2007

Equine obesity-related hyperleptinemia

Nan Killen Huff

Louisiana State University and Agricultural and Mechanical College

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EQUINE OBESITY-RELATED HYPERLEPTINEMIA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Animal and Dairy Sciences

by

Nan Killen Huff
B.S., Louisiana State University, 1979
M.S., Colorado State University, 2003
December 2007
ACKNOWLEDGMENTS

“We must believe in ourselves or no one else will believe in us; we must match our aspirations with the competence, courage and determination to succeed.”
Rosalyn Sussman Yalow

So many professors, family, and friends have encouraged me along my personal and academic journey. I am extremely grateful for all of the local and long-distance support I have received while researching and writing this dissertation. Firstly, I want to thank my doctoral advisor, Dr. Donald L. Thompson, Jr., for his supervision and direction. Secondly, I want to thank the professors who served on my Doctoral Committee: Dr. Kenneth R. Bondioli, Dr. Robert A. Godke, Dr. Dale L. Paccamonti, Dr. Cathleen C. Williams, and Dr. Tin-Wein Yu, for good teaching and intellectual challenge. Thirdly, I want to thank Dr. Laura Gentry for her immense help, encouragement, and advice.

I have also received significant emotional support and advice from my fellow graduate students Bill Storer, Josh Cartmill, Pam Mitcham, Kristian Kelly, Cara Waller, and Maxon Graham. On many occasions, each of them offered the brilliant idea, the helpful suggestion, or the coldest beer. All members of my work family at the LSU School of Veterinary Medicine have also been exceptionally supportive and encouraging. A special thank-you to Leslie Talley, Claudette Leake, Catherine Koch, Frank Garza, Dr. Ashley Stokes, Mike Keowen, Dr. Rustin Moore, Dr. Susan Eades, and Dr. Britta Leise.

My husband, Jim, has unwavering faith in me. He has carried more than half the burden in terms of his emotional support, and I cannot thank him enough for the encouragement that he has given me. Our daughter, Jimmie, has always believed in me and has been remarkably tolerant of my absence. Three generations of family have supported me throughout my doctoral program with their enthusiasm and generosity, and I thank them dearly.
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ABSTRACT

Plasma leptin concentrations in obese adult horses have been shown to vary widely, and horses tend to fit into two groups: low leptin (<10 ng/mL) and hyperleptinemic (10 to 50 ng/mL). Observations over time revealed that the hyperleptinemic condition was consistent, possibly indicating a relatively permanent underlying cause. Based on these observations, three experimental approaches were used to further study equine obesity-related hyperleptinemia. The first experiment determined the prevalence of hyperleptinemia among postpartum, lactating mares, evaluated its consequence on their re-breeding success, and investigated correlations between leptin levels in lactating and non-lactating mares. Postpartum mares (n = 198) and non-foaling mares (n = 31) were categorized based on their leptin status: normoleptinemic or hyperleptinemic. Leptin in the lactating mares averaged 4.8 ng/mL, and 11 of the 198 (13%) displayed hyperleptinemia. Leptin in the non-lactating mares averaged 7.5 ng/mL, with 9 mares (29%) displaying hyperleptinemia. Of the 198 lactating mares bred, 81% became pregnant; there was no effect of leptin status on re-breeding success. To study one possible cause for hyperleptinemia in well-fed horses, a second experiment explored polymorphism(s) within exon 2 of the equine leptin gene. The DNA from five hyperleptinemic and five normal mares of high body condition was used to analyze exon 2 of the leptin gene for polymorphisms. Based on the 10 mares tested, there was no polymorphism in exon 2 of the equine leptin gene; therefore, polymorphism is not a likely explanation for the high vs. low leptin difference. The third experiment explored the possible effects of hyperleptinemia on the endocrine and immune systems. Endotoxin was given to mares and geldings to investigate the role and/or regulation of leptin in the pro-inflammatory cytokine response. Of the endpoints measured, only platelet count differed between normal and hyperleptinemic horses. Endotoxin infusion caused the
expected pro-inflammatory cytokine and endocrine responses, but leptin status was not a
significant factor for any endpoint. It is concluded that hyperleptinemia in mares is not
associated with polymorphism in exon 2 of the leptin gene, does not affect re-breeding rates of
foaling mares, and does not alter the endotoxin-induced responses of the endocrine and immune
systems.
CHAPTER 1
INTRODUCTION

Human obesity has become a widespread nutritional disorder in the United States. Moreover, increased body weight is associated with type II diabetes, hypertension, hyperlipidemia, and certain cancers. Although obesity is often considered to be a psychological problem, there is increasing evidence that body weight is physiologically regulated (Zhang et al., 1994). Equine obesity has become a common nutritional and financial concern for horse owners. In the past, horses were able to continuously graze on native grasses and forage in a manner that efficiently stored fat in the summer months. This resourceful storage of energy would sustain the horse through the leaner months of scarce forage (Johnson, 2004). Today, forage is rarely scarce, even for pastured horses. Furthermore, many horses are stalled and grain-fed twice daily, with unlimited hay and water. As a result, the modern horse continuously stores fat throughout the year.

Proper regulation of feed intake is important for optimizing growth, performance, reproduction, lactation, and overall health in the equine species. The present problem of overfeeding horses generally contributes to numerous clinical disorders, including hyperleptinemia, hyperinsulinemia, hyperglycemia, the metabolic syndrome, and laminitis. Therefore, investigation into the basic systems that regulate adiposity, as well as the interactions between those systems, may lead to new knowledge that will further enhance equine performance and health.

Leptin, a 16-kDa protein, has been reported to be primarily derived from adipose tissue, thus circulating concentrations are generally correlated to body fat mass in animal species and humans (Prolo et al., 1998; Chilliard et al., 2000). Similar results have been reported for the
horse, in which body fat mass was estimated by ultrasonography of backfat (Fitzgerald and McManus, 2000) or visually by body condition score (BCS; Buff et al., 2002; Gentry et al., 2002). Additionally, Gentry et al. (2002) reported that leptin concentrations in adult mares with high BCS in September varied widely, and mares tended to fit into two distinct groups based on leptin concentrations: low (<5 ng/mL) or high (7 to 20 ng/mL). Subsequent observations of 18 of those mares indicated that the high versus low distinction was consistently observed 2 yr later, indicating that the underlying cause was likely permanent (Cartmill et al., 2003).

Leptin secretion by adipocytes has been reported to be affected by various other hormones, including insulin (Sivitz et al., 1998; Ramsay and White, 2000), growth hormone (Isozaki et al., 1999), glucocorticoids (Cartmill et al., 2003), epinephrine (Cammisotto and Bukowiecki, 2002), prolactin (Mastronardi et al., 2000), and thyroid hormones (Cartmill et al., 2003). In addition to having a role in endocrine function, leptin also regulates immune functions. Research by Faggioni et al. (1998, 2001) indicated that leptin levels are increased by pro-inflammatory cytokines, such as IL-1, TNF-α, and IL-6. Leptin levels increase acutely during infection and inflammation, and may represent a protective component of the host response to inflammation.

This dissertation sought to improve on the knowledge of the physiologic and genetic differences between horses in good body condition that are hyperleptinemic vs. those with normal plasma leptin concentrations. The first objective was to investigate the occurrence of high leptin mares and the effect of high plasma leptin levels on fertility. In a low leptin environment, GnRH release is decreased, possibly to prevent energy being wasted for reproduction (Caprio et al., 2001). Research conducted by Gentry et al. (2002) revealed that low BCS and leptin concentrations were associated with inactive ovaries during winter and early
spring, although the mares with low BCS did eventually ovulate. In contrast, hyperleptinemia can interfere with gonadotropin stimulation of peripheral targets. Spicer (2001) concluded that in a high leptin environment, the ovary is kept from overproducing estradiol via leptin inhibition of insulin-induced steroidogenesis. Given the possibility that different concentrations of leptin exert both inhibitory and stimulatory actions on the hypothalamic-hypophyseal-gonadal axis, the first investigation was designed to determine any effect of leptin level on the re-breeding capabilities of high versus low leptin mares (as described by Cartmill et al., 2003), as well as to examine the relative leptin concentrations in lactating and non-lactating mares.

The second objective was to consider the occurrence of polymorphisms in the equine leptin gene. The wide variation in leptin values in well-fed horses indicated that some other factor besides body condition affects leptin concentrations in obese horses. Animal studies have shown that polymorphisms associated with fatness exist within the leptin gene. An allelic variation within exon 2 of the leptin gene is associated with increased fat deposition in beef cattle and increased milk output in dairy cattle (Buchanan et al., 2002, 2003). The second experiment was designed to investigate the possible occurrence of an analogous single nucleotide polymorphism in exon 2 of the equine leptin gene.

The third objective was to further consider the high leptin versus low leptin condition, and the interaction of leptin secretion between the endocrine and immune systems. Using the resident herd of horses with high and low plasma leptin concentrations, a slow-infusion, low dose of endotoxin was administered to the horses in order to measure the endocrine response. Hormones measured were leptin, insulin, cortisol, prolactin, and growth hormone; the cytokines measured were TNF-α and IL-6.
CHAPTER 2
REVIEW OF LITERATURE

Discovery of the Leptin Gene

During the summer of 1949, Ann Ingalls made an astounding discovery at the Jackson Laboratory in Bar Harbor, Maine. Ingalls came across some plump, young mice in the research breeding stock of Jackson Laboratory mice on the C57BK/6J background. This early obese model, later designated the \textit{ob/ob} mouse, was observed to gain weight rapidly until the animal was about four times the weight of normal animals. The recessive \textit{ob} gene was also discovered to cause sterility in the homozygote (Ingalls et al., 1950). A \textit{db} (diabetes) mouse was later discovered on the C57BL/KsJ background. The \textit{db} mouse model was similarly obese, but also hyperglycemic (Hummel et al., 1966). Recessive mutations in the \textit{ob} and \textit{db} genes were found to result in obesity and diabetes in a syndrome resembling morbid obesity; \textit{ob/ob} and \textit{db/db} mice have identical phenotypes, each weighing three to four times more than normal mice (Friedman and Halaas, 1998).

The concept of a biological factor in weight regulation has been researched and reviewed for decades. In 1840, a clinician by the name of Mohr gave the first detailed description of hypothalamic obesity. Mohr’s patient displayed a rapid weight gain, and upon autopsy, Mohr found that a tumor had invaded the pituitary gland and hypothalamus. One hundred years later, Hetherington and Ranson (1942) identified the hypothalamus as a principal site in the regulation of body weight. The research team lesioned the ventromedial hypothalamus (VMH) in the laboratory rats without damaging the rat’s pituitary. Subsequently, the lesioned laboratory rats developed hyperphagia and obesity (Hetherington and Ranson, 1942). Anand and Brobeck (1951) suggested that the hypothalamus contained nervous centers which normally control the
intake of food, and that these centers are damaged by hypothalamic lesions. Early research suggested that bilateral lesions in the region of the VMH cause obesity when the animal has free access to palatable food (Hervey, 1959). Of the brain regions implicated in the regulation of feeding behavior, the VMH is considered to be the most important satiety center in the central nervous system (Zhang et al., 1994).

Over the next decade, three theories were introduced to explain a feedback system in relation to the control of an animal’s food intake. The first theory, postulated by Brobeck (1948), proposed that a rise in temperature after eating is the signal for cessation of eating; therefore, regulated feeding is part of body temperature control. Brobeck summarized his hypothesis as “animals eat to keep warm, and stop eating to prevent hyperthermia.” Mayer (1952) proposed the “glucose theory,” suggesting that the difference between the arterial and venous blood levels of glucose represents the key factor in the feedback system for appetite control. Kennedy (1953) suggested that adjustment of feeding is made in relation to the amount of stored fat in the body. Kennedy’s “lipostasis theory” proposed that there was a product of adipose tissue that circulated in the blood and acted as a feedback signal to the hypothalamus. As fat accumulates, appetite is depressed; when fat stores fall below a critical threshold, appetite is increased.

The lipostatic theory was later supported by an experiment carried out by Hervey (1959). Hervey investigated the mechanism and pathways by which normal animals regulate their food intake over long periods and thus maintain energy balance. Hervey performed a series of parabiotic experiments on rats at the University of Cambridge, surgically joining rodents that displayed bilateral lesions of the ventromedial nucleus with rodents that had an intact VMH. In the conjoined circulatory systems, the animal with a damaged hypothalamus rapidly became
obviously obese as a result of hyperphagia, while intact control animals lost body weight due to decreased food intake. Hervey suggested that the partner with a normal hypothalamus became thin because the animal ate less. This result was due to a response on the part of the hypothalamic controlling centers to overfeeding by the operated animals. Hervey’s classic parabiotic experiments supported Kennedy’s lipostasis theory, but the assumed satiety factor still remained unknown (Henson and Castracane, 2003).

Coleman (1973), in his notable parabiosis studies, provided evidence that such a circulating signal exists. Several decades after Ingall’s obese mouse discovery, Coleman (1973) conducted parabiotic studies with ob/ob and db/db mice at the Jackson Laboratory. Both the obesity and diabetic syndromes are characterized by hyperphagia, obesity, hyperglycemia and hyperinsulinemia. Parabiosis of ob/ob with db/db mice caused the obese partner to become hypoglycemic, to lose weight, and to die of starvation, once tissue reserves were exhausted. No abnormal changes were observed in the diabetic partner. Coleman (1973) postulated that the ob/ob mouse is unable to produce sufficient satiety factor to regulate its food consumption, whereas the db/db mouse produces a satiety factor, yet cannot respond to it due to a defective satiety center. The data from the parabiosis experiments suggested that the db gene encoded for, and was therefore was responsible for, the production of a circulating factor that regulated energy balance. The finding was that the ob gene encoded the receptor for this factor.

A team led by Jeffrey Friedman at Rockefeller University published the specific gene defect of ob/ob mice in December of 1994 (Zhang et al., 1994), after an eight-year search. The ob gene, isolated by positional cloning, was found to encode a 167-amino acid secreted protein, including a 21 amino acid secretory sequence. The name of the hormone, leptin, was taken from
the Greek *leptos*, meaning *thin or lean*, and was descriptive of the body weight-reducing quality of the hormone (Henson and Castracane, 2003).

A nonsense mutation in codon 105 of the leptin gene was found in the original C57BL/6J *ob/ob* mouse strain. In the *ob/ob* mouse, a mutation in the leptin gene results in a premature stop codon at position 105, which results in leptin deficiency, hyperphagia, and obesity (Boute et al., 2004). The increased level of *ob* RNA in the C57BL/6J mutant, which has greatly increased fat cell mass, raised the possibility that the level of expression of the *ob* gene signals the size of the adipose depot (Houseknecht et al., 1998).

Although leptin is mainly produced and secreted by white adipocytes, this is not the only potential source of the hormone. Placenta, gastric mucosa, bone marrow, mammary epithelium, skeletal muscle, pituitary, hypothalamus, and bone have also been shown to produce small amounts of leptin in certain circumstances (Frühbeck, 2006). Circulating leptin may be found in free form or complexed with leptin-binding proteins. The half-life of free leptin is about 30 minutes, with the kidneys being responsible for approximately 80% of leptin clearance from the peripheral circulation (Henson and Castracane, 2003).

**Molecular Organization of the Leptin Gene and Receptor**

The leptin gene consists of three exons, which are separated by two introns. The first exon is not transcribed into the leptin protein. The first 21 amino acids of the leptin gene function as a signal peptide, and are cleaved off before the 146 amino acid protein is released into the blood as a circulating protein (Zhang et al., 1997). Leptin has a 67% sequence identity among the humans, gorillas, chimpanzees, orangutans, rhesus monkeys, dogs, cows, pigs, rats, and mice. Leptin shares many structural similarities to other helical cytokines, including IL-6 and growth hormone (Henson and Castracane, 2003).
Modeling of the leptin structure is difficult as the wild type aggregates extensively in vitro, so it cannot readily be crystallized. Zhang et al. (1997) substituted glutamine for tryptophan at position 100, which resulted in a protein (E-100) with comparable biological activity. The leptin E-100 mutation was crystallized to determine the molecular structure. The leptin protein is an elongated molecule with approximate dimensions of 20 x 25 x 45 Å. Leptin consists of four anti-parallel α-helices (A, B, C, and D), connected by two long crossover loops (AB and CD) and one short loop (BC), arranged in a left-handed, twisted, helical bundle. The four-helix bundle takes an unusual up-up-down-down fold, which forms a two-layer packing of anti-parallel helix pairs A and D against B and C. A large, hydrophobic, cylindrical core, parallel to the helical bundle axis, is formed from the mostly conserved residues of the four α-helices that face one another (Zhang et al., 1997).

An interesting feature of leptin is the small, helical, segment E, found in the CD loop and packed tightly against the helical bundle. The E helix is distorted, bending sharply in the middle, and almost perpendicular (87 degrees) to the helix bundle. Helix E serves as a hydrophobic cap to bury the lipophilic residues on the surface of the BD helical bundle (Zhang et al., 1997).

An intrachain disulfide bond maintains stability of the native state folded protein, and is essential for bioactivity. Although disulfide bonding patterns are not highly conserved within the cytokine family, it seems that leptin’s single disulfide bridge is critical for the development of its helical cytokine folding. The leptin protein contains two cysteine residues, Cys 96 and Cys 146, which form a disulfide bond between the C-terminus of the protein and the beginning of the CD loop. Since the disulfide bond is exposed to solvent at one end, it moves the last turn of the D helix 36 degrees towards the CD loop, causing a kink in the D helix. The distinctive kink may serve to maximize the close contact area between the helices in the structure. Both of the
cysteines in leptin are conserved among species. Mutation of either renders the protein biologically inactive, indicating that the disulfide bridge and the kink in the D helix are essential for proper folding and receptor binding (Zhang et al., 1997). In \textit{ob/ob} mice, this intrachain disulfide bond cannot be formed, due to the appearance of a premature stop codon at position 105 (Boute et al., 2004). Research conducted by Boute et al. (2004) demonstrated that lack of the disulfide bond is sufficient to impair leptin secretion, because that lack results in accumulation of macromolecular aggregates in the cell.

The crystal structure of leptin-E100 suggests that leptin may be classified in the long-chain, helical, cytokine family, inclusive of IL-6, IL-11, and IL-12. Although the information about leptin’s structure places it in the long-chain, helical, cytokine category, several distinguishing characteristics of leptin remain that fall into no category. Other long-chain, helical cytokines have extra helices in the AB loop, whereas leptin has a distorted E helix in the CD loop. For example, Helix B in other cytokines is two turns longer than that of leptin; furthermore, other helices in cytokines have kinks in the middle, rather than at the end, similar to leptin. Understanding these differences and the other distinguishing characteristics of leptin provides insight into its specific function (Zhang et al., 1997).

\textbf{Leptin Receptor.} Parabiosis studies with \textit{ob/ob} and \textit{db/db} mice indicated that \textit{db/db} mice were defective in reception of the \textit{ob} gene product signal (Coleman, 1973). Later studies by Tartaglia et al. (1995) led to the identification of the leptin receptor from mouse choroid plexus, using an expression-cloning strategy. In the \textit{db/db} mouse, the mRNA for the long form of the receptor is abnormal and yields a receptor with a truncated, intracellular domain that is unable to appropriately signal, thus confirming the prediction made by Coleman, based upon his classic parabiosis studies (Houseknecht et al., 1998).
The leptin receptor (OB-R), is a member of the class I cytokine receptor superfamily, and is closely related to: a) gp130, the signal-transducing membrane protein of the IL-6 signaling complex, b) the leukemia inhibitory factor (LIF) receptor, and c) the granulocyte colony-stimulating factor (G-CSF) receptor (Tartaglia et al., 1995). Members of this family have characteristic extracellular motifs of four cysteine residues and two highly conserved cytokine domains with the sequence motif of WSXWS (Trp-Ser-Xaa-Trp-Ser), containing a different number of fibronectin type III domains (Frühbeck, 2006).

Leptin acts through its receptor, which has six isoforms. The OB-R is a glycoprotein with a single transmembrane-spanning region (Tartaglia, 1997). All isoforms share an identical extracellular ligand-binding domain, with the characteristic motif of four cysteine residues and WSXWS. Five isoforms possess transmembrane and cytoplasmic regions; one is a circulating, soluble isoform.

The soluble leptin receptor circulates in the blood and can bind leptin with a high affinity. Thus, it plays a role in regulating the plasma levels of free leptin, the biologically active form. In obese individuals, soluble receptor levels are reduced, whereas the receptor is up-regulated in emaciation (Hegyi et al., 2004).

The membrane bound isoforms consist of a triad of distinct domains: a) an extracellular, leptin binding domain, b) a transmembrane domain, and c) an intracellular, cytoplasmic tail (Henson and Castracane, 2003). The transmembrane and proximal 29 intracellular amino acid residues, including an intracellular proline-rich “box1” motif, are the same in all forms. The intracellular, cytoplasmic domain of the leptin receptor varies in length with each isoform. Membrane bound isoforms originate from a single mRNA transcript and are produced as a result of alternative splicing. Amino acid 29 of the intracellular domain provides the point of
divergence. The OB-Rb form contains about 303 intracellular amino acids, whereas the short forms OB-Ra, OB-Rc and OB-Rd have 34, 32, and 40 amino acids, respectively (Henson and Castracane, 2003).

The leptin receptor is present in many tissues. In fact, there are very few tissues that do not express mRNA for at least one isoform of the leptin receptor. This would indicate that many tissues and organs are primed for leptin action, and that the polypeptide may exert physiological influences via paracrine mechanisms. However, the hypothalamus constitutes a high expression of OB-Rb (Henson and Castracane, 2003; Frühbeck, 2006).

Since only the long form (OB-Rb) has both the box1 and box2 motifs, as well as three intracellular tyrosine residues, this seems to be the functional signaling isoform (Tartaglia, 1997; Henson and Castracane, 2003; Hegyi et al., 2004), and is thought to be responsible for the central actions of leptin (Tartaglia et al., 1995). Tyrosine residues are located at positions 985, 1077, and 1138, and are conserved among known species of long-form leptin receptors (Bjørbæk et al., 2001). The OB-Ra form is present at the blood brain barrier (BBB), with the highest levels in the choroid plexus and in microvessels of the BBB (Tartaglia et al., 1995; Frühbeck, 2006). Research has shown that leptin enters the brain by a specific mechanism, independent of insulin (Banks et al., 2000), and it seems that OB-Ra plays a modulating role in transporting leptin across the BBB (Tartaglia et al., 1995; Banks et al., 2000). The OB-Rc form was found in brain tissues taken from cortex and cerebellum. This indicates that OB-Rc might also function as a leptin transporter in the brain, as either a different or additional function to OB-Ra. The OB-Re form, a circulating and soluble receptor, lacks the transmembrane and cytoplasmic parts, and therefore functions as a binding protein for leptin. A change in OB-Re levels regulate, in part, the biological activity of leptin in the circulation (Frühbeck, 2006).
Receptor Pathways

A complex network of interacting signaling pathways appears to regulate food intake, fuel balance, and body weight. Leptin receptors, members of the class I cytokine receptor superfamily, do not have activity inside the cell; therefore, the leptin receptor does not have endogenous kinase activity. Cell-based in vitro studies and in vivo experiments have led to a relatively detailed understanding of intracellular signaling regulation by the leptin receptor. Four important and different pathways include JAK/STAT, MAPK, PI-3K, and AMPK (Bjørbaek and Kahn, 2004).

**JAK/STAT.** Early recognition of OB-R as a member of the cytokine receptor superfamily resulted in the prompt identification of the JAK/STAT pathway as one of the main signaling cascades activated by leptin (Hegyi et al., 2004; Frühbeck, 2006). Leptin signaling via the JAK/STAT pathway has been well documented and is associated largely with the OB-Rb isoform (Tartaglia, 1997; Houseknecht et al., 1998).

The JAK/STAT pathway consists of four non-receptor tyrosine kinases (JAK) and seven 85 to 95 kDa transcription factors (STAT) that are regulated by phosphorylation on specific serine and tyrosine residues (Frühbeck, 2006). The pathway enables the leptin hormonal signals, which are not fat soluble, to penetrate the cell membrane and be delivered to the cell nucleus to affect gene expression. The JAK/STAT is a simple pathway with two major players: janus kinase, a family of intracellular tyrosine kinases; and signal transducers and activators of transcription proteins. The STAT proteins are initially present in inactive forms in the cytoplasm, while JAK are constitutively associated with the cytoplasmic membrane-proximal regions of various receptors (Frühbeck, 2006).
Of the four known members of the JAK family, JAK1, JAK2, and tyrosine kinase2 (TYK2) are widely expressed, while JAK3 is found only in cells of the hematopoietic immune systems. The OB-R does not have an intrinsic tyrosine kinase domain, and therefore binds cytoplasmic kinases, mainly JAK2. Box1 and Box2 motifs are known to recruit and bind JAK. However, for leptin signaling, it was reported that only Box1 and the immediate surrounding amino acids are essential for JAK activation. The intracellular domain of all OB-R isoforms contains the Box1 JAK-binding domain, whereas OB-Rb also includes the Box2 motif and specific binding sites (Frühbeck, 2006). Tyrosine phosphorylation sites provide binding motifs for src homology 2 (SH2)-domain containing proteins, such as STAT (Bjørbæk et al., 2001).

Once bound to the leptin receptor, JAK become catalytically activated. At the same time, tyrosine residues in the cytoplasmic domain of the leptin receptor become phosphorylated at amino acid residues Tyr985 and Tyr1138 (Banks et al., 2000). This phosphorylation leads to the recruitment of STAT proteins via recognition of the receptor phosphotyrosines by the STAT SH2 domains. The function of SH2 domains specifically recognizes the phosphorylated state of tyrosine residues. STAT3 proteins bind to phosphorylated TYR1138, become tyrosine-phosphorylated by JAK2 (Bjørnæk et al., 2000), then dissociate and form dimers in the cytoplasm. Finally, the cytoplasmic dimers translocate to the nucleus to regulate gene transcription (Hegyi et al., 2004; Frühbeck, 2006).

The JAK/STAT signaling pathway can be inhibited by a suppressor of cytokine signaling-3 (SOCS-3) molecule, which was the first identified negative regulator of leptin signaling. This molecule is a member of the SH2 domain-containing protein family, which operates by binding to the phosphorylated tyrosine residues of signaling molecules and thus mediates either degradation or inhibition. Bjørnæk et al. (2000) suggested that SOCS-3, via its
SH-2 domain, binds to Tyr985 of OB-Rb and this residue is required for maximal inhibition of signaling. There is also evidence that SOCS-3 acts via a direct binding to JAK2. Bjørbæk et al. (2000) further suggested that the Tyr985/SOCS-3 interaction mediates leptin-induced feedback inhibition of OB-Rb. Based on the evidence, Bjørbæk et al. (2000) hypothesized that over-reactivity of the SOCS pathway may be a potential, causal mechanism for leptin-resistant obesity.

Mitogen Activated Protein Kinase (MAPK). Signal transduction by members of the class I cytokine receptor superfamily is not limited to the JAK/STAT pathway. Some receptors are linked to the MAPK pathway, which is downstream from JAK (Houseknecht et al., 1998).

The MAPK are serine/threonine-specific protein kinases that regulate gene expression. Although the distal portion of OB-R is not essential for MAPK signaling, the intact, intracellular portion of the long receptor is needed to achieve maximal activation. The MAPK pathway can be stimulated by either OB-Rb or OB-Ra, but to a lesser extent by the latter (Banks et al., 2000). Ligand binding to OB-Rb leads to activation of the MAPK cascade via two pathways: 1) binding of SHP-2 to Tyr985, followed by tyrosine phosphorylation of SHP-2; or 2) independently of tyrosine phosphorylation of OB-Rb, which requires the phosphatase activity of SHP-2. Both pathways require an intact catalytic domain of SHP-2 (Bjørbæk et al., 2001; Hegyi et al., 2004).

Phosphoinositol-3 Kinase (PI3-K). Leptin can also act through some of the components of the insulin-signaling cascade, although there are conflicting reports concerning its importance in modifying insulin-induced gene expression (Hegyi et al., 2004). Many researchers support the idea that leptin and insulin pathways may be connected (Frühbeck, 2006; Hegyi et al., 2004), although the exact mechanisms by which OB-Rb activates PI3-K and the cAMP pathway are unknown (Bjørbæk and Kahn, 2004).
5’-AMP-Activated Protein Kinase (AMPK). Recent research indicated that the AMP kinase pathway is involved in leptin’s effect on food intake (Bjørbæk and Kahn, 2004). This enzyme is a heterotrimer that functions to monitor cellular energy status; it regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Activation of AMPK represents a signal to shut down anabolic pathways and to promote catabolic processes in response to a decrease in the ATP/AMP ratio by phosphorylation of key enzymes of intermediary metabolism. It is not yet clear how leptin increases AMP levels and activates AMPK (Hegyi et al., 2004; Frühbeck, 2006).

Diurnal Profile

Leptin concentrations in the peripheral circulation exhibit a 24-h rhythm in both children and adults, with peak levels in the early morning hours and a nadir in the late morning or early afternoon (Licinio et al., 1998). Other studies in humans (Licinio et al., 1998), rodents (Saladin et al., 1995), and horses (Piccione et al., 2004) demonstrated a significant overnight increase in plasma leptin concentrations. This circadian pattern has been found in lean and obese subjects, with the only exception being pulse amplitude, which is higher in obese subjects.

The circadian rhythm of leptin is similar to that of prolactin (Matkovic et al., 1997), but inversely related to that of ACTH and cortisol (Licinio et al., 1997). Thyrotropin (TSH) rhythms have been observed to be similar to that of leptin (Mantzoros et al., 2001). Leptin pulsatility is synchronous to the pulsatility of serum luteinizing hormone (LH) and estradiol levels in normal women, especially during the night when leptin levels are relatively high (Licinio et al., 1998).

Several studies have shown that increases in plasma leptin can be attributed to feeding in humans (Schoeller et al., 1997), sheep (Marie et al., 2001), and horses (Cartmill et al., 2003). Cartmill et al. (2003) showed that increases in plasma leptin concentration occur 8 h after
increases in insulin, after a single morning meal in horses. Additional observations from the same laboratory indicated that serum leptin concentrations increase postprandially in the equine (Cartmill et al., 2005; Crowley et al., 2005; Storer et al., 2005), perhaps stimulated by a rise in serum insulin concentration. Similarly, Gordon and McKeever (2005) showed that plasma leptin concentration increased approximately 7 h after an observed increase in plasma insulin concentration. Cartmill et al. (2005) further showed that shifting the time at which stallions receive the concentrate portion of their diet 12 h will also shift the peak in concentrations of leptin approximately 12 h and is most likely a direct result of increasing insulin concentrations following the meal. Similarly, it has been reported by Gentry et al. (2002) that mares in low body condition allowed to graze only 2 h each morning have a similar fluctuation in plasma leptin, further indicating that meal feeding may result in a diurnal leptin secretory pattern. Increased plasma leptin concentration noted in the afternoon could be attributed to the morning feeding, with its subsequent insulin response (Gordon and McKeever, 2005).

Piccione et al. (2004) reported a daily rhythm of leptin in both athletic and sedentary horses, with a daytime trough and a dark-phase peak. Food deprivation did not abolish the daily rhythm of serum leptin, but daily mean leptin levels in fasted horses were significantly lower than in regularly fed horses (Piccione et al., 2004). In other research, high-body-condition mares and geldings (Gentry et al., 2002; Cartmill et al., 2003), free to graze throughout the day, did not exhibit diurnal changes in plasma leptin. Sheep that grazed and ruminated continuously were found to have no nocturnal increases in plasma leptin concentration (Marie et al., 2001). Although the horse is not a ruminant, the role of grazing and hindgut fermentation supports speculation that a more stable, continuous uptake of energy rather than fluctuations associated
with bolus feeding could be responsible for the relatively constant nature of plasma leptin concentrations (Gordon and McKeever, 2005).

**Leptin and Nutrition**

In many species, including the horse, leptin circulates in the blood at concentrations paralleling the amount of adiposity. Circulating concentrations of leptin are directly correlated to body mass index and percentage of body fat in humans (Prolo et al., 1998; Mantzoros et al., 2000; Agarwal et al., 1999) and in ruminants (Chillard et al., 2000; Delavaud et al., 2000). Studies with horses (Kearns et al., 2005; Buff et al., 2002; Ferreira-Dias et al., 2005) and sows (Estienne et al., 2000) also reveal that peripheral concentrations of leptin are proportional to adiposity.

Inadequate nutrition and/or reduced body fat are associated with impaired reproductive efficiency in beef cattle (Selk et al., 1988; Schillo et al., 1992) and sheep (Schillo, 1992). A similar relationship has been noted for horses (Henneke et al., 1983; Gentry et al., 2002). Mares entering the breeding season or foaling in a nutritionally deprived state had prolonged postpartum intervals and reduced conception rates, and required more cycles per conception than mares entering the breeding season in fatter condition (Henneke, 1983). Research by Gentry et al. (2002) concluded that nutrient restriction resulting in low BCS in mares resulted in a profound seasonal anovulatory period that was accompanied by lower leptin levels.

Body weight influences fertility in mice; studies have indicated that leptin is one of the mediators of this effect (Greisen et al., 2000). In mares in good body condition, leptin secretion increases from the anovulatory period to the first follicular and luteal phases of the breeding season (Ferreira-Dias et al., 2005). These observations are consistent with previous findings by Gentry et al. (2002), who concluded that in mares with low body condition had low leptin levels,
and both were accompanied by a deep seasonal anovulatory period. In the Lusitano mare, a transition from ovarian activity to a seasonal anovulatory state is preceded by a decrease in plasma leptin concentrations. Resumption of ovarian activity seems to require that leptin rises again to a threshold level (Ferreira-Dias et al., 2005).

In contrast to low leptin concentrations, chronically persistent hyperleptinemia, as seen in obesity, can interfere with gonadotropin stimulation of peripheral targets. In obesity, leptin central receptors, which are sensitive to extremely low ligand concentrations, are protected from hyperleptinemia by the saturable transport system of the blood-brain barrier, whereas peripheral leptin receptors are directly exposed to high ligand concentrations, with possible negative effects on gonadal steroidogenesis (Caprio et al., 2001). Spicer (2001) suggested that, in a high leptin environment, as observed with obesity, the ovary is kept from over-producing estradiol by leptin’s inhibition of insulin-induced steroidogenesis. Leptin has been shown to inhibit insulin-induced androstenedione production by bovine thecal cells in vitro (Spicer, 2001).

**The Hypothalamic-Pituitary-Gonadal Axis**

**The Estrous Cycle.** The estrous cycle of the mare is divided into the follicular phase and the luteal phase (McKinnon and Voss, 1993). Estrus is the period of sexual receptivity (Higgings and Snyder, 2006). The average length of the estrous cycle in the mare population during the physiologic breeding season is 21 d, with estrus comprising 5 to 7 of these days (McKinnon and Voss, 1993).

**The Follicular Phase.** The follicular phase is characterized by follicular growth with estrogen production. The dominant follicle develops and secretes estradiol-17β, which induces sexual receptivity. Estradiol reaches peak concentrations of greater than 30 to 40 pg/mL 2 to 3 d before ovulation (Sharp and Porter, 2004) and is thought to contribute to the final maturation of
the pre-ovulatory follicle by stimulating further LH release. A preovulatory LH surge develops progressively over approximately a week, beginning near deviation and extends for an average of 1 d after ovulation; the LH surge then gradually recedes over several days (Ginther et al., 2005). Ovulation, the release of the oocyte, occurs approximately 24 to 28 h before the end of sexual receptivity (McKinnon and Voss, 1993).

**The Luteal Phase.** The luteal phase is initiated at ovulation by the formation of a progesterone-secreting corpus luteum (CL). Elevated concentrations of plasma progesterone are detected within 24 h and estrus behavior ceases (Higgins and Snyder, 2006). Maximum circulating progesterone concentrations are reached by 6 d postovulation and then plateau. Hypothalamic GnRH is suppressed by the negative feedback action of progesterone. The life span of the CL depends on the endogenous release of prostaglandin-F2α (PGF2α), released in bursts from the endometrium between d 13 and 16 postovulation. The PGF2α is absorbed into the uterine venous drainage, enters the circulation, and reaches the ovaries by a systemic route. Rapid luteolysis of the CL is caused by PGF2α, resulting in a decline in circulating progesterone concentration. This decrease in circulating progesterone removes negative feedback on the pituitary and hypothalamus; consequently, plasma concentrations of GnRH increase and the mare returns to estrus (Higgins and Snyder, 2006).

**Gonadotropin Releasing Hormone.** In the horse, GnRH is relatively evenly distributed throughout the hypothalamus. The release of GnRH into the hypothalamic-hypophyseal portal system stimulates the synthesis and release of LH and follicle-stimulating hormone (FSH) from the adenohypophysis. Although the major function of GnRH is to stimulate gonadotropin secretion, it also stimulates production of its own receptor. Inhibitory influences on GnRH secretion include corticotropin-releasing factor and progesterone (McKinnon and Voss, 1993).
**Follicle Stimulating Hormone and Luteinizing Hormone.** The two gonadotropins, LH and FSH, enter the systemic circulation and travel to the ovary, where FSH is responsible for follicular recruitment, while LH is responsible for follicular maturation and production of estrogen, ovulation, and luteinization of the CL. Receptors for FSH are found mainly on the granulosa cells in ovarian follicles. Receptors for LH are confined to the thecal cells and other types of ovarian interstitial cell. However, after antrum formation, LH receptors also appear on granulosa cells. Both antrum formation and the induction of LH receptors on granulosa cells require the presence of FSH and estradiol. As the follicle matures, theca and granulosa cells cooperatively secrete estradiol (McKinnon and Voss, 1993).

In the mare, LH concentrations are low during the mid-luteal phase, but rise in a prolonged ovulatory surge, beginning a few days before the onset of estrus, peaking on the day after ovulation, and returning gradually over several days to mid-luteal phase values. Unlike LH, FSH shows a biphasic profile during the cycle, with surges at 10 to 12 d intervals. One surge occurs at or just after ovulation, and the second surge occurs during mid- to late-luteal phase, approximately 10 d before the next ovulation. In early estrus, FSH concentrations reach their lowest value in the cycle (McKinnon and Voss, 1993).

**Inhibin.** Inhibin is a glycoprotein hormone, produced predominantly by granulosa cells of the ovary. The major physiologic role of inhibin is the specific suppression of FSH secretion from the adenohypophysis (Roser et al., 1994; Ginther et al., 2005). The production of inhibin is stimulated by FSH, thereby forming a negative feedback circuit. In the mare, inhibin may not be the sole inhibitor of FSH, but likely acts synergistically with estradiol (McKinnon and Voss).

**Progesterone.** Progesterone is secreted by the CL, and can act at either the hypothalamus or pituitary to influence gonadotropin secretion. Progesterone inhibits GnRH release.
Concentrations of progesterone rise after ovulation and maintain high levels until approximately 3 d before estrus, when the CL is lysed (McKinnon and Voss).

**Estradiol.** Estradiol can act at either the hypothalamus or pituitary to influence gonadotropin secretion. Estrogen activates GnRH release. When estradiol reaches a certain threshold level, large quantities of GnRH cause the release of large quantities of LH that stimulate ovulation (Senger, 2003). Estradiol is produced by the maturing ovarian follicle. Estradiol begins to increase 6 to 8 d before ovulation, rising progressively to peak about 2 d before ovulation. High estradiol concentrations initially inhibit LH, but then trigger an ovulatory LH surge. Most research suggests that the inhibitory effect of estradiol is exerted directly on the gonadotropes (McKinnon and Voss, 1993).

**Insulin-like Growth Factor-I.** In mares, plasma insulin-like growth factor I (IGF-I) increased continuously during late gestation, peaked 2 d after parturition, and then gradually declined until weaning. Circulating IGF-I in mares was maintained at high concentration during early lactation, before decreasing (Hess-Dudan et al., 1994). Research by Heidler et al. (2003) revealed that the highest IGF-I levels were found in lactating mares in the week of foaling, followed by a continuous decrease in IGF-I concentrations. Heidler et al. (2003) suggested that increased IGF-I concentrations during early postpartum might contribute to ovarian stimulation. This may explain why most mares ovulate reliably in foal heat.

**Leptin.** Leptin has emerged as a potential regulator of reproduction (Spicer, 2001). Leptin can affect the hypothalamic-pituitary-gonadal axis by direct actions in the hypothalamus, the pituitary gland, and the gonads (Mantzoros et al., 2000; Spicer, 2001). In the hypothalamus, leptin stimulates the release of GnRH; in the pituitary, leptin stimulates LH and FSH release; and
in the ovary, leptin impairs the IGF-I mediated augmentation of estradiol-17β synthesis by granulosa cells (Licinio et al., 1998).

The effect of leptin on the reproductive system is primarily mediated by its hypothalamic receptor, eliciting the release of GnRH, which subsequently induces the synthesis and release of pituitary FSH and LH. Recent research demonstrated that leptin induces the release of GnRH through inhibition of hypothalamic neuropeptide Y (NP-Y) production. Research in healthy women also indicates that serum leptin rises during the follicular phase, and then reaches a peak at the luteal phase of the reproductive cycle - a finding that strongly links leptin with ovulation (Barkan et al., 2004; Spicer, 2001).

During pregnancy, especially in the second and third trimesters, serum leptin levels rose in both animal models and humans, and dropped sharply after parturition (Caprio et al., 2001). Placental production of leptin may be a major contributor to the increase in maternal leptin; leptin receptors exist in placental tissues and may regulate metabolism of the fetal-placental unit (Spicer, 2001).

Research with dairy cows showed that the plasma concentration of leptin decreased by approximately 50% after parturition and remained depressed during lactation, despite a gradual improvement in energy balance. The reduction could benefit early lactating dairy cows by promoting a faster increase in feed intake and by diverting energy from non-vital functions such as reproduction (Block et al., 2001). In mares, plasma leptin concentrations decreased after foaling, and for 4 wk were lower in lactating than in non-lactating mares. Reduced leptin concentrations may promote feed intake and allow lactating mares to avoid an energy deficit (Heidler et al., 2003). In contrast to cattle, most lactating mares resume follicular growth and ovulation in early postpartum (Ginther, 1993), and ovulation is not impeded by suckling or the
presence of a foal. The majority of foaling mares show estrous behavior and ovulate within 15 days after foaling (Neuschaefer et al., 1990), therefore lactational anestrous does not exist as a principal physiological condition in the horse (Heidler et al., 2004). Lactation, on average, is not associated with weight loss in mares, as the mare seems able to compensate energy losses mainly by increasing feed intake, rather than by mobilization of body reserves. Recent research by Heidler et al. (2004) suggested that a negative energy balance during lactation in most horse breeds may be prevented by adequate feeding. The reduced leptin concentrations in postpartum mares may therefore encourage feed intake, which would allow the lactating mare to avoid an energy deficit.

Leptin seems to have both a central stimulatory and a peripheral inhibitory effect on ovarian function. Much of the available data indicates that leptin acts centrally to stimulate pituitary gonadotropin secretion (Greisen et al., 2000). In contrast, peripheral leptin receptors, directly exposed to high ligand concentrations, can have negative effects on gonadal steroidogenesis (Spicer, 2001). Caprio et al. (2001) hypothesized that a specific and narrow range of leptin concentrations is necessary to maintain a normal, reproductive function in both sexes, and that concentrations below or above these thresholds might interfere in opposing ways with the function of the hypothalamic-pituitary-gonadal axis.

Leptin is present in follicular fluid, and the receptor is expressed in the ovaries of the human (Greisen et al., 2000; Mantzoros et al., 2000) and the cow (Spicer and Francisco, 1998). The identification of OB-R expression in the ovary indicates that leptin might have a direct effect on downstream endocrine targets of the reproductive axis. Both ovarian granulosa and theca cells have high affinity receptors for leptin (Spicer, 2001). There seems to be no physiologically important difference between circulating leptin levels and follicular fluid leptin concentrations.
(Agarwal et al., 1999; Bützow et al., 1999). However, research by Mantzoros et al. (2000) found that lower follicular fluid leptin concentrations were a marker of assisted reproduction treatment success in normal women, indicating that high leptin concentrations in the ovary may suppress estradiol production and interfere with the development of dominant follicles and oocyte maturation.

Spicer and Francisco (1998) found that insulin and IGF-I represent potent stimulators of theca cell proliferation and steroidogenesis. Research with rats (Hernandez et al., 1988), pigs (Caubo et al., 1989), and humans (Bergh et al., 1993) confirmed a role for IGF-I and insulin as regulators of steroidogenesis by theca cells. Insulin and IGF-I enhanced FSH-induced aromatase activity in cultured bovine and rat granulosa cells, in part, by increasing numbers of FSH receptors and mRNA for the FSH receptor. Similarly, insulin and IGF-I also enhance LH-induced androstenedione production by bovine thecal cells by increasing the number of LH receptors (Stewart et al., 1995; Spicer et al., 2002).

Further studies by Spicer and Francisco (1998) indicated that leptin inhibits insulin-induced progesterone and androstenedione production by theca cells. In vitro studies, conducted on theca and granulosa cells, showed that leptin has a negative effect on ovarian steroid output. Human research by Agarwal et al. (1999) indicated that in cultured granulosa cells, leptin had no effect on estradiol production, alone or in the presence of FSH, but caused a concentration-related inhibition of insulin-like growth factor I (IGF-I) augmentation of FSH-stimulated estradiol production. Consistent with these findings, it has been shown that the incubation of granulosa cells from fertile women with leptin, at concentrations between 10 and 100 ng/mL, inhibits FSH- and IGF-1-stimulated estradiol production. Greisen et al. (2000) suggested that leptin acts directly to inhibit basal and FSH-stimulated estradiol and progesterone production in
cultured human granulosa cells. These observations indicate that leptin, at concentrations commonly found in obese women, has the potential to interfere with estradiol production by the dominant follicle in vivo, either directly or through a reduction of the androgenic substrate derived by theca cells. If elevated leptin levels interfere with the development of the dominant follicle and reduce estradiol production, there would be no adequate stimulus for LH secretion, which would result in no ovulation (Caprio et al., 2001; Greisen et al., 2000).

The aforementioned research indicates that high physiological concentrations of leptin could significantly interfere with the ability of the dominant follicle to produce estradiol, both by inhibiting the production of androgen substrate and by decreasing the aromatizing capacity of granulosa cells. Such an effect could result in an inadequate stimulus for the LH surge and may also lead to an immature preovulatory follicle, or in extreme cases, no preovulatory follicle at all. This type of mechanism is consistent with clinical observations that obese women have a higher incidence of infertility, and that weight loss often causes significant clinical improvement (Agarwal et al., 1999).

Kitawaki et al. (1999) reported that a leptin concentration of 1 ng/mL augments basal, FSH and FSH- and IGF-I-stimulated aromatase activity, and estrogen production in human granulosa luteal cells. Greisen et al. (2000) reported an inhibitory effect of leptin on basal and FSH-stimulated progesterone production at 20 ng/mL, whereas 100 ng/mL had no significant effect. Leptin, in a concentration of 30 ng/mL, may impair basal, but not FSH-stimulated, progesterone production in bovine granulosa cells in vitro (Spicer and Francisco, 1997).

Most immunologic, hormone, and cytokine receptors respond to stimulation with a bell-shaped dose-response curve, and this may well be the case for the inhibitory actions of leptin. In a study by Zarkesh-Esfahani et al. (2000) on the effects of a high dose of growth hormone, it was
shown that high concentrations of growth hormone actually prevented dimerization of the receptors, as well as signaling (Waters et al., 2006). Both OB-R and the growth hormone receptor (GHR) belong to the cytokine receptor superfamily and are mediated by JAK2 upon ligand-receptor interaction. In responsive cells, receptor dimerization is the rate-limiting step in GH signaling. Yet a lesser number of available JAK2 molecules may also make activation of JAK2 into a rate-limiting step. In cells where the availability of the effector kinase (JAK2) is high, the effect will become a bell-shaped, dose-response curve, due to complete dependency upon receptor dimerization (Ridderstråle, 2005). It is possible that the overall effect of leptin may depend on the concentration in the circulation. The central stimulatory role of leptin seems to be predominant in non-obese states, where leptin may play a permissive role for both sexual maturation and reproductive function. The direct negative peripheral action of leptin on the ovary may be expressed during conditions of elevated circulating levels, such as in obesity (Greisen et al., 2000).

The Hypothalamic-Pituitary-Adrenal Axis

The end result of the hypothalamic-pituitary-adrenal (HPA) axis activation is a rise in plasma corticosteroid concentrations (Alexander and Irvine, 1998). The HPA axis is a key component of the host response to sepsis. During an acute illness, such as sepsis, circulating pro-inflammatory cytokines, including IL-6 and TNF-α, stimulate the production of corticotrophin-releasing hormone (CRH) and subsequently adrenocorticotropic hormone (ACTH).

Upon ACTH stimulation, glucocorticoids are synthesized by the adrenal cortex from cholesterol. A small amount of corticosterone is stored as a sulphate conjugate in the adrenal cortex. However, the amount of glucocorticoid found in adrenal tissue is not sufficient to
account for the initial rise in cortisol that occurs following stress, and it is not sufficient to maintain normal rates of secretion for more than a few minutes in the absence of continuing biosynthesis. In other words, any disruption in glucocorticoid synthesis will immediately result in glucocorticoid insufficiency (Prigent et al., 2004).

Adrenocorticotrophic hormone increases cortisol release from the adrenal glands, which then binds to a specific carrier, cortisol-binding globulin (CBG), which is synthesized by the liver. The percentage of CBG-bound corticosteroid ranges between 67 and 87% in several domestic species, including the horse (Gayrard et al., 1996). Elastase, produced by neutrophils, liberates cortisol from CBG, allowing localized delivery of cortisol. Then, cortisol can freely cross the cell’s membrane, or it may interact with specific membrane binding sites (Prigent et al., 2004).

Tissue levels of cortisol are also regulated by enzymatic conversion of cortisol to its inactive form, cortisone, by 11β-hydroxysteroid dehydrogenase (11 β-HSD) type 2. Conversely, the conversion from inactive cortisone to the active cortisol is by 11β-HSD type 1. Research has shown that TNF-α up-regulates 11β-HSD-1 and decreases 11β-HSD-2 activity (Prigent et al., 2004).

Cortisol is the physiological glucocorticoid in horses. Production of cortisol is increased with stress and represents an essential, physiological adaptation that promotes survival. Stress-induced cortisol secretion ensures that adequate nutrients are supplied to the brain and other areas of the body that might be compromised by the stressful event. Cortisol secretion releases amino acids from the muscle, glucose from the liver, and fatty acids from adipose tissue into the bloodstream for use as energy. Cortisol also reverses and suppresses the inflammatory responses which accompany stress (Johnson, 2002).
Glucocorticoids specifically inhibit the production of pro-inflammatory cytokines, whereas anti-inflammatory cytokines remain unaffected or are even stimulated. By these effects, the HPA axis acts as a regulatory feedback loop that shuts off inflammatory responses to invading antigens after the initial stress response (Rohleder et al., 2001).

Normal cortisol secretion undergoes a diurnal rhythm, with the highest levels typically observed in the early morning and the lowest levels in the early hours (about midnight to 2 am). According to Irvine (1994), a circadian rhythm in plasma cortisol concentrations occurred in some, but not all, horse studies. When a rhythm occurred, horses were accustomed to a management routine, which may entrain a circadian pattern. Irvine concluded that a circadian cortisol rhythm exists in horses in the absence of any known cues imposed by humans. However, this rhythm can be obliterated by the minor perturbation of removing the horse from its accustomed environment.

In animal studies, the female sex steroids, especially estrogens, stimulated glucocorticoid secretion, whereas the male sex steroid, testosterone, had an inhibitory impact (Rohleder et al., 2001). Animal studies showed that ovariectomy leads to attenuated HPA responses, where estradiol substitution induces HPA stimulation. In rodents, basal ACTH and corticosterone levels, as well as response to various stimuli, are uniformly greater in females (Kirschbaum et al., 1999).

**Leptin and the Immune System**

To further understand the cause behind the increase in equine obesity, understanding the underlying relationship between adiposity and chronic inflammation becomes essential. Cytokines, including IL-6, TNF-α, and leptin, play important roles in the inflammatory processes. During infection, pro-inflammatory cytokines are released that induce regulated
changes in the host’s internal environment, which in turn creates a hostile environment to the invading pathogen. However, recent evidence indicates that certain cytokines are produced at a low, constant rate in a non-pathological, obese state (Cannon, 2000). In obesity, the normal balance of certain adipose cytokines is perturbed (Rajala and Scherer, 2006). According to Rudin and Barzilai (2005), low-grade inflammation is a predominant feature of obesity and may be linked to chronic activation and secretion of cytokines IL-6, TNF-α, and leptin. Additionally, adipose tissue is infiltrated by macrophages, which may also be a major source of locally-produced pro-inflammatory cytokines.

**Tumor Necrosis Factor-α.** While predominantly synthesized and secreted by phagocytic cells of the immune system, TNF-α has also been shown to be synthesized and secreted by the adipocytes (Qi and Pekala, 2000). The expression and circulating levels of TNF-α are elevated in obese subjects. In the basal state, TNF-α is directly proportional to fat mass and has been shown to be involved in the development of insulin resistance (Badman and Flier, 2007).

Tumor necrosis factor-α is believed to be involved in the wasting that occurs during acute and chronic illness and malignancy of human patients. Levels of TNF-α are elevated with various cancers, AIDS, and sepsis, all of which cause a reduction in skeletal muscle protein synthesis and a loss of muscle mass (Williamson et al., 2005). While insulin stimulates protein synthesis in skeletal muscle, elevated concentrations of TNF-α in blood have been shown to inhibit insulin-stimulated pathways (Williamson et al., 2005). *In-vitro* studies have demonstrated that TNF-α decreases the insulin receptor tyrosine phosphorylation by down-regulating several steps in the insulin signaling pathway. Neutralizing agents for TNF-α, however, improve insulin resistance (Rudin and Barzilai, 2005).
In studies of experimentally induced endotoxemia in horses, serum concentrations of TNF-α activity increased to peak values within the first 2 h after the onset of endotoxemia, and then returned to baseline values by 6 h (Morris et al., 1990; MacKay et al., 1991). The early increase in serum TNF-α activity was directly associated with the onset of signs of abdominal pain or depression, fever, and leucopenia (Morris et al., 1990).

During systemic bacterial infection, animals achieve an internal homeostasis by balancing the activities of pro-inflammatory and anti-inflammatory pathways. The toxicity and lethality induced by TNF-α is largely dependent on the presence and absence of endogenous sensitizing or protective factors (Takahashi et al., 1999). One of the cytokines that is induced by TNF-α is leptin (Sarraf, et al., 1997; Grunfeld, et al., 1996). Leptin production in rodents is increased after administration of TNF-α (Grunfeld, et al., 1996). Leptin has protective properties against the toxicity exerted by TNF-α, thus leptin might be an anti-stress cytokine (Takahashi et al., 1999).

Interleukin-6. Interleukin-6 is another cytokine derived from adipose tissue. Its expression and circulating levels correlate directly with obesity; weight loss will lower circulating levels. Indeed, adipose tissue has been estimated to yield approximately a third of circulating IL-6, with visceral fat contributing more than subcutaneous adipose tissue (Badman and Flier, 2007). Elevation of circulating IL-6 is a predictor of the development of cardiovascular disease and diabetes (Rudin and Barzilai, 2005).

Leptin. Leptin is a member of the cytokine family. Although a cytokine itself, leptin synthesis is stimulated by cytokines. The leptin receptor is highly expressed in the hypothalamus, but is also present in peripheral tissues, including those of the immune system such as spleen, thymus, lung and leukocytes (Faggioni et al., 2001). Leptin levels increase
acutely during infection and inflammation, and may serve a protective role against damage by pro-inflammatory cytokines such as TNF-α (Henson and Castracane, 2003). Leptin deficiency is known to increase susceptibility to lipopolysaccharide (LPS) and TNF-α induced lethality; ob/ob mice have a greater number of monocytes and dysfunctional macrophages, together with higher levels of pro-inflammatory cytokines and lower concentrations of anti-inflammatory cytokines (Takahashi et al., 1999).

Studies in ob/ob and db/db mice (Loffreda et al., 1998) have demonstrated that the absence of leptin, or of a functional leptin receptor, leads to impaired macrophage phagocytosis and abnormal cytokine gene expression, rendering these animals more susceptible to infection by various pathogens. Using the acute starvation model, Lord et al. (1998) reported that leptin administration to leptin-deficient mice reversed the immunosuppressive effects of acute nutrient deprivation. Together, these findings suggest a novel role for leptin in relating nutritional status to the regulation of cellular immune function (Henson and Castracane, 2003).

In contrast to leptin deficiency, an excess of leptin, correlating with overweight in obese subjects, may play a role in pathological conditions mediated by an excess of immune response. Elevated leptin levels, along with proinflammatory cytokines, have been associated with an increased risk for coronary heart disease (Yudkin et al., 1999) and type II diabetes (Matarese et al., 2002). Leptin, along with other cytokines, such as TNF-α and IL-6, may contribute to the biochemical and clinical features of the metabolic syndrome X, including accelerated atherosclerosis associated with insulin resistance, glucose intolerance, and central obesity (Pickup et al., 1997).

**Endotoxemia.** Endotoxemia remains the leading cause of death in horses, being directly involved in the pathogenesis of gastrointestinal disorders that cause colic and neonatal foal
septicemia (Morris, 1991; Ouellette et al., 2004; Sykes and Furr, 2005). Compared to other animal species, horses display extreme sensitivity to endotoxin-induced, cardio-pulmonary shock; however, the mechanisms behind increased sensitivity of the horse to endotoxins remain largely unknown (Morris, 1991; Parbhakar et al., 2005). During experimental studies, both horses and humans have shown to be more sensitive to the effects of endotoxin than rodents (Van Miert, 2002). Previous experiments with the horse have even shown large inter-individual variations in the response to endotoxins (Werners et al., 2005).

Endotoxin, or LPS, is a glycolipid that constitutes the major portion of the outermost membrane of gram-negative bacteria. Endotoxemia occurs when gram-negative bacteria, during rapid growth or death, release their endotoxins, which subsequently gain access to the systemic circulation (Fenton and Golenbock, 1998). Endotoxemia is characterized by global activation of inflammatory cells, secretion of pro-inflammatory mediators, and possible development of shock and multiple organ failure (Parbhakar et al., 2005).

Lipopolysaccharide is composed of three parts, each of which has important biologic characteristics. The innermost portion is termed the lipid A, unique because it is well-conserved among different species of gram negative bacteria, and because lipid A imparts toxic qualities to the endotoxin molecule. The middle region of endotoxin is the core oligosaccharide, which links the lipid A with the outer polysaccharide portion. The core region is well conserved in gram-negative bacteria. The outermost portion is comprised of repeating polysaccharides. The composition of this portion is especially diverse and accounts for the serologic differentiation among bacterial species. The interaction of the lipid A moiety of LPS with macrophages appears to be especially important, because subsequent cellular activation results in the release of
systemically active pro-inflammatory molecules, which in turn mediate systemic toxicity (Hardie and Kruse-Elliot, 1990; Fenton and Golenbock, 1998).

Bacteria do not actively secrete endotoxin. Rather, when gram negative bacteria undergo periods of rapid proliferation or lyse upon bacterial cell death, endotoxin is released from the outer cell membrane (Morris, 1991; Moore and Barton 1999). The lumen of the equine gastrointestinal tract harbors large quantities of gram-negative bacteria and free endotoxin. In fact, it has been estimated that at least 2.25 g of free endotoxin exists in the cecum and ventral colons of healthy mature horses (Moore and Barton, 1998).

Once in the systemic circulation, LPS associates with high-density lipoproteins (HDL) or lipopolysaccharide-binding protein (LBP), which has a strong affinity for the lipid A region of endotoxin (Fenton and Golenbock, 1998; Peppelenbosch et al., 1999). Lipopolysaccharide-binding protein serves as a transporter, shuttling molecules of endotoxin to various locations. Lipopolysaccharide-binding protein is an acute-phase reactant, predominantly derived from the liver, and plasma levels rise dramatically after inflammatory challenge, including bacterial sepsis. It can also be synthesized in the lung; local production of LBP might account for many of its biological effects. Through its interaction with lipid A, LBP effectively imprisons endotoxin, with its potential for toxicity determined by the complex’s final destination. Lipopolysaccharide-binding protein can rapidly deliver monomers of endotoxin to the cell surface of host inflammatory cells to evoke an inflammatory response. A second accepted role of LBP is to shuttle LPS into HDL particles, resulting in LPS neutralization. The plasma half-life of LPS is generally very short; however, this varies with animal species and functional capacity of the mononuclear phagocyte system (Morris, 1991).
Macrophages. Macrophages play a pivotal role in the pathophysiology of many diseases by mediating the host immune response to infections and toxins. Werners et al. (2004) concluded that, in response to inflammatory stimuli, macrophages produce the pro-inflammatory cytokine TNF-α, which plays an important role in the network of mediators, and is released during or after the onset of the acute phase response. Stimulation of an equine macrophage cell line, derived from equine bone marrow cells, with increasing concentrations of LPS resulted in a dose-dependent increase in TNF-α production (Werner et al., 2004).

Hematologic Variables. Red blood cell (RBC) counts, reported in units of millions/μl, measure the number of circulating erythrocytes in circulating blood. Packed cell volume (PCV) measures that space in the blood which is taken up by RBC; PCV is reported as a percentage. A high PCV (greater than 45%) generally indicates dehydration. Stress, excitement, or pain may elevate red cell values, as RBC are mobilized from the spleen. This effect may last for approximately 60 min, depending on the level of stress. (Rose and Hodgson, 2000).

Total protein (TP) measures the total amount of protein in serum, and is unaffected by excitement. An increase in TP occurs in conditions of dehydration, with values greater than 7.5 g/dL, indicating sodium and water loss. Greater than normal values may indicate chronic inflammation or infection (Rose and Hodgson, 2000).

White blood cell counts (WBC) measure the total number of leukocytes, and are reported in units of millions/μl. A decrease in total WBC to below normal range (leukopenia) is always a pathologic event, and may be associated with stress or endotoxemia. Absolute counts of the different leukocyte populations are more meaningful than total WBC counts (Sprayberry, 1999).

Neutrophils are the first line of defense against microbial infection. Neutrophils are attracted to sites of infection and inflammation by soluble chemotactic factors released during a
pro-inflammatory reaction. Neutrophils ingest and kill invading microorganisms at the site of tissue injury (Reed et al., 2004). The mature neutrophil has a segmented nucleus, while the immature neutrophil has a band-shaped nucleus. The most notable indicator of infectious, inflammatory disease is the presence of increased numbers of circulating immature neutrophils in the circulation. A “degenerative left shift” occurs when the immature cells are more prevalent in the circulation, as the bone marrow cannot keep up with adequate production (Sprayberry, 1999). Neutropenia occurs when the demand for neutrophils exceeds bone marrow production. Experimentally, administration of endotoxin to horses results in neutropenia within 90 min with a return to baseline numbers within 6 to 18 h (Reed et al., 2004). Endotoxemia causes increased expression of adherence molecules on the vascular endothelium, making them stickier so that neutrophil margination (flowing nearer to the endothelial lining of blood vessels) increases, thus decreasing neutrophil cell numbers in peripheral blood samples. Gram-negative sepsis is one type of bacterial infection that can result in neutropenia, rather than neutrophilia (Sprayberry, 1999).

Lymphocytes are the primary mediators of humoral and cell-mediated immune responses. Two major types of lymphocytes are T lymphocytes that mature in the thymus, and B lymphocytes that mature in the bone marrow and then migrate to lymph nodes. Circulating lymphocytes are predominantly of the T-cell type; however, they represent only a small fraction of the total pool of lymphocytes. Most lymphocytes reside in the spleen, lymph nodes, and other lymphoid tissue of the body. The spleen plays a major role in immune defense, and contains the greatest number of lymphocytes in the adult horse (Reed et al., 2004). Lymphopenia is frequently associated with glucocorticoid administration, severe systemic stress, and acute systemic infections in the horse (Rose and Hodgson, 2000).
Platelets are formed in the bone marrow from megakaryocytes. Platelets are irregularly-shaped, circulating fragments of megakaryocytes that are essential for the formation of primary hemostatic plugs (Guyton and Hall, 2000). Normal equine platelets have a life span of 4 to 5 d (Reed et al., 2004). Vikenes et al (1999) reported that blood platelets are involved in the formation of plaque on the inner lining of arteries. Experimental animal studies have demonstrated that platelets are activated and aggregate at the sites of coronary artery stenosis and endothelial injury. Activated platelets release serotonin in substantial quantities, causing further vasoconstriction and recurrent aggregation of platelets with cyclic flow reductions (Vikenes et al., 1999). The release of the vasoconstrictor substance thromboxane A2 by platelets (Guyton and Hall, 2000) has been shown to act synergistically with serotonin to cause vasoconstriction, platelet aggregation, and reduction in blood flow (Vikenes et al., 1999).

Nakata et al. (1999) and Konstantinides et al. (2001) reported that platelets express the long form of the leptin receptor (Ob-R). Research indicates that leptin may be responsible for the association between obesity and blood clots, which can lead to heart attacks and strokes. Research has shown that at the lower leptin concentrations, as are observed in normal individuals, leptin had no effect on platelet aggregation. However, when leptin concentrations were high, as seen in obese individuals, there was a significant increase in platelet aggregation (Nakata et al., 1999). Konstantinides et al. (2001) stated that leptin potentiates the aggregation of platelets from ob/ob, but not db/db, mice in response to known agonists. The authors suggested that there exists a receptor-dependent effect of leptin on platelet function and hemostasis. These results provided evidence for a link between leptin and the risk for thrombotic complications in obese individuals.
Thrombocytosis is an increase in the number of circulating platelets. Increased bone marrow production of megakaryocytes may occur as a result of acute or chronic inflammatory disorders (Reed et al., 2004). Increased IL-6 production, as part of the acute-phase inflammatory response, may stimulate increased bone marrow platelet production. Extremely high platelet counts could lead to thrombosis, possibly increasing the risk of equine laminitis or organ dysfunction (Brown and Bertone, 2002).

Clinical Signs of Disease. Moore and Barton (1998) reported that the most common clinical effects of endotoxemia are increased heart and respiratory rates, alterations in the color of the horse’s mucous membranes, prolongation of the capillary refill time, fever, and reduced gastrointestinal sounds. The presence of endotoxins in the blood simultaneously initiates numerous processes involving the cardiovascular and the pulmonary systems. The degree of dysfunction within these two systems is determined by the severity and rate of development of endotoxemia.

Research with horses that were given various doses of LPS by several routes of administration showed that endotoxin causes early systemic vasoconstriction, tachycardia, and pulmonary hypertension (Morris, 1991). Interestingly, the responses to bolus administration of high dosages of endotoxin and slow infusion of low doses of endotoxin differ. It has been shown that bolus administration of endotoxin elicits the release of overwhelming quantities of mediators, resulting in severe insult. In contrast, slow infusion is a pathophysiologically more relevant method of study (Clark and Moore, 1989). Clark and Moore (1989) indicated that all horses in the experiment that received a slow infusion of a low dosage of endotoxin became depressed, displayed dark red and dry mucous membranes, and showed a significant increase in heart rate, respiratory rate and body temperature. Congestion and discoloration of mucous
membranes, along with a prolongation of the capillary refill time, are normally found in horses with endotoxemia (Morris, 1991).

At rest, the equine heart rate is usually in the range of 30 to 40 beats per minute (Rose and Hodgson, 2000). The normal respiration rate for a horse is 8 to 16 respirations per minute, while the normal rectal temperature is 38º C for adult horses (Evans, 2001). The mucous membranes of the horse are normally pale pink and should feel moist to the palpating finger. Capillary refill time is 1 to 2 sec (Rose and Hodgson, 2000). Gut sounds are caused by the normal contracting and relaxing movements of the digestive tract during the digestion process. Absence of gut sounds is usually more critical than excessive sounds (Evans, 2001).

**Pro-inflammatory Cytokines.** Cytokines are not produced solely from the consequences of endotoxemia. Obesity is associated with a low-grade inflammation of white adipose tissue resulting from chronic activation of the innate immune system. In obesity, white adipose tissue is characterized by an increased production and secretion of a wide range of inflammatory molecules including TNF-α and IL-6, which may have local effect on white adipose tissue physiology, but also systemic effects on other organs. A recent study indicated that white adipose tissue in obesity is infiltrated by macrophages, which may be a major source of locally-produced pro-inflammatory cytokines (Bastard et al., 2006).

**Prolactin.** Prolactin is a polypeptide protein hormone that is predominantly synthesized in and secreted from specialized cells of the anterior pituitary gland and the lactotropes (Freeman et al., 2000). Recent research concluded that prolactin is a multi-functional hormone and is secreted not only by the anterior pituitary gland, but also by many extra-pituitary sites, including the immune cells (Ben Jonathan et al., 1996; Matera et al., 2000; De Bellis et al., 2005; Malaguarnera et al., 2005). Chikanza and Grossman (1998) categorized prolactin as a pro-
inflammatory neuropeptide, capable of modulating immune and inflammatory responses. Prolactin, released by both the pituitary gland and by some types of lymphocytes, exerts its influence on the immune system by endocrine and paracrine/autocrine mechanisms (Matera et al., 2000; DeBellis et al., 2005).

Proof of specific involvement of prolactin in the immune system comes from animal models in which pituitary deficiency was experimentally induced by surgical ablation of the gland or treatment with bromocriptine (Hiestand et al., 1986; Russell et al., 1988). In both studies, lympho-hemopoietic function was restored when either prolactin was injected or a syngenic pituitary gland was implanted under the kidney capsule (Matera et al., 2000). Other research by Rettori et al. (1994) showed that LPS altered the release of pituitary hormones in rats, increasing prolactin release and decreasing growth hormone, thyroid-stimulating hormone, and luteinizing hormone release. More recently, Mastronardi et al. (2001) suggested that LPS acts on the central nervous system to inhibit the secretion of dopamine, removing the dopamine inhibition on the secretion of prolactin. Consequently, the secretion of prolactin is increased from the lactotropes.

Mastronardi et al. (2000) reported that prolactin stimulated leptin release, and that bromocriptine decreased plasma leptin concentrations. Circulating prolactin, acting on its receptors on the adipocytes, increases the release of leptin that is stored in pinocytotic vesicles in the cytoplasm adjacent to the cell membrane. The blocking of prolactin by bromocriptine produced a rapid and long-lasting lowering of plasma leptin concentrations. The authors concluded that an increase in prolactin secretion during the night may be responsible, at least in part, for the nocturnal elevation of leptin concentrations observed in rats and in humans.
Gualillo et al. (1999) reported that several pro-inflammatory cytokines, most notably TNF-\(\alpha\) and IL-1, both induced by prolactin, produce a prompt and dose-dependent increase in serum leptin levels and leptin mRNA expression in mice adipose tissue. However, the stimulatory effect of prolactin on serum leptin levels was significantly reduced by food deprivation.
CHAPTER 3

BREEDING OUTCOME OF LACTATING MARES WITH HIGH VS. LOW PLASMA LEPTIN CONCENTRATIONS

Introduction

Research in swine (Estienne et al., 2000), sheep (Delavaud et al., 2000), cattle (Delavaud et al., 2002), and horses (Buff et al., 2002; Gentry et al., 2002) showed that concentrations of plasma leptin increase as animal fat mass increases. In general, obesity is a leptin-resistant state in that higher leptin concentrations are found in more obese individuals (Mantzoros et al., 2000). However, Gentry et al. (2002) concluded that although leptin concentrations are very low in mares with low BCS and higher in well-fed mares, there is a wide variation in leptin levels among well-fed mares. Subsequent research by Cartmill et al. (2003) indicated that horses with high BCS could have resting plasma leptin concentrations ranging from low (1 to 5 ng/mL) to very high (10 to 50 ng/mL). Cartmill et al. (2003) further reported that the high vs. low leptin distinction was consistent over a 2-yr period, indicating that the underlying cause was likely a permanent trait.

Inadequate nutrition and/or reduced body fat are associated with impaired reproductive efficiency in beef cattle (Selk et al., 1988; Schillo et al., 1992) and sheep (Schillo, 1992). A similar relationship has been noted for horses (Henneke et al., 1983; Gentry et al., 2002). Nutritionally restricted broodmares sometimes exhibit abnormal estrous cycling and may be difficult to breed successfully. Research conducted by Gentry et al. (2002) revealed that low BCS and low plasma leptin concentrations were associated with inactive ovaries during winter and early spring; mares with low BCS eventually ovulated in April and May, even while leptin concentrations remained low.
During pregnancy, serum leptin concentrations increase in horses and humans, and drops sharply after delivery (Heidler et al., 2003). In a low leptin environment, GnRH release is decreased, possibly to prevent energy from being wasted for reproduction (Caprio et al., 2001) or to encourage a faster increase in feed intake to prevent a negative energy balance (Block et al., 2001). Although short-term reductions in plasma leptin concentrations are not associated with changes in the reproductive axis in mares (McManus and Fitzgerald, 2000), prolonged exposure to lower leptin concentrations would be expected to decrease GnRH release (Yu et al., 1997).

In contrast, chronically persistent hyperleptinemia can interfere with gonadotropin stimulation of peripheral targets. In obesity, leptin central receptors, which are sensitive to extremely low ligand concentrations, are protected from hyperleptinemia by the saturable transport system of the blood-brain barrier, whereas peripheral leptin receptors are directly exposed to high ligand concentrations, with possible negative effects on gonadal steroidogenesis (Caprio et al., 2001).

Leptin stimulates GnRH from the hypothalamus, which in turn stimulates LH and FSH from the anterior pituitary gland (Yu et al., 1997). Although it was originally thought that leptin affected reproduction by regulating the hypothalamic-pituitary-gonadal axis, recent in vitro observations indicated that leptin may also have direct intra-ovarian actions (Mantzoros et al., 2000). In vitro bovine studies showed that leptin at high doses (300 ng/mL) weakly inhibited LH-induced androstenedione production (Spicer et al., 2000). More recent research (Spicer, 2001) indicated that in a high leptin environment, the ovary is kept from over-producing estradiol via leptin inhibition of insulin-induced androstenedione production by bovine thecal cells and aromatase activity by bovine granulosa cells in vitro. Greisen et al. (2000) suggested that leptin acts directly to inhibit basal and FSH-stimulated estradiol and progesterone production.
in cultured human granulosa cells. These results introduce the possibility that high circulating leptin levels, as seen in obese women and mares, may compromise fertility through peripheral mechanisms. If persistently high concentrations of leptin interfere with the development of the dominant follicle’s ability to produce estradiol, such an effect could result in an inadequate stimulus for the LH surge.

Given the possibility that different concentrations of leptin exert both inhibitory and stimulatory actions on the hypothalamic-pituitary-gonadal axis, the present two studies were performed to examine further the possible role of hyperleptinemia on the re-breeding capabilities of mares and the relationship between leptin levels in lactating and non-lactating mares. The specific objectives were 1) to determine the prevalence of high leptin secretors, as described by Cartmill et al. (2003), in postpartum, lactating mares and to evaluate any consequence of high versus low leptin levels on re-breeding capabilities, and 2) to examine possible relationships among BCS, age of mare, breed, feeding regime and reproductive history with plasma leptin concentrations in lactating mares and compare them with those in non-lactating mares.

Materials and Methods

Study 1. A total of 198 light-horse and draft-type postpartum mares residing at eight individual and independent horse-breeding farms within Louisiana were used in this study. Mares were sampled during the month of April. Breeds represented were Quarter Horses (n = 72), Thoroughbreds (n = 86), draft-type (n = 18), and warm-bloods (n = 24). The mares were grouped into finite age groups: less than 6, 7 to 15, and over 15 yr of age, and were assigned to one of two groups based on their plasma leptin concentrations: low to normal leptin (< 10 ng/mL) or hyperleptinemic (> 10 ng/mL). The origin of the data used for this assignment is described below.
Information provided by each farm included breed, mare age, feeding schedule, type of feed, medical treatments, number of years the mare had been bred, number of live foals, and last foaling date. A confirmation of pregnancy (or lack of pregnancy) was provided by each farm after the breeding season had concluded. Body condition score was determined directly on each mare by one technician by visual appraisal and palpation of the six areas described by Henneke et al. (1983). This was done at the time of the first blood sampling for each mare. (Table 3.1).

For assessment of leptin status, two samples of jugular blood were collected from each mare approximately 2 wk apart. The samples were collected in the morning (before 0900) by

**Table 3.1.** Breed prevalence, feeding regimen, mean mare and foal ages, mean body condition scores, and mean leptin concentrations for mares on the eight farms throughout Louisiana in Experiment 1

<table>
<thead>
<tr>
<th>Farm</th>
<th>QH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TB&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WB&lt;sup&gt;a&lt;/sup&gt;</th>
<th>D&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Feeding&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mare Age, yr</th>
<th>Foal Age, d</th>
<th>BCS</th>
<th>Leptin, ng/mL</th>
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<td>24</td>
<td>6</td>
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<td>42</td>
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<td>14</td>
<td>0</td>
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<td>B</td>
<td>9</td>
<td>39</td>
<td>5.5</td>
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<td>64</td>
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<td>9</td>
<td>B</td>
<td>9</td>
<td>37</td>
<td>5.5</td>
<td>7.28</td>
</tr>
</tbody>
</table>

<sup>a</sup>QH = Quarter Horse; TB = Thoroughbred; WB = Warmblood; D = Draft Horse.

<sup>b</sup>Feeding regimen: A = fed concentrate once per day plus pasture; B = fed concentrate twice per day plus pasture; C = pasture only.
jugular venipuncture into 7-mL evacuated tubes containing sodium heparin (Vacutainer, Becton and Dickinson, Franklin Lakes, NJ). Previous studies of leptin in horses (Cartmill et al., 2003, 2005; Crowley et al., 2005; Storer et al., 2005) revealed that leptin concentrations rise about 8 to 10 h after a meal, thus morning samples would represent a non-stimulated state in the case of mares fed once per day (morning). Also, the hyperleptinemic condition persists in the long-term, literally year-to-year in most cases. Thus, to be considered hyperleptinemic, the leptin concentrations in both samples were averaged so that no one sample would determine the status of the mare.

All blood samples were placed on ice and transported to the laboratory for analysis. They were centrifuged at 1,500 x g at 4º C for 15 min and the plasma harvested and stored at -15º C. Plasma leptin concentrations were determined by means of a double-antibody leptin radioimmunoassay procedure 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-μL sample size, was 0.1 ng/mL (Cartmill et al., 2003). Concentrations of progesterone were determined by radioimmunoassay with commercially obtained reagents (Diagnostic Laboratory Systems, Webster, TX, USA). Intra- and inter-assay coefficients of variation and assay sensitivities for progesterone were 5%, 9%, and 0.05 ng/mL, respectively.

Data were analyzed by the SAS Proc Mixed procedure (SAS Institute, Inc., Cary, NC.). All factors were included in the model: breed, farm, day, body condition score of the mare, age of the mare, progesterone concentrations, feeding schedule, type of feed, medical treatments, number of years the mare had been bred, number of live foals, and last foaling date. Insignificant factors were removed one at a time to simplify the model, using a backward
elimination stepwise regression analysis. The factors of interest in the final model were breed, farm, and BCS of mare. The SAS Logistic Regression procedure (SAS Inst. Inc., Cary, NC) was used to determine whether leptin levels affected the re-breeding of the mare.

**Study 2.** For comparison with the lactating mare data obtained in Study 1, data were collected within the same time period from 31 non-foaling, non-lactating mares of light horse breeds from the resident herd at the Louisiana Agricultural Experiment Station Horse Farm. Mares were housed on pasture of native grasses during the late spring, summer, and early fall, and on winter ryegrass pasture during the colder months of the year. Mares were grouped by age and leptin status, and were assigned a BCS, as described for Study 1.

Data were analyzed by the SAS Proc Mixed procedure (SAS Institute, Inc., Cary, NC.). The factor of interest in the final model was BCS of mare.

**Results**

**Study 1.** A scatterplot of the data from the two samples for each mare is presented in Figure 3.1. The trendline \( Y = 0.89X + 0.37 \) showed a positive and strong correlation \( R^2 = 0.826 \). Based on these data, it was decided to average the two leptin samples and to not use day as a repeated measure in the analyses.

Mean leptin concentration of the 198 lactating mares was 4.8 ng/mL, with a range of 0.41 ng/mL to 14.9 ng/mL. A frequency diagram, with mean leptin values categorized at 0.6 ng/mL intervals, is shown in Figure 3.2. There was an apparent normal distribution of mares around a mean of approximately 3 ng/mL, and another grouping of mares on the high end of the scale, around 10 ng/mL and greater. Based on the 10 ng/mL cutoff, 26 mares (13%) were classified as hyperleptinemic. Mean leptin level in these mares was 12.2 ng/mL.
Figure 3.1. Leptin in the two morning blood samples 1 vs. 2. The R² value for the regression equation was 0.826.

Figure 3.2. Frequency diagram for mean leptin concentration. Mares with mean concentration of 10 ng/mL or greater were classified as hyperleptinemic.
The mean BCS of the lactating mares was 5.5, with a range of 3.0 to 7.5 (Figure 3.3). Scores appeared to be normally distributed. The BCS of the mare had a significant effect on leptin levels \( (P = 0.08) \), with leptin levels increasing in general as BCS increased (Figure 3.4).

Feeding regimen affected leptin levels \( (P < 0.0083) \). Compared to mares strictly on pasture, mares fed once each morning and on pasture otherwise had the lowest leptin concentrations; the morning and evening-fed mares displayed intermediate leptin levels (Figure 3.5).

Farm was also a significant source of variation for leptin levels \( (P < 0.0001) \). Farm 1 mares had the lowest leptin levels; those mares were meal-fed once a day. Farm 8 had the highest leptin levels; those mares were meal-fed twice a day (Figure 3.6).

![Figure 3.3. Frequency diagram for body condition score. There were no mares with condition scores < 3 or > 7.5 (on a scale of 1 = emaciated and 9 = grossly obese).](image-url)
Figure 3.4. Leptin as a function of body condition score. The numbers of mares per BCS are indicated within the bars. Body condition score affected ($P = 0.08$) mean leptin concentrations.

Figure 3.5. Feeding regimen affected leptin levels. Compared to the mares on pasture, the morning-fed mares had the lowest leptin concentrations, with the morning and evening-fed mares intermediate ($n = 198; P < 0.0083$).
Figure 3.6. Farm was a significant source of variation for leptin concentrations ($P < 0.0001$). The eight farms were ranked from the lowest to the highest leptin concentrations.

Of the 198 lactating mares that were re-bred, 162 mares (81%) became pregnant. The percentages for hyperleptinemic (81.34) and normal mares (81.44) were virtually identical (Figure 3.7) and were not different from each other.

Figure 3.7. Percentage of mares pregnant at the end of the breeding season. Leptin status (hyperleptinemic vs. normal) did not affect pregnancy rate.
Study 2. A scatterplot of the data from the two samples for each mare is presented in Figure 3.8. The trendline \( Y = -0.298 + 1.04X \) showed a positive and strong correlation, with \( R^2 = 0.857 \).

The mean leptin level of the non-lactating mares was 7.5 ng/mL, with a range of 2.3 ng/mL to 20.2 ng/mL. Of the 31 mares sampled, 9 mares (29%) displayed hyperleptinemia, with plasma leptin concentrations above 10 ng/mL. The mean leptin level of the hyperleptinemic mares was 13.9 ng/mL.

The mean BCS of the non-lactating mares was 7.5, with a range of 5.5 to 9.0. BCS of the mare had a significant effect on leptin concentrations \((P = .02)\), as leptin levels increased as BCS increased (Figure 3.9).

![Figure 3.8](image_url) Leptin samples 1 vs. 2. Leptin concentrations (ng/mL) in jugular blood samples collected approximately 2 wk apart. The \( R^2 \) value for the regression equation was 0.857.
Figure 3.9. Body condition scores for non-lactating mares relative to leptin levels. Leptin levels increased ($P = .02$) as BCS increased ($n = 31$).

When the data were analyzed by the SAS Frequency Procedure, the chi-square tests of independence between BCS and leptin were of particular significance ($P < 0.0001$), indicating that within this group of mares, leptin concentrations were more affected by BCS than the group of lactating mares in Study 1.

Discussion

Contrary to human studies (Licinio et al., 1998; Caprio et al., 2001), recent research by Buff et al. (2005) showed that leptin in horses is not secreted in a pulsatile manner. However, further studies have shown that a circadian pattern in the horse does exist, with leptin concentrations increasing during the night (Cartmill et al., 2005; Buff et al., 2005). Further research with horses (Steelman et al., 2006; Cartmill et al., 2003) indicated that feeding concentrate meals to horses daily can increase mean serum leptin concentrations and may cause
fluctuations in leptin production over a 24-h period. Additional observations have indicated that plasma leptin concentrations increase postprandially in the equine (Cartmill et al., 2005; Crowley et al., 2005; Storer et al., 2005), perhaps stimulated by a rise in plasma insulin concentrations. To avoid possible discrepancies in sampling, the blood samples in the present two studies were obtained before the morning feeding.

Average leptin levels from the Study 1 lactating mares were 4.8 ng/mL, while the average leptin levels from the Study 2 non-lactating mares was 7.5 ng/mL. This discrepancy between experiments is consistent with recent studies (Heidler et al., 2003), which indicated that plasma leptin concentrations decrease after foaling and for 4 wk are lower in lactating than in non-lactating mares. A reduction in plasma leptin concentrations in lactating mares is also in agreement with findings in dairy cattle (Block et al., 2001; Heidler et al., 2003). Block et al. (2001) reported that the plasma concentrations of leptin in dairy cows is reduced by ~50% after parturition, and remains depressed during lactation, despite a gradual improvement in energy balance. The authors suggested that this reduction could benefit early lactating dairy cows by promoting a faster increase in feed intake and by diverting energy from non-vital functions such as reproduction.

Considering leptin at high physiologic doses (>10 ng/mL), out of the 198 lactating mares sampled in Study 1, only 26 mares had leptin levels above 10 ng/mL. The average leptin concentration from the 26 high leptin mares was 12.2 ng/mL. Nine non-lactating mares from Study 2 had leptin levels above 10 ng/mL. The average leptin concentration from the nine high leptin mares was 13.9 ng/mL. This finding from Study 2 is consistent with previous research, which showed that within this particular resident mare herd, approximately one-third of the mares with high body condition scores consistently displayed high leptin concentrations, relative
to their herd mates (Gentry et al. 2002). Subsequent monitoring of these hyperleptinemic mares and their herd-mates up to 2 yr later indicated the same high versus low leptin concentration distinction.

Mares in the two studies had comparable ages, with most mares in the 7-15 year range. Previous studies (Buff et al., 2002; Kearns et al., 2006) have reported lower leptin values for younger mares and weanling fillies than mature mares. Weanling fillies had significantly lower plasma leptin concentrations than mature mares (Kearns et al., 2006). Buff et al. (2002) concluded that young horses are in the growth phase of development; thus, lower concentrations of leptin would be indicative of an animal with lower body fat and thus requiring greater nutritional resources. Additionally, the mature mares used in the above studies (Buff et al., 2002; Kearns et al., 2006) were non-lactating mares. Older, lactating mares might be expected to have lower leptin levels to prevent a stimulation of the hypothalamic-pituitary-gonadal axis, which would allow the mare to more fully recover from the demands of parturition and lactation. However, within our study there were not a sufficient number of aged mares to compare. These data are consistent with more recent research by Ferreira-Dias et al. (2005), who reported no significant difference between the plasma leptin concentrations of younger mares and older mares.

In both studies, the effect of body condition scores on leptin levels indicated that as BCS increases, leptin levels increase. The average BCS of the lactating mares was 5.5, while the BCS of the nonlactating mares was 7.5. These results support previous research with horses (Buff et al. 2002; Gentry et al., 2002; Ferreira-Dias et al., 2005; Kearns et al., 2006) indicating that body weight has a direct relationship with plasma leptin levels. The higher BCS of the mares in Study
2 might be explained by the good body condition initially presented by the mares, in combination with no body weight fluctuations due to gestation, parturition, or demands of a foal.

In high-yielding dairy cattle, the energy loss through lactation cannot be compensated by food intake during the first weeks of lactation. Cows are in a constant state of negative energy balance; fat stores are first used for lactation, maintenance, and growth with reproductive processes receiving the lowest priority (Liefers et al., 2003). The resulting negative energy balance is reported to delay the occurrence of ovarian cyclicity. Reduced concentrations of peripheral IGF-I and estradiol are associated with ovulation failure during the first follicular wave postpartum in non-cystic dairy cows (Beam et al., 1999).

In contrast to cattle, most lactating mares resume follicular growth and ovulation in early postpartum (Ginther, 1993), and ovulation is not impeded by suckling or the presence of a foal. The majority of foaling mares show estrous behavior and ovulate within 15 d after foaling (Neuschaefer et al., 1990), therefore lactational anestrous does not exist as a principal physiological condition in the horse (Heidler et al., 2004). Lactation, on average, is not associated with weight loss in mares, as the mare seems able to compensate energy losses mainly by increasing feed intake, rather than by mobilization of body reserves. Recent research by Heidler et al. (2004) suggested that a negative energy balance during lactation in most horse breeds may be prevented by adequate feeding. The reduced leptin concentrations in postpartum mares may therefore encourage feed intake, which would allow the lactating mare to avoid an energy deficit. The feeding regime at each stud farm was adequate; therefore, a negative energy balance was not evident in any of the mares studied.

Adipocytes have been shown to be a major source of leptin in the body, but leptin synthesis has also been demonstrated in ovarian granulosa cells (Bützow et al., 1999). Research
reported that leptin is present in follicular fluid, and the leptin receptor is expressed in the human ovary (Greisen et al., 2000; Mantzoros et al., 2000). More recent research suggests that identification of Ob-R expression in the ovary may cause leptin to have a direct effect on downstream endocrine targets of the reproductive axis (Caprio et al., 2001). *In vitro* studies with rats (Zachow and Magoffin, 1997; Zachow et al., 1999), porcine (Gregoraszczuk and Ptak, 2005), bovine (Spicer and Francisco, 1997), and human (Agarwal et al., 1999) ovarian cells have demonstrated that leptin can exert direct inhibitory effects on steroidogenesis in granulosa cells and thecal cells obtained from developing follicles. Overall, these data suggest that high leptin concentrations in the ovary may negatively control estradiol production and interfere with the development of dominant follicles and oocyte maturation.

Graafian follicles are capable of concentrating steroids and gonadotropins in the follicular fluid. Certain molecules present in serum are excluded from the follicular fluid, leading to the conclusion that the follicular fluid is a semi-protected environment (Agarwal et al., 1999). When a follicle is selected to become dominant, FSH bioactivity in the follicular fluid begins to increase. High physiological concentrations of leptin could significantly interfere with the ability of the dominant follicle to produce estradiol. Such an effect could result in an inadequate stimulus for the LH surge and may also lead to an immature pre-ovulatory follicle, or in extreme cases, no pre-ovulatory follicle at all. This type of mechanism is consistent with clinical observations that obese women have a higher incidence of infertility and that weight loss often causes significant clinical improvement (Kiddy et al., 1992; Bützow et al., 1999; Caprio et al., 2001).

Leptin, alone, has no effect on gonadotropin-stimulated steroidogenesis, but leptin is a potent inhibitor of the insulin-like growth factor IGF-I augmentation of gonadotropin action in
rat and bovine cells (Spicer, 2001). Agarwal et al. (1999) reported that in human cultured granulosa cells, leptin had no effect on estradiol production, either alone or in the presence of FSH. However, leptin in humans did cause a concentration-related inhibition of the IGF-I augmentation of FSH-stimulated estradiol production. The effect of leptin was specific, because there was no effect on progesterone production. In cultured human theca cells, leptin did not alter androstenedione production, either alone or in the presence of LH. However, leptin caused a concentration-related inhibition of the IGF-I augmentation of LH-stimulated androstenedione production. Additional research (Bützow, et al., 1999) supported the notion that leptin may inhibit the synergistic action of IGF-I and FSH on granulosa cell estradiol production. Leptin synthesis has been expressed in ovarian granulosa cells; therefore Bützow et al. (1999) suggested that the inhibitory action of leptin in the ovary may partially explain why obese individuals require higher doses of gonadotropin for ovarian hyperstimulation.

Research by Heidler et al., (2003) reported that plasma leptin concentrations decrease after foaling, and for 4 wk plasma leptin concentrations were lower in lactating than in non-lactating mares ($P < 0.05$). Although short-term reductions in plasma leptin concentrations are not associated with changes in the reproductive axis in mares (McManus and Fitzgerald, 2000), prolonged exposure to lower leptin concentrations would be expected to decrease GnRH release (Yu et al., 1997). The reduced leptin concentrations may promote feed intake and allow lactating mares to avoid an energy deficit. Previous research (Hess-Dudan et al., 1994) reported that in mares, concentrations of IGF-I increased during late pregnancy, peaked two days after parturition, and declined gradually until weaning. More recent research (Heidler et al., 2003) confirmed that IGF-I concentrations increased continuously in late gestation, reached a maximum in the week of foaling, and decreased thereafter during lactation.
Consequently, in a low leptin, high IGF-I postpartum *in vivo* environment, leptin would not be able to inhibit the IGF-I augmentation of FSH-stimulated estradiol production. Therefore, resumption of ovarian activity by the lactating mare is resumed during early postpartum and most mares will ovulate reliably in foal heat. Out of the 199 mares from Study 1 that were re-bred, 162 mares (81%) became pregnant. However, no significant difference was found between the re-breeding capabilities of a high leptin mare versus a low leptin mare. Our results are consistent with more recent research from our laboratory (Waller et al., 2006), which suggested that the reproductive system in the mare appears to be unaffected by the changes in leptin. Waller et al. (2006) reported that the mares studied had no apparent alteration in reproductive characteristics: ovarian activity, day of ovulation; gonadotropin and progesterone concentrations were similar for mares of high BCS with high versus low leptin concentrations.

Due to the fact that the non-lactating mares were not bred, a direct comparison between these two experiments cannot be made. However, these results concerning BCS with leptin levels are consistent with previous research, in that with an increase in BCS, leptin levels also increase. These results are consistent with research showing that postpartum mares have lower leptin levels.

It is likely that within the various sites of leptin action, a specific and narrow range of leptin concentration must be maintained for normal hypothalamic-pituitary-gonadal function, with chronic deviations interfering with the hypothalamic-pituitary-gonadal axis. Additionally, research supported the finding that leptin by itself is not the sole determinant of reproductive activity. The lactating mares were well cared for on each stud farm, so no mares with chronically low leptin levels were nutritionally deprived. Although the mean leptin level and BCS for the lactating mares was much lower than the non-lactating mares, this concentration
apparently was within the threshold for reproductive success. For the 37 mares that did not re-
breed, chronically low or high leptin levels cannot be considered as the reason. Furthermore, the
11 hyperleptinemic lactating mares were also within their threshold for reproductive success.
For leptin to have a negative effect on the gonadal steroidogenesis of these hyperleptinemic
mares, the expected threshold would have to exhibit leptin levels persistently higher than 11
ng/mL.

It may be hypothesized that a specific and narrow range of leptin concentrations is
necessary to maintain a normal reproductive function, and that concentrations below or above
these thresholds might interfere in opposing ways with the function of the HPG axis (Caprio et
al., 2001). However, to assign a global threshold number for infertility would be unrealistic.
The high and low boundaries would likely be on an individual basis, due to the complex
interaction with other molecules such as hormones, growth factors, and cytokines.

Mantzoros et al., (2000) found that women who succeeded in becoming pregnant with
artificial reproductive techniques had significantly lower leptin concentrations in follicular fluid,
when compared with women who failed to become pregnant. Future research would determine
whether there are significant differences between serum leptin and follicular fluid leptin, and
whether the functional state of the ovary might affect serum leptin concentrations. Direct
measurement and interactions of other follicular fluid hormonal factors, such as androgens,
estrogens, and growth factors also remains to be studied.
CHAPTER 4

SEARCH FOR POLYMORPHISM IN EXON 2
OF THE EQUINE LEPTIN GENE

Introduction

Obesity, a growing dilemma within the horse industry, is associated with numerous medical conditions. These conditions include chronic laminitis (Buff et al., 2002; Johnson, 2002), hyperinsulinemia, glucose intolerance (Johnson, 2002; Cartmill et al., 2003), and variations in thyroid hormone concentrations (Cartmill et al., 2003). The leptin-signaling pathway may play an important role in the pathophysiology of the obese condition. Leptin appears to modulate feeding behaviors and energy expenditure, and therefore plays an important role in body weight regulation (Henson and Castracane, 2003).

Leptin is expressed primarily in the white adipocytes; both its expression and its secretion are highly correlated with body fat and adipocyte size (Henson and Castracane, 2003; Paracchini et al., 2005). The primary factors affecting plasma leptin concentrations include body fat mass and energy balance (Block et al., 2001) and gender (Henson and Castracane, 2003). Plasma leptin levels in dairy cattle and sheep increase linearly with increased body fat mass and plane of nutrition (Blache et al., 2000; Delavaud et al., 2000; Ehrhardt et al., 2000), and these results are consistent in humans and rodents (Henson and Castracane, 2003).

Previous work focusing on the interaction between BCS and leptin concentrations in horses indicated that a wide variation in leptin values in well-fed horses revealed some other factor besides body condition affecting leptin concentrations. Polymorphisms in the leptin gene might possibly modify obesity. A mutation in the mouse LEP gene was first described by Ingalls et al. (1950). Zhang et al. (1994) later described the ob mutation as a cytosine (C) to thymine (T) substitution at codon 105, which results in a premature stop codon. Therefore, leptin production
This mutation results in a total lack of leptin in the blood, which results in obesity, hyperphagia, hypothermia, extreme insulin resistance, and infertility in the ob/ob mouse (Paracchini et al., 2005). Four genetic polymorphisms found in the porcine leptin gene were associated with fatness in four pig breeds (Jiang and Gibson, 1999). Subsequent research, however, indicated that the four polymorphisms are generally of low frequency or are absent in pig populations (Kennes et al., 2001). A polymorphism within exon 2 of the bovine leptin gene has been described (Pomp et al., 1997; Fitzsimmons et al., 1998; Haegeman et al., 2000) and is associated with fat deposition in beef cattle (Fitzsimmons et al., 1998). Researching beef breeds, Buchanan et al. (2001) reported a cytosine (C) to thymine (T) nucleotide base transition that encoded an amino acid change of an arginine to a cysteine in exon 2 of the bovine leptin gene. The T allele was associated with fatter carcasses, while the C allele was associated with leaner carcasses. More recent research by Buchanan et al. (2003) revealed the same genetic variant present in dairy breeds. Dairy cows homozygous for the T allele produced more milk, without any significant alteration in milk fat or protein percent, over the entire lactation.

The aforementioned experiments provide evidence that leptin is a credible candidate gene for the evaluation of polymorphisms that could have an effect on equine obesity. The aim of the present experiment was to investigate the possible occurrence of an analogous single nucleotide polymorphism (SNP) in exon 2 of the equine leptin gene that would explain the wide variation of leptin levels in high BCS mares.

Materials and Methods

Phase 1. Horses and Sampling Procedures. All horses were of light breeds from the resident herd at the Louisiana Agricultural Experiment Station, Central Stations Horse Farm. The horses were routinely maintained on native grass pastures during the spring, summer, and
fall and on ryegrass pasture in winter. Leptin, age, and BCS data were compiled over a 2-yr period on a total of 31 mares, 16 geldings, and 11 stallions. Each year a BCS, as described by Henneke et al (1983; 1= extremely emaciated through 9 = extremely fat), was assigned to each horse by two independent, experienced technicians; the mean of the two scores was used for statistical analyses. Jugular blood samples were collected from each horse into heparinized tubes for determination of plasma leptin concentration.

**Laboratory and Statistical Analyses.** Jugular blood samples were immediately centrifuged at 1,500 x g at 5º C, and the plasma was harvested and stored at -15º C. Concentrations of leptin were measured using the double-antibody leptin radioimmunoassay procedure 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-μL sample size, was 0.1 ng/mL. Data were analyzed by the Proc Mixed procedure of SAS (SAS Institute, Inc., Cary, NC.). All factors (age, gender, BCS, and all possible interactions) were initially included in the model. The results of the model with the log transformed leptin values and all possible interactions showed that none of the interactions were significant. Therefore, the model was subsequently run with just the main effects (gender, BCS, and age).

**Phase 2. Purification of DNA.** A jugular venous blood sample was collected from one hyperleptinemic mare into an evacuated tube (Becton and Dickinson, Franklin Lakes, NJ) containing lithium heparin. Genomic DNA was extracted from the white blood cells and purified, using the PUREGENE DNA Purification Kit (Gentra Systems, Inc. Minneapolis, MN). Forward and reverse primers were designed based upon the nucleotide sequences of the Bos taurus leptin (obese) gene (GenBank Accession # U50365).
The Forward primer was 5’-TATCTTGTCCTTCTTGCAGAGCTCT-3’ (bp 217-241). The Reverse primer was 3’-GTCAGTCACAGTAGAAAACAAAGCCAG-5’ (bp 3960-3985). Polymerase chain reaction (PCR) was performed to amplify the specific region of the equine leptin gene containing exon 2 and partial fragments of introns 1 and 2. Amplifications via PCR were carried out in 50-µl reactions containing 5-µl genomic DNA, 1-µl of forward primer at 20-µM concentration, 1-µl of reverse primer at 20-µM concentration, 1-µl 50X dNTP mix, 1-µl Advantage Genomic Polymerase Mix in 5-µl 10X Genomic PCR Reaction Buffer with 2.2-µl mM Mg(OAc)$_2$ (CLONTECH Laboratories, Inc., Mountain View, CA). The PCR was performed in a BioRad thermocycler (Bio-Rad Laboratories, Hercules, CA). Thermal cycling parameters included an initial denaturation at 94º C for 1 min, followed by 30 cycles of denaturing at 94º C for 30 sec, and annealing at 68º C for 4 min. A final extension at 68º C for 5 min was performed to reduce background.

**Agarose Gel Electrophoresis.** Positive and negative samples were analyzed, along with a suitable DNA sized marker, by electrophoresis on agarose gel. The percentage of agarose used was 0.8%, with a voltage of 70V. To avoid damage to the PCR product by UV light, a crystal violet agarose gel was used. The buffer was poured off of the gel and the gel was transferred to the UV photo machine where the band was carefully excised from the gel with a sharp razor blade. Next, the excised PCR product was isolated.

**Isolating the PCR Product.** A SNAP (Invitrogen, Carlsbad, CA) purification column and collection vial was used for rapid purification of the DNA fragments from the agarose gel. The final isolated DNA product was analyzed by electrophoresis on an agarose gel to estimate the DNA concentration. Ethidium bromide was used in the agarose gel.
Chemical Transformation and Cloning Reaction. The gel-purified long PCR product was inserted into pCR®-XL-TOPO® plasmid vector (Invitrogen) by topoisomerase I from Vaccinia virus. Transformation of chemically competent *E. coli* with the plasmid DNA was accomplished, according to TOPO XL PCR Cloning Kit (Invitrogen). After an overnight growth on plain nutrient lysogeny broth (LB) agar plates, selection of host cells harboring the recombinant DNA was performed by inoculating the cells in a very rich culture media Terrific Broth (TB) containing kanamycin, and incubating the cells overnight at 37°C with vigorous shaking.

Plasmid DNA Isolation, Analysis, and Sequencing. The resulting DNA plasmid was enzymatically digested by restriction analysis to confirm the presence of the segment of interest of the leptin gene. Restriction enzymes EcoRI (sequence 5’-G^AATTC-3’) and Not1 (5’-GC^GGCCGC-3’) were used. The restriction fragments were amplified by PCR and the resulting PCR product was sequenced on an automated DNA sequencer using the M13 Reverse primer, which is (5’-CAGGAAACAGCTATGAC-3’) and the M13 Forward primer, which is (5’-GTAAAACGACGGCCAG-3’).

Sequence of Exon 2 and Flanking Introns. Using the leptin mRNA (GenBank AY462242), a partial sequence of the equine leptin gene, forward and reverse primers were designed to partially sequence intron 1 and intron 2. Exon 2 is from bp 1-118; exon 3 is 119-421. The forward primer (5’-CTGTGGCTTTGGCCTATCTG-3’) is at bp 1 – 21, and the reverse primer, which is (3’-ACTAGGACCACCTGTTAGCAG-5’), is at bp 80 – 100 (Figure 4.1). Sequencing reactions were performed at the Louisiana State University School of Veterinary Medicine’s GeneLab, using Applied BioSystems BigDye Terminator version 3.1 chemistries on an Applied Biosystems Genetic Analyzer (Applied Biosystems, Foster City, CA).
Figure 4.1. Equine leptin mRNA (GenBank AY462242). Exon 2 (bp1-118) and exon 3 (bp119-421). Forward primer (5' TGTGGCTTTGGCCCTATCTG 3') is at bp 1 – 21. Reverse primer (3' ACTAGGACCACTGTTAGCAG 5') is at bp 80 – 100.
Phase 3. Single Nucleotide Polymorphism Assessment. Five mares with the lowest leptin concentrations and five with the highest leptin concentrations, with a BCS of at least 7.5, were selected from the available mares in Phase 1 to study the prevalence of polymorphisms in exon 2 of the equine leptin gene. A jugular blood sample was collected from each mare into a tube containing lithium heparin. Genomic DNA was extracted from the white blood cells of each mare and purified, using the PUREGENE DNA Purification Kit as described in Phase 2. Purified DNA samples were then quantified by UV spectrophotometry to estimate their concentration and purity. Forward and reverse primers were designed from the flanking introns of the genomic fragment that were isolated and sequenced in Phase 2. The forward primer (5’-CAGCGACTTGCAATGTATGG-3’) is located 16 bp upstream of the untranslated region (UTR) of exon 2. The reverse primer (3’-TCTATGTGGGTCCAGTGT-5’) is located 18 bp downstream from the end of exon 2 (Figure 4.2). The PCR was performed in a BioRad Thermocycler (Bio-Rad Laboratories, Hercules, CA), using the TOPO plasmid as the positive control. The PCR amplifications were carried out using Platinum Taq DNA Polymerase (Invitrogen) in 50-μl reactions following the basic PCR protocol included in the kit. The reaction contained 1-μl template DNA, 1-μl of forward primer at 20-μM concentration, 1-μl of reverse primer at 20-μM concentration, 1-μl Taq DNA Polymerase (5 U/μl), 1-μl 10mM dNTP mixture, 1.5-μl MgCl₂ (50mM), and 5-μl 10X PCR Buffer, minus Mg. Thermal cycling conditions included an initial denaturation at 94°C for 2 min to completely denature the template, followed by 30 cycles of denaturing at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec.
Figure 4.2. Forward and reverse sequencing primers. Forward primer (5’-CAGCGACTTGCAATGTATGG-3’) is located in intron 1, 16 bp upstream of the untranslated region (UTR) of exon 2. Reverse primer (3’-TCTATGTGGGTTCCAGTG-5’) is located in intron 2, 18 bp downstream from the end of exon 2.

**Agarose Gel Electrophoresis.** The samples were analyzed, along with a suitable DNA sized marker, by electrophoresis on a 1.2 % agarose EtBr gel. The voltage was at 80V. The buffer was poured off of the gel and the gel was transferred to the UV photo machine, where the band was carefully excised from the agarose gel with a sharp razor blade.

**Isolating the PCR Product.** The PureLink Quick Gel Extraction Kit (Invitrogen) was used for the purification of the DNA fragments from the agarose gel. Purified samples were transported to the Gene Lab at Louisiana State University School of Veterinary Medicine for automated DNA sequencing. Sequencing reactions were performed using Applied BioSystems BigDye Terminator version 3.1 chemistries on an Applied Biosystems Genetic Analyzer.

**Computer-assisted Analysis.** The Basic Alignment Search Tool (BLAST; Altschul et al., 1990) was used to translate DNA sequences and to conduct homology searches of the protein databases available at the National Center for Biotechnology Information, Bethesda, MD.
Results

**Phase 1.** A positive correlation between leptin and BCS was detected ($P = 0.0002$), with leptin concentrations being greater in horses with higher body condition scores (Figure 4.3). A positive correlation was also detected between leptin and gender ($P = 0.015$). The geldings were significantly different than the mares ($P = 0.005$), but the stallions were not significantly different from either the geldings ($P = 0.29$) or the mares ($P = 0.50$). The mean leptin value for the geldings was 3.6 ng/mL; for the mares, 7.6 ng/mL; and for the stallions, 5.6 ng/mL (Figure 4.4). Age was not a significant factor in the analysis ($P = 0.16$).

![Figure 4.3](image-url)  
**Figure 4.3.** Relationship between serum leptin concentrations and BCS. Individual concentrations of leptin are plotted against body condition scores, raw data ($n = 58; P = 0.0002$).
**Figure 4.4.** Leptin concentrations in mares, stallions and geldings. The geldings were significantly different than the mares \((n = 58; P = 0.005)\), but the stallions were not significantly different from either the geldings \((n= 58; P = 0.29)\) or the mares \((n= 58; P = 0.50)\).

**Phase 2.** Exon 2 and partial sequences of intron 1 and intron 2 were sequenced. Using the BLAST program (Altschul et al., 1990), our sequenced exon 2 was confirmed with published (partial) equine exon 2 (GenBank AY462242) and with the Bos taurus leptin (obese) gene (GenBank Accession # U50365). Exon 2 and the junctions of intron 1 and intron 2 are shown (Figure 4.5). There is a 26 bp UTR preceding the start codon of ATG in exon 2. Splice sites at the intron/exon boundaries obey the AG/GT consensus rule.
Figure 4.5. Exon 2 with partial sequence of flanking introns. Splice sites obey the AG/GT consensus rule at the intron/exon boundaries. Twenty-six bp UTR before start codon of ATG.
begins after exon 2 and ends upstream of glutamine. Exon 3 is a coding exon with an open reading frame coding of 199 amino acids.

Comparative genomics was used to locate potential regulatory elements within the 5’ and 3’ flanking regions of the equine ob gene. The potential transcription initiation sites were mapped in the 5’-flanking region of the leptin gene. A computer search of the 5’-flanking region for cis-acting regulatory elements revealed the presence of three copies of GC boxes (GGGCGG) a binding site for CCAAT/enhancer-binding protein (C/EBP) and an E box (CANNTG, N=G/T) (Prestridge, 1995). A TATA box-like sequence (TATAAAG) is located 30 bp upstream of exon 1.

Splicing donor and acceptor consensus sequences were located at the putative exon/intron borders. The predicted splice sites at the intron/exon boundaries were analyzed using the Softberry computer program (Softberry, Inc, Mount Kisco, NY). At the intron/exon boundaries, splice sites obey the AG/GT consensus rule, as these dinucleotides are at the first two and last two positions of the introns. The exon/intron borders were also determined by genomic comparisons through BLAST (Altschul et al., 1990) of the nucleotide sequences of the human, bovine and mouse ob gene (GenBank Accession No. U43653, U50365 and U18812 respectively).

The FGeneSH computer program (Softberry, Inc.) was used for predicting exon regions in the genomic DNA sequence; BLAST (Altschul et al., 1990) was also used to search for exons. Nucleotide identities of other species (Bos taurus, Homo sapiens, Mus musculus, Sus scrofa and Canis familiaris) were compared with the equine leptin gene by BLAST using NCBI genome databases. The equine nucleotide sequence was 78 to 87% identical to the leptin nucleotide sequences of the above mentioned species. The highest identity was found to mouse leptin (Table 4.1).
Table 4.1. Comparison of equine nucleotide sequence. The equine nucleotide sequence was 78 to 87% identical to leptin nucleotide sequences in other species. The highest identity was found to mouse leptin.

<table>
<thead>
<tr>
<th>Species</th>
<th>Identity %</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bos Taurus</td>
<td>78</td>
<td>U50365</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>78</td>
<td>U43653</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>87</td>
<td>U18812</td>
</tr>
<tr>
<td>Sus scrofa</td>
<td>82</td>
<td>U63540</td>
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<tr>
<td>Canis familiaris</td>
<td>78</td>
<td>AB020986</td>
</tr>
</tbody>
</table>

The FGeneSH computer program (Softberry, Inc.) was used for the prediction of the polyadenylation signal. The AAUAAA hexamer motif, the cleavage site (CA) and the GU-rich downstream element (DSE) was found. The AAUAAA hexamer is positioned 20 bp upstream of the CA dinucleotide, which precedes the actual site of transcript cleavage. A GU-rich element is positioned 26 bp downstream from the hexamer.

Phase 3. Based on the 10 mares tested herein, there does not appear to be a polymorphism in exon 2 of the equine leptin gene. The DNA sequences for all 10 mares were homologous; therefore, polymorphism is not a likely explanation for the high vs. low difference.

Discussion

Results from Phase 1 indicated that gender and BCS significantly affect leptin concentrations in horses, whereas age does not. These results regarding the effects of gender and age on leptin are inconsistent with previous research by Buff et al. (2002), who concluded that concentrations of leptin were greater in geldings and stallions than in mares, and tended to increase with the age of the animal. The discrepancy with regard to age may be due to the fact that the horses in our study ranged in age from 4 years to 23 years, while the horses in the Buff et
al. (2002) study ranged in age from 8 d to 24 yr. Furthermore, Buff et al. (2002) reported that the
greatest concentration of leptin was observed in horses greater than 12 yr old. The average age of
horse in our study was 12 years; consequently, the majority of the horses would have passed
their principal growth stages. Young horses are in the growth phase of development and thus
lower concentrations of leptin would be indicative of an animal with lower body fat and
requiring greater nutritional resources (Buff et al., 2002). More recent research (Chapter 3
herein) indicates no significant age effect on leptin levels.

The effect of gender in Phase 2 data is consistent with research by Cartmill et al. (2003),
who reported that mares had greater concentrations of leptin than geldings (10.4 ng/mL vs. 6.6
ng/mL). Moreover, the geldings in the Cartmill et al. (2003) study had an average BCS slightly
higher than the mares, indicating even a lesser leptin output per unit of fat mass. The mares and
geldings in the present experiment both had similar BCS (7.0 and 7.5, respectively), while the
stallions had an average BCS of 5.5. As suggested by Henson and Castracane (2003), after
accounting for body fat, the single most important determinant of serum leptin is gender, with
more circulating leptin in women than men of equivalent body fat. The authors (Henson and
Castracane, 2003) proposed that the gonadal steroids are responsible for this gender dichotomy
in serum leptin, influencing the site of fat deposition and directly acting on leptin synthesis in
adipose tissue.

These results regarding BCS and leptin were consistent with previous research showing
that circulating leptin concentrations are generally correlated to body fat mass (Fitzgerald and
McManus, 2000; Buff et al., 2002; Gentry et al., 2002; Cartmill et al., 2003). As seen in Chapter
3 herein, the relationship between BCS and leptin concentrations appears greatest in non-
lactating mares of high BCS and less evident in lactating mares.
The leptin protein is highly conserved in molecular structure among species. To further understand the physiologic and pathophysiologic roles of the equine leptin gene, it is important to clarify the structural organization of the gene (Isse et al., 1995). Comparative genomics was used to further identify and characterize potential regulatory elements in the 5’ and 3’ flanking regions of the equine leptin gene (BLAST, Altschul et al., 1990). The potential structural organization of the equine leptin gene is reported herein, supported by EST and cDNA data from bovine, human, mouse, pig and dog species (GenBank Accession No.U50365, U43653, U18812, U63540, and AB020986, respectively) and with data from the equine whole genome (GenBank Accession No. AAWR00000000). Regulatory sites within the above mentioned orthologs showed significant correlations.

One of the most common regulatory elements is the GC box, which is widely distributed in promoter regions of tissue-specific genes (Philipsen and Suske, 1999). A computer search (PROMOTER SCAN, Prestridge, 1995) of the 5’-flanking region for cis-acting regulatory elements revealed the presence of three copies of GC boxes (GGGCGG). It was generally thought that Sp1 (named according to the original purification scheme that included Sephacryl and phosphocellulose columns) was the major factor acting through the GC box motif. Recent discoveries determined that Sp1 is only one of many transcription factors binding and acting through these elements (Philipsen and Suske, 1999).

A TATA box-like sequence (TATAAG) is located 30 bp upstream of the exon 1. A TATA box is considered to be the core promoter sequence in eukaryotes (Smale and Kadonaga, 2003).

A computer search (PROMOTER SCAN, Prestridge, 1995) of the 5’-flanking region also revealed the presence of an E box (CANNTG, N=G/T). The cis-element E box is present in the
regulatory regions of many tissue-specific genes (Kim et al., 1995) and is the binding site for the basic-helix-loop-helix proteins (bHLH). The E box is a highly conserved DNA element (CANNTG) recognized by the bHLH family of transcription factors, and acts as a cell type-specific enhancer of class I promoter activity. Precise DNA binding site selection by individual bHLH family members is determined both by the central dinucleotide contained in the core hexamer sequence and the flanking nucleotides (Howcroft et al., 1999). The central dinucleotide found in the equine leptin gene E box is GT.

One putative binding site for members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors was identified immediately upstream from the TATA box (PROMOTER SCAN, Prestridge, 1995). The observation that \( ob \) gene expression is positively influenced by C/EBP\( \alpha \) is consistent with numerous studies showing the role of this transcription factor in adipogenesis and energy homeostasis (De La Brousse et al., 1996). In mouse research, the C/EBP\( \alpha \) transcription factor has been implicated as an indispensable transcriptional activator of adipocyte genes during preadipocyte differentiation. The obese gene, like many other adipocyte genes, is most likely transcriptionally activated by C/EBP\( \alpha \) during preadipocyte differentiation. Expression of the mouse obese (\( ob \)) gene is immediately preceded by the expression of C/EBP\( \alpha \). While the 5’ flanking region of the mouse \( ob \) gene contains several consensus C/EBP binding sites, only one of the sites appears to be functional (Hwang et al., 1996). Further studies with the equine leptin gene are needed to elucidate the functional significance of this 5’ \emph{cis}-acting regulatory element.

Polyadenylation is one of the mRNA maturation steps in eukaryotic cells. The AAUAAA hexamer is the almost invariant poly(A) signal that is positioned ~ 15 nt upstream of the site of cleavage. The sequence AAUAAA is one of the most highly conserved sequence
elements known (Zhao, et al., 1999). A poorly conserved GU-rich or U-rich element is usually positioned 20 – 30 nt downstream of the hexamer. These two elements form the core poly(A) site (Klasens et al., 1998; Takagaki and Manley, 1997). The cleavage site itself, which becomes the point of poly(A) addition, is determined mainly by the distance between the upstream AAUAAA sequence and the downstream elements (DSE). A CA dinucleotide defines the poly(A) site for most genes (Zhao, et al., 1999). We used comparative genomics to identify and characterize functional polyadenylation sites in the human, bovine, and equine leptin genes (BLAST, Altschul et al., 1990). To compare poly(A) sites located on ortholog gene pairs in human and bovine to equine, we aligned 3’ UTR regions of the three leptin genes, and defined these as “conserved” poly(A) sites displaying aligned poly(A) signals.

This study provides a better understanding of the molecular mechanisms underlying the leptin gene and should aid in leptin gene research within the equine species. Single gene polymorphisms resulting in obesity are very rare (Mergen et al., 2006), and therefore it appears more likely that a combination of polymorphisms in one or more candidate genes may contribute to the development of obesity. Nevertheless, distinct differences in leptin secretion rates do indeed persist in a percentage of obese mares, and as Cartmill et al. (2003) reported, these differences are associated with perturbations in insulin, glucose, and thyroid hormone concentrations. Based on the five high leptin and five low leptin mares tested, there was no apparent polymorphism in exon 2 of the equine leptin gene. Therefore, polymorphism is not a likely explanation for the high versus low difference. Larger studies, including testing of multiple genes in both obese and lean subjects, will likely be needed to better understand the role of leptin in regulating weight. Because of the complex interaction with other molecules such as hormones, growth factors, and cytokines, future studies should also consider possible
interactions with other genetic polymorphisms. Polymorphisms in the 5’ promoter region and exon 3 should also be considered. The pathogenesis of obesity is complex and the interaction between the large number of both genetic and environmental factors needs to be considered.
CHAPTER 5

ENDOCRINE RESPONSES TO ADMINISTRATION OF A LOW DOSE OF ENDOTOXIN LIPOPOLYSACCHARIDE IN MARES AND GELDINGS WITH HIGH VS. LOW PLASMA LEPTIN CONCENTRATIONS

Introduction

During periods of immune challenge, pro-inflammatory cytokines orchestrate a homeorhetic response within the body. Organisms respond to the inflammation by mounting a complex and coordinated sequence of events involving the immune, nervous, and endocrine systems in order to restore homeostasis (Spurlock, 1997).

Leptin has been identified as a crucial endocrine factor in the regulation of the immune response (Henson and Castracane, 2003). Research (Faggioni et al., 1998, 2001) has shown that leptin levels are increased by pro-inflammatory cytokines TNF-α and IL-6. Leptin concentrations acutely increase during infection and inflammation, and may represent a protective component of the host response to inflammation. Gainsford et al. (1996) report that leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells and can enhance the production and phagocytic activity of macrophages. Faggioni et al. (1999) reported that leptin deficiency is accompanied by increased susceptibility to lipopolysaccharide (LPS)-induced lethality and decreased induction of anti-inflammatory cytokines, supporting the hypothesis that leptin is an important cytokine component of the acute phase response. From the aforementioned research, one may speculate that hypoleptinemia might result in an impaired host defense that would increase susceptibility to infection.

Understanding the protective role of leptin, with leptin’s ability to influence cytokine production, can help in understanding the biological basis for the communication between the neuroendocrine system and the immune system. The focus of the present experiment was to
investigate the role and/or regulation of leptin during a low-dose infusion of endotoxin to mares and geldings with high vs. low plasma leptin concentrations.

**Materials and Methods**

**Selection of Horses.** All horses were of light horse breed from the resident herd at the Louisiana Agricultural Experiment Station-Central Stations Horse Farm. The horses were routinely maintained on native grass pastures during the spring, summer, and fall, and on ryegrass pasture in winter. Two weeks before to the experiment, the total population of 50 mares and geldings was assigned a BCS as described by Henneke et al (1983; 1 = extremely emaciated through 9 = extremely fat) by one experienced technician. Two early morning (before 8:00 a.m.) jugular blood samples were collected from each horse during a two week interval into heparinized tubes for determination of initial leptin and insulin concentrations. From the mares and geldings with a mean BCS of at least 7.5, 16 horses were selected that had the lowest (low leptin; 4 mares and 4 geldings) and highest (high leptin; 4 mares and 4 geldings) leptin concentrations.

**Daily Monitoring.** To establish baseline daily concentrations of the various cytokines and hormones of interest, samples of jugular blood were collected at 48-hr intervals, beginning 6 d before the experiment. The horses were brought from the pasture on each occasion and sampled within 1 h or less. These samples were collected into evacuated, heparinized tubes, and the plasma was harvested by centrifugation (1,200 x g for 20 min at 5°C) and stored at -15°C.

**Experimental Design.** The experiment was performed as a switch-back with eight mares and eight geldings, half of which from each gender were hyperleptinemic (as defined by Cartmill et al., 2003) and the rest were normoleptinemic. Due to restrictions in equipment and facilities, the experiment was conducted in two replications of eight horses each, balanced as to gender and
leptin status. The first replicate was initiated on July 28, when half of the horses received endotoxin infusion and the other half received vehicle infusion, and reached completion on August 1, when the treatments were reversed. The second replicate was performed between August 5 and 11.

Instrumentation. On the morning of the infusions, eight horses (2 high leptin mares; 2 high leptin geldings; 2 low leptin mares; 2 low leptin geldings) were brought in from the pasture at 0600, placed in individual stalls, and tethered. A 14-guage, 13.3-cm Teflon coated catheter (BD, Franklin Lakes, NJ) was inserted into the left jugular vein for collection of blood. Another catheter was inserted into the right jugular vein for infusion of endotoxin or saline solution. Two randomly selected horses were moved from their stall at 0700, placed in individual stocks, and cross-tied. The horses were then prepared for the infusion. This protocol was repeated each hour, until all 8 horses had been infused.

Hematologic Variables. Blood samples were collected before endotoxin or saline infusion at -60, -30, -20, -10 min. Frequent blood samples were taken after endotoxin or saline infusion (t = 0) at 20, 30, 60, 90, 120, 150, 180, 210, 240 min, and 6, 8, 12, 16, 20, and 24 h. Blood samples were collected into tubes containing heparin and were centrifuged within 30 min and the plasma was stored at -15° C.

Jugular venous blood was collected into tubes containing EDTA for CBC determination. CBC blood samples were taken at t = 0 and 1 and 2 h post-infusion. The tubes were transported, on ice, to the LSU School of Veterinary Medicine's Clinical Pathology Laboratory for evaluation. Variables determined were: total white blood counts, segmented neutrophils, neutrophil bands, lymphocytes, platelets, packed cell volume, and total protein.
Clinical Signs of Disease. Clinical signs of disease were monitored and recorded during the 24-hour time period. Heart rate (beats per minute), respiratory rate (breaths per minute), mucous membrane color, capillary refill time (seconds), rectal temperature (° F), and gastrointestinal sounds were monitored and recorded at -60, 0, 1, 2, 3, and 4 h, then every 2 h until 16 h after infusion.

Blood Analyses. Concentrations of insulin (Diagnostic Systems Laboratory, Webster, TX), growth hormone (Thompson et al., 1992) and cortisol (DSL) were determined by radioimmunoassay previously validated for horse samples (Cartmill et al., 2003). Assay sensitivities and intra- and inter-assay coefficients of variation were 0.1 ng/mL, 5% and 8% for insulin; 0.5 ng/mL, 8% and 11% for GH; and 0.11 μg/dL, 5% and 8% for cortisol. Concentrations of leptin were measured with a double-antibody leptin radioimmunoassay procedure 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-μL sample size, was 0.1 ng/mL. Prolactin concentrations were estimated in plasma by radioimmunoassay previously validated for horses (Colborn et al., 1991). Intra- and interassay CV and assay sensitivity were 7%, 12% and 0.2 ng/mL.

Statistical Analyses. The data was analyzed by ANOVA via the GLM procedure of SAS (SAS Institute, Cary, NC). Main effects of leptin status and gender were factors in the analyses, as well as the interaction; these latter effects were tested with a horse (leptin status x gender) term. Endotoxin treatment, day, square and the horse (leptin status x gender) term were analyzed in the same ANOVA as replicated (n = 4) 2 x 2 Latin squares, using the 4-way interaction (square, treatment, day and horse) term as the error term. Time (minutes relative to endotoxin treatment) and the treatment x time interaction were tested with residual error.
Results

Total Leukocytes. There was no difference in total leukocyte counts between the high leptin group and the low leptin group, or between gender (Figure 5.1). Endotoxin administration resulted in the activation of leukocytes. Endotoxin-treated horses had fewer total leukocytes ($P = <.0001$) from post-infusion hour 1 through 2.

Total Lymphocytes. There were no differences in total lymphocyte counts between the high leptin group and the low leptin group. Mares had a significantly higher ($P = 0.0447$) lymphocyte number as compared to the geldings. Changes in lymphocyte counts mimicked changes in total leukocyte counts. Endotoxin-treated horses had total lymphocyte numbers decrease ($P = 0.001$) at post-infusion hour 1 through 2 (Figure 5.2).

Figure 5.1. Mean total blood leukocyte counts. Endotoxin-treated horses had fewer total leukocytes ($P < .0001$) than saline-infused horses.
Figure 5.2. Mean total blood lymphocytes counts. Endotoxin-treated horses had fewer total lymphocytes \((P = .0001)\) than saline-infused horses.

**Total Neutrophils.** There were no differences in total neutrophils counts between the high leptin group and the low leptin group, nor between gender. Neutrophil migration increased dramatically during the first hour of LPS exposure. Endotoxin-treated horses had segmented neutrophil numbers decrease significantly \((P < 0.0001)\) at post-infusion hour 1 through 2 (Figure 5.3). No neutrophil bands were observed during this time-frame.

**Platelet Counts.** Endotoxin-treatments had no effect on platelet counts (Figure 5.4), although there was a difference in leptin \((P = 0.012)\), gender \((P = 0.06)\), and leptin x gender \((P = 0.07)\).
Figure 5.3. Mean total blood neutrophil counts. Endotoxin-treated horses had segmented neutrophil numbers decrease significantly ($P < 0.0001$) as compared to saline-infused horses.

Figure 5.4. Mean total platelet counts. Mean total platelet counts (cells/μl) in horses given a low dose of endotoxin.
Packed Cell Volume and Total Protein. Endotoxin-treated horses had packed cell volume (PCV) increase ($P < 0.0001$) at hour 1 through hour 24 (Figure 5.5). Total protein was also increased ($P = 0.0012$) during the 24-hour period (Figure 5.6). However, there were no differences between the high leptin group and the low leptin group, nor between gender.

With the exception of the platelet counts, changes in all of the hematologic variables were observed after LPS-infusion. Additionally, with the exception of the platelet counts, there was no leptin status effect. (Table 5.1). With the exception of lymphocyte counts, there was no gender effect. Mares had a significantly higher ($P = 0.0447$) lymphocyte number as compared to the geldings.

![Figure 5.5. Mean packed cell volume. Mean packed cell volume (PCV) in horses given a low dose of endotoxin.](image-url)
Figure 5.6. Mean total protein. Mean total protein (TP) in horses given a low dose of endotoxin.

Table 5.1 Sources of variation in the ANOVA and associated P-values for each hematologic variable in the 24-h sampling period

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>PCV</th>
<th>TP</th>
<th>WBC</th>
<th>Lymphocytes</th>
<th>Platelets</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>LepStatus</td>
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<td>0.2837</td>
<td>0.2836</td>
<td>0.8249</td>
<td>0.7378</td>
<td>0.0126</td>
<td>0.8567</td>
</tr>
<tr>
<td>Gender</td>
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<td>0.0912</td>
<td>0.1384</td>
<td>1.000</td>
<td>0.0447</td>
<td>0.0618</td>
<td>0.6919</td>
</tr>
<tr>
<td>Gender*LepStatus</td>
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<td>0.8993</td>
<td>0.5742</td>
<td>0.6879</td>
<td>0.1338</td>
<td>0.0700</td>
<td>0.4533</td>
</tr>
<tr>
<td>Time</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender*Time</td>
<td>2</td>
<td>0.9198</td>
<td>0.9152</td>
<td>0.5752</td>
<td>0.9072</td>
<td>0.4818</td>
<td>0.4561</td>
</tr>
<tr>
<td>LepStat*time</td>
<td>2</td>
<td>0.0678</td>
<td>0.1263</td>
<td>0.9925</td>
<td>0.0677</td>
<td>0.8362</td>
<td>0.9705</td>
</tr>
<tr>
<td>Gender<em>LepStat</em>time</td>
<td>2</td>
<td>0.9722</td>
<td>0.4264</td>
<td>0.8149</td>
<td>0.6114</td>
<td>0.5548</td>
<td>0.6126</td>
</tr>
<tr>
<td>Trt*time</td>
<td>2</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.1512</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Clinical Signs of Disease. Heart rate was increased significantly ($P < .0001$) from post-infusion hour 1 through 8 in endotoxin-treated horses (Figure 5.7). Respiratory rate also increased ($P < .0001$) in endotoxin-treated horses (Figure 5.8). Temperature accordingly increased ($P < .0001$) in endotoxin-treated horses (Figure 5.9). Mucous membrane color was not consistent among horses; however, all treated horses developed tacky and injected mucous membranes during the first few hours after endotoxin infusion. Capillary refill time increased to three seconds on endotoxin-treated horses, compared with the baseline value of <2 seconds. Endotoxin-treated horses showed signs of lethargy, transient abdominal pain and mild depression from post-infusion hour 0.5 to 8. With regard to clinical signs of disease, there were no significant differences between the high leptin group and the low leptin group, nor between gender.

Figure 5.7. Mean heart rate. Horses were administered an infusion of endotoxin (lipopolysaccharide; 35 ng/kg during a 30-minute period) or an equivalent volume of saline (0.9% NaCl) solution during the same time period.
Figure 5.8. Mean respiratory rate. Horses were administered an infusion of endotoxin (lipopolysaccharide; 35 ng/kg during a 30-minute period) or an equivalent volume of saline (0.9% NaCl) solution during the same time period.

Figure 5.9. Mean rectal temperature. Horses were administered an infusion of endotoxin (lipopolysaccharide; 35 ng/kg during a 30-minute period) or an equivalent volume of saline (0.9% NaCl) solution during the same time period.
Insulin. Insulin levels were measured two weeks before the start of the experiment. Insulin levels were different ($P = 0.0026$) with regard to leptin status, with high leptin horses having higher insulin levels than the low leptin horses (Figure 5.10).

Cortisol. The overall mean cortisol level during the 24-hour period was significantly different ($P = 0.0034$) between the endotoxin-treated horses and the saline-infused horses (Figure 5.11). Cortisol levels began to rise at one hour post-treatment and remained high until post-infusion hour 8. However, there was no leptin or gender effect.

Growth Hormone. The overall mean growth hormone (GH) concentrations during the 24-hour period was significantly different ($P = 0.0086$) between the endotoxin-treated horses and the saline-infused horses (Figure 5.12). Although, endotoxin-treated horses had higher GH levels than the control horses, there was no leptin or gender effect.

![Graph showing resting concentrations of insulin.](image)

**Figure 5.10.** Resting concentrations of insulin. Resting concentrations of insulin (mIU/L) in horses selected for high vs. low leptin concentrations.
Figure 5.11. Mean concentrations of cortisol. Concentrations of cortisol (μU/mL) in horses administered an infusion of LPS or an equivalent volume of saline solution.

Figure 5.12. Mean concentrations of GH. Concentrations of GH (ng/mL) in horses administered an infusion of LPS or an equivalent volume of saline solution.
**Prolactin.** The overall mean prolactin concentration during the 24-hour period was significantly different ($P = 0.0073$) between the endotoxin-treated horses and the saline-infused horses (Figure 5.13). Endotoxin-treated horses had higher PRL levels than the control horses, but there was no leptin or gender effect.

**Leptin.** The overall mean plasma leptin concentrations during the 24-hour period was significantly different ($P = 0.0076$) between the endotoxin-treated horses and the saline-infused horses (Figure 5.14). Endotoxin-treated horses had higher leptin levels than the control horses, although there was no gender effect.

![Figure 5.13.](image_url) Mean concentrations of prolactin. Concentrations of PRL (ng/mL) in horses administered an infusion of LPS or an equivalent volume of saline solution.
Figure 5.14. Mean concentrations leptin. Concentrations of plasma leptin (ng/mL) in horses administered an infusion of LPS or an equivalent volume of saline solution.

During the 24-h experiment, all horses receiving the low-dosage of endotoxin had significant clinical signs of endotoxemia. However, there were no differences with regard to leptin status or gender. From the results of this experiment, it appears that hyperleptinemia does not alter the pro-inflammatory cytokine response after administration of a low-dose of endotoxin to healthy, conscious, adult horses (Table 5.2).

Discussion

During the experiment an initial leucopenia, neutropenia and lymphopenia were observed within 1 to 2 h after LPS infusion. Experimentally, administration of endotoxin to horses results in neutropenia within 90 min with a return to baseline numbers within 6 to 18 h (Reed et al., 2004). No neutrophil bands were observed, indicating no release of immature neutrophils from the bone marrow. Lymphopenia is frequently associated with severe systemic stress and acute systemic insult.
Table 5.2. Sources of variation in the ANOVA and associated P-values for each hormone or cytokine measured in the 24-h sampling period

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>Leptin</th>
<th>Insulin*</th>
<th>Cortisol</th>
<th>PRL</th>
<th>GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LeptinStatus</td>
<td>1</td>
<td>0.0018</td>
<td>0.0026</td>
<td>0.3516</td>
<td>0.7631</td>
<td>0.7082</td>
</tr>
<tr>
<td>Gender</td>
<td>1</td>
<td>0.7355</td>
<td>0.2561</td>
<td>0.3788</td>
<td>0.8421</td>
<td>0.4828</td>
</tr>
<tr>
<td>Gender*LeptinStatus</td>
<td>1</td>
<td>0.3189</td>
<td>0.9901</td>
<td>0.1126</td>
<td>0.1953</td>
<td>0.5706</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
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<td>*</td>
<td>&lt;0.0001</td>
<td>0.0030</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender*Time</td>
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<td>0.2311</td>
<td>*</td>
<td>0.2268</td>
<td>0.2611</td>
<td>0.2444</td>
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<tr>
<td>LeptinStat*time</td>
<td>2</td>
<td>0.3926</td>
<td>*</td>
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<td>0.1956</td>
<td>0.9576</td>
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<tr>
<td>Gender<em>LeptinStat</em>time</td>
<td>2</td>
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<td>*</td>
<td>0.8705</td>
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<tr>
<td>Trt*time</td>
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<td>*</td>
<td>&lt;.0001</td>
<td>0.0031</td>
<td>0.1420</td>
</tr>
</tbody>
</table>

* Insulin was measured 2 wk before the experiment and not during the endotoxin-infusion.

During the first 2 h of the experiment, LPS treatments had no effect on platelet counts, although the high leptin horses exhibited higher platelet counts than the low leptin horses. Geldings also had higher platelet counts than the mares. Studies in humans (Nakata, et al., 1998; Konstantinides et al., 2001) suggested that platelets express the long form of the leptin receptor, and high concentrations of leptin are reported to act synergistically with ADP to promote platelet aggregation. Nakata et al. (1999) concluded that a high concentration of leptin has the function of promoting platelet aggregation, which may explain why obese people develop dangerous
blood clots. A main theory for the pathogenesis of equine acute laminitis involves the
development of vasoconstriction within the foot, which leads to ischemic and/or inflammatory
tissue damage (Elliot and Bailey, 2006). Extremely high platelet counts could lead to
thrombosis, possibly increasing the risk of equine laminitis or organ dysfunction (Brown and
Bertone, 2002).

The PCV and TP of endotoxin-treated horses rose above the baseline at 1 h and continued
through 24 h of the experiment. The PCV is a useful monitor of dehydration. In general terms, a
PCV greater than 45% indicates a reduction in the fluid fraction of the blood, thereby raising the
relative level of red blood cells. Pain, excitement, or other stresses, can increase the PCV
markedly, but generally does not influence TP.

With the exception of the platelet counts, all of the changes observed in the hematologic
variables were significant in regard to LPS-infusion. Additionally, with the exception of the
platelet counts, there was no leptin status effect. With the exception of the lymphocyte counts,
there was no gender effect in the hematologic variables studied. Mares had a significantly higher
\( P = 0.0447 \) lymphocyte number as compared to the geldings.

During the 24-h experiment, all horses receiving the low-dosage of endotoxin had
significant clinical signs of endotoxemia. However, there were no differences with regard to
leptin status or gender. Moore and Barton (1998) reported that common clinical effects of
endotoxemia are increased heart and respiratory rates, alterations in the color of the horse’s
mucous membranes, prolongation of the capillary refill time, fever, and reduced gastrointestinal
sounds. Results of the present experiment were consistent with Clark and Moore (1989), which
reported that all horses in the experiment, having received a slow infusion of a low-dosage of
endotoxin, became depressed, displayed injected mucous membranes, and showed increases in heart rate, respiratory rate and temperature.

Plasma insulin concentrations were measured 2 wk before the start of the experiment. Insulin levels were significantly different with regard to leptin status, with high leptin horses having higher insulin levels than the low leptin horses. This finding is consistent with Cartmill et al. (2003), which showed that obese hyperleptinemic mares and geldings had elevated resting insulin concentrations as well as an exaggerated insulin response to glucose infusion. In a number of species, the two factors most often reported to stimulate leptin secretion either in vivo (Larsson and Ahren, 1996; Miell et al., 1996; Ramsay and White, 2000) or in vitro (Cammisotto and Bukowiecki, 2002) are glucocorticoids and insulin.

Cortisol, a steroid hormone produced and secreted by the adrenal cortices, is the physiological glucocorticoid in horses. In the present experiment, the overall mean cortisol level during the 24-h period was significantly different between the endotoxin-treated horses and the saline-infused horses, although there was no leptin status or gender difference. These results are conflicting to what was previously observed by Cartmill and co-workers (2003), in that mares selected for high leptin had greater cortisol concentrations than mares selected for low leptin, with the opposite being true for geldings.

Toutain et al., (1987) reported that the most typical feature of hydrocortisone profiles in horses is a relatively regular decrease in plasma hydrocortisone concentrations during the afternoon and the beginning of the dark period. In the present experiment, no diurnal rhythm in total cortisol concentrations was apparent in individual horses or in the group mean. According to Irvine and Alexander (1993) the diurnal rhythm can be disrupted by removing the horse from
its accustomed environment. The horses in the present experiment were moved from their pasture into stalls in a covered barn.

Overall mean GH levels during the 24-h experiment differed between the LPS-infused horses and saline-infused horses; however, there was no leptin status effect nor gender effect. This is inconsistent with previous research by Cartmill et al. (2003), who reported that horses selected for high leptin had lower concentrations of growth hormone. Reports in other species indicated a negative relationship between leptin and GH concentrations (Spurlock et al., 1998; Isozaki et al., 1999; Elimam et al., 2001), perhaps via a direct inhibitory effect of GH on adipocytes. Nevertheless, this negative relationship was not shown in the present experiment.

In the present experiment, the overall mean prolactin levels during the 24-h period differed between endotoxin-treated horses and saline-infused horses; nonetheless, there was no leptin status nor gender effect. Recent research concluded that prolactin is a multi-functional hormone and is secreted not only by the anterior pituitary gland, but also by many extra-pituitary sites, including the immune cells (Ben-Jonathan et al., 1996; Matera et al., 2000; De Bellis et al., 2005; Malaguarnera et al., 2005). Chikanza and Grossman (1998) categorized prolactin as a pro-inflammatory neuropeptide, capable of modulating immune and inflammatory responses. Inflammatory cytokines IL-6 and TNF-α are also able to induce release of pituitary prolactin (Chikanza and Grossman, 1998; DeBellis et al., 2005).

In the present experiment, overall mean plasma leptin levels during the 24-h period differed between endotoxin-treated horses and saline-infused horses; however, there was no gender effect. This is in contrast to previous reports of Buff et al. (2002), who indicated that serum leptin concentrations were greater in stallions and geldings, compared to mares. Others (Cartmill et al., 2003) reported that mares displayed greater average plasma leptin concentrations
than geldings. Nevertheless, the present experiment found no gender effect with regard to leptin levels.

The horses in the present experiment were allowed free access to water, but no feed or hay. This fact would account for the decrease in leptin levels in both the LPS-infused and saline-infused horses at 5 h into the experiment.

From the results of this experiment, it appears that hyperleptinemia does not alter the pro-inflammatory cytokine response after administration of a low-dose of endotoxin to healthy, conscious, adult horses. Despite the fact that all of the horses developed clinical signs and showed hematologic changes associated with an acute phase response, leptin status had no effect on the production of TNF-α or IL-6. The results do not exclude an effect of leptin on the immune response, but they suggest that obese, hyperleptinemic horses respond the same as obese normoleptinemic horses to a low-dose of endotoxin.
CHAPTER 6
SUMMARY AND CONCLUSIONS

The goal of the research herein was to investigate the physiologic and genetic differences between obese hyperleptinemic horses and obese normoleptinemic horses. Distinct differences in leptin secretion rates do persist in a percentage of obese mares; therefore, three experiments were designed and performed to study and possibly provide an explanation as to the cause.

The first study concluded that the occurrence of pregnancy was shown to be equally likely between hyperleptinemic mares and normoleptinemic mares. Leptin levels in lactating mares were reported to be generally lower than leptin levels in non-lactating mares, which is consistent with other research. It may be hypothesized that a specific and narrow range of leptin concentrations is necessary to maintain a normal reproductive function, and that concentrations below or above these thresholds might interfere in opposing ways with the function of the hypothalamic-pituitary-gonadal axis. However, to assign a global threshold number for infertility would be unrealistic. The high and low boundaries would likely be on an individual basis, due to the complex interaction with other molecules such as hormones, growth factors, and cytokines.

The second experiment explored the possibility of a polymorphism within exon 2 of the equine leptin gene. No polymorphism within exon 2 was observed between mares that were high vs. low secretors of leptin; therefore, polymorphism is not a likely explanation for the high vs. low difference. Single gene polymorphisms resulting in obesity are very rare, thus the conclusion appears more likely that a combination of polymorphisms in one or more candidate genes may contribute to the development of obesity.
The objective of the third experiment was to further consider the high leptin versus low leptin condition, and the interaction of leptin secretion between the endocrine and immune systems. Although there were significant differences between the endotoxin-infused group and the saline-infused group of horses, with the exception of the platelet counts, there were no significant differences between the obese hyperleptinemic group and the obese normoleptinemic group of horses or between mares or geldings.

Distinct differences in leptin secretion rates do indeed persist in a percentage of obese mares, and as reported previously, these differences are associated with perturbations in insulin, glucose, and thyroid hormone concentrations. Because of the complex interactions among leptin and other molecules such as hormones, growth factors, and cytokines, future studies should again consider possible interactions with other body systems that regulate obesity. The pathogenesis of obesity is complex and interactions between the large number of both genetic and environmental factors must be considered. The knowledge of the interactions among these factors will further enhance equine performance and health.
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