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Effect of Energy Balance and Somatotropin on Serum Insulin-like Growth Factor-I and on Weight and Progesterone of Corpus Luteum in Heifers

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EFFECT OF ENERGY BALANCE AND SOMATOTROPIN ON SERUM INSULIN-LIKE GROWTH FACTOR-I AND ON WEIGHT AND PROGESTERONE OF CORPUS LUTEUM IN HEIFERS

By

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ABSTRACT

EFFECT OF ENERGY BALANCE AND SOMATOTROPIN ON SERUM INSULIN-LIKE GROWTH FACTOR-I AND ON WEIGHT AND PROGESTERONE OF CORPUS LUTEUM IN HEIFERS

By

Mee-Chiann Yung

Holstein heifers (≈13 months old) were synchronized for estrus and fed individually 68% (n=14; NEB) or 139% (n=16; PEB) of maintenance energy requirement during four estrous cycles. On day ≈12 of third estrous cycle of each heifer, prostaglandin F2α was given, and starting the next day, half the heifers were injected daily with bST (14 mg/d) until the corpus luteum was collected on day 10 after estrus. Concentration of IGF-I in serum was 29% less ($P < 0.01$) in control NEB heifers than in control PEB heifers. Injection of bST increased serum IGF-I in both energy balance groups, but the increase was greater ($P < 0.01$) in heifers in PEB (73%) than those in NEB (35%). Weight of corpus luteum in PEB control animals was greater ($P < 0.01$) than that of NEB controls but not affected by exogenous bST. Serum IGF-I and luteal weight were not correlated significantly. In summary, bST injection during luteal

development restored serum IGF-I of NEB heifers to the concentration observed in PEB controls but it had no effect on luteal growth in NEB or PEB heifers. We conclude that low serum IGF-I concentration during luteal growth is not the reason that NEB depresses luteal growth.

To

My Parents

&

Yu-Tsai Wang

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LIST OF ABBREVIATIONS

bST	Bovine somatotropin
BW	Body weight
CL	Corpus luteum or corpora lutea
DM	Dry matter
EB	Energy balance
FSH	Follicle stimulating hormone
IGF-I	Insulin-like growth factor-I
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
NEB	Negative energy balance
NEFA	Non esterified fatty acids
NEm	Net energy for maintenance
PEB	Positive energy balance
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
ST	Somatotropin

INTRODUCTION

Delayed conception extends the proportion of time cows spend in late lactation and the dry period and thus decreases milk profits. A causative factor of delayed conception is negative energy balance (Houghton et al., 1990; Villa-Godoy et al., 1988). At least 92% of dairy cows experience negative energy balance during early lactation (Reid et al., 1966; Coppock et al., 1974). During negative energy balance, homeorhetic mechanisms ensure metabolic support to the mammary gland for milk synthesis. This focus on mammary support could limit nutrient supply to ovaries and thus decrease the activity of the corpus luteum. The corpus luteum produces progesterone, which is necessary for maintenance of pregnancy. Negative energy balance also decreases the concentration of progesterone in blood and milk of lactating cows (Spicer et al., 1990; Villa-Godoy et al., 1988). Decreased luteal activity is indicated by decreased luteal weight, luteal concentration of progesterone and serum progesterone concentration in heifers (Terhune, 1992; Villa-Godoy et al., 1990).

A possible mediator of negative energy balance on reproduction is insulin-like growth factor-I (IGF-I), a peptide that mediates many actions of somatotropin (Baxter, 1986). Somatotropin and IGF-I are regulated by nutritional status (Ronge et al., 1988), and the concentration of IGF-I is positively correlated with the concentration of progesterone in serum of cows (Spicer et al., 1990). Furthermore, adding IGF-I to granulosa cells *in vitro* enhances some of the effects of follicle stimulating hormone and luteinizing hormone (Adashi et al., 1991; Hammond et al., 1991; Giudice, 1992).

The major source of serum IGF-I is the liver, but IGF-I is also produced by many other tissues including granulosa cells and luteal cells (Hammond et al., 1985; Einspanier et al., 1990; VandeHaar et al., 1995). Synthesis of IGF-I by ovarian cells raises the possibility that IGF-I may act through an autocrine and/or paracrine as well as an endocrine role. However, in this thesis, only the endocrine role will be examined (Figure 1). In vitro, IGF-I enhances progesterone secretion in murine (Adashi et al., 1985), bovine (Schams et al., 1988) and porcine (Veldhuis et al., 1985) granulosa cells. IGF-I also stimulates progesterone secretion of bovine luteal cells collected within 1-5 day post-estrusand incubated for 72 hours (McArdle and Holtorf, 1989). In addition, *in vitro* perfusion of IGF-I to bovine luteal cells increases progesterone secretion

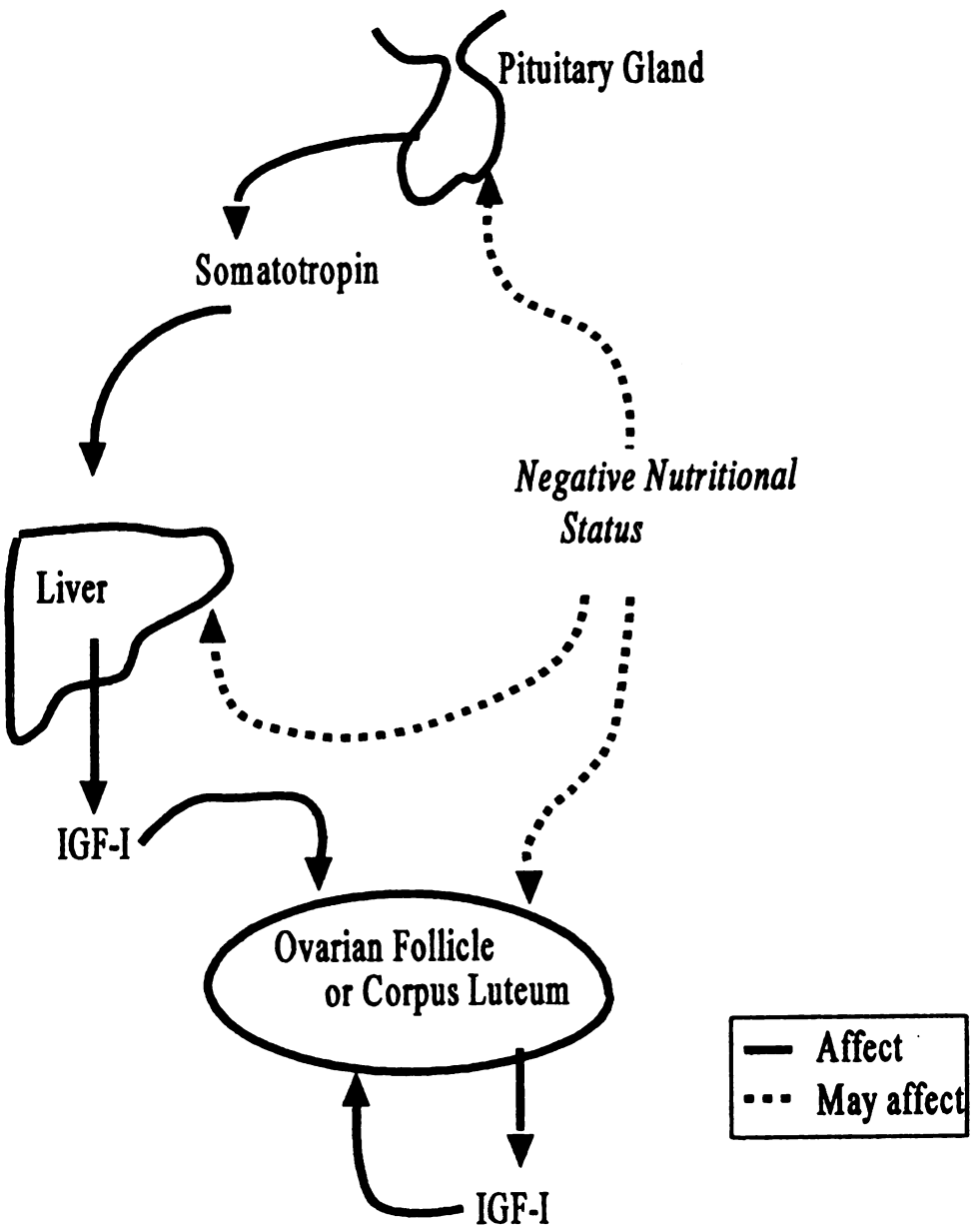

Figure 1. The somatotropic cascade

(Sauerwein et al., 1991). In lactating cows, exogenous bovine somatotropin (bST) increases the concentration of IGF-I and progesterone in blood (Gallo and Block, 1989; Schemm et al., 1990).

The objective of this thesis was to test the hypothesis that serum IGF-I mediates the effect of energy balance on ovarian function and to determine the effect of exogenous bST during the luteal phase on luteal weight and luteal progesterone content. I postulated that bST would increase the concentration of IGF-I in serum of heifers in negative energy balance to the normal concentration of heifers in positive energy balance and thus provide enough IGF-I to the ovary to support normal luteal growth. If so, then perhaps I could alleviate the impaired luteal development in cattle in negative energy balance by injection of bST just before estrus to restore concentrations of IGF-I in serum to normal. To avoid the effect of energy balance confounding with postpartum interval, lactation, sensitivity of the anterior pituitary gland to luteinizing hormone releasing hormone, or reproductive diseases in dairy cows, pubertal heifers were used to determine these effects of bST and negative energy balance on luteal development.

LITERATURE REVIEW

Negative Energy Balance

Energy balance (EB) is defined as the difference between the energy intake of an animal and its energy expenditure. Energy lost from the body includes the loss of heat, urine, feces, and hair growth. When the energy intake exceeds the losses, an animal is in positive energy balance (PEB). On the other hand, when the energy intake is lower than the animal's energy expenditure, an animal is in negative energy balance (NEB).

The Effects of Negative Energy Balance on Conception

Most dairy cattle experience NEB in early lactation because of the insufficient intake of energy for production of milk (Coppock, 1985). Cows which are in severe NEB take a longer time to return to normal cycling in the early postpartum period (Staples et al., 1990). Butler et al. (1981) reported that ovulation and initiation of the first normal luteal phase occurred approximately 10 days after EB began returning toward a positive value. This result of Butler

et al. (1981) agrees with the observation by Lucy et al. (1991) that higher EB is associated with more ovulation in dairy cows in early lactation. Moreover, restricted dietary intake in postpartum cows increases the interval from calving to first ovulation (Butler and Smith, 1989), first estrus (Whitemore et al., 1974) and conception (Haresign, 1979) when compared to ad libitum intake. Also in another study, postpartum cows that were fed a higher energy diet required fewer inseminations per conception and conceived 19 days earlier than those fed a lower energy diet (Folman et al., 1973). Thus, NEB could delay conception in lactating cows.

Negative Energy Balance, Insulin-like Growth Factor-I and Ovaries

Possible Role of Insulin-like Growth Factor-I

Mature IGF-I is a single-chain polypeptide of 70 amino acids that is structurally similar to proinsulin and contains B and A domains which are homologous to those of insulin. Liver is the main source of circulating IGF-I, and IGF-I from liver was originally proposed to mediate the somatogenic actions of somatotropin (ST) (Salmon and Daughaday, 1957). However, IGF-I protein and IGF-I mRNA are also found in many other tissues (D'Ercole et al., 1984;

Lund et al., 1986). The local production of IGF-I is not only regulated by ST but also by other modulators such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) in ovaries (Giudice, 1992) or is independent of ST (Murphy and Friesen, 1988). Multiple production sites and various regulators of IGF-I raise the possibility that in addition to endocrine actions, IGF-I may have autocrine and/or paracrine roles in tissues.

Actions of IGF-I tentative influence both positively and negatively by a family of binding proteins that are found in the circulation and in extracellular fluids (Baxter et al., 1989). Binding proteins modulate the action of IGF-I through prolonged half-life of IGF-I and variation of binding of hormone to its receptor (Giudice. 1992). Thus, concentrations of IGF-binding proteins can modulate the action of IGF-I.

In lactating dairy cows, the level of IGF-I in serum is positively correlated with EB (Ronge et al., 1988). Steers (Breier et al., 1986), heifers (VandeHaar et al., 1995), and cows (Sharma et al., 1994; Rutter et al., 1989) that are in NEB have lower concentrations of IGF-I in serum than those animals in PEB. As in cattle, concentrations IGF-I in plasma are decreased in underfed humans (Clemmons et al., 1981), rats (Maes et al., 1983), dogs (Eigenmann et al., 1985) and sheep (Pell et al., 1993). Decreased concentrations of IGF-I in blood during

underfeeding may signal to reduce anabolic reactions and to focus on survival rather than production.

IGF-I and Follicular Biology

Granulosa and thecal cells are the steroidogenic cells of a dominant ovulatory follicle. After ovulation and during luteinization these follicular cells become the large and small steroidogenic cells, respectively, of the corpus luteum (CL; Median et al., 1990). During early diestrus (d 4 to d 6), granulosa cells develop into large luteal cells and thecal cells develop into small luteal cells. In mid-diestrus (d 10 to d 12), a portion of the small luteal cells develop into large luteal cells. Since granulosa and thecal cells are the parental cells for luteal cells, the variation in number or function of the parental cells could contribute to the variation in number or steroidogenic ability of luteal cells. Removal of granulosa cells from preovulatory follicles in heifers (Milvae et al., 1991) and monkeys (Kreitmann et al., 1981; Marut et al., 1983) reduces serum progesterone concentration in the succeeding luteal phase. Furthermore, precocious induction of ovulation decreases the concentration of progesterone in serum in the succeeding luteal phase of ewes compared to control group (Murdoch et al., 1983). Presumably, decreased development of preovulatory

follicles would decrease the number of follicular cells and would decrease subsequent luteal development.

Bovine (Spicer et al., 1993) and swine (Hsu et al., 1987; Hammond et al., 1985) ovarian granulosa cells synthesize immunoreactive IGF-I and specific binding proteins for IGF-I. In addition, there is a positive correlation ($r = 0.69$) between IGF-I in serum and follicular fluid (Echternkamp et al., 1990), and concentrations of IGF-I are significantly lower in follicular fluid than in serum of cattle (Echternkamp et al., 1990), pigs (Hammond et al., 1988) and humans (Owen et al., 1991). Thus, IGF-I from serum may also be a source of IGF-I in follicular fluid.

Concentrations of IGF-I in blood are unrelated to the stage of the estrous cycle (Huang et al., 1992); however, concentrations of IGF-I in follicular fluid increase with follicular size (Spicer et al., 1988; Hammond et al., 1988). Hammond et al. (1985) observed that the concentration of IGF-I in follicular fluid is greater in preovulatory follicles than in immature follicles. In contrast, Rutter and Manns (1991) observed no relationship between concentrations of IGF-I in follicular fluid and size of follicles in postpartum beef cows. However, most studies show that the size of follicles is positively correlated with the

concentration of IGF-I in follicular fluid but the cause-effect relationship between this two is not clear.

For IGF-I to act on a tissue, receptors for IGF-I must be present. Type I IGF receptors are found in rat and ovine granulosa cells (Monget et al., 1989; Adashi et al., 1988). Type I IGF receptors bind IGF-I, IGF-II and insulin, but the affinity for IGF-I is 50 to 100 times greater than the affinity for IGF-II or insulin (Rechler and Nissley, 1985). The number of IGF-I receptors per cell on granulosa cells of ovine antral follicles is not influenced by the size of follicles (Monget et al., 1989).

In vitro, IGF-I increases ovine granulosa cell multiplication both in small and large follicles (Monniaux and Pisselet, 1992). IGF-I also stimulates the secretion of progesterone of bovine granulosa cells (Schams et al., 1988). Cholesterol is the precursor of progesterone and its uptake also is positively affected by IGF-I. IGF-I increases FSH-stimulated aromatase activity and binding of the LH receptor to LH of rat granulosa cells (Adashi et al., 1985). Additionally, IGF-I also increases basal and FSH-induced secretion of progesterone of ovine granulosa cells, (Monniaux and Pisselet, 1992). Furthermore, both FSH and LH enhance IGF-I binding to its receptor in rat granulosa cells (Adashi et al., 1986). Thus, IGF-I, through changes in receptor

binding, may modulate some of the effects of FSH and LH on ovaries. IGF-I augments the absolute rates of biosynthesis of progestin in swine granulosa cells by enhancing four mechanisms: 1) de novo synthesis of cholesterol in granulosa cells, 2) cellular uptake of low and high density lipoprotein-borne sterol substrate, 3) activity of cholesterol side-chain cleavage, and 4) turnover of cellular cholesteryl esters (Veldhuis et al., 1989).

Concentrations of IGF-I in dairy cattle vary with age and stage of lactation (Abribat et al., 1990). In heifers, IGF-I in serum increased at puberty (Jones et al., 1991), but heifers that have delayed puberty because of malnutrition have lower concentrations of IGF-I (Granger et al., 1989). In addition, cattle selected for high twining frequency have 47% greater concentrations of IGF-I in peripheral blood than those of normal cattle (Echternkamp et al., 1990). These studies suggest that IGF-I is associated with reproductive function in cattle.

Follicular IGF-I System During Negative Energy Balance

Severe restriction of dietary energy intake in postpartum cows decreased follicular fluid volume in medium-sized (7-9.9 mm in diameter) and large-sized (> 10 mm) follicles (Rutter and Manns, 1991). Restricted dietary energy also negatively influences the development of dominant follicles. Low dietary intake

decreases the diameter and persistency of dominant follicles during the estrous cycle of beef heifers (Murphy et al., 1991; Burns et al., 1994). Savio et al. (1993) suggested that the decreased persistency of the dominant follicle is caused by low concentrations of progesterone in plasma and is partly responsible for the impaired fertility of heifers during NEB.

Short-term fasting significantly decreases concentrations of IGF-I in plasma but does not affect concentrations of IGF-I in follicular fluid of heifers (Spicer et al., 1992). Severe NEB for 35 days, which normally would decrease serum IGF-I, does not affect concentrations of IGF-I and progesterone in follicular fluid of beef cows (Rutter and Manns, 1991). Granulosa cells of rats (Oliver et al., 1988), cattle (Spicer et al., 1993) and swine produce IGF-I (Hsu et al., 1987; Hammond et al., 1985). If local production of IGF-I is a major source of intraovarian IGF-I, these data suggest that the regulation of IGF-I synthesis is different in ovary and liver, so that the concentrations of IGF-I in serum and follicular fluid do not decrease in parallel during NEB.

IGF-I and Luteal Biology

After ovulation, the CL is formed. The number of steroidogenic cells in a bovine CL increases until 8 d after estrus (Lei et al., 1991). Bovine CL express

the mRNA and protein for IGF-I and the abundance of mRNA for IGF-I in luteal tissue is increased from d 1-5 until d 12-17 of the luteal phase (Einspanier et al., 1990). IGF-I binding sites are in CL and the specificity, affinity and capacity of IGF-I binding sites are constant throughout the estrous cycle (Sauerwein et al., 1991), in contrasts to granulosa cells (Adashi et al., 1986). In addition, the number of IGF-I receptors in CL does not correlate with serum or luteal progesterone concentrations in humans (Obasiolu et al., 1992). Incubating a primary culture of bovine luteal cells collected 4 - 10 d after estrus with IGF-I for 4 h does not affect the secretion of progesterone (Schams et al., 1988). However, adding insulin or IGF-I for more than 24 h to bovine luteal cells collected d 1-5 after estrus does increase the secretion of progesterone (McArdle and Holtorf, 1989). Also, adding IGF-I to luteal tissue *in vitro* increases the secretion of progesterone (Sauerwein et al., 1991), and IGF-I increases DNA synthesis by luteal cells (Chakravorty et al., 1993). Furthermore, the stimulative effect of IGF-I on luteal cells is dose dependent (McArdle and Holtorf, 1989). Thus, IGF-I has a positive effect on luteal development.

Luteal IGF-I System During Negative Energy Balance

During NEB, the abundance of IGF-I mRNA is decreased in liver. But the abundance of IGF-I mRNA in CL is not changed by NEB (VandeHaar et al., 1995). The regulation of IGF-I synthesis is on the mRNA level (Pell et al., 1993). This supports our previous suggestion that the regulation of IGF-I synthesis is different in ovary and liver. It is also suggested that autocrine and/or paracrine IGF-I does not mediate effect of NEB on CL.

Possible Role of Other Hormones and Metabolites

Somatotropin

Somatotropin (ST) is stored in and secreted by the somatotrophs in the anterior pituitary gland. The protein and mRNA of ST receptors are found on bovine granulosa cells and luteal cells (Lucy et al., 1992). *In vitro*, ST increases the synthesis of protein in granulosa cells and increases the number of granulosa cells in the presence of insulin (Langhout et al., 1991). Moreover, ST stimulates progesterone biosynthesis in cultured human luteal cells (Lanzone et al., 1992).

Dietary restriction increases pituitary ST mRNA and concentrations of ST in serum of cows (Kirby et al., 1993). Reducing feed intake in steers and heifers

to the point that they lost body weight significantly increases mean concentrations of ST in plasma (Breier et al., 1986; Villa-Godoy, 1987; VandeHaar et al., 1995). This increase of mean ST is due to the increased amplitude and duration of pulses of ST but not the baseline concentrations or the frequency of pulses of ST (Breier et al., 1986; Villa-Godoy, 1987).

Progesterone

Small and large cells are defined within a CL according to their morphological and biochemical difference. Small bovine luteal cells range from 10 to 25 μm in diameter and large luteal cells range from 25 to 50 μm in diameter, which is 13 times bigger than small luteal cell in cell volume (Koos and Hansel, 1981). Without the stimulation of LH, large luteal cells secrete 20-fold more progesterone on a per cell basis than small luteal cells in cattle and sheep (Koos and Hansel, 1981; Fitz et al., 1982). Large luteal cells are responsible for approximately 78% of the progesterone secreted into serum (Niswender et al., 1985; O'Shea et al., 1987).

Progesterone is necessary for three events that influence fertility in dairy cattle: estrus detection (Melampy et al., 1957), conception (Folman et al., 1973; Fonseca et al., 1983), and embryonic survival (Hill et al., 1970). Furthermore,

concentrations of progesterone in the estrous cycle before insemination are also associated positively with conception rate (Carstairs et al., 1980; Fonseca et al., 1983). Thus, low concentrations of progesterone in serum may limit fertility.

Studies show that the diameter of ovulatory follicles and CL are decreased by dietary energy restriction (Burns et al., 1994; VandeHaar et al., 1995). Moreover, the fact that NEB decreased the ratio of large to small cells in the CL (Terhune, 1992) indicate that there are fewer large cells for progesterone production in NEB animals.

Villa-Godoy (1987) found that in lactating dairy cows, the concentration of progesterone in milk is reduced proportional to the magnitude of the energy deficiency. Furthermore, Spicer et al. (1990) reported that the concentration of progesterone in serum during early postpartum period was positively associated with EB. However, other studies have observed different results. Restricted dietary intake in cattle also has been reported to increase (Donaldson et al., 1970; McCann and Hansel, 1986) or have no effect (Lucy et al., 1993; Spitzer et al., 1978) on concentrations of progesterone in blood. Thus, the effect of restricted dietary intake on the concentration of progesterone in serum of cows has no consistent results. During overnutrition, cattle either have greater (Folman

et al., 1973) or no change (Spicer et al., 1984) in concentrations of progesterone in blood. What causes these different results is not clear.

Luteinizing Hormone

Luteinizing hormone is the primary luteotropin in most domestic animals (Channing et al., 1974). The frequency of pulsatile LH secretion is important for the final phase of maturation of ovarian follicles (Randel, 1990) and thus for induction of estrus and ovulation. In addition, LH is required for maintenance of CL (Hoffman et al., 1974) and stimulates the secretion of progesterone from CL. Thus, the concentrations and pulses of LH are associated with the development of follicles and the production of progesterone in CL.

McCann and Hansel (1986) found that during the luteal phase, concentrations of LH are lower in fasted animals than in fed cattle. In support of this report, Imakawa et al. (1986) reported that dietary energy restriction suppresses mean concentrations of LH and amplitude of LH pulses in heifers. Furthermore, even small differences in energy intake influence the timing of the prepubertal surge of LH (Hall et al., 1994). In contrast, Gombe and Hansel (1973) found that progressive increases in mean LH occur when heifers are fed low energy diet for three successive estrous cycles. However, Terhune (1992)

found that NEB does not affect basal concentrations of LH. As with progesterone, the effect of restricted dietary intake on LH is not clear but the variance in methods of studies may be the cause.

Insulin

In woman, diabetes is associated with low fertility and hyperinsulinemia is associated with polycystic ovaries (Poretsky and Kalin, 1987). Concentrations of glucose and insulin in plasma of fasting heifers are lower than in fed heifers (McCann and Hansel, 1986). Additionally, low energy intake can decrease concentrations of insulin in plasma even when no change in glucose is observed (McGuire et al., 1991). In general, concentrations of insulin are positively associated with energy balance (Lucy et al., 1991; VandeHaar et al., 1995).

Non Esterified Fatty Acids

High concentrations of non esterified fatty acids (NEFA) in plasma are associated with poor fertility in cows (Ducker et al., 1985). Heifers in NEB have higher concentrations of NEFA in plasma than those in PEB (VandeHaar et al., 1995). Exogenous NEFA decreases the binding of progesterone by albumin (Ramsey and Westphal, 1978). Thus, increased concentrations of NEFA during

NEB may reduce the binding of progesterone to albumin and increase the concentration of free progesterone. As a result, the metabolic clearance rate of progesterone may be increased during NEB, which, in turn, would decrease the concentration of total progesterone in blood of NEB animals.

The Effect of Exogenous Somatotropin on Reproductive Function

Somatotropin and IGF-I

Administration of bST to dairy cattle increases the concentrations of somatotropin and IGF-I in serum (Davis et al., 1984; Gong et al., 1991) and increases the mobilization of NEFA from adipose tissue (Peel et al., 1981). ST acts partly through specifically binding to receptors in liver, and thus increasing the synthesis of IGF-I and the concentration of circulating IGF-I. Plasma insulin level is also increased by exogenous bST in heifers (Gong et al., 1993) and in lactating cows (VanderKooi, 1993).

The specific binding of ST in liver is greater in steers fed a high energy diet because of the appearance of a high-affinity binding site for ST compared to those steers which were fed a lower energy diet (Breier et al., 1988). At restricted energy intake, bST does not increase liver weight, but it does at high

energy intake (Pell et al., 1993). Breier et al. (1988) and Ronge and Blum (1989) demonstrated that undernutrition decreased concentration of IGF-I in blood and abolished the response of IGF-I to exogenous bST. However, other studies have shown that bST increases IGF-I in serum of early lactation cows in NEB (Gallo and Block, 1991; Ronge and Blum, 1989; Vicini et al., 1991). In addition, exogenous somatotropin increases IGF-I in serum of lambs in NEB (Bass et al., 1991) and of humans in NEB (Snyder et al., 1990). Therefore, the effect of bST on IGF-I in serum is diminished but not absent during NEB.

Somatotropin and Reproduction

Studies on the effects of bST on reproductive function are not conclusive. Administration of bST, in a sustained-release vehicle, to seven-month old heifers does not alter the age at puberty (McShane et al., 1989) or the pattern of LH release (Hall et al., 1994). In contrast, administration of bST exerts some negative effects on reproductive function in cows. Days open and the number of cystic ovaries tends to increase as the dose of bST increased in dairy cows (Hansen et al., 1994). BST-treated cows, compared to those not treated, need twice as many inseminations for every conception (McGuffey et al., 1991).

BST increases the frequency of LH pulses but decreases the baseline and average concentrations of LH during the first follicular phase after treatment started (Schemm et al., 1990). Long-term administration of bST also increases the concentration of LH in serum in response to gonadotropin releasing hormone at 14 d postpartum in cattle (Gallo and Block, 1991).

Gallo and Block (1989) and Schemm et al. (1990) found that bST treatment increases plasma progesterone concentrations in lactating cows. Furthermore, progesterone in plasma was increased during the first two estrous cycles post-treatment and during pregnancy by long-term bST treatment (Gallo and Block, 1991). However, short-term administration of bST from ~14 d before ovulation until the end of next cycle did not affect the concentration of progesterone in serum in the following estrous cycle (Gong et al., 1993).

Long-term treatment with bST does not affect lifespan of the CL, length of the follicular phase, length of the estrous cycle or the diameter of the largest follicle in cows (Schemm et al., 1990). Thus, the effects of bST treatment are not the result of changes in the time required for follicular and luteal development but of changes in the activity of the follicle or CL.

Treatment with bST doubles the population of small follicles (2-5 mm) (Gong et al., 1991) in the ovary of heifers. This increase in small follicles is

most likely the result of increased recruitment of small follicles (< 5 mm) because bST does not alter the turnover of follicular waves nor the inhibitory action of the dominant follicle on its subordinate follicles in heifers (Gong et al., 1993). Furthermore, this increase in small follicles occurred after the concentration of ST in serum had returned to control values but the concentration of IGF-I in serum was still high, suggesting that the effects of bST on follicles are through circulating IGF-I.

Studies in cattle (Davoren et al., 1986), gilts (Spicer et al., 1992), and humans (Owen et al., 1991) suggested that ST increases intraovarian concentration of IGF-I. Concentrations of IGF-I in follicular fluid of the dominant follicle are decreased in cows immunized against growth hormone-releasing factor with low concentrations of ST and IGF-I in serum (Kirby et al., 1993). However, porcine somatotropin did not affect estradiol or progesterone concentrations in follicular fluid of pubertal gilts (Bryan et al., 1989).

Summary

Negative energy balance in cattle reduces the concentration of progesterone in serum, which in turn may decrease fertility by impairing the detection of estrus, the ability to conceive, and (or) the survival of embryo.

Factors that may be responsible for this decrease in serum progesterone include: 1) changes in the concentration of luteotropic hormones in serum and the number of receptors for these hormones on luteal cells, 2) changes in the cell population of the CL, 3) decreases in growth of the CL, and 4) decreases in activity of luteal cells. NEB also decreases the concentration of IGF-I in serum, and IGF-I promotes both growth and differentiation of granulosa cells and secretion of progesterone by luteal cells. We postulate that, during NEB, the decreased concentration of IGF-I in serum impairs luteal development. If so, increasing IGF-I by injection of bST during the time of luteal development might alleviate this impaired luteal growth. Thus, objectives for this study are to determine if: 1) exogenous bST during luteal development will restore luteal weights of NEB heifers to normal, and 2) exogenous bST during luteal development will increase luteal weights in PEB heifers.

MATERIALS AND METHODS

Animals and Diets

Thirty-two postpubertal Holstein heifers (≈13 months old) were group-housed indoors at the Michigan State University Dairy Research Center between January and July. Heifers were adapted to a feeding protocol in which they were fed all of their daily ration while locked in stanchions between 1000 and 1430 h each day. For the remainder of the day, animals could not eat but had free access to water and were free to allow social interaction and exhibition of estrus behavior. For at least two weeks before treatments started, heifers were fed a ration that supplied 140% of maintenance energy requirements. Heifers were allocated into two blocks by body weight (heavy and medium; n = 16). The heavy and medium groups began the experiment at different times so the average body weighs at the beginning of the treatments were similar for all heifers. Within each block heifers were assigned randomly to one of two dietary treatment groups (n = 8). At the start of the experiment, heifers were given two

injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 5 CC.= 25 mg/head; Lutalyse®; Upjohn company, Kalamazoo, MI) eleven days apart to synchronize estrus. Beginning 2 d after the second $PGF_{2\alpha}$ injection half the heifers were switched to a low energy diet (≈70% of maintenance energy requirement) and the other half continued on the diet at 140% of maintenance energy requirements (Figure 2). Body condition scores were recorded at the beginning and the end of the experiment by two persons. Body weights were measured on three consecutive days weekly and averaged by week for each heifer.

Diet ingredients were sampled and chemically analyzed (Northeast DHIA Forage Testing Laboratory, Ithaca, NY), and the ingredients and chemical composition are summarized in Table 1. Dietary ingredients were also sampled twice a week for the analysis of dry matter (DM). Samples (≈20 g) of alfalfa silage, corn silage, and silage mixture were used for weekly DM analysis. Samples were dehydrated at 100°C oven to achieve constant weight, and the DM percentage was calculated. Diets for each heifer were adjusted every week according to DM content of diets and individual body weight to ensure that each heifer was fed at the appropriate energy intake (see Appendix A). The orts were collected (if any) every day and weighed to calculate feed intake.

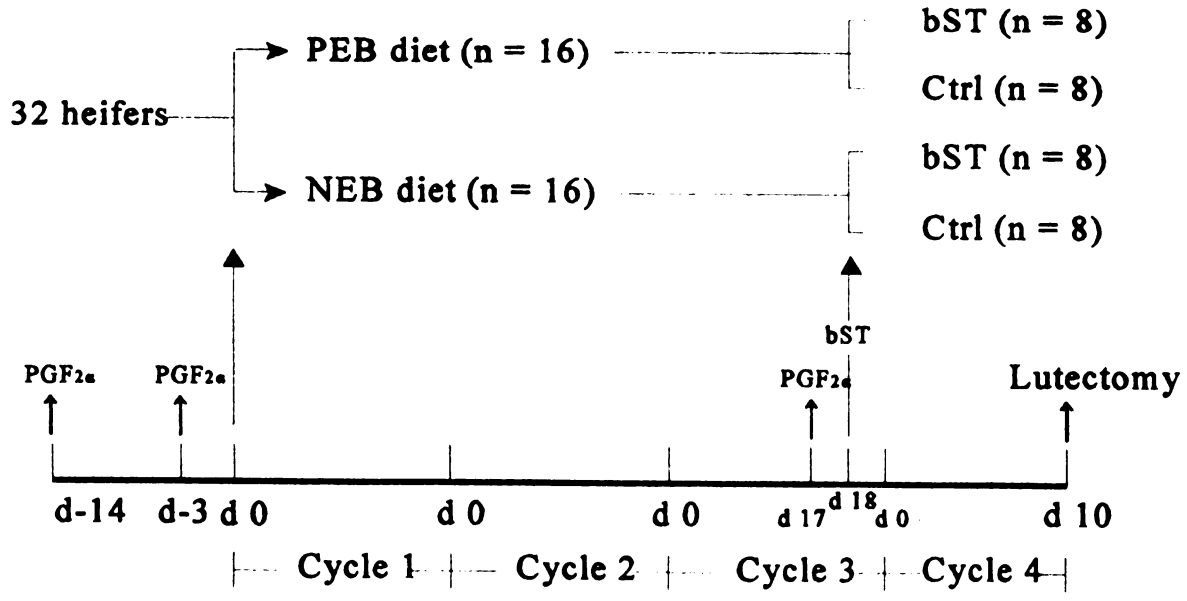

Figure 2. Time-line of experiment (d 0 = estrus; Ctrl = control)

Table 1. Composition of diet (DM basis)[a]

	PEB	NEB
Silage mixture, kg	3.7	2.6
Cracked corn, kg	2.4	0.2
Protein supplement[b], kg	0.5	0.3
Total intake, kg	6.6	3.1
Nutrient composition of total diet[c]		
NE_m, Mcal/kg DM	1.6	1.4
Crude protein % of DM	15	16
Feed consumed, % of ME requirement	139	68
Energy Balance Mcal/d	1.7	-2.2

[a]Daily average for four cycles.
[b]Contained soybean meal, trace-mineral and vitamin premixes and was formulated to provide 100% of protein, mineral and vitamin NRC requirement.
[c]Calculated based on laboratory analyses. (Northeast DHIA Forage Testing Laboratory, Ithaca, NY)

Heifers were observed for estrus behavior for periods of 30 minutes three to five times a day. An animal was considered in estrus if she stood to be mounted by others for at least two seconds. Visual estrus was defined as d 0 of an estrous cycle. After 2.5 estrous cycles, prostaglandin $F_{2\alpha}$ was injected. One day after prostaglandin $F_{2\alpha}$ injection, bST injection was started. Within each dietary treatment group, half animals were randomly selected for daily subcutaneous recombinant bST injection (14 mg/day; courtesy of The Upjohn Company, Kalamazoo, MI). On d 10 of the fourth cycle (d 0 = estrus), CL of heifers were collected. All processes were approved by the University Committee on Animal Use and Care of Michigan State University.

Energy Balance

Dietary energy intake and body weight were used to estimate energy balance. For NEB heifers, it was calculated as in the *Nutrient Requirements of Dairy Cattle* (National Research Council; 1989):

$$EB = NE_{m_{intake}} - NE_{m_{req.}}$$

Where $NE_{m_{intake}}$ is the intake of net energy (NE) for maintenance and $NE_{m_{req}}$ is the NE_m requirement of the animal. $NE_{m_{intake}}$ was calculated as:

$$NE_{m_{intake}} = feed\,intake(\ kg\ dry\ matter) \times NE_m\ (\ Mcal/\ kg\ dry\ matter)$$

where the NE_m value of the feed was calculated based on laboratory analyses of each ingredient. $NE_{m_{req.}}$ is based on average weekly body weight (BW) and calculated as:

$$NE_{m_{req.}} = 0.086 \times BW\ (kg)^{0.75}$$

Because energy is used less efficiently for gaining weight than for maintenance, net energy intake that exceeded NE_m requirement was multiplied by 0.66 (the approximate ratio of NE for gain to NE_m for the diet). Thus, the EB in PEB heifer was calculated as:

$$EB = (NE_{m_{intake}} - NE_{m_{req.}}) \times 0.66$$

Collection of Blood Samples

Beginning with the second estrous cycle, blood was sampled by venipuncture daily at 1400 h. After the injection of prostaglandin $F_{2\alpha}$ in the third estrous cycle, blood samples were taken twice a day at 1000 and 1400 h. On day 9 of the fourth estrous cycle, blood was collected four times a day (0700, 1100, 1500, and 1900h). Blood samples were stored at 4°C overnight, and serum was harvested by centrifugation for 20 min at 800×*g* and frozen at -20°C.

Collection of Corpora Lutea

On d 10 of the fourth estrous cycle, CL were collected. Animals were standing and restrained in a chute. The external genitalia and vagina were sanitized with 0.005% chlorhexidine diacetate. An incision was made in the anterodorsal wall of vagina. By digital pressure, the CL was enucleated from ovarian stroma. All samples were bisected, blotted dry, and frozen immediately in dry ice and weighed. Samples were stored at -70°C for later assay.

Radioimmunoassay of Insulin-like Growth Factor-I

Concentration of serum IGF-I was determined by radioimmumoassay (see Appendix B). Binding proteins for IGF-I were removed from serum by acid

ethanol extraction (Bruce et al., 1991). Recombinant human IGF-I (Bachem, Inc., Torrance, CA) was radioiodinated by the chloramine-T procedure for use as tracer (Etherton et al., 1987). The international human IGF-I reference was used to make standards (Bristow et al., 1990). Rabbit anti-hIGF-I (UBK 487 supplied by Dr. Underwood, University of North Carolina, Chapel Hill) served as the antibody. After overnight incubation of samples and antisera at 4°C, labeled IGF-I (15,000 cpm/tube) was added, and the tubes were incubated for another 48 hours. Bound IGF-I was precipitated using *Staph aureus* protein (0.5 mg/tube; BM 100 061; Boehringer Mannheim Biochemicals, IN; Sharma et al., 1994).

Radioimmunoassay of Progesterone

Luteal tissue was processed much as described by Terhune (1992). CL were crushed into small pieces in a mortar and pestle while frozen. Tissue (≈ 140 mg/sample) and 4 ml phosphate-buffered saline (PBS; pH 7.4) were homogenized (20 strokes) with a teflon pestle connected to a stirrer. Half of the homogenized luteal tissue (2 ml) was saved for later assay of protein content. For the remaining half, 20,000 cpm ^{3}H-progesterone ([1,2,6,7-3H] progesterone, Amersham, Arlington Heights, IL) were added to enable to

determine of the efficiency of extraction. Homogenates were centrifuged (180×*g*, 10 minutes) and supernatants were saved. The remaining pellet was homogenized (20 strokes) in 5 ml diethyl ether and was centrifuged (180×*g*, 10 minutes), and the ether supernatant was saved. After three extractions with ether, all supernatants were combined and vortexed for 30 seconds and centrifuged (180×*g*, 10 minutes). The aqueous (lower) phase was frozen in a dry ice and methanol bath. The ether (upper phase) was decanted, evaporated and resuspended in 6 ml of ether to ensure that samples were in same volume of ether. The recovery of extraction of progesterone is 87 %. After evaporation of ether, the extractions were reconstituted to the equivalent of 1.3 mg luteal tissue per ml of phosphate-buffered saline (pH 7.4) with 0.1% gelatin for later assay.

Concentrations of progesterone in serum and CL were determined by using a validated (Srikandakumar et al., 1986) solid-phase radioimmunoassay (Coat-A-Count®; Diagnostic Products Corporation; Los Angeles, CA). In the assay, 100 µl of sample was added in an antibody-coated tube and 1 ml of ^{125}I-labeled progesterone was added and incubated for 3 - 6 hours at room temperature. After the incubation, liquid was decanted and gamma counter used to determine the radioactivity of tube for 1 minute. The intraassay and interassay coefficients of variation (CV) were 9.5 % and 8.1 %, respectively.

Radioimmunoassay of Somatotropin

Concentration of ST in serum was quantified by radioimmunoassay as described by Sharma et al.(1994). Purified bovine ST (Upjohn) was used for standards and radioiodination. Antisera against purified ST was rabbit anti-bovine ST antibody. The second antibody was sheep anti-rabbit gamma globulin.

Enzymatic Analysis of Non Esterified Fatty Acids

Concentrations of serum NEFA were determined by using an enzymatic kit (Wako Chemicals Inc. Richmond, VA) with modifications suggested by McCutcheon and Bauman (1986). Briefly, 25 ul of serum sample was added with 350 ul diluted Reagent A of the original kit and incubated for 60 minutes at 4°C. Following incubation, 750 ul of diluted Reagent B of the original kit was added, vortexed and incubated for another 20 minutes at 60°C. Absorbance at 550 nm was determined by the spectrophotometer.

Determination of Luteal Protein

To determine the protein quantity of luteal homogenates, the modified Lowry method was used (Markwell et al., 1978). Crystallized bovine serum

albumin was used as standard. Homogenized luteal tissue (10 µl) was diluted to make 1 ml volume before assay. Stock solutions of reagent A (2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate and 1% sodium dodecyl sulfate) and reagent B (4% $CuSO_4 \cdot 5H_2O$) were mixed 100:1 to make reagent C. Samples were added to 3 ml of reagent C and incubated at room temperature for 10 minutes. After the first incubation, samples were mixed vigorously with 0.3 ml of 1:1 diluted Folin-Ciocalteu phenol and incubated for another 10 minutes at 60°C. Absorbance at 660 nm was determined by the spectrophotometer.

Statistical Analysis

Three heifers were removed from the experiment. One was not cycling and the fourth estrus was not detected in two. Therefore, data used in analyses are from seven heifers that were in PEB and treated with bST (PEB-bST), eight heifers that were in PEB and not treated (PEB-control), six heifers that were in NEB and treated with bST (NEB-bST) and eight heifers that were in NEB and not treated (NEB-control).

The experimental design was a randomized block. Interactions and differences between EB and bST treatment means were analyzed by General Linear Models procedure of Statistical Analysis System (SAS Institute, 1989,

Cary, NC). Bonferoni *t* test was used for mean comparison of 1) PEB-control vs. NEB-control, 2) PEB-control vs. NEB-bST, 3) PEB-control vs. -bST, and 4) NEB-control vs. -bST. Pearson correlation coefficients were calculated for selected variables.

RESULTS

Body Weight and Energy Balance

For the duration of the experiment, calculated energy balances of PEB and NEB heifers were 1.7 and -2.2 Mcal/d of net energy, respectively. The actual average energy intakes in PEB and NEB heifers were 139% and 68% of maintenance requirements. Heifers fed PEB diets gained 820 g/d of BW and heifers fed NEB diets lost 414 g/d over the 10-wk experimental period (Figure 3). Final body weights of PEB heifers were greater than those of NEB heifers ($P < 0.01$). Also final body condition scores were greater in PEB heifers than in NEB heifers ($P < 0.01$; Table 2) but were not affected by bST injection (data not shown).

Concentration of Non Esterified Fatty Acids in Serum

NEB heifers had more ($P < 0.01$) NEFA in serum on d 9 of the fourth estrous cycle than did PEB heifers. Treatment with bST did not affect the concentration of NEFA in serum of both EB groups (Figure 4).

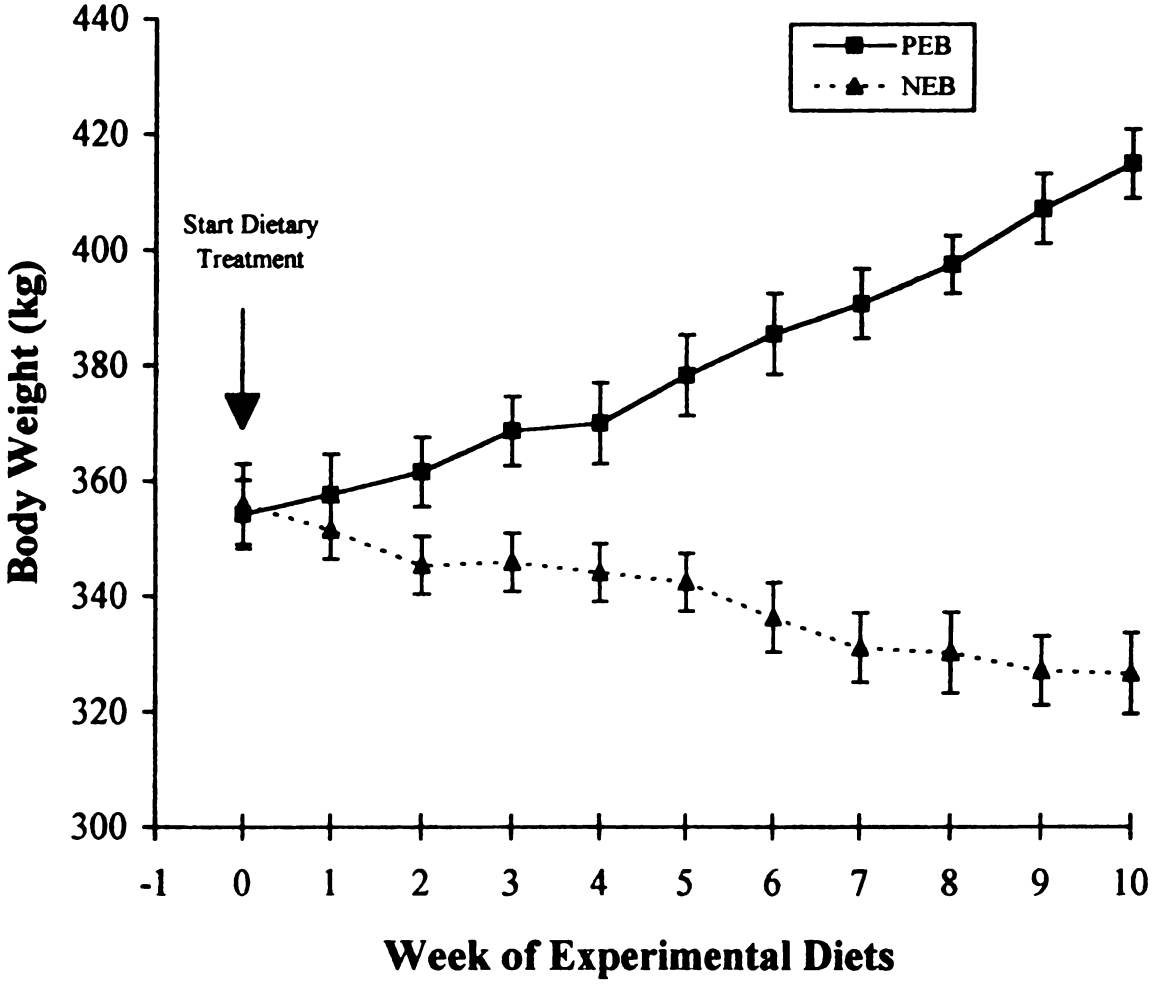

Figure 3. Average weekly body weight of heifers in positive (PEB) or negative (NEB) energy balance diets. Pooled standard error of the means among treatments was 7.

Table 2. Body weight (BW), body weight change and final body condition score of heifers in positive (PEB) or negative (NEB) energy balance for four consecutive estrous cycles.

Parameter	Energy Treatment			P > F
	PEB	NEB	Pooled SEM	
Initial BW, kg	354	356	7	NS
Final BW, kg	415	327	7	0.01
Weight change, g/d	820	-414	33	0.01
Final body condition score	3.8	2.1	0.1	0.01

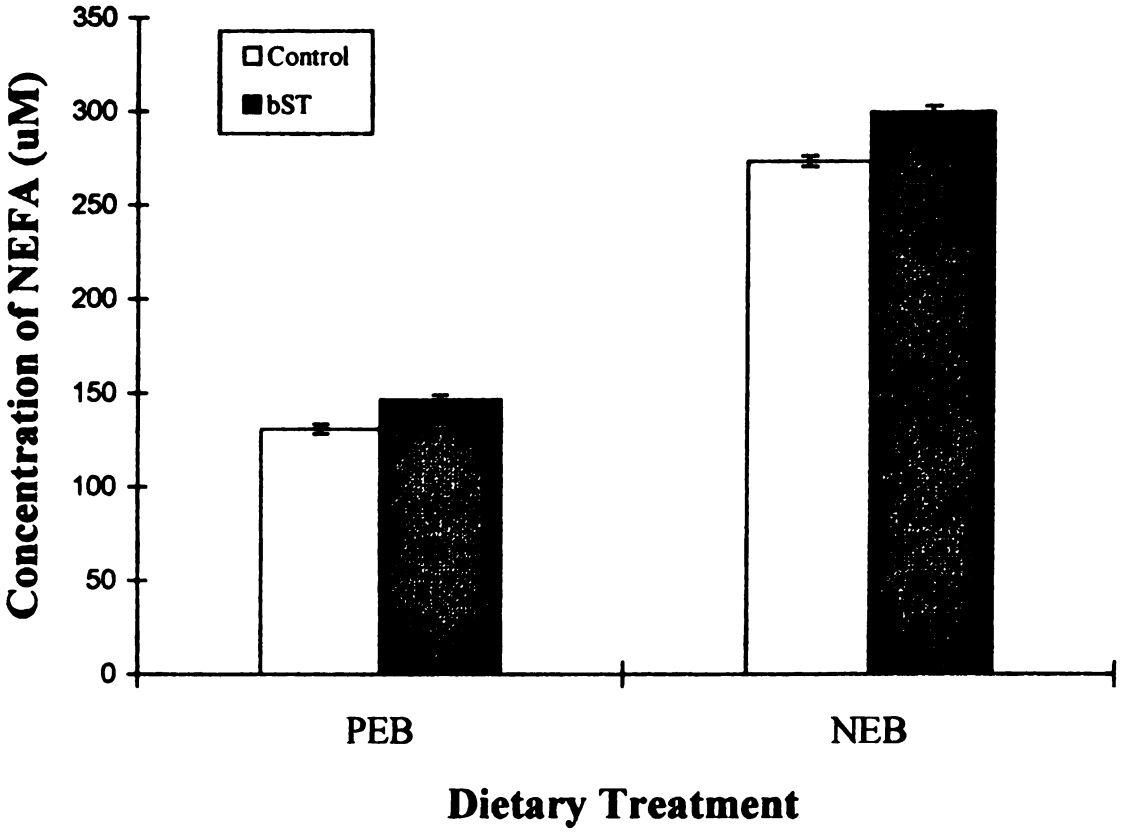

Figure 4. Least squares means of the concentration of NEFA in serum at d 9 of the fourth estrous cycle. Data are from eight PEB-Control, seven PEB-bST, eight NEB-Control, and six NEB-bST heifers. Pooled standard error of the means among treatments was 3.38.
Statistical analysis of mean comparisons:
PEB-Control vs. NEB-Control; $P < 0.01$.
PEB-Control vs. NEB-bST; $P < 0.01$.
PEB-Control vs. PEB-bST; $P = 0.63$.
NEB-Control vs. NEB-bST; $P = 0.44$.

Concentration of Somatotropin in Serum

Concentration of somatotropin in serum on d 9 of the fourth estrous cycle was higher for NEB than PEB heifers (3.43 ng/ml vs. 1.06 ng/ml; $P < 0.01$). Exogenous bST increased ($P < 0.01$; Figure 5) concentration of somatotropin in serum 27 fold in PEB and 15 fold in NEB heifers compared to their respective controls. The interaction between dietary treatment and bST treatment was significant ($P < 0.01$).

Concentration of Insulin-like Growth Factor-I in Serum

The mean concentration of IGF-I in serum from d 0 to d 10 of the fourth estrous cycle was 27% less ($P < 0.01$) in NEB than in PEB heifers. Exogenous bST increased ($P < 0.01$; Figure 6) the concentration of serum IGF-I in PEB and NEB heifers by 91 and 36%, respectively. The interaction between dietary treatment and exogenous bST was significant ($P < 0.02$). As we had planned, mean concentrations of IGF-I in serum were nearly identical for PEB-control and NEB-bST heifers ($P = 0.99$).

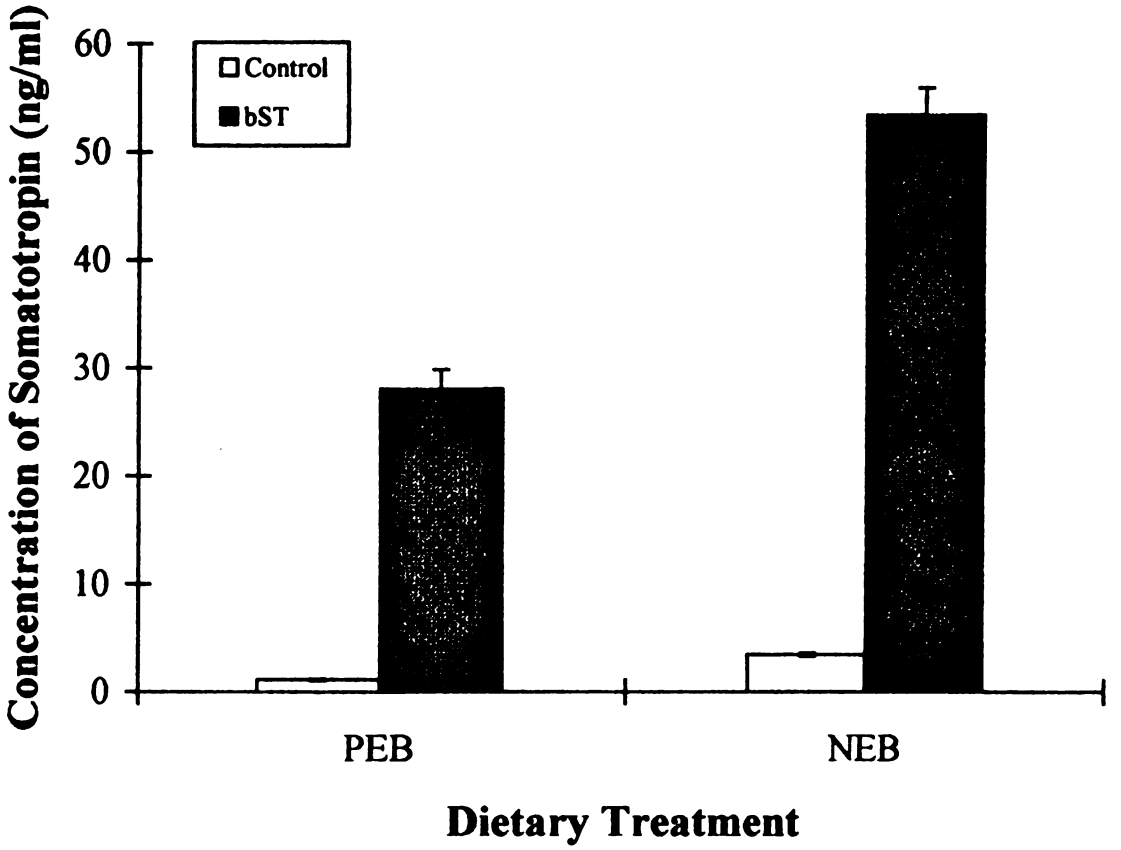

Figure 5. Least squares means of the mean concentration somatotropin at d 9 of the fourth estrous cycle. Data are from eight PEB-Control, seven PEB-bST, eight NEB-Control, and six NEB-bST heifers. Pooled standard error of the means among treatments was 2.5. Statistical analysis of mean comparisons:
PEB-Control vs. NEB-Control; $P = 0.68$.
PEB-Control vs. NEB-bST; $P < 0.01$.
PEB-Control vs. PEB-bST; $P < 0.01$.
NEB-Control vs. NEB-bST; $P < 0.01$.

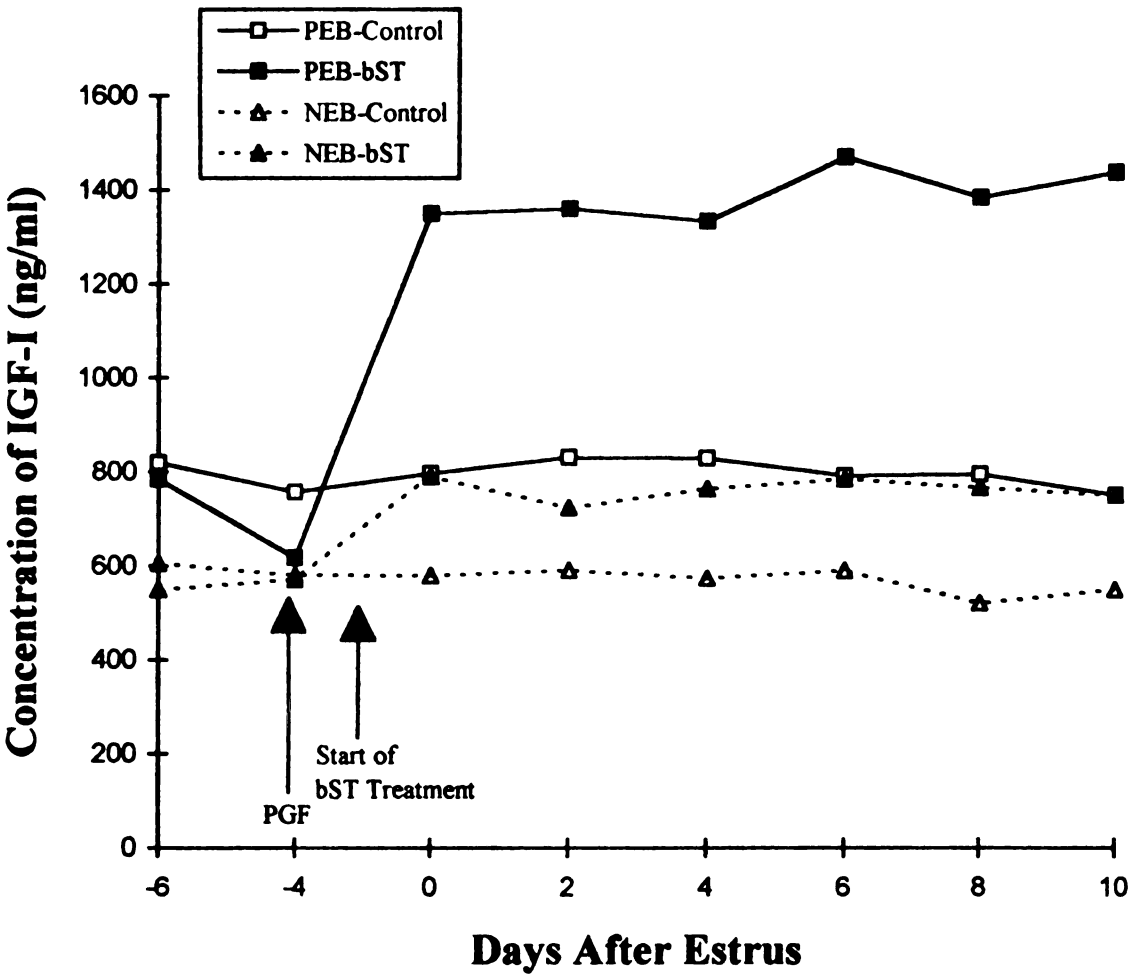

Figure 6. Concentration of IGF-I in serum during the fourth estrous cycle. Data are from eight PEB-Control, seven PEB-bST, eight NEB-Control, and six NEB-bST heifers.
Pooles standard error of the means among treatment was 170.
d 0 = estrus
Statistical analysis of mean comparisons for means from d0 to d10:
PEB-Control vs. NEB-Control; $P < 0.03$.
PEB-Control vs. NEB-bST; $P = 0.99$.
PEB-Control vs. PEB-bST; $P = 0.01$.
NEB-Control vs. NEB-bST; $P < 0.09$.

Concentration of Progesterone in Serum

Mean concentrations of progesterone in serum from d 0 to d 10 and d 10 alone of the fourth estrous cycle were not affected by either dietary treatment or bST treatment (Table 3). The concentration of progesterone in serum was not correlated with the concentration of IGF-I in serum within treatments or across all heifers.

Weights of Corpora Lutea

Weight of the CL was decreased from 6.9 g in PEB to 4.2 g in NEB heifers ($P < 0.01$). Treatment with bST did not alter luteal weight in NEB or PEB heifers (Figure 7), and luteal weights of NEB-bST heifers were smaller than those of PEB-control heifers ($P < 0.01$). Weight of the CL was not correlated significantly with the concentration of IGF-I in serum (Figure 8). For PEB-control heifers, but not other groups, weight of the CL was correlated positively with mean concentration of progesterone in serum ($r = 0.82$, $P = 0.01$). Among all heifers, luteal weight was correlated negatively with the concentration of NEFA in serum ($r = -0.32$, $P = 0.09$).

Table 3. Least square means of serum progesterone, luteal progesterone, and luteal protein content

	Treatment				
	PEB-Control	PEB-bST	NEB-Control	NEB-bST	Pooled SEM
Serum progesterone					
Mean from d 2 to d 10 (ng* ml^{-1})	1.2	1.1	1.2	1.1	0.1
Day 10 (ng/ml)	3.2	2.8	3	2.6	0.16
Luteal progesterone					
Concentration (μg/g) on tissue basis[a]	33.7	29.7	24.5	23.4	5.1
Concentration (μg/g) on protein basis[b]	0.14	0.14	0.11	0.1	0.01
Luteal protein content (ng/mg wet tissue)	241	216	224	231	6.3

[a]Tissue basis is based on the weight of luteal tissue. PEB heifers tended ($p < 0.09$) to have higher luteal progesterone concentration than NEB heifers.

[b]Protein basis is based on luteal protein content. PEB heifers tended ($p < 0.07$) to have higher luteal progesterone concentration than NEB heifers.

[c]Luteal content of progesterone is calculated as: luteal progesterone concentration (on tissue basis) × luteal weight. PEB-Control heifers have higher ($p < 0.01$) luteal progesterone content than NEB heifers in both control and bST heifers.

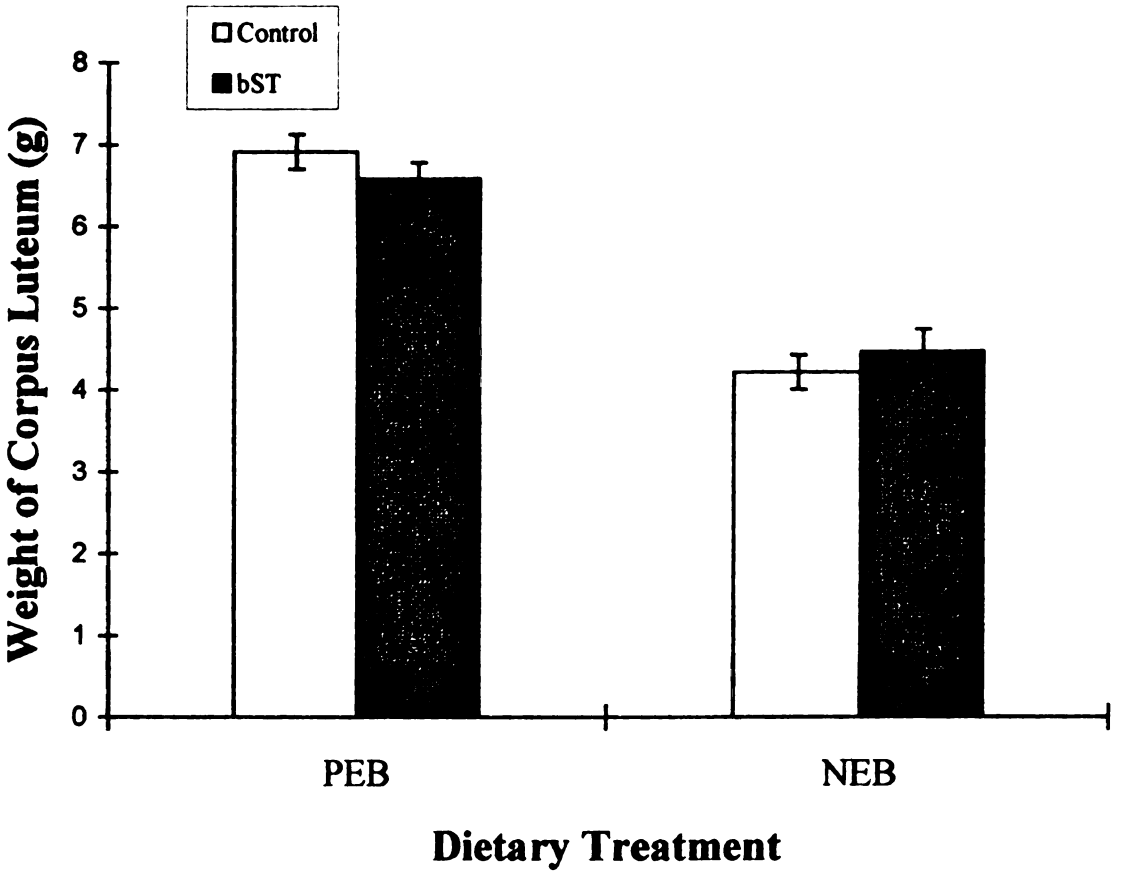

Figure 7. Least squares means of the weight of corpus luteum at d 10 of the fourth estrous cycle. Data are from eight PEB-Control, seven PEB-bST, eight NEB-Control, and six NEB-bST heifers. Pooled standard error of the means among treatments was 0.27.
Statistical analysis of mean comparisons:
PEB-Control vs. NEB-Control; $P < 0.01$.
PEB-Control vs. NEB-bST; $P < 0.01$.
PEB-Control vs. PEB-bST; $P = 0.69$.
NEB-Control vs. NEB-bST; $P = 0.65$.

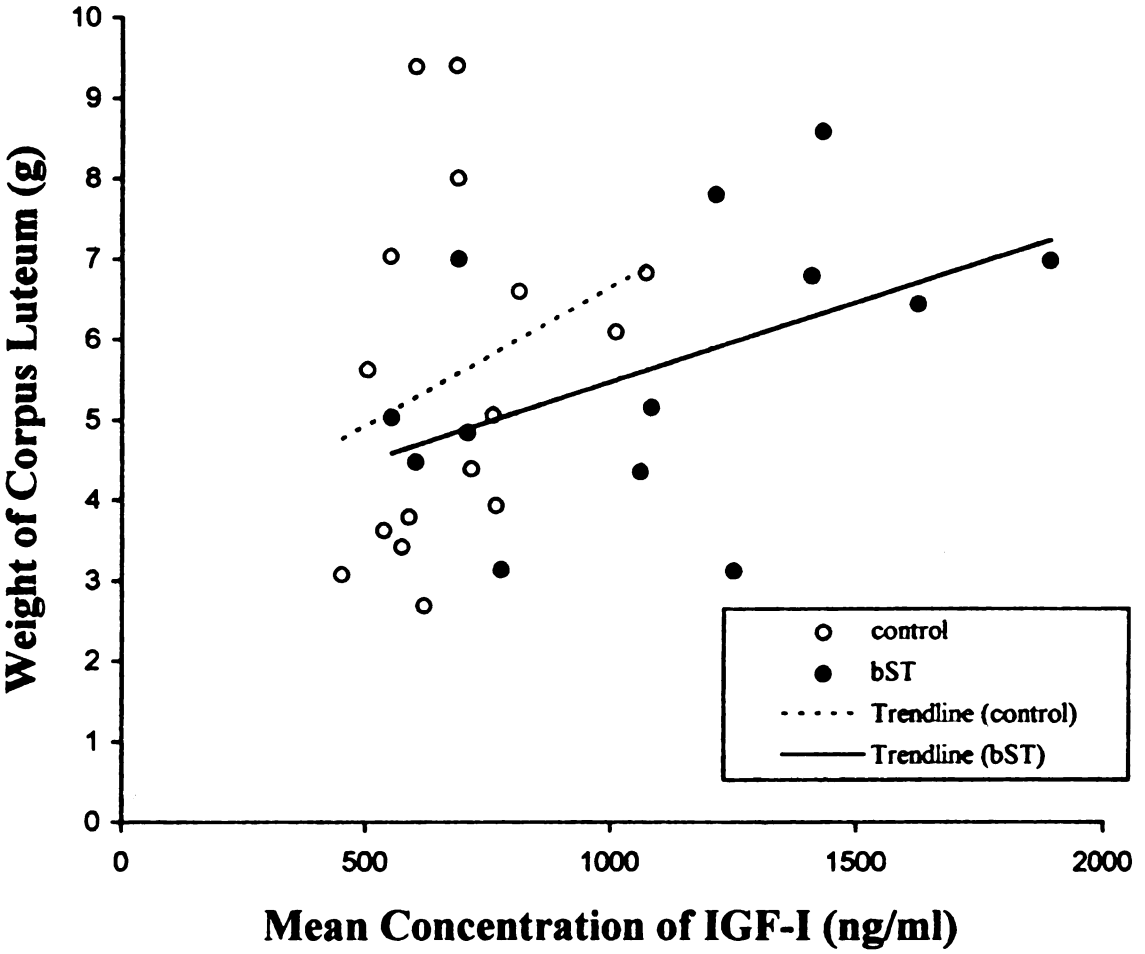

Figure 8. The correlation of the weight of corpus luteum with average concentration of IGF-I in serum from d 0 to d 10 of the fourth estrous cycle in control and bST-treated heifers. Data are from sixteen control and thirteen bST-treated heifers.
The correlation coefficient of:
control heifers is r = 0.27, P = 0.3.
bST heifers is r = 0.48, P = 0.1.

Content of Protein in Corpus Luteum

Negative energy balance did not alter the concentration of protein in CL (mg protein/g wet tissue; P = 0.38; Table 3). Protein content (mg/CL) of the CL was 45.4% less (P < 0.01) in control heifers in NEB than those in PEB. Exogenous bST did not alter the concentration or content of protein of CL.

Concentration of Progesterone in Corpus Luteum

Compared to PEB, NEB decreased the concentration of progesterone in luteal tissue on protein basis by 23%, but this decrease was not statistically significant (P = 0.19). Exogenous bST did not alter the concentration of luteal progesterone (Table 3). Among all heifers, the concentration of luteal progesterone did not correlate with the mean concentration of progesterone in serum or the mean concentration of IGF-I in serum (d 2 to d 10). The concentration of luteal progesterone was negatively correlated with the concentration of NEFA in serum (r = -0.45, P < 0.01).

Content of Progesterone in Corpus Luteum

The content of luteal progesterone was greater in PEB than NEB heifers (P < 0.01, Figure 9). Among all heifers, luteal progesterone content was

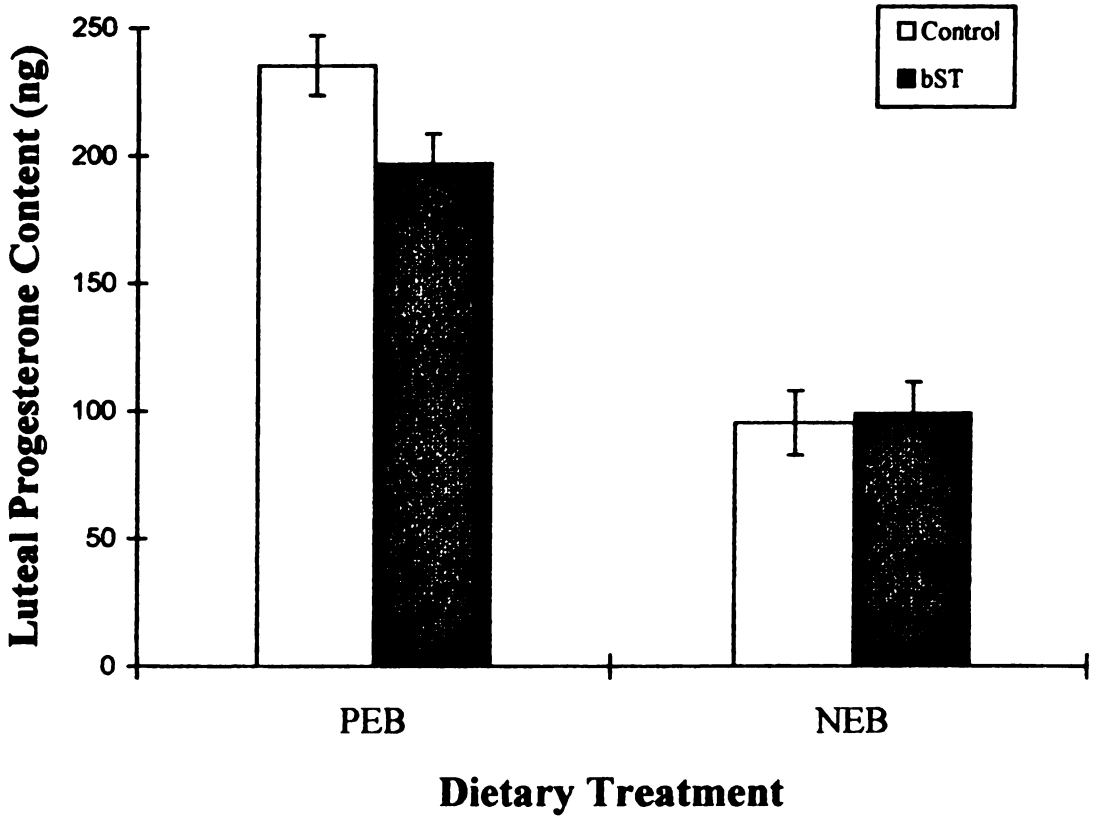

Figure 9. Least squares means of luteal progesterone content at d 10 of the fourth estrous cycle. Data are from eight PEB-Control, seven PEB-bST, eight NEB-Control, and six NEB-bST heifers. Pooled standard error of the means among treatments was 12.56.
Statistical analysis of mean comparisons:
PEB-Control vs. NEB-Control; $P < 0.01$.
PEB-Control vs. NEB-bST; $P < 0.01$.
PEB-Control vs. PEB-bST; $P = 0.40$.
NEB-Control vs. NEB-bST; $P = 0.94$.

correlated positively with the concentration of IGF-I in serum at d 10 of the fourth estrous cycle ($r = 0.34$; $P = 0.07$; Figure 10). The concentration of progesterone in serum at d 10 of the fourth estrous cycle was positively correlated with luteal progesterone content ($r = 0.47$, $P < 0.01$).

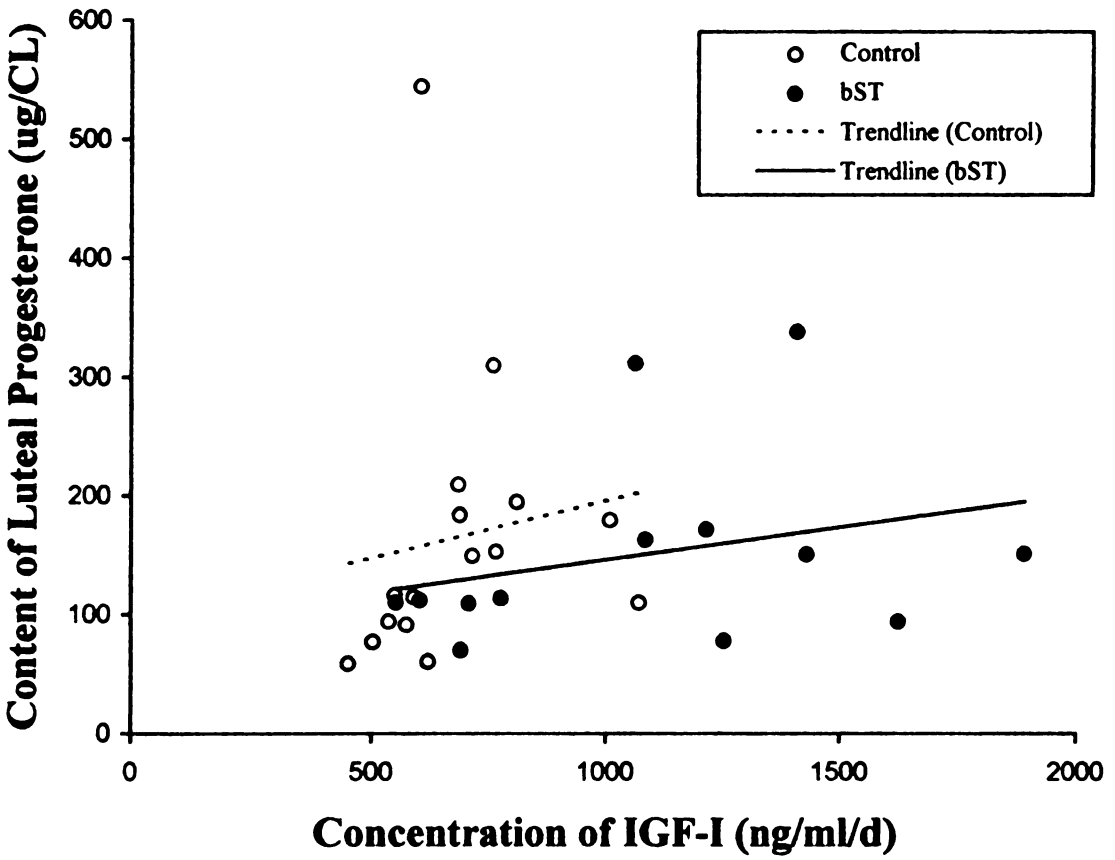

Figure 10. The correlation of content of luteal progesterone with average concentration of IGF-I in serum from d 2 to d 10 of the fourth estrous cycle in control and bST-treated heifers. Data are from sixteen control and thirteen bST-treated heifers.
The correlation coefficient of:
control heifers is $r = 0.24$, $P = 0.37$.
bST heifers is $r = 0.70$, $P < 0.01$.

DISCUSSION

Weights of Corpora Lutea

Our hypothesis was that NEB decreases luteal weight by decreasing the concentration of IGF-I in serum. To test this hypothesis, we assessed the relationship of concentrations of IGF-I in serum during the development of a CL with its weight on d 10 using diet and exogenous bST to manipulate the concentration of IGF-I. As expected, NEB heifers had less IGF-I in serum and smaller CL in confirmation of previous data (VandeHaar et al., 1995). Administration of bST elevated concentrations of IGF-I in NEB heifers to a level similar to that of PEB-control heifers. In PEB-bST heifers, the injection of bST increased the concentration of IGF-I to twice that of PEB-control heifers. However, the increased IGF-I in serum was not associated with increased weight of CL. Furthermore, weight of CL was not correlated with serum concentrations of IGF-I in heifers. Thus, our data suggest that injection of bST and changes in circulating concentrations of IGF-I during the development of a CL do not affect its weight. This is not consistent with the data of Chakravorty et al. (1993) that

IGF-I increases DNA synthesis in bovine luteal cells *in vitro*. Two possible reasons for this discrepancy are that: 1) IGF-I may act through an autocrine or paracrine rather than endocrine pathway, and 2) IGF-binding proteins modulate the action of IGF-I in ovaries.

In addition to endocrine actions, IGF-I may act through autocrine and paracrine pathways. The presence of IGF-I and IGF-I mRNA in luteal tissues suggests that IGF-I may act through these other pathways in the CL (Einspanier et al., 1990). However, in the present study, we observed no difference in the abundance of IGF-I mRNA in CL (data not shown; Sharma et al., 1994). This confirms the lack of effect of NEB on luteal IGF-I mRNA observed previously (VandeHaar et al., 1995). Furthermore, Spicer et al.(1992) found no change in the concentration of IGF-I in follicular fluid during fasting in heifers, suggesting that local production of IGF-I in a dominant follicle is not affected by underfeeding. Thus, the autocrine and paracrine IGF-I do not seem to mediate the effects of EB on the development of CL.

A second possible explanation for why increased IGF-I failed to increase luteal weight is that IGF-binding proteins may modulate the action of IGF-I. The IGF binding protein-2 binds with IGF-I and inhibits DNA synthesis in cultured rat preovulatory granulosa cells, and IGF binding protein-3 extends the half-life

of IGF-I (Giudice, 1992). In this study, IGF binding protein-2 was increased in NEB heifers (data not shown), this increased IGF binding protein-2 might have further decreased DNA synthesis in granulosa and luteal cells and caused a smaller CL in NEB heifers. Injections of bST decreased the concentration of IGF binding protein-2 in both EB groups and increased IGF binding protein-3 in PEB heifers, but neither of these changes was associated with an increase in weight of CL. Thus, the differences in IGF binding protein-2 and IGF binding protein-3 in heifers did not seem to be the cause of the decreased luteal weight in NEB heifers. However, there are six types of IGF binding proteins found in follicular fluid (Giudice, 1992); perhaps another IGF binding protein may affect the action of IGF-I in ovary and help explain the difference between *in vivo* and *in vitro* studies.

Another explanation for why increased IGF-I in response to bST in this study failed to increase luteal weight is that endocrine IGF-I may indirectly alter the development of the CL. Perhaps decreased IGF-I limits development of the dominant ovulatory follicle and thereby decreases the weight of the subsequent CL. In support of this, Terhune (1992) observed that NEB decreases the duration of the preovulatory period. NEB also decreases the persistency of dominant follicles and the maximum diameter of dominant follicles (Burns et

al., 1994). EB and IGF-I are positively correlated and IGF-I increases DNA synthesis in granulosa cells of the follicle (Monniaux and Pisselet, 1992). In our study, concentrations of IGF-I in the previous cycle were decreased in NEB heifers compared to PEB heifers, and the difference in IGF-I may have affected the development of the dominant ovulatory follicle. Treatment with bST increases the recruitment of small follicles and stimulates the growth of the preovulatory follicle in cattle (Gong et al., 1991; De La Sota et al., 1993), but, in the present study, injection of bST and subsequent increases in IGF-I did not occur until 2 d before ovulation, long after the ovulatory follicle had started its development. Since follicular cells are the parental cells of luteal cells, it is possible that decreased follicular development in NEB heifers decreased the development of the subsequent CL, and the increase in IGF-I beginning 2 d before ovulation was not enough to change it.

Progesterone in Corpora Lutea and Serum

Luteal Progesterone

PEB heifers had greater concentration of progesterone in the CL than NEB heifers. The ratio of large to small luteal cells in mid-diestrus (d 7) was

decreased by NEB (Terhune, 1992). Large luteal cells secrete 20-fold greater progesterone than small luteal cells (Koos and Hansel, 1981). Thus, the smaller ratio of large to small cells during NEB would result in less luteal progesterone. Our finding that progesterone content is reduced in the CL of NEB heifers is consistent with the previous study showing decreased progesterone content in CL during NEB (Terhune, 1992). In our study, the concentration of IGF-I was positively correlated with the luteal progesterone content, supporting the report that the concentration of IGF-I in serum is positively correlated with the concentration of progesterone in serum (Spicer et al., 1990), although in neither case was the correlation very strong. In addition, IGF-I increases the production of progesterone by granulosa and luteal cells *in vitro* (Schams et al., 1988; Monniaux and Pisselet, 1992; McArdale and Holtorf, 1989) although some studies using shorter culture times have not shown the increase (Schams et al., 1988; Ealy, 1990). Thus, these studies generally support the idea that IGF-I may mediate the effect of EB on luteal progesterone content. However, in our study, treatment with bST during the luteal phase, and subsequent increases in IGF-I in serum, did not affect the concentration or content of progesterone in the CL. Thus, we conclude that IGF-I in serum during luteal phase does not affect the synthesis of progesterone in the CL. Rather, as we found for luteal growth,

concentrations of IGF-I during growth of follicles before ovulation may be more important for progesterone synthesis by the CL.

Serum Progesterone

Energy balance did not altered mean concentration of progesterone in serum from d 2 to d 10 nor the concentration of progesterone on d 10 alone. In previous studies using similar experimental protocols, Villa-Godoy et al. (1988) observed a decrease in progesterone in serum of NEB compared to PEB heifers but another observed no change (Terhune, 1992). We found that the luteal progesterone content was decreased in NEB heifers compared to PEB heifers. Furthermore, the concentration of progesterone in serum on d 10 was not closely correlated to that in CL; within group, serum and luteal progesterone were positively correlated in PEB but not NEB heifers ($r = 0.65$, $P = 0.01$; $r = -0.02$, $P = 0.93$). Possible reasons that NEB decreased luteal but not serum progesterone are: 1) NEB heifers had less reserves of body fat compared to PEB heifers, and 2) NEB heifers had less hepatic blood flow than PEB heifers.

First, because progesterone is a steroid hormone, it distributes in adipose tissue. During luteal phase, fat tissue contains ~36 ng/g of progesterone (Tsujioka, 1992), which is 10 times more than the concentration of progesterone

in blood. Assuming that blood is about 10% of the body and that adipose tissue is at least 10%, then more than 90% of progesterone in the body will be found in adipose tissue. Thus, most progesterone in the body is in adipose tissue and only 5-10% of progesterone is in blood. Furthermore, NEB heifers had lower final body condition scores than PEB heifers, indicating that NEB heifers had less adipose tissue for distribution of progesterone than did PEB heifers. Thus the clearance of progesterone from blood likely was slower in NEB than PEB heifers. A slower clearance rate for progesterone from serum of NEB heifers would increase the concentration of progesterone in serum and might mask the effects of a decrease in progesterone synthesis by CL. Thus, the different reserves of body fat in the two EB groups likely minimized the concentration of progesterone in serum.

Second, liver is a primary site of clearance of progesterone (Bedford et al., 1974; Freetly and Ferrell, 1994). *In vivo* studies indicated that increased feed intake increases liver oxygen consumption and hepatic blood flow (Burrin et al., 1989; Parr et al., 1993; Freetly and Ferrell, 1994). Parr et al.(1993) reported that increased feed intake increased the metabolic clearance rate of progesterone in liver, although Freetly and Ferrell (1994) did not find the change in metabolic clearance rate of progesterone. In present study, feed intakes of NEB heifers

were half of those of PEB heifers; consequently the hepatic blood flow and metabolic clearance rate of progesterone may have been slower in NEB than PEB heifers. A decrease in metabolic clearance rate may have disguised the decreased synthesis of progesterone of NEB heifers.

In lactating cows, NEB is associated with decreased serum progesterone (Spicer et al., 1990). However, in lactating cows, NEB during early lactation is more often the result of high milk production than low feed intake compared to a cow in late lactation. Some serum progesterone ends up in milk. Thus, spontaneous NEB in lactating cows may be associated with increase clearance of progesterone from blood, whereas NEB may have caused decrease progesterone clearance in the present experiment in which heifers were fed a restricted diet. Differences in clearance would explain why serum progesterone is decreased during NEB in cows, but was not decreased by NEB in this experiment.

Treatment with bST did not affect the concentration of progesterone in serum or in the CL in this study. This seemingly conflicts with reports that long-term administration of bST increases the concentration of progesterone in serum of lactating cows (Gallo and Block, 1989; Schemm et al., 1990) but agrees with the study of Gong et al. (1993) that administration of bST beginning ~14 d

before ovulation did not increase the concentration of progesterone in the subsequent estrous cycle in heifers. Two obvious differences among these studies are the timing of bST administration and the physiological state of animals. Therefore, treatment with bST during luteal phase does not affect luteal development of heifers.

Somatotropin and Insulin-like Growth Factor-I

In the present study, concentrations of ST in serum were higher and concentrations of IGF-I were lower in heifers in NEB than those in PEB, as expected based on previous reports (Ronge et al., 1988; Rutter et al., 1989; VandeHaar et al., 1995). Administration of 14 mg/d of bST increased concentrations of ST in serum of PEB and NEB heifers, but the increase was greater in heifers in NEB than in PEB. Because heifers in NEB weighed less than those in PEB at the end of the study, the reason for the greater serum ST response in NEB heifers was likely in part that they were injected with a higher dose of bST per kg of body weight (0.03 vs. 0.04 mg/kg/day for PEB vs. NEB; respectively). Also, during underfeeding, the abundance of mRNA for ST receptors and the number of high-affinity binding sites for ST in liver are lower (VandeHaar et al., 1995; Breier et al., 1988); decreased ST receptors in liver

might decrease the metabolic clearance rate of ST from blood and thereby increase concentrations of ST in NEB heifers compared to PEB heifers.

Despite their greater concentration of ST, NEB-control heifers had less IGF-I in serum than PEB-control heifers, as previously shown (VandeHaar et al., 1995; Breier and Gluckman, 1991). Furthermore, exogenous bST increased the concentration of IGF-I in serum less in heifers in NEB than in heifers in PEB. This decreased sensitivity to ST in NEB heifers suggests that ST and IGF-I in serum are partly but not entirely uncoupled during NEB. Heifers in NEB have less hepatic mRNA for ST receptors than those in PEB, so the mechanism for this uncoupling may be partly through fewer ST receptors (VandeHaar et al., 1995; Breier and Gluckman, 1991). In addition, heifers in NEB have less IGF-I mRNA in liver than those in PEB (VandeHaar et al., 1995). Thus, in spite of more ST in serum, NEB-bST heifers had less IGF-I in serum than PEB-bST animals likely because of decreased binding of ST in liver and decreased synthesis of IGF-I in liver.

Another possible reason that bST increases IGF-I more in PEB heifers than in NEB heifers is that the concentrations of IGF-binding proteins are different in the two EB groups. Administration of bST decreased the level of serum IGF binding protein-2 in all heifers but increased IGF binding protein-3 only in PEB

heifers (data not shown; Sharma et al., 1994). The increased IGF binding protein-3 of PEB heifers in response to bST likely extended the half-life of IGF-I (Giudice. 1992) and, in turn, increased the concentration of IGF-I in serum of PEB-bST heifers. In NEB heifers, this increase in IGF-binding protein-3 did not occur and therefore the increase in IGF-I was not as great as in PEB heifers. Furthermore, serum IGF binding protein-2 was greater in NEB-control than PEB-control heifers (data not shown; Sharma et al., 1994), and IGF binding protein-2 may facilitate the transport of IGF-I to target tissues (Giudice, 1992). This increased IGF binding protein-2 in NEB heifers might have increased the uptake of IGF-I by target cells and thereby increased the metabolic clearance rate of IGF-I and decreased the concentration of IGF-I in serum. Conversely, the decreased IGF binding protein-2 of bST-treated groups might have decreased the metabolic clearance rate of IGF-I and increased the concentration of IGF-I.

CONCLUSIONS

Administration of bST increased the concentration of IGF-I in serum of both PEB and NEB heifers although the increase was less in NEB heifers. This indicates that, during NEB, the stimulation of IGF-I by ST was partly but not entirely uncoupled.

The main objective of this thesis was to determine the effect of bST injection during luteal phase on growth of the CL in heifers. Results from the present study indicate that administration of bST beginning 2 days before ovulation does not affect luteal development in NEB or PEB heifers. Furthermore, although bST injections increased concentrations of IGF-I of NEB heifers to those of PEB control heifers, weight of the CL was not restored to normal nor was the content of progesterone in the CL affected, indicating that IGF-I in serum during the luteal phase has no effect on luteal development. Thus, my original hypothesis that serum IGF-I mediates the effect of EB on luteal development is rejected.

APPENDICES

Appendix A. The Daily feeding sheet.

Animal	BW Change of Previous Wk (Kg)	Wk 9 BW (Kg)	NEm Requirement (Mcal)	Diet NEm% Requirement	Diet to feed for Wk 10	
					Silage Mixture (Kg; DM%: 28.6%) As Fed Amount	Cracked Corn (Kg) As Fed Amount
PEB						
133	9.5	412	7.9	138%	14	3.30
134	3.6	396	7.6	135%	14	3.11
137	5.4	382	7.4	138%	14	2.95
142	4.5	415	7.9	137%	14	3.34
147	9.5	389	7.5	138%	14	3.03
164	9.1	412	7.9	139%	14	3.30
168	8.6	388	7.5	138%	14	3.01
183	8.2	396	7.6	138%	14	3.12
NEB						
123	1.8	320	6.5	57%	8	0.13
139	-7.3	321	6.5	59%	8	0.13
149	-5.0	317	6.5	61%	8	0.11
154	-4.5	308	6.3	60%	8	0.07
155	-9.1	305	6.3	54%	8	0.05
181	0.0	337	6.8	58%	8	0.21
635	-5.4	353	7.0	59%	8	0.29
648	1.8	321	6.5	57%	8	0.14

Appendix B. The radioimmumoassay of IGF-I

A. Iodination of IGF-I

1. Prepare a 10-ml sephadex G-50 (coarse: medium = 2:1) column that uses glass wool to regulate the flow of degassed phosphate buffer (0.25% BSA; 0.03 M phosphate; 0.02 M sodium azide, and pH 7.5) for about 1 ml/5 min.
2. Run 1 ml of 8% BSA through the column.
3. Prepare fresh solutions (keep in dark) of:
    a. 5 mg/ml sodium metabisulfite in elution buffer (0.03 M sodium phosphate; 0.01 M EDTA; 0.02% protamine sulfate; 0.02% sodium azide; 0.05% Tween-20; 0.25% BSA; pH 7.5).
    b. 1 mg/ml chloramine T in assay buffer (0.03 M sodium phosphate; 0.01 M EDTA; 0.02% protamine sulfate; 0.02% sodium azide; 0.05% Tween-20; pH 7.5).
4. Add in a epp. tube (500 µl) :
    a. 2 µl IGF-I in 10 µl 0.1 N HCl and 35 µl 0.5 M PBS.
    b. 1 mCi ^{125}I in 1-10 µl volume and mix.

c. 25 µl chloramine T (use Hamilton syringe), mix and allow exact 15 seconds for reaction.

d. Add 50 µl sodium metabisulfite (use Hamilton syringe), mix and allow exact 20 seconds for reaction.

e. Add 100 µl transfer solutions (0.5 M PBS; pH 7.4).

5. Apply the mixture to the column bed carefully.
6. Run plenty of elution buffer and collect 30 fractions (0.5 ml/tube) in 12 × 75 mm tubes.
7. Take 10 µl from each fraction in 12 × 75 tube, capped, and count for 0.1 min.
8. Save the fraction comprising the first peak.

B. Extraction of serum IGF-I from binding proteins

1. Take 50 µl serum, add 12.5 µl 2.4 molar formic acid, 250 µl absolute ETOH and vortex and incubated in room temperature for 30 min.
2. Centrifuge for 30 min at 4°C and 600 g.
3. Remove 50 µl supernatant and dilute it into 1.0 ml total volume with neutralizing buffer (0.11 M Na_2HPO_4; 0.154 M NaCl; 0.01 M Na_2EDTA; 0.015 M Sodium azide; 0.5% Tween-20).

C. Radioimmunoassay of IGF-I

1. Label 12 × 75 mm tubes at the following order:

   Total count (TC)

   Non-specific binding (NSB)

   Standard curve

   Samples

   which standard curve and samples have total volume of 200 µl.

2. Add 250 µl first antibody [NIH-AB UBK 189 (1:10000)] to the standard curve and samples (not TC and NSB).
3. Incubate all tubes at 4°C for 24 hours.
4. Add 50 µl iodinated IGF-I (15-18,000 cpm) in all tubes, vortex, and incubate for 48 hours at 4°C.
5. Add 200 µl of 0.25% of *Staph A* in all tubes expect TC, vortex, and incubate for 4 hours at room temperature.
6. Add 2 ml assay buffer (0.03 M sodium phosphate; 0.01 M EDTA; 0.02% protamine sulfate; 0.02% sodium azide, and 0.05% Tween-20. pH 7.5) and centrifuge at 3000 rpm and 4°C for 30 minutes.

7. Pour out the supernatant of all tubes except TC and dry tubes before counting.

8. Count tubes at gamma counter for 1 min.

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