

THE HYPERINSULINEMIA-HYPERLEPTINEMIA SYNDROME IN HORSES:
ASSESSMENT OF METHODS OF DIAGNOSIS AND DIFFERENTIAL EFFECTS OF
INSULIN INJECTION ON GLUCOSE, GLUCAGON AND NONESTERIFIED FATTY
ACIDS IN PLASMA

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“If I have seen far, it is by standing on the shoulders of giants.”

Sir Isaac Newton (1643 – 1727)

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ABSTRACT

Plasma leptin concentrations in horses have been shown to vary widely. However, many factors may perturb these measurements. Previous research has documented a link between hyperinsulinemia and hyperleptinemia in horses. This condition has been speculated to be associated with components of equine metabolic syndrome, such as insulin resistance and laminitis. However, many of the methods accepted in diagnosing these metabolic abnormalities are expensive and often difficult to perform. Thus, the objectives of the experiments in this thesis were 1) to determine if it is possible to differentiate hyperleptinemic horses from normal ones by hormone manipulation with dexamethasone, a glucocorticoid analog; 2) to evaluate intravenous injection combinations of insulin and glucose to assess insulin sensitivity; and 3) to assess insulin sensitivity in hyperleptinemic and normal horses using two concentrations of exogenous insulin. In the first experiment, it was determined that a single injection of dexamethasone did not enhance the ability to differentiate between hyperleptinemic and normal horses. In the second experiment, the combined intravenous insulin and glucose tests proved to be very simple and inexpensive. However, repeatability among estimates on the same horses was not strong enough to be conclusive or predictive in assessing insulin sensitivity in horses. In the third experiment, a single injection of exogenous insulin at 0.02 IU/kg of body weight proved to be a consistent and efficacious method of differentiating hyperleptinemic from normal horses. Glucose concentrations in hyperleptinemic horses decreased very little (14%) at this insulin dosage compared to those in normal horses that dropped ($P < 0.01$) 46% from a pre-injection average of 85 to 46 mg/dL 40 min after injection. It was concluded that a low-level insulin injection coupled with routine plasma leptin estimates constitute a simple method of identifying hyperleptinemic horses.

CHAPTER 1

INTRODUCTION

Hyperleptinemia in horses was first reported for obese horses by Gentry et al. (2002), studying the relationship between body condition score (BCS), leptin, and reproductive hormones in mares. Approximately 30% of the mares studied were hyperleptinemic (concentrations >10 ng/mL). Those mares had high BCS and prolonged ovulatory activity, compared to mares of moderate BCS that became anovulatory in winter. Cartmill et al. (2003) observed that the same mares studied 2 yr later were still hyperleptinemic, indicating that the condition was relatively permanent in these horses. Furthermore, Cartmill et al. (2003) reported that these hyperleptinemic horses also had elevated plasma insulin concentrations. This dynamic of elevated plasma insulin and leptin concentrations is believed to play a role in metabolic disturbances due to hormonal abnormalities in the horse, such as equine metabolic syndrome (EMS) and laminitis (Johnson, 2002). The current research was designed to expand the knowledge base of hyperleptinemia in horses by testing new methods of differentiating hyperleptinemic horses from normal horses.

The first objective was to determine if the detection process for hyperleptinemia in horses could be improved by hormone manipulation. Huff et al. (2008) measured resting leptin concentrations in 2 blood samples obtained from the jugular vein 2 wk apart. A frequency distribution for leptin concentrations indicated that the range of leptin concentrations in horses tended to be bimodally distributed. Starting with the entire data set, the highest increments were truncated one at a time until a normal distribution curve was obtained. Horses with leptin values beyond the 95% confidence interval were considered mildly hyperleptinemic, whereas those with values beyond 10 ng/mL (+5.4 SD from mean) were classified as hyperleptinemic.

Cartmill et al. (2006) reported a dramatic rise in insulin and leptin concentrations following administration of dexamethasone (DEX), a substituted glucocorticoid analogue commonly used by clinicians to assess the presence of Cushing's syndrome and equine pituitary pars intermedia dysfunction (PPID; Messer, 1999). Administration of DEX also exaggerated the insulin response to glucose infusion. The first experiment herein was designed to test the hypothesis that a single injection of DEX would enhance the differences between hyperleptinemic horses and normal horses, thereby increasing our ability to differentiate between the two groups.

The next objective was to determine if the combined intravenous glucose and insulin test (CGIT), a new test designed with simplicity and accuracy in mind (Eiler et al., 2005; Frank et al., 2006), could be used to assess insulin sensitivity in hyperleptinemic horses. Hyperleptinemic horses also tend to be hyperinsulinemic, and thus are suspected to be insulin insensitive, but this has yet to be reported. The most direct and widely accepted methods of assessing insulin sensitivity in horses are clamp techniques and minimal model assessments (Pratt et al., 2005; Treiber et al., 2006). However, these methods are not practical for clinicians due to their invasiveness and difficulty. Therefore, the CGIT was applied to 107 horses to evaluate its use as a predictive test and to study its repeatability in assessing insulin sensitivity.

The final objective was to assess insulin sensitivities of horses with high and low plasma leptin concentrations by evaluating glucose response curves to the direct administration of two doses of insulin. The clamp techniques and minimal model assessment of glucose tolerance tests depend on artificially induced hyperglycemia, via glucose infusion, and its disappearance from the blood in response to insulin. It is unclear why testing within normal physiologic ranges of blood glucose, with smaller, more physiologic doses of insulin, has not been done. Moreover, other metabolites responsive to insulin, such as nonesterified fatty acids (NEFA) and free amino

acids, have not been taken into consideration when assessing insulin sensitivity by the traditional methods. Plasma fatty acid concentration has been shown to be elevated in insulin insensitive horses (Frank et al., 2006), and glucagon is a hormone which has been shown to be elevated subsequent to increased NEFA in humans with type II diabetes mellitus (Reaven et al., 1987), a condition similar to insulin resistance in horses. In the third experiment, insulin injections were administered to 16 horses to study the relationship between glucose, NEFA, and glucagon responses in hyperleptinemic and normal horses with similar BCS.

CHAPTER 2

REVIEW OF LITERATURE

Metabolic Syndrome

Obesity has become a central causative component in the development of many diseases in the United States in humans and in pet populations. Metabolic and hormonal abnormalities are resultant maladies from sustained obesity and often lead to cardiovascular disease (CVD) or death. In 1988, Gerald Reaven described Syndrome X, an aggregate of disorders believed to strongly predispose a person to the development of CVD, kidney disease, and diabetes mellitus. Syndrome X was comprised of insulin resistance (IR), hyperglycemia, hypertension, low high-density-lipoprotein (HDL)-cholesterol, and raised very-high-density-lipoprotein (VLDL)-triglycerides (Reisin and Alpert, 2005). Today, in human medicine, this condition is more commonly referred to as the metabolic syndrome, consisting of dyslipidemia and obesity as additional key components. Prevalence of metabolic syndrome is projected to affect 40% of the population by 2020 (Alberti et al., 2005; Hutley and Prins, 2005)

Johnson (2002) related this information to the horse when he described EMS as a precursor to many debilitating and sometimes fatal diseases, such as laminitis, in the horse. A clustering of obesity (generalized, regional, or both), IR, hyperinsulinemia, and hypertriglyceridemia are more commonly observed in horses with EMS. Equids are not commonly diagnosed with diabetes mellitus, but rather IR and hyperinsulinemia (Geor and Frank, 2008). This is believed to be due to a lack of beta-cell failure and the exorbitant amount of insulin the horse is able to produce. Furthermore, laminitis, rather than CVD, is more likely to result in the horse compared to human models (Johnson, 2002; 2004). Hence, Treiber et al. (2006b) proposed pre-laminitic metabolic syndrome as a more specific term for such horses with increased susceptibility.

Horses or ponies with a BCS of 7 or greater (Henneke et al., 1983) are considered obese and are at most risk for developing metabolic abnormalities. In 1998, prevalence of equine obesity was assessed by the National Animal Health Monitoring System of the Department of Agriculture. Their survey estimated that 4.5% of the national horse population was overweight or obese based on horse-owner reporting. This number is considered to be inaccurately low by many researchers (Geor, 2008).

Breed and sex predispositions to EMS tend to implicate mares and “easy keeper” breeds, such as Morgans, Paso Finos, Spanish Mustangs, and draft horses. These horses will most often have high BCS, though some horses may be of a moderate score and still exhibit signs of EMS. Fat deposits on the neck (“cresty neck”) and in the abdominal (omental) and rump regions are most commonly associated with horses of higher risk. A genetic predisposition has yet to be proven but is also likely to be involved in some bloodlines (Johnson, 2002).

Insulin Sensitivity and Resistance

The normal physiologic response to glucose in the absorptive state may be compromised by many factors in the body. Blood glucose concentrations are narrowly regulated in the healthy person, usually between 80 to 90 mg/dL in a fasted state. Concentrations increase to 120 to 140 mg/dL in the first hour after a meal, and usually return to resting concentrations within 2 h. Similar concentrations are evident in the horse; blood glucose concentrations after feed deprivation are normally 60 to 90 mg/dL (Ralston, 2002).

In the horse, different scenarios are possible depending on diet, metabolic demands, and the daily routine of the horse. Typically, following a high carbohydrate grain meal, intestinal microvilli brush border enzymes of the duodenum will hydrolyze disaccharides into monosaccharides. The final product is predominantly glucose, which enters portal blood and is delivered directly to essential tissues (nervous, fetal, etc.). In response to glucose stimulation,

the pancreas secretes preformed (by β cells of the islets of Langerhans) and stored insulin, which inhibits gluconeogenesis by the liver. In humans, insulin has a plasma half-life of approximately 6 min and circulates almost entirely unbound, except for that which reaches a target cell (Guyton and Hall, 2006).

Insulin is required as a mediator for glucose uptake by about 80% of the body, especially muscle, adipose, and hepatic tissues. The insulin receptor is a combination of 2 subunits, α and β , held together by disulfide linkages. The receptor is a tyrosine kinase, enzyme-linked receptor that becomes immediately autophosphorylated following binding of insulin with the α subunit of the receptor on the cell membrane of the target cell. Autophosphorylation activates tyrosine kinase and causes phosphorylation of multiple intracellular enzymes and insulin receptor substrates (IRS) responsible for release of glucose transport (GLUT) proteins to the cell membrane to absorb glucose from the interstitial fluid. Glucose that is not metabolized by the body for energy can be converted to glycogen and stored in the liver and muscle cell until needed. Upon insulin stimulation, excess carbohydrates are converted to fats and stored in adipose tissue (Guyton and Hall, 2006). Because horses are usually grazing for most of the day, insulin secretions tend to be consistent as they graze (Storer et al., 2007).

Impaired insulin sensitivity is a major component of the metabolic syndrome cascade, disrupting homeostasis and opening the door to a multitude of serious and potentially fatal ailments. Insulin resistance or insensitivity is a condition in which one requires excessive or prolonged secretions of insulin to signal target tissues to release GLUT proteins during hyperglycemia (Frank, 2006). Furthermore, hyperinsulinemia is even required between meals to maintain homeostatic blood glucose (euglycemia). This condition is commonly referred to as type 2 diabetes, or noninsulin-dependent diabetes. Over time, human islet β cells may become

exhausted during this hyperinsulinemic state, leading to frank diabetes (total insulin lack). In equids, pancreatic islet β cell exhaustion is rarely observed for indefinite reasons.

Insulin resistance is not completely understood, and a paucity of data exist concerning its manifestation in the horse. Proposed mechanisms include a reduction in the number of insulin receptors, a change in receptor affinity, or defective intracellular signaling (Johnson, 2002). Many have linked the condition to obesity, though IR has been predicted to afflict horses of moderate BCS as well (Johnson, 2002; Bailey et al., 2008; Geor, 2008). Grain diets have a high glycemic index, which cause a spike in glucose and insulin concentrations that are believed to potentially lead to insulin insensitivity (Kronfeld, 2004; 2005b; Kronfeld et al., 2005). Insulin resistance is also a risk to young developing horses, because there is speculative evidence supporting a link between IR and osteochondrosis dessicans (Ralston, 2002).

Prevalence of IR and its documented link to laminitis in the horse has warranted a demand for increased research in the area. Hyperinsulinemia has been questioned as a common thread for horses with IR and has been proven to be dangerous alone in excessive amounts. Asplin et al. (2007) induced laminitis with infusion-induced hyperinsulinemia in clinically healthy ponies with moderate BCS while maintaining euglycemia. Using the prolonged euglycemic hyperinsulinemic clamp (pEHC) technique, a bolus of insulin was infused and hyperinsulinemia maintained while regulating euglycemic conditions for a prolonged period of time. In that experiment, all horses exhibited signs of Obel grade 2 laminitis (Obel, 1948) in all 4 feet within 72 h following administration.

Diagnosing Insulin Resistance

Intravenous Dextrose Challenge. Commonly referred to as the glucose tolerance test, this procedure is most used clinically in human and equine medicine to predict insulin sensitivity to glucose (β cell function). The test is performed by administering a large bolus dose of

dextrose solution after an overnight period of feed deprivation (10 to 12 h) and collecting blood samples both shortly before and 2 to 5 h after injection. Glucose values normally peak <15 min following the injection, and insulin at approximately 30 min. In normal horses, both hormones should return to baseline within 1 h following administration. Thus, a prolonged or excessively high response is indicative of abnormal insulin signaling and glucose intolerance (Ralston, 2002).

This test has been referred to as the “gold standard” for decades as a means to assess insulin sensitivity. However, many scientists have criticized the test as being unreliable, unstandardized, ambiguous, and non-specific in horse models. Therefore, more complex and expensive tests have been used to achieve a more accurate, precise, and specific assessment of insulin sensitivity.

Minimal Model Assessment. This test is 1 of the 2 most accepted methods used today for diagnosing failures in insulin signaling in the horse. The minimal model method is a set of software-calculated mathematical equations used to estimate the time course of changes in glucose and insulin following an i.v. glucose load (Pratt et al., 2005). Most often used in conjunction with the frequent sampling intravenous glucose tolerance test (FSIGT), this test has the advantages of being able to measure both insulin-dependent and insulin-independent glucose utilization in the horse and also to assess the sensitivity of pancreatic β cells to glucose. It has been used in human, dog, and cat models (Saad et al., 1994; Petrus et al., 1998). Nonetheless, this test does have some limitations. Because the minimal model is based on mathematical data implicating assumptions of glucose and insulin kinetics, systematic errors in its estimates may result. Furthermore, the test is complex and requires costly software for minimal model calculations, which limits its clinical use. Also, the FSIGT requires a large insulin response to obtain a precise estimate of insulin sensitivity that may be compromised in diseased horses.

However, this test is considered by many to be one of the most specific tests for accurately assessing insulin sensitivity.

Horses are restricted to box stalls for 13 h (overnight) before beginning the test. Baseline samples are obtained to determine resting insulin and glucose values. A dextrose bolus is then infused and frequent blood samples are obtained in short intervals to 180 min. In some variations of the FSIGT, a single dose of insulin or an insulin secretagogue, such as tolbutamide, may be administered 20 min following glucose infusion (Hoffman et al., 2003). This is due to the need for a well-defined 2nd-phase insulin response to increase precision of insulin sensitivity in horses. Data are analyzed using minimal model calculations using computerized algorithms to estimate 3 primary variables: 1) the insulin sensitivity index (S_i), which calculates an estimate of determining net fractional glucose clearance rate per unit change of insulin following glucose infusion; 2) glucose effectiveness (S_g), the net fractional glucose rate independent of insulin; and 3) the acute insulin response to glucose (AIR_g), which computes the incremental area under the insulin curve, a calculation measured in the first 20 min of sampling following administration. The incremental areas under the concentration versus time curves (AUC) for both glucose (AUC_g) and insulin (AUC_i) are also calculated using the computerized trapezoidal method. Plasma half-life for glucose clearance ($T_{1/2g}$) is estimated by use of non-linear regression analysis (glucose concentration versus time) between 1 and 180 min after glucose administration.

Proxies and Reference Quintiles. The use of proxies and reference quintiles to assess insulin sensitivity has more of a robust implication in humans than in horses. Treiber et al. (2005) attempted to lead the horse world in this direction by comparing basal proxies used in horses to those used in human medicine. The use of basal proxies offers a simple, single-sample approach to predict insulin sensitivity based on steady-state sample plasma concentrations of the feed-deprived horse (12 h). Basal samples are believed to provide the investigator with a more

accurate representation of glucose and insulin status in the horse due to the unperturbed state of the subject.

Baseline samples are obtained following a 12-h (overnight) period of feed deprivation, with the exception of *ad libitum* water and trace minerals. Glucose and insulin values are analyzed using the same methods used with the minimal model (see above). The reciprocal inverse square root to basal insulin (RISQI) and the modified insulin-to-glucose ratio (MIRG) are proxies used in comparison with S_i and AIR_g , respectively. These values are calculated for each as follows: $RISQI = 1/\sqrt{insulin}$; $MIRG = (800 - 0.30 \times [insulin - 50]^2)/(glucose - 30)$. When used together, these proxies enable assessment of compensatory insulin secretion in healthy horses and insulin signaling failure in hyperglycemic horses. The homeostasis model assessment (HOMA) and the quantitative insulin sensitivity check index (QUICKI) are also insulin sensitivity proxies developed for use in humans but have been correlated in horse models with S_i using minimal model assessment (Treiber et al., 2005). Calculations for HOMA and QUICKI are as follows: $HOMA = (glucose - insulin)/22.5$ and $QUICKI = (\log [glucose \times insulin])^{-1}$. The HOMA proxy takes advantage of the reciprocal relationship between basal insulin and glucose concentrations to avoid the ambiguity of low insulin concentrations alone. This proxy has a good correlation with pEHC data in humans. Use of the QUICKI proxy improves precision in insulin sensitivity when compared with the pEHC technique. However, neither HOMA nor QUICKI are as highly correlated with insulin sensitivity in the horse as the RISQI and MIRG proxies.

Treiber et al. (2005) assessed these proxies in a herd of 46 healthy horses containing 1 hyperlipidemic laminitic pony and 1 obese insulin-resistant thoroughbred gelding. Data from the minimal model were used to test for normalcy using the Shapiro-Wilk statistic. Reference ranges were calculated and data divided into quintiles to compare RISQI and MIRG to S_i and

AIR_g, respectively. The reference quintiles were used to characterize individual deviations and to monitor the progress of affected horses. From a clinical perspective, the laminitic pony was in the lowest quintile for S_i and RISQI, suggesting very low insulin sensitivity compared to normal, healthy horses. This horse tested into the lowest quintile for MIRG as well, suggesting that despite elevated basal insulin, this horse may have reduced β cell activity. This decompensation from the β cells of the pancreas is noted by basal glucose appearing in the highest quintile among all horses tested.

For the clinically insulin-resistant gelding, calculations from the minimal model indices provided no solution for this horse. However, based on RISQI, the horse was in the lowest quintile of all horses, suggesting low insulin sensitivity. However, as for MIRG, the horse tested in a moderately high quintile indicative of effective β cell compensation with normal basal glucose concentrations. Based on this information, this horse was placed on a feed of a low glycemic index in which RISQI was increased by 11% and MIRG by 10% indicative of improved sensitivity and β cell function.

Clamp Methods: pEHC Technique. Clamp techniques include the pEHC technique, the hyperglycemic clamp (DeFronzo et al., 1979), and the insulin sensitivity test. These methods are the second (see minimal model above) of the 2 most widely accepted and specific models for assessing insulin sensitivity in the horse, although also the most laborious, expensive, and impractical to veterinarians. These techniques measure the degree of insulin insensitivity via infusing either glucose or insulin to excess while monitoring the other at steady-state concentrations.

The pEHC technique is a common clamp method used in equine research. This test, as described by Pratt et al. (2005), again requires a 13 h overnight period of feed deprivation with only water and trace minerals available *ad libitum*. On the morning of the test, 2 mL of the

horse's serum is mixed with 5 mL recombinant human DNA insulin (100 IU/mL) and 493 mL of 0.9% NaCl. Baseline samples are collected previous to commencing the test. A peristaltic pump is used to infuse insulin at a constant rate (3 mL/min/kg BW) to achieve prolonged hyperinsulinemia in the horse for the entire 180 min of the test. Small (~2 mL) blood samples are collected throughout the test at 5-min intervals for glucose measurements with a portable glucometer. A calibrated syringe pump is also used to deliver and maintain glucose at euglycemia (5 mmol/L) and adjusted as necessary throughout the test. Additional samples are obtained at 15-min intervals to measure insulin concentrations.

The first 90 min of the test are used to equilibrate the infusions so that insulin is maintained at excessive concentrations and glucose at euglycemia. The final 60 min of the test are used for calculations of whole body glucose uptake (M) and insulin sensitivity index, calculated as glucose uptake per unit of insulin. These calculations are based on the assumptions that endogenous glucose production is completely suppressed by the hyperinsulinemia and so M should equal the amount of glucose infused. This information is indicative of insulin sensitivity in the horse. However, due to the fact that glucose is not measured perfectly constant during the clamp, a correction factor must be used to account for glucose that has been added/removed from the glucose space other than by metabolism. This space correction (SC) is calculated as follows: $SC = (G_2 - G_1)(0.19 \times BW)/(T \times BW)$, where G_1 and G_2 are glucose measurements obtained at the beginning and end, respectively, of the 5 min time interval (T), and $(0.19 \times BW)$ is the glucose space in liters. The final calculation with the SC accounted for is therefore: $M = GIR - SC$, where M is equal to GIR (the glucose infusion rate; mmol/kg BW/min) minus the space correction.

Steady-state insulin (I) is measured in the final 60 min of the clamp test and is used to calculate the M/I ratio; a calculation that reflects the rate of glucose disposal per unit insulin.

This calculation is an index for tissue sensitivity to exogenous insulin. Despite the test's complexity, many have regarded it to be very reliable and specific for classifying insulin sensitivity.

The CGIT. Due to characteristics that may limit clinicians from implementing any of the variety of tests previously described, a clinical test for horses that is both practical and specific is still very much needed. The CGIT is a simple test designed with such an objective in mind. The procedure, first described by Eiler et al. (2005), consists of an overnight period of partial feed deprivation (access to hay only), with water and trace minerals available *ad libitum*. On the morning of the procedure, animals are catheterized in one jugular vein and administered an i.v. bolus dose of glucose (150 mg/kg BW as a 50% dextrose solution) immediately followed by a bolus of insulin (0.1 IU/kg BW). Blood samples are collected at 1, 5, 15, 25, 35, 45, 60, 74, 90, 105, 135, and 150 min relative to glucose and insulin injections. Plasma glucose concentrations are analyzed in all samples and insulin in selected samples.

Frank et al. (2006) used the CGIT as a means to confirm differences in glucose dose response curves of obese clinically insulin-resistant horses versus normal healthy horses of moderate BCS. In healthy horses, glucose concentrations returned to baseline concentrations at 45 min or less following administration. A delayed or prolonged response may be attributable to impaired insulin sensitivity or glucose intolerance. In their experiment, measuring area under the glucose and insulin curves detected 68% and 81% higher values for insulin resistant horses compared to clinically healthy horses.

Oral Dextrose and Grain Challenges. Oral challenges involving the administration of glucose in the form of dextrose via nasogastric intubation are less frequently used in the clinical equine research community. Similarly, grain challenges consisting of a carbohydrate-rich concentrate have also been studied. Although not completely specific, these techniques are no

substitutes for direct administration of glucose into the blood. However, oral challenges permit beneficial measurements of intestinal absorption ability of the animal and how this may relate to insulin sensitivity. These variables are not measured in intravenous tests.

The oral dextrose challenge traditionally involves a 1 g/kg BW dose of 20% dextrose solution administered through a nasogastric tube into the stomach following a 12 to 16 h period of feed deprivation. A pre-treatment blood sample is obtained followed by treatment administration and short interval sampling (every 0.5 to 1 h). Glucose and insulin response curves tend to be more prolonged, with a delayed peak in comparison to direct methods of i.v. glucose infusion. Glucose concentrations are expected to return to baseline amounts within 4 to 5 h (Roberts and Hill, 1973; Ralston, 2002).

Dyslipidemia in the Insulin Resistant Horse

Fats, in their many forms, are very important to sustain homeostasis throughout the day. Between meals, human and equine tissues (with the exception of nervous tissue) are only slightly permeable to glucose and therefore depend on fats in the forms of free and volatile fatty acids for energy. In a healthy state, high insulin in the blood from the pancreas following a meal inhibits gluconeogenesis and triglyceride hydrolyzing enzymes, such as hormone-sensitive lipase, and instead switches to glucose as a substrate for energy (Guyton and Hall, 2006). In the absorptive state, the same IRS that are responsible for release of GLUT proteins also facilitate protein and fat synthesis. Promoted by insulin, fatty acids (FA) are synthesized mostly in the liver and packaged as triglycerides (TG) in lipoproteins and transported in this form to adipose tissue. The enzyme lipoprotein lipase from capillary walls of the adipose tissue splits the FA complex and stores the TG. Excess glucose and high insulin, therefore, drive fat metabolism from a catabolic state (breakdown and utilization) to an anabolic state (storage) converting glucose to fats (Hadley and Levine, 2006).

Conversely, at times of insulin deficiency, as in type-1 diabetes mellitus, insulin-dependent tissues become starved of glucose and must rely on lipolysis and gluconeogenesis to survive. Plasma NEFA concentrations increase almost immediately as well as plasma lipoproteins lipids. This compensation presents the very dangerous causative factors of atherosclerosis and CVD in people with serious diabetes (Alberti et al., 2005).

Compensated hyperinsulinemia, as a result of insulin resistance, has been reported to perturb the “switching” mechanism of lipid metabolism in humans and in horses, resulting in dyslipidemia (DL). Dyslipidemia is characterized by increased circulating VLDL, elevated TG, and low HDL. The exact mechanism of how this occurs has yet to be determined. Obesity is believed to have key etiological implications for DL and IR. Adipose tissue is responsible for the release of cytokines (adipokines), such as tumor necrosis factor alpha (TNF- α), a main autocrine/paracrine factor that triggers secretion of NEFA from adipose tissue into circulation. Tumor necrosis factor- α is believed to mediate the repression of many genes responsible for glucose and NEFA uptake and storage. The Randle hypothesis (Randle et al., 1963) suggests that overproduction of TNF- α and NEFA impair insulin signaling especially in muscle tissue (i.e., glucose uptake is reduced when tissue energy needs are being met by NEFA oxidation). Also, visceral adipose tissue secretes its products directly into portal blood and can have potent liver effects. This is in opposition to subcutaneous adipose tissue that secretes into peripheral circulation (Mlinar et al., 2007). Excessive NEFA exposure is also believed to have a direct effect on β cells of islets of Langerhans. Free fatty acids have been shown to be insulin secretagogues in obese people with normal pancreatic β cells. Experimentally-induced, chronic elevation of NEFA (2 to 4 d) caused elevated insulin secretion in humans (Kashyap et al., 2003; Boden and Laakso, 2004).

Clinically insulin resistant and obese horses were shown to have greater circulating NEFA (86% greater), VLDL (104% greater), and HDL-cholesterol (29% greater) concentrations compared to nonobese, clinically healthy horses (Frank et al., 2006). Non-esterified fatty acids are markers of fat metabolism and mobilization. Plasma NEFA concentrations increase with lack of physical activity in humans, because adipose tissues reach their maximum capacity for fat storage and the inhibitory effects of insulin on hormone-sensitive lipase are reduced (Boden and Laakso, 2004; Frank et al., 2006). This shunting of NEFA results in storage in other tissues such as the liver and muscle cells, the effects of which have been shown to cause local IR in tissues due to products of fat metabolism, such as diacylglycerol, perturbing intracellular insulin signaling pathways (Boden and Laakso, 2004). Increased VLDL and TG are also a markers of NEFA liver uptake in humans (Carr and Brunzell, 2004; Frank et al., 2006).

Although infrequently measured in horses, high blood pressure as a result of hypertriglyceridemia is a common factor of human metabolic syndrome, obesity, and IR. Clinically insulin resistant and previously laminitic pony breeds with moderate BCS have been studied and have shown to exhibit elevated insulin, blood pressure, VLDL, and TG concentrations in summer months when high-fructan pastures are abundant. Fructans are high-glycemic, non-structural carbohydrates that have been linked to IR and laminitis in the horse (Bailey et al., 2008). Similarly, a positive correlation has been reported between insulin and triglyceride concentrations in experimentally-induced and naturally hypertriglyceridemic donkeys (Forhead et al., 1994).

Hyperleptinemia in the Horse

Adipose tissue is no longer perceived as a passive storage depot for excess energy as TG. Adipose cells, including adipocytes, preadipocytes, and macrophages have been found to secrete multiple bioactive molecules, collectively referred to as adipokines. Upon further investigation,

adipokines have been claimed to be the “missing link” between IR and CVD (Hutley and Prins, 2005). Leptin is a hormone secreted by adipocytes and has been the focus of study in many laboratories in equine research to further elucidate the hormone’s relationship to insulin and insulin sensitivity.

Leptin is secreted into the blood and has receptors in many peripheral and central nervous system tissues, but particularly in the hypothalamus and brainstem that control satiety, energy expenditure, and neuroendocrine function (Buff et al., 2002; 2005). Naturally, in many species, including the horse, leptin is secreted proportionally to body mass index (Wild and Byrne, 2006) and BCS, due to its secretion by adipocytes (Prolo et al., 1998; Buff et al., 2002). Gentry et al. (2002) further investigated the relationship between leptin concentrations and BCS in horses of high and low BCS and reported low concentrations of circulating leptin and a prolonged seasonal anovulatory period following induction of low BCS by diet restriction. Leptin has been shown to play a role in FA metabolism in humans by stimulating FA oxidation in muscle and in rats by shunting lipids away from non-adipose tissue, thus preventing lipotoxicity and ectopic fat accumulation. Leptin has also been shown to inhibit hepatic TG accumulation by directly activating phosphoinositol-3-kinase in humans (Hutley and Prins, 2005).

Gentry et al. (2002) and Cartmill et al. (2003) described a consistent low (<5 ng/mL) or high (7 to 20 ng/mL) dichotomy of mares based on leptin concentrations documented over a 2-yr period. In those horses, insulin concentrations were highly correlated with leptin concentrations. This relationship has previously been documented in rodent (Sivitz et al., 1998) and swine (Ramsay and White, 2000) models. Hyperinsulinemia has been shown to increase leptin concentrations within 3 to 5 h in rats (Cusin et al., 1995); leptin was reduced under conditions of insulin deficiency (Sivitz et al., 1998). Cartmill et al. (2003) reported that hyperinsulinemic-hyperleptinemic horses had greater and prolonged insulin response to glucose infusion.

Therefore, leptin is also believed to have a role in insulin signaling and sensitivity in the horse. This condition was also reported in clinically IR horses of high BCS (Frank et al., 2006). Further research is needed to more clearly elucidate the relationship between the hyperinsulinemic-hyperleptinemic condition and IR in the horse.

Rationale for Current Experiments

The present knowledge of the hyperinsulinemic-hyperleptinemic syndrome in horses, first reported by Gentry et al. (2002), does not include a documented report of decreased insulin sensitivity associated with the syndrome. Although indirect indices point to an insensitive state (elevated resting insulin and glucose concentrations and exaggerated insulin response to glucose infusion; Cartmill et al., 2003), direct assessments (Cartmill, 2006) were inconclusive. Several technologies are needed for further study of the syndrome, including a better method for assessing hyperleptinemic vs normal horses, and a simpler, more direct method of assessing insulin sensitivity in these horses. The experiments that follow were designed to test the hypotheses that 1) a single injection of DEX, a potent glucocorticoid analog, would enhance the differentiation of hyperinsulinemic-hyperleptinemic horses from normal horses, 2) the CGIT would provide a reliable assessment of insulin sensitivity adequate to differentiate between hyperinsulinemic-hyperleptinemic and normal horses, and 3) a direct single shot, or combined 2-dose regiment, of insulin provide a reliable assessment of insulin sensitivity adequate to differentiate between hyperinsulinemic-hyperleptinemic and normal horses.

CHAPTER 3

HYPERLEPTINEMIA IN HORSES: DOES A SINGLE INJECTION OF DEXAMETHASONE ENHANCE THE IDENTIFICATION PROCESS?

Introduction

Endocrine disturbances in the horse are associated with an array of maladies. Equine metabolic syndrome (Johnson, 2002) is a collection of disorders that disrupt endocrine homeostasis. These disorders have been extensively studied due to the increasing prevalence of IR and obesity horses. The dangers associated with EMS can be fatal, if left untreated.

Obesity is a central component of EMS. Once understood to only play a minor role in physiology as a storage depot for TG, adipose tissue is now believed to have very important implications as an endocrine organ and a regulator of metabolism. Increased adiposity has been studied with associations of IR, hypertension, hyperinsulinemia, and DL in humans. In equids, IR with compensated hyperinsulinemia is often prevalent. Gentry et al. (2002) first showed that the satiety hormone, leptin, produced by adipocytes was elevated in mares of higher BCS. Conversely, horses of low BCS also had lower concentrations of leptin coupled with prolonged seasonal anestrus. Subsequently, Cartmill et al. (2003) reported that the same horses observed by Gentry et al. (2002) had sustained hyperleptinemia 2 yr later, suggesting a relatively permanent condition. The horses studied in these experiments seemed to fit into 2 distinct groups based on leptin concentrations: low (<5 ng/mL) or high (7 ng/mL or greater).

Cartmill et al. (2003) reported hyperinsulinemia, hyperglycemia, and a prolonged and exaggerated insulin response to glucose in hyperleptinemic horses. These characteristics are often associated with type 2 diabetes in humans (adult-onset diabetes) due to failures in insulin signaling. This hyperinsulinemic-hyperleptinemic coupling has been the basis of further study in

our lab. Huff et al. (2008) further elucidated this condition in horses by measuring resting leptin in 2 blood samples obtained from the jugular vein 2 wk apart. A frequency diagram for leptin concentrations provided evidence of a bimodal distribution of horses across a range of leptin concentrations. This frequency diagram was used to differentiate high leptin horses from low.

Cartmill et al. (2006) reported a dramatic rise in insulin and leptin concentrations following administration of DEX, a substituted glucocorticoid analogue commonly used in clinical settings to assess the presence of Cushing's syndrome and equine PPID (Messer, 1999). Administration of DEX also exaggerated the insulin response to glucose infusion. Given these responses to DEX, the present experiment was designed to test the hypothesis that a single injection of DEX would enhance the differences between hyperinsulinemic-hyperleptinemic horses and normal horses, thereby increasing our ability to differentiate between the 2 groups.

Materials and Methods

One-hundred-seven horses (15 geldings and 92 nonpregnant, nonlactating mares) from 2 separate farms in Louisiana were screened in December for hyperleptinemia using a single-shot injection of DEX. The horses were of various light-horse breeds, ages, and BCS (mean and range: 6.27 and 3.5 to 8, respectively). Horses were maintained at each farm throughout the experiment on pasture, *ad libitum* availability of water, and supplemental grass hay as needed to maintain body condition.

On d 0 of the experiment, all horses were administered a single i.m. injection of DEX (40 µg/kg BW; Sigma Chemical Co., St. Louis, MO) in aqueous solution, and BCS was estimated and recorded. Jugular blood samples were collected in 7-mL, evacuated blood collection tubes containing potassium oxalate and sodium fluoride (Tyco Healthcare Group LP, Mansfield MA) for pre-DEX (d -2, -1, 0) and post-DEX (d 1, 2, 3) plasma analysis by radioimmunoassay (RIA) of leptin, insulin, and cortisol concentrations. All blood samples were collected in the morning

between 0700 and 0900 and were kept refrigerated until all samples were collected. Following blood collection each day, all samples were centrifuged at 1,500 x g at 4°C for 15 min; plasma was harvested and stored at -15°C. Leptin concentrations were measured using the double-antibody RIA technique described by Cartmill et al. (2003) using a 200- μ l sample size. Plasma concentrations of insulin were measured in all samples, and cortisol in d 0, 1, and 2 samples only, with commercially available reagents (Diagnostic Systems Laboratories, Webster, TX). Cortisol was measured in these samples to assess any occurrence of PPID or Cushing's syndrome in the horses (Messer, 1999).

Data were analyzed using the MIXED procedure in SAS (SAS Institute, Inc., Cary, NC) to detect pre- and post-DEX differences with the main variables being leptin, insulin, and BCS. Normalcy, regression, and frequency distribution analyses were analyzed for all variables using the UNIVARIATE, REG, and FREQ procedures, respectively.

Results

Metabolite concentrations in the pre-treatment samples were averaged and are presented as d 0 means. No horse showed signs of PPID or Cushing's syndrome following injection of DEX. Cortisol concentrations were suppressed ($P < 0.01$) in all horses within 24 h of DEX administration and remained suppressed for at least 48 h in all horses (Fig. 3.1). Differences existed ($P < 0.05$) for concentrations of leptin and insulin in pre- versus post-DEX samples, with sustained elevation of both hormones on d 1, 2, and 3 (Fig. 3.2 and 3.3).

There was no evidence of a bimodal distribution of horses for leptin or insulin concentrations. Data were positively-skewed ($P < 0.05$) toward higher concentrations for mean pre- (Fig. 3.4 and 3.5) and post- (Fig. 3.6 and 3.7) DEX samples for both leptin and insulin.

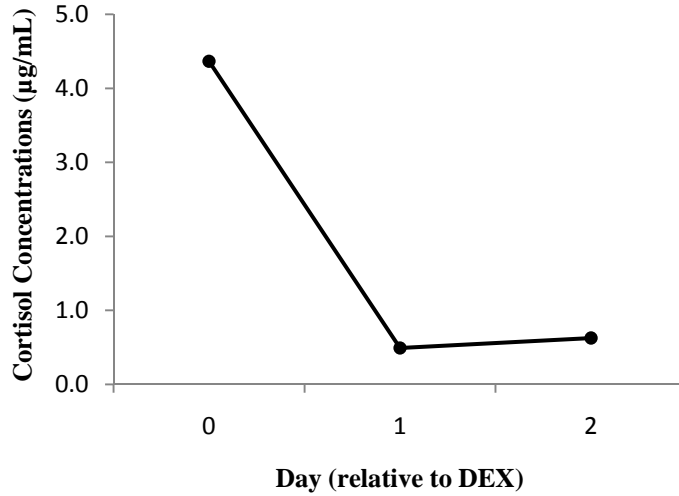


Figure 3.1. Mean cortisol concentration relative to DEX treatment. Cortisol remained suppressed ($< 1.0 \mu\text{g/mL}$) in all horses for at least 48 h ($P < .01$). Pooled SEM for cortisol was $0.08 \mu\text{g/mL}$.

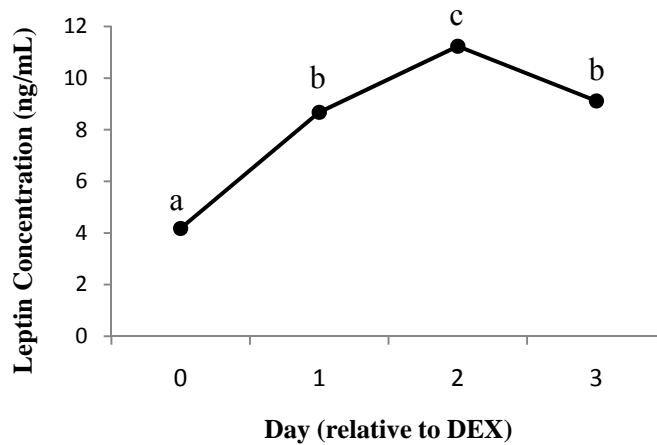


Figure 3.2. Mean leptin concentration relative to DEX treatment. Concentrations were highest on d 2 post-treatment ($P < 0.05$). Pooled SEM for leptin was 1.15 ng/mL .

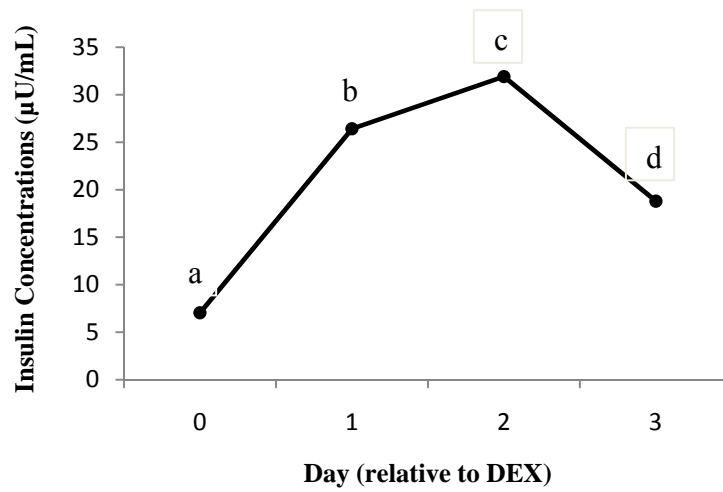


Figure 3.3. Mean insulin concentration relative to DEX treatment. Concentrations were highest on d 2 post-treatment ($P < 0.05$). Pooled SEM for insulin was $2.56 \mu\text{U/mL}$.

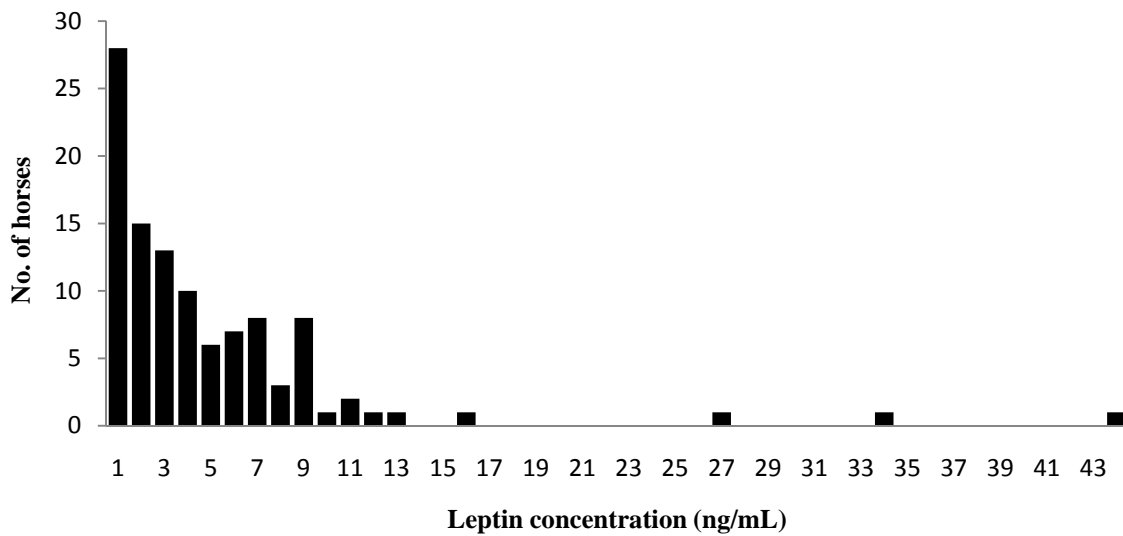


Figure 3.4. Frequency diagram for pre-DEX leptin concentration. Distribution of horses was positively-skewed ($P < 0.05$) toward higher concentrations.

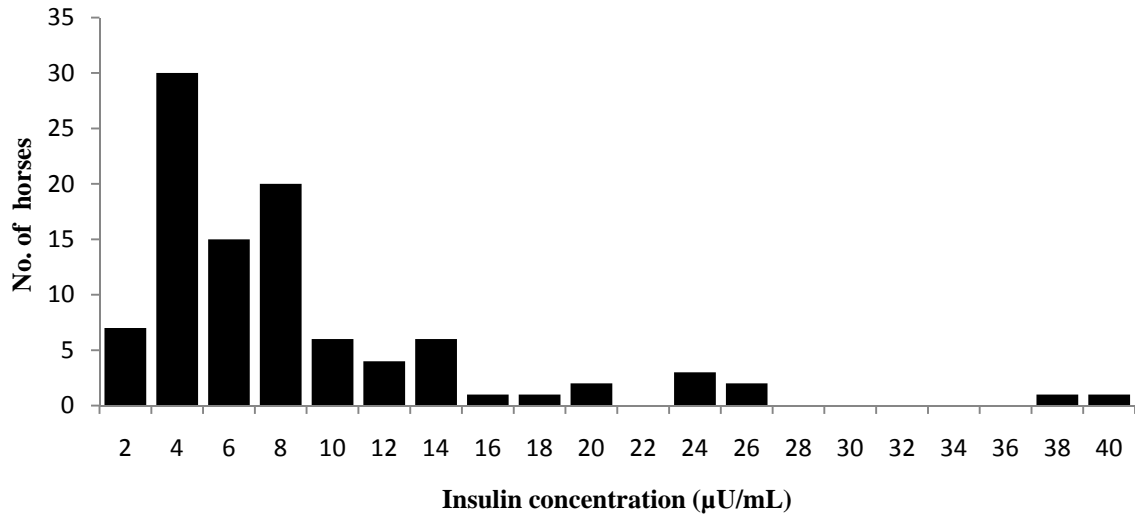


Figure 3.5. Frequency diagram for pre-DEX insulin concentration. Distribution of horses was positively-skewed ($P < 0.05$) toward higher concentrations.

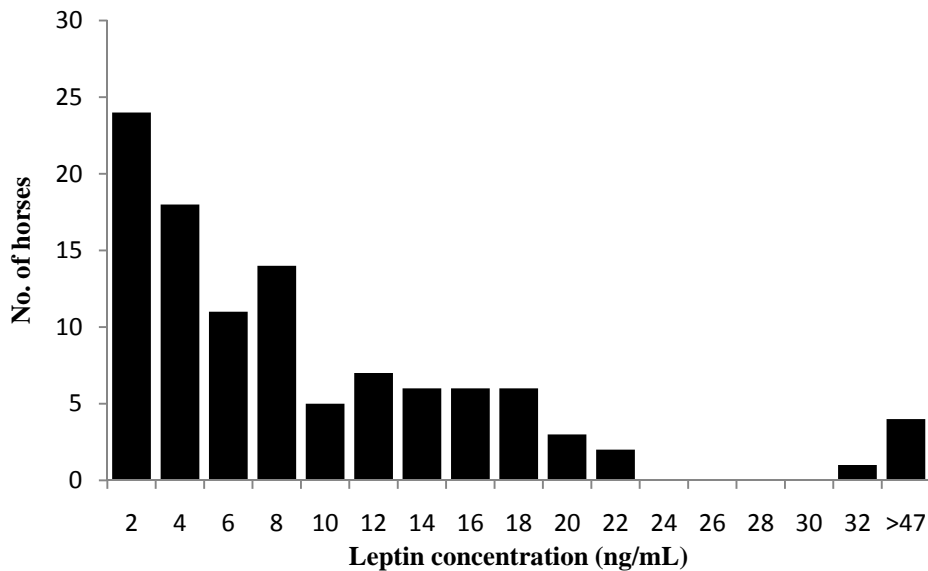


Figure 3.6. Frequency diagram for post-DEX leptin concentration. Distribution of horses was positively-skewed ($P < 0.05$) toward higher concentrations.

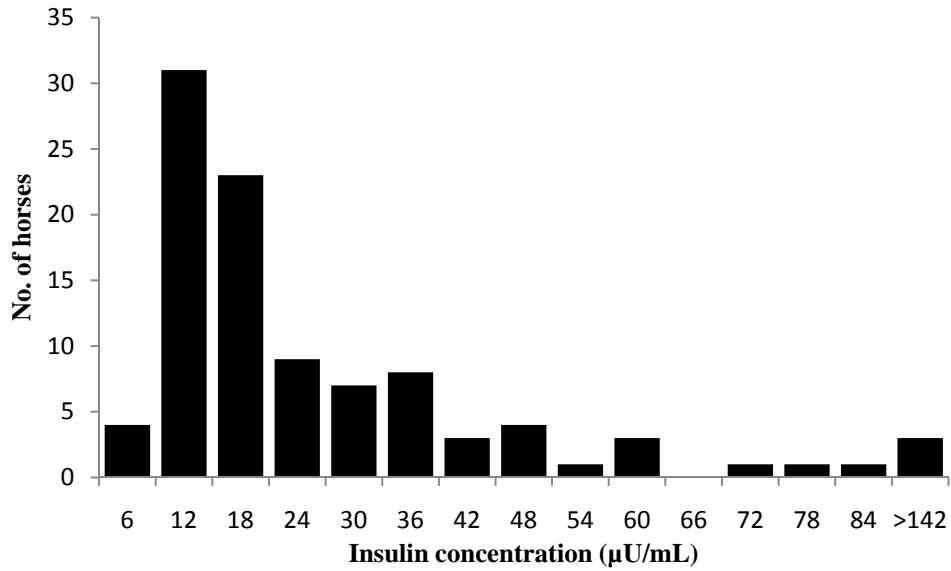


Figure 3.7. Frequency diagram for post-DEX insulin concentration. Distribution of horses was positively-skewed ($P < 0.05$) toward higher concentrations.

Pre- versus post-DEX samples were highly correlated ($P < 0.01$) for leptin concentration (Fig. 3.8) and significantly correlated ($P < 0.05$) for plasma insulin concentration (Fig. 3.9).

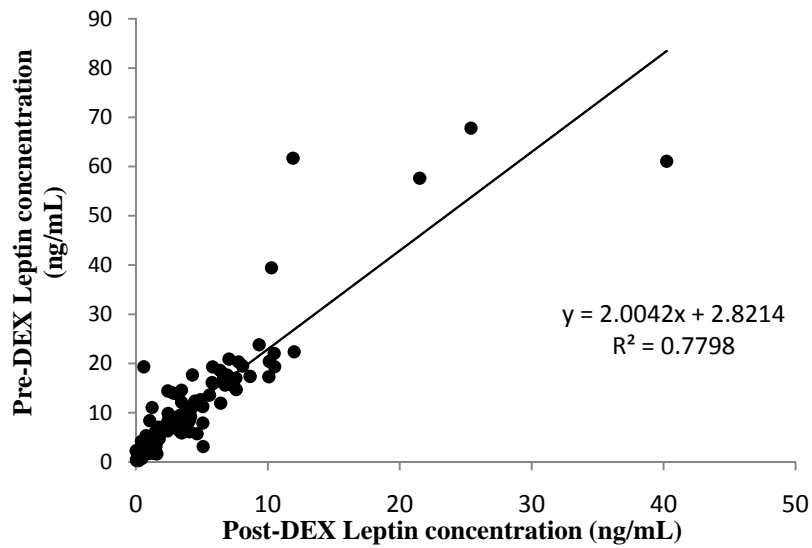


Figure 3.8. Pre- versus post-DEX leptin correlation. Data were highly correlated ($R^2 = 0.78$; $P < 0.001$).

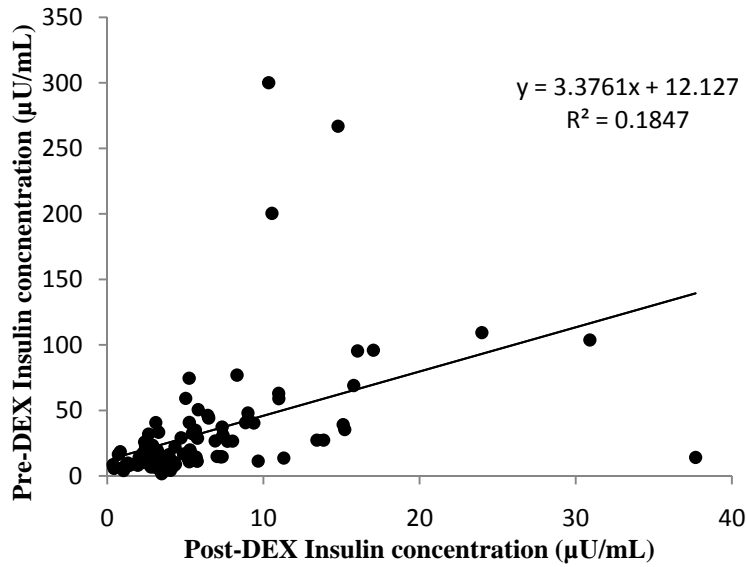


Figure 3.9. Pre- versus post-DEX insulin correlation. Data were correlated ($R^2 = 0.1847$; $P < 0.01$).

Horses were ranked based on concentrations of leptin and insulin to determine correlations and to remove the differences in scales for the 2 variables. Rank correlations were positively correlated ($P < 0.05$) for both pre- (Fig. 3.10) and post- (Fig. 3.11) DEX samples.

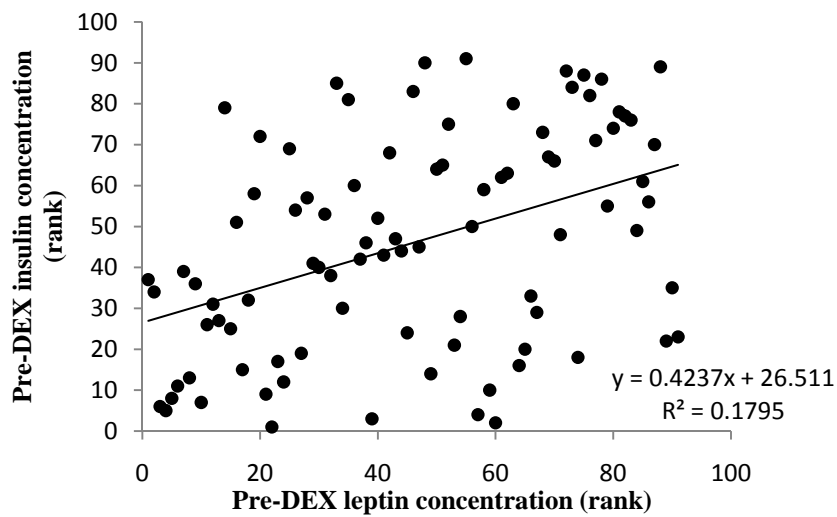


Figure 3.10. Ranked pre-DEX leptin and insulin correlation. Data were correlated ($R^2 = 0.1795$; $P < 0.01$).

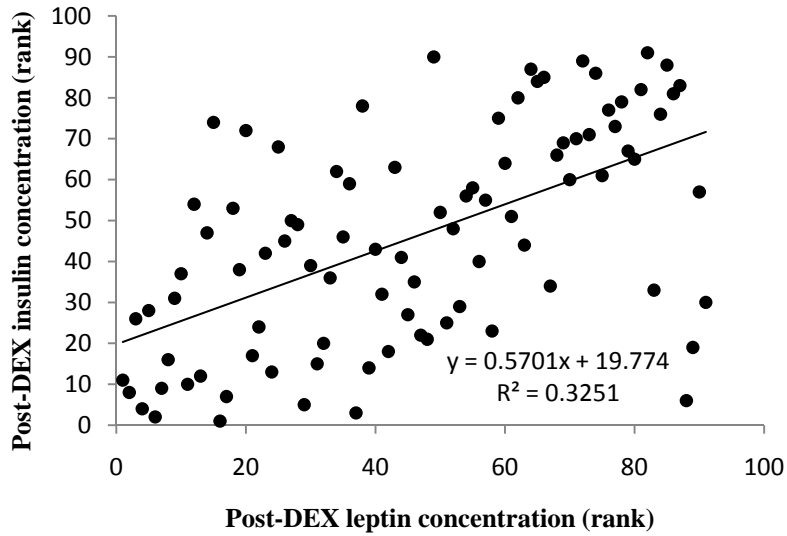


Figure 3.11. Ranked post-DEX insulin versus leptin correlation. Data were correlated ($R^2 = 0.3251$; $P < 0.01$).

Correlations were also used to determine if post-DEX leptin concentrations were correlated with BCS, both by rank (Fig. 3.12) and by actual leptin values (Fig. 3.13).

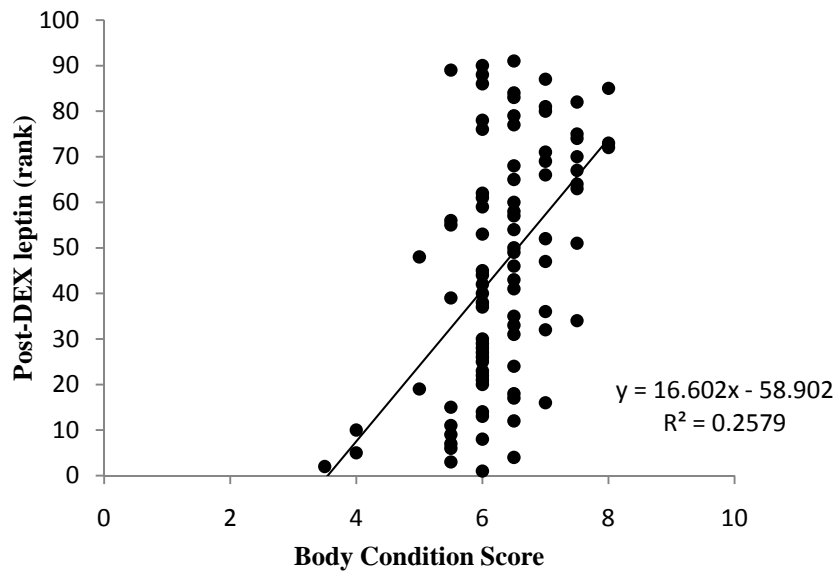


Figure 3.12. Ranked post-DEX leptin versus. body condition score. Data were correlated ($R^2 = 0.2579$; $P < 0.01$).

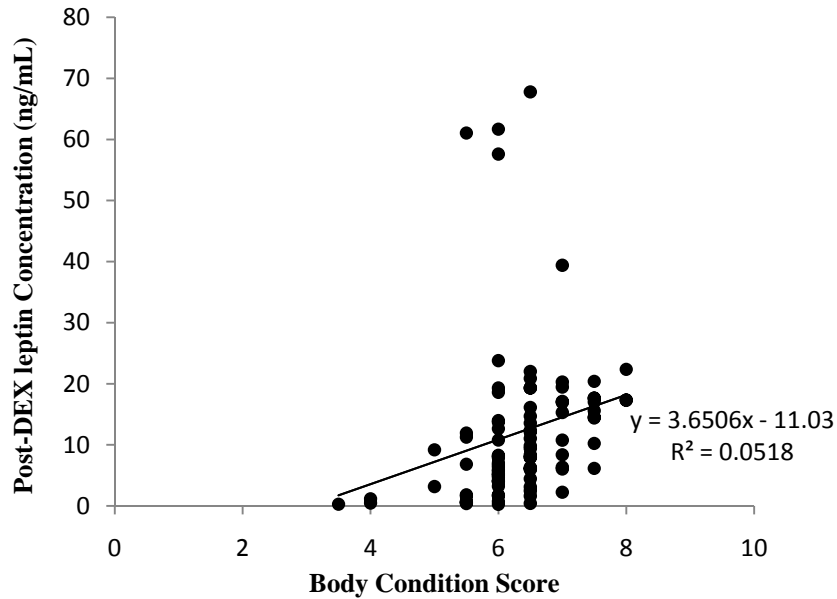


Figure 3.13. Post-DEX leptin concentration versus body condition score. Data were correlated ($R^2 = 0.0518$; $P < 0.05$), but less so than using ranked leptin concentrations (above).

Discussion

Use of the DEX suppression test in horses is a readily applied technique used by veterinarians to assess adrenal response. Equine Cushing's syndrome is a result of hyperadrenocorticism, often caused by PPID. Adenomas of the pars intermedia will often result in dysregulated ACTH production causing hypersecretion of glucocorticoids by the adrenal glands (Hadley and Levine, 2006). Increased glucocorticoid actions in the horse have been associated with increased circulating insulin, glucose intolerance, and elevated plasma lipids (Johnson, 2002). In normal horses, intravenous delivery of DEX results in suppressed endogenous ACTH and cortisol as a result of negative feedback to the pituitary within 24 h. In abnormal or Cushingoid horses, suppression of ACTH is absent (or reduced) and horses continue to maintain elevated concentrations of plasma cortisol (Messer, 1999).

The stimulatory effects of DEX on insulin and leptin have been reported in humans (Larsson and Ahren, 1996) and in horses (Freestone et al., 1991; Cartmill et al., 2003; Tiley et

al., 2007; Tiley et al., 2008). The DEX suppression test was implicated in this experiment to avoid the ambiguity of hyperinsulinemia due to Cushing's syndrome as opposed to compensation due to decreased insulin sensitivity. Even though Cushing's was absent in these horses, leptin and insulin were not stimulated enough to observe differences in horses. Cartmill (2003) used a high dose (125 $\mu\text{g}/\text{kg}$ BW) of DEX to invoke a similar response in horses. This resulted in persistent hyperglycemia and hyperinsulinemia for 19 d following administration. In contrast to previous experiments in horses, the present experiment used a smaller dosage of DEX to promote an acute response that a clinical screening could detect while avoiding further perturbations.

The increased plasma leptin and insulin concentrations following DEX administration in the present experiment is in agreement with previous research. However, identification of hyperleptinemic horses was not as obvious as that described by Huff et al. (2008), who described a normal population of mares between 0 and 6 ng/mL leptin. In that study, the normal range was determined by truncating the higher increments in the leptin frequency diagram until the remaining distribution was no longer significantly skewed towards higher values. Distribution of leptin values in the present experiment were also highly skewed towards higher values, but no amount of truncation resulted in a normal distribution. Certainly those horses with leptin values above 10 ng/mL in Fig. 3.4 are hypersecretors of leptin; however, no distinct demarcation from a normal distribution was obvious. Accurate diagnosis of hyperleptinemia in horses is important, because the hormone is often associated with metabolic and hormonal disturbances in the horse. Gentry et al. (2002) was the first to report a dichotomy of leptin concentrations in horses based on BCS. Cartmill et al. (2003) expanded on this research and reported elevated insulin in parallel to hyperleptinemia in such horses.

Elevated insulin is commonly reported in horses with insulin resistance or glucose intolerance. Such horses often exhibit a delayed or prolonged response to glucose infusion, believed to be caused by failures in insulin signaling of glucose transport proteins that promote glucose uptake by peripheral tissues (primarily muscle). Prolonged hyperinsulinemia can be dangerous and result in laminitis. Asplin et al. (2007) induced laminitis in ponies using the pEHC technique, in which sustained hyperinsulinemia is maintained while glucose concentrations are kept constant (euglycemia). However, leptin concentrations were not reported in that experiment.

Previous research by Cartmill et al. (2002) reported prolonged glucose response to i.v. dextrose injection in hyperleptinemic horses. Frank et al. (2006) reported higher resting plasma leptin concentrations in obese (BCS >7), insulin-resistant horses versus horses with moderate BCS (4 to 6); obviously, the relationship between leptin concentrations and insulin resistance was confounded by the mares' body condition, which itself correlates to leptin concentrations. Obese, insulin-resistant horses in that experiment also had elevated resting plasma insulin, glucose, and VLDL concentrations. Body condition score and leptin and insulin concentrations in the present experiment were also correlated. Although not measured in this experiment, hyperglycemia may (Cartmill et al., 2003) or may not (Storer et al., 2007) exist in hyperleptinemic horses.

This experiment indicates that a single, low-dose injection of DEX did not enhance the identification process in differentiating hyperleptinemic horses from normal, healthy horses. Evidence of a bimodal distribution of horses on a frequency diagram was not observed for plasma leptin and insulin concentrations either before or after DEX administration. The difference between the present results and those of Huff et al. (2008) may be due to the difference in metabolic demands of the horses studied. That is, Huff et al. (2008) studied brood

mares that had recently foaled. The combination of late pregnancy and early lactation would put demands on those horses that the horses in the present experiment would not have experienced. Nutrient availability and environmental conditions may also have contributed to the difference. Data from the present experiment were collected in December, whereas those of Huff et al. (2008) were collected in the spring and early summer. Nutrient availability (Gentry et al., 2002; Storer et al., 2007; Huff et al., 2008) and feed status (feed-deprived versus fed; Buff et al., 2005) have been shown to influence leptin and insulin concentrations. Furthermore, horses maintained on pasture have previously been reported to have the highest leptin concentrations relative to hay and/or grain-fed horses (Storer et al., 2007; Huff et al., 2008). Previous studies in mares have also shown that leptin concentrations are greatest in summer months and lowest in the winter (Fitzgerald and McManus, 2000).

Data from the DEX injections proved to be helpful in determining the absence of PPID and Cushing's syndrome in these horses; however, it was not helpful in differentiating hyperleptinemic horses in our experiment. Further research is needed to more clearly describe the relationships between insulin and leptin concentrations, equine metabolic syndrome, and ways to accurately diagnose and efficiently alleviate these conditions. Because obesity seems to be the central component, "easy keeper" breeds have been the focus of attention. Therefore, diet and exercise have also been main factors of consideration for alleviating the problem (Gordon and McKeever, 2005). However, not only obese horses have been shown to be affected (Huff et al., 2008), and a genetic component is speculated, but yet to be confirmed.

CHAPTER 4

EVALUATION OF THE COMBINED GLUCOSE AND INSULIN TEST FOR ASSESSING INSULIN SENSITIVITY

Introduction

Impaired insulin sensitivity is a major component of EMS, disrupting homeostasis and opening the door to a multitude of serious and potentially fatal ailments in horses (Johnson, 2002). A multitude of tests have been used to assess the effectiveness of insulin to signal muscle tissue to take up glucose from the interstitium. These tests range in ambiguity, invasiveness, and practicality. Albeit simple and less technically-involved than other tests, intravenous glucose tolerance tests are indirect and ambiguous in assessing insulin sensitivity. Thus, the advent of an accurate and practical test is an absolute necessity to clinicians (Frank, 2006).

The most direct and specific methods are clamp techniques and minimal model methods. Considered to be the “gold standard” in assessment of insulin sensitivity, clamp techniques, such as the pEHC, infuses large, constant amounts of insulin and enough glucose to maintain glucose concentrations in a steady state. The amount of glucose required to keep glucose concentrations at euglycemia in the face of high insulin infusion is proportional to the sensitivity to insulin (that is, more sensitive horses will require greater glucose infusion; Pratt et al., 2005).

Minimal model assessments use a frequent sampling intravenous glucose tolerance test in which both glucose and insulin are infused to invoke a response. Computer software is then used to calculate the degree of sensitivity and insulin effectiveness (Treiber et al., 2006a). However specific and accurate, these methods are invasive, difficult, and expensive, and are therefore minimally useful in a clinical setting.

The CGIT is a qualitative, nonspecific test used to assess insulin sensitivity in horses (Eiler et al., 2005; Frank et al., 2006). However, a paucity of information exists concerning the

repeatability and implications of the CGIT in horses, and the possible interactions in horses of varying BCS, insulin and leptin status, and sex. Previous research documented a hyperinsulinemic-hyperleptinemic syndrome in approximately 30% of the horses in the LSU Agricultural Center herd. This condition has been the topic of an abundance of research contending that the condition may have a role in IR and compensated hyperinsulinemia (Gentry et al., 2002; Cartmill et al., 2003). The present experiment was designed to study the utility and repeatability of the CGIT in horses for assessing insulin sensitivity.

Materials and Methods

Combined intravenous glucose and insulin tests were performed on 55 horses of various breeds (Quarterhorse, Arabian, and Thoroughbred), sex (33 mares, 15 geldings, and 7 stallions), and BCS (4.5 to 8). Tests were completed within a 2 wk period during November, 2008. Horses were randomly assigned to groups with a fixed number of stallions, geldings, and mares tested each day. Mares and geldings were placed in a dry lot paddock, and stallions were assigned to box stalls overnight, for at least 13 h before commencing the CGIT. Horses were allowed *ad libitum* access to grass hay and water during the 13-h period.

On the morning of testing, mares and geldings were loaded into an open chute; stallions were restrained in stocks. Stallions were kept separate from other horses to avoid excitability, which might have influenced the results. All horses were catheterized in one jugular vein; catheters were kept patent with 6% (w/v) sodium citrate solution. Blood samples were obtained for 3 d before the CGIT (d -3, -2, -1) and immediately preceding treatment injections (-15 and 0 min) to determine baseline plasma concentrations of glucose, insulin, and leptin. All horses were then administered an i.v. bolus of glucose (150 mg/kg BW as a 50% dextrose solution; Durvet, Inc., Blue Springs, MO) followed by bovine insulin (Sigma; 0.1 IU/kg BW in saline) followed by 3 mL of sodium citrate solution.

Post-treatment blood samples were obtained at 1, 5, 15, 30, 45, 60, 90, and 120 min. All samples were kept chilled until centrifuged (1,500 x g) for 15 min; plasma was harvested and stored frozen until analyzed. Plasma glucose concentrations were measured by the ortho-toluidine method (Anderson and Cockayne, 2003); insulin concentrations were measured by RIA (Diagnostic Systems Laboratories). Glucose response curves were generated for each horse.

Horses were classified by sensitivity based on the ability of glucose concentrations to return to baseline at 45 min following glucose and insulin injections (Eiler et al., 2005). The calculation used to diagnose insulin sensitivity following the tests was the difference in plasma glucose concentration at the 45- and 0-min sampling relative to glucose and insulin infusion (insulin sensitivity = $t_{45} - t_0$). If the difference in concentration between these 2 sampling intervals was positive, the horse was classified as insensitive. Conversely, if the difference resulted in a negative number the horse was considered to exhibit normal, or sensitive, glucose response to insulin. Using this classification system, horses were divided into their respective groups based on sensitivity. The procedure was repeated one month later and again 1.5 months after the original trial with a fixed group of 18 horses. These repeat samplings included horses exhibiting a range of sensitivities in the original trial. Data correlations and differences were analyzed by regression analysis and ANOVA using SAS (SAS Inst., Cary, NC).

Results

Data from the CGIT revealed differences ($P < 0.05$) in sensitivities to glucose and insulin administration. Glucose response curves were generated to display the differences in normal (sensitive) and insensitive horses (Fig. 4.1). Least significant difference was calculated and is displayed on the diagram for determining differences between groups.

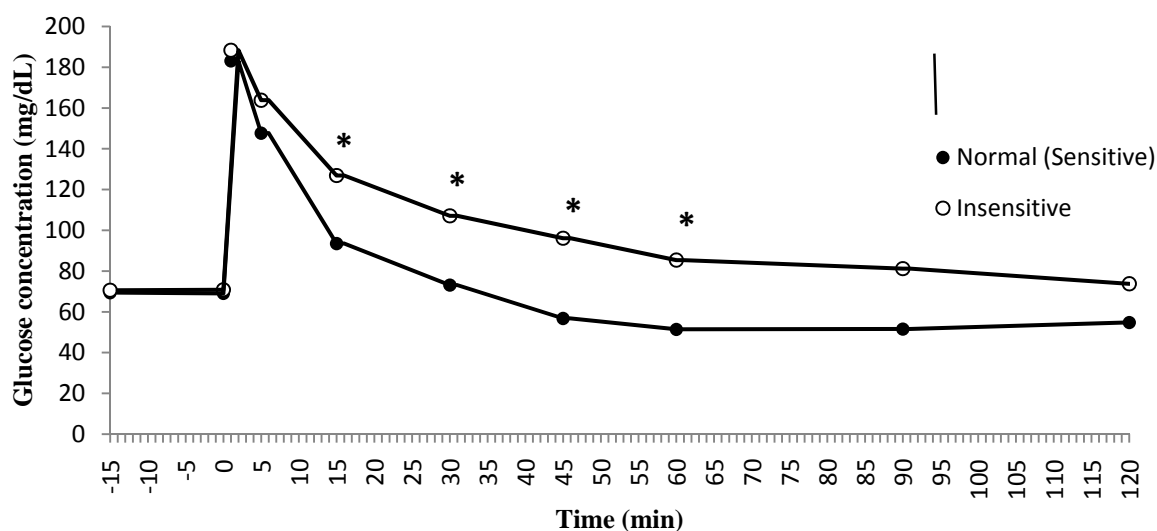


Figure 4.1. Glucose response curves to combined glucose and insulin tests. Plasma glucose concentrations were greater ($P < 0.05$) for insensitive horses compared with normal at times indicated by asterisks (*). Pooled SEM for glucose was 6.37 mg/dL. The vertical bar represents the LSD value ($P < 0.05$) for determining differences between groups within a time period.

Horses were ranked based on sensitivities following CGIT trials to standardize the sensitivity scale between trials and to determine if insulin sensitivity was maintained in the repeat trials. The repeated CGIT (Trials 2 and 3) proved to be significantly correlated ($P < 0.01$) by sensitivity ranks with original (Trial 1) data (Fig. 4.2 and 4.3). Approximately 75% of the horses in each repeat were highly correlated. However, these correlations were not strong enough to classify the CGIT as a predictive test of insulin sensitivity in these horses.

Mean pre-treatment leptin, insulin, and glucose concentrations for insulin sensitive horses (2.84 ng/mL, 13.48 μ U/mL, and 69.93 mg/dL, respectively) were not different ($P > 0.05$) from insulin insensitive horses (4.67 ng/mL, 15.56 μ U/mL, and 73.15 mg/dL, respectively; Fig. 4.4, 4.5, and 4.6).

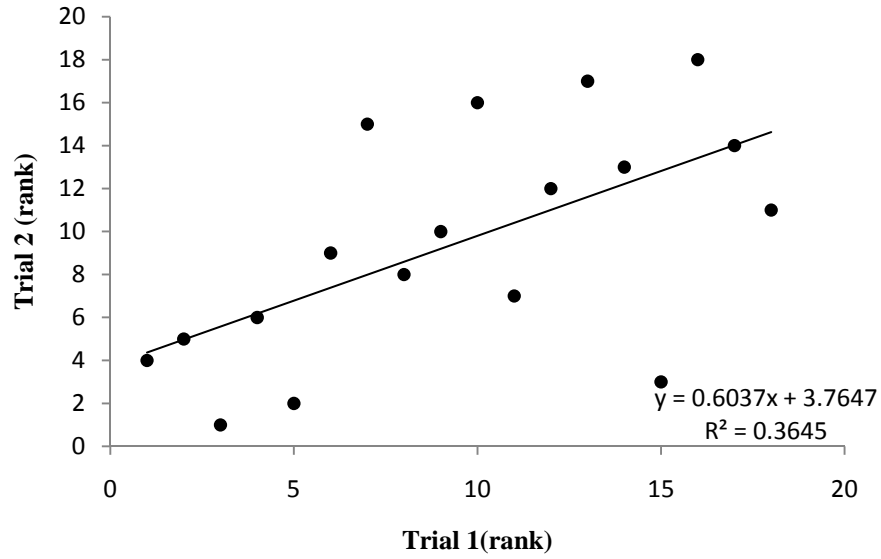


Figure 4.2. Trial 1 versus Trial 2 insulin sensitivity correlation. Data were correlated ($R^2 = 0.3645$; $P < 0.01$).

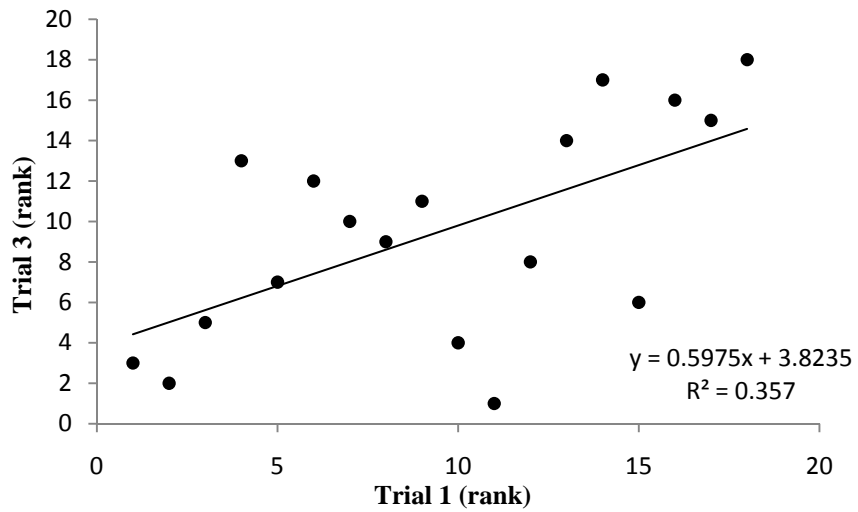


Figure 4.3. Trial 1 versus 3 insulin sensitivity correlation. Data were correlated ($R^2 = 0.357$; $P < 0.01$).

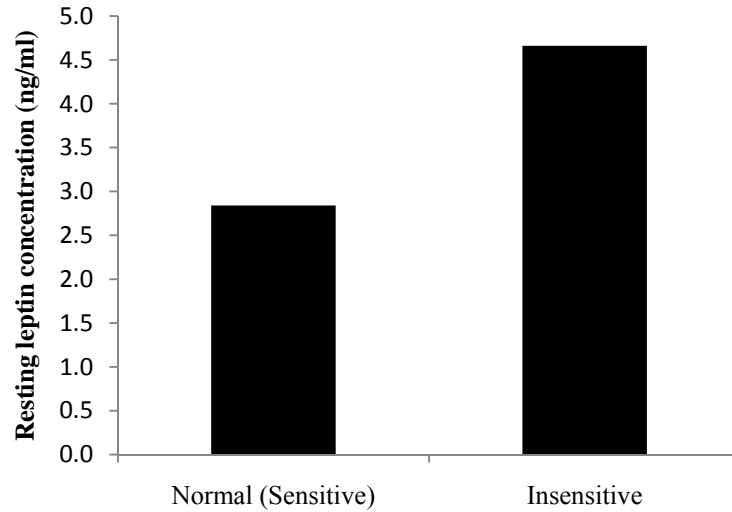


Figure 4.4. Mean pre-treatment plasma leptin concentration in sensitive and insensitive horses. Data were not different for horses classified as insensitive by CGIT ($P > 0.05$) compared to normal horses. Pooled SEM for leptin was 0.75 ng/mL.

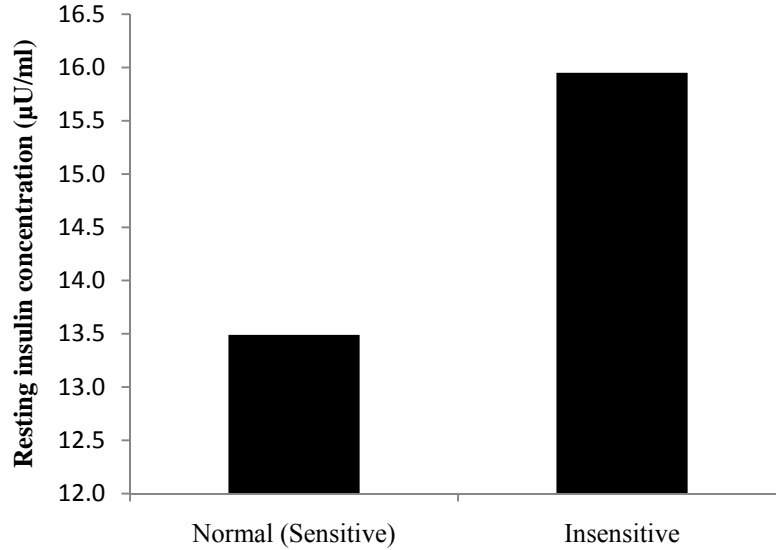


Figure 4.5. Mean pre-treatment plasma insulin concentration in sensitive and insensitive horses. Data were not different ($P > 0.05$) for horses classified as insensitive by CGIT compared to normal horses. Pooled SEM for insulin was 5.57 µU/mL.

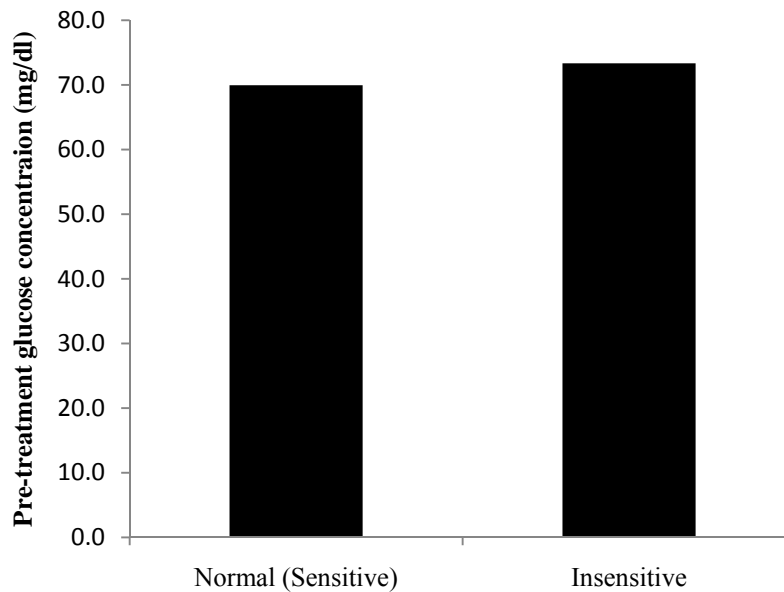


Figure 4.6. Mean pre-treatment plasma glucose concentration in sensitive and insensitive horses. Data were not different ($P > 0.05$) for horses classified as insensitive by CGIT compared to normal horses. Pooled SEM for glucose was 1.94 mg/dL.

Mean glucose concentrations were, however, different ($P < 0.0001$) between normal (46.12 ± 3.76 mg/dL) and insensitive (97.50 ± 5.71 mg/dL) horses at the 45 min post-treatment sampling interval (Fig. 4.7). Furthermore, mean body condition score (Fig. 4.8) was also higher ($P = 0.0057$) for horses classified as insensitive by CGIT (mean BCS = 6.76 ± 0.15) compared with horses with normal insulin sensitivity (mean BCS = 6.09 ± 0.18).

Resting leptin concentration and insulin sensitivity (Fig. 4.9) were significantly correlated ($P < 0.01$), as well as resting leptin and insulin concentrations (Fig. 4.10; $P < 0.05$). Body condition score was also significantly correlated with ranked baseline plasma leptin concentration (Fig. 4.11; $P < 0.01$) and insulin sensitivity (Fig. 4.12; $P < 0.05$).

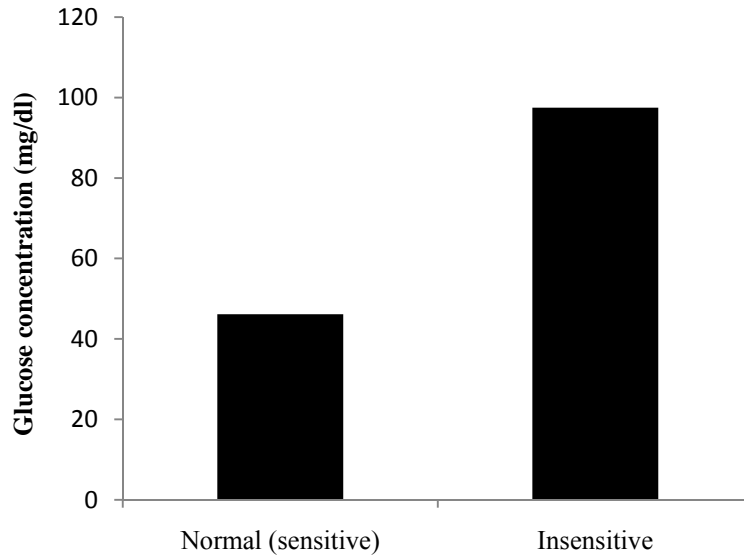


Figure 4.7 Mean plasma glucose concentration at the 45 min in sensitive and insensitive horses. Data were higher ($P < 0.05$) for horses classified as insensitive by CGIT. Pooled SEM for glucose was 4.74 mg/dL.

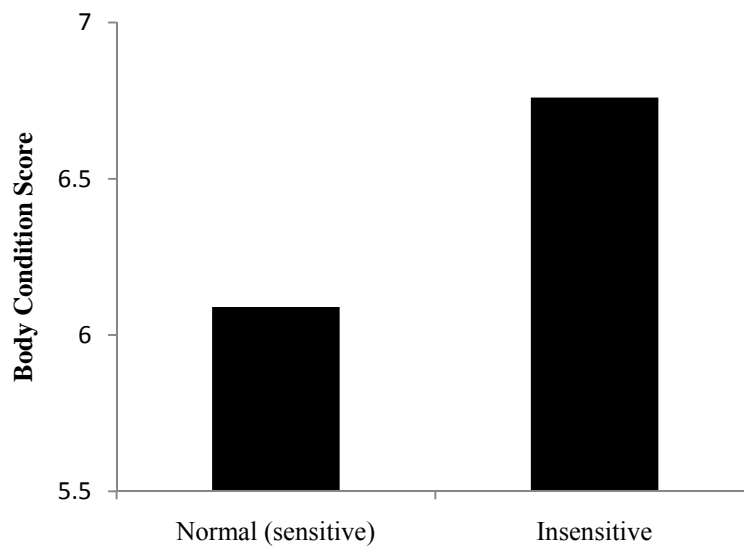


Figure 4.8. Mean body condition score in sensitive and insensitive horses. Data were higher for horses classified as insensitive by CGIT ($P < 0.05$) compared to normal horses. Pooled SEM. for BCS was 0.17.

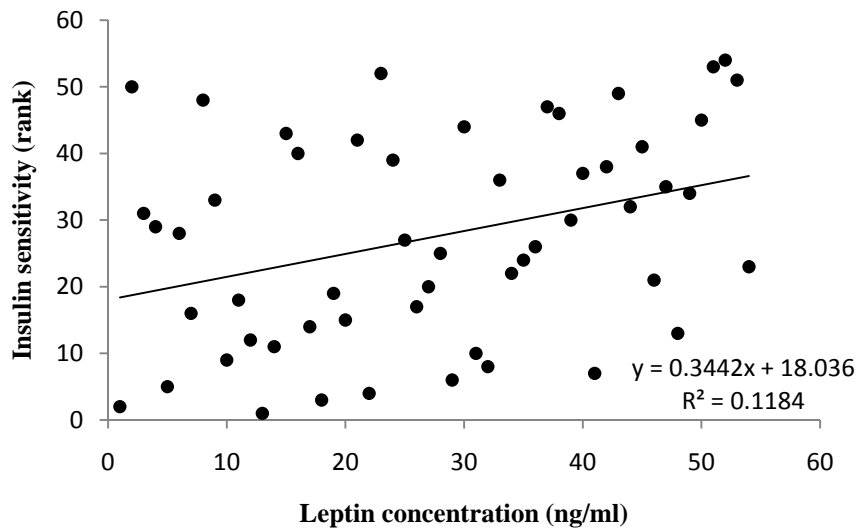


Figure 4.9. Insulin sensitivity ranks versus leptin concentration correlation. Data were significantly correlated ($P < 0.05$; $R^2 = 0.1184$). Ranked insulin sensitivities were used to standardize the scale differences between units of the two variables.

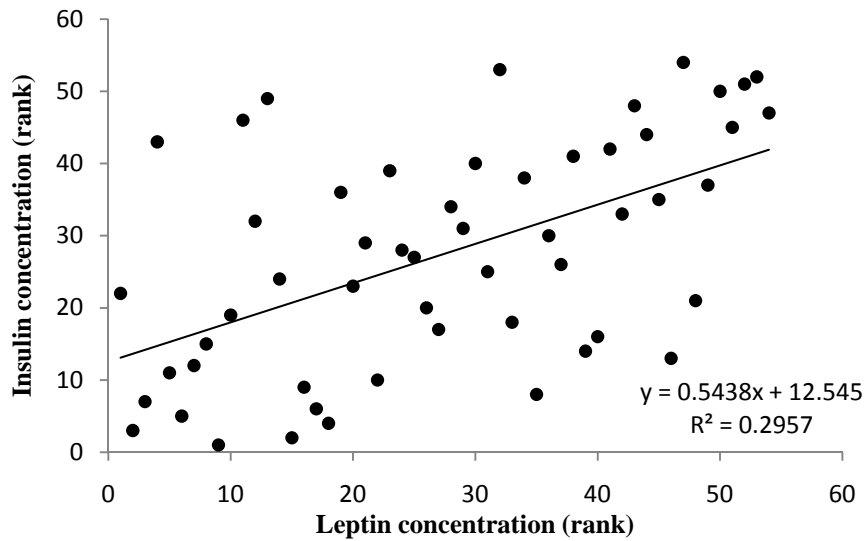


Figure 4.10. Insulin versus leptin rank correlation. Data were correlated ($P < 0.01$; $R^2 = 0.2957$). Rank correlations were used to standardize the scale differences between units of the 2 variables.

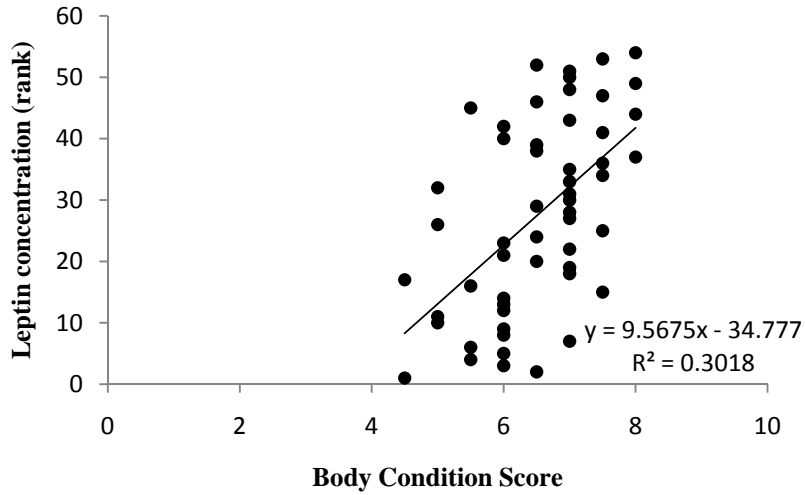


Figure 4.11. Pre-treatment leptin rank versus body condition score correlation. Data were correlated ($P < 0.01$; $R^2 = 0.3018$). Rank correlations were used to standardize the scale differences between units of the 2 variables.

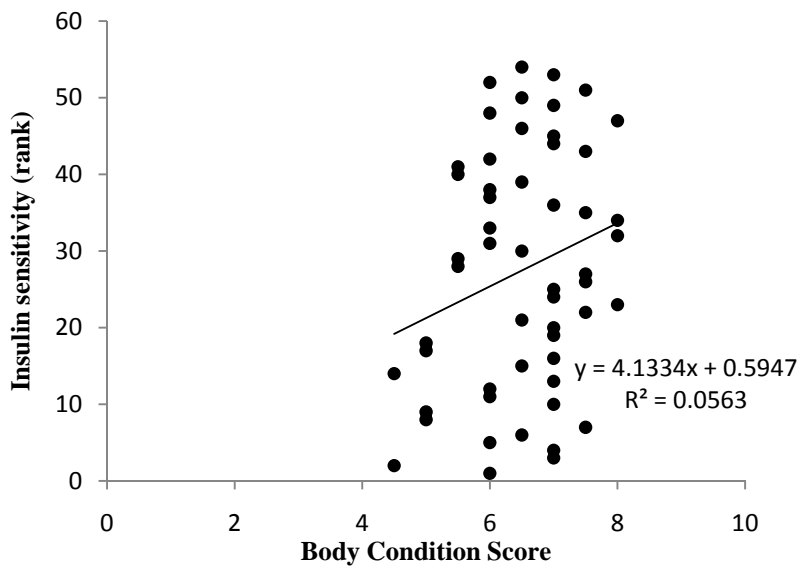


Figure 4.12. Pre-treatment insulin sensitivity rank versus body condition score correlation. Data were marginally correlated ($P = 0.07$; $R^2 = 0.0563$). Rank correlations were used to standardize the scale differences between units of the two variables.

Discussion

Glucose response curves for insensitive horses were prolonged and took longer to reach baseline concentrations compared with normal, sensitive horses. These data are comparable to that described by Frank et al. (2006), in which glucose response curves for horses pre-screened as obese and diagnosed with insulin resistance had a more prolonged response curve compared with normal horses that returned or fell below baseline concentration by 45 min. In the present experiment, horses classified as insensitive not only failed to fully recover by 45 min, but failed to return to pre-treatment concentration by the end of the 120-min test.

Although glucose response curves were different for the 2 groups of horses, the distinguishing factor of this experiment was to assess the viability of the CGIT as a predictive test on multiple testing occasions. Correlations between the multiple trials proved to be significant ($P < 0.05$), although not high enough to be predictive. Approximately 75% of the horses tested in the repeated tests were highly correlated with original data. These poor correlations are far inferior to other tests such as clamp and minimal model techniques. Pratt et al. (2005) reported high (>0.70) intra-class correlation coefficients in testing the repeatability of the pEHC technique and minimal model analysis of the FSIGT. These high intra-class values are indicative of acceptable repeatability for these tests.

Inconsistent with data reported by Frank et al. (2006), horses classified as insensitive by CGIT in the present experiment did not have higher ($P < 0.05$) baseline plasma concentrations of leptin, glucose, or insulin in samples collected prior to testing. Insulin resistance is typically characterized in horses by hyperinsulinemia as a compensatory mechanism (Kronfeld, 2005a; Kronfeld et al., 2005; Geor and Frank, 2008). Therefore, insensitive horses may show elevated baseline plasma insulin concentrations relative to normal horses. Data from our laboratory have previously shown a hyperinsulinemic-hyperleptinemic condition afflicting approximately 30% of

our herd (Gentry et al., 2002; Cartmill et al., 2003, Storer et al., 2007). Based on leptin and insulin data from the present study, it does not appear that this aforementioned condition played a factor in the results of the experiment, even though baseline plasma leptin and insulin concentrations were significantly correlated.

Similar to CGIT results reported by Frank et al. (2006), horses classified as insensitive by the CGIT had higher BCS than those classified as normal. This supports the claim that obesity is a central factor in assessing insulin sensitivity in horses. Obesity and insulin response to glucose have been widely studied in horses as to their roles in the manifestation of laminitis (Johnson et al., 2004; Treiber et al., 2006a,b; Bailey et al., 2008). Obese ponies predisposed to laminitis often show signs of attenuated insulin sensitivity and insulin resistance, which is believed to play a role in laminitis susceptibility (Bailey et al., 2008; Geor, 2008).

Similar to humans, there is also evidence that obesity may lead to hypertension in horses. Fat deposits in horses may not only build up in the omental (abdominal) region of the body, but also on the neck and tail heads. This can sometimes be an elusive property, because many horses may show good to moderate BCS with an overlooked buildup of fat on the neck and tail head. Ponies predisposed to recurrent laminitis with moderate BCS and similar neck adiposity show a significant elevation in systolic and diastolic blood pressure compared to control ponies (Bailey et al., 2008). Although not measured in our experiment, insulin resistant horses tend to exhibit signs of elevated plasma free fatty acids and increased lipoproteins, which may lead to hypertension (Frank et al., 2006).

Predispositions and physical characteristics believed to be associated with insulin resistance have been studied in horses (Johnson et al., 2004; Frank et al., 2006; Treiber et al., 2006a; Geor, 2008). A large majority of claims implicate “easy-keeper” breeds and sedentary horses as having a higher susceptibility. Nutritional countermeasures consisting of low-

carbohydrate, high-fiber diets have been shown to improve insulin sensitivity, while carbohydrate-rich diets of high glycemic index have been shown to exacerbate IR in horses (Hoffman et al., 2003; Kronfeld, 2004). Regular exercise has also been shown to improve insulin and glucose dynamics in the horse (Treiber et al., 2006c).

The CGIT was a simple test with a conceptual approach that would be conducive to a clinical practice; it's prompt, inexpensive, and simple. However, based on the apparent repeatability in the CGIT trials performed in this experiment, use of this test is questionable at this time. Further research is needed to identify a diagnostic testing procedure that is clear, accurate, and easily-performed.

CHAPTER 5

HYPERLEPTINEMIA IN HORSES: DIFFERENTIAL EFFECTS OF INSULIN INJECTION ON GLUCOSE, NONESTERIFIED FATTY ACIDS, AND GLUCAGON IN PLASMA

Introduction

Metabolic syndrome (also referred to as Syndrome X) was first proposed by Reaven (1988) as a collection of metabolic and hormonal abnormalities in humans believed to increase one's chances of diabetes mellitus and CVD. Today, much debate has led to some additional components that largely revolve around obesity. Insulin signaling is a main component of the metabolic syndrome. Johnson (2002) adapted these criteria to horses as part of the EMS, which is believed to largely predispose a horse to chronic and potentially fatal laminitis and failures in insulin signaling pathways.

Insulin resistance and glucose intolerance is becoming a serious problem in sedentary horses. To that end, clinicians have sought practical tests which accurately assess insulin signaling and effectiveness in the horse. Testing procedures, such as the pEHC technique (Saad et al., 1994; Petrus et al., 1998) and the minimal model assessment (Hoffman et al., 2003; Pratt et al., 2005) have been identified as “gold standards” in assessing insulin sensitivity, but are arduous and impractical from a clinical standpoint. The insulin sensitivity test is an uncommonly used procedure involving the infusion of insulin alone following a period of fasting to observe glucose response curve in assessing insulin sensitivity. There is a paucity of data concerning the test and its use in horses.

Hyperinsulinemia is common in insulin resistant horses as a compensatory mechanism of the pancreas, which detects hyperglycemia due to failed or prolonged glucose clearance (Johnson, 2002; Geor and Frank, 2008). Other perturbed substances (hormones and metabolites) of the body as a result of failures in insulin signaling are leptin, a satiety hormone produced by

adipocytes, and fatty acids, synthesized by the liver and stored as triglycerides in various tissues. Gentry et al. (2002) was the first to describe a hyperinsulinemic-hyperleptinemic condition evident in approximately 30% of the LSU Agricultural Center research herd. Though believed to be in association with insulin resistance, no conclusive correlation of the hyperinsulinemic-hyperleptinemic condition with insulin resistance by accepted testing procedures has been performed. Fatty acids have yet to be studied in hyperleptinemic horses, but are believed to be elevated based on previous research in horses.

Glucagon is a pancreatic hormone that is secreted between meals when glucose levels in the blood decline (Hadley and Levine, 2006). This hormone signals the liver to synthesize glucose via glycogenolysis for use by tissues. To date, glucagon concentrations have not been measured in hyperleptinemic horses. The goal of the present experiment was to compare a low dose of insulin administration (0.02 IU/kg BW) to the standard dose given in insulin suppression tests (0.1 IU/kg BW) with regard to glucose and NEFA responses to determine whether this direct method would be able to detect differences between hyperleptinemic and normal horses. Glucagon was also measured to determine whether hyperleptinemic horses differed from normal horses 1) in the resting (untreated) condition or 2) after insulin-induced hypoglycemia.

Materials and Methods

Sixteen horses (8 mares and 8 geldings) of light horse breeds (Quarterhorse, Arabian, and Thoroughbreds) with moderate to high BCS (range = 5 to 7.5; Henneke, 1983) were designated “High” (HL) or “Low” (LL) horses based on mean plasma leptin concentrations recorded on 3 separate occasions in 3 different seasons of the same year before performing this experiment. The horses were assigned to 4 groups based on sex, BCS, and mean leptin concentrations: LL mares (mean BCS = 6.5; mean leptin = 0.91 ng/mL), HL mares (mean BCS = 7.0; mean leptin =

17.5 ng/mL), LL geldings (mean BCS = 6.5; mean leptin = 3.7 ng/mL), and HL geldings (mean BCS = 7.0; mean leptin = 22.4 ng/mL).

Two different dosages of bovine insulin (0.02 IU/kg BW and 0.1 IU/kg BW; bovine insulin, Sigma) were administered on 2 d in a double split-plot design with 2 d of rest between the 2 administrations. Horses were randomly assigned to treatment days (2 from each group) and all tests were completed within 1 wk. Horses were deprived of feed overnight (~13 h) preceding treatment in a drylot paddock with water available *ad libitum*. On the morning of testing, mares and geldings were loaded into an open chute together and stood quietly. An i.v. 14-ga catheter (Becton-Dickson, Nutley, NJ) was placed into the left jugular vein of each horse and was kept patent with 6% sodium citrate solution. The insulin injections were administered via bolus i.v. dose and flushed with 3 mL of the citrate solution.

Following injections, blood samples were collected into 7-mL, blood collection tubes containing potassium oxalate and sodium fluoride (Tyco Healthcare Group LP, Mansfield MA) at -20, 0, 20, 40, 60, 90, 120, 180, and 240 min relative to administration of insulin. The first 3 mL of blood was discarded at each withdrawal to eliminate residual citrate in catheters, which were flushed with the citrate solution following each sample collection.

Immediately following the blood collection and inversion to mix with anticoagulant at 0, 60, 120, 180, and 240 min relative to insulin injection, 1 mL aliquots of whole blood was withdrawn from the tube and placed in a separately prepared polystyrene tube containing aprotinin (protease inhibitor; A.G. Scientific, Inc., San Diego, CA) for glucagon measurement. All samples were kept refrigerated until centrifuged (15 min at 1,500 x g at 4°C) and the plasma fraction was harvested. Samples were stored frozen until subsequent analysis.

Plasma leptin concentrations were determined by RIA described by Cartmill et al. (2003). Estimates of the intra- and inter-assay CV averaged 6% and 4%, respectively.

Sensitivity of the leptin assay, based on a 200- μ L sample size, was 0.1 ng/mL (Cartmill et al., 2003). Concentration of glucose in whole blood was determined in duplicate via a portable glucometer (Accu-Chek, Roche Diagnostics, Nutley, NJ). Plasma glucagon concentrations were determined by RIA kit validated for equine plasma (LINCO Research, St. Charles, MO). Concentration of NEFA was determined via enzyme-linked, colorimetric assay (WAKO Diagnostics, Richmond, VA).

All measured variables were analyzed by ANOVA using the GLM procedure for a double split-plot design using SAS (SAS Inst., Cary, NC). Factors included in the model were: sex, status (HL vs. LL), dose, and time; BCS was included in the ANOVA model as a covariate.

Results

Baseline data for horses are presented in Table 5.1. Body condition score was not different between groups ($P > 0.05$). Pre-screened plasma leptin concentration was very different between groups, confirming that high leptin horses had significantly higher baseline plasma leptin concentrations compared with normal (LL) horses. Resting plasma NEFA were not different, as well as baseline glucose and glucagon concentrations ($P > 0.05$).

Glucose response curves were different for the 2 groups of horses (Fig. 5.1 and 5.2) for the 0.02 IU/mL/kg BW dosage. Normal mares and geldings had a much sharper drop in plasma glucose concentration relative to hyperleptinemic horses, which remained fairly constant throughout sampling intervals. Glucose response curves to the 0.1 IU/mL/kg BW dosage were also different across groups ($P < 0.05$). However, all four groups appeared to be suppressed by insulin injections, though hyperleptinemic mares were not as sensitive to the injection as the other 3 groups.

Table 5.1. Pre-treatment measurements of body condition score (BCS), leptin, non-esterified fatty acids (NEFA), glucose, and glucagon. Only resting plasma leptin concentrations differed between groups before the start of the experiment. Body condition and resting glucose, NEFA, and glucagon concentrations were similar.

Variable	High leptin		Low leptin		P-value
	Mares	Geldings	Mares	Geldings	
n	4	4	4	4	
BCS (scale, 1 to 9)	7 (6.5-7)	7 (6-7.5)	6.5 (6-7)	6.5 (5-7.5)	0.350
Pre-treatment plasma leptin (ng/mL)	17.47 (15.9-23.08)	22.35 (11.6-34.32)	0.91 (0.14-1.25)	3.74 (2.72-3.43)	<0.001
Pre-treatment plasma NEFA (mmol/L)	697.4 (633.9-753.1)	519.2 (477.9-597.2)	531.0 (406.2-622.3)	787.0 (641.7-837.3)	0.405
Pre-treatment plasma glucose (mg/dL)	84.87 (80.25-87.0)	78.27 (72.71-83.38)	86.42 (80.69-93.13)	85.7 (77.16-91.38)	0.256
Pre-treatment plasma glucagon (pg/mL)	26.88 (23.23-30.53)	24.78 (23.23 - 26.33)	26.75 (22.03-31.46)	40.0 (39.15-40.85)	0.710

Response curves for plasma concentration of NEFA were not different across any of the groups to the 0.1 IU (Fig. 5.3) or 0.02 IU/kg BW (Fig. 5.4) injection. However, there was a time effect ($P < 0.01$) at the 20, 40, and 60 min sampling interval that was similar to all 4 groups.

Plasma glucagon concentrations remained relatively stable and did not differ throughout sampling ($P > 0.05$) for the 0.02 IU (Fig. 5.5) or 0.1 IU/kg BW (Fig. 5.6) insulin injection.

Discussion

The major finding of this experiment was the clear dichotomy of glucose response curves after insulin administration in hyperleptinemic and normal horses of similar BCS. This dichotomy was most evident using a relatively low dose of insulin (0.02 IU/kg of BW). Hyperleptinemic horses had little or no plasma glucose response to insulin injections compared to normal (LL) horses. These data support previous speculation that hyperleptinemic horses have decreased insulin sensitivities (Cartmill et al., 2002; Huff et al., 2008). However, this is the

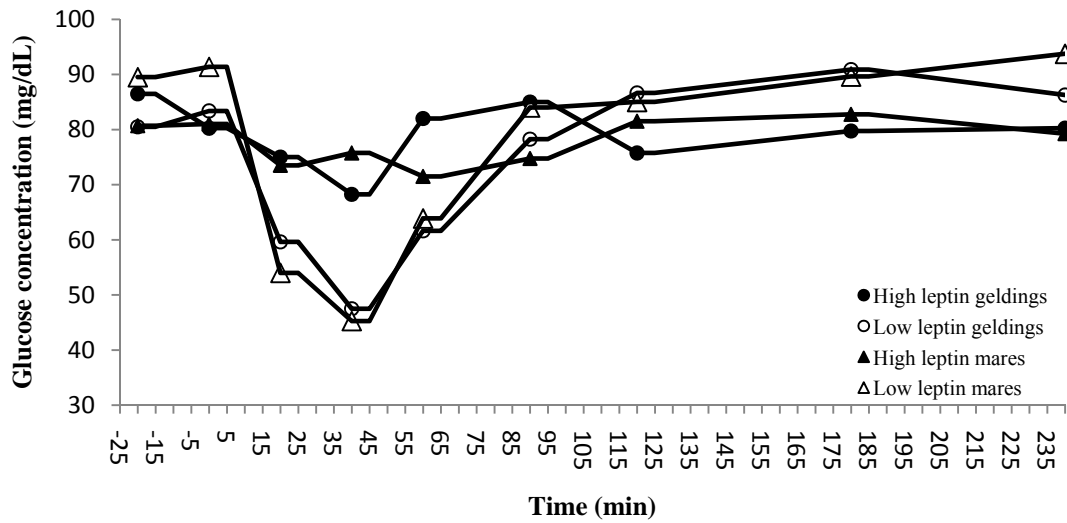


Figure 5.1. Whole blood glucose response to 0.02 IU/kg of body weight insulin injection in hyperleptinemic and normal (low leptin) mares and geldings. Normal horses exhibited a sharp drop in glucose ($P < 0.05$) for 40 min before recovering at 90 min. Hyperleptinemic horses sustained relatively constant glucose concentrations throughout the experiment. Pooled SEM for glucose was 4.7 mg/dL.

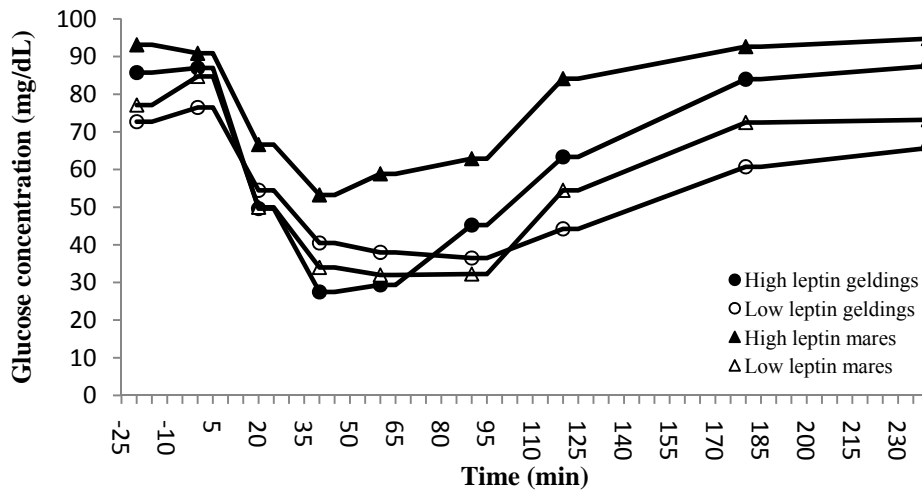


Figure 5.2. Whole blood glucose response curves to a 0.1 IU/kg body weight insulin injection in hyperleptinemic and normal (low leptin) mares and geldings. Normal mares and geldings exhibited a sharp drop in glucose, as did hyperleptinemic geldings for 40 min following the injection. Hyperleptinemic mares, however, were not as suppressed and recovered quicker than the other groups ($P < 0.05$). Pooled SEM for glucose was 4.7 mg/dL.

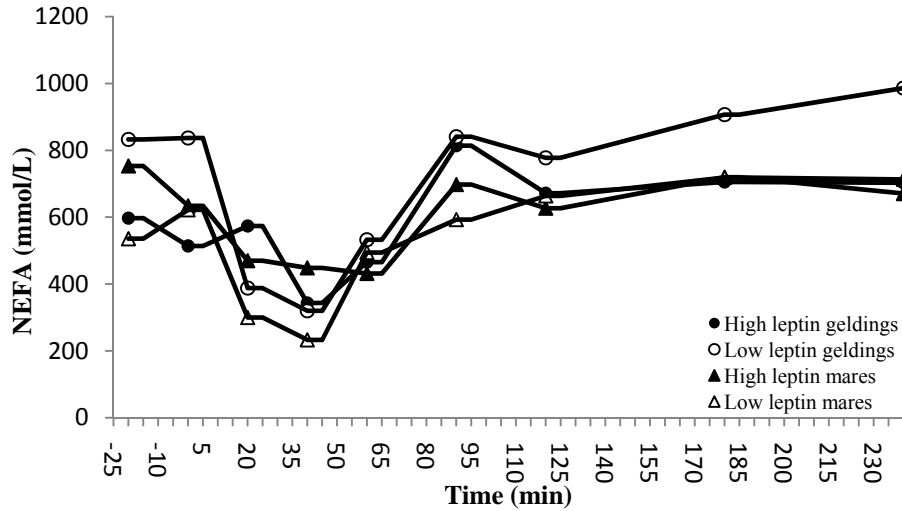


Figure 5.3. Plasma non-esterified fatty acid (NEFA) response to 0.02 IU/kg body weight insulin injection in hyperleptinemic and normal (low leptin) mares and geldings. Data were not different ($P > 0.05$) across the 4 groups.

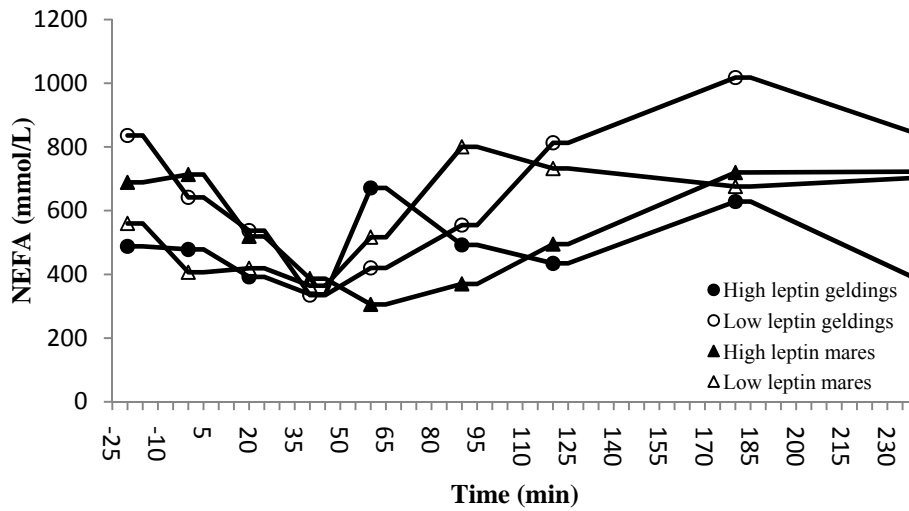


Figure 5.4. Plasma non-esterified fatty acid (NEFA) response to 0.1 IU/kg body weight insulin injection in hyperleptinemic and normal (low leptin) mares and geldings. Data were not different ($P > 0.05$) across the 4 groups.

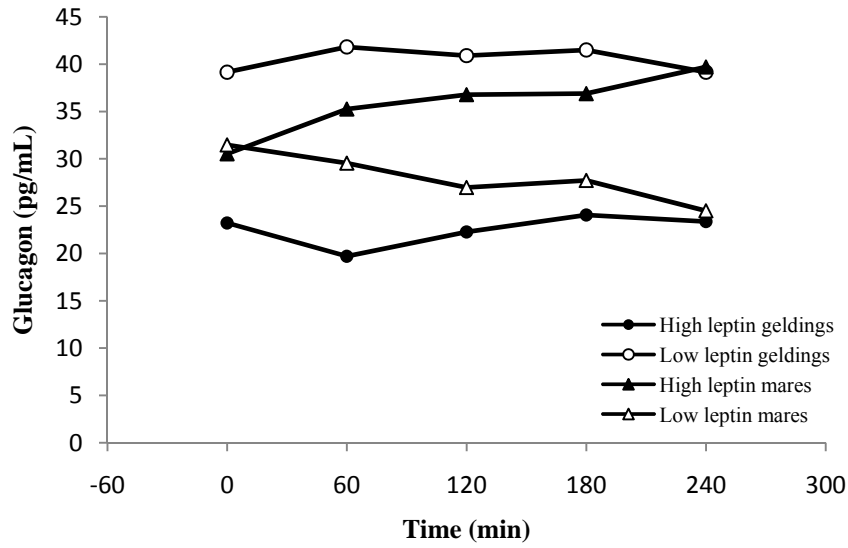


Figure 5.5. Plasma glucagon response to 0.02 IU/kg body weight insulin injection in hyperleptinemic and normal (low leptin) mares and geldings. Data were similar ($P > 0.05$) across groups and remained relatively stable throughout sampling.

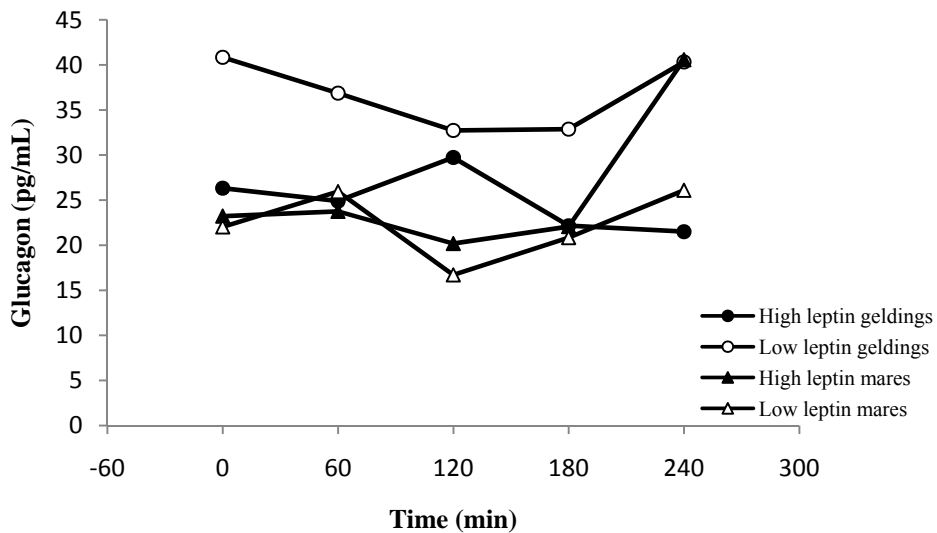


Figure 5.6. Plasma glucagon response to 0.1 IU/kg body weight insulin injection in hyperleptinemic and normal (low leptin) mares and geldings. Data were similar ($P > 0.05$) across groups and remained relatively stable throughout sampling.

first test to clearly provide evidence of IR in horses of similar BCS categorized by high resting leptin concentrations alone. Insulin resistant horses require a higher than normal insulin response to maintain euglycemia in the post-absorptive state, when blood glucose concentrations are elevated (Johnson, 2002; Frank, 2006; Treiber et al., 2006a,b). Although Frank et al. (2006) reported higher leptin concentrations in obese (BCS >7), clinically insulin resistant horses relative to horses with low to moderate BCS (3 to 6), those comparisons were confounded by the differences in BCS.

The difference in response among the groups to the higher insulin dose, as opposed to the low dose, was unexpected in that it differed between mares and geldings. One reason for using more than one dose in the present experiment was the possibility that the commonly used 0.1 IU/kg BW may have been saturating, and therefore less discriminatory of insulin sensitivity. This would be analogous to the naturally occurring situation of compensated insulin resistance, in which slightly insulin resistant horses over secrete insulin to make up for the reduced sensitivity, and thereby maintain euglycemia. The fact that HL geldings but not mares displayed sensitivity to the high insulin dose may indicate that the mares were even more insensitive than the geldings, if assessed by more traditional methods.

Overnight feed deprivation is used to ensure that metabolites are measured in a steady-state situation, as opposed to variable times after a meal. Although feed deprivation minimizes insulin concentrations and stabilizes glucose concentrations, some have argued against it in studies of insulin sensitivity because the animal is dependent on gluconeogenesis to maintain glucose concentrations in blood (Treiber et al., 2005; Hadley and Levine, 2006). It has also been shown that insulin sensitivity decreases in feed-deprived horses, supposedly as a glucose-sparing mechanism for essential tissues such as the brain (Forhead and Dobson, 1997). It is likely that overnight feed deprivation is not long enough to cause extreme changes in insulin sensitivity.

Forhead and Dobson (1997) showed differences in glucose response curves to insulin injections in donkeys deprived of feed overnight compared to donkeys feed-deprived for 3 d. The overnight-deprived group was much more sensitive to glucose administration compared to the 3-d group, indicating that the latter group was less sensitive and more dependent on gluconeogenesis for energy.

Insulin and glucagon are hormones with contrasting effects in normal patients. At times of abundance, when glucose is high in the blood, glucagon is suppressed and insulin mediates the metabolism of glucose for energy and storage as glycogen, fats, and amino acids (Hadley and Levine, 2006). Between meals and during times of fasting or starvation, glucose and insulin are low in the blood, and tissues rely heavily on glucagon to signal glycogenolysis in the liver and hormone-sensitive lipase of fat cells to propagate lipolysis for energy (Hadley and Levine, 2006). In humans, it has been reported that hyperglucagonemia and elevated NEFA accompany elevated plasma glucose and insulin concentrations (Reaven et al., 1987). Although serum glucagon levels are normally suppressed by hyperglycemia and food intake, glucagon is unable to decrease appropriately in type II diabetic humans. Thus, hyperglucagonemia is believed to exacerbate the hyperglycemic state in diabetic humans (Ørskov et al., 1991; Shah et al., 2000; Li and Zhuo, 2007). Given the lack of differences in the present experiment, glucagon does not seem to be a major causative factor in the hyperleptinemia or hyperinsulinemia in these horses.

In equine models, horses diagnosed as insulin resistant are often hyperinsulinemic and hyperglycemic, though hyperglycemia alone is ambiguous and inconclusive (Firshman and Valberg, 2007). This may explain why plasma glucagon concentrations measured in this experiment remained fairly constant. Furthermore, in humans afflicted with type II diabetes mellitus, pancreatic abnormalities such as β cell failure arise due to insulin depletion (Johnson, 2002). Thus, it is apparent that the mechanism of insulin resistance in humans is in part a

disorder implicating both the pancreas as well as molecular signaling of insulin-dependent tissues. To date, no data have been published documenting changes in glucagon or β cell depletion in horses with impaired insulin signaling.

The lack of differences of plasma NEFA concentrations measured at rest across groups and during the challenge was surprising. Non-esterified fatty acids decreased during the test and recovered in all groups, many with overcompensation evident by higher than baseline concentration at 240 min. This overshoot may have been the product of increased sympathetic activity as a result of hypoglycemia, which causes fatty acid mobilization (Guyton and Hall, 2006). Previous research has reported an association of dyslipidemia and hyperlipemia with insulin resistance in various species including equids (Fraze et al., 1985; Reaven et al., 1987; Frank et al., 2006). Hypertension resulting from dyslipidemia has also been documented in ponies (Bailey et al., 2008). It may be that this condition is only characteristic to insensitive horses with very high BCS indicative of adiposity accretion.

Results from the present experiment indicate that insulin injections may be a viable alternative for assessing insulin sensitivity in the horse. Although it does not estimate insulin sensitivity directly via modeling, as do the clamp and minimal modeling techniques, it likely does reflect insulin sensitivity as determined by those more laborious and invasive methods. In spite of its simplicity, clinicians may be hesitant to implement a testing procedure which administers intravenous insulin alone due to the potential fatal risk of hypoglycemia. However, previous studies (Alexander et al., 1997; Forhead and Dobson, 1997) have safely used insulin concentrations in horses at 20-times (0.4 IU/kg BW) that of the low dose used in the present experiment (0.02 IU/kg BW) without any reported emergencies. Moreover, the use of a portable glucometer to quickly measure glucose would allow a clinician to immediately administer i.v. glucose solution if needed to counteract severe hypoglycemia.

Glucose tolerance tests are simple and most clinically-implicated, but inconsistent and, therefore, unreliable. The pEHC technique and minimal model assessments may be accurate in quantifying the degree of insulin sensitivity, but are technically-involved and expensive techniques, thus, impractical from a clinical perspective (Pratt et al., 2005; Geor et al., 2008). A single, low dose of insulin may provide a meaningful index of insulin sensitivity in horses, and from the current results, only 2 blood samples would need to be collected (0 and 40 min relative to insulin injection). Further studies are needed to confirm that known factors affecting insulin sensitivity (e.g., diet, adiposity, exercise, and anti-diabetic drugs) affect the response to the low dose of insulin in a similar manner.

SUMMARY AND CONCLUSIONS

The goal of the research herein was to investigate the hyperinsulinemic-hyperleptinemic syndrome in horses. This was done via assessing methods of diagnosis and evaluating differences in plasma metabolites to develop relationships between leptin concentration, insulin sensitivity, and other metabolites. A dichotomy of horses is apparent in the native herd at LSU based on leptin and insulin concentrations in plasma. Based on these known characteristics, three experiments were conducted to further elucidate the relationships and repercussions of these afflictions.

The first study concluded that a single injection of DEX did not result in a more lucid approach to differentiating hyperleptinemic horses from normal ones, although DEX administration did increase insulin and leptin in the horses. When analyzed with the aid of a frequency distribution, the data were highly-skewed toward higher leptin concentrations rather than bimodal. This distribution may be explained by season or reproductive status in the horse.

The second experiment assessed the viability of the CGIT procedure in evaluating insulin sensitivity in horses. Differences did exist in the horses tested and correlations between insensitivity, BCS, leptin, and insulin concentrations were significant. However, though simple and inexpensive, insulin sensitivity estimates from the CGIT proved to be only moderately repeatable. A simple, accurate and reliable test to be used by clinicians is therefore still needed.

Insulin injections administered in the third experiment at two different dose concentrations were very meaningful. Glucose response curves indicated decreased insulin sensitivity in horses of higher plasma leptin concentration using a 0.2 IU/mL/ kg BW insulin dose. This is the first experiment to report a relationship between leptin and insulin sensitivity in horses of similar BCS using insulin injections. Glucagon concentration remained relatively

stable throughout the experiment in all horses. Plasma concentration of NEFA were suppressed with insulin injections, but were variable and not informative.

Data from these experiments should help diagnose, treat, and understand the role of leptin and insulin in metabolic disturbances. Although obesity is often a central component in the pathogenesis of disease, genetic and nutritional predispositions may also increase susceptibility. Further research is essential to more clearly illuminate the factors involved in the predisposition and manifestation of these hormonal abnormalities.

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VITA

Thomas Joseph Caltabilota is the third child of four to Frank and Joanne Caltabilota. Raised in West Long Branch, New Jersey, Thomas is the younger brother of Tracey and Frank, Jr., and the older brother of Gina. Thomas earned his Bachelor of Science degree from Cook College at Rutgers, the State University of New Jersey. While at Rutgers, Thomas conducted undergraduate research in the field of equine exercise physiology under the tutelage of Dr. Kenneth H. McKeever and graduated a G.H. Cook Scholar in May of 2007. The following August, Thomas began working on a Master of Science degree in animal science at Louisiana State University in Baton Rouge, Louisiana, under the mentorship of Dr. Donald L. Thompson, Jr., in the field of equine endocrinology. He plans to graduate from Louisiana State University with a Master of Science degree in May of 2009. Following graduation, Thomas plans to settle in Pennsylvania to begin a degree program in veterinary medicine.