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This is to certify that the

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The Association of Preovulatory Follicular Events with Morphology and Progesterone of Corpora Lutea in Heifers Fed High or Low Energy Diets

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THE ASSOCIATION OF PREOVULATORY FOLLICULAR EVENTS WITH MORPHOLOGY AND PROGESTERONE OF CORPORA LUTEA IN HEIFERS FED HIGH OR LOW ENERGY DIETS

By

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ABSTRACT

THE ASSOCIATION OF PREOVULATORY FOLLICULAR EVENTS WITH MORPHOLOGY AND PROGESTERONE OF CORPORA LUTEA IN HEIFERS FED HIGH OR LOW ENERGY DIETS

By

Amy Lynn Frith Terhune

My goal was to determine if preovulatory follicular development was associated with subsequent development of corpora lutea (CL) in heifers fed high or low energy diets. Two groups of heifers were fed either high (PEB) or low (NEB) energy diets for 3.3 estrous cycles while a third group was fed the low energy diet for 2.0 and the high energy diet for 1.3 (NEB-PEB) estrous cycles. Preovulatory follicular development was estimated from concentrations of preovulatory serum estradiol in third estrous cycle. Luteal development was estimated from concentration of progesterone in serum and in CL in the fourth estrous cycle. NEB reduced duration of the preovulatory period. Independent of diet, duration of the preovulatory period was associated positively with percentage of large cells in CL. As peak estradiol increased, there was an increase in percentage of small cells in CL of PEB heifers, an increase in percentage of large cells in CL of NEB heifers, and an increase in ratio of large to small luteal cells in NEB-PEB heifers. In conclusion, development of CL increased as preovulatory follicular development increased. In addition, shorter duration of the preovulatory period due to NEB may limit preovulatory follicular development, thereby, reducing concentration of large luteal cells.

To my mother

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Introduction

Progesterone prepares the uterus for implantation of embryos and is necessary for maintenance of pregnancy in cattle (Catchpole, 1991). In the estrous cycle preceding or following insemination, serum concentration of progesterone was associated positively with conception and pregnancy rate (Carstairs et al., 1980; Folman et al., 1973; Fonseca et al., 1983; Kimura et al., 1987; Robinson et al., 1989). Most progesterone in serum is synthesized and released from the corpus luteum in postpubertal female cattle (Keyes and Wiltbank, 1988). Thus, progesterone from the corpus luteum is a major determinant of fertility.

Luteal cells that synthesize progesterone originate from steroidogenic cells of the preceding ovulatory follicle (Alila and Hansel, 1984). Thus, number of steroidogenic cells in an ovulatory follicle defines the number of steroidogenic luteal cells at the beginning of corpora lutea development. Our hypothesis is that increased development of an ovulatory follicle will increase development of a corpus luteum.

Ovulatory follicles and corpora lutea vary in size, number of steroidogenic cells and steroid production (Ireland and Roche, 1984; McNatty et al., 1984; O'Shea et al., 1986; 1989; Webb and England, 1982). Among heifers, maximal serum concentration of estradiol during the preovulatory period varies five-fold (Walters and Schallenberger, 1984), while maximal serum concentration of progesterone during diestrus varies four-fold (Peters, 1991). Many hormones that stimulate follicular development also stimulate luteal development, such as: luteinizing hormone (Ireland, 1985; Murphy and Silivan, 1988), insulin (May et al., 1981; Murphy and Silivan, 1989), insulin-like growth hormone-I (Mondschein et al., 1989; Sauerwein et al., 1992), and growth hormone (Langout et al., 1991; Lanzone et al., 1992). Thus, corpora lutea are developmentally related to ovulatory follicles and have similar regulatory factors. The broad objective of this study was to determine if development of a preovulatory follicle is associated with development of the subsequent corpus luteum.

Negative energy balance in postpartum dairy cows reduced serum and milk progesterone (Spicer et al., 1990; Villa-Godoy et al., 1989). Similarly, NEB in heifers reduced serum progesterone, luteal concentration of progesterone and luteal weight (Gombe and Hansel, 1973; Harrison and Randel, 1986; Hill et al., 1970; Murphy et al., 1990; Villa-Godoy et al., 1990). Thus, negative energy balance may adversely affect fertility because of adverse effects on corpora lutea. There are three possible mechanisms by which negative energy balance may reduce luteal development: 1) reduced luteotropic factors, 2) reduced development of antecedent ovulatory follicle(s) or 3) a combination of the above. Luteotropic factors (luteinizing hormone & insulin) were not associated with luteal development in heifers experiencing negative energy balance (Schrick et al., 1992; Villa-Godoy et al., 1990). Thus, another objective was to determine if preovulatory follicular development is associated with luteal development in heifers experiencing negative energy balance.

In the following review of literature, I will discuss: 1) factors that affect serum concentration of progesterone, 2) cellular continuity of ovulatory follicles with ensuing corpora lutea, 3) effects of negative energy balance on follicles and corpora lutea and 4) effects of negative energy balance on hormones that regulate follicular and luteal development.

Review of Literature

Factors That Affect Secretion of Progesterone

Maximal serum concentration of progesterone varies four-fold among normal heifers during the estrous cycle (Peters, 1991). Variation of serum progesterone is explained largely by variation in luteal weight (Garverick et al., 1985; Peters, 1991; Spicer et al., 1981). Percent of dry matter of luteal tissue does not vary among corpora lutea, therefore, tissue edema does not explain variation in luteal weight (Peters, 1991). The other major determinant of luteal weight is number of cells in corpora lutea. Hyperplasia increases number of steroidogenic cells in bovine corpora lutea until d8 postestrus (Donaldson and Hansel, 1965; Lei et al., 1991; Moss et al., 1954; Niswender et al., 1985). Weight of luteal tissue, number of capillaries and number of steroidogenic cells in corpora lutea increase coincidently and are associated positively with serum progesterone (Lei et al., 1991; Moss et al., 1954; Peters, 1991; Spicer et al., 1981).

Small and large cells are found within a corpus luteum. These cells have distinct morphological and biochemical differences (Koos and Hansel, 1981; Ursely and Leymarie, 1979). For example, small bovine luteal cells range from 10 to 25μm in diameter and respond to luteinizing hormone with a 6 to 11-fold increase in progesterone secretion (Koos and Hansel, 1981; Meidan et al., 1990). In contrast, large bovine luteal cells range from 25 to 50μm in diameter and respond to luteinizing hormone with a 1.4-fold increase in progesterone secretion (Koos and Hansel, 1981; Niswender et al., 1985). In sheep and cattle (Fitz et al., 1982; Koos and Hansel, 1981), large luteal cells secrete 20-fold more progesterone than small luteal cells when not stimulated by luteinizing hormone. In ewes, small luteal cells ($\approx$ 76.8 x 10^6 cells/corpus luteum) contribute approximately 22% of progesterone to serum while large luteal cells ($\approx 9.6 \pm 0.9$ x 10^6 cells/corpus luteum) contribute approximately 78% of progesterone to serum (Niswender et al., 1985; O'Shea et al., 1987). Thus, total number and proportion of each type of steroidogenic cell in corpora lutea contribute to variation in serum progesterone.

The primary luteotropin in cattle is luteinizing hormone (Smith, 1986). Luteinizing hormone is synthesized then released in a pulsatile manner from the anterior pituitary gland in response to luteinizing hormone releasing hormone (McCann, 1974). After luteinizing hormone binds to receptors on luteal cells (Spicer et al., 1981), secretion of progesterone increases (Hansel, 1971; Mason et al., 1962; Rodgers et al., 1988). Between d4 and d11 postestrus frequency of luteinizing hormone pulses decreases while amplitude of pulses and basal luteinizing hormone does not change (Schallenberger et al., 1985; Walters et al., 1984). During diestrus, concentration of luteinizing hormone and progesterone in serum are not correlated (Spicer et al., 1981). But, number of luteinizing hormone receptors per luteal cell is associated positively with serum progesterone (Garverick et al., 1985; Spicer et al., 1981). However, luteinizing hormone in serum is important for normal luteal development because passive immunization against luteinizing hormone reduced luteal weight and progesterone content in corpora lutea of heifers (Snook et al., 1969).

Relationship Between Ovulatory Follicles and Subsequent Corpora Lutea

Two to three days before estrus, the ovulatory follicle can be identified as the largest follicle in either ovary of cattle (Dufour et al., 1972; Sirois and Fortune, 1988). The preovulatory follicle produces 500 to 1000-fold more estradiol than other nondominant follicles (Staigmiller et al., 1982) and is responsible for increased serum estradiol during the bovine and ovine preovulatory period (England et al., 1981; Ireland et al., 1984; Murdoch and Dunn, 1982; Webb and England, 1982). Granulosal and thecal cells are the steroidogenic cells of a dominant ovulatory follicle which after luteinization become the steroidogenic cells of a corpus luteum (Meidan et al., 1990; Figure 1). In preovulatory follicles, luteinization of granulosal and thecal cells begins with the preovulatory surge of luteinizing hormone. Luteinization changes the primary product of steroidogenesis from estradiol to progesterone (Ireland and Roche, 1982; Murdoch and Dunn, 1982). During early diestrus (d4 to d6) large luteal cells are derived from granulosal cells and small luteal cells are derived from thecal cells. In mid-diestrus (d10 to d12) a portion of small luteal cells become large luteal cells (Alila and Hansel, 1984). Consequently, the ratio of large to small luteal cells increases between early (d6) and mid-diestrus (d12) (Lei et al., 1992). Since granulosal and thecal cells in ovulatory follicles are the parental population for luteal cells, variation in number of either or both type of parental cells could contribute to variation in number and ratio of steroidogenic luteal cells. The effect of total number and proportion of granulosal and thecal cells in a preovulatory follicle on number of luteal cells has not been determined. But, removal of granulosal cells from preovulatory follicles in monkeys (Kreitmann et al.,

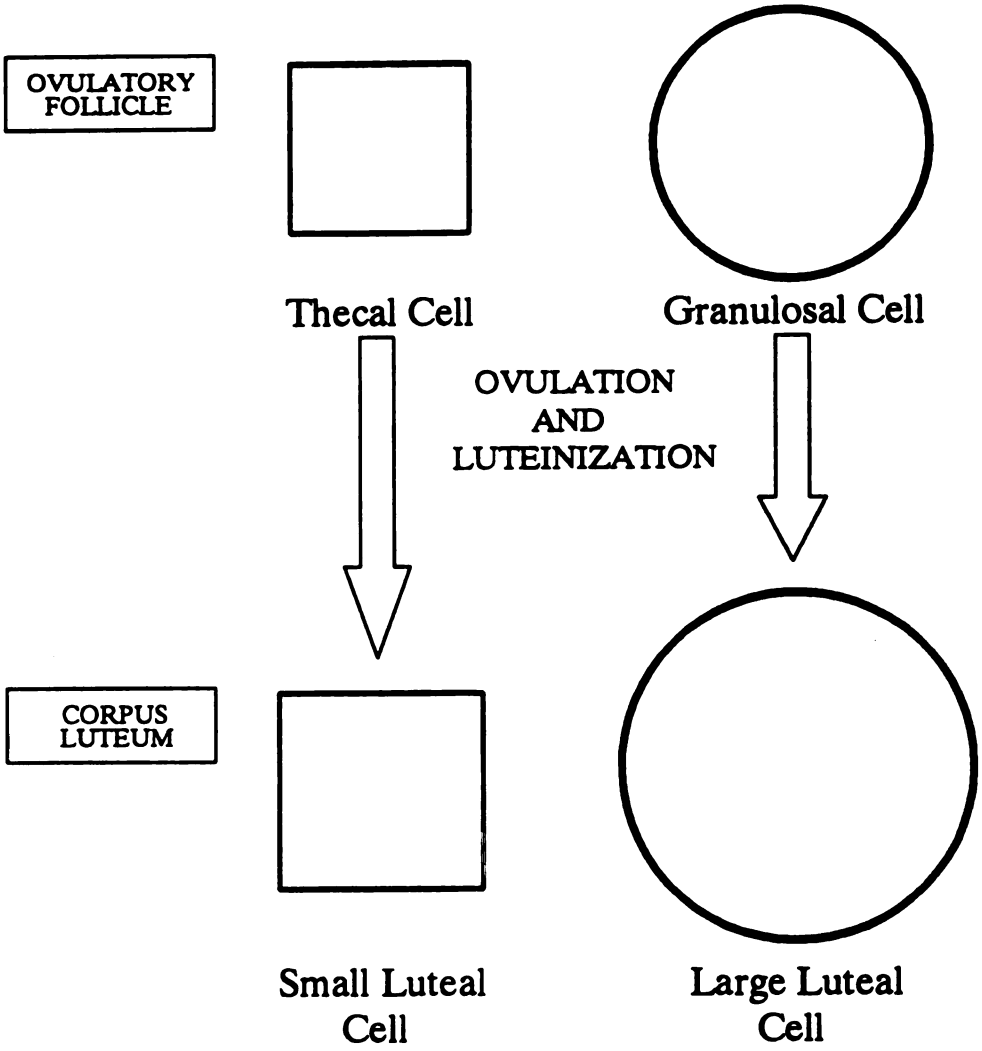


Figure 1. A model of the cellular continuity between an ovulatory follicle and a corpus luteum.

1981; Marut et al., 1983) and heifers (Milvae et al., 1991) reduced serum concentration of progesterone in the ensuing luteal phase. Number of granulosal cells removed was associated negatively with concentration of progesterone serum in the subsequent luteal phase of monkeys (Krietmann et al., 1981). An interpretation of these data is that removal of granulosal cells reduced the number of large luteal cells and reduced luteal mass which reduced progesterone.

Precocious induction of ovulation with intrafollicular injection of follicle stimulating hormone or luteinizing hormone reduced concentration of progesterone in serum of the ensuing luteal phase of ewes (Murdoch et al., 1983). In monkeys, reduction of serum follicle stimulating hormone during the early follicular phase reduced preovulatory serum estradiol and subsequent luteal phase progesterone (Stouffer et al., 1981). Thus, manipulations that shortened lifespan of or reduced development of preovulatory follicles, and presumably reduced number of follicular cells, decreased luteal development. But, it is not known if spontaneous variation in development of ovulatory follicles contributes to variation in luteal development.

Energy Balance

A definition of gross energy balance (EB) for dairy cows is net energy consumed minus net energy required for maintenance, growth and lactation (Moe et al., 1972). When energy intake exceeds energy requirements, an animal is in positive energy balance (PEB). Alternatively, when energy requirements exceed energy intake, an animal is in negative energy balance (NEB).

Approximately 80% of postpartum dairy cows experience negative energy balance in early lactation (Coppock et al., 1974; Reid et al., 1966; Villa-Godoy et al., 1988).

During early lactation, when appetite is low and milk yield is high, most dairy cows do not consume enough energy to satisfy total requirement for net energy (Coppock et al., 1974). Nadir of negative energy balance in lactating cows ranges from -9 to -16Mcal/d and occurs within 14d postpartum (Bauman and Currie, 1980; Villa-Godoy et al., 1988). Duration of negative energy balance can extend from calving until 16 weeks postpartum (Butler and Smith, 1989; Coppock et al., 1974; Villa-Godoy et al., 1988). Magnitude and duration of negative energy balance are highly variable and are explained largely by variation in energy ingested by cows (Villa-Godoy et al., 1988).

To maintain a 12 to 13 month calving interval, conception must occur 60 to 100d postpartum. Dairy cows with high milk yields often experience low conception rates between d60 and d120 postpartum (Faust et al., 1988; Ferguson, 1991) and frequently do not achieve a conception interval of 12 to 13 mo. But, the association between milk yield and fertility is inconsistent (Butler and Smith, 1989; Fonseca et al., 1983; Staples et al., 1990) so the adverse effect of milk yield on fertility is equivocal. Many cows are still in negative energy balance during the first third of lactation and limited voluntary energy intake, not milk yield, is the primary cause of negative energy balance. Thus, EB, not milk yield may affect conception rates. Unfortunately, there are no studies that combine detailed data on EB with adequate number of cows for reliable fertility data. So effects of EB on fertility have not been tested directly. However, EB in early lactation was associated positively with concentration of progesterone in serum and milk during the second and third postpartum estrous cycle (Spicer et al., 1990; Villa-Godoy et al., 1988; Table 1). The significance of this is that progesterone in the estrous cycle before or after insemination was associated positively with conception rate (Carstairs et al., 1980; Fonseca et al., 1983). An interpretation is that low progesterone can limit fertility. Thus, the effect of EB on fertility in early lactation may be mediated by effects of EB on progesterone. To address the effect of negative energy balance on luteal development and fertility, some investigators have used heifers fed low energy diets to avoid confounding effects of EB with interval postpartum, suckling, sensitivity of anterior pituitary gland to luteinizing hormone releasing hormone and reproductive diseases.

Endocrine and Metabolic Milieu during Negative Energy Balance

Luteinizing Hormone

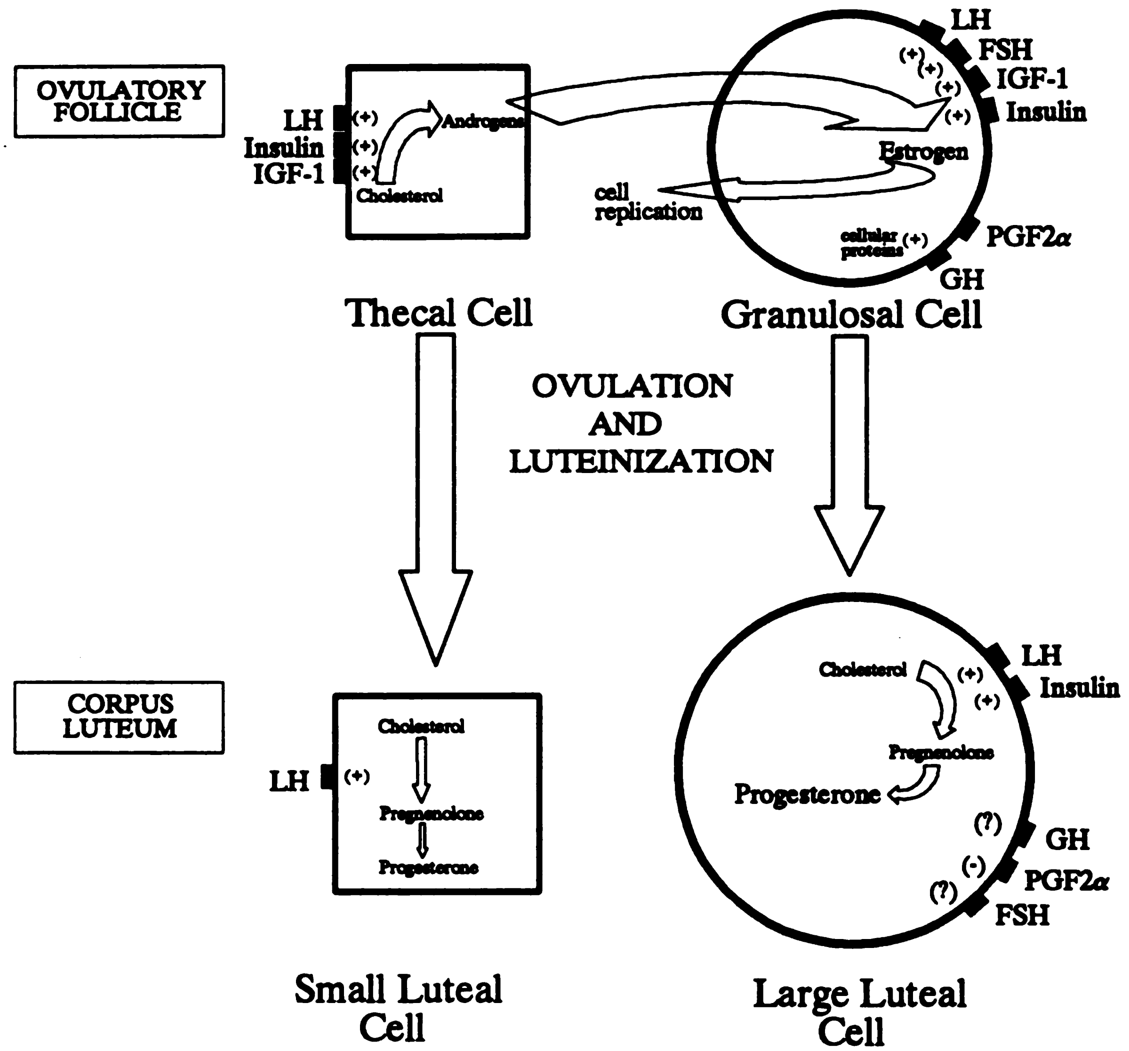
During luteal development, negative energy balance increased (Gombe and Hansel, 1973), decreased (Apgar et al., 1974; Imakawa et al., 1984; reviewed by Schillo, 1992) or had no effect on serum luteinizing hormone (Beal et al., 1978; Harrison and Randel, 1986; Hill et al., 1970; Schrick et al., 1992; Villa-Godoy et al., 1990) (Table 1). In heifers during proestrus and estrus, negative energy balance increased concentration of luteinizing hormone in serum (Gombe and Hansel, 1973; Table 1) or reduced amplitude of luteinizing hormone pulses during proestrus (Imakawa et al., 1986).

Corpora lutea and antral follicles are target tissues for luteinizing hormone. The plasma membrane of granulosa, theca and both types of luteal cells contain receptors for luteinizing hormone (Garverick et al., 1985; Ireland, 1987; Spicer et al., 1981; Figure 2). In fact, there are similar effects of luteinizing hormone on follicular and luteal cells including: 1) activation of adenylate cyclase, 2) activation of Ca^{2+}

Table 1. Effects of restricted dietary energy (NEB) on hormones and metabolites in bovine serum.

Hormones & Metabolites	Effects of NEB on Serum Concentrations	References
Progesterone	↓	Gombe and Hansel, 1973
		Imakawa et al., 1983
		Villa-Godoy et al., 1990
		Spicer et al., 1990
	→	Murphy et al., 1991
LH	↑	Gombe and Hansel, 1973
	↓	Imakawa et al., 1984
	→	Schrick et al., 1992
FSH	↓	Gauthier et al., 1983
	→	Dunn et al., 1974
		Perry et al., 1990
GH	↑	Houseknecht et al., 1988
		Villa-Godoy et al., 1990
IGF-I	↓	Spicer et al., 1991
Insulin	↓	Harrison and Randel, 1986
		Schrick et al., 1992
		Villa-Godoy et al., 1990
Corticosteroids	↓ or →	reviewed by I'Anson et al., 1991
NEFA	↑	Holmes and Lambourn, 1970
		Chillard et al., 1984

Figure 2. A model of the cellular continuity and regulatory homology between follicular and luteal cells. Receptors of the following: LH = luteinizing hormone; FSH = follicle stimulating hormone; IGF-I = insulin-like growth factor-I; GH = growth hormone; PGF2α = prostaglandin F2α. Effect of hormones on steroidogenesis indicated by +, -, or ?.


OVULATORY FOLLICLE
LH
Insulin
IGF-1
(+)
Androgens
Cholesterol
cell replication
Estrogen
LH
FSH
IGF-1
Insulin
PGF2α
GH
cellular proteins
Thecal Cell
Granulosal Cell
OVULATION AND LUTEINIZATION
CORPUS LUTEUM
LH
Cholesterol
Pregnenolone
Progesterone
LH
Insulin
GH
PGF2α
FSH
(?)
(-)
Small Luteal Cell
Large Luteal Cell

polyphosphoinositol-protein kinase C second messenger systems, 3) stimulation of steroidogenesis, 4) transport of cholesterol to cholesterol side chain cleavage enzyme complex in luteal cells and 5) uptake of lipoproteins (reviewed by Alila and Dowd, 1991; Ireland, 1987; Murphy and Silavin, 1989). High frequency pulses of luteinizing hormone are required for ovulation of dominant ovulatory follicles (Randel, 1990), and basal luteinizing hormone is essential for luteal development (Snook et al., 1969). Thus, effects of negative energy balance on follicles or corpora lutea could be direct effects of changes in luteinizing hormone.

Follicle Stimulating Hormone

Follicle stimulating hormone is synthesized in and released from the anterior pituitary gland in response to luteinizing hormone releasing hormone (Findley and Clark, 1987; Kesner and Convey, 1982). Restriction of dietary energy did not affect mean daily concentration of follicle stimulating hormone in beef cows during an estrous cycle (Dunn et al., 1974) or on d14, d42 and d70 after calving (Perry et al., 1990). In contrast, beef cows fed diets restricted in energy and protein pre- and postpartum had reduced serum follicle stimulating hormone 5, 15 and 30d after calving (Gauthier et al., 1983; Table 1). Restriction of dietary energy in ewes reduced pituitary and serum follicle stimulating hormone during preovulatory gonadotropin surge and during diestrus (I'Anson et al., 1990). However, effect of energy restriction on serum follicle stimulating hormone during the preovulatory follicular phase has not been tested in cattle.

Follicle stimulating hormone is essential for follicular development (Hisaw, 1947). Exogenous follicle stimulating hormone stimulates follicular growth (Mariana et al., 1991). Plasma membrane of granulosal cells in preantral and antral follicles contain receptors for follicle stimulating hormone (Richards, 1980; Figure 2). The actions of follicle stimulating hormone on bovine and rat granulosal cells include: 1) increasing the number of follicle stimulating hormone and luteinizing hormone receptors, 2) activation of adenylate cyclase and the cyclic AMP second messenger system, 3) proliferation of granulosal cells and 4) activation of aromatase (Baird, 1983; Hsueh et al., 1983; Ireland, 1987). Reduction of serum follicle stimulating hormone with exogenous follicular fluid delays ovulation (Quirk and Fortune, 1986). Since follicle stimulating hormone is necessary for follicular development, it was of interest to determine if negative energy balance affects preovulatory follicle stimulating hormone in serum. Although plasma membrane of bovine luteal cells contains follicle stimulating hormone receptors (Manns et al., 1984; Figure 2), no physiological function has been demonstrated.

Growth Hormone

Growth hormone is synthesized and secreted from the anterior pituitary gland in response to growth hormone releasing factor (McCann, 1974). Prepubertal and postpubertal heifers fed low energy diets had increased basal concentration of growth hormone, increased duration and amplitude of growth hormone pulses (Houseknecht et al., 1988; Park et al, 1989; Villa-Godoy et al., 1990; Table 1).

Previous investigations have focused on effects of growth hormone on growth or lactation but not reproduction. Effects of growth hormone on growth are mediated partially by insulin-like growth factor-I (IGF-I) (Boyd and Bauman, 1989). Similarly, some effects of growth hormone on follicles are mediated by IGF-I (Mondschein et al., 1989). However, there are growth hormone receptors on granulosal cells (Hsueh et al., 1983; Lucy et al., 1992a; Nett, unpublished; Figure 2) and there are recent reports that growth hormone has direct effects on follicles (Christman and Halme, 1992; Fowler and Templeton, 1991). In vitro, growth hormone increased synthesis of proteins (≥ 10 Kd) in bovine granulosal cells while growth hormone plus insulin increased number of granulosal cells (Laughout et al., 1991). In addition, growth hormone stimulated estradiol production in human granulosal cells (Mason et al., 1990). Administration of recombinant bovine somatotropin (rbST) increased number of antral follicles (2 to 5 mm) in heifers (Gong et al., 1991). Women with high endogenous growth hormone levels had increased serum estradiol during the preovulatory phase and more oocytes were recovered after ovarian hyperstimulation (Stone and Marrs, 1992). Exogenous growth hormone reduced the dose of human menopausal gonadotrophin required to induce ovulation in women (Homburg et al., 1988; 1990). Clearly, growth hormone has positive effects on selection of follicles. But, the mechanism by which growth hormone increases number, steroid production or sensitivity of follicles to gonadotropin is not known. When dietary energy is restricted endogenous growth hormone is increased (Houseknecht et al., 1988; Villa-Godoy et al., 1990). Whether negative energy balance and increased endogenous growth hormone affect preovulatory follicles has not been reported.

Large luteal cells contain growth hormone receptors (Lucy et al., 1992a). In lactating dairy cows, exogenous rbST increased (Schemm et al., 1990; Gallo and Block, 1991) or did not affect (Lefebvre and Block, 1992) serum concentration of progesterone during diestrus. In heifers, rbST during the luteal phase did not affect concentration of serum progesterone (Gong et al., 1991). Thus, effects of growth hormone on luteal development are equivocal.

Energy Balance and Follicles

Prolonged and severe restriction of dietary energy abolished ovulation in heifers (Imakawa et al., 1983; 1986a). Thus, extreme negative energy balance adversely affects folliculogenesis. In early postpartum dairy cows (Butler et al., 1981; Canfield and Butler, 1981) and beef cows (Perry et al., 1991; Short and Adams, 1988; Wiltbank et al., 1962; Wright et al., 1992), severe negative energy balance increased days to first ovulation. Dairy cows with the most severe negative energy balance had decreased number of large follicles (≥ 15 mm) before first postpartum ovulation (Beam et al., 1992). Short term (4d) restriction of dietary energy reduced the growth rate of preovulatory follicles in lactating dairy cows (Lucy et al., 1992b). Restriction of dietary energy reduced volume of follicular fluid in medium (7 to 9 mm) and large (≥ 10 mm) follicles and decreased size of medium follicles, but increased number of estrogen-active (non-atretic) medium follicles and concentration of estradiol in follicular fluid of large and small (4 to 6.9 mm) follicles at 20 and 35 days after calving (Rutter and Manns, 1991). Compared with heifers that maintained or gained body weight, energy restriction (10 wk) in heifers did not alter number of follicles (Spicer et al., 1991), but reduced diameter and persistence of preovulatory follicles (Murphy et al., 1991). Heifers that maintained or lost body weight had no change in concentration of estradiol or IGF-I in follicular fluid, but had reduced progesterone in follicular fluid of small (1 to 5.9 mm) and medium follicles (6.0 to 9.9 mm) compared to heifers which gained body weight (Spicer et al., 1991). Ratio of estradiol to progesterone in follicular fluid of small and medium follicles was increased in heifers that maintained or lost body weight compared to heifers which gained body weight. This suggests increased selection of follicles. However, heifers fasted for 24 or 48 hours had reduced concentration of estradiol, but not IGF-I, in follicular fluid of large follicles (≥ 10mm) during proestrus (Spicer et al., 1992). In heifers, the effects of negative energy balance on steroid concentration in follicular fluid are dependent upon duration of negative energy balance.

In postpartum cows, negative energy balance delayed resumption of ovulation, reduced number of large follicles, increased number of estrogen-active medium follicles and reduced growth rate of preovulatory follicles. In heifers, long-term (10 wk) negative energy balance decreased size and lifespan of preovulatory follicles, decreased concentration of progesterone in follicular fluid of small and medium follicles, but did not affect estradiol in follicular fluid of follicles. Different effects of negative energy balance on follicles in postpartum cows versus heifers may be due to variable magnitude of negative energy balance, physiological state or age.

Energy Balance and Corpora Lutea

Restricted dietary energy reduced development of corpora lutea. During the first two postpartum estrous cycles, peak progesterone concentration in positive energy balance cows was 1.8-fold greater than in negative energy balance cows (Spicer et al., 1990). Energy balance was associated positively with milk concentration of progesterone in second and third postpartum estrous cycles (Villa-Godoy et al.,1988). In heifers, restriction of dietary energy either decreased (Hill et al., 1970; Gombe and Hansel, 1973; Imakawa et al., 1983; Villa-Godoy et al., 1990) or did not affect

(Beal et. al., 1978; Murphy et al., 1991; Spitzer et al., 1978) serum concentrations of progesterone (Table 1). Similarly, restriction of dietary energy either reduced (Harrison and Randel, 1986) or did not affect (Gombe and Hansel, 1973) progesterone in corpora lutea. Luteal cells collected from heifers in negative energy balance did not produce as much progesterone in vitro when induced with luteinizing hormone as luteal cells collected from heifers in positive energy balance (Imakawa et al., 1983; Villa-Godoy et al., 1990). Inconsistent effects of negative energy balance on serum or luteal progesterone are due, in part, to variation among studies in magnitude and duration of negative energy balance. Independent of nutritional management in previous studies, negative energy balance reduced luteal weight in all previous studies (Dunn et al., 1974; Harrison and Randel, 1986; Hill et al., 1970; Gombe and Hansel, 1973; Villa-Godoy et al., 1990) except one (Imakawa et al., 1983). Thus, negative energy balance reduced serum progesterone in cattle because of: 1) decreased synthesis of progesterone per luteal cell and 2) decreased number of luteal cells.

Summary

During the luteal phase of an estrous cycle, there is 2 to 4-fold variation in concentration of progesterone in serum among healthy, well nourished cattle. Negative energy balance reduces serum progesterone in cattle. Some factors that cause spontaneous variation of serum progesterone are: concentration of luteotropic hormones in serum, luteal weight, number of receptors on luteal cells and cellular population of corpora lutea. Among these factors, luteal weight explains most of the variation in serum progesterone. As luteal weight increases there is an increase in number of luteal cells and an increase in concentration of progesterone in serum. There is an increase in number of steroidogenic cells in bovine corpora lutea until d10. But, follicular cells from ovulatory follicles contribute substantially to final number of steroidogenic cells in corpora lutea. For example, granulosal-lutein cells have to undergo one mitotic division to obtain the estimated number of large luteal cells ($\approx 45.0 \times 10^6$) in a mature corpus luteum. Therefore, a major question posed in this thesis is whether preovulatory follicular development is associated with subsequent luteal development? Specific aims of this research were: 1) to determine if preovulatory serum estradiol is associated with progesterone production, weight or cellular inventory of ensuing corpora lutea and 2) to determine if effects of negative energy balance on corpora lutea are associated with preovulatory serum estradiol.

Materials and Methods

Animals and Diets

Twenty-seven postpubertal nulligravid Holstein heifers with average body weight of 424 kg and age of 13 mo were group housed and exposed to ambient light and temperature between late April and early August. Heifers had individual access to feed between 1000 and 1400 h each day of the experiment. Heifers had free access to water for the remainder of the day. Heifers were adapted to facilities four weeks prior to the experiment. During the adaptation period heifers were fed diets to support growth (0.5 kg/d). Three days before the experimental phase, heifers were blocked (n=9) by body weight and assigned randomly from within block to three treatment groups: 1) positive energy balance (PEB), 2) negative energy balance (NEB), and 3) NEB followed by PEB (NEB-PEB). Each ration (PEB and NEB) was sampled weekly, composited and chemically analyzed (DHIA, Ithaca, NY). Ingredients and chemical analysis of diets are presented in Table 2. Diets were not isonitrogenous (PEB = 15% CP; NEB = 18% CP); but dietary crude protein was adequate without being excessive. During the adaptation period heifers received two injections of prostaglandin F2α 11d apart to synchronize estrus (Figure 3). Therefore, the experimental period started with heifers at estrus (d=0).

Duration of the experimental period was 3.3 estrous cycles. Heifers in PEB and NEB group were fed their respective diets throughout the experiment; NEB-PEB heifers received NEB diet for two and PEB diet for 1.3 estrous cycles. Individual body weights of heifers were measured on three consecutive days (1500h) weekly and

Table 2. Composition of diets.

	Diets[a]	
Ingredients(kg/d)	PEB	NEB
Alfalfa Hay	4.20	2.90
Cracked Corn	2.5	0.20
Soy Bean Meal	0.34	0.27
Minerals/vitamins	0.08	0.07
Chemical Analysis[b,c]		
Dry Matter %	89.40	90.00
Crude Protein (CP), %DM	15.00	18.00
Undeg. Protein,%CP	36.28	33.66
NDF, %DM	33.3	43.7
Intake		
DM Intake (kg/d)	7.22	3.51
DM Fed(kg)/MBW[d], %	7.2	3.7
NEm Fed (Mcal/d)	11.1	4.7
NE Fed, % maint.	132	57

[a]Dry matter (DM) basis.

[b]Chemical analysis by DHIA, Ithaca, NY.

[c]Concentration of minerals in diets were: Ca (1.15%), P (.37%), K (.89%), Mg (.28%), S (.26%), Na (.32%), Mn (48ppm), Fe (130ppm), Cu (12ppm), and Zn (46ppm).

[d]Metabolic body weight.

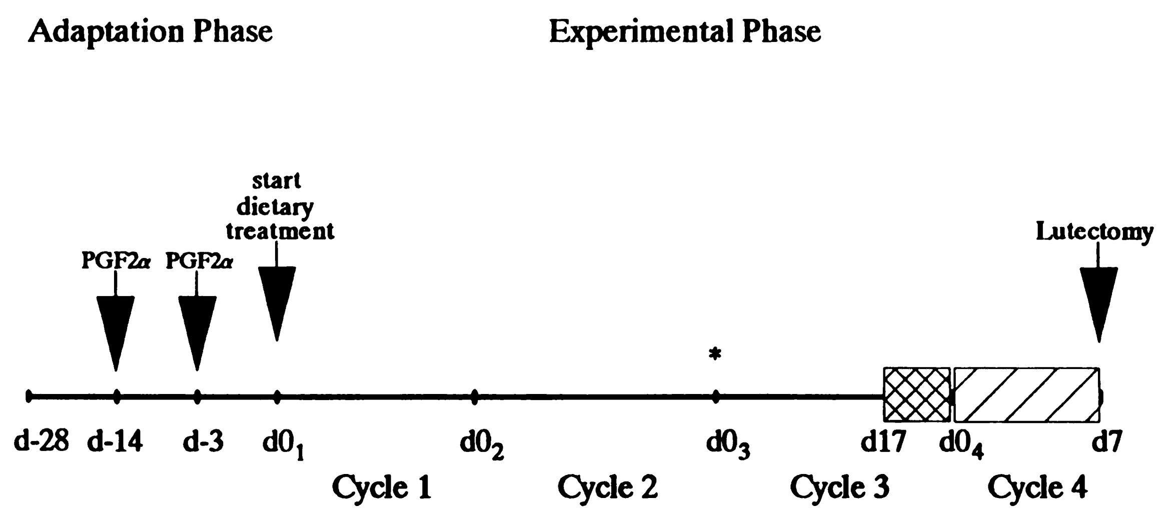


Figure 3. Time-line of experiment.
d0= estrus; Numeric subscript designates estrous cycle during experimental phase
= 2-h sampling of blood
= twice daily sampling of blood
* NEB-PEB heifers switched to PEB diet.

averaged for each heifer. Heifers were observed for signs of estrus 2 or 3 times daily for periods of 30 min each. Starting on d17 of the third estrous cycle heifers were observed six times daily for periods of 30 min each for signs of the fourth estrus. A heifer was judged in estrus if she stood $\geq$ 2 sec while being mounted by another heifer.

Energy Balance

Energy ingested and body weight were used to estimate energy balance (EB). Net energy was calculated as described by the National Research Council (1989). Energy balance was calculated as intake of net energy (NE) minus net energy required for maintenance (NEm). Daily intake of dry matter (DM) was measured. Daily energy intake was calculated as: daily energy intake = weight of feed (kg) $\cdot$ DM of feed $\cdot$ NEm for feed (from chemical analysis). NEm required was based on average weekly body weight and calculated as follows: NEm = .086 $\cdot$ body weight (kg)$^{.75}$ (National Research Council, 1989). Net energy that exceeded NEm was multiplied by .68 because use of energy for gain is less efficient than energy for maintenance (National Research Council, 1989). For individual heifers, NEm was calculated and amount of feed offered was adjusted weekly based on change in body weight. For example, in NEB heifers as body weight decreased total dry matter offered was decreased, but dry matter per unit of body weight was constant. Amount of DM offered per unit of metabolic body weight was equal among heifers within treatment.

Sampling of Blood

For each heifer, the preovulatory period began when serum concentration of progesterone was < 1 ng/ml for 4 h. The preovulatory period ended at peak of the preovulatory luteinizing hormone surge. On d16 of the third estrous cycle a polyvinyl chloride cannula (Ico-rally Co., Palo Alto, CA) was inserted into a jugular vein of each heifer. Blood was sampled (10 ml) every 2 h between d17 of the third estrous cycle until 14 h after onset of the fourth estrus. Blood was sampled (10 ml) twice daily (0900 and 1700 h) d1 to 7 postestrus of fourth estrous cycle to monitor luteal development. Blood was stored immediately at 4°C, allowed to clot, centrifuged and serum was decanted and frozen (-20°C) within 24 h. Concentrations of estradiol-17β, luteinizing hormone, follicle stimulating hormone, growth hormone and progesterone in serum were determined by radioimmunoassays (RIA) described below.

Collection and Sampling of Corpora Lutea

On d7 of the fourth estrous cycle corpora lutea were collected supravaginally. The ovary bearing the corpus luteum was located and the corpus luteum was enucleated from ovarian stroma by digital pressure (Villa-Godoy, 1987). Corpora lutea were rinsed twice in Ham's F-12 medium, blotted dry, adhering connective tissue was removed and luteal tissue was weighed. Corpora lutea were bisected perpendicular to the stigmata. Excluding the stigmata, luteal tissue was sampled (≈100 mg/sample) twice from medullary and cortical regions of a corpus luteum (4 total samples). One medullary and cortical sample from each corpus luteum was fixed separately in 1% glutaraldehyde in .1M phosphate buffer for morphometric analysis. Medullary and cortical samples that were not fixed, were frozen in Ham's F-12 medium until progesterone was quantified by RIA. Corpora lutea were sampled and processed for storage as described within 3 min after they were removed from ovaries.

Radioimmunoassay for Estradiol

Concentration of estradiol-17β in jugular serum was used to monitor preovulatory follicular development. Serum concentrations of estradiol-17β were determined using a double antibody RIA (Diagnostic Products Corporation; Los Angeles, Ca.) with modifications by Turzilla et al. (1989) (Appendix; validation and procedure). Modifications of the RIA included: 1) extraction of serum twice with diethyl ether, 2) reconstitution of estradiol ether extracts in assay buffer (0.1% gelatin in phosphate-buffered saline, pH 7.4) for 8 h at 4 °C, 3) estradiol standards were diluted in assay buffer and 4) reduced amount of antisera and radiolabeled ligand to increase sensitivity. Intra-assay (10.8%) and interassay (12.5%) coefficients of variation (CV) for estradiol were based on a pool of serum from an ovariectomized heifer containing exogenous estradiol (mean = 25 pg/ml).

Radioimmunoassay for Luteinizing Hormone, Follicle Stimulating Hormone, and Growth Hormone

Concentrations of luteinizing hormone in jugular serum were quantified by double antibody RIA as described by Convey et al. (1976). Intra-assay (5.8%) and interassay (10.3%) CV's for luteinizing hormone were from bovine standard serum (mean = 6 ng/ml). Concentrations of follicle stimulating hormone in jugular serum were quantified by double antibody RIA as described by Garcia-Winder et al. (1986). Intra-assay (2.2%) and interassay (3.3%) CV's for follicle stimulating hormone were from bovine standard serum (mean = 8.5 ng/ml). Concentrations of growth hormone in jugular serum were quantified by RIA as described by Zinn et al. (1989). Intra-assay (4.6%) and interassay (3.6%) CV's for growth hormone were from pooled bovine standard serum (mean = 5.5 ng/ml).

Radioimmunoassay for Progesterone

To monitor luteal development, concentrations of progesterone in serum and corpora lutea was quantified by RIA as described by Louis et al. (1973) and modified by Convey et al. (1976). Intra-assay (6.1%) and interassay (8.4%) CV's were from bovine standard serum collected during estrus (mean = 0.17 ng/ml), and intra-assay and interassay CV's were 5.7% and 8.4% for bovine standard serum collected in diestrus (mean = 2.5 ng/ml).

The procedure to process luteal tissue for quantification of progesterone is shown in Figure 4. Luteal tissue (≈100 mg) and 2 ml of Ham's F-12 medium were homogenized (20 strokes) with a teflon pestle connected to a stirrer (Sargent, Chicago, IL). To account for procedural losses, 20,000 cpm ^{3}H-progesterone ([1,2,6,7-^{3}H]Progesterone, Amersham, Arlington Heights, IL) was added to each homogenization tube. Homogenates were centrifuged (250 x g for 10 min) and supernatants were decanted and saved. To extract progesterone, tissue samples were homogenized (20 strokes) thrice in 5 ml ether. Ether supernatants were added to the previously decanted aqueous supernatant. Combined supernatant was vortexed for 30 seconds and centrifuged (250 x g for 10 min). The aqueous phase was frozen in a bath of dry ice and methanol. The ether was decanted and evaporated and extracts were resuspended in 6 ml of ether. Three 500 μl aliquots were used to determine efficiency of extraction of progesterone (77.6%). After evaporation of ether, extracts

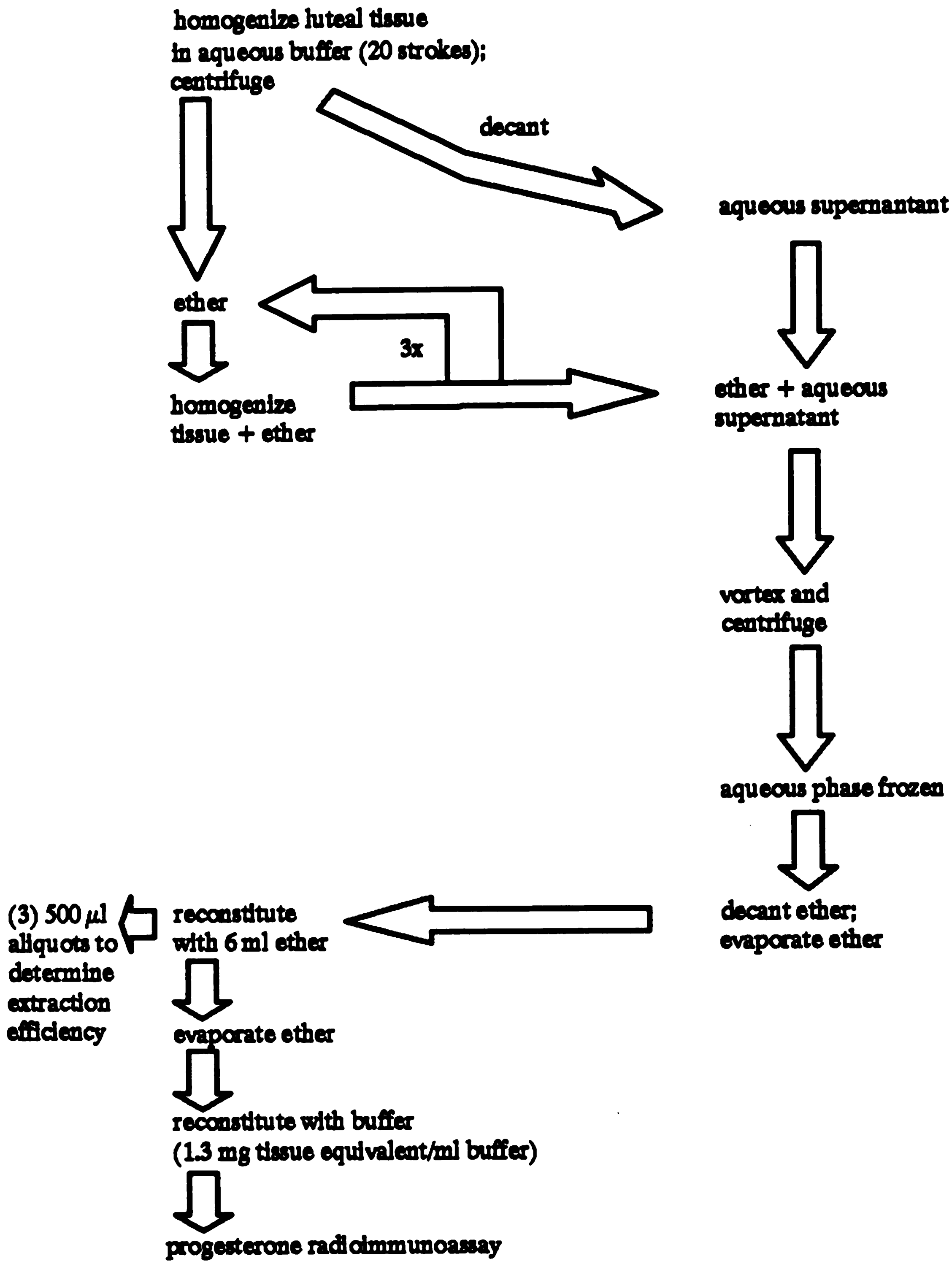


Figure 4.Preparation of bovine luteal tissue for quantification of progesterone.

of homogenates were reconstituted to the equivalent of 1.3 mg luteal tissue per ml of buffer (0.1% gelatin in phosphate-buffered saline, pH 7.4) and progesterone was quantified by RIA. Intra-assay (3.8%) and interassay (5.2%) CV's were based on a pool of bovine luteal tissue (3.5 ng progesterone/ml) collected on d7 of an estrous cycle.

Histology and Morphometric Analysis

The primary purpose of morphometric analysis of luteal tissue was to determine number and ratio of large to small luteal cells. Luteal tissue (≈100 mg/sample) was fixed for 24 h in 1.0% glutaraldehyde (Sigma, St. Louis, MO) 0.1 M phosphate buffer at 4°C. Luteal tissue was rinsed thrice and stored in 0.1M phosphate buffer at 4°C for approximately 5 mo. Samples were dehydrated in increasing concentrations of ethanol and Hemo-de (Fisher, Pittsburg, PA) and embedded in paraffin. Paraffin embedded samples were sectioned (10 μm). Sections were selected randomly from each sample and stained with hematoxylin and eosin followed by Mallory Triple Stain (Humason, 1989). Mallory Triple Stain stained fibroblastic cells blue while steroidogenic cells were stained red. Thus, small luteal cells were distinguished from connective tissue.

A sample from the central and polar region of each corpus luteum was processed as above. Two sections from each sample of luteal tissue, separated by ≥ 50 μm, were selected for examination by light microscopy (400x). Within each section, two fields (≈2.51×10^5 μm^2 per field) were examined. Thus, for each corpus luteum eight fields (two regions x two sections x two fields) were examined for a total area of ≈2.01×10^6 μm^2. Structures had to be completely within the microscopic field to be included in a data set. Diameter and number of large and small cells were determined in each field. Large and small luteal cells were identified using criteria of Koos and Hansel (1981). An image analyzer (Bioquant System IV, R and M Biometrics, Nashville, TN) coupled to a digitizer pad was used to measure diameter and to inventory cells. Ratio of large to small luteal cells was number of large luteal cells divided by number of small luteal cells. Data for steroidogenic cell types were also expressed as a percentage of total number of cells counted. Total number of cells counted did not include erythrocytes, but did include fibroblasts, steroidogenic cells, endothelial cells and a small percentage ($< 10\%$) of unidentified nucleated cells.

Assimilation of Data

Estradiol was quantified in serum samples collected during the defined preovulatory period and data were expressed as mean estradiol, area under estradiol profile and peak estradiol. Blood collected at 2 h intervals for 6 hours after progesterone was < 1 ng/ml (third estrous cycle) was used as a measure of basal luteinizing hormone. Area of preovulatory luteinizing hormone surge was determined from samples collected at 2 h intervals during the preovulatory period that were ≥ 3 standard deviations above basal luteinizing hormone. Mean concentration of follicle stimulating hormone and growth hormone in serum were quantified in samples collected during the preovulatory period. Serum progesterone was expressed as area under progesterone profile d1 to d7 and concentration of progesterone on d7. Data were excluded from one heifer in NEB group because of excessive weight loss due to illness and from one heifer in PEB group and four heifers in NEB-PEB group because the third or fourth estrus was not detected.

Statistical Analysis

The experimental design was randomized block; however, one-way analysis of variance (GLM SAS, 1989) was used because of incomplete blocks. Homogeneity of variance was tested using the f_{max} test (Gill, 1978). Area of estradiol profile and luteal weight were transformed to natural log to reduce heterogenous variance. Heifers were blocked by body weight at the beginning of experiment, but because of incomplete blocks initial body weight was used as a covariate. The covariate was not significant ($P > .20$) for any variable except for a treatment by initial body weight interaction with mean follicle stimulating hormone. Thus, the covariate term was removed except that mean follicle stimulating hormone was divided by initial body weight before means were compared. Treatment means were compared using the Bonferroni t test (Gill, 1978). Pearson correlation coefficients were calculated to determine associations of: 1) preovulatory follicular development with corpora lutea development 2) body weight change with preovulatory follicular development or corpora lutea development and 3) serum progesterone with cellular inventory of corpora lutea.

Results

Animals

Data used in analyses are from eight PEB, eight NEB and five NEB-PEB heifers.

Energy Balance

For 3.3 estrous cycles PEB heifers were in positive energy balance (2.2 ± .1 Mcal/d) and NEB heifers were in negative energy balance (-3.4 ± .1 Mcal/d). For 2.0 estrous cycles NEB-PEB heifers were in negative energy balance (-3.0 ± .1 Mcal/d) and for 1.3 estrous cycles were in positive energy balance (2.1 ± .1 Mcal/d).

Initial body weight did not differ among treatments and averaged 420 kg. Heifers fed NEB diets lost body weight (NEB = -0.5 kg/d; NEB-PEB = -0.43 kg/d) (Figure 5). Heifers fed PEB diets gained body weight (PEB = 0.5 kg/d; NEB-PEB = 0.6 kg/d) (Figure 5). Based on ingested energy, calculated energy balance and associated changes in body weight, the metabolic states that were planned were achieved. For the remainder of this thesis, treatments will be identified as follows: PEB heifers were in positive energy balance throughout the experiment; NEB heifers were in negative energy balance throughout the experiment (long-term) and NEB-PEB heifers were in short-term negative energy balance followed by positive energy balance.

Preovulatory Estradiol

Mean preovulatory estradiol did not differ between PEB and NEB-PEB heifers. But, relative to positive energy balance, long-term negative energy balance increased ($P < .05$) mean preovulatory estradiol during third estrous cycle (Figure 6).

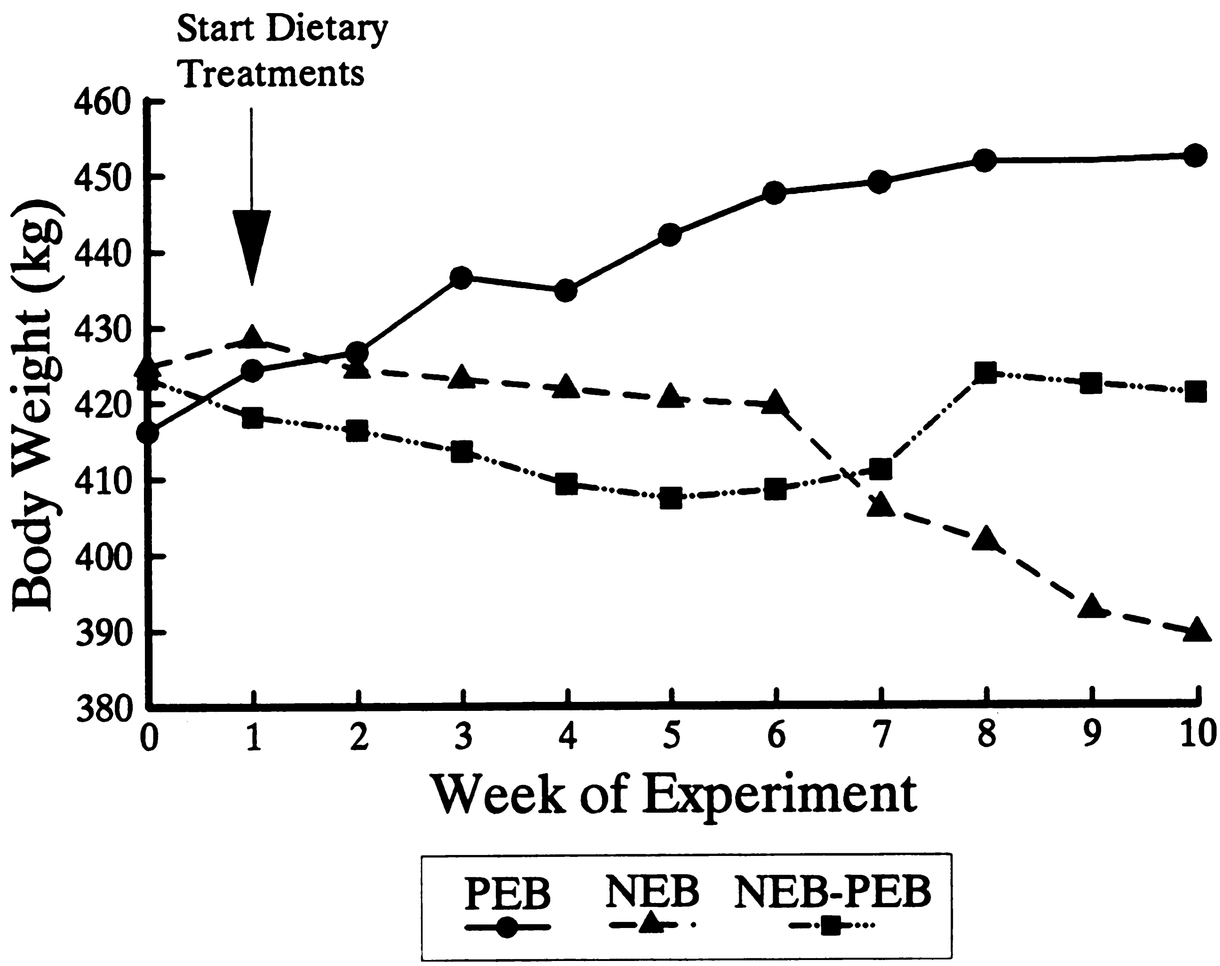
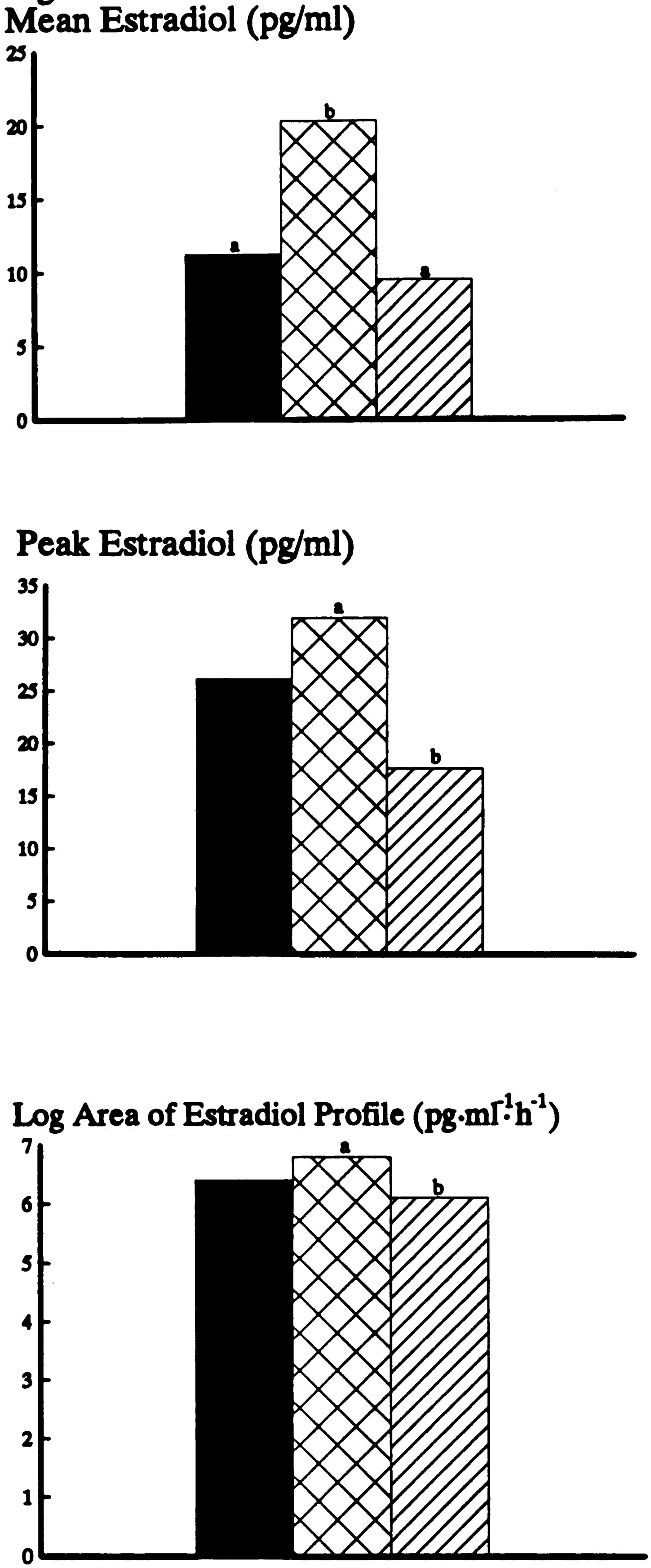


Figure 5. Mean weekly body weight of heifers fed a high energy diet (130% NEm; PEB), a low energy diet (60% NEm; NEB), or low switched to high energy diet (NEB-PEB). On the day of estrus between weeks 6 and 7, NEB-PEB heifers were switched to PEB diet.

Figure 6. Effects of diets (high energy [PEB], low energy [NEB], low energy followed by high energy [NEB-PEB]) on mean, peak, and log area of profile of estradiol during the preovulatory period of third estrous cycle. Values represent means within treatment. Mean estradiol, PEB and NEB (SEM = ± 2.0) and NEB-PEB (SEM = ± 2.5). Peak estradiol tends ($P < .15$) to be higher in NEB than PEB heifers; PEB and NEB (SEM = ± 2.9) and NEB-PEB (SEM = ± 3.7). Log area of estradiol profile tends ($P < .15$) to be higher in NEB than PEB heifers; PEB and NEB (SEM = ± .13) and NEB-PEB (SEM = ± .16).
[a,b] Means within graph with superscripts that do not have a common letter differ ($P < .05$).

Figure 6.


Mean Estradiol (pg/ml)
25
20
15
10
5
0
a
b
a
Peak Estradiol (pg/ml)
35
30
25
20
15
10
5
0
a
b
Log Area of Estradiol Profile (pg·ml⁻¹·h⁻¹)
7
6
5
4
3
2
1
0
a
b

Preovulatory Luteinizing Hormone, Follicle Stimulating Hormone, and Growth Hormone

Follicle stimulating hormone, basal luteinizing hormone and area under profile of luteinizing hormone surge were not affected by energy balance (Table 3). Relative to positive energy balance, long-term negative energy balance increased ($P < .05$) and short-term negative energy balance tended to increase ($P < .1$) mean growth hormone (Table 3). But, mean growth hormone in NEB heifers did not differ from NEB-PEB heifers (Table 3).

Duration of Preovulatory Period

Preovulatory period of the third estrous cycle began when serum progesterone was ≤ 1 ng/ml and ended at peak of the luteinizing hormone surge. Duration of the preovulatory period was not different in NEB and NEB-PEB heifers. Compared to positive energy balance, short-term negative energy balance did not affect, but long-term negative energy balance tended ($P < .11$) to shorten duration of the preovulatory period (Table 3).

Serum Progesterone

Energy balance did not affect area of progesterone profile (d1 to d7) or mean serum progesterone on d7 (Table 4).

Corpora Lutea

Long-term negative energy balance reduced ($P < .05$) luteal weight compared with positive energy balance (Table 5). Because of heterogeneous variance, the natural log of luteal weight was used for treatment comparisons. Compared with positive energy balance, long and short-term negative energy balance decreased (P

Table 3. Effects of diet on duration of preovulatory period and serum concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), and growth hormone (GH)[a].

	Energy Balance of Heifers		
	PEB	NEB	NEB-PEB
Preovulatory Period (h)[b]	61 ± 6	47 ± 6	59 ± 8
Basal LH (ng/ml)	1.3 ± .2	1.5 ± .2	1.4 ± .3
Area of LH surge Profile ($ng \cdot ml^{-1} \cdot h^{-1}$)	121.4 ± 15.7	150.9 ± 15.7	128.4 ± 20.0
LH Peak (ng/ml)	25.7 ± 3.6	32.0 ± 3.6	28.2 ± 3.7
FSH (ng/ml)	14.2 ± 1.6	14.1 ± 1.6	15.1 ± 2.0
GH (ng/ml)	4.4 ± .5[c]	7.0 ± .5[d]	6.0 ± .7

[a] Data are expressed as mean ± SEM.

[b] Duration of preovulatory period tended ($P < .11$) to be less in NEB heifers than in PEB heifers.

[c,d] Means within row with superscripts that do not have a common letter differ ($P < .05$).

Table 4. Effect of diet on serum and luteal progesterone[a].

	Energy Balance of Heifers		
Progesterone	PEB	NEB	NEB-PEB
Serum:			
Area of Profile ($ng \cdot ml^{-1} \cdot d^{-1}$)b	10.8 ± 1.0	9.5 ± 1.0	13.0 ± 1.3
Day 7 (ng/ml)	1.9 ± .2	1.7 ± .2	2.2 ± .2
Luteal:			
Concentration (µg/g)	82.6 ± 5.2[d]	46.9 ± 5.2[e]	45.7 ± 6.6[e]
Content (µg/CL)[c]	405.2 ± 36.1[d]	172.8 ± 36.1[e]	196.1 ± 45.7[e]

[a] Data are expressed as mean ± SEM.

[b] Data are expressed as area under the profile of progesterone (P_4) in serum ($ng \cdot ml^{-1} \cdot d^{-1}$) from d1 to 7 of the fourth estrous cycle. Area of P_4 profile tended ($P<.15$) to be less in NEB heifers than NEB-PEB heifers.

[c] Luteal content of P_4 = luteal concentration of P_4 · luteal weight.

[d,e] Means within a row with superscripts that do not have a common letter differ ($P<.01$).

Table 5. Effect of diet on weight and steroidogenic cell composition of corpora lutea (CL)[a].

	Energy Balance of Heifers		
	PEB	NEB	NEB-PEB
CL weight (g):	4.82 ± .22	3.74 ± .22	4.30 ± .30
log CL weight[b]	1.55 ± .06[e]	1.31 ± .06[f]	1.45 ± .08
Percent of total cells ($\overline{x}$±SEM)[c]:			
small luteal cells (%)	27.3 ± 1.6	30.4 ± 1.6	31.9 ± 2.1
large luteal cells (%)	11.5 ± .51[e]	9.4 ± .51[f]	9.9 ± .64
Ratio large to small luteal cells[d]:			
ratio in polar section of CL	.44 ± .045	.34 ± .045	.33 ± .053
ratio in central section of CL	.44 ± .035[f]	.28 ± .035[g]	.28 ± .041[g]
overall ratio	.44 ± .03[f]	.31 ± .03[g]	.30 ± .04[g]

[a] Data are expressed as mean ± SEM.

[b] Because of heterogenous variance, the natural log of CL weight was used for treatment contrasts.

[c] Seven hundred to 800 luteal cells were counted in a total area of ≈ 2.01×10^6 μm^2 from each CL. Percentage of large luteal cells tended ($P<.15$) to be less in NEB-PEB heifers than PEB heifers.

[d] Ratio of large to small luteal cells = number of large luteal cells ÷ number of small luteal cells. Overall ratio = average of ratio in polar and central section of CL.

[e,f,g] Means within a row with superscripts that do not have a common letter differ ([e,f] $P<.05$; [f,g] $P\leq.01$).

< .01) concentration and content of progesterone in corpora lutea (Table 4). But luteal progesterone did not differ between NEB and NEB-PEB heifers (Table 4). Relative to positive energy balance, long and short-term negative energy balance decreased ($P \leq .01$) ratio of large to small luteal cells in the central, but not polar, region of corpora lutea (Table 5). Independent of region within corpora lutea, ratio of large to small luteal cells in NEB and NEB-PEB heifers was less ($P < .01$) than in PEB heifers (Table 5). Since percentage of cells inventoried that were small luteal cells was not different among treatments (Table 5), the decreased ratio of large to small luteal cells was due to decreased number of large luteal cells.

Association of Changes in Body Weight with Estradiol, Progesterone and Cellular Inventory of Corpora Lutea

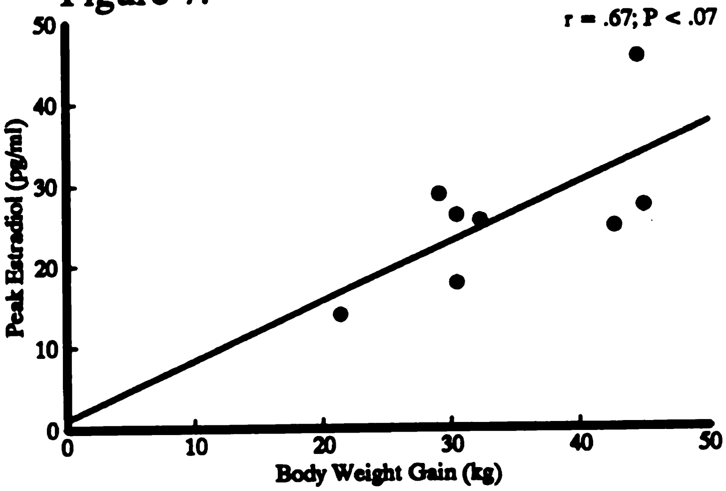
In PEB heifers, body weight gain was associated positively with peak estradiol, area of progesterone profile and luteal content of progesterone (Figure 7). Thus, as body weight gain increased in PEB heifers, there was increased peak secretion of estradiol and increased production of progesterone. As NEB heifers lost body weight duration of preovulatory period, luteal concentration of progesterone and ratio of large to small luteal cells decreased (Figure 8).

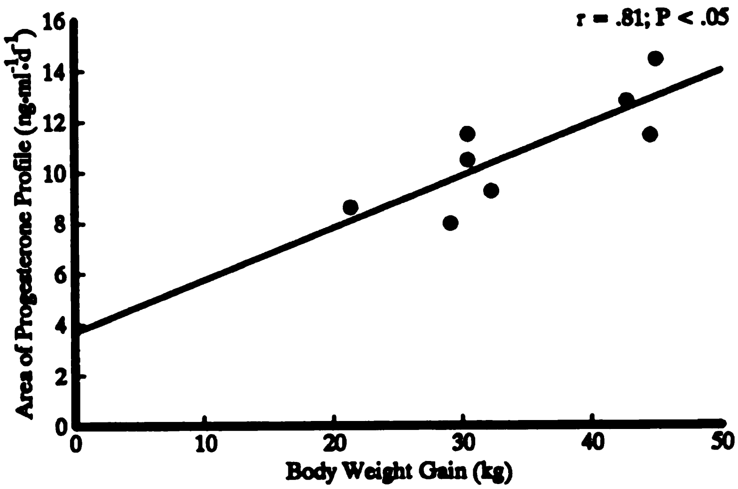
Association of Corpora Lutea and Progesterone

Among all heifers, luteal weight was associated positively ($r = .551$; $P < .01$) with serum concentration of progesterone on d7 of the fourth cycle. Luteal weight was associated positively with the profile of serum progesterone in PEB ($r = .737$, $P < .05$) and NEB-PEB heifers ($r = .842$, $P < .08$), but not in NEB heifers ($r = .450$; $P > .2$).

Figure 7. Association of body weight gain during experiment with peak serum estradiol, area of progesterone profile in serum or progesterone content of corpora lutea in positive energy balance (PEB) heifers. Values for peak estradiol are from the preovulatory period of the third estrous cycle. Values for area of progesterone are from d1 to 7 of the fourth estrous cycle. Values for progesterone content are from corpora lutea collected on d7 of the fourth estrous cycle.

Figure 7.


r = .67; P < .07
Peak Estradiol (pg/ml)
Body Weight Gain (kg)


r = .81; P < .05
Area of Progesterone Profile (ng·ml⁻¹·d⁻¹)
Body Weight Gain (kg)

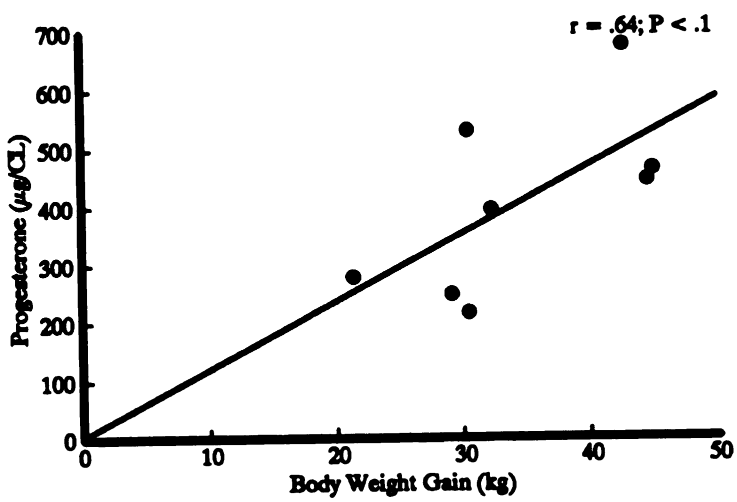
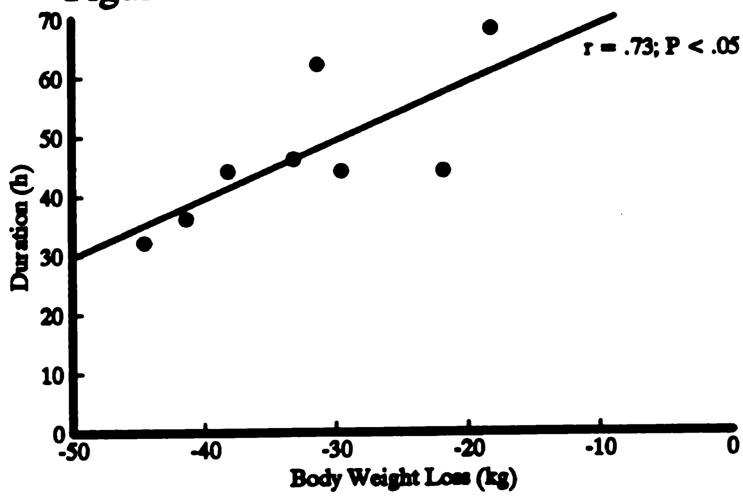
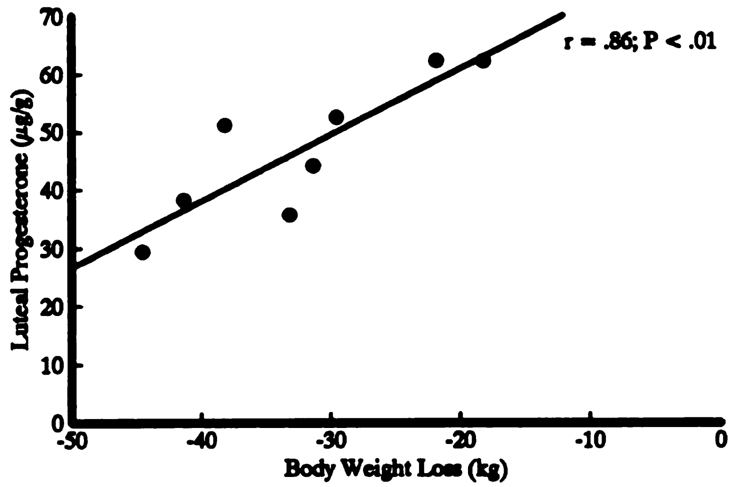
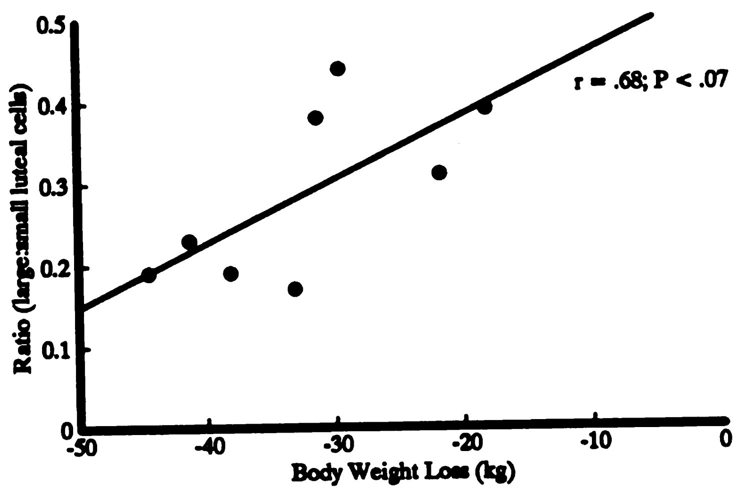

r = .64; P < .1
Progesterone (µg/CL)
Body Weight Gain (kg)

Figure 8. Association of body weight loss during experiment with duration of the preovulatory period, concentration of progesterone in corpora lutea or ratio of large to small luteal cells in negative energy balance (NEB) heifers. Values for duration of the preovulatory period are from the third estrous cycle. Concentration of progesterone and ratio of large to small luteal cells are from corpora lutea collected on d7 of the fourth estrous cycle.

Figure 8.


r = .73; P < .05
Duration (h)
Body Weight Loss (kg)


r = .86; P < .01
Luteal Progesterone (µg/g)
Body Weight Loss (kg)


r = .68; P < .07
Ratio (large:small luteal cells)
Body Weight Loss (kg)

Association between Preovulatory Phase and Luteal Development

Independent of diet, duration of the preovulatory period was associated positively ($r = .490$; $P < .05$) with percentage of large luteal cells (Figure 9) and with ratio of large to small luteal cells in the central region of corpora lutea ($r = .373$; $P < .1$). Compared with other dietary groups, NEB heifers had the strongest positive association ($r = .776$; $P < .02$) between duration of the preovulatory phase and ratio of large to small luteal cells in the central region.

Peak or mean preovulatory serum estradiol was not associated with subsequent mean progesterone (d7) or area of progesterone profile (d1 to d7). However, in PEB heifers, peak estradiol was associated positively with percentage of small cells in corpora lutea (Figure 10). In NEB heifers, peak estradiol was associated with percentage large cells in corpora lutea (Figure 10). In addition, peak estradiol was associated positively with ratio of large to small luteal cells in NEB-PEB heifers (Figure 10).

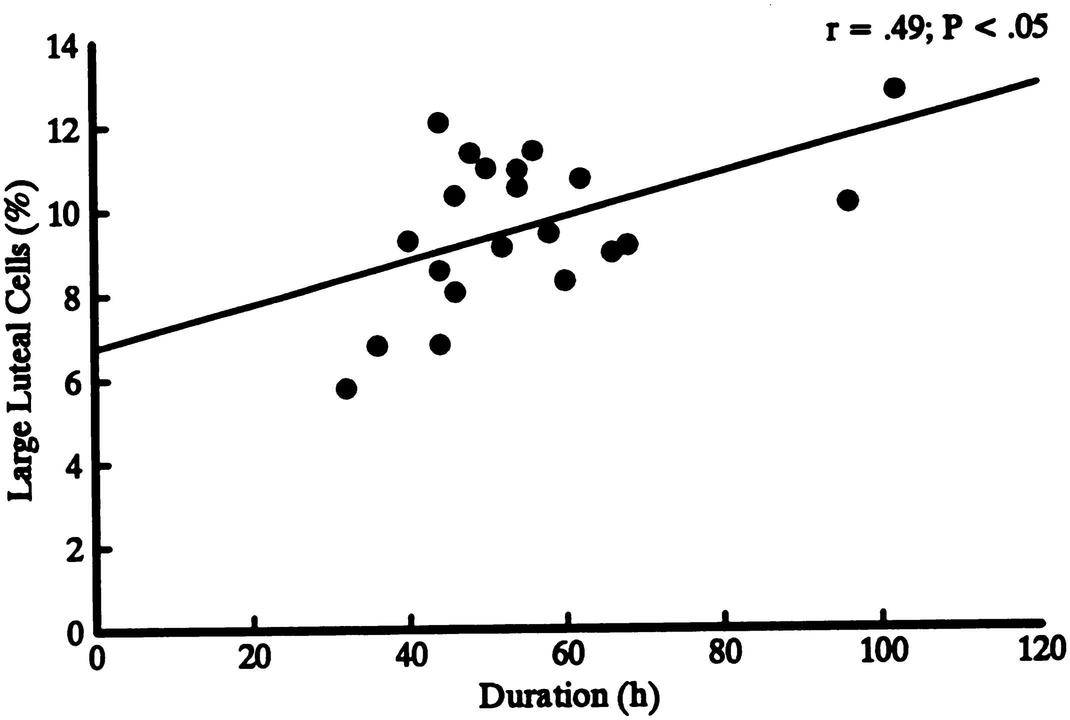
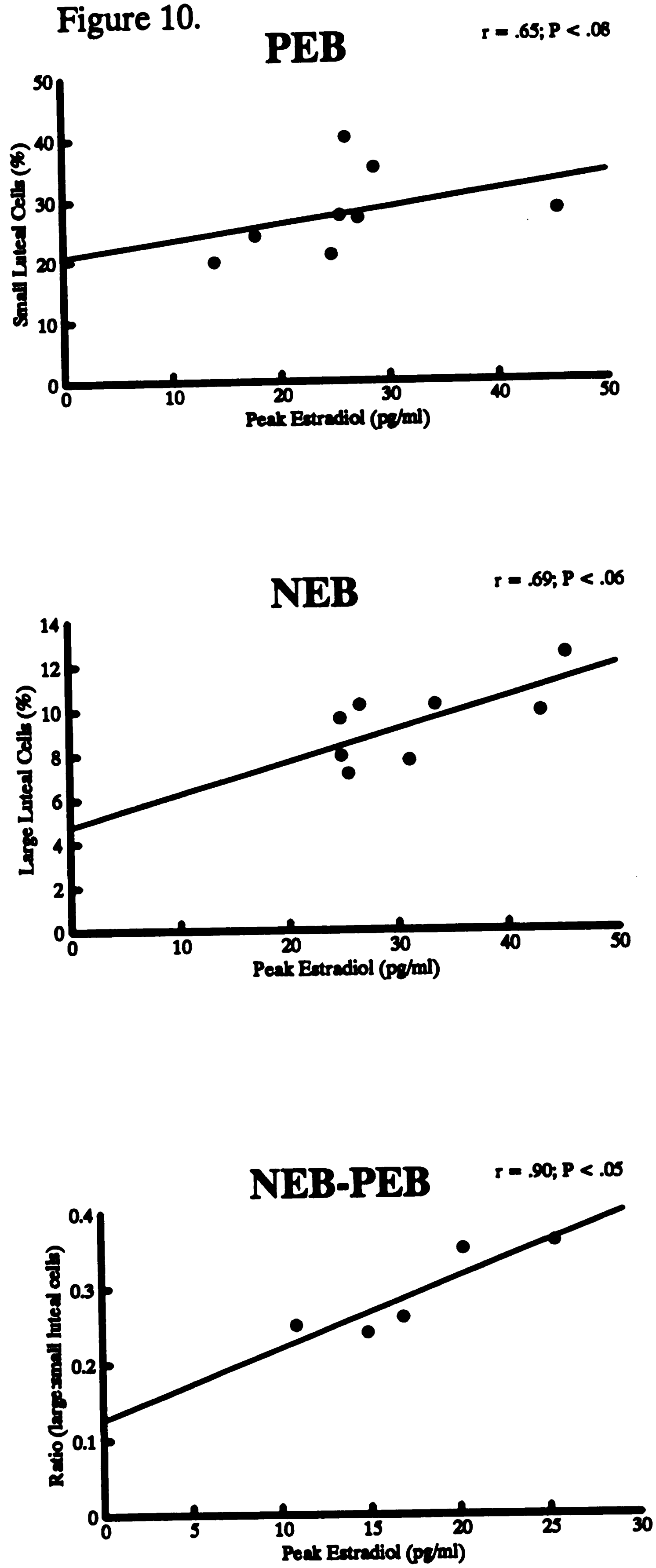


Figure 9. Association of duration of the preovulatory period with percentage of cells that are large luteal cells in all heifers. Values for duration of the preovulatory period are from the third estrous cycle. Percentage of total cells inventoried that were large luteal cells are from corpora lutea collected on d7 of the fourth estrous cycle.

Figure 10. Association of peak serum estradiol with percentage of small luteal cells in positive energy balance (PEB) heifers, percentage of large luteal cells in negative energy balance (NEB) heifers or ratio of large to small luteal cells in short-term negative energy balance (NEB-PEB) heifers. Values for peak estradiol are from the preovulatory period of the third estrous cycle. Percentage of total cells inventoried that were small or large luteal cells and ratio of large to small luteal cells are from corpora lutea collected on d7 of the fourth estrous cycle.

Figure 10.


PEB
r = .65; P < .08
Small Luteal Cells (%)
Peak Estradiol (pg/ml)
0
10
20
30
40
50
NEB
r = .69; P < .06
Large Luteal Cells (%)
Peak Estradiol (pg/ml)
0
2
4
6
8
10
12
14
NEB-PEB
r = .90; P < .05
Ratio (large:small luteal cells)
Peak Estradiol (pg/ml)
0
0.1
0.2
0.3
0.4
5
15
25
30

Discussion

The broad objective of this study was to determine if preovulatory follicular development was associated with subsequent development of bovine corpora lutea. Preovulatory serum estradiol and postovulatory serum progesterone were not related. But, within treatment, heifers with increased estradiol or independent of treatment, heifers with increased duration of the preovulatory period had increased concentration of large or small cells in corpora lutea.

Independent of diet, with longer duration of the preovulatory period there was increased percentage of cells that were large luteal cells. During the preovulatory period, size of preovulatory follicles and number of granulosal cells within a preovulatory follicle increases (Ireland and Roche, 1984). Granulosal cells in selected follicles undergo mitosis until atresia or until the preovulatory luteinizing hormone surge (Marion et al., 1968). Based on duration of the cell cycle in mammalian cell lines, the shortened preovulatory phase in NEB heifers would result in .4 to .6 fewer mitotic cycles than in PEB heifers (Lewin, 1990). A major consequence of fewer mitotic cycles would be fewer granulosal cells (≈ 3 to 4.4×10^6). Decreased granulosal cell mitosis is consistent with our observation that as percentage of large cells in corpora lutea decreased, duration of preovulatory period decreased.

In NEB heifers, as peak estradiol increased, percentage of large cells in corpora lutea increased. Similarly, as peak estradiol increased, ratio of large to small luteal is a function of number of steroidogenic cells and amount of steroidogenesis per cell. In preovulatory follicles, granulosal cells synthesize and secrete estradiol (McNatty et al., 1984). Thus, a possible explanation for the positive relationship between peak estradiol and percentage of large luteal cells is that heifers with more granulosal cells (indicated by increased peak estradiol) produce subsequent corpora lutea with a greater concentration of large luteal cells.

In PEB heifers, as peak estradiol increased, percentage of small cells in corpora lutea increased. Small luteal cells are derived from thecal cells of the ovulatory follicle (Alila and Hansel, 1984). Thecal cells synthesize and secrete androgens which are the precursors for estradiol synthesis by granulosal cells (McNatty et al., 1984). Theoretically, increased estradiol could be due to increased androgen availability. Increased androgen availability could be due to increased number of thecal cells in the preovulatory follicle. Consequently, there would be increased concentration of small luteal cells. Thus, a positive relationship between preovulatory estradiol and concentration of small luteal cells is another example of the positive relationship between the ovulatory follicle and development of the ensuing corpus luteum.

Another objective of this experiment was to determine if adverse effects of negative energy balance on corpora lutea are mediated by preovulatory follicles. Long-term negative energy balance increased serum estradiol and growth hormone during the preovulatory period and reduced the duration of the preovulatory period. Increased estradiol in NEB heifers was not expected. The present experiment was not designed to determine mechanisms for changes in serum hormones so only possibilities can be considered here. Three possible explanations for increased estradiol in NEB heifers are: 1) increased estradiol from one preovulatory follicle, 2) decreased clearance of estradiol from serum or 3) selection of multiple follicles which collectively produced more estradiol.

What is the evidence that negative energy balance will increase estradiol from an individual follicle? Restriction of dietary energy did not alter the rate of estradiol secretion from bovine preovulatory follicles in vitro (Staigmiller et al., 1982). Restriction of dietary energy in postpartum beef cows reduced follicular fluid volume of large follicles (≥ 10mm), but did not affect content of estradiol in follicular fluid (Rutter and Manns, 1990). In heifers, energy restriction for 10 weeks did not affect concentration of estradiol in follicular fluid from small, medium, or large follicles, but decreased concentration of estradiol in follicular fluid of estrogen-active follicles (Spicer et al., 1991). Thus, in the present study, it is not likely that increased estradiol in NEB heifers was due to increased estradiol secretion from a single preovulatory follicle.

A second possible explanation for increased serum estradiol in NEB heifers is due to decreased metabolic clearance rate (MCR) of estradiol. Metabolic clearance rate is controlled largely by sex hormone binding proteins in blood. As sex hormone binding proteins increases, MCR of estradiol decreases (Siiteri et al., 1982). Restriction of dietary energy in heifers decreased concentration of sex steroid binding proteins in blood (Lermite and Terqui, 1991). Decreased sex hormone binding proteins should increase MCR of estradiol and would decrease serum concentration of estradiol. In the present study, negative energy balance did not reduce, but rather increased, serum estradiol. Thus, it is not a likely that negative energy balance increased sex hormone binding proteins and, consequently, decreased MCR of estradiol. The effect of diet on MCR of steroids should be interpreted with caution, however, because there are no data directly from cattle.

A third possible explanation for increased serum estradiol in NEB heifers is that multiple follicles produced increased total amount of estradiol before one follicle ovulated. Evidence for this possibility is that in cows fed low energy diets, exogenous follicle stimulating hormone induced more large (> 10mm) follicles than in cows receiving high energy diets (Staigmiller et al., 1982). On d25 or d35 postpartum beef cows fed low energy diets since calving had more estrogen-active medium (7 to 9.9 mm) follicles compared with cows fed ad libitum (Rutter and Manns, 1990). Heifers fed a low energy diet (10 wk) had 40% more large follicles during proestrus than heifers fed a maintenance energy diet. But, the authors did not report whether these additional large follicles were estrogen-active (non-atretic) (Spicer et al., 1991). Thus, it is possible that NEB heifers had multiple estrogen-active follicles which collectively increased concentration of estradiol in serum during the preovulatory period, but only one follicle ovulated.

Luteinizing hormone and follicle stimulating hormone are the major extra-ovarian hormones that stimulate folliculogenesis (Hisaw, 1947). Diet did not affect concentration of luteinizing hormone and follicle stimulating hormone in serum. Since there was no change in concentrations of gonadotropins in serum, how might selection of follicles be increased in NEB heifers? Concentration of growth hormone in serum was increased in NEB heifers. There is evidence that growth hormone can affect folliculogenesis. Growth hormone receptors are present on bovine (Lucy et al., 1992a) and ovine granulosal cells (Nett, unpublished). Exogenous growth hormone increased the number of small (2 to 5mm) antral follicles in heifers (Gong et al, 1991) and number of follicles in cows (Herrler and Niemann, 1990). In women, exogenous growth hormone decreased the dose of human menopausal gonadotropin necessary to induce ovulation (Homburg et al., 1988; 1990). Exogenous and high endogenous growth hormone increased preovulatory serum estradiol (Hugues et al., 1991; Stone and Marrs, 1992). In the present study, NEB increased growth hormone and preovulatory serum estradiol. From previous data, growth hormone enhanced selection of follicles. Thus, it is possible that increased estradiol in NEB heifers is due to multiple follicles selected by increased secretion of growth hormone.

Long-term negative energy balance tended to decrease duration of the preovulatory period. Similarly, restriction of dietary energy (10 wk) reduced persistence of preovulatory follicles as determined by ultrasound in heifers (Murphy et al., 1991). Since preovulatory estradiol is a major stimulus for the preovulatory surge of luteinizing hormone (Kesner et al., 1981), higher concentration of estradiol in NEB heifers during proestrus likely hastened the preovulatory surge of luteinizing hormone compared to PEB or NEB-PEB heifers.

Energy balance did not affect serum concentration of luteinizing hormone or follicle stimulating hormone during the preovulatory period. This agrees with effect of negative energy balance on luteinizing hormone in mid-diestrus (Harrison and Randel, 1986; Villa-Godoy, 1990). Gombe and Hansel (1973) reported that restriction of dietary energy increased preovulatory luteinizing hormone while

Imakawa et al. (1986b) reported decreased luteinizing hormone during proestrus. Recently, Roberson et al. (1992) reported that the effect of negative energy balance on serum luteinizing hormone is dependent upon body condition of heifers. Differences in body condition of heifers may explain inconsistent data in the literature.

Energy balance did not affect serum concentration of progesterone from d1 to d7 of the fourth estrous cycle in the present study. But, concentration and content of progesterone in luteal tissue was decreased by long and short-term negative energy balance. Therefore, synthesis of progesterone per gram of luteal tissue and per corpus luteum was reduced by negative energy balance. There are several possible explanations for non-parallel effects of negative energy balance on progesterone in serum and corpora lutea. Volume of blood is associated positively with body weight. Thus, PEB heifers would have greater volume of blood than NEB or NEB-PEB heifers. When calculated volume of blood and concentration of progesterone in serum is used to estimate serum content of progesterone, the small difference (≈ 10%) between PEB and NEB heifers in serum concentration of progesterone is magnified (≈ 23%; PEB = 32.6 mg progesterone; NEB = 25.7 mg progesterone) in content of serum progesterone. Although serum concentration of progesterone did not reflect differences in progesterone observed in luteal tissue, content of serum progesterone paralleled effects of diet on luteal progesterone.

As discussed for estradiol, clearance of progesterone from blood also can affect concentration of progesterone in serum. During diestrus, McCracken (1964) estimated that adipose tissue contains 5 to 10 mg of progesterone per cow. This is

15 to 25% of the estimated content of progesterone in blood (25.0 to 35.0 mg progesterone) of heifers in the present study. Although not measured, PEB heifers probably had more fat in which to store progesterone. Increased body fat would reduce the amount of progesterone in serum even with increased synthesis of progesterone in PEB heifers. Progesterone in serum is also regulated by MCR. Does negative energy balance decrease MCR? Decreased MCR would occur if corticosteroid binding globulin was increased. Undernutrition decreased corticosteroid binding globulin in humans (Siiteri et al., 1982) and ewes (Barnett and Star, 1981). Decreased corticosteroid binding globulin would increase, not decrease, MCR of progesterone. However, the effect of dietary energy restriction on corticosteroid binding globulin is not known in cattle.

Negative energy balance adversely affected luteal development. Reduced luteal weight in NEB heifers compared with PEB heifers is consistent with previous data (Dunn et al., 1974; Harrison and Randel, 1986; Hill et al., 1970; Gombe and Hansel, 1973; Villa-Godoy et al., 1990). Although short-term negative energy balance did not affect luteal weight, ratio of large to small luteal cells was reduced by long and short-term negative energy balance. Ratio of large to small luteal cells in mid-diestrus (d10 to d12) was reduced by negative energy balance in moderately conditioned heifers (Villa-Godoy et al., 1990). Reduced ratio of large to small luteal cells could be due to fewer granulosal cells in the preceding ovulatory follicle, decreased mitosis of small or large luteal cells or both. Since number of granulosal cells ($\approx$18.0 to 20.0 x 10^6; Milvae et al., 1991) at the preovulatory surge of luteinizing hormone is approximately 40% the number of large cells in mature corpora lutea ($\approx$45.0 to 51.0

x 10^6; O'Shea et al., 1989), and growth of luteal cells is exponential; it is likely that long and short-term negative energy balance reduced number of granulosal cells in the preovulatory follicle. As ratio of large to small luteal cells decreased, concentration and content of luteal progesterone decreased. Basal secretion of progesterone from large luteal cells is 20-fold greater than from small luteal cells (Koos and Hansel, 1981). Reduced progesterone in luteal tissue from NEB and NEB-PEB heifers is consistent with a decreased ratio of large to small luteal cells.

In PEB and NEB-PEB heifers, as luteal weight increased, serum progesterone increased. But in NEB heifers, only luteal content of progesterone (luteal weight · concentration of luteal progesterone) was associated with serum concentration of progesterone. Thus, studies which involve different levels of dietary energy need to measure more than serum concentration of progesterone to accurately assess luteal development.

Since negative energy balance affected follicular function and luteal development, does amount of body weight loss affect these variables? Among NEB heifers those that lost the most body weight had the shortest preovulatory period and had the lowest ratio of large to small luteal cells. Thus, excessive weight loss reduces the amount of time available for preovulatory mitosis of granulosal cells and may reduce number of large luteal cells in an ensuing corpus luteum. An unanswered question is whether loss of body weight will reduce number of granulosal cells in the ovulatory follicle.

Summary and Conclusions

As duration of the preovulatory phase increased, there was increased percentage of large luteal cells in ensuing corpora lutea. In PEB heifers, with increased peak estradiol during the preovulatory period, there was increased percentage of small cells in corpora lutea. In addition, as peak preovulatory estradiol increased in heifers that experienced negative energy balance, there was increased concentration of large luteal cells. In heifers experiencing short-term NEB, as peak estradiol increased, there was increased ratio of large to small luteal cells. In conclusion, preovulatory follicular development is associated positively with luteal development.

Negative energy balance increased preovulatory estradiol, increased serum growth hormone and reduced duration of the preovulatory phase. In addition, negative energy balance reduced a variety of measures of luteal development including: luteal progesterone, luteal weight, ratio of large to small luteal cells and percentage of large cells in corpora lutea. But, negative energy balance did not reduce serum concentration of progesterone. Excessive body weight loss shortened the duration of the preovulatory period and reduced ratio of large to small luteal cells. Thus, in heifers losing body weight, a shorter preovulatory period may limit the number of steroidogenic cells in a preovulatory follicle which subsequently luteinizes to become a corpus luteum.

Future investigations should examine the effects of negative energy balance on number of steroidogenic cells in preovulatory follicles. In addition, does negative energy balance increase selection of follicles by increasing endogenous growth hormone? Also, can follicular growth and development be promoted in order to increase development of corpora lutea in early postpartum cows?

General Discussion

Most lactating dairy cows undergo a period of NEB. Negative energy balance adversely affects follicular and luteal growth in cattle. Energy balance in early lactation is positively associated with serum progesterone (Spicer et al., 1991; Villa-Godoy et al., 1988), thus, cows in severe NEB have reduced concentration of progesterone in serum. Changes in metabolites and hormones due to NEB may affect follicular and luteal growth directly (ovarian) or indirectly (through central nervous system). Signals, such as low serum concentrations of luteinizing hormone, insulin, insulin-like growth hormone-I (IGF-I), glucose, tyrosine and high growth hormone and opiates are possible links between nutritional deficiency and reproductive performance. In the present thesis, peak preovulatory estradiol was associated positively with morphological development of corpora lutea. Thus, it is of interest to speculate on potential mechanisms by which NEB affects follicular and corpora lutea growth. Three predominant changes in hormones which may link NEB and ovarian development include: 1) reduced luteinizing hormone, 2) reduced insulin 3) reduced IGF-I and 4) interactive or associative effects.

Luteinizing hormone positively regulates granulosal and small luteal cell steroidogenesis and growth. Reduced pulsatile secretion of luteinizing hormone and reduced ability of the ovary to respond to luteinizing hormone are two primary causes of extended postpartum anestrous in cattle experiencing NEB (Schillo, 1992). In contrast, others report that in heifers and lactating dairy cows, NEB reduced serum progesterone, luteal weight or growth of dominant follicles with no concurrent change in concentration of serum luteinizing hormone (Lucy et al., 1992b; Schrick et al., 1992; Villa-Godoy et al., 1990). Although reduced concentration of luteinizing hormone in serum mediates the effect of NEB on the ovary in anestrous postpartum cows, other metabolites or hormones may cause reduced ovarian function in cycling cattle.

Negative energy balance reduced concentration of insulin in serum of heifers and lactating dairy cows (Hart et al., 1978; Schrick et al., 1992; Villa-Godoy et al., 1990). Granulosal and thecal cells are targets for insulin. In these cells, insulin increased steroidogenesis, increased cell viability, mitosis and synergized with gonadotropins. Thus, even if gonadotropins (luteinizing hormone and follicle stimulating hormone) are not altered by NEB, lower insulin due to NEB may limit ovarian follicles responsiveness to gonadotropic stimuli. Insulin is a potent luteotropin. Insulin increased degradation of low density lipoproteins which liberates precursors for progesterone production (Murphy and Silivan, 1989). Furthermore, insulin increased progesterone production by increasing cholesterol side-chain cleavage enzyme activity and by stimulating endothelial cell proliferation (Keyes and Wiltbank, 1988). Increased proliferation of endothelial cells increases vasculature of corpora lutea. Increased vasculature of corpora lutea interacted with luteal weight to increase concentration of luteal progesterone (Peters, 1989). Restriction of dietary energy in heifers reduced serum concentration of insulin and luteal weight. But, luteal weight was not reduced in heifers restricted in dietary energy that received exogenous insulin (Harrison and Randel, 1986).

Insulin stimulates glucose transport into cells. Low insulin may indirectly affect ovarian cells by limiting glucose transport into cells. Glucose transport into cells is critical because administration of 2-deoxyglucose, which decreases intracellular glucose, to cycling heifers before or during estrus, prevented estrus and corpora lutea development (McClure et al., 1978). Thus, reduced insulin may directly or indirectly reduce follicular growth, luteal growth or both.

Negative energy balance reduced IGF-I in heifers (Houseknecht et al., 1988; Spicer et al. 1990; VandeHaar et al., 1992) and lactating dairy cows (Spicer et al., 1991). Granulosal and luteal cells contain IGF-I receptors (Adashi et al., 1985; Sauerwein et al., 1992; Talavera and Menon, 1991). Insulin-like growth factor-I stimulated granulosal cell replication and enhanced action of follicle stimulating hormone on granulosal cells (Hammond et al., 1988; Ireland, 1987). Thus, low concentration of IGF-I in serum due to NEB could limit number and responsiveness to follicle stimulating hormone of granulosal cells. It is not known, however, if IGF-I affects replication of bovine luteal cells. But, IGF-I increased progesterone production by bovine luteal cells (Sauerwein et al., 1992). Thus, in early lactating dairy cows and heifers restricted in dietary energy, reduced concentration of IGF-I in serum is likely to limit follicular growth, luteal function or both.

Concentration of growth hormone in plasma is higher in underfed compared with adequately fed lactating dairy cows (Gluckman et al., 1987) and heifers (Spicer et al., 1991). In lactating dairy cows, several studies have examined effects of bovine somatotropin (bST) on reproductive traits (conception rate, pregnancy rate and days open) (for review Baumen, 1992). Bovine somatotropin reduced pregnancy rate, but did not alter conception rate or days open. There are growth hormone receptors on bovine granulosal and large luteal cells (Lucy et al., 1992a). In vitro, growth hormone plus insulin increased number of bovine granulosal cells (Langout et al., 1991). However, administration of bST to lactating dairy cows did not alter concentration of estradiol in serum or size of the dominant follicle (Schemm et al., 1990). Furthermore, the effect of bST on concentration of progesterone in serum is equivocal (Schemm et al., 1990; Lefebvre and Block, 1992). Thus, growth hormone should increase follicular growth by increasing number of granulosal cells, but reduced serum insulin due to NEB may limit growth hormone's positive effect.

Nutrition influences ovarian function and development. The NEB heifers in the present study, had reduced concentration of insulin and IGF-I in serum compared to PEB heifers (VandeHaar et al., 1992). Thus, reduced serum insulin and reduced IGF-I may have directly (via corpora lutea) and (or) indirectly (via ovulatory follicle) mediated effects of NEB on luteal morphology and weight and may also mediate effects of NEB in lactating dairy cattle. Future research should include further identification of changes in hormones, growth factors and metabolites due to NEB, and the mechanisms by which these factors, singly or in concert with one another, influence the ovary.

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Appendix

Validation of Estradiol Radioimmunoassay for Bovine Serum

Estradiol antisera, labeled ligand, and goat anti-rabbit gamma globulin were purchased from Diagnostic Products Corporation (Los Angeles, CA). These reagents as purchased were developed to quantify estradiol in human serum. Validation of these reagents for bovine serum included recovery of estradiol from bovine serum, and parallelism of estradiol in bovine serum to estradiol standard curve. Recovery of exogenous estradiol-17β (0.25, 0.625, or 2.5 pg/100 μl) was 0.23 ± 0.04 pg, 0.61 ± 0.13 pg, and 2.5 ± 0.13 pg/100 μl (r=8) in charcoal-extracted serum of an ovariectomized heifer. Concentration of estradiol in charcoal-extracted serum of the ovariectomized heifer was below the detection limit of the assay. Sensitivity of the assay was 1.31 ± 0.004 pg/ml.

Displacement of ^{125}I-estradiol from the antisera by exogenous estradiol (2.5, 10.0, 25.0, and 50.0 pg/ml) in 200 μl and 400 μl of bovine serum was parallel (r^2 = .9; r^2 =.9) to displacement in buffer standards (n = 8). Pooled serum from PEB or NEB heifers (r = 8) were parallel (r^2 = .9) to the estradiol buffer standard (n = 4). The estradiol antisera exhibited low crossreactivity with other steroids (progesterone ND, estriol 0.235%, androstenedione 0.004%).

Procedures for Estradiol Radioimmunoassay

Iodinated estradiol was the labeled ligand. Goat anti-rabbit gamma globulin with polyethyleneglycol in saline was used to separate bound and free ligand. Estradiol standards diluted in assay buffer (0.1% gelatin in phosphate-buffered saline, pH 7.4) were prepared in our laboratory and substituted for the commercially available standard. Duplicate aliquots of 200 or 400 μl of serum were extracted twice. To extract, duplicates of 200 μl or 400 μl of serum were vortexed for 2 minutes with 2 ml ethyl ether, the aqueous phase was frozen in dry ice methanol bath, and ether was decanted and saved. This procedure was repeated once. Ether extracts were combined and evaporated. Dried extracts were reconstituted in 200 μl assay buffer and stored 8 to 12 hours at 4°C. A 100 μl aliquot from each duplicate, estradiol standards and standard sera were assayed in each RIA. Estradiol antisera to cause 40% total binding was added to samples, standards, standard sera and total binding tubes. Assay buffer (30 μl) was substituted for estradiol antibody in nonspecific binding tubes. After addition of reagents to all tubes incubation was at room temperature for 2 h. Iodinated estradiol (specific activity = 2900 μCi/μg estradiol; ≈25,000 cpm) was added to all tubes and incubated at room temperature for 1 h. Goat anti-rabbit gamma globulin (1 ml) was added and tubes were incubated for 10 min at room temperature. Tubes were centrifuged at 3200 x g for 20 min at 4°C. Supernatants were decanted, tubes were dried and pellets were counted for 1 minute in a gamma counter (Tm Analytic, Elk Grove Village, IL).

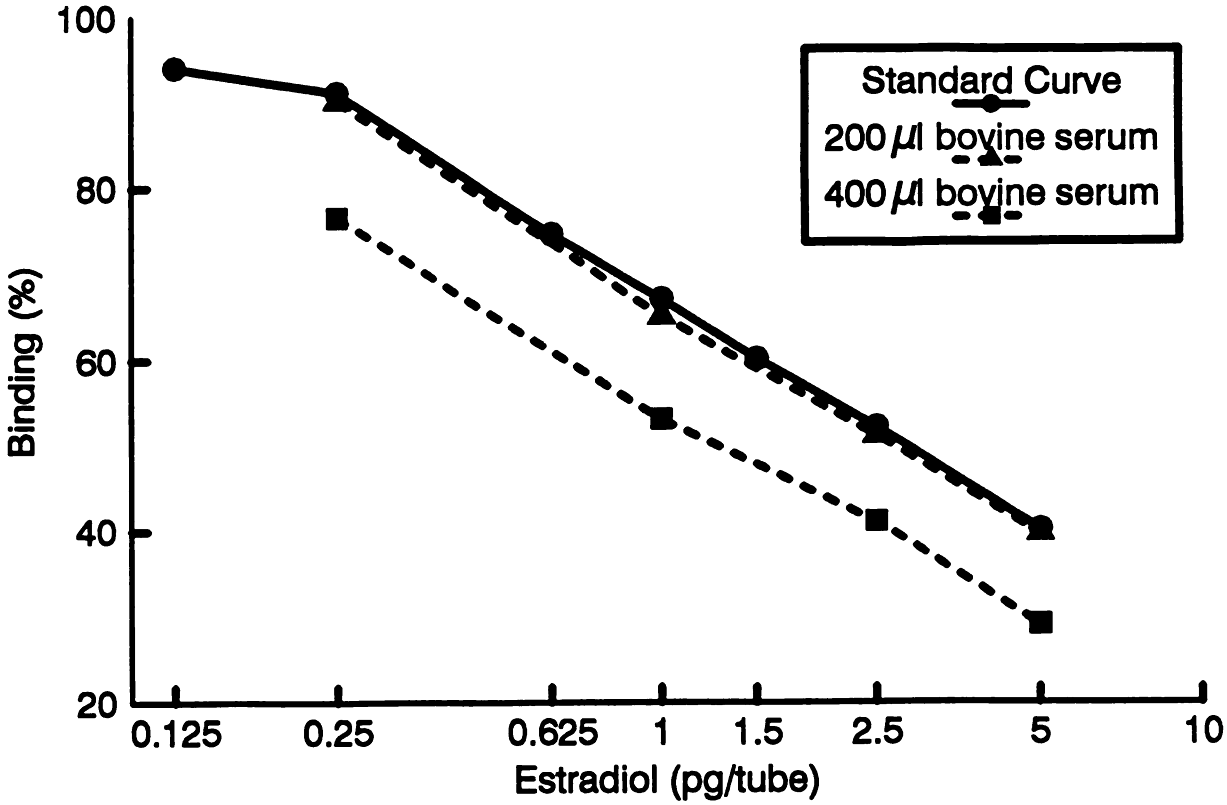

Figure 11. Displacement of ^{125}I-estradiol from antibody for estradiol by different dilutions of pooled bovine sera. Slope for eight standard curves of estradiol paralleled (r^2 = .9) slope from regression line of estradiol in dilution of pooled bovine sera (n = 20/dilution).