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INFLUENCE OF NEGATIVE ENERGY BALANCE AND BODY CONDITION ON LUTEAL FUNCTION AND ESTROUS BEHAVIOR IN DAIRY CATTLE

presented by

Alejandro Villa-Godoy

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Animal Science

R. H. Foquay
Major professor

Date July 13, 1987

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ABSTRACT

INFLUENCE OF NEGATIVE ENERGY BALANCE AND BODY CONDITION ON LUTEAL FUNCTION AND ESTROUS BEHAVIOR IN DAIRY CATTLE

By

Alejandro Villa-Godoy

Three studies were conducted to determine independent and associative effects of energy balance (EB) and body condition (BC) on luteal function and behavior at estrus of dairy cattle.

In experiment I, 32 Holstein cows were studied from parturition to day 100 postpartum or conception, whichever occurred first. Energy balance was estimated daily (energy intake–energy required for maintenance and lactation) and luteal function was monitored by quantifying progesterone in milk sampled every third day. Cows were fed ad libitum but, within 100 days postpartum, 81% of the cows experienced negative EB (NEB). Most variation in EB was explained by intake of dietary energy (r = .7). Energy balance and progesterone in milk were correlated positively. Thus, NEB may reduce luteal function in lactating dairy cows.

In Experiments II and III, 20 postpubertal Holstein heifers were used in a 2 x 2 factorial experiment. Main effects were: 1) EB, positive or negative and 2) BC, moderate (MOD) or fat (FAT). Heifers were studied for 3.5 estrous cycles. Energy balance (energy intake–energy required for maintenance) was calculated daily, BC was scored (1 to 4; 4 = fat) every two weeks and luteal function was monitored in vivo by progesterone in serum sampled daily. Corpora lutea were removed from heifers between days 10 and 12 of the last estrous cycle for in vitro studies.

In Experiment II, negative EB and FAT reduced luteal function in vivo. Adverse effects of NEB were observed as early as the second estrous cycle in MOD heifers, but were not detected until the fourth estrous cycle in FAT heifers. Adverse effects of NEB on luteal function were delayed until BC of heifers declined below MOD. Concentrations of luteinizing hormone (LH) in serum were not altered by NEB or FAT. Thus, NEB and FAT do not limit luteal function by reducing luteotropic support. Negative EB but not FAT reduced LH-induced secretion of progesterone by luteal cells in vitro.

In Experiment III, to monitor estrous behavior, heifers were observed for periods of 30 minutes at intervals of 3 hours. Energy balance and BC independently or combined did not reduce duration or intensity of standing and mounting behavior.

I conclude that EB and BC do not reduce expression and detectability of estrus in heifers. But, FAT reduces luteal function in heifers and NEB exerts limitations on luteal function of heifers and lactating dairy cows. Apparently, effects of NEB and FAT are exerted through different mechanisms. Moreover, adverse influence of NEB on luteal function of heifers was not detected until BC declined below MOD.

Esta disertación fue lograda en gran parte gracias al apoyo y cariño que mi esposa, María Luisa Parkman de Villa, me ha brindado constantemente. Dedico esta tesis a Ella y a mi hija Lorena, quienes lograron que una dura labor se transformara en la mejor experiencia de mi vida.

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INTRODUCTION

Causes of infertility in individual dairy herds are not easily determined and once identified, they are not easily corrected. As a result, infertility is one of the leading factors which limit productivity in dairy farms. Overall, poor fertility diminishes gross income and increases cost of production resulting in a significant reduction net income for dairy farmers. Reduction of gross income is largely due to decreased yield of milk per productive life of cows in herds with low fertility. Production of milk is reduced in these herds, because non-pregnant postpartum intervals are prolonged and cows spend more days in late lactation when yield of milk and feed to milk convertion are low. Furthermore, for each month added to non-pregnant intervals, production of calves is reduced by 8.3% (Pelissier, 1972) and cows sold because of reproductive failure do not produce offspring. Ferris and Fogwell (1984) estimated that for each day that non-pregnant intervals are prolonged beyond the optimal (82 days postpartum), gross income is reduced between $2.37 and $4.63. Applying these figures to national herd averages (10 million dairy cows; 45 days beyond optimal), indicate that dairy farmers lose between 1.1 and 2.2 billion dollars a year. Infertility increases costs of milk production largely due to increased costs of veterinary services, medication, additional artificial inseminations and the need of producing more replacements (Pelissier, 1972).

Despite the magnitude of economic losses that poor reproductive performance represents to the dairy industry knowledge about causes of infertility is fragmentary.

Fertility in dairy cows is determined by detection of estrus, fertilization and survival of embryos. Interestingly, these three reproductive events that largely determine reproductive performance are correlated positively with concentrations of progesterone in serum of cattle (De Silva et al., 1981; Folman et al., 1973; Fonseca et al., 1983; Hill et al., 1970; Melampy et al., 1975). Therefore, it is important to determine sources of variation of luteal function in dairy cows.

Undetected estrus is a major cause of prolonged postpartum intervals to conception (Pelissier, 1972). Known sources of failure to detect estrus are: uninformed farmers, limited number of observations of cows and variation in duration and intensity of estrus within and among cows. In addition, erroneously identified estrus and poor detection of onset of estrus contribute to low conception rates after artificial insemination (Pelissier, 1978). Consequently, identification of sources of variation for duration and (or) intensity of behavior at estrus in cattle has practical and biological implications.

At least 92% of dairy cows experience negative energy balance during early lactation (Reid et al., 1966). During negative energy balance, homeorhetic mechanisms insure metabolic support to the lactating mammary gland and sustain synthesis of milk (Bauman and Currie, 1980). Perhaps homeorhetic controls which sustain lactation limit luteal function or behavior at estrus.

In herds of dairy cattle, subclinical hepatic lipidosis has been diagnosed in as many as 35% of the lactating cows (Reid, 1980). This condition is associated with obesity of cows at parturition and has been implicated as a source of infertility (Reid and Roberts, 1983; Roberts et al., 1979). Fatness at parturition reduces intake of feed and dietary energy by cows (Garnsworthy and Topps, 1982). Therefore, fat periparturient cows may experience more severe negative energy balance than lean periparturient cows.

The primary goal of this research was to determine whether energy balance and (or) body condition reduce luteal function or limit estrous behavior in dairy cattle. If so, a second goal was to gain knowledge about mechanisms mediating effects of energy balance and(or) body condition on luteal function and(or) estrous behavior.

REVIEW OF LITERATURE

Introduction

Although lactating dairy cows are the target animal of this research, postpubertal Holstein heifers were also used to address some specific objectives. Consequently, aspects of energy balance (EB), body condition (BC), luteal function and estrous behavior pertaining to dairy heifers and lactating dairy cows will be reviewed. Occasionally, information from other species will be used to illustrate or support some concepts.

Variation of EB during growth and during lactation in cattle will be described. Importance of individual components of EB will also be addressed for growing heifers and lactating cows. Emphasis will be given to components of EB related to physiology of non-pregnant cattle (i.e. voluntary intake of feed, body weight, yield of milk, BC). If components of EB related to feeds, diets or environment are discussed attention will be minor.

Most dairy cows experience negative EB (NEB) during early lactation. Due to homeorhetic controls, cows sustain milk production despite NEB. Variations in peripheral concentrations of insulin and GH are consistently associated with homeorhetic control of growth and lactation. Changes in concentrations of non esterified fatty acids (NEFA) are associated positively with beginning of lactation and with NEB. Thus association of EB with secretory patterns of insulin and GH, and concentrations of NEFA in plasma of growing heifers and lactating cows will be described.

In late lactation, dairy cows may ingest excessive dietary energy. Thus, at parturition cows are in positive EB (PEB) and many are fat. Peripartum fat BC reduces postpartum intake of feed and exaggerates NEB. But fat BC affects metabolism independent of EB in many species. Consequently, BC will

be discussed separate from other components of EB but in association with reproduction, insulin, GH and NEFA.

Two reproductive functions are the dependent variables throughout this research: 1) luteal function and 2) estrous behavior. Control of development and maintenance of corpora lutea in non-pregnant, ovulatory cattle will be addressed in this review. Discussion will focus on actions of luteinizing hormone (LH).

Behavior at estrus will be described and related with sources of variation. Hormonal control of estrous behavior in dairy cattle will be reviewed.

The relationships of EB or BC with luteal function or estrous behavior in cattle have not been addressed. But effects of some components of EB on these two reproductive functions have been examined. Available information addressing the association between components of EB and luteal function or expression of estrus is discussed.

Finally, evidence indicating that insulin, GH or NEFA may be involved in luteal function is reviewed.

Energy Metabolism in Cattle

Requirements of Energy

Nutritional value of feeds is expressed in several units of energy. Estimates of energy in feeds are based on productive values (i.e. meat and milk) of feeds ingested by cattle. Each unit estimates a different level of utilization of energy but all units are interrelated and have a hierarchical nature. The relationship among several measures of energy is established in the following expression:

Gross energy - energy lost in feces = digestible energy (DE)

DE - energy lost in urine, heat of fermentation and methane = metabolizable energy (ME)

ME - energy lost as heat increment = net energy (NE)

All systems currently used to determine energy values of feeds are based on principles of NE. Net energy actually indicates partial efficiencies in which ME of feeds is used for different physiological functions (Garrett and Johnson, 1983).

Fasting metabolism is the major determinant of requirements of NE for maintenance (NEm). But to define NEm, energy systems adopted by the National Research Council (NRC) includes sources of variation of NEm such as: physical activity, age, gestation and lactation (Lofgreen and Garrett, 1968; Milligan and McBride, 1985; Moe et al., 1972; NRC, 1978). Thus energy values of feeds or requirements of energy by cattle stated in review of literature or used in this research will be expressed in NE values.

Partial efficiency of energy used for maintenance is higher than partial efficiency of energy used for production in growing cattle. Therefore two values are estimated for each feed: NEm and NEg for energy retained in tissues during growth (Garrett and Johnson, 1983). Similar terminology is used to state requirements of energy by animals: maintenance (NEm) and gain (NEg). Because

dietary energy is used with similar efficiency for maintenance and for production of milk in dairy cows, a single value of NE, net energy for lactation (NEl) is adequate to calculate rations or to determine requirements for maintenance or production of milk (Moe, 1981).

In Holstein heifers within a range of 100 to 550 kg of body weight, NEg required for daily gains of .5 kg represents 37.5 to 22.4% of NE required for maintenance (NRC, 1978). But in Holstein cows, total requirements for lactation frequently exceed two to three fold the requirements of energy for maintenance (Moe, 1981; NRC, 1978).

Thus, in heifers the major proportion of ingested energy is used to satisfy requirements for maintenance. In contrast, a large proportion of energy ingested by dairy cows during early postpartum is used to satisfy requirements of energy to support lactation.

Energy Balance

Energy balance is generally defined as input of energy minus output of energy. Thus in lactating dairy cows, EB is estimated by subtracting requirements of energy for maintenance and lactation from intake of dietary energy. In heifers, EB results from subtracting energy required for maintenance from intake of dietary energy. In these calculations growth or gain is excluded because energy is stored not consumed by body tissues of cattle.

Maintenance constitutes the largest proportion of total energy required by growing cattle (Ferrel and Jenkins, 1985). Large variation in requirements of NEm exists among cattle of similar age and weight (Ferrel and Jenkins, 1985; Ledger and Sayers, 1977). Previous and current nutritional level, body weight and body composition have been suggested as sources of variation of NEm. But results are inconsistent and causes of variation of NEm among growing cattle remain unresolved (Ferrell and Jenkins, 1985).

At recommended rates of growth (.5 kg/d) between 62.5 and 77.6% of total dietary energy ingested by heifers is used for maintenance and thus to achieve EB (NRC, 1978). Dietary energy exceeding requirements for maintenance of heifers is used for growth. Thus growing heifers are in PEB. Negative EB may be induced in heifers only by reducing dietary energy to levels below maintenance.

In lactating dairy cows, requirements of energy for synthesis of milk exceed requirements for maintenance (Moe, 1981; NRC, 1978). Thus, to achieve EB lactating cows must ingest sufficient energy to satisfy requirements for maintenance and lactation. Reid et al. (1966) determined that coincident with peak lactation, 92% of lactating dairy cows were in NEB. Coppock et al. (1974) observed that during the first 6 to 14 weeks postpartum 100% of lactating dairy cows experienced NEB. Thus at least 92% of lactating cows experience NEB at or before peak lactation.

Duration and magnitude of NEB vary greatly within and among lactating cows. Average postpartum interval to nadir of EB was 4 weeks and ranged from 2 to 6 weeks (Bauman and Currie, 1980; Gravert, 1985). Average deficit of energy of lactating cows was 9 Mcal of NEl at nadir of EB (Bauman and Currie, 1980) but maximal deficit of energy for an individual cow was 32 Mcal of NEl (Butler et al., 1981). After nadir, average EB of cows increased and became positive by 6 to 16 weeks postpartum (Bauman and Currie, 1980; Coppock et al., 1974; Gravert, 1985). Among cows experiencing energy deficit postpartum, duration of NEB in individual animals ranged from 4 to 14 weeks (Butler et al., 1981).

Sources of Variation of Energy Balance

Some sources of variation for EB of lactating dairy cows have been proposed: age (parity), body weight, peripartum body condition, yield of milk

and voluntary intake of feed (Blake and Custodio, 1984; Flatt, 1966). Effects of parity on EB is equivocal. Braund and Steel (1972) reported that cows in first lactation reached EB later postpartum than older cows. But in other study, parity did not affect magnitude or duration of NEB in Holstein cows (Coppock et al., 1974).

Body weight did not alter efficiency of cows to use dietary energy for production of milk (Gravert, 1985; Hooven et al., 1968). In addition, body weight at the beginning of lactation or changes in body weight throughout lactation were not correlated significantly with EB of dairy cows (Flatt, 1966; Moe et al., 1972). Consequently, variation of energy needed for maintenance irrespective of body weight, becomes a relatively small proportion of the total variation of requirements for energy of lactating dairy cows. This is consistent with the fact that intake of dietary energy exceeds up to three fold the requirements of energy for maintenance in lactating dairy cows (Reid et al., 1966).

Yield of milk and EB during the first 20 d postpartum were correlated negatively in Holstein cows (Butler et al., 1981). At least 92% of cows with high individual yields (> 30 kg/d) were in NEB at peak lactation (Reid et al., 1966). This implies that while cows with modest yields of milk would have no difficulty to sustain PEB, cows with high yields of milk would be unable to maintain PEB during early lactation (Butler et al., 1981; Reid et al., 1966). But, Coppock et al. (1974) observed that Holstein cows with relatively low individual yields ($\leq$ 20.1 kg/d) and fed ad libitum were in NEB for at least the first 6 weeks postpartum. Thus cows with low yields of milk are as likely to experience NEB as cows with high yields of milk.

Cows with similar body weights and intakes of feed differ in EB due to variation in yield of milk (Bauman and Currie, 1980), thus yield of milk affects EB. But a general concept is that intake of feed is correlated positively with

yield of milk (Grainger et al., 1985; Kazmer et al., 1986) and with EB (Kazmer et al., 1986). Therefore, increased intake of dietary energy compensates, at least partially, for greater demands of energy to support increased yields of milk.

Moe et al. (1972) observed a high positive correlation (.9) between intake of dietary energy and EB of cows. Cows of high genetic potential for milk fed ad libitum produced more milk but ingested more feed and had similar EB than equally fed cows with low potential for production (Kazmer et al., 1986). Although daily yield of milk was negatively associated with EB (-.34), at least similar amount of variation in EB of lactating cows was explained (.45) by intake of feed (Kazmer et al., 1986).

In summary, at least 92% of dairy cows fed ad libitum are in NEB during early lactation. Despite NEB, dairy cows sustain synthesis of milk. Thus while heifers must be in PEB to grow, NEB in dairy cows is a common, spontaneous condition which does not limit synthesis of milk.

Under farm conditions, dairy heifers are able to ingest sufficient feed to satisfy requirements of energy for maintenance and growth. Thus, voluntary intake does not represent a limitation to achieve PEB in heifers. But in cows, voluntary intake of feed is the principal component of EB.

Homeorhetic Control of Growth and Lactation

Optimal growth and lactation by cattle depend on proper nutritional management. Central to this aspect of animal husbandry is prediction of how energy in feeds is used by cattle for the frequently competing processes of growth, lactation and reproduction.

The existence of biological mechanisms that prioritize metabolic support to some functions (growth, lactation, pregnancy) have been suggested (Bauman and Currie, 1980; Kennedy, 1967; Wood, 1979). Bauman and Currie (1980)

proposed that utilization of nutrients by various body tissues is regulated by homeostasis and homeorhesis. Homeostatic control involves maintenance of physiological equilibrium in the internal environment. Homeorhesis was defined as the coordinated changes in metabolism of body tissues to support a physiological state (i.e. lactation or growth). An example of homeorhesis is that in cattle at early stages of growth, deposition of protein exceeds deposition of fat, while in similarly fed cattle approaching mature weight, deposition of fat exceeds deposition of proteins (Vernon, 1986). The importance of homeorhetic control is more obvious during lactation than during growth because of marked variation in EB of cows. When lactation begins, metabolism is rapidly altered to satisfy the enhanced demands for energy of the mammary gland. If cows are unable to alter energy metabolism to support synthesis of milk, yield of milk is reduced and(or) cows experience metabolic disorders (Bauman and Currie, 1980).

Current understanding of factors controlling utilization of nutrients by tissues during growth and lactation is fragmentary. Control of growth and lactation is exerted by homeorhetic mechanisms which are concurrent with short-term homeostatic factors. Metabolism is controlled by interactions among endocrine factors that are homeorhetic and(or) homeostatic. This review will focus on insulin and growth hormone (GH) which are presently considered as major homeorhetic regulators of growth and lactation. For detailed discussion on metabolic control of growth, the reader is referred to recent reviews (Brockman and Laarveld, 1986; Hart and Johnsson, 1986; Trenkle, 1981; Weekes, 1986). Metabolic control of lactation has been thoroughly discussed by Bauman and Currie (1980), Bauman et al. (1985),Collier et al. (1984), Trenkle (1981).

Discussion will focus on effects of EB and its components on secretory patterns of insulin and GH and on concentrations of NEFA in blood.

Insulin in Cattle

A major role of insulin in concert with glucagon and catecholamines is homeostasis regarding glucose (Weekes, 1986). Insulin stimulates uptake and oxidation of glucose by several tissues (Weekes, 1986). In addition to the universally recognized homeostatic effects of insulin, a chronic, homeorhetic role of insulin in regulation of growth (Weekes, 1986) and lactation in farm animals has been recognized recently (Bauman et al., 1985; Trenkle, 1981). In coordination with other anabolic and catabolic hormones, insulin controls partitioning of available nutrients during growth and lactation. Overall, insulin stimulates deposition of glycogen, triglyceride and protein in body depots (Weekes, 1986).

Secretory Patterns of Insulin in Cattle

In growing cattle (McAtee and Trenkle, 1971a), feeding induces a biphasic secretory pattern of insulin. Coinciding with feeding there is a rapid but transient increase of circulating insulin followed by a second rise of insulin which lasts between 2 and 6 h. Concentrations of insulin in jugular serum of lactating cows increase after feeding. But postprandial secretion of insulin is not biphasic as in heifers (McAtee and Trenkle, 1971a). Instead concentrations on insulin increased about 2 h after lactating cows ingested feed. This rise of insulin was sustained for 2 or 3 h and then declined to preprandial concentrations (Jenny and Polan, 1975).

The first postprandial secretory increase of insulin in heifers, is caused by vagal reflexes (Weekes, 1986). Because in lactating cows a rapid increase of insulin is not observed after feeding, vagal stimuli may be reduced in cows. The second postprandial peak of insulin observed in heifers and the single rise of insulin in cows coincide with maximal postprandial absorption of digestive products.

What products of digestion induce postprandial release of insulin by pancreas is not clear in ruminants. Due to microbial activity in rumen, dietary carbohydrate is fermented into acetate, propionate and a small percentage of butyrate. Thus, amount of glucose derived from diet that reaches and is absorbed by intestine is negligible. But large quantities of glucose are synthesized and released at all times by liver in ruminants. Glucose injected into jugular (McAtee and Trenkle, 1971a; McCann et al., 1986) or mesenteric arteries of ruminants (Manns et al., 1967) increased concentrations of insulin in jugular (Manns et al., 1967; McAtee and Trenkle, 1971a; McCann et al., 1986) and portal blood (Lomax et al., 1979). Thus glucose may be involved in control of insulin secretion.

Among products of digestion that induce release of insulin in ruminants when injected into jugular and (or) portal veins of ruminants are propionate and butyrate (Horrino et al., 1968; Lomax et al., 1979; Manns et al., 1967; McAtee and Trenkle, 1971a) and perhaps free amino acids (McAtee and Trenkle, 1971a).

Energy Balance and Insulin

Heifers fasted for intervals of two to eight days had lower basal concentrations of insulin in jugular blood than during fed state (McAtee and Trenkle, 1971a; McCann and Hansel, 1986). Lambs with restricted dietary energy for > 30 days that lost weight and that were presumably in NEB, had lower concentrations of insulin in serum than lambs fed adequately (Hart et al., 1985). Steers maintained in NEB for 120 days had lower circulating insulin during the entire period than steers in PEB (Blum et al., 1985). Consequently, deprivation of feed for relatively short periods, and NEB for long intervals decrease concentrations of insulin in growing ruminants.

Concentrations of insulin in Holstein cows are lower in early lactation (< 40 d) than in late lactation (Bines and Hart, 1981; Koprowski and Tucker,

1973; Smith et al., 1976). At least 92% of dairy cows are in NEB during early lactation but all cows are in PEB by late lactation (Reid et al., 1966; Coppock et al., 1974). Therefore, low concentrations of insulin in serum of lactating dairy cows coincide with intervals of NEB whereas high concentrations of insulin concur with PEB. Confirming this, Vasilatos and Wangness (1981) determined that basal concentrations and amplitude of pulses of insulin were lower at 30 d of lactation when cows were in NEB than at 90 d of lactation when in average cows were in PEB.

Thus NEB is associated with low concentrations of insulin in serum of growing cattle and lactating dairy cows.

Components of Energy Balance and Insulin

Increased proportion of concentrate in diets enhanced magnitude of postprandial rise of insulin in growing sheep (Weekes, 1986). But, homeorhetic controls during growth are superimposed on homeostatic factors controlling secretion of insulin in response to changes in diet. For example, in lambs receiving constant amounts of feed per unit of metabolic weight, the second postprandial rise of insulin in serum increased with age and body weight (Weekes, 1986). Steers approaching mature size had higher concentrations of insulin than younger steers (Verde and Trenkle, 1987), and insulin in serum increased as heifers aged from 2 to 4 years (McCann and Reimers, 1986). But cattle approaching mature size have greater deposition of body fat than animals in early stages of growth. Thus actual source of variation for postprandial concentrations of insulin in heifers may be body composition, body weight or age.

In lactating dairy cows, influence of yield of milk on serum concentrations of insulin is equivocal. Hart et al. (1978) reported that high yielding dairy cows had lower concentrations of insulin in serum than low producing cows. However,

the low producing cows were crosses of dairy and beef breeds. When Holstein cows with superior yields were contrasted with Holstein cows with good yields of milk, concentrations of insulin were not different between groups (Barnes et al., 1985) or tended to be higher in superior cows (Kensinger et al., 1984).

Dairy cows ingesting a diet with low proportion of grain did not have a postprandial increase of insulin while cows receiving a diet with a high proportion of grain had a normal rise of insulin after feeding (Jenny and Polan, 1975). Cows fed a low-grain diet had also lower basal concentrations of insulin in serum than cows fed a high-grain diet (Jenny and Polan, 1975). Thus, composition of diet may alter serum insulin in cows. However, concentrations of insulin were not influenced by composition of diets but were positively associated with individual intake of DM or energy by lactating dairy cows (Smith et al., 1976). Consequently, composition of diet have some effect but amount of DM or energy ingested may exert a greater influence on secretion of insulin by dairy cows.

More important than composition of diet or level of intake of DM or dietary energy on insulin is EB and(or) lactation. For example, cows in early lactation (NEB) received a diet with higher concentration of energy and ingested more DM and energy than non-lactating cows (PEB). But cows in early lactation secreted less insulin (portal vein concetrations) in response to feeding or to exogenous propionate than non-lactating cows (Lomax et al., 1979).

Summarizing, EB is associated positively with concentrations of insulin in heifers and lactating cows. Among components of EB; age and body weight are correlated positively with insulin, but body composition is confounded with these effects in heifers. In dairy cows amount of DM and(or) energy ingested exert the greatest influence on secretion of insulin.

Body Condition and Insulin

Associations between BC and insulin in dairy cows have not been examined. But McCann and Reimers (1985a) observed that basal concentrations of insulin in jugular blood were higher in fat heifers than in heifers with moderate BC. After intravenous injection of glucose, insulin was higher in fat heifers and lambs than in lean individuals (McCann and Reimers, 1985b; McCann et al., 1986). Weekes (1986) determined that relative to lean lambs, increased circulating insulin in fat lambs was partially due to decreased metabolic clearance rate of insulin. But relative to lean animals, release of insulin from isolated islets of Langerhans and perfused pancreas from obese rodents is enhanced (Bray and York, 1979). Thus increased secretion and reduced metabolic clearance rate of insulin result in greater concentrations of peripheral insulin in obese than in lean individuals.

Growth Hormone in Cattle

Growth hormone exerts a variety of effects on metabolism. Directly or indirectly GH stimulates anabolic processes such as cell division, skeletal growth and synthesis of protein. GH also exerts catabolic actions by stimulating oxidation of lipids and by inhibiting transport of glucose into cells (Hart and Johnsson, 1986; Spencer, 1985).

Growth hormone is stored in and secreted by the somatotrophs in the anterior pituitary. Secretion of GH by pituitary is controlled by multiple factors which have been summarized by Bennett and Whitehead (1983).

Secretory Patterns of Growth Hormone

Mean concentrations, magnitude and frequency of episodic pulses of GH in blood vary considerably among animals. Release of GH by pituitary of young ruminants seems to be inherently episodic and is not influenced by time of feeding (Breier et al., 1986; Zinn et al., 1986). Secretory pulses of GH are asynchronous

among steers but pulsatile patterns within an animal are repeatable over time (Breier et al., 1986).

Lactating dairy cows have higher concentrations of GH in serum than non-lactating cows (Hart et al., 1978; Sartin et al., 1985). Growth hormone in lactating dairy cows is secreted episodically. As in heifers, pulses of GH in serum are asynchronous, do not follow diurnal patterns (Phillips and Athanasiou, 1978) and are unrelated with times of feeding (Vasilatos and Wangsness, 1981) or milking (Bauman et al., 1979; Phillips and Athanasiou, 1978). Great variation in pulsatile patterns of GH is observed among cows (Enright et al., 1986) but as in growing cattle, episodic pulses are characteristic of individual cows on a day to day basis (Vasilatos and Wangsness, 1981).

Energy Balance and Growth Hormone

Short-term deprivation of food (12 to 15 h) increased concentrations of GH in humans (Roth et al., 1963). However, relative to values in fed animals, concentrations of GH in serum of heifers did not vary after an interval of 60 h of fasting (McAtee and Trenkle, 1971b). Similarly, concentrations of GH did not vary in plasma collected from growing sheep at 12 h postprandial or at 96 h of fasting (Wallace and Bassett, 1970). Therefore, unlike humans and pigs, GH may not be involved in short-term metabolic control of energy in growing ruminants. In contrast, submaintenance levels of dietary energy for prolonged periods increase secretion of GH in growing ruminants. Lambs who received a diet containing about 50% below their requirements of energy for maintenance during 42 days had higher circulating GH than lambs ingesting adequate levels of energy (Hart et al., 1985). Increased concentrations of GH resulting from reduced intake of dietary energy by steers who lost weight was due to enhanced amplitude of pulses of GH but not to variation in baseline concentrations or frequency of pulses of GH (Breir et al., 1986). Influence of NEB on secretion

of GH has not been examined in heifers but steers maintained in NEB for 120 days had higher circulating GH than steers in PEB (Blum et al., 1985).

Dairy cows in early lactation (< 40 d) had higher concentrations of GH in serum than cows in later (> 60 d) stages of lactation (Koprowski and Tucker, 1973; Smith et al., 1976; Vasilatos and Wangsness, 1981). The decline of circulating GH as lactation progresses results from reduced amplitude of pulses rather than in basal concentrations or frequency of pulses of GH (Vasilatos and Wangsness, 1981). Apparently decreased concentrations and amplitude of pulses of GH with advancing lactation is due to a reduced ability of pituitary to secrete GH. This was supported further when Kazmer et al. (1986) determined that mean concentrations and thyrotropin releasing hormone (TRH)-induced secretion of GH were lower at late than at early lactation in dairy cows. At least 92% of dairy cows were in NEB during early lactation but 100% were in PEB at late lactation (Reid et al., 1966; Coppock et al., 1974). Thus, circulating GH is high when cows are in NEB and it is low when cows are in PEB. Indeed, dairy cows were in NEB and had higher concentrations of GH in serum at day 30 than at day 90 of lactation when they were in PEB (Vasilatos and Wangsness, 1981).

Thus, in heifers and lactating dairy cows EB is negatively associated with concentrations of GH in serum.

Components of Energy Balance and Growth Hormone

Prepubertal heifers had higher concentrations of GH in serum than postpubertal heifers (Sejrsen et al., 1983; Zinn et al., 1986). Moreover, circulating GH after exogenous administration of TRH was higher in pre than in postpubertal heifers (Sejrsen et al., 1983).

Level of dietary energy ingested by growing cattle also affects circulating GH. Sejrsen et al. (1983) determined that prepubertal PEB heifers with restricted

intake of dietary energy had higher GH in serum than heifers fed identical feeds but offered ad libitum. But this influence of intake of energy on circulating GH was not evident in postpubertal heifers (Sejrsen et al., 1983).

Body composition may also alter concentrations of GH in serum. For example, concentrations of GH in jugular blood are correlated negatively with proportion of fat in carcass of cattle (Hart and Johnson, 1986). Greater deposition of fat in body depots of postpubertal heifers (Sejrsen et al., 1983; Zinn et al., 1986) might explain lower levels of GH in serum and reduced response of GH to TRH than in prepubertal heifers (Sejrsen et al., 1983). However, while rate of body weight gain or deposition of fat in body tissues was associated negatively with circulating GH of prepubertal heifers, in postpubertal heifers neither intake of feed, rate of weight gain or fat deposited in tissues affected mean concentrations or secretory patterns of GH (Sejrsen et al., 1983; Zinn et al., 1986).

It is apparent from the previous discussion that regardless of gonadal status, circulating GH or response to secretagogues of GH in cattle diminishes with age. But effects of age on secretion of GH are confounded with effects of body weight and body composition. An interesting point is that secretion of GH in heifers at late stages of growth are not altered by factors which affect secretion of GH in heifers during early stages of growth. It can be speculated that once a "critical" age or weight or body composition is reached by heifers maintained in PEB, variations in intake of feed above requirements for maintenance, rate of gain or deposition of fat in tissues will not alter secretion of GH.

High yields of milk were correlated positively with concentrations of GH in serum (Barnes et al., 1985; Kazmer et al., 1986; Kensinger et al., 1984). Independent of EB, concentrations of GH in serum were greater in high yielding than in low yielding Holstein cows (Kazmer et al., 1986).

Composition of diet did not alter mean concentrations of GH in plasma from dairy cows at early lactation (Smith et al., 1976). But intake of DM or intake of dietary energy was associated negatively with GH (Smith et al., 1976) and with magnitude of TRH-induced pulses of GH in lactating dairy cows (Bauman et al., 1979).

The previous discussion indicates that NEB increases GH in serum of heifers and cows. But, among components of EB, only intake of dietary energy sufficiently reduced to produce NEB affects GH in postpubertal heifers. In dairy cows, components of EB which are associated with GH are yield of milk and intake of DM or dietary energy. Yield of milk is correlated positively but intake of DM or energy is correlated negatively with GH.

Non Esterified Fatty Acids (NEFA) in Cattle

In heifers fed once or twice daily, concentrations of NEFA in plasma were affected by time of feeding. Concentrations of NEFA were maximal before feeding and declined rapidly after feeding (Holmes and Lanbourne, 1970). In these heifers concentrations of NEFA were maintained low for 8 h postprandial and increased thereafter. Fluctuations of NEFA in plasma due to time of feeding were suppressed when heifers received diets with high proportion of grain (Holmes and Lanbourne, 1970).

In lactating dairy cows fed once daily, concentrations of NEFA in plasma were maximal shortly before feeding and were maintained low for at least 12 h after feeding (Radloff et al., 1966). But, concentrations of NEFA in plasma of cows fed more than once per d did not follow diurnal patterns and were not affected by time of feeding or milking (Phillips and Athanasiou, 1978). In cows, concentrations of NEFA increase as parturition approaches and peak during the first 7 d postpartum (Athanasiou and Phillips, 1978a; Radloff et al., 1966). Subsequently, NEFA decline to reach prepartum concentrations between 20 and 35 d postpartum (Radloff et al., 1966).

Energy Balance and NEFA

Steers in NEB had greater concentrations of NEFA in plasma than PEB steers (Blum et al., 1985).

Dairy cows in early lactation (40 to 80 d) had higher concentrations of NEFA in plasma than cows at late (> 120 d) lactation (Bauman and Currie, 1980; Coppock et al., 1974). Because EB in cows changes from negative to positive after peak of lactation, it is reasonable that EB could be a major source of variation on NEFA. But Vasilatos and Wangsness (1981) determined that NEB cows at 30 d postpartum had similar concentrations of NEFA than at 90 d postpartum when cows were in PEB. In contrast, dairy cows in severe NEB during the first 56 d postpartum had two-fold higher concentrations of NEFA than cows with less severe NEB (Chilliard et al., 1984). Thus, in growing heifers and in cows within same postpartum stage, concentrations of NEFA and EB are associated negatively.

Components of Energy Balance, Body Condition and NEFA

Submaintenance levels of dietary energy increased concentrations of NEFA in plasma of heifers (Holmes and Lanbourne, 1970). Daily changes in concentrations of NEFA in plasma were unrelated with levels of dietary energy offered to lactating cows (Ducker et al., 1985a, b). But effects of actual intake of energy on NEFA are untested in lactating cows. In cows, deprivation of feed for 2 or 5 d (Athanasiou and Phillips, 1978b; Brumby et al., 1975) increased concentrations of NEFA in plasma. In addition, yield of milk is correlated positively with NEFA (Radloff et al., 1966).

Concentrations of NEFA in plasma from obese pigs are the same or lower than in non-obese pigs (Bakke, 1975; Weisemberger and Allen, 1973). But obese rats have higher NEFA in plasma than lean rats (Zucker, 1972). In cattle affects of BC on NEFA are untested.

In summary, EB and NEFA in plasma are correlated negatively in heifers and cows. In heifers dietary energy below requirements of maintenance increases concentrations of NEFA. In cows, fasting and yield of milk enhance concentrations of NEFA in plasma.

Luteal Function in Cattle

Corpora Lutea of Estrous Cycles

After puberty but before conception, sexual behavior in bovine females follows a rhythmic pattern (estrous cycle) that peaks regularly every 21 d with estrus and ovulation. With ovulation a new corpus luteum develops. Luteal regression is prerequisite to the next ovulation and cycle. The primary hormonal product of bovine corpora lutea is progesterone (Niswender et al., 1980). Concentrations of progesterone in blood parallel function and lifespan of corpora lutea. Progesterone blocks ovulation by exerting negative feedback on luteinizing hormone (LH). Thus, enucleation of corpora lutea during mid-diestrus reduces concentrations of progesterone and allows precocious estrus and ovulation in cattle (Snook et al., 1969; Hobson and Hansel, 1972). In contrast, continuous administration of progesterone prevents estrus and ovulation, and extends estrous cycles in cows (Christian and Casida, 1948). Estrus and ovulation follow withdrawal of exogenous progesterone in postpubertal cattle (Roche, 1976). Clearly, timing of events associated with estrous cycles is influenced by progesterone and thus by corpora lutea. Thus, progesterone and corpora lutea are associated temporally and functionally with behavior at estrus, fertilization of oocytes and survival of embryos.

Control of Development and Maintenance of Corpora Lutea.

Function of corpora lutea depends on a balance between luteotropic and luteolytic factors. During development and maintenance of corpora lutea, availability of luteotropic factors exceeds availability of luteolytic substances.

Factors secreted by the anterior pituitary gland (LH) and uterus (prostaglandins E_2 and I_2) affect luteal function positively.

Luteinizing hormone is involved in luteinization of thecal and granulosal cells (Channing, 1980). Differentiation of follicular cells into luteal cells is

marked by change of the major end product of steroidogenesis from estradiol-17 to progesterone (Channing, 1980; Ireland and Roche, 1983). Presence of LH is required for maintenance of morphological and functional differentiated state of bovine luteal cells in vitro (Gospodarowicz and Gospodarowicz, 1975). Furthermore, LH increases synthesis and release of progesterone by corpora lutea of cattle in vivo (Schoemberg et al., 1967), or when incubated as luteal slices or as dissociated luteal cells in vitro (Armstrong and Black, 1966; Williams and Marsh, 1978). Moreover, removal of LH by hypophysectomy (Kaltenbach et al., 1968) or neutralization of LH by passive immunization (Hoffman et al., 1974; Snook et al., 1969) accelerated luteal regression in cycling ewes and cows. In addition, exogenous LH extends the functional lifespan of corpora lutea in sheep (Karsch et al., 1971; Donaldson and Hansel, 1965) and cattle (Wiltbank, 1961 a, b). Thus, luteotropic support to corpora lutea in sheep and cattle is provided largely by LH. In this review of literature all mention to luteotropic stimuli or support will refer to LH.

Specific receptors for LH are located in plasma membrane of bovine luteal cells (Gospodarowicz, 1973). Binding of LH to these specific receptors activates the adenylate cyclase system (Condon and Black, 1976; Marsh, 1976). Activated adenylate cyclase induces synthesis of cyclic adenosine 3',5'-monophosphate (cAMP; Marsh, 1976) which ultimately stimulates synthesis (Marsh, 1976; Williams and Marsh, 1978) and release of progesterone (Gemmell et al., 1974; Niswender et al., 1980).

Relative to other stages of a bovine estrous cycle, mean concentrations of LH are low and constant throughout metestrus and diestrus (Butler et al., 1983; Walters et al., 1984). But during luteal development, LH is secreted as pulses of higher frequency and lower amplitude than when corpora lutea are fully developed at mid-diestrus (Rahe et al., 1980; Walters et al., 1984). Changes

in pulsatile secretion of LH may be more important for luteal development than total concentrations of LH in serum (Hansel and Convey, 1983).

Once corpora lutea develop, binding of human chorionic gonadotrophin (hCG) to luteal receptors increases progressively until about d 12 postestrus when progesterone in serum, luteal weight and binding of hCG to luteal cells are maximal (Spicer et al., 1981). During luteal development in cattle, concentrations of progesterone in serum, binding of hCG or LH to luteal cells and luteal weight are correlated positively (Rao et al., 1979; Spicer et al., 1981). Between d 12 and 16 postestrus, binding of hCG to corpora lutea declined, while progesterone in serum and luteal weight were maintained (Spicer et al., 1981). Thus, during maintenance of fully developed corpora lutea, binding of LH is not associated with luteal weight or secretion of progesterone.

Effects of Components of Energy Balance on Luteal Function

Influence of EB on luteal function of farm animals has not been examined. But effects of some components of EB on luteal function of cattle have been tested. Discussion will focus on influence of a) restricted intake of energy, b) body weight, c) body condition and d) yield of milk on luteal function.

Restricted Intake of Energy

Influence of restricted intake of dietary energy on luteal weight has been examined. Some researchers did not detect effects of undernutrition on weight (Imakawa et al., 1983) or size of corpora lutea from heifers (Spitzer et al., 1978). But other workers observed that reduced intake of energy diminished luteal weight in heifers (Apgar et al., 1975; Gombe and Hansel, 1973; Hill et al., 1970). In addition, concentrations of progesterone in corpora lutea from underfed heifers were lower than in corpora lutea from adequately fed animals (Gombe and Hansel, 1973). Relative to values in adequately fed heifers, progesterone in serum was increased, lowered or not changed with restricted dietary energy. For example,

during the first diestrus after start of fasting, heifers had higher concentrations of progesterone than fed heifers (McCann and Hansel, 1986). Progesterone in serum of heifers receiving dietary energy below requirements for maintenance was not reduced for three (Spitzer et al., 1978) or four consecutive estrous cycles (Beal et al., 1978). But in other studies, restricted dietary energy reduced progesterone in serum of heifers immediately after (Hill et al., 1970; Imakawa et al., 1983) or at the second estrous cycle after onset of restriction (Gombe et al., 1973).

In cows effects of restricted dietary energy are also inconclusive. Concentrations of progesterone during first postpartum estrous cycle did not differ between lactating dairy cows receiving restricted or ad libitum dietary energy (Carstairs et al., 1980). Corah et al. (1974) could detect no effect of prolonged under-nutrition on progesterone in postpartum beef cows. But, Dunn et al. (1974) observed a progressive decline of progesterone during four consecutive estrous cycles in underfed postpartum beef cows.

Slices of luteal tissues (Apgar et al., 1983) or dispersed luteal cells (Imakawa et al., 1983) from underfed heifers had similar basal secretion of progesterone than cells from heifers fed adequately. But, luteal cells from underfed heifers secreted less hCG-or LH-induced progesterone than luteal cells from heifers receiving adequate diets (Apgar et al., 1975; Imakawa et al., 1983). Conclusions from these studies are limited because concentrations of hCG in medium with luteal cells were not reported (Imakawa et al., 1983) or LH-induced secretion of progesterone by luteal cells from heifers under restricted diet, was affected only at very high concentrations (1000 ng/ml) of LH in medium (Apgar et al., 1975). But basal secretion of progesterone by luteal cells in vitro is not affected by dietary restrictions to heifers in vivo. And ability of luteal cells to respond to LH may be reduced in vitro by restricted intake of dietary energy.

Reduced concentrations of LH in blood as cause of adverse effects of restricted diets on luteal function has been tested. In ovariectomized heifers, some authors observed that underfeeding increased LH (Imakawa et al., 1987). But other workers did not detect effects of dietary restrictions on LH in serum (Beal et al., 1978). In cycling heifers, effects of restricted dietary energy on serum LH vary with stage of estrous cycles. Relative to adequately fed heifers, peri-ovulatory LH was high in underfed and fasted heifers (Gombe and Hansel, 1973; McCann and Hansel, 1986). Similarly, gonadotropin-releasing hormone (GnRH)-stimulated secretion of LH was higher in heifers with deficit of dietary energy than in properly fed heifers at estrus (Bale et al., 1978; Rasby et al., 1986). Dietary restriction did not affect secretion of LH during diestrus (Hill et al., 1970; Spitzer et al., 1978).

Changes in Body Weight

The relationship between changes in body weight and luteal function is not clear. Losses in body weight were associated with decreased concentrations of progesterone in serum of heifers (Beal et al., 1978; Hill et al., 1970; Imakawa et al., 1983, 1986) and postpartum beef cows (Donaldson et al., 1970). But in other studies, declining body weight of heifers (Spitzer et al., 1978) or cows (Corah et al., 1974) was not accompanied by changes in progesterone.

Relative to values in animals gaining weight, concentrations of LH were reported to be reduced (Imakawa et al., 1986), increased (Beal et al., 1978) or unchanged (Hill et al., 1970; Spitzer et al., 1978) in serum from heifers losing weight.

Body condition

In women (Frisch, 1984; Hartz et al., 1979) and rodents (Bray and York, 1979) obesity has been associated with infertility. In dairy cows it was suggested that obesity at parturition is related with postpartum reproductive problems

(Reid and Roberts, 1983; Roberts et al., 1979). Although the link between obesity and infertility exists, the underlaying mediating effects of obesity and specific reproductive functions affected in obese individuals have not been determined.

McCann and Reimers (1986) and Spicer et al. (1984) observed that concentrations of progesterone at mid-diestrus in fat heifers was similar than in moderately conditioned heifers.

In rodents, obesity has been associated with low concentrations of LH in blood (Bray and York, 1979). But in heifers, fatness did not alter mean concentrations or GnRH-induced secretion of LH (Rasby et al., 1986; Spicer et al., 1984).

Existing information is not conclusive regarding association between fatness and luteal function in cattle. But luteal function is associated positively with fertility and fatness is associated with infertility. Does limited luteal function mediate adverse effects of fat BC on fertility? If so, fat BC would be expected to affect luteal function.

Yield of Milk

The relationship between yield of milk and reproductive functions in dairy cattle is equivocal. Some researchers reported a negative influence of high yield of milk on fertility (Berger et al., 1981; Laben et al., 1982; Morrow 1969; Spalding et al., 1975). But other workers did not find any association between reproductive performance and level of milk production of dairy cows (Carstairs et al., 1980; Dachir et al., 1984; Fonseca et al., 1983). Yield of milk and progesterone in serum during first postpartum diestrus were not associated in dairy cows (Carstairs et al., 1980). But first postpartum corpora lutea are functionally subnormal in cattle and subjected to many negative influences (Rutter et al., 1985). Therefore results obtained by Carstairs et al. (1980) are not conclusive.

Insulin, Growth Hormone and Non Esterified Fatty Acids on Luteal Function

During NEB concentrations of insulin decrease but GH and NEFA increase in jugular blood of cattle. If NEB reduces luteal function in cattle, do changes in concentrations of insulin, GH and(or) NEFA mediate effects of NEB on luteal function? Evidence supporting these assumptions will be discussed.

Insulin

Uptake and utilization of glucose are necessary for viability and function of bovine luteal cells in vitro (Armstrong and Black, 1966, 1968; Flint and Denton, 1969; Savard et al., 1963). Uptake and utilization of glucose by luteal cells are mediated by insulin in cattle (Armstrong and Black, 1966; 1968) and rats (Flint and Denton, 1969). Increased uptake of glucose by luteal cells stimulates incorporation of 14C from [1-^{14}C] acetate into sterols and steroids (Flint and Denton, 1969) and enhances supply of reducing equivalents; both essential for de novo synthesis of cholesterol and production of progesterone by luteal cells of ruminants (Armstrong and Black, 1966, 1968; Savard et al., 1963). Specific receptors for insulin have been identified in luteal cells of rats (Ladenheim et al., 1984) and in luteinized granulosal cells of gilts (Veldhuis et al., 1984). These observations provide evidence for direct actions of insulin in luteal cells. Effects of insulin on luteal function are not restricted to control of glucose metabolism. Insulin may affect steroidogenesis. Steroidogenic actions of insulin include increased supply of extraovarian cholesterol. Cholesterol is the main precursor for progesterone and is delivered to bovine corpora lutea by low density lipoproteins (LDL; Savion et al., 1982). Binding sites for LDL were identified in corpora lutea of pigs (Veldhuis et al., 1986) and rats (Christie et al., 1979; Rajendran et al., 1983). Insulin increases binding of LDL to adrenal glands of mammals (Brown et al., 1981) and porcine granulosal cells (Veldhuis et al.,

1986). Thus insulin may increase binding of LDL to corpus luteum, increase supply of cholesterol and support, if not stimulate, synthesis of progesterone by luteal cells. Insulin also increases binding of hCG in porcine granulosal cells (May and Schomberg, 1981). Overall, responsiveness of luteal cells to luteotropic stimuli should be increased by insulin. Accordingly, in vitro insulin induced luteinization of porcine granulosal cells (May and Schoemberg, 1981), increased proliferation of bovine granulosal cells (Savion et al., 1981) and enhanced secretion of progesterone by porcine and bovine granulosal cells (Channing et al., 1976; Savion et al., 1981). Moreover, insulin is required for maximal steroidogenic actions of gonadotropins on cultured granulosal cells from pigs (May and Schoemberger, 1981). In vivo, exogenous insulin blocked adverse effects of restricted dietary energy on FSH-induced ovulation in cattle (Harrison and Randel, 1986) and on spontaneous ovulation in gilts (Jones et al., 1983). Basal and LH-induced secretion of progesterone were depressed in streptozotocin induced diabetic rats (Tesone et al., 1983). Injections of xyalazine, an inhibitor of insulin secretion, reduced insulin and progesterone in serum of heifers (McCann, 1984). Therefore, insulin exerts positive effects on ovarian and luteal function. In cattle experiencing NEB, concentrations of insulin are reduced. Thus, NEB could affect luteal function adversely because of reduced concentrations of insulin in serum.

Growth Hormone

Growth hormone antagonizes actions of insulin on uptake of glucose by adipose tissue of sheep (Vernon, 1978). But in liver of rats, GH and insulin synergize to induce synthesis of somatomedin-C (Maes et al., 1986). Furthermore, GH stimulates formation of receptors for LH and synthesis of progesterone by granulosal cells of rats (Jia et al., 1986). These roles of GH are perhaps mediated by increasing ovarian somatomedin-C (Davoren and Hsueh, 1986) which

mimics actions of GH in granulosal cells of rats (Adashi et al., 1985). Study of actions of GH on luteal functions has been neglected. Thus there is no information indicating the direction of GH effects, if any, on luteal function. Based on actions of GH on other tissues, increased GH during NEB in cattle might affect luteal function in at least three ways: a) should GH act on corpus luteum, as it does on adipose tissue, then GH might oppose actions of insulin and reduce luteal function; b) if GH acts on corpus luteum as in granulosal cells, greater concentrations of GH during NEB might affect luteal function positively; and c) if as occurs with hepatic synthesis of somatomedin, actions of GH on corpora lutea require presence of insulin at concentrations observed in non-obese, adequately fed individuals; incresed GH during NEB would not alter luteal function due to concommitant reductions of insulin.

NEFA

High concentrations of NEFA in plasma were associated with poor fertility in dairy cows (Ducker et al., 1985b). But cause-effect relationship between increased NEFA and infertility has not been tested. Addition of NEFA to human serum albumin devoid of lipids, reduced binding of progesterone by albumin (Ramsey and Westphal, 1978). Decreased binding affinity of albumin for progesterone was proportional to the amount of NEFA.

Thus increased NEFA during NEB may reduce binding of albumin to progesterone and increase free progesterone in blood. Consequently, metabolic clearance rate of progesterone may be increased and concentrations of progesterone in serum reduced.

Estrous Behavior in Cattle

In most mammalian females, sexual receptivity is confined to estrus, the stage of an estrous cycle immediately preceding ovulation. At or near estrus, females will seek out a male and engage in behaviors which enhance the probability of copulation. These behaviors have been defined as proceptivity when estrous behavior of laboratory species is described (Clemens and Gladue, 1979). Proceptivity corresponds to a group of behavioral signs classified as "initiating behavior" in cattle (Esslemont et al., 1980; Glencross et al., 1981). If a sexually active male is present, the receptive female stands so the male can achieve vaginal penetration (penile intromission; Clemens and Gladue, 1979). The group of behaviors of a sexually receptive cow has been classified as "receiving behavior" (Esslemont et al., 1980; Glencross et al., 1981). In cattle, a female in estrus stimulates other females who mimic the sexual behavior of males. Thus, in groups of bovine females, individuals in estrus have the opportunity to display the receptive as well as initiating behaviors in absence of males. The practical importance of detecting estrus in groups of bovine females is to appropriately schedule artificial insemination relative to ovulation. Estrous behavior of dairy heifers and cows in absence of males will be discussed in this review.

Expression of Estrus

Esslemont et al. (1980) classified the most frequently observed components of estrous behavior as: a) agressive behavior which is expressed mainly as butting and chasing; b) investigatory behavior that includes sniffing, rubbing, licking and chin-resting; c) disordered mounting, consisting in mounting cows not in estrus or disoriented mounting (i.e. head to head mounting), and d) oriented mounting with standing. Clearly each component of behavior includes an initiating and a receiving individual.

With the possible exception of standing, and in presence of a male, penile intromission, all behavioral signs of estrus may be displayed at all stages of an estrous cycle by bovine females. But all signs of estrus are displayed most frequently in proestrus and estrus (Esslemont et al., 1980; Glencross et al., 1981).

Standing and mounting are the behavioral components most definitive of estrus. For example, in all heifers studied by Glencross et al. (1981), onset of standing behavior coincided with estrus (peak concentrations of estradiol-17β) in serum. Based on concentrations of progesterone and estradiol, other authors determined that few standing events occur at non-estrus stages in heifers (Esslemont et al., 1980). Therefore, near 100% of standing events occur at estrus in heifers. Between 70 (Helmer and Britt, 1985) and 79% (Hurnick et al., 1975) of all mounting events were exhibited at estrus in cattle.

Duration of standing estrus in individual heifers ranged from 3 to 21 h (Glencross et al., 1981) and averaged 10.2 h (Esslemont et al., 1980). For lactating dairy cows, duration of standing behavior ranged from 7.5 to 10.1 h (Hurnick et al., 1975). Estrous behavior is a continuum throughout an estrous cycle, but frequency and intensity of estrous activity, especially standing and mounting, peak immediately before and during estrus.

Factors Affecting Estrous Behavior

Many environmental factors affect expression of estrus in dairy cattle. Time of day when cattle are observed alters efficiency on detection of estrus (De Silva et al., 1981; Helmer and Britt, 1985; Hurnick et al., 1975) as does duration (Hurnick et al., 1975) and frequency of observations (Donaldson, 1968). High (> 25°C) ambient temperatures reduce expression of estrus (Bond and McDowell, 1972; Ganwor et al., 1965) and percentage of cows detected in estrus is altered by type of housing (De Silva et al., 1981; King et al., 1976). As numbers

of animals simultaneously in estrus increased up to 4 or 5, mounts per period of observation increase proportionally (Esslemont et al., 1980; Helmer and Britt, 1985; Hurnik et al., 1975).

Effects of EB, intake of dietary energy, body weight or BC on estrous behavior of cattle have not been examined. But, influence of other components of EB on expression of estrus were tested. Carstairs et al. (1980) did not observe any association between levels of dietary energy offered to dairy cows and detection of estrus. Some workers found a negative correlation between yield of milk and detection of estrus (Morrow et al., 1966) but others observed that estrous behavior was not influenced by yield of milk in dairy cows (Carstairs et al., 1980; De Silva et al., 1981; Fonseca et al., 1983).

Experiments addressing the association between estrous behavior and levels of dietary energy offered to cows (Carstairs et al., 1980) or yield of milk (Morrow et al., 1966; De Silva et al., 1981; Fonseca et al., 1983), do not allow definitive conclusions because estrus was considered a discrete rather than a continuous variable. To draw valid conclusions from those data, many more animals must be observed. In addition, duration of estrus averages approximately 10 h in heifers and lactating cows (Esslemont et al., 1980; Hurnick et al., 1975). However in previous reports, cattle were only observed for periods of 1 h at intervals of 12 h. Thus in existing research, number of cattle and number of periods of observation were insufficient to characterize adequately variation of estrous behavior under different conditions.

Hormonal Control of Estrus

Removal of gonads reduced or suppressed sexual activity in mammals. With the exception of primates, mammalian females become sexually unresponsive to males after removal of ovaries (Clemens and Gladue, 1979). In cattle, removal of ovaries eliminates estrus (Katz et al., 1980). Manifestation of estrous behavior

in ovariectomized cattle is restored by exogenous estradiol-17β (Katz et al., 1980; Melampy et al., 1957), estrone (Wiltbank et al., 1961) or testosterone (Katz et al., 1980; Kiser et al, 1977). Estradiol is several fold more potent than testosterone or estrone in eliciting estrous behavior in ovariectomized cattle (Katz et al., 1980; Wiltbank et al., 1961). Thus among the ovarian steroids capable of restoring sexual activity in ovariectomized cattle, estradiol-17β is the most potent and thus may play the most important role in regulating estrous behavior of gonadally intact cattle. Concentrations of estradiol in serum increase during estrus (Chenault et al., 1975; Glencross et al., 1981). Passive immunization of ewes against estradiol inhibited estrus (Scaramuzzi et al., 1975). Large doses of progesterone antagonize stimulatory effects of exogenous estradiol on estrous behavior of ovariectomized cows (Melampy et al., 1957). But positive effects of exogenous estradiol on estrous behavior are enhanced when given simultaneously with progesterone in low doses (Melampy et al., 1957). In cycling cows, concentrations of progesterone at estrus ranged between 0.4 and 1.0 ng/ml of serum (De Silva et al., 1981). Within this range, circulating progesterone at estrus was correlated positively with mounting activity in dairy cows (De Silva et al., 1981). Thus, concentrations of progesterone in peripheral blood of female cattle at estrus may enhance estrous behavior.

Repeated administration of estradiol suppressed estrous behavior in ovariectomized cows. But pretreatment with progesterone abolished refractoriness caused by repeated injections of estradiol (Carrick and Shelton, 1969). In addition, pretreatment with progesterone enhanced display of estrous behavior induced by single injection of estradiol in ovariectomized heifers (Melampy et al., 1957). Thus, variation in concentrations of progesterone during luteal phases preceding estrus may also influence estrous behavior in cycling female cattle.

Estradiol is the major hormone determining estrous behavior but progesterone modulates effects of estradiol on expression of estrus.

Summary

At least 92% of dairy cows fed ad libitum experience NEB in early lactation. Large variation in magnitude and duration of NEB is observed among cows. Yield of milk and intake of feed have been implicated as factors with major impact on EB in dairy cows. But the fact that NEB is equally frequent among cows with high and low yields of milk, indicates that the major determinant of EB is intake of feed. Homeorhetic mechanisms support synthesis of milk during NEB. Perhaps homeorhetic mechanisms that support lactation alter reproductive functions in dairy cows.

Luteal function is correlated positively with expression of estrus, conception and embryo survival. Associations between individual components of EB (i.e. reduced dietary energy, changes in body weight or yield of milk) and luteal function in cattle are not consistent. Perhaps these inconsistencies are due to unexplained or uncontrolled variation in other components of EB. As a single variable, EB should integrate multiple components of variables such as diet, feed intake, body weight and yield of milk. Thus EB is integrative. A high proportion of dairy cows experience NEB during early lactation and normal luteal function is important to fertility in cattle. The first objective of this dissertation was to determine the relationship between EB and luteal function in lactating dairy cows.

Fat periparturient dairy cows ingest less DM and dietary energy during early lactation than lean cows. Thus fatness at parturition may accentuate postpartum NEB of cows and further limit luteal function. Fat BC may affect luteal function in cattle by enhancing NEB or by mechanism unrelated with EB. Thus the second objective of this research was to determine the independent and associative effects of EB and BC on luteal function in dairy heifers.

Luteinizing hormone is the main luteotropic factor in cattle. Effects of reduced intake of dietary energy on LH are equivocal. But it is possible that reduced luteotropic support to corpora lutea might mediate effects of EB or BC on luteal function. Thus, a third objective of the present dissertation was to determine independent or associative effects of EB and BC on LH in dairy heifers.

Alternatively, responsiveness of luteal cells to luteotropic stimuli might be reduced directly or indirectly by energy deficit. The fourth objective of this research was to determine effects of EB, BC and (or) their interactions on basal and (or) LH-induced secretion of progesterone from luteal cells in vitro.

From reviews cited, it is apparent that in cattle insulin and GH are involved in homeorhetic control of growth and lactation. Additional literature indicates that secretion of insulin and GH vary with changes in EB and BC. Concentration of NEFA changes in response to various stimuli associated with lactation or EB. Thus, the fifth objective was to determine effects of EB, BC and (or) their interactions on profiles of insulin and GH and in concentrations of NEFA in blood of dairy heifers.

Insulin exerts an important role in luteal function of cattle and other species. Whether or not GH exerts any effects on corpora lutea has not been addressed. But known effects of GH on ovarian follicles or on non-reproductive tissues provide rationale that GH might be involved in luteal function. Increased NEFA have been associated with infertility in cattle. Because luteal function and fertility are positively associated, NEFA may alter luteal function. Thus changes of insulin, GH and(or) NEFA associated with homeorhetic control of lactation might mediate effects of NEFA on luteal function. The sixth objective of the present dissertation was to determine associations between luteal function and changes of circulating insulin, GH or NEFA.

Knowledge of hormonal control of estrous behavior in cattle is fragmentary. However, behavior of bovine females at estrus or other stages of an estrous cycle has been assessed and described thoroughly. Many environmental factors which alter estrous behavior of cattle have been identified. But whether metabolic status of cattle affects expression of estrus has not been tested adequately. Therefore, the seventh objective of the present dissertation was to determine the independent and associative effects of EB and BC on estrous behavior in dairy heifers.

EXPERIMENT I:

Association Between Energy Balance and Luteal Function in Lactating Dairy Cows

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INTRODUCTION

Yield of milk and levels of dietary energy have been implicated as causes of infertility in lactating dairy cows. But effects of yield of milk on fertility of cows are equivocal. Some researchers observed that reproductive efficiency declines as yield of milk increases (Berger et al., 1981; Morrow, 1969; Spalding et al., 1975), whereas other workers did not confirm this association (Dachir et al., 1984; Fonseca et al., 1983; Hansen et al., 1983). Similarly, there are inconsistent effects of low dietary energy on reproductive performance. Ducker et al. (1985a) and King (1968) observed that diets with low energy were detrimental to fertility of dairy cows. In contrast, other workers did not find a relationship between diet and reproductive performance (Carstairs et al., 1980; Ducker et al., 1985b; Gardner, 1969).

Energy balance is the net result of associations among diet, intake and use of nutrients by cows and yield of milk. Thus energy balance as a single measure should integrate homeorhetic changes that occur during lactation. At least 92% of dairy cows experience negative energy balance during early lactation (Coppock et al., 1978; Reid et al., 1966), but magnitude and duration of negative energy balance are variable among cows (Butler et al., 1981).

In dairy cattle, luteal function is associated with three events that determine fertility: 1) success of detecting estrus (Melampy et al., 1957), 2) rate of conception (Folman et al., 1973; Fonseca et al., 1983) and 3) embryonic

survival (Hill et al., 1970). Therefore, it is important to identify factors that affect luteal function in dairy cows.

The main objective of this study was to determine the relationship between energy balance and luteal function in lactating dairy cows.

MATERIALS AND METHODS

General. Eight primiparous and 24 multiparous lactating Holstein cows were studied from parturition to 100 day (d) postpartum or conception, whichever occurred first. Cows calved normally and remained healthy throughout the study. Cows were housed in stanchion stalls and received water and feed ad libitum. Cows were fed a total mixed ration (TMR; 50% roughage: 50% grain on DM basis) at 0200 and 1400 h (table 1) formulated to satisfy all requirements of nutrients for maintenance and lactation. Intake of feed by individual cows was recorded daily. Throughout the study ingredients of TMR were sampled and DM of ingredients was determined weekly. Amount of ingredients in TMR were adjusted according to variation in DM to maintain a 50:50 ratio of roughage to grain. On alternate weeks during the experiment, TMR was sampled for chemical analysis (table 1).

Milk Yield, Body Weight and Energy Balance. Cows were milked twice daily (0400 and 1500 h) and yields of milk were recorded. Concentrations of fat in milk were determined monthly by Michigan Dairy Herd Improvement Association (East Lansing) using an infrared analyzer (Multipec, Wheldrake, England). For each cow, it was assumed that samples of milk within ± 15 d from sampling d had the same concentrations of fat than the analyzed sample of milk. Milk was adjusted to 4% fat (4% FCM) by formula derived from Tyrrell and Reid (1965): 4% FCM = yield of milk [136 + 5 (actual % of fat)/340].

TABLE 1. COMPOSITION OF TOTAL MIXED RATION.

Item	Concentration[a]
Ingredient, %	
Alfalfa hay	12.5
Corn silage	37.5
High moisture ear corn	30.0
Protein supplement (44%)	17.0
Dicalcium phosphate and vitamin premix	3.0
Chemical analyses[b,c]	
Dry matter, %	52.9
Energy, Mcal/kg[d]	1.6
Crude protein, %	17.8
Acid detergent fiber, %	21.0

[a]Dry matter basis. Values are means from 10 samples of total mixed ration taken in alternate weeks during the study.
[b]Methods for chemical analyses in Pritchard and Staubus (1978).
[c]Concentrations of minerals in total mixed ration were Ca (.6%), P (.4%), K (1%), Mg (.2%), S (.2%), Na (.15%), Mn (32 ppm), Fe (217 ppm), Cu (10 ppm) and Zn (45 ppm).
[d]As NEl (estimated from total mixed ration).

From d 4 to 7 postpartum until the end of the study, body weight of cows was measured (6 to 7 h after 0200 h feeding) on two successive d per week. Weekly changes in body weight were extrapolated to estimate daily body weight (weekly change/7 d).

Energy balance (EB) of cows was estimated daily by subtracting net energy required for maintenance and lactation from intake of NEl. Requirements of NEl for maintenance were based on daily body weight and calculated as suggested by NRC (1978): NEl (Mcal) = 80 (Kcal NE/kg$^{.75}$). Due to requirements for growth, cows in first and second lactation were fed 20 and 10%, respectively, above requirements of NE for maintenance indicated for mature cows (NRC, 1978). To determine NEl for lactation, daily yield of 4% FCM (kg) was multiplied by .74 Mcal (NRC, 1978). Daily intake of NEl (DM basis) was calculated by multiplying NEl (Mcal) per kg of TMR (table 1) by kg of TMR ingested by cows.

Luteal Function and Other Reproductive Measures. Concentrations of progesterone in fat-free milk parallel concentrations of progesterone in serum of cows (Appendix A; Pope et al., 1976). Therefore, luteal function can be monitored by progesterone determined in milk or serum. To quantify progesterone in milk, samples of milk were obtained every third d from d 5, 6 or 7 postpartum until the end of the experiment. Collection and processing of samples of milk and radioimmunoassay techniques used to quantify progesterone are described in Appendix A.

To detect estrus, cows were observed for three daily periods of 30 min (0600, 1800 and 2400 h). Estrus was when a cow stood to be mounted ($\geq$ 2 sec) during intervals of low concentrations of progesterone in milk ($<$ 1 ng/ml). Day of ovulation was recorded as 3 d before concentrations of progesterone in milk exceeded two standard deviations above basal progesterone. Basal progesterone (.175 $\pm$ .01 ng/ml) was the mean of progesterone in milk sampled

during the first week postpartum, when all cows were anovulatory. Estrous cycle was defined as the interval between two consecutive periods of estrus. When estrous behavior did not accompany ovulation, d 0 of an estrous cycle was recorded as one d before ovulation.

At weekly intervals from d 5, 6 or 7 postpartum to first artificial insemination, reproductive organs of cows were examined rectally to determine presence of corpora lutea in ovaries and to determine uterine involution. Presence of corpora lutea indicated resumption of ovarian functions postpartum. To determine uterine involution, diameter and length of uterine horns were estimated. Uterine involution was when previously gravid uterine horn had diameter and length similar to opposite uterine horn and neither horn declined further.

Assimilation of Data and Statistical Analysis. To characterize changes in EB of cows throughout the study, EB was expressed as Mcal of NEl/d and could be positive (PEB) or negative (NEB). To explain variation of EB, daily EB was the dependent variable in multiple linear regression using backward elimination procedures (Gill, 1978). Independent variables were daily yield of 4% FCM, daily body weight, daily intake of DM and parity (1, 2 or $\geq$ 3 parturitions).

Concentrations of progesterone in milk were plotted over time within an estrous cycle per cow and area under the curve was calculated. Area included values that were 2 standard deviations above basal progesterone and that were sustained for $\geq$ 3 consecutive samples of milk. Maximal concentration of progesterone within an estrous cycle (peak) and duration of luteal phase (interval of progesterone exceeding basal progesterone by 2 standard deviations) were calculated for first to third postpartum estrous cycles. Together, peak progesterone and duration of luteal phase accounted for 84% of the variation

in area under the curve of progesterone (r^2 = .84; P < .001). Area, peak and duration were used as measures of luteal function.

To determine sources of variation in luteal function, area, peak or duration were used as dependent variables in multiple linear regression analyses, including backward elimination procedures (Gill, 1978). Independent variables were mean EB per estrous cycle, mean intake of DM per estrous cycle, mean yield of 4% FCM during the first 100 d postpartum, mean body weight per estrous cycle and parity (1, 2 or $\geq$ 3 parturitions). Concentrations of progesterone during first postpartum estrous cycle are lower than in subsequent cycles in cattle (Edgerton and Hafs, 1973). Thus by regression analysis, I examined the association between progesterone (area, peak or duration) as dependent variable and consecutive estrous cycles (first, second or third) as an independent variable.

To determine sources of variation on duration of postpartum anovulation I used interval from parturition to first ovulation postpartum as dependent variable and used same analysis and independent variables described in the previous paragraph.

The preceding analysis indicated confounded effects of EB, duration of postpartum anovulation and number of consecutive estrous cycles postpartum on progesterone (see Results and Discussion). To reduce these confounding effects, concentrations of progesterone were blocked within first, second and third estrous cycle and mean EB was limited to the interval when all cows were anovulatory (d 1 to 9 postpartum). Additional advantage of this reorganization of data was that we were able to address whether or not EB and progesterone were associated within and among estrous cycles per cow. Thus with cow as experimental unit, we examined the regression of progesterone (area, peak or duration) during first, second or third estrous cycle on mean EB during anovulation ($\geq$ 0, -0.1 to -3.0, -3.1 to -6.0 and < -6 Mcal of NEl). Unless stated,

all reference to EB will be mean during anovulation and will be discussed as mean EB. For these data, specific comparisons were between means of progesterone for control ($\geq$ 0.0 Mcal) and means of progesterone for other levels of mean EB during anovulation (-0.1 to -3.0, -3.1 to -6.0 or $\leq$ -6 Mcal). Contrasts were made by Dunnett's test (Gill, 1978). To determine association between EB and luteal function over time, we examined changes of progesterone within level of mean EB across estrous cycles as follows. Slopes of regressions of progesterone (area, peak or duration) within level of EB were contrasted with zero slope (Gill, 1978). Significant increases of progesterone were indicated by positive slopes while negative slopes indicated decreases of progesterone over time. In addition, slope for regression lines of progesterone within level of NEB (-0.1 to -3.0; -3.1 to -6.0 or < -6 Mcal) were contrasted with corresponding slopes of cows in PEB during anovulation ($\geq$ 0.0 Mcal). Lack of parallelism between slopes indicated difference in rate of change of progesterone over time.

To further examine the association between EB during anovulation and luteal function of cows, duration and magnitude of EB were determined for cows grouped by level of EB (>0.0, -0.1 to 3.0, -3.1 to -6.0 or < -6 Mcal) during postpartum anovulation. For this we defined onset of NEB as the first 3 consecutive d postpartum when cows were in NEB. End of NEB was when cows were in PEB for at least 8 d of two consecutive weeks. Duration of NEB was the interval between the first d of onset and the first d of end of NEB. Magnitude of NEB was indicated by nadir of EB within the interval of NEB. To determine the relationship between these aspects of EB and luteal function, area of progesterone within first, second or third estrous cycles was the dependent variable in a regression analysis by backward elimination, in which the independent variables were day of nadir, nadir and duration of NEB.

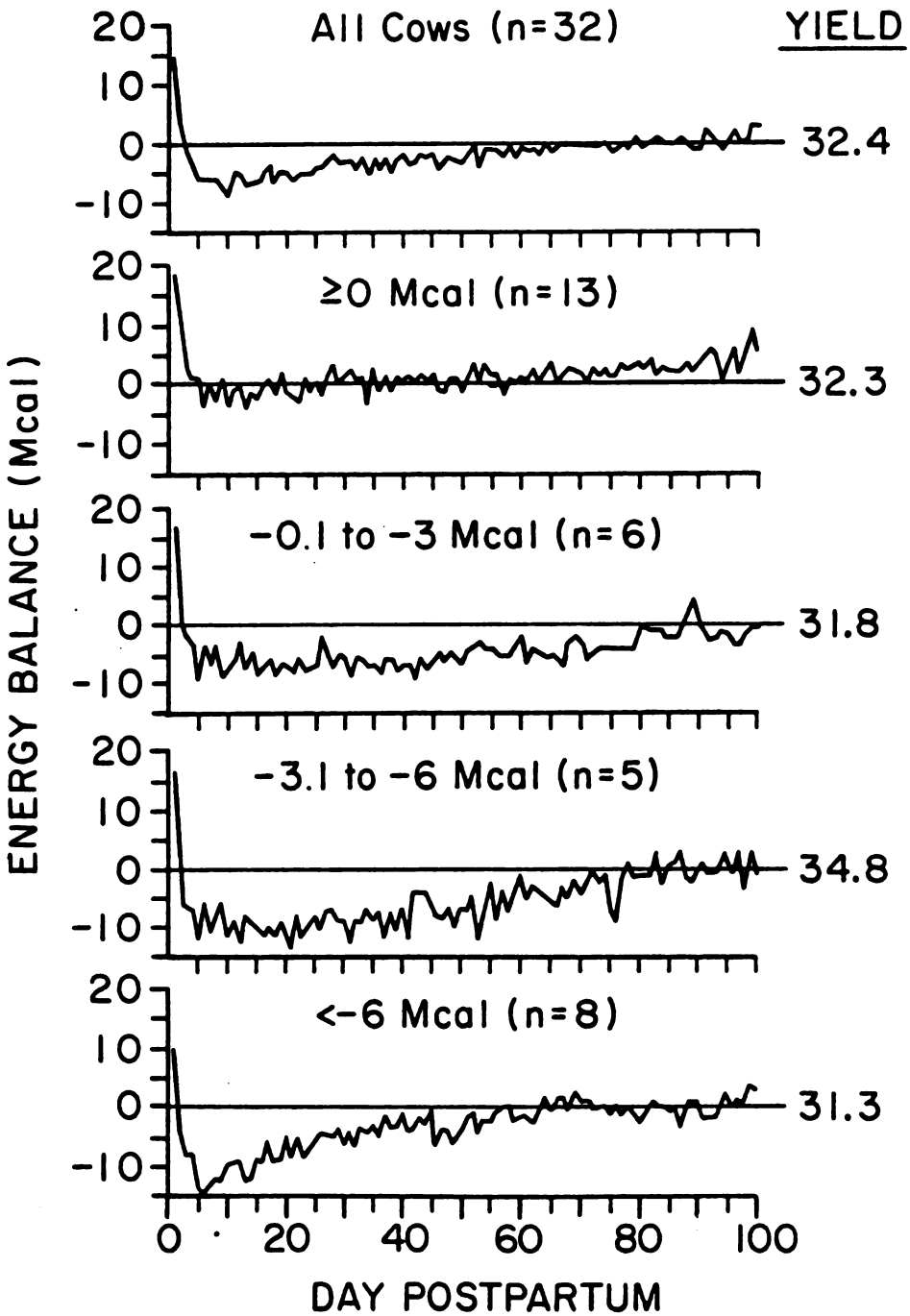
Using chi square (Gill, 1978), proportions of ovulations associated with estrous behavior were contrasted among levels of mean EB, mean intake of DM per estrous cycle, mean body weight per estrous cycle, mean yield of 4% FCM during first 100 d postpartum or parity (1, 2 or $\geq$ 3 parturitions).

RESULTS AND DISCUSSION

Yield of Milk, Body Weight and EB. Mean yield of 4% FCM during the experiment was 32.4 ± .9 kg/d (range: 20.8 to 40.7 kg). Peak yield of milk (40.2 ± 3.4 kg) occurred at 44.8 ± 3.5 d postpartum. Initial body weight of cows averaged 601 ± 30 kg. Four cows maintained or gained weight throughout the study. All other cows lost 29.4 ± 3.9 kg (range: 8.6 to 106 kg) from first week to 43.4 ± 4.2 d postpartum when nadir for body weight occurred. From nadir, body weight increased and by the end of the study all cows averaged 591 ± 31 kg. Mean intake of DM increased from 19.1 ± .9 kg in first week to 27 ± 2.2 kg by d 100 postpartum.

When EB was averaged for all cows (figure 1), mean EB was positive during the first 2 d postpartum, became negative on d 3 and reached nadir by d 10 postpartum. Starting on d 11 postpartum, mean EB increased from nadir and was zero or positive by 80 d postpartum. Approximately 81% (n = 26) of the cows were in NEB for at least 4 consecutive d postpartum. But at the end of the study 69% (n = 22) of the cows were in net energy loss. Thus by d 100 postpartum, 69% of cows did not recover energy lost at earlier stages of lactation. Overall, daily EB was highly variable within and especially among cows. For all cows, the observed range of daily EB was -35 to 31 Mcal of NE. Among the 26 cows experiencing NEB, nadir of EB (-16.3 ± 1.2; range: -4 to -35 Mcal) and duration of NEB (69.6 ± 5.9, range 4 to 98 d) were similar to values reported previously (Butler et al., 1981; Kazmer et al., 1986). In the present study, cows

Figure 1. Energy balance and yield of milk during the first 100 d postpartum. Daily mean of energy balance (Mcal of net energy for lactation) was calculated for all cows (top panel) or for cows grouped according to mean energy balance during postpartum anovulation (1 to 9 d). In parenthesis are numbers of cows. Yield of 4% FCM is the mean during first 100 d postpartum. Pooled standard error of energy balance for all cows was 1.3. Pooled standard error for yield of milk was .9.


All Cows (n=32)
YIELD
32.4
≥0 Mcal (n=13)
32.3
-0.1 to -3 Mcal (n=6)
31.8
-3.1 to -6 Mcal (n=5)
34.8
<-6 Mcal (n=8)
31.3
20
10
0
-10
0
20
40
60
80
100
ENERGY BALANCE (Mcal)
DAY POSTPARTUM

experienced NEB even when feed offered was not limiting. Thus the large variation in EB among cows was spontaneous. Consequently I was able to examine the relationship between luteal function and different levels of EB without dietary manipulation. This is consistent with examining effects of homeorhetic control of lactation on luteal function.

To further examine EB throughout the experiment, cows were grouped according to mean EB during postpartum anovulation. In cows which averaged $\geq$ 0 Mcal of NE during anovulation, magnitude of energy deficit (nadir: -8.7 ± 1.6 Mcal) was less ($P < .01$) and interval of NEB was shorter (38.6 d) than in cows with <0.1 Mcal during anovulation (figure 1). Magnitude and duration of NEB did not differ among groups of cows that were in energy deficit during anovulation. But, average EB throughout the study was lower ($P < .05$) in groups of cows with $\leq$ -3.1 Mcal than in cows with marginal energy deficit (-0.1 to -3 Mcal) during anovulation (figure 1). For all cows with $\leq$ -0.1 Mcal during anovulation, EB at nadir averaged -19.1 ± 1.3 Mcal and mean duration of NEB was 78 ± 6 d. But day of nadir was earlier ($P < .01$) in cows with < -6 Mcal during anovulation (d 12 ± 4.4 postpartum) than in cows with -3.1 to -6 Mcal (d 30.2 ± 8.5) or with -0.1 to -3 Mcal during anovulation (d 40 ± 10.5). Thus, level of energy deficit during anovulation seems to be determined primarily by when nadir occurs postpartum and, to a lesser extent by actual of nadir.

Factors Associated with Variation in EB. Parity and body weight were not significantly associated with EB (table 2). But variation in EB of cows was explained largely (table 2) by intake of energy ($P < .01$) and, to a lesser extent by yield of milk ($P < .05$). Throughout the study, only 19% (n = 6) of the cows were in PEB. Yield of 4% FCM (32.8 ± 1.9 kg) of cows always in PEB was not different than mean yield of 4% FCM for the 26 cows in NEB (32.3 ± 1.01 kg). Interestingly, the cow with highest yield (40.7 kg 4% FCM/d) ingested more

TABLE 2. COEFFICIENTS OF CORRELATION FOR ENERGY BALANCE WITH PARITY, BODY WEIGHT, YIELD OF MILK AND INTAKE OF ENERGY.[a]

	Parity	Body Weight	Yield of Milk	Intake of Energy
Energy balance	.10	-.13	-.25[b]	.73[c]

[a]Daily energy balance was regressed on estimated daily body weight, daily yield of milk (4% FCM), daily intake of dietary energy and parity (1, 2 or $\geq$ 3 parturitions).

[b]Significant correlation ($P < .05$).

[c]Significant correlation ($P < .01$).

DM (30.9 kg/d) than any other cow and sustained PEB during the study. These observations on individuals and the fact that yield of milk did not differ among cows with distinctly different EB (figure 1), illustrate that some cows with large yields of milk ingest sufficient energy to satisfy requirements for high yields of milk and to sustain PEB. Conversely, some cows with average or low yields of milk do not ingest enough energy to maintain PEB even with modest yields of milk. In general our observations are consistent with results reported by Kazmer et al. (1986) and indicate that variation in EB is largely due to voluntary intake of DM by cows. Yield of milk, although associated negatively, has a minor effect on variation in EB of cows. Thus, yield of milk would not be highly predictive of EB or metabolic status of lactating cows.

Factors Associated with Luteal Function. All cows ovulated at least twice during the study. The interval from parturition to first ovulation averaged 23.9 ± 2.2 d (range 10 to 52 d). Behavioral signs of estrus were detected in association with 64.9% of ovulations. A lower proportion ($P < .01$) of behavioral estrus was associated with first postpartum ovulation (34.4%) than with second (71.9%) or third (83.3%) postpartum ovulations. Duration of postpartum anovulation and detection of estrus associated with ovulation were not correlated significantly with parity, body weight, yield of 4% FCM, intake of DM or EB. My observation that all cows ovulated and resumed estrous cycles by 23.9 ± 2.2 d postpartum is consistent with previous reports (Butler et al., 1981; Carstairs et al., 1980; Rosemberg et al., 1977). Thus, postpartum anovulation is not a source of extended intervals non pregnant in most dairy cows. Success of detecting estrus associated with ovulation in the present study was low as in previous works (Butler et al., 1981; Carstairs et al., 1980) and confirms the importance of understanding sources of variation for expression of estrus.

First, second and third estrous cycles began at 23 ± 2, 42 ± 2 and 63 ± 3 d postpartum, respectively. Duration did not differ among postpartum estrous cycles and averaged 21.3 ± 8 d. Mean duration of luteal phases was 12.8 ± .6 d and did not vary among postpartum estrous cycles. But concentrations of progesterone (area and peak) increased ($P < .05$) from first to third postpartum estrous cycle. This agrees with previous reports that concentrations of progesterone in serum during luteal phases after first ovulations are lower than during subsequent luteal phases of postpartum dairy cows (Edgerton and Hafs, 1973).

Yield of 4% FCM, mean body weight per cycle and parity were not correlated significantly with area and peak of progesterone or with duration of luteal phase (table 3). In addition changes in body weight from parturition to nadir of body weight were not associated with progesterone in milk (data not shown). Yield of milk affects EB and changes in body weight are altered by EB. Thus these variables are associated with EB and might be expected to affect reproductive events. But in the present study, there was no evidence that duration of postpartum anovulation, detection of estrus or luteal function were influenced by yield of milk or body weight. Thus, as independent factors, yield of milk or body weight may not influence reproductive performance in lactating dairy cows. Alternatively, yield of milk and (or) body weight may affect reproductive performance by mechanisms related to reproductive variables not examined in this study.

Since duration of luteal phases did not vary among cycles, it is not surprising that duration of luteal phase was not associated with EB or intake of DM. But variation in area and peak of progesterone were correlated positively ($P < .05$) with mean EB and intake of DM per estrous cycle (table 3). However, intake of DM was the most important component of EB (table 2). Thus, coefficients

TABLE 3. COEFFICIENTS OF CORRELATION OF PROGESTERONE WITH ENERGY BALANCE, INTAKE OF DRY MATTER, YIELD OF MILK, BODY WEIGHT AND PARITY.

Independent variables[a]	Progesterone[b]		
	Area	Peak	Duration
Energy balance	.28[c]	.25[c]	.07
Intake of DM	.23[c]	.13	.04
Yield of milk	-.01	-.04	-.01
Body weight	.07	.09	.05
Parity	-.02	-.01	.03

[a]Energy balance, intake of DM and body weight were averaged per estrous cycle. Yield of milk is average of 4% FCM during the first 100 d postpartum. Parity was 1, 2 or $\geq$ 3 parturitions.

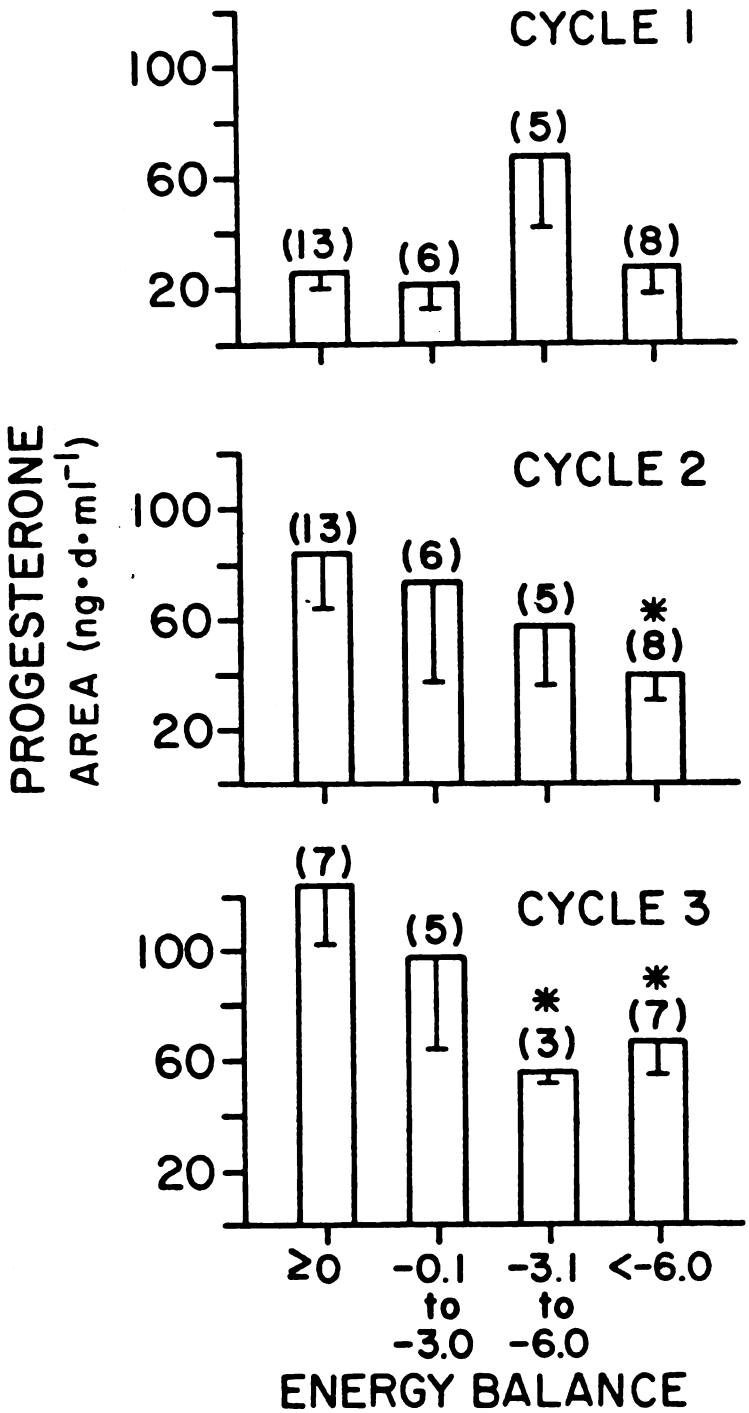
[b]Variables describing progesterone were means per estrous cycle of area under the curve of progesterone (progesterone > basal concentrations), maximal concentration of progesterone (peak) and duration of luteal phase.

[c]Significant correlation with independent variable in same line ($P < .05$).

of determination were not strengthened by including intake of DM or NE with EB as an independent variable in statistical models (data not shown). Associations of intake of DM with EB and luteal function observed in this experiment may explain inconsistent results about effects of dietary energy on reproduction (Carstairs et al., 1981; Ducker et al., 1985a, 1985b; Gardner, 1969; King, 1968). Generally, researchers assume that cows fed ad libitum are in PEB or in less NEB than cows which receive restricted amounts of feed. But, based on evidence from this study, intake cf feed or appetite are not related linearly with feed offered to cows above 100% of requirements of NE for maintenance and lactation. Therefore, actual EB and reproductive performance of cows may be similar although feed offered to cows varies.

Energy balance per estrous cycle and progesterone in milk were correlated positively (table 3). But successive postpartum estrous cycles were correlated positively with EB ($r = .5$; $P < .05$) and with progesterone in milk ($r = .3$; $P < .05$). Furthermore, duration of postpartum anovulation determines when estrous cycles occur and EB varies colinearly with postpartum interval. Therefore, effects of EB and successive postpartum estrous cycles on luteal function of cows are confounded. To reduce these confounding effects, cows were segregated among 4 categories of mean EB during postpartum anovulation (1 to 9 d). Among these four groups of cows we contrasted progesterone blocked within postpartum estrous cycle. During the first estrous cycle postpartum, variation of progesterone in milk (area) was not correlated significantly with mean EB (figure 2). But within second or third estrous cycle, variation of progesterone (area) was correlated positively ($P < .08$) with changes in EB during anovulation (figure 2). In fact, during second and third cycles, cows with most severe NEB (< -6 Mcal) during anovulation had lower ($P < .05$) progesterone in milk (area) than PEB cows (figure 2). In addition, cows with EB between -3.1 and -6 Mcal during

Figure 2. Association between energy balance and concentrations of progesterone in milk. Progesterone was measured in free-fat milk collected every third d. Energy balance (Mcal of net energy) was averaged during interval of postpartum anovulation (1 to 9 d). Association examined was between levels of EB during anovulation and progesterone within first, second or third postpartum estrous cycle. In parenthesis are numbers of cows within estrous cycle per level of energy balance. Values are mean ± SE. Energy balance and progesterone were correlated positively within second and third cycle ($r = .3$; $P = .07$). *This value differs ($P < .05$) from value for ≥ 0 within estrous cycle.


CYCLE 1
100
60
20
(13)
(6)
(5)
(8)
PROGESTERONE
AREA (ng·d·ml⁻¹)
CYCLE 2
100
60
20
(13)
(6)
(5)
*
(8)
CYCLE 3
(7)
(5)
*
(3)
*
(7)
100
60
20
≥0
-0.1
to
-3.0
-3.1
to
-6.0
<-6.0
ENERGY BALANCE

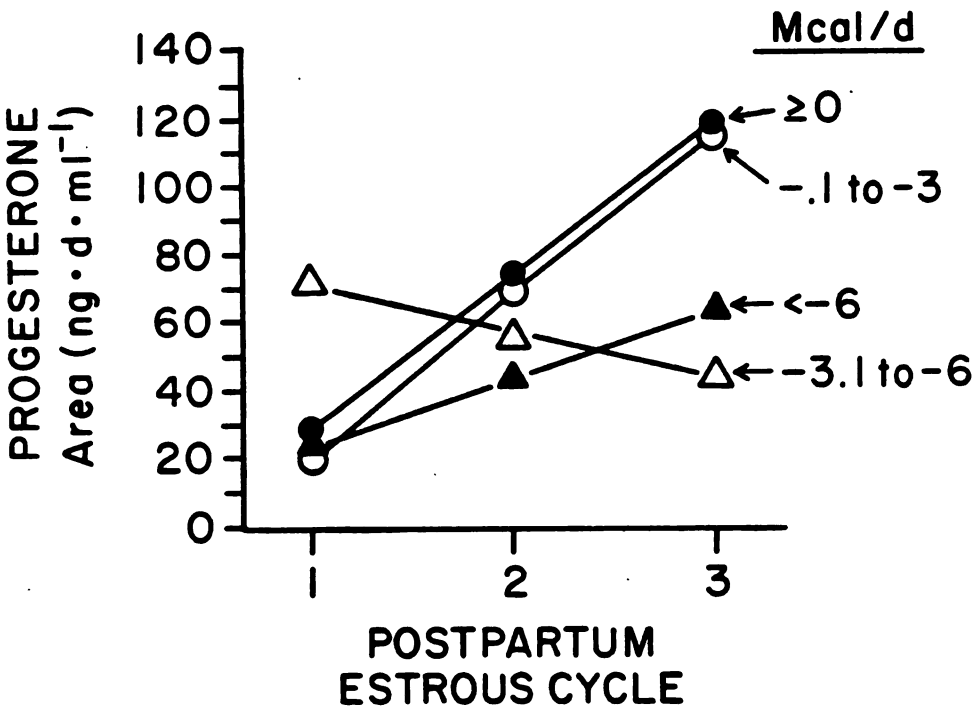
anovulation had less progesterone ($P < .05$) during the third estrous cycle than PEB cows. Duration of luteal phases and EB during anovulation were not correlated significantly, but associations between peak progesterone and mean EB (data not shown) were similar than for area and EB. Similarly, in underfed heifers length of diestrus was normal but progesterone in serum was lower than in adequately fed heifers (Hill et al., 1970; Imikawa et al., 1983). One interpretation of these data is that second and third postpartum corpora lutea in cows which were in severe NEB during anovulation, had normal lifespan but secreted less progesterone. Effects of NEB on magnitude of peak progesterone could be due to a) reduced luteal development, b) decreased secretory activity per luteal cell or c) a combination of these effects.

Why was EB during anovulation not related with luteal function during first estrous cycle postpartum? Why was apparent influence of NEB delayed for 40 to 70 d when second and third postpartum corpora lutea developed? Concentrations of progesterone in serum (Edgerton and Hafs, 1973) and in milk (this study) during the first postpartum diestrus were lower than during subsequent estrous cycles. It is possible that the first postpartum corpus luteum is inherently limited independent of NEB. Thus adverse effects of NEB observed on second and third postpartum corpora lutea are inconsequential or undetectable during first postpartum diestrus. In contrast, modulating factors present during first diestrus (Rutter et al., 1985) are absent or reduced by 40 to 70 d postpartum, when adverse effects of NEB on luteal function in second and third postpartum diestrus are exerted or detectable. Alternatively, NEB may not have immediate influence on luteal function of cows. This is possible because in heifers with restricted dietary energy and presumably in NEB, progesterone did not decline until second and third diestrus after dietary restriction (Gombe and Hansel, 1973).

In cows that were in PEB, marginal NEB (-.1 to -3 Mcal) or most severe NEB (<-6 Mcal), contrasts between zero slope and slopes of regression of progesterone within level of EB during anovulation over time postpartum (figure 3), indicated that progesterone increased ($P < .05$) from first to third estrous cycle. But in cows with EB of -3.1 to -6 Mcal during anovulation, progesterone tended ($P = .09$) to decline overtime (figure 2). When contrasted with PEB cows, slopes of progesterone in cows with marginal NEB (-.1 to -3 Mcal) were parallel. But slopes of cows in severe NEB (-3.1 to -6 or <-6 Mcal) during anovulation did not parallel ($P < .05$) slopes of cows in PEB or in marginal NEB. Thus in cows with severe NEB ($\leq$ -3.1 Mcal), progesterone did not increase with sequential postpartum estrous cycles as in cows with marginal (-.1 to -3 Mcal) and PEB during anovulation, or increased at a lower rate. Based on results from cows in PEB and moderate NEB, luteal function is affected positively by progressive postpartum estrous cycles or advancing postpartum interval. But severe NEB appears to modulate adversely the positive effect of progressive postpartum estrous cycles on luteal function of dairy cows.

Variation in concentrations of progesterone in milk (area) was not associated with changes in nadir or duration of NEB in cows. But progesterone during second estrous cycle was correlated positively ($r = .51$; $P < .01$) with postpartum interval when nadir of EB occurred. Moreover, changes in progesterone during second and third estrous cycles were associated positively ($r^e = .69$; $P < .01$) with interactions between postpartum interval to nadir and EB at nadir. An interpretation of these results is that luteal function in second and third postpartum diestrus will be most limited in cows with early postpartum nadir of EB and severe NEB at nadir. Therefore early occurrence of nadir relative to parturition and severity of NEB at nadir are components of EB during anovulation that potentially limit luteal function in dairy cows.

Figure 3. Regression of energy balance on concentrations of progesterone in milk during 3 successive estrous cycles postpartum. Energy balance was averaged during postpartum anovulation (1 to 9 d postpartum) and cows were grouped as $\geq$ 0 (●; n = 13), -.1 to -3 (O; n = 6), -3.1 to -6 (△; n = 5) or < -6 Mcal (▲; n = 8). Relative to zero slope, slopes of regression lines for $\geq$ 0 and -.1 to -3 were positive ($P < .01$), slope for < -6 was positive ($P < .05$) and slope for -3.1 to -6 Mcal tended ($P = .09$) to be negative. Slopes of regression lines of $\geq$ 0 or -.1 to -3 differed ($P < .05$) from slopes of -3.1 to -6 ($P < .01$) or from < -6.


Mcal/d
≥0
-.1 to -3
<-6
-3.1 to -6
PROGESTERONE
Area (ng • d • ml⁻¹)
140
120
100
80
60
40
20
0
1
2
3
POSTPARTUM
ESTROUS CYCLE

In this experiment, diet was formulated to satisfy all requirements for energy and was offered ad libitum. But NEB occurred in 81% of the cows and was spontaneous rather than due to dietary restriction. Although yield of milk was associated negatively with EB, most variation of EB was determined by intake cf energy. There was no evidence that parity, body weight or yield of milk affect reproduction because these variables were not correlated significantly with duration of postpartum anovulation, detection of estrus or luteal function. Mean EB during postpartum anovulation was not associated with duration of luteal phases. But EB during anovulation was correlated positively with 1) luteal function within second and third postpartum estrous cycles and 2) changes of luteal function among successive postpartum estrous cycles. My primary conclusion is that severe NEB ($\leq$ -3.1 Mcal) during postpartum anovulation may reduce level of luteal function during second and third postpartum diestrus.

Nadir of EB occurred earlier postpartum in cows with severe NEB (< -6 Mcal) than in cows with less severe NEB during anovulation. Moreover, d of nadir but not EB at nadir was correlated positively with luteal function within second estrous cycle. Interactions between d of nadir and EB of nadir were associated positively with luteal function within second and third cycle. Therefore, timing and magnitude of NEB appear to be important determinants of the extent that NEB limits luteal function in cows. Severe NEB was associated with reduced luteal function during second and third estrous cycles, when most dairy cows are artificially inseminated for first time postpartum. Luteal function is associated with reproductive events that determine fertility in cattle (Folman et al., 1973; Hill et al., 1970; Melampy, 1957; Rosemberg et al., 1977). Consequently I suggest that NEB is a potential source cf infertility in cattle.

EXPERIMENT II:

Influence of Energy Balance and Body Condition on Luteal Function in Heifers

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Introduction

Negative energy balance (NEB) is associated with reduced luteal function in lactating dairy cows (Experiment I). This is particularly significant since $\geq$ 80% of cows are in NEB during early lactation (Coppock et al., 1974; Reid et al., 1966; Experiment I) and reduced concentrations of progesterone in serum are associated with low rates of conception (Folman et al., 1973), embryonic death (Hill et al., 1970) and failure to detect estrus (Melampy et al., 1957). Thus NEB may be a source of infertility in lactating cows.

Intake of dietary energy is an important source of variation of energy balance in lactating dairy cows fed ad libitum (Experiment I). Cows that are overconditioned at parturition consume less feed and energy during early lactation than moderately conditioned herdmates (Garnsworthy and Topps, 1982). Consequently overconditioning may enhance adverse effects of NEB on luteal function.

In dairy cows, influences of NEB, time postpartum and sequential estrous cycles on luteal function are confounded (Experiment I). Therefore, I used postpubertal Holstein heifers to determine the independent and associative effects of body condition and energy balance on luteal function. In addition, I examined the relationship between body condition and energy balance with concentrations of insulin, growth hormone (GH) and non-esterified fatty acids (NEFA) in blood serum.

Materials and Methods

Design and General Procedures. Twenty postpubertal Holstein heifers were grouped by body weight and assigned to treatments within a 2 x 2 factorial experiment. Main effects were: 1) body condition: moderate or fat, and 2) energy balance: positive or negative. Heifers were housed in a free stall barn that was divided into two pens. Each pen was equipped with 16 gang-lock stanchions at the feed bunk. In addition, feed bunks were partitioned into 16 individual mangers. Thus, heifers could be fed individually. Throughout the study heifers were fed total mixed rations (TMR; table 4) at 0500 and 1700 h and had free access to water and salt supplemented with trace minerals. The study consisted of three phases: conditioning, adaptation and experimental. The conditioning phase lasted about 6 mo during which heifers were fed to produce two groups of animals with distinct body conditions (BC), moderate or fat. To achieve this goal, 12 heifers with an average body weight of 275.8 ± 5.6 kg received diet A (table 4) in amounts that supported daily gains of .78 kg and maintained moderate BC (MOD). In addition, 8 heifers with average body weight of 279.1 ± 7.6 kg received diet B (table 4) ad libitum to support daily gains of .9 kg and to produce fat BC (FAT). The conditioning phase ended when BC of the two groups was different ($P < .05$).

To determine energy balance (EB), heifers were fed individually. Thus during an interval of 20 d, heifers were adapted to gang-lock stanchions and trained to consume their daily dietary allowance within two intervals of 90 min (0500 to 0630 and 1700 to 1830 h). Variation in intake of dry matter (DM) per unit of body weight or in levels of starch in diets alters patterns of rumen fermentation in cattle (Robinson et al., 1986). Because changes in rumen fermentation may affect ovarian functions in cattle (McCartor et al., 1979), during the period of adaptation MOD and FAT heifers received the same diet

TABLE 4. COMPOSITION OF TOTAL MIXED RATIONS.

Item	Diet[a] A	B	C
Ingredients, %			
Alfalfa haylage	24.9	4.4	22.4
Corn silage	53.4	16.9	48.9
High moisture ear corn	16.2	73.1	12.0
Protein supplement (44%)	5.0	5.1	16.2
Vitamin premix	.5	.5	.5
Chemical analysis[b,c]			
Dry matter, %	40.7	50.9	46.4
Energy, Mcal/kg[d]	1.5	1.6	1.5
Crude protein, %	13.6	13.3	17.7
Acid detergent fiber, %	21.7	14.3	15.5

[a]Dry matter basis.

[b]Methods for chemical analysis in Pritchard and Staubus (1978).

[c]Concentration of minerals in total mixed rations were: Ca (.71%), P (.39%), K (1.1%), Mg (.26%), S (.21%), Na (.13%) Mn (57 ppm), Fe (228 ppm), Cu (8 ppm) and Zn (48 ppm).

[d]As NE_m (estimated from total mixed rations).

(A, table 4) and received similar amounts of DM per unit of body weight. Therefore, at the end of the adaptation phase, all heifers received the same diet for at least 20 d but BC of heifers was still different.

The experimental phase lasted 3.5 estrous cycles and ended when corpora lutea of heifers were removed surgically (lutectomy) 10 to 12 d postestrus of the fourth estrous cycle. From the first d and throughout the experimental phase, 6 MOD and 5 FAT heifers individually received diet A (table 4) to sustain daily gains of .78 kg and positive EB (PEB). The remaining MOD (n = 6) or FAT (n = 3) heifers individually received diet C (table 4) in amounts calculated to cause NEB without deficiences in protein, vitamins or minerals. Thus at the beginning of the experimental phase four treatment combinations were established: MOD-PEB (n = 6), FAT-PEB (n = 6), MOD-NEB (n = 5) and FAT-NEB (n = 3).

Twice daily throughout adaptation and experimental phases, heifers were restrained to receive TMR individually and to record daily intake of feed. During the entire study, weekly samples of ingredients in TMR were collected and dried in oven at 100°C for 12 h to determine DM. Based on changes in DM of ingredients, TMR were adjusted to maintain a constant ratio of ingredients. During the entire experiment, TMR was sampled weekly. For each TMR, aliquots from individual samples were obtained and pooled so at the end of the study there was a pooled sample from each TMR. Duplicates from each pool of TMR were analyzed chemically (table 4).

Body Weight and Body Condition. Throughout the study, body weight and BC of heifers were used to monitor diets. Heifers were weighed (1500 h) on two consecutive d per week. Weekly changes in body weight were extrapolated to estimate daily changes in body weight per d of the experiment. Methods devised by Mulvany (1981) were modified and used to determine BC of heifers.

Body condition of heifers was scored every other week in a scale of 1 to 4 (4 = fat), including half points. For scoring BC of heifers, thickness of subcutaneous adipose tissue was estimated by palpating loin and tailhead. Loin consisted of spinal and transverse processes of lumbar vertebrae. Tailhead was a triangular area defined by anterior coccygeal vertebrae and both Tuber ischia (pin bones). Average scores for loin and tailhead indicated BC of heifers. Scores used for statistical analyses of BC were mean of 4 observers.

Energy Balance. During the experimental phase, EB was estimated daily for each heifer. Energy balance was calculated by subtracting energy required for maintenance from intake of energy. Net energy required for maintenance (NEm) were based on daily body weight and calculated by: NEm (Mcal) = (.077) body weight$^{.75}$ (National Research Council, 1984). Daily intake of energy was calculated by multiplying NEm in feeds (table 4) by intake of DM.

Luteal Function in vivo. To determine the influence of EB, BC and(or) their interactions on progesterone, jugular blood was sampled daily (1500 h) for experimental phase (3.5 estrous cycles). Progesterone was quantified in serum by radioimmunoassay.

Luteal Function in vitro. To determine effects of EB, BC and (or) their interactions on basal and LH-induced secretion of progesterone from luteal cells in vitro, corpora lutea were collected via supravaginal incision between d 10 and 12 postestrus of the last estrous cycle. Immediately after collection, corpora lutea were rinsed with medium 199 (Gibco Laboratories, Grand Island, NY), pH 7.35 with Hank's salts, $NaCHO_3$ (.36 g/liter), HEPES (4.7 g/l), bovine serum albumin fraction V (1 mg/ml), penicillin G (sodium salt, .7 g/l), streptomycin sulfate (.1 g/l) and neomycin sulfate (.05 g/l). Rinsed corpora lutea were placed in tubes with fresh medium and transported on ice to our laboratory. Subsequently, adherent interstitial tissue was removed and weights

of corpora lutea were determined. Corpora lutea were bisected and sliced (Stadie-Riggs microtome). For dissociation of cells, slices of luteal tissue were placed in Hank's medium devoid of Ca^{++} and Mg^{++} but containing collagenase (Worthington type IV, 125 U/ml, .05 to .3%), deoxyribonuclease (.005%) and bovine serum albumin (.05%; Sigma Chemical Co., St. Louis, MO). Luteal slices were incubated at 37°C in an atmosphere of 95% 0_2 and 5% CO_2 for 4 to 6 h with frequent agitation by aspiration to disperse cells. Viability of luteal cells (> 90% in all corpora lutea) was determined by Trypan blue exclusion (Patterson, 1979) and cells were counted by hemocytometer. Dissociated luteal cells from each corpus luteum were suspended in medium 199 and incubated at 37°C in an atmosphere of air for 2 h. Incubation medium contained 0, .1, 1, 10 or 100 ng/ml of bovine luteinizing hormone (NIH-LH-B8) and each dose was replicated 5 times per corpus luteum. After incubation, cells and medium were frozen together in bath of dry ice-methanol and stored at -60°C until assayed for progesterone.

Detection of Estrus. To monitor intervals between estrus, heifers were observed daily for signs of estrus for at least two periods of 30 min. A heifer was considered in estrus when she stood to be mounted for at least 2 sec and concurrently had < 1 ng of progesterone per ml of serum. In addition, ovaries and uteri of heifers were examined rectally at least twice before experimental phase to confirm postpubertal status. Only postpubertal heifers were included in the experiment.

LH, GH, Insulin and NEFA. Energy balance, BC and(or) their interactions may alter luteotropic support to corpora lutea during diestrus. To determine effects of EB and (or) BC on LH, polyvinyl cannulae were installed in a jugular vein of heifers and on d 9, 10 or 11 postestrus of fourth estrous cycle, approximately 24 h before lutectomy. Samples of blood were collected every

15 min during an interval of 12 h (0430 to 1630 h) for quantification of LH. In addition, GH and insulin were measured in serum from these same samples. Feed was presented to heifers immediately after collecting blood at 0500 h. Jugular blood was taken before (0430 and 0500 h) and every h (0600 through 1600 h) after feeding, placed in heparinized tubes and resulting plasma was assayed for NEFA.

Assays. Concentrations of progesterone in serum were quantified by a solid-phase radioimmunoassay described in Appendix A. From 21 assays, concentrations of progesterone in serum from heifers in estrus was .15 ± .002 ng/ml. The intraassay coefficient of variation (CV) was 9.6% and the interassay CV was 12.7%. In serum from pregnant cows concentrations of progesterone were 6.7 ± .05 ng/ml (intraassay CV = 4.2%; interassay CV = 6.7%).

Tubes containing luteal cells and medium were thawed and frozen repeatedly to disrupt membranes of cells. Media and disrupted luteal cells were extracted twice (benzene:hexane, 1:2) and quantified for progesterone (Louis et al., 1973). From 25 radioimmunoassays, concentrations of progesterone in a pool of media plus luteal cells from heifers in late diestrus was 13.7 ± .14 ng/ml (intrassay CV = 7.45%; interassay CV = 9.99%). Extraction efficiency among assays was 95.3 ± .5%.

Concentrations of LH and GH in serum were determined by radioimmunoassays described by Convey et al. (1976) and Purchas et al. (1970), respectively. From a single assay, concentrations of LH in serum from cows in estrus were 10.5 ± .3 ng/ml (CV = 8.5%). Concentrations of GH in serum from lactating cows was 5.4 ± .1 ng/ml (CV = 6.2%). Methods to quantify insulin are described in appendix. Concentrations of insulin in serum from 5 h fasted beef heifers was 66.7 ± 1.9 μU/ml (CV = 6.8%) but was 177.5 ± 5.9 μU/ml (CV = 4.5%) in serum from recently fed beef heifers. Concentrations of NEFA in

plasma were determined by colorimetric techniques (Brunk and Swanson, 1981). From 13 assays, concentrations of NEFA in plasma from fed heifers were 135.8 ± 4.3 μEq/l (intraassay CV = 2%; interassay CV = 16.4%).

Statistical Analyses. Data on EB, BC and body weight change were averaged per estrous cycle and analyzed by split-plot analysis of variance with estrous cycles as subplots (Gill and Hafs, 1971). In addition slopes of regression lines of EB, BC or body weight change across estrous cycles were determined. Positive slopes indicated that EB, BC or body weight increased. Negative slopes indicated that EB, BC or body weight decreased. Lack of parallelism of slopes between treatment combinations (Gill, 1978) was interpreted as a difference in rates of change of EB, BC or body weight. Results from this analysis will indicate whether or not the treatment combinations needed to address our objective were established. To analyze concentrations of progesterone in serum, area under the curve of progesterone was calculated for first 10 to 12 d of 4 estrous cycles within heifer. Area included values that were 2 standard deviations above baseline (.125 + (2) .089 = 0.3 ng/ml). Baseline (.125 ng/ml) was the mean concentrations of progesterone in samples of blood taken during estrus ± 1 d. These data were arranged in a 2-factor design with 4 estrous cycles as a subplot and examined by a split-plot analysis of variance (Gill, 1978). Area under the curve is determined largely by maximal concentration of progesterone (peak) and duration of diestrus (interval that progesterone exceeds baseline by 2 standard deviations). Therefore, for all four periods of diestrus during experimental phase, peak concentrations of progesterone and duration of luteal phases during first 10 to 12 d postestrus were calculated and were analyzed in a 2-factor design with 4 estrous cycles as a subplot.

To evaluate luteal development I used rate of increase (slope) of progesterone in serum during first 10 d postestrus of each cycle. Origin of

these slopes (onset of diestrus) was day when concentrations of progesterone exceeded baseline by 2 standard deviations. Slopes for progesterone within estrous cycle were contrasted among treatment combinations according to methods described by Gill (1978). Parallelism between slopes of two treatment combinations was interpreted as similar rates and (or) extent of luteal development. In contrast, lack of parallelism between slopes of 2 treatment combinations indicated different rates and (or) extent of luteal development. Concentrations of progesterone in medium were analyzed by split-plot analysis of variance with doses of LH as subplots. Results of this analysis will determine existence of independent or associative effects of EB and BC on luteal function in vivo and(or) in vitro.

Luteotropic support may be altered by EB and(or) BC. Insulin, GH and (or) NEFA likely are affected by EB, BC and(or) their interaction. Therefore, concentrations of NEFA in plasma and concentrations of LH, GH and insulin in serum were analyzed by analysis of variance for repeated sampling over time (Gill and Hafs, 1971). Furthermore, baseline concentrations, frequency and amplitude of pulses of LH, GH and insulin were calculated and then analyzed by analysis of variance for 2-factor experiments with fixed effects (Gill, 1978). Pulse of LH (Hughes et al., 1987) was an increase in concentrations of LH that exceeds a value of LH in previous 30 min by twice the within assay standard deviation (2 (0.55) = 1.1 ng/ml). Amplitude of pulses was the difference between nadir and subsequent maximal value reached during a pulse of LH. Frequency of pulses was the number of pulses per heifer occurring during an interval of 12 h. Basal LH was mean concentration of LH in all samples not included in pulses. Amplitude, frequency and duration of pulses of GH and insulin and basal GH were identified by the Pulsar program which uses alogarithms for pattern recognition (Merriam and Watcher, 1982). Basal insulin was set at mean concentrations in preprandial samples.

Energy balance and(or) BC may affect luteal function and metabolic factors (GH, insulin, NEFA). Are luteal function and metabolic factors associated? To determine associations between luteal function and GH, insulin or NEFA in heifers, regression analyses were used in which mean concentrations of progesterone in serum at d of lutectomy was the dependent variable. Independent variables were mean concentration of GH, insulin and NEFA in blood of heifers.

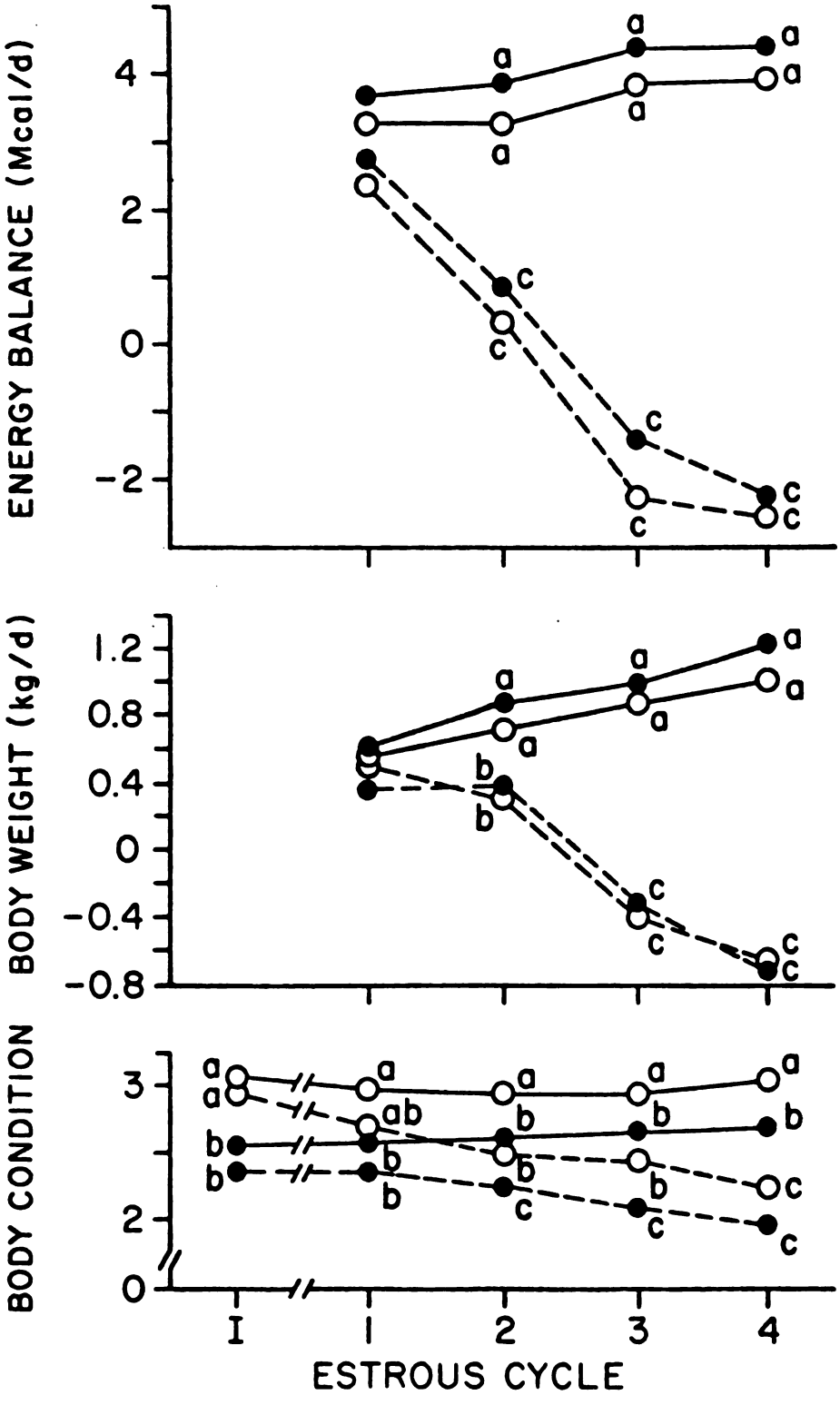
For area and peak of progesterone and duration of luteal phases within treatment combination, specific contrasts among estrous cycles were made by Bonferroni t statistics (Gill, 1978). These contrasts will identify specific interactions of EB with BC that alter progesterone over time in vivo. But for area, peak and duration of luteal phases within cycle, Dunnett's test (Gill, 1978) was used to contrast controls (MOD-PEB heifers) versus other treatment combinations. Results from these comparisons will be to know specific interactions of EB with BC that alter progesterone within estrous cycle in vivo. To determine whether specific interactions of EB with BC affected luteal function in vitro, basal and LH-induced progesterone in controls (MOD-PEB) were contrasted with other treatment combinations by Dunnett's test.

Effects of specific interactions between EB and BC on luteal weight, LH, GH, insulin and NEFA were determined by contrasting treatment combinations with Bonferroni t test. For statistical models with split-plot structure, error terms and critical values for Dunnett's and Bonferroni t tests were modified as recommended by Gill (1986).

Results

Energy Balance and Body Measurements. As expected, EB was positive throughout the experimental phase (figure 4) in heifers that received diet A (table 4). In contrast, heifers offered diet C (table 4) were in PEB during the

Figure 4. Changes in energy balance, body weight and body condition of heifers over four estrous cycles. Heifers had moderate (●) or fat (O) body condition and were in positive (—) or negative (- - -) energy balance. Body condition at the beginning (I) of the experimental phase is included in the bottom panel. For each variable, treatment combination means within estrous cycle (or I) with different superscripts differ (^{a,b} $^{b,c}P < .05$ or $^{a,c}P < .01$). Pooled standard errors are: .6 for energy balance, .1 for body weight and .06 for body condition.


ENERGY BALANCE (Mcal/d)
4
2
0
-2
BODY WEIGHT (kg/d)
1.2
0.8
0.4
0
-0.4
-0.8
BODY CONDITION
3
2
0
I
1
2
3
4
ESTROUS CYCLE

first experimental estrous cycle, approached EB during the second cycle and were in NEB during the third and fourth estrous cycles (figure 4). Parallelism between slopes indicates that rates of decline in EB were not different between MOD-NEB and FAT-NEB heifers. However, duration of NEB (46 ± .6 d) and net accumulated loss of energy (-98.1 ± 12.6 Mcal) for FAT-NEB heifers were greater ($P < .01$) than for MOD-NEB heifers (38 ± 2.8 d; -57.3 ± 4.1 Mcal).

In agreement with changes in EB, MOD-PEB and FAT-PEB heifers gained weight during the experimental phase (figure 4). In contrast, MOD-NEB and FAT-NEB heifers gained weight during the first two estrous cycles, but lost weight during the third and fourth estrous cycles (figure 4).

As indicated previously, BC at the beginning of the experimental phase (figure 4) differed ($P < .05$) between FAT (3 ± .06; range 2.8 to 3.5) and MOD heifers (2.5 ± .03; range 2.3 to 2.6). Heifers in PEB maintained initial BC with no significant change throughout the experimental phase (figure 4). In contrast, BC of MOD-NEB and FAT-NEB heifers declined ($P < .05$) and was less ($P < .05$) than BC of corresponding control heifers (MOD-PEB and FAT-PEB, respectively) by the second experimental estrous cycle.

Luteal Function in vivo. Discussion of effects of BC on luteal function or on any other dependent variable will refer to BC of heifers at onset of experimental phase. Otherwise, it will be indicated. Luteal weight was not influenced by EB or BC independently. But corpora lutea of FAT-PEB and MOD-NEB heifers weighed less ($P < .05$) than corpora lutea of MOD-PEB heifers (table 5). As single effects, EB or BC did not alter luteal function among estrous cycles (figure 5). Within first, second and third estrous cycle, EB or initial BC did not influence luteal function. But during the fourth estrous cycle, NEB heifers had less ($P < .05$) area and peak progesterone than PEB heifers and FAT heifers had less ($P < .05$) area and duration of luteal phase than MOD heifers

TABLE 5. EFFECTS OF ENERGY BALANCE AND BODY CONDITION ON WEIGHTS OF BOVINE CORPORA LUTEA.[a]

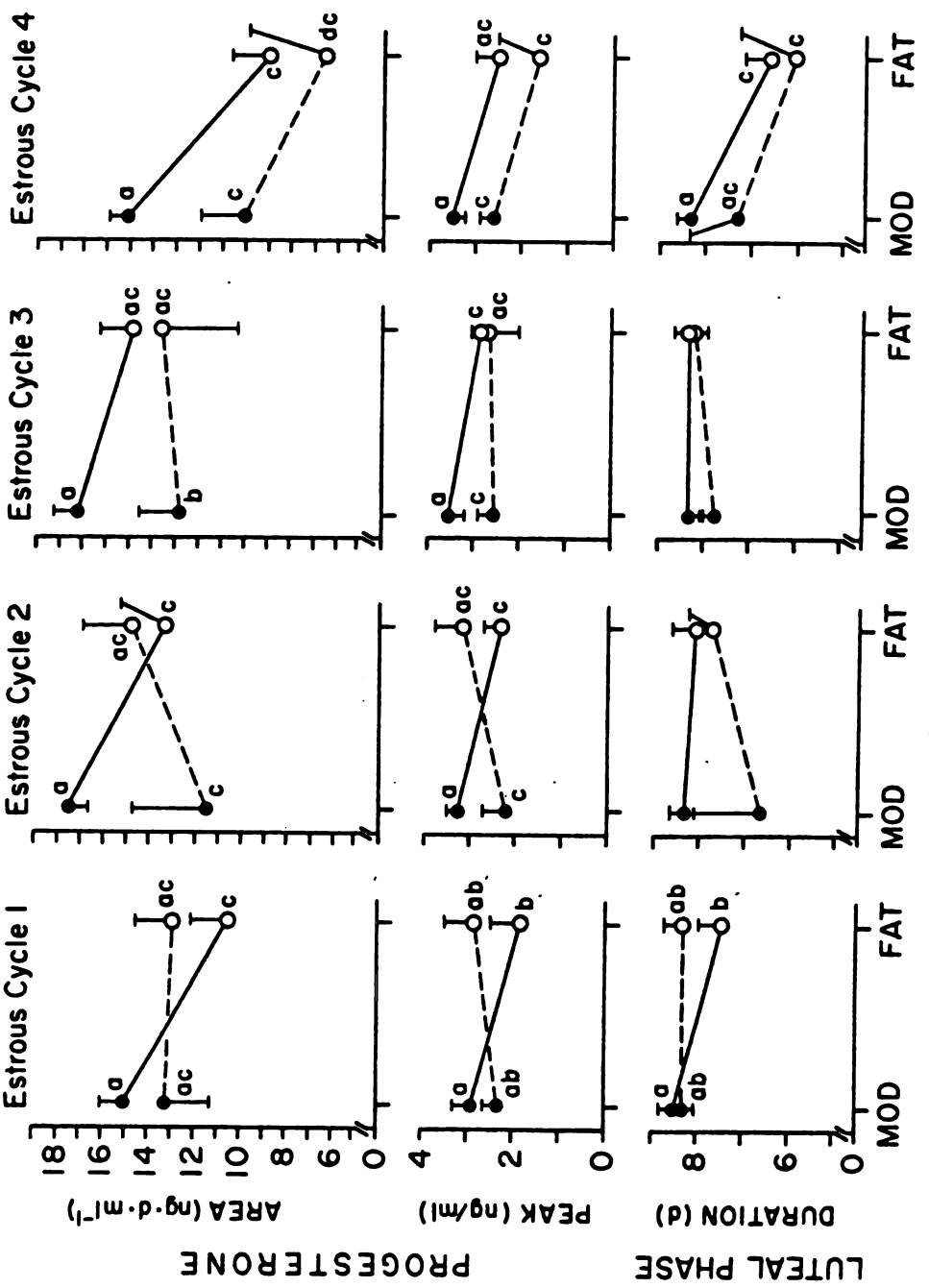
Treatment combination	n	Luteal weight[b], g
MOD-PEB	6	6.2 ± 0.7[c]
FAT-PEB	6	4.6 ± 0.6[d]
MOD-NEB	5	4.5 ± 0.6[d]
FAT-NEB	3	5.1 ± 1.0[c,d]

[a]Corpora lutea were collected on d 10, 11 or 12 postestrus of the fourth estrous cycle.

[b]Data are expressed as mean ± standard error.

[c,d]Means with different superscript differ ($P < .05$).

Figure 5. Effects of energy balance and body condition on progesterone in serum and duration of luteal phase during the first 10 to 12 d postestrus of four estrous cycles. Heifers had moderate (●) or fat (O) body condition and were in positive (—) or negative (- - -) energy balance. Data are mean ± standard error. For area, peak or duration means within an estrous cycle with different superscript differ (^{a,b} $P < .10$; ^{a,c} $P < .05$; ^{a,d} $P < .01$).


Estrous Cycle 1
Estrous Cycle 2
Estrous Cycle 3
Estrous Cycle 4
PROGESTERONE
LUTEAL PHASE
AREA (ng·d·ml⁻¹)
PEAK (ng/ml)
DURATION (d)
MOD
FAT
BODY CONDITION

(figure 5). Therefore, during fourth cycle NEB and FAT independently exerted adverse effects on luteal function and there were no interactions. In contrast, during the first three cycles EB and BC did not exert independent effects on luteal function but several interactions were observed: relative to MOD-PEB heifers, MOD-NEB heifers had less ($P < .05$) area and peak progesterone from second throughout fourth estrous cycle (figure 5). But FAT-NEB heifers had less ($P < .05$) area, peak and duration of luteal phase than MOD-PEB heifers only during the fourth estrous cycle (figure 5). Except for third cycle, FAT-PEB had less ($P < .05$) area and peak progesterone during the study than MOD-PEB heifers. In addition, luteal phases of FAT-PEB heifers were shorter during first ($P < .10$) and fourth ($P < .05$) cycles than for MOD-PEB heifers (figure 5).

Rate of increase of progesterone during the first 10 d postestrus (figure 6) did not differ among estrous cycles in MOD-PEB heifers. During the first two estrous cycles, MOD-NEB heifers had similar rates of increase of progesterone than MOD-PEB heifers. But during third and fourth estrous cycles progesterone of MOD-NEB heifers increased slower ($P < .05$) than in MOD-PEB heifers (figure 6). Relative to values for MOD-PEB heifers, progesterone increased slower ($P < .05$) during all estrous cycles in FAT-PEB heifers (figure 6). Progesterone in FAT-NEB heifers increased at similar rates than in MOD-PEB heifers during the first three estrous cycles. But for the fourth estrous cycle, rate of increase of progesterone of FAT-NEB heifers was slower ($P < .005$) than in MOD-PEB heifers (figure 6).

Luteal Function in vitro. Energy balance or initial BC of heifers did not influence basal secretion of progesterone by luteal cells in vitro (figure 7). But the interaction between MOD and NEB affected adversely the ability of luteal cells to secrete basal progesterone in vitro. Evidence of this was that luteal cells from MOD-NEB heifers secreted less ($P < .01$) basal progesterone

Figure 6. Effects of energy balance and body condition on luteal development. Luteal development was measured by rate of increase of progesterone in serum during the first 10 d of four estrous cycles (for clarity only values for d 4 and 10 postestrus were plotted). Heifers had moderate (●) or fat (O) body condition and were in positive (—) or negative (- - -) energy balance. Coefficients of determination (r^2) for regression equations of estrous cycles 1, 2, 3 and 4, respectively, were: .9, .8, .7 and .8 for MOD-PEB heifers; .4, .6, .7 and .2 for MOD-NEB heifers; .4, .5, .6 and .4 for FAT-PEB heifers; .6, .6, .6 and .1 for FAT-NEB heifers. Within estrous cycle, slopes with different superscript differ (^{a,b} $P < .05$).

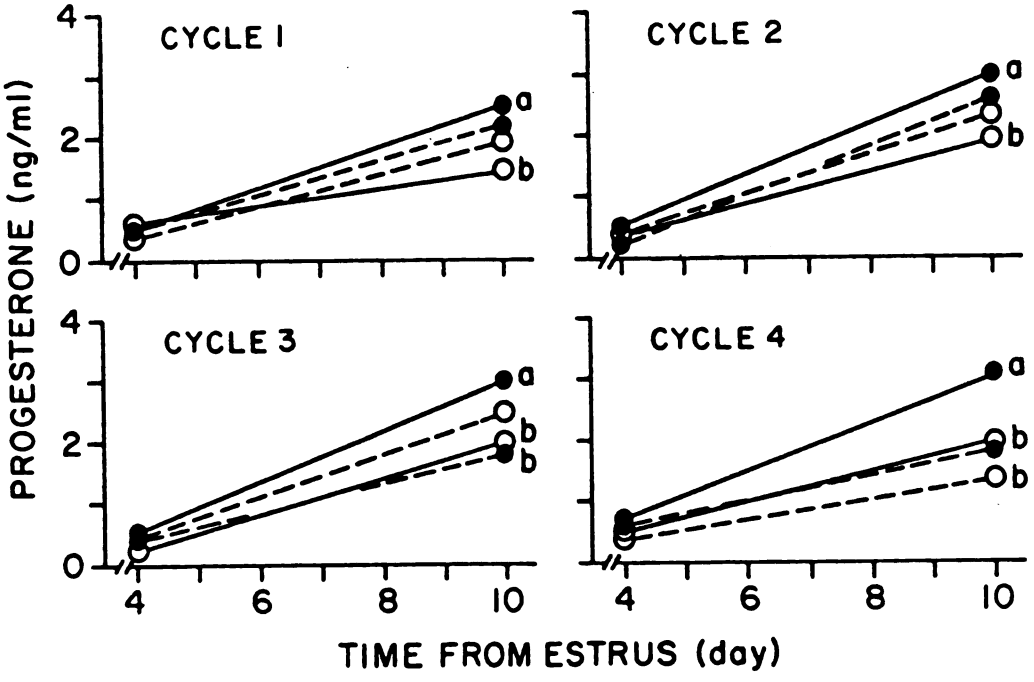
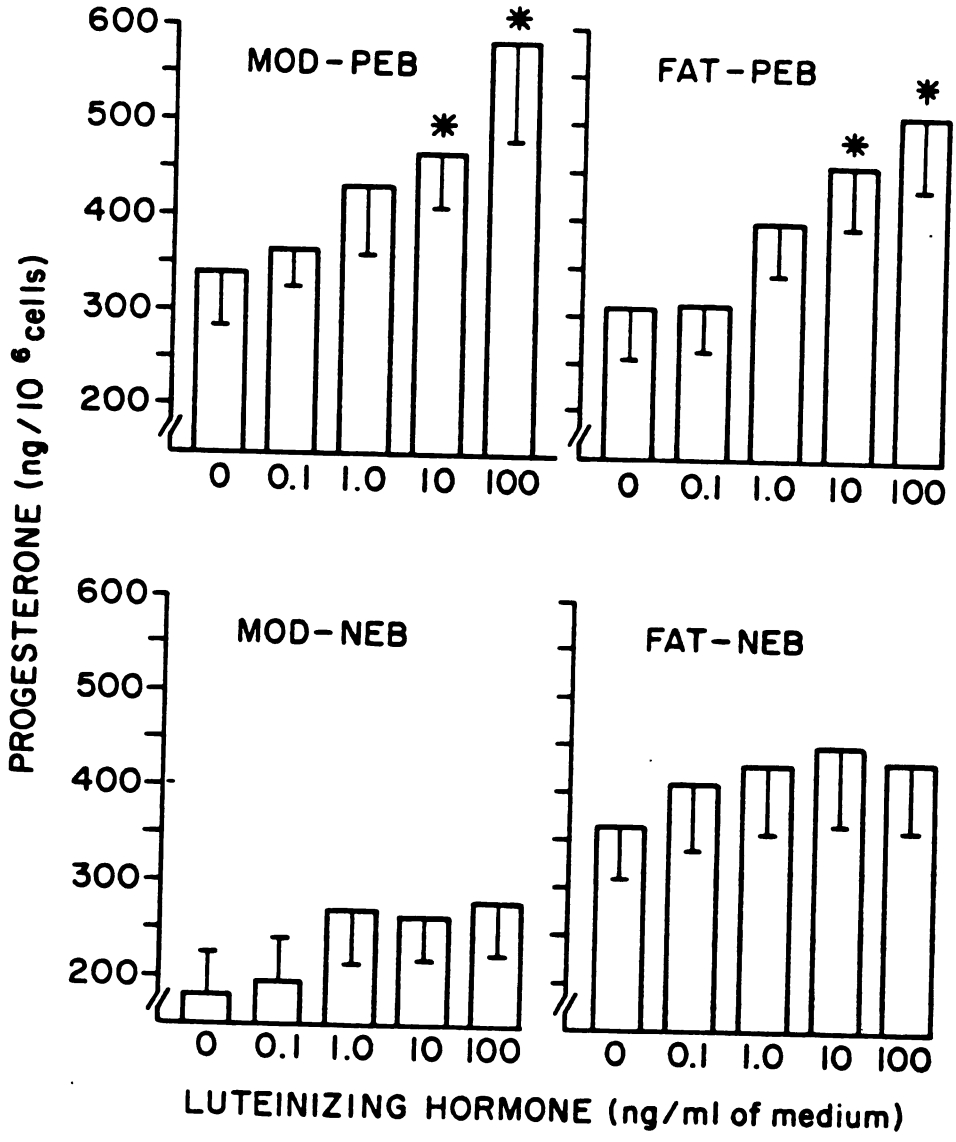

CYCLE 1
CYCLE 2
CYCLE 3
CYCLE 4
a
b
b
PROGESTERONE (ng/ml)
0
2
4
4
6
8
10
TIME FROM ESTRUS (day)

Figure 7. Effects of energy balance and body condition on secretion of progesterone by luteal cells in vitro. Corpora lutea were collected from heifers on d 10, 11 or 12 postestrus of fourth estrous cycle. MOD-PEB, FAT-PEB, MOD-NEB or FAT-NEB. Luteal cells were dissociated and suspended in medium with different concentrations of LH. Data are means ± standard error. Basal secretion of progesterone (0 ng of LH) in MOD-NEB was lower ($P < .01$) than basal secretion of progesterone in other treatment combinations. * indicates difference ($P < .01$) from basal secretion within treatment combination.


MOD-PEB
FAT-PEB
MOD-NEB
FAT-NEB
600
500
400
300
200
0
0.1
1.0
10
100
PROGESTERONE (ng/10 6 cells)
LUTEINIZING HORMONE (ng/ml of medium)

in vitro than luteal cells from MOD-PEB, FAT-PEB or FAT-NEB heifers (figure 7). Ability of luteal cells to respond to LH in vitro was not altered by BC of heifers or interaction of EB and BC (figure 7). But NEB of heifers reduced ($P < .01$) LH-induced secretion of progesterone in vitro (figure 7).

Luteotropic Support. Mean concentrations and pulsatile patterns of LH in serum of heifers at mid-diestrus of fourth estrous cycle were not altered by EB or BC and there were no interactions (table 6).

GH, Insulin and NEFA. Changes in concentrations of GH in serum (figure 8) were not associated with feeding or eating. Mean concentrations of GH in serum were higher ($P < .01$) in NEB heifers (5.3 ± 1.4 ng/ml) than in PEB heifers (2.9 ± .3 ng/ml). Basal concentrations (2.6 ± .14 ng/ml) and frequency of pulses (4 ± .4 pulses/12 h) of GH were not altered by EB. But pulses of GH in NEB heifers had greater ($P < .001$) amplitude (8.1 ± 1.9 ng/ml) and duration (101.8 ± 7.5 min) than pulses of PEB heifers (amplitude = 2.1 ± .4 ng/ml; duration = 56.2 ± 7.1 min). Mean concentrations and secretory patterns of GH in serum (figure 8) of heifers were influenced by EB but were not affected by initial BC and there were no interactions.

Basal and mean concentrations of insulin in serum were not altered by EB, BC or their interactions. But profiles of insulin were affected negatively by NEB (figure 9). Fifteen min after offering feed to heifers, insulin increased ($P < .05$) above basal concentrations (23.8 ± 3.2 μU/ml) in PEB heifers. Postprandial rise of insulin (39.1 ± 4.2 μU/ml) in PEB heifers was maintained for 3 h and then returned to basal levels. In contrast, within 3 h from feeding, concentrations of insulin of NEB heifers (27.2 ± 2.8 μU/ml) did not increase above baseline values (22.8 ± 3.2 μU/ml). Frequency of pulses of insulin during the entire sampling interval (2.7 ± .4 pulses/12 h) or during the first 3 h postprandial (1.4 ± .2 pulses) were not affected by EB or BC. No significant

TABLE 6. EFFECTS OF ENERGY BALANCE AND BODY COMPOSITION ON SECRETORY CHARACTERISTICS OF LUTEINIZING HORMONE IN SERUM OF HEIFERS.[a]

Treatment combinations	n	Concentration (ng/ml)		Pulse	
		Mean	Baseline	Amplitude (ng/ml)	Frequency (pulses/12 h)
MOD-PEB	6	1.0	.8	2.8	2.2
FAT-PEB	6	1.0	.8	3.0	2.4
MOD-NEB	5	1.1	.9	2.5	1.8
FAT-NEB	3	1.4	1.2	3.5	1.7
		(.2)	(.2)	(.3)	(.3)

[a]Blood was sampled every 15 min for 12 h on d 9, 10 or 11 postestrus of fourth estrous cycle. Values in parentheses are pooled standard errors.

Figure 8. Effects of energy balance and body condition on secretory profiles of growth hormone in serum. Heifers were in MOD-PEB, FAT-PEB, MOD-NEB or FAT-NEB. Jugular blood was sampled on d 9, 10 or 11 after the fourth estrus, during a 12 h period at intervals of 15 min. Pooled standard error was .2 ng/ml serum.

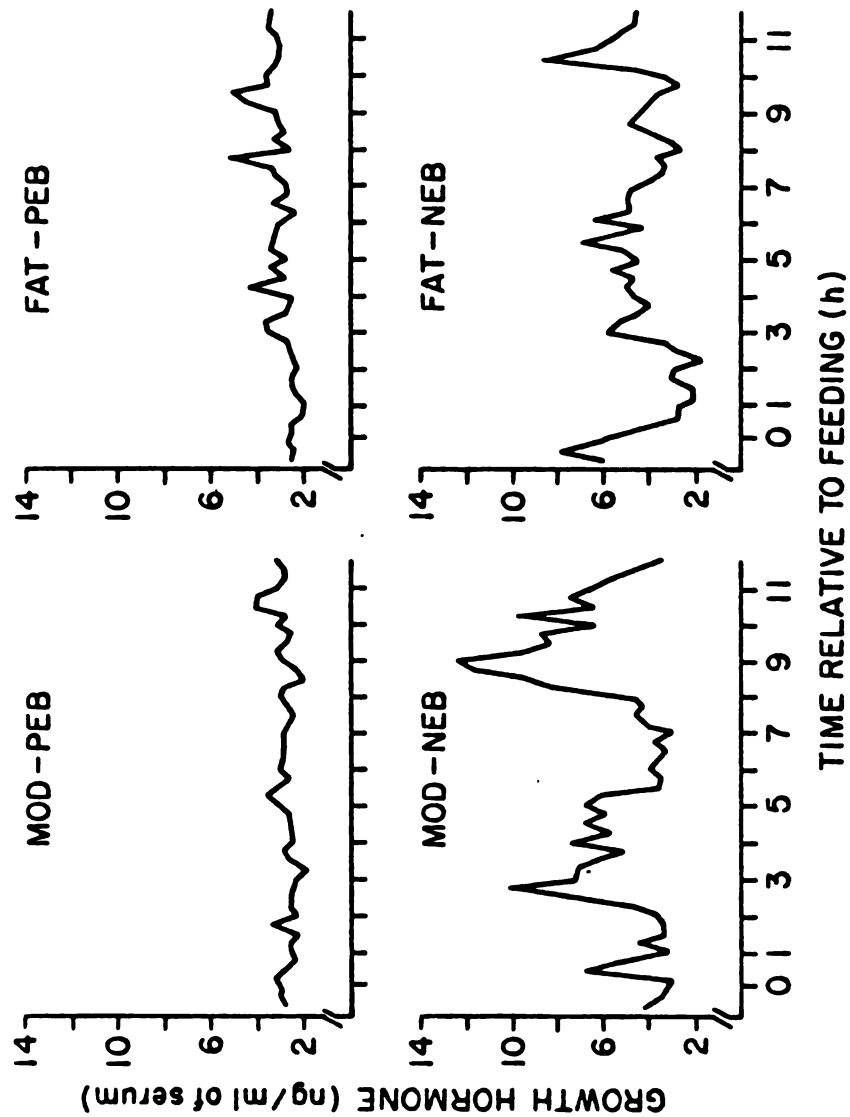
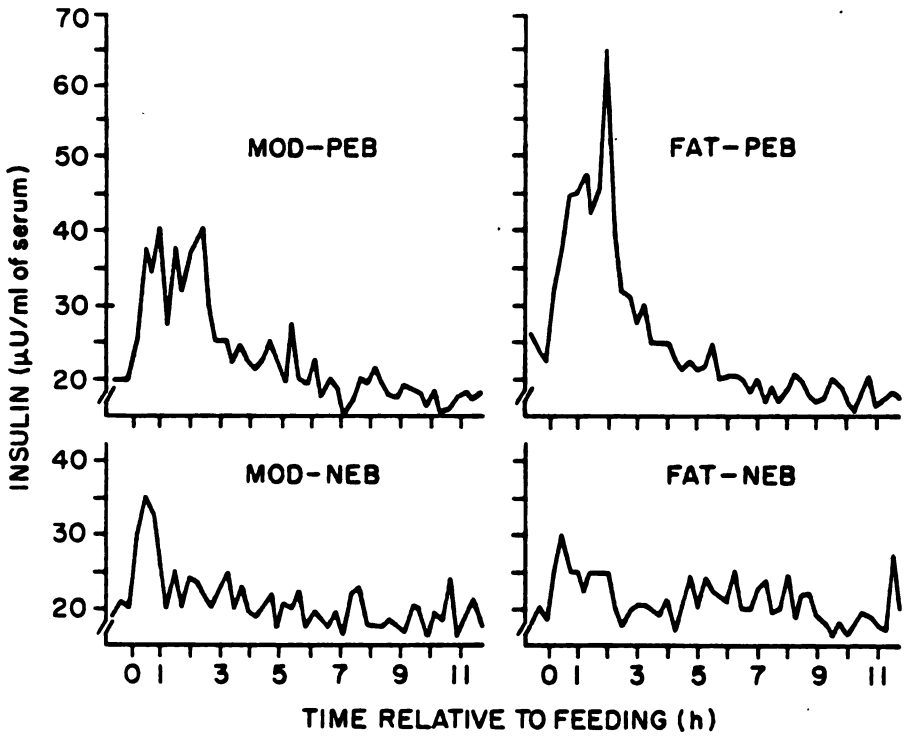

MOD-PEB
FAT-PEB
MOD-NEB
FAT-NEB
14
10
6
2
0 1 3 5 7 9 11
GROWTH HORMONE (ng/ml of serum)
TIME RELATIVE TO FEEDING (h)

Figure 9. Effects of energy balance and body condition on profiles of mean concentrations of insulin in serum. Heifers were in MOD-PEB, FAT-PEB, MOD-NEB or FAT-NEB. Jugular serum was taken on d 9, 10 or 11 after the fourth estrus, during a 12 h period at intervals of 15 min. Pooled standard error was 2.9 μU/ml serum.


MOD-PEB
FAT-PEB
MOD-NEB
FAT-NEB
INSULIN (μU/ml of serum)
70
60
50
40
30
20
40
30
20
0 1 3 5 7 9 11
0 1 3 5 7 9 11
TIME RELATIVE TO FEEDING (h)

interactions between EB and BC were observed for any measurements of insulin in serum. But during the first 3 h postprandial, amplitude (42.9 ± 4.2 μU/ml) and duration (137.5 ± 39.8 min) of pulses of insulin in FAT-PEB heifers tended ($P = .08$) to be greater than in MOD-PEB heifers (amplitude = 30.6 ± 6.4 μU/ml; duration = 76.3 ± 20.1 min). As single effect, EB but not BC altered amplitude and duration of pulses of insulin during the first 3 h postprandial. During this interval, amplitude (36.7 ± 6.4 μU/ml) and duration (116.7 ± 3.1 min) of pulses of insulin in PEB heifers were greater ($P < .01$) than for NEB heifers (amplitude = 15.9 ± 4.2 μU/m; duration = 72.3 ± 15 min).

Body condition did not influence NEFA in plasma and there were no interactions of EB and BC. But heifers in NEB had higher ($P < .01$) mean concentrations of NEFA (404 ± 120 μEq/l) in plasma than PEB heifers (84 ± 36.4 μEq/l)

Insulin and GH were not correlated significantly with concentrations of progesterone in serum at lutectomy. In contrast, concentrations of NEFA in plasma were correlated negatively ($r = .52$; $P < .01$) with concentrations of progesterone in serum at lutectomy.

Discussion

Heifers in PEB maintained BC and sustained daily gains with minimal variation throughout the experimental phase. Independent of initial BC, PEB heifers received the same diet and similar amounts of feed per unit of body weight. Therefore, I was able to determine the independent influence of BC, MOD or FAT, on luteal function without bias by diet or EB. Within each category of initial BC, I produced heifers in sustained PEB during the entire study or in NEB for the last two estrous cycles. Thus it was possible to determine independent effects of EB and associative effects of EB with BC on luteal function.

In the present study, diets were formulated based on requirements for growth and maintenance from NRC (1984). Accordingly, diets given to NEB heifers were deficient in energy from the beginning of the experimental phase. But, during the first estrous cycle, heifers committed to NEB gained weight but when amounts of feed offered were reduced further, heifers lost weight. Retrospective chemical analysis of feeds indicated that TMR used contained more NEm than averages estimated from NRC (1984). Thus, calculated restrictions of dietary energy may not cause NEB in all cases due to variation of nutrients in feeds and(or) in requirements for maintenance of heifers. These observations may explain variable effects of restricted intake of dietary energy on luteal function in previous studies in which NEB of heifers was presumed (Apgar et al., 1975; Beal et al., 1978; Gombe and Hansel, 1973; Hill et al., 1970; Spitzer et al., 1978). During NEB, losses of body weight paralleled decreases in EB of heifers ($r = .72$; $P < .01$). Therefore, if heifers are losing weight, dietary energy is insufficient to satisfy requirements for maintenance. Thus, a discrete marker for positive or negative EB is gain or loss of weight.

Declines of BC paralleled decreases of EB of heifers ($r = .54$; $P < .05$) during NEB. Because BC is a measurement of subcutaneous fat, decreased BC during NEB indicates that concomitant losses of body weight are due, at least in part, to decreased fat. In fact, as cattle lose weight, up to 70% of tissue lost is fat (Reid and Robb, 1971).

During the fourth estrous cycle, NEB heifers had less progesterone in serum than PEB heifers and FAT heifers had less progesterone than MOD heifers. Therefore, NEB and FAT have independent adverse effects on luteal function. Because rate of increase of progesterone was reduced in MOD and FAT heifers, it is likely that NEB and FAT as independent effects inhibit development of corpora lutea. Moreover, luteal phases were shortened in FAT heifers. Reduced

duration of luteal phases during fourth estrous cycle was due to delayed onset of diestrus indicating that ovulation could be delayed in FAT heifers. Our results are consistent with earlier reports that spontaneous NEB reduces progesterone in serum of lactating dairy cows without altering duration of luteal phases (Experiment I). Whether induced in heifers or spontaneous in lactating cows, NEB exerts adverse effects on luteal function.

Reduced luteal weight at mid-diestrus from MOD-NEB and FAT-PEB heifers is consistent with adverse effects of NEB or FAT on luteal development. Perhaps reduced mass of luteal tissue diminishes total amount of progesterone secreted from corpora lutea and in part explains reduced concentrations of progesterone in serum of NEB and FAT heifers.

From previous reports, effects of restricting dietary energy on luteal function were equivocal in heifers. Some researchers observed negative association between restricted dietary energy and progesterone in serum (Gombe and Hansel, 1973; Hill et al., 1970; Imakawa et al., 1983) but other workers did not (Beal et al., 1978; Spitzer et al., 1978). I have shown that restricting dietary energy does not guarantee NEB in heifers. Perhaps in studies where luteal function was normal during restricted dietary energy, heifers were not in NEB. But, in studies where luteal function was reduced, dietary energy was restricted sufficiently that heifers were in NEB. Thus, luteal function will probably be normal unless diet is restricted sufficiently to cause NEB in heifers. Duration of NEB and net energy deficit in FAT-NEB heifers were greater than in MOD-NEB heifers. During fourth cycle, it is interesting that FAT-NEB heifers had lowest numeric increase, smallest area and peak of progesterone and the shortest luteal phase of all treatment combinations. Consequently, secretion of progesterone is associated negatively with severity and(or) duration of energy deficit in heifers.

In NEB heifers, loss of body weight paralleled declines in EB. Large losses of body weight should indicate great deficits of energy and should be associated with more severely limited luteal function. However, I detected adverse effects of NEB on luteal function (area, peak and rate of increase of progesterone) as early as the second estrous cycle in MOD-NEB heifers, but not until the fourth estrous cycle in FAT-NEB heifers. Therefore, temporal associations between NEB and reduced luteal function depends apparently on BC of heifers when NEB starts.

Interactions between levels of EB and FAT indicate other potential sources of variation for progesterone in serum of heifers. For example, compared to MOD-PEB heifers, FAT-PEB heifers had slower increases and lower peaks of progesterone during three estrous cycles, supporting the concept that FAT reduces luteal function independently. But in FAT-NEB heifers, adverse effects of FAT on luteal function during first to third estrous cycles did not exist or were masked by NEB. Perhaps the key to understand interactions between FAT and NEB is variation of BC throughout the study. Body condition and subnormal luteal function of FAT-PEB heifers were sustained during the experimental phase. But reduced intake of dietary energy caused BC of FAT-NEB heifers to decline to MOD. Because BC and consequently amount of fat in depots were reduced, adverse effects of excess fat on luteal function were removed. But NEB continued, so by fourth estrous cycle BC of FAT-NEB heifers decreased below MOD, with a concomitant reduction of all variables used to examine luteal function *in vivo*. Thus effects of NEB on luteal function of FAT-NEB heifers were delayed until BC declined below MOD. An interpretation of these data is that effects of EB on luteal function are exerted indirectly by altering BC of cattle.

There were no independent or associative effects of EB or BC on mean concentrations and pulsatile patterns of LH. Thus, adverse effects of NEB and FAT on luteal function are not due to limited luteotropic support. These results agree with previous reports that LH in serum of heifers was not reduced by restricted dietary energy (Hill et al., 1970; Spitzer et al., 1978) or FAT (Spicer et al., 1984). But our results were determined at mid-diestrus and do not examine the possibility that periestrus secretion of LH is altered by NEB and(or) FAT as with restricted intakes of energy (Gombe and Hansel, 1973) or fasting in heifers (McCann and Hansel, 1986).

As single effects EB or BC of heifers did not influence basal secretion of progesterone by luteal cells in vitro. Body condition of heifers did not alter LH-induced secretion of progesterone from luteal cells in vitro. But NEB in heifers reduced secretion of progesterone in response to LH in vitro. How might NEB reduce LH-induced secretion of progesterone in vitro? Corpora lutea are composed by small and large luteal cells (Alila and Hansel, 1984). Large luteal cells secrete most basal progesterone. Numbers of receptors for LH and LH-induced secretion of progesterone in vitro are greater in small than in large luteal cells (Fitz et al., 1982). Because NEB did not alter basal secretion of progesterone from luteal cells in vitro, it is not likely that NEB affects steroidogenic activity of large luteal cells. But potential mechanisms by which NEB reduced LH-induced secretion of progesterone in vitro are: a) reduced numbers of small luteal cells, b) reduced numbers and(or) affinity of receptors for LH on small and possibly large luteal cells, c) altered postreceptor event(s) involved in LH-induced steroidogenesis or d) a combination of some or all the above mechanisms.

Basal progesterone secreted in vitro by luteal cells from MOD-NEB heifers was less than in other treatment combinations. Thus, BC of heifers at the

beginning of dietary restrictions modulates effects of NEB on basal secretion of progesterone from luteal cells in vitro. Consequently, NEB limits LH-induced secretion of progesterone and NEB interacts with MOD to reduce basal steroidogenic activity in vitro.

In the present study, NEB decreased progesterone in serum, reduced luteal weight and development, and reduced LH-induced secretion of progesterone in vitro. Similarly, FAT decreased progesterone in serum reduced luteal weight and slowed luteal development but did not alter basal or LH-induced secretion of progesterone in vitro. Thus, NEB and FAT affected luteal function in vivo but only NEB affected luteal function in vitro. Because NEB but not FAT reduced luteal function in vitro, adverse effects of NEB and FAT on luteal function are apparently exerted through different mechanisms. In FAT heifers, low concentrations of progesterone in serum were associated with reduced luteal development with no apparent reduction in secretory activity of luteal cells. But in NEB heifers, low concentrations of progesterone in serum were associated with reduced luteal development and with reduced secretory activity of luteal cells.

Pulsatile secretory activity of GH in serum from PEB heifers was unrelated with time of feeding and followed similar patterns than those reported previously (Sejrsen et al., 1981; Zinn et al., 1986). In the present study, FAT did not alter mean concentrations or pulsatile secretory patterns of GH in heifers. These results support previous observations that body composition does not influence GH in post-pubertal heifers (Sejrsen et al., 1983; Zinn et al., 1986). But NEB increased concentrations of GH in heifers as in steers (Blum et al., 1985). Secretion of insulin in meal-fed cattle increased shortly after feeding (McAtee and Trenkle, 1971). In the present study, insulin increased to maximal concentrations within 3 h postprandial in PEB heifers. In contrast, relative

to basal levels, postprandial insulin did not change in NEB heifers. Thus NEB blocks postprandial increase of insulin in heifers. Response of insulin to feeding is modulated by energy status of cattle and by amount of energy in diet (Brockman and Laarveld, 1986; Lomax et al., 1979). McCann and Reimers (1986) observed that glucose-induced secretion and basal concentrations of insulin were greater in fat than in moderately conditioned heifers. In the present study, postprandrial concentrations of insulin tended (P = .08) to be greater in FAT than in MOD heifers. Heifers studied by McCann and Reimers (1986) were older (2.5 to 4 yr of age) than heifers in our experiment (< 2 yr of age). In obese individuals hyperinsulinemia progresses with advancing age and increasing obesity (McCann and Reimers, 1986). Thus FAT heifers used in the present study could be in early stages of hyperinsulinemia.

Concentrations of NEFA in plasma of NEB heifers were greater than in PEB heifers. This is in agreement with earlier research in steers (Blum et al., 1985). Body condition did not alter concentrations of NEFA in plasma of heifers and there were no interactions of BC with EB. To my knowledge this is the first report of effects of BC on NEFA in plasma of cattle.

Consistent with adjustments expected during energy deficit, NEB heifers had increased concentrations of GH and NEFA but reduced insulin in blood. It was of interest to examine, by correlation, the possibility that GH, insulin and (or) NEFA mediate adverse effect of NEB on luteal function. Lack of significant correlations between progesterone in serum and insulin or GH could be interpreted that changes in GH and insulin are not involved in reduced luteal function during NEB. But insulin exerts positive effects on luteal function in vivo (McCann, 1984) and in vitro (Savion et al., 1982), and GH antagonizes actions of insulin in some tissues (Rizza et al., 1982). Thus, it would seem fruitful to test directly the hypotheses that decreased secretion of insulin or antagonism of insulin by GH mediate adverse effects of NEB on luteal function.

Increased concentrations of NEFA in plasma of NEB heifers correlated negatively with progesterone in serum. This agrees with data indicating a negative association between NEFA in plasma and reproductive performance in dairy cows (Ducker et al., 1985). Negative associations between NEFA and progesterone may be causative or coincidental.

Overall I determined that NEB and FAT independently reduced luteal function of heifers in vivo. Because LH in serum was not reduced by EB or BC, adverse effects of NEB and FAT on luteal function are not due to reduced luteotropic support. Negative EB and FAT slowed rate of increase of progesterone in serum and this was interpreted as reduced luteal development. Adverse effects of NEB were first detected during second cycle in MOD heifers but not until fourth cycle in FAT heifers. Basal secretion of progesterone in vitro was not influenced by EB or BC. But basal progesterone in vitro was reduced in MOD-NEB heifers. Thus initial BC of heifers determined time from initial dietary restriction when adverse effects of NEB on luteal function occurred in vivo and determined effects of NEB on basal secretion of progesterone in vitro. FAT had no effect but NEB reduced LH-induced secretion of progesterone in vitro. In FAT heifers, low progesterone in serum was associated only with reduced luteal development with no apparent effect on activity of luteal cells. But in NEB heifers, low progesterone in serum was associated with reduced luteal development and reduced secretory activity of luteal cells. Because NEB but not FAT reduced luteal function in vitro, effects of NEB and FAT on luteal function are apparently exerted through different mechanisms.

My primary conclusion is that NEB and FAT exert independent limitations to luteal function. Apparently, adverse effects of NEB and FAT on luteal function are exerted through different mechanisms. In addition, BC of heifers determines temporal manifestation of adverse effects of NEB on luteal function.

EXPERIMENT III:

Influence of Energy Balance and Body Condition on Behavior of Heifers During Estrus

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Introduction

Estrus is detected in association with 50 to 64% of ovulations when dairy cows are observed two (King et al., 1976; Williamson et al., 1972) or three times per d (Experiment I). Under constant surveillance, estrus is associated with 94 to 100% of second and third postpartum ovulations (Hurnik et al., 1975). Because farmers commonly observe cows twice daily, a high proportion of estrus is undetected in dairy herds. Together, undetected and inaccurately detected estrus lower fertility (Macmillan and Watson, 1971; Pelissier, 1978), prolong postpartum intervals to conception and represent a major source of economic loss to dairy farmers (Barr, 1974; Esslemont, 1974; Williamson et al., 1972). Many methods to improve detection of estrus have been developed (Foote, 1975; Kiddy, 1977; Holman et al., 1987). But failure to detect estrus still limits reproductive performance in dairy cows. Some workers proposed that inefficiency of farmers is the leading cause of undetected estrus (Pelissier, 1976; Zemjanis et al., 1969). But a large range in duration of estrus among dairy cattle, 3 to 21 h (Glencross et al., 1981; Hurnick et al., 1975), indicates that there are biological sources of variation for expression and detection of estrus.

At least 80% of dairy cows are in negative energy balance during early lactation (Reid et al., 1966; Experiment I). Homeorhetic mechanisms insure metabolic support for lactation during negative energy balance (Bauman and Currie, 1980). My hypothesis is that homeorhetic events of postpartum dairy cows may limit expression and detection of estrus.

Cows that are overconditioned at parturition ingest less feed and energy during early lactation than moderately conditioned cows (Garnsworthy et al., 1982). Intake of dietary energy is a major source of variation of energy balance in postpartum dairy cows fed ad libitum (Experiment I). Thus overconditioning may enhance negative energy balance and indirectly alter expression of estrus in lactating cows.

In lactating dairy cows, display of behavioral estrus in association with ovulation varies among postpartum estrous cycles (Experiment I). Effects of successive estrous cycles may interfere with detection of other sources of variation of expression of estrus. In heifers these interfering factors do not exist. Thus, this experiment was designed to determine independent and associative effects of energy balance and body condition on estrous behavior of heifers. Collaterally, I examined the influence of energy balance, body condition and their interactions on duration of estrous cycles.

Materials and Methods

Twenty nulliparous, Holstein heifers were grouped by body weight and assigned to treatments within a 2 x 2 factorial experiment. Main effects were: 1) body condition (BC): moderate or fat, and 2) energy balance (EB): positive or negative. Materials, and general procedures regarding housing, nutrition and measuremetns of EB, BC and body weight were as described previously (Experiment II). Briefly, heifers were housed in a free stall barn equipped for individual feeding. Before the study heifers were fed so BC (1 to 4; 4 = fat) remained moderate (MOD = 11) or became fat (FAT; n = 9). After approximately 6 mo of these regimens, BC of MOD and FAT heifers differed ($P < .05$). Subsequently, during 20 d of adaptation, FAT and MOD heifers were trained to use gang-lock stanchions and to consume their daily dietary allowance within

2 intervals of 90 min (0500 to 0630 and 1700 to 1830 h). This allowed me to determine intake of DM and EB daily. But during 21 h per d, heifers were not confined and were free to exhibit all signs of estrus. Pubertal status of heifers was determined by two daily observations for estrus (0700 to 0730 and 1900 to 1930 h), and by rectal examination of reproductive organs at least twice during the adaptation period. Thus at the end of the adaptation period, heifers with two distinct BC (MOD or FAT) and that were postpubertal were available.

The experiment lasted 3.5 estrous cycles and ended between d 10 and 12 after the last estrus. Throughout the experiment, 6 MOD heifers and 6 FAT heifers were fed (Experiment II; table 4) to gain weight and to be in positive EB (PEB). The remaining MOD (n = 5) and FAT (n = 3) heifers received a diet (Experiment II; table 4) to satisfy requirements for protein, vitamins and minerals but was limited in energy to produce and sustain negative EB (NEB). Thus, from the beginning of the experiment four treatment combinations were established: MOD-PEB (n = 6), FAT-PEB (n = 6), MOD-NEB (n = 5) and FAT-NEB (n = 3).

To estimate EB, feed intake was measured daily and body weight weekly throughout the experiment. Daily body weight was calculated by extrapolation of weekly changes in body weight (weekly change/7). Throughout the experiment, EB was estimated daily for each heifer. Energy balance was calculated by subtracting requirements of energy for maintenance from intake of energy. Requirements of net energy for maintenance (NEm) were based on daily body weight and calculated as follows: NEm (Mcal) = (.077) body weight$^{.75}$ (NRC, 1984). Daily intake of energy resulted from multiplying NEm in feeds by intake of DM. Body condition was scored in alternate weeks using a scale of 1 to 4 (4 = fat) according to methods developed by Mulvany (1981) and modified to determine BC in heifers (Experiment II).

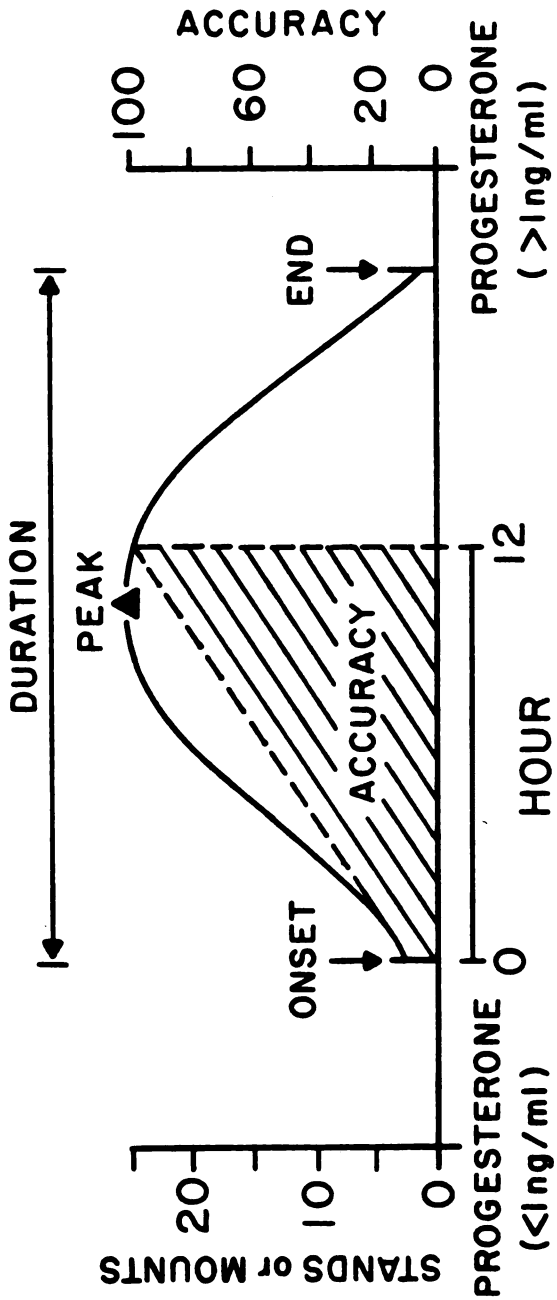
Progesterone in Serum. Jugular blood was sampled daily by venipuncture. Progesterone was quantified in serum from all these samples by radioimmunoassay (Appendix A). Concentrations of progesterone in blood were used to define diestrus (see Duration of Estrous Cycles) and to define estrus (see Estrous Behavior).

Estrous Behavior. Heifers were housed in two separate but adjacent pens so heifers in the two pens were observed simultaneously. One pen contained MOD-PEB and FAT-PEB heifers and the other pen held MOD-NEB and FAT-NEB heifers. For 3 consecutive estrous cycles (3 periods of estrus), heifers were observed daily for periods of 30 min at intervals of 3 h (beginning at 0100 h). Events recorded during periods of observation included standing to be mounted ($\geq$ 2 sec), mounting and mounted but not standing. A heifer was considered to be in estrus when standing behavior coincided with concentrations of progesterone < 1 ng/ml of serum.

Duration and frequency of mounting is correlated positively with number of animals up to 5 in estrus simultaneously (Helmer and Britt, 1985; Hurnick et al., 1975). Number of animals in estrus simultaneously could bias analysis of effects of EB and(or) BC on estrous behavior. Therefore, during each period of observation, number of heifers standing and(or) mounting during each period of observation was recorded and used as a covariate in statistical analyses of behavioral data related with estrous behavior.

Duration of Estrous Cycles. I examined the influence of EB, BC and their interactions on duration of complete estrous cycles and selected stages of estrous cycles. An estrous cycle was the interval between onset of two consecutive periods of estrus. Diestrus was the interval within an estrous cycle when progesterone in serum exceeded basal concentrations of progesterone by two standard deviations (.125 + .09 (2) = .3 ng/ml). Basal progesterone was the mean

Figure 10. Characterization of total activity, intensity, duration and accuracy of estrus in heifers. Onset of estrus was when first standing event was observed during basal progresterone. Duration was the interval from first to last (END) standing or mounting event during basal progesterone per estrus. Area under frequency distribution of standing or mounting was used to measure total activity. Intensity (PEAK) was maximal number of events (standing or mounting) within area. Accuracy was the percentage of standing or mounting events within 12 h from onset of estrus. All behavioral data were collected when progesterone in serum was basal (< 1 ng/ml).


ACCURACY
100
60
20
0
PROGESTERONE
(>1ng/ml)
END
DURATION
PEAK
ACCURACY
12
HOUR
ONSET
0
PROGESTERONE
(<1ng/ml)
STANDS or MOUNTS
20
10
0

concentration of progesterone (.125 ng/ml) during estrus ± 1 d. Estrus and metestrus were pooled and defined as the interval from onset of estrus to onset of diestrus. Onset of estrus was the first standing event within the low progesterone phase per estrous cycle (figure 10). Onset of diestrus was the first sample of serum within an estrous cycle with concentrations of progesterone exceeding basal progesterone by two standard deviations.

Ambient Temperature. Ambient temperatures above 28°C reduced duration of estrus (DeKruif, 1978) and prolonged estrous cycles in cattle (Ganwar, et al., 1965). Therefore before each period of observation ambient temperature was recorded and used as a covariate in analyses of behavioral data.

Statistical Analyses. Standing (98.2%) and mounting (91.7%) events occurred largely during intervals when concentrations of progesterone were basal. Mounted but not-standing (64%) occurred less frequently during low concentrations of progesterone and was less associated with estrus than standing or mounting. Therefore, only standing and mounting behavior during basal progesterone were analyzed and will be discussed (figure 10).

Frequency distributions of standing and mounting were constructed and area under these curves was calculated and used as a measure of total behavioral activity (figure 10). Key components of area: peak and duration were also examined (figure 10). Peak was the maximal number of standing or mounting events during a period of observation within an estrus and was used as an indicator of intensity of behavior (figure 10). Duration was the interval between first and last standing or mounting events during basal progesterone (figure 10). Intensity and duration were measures of detectability of estrus. Variation of intervals between onet of estrus and ovulation is low in cattle (Bernard et al., 1983). Thus, relative to ovulation, artificial insemination will be more accurately timed in cattle detected in estrus shortly after onset of estrus than cattle

detected later. To determine effects of EB and(or) BC on variation of estrous activity relative to onset of estrus, we used the percentage of standing and mounting events occurring within 12 h from onset of estrus as a measure of accuracy (figure 10). Twelve h from onset of estrus was selected because most farmers apparently observe cattle for estrus twice daily at intervals of 12 h.

Data related with duration of estrous cycles, interval from onset of estrus to onset of diestrus, duration of diestrus and total, peak or duration of standing or mounting activity, were examined by a 2-factor analysis of variance with periods of estrus as subplot (Gill, 1978). Means of all above variables within main effect (EB and BC) among periods of estrus were contrasted with Bonferroni t statistics (Gill, 1978). For means of all the above variables among treatment combinations within period of estrus, contrasts were made with Dunnett's test (Gill, 1978). For these contrasts MOD-PEB heifers were considered as controls. Contrasts of means within treatment combination among periods of estrus were made with Bonferroni t statistics. Accuracy of standing or mounting behavior (percent of events within 12 h from onset of estrus) was examined within main effect and among treatment combinations per estrus, or within treatment combination among periods of estrus by chi-square contingency tables (Gill, 1978). For statistical models with split-plot structure, error terms and critical values for Dunnett's and Bonferroni t test were modified as recommended by Gill (1986).

Results and Discussion

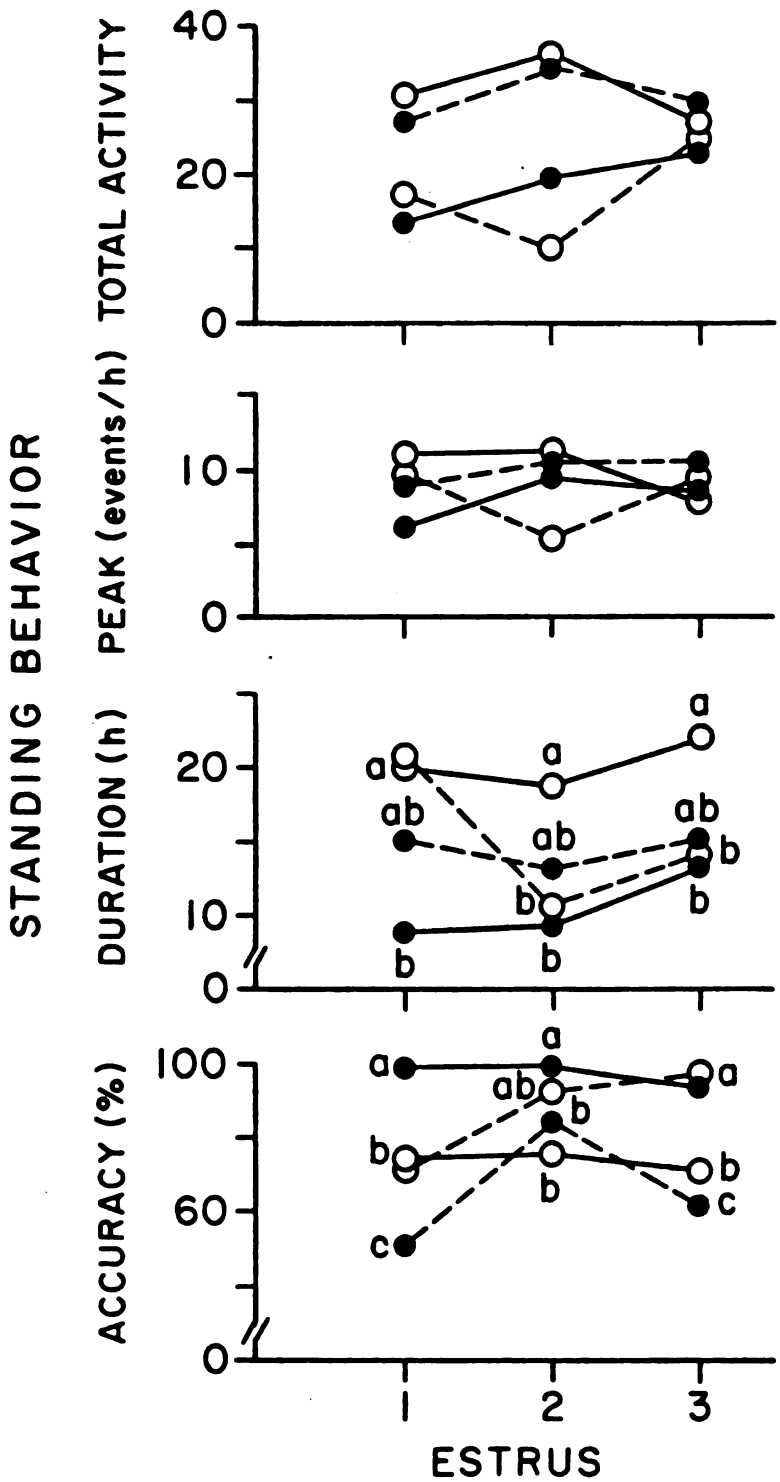
All heifers that received diet A (Experiment I; table 4) were in PEB throughout the experiment. In contrast, heifers that received diet C were in PEB during the first estrus but were in NEB during second and third estrus (Experiment II; figure 4). Independent of initial BC, PEB heifers gained weight

during the first estrus but lost weight during second and third estrus (Experiment II; figure 4). Initial BC (MOD or FAT) was sustained in PEB heifers throughout the study. But BC of NEB heifers declined ($P < .05$) and was below MOD from first to third estrus in MOD-NEB heifers or at third estrus in FAT-NEB heifers (Experiment II; figure 4). These results indicate that PEB heifers maintained BC and sustained body weight within narrow range during the study. Independent of BC, PEB heifers received same diet and similar amounts of feed per unit of body weight. Consequently we determined the influence of sustained MOD or FAT on estrous behavior without bias by diet or EB. Within level of BC (MOD or FAT) we produced heifers in sustained PEB during the study or in NEB for the last two periods of estrus. Therefore, it was possible to determine independent effects of EB and associative effects of EB with BC on behavior during estrus.

Total activity of standing and intensity of standing behavior were not influenced by EB or BC and there were not interactions (figure 11). As single effect EB did not alter duration of standing activity. Duration of standing behavior for MOD-PEB, MOD-NEB and FAT-PEB heifers did not change over time (figure 11). But relative to first cycle, duration of standing behavior in FAT-NEB heifers declined ($P < .01$) during second and third cycles (figure 11). This seems to indicate an adverse effect of interaction between FAT and NEB on duration of standing behavior. But duration of standing behavior in FAT-NEB heifers was longer ($P < .01$) than for MOD-PEB (controls) during first estrus and not different during second and third estrus. This and the fact that FAT-PEB exhibited standing behavior for longer ($P < .01$) intervals than MOD-PEB heifers actually indicates a positive effect of FAT on duration of standing activity.

Mounting behavior (total activity, peak, duration and accuracy) did not vary among periods of estrus. Therefore mounting activity was averaged among

Figure 11. Effects of energy balance and body condition on standing behavior of heifers during three periods of estrus. Heifers had moderate (●) or fat (O) body condition and were in positive (——) and negative energy balance (- - -). Estrus was when standing behavior of heifers coincided with progesterone < 1 ng/ml of serum. Total activity was the area (event·h·h^{-1}) calculated from curves describing frequency distribution of standing events per estrus. Peak was the maximal frequency of standing events within estrus. Duration was the interval between first and last standing events within estrus. Accuracy was the percentage of standing events within 12 h of onset of estrus. ^{a,b,c}For each variable (area, peak, duration or accuracy) values within estrus with different superscript differ ($P < .05$). Pooled standard errors are: 4.7 for total activity, 1.5 for peak and 1.9 for duration.

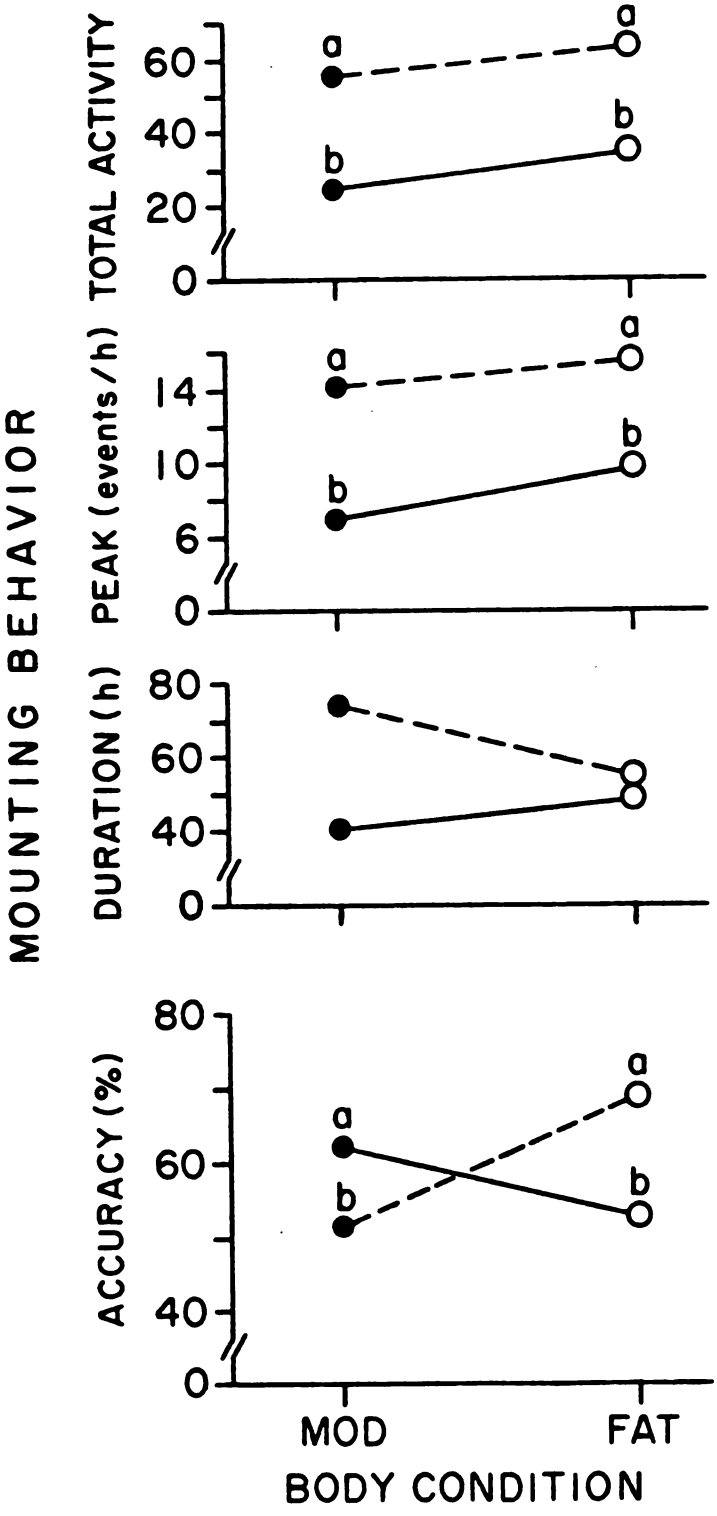

STANDING BEHAVIOR
TOTAL ACTIVITY
PEAK (events/h)
DURATION (h)
ACCURACY (%)
40
20
0
10
0
20
10
0
100
60
0
a
a
a
ab
ab
ab
b
b
b
b
b
b
a
a
a
ab
b
b
b
b
c
c
1
2
3
ESTRUS

all periods of estrus. These averages were then contrasted to determine influence of main effects and treatment combinations. Body condition did not influence but NEB increased ($P < .01$) total activity and intensity of mounting behavior (figure 12).

A salient result of this experiment is that intensity and duration of standing or mounting events are not reduced by NEB, FAT or interactions between EB and BC. Thus changes in EB and BC are not important sources of variation in expression of estrus by heifers. Because intensity and duration of estrous behavior were used as measures of detectability of estrus, it is likely that NEB and FAT do not reduce success of detecting estrus in heifers. To my knowledge, influence of FAT on expression of estrus has not been addressed previously in cattle. But detection of estrus in association with ovulation in lactating dairy cows observed three times per d was not affected by EB (Experiment I). Therefore it is possible that as in heifers, NEB does not reduce expression of estrus in postpartum dairy cows. Yield of milk and intake of feed are major determinants of EB (Experiment I). Since EB does not affect estrus, voluntary intake of feed and yield of milk may not limit success of detecting estrus in cows.

Independent of BC, accuracy of standing events for PEB heifers did not change over time (figure 11). But accuracy of standing events in FAT-PEB heifers was lower ($P < .01$) throughout the study than in MOD-PEB heifers (figure 11). Accuracy of standing events in FAT-NEB heifers was lower ($P < .01$) during first cycle but as BC declined during second and third cycle ($r = .58$; $P < .05$) accuracy of FAT-NEB increased to accuracy observed in MOD-PEB heifers (figure 11). An inference from these data is that sustained FAT (FAT-PEB heifers) reduces accuracy of standing activity. But, when BC is reduced from FAT to $\leq$ MOD as in FAT-NEB heifers, accuracy of standing behavior increases.

Figure 12. Effects of energy balance and body condition on mounting behavior of heifers. Heifers with moderate (●) or fat (O) body condition were in positive (——) or negative (- - -) energy balance. Values for total activity, peak and duration are means and for accuracy are percentage for three periods of estrus. Estrus was when standing behavior of heifers coincided with progesterone < 1 ng/ml of serum. Total activity was the area (event·h·h^{-1}) calculated from curves describing frequency distribution of mounting events per estrus. Peak was the maximal frequency of mounting events within basal progesterone per estrus. Duration was the interval between first and last mounting events within basal progesterone per estrus. Accuracy was the percentage of mounting events within 12 h from onset of estrus. ^{a,b}For each variable, values with different superscript differ ($P < .01$). Pooled standard errors are: for area 6.0; for peak 1.3 and for duration 8.6.


MOUNTING BEHAVIOR
TOTAL ACTIVITY
60
40
20
0
a
b
PEAK (events/h)
14
10
6
0
DURATION (h)
80
60
40
0
ACCURACY (%)
80
60
40
0
MOD
FAT
BODY CONDITION

Energy balance did not influence accuracy of standing (figure 11) or mounting events (figure 12). There were no interactions between NEB and FAT on accuracy of standing during the last two cycles (figure 11) or accuracy of mounting throughout the study (figure 12). But in MOD heifers, NEB reduced ($P < .01$) accuracy of standing (figure 11) and mounting behavior (figure 12) during all periods of estrus. Negative EB reduces accuracy of estrous behavior only when BC at the beginning of dietary restrictions is MOD. Therefore BC at onset of NEB determines whether or not NEB affects accuracy of estrous behavior.

Sustained FAT or NEB associated with MOD reduced accuracy of standing and mounting behavior. Consequently, sustained FAT or NEB-MOD may affect accuracy of timing insemination relative to ovulation due to increased variation in detected estrous behavior relative to onset of estrus, and(or) potentially, altered intervals from onset of estrus to ovulation.

Before the experiment, duration of an estrous cycle averaged 20.4 ± .7 d and did not differ among heifers assigned to either of the four treatment combinations. During the study, EB or BC as single effects did not influence duration of estrous cycles (table 7), intervals between onset of estrus and onset of diestrus (table 8) or length of diestrus (table 9). But NEB and FAT interacted to prolong estrous cycles (table 7). Pelissier (1978) observed that duration of estrous cycles varied widely among dairy cows, and assumed that inaccuracy of farmers to identify estrus was the main source of variation in length of estrous cycles. But the present study indicates that duration of estrous cycles is influenced by metabolic status of cattle. Estrous cycles of FAT-NEB heifers were prolonged because: a) intervals from onset of estrus to onset of diestrus (table 8) and b) duration of diestrus (table 9) were extended ($P < .05$). Long diestrus likely results from retarded luteolysis. Delayed onset of diestrus may

TABLE 7. ENERGY BALANCE AND BODY CONDITION ON DURATION OF ESTROUS CYCLES IN HEIFERS.[a]

Treatment combination	n	Estrous cycle (day)[b] 1	2
MOD-PEB	6	21.3 ± .8	21.3 ± .8
FAT-PEB	6	20.7 ± .9	20.7 ± .7
MOD-NEB	5	21.2 ± .5	22.7 ± .9
FAT-NEB	3	24.3 ± .7[c]	27.0 ± 2.1[d]

[a]Estrous cycles were numbered according to number of preceding estrus and were defined as intervals between two consecutive onsets of estrus. By design third estrous cycle ended between d 10 and 12 postestrus, so duration of third cycle was not determined.

[b]Values are mean ± standard error.

[c]Within column and within line, this value differs from others ($P < .05$).

[d]Within column, this value differs from others ($P < .01$).

TABLE 8. EFFECTS OF ENERGY BALANCE AND BODY CONDITION ON DURATION OF INTERVALS FROM ONSET OF ESTRUS TO ONSET OF DIESTRUS IN HEIFERS.[a]

Treatment Combination	n	Estrous cycle (d)[b]		
		1	2	3
MOD-PEB	6	4.5 ± .2	4.7 ± .2[c]	4.2 ± .3[d]
FAT-PEB	6	5.2 ± .3	5.0 ± .3	5.2 ± .4
MOD-NEB	5	6.6 ± 1.9	5.2 ± .2	5.0 ± .4
FAT-NEB	3	5.0 ± .6	5.3 ± .3[c]	6.0 ± .6[d]

[a]Interval from onset of estrus to onset of diestrus was the interval between first standing event within basal progesterone and first sample with concentrations of progesterone exceeding basal progesterone by two standard deviations.

[b]Estrous cycles were numbered according to number of preceding estrus and were defined as intervals between two consecutive onsets of estrus. Values are means ± standard error.

[c]Within estrous cycle, means with common superscript differ ($P < .05$).

[d]Within estrous cycle, means with common superscript differ ($P < .01$).

TABLE 9. EFFECTS OF ENERGY BALANCE AND BODY CONDITION ON DURATION OF DIESTRUS IN HEIFERS.[a]

Treatment Combination	n	Estrous cycle (d)[b] 1	2
MOD-PEB	6	14.2 ± .5	14.7 ± .8
FAT-PEB	6	13.7 ± .8	13.5 ± .9
MOD-NEB	5	13.8 ± 1.1	15.6 ± 1.0
FAT-NEB	3	17.0 ± 1.3[c]	20.0 ± 2.1[d]

[a]Diestrus was the interval within an estrous cycle when concentrations of progesterone in serum exceeded by two standard deviations mean progesterone at estrus ± 1 d.

[b]Estrous cycles were numbered according to number of preceding estrus and were defined as intervals between two consecutive onsets of estrus. By design third estrous cycle ended at mid-diestrus, so duration of diestrus for third cycle was not determined.

[c]Within estrous cycle this mean differs from others ($P < .05$).

[d]Within estrous cycle this mean differs from others ($P < .01$).

indicate delayed ovulation or limited luteal development. Variation in intervals from onset of estrus or onset of preovulatory surges of LH to ovulation was minimal among heifers fed adequately (Bernard et al., 1983). But in the present study, onset of diestrus was delayed in FAT-NEB heifers indicating that metabolic status may delay ovulation in cattle as fasting delays ovulation and prolongs estrous cycles in mice (Bronson and Marsteller, 1985).

In summary I found no evidence for independent or associative effects of EB and BC to reduce intensity of standing or mounting behavior. Sustained FAT in heifers prolonged intervals of standing behavior but reduced accuracy of standing and mounting behavior. Independent of BC, NEB increased intensity of mounting behavior. But in heifers with limited stores of fat (MOD), NEB reduced accuracy of standing and mounting events whereas in FAT heifers, NEB delayed onset of diestrus and luteolysis and prolonged estrous cycles.

Because intensity and duration of standing and mounting behavior were not reduced by NEB or FAT, my first conclusion is that NEB or FAT does not diminish detectability of estrus in heifers. But FAT decreased accuracy of estrous behavior and initial BC determined whether NEB reduced accuracy of estrous behavior or potentially altered temporal associations of onset of estrus and ovulation. In both cases, NEB or FAT might decrease accuracy of timing artificial insemination relative to ovulation and then lower fertility of cattle.

GENERAL DISCUSSION

The overall goal of this research was to study effects of homeorhetic changes that occur during early lactation on reproduction of dairy cows. I proposed EB as an integrative measure of metabolic adjustments associated with lactation. Luteal function and behavior of cattle at estrus were the aspects of reproduction selected to study.

A salient finding of this research was that spontaneous NEB was associated with reduced luteal function in lactating dairy cows. Furthermore, induced NEB in dairy heifers limited luteal function in vivo and in vitro. Because NEB did not reduce luteotropic support, but diminished responsiveness of luteal cells to LH in vitro, it is likely that NEB inhibits luteal function locally. These results are consistent with the concept that homeorhetic mechanisms which provide metabolic support to lactating mammary glands reduce luteal function in cows. Apparently, NEB and reduced luteal function are associative effects of homeorhetic controls of lactation.

What are the homeorhetic mechanisms that mediate adverse effects of NEB on luteal function? At parturition, synthesis of milk achieves metabolic priority and, through homeorhesis, non-mammary tissues undergo adaptations to insure metabolic support to mammary tissues (Bauman and Currie, 1980; Collier et al., 1984). At least 81% of dairy cows experience NEB during early lactation (Coppock et al., 1966; Reid et al., 1966; Experiment I). This deficit of energy is largely due to insufficient ingestion of dietary energy (Experiment I). Precursors of energy (amino acids, NEFA) stored in body depots (muscle, adipose) provide additional energy required to synthesize milk. Precursors of energy are mobilized from depots and are used by mammary gland directly or after processing by liver or kidney (Bauman and Currie, 1980; Collier et al., 1984). The major changes that determine metabolic priority of mammary gland

include: increased blood flow to mammary gland, enhanced metabolic activity of mammary tissue and decreased utilization of nutrients by non-mammary tissues (Collier et al., 1984).

Homeorhetic regulation of metabolism to support synthesis of milk depends critically on changes in serum concentrations of hormones and altered responsiveness of mammary and non-mammary tissues to hormones during early lactation. As discussed earlier, hormonal changes associated consistently with homeorhetic support of lactation and with NEB in dairy cows, are increased concentrations of GH and reduced concentrations of insulin in serum. Concommitantly, binding of insulin to mammary gland increases but binding of insulin to non-mammary tissues decreases (Collier et al., 1984). In contrast to insulin, responsiveness of adipose tissue to GH and numbers of β-adrenergic receptors in adipose tissue increase (Bauman and Currie, 1980; Collier et al., 1984). These adaptations decrease uptake of nutrients (glucose, acetate, amino acids) by non-mammary tissues and increase availability of precursors for energy and milk constituents (glucose, amino acids, NEFA and other lipids) to mammary gland.

Metabolic adaptations of luteal tissue during early lactation and NEB have not been studied. But, observations in other tissues provide basis to hypothesize that responsiveness of luteal cells to insulin may be reduced. In the review of literature, it was established that insulin binds to corpora lutea and exerts luteotropic actions. Consequently, decreased serum concentrations of insulin, reduced responsiveness of corpora lutea to insulin, or both may mediate adverse effects of NEB on luteal function during the homeorhetic milieu of early lactation. Whether or not GH affects corpora lutea is untested. But, based on effects of GH in adipose tissue, high concentrations of GH in serum during early lactation and NEB may oppose the anabolic influence of insulin on corpora lutea and then reduce luteal function.

In heifers, I determined that FAT reduced luteal function in vivo. What mechanisms mediate negative effects of FAT on luteal function? Development of hyperinsulinemia accompanied by reduced responsiveness of muscle, adipose and hepatic tissues to insulin are consistently observed in obese individuals (Bray and York, 1979; McCann and Reimers, 1986). In obese rodents reduced binding of insulin and altered postreceptor events are involved in reduced response of tissues to insulin (Bray and York, 1979). I propose that retarded luteal development in FAT heifers (Experiment II) is due to reduced responsiveness of luteal cells to insulin. Thus, adverse effects of NEB and FAT on luteal function may ultimately be mediated by limited availability and action of insulin on corpora lutea. If so, why was secretory activity of luteal cells in vitro reduced by NEB but not by FAT? Increased availability of potential inhibitors of luteal activity (GH, NEFA) during NEB, which are not increased in FAT heifers, may account for the difference in effects of NEB and FAT on luteal function.

Negative EB was associated with reduced luteal function in lactating dairy cows and heifers. Due to confounded effects of duration of postpartum anovulation, postpartum estrous cycles and EB on luteal function of cows, I used heifers to explore potential mechanisms by which NEB altered luteal function. A question is: are heifers an appropriate model for lactating cows?

Due to intense genetic selection for high yields of milk, some metabolic aspects of dairy cows are unique among farm animals. One of these aspects is that cows fed ad libitum commonly experience spontaneous NEB. Magnitude of NEB in cows (Experiment I) was three to four fold greater than magnitude of induced NEB in heifers (Experiment II). In addition, cows with nadir of EB between 12 and 30 d postpartum had the lowest concentrations of progesterone in milk. But, NEB heifers, who had reduced serum progesterone, had lowest EB between 60 and 80 d after initial dietary restrictions. Moreover, postpartum

interval to nadir EB and magnitude of nadir but not duration of NEB, were components of EB which were most associated with luteal function in cows. But in heifers, magnitude and duration of NEB affected adversely luteal function. These differences between cows and heifers indicate that knowledge gained from heifers may not be applicable to lactating dairy cows. However, NEB did not alter duration of luteal phases but was associated with reduced area and peak concentrations of progesterone in serum of lactating cows (Experiment I) and heifers (Experiment II). Consequently, secretion of progesterone but not lifespan of corpora lutea is reduced in cows and heifers.

From literature cited, high concentrations of GH and NEFA but low concentrations of insulin in blood coincide with NEB in dairy cows during early lactation. In heifers, NEB reduced postprandial concentrations of insulin and increased concentrations of GH and NEFA in blood (Experiment II). If changes in concentrations of insulin, GH and(or) NEFA mediate adverse effects of NEB on luteal function, NEB would limit luteal function through similar mechanisms in cows and heifers. Negative EB did not reduce expression and detectability of estrus in heifers (Experiment III). Based on three daily periods of observation, variation of estrous behavior of lactating dairy cows was not associated with EB (Experiment I). Thus, it is likely that NEB does not reduce detectability of estrus in dairy cows. But, three daily periods of observation are insufficient to determine influence of NEB on estrous behavior of cows. In addition, magnitude of NEB in cows was greater than magnitude of NEB in heifers. Thus severity of NEB could affect adversely estrous behavior in cows while relatively moderate NEB of heifers did not. In dairy cows, NEB occurs spontaneously. Therefore, appetite in cows is satisfied since feed is available and cessation of DM ingestion is voluntary. But, in heifers, NEB was induced by dietary restrictions and appetite for volume of feed and(or) nutrients was not satisfied.

This difference in satiety between heifers and cows might determine distinct patterns of behavior in response to NEB. Thus, the hypothesis that spontaneous NEB alters expression of estrus needs to be tested in lactating dairy cows.

SUMMARY AND CONCLUSIONS

Eighty one % of lactating dairy cows fed ad libitum experienced NEB within 100 days postpartum. Although yield of milk was associated negatively with EB, most of the variation in EB was explained by intake of energy. Yield of milk did not affect luteal function. But, EB and luteal function were associated positively in cows. Therefore, NEB may reduce luteal function in lactating dairy cows.

In heifers, NEB and FAT independently reduced luteal function in vivo. Reduced rate of increase of progesterone in serum and reduced luteal weight in NEB and FAT heifers indicate that NEB and FAT retarded luteal development. Negative EB but not FAT reduced LH-induced secretion of progesterone by luteal cells in vitro. Consequently, FAT retarded luteal development but NEB retarded luteal development and reduced secretory activity per luteal cell.

Concentrations of LH in serum of heifers were not affected by EB and BC or their interactions. Thus, adverse influence of NEB and FAT on luteal function are not due to reduced luteotropic support.

Adverse effects of NEB on luteal function were first detected during the second estrous cycle in MOD heifers, but were not detected until the fourth estrous cycle in FAT heifers. Coincidentally, BC of MOD-NEB heifers declined and was below MOD by the second estrous cycle while BC of FAT-NEB heifers was not below MOD until the fourth estrous cycle. Thus, adverse effects of NEB on luteal function were not detected until BC declined below MOD.

Intensity and duration of standing and mounting behavior were not reduced in NEB or FAT heifers. But, FAT decreased accuracy of standing and mounting activity. Body condition determined whether NEB reduced accuracy of estrous behavior (in MOD heifers) or altered temporal associations between onset of estrus and onset of diestrus or potentially ovulation (in FAT heifers).

The first conclusion is that NEB exerts limitations to luteal function in heifers and lactating dairy cows. In addition, FAT limits luteal function in heifers. Apparently, effects of NEB and FAT on luteal function are exerted through different mechanisms.

A second conclusion is that BC determines effects of NEB on luteal function. Apparently, adverse influence of NEB on luteal function is not detected until BC of heifers declines below MOD.

Finally, I conclude that NEB and FAT do not reduce expression and detectability of estrus in heifers. But NEB and FAT diminish accuracy of estrous behavior and delay onset of diestrus.

If extended intervals from onset of estrus to onset of diestrus indicate delayed ovulation, artificial insemination scheduled according to onset of estrus would have reduced success. Furthermore, reduced luteal function due to NEB and FAT, could directly or indirectly limit fertility. Consequently, I propose that NEB and FAT are potential sources of infertility in dairy cattle.

APPENDIX A

APPENDIX A

Validation of a Solid Phase Radioimmunoassay Developed to Quantify Progesterone in Milk

Samples of milk were collected from individual cows immediately after milking. Samples of milk were allowed to cool at 5°C for 2 or 3 h. To separate fat from milk, samples were centrifuged at 704 x g for 15 min. The aqueous fraction (fat-free milk) from each sample was aspirated and stored at -20°C until assayed. Concentrations of progesterone in milk were determined by a solid phase radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). Specificity of antibody against progesterone was determined previously (Archbald et al., 1984). Among 11 steroids tested, 11-deoxicortisol had the greatest cross-reactivity (2.4%) with this antibody. To remove potential bias due to protein milieu of milk on binding of ^{125}I-progesterone to specific antibody, standard for progesterone were diluted with fat-free milk from cows in estrus. To remove steroids from milk used as diluent for standards, free-fat milk was stirred with 25 mg of washed neutralized Norit-A charcoal (Fisher Scientific Co., Fair Lawn, NJ)/ml at 37°C for 20 min and centrifuged at 105,000 x g for 30 min. The supernatant was aspirated and stored at -20°C. Standard curves of progesterone (Δ^4-pregnen-3, 20-dione; Sigma Laboratories) diluted with fat-free, steroids-free milk, were contrasted with standards diluted with phosphate buffer-gelatin. Standard curves (figure 13) prepared with milk (50% intercept = 1.2 ng/ml) paralleled (r^2 = .99) standard curves prepared with

phosphate buffer-gelatin (50% intercept = 1.1 ng/ml). Therefore, in all subsequent validation procedures and assays, standards for progesterone were diluted with fat-free, steroid-free milk. To determine the need for extraction, fat-free samples of milk (8 samples, 4 replicates; range .2 to 3.7 ng of progesterone/ml) were extracted (toluene:hexane; 1:2) and assayed for progesterone, or assayed directly with no extraction. The coefficient of correlation between extracted and no extracted samples was .99. Thus progesterone was assayed directly without extraction in all samples of milk collected during the experiment. To determine parallelism of concentrations of progesterone in milk and in serum, samples of milk and samples of jugular blood were collected every third d during an estrous cycle of two cows (12 samples). Fat-free milk or sera were obtained from samples and assayed in duplicate. Coefficient of correlation between concentrations of progesterone in serum and in milk was .9 (figure 15). Thus, concentrations of progesterone in milk indicate accurately changes in concentrations of progesterone in serum of cows. Efficiency of recovery for the assay was determined by adding 1, 2 or 10 ng of progesterone/ml (12 replicates each) to tubes containing fat-free milk known to have low concentrations of progesterone. After subtracting initial concentrations, .9 ± .02, 1.8 ± .01 and 9.1 ± .13 ng of progesterone/ml of milk were detected, respectively. Thus, recovery was 90.7% for the 3 concentrations of progesterone. Immunological similarity and (or) potential interference with binding, were tested by determining parallelism between progesterone in samples of milk and standard curves (figure 13). For this, dilutions were prepared from a pool of milk from pregnant cows (85, 75, 50, 25 and 15% in fat-free, steroid-free milk; 4 replicates each). Slope of standard curves of progesterone ($b = -.65$; $r^2 = .99$) and slope for the regression line of progesterone quantified in diluted milk ($b = -.51$; $r^2 = .94$) were parallel. Concentrations of progesterone in milk determined by the solid phase assay,

were contrasted with concentrations of progesterone determined in the same samples of milk by an assay validated in this laboratory (Louis et al., 1973). The coefficient of correlation between progesterone in milk (8 samples, 3 replicates) determined by the solid phase and the previously validated assay was .98 (figure 14). To determine precision of the assay, milk from cows in metestrus (.465 ± .006 ng/ml) and milk from pregnant cows (9.56 ± .09 ng/ml) was used. From 13 assays, milk with low progesterone (n = 74) had an intraassay coefficient of variation (CV) of 10.9% and interassay CV of 12.9%. For milk with high progesterone (n = 78), intrassay CV was 5.2% while interassay CV was 8.5%.

Figure 13. Standard curves of progesterone prepared with milk, and displacement of ^{125}I-progesterone from specific antibody against progesterone by different dilutions of a milk pool. Standard curves prepared with phosphate buffer–gelatin (●) paralleled (r^2 = .99) standard curves diluted with fat-free, steroid-free milk (O). Slope for regression line of progesterone in different dilutions of a pool of fat-free milk from pregnant cows (△) paralleld (r^2 = .9) slopes for standard curves prepared with milk. Each point in standard curves is the mean from six replicates. Each point of pooled milk is the mean from four replicates.

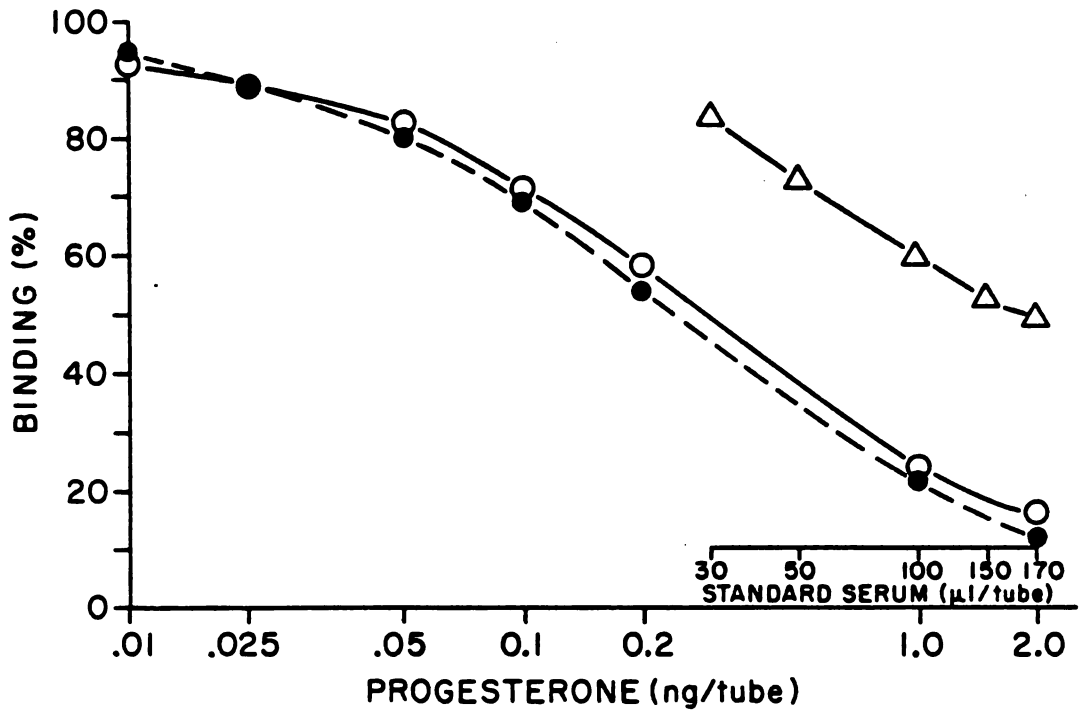

100
80
60
40
20
0
BINDING (%)
.01
.025
.05
0.1
0.2
1.0
2.0
PROGESTERONE (ng/tube)
30
50
100
150
170
STANDARD SERUM (µl/tube)

Figure 14. Concentrations of progesterone determined in samples of milk by solid phase or by a previously validated, liquid phase assay. Concentrations of progesterone were determined by liquid (——) or solid (- - -) phase radioimmunoassay in samples of fat-free milk. Coefficient of correlation between both assays was .99. Each point is the mean from 3 replicates. Pooled standard error of progesterone determined by solid phse or by liquid phase assays was .5 and .8, respectively.

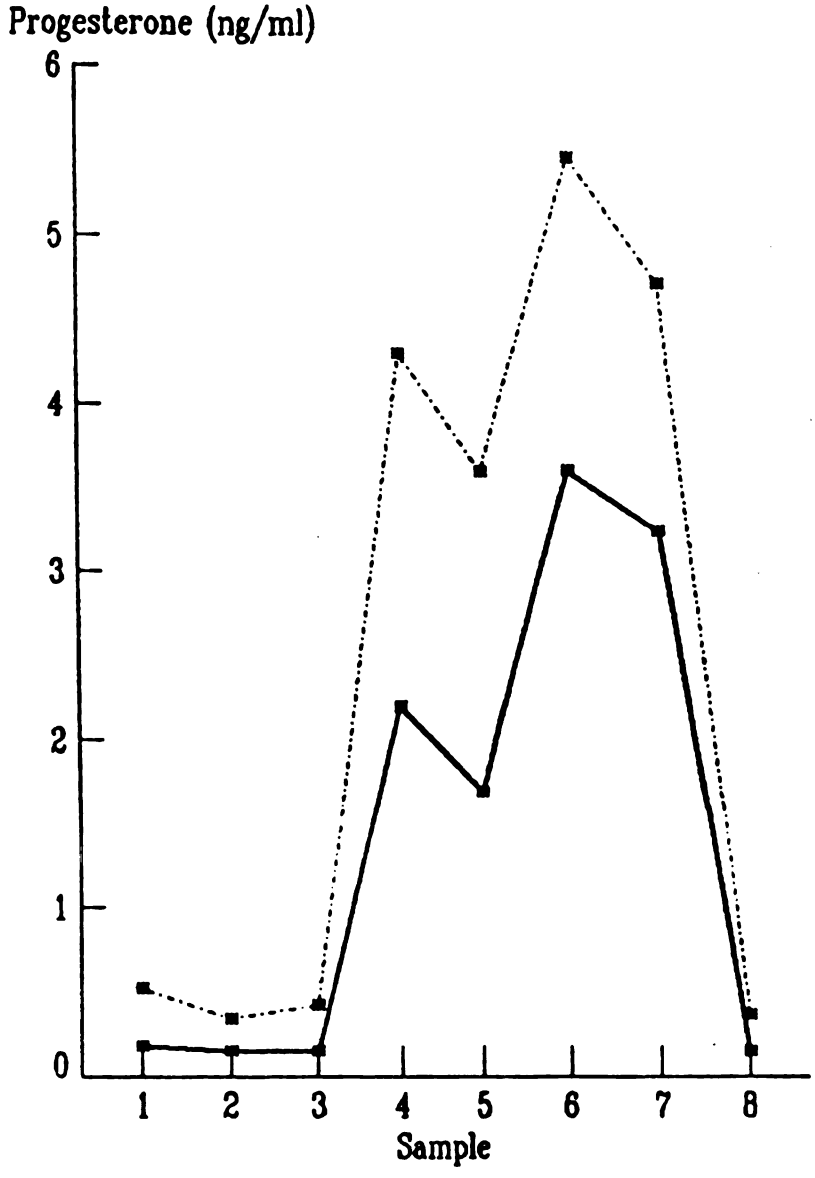
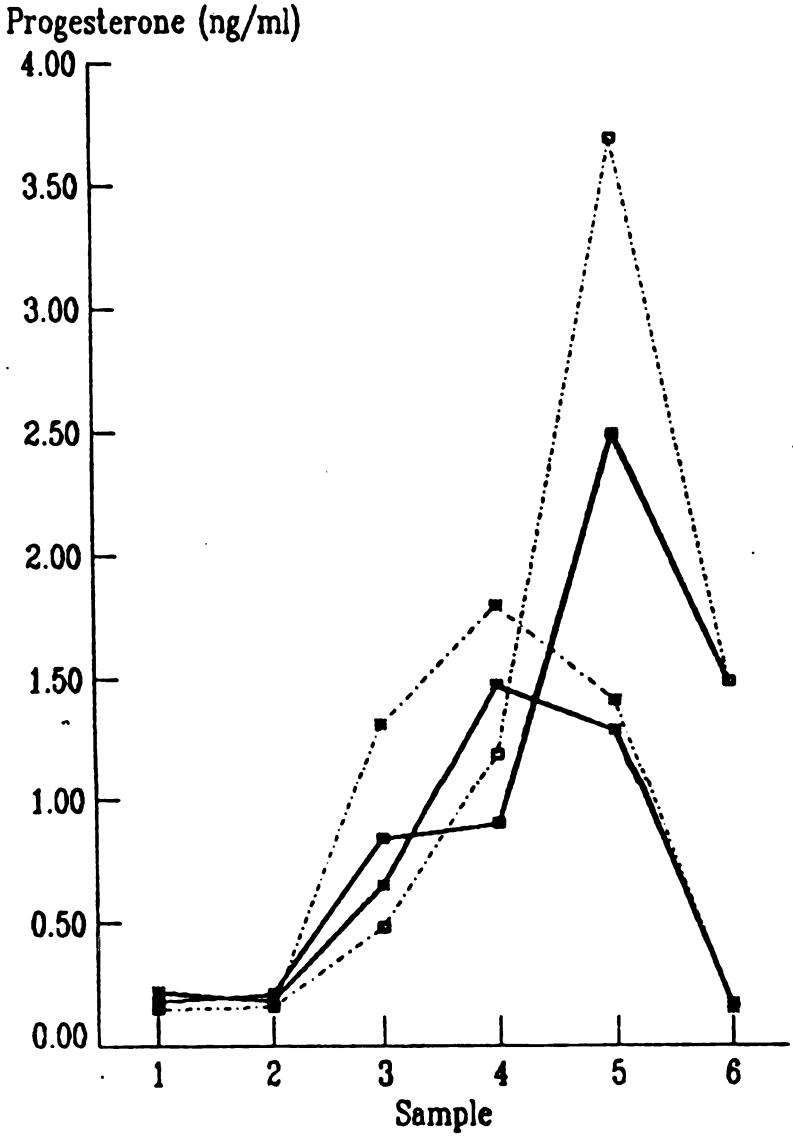

Progesterone (ng/ml)
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
Sample

Figure 15. Parallelism between concentrations of progesterone in serum and milk. Progesterone was determined in serum (- - -) and fat-free milk (——) sampled every third d during an estrous cycle from cow 1 (□) and cow 2 (*). Coefficient of correlation between progesterone in serum and milk was .90. Each point is the mean from two replicates. Pooled standard error for progesterone in serum and milk was .3 and .2, respectively.


Progesterone (ng/ml)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1
2
3
4
5
6
Sample

APPENDIX B

APPENDIX B

Validation of a Homologous Radioimmunoassay Developed to Quantify Bovine Insulin in Serum

A homologous, double antibody radioimmunoassay was developed to quantify insulin in serum of cattle. Specific antibodies against bovine insulin (first antibody) were produced in guinea pig (lot GP23; Miles Scientific, Naperville, IL). Antibodies against gamma globulin of guinea pig (second antibody) were produced in sheep as described previously (Oxender et al., 1972) and diluted 1:20 in .5 M EDTA-phosphate buffer (pH 7.3) for this study. Crystalized monocomponent bovine insulin (99.99% pure, biological potency of 26.9 μU/ng; Novo BioLabs, Wilton, CT) was used to prepare radioiodinated insulin and to construct standard curves.

Insulin was radioiodinated by a modification of methods described by Niswender et al. (1969). Five μg of insulin were diluted in 25 μl of .05 M phosphate buffer, .85% NaCl (pH 8.6). One mCi of sodium ^{125}I-iodide (Amersham, Arlington Heights, IL) was added to the vial containing insulin and mixed. Twenty five μl of chloramine-T (1 μg/μl) in .05 M phosphate buffer (pH 7.5) were added and the mixture was agitated for 15 sec. The reaction was stopped by adding 50 μl of .05 M phosphate buffer containing 50 μg of sodium metabisulfate. This mixture was agitated for 15 to 20 sec. Transfer solution and column of bio-gel P-60 were the same as described by Niswender et al. (1969). The column eluate (figure 16) was collected in fractions of 1 ml into tubes containing 1 ml of .5

M phosphate buffer, 2% bovine serum albumin, .9% NaCl (pH 7.3). To prepare standard curves, insulin was prediluted (1 μg/μl) in .05 M phosphate buffer, .85% NaCl (pH 8.6) and subsequently diluted (.025 ng/μl) in .5 M phosphate buffer, .5% bovine serum albumin, .9% NaCl, .025% thimerosal (pH. 7.3). Standard curves contained .025, .05, .10, .15, .2, .30, .40, .50, .60, 1.0, 2.0, 3.0 and 4.0 ng/tube. Standard curves were calculated from multiple regression equations with linear, quadratic and cubic components. From 8 standard curves, the coefficient of determination (r^2) was .996 and, based on intercepts of fitted standard curves on y axis (% binding), the assay was optimal between .025 to 1.77 ng/tube. Lyophilized guinea pig serum containing antibodies to bovine insulin was reconstituted with 1 ml of deionized, double distilled water, diluted to 1:400 in .05 M EDTA-phosphate buffer, .9% NaCl (pH 7.0) and titered. A dilution of antibody against insulin of 1:30,000 provided approximately 40% specific binding of ^{125}I-insulin and was used in assay and validation procedures. Slope of the regression line of insulin in multiple dilutions (range in μl; 20:480 to 200:300, sera:buffer) from a pool of serum (42 replicates) from fasted heifers ($b = -8.0$; $r^2 = .98$) and from a pool of serum (36 replicates) from fed heifers ($b = -11.6$; $r^2 = .93$) paralleled ($P > 0.10$) slope of standard curves (figure 17) between .02 and .4 ng of insulin/tube ($b = -11.0$; $r^2 = .99$). Coefficient of variation (CV) of insulin within dilution of serum from fasted heifers was 6.2% whereas interdilution CV was 6.5%. For insulin in serum of fed heifers, within dilution CV was 4.5% and interdilution CV was 13.3%. From five assays, intraassay CV was 7.6% and interassay CV was 13.7%. Specificity of antibody against bovine insulin was determined by testing the degree of cross-reactivity with 6 hormones (figure 18). Bovine GH (purified, 15825-AJP-152, Upjohn, Kalamazoo, MI), bovine LH (NHI-LH-B8), bovine FSH (BP3, USDA), bovine TSH (NIH-TSH-B5), bovine prolactin (NIH-PRL-B4) or bovine-porcine glucagon (Sigma Chemical

Co., St. Louis, MO), in amounts up to 50 ng/tube did not cause significant displacement of ^{125}I-insulin (<1% cross-reaction). Recoveries from serum supplemented with .1, .5 and 1.0 ng of insulin/tube (4 replicates each) averaged 97.6 ± 2.1. Samples of jugular blood were collected from heifers every 15 min during an interval of 12 h surrounding a meal. From each sample (n = 1000), duplicates of 200 µl of serum (diluted in 0.5 M phosphate buffer, .5% bovine serum albumin, .9% NaCl, pH 7.3) were quantified in a single radioimmunoassay. Average concentration of insulin for all samples was .93 ± .02 ng/ml (25.1 µU/ml) and range was .26 to 5.01 ng/ml (6.99 to 134.8 µU/ml).

Figure 16. Elution pattern of ^{125}I-bovine insulin. Bovine insulin and ^{125}I-iodide were mixed and eluted through a 10 (1/10) ml column of bio-gel P-60. Before elution, iodination reaction was initiated by chloramine-T and stopped 15 sec later by adding sodium metabisulfate. Volume of elution fractions was 1 ml. Radioactivity was determined during .10 of a min in a 20 µl aliquot from each elution fraction. Fractions 7 and 8 contained ^{125}I-bovine insulin whereas fractions 10 and 11 contained free ^{125}I.

Figure 16. Elution pattern of ^{125}I-bovine insulin. Bovine insulin and ^{125}I-iodide were mixed and eluted through a 10 (1/10) ml column of bio-gel P-60. Before elution, iodination reaction was initiated by chloramine-T and stopped 15 sec later by adding sodium metabisulfate. Volume of elution fractions was 1 ml. Radioactivity was determined during .10 of a min in a 20 µl aliquot from each elution fraction. Fractions 7 and 8 contained ^{125}I-bovine insulin whereas fractions 10 and 11 contained free ^{125}I.

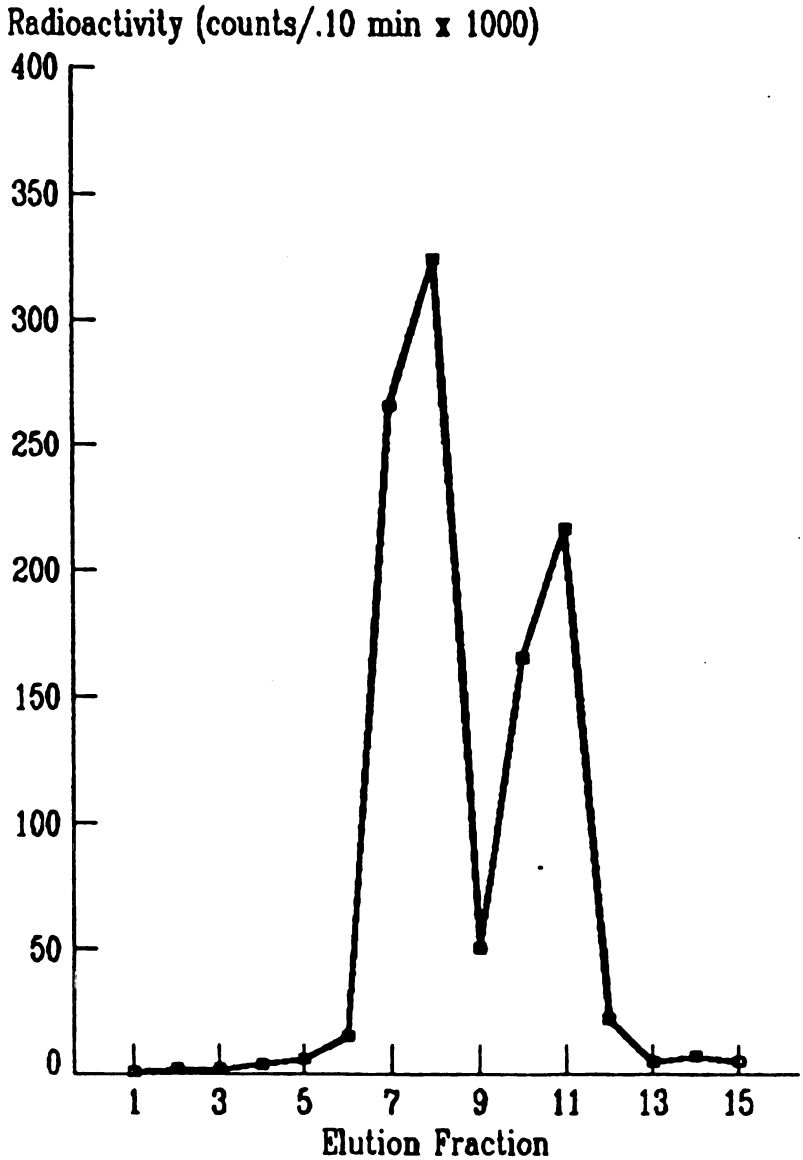

Radioactivity (counts/.10 min x 1000)
400
350
300
250
200
150
100
50
0
1
3
5
7
9
11
13
15
Elution Fraction

Figure 17. Displacement of ^{125}I-bovine insulin from antibody for bovine insulin by different dilutions of pooled bovine sera. Slope for eight standard curves of insulin (O) paralleled (r^2 = 9) slope for regression line of insulin in dilutions of pooled serum from fasted (□; 42 replicates) or fed heifers (△; 36 replicates).

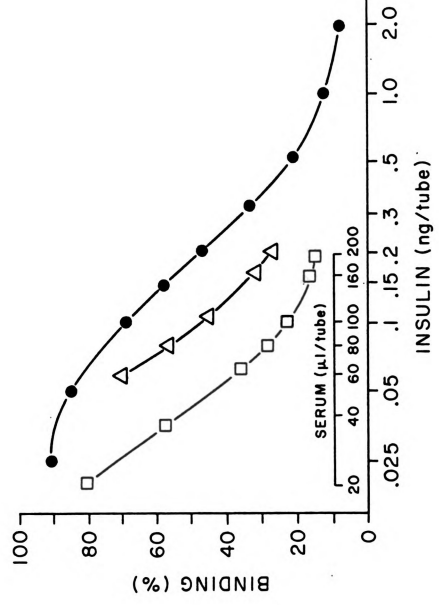
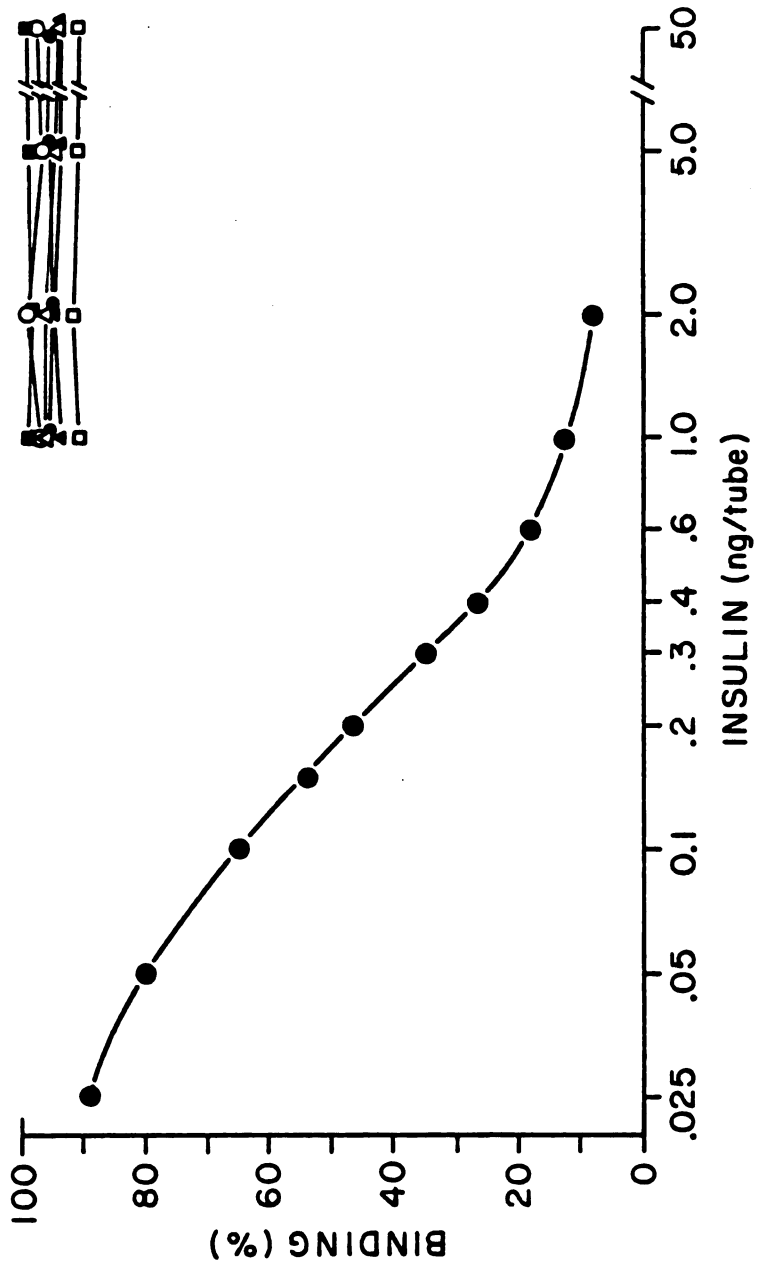

BINDING (%)
100
80
60
40
20
0
.025
.05
.1
.15
.2
.3
.5
1.0
2.0
INSULIN (ng/tube)
SERUM (µl/tube)
20
40
60
80
100
160
200

Figure 18. Specificity of antibody against bovine insulin. Displacement of ^{125}I-bovine insulin from antibody against insulin by other protein hormones was tested by adding into reaction tubes 1, 2, 5 or 50 ng of bovine GH (■), bovine LH (○), bovine FSH (△), bovine TSH (□), bovine prolactin (▲) or bovine-porcine glucagon (●). Cross reactivity of antibody against insulin with any of the tested hormones was < 1%.


BINDING (%)
100
80
60
40
20
0
.025
.05
0.1
.2
.3
.4
.6
1.0
2.0
5.0
50
INSULIN (ng/tube)

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