

EFFECT OF AGE, BODY CONDITION, PREGNANCY AND LACTATION ON
CIRCULATING LEPTIN CONCENTRATIONS IN BEEF CATTLE

A Dissertation

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ABSTRACT

A series of experiments were conducted to evaluate the potential role of leptin in bovine reproduction. In Experiment 1, mean circulating leptin concentrations of postpartum cows were not affected by exogenous dexamethasone treatments. In Experiment 2, mean leptin concentrations were not correlated with female age or body weight but were positively correlated with body condition scores of beef cattle. Leptin concentrations were higher in 1 year old heifers (8.9 ng/ml) compared with 2 year old cows (6.0 ng/ml), but heifer leptin concentrations were not different than 4 to 6 year old cows (8.0 ng/ml) and cows \geq 7 years of age (10.5 ng/ml). Mean leptin concentrations were negatively correlated with age in heifers and cows \leq 2 years of age and positively correlated with age in cows $>$ 3 years of age. In Experiment 3, there were no differences in mean leptin concentrations for 56 days starting 14 days following AI among 2-year old and 3-year old cows pregnant to AI (1.2 ng/ml), the clean-up bulls (1.2 ng/ml) and nonpregnant females (2.2 ng/ml) after a 60-day breeding season. Plasma leptin concentrations were lower for lactating cows (1.0 ng/ml) compared with nonlactating cows (2.1 ng/ml). Female age did not affect circulating leptin concentrations. In Experiment 4, oviduct and uterine epithelial cells from mid-luteal phase females stained positive for the long form of the leptin receptor, and uterine biopsies revealed intense staining for the long form of the leptin receptor on the luminal side of the uterine endometrium. Bovine blastocysts stained positive for the long form of the leptin receptor in the trophoblast cells. In Experiment 5, addition of leptin to culture medium at 0, 100 and 1,000 ng/ml did not affect the percentage embryos developing to the blastocyst stage. Also, leptin did not affect the ratio of blastocysts:8- to 16-cell embryos among the 0 ng/ml treatment group, the 100 ng/ml treatment and the 1,000 ng/ml treatment groups. Results indicate that in the beef cow, the release of leptin and subsequent role(s) of leptin in reproductive processes are likely different than those that have been reported for mice, rats and humans.

CHAPTER I

INTRODUCTION

Zhang et al. (1994) first described leptin as a 146 amino acid, 16 kDa protein that had a structure similar to that of cytokines and was produced by the obesity (*ob*) gene. The *ob/ob* mice are characterized by obesity and infertility, however, when these *ob/ob* mice are administered leptin, intake is decreased, weight is normalized and fertility is restored. Chen et al. (1996) and Lee et al. (1996) reported that *db/db* mice (mice that are similar in appearance and physiology as *ob/ob* mice) expressed mutated leptin receptors, and exogenous leptin had no effect on energy homeostasis or fertility. With the discovery of leptin a considerable amount of research has focused on its effects on nutrition and energy metabolism, particularly in mice and humans. Most leptin research has been directed toward the understanding of the role of leptin in body adiposity and energy balance. It is now generally accepted that leptin is produced mainly by adipose tissue and relays information to the brain concerning the amount of energy stores and activates the hypothalamic centers that regulate energy uptake and expenditure (Flier, 1997). The effect(s) of body fat and body mass on the circulating levels of leptin has been extensively studied (Maffei et al., 1995; Schwartz et al., 1996; Blum, 1997; Perry et al., 1997; Shimizu et al., 1997; Langendonk et al., 1998; Blache et al., 2000), all with similar findings. As body fat increases, circulating leptin also increases.

In nutritionally restricted ruminants decreased serum concentration of luteinizing hormone (LH) and decreased LH pulse frequency characterize nutritional anestrus (Richards et al., 1989), thus negatively impacting follicular growth. In a review of reproduction, Randel (1990) speculated that the nutritional mechanism controlling ovarian activity may have its effect on the hypothalamus, pituitary or ovary directly. Because ovarian function is controlled by the hypothalamic release of GnRH causing a release of gonadotropins from the pituitary, the hypothalamic-pituitary axis is where control of the nutritional mechanism is most likely located. Strauch et al. (2002) first reported that leptin may impact the postpartum interval in cows. They found that females having a short (30 to 37 days) postpartum interval exhibited higher serum leptin concentration than did cows having a longer (78 to 132 days) postpartum interval.

The human blastocyst produces leptin, and the production of leptin in healthy blastocysts is greater than in arrested blastocysts (Gonzalez et al., 2000). In mice, Malik et al. (2001) reported that using minimum daily doses of leptin past either 6.5 days postcoitus or 14.5 days postcoitus when *ob/ob* females were mated resulted in normal pregnancies. However, when leptin treatment was withdrawn at 3.5 days postcoitus, females failed to achieve a pregnancy.

Ovarian tissues, including oocytes, express leptin receptors in several species (Cioffi et al., 1997). In addition, both the short and long form of the leptin receptor have been isolated in a number of fetal/placental tissues in the mouse (Hoggard et al., 1997), baboon (Green et al., 2000), human (Bodner et al., 1999) and rat (Smith et al., 2002). Furthermore, leptin has direct effects through steroidogenesis on the ovary (Francisco, 1997; Zachow and Magoffin, 1997; Spicer and Spicer and Francisco, 1998; Agarwal et al., 1999; Brannian et al., 1999; Ryan et al., 2002; Ryan et al., 2003) in cell types such as theca and granulosa cells. Research has shown the leptin receptor gene varies in the rat ovary throughout the estrous cycle (Duggal et al., 2002), and it has been hypothesized that these variations modulate the sensitivity of the ovary to the leptin protein. Both the long and short forms of the receptor are expressed in the adult rat ovary. RT-PCR results indicated that long form receptor gene expression was low during proestrus and diestrus II, while expression was high in estrus and diestrus I. The short form receptor gene expression was highest in diestrus I and elevated in estrus, while expression was low at proestrus and diestrus II.

Antczak et al. (1997) reported that leptin receptors are expressed in early human and mouse embryos and oocytes. Using immunohistological techniques, Gonzalez et al. (2000) reported that both leptin and its receptor are expressed in the embryo and the luminal and glandular endometrium. Kitawaki et al. (2000) was also able to use Northern and Western blot analysis to isolate leptin receptors from human endometrial tissues. Results have indicated that leptin may facilitate invasion of the cytotrophoblast into the endometrium and that leptin may play an autocrine or paracrine role in this process (Gonzalez and Leavis, 2001). It has also been shown that the addition of exogenous leptin to embryo culture medium increased the development of embryos

from the 2-cell stage to the blastocyst, expanded blastocyst and hatched blastocyst stages in a dose dependent manner (Kawamura et al., 2002).

Therefore, in the present study, we characterized leptin in the beef cow as it relates to age, body condition score, reproductive status and lactational status and investigated the expression of the long form of the leptin receptor in both endometrial epithelial cells and embryos. Also, we examined the effect of leptin on the culture of in vitro-produced bovine embryos. Our hypothesis is that increased exogenous leptin concentrations will increase embryo development.

CHAPTER II

LITERATURE REVIEW

Leptin

Kennedy et al. (1953) proposed the 'Lipostatic' theory, in which adipose tissue must produce a substance that circulates through the body to regulate body weight. Hervey et al. (1959) and Coleman et al. (1978) later verified Kennedy's theory using parabiosis experiments with mice and rats. These researchers proposed that mice with the inability to synthesize the hormone leptin (*ob/ob* mice) did not possess this 'factor' and mice which lacked the receptor for leptin (*db/db* mice) were resistant to its action.

Zhang et al. (1994) first described leptin as a 146 amino acid, 16 kDa protein that had a structure similar to that of cytokines and was produced by the obesity (*ob*) gene. The 'Lipostatic' theory proved true when the leptin gene was identified, and it was determined that *ob/ob* mice lacked this gene. Later, Chen et al. (1996) and Lee et al. (1996) reported that *db/db* mice were resistant to leptin because of mutations in the leptin receptor. With the discovery of a new protein hormone, a considerable amount of research has focused on its effects on nutrition and energy metabolism, particularly in mice and humans. Leptin has been reported to circulate bound to serum proteins with a $t_{1/2}$ life of ~1.6 hours (Houseknecht et al., 1996). In humans, secretion of leptin from adipocytes occurs in a pulsatile pattern with peaks occurring every 32 minutes (Licinio et al., 1997).

Most research pertaining to leptin has been directed toward the understanding of the role of leptin in body adiposity and energy balance. This hormone is produced mainly by adipose tissue and relays information to the brain about energy stores and activates the hypothalamic centers that regulate energy uptake and expenditure (Flier, 1997). In addition, it has been suggested that this hormone affects neuroendocrine mechanisms and regulates multiple hypothalamic functions (Flier, 1997).

Leptin and Body Energy Stores

Most experimental results that have been published pertain to the effect(s) of body fat and body mass on the circulating levels of leptin (Maffei et al., 1995; Schwartz et al., 1996; Blum, 1997; Perry et al., 1997; Shimizu et al., 1997; Langendonk et al., 1998; Blache et al., 2000) all with similar findings, as body fat increases circulating

leptin also increases. Thong et al. (2000) measured circulating leptin levels in female athletes and reported that circulating leptin concentrations were significantly lower in noncyclic female athletes than in cyclic female athletes. These researchers hypothesized that leptin was the signal to the hypothalamus of available energy for reproduction, and thus was lower in athletes lacking significant amounts of energy storage.

Animal studies have repeatedly shown that inadequate nutrition resulting in diminished body condition results in a decrease in reproductive competence at the hypothalamic level. Day et al. (1986) found no difference in LH pulse frequency of prepubertal heifers compared with pubertal heifers fed a low energy diet, while a contemporary group fed adequate energy exhibited increased LH pulse frequency and attained a normal onset to puberty. Likewise, heifers receiving a low energy diet failed to attain puberty during a reasonable period of time. Pulse frequency of LH in ovariectomized ewe lambs primed with estradiol is also diminished following nutritional restriction (Foster and Olster, 1985).

Mature bovine females have been shown to achieve an anovulatory and anestrous state when maintained on a restricted diet (Schillo, 1992). Sansinanea et al. (2001) reported that leptin was significantly higher in post-fed Hereford cows compared with pre-feeding values. Serum free fatty acid, β -OH-butyrate and protein concentrations were used to assess energetic indicators. Those females receiving a restricted diet showed depressed free fatty acid, β -OH-butyrate and protein concentrations indicating impaired adipogenesis, and also exhibited significantly lower serum leptin levels.

It has also been shown that follicular development can be negatively impacted by a reduction in energy reserves. Day et al. (1986) noted that follicular development was arrested with follicles not progressing past Class II size in Hereford crossbred beef cows fed a restricted diet. It is generally accepted that the incompetence of the reproductive system in nutritionally restricted ruminants is a result of decreased circulating LH concentrations as well as a decrease in LH pulse frequency (Richards et al., 1989), thus negatively impacting folliculogenesis. Schillo et al. (1992) proposed that the negative

effects of inadequate nutrition on the release of LH were due to the reduction in the release of GnRH secretion.

Postpartum intervals in beef females have also been shown to be affected by the lack of energy stores prior to breeding or at calving. Lalman et al. (1997) reported a negative correlation between dietary energy and postpartum interval. In thin beef heifers, as dietary energy increased the postpartum interval decreased. Later, Lalman et al. (2000) noted that body condition scores were positively associated with IGF-1 and insulin concentrations, suggesting both are indicators of amounts of energy storage in primiparous beef heifers. Strauch et al. (2002) first reported a relationship of leptin to the postpartum interval in cows. They found that females having a short (30 to 37 days) postpartum interval exhibited higher circulating leptin levels than did cows having a longer (78 to 132 days) postpartum interval. However, the authors offered no further explanation of this finding.

An association between circulating leptin concentrations in Merino sheep and backfat thickness and the ratio of backfat thickness to live weight have been reported (Blache et al., 2000). Circulating leptin levels were highly correlated with backfat thickness and the ratio of backfat thickness to live weight in both female and castrated animals. It was also noted that the levels of leptin were higher in females than either the castrated or intact males. These authors go on to state that the secretion of leptin (based on frequent sampling) is episodic and is not pulsatile as found in humans (Licinio et al., 1997).

It would appear that leptin may be the 'adipostat' in cattle and possibly sheep (Kennedy et al., 1953). In a review of reproduction mechanisms, Randel (1990) speculated that the nutritional mechanism controlling ovarian activity may have its effect directly on the hypothalamus, the pituitary and/or the ovary. Since ovarian function is controlled by the hypothalamic release of GnRH causing a release of pituitary gonadotropins, the nutritional control mechanism is most likely located in the hypothalamic-pituitary axis.

The effects of leptin on the bovine hypothalamic-pituitary-gonadal axis likely is limited to the control at the hypothalamic level. Therefore, the regulation of reproductive function based on the nutritional status of the animal may be one of hypothalamic origin.

Very little research has been reported where researchers have tried to unlock some of the potential roles leptin may play on embryo development. Because leptin appears to be a regulator of the hypothalamic-pituitary axis, one should not overlook the effect of leptin on the uterus and embryo interaction in beef cattle.

Hypothalamic-Pituitary Axis

The brain has been shown to monitor the body energy reserves to signal the system when energy stores are adequate or low. The level of energy stores in the body are thought to be the regulatory mechanism controlling various aspects of reproduction (Day et al., 1986; Richards et al., 1989; Schillo, 1992). Ablation of the ventrobasal hypothalamus has been shown to cause hyperphagia and obesity, while lateral hypothalamic lesions can lead to inanition and death due to starvation (Heterington, 1940; Anand and Brobeck, 1951). Based on these results the ventromedial hypothalamus was proposed to contain a 'feeding center' which when stimulated can cause satiety in the rat (Anand and Brobeck, 1951). For a species to propagate, the energy reserves must be adequate enough to support gestation and subsequent lactation.

Numerous researchers have reported that decreased levels of body fat result in an anestrous state, where the reproductive system essentially becomes quiescent. As the animal becomes nutritionally stressed there is a reduction in the secretion of GnRH, however, as the level of nutrition increases the frequency of LH pulses and an increase in serum FSH results, indicating that the release of GnRH is altered by nutritional stress (Martin et al., 1994).

Anabolic neuropeptides (neuropeptide Y, agouti-related peptide, melanin-concentrating hormone, galanin and orexins) and catabolic neuropeptides (corticotrophin-releasing hormone, α -melanocyte stimulating hormone, cocaine regulated transcript and amphetamine regulated transcript) help regulate nutritional intake and energy homeostasis in the brain (Aroa et al., 2006). Research has shown that acute injections of neuropeptide Y results in increased food intake and daily gains in mice (Stanley et al., 1986). It has also been shown that acute injections of neuropeptide Y results in hyperphagia in satiated rats (Clark et al., 1984; Billington et al., 1991; McMinn et al., 1998) and sheep (Miner et al., 1989; Sartin et al., 2001). However, chronic injections of

neuropeptide Y over 7 days results in a sustained increase in food intake and body fat in rats (Zarjevski et al., 1993).

Both the neuropeptide Y and leptin receptors are expressed in the arcuate nucleus (Mercer et al., 1996), and it has been reported that administration of leptin reduces neuropeptide Y expression and synthesis in the hypothalamus of both food-deprived and obese rodents (Cusin et al., 1996; Schwartz et al., 1996; Flynn et al., 1998; Sahu, 1998b) and sheep (Henry et al., 1999). Results have shown that neuropeptide Y secretion in the rat increases in the arcuate nucleus during fasting and decreases during feeding (Sahu et al., 1988). Studies have shown that leptin receptors are co-localized with melanin-concentrating hormone on the LH neuron (Bittencourt et al., 1992), although not in as great a number as are expressed on neuropeptide Y neurons (Schwartz et al., 1996). When leptin is administered, a decrease in hypothalamic expression of melanin-concentrating hormone and the melanin-concentrating hormone induced increase in food intake is ablated (Sahu, 1998a).

Administration of galanin to rats and mice results in increased food intake but to a lesser extent than that of neuropeptide Y (Kyrkouli et al., 1986; Sahu, 1998b). However, when leptin is administered intracerebroventricularly (icv) the galanin mRNA expression is decreased (Sahu, 1998a; Sahu, 1998b; Cheung et al., 2001). Galanin-like peptide is reduced in the arcuate nucleus during fasting, but, upon administration of leptin the number of cells expressing galanin-like peptide is increased 4-fold (Jureus et al., 2000).

Like leptin, corticotrophin-releasing hormone reduces intake in rodents (Arase et al., 1988; Hotta et al., 1991; Richard, 1993) monkeys (Glowa and Gold, 1991) and cattle (Ruckebusch and Malbert, 1986). Costa et al. (1997) reported that in rat hypothalamic explants the secretion of corticotrophin-releasing hormone increases in response to leptin administration and icv infusion of leptin has been shown to increase the expression of corticotrophin-releasing hormone in the hypothalamus (Schwartz et al., 1996).

Release of Leptin and Control at the Hypothalamic-Pituitary Axis

The mechanisms of leptin release from the adipose cells have apparently not been conserved across species. Gentry et al. (2001) reported increased levels of

circulating leptin in mares that were treated with dexamethasone, while Maciel et al. (2001) reported dairy cows treated with dexamethasone showed no increase in endogenous leptin levels. Therefore, the mode of release across species may be completely different.

Nitric oxide synthase (NOS) is needed to stimulate the production and release of nitric oxide (NO) from the arcuate nucleus. Earlier, Rettori et al. (1993) reported that NO controls the release of luteinizing hormone releasing hormone (LHRH) both in vivo and in vitro. Yu et al. (1997) found that in the rat leptin not only stimulates LHRH but also stimulates the release of LH and FSH from anterior pituitary cells in vitro. Furthermore, it has been previously shown that leptin reduces NOS in the hypothalamus of mice (Calapai et al., 1999; Morley et al., 1999), however, Baratta et al. (2002) reported that the incubation of pig pituitary cells in leptin (10 nM and 1 μ M) for 4 or 24 hours increased the amount of NO released for these cells.

There are only limited reports in the literature on the role of leptin in the release of the pituitary gonadotropins. A general consensus is needed to explain this action and certainly further experimentation is needed. To date, no research has addressed whether leptin is involved in hypothalamic pituitary function in cattle. Monitoring circulating leptin levels in cattle would be an important step in understanding if leptin is involved in the nutritional regulatory control of reproduction function in cattle.

Although early studies have produced contrasting results, functional leptin receptors have been identified in ovarian tissues (Karlsson et al., 1997). Shimizu et al. (1997) reported that increased levels of circulating estrogen directly increased circulating levels of leptin in mice. This may be a control mechanism regulating the ovulatory release of pituitary LH. Shimizu et al., also showed that increased adipocyte aromatase has been shown to increase the conversion of androstenedione to estrone in mice, which in turn increases the amount of LH to FSH ratio of gonadotropins secreted. There is a possibility that this may play a role in the reduced fertility often noted in obese beef females.

Leptin Sexual Dimorphism

Significant differences in circulating leptin levels have been well documented across gender in humans (Montague et al., 1997; Licinio et al., 1998; Saad et al., 1998),

in human chord blood (Tome et al., 1997) and sheep (Blache et al., 2000). Montague et al. (1997) reported that leptin mRNA levels in nonobese and mildly obese patients was higher in women compared with men. It has been generally accepted that circulating levels of leptin are 'individualistic' in humans and nonprimates meaning, the variation within a population with similar amounts of adiposity may be quite large. Saad et al. (1998) reported that in 267 human subjects, fasting leptin levels ranged from 1.8 to 79.6 ng/ml with a mean of 12.4 ng/ml, and that women had 40% higher leptin levels than men at any level of adiposity, even after controlling for body fat in women. These researchers go on to state that following regression analysis adiposity, gender and insulinemia were the major determinants of leptin concentrations accounting for 42%, 29% and 2% of the variance, respectively.

In a study of frequent sampling in humans over a 24-hour period, women had leptin levels that were twice as high as men, with pulse amplitude twice as high as men (Licinio et al., 1998). However, both men and women exhibited a similar 24-hour pattern. One interesting note was that the most distinctive difference between the sexes was not the leptin concentrations, level of pulses or oscillation frequency, but the amount of leptin released (or removed) per unit of time. These researchers implied that women may be more resistant to the effects of leptin than men which could explain the sexual dimorphism pattern.

Evidently the difference in leptin levels between the sexes do not only occur in postnatal humans. Tome et al. (1997) reported significant differences in levels of umbilical cord blood leptin between male and female infants which was not different than the leptin levels of the mothers. They showed that irrespective of body weights, cord blood concentrations of leptin from female infants were significantly higher (12.9 vs. 6.8 ng/ml) when compared with male infants. Also, the concentration of leptin in cord blood was disproportionately higher in infants (18.7 ng/ml) compared with that of the mothers (9.4 ng/ml). These findings suggest that the regulation of leptin levels from fetal adipose tissues is likely different than that of adult adipose tissue.

Photoperiod and Diurnal Patterns

Circulation of serum leptin has been shown to be affected by photoperiod (Marie et al., 2001). Rams exposed to 16 hours of light and 8 hours of darkness had higher

serum leptin levels than rams exposed to 8 hours of light and 16 hours of darkness. This is in agreement with an earlier report by Bocquier et al. (1998) where Lacaune ewes subjected to short days exhibited decreased leptin levels irrespective of nutritional state.

A leptin diurnal pattern has been reported in rats (Saladin et al., 1995), mice (Ahren, 2000) and humans (Langendonk et al., 1998). On the other hand, Blache et al. (2000) and Gentry et al. (2001) have reported no diurnal pattern in either sheep or mares. However, Blache et al. (2000) did note that there were no feeding related leptin fluctuations found in rams, while Marie et al. (2001) reported that leptin increased 2 to 8 hours after feedings in Soay rams. Feeding related fluctuations have also been reported in beef cattle (Amstalden et al., 2000; Sansinanea et al., 2001) and are thought to be controlled by insulin release following food intake.

The Embryo and Endometrium

It has been reported that the human blastocyst produces leptin (Gonzalez et al., 2000). Furthermore, it was noted that the production of leptin in arrested blastocyst stage is lower than those embryos that are developing normally. It has been proposed that leptin may mediate implantation in the human based on the protein's ability to physiologically change the uterine epithelium to facilitate invasion by trophoblast cells.

Malik et al. (2001) reported that when leptin treatment (5 mg/kg / day) in *ob/ob* mice was withdrawn at 3.5 days postcoitus, pregnancies did not result, however, if leptin treatment was continued to either 6.5 days postcoitus or 14.5 days postcoitus then normal pregnancies resulted. These researchers also reported that when *ob/ob* leptin-treated females were mated to *ob/ob* leptin-treated males the newborn pups failed to survive in the 6.5 day postcoitus and 14.5 day postcoitus treated groups, even with leptin supplementation after birth of litters due to lack of maternal mammary gland development. It was hypothesized that leptin is essential for normal pre-implantation and/or implantation processes and for normal mammary gland development, but is not essential for either pregnancy or parturition once implantation has taken place.

Researchers have demonstrated that leptin affects the expression of " 2, " 5 and " 6 integrin subunits in the endometrium (Gonzalez et al., 2001), as well as the up regulation of MMP9. These results indicate that leptin may facilitate invasion of the

cytotrophoblast into the endometrium and that leptin may play an autocrine or paracrine role in this process (Gonzalez and Leavis, 2001).

It has been proposed that the IL-1 system may be an important factor in the embryo maternal molecular interactions (communications) during the implantation process (Sheth et al., 1991; Simon et al., 1993). Leptin has been shown to up regulate $\alpha 3$ integrin expression (a molecular marker for endometrial receptivity) by endometrial epithelial cells (Gonzalez and Leavis, 2001). These researchers also showed that leptin exerts a greater effect on $\alpha 3$ integrin than its own ligand IL-1 β at similar concentrations, and IL-1 β was shown to stimulate leptin secretion and Ob-Rb expression in endometrial epithelial cells.

It has also been reported that leptin stimulated IL-1 β secretion and expression of IL-1 receptor in both endometrial epithelial cells and endometrial stromal cells (Gonzalez et al., 2003). Using specific antibodies, IL-1 β secretion was reduced and the addition of leptin to the medium neutralized this effect. These researchers also reported that when OB-R was blocked the effect of leptin and IL-1 β on the expression of the IL-1 β system and $\alpha 3$ integrin phosphorylation of STAT3 were neutralized. It was concluded that leptin regulates the IL-1 system and that blocking of OB-R impairs both leptin and IL-1 β functions at the endometrial level.

Only one report could be found describing the ability of the embryo to produce and secrete leptin in the human (Gonzalez et al., 2000). These researchers reported the detection of leptin in conditioned medium from both human blastocysts and endometrial epithelial cells (EEC). Hatched blastocysts cultured alone secreted higher levels (453.3 ± 43.3 pg/ml) compared with arrested blastocysts alone, blastocysts co-cultured with EEC or EEC cultured alone. Furthermore, when blastocysts were co-cultured with EEC, leptin concentrations were lower compared with arrested embryos co-cultured with EEC (257 ± 14.5 pg/ml vs. 360 ± 30 pg/ml, respectively).

Gonzalez et al. (2000) hypothesized that expression of leptin receptors and leptin protein within the secretory endometrium and cultured EEC could be interpreted as a molecular communication between the embryo and uterine environment and may be an important factor for implantation to proceed. However, it was theorized that because leptin is produced and secreted locally by epithelial cells, leptin could act in an autocrine

or paracrine manner to regulate biological functions that may mediate endometrial receptivity to facilitate the implantation process. While regulation of leptin and leptin receptor is not completely understood, expression of both in EEC and the human blastocyst likely coordinates the molecular events allowing implantation to occur.

The first evidence that leptin influenced oocyte maturation and developmental competence was reported by Swain et al. (2004). It was shown that fewer blastocysts developed compared with controls when porcine oocytes were cultured with leptin during in vitro maturation. Leptin has also been shown to enhance the resumption of meiosis in preovulatory follicle enclosed oocytes with no effects on meiotic resumption of denuded or cumulus-enclosed oocytes (Ryan et al., 2002).

Kawamura et al. (2002) reported that leptin mRNA was expressed in mouse blastocysts and hatched blastocysts, with 2 isoforms of the OB-R (Ob-Ra and Ob-Rb) found in oocytes, 1-cell, 2-cells, morulae, blastocysts and hatched blastocysts. Leptin was detected in the epithelium of the oviduct and the uterine endometrium in pregnant mice. It was also noted that leptin levels were higher in the uteri of pregnant mice than nonpregnant mice.

It has also been shown by Kawamura et al. (2002) that the addition of exogenous leptin to embryo culture medium increased the development of embryos from the 2-cell stage to the blastocyst, expanded blastocyst and hatched blastocyst stages in a dose dependent manner. These authors also reported that the addition of an antibody against the extracellular domain of the Ob-Rb isoform ablated this increased growth response.

Genomic activation is generally accepted to occur during the 2-cell stage in the mouse (Flach et al., 1982). However, Kawamura et al. (2002) reported that leptin mRNA was detected after the blastocyst stage using a commercial ELISA kit, indicating that leptin mRNA is likely originating from an embryonic gene. These authors also noted that Ob-Ra and Ob-Rb isoforms mRNA were detected from oocytes through the hatched blastocyst stage, which would indicate that they may be maternally derived since they are expressed prior to genomic activation.

Kawamura et al. (2002) also reported that leptin mRNA was expressed in the oviduct and uterus endometrium of pregnant mice; however, there were differences in concentrations at different physiological sites. Using immunohistochemistry, it was

shown that leptin protein was more strongly expressed in the glandular and luminal endometrial epithelial cells of pregnant mice on day 1 and day 4.5, which correspond to the 2-cell and blastocyst stages in the mouse. These researchers hypothesized that leptin is produced by the oviduct and uterine endometrium epithelium during early pregnancy. These researchers also showed that circulating leptin concentrations were not different in virgin, nonpregnant and pregnant mice. However, the uterine fluid of pregnant mice had higher leptin concentrations than uterine fluid of nonpregnant mice. Levels of leptin in the oviduct appeared to be below the sensitivity of the ELISA used in their study.

The hypothesis that leptin plays a role in implantation of the human embryo may be valid, however, one should not overlook that leptin may also play a role in the communication between the blastocyst and endometrium and for enhancement of maternal recognition or possibly for direct stimulation of embryo development.

Tumor Necrosis Factor (TNF) increases leptin levels while cytotrophoblastic cells appear to mediate the release of leptin through the cytokine Interleukin 1 (IL-1) (Bischof, 2001). The secretion of TNF produced by the early embryo may be a signaling factor to the endometrium that pregnancy has begun.

More research is needed to determine the effects of leptin on the early developing embryo. To date most of the research centers on polycystic ovarian syndrome (PCOS) and levels of circulating leptin in obese humans. More specifically, the secretion of leptin from early developing embryos through to the blastocyst stage needs further investigation.

Granulosa and Theca Cell Culture

Agarwal et al. (1999) reported that the administration of leptin to cultured murine theca cells inhibited the production of androstenedione normally associated with addition of IGF-1 to the culture system. They indicated that when LH was added to murine theca cells in culture, it increased the amounts of androstenedione in the medium. Furthermore the addition of IGF-1 to the culture further increased the androstenedione secretion. When leptin was added, the increase of androstenedione resulting from addition of IGF-1 was ablated.

Spicer et al. (1998) have previously reported that when insulin was added to the culture medium, the number of bovine theca cells increased 8% to 16%. However, when leptin was added to the system there was a significant reduction in both progesterone and androstenedione production. In contrast, Zachow et al. (1999) reported that leptin had no effect on theca cell androgen production.

Other studies have described the effects of leptin on granulosa cell production of progesterone and estradiol (Agarwal et al., 1999; Zachow et al., 1999). Agarwal et al. (1999) reported that the addition of leptin to granulosa cell culture resulted in a decrease in the amounts of progesterone and estradiol secreted in the rat. Although it must be stated that the leptin levels used in this study were high (100 ng/ml), these researchers reported similar results occurred with estradiol on granulosa cell culture from the rat.

Leptin can reduce aromatase cytochrome needed for the production of estrone and estradiol in rat granulosa cell cultures (Zachow et al., 1999). When granulosa cell cultures were supplemented with FSH and TGF β the amounts of estrone and estradiol increased in the culture medium. However, when compared with similarly supplemented cultures, the addition of leptin abolished the augmentation of estrone and estradiol caused by TGF β .

Barkan et al. (1999) reported that the addition of leptin to rat and human granulosa cell cultures had no effect on progesterone production, but increased progesterone secretion was reported when FSH and dexamethasone both were added to the cultures. However, when leptin was added to the cultures the increase in progesterone from the dexamethasone was ablated. These researchers went on to report that there was a significant reduction in estradiol production in LH and leptin supplemented granulosa cell cultures when compared with cultures supplemented with LH alone.

Lactation

Lactation is a continuous physiological function that requires increased energy demands in the mouse and rat (Vernon and Flint, 1984; Malabu et al., 1994; Barber et al., 1997; Flint and Vernon, 1998) and the cow (Blum et al., 1983; Kunz et al., 1985;

Bauman et al., 1988). There are a lot of endocrine and metabolic changes that increase metabolic efficiency and decrease energy expenditure during lactation.

During lactation rats exhibit decreased thermogenesis of brown adipose tissue (Trayhurn, 1989) and become hypoinsulinemic (Vernon and Pond, 1997). It has been shown that rats mobilize approximately 1 g of fat/day at the peak of lactation (Barber et al., 1997). Similar endocrine and metabolic changes are found in high producing dairy cows (Blum et al., 1983; Kunz et al., 1985; Chilliard, 1988). It is generally accepted, due to the high energy demand of lactation, that cyclicity is decreased in most animals due to low circulating levels of LH.

In mice and rats the hormonal and metabolic changes that occur during lactation are reflected by an increase in insulin receptors (Flint, 1982), decrease in thyroid hormone levels (Oberkotter and Rasmussen, 1992; van Haasteren et al., 1996), increase in food intake (Ota and Yokoyama, 1967) and an increase in neuropeptide Y concentrations in the hypothalamus (Smith, 1993; Li et al., 1998).

Brogan et al. (1999) conducted a series of studies to determine whether there are changes in leptin levels during lactation in the rat. These researchers reported that leptin levels were significantly reduced in ovariectomized rats that were chronically nursed by pups and also noted that this decrease in leptin paralleled the suppression of pulsatile LH secretion. These authors also showed that if pups were removed on day 9 for 48 hours and then returned and allowed to nurse, the leptin and insulin levels were reduced following nursing. These results indicate that after 48 hour of nursing pulsatile LH secretion was significantly reduced compared with nonsuckled females.

Local production of leptin by mammary tissues in mice has been documented (Aoki et al., 1999). Using virgin, pregnant, lactating and post-lactating mice, leptin gene expression in the mammary glands and adipose tissues were detected before pregnancy and decreased 30% to 50% after parturition. These levels remained low during lactation and rebounded to pre-pregnancy levels following weaning of the pups. Milk leptin measured just before weaning was 2-fold higher than milk collected later in lactation, and there were no differences in serum leptin levels between mid-lactating and late lactating mice. These researchers proposed that down regulation of leptin due to lactation was associated with an autocrine/paracrine action of leptin in both the

mammary and adipose tissue. Also, leptin was not only diffused from the maternal blood stream into the mammary, but regional production and secretion by the mammary epithelial cells was thought to be responsible for leptin level changes expressed in the milk.

In dairy cows, similar endocrine and metabolic changes are indicative of lactation as has been reported in mice. It is generally accepted that the onset of negative net energy balance at parturition causes a reduction in circulating leptin and is also associated with increased concentrations of growth hormone (GH). Block et al. (2003) reported that in late lactating dairy cows fed either 120% of their nutrient requirement or restricted to 33% of maintenance requirement, circulating GH increased and insulin decreased following a 24-hour and a 48-hour feed restriction period. However, when these dairy cows were treated with excipient (vehicle control) or bovine somatotropin in early or late lactation there was no effect on circulating leptin concentrations irrespective of stage of lactation. These authors also showed after 96 hours of hyperinsulinemia, using euglycemic hyperinsulinemic clamps in mid-lactating dairy cows, that circulating leptin was significantly increased. It was concluded that insulin regulated circulating leptin in lactating dairy cows and that elevated circulating GH was unlikely to have a major effect on circulating leptin levels.

Research has also shown that food intake restriction increased metabolic stress and negative energy balance in dairy cattle fed concentrates at 30% of total dry matter intake, compared with females fed concentrates at 50% of total dry matter intake with those animals receiving 30% concentrated having lower circulating leptin levels (Reist et al., 2003). It was noted that the metabolic stress in the 30% concentrate group was expressed by lower glucose, insulin, IGF-1, triiodothyronine, milk protein and lactose concentrations and higher nonesterified fatty acid, β -OH-butyrate and GH concentrations and accelerated a decline in body condition score.

Reist et al. (2003) also found circulating leptin concentrations were positively associated with body condition score, energy balance body weight, cholesterol, albumin, insulin and IGF-1, but were negatively associated with dry matter intake and triiodothyronine and was higher in the spring than in the fall during the first 20 weeks of lactation.

One report has shown that leptin was not different between lactating and nonlactating women, but circulating leptin may have affected milk production indirectly through its negative effect on serum prolactin (Butte et al., 1997). Serum leptin, body fat, energy expenditure and milk production was measured in 65 women at 36 weeks of gestation and at 3 and 6 months postpartum. When adjusted on a per unit of fat mass, serum leptin was higher at 36 weeks of gestation than either 3 or 6 months postpartum. It was shown that leptin levels were associated with changes in body weight, fat mass and circulating insulin.

Sir-Petermann et al. (2001) made an effort to determine if leptin modulates hypothalamic-pituitary-gonadal axis function in lactational amenorrhea in women, by monitoring LH concentrations in lactating women with PCOS and lactating normal women on the 4th and 8th week postpartum. It was reported that LH concentration increased between the 4th and 8th week postpartum, however serum leptin concentrations remained unchanged. It was also shown that leptin pulse frequency was higher than LH pulse frequency in both groups, with no synchronicity between the hormone pulses. It was concluded that during lactational amenorrhea circulating leptin is probably not involved in reactivation of pulsatile LH secretion in women.

Leptin Receptor

Tartaglia et al. (1995) was first to describe the leptin receptor (OB-R) and subsequently identified 5 isoforms. These 5 isoforms have been isolated in a multitude of tissues and subsequently divided into 3 categories: short form (OB-R_S), soluble form (OB-R_e) and long form (OB-R_L). The extracellular domains of all isoforms are identical and contain 816 amino acids, while the intracellular domains are quite different and have been used to identify the different isoforms. The OB-R_S have 4 different isoforms consisting of significantly different transmembrane domains (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf; (Tartaglia et al., 1995).

Tartaglia et al. (1995) was first to report that the OB-R was a single membrane-spanning receptor of the Class I cytokine receptor family. The early predictions of extra and intracellular domains were 816 and 34 amino acids, respectively. However, upon complete analysis of cDNA libraries, deviations from the original OB-R isoform were identified based on the length of the intracellular and transmembrane domain. An

isoform containing 303 amino acids was identified (Tartaglia et al., 1995; Chen et al., 1996; Cioffi et al., 1996; Lee et al., 1996) as the OB-R_L and was thought to be the only isoform having intracellular signaling capabilities.

As previously stated, the extracellular domains of both the OB-R_L and OB-R_S are identical throughout the entire length of the amino acid sequence, with the differences in the isoforms being determined by the differing lengths of the transmembrane and intracellular domains. The intracellular domains of all known isoforms are also identical up to 30th amino acid of the intracellular domain, at which point divergence in amino acid sequence occurs.

There has also been a report of an OB-R without a transmembrane domain that is considered soluble (Ob-Re) and its function remains unclear (Lee et al., 1996). The sequence of the OB-R has been highly conserved across species with murine and human receptors having similar homology of both the intracellular (71%) and extracellular (78%) domains. Because of homology across species and isoforms, any analysis from probes generated against the extracellular domain must be carefully considered because results have indicated that expression has been identified in nearly all tissues at varying amounts (Tartaglia et al., 1995; Lee et al., 1996; Schwartz et al., 1996).

Long Form Receptor (OB-Rb)

The nomenclature for the long form of the leptin receptor through out the literature is inconsistent and the acronyms OB-R_L, Ob-Rb and Lep R are all the considered to be the same receptor. For the remainder of this paper the acronym Ob-Rb will be used for the long form of the leptin receptor. Class I cytokine receptors are known to transduce signals through the Janis Kinase (JAK) and Signal Transduction and Transcription (STAT) pathway (Kishimoto et al., 1994; Heldin, 1995). Briefly, JAK upon ligand binding phosphorylates membrane-proximal sequences of the receptor.

After STAT phosphorylation by JAK, STAT translocates to the nucleus and initiates transcription. Two reports (Baumann et al., 1996; Ghilardi et al., 1996) have shown that Ob-Rb transient co-transfected proteins are capable of activation of STAT signaling, while the OB-R_S proteins remain inactive. Ob-Rb proteins are also capable of activating both the mitogen-activated protein and the phosphatidylinositol 3-kinase

pathways as well, and it is believed that these pathways may be involved in the control of body weight (Kishimoto et al., 1994; Heldin, 1995). Baumann et al. (1996) showed that transcription could be initiated by Ob-Rb in hepatoma cells via response elements of the Class I cytokine receptor family, while the OB-R_S has been shown to be incapable of a similar response.

The full scope of the pathway(s) utilized by the Ob-Rb has yet to be determined. Tartaglia et al. (1997) suggested that Ob-Rb could interact with a novel signaling chain or may accomplish signal transduction through homodimerization or homo-oligomerization as is reported in the literature for the GH receptor, erythropoietin receptor and G-SCFR (Kishimoto, 1994; Heldin, 1995).

White et al. (1997) illustrated this signal transduction using G-CSFR/Ob-Rb chimeric receptors. Fusing extracellular and intracellular domains of the receptors, these researchers reported that these chimeric receptors had a signaling capability and potency that was not different from that of the native receptors. This supports the hypothesis that a regulatory chain may not be needed for signal transduction and activation may be accomplished by simple homodimerization or homo-oligomerization.

Research has shown that there are at least 2 regions of the intracellular domain that induce signal transduction in the Ob-Rb (White et al., 1997). An OB-R receptor with the most C-terminal 50 amino acids removed cannot initiate transcription via an IL-6 response element. However, transcription is not interrupted when a hematopoietic response element is used, even with the C-terminal 50 amino acids truncated. STAT1 and STAT3 activities are significantly lowered when Tyr 1141 was mutated to phenylalanine, while STAT5 is only slightly affected (Baumann et al., 1996). These results show that the Ob-Rb uses multiple signaling paths and suggests that the complete pathway(s) have not been fully described.

Short Form Receptor (OB-R_S)

Five isoforms of the short form of the leptin receptor have been identified (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf). However, it has been suggested that the short intracellular domain of the OB-R_S may play a role in transporting leptin from the blood into the cerebral spinal fluid (CSF) where it can diffuse into the brain center(s) responsible for the regulation of body weight (Tartaglia, 1997). Banks et al. (1996)

reported that leptin enters the brain by a specific and saturable transport mechanism. It has also been shown that there are differences in leptin levels of obese and lean individuals, where in obese individuals leptin concentrations are greater in the blood than in CSF (Caro et al., 1996; Schwartz et al., 1996). Tartaglia et al. (1997) suggested that adipose derived leptin levels may not be indicative of increased CSF leptin levels.

One receptor is considered a soluble receptor (Ob-Re) and is transported through circulation and has no transmembrane domain, only an extracellular domain. This Ob-Re isoform is also called a leptin binding protein (LBP) and is thought to act as a biomodulator of receptor signaling or as a transporter of cytokines in circulation to prevent their degradation and clearance (Huang et al., 2000). The LBP has been linked to an mRNA encoding for the Ob-Re isoform in the mouse (Lee et al., 1996); however, in humans no such mRNA has been found.

In mice a 20-fold to 40-fold increase in circulating leptin levels occurs during pregnancy (Gavrilova et al., 1997). In these mice leptin mRNA and protein levels of maternal adipose tissue were not elevated and results from binding assays indicated that leptin was in a high molecular weight complex, indicative of the presence of a binding protein, and subsequent quantitative assays showed increases in LBP of 40-fold.

Early on, researchers hypothesized that the LBP may be a result of proteolytic cleavage of the OB-R_S isoform Ob-Ra. Maamra et al. (2001) reported that ligand mediated immunofunctional and immunofluorometric assays showed that LBP would bind to leptin and OB-R specific antibodies. It was noted that levels of LBP were correlated with receptor presence at the cell surface where Ob-Ra exhibited a higher expression than Ob-Rb. Subsequently, it was concluded that human LBP is a result of proteolytic cleavage of membrane bound leptin receptors by metalloprotease.

To further verify the findings of Maamra et al. (2001), that same year Lammert et al. (2001) reported that LBP is a major leptin binding protein in circulating human blood. Following size exclusion chromatography, a Western blot was used to visualize bands of partially purified leptin binding activity of LBP. Crosslinking studies using ¹²⁵I-leptin indicated that there were formations of heterodimers and homodimers of LBP complexed with and without leptin.

Tartaglia et al. (1997) also proposed that the OB-R_S may be a source of the soluble receptor playing a role in the clearance of leptin. These researchers went on to state that there are distinct tissue distributions of the different short forms, but it is unlikely that any marked differences in functionality between the different short forms exist.

Hypothalamus and the Leptin Receptor

Shortly after the discovery of the leptin hormone studies began in earnest to determine the pathways and signaling mechanisms of this new hormone. Tartaglia et al. (1995) was the first to identify the Ob-Rb isoform. Using leptin alkaline phosphatase fusion proteins and ¹²⁵I-leptin these researchers reported high affinity binding sites in the choroid plexus of the mouse. These researchers also were the first to describe this isoform as a single membrane spanning receptor most related to the gp130 signal transducing component of the IL-6 receptor, the G-CSF receptor and the LIF receptor. These results were subsequently verified by Mercer et al. (1996) who used in situ hybridization and found mRNA of known splice variants of the Ob-Rb isoform in the cortex, hippocampus, thalamus with high expression in the hypothalamus (arcuate, ventromedial, paraventricular and ventral premammillary nuclei), choroid plexus and leptomeninges of the mouse.

To substantiate previous studies, Schwartz et al. (1996) reported Ob-Rb isoform mRNA was concentrated around the arcuate nucleus, with the ventromedial and dorsomedial hypothalamic nuclei, but with lower concentrations. These authors suggested at that time that leptin action in the rat hypothalamus involved altered expression of key neuropeptide genes, and implicated leptin in the hypothalamic response to fasting. Mercer et al. (1997) used in situ hybridization to show that OB-R mRNA was higher in the hypothalamus in obese mice than in lean mice. Twice daily injections of leptin for 7 days profoundly reduced both food intake and OB-R mRNA in the arcuate nucleus, while single leptin injections were ineffective in inducing any physiological response.

Using immunohistochemistry, Hakansson et al. (1998) reported similar findings where OB-R isoforms were visualized in the choroid plexus, cerebral cortex, hippocampus, thalamus and hypothalamus; whereas, the 'leptin receptor like

immunoreactivity' (LR-LI) were strongly expressed in the supraoptic nucleus and paraventricular nucleus. Only weak signals following staining were noted in the lateral and medial preoptic nuclei, suprachiasmatic nucleus, ventromedial and dorsomedial nuclei and tuberomammillary nucleus.

Using confocal laser scanning microscopy these authors went on to show that LR-LI occurred on the periphery of cells in magnocellular neurons of both the supraoptic nucleus and paraventricular nucleus. Also of note, was the expression of LR-LI staining that occurred in many corticotropin releasing neurons, vasopressin and oxytocin containing neurons, with most of the staining occurring in the ventromedial aspect of the arcuate nucleus where LR-LI were co-localized with neuropeptide Y. One should note at this point that antisera used in these studies recognized both the Ob-Rb and OB-R_S isoforms of the leptin receptor. LR-LI staining was also expressed in large adrenocorticotrophic hormone-IR, proopiomelanocortin containing neurons and in galanin, neurotensin and GHRH containing neurons.

All the evidence to date shows that when in situ hybridization methods are used with probes that are specific for both the Ob-Rb and OB-R_S isoforms, hybridization is found in the hypothalamus (Mercer et al., 1996; Fei et al., 1997; Hakansson and Meister, 1998). Likewise, probes against the Ob-Rb isoform show only minimal labeling in the choroid plexus (Hakansson et al., 1996; Mercer et al., 1996). Using RT-PCR, Lee et al. (1996) have shown that Ob-Rb is located primarily in the hypothalamus. Considering these results it appears that the hypothalamus and choroid plexus both show an affinity for OB-R with the Ob-Rb isoform and Ob-Ra isoform simultaneously occupying different regions.

In pigs, Ob-Rb isoform expression increased in hypothalami from 106 day old fetus to 3.5 month postnatal piglets based on semiquantitative reverse transcription polymerase chain reaction compared with leptin mRNA expression increasing only to day 7 postnatal piglets. Expression of preproorexin, somatostatin and GnRH peaked at 3.5 months of age, while proopiomelanocortin mRNA continued to increase through 6 months of age (Lin et al., 2001). It was proposed that possible relationships might exist between Ob-Rb isoform and other hypothalamic and peripheral peptides during the development of the neuroendocrine axis in the neonatal pig.

Pregnancy and the Leptin Receptor

Because pregnancy and subsequent lactation places an increased demand on physiological systems, one would expect a decrease in leptin levels during pregnancy to enhance food intake to meet this demand. However, research has shown that leptin increases in the rat from mid-gestation through parturition (Kawai et al., 1997; Tomimatsu et al., 1997; Lewandowski et al., 1999), while food consumption remains unchanged (Shirley, 1984) or increases (Rosso, 1987; Seeber et al., 2002). Thus researchers hypothesize that a state of leptin resistance is initiated in these pregnant animals to ensure that energy demands are met for fetal growth.

As previously stated Maamra et al. (2001) and Lammert et al. (2001) showed that the Ob-Re isoform was a result of Ob-Ra isoform being cleaved from the cell surface. It appears as though this is a mechanism for inducing the leptin resistance in pregnant females in at least 2 species (mouse and baboon). Because the placenta is a site that is rich in the Ob-Ra isoform (Hoggard et al., 1997; Green et al., 2000; Seeber et al., 2002; Smith and Waddell, 2002) and increases in Ob-Re isoforms occur at the same time as increased circulating leptin levels (Seeber et al., 2002), it has been proposed that leptin may play a role in fetal development and is sequestered by Ob-Re isoform so energy intake is not decreased by low levels of hypothalamic neuropeptide Y.

OB-R_S and Ob-R_b isoforms have been isolated in a number of fetal/placental tissues in the mouse (Hoggard et al., 1997), baboon (Green et al., 2000), human (Bodner et al., 1999) and most recently in the rat (Smith et al., 2002). In the baboon, Green et al. (2000) used reverse transcriptase polymerase chain reaction and in situ hybridization and found that both Ob-R_b and OB-R_S isoforms were expressed in the placenta (anmiochorion and decidua) and the corpus luteum throughout gestation. It was also noted that OB-R_S isoforms were expressed in greater abundance than the Ob-R_b isoform in all tissues. Henson et al. (2004) showed that transcripts for both Ob-R_b and OB-R_S isoforms increased several fold in baboon fetal lungs between mid and late gestation.

Hoggard et al. (1997) reported that high levels of leptin and its receptor were expressed in fetal bone and cartilage and may be linked to hematopoiesis actions of leptin in adults. Using the Western blot and immunocytochemistry, these researchers

reported that expression of both mRNA and protein occurred in the ribs, scapula, clavicle, humerus, ulna radius, femur and mandible, especially while these structures were undergoing ossification in 14.5-day postcoitus mouse fetuses.

Several isoforms of the OB-R_S (short form of the leptin receptor) have been reported in the rat placenta (Smith et al., 2002). Ob-Ra, Ob-Rb and Ob-Re mRNAs are expressed in the functionally distinct basal and labyrinth zones of the rat placenta from day 16 to day 22 postcoitus by real time quantitative reverse transcription polymerase chain reaction. Also, Smith et al. (2002) reported that the spacial placental expression of the Ob-Rb isoform was confirmed by the Western blot and immunolocalization, and was most prominent in trophoblast and vascular tissues. The latter group goes on to report that Ob-Ra and Ob-Re mRNA expression increased from day 16 to day 22 in the labyrinth but not in the basal zone, while Ob-Rb remained stable through all sampling periods.

Previous research findings not only support the hypothesis that females enter a state of leptin resistance, but also provide experimental results that explain how physiological mechanisms work to provide protection from the high maternal circulating leptin concentrations on energy intake. Research has shown that leptin plays a critical role in the fetal/placental unit late in pregnancy, and scientists are just starting to unravel its mechanisms and impacts on the growing fetus.

Ovary and the Leptin Receptor

Studies have shown that OB-R are present in the ovarian tissues including preovulatory oocytes in chickens (Cassy et al., 2004), in the rat (Duggal et al., 2002) and humans (Cioffi et al., 1997; Karlsson et al., 1997; Ryan et al., 2002; Ryan et al., 2003) However, at this time no studies have been found identifying mRNA for leptin within the oocyte, thus, the mechanism by which leptin enters the oocyte remains to be determined.

Cassy et al. (2004) reported significant changes in the Ob-Rb isoform in slow growing domestic hens compared with fast growing hens. Ob-Rb mRNA (determined by RT-PCR) expression in chicken theca cells did not change. However, in slow growing hens Ob-Rb receptor isoforms decreased with follicular differentiation in the granulosa cells, while in fast growing hens up-regulation of the OB-R isoform occurred in the

granulosa cells. When food intake was restricted the overall expression of OB-R mRNA was decreased. Also, these authors reported that circulating leptin and other metabolic factors were not affected by altering nutritional status.

Furthermore, it has been shown that leptin has direct effects on the ovary, more specifically, in ovarian cell types such as theca and granulosa cells, through steroidogenesis pathways (Spicer and Francisco, 1997; Zachow and Magoffin, 1997; Spicer and Francisco, 1998; Agarwal et al., 1999; Brannian et al., 1999; Ryan et al., 2002; Ryan et al., 2003). Ryan et al. (2002) reported that the OB-R isoform is expressed in the mouse in all of the primary ovarian tissue cell types, with the highest staining levels occurring in the theca cells. In another study, Ryan et al. (2003) again showed Ob-Rb isoforms present in theca cells, endothelial cells, oocytes and corpora lutea in immature gonadotropin primed rat ovaries following human chorionic gonadotropin administration.

Also, the leptin protein has been found in both mouse and human oocytes (Cioffi et al., 1997; Matsuoka et al., 1999) but no studies to date have been able to show mRNA for leptin in either of these species (Antczak et al., 1997; Cioffi et al., 1997). Antczak and Van Blerkom (1997) hypothesized that leptin is produced elsewhere and transported into the oocyte by endocytosis. It has been suggested that the amount of leptin increases in the oocyte as it matures from germinal vesicle stage to the second metaphase (Matsuoka et al., 1999).

Research has shown that the OB-R gene varies in the rat ovary throughout the follicular wave cycle (Duggal et al., 2002), and it has been suggested that these variations modulate the sensitivity of the ovary to the leptin protein. These researchers reported that both Ob-Rb and OB-R_S isoforms were present in the adult rat ovary. RT-PCR results indicated that Ob-Rb gene expression was low during proestrus and diestrus II, while expression was high in estrus and diestrus I of the mouse. The OB-R_S isoform gene expression was highest in diestrus I and elevated in estrus, while expression was low during proestrus and diestrus II.

In the human ovary, transcripts encoding for both Ob-Rb and OB-R_S isoforms were detected in granulosa and theca cells (Cioffi et al., 1996; Karlsson et al., 1997). Karlsson et al. (1997) reported that the OB-R_S isoform is expressed at much higher

levels than the Ob-Rb isoform. Also, while no leptin gene expression was detected in these ovaries, leptin was present in follicular fluid at levels similar to those found in the serum. In contrast, Cioffi et al. (1997) reported leptin gene expression at both the mRNA and protein levels demonstrated production by the granulosa and cumulus cells.

Zerani et al. (2004) examined the expression of the Ob-Rb isoform on day 9 of pseudopregnancy in rabbits. Positive Western blot analysis confirmed the presence of Ob-Rb isoforms in large luteal cells of the corpus luteum (CL) and granulosa cells of follicles and oocytes. These researchers reported increased prostaglandin F_2 production with decreased basal progesterone. The increased prostaglandin F_2 was a result of activation of the JAK pathway, while the decreased prostaglandin was a result of the activation of mitogen-activated kinase and cAMP specific pathways.

Therefore, leptin may play a significant role in follicle somatic cells in the regulation of oocyte maturation, CL formation, prostaglandin release and the decline in circulating progesterone in farm animals. Further studies are needed to fully determine and understand the role of leptin and its receptor isoforms in ovarian function.

The Embryo and Endometrium and the Leptin Receptors

Studies have shown that Ob-R are expressed in early human and mouse embryos and oocytes (Antczak and Van Blerkom, 1997). Through immunofluorescence these researchers have shown that the Ob-R receptor is expressed in mouse oocytes, fertilized ova and early cleavage stage embryos. Using immunohistological techniques Gonzalez et al. (2000) reported the Ob-R is expressed in the luminal and glandular endometrial epithelium in the human. Both leptin and OB-R were detected in EEC when cells were cultured with and without embryos, indicating that the embryos were not the only source of leptin but the EEC themselves were able to produce this protein.

Kitawaki et al. (2000) used Northern and Western blot analyses to isolate leptin receptors from human endometrial tissues. They went on to state that there were different profiles of expression with leptin receptors gradually increasing during the early proliferative phase toward ovulation and peaking during the early secretory phase then declining during the middle and late secretory phase. However, no leptin protein was detected in these human endometrial samples.

The control for expression of the Ob-Rb gene in the human endometrium has been proposed to be the ovarian steroid hormones (Koshiba et al., 2001). These researchers reported that mRNA of the Ob-Rb isoform were variable throughout the menstrual cycle and peaked in the early secretory phase. When proliferative endometrium samples were supplemented with estradiol, no changes were found in the expression of mRNA for the Ob-Rb isoform. However, when estradiol was combined with progesterone or medroxyprogesterone acetate the expression of mRNA then declined in the medium. When the progesterone antagonist (mifepristone) was added to the medium the progesterone suppression was inhibited. These results indicate that steroid hormones of ovarian origin have significant effects on the expression of the Ob-Rb isoform.

Lactation and Leptin Receptors

Lactation requires increased energy resulting in increased food intake. Nutrients are channeled to the mammary gland through various metabolic and hormonal changes. It has been shown that leptin acts at the hypothalamic level to decrease appetite and increase energy expenditure (Friedman, 1998; Ahima et al., 2000; Havel, 2000; Spiegelman and Flier, 2001; Williams et al., 2001). Research has also shown that circulating leptin concentrations decrease when negative energy balance is achieved (Friedman, 1998; Houseknecht and Portocarrero, 1998; Ahima, 2000; Ahima and Flier, 2000; Ahima et al., 2000; Vernon et al., 2001) as is the case during lactation.

OB-R of hypothalamic origin is crucial for modulating feeding behaviors in rats. Brogan et al. (2000) showed that the Ob-Rb isoform mRNA in the ventromedial hypothalamus decreased in lactating animals when compared with increases in the supraoptic nucleus in animals in diestrus. These authors propose that increases in neuropeptide Y and agouti-related protein with down-regulation of pro-opiomelanocortin are responsible for sustaining the chronic hyperphagia that occurs during lactation.

Denis et al. (2003) showed that circulating leptin concentration in nonlactating rats was increased at night, while hypothalamic mRNA levels of Ob-Rb, Ob-Rc, and Ob-Re isoforms were decreased. It was also reported that serum leptin and mRNA expression of Ob-Rb gene and Ob-Re gene were negatively correlated regardless of time of day. There was no nocturnal rise in circulating leptin concentrations in lactating

rats and daytime mRNA levels of hypothalamic Ob-Rb isoforms were lower in nonlactating females than in lactating females.

The relationship between leptin and the soluble receptor Ob-Re isoform expression was not changed by lactation. Thus, Denis et al. (2003) hypothesized that selective changes in expression of OB-R isoforms may contribute to the hyperphagia during lactation in rats because decreased daytime expression of the Ob-Rb isoform could reduce the hypothalamic sensitivity to leptin and cause an increase in daytime appetite in lactating rats. Also, because Ob-Re isoform expression was high during lactation, the binding of free leptin in circulation would further accentuate hyperphagia.

In summary, scientists are only in the early stages of understanding the role of leptin and its receptors. Most published research has focused on leptin and obesity in the mouse, rat and human. Very few studies have concentrated on the effects of leptin on reproduction, especially in ruminants, where results have been reported they are at best conflicting from study to study. Clearly, more experimentation is needed to determine the effects of leptin on the early embryo and subsequent pregnancy in farm animals.

CHAPTER III

EFFECTS OF DEXAMETHASONE ON PLASMA LEPTIN LEVELS, LH, FSH, PROGESTERONE AND FOLLICULAR GROWTH IN EARLY POSTPARTUM BEEF CATTLE

Introduction

Leptin is a small polypeptide with physiological properties, much like cytokines, and has been shown to regulate body weight through intake reduction and increased energy expenditure (Maffei et al., 1995; Schwartz et al., 1996; Shimizu et al., 1997; Perry et al., 1997; Blum, 1997; Langendonk et al., 1998; Blache et al., 2000). Nutrition has been implicated in the indirect control of LH compromising reproduction through delayed puberty in heifers (Day et al., 1986), anestrus in adult beef cows (Richards et al., 1989) and reduced LHRH in sheep (Schillo et al., 1992). More recently, researchers have reported that leptin was highly correlated to available body energy (Sansinanea et al., 2001) and exogenous leptin has been found to increase the release of LH in rats (Yu et al., 1997; Gonzalez et al., 2000).

Glucocorticoids have also been reported to increase leptin secretion in the horse (Gentry et al., 2002; Cartmill et al., 2003a; Cartmill et al., 2005a) and in humans (Laferrere et al., 2000). Research has shown that the addition of 100 nM dexamethasone to isolated rat adipocytes rapidly induced a 4-fold to 8-fold increase in leptin receptor mRNA (Murakami et al., 1995). The increase in leptin receptor mRNA level was apparent within 1 hour, and reached a maximum at ~7 hours after stimulation.

In children undergoing chemotherapy for lymphoblastic leukemia, a common component of the MRC-ALL97/99 induction chemotherapy is the administration of dexamethasone (Wallace et al., 2003). The administration of dexamethasone to the children for 5 weeks resulted in significant increases in body mass index (BMI), leptin (corrected for BMI), the leptin:sex hormone binding globulin ratio and a reduction in the circulating levels of sex hormone binding globulin.

Miell et al. (1996) reported that administration of dexamethasone (2 mg twice daily) in humans resulted in a rapid and sustained increase in leptin levels from basal values after 24 hours of treatment. These researchers also noted that following placebo administration 24-hour hormone profiles confirmed a nocturnal rise in leptin levels with

an increase of 73% at midnight. After dexamethasone treatment, mean circulating leptin levels increased by 123%.

Putignano et al. (2003) monitored overnight plasma leptin level changes in normal and obese men and women being administered different doses of dexamethasone. These researchers reported that basal leptin levels were significantly higher in obese than in normal subjects. In obese subjects, leptin levels increased significantly after administration of either 1 mg of dexamethasone or 0.015 mg/kg of body weight of dexamethasone, while in normal subjects plasma leptin levels remained unchanged by each dose of dexamethasone.

However, after splitting subjects by gender, mean leptin levels rose after dexamethasone doses of either 1 mg, 0.015 mg/kg of body weight, 0.007 mg/kg of body weight or 0.0035 mg/kg of body weight in obese women. However, no changes in circulating leptin concentrations were detected in the obese men or normal men and women groups. These researchers proposed that in obese women but not in obese men and in normal weight subjects, small overnight increases in plasma glucocorticoid concentrations induced gender-related plasma leptin elevations that were unrelated to body fat distribution and insulin sensitivity. This study showed that there is a greater sensitivity of female adipose tissue to glucocorticoids compared with male adipose tissue, regardless of level of obesity.

Trujillo et al. (2004) reported that in both human omental and abdominal subcutaneous adipose tissue cultures, the addition of exogenous IL-6 and dexamethasone for 2 days increased leptin compared with tissue incubated with IL-6 alone. These researchers concluded that high local concentrations of IL-6 can modulate leptin production and lipid metabolism in human adipose tissue.

Research has shown glucocorticoids can either stimulate (Hardie et al., 1996; Wabitsch et al., 1996) or inhibit (Considine et al., 1997; Reul et al., 1997) the release of leptin. Faulconnier et al. (2003) have reported that after incubation of perirenal ovine adipose tissue extracts in dexamethasone, leptin production was increased in tissue extracts. The authors also noted that when ovine adipose tissue was incubated in dexamethasone and insulin that the leptin production was substantially increased compared with adipose tissue incubated in dexamethasone alone. Gentry et al. (2002)

reported that high body condition score mares given exogenous dexamethasone exhibited increased plasma concentrations of leptin following a 4-day treatment interval. They also report that this effect was also found in low body condition score mares, but to a lesser degree.

It has been reported (Cartmill et al., 2003b) that administration of dexamethasone (125 : g/kg of body weight) to light horse geldings and mares once per day for 4 days resulted in increased glucose, insulin and leptin. Furthermore, administration of 200 IU of adrenocorticotropin (ACTH) to light horse geldings twice daily for 4 days resulted in increased plasma cortisol, glucose, insulin and leptin concentrations.

Cartmill et al. (2005a) have evaluated the effects of dexamethasone, gender and testosterone on plasma leptin concentrations in horses. These researchers reported increased plasma leptin, insulin, glucose and IGF-1 concentrations in stallions following five daily injections of dexamethasone (125 : g/kg of body weight). It was also noted that plasma leptin concentrations increased in mares, geldings and stallions following a single injection of dexamethasone, and this response was greater in mares and geldings than in stallions.

It has been reported that the release of glucocorticoids resulting from acute stress during the follicular phase of the estrous cycle in dairy heifers resulted in prolonged estrous cycles due to the inhibition of LH secretion (Stoebel and Moberg, 1982; Pool et al., 1983). In vitro studies have also shown that pituitary cells from luteal phase cows exhibit decreased LH secretion when incubated with dexamethasone and cortisol (Stoebel and Moberg, 1982; Padmanabhan et al., 1983). Research has also shown that treatment of cattle for 14 days during the luteal phase with ACTH resulted in decreased mean circulating concentrations of LH and FSH, and the pre-ovulatory surges of LH and FSH did not occur (Kawate et al., 1996).

Therefore, the objectives of this experiment were to determine if exogenous glucocorticoid (dexamethasone) administration would affect circulating plasma leptin concentrations in early postpartum cows and to determine if exogenous dexamethasone would affect gonadotropins released from the anterior pituitary.

Materials and Methods

Experimental Animals

Brahman x Hereford crossbred cows (n = 22) , 8 to 9 years of age, with a mean body weight of 553 ± 12 kg and a mean body condition score of 4.9 ± 0.09 (1 = emaciated and 9 = obese) were used. These cows had a mean postpartum interval of 32 days (range of 8 days to 40 days). All females were allowed free choice to ryegrass forage, *ad libidum* bermudagrass hay and water in 2 hectare pastures for the duration of the study.

Experimental Design

Females were stratified by days postpartum, body condition score and body weight and randomly assigned to two groups in the early part of the calving season (February). The two treatment groups were (A) control with a daily intramuscular injection (im) of an oil (Louana vegetable oil, Ventura Foods, Opelousas, LA) vehicle for 4 days and (B) dexamethasone with a daily injection (im) of dexamethasone (Sigma Aldrich, St. Louis, MO) at 125 : g/kg of body weight for 4 days. The experimental protocol is shown in Figure 3.1. Briefly, dexamethasone treatments were begun on day 0 at 0800 hours and continued daily through day 4. Blood sampling for circulating leptin, FSH, LH, progesterone and ovarian ultrasound examinations began on pretreatment day -6 and were conducted every other day until day 0. Starting on day 0 ovary examinations and blood sampling occurred daily, continuing for 8 days. After day 8 females were examined and blood samples were collected every other day through day 16. In this experiment, pretreatment ovary size, follicle size, plasma leptin, plasma LH and plasma FSH were compared with the post-treatment values (starting on day 0).

Experimental Procedure

Jugular blood samples were collected into 7-ml evacuated tubes containing sodium heparin (Vacutainer, Becton and Dickinson, Franklin Lakes, NJ) 6 days prior to the first dexamethasone treatment at 0800 hours and continued for the duration of the experiment. Blood samples were centrifuged at $1,500 \times g$ at 4°C for 15 minutes and the plasma harvested and stored at -15°C . Concentrations of LH and FSH were determined by previously validated RIA (Thompson et al., 1983). Plasma leptin concentrations were

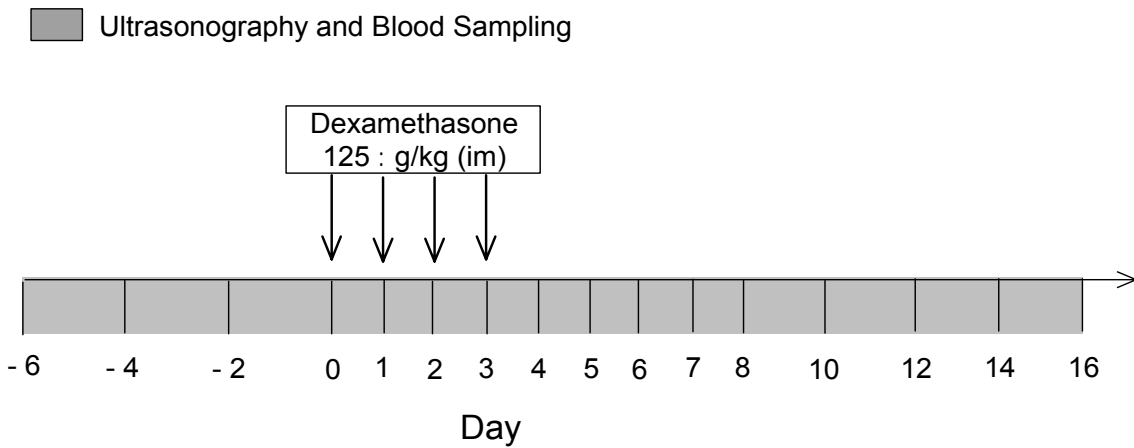


Figure 3.1. Experimental protocol for postpartum cow dexamethasone treatment, ultrasonography and blood sampling. Similarly, the control cows received an oil vehicle treatment on days 0, 1, 2 and 3.

determined by RIA using commercially available reagents (Linco Multispecies Kit, Linco Research, St. Louis, MO). The intrassay and interassay coefficients of variation and assay sensitivities were 6%, 9% and 0.2 ng/ml for LH, respectively. The intrassay and interassay coefficients of variation and assay sensitivities were 7%, 11% and 1.4 ng/ml for FSH, respectively. The intrassay and interassay coefficients of variation and assay sensitivities were 4%, 8% and 0.8 ng/ml for leptin, respectively.

Ultrasonography

Ovaries from each female were examined using transrectal ultrasonography (Aloka 550V with 5 MHz linear-array transducer; Aloka, Wallingford, CT). Ovary size, follicle number and size, and number of corpora lutea (CL) were assessed daily or every other day (Figure 3.1). Follicles were assigned categories based on ultrasonographic measurements as described previously for cattle by Lucy et al. (1991). Ovary size was determined by rectal palpation and were assigned a score of either: (1) 5 mm or less, (2) 6 to 10 mm, (3) 11 to 25 mm, (4) 26 to 40 mm or (5) greater than 40 mm.

Statistical Analysis

Data were analyzed by the GLM procedure of SAS (SAS Institute, Inc., Cary, NC) as a randomized block design with repeated measures. Main effects of treatment, day and phase (pretreatment or post-treatment) were analyzed with the animal (treatment) as the error term. Treatment by day and treatment by phase interactions were analyzed using the Type III sums of squares. Means and standard errors reported were generated using the LSMEANS statement.

Results

There was no significant difference in the mean number of total follicles in the post-treatment phase (3.57 ± 0.27) in the control group compared with the post-treatment phase (3.78 ± 0.29) in the dexamethasone treatment group (Figure 3.2). Also, there was no difference in the mean number of total follicles between the pretreatment phase (3.20 ± 0.88) and post-treatment phase (3.78 ± 0.29) in the dexamethasone treatment group.

There was no difference in mean number of Class 2 follicles in the post-treatment phase (2.13 ± 0.24) of the control group compared with the post-treatment phase (2.07 ± 0.26) of the dexamethasone treatment group (Figure 3.3). Also, there was no

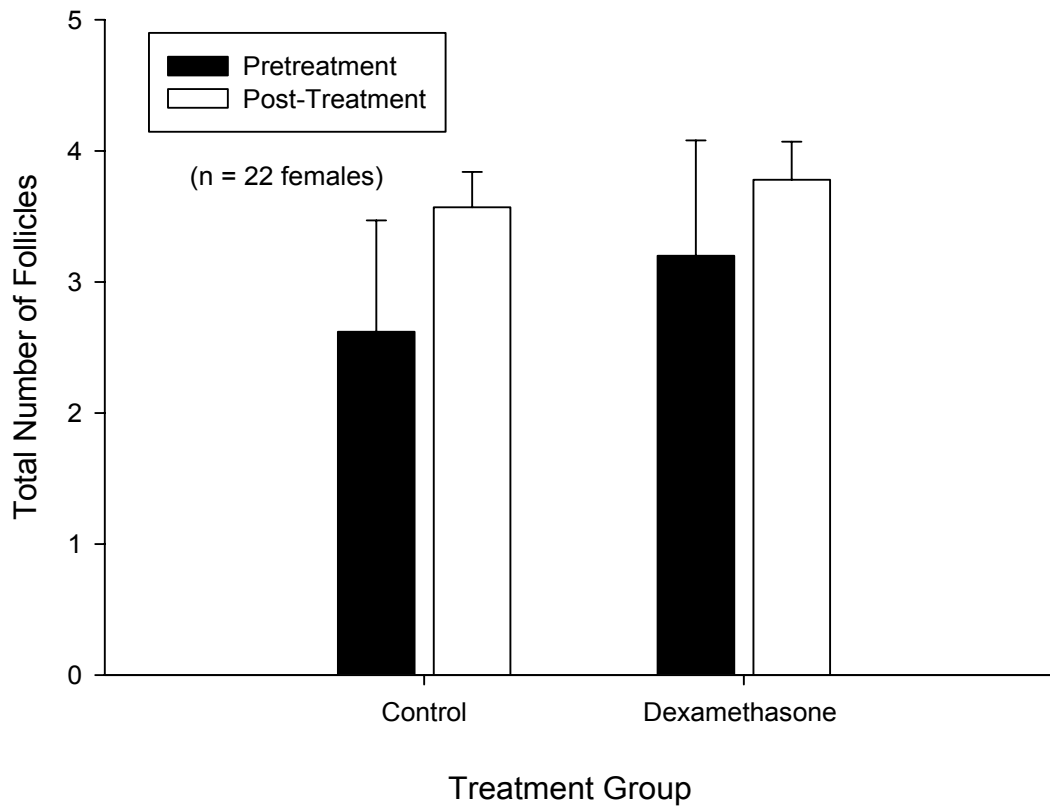


Figure 3.2. Mean number of follicles (>3mm) per female for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3) pretreatment and post-treatment. There were no significant differences between pretreatment and post-treatment means within treatment groups.

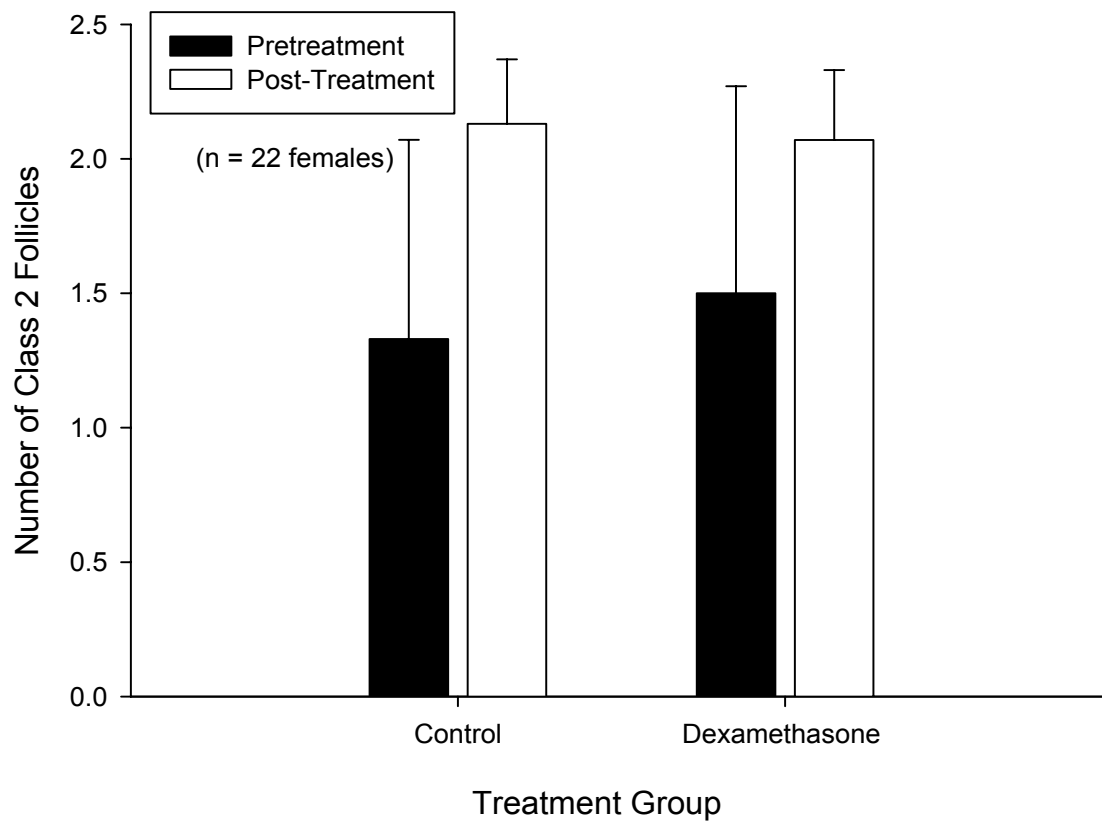


Figure 3.3. Mean number of Class 2 follicles (5 mm to <10 mm) per female for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3) pretreatment and post-treatment. There were no significant differences between pretreatment and post-treatment means within treatment groups.

difference in mean number of Class 2 follicles in the pretreatment phase (1.5 ± 0.77) compared with the post-treatment phase (2.07 ± 0.26) of the dexamethasone treatment group.

There was no difference in mean number of Class 3 follicles in the pretreatment phase (1.70 ± 0.43) compared with the post-treatment phase (1.44 ± 0.13) of the control group. Also, there was no difference in mean number of Class 3 follicles in the pretreatment phase (1.33 ± 0.74) compared with the post-treatment phase (1.71 ± 0.14) of the dexamethasone treatment group (Figure 3.4). Likewise, there was no difference in mean number of Class 3 follicles in the post-treatment phase (1.44 ± 0.13) of the control group compared with the post-treatment phase (1.71 ± 0.14) of the dexamethasone treatment group

In this study, dexamethasone treatment did not affect ovary size. There was no difference in the mean size of the left ovary per female between the post-treatment phase (2.84 ± 0.12) of the control group compared with the post-treatment phase (2.73 ± 0.13) of the dexamethasone treatment group (Figure 3.5). Furthermore, there was no significant difference in mean left ovary size per female between the pretreatment phase (2.70 ± 0.41) and post-treatment phase (2.73 ± 0.13) for the dexamethasone treatment group.

There was no difference in the mean size of the right ovary per female between the post-treatment phase (2.80 ± 0.11) of the control group compared with the post-treatment phase (2.88 ± 0.13) of the dexamethasone treatment group (Figure 3.6). Also there was no difference in mean right ovary size per female between the pretreatment phase (2.80 ± 0.38) and post-treatment phase (2.88 ± 0.13) for the dexamethasone treatment group.

Mean circulating LH concentrations were not affected by dexamethasone treatment. However, mean circulating LH levels tended to be higher ($P = 0.08$) for the post-treatment phase (0.12 ± 0.02 ng/ml) of the control treatment group compared with the post-treatment phase (0.06 ± 0.02 ng/ml) of the dexamethasone treatment group (Figure 3.7). There was no difference between the pretreatment phase (0.08 ng/ml ± 0.06) and the post-treatment phase (0.06 ± 0.02 ng/ml) of the dexamethasone treatment group for circulating levels of LH.

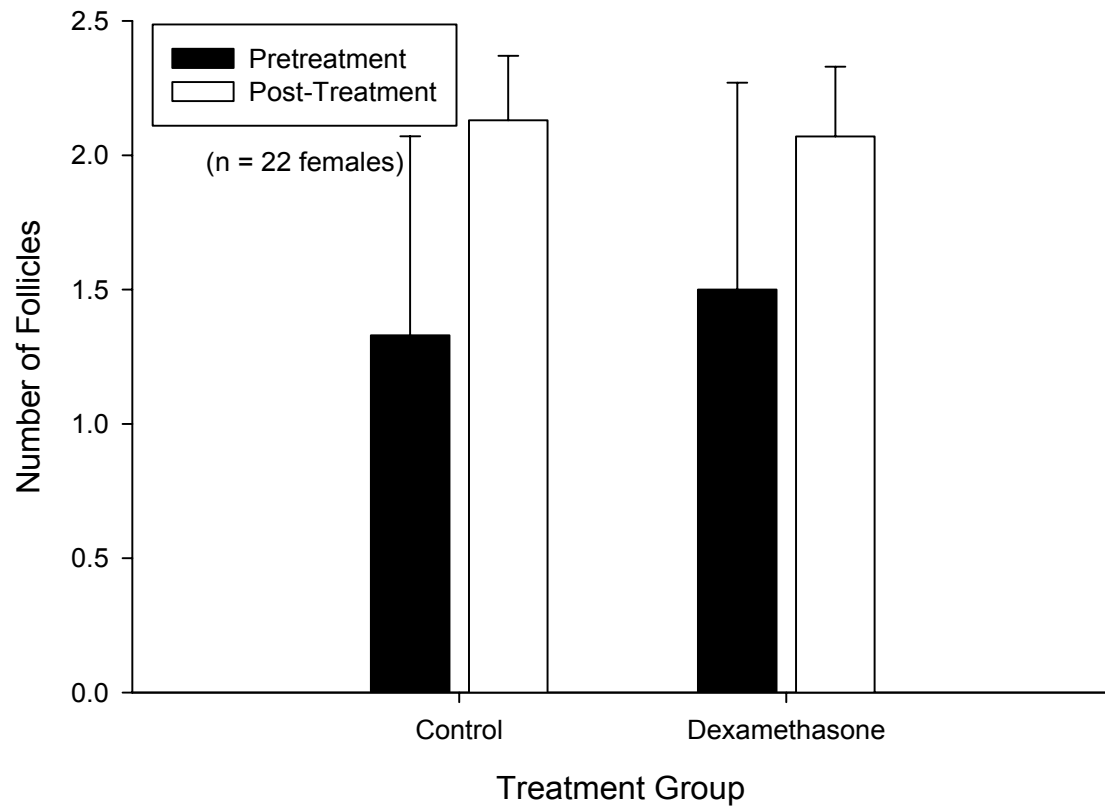


Figure 3.4. Mean number of follicles greater than Class 2 follicles (≥ 10 mm) per female for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3) pretreatment and post-treatment. There were no significant differences between pretreatment and post-treatment means within treatment groups.

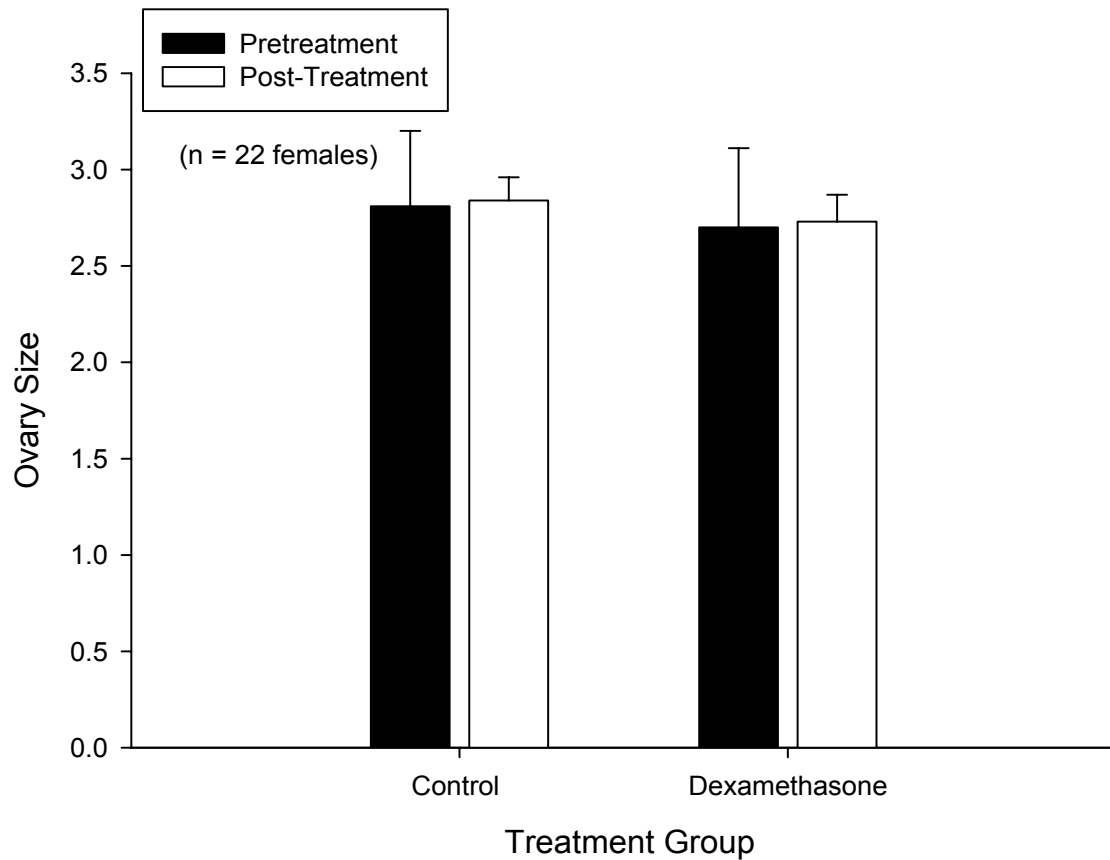


Figure 3.5. Mean left ovary size per female for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3) pretreatment and post-treatment. There were no significant differences between pretreatment and post-treatment means within treatment groups.

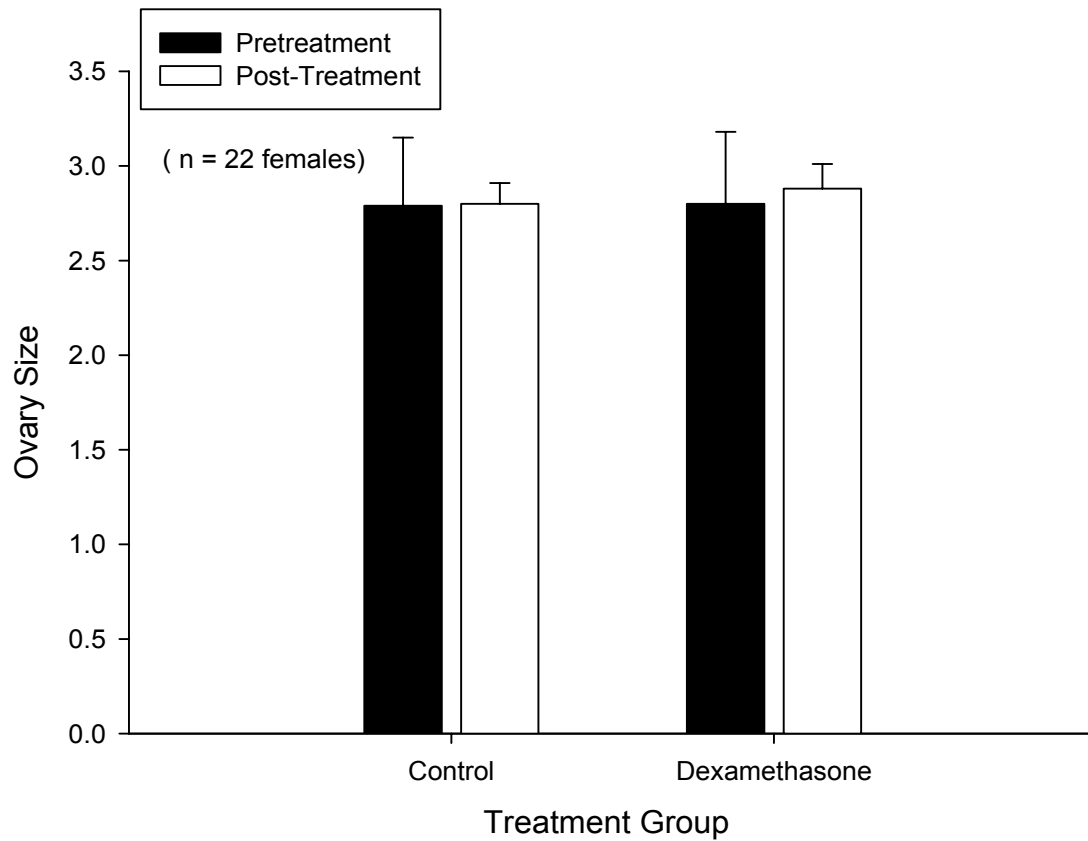


Figure 3.6. Mean right ovary size per female for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3) pretreatment and post-treatment. There were no significant differences between pretreatment and post-treatment means within treatment groups.

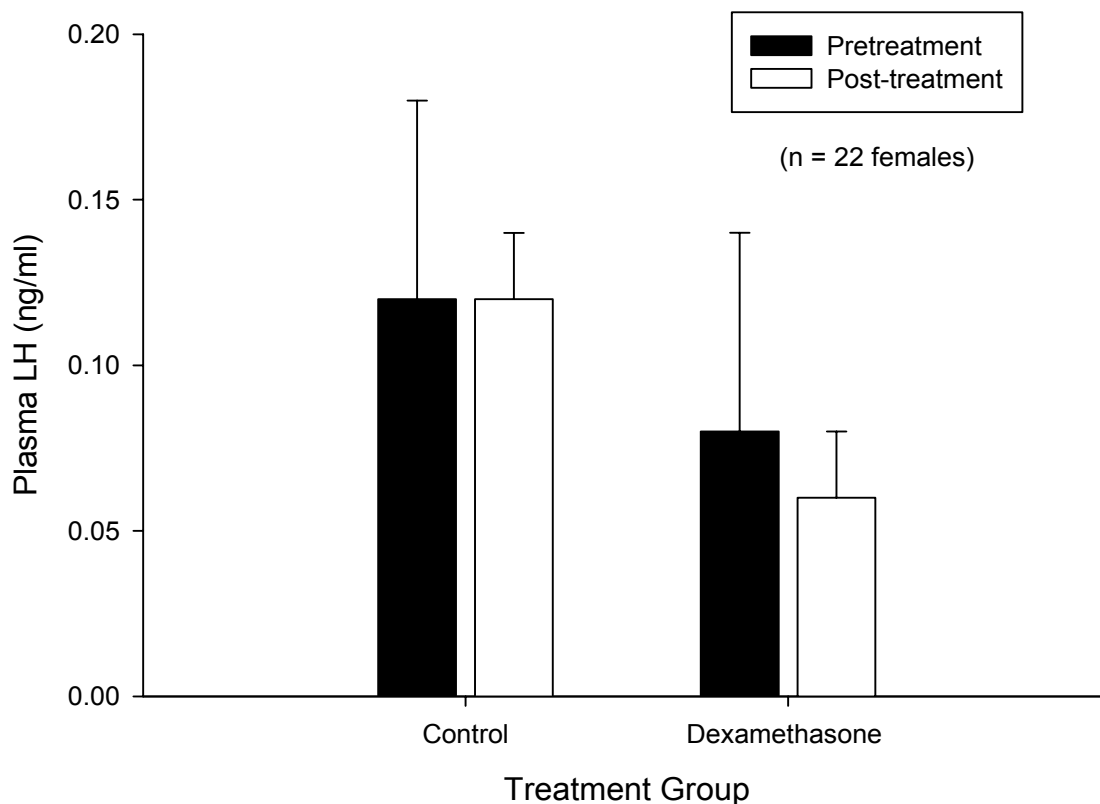


Figure 3.7. Mean plasma LH concentrations for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3) pretreatment and post-treatment. There were no significant differences between pretreatment and post-treatment means within treatment groups.

Mean circulating FSH levels were significantly higher ($P < 0.05$) in the post-treatment phase (5.31 ± 0.63 ng/ml) of the control group compared with the post-treatment phase (3.38 ± 0.63 ng/ml) of the dexamethasone treatment group (Figure 3.8). Pretreatment levels of FSH were not different between the control treatment group (5.29 ± 1.59 ng/ml) compared with the dexamethasone treatment group (3.73 ± 1.63 ng/ml). There was no significant change in mean circulating FSH concentrations from the pretreatment phase (3.73 ± 1.63 ng/ml) to the post-treatment phase (3.39 ± 0.63 ng/ml) in the dexamethasone treatment group.

There was no difference in mean plasma progesterone levels between the post-treatment phase (0.64 ± 0.26 ng/ml) in the control treatment group and the post-treatment phase (0.15 ± 0.26 ng/ml) in the dexamethasone treatment group (Figure 3.9). Also, mean circulating progesterone levels were not different from the pretreatment phase (0.34 ± 0.67 ng/ml) to the post-treatment phase (0.15 ± 0.26 ng/ml) in the dexamethasone treatment group.

Mean circulating leptin concentrations were not affected by dexamethasone treatments. There was no difference in plasma leptin concentrations in the post-treatment phase (6.02 ± 0.89 ng/ml) of the control treatment group compared with the post-treatment phase (7.80 ± 1.04 ng/ml) of the dexamethasone treatment group (Figure 3.10). Also, mean plasma leptin levels for the pretreatment phase (6.48 ± 2.73 ng/ml) were not different from post-treatment phase (7.80 ± 1.04 ng/ml) in the dexamethasone treatment group.

Discussion

In the present study there were no differences in follicular development for those animals treated with dexamethasone (125 : g/kg of body weight) for 4 days compared with those females in the control group receiving an oil vehicle for 4 days. These data are in agreement with those of Maciel et al. (2001), who reported that dairy cows receiving exogenous dexamethasone (44 : g/kg of body weight) for 12 days following a synchronized estrus, exhibited no significant difference in follicular development compared with control cows.

In our study, the dosage of dexamethasone (125 : g/kg) was likely high enough to induce an ovarian response of reducing follicle size as was reported in horses (Asa

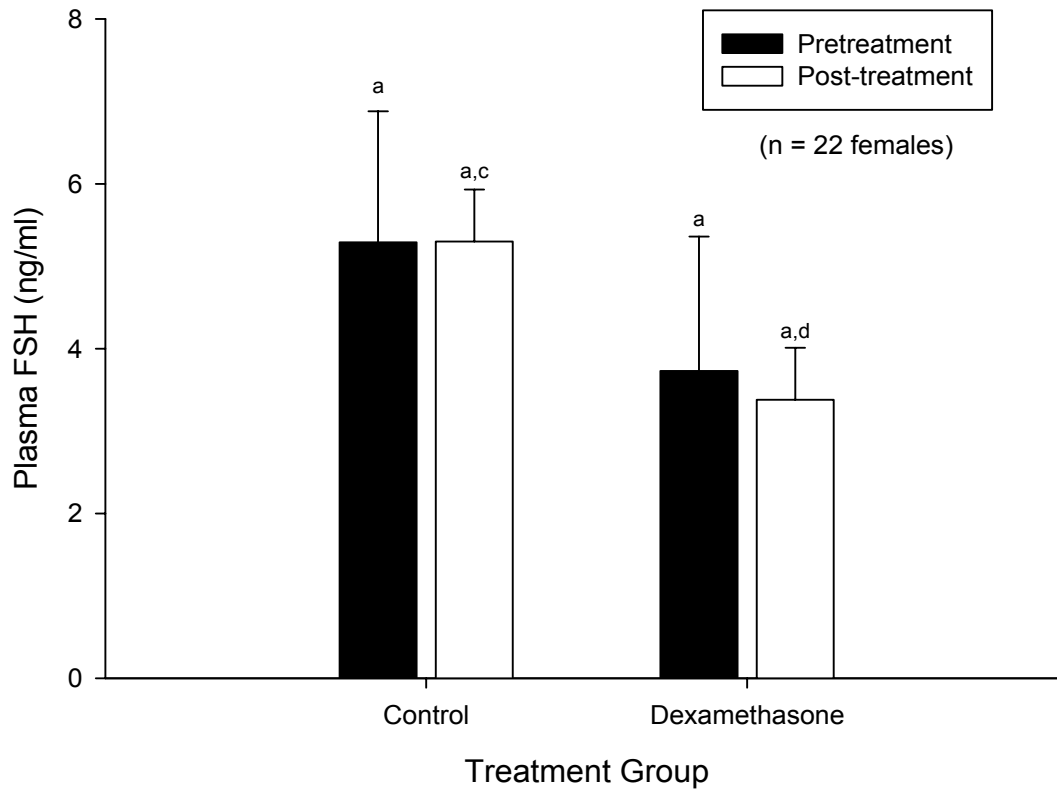


Figure 3.8. Mean plasma FSH concentrations for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3). ^{a,b}Bars with different superscripts within treatment groups are significantly different (P<0.05). ^{c,d}Bars with different superscripts across treatment groups are significantly different (P<0.05).

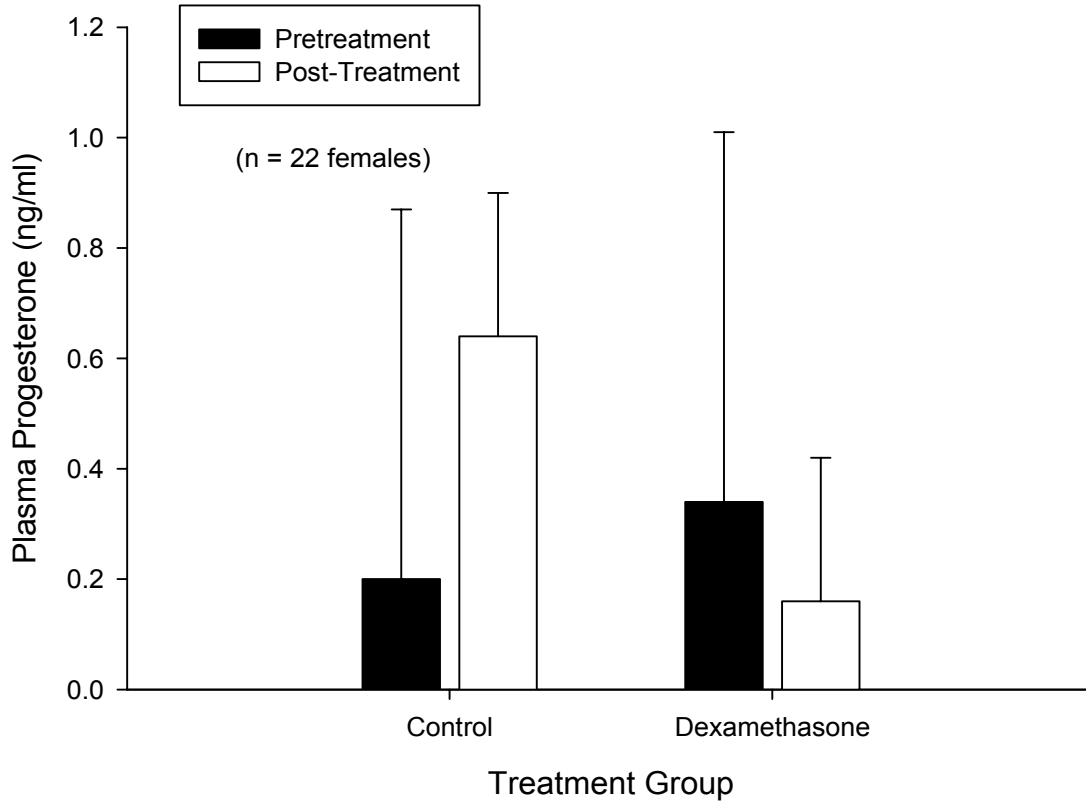


Figure 3.9. Mean plasma progesterone concentrations for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3) pretreatment and post-treatment. There were no significant differences between pretreatment and post-treatment means within treatment groups.

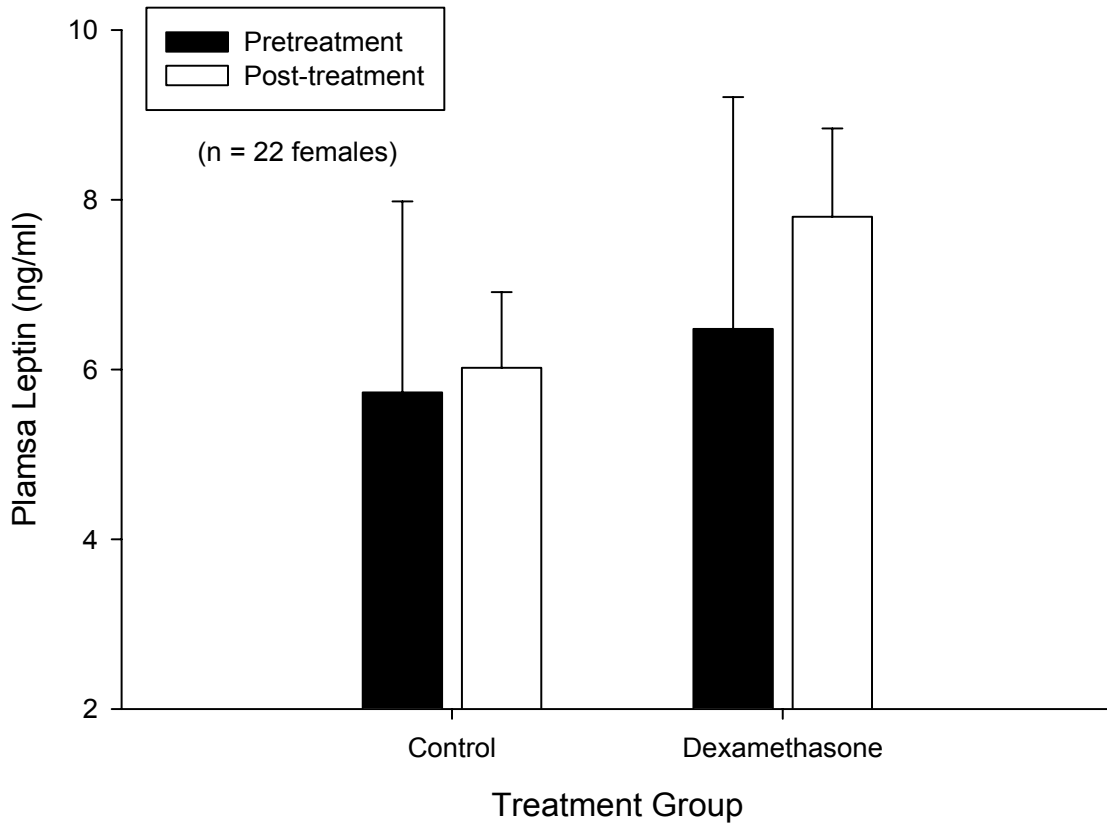


Figure 3.10. Mean plasma leptin concentrations for the control treatment group (received oil vehicle on days 0, 1, 2, and 3) and the dexamethasone treatment group (received 125 : g/kg of dexamethasone on days 0, 1, 2 and 3) pretreatment and post-treatment. There were no significant differences between pretreatment and post-treatment means within treatment groups.

and Ginther, 1982; Gentry et al., 2001). However, the duration of exogenous dexamethasone treatment in our study may not have been long enough to elicit a response. The females in our study averaged 32 days postpartum, and this postpartum interval may not have been long enough for recovery of the hypothalamic-reproductive axis from the previous pregnancy for these females to respond to dexamethasone treatment (Wathes et al., 2003).

In the present study, dexamethasone treatment for 4 days did not alter circulating LH concentrations. These results are in agreement with previously reported results in dairy cattle (Hockett et al., 2000), where cows with mastitis during the luteal phase had increased cortisol levels, but circulating LH levels were not different from control cows with normal circulating cortisol levels. Maciel et al. (2001) also reported that plasma LH concentrations did not change in Holstein cows when exogenous dexamethasone was administered during the luteal phase.

In contrast, both in vitro and in vivo studies have shown exogenous glucocorticoids decrease LH secretion and LH pulsatile release in cattle (Padmanabhan et al., 1983; Echternkamp, 1984). Pituitary cells harvested from cows in the luteal phase of their cycle incubated with dexamethasone (1, 5 and 10 ng/ml) had decreased LH concentrations of 60%, 71% and 88%, respectively, when compared with control pituitary cells (Padmanabhan et al., 1983). Echternkamp et al. (1984) reported that pulsatile release of LH in Hereford cows was suppressed when circulating cortisol concentrations increased 10-fold to 20-fold, resulting from intense stress.

Hockett et al. (2000) have reported in frequent samples from dairy cattle (samples collected every 20 minutes for 8 hours) there were no differences in LH secretion in dexamethasone-treated animals compared with control animals. In our study, sampling was not frequent enough to evaluate the pulsatile release of LH in these females.

Daley et al. (2000) reported that wethers that received cortisol (90 : g/kg/hour) by continuous infusion for 48 hours exhibited no differences in circulating concentrations of LH or LH pulse frequency pattern compared with vehicle-treated controls. However, circulating LH and LH pulse frequency patterns were significantly reduced in wethers

receiving a combination cortisol and estradiol for 3 hours starting 21 hours after the cortisol infusion.

It has been reported that increased circulating glucocorticoids stimulate FSH synthesis and secretion in rats (Suter and Schwartz, 1985; Mahesh and Brann, 1998; Tohei and Kogo, 1999). However, in our study, the administration of exogenous dexamethasone decreased plasma FSH in early postpartum cows.

Our results are in agreement with those of Kawate et al. (1996), who demonstrated that treatment of cattle during late luteal phase with exogenous ACTH (3 mg/day) for 14 days resulted in decreased circulating FSH levels. These authors indicated that LH pulse frequency following ACTH treatment may interact with increased estradiol concentration to induce ovarian cyst formation in heifers. In ewes (Phillips and Clarke, 1990) there was no difference in circulating FSH levels after receiving high doses (2mg/day) of dexamethasone.

Pool et al. (1983) reported increased estrous cycle lengths for beef cattle stressed by penning. These researchers also reported that cattle that were penned and received 200 IU ACTH daily on day 17 to day 21 of the estrous cycle exhibited suppressed follicular growth and extended cycle length compared with cattle left in the field.

Since this experiment was conducted, reports have indicated (Ehrhardt et al., 2000; Chilliard et al., 2001) that the multi-species leptin kit may not accurately estimate extreme physiological leptin values in ruminants. In some cases, this kit either truncates or exaggerates leptin values (Chilliard et al., 2001), and often values were lower and unresponsive to changes in nutrition or adiposity (Ehrhardt et al., 2000). The standards of this kit are based on human leptin molecule rather than the ruminant leptin.

Conclusions

Our study suggests that dexamethasone administered at relatively high dosage levels on 4 consecutive days does not significantly alter the physiological levels of LH, FSH or progesterone in early postpartum beef cows. We have also shown that exogenous dexamethasone does not affect follicle development or ovary size in the early postpartum cow. However, reports have shown that dexamethasone is a potent regulator of the release of leptin in sheep and goats (Chilliard et al., 2001), horses

(Gentry et al., 2002; Cartmill et al., 2003b; Cartmill et al., 2005b), humans (Papasprou-Rao et al., 1997; Considine et al., 1997) and rats (Murakami et al., 1995; Larsson and Ahren, 1996; Papasprou-Rao et al., 1997; Reul et al., 1997; Considine et al., 1997; Reul et al., 1997; Chilliard et al., 2001).

Therefore, we believe that the hypothalamic-pituitary axis of early postpartum cows may be resistant to glucocorticoids or possibly that the gonadotropins are depleted from the pituitary following pregnancy. Also, it appears that adipocytes (site of leptin synthesis and release) in the early postpartum cow are either resistant to glucocorticoid stimulation, or the early postpartum cow utilizes pathways for leptin release that are different than has been reported in horses, sheep and humans.

CHAPTER IV

INFLUENCE OF AGE, BODY WEIGHT AND BODY CONDITION ON PLASMA LEPTIN CONCENTRATIONS IN CROSSBRED BEEF CATTLE

Introduction

Reproductive competence is one of the most important components in the overall management of a cow-calf operation. The ability to maintain a 365-day calving interval in a high percentage of the females influences the profitability in breeding operations (Lemenager and Martin, 1982). Poor nutrition has been shown to negatively impact production efficiency in both the prepartum cow (Whitman, 1975; Killen et al., 1989) and the postpartum cow (Wiltbank et al., 1962). Poor nutrition results in longer intervals from calving to first estrus and subsequently compromises the annual reproduction cycle (Rutter and Randel, 1984; Randel, 1990).

Researchers have long known that the hypothalamus is the primary brain center responsible for body weight regulation (Hetherington and Ranson, 1940). Research has shown that decreased body energy reserves result in decreased reproductive competence in mares (Voss and Pickett, 1974; van Niekerk and van Niekerk, 1997; Fitzgerald and McManus, 2000; Fitzgerald et al., 2002), beef cows (Houghton et al., 1990; Leers-Sucheta et al., 1994) and sheep (Mukasa-Mugerwa et al., 1993).

Most researchers agree that reproduction is compromised at the hypothalamic level through decreases in the release of LH (Shevah et al., 1975; Randel, 1990; Schillo, 1992; Maciel et al., 2001). Furthermore, research has shown that decreases in body fat stores are usually associated with decreased daily LH pulse frequency (Foster and Olster, 1985; Day et al., 1986; Richards et al., 1989).

Prolonged restriction of dietary energy delays onset of puberty, disrupts cyclicity in sexually mature animals and lengthens the postpartum anestrous period in domestic ruminants (Schillo et al., 1992). Researchers have shown that the suppression of daily LH pulse frequency is an important mechanism by which dietary energy restriction subsequently impairs reproductive function. It is generally understood that increased LH pulse frequency is needed for growth of ovarian pre-ovulatory follicles.

Day et al. (1986) have shown that in heifers fed a control diet (allowing spontaneous onset of puberty) or an energy deficient diet (delaying onset of puberty) during the prepubertal period resulted in puberty occurring at 428 days of age in control fed heifers, whereas, none of the delayed heifers reached puberty during the feeding period. These researchers went on to report that circulating LH concentrations and the frequency of LH pulses increased markedly during the 175-day feeding period in control heifers, while the LH concentrations increased less rapidly with no increase in pulse frequency was detected in the delayed heifers during the feeding period. It was also noted that LH pulse amplitude tended to be higher in control than delayed puberty heifers.

Richards et al. (1989) found when multiparous Hereford cows in good body condition were fed either a restricted diet (until luteal activity ceased) or a maintenance diet (body condition maintained) within 5 weeks the restricted fed cows had significantly reduced body weight and within 15 weeks exhibited significantly reduced body condition scores. It was noted that luteal activity ceased in 91% of the restricted cows by week 26 of feeding. Estrous cycles resumed 9 weeks after the diet of restricted cows was replaced with the maintenance diet. Concentrations of LH in serum samples obtained weekly were significantly lower in cows on the restricted diet compared with cows on the maintenance diet. It was also noted that LH pulse frequency was significantly lower when cows on the restricted diet were in a state of anestrus.

One of the earliest reports on animal body condition scoring was reported in sheep (Jefferies, 1961). Research has subsequently shown that, in cattle, body condition scoring was a good indicator of body energy reserves in animals (Wagner et al., 1988; Richards et al., 1989; Spitzer et al., 1995; Ciccioli et al., 2003). It has now been established that cattle scoring 5 to 7 on Wagner's Scoring System (Wagner et al., 1988) at calving will rebreed earlier the following breeding season (Richards et al., 1986; Selk et al., 1988; DeRouen et al., 1994; Morrison et al., 1999).

DeRouen et al. (1994) reported that body weights and body condition scores of crossbred beef cattle at calving were significantly higher for cows with higher body condition score at 90 days prepartum and for cattle fed high energy diets 90 days prepartum until parturition compared with cattle fed low energy diets from 90 days

prepartum until parturition. It was also noted that prepartum changes in body weight and body condition scores did not affect postpartum reproduction. However, these researchers concluded that increased body condition scores at calving increased pregnancy rates and reduced the postpartum interval.

Morrison et al. (1999) grouped crossbred beef cows by different body condition scores (4 to 5, 5 to 6, and ≥ 7) and managed these females to calve at a body condition score of 5 to 6. It was found that late gestation management had no effect on luteal activity at the start of the next breeding season and little effect on subsequent pregnancy rates. Also, mean pregnancy rates at 20, 40 and 60 days of the breeding season were 55%, 76% and 89%, respectively, for cows that had body condition scores of 4 and 5 at late gestation and 64%, 79% and 89%, respectively, for cows that had body condition scores of ≥ 7 , then managed to reach a body condition score of 5 to 6 at calving. It was found that mean pregnancy rates at 20, 40 and 60 days of the breeding season were 51%, 67% and 82%, respectively, for cows managed to maintain a body condition score of 5 to 6 from late gestation to calving and then managed to calve at a body condition score of 5 to 6.

Strauch et al. (2002) have reported that circulating leptin levels can influence the postpartum interval in Brahman cows, noting that females having a short postpartum interval (30 to 37 days) exhibited higher circulating leptin levels than did cows having a longer postpartum interval (78 to 132 days). Therefore, the objective of this experiment was to quantify circulating leptin levels based on female age, body weight and body condition in beef cattle.

Materials and Methods

Experimental Animals

At 60 days after calves were weaned (November) at the Idlewild Research Station, Clinton, Louisiana. A group of 63 beef females ($\frac{1}{4}$ Hereford, $\frac{1}{4}$ Brahman, $\frac{1}{2}$ Angus and Hereford) were selected for this study. All females were palpated for pregnancy in early fall and all open cows were removed from the group at weaning. The experimental groups included beef heifers (n = 15) that were <2 years of age, with a mean (\pm SEM) body weight of 227 ± 4.98 kg and a mean body condition score of 5.13 ± 0.13 , a group of cows ≥ 2 years of age and < 3 years of age (n = 18), with a mean body

weight of 299 ± 4.45 kg and a mean body condition score of 4.44 ± 0.12 , and a group of multiparous cows ($n = 30$) with a mean age of 6.7 ± 0.38 years, a mean body weight of 615 ± 20.17 kg and a mean body condition score of 5.9 ± 0.24 . All females were weighed and visually assigned a body condition score (body condition score; 1 = emaciated and 9 = obese) at the onset of this study. The overall means for all females by production groups used in the study are shown in Table 4.1.

Experimental Design

Experimental females were evaluated for circulating leptin levels by age and female production groups. For this study, females <2 years of age were classified as 'heifers' and females ≥ 2 years of age were classified as 'cows'. These females were assigned to the production groups and were blocked by age as heifers (<2 years of age), 2-year old cows (between 2 and 3 years of age), young mature cows (between 3 and 6 years of age) and older mature cows (older than 6 years of age). Females assigned a body condition score of <6 were classified as the 'lower' body condition group (low) and females that received a body condition score of ≥ 6 were classified as the 'higher' body condition group (high). All females were maintained on bahia pastures and during this study.

Sample Collection and Laboratory Analysis

At 60 days following calf weaning, a blood sample was collected from each female via jugular venipuncture into heparinized evacuated tubes and placed on ice (<2 hours) until centrifugation at $1,200 \times g$ for 15 minutes at 5°C . Plasma was harvested and stored at -20°C until assayed for plasma leptin concentrations (Cartmill et al., 2003a).

Statistical Analysis

Data were analyzed by the GLM procedures outlined in SAS (SAS Institute, Cary, NC). Least Squares means were determined and all possible comparisons among the means were computed by using a protected *t*-test. Linear and orthogonal comparisons of dependent variables on independent variables were also tested using the GLM procedure of SAS. Correlations were determined using the CORR procedure of SAS. Differences in mean body condition scores were determined using the Mann-

Table 4.1. Overall mean body weights and body condition scores for the different female production groups used in this experiment.

Age (Years)	n	Mean ¹	Range
<u>1 to <2 (Heifers):</u>	15		
Weight (kg)		227 ± 5.00	197 - 260
Body condition scores ²		5.1 ± 0.13	4 - 6
<u>2 to <3 (Cows):</u>	18		
Weight (kg)		299 ± 4.45	272 - 345
Body condition scores ²		4.4 ± 0.12	4 - 5
<u>4 to 6 (Cows):</u>	17		
Weight (kg)		611 ± 30.0	466 - 820
Body condition scores ²		6.1 ± 0.30	4 - 8
<u>7 to 11 (Cows):</u>	13		
Weight (kg)		612 ± 30.0	505 - 791
Body condition scores ²		5.6 ± 0.40	4 - 8

¹ Mean ± SEM.

² Body condition score (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988).

Whitney Rank Sum Test of Sigma Stat (Systat Software, Inc., San Jose, CA). An alpha level of 0.05 was used to determine statistical significance among mean values.

Results

All Females

In this study, plasma leptin levels were not correlated with female age (Correlation Coefficient = +0.21, $P = 0.09$) or body weight (Correlation Coefficient = +0.21, $P = 0.10$). However, plasma leptin concentrations were positively ($P = 0.0009$) correlated to body condition scores (Correlation Coefficient = +0.41) (Figure 4.1).

Plasma leptin concentrations were significantly different across female production groups ($P < 0.003$) (Table 4.2). Plasma leptin concentrations were higher ($P < 0.05$) in 1 year old heifers (8.9 ± 0.96 ng/ml) compared with 2 year old cows (6.0 ± 0.70 ng/ml), but heifers were not different from younger mature cows (8.0 ± 0.73 ng/ml) and older mature cows (10.5 ± 0.85 ng/ml). Also, mean plasma leptin concentrations were higher ($P < 0.05$) for older mature cows (10.5 ± 0.85 ng/ml) compared with 2 year old cows (6.0 ± 0.70 ng/ml) but were not different from younger mature cows (8.0 ± 0.73 ng/ml) or 1 year old heifers (8.9 ± 0.96 ng/ml). Mean plasma leptin levels of younger mature cows (8.0 ± 0.73) were not different from 1 year old heifers (8.9 ± 0.96 ng/ml), 2 year old cows (6.0 ± 0.70 ng/ml) or older mature cows (10.5 ± 0.85 ng/ml).

Mean plasma leptin concentrations were higher ($P < 0.05$) for females with a body condition score of 8 (11.29 ± 1.5 ng/ml) compared with females with body condition scores of 4 (7.30 ± 1.07) and 5 (7.91 ± 0.70), but was not different from females with a body condition score of 6 (8.80 ± 2.0) and 7 (10.22 ± 1.51) (Figure 4.2). Mean plasma leptin was not significantly different among females with body condition scores of 4 (7.30 ± 1.07), 5 (7.91 ± 0.70), 6 (8.80 ± 2.0) and 7 (10.22 ± 1.51). Also, mean plasma leptin was not different for females with body condition scores of 6 (8.80 ± 2.0), 7 (10.22 ± 1.51) and 8 (11.29 ± 1.5 ng/ml).

Overall mean plasma leptin concentrations were higher ($P = 0.01$) for females with a mean body condition score of ≥ 6 (10.1 ± 0.96) compared with females with a body condition score of < 6 (7.4 ± 0.65 ng/ml) (Figure 4.3).

Body condition scores were significantly different ($P < 0.0001$) for production groups. Body condition scores were lower ($P < 0.05$) in 2 year old cows (4.4 ± 0.13)

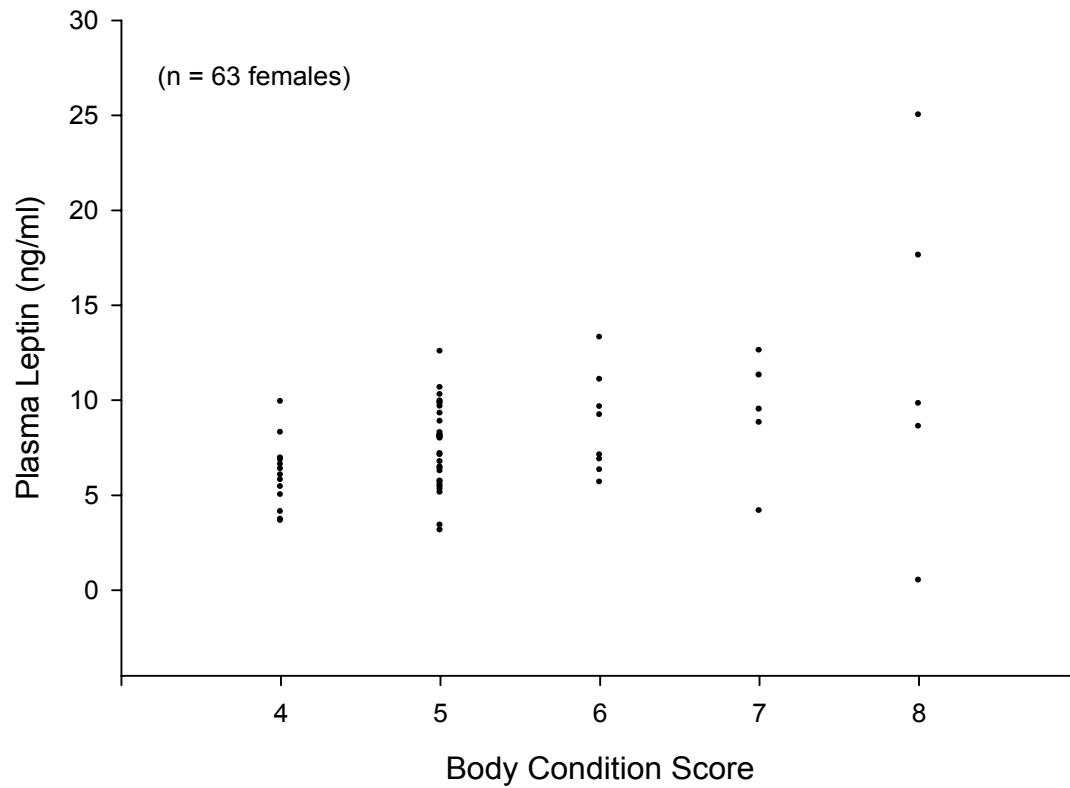


Figure 4.1. Correlation between plasma leptin concentrations and body condition scores for all beef heifers and cows. Correlation Coefficient = +0.41 (P = 0.0009). Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988).

Table 4.2. Overall mean plasma leptin concentrations by female production groups.

Age (years)	n	Mean plasma leptin (ng/ml) ¹
<u>Heifers:</u>		
1 to <2	15	8.9 ± 0.96 ^a
<u>Cows:</u>		
\$2 - <3	18	6.0 ± 0.70 ^b
4 - 6	17	8.0 ± 0.73 ^{a,b}
7 - 11	13	10.5 ± 0.85 ^a

¹ Mean ± SEM.

^{a,b} Numbers across production groups with different superscripts are significantly different (P<0.05).

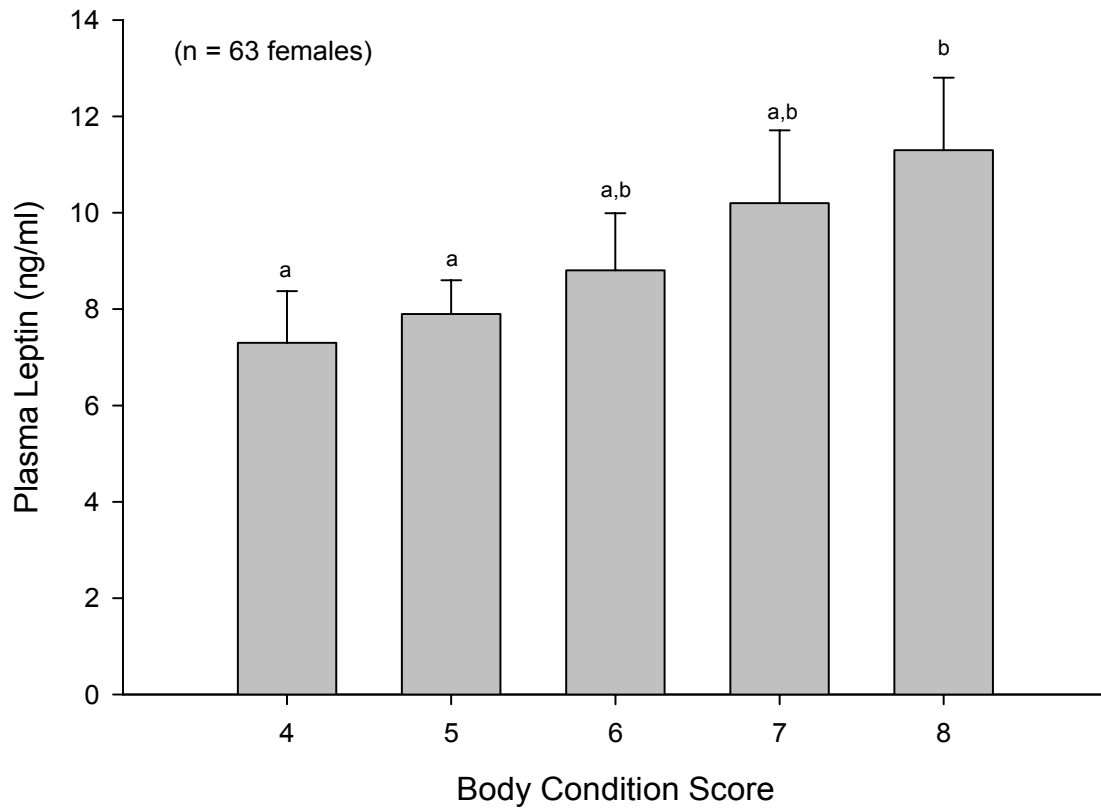


Figure 4.2. Overall mean plasma leptin concentrations of beef heifers and cows for different body condition scores. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 198^{a,b} Bars with different superscripts are significantly different (P<0.05).

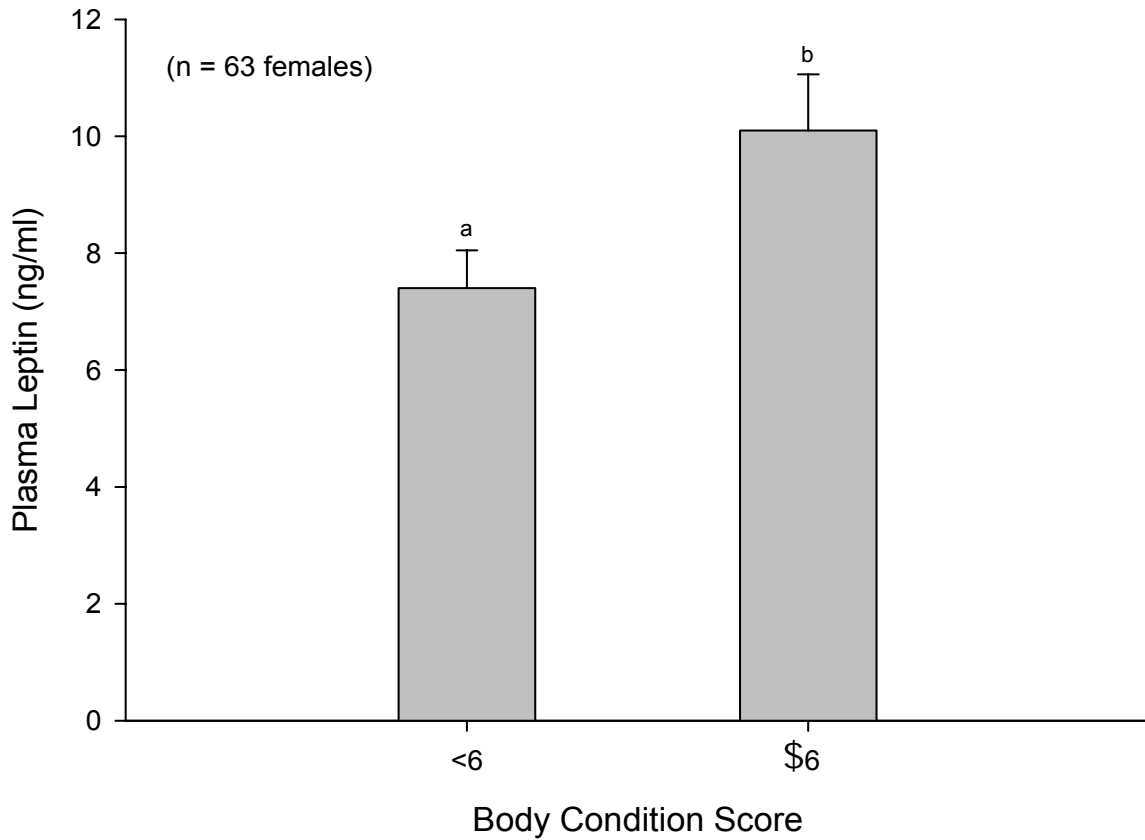


Figure 4.3. Overall mean plasma leptin concentrations of beef heifers and cows with a body condition score higher and lower than the suggested body condition score at calving. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988).^{a,b} Bars with different superscripts are significantly different (P=0.01).

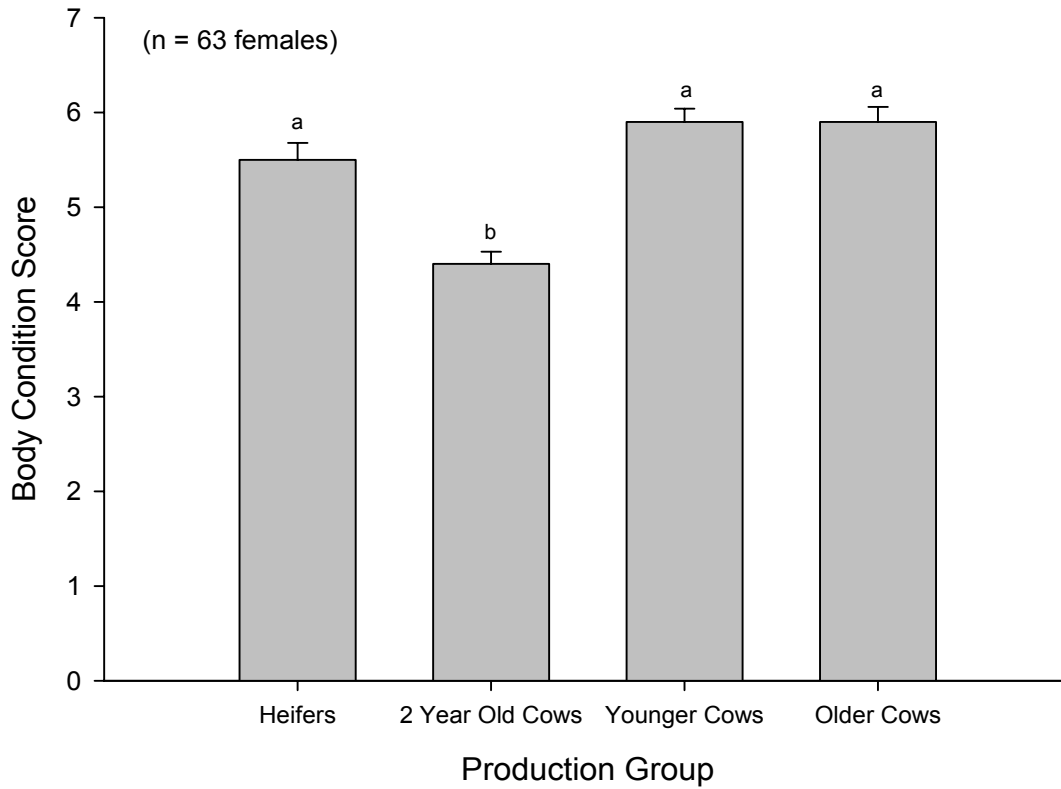


Figure 4.4. Overall mean body condition scores across female production groups. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988). ^{a,b} Bars with different superscripts are significant different ($P < 0.05$).

compared with heifers (5.5 ± 0.18), younger mature cows (5.9 ± 0.14) and older mature cows (5.9 ± 0.16) (Figure 4.4). There was not a significant difference in body condition scores among heifers (5.5 ± 0.18), younger mature cows (5.9 ± 0.14) and older mature cows (5.9 ± 0.16).

None of the 2 year old cows met the criteria of the higher body condition score group and were not included in the following analysis. Mean plasma leptin concentrations were higher ($P < 0.05$) in the higher body condition older cow group (14.3 ± 1.3 ng/ml) compared with the lower body condition group of heifers (9.1 ± 0.9), younger mature cows (8.0 ± 1.1) and older mature cows (6.6 ± 1.1) and the higher body condition score group of younger mature cows (7.8 ± 0.9) (Figure 4.5). There were no differences in mean plasma leptin concentrations between heifers (8.5 ± 1.7 ng/ml vs. 9.2 ± 0.9) and younger mature cows (7.8 ± 0.9 vs. 8.0 ± 1.1) for higher and lower body condition score groups, respectively.

The mean body condition score was greater ($P < 0.05$) for the higher body condition score group of older mature cows (7.2 ± 0.25) compared with the lower body condition score group of older mature cows (4.6 ± 0.20), the higher (6.0 ± 0.3) and lower (4.9 ± 0.16) body condition score groups of heifers and the lower body condition score group of younger mature cows (4.9 ± 0.21) (Figure 4.6). However, the mean body condition scores were not different between higher body condition score group of older mature cows (7.2 ± 0.25) compared with the higher body condition score group of younger mature cows (6.9 ± 0.18)

1 Year Old Heifers and 2 Year Old Cows

Mean plasma leptin concentrations were higher ($P = 0.0007$) for 1 year old heifers (8.9 ± 0.58 ng/ml) compared with 2 year old cows (5.8 ± 0.64 ng/ml) (Figure 4.7). Also, there was a significant negative correlation between plasma leptin concentrations and female age (Correlation Coefficient = -0.64 , $P < 0.0001$) (Figure 4.8).

Mean body condition score was higher ($P < 0.01$) for 1 year old heifers (5.1 ± 0.13) compared with 2 year old cows (4.4 ± 0.12) (Figure 4.9). Body condition score was positively correlated ($P = 0.048$) with female age (Correlation Coefficient = $+0.35$) in 1 year old heifers and 2 year old cows (Figure 4.10). There was no difference in mean plasma leptin concentrations in 1 year old heifers and 2 year old cows for body

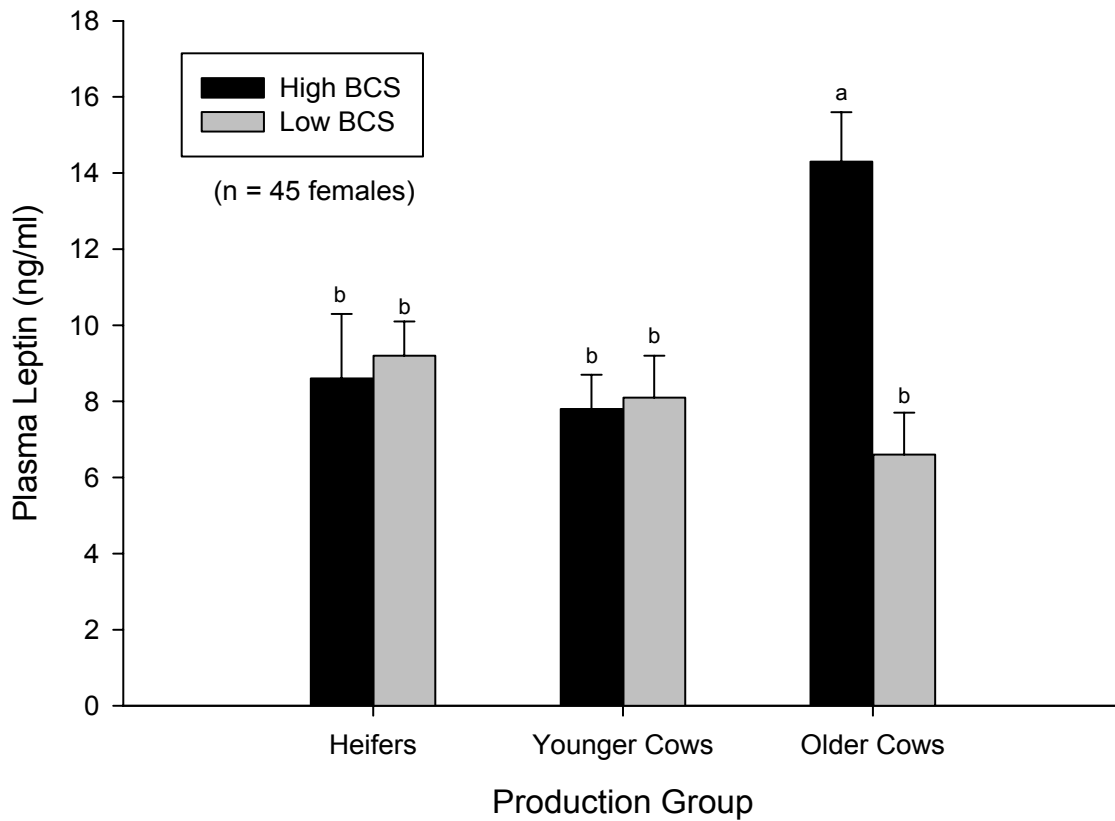


Figure 4.5. Mean plasma leptin levels across female production groups. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988). ^{a,b} Bars with different supercripts within and a production groups are significantly different ($P < 0.05$).

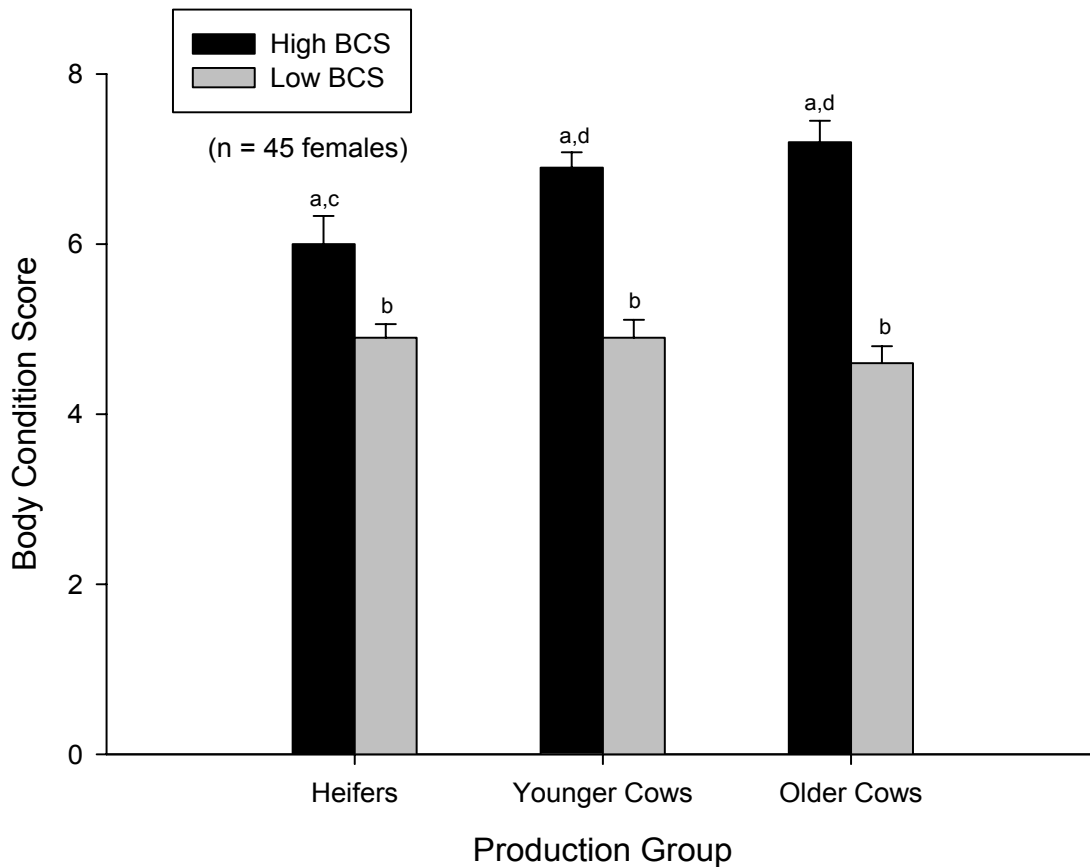


Figure 4.6. Mean body condition scores for female production groups. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988). ^{a,b}Bars with different superscripts within production groups are significantly different (P<0.05). ^{c,d}Bars with different superscripts across production groups are significantly different (P<0.05).

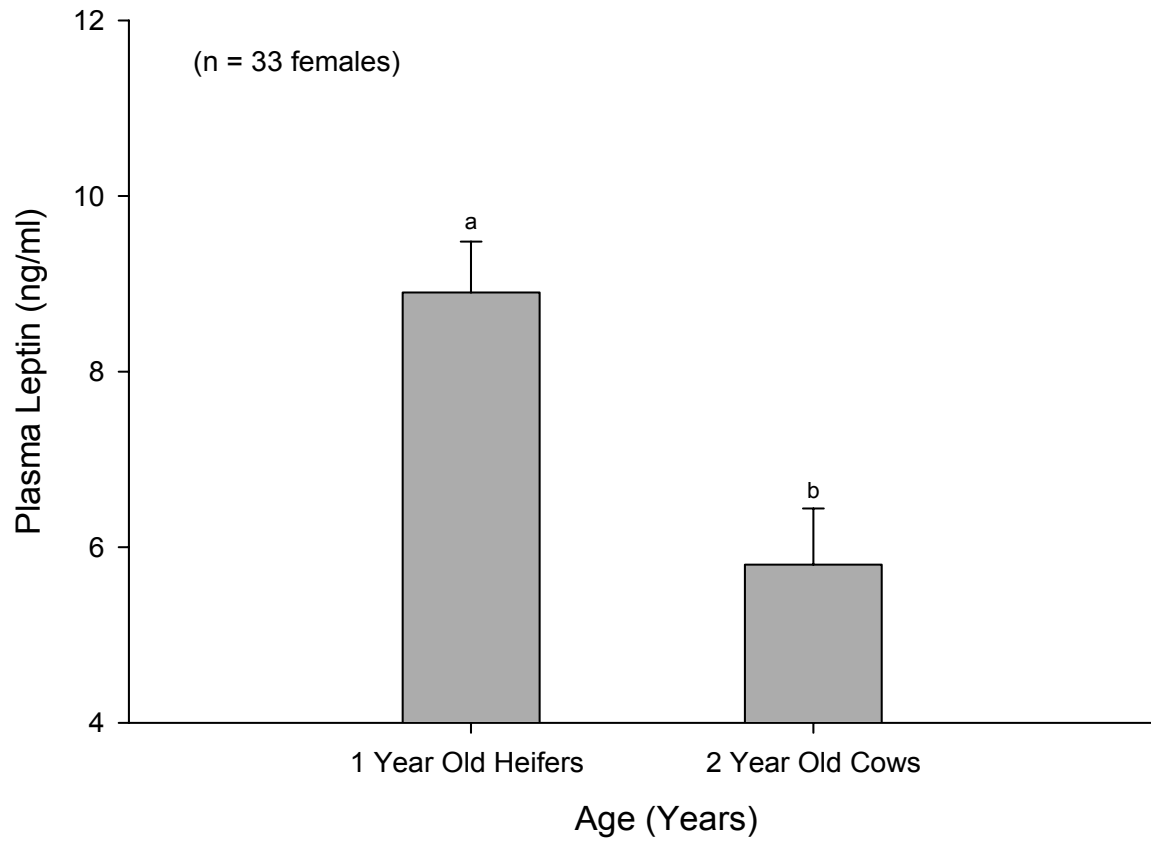


Figure 4.7. Mean plasma leptin concentrations of 1 year old heifers and 2 year old cows. ^{a,b} Bars with different superscripts are significantly different ($P = 0.0007$).

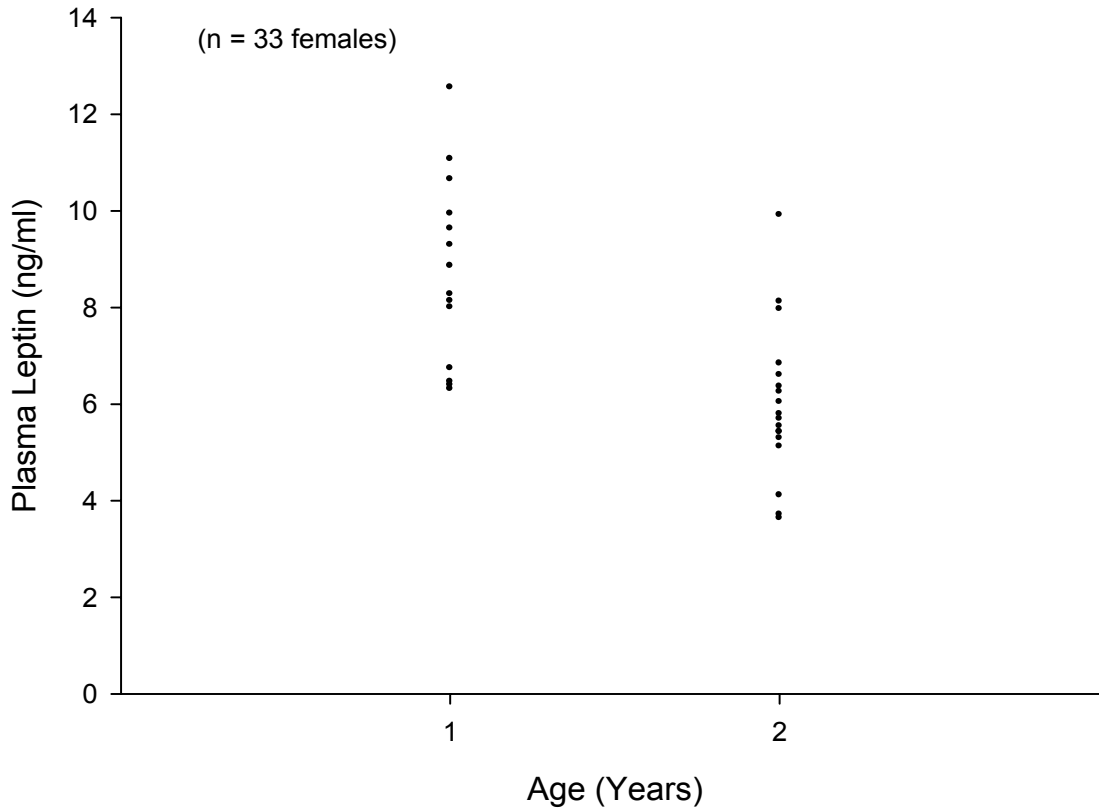


Figure 4.8. Correlation between plasma leptin concentrations and age for 1 year old heifers and 2 year old cows. Correlation Coefficient = -0.64 (P = 0.0001).

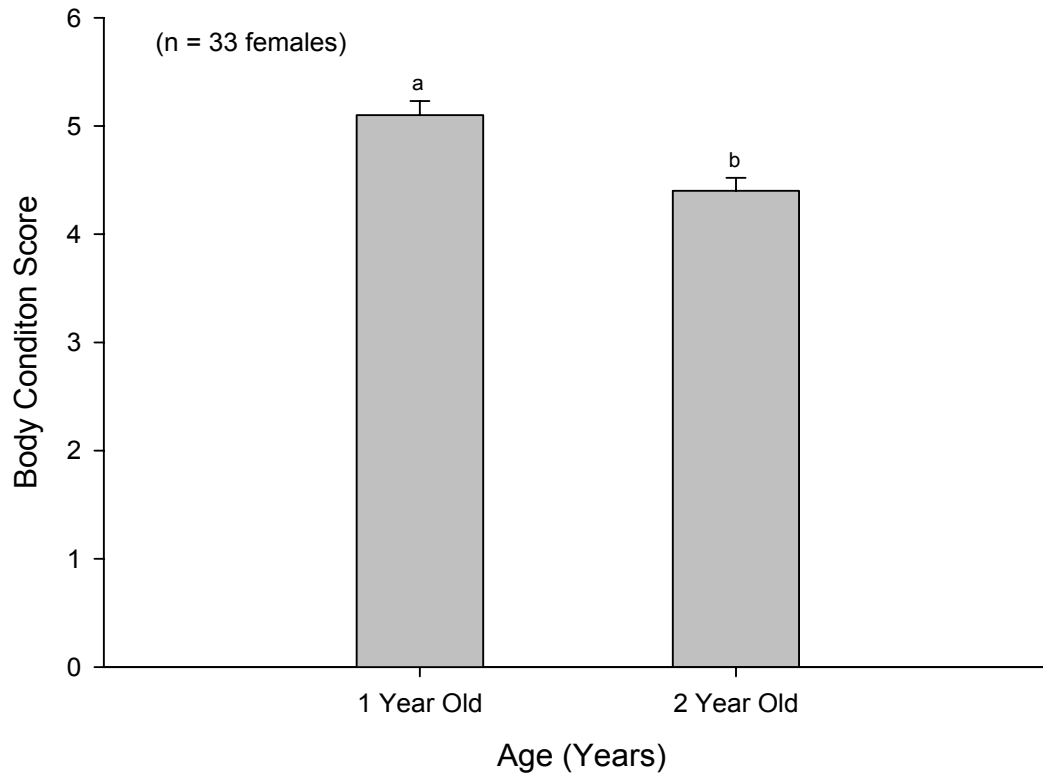


Figure 4.9. Mean body condition scores for 1 year old heifers and 2 year old cows. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988). ^{a,b}Bars with different superscripts a significantly different (P<0.01).

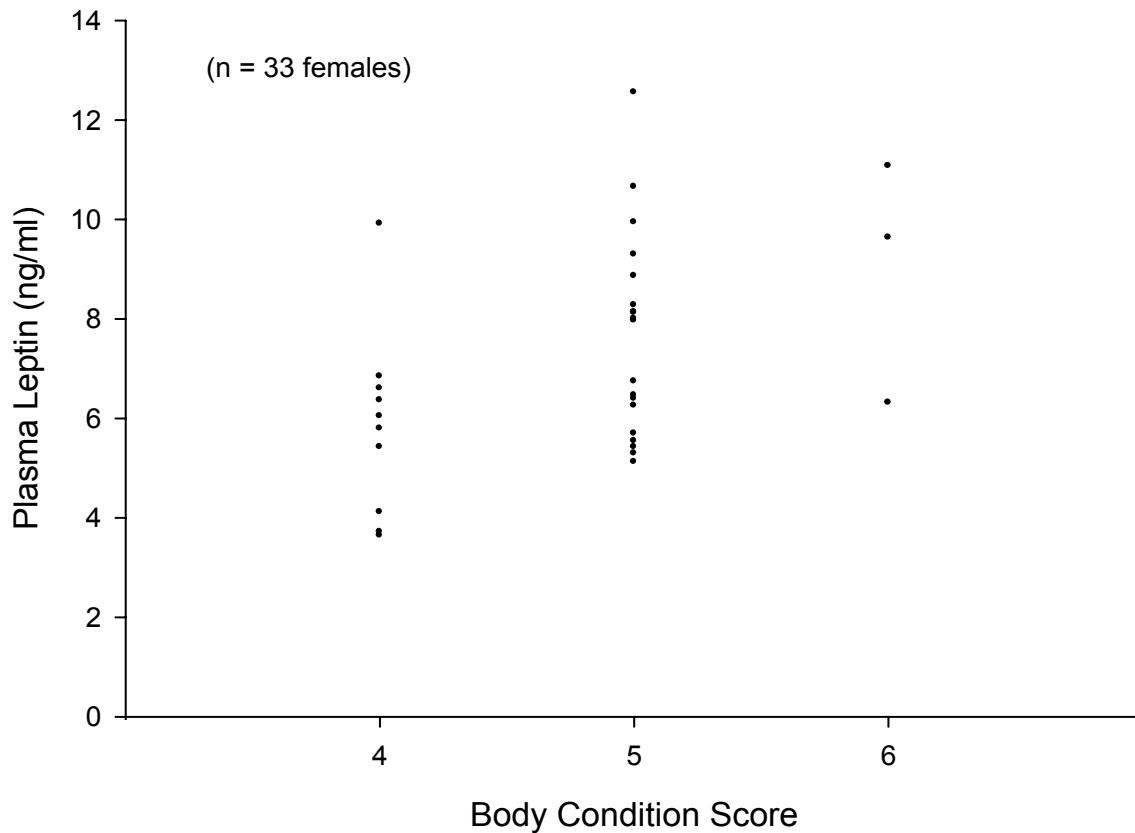


Figure 4.10. Correlation between plasma leptin concentrations and body condition scores for 1 year old heifers and 2 year old cows. Correlation Coefficient = +0.35, (P = 0.048). Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988).

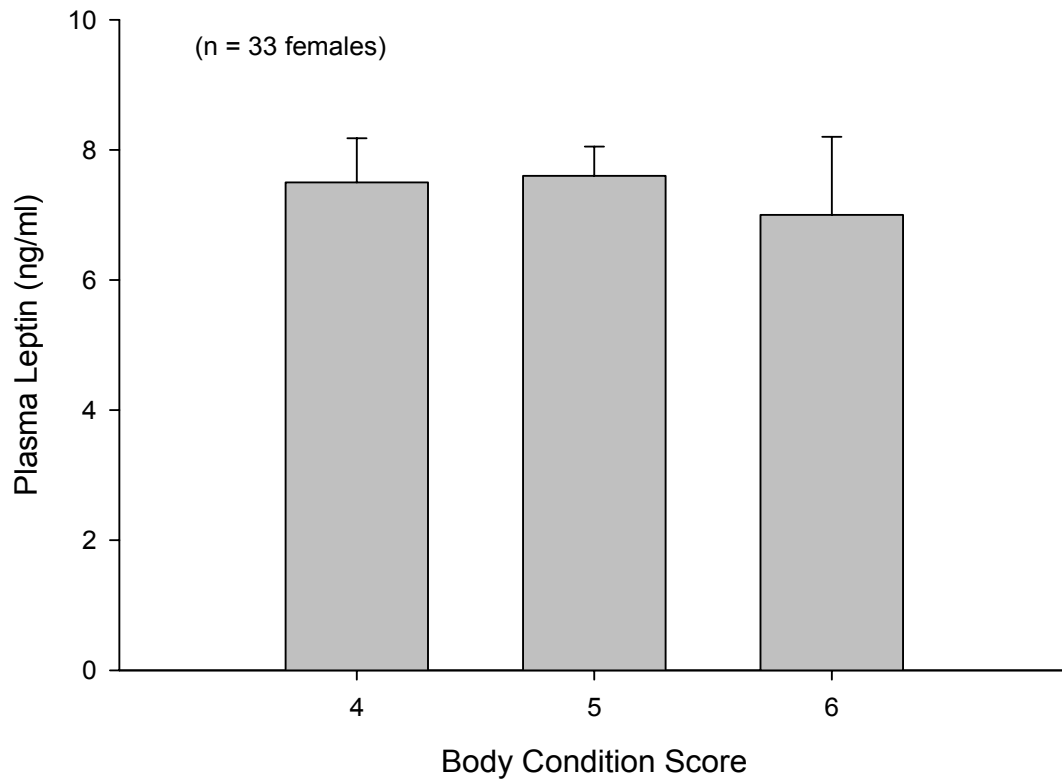


Figure 4.11. Overall mean plasma leptin concentrations for 1 year old heifers and 2 year old cows of different body condition scores. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988).

condition scores 4 (7.5 ± 0.68 ng/ml), 5 (7.6 ± 0.45 ng/ml) and 6 (7.0 ± 1.20 ng/ml) (Figure 4.11).

Young and Older Mature Cows (>3 Years Old)

Female age did not influence plasma leptin concentrations in 4 year old cows (9.8 ± 2.30 ng/ml), 5 year old cows (7.5 ± 1.40 ng/ml), 6 year old cows (6.0 ± 2.63), 7 year old cows (8.7 ± 2.39 ng/ml), 8 year old cows (13.0 ± 2.62 ng/ml), 9 year old cows (6.6 ± 2.72 ng/ml) and 11 year old cows (12.6 ± 2.64 ng/ml) (Figure 4.12).

Body condition score was positively ($P = 0.04$) correlated with circulating levels of leptin (Correlation Coefficient = $+0.38$) in cows >3 years old (Figure 4.13). Plasma leptin concentrations were not influenced by body condition scores for cows >3 years old with body condition scores of 4 (6.5 ± 2.52 ng/ml), 5 (8.0 ± 1.35 ng/ml), 6 (9.7 ± 2.09 ng/ml), 7 (10.4 ± 2.09 ng/ml) and 8 (11.4 ± 2.09 ng/ml) (Figure 4.14).

Discussion

In this study, we have shown that circulating levels of leptin were not influenced by female age or body weight in beef cattle. However, results did show that circulating leptin concentrations decreased as young females progressed into the calf production phase (from 1 year old heifers to 2 year old cows), while the plasma leptin concentrations in beef females, during the most productive phase were not significantly different from 4 to 6 years of age. Research has shown that prolonged energy restriction delays puberty, disrupts cyclicity in sexually mature animals and lengthens the postpartum anestrous period in domestic ruminants (Shillo et al., 1992). It has been reported that over 24% of beef cattle culled annually are culled as a result of pregnancy status (NAHMS, 1997). As females progress through production cycles and culling practices are initiated, the less productive females are removed from the herd leaving the most productive females. In the present study, heifers and cows were pregnancy checked in September, and open females were removed from the herd at calf weaning.

Houghton et al. (1990) reported that reproductive efficiency in mature Charolais x Angus crossbred cows (in moderate body condition) was influenced by level of energy supplementation prior to parturition. These researchers reported that cows receiving a lower energy diet had lighter calves at birth and at 105 days. Also, cattle on a higher energy diet had a significantly higher pregnancy rate and heavier calves at 105 days

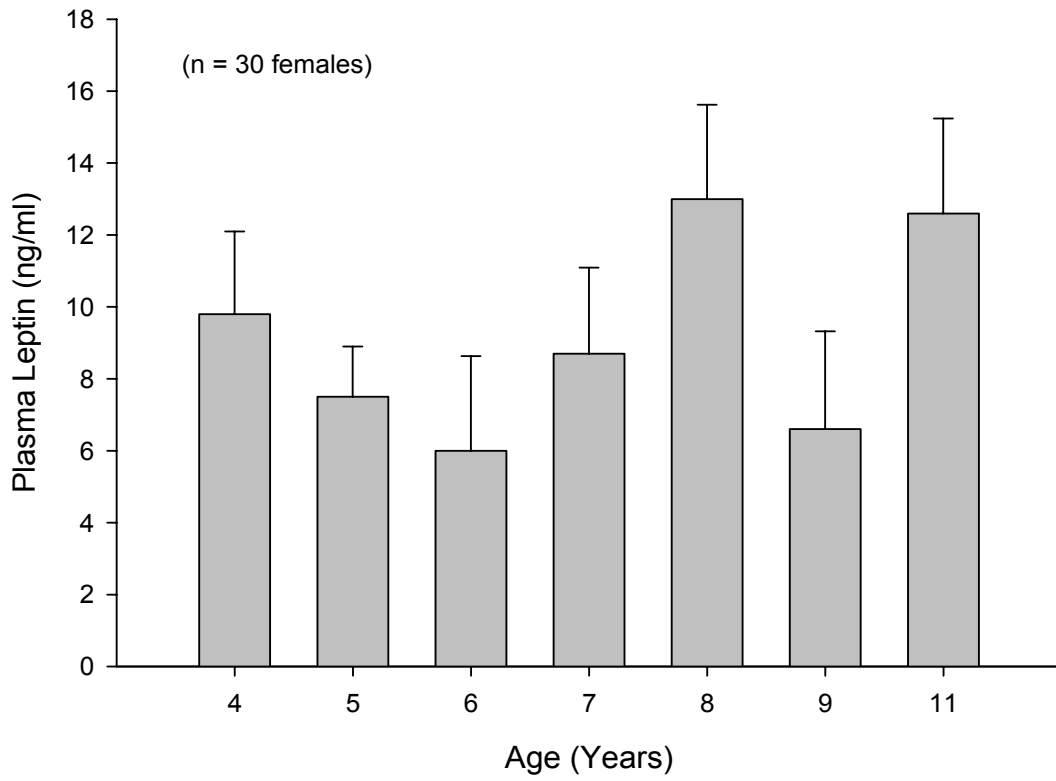


Figure 4.12. Mean plasma leptin concentrations of mature beef cows of different ages.

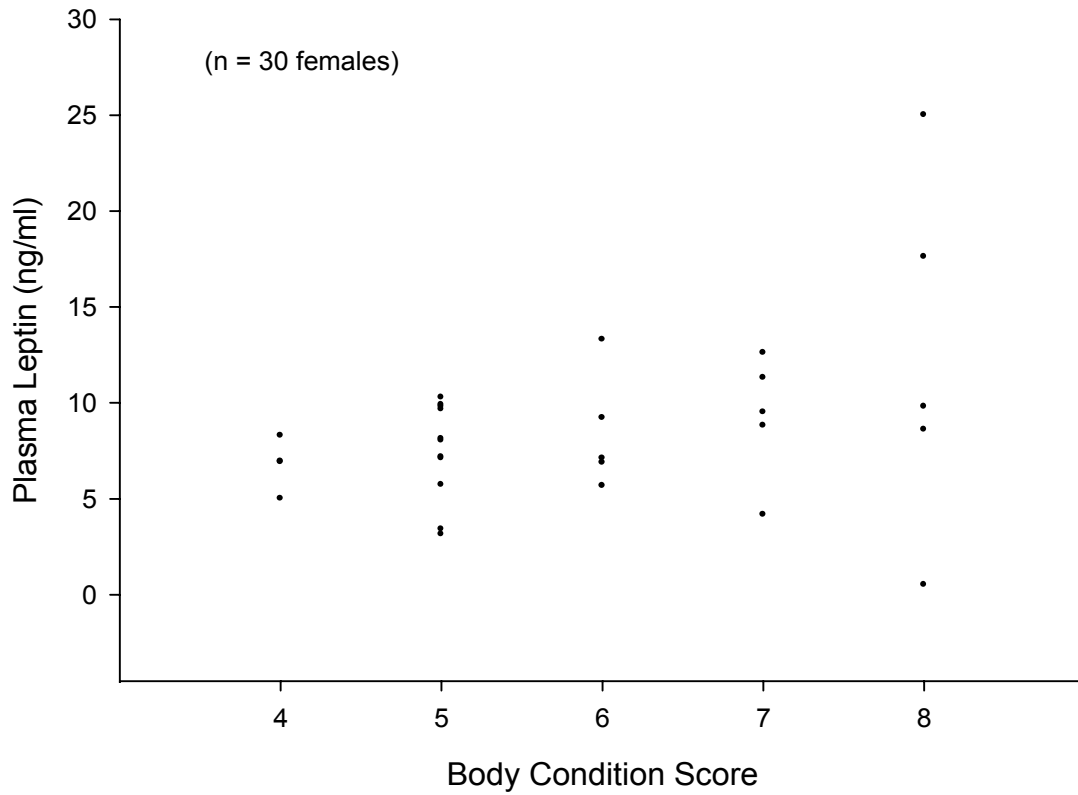


Figure 4.13. Correlation between plasma leptin concentrations and body condition scores of mature beef cows. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988). Correlation Coefficient = +0.38, (P = 0.04).

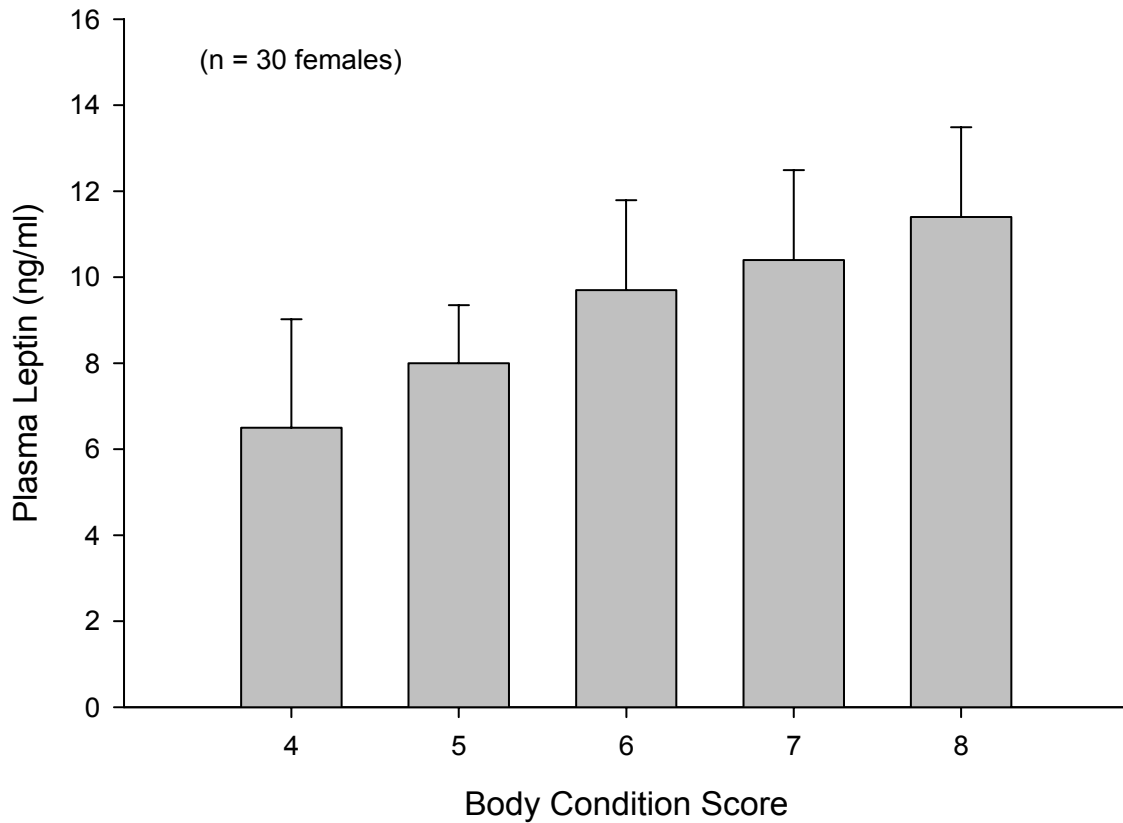


Figure 4.14. Mean plasma leptin concentrations of mature beef cows with different body condition scores. Body condition scores (1 = emaciated to 9 = obese) using the Wagner Body Condition Scoring System (Wagner et al., 1988).

postpartum, while the thin cows had a longer postpartum interval. However, it was noted that thin cows had a higher first service pregnancy rate than moderate to more fleshy cows. It was noted that higher pregnancy rates were found in cows approaching or maintaining average body condition from parturition to pregnancy than for cows that had been at a moderate body condition and were either increasing body condition or decreasing body condition at parturition.

Since ~25% of all animals culled are done so based solely on reproduction parameters, most females that lack the body condition following lactation to initiate reproduction are removed early as 2 year olds and 3 year olds, leaving older more fertile females in the breeding herd. Therefore, it is not surprising that circulating leptin concentrations are not different in mature cows ranging from 4 years to 6 years of age. There was also a positive correlation between plasma leptin concentrations and age in mature cows, ranging from 4 years to 6 years of age. It is proposed that because of culling practices in this herd, females that have lower leptin levels have been culled earlier and were not in the herd for sampling during this study.

Our results show that an increase in body condition score correlates with circulating leptin concentrations, and the correlation of plasma leptin and body condition scores shows that as body condition scores increase a rise in circulating leptin concentrations can be expected. These results are in agreement with other research, which reported an increase in the circulating levels of leptin as body fat mass increases in mice (Maffei et al., 1995), rats (Shimizu et al., 1997), sheep (Blache et al., 2000a) and humans (Campfield et al., 1996; Perry et al., 1997; Blum et al., 1997; Langendonk et al., 1998).

Spitzer et al. (1995) evaluated beef cattle allotted to management groups and fed to calve at either a body condition score of 4, 5 or 6 at parturition. These researchers noted that the higher the body condition score at calving the more cows exhibited estrus, resulting in greater pregnancy rates at 40 days and 60 days into the breeding season.

In sheep, Blache et al. (2000b) found that circulating leptin concentrations were highly correlated with back-fat thickness and to the ratio of back-fat thickness to live weight in female and castrated male sheep. The plasma leptin concentrations were

higher in female sheep than in castrated or intact male sheep. These researchers also noted that leptin concentrations in both plasma and cerebrospinal fluid increased within 5 days in male sheep fed a diet with a higher content of energy and protein, subsequently resulting in increased LH secretion. It was concluded that leptin production in sheep was correlated with the mass of adipose tissue and that the hormone passes from the circulation to the cerebrospinal fluid and then into the hypothalamic region.

Sansinanea et al. (2001) reported similar findings for circulating leptin levels in beef cattle. Polled Hereford heifers were fed in optimum conditions and either grazed, grazed and supplemented, or fed in a feedlot. The circulating leptin values were significantly lower in prefeeding compared with post-feeding intervals in all three management groups. It was concluded that these changes in blood leptin levels were associated with control of food intake in sheep.

In addition, Sansinanea et al. (2001) have also reported that when cattle were subjected to feed restriction, circulating leptin levels were significantly reduced in the feed-restricted group compared with the nonrestricted group. These researchers also noted that the poor energetic status of animals from the restricted group influenced the circulating FFA, β -OH-butyrate and protein concentrations (all serum energetic indicators). It was indicated that the energetic status of animals is correlated with the circulating leptin levels.

In our study, cows with body condition scores of ≥ 6 had higher plasma leptin concentrations when compared with cows with body condition scores of < 6 . These results support findings that beef females need to attain a body condition score of 5 to 7 at calving to successfully rebreed during the subsequent breeding season (Richards et al., 1986; Selk et al., 1988; DeRouen et al., 1994; Morrison et al., 1999). Perhaps, there is a critical level of leptin needed to trigger the release of LH. It is generally accepted that the functionality of the reproductive system is reduced in nutritionally restricted ruminants. It has been proposed that decreased circulating LH concentrations and decreased LH pulse frequency negatively impacts folliculogenesis (Richards et al., 1989).

Schillo (1992) has noted energy restriction effects on the onset of puberty and cyclicity in sexually mature animals and that energy restriction lengthens the postpartum anestrus period in domestic ruminants. This author proposed that energy restriction inhibits pulsatile secretion of LH by reducing LHRH secretion and that the ability of an animal to sustain a higher-frequency mode of pulsatile LH release is negatively impacted.

Prior studies have not fully characterized the mechanisms linking metabolic status to LHRH secretion but have shown that changes in body fat have been associated with changes in reproductive activity. Schillo (1992) proposed that it is possible that pulsatile LHRH release is regulated by specific metabolites and/or metabolic hormones that reflect nutritional status. He stated that our understanding of how the central nervous system transduces information relating to nutritional status into neuroendocrine signals that control reproduction in cattle and sheep is limited by a lack of information on the nature of neurons controlling LHRH release in these species.

In our study, 2 year old cows had lower circulating leptin concentrations when compared with 1 year old heifers, along with a significant negative correlation between circulating leptin concentration and female age. This finding was not surprising. Even though blood samples were taken 60 days after the calves had been weaned, more 2 year old cows were suckled the previous calving season compared with 1 year old heifers. Thus, the 2 year old cows had not yet regained body adiposity that had been lost during lactation. While these 2 year old cows were still in a body growth phase, available energy was partitioned into growth and lactation during the previous months, whereas, the 1 year old heifers did not have the burden of lactation and all energy was utilized for growth and development. This hypothesis is further supported by higher body condition scores in the 1 year old heifers compared with the 2 year old cows, and a positive correlation between plasma leptin concentration and body condition scores.

In the present study, plasma leptin concentrations in mature cows (4 to 11 years old) were not influenced by body condition score, nor was there a correlation between female age and plasma leptin concentrations. However, there was a significant positive correlation between body condition scores and circulating leptin concentrations. Using practical culling practices the nonproductive cattle with lower body condition scores

were removed from the herd by the time the females reached 4 years of age. Therefore, increased leptin should positively influence reproductive competence in the remaining females.

In older cows, nutritional efficiency is primarily affected by the status of their teeth, and 39% of all cattle culled are culled based on female age or bad teeth (NAHMS, 1992). Because most of the nonproductive females are removed from the herd by 3 years of age the vast majority of the remaining females remain efficient and productive until their ability to digest forage becomes diminished due to tooth loss. Therefore, fewer differences in circulating leptin concentrations would be expected in mature beef cows.

Conclusions

We have shown that body condition scores are significantly correlated to circulating levels of plasma leptin concentrations in both heifers and cows. Also, we have shown that 2 year old cows have significantly lower circulating leptin concentrations than 1 year old heifers, which also were indicated by significantly lower body condition scores in these 2 year old cows. It appears that younger cattle are more sensitive to changes in nutrition due to energy requirements when compared with more mature older cows.

We believe that culling practices may have indirectly select for females with levels of leptin high enough to meet the critical demand for the release of LH and subsequent ovarian function. It appears that earlier studies reporting increased pregnancy rates for females with at least a body condition score of 5 or greater at calving may have been indirectly selecting for those females that have met a critical level of circulating level of plasma leptin to ensure proper follicular development and rebreeding. More studies are needed to determine the role leptin plays in reproduction of beef cattle.

CHAPTER V

CIRCULATING LEPTIN LEVELS IN LACTATING AND NONLACTATING BEEF FEMALES FOLLOWING ARTIFICIAL INSEMINATION

Introduction

Zhang et al. (1994) first described the leptin molecule as having a structure similar to that of cytokines with 146 amino acids and was produced by the obesity gene (*ob*). These researchers went on to state that when leptin was administered to *ob/ob* mice (mice that lack the leptin gene) the obesity and infertility in these animals was reversed. These researchers subsequently concluded that this new hormone was the 'factor' needed to substantiate the 'Lipostatic' theory.

Studies have repeatedly shown that inadequate nutrition resulting in diminished body condition results in a decrease in reproductive competence at the hypothalamic level (Mantzoros and Moschos, 1998; Sansinanea et al., 2001). Lalman et al. (1997) reported a negative correlation between dietary energy and length of the postpartum interval in first calving beef heifers. As dietary energy increased in thin postpartum beef heifers the postpartum interval decreased. Furthermore, these researchers also noted that body condition scores were positively associated with circulating IGF-1 and insulin concentrations, suggesting that both hormones were indicators of the amount of energy storage in the animal.

Strauch et al. (2002) was first to report that circulating leptin may be associated with the postpartum interval in beef cows, noting that females having a short postpartum interval (30 to 37 days) had higher circulating leptin levels than did beef cows having a longer postpartum interval (78 to 132 days).

Scientists are still unsure what role(s) leptin plays in reproduction. However, Antczak and Van Blerdom (1997) have reported that the leptin receptor was expressed in oocytes and early mouse and human embryos. Subsequently, immunofluorescence was used to detect the mouse leptin receptor in oocytes, fertilized ova and cleavage stage embryos (Antczak and Van Blerkom, 1999). Furthermore, the long form of the leptin receptor has been isolated in both theca and granulosa cells of the ovary in the human (Karlsson et al., 1997; Cioffi et al., 1997).

Gonzalez et al. (2000) reported that leptin mRNA is present in human epithelial endometrium cells (EEC), secretory endometrium, granulosa cells (GC) and placental tissue. Immunohistological techniques were used to isolate and stain leptin receptor in the human luminal and glandular endometrial epithelium. It was also reported that both the leptin protein and the leptin receptor were detected in human EEC and embryos. It was noted that both EEC and embryos were a site for leptin production and secretion. Furthermore, leptin was detected in conditioned media from cultured human blastocysts and EEC. Hatched blastocysts secreted higher levels of leptin compared with arrested blastocyst-stage embryos, blastocysts co-cultured with EEC or EEC cultured alone. This study suggested the early stage embryo could produce and secrete leptin.

In addition, Gonzalez et al. (2000) suggested that the expression of leptin receptors and leptin protein within the secretory endometrium and cultured EEC might be interpreted as a molecular communication mechanism between the human embryo and uterine environment, which may be an important factor in the process of implantation. These researchers proposed that because leptin is produced and secreted locally by epithelial cells, leptin may act in an autocrine or a paracrine manner to regulate biological functions that mediate endometrial receptivity to facilitate the implantation process in the mouse and human.

Kitawaki et al. (2000) used Northern and Western blot analyses to isolate leptin receptors in human endometrial tissues, and noted that there were different patterns of expression for leptin receptors. Leptin receptor expression was found to gradually increase in women during the early proliferative phase towards the time of ovulation, then peak during the early secretory phase and subsequently decline during the middle and late secretory phase. Contrary to previous reports on sites of leptin production, Kitawaki et al. (2000) failed to detect any leptin protein in human endometrial tissue.

It has become evident that leptin may play a role in the early pregnancy of females but to what extent is still unknown (Gonzalez et al., 2000). However, the presence of leptin and its receptor in the uterus and early embryo leads to the hypothesis that leptin may be an important factor in maintaining pregnancy. Thus, the objective of this experiment was to determine how pregnancy and lactation influence

circulating leptin concentrations in crossbred beef heifers and early postpartum beef cows.

Materials and Methods

Experimental Animals and Estrus Synchronization Protocol

Data were collected on a group of 63 crossbred beef females ($\frac{1}{4}$ Brahman, $\frac{1}{4}$ Hereford, $\frac{1}{2}$ Angus), with a mean body weight of 372 kg, a mean age of 1.97 years and a mean body condition score of 4.8 (1 = emaciated and 9 = obese) (as described by Wagner et al., 1988) for 56 days following artificial insemination. All females were allowed free choice access to dormant Alicia bermudagrass pasture in 4 hectare paddocks and were supplemented with good quality hay as needed at the Idlewild Research Station in Clinton, Louisiana.

All females were synchronized during the spring breeding season (April) for fixed timed AI using a 7 day CIDR + GnRH protocol with AI occurring 48 hours after CIDR removal. All females were administered a 100 : g intramuscular injection (im) of GnRH (Cystorellin, Merial, Athens, GA) and received a bovine Easi-Breed CIDR (Pfizer Animal Health, New York, NY) on day 0. On day 7 all females received a 25 mg injection (im) of dinoprost tromethamine (Lutalyse, Pfizer Animal Health, New York, NY), and given a 100 : g injection (im) of GnRH when the CIDR was removed on day 7. Then 48 hours following the CIDR removal all females were brought to the animal handling facility for fixed timed AI. All females were inseminated with frozen-thawed semen from the same Angus sire by the same AI technician. Location of the semen deposition and presence or absence of mucus at AI were also recorded.

Experimental Design

All females were weighed and body condition scored at initial processing before estrus synchronization (April). In this study, females were blocked and assigned to production groups based on age and lactation status (Table 5.1). Females that were <2 years of age were classified as 'heifers' and females that were \geq 2 years of age were considered 'cows'. Females were grouped for circulating blood leptin concentration comparisons as 'lactating' or 'nonlactating' females for this study. Female production groups were used for monitoring circulating leptin levels in this study and were females that became pregnant from artificial insemination, females that became pregnant from

Table 5.1. Experimental design for the different female production groups used in this experiment.

Lactating and nonlactating females (cows)	n
AI	17
Clean-up bulls	8
Open	17
Lactating	34
Nonlactating	8
Nonlactating females (heifers and cows)	n
AI	10
Clean-up bulls	10
Open	9

clean-up bulls (Clean-up bull) and females that did not become pregnant following AI and clean-up bulls (Open) after pregnancy determination.

Starting 14 days post-AI, every 7 days (at 0800) all females were weighed, assigned a body condition score and a blood sample collected. Calves were allowed to nurse cows at all times except during blood collection. Females were placed with clean-up bulls on day 7 post-AI for a 60-day breeding season. Females were ultrasonographically (Aloka, Wallingford, CT) pregnancy checked at 30 days post-AI and again at 105 days post-AI.

Blood Collection

Starting 14 days following AI and every 7 days thereafter, until day 56 post-AI, all females were brought to the cattle handling facility for blood sample collection. Blood samples were collected via jugular venipuncture and collected into heparinized vacutainers and placed on ice until centrifugation at 1,200 x g for 15 minutes at 5°C. Plasma was harvested and stored at -15°C until assayed for plasma leptin concentrations (Cartmill et al., 2003a).

Statistical Analyses

Data were analyzed by the MIXED procedure of SAS (SAS Institute, Cary, NC). Least significant differences for means were determined and comparisons among the means were reported with a significance level of 0.05. Two statistical models were developed to identify variation in plasma leptin concentrations across age classes, body condition scores and lactation status of the crossbred beef females.

Because none of the heifers were lactating, they were not included in the model with lactating cows. Therefore, 2 year old and 3 year old lactating and nonlactating cow values were used in Model 1 and heifers, 2 year old and 3 year old nonlactating cows were used in Model 2. Class variables in Model 1 included animal, female production group, sampling day, age, lactation status and pregnancy status. Independent variables included in Model 2 were animal, female production group, sampling day, age and pregnancy status. Body condition score (BCS) and plasma leptin concentrations were used as dependent variables in both models.

Results

Lactating and Nonlactating Beef Cows

Overall there were no significant differences in mean plasma leptin concentrations among 2 year old and 3 year old cows in the AI production group ($1.17 \pm$

0.32 ng/ml), the clean-up bulls production group (1.18 ± 0.39 ng/ml) and the open production group (2.21 ± 0.36 ng/m) for 56 days starting 14 days following AI (Figure 5.1).

Mean plasma leptin concentrations were higher ($P = 0.013$) for nonlactating cows (2.07 ± 0.37 ng/ml) compared with lactating cows (0.97 ± 0.18 ng/ml) (Figure 5.2). Age did not affect plasma leptin concentrations with 2 year old cows exhibiting similar leptin values (1.4 ± 0.31 ng/ml) compared with 3 year old cows (1.7 ± 0.28 ng/ml).

There was a sample day effect ($P < 0.0001$), with mean plasma leptin being higher ($P < 0.05$) on sample day 35 (2.07 ± 0.24 ng/ml), day 42 (1.48 ± 0.21 ng/ml), day 49 (1.43 ± 0.21 ng/ml) and day 56 (2.12 ± 0.35 ng/ml) than on sample day 14 (1.07 ± 0.17 ng/ml) and day 21 (1.12 ± 0.16 ng/ml) (Figure 5.3).

Mean plasma leptin was significantly higher ($P < 0.05$) in nonlactating cows on day 35 (3.06 ± 0.43 ng/ml) and day 56 (2.95 ± 0.63 ng/ml) compared with lactating cows on day 35 (1.08 ± 0.21 ng/ml) and day 56 (1.28 ± 0.31 ng/ml) (Figure 5.4).

There was a sample day by production group interaction ($P < 0.0001$) in this data set. The change in plasma leptin concentrations in open (1.07 ng/ml) and clean-up bulls (0.80 ng/ml) treatment groups from day 28 to day 35 were greater than the AI production group (0.28 ng/ml) (Figure 5.5).

There was a lactation by production group interaction ($P = 0.02$). The difference in mean plasma leptin concentrations between lactating and nonlactating cows were greater in the open production group (2.77 ng/ml) and clean-up bull production group (0.61 ng/ml) compared with the AI production group (-0.08 ng/ml) (Figure 5.6).

There was a sample day x production group interaction for lactating cows ($P = 0.001$). Changes in blood levels of leptin for lactating cows from sample day 21 to sample day 28 were greater for both the clean-up bull production group (0.51 ng/ml) and the open production group (0.33 ng/ml) compared with the AI production group (0.17 ng/ml) (Figure 5.7).

Body condition scores were significantly higher ($P < 0.05$) on sample day 14 (5.01 ± 0.12), day 21 (4.89 ± 0.09), day 28 (5.02 ± 0.12) and day 56 (5.25 ± 0.10) compared with day 35 (4.64 ± 0.11) and day 49 (4.51 ± 0.12) (Figure 5.8). Also, body condition scores were higher ($P < 0.05$) on sample day 14 (5.01 ± 0.12), day 28 (5.02 ± 0.12) and

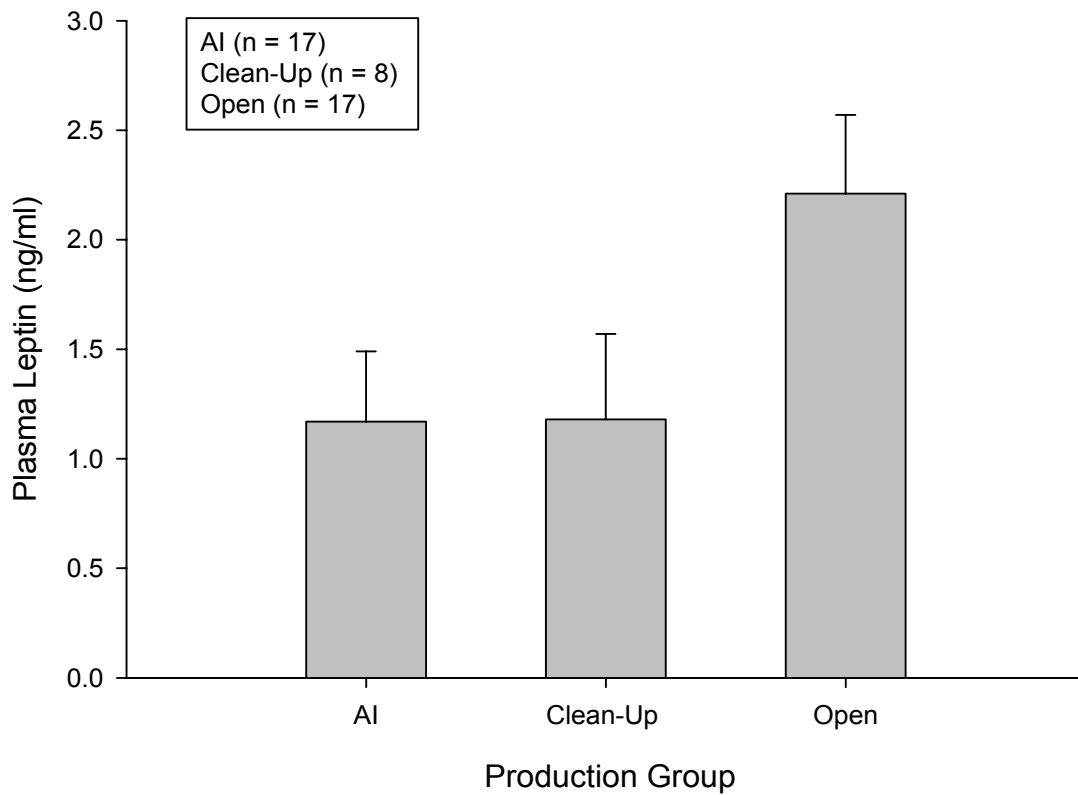


Figure 5.1. Overall mean plasma leptin concentrations of 2 year old and 3 year old lactating and nonlactating crossbred postpartum beef cows either pregnant from artificial insemination (AI), clean-up bulls or remaining open following the breeding season.

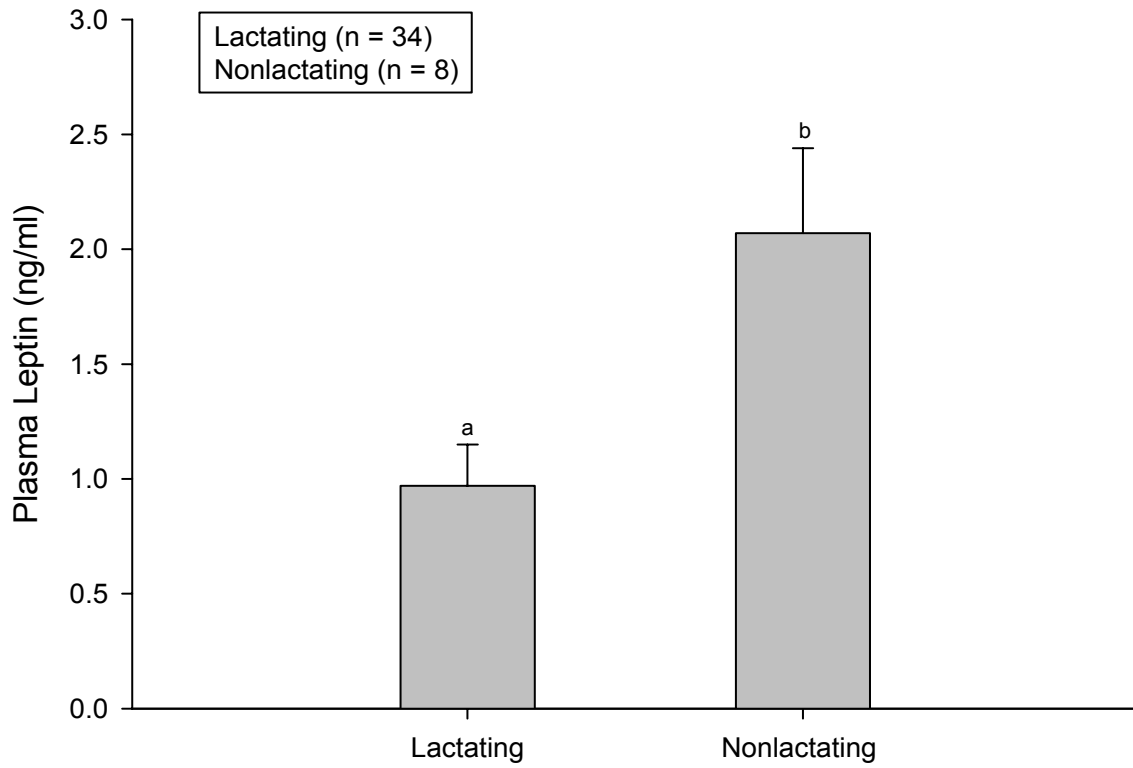


Figure 5.2. Overall mean plasma leptin concentrations of 2 year old and 3 year old lactating and nonlactating crossbred postpartum beef cows. ^{a,b}Bars with different superscripts are significantly different (P = 0.013).

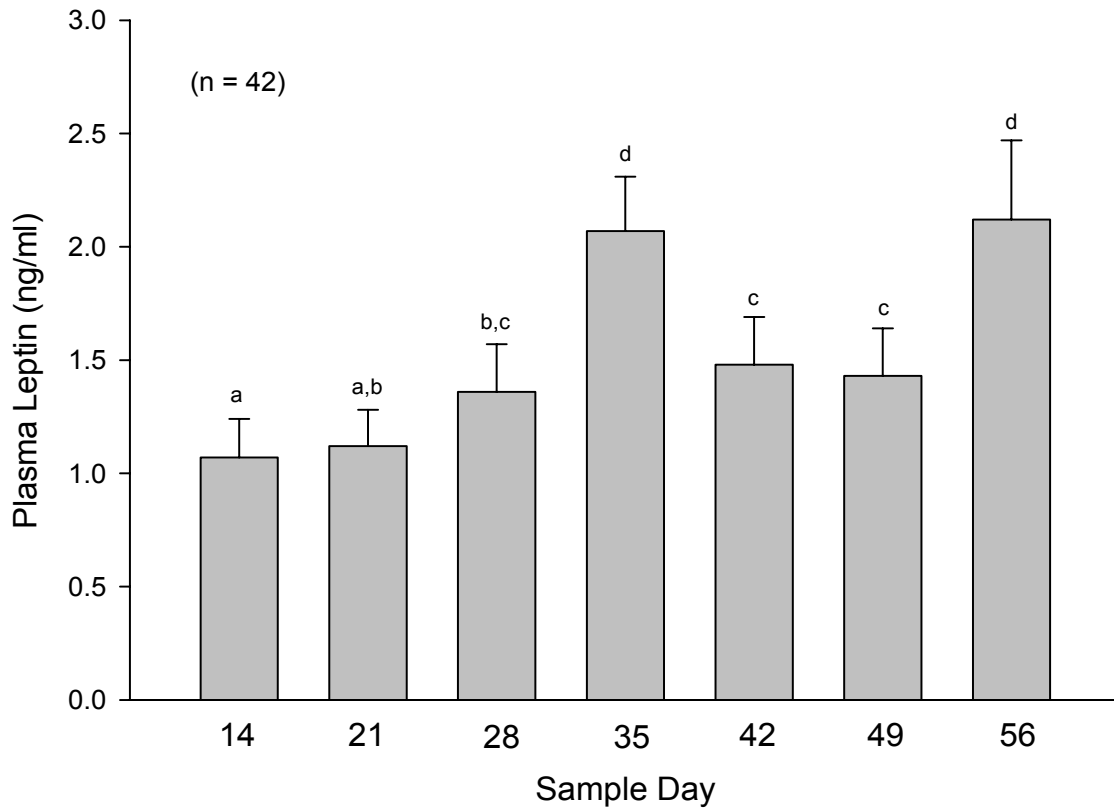


Figure 5.3. Weekly mean plasma leptin concentrations in 2 year old and 3 year old lactating and nonlactating crossbred postpartum beef cows across sample days. ^{a,b,c,d} Bars with different superscripts are significantly different ($P < 0.05$).

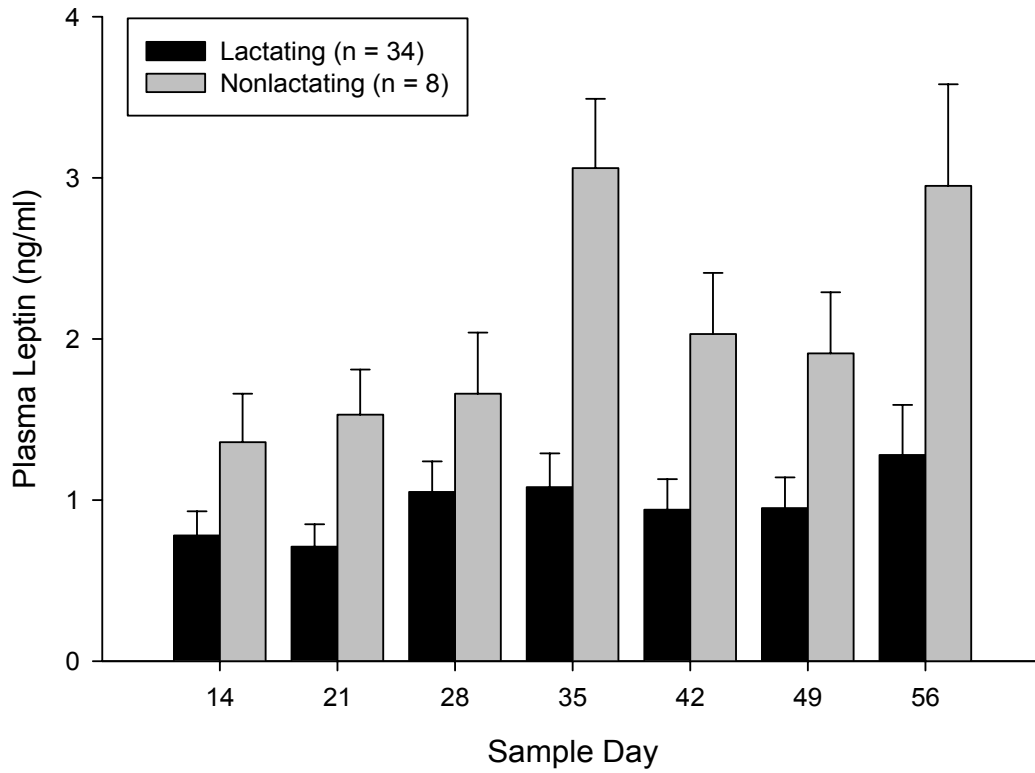


Figure 5.4. Weekly mean plasma leptin concentrations for 2 year old and 3 year old lactating and nonlactating crossbred postpartum beef cows. There was a sample day x lactation interaction in the analysis ($P < 0.0001$).

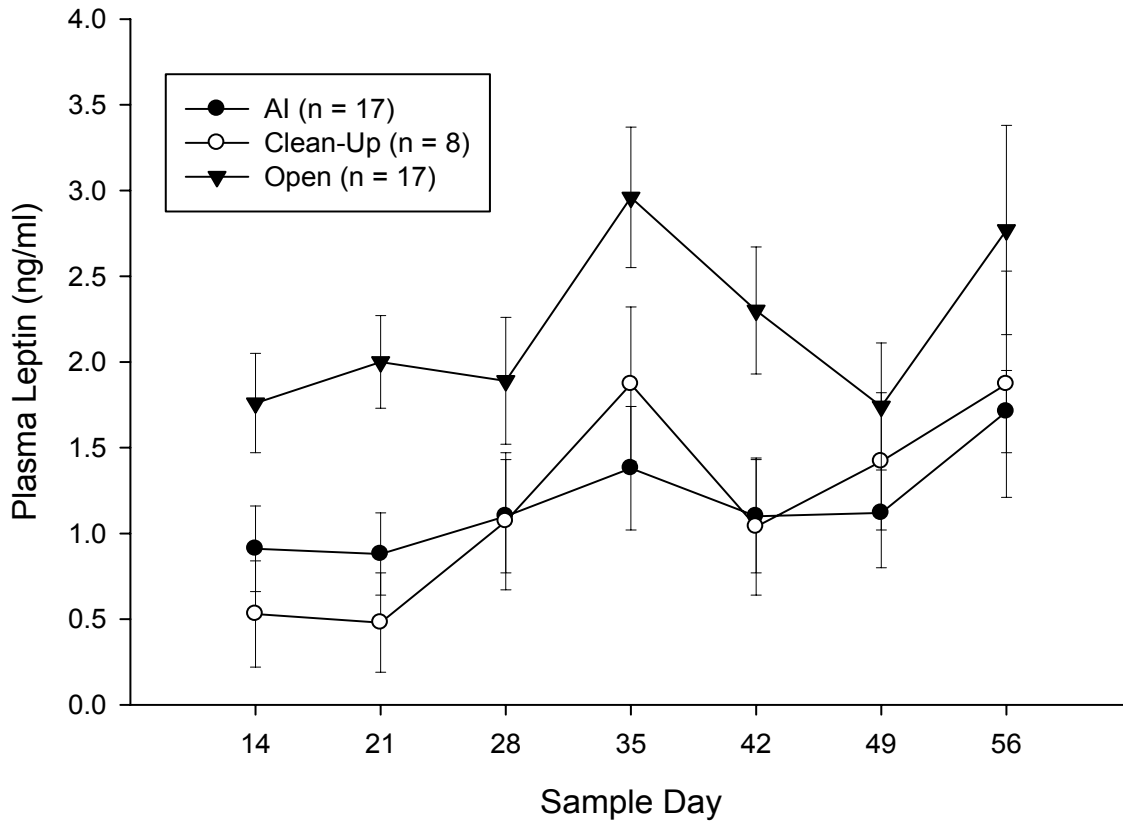


Figure 5.5. Weekly mean plasma leptin concentrations in 2 year old and 3 year old lactating and nonlactating crossbred postpartum beef cows. There was a sample day x production group interaction in the analysis ($P < 0.0001$).

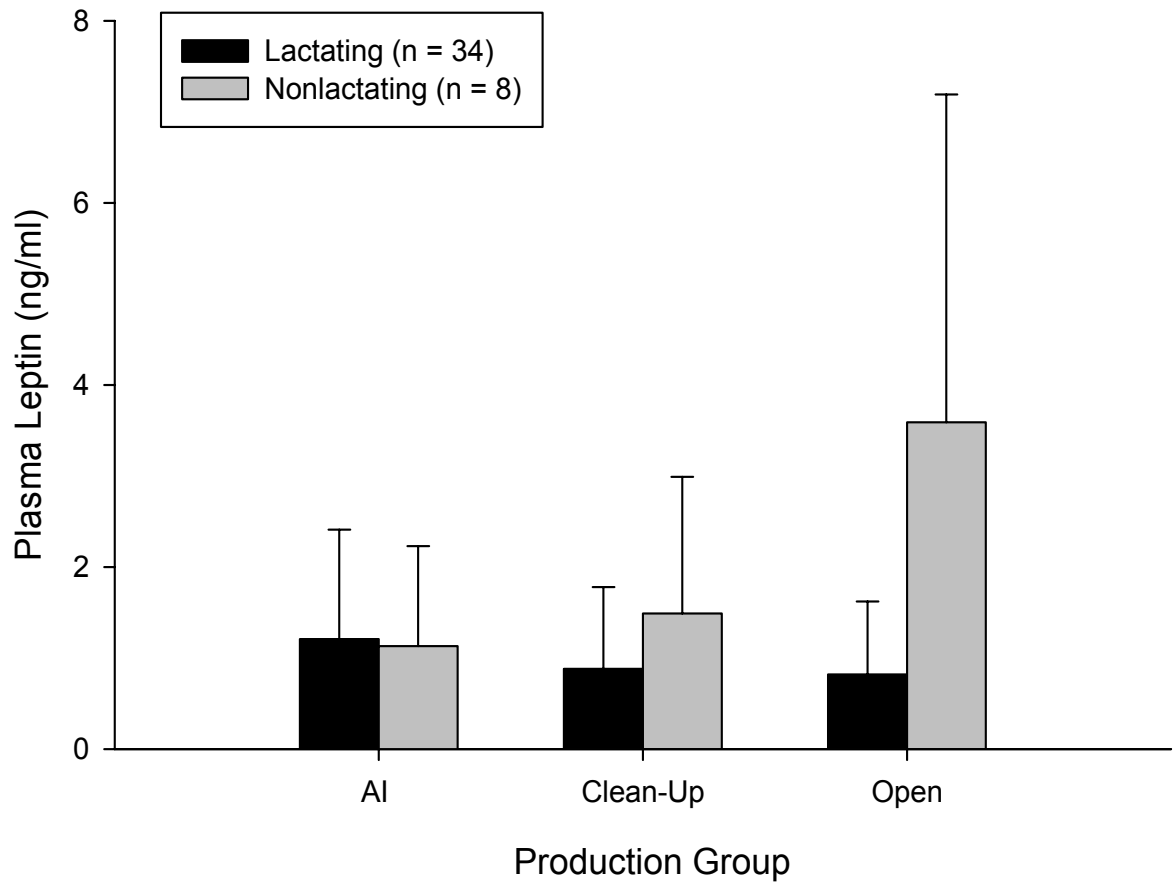


Figure 5.6. Overall mean plasma leptin concentrations in 2 year old and 3 year old lactating and nonlactating postpartum crossbred beef cows for different production groups. There was a lactation x production group interaction in the analysis ($P = 0.02$).

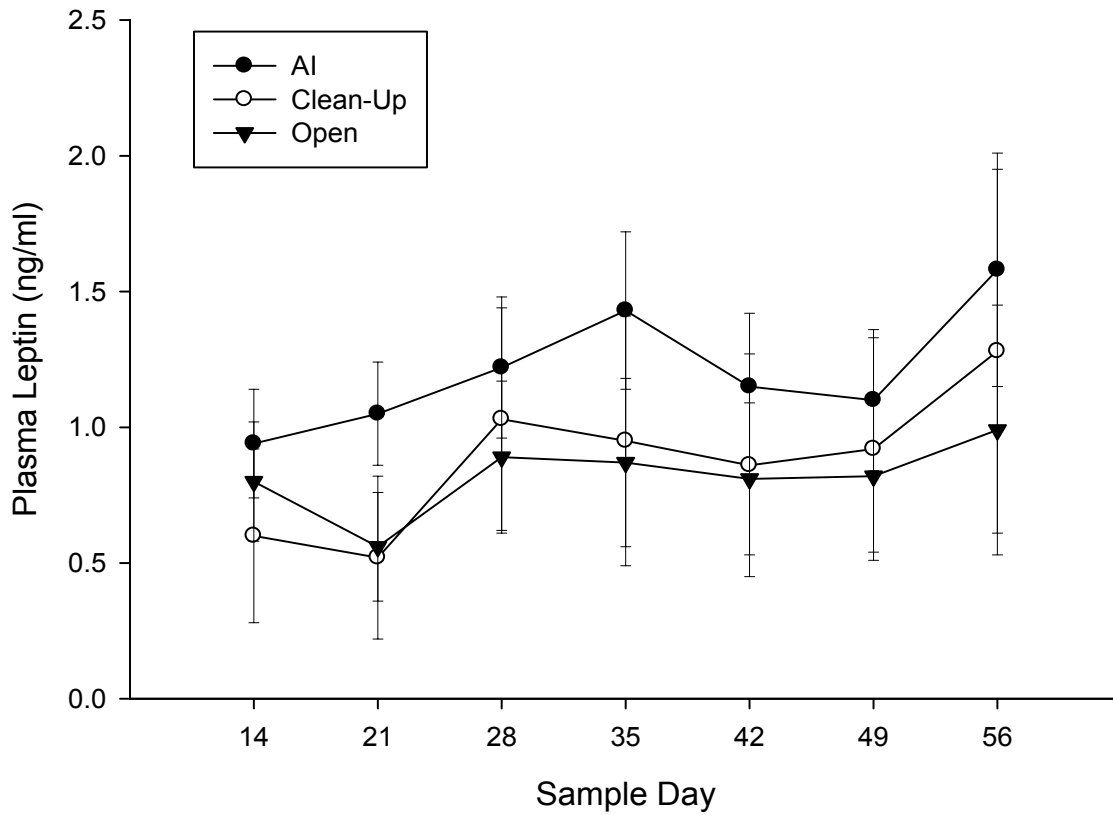


Figure 5.7. Weekly mean plasma leptin concentrations for 2 year old and 3 year old lactating crossbred postpartum beef cows across sample days. Day 14 would be equivalent to day 14 of gestation for the AI production group. There was a sample day x production group interaction in the analysis ($P = 0.001$).

day 56 (5.25 ± 0.10) compared with sample day 35 (4.64 ± 0.11), day 42 (4.67 ± 0.12) and day 49 (4.51 ± 0.12).

Body condition scores were lower ($P = 0.0002$) for nonlactating cows (4.5 ± 0.07) compared with lactating cows (5.2 ± 0.15) (data not shown). There was an interaction ($P = 0.003$) for lactation by production group. Differences in the mean body condition score for the open production group were greater (1.6) than differences in both the AI (0.09) and clean-up bull production groups (0.5) (Figure 5.9).

Nonlactating Heifers and Cows

There were no statistical differences among mean plasma leptin concentrations for heifers (1.4 ± 0.19 ng/ml), nonlactating 2 year old cows (2.1 ± 0.52 ng/ml) and nonlactating 3 year old cows (2.1 ± 0.46 ng/ml). Mean plasma leptin concentrations were significantly different ($P < 0.0001$) by sample day and were higher ($P < 0.05$) on sample day 35 (2.5 ± 0.25 ng/ml) and day 56 (2.4 ± 0.32 ng/ml) compared with sample day 14 (1.4 ± 0.32 ng/ml), day 21 (1.5 ± 0.32 ng/ml), day 28 (1.6 ± 0.28 ng/ml), day 42 (1.7 ± 0.23 ng/ml) and day 49 (1.6 ± 0.21 ng/ml) (Figure 5.10).

Mean plasma leptin concentrations were higher ($P = 0.04$) in the open production group (2.8 ± 0.43 ng/ml) compared with the AI production group (1.3 ± 0.37 ng/ml) and the clean-up bull production group (1.4 ± 0.42 ng/ml) (Figure 5.11). There was a sample day by production group interaction ($P < 0.0001$). Differences in mean plasma leptin concentrations from sample day 28 to day 35 were greater in both the AI production group (1.11 ng/ml) and the open production group (1.35 ng/ml) compared with mean plasma leptin concentrations in the clean-up bull production group (0.22 ng/ml) (Figure 5.12).

There was a sample day effect ($P < 0.0001$) with body condition scores being higher ($P < 0.05$) on sample day 14 (5.4 ± 0.12), day 28 (5.4 ± 0.17) and day 56 (5.6 ± 0.14) compared with sample day 35 (5.0 ± 0.14), day 42 (5.0 ± 0.19) and day 49 (4.9 ± 0.10). Also, body condition scores were higher ($P < 0.05$) on sample day 21 (5.3 ± 0.13) compared with day 35 (5.0 ± 0.14) and day 49 (4.9 ± 0.10) (Figure 5.13). Body condition scores were higher ($P = 0.013$) in the open production group (5.7 ± 0.18) compared with both the AI production group (4.9 ± 0.15) and clean-up bull production group (5.1 ± 0.17) (Figure 5.14).

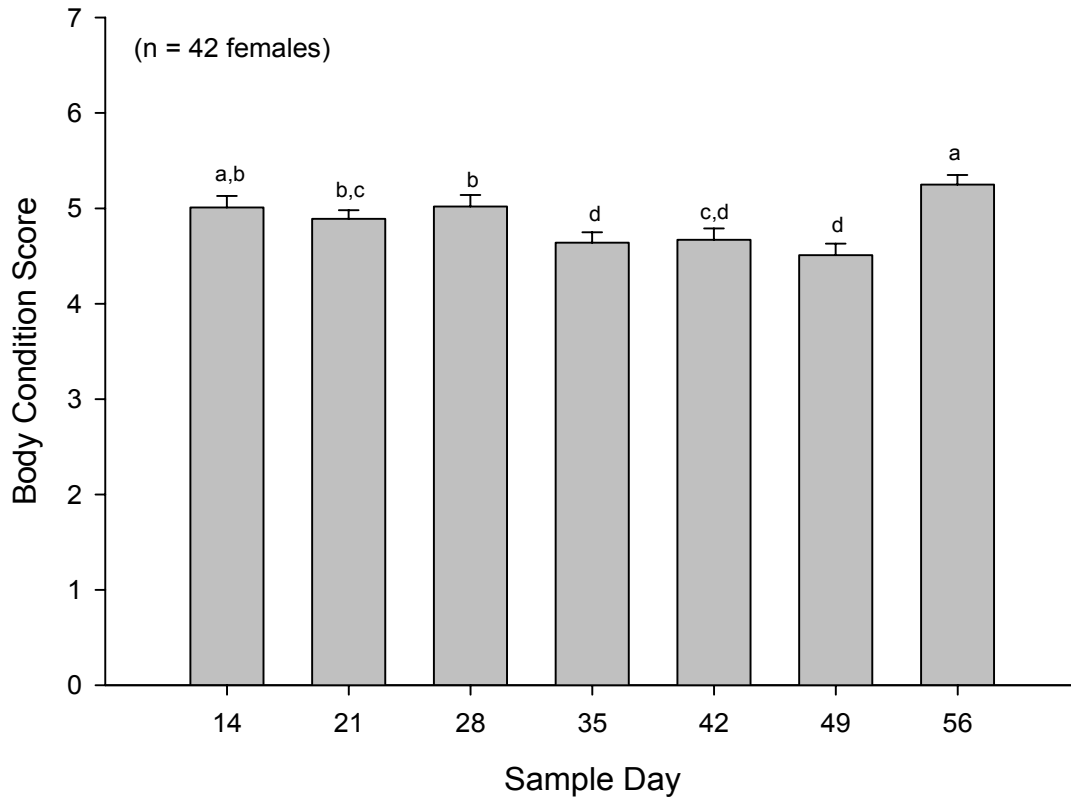


Figure 5.8. Weekly mean body condition scores of 2 year old and 3 year old lactating and nonlactating crossbred postpartum beef cows. ^{a,b,c,d} Bars with different superscripts are significantly different ($P < 0.05$)

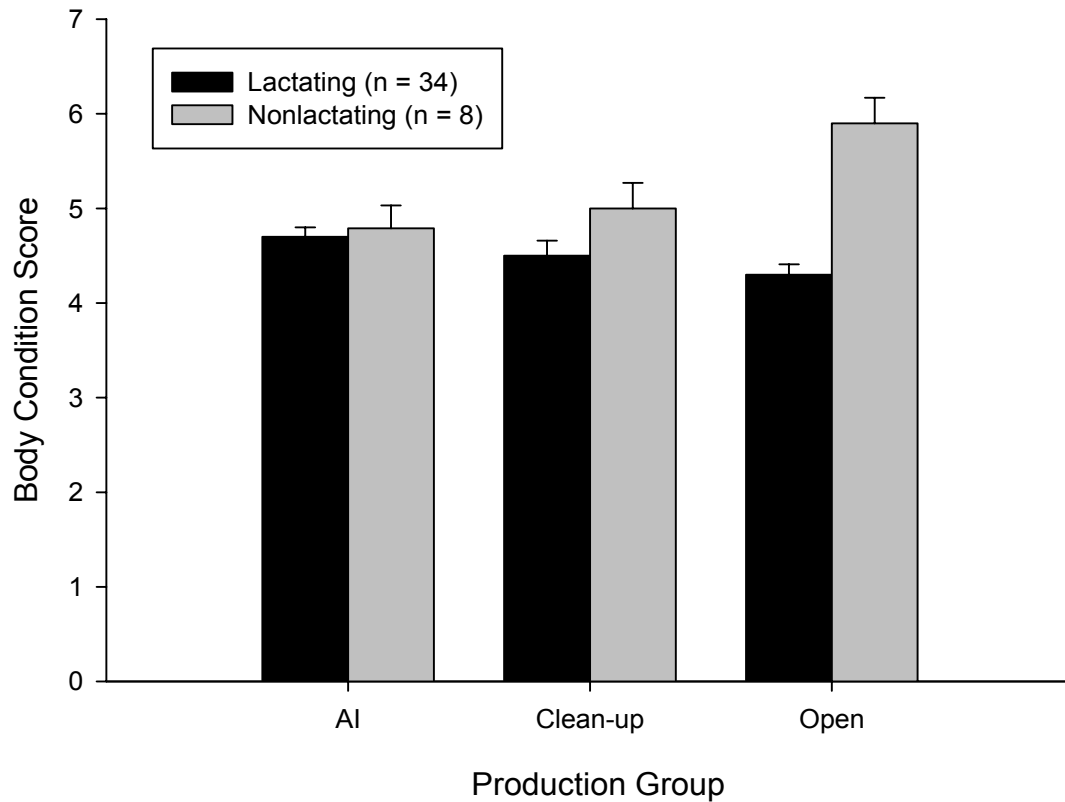


Figure 5.9. Overall mean body condition scores of 2 year old and 3 year old lactating and nonlactating postpartum beef cows. There was a lactation x production group interaction in the analysis ($P = 0.003$).

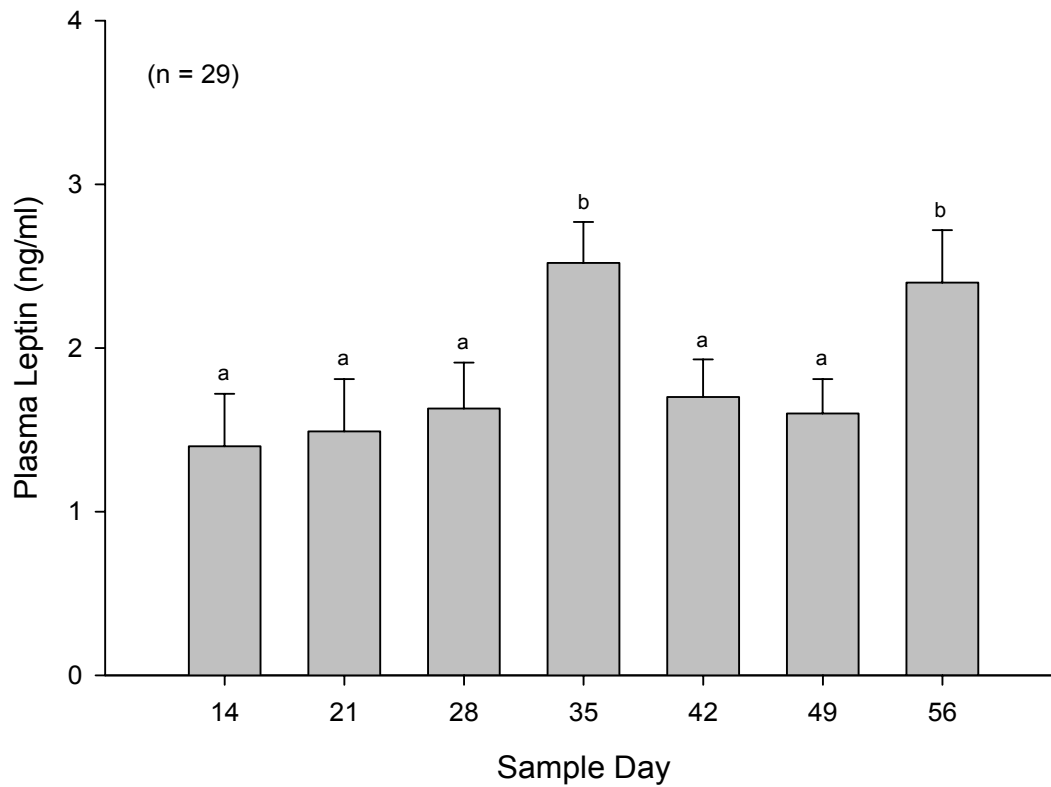


Figure 5.10. Overall weekly mean plasma leptin concentrations of nonlactating heifers, 2 year old and 3 year old cows. ^{a,b}Bars with different superscripts are significantly different ($P < 0.05$).

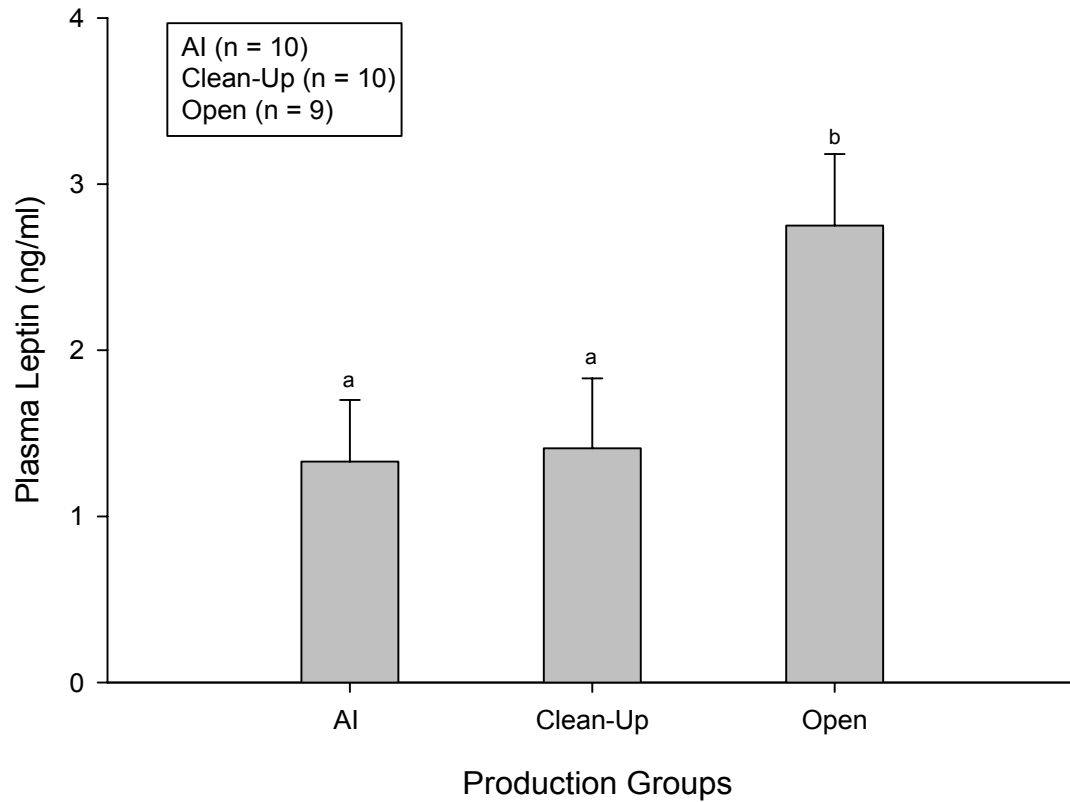


Figure 5.11. Overall mean plasma leptin concentrations by production groups of nonlactating heifers, 2 year old and 3 year old cows. ^{a,b}Bars with different superscripts are significantly different ($P = 0.04$).

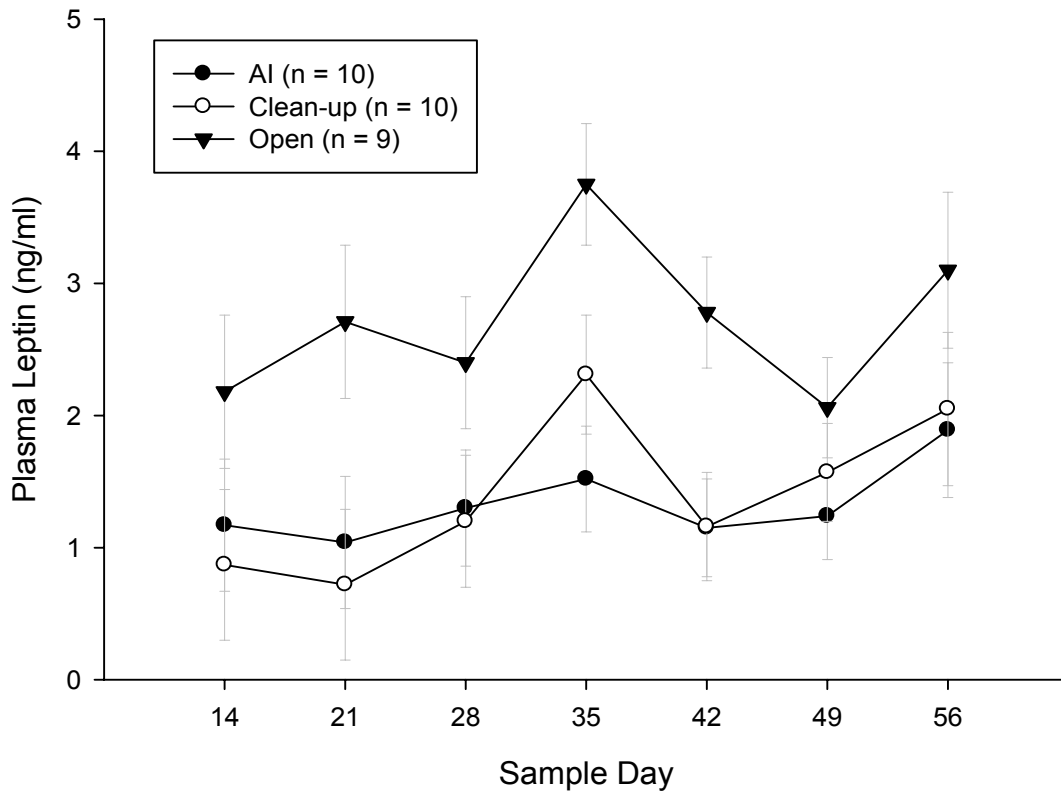


Figure 5.12. Weekly mean plasma leptin concentrations for nonlactating heifers, 2 year old and 3 year old cows. There was a sample day x production group interaction in the analysis ($P < 0.0001$).

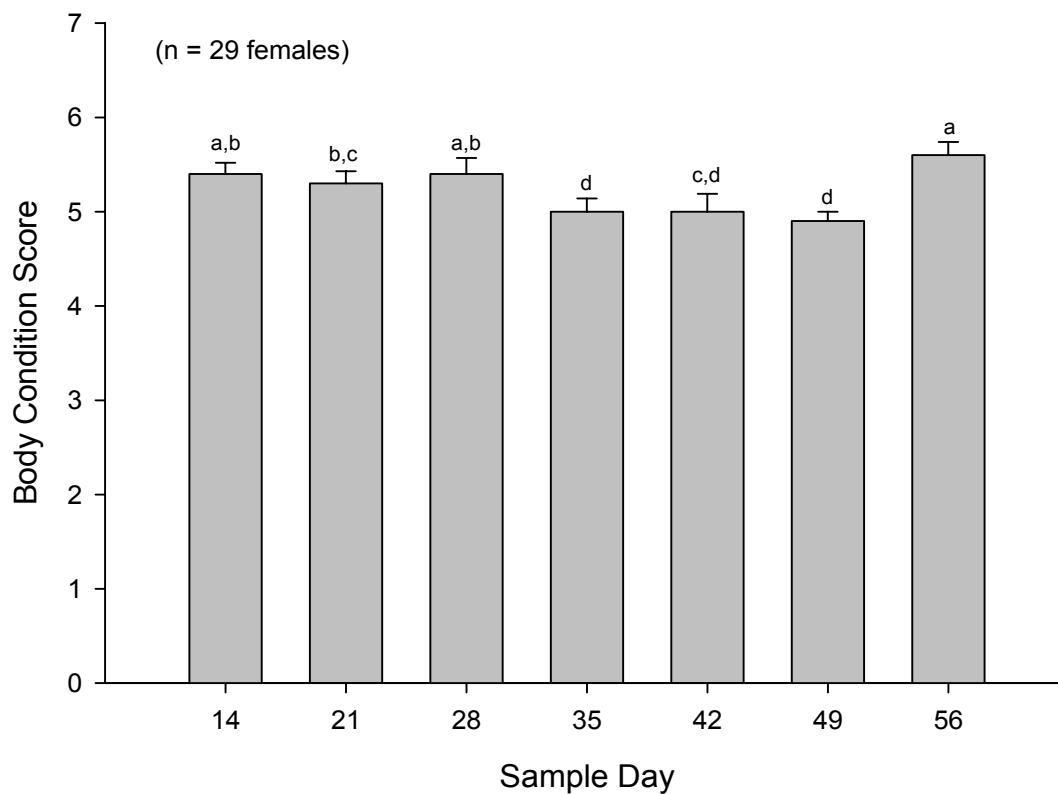


Figure 5.13. Weekly mean body condition scores for crossbred beef heifers, 2 year old and 3 year old nonlactating cows. ^{a,b,c,d} Bars with different superscripts are significantly different ($P < 0.05$).

Discussion

In our study age did not affect circulating plasma leptin level in heifers or early crossbred postpartum cows. These results agree with other reports that show that age does not affect circulating leptin concentrations in human females (Castracane et al., 1998; Baumgartner et al., 1999). Castracane et al. (1998) reported circulating leptin concentrations expressed as a measure of adiposity were greater in young normally cycling females (20 to 35 years old) than in similarly aged males. Also there was no difference in levels of circulating leptin between young and post-menopausal (50 to 65 years old) women.

Baumgartner et al. (1999) reported that circulating leptin adjusted for total body fat had a significant positive association with age in men and a negative association (nonsignificant) with age in women in a study of 106 men and 166 women aged 62 to 98 years. These researchers reported that circulating testosterone levels had a significant negative relationship with circulating leptin levels in men. These scientists went on to report that in a sample of elderly men and women, circulating leptin levels increased significantly over a 14-year period in men, but not in women. These increases in leptin were significantly correlated with decreases in circulating testosterone levels but not with changes in the body mass index in men. In contrast, changes in leptin were associated with changes in the body mass index but not with estrone levels in women. These researchers hypothesized that differences among men and changes in leptin with age are associated with levels of testosterone, and that elderly men become progressively 'hyperleptinemic' with age regardless of body fatness, likely due to decreasing testosterone levels.

In our study, the lack of differences in mean plasma leptin across production group is somewhat surprising. Unkila et al. (2001) reported that women who became pregnant following IVF cycles had higher plasma leptin concentrations than those who either miscarried or did not become pregnant. Bonnet et al. (2005) studied leptin levels in primiparous and nulliparous goats starting 165 days prior to parturition and continuing to 59 days after parturition. These researchers reported plasma leptin increased 49% up to mid-pregnancy in nulliparous goats, then decreased from mid-pregnancy to parturition.

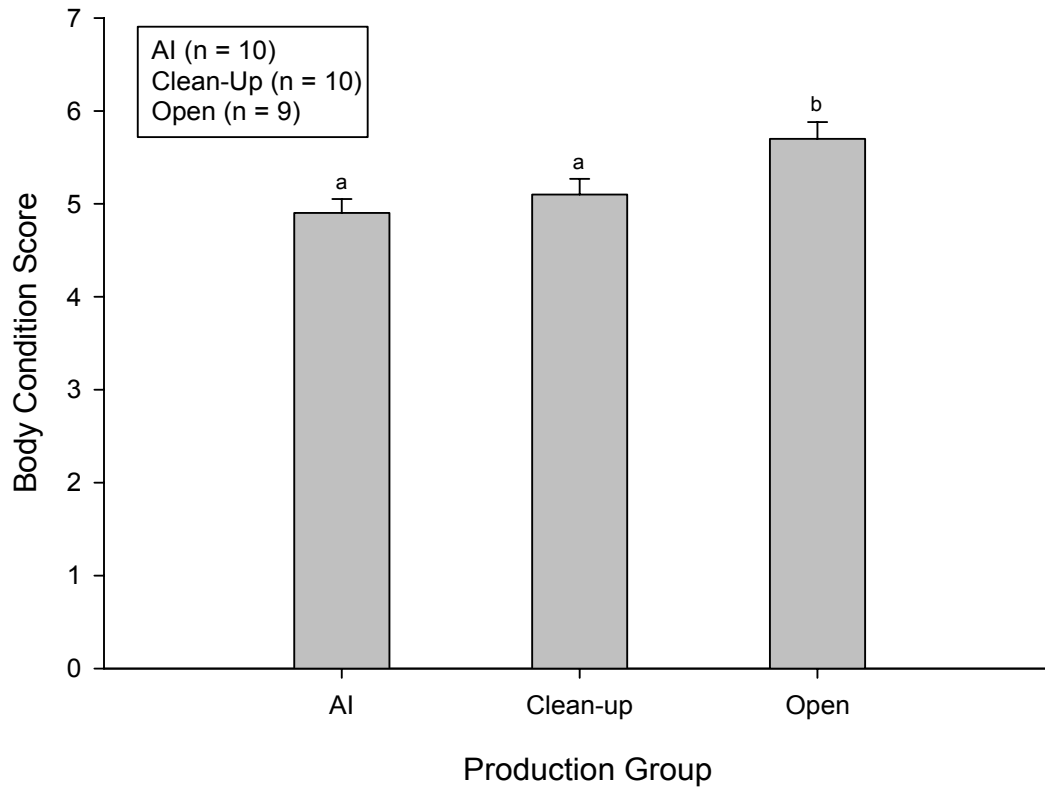


Figure 5.14. Overall mean body condition scores across reproductive status for heifers, 2 year old and 3 year old nonlactating cows. ^{a,b} Bars with different superscripts are significantly different ($P = 0.013$).

Our data shows that nonlactating open females had higher plasma leptin concentrations compared with lactating open females, but there was no difference in mean plasma leptin concentrations between lactating and nonlactating females that became pregnant either by AI or to clean-up bulls. These data are in agreement with those reported in goats (Bonnet et al., 2005), rats (Brogan et al., 1999; Denis et al., 2003), humans (Butte et al., 1997) and dairy cows (Block et al., 2001, 2003).

In goats, Bonnet et al. (2005) reported that circulating leptin concentrations were similar between pregnant and nonpregnant lactating primiparous goats. Leptin levels decreased similarly during early lactation in both primiparous and nulliparous females. These researchers noted that decreased circulating leptin levels in late pregnancy remained low after drying off lactation in pregnant primiparous goats, while leptin levels increased in nonpregnant goats. These scientists proposed that in both primiparous and nulliparous females the low circulating leptin concentrations during transition from late pregnancy to lactation, and during late lactation, may be important for the adaptations that occur during early lactation, such as the partitioning of energy and nutrients towards essential functions and/or hyperphagia.

Block et al. (2001) reported that circulating leptin concentrations from 35 days before to 56 days after parturition were reduced by ~50% after parturition in dairy cows, and then concentrations remained low during lactation. These researchers also noted nonmilked cows had twice the circulating leptin concentration as milked cows. Furthermore, circulating leptin concentrations were positively correlated with circulating concentrations of insulin and glucose, and negatively correlated with concentrations of GH and nonesterified fatty acids.

Liefers et al. (2003) reported that in Holstein cows circulating leptin concentrations were high during pregnancy and then declined to the lowest levels measured at parturition. These researchers hypothesized that circulating leptin concentrations reflect the state of energy balance during lactation, since circulating leptin concentrations were lower in cows with a negative energy balance during lactation. These researchers also noted that cows with lower leptin levels that were in a negative energy balance produced more milk, consumed less feed and had a lower live weight compared with cows having a positive energy balance.

It is our hypothesis that in pregnant postpartum heifers and cows leptin may be repartitioned away from the general circulation, possibly to support a growing fetus. Circulating leptin levels across sample days were more variable for nonlactating 2 year old and 3 year old females compared with the same aged lactating females, suggesting that lactation moderates circulating leptin concentrations. These results may indicate that available leptin is being repartitioned for increased metabolism for fetal growth resulting in lower leptin levels needed for increased feed intake to support pregnancy and/or lactation. Because the placenta is a site that is rich in the leptin receptors (Hoggard et al., 1997; Green et al., 2000; Smith and Waddell, 2002; Seeber et al., 2002) and increases in leptin receptor number occur at the same time as increased leptin levels (Seeber et al., 2002), it has been hypothesized that leptin plays a critical role in fetal development, and that leptin is sequestered by leptin receptors so that energy intake is not decreased by reduced hypothalamic function.

Leptin receptors have been isolated in a number of fetal/placental tissues in the mouse (Hoggard et al., 1997), baboon (Green et al., 2000), human (Bodner et al., 1999) and rat (Smith et al., 2002). In the baboon, Green et al. (2000) used reverse transcriptase polymerase chain reaction and *in situ* hybridization and found that leptin receptors were expressed in the placenta throughout gestation. Edwards et al. (2004) showed that transcripts for the leptin receptor increased several fold in baboon fetal lungs between middle and late gestation.

Hoggard et al. (1997) have reported that high levels of both leptin and its receptor were expressed in fetal bone/cartilage and may be linked to hematopoiesis actions of leptin in adults. Using Western blot and immunocytochemistry, these researchers reported that expression of both mRNA and protein occurred in the ribs, scapula, clavicle, humerus, ulna radius, femur and mandible, while these structures were undergoing ossification in 14.5-day postcoitus mouse fetuses.

In the pregnant female, increases in leptin receptors in the fetal/placental unit could explain reductions in circulating leptin concentrations in the pregnant beef females compared with the open females in our study.

Day-14 blood samples would be equivalent to day 14 of gestation for the AI production group. The females pregnant from the clean-up bulls were not considered to

be pregnant during the first palpation of this study. Several isoforms of the leptin receptor have been reported in the rat placenta (Smith et al., 2002). Leptin receptor mRNAs are expressed in the functionally distinct basal and labyrinth zones of the rat placenta from day 16 to day 22 postcoitus by real time quantitative reverse transcription polymerase chain reaction. Also, these researchers reported that the spacial placental expression of the leptin receptor was confirmed by Western blot and immunolocalization and was most prominent in trophoblast and vascular tissues. This group goes on to report that leptin receptor mRNA expression increased from day 16 to day 22 in the labyrinth zone of the rat placenta but not in the basal zone of the rat placenta. However, in both the labyrinth and basal zone of the placenta, leptin receptor numbers remained unchanged from day 16 to day 22 in the rat placenta.

In our study, all treatment groups exhibited similar mean circulating leptin patterns across sampling days regardless of lactation or production group, with increases in blood leptin levels starting on sampling day 28 and peaking on day 35, followed by a decrease on day 42 and day 49 and then with an increase again on day 56. Drought conditions did occur during this study, and may have contributed to the differences in circulating leptin concentrations across sample days. Precipitation occurred 3 times during the sampling, on days 12, 37 and 40 followed by circulating leptin increases, suggesting that available forage and forage intake may have increased after precipitation. Subsequent grass growth may have altered intake thereby changing circulating leptin levels in both pregnant and nonpregnant beef females.

Leptin release has been shown to closely follow circulating insulin levels in cattle (Block et al., 2001, 2003), where in postpartum dairy cows the circulating concentration of leptin was positively correlated with the circulating concentrations of insulin and glucose. Block et al. (2003) reported that circulating leptin concentrations in postpartum dairy cows was significantly reduced following 24 hours of feed restriction. These authors also reported that after a 96 hour state of hyperinsulinemia in dairy cattle circulating leptin increased significantly. Therefore, we believe that sample day interactions found by all female production groups over the course of the experiment was partly due to available forage following precipitation during a period of drought.

In our study body condition score followed a similar pattern across sample days as did circulating leptin levels. We have also shown that nonlactating females had significantly higher body condition scores and leptin levels compared with lactating females. Reist et al. (2003) reported that circulating leptin concentrations are positively associated with body condition score, energy balance and body weight in dairy cows. Ciccioli et al. (2003) reported that early postpartum beef cows fed to gain 0.90 kg/day exhibited increased body weight and body condition scores resulting in increased concentrations of IGF-1, leptin and glucose compared with cows fed to gain only 0.45 kg/day.

Conclusions

Our data show that age does not affect circulating leptin concentrations in yearling heifers and 2 and 3 year old crossbred postpartum beef cows. We have also shown that lactation significantly affects circulating leptin levels in beef females. Production group (females pregnant from AI, clean-up bulls or remained open) can affect circulating blood levels of leptin, although not quite as expected. Our study also indicates that environmental factors, such as drought and subsequent rainfall can drastically affect circulating levels of leptin in these young beef females. Subsequent experiments need to be conducted to determine how involved leptin is in the maintenance of early pregnancy in cattle.

CHAPTER VI

EXPRESSION OF THE LONG FORM OF THE LEPTIN RECEPTOR IN BOVINE OVIDUCT EPITHELIAL, UTERINE EPITHELIAL CELLS AND BOVINE BLASTOCYSTS

Introduction

In 1953, Kennedy proposed the 'Lipostatic' theory, stating that adipose tissue must produce some 'substance' that circulates through the body to regulate body weight. Hervey (1958) and Coleman (1969) later proved Kennedy's theory through parabiosis experiments in mice and rats.

Zhang et al. (1994) first described leptin as a 146 amino acid protein that has a structure similar to that of cytokines and is produced by the obesity (*ob*) gene. The 'Lipostatic' theory was further enhanced when the leptin gene was identified and it was determined that *ob/ob* mice lacked this gene. Subsequently, Chen et al. (1996) and Lee et al. (1996) reported that *db/db* mice were resistant to leptin because of mutations in its receptor (see review by Mantzoros and Moschos, 1998). The discovery of this new protein hormone has led to a considerable amount of research focused on its effects on nutrition and energy metabolism, particularly in mice and humans. Leptin has been reported to circulate bound to serum proteins with a $t_{1/2}$ life of ~1.6 hours (Houseknecht et al., 1996). In humans, cellular secretion of leptin occurs in a pulsatile pattern with peaks occurring every 32 minutes (Licinio, 1997).

Most of the previous research with leptin has been directed toward the understanding of leptin's role in body adiposity and energy balance. This hormone is produced mainly by adipose tissue and relays information to the brain concerning energy storage and activates the hypothalamic centers that regulate energy uptake and expenditure (Flier, 1997). It was suggested that leptin may affect various neuroendocrine mechanisms and might regulate multiple hypothalamic functions. However, only recently have scientists begun to study the effects of leptin on reproductive functions in mammals and the effects that neuroendocrine mechanisms, hormones and growth factors have on leptin function (Blum, 1997).

Six isoforms of the leptin receptor have been reported in the scientific literature to date: OB-Rb (long form), OB-Ra, OB-Rc, OB-Rd (short forms), OB-Re (soluble form) and OB-Rf (Tartaglia, 1997). Researchers agree that the only receptor that conducts

signal transduction within the cell is the OB-Rb form of the receptor, which has now been identified in a wide range of tissues. The short form OB-Ra receptor has been found to be a carrier molecule (Stephens and Caro, 1998) that helps stabilize leptin when transported from adipose tissue via the blood to target tissues.

Antczak and Van Blerkom (1997) reported that the Ob-Rb isoform was found in oocytes and early mouse and human embryos, and in both theca and granulosa cells of the ovary (Karlsson et al., 1997; Cioffi et al., 1997). The Ob-Rb isoform has been detected through immuno-fluorescence in mouse oocytes, fertilized ova and cleavage stage embryos (Antczak and Van Blerkom, 1997). Gonzalez et al. (2000) reported that leptin mRNA was present in human epithelial endometrium cells (EEC), secretory endometrium, granulosa cells (GC) and placental tissue. These authors reported that leptin and the OB-Rb isoform are found in EEC when cultured with and without embryos. This supports the hypothesis that the oocytes and early stage embryos could be a source of leptin and that EEC may also produce leptin.

Gonzalez et al. (2000) reported human hatched blastocysts cultured alone secreted more leptin compared with arrested blastocyst stage embryos cultured alone, and that hatched blastocysts secreted greater amounts of leptin compared with blastocysts co-cultured with EEC or EEC cultured alone. However, when blastocysts were co-cultured with EEC, leptin concentrations were lower than arrested blastocysts co-cultured with EEC.

Kitawaki et al. (2000) used Northern and Western blot analysis to isolate the leptin Ob-Rb isoform from human endometrial tissues and went on to state that there were different profiles of expression with the Ob-Rb isoform, gradually increasing during the early proliferative phase toward ovulation and peaking during the early secretory phase, then declining during the middle and late secretory phases. However, Kitawaki et al. (2000) have failed to detect any leptin protein in human endometrial cells.

Gonzalez et al. (2000) hypothesized that expression of leptin receptors and leptin protein within the secretory endometrium and cultured EEC might be interpreted as a molecular communication between the human embryo and uterine environment and could be an important factor for implantation to proceed. However, Gonzalez et al. (2000) went on to state that leptin is produced and secreted locally by epithelial cells

and leptin may act in an autocrine or paracrine manner to regulate biological functions to mediate endometrial receptivity to facilitate implantation. Also, regulation of leptin and leptin receptor expression in the EEC by the human embryo could represent an event to allow implantation to occur.

One hypothesis is that leptin may play a role in implantation of the human embryo. Another is that the communication between the blastocyst and endometrium may be needed for enhancement of maternal recognition or for stimulating embryo development.

The objective of this experiment was to determine if the long form of the leptin receptor was present in bovine oviduct epithelial cells, uterine epithelial cells and bovine blastocysts.

Materials and Methods

Harvesting Cells

Reproductive tracts (n = 5) were obtained from mature cows of various breed types (Holstein and Angus crossbred) at a local abattoir and transported on ice to the reproductive laboratory at the Idlewild Research Station. The oviducts and upper 1/3 of the uterine horns were dissected and placed in phosphate-buffered saline (PBS). Based on ovarian structures, the stage of the estrous cycles for these tracts were mid-luteal phase. Oviducts and uterine horns were opened by longitudinal incisions exposing the lumen of both body parts. Cells were harvested using a scalpel blade, and cells (from both oviducts and uterine horns) were placed in 10 ml of mTCM 199 (TCM 199, 10% fetal bovine serum and 1 µg/ml Gentamicin) (TCM 199 and fetal bovine serum; Gibco, Carlsbad, CA) and centrifuged at 200 x g for 5 minutes. Supernatant was aspirated from the cell pellet and the pellet was resuspended in 6 ml mTCM 199. Cells harvested from the oviduct and uterine horns of each reproductive tract were divided into 3 replicates.

Cells from the oviduct and uterine horns were then seeded into 3 labeled 25 cm² flasks (2 ml per flask) and placed in an incubator at 39°C in a 5% CO₂ and 95% air atmosphere. Medium mTCM 199 was used to culture cells, with culture medium change occurring every 2 days. When cells became 80% to 90% confluent, cells were washed 4 times in Ca²⁺ and Mg²⁺ free PBS (Invitrogen Cooperation, Carlsbad, CA). Cells were then trypsinized using 2 ml of 1X Trypsin solution (Sigma Aldrich, St. Louis,

MO) on a heated orbital shaker for 6 minutes for oviduct cells and 2 minutes for uterine cells. The trypsin was stopped by adding mTCM. Cells were then seeded into 4 wells on a chamber slide (Lab Tek II Chamber Slide System, Nalge Nunc International). Cells were allowed 48 hours to adhere to the slide in a 5% CO₂, 95% air atmosphere at 39°C.

Cell Immunological Staining

Both cells types were washed 3 times with 500 µl of pre-warmed PBS to remove serum followed by fixation with 2% paraformaldehyde and allowed to incubate for 10 minutes at room temperature. Cells were then washed 3 times in PBS and then incubated in 1% bovine serum albumin (BSA, Sigma, St. Louis, MO), 99% PBS (blocking buffer) for 15 minutes at room temperature and 15 minutes in a 5% CO₂ and 95% air atmosphere at 39°C. Paraformaldehyde (2%) was then added to the cells and allowed to incubate for 10 minutes, followed by 3 washes with pre-warmed PBS. Cells were then allowed to incubate in the blocking buffer for 30 minutes at room temperature.

The blocking buffer was then removed and cells were incubated in rabbit anti-human leptin receptor antibody (Linco Research, St. Charles, MO) at a concentration of 1:30 in the blocking buffer for 1 hour in a 5% CO₂ 95% air atmosphere at 39°C. Cells were then washed with the blocking buffer 3 times and then incubated in Alexa Fluor 488 anti-rabbit IgG conjugate (Molecular Probes, Carlsbad, CA) at a concentration of 5 µg/ml for 4 hours at room temperature. Cells were then washed 3 times in the blocking buffer and allowed to incubate in the blocking buffer for 12 hours at 5°C. The blocking buffer was then aspirated and cells were mounted in a 50:50 solution of glycerol and PBS with the cover-glass sealed with fingernail polish. Slides were placed on a Nikon Microphot FXA (Nikon, Japan) upright microscope and images were captured using a SPOT digital camera and SPOT Advanced software (Diagnostic Instruments Inc, Sterling Heights, MI).

Tissue Biopsies

Uterine biopsies from 4-year old mature beef cows (n = 4) were collected on day 10 of the estrous cycle (day 0 = estrus) using an equine 'basket type' uterine biopsy instrument. Uterine biopsies (n = 5) were taken from each cow at the greater curvature ipsilateral to the corpus luteum and the contralateral uterine horn to the corpus luteum. Biopsies were individually placed in an Ominsette tissue cassette (Fischer Scientific,

Pittsburgh, PA) and labeled with a #2 pencil with animal identification, date and day of estrous cycle. The tissue cassettes were placed in 10% neutral-buffered formalin for ~24 hours. Cassettes were then delivered to the Pathobiological Sciences Department at the Louisiana State University Veterinary School. Tissues were paraffinized and sliced to a thickness of 5 : m and mounted with 2 tissue sections per glass slide.

Biopsy Immunological Staining

Tissue sections were then transported back to the Biological Sciences laboratory and deparaffinized using 2 washes of xylene (Sigma, St. Louis, MO) for 3 to 5 minutes each, followed by 2 washes in absolute ETOH for 2 to 3 minutes each, dipping during second wash until alcohol sheeted. Sections were then washed in 95% ETOH for 2 to 3 minutes each, followed by incubation for 15 minutes in the blocking buffer (1% BSA in PBS) in an incubator at 39°C. The blocking buffer was removed by blotting the edge of the slide.

Tissue sections were then incubated in rabbit anti-human leptin receptor antibody (Linco Research, St. Charles, MO) diluted 1:30 in the blocking buffer for 1 hour in an incubator at 39°C. Sections were then washed 3 times in the blocking buffer followed by incubation in Alexa Fluor 488 anti-rabbit IgG antibody (Molecular Probes, Carlsbad, CA) at a dilution of 5 : g/ml in the blocking buffer for 2 hours at room temperature.

Tissue sections were then washed 3 times in the blocking buffer and mounted with antifade, coverslip and sealed with fingernail polish. Tissue sections were then viewed on a Nikon Microphot FXA (Nikon, Japan) upright microscope and images were captured using a SPOT digital camera and SPOT Advanced software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Morphological Measurements

Cytoplasmic thickness was measured using a Nova IN834 inverted phase contrast microscope (Nova Instruments, Portland, OR) during cell culture in 25 cm² flasks. The following formula was used to determine cytoplasmic thickness:

$$) Z = [(Z_1 - Z_2) * C](n/n')$$

Where:

Z₁ = Starting calibration mark on fine focal adjustment

Z₂ = Ending calibration mark on fine focal adjustment

C = Graduation of calibration marks on fine focal adjustment
n = Refractive index of medium where sample is mounted
n' = Refractive index of medium between sample and objective lens

The refractive index of the culture medium was determined using a Reichert-Jung refractometer (Reichert-Jung, Buffalo, NY). Cell diameter was determined using the SPOT digital camera and measuring package in the SPOT Advanced software (Diagnostic Instruments, Inc, Sterling Heights, MI). Five samples (20 cells/sample) were evaluated for cell diameter and cell thickness for both oviduct and uterine epithelial cells.

Blastocyst Staining

Forty expanded blastocysts were obtained from the Embryo Biotechnology Laboratory and were washed 3 times in PBS and then incubated in 1% bovine serum albumin (BSA, Sigma, St. Louis, MO), 99% PBS (blocking buffer) for 15 minutes at room temperature and 15 minutes in a 5% CO₂ and 95% air atmosphere at 39°C. Paraformaldehyde (2%) was then added to the embryos and allowed to incubate for 10 minutes, followed by 3 washes with pre-warmed PBS. Embryos were then allowed to incubate in the blocking buffer for 30 minutes at room temperature.

The blocking buffer was then removed and embryos were incubated in rabbit anti-human leptin receptor antibody (Linco Research, St. Charles, MO) at a concentration of 1:30 in the blocking buffer for 1 hour in a 5% CO₂ 95% air atmosphere at 39°C. Embryos were then washed with the blocking buffer 3 times and then incubated in Alexa Fluor 488 anti-rabbit IgG conjugate (Molecular Probes, Carlsbad, CA) at a concentration of 5 µg/ml for 4 hours at room temperature. Embryos were then washed 3 times in the blocking buffer and allowed to incubate in the blocking buffer for 12 hours at 5°C. The blocking buffer was then aspirated and embryos were mounted in a 50:50 solution of glycerol and PBS with the cover-glass sealed with fingernail polish. Slides were placed on a Nikon Microphot FXA (Nikon, Japan) upright microscope and images were captured using a SPOT digital camera and SPOT Advanced software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Experimental Design

For morphological measurements, cell diameter and cytoplasmic thickness of oviduct epithelial cells (n = 100) and uterine epithelial cells (n = 100) per sample were compared during this study. Experimental design for cell staining, biopsy cell staining and blastocyst staining is shown in Table 6.1. Briefly, cells (n = 2 slides/treatment), biopsies (n = 5 sections/treatment) and blastocysts (n = 10 embryos/treatment) were incubated in (1) blocking buffer only (Control -), (2) rabbit anti-human leptin receptor antibody only (1st antibody), (3) Alexa Fluor 488 anti-rabbit IgG conjugate only (2nd antibody) and (4) rabbit anti-human leptin receptor antibody followed by Alexa Fluor 488 anti-rabbit IgG conjugate (Complete).

Statistical Analysis

Morphological measurements were analyzed using the GLM procedure of SAS (SAS Institute, Cary, NC). Cells harvested from the oviducts and uterine horns of each reproductive tract (n = 5) were divided into 3 replicates. Tissue biopsies (n = 5) were collected from each cow (n = 4) at the greater curvature from both the ipsilateral uterine horn to the corpus luteum and the contralateral uterine horn to the corpus luteum.

Results

Mean cell diameter was significantly greater ($P < 0.0001$) in bovine oviduct epithelial cells ($25.7 \pm 2.03 \mu\text{m}$) compared with uterine epithelial cells ($166.3 \pm 8.76 \mu\text{m}$) (Figure 6.1). The cytoplasm of the cells was thicker ($P = 0.011$) for oviduct cells ($7.50 \pm 0.37 \mu\text{m}$) compared with uterine cells ($6.15 \pm 0.35 \mu\text{m}$) (Figure 6.2).

Results for oviduct and uterine cell, uterine biopsy and embryo staining are presented in Table 6.2. All oviduct and uterine cells, biopsies and embryos that were incubated in only blocking buffer exhibited no fluorescence. Also, all oviduct and uterine cells, biopsies and embryos that were incubated in only rabbit anti-human leptin receptor antibody exhibited no fluorescence. Furthermore, all oviduct and uterine cells and uterine biopsies that were incubated in only Alexa Fluor 488 anti-rabbit IgG conjugate exhibited no fluorescence. However, oviduct and uterine cells, uterine biopsies and embryos that were incubated in both rabbit anti-human leptin receptor antibody followed by incubation in Alexa Fluor 488 anti-rabbit IgG conjugate exhibited differing degrees of fluorescence.

Table 6.1. Experimental design and treatments for bovine oviduct epithelial cells, uterine epithelial cells and embryos.

Treatment	Blocking buffer	Antibody	
		Rabbit anti-human leptin receptor	Alexa Fluor 488 anti-rabbit IgG
Oviduct epithelial cells:			
Control (-)	+	-	-
1 st antibody	+	+	-
2 nd antibody	+	-	+
Complete	+	+	+
Uterine epithelial cells:			
Control (-)	+	-	-
1 st antibody	+	+	-
2 nd antibody	+	-	+
Complete	+	+	+
Blastocysts:			
Control (-)	+	-	-
1 st antibody	+	+	-
2 nd antibody	+	-	+
Complete	+	+	+

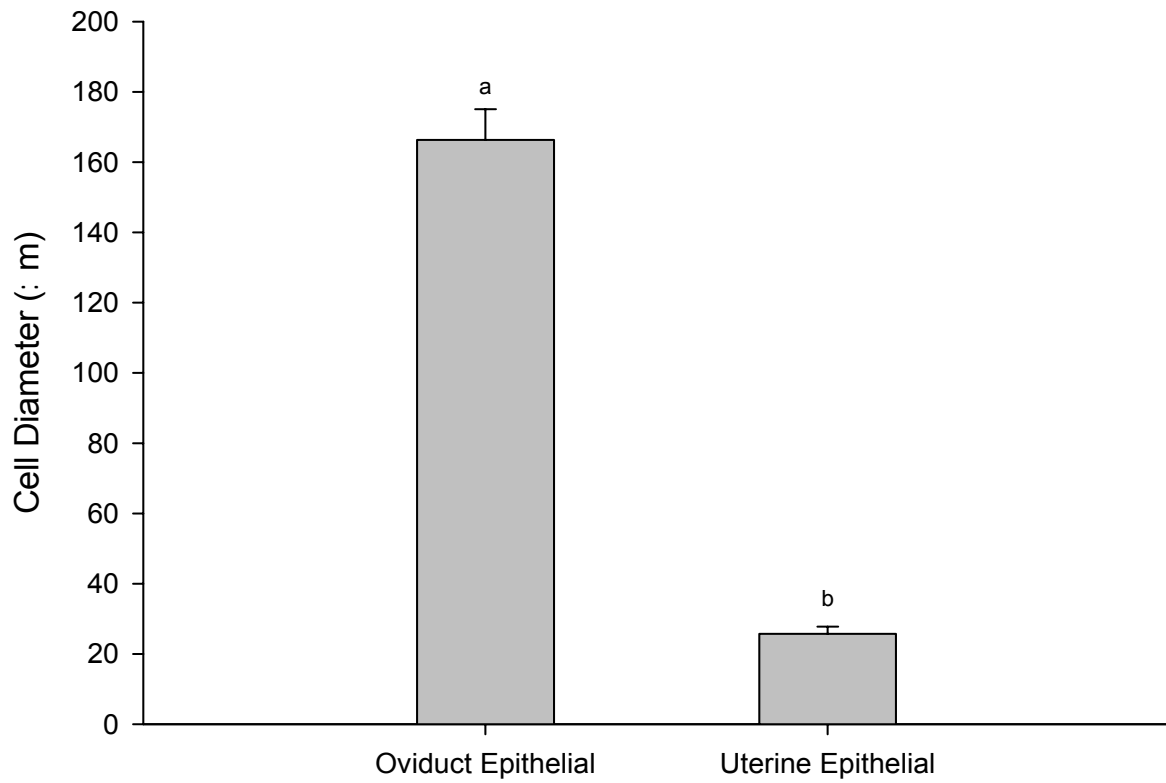


Figure 6.1. Mean cell diameter for bovine oviduct epithelial and uterine epithelial cells. ^{a,b}Bars with different superscripts are significantly different ($P < 0.0001$).

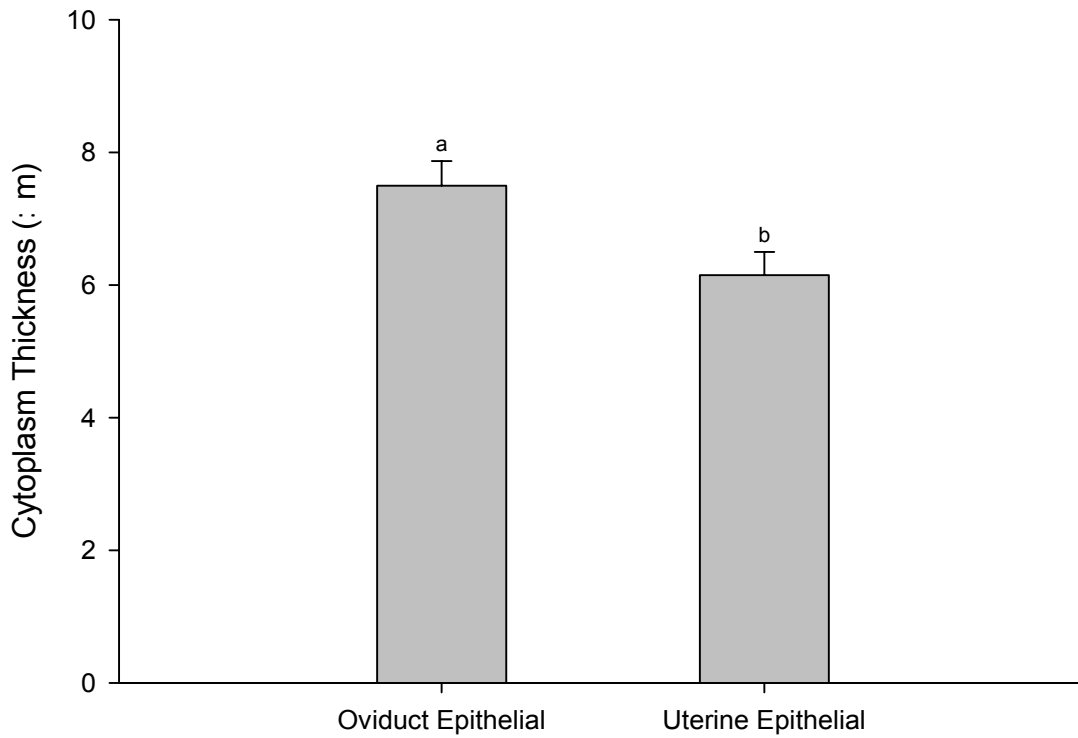


Figure 6.2. Mean cell cytoplasm thickness for bovine oviduct epithelial and uterine epithelial cells. ^{a,b}Bars with different superscripts are significantly different ($P < 0.011$).

Table 6.2. Immunostaining results for bovine oviduct epithelial cells, uterine epithelial cells, uterine biopsies and blastocysts.

Tissue/cell type	Treatment			
	Control (-)	1 st antibody	2 nd antibody	Complete ¹
Oviduct epithelial	-	-	-	+++
Uterine epithelial	-	-	-	++
Uterine biopsy	-	-	-	++++
Blastocysts	-	-	-	+++

¹ Indicates fluorescent intensity (+ = some fluorescence to ++++ = bright fluorescence).

All oviduct epithelial cells were positive for staining for the long form of the leptin receptor (Figure 6.3), however, not all uterine epithelial cells stained positive (~30%) for the long form of the leptin receptor (Figure 6.4). All uterine biopsies showed intense staining for the long form of the leptin receptor along the luminal side of the uterine endometrium (Figure 6.5) and no staining in the myometrium. All the bovine blastocysts evaluated (n = 10) stained positive for the long form of the leptin receptor, with intense staining occurring in the trophoblast cell type (Figure 6.6).

Discussion

Our data show that cell diameter was greater in bovine oviduct epithelial cells compared with uterine epithelial cells. The cytoplasm of the cells was thicker for oviduct cells compared with uterine cells. This difference in cell diameter and thickness was unexpected.

Several studies have shown that during 'classic' culture conditions (on glass or on plastic) epithelial cells lose their cuboidal shape (Mulholland et al., 1988; Streuli et al., 1991). According to these studies, cultivated epithelial cells lose their gap junctions and their ability to react to hormones that would be considered organotypical by the reduction in the expression of specific receptor proteins, typically for the steroid hormones. Classen-Linke et al. (1997) cultured endometrial cells from human tissues collected during the proliferative phase and reported that when cells were cultivated on a basement membrane system using Matrigel they formed polarized monolayers or gland-like structures that responded to hormone concentrations typical to cells in an in vivo environment. In our study, the cells were cultivated in the classical sense and did lose their cuboidal shape, however, no hormone challenges or steroid receptor staining was performed.

Results from our study show that both oviduct and uterine epithelial and uterine biopsies from mid-luteal phase reproductive tracts stained positive for the long form of the leptin receptor. The leptin receptor is considered a membrane bound protein and upon visualization the receptor did appear to be membrane bound in both the oviduct and uterine epithelial cells.

Not all uterine epithelial cells stained positive for immunofluorescence for the leptin receptor. Numerous uterine cells did not take up the stain (~30%), while all

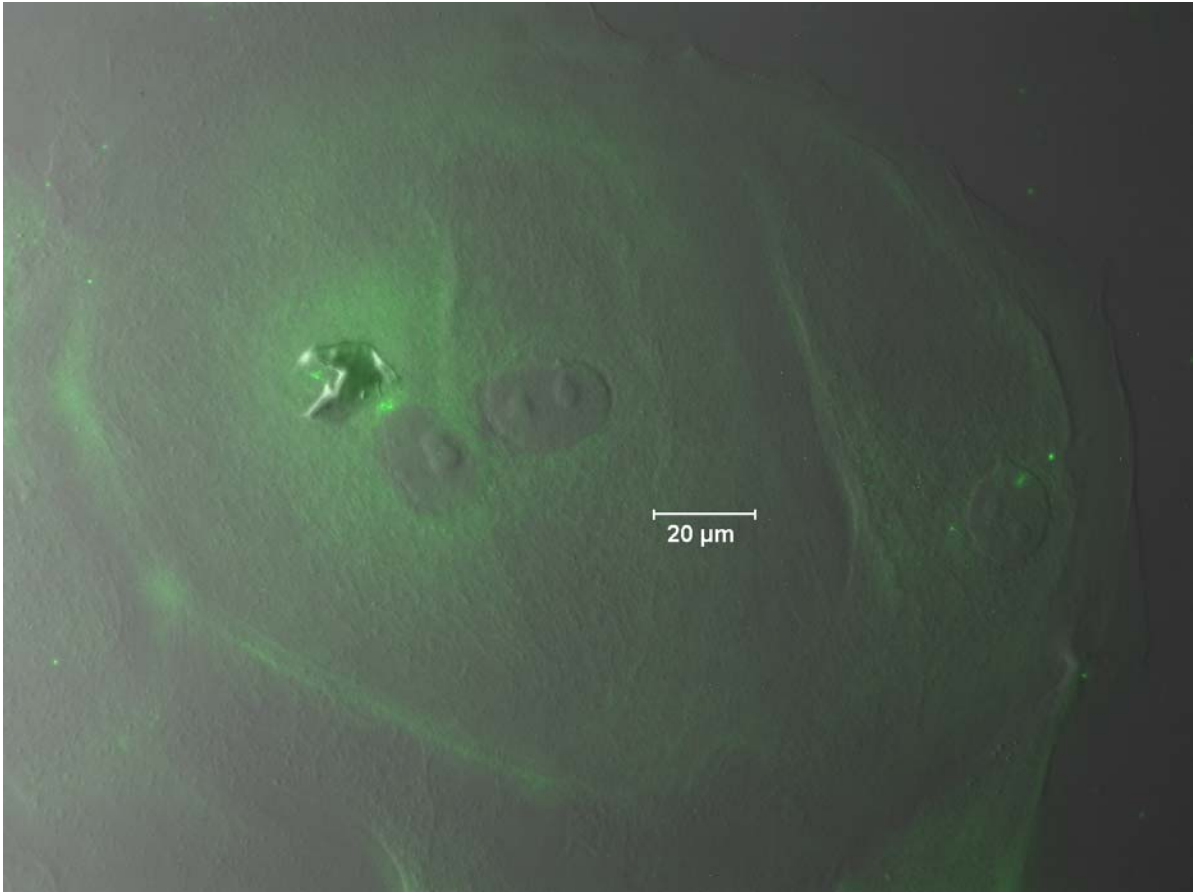


Figure 6.3. Immunofluorescence image overlaid with a differential interference contrast image of bovine oviduct epithelial cells stained for the long form of the leptin receptor.

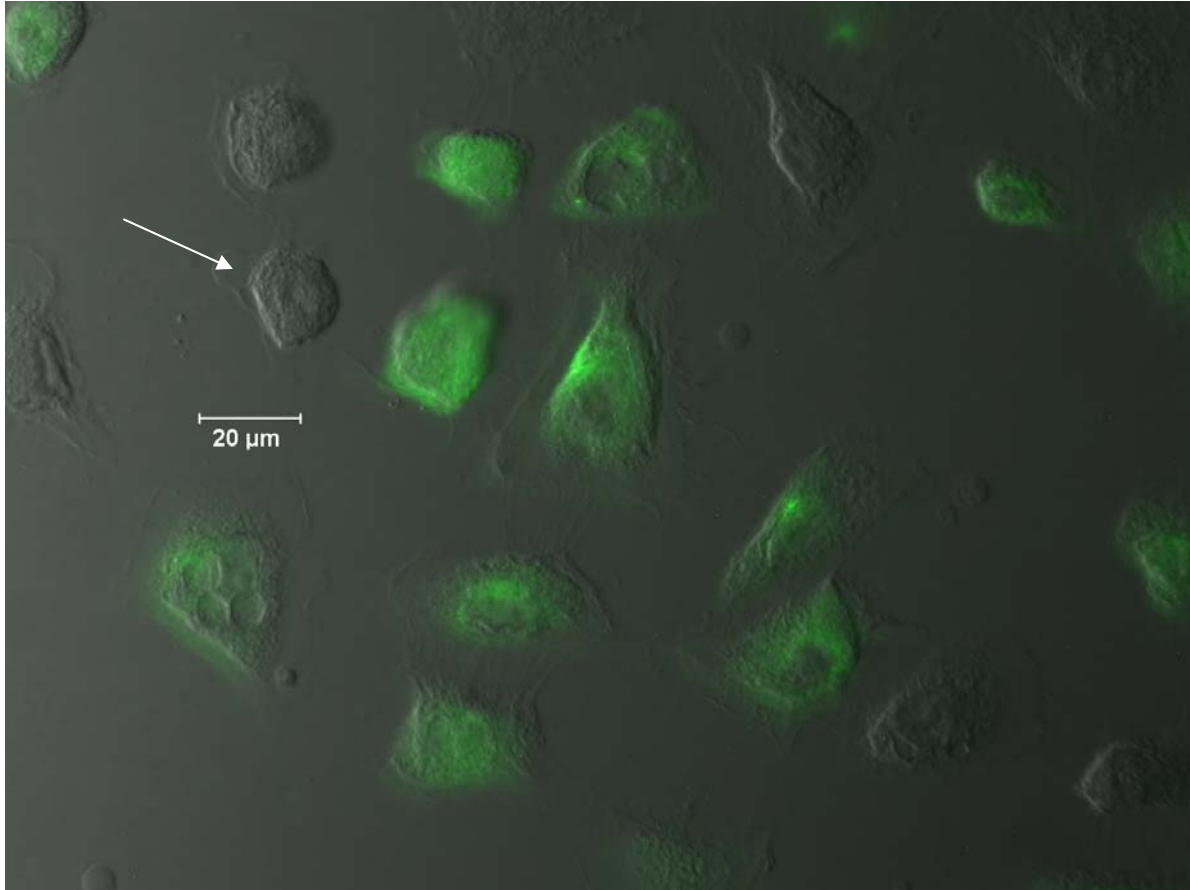


Figure 6.4. Immunofluorescence image overlaid with a differential interference contrast image showing bovine uterine epithelial cells stained for the long form of the leptin receptor. Arrow indicates uterine epithelial cells not staining for the leptin receptor.

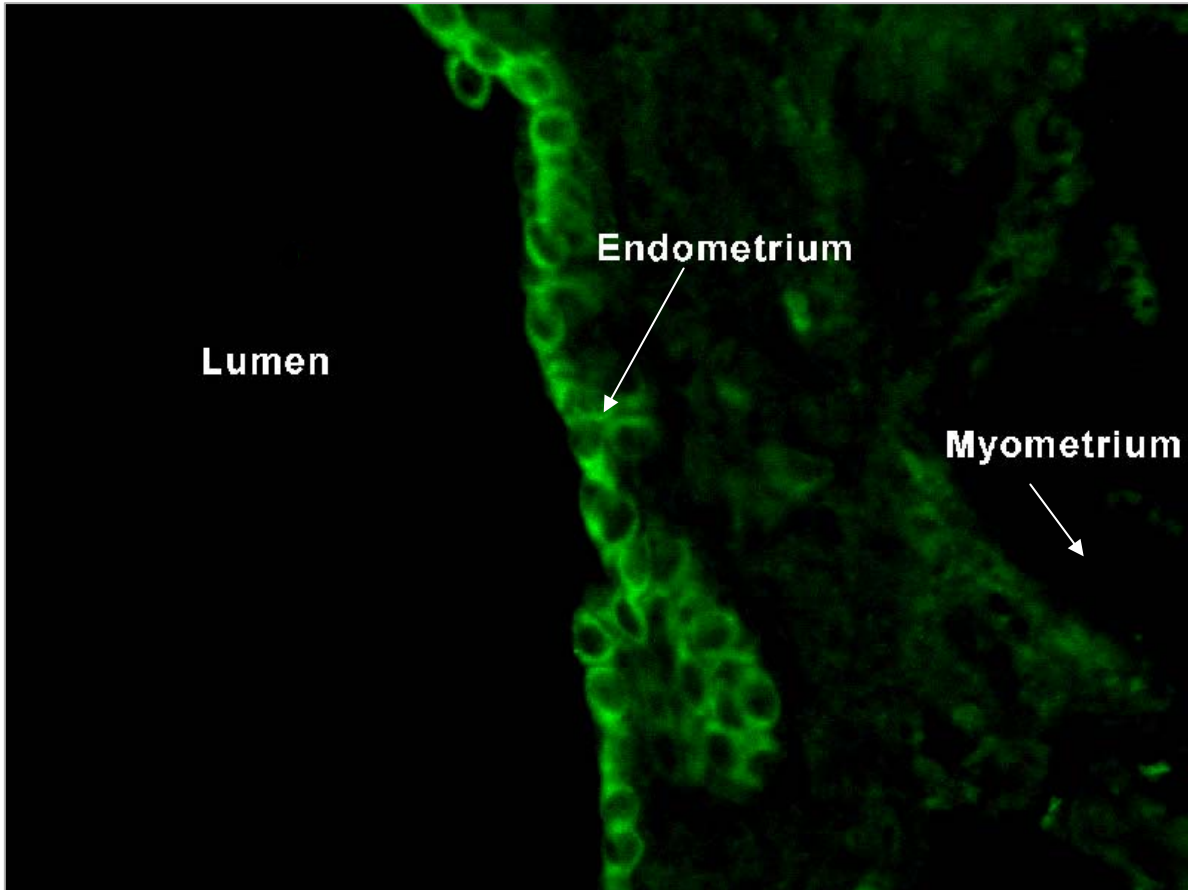


Figure 6.5. Immunofluorescence image of bovine uterine biopsy taken from beef cow on day 10 of the estrous cycle (day 0 = estrus) stained for the long form of the leptin receptor.

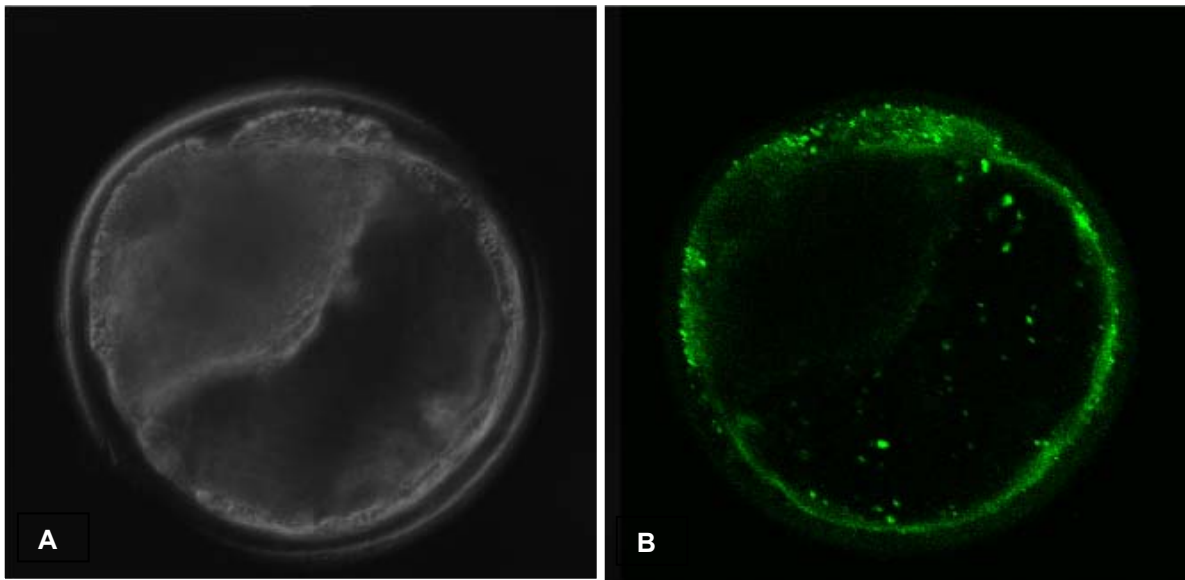


Figure 6.6. Differential interference contrast image (Panel A) and immunofluorescence image (Panel B) of a bovine blastocyst stage embryo stained for the long form of the leptin receptor.

oviduct cells appeared to stain positive. These data provide the first evidence that oviduct epithelial cells of any mammal express the long form of the leptin receptor. This study is also the first to report that this isoform exists in bovine uterine epithelial cells. Our study also shows that the OB-Rb isoform is a membrane bound receptor in both oviduct and uterine epithelial cells. There were highly localized numbers of this isoform fluorescing in close proximity to the nuclei. Since the leptin receptor is a protein, we believe that this localization may be occurring in the endoplasmic reticulum or golgi apparatus, where proteins are formed and packaged. This finding needs further exploration.

Gonzalez et al. (2000) reported that the human endometrium expresses the OB-Rb isoform of the leptin receptor and that the human blastocyst possibly regulates the secretion of leptin by the secretory endometrium. These researchers go on to state that the human endometrium likely secretes leptin in an autocrine and paracrine fashion. Their hypothesis suggests that the leptin system may be implicated in the human implantation process.

The relationship between circulating leptin levels and different phases of the menstrual cycle is not clear from the scientific literature. In one study, Stock et al. (1999) reported that leptin levels varied throughout the menstrual cycle and there were no differences among cycles of 13 healthy, cyclic females. However, Hardie et al. (1997) reported that when the cycle was subdivided into functional stages, leptin levels were associated with stage of the menstrual cycle, but were also found to vary across women. These researchers reported that leptin levels rose during the transition from the early follicular phase to the peri-ovulatory phase and then peaked in the luteal phase with values 1.5-fold higher than those noted in the follicular phase.

Using the semiquantitative RT-PCR method, Kitawaki et al. (2000) reported that expression of the OB-Rb isoform was the dominate leptin receptor being expressed by the human endometrium. Its expression was found to be the lowest during the proliferative phase and then gradually increased toward ovulation with the greatest expression during the early secretory phase. The results presented by Hardie et al. (1997) and Kitawaki et al. (2000) suggest that there is increased expression of the OB-Rb isoform and increased circulating levels of leptin shortly before and increasing

several days after ovulation. Our uterine biopsy cells were harvested during mid-luteal phase of the cow, and results indicate that leptin receptors are expressed during this phase of the estrous cycle. Our study also showed that the epithelial cells in the oviduct express the OB-Rb receptor.

The results from blastocyst staining in the present study are in agreement with those of Antczak and Van Blerkom (1997), who reported spacial polarized domains in mouse embryos of both leptin and STAT3 proteins in mouse and human oocytes and early stage embryos. Those researchers showed that differences in allocation of leptin between blastomeres occur at the first cell division and by the 8-cell stage within each embryo unique cellular domains consisting of rich leptin/STAT3 domains and poor leptin/STAT3 domains were present. A concentration gradient of these proteins along the surface of the embryo was noted by the morula stage. During this stage of embryo development the 'inner' cells were composed of blastomeres that contain little leptin/STAT3 proteins, whereas, the 'outer' cells exhibit cell populations both rich and poor in the leptin/STAT3 proteins. These researchers noted that this pattern persists through the hatched blastocyst stage with the inner cell mass containing little, if any, leptin/STAT3 protein and trophoblast having both rich and poor leptin/STAT3 cell populations. These observations are consistent with our findings on later stage bovine embryos.

Conclusions

Our study shows for the first time that bovine oviduct and uterine epithelial cells contain the long form of the leptin receptor. Also, our immunofluorescence results support earlier findings that blastocyst stage embryos contain the long form of the leptin receptor. Our findings suggest that if intercellular communication does exist between epithelial cells and the developing embryo, this communication starts much earlier than the blastocyst stage (day 7). Further research is needed to determine what role(s) leptin plays in oviduct and uterine tissues and by what mechanisms leptin regulates its receptor presence in the oocyte and the early stage embryo.

Based on observations from our study, we propose that there may be cellular communication between the oviduct epithelial cells and early embryo. This could act as an activation or an inhibitory mechanism for the early stage embryo in vivo, and may be

a part of an 'developmental block' in the embryo, if maternal nutritional status becomes critical after ovulation but before implantation occurs.

CHAPTER VII

DEVELOPMENT OF IN VITRO-DERIVED EMBRYOS CULTURED IN DIFFERENT CONCENTRATIONS OF LEPTIN

Introduction

Development of an effective embryo culture system is needed to study early embryo development. It has recently been shown that the human blastocyst produces leptin (Gonzalez et al., 2000) and that the production of leptin is increased in normally developing blastocysts compared with arrested embryos. Scientists believe that leptin may play a critical role in maintaining early pregnancy in the mouse (Malik et al., 2001) and the human (Gonzalez et al., 2000).

In 1953, the 'Lipostatic' theory was proposed stating that adipose tissue must produce some 'substance' that circulates through the body to regulate body weight (Kennedy, 1953). Hervey (1958) and Coleman (1969) later proved Kennedy's theory through parabiosis experiments in mice and rats. These researchers hypothesized that *ob/ob* mice (characterized by obesity and sterility) did not possess this 'substance', and *db/db* mice (characterized by obesity and hyperglycemia) were resistant to its action.

Zhang et al. (1994) first described leptin as a 146 amino acid protein that has a structure similar to that of cytokines. It was reported that leptin was produced by the obesity (*ob*) gene and that *ob/ob* mice lacked this gene. Later, Chen et al. (1996) and Lee et al. (1996) reported that *db/db* mice were resistant to leptin because of mutations in the leptin receptor (Mantzoros and Moschos, 1998).

Leptin receptors have been found in both mouse and human oocytes and embryos (Antczak and Van Blerkom, 1997). Gonzalez et al. (2000) reported leptin mRNA was present in human epithelial endometrium cells, secretory endometrium, granulosa cells and placental tissue. These researchers showed that hatched blastocysts (cultured alone) secreted higher levels of leptin compared with arrested embryos (cultured alone). However, when developing hatched blastocysts were co-cultured with epithelial endometrial cells, leptin concentrations were lower than in arrested blastocyst stage embryos co-cultured with epithelial endometrial cells.

Kitawaki et al. (2000) used Northern and Western blot analysis to isolate leptin receptors from human endometrial tissues. These researchers showed that there were different leptin receptor expression profiles in humans, which gradually changed (increased) during the early proliferative phase toward ovulation and peaking during the early secretory phase, then declining during the middle to late secretory phase. However, these researchers failed to detect any leptin protein in human endometrial samples.

Gonzalez et al. (2000) hypothesized that expression of leptin receptors and leptin protein within the secretory endometrium and cultured endometrial epithelial cells might be interpreted as a molecular communication between the mouse and human embryo and uterine environment, and may be an important factor for implantation in the mouse and human to proceed. However, it was noted that because leptin is produced and secreted locally by epithelial cells, it could act in an autocrine or paracrine manner to regulate biological functions that may mediate endometrial receptivity to facilitate the implantation process. Furthermore, regulation of leptin and leptin receptor expression in endometrial epithelial cells by the human embryo could represent a coordinate molecular event to allow implantation to occur.

Malik et al. (2001) administered minimum daily doses of leptin (5 mg/kg, intraperitoneal) and then *ob/ob* females were mated with either wild type or leptin treated *ob/ob* males. When leptin treatment was withdrawn at 3.5 days postcoitus, no pregnancies resulted. However, if leptin treatment was continued to either 6.5 days postcoitus or 14.5 days postcoitus then normal pregnancies resulted.

The first evidence that leptin may influence oocyte maturation and developmental competence of embryos was reported by Swain et al. (2004). Fewer porcine blastocysts resulted when compared with controls, when porcine embryos had been exposed to leptin during in vitro maturation. In contrast, leptin has also been reported to enhance the resumption of meiosis in pre-ovulatory follicle enclosed oocytes with no effects on meiotic resumption of denuded or cumulus-enclosed oocytes in the mouse (Ryan et al., 2002).

Kawamura et al. (2002) reported that leptin mRNA was expressed in blastocysts and hatched blastocysts in the mouse, with 2 isoforms of the leptin receptor (Ob-Ra and

Ob-Rb) in oocytes, 1-cell, 2-cell, morula, blastocyst and hatched blastocyst stages. Leptin protein was detected in the epithelium of the oviduct and the uterine endometrium in pregnant mice. It was also noted that leptin levels were higher in the uteri of pregnant mice than nonpregnant mice.

It has also been shown by Kawamura et al. (2002) that the addition of exogenous leptin to embryo culture medium increased the development of mouse embryos from the 2-cell stage to the blastocyst, expanded blastocyst and hatched blastocyst stages in a dose dependent manner. These researchers also reported that the addition of an antibody against the extracellular domain of the leptin receptor ablated this stimulatory response.

Genomic activation in the mouse occurs at the 2-cell stage (Flach et al., 1982). Kawamura et al. (2002) have reported that leptin mRNA was detected after the blastocyst stage (using a commercial ELISA kit), indicating that leptin mRNA may be originating from an embryonic gene. The latter authors also noted that mRNA for leptin receptors in mouse oocytes was found all the way through to the hatched blastocyst stage. This would suggest that these receptors may be maternally derived because receptor mRNA are expressed prior to genomic activation of the embryo.

To date there have been no reports on the effects of exogenous leptin on the bovine embryo. Therefore, the objective of this study was to determine if the addition of exogenous leptin would affect the development of bovine embryos or change the growth patterns in vitro.

Materials and Methods

Leptin Stock Solution Preparation

Prior to obtaining the bovine zygotes, recombinant rat leptin (No. L 5037, Sigma, St. Louis, MO) was reconstituted in 0.5 ml of 0.2 : m-filtered 15 mM HCl. After the protein was dissolved, 0.3 ml of 7.5 mM 0.2 : m-filtered NaOH was added to bring pH to 5.2. Volume was brought to 4.755 ml with the addition of double distilled water. Aliquots of leptin stock solution of 10 : l were then stored at -80°C.

Day 1 to 3 Culture Medium Preparation

Prior to embryo culture the holding and culture media were prepared. HEPES Talp holding medium (Appendix A) was prepared followed by CR1aa Stock Medium

(Rosenkrans and First, 1994) (Appendix B), then CR1aa medium (day 0 to 3) was supplemented with 20 : l/ml Basal Medium Eagle (BME, Sigma, St. Louis, MO), 10 : l/ml Modified Eagle's Medium (MEM, Sigma, St. Louis, MO), 10 : l/ml of penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA), 5 : l/ml of L-gutamine (Sigma, St. Louis, MO), 10 : l/ml of glycine (Sigma, St. Louis, MO) and 10 : l/ml of alanine (Sigma, St. Louis, MO) (Appendix C).

Experimental Design

Bovine zygotes were transferred to mCR1aa medium containing either 0 ng/ml, 100 ng/ml or 1,000 ng/ml of recombinant rat leptin in two replications (Replicate I, n = 177; Replicate II, n = 165). Development to the <8 cell, 8- to 16-cell, morula and blastocyst (total number of blastocysts, number of expanded and hatched blastocysts) stages was examined on day 4, day 7 and day 8 post-insemination, respectively. Subsequent inner cell mass, trophoblast and total number of cells per embryo were also evaluated on day 7 post-insemination.

Prior to in vitro culture, 0 ng/ml of leptin (Treatment A), 100 ng/ml of leptin (Treatment B) and 1,000 ng/ml of leptin (Treatment C) were prepared by adding 0 : l, 0.8 : l and 8 : l of leptin stock to 10 ml of CR1aa medium (days 0 to 3) (mCR1aa) for culture from day 1 to day 3. At this time, 3 ml of HEPES Talp was added to a 35 mm x 10 mm Falcon plastic petri dish (35 mm dish) (Becton Dickinson & Co., Lincoln Park, NJ) and four 50 : l drops of each treatment of mCR1aa (Treatments A, B and C) were added to each of three 35 mm plastic petri dishes to be used as the washing medium. Then, 30 : l drops of each treatment of mCR1aa (Treatments A, B and C) were added to 35 mm dishes (9 drops per dish, 2 dishes per treatment) and covered with embryo-tested mineral oil (Sigma Chemical Co., St. Louis, MO). All petri dishes were placed in a 38°C and 5% CO₂ in air humidified incubator for at least 20 minutes to equilibrate. The leptin concentrations for the treatments used in this experiment were based on those used in mice (Kawamura et al., 2002).

Day 3 to 7 Culture Medium Preparation

On day 3, CR1aa medium (day 3 to 7) (Appendix D) was supplemented with 20 : l/ml of Basal Medium Eagle (BME, Sigma, St. Louis, MO), 10 : l/ml of Modified Eagle's Medium (MEM, Sigma, St. Louis, MO), 10 : l/ml of penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA), 5 : l/ml of L-glutamine (Sigma, St. Louis, MO), 50 : l/ml of fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA), 10 : l/ml of glycine (Sigma, St. Louis, MO) and 10 : l/ml of alanine (Sigma, St. Louis, MO) (Appendix C).

Treatment A with 0 ng/ml (control), Treatment B with 100 ng/ml and Treatment C 1,000 ng/ml of leptin were prepared by adding 0 : l, 0.8 : l and 8 : l of leptin stock to 10 ml of CR1aa medium (day 3 to 7) (mCR1aa2) for culture from day 3 to day 7. New 35 mm petri dishes were labeled with the appropriate treatment and date, then 30 : l drops of each treatment of mCR1aa2 (Treatments A, B and C) were added to the dishes (9 drops per dish, 2 dishes per treatment) and covered with embryo-tested mineral oil (Sigma, St. Louis, MO). All dishes were placed in a 38°C and 5% CO₂ in air humidified incubator for at least 20 minutes to equilibrate.

In Vitro Culture

Bovine zygotes were obtained from a commercial source (BOMED, Inc., Madison, WI) and shipped to the laboratory in 2 ml of BOMED maturation medium in a battery-powered incubator via an overnight express mail service. These zygotes arrived within 24 hours of exposure to culture medium pre-equilibrated at 39°C in 5% CO₂ in air. Upon arrival, zygotes were removed from 5 mm shipping tubes and placed in holding medium. Each tube was randomly assigned to a treatment and all zygotes from each tube were then washed 4 times in 50 : l washing drops and then placed (10 to 15 embryos per 30 : l culture drop) in mCR1aa (day 0 to 3) medium containing 0 ng/ml, 100 ng/ml and 1,000 ng/ml of leptin the labeled 35 mm dishes. These dishes were then transferred to a modulator incubator chamber. The modular incubator was then purged with a mixture of 5% CO₂, 5% O₂ and 90% N₂ at 1.4 kg/cm² (equivalent to 20 psi) for 2 minutes. The chamber was sealed and placed in an incubator maintained at 38°C and 5% CO₂ in air.

Embryos were first evaluated on day 3 post-fertilization and morphological development was recorded. The total number of degenerate embryos, <8-cell embryos and 8- to 16-cell embryos were noted. Embryos that had cleaved but were evaluated as having <8 cells were transferred to a labeled 35-mm dish containing 30 : l drops of respective treatments in mCR1aa drops, containing 10 to 15 embryos each. Likewise, embryos that were evaluated as having 8- to 16-cells were transferred into a separate 35 mm dish containing 30 : l drops of respective treatments in mCR1aa, with drops containing 10 to 15 embryos each. Petri dishes were then placed back into the airtight modulator incubator where they were again charged with a 5% CO₂, 5% O₂ and 90% N₂ gas mixture for 2 minutes. The chamber was placed in a humidified 5% CO₂ incubator at 38°C and embryos were allowed to develop until day 7 post-insemination.

Embryo Development

On day 7 post-insemination, embryos were removed from the incubator and the developmental stage and quality of all embryos was recorded. The ratio of blastocyst to 8- to 16-cell embryos by treatment were then calculated. Also, the trophoblast to ICM ratios by treatment were calculated. An embryo quality grade, ranging from 1 to 4 (1 = excellent to 4 = poorest quality), was assigned to each embryo. Symmetrical appearances of the embryo, presence of extruded blastomeres, degeneration of the inner cell mass and developmental synchrony to day of culture were factors accessed in assigning embryo quality grades. In addition, embryos were assigned a modified RED score (mRED) based on the developmental stage and quality grade (Table 7.1), as previously described by Collins (2002).

Embryo Morphology Measurements

The inner cell mass and trophoblast cell numbers were counted in 10 randomly selected blastocysts from each treatment for each replication. Expanded blastocysts were selected for counts so the staining protocol could be standardized for repeatable results. Ten embryos from each treatment (in groups of 5) were selected from cultures on day 7 and placed in 500 : l of Hoechst 33342 (Sigma, St. Louis, MO) for 10 minutes. Afterwards, embryos were transferred to 500 : l of 0.1% Triton (Sigma, St. Louis, MO)

Table 7.1. The mRED¹ scoring system for bovine IVF-derived embryos.

Stage of development ²	Degree of development				
	Compacted morulae	Early blastocysts	Blastocysts	Expanded blastocysts	Hatched blastocysts
1	1.00	2.00	3.00	4.00	5.00
2	0.66	1.66	2.66	3.66	4.66
3	0.33	1.33	2.33	3.33	4.33
4	0.01	0.01	0.01	0.01	0.01

¹ mRED used for the IVF-derived embryos was previously described by Collins (2002).

² Embryo quality grades (1 = excellent to 4 = very poor).

for 140 seconds, followed by a 140-second incubation in 500 : l of 1% propidium iodide (Sigma, St. Louis, MO). Embryos were then transferred to 500 : l of HEPES Talp medium for 1 minute. Embryos were mounted on slide with 25% glycerol and visualized for cell counts.

Statistical Analysis

This experiment included two replications. Shipping tubes containing between 50 and 75 zygotes were randomly assigned to leptin treatment groups. Zygotes that developed to the morula, early blastocyst, blastocyst, expanded blastocyst and hatched blastocyst stages were individually scored based on the mRED scale on day 7 post-fertilization. Differences in replications for cell counts (ANOVA), developmental rates (Chi square) and mRED scores (Mann-Whitney Rank Sum Test) were analyzed using Sigma Stat (Systat Software, Inc., San Jose, CA). If no significant differences between replicates were detected, then the replicates were pooled and another analysis was initiated. If differences were detected between replicates then comparisons were made within each replicate and across replicates.

Results

Embryo Development

Embryonic development was reduced with an increase ($P < 0.05$) in percentage of embryos with < 8 cells on day 3 in the 1,000 ng/ml of leptin treatment group (32%) compared with the 0 ng/ml treatment group (22%) (Table 7.2). There was no difference on day 3 between the 0 ng/ml treatment group (22%) and the 100 ng/ml treatment group (23%) for embryos that had cleaved but were < 8 cells. Also, there was no difference between the 100 ng/ml treatment group (23%) compared with the 1,000 ng/ml treatment group (32%) for cleaved embryos that were < 8 cells on day 3. There were no differences in percentage of embryos developing to the 8- to 16-cell stage among the 0 ng/ml treatment group (46%), the 100 ng/ml treatment group (49%) and the 1,000 ng/ml treatment group (44%) (Table 7.2).

On day 7 of in vitro culture, there was no significant difference between the 0 ng/ml treatment group (6%), the 100 ng/ml treatment group (5%) and the 1,000 ng/ml treatment group (4%) for embryos developing to the morula stage. Likewise, there was

Table 7.2. Development of bovine embryos after in vitro culture in a modified bovine culture medium supplemented with different concentrations of leptin.

Leptin ² Supplement (ng/ml)	No. of zygotes cultured	No. and (%) of zygote developed to ¹				Ratio of blastocysts: 8- to 16-cell embryos
		<8 cells	8- to 16-cells	Morulae	Blastocysts	
0	342	76 (22) ^a	158 (46)	20 (6)	138 (40)	0.87
100	342	79 (23) ^{a,b}	166 (49)	18 (5)	128 (37)	0.77
1,000	342	110 (32) ^b	149 (44)	14 (4)	136 (40)	0.91

¹ Percentage of the number of zygotes cultured.

² Recombinant rat leptin (Sigma Aldrich, St. Louis, MO).

^{a,b} Different superscripts in a column of each parameter were significantly different (P<0.05).

no difference in percentage of embryos that developed to the blastocyst stage for the 0 ng/ml treatment group (40%), the 100 ng/ml treatment group (37%) and the 1,000 ng/ml treatment group (40%). Furthermore, there were no differences in the ratio of blastocysts:8- to 16-cell stage between the 0 ng/ml treatment group (0.87), the 100 ng/ml treatment group (0.77) and the 1,000 ng/ml treatment group (0.91).

In addition, there was no difference in the percentage of embryos developing to the expanded blastocyst stage from cleaved 8- to 16-cell embryos between the 0 ng/ml treatment group (43%), the 100 ng/ml treatment group (52%) and the 1,000 ng/ml treatment group (56%) (Figure 7.1). Likewise, there was no difference in the percentage of embryos developing to the hatched blastocyst stage from cleaved 8- to 16-cell embryos between the 0 ng/ml treatment group (7%), the 100 ng/ml treatment group (8%) and the 1,000 ng/ml treatment group (4%).

Embryo Stage and Quality Grade (mRED)

Mean mRED scores by replicate and treatment group are presented in Table 7.3. There was a significant difference ($P < 0.05$) in the mean mRED score between the 1,000 ng/ml treatment group (1.23 ± 0.12) in Replicate I compared with the 1,000 ng/ml treatment group (155 ± 0.13) in Replicate II. However, there was no difference in the mean mRED scores among the 0 ng/ml treatment group (153 ± 0.13), the 100 ng/ml treatment group (132 ± 0.13) and the 1,000 ng/ml treatment group (123 ± 0.12) in Replicate I. Likewise, there were no differences in the mean mRED scores among the 0 ng/ml treatment group (1.28 ± 0.13), the 100 ng/ml treatment group (1.14 ± 0.12) and the 1,000 ng/ml treatment group (1.55 ± 0.13) in Replicate II.

Morphological Analysis

Mean inner cell mass, trophoblast and total cell numbers for embryos cultured for 6 days in modified culture media supplemented with 0 ng/ml, 100 ng/ml and 1,000 ng/ml of recombinant rat leptin are presented in Table 7.4. In Replicate I, stained expanded blastocysts in the 0 ng/ml treatment group (34 ± 3.3) and the 100 ng/ml treatment group (28 ± 2.8) had a greater ($P < 0.05$) number of cells in the inner cell mass compared with those in the 1,000 ng/ml treatment group (16 ± 1.9). There was no difference in number of cells in the inner cell mass of expanded blastocysts in Replicate II among the 0 ng/ml

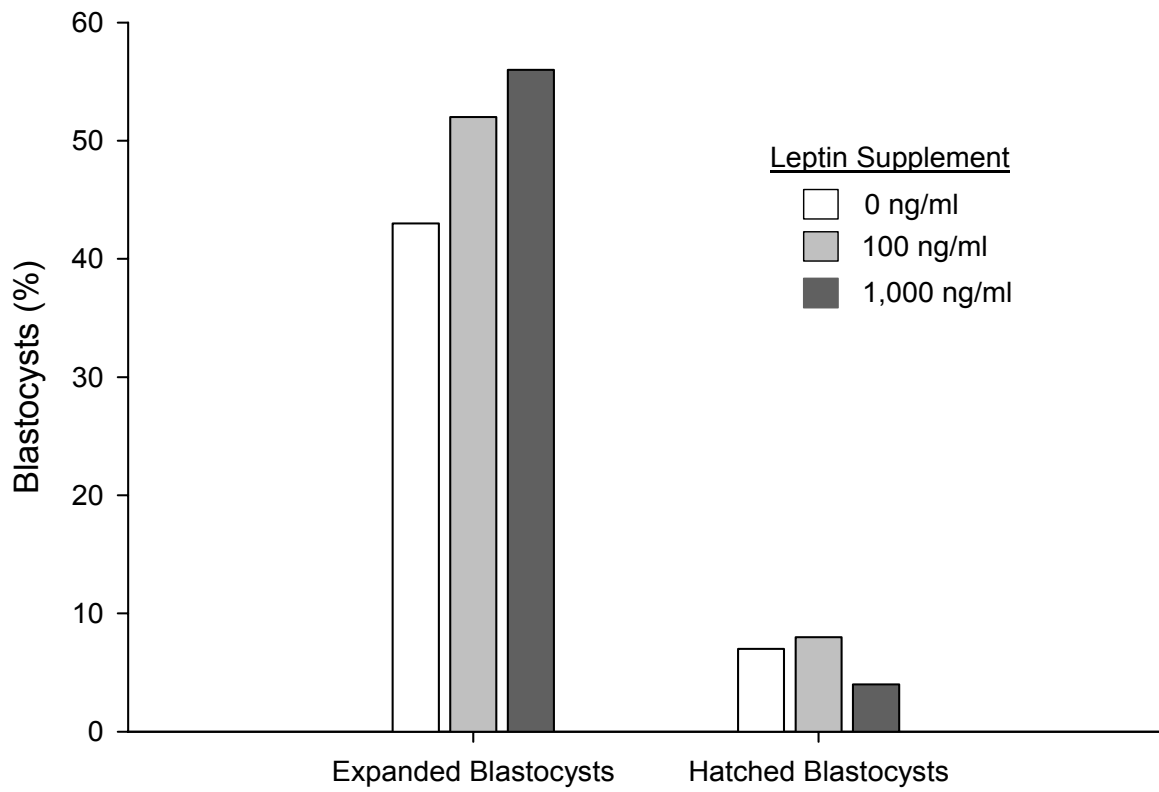


Figure 7.1. Development of blastocysts after culture in a modified bovine culture medium with supplemented with different concentrations of leptin.

Table 7.3. Mean mRED¹ scores of bovine embryos after 6 days of in vitro culture in a modified bovine culture medium supplemented with different concentrations of leptin.

Replicate	Leptin supplement ² (ng/ml)	n	mRED score ³
I	0	177	1.53 ± 0.13
	100	177	1.32 ± 0.13
	1,000	177	1.23 ± 0.12 ^a
II	0	165	1.28 ± 0.13
	100	165	1.14 ± 0.12
	1,000	165	1.55 ± 0.13 ^b

¹ mRED score as previously described by Collins (2000).

² Recombinant rat leptin (Sigma, L-5037, Sigma Aldrich, St. Louis, MO).

³ Mean ± SEM.

^{a,b} Numbers across replicates with different superscripts are significantly different (P<0.05).

Table 7.4 Mean inner cell mass, trophectoderm and total cells for bovine embryos after 6 days of in vitro culture in a modified bovine culture medium supplemented with different concentrations of leptin.

Replicate	Leptin supplement ¹ (ng/ml)	n	Cell counts ²		
			ICM ³	TE ⁴	TC ⁵
I	0	10	34 ± 3.3 ^a	80 ± 6.8 ^a	113 ± 8.5 ^a
	100	10	28 ± 2.8 ^a	53 ± 8.6 ^{a,b}	87 ± 10.7 ^{a,b}
	1,000	9	16 ± 1.9 ^b	42 ± 5.5 ^b	69 ± 8.4 ^b
II	0	10	30 ± 13.3	61 ± 5.3	90 ± 0.5
	100	10	35 ± 12.0	69 ± 8.4	104 ± 30.9
	1,000	11	24 ± 10.9	86 ± 12.1	110 ± 42.7

¹ Recombinant rat leptin (Sigma Aldrich, St. Louis, MO).

² Mean ± SEM.

³ ICM = Inner cell mass.

⁴ TE = trophectoderm.

⁵ TC = Total number of cells.

^{a,b} Means with different superscripts within columns are significantly different (P<0.05).

treatment group (30 ± 13.3), the 100 ng/ml treatment group (35 ± 12.0) and the 1,000 ng/ml treatment group (24 ± 10.9).

In Replicate I, there were significantly more total cells in stained expanded blastocysts in the trophoblast in the 0 ng/ml treatment group (80 ± 6.8) compared with the 1,000 ng/ml treatment group (42 ± 5.5). However, there was no difference among the 0 ng/ml treatment group (80 ± 6.8) and the 100 ng/ml treatment group (53 ± 8.6); nor was there a significant difference between the 100 ng/ml treatment group (53 ± 8.6) and the 1,000 ng/ml treatment group (42 ± 5.5). In Replicate II, there were no significant differences in the number of trophoblast cells in stained expanded blastocysts among the 0 ng/ml treatment group (61 ± 5.3), the 100 ng/ml treatment group (69 ± 8.4) and the 1,000 ng/ml treatment group (86 ± 12.1).

There were more ($P < 0.05$) total cells in stained expanded blastocysts in Replicate I from the 0 ng/ml treatment group (113 ± 8.5) when compared with the 1,000 ng/ml treatment group (69 ± 8.4). However, there was no difference in total number of cells from stained expanded blastocysts between the 0 ng/ml treatment group (113 ± 8.5) when compared with the 100 ng/ml treatment group (87 ± 10.7). Also, there were no significant differences in total number of cells from stained expanded blastocysts between the 100 ng/ml treatment group (87 ± 10.7) when compared with the 1,000 ng/ml treatment group (69 ± 8.4). In Replicate II, there were no differences in total number of cells from stained expanded blastocysts among the 0 ng/ml treatment group (90 ± 0.5), the 100 ng/ml treatment group (104 ± 30.9) and the 1,000 ng/ml treatment group (110 ± 42.7).

Stained expanded blastocysts the 1,000 ng/ml treatment group (3.8 ± 0.9) had a greater ($P < 0.05$) trophoblast:inner cell mass ratio compared with the 100 ng/ml treatment group (2.0 ± 0.2) but was not different from the 0 ng/ml treatment group (2.6 ± 0.3) (Table 7.5).

Discussion

In our study, the addition of leptin to the culture medium of bovine embryos did not enhance in vitro embryo developmental rates. In fact, the addition of leptin at 1,000 ng/ml negatively impacted early embryo cleavage rates, when fewer embryos cleaved to the 8- to 16-cell stage in the 1,000 ng/ml treatment group compared with the control

Table 7.5. Mean trophoblast cell to inner cell mass ratio for bovine embryos after 6 days of culture in a modified bovine culture medium supplemented with different concentrations of leptin.

Leptin supplement (ng/ml)	n	TE/ICM ¹
0	20	2.6 ± 0.3 ^{a,b}
100	20	2.0 ± 0.2 ^a
1,000	18	3.8 ± 0.9 ^b

¹ TE = trophoblast cells, ICM = inner cell mass cells.

^{a,b} Numbers with different superscripts are significantly different (P<0.05).

treatment group (no leptin). These results are not in agreement with those reported by Kawamura et al. (2002), who reported addition of exogenous leptin added to embryo culture medium increased the development of mouse embryos from the 2-cell stage through to the hatched blastocyst stage. It should be noted that these researchers also reported that mRNA for the leptin receptor was detected in mouse oocytes. However, leptin mRNA was detected after the blastocyst stage suggesting that the receptors were of maternal origin and the leptin protein per se may be originating from the embryonic gene. In our study, we did not quantify leptin production from the developing embryos, so this finding could not be substantiated for cattle embryos.

Gonzalez et al. (2000) hypothesized that leptin and leptin receptors in the human epithelial endometrial cells may work in concert to direct implantation and subsequent embryonic development in the mouse and human. In our study, the lack of enhanced development of embryos cultured with increased in vitro levels of leptin may be explained by the lack of other stimulatory factors produced by the uterine epithelial cells are needed to encourage embryonic development and growth. In the future, the circulating physiological leptin levels in the cow need to be evaluated with in vivo embryo development.

Our previous results (Chapter VI) show that both oviduct and uterine epithelial cells possess the signaling form of the leptin receptor. Paria and Dey (1990) have previously shown that Epidermal Growth Factor (EGF) and Transforming Growth Factor (TGF) (" and \$1) improved in vitro developmental rates of early stage mouse embryos. Also, these researchers reported that detection of EGF receptors on the embryonic cell surface suggests beneficial effects of EGF or TGF on pre-implantation embryo development in the mouse.

More recently, Watson et al. (2005) reported that transcripts for TGF" and Platelet-Derived Growth Factor (PDGF) were detectable in all prehatching bovine stage embryos, as has been reported in the mouse. Also, transcripts for TGF\$2 and IGF and receptors for PDGF" , insulin, IGF-I and IGF-II were detectable throughout bovine prehatching development. It was suggested that the mRNAs are products of both the maternal and embryonic genomes in the cow, whereas, in the mouse they are only present following activation of the embryonic genome at the 2-cell stage.

Two decades ago, it was hypothesized that a growth factor loop existed between the early conceptus and the uterus for increased cell differentiation, cell proliferation and cell migration in the early pregnancy (Simmen and Simmen, 1991). There are at least two possible reasons for the lack of influence of leptin on bovine embryos: (1) for leptin to influence embryonic development, the uterine tissues may be necessary to complete the growth factor loop or (2) the roles and biologic pathways for leptin in the mouse and human may be different in the cow. Our study certainly indicates that when using standard IVF procedures the addition of greater than physiological leptin levels to the culture medium was not beneficial in developing bovine embryos to the blastocyst stage in vitro. It is also possible rat leptin does not stimulate growth of bovine embryos and may be somewhat detrimental to the <8 cell embryos.

The differences in mRED scores across replications cannot be explained at this time. However, because there were no differences across treatment groups within each replicate, the addition of rat leptin to the culture medium of bovine embryos at the levels used in this experiment, does not enhance embryo development to the blastocyst stage in vitro.

In our study, the decrease in the number of cells in the inner cell mass from the 0 ng/ml group to the 1,000 ng/ml leptin group was somewhat surprising. Based on the results that we reported in Chapter VI, the lower cell number per embryo in the 1,000 ng/ml group for both trophoctoderm and total cells per embryo was unexpected. Our earlier results (Chapter VI) showed increased leptin receptor numbers in the trophoctoderm compared with that of the inner cell mass. The cell numbers we found in our study were lower than those reported by Knijn et al. (2003) for both in vivo (205 cells) and in vitro-produced (160 cells) bovine embryos.

We expected to see increased proliferation of trophoblast cells. It should not be overlooked that the time of implantation is much earlier in the mouse and human than in the cow. Furthermore, placental attachment types are also morphologically different in the mouse and human than that of the cow. This could suggest that leptin may influence conceptus development much later in the cow.

Conclusions

The results of the present study show that the addition of leptin to the culture medium of in vitro-produced embryos does not influence development to the blastocyst stage, but can negatively influence cell proliferation in the inner cell mass, trophoblast and total cell numbers of the bovine embryo. Also, the addition of leptin to the culture medium of in vitro-produced embryos did not influence the mRED score (characterizes both stage and quality grade of the embryo). It is apparent that more studies must be conducted to determine if different pathways are utilized by leptin in the cow compared with other more studied species, such as the mouse and human.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

The primary objectives of this study were to determine the circulating levels of leptin in different female production groups in beef cattle and to determine the effects of leptin on bovine embryo development in vitro. Information on the effects of leptin on pregnancy in the beef cow is limited, and development of an in vitro culture (IVC) protocol that would increase the number of transferable embryos would enhance both the experimental and commercial use of IVF and IVC techniques for the beef female.

In Experiment 1, the effect of exogenous dexamethasone on circulating LH, FSH, leptin and progesterone in postpartum beef cows was assessed. Results showed that mean circulating LH, leptin and progesterone concentrations were not affected by dexamethasone treatment. Mean circulating FSH levels were significantly higher in the post-treatment phase of the control group only when compared with the post-treatment phase of the dexamethasone treatment group, which suggests that in the early postpartum beef cow, administration of dexamethasone may not have an effect on circulating levels of leptin levels as have been reported in the horse and human. Therefore, the mechanism of leptin release in the beef cow may be different from those that have been described in the literature for horses and humans.

In Experiment 2, we assessed the influence of female age, body weight and body condition on circulating leptin levels in crossbred beef cattle. Results showed that circulating leptin levels were not correlated with female age or body weight. However, circulating leptin concentrations were positively correlated with body condition scores in heifers and cows up to age 11. We showed that circulating leptin concentrations were higher in heifers compared with 2 year old cows, but were not different among heifers, cows between 3 and 6 years old and cows >6 years of age. Results also indicated that circulating leptin levels were higher in cows older than 6 years compared with 2 year old cows. Furthermore, there was a significant negative correlation between circulating leptin concentrations and female age in heifers and cows <3 years of age. Our results also indicate that in cows older than 3 years of age, their body condition score is positively correlated with circulating levels of leptin. Therefore, we concluded that culling open females at the end of each breeding season may have indirectly select for females

with levels of leptin high enough to meet the critical demand for the release of LH and subsequent ovarian function, leaving the most productive females in the breeding herd.

In Experiment 3, circulating leptin levels in lactating and nonlactating beef females following artificial insemination were evaluated in heifers, 2 year old and 3 year old cows. We showed that there were no differences in circulating leptin concentrations among 2 year old and 3 year old cows pregnant from AI, the clean-up bulls or those that remained open after a 60-day breeding season. Our results point out that circulating leptin concentrations were lower for lactating cows compared with nonlactating cows. We also showed that circulating leptin levels were different across sample days and may have been influenced by precipitation and pasture growth during the experimental period. We concluded that in pregnant postpartum cows and heifers, leptin may be repartitioned nutrients away from circulation possibly to support a growing fetus. Because circulating leptin levels were more variable for nonlactating 2 year old and 3 year old females compared with the same age lactating females, lactation may moderate circulating leptin levels so that food intake is not reduced during this period of increased energy demand.

In Experiment 4, the expression of the long form of the leptin receptor in bovine oviduct epithelial, uterine epithelial cells and bovine blastocysts were evaluated. Results from our study show that both oviduct and uterine endometrial epithelial cells, and uterine biopsies from mid-luteal stage reproductive tracts, stained positive for the long form of the leptin receptor. However, not all uterine endometrial epithelial cells showed uptake of the leptin receptor stain. Our findings suggest that if communication does exist between endometrial epithelial cells and the developing embryo it starts much earlier than the blastocyst stage. Based on this study, there may be cellular communication between the oviduct epithelial cells and early bovine embryo. This could act as a control of pregnancy for the female, and may be an 'in vivo developmental block' if nutritional status becomes critical after ovulation but before implantation occurs.

In the final experiment, development of in vitro-derived bovine embryos cultured in different concentrations of leptin was evaluated. Our results indicate that the addition of leptin to the culture medium of in vitro-produced embryos does not influence development to the blastocyst stage, but can negatively influence cell proliferation in the

inner cell mass, trophoblast and total cell numbers of the bovine embryo. Also, the addition of leptin to the culture medium of in vitro-produced embryos did not influence the mRED score. It is apparent that more studies must be conducted to determine if different pathways are utilized by leptin in the cow compared with other more studied species, such as the mouse and human.

Therefore, we conclude that the pathways for the control and release of leptin in the beef cow may be different from those reported of other farm animals, mice, rats and humans. Also, it appears that even though leptin receptors are expressed in oviduct epithelial cells, uterine epithelial cells and the trophoblast cells of the developing bovine blastocyst, the addition of exogenous rat leptin does not directly influence in vitro embryo developmental rates in the cow. However, we did show that there were increases in circulating leptin levels in heifers and cows 35 days following AI, which may be involved in implantation as has been previously hypothesized for the mouse and the human. Therefore, more studies must be conducted to fully understand the role(s) of leptin in cattle reproduction.

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APPENDIX A
COMPOSITION OF MODIFIED BOVINE EMBRYO HOLDING MEDIUM

Components	Catalogue no.	Amount
BSA Fraction V	A-3311	4.8 mg/ml
HEPES-TL	H-3784	1.568 ml/ml
Na Pyruvate	P-5280	16.0 : l/ml
Penicillin/Streptomycin	15140-122	16.0 : l/ml

APPENDIX B
COMPOSITION OF CR1AA STOCK MEDIUM

Components	Catalogue no.	mg/ml
NaCl	S-5886	6.7
KCl	P-5405	0.231
NaHCO ₃	S-5761	2.2
Na Pyruvate	P-4562	0.44
Na Lactate	L-4388	0.546
BSA Fraction V	A-4503	3.0

Rosenkrasen and First (1994).

APPENDIX C

COMPOSITION OF MODIFIED BOVINE EMBRYO CULTURE MEDIUM USED TO CULTURE EMBRYOS FROM DAY 0 TO 3

Components	Catalogue no.	mg/ml
NaCl	S-5886	6.7
KCl	P-5405	0.231
NaHCO ₃	S-5761	2.2
Na Pyruvate	P-4562	0.44
Na Lactate	L-4388	0.546
BSA Fraction V	A-4503	3.0
		: l/ml
Phenol Red	P-2090	1
BME ¹ Amino Acid Solution (X50)	B-6766	20
MEM ² Nonessential Amino Acid Solution (X100)	M-7145	10
Penicillin/Streptomycin	10437-101	10
L-Glutamine	G-5763	5
Glycine (X100)	G-8790	10
Alanine (X100)	A-7469	10

¹BME = Basal Medium Eagle.

²MEM = Modified Eagle's Medium.

APPENDIX D

COMPOSITION OF MODIFIED BOVINE EMBRYO CULTURE MEDIUM USED TO CULTURE ZYGOTES FROM DAY 3 TO 7

Components	Catalogue No.	mg/ml
NaCl	S-5886	6.7
KCl	P-5405	0.231
NaHCO ₃	S-5761	2.2
Na Pyruvate	P-4562	0.44
Na Lactate	L-4388	0.546
BSA Fraction V	A-4503	3.0
		: l/ml
Phenol Red	P-2090	1
BME ¹ Amino Acid Solution (X50)	B-6766	20
MEM ² Nonessential Amino Acid Solution (X100)	M-7145	10
Penicillin/Streptomycin	10437-101	10
Fetal Bovine Serum	10437-101	50
L-Glutamine	G-5763	5
Glycine (X100)	G-8790	10
Alanine (X100)	A-7469	10

¹BME = Basal Medium Eagle.

²MEM = Modified Eagle's Medium.

VITA

Glen T. Gentry, Jr., the son of Margaret and Glen Gentry, was born February 9, 1966, in Baton Rouge, Louisiana. Upon graduation from Central High School in 1984, he attended Louisiana State University, and received his Bachelor of Science degree in animal science in 1989. During his undergraduate program he married Laura T. Roland and they have one son, Jacob Cameron.

Upon graduation he accepted the position of Divisional Manager (Baker Division) at J. B. Hunt Ranches in Fayetteville, Arkansas. In 1991, he accepted the position of Research Associate at the LSU AgCenter's Idlewild Research Station, in Clinton, Louisiana, where he is currently employed.

Glen received his master's degree from Louisiana State University in reproductive physiology under the direction of Dr. Robert A. Godke in 1999. He is now a candidate for the degree of Doctor of Philosophy in reproductive physiology in the Department of Animal Sciences under the direction of Dr. Robert A. Godke.