LONG TERM AND SHORT TERM CHANGES IN LEPTIN, INSULIN AND GLUCOSE IN GRAZING THOROUGHBRED MARES

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(ABSTRACT)

The central objective of this dissertation was to evaluate the insulin-leptin interactions in grazing mares and their impact on aspects of reproductive function. A series of four studies was conducted to evaluate this objective. Blood samples were collected from mares for which pasture was the only source of nutrition, and compared to pasture kept mares supplemented with sugar and starch (SS) or fat and fiber (FF). Fourteen mares were first examined, 10 grazing and 4 confined to stalls in a series of four 22-h studies in April, August, October (2005) and January (2006). There was a positive relationship between plasma insulin and leptin concentrations ($r = 0.50$; $P < 0.001$). In the second study 24 mares maintained at pasture and fed supplements rich in either FF, or SS, or pasture forage only (P) had blood samples collected twice weekly from January to June to evaluate hormonal and metabolic patterns . Nonstructural carbohydrate (NSC) content of pasture forage was correlated to plasma insulin concentration ($r = 0.55$; $P < 0.01$). Plasma insulin was associated with plasma leptin ($r = 0.55$; $P < 0.001$) and plasma progesterone ($r = 0.48$; $P < 0.001$). In the third study nine mares adapted to FF, SS or pasture forage only underwent two frequently samples i.v. glucose tolerance tests; once during the luteal phase and again during the follicular phase of the estrous cycle. Minimal model analysis was used to describe insulin sensitivity (SI), glucose effectiveness (Sg), acute insulin response to glucose (AIRg) and the disposition index (DI). Progesterone was higher in the luteal phase (18.2 ± 1.8 ng/mL) when compared the follicular phase (5.5 ± 2.1 ng/mL; $P < 0.05$). Insulin sensitivity was lower ($P < 0.001$) in the luteal phase (3.1 ± 0.6) compared to the follicular phase (5.0 ± 0.6) of the estrous cycle. In the fourth study 15 mares adapted to FF, SS or pasture forage

had all of their accessible follicles ablated and fluid collected during the luteal and follicular phase of the estrous cycle. Insulin concentration (ln (x+1)) was 52% higher ($P < 0.01$) in large (> 25 mm) follicles (1.4 ± 0.1 mIU/L) than either medium (16 to 25 mm) or small (≤ 15 mm) follicles (0.9 ± 0.1; 0.9 ± 0.1 mIU/L, respectively) irrespective of estrous cycle phase. A correlation was observed between follicular fluid (FFL) leptin and plasma leptin (r = 0.30; $P < 0.001$). A similar relationship was observed between FFL insulin and plasma insulin (r = 0.25; $P < 0.001$). Plasma insulin and leptin were positively associated (r = 0.45, $P < 0.0001$), along with FFL insulin and FFL leptin (r = 0.46, $P < 0.0001$). Relationships between leptin and insulin were observed in the first two longitudinal studies and in the final study evaluating follicular fluid and plasma. This is the first study to evaluate this relationship in grazing mares and in follicular fluid. Both leptin and insulin were affected by season and this observation contradicts the use of single sample analysis for determining detrimental concentrations of these hormones.

Keywords: Mare, leptin, glucose, insulin, estrous cycle, ovary, season

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GLOSSARY OF TERMS

Nonstructural carbohydrates (NSC)-Sugars, starches and fructans that accumulate in plant cells and are then readily mobilized for metabolism or translocation to other plant parts.

Water-Soluble Carbohydrate (WSC)-The carbohydrate fraction in plants that is soluble in water including mono- and disaccharides and fructans.

Sugar- Mono- and di-saccharides, such as glucose and fructose, and sucrose respectively. Sugars are water soluble and hydrolysable.

Fructan- Collective term for all oligo- and poly-fructosyl sucrose that consists of one or more fructosyl-fructose links. I Fructans are water soluble. Animals do not have the enzymes to digest fructans so they are fermented by microbes in the hindgut

Starch- Polymer of glucose, composed of D-glucopyranose units with α (1→ 4) glycosidic links, readily hydrolyzed by digestive enzymes. Starches are not water soluble. Starches are reserve carbohydrates in forages, and the *primary* reserve carbohydrate in legumes.

Non-Fiber Carbohydrate (NFC)- Nonstructural carbohydrate fraction estimated by forage laboratories as NFC = 100- (CP % + (NDF % -NDICP %) + Fat % + Ash %)

Estrous Cycle- (also **oestrous cycle**; originally derived from Latin **oestrus**) comprises the recurring physiologic changes that are induced by reproductive hormones in most mammalian placental females. Estrous cycles start after puberty in sexually mature females and are interrupted by anestrous phases. The estrous cycle is made up of two phases: follicular and luteal.

Follicular Phase- refers to the "heat" stage of the cycle when the mares is receptive to the stallion's advances. During this phase follicles mature and it ends with ovulation. The main hormone controlling this phase is estradiol

Luteal Phase- begins following ovulation, with the formations of the corpus luteum and ends in either pregnancy or regression of the corpus luteum. The main hormone controlling this phase in progesterone.

Anestrus- Anestrus refers to the phase when the reproductive cycle rests. This is typically a seasonal event and controlled by light exposure through the pineal gland that releases melatonin. Anestrus is induced by time of year, pregnancy, lactation, nutritional status, significant illness, and possibly age.

Circannual-describes events (reproductive activity) that take place over the course of one year and repeats year after year.

CHAPTER ONE

INTRODUCTION

The horse has evolved as a grazing animal and has the ability to adapt to changes in environmental conditions, in particular, to forage availability and quality. In the mare this adaptive strategy has affected when she is reproductively active. Mares are considered seasonally polyestrous, where onset of the breeding season occurs in spring and is associated with increase in daylight, temperature, and availability of nutrients (forage). This strategy has ensured that offspring were born in favorable climatic and environmental conditions. Pasture is a pivotal part of the equine diet and changes in availability and quality are primarily driven by long term (photoperiod, temperature) and short term (fertilization) environmental conditions.

The primary energy components of forages are nonstructural carbohydrates (NSC) which are a combination of simple sugars, fructans and starches. It was shown that changes in pasture NSC directly relate to changes in circulating metabolites in the horse (Byrd et al., 2006). These changes in NSC and resulting perturbations in circulating hormones and metabolites, are implicated in the development of resistance and laminitis (Hoffman et al., 2003b; Kronfeld and Harris, 2003).

Leptin followed a seasonal pattern with circulating concentrations higher in summer than in winter (Buff et al., 2006). Fitzgerald and associates (2002) suggest a role of leptin in the regulation of seasonal reproductive activity. Leptin may act by indicating information on nutritional status of the animal to the brain. Another study showed that circulating leptin concentrations increased approximately 8 h after a constant insulin infusion or grain meal that elicits a marked insulinemic response (Cartmill et al.,

2005). These observations raise questions of the possible influence of high NSC pasture forage on circulating leptin in mares, and the implications for reproductive activity during the transition from seasonal anestrous to estrous. To date there are no published reports describing a relationship between circulating insulin and leptin in the grazing mare.

While a large number of horses have access to pasture, many are provided supplemental sources of dietary energy. Until recently these supplements were mainly concentrates of grain and molasses, which were high in hydrolysable carbohydrates (i.e., NSC; NAHMS, 1998). These diets elicited high glycemic and insulinemic responses which were associated with development of impaired insulin sensitivity (Hoffman et al., 2003a; Treiber et al., 2005a). Research has focused on substituting NSC with fat and fiber. These fat and fiber feeds elicit lower glycemic/insulinemic response compared to the response following a grain and molasses meal (Williams et al., 2001a).

It was clearly demonstrated that diet affects glucose and insulin metabolism in horses. Studies in horses have evaluated the affect of dietary energy source on glucose and insulin metabolism in different physiological states. Mares in late gestation and early lactation exhibit a more rapid clearance of glucose and lower insulin sensitivity when fed a diet high in hydrolysable carbohydrates (SS) compared to fat and fiber (FF; Hoffman et al., 2003b). Evidence in other species suggested that stage of estrous cycle can influence glucose and insulin dynamics (Robinson et al., 1993; Scaramal et al., 1997). In women insulin sensitivity is decreased during the luteal phase of the estrous cycle (Pulido and Salazar, 1999). Interestingly, circulating leptin was altered depending on the stage of menstrual cycle, with increased concentrations observed in the luteal phase compared to the follicular phase (Hardie et al., 1997).

Diet in other species affected follicular fluid concentrations of hormones, oocyte quality, and fertility. Injection of glucose and insulin directly into the ovarian artery resulted in decreased estradiol in follicular fluid (Downing et al., 1999), which caused inhibition of LH-stimulated steroidogenesis by the ovarian follicle which occurs in the absence of any detectable changes in circulating plasma concentrations of FSH. Cyclic ewes fed a high-energy, high-protein diet demonstrated increased plasma insulin and glucose concentrations along with elevated follicular fluid insulin and glucose concentrations (Somchit et al., 2007) and follicular fluid glucose was correlated to plasma glucose. This indicated a relationship between diet and follicular environment. In women serum leptin was correlated to follicular fluid leptin concentrations (Butzow et al., 1999).

The central focus of this dissertation was to evaluate the insulin-leptin interactions in grazing mares and their impact on aspects of reproductive function. Blood samples were taken from mares for which pasture was the only source of nutrition, and compared to pasture kept mares supplemented with sugar and starch (SS) or fat and fiber (FF).

CHAPTER TWO

REVIEW OF LITERATURE

SEASONALITY IN MARES

Animals have developed many strategies for seasonal breeding that ensure that their offspring are born in favorable climatic conditions and environmental conditions. The annual change in photoperiod is the primary environmental cue for timing of the reproductive cycle (Kooistra and Ginther, 1975). This environmental signal is translated to an endocrine signal in the pineal gland, which secretes melatonin during the phase of darkness. In the mare, short day length is associated with a decreased gonadotropin secretion and consequently decreased ovarian activity (Sharp and Ginther, 1975).

The horse is seasonally polyestrous, where onset of the breeding season occurs in spring and is associated with increase in daylight, temperature, and availability of nutrients (forage). The natural breeding season occurs from April to September in the Northern Hemisphere (Ginther et al., 1972). The arbitrary birth date in horses (Jan 1st, Northern Hemisphere, Aug 1st Southern Hemisphere; Ginther, 1992) has stimulated researchers to determine the mechanisms of reproductive seasonality in mares and develop methods for induction of an early onset of the breeding season. Most of the work has focused on photoperiod and it was demonstrated that artificial photoperiod, simulating long days, can advance the time of the first ovulation in mares (Guillaume and Palmer, 1991).

Photoperiod

It is well documented that photoperiod is one of the most important external factors that influences the circannual endogenous reproductive rhythm in the horse (Fitzgerald and McManus, 2000; Ginther, 1992; Guillaume and Palmer, 1991; Kooistra and Ginther, 1975). Additional light exposure during winter and early spring stimulates ovarian activity in anestrous mares and was commonly used to advance the onset of the breeding season (Sharp et al., 1975). The retina of the eye is stimulated by light. The photoreception is transferred by the optic nerve to the suprachiasmatic nucleus. From here, signals travel to the superior cervical ganglion (Karsch et al., 1986). These presynaptic neurons cause the postganglionic neurons to fire. These neurons synapse with inhibitory neurons that make contact with cells in the pineal gland (pinealocytes). The pinealocytes secrete the hormone melatonin (Sharp and Ginther, 1975). During the daylight hours (increased photostimuli) the light sensed by the retinal cells of the eye activates an excitatory neural pathway at the level of the pineal gland where inhibitory neurons continue to fire, thus inhibiting the release of melatonin from pinealocytes. Melatonin inhibits GnRH secretion (Vanecek, 1999) therefore, increased day-length, which inhibits melatonin, causes an increase in GnRH secretion, and thus promotes cyclicity. The opposite occurs in response to decreased day-length (photoperiod) during which the increased synthesis and secretion of melatonin inhibits cyclicity (Sharp and Ginther, 1975).

Ambient Temperature

A 10-year survey of breeding records of one Thoroughbred farm reported significant difference between years in the time of the first ovulation. The onset of reproductive activity was closely related to minimum and maximum environmental temperatures. The minimum (0°C) and maximum (20°C) temperatures in the weeks immediately prior to the first ovulation were similar in all years (Nagy et al., 2000). Field data for thoroughbred mares in the UK suggest that the spring transition is slowed by cold weather (Allen, 1987). Thus, it appears that under similar conditions of photoperiod, nutrition and management system, ambient temperature plays a role in the timing of the circannual reproductive rhythm.

Nutrition and Body Condition Score

The effect of nutrition and body condition on seasonal reproduction in mares was described by several authors (Henneke et al., 1983; Robinson, 1996). Mares that receive supplementary dietary energy above maintenance requirements ovulate earlier after winter anestrous than control mares without supplementation (Ginther and Wentworth, 1974). In mares with a body condition score < 5.0 on a scale of 1 to 9, compared to mares with a condition score > 5.0 had an interval to first ovulation was significantly longer during the spring transition from winter anestrus to reproductive cyclicity (Henneke et al., 1984). Conversely, obese mares continued to cycle through the winter anestrous period (McManus and Fitzgerald, 2003). The impact of energy intake and body condition on the reproductive performance of non-pregnant mares was further demonstrated in a study where high-energy intake shortened the interval to first ovulation

in thin transitional mares, but does not benefit mares in moderate or fat body condition. Mares with a body fat content greater than 15% of body weight had shorter interval to first ovulation compared to those with a body fat content lower than 15% (Kubiak et al., 1987). The quality of dietary protein influenced the onset of the breeding season. Animals receiving a high-quality protein diet had increased FSH secretion and ovulated approximately 3 to 6 wk earlier than mares on low-quality protein diet (van Niekerk and van Niekerk, 1997). The stimulatory effect of pasture grazing on the time of first ovulation has been reported. First ovulation occurred over a longer period of time in Thoroughbred mares that were housed inside at night and were allowed to eat grass on pasture for 4 to 6 h/d when compared to pony mares that were kept in concrete yards during winter, but then allowed access to lush spring grass (Allen, 1987; Carnevale et al., 1997). Anestrous mares pastured on green grass from early May ovulated sooner than mares housed on dry lot and fed hay (Carnevale et al., 1997).

INSULIN

Insulin is a 51 amino acid peptide hormone with a molecular weight of 5808 Da. It is produced in the in the pancreas and controls the uptake of glucose into the cells. Increased plasma glucose concentration stimulates the β-cells of the pancreas to secrete more insulin into the bloodstream (Holley and Evans, 1979). Plasma insulin eventually enters the interstitial fluid where it binds to insulin receptors at the cell membrane and stimulates GLUT-4 insulin-dependant glucose transporters to aggregate at the plasma membrane as shown in human (Farese, 2001). Insulin may also increase the glucose-shuttling activity of GLUT-4 transporters as has been shown in rat, mouse and human

muscle cells (Furtado et al., 2002). As a consequence, glucose is more efficiently transported from the plasma compartment into the cells, reestablishing normo-glycemia. Insulin stimulates bovine granulosa and thecal cell proliferation, as well as steroidogenesis in vitro (Spicer and Echternkamp, 1995). The effects of insulin on estrogen production by granulosa cells seem to depend on the species studied. In rats and primates, studies indicate that insulin can stimulate granulosa cell estradiol production in vitro (Spicer and Echternkamp, 1995). Hyperinsulinemia, however is associated with impaired oocyte quality in ruminants (Adamiak et al., 2005).

LEPTIN

Leptin is a protein encoded by the Ob gene in adipose cells and thought to play a role in the regulation of food intake and metabolism. This adipocyte derived hormone plays a key role in body weight homeostasis. Leptin has emerged as a neuroendocrine mediator in several systems, including the reproductive axis. 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). Interest in the role of leptin in reproduction was initiated after the demonstration that infertile ob/ob mutant mice, which lack the ability to produce leptin, could be made fertile with leptin injections (Chehab et al., 1996; Chehab et al., 1997).

An apparent association between leptin and reproductive activity in horses was inferred from the observation that in mature mares, higher amounts of body fat were 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 high in horses between 5 and 12 yr compared to those < 5 yr and are higher in stallions, but not different between gelding and mares. Fat 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 high body condition scores 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.

Fitzgerald et al. (2002) administered recombinant human leptin (50 μg/kg) to lean, anestrous mares and used plasma FSH as an index of GnRH secretion. This treatment did not result in FSH secretion; however, a similar dose of leptin administered to feed restricted, steroid treated wethers resulted in a marked increase in pulsatile gonadotrophin secretion (Nagatani et al., 2000). Fitzgerald et al. (2002) hypothesized that one aspect of the long-term regulation of seasonal reproductive rhythms, specifically in anestrous mares, was the recognition of the availability of metabolic fuels before perception of a change in photoperiod. Therefore, energy availability may need to reach a critical value before presumptive inhibitory day-length signals initiate termination of the breeding season.

Leptin and its actions were linked with insulin. An increase in insulin prompted an increase in leptin in both humans and rats (Cusin et al., 1995; Saladin et al., 1995). Cartmill et al, (2003b) 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 diabetics. 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. It is likely that studies related to the role of leptin will improve our understanding of the interaction between nutrition and seasonality.

NUTRIENT COMPOSITION OF FORAGES

Pasture forage plays a pivotal role in equine nutrition. About 80% of the horses in Virginia receive all or part of their nutrition from pasture (NAHMS, 1998). Pasture plant nutrient composition is highly variable. It is affected by plant species, soil fertility, climate, stage of growth and management practices (Blaser, 1986). Plant growth is most rapid in spring between the months of April and May. Plant crude protein (CP) content peaks in the spring and in the fall and optimal concentrations in pasture for growing horses and broodmares occurs at a mean ambient temperature of approximately 12°C (Cubitt et al., 2007). Seasonal variation in CP concentration may be caused in part by differences in day length. The CP concentration in forage is decreased by high light intensity (Bathurst and Mitchell, 1958), and this reduction was associated with an increase in forage yield and dilution of the available CP. Variation in the pasture due to season occured as grasses grow from leafy to stemmy stages. With advancing plant maturity there are increases in fiber and lignin and decreases in CP and non-structural carbohydrates (NSC; Blaser, 1986). A study summerizing 5 consecutive years of pasture nutrient analysis data at the MARE Center revealed dry matter (DM) content of the

pasture was highest in the winter, lowest in spring and autumn and moderate in the summer. Patterns of minerals, including phosphorus, zinc, copper and calcium revealed no consistent seasonal pattern. However, NSC content followed a similar pattern to CP and DM, with highest values in spring compared to summer and winter, and intermediate values in the autumn (Cubitt, 2004). This seasonal pattern in NSC was reported by others (Longland, 1999; McIntosh, 2007). Seasonal changes in pasture NSC impact hormonal and metabolite regulation in grazing horses (Byrd et al., 2006; Staniar et al., 2007). The rise in pasture NSC was associated with a rise in circulating insulin concentrations (Byrd et al., 2006) and was related to the onset of disease in particular insulin resistance (Treiber et al., 2006b) and laminitis (Kronfeld et al., 2006); and therefore, pasture NSC will be the focus for this section of nutrient availability in forages.

Pasture Carbohydrate Composition

Forage plants produce simple sugars through the biochemical process known as photosynthesis. During photosynthesis, plants take in carbon dioxide from the atmosphere, and use sunlight for energy to produce simple sugars, which are used for growth and reproduction. When sugar production exceeds the intermediate energy requirement of the plant for growth and development, it is converted into storage forms of carbohydrates within the vegetative tissues. Cool-season pasture grasses accumulate fructan as their storage carbohydrate (Longland et al., 1999). Fructan is stored in the vacuoles of the leaves and transported to vacuoles of the stem, where it is stored until the plant needs it as an energy source. Starches are the primary storage carbohydrate of

warm-season grasses and legumes; these plants do not produce fructans (Bailey et al., 2004; Chatterton, 1989).

Circadian utilization of the sugars produced from photosynthesis typically results in lowest NSC content in the early morning, a peak in the late afternoon, and a decline in NSC content overnight (Bowden, 1968; Holt, 1969; Longland, 1999; McIntosh, 2007). Seasonal variations in NSC content of grasses and legumes results in concentrations highest in late spring, lowest in the summer and winter, and intermediate in autumn (McIntosh, 2007; Smith, 1973; Waite, 1953).

Factors Affecting Pasture NSC

Studies have shown that environmental conditions can lead to significant fluctuations in the amounts of NSC that accumulate in forage plants. The water soluble carbohydrate (WSC) content, which is comprised of simple sugars and fructan, of a given plant species ranged from 95 to 560 g/kg DM with corresponding fructan amounts of 32 to 439 g/kg DM, depending on the temperature at which it was grown, with higher and lower values being associated with cooler (5 to 10º C) and warmer (15 to 25º C) temperatures, respectively (Chatterton, 1989). Light intensity, or solar radiation, affects the NSC content of forages, thus shading *Phalaris aqautica* L. pastures for an average of 43 h resulted in NSC contents of 62 and 126 g/kg NSC DM for shaded vs. unshaded pastures, respectively (Ciavarella, 2000).

DIETARY ENERGY SOURCE: METABOLIC AND HORMONAL RESPONSES IN THE HORSE

It has been demonstrated in grazing horses that a positive association exists between plasma insulin concentration and the NSC content of pasture exists (Byrd et al., 2006; Staniar et al., 2007). While a large proportion of horses have access to pasture, over 90% are provided a supplementary dietary energy source and almost 60% of these supplements are based on concentrates of grain and molasses, which contain hydrolysable carbohydrates (NAHMS, 1998). Meals containing high percentages of a grain-based concentrates were associated with increased plasma glucose and insulin, and insulin resistance (Treiber et al., 2005a; Williams et al., 2001a). Research has focused on substituting fat in place of carbohydrates as a source of concentrated energy and incorporating fiber to complement the horse's natural diet (Hoffman and Kronfeld, 1999; Kronfeld, 1996).

These FF supplements are designed to mimic the nutrient profile of pasture, providing concentrated energy and essential vitamins and minerals without upsetting the natural digestion and metabolism of the horse. Sugar and starch vs. FF results in very different post feeding glycemic and insulinemic responses. Thoroughbred mares fed a FF had a lower glycemic/insulinemic response compared to the response following an SS meal (Williams et al., 2001a).

Horses supplemented with meals rich in hydrolysable carbohydrates displayed postprandial increases in plasma glucose and insulin concentration in circulation (Treiber et al., 2005a). Insulin resistance may develop with chronic adaptation to meals high in SS, resulting from the effects of repeated large fluctuations in glycemia and insulinemia after these meals (Hoffman et al., 2003a; Treiber et al., 2005a). Insulin resistance

affected numerous physiological states including the pregnancy (Bell and Bauman, 1997), training (Pratt et al., 2005) and stage of estrous cycle (Pulido and Salazar, 1999), and was implicated in the onset of disease, specifically laminitis in the horse (Treiber et al., 2006a).

EQUINE ESTROUS CYCLE

Hormones from the hypothalamus, pituitary, ovary and uterus control the dynamic changes in the reproductive tract and sexual behavior through complex interactions. The mare's estrous cycle the period between one ovulation and the next, is about 21 d and can be divided into 2 phases: the follicular phase and the luteal phase. During the follicular phase, FSH stimulates the development of a dominant follicle which secretes estrogen that, in turn, causes the mare to be receptive to the stallion. Luteinizing hormone is produced in the pituitary and is secreted during the follicular phase and suppressed during the luteal phase. Peak concentrations occur slightly after ovulation. The luteal phase occurs after ovulation, where the ruptured follicle develops into a corpus luteum (CL) which secretes progesterone and causes a cessation in receptive behavior toward the stallion. The end of the luteal phase is marked by a surge in prostaglandin $F_2\alpha$ which causes luteolysis (regression of the CL) 10 to 15 d after ovulation (Daels and Hughes, 1993).

Follicle Structure and Growth

The follicle is the fundamental structural and functional unit of the ovary and has both endocrine and exocrine functions. The activities of the ovaries are controlled by

hormones released into the circulation, local intercellular diffusion of substances, and auto-regulation by release of substances that bind to the cell's own receptors (Pierson, 1992). The ovarian follicle consists of the oocyte enveloped within a supportive layer of granulosa cells and surrounded by an adjacent layer of thecal cells. The primordial follicle is defined as the oocyte surrounded by a single layer of granulosa cells. As the follicles grow, the quantity of granulosa cells multiply, signifying the transformation of the primary follicle to a secondary follicle (collectively referred to as preantral follicles). Small pockets of fluid secreted from granulosa cells accumulate and mark the formation of an antrum and the transition from secondary follicle to tertiary follicle (Pierson and Ginther, 1985).

Further development of the follicle is marked by a dramatic increase in volume of the antrum and differentiation and thickening of the follicular wall (Stabenfeldt et al., 1972). Follicle stimulating hormone secreted from the anterior pituitary gland acts on the ovaries, stimulating follicular growth and development. Its action is via binding to FSH receptors on granulosa cells, stimulating the release of estradiol. Preantral follicles acquire receptors for LH in the thecal cell membranes and for FSH in the granulosa cell membranes. A critical point in the life of the follicle is the acquisition of membrane receptors for LH by the granulosa cells.

Luteinizing hormone receptors on granulosa cells of the pre-ovulatory follicle allow the follicle to respond to the pre-ovulatory rise in LH (Ginther et al., 2001). These estrogen induced LH receptors prepare the follicle for the final stages of preovulatory follicular maturation. These dominant follicles have approximately 500- to 1000-fold more estrogen than small follicles.

After ovulation, the granulosa cells begin to luteinize and by 3 d following ovulation a CL will be completely formed (van Niekerk et al., 1973). Luteinizing hormone supports the lifespan of the CL during the estrous cycle in the mare. The CL produces progesterone, which increases receptors for LH in the CL. Progesterone concentrations increase and reach maximal secretion by d 9, which corresponds to the maximum size of the CL. Mares are unique in that they exhibit considerable follicular development during the luteal phase. The majority of follicles that develop during this phase will become atretic and regress during or at the end of the luteal phase (Niswender, 2002).

Effect of Diet on the Estrous Cycle and Folliculogenesis

Exogenous administration of insulin during the follicular phase enhanced ovulation rate and increased the episodic release of LH in gilts (Cox et al., 1994). Insulin binding sites were identified on pig granulosa cells in vitro (Otani et al., 1985) and insulin increased gonadotrophin-stimulated steroidogenesis by granulosa cells in vitro (May and Schomberg, 1981).

High energy and high protein supplementation in cyclic ewes increased plasma concentrations of insulin and glucose and the follicular fluid concentrations of glucose in some, but not all follicles (Somchit et al., 2007) . Both the infusion of glucose and the feeding of a supplement of lupin grain suppressed estradiol secretion during the follicular phase of the estrous cycle and this appears to be a direct effect on the follicle because it took place in the presence of either unchanged or slightly increased concentrations of FSH.

It has been established in several species, including horses, that diet can affect circulating concentrations of glucose and insulin (Holemans et al., 2004; Williams et al., 2001a). Several studies have demonstrated alterations in glycemic and insulinemic regulation during the luteal phase of the menstrual cycle in women with insulin-dependent diabetes mellitus (Moberg et al., 1995; Widorn et al., 1992). 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). No such studies exist in the horse regarding the characterization of insulin sensitivity during the estrous cycle in mares fed different levels of energy as well as different sources of energy. To date the only study describing the effect of insulin resistance on equine estrous reported a lengthened luteal phase. Mares were induced into transient insulin resistance using an infusion of a heparinized lipid solution, and the length of the luteal phase of their estrous cycle was marginally longer then control mares (Sessions et al., 2004; Vick et al., 2006). Leptin may serve as a signal of nutritional status and may act directly on the brain or may possibly have a direct effect on the ovary, as leptin receptors were found on the pig ovary (Mendoza et al., 2002).

Many of the hormonal systems that respond to nutrition affect the ovary and it is from among these that several research teams around the world are seeking to unravel the mechanisms that link nutrition and the follicle. Contenders among many for the “link” between nutrition and the follicle are the glucose-insulin system, and leptin system (Scaramuzzi et al., 2006).

OBJECTIVES

The specific objectives and hypothesis for the work contained herein were:

1. To examine the circadian and seasonal fluctuations of circulating leptin concentrations in relation to insulin in grazing horses and those fed hay, and to identify possible relationships between insulin, leptin and pasture carbohydrate content. Hypothesis: Our hypothesis was that there would be a relationship between plasma insulin concentration and plasma leptin in grazing mares and this relationship would be influenced by forage NSC content.

2. To characterize changes in circulating glucose, insulin and leptin concentrations in pastured mares during the transition from winter to spring and assess the possible association with changes in pasture forage NSC content. These observations were made in mares adapted to feeds rich in FF or SS and in mares maintained on pasture only. Our hypothesis was that the seasonal transition from winter to spring would result in increased NSC content and subsequent increases in plasma insulin and leptin concentration.

3. To characterize glucose and insulin dynamics using the minimal model approach in non-pregnant Thoroughbred mares during the luteal and follicular phases of the estrous cycle. A secondary objective was to determine whether supplementation with feeds rich in either SS or FF modified the influence of phase if estrous cycle on glucose and insulin dynamics. Hypothesis: Our hypothesis was that mares

would have decreased insulin sensitivity during the luteal phase compared to the follicular phase of the estrous cycle.

4. To compare effects of SS and FF feeds on plasma and follicular fluid concentrations of insulin, glucose, leptin, progesterone and estradiol in Thoroughbred mares during the luteal and follicular phases of the estrous cycle. Hypothesis: Our hypothesis was that feeding supplementary dietary energy in the form of SS would increase follicular fluid insulin and glucose. It was also hypothesized that concentration of insulin, glucose and leptin in follicular fluid may mimic plasma.

CHAPTER THREE
MANUSCRIPT 1

CIRCADIAN AND SEASONAL PATTERNS OF PLASMA LEPTIN IN GRAZING THOROUGHBRED MARES

ABSTRACT: The objectives of this study were to examine the circadian and seasonal patterns of plasma leptin concentrations in relation to circulating insulin in grazing horses and those fed hay, and to identify possible relationships between insulin, leptin and pasture carbohydrate content. Fourteen Thoroughbred mares were used in a study conducted in April, August, October 2005, and January 2006, each for a 22 h period. Ten mares were maintained on a 4.8 hectare pasture and 4 horses were housed in stalls. Hourly pasture and blood samples were taken for a 22 h period to measure carbohydrates in the forage and leptin and insulin in the plasma. Plasma leptin was higher ($P < 0.01$) in the grazing vs. hay fed mares in April (2.87 ± 0.1; 1.12 ± 0.2 ng/mL, respectively) and October (3.00 ± 0.1; 1.3 ± 0.2 ng/mL, respectively). In grazing mares plasma leptin was higher ($P < 0.05$) in April and October (2.87 ± 0.1; and, 3.0 ± 0.1 ng/mL, respectively) compared to August and January (1.98 ± 0.1; and, 1.91 ± 0.1 ng/mL, respectively). Plasma leptin in hay fed mares in April (1.12 ± 0.2 ng/mL) was only different ($P < 0.01$) from January (0.63 ± 0.2 ng/mL). Plasma leptin in hay fed mares in October was higher ($P < 0.01$) than leptin in August and January (October, 1.3 ± 0.2 > August, 0.94 ± 0.2 > January, 0.63 ± 0.2 ng/mL). Plasma insulin was higher in the grazing vs. hay fed mares in both April and October ($P < 0.001$). In grazing mares, plasma insulin concentration was higher ($P < 0.001$) in April (51.2 ± 0.8 mIU/L) than all other months (August, 11.1 ±

0.8; October, 13.8 ± 0.8; January, 11.5 ± 0.8 mIU/L). No differences ($P > 0.1$) in plasma insulin were detected between months for the hay fed horses. Plasma insulin concentrations were affected ($P < 0.01$) by pasture carbohydrates in all months except August (April, r = 0.66; October, r = 0.13; January, r = 0.47) in grazing mares. Correlations between plasma insulin and leptin in grazing mares revealed significant ($P < 0.001$) associations in all months (April, r = 0.69; August, r = 0.32; October, 0.37; January, r = 0.44). In conclusion a relationship between plasma insulin and leptin was apparent in all seasons in grazing mares. In spring and autumn plasma insulin and leptin where higher than in summer and winter, suggesting a seasonal effect on plasma insulin and leptin. This data provides evidence for a potential role of pasture forage carbohydrates and plasma insulin in the perturbations in circulating leptin in grazing mares.

Introduction

Seasonal adjustments in body composition and reproductive activity were demonstrated in many species including the horse (Kuntz et al., 2006). These seasonal changes may be due to alterations in hormonal signals that reflect metabolic fuel availability (Forcada and Abecia, 2006). Leptin was suggested as a primary signal linking body condition (adiposity) and the hypothalamus (Clarke and Henry, 1999).

Several studies in mares found differences in leptin concentrations between autumn, winter, and spring in relation to reproductive seasonality (Fitzgerald and McManus, 2000; Gentry et al., 2002). One study of Quarter horse mares showed a higher leptin concentration in the summer when compared to the winter (Buff et al., 2006).

Previous research at the MARE Center has shown seasonal patterns in pasture forage non-structural carbohydrate (NSC) content, where NSC is higher in the spring and autumn compared to the summer and winter (Hoffman et al., 2001) and these changes were directly related to changes in circulating insulin concentrations of mares grazing these pastures (Byrd et al., 2006). This relationship between pasture NSC and circulating insulin was implicated in the pathogenesis of laminitis (Kronfeld et al., 2006; Longland and Byrd, 2006).

Leptin secretion was stimulated by insulin (Patel et al., 1998). Recent studies in horses have indicated a positive relationship between plasma insulin and leptin following a grain meal or constant insulin infusion (Cartmill et al., 2005). Whether relationships exist between plasma insulin and leptin in grazing mares is yet to be established. Our hypothesis was that plasma leptin would rise in response to increases in plasma insulin, which were related to elevations in pasture NSC content.

Photoperiod influences seasonal changes in leptin concentration (Li and Wang, 2007). In lactating dairy cows, exposure to long photoperiod (18:6 h light:dark) increased circulating leptin and its receptor mRNA in adipose tissue. Short photoperiod (6:18 h light:dark) reduced expression of leptin receptor mRNA. These changes were not related to feed intake, adiposity or energy metabolism (Bernabucci et al., 2006). Studies in horses (Fitzgerald and McManus, 2000) and obese ponies (Buff et al., 2005) have provided evidence that continuous melatonin treatment does not alter plasma concentrations of leptin.

The objectives of this study were to examine the circadian and seasonal fluctuation of leptin concentrations in relation to insulin in grazing horses and those fed

hay, and to identify possible relationships between insulin, leptin and pasture carbohydrate content.

Materials and Methods

Horses

Fourteen Thoroughbred mares were used for a 22 h period, which was conducted in April, August, October 2005, and January 2006. Ten mares were housed on a 4.8 hectare pasture and 4 horses were housed in temporary 4 X 4 m stalls in a run-in shed within the same pasture, and offered timothy/alfalfa hay only. All horses had ad libitum access to white salt and fresh water. The horses were 11 ± 5 yr old (range 6 to 16 yr). Body weight of the horses over the 4 mo was 596.0 ± 14.5 kg, and body condition score was 6.2 ± 0.2 (range 4.5 to 7.5 on a scale of 1 to 9) (Henneke et al., 1983) (Table 3.1). The horses were acclimated to the pasture for 7 d before sampling began and the hay fed horses were placed in the stalls 36 h before each trial to acclimate. Hourly blood samples were collected from all 14 horses during the four 22 h trials. This study was approved by the Institutional Animal Care and Use Committee of Virginia Tech.

Pasture

The pasture consisted of 75% Max Q tall fescue, 20% Kentucky bluegrass, and 5% white clover. Forage samples were collected hourly from 0900 to 2200 the first day, then overnight at 2400, 0200, 0400, 0600 and 0700. Samples were collected by walking in a zig-zag pattern from edge to edge of the pasture and clipped every 5 m. Samples were submitted to a commercial laboratory for proximate analysis (Dairy One,

Ithaca, NY, USA). Sugar represents water soluble sugars (glucose, sucrose, fructose) that were extracted prior to analysis for starch (Hall et al., 1999) and included fructans. Starch content was determined following enzymatic digestion (glucoamylase) with measurement of dextrose in an automated biochemical analyzer (YSI 2700 SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA, Application Note Number 319) (Table 3.2).

Sample Collection and Analysis

On the morning (0500 h) of each trial, a jugular catheter (13 cm, 14 guage Milacath, Mila International Inc., Florence KY, USA) was inserted after aseptic preparation and desensitization (lidocaine 2%) of the overlying skin. Blood sampling began at 0930 and continued hourly until 0730 the second day. Blood samples were drawn from the catheter, and immediately transferred into two 7 ml tubes containing sodium heparin as an anticoagulant (Vacutainer; Fisher Health Care, Chicago, IL, USA) and placed on ice until centrifugation (3,000 x g for 10 min at 4°C). Plasma was removed within 30 min of collection and frozen at -20ºC until analysis. Plasma insulin (mIU/mL) was measured by a validated chemiluminescent immunoassay previously validated for horses (Staniar et al., 2007). Interassay CV for insulin was 5.6% and intraassay CV was 2.0%. Plasma leptin concentrations were analyzed by use of a previously validated multi-species leptin radioimmunoassay (Linco Research Inc., St. Charles, MO, USA) (Cartmill et al., 2003; McManus and Fitzgerald, 2000). Interassay CV for leptin was 4.1% and intraassay CV was 6.0%. All analyses were performed in duplicate. Ambient

temperature (ºC) and solar radiation (watts/m^{-2}) were measured and recorded hourly by an on site weather station (Texas Weather Instruments, Dallas, TX, USA).

Statistical analysis

Data for body weights and body condition scores are summarized as means ± SE. Temperature, solar radiation and NSC are presented as actual values for each time of day measured. Plasma leptin and insulin concentrations are summarized as least squares means ± SEM. Plasma leptin and insulin as well as pasture NSC and ambient temperature data were tested for normality using the Shapiro-Wilkes statistic (Intercooled Stata 9.2, Stata Corp., College Station, TX, USA). These variables approximated a normal distribution. Plasma leptin and insulin concentrations were analyzed using a mixed model analysis of variance (ANOVA) with repeated measures obtained with the MIXED procedure of SAS (SAS, 2001). Main effects of treatment and month and the interaction between them were included in the model. When the model had a significant effect of time, treatment or their interaction, preplanned comparisons where made between time zero and all other time points. Pearson correlation coefficients were calculated to evaluate relationships between variables. Correlation coefficients analysis was used to determine the relationship between plasma leptin and insulin. Significance was designated at a value of $P \leq 0.05$.

Results

Body weights and body condition scores did not differ between the trial months ($P > 0.05$, Table 3.1) or among horses within each trial ($P > 0.05$). Solar radiation

(Watts/m^2), temperature (°C) and NSC (% of DM) for each trial month are graphically reported in Figure 3.1. Overall (22-h average) environmental conditions differed between months. April was sunny and cool (10.7 ± 0.2 °C, solar radiation 314 ± 19 W/m^2, NSC 16.3 ± 0.3 % of DM), August was sunny and warm (25.6 ± 0.2 °C, solar radiation 320 ± 18 W/m^2, NSC 8.4 ± 0.1 % of DM), October was cool, cloud covered and rainy (9.9 ± 0.1 °C, solar radiation 88 ± 8.5 W/m^2, NSC 7.7 ± 0.6 % of DM), and January was sunny and cool (8.2 ± 1.8 °C, solar radiation 118 ± 9.7 W/m^2, NSC 7.8 ± 0.1 % of DM). Pasture NSC content was significantly ($P < 0.01$) associated with ambient temperature ($r = 0.78$). In the October trial, it began raining and temperature and solar radiation began to decrease at approximately 12:30 pm, this continued until the end of the study period (0730 following day). These changes in environmental conditions coincided with a decrease grazing behavior which continued until the end of the trial (0730 following day).

Plasma leptin in all months is depicted in Figure 3.2. The main effect of month and the interaction between treatment and month affected ($P < 0.0001$) plasma leptin. Plasma leptin concentration was higher in grazing vs. hay fed mares in April and October ($P < 0.001$) (Table 3.3). In grazing mares plasma leptin was higher ($P < 0.05$) in April and October (2.87 ± 0.1; and, 3.0 ± 0.1 ng/mL, respectively) compared to August and January (1.98 ± 0.1; and, 1.91 ± 0.1 ng/mL, respectively). In the hay fed mares plasma leptin in April (1.12 ± 0.2 ng/mL) was higher ($P < 0.01$) than January (0.63 ± 0.2 ng/mL) and October was higher ($P < 0.01$) than August and January (October, 1.3 ± 0.2; August, 0.94 ± 0.2). In April, August and October ($P < 0.001$), but not January ($P = 0.60$), the 22-h pattern in plasma leptin was similar in the grazing and hay fed mares (Pearson

correlation coefficients: April, r = 0.70; August, r = 0.83; October, r = 0.75; January, r = 0.18).

In grazing mares, plasma leptin concentrations were not significantly ($P > 0.10$) affected by changes in pasture NSC (Table 3.4).

Plasma insulin in all months is depicted in Figure 3.3. The main effects of month, treatment, and the interaction between month and treatment affected ($P < 0.01$) plasma insulin. Plasma insulin concentration was higher in the grazing compared to hay fed mares in both April and October ($P < 0.001$) (Table 3.3). In grazing mares, plasma insulin concentration was higher ($P < 0.001$) in April (51.2 ± 0.8 mIU/L) than all other months (August, 11.0 ± 0.8; October, 13.8 ± 0.8; January, 11.5 ± 0.8 mIU/L). In hay fed mares, plasma insulin concentration was higher ($P < 0.01$) in April (12.0 ± 1.3 mIU/L) than all other months (August, 8.8 ± 1.3; October, 8.5 ± 1.3; January, 9.5 ± 1.3 mIU/L). In April (51.2 ± 0.8) and October (13.8 ± 0.8) plasma insulin was higher ($P < 0.05$) in grazing mares than in mares fed hay only (12.0 ± 1.3; 8.5 ± 1.3, respectively). Plasma insulin concentration was associated ($P < 0.01$) with pasture NSC in all months except August (April, r = 0.66; October, r = 0.13; January, r = 0.47). Ambient temperature and plasma insulin were associated in October (r = 0.28) and January (r = 0.22; $P < 0.05$) (Table 3.4).

Significant ($P < 0.001$) relationships between plasma insulin and leptin in grazing mares were evident in all months (April, r = 0.69; August, r = 0.32; October, r = 0.37; January, r = 0.44). In hay fed mare mares relationships between leptin and insulin only existed in August (r = 0.65) and October (r = 0.42). Scatterplots illustrate the

relationships between leptin and insulin in all months for grazing and hay fed mares combined (Figure 3.4).

Discussion

This study is the first to characterize seasonal patterns plasma leptin in grazing Thoroughbred mares as well as daily patterns within each season and the relationship between plasma insulin and leptin concentrations in grazing mares.

The main findings were: no consistent pattern in plasma leptin was observed between months; higher plasma leptin and insulin concentrations in grazing compared to hay fed mares in April and October; and plasma insulin and leptin concentrations in grazing mares were higher in April and October compared to August and January.

In all months except January the 22-h pattern in plasma leptin was similar in the grazing mares compared to those confined to stalls. These findings suggest that multiple factors may influence plasma leptin concentrations in mares for example photoperiod or ambient temperature (Li and Wang, 2007) in addition to pasture nutrient content.

The current study demonstrated that plasma leptin concentrations in the grazing mares were consistently higher in April and October compared to August and January. These findings were consistent with a study conducted in Lusitano mares that observed peak leptin concentrations in April and November (Ferreira-Dias et al., 2005). In other studies an increase in leptin during spring and a decrease during the winter months has been observed (Buff et al., 2006). Fitzgerald and McManus (2000) reported elevated leptin concentrations in older mares (> 10 yr) from July (summer) through November (autumn) with a peak in August (summer) and low concentrations from December to

March (winter) with a slight increase in April and May (spring). This report differs slightly from the current study in that peak leptin was observed in summer and only a slight increase was observed in spring. In the current study leptin was highest in the spring and moderately higher autumn compared to summer and winter. In our study no differences in body condition were reported between months, in the above mentioned study however, peak leptin coincided with a peak estimated percent body fat. This relationship between leptin and percent body fat may account for the differences between the two studies.

Body weight and BCS were correlated to circulating leptin concentrations (Ahima and Flier, 2000). In the present study, no differences in BW or BCS were observed between seasons or between pasture and control mares. Thus, the seasonal changes in leptin observed in the present study were not explained by variations in adiposity. These alterations in leptin irrespective of changes in BCS were reported previously in the horse (Buff et al., 2006).

In several species, including the horse, insulin appears to be a stimulus for leptin secretion (Cartmill et al., 2005; Patel et al., 1998). In the present study, there was a positive linear relationship between plasma insulin and leptin in all months (Figure 3.4). Treatment (grazing or hay fed) affected plasma insulin concentration in April and October. A higher response in plasma insulin in April for grazing mares compared to mares in stalls was observed compared to October. However, weather conditions in April were cool and sunny which are optimal for increased accumulation of NSC in the pasture (Byrd et al., 2006). In October the study period was cool but overcast and rainy, and plasma insulin concentration in the grazing mares was lower than in April, perhaps due to

lower plant NSC content (Table 3.2) and or decreased grazing behavior. Plasma insulin and pasture NSC were correlated in all months except August (Table 3.4). These findings along with the results of previous research (Byrd et al., 2006; Longland et al., 1999; Longland and Byrd, 2006) support the hypothesis that changes in pasture NSC directly influence plasma insulin concentration.

In conclusion an association between circulating insulin and leptin concentrations was evident. In spring (April) and autumn (October) plasma insulin and leptin concentrations were higher than in summer (August) and winter (January) for pasture fed horses. This corresponds with higher pasture forage NSC concentrations in spring and fall, and lower pasture forage fiber content.

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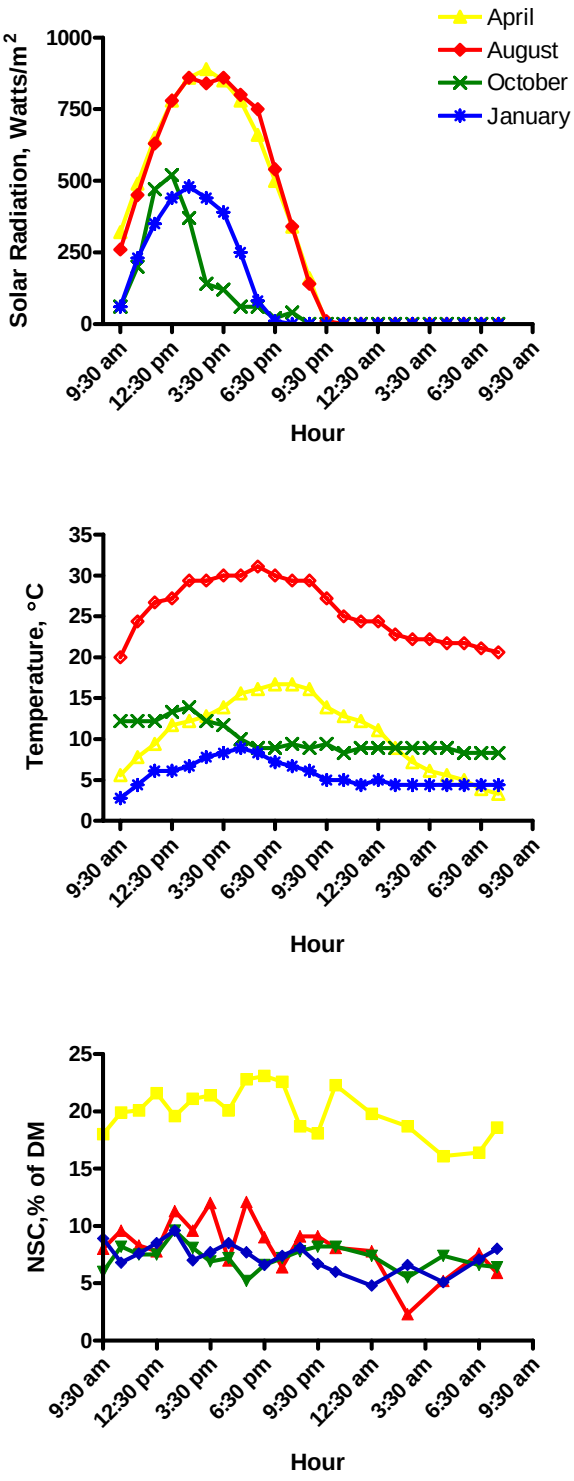

Figure 3.1. Temperature, solar radiation and non-structural carbohydrates (NSC) for each trial period from the on site weather station at the MARE Center.

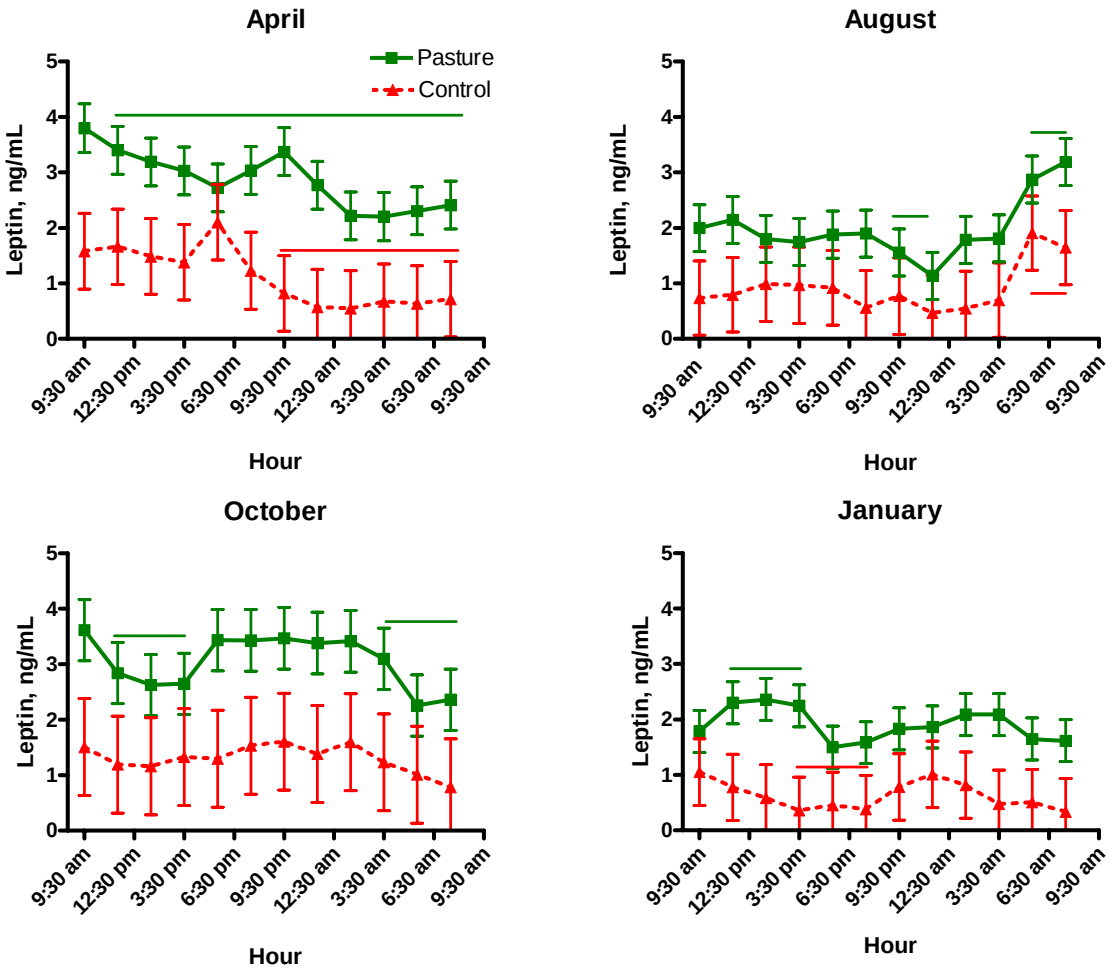

Figure 3.2. Least squares means ± SEM plasma leptin concentrations from grazing and hay fed mares for each month.

Bars denote differences ($P < 0.05$) from baseline (hour = 9:30 am).

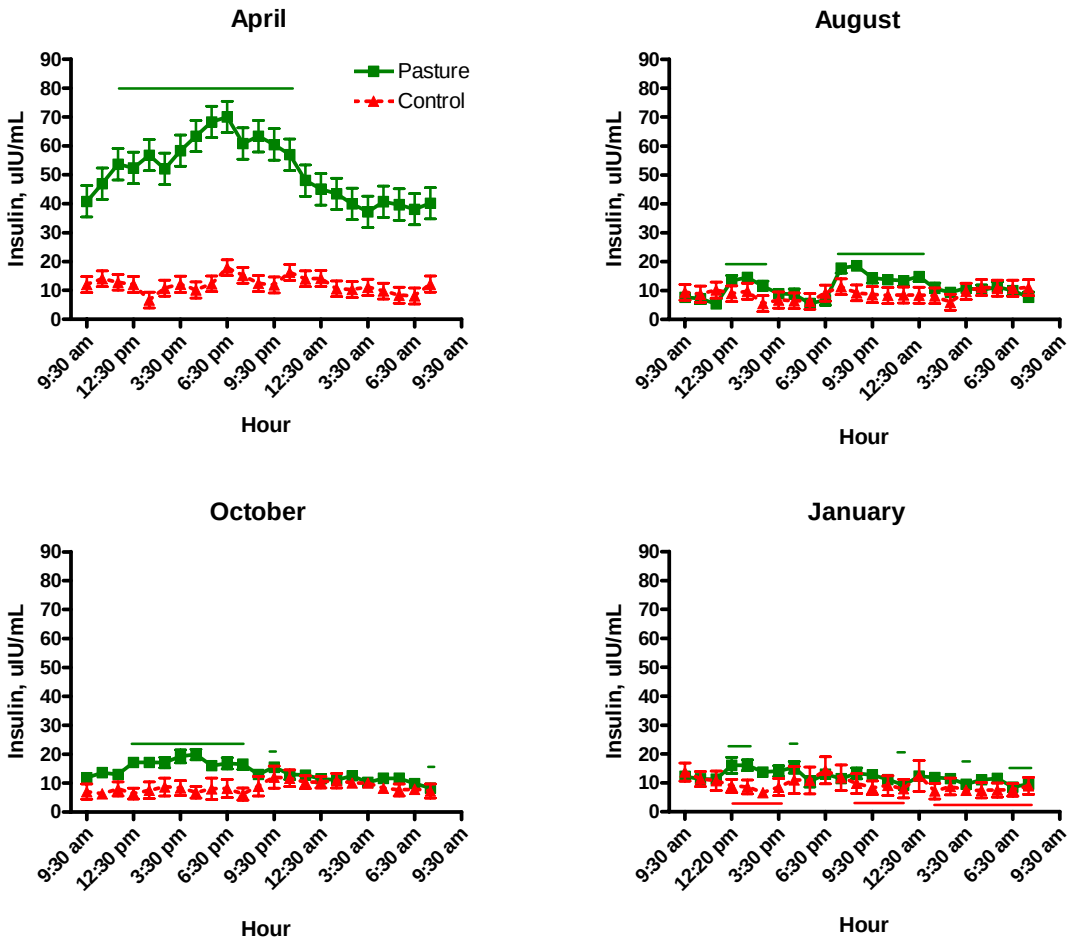

Figure 3.3. Least squares means ± SEM, plasma insulin concentration from grazing and hay fed mares for each month.

Bars denote differences ($P < 0.05$) from baseline (hour = 9:30 am).

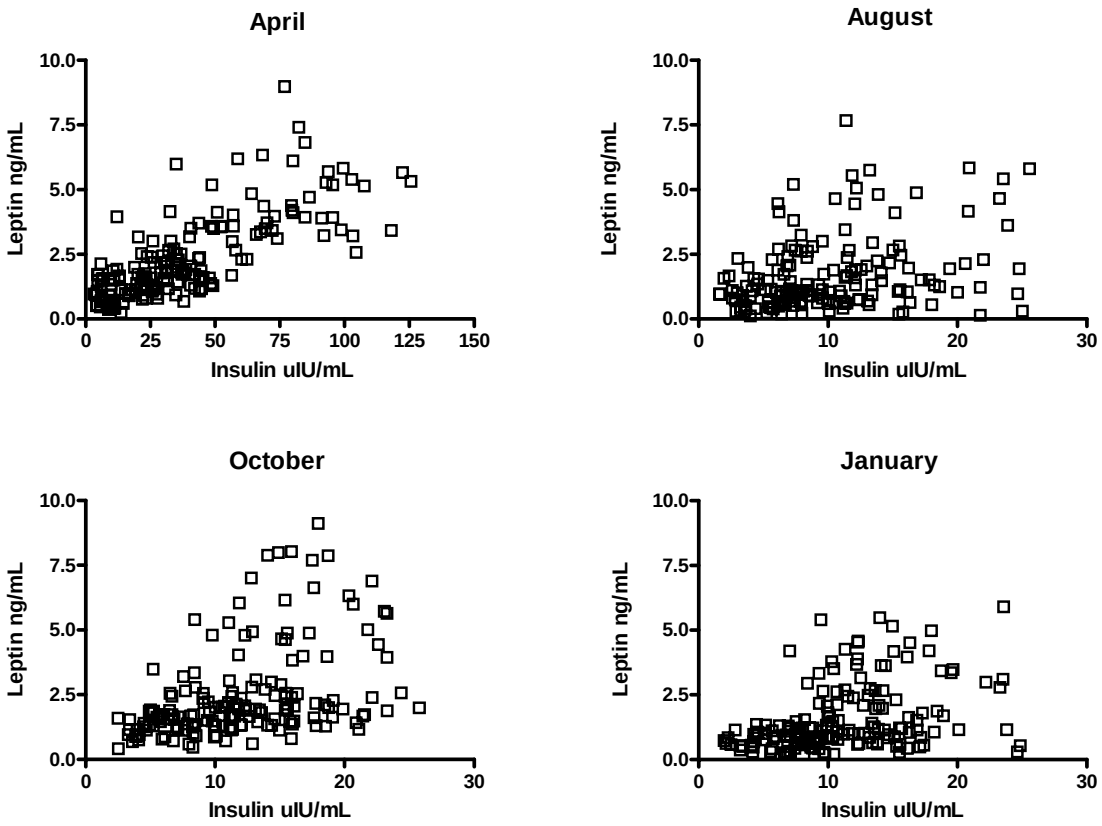

Figure 3.4. Scatterplots of the relationship between plasma leptin and plasma insulin for each month

Table 3.1. Weights (kg) and body condition scores (BCS) of horses (n = 14) for each month. Data are summarized as means ± SEM.

	April	August	October	January
Weight*	572.7 ± 9.9	605.4 ± 8.9	596.0 ± 8.3	610.3 ± 10.1
BCS*	5.7 ± 0.2	6.4 ± 0.1	6.4 ± 0.1	6.3 ± 0.2

*No effect of treatment or trial period was detected ($P > 0.1$).

Table 3.2. Nutrient analysis of the pasture (n = 33) for each trial period and hay (n = 2) summarized as means ± SEM

Nutrient[b]	April	August	October	January	Hay
DE, Mcal/kg [c]	2.8 ± 0.01[f]	2.1 ± 0.01[g]	2.1 ± 0.01[g]	2.1 ± 0.01[g]	1.9 ± 0.02[h]
Starch, %	1.4 ± 0.04[f]	1.5 ± 0.06[f]	1.1 ± 0.05[g]	1.0 ± 0.03[g]	2.8 ± 0.1[h]
Sugar, %	18.9 ± 0.40[f]	7.6 ± 0.5[g]	5.7 ± 0.2[h]	6.1 ± 0.2[h]	6.1 ± 0.2[h]
NSC, % [d]	20.3 ± 0.41[f]	9.2 ± 0.5[g]	6.9 ± 0.2[h]	7.1 ± 0.2[h]	8.9 ± 0.1[g]
EE, %	2.8 ± 0.04[f]	4.3 ± 0.1[g]	3.8 ± 0.06[f]	2.2 ± 0.09[h]	1.6 ± 0.3[h]
ADF, %	25.4 ± 0.24[f]	36.9 ± 0.5[g]	37.9 ± 0.2[g]	39.1 ± 0.3[h]	41.7 ± 0.4[g]
NDF, %	46.4 ± 0.33[f]	64.0 ± 0.7[g]	66.2 ± 0.4g	66.5 ± 0.5[g]	62.1 ± 1.3[h]
CP, %	21.3 ±1.4[f]	12.7 ± 0.2[g]	12.9 ± 0.2[g]	14.2 ± 0.3[h]	11.3 ± 0.5[g]

[a] Analysis were performed at Dairy One DHIA Forage Testing Laboratory, Ithaca, NY.

[b] Digestible energy (DE), non-structural carbohydrates (NSC), ether extract (EE), acid detergent fiber (ADF), neutral detergent fiber (NDF), Crude protein (CP).

[c] Calculated using equation from (Harris and Kronfeld, 2002).

[d] NSC = starch + sugar, sugar = water soluble carbohydrates.

[f,g,h] Groups with different letter superscripts (within row) differ ($P < 0.01$)

Table 3.3 Least squares means ± SEM for plasma leptin and insulin concentrations in pasture and hay fed horses for each month

Month	Leptin (ng/mL)		Insulin (mIU/mL)	
	Pasture	Hay	Pasture	Hay
April	2.87 ± 0.1[a]	1.12 ± 0.2*[ab]	51.2 ± 0.8[a]	12.0 ± 1.3*
August	1.98 ± 0.1[b]	0.94 ± 0.2[b]	11.0 ± 0.8[b]	8.8 ± 1.3
October	3.00 ± 0.1[a]	1.30 ± 0.2*[a]	13.8 ± 0.8[c]	8.5 ± 1.3*
January	1.91 ± 0.1[b]	0.63 ± 0.2[c]	11.5 ± 0.8[b]	9.5 ± 1.3

[*] Denotes a significant difference ($P < 0.05$) between pasture and hay fed mares for each month within leptin and insulin.

[a,b,c,] Means with different letter superscripts denote differences between month within each leptin and insulin ($P < 0.05$).

Table 3.4. Pearson correlation coefficients (r) between environmental variables and plasma leptin and insulin for all months in grazing horses only

		Leptin (ng/mL)	Insulin (mIU/L)
NSC (% DM)[†]	April	-0.10	0.66*
	August	-0.17	-0.03
	October	-0.09	0.13*
	January	0.07	0.47*
Ambient Temperature (°C)	April	0.09	0.35*
	August	-0.15	0.09
	October	-0.01	0.28*
	January	0.03	0.22 *

[†] NSC = starch + sugar, sugar = water soluble carbohydrates.

[*] Denote significant ($P < 0.01$) associations between environmental variables and blood parameters within each trial period for leptin and insulin.

Table 3.5. Pearson correlation coefficients (r) between plasma leptin and insulin during each month for grazing horses and horses fed hay and confined to stalls.

Blood Parameters		Insulin (mIU/L)	
		Pasture	Hay
Leptin (ng/mL)	April	0.69*	0.12
	August	0.32*	0.65*
	October	0.37*	0.42*
	January	0.44*	0.02

* Denote significant ($P < 0.001$) relationships between blood parameters in each trial period, within treatment

CHAPTER 4
MANUSCRIPT 2

EFFECTS OF DIETARY ENERGY SOURCE ON PATTERNS OF GLUCOSE, INSULIN AND LEPTIN IN GRAZING THOROUGHBRED MARES TRANSITIONING FROM WINTER TO SPRING

ABSTRACT: The objective of this study was to characterize changes in circulating glucose, insulin and leptin concentrations during the transition from winter to spring in mares fed supplements containing different energy sources. Twenty-four mares were divided into three dietary treatment groups; high fat and fiber (FF), high sugar and starch (SS), and pasture only (P). Horses were maintained on a single 4.8 hectare pasture divided into three sections from December to June. Twice weekly blood samples were collected for glucose, insulin, leptin and progesterone measurements. Baseline measurements for all physical and blood variables were not different at the commencement of the study. Nonstructural carbohydrate (NSC) content of pasture forage was positively correlated to plasma insulin concentration ($r = 0.55$, $P < 0.01$). Plasma insulin was affected by diet, day of year (DOY) and the interaction between diet and DOY ($P < 0.001$). In all dietary groups plasma insulin peaked at DOY 110 (April 20th) and was highest in FF (44.8 ± 2.3 mIU/L) than SS (31.4 ± 2.1 mIU/L) and lowest in P (23.2 ± 2.1 mIU/L; $P < 0.01$). Glucose was not affected by diet ($P > 0.1$), but changed over time with peak concentrations reached at day of year DOY 144 (May 24th, 103.3 ± 2.3 mg/dL; $P < 0.001$). Plasma leptin was affected by diet ($P = 0.02$), DOY ($P < 0.0001$) and the interaction between diet and DOY ($P < 0.0001$). In FF and SS but not P plasma

leptin increased to a peak at DOY 144 (FF, 5.8 ± 0.7, SS 5.8 ± 0.6 ng/mL; $P < 0.01$). There was a significant correlation between plasma insulin and leptin ($r = 0.55$, $P < 0.001$). Plasma progesterone increased above baseline values in all groups by DOY 124 (May 4th), indicating the resumption of regular ovarian activity. In conclusion, plasma insulin in pastured mares was related to forage NSC content. Plasma leptin was higher in spring than winter and a relationship between plasma insulin and leptin was evident in horses adapted to supplements high in FF or SS.

Introduction

Forage is a critical source of nutrients and energy for the horse. Approximately 80% of horses in the United States receive all or part of their nutrition from pasture (NAHMS, 1998). There is interest in pasture forage carbohydrate fractions and how the consumption of these may impact metabolites and hormones within the animal, with possible implications for animal health, for example laminitis and insulin resistance (Kronfeld et al., 2006; Longland and Byrd, 2006). Seasonal and circadian patterns in pasture nutritive variables have been demonstrated (Byrd et al., 2006; Cubitt et al., 2007). Relationships between these seasonal and circadian patterns of forage nutrient composition and circulating concentrations of plasma metabolites and hormones in grazing mares have been partially elucidated in recent studies (Byrd et al., 2006; McIntosh, 2007).

Studies characterizing glycemic and insulinemic responses in weanling and mature horses maintained at pasture and adapted to differing to feeds with dietary energy sources, either high sugar and starch (SS) or high fat and fiber (FF), have demonstrated a

higher basal plasma insulin concentration in horses fed SS compared to FF (Treiber et al., 2005a; Williams et al., 2001a). Grazing mares fed the same supplements and sampled over a one day period in May showed no difference in basal insulin concentrations between the dietary groups or between the dietary groups and horses fed pasture only. However, peak insulin after a meal was significantly higher in the SS fed mares than the FF or pasture only mares (Staniar et al., 2007). Increased NSC in the diet either from supplement or pasture increased circulating insulin concentrations in horses (Williams et al., 2001a).

A rise in insulin following a grain meal was shown in horses to elicit a rise in leptin concentrations (Cartmill et al., 2005). Circulating leptin concentrations in the mare decrease during the winter months (Fitzgerald and McManus, 2000). In sheep and hamsters this decrease in leptin during short photoperiod was independent of feed intake and body fatness, suggesting a role for other metabolic or environmental signals, such as melatonin or ambient temperature (Bocquier et al., 1998).

The effect of seasonal transition, including alterations in environment and pasture nutrient composition on circulating metabolites and hormones in pastured mares adapted to different energy sources has not been reported. Our hypothesis was that the transition from winter to spring would result in increased NSC content. Therefore, the objective of the study reported here was to characterize changes in circulating glucose, insulin and leptin concentrations in pastured mares during the transition from winter to spring and assess the possible association with changes in pasture forage NSC content. These observations were made in mares adapted to feeds rich in FF or SS and in mares maintained on pasture only.

Materials and Methods

Animals and management

Twenty-four non-pregnant Thoroughbred mares (11.1 ± 0.1 yr of age (range 4 to18); body condition score (BCS), 6.3 ± 1.0 (Table 4.1; range 4.5 to 8.5) (Henneke et al., 1983) and body weight (BW), 572 ± 4.0 kg (Table 4.1; range 460 to 725)) were maintained on a 12 ha mixed grass-legume (bluegrass, fescue, clover) pasture at the Middleburg Agricultural Research and Extension (MARE) Center. The pasture was divided into 3 even sections using temporary electric fencing. The mares were divided into three dietary treatment groups: FF, SS, or pasture only (P) group (n = 8/group). Each dietary group was kept in a separate section to allow for daily supplementation in their natural herd environment with minimal disturbance at feeding times (Hoffman et al., 2003b; Treiber et al., 2005a). Mares in FF and SS received meals 3 times daily (0730, 1100, and 1400hr). Horses were fed in a 30 m circle of fed pans containing individual portions. The mares were observed to ensure each consumed the allotted amount of supplement. All horses consumed the total amount of the feed provided to them within approximately 45 mins. No measures of pasture intake were recorded. This study was approved by the Institutional Animal Care and Use Committee of Virginia Tech.

Dietary Treatments

The dietary supplements FF and SS were formulated to be isocaloric and isonitrogenous and provided the mares with 67% of their daily DE requirements (NRC, 1989). Water was available ad libitum. A composite sample of each section of the pasture was collected between 0800 and 1000 hr weekly by following a zig-zag pattern

between fences covering the entire section with samples taken at approximate 20 m intervals. Samples of the supplements (Table 4.2) and pasture (Table 4.3) were submitted to a commercial laboratory for proximate and mineral analysis (Dairy One, Ithaca, NY). Sugar represented water soluble sugars (WSC; glucose, sucrose, fructose) that were extracted prior to analysis for starch (Hall et al., 1999) and included fructans. Starch content was determined following enzymatic digestion (glucoamylase) with measurement of dextrose in an automated biochemical analyzer (YSI 2700 SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA, Application Note Number 319). The pasture harvested and analyzed from each section, at each time point was not significantly different in contents of DE and major nutrients according to equivalence tests ($P > 0.05$) (Byrd et al., 2005). Therefore the data from each sub-section of pasture were pooled. Pasture data are summarized in Table 4.3 for each DOY sampled.

Blood Collection and Analysis

Blood sampling began in late December 2005 prior to mares being allocated to a dietary treatment. Once mares had been divided into dietary groups sampling continued twice weekly (Monday and Friday) from early January until the end of March 2006. Once weekly sampling continued until early June. Samples were collected prior to the morning feeding between 0700 and 0800 hr. Blood samples were drawn via jugular venipuncture into two 7 ml tubes containing sodium heparin as an anticoagulant (Vacutainer; Fisher Health Care, Chicago, IL) and placed on ice until centrifugation (3,000 x g for 10 min at 4°C). Plasma was removed within 60 min of collection and frozen at -20ºC until analysis.

Plasma glucose concentrations were measured by the glucose oxidase method using a chemical autoanalyzer (Kit # 442640, Beckman Synchron CX5CE, Brea, CA). Inter- and intra-assay CV were < 1% for glucose. Plasma insulin and progesterone were determined by use of radioimmunoassays (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA, USA) previously validated for equine plasma (Freestone et al., 1991a; Freestone et al., 1991b; Ginther et al., 2005). Inter- and intra-assay CV for insulin were 5.3% and 5.5%, respectively. Inter- and intra-assay CV for progesterone were 7.1% and 4.9%, respectively Plasma leptin concentrations were analyzed by use of a previously validated multi-species leptin radioimmunoassay (Linco Research Inc., St. Charles, MO) (Cartmill et al., 2003; McManus and Fitzgerald, 2000). Inter- and intra-assay CV for leptin was 4.1% and 6.0%, respectively. All analyses were performed in duplicate.

Statistical Analysis

Nutrient analysis for supplements and pasture are summarized as means ± SE (Table 4.2 and 4.3). Plasma insulin, glucose, leptin and progesterone concentrations and BW and BC are summarized as least squares means ± SE. Analysis of variance (ANOVA) was used to compare dietary treatments. A mixed ANOVA with repeated measures was performed with the MIXED procedure of SAS (SAS, 2001). Main effects of diet, time and their interaction were analyzed. When the model had a significant effect of time, preplanned comparisons where made within each diet, comparing time zero (insulin, glucose, BC and BW time 0 = DOY-17; Dec 15th, leptin and progesterone time 0 = DOY 5; Jan 5th) with all other time points. When there was a significant diet by DOY interaction, comparisons where made between diets at each time point. Spearman rank

correlation coefficients were calculated to evaluate relationships between and within blood variables and NSC. For all analyses, significance was designated at a value of $P \leq 0.05$.

Results

The first pasture samples was collected on DOY 20 (Jan 20th) and pasture NSC concentration was 5.7 ± 0.3 % DM. Pasture NSC remained at basal levels until an increase at DOY 41 (Feb 10th; 8.7 ± 0.5 % DM), which corresponded with an increase in plasma insulin concentration (Figure 4.2). Nonstructural carbohydrate concentrations returned to below baseline at the next sample point (DOY 48; Feb 17th) and remained there until DOY 90 (Mar 31st). The observed peak in NSC was at DOY 118 (15.7 ± 0.1 % DM). Pasture NSC values remained significantly higher than baseline until the end of the study (Figure 4.1). No association (r = 0.23; $P = 0.07$) was found between NSC and starch, but a positive relationship was observed between NSC and WSC (r = 0.99; $P < 0.0001$). Water soluble carbohydrates and NSC were negatively associated with ADF (WSC r = -0.78; NSC r = -0.76; $P < 0.0001$). Starch was not associated with any other variable ($P > 0.1$). Crude protein showed positive associations with WSC and NSC (r = 0.63; r = 0.61; respectively, $P < 0.0001$) and a negative relationship with ADF (r = -0.93; $P < 0.0001$).

Two mares were removed from the study, one from the P group because ultrasound evaluation revealed a pregnancy and the other from the FF group due to the development of laminitis (in early April). At the commencement of the study there were no differences among dietary groups ($P > 0.05$) for any of the variables measured

(insulin, 7.6 ± 1.9 mIU/L; glucose, 90.8 ± 1.1 mg/dl; leptin, 3.1 ± 0.4 ng/mL; progesterone 1.3 ± 0.9 ng/mL).

The main effects of diet, DOY and the interaction between diet and DOY significantly affected BCS and BW ($P < 0.05$; Table 4.1). An increase above baseline was observed for BW and BCS in both FF and SS groups at DOY 90 ($P < 0.01$). Body weight and BCS in the P group did not change ($P > 0.05$) throughout the study.

Diet, DOY and the interaction between diet and DOY significantly affected plasma insulin concentration ($P < 0.001$; Table 4.4). At DOY 41, plasma insulin concentration was significantly elevated above baseline in all dietary groups (14.1 ± 1.1 mIU/L; $P < 0.01$; Figure 4.2). This rise in plasma insulin corresponded with a rise in pasture NSC concentration (Figure 4.1). Insulin returned to baseline at the next sample time (DOY 44; Feb 13th) and remained there until DOY 110, when a significant increase above baseline was observed in all dietary groups ($P < 0.001$). Day of year 110 corresponded with the observed peak in plasma insulin concentration in all diet groups, which was significantly different among groups (FF, 44.8 ± 2.3 > SS, 31.4 ± 2.1 > P, 23.1 ± 2.1 mIU/L; $P < 0.01$; Figure 4.2). Plasma insulin concentrations in all dietary groups had returned to baseline values by the end of the study (DOY 163; June 12th).

There was a significant effect of DOY ($P < 0.001$; Table 4.5) but not diet or the interaction between diet and DOY on plasma glucose concentrations. Plasma glucose was not different from baseline in all dietary groups until DOY 110. In all groups, plasma glucose concentration was significantly increased after DOY 110 and reached a peak in all groups on DOY 144 (FF, 101.9 ± 2.4; SS, 104.9 ± 2.2; P, 103.2 ± 2.2 mg/dL).

By the end of the study (DOY 163), plasma glucose concentrations in all groups had returned to baseline values (Figure 4.3).

There was a significant effect of diet, DOY and the interaction between diet and DOY on plasma leptin concentrations ($P < 0.01$). Plasma leptin remained at baseline concentrations until DOY 110 when an increase was observed in FF and SS ($P < 0.01$) but not in P. Observed peak plasma leptin concentrations occurred at DOY 144 (FF, 5.8 ± 0.7; SS, 5.8 ± 0.6 ng/mL). Leptin concentrations in P did not rise above baseline concentrations until the last sample of the study (DOY 163; Table 4.4; Figure 4. 4). The observed peak in plasma leptin concentration for both the FF and SS groups was approximately 34 d after the observed peak in plasma insulin (Figure 4.6).

Day of year, but not diet or the interaction between diet and DOY, had a significant effect on plasma progesterone concentration ($P < 0.001$). Peak plasma progesterone concentration in all groups was reached on DOY 124 ($P < 0.05$; Figure 4.6).

Spearman rank correlation analysis revealed positive relationships ($P < 0.001$) between insulin and glucose (r = 0.55), leptin (r = 0.55), progesterone (r = 0.48) and NSC (r = 0.55). In addition to insulin, glucose was positively correlated ($P < 0.001$) with leptin (r = 0.43), progesterone (r = 0.50) and NSC (r = 0.51) and leptin was associated with progesterone (r = 0.35) and NSC (r = 0.37).

Discussion

Seasonal variation in pasture NSC and its constituents is well documented (Smith, 1973). Studies have shown that environmental conditions, including temperature (Chatterton, 1989) and solar radiation (Ciavarella, 2000), can lead to substantial

fluctuations in the amount of NSC that accumulates in forage plants. Consistently, NSC is higher in the spring, lowest in summer and winter, and intermediate in the fall (Vervuert et al., 2005). Previous studies at the MARE Center found peak pasture forage NSC concentrations in April and November (Cubitt et al., 2007). It is interesting to note the relationships between the pasture NSC fractions (WSC and starch) and total pasture NSC. There was no significant relationship between NSC and starch, whereas a positive association between NSC and WSC was detected. Overall, the seasonal changes in forage NSC can be attributed to alterations in WSC content. Fructans, the most abundant component of WSC have been implicated in the association between pasture carbohydrate content and incidence of pasture associated laminitis in horses (Longland et al., 1999).

Total NSC content and WSC were both positively associated with CP, insulin, glucose, leptin and progesterone and negatively correlated with pasture forage fiber content. Starch content was not associated with any of the pasture or plasma variables. This suggests that the WSC fraction and not the starch fraction of NSC was primarily responsible for interactions between pasture carbohydrate composition and plasma variables observed in this study.

Previous work from this laboratory reported a positive relationship between pasture forage NSC content and plasma insulin concentration in grazing mares (Byrd et al., 2006; McIntosh, 2007). The current study also reported associations positive associations between pasture forage NSC content and plasma insulin in grazing mares. Interestingly in the present study forage CP had a significant association with plasma insulin. A similar relationship between dietary protein intake and circulating insulin has

been shown previously in mice (Schneider et al., 1996). These observations suggest that alterations in plasma insulin in grazing horses may be in part due to changes in pasture forage CP. However, in mid-February a significant increase in forage NSC but not CP (Figure 4.1) was observed coincident with an increase in circulating insulin concentration (Figure 4.2) in all dietary groups. Both NSC and insulin returned to baseline until mid April when peak NSC and plasma insulin (in all diet groups) was observed. These data support a role for pasture NSC in the perturbation of circulating insulin in the grazing mare.

Glucose concentrations began to rise above baseline at DOY 110. However, peak concentrations were not observed until DOY 144, approximately 34 d after peak insulin. Glucose response was not different between dietary groups, an observation in accordance with previous findings in pastured horses (Byrd et al., 2006; Treiber et al., 2006b). The lack of response in circulating glucose to dietary energy source (FF or SS) may indicate that WSC and not starch was responsible for eliciting a response in plasma glucose in pastured animals.

Leptin followed a similar pattern to glucose, rising at DOY 110 and peaking at DOY 144. This increase was only observed, however, in the FF and SS groups. The rise in leptin corresponds to changes in BW and BCS where significant elevations were detected in FF and SS, but not P. However, BW and BCS remained elevated until the completion of the study, while plasma leptin concentrations returned to baseline. Moreover, the pattern of leptin in FF and SS was parallel to that of circulating insulin and there was a positive relationship between insulin and leptin. Previous work has demonstrated a stimulatory effect of circulating insulin on leptin (Cartmill et al., 2005;

Patel et al., 1998) and the positive association between insulin and leptin observed in the present study is consistent with this finding. The lack of response in plasma leptin to a rise in insulin in P may indicate that a threshold level of insulin is needed before a response in leptin is observed. In the study by Cartmill and associates (2005) a post grain meal rise in plasma insulin to approximately 25 mIU/L was required to elicit a response in plasma leptin concentrations. The insulin concentration data at DOY 41 provide support for this threshold theory. Plasma insulin rose in the SS group to similar concentrations when compared to the P group, and in both groups circulating leptin remained at baseline levels.

The rise in circulating progesterone observed by DOY 124 indicated the resumption of ovarian activity in the mares after the winter anestrous period. The rise in progesterone was independent of leptin as the increase in progesterone in the P mares occurred without a concomitant change in leptin concentrations. This finding contrasts with a recent study in Lusitano mares that reported leptin to be a potential metabolic signal stimulating the resumption of ovarian activity (Ferreira-Dias et al., 2005). Mares generally exhibit reproductive activity that is entrained to photoperiod. However, energy balance either negative or positive can affect this pattern. In obese mares, continued reproductive activity during the non-breeding (winter) season was reported (Gentry et al., 2002; Vick et al., 2006). On the other hand, emaciated mares did not resume ovarian activity in response to photo-stimulation (Guillaume et al., 2002). These observations suggest that nutritional status, in addition to environmental cues, stimulates the onset of reproductive cyclicity and that, in the extreme of obesity or emaciation, leptin may influence in reproductive cyclicity.

In conclusion, a rise in plasma insulin was associated with a rise in pasture forage NSC. The relationship between increased insulin as a response to pasture carbohydrates was directly related to WSC content of the plant and not starch. The findings of this study add to the previous report (chapter 3) by outlining a relationship in grazing mares between plasma insulin and leptin which seems to be driven by seasonal changes in pasture forage NSC and is only evident when insulin rises above a threshold concentration. The seasonal changes in glucose and insulin have implications for use of single sample measurements of these variables in the diagnosis of insulin resistance in horses and ponies. Specifically, increase plasma insulin concentration in animals grazing spring pasture may reflect high forage NSC rather than insulin resistance.

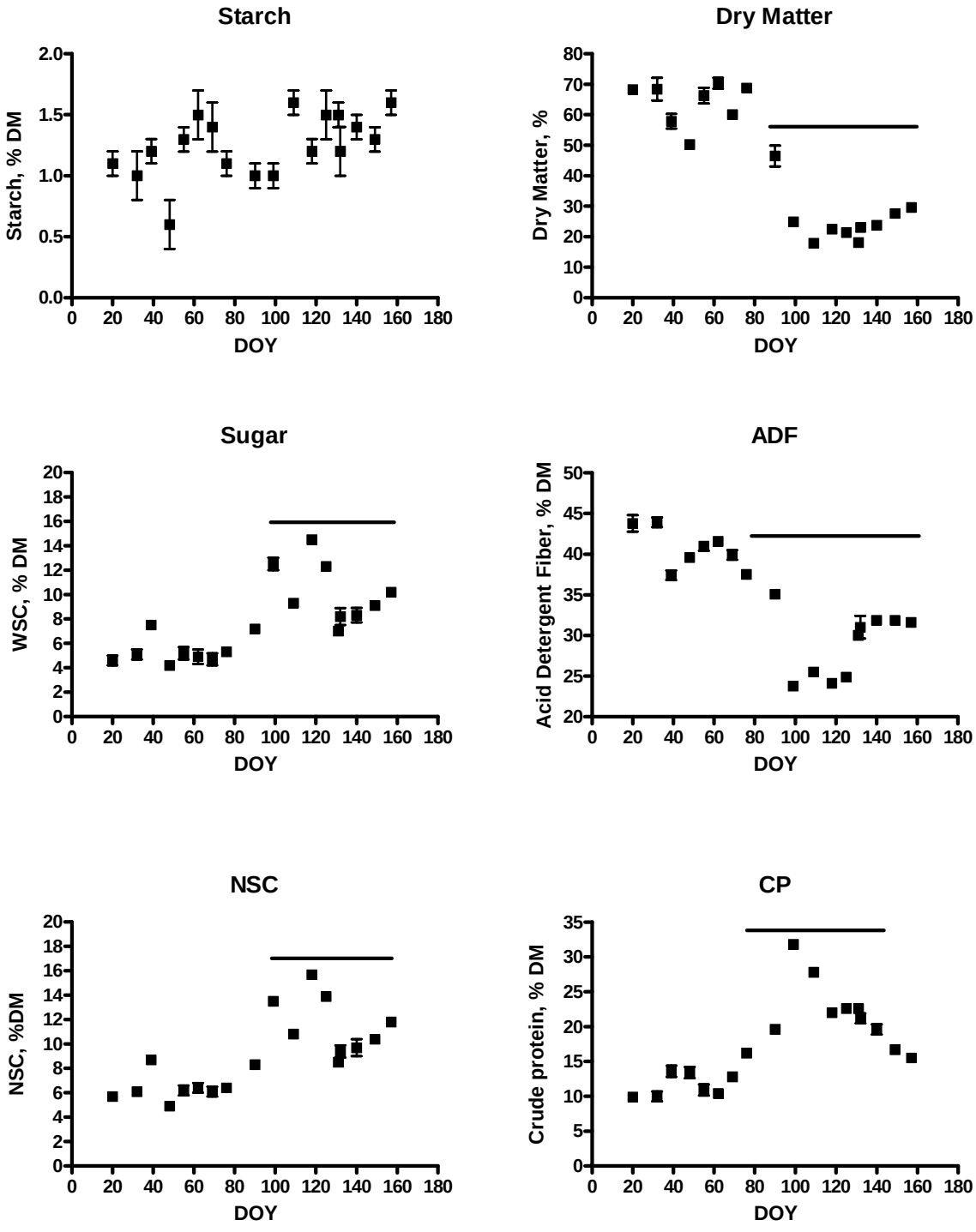

Figure 4.1. Fluctuations in pasture variables during the transition from winter to spring. Bars denote significant deviations ($P < 0.05$) from baseline (Day of year [DOY 20]).

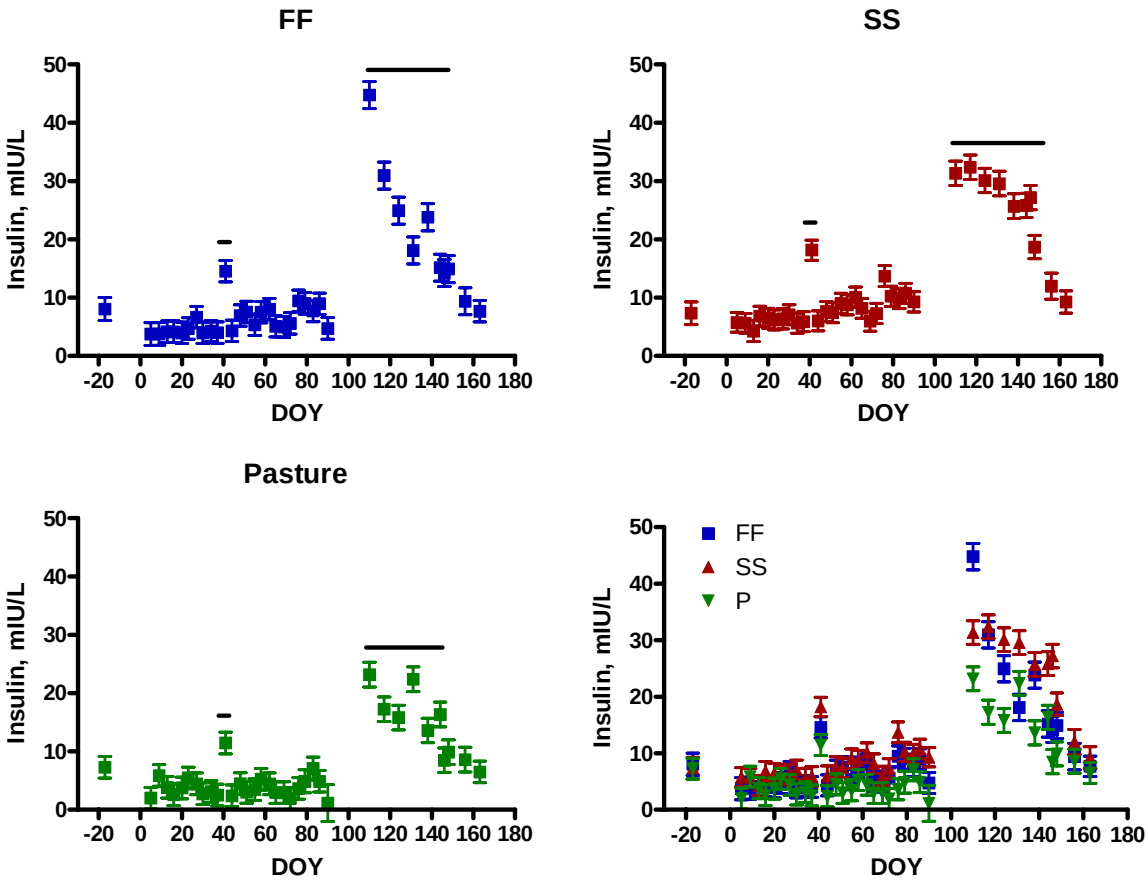

Figure 4.2. Mean plasma insulin concentration for each dietary treatment and all treatments plotted against day of year (DOY).

Bars denote significant ($P < 0.05$) differences greater than baseline samples, DOY -17.

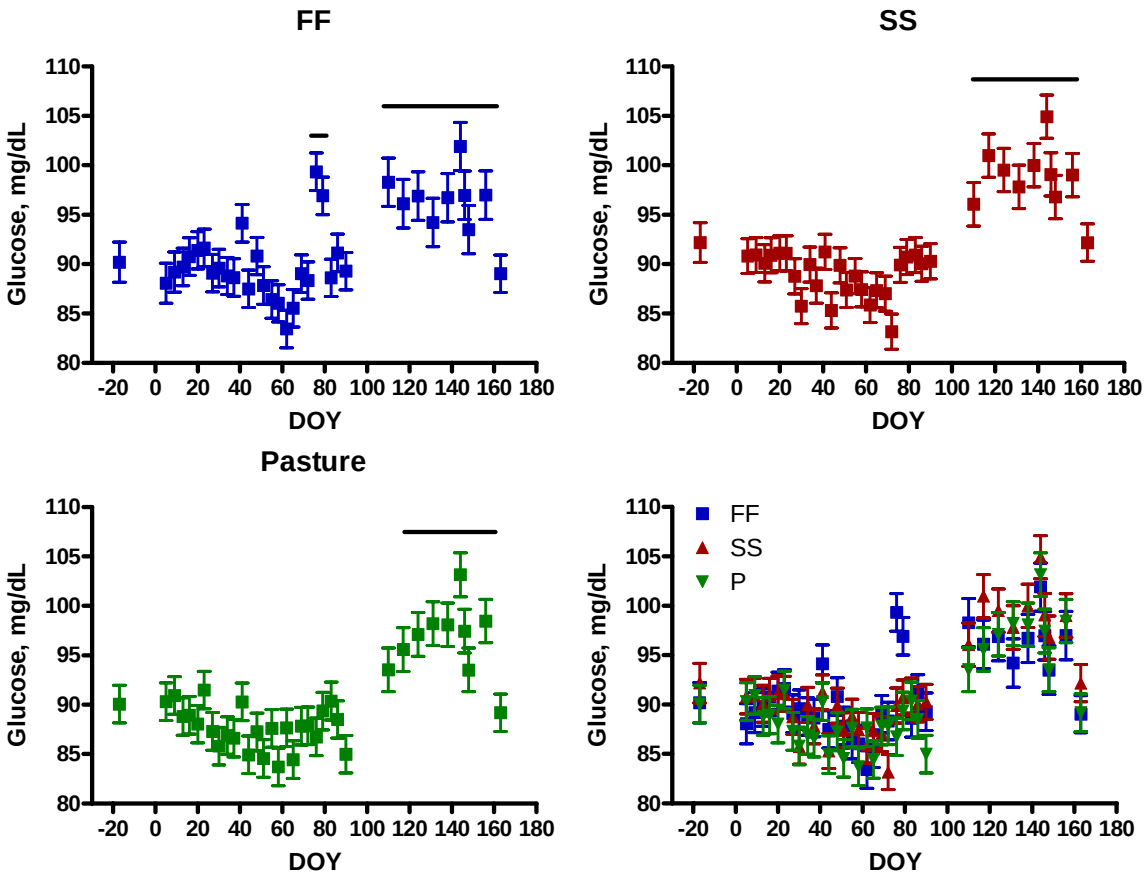

Figure 4.3. Mean plasma glucose concentration for each dietary treatment and all treatments plotted against day of year (DOY).

Bars denote significant ($P < 0.05$) differences greater than baseline samples, DOY -17.

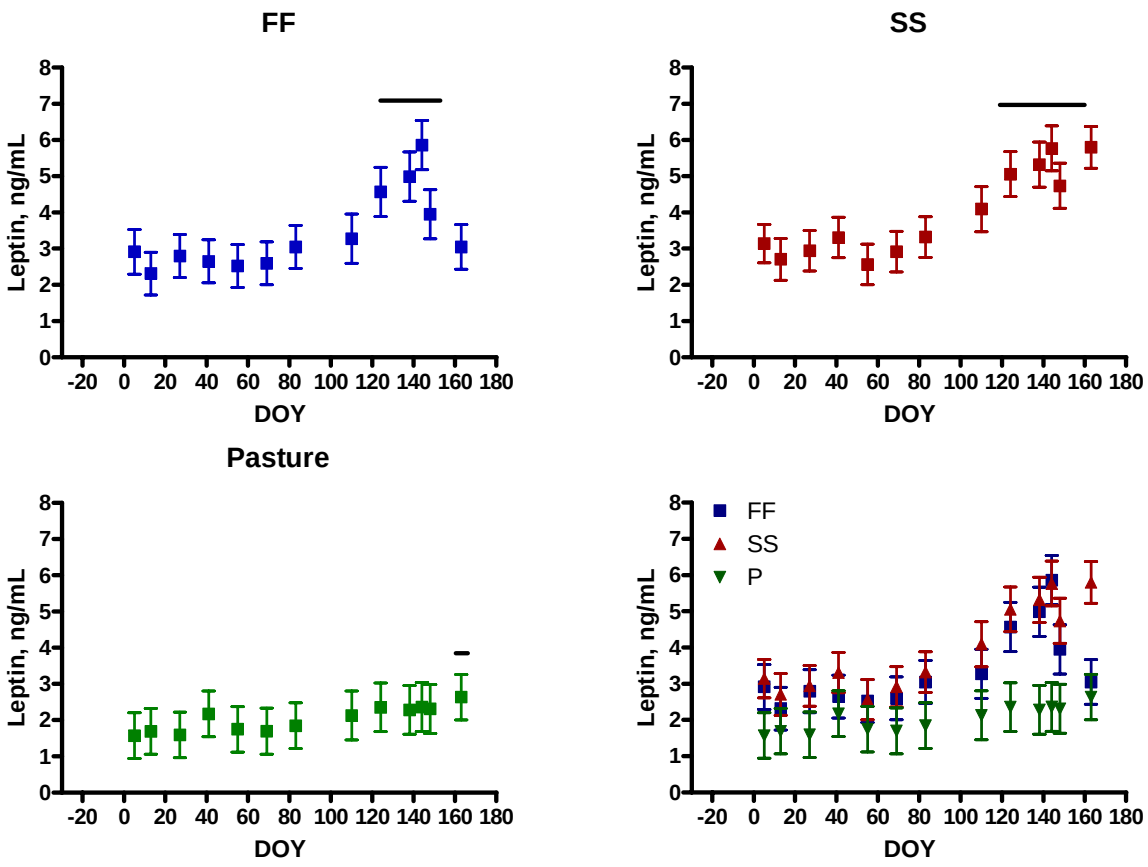

Figure 4.4. Mean plasma leptin concentration for each dietary treatment and all treatments plotted against day of year (DOY).

Bars denote significant ($P < 0.05$) differences greater than baseline samples, DOY 5.

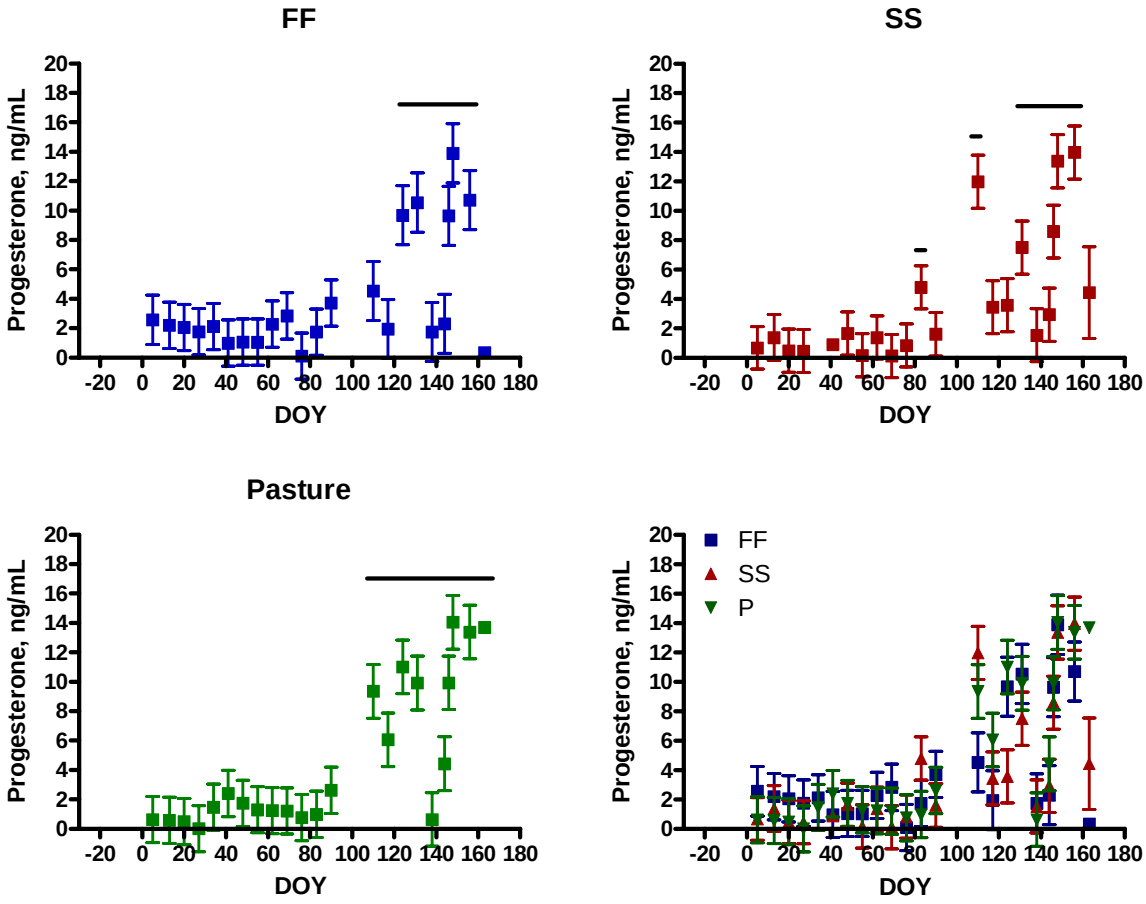

Figure 4.5. Mean plasma progesterone concentration for each dietary treatment and all treatments plotted against day of year (DOY).

Bars denote significant ($P < 0.05$) differences greater than baseline samples, DOY 5.

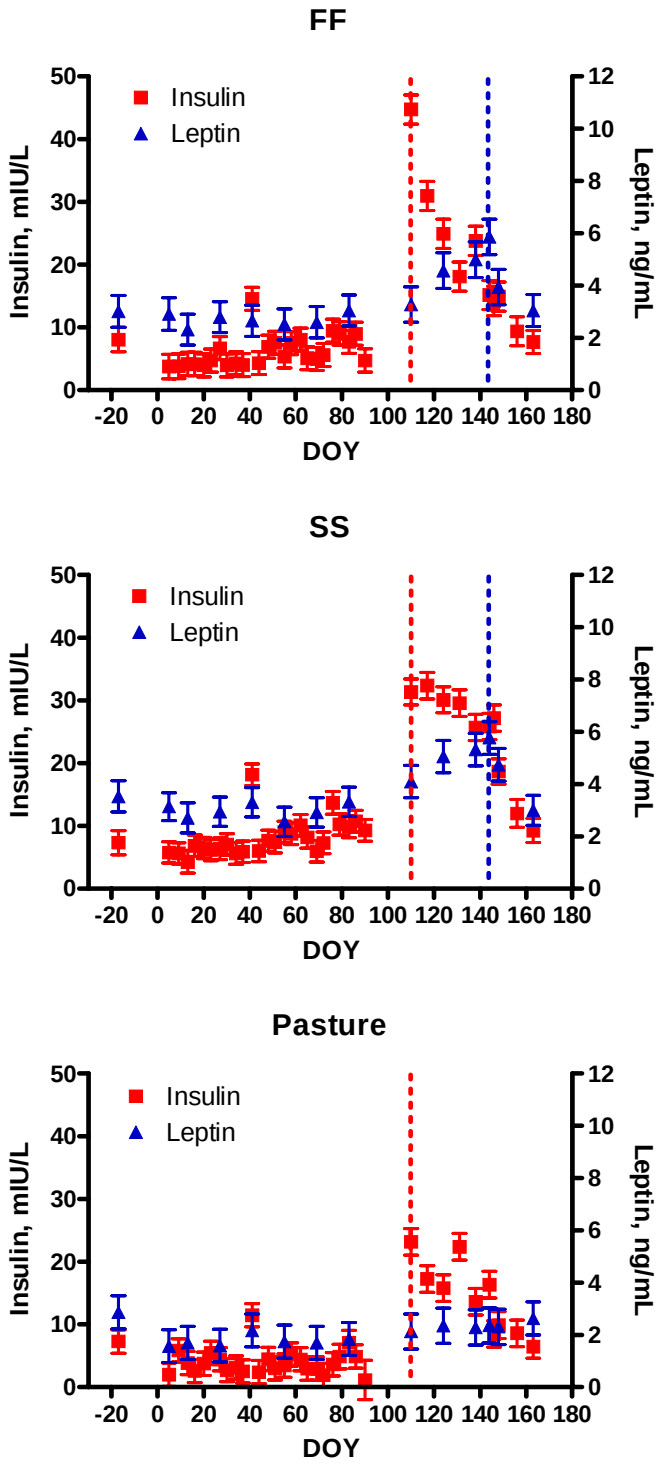

Figure 4.6. Day of year (DOY) for observed peak in plasma insulin and leptin. Dotted lines represents observed peak in plasma insulin and leptin concentration (red line, insulin; blue line, leptin)

Table 4.1. Weight (kg) and body condition score (BCS) of horses over the six month sampling period for FF, SS and pasture. Data are summarized as means ± SEM.

Day of Year	Body Weight			Body Condition		
	FF	SS	Pasture	FF	SS	Pasture
-17	580 ± 18	573 ± 73	564 ± 17	6.0 ± 0.3	6.0 ± 0.3	5.8 ± 0.2
90	600 ± 18 [a*]	600 ± 17 [a*]	548 ± 13 [b*]	6.6 ± 0.4[a*]	6.9 ± 0.3[a*]	5.8 ± 0.2[b]
163	618 ± 19 [a*]	608 ± 17 [a*]	574 ± 10 [b]	6.9 ± 0.3[a*]	7.1 ± 0.4[a*]	5.7 ± 0.1[b]

[ab] Within a row, means with different superscript letters significantly differ ($P < 0.05$)

* Denotes differences from baseline (DOY -17).

Table 4.2. Nutrient analysis of supplements high in sugar and starch (SS, n = 18), or fat and fiber (FF, n = 18) summarized as means ± SEM.

Nutrient [b]	FF	SS
DM %	91.5 ± 0.4	87.7 ± 0.8
DE, Mcal/kg [c]	2.9 ± 0.1	3.0 ± 0.1
Starch	5.8 ± 1.9	38.3 ± 2.5
Sugar	8.8 ± 0.4	10.7 ± 0.5
NSC [d]	14.6 ± 2.1	49.1 ± 2.6
NFC [e]	23.2 ± 2.3	55.3 ± 2.7
EE	12.5 ± 0.9	4.3 ± 0.7
ADF	27.2 ± 1.2	11.0 ± 1.1
NDF	40.9 ± 1.6	18.9 ± 1.5
CP	15.5 ± 0.2	14.4 ± 0.37

[a] Analysis were performed at Dairy One DHIA Forage Testing Laboratory, Ithaca, NY.

[b] (% of Dry matter), digestible energy (DE), non-structural carbohydrates (NSC), non-fiber carbohydrates (NFC), ether extract (EE), acid detergent fiber (ADF), neutral detergent fiber (NDF), Crude protein (CP).

[c] Calculated using equation from Harris and Kronfeld, 2002.

[d] NSC = starch + sugar, sugar = water soluble carbohydrates.

[e] Nonfiber carbohydrate, NFC = 100 – CP – fat – (NDF + neutral detergent insoluble CP) – ash.

Table 4.3. Longitudinal nutrient analysis of pasture variables over day of year (DOY) summarized as means ± SEM.

DOY	Pasture Variable [a,b]									
	DM %	DE, Mcal/kg[c]	Starch %	Sugar %	NSC %[d]	NFC %[e]	EE %	ADF %	NDF %	CP %
20	68.3 ± 0.8	2.3 ± 0.1	1.1 ± 0.1	4.6 ± 0.4	5.7 ± 0.3	15.6 ± 1.1	1.8 ± 0.1	43.8 ± 1.0	72.1 ± 1.3	9.9 ± 0.2
32	68.4 ± 3.7	2.4 ± 0.2	1.0 ± 0.4	5.1 ± 2.0	6.1 ± 1.7	12.0 ± 0.8	1.7 ± 0.1	43.9 ± 0.6	74.9 ± 0.5	10 ± 0.7
39	57.9 ± 2.4	2.4 ± 0.1	1.2 ± 0.1	7.5 ± 1.3	8.7 ± 1.3	16.1 ± 0.2	2.4 ± 0.1	37.4 ± 2.2	66.8 ± 1.4	13.6 ± 1.8
48	50.3 ± 1.4	2.3 ± 0.1	0.6 ± 0.2	4.2 ± 0.3	4.9 ± 0.1	12.9 ± 2.4	2.4 ± 0.2	39.6 ± 0.5	69.4 ± 0.8	13.4 ± 0.8
55	66.3 ± 2.6	3.0 ± 0.1	1.3 ± 0.1	5.2 ± 0.5	6.2 ± 0.6	17.8 ± 0.9	2.1 ± 0.2	41.0 ± 0.6	68.3 ± 0.3	10.9 ± 0.8
62	70.3 ± 1.8	2.3 ± 0.1	1.5 ± 0.2	4.9 ± 0.6	6.4 ± 0.4	16.0 ± 1.6	2.2 ± 0.1	41.6 ± 0.5	70.3 ± 1.4	10.4 ± 0.4
69	60.1 ± 0.9	2.3 ± 0.1	1.4 ± 0.2	4.7 ± 0.5	6.1 ± 0.7	15.4 ± 0.9	2.1 ± 0.1	39.9 ± 1.6	68.9 ± 1.3	12.8 ± 0.1
76	68.8 ± 0.5	2.4 ± 0.1	1.1 ± 0.1	5.3 ± 0.2	6.4 ± 0.2	16.5 ± 2.0	2.0 ± 0.2	37.5 ± 1.2	63.4 ± 2.0	16.2 ± 0.6
90	46.5 ± 3.4	2.4 ± 0.1	1.0 ± 0.1	7.2 ± 0.2	8.3 ± 0.1	14.3 ± 0.6	2.7 ± 0.1	35.1 ± 0.4	62.9 ± 0.4	19.6 ± 0.2
99	24.9 ± 0.9	2.7 ± 0.2	1.0 ± 0.1	12.5 ± 0.5	13.5 ± 0.1	17.9 ± 1.0	3.2 ± 0.1	23.8 ± 0.4	46.0 ± 0.4	31.8 ± 0.3
109	17.9 ± 0.5	2.5 ± 0.3	1.6 ± 0.1	9.3 ± 0.2	10.8 ± 0.1	15.0 ± 0.5	4.1 ± 0.1	25.5 ± 0.4	50.8 ± 0.5	27.8 ± 0.3
118	22.5 ± 0.9	2.8 ± 0.1	1.2 ± 0.1	14.5 ± 0.2	15.7 ± 0.1	19.7 ± 0.6	3.5 ± 0.1	24.1 ± 0.4	52.0 ± 6.0	22.0 ± 0.2
125	21.4 ± 0.5	2.7 ± 0.2	1.5 ± 0.2	12.3 ± 0.3	13.9 ± 0.1	20.0 ± 0.7	3.6 ± 0.1	24.9 ± 0.4	50.2 ± 0.5	22.6 ± 0.28
131	18.1 ± 0.7	2.4 ± 0.1	1.5 ± 0.1	7.0 ± 0.3	8.5 ± 0.3	13.9 ± 0.9	4.9 ± 0.7	30.0 ± 0.4	56.3 ± 0.6	22.6 ± 0.5
132	23.0 ± 0.5	2.5 ± 0.1	1.2 ± 0.2	8.2 ± 0.7	9.4 ± 0.5	15.6 ± 1.0	3.8 ± 0.2	31.0 ± 1.4	57.7 ± 1.1	21.2 ± 0.7
140	23.8 ± 0.7	2.5 ± 0.1	1.4 ± 0.1	8.3 ± 0.6	9.7 ± 0.7	16.3 ± 0.5	3.2 ± 0.2	31.9 ± 0.4	59.2 ± 0.7	19.6 ± 0.7
149	27.6 ± 0.9	2.5 ± 0.2	1.3 ± 0.1	9.1 ± 0.2	10.4 ± 0.1	18.3 ± 0.5	1.8 ± 0.1	31.9 ± 0.4	59.9 ± 0.4	16.7 ± 0.4
157	29.6 ± 0.9	2.6 ± 0.2	1.6 ± 0.1	10.2 ± 0.2	11.8 ± 0.1	18.2 ± 0.7	3.7 ± 0.1	31.6 ± 0.4	59.3 ± 0.4	15.5 ± 0.4

[a] Analysis were performed at Dairy One DHIA Forage Testing Laboratory, Ithaca, NY.
[b] (% of Dry matter), digestible energy (DE), non-structural carbohydrates (NSC), non-fiber carbohydrates (NFC), ether extract (EE), acid detergent fiber (ADF), neutral detergent fiber (NDF), Crude protein (CP).
[c] Calculated using equation from (Harris and Kronfeld, 2002).
[d] NSC = starch + sugar, sugar = water soluble carbohydrates.
[e] Nonfiber carbohydrate, NFC = 100 – CP – fat – (NDF + neutral detergent insoluble CP) – ash.

Table 4.4. Least squares means ± SEM for plasma insulin and leptin concentrations over day of year (DOY).

DOY	Insulin			Leptin		
	FF	SS	Pasture	FF	SS	Pasture
-17	8.1 ± 2.0	7.3 ± 1.9	7.3 ± 1.9			
5	3.8 ± 2.0	5.8 ± 1.7	2.0 ± 1.9	2.9 ± 0.6	3.1 ± 0.5	1.6 ± 0.6
9	3.8 ± 2.0	5.6 ± 1.7	5.9 ± 1.9			
13	4.2 ± 1.8	4.3 ± 1.8	3.9 ± 1.9	2.3 ± 0.6	2.7 ± 0.6	1.7 ± 0.6
16	4.2 ± 1.8	6.9 ± 1.7	2.6 ± 1.9			
20	4.0 ± 1.8	6.5 ± 1.7	3.8 ± 1.9			
23	4.7 ± 1.8	6.2 ± 1.7	5.5 ± 1.9			
27	6.7 ± 1.8	6.4 ± 1.7	4.4 ± 1.9	2.8 ± 0.6	2.9 ± 0.6	1.6 ± 0.6
30	4.0 ± 1.8	7.1 ± 1.7	2.7 ± 1.9			
34	4.3 ± 1.8	5.6 ± 1.7	3.1 ± 1.9			
37	4.0 ± 1.8	5.9 ± 1.7	2.5 ± 1.9			
41	14.6 ± 1.8^{ab}	18.2 ± 1.7^{a}	11.5 ± 1.9^{b}	2.7 ± 0.6	3.3 ± 0.6	2.2 ± 0.6
44	4.3 ± 1.8	6.0 ± 1.7	2.4 ± 1.9			
48	6.9 ± 1.8	7.7 ± 1.7	4.5 ± 1.9			
51	7.6 ± 1.8	7.4 ± 1.7	3.0 ± 1.9			
55	5.4 ± 1.8^{ab}	9.0 ± 1.7^{a}	3.5 ± 1.9^{b}	2.5 ± 0.6	2.6 ± 0.6	1.7 ± 0.6
58	7.5 ± 1.8	8.8 ± 1.7	5.3 ± 1.9			
62	8.1 ± 1.8^{ab}	10.1 ± 1.7^{a}	4.4 ± 1.9^{b}			
65	5.1 ± 1.8^{ab}	8.2 ± 1.7^{a}	3.0 ± 1.9^{b}			
69	5.0 ± 1.8	6.0 ± 1.7	3.0 ± 1.9	2.6 ± 0.6	2.9 ± 0.6	1.7 ± 0.6
72	5.6 ± 1.8^{a}	7.3 ± 1.7^{ab}	1.9 ± 1.9^{b}			
76	9.5 ± 1.8^{a}	13.7 ± 1.8^{a}	3.6 ± 1.9^{b}			
79	9.0 ± 1.8^{ab}	10.3 ± 1.7^{a}	4.9 ± 2.0^{b}			
83	7.7 ± 1.8	9.9 ± 1.7	7.1 ± 2.0	3.0 ± 0.6	3.3 ± 0.6	1.8 ± 0.6
86	9.0 ± 1.8^{ab}	10.8 ± 1.7^{a}	4.9 ± 1.9^{b}			
90	4.7 ± 1.8^{a}	9.3 ± 1.7^{ab}	1.1 ± 3.2^{b}			
110	44.8 ± 2.3^{a}	31.4 ± 2.1^{b}	23.2 ± 2.1^{c}	3.3 ± 0.7^{ab}	4.1 ± 0.6^{a}	2.1 ± 0.7^{b}
117	31.0 ± 2.3^{a}	32.4 ± 2.1^{a}	17.3 ± 2.1^{b}			
124	24.9 ± 2.3^{a}	30.1 ± 2.1^{a}	15.8 ± 2.1^{b}	4.6 ± 0.7^{a}	5.1 ± 0.6^{a}	2.4 ± 0.7^{b}
131	18.1 ± 2.3^{a}	29.6 ± 2.1^{b}	22.4 ± 2.1^{a}			
138	23.8 ± 2.3^{a}	25.7 ± 2.1^{a}	13.6 ± 2.1^{b}	5.0 ± 0.7^{a}	5.3 ± 0.6^{a}	2.3 ± 0.7^{b}
144	15.2 ± 2.3^{a}	25.9 ± 2.1^{b}	16.4 ± 2.1^{a}	5.9 ± 0.7^{a}	5.8 ± 0.6^{a}	2.4 ± 0.7^{b}
146	14.2 ± 2.3^{a}	27.2 ± 2.1^{b}	8.5 ± 2.1^{a}			
148	14.9 ± 2.3^{ab}	19.2 ± 2.1^{a}	9.9 ± 2.1^{b}	3.9 ± 0.7^{ab}	4.7 ± 0.6^{a}	2.3 ± 0.7^{b}
156	9.4 ± 2.3	12.5 ± 2.1	8.6 ± 2.1			
163	7.7 ± 1.8	9.2 ± 1.8	6.5 ± 1.9	3.0 ± 0.6^{a}	5.8 ± 0.6^{b}	2.6 ± 0.6^{a}

[abc] Within and insulin or leptin superscripts denotes dietary differences (between columns; $P < 0.05$) at each DOY.

Table 4.5. Least squares means ± SEM for glucose and progesterone concentrations over day of year (DOY).

DOY	Glucose	Progesterone
-17	90.8 ± 1.1	
5	89.7 ± 1.1	1.3 ± 0.9
9	90.4 ± 1.1	
13	89.5 ± 1.1	1.4 ± 0.9
16	90.2 ± 1.1	
20	90.2 ± 1.1	1.0 ± 0.9
23	91.4 ± 1.1	
27	88.4 ± 1.1	0.7 ± 0.9
30	87.0 ± 1.1	
34	88.5 ± 1.1	1.2 ± 0.9
37	87.7 ± 1.1	
41	91.9 ± 1.1	1.4 ± 0.9
44	85.9 ± 1.1	
48	89.3 ± 1.1	1.5 ± 0.9
51	86.6 ± 1.1	
55	87.6 ± 1.1	0.8 ± 0.9
58	85.7 ± 1.1	
62	85.7 ± 1.1	1.6 ± 0.9
65	85.8 ± 1.1	
69	88.0 ± 1.1	1.4 ± 0.9
72	86.5 ± 1.1	
76	92.0 ± 1.1	0.6 ± 0.9
79	92.3 ± 1.1	
83	90.0 ± 1.1	2.5 ± 0.9
86	89.9 ± 1.1	
90	88.2 ± 1.1	2.6 ± 0.9
110	96.0 ± 1.3	8.6 ± 1.1
117	97.6 ± 1.3	3.8 ± 1.1
124	97.8 ± 1.3	8.1 ± 1.1
131	96.7 ± 1.3	9.3 ± 1.1
138	98.3 ± 1.3	1.3 ± 1.1
144	103.3 ± 1.3	3.2 ± 1.1
146	97.8 ± 1.3	9.4 ± 1.1
148	94.6 ± 1.3	13.8 ± 1.1
156	98.2 ± 1.3	12.7 ± 1.1
163	90.1 ± 1.1	

CHAPTER FIVE
MANUSCRIPT 3

PHASE OF THE ESTROUS CYCLE AFFECTS INSULIN SENSITIVITY IN THOROUGHBRED MARES

ABSTRACT: The objective of this study was to examine glucose and insulin dynamics in mares during the follicular and luteal phase of the estrous cycle by minimal model analysis of a frequently sampled i.v. glucose tolerance tests (FSIGT). Minimal model analysis was applied to the plasma glucose and insulin data, providing estimates of insulin sensitivity (SI), non-insulin dependent glucose disposal (glucose effectiveness, Sg), acute insulin response to glucose (AIRg), and the disposition index (DI). Nine non-pregnant Thoroughbred mares (11.5 ± 2.5 yr of age (range 7 to 15); body condition score, 7.0 ± 0.7 (range 6.5 to 8.5) and BW, 619 ± 52.9 kg (range 550 to 630)) were studied in three dietary treatments: fat and fiber (FF), sugar and starch (SS) and pasture (P). The FSIGT was performed in these mares at 9 (luteal phase) and 17 (follicular phase) days post ovulation during one complete estrous cycle. Plasma insulin was higher ($P < 0.01$) in FF (17.6 ± 2.7 mIU/L) than SS (7.7 ± 2.7mIU/L) or P (8.2 ± 2.7 mIU/L). Plasma glucose was not different between dietary groups or estrous cycle phases (100.8 ± 2.3 mg/dL; $P > 0.1$). Progesterone was higher in the luteal phase (18.2 ± 2.3 ng/mL) when compared to the follicular phase (4.2 ± 2.3 ng/mL; $P < 0.01$). Plasma leptin was not different between dietary groups or estrous cycle phases (4.1 ± 0.9 ng/mL; $P > 0.1$). Diet did not affect insulin sensitivity ($P < 0.1$). Insulin sensitivity (SI x 10^{-4} $L \cdot min^{-1} \cdot mIU^{-1}$) was lower in the luteal phase (3.1 ± 0.6) compared to the follicular phase (5.0 ± 0.6; $P <$

0.001) of the estrous cycle. Disposition index was lower ($P < 0.001$) in the luteal phase (713.2 ± 190) compared to the follicular phase (1132 ± 190). Glucose effectiveness (0.02 ± 0.01) and AIRg (269.5 ± 36), were not different ($P > 0.1$) between estrous cycle phases or among dietary treatments. The results of the present study indicate that stage of estrous affects insulin sensitivity and disposition index in cyclic mares and should be monitored when mares are used in studies characterizing glucose and insulin dynamics.

Introduction

There is interest in the measurement of insulin sensitivity in horses due to the association between insulin resistance and risk of laminitis (Treiber et al., 2006a). However, evaluation of insulin sensitivity values requires knowledge of physiological factors affecting glucose and insulin dynamics. Examples of these factors would include physiological state (e.g., pregnancy, training state) or stage of the estrous cycle.

Several studies have demonstrated alterations in glycemic regulation during the luteal phase of the menstrual cycle in women with insulin-dependent diabetes mellitus (Moberg et al., 1995; Widorn et al., 1992). In both non-diabetic and diabetic women, insulin sensitivity was lower during the luteal phase when compared to the follicular phase (Pulido and Salazar, 1999). However, another study in women reported no difference in insulin sensitivity between the luteal and follicular phases (Toth et al., 1987).

Progesterone stimulates both α- and β-cell proliferation and has been suggested to be responsible for increasing insulin secretion in pregnancy (Batista et al., 2005). Elevated leptin concentrations were associated with decreased insulin sensitivity and both

result in marked disturbances in the equine estrous cycle (Vick et al., 2006). Studies in women demonstrated elevated serum leptin during the luteal phase compared to the follicular phase of the menstrual cycle (Cella et al., 2000).

To the author's knowledge, there are no published data specifically relating stage of the estrous cycle to insulin sensitivity in the mare. It was hypothesized that mares would show similar patterns in glucose and insulin dynamics during the estrous cycle as rodents and women, with decreased insulin sensitivity during the luteal phase compared to the follicular phase. Therefore, the objective of this study was to characterize glucose and insulin dynamics using the minimal model approach in non-pregnant Thoroughbred mares during the luteal and follicular phases of the estrous cycle. Previous equine studies have shown that diets with increased sugar and starch increases basal insulin and decreases insulin sensitivity in late gestation and lactating mares (Hoffman et al., 2003b), geldings (Hoffman et al., 2003a), and weanlings (Treiber et al., 2005a). Therefore, a second objective was to determine whether supplementation with feeds rich in either SS or FF modified the influence of phase of estrous cycle on glucose and insulin dynamics.

Materials and Methods

Animals and management

Nine non-pregnant Thoroughbred mares (11.5 ± 2.5 yr of age (range 7 to 15) and 631 ± 16 kg BW (range 585 to 725)) were maintained on a 12-ha mixed grass-legume (bluegrass, fescue, clover) pasture at the Middleburg Agricultural Research and Extension (MARE) center. The pasture was divided into 3 even sections using temporary

electric fencing. The mares had been previously (January) separated into three dietary treatment groups which included a fat and fiber supplement (FF), a sugar and starch supplement (SS), or a pasture only (P) group (n = 3 in each) and acclimated to the diet for approximately 5 mo. Each dietary group was kept in a separate section to allow for daily supplementation in their natural herd environment with minimal disturbance at feeding times (Hoffman et al., 2003b; Treiber et al., 2005a). The mares received meals 3 times daily (0730, 1100, and 1400). Horses in the SS and FF groups only were collectively fed in a 30 m circle in pans containing individual portions. The mares were observed to ensure each consumed their allotted amount of supplement (Hoffman et al., 2003b). All horses consumed the total amount of the feed provided to them. No measures of pasture intake were recorded. This study was approved by the Institutional Animal Care and Use Committee of Virginia Tech.

Dietary Treatments

The dietary supplements FF and SS were formulated to be isocaloric and isonitrogenous and provided the mares with 67% of their DE requirements (NRC, 1989). Water and pasture forage were available ad libitum. A composite sample of each sub-section of the pasture was collected between 0800 and 1000 hr weekly, by following a zig-zag pattern between fences covering the entire section with samples taken at approximate 20 m intervals. Samples of the pasture and the supplements (Table 5.1) were submitted to a commercial laboratory for proximate and mineral analysis (Dairy One, Ithaca, NY, USA). Sugar represents water soluble sugars (glucose, sucrose, fructose) that were extracted prior to analysis for starch (Hall et al., 1999) and includes fructans. Starch

content was determined following enzymatic digestion (glucoamylase) with measurement of dextrose in an automated biochemical analyzer (YSI 2700 SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA, Application Note Number 319). The pasture harvested and analyzed from each section was not significantly different in contents of DE and major nutrients according to equivalence tests ($P > 0.05$) (Byrd et al., 2005). Therefore the data from each sub-section of pasture were pooled.

Experimental Design and Sample Collection

The estrous cycles were synchronized one estrous cycle prior to sampling using a progesterone and estradiol treatment regimen (Loy et al., 1981). Progesterone (150 mg) and estradiol 17 ß (10 mg) were administered i.m. daily for 10 d. On the 10^{th} d prostaglandin $F_2\alpha$ (10 mg) was also administered. Palpation per rectum and ultrasonic evaluation of the ovaries verified that mares ovulated within 9 to 10 d of the last treatment.

Stage of the estrous cycle and subsequent ovulation was determined by daily palpation and ultrasonography of the ovaries. The day of ovulation was defined as d 0 of the cycle. Mares were considered in the luteal phase of the estrous cycle at 9 d post ovulation and the follicular phase at 17 d post ovulation (Daels and Hughes, 1993) . Frequently sampled i.v. glucose tolerance tests (FSIGT) were administered to each horse at 9 d and 17 d post ovulation during one complete estrous cycle (Spicer et al., 2005).

On the morning of the test (0700 hr) a catheter (13 cm, 14 guage Milacath, Mila International Inc., Florence KY, USA) was inserted into a jugular vein after aseptic preparation and desensitization (lidocaine 2%) of the overlying skin. The mares were

weighed on an electronic scale (Tyrel Platform, model TC-10S; Allweights Hamilton Scale Corp., Richmond, VA, USA) before being placed in stalls with free access to grass hay and water. Hay consumption was of short duration and was not expected to cause perturbations in plasma glucose or insulin (Treiber et al., 2005a). At 0830 hr 300 mg/kg BW of glucose (Dextrose Solution 50%; Phoenix Pharmaceutical, Inc., St. Joseph, MO, USA) was rapidly administered (within 2 min) through the catheter. Twenty minutes after glucose administration, a bolus dose of insulin (20 mIU/kg BW; Humulin R, Eli Lilly and Co., Indianapolis, IN, USA) was administered through the catheter.

Basal blood samples were taken 15 and 0 min prior to the glucose dose. Additional samples were drawn at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, 180, 210, and 240 min after glucose administration. Samples were immediately transferred to tubes containing sodium heparin as anticoagulant (Vacutainer; Fisher Health Care, Chicago, IL, USA) and placed on ice until centrifugation (3,000 x g for 10 min at 4°C). Plasma was removed within 30 min of collection and frozen at -20ºC until analysis.

Blood Analysis

Plasma glucose concentrations were measured by the glucose oxidase method using a chemical autoanalyzer (Kit # 442640, Beckman Synchron CX5CE, Brea, CA, USA). Inter- and intra-assay CV were < 1% for plasma glucose. Plasma insulin and progesterone were determined by use of radioimmunoassays (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA, USA) previously validated for equine plasma (Freestone et al., 1991b; Ginther et al., 2005). Inter- and intra-assay CV for insulin were

5.3% and 5.5%, respectively. Inter- and intra-assay CV for progesterone were 7.1% and 4.9%, respectively. Plasma leptin concentrations were analyzed by use of a previously validated multi-species leptin radioimmunoassay (Linco Research Inc., St. Charles, MO) (Cartmill et al., 2003; McManus and Fitzgerald, 2000). Interassay CV for leptin was 4.1% and intraassay CV was 6.0%. All analyses were performed in duplicate. .

Minimal Model Analysis

Results of the frequently sampled i.v. glucose tolerance test (FSIGT) for each mare were analyzed according to the minimal model of glucose and insulin dynamics (Boston et al., 2003). The minimal model approach includes the following variables; insulin sensitivity (SI), non-insulin dependent glucose disposal (glucose effectiveness, Sg), acute insulin response to glucose 0 to 10 min (AIRg) and disposition index (DI). Insulin sensitivity is the efficiency of insulin secretion by the pancreas to accelerate glucose uptake. Glucose effectiveness is the capacity of tissue to uptake glucose independent of insulin. Acute insulin response to glucose is the endogenous insulin secretion increase from baseline between 0 and 10 min following IV glucose bolus. The disposition index (SI x AIRg) represents the ability of the pancreatic islets to compensate for insulin resistance (Boston et al., 2003; Treiber et al., 2005b).

Statistical Analysis

Nutrient analysis for supplements and pasture are summarized as means ± SEM. Plasma progesterone, glucose, insulin and leptin data were tested within diet for normality by the Shapiro-Wilkes statistic using Intercooled Stata 9.2 (Stata Corp.,

College Station, TX, USA). Plasma progesterone, glucose, insulin and leptin were analyzed using mixed model analysis of variance (ANOVA) obtained with the MIXED procedure of SAS (SAS, 2001). Main effects, consisting of diet and estrous cycle phase (luteal and follicular) and their interactions were analyzed. Differences between least squares means were compared if significant main effects or interactions were observed using the pairwise differences option in SAS. Data are presented as least squares means ± SEM. Pearson correlation coefficients were calculated to evaluate relationships between variables. For all analyses, significance was designated at a value of $P \leq 0.05$.

Results

Mean age, 11.5 ± 2.5 yr (range 7 to 15), BW, 619 ± 12 kg (range 532 to 725), and BCS, 7.3 ± 0.2 (range 6.5 to 9) were not different among dietary treatment groups ($P > 0.05$; Table 5.2). No difference was detected between the luteal and follicular phase when using pooled data for BW or BCS ($P > 0.05$). In all mares ovulation occurred 9 to 10 d post prostaglandin $F_2\alpha$ treatment. Plasma progesterone was significantly ($P < 0.001$) higher at d 9 post-ovulation (luteal, 18.2 ± 1.8 ng/mL) in comparison to 17 d post ovulation (follicular, 5.5 ± 2.1 ng/mL).

Diet ($P = 0.002$) but not phase of estrous cycle ($P = 0.98$) or the interaction between them ($P = 0.97$) affected basal insulin. Mares supplemented with FF (17.6 ± 2.7 mIU/L) had higher basal insulin than those supplemented with SS (7.7 ± 2.7 mIU/L) or those grazing pasture only (8.2 ± 2.7 mIU/L; $P < 0.01$). Basal glucose (100.8 ± 2.3 mg/dl) was not affected by diet ($P = 0.15$), by phase of the estrous cycle ($P = 0.26$) or by the interaction between them ($P = 0.19$). Similarly plasma leptin (4.1 ± 0.9 ng/mL) was

not affected by diet ($P = 0.16$), by phase of the estrous cycle ($P = 0.25$) or by the interaction between them ($P = 0.33$). Plasma progesterone was affected by the phase of the estrous cycle ($P < 0.01$) but not diet ($P = 0.95$) or the interaction between diet and estrous cycle phase ($P = 0.63$).

. Glucose effectiveness (Sg, 0.02 ± 0.01 min^{-1}) was not affected by diet ($P = 0.98$), phase of estrous cycle ($P = 0.27$) or the interaction between them ($P = 0.15$). Insulin sensitivity (SI x 10^{-4} $L \cdot min^{-1} \cdot mIU^{-1}$) was affected by estrous cycle phase ($P < 0.001$), but not diet ($P < 0.1$) or the interaction between diet and estrous cycle phase ($P = 0.36$). During the luteal phase (3.1 ± 0.6) of the estrous cycle insulin sensitivity was lower ($P < 0.01$) than during the follicular phase (5.0 ± 0.6).

Acute insulin response to glucose (AIRg, 269.5 ± 36 $(mIU/L) \cdot min^{-1}$) was not affected by diet ($P < 0.1$), phase of estrous cycle ($P = 0.43$) or the interaction between them ($P = 0.32$). Disposition index was affected by estrous cycle phase ($P < 0.001$), but not diet ($P = 0.96$) or the interaction between diet and estrous cycle phase ($P < 0.1$). During the luteal phase (713.2 ± 190) of the estrous cycle DI was lower ($P < 0.01$) than during the follicular phase (1132 ± 190).

Spearman correlation coefficients revealed a negative relationship between basal insulin and SI ($r = - 0.65$, $P < 0.05$) and positive relationships ($P < 0.05$) between insulin and glucose ($r = 0.70$), leptin ($r = 0.56$) and AIRg ($r = 0.72$). Glucose was negatively correlated with SI ($r = -0.61$) and DI (-0.57; $P < 0.05$). Leptin was negatively correlated with SI ($r = -0.47$) and positively correlated with AIRg ($r = 0.66$; $P < 0.05$). Insulin sensitivity was negatively correlated with AIRg ($r = -0.50$) and positively correlated with DI ($r = 0.65$, $P < 0.05$; Table 5.3).

Discussion

To the author's knowledge, this is the first study in mares to report lowered insulin sensitivity in the luteal phase of the estrous cycle compared to the follicular phase. Disposition index, an indication of the ability of the pancreas to adapt to lowered insulin sensitivity, also was lower in the luteal phase. However, Sg, AIRg, basal glucose and leptin concentrations did not differ between phases or among dietary groups. Basal insulin concentrations were higher in the FF mares compared to SS and P. It should be noted that all values for blood variables and the minimal model parameters were well within published reference ranges for Thoroughbred horses (Treiber et al., 2005b), including values for SI in the luteal phase.

Several lines of investigation have provided evidence that elevated circulating progesterone contributes to decreased insulin sensitivity. In women, lower insulin sensitivity during the luteal phase was related to a high progesterone state during this phase of the menstrual cycle (Pulido and Salazar, 1999). Decreased insulin sensitivity in women during pregnancy (Cousins, 1991) and in girls immediately prior to the onset of puberty also has been attributed to increased progesterone (Hannon et al., 2006). Moreover, the administration of exogenous progesterone to women and mice results in decreased insulin sensitivity (Livingstone and Collison, 2002). Accordingly, it is reasonable to speculate that the greater than threefold higher plasma progesterone concentration in the luteal when compared to follicular phase contributed to the variation in SI observed in the mares of the present study. The mechanism by which progesterone may alter insulin sensitivity is not known. Treatment of ovariectomized rats with natural progesterone for 5 to 7 d did not change insulin-mediated glucose intake in peripheral

tissues, but did reduce the ability of insulin to suppress endogenous glucose production (Nelson et al., 1994). In pregnant women progesterone significantly reduced insulin binding and glucose transport in adipose tissue (Ryan and Enns, 1988).

In the study reported here, no significant change in glucose effectiveness was detected, a finding in accordance with previous research in women (Pulido and Salazar, 1999). It can be concluded that variation in SI during the estrous cycle is independent of variation in glucose effectiveness.

Surprisingly, the lower SI in the luteal phase was not associated with a compensatory increase in pancreatic (i.e., AIRg), and DI was lower during the luteal phase compared to the follicular phase. In this and previous equine studies (Treiber et al., 2005a)), an inverse relationship between SI and AIRg has been observed, while a decrease in SI associated with weight gain or adaptation to a high starch diet has been accompanied by an increase in AIRg such that DI is unchanged (Treiber et al. 2005a). It is possible that the β-cell response to lowered insulin sensitivity is slow to develop. Alternatively, the small difference in SI between the luteal and follicular phase may have been insufficient to elicit a compensatory β-cell response.

The correlation between basal insulin and plasma leptin is consistent with previous observations on horses in different physiological states (Buff et al., 2006; Cartmill et al., 2005). This study also reported an inverse relationship between plasma leptin and SI. Pratt et al., 2005 also reported this – leptin may directly affect insulin sensitivity or the association may reflect other factors such as adiposity.

Many equine research groups have used the minimal model of glucose and insulin dynamics to evaluate the effects of physiological state (e.g., exercise) and other

environmental influences (e.g., diet) on the glucoregulatory system. The data reported here indicate that stage of the estrous cycle is a significant cause of variation in insulin sensitivity. This source of variation should be accounted for in the design of experiments utilizing mares for the assessment of glucose and insulin dynamics.

Table 5.1. Nutrient analysis of supplements high in sugar and starch (SS, n = 16), fat and fiber (FF, n = 16) or pasture (P, n = 2) summarized as means ± SEM.

Nutrient [b]	FF	SS	Pasture
DM %	91.5 ± 0.4[f]	87.7 ± 0.8[f]	22.5 ± 0.9[g]
DE, Mcal/kg [c]	2.9 ± 0.1[f]	3.0 ± 0.1[f]	2.4 ± 0.1[g]
Starch %	5.8 ± 1.9[f]	38.3 ± 2.5[g]	1.3 ± 0.1[f]
Sugar %	8.8 ± 0.4	10.7 ± 0.5	9.2 ± 0.6
NSC % [d]	14.6 ± 2.1[f]	49.1 ± 2.6[g]	10.6 ± 0.5[f]
NFC % [e]	23.2 ± 2.3[f]	55.3 ± 2.7[g]	16.4 ± 0.6[h]
EE %	12.5 ± 0.9[f]	4.3 ± 0.7[g]	3.7 ± 0.3[g]
ADF %	27.2 ± 1.2[f]	11.0 ± 1.1[g]	29.3 ± 0.8[h]
NDF %	40.9 ± 1.6[f]	18.9 ± 1.5[g]	55.8 ± 1.1[h]
CP %	15.5 ± 0.2[f]	14.4 ± 0.37[f]	21.8 ± 1.0[g]

[a] Analysis were performed at Dairy One DHIA Forage Testing Laboratory, Ithaca, NY.

[b] (% of Dry matter), digestible energy (DE), non-structural carbohydrates (NSC), non-fiber carbohydrates (NFC), ether extract (EE), acid detergent fiber (ADF), neutral detergent fiber (NDF), Crude protein (CP).

[c] Calculated using equation from (Harris and Kronfeld, 2002).

[d] NSC = starch + sugar, sugar = water soluble carbohydrates.

[e] Nonfiber carbohydrate, NFC = 100 – CP – fat – (NDF + neutral detergent insoluble CP) – ash.

[gfh] Groups with different letter superscripts (within row) differ ($P < 0.01$)

Table 5.2. Body weight (kg) and body condition score (BCS) of mares (n = 18) within dietary groups. Data are summarized as means ± SEM.

	FF	SS	Pasture
Weight	606 ± 34	639 ± 17	611 ± 25
BCS	7.8 ± 0.4	7.5 ± 0.3	6.5 ± 0.2[b]

Table 5.3. Spearman correlation coefficients (r) between blood variables and minmod parameters.

	Insulin	Glucose	Leptin	Progesterone	Sg	SI	AIRg	DI
Insulin	1.0	0.70	0.56	NS	NS	-0.65	0.72	NS
Glucose		1.0	NS	NS	NS	-0.61	NS	-0.57
Leptin			1.0	NS	NS	-0.47	0.66	NS
Progesterone				1.0	NS	NS	NS	NS
Sg					1.0	NS	NS	NS
SI						1.0	-0.50	0.65
AIRg							1.0	NS
DI								1.0

NS denotes not significant, all other ($P < 0.05$).

CHAPTER SIX
MANUSCRIPT 4

FOLLICULAR FLUID HORMONES AND METABOLITES ARE AFFECTED BY ESTROUS CYCLE PHASE, FOLLICLE SIZE AND DIET IN THOROUGHBRED MARES

ABSTRACT: The objective of this study was to compare effects of sugar and starch (SS) and fat and fiber (FF) feeds on plasma and follicular fluid concentrations of glucose, insulin, leptin, progesterone and estradiol in Thoroughbred mares during the luteal and follicular phases of the estrous cycle. Fifteen non-pregnant Thoroughbred mares 11.1 ± 0.5 yr of age (range 6 to 13); 610.2 ± 16 kg BW (range 532 to 725) and BC, 7.1 ± 0.2 (range 5.5 to 9) were fed in three dietary treatments FF, SS, and pasture only (P). Body condition score was higher in the FF and SS groups compared to the pasture group (7.6 ± 0.2 and 7.5 ± 0.2 v.s. 6.1 ± 0.1; $P < 0.001$ respectively). Plasma progesterone was higher ($P < 0.001$) 9 d after ovulation (luteal, 16.1 ± 1.6 ng/mL) in comparison to 17 d post ovulation (follicular, 3.5 ± 1.9 ng/mL). Estradiol concentration (ln (x+1)) was higher ($P < 0.001$) in large follicles (7.3 ± 0.3 ng/mL) in the follicular phase than large follicles (5.5 ± 0.5 ng/mL) in the luteal phase. Insulin concentration (ln (x+1)) was 52% higher ($P < 0.01$) in large (> 25 mm) follicles (1.4 ± 0.1 mIU/L) than either medium (16 to 25 mm) or small (≤ 15 mm) follicles (0.9 ± 0.1; 0.9 ± 0.1 mIU/L, respectively) irrespective of estrous cycle phase. A positive correlation was observed between follicular fluid (FFL) leptin and plasma leptin (r = 0.30; $P < 0.001$). A similar relationship was observed between FFL insulin and plasma insulin (r = 0.25; $P < 0.001$). Plasma insulin and leptin

were positively associated ($r = 0.45$, $P < 0.0001$), along with FFL insulin and FFL leptin ($r = 0.46$, $P < 0.0001$). Diet may have an indirect effect on FFL concentrations of insulin and leptin via its action plasma concentrations of these variables.

Introduction

The interaction between nutrition and reproduction has major implications for the reproductive performance of the mare (Harris and Kronfeld, 2002). Research regarding nutrition and reproduction in the mare has primarily focused on the impact of body condition (Henneke et al., 1984; Waller et al., 2006) and energy availability or restriction (McManus and Fitzgerald, 2000), with minimal work on the influence of different energy source on the ovary in mares.

A review (Scaramuzzi et al., 2006) of nutrition, reproductive and metabolic hormones and folliculogenesis in the ewe suggested that insulin, glucose and leptin may be key links between diet and the follicle. Insulin had specific effects on granulosa and theca cell function (Campbell et al., 1996; Yen et al., 2004). In sheep, insulin-dependent glucose transporter (GLUT-4) was identified in follicular granulosa and theca cells (Williams et al., 2001b) and changes in insulin-mediated glucose uptake within the ovary modulated ovarian function. Insulin may regulate folliculogenesis via effects on glucose uptake or by direct action on folliculogenesis (Downing and Scaramuzzi, 1997; Somchit et al., 2007).

Leptin secretion was stimulated by insulin (Patel et al., 1998). Recent studies in horses have demonstrated a positive relationship between plasma insulin and leptin following a grain meal or constant insulin infusion (Cartmill et al., 2005). Leptin may

exert direct effects on ovarian function (Barkan et al., 2005). The functional long form of the leptin receptor (OB-Rb) is expressed in ovarian follicles of mice, and leptin inhibits progesterone synthesis in ovarian granulosa cells in vitro (Zachow and Magoffin, 1997).

Previous research reported that feeds abundant in sugars and starches (SS) or fat and fiber (FF) elicit different responses in insulin and glucose dynamics (Staniar et al., 2007; Treiber et al., 2005a; Williams et al., 2001a). Diets high in SS increase basal insulin concentrations and reduced oocyte quality and embryo development when at elevated concentrations (Adamiak et al., 2006) in bovine oocytes in vitro.

Our hypothesis was that feeding supplementary dietary energy as either FF or SS would increase follicular fluid insulin, glucose and leptin. It was also hypothesized that concentration of insulin, glucose and leptin in FFL may mimic plasma. The objective of this study was to compare effects of SS and FF feeds on plasma and follicular fluid concentrations of glucose, insulin, leptin, progesterone and estradiol in Thoroughbred mares during the luteal and follicular phases of the estrous cycle.

Materials and Methods

Mares and management

Fifteen non-pregnant Thoroughbred mares (11.1 ± 0.5 yr of age (range 6 to 13); 610.2 ± 16 kg BW (range 532 to 725) and BCS, 7.1 ± 0.2 (range 5.5 to 9)) were maintained on a 12-ha mixed grass-legume (bluegrass, fescue, clover) pasture at the Middleburg Agricultural Research and Extension (MARE) center. The pasture was divided into 3 even sections using temporary electric fencing. Five months prior to the commencement of the study, mares were separated into three dietary treatment groups:

FF, SS, or pasture only (P) (n = 5 in each). Each dietary group was kept in a separate section to allow for daily supplementation in their natural herd environment with minimal disturbance at feeding times (Hoffman et al., 2003b; Treiber et al., 2005a). Mares received meals 3 times daily (0730, 1100, and 1400). Horses were fed in a 30 m circle of feed pans containing individual portions. The mares were observed to ensure each consumed their allotted amount of supplement. All horses consumed the total amount of the feed provided to them. No measures of pasture intake were recorded.

The mare's estrous cycles were synchronized, one estrous cycle prior to sampling using a progesterone and estradiol treatment regimen (Loy et al., 1981). Progesterone (150 mg) and estradiol 17 ß (10 mg) were administered i.m. daily for 10 days. On the 10^{th} day, prostaglandin $F_2\alpha$ (5 mg) was administered. Palpation per rectum and ultrasonic evaluation of the ovaries verified that mares ovulated within 9 to 10 days of the last treatment. This study was approved by the Institutional Animal Care and Use Committee of Virginia Tech.

Dietary Treatments

The dietary supplements FF and SS were formulated to be isocaloric and isonitrogenous and provided the mares with 67% of their daily DE requirements (NRC, 1989). Water and pasture forage were available ad libitum. A composite sample of each sub-section of the pasture was collected between 0800 and 1000 hr weekly by following a zig-zag pattern between fences covering the entire section and with samples taken at approximate 20 m intervals. Samples of pasture and the supplements (Table 6.1) were submitted to a commercial laboratory for proximate and mineral analysis (Dairy One,

Ithaca, NY). Sugar represents water soluble sugars that were extracted prior to analysis for starch (Hall et al., 1999) and includes fructans. Starch content was determined following enzymatic digestion (glucoamylase) with measurement of dextrose in an automated biochemical analyzer (YSI 2700 SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA, Application Note Number 319). The pasture harvested and analyzed from each section was not significantly different in contents of DE and major nutrients according to equivalence tests ($P > 0.05$) (Byrd et al., 2005). Therefore, the data from each sub-section of pasture were pooled.

Experimental Design and Aspiration Procedure

The experiment was conducted during the mid-ovulatory season (June; Northern Hemisphere) (Utt et al., 2007). Ovaries were examined once daily by transrectal ultrasound throughout the synchronization protocol to determine the day of ovulation (Day 0). Mares were considered in the luteal phase of the estrous cycle at approximately 9 d post ovulation and the follicular phase at approximately 17 d post ovulation (Daels and Hughes, 1993).

The aspiration procedure was carried out on all mares on both d 9 and d 17 post ovulation in the same estrous cycle. An ultrasound (Aloka 500 SSD, Wallingford, CT) machine with a 5MHz curvilinear probe housed in a hard plastic casing with a needle guide was used for visualization and aspiration of follicles. Each follicle was aspirated with an 18-gauge needle attached to a 60 cc syringe. The probe was inserted transvaginally and placed lateral to the exterior cervical os; the ovary was then manipulated transrectally and placed next to the end of the ultrasound probe to visualize

follicles during the aspiration procedure (Carnevale and Ginther, 1993; Purcell et al., 2007). All accessible follicles were aspirated and where were categorized as being small (≤ 15 mm), medium (16-25 mm), or large (> 25 mm) using the external diameter of the follicle (Spicer et al., 2005).

Sample Collection and Analysis

Basal blood samples were taken prior to the each aspiration. Samples were immediately transferred to tubes containing sodium heparin as anticoagulant (Vacutainer; Fisher Health Care, Chicago, IL) and placed on ice until centrifugation (3,000 x g for 10 min at 4°C). Plasma was removed within 30 min of collection and frozen at -20ºC until analysis. Follicular fluid samples were collected into a 60 cc syringe, volume was recorded and then fluid was transferred into plain glass tubes and centrifuged (200 x g for 5 min) to remove granulosa cells as previously described (Davidson et al., 2002). Samples were then frozen at -20ºC until analysis.

Plasma and follicular fluid (FFL) glucose concentrations were measured by the glucose oxidase method using an autoanalyzer (Kit # 442640, Beckman Synchron CX5CE, Brea, CA). Inter- and intra-assay CV were < 1% for glucose. Plasma insulin and progesterone were determined by use of radioimmunoassays (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA, USA) previously validated for equine plasma (Freestone et al., 1991b; Ginther et al., 2005). Inter- and intra-assay CV for insulin were 5.3% and 5.5%, respectively. Inter- and intra-assay CV for progesterone were 7.1% and 4.9%, respectively. Plasma leptin concentrations were analyzed by use of a previously validated multi-species leptin radioimmunoassay (Linco Research Inc., St.

Charles, MO) (Cartmill et al., 2003; McManus and Fitzgerald, 2000). Inter- and intra-assay CV for leptin was 4.1% and 6.0%, respectively. Follicular fluid progesterone and estradiol were determined by use of radioimmunoassays (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA, USA) previously validated for equine follicular fluid (Spicer et al., 1991). Follicular fluid insulin was determined by use of a radioimmunoassay (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA, USA) and FFL leptin was also determined by a radioimmunoassay (Linco Research Inc., St. Charles, MO). All analyses were performed in duplicate.

Statistical Analysis

Nutrient analysis for supplements and pasture are summarized as means ± SEM. All other data are summarized as least squares means ± SEM. Follicular fluid (FFL) estradiol, progesterone, glucose, insulin and leptin data were tested for normality by the Shapiro-Wilkes statistic (Intercooled Stata 9.2, Stata Corp., College Station, TX). This test showed that all variables were non-normally distributed. Data were transformed to [ln(x + 1)] which revealed normal distributions in estradiol, progesterone and insulin. These variables were analyzed using mixed model analysis of variance (ANOVA) obtained with the MIXED procedure of SAS (SAS, 2001). Main effects of diet, follicle size (small, medium and large) and estrous cycle phase (luteal and follicular) and their various interactions were evaluated. Differences between least squares means were compared if significant main effects or interactions were observed. Non-normal data (FFL glucose and leptin) were analyzed by the nonparametric Kruskal-Wallis test for differences between diet, follicle size and estrous cycle phase. Plasma concentrations of

insulin, glucose, leptin and progesterone were analyzed using the MIXED procedure of SAS where main effects were diet and estrous cycle phase and the interaction between diet and estrous cycle phase.

Spearman rank correlation coefficients were calculated to evaluate relationships between variables. Mean FFL variable concentrations for each horse were compared to plasma concentrations of the same variable using correlation coefficients. Relationships between variables were tested within plasma and FFL using correlation coefficients. Significance was designated at a value of $P \leq 0.05$.

Results

Body weight was not different between the dietary groups (Table 6.2). Body condition score was higher in the FF and SS groups compared to the pasture group (7.6 ± 0.2 and 7.5 ± 0.2 v.s. 6.1 ± 0.1; $P < 0.001$, respectively; Table 6.2). Follicle dynamics and FFL volume are presented in Table 6.3.

Palpation per rectum and ultrasound evaluation of the ovaries was performed daily from the beginning of the synchronization treatment and revealed an ovulation 9 to 10 d post prostaglandin $F_2\alpha$ treatment and elevated ($P < 0.001$) plasma progesterone by d 9 after ovulation (luteal, 16.1 ± 1.6 ng/mL) in comparison to 17 d post ovulation (follicular, 3.5 ± 1.9 ng/mL; $P < 0.01$).

Follicular fluid data for estradiol, progesterone and insulin are presented as transformed (ln(x+1)) values. Follicular fluid leptin and glucose are presented as actual values. There was no significant effect of diet on ($P > 0.1$) any of the FFL variables. Therefore, diet was removed from the model. Main effects of follicle size (large, 6.4 ±

0.29; medium, 5.1 ± 0.29; small, 4.5 ± 0.29 ng/mL; $P < 0.0001$) and estrous cycle phase (luteal, 4.9 ± 0.23; follicular, 5.8 ± 0.23 ng/mL; $P < 0.001$) and the size x phase interaction affected FFL estradiol concentrations ($P = 0.02$; Table 6.4). Estradiol concentration was higher ($P < 0.001$) in large follicles (7.3 ± 0.3 ng/mL), in the follicular phase than large follicles (5.5 ± 0.5 ng/mL) in the luteal phase (Table 6.4). Follicle size (P = 0.79) or the interaction between follicle size and estrous cycle phase ($P = 0.17$) did not affect FFL progesterone concentrations. Luteal follicles had higher ($P < 0.01$) progesterone concentrations (4.1 ± 0.2 ng/mL) than follicular follicles (3.4 ± 0.2 ng/mL). Follicular fluid insulin concentrations were affected by follicle size ($P < 0.001$) but not estrous cycle phase ($P = 0.80$) or the interaction between follicle size and estrous cycle phase ($P = 0.20$). Large follicles (1.4 ± 0.1 mIU/L) contained 52% more insulin than either medium or small follicles (0.9 ± 0.1, 0.9 ± 0.1 mIU/L, respectively). Follicular fluid glucose (97.4 ± 1.07 mg/dL) and leptin (2.4 ± 0.3 ng/mL) concentrations were not affected ($P > 0.1$) by follicle size, estrous cycle phase or the size x phase interaction.

Plasma insulin was affected by diet ($P < 0.05$), estrous cycle phase ($P < 0.01$) and the interaction between diet and estrous cycle phase ($P < 0.01$). In the luteal phase mares in the SS group (15.9 ± 2.4 mIU/L) had higher ($P < 0.05$) insulin than P (7.5 ± 2.4 mIU/L). In the follicular phase plasma insulin was higher in SS (13.6 ± 2.4 mIU/L) than P (8.6 ± 2.4 mIU/L; Table 6.5). Plasma leptin was affected by diet ($P < 0.01$) and the interaction between diet and estrous cycle phase (P < 0.001; Table 6.5), but not estrous cycle phase alone ($P = 0.18$). Plasma leptin was higher ($P < 0.01$) in the SS mares (6.0 ± 1.1 ng/mL) compared to the P mares (2.4 ± 1.1 ng/mL). Plasma progesterone was affected by estrous cycle phase (P < 0.001) and the interaction between diet and estrous

cycle phase ($P < 0.001$), but not diet alone ($P = 0.39$). Luteal phase plasma progesterone (15.2 ± 1.5 ng/mL) was higher than follicular phase (4.1 ± 1.5 ng/mL; $P < 0.01$) progesterone. Plasma glucose (99.4 ± 1.1mg/dl) was not affected by diet ($P = 0.93$), estrous cycle phase ($P = 0.38$) or the diet x estrous cycle phase interaction ($P = 0.19$).

Spearman correlations revealed a relationship between plasma and FFL leptin ($r = 0.30$; $P < 0.01$) and plasma and FFL insulin ($r = 0.25$; $P < 0.05$; Figure 6.1). A relationship was evident between plasma leptin and insulin ($r = 0.45$; $P < 0.001$) and FFL leptin and insulin ($r = 0.46$; $P < 0.001$; Figure 6.2). Follicular fluid insulin and estradiol were also correlated ($r = 0.52$; $P < 0.001$; Table 6.6).

Discussion

To the authors knowledge the present study is the first to report concentrations of insulin, glucose and leptin in equine follicular fluid. The present study is also the first to examine dietary influences on follicular fluid concentrations of metabolites and hormones in mares. The main findings were: 1) FFL estradiol was affected by size of the follicle and FFL progesterone was affected by phase of the estrous cycle. 2) Larger follicles had higher concentrations of insulin compared to medium and small follicles. 3) Diet did not have an effect on follicular fluid concentrations of hormones or metabolites, but did influence leptin and insulin concentrations in plasma. 4) Relationships were evident between plasma and follicular fluid concentrations of leptin and insulin. 5) Associations were apparent between insulin and leptin in both plasma and follicular fluid.

The effect of dietary energy source on plasma concentrations of insulin and leptin was shown in chapter 1 and 2. This study reported insulin concentrations 52%

higher in large when compared to medium and small follicles. These results disagree with a recent study that measured insulin in bovine follicular fluid did not detect a difference in FFL insulin between follicle sizes (Spicer et al., 2004). Spicer et al., (2004) did however report an increase in the expression of the insulin receptor (IR) in the large follicles along with high concentrations of estradiol and suggested that high expression of IR is one of the crucial factors promoting development of the follicle to the pre-ovulatory stage (Shimizu et al., 2007).

Leptin plays a role in the central regulation of reproduction (Spicer, 2001). It is known that in healthy cyclic women serum leptin concentrations peak in the luteal phase (Cella et al., 2000). In contrast, the present study found that follicular fluid and plasma leptin concentrations were not significantly higher in the luteal compared to the follicular phase, findings that are in accordance with a study in dairy cattle (Dayi et al., 2005).

In plasma, both insulin and leptin were affected by diet, but not by estrous cycle phase. Leptin followed the same pattern as insulin with elevated concentrations in SS and FF as compared to pasture. This difference between dietary treatment groups and the control group may be due to increased body condition score in the FF and SS mares compared to the pasture group. Leptin has been clearly shown to relate to fat mass in the horse (Buff et al., 2002) and obesity in mares is related to insulin resistance (Vick et al., 2006). However, the relationship between insulin and leptin has been reported in horses where there is no difference between BC in the horses (Buff et al., 2006).

In this study there was a positive relationship between FFL insulin and FFL estradiol and higher concentrations of both insulin and estradiol in large follicles. In agreement with others, the measurement of estradiol in FFL from individual follicles

indicated that as follicle size increased, concentrations of estradiol increased (Chang et al., 1976; Somchit et al., 2007). An *in vitro* study using cultured granulosa cells showed that insulin readily stimulated granulosa cell estradiol production (Spicer et al., 1993).

Whilst diet may not have directly affected follicular fluid variables, there was a relationship between diet and plasma concentrations of insulin and leptin and in turn direct relationships between plasma and FFL concentrations of both insulin and leptin, which had associations with estradiol and progesterone. Therefore, it could be concluded that diet has an indirect influence on FFL concentrations of insulin, glucose, leptin, progesterone and estradiol.

A more complete understanding of how and when nutrition affects folliculogenesis will enable more targeted nutritional strategies that will optimize reproductive efficiency.

Table 6.1. Nutrient analysis of supplements high in sugar and starch (SS, n = 16), or fat and fiber (FF, n = 18) and pasture summarized as means ± SEM.

Nutrient [b]	FF	SS	Pasture
DM %	91.5 ± 0.4[f]	87.7 ± 0.8[f]	22.5 ± 0.9[g]
DE, Mcal/kg [c]	2.9 ± 0.1[f]	3.0 ± 0.1[f]	2.4 ± 0.1[g]
Starch %	5.8 ± 1.9[f]	38.3 ± 2.5[g]	1.3 ± 0.1[f]
Sugar %	8.8 ± 0.4	10.7 ± 0.5	9.2 ± 0.6
NSC % [d]	14.6 ± 2.1[f]	49.1 ± 2.6[g]	10.6 ± 0.5[f]
NFC % [e]	23.2 ± 2.3[f]	55.3 ± 2.7[g]	16.4 ± 0.6[h]
EE %	12.5 ± 0.9[f]	4.3 ± 0.7[g]	3.7 ± 0.3[g]
ADF %	27.2 ± 1.2[f]	11.0 ± 1.1[g]	29.3 ± 0.8[h]
NDF %	40.9 ± 1.6[f]	18.9 ± 1.5[g]	55.8 ± 1.1[h]
CP %	15.5 ± 0.2[f]	14.4 ± 0.37[f]	21.8 ± 1.0[g]

[a] Analysis were performed at Dairy One DHIA Forage Testing Laboratory, Ithaca, NY.

[b] (% of Dry matter), digestible energy (DE), non-structural carbohydrates (NSC), non-fiber carbohydrates (NFC), ether extract (EE), acid detergent fiber (ADF), neutral detergent fiber (NDF), Crude protein (CP).

[c] Calculated using equation from (Harris and Kronfeld, 2002).

[d] NSC = starch + sugar, sugar = water soluble carbohydrates.

[e] Nonfiber carbohydrate, NFC = 100 – CP – fat – (NDF + neutral detergent insoluble CP) – ash.

[gfh] Groups with different letter superscripts (within row) denote differences between treatments ($P < 0.01$)

Table 6.2. Body weight (kg) and body condition score (BCS) of mares (n= 15) within dietary groups. Data are summarized as means ± SEM.

	FF	SS	Pasture
Weight	602 ± 17	628 ± 16	599 ± 10
BCS	7.6 ± 0.2[a]	7.5 ± 0.2[a]	6.1 ± 0.1[b]

[ab] Denotes differences between dietary groups

Table 6.3. Follicle dynamics during the luteal and follicular phase of the estrous cycle.

Summarized as means ± SEM (range).

		Follicle Diameter (mm)	Follicular Fluid Volume (mL)
Luteal	Small (≤ 15 mm) n = 81	9.3 ± 0.4 5 to 15	1.4 ± 0.1 0.1 to 6.0
	Medium (16 to 25 mm) n = 35	21.5 ± 0.4 17 to 25	3.3 ± 0.3 0.6 to 10
	Large (> 25mm) n = 4	30.0 ± 1.0 30 to 35	7.8 ± 1.1 5.3 to 11
Follicular	Small (≤ 15 mm) n = 46	11.9 ± 0.4 5 to 15	1.55 ± 0.2 0.2 to 4.3
	Medium (16 to 25 mm) n = 29	21.9 ± 0.5 17 to 25	3.8 ± 0.4 0.5 to 10
	Large (> 25mm) n = 16	35.8 ± 1.5 30 to 50	12.2 ± 1.9 3.1 to 40

Table 6.4. Least square means ± SEM results for follicular fluid estradiol within a follicle size during the luteal or follicular phase of the estrous cycle (all values are presented as ln(x+1)).

Follicular Fluid Variable[†]		Luteal	Follicular
Estradiol, ng/mL	large	5.5 ± 0.5*[a]	7.3 ± 0.3*[a]
	medium	4.7 ± 0.4[ab]	5.4 ± 0.4[b]
	small	4.5 ± 0.2[b]	4.7 ± 0.3[b]

* Significant differences ($P < 0.05$) between luteal and follicular phase within a follicle size (within row).

[a,b] Superscripts denote significant ($P < 0.05$) differences between follicle sizes (within column).

Table 6.5. Least square means ± SEM results for plasma variables within diet during the luteal or follicular phase of the estrous cycle.

Blood Variable[†]		Luteal	Follicular
Insulin, mIU/L	FF	12.0 ± 2.4^{b}	$8.9 \pm 2.4^{ab*}$
	SS	15.9 ± 2.4^{a}	$17.2 \pm 2.4^{a*}$
	Pasture	7.2 ± 2.4^{c}	7.3 ± 2.4^{b}
Leptin, ng/mL	FF	4.9 ± 1.1^{ab}	4.03 ± 1.1^{ab}
	SS	6.11 ± 1.1^{a}	5.9 ± 1.1^{a}
	Pasture	2.4 ± 1.1^{b}	2.3 ± 1.1^{b}
Progesterone, ng/mL	FF	17.8 ± 3.1*	1.02 ± 3.1*
	SS	14.0 ± 3.1*	3.3 ± 3.1*
	Pasture	17.9 ± 3.1*	5.8 ± 3.1*

* Significant differences ($P < 0.05$) between luteal and follicular phase within a diet (within row).

[a,b] Superscripts within a blood variable denotes a significant ($P < 0.05$) difference between diets (within column).

Table 6.6. Spearman correlation coefficients (r) between follicular fluid (FFL) and plasma variables.

	Insulin	Glucose	Leptin	Progesterone	FFL Insulin	FFL Glucose	FFL Leptin	FFL Progesterone
Insulin	1.0							
Glucose	0.60	1.0						
Leptin	0.45	0.27	1.0					
Progesterone	ns	ns	ns	1.0				
FFL Insulin	0.25	ns	0.22	ns	1.0			
FFL Glucose	ns	0.15	ns	ns	0.26	1.0		
FFL Leptin	0.20	ns	0.30	ns	0.46	0.22	1.0	
FFL Progesterone	ns	ns	ns	0.30	0.36	0.20	ns	1.0
FFL Estradiol	ns	ns	ns	ns	0.52	0.21	0.20	0.22

Significance $P < 0.05$, ns denotes not significant.

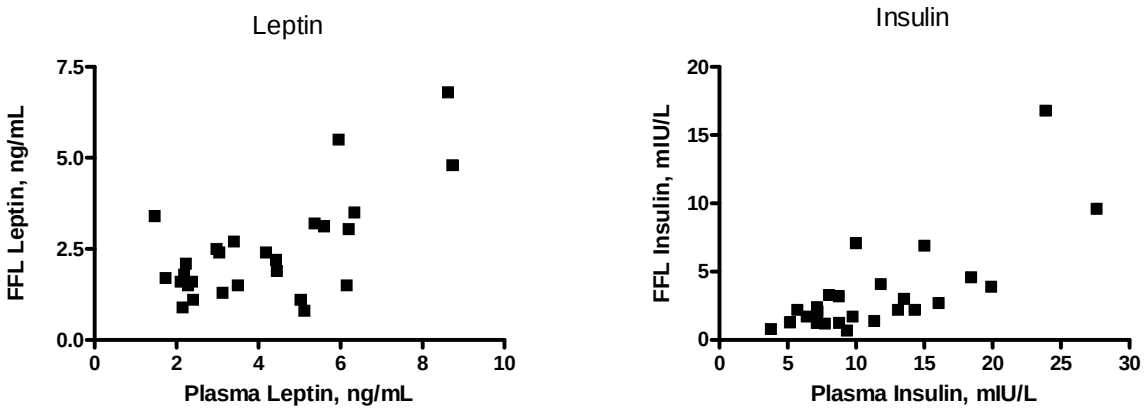

Figure 6.1. Scatterplots showing relationships between follicular fluid leptin and plasma leptin and follicular fluid insulin and plasma insulin.

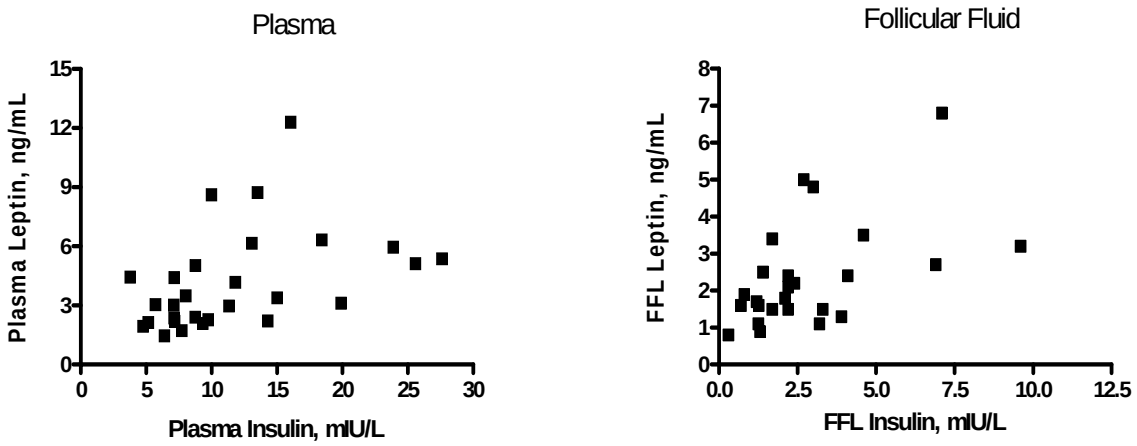

Figure 6.2. Scatterplots of relationships between leptin and insulin concentrations in both plasma and follicular fluid.

CHAPTER SEVEN

SUMMARY AND CONCLUSIONS

The following summaries key findings from these studies:

The objective of the first study was to examine seasonal and circadian fluctuations of plasma leptin concentrations in relation to insulin in grazing mares, and to identify possible associations with pasture NSC content. Plasma leptin concentrations were consistently higher in grazing mares compared to those confined to stalls. No circadian pattern was evident in plasma leptin concentrations, but there was an association between circulating insulin and leptin concentrations. In spring (April) and autumn (October) plasma insulin and leptin concentrations were higher than in summer (August) and winter (January).

The first study involved observation of mares over a 22 h period within each season. These short windows of observation did not capture the transition between seasons. Therefore, the objectives of the second study were to characterize insulin, glucose and leptin during the transition from winter to spring in pasture kept mares. Additionally, the effect of adding supplementary dietary energy as either FF or SS was examined. As in the first study, and in agreement with the findings of previous reports (Buff et al., 2006), plasma leptin was higher in spring compared to winter. The increase in plasma leptin concentration was associated with an increase in plasma insulin concentrations in mares supplemented with SS or FF. Earlier studies at the MARE Center showed that increases in plasma insulin concentrations were directly related to elevations in pasture NSC (Byrd et al., 2006). In the present study, alteration in the WSC fraction of NSC was primarily associated with variations in circulating plasma insulin. A

relationship between plasma insulin and leptin was evident in this study, but only in mares supplemented FF or SS, not in those maintained at pasture alone. Plasma insulin concentration increased in all groups at the same time (coincident with an increase in pasture forage WSC content), but the peak concentration in non-supplemented mares (pasture only) was lower when compared to the SS and FF fed mares. One interpretation is that there may be a threshold effect of plasma insulin on leptin, potentially explaining the lack of rise in plasma leptin in the pasture group. Further investigation is warranted to test this hypothesis.

The second study showed that supplementary dietary energy as either FF or SS fed to grazing mares elicited a more heightened response in plasma insulin when compared to mares on pasture only. Previous work in the horse has shown that diet and physiological state (Hoffman et al., 2003b) impact glucose and insulin dynamics. In women it has been reported that insulin sensitivity was decreased during the luteal phase compared to the follicular phase of the menstrual cycle (Pulido and Salazar, 1999). Mares are commonly used in research protocols examining aspects of glucoregulation, but to date there have been no reports on the effect of stage of estrous cycle on insulin sensitivity or leptin concentration in the mare. My study (Chapter 3) demonstrated that, as in women, insulin sensitivity in mares is decreased in the luteal phase compared to the follicular phase of the estrous cycle. This finding has major implications for the use of mares in studies evaluating glucose and insulin dynamics.

A logical progression from evaluating the effect of the estrous cycle and diet on insulin sensitivity was to investigate possible interactions between diet and estrous cycle phase on the ovary (Chapter 4). The specific objective was to compare the effects of

supplementation with FF or SS (vs. pasture only) on follicular fluid concentrations of insulin, glucose and leptin and subsequently the effect of these dietary treatments on follicular fluid steroid concentrations and follicle development in mares during the luteal and follicular phase of the estrous cycle. Follicle size affected follicular fluid concentrations of estradiol and insulin, with the concentrations of both hormones higher in large follicles compared to medium and small follicles.

Whilst diet did not have a detectable direct effect on follicular fluid variables, there was a relationship between diet and plasma concentrations of insulin and leptin and in turn direct relationships between plasma and FFL concentrations of both insulin and leptin, which had associations with estradiol and progesterone. Therefore, it could be argued that diet has an indirect influence on FFL concentrations of insulin, glucose, leptin, progesterone and estradiol.

One limitation of the studies presented in Chapters 3 and 4 was the small number of mares in the 3 dietary treatments (n = 3 in Chapter 3; n = 5 in Chapter 4). The associated low statistical power may have precluded detection of differences among the dietary treatments. Further studies utilizing larger group sizes are recommended to confirm the findings reported in this dissertation. Future studies evaluating the amount of insulin required to elicit a leptin response in the horse would add to the information reported here and determine whether a threshold effect exists. Finally, it should be noted that basal insulin concentrations were significantly different in mares between seasons and evidence suggested this was due to seasonal perturbations in pasture WSC. Insulin sensitivity values, however, fell well within the normal references ranges for horses described by Treiber and associates (2005b). Single sample measurement of plasma or

serum insulin concentration has been advocated as a screening test for insulin resistance in horses and ponies. Several laboratories use a cutoff of 30 mU/L, with values above this concentration taken as evidence of insulin resistance. However, the present studies have demonstrated that this interpretation would be inappropriate in pasture kept equids, wherein insulin values > 30 mU/L may reflect an appropriate response to nutrient intake (i.e., forage WSC) rather than insulin resistance with compensatory hyperinsulinemia.

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VITAE

Tania A. Cubitt was raised in rural Queensland Australia. Her family has been long time sheep and cattle farmers in the South Island of New Zealand. She received her B.S. from the University of Queensland in animal science. She was then awarded a Rotary International Ambassadorial Scholarship and traveled to the USA to study with the late Dr. David S. Kronfeld. She received her M.S. from Virginia Tech in equine nutrition and growth, this work focused on changes in environmental influences, the somatotropic axis, and hormonal and physical characteristics in Thoroughbred fillies at the onset of puberty. She continued her graduate career pursuing a Ph.D. under the guidance of Dr. Raymond J. Geor in equine nutrition and reproduction. She hopes to pursue a career in equine nutritional consulting.