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Reproductive characteristics of high body condition mares with high versus low leptin concentrations

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REPRODUCTIVE CHARACTERISTICS OF HIGH BODY CONDITION MARES
WITH HIGH VERSUS LOW LEPTIN CONCENTRATIONS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirement for the degree of
Master of Science

in

The Interdepartmental Program in Animal and Dairy Sciences

by

Cara Alexandra Waller
B.S., University of Maryland, College Park, 2003
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ABSTRACT

Two experiments were performed to discover what, if any, reproductive differences exist in high body condition (BCS) mares with varying concentrations of leptin. Previous research showed that mares with high body condition scores can have a range of leptin levels, from very low (<5 ng/mL) to very high (>10 ng/mL). Earlier results indicated that most mares with high body condition scores maintain estrous cycles or show significant follicular activity during the winter. Among these high BCS mares, about 30% of them exhibit hyperleptinemia and hyperinsulinemia. The first experiment was designed to compare the reproductive characteristics of high BCS mares with high versus low leptin levels during vernal transition and the first estrous cycle. Also, an IVGTT, insulin challenge, and two sulpiride challenges were performed to characterize endocrine profiles of these mares. Results of these challenges were similar to previous work, in that hyperleptinemic mares had greater insulin responses to glucose and greater clearance rates of infused glucose compared to low leptin mares. These mares also showed a slightly greater prolactin response to sulpiride than their low leptin counterparts. When analyzing the reproductive traits of these mares, no differences between groups existed for follicular sizes or distributions, gonadotropin levels, or date of first ovulation. The second experiment was designed to assess what differences may exist between high BCS mares with high versus low leptin levels with regard to their gonadotropin and ovarian responses to several analogs of gonadotropin releasing hormone (GnRH). Histrelin and deslorelin appeared to be the most potent of the analogs, followed by buserelin and then GnRH. Leptin status did not appear to affect the gonadotropin response or time to ovulation for any of the analogs used in this trial. In
conclusion, although hyperleptinemic mares showed altered insulin and glucose characteristics, no significant reproductive differences were observed between these mares and their normal counterparts in terms of ovarian function or the hypothalamic-pituitary axis during vernal transition or the estrous cycle.
INTRODUCTION

Leptin is thought to be a signal communicating the nutritional status of the body to the brain and eventually to the reproductive system. In horses, leptin levels are correlated with body condition, age, season and gender (Fitzgerald and McManus, 2000; Gentry et al., 2002a,b; Buff et al., 2002; Cartmill et al., 2003b). Mares and geldings have greater leptin levels than stallions (Cartmill et al., 2003b), and aged mares have increased leptin concentrations compared to young mares (Fitzgerald and McManus, 2000; Buff et al., 2002). In both aged and young mares, circulating leptin levels tend to be higher in the summer than in the winter (Fitzgerald and McManus, 2000; Gentry et al., 2002b). Both short (McManus and Fitzgerald, 2000) and long term (Gentry et al., 2002b) feed restriction cause a decline in leptin levels.

Leptin has several effects on reproduction in mammals. It can restore normal reproductive function in sterile \textit{ob/ob} mice (Barash et al., 1996; Chehab et al., 1996; Mounzih et al., 1997) and it is necessary for onset of puberty in humans, mice, and cattle (Ahima et al., 1997; Chehab et al., 1997; Garcia et al., 2002). In horses, a large proportion of mares with high body condition do not experience a traditional anovulatory season in the winter (Gentry et al., 2002b). Interestingly, among those mares with high body condition, a wide variation of leptin levels existed: from very low (<5 ng/mL) to very high (>10 ng/mL) (Gentry et al., 2002b). The range in differences of leptin levels in the mares of high body condition were consistently observed up to three years later (L. R. Gentry, unpublished), which implies some innate factor in determining leptin levels in the mare.
The question arose as to what, if any, reproductive, transitional, or estrous cycle differences might exist among mares of high body condition score with varying levels of leptin. The first experiment described herein was designed to observe mares throughout vernal transition and through one estrous cycle to determine if any underlying reproductive differences exist in high body condition mares with very high or low leptin concentrations.

Manipulation of the estrous cycle is one of the major focuses of the horse industry. Bringing mares into estrus earlier, inducing ovulation, and shortening the estrous cycle are all goals of the industry. Human chorionic gonadotropin (hCG) is a protein that shortens estrus and increases the number of ovulations occurring within 48 h of treatment (Voss, 1993), but because it is a human protein, antibodies are formed in the mare and thus responsiveness after subsequent uses is diminished (Sullivan et al., 1973; Voss, 1993). Gonadotropin releasing hormone (GnRH) and its analogs are becoming popular objects of study for their potential ability to increase follicular growth and induce ovulation without the problems associated with hCG. Frequent administration of GnRH itself can induce follicular development in anestrous mares, and can also cause deviation of the pre-ovulatory follicle (Irvine, 1993b). Deslorelin acetate is an analog of GnRH that can cause ovulation within 48 h of treatment if given to an estrous mare exhibiting a follicle of 35 mm or greater (McKinnon et al., 1993; Meinart et al., 1993; Squires et al., 1994). Buserelin and histrelin are other GnRH agonists that can encourage ovulation and an increase in gonadotropin levels (Squires et al., 1981; Barrier-Battut et al., 2001).
However, large doses of these analogs can cause a prolonged interovulatory interval and potential inhibition of follicular development (Johnson et al., 2000; Farquhar et al., 2001; Blanchard et al., 2002).

The second experiment described herein was originally designed to compare the efficacy of deslorelin, histrelin, buserelin, and GnRH itself at two doses (166.5 and 500 µg) for stimulating gonadotropin release and inducing ovulation. To determine whether leptin status (high vs low) affected the responsiveness to these secretagogues, mares of both leptin classifications were interspersed throughout the treatment assignments. Overall, these two experiments provide information on the impact of hyperleptinemia and hyperinsulinemia on the reproductive traits of high body condition mares during the vernal transition, the estrous cycle, and during treatment with GnRH analogs.
CHAPTER I

REVIEW OF LITERATURE

Hypothalamic-Hypophyseal-Gonadal Axis

It is general knowledge that the hypothalamus controls the reproductive axis through release of GnRH. In the horse, GnRH is synthesized evenly from all areas of the hypothalamus (Irvine and Alexander, 1993). GnRH is stored in secretory granules in the median eminence which are released into the capillary beds of the hypothalamic-hypophyseal portal system when stimulated by neuronal activity (Irvine and Alexander, 1993). The target tissue of GnRH in the mare is the adenohypophysis, which releases quantities of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), in response to GnRH. Gonadotropin releasing hormone is released from the hypothalamus in a pulsatile fashion, the frequency of which determines the relative secretion rates of the two gonadotropins (Alexander and Irvine, 1993; Ginther, 1992). Pulses of GnRH every 45 min favor the production and secretion of LH, while less frequent pulses (every 6 h) encourage FSH secretion (Alexander and Irvine, 1993).

Luteinizing hormone and FSH are glycoprotein molecules made up of identical alpha chains and a differing, function-specific beta chain (Alexander and Irvine, 1993). Effects of the gonadotropins occur when they bind to their specific ovarian receptors. Receptors for FSH are found mainly on the granulosa cells of the ovarian follicles, while receptors for LH are generally found on the thecal cells of the preantral follicles (Alexander and Irvine, 1993). After antrum development, LH receptors are also found on the granulosa cells (Alexander and Irvine, 1993). The main function of FSH is to stimulate follicle growth (Guyton and Hall, 2000), but the final follicular growth phase
and ovulation are driven by LH activity (Alexander and Irvine, 1993). Follicular growth is accompanied by production of estradiol and inhibin, and ovulation leads to formation of a corpus luteum (CL), which secretes progesterone. Each of those hormones exerts a negative feedback mechanism on both the hypothalamus and the adenohypophysis (Alexander and Irvine, 1993). So, a tight feedback mechanism exists to keep all hormones and their actions balanced.

**Estrous Cycle**

The estrous cycle is defined as the repetitive sequence of events that prepares a mare for conception (Daels and Hughes, 1993). Another working definition of the estrous cycle is the interval from the beginning of one estrus to the next (Daels and Hughes, 1993). The average length of the estrous cycle in the mare, as stated by Ginther (1992), is 21.7 days, with a range of 19.1 to 23.7 days, depending upon the study. The estrous cycle is broken up into two distinct parts, the follicular phase, also known as estrus, and the luteal phase, known as diestrus (Daels and Hughes, 1993). During estrus, follicles develop under the influence of FSH and begin to produce estrogen. Estrogen encourages the mare to be sexually receptive to the stallion and to exhibit sexual behavior (Daels and Hughes, 1993). The average length of estrus in the mare is 6.5 days, with a range of 4.5 to 8.9 (Ginther, 1992). Elevated LH levels lead to ovulation (expulsion) of the oocyte from the dominant follicle. Ovulation occurs within 24 to 48 h of the end of estrus (Daels and Hughes, 1993).

Once ovulation has occurred, a CL develops in place of the ruptured follicle. The CL secretes progesterone, which is the dominant hormone of diestrus. Progesterone causes the mare to be unresponsive to sexual behavior from a stallion (Daels and Hughes,
According to Ginther (1993), the diestrus period lasts 12.1 to 16.3 days after ovulation. Around day 14 after development of the CL, it begins to regress, at which point estrus begins again (Daels and Hughes, 1993).

A reciprocal pattern exists describing the actions of the gonadotropins during the estrous cycle. Follicle-stimulating hormone secretion is characterized by surges that occur at 10 to 11 day intervals (Evans and Irvine, 1975). One surge occurs during early diestrus, which causes the initial development of up to 20 follicles (Evans and Irvine, 1975). The highest concentration of FSH occurs during a surge that occurs during the mid-diestrus period. This rise in FSH stimulates further development of dominant follicles (Evans and Irvine, 1975). As the follicular phase begins, FSH levels begin to decrease, while LH levels experience a sharp rise and peak approximately one day after ovulation (Miller et al., 1980). In general, LH levels are high during estrus and low during diestrus, while the opposite holds true for FSH (Miller et al., 1980). It is thought that the low levels of LH during diestrus are due to a negative feedback from progesterone produced by the CL, while the high levels of FSH are due to a lack of negative feedback from the ovaries during diestrus (Freedman et al., 1979).

Seasonality

Mares are classified as seasonally polyestrous. Most non-pregnant mares exhibit repeated typical cyclic sexual activity during the spring and summer months and are reproductively quiet during the fall and winter (Daels and Hughes, 1993). Ginther (1993) divides the anovulatory season into three phases: receding, inactive, and resurging. The receding phase, also known as fall transition, is characterized by failure of a large follicle to ovulate at the expected time following luteolysis (Ginther, 1992). This failure to
ovulate is due to the missing LH surge that is necessary for ovulation. Silvia et al. (1986) found that the pituitary content of LH steadily decreases from the middle of the ovulatory season until the middle of the seasonal anovulatory period. After the last ovulation of the year, the mare slowly enters an anestrous state, more correctly defined as an anovulatory state, due to the lack of adequate LH secretion and failure of final growth of a preovulatory follicle (Snyder et al., 1979).

The seasonal anestrous, or anovulatory period, is described by Sharp and Davis (1993) as the period of sexual incompetence and indifference that occurs during the winter months. This lack of reproductive activity has been reported to be due to a lack of GnRH secretion by the hypothalamus (Sharp and Davis, 1993). There is a distinct suppression of secretion of the gonadotropins during the winter (Thompson et al., 1986, 1987). As a result of this diminished secretion of GnRH and the gonadotropins, ovarian activity is minimal. Follicular growth is drastically reduced, often with only one or two follicles reaching the size of 10 to 15 mm (Sharp and Davis, 1993). Although mares do not usually exhibit signs of sexual receptivity to stallions during this time, weak signs of estrus may be observed in some mares in the absence of follicular activity (Ginther, 1992; Gentry et al., 2002b).

The resurging phase, more commonly called vernal transition, is the phase marked by the gradual return of ovarian activity and eventually ovulation. Increases in follicular development, concentrations of circulating gonadotropins, and displays of sexual behavior occur during vernal transition (Sharp and Davis, 1993). Plasma FSH concentrations and pulse amplitude actually decrease during spring transition as ovulation is approached (Freedman et al., 1979; Silvia et al., 1986; Hines et al., 1991), apparently
due to the production of inhibitory factors from the newly present large follicles (Miller et al., 1981). In contrast, LH levels remain low until the first preovulatory LH surge (Hines et al., 1991). Behaviorally, mares often exhibit long periods of erratic and unpredictable responsiveness to stallions (Hines et al., 1991).

Folliculogenesis and Ovulation

The fundamental functional and structural unit of the ovary is the follicle. Folliculogenesis is the growth and differentiation process of the ovarian follicles. Three phases of folliculogenesis exist: selection, dominance, and ovulation or atresia (Pierson, 1993). The follicle has several functions that fall into both the endocrine and exocrine categories. The follicle produces estrogens and protein hormones and also releases the oocyte at ovulation. The activity of the ovary itself is governed in three different ways: endocrine, or systemic hormone release; paracrine, or local intracellular diffusion; and autocrine, which is the autoregulation and release of substances that bind to the cell’s own receptors (Pierson, 1993).

According to Ginther (1992), each mare has a finite pool of primordial follicles on her ovaries. These primordial follicles are simply an oocyte surrounded by a single layer of cells. Ginther (1992) also states that small follicles (<10 mm) are continuously growing and regressing, regardless of the reproductive status of the mare, thus providing a pool from which large follicles are selected. The next stage in follicular growth is the development of pre-antral follicles, which is defined by oocyte growth, development of a zona pellucida, and division of granulosa cells into cuboidal epithelium (Pierson, 1993). Tertiary follicles are defined as those with antrum development, which occurs around 300 µm in diameter in the mare. Further development of this follicle includes an increase in
antral volume and a thickening of the follicle wall (Pierson, 1993). Over 99% of the follicles that grow to antrum stage fail to ovulate, but rather undergo atresia, which is a gradual reduction in size and activity and disappearance from the ovary. The small fraction of follicles that do not experience atresia ovulate (Pierson, 1993).

The gonadotropins are the major regulators of follicular growth, although the mechanisms that control early growth and entry into the growth pool are not as well researched (Pierson, 1993). Follicles develop to the antral stage in absence of the gonadotropins, but these hormones are necessary for continued growth. Pre-antral follicles acquire LH receptors in the thecal cell membrane and FSH receptors in the granulosa cell membrane (Pierson, 1993). The thecal cells produce androgens under the influence of LH. These androgens then pass through the basal lamina of the follicle to the granulosa cells where they are aromatized to estrogens under the influence of FSH. The rising concentration of estrogen stimulates LH release, which in turn encourages more estrogen production. An increase in LH receptors is also detectable, apparently due to the rise in estrogen levels. The transition from the antral stage to the preovulatory stage is marked by this drastic increase in estrogen production. Without the ability to respond to the preovulatory surges in the gonadotropins, the follicle does not reach the final stages of maturation (Pierson, 1993).

Follicular changes occur during the entire estrous cycle due to changing levels of hormones. Follicles can be classified into categories based on size: small (2 to 10 mm), medium (11 to 24 mm), and large (≥25 mm) (Ginther, 1992). The number of small follicles decreases during the preovulatory stage of the cycle and increases during the postovulatory phase (Pierson, 1993).
The number of medium follicles appears relatively constant throughout the cycle. The number of large follicles is dependant on a phenomenon called the follicular wave (Pierson, 1993).

Ginther (1992) defines a major follicular wave as several follicles that initially grow in synchrony (approximately the same diameter and growth rate) but eventually dissociate. Follicular waves are associated with an increase in FSH concentrations (Ginther and Bergfelt, 1993), which precedes the change in the number of large follicles by approximately 6 days (Snyder et al., 1979). Dissociation (also referred to as divergence) occurs when one dominant follicle is selected for continued growth. The other (or subordinate) follicles are fated for atresia (Ginther, 1992). All mares have at least one follicular wave, while some may exhibit two, per estrous cycle. In mares that have two waves, the first is the secondary wave which occurs during late estrus or early diestrus. Any dominant follicle from this wave normally becomes static and regresses, although a rare ovulation during diestrus occurs. The second wave is the primary wave during mid-diestrus from which the dominant follicle is selected (Ginther, 1992).

Ginther and his team provide a good description of selection of the dominant follicle (2001). When follicles in mares reach approximately 13 mm, the FSH surge has reached its peak. After this peak, the growing follicles cause a decrease in FSH levels. In horses, estradiol, free IGF-1, activin-A, and inhibin-A begin to increase differentially in the future dominant follicle about 1 day before deviation. These changes are associated with a greater responsiveness to LH and FSH by the developing dominant follicle than by other follicles, thereby accounting for deviation. Deviation begins 3 days
later, when the two largest follicles are an average of 22.5 and 19.0 mm, respectively. The onset of the deviation mechanism occurs when FSH levels have dropped considerably, and only the most developed follicle will be able to continue to grow in that condition. The dominant follicle continues to produce estradiol and inhibin, thereby causing atresia of the other follicles (Ginther, 1993; Pierson, 1993).

Luteinizing hormone also plays a substantial role in the selection of the dominant follicle. The rate of increase in LH concentrations is similar to the growth in diameter of the selected follicle, and this increase occurs at the same time that selective growth begins (Pierson, 1993). An increase in LH is observed when the levels of FSH begin to decrease. This increase is also thought to be responsible for increased follicular development and growth of the follicle wall (Pierson, 1993).

Ovulation in the mare is different than that of other farm species. The dominant follicle has LH receptors present in the granulosa cells which allow for a response to the LH increase during estrus (Ginther, 1992). In mares, it is noted that the peak of LH activity does not occur until one day after ovulation, so the increase in LH levels during midestrus is thought to encourage ovulation, as opposed to the surge that is present in other species (Pierson, 1993). Mares are also unique because they ovulate from an ovulation fossa, instead of from random spots on the exterior of the ovary (Ginther, 1992). The wall of the ovary ruptures at the fossa, expelling the oocyte and follicular fluid (Pierson, 1993).

Niswender and Nett (1993) provide a comprehensive description of the luteal phase of the mare. Ovulation starts the onset of the luteal phase of the mare's estrous cycle. This phase is dominated by the CL, which secretes progesterone. After ovulation,
the granulosa cells begin to luteinize. The CL in the mare reaches maximum diameter within 3 days after ovulation. However, maximum secretion of progesterone does not occur until about 9 days after ovulation. The lifespan of the CL is supported by LH in the mare. If the mare is not pregnant, the mare secretes prostaglandin-$F_{2\alpha}$ (PGF$_{2\alpha}$) on approximately day 14, which causes lysis of the CL. Progesterone levels decrease as the CL regresses, and the cycle begins again.

**Manipulation of the Estrous Cycle**

Manipulation of the estrous cycle has profound implications in the horse industry. Regulation and suppression of estrus, increasing follicular growth, and controlling and inducing ovulation are all major goals of manipulating the estrous cycle. A range of pharmaceuticals exist for control of the estrous cycle in the mare.

Progestins are a popular means for suppression of estrus and the regulation of estrus in vernal transitional mares (Squires, 1993). The most common progestin in use today is an orally active synthetic progestin, altrenogest (trade name Regu-Mate). Once removed from altrenogest treatment, mares typically return to estrus within 3 to 4 days (Squires, 1993).

Prostaglandins, specifically PGF$_{2\alpha}$, are used to control the estrous cycle due to their luteolytic properties. Prostaglandin-$F_{2\alpha}$ is most often used to ‘short cycle’ mares, or shorten their normal luteal phase, thereby bringing them back into estrus sooner (Irvine, 1993a). A single injection of PGF$_{2\alpha}$ to a diestrous mare causes rapid luteolysis and return to estrus in about 3 days. Prostaglandin can be given in a variety of ways, such as intramuscular, intravenous, intrauterine, and intraluteal. For practical purposes, intramuscular injections are the most common (Irvine, 1993a). Prostaglandins are most
effective if given at day 5 or later post-ovulation (Oxender et al., 1975). Side effects, such as sweating, diarrhea, and abdominal cramping, are common with use of PGF$_{2\alpha}$. An analog of PGF$_{2\alpha}$ without the side effects was eventually developed and proven equally effective (Nett et al., 1979).

Human chorionic gonadotropin shortens the duration of estrus and increases the number of ovulations occurring within 48 h of treatment (Voss, 1993). Induction of ovulation is more precise when hCG is given after a follicle reaches a diameter of 30 to 35 mm. Human chorionic gonadotropin exhibits LH-like activity and also shows some minor effects similar to FSH. However, since hCG is a protein derived from humans, injection into mares may cause formation of antibodies (Roser et al., 1979). This antibody production causes a decrease in effectiveness and response after each subsequent use (Sullivan et al., 1973; Voss, 1993) but does not appear to alter fertility rates (Wilson et al., 1990).

Gonadotropin-releasing hormone and its analogues are another means of inducing ovulation and initiating follicular growth which do not have the immunological problems seen with hCG. Pulsatile injections of low doses of GnRH can induce deviation of the preovulatory follicle (Irvine, 1993b). Continuous application of GnRH can cause refractoriness of the gonadotropes in many species; however, horses appear somewhat resistant to this reaction. Intermittent administration of GnRH can induce follicular development in seasonally anovulatory mares (Irvine, 1993b). Several analogues exist although none have proved ideal. Most GnRH analogs are produced by the substitution or removal of one or more amino acids. Substitution of the glycine at position 6 with D-alanine, tryptophan, or serine confers more structural and metabolic stability, which
prolongs the half-life and action of the analog (Monahan et al., 1973; Sandow, 1978). Removal of the glycine at position 10 and amidation of the proline at position 9 also results in an increase in agonist activity (Sandow, 1978).

Deslorelin acetate has been reported to effectively induce ovulation when given to mares exhibiting estrus and with a follicle >30 mm; these mares ovulate within 48 h (McKinnon et al., 1993; Meinart et al., 1993; Squires et al., 1994). However, it has also been noted that treatment with deslorelin results in a prolonged interovulatory interval, with reduced concentrations of FSH and decreased follicular development (Johnson et al., 2000; Farquhar et al., 2001; Blanchard et al., 2002). When given as an implant, removal of the implant at 48 hrs eliminated the decreased FSH secretion and the increased interovulatory interval associated with implant administration (Farquhar et al., 2002). Another observed side effect of deslorelin implants is the fact that some mares enter an anovulatory state and do not return to estrus throughout the rest of the breeding season (Johnson et al., 2000). A lower dose (1.5 mg) injectable, short-term release formulation of deslorelin has also been proven to induce ovulation within 24 h. This injectable did not cause suppression of follicular activity, perhaps indicating that at smaller doses the negative effects of deslorelin are somewhat abated (Stich et al., 2004).

Attempts to hasten ovulation have also been tried with other GnRH agonists. Buserelin has been shown as effective at hastening ovulation as hCG, and also showed an increased level of LH prior to ovulation (Barrier-Battut et al., 2001). Twice daily injections of buserelin encouraged ovulation and increased FSH levels if given with a follicle >35 mm (Squires et al., 1981). Histrelin is yet another synthetic GnRH agonist
which hastens ovulation. The potential suppression of the gonadotropins and prolonged interovulatory interval are side effects that must be avoided when using GnRH analogs.

**Leptin**

**History and Discovery of Leptin.** One of the earliest models of animal obesity was the \textit{ob/ob} mouse, which was identified in 1950 (Ingalls et al., 1950). These mice have a recessive mutation of the obese gene that creates sterile adult mice with over 50% body fat (Houseknecht et al., 1998). In addition to the \textit{ob/ob} gene, a related recessive mutation called \textit{db/db} (diabetic) was discovered. These mice were not phenotypically unlike their \textit{ob/ob} counterparts, but they also exhibited extreme hyperglycemia (Hummel et al., 1966). These genes were not cloned and identified until 1994 (Zhang et al., 1994), but studies were done with these two types of mice much earlier then that.

Hervey (1958) and Coleman (1978) both performed parabiosis studies with the \textit{ob/ob} and \textit{db/db} mice. By joining the vascular systems of these mice together, they drew several conclusions. By pairing an \textit{ob/ob} mouse with a normal mouse, the \textit{ob/ob} reduced their food intake and lost weight. Joining a \textit{db/db} mouse and a normal mouse caused the normal mouse to stop eating and die, while joining the \textit{ob/ob} mouse and the \textit{db/db} mouse did not affect the \textit{db/db} mouse but did cause the \textit{ob/ob} mouse to stop eating and lose weight. Hervey (1958) concluded that there was some factor that the \textit{ob/ob} mice did not have, but were receptive to when exposed, while the \textit{db/db} mice had plenty of this factor but could not utilize it. This factor was eventually found to be leptin.

Leptin is a 16 kDa protein that is primarily produced and secreted by the white adipocytes. Its name comes from the Greek word ‘leptos,’ meaning thin. In addition to
the adipocytes, leptin is secreted by the stomach and the placenta in lesser amounts
(Houseknecht et al., 1998).

Receptors for leptin, which would indicate target tissues, are located in numerous
tissues in the body. However, the only fully functional receptors are located in the
arcuate ventromedial nuclei of the hypothalamus. These receptors are thought to be
responsible for the central actions of leptin (Houseknecht et al., 1998). The leptin
receptor bears striking similarity to the cytokine-growth hormone family of receptors
(Keisler et al., 1999). The signal transduction mechanism of the hypothalamic leptin
receptor is similar to the G proteins, and is activated by the JAK-STAT pathway (Keisler
et al., 1999). Although signal transduction by members of the class I cytokine family
vary beyond the JAK-STAT pathway, it is yet to be determined if leptin shares some of
these other systems (Houseknecht et al., 1998). The short forms of the receptors are
thought to exhibit limited or weakened signal transduction abilities (Houseknecht et al.,
1998). Shortened forms of the leptin receptor are located in numerous tissues, including
but not limited to the anterior pituitary, pancreas, hair follicles, gonads, heart, and skeletal
muscle (Keisler et al., 1999). The function of leptin on each of these tissues is yet to be
determined.

Once leptin is produced and secreted by the adipocytes, it travels bound through
the blood. Leptin must be bound or else it cannot cross the blood-brain barrier and exert
its functions on the hypothalamus (Houseknecht et al., 1998).

The major function of leptin is to act as a signal to the body concerning its energy
reserves. However, its more specific activities are to decrease food consumption and
appetite while increasing thermogenesis, which in turn causes weight loss. Leptin is
thought to act centrally and inhibit synthesis and release of neuropeptide Y (NPY; Houseknecht et al., 1998).

Neuropeptide Y (NPY) is a 36-amino acid protein which acts as a transmitter in the central nervous system. Neuropeptide Y is thought to be involved with feeding behavior and development of obesity, since it stimulates appetite while decreasing energy use and thermogenesis. However, NPY does not appear to be essential for these activities, given that knockout mice lacking NPY expression do not show any adverse traits, although they do exhibit an increased sensitivity to leptin (Erickson et al., 1996).

Upon its discovery, leptin was thought to be a potential "miracle cure" for obesity in humans, because animals treated with leptin experienced losses of body weight and fat depots and a decreased appetite (Pelleymounter et al., 1995). However, leptin and its actions are much more complex than simple appetite reduction and weight loss. In the realm of animal sciences, learning about the relation of nutrient use and production efficiency are major concerns (Houseknecht et al., 1998). Energy balance and its relation to reproductive and lactational efficiency are important in livestock species to optimize performance.

Leptin levels vary among species, gender, nutritional status, and season. In humans, ruminants, and horses, leptin levels are strongly correlated to amount of body fat or body condition (Prolo et al., 1998; Chilliard et al., 2000; Gentry et al., 2002a,b). However, Cartmill and associates (2003a) found that a permanent variation in leptin levels exists among obese mares. This indicates a possible genetic determination for leptin levels. In horses, mares and geldings exhibit higher leptin levels than stallions (Cartmill, 2004), which is thought to be due to stallions having more lean mass. In
contrast, Buff et al. (2002) found no difference between geldings and stallions. In that report, body condition was assessed and related in general to leptin levels, however the specific averages for stallions and geldings were not given.

Age is another factor that influences leptin levels in horses. Younger mares (<5 years of age) are generally less fat and have less leptin than older (>10 yrs) mares (Fitzgerald and McManus, 2000; Buff et al., 2002). Gentry et al. (2002b) found that long term feed restriction lowered body condition (Henneke et al., 1983) and leptin levels in mares. Also, through the course of that study (September through January), leptin levels declined in all mares regardless of body condition (low or high). Similarly, short term feed restriction decreased leptin levels in both aged and young mares (McManus and Fitzgerald, 2000) without any change in body condition; this is in contrast to studies with rodents that showed a change in leptin directly following a decrease in body fat (Ahima et al., 1996).

In livestock species, few studies have been performed on the direct effects of leptin on appetite and body weight. In two separate studies (Henry et al., 1998; Morrison et al., 2001) of well-fed and feed-restricted ewes that were given leptin injections, similar results were found. In the well fed animals, in which energy reserves could be met by body fat stores, leptin suppressed appetite. Conversely, the feed restricted ewes, which lacked body fat, did not stop eating until leptin levels were at a maximum. This confirmed that the signal to the brain about the availability of body fuel (i.e., leptin) did not override the actual lack of body fuel in the ewes. Therefore the feed restricted ewes continued to eat for survival (Henry et al., 1998; Morrison et al., 2001).
The first indications that leptin was related to reproductive function came with a rash of studies in the mid-1990’s. Obese (ob/ob) mice are sterile until they are given leptin, at which time normal reproductive function is restored. Upon administration of leptin, both males and females showed a marked increase in gonadal weight, an increase in circulating amounts of gonadotropins, and an increase in the number of ovarian follicles or sperm count (Barash et al., 1996; Chehab et al., 1996; Mounzih et al., 1997).

Puberty is also affected by leptin. Treatment with leptin advanced puberty in both malnourished and well-fed mice (Chehab et al., 1997). There is also evidence that leptin is necessary for onset of normal reproductive function (Chehab et al., 1997). In humans, females with abnormally low body condition, and therefore low leptin, show an absence or a severe delay in menarche (Frisch, 1980). Both young boys and young girls experience an increase in leptin concentrations before puberty, although girls are the only ones to maintain these high levels. Males experience a decline in leptin after puberty (Mantzoros et al., 1997; Cunningham et al., 1999). In cattle, leptin levels increase linearly during the 16 weeks preceding the pubertal ovulation (Garcia et al., 2002). Moreover, leptin injections in normal mice trigger early puberty (Ahima et al., 1997; Chehab et al., 1997).

Leptin appears to be affected by season. In cattle, leptin increases from early winter through the summer solstice (Garcia et al., 2002). In mares, a marked decrease in leptin levels occurs between fall and winter (Fitzgerald and McManus, 2000; Gentry et al., 2002b); leptin levels increase from July until September. Interestingly, Gentry and coworkers (2002b) found that mares with high body condition, and relatively high leptin,
continued to cycle and remain reproductively active during the winter months, which is traditionally the time of seasonal anovulation.

Leptin and its effects in the male tend to vary among species. Rats, which have receptors for leptin on the Leydig cells in the testes, experience inhibited steroid production in response to leptin. However, research in primates and mice showed that leptin may not in fact affect testicular steroid production (Lado-Abeal et al., 1999). In men, androgen treatment decreased concentrations of leptin (Simon et al., 2001).

**Hyperleptinemia and Diabetes.** Leptin and its actions have been closely linked with insulin. An increase in insulin prompts a rapid increase in leptin in both humans and rats (Cusin et al., 1995; Saladin et al., 1995). Sivitz and others (1998) reported that leptin decreases in response to a decrease in insulin, and increases with a restoration of insulin levels. Cartmill and coworkers (2003b) found that horses of a similar high body condition fell into two distinct groups based on leptin levels: hyperleptinemic or normal. The hyperleptinemic horses had metabolic profiles similar to that of human Type II diabetics. These horses were characterized by high glucose and insulin levels and exaggerated insulin response to glucose; they also had exaggerated glucose and insulin responses to dexamethasone treatment. Type II diabetes in humans is also known as non-insulin dependant diabetes (Guyton and Hall, 2000). Insulin resistance is the primary symptom; other traits of the disease are an increase in insulin, abnormal blood lipid profiles, and elevated blood glucose levels (Guyton and Hall, 2000).

**Diabetes, Insulin Resistance, and Gonadal Dysfunctions.** Approximately 10 to 25% of the general population is affected by insulin resistance (Ovalle and Azziz, 2002). Of these people, 4 to 6% of reproductive-aged women are affected with another related
disorder called polycystic ovarian syndrome (PCOS; Dunaif, 1994; Franks, 1995; Ovalle and Azziz, 2002). Polycystic ovarian syndrome is characterized by menstrual dysfunction, chronic anovulation, hyperandrogenism, and ovarian cysts (Dunaif, 1994; Franks, 1995). Besides the classic symptoms of PCOS, patients also have an increased risk of endometrial cancer, spontaneous abortion and development of gestational diabetes (Dahlgren et al., 1992; Norman and Clark, 1998; Schroder et al., 2004). In humans, the ovarian insulin resistance seen in PCOS patients is thought to be caused by a defect in the actions of the granulosa cells (Wu et al., 2003). Insulin resistance disrupts steroidogenesis and the insulin signaling pathways of the central nervous system (Sam and Dunaif, 2003).

Insulin resistance is a warning sign of both Type II diabetes and PCOS. Polycystic ovarian syndrome is closely related to Type II diabetes in that patients of one disease have a five to ten-fold greater risk of acquiring the other (Schroder et al., 2004). In fact, 7.5 to 10% of females with PCOS also have Type II diabetes (Schroder et al., 2004).

Diabetic female rats show a decrease in ovulation, sexual behavior, and LH surges, which were all restored and corrected after peripheral insulin treatment (Kovacs et al., 2003). Humans treated with insulin sensitizing agents such as metformin have shown decreased signs of insulin resistance, decreased hyperinsulinemia, and corrected reproductive effects of the disorder (Glueck et al., 2002). Exercise, diet, and other lifestyle changes have proven helpful for altering the short- and long-term effects of PCOS, insulin resistance, and Type II diabetes (Moran and Norman, 2004).
Rationale for Present Experiments

Several reports state a relationship between body condition and reproductive status. Leptin has been studied as a major link between nutritional status and the reproductive system in several species. Prior research also indicates that mares of high body condition can have widely ranging levels of leptin, and may not exhibit the textbook signs of anestrous during the winter. However, little data exists about leptin and its relationship to the reproductive cycle in the horse. The first experiment described herein was designed to determine whether the hyperleptinemic condition in mares described by Cartmill et al. (2003b) in high body condition mares had any impact, relative to mares with normal leptin levels, on reproductive characteristics of high body condition mares during the vernal transition period and first estrous cycle.

Given the efficacy with which deslorelin implants induced ovulation in mares, its use became widespread quickly after its commercial availability. However, due to the undesirable side effects of the implants, such implants are no longer available, and alternative products are needed. The second experiment described herein was designed to determine the relative potencies of GnRH, deslorelin, and other analogs on LH and FSH release in follicular phase mares. The effects of leptin status on the responsiveness to these secretagogues was studied by interspersing hyperleptinemic mares and normal mares throughout the treatment assignments.
CHAPTER II

REPRODUCTIVE CHARACTERISTICS OF HIGH BODY CONDITION MARES WITH HIGH VERSUS LOW LEPTIN CONCENTRATIONS DURING VERNAL TRANSITION AND THE FIRST ESTROUS CYCLE

Introduction

A major goal of equine reproductive management is to be able to induce ovarian activity in the mare earlier in the year, thus bringing the day of first ovulation closer to January 1. Many factors affect the mare’s ability to begin cycling earlier than the literature-stated norm of early April (Ginther, 1992).

Leptin is an adipocyte-derived hormone that acts as a signal to the brain regarding the body’s nutritional status (Houseknecht et al., 1998). In other species, concentrations of leptin vary directly with percent body fat (Prolo et al., 1998; Chilliard et al., 2000). In the horse, leptin levels are not only related to body condition but also to age, gender, and season (Fitzgerald and McManus, 2000; Gentry et al., 2002a,b; Cartmill, 2004). Gentry et al. (2002b) showed that some mares with high body condition score continued to ovulate or show significant follicular activity during the winter, which is the normal anestrous season for the mare. That same group (Gentry et al., 2002b) reported that among mares with high body condition, a wide variation in leptin levels exists. Later analysis of these horses up to 3 years later showed the same high versus low distinction (L. R. Gentry, unpublished).

Cartmill et al. (2003b) studied differences between mares with hyperleptinemia and mares of similar high body condition with low leptin levels. Hyperleptinemic mares displayed elevated blood glucose and insulin levels, elevated triiodothyronine levels, and exaggerated insulin and glucose responses to dexamethasone treatment. These symptoms are similar to those exhibited by humans with Type II diabetes. Given that women with Type II diabetes often
experience ovarian abnormalities (Schroder et al., 2004), the present experiment was designed to compare the reproductive characteristics of high body condition mares with high versus low leptin concentrations through the spring transition period and the first estrous cycle.

**Materials and Methods**

**Mares and Monitoring.** Sixteen light horse mares with high body condition scores (range 6.0 to 8.5, average = 7.4; Henneke et al., 1983) were allotted equally to two groups based on leptin levels: low leptin (<5 ng/mL) and high leptin (>10 ng/mL). Mares were routinely maintained on pasture (available native grasses and winter ryegrass) and were supplemented with good quality bermudagrass hay as needed to maintain their body condition.

Beginning January 7, 2004, ovarian activity was evaluated in each mare every 3 days by transrectal ultrasonography (Aloka 550V with 5-MHz linear-array transducer; Aloka Science and Humanity, Wallingford, CT) until a follicle 25 mm or greater was detected, at which time the mare was examined every day until ovulation or regression of the follicle to <25 mm. Activity of the ovaries was characterized by follicle size and numbers. Follicles were classified into three groups, small (≤10 mm), medium (11 to 24 mm), and large (≥25 mm). Once ovulation occurred, mares were scanned every 3 days according to the procedure described above until a second ovulation was observed.

Jugular blood samples were collected via venipuncture into sodium heparin-coated tubes (Vacutainer, Becton and Dickinson, Franklin Lakes, NJ) on the same days as (and immediately before) the ultrasound exams. After the first ovulation, blood samples were collected daily until a second ovulation occurred. Blood samples were routinely centrifuged within 30 min of collection and plasma was harvested and stored frozen. Plasma levels of LH, FSH, and progesterone were measured to characterize their patterns in each group of mares.
Hormonal and Metabolic Challenges. Several challenges were performed throughout the course of the experiment. An intravenous glucose tolerance test (IVGTT) was administered on January 15, 2004, to measure glucose tolerance and insulin response to glucose in the two groups of mares. Mares were fitted with indwelling jugular catheters and blood samples were collected at -10, 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150, and 180 min relative to an i.v. injection of glucose (0.2 g/kg BW as a 50% solution in saline) at time 0.

An insulin challenge was administered on January 24, 2004, to determine the blood glucose response to insulin. Mares were fitted with jugular catheters and given a bolus injection of insulin (human recombinant 0.1 IU/kg BW; Sigma Chem. Co., St. Louis, MO); blood samples were collected at -10, 0, 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, and 210 min relative to the injection of insulin.

Two sulpiride challenges (February 2 and March 24, 2004) were administered to determine the relative prolactin responses in the two groups of mares. All mares were given sulpiride (Sigma), a dopamine receptor antagonist and prolactin secretagogue. Blood samples were collected at -15, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min relative to injection of sulpiride (0.1 mg/kg BW) in saline. Plasma was harvested for determination of prolactin concentrations.

Sample Analyses. Radioimmunoassay was performed on selected samples to determine plasma LH (Thompson et al., 1983b) and FSH (Thompson et al., 1983a) levels throughout the estrous cycle. Leptin levels were measured every 3 days throughout the term of the project with a radioimmunoassay described by Cartmill et al. (2003b). Plasma insulin from the IVGTT and insulin challenges was measured with radioimmunoassay as described by Depew et al. (1994). Progesterone levels were measured using commercially obtained reagents (Diagnostic
Laboratory Systems, Webster, TX). Plasma glucose was determined spectrophotometrically (method no. 315: Sigma Chemical Co., St. Louis, MO, USA).

Statistical Analyses. Data were analyzed by the GLM procedure of SAS (SAS Institute Inc., Cary, NC). Data from daily and frequent sampling periods were analyzed for effects of treatment, time, and treatment by time interactions via split-plot ANOVA (Gill and Hafs, 1971). Single point variables were analyzed via a one-way ANOVA. For the challenges, the main effect of group and its interaction with time for concentrations of insulin, glucose and prolactin were analyzed using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC). When a significant F was detected (P > 0.05) the least significant difference (LSD)-test (Steel and Torrie, 1980) was used to determine differences between groups.

Results

Leptin levels between groups were different (P < 0.0003) over the course of the experiment (Figure 2.1). Leptin levels also tended to decrease slightly (P = 0.062) over time through day 40 (January 7 to February 15) of the project in each group.

There was no difference between groups for the size of ovulatory follicle 1 day prior to ovulation (P=.71) or between the first versus second ovulation (P = 0.72) (Table 2.1). Likewise, there was no difference between groups for the number of follicles in either the small (P = 0.32), medium (P = 0.51), or large (P = 0.72) categories at any point during the estrous cycle (data not shown). Nine of the sixteen mares showed significant follicular activity and four of those mares also showed a CL, which indicates cyclicity, during the first week of the project (January 7 to 14). Five of these mares were in the high leptin group and four were in the low leptin group.
Figure 2.1. Mean leptin concentrations of high versus low leptin mares measured every three days from January 7 (day 1) through February 15 (day 40). Leptin levels between groups were different ($P < 0.0003$) over the course of the experiment. Levels also decreased slightly ($P = 0.0624$) in both groups over time. The least-significant difference (LSD) bar at $\alpha = 0.05$ is indicated by the vertical bar for comparison of values.
Table 2.1. Follicle size 1 day before ovulation, day of first ovulation relative to January 7 (day 0), and interovulatory interval for high versus low leptin mares. Data are expressed as mean (range).

<table>
<thead>
<tr>
<th>Item</th>
<th>High Leptin</th>
<th>Low Leptin</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicle size prior to ovulation, mm</td>
<td>44.2 (33-56)</td>
<td>44.3 (35-52)</td>
<td>.7124</td>
</tr>
<tr>
<td>Day of first ovulation</td>
<td>54.2 (11-111)</td>
<td>56.7 (12-110)</td>
<td>.9036</td>
</tr>
<tr>
<td>Interovulatory interval, days</td>
<td>26.4 (21-48)</td>
<td>26.6 (21-42)</td>
<td>.9653</td>
</tr>
</tbody>
</table>

The day of first ovulation relative to the start of the project (January 7) for the high mares did not differ (P = 0.90) between high and low leptin mares (Table 2.1), nor did the length of the interovulatory interval between the first and second ovulations (P = 0.96).

Hormonal data were expressed relative to day of the first ovulation (designated day 0 in Figure 2.2). There was no difference on days -9, -6, -3, 0, 3, 6, and 9 for the first ovulation between groups for levels of LH (P = 0.31), FSH (P = 0.53), or progesterone (P = 0.61). Also, no difference was detected between groups for the same days relative to the second ovulation in LH (P = 0.72), FSH (P = 0.28) or progesterone (P = 0.99) (Fig. 2.2).

During the IVGTT, the decline in blood glucose in response to insulin was faster (P = 0.049) in mares with high leptin levels relative to mares with low leptin levels (Figure 2.3). The insulin response in mares with high leptin was also greatly exaggerated (P < 0.0001) relative to mares with low leptin (Fig 2.3). In contrast, no difference was found between groups for either the insulin (P = 0.34) or glucose (P = 0.10) responses during the insulin challenge (Fig. 2.4).
Figure 2.2. Mean plasma concentrations of progesterone (a), LH (b), and FSH (c) for high versus low mares from day -9 from first ovulation to day 3 after the second ovulation. No effect (P > 0.1) of treatment was observed.
Figure 2.3. Mean plasma concentrations of glucose (a) and insulin (b) in high and low leptin mares after administration of an intravenous glucose tolerance test. The decline in blood glucose in response to insulin was faster ($P = 0.049$) in the high leptin group. The insulin response in the high group was also greatly exaggerated ($P < 0.0001$). The least-significant difference (LSD) bar at $\alpha = 0.05$ is indicated by the vertical bar for comparison of values.
Figure 2.4. Mean plasma concentrations of glucose (a) and insulin (b) after administration of an insulin challenge. When comparing the group by time interaction, a significant difference existed for both insulin (P < 0.0001) and glucose (P < 0.0001). The least-significant difference (LSD) bar at $\alpha = 0.05$ is indicated by the vertical bar for comparison of values.
Prolactin levels were higher ($P < 0.0001$) in both groups during the second of the two sulpiride challenges. When comparing the interaction of group, challenge, and time, mares in the high leptin group had a greater ($P = 0.014$) prolactin response to the sulpiride at 15 and 30 min after sulpiride injection (Fig 2.5).

**Discussion**

Over the course of this experiment (January to May), leptin concentrations between the high and low groups remained different. This agrees with previous observations that mares determined to be in high versus low leptin classifications were generally classified the same up to two years later (Cartmill et al., 2003b). Leptin levels decreased slightly in both groups from the beginning of January through the middle of February, which is again in agreement with previous reports (Fitzgerald and McManus, 2000; Gentry et al., 2002b). It should be noted that all mares maintained similar, high body condition and weight throughout this experiment. The presence of a continual difference in leptin levels among mares with similar high body condition score strengthens the idea that an intrinsic factor, perhaps genetics, affects leptin levels among mares.

Previous research has shown that many mares with high body condition scores continue to cycle or have significant follicular activity during the winter (Gentry et al., 2002b), thus it was predicted that the majority of mares on this project would show significant follicular activity throughout the winter. Nine of the sixteen mares on the project showed large follicles during the first week of ultrasound scanning. Four of those mares had also evidence of a CL. However, no difference between groups existed for the number of small, medium, or large follicles present at any point during the course of the experiment.
Figure 2.5. Mean plasma concentrations of prolactin in high versus low mares; first challenge February 2 and second challenge March 24. Prolactin levels were higher ($P < 0.0001$) in both groups during the second of the two sulpiride challenges. When comparing the interaction of group, challenge, and time, mares in the high leptin group had a greater ($P = 0.014$) prolactin response to the sulpiride at 15 and 30 min after injection. The least-significant difference (LSD) bar at $\alpha = 0.05$ is indicated by the vertical bar for comparison of values.
This indicates that the follicular activity observed by Gentry et al. (2002b) for mares with high body condition was due to the high body condition in those mares, not to the elevated leptin levels exhibited by some of those mares. However, given that mares with low body condition exhibited a prolonged anovulatory season (Gentry et al., 2002b) as well as lower leptin levels than mares of high body condition, further research in which low body condition mares receive exogenous leptin during the anovulatory season is needed.

No significant difference existed between groups for day of first ovulation, although both high and low groups experienced their first ovulation (February 29 and March 2, respectively) more than one month earlier than the predicted date of early April reported in the literature (Henneke et al., 1983; Sharp and Davis, 1993). All of the mares had high body condition throughout the project (mean = 7.4) regardless of leptin levels, which is in agreement with the concept that nutritional status and body condition are primary indicators of reproductive ability. Given that no difference existed between groups for day of first ovulation, it can be concluded that excessive leptin levels do not modulate the time of first ovulation given a constant high body condition.

There was no difference between groups regarding the size of the preovulatory follicle one day before ovulation (mean 44.3 mm), which agrees with the average diameter given in the literature of approximately 45 mm (Pierson, 1993). It was observed that some mares obtained a relatively large follicle (>30 mm) and maintained it for several days and up to two weeks before regression or ovulation. According to Sharp and Davis (1993), pony mares develop an average of 3.7 consecutive anovulatory follicles of 30 mm or greater during vernal transition, which is consistent with these mares exhibiting these large, anovulatory follicular waves during the transition season.
The mean interovulatory interval for all mares on this project, high or low leptin, was 26.5 days (26.4 for high mares and 26.6 for low mares); the median was 22 days for high and 24 days for low. The range for high mares was 21 to 48 days, and for low mares was 21 to 42 days. Data from one mare in the low group was not used because radioimmunoassay for progesterone did not confirm the estimate of ovulation gathered from ultrasound exam. Although these ranges are notably longer than those reported by Ginther (1992; 21.7 days, with a range of 19-23 days), these mares experienced their first ovulation over one month sooner than the reported date of April 1 (Ginther, 1992). Ginther (1992) notes that during the early spring, both the entire estrous cycle and the estrus period lengths can be quite long, and that as the season progresses the lengths will stabilize and become shorter.

When analyzing concentrations of the gonadotropins and progesterone over the course of this experiment, no differences were observed between groups at any point of the transition period or estrous cycle. In vivo studies in ruminants have shown that leptin can modulate ovarian steroidogenesis directly and acutely (Kendall et al., 2004). In those experiments, infusion of physiological doses of leptin (2 µg/h) in vivo caused a decrease in estradiol production, while passively immunizing sheep against leptin increased in ovarian estradiol. No changes in the gonadotropins were noted. Interestingly, infusion of a supraphysiologic dose of leptin (20 µg/h) had no effect on either estradiol or the gonadotropins. No mention of body condition or nutritional status was mentioned in that study.

The various challenges performed in this experiment were designed to confirm the presence of previously noted symptoms of this hyperleptinemic syndrome (Cartmill et al., 2003b): hyperleptinemia, hyperinsulinemia, and hyperglycemia in horses similar to Type II diabetes in humans. The IVGTT revealed that the insulin response in high leptin mares was
exaggerated compared to the low leptin mares, which agrees well with the report of Cartmill et al. (2003b). However, the faster decline in blood glucose levels in high leptin mares noted in the present experiment was in contrast with other results of Cartmill (2004), in which mares with high leptin had no difference in glucose clearance relative to mares with low leptin. In general, exaggerated insulin responses to glucose infusion are associated with insulin insensitivity, although Cartmill (2004) did not detect any difference in insulin sensitivity between mares with high versus low leptin levels. In the present case, the increased insulin appears to be resulting in faster glucose clearance, indicating a sensitivity of the mares to their secreted insulin.

An insulin challenge was also performed to determine the relative glucose response in the two groups of mares. Interestingly, a standard dose of insulin, based on body weights of the mares, resulted in a greater plasma insulin concentration in low leptin mares than in high leptin mares. The glucose drop in response to the insulin bolus was significantly greater in the low leptin mares, which may have been due to the greater insulin levels achieved by the injection. Given that blood volume, and hence plasma volume, is a relatively consistent percentage of body mass, the reason for the difference in insulin levels after injection needs further study.

Two sulpiride challenges were performed to measure prolactin activity, one on February 2 and one on March 24. A significant difference in prolactin response existed between the first and second challenge. The rise in prolactin over the 2 to 3 month period reflects the natural seasonal changes in prolactin secretion (Johnson and Becker, 1987; Thompson and Johnson, 1987). The significant three-way interaction of group, challenge, and time was due mainly to the fact that mares with high leptin generally had higher prolactin levels after sulpiride injection, although the magnitude of the differences was small. The previous report by Gentry (2002b) indicated no difference in prolactin response between mares of high versus low BCS after
sulpiride; however, the prolactin response to TRH was higher in mares with high BCS.

In conclusion, mares of high BCS exhibiting hyperleptinemia as well as other alterations in glucose and insulin characteristics had no apparent alteration in reproductive characteristics in the transition from the anovulatory state up through the first estrous cycle. Ovarian activity, day of first ovulation, and gonadotropin and progesterone levels were similar for the mares with high versus low leptin levels. Thus, unlike their human counterparts with Type II diabetes who often develop ovarian pathologies, the reproductive system in the mare appears to be unaffected by the changes in leptin and insulin characteristics.
CHAPTER III
HIGH BODY CONDITION MARES WITH HIGH VERSUS LOW LEPTIN CONCENTRATIONS: RESPONSES TO VARYING DOSES OF SEVERAL GNRH ANALOGS

Introduction

The ability to predict the time of ovulation is a major goal of the horse industry. This capability can lead to getting mares in foal earlier in the year, reducing the number of breedings before successful implantation, and shortening the estrous cycle. Mares are unique among livestock species in that their estrus averages 6 to 7 days in duration. This can lead to difficulty predicting the exact day and time of ovulation, which can lead to expensive breeding costs to both the mare and stallion owners.

Several products exist to induce ovulation in the mare. Human chorionic gonadotropin shortens the duration of estrus and also increases the number of ovulations occurring within 48 h of treatment (Voss, 1992). Because it is a human protein, injection of hCG into mares causes formation of antibodies (Roser et al., 1979), therefore reducing effectiveness with subsequent use (Sullivan et al., 1973; Wilson et al., 1990). Gonadotropin-releasing hormone and its analogs are alternatives to hCG that do not present the antigenic problems while hastening ovulation and increasing follicular development.

Deslorelin is one GnRH agonist that has been shown to be effective for inducing ovulation in the mare when given to mares exhibiting estrus and having a follicle ≥30 mm in diameter; the vast majority of such mares ovulate within 48 h of injection (McKinnon et al., 1993; Meinart et al., 1993; Squires et al., 1994). Unfortunately, the commercial availability of deslorelin implants revealed unwanted side effects: prolonged interovulatory interval, reduced concentrations of LH and FSH, and decreased follicular development (Johnson et al., 2000;
administered a deslorelin implant entered prolonged anestrus lasting the entire breeding season (Johnson et al., 2000). Because of these problems, an effective alternative to the deslorelin implants has been sought.

The GnRH-agonist buserelin has been shown as effective at hastening ovulation as hCG and also shows an increased level of LH prior to ovulation (Barrier-Battut et al., 2001). Histrelin is yet another GnRH agonist which hastens ovulation. However, prolonged use of buserelin and histrelin, similar to deslorelin, can lead to a decrease in gonadotropic hormones (Johnson et al., 2000; Farquhar et al., 2001; Blanchard et al., 2002).

The present experiment was primarily designed to compare the efficacy of two doses of several GnRH analogs (deslorelin, buserelin, and histrelin) for increasing plasma levels of LH and FSH and inducing ovulation in preovulatory mares. Based on the descriptions and results in Chapter II regarding mares of high versus low leptin status, it was of interest to determine whether leptin status affected the response to such GnRH analogs during the normal breeding season. Thus, the effects of leptin status on the responsiveness to GnRH secretagogues was studied by evaluating hyperleptinemic mares and normal mares interspersed within the treatment assignments.

**Materials and Methods**

Twenty nine light horse mares from the herd at the Horse Farm at the LSU Agricultural Center were used. On day 1, all mares were teased with a stallion and observed for signs of estrus. Mares in estrus had their ovaries examined by transrectal ultrasonography (Aloka 550V with 5-MHz linear-array transducer, Aloka Science and Humanity, Wallingford, CT), and follicle sizes were recorded. Mares that did not exhibit signs of estrus were treated i.m. with 2 mL of
PGF$_{2\alpha}$ (Lutalyse 5 mg/mL, Pharmacia and Upjohn Company, Kalamazoo, MI) or fluprostenol (50 µg/mL, BET Pharm LLC, Lexington, KY) to cause luteolysis.

Once a mare showed a follicle $\geq$ 30 mm in diameter, she was randomly assigned to one of 10 treatments. The treatments were as follows: deslorelin 500 µg, deslorelin 166.5 µg, histrelin 500 µg, histrelin 166.5 µg, buserelin 500 µg, buserelin 166.5 µg, GnRH 500 µg, GnRH 166.5 µg, saline (negative control), and GnRH 1.5 mg (positive control). The solutions of GnRH and analogs were provided by BET Pharm LLC, Lexington, KY. All injections were 2 mL with saline as the vehicle.

For each treatment injection, a blood sample was collected at 0, 1, 2, 4, 6, 12, and 24 h relative to injection, and then daily at 0800 through day 7. Jugular blood was collected in sodium heparin-coated tubes (Vacutainer, Becton and Dickinson, Franklin Lakes, NJ). Blood was then centrifuged and plasma was harvested and stored frozen. Blood was assayed with radioimmunoassay for levels of LH (Thompson et al., 1983b) and FSH (Thompson et al., 1983a). Progesterone levels were measured using commercially available reagents (Diagnostic Laboratory Systems, Webster, TX).

Once treated, mares were examined daily until ovulation occurred and a CL formed. On day 7 after ovulation, mares were treated with a 2 mL of either PGF$_{2\alpha}$ or fluprostenol to lyse the CL and to bring her back into heat. Mares that returned to estrus were again randomly assigned to treatment with the following restrictions: 1) no mare received the same analog twice and 2) no mare received the same dose level (500 vs 166.5) twice.

Jugular blood was drawn before the start of the experiment for determination of leptin levels. Levels were characterized as low (<10 ng/ml) or high (>20 ng/ml) at the end of the experiment. Unfortunately, due to the random nature of the treatment assignments and the fact
that only about one third of obese mares are Hyperleptinemic, there were an uneven number of high or low mares per group.

Data were analyzed using the MIXED procedure of SAS (SAS Institute, Cary, NC). Treatments were arranged as split plot analysis with repeated measures (Gill and Hafs, 1971). Differences between treatment groups were compared with the LDS test (Steel and Torrie, 1980). The LSD values were calculated for $\alpha = 0.05$.

**Results**

Means for ovulation and follicular size data are presented in Table 3.1. There was no difference ($P > 0.1$) among treatments for the day of ovulation relative to the injection or for the size of the follicle 1 day prior to ovulation. One-third (33.3%) of treated mares did not ovulate within 72 h of treatment; the mean day after treatment for their ovulation was 3.01. Only 17% of control mares did not ovulate before the 72-h mark; their mean day of ovulation compared to treatment was day 4.5.

Follicle stimulating hormone levels varied ($P < 0.0001$) among treatments over time (Figure 3.1). There were two phases of FSH secretion: 1) the immediate rise after injection of saline (no rise), GnRH, or an analog and 2) the rise (or lack thereof indicating suppression) several days later, representing the postovulation release from follicular products.

Luteinizing hormone levels were also different ($P < 0.0001$) among treatments over time (Figure 3.2). In control mares, LH levels continued to rise to their normal peri-ovulatory peak 4 days later. In most mares receiving GnRH or an analog, there was an immediate rise in LH levels, followed by some degree of suppression (e.g., after 500 µg of histrelin or 1.5 mg GnRH) or lack of suppression (e.g., after the lower doses of GnRH).
Table 3.1. Mean follicular diameter and ovulation data for mares receiving GnRH analog treatments or controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Mares</th>
<th>Follicle size before ovulation, mm</th>
<th>Treatment to ovulation, days</th>
<th>Number ovulated by 48 h (%)</th>
<th>Number ovulated by 72 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deslorelin, 500 µg</td>
<td>6</td>
<td>42.2</td>
<td>3.7</td>
<td>2 (33)</td>
<td>4 (67)</td>
</tr>
<tr>
<td>Deslorelin, 166.5 µg</td>
<td>6</td>
<td>40.3</td>
<td>2.3</td>
<td>4 (67)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>Histrelin, 500 µg</td>
<td>6</td>
<td>39.0</td>
<td>2.0</td>
<td>5 (83)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>Histrelin, 166.5 µg</td>
<td>5</td>
<td>38.7</td>
<td>3.5</td>
<td>3 (60)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>Buserelin, 500 µg</td>
<td>6</td>
<td>41.3</td>
<td>2.5</td>
<td>5 (83)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>Buserelin, 166.5 µg</td>
<td>6</td>
<td>41.2</td>
<td>4.5</td>
<td>3 (50)</td>
<td>3 (50)</td>
</tr>
<tr>
<td>GnRH, 500 µg</td>
<td>7</td>
<td>38.7</td>
<td>3.0</td>
<td>3 (43)</td>
<td>4 (57)</td>
</tr>
<tr>
<td>GnRH, 166.5 µg</td>
<td>6</td>
<td>41.5</td>
<td>3.2</td>
<td>2 (33)</td>
<td>3 (50)</td>
</tr>
<tr>
<td>Saline</td>
<td>6</td>
<td>40.3</td>
<td>3.0</td>
<td>1 (17)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>GnRH, 1.5 mg</td>
<td>5</td>
<td>39.6</td>
<td>2.4</td>
<td>3 (60)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.40</td>
<td>0.24</td>
<td>0.41</td>
<td>0.79</td>
</tr>
</tbody>
</table>
Figure 3.1. Mean plasma concentrations of FSH over time for GnRH analog treatments. Levels between treatments were significantly different (P < 0.0001) over time. The least significant difference (LSD) value at $\alpha < 0.05$ for comparison of means is indicated by the vertical bar.
Figure 3.2. Mean plasma concentrations of LH over time for GnRH analog treatments. Levels between treatments were significantly different (P < .0001) over time. The least significant difference (LSD) at $\alpha = 0.05$ for comparison of means is indicated by the vertical bar.
Breakdown of the LH and FSH responses between high leptin mares and low leptin mares (Figures 3.3 and 3.4, respectively) resulted in significant interactions (P < 0.0001) for both gonadotropins, indicating that high and low mares did not respond in each case the same. These comparisons were complicated by the fact that some groups had no or only one mare in the group (buserelin 500 µg and saline groups). The LSD value is indicated on each graph for comparison of means when a significant F value was detected.

Discussion

Gonadotropin responses varied greatly among mares within each treatment and dose. In general, it might be expected that the 500 µg dose would result in a greater gonadotropin response than the 166.5 µg dose. The fact that some means for the lower dose were higher than for the higher dose indicates that both doses were likely near the plateau region of the dose-response curve. Administering the injections on the day that a ≥30 mm follicle was detected resulted in considerable variation in starting LH levels, with some mares starting at 1 to 2 ng/mL while others started at 10 ng/mL.

Relative to the potency of GnRH and the analogs for increasing gonadotropin levels, two aspects were clear. First, the immediate response (in the first 12 h) should be proportional to the potency, allowing for the variations discussed in the previous paragraph. Secondly, for each gonadotropin, the pattern of levels at 96 to 144 h after injection (4 to 6 days) was also very revealing. That is, in mares receiving saline, LH levels at these times were rising or peaking, as expected from the normal peri-ovulatory surge, whereas FSH levels were rising from low levels due to the release from follicular products around the time of ovulation (Ginther, 1992; Johnson et al., 2000). In contrast, the more potent analogs suppressed both of these rises at 96 to 144 h, as has been previously reported for deslorelin implants (Johnson et al., 2000). Thus, even though...
Figure 3.3. Mean plasma concentrations of FSH for each GnRH treatment by high versus low leptin mares. High leptin results (top row) are indicted with an “H” on the graph, and low leptin results (bottom row) are marked with an “L.” Number of mares per group is indicated by N.
Figure 3.4. Mean plasma concentrations of LH for each GnRH treatment by high versus low leptin mares. High leptin results (top row) are indicated with an “H” on the graph, and low leptin results (bottom row) are marked with an “L.” Number of mares per group is indicated by N.
the initial FSH response might be similar between doses of deslorelin, subsequent low levels of FSH for the high dose indicated a greater downregulation, which has been noted by several groups (Johnson et al., 2000; Blanchard et al., 2002; Farquhar et al., 2001, 2002).

Histrelin appeared to have the greatest LH and FSH responses of all the treatments, and the later suppressive effects were evident for the 500 µg dose. Neither of the buserelin doses showed a remarkable initial LH or FSH response to the injections, and neither dose seemed to produce much long-term suppression. Although the LH response to the low dose of buserelin was consistently higher than that of the high dose, the initial LH levels were quite different.

The FSH response to the 1.5 mg injection of GnRH was noteworthy in that the initial responses of LH and FSH were minimal (relative to the analogs) but there was obvious inhibition of LH and FSH in the 96- to 144-h samples. The large, prolonged gonadotropin responses after analog injection are supposedly due to longer half-lives of the analogs. Increasing the dose of GnRH from 166.5 µg to 1.5 mg had very little effect on its short-term efficacy, whereas it did appear to result in downregulation not evident at the lower doses.

Previous studies had indicated that buserelin increased FSH and LH secretion in mares before ovulation (Squires et al., 1981; Barrier-Battut et al., 2001). In the present experiment, buserelin elicited an immediate LH and FSH response, but exhibited no inhibitory activity in later samples. Of the three analogs tested, buserelin appeared to have the lowest efficacy for gonadotropin release.

When adding the high versus low leptin variable to the analyses, there was generally no difference in response to any treatment by high or low leptin mares. There were a few instances in which the high mares appeared to have higher responses, but there were also instances in
which the response in low leptin mares was higher than in the high leptin mares. Most of the responses were similar for both groups. Moreover, assessments of response were hindered (and perhaps confused) by the small number of high or low leptin mares in any given group, given that only approximately one-third of mares with high body condition score exhibit the hyperleptinemic state (Cartmill, 2004). Regardless, based on the responses overall, it appears that leptin status has little or no impact on the average response of LH or FSH to GnRH or these analogs at these doses in the preovulatory mare.

In summary, comparison of the LH and FSH responses in preovulatory mares to GnRH and three of its analogs at 500 and 166.5 µg doses revealed that histrelin was a potent secretagogue for both gonadotropins followed closely by deslorelin. Buserelin was generally more potent than GnRH itself but seemed less potent than histrelin or deslorelin. Increasing the dose of GnRH to 1.5 mg did little to improve its secretagogue activity, but this high dose did exhibit downregulation activity on LH and FSH secretion 4 to 6 days after injection. Averaged across all secretagogues, there appeared to be little or no impact of leptin status on the LH or FSH responses of mares.
SUMMARY AND CONCLUSIONS

In the first experiment, no significant reproductive differences were found in mares of high body condition displaying hyperleptinemia relative to mares of equivalent body condition with low leptin levels. Hyperleptinemic mares did have greater insulin responses to glucose and a greater clearance rate of infused glucose compared to low leptin mares. Although there was a large variation in day of first ovulation, means for the high and low leptin mares were essentially the same. Both groups experienced their first ovulation by the first week in March, which is nearly one month earlier than the previously reported day of April 1 (Ginther, 1992). This was likely due to the high body condition of the mares, which has been shown to be a factor affecting the length of the seasonal anovulatory period (Gentry et al., 2002b). Comparison of high and low leptin mares during the first estrous cycle of the season revealed no differences in follicular sizes or distributions or in LH or FSH levels. The prolactin responses after sulpiride injection indicated that high leptin mares had a slightly greater prolactin response relative to low leptin mares.

In the second experiment, responses to GnRH and three of its analogs in mares with high versus low leptin were studied. Differences were found among GnRH analogs, with histrelin and deslorelin appearing to be most potent for the secretion of LH and FSH in preovulatory mares; buserelin was less potent than these latter two analogs, but was more potent than GnRH. Averaged over all the secretagogues, there did not appear to be any impact of leptin status of the mares on their LH or FSH responses or on the time to ovulation.

In conclusion, mares of high body condition previously identified as hyperleptinemic do indeed have altered insulin and glucose characteristics that are similar to those described for humans with type II diabetes. However, unlike their human counterparts, hyperleptinemic mares
do not seem to have any alteration in ovarian function, in the hypothalamic-pituitary-gonadal axis, or in the ability to transition from the anovulatory season to the breeding season.


VITA

Cara Alexandra Waller, daughter of Bill and Ania Waller, was born in Silver Spring, Maryland, on April 7, 1981. Cara is the older sister of Erin and Davina Waller. Cara graduated from John F. Kennedy High School in Silver Spring, Maryland, in 1999. In May, 2003, she completed her Bachelor of Science degree in animal science at the University of Maryland, College Park. During her career at Maryland, Cara graduated from the Life Science Scholars program and taught riding lessons with the University Equestrian Club. She also founded and presided over the Maryland Club Swim Team for three years. Cara began working towards her Master of Science degree at Louisiana State University in the fall of 2003. Cara married Robert Leland Wright in February, 2005. Upon successful completion of her Master of Science degree, Cara will attend veterinary school at the Virginia-Maryland Regional College of Veterinary Medicine.