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Reproductive and metabolic effects of recombinant equine leptin on seasonally anovulatory mares

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REPRODUCTIVE AND METABOLIC EFFECTS OF RECOMBINANT EQUINE LEPTIN ON SEASONALLY ANOVULATORY MARES

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ABSTRACT

Mares of poor body condition have low plasma leptin concentrations, while obese mares have the highest concentrations. Leptin is a primary signal of body condition to the brain in other species; therefore, low leptin concentrations in thin mares could contribute to their extended anovulatory period in winter compared to obese mares. The current experiment was designed to determine whether recombinant equine leptin, administered to seasonally anovulatory mares, would induce ovarian activity and ovulation in the winter. Leptin effects on metabolism were also studied. Beginning January 7, leptin-treated mares (n = 9) received daily i.m. injections of 10 mg recombinant equine leptin in saline and control mares (n = 10) received equivalent injections of gelatin. Reproductive effects were assessed by daily blood samples and regular ultrasound examination of the ovaries. Weights were also collected routinely. In addition, mares were confined to individual pens and hay consumption was measured as a means of evaluating appetite. A 24-h period of 30-min blood sampling was used to characterize hormone patterns. Over the course of the experiment, there was no difference between groups in follicular activity, date of first ovulation, or prolactin in either daily or frequently collected blood samples. Leptin-treated mares lost more ($P < 0.0001$) weight than control mares in the first 31 d; however, there was no difference in appetite as indicated by 24-h hay consumption. Post-experimental analysis revealed that leptin-treated mares developed antibodies ($P < 0.001$) against the injected leptin beginning around d 16. Subsequent leptin treatment did not affect growth hormone secretion during the frequent blood sampling window. Leptin treatment did not affect daily triiodothyronine concentrations; however, treated mares had lower ($P < 0.016$) daily thyroxine concentrations than control mares. Treated mares also had a tendency ($P = 0.11$) to have lower insulin concentrations during the frequent sampling period. In conclusion, daily treatment with recombinant equine leptin had an immediate effect on body weight without any

effect on hay consumption. Although leptin-treated mares had lower thyroxine concentrations and a tendency for lower insulin, no effect was observed on reproductive endpoints in the time period studied.

INTRODUCTION

Leptin is a protein hormone that is secreted by adipocytes and serves several functions, including regulation of feed intake. Another function of the hormone is as a signal to the brain regarding nutritional status of the individual (Houseknecht et al., 1998) and thereby to the reproductive system as well.

Research has shown correlation of body condition, age, season, and gender with leptin concentrations in horses (Fitzgerald and McManus, 2000; Gentry et al., 2002a, b; Buff et al., 2002; Cartmill et al., 2003a). Leptin concentrations vary with season, with lower circulating concentrations occurring in winter regardless of age or nutritional status and body condition (Fitzgerald and McManus, 2000; Gentry et al., 2002a). While aged mares have increased leptin concentrations compared to younger mares (Fitzgerald and McManus, 2000; Buff et al., 2002), leptin concentrations are higher in both groups in summer than winter (Fitzgerald and McManus, 2000; Gentry et al., 2002a). Additionally, mares and geldings have higher circulating leptin concentrations than stallions (Cartmill et al., 2003a). Feed restriction, both long term (Gentry et al., 2002a) and short term (McManus and Fitzgerald, 2000), causes leptin concentrations to decline.

Multiple effects of leptin on reproduction have been observed. It can restore reproductive function in sterile *ob/ob* mice (Barash et al., 1996; Chehab et al., 1997; Mounzih et al., 1997) and is required for the normal onset of puberty in humans, mice, and cattle (Ahima et al., 1996; Chehab et al., 1997; Garcia et al., 2002). Research with horses has shown that a large number of mares of high body condition fail to enter an anovulatory state; some cycle normally for the duration of the traditional anovulatory season (Gentry et al., 2002a).

Given that manipulation of the estrous cycle is a primary focus of the horse industry to meet the demand for foals born as near to January 1 as possible, researchers are continually looking for new and innovative ways to accomplish this goal. The question arose whether or not treatment with exogenous leptin would induce early cyclicity in mares of normal body condition and leptin concentrations. However, the lack of an economically viable source of leptin prevented the testing of this hypothesis in the past. Recent production of recombinant equine leptin (A. Gertler, Hebrew University of Jerusalem, Rehovot, Israel) made it possible to pursue this line of research.

The experiment described herein was designed to determine if administration of recombinant equine leptin to seasonally anovulatory mares of normal body condition and leptin concentrations would, in fact, induce early onset of cyclicity and ovulation in winter. At the same time, data were collected to determine, what, if any, metabolic effects might be observed. This would also provide additional methods for determining the efficacy of the recombinant equine leptin if no reproductive effects were observed.

REVIEW OF LITERATURE

Hypothalamic-pituitary axis

Extensive research has led to a common understanding that the reproductive axis is managed by the production of gonadotropin releasing hormone (GnRH) by the hypothalamus. In the horse, production of GnRH occurs throughout the hypothalamus (Irvine and Alexander, 1993). Once synthesized, GnRH is packaged and stored in secretory granules within the median eminence, which, when stimulated by neuronal activity, are released into capillaries of the primary plexus; GnRH travels to and acts upon receptors on the gonadotropes within the pars distalis of the adenohypophysis (Alexander and Irvine, 1993; Kainer, 1993).

Gonadotropin releasing hormone is responsible for stimulating the production of its own receptors on the gonadotropes (Clayton, 1989; Alexander and Irvine, 1993). It is also responsible for the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the target cells of the adenohypophysis. The release of GnRH is in a pulsatile fashion, the frequency of which may regulate the secretion of LH and FSH (Ginther, 1992; Alexander and Irvine, 1993). More frequent pulses of GnRH (every 45 min) favor secretion of LH, while less frequent pulses (every 6 h) favor FSH (Alexander and Irvine, 1993). This is supported by the fact that during estrus, GnRH pulses occur at a rate of 2/h, resulting in high circulating concentrations of LH and low concentrations of FSH. Conversely, during diestrus, GnRH pulses occur at a much less frequent rate (around 0.1/h), resulting in high FSH and low LH secretion (Alexander and Irvine, 1993).

Luteinizing hormone and FSH are glycoprotein hormones consisting of matching alpha chains and differing function-specific beta chains (Alexander and Irvine, 1993). Effects of the gonadotropins are exerted through binding with their specific ovarian receptors. The receptors

for FSH are found mainly on the granulosa cells of the ovarian follicles, whereas LH receptors are located on the thecal cells of the pre-antral follicles (Alexander and Irvine, 1993). Following antral development, LH receptors can also be found on the granulosa cells (Alexander and Irvine, 1993). The primary function of FSH is stimulation of follicular growth (Guyton and Hall, 2000); however, the final stages of follicular growth and ovulation are controlled by LH (Alexander and Irvine, 1993). Accompanying follicular growth is the production of estradiol and inhibin as well as the release of progesterone by the corpus luteum (CL), which forms following ovulation. Each of these hormones provides a negative feedback mechanism on the hypothalamus and the adenohypophysis (Alexander and Irvine, 1993). Therefore, a closely regulated feedback loop exists to assure balance of all hormones and their actions.

Estrous cycle of the mare

The estrous cycle is defined as the period of time from the beginning of one estrus to the start of the next (Daels and Hughes, 1993). According to Ginther (1992), the average estrous cycle is 21.7 d, with a range of 19.1 to 23.7 d. The estrous cycle consists of 2 distinct phases: the follicular phase, commonly known as estrus, and the luteal phase, or diestrus (Daels and Hughes, 1993). Circulating FSH causes the follicular growth that occurs during diestrus and early estrus. Estrogen is then released by the follicles, resulting in the exhibition of sexual behavior and receptivity to the stallion (Daels and Hughes, 1993). The average length of estrus is 6.5 d with a range of 4.5 to 8.9 d (Ginther, 1992). The estrogen released by the growing follicles serves the dual purpose of stimulation of LH release through a positive feedback mechanism. The follicles also release the hormone inhibin from the granulosa cells, which, along with estradiol, has a negative feedback effect on FSH release (Bergfelt and Ginther, 1985). The subsequent rise in LH leads to ovulation, or expulsion, of the oocyte from the dominant

follicle. Ovulation typically occurs 24 to 48 h before the end of estrus (Daels and Hughes, 1993), but can be quite variable among mares.

Following ovulation, the evacuated follicle develops into a CL, which secretes the progesterone that dominates the diestrus phase. Ginther (1992) found that the duration of diestrus is 12.1 to 16.3 d following ovulation. Under the influence of progesterone, a mare will not be sexually receptive to the stallion (Daels and Hughes, 1993). The CL will persist for about 14 d, and then begin to regress, allowing the mare to return to the estrus phase once more (Daels and Hughes, 1993).

There is a reciprocal pattern to the gonadotropins during the estrous cycle. Generally speaking, when LH concentrations are high, FSH concentrations are low. It is believed that LH is low during diestrus due to negative feedback from progesterone secreted by the CL, whereas the high concentrations of FSH are allowed because of a lack of negative feedback from the ovaries (Freedman et al., 1979). Secretion of FSH occurs in surges at 10- to 11-d intervals (Evans and Irvine, 1975). Initial development of multiple follicles (up to 20) is caused by the first FSH surge in early diestrus (Evans and Irvine, 1975). The highest concentration of FSH occurs during mid-diestrus surge. It is this surge that allows dominant follicles to develop further (Evans and Irvine, 1975). Secretion of FSH begins to decrease as the follicular phase progresses, and LH secretion undergoes a sharp increase, peaking approximately 1 d after ovulation (Miller et al., 1980).

Seasonality

Mares are considered to be seasonally polyestrous. Most non-pregnant mares will exhibit repeated estrous cycles throughout the spring and summer months, commonly referred to as the breeding season, with many becoming reproductively quiescent during the winter months (Daels

and Hughes, 1993). This phenomenon is modified by several factors, including nutrition and climate, but is controlled primarily by photoperiod (Daels and Hughes, 1993). This period of decreased to absent reproductive activity in winter is best described as the anovulatory period.

Following the last ovulation of the breeding season, the mare will slowly enter an anovulatory state due to inadequate LH secretion and lack of final growth of an ovulatory follicle (Snyder et al., 1979). The anovulatory period is divided into 3 phases by Ginther (1992). The first is the receding phase, also called fall transition, which coincides with the autumnal equinox (September 21). This phase is characterized by the failure to ovulate large follicles due to the absence of an LH surge (Ginther, 1992). The pituitary reserves of LH decline steadily from mid-breeding season to mid-anovulatory season (Silvia et al., 1986). Following the receding phase is the inactive phase, during which few follicles develop beyond 10 to 20 mm (Ginther, 1992). The last phase is known as the resurging phase, or vernal transition. At this time the mare begins to recover from the anovulatory state (Ginther, 1992).

The anovulatory period is defined by Sharp and Davis (1993) as a time of sexual incompetence and indifference during the winter months. The reason for this inactivity is believed to be the sharp decrease in GnRH secretion by the hypothalamus (Sylvia et al., 1986). Sharp and Grubaugh (1987) measured hypothalamic GnRH in pony mares using push-pull perfusion technique and found no detectable GnRH secretion in winter. Additionally, Thompson et al. (1986, 1987) reported a distinct suppression of gonadotropins in the winter months. It has been found, however, that some mares will display weak signs of estrus despite an absence of follicular activity (Ginther, 1992; Gentry et al., 2002a).

During the resurging phase, the span of time between the initial rise in FSH and first ovulation can be lengthy (Sharp and Davis, 1993). Secretion of FSH will fluctuate, but increases

in LH remain absent until just before ovulation (Hines et al., 1991). Also, as the first ovulation is approached, FSH secretion and pulse amplitude will actually decrease (Freedman et al., 1979; Silvia et al., 1986; Hines et al., 1991) due to the production of inhibitory factors (estradiol and inhibin) from large follicles (Miller et al., 1981). Secretion of LH at this time resurges, due to the rise in GnRH secretion coupled with the development of steroidogenically competent follicles, which elevate peripheral estrogen concentrations (Sharp and Davis, 1993). Mares at this time may exhibit long periods of erratic estrus, and their receptivity to a stallion can be ambiguous (Hines et al., 1991).

Folliculogenesis and follicular waves

The primary functional unit of the ovary is the follicle. The process of growth and differentiation of ovarian follicles is called folliculogenesis. There are 3 phases to this process: selection, dominance, and then ovulation or atresia (Pierson, 1993). Follicles have both endocrine and exocrine functions. Both estrogens and protein hormones are produced and released by follicles, as well as expulsion of the oocyte at ovulation. Control of ovarian activity is exerted through a myriad of processes: endocrine (systemic hormone release); paracrine (local intracellular diffusion); and autocrine (release of substances that bind to the cell's own receptors) (Pierson, 1993).

The mare, like other mammalian females, is born with a finite number of primordial follicles on her ovaries (Ginther, 1992). A primordial follicle is a simple oocyte surrounded by one layer of cells. Ginther (1992) classifies antral follicles into 3 categories based on size: small (2 to 10 mm), medium (11 to 24 mm), and large (≥ 25 mm). During the preovulatory stage, the number of small follicles decreases; they subsequently increase during the postovulatory stage (Pierson, 1993). There is a relatively constant number of medium follicles throughout the cycle,

and the number of large follicles is dependent upon follicular waves (Pierson, 1993), which will be explained further.

A group of small follicles is constantly growing and regressing independent of reproductive status, providing a cohort from which large follicles are selected (Ginther, 1992). From here, pre-antral follicles develop. This stage is marked by growth of the oocyte, formation of the zona pellucida, and division of granulosa cells into cuboidal epithelium (Pierson, 1993). Upon development of the antrum, which occurs around 300 μm in the mare, the follicle is said to be a tertiary follicle. Further development will occur, including an increase in volume of the antrum and thickening of the follicular wall (Pierson, 1993).

The vast majority, 99%, of follicles that develop to the antral stage are not destined for ovulation, but rather for atresia. When a follicle goes atretic, it undergoes a gradual reduction in size and activity, eventually disappearing from the ovary. The rest ovulate (Pierson, 1993).

The gonadotropins are highly involved in regulation of follicular growth, despite the fact that they are not needed for development to the antral stage. They are, however, necessary for continued growth. In pre-antral follicles, LH and FSH receptors are acquired in the thecal and granulosa cell membranes, respectively (Pierson, 1993). Under the influence of LH, the thecal cells produce androgens, which then pass through the basal lamina and are aromatized to estrogens by the granulosa cells under the control of FSH. The rise in estrogen feeds back positively on LH secretion, which further increases estrogen production. There is a corresponding increase in LH receptors. This marked increase in estrogen production indicates the transition from the antral stage to the preovulatory stage. The follicle will fail to reach the final stages of development without the capability to respond to the gonadotropins (Pierson, 1993).

Growth and selection of large follicles seems to occur in "waves" in mares, as in other species (Ginther, 1992; Pierson, 1993). Follicular waves can be attributed to bimodal and unimodal FSH surges, according to Pierson (1993). Ginther and Bergfelt (1993) also attribute follicular waves to increases in FSH concentrations. When several follicles grow in synchrony (equal size and rate of growth), with eventual dissociation, this constitutes a major follicular wave (Ginther, 1992). Dissociation, also known as divergence, is the selection of a dominant follicle for continued development. Subordinate (smaller) follicles will begin to undergo atresia at this point (Ginther, 1992). All mares will experience at least one follicular wave per cycle, with some having two. When two waves occur, the first wave is the secondary wave, happening during late estrus to early diestrus. Dominant follicles formed in this wave typically become static and regress; however, occasional diestrus ovulations do occur. The primary wave is the second wave during mid-diestrus. A dominant follicle from this wave will become the ovulatory follicle (Ginther, 1992).

Ginther et al. (2001) explain that FSH concentrations decrease once a follicle reaches approximately 13 mm. The follicles themselves cause this decrease. Estradiol, free IGF-1, activin-A, and inhibin-A begin to increase differentially in the dominant follicle just before deviation. Deviation occurs due to higher responsiveness by the dominant follicle to the gonadotropins. Deviation is marked by the two largest follicles reaching an average size of 19.0 to 22.5 mm. Since FSH concentrations have already decreased, only the most developed follicles will continue to grow. Continued production of estradiol and inhibin by the dominant follicle causes atresia of the subordinate follicles (Ginther, 1992; Pierson, 1993).

Ovulation and the luteal phase

Mares differ from females of other species in how they ovulate the dominant follicle. Rather than expelling the oocyte and follicular fluid from the exterior surface of the ovary, mares ovulate from an ovulation fossa (Ginther, 1992; Pierson, 1993). Mares also have LH receptors in the granulosa cells of the dominant follicle, allowing for response to increasing LH concentrations during estrus (Ginther, 1992). The LH peak does not occur in the mare until approximately 1 d post-ovulation, suggesting it is the rise in LH, rather than the peak as in other species, that causes ovulation in the mare (Pierson, 1993).

Ovulation is the start of the luteal phase. This phase of the cycle is dominated by progesterone secreted by the CL, which is fully formed by d 3 (Niswender and Nett, 1993). Luteinizing hormone is required by the CL in the mare for continued function. Progesterone increases receptors for LH. Around d 14, if the mare is not pregnant, the uterus will secrete prostaglandin-$F_{2\alpha}$, which lyses the CL and causes progesterone to drop, allowing the estrus cycle to begin again.

Leptin

Leptin is a 16-kDa protein hormone secreted primarily by white adipocytes. It plays a role in multiple bodily processes including regulation of food intake, energy expenditure, and energy balance in rodents and humans (Houseknecht et al., 1998). Many of leptin's effects on food intake and energy regulation are thought to be controlled by neurotransmitters such as neuropeptide Y (NPY; Houseknecht et al., 1998). Leptin has also been found to play a regulatory role in insulin secretion by β cells of the pancreas and insulin action and metabolism in adipocytes and skeletal muscle as well as regulation of nutritional effects on reproduction

(Houseknecht et al., 1998). Its potential implications on the treatment of obesity could be quite profound.

Leptin's discovery process began in 1950 with the identification of a model for obesity, the *ob/ob* mouse (Ingalls et al., 1950). These mice possessed a recessive mutation resulting in sterility in adult individuals and over 50% body fat. Later, a similar mutation was identified in another strain of mice, and they were termed *db/db* mice (Hummel et al., 1966). These animals were also obese, but were also hyperglycemic. Subsequently, parabiosis studies (cross-circulation) were conducted by Coleman (1973). A pairing of *ob/ob* mice with *db/db* mice resulted in decreased food intake and weight loss in the *ob/ob* individuals and a sustained increase in food intake and weight in the *db/db* individuals. Coleman concluded that the *ob/ob* mice were deficient in some factor regulated and produced by adipose tissue, but their brains could respond when exposed to it. Conversely, the *db/db* mice produced the factor, but were immune to it. Due to a lack of the necessary biotechnology available at the time, it would not be until much later that these hypotheses could be tested and verified.

Twenty years later, the leptin gene was finally identified and cloned by Zhang et al. (1994). The gene encoding the leptin receptor was cloned a year later (Tartaglia et al., 1995). Leptin has a helical structure that suggested its receptors would be similar to those of cytokines, which was confirmed when it was determined that the receptors were like those of class I cytokines (Tartaglia et al., 1995). The signal transduction mechanism of leptin receptors in the hypothalamus is activated via the JAK-STAT pathway (Houseknecht et al., 1998). It's unclear at this point if any similarities in signaling exist beyond the JAK-STAT pathway (Houseknecht et al., 1998). Leptin receptors are found throughout the body despite the fact that leptin is primarily produced and secreted by adipocytes (Kline et al., 1997); other tissues, including the placenta

(Hoggard et al., 1997), stomach (Bado et al., 1998), and muscle (Wang et al., 1998), produce much lesser amounts. The long form of the receptor is almost solely located in the hypothalamus and absent in the peripheral bodily tissues (Mercer et al., 1996; Schwartz et al., 1996), while the short form is more diffusely expressed (Houseknecht et al., 1998). It is possible that some of these short receptor forms are involved in transporting leptin through the bloodstream and across the blood-brain barrier (Devos et al., 1996).

Research has shown that leptin serves not only as a signal of energy stores to the brain, but also as a signal of energy balance. Leptin expression and secretion reflect body mass in both rodents and humans (Frederich et al., 1995a; Maffei et al., 1995; Considine et al., 1996) and are highly correlated with adipocyte size (Houseknecht et al., 1996a,b) when in a fed, zero energy balance state. However, slight alterations to energy states cause drastic changes in correlation. Short spans of feed deprivation (12 to 48 h) result in rapid and severe decreases in leptin gene expression (Cusin et al., 1995; Frederich et al., 1995b; Trayhurn et al., 1995; Kolaczynski et al., 1996a). Even subtle changes have dramatic effects. A mere 10% loss of body weight will cause serum leptin concentrations to fall 53% (Considine et al., 1996). Likewise, a 10% weight gain causes a 300% increase (Kolaczynski et al., 1996b).

Dramatic results have been observed in studies where leptin was administered to test subjects. Decreases in feed intake (dependent upon dose), weight loss, loss of fat depots, and increases in energy metabolism have all been observed (Pelleymounter et al., 1995; Levin et al., 1996). It is remarkable that the weight losses following leptin treatment are sustained for several weeks following cessation of treatment (Chen et al., 1996; Azain et al., 1997). This may mean that the loss comes not only from decreases in food intake, but also from an increased metabolism (Houseknecht et al., 1998).

Leptin has an inhibitory effect on NPY. Apparently, this effect is manifested through an inhibition of synthesis of NPY in the hypothalamus (Houseknecht et al., 1998). This 36-amino acid neuropeptide is a potent stimulator of food intake and an inhibitor of gonadotropin secretion (Houseknecht et al., 1998). Neuropeptide Y increases in the hypothalamus and spinal fluid in cases of undernourishment (Houseknecht et al., 1998). However, there is evidence that NPY is not the sole target of leptin. Erickson et al. (1996b) produced *ob/ob* mice with no NPY (knock-out mice) resulting in reduced phenotypic obesity, but body weight was not completely normalized. Normal mice with knocked out NPY also had normal control of food intake and body weight with normal response to leptin (Erickson et al., 1996a).

Multiple reports indicating that treatment with leptin led to a reduction in body weight led to speculation that leptin would be a "cure all" for obesity (Houseknecht et al., 1998). However, subsequent research has led to the knowledge that leptin's effects are much more complicated than just on weight loss. In the realm of agriculture, obesity is not a primary concern for producers, but improving production efficiency is. Optimization of reproductive performance through regulation of energy metabolism could be economically beneficial (Houseknecht et al., 1998).

Research has shown that leptin concentrations are affected by multiple factors, including species, age, gender, nutritional status, and season. Leptin concentrations are strongly correlated to body condition in humans, ruminants, and horses (Prolo et al., 1998; Chilliard et al., 2000; Fitzgerald and McManus 2000; Gentry et al., 2002a,b). Furthermore, Cartmill and associates (2003b) found that obese mares possess a permanent variation in leptin concentrations. These findings indicate a possibility that a genetic determination exists for hyperleptinemia. In horses, Cartmill (2004) determined that mares and geldings have higher leptin concentrations than

stallions, which could not be totally attributed to stallions just being leaner. Conversely, Buff et al. (2002) found no differences between geldings and stallions in leptin concentrations. In that study, body condition was examined and compared to leptin concentrations in general; however, specific averages were not given.

Leptin is also influenced by age in horses. Younger mares (< 5 years of age) are usually leaner with lower leptin concentrations than their older (> 10 years) counterparts (Fitzgerald and McManus, 2000; Buff et al., 2002). As mentioned earlier, feed restriction affects circulating leptin concentrations. Gentry et al. (2002a) found that long-term feed restriction resulted in both lower body condition scores and lower leptin concentrations. In addition, throughout the duration of the study (September through January), leptin concentrations declined in all mares, regardless of body condition. Likewise, a decrease in leptin concentrations was seen following short-term feed restriction in aged and young mares (McManus and Fitzgerald, 2000), with no corresponding change in body condition. These results were in contrast to a previous study finding changes in leptin following a decrease in fat mass (Ahima et al., 1996).

Initial evidence that leptin was related to reproductive function began in the mid-1990's. Recall that *ob/ob* mice with greater than 50% body fat were sterile until cross-circulated with *db/db* mice and exposed to leptin. The exposure to leptin restored normal reproductive function in these animals. In addition, both males and females experienced a gain in gonadal weight, increased concentrations of circulating gonadotropins, and an increase in the number of ovarian follicles or sperm count (Barash et al., 1996; Chehab, 1997; Mounzih et al., 1997).

Leptin not only restores fertility in deficient animals, but is also involved in the onset of puberty. Leptin has been reported to be required for the normal onset of puberty (Chehab et al., 1997). Puberty was hastened in both well-fed and malnourished mice by treatment with leptin

(Ahima et al., 1996; Chehab et al., 1997). In humans, there is a severe delay in menarche in individuals with severely low body condition and corresponding low leptin concentrations (Frisch et al., 1980). Both boys and girls experience a rise in leptin concentrations around the time of onset of puberty, but only the females maintain this increase. Males, on the other hand, experience a decline in leptin concentrations after the onset of puberty (Mantzoros et al., 1997; Cunningham et al., 1999).

Leptin also varies with season. In mares, leptin concentrations decrease drastically between fall and winter (Fitzgerald and McManus, 2000; Gentry et al., 2002a) and rise from July until September. Likewise, in cattle, leptin increases from early winter through the summer solstice (Garcia et al., 2002).

In a study previously mentioned, long-term feed restriction (5 months) led to lower plasma leptin concentrations as well as decreased body condition in mares (Gentry et al., 2002a). In that same study, mares with low body condition scores (3.0 to 3.5) underwent a longer winter anovulatory period, while all but one of their high body condition counterparts continued to cycle or have significant follicular activity throughout the winter. The mares that became reproductively quiescent also had decreased leptin, IGF-1, and prolactin concentrations. Also of note was that a dichotomy of leptin concentrations was observed (mares with <5 ng/mL vs. those 7 to 20 ng/mL), even though body conditions were similar, indicating other factor(s) may be influencing leptin concentrations under conditions of high body condition (Gentry et al., 2002a). Later observations of the same mares up to 3 yr later showed sustained differences of circulating leptin concentrations (L. R. Gentry, unpublished), indicating that some innate factor, perhaps genetic, affected leptin concentrations.

Rationale for present research

Numerous reports have indicated a strong relationship between leptin and reproductive function. Due to pressure on the equine industry to breed mares to foal as near to January 1 as possible, various methods have been and are being studied to facilitate this demand. Leptin has long been suspected as a signal to the brain of reproductive status. Additionally, Waller and co workers (2006) showed that no reproductive differences exist between hyperleptinemic vs. normal leptin mares of high body condition with respect to day of first ovulation after January 7 or number of follicles. These researchers also saw evidence that numerous mares on the project had luteal tissue prior to d 0, indicating ovarian activity during winter. This, coupled with the fact that many high body condition, hyperleptinemic mares fail to enter an anovulatory state during winter (Gentry et al., 2002a), gave rise to the question as to whether or not treatment with leptin in mares with normal leptin concentrations would send a signal to the brain allowing the mare to return to normal cyclicity earlier in the year. The experiment described herein was designed to determine if administration of exogenous recombinant equine leptin would induce early cyclicity in seasonally anovulatory mares. Paralleling these observations, data were collected to determine any coincident metabolic effects that might be attributed to the leptin treatment.

MATERIALS AND METHODS

Mares and Monitoring. Beginning on December 22, 2005, 47 light horse mares were examined via transrectal ultrasonography (Aloka 550V with 5-MHz linear-array transducer; Aloka Science and Humanity, Wallingford, CT) to determine follicular activity. Any mares with follicles >25 mm were eliminated from the pool of mares eligible for the project. A blood sample was also collected via jugular venipuncture into 7 mL sodium heparin-coated tubes (Vacutainer, Becton and Dickinson, Franklin Lakes, NJ) from mares with no follicles <25 mm. Following the initial ultrasound exam and blood sample, a twice-weekly regimen of ultrasound and blood sampling was implemented.

After 3 wk of sample collection, radioimmunoassay was performed on all blood samples to determine plasma progesterone concentrations using commercially available reagents (Diagnostic Laboratory Systems, Webster, TX). A mare was classified as anovulatory if all blood samples were < 1 ng/mL. Only mares with < 25 mm follicles that were also anovulatory were considered for the experiment.

Twenty light horse mares were randomly selected from the pool of eligible horses. On January 5, 2006, the horses were assessed for body condition score (Henneke et al., 1983). Body condition scores ranged from 4.5 to 6.0, with an average of 5.4. The horses were also weighed. Weights ranged from 339 kg to 516 kg, with an average of 462 kg. Ages of the mares ranged from 6 to 17 yr, with an average of 11.8 yr. The 20 horses were paired (matched) as closely as possible based on age, body condition score, and weight; one mare from each pair was then randomly assigned to one of two treatment groups: leptin-treated (n = 10) and controls (n = 10).

Mares were maintained together on pasture (available native grasses) and were supplemented with good quality bermudagrass hay as needed throughout the duration of the

project. Beginning on January 7 (d 0), leptin-treated mares received i.m. injections of 10 mg of recombinant equine leptin (provided by Dr. A. Gertler, Hebrew University of Jerusalem, Rehovot, Israel) in saline (1 mL); injections were repeated daily for 45 d. Control mares received injections of 10 mg of inert protein (gelatin) in 1 mL saline daily for 45 d.

Blood samples were collected every 3 d before treatment starting on d -2. Mares were also examined via ultrasonography twice weekly to monitor ovarian activity. When a mare was found with a follicle ≥ 25 mm, she was scanned daily until ovulation. Blood samples were also collected daily once a follicle ≥ 25 mm was present until 5 d post-ovulation.

Body weights were recorded for all mares on d -2, 16, 31, 47, 107 and 121 as a means of assessing the potential effect of leptin on weight loss. On d 26 or 27, mares were confined to individual pens and hay consumption was recorded over a 24-h period to assess appetite. On d 35 or 39, a period of frequent blood sampling (every 30 min) was included to characterize 24-h hormonal patterns. Blood samples were collected via indwelling jugular catheters and placed in 12 x 75 mm borosilicate glass tubes containing 50 IU sodium heparin (Sigma Chemical Co., St. Louis, MO). All blood samples were routinely centrifuged within 30 min of collection; plasma was harvested and stored at -20˚C.

Sample Analyses. Leptin was measured in daily plasma samples via radioimmunoassay previously validated for equine samples (Cartmill et al., 2003a). Daily concentrations of triiodothyronine and thyroxine were evaluated using commercially available reagents (ICN Pharmaceuticals, Costa Mesa, CA; Kits 07-290102 and 07-292102, respectively). Prolactin, growth hormone, and insulin concentrations were measured in both daily and frequently collected samples using radioimmunoassays previously validated for horse samples (Colborn et al., 1991; Thompson et al., 1992; Depew et al., 1994). Intra- and interassay CV and assay

sensitivities were 6%, 4%, and 0.2 ng/mL for leptin; 5%, 8%, and 3.7 ng/dL for triiodothyronine; 5%, 9%, and 1.2 μg/dL for thyroxine; 7%, 12%, and 0.2 ng/mL for prolactin; 8%, 11%, and 0.5 ng/mL for GH; and 5%, 8%, and 0.5 mIU/L for insulin.

After completion of the experiment, aberrations in the leptin assay consistent with antibody formation against the recombinant equine leptin were observed. To test this possibility, 4-μL samples of plasma from all mares from before treatment and then weekly after treatment were mixed with 200 μL (18 nCi) of I^{125}-labeled human recombinant leptin (about 2 ng in 200 μL phosphate buffered saline; Sigma). After 24 h incubation at 4˚C, the equine gamma globulin fraction was precipitated with 100 μL of rabbit anti-equine-gamma-globulin serum. The pellet was harvested by centrifugation (1,200 x g for 30 min), washed once with 1 mL of cold buffer, and then assessed for radioactivity in a solid scintillation counter.

Statistical Analyses. Data were analyzed by the Proc Mixed procedure of SAS (SAS Institute Inc., Cary, NC). Data from both daily and frequent samples were analyzed for effects of treatment, time, and treatment by time interactions with repeated measures. Single point variables were analyzed via one-way ANOVA. For the period of frequent sampling (24-h window), the main effect of treatment and its interaction with time were analyzed using the Proc Mixed procedure of SAS. When a significant F was detected ($P < 0.05$) the least significant difference (LSD) test (Steele et al., 1997) was used to determine differences between groups within times.

RESULTS

On d 26 after the start of the project, one mare from the leptin-treated-group was euthanized as a result of a non-treatment related infection. Due to the fact that she did not complete the treatment, her information is not included in any of the results, bringing the number of treated mares to 9.

Leptin-treated mares lost more weight ($P < 0.0001$) as a percentage of pretreatment body weight than did control mares during the first 31 d of the experiment (Figure 1). However, there was no difference ($P = 0.47$) in hay consumed between groups under the conditions of the 24-h assessment period (Table 1). Mean ages, body condition scores, and dates of first ovulation are presented in Table 2. Ages and body condition scores were similar ($P > 0.1$) between groups. The day of first ovulation relative to the start of the project (January 7) for the leptin-treated mares did not differ ($P = 0.167$) from that control mares (Table 2). Additionally, there were no differences ($P > 0.1$) in follicular activity (based on size of largest follicle) between groups during treatment (Figure 2).

There was no effect ($P > 0.1$) of leptin treatment on daily prolactin concentrations (Figure 3). Daily treatment with leptin also did not affect ($P > 0.1$) daily triiodothyronine concentrations (Figure 4). However, leptin-treated mares did have lower ($P = 0.016$) daily thyroxine concentrations than controls (Figure 5).

There was no effect ($P > 0.1$) of leptin treatment on plasma concentrations of either growth hormone (Figure 6) or prolactin (Figure 7) during the 24-h blood-sampling period. There was a tendency ($P = 0.11$) for leptin-treated mares to have lower insulin concentrations during the blood-sampling window (Figure 8).

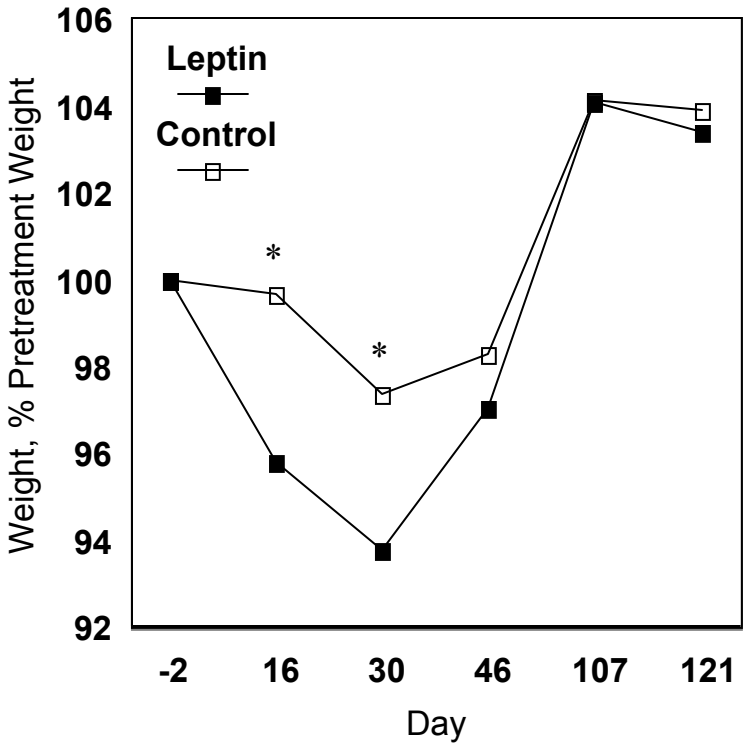

Figure 1. Mean body weights collected on d –2, 16, 30, 46, 107, 121, expressed as a percent of pretreatment weight. Leptin-treated mares lost more weight ($P < 0.0001$) during the first 30 days of the experiment.

Table 1. Mean hay consumption, in kg and as a percent of body weight, consumed by leptin-treated and control mares

Item	Treatment group		P-value
	Control [a]	Leptin-treated [b]	
Kg, hay consumed	5.31	4.45	0.29
Percent body weight consumed	1.16	1.05	0.47

[a]N = 10
[b]N = 9

Table 2. Mean age, body condition score (BCS), and day of first ovulation relative to the start of treatment (January 7). Data are expressed as mean (range).

Item	Treatment group		P-value
	Control[a]	Leptin-treated[b]	
Age	11.3 (6 – 17)	12.4 (9 – 16)	0.39
BCS	5.5 (4.5 – 6.0)	5.4 (4.5 – 6.0)	0.81
Day of first ovulation	81.0 (30.0 – 103.0)	93.0 (72.0 – 106.0)	0.17

[a]N = 10
[b]N = 9

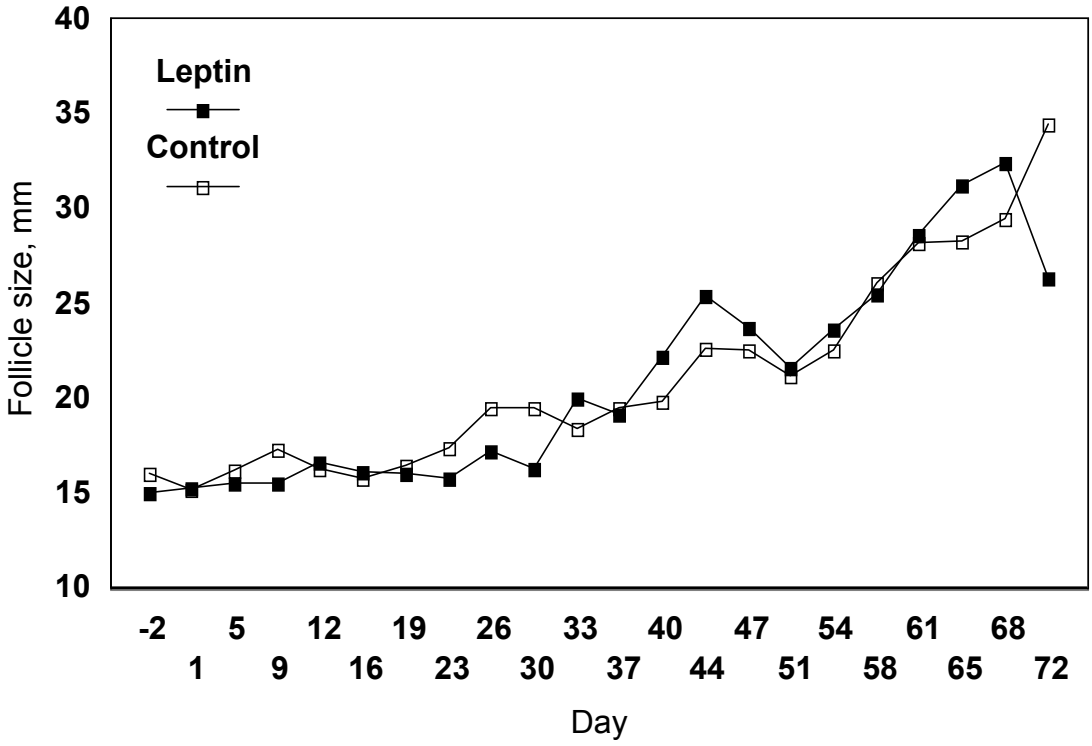

Figure 2. Mean size of largest follicle of leptin-treated versus control mares from day –2 relative to the start of scanning (January 5) through day 72 (March 19). There was no difference (P = 0.45) between treatment groups.

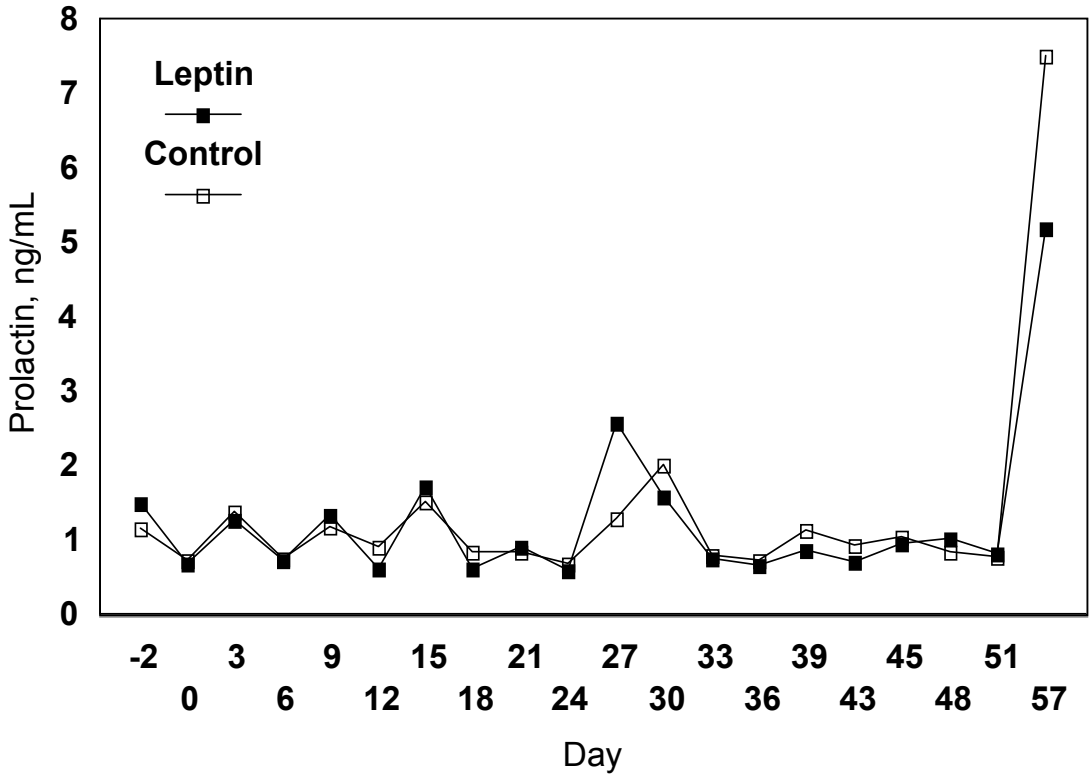

Figure 3. Mean plasma concentrations of prolactin for leptin-treated mares versus control mares from day –2 relative to the start of project (January 7) through day 57. No effect ($P > 0.1$) of treatment was observed.

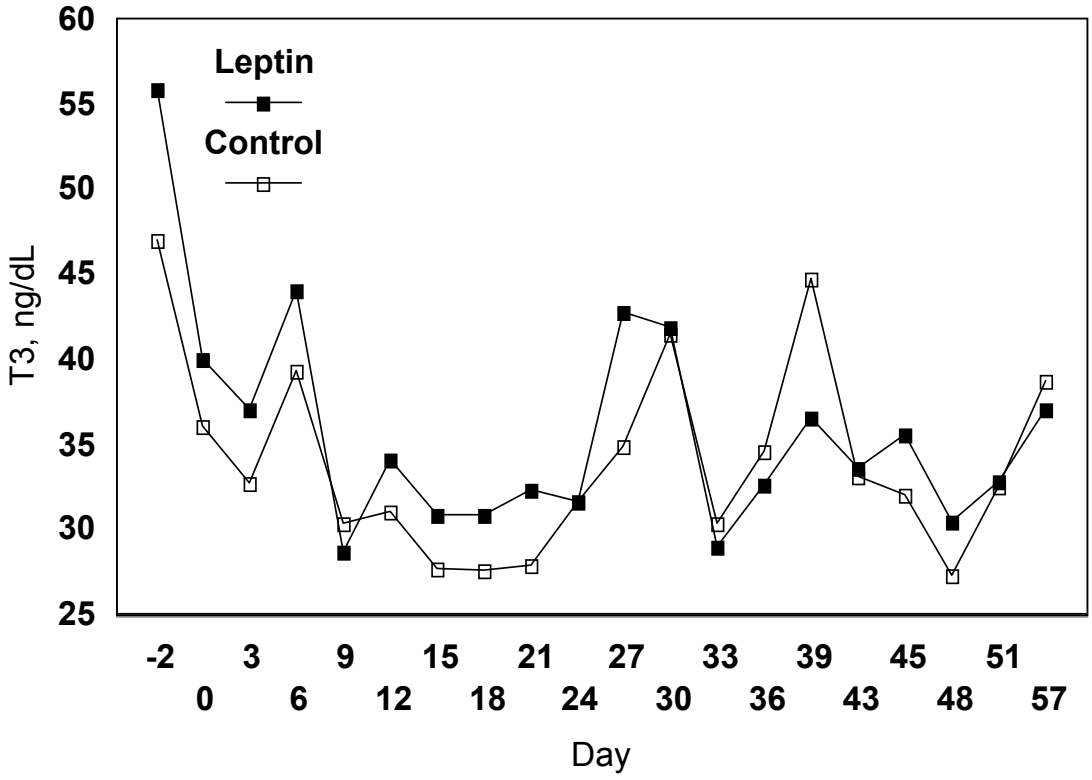

Figure 4. Mean plasma concentrations of triiodothyronine (T3) in leptin-treated mares versus control mares from d –2 relative to the start of the project (January 7) through day 57. No effect ($P > 0.1$) of treatment was observed.

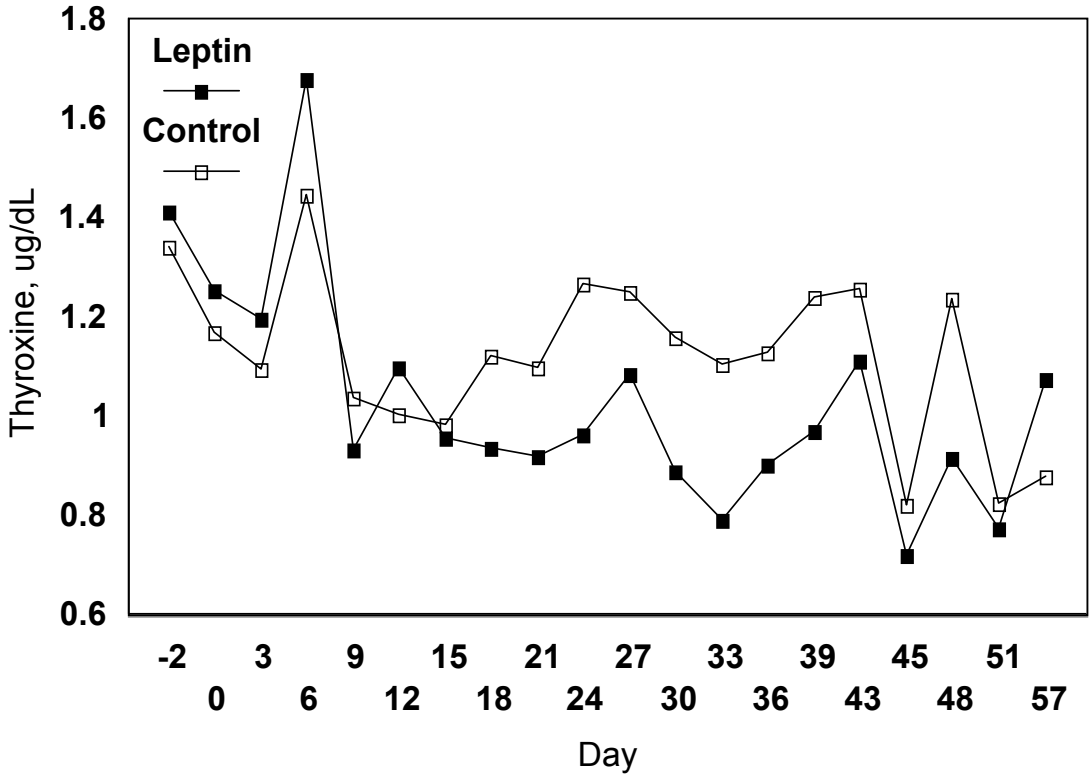

Figure 5. Mean plasma concentrations of thyroxine for leptin-treated and control mares from day –2 relative to the start of the project (January 7) through day 57. Leptin-treated mares had lower (P = 0.016) daily thyroxine concentrations than control mares.

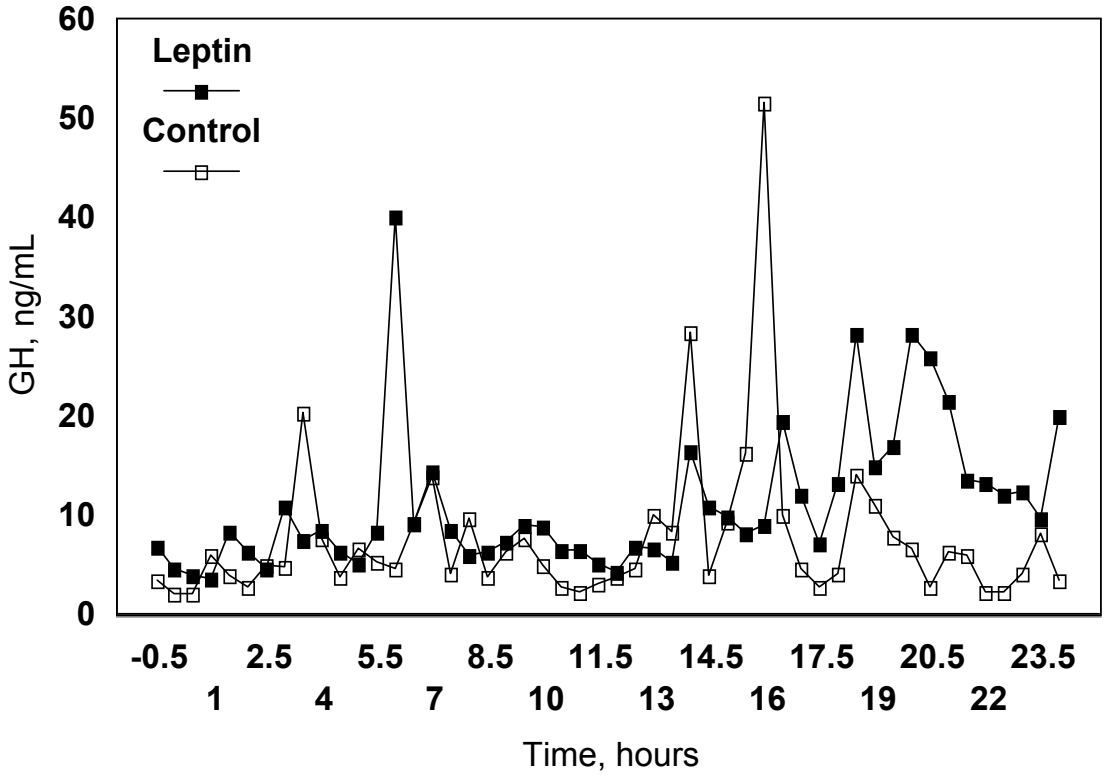

Figure 6. Mean plasma concentrations of growth hormone (GH) in leptin-treated mares versus control mares during a 24-h, frequent (every 30 min) blood-sampling window. No effect of treatment was observed ($P > 0.1$).

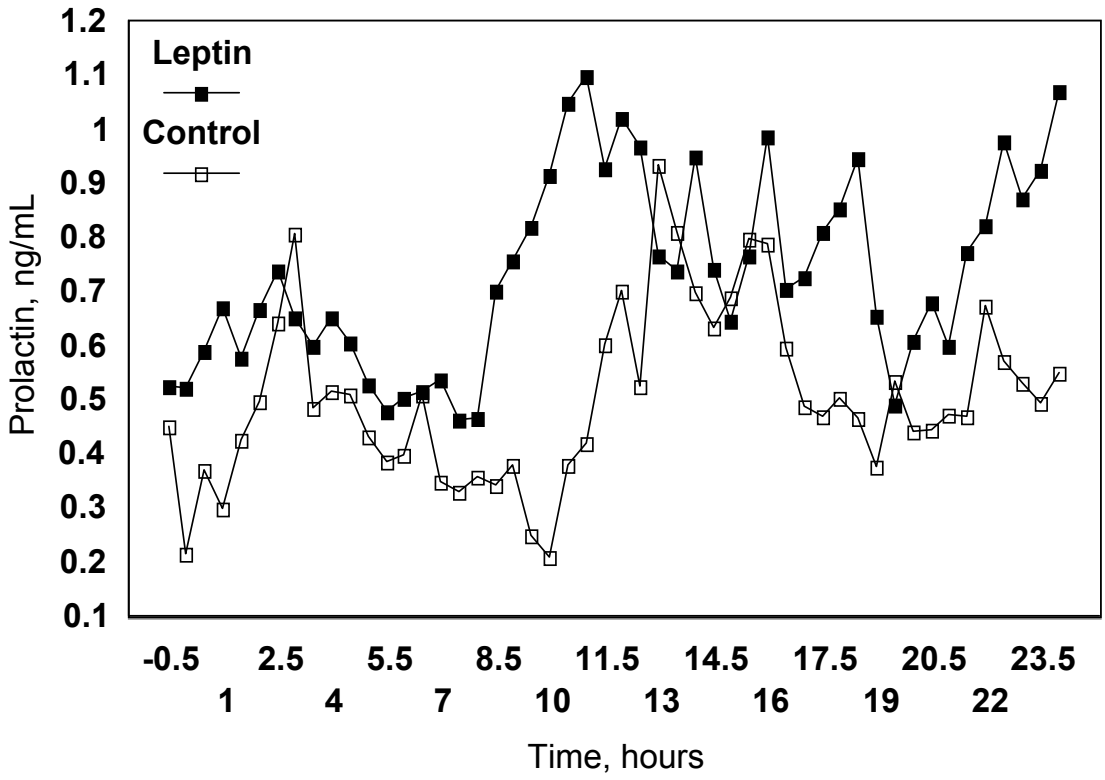

Figure 7. Mean plasma concentrations of prolactin in leptin-treated mares versus control mares during a 24-h, frequent (every 30 min) blood-sampling window. No effect of treatment was observed ($P > 0.1$).

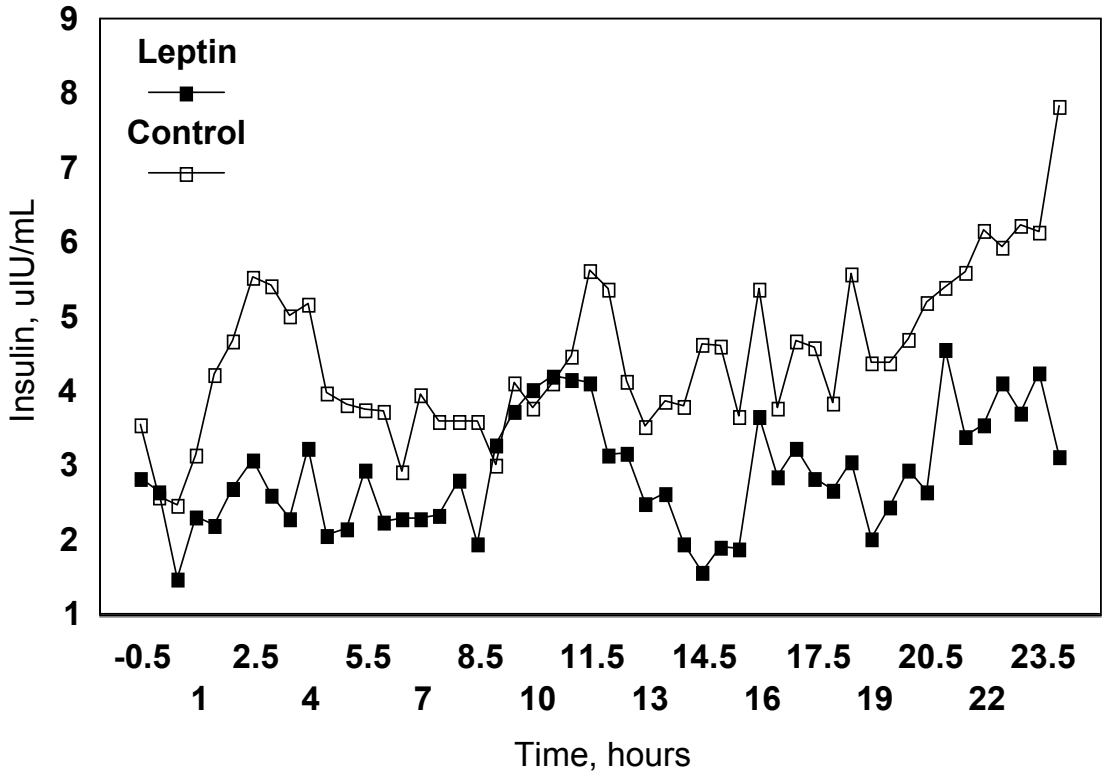

Figure 8. Mean plasma concentrations of insulin in leptin-treated mares versus control mares during a 24-h, frequent (every 30 min) blood-sampling window. There was a tendency (P = 0.11) for leptin-treated mares to have lower insulin concentrations during the sampling window.

Assessment of possible antibody formation against the injected recombinant equine leptin by means of radiolabeled leptin and immunoprecipitation of the horses' IgG fraction in a 4-μL plasma sampled revealed that leptin-treated mares had elevated ($P < 0.001$) antibody concentrations relative to control mares (Figure 9). This elevation was rapid, occurring within the first 2 wk of treatment.

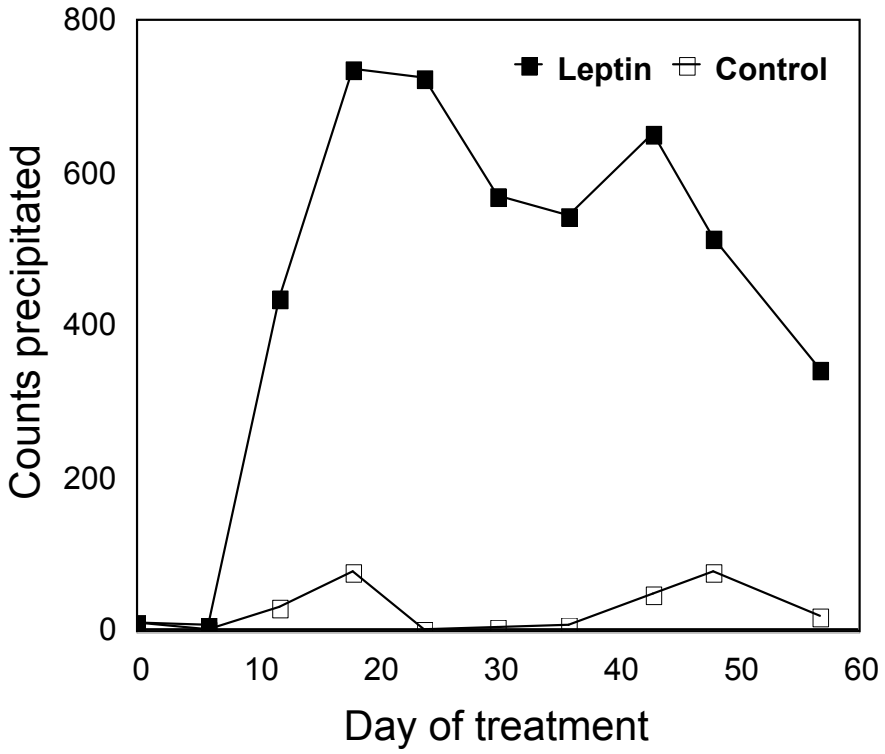

Figure 9. Mean number of counts of radiolabeled human leptin immunoprecipitated from 4-μL samples of plasma from leptin-treated and control mares in the first 57 d of treatment. There were more ($P < 0.001$) counts precipitated with the IgG fraction of the sample in leptin-treated mares than in control mares beginning on d 12.

DISCUSSION

Immunoprecipitation of the gamma globulin fraction of selected plasma samples from all mares, after incubation with radioiodinated leptin, revealed that leptin-treated mares had produced significant antibodies to the injected recombinant equine leptin by d 16 of the experiment. This possibility was first raised by perturbations in the leptin radioimmunoassays performed at the end of the experiment. That is, assay tubes with samples from treated mares around d 10 to 19 had much more radioactivity in them after the final centrifugation step than was in the buffer control (zero standard) tubes. These latter tubes theoretically should contain the highest amount of labeled, precipitated leptin, bound by the primary antibody of the assay. The higher counts indicated that some other factor was causing precipitation of the labeled hormone. Given that the initial humoral immune response to a foreign protein involves mainly IgM antibodies (Guyton and Hall, 2000), which have 10 binding sites and a MW approximately 5 times that of IgG, it is likely that the high counts in the assay were due to IgM forming immune complexes with the labeled hormone. These complexes, just like the primary-secondary antibody complexes normally formed in the assay, would precipitate due to their large size, and would carry with them the labeled leptin. As the immune response to the injected equine leptin proceeded, IgM antibodies would diminish and IgG antibodies would become predominant in the plasma samples (Guyton and Hall, 2000). These antibodies do not typically precipitate in the presence of antigen such as leptin, thus their effect on the leptin radioimmunoassay would be quite different. Rather than observing higher than expected counts, the anti-leptin IgG in the samples would compete with the primary antibody in the assay, tying up large amounts of the labeled hormone that would normally bind to the primary antibody. Given that the second antibody only precipitates the primary (rabbit) antibody, the amount of labeled leptin precipitated

would be much less than normal, which is interpreted in the assay calculation as very high leptin concentration in the sample. This is exactly what was observed in the leptin assays on samples from treated mares: an initial odd "extra counts" period in samples from around d 10 to 19, followed by a decrease to somewhat normal, and then a rapid skyrocketing of calculated leptin levels to values well beyond the expected physiologic range, even for injected mares. The timing of this apparent immunological response to the injected recombinant equine leptin would indicate that the protein was immediately detected as "foreign" from d 1.

This immunological response to injected recombinant leptin is similar to that described by Thompson et al. (1997) for recombinant porcine prolactin. In that experiment, mares treated with prolactin during the seasonal anovulatory period immediately began to shed their winter hair coat, and most ovulated within a few weeks. However, the hair shedding slowed by 5 wk of treatment and totally ceased by 7 wk; thereafter, the mares did not lose any more hair even through May (control mares shed around April 1 and were complete by mid-April). Post-experimental testing showed that the prolactin-treated mares had developed antibodies against the recombinant protein, even though the homology with the known equine protein was greater than 93% (Thompson et al., 1997). Thus, the question in the present experiment, just like in that of Thompson et al. (1997), is when the treated mares should be considered "leptin-treated" as opposed to when they might be considered "neutralized" due to the presence of antibodies against the injected leptin. The presence of antibodies does not necessarily mean neutralization of the biological activity of the injected leptin; however, it is a likely result. Based on the immunoprecipitation data, the time of transition from treated to start of neutralization was around d 16, thus discussion of the results will reflect this limitation in the experiment.

The increased weight loss observed in the leptin-treated mares was consistent with previous research in other species indicating that treatment with leptin causes weight loss, loss of fat, and increases in energy metabolism (Pellymounter et al., 1995; Levin et al., 1996). Moreover, the weight loss was apparent by the first assessment on d 16, about the time when antibodies to leptin were beginning to rise in these mares. The leptin-treated mares continued to lose weight, however so did the control mares, thus the drop from d 16 to 31 could reflect only the environmental conditions of the time. Previous research in other species has shown that exogenous leptin decreases food intake (Pellymounter et al., 1995; Levin et al., 1996). The fact that there was no difference between groups when intake was assessed on d 26 and 27 could be due to a lack of effect of leptin on intake, or more likely, the fact that neutralization of the injected leptin had masked any possible effects. Although leptin treatment reduces food intake in smaller species, it also increases metabolic rate (Houseknecht et al., 1998), which contributes to weight loss as well.

Previous research has indicated that many mares with high body condition score and high leptin concentrations fail to enter an anovulatory state, continuing to display estrous cycles or having significant follicular activity during the winter (Gentry et al., 2002a); therefore the absence of differences in reproductive characteristics observed between treatment groups was in contradiction to previous studies. Date of first ovulation did not differ between groups, nor were there any differences in the size of the largest follicles at any point during the project. The mean dates of ovulation were d 81 (March 29) and d 93 (April 10) for controls and leptin-treated mares, respectively, meaning that all of the mares ovulated close to what is considered to the be predicted date of early April (Henneke et al., 1983; Sharp and Davis, 1993). The mares on this project were not of high body condition, so the lack of a difference could be attributed to their

lower body condition. Additionally, research has shown that even slight reductions in weight dramatically affect leptin concentrations. A loss of only 10% of body weight will cause serum leptin concentrations to fall by 53% (Considine et al., 1996). Leptin-treated mares lost an average of 6.3% of their pretreatment body weight.

Leptin has a stimulatory effect on prolactin in other species (Yu et al., 1997; Macajova et al., 2004). Kelley et al. (2006) showed that prolactin is a key component in the early onset of cyclicity in mares. In the present experiment, there was no difference in prolactin in either frequent or daily blood samples, nor was there any elevation of prolactin to levels consistent with those seen in previous work where there was an early onset of cyclicity (Kelley et al., 2006).

Previous results have indicated that hyperleptinemic horses have higher concentrations of triiodothyronine than normal horses, but similar concentrations of thyroxine (Cartmill et al., 2003a). Triiodothyronine is the more biologically active of the thyroid hormones, however thyroxine is thought to exert negative feedback on the hypothalamus (Hadley, 2000). Higher levels of thyroid hormones are associated with weight loss. We would expect to see higher levels of both thyroid hormones in treated horses due to the weight loss, but this was not the case as there was no effect of treatment on triiodothyronine, and thyroxine was lower in treated animals.

Growth hormone is secreted in a pulsatile fashion; therefore it was analyzed over the 24-h blood-sampling window. In previous studies, high leptin horses have exhibited lower circulating growth hormone (Gentry et al., 2002b; Cartmill et al., 2003a). Our findings were in contrast to these previous finding, which may be due to a lack of effect on growth hormone secretion or may be attributable to antibody formation against the recombinant leptin.

Leptin is known to directly inhibit β-cell secretion of insulin via alteration of ion channel function (Houseknecht et al., 1998). The tendency for a reduction in insulin concentrations in

the 24-h blood sampling window in leptin-treated mares would be consistent with this model. Horses of high body condition score with hyperleptinemia also have relatively high circulating insulin concentrations (mares; Gentry et al., 2002b; Waller, 2005; mares and geldings; Cartmill et al., 2003a). However, Cartmill et al. (2003a) showed that the cause-and-effect is likely the reverse; that is, infusion of insulin with a maintenance of normal glucose levels stimulated leptin secretion 8 h later, identical to what is seen normally after horses are fed a grain meal.

In conclusion, treatment of seasonally anovulatory mares of average body condition with recombinant equine leptin reduced body weight and thyroxine concentrations, and tended to reduce insulin concentrations, relative to control mares. The formation of circulating antibodies to the injected leptin within approximately 16 d complicates the interpretation of the results, because it is not known for sure whether the antibodies did in fact neutralize the biological activity of the injected leptin. Thus, the lack of effect of treatment on prolactin, growth hormone, triiodothyronine, feed intake, ovarian activity, and day of first ovulation may be due to a true lack of effect of leptin, or may be due to antibody formation. The reduction in body weight was an expected result, based on reports in other species, and confirms an initial positive biological response to the injected leptin. The alterations in thyroxine, and perhaps insulin, may indicate that there was still a biological response through d 35 to 39.

SUMMARY AND CONCLUSIONS

In this experiment, no significant reproductive differences were found as a result of treatment with recombinant equine leptin. This could be a result of the formation of antibodies, although their ability to neutralize the injected leptin was not determined. Prior to antibody formation, leptin-treated mares lacked any advantage of significant follicular activity based on size of largest follicle. However, the biological activity of the injected leptin was confirmed by the expected effects body weight in the first 16 d of treatment. Control mares also lost weight after d 16, likely due to the winter conditions under which the project was conducted, but not as much as their leptin-treated counterparts. It is reasonable to assume that previous results indicating continued cyclicity and ovarian activity through the traditional anovulatory season observed by Gentry and coworkers (2002a) were perhaps attributable more to the high body condition of the mares as opposed to their leptin status. However, it is still possible that leptin plays a role, as we were not successful in inducing such an extreme difference. With an altered recombinant equine leptin, the project could be attempted again with very different results if no antibody formation occurs. It was also interesting that all mares ovulated within the normal, expected time period (April 1; Ginther, 1992), despite their lower body condition.

From a metabolic standpoint, effects on thyroxine and insulin secretion were in contradiction to results seen in hyperleptinemic, high body condition horses (Gentry et al., 2002a; Cartmill et al., 2003a), but were consistent with the role of leptin in smaller species.

In conclusion, treatment with recombinant equine leptin did not induce early onset of cyclicity and ovarian activity in winter, perhaps due to antibody formation against the injected leptin. However, multiple metabolic effects were observed, including significant weight loss and altered thyroxine and insulin secretion.

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VITA

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