs to be handled appropriately (Couetil et al., 1996) and caution must be used when interpreting a high concentration of ACTH in autumn samples. Laboratory results of plasma ACTH concentrations being higher than 27 pg/ml in ponies and 50 pg/ml in horses strongly supports a clinical diagnosis of PPID (Couetil et al., 1996). Measurement

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of basal levels of ACTH has been validated for horses by radioimmunoassay (Couetil et al., 1996) and chemiluminescent assay (Perkins et al., 2002).

Factors Affecting Test Results for PPID

Seasonal Variation in Plasma Concentrations of Adrenocorticotropic Hormone:

The plasma concentrations of ACTH vary in healthy horses at different times of the year (Donaldson et al., 2005; Beech et al., 2009; Frank et al., 2010; Lee et al., 2010; Place, et al., 2010; Copas and Durham, 2011; McFarlane et al., 2011). Seasonal variations can affect not only the use of baseline ACTH as a diagnostic test for PPID, but also its usefulness for monitoring horses with PPID (Beech et. al., 2007). The reasons plasma ACTH and α-MSH concentrations are highest when length of day is waning from its longest period and then decrease and remain low when the photoperiod is short are unknown. Additionally, whether the duration or amplitude of the melatonin signal during darkness is a factor is also unknown. During the fall season, particularly September, there is an increase in ACTH, α-MSH, and plasma cortisol levels in normal (Donaldson et al., 2005; Copas and Durham, 2011) and PPID-affected horses (Frank et al., 2010; Copas and Durham, 2011; McFarlane et al., 2011). This physiological change adversely affects the results of diagnostic tests for PPID that use measurement of plasma ACTH and plasma cortisol such as DST, basal plasma ACTH, TRH stimulation test, and DST/TRH test (Donaldson et al., 2005). For instance, when measuring horses tested in September using DST, the number of false-positive tests for PPID is greater than other times of the year. Similarly, when measuring basal levels of ACTH in September, the number of false-positive tests for PPID is great than other times of the year (Donaldson et al., 2005); however, evaluation of resting (baseline) endogenous plasma ACTH concentration has

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been more widely utilized because of its convenience and 84% to100% sensitivity in

diagnosing PPID in the horse (van der Kolk et al., 1995; Couetil et al., 1996; Donaldson

et al., 2002; Perkins et al., 2002). Plasma ACTH can be used for the diagnosis and

monitoring of PPID throughout the year with the use of appropriate reference intervals

(Copas and Durham, 2011).

Other Factors that can produce False-Positive Results for PPID:

Even though high plasma ACTH concentrations appear to be a specific indicator

of PPID, other factors may contribute, such as stress. False-positive results for PPID can

be due to normal horses being stressed; such as immediately after transport, during

anesthesia (Taylor 1989; Luna et al., 1996), acute strenuous exercise (Alexander et al.,

1991; McCarthy et al., 1991; Nagata et al., 1999), colic (Hodson et al., 1986), illness

(Aron and Tyrell, 1994), and chronic pain, possibly due to chronic laminitis.

Treatment for Equine Cushing's Disease:

Treatment of equids with PPID initially involves greater attention to general

health care along with a variety of management changes to improve the condition of older

horses (Garcia and Beech, 1986; Hillyer et al., 1992; van der Kolk, 1998; Johnson and

Scherding, 2000). In horses suffering from PPID, it seems important to reduce the insulin

resistance, thereby potentially decreasing the risk of laminitis as being a major

complication of PPID (Klinkhamer et al., 2011). Furthermore, management of PPID-

affected horses is a daily commitment (McCue, 2002) and can be treated

pharmacologically by administering oral medication, such as Pergolide (a dopaminergic

agonist) (Orth et al., 1982; Donaldson et al., 2002; Perkins et al., 2002; Schott, 2002),

which has been shown to produce significant reductions in concentrations of POMC and

ACTH in horses affected with PPID and is the current treatment of choice (Orth et al., 1982; Donaldson et al., 2002; Perkins et al., 2002; Schott, 2002).

Systemic supplementation of dopamine or a dopamine agonist, such as pergolide, to a horse with PPID, resulted in a decrease in plasma concentration of POMC peptides and an improvement in clinical signs and biochemical abnormalities associated with the disease (Orth et al., 1982; Schott et al., 2001; Donaldson et al., 2002; Perkins et al., 2002). Since ACTH appears to decrease significantly in horses with PPID treated with pergolide and actually return to normal in a significant number of affected horses, measurement of ACTH levels provides a better way of monitoring effects of pergolide treatment than the DST (Couetil et al., 1996; Donaldson et al., 2002; Perkins et al., 2002).

The recommended dose of pergolide ranges from 1.7 to 5.5μg/kg, varying based on the severity of the disease in the individual. If clinical improvement is not seen within 6 to 8 weeks of pergolide therapy at the above dose, then it can be increased slowly to 3 to 5 mg/day. It is important to increase the pergolide dose slowly, using increases such as 0.006 to 0.01 mg/kg of bwt/day or 0.5 mg/day, otherwise side-effects such as anorexia and colic may occur (Beech, 2009).

Differentiating Equine Metabolic Syndrome from equine Cushing's disease:

Laminitis and regional adiposity (Figure 2.2 and 2.4) are clinical signs shared for both EMS and PPID-affected horses (Schott, 2002), so both endocrine disorders should be considered when these problems are detected. Differentially, the EMS phenotype is typically first recognized in younger horses, whereas PPID is more common in older horses. In PPID-affected horses, a rise in ACTH is detected and positive diagnostic test results for PPID can deduce PPID from EMS (*See: Equine Cushing's Disease:*

Diagnoses). Upon necropsy, it was discovered that the pituitary gland of horses affected by PPID was enlarged, unlike those seen by horses with EMS (Johnson, 2002). Furthermore, some equids with EMS subsequently develop PPID, so both conditions can occur concurrently, so it is recommended that equids with EMS be closely monitored for clinical signs of PPID and undergo regular testing. In addition, clinical signs suggestive of PPID, but not EMS, include delayed or failed shedding of the winter hair coat (hirsutism), excessive sweating, polyuria/polydipsia, and skeletal muscle atrophy (Schott, 2002). Finally, if PPID is causing and/or exacerbating IR, treatment should improve insulin sensitivity. Pergolide is recommended for the treatment of PPID in equids (Schott, 2002).

Effects of Endocrine Disorders on Reproduction in Mares

Older mares are an important pool of genetic material for breeders and could require alternative reproduction program procedures. Significant effort can be required to obtain embryos from the older mares, and could require corresponding money to obtain offspring in an oocyte transfer program. Human (Pulido and Salazar, 1999) and horse (Cubitt, 2007) studies have shown that insulin sensitivity is lower in the luteal phase in women, when compared to the follicular phase of the menstrual cycle. In women, long-term insulin resistance is frequently associated with an increase in the duration of the follicular phase of the menstrual cycle and is well documented among women with polycystic ovarian syndrome. Some hypothesize that the small follicles that have just attained LH receptors, in the presence of insulin, display enhanced estradiol production equal to large antral follicles, thereby inhibiting further growth and promotins arrest of the mature follicles (Diamanti-Kandarakis and Bergiele, 2001).

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Many mares fail to become pregnant despite good reproductive management techniques. Depending on the cause of subfertility and breed registry restrictions, most sub-fertile mares can still produce offspring through the use of assisted reproductive techniques (ART). There is substantial variation among specific ART in complexity, cost, and success rates. The cost of the procedure is directly related to the laboratory costs, personnel training, and complexity of the procedure. Diagnoses of the underlying causes of subfertility are crucial for an appropriate referral of a mare for ART (Coutinho da Silva, 2008).

Age-Associated Assisted Reproductive Techniques – Oocyte Transfer:

Oocyte transfer (OT) has been used to obtain pregnancies from valuable mares from which viable embryos cannot be obtained for transfer (Carnevale et al., 2001). The indications for OT include: ovulatory failure, failure of oocyte pickup, and severe pathology of the oviduct, uterus or cervix, including chronic uterine infections, cervical lacerations, or uterine/oviduct scarring (Coutinho da Silva, 2008). Success rates for OT depend on the oocyte collection rate and pregnancy rate per transferred oocyte. Oocyte collection rate from pre-ovulatory follicles range from 65 to75%, and the mean pregnancy rate after transfer of oocytes from older donor mares is approximately 35% (Carnevale et al., 2005). The most popular method of collecting oocytes from ovarian follicles in the mare is transvaginal, ultrasound-guided aspirations (TVA) (Cook et al., 1993). TVA has advantages because it is non-surgical, and repeated aspiration attempts did not affect subsequent fertility of the donor mare (Vanderall et al., 2006). However, oocyte quality is affected by age (Carnevale et al., 2005).

In a study that demonstrates an age-associated decline in oocyte viability, embryo-development rates were higher for oocytes from young (6 to10 years of age) than old (20 to 26 years of age) donors (11/12, 92%; 8/26, 31%, respectively) (Carnevale and Ginther, 1995). Furthermore, oocyte transfers for mares less than 15 years of age resulted in a 50% pregnancy rate (28/56), while transfer of oocytes from donors greater than 23 years of age resulted in a 16% (12/77) pregnancy rate (Carnevale, 2004). One 16-day pregnancy and no 50-day pregnancies were achieved for mares greater than 27 years of age after 12 transfers (Carnevale et al., 2001).

Nutrition Requirements for Aged Horses

The energy requirements for old horses are unknown (NRC, 2007). Energy requirements are a function of energy expenditure and the efficiency with which gross energy present in feeds is converted to net energy can theoretically be factors affected by age (NRC, 2007). A group of researchers compared digestibility of various feedstuffs in healthy adult (5 to 12 years of age) and aged (19 to 28 years of age) horses of similar breeding and from a similar management background (Elzinga et al., 2011). Horses were dewormed, underwent remedial dental work, and were randomly assigned to diets: hay only, hay plus a cereal-based feed, and hay plus a fat and fiber-rich feed. Horses were weighed and assigned a body condition score (BCS; Figure 2.3) approximately every three weeks throughout the study. Feed and fecal samples were analyzed in order to determine neutral detergent fiber (NDF) digestibility plus gross energy, and fat apparent digestibility. The study found that although aged horses weighed less (455 ± 12 kg) than adult horses (500 ± 13 kg; $P = 0.02$), there were no differences in BCS between the two age groups ($P = 0.20$). There were no differences in daily feed or hay intake ($P = 0.73$)

between the two age groups on a g/kg body weight basis, just as there were no differences in fecal ($P = 0.29$) or urine output ($P = 0.29$). There was no effect of age on NDF digestibility nor crude protein, calcium, phosphorus or magnesium retention. This study did not evaluate micronutrient digestibility and retention. All horses used in the study were healthy and nutrient requirements of compromised older horses and those with dental disorders may differ from these reported findings (Elzinga et al., 2011).

An age-related increase or decrease in maintenance energy requirements for aged horses is unknown (NRC, 2007). In aged humans and dogs, the maintenance energy requirements are reported to be 15 to 20% lower when compared to younger populations (Harper, 1998; Bosy-Westphal et al., 2003). Theoretically, a decrease in physical activity is estimated to be the primary factor (Harper, 1998; Roubenoff, 1999) in the decline in maintenance energy expenditure, which is thought to be a function of declining fat-free mass associated with aging (Bosy-Westphal et al., 2003). Although it is unknown whether a horse's energy requirements decrease, an apparent loss of muscle mass in old horses is observed (Ralston et al., 1989). Caloric restriction is proven to extend the lifespan in a variety of species (Heilbronn and Ravussin, 2003); however, it is unknown whether a dietary caloric restriction on aged horses may be beneficial. A lifetime dietary restriction improved glucose tolerance and had favorable effect on disease and survival in dogs (Larson et al., 2003).

The mean resting energy expenditure was increased in 41% of horses suffering from recurrent airway obstruction, which could mean older horses suffering from that disease may actually have increased energy requirements (Mazan et al., 2004).

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The effect of aging and age-related disease on protein requirements in horses is unknown. Healthy humans appear to have a decreased protein requirement with aging (Millward et al., 1997). In healthy elderly humans, a 33% reduction in protein metabolic demand (with no significant impairment in efficiency of protein utilization) suggested a decline in protein requirements with aging (Millward et al., 1997). Controversy surrounds whether increased protein intake is beneficial or detrimental to renal and hepatic function in geriatric animals in other species (Bauer, 1986; Grauer et al., 1994).

Comparing results of digestion trials which used different feeds, horses, and conditions is somewhat risky and controversy surrounds whether the following study's finding reflects the old horse population. Ralston and others (1989) reported lower crude protein apparent digestibility (67% ± 3% vs. 73% ± 3%) in aged horses (26 ± 5 years of age) when compared to younger horses (2.3 ± 0.5 years of age). The digestive profile of the aged horses was very similar to that reported for horses which had had 90% resection of the large colon (Bertone et al., 1989a,b). After more research on feed digestion in old horses was done by the same investigator (Sarah L. Ralston) in the 1990s, the reductions of digestibility in protein were not as apparent. In fact, in trial 1, when old mares (> 19 years of age) were fed a grain concentrate containing 12% crude protein, protein, calcium and phosphorus intakes were higher than mares fed grain containing 10% crude protein (Ralston and Breuer, 1996). Again, in trial 2, where 15 aged Standardbred mares were used in a trial comparing daily injections with equine somatotropin (eST), regardless of the treatment, protein, phosphorus, and calcium apparent digestibility were within normal limits and the reduction in large intestinal absorption observed previously (Ralston et al., 1989) was not apparent, despite similar types of hay and grain (Ralston et al., 2001).

Differences between the two populations of aged horses used in the 1989 and 1990 trials may significantly adjust the apparent digestibility of protein. For example, horses used in both trials in the 1990s were in fair to good body condition with no major dental abnormalities, whereas in the 1989 report, 3 of 7 aged horses were reported to have weight loss and/or poor dentition. In addition, improved gastrointestinal parasite control in the horses used in the later studies which had the benefit of larvicidal anthelminthic administration for most of their lives could have played a role in increasing apparent protein digestibility.

Conclusion

Knowledge of managing older horse care, dealing with endocrine diseases common in older horses, and learning how to properly feed older horses is important for the future owners and managers of a growing old equid demographic in the United States and United Kingdom. Properly diagnosing and managing endocrine disorders in the old horse can provide a higher quality of life for that animal. By increasing insulin sensitivity a decrease in the prevalence of laminitis, a devastating disease, can be achieved. Awareness to owners of proper nutrition for equine suffering from metabolic disorders and endocrine disease will better the quality of life for older equine.

Objectives and Hypothesis

The objectives of the current studies were:

1) To compare dietary intake in a group of aged mares to current recommendations for feeding old horses (NRC, 2007).

2) To investigate the effects of a fatty acid supplement containing α-linoleic acid and chromium yeast in a group of old mares (21.7 ± 3.2 years) on insulin sensitivity and circulating inflammatory markers.

The hypothesis of the current studies:

1) We hypothesize that older horses with physical activity will require more energy and crude protein when compared to the current nutritional guidelines, and those who are idle will meet the nutritional recommendations from the National Research Council (2007).

2) We hypothesize our research will show an increase in insulin sensitivity when horses are supplied with an n-3 fatty acid (ALA) supplement containing chromium yeast.

CHAPTER III: MATERIALS & METHODS

Part I: 2009

Horses:

Twelve healthy, old (mean ± SE, 22.3 ± 0.08 yr of age), non-pregnant mares (mean ± SE, body mass [BM], 492.5 ± 0.6 kg) of American Quarter Horse (n = 10), Arabian (n = 1), and Saddlebred (n = 1) breeds were chosen to be a part of a metabolic supplement study. Mares were enrolled in an alternative reproductive program at the Equine Reproduction Laboratory (ERL) of Colorado State University (CSU) and studied between six and twelve months, depending on their time at the ERL. In July 2009, old mares that appeared to have equine metabolic syndrome and tested negative for equine Cushing's disease were chosen to be supplemented with Equine Platinum Plus Metabolic Support™ (EPPMS™): a fatty-acid formula (Platinum Performance™; Buellton, CA) containing n-3 α-linolenic acid (ALA) with an addition of chromium yeast, magnesium, and other minerals and vitamins. The composition of the EPPMS™ supplement can be seen in Tables 3.1a and 3.1b. Prior to testing, all horses were dewormed, underwent routine vaccinations, and remedial dental work as required. Mares served as their own controls. There were various stalling situations, all had an automatic water tank and a salt block, but some mares were stalled in a 3.6 x 3.6 m stall with access to a 3.6x 12.2 m dry run; some were stalled in a 3.6 x 5.2 m stall with access to a 3.6 x 6.1 m dry run, and others were stalled in a 3.6 x 3.6 m stall and turnedout into large dry lot paddocks, a 120 x 120 m pasture 6 h/d, or hand-walked daily.

An automatic walker (Priefert® 6-Horse Panel Walker; Priefert®, Mount Pleasant, TX) was used five days a week for eight mares that were able to comfortably walk without pain and was set at a moderate pace (between 2 and 3 mph) for 30 to 40 min.

Body weight was measured on a monthly basis using a calibrated electronic livestock scale (Cardinal Scale Manufacturing Company; Webb City, MO). Protocols were approved by the Colorado State Institutional Animal Care and Use Committee. Written consent from the mare owners was obtained.

Treatment Diets:

Mares were fed to meet nutritional requirements of idle maintenance horses and fed 1.43% ± 0.10% dry matter (DM) of BW/d. The diet contained an average of 1.5 kg/d of a commercial concentrate (Nutrena® Safe Choice®), and 7.2 kg/d of mixed alfalfa and orchard brome grass hay that was divided into two feedings; morning and late afternoon. EPPMS™ was provided daily (190 g/d) to the horses on top of the grain concentrate. Three mares were given an average of 0.45 kg/d (as-fed basis) of a commercial pelleted high-fat rice bran supplement (Nutrena® Empower® Boost; Cargill, Inc. ©, Minneapolis, MN) and the nutritional composition can be seen in Table 3.2. Feed and supplements were never refused. Nutritional analysis of grain was provided by the feed company (Nutrena®; Cargill, Inc. ©, Minneapolis, MN) and the hay was analyzed by a commercial laboratory (Equi-analytical Laboratories©; Ithaca, NY) who combined a high tech near infrared and plasma spectroscopy for a complete nutritional profile. Nutritional analysis of diet is shown in Table 3.3.

Classification of exercise groups:

According to the NRC (2007), walking without the incorporation of the trot and canter is considered idle and considerations for exercise and the old horse have yet to been made. We classified the mares into two groups; the first was for mares that were walked at a moderate speed (between 2 and 3 mph) for 30 to 40 min on the automated walker (Priefert® 6-Horse Panel Walker; Priefert®, Mount Pleasant, TX) 5 d/wk. The second group was for mares that were stall-bound and unable to travel at a walk due to health ailments such as laminitis and osteoarthritis. Those mares stall-bound had access to a dry run and were able to be mobile under voluntary conditions.

Mares who participated on the automated walker panel varied between July and May; although, for those who were maintained at the ERL, their exercise regimen was tracked on a daily basis. Monthly averages of exercise were accounted. Mares were classified in the average maintenance category is she was walked using the automated walker 3 to 4 d/wk; and was predicted to require 33.3 kcal/kg a day (NRC, 2007). Mares were classified under minimum average maintenance requirements if she was stall-bound with access to a dry run or turned out to ao dry paddock; and was predicted to require 30.3 kcal/kg a day (NRC, 2007).

Blood Sample Collection:

In July (n = 9) and November (n = 3) of 2009, after 30 days of acclimation to diet and environment at the ERL, a baseline blood sample was collected during fasting (8 to 10 h post-feed). Blood samples were collected through venipuncture from the jugular vein using a 25 G needle and a 20 mL syringe. The whole blood was transferred into two

10 mL collection tubes: one serum separator tube containing polymer gel for the collection of insulin and one sterile blood collection tube containing dried sodium heparin for the collection of glucose. Blood tubes were manually inverted several times and then cooled and maintained immediately at 4° C in a portable cooler. The plasma from the sterile blood collection tube containing heparin was centrifuged at room temperature at 1096 x *g* for 15 min within 20 min of blood collection. Aliquots of plasma (1.5 mL) were stored at -20° C until assayed. Blood in the serum separator tube was allowed at least 30 min to clot at room temperature and then spun for 15 min at 1096 x *g*. Aliquots of serum (1.5 mL) were stored at -20° C until assayed.

Two hours after the fasting blood collection, a post-prandial blood sample was collected for the measurement of inflammatory cytokines. A blood sample was collected through venipuncture from the jugular vein. Whole blood was transferred into a PAXgene® Blood RNA tube (PreAnalytiX GmbH™; Qiagen, Valencia, CA) and a 10 mL serum separator tube, containing polymer gel. The PAXgene® Blood RNA tubes (PreAnalytiX GmbH™; Qiagen, Valencia, CA) were maintained upright; immediately refrigerated at 4° C for 2 h, and then maintained in -20° C until time of RNA isolation to assess gene expression. The serum separator tube was allowed 30 min to clot at room temperature and then spun for 15 min at 1096 x *g*. Aliquots (1.5 mL) of serum were stored at -20° C until assayed.

Treatment commenced after the initial blood samples were collected and each mare weighed. At the end of the summer horses (n = 4) were returned to their facilities of origin and continued treatment at the direction of management. Mares that departed were

assumed to be maintained in a similar daily routine experienced at the ERL: two feedings per day, a stall with a run, and no work.

In October and November three American Quarter horse mares joined the project under the direction of the veterinarians of the ERL and were housed under the same conditions of the previously sampled mares (n = 9). The mares were given 2 wk of acclimation to the environment at the ERL before blood was collected and parameters measured in the previous mares and methods of sampling were followed exactly. Those three mares began supplementation immediately following the baseline blood collection.

In March 2010, mares that had departed (n = 4) returned to the ERL, and by April 2010, all mares were present at the ERL. Weights were measured monthly and diets were tracked. In May 2010, fasting blood samples were collected on all mares and processed as described above.

Laboratory Analysis:

Insulin Sensitivity:

Plasma was analyzed for glucose by enzymatic assay using the YSI® 2700 SELECT™ Biochemistry Analyzer (YSI® Inc., Life Sciences; Yellow Springs, OH) with YSI® 2365 glucose membranes (YSI® Inc., Life Sciences; Yellow Springs, OH), YSI® 2747 Glucose/L-Lactate Standard (YSI® Inc., Life Sciences; Yellow Springs, OH) and YSI® 2357 Buffer Concentrate Kit (YSI® Inc., Life Sciences; Yellow Springs, OH) in two laboratories. An enzyme for the specific substrate, glucose, was immobilized between two membrane layers: one polycarbonate and one cellulose acetate. The substrate was oxidized as it entered the enzyme layer, producing hydrogen peroxide, which then passed though the cellulose acetate layer to a platinum electrode where the

hydrogen peroxide was oxidized. The current that resulted was proportional to the concentration of glucose in the sample.

Insulin was analyzed using Insulin Coat-A-Count® Radioimmunoassay Kit (Siemens™; Dublin 12, Ireland) in two laboratories. Serum is combined with iodinated insulin in insulin antibody coated tubes. The iodinated insulin competed for a fixed time with insulin in the sample for sites on the insulin specific antibodies. The antibody was then immobilized. The insulin in the sample displaced some of the tagged insulin, and the free tagged insulin was measured with isotope detectors in a gamma counter.

Basal proxies (Table 3.4) were calculated from baseline blood samples as well. Beta-cell responsiveness (MIRG) was measured as ([800-0.30(insulin-50)2]/ (glucose-30)). The reciprocal inverse square of basal insulin (RISQI) is an indication of insulin sensitivity and was measured as insulin$^{-0.5}$ (Treiber et al., 2005)

Classification used to define normal or insulin-resistant groups:

Mares were classified into either a normal group (non-IR), or a metabolic group (IR) based on their baseline insulin sensitivity (SI) values. Horses with RISQI basal proxies less than 0.29 (Table 3.4) were classified into the IR group, based on a previous study's quantification of quintiles (Treiber et al., 2005).

Cytokine Expression:

Analysis of mRNA expression of cytokines: Blood samples will be analyzed for levels of mRNA expression Cox-2, TNF-α, IL-1, IL-6, IL-10, and INF-γ. Peripheral blood (2.5 ml) was collected into PAXgene® Blood RNA tubes (Ref #762165; PreAnalytiX GmbH™; Qiagen, Valencia, CA), which were maintained upright and immediately refrigerated at 4° C for two hours; then maintained in -20° C until time of

RNA isolation to assess gene expression. RNA was isolated following PAXgene® Blood

RNA kit protocol (Ref 762164; PreAnalytiX GmbH™; Qiagen, Valencia, CA). cDNA

was created using SuperScript® III First-Strand Synthesis SuperMix (Ref #18080;

Invitrogen™; Life Technologies Corporation™, Grand Island, NY).   Gene expression

was run using the 7500 Fast Real-Time PCR System™ (Applied BioSystems®; Life

Technologies Corporation™, Grand Island, NY). Power SYBR® Green PCR Master

Mix (Ref #4367659; Applied BioSystems®; Life Technologies Corporation™, Grand

Island, NY) was the assay used.   Primers were obtained from Integrated DNA

Technologies® (Coralville, IA).

Calculation of cytokine fold-change:

Cytokine expression results are reported as fold change, according to the 2-ΔΔCT

method (Livak and Schmittgen, 2001).

Cortisol:

Chemiluminescent immunoassays for the diagnosis of endocrine diseases in

animals have been validated in dogs, cats, and horses (Reimers et al., 1996; Singh et al.,

1997). This method involves modular systems consisting of a mechanical shaker, sample

washer, and chemiluminescence photometer. The kits utilized antibody-coated beads for

solid-phase immunoassay and an acridinum ester as the substrate. The hydrolysis of this

substrate produces a flash of light. For analysis, a 25-μl aliquot of each sample was added

to a plain 12- x 75-mm glass tube. Then, each tube received one antibody-coated bead.

The samples were shaken at room temperature for 60 min and then mixed with 1 ml of a

wash solution. The samples were washed 4-5 times with water. The washed tubes were

placed in the photometer, and the intensity of light was measured. The bound complex

was directly proportional to the photon output but inversely proportional to the hormone concentrations. A calibration curve was generated by using the standards samples provided with the kit (Singh et al., 1997). Samples for the 2009 and 2010 study were run by IDEXX Laboratories (Westbrook, ME).

Statistical Analysis:

Treatments were evaluated by an analysis of variance (ANOVA) in a mixed model, with horses nested with metabolic (IR) as a random variable and treatment and exercise as main factors. Significant results were analyzed by the least square means analysis.

Results

Insulin sensitivity, weights, and exercise:

Overall, 7 out of 12 mares were considered normal (non-IR) at baseline and after treatment. Five out of 12 mares were considered metabolic (IR), having basal proxies below 0.29 RISQI and 4 of those 5 mares improved to normal (non-IR) status after treatment. A treatment effect ($P < 0.04$) occurred between baseline samples taken in July 2009 and measured again in May 2010 for plasma glucose. Plasma glucose levels were lower (92.1 ± 2.24 mg/dL) in May 2010 compared to the baseline samples (97.9 ± 1.99 mg/dL). No effect was found for plasma glucose levels by exercise ($P = 0.49$) or exercise by treatment ($P = 0.09$). A treatment effect ($P < 0.001$) occurred between sample periods for serum insulin and was lower (6.6 ± 1.3 µU/mL) in May 2010 compared to the baseline sample (13.3 ± 1.2 µU/mL). No effect on serum insulin levels by exercise ($P = 0.10$) or exercise by treatment ($P = 0.59$) was found. A treatment effect ($P < 0.013$) was found in insulin sensitivity (RISQI) as it increased with treatment (0.51 ± 0.04) compared

to baseline (0.28 ± 0.04). No effect on insulin sensitivity was seen by exercise ($P = 0.47$)

or exercise by treatment ($P = 0.55$). A treatment effect ($P < 0.001$) was found in an

increase of pancreatic β-cell responsiveness (MIRG) by May 2010 (3.56 ± 0.42)

compared to baseline (5.83 ± 0.38). No effect on MIRG was seen by exercise ($P = 0.25$)

or exercise by treatment ($P = 0.42$). Mares tended to increase in weight ($P = 0.07$) from

baseline (454.3 ± 9.88 kg) to May 2010 (480.0 ± 11.12 kg). No effect on weight by

exercise ($P = 0.48$) or exercise by treatment ($P = 0.86$) was found. No effect on cortisol

by treatment ($P = 0.44$), exercise ($P = 0.95$), or exercise by treatment ($P = 0.59$) was

found.

Inflammatory Markers:

Cytokine Cox-2 increased with treatment ($P < 0.05$) from baseline (1.1 ± 0.97) to

sampling in 2010 (5.1 ± 1.2). No effect on cytokine Cox-2 fold change by exercise ($P =$

0.14) or exercise by treatment ($P = 0.11$) was found. Cytokine IL-1 fold change increased

with treatment ($P < 0.05$) from baseline (1.96 ± 4.47) to post-treatment (23.7 ± 6.2). An

exercise trend ($P = 0.06$) and exercise by treatment effect ($P < 0.05$) was found for

cytokine IL-1. Those with voluntary exercise at baseline had lower IL-1 fold change

levels (1.7 ± 6.3; $P = 0.68$) compared to those under voluntary exercise after treatment

(43.9 ± 10.9; $P < 0.019$).

A treatment effect trend on cytokine IL-10 ($P = 0.06$) occurred and IL-10 fold

change levels increased with treatment ($972{,}921 \pm 259{,}528$) compared to baseline

($137{,}079 \pm 186{,}881$). An exercise trend effect on cytokine IL-10 ($P < 0.06$) occurred and

was lower for those who exercised on the automated panel walker ($142{,}181 \pm 180{,}083$)

compared to those who were under voluntary exercise or stall-bound with a dry run

(967,818 ± 264,290). An exercise by treatment effect ($P < 0.05$) was found on cytokine IL-10. At baseline, those that were under voluntary exercise had lower levels of cytokine IL-10 (110,394 ± 264,290; $P = 0.7$) compared to those under voluntary exercise after treatment (1,825,242 ± 457,764; $P < 0.02$). Conversely, those who were exercised on the automated panel walker at baseline had higher levels of cytokine IL-10 (163,763 ± 264,290; $P = 0.56$) compared to those exercised on the automated panel walker after treatment (120,600 ± 224,685; $P = 0.65$).

Cytokine TNF-α showed a treatment trend ($P = 0.07$) and was higher after treatment (4.82 ± 1.01; $P < 0.009$) compared to baseline (1.54 ± 0.84; $P = 0.14$). No effect on cytokine TNF-α by exercise ($P = 0.23$) or exercise by treatment ($P = 0.18$) was found.

No effect on cytokine IL-6 by treatment ($P = 0.71$), exercise ($P = 0.71$) or exercise by treatment ($P = 0.77$) was found. No effect for cytokine by treatment ($P = 0.26$), exercise ($P = 0.84$) or treatment by exercise ($P = 0.90$) was found.

Discussion

In this study, old mares who were initially metabolically IR or normal, had achieved lower plasma glucose, serum insulin, and consequently, an increase in both insulin sensitivity (RISQI) and pancreatic β-cell responsiveness (MIRG) after supplementation with an n-3 α-linolenic acid fatty-acid formula (Platinum Performance™; Buellton, CA) containing chromium yeast, magnesium, and other minerals and vitamins for at least 6 mo.

Overall, 7 out of 12 mares were considered normal (non-IR) at baseline and after treatment. Five out of 12 mares were considered metabolic (IR), having basal proxies

below 0.29 RISQI and 4 of those 5 mares improved to normal (non-IR) status after treatment. Specifically, ten of the twelve mares actually increased insulin sensitivity and moved up into higher quintiles (Treiber et al., 2005). Five of those ten mares increased insulin sensitivity by three quintiles and four of those five mares increased from quintile #2 to #5, where quintile #5 is considered the most insulin sensitive. Also, two mares were able to increase insulin sensitivity by two quintiles, while three mares increased insulin sensitivity by one quintile.

Mares in this study were given the EPPMS supplement that contained 17.6 g/d (36.5 mg/kg BW) of n-3 α-linolenic acid and 7.9 g/d (16.4 mg/kg BW) of n-6 α-linoleic acid. In another study, by Rexford and others (2012), horses were supplemented with n-3 fatty acids, such as, 38 g of n-3 long chain highly unsaturated fatty acids (LCHUFA) per day, of which 38 g consisted of n-3 α-linolenic acid provided by a supplement containing flaxseed meal. The second group was supplemented with 38 g of n-3 LCHUFA provided by a supplement containing algae and fish oil, of which consisted of 2 g of alpha linolenic acid, 7.6 g of eicopentaenoic acid (EPA), 26.6 g of docosahexaenoic acid (DHA), and 1.7 g of docosapentaenoic acid (DPA). Those supplemented with n-3 α-linolenic acid showed an increase in insulin sensitivity using the reciprocal inverse square of basal insulin, RISQI, an indication of insulin sensitivity measured as insulin-0.5, from 30 d (0.23 ± 0.012) to 60 d (0.25 ± 0.012) (Rexford, 2010). In addition, when pancreatic β-cell function for the mares in this study is compared from baseline to post-supplementation, four of the twelve mares maintained their rank in quintile #5, which reflects an increase in β-cell function; while, three mares dropped by three quintiles. In addition, four mares dropped by two quintiles according to MIRG and one mare dropped by one quintile,

which reflects a decrease in pancreatic β-cell function. Some of the variation in RISQI and MIRG parameters could be attributed to differences in laboratories used for analysis of plasma glucose and serum insulin; however, the same techniques were used in both.

Mares in this study received a total diet of 4.87 mg of chromium daily or 0.01 mg/kg BW. Other research studies confirm our findings with increased insulin sensitivity by supplementing chromium and have shown that chromium significantly altered glucose and insulin concentrations in horses (Pagan et al., 1995; Ralston et al., 1997; Ott and Kivipelto, 1999). Aged mares (> 20 yr) that were supplemented with 0.02 mg/kg BW chromium-L-methionine for 4 wk had a lower peak insulin response to a concentrate meal after four weeks of supplementation (Ralston et al., 1997). In another study, Quarter horse and Thoroughbred yearlings that received chromium at the highest dosage (175.35 mg/ kg BW) had faster glucose clearance rates than animals in the control group. Pagan and others (1995) used exercising Thoroughbreds and results showed a positive effect of supplementing Cr-enriched yeast (5mg Cr per horse over a 14-day period) to mature horses had some beneficial effect on glucose metabolism. Significantly lower levels of insulin, cortisol, and glucose during exercise were measured. Lower plasma glucose concentrations were also detected one hour post-feeding in the same horses. Yet, in other studies, chromium supplementation showed no beneficial effects. Obese, laminitic adult 500-kg horses supplemented with 0.01 mg/kg BW of chromium yeast (5 mg/day) for 16 wk and no effects were found on blood variables or insulin sensitivity. Also, in a study using healthy, trained Standardbreds elicited no beneficial effects on glucose and insulin metabolism during both rest and exercise (Vervuert et al., 2005). Unfortunately, the mechanism for chromium from the cellular-level is unknown and its effects in old horses

would require further investigation because we did not measure chromium levels in the blood.

Mares in this study received an average of 11.3 g/d of magnesium or 24 mg/kg BW in the total diet at baseline. Subsequently, the mares received an average of 15.9 g/d of magnesium or 32.1 mg/kg BW in the total diet post-supplementation. According to the NRC (2007), a 500-kg horse at maintenance requires 7.5 g of dietary Mg/d or 15 mg/kg BW. The amount of magnesium provided in this study at both baseline and post-supplementation exceeded maintenance requirements. In the study by Chameroy and others (2011), obese, laminitic adult (8 to 20 yr of age) 500-kg horses were supplemented with 17.6 mg/kg BW of magnesium (8.8 g/d) and no effects of insulin sensitivity were found. These differences suggest that a larger dose of magnesium may be needed in order to find an increase in insulin sensitivity in horses.

In this study, old mares increased (from 2009 to 2010) the cytokine expression of Cox-2, IL-1, and IL-10 and showed a trend for an increase in pro-inflammatory cytokine TNF-α, after supplementation with a fatty-acid formula containing PUFA. This result conflicts with other studies where n-3 LCHUFA from plant and animal sources reduced cytokine production *in vitro* (De Caterina et al., 1994) and *in vivo* (Grimm et al., 1994; McCann et al., 2000). In contrast with other findings, Holbrook and others (2012) showed peripheral blood cells of obese, hyperinsulinemic horses had decreased endogenous pro-inflammatory cytokine gene expression (IL-1 and IL-6), which suggests that unlike in people, cytokine-mediated inflammation does not increase in direct response to obesity or insulin resistance in horses. Another study showed that age-related obesity potentially plays a role in the dysregulation of inflammatory cytokine IFNɣ and

TNF-α production by the immune system with increased age or "inflamm-aging" in the horse (Adams et al 2008). In a study by Vick and others (2007), increased levels of IL-1 and TNF-α were associated with decreased insulin sensitivity and TNF-α expression was associated with increased BCS and body fat composition, in mares 20 yr of age. Plasma TNF- α concentration was also increased in ponies on spring pasture that developed laminitis (Treiber et al. 2006c). Pro-inflammatory cytokine TNF- α may play a role in muscle damage (Kimura et al., 2001), sarcopenia or loss of muscle mass associated with aging (Petersen and Pedersen, 2005), insulin resistance, obesity, and diabetes (Saghizadeh et al., 1996; Ferrier et al., 2004; Keller et al., 2004; Petersen and Pedersen, 2005).

Adipocytes are capable of secreting endocrine signals (adipokines) that cause insulin resistance (Lyon et al., 2003). White adipose tissue is composed of adipocytes, fibroblasts, endothelial cells and macrophages (Weisberg et al., 2003; Berg and Scherer, 2005). When adipose tissues reach their capacity for fat storage, they can become stressed and release cytokines, causing a pro-inflammatory state. Omental adipocytes contain an enzyme (11-Beta, hydroxysteroid DH; 11-Beta, HSD) capable of converting inactive cortisone to active cortisol; which can play a role in the pathogenesis of insulin resistance (Masuzaki & Paterson, 2001). However, in this study, no supplementation effect was seen for cortisol. Data reported in another study suggests that the nuchal ligament fat depot has unique biological behavior in the horse and is more likely to adopt an inflammatory phenotype than other depots examined, such as the tail head and mesenteric adipose tissue (Burns et al., 2010). Expression of inflammatory cytokines IL-1 beta, IL-6, and TNF- α were higher in nuchal ligament adipose tissue than in other depots

and both insulin resistant and insulin sensitive mares were similar in their expression of these cytokines (Burns et al., 2010). These findings suggest that visceral fat may not contribute to the pathogenesis of obesity-related disorders in the horse as in other species (de Koning et al., 2007; Lee et al., 2007). An increased inflammatory status in older horses is also thought to be a possible cause of PPID (McFarlane & Holdbrook, 2008; Bertone, 2006), although the mares used in this study were tested for PPID and the results were negative. High levels of TNFα, IL-1 and IL-6 have may be a result of the trend of an increase in weight observed in these mares.

In other species, research shows that prostaglandins and IL-6 up-regulate the production and secretion of IL-10 which in turn, inhibits pro-inflammatory cytokines: TNF-α, IL-1β, and IFN- ɣ production (Moore et al., 1993; Goldman et al., 1997; Petersen and Pedersen, 2005; Calder, 2006). In addition, regulatory cytokine IL-10 has shown to decrease the mRNA stability for several other cytokines, including IL-6, IL-8, and IL-10 itself (Wang et al., 1994a,b; Brown et al., 1996; Takeshita et al., 1996). Since mares that were under voluntary exercise conditions showed an increase in the expression of regulatory cytokine IL-10, the results may show that those mares were under voluntary exercise had lower inflammation than those walked on the automated walker.

No supplementation effect was seen for weight, plasma glucose, serum insulin, insulin sensitivity (RISQI) or pancreatic β-cell responsiveness (MIRG) by exercise and treatment. A trend for treatment to have increased weight from baseline (454.3 ± 9.88 kg) to May 2010 (480.0 ± 11.12 kg) was found. Dietary caloric intake increased from an average of 14981.7 kcal/kg of BW at baseline to an average of 16922 kcal/kg of BW post-supplementation. Simply put, the mare's daily dietary intake averaged 1.35% of BW

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(DM) at baseline and increased to an average of 1.47% of BW (DM) post-supplementation, so it is conceivable that the weight increase is not due to the fatty-acid supplementation, but possibly due to the increase in caloric energy. According to the NRC (2007), each unit of condition score increase requires about 16 to 20 kg of weight gain in a mature horse. In this study, BCS was not assessed at baseline sampling so results are officially inconclusive, however, if we were to compare the average weight gain of mares in this study post-supplementation to the NRC (2007) predictions, we can predict that they gained approximately one body condition score. Studies show a decrease in insulin sensitivity when percent fat and BCS increase (Vick et al., 2007, 2008). When these same mares were utilized in another evaluation (Part 3) and BCS was measured during the spring and summer of 2010, these specific mares averaged a BCS of 5.3 when post-supplementation sampling was taken. According to Hoffman and others (2003), very fat horses (≥ 7 BCS) may have disturbed metabolic and endocrine regulation. Additionally, a condition score of 5 is considered moderate and a BCS of at least 5 as shown optimized reproductive efficiency (NRC, 2007. Therefore, the mares in this study may have become more ideal in BCS with the increase in caloric intake and gain in bodyweight.

Although we found that by using an n-3 α-linolenic acid fatty-acid formula (Platinum Performance™; Buellton, CA) containing chromium yeast, magnesium, and other minerals and vitamins lowered basal glucose and insulin levels, and thus, increased insulin sensitivity, we did not measure BCS, which would have been an important element to the study because of its implications with age, obesity, and insulin sensitivity (Vick et al., 2007, 2008; Adams et al., 2009). In addition, we assumed multiple factors

were consistent for those mares who returned home after the breeding season (n = 4). We assumed those mares were under no working conditions of exercise, that they were given the EPPMS supplement twice daily without missing a feeding, and that they were on a similar diet to what they experienced at the ERL. Ideally, this study would have been done under controlled conditions. More frequent blood sampling and condition scoring may also have helped to confirm the results found in this study.

Part II: Short-term effects of a fatty acid supplement

Horses:

Eleven old (mean ± SE, 21.1 ± 0.68 yr of age), non-pregnant, client mares (mean ± SE, body mass [BM], 497.5 ± 2.1 kg) of stock horse breeds were enrolled in an alternative reproductive program at the Equine Reproduction Laboratory (ERL) of Colorado State University (CSU). Mares were selected on the basis of negative results for equine Cushing's disease and to be healthy, aged (> 16 yr), and non-pregnant. Mares that appeared metabolic (n = 7) were chosen to be supplemented with Equine Platinum Plus Metabolic Support™ (EPPMS™): a fatty-acid formula (Platinum Performance™; Buellton, CA) containing n-3 α-linolenic acid (ALA) with an addition of chromium yeast, magnesium, and other minerals and vitamins. The composition of the EPPMS™ supplement can be seen in Tables 3.1a.and 3.1b. Prior to testing, all horses were dewormed and underwent remedial dental work as required. There were various stalling situations, but all had access to an automatic water tank and a salt block; however, some were stalled in a 3.6 x 3.6 m stall with access to a 3.6 x 12.2 m dry lot run; some were stalled in a 3.6 x 5.2 m stall with access to a 3.6 x 6.1 m dry lot run, and others were

stalled in a 3.6 x 3.6 m stall and turned out into large dry lot paddocks, a 120 x 120 m

pasture, or hand-walked daily.

An automatic walker (Priefert® 6-Horse Panel Walker; Priefert®, Mount

Pleasant, TX) was used 5 d/ wk for 11 mares that were able to comfortably walk without

pain and was set at a moderate pace (between 2 and 3 mph) for 30 to 40 min.

Five mares had been maintained at CSU during the year prior to this study. The

other mares (n = 6) were present by April 2010. All mares were adjusted to the

environment for at least one month prior to sampling. Assessment of diet, weight, body

condition score (BCS; Figure 2.3) and neck condition score (NCS; Figure 2.4) began

upon arrival to the ERL and were assessed monthly. Body weight was measured using a

calibrated electronic livestock scale (Cardinal Scale Manufacturing Company; Webb

City, MO). Body condition score (Figure 2.3) was based on a scale of 1 to 9, with 1

being emaciated and 9 representing extreme obesity (Henneke et al., 1983)(See: *Physical

Measurements of Fat Indication and Body Condition)*. Neck condition score (Figure 2.4)

was based on a scale from 0 to 5 with 0 being no palpable crest and 5 meaning crest is so

large it permanently droops to one side (Carter, et al., 2009a) *(See: Regional Adiposity*;

Figure 2.2*)*. Final marks were an average based on scores from two evaluators. Protocols

were approved by the Colorado State Institutional Animal Care and Use Committee.

Written consent from the mare owners was obtained.

Treatment Diets:

Mares were fed to meet nutritional requirements of idle maintenance horses and

fed 1.56% ±.0.25% dry matter (DM) of BW/d. Those supplemented with EPPMS™ (n =

7) served as the treatment group and the other mares (n = 4) served as the control group.

Both group's diet included a mix of alfalfa hay and orchard brome grass hay in addition to a grain concentrate that was divided into two feedings: morning and late afternoon. An average of 7.15 kg/d (as-fed basis) of forage (an average of 3.9 kg/d of orchard brome grass hay and 3.3 kg/d of alfalfa) was fed. Mares (n = 10) were fed a controlled-starch grain concentrate (Nutrena® Safe Choice®; Cargill, Inc.©, Minneapolis, MN) except for one mare who was maintained in the control group (n = 4), and fed an average of 3.63 kg/d (as-fed basis) a low-NSC grain concentrate designed for senior horses (Purina® Equine Senior®; Purina Mills, LLC®, St. Louis, MO). Of those supplemented with EPPMS™ (n = 7), two mares were fed (starting in March, n = 1; starting in April, n = 1) an average of 0.453 kg/d of a commercial pelleted high-fat rice bran supplement (Nutrena® Empower® Boost; Cargill, Inc. ©, Minneapolis, MN) and the nutritional composition can be seen in Table 3.3. Mares supplemented with EPPMS™ were provided 190 g/d of the supplement on top of their grain concentrate. The nutritional composition of the EPPMS™ supplement can be seen in Tables 3.1a and 3.1b. In February 2010, both forages were sampled using a manual probe to core several bales and then analyzed by a commercial laboratory (Equi-analytical Laboratories©; Ithaca, NY) who combined a high tech near infrared and plasma spectroscopy for a complete nutritional profile. The analysis of grain concentrate was provided by commercial feed companies (Nutrena®; Cargill, Inc. ©, Minneapolis, MN and Purina Mills, LLC. ®, St. Louis, MO). Nutritional analysis of diet is shown in Table 3.3.

Classification of exercise groups:

According to the NRC (2007), walking without the incorporation of the trot and canter is considered idle and considerations for exercise and the old horse have yet to

been made. We classified the mares into two groups, the first were walked at a moderate speed (between 2 and 3 mph) for 30 to 40 min on the automated walker (Priefert® 6-Horse Panel Walker; Priefert®, Mount Pleasant, TX) 5 d/wk. The second group was for mares that were stall-bound and unable to travel at a walk due to health ailments such as laminitis and osteoarthritis (n = 5). Those mares stall-bound had access to a dry run and were able to be mobile under voluntary conditions. Mares who participated on the automated walker panel varied between May and July, their exercise regimen was tracked on a daily basis. Monthly averages of exercise were accounted. Mares were classified in the average maintenance category is she was walked using the automated walker 3 to 4 d/wk; and was predicted to require 33.3 kcal/kg a day (NRC, 2007). Mares were classified under minimum average maintenance requirements if she was stall-bound with access to a dry run or turned out to ao dry paddock; and was predicted to require 30.3 kcal/kg a day (NRC, 2007).

Blood Sample Collection:

In May 2010, blood collection for the assessment of glucose, insulin, and endogenous adrenocorticotropic hormone (ACTH) was analyzed for both groups of mares. The mares were tracked and chosen to have their blood drawn when they were in the luteal phase of their reproductive cycle, at 5 to 7 d post-ovulation or 6 to 8 d post-aspiration, and this is the period of time mares are considered to be less insulin sensitive (Cubitt, 2007). Mares were sampled based on an ultrasound examination confirming the mare was in the luteal phase (Cubitt, 2007). Both groups had their baseline sample taken in May, after at least 30 d of acclimation to diet and environment at the ERL, during a period of fasting (10 h post-feed). Blood samples were collected through venipuncture

from the jugular vein, using the exact measures listed in Part I of Materials and Methods, and collected again in July 2010. One sterile blood collection tube (7 mL) containing Potassium EDTA (0.10 mL, 15% 14.3 mg buffer) was used to collect blood for the assessment of endogenous ACTH; which was immediately cooled and maintained at 4°C in a portable cooler and centrifuged at room temperature at 1096 x g for 15 min within 15 min of collection. No post-prandial blood sampling took place, and inflammatory cytokines and cortisol was not assessed (as seen in Part I of Materials and Methods).

Laboratory Analysis:

Classification of Equine Cushing's Disease:

Frozen serum samples were taken to a laboratory at the Colorado State University Veterinary Teaching Hospital to determine the level of endogenous ACTH released from the pars intermedia of the pituitary gland. Samples were analyzed using chemiluminescent technology on the IMMULITE® 1000 Immunoassay System (Siemens™; Dublin 12, Ireland). The wash technique offers automated bead washing of the Test Unit. The Test Unit contains an assay-specific coated bead and serves as the reaction vessel for all sampling processing. Spinning the Test Unit at high speed efficiently expels fluid into the integral sump chamber. The tube design allows for multiple discrete washes within seconds, ensuring excellent separation of unbound material for highly sensitive assays. Consistent and extremely low nonspecific binding is produced (Siemens™; Dublin 12, Ireland). Endogenous ACTH levels between 18-25 pg/ml in May were considered normal; higher than that was considered positive for equine Cushing's disease. Accommodations for seasonal variation in July were

considered; thus, mares whose endogenous ACTH levels tested above 54 pg/ml in the summer were classified as having equine Cushing's disease.

Insulin Sensitivity:

Plasma was analyzed for glucose by enzymatic assay using the YSI® 2700 SELECT™ Biochemistry Analyzer (YSI® Inc., Life Sciences; Yellow Springs, OH) with YSI® 2365 glucose membranes (YSI® Inc., Life Sciences; Yellow Springs, OH), YSI® 2747 Glucose/L-Lactate Standard (YSI® Inc., Life Sciences; Yellow Springs, OH)and YSI® 2357 Buffer Concentrate Kit (YSI® Inc., Life Sciences; Yellow Springs, OH). An enzyme for the specific substrate, glucose, was immobilized between two membrane layers: one polycarbonate and one cellulose acetate. The substrate was oxidized as it entered the enzyme layer, producing hydrogen peroxide, which then passed though the cellulose acetate layer to a platinum electrode where the hydrogen peroxide was oxidized. The current that resulted was proportional to the concentration of glucose in the sample.

Insulin was analyzed using Insulin Coat-A-Count® Radioimmunoassay Kit (Siemens™; Dublin 12, Ireland). Serum is combined with iodinated insulin in insulin antibody coated tubes. The iodinated insulin competed for a fixed time with insulin in the sample for sites on the insulin specific antibodies. The antibody was then immobilized. The insulin in the sample displaced some of the tagged insulin, and the free tagged insulin was measured with isotope detectors in a gamma counter.

Basal proxies (Table 3.4) were calculated from baseline blood samples as well. Beta-cell responsiveness (MIRG) was measured as ([800-0.30(insulin-50)2]/ (glucose-30)). The reciprocal inverse square of basal insulin (RISQI) is an indication of insulin sensitivity and was measured as insulin$^{-0.5}$ (Treiber et al., 2005).

Classification used to define normal or insulin-resistant groups:

Mares were classified into either a normal group (non-IR), or a metabolic group (IR) based on their baseline insulin sensitivity (SI) values. Horses with RISQI basal proxies less than 0.29 (Table 3.4) were classified into the IR group, based on a previous study's quantification of quintiles (Treiber et al., 2005).

Statistical Analysis:

Treatments were evaluated by ANOVA, with horses nested with metabolic (IR) as a random variable and treatment as main factor. Significant results were analyzed by the least square means analysis. ANOVAs were run to compare mares that could walk on the automatic panel walker every day to mares that had voluntary activity or resided in their stall with access to a dry run.

Results

Overall, seven of the eleven mares were supplemented with EPPMS and none of them were considered IR in May. Four of the eleven were not supplemented and of the four only one was considered IR in May. In July one out of the seven supplemented mares was considered IR, where the rest were considered normal. Also, the same mare in the non-supplemented group who was considered IR in May remained IR in the July sampling. Nutritional tracking revealed that dietary energy intake increased ($P < 0.01$) from May (33.3 ± 2.8 kcal/kg BW) to July (39.3 ± 2.8 kcal/kg BW). No effect on dietary energy by treatment ($P = 0.71$) or treatment by sampling ($P = 0.34$) was found. Body condition scores for supplemented mares averaged 5.4 ± 0.48 and 6.1 ± 0.29, in May and July, respectively. Body condition scores for non-supplemented mares averaged $5.8 \pm$

0.32 and 6.4 ± 0.17, in May and July, respectively. Monthly measurements of weight, NCS, and BCS can be seen in Tables 3.5 and 3.6.

Physical parameters analyzed by monthly sampling and treatment:

A monthly effect on weight ($P < 0.006$) was found and weight increased on average with both sample groups combined from May (482.9 ± 14.9 kg; $P = 0.12$) to July (493.1 ± 14.9 kg; $P < 0.006$). Specifically, the supplemented group increased in body weight on average from 495.3 ± 2.1 kg of BW in May to 507.7 ± 3.2 kg of BW in July. Additionally, the non-supplemented group increased in body weight on average from 490.6 ± 2.2 kg of BW in May to 498.6 ± 1.9 kg of BW. However, no effect on weight by treatment ($P = 0.83$) or treatment by monthly sampling (P = 0.44) was found. A monthly effect on BCS ($P < 0.003$) was found and BCS increased from May (5.63 ± 0.21) to July (6.25 ± 0.21). No treatment effect on BCS ($P = 0.47$) or treatment by monthly sampling ($P = 0.68$) was found. No treatment effect on NCS ($P = 0.94$) was found and likewise, no monthly sampling effect ($P = 0.57$) or treatment by monthly sampling effect ($P = 0.95$) was found.

Blood parameters analyzed by monthly sampling and treatment:

No monthly effect on endogenous ACTH ($P = 0.10$), treatment ($P = 0.78$), or treatment by monthly sampling ($P = 0.83$) was found. A monthly trend effect on plasma glucose ($P = 0.09$) was found; however, no treatment effect was seen for plasma glucose ($P = 0.14$) or treatment by monthly sampling ($P = 0.14$). Overall, lower levels of plasma glucose were measured in July (90.9 ± 4.3 mg/dL) compared to May (101.9 ± 3.8 mg/dL). No treatment effect on serum insulin ($P = 0.23$), sampling ($P = 0.23$) or treatment by monthly sampling ($P = 0.85$) was found. No treatment effect on insulin

sensitivity (RISQI) ($P = 0.58$), sampling ($P = 0.14$) or treatment by monthly sampling ($P = 0.94$) was found. No treatment effect on pancreatic β-cell responsiveness ($P = 0.38$), sampling ($P = 0.43$), or treatment by monthly sampling ($P = 0.39$) was found.

Physical parameters analyzed by monthly sampling and exercise:

A monthly effect on BCS ($P < 0.003$) was found and BCS increased from May (5.6 ± 0.22) to July (6.25 ± 0.22). No exercise effect on BCS ($P = 0.95$) or exercise by monthly sampling ($P = 0.28$) was found. A monthly effect on weight ($P < 0.004$) was found and weight increased from May (484 ± 13.91 kg) to July (495.24 ± 13.91 kg). No exercise effect ($P = 0.94$) on weight was found; however, a monthly sampling effect ($P < 0.004$) and an effect of exercise by monthly sampling ($P < 0.05$) had an effect. Those whom were stall-bound or under voluntary exercise activity weighed less in May (487.2 ± 14.4 kg) compared to July (491.9 ± 14.0 kg). Those who were exercised at a moderate walk on the automated panel walker also gained weight from May (481.4 ± 14.0 kg) to July (498.5 ± 14.4 kg).

Blood parameters analyzed by monthly sampling and exercise:

No monthly effect on plasma glucose ($P = 0.31$), exercise ($P = 0.59$) or sampling by exercise ($P = 0.37$) was found. No monthly effect on serum insulin ($P = 0.67$) or monthly sampling by exercise ($P = 0.20$) was found; yet an effect by exercise ($P < 0.05$) on serum insulin was found and was lower for those who were stall-bound or under voluntary exercise activity in May (3.37 ± 1.75 μU/mL) compared to those who were stall-bound or under voluntary exercise activity in July (7.39 ± 1.71 μU/mL). No monthly effect on insulin sensitivity (RISQI) ($P = 0.16$), exercise ($P = 0.15$), or exercise by monthly sampling ($P = 0.76$) was found. A monthly effect on endogenous ACTH ($P <$

0.005) and exercise effect ($P < 0.016$) was found. Overall, those that exercised at a moderate walk on the automated panel walker had higher levels of ACTH (31.5 ± 2.8 pg/ml) compared to those that were left to voluntary exercise or stall-bound with access to a dry run (19.04 ± 2.8 pg/ml). Higher levels of ACTH were found in July (30.8 ± 2.6) compared to May (19.7 ± 2.4 pg/ml). No monthly exercise effect on endogenous ACTH ($P = 0.20$) was found.

Discussion

For purposes of knowing when the mares were more likely to be IR, and to be consistent in our evaluation of insulin sensitivity, we consistently sampled blood in May and July when the mares were in the diestrus or luteal phase which has been shown to be the more IR time of the estrous cycle in the mare (Cubitt, 2007), which was confirmed via ultrasonography. The eleven mares selected (out of a possible 32) for this study was based on the fact that they were eligible to continue with reproductive procedures at the ERL in both May 2010 and July 2010, and thus, we were able to track their reproductive cycle and to know exactly when they were in the luteal phase of the estrous cycle. Supplemented mares were given 17.57 g/d of n-3 α-linolenic fatty acid which averaged to be 34.6 mg/kg of BW. In addition, supplemented mares were given 16.8 g/d of magnesium (averaged 33.2 mg/kg of BW) and 4.87 mg/d of chromium yeast (0.01 mg/kg of BW). Non-supplemented mares received on average 15.1 g/d of magnesium (31.6 mg/kg of BW) through hay and grain. Omega-3 α-linolenic fatty acid and n-6 α-linoleic fatty acid were not measured in the hay or grain, so if the horses in this study received any additional amount of fatty acids in the diet is unknown.

Overall, none of the mares supplemented with EPPMS, an n-3 α-linolenic fatty acid supplement, were considered IR in May and one supplemented mare decreased insulin sensitivity enough to be considered IR in the July sampling. Of the non-supplemented mares, only one was considered IR in May, and that same mare remained IR when sampled in July. For this study, the choice of mares may not have been the most optimal to study a change in insulin sensitivity using the EPPMS fatty-acid formula containing n-3 α-linolenic acid with an addition of chromium yeast, magnesium, and other minerals and vitamins. More IR mares that were similar in BCS and NCS used in a randomized fashion would have provided us a stronger basis of analysis of the short-term effects of the supplement in a group of old mares. In another study, by Rexford and others (2012), horses were supplemented with n-3 fatty acids, such as, 38 g of n-3 long chain highly unsaturated fatty acids (LCHUFA) per day, of which 38 g consisted of n-3 α-linolenic acid provided by a supplement containing flaxseed meal. The second group was supplemented with 38 g of n-3 LCHUFA provided by a supplement containing algae and fish oil, of which consisted of 2 g of alpha linolenic acid, 7.6 g of eicopentaenoic acid (EPA), 26.6 g of docosahexaenoic acid (DHA), and 1.7 g of docosapentaenoic acid (DPA). Those supplemented with n-3 α-linolenic acid showed an increase in insulin sensitivity using the reciprocal inverse square of basal insulin, RISQI, an indication of insulin sensitivity measured as insulin-0.5, from 30 d (0.23 ± 0.012) to 60 d (0.25 ± 0.012) (Rexford et al., 2012). Also, horses in the non-supplemented control group had lower n-3 α-linolenic acid levels in plasma compared to those supplemented with a flaxseed meal, but not in muscle or red blood cells, indicating a dose effect for dietary n-3 α-linolenic acid on plasma (Rexford et al., 2012).

In addition, the increase in dietary energy, BCS, and weight may have affected insulin sensitivity and the lack of seeing any difference amongst the groups. Dietary intake of non-structural carbohydrates (NSC) in supplemented mares averaged 13.9% NSC, while non-supplemented mares averaged 17.5% NSC. A study using a diet with less than 10% NSC showed a reduced insulin and glucose response (Cottrell et al., 2005). A study which had more control to the diet and the percent NSC may be advantageous. Also, part of the mares supplemented with EPPMS (n = 7) were utilized from the previous evaluation (Part 1) so while most mares (n = 4) were supplemented for an entire year by the July 2010 sampling, other mares (n = 3) began supplementation in the fall of 2009, which is approximately 7 to 9 mo of supplementation. The mare who started out metabolically normal and supplemented with EPPMS and then changed to IR in the July sampling may have been in part to her only being supplemented for 9 mo, but could have been associated with other factors such as a dietary change or seasonal increase.

No exercise effect on weight was found, as all mares in this study averaged a body weight gain of from May to July. No monthly effect with exercise as a consideration was found for plasma glucose, serum insulin, and RISQI. Dietary changes in caloric intake increasing, the mares gaining bodyweight, and almost one unit of body condition (0.6 in supplemented, 0.7 in non-supplemented), while decreasing insulin sensitivity easily correlates with other findings of increased body condition and a decreased insulin sensitivity in horses (Vick et al., 2007, 2008; Adams et al., 2009).

A monthly effect on endogenous ACTH and exercise effect was found. Overall, those that exercised at a moderate walk on the automated panel walker had higher levels of ACTH (31.5 ± 2.8 pg/ml) compared to those that were left to voluntary exercise or

stall-bound with access to a dry run (19.04 ± 2.8 pg/ml). Higher levels of ACTH were found in July (30.8 ± 2.6) compared to May (19.7 ± 2.4 pg/ml) and this supports other studies which found an increase level of ACTH in summer months due to seasonal variation (Donaldson et al., 2005; Beech et al., 2009; Frank et al., 2010; Lee et al., 2010; Place, et al., 2010; Copas and Durham, 2011; McFarlane et al., 2011).

The most likely reason for the lack of an n-3 α-linolenic fatty acid supplement effect is the choice of the treatment groups, the increase in caloric intake, and the consequent increase in weight and BCS. Studies show a decrease in insulin sensitivity when percent fat and BCS increase (Vick et al., 2007, 2008). Those variables included in this observational study may have confounded and masked any possible effects of the n-3 α-linolenic fatty acid supplementation. A more controlled study where mares are given a consistent, low-starch diet with EPPMS supplementation, and sampled year-round, may produce more conclusive results on insulin sensitivity. Ideally, the mares would be scored on neck and body condition frequently, and weighed year-round to track any fluctuations. In this study, mares did not have access to pasture, so daily changes in plant/pasture NSC would not have played a role in affecting the mare's insulin sensitivity. If we conducted a study where we sampled from a group of old mares year-round, we could investigate any seasonal variation on insulin sensitivity for mares in a controlled environment.

Part III: Nutritional Intake of Old Horses and Body Condition Score

In this observational study, the design was to compare predicted dietary intake values for idle maintenance horses published in the NRC (2007) against actual dietary intake values in old idle maintenance horses. Diet, weight, body and neck condition scores were tracked monthly.

Horses:

Thirty-two old, non-pregnant, client mares of American Quarter Horse (n = 21), Standardbred (n = 5) Arabian (n = 3), Morgan (n = 2) and Saddlebred (n = 1) breeds were enrolled in an alternative reproductive program at the Equine Reproduction Laboratory (ERL) of Colorado State University (CSU). Protocols were approved by the Colorado State Institutional Animal Care and Use Committee. Written consent from the mare owners was obtained. Mares were selected on the basis of age (> 15 yr; ranged from 16 to 26 yr; 21.9 ± 0.65) and being of non-pregnant reproductive status. Mares were dewormed and underwent remedial dental care if needed, such as floating. Mares served as their own controls. Mares began arriving to the ERL in the fall of 2009 and all mares were present by April 2010. Nutritional intake of all mares was tracked upon their arrival to the ERL (Feb, n = 15; Mar, n = 28; Apr, n = 32; May, n = 32; June, n = 32; July, n = 24). Assessment of diet, weight, body condition score (BCS; Figure 2.3) and neck condition score (NCS; Figure 2.4) began upon arrival. Weight was obtained using a calibrated electronic livestock scale (Cardinal Scale Manufacturing Company; Webb City, MO). Body condition score (Figure 2.2 and 2.3) was based on a scale of 1 to 9 with 1 being emaciated and 9 representing extreme obesity (Henneke et al., 1983). Neck condition score (Figure 2.4) was based on a scale from 0 to 5, with 0 being no palpable crest, and 5 meaning crest is so large it permanently droops to one side (Carter, et al., 2009a). Final marks were an average based on scores from two evaluators.

There were various stalling situations, some were stalled in a 3.6 x 3.6 m stall with access to a 3.6 x 12.2 m dry lot run; some were stalled in a 3.6 x 5.2 m stall with access to a 3.6 x 6.1 m dry lot run, and others were stalled in a 3.6 x 3.6 m stall and

turned out into large dry lot paddocks, a 120 x 120 m pasture, or hand-walked daily. All horses had access to an automatic water tank and a salt block.

An automatic walker (Priefert® 6-Horse Panel Walker; Priefert®, Mount Pleasant, TX) was used on a daily basis for 22 mares that were able to comfortably walk without pain and was set at a moderate pace (between 2 and 3 mph) for 30 to 40 min. Mares had varying health ailments; including positive test results for equine Cushing's disease (n = 4), PPID-associated laminitis (varied), and osteoarthritis (n = 1), which ultimately effected the ability for them to walk. Mares that tested positive for equine Cushing's disease received a dopamine receptor agonist (Pergolide) every day, and the mare with osteoarthritis was treated with a non-steroidal anti-inflammatory drug (Equioxx®; Merial, Duluth, GA).

Dietary Intake:

Dietary intake was assessed on a weekly basis from February to July 2010. In changes which occurred during a month, the averages were calculated for the month. Hay and grain was weighed on a daily basis and grain was given with a pre-weighed scoop. In February 2010, both forages were sampled using a manual probe to core several bales and then analyzed by a commercial laboratory (Equi-analytical Laboratories©; Ithaca, NY) who combined a high tech near infrared and plasma spectroscopy for a complete nutritional profile. The analysis of grain concentrate was provided by feed companies (Nutrena®; Cargill, Inc. ©, Minneapolis, MN and Purina Mills, LLC. ®, St. Louis, MO). Calories per kilogram were calculated by dividing the total calorie intake by the body weight (kg).

Weather:

Average monthly temperature, average monthly high and low temperature, and maximum and minimum temperature reached throughout the day were recorded and obtained from the Colorado Climate Center readings from Colorado State University's Foothills Campus and can be seen in Table 3.8.

Laboratory Nutrient Analysis:

Crude Protein was analyzed by Leco FP-528 Nitrogen/Protein Analyzer (Leco Corporation, St. Joseph, MI). Crude protein is simply nitrogen x 6.25 (NRC, 2007).

Acid Detergent Fiber was analyzed by ANKOM Technology Method 5 (Filter Bag Technique for A200) (ANKOM Technology, Macedon, NY) and the samples were individually weighed at 0.5g into filter bags and digested for 75 min as a group of 24 in 2L of ADF solution in ANKOM A200 Digestion Unit (ANKOM Technology, Macedon, NY). Samples were rinsed three times with boiling water for 5 min in filter bags followed by a 3 min acetone soak and drying at 100°C for 2 hr.

Neutral Detergent Fiber was analyzed by ANKOM Technology Method 6 (Filter Bag Technique for A200) (ANKOM Technology, Macedon, NY) and samples were weighed at 0.5g into filter bags and digested for 75 min as a group of 24 in 2L of NDF solution in ANKOM A200 Digestion Unit (ANKOM Technology, Macedon, NY). Four ml of Alpha Amylase and 20g sodium sulfite are added at the start of digestion. Samples are rinsed 3 times with boiling water for 5 min. Alpha Amylase is added to the first 2 rinses. Water rinses are followed by a 3 min acetone soak and drying at 100°C for 2 hr.

Lignin was analyzed by ANKOM technology Method 9 (ANKOM Technology, Macedon, NY) and ADF residue digested as a group of 24 in 72% w/w sulfuric acid for 3 hr in ANKOM DaisyII Incubator (ANKOM Technology, Macedon, NY) at ambient temperature.

Ethanol Soluble Carbohydrates (ESC) was analyzed using a method for partitioning neutral detergent soluble carbohydrates. Samples were shaken with 80% ethanol to extract ESC comprised of simple sugars. ESC was determined colorimetrically using a phenol-sulfuric acid reaction.

Water Soluble Carbohydrates (WSC) was analyzed by a method for partitioning neutral detergent soluble carbohydrates. Samples were incubated with water in a 40°C bath extracting WSC comprised of simple sugars and fructan. WSC determined after acid hydrolysis with sulfuric acid and colorimetric reaction with potassium ferricyanide.

Starch was analyzed using the YSI 2700 SELECT Biochemistry Analyzer (YSI Incorporated Life Sciences, Yellow Springs, OH). Samples are pre-extracted for sugar by incubation in 40°C water bath and filtration on Whatman 41 filter paper. Residues are thermally solubilized using an autoclave, and incubated with glucoamylase enzyme to hydrolyze starch to produce dextrose (glucose). Prepared samples injected into sample chamber of YSI Analyzer (YSI Incorporated Life Sciences, Yellow Springs, OH) where dextrose diffuses into a membrane containing glucose oxidase. The dextrose is immediately oxidized to hydrogen peroxide and D-glucono-4-lactone. The hydrogen peroxide is detected amperometrically at the platinum electrode surface. The current flow at the electrode is directly proportional to the hydrogen peroxide concentration, and hence to the dextrose concentration. Starch is determined by multiplying dextrose by 0.9.

Crude Fat was analyzed by an ether extraction (AOAC 2003.05; AOAC International, Gaithersburg, MD). Extraction was done by Soxtec HT6 System using anhydrous diethyl ether. Crude fat residue was determined gravimetrically after drying.

Ash was analyzed by the AOAC method 942.05 (AOAC International, Gaithersburg, MD). This method quantitatively determines the amount of ash in feed materials based on the gravimetric loss by heating to 550 °C for a period of ≥ 3 hr. The method has a detection limit of 0.01%.

Minerals which were analyzed include: Calcium (Ca), Phosphorus (P), Magnesium (Mg), Potassium (K), Sodium (Na), Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn), Molybdenum (Mo), cobalt (Co), Sulfur (S), and Chromium (Cr). Minerals were analyzed using a Thermo IRIS Advantage HX or Intrepid Inductively coupled Plasma (ICP) Radial Spectrometer (Thermo Fisher Scientific Inc., Waltham MA) after microwave digestion. A CEM Microwave Accelerated Reaction System (CEM, Matthews, NC) with MarsXpress Temperature Control (CEM, Matthews, NC) using 50ml calibrated Xpress Teflon PFA vessels with Kevlar/fiberglass insulating sleeves was used. Samples weighed 0.5g for forages. Samples predigested at ambient temperature 15 min with 8ml nitric acid (HNO3) and 2ml hydrochloric acid (HCl) then ramped to 190ºC in 15 min and finally held at digestion temperature of 190ºC for 15 min at 1600W. Vessels brought to 50-ml volume, aliquot used for analysis.

Chloride Ion (Cl⁻) was analyzed using the Brinkmann Metrohm 716 Titrino Titration Unit. A dried and ground sample of 0.5g was extracted in 50ml of 0.1N HNO3 followed by potentiometric titration with AgNO3 using Brinkmann Metrohm 716 Titrino Titration Unit with silver electrode (Metrohm USA, Riverview, FL).

Statistical Analysis:

Predicted dietary intake of idle maintenance horses using requirements published in the NRC (2007) were compared against actual intake of idle maintenance horses between the months of February to July 2010 by ANOVAS and can be seen in Table 3.7. Regressions were prepared on BCS, NCS, weight, and dietary intake. All nutritional components were analyzed by ANOVA using SAS/STAT 9.2 (SAS Inst. Inc., Cary, NC). Regressions by ANOVA were calculated between predicted and actual nutrient intake. For all analyses, a P-value < 0.05 was accepted as statistically significant. Significant differences were then further analyzed using Fisher's least square means differences. Results are reported as mean $\pm$ SE.

Results

Weights and condition scores:

Weights, BCS, and NCS were evaluated on a monthly basis and can be seen in Figure 3.7. Weight initially ranged from 359.2 kg to 599.1 kg (493.5 ± 2.5 kg). Body and neck condition scores (Figure 2.3 and 2.4) initially ranged from 4.25 to 7.0 (5.5 ± 0.33) and 1 to 4 (2.4 ± 0.29), respectively. There was a sample effect on total monthly BCS ($P < 0.05$). Body condition score was on average 6.2 in February, 5.9 in March, 5.8 in April, 5.8 in May, 5.9 in June, and 6.4 in July. An increase in BCS started from June at 5.9 to 6.4 in July. In comparing July to other months, July was higher in BCS compared to March (0.5 BCS; $P < 0.01$), April (0.6 BCS; $P < 0.005$), May (0.6 BCS; $P < 0.005$), and June (0.5 BCS; $P < 0.02$). In July, BCS had increased by 0.6 from April and May; yet, NCS ($P = 0.71$) and BW ($P = 0.99$) did not vary. Body weight averaged across the

months by 511.9 kg BW in February, 514.01 kg BW in March, 508.7 kg BW in April, 509.1 kg BW in May, 512.9 kg BW in June, and 506.7 kg BW in July.

Using Differences of Least Squares Means Analysis, BCS was the highest in July compared to sample months March ($P = 0.01$) April ($P = 0.01$), May ($P = 0.01$), and June ($P = 0.017$).

Exercise on Automated Walker vs Voluntary Exercise:

Mares who participated on the automated walker panel varied between February and July due to health ailments such as laminitis, arthritis, and recurrent airway obstruction. The exercise regimen was tracked on a daily basis. Monthly averages of exercise were accounted. Mares were classified in the average maintenance category is she was walked using the automated walker 3 to 4 d/wk; and was predicted to require 33.3 kcal/kg a day (NRC, 2007). Mares were classified under minimum average maintenance requirements if she was stall-bound with access to a dry run or turned out to ao dry paddock; and was predicted to require 30.3 kcal/kg a day (NRC, 2007).

Diets:

The diet included a mix of alfalfa hay and orchard brome grass hay in addition to a grain concentrate (Nutrena® Safe Choice®, Nutrena® Empower® Boost , Purina® Equine Senior®, Purina® Strategy® Professional Formula GX) that was divided into two feedings: morning and late afternoon. Nutritional composition can be seen in Table 3.3. Thirty mares were fed an average of 1.3kg/d (as-fed basis) of a controlled-starch grain concentrate (Nutrena® Safe Choice®; Cargill, Inc. ©, Minneapolis, MN). Of those thirty mares, eight were given an average of 0.56kg/d (as-fed basis) of a high-fat rice bran supplement (Nutrena® Empower® Boost; Cargill, Inc. ©, Minneapolis, MN). One mare

was fed an average of 3.63kg/d (as-fed basis) a grain concentrate designed for senior horses (Purina® Equine Senior®; Purina Mills, LLC.®, St. Louis, MO) and another mare was fed an average of 1.36 kg/d (as-fed basis) a grain concentrate designed for performance horses (Purina® Strategy® Professional Formula GX; Purina Mills, LLC.®, St. Louis, MO). Fourteen of the 32 mares who appeared metabolic were supplemented for at least 1 mo before sampling in May 2010 with Equine Platinum Plus Metabolic Support™ (EPPMS™): a fatty-acid formula from Platinum Performance™ containing n-3 α-linolenic acid (ALA) with an addition of chromium yeast, magnesium, and other minerals and vitamins. For those supplemented with EPPMS™ the nutritional supplementation can be seen in Tables 3.1a and 3.1b. Horses given EPPMS™ were provided 190 g/d of the supplement on top of the grain concentrate. The nutritional composition analysis for forage and grain concentrates can be seen in Table 3.3.

Predicted nutrient intake vs. actual nutrient intake values for DE caloric intake, CP, phosphorous, Ca, Cu, Mg, and Zn can be seen in Tables 3.7a and 3.7b.

Overall, actual caloric intake was on average 13.8% higher than predicted caloric intake ($P < 0.0001$). Specifically, in February DE was 9.4% higher than predicted maintenance requirements, in March DE was 5.0% higher than predicted maintenance requirements, in April DE was 9.2% higher than predicted maintenance requirements, in May DE was 18.5% higher than predicted maintenance requirements, in June DE was 21.6% higher than predicted maintenance requirements, and in July DE was 16.9% above predicted maintenance requirements. Digestible energy was higher in June (18,679 kcal) than March (16,887 kcal; $P < 0.05$) and April (16,912 kcal; $P < 0.04$). Actual and predicted values for DE were compared and can be seen in Tables 3.7a., 3.7b, and Figure

3.8. Actual DE intake in May was higher than predicted DE intake values in February ($P < 0.01$), March ($P < 0.003$), April ($P < 0.001$), May ($P < 0.0002$) and June ($P < 0.002$). May actual DE intake was higher than March actual intake DE ($P < 0.03$). Predicted DE intake in May was lower than June actual DE intake ($P < 0.0001$). Actual intake of DE was higher in June compared to actual intake DE in February ($P < 0.02$), March ($P < 0.001$), April ($P < 0.003$) and predicted values of February ($P < 0.001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), and June ($P < 0.0001$).

The monthly sample effect for energy (Mcal/kg of BW) showed a trend for variation ($P = 0.08$) with energy intake increasing in May, and being highest in June. Actual energy intake (Mcal/kg of BW) was higher in June compared to actual intake in February ($P < 0.03$), March ($P < 0.0003$), and April ($P < 0.002$) while showing a trend for being higher in June compared to July ($P = 0.08$). Actual energy intake (Mcal/kg of BW) was higher May compared to March ($P < 0.03$) and April ($P < 0.01$) while showing a trend to be higher than actual intake in February ($P = 0.09$).

An effect on dietary energy (kcal/kg BW) with sample by predicted vs. actual nutrient intake values ($P < 0.05$) and predicted vs. actual nutrient intake values ($P < 0.0001$) was found, with actual intake being higher than predicted. No monthly sampling effect ($P = 0.26$) was found for dietary energy (kcal/kg BW).

Overall, actual CP intake was higher than predicted CP intake ($P < 0.0001$), and was highest in June compared to all other months ($P < 0.0001$), which can be seen in Tables 3.7a, 3.7b, and Figure 3.9. Actual CP intake in February was higher than predicted CP intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual CP intake was higher in

March compared to predicted CP intake for February ($P < 0.0004$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$) and July ($P < 0.0001$). Actual CP intake for April was higher than predicted CP intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual CP intake in May was higher than predicted CP intake in February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual CP intake in June was higher than predicted CP intake in February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual CP intake for July was higher than predicted CP intake for February ($P < 0.001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual CP intake for June was higher than actual CP intake for March ($P < 0.03$), April ($P < 0.05$), and July ($P < 0.02$).

Overall, actual Ca intake was higher than predicted Ca intake in all other months ($P < 0.0001$), and can be seen in Figure 3.10. Actual Ca intake in February was higher than predicted Ca intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Ca intake was higher in March compared to predicted Ca intake for February ($P < 0.0004$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$) and July ($P < 0.0001$). Actual Ca intake in April was higher than predicted Ca intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Ca intake in May was higher than predicted Ca intake in February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June

($P < 0.0001$), and July ($P < 0.0001$). Actual Ca intake in June was higher than predicted Ca intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Ca intake in July was higher than predicted Ca intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Ca intake in May was higher than actual Ca intake in July ($P < 0.05$). Actual Ca intake in June showed a trend ($P < 0.06$) to be higher than actual Ca intake in March, and June actual Ca intake was also higher than actual Ca intake in July ($P < 0.02$).

Overall, actual P intake was higher than predicted phosphorus intake in all other months ($P < 0.0001$). Actual P intake in February was higher than predicted P intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual P intake was higher in March compared to predicted P intake for February (P < 0.0004), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$) and July ($P < 0.0001$). Actual P intake in April was higher than predicted P intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual P intake in May was higher than predicted P intake in February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual P intake in June was higher than predicted P intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual P intake in July was higher than predicted P intake for February (P < 0.0001), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual P intake in May was higher than actual P intake in March

($P < 0.02$) and April ($P < 0.05$). Actual P intake in June was higher than actual P intake in February ($P < 0.04$), March ($P < 0.001$), April ($P < 0.005$), and July ($P < 0.03$).

Overall, actual Mg intake was higher than predicted Mg intake in all other months ($P < 0.0001$). Magnesium (mg/kg BW) trended towards significance in the total monthly sampling effect ($P < 0.067$) and increased from April (30.2 mg/kg BW) to June (34.6 mg/kg BW). Sample month May (33.3 mg/kg BW) trended higher in intake compared to sample months March (30.2 mg/kg BW; $P < 0.07$) and April (30.2 mg/kg BW; $P < 0.06$). In addition, magnesium intake was higher in June (34.6 mg/kg BW) compared to March (30.2 mg/kg BW; $P < 0.011$) and April (30.2 mg/kg BW; $P < 0.01$). Actual Mg intake in February was higher than predicted Mg intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Mg intake was higher in March compared to predicted Mg intake for February ($P < 0.0004$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$) and July ($P < 0.0001$). Actual Mg intake in April was higher than predicted Mg intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Mg intake in May was higher than predicted Mg intake in February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Mg intake in June was higher than predicted Mg intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Mg intake in July was higher than predicted Mg intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Mg intake was higher in May than actual Mg intake in April ($P < 0.05$).

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Actual Mg intake was higher in June compared to actual Mg intake in February ($P <$ 0.05), March ($P < 0.003$), April ($P < 0.001$), and July ($P < 0.05$).

Overall actual Cu intake was higher than predicted Cu intake in all other months ($P < 0.0001$). Copper (mg/kg BW) had a total monthly sampling effect ($P < 0.032$) and increased from sample month May (0.27 mg/kg BW) to June (0.28 mg/kg BW). When copper intake (mg/kg BW) in May (0.27 mg/kg BW) is compared to sample months March (0.23 mg/kg BW; $P < 0.03$) and April (0.23 mg/kg BW; $P < 0.04$) an effect can be seen. In addition, copper intake in June (0.28 mg/kg BW) was higher compared to sample months February (0.24 mg/kg BW; $P < 0.06$), March (0.23 mg/kg BW; $P < 0.008$) and April (0.23 mg/kg BW; $P < 0.01$). Actual Cu intake in February was higher than predicted Cu intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Cu intake was higher in March compared to predicted Cu intake for February ($P < 0.0004$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$) and July ($P < 0.0001$). Actual Cu intake in April was higher than predicted Cu intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Cu intake in May was higher than predicted Cu intake in February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Cu intake in June was higher than predicted Cu intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Cu intake in July was higher than predicted Cu intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Cu intake

in May was higher than actual Cu intake in February ($P < 0.05$), March ($P < 0.01$), and April ($P < 0.015$). Actual Cu intake was higher in June compared to actual Cu intake in February ($P < 0.005$), March ($P < 0.0003$), and April ($P < 0.0005$). Actual Cu intake was higher in July compared to actual Cu intake in March ($P < 0.04$).

Overall actual Zn intake was higher than predicted Zn intake in all other months ($P < 0.0001$). Actual Zn intake in February was higher than predicted Zn intake for February ($P < 0.011$), April ($P < 0.04$), May ($P < 0.04$), June ($P < 0.05$), and July ($P < 0.014$). Actual Zn intake was higher in March compared to predicted Zn intake for February ($P < 0.014$), March ($P < 0.03$), April ($P < 0.025$), May ($P < 0.025$), June ($P < 0.04$) and July ($P < 0.01$). Actual Zn intake in April was higher than predicted Zn intake for February ($P < 0.013$), March ($P < 0.04$), April ($P < 0.013$), May ($P < 0.023$), June ($P < 0.034$), and July ($P < 0.007$). Actual Zn intake in May was higher than predicted Zn intake in February ($P < 0.0001$), March ($P < 0.0002$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Zn intake in June was higher than predicted Zn intake for February ($P < 0.0001$), March ($P < 0.0001$), April ($P < 0.0001$), May ($P < 0.0001$), June ($P < 0.0001$), and July ($P < 0.0001$). Actual Zn intake in July was higher than predicted Zn intake for February ($P < 0.0005$), March ($P < 0.0013$), April ($P < 0.0005$), May ($P < 0.0005$), June ($P < 0.001$), and July ($P < 0.0001$). Actual Zn intake in June was higher than actual Zn intake in March ($P < 0.02$) and April ($P < 0.01$), while showing a trend for being higher than actual Zn intake in February ($P = 0.08$).

No monthly sample effect for percent starch ($P = 0.40$) and non-structural carbohydrates (NSC) was seen ($P = 0.17$); however, the highest intake for starch was in

the month of June (2.05%). When percent starch in June is compared to March (1.34%; $P < 0.08$) and July (1.31%; $P < 0.09$) it shows a trend for an effect. Percent NSC is higher in June (18.8%) compared to sampling months March (13.5%; $P < 0.03$), April (14.1%; $P < 0.04$) and July (14.3%; $P < 0.07$).

There was a monthly sample effect of WSC ($P < 0.028$), and WSC began increasing in from May (8.4%) to July (7.6%), with its highest level of intake being in June (9.5%). When WSC intake in June is compared to February (6.9%; $P < 0.04$), March (6.5%; $P < 0.004$) and April (6.7%; $P < 0.005$) a sample effect is seen. A trend towards significance for WSC can be seen when May (8.4%) is compared to March (6.5%; $P < 0.061$) and April (6.7%; $P < 0.082$), and trends higher in June (9.5%) compared to July (7.6%; $P < 0.085$). A monthly sample effect for DMI % (kg/BW) was found ($P < 0.028$) and increased from April (1.36 %) to June (1.60 %), with the highest level being in June. When DMI % (kg/BW) in May (1.54%) is compared to March (1.33 %; $P < 0.026$) and April (13.6 %; $P < 0.04$) a sample effect can be seen. In addition, DMI % (kg/BW) trends higher in June (1.60 %) compared to February (1.4%; $P < 0.08$), March (1.33%; $P < 0.005$), and April (1.36%; $P < 0.008$).

Crude fat percent (kg/BW) showed a total monthly sample effect ($P < 0.022$), and increased from May (0.56%) to June (0.59%). When crude fat % (kg/BW) intake in May (0.56%) is compared to March (0.49%; $P < 0.024$) and April (0.50%; $P < 0.04$) an effect can be seen. Sample month June (0.59%) had the highest level of crude fat % (kg/BW) intake and when compared to March (0.49%; $P < 0.004$) and April (0.50%; $P < 0.006$) an effect can be seen. Crude fat % trended higher in July (0.55%) compared to March (0.49%; $P < 0.08$). As mentioned earlier, mares were provided a salt block in individual

feeders, and total sodium intake cannot be accounted for statistically; however, the sodium in the grain and hay was analyzed, and a monthly sampling effect ($P < 0.001$) was found.

Sodium (mg/kg BW) intake from a feedstuff standpoint increased from May (0.004 mg/kg BW) to June (0.005 mg/kg BW). When sample month June (0.005 mg/kg BW) is compared to months February (0.004 mg/kg BW; $P < 0.03$), March (0.004 mg/kg BW; $P < 0.0006$), and April (0.004 mg/kg BW; $P < 0.0003$) a sample effect can be found. In addition, sample month May (0.004 mg/kg BW) was higher in sodium intake compared to sample months March (0.004 mg/kg BW; $P < 0.02$) and April (0.004 mg/kg BW; $P < 0.015$). Sodium intake was also higher in July (0.0045 mg/kg BW) compared to March (0.004 mg/kg BW; $P < 0.016$) and April (0.004 mg/kg BW; $P < 0.013$).

Chlorine (mg/kg BW) had total monthly sampling effect ($P < 0.0001$) and increased from May (65.4 mg/kg BW) to June (70.1 mg/kg BW). When sample month May (65.4 mg/kg BW) is compared to sample month March (56.8 mg/kg BW; $P < 0.01$) an effect can be seen. Also, when sample month May is compared with sample month April (56.9 mg/kg BW; $P < 0.009$) a trend can be seen. In addition, a trend can be seen when sample month June (70.1 mg/kg BW) is compared to February (58.6 mg/kg BW; $P < 0.006$) and then in April (56.9 mg/kg BW; $P < 0.0001$) an effect is seen. Chlorine was also higher in sample month July (68.5 mg/kg BW) compared to sample months February (58.6 mg/kg BW; $P < 0.02$), March (56.8 mg/kg BW; $P < 0.002$) and April (56.9 mg/kg BW; $P < 0.001$).

A total monthly sampling effect was found for iron (mg/kg BW) ($P < 0.002$) and an increase was seen from May (2.77 mg/kg BW) to June (2.96 mg/kg BW). When

sample month June (2.96 mg/kg BW) was compared to sample months February (2.6 mg/kg BW; $P < 0.05$), March (2.5 mg/kg BW; $P < 0.002$), and April (2.5 mg/kg BW; $P < 0.001$) a sample effect can be seen. Iron intake trended higher in May (2.77 mg/kg BW) compared to March (2.5 mg/kg BW; $P < 0.07$) and also in July (2.94 mg/kg BW) compared to February (2.61 mg/kg BW; $P < 0.09$). Iron intake was higher in May (2.77 mg/kg BW) compared to April (2.5 mg/kg BW; $P < 0.03$) and also in July (2.94 mg/kg BW) compared to March (2.5 mg/kg BW; $P < 0.01$) and April (2.5 mg/kg BW; $P < 0.003$).

A total monthly sampling effect was found in cobalt (mg/kg BW) ($P < 0.005$), and increased from May (0.017 mg/kg BW) to July (0.019 mg/kg BW). When sample month June (0.018 mg/kg BW) is compared to sample months February (0.015 mg/kg BW; $P < 0.05$), March (0.015 mg/kg BW; $P < 0.01$), and April (0.014 mg/kg BW; $P < 0.005$), an effect can be seen. In addition, cobalt was highest in July (0.019 mg/kg BW) and when compared to sample months February (0.015 mg/kg BW; $P < 0.03$), March (0.015 mg/kg BW; $P < 0.005$), and April (0.014 mg/kg BW; $P < 0.002$), an effect can be seen.

A total monthly sample effect was not found in CP intake ($P = 0.48$), calcium ($P = 0.64$), phosphorous ($P = 0.15$), potassium ($P = 0.59$), sulfur ($P = 0.62$), Zinc ($P = 0.27$) or selenium ($P = 0.98$); although, an overall increase in dietary intake was seen during May (1.5%) and June (1.6%). Even though a total monthly sample effect for phosphorus did not occur, phosphorus was higher in May (47.0 mg/kg BW) compared to March (39.5 mg/kg BW; $P < 0.05$); and, was also higher in June (48.3 mg/kg BW) compared to March (39.5 mg/kg BW; $P < 0.02$) and April (41.05 mg/kg BW; $P < 0.05$). Zinc had no total

monthly sample effect; however, intake in June (1.022 mg/kg BW) tended to be higher than March (0.88 mg/kg BW; $P = 0.06$), and was higher than April (0.88 mg/kg BW; $P < 0.05$).

Regressions analyzed predicted energy and an intercept with BCS as an independent variable against actual energy intake ($P < 0.0001$; $r^2 = 0.18$). Regressions analyzed predicted values of intake and intercept as an independent variable for kcal/kg BW ($P = 0.9$), P ($P = 0.22$), zinc ($P = 0.52$), and Cu ($P = 0.37$), which were weakly correlated. Regressions that analyzed predicted values of intake and intercept as independent variables for CP ($P < 0.003$; $r^2 = 0.05$), Ca ($P < 0.005$; $r^2 = 0.05$), and Mg ($P < 0.0001$; $r^2 = 0.09$) were significant, yet weakly predicted using the regression model.

Weather:

Average monthly temperature, average monthly high and low temperature, and maximum and minimum temperature reached throughout the day can be seen in Table 3.8.

Discussion

Overall, old mares in this study had an actual caloric intake that was on average 13.8% higher than predicted caloric intake for mature maintenance horses from February to July 2010. Dietary caloric intake increased by an average of 20% from May to July and BCS increased from 5.8 to 6.4 on average (out of a 1-9 possible range); yet NCS and weight did not vary even though most of the time the mares were fed on average, 13.8% above DE requirements. In addition, actual intake of CP, Ca, P, Mg, Zn, and Cu was higher than predicted values in all months; and was the highest in June. Although an increase in total diet WSC occurred with the increase in DMI from May (8.4%) to June

(9.5%) the amount was well within the recommendations ($\leq 12\%$) for feeding IR horses from other researchers (Cottrell et al., 2005; Longland et al., 2011). Furthermore, no monthly sample effect for percent starch and NSC was seen.

An increase in BCS by 0.5 from June to July may have been due to the even greater increase (21.6%) in calories during the month of June. According to the NRC (2007), BCS is often reported in equine nutrition studies; however, the amount of weight loss or gain necessary to achieve a change in BCS has not been well studied. It appears that each unit of BCS increase requires about 16 to 20 kg of weight gain (NRC, 2007). However, the mares in this study gained 0.6 in BCS without a significant change in weight. In fact, the mares in this study never came close to gaining 16 to 20 kg of weight, although the NRC (2007) states that the amount of weight needed to change condition score by one unit will vary with the mature weight of the horse. Previous research varies in nutrient maintenance requirements for old horses and is conflictive. Specifically, one report has indicated that nutrient intake is similar between old and young horses (Elzinga et al., 2011), yet in the current study, old mares who were fed above maintenance requirements standardized for mature horses from the NRC (2007), which includes adult and geriatric horses, did not increase in body weight. Another study reports that maintenance energy requirements are lower for adult horses (approximately 11 years old) than for horses approximately 4 years of age (Martin-Rosset and Vermorel, 1991).

A recommendation from the NRC (1989) to increase BCS for the average adult maintenance horse is to increase DE requirements by 10 to 15% above maintenance requirements (NRC, 2007). When BCS in July is compared to previous months, March, April, May and June, statistical significance is found. In February, the mares in this study

were fed an average of 9.4% above DE maintenance requirements and were measured to be an average of 6.2 in BCS. However, although not statistically significant, the numerical results are as follows: in March, mares were fed 5% above DE maintenance requirements and actually decreased in BCS by 0.3. Then in April, mares were fed 9.2% above DE maintenance requirements and decreased again in BCS by 0.1. In May, mares were given 18.5% above DE maintenance requirements and they maintained the same BCS from April of 5.8. The mares were fed 21.6% above DE maintenance requirements in June, and gained 0.1 in BCS from May. Finally, in July, mares gained 0.5 in BCS when fed 16.9% above DE maintenance requirements. According to the NRC (2007), if horses are fed 16 to 20.5% above DE maintenance requirements increases in BCS by 0.5 in 60 days should be seen. Along with this, an increase by 32 to 41% of DE above maintenance requirements should increase BCS by one unit in 60 days (NRC, 2007). If we compare DE intake for 60 days from months May to July where an increase in BCS by 0.6 occurred, we would be slightly above the NRC (2007) recommendations of increasing DE maintenance requirements from 16 to 20.5 % by 1.6 % (fed at 21.6 % above DE); however, this projection is very close to the recommendation from the NRC (2007).

One report showed a decrease in protein, phosphorus, and fiber digestion in Quarter horse and Thoroughbred horses (Ralston et al., 1989). Yet, a later report by the same author hypothesizes that the reduction in apparent digestion of protein, phosphorus and fiber reported previously in aged horses may have been due in part to chronic parasitic damage to the large colons and/or abnormal dentition rather than caused by aging per se in the equine animal (Ralston et al., 2001). Furthermore, the mares in this

study had routine dental exams and they were conducted prior to the start of breeding season and largely before March. The dental records showed that those that needed dental work had minor (floating) and routine maintenance, but nothing major. Mares were never restricted calorically and finished their grain and supplement. One mare was observed to have her hay feeder full most of the day and may have had a slower time finishing her ration. In relation to condition scoring, five evaluators trained in using the BCS and NCS system were used and averages taken. The author served as one of the evaluators for each evaluation every month and two evaluators were present for each evaluation and averages taken.

Overall, the mares were exposed to climate conditions that averaged within the lower and upper critical temperatures for horses (NRC, 2007) and results can be seen in Tables 3.7a and 3.7b. However, in February the mares experienced 14 d of temperature below freezing; and, the average minimum temperature reached throughout the day was -7.5 °C. Still, the lower critical temperature (LCT) range for horses is -9.4 to -20 °C; thus, on average, the mares were not in the LCT range. The NRC reports horses who are exposed to cold conditions such as rain and wind have been reported to have DE requirements elevated as much as 50 % above maintenance (Kubiak et al., 1987) which may have been a challenge to the mares part of the time in February and could have affected their lack of gain in weight or BCS. However, mares in this study had access to shelter and were closed into their stalls on high-wind days by ERL staff. In addition, barn doors located at each end of the barn were manually closed during high-wind times and at night. A dramatic difference in weather was seen after March; specifically, between

April and July, compared to February; as none of the days had temperature fall below freezing.

We did not consider the mares to be of elevated DE maintenance requirements for voluntary activity, including access to a turnout or dry run. We also did not consider the mares elevated or on "light" exercise when they participated on the automated walker panel exercise, because of the fact that they were walking and not trotting or cantering. In addition, elevated maintenance requirements are derived for adult horses with nervous temperaments or high levels of voluntary activity. Members of this group might include stallions or young adult horses that are noticeably active in their stalls or during periods of turnout (NRC, 2007). None of the mares in this study displayed nervous or noticeably active activity in turnout, or in their stalls; therefore, we decided to categorize the mares at average maintenance requirements.

Another important consideration is the strong relationship between heart rate and oxygen utilization (Eaton et al., 1995; Coenen, 2005). According to the NRC (2007), the maximal heart rate of an individual horse may vary with age and breed, and, therefore, to estimate the percentage of heart rate maximum achieved during a specific work bout, it is necessary to know the maximal heart rate of an individual horse. However, because of the challenges included with testing maximal heart rate, it is more practical to use heart rate as a guide to oxygen consumption than percentage of heart rate maximum achieved during various work bouts (NRC, 2007).

Unfortunately, we did not measure heart rates at rest, voluntary exercise, or when they were walked on the automated walker. It is possible that if the mares averaged 80 beats/min 1 to 3 hr/wk, they could have been considered under the light work category,

and not maintenance. Light work is defined, as "requiring an increase of DE by 20% above maintenance requirements." If the mares were achieving "light" work on the automated walker or during turnout, then that may explain the lack in increased weight or BCS in the months prior to July.

Initially the BCS ranged from 4.25 to 7.0 (5.5 ± 0.33). According to the NRC (2007), mares entering the breeding season with a mean BCS of 5.3 ovulated sooner than mares that entered the breeding season below 5 (Kubiak et al., 1987). In addition, mares entering the breeding season in a moderate BCS (BCS 4 to 6) required fewer cycles for conception and had higher conception rates than mares entering the breeding season in thin BCS (BCS 1-3) (Henneke et al., 1984). Also, open mares maintained in moderately fat to fat BCS (BCS 6.5 to 8) during the fall and winter months often continue to cycle throughout the winter (Gentry et al., 2002). Some studies have shown no disadvantages to very high BCS in broodmares (Henneke et al., 1983; Kubiak et al., 1988; Cavinder et al., 2005). However, Hoffman and others (2003) suggested that very fat horses may have disturbed metabolic and endocrine regulation, although specific consequences to animal health were not reported (NRC, 2007). Therefore, at this time the optimal body condition for a horse is not known (NRC, 2007). Since the mares in this study averaged an initial BCS of 5.5, we may have seen a positive trend for ovulation; however, these mares were given hormonal therapies in order to control their estrous cycles, so comparisons to the previous studies are inconclusive. In addition, conception rate is inconclusive due to the mares having their oocytes fertilized after transvaginal aspiration of follicles; and with the use of a variety of stallions utilized for sperm injection.

An improvement to the current study would be conducting a long-term controlled study measuring dietary nutrient inputs and outputs with more frequent weighing for tracking body weight fluctuations and also, more evaluations of BCS. In addition, due to the possibility of differences in nutrient digestion from horse-to-horse, DE maintenance variation may be relatively high (NRC, 2007), and DE maintenance requirements could be accounted for by recording inputs and outputs from each horse. Finally, utilizing a group of old mares without reproductive hormone therapy treatments, while tracking their natural ovulation and estrous cycles would be worth the investigation because of the lack of conclusive results of BCS and reproductive efficiency in old mares.

Actual caloric intake was on average 13.8% higher than predicted caloric intake for mature maintenance horses from February to July 2010. Also, actual intake of CP, Ca, P, Mg, Zn, and Cu was higher than predicted values using guidelines in the NRC (2007) in all months. Water-soluble carbohydrates increased from May (8.4%) to June (9.5%), which was within the recommendations (below 12%) for feeding IR horses from other researchers (Cottrell et al., 2005; Longland et al., 2011). No monthly sample effect for percent starch and NSC was seen. Dietary caloric intake increased by an average of 20% from May to July and BCS increased from 5.8 to 6.4 on average. When BCS in July is compared to previous months, March, April, May and June, statistical significance is found. From June to July, mares increased by half a BCS when fed 16.9 % above DE maintenance requirements for mature horses; yet NCS and weight did not vary. From months May to July, an increase in BCS by 0.6 occurred, and is similar to the projection from the NRC (2007); however, an increase in weight was not found which contradicts

other findings from the NRC (2007), stating that each unit of BCS increase requires about 16 to 20 kg of weight gain.

Conclusion

Although previous research has indicated that nutrient intake is similar between old and young horses, in the current study mares did not increase in body weight or condition when fed 10% above energy requirements. Overall, mares came close to the NRC recommendation for increased BCS by being fed 21.6% above dietary requirements for adult average maintenance horses. A long-term controlled study measuring dietary nutrient inputs and outputs as well as left-over feed in addition to more frequent body weighing for tracking body weight fluctuations is needed to confirm current results. Also, analysis of heart rate and "working" heart rate for mares on the walker exercise schedule would be helpful in terms of determing if horses were true to needing maintenance requirements or if they may have been considered "light" exercise by achieving increased heart rate for 1-3 hours a week, according to the NRC (2007). Older horses may not be as fit as a younger adult maintenance horse; therefore, their heart rate may increase to the point of fitting into the "light" exercise category, even at without any additional gate included besides a moderately-paced walk.

Mares fed a fatty acid supplement with chromium increased in insulin sensitivity and pancreatic-beta cell function from July 2009 to May 2010. However, when BCS and weight increased due to a dietary calorie increase in May 2010, no effect was found on insulin sensitivity in the short-run study of those supplemented versus non-supplemented and weight and BCS increased. In terms of improvement, a more controlled study

including insulin resistant old horses with simiar BCS given a consistent diet would be

needed to evaluate the effects of a fatty acid supplement with chromium.

Table 3.1a: Fatty Acid Supplement Formula

Item	Guaranteed Analysis[2]	
Ingredient List[1]	A	B
Energy, Cal	-	620
Carbohydrate, g *	19.0	38.0
CP, g *	12.6	25.1
Crude Fiber, g **	4.0	9.2
Ash, g *	7.5	14.9
Crude Fat, g *	20.4	40.8
Linolenic Acid (Omega-3), g	9.5	18.9
Linoleic Acid (Omega-6), g	4.3	8.5
Amino Acids		
Arginine, mg *	1,010	2,020
Glycine, mg *	647	1,294
Methionine, mg *	218	436
Isoleucine, mg *	422	845
Leucine, mg *	700	1,399
Valine, mg *	627	1,254
Lysine, mg *	1,089	1,122
Tyrosine, mg *	290	581
Cystine, mg *	211	422
Glutamine, mg *	2,264	4,528
Phenylalanine, mg *	508	1,016
Threonine, mg *	429	858
Aspartic Acid, mg *	1,148	2,297
Alanine, mg *	548	1,096
Histidine, mg *	541	1,082
Tryptophan, mg *	191	383
L-Carnitine, mg *	26	26
Proline, mg *	350	700
Chondroprotective		
Glucosamine Sulfate, mg *	-	850

[1]Ingredient list: Equine Platinum Plus Metabolic Formula, Platinum Performance; Buellton, CA
[2]Guaranteed Analysis: A = per 66 g (1 scoop); B = per 190 g (total daily amount)
* = Minimum ** = Maximum

Table 3.1a: Fatty Acid Supplement Formula

Item	Guaranteed Analysis[2]	
	A	B
Macrominerals		
Calcium, mg *	264	396
Phosphorus, mg *	530	1,060
Sodium, mg *	133	130
Magnesium, mg *	2700	6,500
Potassium, mg *	660	1320
Chloride, mg *	125	250
Sulfur, mg *	238	475.2
Trace Minerals		
Copper, mg *	6.6	13.2
Iron, mg *	132	264
Manganese, mg *	66	132
Silicon, mg *	1,320	2,640
Zinc, mg *	66	132
Cobalt, mcg *	396	792
Chromium, mcg *		5,200
Selenium, mcg *	396	792
Iodine, mcg *	330	660
Vitamins		
Vitamin A, IU *	3,750	7,500
Vitamin D, IU *	750	1,500
Vitamin E, IU *	370	740
Vitamin C, mg *	125	250
Folic Acid, mg *	5	10
Thiamin, mg *	9.9	19.8
Riboflavin, mg *	7.7	15.4
Niacin, mg *	9.9	19.8
Choline, mg *	3	6
Pyridoxine B6, mg *	15.9	31.8
Pantothenic Acid, mg *	24.3	48.6
Biotin, mg *	1.25	2.5
Cyanocobalamin, mcg *	22	44

Header: Ingredient List[1]

[1]Ingredient list: Equine Platinum Plus Metabolic Formula, Platinum Performance; Buellton, CA
[2]Guaranteed Analysis: A = per 66 g (1 scoop); B = per 190 g (total daily amount)
* = Minimum ** = Maximum

Table 3.2: Nutritional Analysis of 2009 Diet

Item[2]	Nutritional Composition of Feedstuffs[1]				
	A	**B**	**C**	**D**	**E**
DM, %	92.2	89.0	87.0	88.0	93.0
DE, Mcal/kg	2.12	2.60	3.15	3.90	4.70
CP, %	7.10	20.30	14.0	12	19.03
Fat, %	2.50	2.50	7.0	22	30.90
Omega-3 Fatty Acids, %	-	-	-	2.7	14.34
Omega-6 Fatty Acids, %	-	-	-	7.7	6.47
WSC[3], %	16.00	13.50	< 12.0	< 12.0	10.32
NSC[4], %	22.30	32.40	22-25	22-25	9.68

[1]Nutritional Composition of Feedstuffs: A = Grass hay; B = Alfalfa hay; C = Low-starch grain (Nutrena Safe Choice; Cargill, Inc., Minneapolis, MN); D = Fat supplement (Nutrena Empower Boost; Cargill, Inc., Minneapolis, MN); E = Metabolic supplement (Platinum Performance; Buellton, CA)
[2]Item reported in terms of DM
[3]Water-soluble carbohydrates
[4]Non-structural carbohydrates

Table 3.3: Nutritional Analysis of 2010 Diet

Item[2]	Nutritional Composition of Feedstuffs[1]						
	A	**B**	**C**	**D**	**E**	**F**	**G**
DM, %	94.1	91.6	87.0	90.0	90.7	88.0	93.0
DE, Mcal/kg	2.16	2.36	3.15	2.71	3.31	3.90	4.70
CP, %	8.3	19.4	14.0	14.0	14.0	12	19.03
Fat, %	3.1	2.3	7.0	5.50	6.50	22	30.9
Omega-3 Fatty Acids, %	-	-	-	-	-	2.7	14.34
Omega-6 Fatty Acids, %	-	-	-	-	-	7.7	6.47
WSC[3], %	15.2	8.90	< 12.0	11	8	< 12.0	10.32
NSC[4], %	22.0	25.7	22 - 25	22	26	22 – 25	9.68

[1]Nutritional Composition of Feedstuffs: A = Grass hay; B = Alfalfa hay; C = Low-starch grain concentrate (Nutrena Safe Choice; Cargill, Inc., Minneapolis, MN); D = Senior horse grain concentrate (Purina Equine Senior; Purina Mills, LLC, St. Louis, MO); E = performance horse grain concentrate (Purina® Strategy® Professional Formula GX; Purina Mills, LLC.®, St. Louis, MO); F = Fat supplement (Nutrena Empower Boost; Cargill, Inc., Minneapolis, MN); G = Metabolic supplement (Platinum Performance; Buellton, CA)
[2]Item reported in terms of DM
[3]Water-soluble carbohydrates
[4]Non-structural carbohydrates

Table 3.4: Reference Quintiles adapted from Treiber et al., 2005

Quintile	1	2	3	4	5
RISQI, $(mU/L)^{-0.5}$	0.152-0.295	0.296-0.335	0.336-0.393	0.394-0.470	0.471-0.953
MIRG, $(mU_{insulin}^{2}/[10 \cdot L \cdot mg_{glucose}])$	1.20-2.12	2.13-3.48	3.49-4.54	4.55-5.27	5.27-10.67

Table 3.5: 2010 May/July Comparisons of Daily Total Dietary Intake

Month	Starch, %	NSC[4], %	WSC[5], %	Ca[6], g	P[7], g	Mg[8], g	K[9], g	Cr[10], mg/kg
May								
A[2]	1.8 ± 0.7	13.2 ± 0.8	7.4 ± 0.5	68.2 ± 2.4	25.0 ± 0.9	16.3 ± 0.6	129.6 ± 3.7	4.76 ± 0.00
B[3]	1.6 ± 1.1	15.8 ± 2.2	7.4 ± 1.2	83.5 ± 4.3	21.6 ± 1.6	14.5 ± 1.1	161.8 ± 4.6	-
July								
A[2]	2.0 ± 0.8	14.6 ± 0.8	8.1 ± 0.6	74.1 ± 2.4	26.1 ± 1.0	17.2 ± 0.5	140.7 ± 3.4	4.76 ± 0.00
B[3]	1.8 ± 1.0	19.2 ± 2.0	9.5 ± 1.3	89.9 ± 3.8	24.1 ± 1.3	16.7 ± 1.0	180.1 ± 4.0	-

[1]Measurement: reported in mean ± SEM; feedstuffs reported in DM
Treatment: [2]A: treated; [3]B: non-treated
[4]NSC: non-structural carbohydrates
[5]WSC: water-soluble carbohydrates
[6]Ca: calcium
[7]P: phosphorous
[8]Mg: magnesium
[9]K: potassium
[10]Cr: Chromium

Table 3.6: 2010 May/July Comparisons of Body and Neck Condition Scores, Weight, and Total Diet Intake

					Measurement[1]					
Month	BCS	NCS[4]	Weight, kg	kcal/kg BW	DE, Kcal/kg	DMI, kg	DM, %	CP, g	Crude Fat, g
May									
A[2]	5.4 ± 0.3	2.3 ± 0.2	495.3 ± 2.1	36.3 ± 0.9	17834.9 ± 16.6	7.5 ± 0.3	1.5 ± 0.2	933.2 ± 7.5	308.4 ± 2.3
B[3]	5.8 ± 0.3	2.2 ± 0.4	490.6 ± 2.2	36.9 ± 1.6	18327.8 ± 45.9	7.7 ± 0.9	1.6 ± 0.3	1023.2 ± 11.8	199.1 ± 4.3
July									
A[2]	6.1 ± 0.3	2.3 ± 0.1	507.7 ± 2.2	37.4 ± 1.0	18799.6 ± 17.8	7.9 ± 0.3	1.6 ± 0.2	996.1± 6.8	316.7 ± 2.6
B[3]	6.4 ± 0.2	2.3 ± 0.2	498.6 ± 1.9	41.3 ± 1.5	20647.8 ± 38.6	8.8 ± 0.7	1.8 ± 0.3	1123.1 ± 10.5	231.3 ± 5.1

[1]Measurement: reported in mean ± SEM; feedstuffs reported in DM
Treatement: [2]A = treated; [3]B = non-treated
[4]NCS = neck condition score

Table 3.7a: Predicted vs. Actual Intake Values of 2010
Diet

	Daily Dietary Intake			P-value		
Item	Predicted	Actual	SEM	TRT	TIME	TRT*TIME
DE, kcal				< 0.0001	= 0.567	< 0.017
February	16162.00 ± 1163.90	17847.00 ± 1126.52	1145.21	-	-	-
March	16453.00 ± 824.53	17320.00 ± 824.53	824.53	-	-	-
April	16097.00 ± 771.28	17728.00 ± 771.28	771.28	-	-	-
May	16148.00 ± 771.28	19812.00 ± 771.28	771.28	-	-	-
June	16419.00 ± 795.19	20939.00 ± 771.28	783.23	-	-	-
July	15329.83 ± 16.14	18448.00 ± 890.59	453.36	-	-	-
Cal/kg BW				< 0.0001	= 0.083	= 0.060
February	32.50 ± 1.80	36.31 ± 1.86	1.83	-	-	-
March	32.87 ± 1.32	34.53 ± 1.32	1.32	-	-	-
April	32.50 ± 1.23	35.68 ± 1.23	1.23	-	-	-
May	32.64 ± 1.23[a]	40.02 ± 1.23[b]	1.23	-	-	-
June	32.55 ± 1.23[a]	41.20 ± 1.27[b]	1.25	-	-	-
July	32.05 ± 1.42[a]	37.79 ± 1.45[b]	1.44	-	-	-
CP, g				< 0.0001	= 0.467	= 0.468
February	608.24 ± 77.19[a]	1022.50 ± 77.19[b]	77.19	-	-	-
March	614.98 ± 54.58[a]	948.83 ± 54.58[b]	54.58	-	-	-
April	601.60 ± 51.05[a]	972.15 ± 51.05[b]	51.05	-	-	-
May	604.31 ± 51.05[a]	1067.47 ± 51.05[b]	51.05	-	-	-
June	615.97 ± 52.68[a]	1112.95 ± 51.05[b]	51.87	-	-	-
July	583.95 ± 60.18[a]	926.44 ± 58.95[b]	59.57	-	-	-
Calcium, g				< 0.0001	= 0.535	= 0.578
February	21.22 ± 6.98[a]	75.01 ± 6.74[b]	6.86	-	-	-
March	21.51 ± 4.93[a]	72.75 ± 4.93[b]	4.93	-	-	-
April	21.22 ± 4.62[a]	75.41 ± 4.62[b]	4.62	-	-	-
May	21.24 ± 4.62[a]	82.10 ± 4.62[b]	4.62	-	-	-
June	21.56 ± 4.77[a]	85.25 ± 4.62[b]	4.69	-	-	-
July	20.23 ± 5.33[a]	68.31 ± 5.33[b]	5.33	-	-	-
Phosphorus, g				< 0.0001	= 0.197	= 0.148
February	13.88 ± 1.58[a]	24.26 ± 1.53[b]	1.55	-	-	-
March	14.00 ± 1.12[a]	22.96 ± 1.12[b]	1.12	-	-	-
April	13.82 ± 1.05[a]	23.78 ± 1.05[b]	1.05	-	-	-
May	13.83 ± 1.05[a]	26.60 ± 1.05[b]	1.05	-	-	-
June	14.01 ± 1.08[a]	28.00 ± 1.05[b]	1.06	-	-	-
July	13.17 ± 1.21[a]	24.54 ± 1.21[b]	1.21	-	-	-
Magnesium, mg/kg				< 0.0001	= 0.258	= 0.112
February	6.97 ± 0.77[a]	15.47 ± 0.77[b]	0.77	-	-	-
March	7.50 ± 0.56[a]	14.97 ± 0.56[b]	0.56	-	-	-
April	7.40 ± 0.52[a]	14.86 ± 0.52[b]	0.52	-	-	-
May	7.37 ± 0.53[a]	16.36 ± 0.52[b]	0.53	-	-	-
June	7.50 ± 0.54[a]	17.29 ± 0.52[b]	0.53	-	-	-
July	7.06 ± 0.61[a]	15.68 ± 0.61[b]	0.61	-	-	-

[a, b] Values within rows lacking common superscripts differ by $P \leq 0.05$
[A,B] Values within columns lacking common superscripts differ by $P \leq 0.05$

Table 3.7b: Predicted vs. Actual Intake Values of 2010 Diet

	Daily Dietary Intake			P-value		
Item	Predicted	Actual	SEM	TRT	TIME	TRT*TIME
Copper, mg/kg				< 0.0001	= 0.086	< 0.029
February	85.46 ± 8.00^a	138.78 ± 7.73^b	7.86	-	-	-
March	86.04 ± 5.66^a	136.27 ± 5.66^b	5.66	-	-	-
April	84.89 ± 5.29^a	138.54 ± 5.29^b	5.29	-	-	-
May	84.96 ± 5.29^a	156.86 ± 5.29^b	5.29	-	-	-
June	85.92 ± 5.46^a	164.93 ± 5.29^b	5.38	-	-	-
July	84.64 ± 6.24^a	153.57 ± 6.11^b	6.17	-	-	-
Zinc, mg/kg				< 0.0001	= 0.413	= 0.277
February	371.74 ± 29.35^a	468.94 ± 29.35^b	29.35	-	-	-
March	400.17 ± 21.48^a	461.26 ± 21.48^b	21.48	-	-	-
April	394.82 ± 20.09^a	460.33 ± 20.09^b	20.09	-	-	-
May	395.18 ± 20.09^a	511.66 ± 20.09^b	20.09	-	-	-
June	398.77 ± 20.73^a	531.17 ± 20.09^b	20.41	-	-	-
July	376.45 ± 23.20^a	502.78 ± 23.20^b	23.20	-	-	-

[a, b] Values within rows lacking common superscripts differ by $P \leq 0.05$
[A,B] Values within columns lacking common superscripts differ by $P \leq 0.05$

Table 3.8: Climate

Item	A, °C	B, °C	C, °C	D, °C	E, °C	F
			Climate Temperatures[1]			
February	4.8	-7.5	-1.4	10.5	-15.0	14
March	13.4	-1.8	5.7	26.1	-12.2	2
April	16.4	2.5	9.4	24.4	-3.8	0
May	19.8	5.6	12.7	32.7	-1.1	0
June	27.6	12.6	20.1	36.1	6.1	0
July	31.3	14.5	22.8	36.1	10.0	0

Climate Temperatures[1]= Produced by the Colorado Climate Center;
http://ccc.atmos.colostate.edu/dataaccess.php
A= Average monthly maximum temperature
B= Average monthly minimum temperature
C= Average mean monthly temperature
D= Maximum temperature reached throughout the day
E= Minimum temperature reached throughout the day
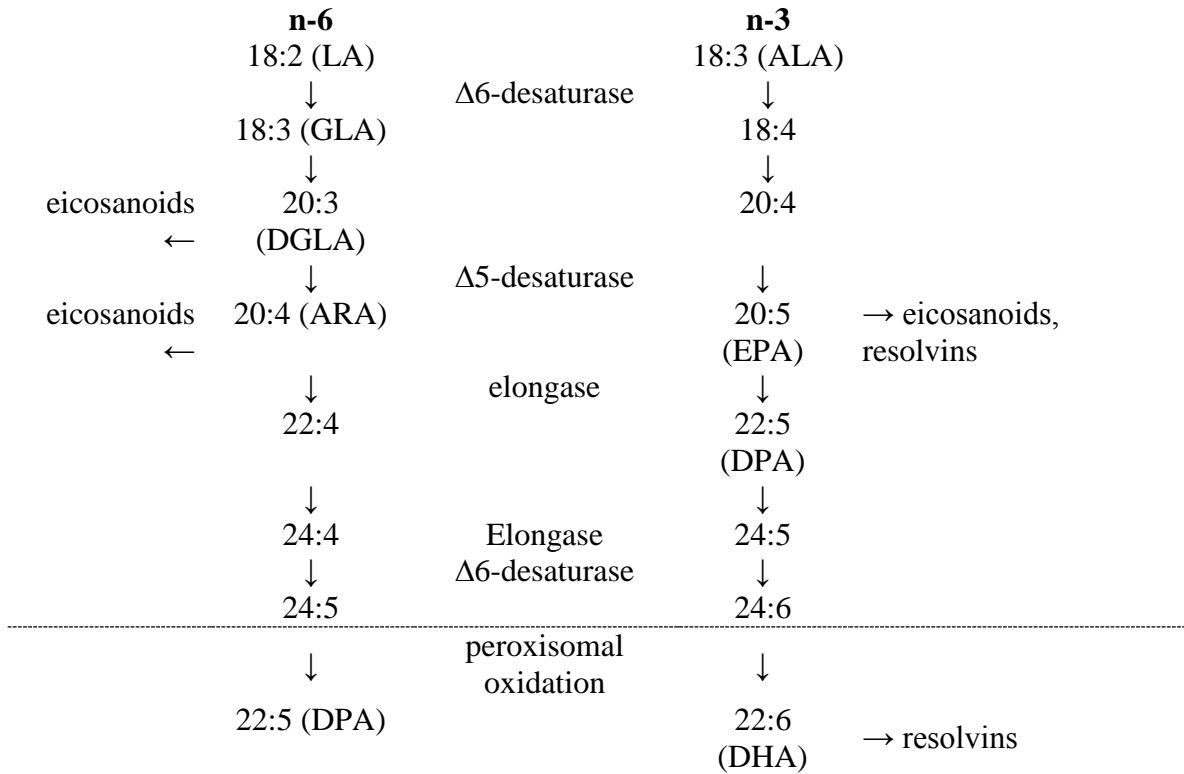F= Number of days at or below freezing temperature

```
          n-6                                    n-3
        18:2 (LA)                             18:3 (ALA)
            ↓           Δ6-desaturase              ↓
        18:3 (GLA)                                18:4
            ↓                                      ↓
eicosanoids   20:3                                20:4
    ←       (DGLA)
            ↓           Δ5-desaturase              ↓
eicosanoids  20:4 (ARA)                           20:5      → eicosanoids,
    ←                                            (EPA)        resolvins
            ↓             elongase                 ↓
          22:4                                    22:5
                                                 (DPA)
            ↓                                      ↓
          24:4          Elongase                  24:5
            ↓           Δ6-desaturase              ↓
          24:5                                    24:6
--------------------------------------------------------------------------------
            ↓           peroxisomal                ↓
                         oxidation
        22:5 (DPA)                                22:6      → resolvins
                                                 (DHA)
```
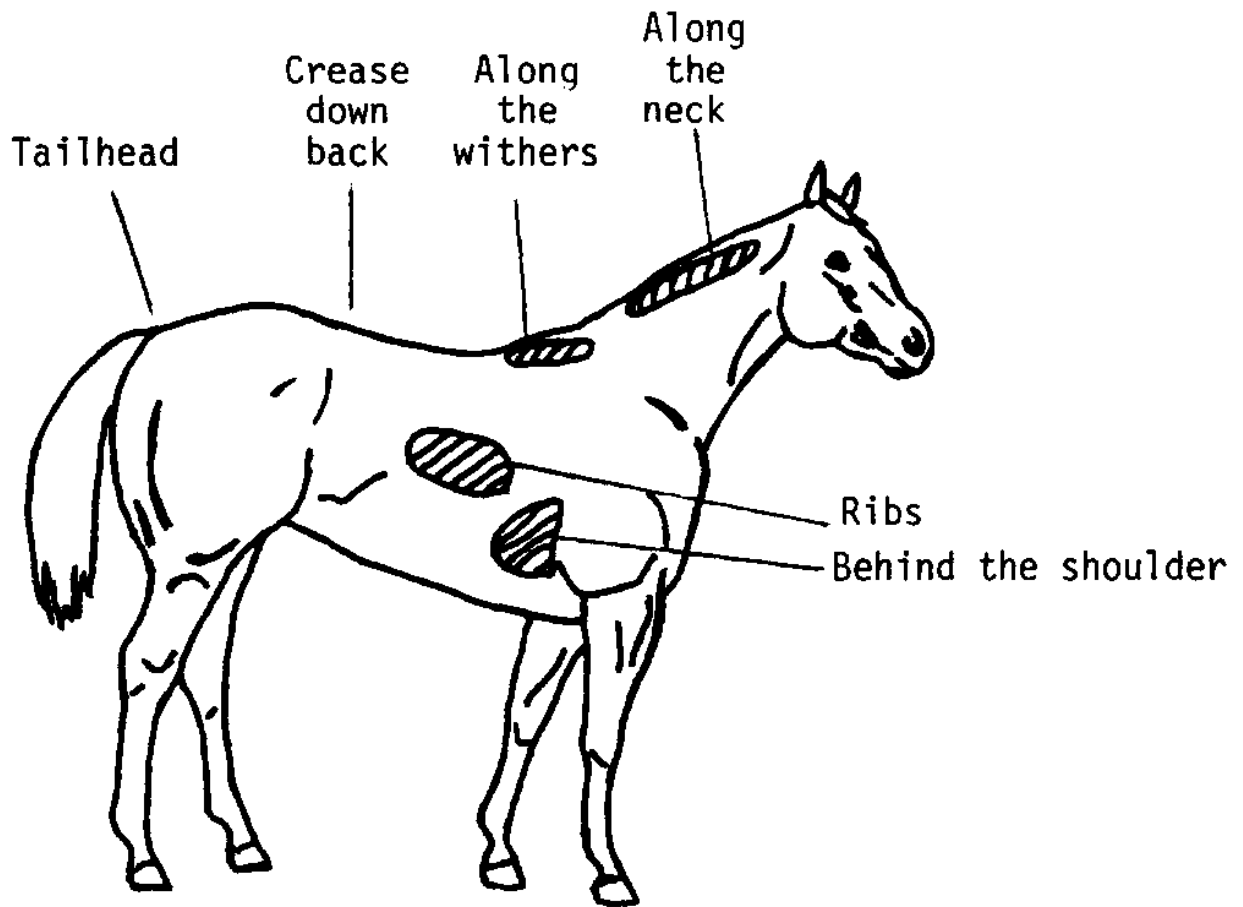
Figure 2.1: n-6 and n-3 fatty acid pathways

Figure 2.2: Regional Adiposity (Henneke et al., 1983)

Score	Description
1 Poor	Animal extremely emaciated. Spinous processes, ribs, tailhead, tuber coxes and ischia 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 ischia prominent. Withers, shoulders and neck structures fairly discernible.
3 Thin	Fat build up about halfway on spinous processes, transverse processes cannot be felt. Slight fat cover over ribs. Spinous processes and ribs easily discernible. Tailhead prominent, but individual vertebrae cannot be visually identified. Tuber coxae appear rounded, but easily discernible. Tuber ischia 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 spinous processes. Shoulders and neck blend smoothly into body.
6 Moderately Fleshy	May have slight crease down back. Fat over ribs feels 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 crease down back. Individual ribs can be felt, but noticeable filling between ribs with fat. Fat around tailhead is soft. Fat deposited along withers, behind shoulders and along the neck.
8 Fat	Crease down back. Difficult to feel ribs. Fat around tailhead very soft. Area along withers filled with fat. Area behind shoulder filled with fat. Noticeable thickening of neck. Fat deposited along the 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 thighs may rub together. Flank filled with fat.

Figure 2.3: Body Condition Score Chart (Henneke et al., 1983)

Score	Description
0	No visual appearance of a crest (tissue apparent above the ligament nuchae). No palpable crest.
1	No visual appearance of a crest, but slight filling felt with palpation.
2	Noticeable appearance of a crest, but fat deposited fairly evenly from poll to withers. Crest easily cupped in one hand and bent from side to side.
3	Crest enlarged and thickened, so fat is deposited more heavily in middle of the neck than toward poll and withers, giving a mounded appearance. Crest fills cupped hand and begins losing side to side flexibility.
4	Crest grossly enlarged and thickened, and can no longer be cupped in one hand or easily bent from side to side. Crest may have wrinkle s/creases perpendicular to topline.
5	Crest is so large it permanently droops to one side.
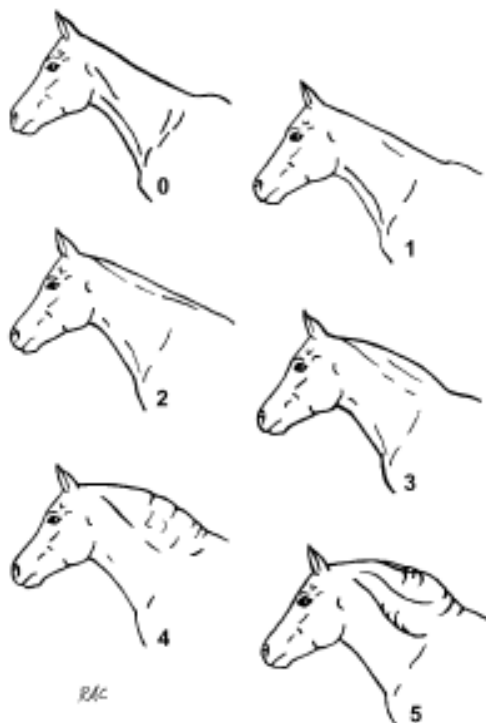
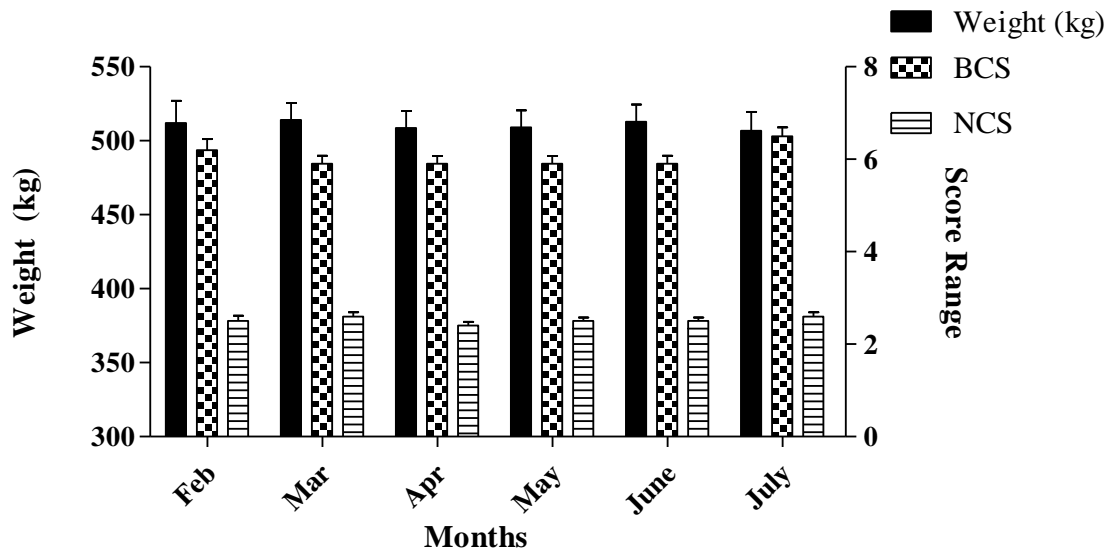**Figure 2.4: Neck Condition Score Chart** (Carter et al., 2009a)

Figure 3.7: 2010 Dietary Intake and Condition Scores
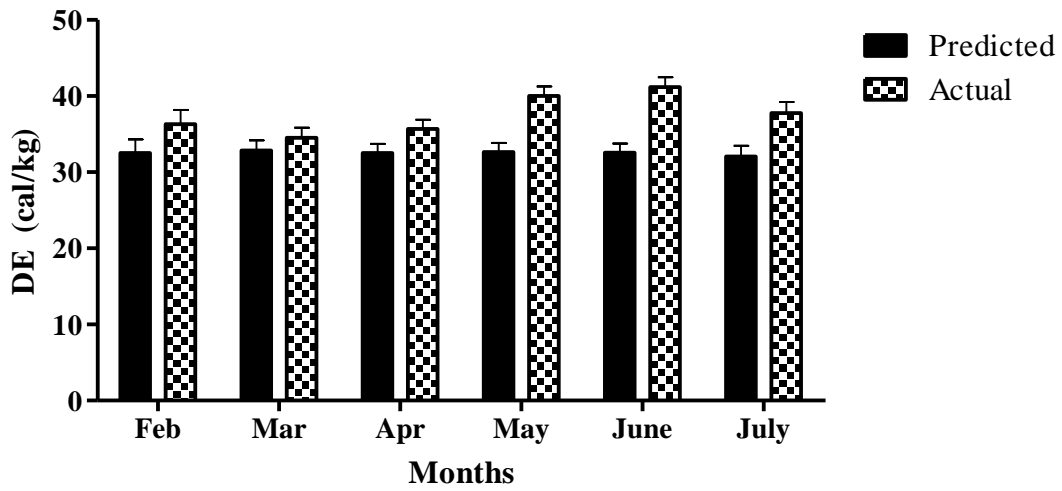
Average Monthly DE Intake

Figure 3.8: Average Monthly Predicted vs. Actual Digestible Energy Intake
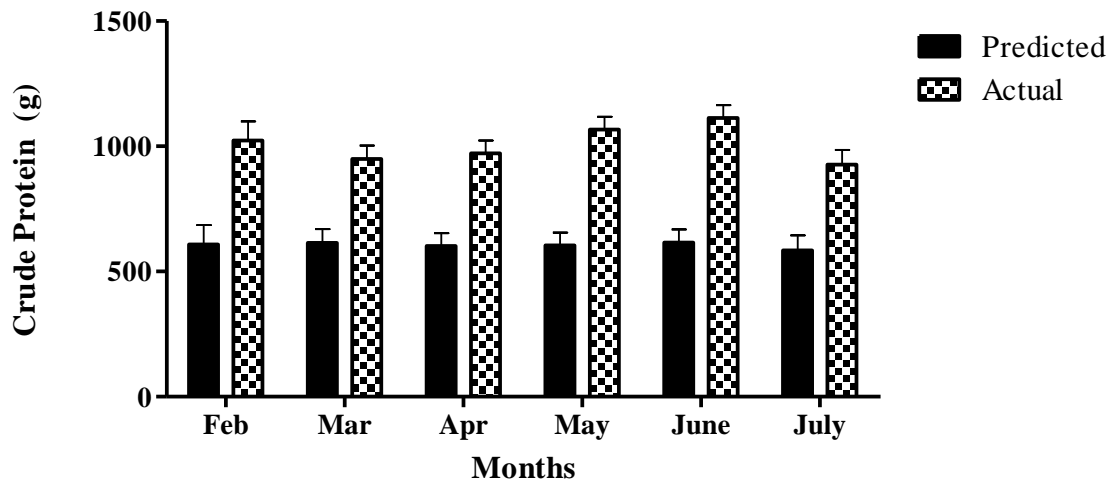
Average Monthly Crude Protein Intake

Figure 3.9: Average Monthly Predicted vs. Actual Crude Protein Intake

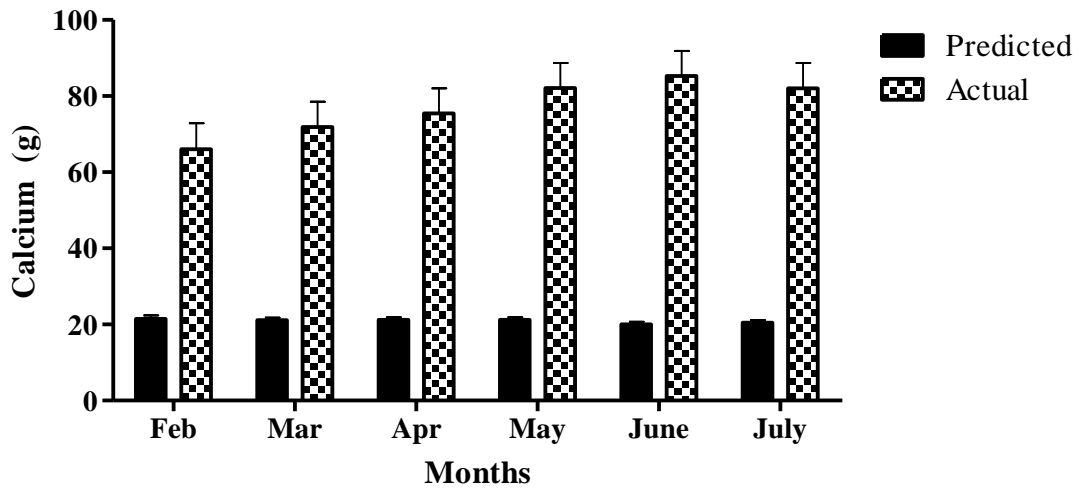**Average Monthly Calcium Intake**

Figure 3.10: Average Monthly Predicted vs. Actual Calcium Intake

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