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NUTRITIONAL INFLUENCES ON BODY AND MAMMARY
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NUTRITIONAL INFLUENCES ON BODY AND MAMMARY GROWTH AND EFFECT OF LEPTIN AND IGF-I IN PREPUBERTAL DAIRY HEIFERS

By

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ABSTRACT

NUTRITIONAL INFLUENCES ON BODY AND MAMMARY GROWTH AND EFFECT OF LEPTIN AND IGF-I IN PREPUBERTAL DAIRY HEIFERS

By

Laurie Ellen Davis Rincker

Feeding high energy diets to prepubertal dairy heifers for 12 wk or more increases daily gain and can reduce the number of non-productive days before first calving, but also can impair mammary growth relative to body growth and decrease subsequent milk production. Recent research indicates that feeding calves a high energy diet prior to weaning for a shorter duration results in an increase in body weight gain without impairing mammary growth. The objective of this research was to determine the effect of feeding a high energy diet to prepubertal heifers for a short and long duration of time on body growth, mammary growth, and protein and mRNA abundance of leptin and IGF-I in serum and mammary parenchymal tissue. Sixty-four heifers (age = 11 wk) were assigned to 1 of 4 treatments and fed 2 diets for a different duration: H0, H3, H6, and H12 were fed a low energy diet for 12, 9, 6, and 0 wk followed by a high energy diet for 0, 3, 6, and 12 wk, respectively. The low and high energy diets were fed to achieve 0.6 and 1.2 kg daily gain, respectively. Heifers were slaughtered at 23 wk of age.

Body, carcass, carcass fat, liver, and perirenal fat weights increased linearly with a longer duration fed the high energy diet. A longer time fed the high energy diet increased weights of total mammary gland, extraparenchymal fat, and intraparenchymal

fat, but did not change the weight of mammary parenchyma. When expressed relative to carcass weight to adjust for treatment differences in physiological maturity, fat-free parenchymal tissue weight and mammary RNA and DNA content decreased as heifers were fed a high energy diet for a longer duration. An increase in body or carcass growth without a proportional increase in mammary growth would result in less mammary parenchyma at puberty because heifers fed for rapid gains reach puberty at a younger age.

The phenomenon of why high energy intake impairs mammary growth relative to body growth is not clearly understood. An increase in fat deposition could play a role in the impairment of mammogenesis. Leptin is produced by adipocytes and impairs mammary epithelial cell proliferation in heifers. To determine whether heifers fed high energy diets had greater amounts of leptin, concentration and mRNA expression of leptin in mammary parenchymal tissue were measured. Heifers fed a high energy diet for a longer duration had increased leptin concentrations in serum and mammary tissue and increased leptin mRNA expression in mammary tissue.

Feeding prepubertal heifers a high energy diet for rapid gains increases serum concentration of IGF-I, a mitogen for mammary epithelial cells. To better understand this apparent contradiction, IGF-I and IGF-I receptor mRNA expression in mammary parenchymal tissue were measured. There was no dietary effect on IGF-I mRNA expression, while a short duration of time fed a high energy diet decreased IGF-I receptor mRNA expression in mammary tissue. A potential inhibition of IGF-I stimulation via leptin, IGFBP-3, or another factor not yet elucidated could explain why high energy intake impairs mammary growth relative to body growth in prepubertal dairy heifers.

In honor of Flora Diehl Williams and Joyce McCune Davis
and in memory of Lydia Driver Diehl.
Mothers and grandmothers are the glue that holds a family together.
Thanks for teaching me how to be a strong-minded woman
in faith,
in family,
and in love.

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TABLE OF CONTENTS

CHAPTER ONE

CHAPTER TWO

LIST OF TABLES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

APPENDIX

LIST OF FIGURES

INTRODUCTION

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

APPENDIX

LIST OF ABBREVIATIONS

ADF = acid detergent fiber

ALS = acid labile subunit

AOAC = association of official analytical chemistry

ADG = average daily gain

BL = baseline

bST = bovine somatotropin

BrdU = bromodeoxyuridine

BW = body weight

C = cubic

CP = crude protein

CW = carcass weight

DM = dry matter

DMI = dry matter intake

FBS = fetal bovine serum

FAR = fractional accretion rate

GAPDH = glyceraldehyde -3 – phosphate dehydrogenase

GLM = general linear model

GH = growth hormone

GHRH = growth hormone releasing hormone

IGF-I = insulin-like growth factor-I

IGFBP = insulin-like growth factor binding protein

L = linear

LS means = least square means

LH = luteinizing hormone

MAC-T = mammary alveolar cell large T-antigen

ME = metabolizable energy

NRC = National Research Council

NE_m = net energy for maintenance

NE_g = net energy for gain

NPY = neuropeptide Y

NDF = neutral detergent fiber

Ob-R = leptin receptor

Q = quadratic

RIA = radioimmunoassay

RT-PCR = reverse transcription polymerase chain reaction

RDP = rumen degradable protein

RUP = rumen undegradable protein

SRIF = somatotropin releasing inhibitory factor

TMR = total mixed ration

TGF-β_1 = transforming growth factor -β_1

Trt = treatment

INTRODUCTION

Raising replacement heifers is costly for the producer and is estimated to be 20% of total dairy herd expenses (Heinrichs, 1993). Growing heifers faster for earlier breeding and calving can reduce these costs. However, feeding a high energy diet to prepubertal heifers for gains of greater than 1 kg/d can impair mammary growth relative to body growth and reduce subsequent milk yield (Radcliff et al., 2000; Sejrsen et al., 1982). Since 1915, research studies have focused on understanding why high dietary energy intakes impair mammary growth (Eckles, 1915).

The importance of understanding mammary gland development in heifers and how this foundation for subsequent development and future milk yield can be affected by factors during early life is the focus of many reviews (Akers, 1990; Sejrsen, 1994; Tucker, 1981). The mammary gland is a unique organ because the epithelial tissue is still rudimentary at birth, and its development can be influenced by management factors, such as nutrition. The critical window for when high energy intake can negatively alter mammary growth is from a few months of age until around the onset of puberty. During this time, growth of the mammary gland is allometric, meaning that the rate of gland growth is faster than that of body growth.

Studies with numerous designs have tested the effects of nutrition on mammary growth. These studies include: 1) ad libitum versus restricted feeding of the same diet; 2) diets differing in energy and protein content; 3) diets with varying levels of protein but similar energy densities; and 4) diets with varying levels of rumen undegradable protein. Results of most studies indicate that gains greater than 1 kg/d can impair mammary growth relative to body growth. However, the influence of protein within the diet on mammary growth is still not clear. Compensatory growth studies indicate that a stair-step feeding regimen of alternating feed intake of heifers by 25 – 30% above recommendations for 2 mo and 20 – 30% below recommendations for 3 to 5 mo in length can positively affect lactation potential of heifers (Park et al., 1998). Whether this influence on mammary growth is due to the stair-step regimen or due to a short time period fed a high energy diet is not known. Data from younger heifers may indicate the latter. For example, increasing the energy and protein intake in calves from 2 to 8 wk of age resulted in an increase in body growth and nearly a doubling of mammary parenchymal DNA (Brown et al., 2005a). Other studies have measured an increase in milk production when heifers were fed for greater gains during the preweaning period (Bar-Peled et al., 1997; Shamay et al., 2005). Whether these positive results of feeding a high energy diet to calves were due to the younger age of the animal or the short time period fed this diet is not known. How a short duration (e.g. ≤ 6 wk in length) of feeding a high energy diet alters mammary growth in older prepubertal heifers is not known.

Several theories have been proposed to explain the nutritional impairment of mammary growth, but the mechanism is still not understood. For example, Swanson (1960) noted that twin heifers fed a high energy diet had undeveloped areas of parenchyma, whereas the glands from control twins appeared normal. Mammary parenchymal tissue accretion rates for heifers fed high or low energy diets were similar, indicating that heifers fed a high energy diet may have a shortened allometric growth phase because they reach puberty at a younger age (Meyer et al., 2004). When heifers are fed high energy diets, serum growth hormone (GH) levels decrease, but serum insulin-like growth factor-I (IGF-I) levels increase. This seems contradictory because IGF-I is a known mitogen for mammary epithelial cells. No difference in mRNA expression or concentration of IGF-I in the mammary gland was noted in prepubertal heifers fed a high or low energy diet (Weber et al., 2000b). Specific binding of IGF-I to mammary tissue was unaffected by feeding level (Purup et al., 1999). However, mammary tissue explants from heifers fed a high energy diet were less sensitive to the mitogenic activity of IGF-I compared to explants from heifers fed a low energy diet (Purup et al., 1996). One explanation for this difference in sensitivity could be that nutrition alters the number of IGF-I receptors. To my knowledge, studies analyzing nutritional effects on IGF-I receptor mRNA expression and/or quantification of IGF-I receptors in mammary epithelial cells have not been published.

In the last few years, our laboratory has focused on earlier observations of higher amounts of mammary fat deposited within the mammary gland of heifers fed high energy diets. Mammary tissue extracts from heifers fed a high compared to a low energy diet were less mitogenic for mammary epithelial cells in vitro (Weber et al., 2000a). Also, bovine mammary fat pad explants inhibited mammary epithelial cell proliferation in vitro (McFadden and Cockrell, 1993). These results indicate that adipocytes may produce a substance that inhibits mammary cell growth. Our laboratory's working hypothesis is that leptin, a protein produced by fat cells, may play a role in this inhibition. When infused into the mammary gland of prepubertal heifers, leptin inhibited the IGF-I stimulation of mammary growth (Silva et al., 2003). Whether protein concentration and mRNA expression of leptin are increased in the mammary tissue of heifers fed a high compared to a low energy diet is not known. Our current working model of the mechanism by which high energy intake impairs mammary growth relative to body growth is illustrated in Figure 1.

The objectives of this project were to determine the effects of feeding a high energy diet to prepubertal heifers for longer durations (0, 3, 6, or 12 wk) on:

1) Body growth and carcass composition
2) Mammary growth and composition
3) Protein levels of IGF-I and IGFBP within serum
4) Messenger RNA expression of IGF-I and IGF-I receptor in mammary tissue
5) Protein concentrations of leptin in serum and mammary tissue
6) Messenger RNA expression of leptin and leptin receptor in mammary tissue

The hypotheses were that:

1) Feeding a high energy diet for a short duration (3 or 6 wk) would increase the growth of mammary parenchyma, but a long duration (12 wk) of feeding a high energy diet would be detrimental to mammary growth relative to body growth in prepubertal heifers.
2) Feeding a high energy diet would increase abundance of IGF-I and IGFBP-3 and decrease IGFBP-2 in serum, but would not change mRNA expression of IGF-I in mammary tissue. Expression of IGF-I receptor mRNA in mammary parenchymal tissue would decrease as heifers were fed a high energy diet for a longer duration.

3) Feeding a high energy diet would increase leptin protein concentrations in serum and mammary parenchymal tissue and mRNA expression of leptin in mammary parenchymal tissue.

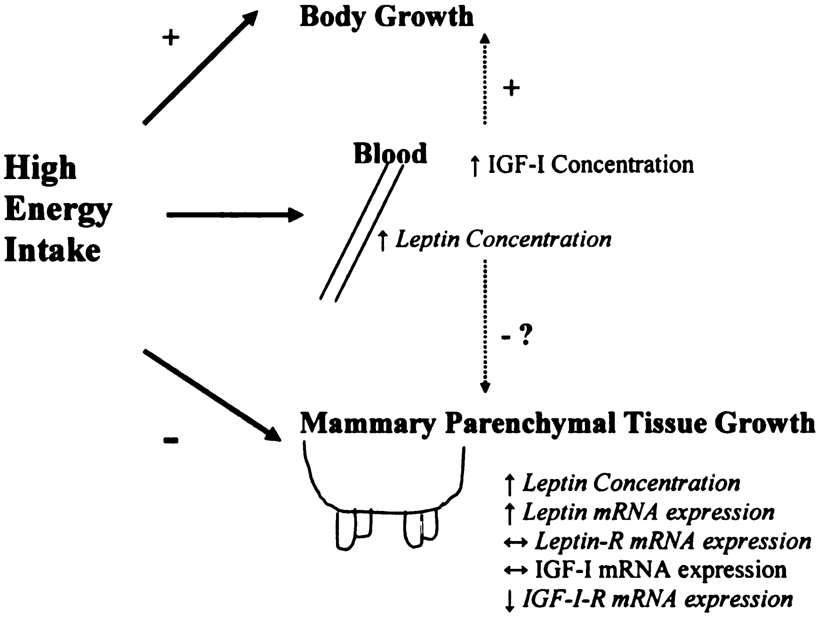


Figure 1. A proposed mechanism for why feeding a high energy diet to prepubertal heifers impairs mammogenesis relative to body growth. Items not in italics are already known, while those in italics are not known and the hypothesized effect of high energy intake on these measurements is indicated.

CHAPTER ONE

LITERATURE REVIEW

Statement of Problem

The most important factors affecting heifer management decisions are economics and how heifer growth will affect both reproduction and lactation performance (Hoffman and Funk, 1992; Swanson, 1960). The cost of raising replacement heifers accounts for approximately 20% of total dairy herd expenses (Heinrichs, 1993). Cost of raising replacement heifers from birth to calving is between $1000 and $1300 per heifer (Cady and Smith, 1996). Costs associated with raising replacements can be decreased if heifers are bred at a younger age for earlier calving, thus decreasing the number of non-productive days.

Heifers must be of adequate size for both reproductive purposes (minimizing dystocia) and lactation potential (Hoffman and Funk, 1992). Research from the mid-1970's indicated that if heifers were of adequate size, the most economical age at first calving was between 22.5 and 23.5 mo (Gill and Allaire, 1976). Recommendations for heifers calving at 22 to 24 mo of age include a pre-calving body weight of 635 kg and post-calving body weight of 570 kg, height of 56 in, and body condition score of 3.0 to 3.5 (Vandehaar, 1998b). Meeting these recommendations for size at a younger age (20 to 21 mo) is achievable if heifers are fed a high energy diet allowing for rapid gains. However, feeding heifers high energy diets for gains of greater than 1 kg/d impaired mammary growth relative to body growth and decreased subsequent milk production (Little and Kay, 1979; Petitclerc et al., 1999; Radcliff et al., 2000; Sejrsen et al., 1982;

Swanson, 1960). In addition, some researchers would argue that even gains of 0.8 to 1 kg/d are detrimental (Sejrsen et al., 2000).

The phenomenon of high energy intake impairing milk production was first published 90 years ago (Eckles, 1915). Eckles noted that excessive fleshing of heifers during the growing period might lower milk production. Although many studies have been performed since Eckles' work, there is still not a clear mechanism for why feeding a high energy diet to prepubertal heifers can hamper mammary growth and reduce subsequent milk yield.

Physiology of Mammary Development

During development of an embryo, a mammary line or ridge is formed from the thickening of epithelial cells (Anderson, 1978). This ridge thickens to a hillock that differentiates into buds that form pairs of glands. The bovine has two pairs of glands that form in the inguinal area. The cells in this region differentiate into what will become the mammary parenchyma. Mammary buds determine the number of glands and teats an organism will have, corresponding to four in the bovine. These sprouts will later give rise to the gland cistern and major ducts of each mammary gland. The mesenchyme (fat pad) grows outwardly creating pressure that forms the shape of the teat. At birth, the epithelial tissue is still rudimentary and the basic structures of the mammary gland are present. The fat pad provides the space for future development, as the parenchymal tissue grows into the fat pad in later stages during prepubertal development. The periphery of the duct in the bovine gland is surrounded by connective tissue (Woodward et al., 1993). During the prepubertal period, the fat pad and ducts that branch into it undergo rapid growth, yet the alveoli are not yet formed. Alveoli are not formed until conception (Tucker, 1987).

Mammary secretory cell numbers increase rapidly during gestation until the onset of lactation (Tucker, 1981). During gestation, mammary ducts elongate, alveoli form, and these replace lipid within the mammary fat pad (Tucker, 1969). Rate of increase in the amount of mammary parenchymal tissue in a heifer is approximately 25% per month during gestation (Swanson and Poffenbarger, 1979). There is a gradual loss of mammary cells during the course of a lactation (Capuco et al., 2001). After peak lactation, the rate of secretory cell atrophy becomes greater than cell division and milk yield begins to decrease (Knight, 2000; Knight and Wilde, 1987).

Much of the recent research concludes that feeding prepubertal heifers a high energy diet for gains of greater than 1 kg/d impairs mammary growth (Sejrsen et al., 1982; Swanson, 1960). However, feeding postpubertal heifers a high energy diet results in no detrimental effects on mammary growth (Harrison et al., 1983; Sejrsen et al., 1982). Therefore, the critical window for nutritional effects on mammary development is during the prepubertal period. During this time from approximately 3 mo until a few estrous cycles past puberty, the mammary gland is growing at an allometric rate, meaning that the gland is growing faster than the rest of the body (Sinha and Tucker, 1969). The growth and development of the mammary gland in heifers is important, as the number of mammary epithelial cells is a major factor limiting milk production (Tucker, 1981). Mammary tissue DNA content was positively correlated ($r = 0.85$) to litter weight gain in rats (Tucker, 1966). Mammary tissue DNA content from 5 mo-old heifers was also positively correlated to milk yield (0 to 30-d; $r = 0.21$) and to mammary tissue DNA collected at 60-d into first lactation (r = 0.25) (Tucker et al., 1973). The milk yield potential is determined partially by the growth of the mammary gland prior to puberty and during pregnancy (Sejrsen, 1994). This is why high energy diets fed during the prepubertal period can have long-lasting detrimental effects on milk yield. At the end of the allometric period, weight of mammary tissue is 2 to 3 kg, with 0.5 to 1 kg of this being parenchymal tissue. This parenchyma consists of 10-20% epithelium, 40-50% connective tissue, and 30-40% adipose tissue (Sejrsen et al., 1982).

Sinha and Tucker (1969) determined that in calves from birth until 2 mo of age, the growth of the mammary gland occurs at the same rate as body growth, which is referred to as isometric growth. The mechanism for the switch from isometric to allometric mammary growth occurring at 2 to 3 mo of age is not known. More recent evidence indicates that mammary growth rate was allometric by 100 kg BW and became isometric shortly before puberty (Meyer et al., 2004). Only a small streak of parenchymal tissue is evident at 1 mo of age, but growth of the mammary gland increases 60-fold by 90 d in calves (Akers et al., 2005). Therefore, recent studies indicate that the allometric phase of mammary growth relative to body growth may occur earlier than originally reported by Sinha and Tucker.

From approximately the third estrous cycle until pregnancy, the growth of the mammary gland is isometric relative to growth of the rest of the body (Sinha and Tucker, 1969). Growth of the mammary gland within an estrous cycle occurs mainly around estrus and is least during the luteal phase (Sinha and Tucker, 1969). The physiological explanation for why the growth of the mammary gland deviates to an isometric rate around puberty is unknown. However, Tucker (1981) suggested that it may be due to the asynchronous secretion of estrogen and progesterone occurring at puberty. As puberty approaches, luteinizing hormone (LH) pulse frequency increases, stimulating development of large follicles and leading to an increase in estrogen production and secretion. After puberty, secretion of progesterone is also thought to contribute to the shift from the allometric to the isometric growth phase (Knight and Peaker, 1982). However, Sejrsen (1994) suggested that termination of the allometric phase is independent of ovarian secretions since heifers that had puberty permanently delayed (immunized for gonadotropin-releasing hormone at 8 mo) had similar amounts of mammary parenchyma as heifers that reached puberty at a normal age.

Length of Prepubertal Period

One reason for why heifers fed a high energy diet have impaired mammary growth may be due to a shorter period of accelerated mammary growth relative to body growth. This allometric phase of mammary growth is concluded around the onset of puberty. Body weight is a factor known to influence the onset of puberty, and the degree of body fatness and serum leptin concentration may also play a role (Garcia et al., 2002; Schillo et al., 1992). Dairy heifers typically reach puberty at about 55% of their mature weight (NRC, 2001). On average, the onset of puberty occurs between 9 and 11 mo of age and 250 to 280 kg BW in large dairy breeds. However, this age range varies tremendously (Sejrsen, 1994). Reproductive development is more closely related to body development than actual chronological age (Schillo et al., 1992). The main source of within breed variation in age at pubertal onset is level of feeding (Schillo et al., 1992). Heifers fed a high energy diet during the prepubertal period reached puberty at a younger age than heifers fed a moderate or low energy diet (Schillo et al., 1992). Van Amburgh and co-workers (1991) suggested that consumption of excess energy before puberty could truncate parenchymal tissue growth because of a shorter period of allometric growth relative to body growth. Rapidly grown heifers had less mammary parenchymal DNA measured soon after puberty than moderate fed heifers (Niezen et al., 1996). In a recent abstract, daily mammary parenchymal DNA accretion rate was not influenced by energy intake, but heifers fed restricted energy diets had more mammary parenchymal DNA content and less fat pad weight measured between 250 to 350 kg BW (Meyer et al., 2004). Meyer et al. suggested that the reduction in mammary parenchymal DNA associated with high energy intake resulted from a shorter time to puberty instead of a decrease in epithelial cell proliferation. However, one study (Silva et al., 2002b) has analyzed age at puberty as a covariate to explain variation in mammary parenchymal DNA and did not find a significant correlation using data from Whitlock et al. (2002). It would be helpful to test other datasets given that only one study was used to test this relationship. That is, do early maturing heifers (irrespective of nutrition) produce less milk during first lactation than later maturing heifers? In addition, the physiological explanation for the switch from allometric to isometric growth and its relationship to puberty has not been determined. Some have suggested that reproductive hormones play a role in this development (Knight and Peaker, 1982; Tucker, 1981). Others have noted that concentrations of serum IGFBP-3 and leptin increase until the onset of puberty in heifers (Luna-Pinto and Cronge, 2000), but whether these factors play a direct role in the switch from allometric to isometric phases is unknown.

Growth Patterns and Requirements of Dairy Heifers

Growth is defined as an increase in tissue mass, either by hyperplasia (increase in cell number) or hypertrophy (increase in cell size). Tissues grow and develop in a sequence, starting with neural tissue, then bone, muscle, and adipose tissue (Owens et al., 1993). This is why bone percentage decreases with age, muscle percentage increases early until deposition of fat occurs, and fat percentage increases over time (Berg and Butterfield, 1976). Deposition of fat typically occurs first around the kidneys, then intermuscular, subcutaneous, and finally intramuscular areas (Owens et al., 1993). Mature size is generally considered to be attained when muscle mass reaches a maximum (Owens et al., 1993). Hyperplasia of muscle primarily occurs prenatally (Allen et al., 1979). Postnatal growth of muscle mass is by hypertrophy and satellite cell replication and incorporation, but the number of muscle fibers remains static (Goldspink, 1991). Body weight from conception until mature size and carcass weight from birth through 2 yr of age plotted against age of the animal depicts a sigmoidal curve (Berg and Butterfield, 1976; Owens et al., 1993). The point of inflection for carcass weight equaled the time point of increased fat deposition in Friesian steers (Berg and Butterfield, 1976). Age of an animal and the level of energy intake determine how dietary energy is partitioned into protein or fat synthesis (Koch et al., 1979).

Mass of visceral organs varies in weight in proportion to nutrient energy intake (Drouillard et al., 1991). As mass of the digestive tract and liver increases, an animal's maintenance energy requirements also increase. This may explain why maintenance requirements decrease during feed restriction, because weight is decreased in organs that typically undergo rapid cell turnover during re-feeding, such as the small intestine and liver. For example, in compensatory growth studies, heifers are alternately fed at below and above requirements (stair-step) or maintained at requirements. In these trials, the stair-step managed heifers gain more body weight, consume less dry matter and therefore, are more efficient compared to those maintained at requirement levels (Choi et al., 1997). During the compensatory growth phase, the stair-step managed heifers have greater gains, partly because of a lower maintenance requirement than control animals and also due to weight reductions in the liver and intestine (Carstens et al., 1991).

Body growth of dairy heifers from birth to first calving has been measured in a number of studies. For example, Kertz et al. (1998) estimated that 50% of the total height increase occurred from birth to 6 mo of age, 25% from 7 to 12 mo of age, and 25% from 13 to 24 mo of age. Approximately 25% of the total BW increase occurred from birth to 6 mo of age, an additional 25% from 7 to 12 mo of age, and the remaining 50% occurred from 13 to 24 mo of age. Feed cost per unit of gain and per unit of height increase was lowest during the first 6 mo. A positive correlation exists between height at the withers and first lactation milk yield (Heinrichs and Hargrove, 1987).

Nutritional requirements for dairy heifers at specific body weights and gaining at 3 different rates (0.6, 0.8, and 1.0 kg/d) are given in Table 1 adapted from the 2001 Nutrient Requirements of Dairy Cattle (NRC, 2001). Heifers with higher rates of gain have greater energy and protein requirements for growth (Table 1). As weight gain increases (e.g., 0.6 to 1.0 kg/d), energy proportion of the gain increases and protein proportion of the gain decreases (NRC, 2001). This is because an increase in growth rate is associated with a higher proportion of gain as fat. Since more fat is deposited at higher rates of gain, the body content of ash, protein, and water is diluted.

Few studies have been published that evaluate nutritional requirements of heifers as outlined in the 2001 NRC (NRC, 2001). However, data from a number of previously published studies were used by the NRC committee to evaluate equations for predicting energy and protein requirements for growth (Fox et al., 1999; Garrett, 1980; Waldo et al., 1997). A number of assumptions must be made using the new NRC. Requirements are given assuming that heifers are equivalent to a body condition score of 3.0. The RUP intestinal digestibility is estimated to be 67%. However, this value could be as low as 60% with mature forages or 75% with supplemental protein from concentrate sources. With mature forage diets, RUP needed for metabolizable protein would need to be increased by 10% and decreased by 10% for high concentrate diets. A potential problem is that CP requirement may be underestimated. For example, the CP requirement recommended by NRC for a large frame heifer weighing 200 kg and gaining 1 kg/d is 15.8%. Kertz and co-workers (1987) suggested that 3 to 6 mo old heifers gaining around 1 kg/d may require diets greater than 17% protein if fattening is a concern. Van Amburgh (2005) suggested that the activity level and energy needs of grazing heifers may be greater than recommended and that actual gains of non-grazing heifers are typically higher than those predicted by the 2001 NRC model.

Table 1. Nutritional Requirements for Dairy Heifers.

Mature Wt*	**Live BW during growth (kg)**						
650 kg Holstein	200	250	300	350	400	450	500
800 kg Holstein	246	308	369	431	493	554	616
SWG (kg/d)#	**NEg required (Mcal/d)**						
0.6	1.34	1.58	1.81	2.03	2.25	2.46	2.66
0.7	1.83	2.17	2.48	2.79	3.08	3.37	3.64
1.0	2.34	2.77	3.17	3.56	3.94	4.30	4.65
SWG (kg/d)	**Net protein required for growth (g/d)**						
0.6	122	114	108	101	95	89	83
0.8	161	151	141	132	124	115	107
1.0	199	187	175	163	152	142	131
SWG (kg/d)	**Metabolizable protein required for growth (g/d)**						
0.6	182	183	185	187	190	194	199
0.8	241	241	243	245	248	253	259
1.0	299	299	300	302	305	310	316

Adapted from NRC, 2001

*Mature weights are full body weights

#SWG = shrunk body weight (96% of full body weight)

Nutrition: Restricted Compared to Ad libitum Feeding

Nutritional studies have been performed to better explain how dietary manipulation can have an effect on mammary growth in prepubertal heifers. Some studies involved feeding of the same diet to both treatments, but restricted intake fed to the heifers on the lower diet. Sejrsen and others (1982) fed a 60:40, concentrate to forage ratio diet either ad libitum or restricted to 60% DMI. Daily gains were 613 and 1218 g for the restricted and ad libitum groups, respectively. All heifers were slaughtered at 320 kg of body weight. Mammary parenchymal DNA content was reduced and mammary adipose tissue weight was increased by ad libitum feeding. In another study (Petitclerc et al., 1999), heifers were given a grass hay and concentrate diet fed for 700 g/d or for ad libitum intake. Actual gains were 615 and 954 g/d for restricted and ad libitum groups, respectively. Heifers started treatments at 6 wk and then were slaughtered at 4 mo of age. After adjusting for BW at slaughter, amount of parenchymal tissue was reduced in heifers fed for ad libitum intake. Milk production was 8% greater (305-d, fat corrected) in heifers grown at standard rates compared to accelerated rates from 4.5 to 9.5 mo of age (Lammers et al., 1999). In this study, heifers were fed the same diet with intakes allowing for 0.7 and 1.0 kg/d of gain. These studies suggest that when heifers are allowed to eat ad libitum and gain approximately 1.0 kg/d or greater they have impaired mammary growth relative to body growth and decreased milk production compared to heifers with restricted intake of the same diet.

Nutrition: Different Diet Composition

Other studies have fed two different diets resulting in a difference in energy and protein concentrations of the diet and in daily intake amounts. During the prepubertal period, Little and Kay (1979) fed a diet consisting of 80% rolled barley and 20% grain to heifers for actual gains of or exceeding 1 kg/d. Heifers fed a low energy diet were grazed during the summer with supplement or were fed a concentrate mix and hay during the winter for gains of approximately 0.6 kg/d. Half of the heifers fed the high energy diet were first mated at an average of 43 wk of age and weighed an average of 302 kg at breeding. The other half of the rapidly reared heifers and all of the low fed heifers were first mated at an average of 78 wk of age and averaged 443 and 353 kg BW, respectively. Milk yield (305-d, fat corrected), during the first lactation was 58% and 97% higher for the heifers fed the low energy diet than the rapidly reared heifers that were mated at 78 and 43 wk, respectively. Early age at calving and high energy intake significantly reduced milk production during the first lactation. Radcliff and others (2000) fed a standard diet of 10% grain and 90% haylage (16.3% CP; 0.6 Mcal/kg NEg), while the high energy diet consisted of 75% grain and 25% haylage (19.4% CP; 1.2 Mcal/kg NEg). Heifers fed the standard and high energy diet gained 0.77 and 1.15 kg/d, respectively, from 3 or 4 mo of age until confirmed pregnant. In the Radcliff study, standard fed heifers produced 15% more milk (projected 305-d; $P < 0.01$) during their first lactation than heifers fed the high energy diet. Prior to this study, Radcliff and co-workers (1997) published a study with a similar design but measured mammary growth of heifers slaughtered during the fifth estrous cycle after the onset of puberty. In that study, there was no effect of diet on mammary parenchymal tissue mass, but high energy intake did increase the amount of extraparenchymal fat. The authors suggested that high energy intake may have not affected parenchymal mass due to the high level of protein (19.4%) within the high energy ration. Other reports have noted a detrimental effect on mammary growth when heifers were fed high energy diets with lower amounts of protein (Petitclerc et al., 1984; Sejrsen et al., 1982). Radcliff concluded that high dietary protein intake might overcome the negative effects of high dietary energy on mammary growth. However, Radcliff and co-authors did not speculate on why there was a detrimental effect of high energy diets on milk production but not on mammary development. In the milk production study, heifers fed the high energy diet during the prepubertal period were only 11 mo old at first insemination, and gained less weight and lost body condition during gestation compared to standard fed heifers. However, in a different study, slow growth rates during gestation did not hamper milk production (Lacasse et al., 1993). In another study, heifers gained 0.6, 0.8, or 1.0 kg/d during the prepubertal period (Van Amburgh et al., 1998b). Post-treatment daily gain was greater for those heifers gaining 0.6 kg/d during the treatment period and resulted in these heifers fed the low diet being larger in BW at calving but less conditioned than heifers on a high plane of nutrition. Although actual 305-d and fat corrected milk yields were reduced by high gains, postcalving BW accounted for more variation in milk yield than prepubertal BW gain. Van Amburgh et al. (1998b) suggested that postpubertal management may have impacted lactation yield and that lighter postcalving BW could have an effect on DMI and nutrient partitioning for growth during first lactation.

Nutrition: Effect of Dietary Protein and Energy

Since mammary development and subsequent milk yield are not always both decreased during high energy intake in heifers, other dietary components or management factors may play a role in these discrepancies. VandeHaar (1998b) noted that one of these factors may be the ratio of protein to energy in the diet. VandeHaar suggested that feeding high protein diets (65 g CP/ Mcal ME) could potentially reduce the detrimental effect of feeding high energy diets on mammary development. To address the question of high compared to low protein fed in conjunction with high energy diets, studies were designed in which the ration was constant in energy but differed in protein concentrations. Increasing CP:ME ratios in the diet (48, 59, 68, 77 g CP/ Mcal ME) resulted in linear increases in feed efficiency and structural growth (Gabler and Heinrichs, 2003). These measurements along with rate of gain and indirect measures of mammary growth (change in teat length) were increased in prepubertal heifers fed diets containing higher ratios of CP:ME (46, 54, and 61 g CP/ Mcal ME) from 200 to 341 kg BW (Lammers and Heinrichs, 2000). But, measurement of teat length as an indirect indicator of mammary growth is questionable (Whitlock et al., 2002). No differences in weight of mammary parenchymal tissue or mammary fat were evident when rapidly grown heifers were fed rations with CP:ME ratios of 48.1, 56.8, or 66.0 g CP/ Mcal ME (Whitlock et al., 2002). In addition, no advantage in gains or skeletal growth with a higher CP diet was evident in the Whitlock study. Further analysis using regression predictions indicated that heifers reaching puberty early had less mammary parenchyma if fed the low protein compared to high protein diet. Dobos et al. (2000) found that heifers fed high CP diets (18.2%) had less mammary fat than heifers fed a low CP diet

(14.2%), but secretory tissue area and milk yield were not altered by dietary CP concentration. In a fourth study, prepubertal heifers fed corn silage to gain 950 compared to 725 g/d had lower amounts of mammary parenchymal DNA and RNA, whereas mammary growth was not changed in heifers fed alfalfa silage to achieve different daily gains (Capuco et al., 1995). No effect of rate of gain on milk production was evident with either the corn or alfalfa silage diet. VandeHaar (1998a) noted that the difference in mammary growth of heifers in the Capuco study that had accelerated versus restricted gains on a corn silage diet could be due to the lower protein content of the corn silage diet (54 g CP/ Mcal ME) compared to the alfalfa silage diet (83 g CP/ Mcal ME). Recent evidence supports feeding of more dietary protein as heifers that were supplemented with fish meal to supply 2% CP in the diet fed from 180 to 270 d of age had 5% greater fat-corrected daily milk yield (Shamay et al., 2005). However, the standard diet contained only 13.2% CP. High dietary protein compared to moderate levels of protein may be one way to increase gains without causing impairment of mammary development, but overall the data are inconclusive and more studies need to be performed to better understand how dietary protein affects mammary development.

The amount of rumen undegradable protein included in the diet has also been tested. The amount of by-pass protein included in the diet (rapeseed meal vs. urea) did not affect mammary development when heifers gained between 0.65 and 0.90 kg/d (Mantysaari et al., 1995). Capuco et al. (2004) fed heifers either a control diet of 14.9% CP and 5.9% RUP or supplemented the diet with an additional 2% RUP (Capuco et al., 2004). Mammary parenchymal DNA, Ki-67 labeling of epithelial cells, and lipid content were not affected by RUP supplementation. Daily gains were between 0.90 and 1.07

kg/d and were significantly higher for RUP supplemented heifers, averaging 0.10 kg/d higher than heifers on the control diet. Increasing the RUP content of the diet may be one way to increase growth rates for earlier breeding without causing a detrimental effect on mammary development.

Nutrition: High Energy Diets Fed for a Short Duration

Results of most studies indicate that feeding prepubertal heifers a high energy diet to promote rapid gains is detrimental to mammary growth relative to body growth and reduces subsequent milk production. Very few of these studies focused on the effects of a high energy diet fed for a short duration on mammary growth. The shortest treatment period discussed so far in this review was about 11 wk (Petitclerc et al., 1999), which resulted in an impairment of mammary growth (adjusted for BW) when heifers were allowed ad libitum intake for gains of approximately 1 kg/d. Most other published studies involve treatment periods of 5 mo in length or longer. A question remaining is how feeding prepubertal heifers a high energy diet for a short duration of time (≤ 6 wk) affects mammary growth.

Compensatory growth studies indicate that a stair-step feeding regimen of alternating feed intake of heifers by 25 – 30% above recommendations for 2 mo and 20 – 30% below recommendations for 3 to 5 mo in length can positively affect the lactation potential of heifers (Park et al., 1998). Whether this influence on mammary growth is a result of the stair-step regimen or a short time period fed a high energy diet is not known. Data from studies that evaluated different levels of energy intake during the preweaning phase suggest the latter may be true, since these studies were performed for a short time period. In one of these studies, calves were either allowed to suckle the dam 3 times a day or were fed milk from a bucket for the first 6 wk of age. Calves allowed to suckle had higher daily gains during the treatment period and also tended to produce more milk during the first lactation (Bar-Peled et al., 1997). In a recent study, calves were either fed milk replacer resulting in gains of 0.59 kg/d or given free access to whole milk for two 30-min intervals/day for gains of 0.88 kg/d (Shamay et al., 2005). Feeding whole milk to calves affected BW but not skeletal size of the adult animal and increased milk yield by 4% during first lactation (daily 3.5% fat corrected; $P < 0.01$) compared to calves fed milk replacer. In a more controlled study, calves were fed two types of milk replacer and starter grain from 2 to 8 wk of age with gains of 0.38 and 0.67 kg/d for moderate and high energy diet, respectively (Brown et al., 2005a). Calves fed the high energy diet during this period had more fat-free tissue, DNA, and RNA in the mammary parenchyma, but also had more extraparenchymal fat than calves fed the moderate diet. Calves not slaughtered during the first period were fed either a low or high energy diet until 14 wk of age during period 2. Diet during the second period did not alter amount of mammary parenchyma. Results from the above studies show that increasing energy intake of calves during the preweaning phase causes no detrimental effects on mammary growth and subsequent milk yield, and may actually be beneficial to mammogenesis and future milk production. These results differ from other studies that observe a detrimental effect of high energy intake on mammary growth relative to body growth in older prepubertal heifers. Whether these positive results of feeding a high energy diet to calves are due to the younger age of the animal is not known. This differential response of high energy intake on mammary development from preweaning to postweaning phases as seen in the Brown study (2005a) and in studies using older heifers may be due to changes in the growth of the mammary gland. The mammary gland grows at an isometric rate compared to overall body growth during the first few mo of life and then switches to an allometric rate until around puberty (Sinha and Tucker, 1969). Recent evidence indicates that the mammary gland is undergoing allometric growth relative to body growth in calves at 100 kg BW and the allometric phase ends shortly before puberty (Meyer et al., 2004). The change in growth patterns from isometric to allometric rates coincides with the time period when high energy intake impairs mammary growth relative to body growth. In these preweaning period studies, heifer calves were also fed a high energy diet for a shorter period of time compared to studies using older prepubertal heifers. However, mammary tissue extracts from prepubertal heifers fed a high energy diet for only 5 wk were less mitogenic than those from heifers fed a low energy diet (Berry et al., 2003; Weber et al., 2000a). How a short duration (e.g. ≤ 6 wk in length) of feeding a high energy diet affects mammary growth in older prepubertal heifers has not been published.

Body Fatness

Another idea that has evolved recently is the relationship between the degree of body fatness and the impairment of mammary development and milk yield. Data collected from 2 studies (Radcliff et al., 2000; Whitlock et al., 2002) were used to identify factors accounting for variation in milk production and mammary growth (Silva et al., 2002b). A significant covariate for milk production was body condition score at breeding with prepubertal BW gain, gestational BW gain, postpartum BW gain, postpartum BW, and BCS at calving within treatment also tested and not significant covariates. A significant covariate for mammary parenchymal DNA was body fat content at slaughter with BW at slaughter, age at puberty, prepubertal BW gain, and body protein at slaughter also tested and not significant covariates. Results suggest that increased body fatness may be a better indicator of impaired mammary growth than daily gain. Obesity is also linked to impaired mammary development and lactogenesis in rodents (Flint et al., 2005; Rasmussen et al., 2001). Whole mount analysis of mammary tissue from pregnant mice showed abnormal ductal and alveolar development and less parenchyma per unit area was evident in obese compared to lean mice (Flint et al., 2005).

Heifers fed high energy diets typically have an increase in fat deposition in locations such as carcass, perirenal, and mammary extraparenchymal tissues (Radcliff, 1995; Swanson, 1960). Swanson (1960) noted that twin heifers fed a high energy diet had undeveloped areas of parenchyma, whereas the glands from control twins appeared normal. The growth of mammary epithelial cell organoids is inhibited when co-cultured with bovine mammary fat pad explants (McFadden and Cockrell, 1993). This finding suggests that mammary fat may secrete a factor that inhibits mammary epithelial cell growth. Also, mammary tissue extracts were less mitogenic for mammary epithelial cells in vitro from heifers fed a high compared to a low energy diet (Berry et al., 2003; Weber et al., 2000a). The idea that high energy intake increases mammary fat, which might secrete an inhibitory factor, has led our laboratory to further investigate potential factors produced by adipose tissue that may inhibit mammary growth. Leptin, a protein mainly produced by adipocytes (Chilliard et al., 2001), but also produced by bovine mammary epithelial cells (Smith and Sheffield, 2002), was chosen as a candidate for further research.

Leptin

The lipostatic theory, proposed by Kennedy (1953), explains that energy balance, body weight, and body composition in mammals are regulated by a hypothalamic feedback loop whereby fat reserves control food intake and energy expenditure. Leptin, a component of this system, has many functions including regulation of appetite, regulation of energy expenditure, nutrient partitioning, hormone secretion, reproduction, immune function, etc. (Chilliard et al., 2001). Leptin, a 16 kDa protein, was discovered over a decade ago as the product of the *ob* gene (Zhang et al., 1994). In *ob/ob* mice, a mutation occurs in the *ob* gene and causes these mice to be obese. This obesity was partially cured when these *ob/ob* mice were parabiosed to lean mice (Hausberger, 1959).

Leptin regulation of energy homeostasis is mediated in the hypothalamus. Leptin regulates the synthesis of neurotransmitters involved in food intake and secretion of growth hormone from the pituitary (Carro et al., 1997). Leptin inhibited the synthesis of neuropeptide Y (NPY), leading to a reduction in food intake (Erickson et al., 1996). Intracerebroventricular infusions of leptin into ewes caused a reduction in appetite (Henry et al., 1999). Regulators of leptin include proopiomelanocortin, melanocortin stimulating hormone, and agouti-related peptide (Houseknecht and Portocarrero, 1998). Leptin may also have a role in regulating the neuroendocrine mechanisms involved in the partitioning of energy (Ahima and Flier, 2000). For example, when animals were fasted or underfed, low plasma leptin supported the conservation of energy at the expense of reproduction and immunity (Ahima and Flier, 2000).

Leptin mRNA expression is highly correlated to fat mass, adipocyte size, and basal metabolic index (Houseknecht and Portocarrero, 1998). Plasma leptin concentration was positively related to adiposity in growing and mature ruminants (Blanche et al., 2000; Delavaud et al., 2000; Ingvartsen and Boisclair, 2001). Bull calves (1 to 8 wk of age) fed for higher rates of gain had higher concentration of plasma leptin and more body fat content than calves fed for lower gains (Block et al., 2003b; Ehrhardt et al., 2000). This effect of energy intake on serum leptin in heifer calves was apparent from 3 to 6 wk of age, but not apparent from 7 to 14 wk of age (Brown et al., 2005b). Fasting for 48 hr lowered leptin gene expression in adipose tissue and concentration of serum leptin in prepubertal beef heifers (Amstalden et al., 2000). In addition, the onset of negative energy balance in lactating dairy cows either at parturition or induced by feed restriction was associated with reduced plasma leptin and insulin and increased plasma growth hormone (GH) concentrations (Block et al., 2001; Block et al., 2003a). Source of fat within the diet (calcium salts of palm fat or conjugated linoleic acid) had no effect on plasma leptin concentrations when prepubertal heifers were fed for gains of 1 kg/d (Block et al., 2003b). It remains to be seen whether dietary energy levels affect plasma leptin and leptin expression in older prepubertal dairy heifers.

Leptin has been proposed as a signal that links body weight and adiposity to the onset of puberty (Chehab, 2000). Plasma leptin remained fairly constant (2.3 ng/ml) until about 1 yr of age in prepubertal dairy heifers at which point leptin increased until 400 d of age (Block et al., 2003b). This age corresponds to the time when nutrients are being increasingly partitioned to fat deposition instead of lean gain. These results may indicate that leptin increased prior to puberty, but this was only true of heifers reaching puberty at a later age (414 d) who were also heavier than those reaching puberty at an earlier age (286 d). In another study, weekly serum leptin concentration and leptin expression in adipose tissue of beef heifers increased from 16 wk prior until the onset of puberty (Garcia et al., 2003; Garcia et al., 2002). Body weight accounted for the most variation associated with onset of puberty, but leptin concentration was closely related to body weight and without body weight in the model was most predictive of pubertal onset.

Our laboratory has recently performed experiments that provide evidence for leptin as a candidate in mediating the inhibitory effect of high energy intake on mammary growth. Leptin protein is present in bovine milk and is produced by a bovine mammary epithelial cell line (MAC-T) (Smith and Sheffield, 2002). Bovine mammary epithelial cells from prepubertal heifers and MAC-T cells express the long form of the leptin receptor (Ob-Rb) but not the short form (Silva et al., 2002a). Physiological concentration (2 – 6 ng/mL) of leptin inhibited IGF-I-stimulation and FBS-stimulation of DNA synthesis in MAC-T cells (Silva et al., 2002a). However, this inhibitory effect of leptin on IGF-I action was not replicated in bovine primary epithelial cells isolated from prepubertal heifers (Purup and Sejrsen, 2000). Intramammary infusion of leptin caused a 48% decrease in the stimulatory effects of IGF-I on Brd-U labeling of mammary epithelial cells in prepubertal heifers (Silva et al., 2003). Intramammary infusion of leptin also decreased Brd-U labeling of mammary epithelial cells by 19% in saline-treated quarters. But, these concentrations used in vivo were supraphysiological, and it is not known if a physiological level of leptin can inhibit mammary growth. Mammary extracts from heifers fed a high energy diet were less mitogenic than mammary extracts from heifers fed a low energy diet when used as treatments with MAC-T and primary bovine mammary epithelial cells (Berry et al., 2003; Weber et al., 2000a). These results suggest that changes in growth factor concentrations in mammary parenchymal tissue are at least partially modulated by feeding level. However, it is not known whether prepubertal heifers fed a high energy diet compared to a moderate or low energy diet have greater leptin gene expression and leptin protein levels in mammary tissue and if this correlates to a decrease in mammary development.

Administration of GH to heifers increased daily gain and mammary parenchymal DNA and RNA content without changing body composition (Radcliff et al., 1997). Recent research indicated that GH may be a potential inhibitor of leptin within mammary tissue. For example, GH treatment in vitro decreased leptin mRNA expression in bovine mammary epithelial cells (Yonekura et al., 2005). Moreover, GH administration to heifers fed a high energy diet decreased leptin mRNA expression in mammary parenchymal tissue compared to heifers fed a high energy diet and not treated with GH (Lew et al., 2005).

Growth Hormone (GH) / Insulin-like Growth Factor-I (IGF-I) Axis

Older experiments have clearly demonstrated that growth hormone (also known as somatotropin, bST) is required for normal mammary development (Forsyth, 1989; Sejrsen, 1994). Exogenous administration of GH increased milk production in cows (Peel and Bauman, 1987) and stimulated peri-pubertal mammary growth in sheep (McFadden et al., 1990) and dairy heifers (Sandles and Peel, 1987; Sejrsen et al., 1986). However, local infusion of GH into the mammary gland did not increase milk production in sheep (McDowell and Hart, 1984) indicating that GH does not act directly on mammary epithelial cells to stimulate milk production. Moreover, mammary tissue did not bind GH, and GH did not stimulate cell proliferation in cultures of mammary epithelial cells (Akers, 1985; Purup et al., 1993; Sejrsen, 1994). However, Glimm and colleagues (1990) used molecular hybridization analysis to detect GH-R mRNA in lactating bovine mammary tissue. In addition, GH-R mRNA was detected via Northern blots in mammary tissue from prepubertal heifers and band density was not changed with feeding level (Purup et al., 1999). The receptor gene may be transcribed but it is not known whether the mRNA is translated.

Much evidence suggests that the effect of GH on the mammary gland is mediated indirectly through the IGF-I system (Akers, 1985; Kleinberg, 1997; Sejrsen, 1994). Insulin-like growth factor-I, a 7.6 kD protein, is primarily synthesized and secreted by the liver when GH binds to hepatic GH receptors (Gluckman et al., 1987; Wong et al., 1989). However, many tissues produce the IGF-I peptide including heart, lung, skeletal muscle, and the gonads (D'Ercole et al., 1984). Vander Kooi et al. (1995) observed increases in serum IGF-I concentration, hepatic IGF-I mRNA abundance, and serum IGFBP-3 abundance after intravenously infusing lactating cows with either GH or GH Releasing Hormone (GHRH). A subcutaneous injection of GH in lactating dairy cows and goats resulted in increased milk yield (Bauman and Vernon, 1993; Faulkner, 1999). A greater increase of IGF-I in milk compared to plasma was noted and it was suggested that changes in IGF-I concentrations within the mammary gland occur prior to the general circulation (Faulkner, 1999). IGF-I (-/-) null mice had significantly less mammary development than same age wild-type controls (Ruan and Kleinberg, 1999). Administration of IGF-I, but not GH or estrogen, had a stimulatory effect on mammary development in these null mice. Thus, even when GH is present, mammary development is reduced unless IGF-I is present. IGF-I is a major mitogen for mammary epithelial cells when infused into the mammary gland of cattle (Collier et al., 1993; Silva et al., 2005).

IGF-I treatment stimulated the growth of primary bovine (Shamay et al., 1988) and ovine (Winder et al., 1989) mammary epithelial cells and MAC-T cells (Zhao et al., 1992). Cell stimulation is caused by IGF-I binding to membrane receptors on secretory epithelial cells and is detected as an increase in DNA synthesis in myoepithelial, ductal, and alveolar epithelial cells (Baumrucker and Stemberger, 1989; McGrath et al., 1991). Bovine mammary epithelial cells express IGF system receptors (IGF-I, -II, and insulin) and do not produce IGF-I (Hadsell et al., 1990). IGF-I is produced in the stromal portion of the mammary gland; therefore, a paracrine role for IGF-I action in the epithelium has been suggested (Hauser et al., 1990). However, data from Hodgkinson and co-workers (1991) suggests that blood is the major source of IGF-I in the mammary epithelium. Weber and co-workers (1999) measured IGF-I concentrations in serum and extracts of mammary tissue of heifers, finding averages of 107 ng/mL and 133 ng/g, respectively.

In 1983, Sejrsen and others suggested that the negative influence of excess feeding on mammary growth in prepubertal heifers might be associated with the decrease in circulating GH concentration noted in these heifers. Mammary growth and milk production are positively correlated with plasma concentration of GH (Bauman and Vernon, 1993; Sejrsen et al., 1983). Serum concentration of IGF-I is generally increased, not decreased as with GH, in steers and heifers fed for high rates of gain compared to low rates of gain (Breier et al., 1986; Vestergaard et al., 1995). This high rate of body growth in heifers is associated with decreased mammary development. Possible explanations for why this contradiction occurs may be due to changes in the IGFBP profile, differences in sensitivity of mammary tissue to IGF-I, local numbers or binding capabilities of IGF-I receptors, local production of IGF-I in the mammary gland, potential inhibition of IGF-I stimulation via leptin (previously discussed) and/or another factor that has not been elucidated.

Administration of somatotropin increased growth rate and decreased carcass fat in beef steers (Moseley et al., 1992). Bovine somatotropin (bST) administration to heifers caused an increase in mammary parenchymal tissue and a decrease in extraparenchymal adipose tissue (Sejrsen et al., 1986). Other studies have also investigated whether bST administration can lessen the negative effects of high energy intake on fat deposition and mammary parenchymal development. Overall, injection of bST increased the content of mammary parenchymal DNA, RNA, and the ratio of RNA: DNA (Radcliff et al., 1997). Injection of bST also increased daily BW gain but had no effect on body condition or age at puberty. Body weight and withers height taken at puberty were increased with administration of bST. Using the same dietary treatments, Radcliff and co-workers (2000) were unable to measure a difference in milk production in heifers fed high energy diets and treated with or without bST. However, heifers fed the standard diet were similar in milk production to heifers fed the high energy diet and treated with bST. Heifers fed the standard diet gave 15% more milk (projected 305-d; $P < 0.01$) than heifers fed the high energy diet without bST treatment. Therefore, injection of bST in conjunction with feeding a high energy diet during the prepubertal period was able to decrease age at calving ($P < 0.01$) without reducing milk production (Radcliff et al., 2000). In a recent study with only 3 heifers per treatment for each age group, bST administration gave no positive effects on prepubertal mammary growth (Capuco et al., 2004), although ADG and skeletal growth were greater with bST injection and the weight ratio of fat:parenchyma was decreased with bST treatment. Therefore, it may be possible to use bST to negate the detrimental effect of feeding prepubertal heifers a high energy diet on mammary growth and milk production.

Another reason for why high energy feeding decreases serum GH but increases serum IGF-I concentrations could be due to a negative feedback mechanism (see Figure 1). GH-releasing hormone (GHRH) is produced by the hypothalamus and regulates the amount of GH synthesized and secreted from the pituitary (Thissen et al., 1994). GH secretion is controlled by both the stimulatory influence of GHRH and the inhibitory effect of somatostatin (SRIF, somatotropin releasing inhibitory factor). In pituitary cells, IGF-I inhibited the GHRH-stimulated GH secretion by 67%. High levels of circulating IGF-I may provide a negative feedback mechanism by either decreasing the amount of GHRH secreted or increasing the amount of SRIF secreted, thereby decreasing the amount of GH synthesized and released from the pituitary (Ceda et al., 1987).

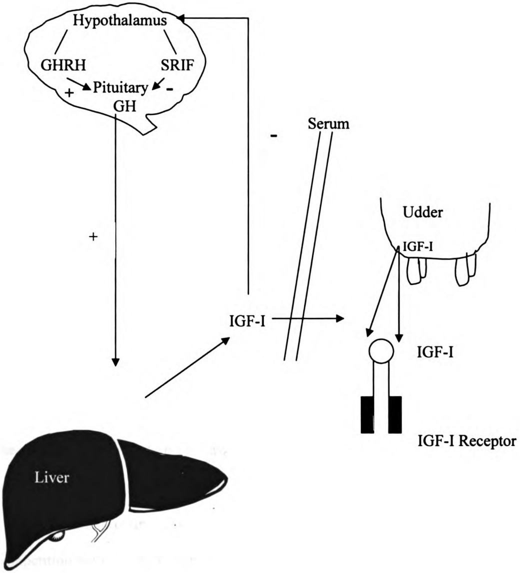


Figure 1. Overview of Growth Hormone (GH) / Insulin-like Growth Factor-I (IGF-I) axis. See text for description.

IGF Binding Proteins (IGFBP)

The IGF-I ligand can be found in the circulation in three different forms. Little free IGF-I is found in the circulation. Approximately 80% of all circulating IGF-I is bound to IGFBP. Of this bound IGF-I, 80% is part of a 140-kD ternary complex, which is formed by binding to the acid-labile subunit (ALS) and either IGFBP-3 or-5. The other 20% is bound to single binding proteins. These binary complexes are small enough to cross the capillary endothelium, but the ternary complex is too large (Baxter, 1993).

The function of a labile pool of IGF-I in the circulation is to provide an available source of IGF-I for delivery to target tissues. Regulation of this labile pool is through the IGFBP. These multifunctional proteins have the capacity to both inhibit and enhance IGF-I actions. The binding proteins assist in transporting IGF-I to target organs and tissue via the blood stream. Binding proteins function to extend the half-life of IGF-I in the circulation (mainly IGFBP-3 associating with the ALS), transport IGF-I from the vasculature to tissues, and to localize IGF-I to specific target tissues and cells (Cohick, 1998). Binding proteins may act by inhibiting the bioactivity of IGF-I through competition with receptors, interacting with other growth factors, or by acting independently of IGF-I (Baumrucker and Erondu, 2000; Oh, 1998). Six high affinity BP (IGFBP 1-6; (Keifer et al., 1991) and lower affinity IGFBP-related proteins (Hwa et al., 1999) are able to bind to IGF and alter its activity. For example, addition of IGFBP-3 reduced the mitogenic activity of serum and IGF-I treatments on mammary epithelial cells (Weber et al., 1999). Addition of IGFBP-3 to basal media also inhibited DNA synthesis in bovine mammary epithelial cells (Weber et al., 1999). Few studies have indicated the effects of other IGFBP on the proliferation of bovine mammary epithelial cells, thus their role in mammary epithelial cell growth is unclear. Bovine mammary epithelial cells from pregnant and lactating cows synthesize IGFBP-2 (34-kD), IGFBP-3 (46 and 42-kD), IGFBP-4 (24-kD), and IGFBP-5 (30-kD) (Gibson et al., 1999). Mammary tissue extracts from prepubertal heifers contained IGFBP-2 (32-kD), IGFBP-3 (40 to 43-kD), and IGFBP of 28-kD and 24-kD (putatively IGFBP-1 and -4) (Weber et al., 2000b). IGFBP-3 constitutes the majority of IGFBP in the serum of heifers (McGrath et al., 1991) and in bovine mammary tissue extracts (Weber et al., 2000b). It was suggested that bioavailability of IGF-I to tissues is regulated by serum IGFBP levels, whereas the IGF-I activity in mammary tissue is controlled by local IGFBP levels (Lemozy et al., 1994).

Circulating levels of IGF-I change over the lifetime of a cow. Plasma concentration of IGF-I is low in the newborn calf and rise following birth, increasing from 50 to 450 ng/mL from 1 to 45 weeks of age (Skarr et al., 1994). The postnatal rise in serum IGF-I is suggested to occur because of the maturation of the somatotropic axis in the liver and the onset of GH-dependent IGF-I release (Cordano et al., 2000). It is difficult to distinguish between the effect of nutrition and body size on IGF-I since there is a positive correlation among ADG, BW, and IGF-I concentration (Kerr et al., 1991). Calves fed a high energy diet from 2 to 8 wk or from 8 to 14 wk of age compared to calves fed a low or moderate diet had increased plasma IGF-I concentration (Brown et al., 2005b). A similar response in serum IGF-I concentration to dietary intake was also seen with older heifers in various studies (Petitclerc et al., 1999; Radcliff et al., 2004). In adult cows, serum IGF-I concentration fluctuates depending on the physiological state of the animal and is inversely related to milk production (Ronge et al., 1988; Vega et al., 1991).

Abundance of serum IGFBP-3 and IGF-I are generally decreased during feed deprivation, while IGFBP-1 and IGFBP-2 are increased. It was suggested that the GH / IGF-I axis is uncoupled during severe feed restriction and this restriction may even abolish the ability of GH to increase IGF-I (Bauman, 1999). Two studies have investigated how feeding level and bST administration regulate the expression and concentration profiles of IGF-I and IGFBP in a number of tissues. These two studies were similar in design, with heifers fed high or low energy diets and with or without inclusion of bST treatment (Radcliff et al., 2004; Weber et al., 2000b), although the treatment period was much shorter in the Weber study. Feeding heifers a high energy diet resulted in an increase in liver IGF-I mRNA abundance and serum IGF-I concentration (Radcliff et al., 2004). Administration of bST also increased IGF-I levels in the serum and liver mRNA (Radcliff et al., 2004). High fed heifers had lower liver IGFBP-2 mRNA abundance and serum IGFBP-2 compared to low fed heifers (Radcliff et al., 2004). However, dietary treatments did not alter the expression or concentration of IGFBP-3. Expression of IGFBP-2 and IGFBP-3 mRNA were unchanged in mammary tissue from bST-treated heifers or high fed heifers (Weber et al., 2000b). High feeding level reduced the expression of IGFBP-1 mRNA in the mammary gland. IGF-I mRNA expression in mammary tissue was not influenced by nutrition. The combination of bST administration and high feeding level increased IGF-I concentration in mammary tissue extracts compared to other treatments. High feeding level decreased protein levels of IGFBP-2 and increased the abundance of a 24-kD IGFBP in mammary tissue extracts.

Somatotropin tended to increase the abundance of IGFBP-3 in mammary tissue extracts. In summary, high feeding level increased IGF-I concentration in the serum and IGF-I mRNA expression in the liver but not in mammary tissue. Feeding level was capable of altering the IGFBP expression profile within the mammary gland and in the liver. Administration of bST increased both mRNA expression of IGF-I in the liver and serum IGF-I concentration. Injection of bST had no effect on mRNA expression of IGF-I in mammary tissue and concentration of IGF-I was only increased in mammary tissue extracts when bST is used in combination with high energy intake.

Sensitivity of Mammary Tissue to IGF-I

Heifers fed high energy diets have impaired mammary growth relative to body growth. Yet, high energy intake increases IGF-I, a known mammary mitogen, in serum and mRNA expression in the liver of prepubertal heifers. This contradiction could be explained by differences in receptor numbers or in the sensitivity of mammary tissue. In addition, no diet-related change in the mRNA expression or concentration of IGF-I in the mammary gland of prepubertal heifers were noted (Weber et al., 2000b). It is possible that the negative effect of high energy intake on mammary growth may be due to decreased sensitivity to IGF-I. A study using mammary tissue explants from prepubertal heifers fed a high plane of nutrition showed a decrease in mammary sensitivity to IGF-I treatment compared to explants from heifers fed a low plane of nutrition (Purup et al., 1996). However, IGF-I and IGFBP are expressed and secreted by mammary tissue and the IGFBP profile is modulated by feeding level (Weber et al., 2000b), so it is difficult to determine if this difference in mitogenic response was due solely to differences in tissue sensitivity. Another study indicated that specific binding of IGF-I to mammary membranes was similar in mammary tissue from heifers fed a low and high energy diet (Purup et al., 1999). Thus, the negative effect of high feeding level on mammary development probably cannot be explained by changes in binding to IGF-I receptors. However, serum IGF-I concentration was not different between dietary treatments in the Purup study (Mantysaari et al., 1995; Purup et al., 1999), and differences in daily gain were marginal (674 and 848 g/d). Further research is needed to more accurately determine the influence of energy intake on IGF-I receptor binding parameters.

Another explanation of the negative effect of high energy feeding on mammary growth could be that the number of IGF-I receptors is altered by nutritional modulation. To my knowledge, studies analyzing IGF-I receptor expression and/or quantifying IGF-I receptors in mammary epithelial cells in heifers fed different diets have not been published.

Other Factors with a Potential Role in Nutritional Modulation of Mammary Growth

Expression of mRNA for all three forms of transforming growth factor-β (TGF-β) were identified in the mammary gland of mature cows and in calves (Maier et al., 1991). Physiologically, TGF-β_1 has a biphasic response. That is, depending on the concentrations used in cell culture, TGF-β_1 can be either inhibitory or stimulatory to mammary epithelial cell proliferation (Purup et al., 2000). Addition of 5 ng TGF-β_1/ mL culture media inhibited the mitogenic response of IGF-I (50 ng/mL) in primary mammary epithelial cells (Purup et al., 2000). When implanted into the mammary gland of pregnant mice, 100 ng of TGF-β_1 completely inhibited ductal elongation and inhibited DNA synthesis in the mammary epithelium (Daniel et al., 1989). Purup and co-workers (2000) cited unpublished results by Plaut that serum concentration of TGF-β_1 tended to be higher in heifers fed at a high feeding level than at a moderate feeding level. The significance of this finding is important because TGF-β_1 is known to have an inhibitory role in mammary development. Further research is needed to determine if TGF-β_1 plays a role in the detrimental effects of feeding prepubertal heifers a high energy diet on mammary growth.

Beta-agonists are well known to increase muscle protein deposition and decrease deposition of body lipid (Reeds and Mersmann, 1991). Examples of β-agonists include cimaterol, clenbuterol, isoproterenol, L-644-969, ractopamine and salbutamol. Since high energy intake increases fat deposition within the mammary gland and fat cells may produce an inhibitory substance, decreasing lipid deposition could be a way to control mammary growth. But when β-agonists were given to prepubertal ewes, weight of the mammary gland, fat-free parenchymal tissue, and parenchymal fat were all reduced (Zhang et al., 1995). In addition, ewes treated with β-agonists tended to have 20% less milk yield compared to control ewes.

Summary and Statement of Objectives

Many factors and hormones regulate mammary development in prepubertal heifers. One of the factors most studied is nutrition, yet the mechanism for why feeding high energy diets impairs mammary growth relative to body growth is not clearly understood. The effect of feeding postweaned dairy heifers a high energy diet for a short time period ($\leq$ 6 wk) has not been reported. The first objective of this study was to evaluate if feeding a high energy diet for a short duration compared to a long duration would result in a positive effect on mammary growth relative to body growth.

IGF-I is a known mitogen for mammary epithelial cells. Serum IGF-I concentration is greater in heifers fed a high energy diet compared to heifers fed a low or moderate energy diet. The mRNA expression of IGF-I within mammary tissue and IGF-I concentration in mammary tissue extracts were similar in heifers fed a high compared to a low energy diet (Weber et al., 2000b). Mammary explants from prepubertal heifers fed a high plane of nutrition were less sensitive to IGF-I treatment compared to explants from heifers fed a low plane of nutrition (Purup et al., 1996). This difference in sensitivity to IGF-I suggests that the number of IGF-I receptors might be altered by nutritional modulation and/or that a potential inhibitor of IGF-I could be more abundant in mammary tissue of heifers fed a high energy diet. Leptin, a protein produced by both adipocytes and mammary epithelial cells, can inhibit the IGF-I stimulation of mammary growth. Serum leptin concentration is increased in calves and sheep fed a high energy diet compared to a low energy diet. Yet, it is not known if prepubertal heifers fed a high energy diet have a greater concentration of leptin and increased mRNA expression abundance of leptin within the mammary tissue than heifers fed a low energy diet. A second objective of this study was to determine the effects of feeding a high energy diet to prepubertal dairy heifers for short and long durations on serum levels of IGF-I, IGFBP, and leptin; leptin concentration in mammary parenchymal tissue; and mRNA expression of IGF-I, IGF-I receptor, leptin, and leptin receptor in mammary parenchymal tissue.

CHAPTER TWO

EFFECT OF FEEDING A HIGH ENERGY DIET TO PREPUBERTAL HEIFERS FOR A LONGER DURATION ON BODY GROWTH, CARCASS COMPOSITION, AND FEED EFFICIENCY

ABSTRACT

Our objective was to determine the effects of feeding prepubertal dairy heifers a high energy diet for a longer duration on body growth, body composition, and carcass composition. We also used feed composition, daily intake, and body growth data to evaluate the 2001 NRC for predicting intakes and gains. Holstein heifers (age = 11 wk; BW = 107 ± 1 kg) were assigned to 1 of 4 treatments (n = 16/trt) and fed 2 diets for different durations of time: H0, H3, H6, and H12 were fed the low energy diet for 12, 9, 6, and 0 wk followed by the high energy diet for 0, 3, 6, and 12 wk, respectively. The low energy diet was fed to achieve 0.6 kg average daily gain (ADG) and contained 0.72 Mcal NE_g/kg, 16.3 % CP, and 46.1 % NDF. The high energy diet was fed to achieve 1.2 kg ADG and contained 1.17 Mcal NE_g/kg, 18.4 % CP, and 22.6 % NDF. Actual daily gains averaged over the 12 wk treatment period were 0.64, 0.65, 0.83, and 1.09 kg for H0, H3, H6, and H12, respectively. Daily gains during the last 2 wk of the treatment period were 0.72, 1.05, 1.34, and 1.19 kg for H0, H3, H6, and H12, respectively. Body weight, withers height, hip width, carcass weight, liver weight, and total carcass protein and fat increased in heifers fed a high energy diet for a longer duration. Percentage of carcass fat increased with a longer duration of feeding a high energy diet, but the opposite was true for percentage of protein in the carcass. The efficiency of converting dietary protein and energy into carcass protein and fat increased as heifers were fed the high energy diet for longer durations. Evaluation of NRC indicated that the 2001 version underestimated daily intake of the high energy diet and underestimated daily gain in heifers fed both the high and low energy diets. The Spartan Dairy Ration Evaluator/Balancer Program (Version 2.02b) underestimated daily intakes of both diets. A more complete evaluation of NRC is needed to more clearly determine where improvements should be made in heifer requirements and diet formulation. We conclude that feeding a high energy diet for a short duration altered body growth and carcass composition in a time-dependent linear manner consistent with feeding a high energy diet for a long duration.

Key Words: heifer, nutrition, growth

Abbreviation Key: **ADG** = average daily gain; **BL** = baseline; **BW** = body weight; **CP** = crude protein; **CW** = carcass weight; **DMI** = dry matter intake; **ME** = metabolizable energy; **NE$_g$** = net energy for gain; **NE$_m$** = net energy for maintenance; **NRC** = National Research Council; **TMR** = total mixed ration

INTRODUCTION

Raising replacement dairy heifers is costly and accounts for approximately 20% of total dairy herd expenses (Heinrichs, 1993). Actual costs until calving range from \$1000 to \$1300 per heifer (Cady and Smith, 1996). Feeding a high energy diet to allow for rapid growth enables heifers to be bred and calve earlier, potentially reducing costs associated with raising replacement heifers. The optimal BW of heifers before calving ranges from 590 to 640 kg (Hoffman, 1997; Keown and Everett, 1986) and can be reached as early as 19 to 20 mo of age if gains average approximately 1 kg/d. However, mammary growth relative to body growth and milk yield potential are reduced when heifers that are approximately 3 to 10 mo of age are fed a high energy diet for periods of 12 wk or longer (Petitclerc et al., 1999; Radcliff et al., 2000; Sejrsen et al., 1982). Feeding heifers for rapid gains also increases the amount of fat deposited within the body, carcass, and mammary gland.

A positive correlation exists between height at the withers at parturition and first lactation milk production, and it was estimated that 50% of the total height increase from birth to first calving occurred during the first 6 mo (Heinrichs and Hargrove, 1987; Kertz et al., 1998). A high plane of nutrition increased skeletal growth in young heifers (Brown et al., 2005b; Shamay et al., 2005) and in older heifers when measured at a similar age (Lammers et al., 1999). However, increasing daily gains of heifers does not seem to alter frame height and width (Radcliff et al., 1997) when measured near the onset of puberty at similar body weights.

Restricting the energy intake of heifers may improve mammary growth, but it also delays puberty and increases the number of non-productive days. However, heifers that are fed according to a stair-step regime grow similar to heifers raised on a continuous moderate diet, have minimal delay in the onset of puberty, and produce more milk (Park et al., 1987; Peri et al., 1993). A stair-step feeding regime involves alternating between high energy diets fed for periods of 2 mo in length and energy restricted diets fed for 3 mo or longer (Choi et al., 1997; Park et al., 1998). These stair-step managed heifers have improved efficiency of growth, dietary energy, and dietary protein compared with control heifers (Park et al., 1987). Therefore, feeding heifers a high energy diet for a short duration may be one way to decrease age at first calving without causing detrimental effects on mammary parenchymal tissue growth.

Body composition of beef steers slaughtered at the end of a compensatory growth period was similar to steers fed a control diet (Carstens et al., 1991; Fox et al., 1972). However, steers undergoing compensatory growth deposit more protein and less fat than controls during the early portion of the compensatory growth phase (Fox et al., 1972). Although carcass composition data of dairy heifers undergoing compensatory growth is lacking, Holstein heifers have a similar percentage of empty body fat at the same body weight as Angus steers (Fox and Black, 1984). Fat deposition is usually not dramatically increased until approximately 12 mo of age (Berg and Butterfield, 1976). Therefore, feeding young heifers a high energy diet for a short duration following low energy intake may be one way to feed heifers for rapid gains without an increase in fat deposition.

Many academic and industry professionals use the NRC Nutrient Requirements of Dairy Cattle (NRC, 2001) as a reference for nutrient analysis, nutrient utilization of the animal, and diet formulation. Few studies have been published that perform a comprehensive evaluation of the growth equations used in NRC (Van Amburgh et al., 1998a) and only one heifer study has briefly evaluated the 2001 edition of NRC (Gabler and Heinrichs, 2003). Van Amburgh (2005) suggested that actual gains of heifers are typically higher than those predicted by the model.

Our objective was to determine how feeding prepubertal dairy heifers a high energy diet for a short duration compared to a low duration affects body growth, carcass composition, and mammary growth. Prior studies that involved treatment periods of 12 wk or greater indicated a detrimental effect of high energy intake on mammary growth relative to body growth. Thus, we selected 12 wk as our long duration time point, 6 and 3 wk as our shorter duration time points, and 0 wk of feeding a high energy diet as our base control treatment. Treatment effects on mammary growth in these heifers are reported in Chapter 3. We also evaluated the 2001 NRC predictions for intakes and gains of heifers using growth and diet composition data from this trial.

MATERIALS AND METHODS

Animals and Dietary Treatments

All procedures were approved by the Michigan State University Animal Use and Care Committee. Sixty-eight Holstein heifers (approximate age = 8 wk) were purchased during 4 consecutive wk in the fall (17 heifers/wk) with each wk classified as a separate purchase group. Heifers were housed at the Michigan State University Beef Cattle Research Center and were exposed to ambient temperatures and lighting during the adaptation and treatment periods, which occurred during late fall and winter. Heifers were housed in an open-sided barn with enough space per pen (dimensions: 14 X 38 ft) to allow for exercise. Each purchase group was allowed a 3-wk adaptation period for adjustment to facilities and diet. During this adaptation period, heifers were gradually transitioned from a diet similar to that fed before purchase to a total mixed ration (**TMR**) similar to a mixture of the treatment diets. At the beginning of the adaptation period, heifers were fed a texturized complete feed (21% CP, ADM Alliance Nutrition, Quincy, IL) and alfalfa hay. Alfalfa silage, oatlage, straw, and corn silage were slowly introduced into the diet during the adaptation period. One heifer within each purchase group was randomly selected and slaughtered at 11 wk of age for baseline (**BL**) measurements used for calculation of carcass accretion rate data.

During the first wk of the adaptation period, body temperatures were taken daily and then only if heifers appeared ill (lethargic appearance, nasal discharge, labored breathing, or coughing). Heifers were treated if body temperatures were greater than 39.7°C, appeared ill, or were lame. Various medications [Nuflor (Schering-Plough), Micotil (Elanco), Recovr (Fort Dodge), A-180 (Pfizer), LA-200 (Pfizer), Excenel (Pfizer)] were used depending on the signs (see Appendix) and previous treatments. During the second week of the adaptation period, heifers were vaccinated against bovine rhinotracheitis, bovine virus diarrhea, parainfluenza type 3, and leptospirosis (BoviShield4, Pfizer); pasteurella (Pfizer); and clostridium perfringens (Ultrabac7/Somubac, Pfizer). No animals died during the adaptation or treatment periods. A total of 6 heifers appeared ill and were medicated during the treatment period. One heifer (trt = H3) had chronic bloat and the other 5 heifers were treated once for respiratory-type symptoms (H0 = 1; H3 = 2; H6 = 2; H12 = 0). All heifers given medication were being fed the low energy diet at the time of apparent illness.

At 11 wk of age (BW = 107 ± 1 kg), 16 heifers within each purchase group were blocked by body weight and randomly assigned within block to 1 of 4 treatments. All heifers within a given treatment in the same purchase group were housed in the same pen. Thus, 4 pens of 4 heifers (1 pen per purchase group) were used in each of the 4 treatments. A timeline for the experiment is depicted in Figure 1. The treatment period lasted 12 wk and treatments were as follows: H0 (low energy diet fed for 12 wk); H3 (low energy diet fed for 9 wk followed by high energy diet for 3 wk); H6 (low energy diet fed for 6 wk followed by high energy diet for 6 wk); and H12 (high energy diet for 12 wk). The low energy diet was fed to achieve 0.6 kg average daily gain (ADG) and consisted of 10% straw, 33% mature alfalfa silage, 33% oatlage, and 24% concentrate on a DM basis. The low energy diet had 0.77 Mcal NE_g/kg DM, 16.4% CP, and 43.6% NDF. The high energy diet was fed to achieve 1.2 kg ADG and consisted of 20% immature alfalfa silage, 20% corn silage, and 60% concentrate on a DM basis. The high energy diet had 1.17 Mcal NE_g/kg DM, 18.4% CP, and 22.6% NDF. Both diets and water were available ad libitum. Composition of diets based on actual individual feedstuff analyses (Dairy One Forage Analysis Laboratory, Ithaca, NY) is given in Table 1. Sodium decoquinate (Deccox®, Alpharma, Fort Lee, NJ) was included in both vitamin/mineral mixes as a coccidiostat to supply approximately 0.5 mg/kg of BW/d. Diets were fed as a TMR once daily between 0900 and 0930 h and refusals were measured daily at 0700 h. Amount of TMR fed was adjusted so that daily refusals were approximately 10%. Daily intakes for each pen were collected and are reported as an average per heifer (Figure 4).

During the treatment period, body weight was measured weekly before feeding. Withers height and hip width were measured initially before treatments began and then on odd wk and on wk 12 of the treatment period. Heifers were slaughtered at the end of the treatment period when heifers were 23 wk of age. Heifers were allowed to consume the TMR from the prior day's feeding until they were transported at 0600 h via trailer to the abattoir at the Michigan State University Meats Laboratory.

Evaluation of NRC

Diet composition, dry matter intake, and daily gains of heifers from the H0 and H12 treatment groups were used to evaluate actual versus predicted values for intakes and gains according to the NRC (NRC, 2001). Treatment groups H3 and H6 were not included due to the short duration of time in which heifers received the low or high diet. Time is required to diminish the carry-over effect of the previous diet on variables measured; therefore inaccurate comparisons of actual versus predicted measurements may have occurred if data from H3 and H6 had been used.

Tissue Collection

Heifers were weighed, stunned by captive bolt, and killed by exsanguination. Heifers were killed on 2 different days each wk for 4 consecutive wk with 8 heifers (2/trt) killed per day. The gallbladder was removed from the liver and the liver was weighed. Four heifers on the H12 treatment had liver abscesses and these livers were condemned at slaughter. Liver abscesses were likely due to acidosis caused by the high grain diet (Nagaraja and Chengappa, 1998). Also, 2 heifers on the H6 treatment had "sawdust liver" or Telaniectasis. After the hide was removed, the carcass was split into halves and weighed (**CW**). Perirenal fat was removed from both sides of the carcass and weighed. The day after slaughter, the left half of the carcass was cut between the 7th and 8th and the 12th and 13th ribs. The 8th through 12th rib section was removed and stored at -20°C until composition was analyzed.

Reproductive tracts were examined to confirm that heifers were not freemartins and had not reached puberty. The uterus and ovaries were removed and weighed. One heifer (trt = H3) was a freemartin and her data were eliminated from the results. Another heifer (trt = H12) was confirmed postpubertal after a corpus lutem was detected and her data were removed from the study. Uterine and ovarian weights, along with liver, perirenal fat, and carcass weights, were taken to determine how short and long durations of high energy intake alter growth of various tissues. These measurements were then also compared to the growth of mammary tissue in prepubertal dairy heifers fed a high energy diet for a longer duration (see Chapter 3).

Estimated Carcass Composition

The rib section containing the 8th through the 12th rib section was slightly thawed. The 9, 10 and 11 ribs were then dissected according to Hankins (1946) and weighed. The soft tissue was dissected from the bone and put into separate piles and then weighed. The soft tissue was then ground, mixed, and subsampled. The tissue was stored at -20°C until fat, protein, and water content was analyzed. Fat was determined by Soxhlet ether extraction (AOAC, 1990). Crude protein was determined using the method of Hach et al. (1987). Water was determined as the difference in weight after drying samples in a 106°C oven for 24 h.

Statistical Analysis

Statistical analysis used the PROC GLM procedure of SAS. Pen (n = 4 heifers per treatment in each purchase group) was used as the experimental unit with purchase group as a random variable and treatment*purchase group as the error term. Comparisons were tested using a linear (L) contrast with coefficients -7, -3, 1, and 9; a quadratic (Q) contrast with coefficients 7, -4, -8, and 5; and a cubic (C) contrast with coefficients -3, 8, -6, and 1 for H0, H3, H6, and H12, respectively. Least square means and standard errors of the mean are presented. Differences were declared to be statistically significant at $P < 0.05$ and tendencies at $P < 0.10$. All data from the 2 heifers that were eliminated from the trial were removed so that final animal numbers were 16, 15, 16, and 15 for treatment groups H0, H3, H6, and H12, respectively.

Data that were collected every wk or every other wk were treated as a repeated measure and analyzed using PROC MIXED with either compound symmetry or first-order autoregressive as the covariance structure. The data for ME consumed, carcass protein percent, amount of carcass fat, ovarian weight relative to BW, and ovarian weight relative to CW were log transformed to achieve homogeneous variance and normality and the results presented were back transformed. The error term for the transformed data is the average of the back transformed lower and upper 68% confidence intervals. Non-transformed means are presented in the Appendix.

RESULTS

Initial BW was not different between treatments (Table 3; all contrasts: $P > 0.10$). Body weights averaged during the treatment period were significant for both a linear and quadratic response as heifers were fed the high energy diet for a longer duration (Figure 2; L: $P < 0.01$; Q: $P = 0.02$). Ending BW averaged 165, 167, 181, and 203 kg for H0, H3, H6, and H12 respectively, and was significant for linear, quadratic, and cubic contrasts (all contrasts: $P < 0.01$). Daily gain averaged during the treatment period increased as heifers were fed the high energy diet for a longer duration (Figure 3; Table 3; L: $P < 0.01$), but also was significant for quadratic and cubic contrasts ($P < 0.01$). During the entire treatment period, daily gain averaged 0.64, 0.65, 0.83, and 1.09 kg for

H0, H3, H6, and H12, respectively. Thus, 0.64 kg/d for the low energy diet and 1.09 kg/d for the high energy diet were very close to the 0.6 and 1.2 kg daily gain formulated for within the diets. Average daily gain during the last 2 wk was also significant for all 3 contrasts tested and averaged 0.72, 1.05, 1.34, and 1.19 kg for H0, H3, H6, and H12, respectively (Table 3; $P < 0.01$). There was a delay of 1 wk in the increase in daily gain after the switch to the high energy diet for both H3 and H6 treatments. Gains calculated during the 2nd and 3rd week after the dietary switch for H6 and H3 were 1.22 and 1.05 g/d, respectively (Table 4). The H3 treatment group never reached the target gain of 1.2 kg/d on the high energy diet. However, there was no delay in daily gain of the H12 treatment when switched to the high energy diet from the adaptation diet.

Average daily DMI increased with a longer duration fed the high energy diet (Figure 4; L: $P < 0.01$). There was an increase in daily DMI until wk 4 of the treatment period. After wk 4, feed consumption was fairly constant for H0 and H12 heifers and increased for H3 and H6 heifers when switched to the high energy diet (Table 4). Daily DMI was also adjusted for BW (Figure 5) and averaged 2.79, 2.99, 3.03, and 3.29 kg/100 kg for H0, H3, H6, and H12, respectively (SE = 0.07; L: $P < 0.01$).

Initial withers height and hip width were not different among treatment groups (Table 3; all contrasts: $P > 0.4$). Hip width and withers height measurements increased with age (Figure 6 and 7) and showed a linear increase with a longer duration fed the high energy diet when measured during the last 2 wk of the treatment period (Table 3.; L: $P < 0.01$). Although significant for a linear increase, the nominal difference between H0 and H12 treatments at the end of the treatment period in withers height and hip width was 5 cm and 4 cm, respectively.

The amount of CP consumed averaged 616, 679, 737, and 932 g/d for H0, H3, H6, and H12, respectively (SE = 15; L: $P < 0.01$), and is depicted per week in Figure 8. Consumption of CP increased for H3 and H6 when switched to the high energy diet (Table 4). The amount of ME consumed averaged 7.67, 8.64, 10.00, and 14.41 Mcal/d for H0, H3, H6, and H12, respectively (confidence interval = 0.20, 0.22, 0.26, and 0.37 for H0, H3, H6, and H12; L: $P < 0.01$) and followed a similar pattern per week as CP (Figure 9). Efficiency of protein and energy deposition were calculated using daily accretion rates of carcass protein and carcass fat as a percentage of dietary consumption of protein and energy (Table 4). Efficiency of both protein and energy deposition increased in a linear manner with a longer duration fed the high energy diet. Energy efficiency was also significant for a quadratic response due to similarities between H6 and H12 ($P < 0.01$).

Carcass weight increased in a linear fashion as heifers were fed the high energy diet for a longer duration (Table 5; L: $P < 0.01$). But, BW at slaughter was significant for all 3 contrasts (Table 3). There was a lack of a BW difference between the H0 and H3 treatment groups. Carcass weight expressed as a percentage of live BW was significant for both linear and quadratic contrasts ($P < 0.01$). Total amounts of estimated carcass protein and fat unadjusted and adjusted for BW increased with a longer duration fed the high energy diet (Table 5; Figure 11; L: $P < 0.01$). Similarly, a longer duration fed the high energy diet increased the percentage of fat in the carcass, but the percentage of protein in the carcass decreased (L: $P < 0.01$). Percentage of carcass fat was also significant for a quadratic effect (Q: $P < 0.01$) due to a small difference in means for H6 and H12 treatments. Relative to baseline measurements, percentage of carcass fat increased and percentage of carcass protein decreased with age (Table 5). Fat-free carcass weight increased in a linear manner and also had a tendency for a quadratic relationship because of the large amount of fat-free carcass of the H12 treatment (L: $P < 0.01$; Q: $P = 0.08$). Daily accretion rates for carcass fat and carcass protein both increased in a linear manner as heifers were fed a high energy diet for a longer duration (Figure 10; $P < 0.01$).

Liver weight increased in a linear fashion with a longer duration fed a high energy diet and also was quadratic and cubic (Table 5; $P = 0.01$) due to the biggest difference between H0 and H3 and similar results for H3 and H6. Liver weight as a proportion of BW was significant for all 3 contrasts ($P = 0.01$) and this was due to the large adjusted liver weight of the H3 treatment. The amount of perirenal fat unadjusted and adjusted for BW increased in a linear fashion with time fed the high energy diet (L: $P < 0.01$).

Uterine and ovarian weights unadjusted and adjusted for BW and CW are presented in Table 6. There was a significant cubic contrast for uterine weight (C: $P = 0.05$). When adjusted for CW and BW, uterine weight was significant for a cubic contrast due to the large uterine weight for the H3 treatment (C: $P = 0.03$ and $P = 0.04$, respectively). Uterine weight adjusted for carcass weight also decreased in a linear response as heifers were fed the high energy diet for a longer duration of time (L: $P = 0.04$). Ovarian weight was not significant for any contrasts (all contrasts: $P > 0.10$). However, when adjusted for BW and CW, ovarian weight decreased as heifers were fed the high energy diet for a longer duration (L: $P = 0.02$ and $P < 0.01$, respectively). Uterine and ovarian weights were also combined and mimicked the response of uterine weight alone. Although follicles were quite large on some ovaries (12 to 15 mm; Davis

Rincker and Kobayashi, observation), the number of follicles and the diameter of follicles were not calculated.

Predicted values for intake and gains by NRC relative to actual values are depicted in Table 7. The Spartan Dairy Ration Evaluator/Balancer Program underestimated daily intakes by 2.91% for the low diet and 28.2% for the high diet. The 2001 version of NRC underestimated daily intakes by 19.3% for the low diet and 28.4% for the high diet. Daily gains, using actual intake data, were underestimated by 31.3% for the low diet and 5.5% for the high diet using the 2001 NRC.

DISCUSSION

A number of researchers (Petitclerc et al., 1984; Vestergaard et al., 2003) have observed an increase in carcass weight and body weight when heifers are fed a high energy diet for rapid gains. In this study both carcass and body weights increased in a linear manner as heifers were fed a high energy diet for a longer duration. However, body weight at slaughter was also significant for quadratic and cubic contrasts. There was a lack of a BW difference between the H0 and H3 treatment groups at slaughter and this was likely because of the increased gut fill of heifers fed the low energy diet (see Figure 12). Empty body weights were not taken, but would have likely been lower for the H0 compared to H3 treatment group.

Previous studies (Ferrell et al., 1978; Petitclerc et al., 1984) noted that at similar body weights, cattle fed on a higher plane of nutrition will have increased fat storage, which was the case in this study when carcass fat and perirenal fat were adjusted to BW. The amount of carcass fat and percentage of fat within the carcass both increased as heifers were fed a high energy diet for a longer duration. Similar results were shown by Petitclerc et al. (1984) and Waldo et al. (1997). However, this is the first study to demonstrate the short term effects of nutrition on body growth and carcass composition of prepubertal dairy heifers. The linear increase in carcass fat and perirenal fat observed in this study as heifers were fed a high energy diet for a longer duration may be a concern for the future performance of heifers fed for rapid gains. Recent evidence indicated that the degree of body fatness is negatively correlated with mammary parenchymal DNA and milk production (Silva et al., 2002b).

In evaluating nutritional impacts on heifer performance it is critical to not only analyze the effects on body weight and body composition but also on skeletal growth. Heifers in this study started treatments at 11 wk of age and were slaughtered at 23 wk of age. It is estimated that 50% of the height gain from birth to calving occurs in the time period from birth until 6 mo of age, while only 25% of the body weight gain occurs during the first 6 mo (Kertz et al., 1998). Withers height measured at 24 mo is positively correlated with first lactation milk yield in Holsteins (Heinrichs and Hargrove, 1987). Previous reports demonstrated that increasing the energy intake of young heifers can increase the daily gain of frame height and width (Lammers et al., 1999; Pirlo et al., 1997), similar to findings of this study. However, dietary intake did not affect growth measurements taken at the onset of puberty (Niezen et al., 1996). Measurements of body weight and withers height of heifers in the present study were within the range of previous reports for heifers of a similar age (Heinrichs and Hargrove, 1987; Hoffman, 1997), except that H12 heifers were heavier than the range reported for 5 to 6 mo-old heifers.

Compensatory growth studies using heifers and steers indicate that this type of feeding can be beneficial to mammary development (Choi et al., 1997) and lean body growth during the early phase (Fox et al., 1972). However, heifers on treatment H3 and H6 did not seem to experience compensatory growth and deposited body fat in a time-dependent manner similar to the H12 treatment. Kabbali et al. (1992) noted that feeding a high energy diet after a moderate diet did not have a compensatory effect in sheep while feeding a high energy diet following a low energy diet yielded higher gains and more efficient feed conversion compared to continuously high fed controls. Carcasses from steers fed at maintenance and then full fed compared to full fed control steers were higher in protein and lower in fat when harvested at similar body weights (364 kg) during the early refeeding period, but were similar in composition at final slaughter weights (454 kg) (Fox et al., 1972). Fox and co-workers suggested that steers deposit lean gain during the early compensatory growth period. The low energy diet fed in the present study may have not been low enough to yield compensatory results after 3 and 6 wk fed the high energy diet. Body weight differences between H6 and H12 treatment groups were steady at 26 to 27 kg from wk 8 to wk 12 (see Figure 1) indicating no compensatory gain in H6 heifers. Although accretion rate of carcass protein increased linearly with a longer duration fed the high energy diet, accretion rate of carcass fat also increased with time fed the high diet.

In the present study, the weight of the liver increased in a linear fashion with time fed the high energy diet. Liver weight was also significant for quadratic and cubic contrasts due to similar values for H3, H6, and H12. However, liver weight expressed as a percentage of body weight was highest for heifers on the H3 treatment, followed by H6, H12, and H0. These results indicate that there is elevated growth of the liver early on in the switch from a low to high energy diet and that when adjusted for body weight this acceleration decreases over time on a high plane of nutrition. Similar results have been observed in compensatory growth studies where the growth coefficient of the liver was higher in beef steers during the refeeding phase compared to continuously grown steers (Carstens et al., 1991). The weight of the liver was greater in lambs that were switched from a low to a high feeding level compared to lambs that were continuously fed a high energy diet (Kabbali et al., 1992). These authors suggested that during the first phase of the compensatory period, energy is diverted to metabolic organs to replenish protein and glycogen reserves.

After a review of recently published reports, there is limited evidence to support a role for nutrition in altering reproductive organ weights in prepubertal heifers. Pritchard et al. (1972) indicated that when heifers were allowed ad libitum intake of corn silage and alfalfa hay and fed either 0.9 or 4.5 kg of grain/d, treatments had similar uterine weights at first estrus. Daily gain averaged 0.83 and 1.08 kg for heifers fed standard or high grain diet, respectively. We hypothesized that weights of uterine and ovarian tissue would have a linear increase with longer durations of time fed the high energy diet and thus, parallel overall body growth. This would seem likely if heifers were to have similar reproductive organ weights at the onset of puberty. Body weight and possibly the degree of body fatness are factors that affect the onset of puberty and heifers fed for rapid growth attain puberty at an earlier age (Schillo et al., 1992; Wiltbank et al., 1969). Evidence from this experiment indicates that reproductive organ weight did not increase in a linear fashion with a longer duration fed a high energy diet. In fact, uterine and ovarian weights relative to carcass weight decreased with a longer duration fed the high energy diet. Overall, these results suggest that heifers fed a high energy diet will have smaller reproductive organs at puberty than heifers fed a moderate or low energy diet. However, high energy intake during the prepubertal period did not negatively alter pelvic area, conception rates, or calving rates of heifers (Radcliff et al., 2000; Radcliff et al., 1997) and may not be a long-term concern.

Similar to liver weight, uterine weight was highest for the H3 treatment group. Uterine weight adjusted for body weight or carcass weight was significant for a cubic contrast due to the large mean for the H3 treatment group. This increase in uterine weight that was evident shortly after switching to a high energy diet may be due to an acute hormonal response since serum levels of IGF-I increased soon after heifers were switched to a high energy diet (Chapter 4). Insulin could also play a role in the large uterine weight for the H3 treatment, but serum insulin concentration was not measured. Plasma concentration of insulin increased three-fold in heifers fed a high energy diet for 5 wk compared to heifers fed a low energy diet (Vestergaard et al., 2003). Both insulin and IGF-I are thought to play a role in follicular growth and development of cows in early lactation (Butler, 2000). Improved nutrition in sheep results in an increase in the number of follicles and in the ovulation rate (Downing and Scaramuzzi, 1991). This increase in number of follicles was later confirmed in Hereford-Fresian heifers fed for increased dietary intake (Gutierrez et al., 1997). Although size and number of follicles were not measured in this study, unadjusted ovarian weight was not different. Less is known about the role of IGF-I and insulin in bovine endometrial cells. In human endometrial cells, IGF-I is thought to mediate mitogenesis through estrogen-mediated proliferation

(Murphy and Ghahary, 1990). However, it is unclear if IGF-I or insulin can stimulate uterine growth in prepubertal heifers. More research is needed to understand the role of dietary energy intake on growth of reproductive tissues relative to body growth and how hormonal mechanisms might alter this growth.

CONCLUSION

Body weight, skeletal growth, and carcass weight in dairy heifers increased in a linear fashion with a longer duration fed a high energy diet. Feeding prepubertal heifers a high energy diet for a longer duration increased the daily accretion rate of both carcass protein and carcass fat. A longer duration of feeding a high energy diet improved the efficiency of converting dietary protein and energy into carcass growth. Uterine and ovarian weights adjusted for carcass weight, decreased as heifers were fed a high energy diet for a longer duration. An increase in body or carcass growth without a proportional increase in reproductive organ weight might result in smaller organs at puberty in heifers fed a high energy diet. Evaluation of NRC for heifer requirements indicates that daily intakes and gains were underestimated compared to actual values for both diets.

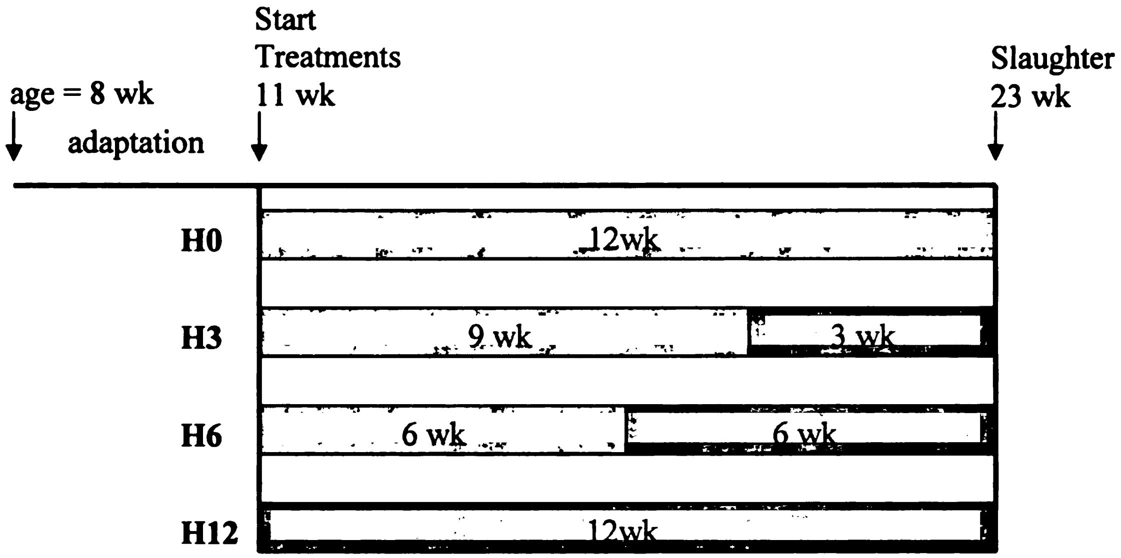


Figure 1. Timeline for experiment. Low energy diet is represented by lighter shaded bar and high energy diet is represented by darker shaded bar. The low and high energy diets were formulated for gains of 0.6 and 1.2 kg/d, respectively. Heifers (n = 15 or 16/trt) on H0, H3, H6, and H12 were fed the low energy diet for 12, 9, 6, and 0 wk followed by the high energy diet for 0, 3, 6, and 12 wk, respectively.

Table 1. Ingredient content of diets.

	Low diet	High diet
Ingredients, % of DM		
Alfalfa silage, late stage	30.8	-
Alfalfa silage, early stage	-	20.0
Corn silage	-	20.0
Oatlage	30.8	-
Straw	9.9	-
Ground corn	17.0	42.9
Solvent-extracted soybean meal	1.6	7.6
Expeller soybean meal[1]	8.6	7.5
Minerals and Vitamins	1.3[2]	2.0[3]
Nutrient Composition, DM basis		
NDF, %	46.1	22.6
ADF, %	29.9	14.1
ME, Mcal/kg	2.07	2.86
NE_m, Mcal/kg	1.30	1.79
NE_g, Mcal/kg	0.72	1.17
CP, %	16.3	18.4
RUP, % of CP	35.6	37.9
RDP, % of CP	64.4	62.1
CP:ME (g CP/kg Mcal ME)	72.1	63.9

[1] The expeller soybean meal was SoyPlus (West Central Cooperative, Ralston, IA).

[2] Composition: 43.1 % salt, 33.3 % sodium decoquinate (5007 mg/kg), 13.6 % calcium: phosphorus (17 %:21 %), 8.61 % mineral mix, 1.38 % vitamin mix. The mineral and vitamin mix was formulated so that the diet provided 100% of mineral and vitamin requirements.

[3] Composition: 74.6 % limestone, 38.0 % salt, 24.8 % sodium decoquinate (5007 mg/kg), 8.28 % calcium: phosphorus (17 %:21 %), 7.46 % mineral mix, 1.19 % vitamin mix. The mineral and vitamin mix was formulated so that the diet provided 100% of mineral and vitamin requirements.

Table 2. Feedstuff analysis.

	CP, %	NDF, %	ME, Mcal/kg[1]
Ingredients			
Alfalfa silage, late stage	17.4	50.0	1.76
Alfalfa silage, early stage	23.9	39.0	2.05
Corn silage	8.00	45.0	2.40
Oatlage	16.4	58.0	1.69
Straw	3.60	85.0	1.20
Ground corn	10.0	9.00	3.35
Solvent-extracted soybean meal	55.0	10.0	3.46
Expeller soybean meal	47.5	15.0	3.39

[1] Calculated using equation: ME (Mcal/kg DM) = NE_m (Mcal/kg DM) * 1.6

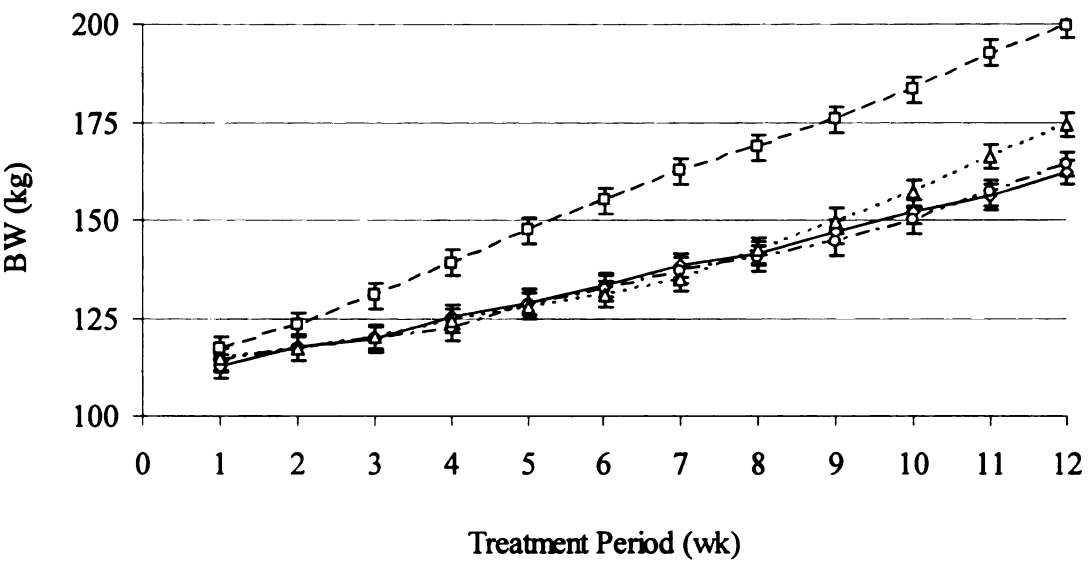


Figure 2. Weekly body weight measurements of heifers on treatment H0 (——◇——), H3 (- — -○- — -), H6 (- - -Δ- - -), H12 (— —□— —). Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Linear: $P < 0.01$; Quadratic: $P = 0.02$.

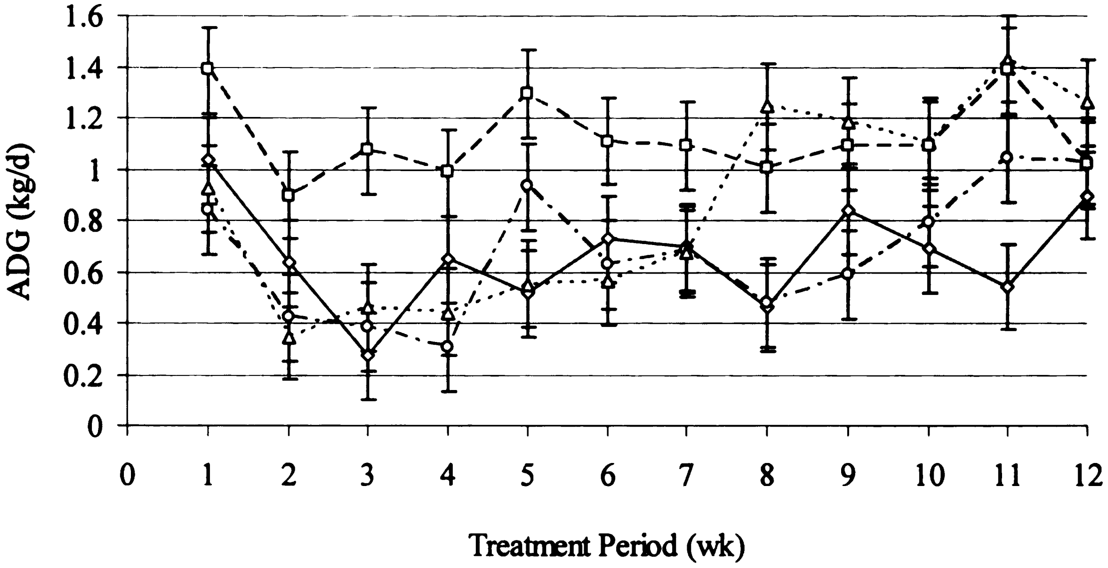


Figure 3. Weekly average daily gain (ADG) of heifers on treatment H0 (——◇——), H3 (- — -○- — -), H6 (- - -Δ- - -), H12 (— —□— —). Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Linear: $P < 0.01$.

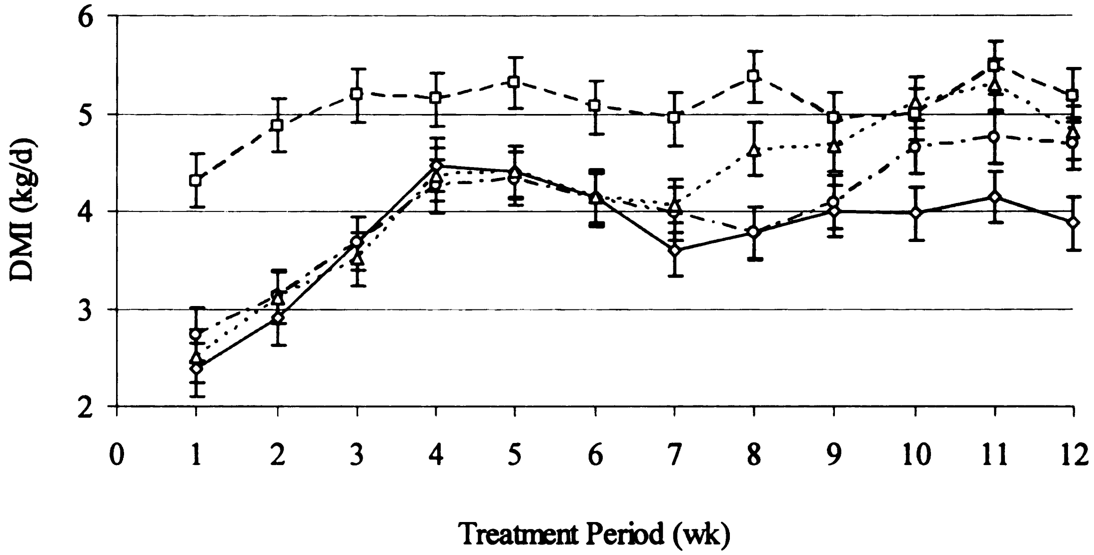


Figure 4. Daily dry matter intake (DMI) averaged each week of heifers on treatment H0 (——◊——), H3 (- — -○- — -), H6 (- - -Δ- - -), H12 (— —□— —). Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Linear: $P < 0.01$.

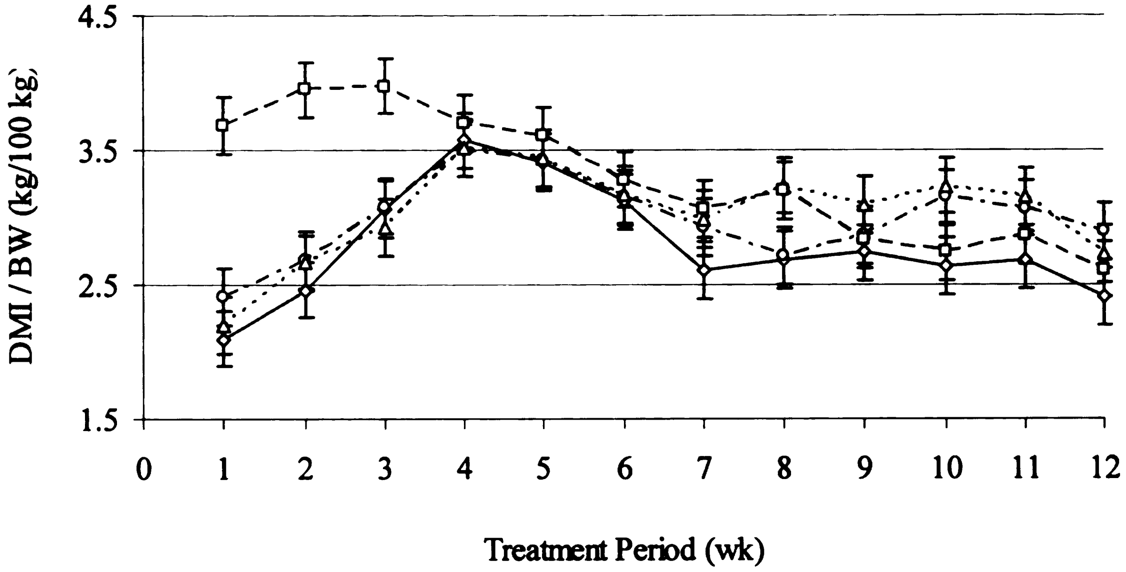


Figure 5. Dry matter intake (DMI) in kg/d as a proportion of body weight (BW, 100 kg) of heifers on treatment H0 (——◇——), H3 (- — -○- — -), H6 (- - -Δ- - -), H12 (— —□— —). Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Linear: $P < 0.01$.

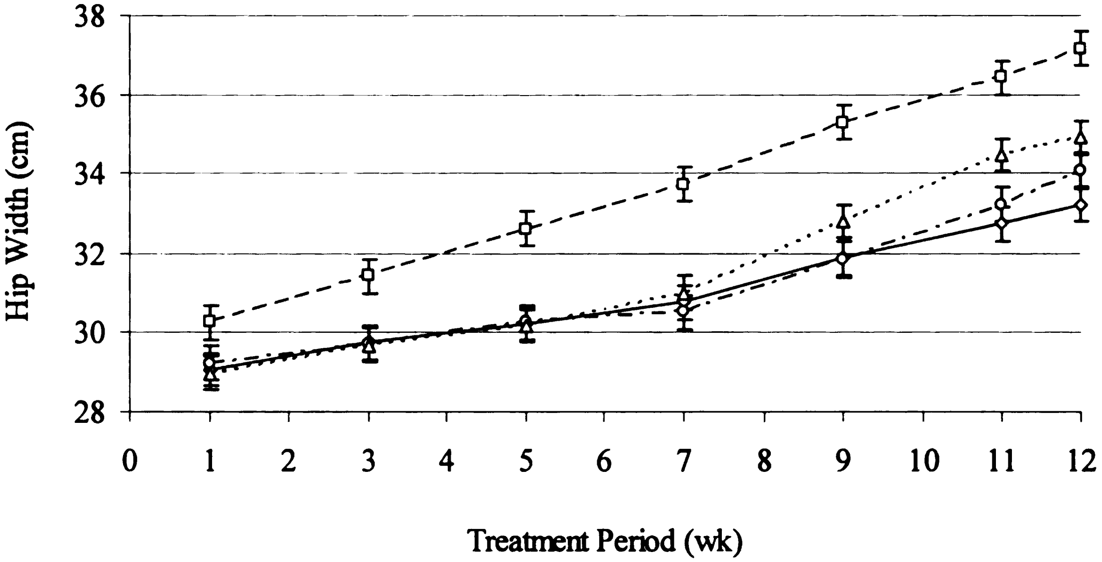


Figure 6. Hip width measurements of heifers on treatment H0 (——◇——), H3 (- — -○- — -), H6 (- - -Δ- - -), H12 (— —□— —). Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Linear: $P < 0.01$; Quadratic: $P = 0.03$.

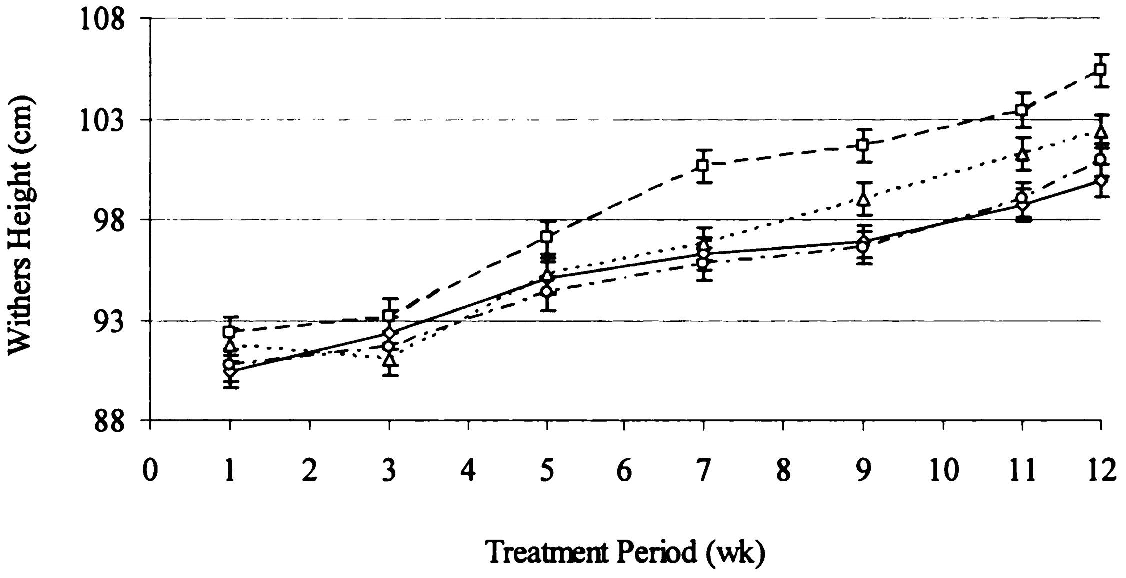


Figure 7. Withers height measurements of heifers on treatment H0 (——◇——), H3 (- — - ○- — -), H6 (- - -△- - -), H12 (— —□— —). Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Linear: $P < 0.01$.

Table 3. Least square means for body growth.

	Treatment Group[1]					*P* for Contrast[2]		
	H0	H3	H6	H12	SE[3]	L	Q	C
Heifers, no	16	15	16	15				
Initial BW, kg	106	108	108	108	0.74	0.25	0.19	0.72
Initial Withers Height, cm	89.9	90.0	90.4	90.1	0.32	0.52	0.41	0.61
Initial Hip Width, cm	29.8	30.0	29.7	30.0	0.28	0.80	0.69	0.44
BW at slaughter, kg	165	167	181	203	1.1	< 0.01	< 0.01	< 0.01
Last 2 wk BW gain, kg/d	0.72	1.05	1.34	1.19	0.09	< 0.01	< 0.01	< 0.01
Overall BW gain, kg/d	0.64	0.65	0.83	1.09	0.01	< 0.01	< 0.01	< 0.01
Last 2 wk Withers Height, cm	99.3	100	102	104	0.32	< 0.01	0.21	0.04
Final Hip Width, cm	32.9	33.6	34.7	36.7	0.22	< 0.01	0.50	0.43

[1] Treatment groups are as follows: Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

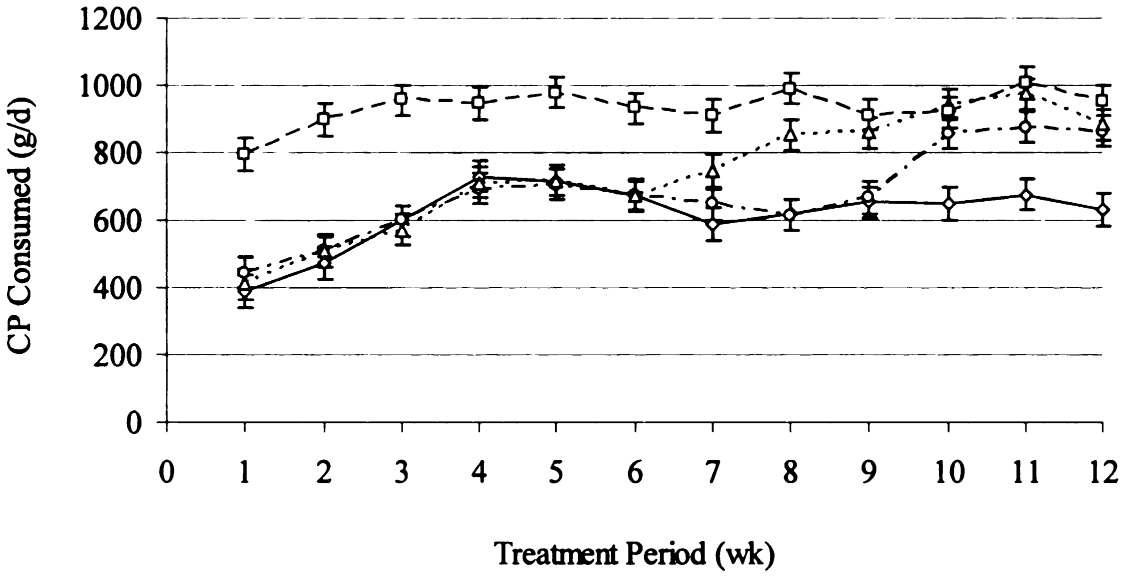


Figure 8. Grams of CP consumed per day of heifers on treatment H0 (——◇——), H3 (- — -○- — -), H6 (- - -Δ- - -), H12 (— —□— —). Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Linear: $P < 0.01$.

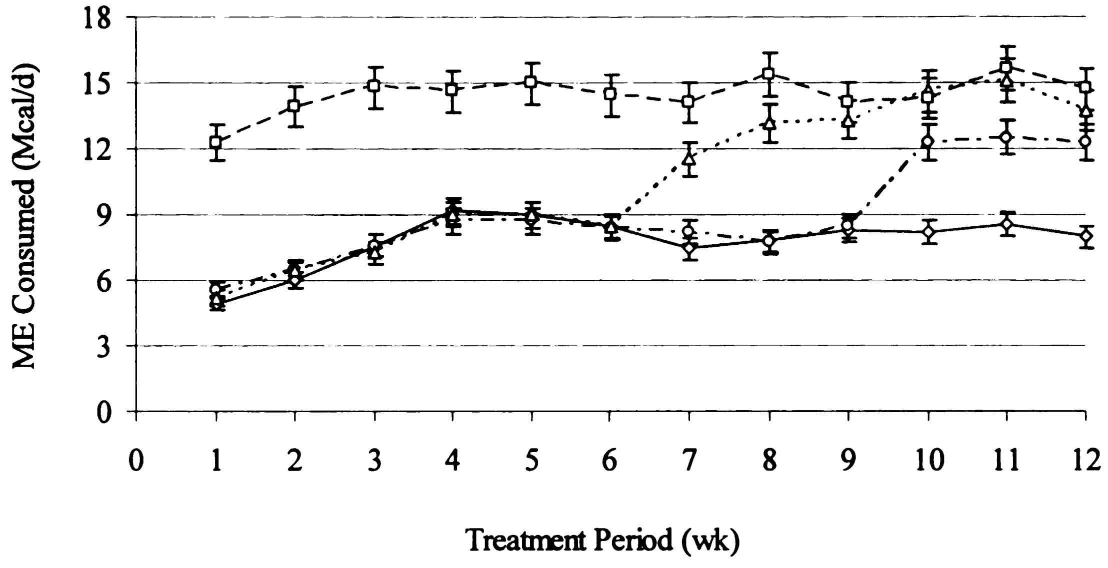


Figure 9. Mcal of ME consumed per day of heifers on treatment H0 (——◇——), H3 (- — - ○- — -), H6 (- - -Δ- - -), H12 (— —□— —). Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. ME consumed values were log transformed to achieve homogeneous variance. ME consumed means presented are back transformed. The error term is the average of the lower and upper confidence intervals. Linear: $P < 0.01$.

Table 4. Least square means for feed intake and efficiency during specific wk of the treatment period.

	Treatment Group[1]					*P* for Contrast[2]		
	H0	H3	H6	H12	SE[3]	L	Q	C
DMI (kg/d)								
2-6 wk	3.92	3.90	3.91	5.12	0.34	< 0.01	< 0.01	0.51
8-9 wk	3.89	3.93	4.65	5.16	0.23	< 0.01	0.97	0.17
11-12 wk	4.01	4.72	5.04	5.32	0.21	< 0.01	0.04	0.64
ADG (kg/d)								
2-6 wk	0.57	0.54	0.48	1.07	0.19	< 0.01	< 0.01	0.06
8-9 wk	0.65	0.53	1.22	1.05	0.13	< 0.01	0.09	< 0.01
11-12 wk	0.72	1.05	1.34	1.19	0.18	< 0.01	< 0.01	0.54
CP consumed (g/d) [4]								
2-6 wk	639	636	637	941	57	< 0.01	< 0.01	0.31
8-9 wk	634	640	856	950	40	< 0.01	0.61	0.02
11-12 wk	653	868	928	979	37	< 0.01	< 0.01	0.20
Protein efficiency (%) [5]								
1-12 wk	7.00	7.78	9.03	10.4	0.31	< 0.01	0.58	0.45
Energy efficiency(%) [6]								
1-12 wk	6.67	9.80	13.1	13.0	0.63	< 0.01	< 0.01	0.30

[1] Treatment groups are as follows: Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

[4] ME consumed followed similar pattern.

[5] Daily accretion rate of carcass protein / daily consumption of dietary protein (%)

[6] (Daily accretion rate of carcass protein*5.6 + daily accretion rate of carcass fat*9.5)/ daily consumption of ME (%)

Table 5. Least square means for body and estimated carcass composition.

		Treatment Group[1]					*P* for Contrast[2]		
	BL	H0	H3	H6	H12	SE[3]	L	Q	C
Heifers, no	4	16	15	16	15				
Carcass weight, kg	50.0[#]	76.6	82.5	91.5	107	0.65	< 0.01	0.40	0.10
Carcass weight, % of BW	48.5[#]	46.4	49.4	50.6	52.6	0.36	< 0.01	< 0.01	0.24
Carcass protein, %	21.6	19.0	18.8	18.1	18.1	A*	< 0.01	0.12	0.38
Carcass fat, %	6.10	7.33	10.1	13.5	14.4	0.61	< 0.01	< 0.01	0.24
Carcass water, %	71.2	71.9	69.4	66.5	65.3	0.52	< 0.01	< 0.01	0.31
Carcass protein, kg	10.8[#]	14.6	15.4	16.6	19.3	0.25	< 0.01	0.22	0.76
Carcass protein, kg/100 kg BW	10.5[#]	8.82	9.22	9.19	9.51	0.14	< 0.01	0.54	0.26
Carcass fat, kg	3.05[#]	5.44	8.26	12.3	14.8	B*	< 0.01	< 0.01	0.40
Carcass fat, kg/100 kg BW	2.96[#]	3.40	5.00	6.85	7.57	0.30	< 0.01	< 0.01	0.25
Fat-free carcass, kg	47.0[#]	71.0	74.2	79.1	91.2	0.98	< 0.01	0.08	0.74
Liver weight, kg	-	2.66	3.62	3.71	3.94	0.04	< 0.01	< 0.01	0.01
Liver weight, % of BW	-	1.62	2.17	2.05	1.97	0.02	< 0.01	< 0.01	< 0.01
Perirenal fat, g	-	693	987	1480	1856	100	< 0.01	0.20	0.27
Perirenal fat, g/100 kg BW	-	418	588	814	914	54	< 0.01	0.06	0.37

[1] Treatment groups are as follows: Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

[*] Data were log transformed to achieve homogeneous variance. Means presented are back transformed. The error term indicated below is the average of the lower and upper confidence intervals for each treatment group.

[#] Baseline carcass weight measurements were estimated using previous results (Brown et al., 2005b).

[A] Average confidence intervals are 0.16, 0.17, 0.16, and 0.16 for H0, H3, H6, and H12 treatment groups, respectively.

[B] Average confidence intervals are 0.31, 0.49, 0.70, and 0.88 for H0, H3, H6, and H12 treatment groups, respectively.

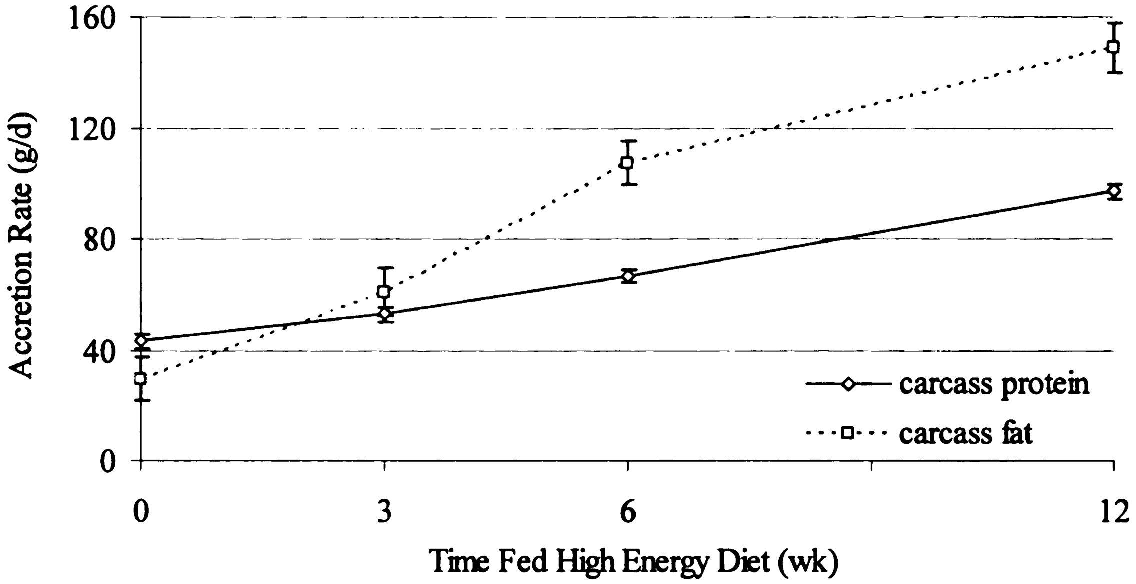


Figure 10. Daily accretion rates of carcass protein and fat averaged during treatment period. Heifers (n = 15 or 16/trt) were fed a high energy diet for 0, 3, 6, or 12 wk in duration. Accretion rates for both protein and fat were significant for a linear effect (*P* < 0.01).

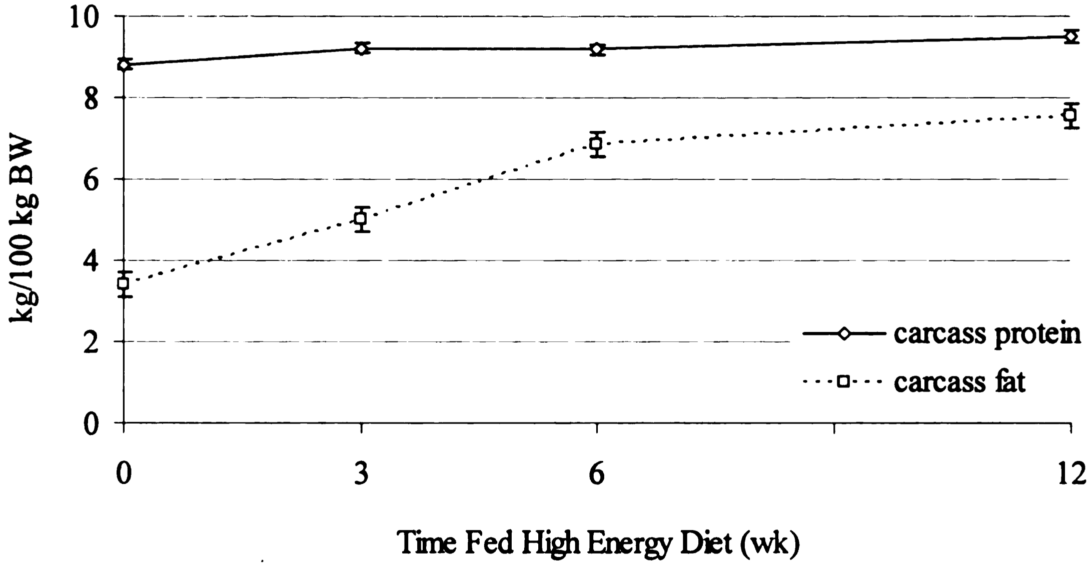


Figure 11. Amount of carcass fat or carcass protein adjusted for BW (kg/100 kg BW). Heifers (n = 15 or 16/trt) were fed a high energy diet for 0, 3, 6, or 12 wk. Carcass fat adjusted for BW was significant for both linear and quadratic effects ($P < 0.01$). Carcass protein adjusted for BW was significant for a linear effect ($P < 0.01$).

Table 6. Least square means for uterine and ovarian weights.

	Treatment Group[1]					*P* for Contrast[2]		
	H0	H3	H6	H12	SE[3]	L	Q	C
Heifers, no	16	15	16	15				
Uterine weight, g	56.3	71.0	60.1	67.4	4.6	0.27	0.54	0.05
Uterine weight, g/100 kg BW	34.5	42.4	33.3	33.2	2.6	0.25	0.37	0.03
Uterine weight, g/100 kg CW	74.2	85.9	66.0	63.1	5.4	0.04	0.57	0.04
Ovarian weight, g	8.30	7.75	6.91	7.75	0.62	0.52	0.18	0.61
Ovarian weight, g/100 kg BW	4.94	4.37	3.63	3.71	A*	0.02	0.14	0.50
Ovarian weight, g/100 kg CW	10.5	8.85	7.17	7.06	B*	< 0.01	0.12	0.56
U + O weight, g	64.6	78.7	65.8	75.2	4.4	0.30	0.81	0.03
U + O weight, g/100 kg BW	39.7	47.1	36.3	37.1	2.6	0.12	0.62	0.02
U + O weight, g/100 kg CW	85.2	95.3	71.9	70.4	4.4	0.02	0.90	0.03

[1] Treatment groups are as follows: Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

[*] Data were log transformed to achieve homogeneous variance. Means presented are back transformed. The error term indicated below is the average of the lower and upper confidence intervals for each treatment group.

[A] Average confidence intervals are 0.39, 0.35, 0.27, and 0.28 for H0, H3, H6, and H12 treatment groups, respectively.

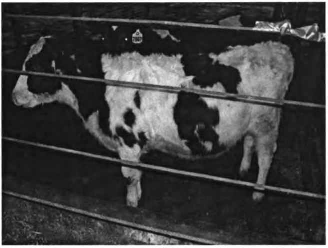
[B] Average confidence intervals are 0.84, 0.77, 0.58, and 0.57 for H0, H3, H6, and H12 treatment groups, respectively.

Table 7. Measurements of predicted versus observed daily intakes and gains.

	H0	H12
Heifers, no	16	15
DMI, kg/d		
Actual DMI	3.78	5.07
Predicted DMI, Spartan	3.05	3.63
Predicted DMI, NRC	3.67	3.64
ADG, kg/d		
Target ADG	0.60	1.20
Actual ADG	0.64	1.09
Predicted ADG, NRC*	0.44	1.03

* Predicted values for ADG with DMI adjusted for actual values.

A.



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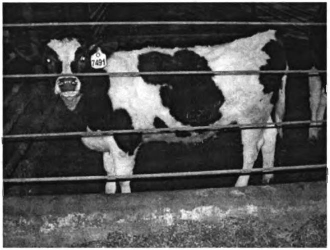

Figure 12. Representative picture of heifers fed low and high energy diets. Picture A is a heifer fed the low energy diet for 12 wk. Picture B is a heifer fed the high energy diet for 12 wk. Note the difference in apparent gut fill, body condition, and hair coat between heifers fed the two different diets.

CHAPTER THREE

EFFECTS OF FEEDING A HIGH ENERGY DIET TO PREPUBERTAL HEIFERS FOR A LONGER DURATION ON MAMMARY DEVELOPMENT

ABSTRACT

Our objective was to determine the effects of feeding prepubertal dairy heifers a high energy diet for a longer duration on mammary growth and composition. Holstein heifers (age = 11 wk; BW = 107 ± 1 kg) were assigned to 1 of 4 treatments (n = 16/trt) and fed 2 diets for different durations: H0, H3, H6, and H12 were fed a low energy diet for 12, 9, 6, and 0 wk followed by a high energy diet for 0, 3, 6, and 12 wk, respectively. The low and high energy diets were fed to achieve 0.6 and 1.2 kg average daily gain (ADG), respectively. Heifers were slaughtered at 23 wk of age and mammary tissue was collected. A longer duration of feeding the high energy diet increased total mammary gland weight, extraparenchymal fat weight, and intraparenchymal fat weight, but did not alter fat-free parenchymal tissue weight. When adjusted for fat-free carcass weight to more accurately reflect differences in physical maturity, fat-free parenchymal tissue weight decreased with a longer duration fed the high energy diet. Total amount of mammary parenchymal DNA and RNA and concentration of DNA were not different. However, after adjustment for carcass weight, the amount of DNA and RNA decreased as heifers were fed the high energy diet for a longer duration. We conclude that feeding prepubertal heifers a high energy diet for a longer duration results in a linear decrease in mammary fat-free parenchymal mass and a linear increase in extraparenchymal fat when data are adjusted for carcass weight. Because heifers fed for rapid gains reach puberty at a younger age, feeding heifers a high energy diet will result in less mammary parenchymal tissue at puberty and potentially lower milk production.

Key Words: mammary growth, heifer, nutrition

Abbreviation Key: ADG = average daily gain; **BL** = baseline; **CW** = carcass weight; **FAR** = fractional accretion rate

INTRODUCTION

The cost of raising replacement dairy heifers accounts for approximately 20% of total dairy herd expenses (Heinrichs, 1993). Feeding a high energy diet to allow for a rapid growth rate enables heifers to be bred and calve earlier, potentially reducing costs associated with raising replacement heifers. However, mammary growth relative to body growth and milk yield potential are reduced when heifers that are approximately 3 to 10 mo of age are fed a high energy diet promoting gains of greater than 1 kg/d for periods of 12 wk or longer (Petitclerc et al., 1999; Radcliff et al., 2000; Sejrsen et al., 1982).

Several recent studies indicate that increasing the energy intake of calves and heifers for a short duration (≤ 8 wk) may improve mammary development and future milk yield. Increasing the energy and protein intake of calves fed milk replacer from 2 to 8 wk of age resulted in increased body growth, mammary parenchymal mass, and content of mammary DNA and RNA (Brown et al., 2005a; Brown et al., 2005b). In another study, calves were either allowed to suckle a cow or were fed milk replacer until 6 wk of age. Calves that suckled gained more (0.86 versus 0.56 kg/d) and tended to yield more milk during first lactation (Bar-Peled et al., 1997). In a recent study, calves were either fed milk replacer resulting in gains of 0.59 kg/d or given free access to whole milk for 2, 30-min intervals/day for gains of 0.88 kg/d (Shamay et al., 2005). Feeding whole milk to calves affected BW but not skeletal size of the adult animal and increased milk yield by 4% during first lactation (daily 3.5% fat corrected; $P < 0.01$) compared to those calves fed milk replacer. Compared to a consistent growth regime, a stair-step feeding regime for heifers, which consisted of feeding high energy diets for 2 mo and energy-restricted diets for 3 mo, resulted in higher concentrations of mammary DNA, RNA, and protein, and increased milk yield in dairy and beef heifers (Choi et al., 1997; Park et al., 1998). However, the mechanism for how a high energy intake during the preweaning period and a stair-step feeding regime for heifers increases mammary growth has not been determined. One possibility is that preruminant calves and heifers respond differently when fed a high energy diet for a short versus a long duration.

The question remains as to how a short duration compared to a long duration of feeding of a high energy diet will affect mammary growth relative to body growth. Prior studies that indicated a detrimental effect of feeding a high plane of nutrition involved treatment periods of 12 wk or greater. Thus, we choose 12 wk as our long duration time point, 6 and 3 wk as our short duration time points, and 0 wk of feeding a high energy diet as our base control treatment. Our objective was to determine the effects of feeding prepubertal dairy heifers a high energy diet for a longer duration on mammary growth and composition. We hypothesized that feeding a high energy diet for a short duration would stimulate growth of mammary parenchyma, but a long duration of feeding a high energy diet would be detrimental to mammary growth relative to body growth in prepubertal heifers.

MATERIALS AND METHODS

Animals and Treatment

All procedures were approved by the Michigan State University Animal Use and Care Committee. Sixty-eight Holstein heifers (approximate age = 8 wk) were purchased within 4 consecutive wk in the fall (17 heifers/wk) with each wk classified as a separate purchase group. Heifers were housed at the Michigan State University Beef Cattle Research Center and were exposed to ambient temperatures and lighting during the adaptation and treatment periods, which occurred during late fall and winter. Heifers were housed in an open-sided barn with enough space per pen (dimensions: 14 X 38 ft) to allow for exercise. Each purchase group was allowed a 3-wk adaptation period for adjustment to facilities and diet. During this adaptation period, heifers were gradually transitioned from a diet similar to that fed before purchase to a TMR similar to a mixture of the treatment diets. One heifer within each purchase group was randomly selected and slaughtered at 11 weeks of age for baseline (**BL**) measurements used for calculation of mammary tissue accretion rates.

At 11 wk of age (BW = 107 ± 1 kg), 16 heifers within each purchase group were blocked by body weight and randomly assigned within block to 1 of 4 treatments. All heifers within a given treatment in the same purchase group were housed in the same pen. Thus, 4 pens of 4 heifers (1 pen per purchase group) were used in each of the 4 treatments. The treatment period lasted 12 wk and treatments were as follows: H0 (low energy diet fed for 12 wk); H3 (low energy diet fed for 9 wk followed by high energy diet for 3 wk); H6 (low energy diet fed for 6 wk followed by high energy diet for 6 wk); and H12 (high energy diet for 12 wk). The low energy diet was fed to achieve 0.6 kg average daily gain (**ADG**) and consisted of 10% straw, 33% mature alfalfa silage, 33% oatlage, and 24% concentrate on a DM basis. The low energy diet had 0.72 Mcal NE_g/kg DM, 16.3% CP, and 46.1% NDF. The high energy diet was fed to achieve 1.2 kg ADG and consisted of 20% immature alfalfa silage, 20% corn silage, and 60% concentrate on a DM basis. The high energy diet had 1.17 Mcal NE_g/kg DM, 18.4% CP, and 22.6% NDF. A more detailed description of diets was presented previously (Chapter 2). Both diets and water were available ad libitum. Diets were fed as a TMR once daily between 0900 and 0930 h. Actual daily gains previously reported (Chapter 2) averaged during the treatment period were 0.64, 0.65, 0.83, and 1.09 kg (± 0.01) for H0, H3, H6, and H12, respectively. During the last 2 wk during the treatment period daily gains were 0.72, 1.05, 1.34, and 1.19 kg (± 0.09) for H0, H3, H6, and H12, respectively.

Heifers were slaughtered at the end of the treatment period when heifers were 23 wk of age. Heifers were allowed to consume the TMR from the prior day's feeding until they were transported at 0600 h via trailer to the abattoir at the Michigan State University Meats Laboratory.

Tissue Collection

Heifers were weighed, stunned by captive bolt, and killed by exsanguination. Heifers were killed on 2 different days each wk for 4 consecutive wk with 8 heifers (2/trt) killed per day for each purchase group. Mammary glands were quickly removed after slaughter, cleaned, and bisected through the median suspensory ligament into right and left halves. The left half was weighed, put into a plastic bag, and frozen by submersion into a dry ice and 95% ethanol mixture. Frozen left hemiglands were stored at -20°C until composition was analyzed. Mammary parenchymal tissue samples were excised from the right front quarter for histology. Samples for histology were processed in the laboratory of Dr. Mike Akers at Virginia Tech and will not be discussed in this dissertation. Body weights, carcass weights (**CW**), and composition of other tissues collected at slaughter were previously reported (Chapter 2)

Reproductive tracts were examined to confirm that heifers were not freemartins and had not reached puberty. One heifer (trt = H3) was a freemartin and her data was eliminated from all results. Another heifer (trt = H12) was confirmed postpubertal after a corpus lutem was detected at slaughter and her data were also removed from the study.

Mammary Gland Composition

The frozen left half of the udder was cut transversely using a band saw into 5- to 10- mm thick slices. Slices on the anterior and posterior ends that did not contain parenchymal tissue were discarded. Slices were then placed on a cutting board and allowed to thaw slightly. Skin, teats, and lymph nodes were removed and discarded. The parenchymal tissue was dissected from the extraparenchymal fat and these 2 types of tissue were then weighed. Parenchymal tissue was ground with liquid nitrogen into a fine powder using a blender (Waring Commercial, New Hartford, CT). The powder was mixed and subsampled for analysis of DNA, RNA, fat, protein, and water. DNA and RNA content were measured as indicators of cell number and metabolic activity, respectively, using the same methods as Tucker (1964). Fat was determined by Soxhlet ether extraction (AOAC, 1990). Crude protein was determined using the method of Hach et al. (1987). Water was determined as the difference in weight after drying mammary parenchymal tissue in an oven at 106°C for 24 h.

Statistical Analysis

Statistical analysis used the PROC GLM procedure of SAS. Pen (n = 4 heifers per treatment in each purchase group) was used as the experimental unit with purchase group as a random variable and treatment*purchase group as the error term. Comparisons were tested using a linear (L) contrast with coefficients -7, -3, 1, and 9; a quadratic (Q) contrast with coefficients 7, -4, -8, and 5; and a cubic (C) contrast with coefficients -3, 8, -6, and 1 for H0, H3, H6, and H12, respectively. Least square means and standard errors of the mean are presented. Differences were declared to be statistically significant at $P < 0.05$ and tendencies at $P < 0.10$. All data from the 2 heifers that were eliminated from the trial were removed so that final animal numbers were 16, 15, 16, and 15 for treatment groups H0, H3, H6, and H12, respectively.

Accretion rates for mammary and body tissues were quantified by calculating the average daily accumulation of mammary tissue using the averages of the 4 baseline heifers as initial value and number of days between slaughter dates for baseline and treatment heifers. These accretion rates were then calculated on a fractional basis (fractional accretion rates: **FAR**) that was compounded over time.

Data for extraparenchymal fat weight, intraparenchymal fat weight, intraparenchymal fat weight adjusted for carcass weight, fat-free parenchymal tissue weight, fat-free parenchymal tissue weight adjusted for carcass weight, fat-free parenchymal tissue weight adjusted for fat-free carcass weight, DNA weight, DNA concentration, DNA weight adjusted for carcass weight, RNA weight, RNA weight adjusted for carcass weight, and the ratio of RNA:DNA were log transformed to achieve homogeneous variance and normality. Means presented for these data points are back transformed. Error is depicted as the average of the back transformed upper and lower 68% confidence intervals. Non-transformed means are presented in the Appendix.

RESULTS

Total weight of the mammary gland increased as heifers were fed the high energy diet for a longer duration (Table 2; L: $P < 0.01$). This was due to a linear increase in extraparenchymal fat (L: $P < 0.01$), as parenchymal tissue weights were not different (all contrasts: $P > 0.10$). When adjusted for carcass weight to more accurately reflect the differences in physiological maturity of the heifers, parenchymal tissue weight tended to decrease as heifers were fed the high energy diet for a longer duration (L: $P = 0.06$). Similar to extraparenchymal fat, intraparenchymal fat mass and also the percent of intraparenchymal fat increased as heifers were fed the high energy diet for a longer duration (L: $P < 0.01$). When adjusted for carcass weight, extraparenchymal fat increased in heifers fed the high energy diet for a longer duration, but there was no effect on adjusted intraparenchymal fat (L: $P < 0.01$; all constrasts: $P > 0.10$, respectively). Fat-free parenchymal tissue weight relative to carcass weight or fat-free carcass weight decreased with a longer duration fed the high energy diet (Table 2 and Figure 1; L: $P < 0.01$ and $P = 0.02$, respectively). Mammary parenchymal protein mass was not different, but the percentage of protein in mammary tissue tended to be less with a longer duration of feeding the high energy diet and for a cubic relationship (L: $P = 0.09$ and C: $P = 0.08$).

The total amount of parenchymal DNA and RNA and concentration of DNA were not different among treatment groups (Table 3; all contrasts: $P > 0.10$). A linear decrease with a longer duration fed the high energy diet was evident when DNA mass was adjusted for carcass weight (L: $P = 0.05$). Both the concentration of RNA and the ratio of RNA:DNA displayed a cubic effect due to the higher abundance of mammary RNA within the H3 heifers (C: $P = 0.07$ and $P = 0.05$, respectively).

Daily compounded FAR of extraparenchymal fat and intraparenchymal fat within the mammary gland increased as heifers were fed the high energy diet for a longer duration (Table 4; L: $P < 0.01$). Body weight and carcass fat FAR were significant for linear and quadratic effects and these were mainly due to the duration of time that heifers received the high energy diet (L: $P < 0.01$ and Q: $P = 0.01$ for both measurements). Daily accretion rates for mammary parenchyma, fat-free parenchyma, mammary DNA, and mammary RNA did not change with time fed the high energy diet (all contrasts: $P > 0.10$).

DISCUSSION

Feeding prepubertal heifers a high energy diet for a longer duration results in a linear decrease in fat-free mammary parenchymal weight and a linear increase in mammary fat when data are adjusted for carcass weight. Our results are in agreement with other studies that have demonstrated an impairment of mammogenesis in prepubertal heifers fed high energy diets for time periods of 12 wk or more (Harrison et al., 1983; Petitclerc et al., 1999). Similar to earlier work (Capuco et al., 1995; Radcliff et al., 1997), these findings indicate that high energy intake in prepubertal heifers results in accelerated body growth rates, but also excessive fattening within the mammary gland. However, this is the first study to demonstrate the effects of feeding a high energy diet for a short duration on mammary growth in prepubertal heifers.

Our original hypothesis was that a short duration of feeding a high energy diet would stimulate mammary parenchymal growth relative to body growth. This idea originated from observations in compensatory growth studies and nutritional studies during the preweaning growth phase. Compensatory growth studies indicate that a stair-step feeding regimen of alternating feed intake of heifers by 25 to 30% above recommendations for 2 mo and 20 to 30% below recommendations for 3 to 5 mo in length can positively affect the lactation potential of heifers (Choi et al., 1997). The mechanism for why this phenomenon occurs is not known. The positive influence on mammary growth could be due to the stair-step regime or potentially the short time period that heifers were fed above recommendation levels. However, some have suggested that the treatment period in these compensatory growth studies is either completely or partly outside the critical window, because feeding high energy diets to postpubertal heifers does not alter mammary growth (Sejrsen et al., 1982; Sejrsen and Purup, 1997). Nutritional studies during the preweaning period indicate that increasing the energy and protein intake in calves for a period of 6 wk in length (2 to 8 wk of age) resulted in an increase in body growth and nearly a doubling of mammary parenchymal DNA (Brown et al., 2005a; Brown et al., 2005b). However, increasing the energy and protein intake of postweaned calves from 8 to 14 wk of age resulted in no difference in mammary parenchymal growth (Brown et al., 2005a). Other studies have measured an increase in 300-d milk production and daily fat corrected milk yield when heifers were allowed greater gains during the preweaning period (Bar-Peled et al., 1997; Shamay et al., 2005). The mechanism for why feeding diets promoting rapid gains might stimulate mammogenesis during the preweaning period, but not during the later prepubertal period is not understood. However, the results from this present study using older prepubertal heifers indicate that high energy diets fed for a short duration of 3 and 6 wk are not stimulatory to mammogenesis and instead inhibit mammary growth relative to body growth in a time-dependent manner consistent with a long duration. Therefore, it seems more likely that the positive influence on mammary growth in the above studies is due to the stair-step regime and due to high energy intake before weaning and not because of short-term high energy intakes. Thus, the mechanisms explaining why these feeding programs are stimulatory to mammary growth are still unknown.

The growth and development of the mammary gland in heifers is crucial to productivity, as the number of mammary epithelial cells is a major factor determining milk production (Tucker, 1981). Mammary tissue DNA content was positively correlated ($r = 0.85$) to litter weight gain in rats (Tucker, 1966). Mammary tissue DNA content from 5 mo-old heifers was positively correlated to milk yield (0 to 30-d; $r = 0.21$) and to mammary tissue DNA collected at 60-d into first lactation ($r = 0.25$) (Tucker et al., 1973). The milk yield potential is partially determined by the growth of the mammary gland prior to puberty and during pregnancy (Sejrsen, 1994). This is why high energy diets fed during the prepubertal period can have long lasting detrimental effects on milk yield. In this study, all heifers were slaughtered at the same age, but there were significant differences in body weight and carcass weight at slaughter. Mammary data were adjusted for carcass weight to more accurately reflect the difference in physiological maturity of the heifers. We chose carcass weight instead of body weight due to the difference in the forage: concentrate ratios in the diets, which resulted in apparent differences in gut fill between the H0 treatment and the other 3 treatment groups at slaughter. Fat-free mammary parenchymal tissue weight adjusted for fat-free carcass weight decreased in a linear, time-dependent manner with a longer duration fed the high energy diet. This indicates impaired mammary development. Heifers fed a high energy diet during the prepubertal period reached puberty at a younger age than heifers fed a moderate or low energy diet (Schillo et al., 1992). The onset of puberty is influenced by body weight, degree of body fatness, and plane of nutrition (Schillo et al., 1992). The rate of mammary growth becomes isometric relative to other tissues around the onset of puberty (Meyer et al., 2004; Sinha and Tucker, 1969). Therefore, this demonstrates that high energy intake did inhibit mammary growth because it is likely that if heifers had been slaughtered at the onset of puberty, the amount of mammary parenchymal tissue would have been greater in the heifers fed the low energy diet than heifers fed the high energy diet.

An increase in the amount of extraparenchymal fat, amount of intraparenchymal fat, intraparenchymal fat percent, and extraparenchymal fat adjusted for carcass weight were all observed in this study when heifers were fed a high energy diet for a longer duration. Heifers fed high energy diets containing corn silage had more fat deposited within the mammary gland (Capuco et al., 1995). An increase in body fatness, which was also observed in these heifers (Chapter 2), is negatively correlated with mammary parenchymal DNA and milk production (Silva et al., 2002b). Similarly, the amount of mammary secretory tissue is inversely related to extraparenchymal fat mass in heifers

(Sejrsen et al., 1982). The growth of mammary epithelial cell organoids is inhibited when co-cultured with bovine mammary fat pad explants (McFadden and Cockrell, 1993). In addition, mammary tissue extracts from prepubertal heifers fed a high compared to a moderate energy diet were less mitogenic for mammary epithelial cells in vitro (Berry et al., 2003; Weber et al., 2000a). These studies demonstrate that heifers fed a high energy diet have an increased deposition of fat and that mammary fat may secrete a factor that inhibits mammary epithelial cell growth. In agreement with this idea, metabolic activity (RNA), cell number (DNA), and fat-free mammary parenchymal mass were all decreased with time fed a high energy diet when adjusted for carcass weight. Previous results indicate that heifers fed a high energy diet had a tendency for decreased parenchymal DNA weight (Sejrsen et al., 1982) and a tendency for decreased parenchymal DNA concentration and total DNA adjusted for body weight (Petitclerc et al., 1984).

Most studies observe that the amount of extraparenchymal fat is increased in heifers fed a high energy diet for rapid gains. But, some have not observed a dietary effect on the amount of intraparenchymal fat (Sejrsen et al., 1982). Sejrsen and co-workers suggested that the slower growth of parenchymal DNA measured in heifers fed a high energy diet was not caused by increased fat infiltration of the gland but due to an inhibitory effect of higher amounts of extraparenchymal fat. Intraparenchymal fat percentage in 14 wk old calves ranged from 7.0 to 13.2 % and calves fed a high energy diet from 8 to 14 wk had a greater percentage of intraparenchymal fat than calves fed a low energy diet, but mammary parenchymal weights were similar (Brown et al., 2005a).

In this study, we were able to detect an increase in both extraparenchymal and intraparenchymal fat with a longer duration fed a high energy diet.

Data collected from 4 baselines heifers were used to calculate compounded FAR of mammary growth. Baseline values (age = 11 wk) for parenchymal tissue weight, DNA content, and RNA content were similar to those previously reported for 14 wk old calves (Brown et al., 2005a). Results clearly indicate that FAR of extraparenchymal fat and intraparenchymal fat were increased with a longer duration fed the high energy diet, but FAR of mammary parenchyma, RNA, and DNA were not altered by diet. There was no change in the FAR of fat-free mammary parenchyma when comparisons between the H12 and the H0 treatment groups were performed, but fractional rate of both body weight and fat-free carcass weight accretion were increased with high feeding by 45% and 78%, respectively (see Table 5). This indicates that although high energy feeding did not reduce the fractional rate of mammary tissue accretion, it did increase the accretion rate of body weight and carcass weight compared to the low plane of nutrition.

CONCLUSIONS

We conclude that increasing the dietary energy intake of prepubertal heifers for a short duration does not improve mammary growth but rather alters growth of mammary tissues relative to body growth in a time-dependent manner, consistent with feeding high energy diets for a long duration. Fat-free mammary parenchymal tissue weight adjusted for fat-free carcass weight decreased in a linear fashion as heifers were fed a high energy diet for a longer duration. An increase in body or carcass growth without a proportional increase in mammary growth would result in less mammary parenchymal tissue at puberty and potentially lower milk yield because heifers fed for rapid gains reach puberty at a younger age. Feeding prepubertal heifers a high energy diet increases the deposition of fat in the mammary gland and may play a role in the impairment of mammogenesis.

Table 1. Least square means for body and carcass characteristics.

	Treatment Group[1]					*P* for contrast[2]		
	H0	H3	H6	H12	SE[3]	L	Q	C
Heifers, no	16	15	16	15				
Initial BW, kg	106	108	108	108	0.74	0.25	0.19	0.72
BW at slaughter, kg	165	167	181	203	1.10	< 0.01	< 0.01	< 0.01
Carcass weight, kg	76.6	82.5	91.5	107	0.65	< 0.01	0.40	0.10
Fat-free carcass, kg	71.0	74.2	79.1	91.2	0.98	< 0.01	0.08	0.74

[1] Treatment groups are as follows: Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment

Table 2. Least square means for mammary gland composition.

		Treatment Group[1]					*P* for contrast		
	BL	H0	H3	H6	H12	SE	L	Q	C
Heifers	4	16	15	16	15				
Mammary gland weight, g	78.1	808	1014	1412	1818	90.0	< 0.01	0.59	0.33
Parenchyma, g	8.05	146	152	166	164	10.5	0.22	0.48	0.63
Parenchyma, g/100 kg carcass weight	14.9	191	185	181	155	12.6	0.06	0.63	0.84
Extraparenchymal fat, g	39.5	210	290	429	588	A*	< 0.01	0.08	0.43
Extraparenchymal fat, g/100 kg carcass weight	72.9	278	358	476	582	47.8	< 0.01	0.52	0.64
Intraparenchymal fat, g	0.46	23.2	26.6	33.6	37.7	B*	< 0.01	0.29	0.45
Intraparenchymal fat, % of parenchyma	5.32	17.2	18.5	22.0	25.3	1.06	< 0.01	0.80	0.37
Intraparenchymal fat, g/100 kg carcass weight	0.84	30.4	32.3	36.8	35.5	C*	0.17	0.33	0.55
Fat-free parenchyma, g	7.59	119	120	123	115	D*	0.93	0.49	0.96
Fat-free parenchyma, g/100 kg carcass weight	14.0	151	146	134	109	E*	< 0.01	0.54	0.91
Parenchymal protein, g	0.77	13.0	13.8	13.9	14.0	0.14	0.53	0.67	0.87
Parenchymal protein, % of parenchyma	9.54	8.87	8.96	8.46	8.59	0.14	0.09	0.40	0.08

[1] Treatment groups are as follows: heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

[*] Data were log transformed to achieve homogeneous variance. Means presented are back transformed. The error term indicated below is the average of the lower and upper confidence intervals for each treatment group.

[A] Average confidence intervals are 15.7, 22.6, 32.0, and 45.7 for H0, H3, H6, and H12 treatment groups, respectively.

[B] Average confidence intervals are 1.79, 2.14, 2.60, and 3.03 for H0, H3, H6, and H12 treatment groups, respectively.

[C] Average confidence intervals are 2.38, 2.63, 2.88, and 2.89 for H0, H3, H6, and H12 treatment groups, respectively.

[D] Average confidence intervals are 8.58, 8.99, 8.82, and 8.62 for H0, H3, H6, and H12 treatment groups, respectively.

[E] Average confidence intervals are 10.7, 10.7, 9.47, and 7.97 for H0, H3, H6, and H12 treatment groups, respectively.

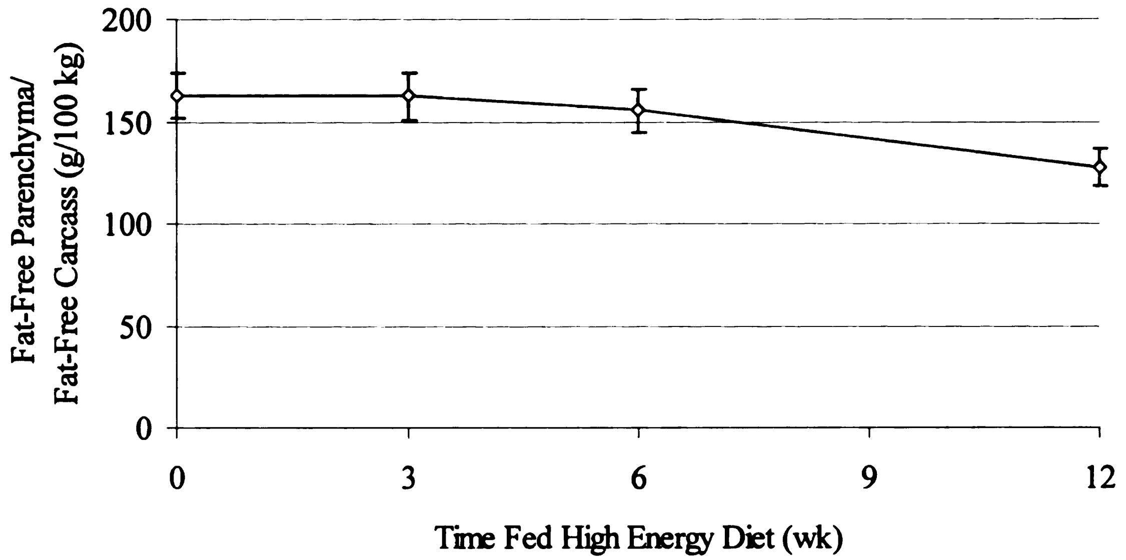

Figure 1. Grams of fat-free parenchymal tissue relative to 100 kg fat-free carcass. Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Data was log transformed to achieve homogeneous variance. Means presented are back transformed. The error term is the average of the lower and upper confidence intervals for each treatment group. Data are significant for a linear effect (Linear: *P* = 0.02).

Table 3. Least square means for mammary gland nucleic acid content.

		Treatment Group[1]					*P* for contrast[2]		
	BL	H0	H3	H6	H12	SE[3]	L	Q	C
Heifers, no	4	16	15	16	15				
Parenchymal DNA, mg	37.5	803	859	916	900	A[*]	0.31	0.43	0.63
DNA, mg/100 kg carcass weight	69.2	1051	1043	1002	848	B[*]	0.05	0.48	0.99
DNA concentration, mg/g	4.59	5.75	5.86	5.82	5.81	C[*]	0.90	0.69	0.72
Parenchymal RNA, mg	64.3	767	875	871	850	D[*]	0.49	0.30	0.63
RNA, mg/100 kg carcass weight	119	1004	1062	953	801	E[*]	0.03	0.34	0.52
RNA concentration, mg/g	8.05	5.43	6.04	5.61	5.53	0.17	0.49	0.20	0.07
RNA:DNA	1.80	0.59	1.02	0.94	0.94	F[*]	0.42	0.48	0.05

[1] Treatment groups are as follows: heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

[*] Data were log transformed to achieve homogeneous variance. Means presented are back transformed. The error term indicated below is the average of the lower and upper confidence intervals for each treatment group.

[A] Average confidence intervals are 58.8, 65.6, 67.2, and 68.7 for H0, H3, H6, and H12 treatment groups, respectively.

[B] Average confidence intervals are 75.6, 78.1, 72.1, and 68.5 for H0, H3, H6, and H12 treatment groups, respectively.

[C] Average confidence intervals are 0.13, 0.14, 0.13, and 0.14 for H0, H3, H6, and H12 treatment groups, respectively.

[D] Average confidence intervals are 57.6, 68.4, 65.4, and 66.5 for H0, H3, H6, and H12 treatment groups, respectively.

[E] Average confidence intervals are 73.8, 81.3, 70.1, and 61.3 for H0, H3, H6, and H12 treatment groups, respectively.

[F] Average confidence intervals are 0.02, 0.03, 0.02, and 0.02 for H0, H3, H6, and H12 treatment groups, respectively.

Table 4. Least square means for daily compounded fractional accretion rates (FAR).

	Treatment Group[1]					*P* for contrast[2]		
	H0	H3	H6	H12	SE[3]	L	Q	C
Heifers, no	16	15	16	15				
Extraparenchymal fat FAR (%)	1.14	1.52	1.98	2.35	0.09	< 0.01	0.08	0.42
Parenchymal FAR (%)	2.53	2.60	2.68	2.65	0.08	0.31	0.44	0.80
Fat-free parenchymal FAR (%)	2.37	2.42	2.44	2.37	0.09	0.92	0.48	0.94
Intraparenchymal fat FAR (%)	3.89	4.05	4.33	4.46	0.10	< 0.01	0.29	0.44
RNA FAR (%)	2.08	2.24	2.23	2.22	0.09	0.50	0.30	0.64
DNA FAR (%)	2.80	2.88	2.96	2.93	0.09	0.32	0.43	0.86
BW FAR (%)	0.49	0.49	0.58	0.71	0.01	< 0.01	0.01	0.02
Carcass Fat FAR (%)	0.69	1.19	1.66	1.88	0.07	< 0.01	< 0.01	0.40

[1] Treatment groups are as follows: heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

Table 5. Difference in daily fractional accretion rates (FAR) [1] of heifers fed high compared to low energy diets for 12 wk.

	H0[1]	H12[2]	Percent Difference
Carcass fat FAR (%)	0.69	1.88	172
Extraparenchymal fat FAR (%)	1.14	2.35	106
Fat-free carcass FAR (%)	0.37	0.66	78
Body weight FAR (%)	0.49	0.71	45
Intraparenchymal fat FAR (%)	3.89	4.46	15
RNA FAR (%)	2.08	2.22	7
DNA FAR (%)	2.80	2.93	5
Parenchymal FAR (%)	2.53	2.65	5
Fat-free parenchymal FAR (%)	2.37	2.37	0

[1] Variable "x" FAR = [ln (x adjusted to 84 d) – ln (x for baseline or at wk 11)]/84 d

[2] Treatment groups are as follows: heifers on H0 were fed the low energy diet for 12 wk and heifers on H12 were fed the high energy diet for 12 wk. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

CHAPTER FOUR

EFFECTS OF FEEDING A HIGH ENERGY DIET TO PREPUBERTAL HEIFERS FOR A LONGER DURATION ON ABUNDANCE OF LEPTIN AND IGF-I IN MAMMARY TISSUE AND SERUM

ABSTRACT

Feeding a high energy diet to prepubertal heifers for a longer duration decreased fat-free mammary parenchymal tissue mass when adjusted for fat-free carcass weight and increased mammary fat. The mechanism by which feeding a high energy diet to prepubertal dairy heifers impairs mammary growth relative to body growth is not clear but may involve leptin and IGF-I synthesis. Our objective was to determine the effects of feeding prepubertal heifers a high energy diet for a longer duration on serum protein levels of leptin, insulin-like growth factor-I (IGF-I), and IGF binding proteins (IGFBP); protein concentration of leptin in extracts of mammary parenchymal tissue; and mRNA expression of leptin, leptin receptor, IGF-I, and IGF-I receptor in mammary parenchymal tissue. Heifers (n = 64; age = 11 wk; BW = 107 kg) were randomly assigned to 1 of 4 treatments and fed 2 diets for different lengths of time: H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, and 0 wk followed by the high energy diet for 0, 3, 6, and 12 wk, respectively. The low and high energy diets were formulated for 0.6 and 1.2 kg daily gain, respectively. Animals were slaughtered at 23 wk of age and mammary parenchymal tissue samples were collected for analysis. Concentrations of leptin protein in serum and mammary tissue and mRNA expression of leptin in mammary tissue increased as heifers were fed the high energy diet for a longer duration. Dietary intake did not alter the abundance of leptin receptor, IGF-I, or IGF-I receptor mRNA expression in mammary tissue. A longer duration fed the high energy diet increased serum levels of IGF-I and decreased abundance of IGFBP-2. Abundance of serum IGFBP-3 increased in a linear fashion in heifers fed a high energy diet for a longer duration, but was also significant for a cubic contrast. These dietary effects on leptin abundance, taken together with prior research indicating that leptin inhibited the proliferation of mammary epithelial cells, show that leptin may in part mediate the inhibitory effects of high energy intake on mammary growth relative to body growth in prepubertal heifers.

Key Words: mammary gland, heifer, nutrition, leptin, IGF-I

Abbreviation Key: GAPDH = glyceraldehyde 3-phosphate dehydrogenase; **IGF-I** = insulin-like growth factor-I; **IGFBP** = insulin-like growth factor binding protein

INTRODUCTION

Raising replacement heifers is costly for the producer and is estimated to be 20% of total dairy herd expenses (Heinrichs, 1993). Growing heifers faster for earlier breeding and calving can reduce these costs. However, feeding a high energy diet for rapid gains of greater than 1 kg/d to prepubertal heifers can impair mammary growth relative to body growth and reduce subsequent milk yield (Petitclerc et al., 1999; Radcliff et al., 2000; Sejrsen et al., 1982). Since 1915, researchers have focused on understanding the link between high energy intake and impairment of mammary growth (Eckles, 1915).

Several theories to explain the nutritional impairment of mammary growth have been suggested, but the mechanism is still not understood clearly. Earlier studies noted that high energy feeding resulted in undeveloped areas of mammary parenchyma and lower milk yields (Swanson, 1960). More recent studies showed an increase in the deposition of fat in the mammary glands of heifers fed for rapid gains during the prepubertal period (Radcliff et al., 1997; Sejrsen et al., 1982). Mammary tissue extracts from heifers fed a high compared to a low energy diet were less mitogenic for mammary epithelial cells in vitro (Weber et al., 2000a). Also, bovine mammary fat pad explants inhibit mammary epithelial cell proliferation in vitro (McFadden and Cockrell, 1993). Taken together, these results indicate that adipocytes within the gland may produce a substance that inhibits mammary epithelial cell growth. Leptin, a protein produced by fat cells, may play a role in this inhibition. When infused into the mammary gland of prepubertal heifers, leptin inhibited the insulin-like growth factor-I (**IGF-I**) stimulation of mammary growth (Silva et al., 2003). Infusion of leptin also decreased the percentage of BrdU-labeled mammary epithelial cells compared to control quarters infused with saline (Silva et al., 2003). Whether leptin protein concentration and mRNA expression of leptin are increased in the mammary tissue of heifers fed a high compared to a low energy diet has not been established.

When heifers are fed a high energy diet associated with impaired mammogenesis, serum growth hormone concentration decreases, but serum IGF-I concentration increases (Vestergaard et al., 2003). This seems contradictory because IGF-I is a known mitogen for mammary epithelial cells (Shamay et al., 1988; Silva et al., 2005). No change in mRNA expression or concentration of IGF-I in the mammary gland was noted in prepubertal heifers fed a high or low energy diet (Weber et al., 2000b). Specific binding of labeled IGF-I to mammary membranes was unaffected by feeding level in heifers (Purup et al., 1999). However, mammary tissue explants from heifers fed a high energy diet was less sensitive to IGF-I treatment compared to explants from heifers fed a low energy diet (Purup et al., 1996). One explanation for this difference could be that nutrition alters the number of IGF-I receptors present on mammary epithelial cells.

The objective of this experiment was to determine the effects of feeding a high energy diet for a longer duration on serum protein levels of IGF-I, IGF binding proteins (**IGFBP**), and leptin; protein concentration of leptin in extracts of mammary parenchymal tissue; and mRNA expression of leptin and IGF-I and their receptors in mammary parenchymal tissue. We hypothesized that feeding a high energy diet would increase abundance of IGF-I and IGFBP-3 and decrease IGFBP-2 in serum, but would not change mRNA expression of IGF-I in mammary tissue. Expression of mRNA for the IGF-I receptor in mammary parenchymal tissue would decrease as heifers were fed a high energy diet for a longer duration. Feeding a high energy diet would increase leptin protein concentrations in serum and mammary tissue and expression of mRNA for leptin in mammary tissue. Results of treatment effects on body growth, carcass composition, and mammary growth are presented elsewhere (Chapters 2 and 3).

MATERIALS AND METHODS

Animals and Treatment

All procedures were approved by the Michigan State University Animal Use and Care Committee. Specific details of the experiment were described earlier (Chapter 2). Briefly, 64 Holstein heifers (approximate age = 8 wk) were purchased within 4 consecutive wk in the fall (16 heifers/wk) with each wk classified as a separate purchase group. Heifers were housed at the Michigan State University Beef Cattle Research Center in an open-sided barn. Each purchase group was allowed a 3-wk adaptation period for adjustment to facilities and diet. During this adaptation period, heifers were gradually transitioned from a diet similar to that fed before purchase to a TMR similar to a mixture of the treatment diets.

At 11 wk of age (BW = 107 ± 1 kg), heifers within each purchase group were blocked by body weight and randomly assigned within block to 1 of 4 treatments. All heifers within a given treatment in the same purchase group were housed in the same pen. Thus, 4 pens of 4 heifers (1 pen per purchase group) were used in each of the 4 treatments. The treatment period lasted 12 wk and treatments were as follows: H0 (low energy diet fed for 12 wk); H3 (low energy diet fed for 9 wk followed by high energy diet for 3 wk); H6 (low energy diet fed for 6 wk followed by high energy diet for 6 wk); and H12 (high energy diet for 12 wk). The low energy diet was fed to achieve 0.6 kg average daily gain (ADG) and consisted of 10% straw, 33% mature alfalfa silage, 33% oatlage, and 24% concentrate on a DM basis. The low energy diet had 0.72 Mcal NE_g/kg DM, 16.3% CP, and 46.1% NDF. The high energy diet was fed to achieve 1.2 kg ADG and consisted of 20% immature alfalfa silage, 20% corn silage, and 60% concentrate on a DM basis. The high energy diet had 1.17 Mcal NE_g/kg DM, 18.4% CP, and 22.6% NDF. Diets were fed as a TMR and both diets and water were available ad libitum. Composition of diets based on actual individual feedstuff analyses are presented elsewhere in more detail (Chapter 2). Actual daily gains previously reported (Chapter 2) averaged during the treatment period were 0.64, 0.65, 0.83, and 1.09 kg (± 0.01) for H0, H3, H6, and H12, respectively. Blood samples were taken on wk 0, 2, 4, 6, 8, 10, 11, and 12 during the treatment period and at slaughter. Blood samples were kept at room temperature for 4 to 6 hr to clot and then refrigerated overnight at 4°C. Blood tubes were then centrifuged at 2700 x g for 20 min at 4°C. Serum was collected and stored at -20°C until analysis.

Heifers were slaughtered at the end of the treatment period when heifers were 23 wk of age. Heifers were allowed to consume the TMR from the prior day's feeding until they were transported at 0600 h via trailer to the abattoir at the Michigan State University Meats Laboratory. Heifers were weighed, stunned by captive bolt, and killed by exsanguination. Mammary glands were quickly removed after slaughter (12 min ± 0.002), cleaned, and bisected through the median suspensory ligament into right and left halves. The left half was placed into a plastic bag and frozen by submersion into a dry ice and 95% ethanol mixture. Frozen left hemiglands were stored at -20°C until further analysis. Mammary parenchymal tissue samples were excised from the right rear quarter for isolation of RNA and stored at -80°C until further analysis. The right rear quarter was visually separated into 3 regions and small pieces of tissues were taken from the outer third region of parenchyma closest to the fat pad, but not including any extraparenchymal fat.

Preparation of Mammary Extracts

The frozen left half of the udder was cut transversely using a band saw into 5- to 10-mm thick slices. The parenchymal tissue was dissected from the extraparenchymal fat. Parenchymal tissue was ground with liquid nitrogen into a fine powder using a blender and stored at -20°C until extracts were prepared. Extracts were prepared by weighing 1 g of parenchymal tissue powder into tarred tubes and then 2 mL of saline (0.9% sodium chloride) was added to the tube. Tissue was homogenized for 1 min using a Polytron (PT 10 20 350D, Switzerland). The tip of the Polytron was rinsed between samples with 1 mL of saline, which was then combined with the homogenate to yield a total of 3 mL, with a 3:1 saline to tissue ratio. Protease inhibitory cocktail (Sigma, 25 µL) was added to the homogenate, and the mixture was vortexed. Homogenate was centrifuged at 10,000 x *g* and 4°C for 45 min. The supernatant was recovered and filtered through a 0.22-micron low protein binding filter unit. Extracts of mammary tissue from each heifer were stored at -20°C in microcentrifuge tubes until leptin concentration was analyzed.

Leptin Radioimmunoassay (RIA)

Leptin concentrations in mammary extracts and serum samples (wk 0, 2, 4, 6, 8, 10, 11, 12, and slaughter) were determined as in Delavaud et al. (2000). Serum samples at wk 0, 2, 4, 6, 8, 10, 11, and 12 were run in a separate assay from serum samples taken at slaughter and mammary extracts. A standard curve (0.08 to 4 ng) prepared from recombinant ovine leptin was included in the assay. For samples, triplicate aliquots of 200 µL were assayed. Both sample and standard tubes were incubated for 24 h at 4°C with 50 µL of a 1:1,500 dilution of rabbit anti-ovine leptin antisera. After this incubation, tubes were incubated an additional 20 h after 20,000 CPM ^{125}I-ovine leptin was added to each tube. Final dilution of antisera was 1:15,000. Bound and free leptin were separated by addition of 100 µL of sheep anti-rabbit plasma and the antibody-antigen complex was precipitated through the addition of 2 mL of 4.4% polyethylene glycol and centrifugation. Radioactivity of the pellet was quantified with a gamma counter (Cobra II Auto Gamma, Packard BioScience Co, Dowers Grove, IL).

IGF-I Radioimmunoassay (RIA)

IGF-I concentration was measured in serum samples (wk 0, 2, 4, 6, 8, 10, 11, 12, and slaughter) from each heifer. A total of 4 assays were performed, each with equal representation of the treatment groups and with each purchase group as a separate assay. Binding proteins were separated from IGF-I by acid-ethanol cryoprecipitation (Breier et al., 1991). Formic acid (2.4 M; 25 µL) and ethanol (100%; 500 µL) were added to each sample (100 µL) and the mixture was vortexed. Samples were incubated for 30 min at room temperature and then centrifuged at 600 x *g* and 4°C. The supernatant (100 µL) was pipetted into a clean tube with 2 mL of neutralizing buffer [53.5% ethanol/HCl mixture (87.5%ethanol, 12.5% 2 *M* HCL) 28.6% 0.855 *M* Tris, 17.9% deionized H_2O]. For samples, duplicate aliquots of 200 µL were assayed. A standard curve (25 to 6400 pg) prepared from recombinant human IGF-I was included in the assay. Both standard and sample tubes were incubated with 20,000 CPM ^{125}I-IGF-I isotope per tube and 250 µL of rabbit anti-human IGF-I antisera (GroPep, Adelaide, SA, Australia) was added to each sample for a final assay dilution of 1 to 100,000. Samples were vortexed and incubated overnight at 4°C. Protein A (*Staphylococcus aureus*, Zymed, San Francisco, CA) was added the next day at 1 mg/tube and tubes were vortexed. After a 2 h incubation at room temperature, 2 mL of assay buffer (0.03 *M* NaH_2PO_4, 0.01 *M* EDTA, 0.02% Na azide, 0.005% Tween 20, 0.02% protamine sulfite; pH to 7.5) was added to each tube and then centrifuged for 30 min at 3070 x *g*. Liquid was decanted and radioactivity of the pellet was quantified in a gamma counter (Cobra II Auto Gamma, Packard BioScience Co, Dowers Grove, IL).

Western Ligand Blot

Western ligand blotting was used to analyze the relative abundance of IGFBP in serum samples taken at wk 0 and wk 12 of the treatment period. Samples from 32 heifers were analyzed using a total of 8 gels. Each gel contained wk 0 and wk 12 samples from 4 heifers, with each treatment group represented on a gel and a total of 2 gels for each purchase group. A 12.5% acrylamide setparating gel solution was prepared and deaerated [24.9 mL monomer solution (30.8% acrylamide, 2.7% bisacrylamide), 15 mL 4X separating buffer (1.5 *M* Tris-Cl, pH 8.8), 600 µL 10% SDS, and 19.2 mL deionized H_2O]. Tetramethylethylenediamine (TEMED, 20 µL, Eastman, Rochester, NY) and 10% ammonium persulfate solution (300 µL, Sigma) was added to the deaerated separating gel solution. Separating gels were allowed to polymerize and were then removed from the gel apparatus to the electrophoresis unit.

A mini vertical gel electrophoresis instrument (Hoefer SE 250, San Francisco, CA) was used. A 4% acrylamide stacking gel solution was prepared and deaerated [2.64 mL monomer, 4.98 mL 4X stacking gel buffer (0.5 *M* Tris-Cl, pH 6.8), 198 µL 10% SDS, and 12.18 mL deionized H_2O]. After adding TEMED (10 µL) and ammonium persulfate (100 µL), the gel solution was pipetted on top of the separating gel and a comb (1.5 mm thick, 16 X 5 mm) was inserted into each stacking gel. The gel was allowed to polymerize for 1 hr and the comb was then removed. Tank buffer (0.025 *M* Tris, 0.192 *M* glycine, 0.1% SDS, pH 8.3) was placed into the upper and lower chambers and into each well. Serum samples were prepared using equal part of sample and 2X treatment buffer (0.125 *M* Tris-Cl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, pH 6.8). The samples were placed in a 70°C waterbath for 2.5 min to denature the protein in the sample. The sample was then loaded at 200 µg of protein per lane. It was assumed that serum samples were approximately 8% protein and were similar among samples. A molecular ladder (BenchMark Prestained Protein Ladder, Invitrogen, Carlsbad, CA) containing proteins from 10 to 190-kD was used as a marker for binding protein sizes. The voltage remained constant at 180 V throughout electrophoresis (~75 min) and was stopped when the bromophenol blue dye reached the bottom of the gel.

Gels and filter paper were placed into transfer buffer (0.025 *M* Tris, 0.192 *M* glycine, 20% methanol, pH 8.3). Polyvinylidene difluoride (PVDF) membranes were pre-wetted in 100% methanol before rinsing in transfer buffer. On the semi-dry transfer cell (BioRad TransBlot SD, Hercules, CA); items were layered in the following order: filter paper, PVDF membrane, gel, and filter paper. Voltage was set constant at 25 V for approximately 130 min. Membranes were removed and placed in Tris buffer (100 mM Tris/HCl, 0.9% NaCl, pH 7.5) with 1% BSA (RIA grade, Sigma, St Louis, MO) and 0.1% Tween 20 for 1 h and then incubated overnight with 500,000 CPM ^{125}I-IGF-I isotope per mL of buffer. The next day membranes were washed using Tris buffer with and without Tween 20, allowed to dry, and placed in a x-ray cassette with film. Film was scanned with a densitometer (Fluor-S MultiImager, BioRad; Quantity One v4.1, BioRad) to quantify differences in the density of the bands corresponding to various IGFBP.

RNA Isolation

RNA was isolated from mammary parenchymal tissue using the Trizol method. Tissue was kept cold using dry ice and 200 mg of mammary tissue was weighed and added directly to 3 mL of Trizol reagent (Invitrogen) in a culture tube. Tissue was then homogenized using a Polytron for 30 s. The tip of the Polytron was rinsed in between samples using diethyl pyrocarbonate (DEPC, Sigma) treated water. Samples were split into 3, 1-mL samples and incubated at room temperature for 5 min. Chloroform (200 µL) was added to each microcentrifuge tube. The tube was vortexed, incubated for 3 min at room temperature, and centrifuged at 10,500 rpm for 15 min at 4°C. The upper phase was transferred to a clean tube. Isopropanol (500 µL) was added to the precipitated RNA. The tube was vortexed, incubated at room temperature for 10 min, and centrifuged at 10,500 rpm for 10 min at 4°C. The isopropanol was decanted and the remaining pellet was washed with 75% ethanol, centrifuged at 8500 rpm for 5 min at 4°C, decanted, and dried. Water free of RNAse (52 µL), DNase buffer (10 µL of 10X; Ambion, Austin, TX), and DNase (1 µL of 2U/µL; Ambion) were added to the pellet and then incubated at 37°C for 30 min. Then, RNase-free water (37 µL) and phenol/chloroform (100 µL) were added. The tube was shaken and centrifuged for 2 min at 14,000 rpm. The upper phase was transferred to a fresh tube, sodium acetate (3 M, 9 µL) and ethanol (250 µL) were added to this phase, and the mixture was incubated overnight at -20°C. The next day, the tube was centrifuged at 14,000 rpm at 4°C for 15 min. The liquid was decanted and the pellet was washed with ethanol (75%, 500 µL). The tube was centrifuged at 14,000 rpm at 4°C for 10 min. The ethanol was decanted and the pellet was dried in the hood for 15 min. The pellet was resuspended in 50 µL of nuclease-free water and incubated at 60°C for 10 min. The tube was then removed, put on ice, and the RNA concentration was determined using a spectrophotometer (NanoDrop, ND-1000 Spectrophotometer, Wilmington, DE). Quality of the RNA was also determined (Agilent 2100 BioAnalyzer, Palo Alto, CA) and samples used for analysis were of high quality.

Quantitative Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

A master mix (4 µL 5X First Strand Buffer, 2 µL 0.1 M DTT, 1 µL SuperScript II, 2 µL H_2O, and 1 µL 10 mM dNTP mix; Invitrogen) was prepared and kept on ice. RNA was removed from the freezer, thawed, and 2 µg RNA was combined with 1 µL dT_{18} primer and RNase-free water to equal 10 µL total volume. Tubes were placed in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA), which was set for the following: 70°C for 5 min, 20°C for 5 min, 10 µL of master mix was added, 42°C for 60 min, 70°C for 5 min, 37°C for 20 min, and 0.5 µL of RNase H was added when the reaction reached 37°C. The reaction tubes were removed from the thermocycler and 0.2 µL of 0.5 M EDTA was added and mixed. Then, 25 µL of water, 5 µL 3M sodium acetate, and 125 µL of ethanol (-20°C) were added to the tube. This was allowed to precipitate overnight at -20°C. Tubes were then centrifuged at 14,000 rpm and 4°C for 20 min and the supernatant was decanted. The pellet was washed with 250 µL of ethanol (75%, -20°C) and the tube was centrifuged at 14,000 rpm and 4°C for 6 min. The supernatant was decanted and the pellet was allowed to dry for 15 min. The pellet was resuspended in 50 µL of water and incubated at 60°C for 5 min. The cDNA concentration was analyzed using a spectrophotometer (NanoDrop), then was diluted to a final concentration of 10 ng/µL, and stored at -80°C until the PCR reaction was initiated.

Primers (Table 1) were designed using Abi Prism Primer Express Version 2.0 (Applied Biosystems) and made by Invitrogen. Control genes [Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**), Hydroxymethyl-bilane synthase (HMBS), Hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP), Succinate dehydrogenase complex, subunit A (SDHA)] were tested to determine the variability between samples for a given gene. Each sample tested was a pool of 2 heifers from the same treatment group with a total of 2 samples per treatment group. The coefficient of variation for samples was low and ranged from 1 to 2% for each of the control genes. GAPDH was chosen because it has previously been used as a control gene is experiments with mammary tissue (Smith and Sheffield, 2002; Song and Oka, 2003).

The amount of primer used was determined by performing an optimization matrix for each primer using three concentrations of primers: 50:50 nM, 300:300 nM, 900:900 nM. Dissociation curves were similar for all concentrations and the 300:300 nM matrix was chosen, thus 3 μL of primer was used for all experiments. Standard curves were performed using different amounts of cDNA (5, 10, 20, 40, 80 ng) and tested primers for both GAPDH and the gene of interest. After normalization to GAPDH, the delta C_T values were plotted against the log amount of cDNA and the slope of this line was less than 0.04 for all of the genes tested. This demonstrates that the efficiencies of the two primers (GAPDH and gene of interest) were similar and that the data could be analyzed using the delta delta C_T method (Livak and Schmittgen, 2001). Each gene of interest (leptin, leptin receptor, IGF-I, IGF-I receptor) and the control gene were measured in duplicate. A total of 4 plates for each gene of interest were assayed with each plate containing all samples from a single purchase group. Therefore, each plate contained samples from 15 or 16 heifers with 3 or 4 heifers per treatment group. Within each well of a 96-well reaction plate (MicroAmp Optical, Applied Biosystems), 20 ng of sample cDNA (2 μL), 7.5 μL DEPC water, 3 μL primer, and 12.5 μL Sybr Green (Applied Biosystems) were added. The PCR system used was the ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Statistical Analysis

Statistical analysis used the PROC GLM procedure of SAS. Pen (n = 4 heifers per treatment within each purchase group) was used as the experimental unit with purchase group as a random variable and treatment*purchase group as the error term. Comparisons were tested using a linear (L) contrast with coefficients -7, -3, 1, and 9; a quadratic (Q) contrast with coefficients 7, -4, -8, and 5; and a cubic (C) contrast with coefficients -3, 8, -6, and 1 for H0, H3, H6, and H12, respectively. Least square means and standard errors of the mean are presented. Differences were declared to be statistically significant at $P < 0.05$ and tendencies at $P < 0.10$. All data from the 2 heifers that were eliminated from the trial were removed so that final animal numbers were 16, 15, 16, and 15 for treatment groups H0, H3, H6, and H12, respectively.

Repeated measures (first-order autoregressive or compound symmetry covariance structure) within the PROC MIXED procedure of SAS was used to test treatment effects on serum IGF-I and leptin concentrations. Initial serum samples (wk 0) were used as a covariate for analysis of leptin and IGF-I concentrations in serum taken during the treatment period. Results from both the leptin and IGF-I RIA were log transformed to achieve homogeneous variance and normality. Both non-transformed data and back transformed data (see Appendix) are presented for serum leptin and IGF-I concentrations.

The error term for the transformed data is the average of the back transformed lower and upper 68% confidence intervals. For abundance of IGFBP-2 and -3, initial serum samples (wk 0) were used as a covariate for analysis of serum samples from wk 12 of the treatment period. The average of the initial serum samples is also presented. Expression of mRNA for leptin, leptin receptor, IGF-I, and IGF-I receptor in mammary parenchymal tissue samples were normalized to the GAPDH expression of the sample that was assayed on the same plate. Results were analyzed using the delta delta C_T method with the H0 treatment group serving as the reference. Pearson correlations using the PROC CORR procedure of SAS were calculated to determine the relationship between the following variables: intraparenchymal fat percent, serum leptin protein concentrations, mammary tissue leptin protein concentrations, and mammary tissue leptin mRNA expression.

RESULTS

Serum leptin concentrations from samples taken during wk 0, 2, 4, 6, 8, 10, 11, and 12 of the treatment period are depicted in Figure 1. Initial serum leptin concentration was not different (all contrasts: $P > 0.10$). Serum leptin concentrations at wk 2, 8, 11 and at slaughter are shown in Table 2. No differences existed in serum leptin concentrations at wk 2 and 8 of the treatment period (all contrasts: $P > 0.10$). At wk 11, serum leptin concentration increased in a linear fashion and was also significant for a quadratic contrast because of the small mean difference between H6 and H12. Leptin protein concentrations in serum collected at slaughter and in mammary parenchymal tissue samples increased as heifers were fed the high energy diet for a longer duration (Figure 2; L: $P < 0.03$ and $P < 0.01$, respectively). These effects were also quadratic for tissue and serum leptin concentrations because of the small mean difference between H6 and H12 (Q: $P = 0.07$ and $P < 0.01$, respectively).

Leptin mRNA expression in mammary parenchymal tissue increased as heifers were fed the high energy diet for a longer duration (Figure 3; L: $P < 0.02$). Feeding a high energy diet for 12 weeks (H12) increased leptin mRNA expression in mammary parenchymal tissue by 2.5-fold compared to feeding a low energy diet (H0). However, diet did not alter the abundance of leptin receptor mRNA expression in mammary parenchymal tissue (Figure 4; all contrasts: $P > 0.10$).

Mammary gland composition was presented previously (Chapter 3). Because leptin is mainly but not exclusively produced by adipocytes (Chilliard et al., 2001), it is important to observe the effects of treatment on the percent of fat interspersed within the parenchyma, called intraparenchymal fat. The percentage of intraparenchymal fat increased as heifers were fed the high energy diet for a longer duration (Figure 5; L: $P < 0.01$). Intraparenchymal fat percent, leptin protein in serum, leptin protein in mammary tissue, and leptin mRNA expression in mammary tissue were all positively correlated with each other (Table 3), but no one relationship explained more than 30% of the variation within a variable. For example, intraparenchymal fat percent explained approximately 15 and 8% of the variation within leptin mRNA expression and leptin protein concentration within mammary parenchymal tissue, respectively. Although not presented in Table 3, carcass fat, as an indicator of body fatness, was positively correlated with serum leptin concentration ($r = 0.52$, $P < 0.0001$).

Serum IGF-I concentrations from samples taken during wk 0, 2, 4, 6, 8, 10, 11, and 12 of the treatment period are depicted in Figure 6. Initial serum IGF-I concentration was not different (all contrasts: $P > 0.10$). Serum IGF-I concentration taken at wk 2 was significant for a linear and quadratic contrast because of the similarities in H0, H3, and H6, which were all consuming the low energy diet at wk 2 (Table 4; $P < 0.01$ for both). At wk 8, serum IGF-I concentration increased in a linear response ($P < 0.01$) and was significant for a cubic contrast ($P < 0.01$) because of the low mean for the H3 treatment compared to H6 and H12. Serum IGF-I concentrations taken at wk 11 and at slaughter were both significant for a linear contrast ($P < 0.01$) and tended to be significant at slaughter for a quadratic response ($P = 0.06$).

When abundance of plasma IGFBP-2 at wk 0 was used as a covariate, protein abundance of IGFBP-2 in plasma samples taken at slaughter (23 wk of age) decreased as heifers were fed a high energy diet for a longer duration (Figure 7; L: $P = 0.03$). Abundance of serum IGFBP-3 increased in a linear fashion as heifers were fed a high energy diet for a longer duration when analyzed using wk 0 as a covariate in the model (Figure 8; L: $P < 0.01$). Serum abundance of IGFBP-3 was also significant for a cubic contrast when wk 0 was used as a covariate (C: $P = 0.02$).

There was no dietary effect on IGF-I mRNA expression in mammary parenchymal (Figure 9; all contrasts: $P > 0.10$). However, a short duration of feeding prepubertal heifers a high energy diet decreased IGF-I receptor mRNA expression in mammary parenchymal tissue (Q: $P = 0.02$).

DISCUSSION

Previous studies have associated the amount of fat deposited within the body and mammary gland with impaired mammary growth. The degree of body fatness is negatively correlated to mammary parenchymal DNA content and milk production in dairy cattle (Silva et al., 2002b). Obesity has also been linked to impaired mammary development and lactogenesis in rodents (Flint et al., 2005; Rasmussen et al., 2001). The growth of bovine mammary epithelial cell organoids was inhibited when co-cultured with mammary fat pad explants (McFadden and Cockrell, 1993). Mammary tissue extracts from prepubertal heifers fed a high compared to a low energy diet were less mitogenic for mammary epithelial cells in vitro (Berry et al., 2003; Weber et al., 2000a). These findings suggest that mammary fat secretes a factor that inhibits mammary epithelial cell growth. A candidate for this factor may be leptin.

This is the first study indicating that protein concentration and mRNA expression of leptin in mammary parenchymal tissue are increased by feeding a high energy diet to prepubertal heifers. This effect is also influenced by the duration of time that heifers are fed a high energy diet. These findings may help to explain why feeding a high energy diet decreases mammary growth relative to body growth in prepubertal dairy heifers. Also, our results may help to explain the reason why high energy diets decrease mammogenesis while increasing serum IGF-I concentration, given that IGF-I is a known mitogen for mammary epithelial cells in prepubertal heifers (Shamay et al., 1988; Silva et al., 2005). Previous research indicated that leptin infusion into the mammary gland of prepubertal dairy heifers decreased BrdU-labeling of mammary epithelial cells in IGF-I treated quarters by 48% and in saline treated quarters by 19% (Silva et al., 2003). Therefore, if heifers fed a high energy diet have greater leptin mRNA expression and leptin protein concentration in mammary parenchymal tissue, then this higher abundance of leptin might hamper mammary development directly or indirectly by inhibiting IGF-I stimulation of mammary growth.

Leptin is mainly, but not exclusively, produced by adipocytes (Chilliard et al., 2001) and deposition of fat within the mammary gland is increased when heifers are fed a high energy diet (Capuco et al., 1995; Radcliff et al., 1997). Leptin mRNA is present in mammary tissue and a bovine mammary epithelial cell line (MAC-T), leptin protein is present in bovine milk (Smith and Sheffield, 2002), and the long form of the leptin receptor is expressed on mammary epithelial cells (Silva et al., 2002a). Also, bovine mammary fat cells likely express mRNA for leptin (Block et al., 2003b). It is not known whether nutrition alters leptin mRNA expression to a different extent in adipocytes versus epithelial cells within the mammary gland. Since there was an increase in the percent of intraparenchymal fat, the increase in leptin mRNA expression in this study could be due to an increase in the number or size of fat cells within the parenchyma. Further studies are needed to better understand nutritional effects on leptin mRNA expression in different cell types within the mammary gland and could be accomplished through the use of laser capture micro-dissection or in situ hybridization techniques.

Another potential question is how leptin delivery from the blood compared to synthesis of leptin in the mammary gland affects leptin concentration in mammary tissue. We found that leptin protein concentrations in both serum and mammary tissue at slaughter increased as heifers were fed the high energy diet for a longer duration. Block et al. (2003b) suggested that synthesis of leptin within the tissue rather than delivery from the blood could determine concentration of leptin within developing mammary tissue. In the present study, serum and mammary tissue leptin protein concentrations were correlated positively and had the highest correlation of all variables tested ($r = 0.55$). But, leptin protein concentration in mammary parenchymal tissue was also positively correlated with mRNA expression of leptin in mammary parenchymal tissue and intraparenchymal fat percent. These results indicate that leptin concentration in mammary tissue is altered by dietary energy intake and that leptin level in the tissue is likely due to leptin delivered to the tissue from the blood and leptin synthesized by mammary epithelial cells and/or fat cells within mammary tissue.

Other studies have clearly demonstrated the nutritional regulation of plasma leptin in young calves. Research indicates that increased energy consumption during the preweaning period results in a greater concentration of leptin within the blood (Block et al., 2003b; Brown et al., 2005b; Ehrhardt et al., 2000). The dietary effect on serum leptin concentration was significant within 4 wk on treatment for preweaned calves (Brown et al., 2005b). It is well established that feed-restricted or fasted animals have reduced leptin concentrations, but variation in plasma leptin may be more related to body fatness than plane of nutrition (Amstalden et al., 2000; Delavaud et al., 2000). Actual serum leptin concentration in the present study averaged approximately 2 ng/mL, which is similar to those reported in dairy heifers of the same age (Block et al., 2003b). Leptin concentration in this study was similar for treatments prior to wk 10 of the treatment period. Similarly, energy intake did not alter plasma leptin concentrations in heifer calves from 8 to 14 wk of age (Brown et al., 2005b). Separation of treatment means for serum leptin began to occur at wk 10 of the treatment period and by wk 11 were significant for linear and quadratic effects. In addition, serum samples taken at slaughter indicated that leptin concentration increased in a linear fashion as heifers were fed the high energy diet for a longer duration. Serum leptin protein concentration at slaughter was also quadratic because of the similar means for H6 and H12 treatments. It is not known why treatment differences in serum leptin were not evident earlier during the treatment period, especially for the H12 treatment. It is also difficult to separate the effects of nutrition from the effects of physiological maturity on serum leptin concentration. Garcia and co-workers (2002) noted that concentration of leptin began to increase 16 wk prior to the onset of puberty in beef heifers. One heifer from the H12 treatment group was removed from the dataset due to the presence of a corpus luteum. It is likely that H12 heifers were closer to the onset of puberty than the H0 heifers, so perhaps differences in physiological maturity may have influenced serum leptin differences at the end of the treatment period. These results also suggest that degree of body fatness may not be as important a factor in determining leptin concentration as with preruminant calves. In this study, carcass fat, as an indicator of body fatness, was positively correlated with serum leptin concentration, but only explained approximately 30% of the variation in serum leptin. Similarly, body condition scores explained 37% of the variation in serum leptin in well-fed cows in late lactation (Ehrhardt et al., 2000), while body fatness explained 83% of the differences in serum leptin in milk-fed calves. The degree of body fatness of heifers in this study may have not been high enough to elicit an increase in leptin concentration in the H12 heifers earlier in the treatment period. Although carcass fat and other measures of body fatness increased as heifers were fed the high energy diet for a longer duration, the time during which fat accretion occurred in these heifers was not measured (Chapter 2). Carcass fat percent nearly doubled and mass of perirenal fat more than doubled in heifers fed the high energy diet for 12 wk compared to heifers fed the low energy diet for 12 wk. Further research is needed to better understand why nutrition seems to alter serum leptin concentration to a greater extent in the preruminant period compared to older prepubertal heifers.

Insulin-like growth factor-I is a known mitogen of mammary epithelial cells and serum IGF-I likely plays a role in the abundance of IGF-I in the mammary gland. Actual serum concentrations of IGF-I were within a similar range to those previously reported in young heifers (Brown et al., 2005b; Petitclerc et al., 1999). As expected, concentration of serum IGF-I increased as heifers were fed the high energy diet for a longer duration and this dietary effect is in agreement with previous research (Elsasser et al., 1989; Radcliff et al., 2004; Vestergaard et al., 2003). Serum IGF-I concentration also dramatically increased in H6 heifers (cubic effect at wk 8) and to a lesser extent in H3 heifers after being switched from the low to the high energy diet (no quadratic effect at wk 11). Serum IGF-I mimicked the response in daily gain after the dietary switch in H6 and H3 heifers, as H6 and H3 treatments averaged 1.15 kg/d and 0.96 kg/d of gain, respectively, during the time period fed the high energy diet (see Chapter 2).

The activity and availability of IGF-I is modulated by the IGF binding proteins. Therefore, the regulation of IGFBP is crucial in understanding the function of IGF-I. Nutrition also altered the protein abundance of serum IGFBP-2 and -3. The abundance of serum IGFBP-2 decreased as heifers were fed a high energy diet for longer durations and this dietary effect is in agreement with previous research (Radcliff et al., 2004; Vestergaard et al., 2003). The major IGFBP within serum is IGFBP-3 (McGrath et al., 1991). The abundance of serum IGFBP-3 increased in a linear fashion with time fed the high energy diet, but was also significant for a cubic effect, due to the high abundance of

IGFBP-3 within the serum from H3 heifers. The abundance of IGFBP-3 in serum typically mimics the response of IGF-I, and increases with a high plane of nutrition (Vestergaard et al., 2003). An acute increase in serum IGFBP-3 in response to feeding a high energy diet is not surprising since heifers in the Vestergaard study were only on treatments for 5 wk. In the present study, the concentration of serum IGF-I increased in heifers as they were fed the high energy diet for a longer duration. The amount of free IGF-I in serum could potentially not differ between treatments because of the increase in serum IGFBP-3. Since IGFBP-3 constitutes the majority of IGFBP within the serum, the higher abundance of IGFBP-3 could possibly bind more serum IGF-I in heifers fed high compared to low energy diets.

It seems contradictory that feeding a high energy diet to prepubertal heifers increases the serum concentration of IGF-I, a known mitogen of the mammary gland, given that high energy intake also decreases mammary growth relative to body growth (Chapter 3). The liver is the primary source of circulating IGF-I in animals (Daughaday and Rotwein, 1989) and serum concentration of IGF-I is increased by high energy intake (Radcliff et al., 2004; Vestergaard et al., 2003). Bovine mammary epithelial cells express IGF system receptors (IGF-I, -II, and insulin); but do not produce IGF-I (Hadsell et al., 1990). IGF-I is produced in the stromal portion of the mammary gland (Hauser et al., 1990). The level of IGF-I protein found within the mammary tissue is due to a combination of IGF-I produced within the tissue and that which travels to the mammary gland via the circulation. A high correlation ($r = 0.84$) existed between IGF-I concentrations in serum and mammary extracts from heifers fed a high or a low energy diet and with or without bST administration (Weber et al., 2000b). There was no dietary effect on IGF-I concentration and abundance of mRNA in mammary parenchymal tissue from prepubertal dairy heifers (Weber et al., 2000b). Therefore, this apparent contradiction of high energy diets and increased concentration of serum IGF-I may be subdued by the lack of a dietary effect on IGF-I concentration in mammary extracts. Furthermore, no significant correlation existed between IGF-I concentration in mammary extracts and the mitogenic response of the mammary extracts used in the Weber study (Purup et al., 2000).

Negative effects on mammary growth that are attributed to feeding a high energy diet may be caused by a decrease in sensitivity within the mammary gland to IGF-I. A study using mammary explants from prepubertal heifers fed a high energy diet showed a decrease in mammary tissue sensitivity to IGF-I treatment compared to explants from heifers fed a low energy diet (Purup et al., 1996). However, because IGF-I and IGFBP are expressed and secreted by mammary tissue and the IGFBP profile is modulated by feeding level (Weber et al., 2000b), the difference in mitogenic response noted in Purup et al. (1996) may not be due solely to differences in tissue sensitivity. Another study reported no effect of diet on labeled IGF-I binding to mammary membranes (Purup et al., 1999). In the present study, mRNA expression of IGF-I in mammary tissue was not altered by nutrition. This is in agreement with Weber et al. (2000b) who reported no dietary effect on IGF-I mRNA expression in the mammary gland of prepubertal heifers. But, a short duration of feeding a high energy diet to prepubertal heifers decreased IGF-I receptor mRNA expression in mammary parenchymal tissue. This could partially explain the dietary difference in sensitivity of explants to IGF-I observed in the Purup study (1996), but expression of mRNA for the IGF-I receptor was only decreased for the H3 and H6 treatment groups and the numerical differences in mRNA expression levels were minimal. Unless the number of IGF-I receptors present on mammary epithelial cells is decreased by translational or post-translational modifications, it is difficult to understand how nutrition can affect the sensitivity of mammary tissue to IGF-I. However, a potential inhibition of IGF-I stimulation via leptin, IGFBP-3, and/or another factor that has not been elucidated could explain why feeding a high energy diet to prepubertal heifers impairs mammary growth relative to body growth, but also increases serum IGF-I.

CONCLUSION

Feeding heifers a high energy diet for a longer duration of the prepubertal period causes a linear increase in leptin protein concentrations at 23 wk of age in serum and mammary parenchymal tissue and increases leptin mRNA expression in mammary parenchymal tissue. These data, along with prior work indicating that leptin reduced the proliferation of bovine mammary epithelial cells, indicate that leptin may play a role in the inhibitory effects of a high plane of nutrition on mammary growth relative to body growth in prepubertal heifers. Serum protein levels of IGF-I and IGFBP-3 were increased with time fed the high energy diet, while IGFBP-2 levels were decreased. A short duration of feeding a high energy diet decreased IGF-I receptor mRNA expression in mammary tissue. But, dietary effects did not alter mRNA expression of leptin receptor and IGF-I in mammary parenchymal tissue. Nutrition clearly affects the concentration of serum IGF-I, but the extent that dietary intake alters IGF-I within mammary tissue seems less profound.

Table 1. Primer Sequences (5' to 3').

Gene Name	Sequence
GAPDH-forward	GCATCGTGGAGGGACTTATGA
GAPDH-reverse	GGGCCATCCACAGTCTTCTG
IGFI-forward	TGCTGCTTCCTGGTCCTCAT
IGFI-reverse	TGTGCCAGTCCCTTTCCATC
IGFI Receptor-forward	TTCTGGACAAGCCGGACAA
IGFI Receptor-reverse	GCTGCTGATGATCTCCAGGAA
Leptin-forward	GGGTGATTTCAGAGCCTTTGG
Leptin-reverse	CCATCGTATGTTGTGTGGGAAT
Leptin Receptor-forward	GGGCACATCCAAGCATTAAAA
Leptin Receptor-reverse	GGCCGGCATCAAAGCTTT

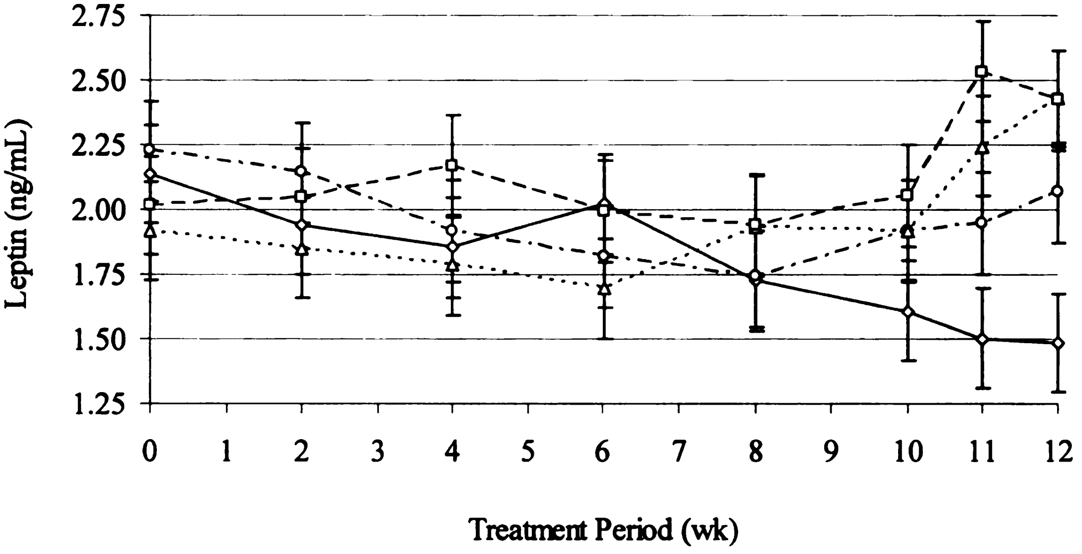

Figure 1. Serum leptin concentrations taken every 2 wk and weekly during the last 3 wk of the treatment period in heifers 11 to 23 wk of age. Heifers (n = 15 or 16/trt) on treatment H0 (——◊——), H3 (- — -○- — -), H6 (- - -Δ- - -), H12 (— —□— —) were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The serum leptin least square means are non-transformed.

Table 2. Least square means for serum leptin concentrations during specific wk of the treatment period.

	Treatment Group[1]					*P* for Contrast[2]		
	H0	H3	H6	H12	SE[3]	L	Q	C
Leptin (ng/mL)								
2 wk	1.78	1.96	1.78	2.02	A*	0.23	0.71	0.15
8 wk	1.66	1.67	1.89	1.85	B*	0.16	0.47	0.37
11 wk	1.49	1.86	2.21	2.45	C*	< 0.01	0.04	0.87
slaughter	1.51	2.14	2.42	2.52	D*	< 0.01	< 0.01	0.46

[1] Treatment groups are as follows: Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

[*] Data were log transformed to achieve homogeneous variance. Means presented are back transformed. The error term indicated below is the average of the lower and upper confidence intervals for each treatment group.

[A] Average confidence intervals are 0.11, 0.13, 0.11, and 0.13 for H0, H3, H6, and H12 treatment groups, respectively.

[B] Average confidence intervals are 0.14, 0.15, 0.16, and 0.17 for H0, H3, H6, and H12 treatment groups, respectively.

[C] Average confidence intervals are 0.12, 0.15, 0.17, and 0.20 for H0, H3, H6, and H12 treatment groups, respectively.

[D] Average confidence intervals are 0.09, 0.11, 0.12, and 0.14 for H0, H3, H6, and H12 treatment groups, respectively.

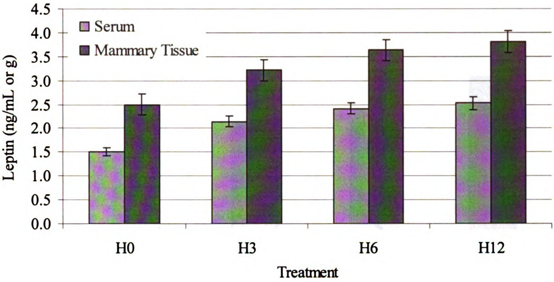

Figure 2. Leptin protein concentrations in mammary parenchymal tissue (ng/g wet tissue) and serum (ng/mL) collected at slaughter from 23 wk old heifers. Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Serum leptin values were log transformed to achieve homogeneous variance. Serum leptin means presented are back transformed. The error term is the average of the lower and upper confidence intervals. Actual values for mammary tissue leptin are not final due to ongoing assay validation. Leptin protein concentrations in both mammary parenchymal tissue and serum samples increased as heifers were fed a high energy diet for longer durations of time (Linear: $P < 0.01$ and $P < 0.01$, respectively). Concentrations of leptin in tissue and serum also had a quadratic effect (Quadratic: $P = 0.07$ and $P < 0.01$, respectively).

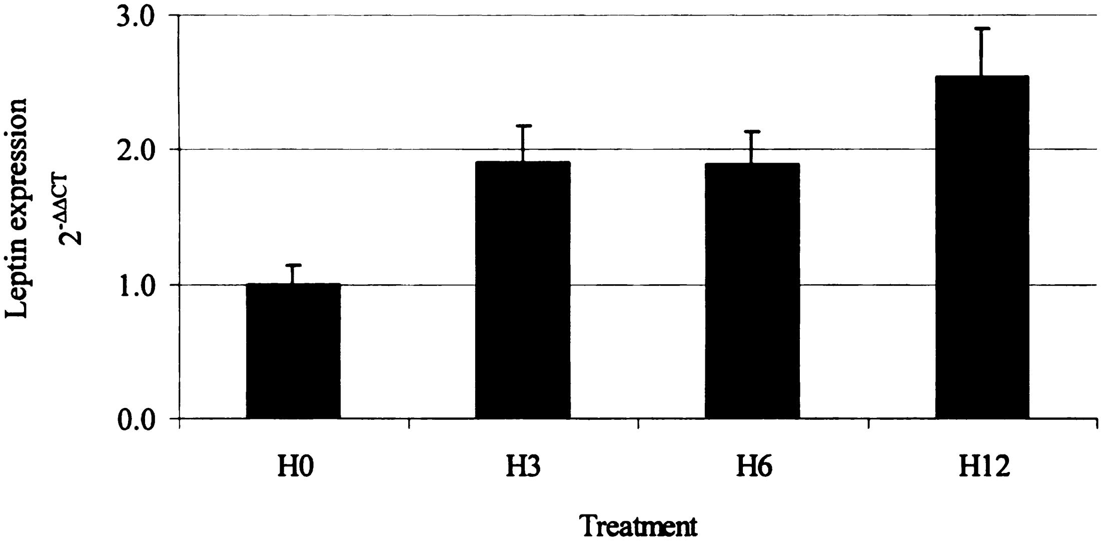

Figure 3. Leptin mRNA expression in mammary parenchymal tissue collected at slaughter from 23 wk old heifers. Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Gene expression is normalized to GAPDH expression. The H0 treatment is the reference group. Leptin mRNA gene expression in mammary parenchymal tissue increased as heifers were fed a high energy diet for longer durations of time (Linear: $P < 0.02$). Feeding a high energy diet for 12 wk (H12) in length increased leptin gene expression in mammary tissue by 2.5-fold compared to the low energy diet control treatment (H0).

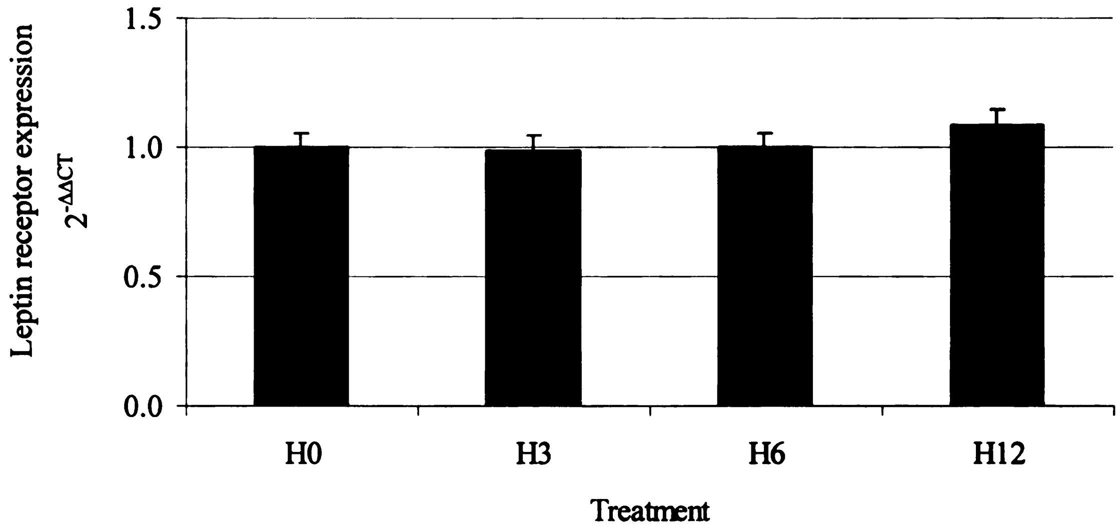

Figure 4. Leptin receptor mRNA expression in mammary parenchymal tissue collected at slaughter from 23 wk old heifers. Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Gene expression is normalized to GAPDH expression. The H0 treatment is the reference group. There was no response in leptin receptor mRNA gene expression in mammary parenchymal tissue as heifers were fed a high energy diet for longer durations of time (All contrasts: $P > 0.10$).

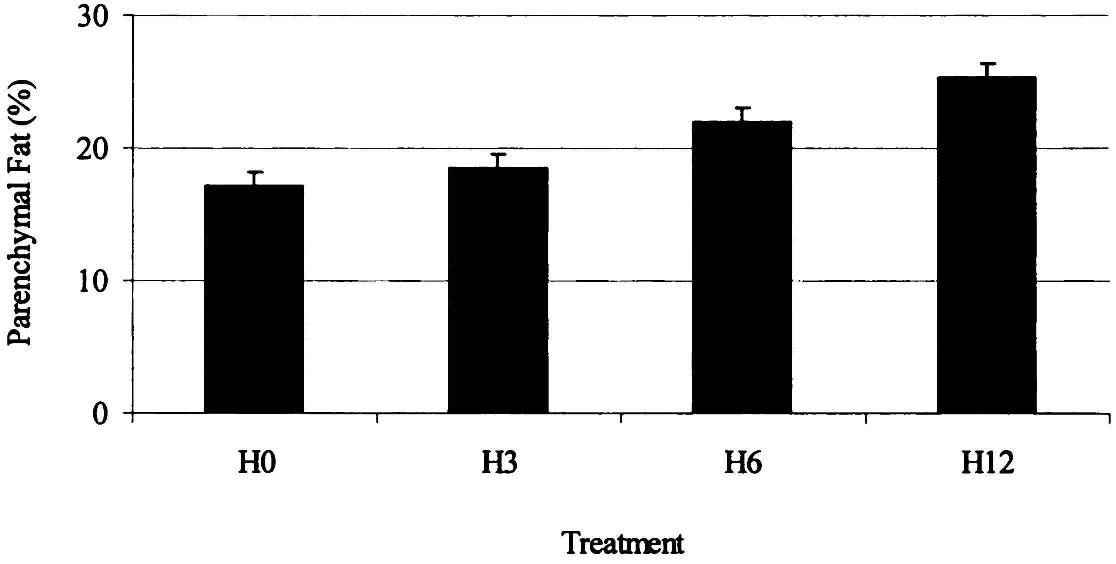

Figure 5. Intraparenchymal fat percent in mammary tissue collected at slaughter from 23 wk old heifers. Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Intraparenchymal fat percent increased as heifers were fed a high energy diet for longer durations of time (Linear: $P < 0.01$)

Table 3. Correlation of leptin variables and mammary intraparenchymal fat percent[1].

	Intramam[2] fat percent	Mam[2] leptin mRNA expression	Mam[2] leptin protein concentrations	Serum[3] leptin protein concentrations
Intramam[2] fat percent	1	0.39*	0.29†	0.40*
Mam[2] leptin mRNA expression		1	0.43**	0.34*
Mam[2] leptin protein concentrations			1	0.55***
Serum[3] leptin protein concentrations				1

[1] Table contains r values.

[2] Mam = Mammary parenchymal tissue

[3] Serum leptin protein concentration data used for correlation were log transformed to achieve homogeneous variance.

*** $P < 0.0001$

** $P < 0.001$

* $P < 0.01$

† $P < 0.03$

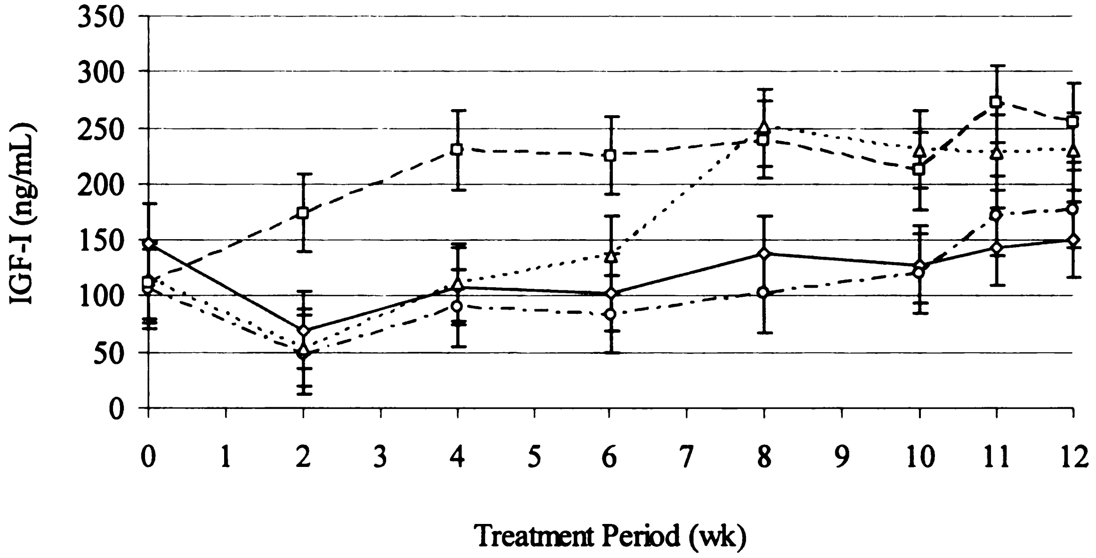

Figure 6. Serum IGF-I concentrations taken every 2 wk and weekly during the last 3 wk of the treatment period in heifers 11 wk to 23 wk of age. Heifers (n = 15 or 16/trt) on treatment H0 (——◇——), H3 (- — -○- — -), H6 (- - -△- - -), H12 (— —□— —) were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The serum IGF-I least square means are non-transformed.

Table 4. Least square means for serum IGF-I concentrations during specific wk of the treatment period.

	Treatment Group[1]					*P* for Contrast[2]		
	H0	H3	H6	H12	SE[3]	L	Q	C
IGF-I (ng/mL)								
2 wk	50.5	39.8	45.6	140	A*	< 0.01	< 0.01	0.86
8 wk	103	85.5	201	213	B*	< 0.01	0.60	< 0.01
11 wk	112	162	189	245	C*	< 0.01	0.27	0.67
slaughter	117	171	227	236	D*	< 0.01	0.06	0.89

[1] Treatment groups are as follows: Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

[*] Data were log transformed to achieve homogeneous variance. Means presented are back transformed. The error term indicated below is the average of the lower and upper confidence intervals for each treatment group.

[A] Average confidence intervals are 7.85, 6.39, 7.26, and 22.3 for H0, H3, H6, and H12 treatment groups, respectively.

[B] Average confidence intervals are 16.3, 13.9, 31.3, and 34.5 for H0, H3, H6, and H12 treatment groups, respectively.

[C] Average confidence intervals are 16.8, 24.9, 27.9, and 37.7 for H0, H3, H6, and H12 treatment groups, respectively.

[D] Average confidence intervals are 11.6, 17.5, 22.1, and 24.0 for H0, H3, H6, and H12 treatment groups, respectively.

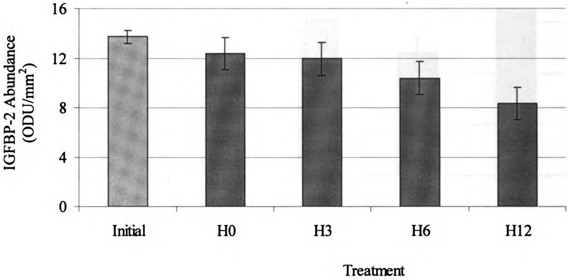

Figure 7. Abundance of IGF-binding protein-2 (IGFBP-2) in serum samples taken at slaughter with wk 0 samples serving as a covariate. Data represent means from 8 heifers per treatment group. Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The abundance of IGFBP-2 in serum decreased as heifers were fed a high energy diet for a longer duration of time (Linear: $P < 0.03$).

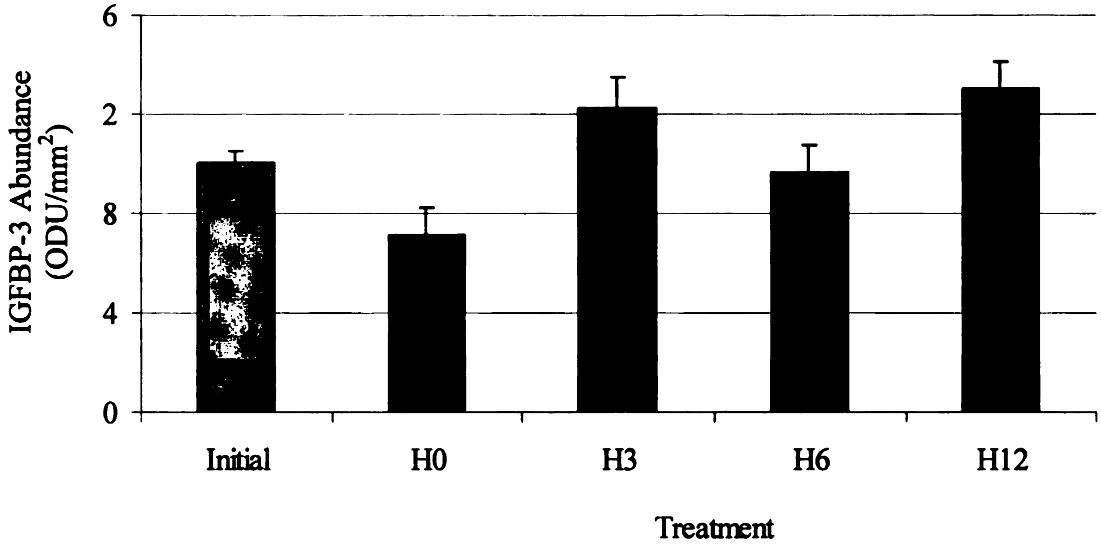

Figure 8. Abundance of IGF-binding protein-3 (IGFBP-3) in serum samples taken at slaughter with wk 0 samples serving as a covariate. Data represent means for 8 heifers per treatment group. Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Abundance of IGFBP-3 increased in a linear fashion with a longer duration fed the high energy diet and was also significant for a cubic contrast ($P < 0.01$ and $P = 0.02$, respectively).

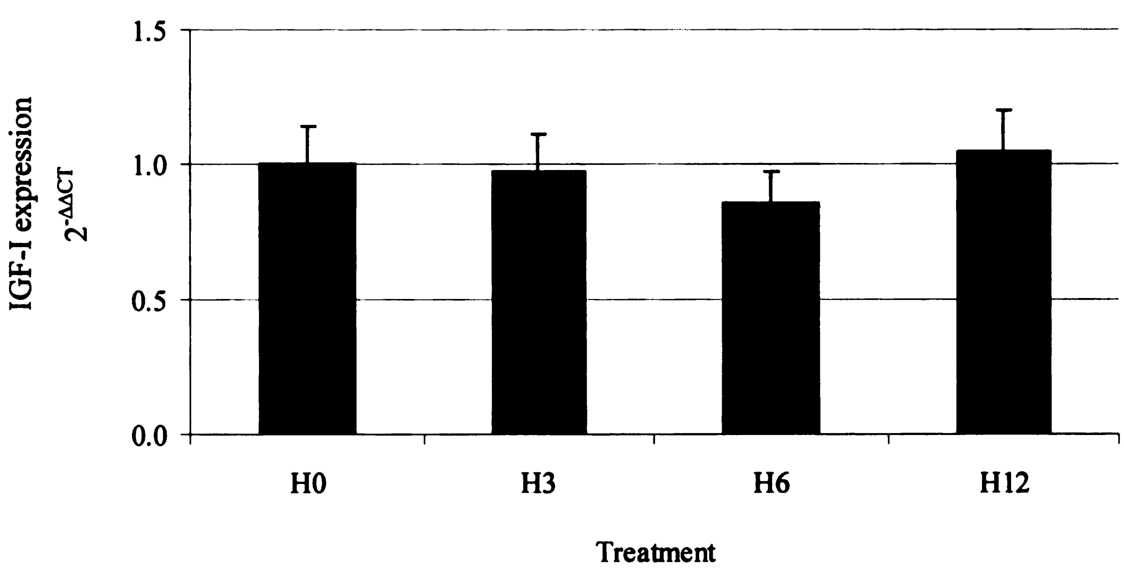

Figure 9. IGF-I mRNA expression in mammary parenchymal tissue collected at slaughter from 23 wk old heifers. Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Gene expression is normalized to GAPDH expression. The H0 treatment is the reference group. There was no response in IGF-I mRNA gene expression in mammary parenchymal tissue as heifers were fed a high energy diet for a longer duration of time (All contrasts: $P > 0.10$).

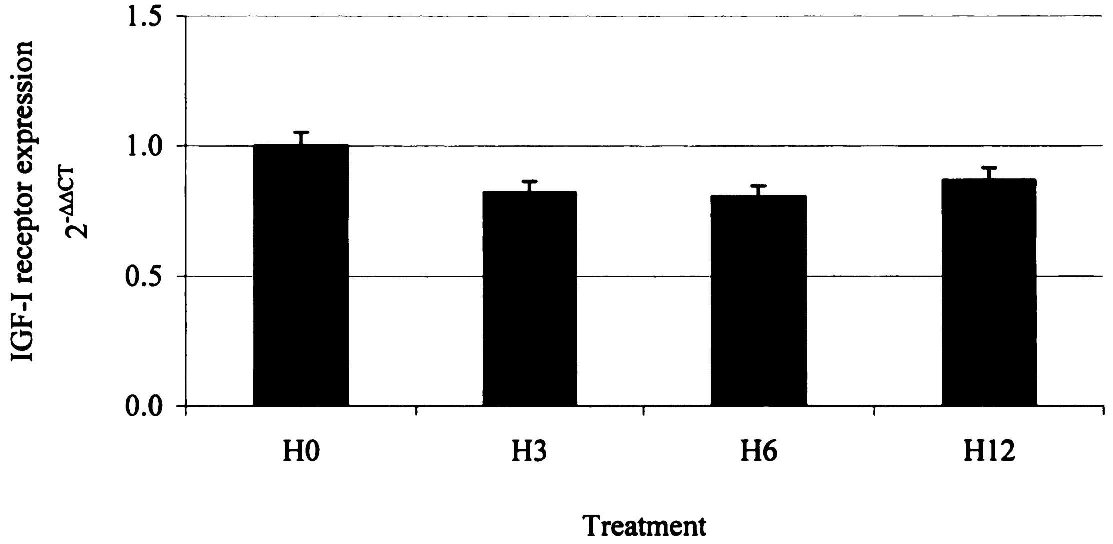

Figure 10. IGF-I receptor mRNA expression in mammary parenchymal tissue collected at slaughter from 23 wk old heifers. Heifers (n = 15 or 16/trt) on H0, H3, H6, and H12 were fed the low energy diet for 12, 9, 6, and 0 wk followed by the high energy diet for 0, 3, 6, and 12 wk, respectively. Gene expression is normalized to GAPDH expression. The H0 treatment is the reference group. A short duration (3 or 6 wk) of feeding a high energy diet to prepubertal heifers decreased IGF-I receptor mRNA expression in mammary parenchymal tissue (Q: *P* = 0.02).

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

My original hypothesis was that feeding a high energy diet to prepubertal heifers for a short duration would increase the growth of mammary parenchyma, but that feeding a high energy diet for a long duration would impair mammary growth relative to body growth. However, the results presented in Chapter 2 and 3 of this dissertation indicate that feeding heifers a high energy diet for a short duration alters body, carcass, and mammary growth in a time-dependent manner, consistent with feeding a high energy diet for a long duration. My hypothesis stemmed from studies that indicated a stimulatory effect on mammary growth when heifers were fed high energy diets for short periods of time, either before weaning (Brown et al., 2005a) or in compensatory growth studies (Choi et al., 1997). In these two studies, the positive influence on mammary growth is more likely due to the stair-step regime and high energy intake before weaning, rather than the duration of high energy intake.

In my study, daily gain, skeletal growth, and fat-free carcass weight increased in a linear fashion as heifers were fed high energy diets for a longer duration. But, feeding a high energy diet for a longer duration also increased fat deposition within the body and carcass. Total weight of the mammary gland increased as heifers were fed the high energy diet for a longer duration, but this was due to greater amounts of extraparenchymal fat, as parenchymal tissue weights were not different. I chose to express mammary tissue weights adjusted for carcass weight to more accurately reflect the differences in physiological maturity of the heifers. Fat-free mammary parenchymal tissue weight adjusted for fat-free carcass weight decreased as heifers were fed the high energy diet for a longer duration. An increase in body or carcass growth without a proportional increase in mammary growth would result in less mammary parenchymal tissue at puberty because heifers fed for rapid gains reach puberty at a younger age and growth of the mammary gland becomes isometric relative to body growth around the onset of puberty.

I also examined how dietary intake affects the accretion of several other tissues in addition to the carcass and mammary gland. Liver weight increased in a linear fashion as heifers were fed a high energy diet for a longer duration. However, similar to weight of mammary parenchyma, uterine and ovarian weights adjusted for carcass weight decreased as heifers were fed the high energy diet for a longer duration. An increase in body or carcass growth without a proportional increase in reproductive organ weights would likely result in smaller reproductive organs at puberty in heifers fed a high energy diet.

A proposed mechanism for why high energy intake in prepubertal heifers impairs mammogenesis relative to body growth is depicted in Figure 1. In support of this model, my research demonstrated that high energy intake in prepubertal heifers increases the amount of fat deposited within the carcass and mammary gland. Leptin is a protein that is mainly, but not exclusively, secreted by adipocytes. I found that feeding heifers a high energy diet for a longer duration increased leptin protein concentrations in serum and in mammary tissue. Silva et al. (2003) found that leptin inhibited the proliferation of bovine mammary epithelial cells. In addition, I found that high energy intake in heifers increases mRNA expression of leptin. There was an increase in the percentage of fat within the parenchymal tissue as heifers were fed the high energy diet for a longer duration.

Therefore, the increase in leptin concentration and mRNA expression in mammary parenchymal tissue could be due to an increased number or size of adipocytes within the mammary parenchyma, or possibly mammary epithelial cells from heifers fed a high energy diet might express more leptin. An increase in leptin concentration in mammary parenchymal tissue and an increase in leptin mRNA expression in mammary parenchymal tissue might explain the impairment of mammogenesis relative to body growth observed when prepubertal heifers are fed high energy diets.

An increase in serum IGF-I concentration due to high dietary intake had previously seemed contradictory since IGF-I is a known mitogen for mammary epithelial cells. But, my work also showed that high energy intake in prepubertal heifers does not alter mRNA expression of IGF-I in mammary parenchymal tissue. Another study indicated that IGF-I concentration in extracts of mammary tissue was not different from heifers fed high compared to low energy diets (Weber et al., 2000b). These results indicate that serum concentration of IGF-I may be increased by high energy intake, but in mammary parenchymal tissue, protein levels and mRNA expression of IGF-I are not altered by diet. This may help to explain the apparent contradiction of high energy intake impairing mammogenesis while also increasing serum IGF-I concentrations.

An important question remains as to why a high energy intake in prepubertal heifers increases muscle and fat accretion, but does not increase the growth of mammary parenchymal, ovarian, and uterine tissues. Dietary intake might cause inhibitory and/or mitogenic growth factors to be synthesized in one tissue differently than in another tissue. Moreover, a potential inhibition of IGF-I stimulation of mammary growth via leptin, IGFBP-3, and/or another factor that has not been elucidated could explain why feeding a high energy diet to prepubertal heifers impairs mammogenesis relative to body growth.

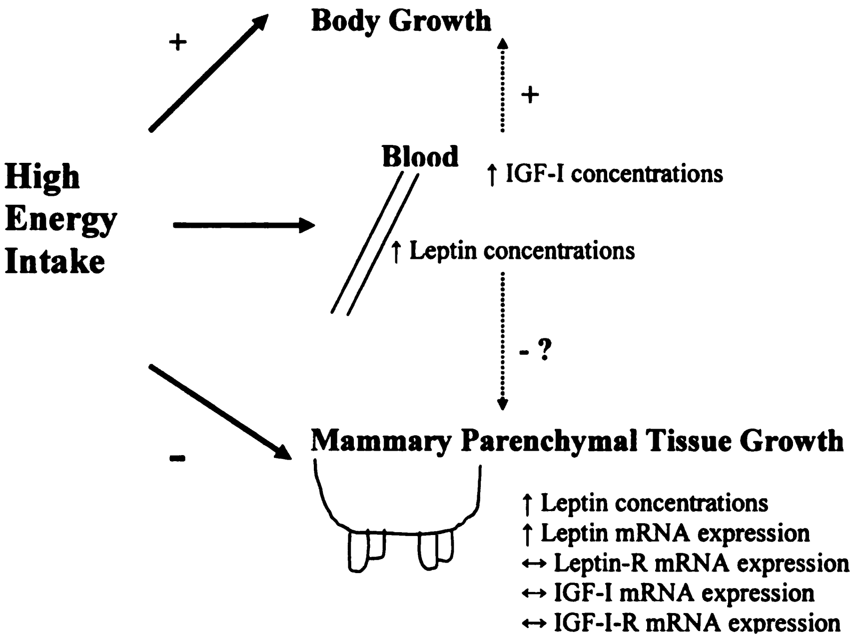

Figure 1. A proposed mechanism for why feeding a high energy diet to prepubertal heifers impairs mammogenesis relative to body growth. Results from this dissertation are listed.

CHAPTER SIX

FUTURE RESEARCH

Many different studies could be carried out using tissues from the heifers in this study.

1.) I measured the abundance of leptin mRNA expression within mammary parenchymal tissue, which contains epithelial cells, stromal cells, and adipocytes. The next step would be to determine if leptin mRNA expression is altered by diet specifically within epithelial cells, fat cells, etc., in mammary tissue and could be determined using laser capture microdissection techniques along with qRT-PCR or using in situ hybridization.

2.) Only four genes of interest were tested for mRNA expression in mammary tissue. Use of microarray techniques would allow one to test possible changes in gene expression in pathways involved with leptin, cell proliferation, cell death, etc. Other factors with potential inhibitory roles in mammary growth (i.e. TGF-β_1, interleukin-6, SOCS-3) could be found to be altered by nutrition or unknown factors could be found within the mammary tissue that are altered by diet.

3.) Mammary tissue extracts from heifers fed high energy diets were less stimulatory to mammary epithelial cells in vitro than extracts prepared from heifers fed low energy diets (Weber et al., 2000a). Mammary tissue extracts from the heifers fed high energy diets likely had lower amounts of a particular stimulatory factor or greater abundance of an inhibitory factor. My dissertation research indicated that leptin, a potential inhibitory factor, was increased in mammary tissue extracts from heifers fed high energy diets. Mammary extracts could be prepared from tissue collected from heifers on this study and used as treatments on mammary epithelial cells. If extracts from H12 heifers are less stimulatory to mammary epithelial cells than extracts from H0 heifers, then it would be interesting to block the action of leptin by adding a leptin antibody to the culture media. If mitogenic activity is not different after the action of leptin is blocked, then it could be concluded that leptin is the inhibitory substance causing a difference in mitogenic activity within the mammary extracts. Also, co-culture of mammary fat pad explants with mammary epithelial cells inhibits the growth of the epithelial cells (McFadden and Cockrell, 1993). A study could also be performed, similar to the one above, but using mammary fat pad explants instead of mammary tissue extracts. This would determine if leptin secretion by fat cells (as opposed to potentially many cell types) could also be blocked and result in growth stimulation of epithelial cells.

Other studies could be performed to better understand the role of nutrition on body and mammary growth in prepubertal dairy heifers.

1.) The allometric phase of mammary growth seems to end, and an isometric phase begins around the onset of puberty (Meyer et al., 2004; Sinha and Tucker, 1969). If the onset of puberty could be delayed, then this might delay the switch to isometric growth, resulting in increased mammary growth. In an abstract, Sejrsen et al. (1994) indicated that GnRH immunization of heifers inhibited the onset of puberty, but did not alter mammary gland weight. However, factors that trigger the switch from allometric to isometric mammary growth are not known. Serum concentrations of leptin increase prior to the attainment of puberty (Garcia et al., 2002). Daily treatment of mice with leptin (2 μg/g BW) accelerated the onset of puberty (Ahima et al., 1997). Blocking the increase in serum concentrations of leptin may be one way to delay the onset of puberty in dairy heifers.

2.) Increasing the energy and protein intake of dairy calves from 2 to 8 wk of age increases mammary parenchymal tissue mass, and content of DNA and RNA (Brown et al., 2005a). The reason for why high energy intake stimulates mammary growth in preruminant calves, but not in older calves or heifers is not known. More research is needed to answer this phenomenon. Whether the increase in mammary growth observed in the Brown study will result in greater milk yield during first lactation will be answered in another experiment currently taking place at MSU.

3.) Finding potential inhibitors of leptin might be one way that producers could feed heifers faster without potential impairment of mammogenesis. Treatment of heifers on a high plane of nutrition with bST decreased leptin mRNA expression in mammary tissue compared to placebo-treated heifers on a high plane of nutrition (Lew et al., 2005). Isoprothiolane treatment alters lipid mobilization and decreases serum lipid concentrations in rats (Katamoto et al., 1991). Isoprothiolane also increased the proliferation of mammary epithelial cells and inhibited the production of IL-1 and IL-6 by mammary epithelial cells (Okada et al., 1999). Pathway similarities exist between leptin and interleukins. More research is needed to understand how bST alters leptin expression and if isoprothiolane is an inhibitor of leptin expression in the mammary gland.

4.) A question remains as to why high energy intake in prepubertal heifers increases muscle and fat accretion, but does not increase mammary growth. Microarrays could be used to quantitate differences in expression between heifers fed high compared to low energy diets using mammary, muscle, and adipose tissue. A comparative analysis between tissues could determine if particular mitogenic factors were upregulated within muscle and adipose compared to mammary tissue. For example, my research determined that IGF-I mRNA expression was not altered by feeding level and IGF-I receptor mRNA expression was lower in heifers fed a high energy diet for a short duration (actual fold change was minimal). However, Vestergaard et al. (2003) showed that IGF-I receptor density in longissimus muscle was increased in heifers fed a high energy diet.

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APPENDIX

Medication

Various medications were used depending on the symptoms and previous treatments [Nuflor (Schering-Plough), Micotil (Elanco), Recovr (Fort Dodge), A-180 (Pfizer), LA-200 (Pfizer), Excenel (Pfizer)]. Listed below was the protocol for which drugs were used depending on symptoms:

Symptom	**Drug**
Lame	LA-200
Respiratory (1st time)	Nuflor or Nuflor + Recovr
Respiratory (2nd time)	Micotil or Micotil + Recovr
Respiratory (3rd time)	A-180
If sick and near slaughter	Excenel

Table 1. Least square means for body and carcass composition (non-transformed).

		Treatment Group[1]					*P* for Contrast[2]		
	BL	H0	H3	H6	H12	SE[3]	L	Q	C
Heifers, no	4	16	15	16	15				
Carcass weight, kg	50.0$^{\#}$	76.6	82.5	91.5	107	0.65	< 0.01	0.40	0.10
Carcass weight, % of BW	48.5$^{\#}$	46.4	49.4	50.6	52.6	0.36	< 0.01	< 0.01	0.24
Carcass protein, %	21.6	19.1	18.7	18.1	18.1	0.16	< 0.01	0.11	0.36
Carcass fat, %	6.10	7.33	10.1	13.5	14.4	0.61	< 0.01	< 0.01	0.24
Carcass water, %	71.2	71.9	69.4	66.5	65.3	0.52	< 0.01	< 0.01	0.31
Carcass protein, kg	10.8$^{\#}$	14.6	15.4	16.6	19.3	0.25	< 0.01	0.22	0.76
Carcass protein, kg/100 kg BW	10.5$^{\#}$	8.82	9.22	9.19	9.51	0.14	< 0.01	0.54	0.26
Carcass fat, kg	3.05$^{\#}$	5.6	8.4	12.4	15.4	0.56	< 0.01	0.04	0.17
Carcass fat, kg/100 kg BW	2.96$^{\#}$	3.40	5.00	6.85	7.57	0.30	< 0.01	< 0.01	0.25
Fat-free carcass, kg	47.0$^{\#}$	71.0	74.2	79.1	91.2	0.98	< 0.01	0.08	0.74
Liver weight, kg	-	2.66	3.62	3.71	3.94	0.04	< 0.01	< 0.01	0.01
Liver weight, % of BW	-	1.62	2.17	2.05	1.97	0.02	< 0.01	< 0.01	< 0.01
Perirenal fat, g	-	693	987	1480	1856	100	< 0.01	0.20	0.27
Perirenal fat, g/100 kg BW	-	418	588	814	914	54	< 0.01	0.06	0.37

[1] Treatment groups are as follows: Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

[*] Data were log transformed to achieve homogeneous variance. Means presented are back transformed. The error term indicated below is the average of the lower and upper confidence intervals for each treatment group.

[#] Baseline carcass weight measurements were estimated using previous results (Brown et al., 2005b).

Table 2. Least square means for mammary gland composition (non-transformed).

		Treatment Group[1]					*P* for Contrast		
	BL	H0	H3	H6	H12	SE	L	Q	C
Heifers	4	16	15	16	15				
Mammary gland weight, g	78.1	808	1014	1412	1818	90.0	< 0.01	0.59	0.33
Parenchyma, g	8.05	146	152	166	164	10.5	0.22	0.48	0.63
Parenchyma, g/100 kg carcass weight	14.9	191	185	181	155	12.6	0.06	0.63	0.84
Extraparenchyma, g	39.5	213	297	438	624	49.6	< 0.01	0.89	0.62
Extraparenchyma, g/100 kg carcass weight	72.9	278	358	476	582	47.8	< 0.01	0.52	0.64
Intraparenchymal fat, g	0.46	24.1	27.8	37.6	42.1	3.09	< 0.01	0.40	0.33
Intraparenchymal fat, % of par.	5.32	17.2	18.5	22.1	25.3	1.06	< 0.01	0.80	0.37
Intraparenchymal fat, g/100 kg carcass weight	0.84	31.4	33.8	41.1	39.3	3.17	0.08	0.24	0.37
Fat-free Parenchyma, g	7.59	122	124	128	122	9.37	0.99	0.60	0.84
Fat-free Parenchyma, g/100 kg carcass weight	14.0	159	151	140	116	11.2	0.02	0.84	0.97
Parenchyma protein, g	0.77	13.0	13.8	13.9	14.0	0.14	0.53	0.67	0.87
Parenchyma Protein, % of parenchyma.	9.54	8.87	8.96	8.46	8.59	0.14	0.09	0.40	0.08

[1] Treatment groups are as follows: heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

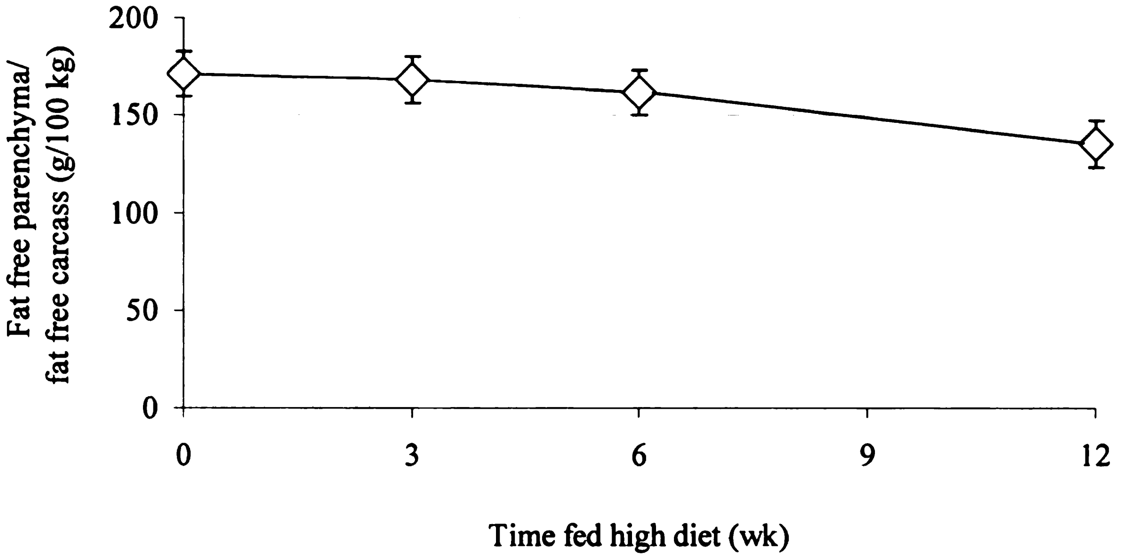

Figure 1. Grams of fat-free parenchymal tissue relative to 100 kg fat-free carcass. Heifers (n = 15 or 16/trt) on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Data presented are non-transformed. Data are significant for a linear effect (Linear: $P < 0.05$).

Table 3. Least square means for mammary gland nucleic acid content (non-transformed).

	Treatment Group[1]						*P* for Contrast[2]		
	BL	H0	H3	H6	H12	SE[3]	L	Q	C
Heifers, no	4	16	15	16	15				
Parenchymal DNA, mg	37.5	874	906	853	967	75.7	0.38	0.76	0.87
DNA, mg/100 kg carcass weight	69.2	1141	1100	1038	919	93.4	0.10	0.91	0.95
DNA concentration, mg/g	4.59	5.81	5.91	5.86	5.87	0.14	0.88	0.78	0.72
Parenchymal RNA, mg	64.3	826	921	925	908	70.3	0.52	0.39	0.74
RNA, mg/100 kg carcass weight	119	1075	1118	1008	867	87.4	0.07	0.54	0.57
RNA concentration, mg/g	8.05	5.52	6.04	5.61	5.53	0.17	0.49	0.20	0.07
RNA:DNA	1.80	0.96	1.04	0.96	0.96	0.03	0.37	0.38	0.05

[1] Treatment groups are as follows: heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. The low energy diet and high energy diet were formulated for gains of 0.6 and 1.2 kg/d, respectively.

[2] L = Linear; Q = Quadratic; C = Cubic

[3] Pooled SE using treatment*purchase group or pen as the error term with 4 pens per treatment.

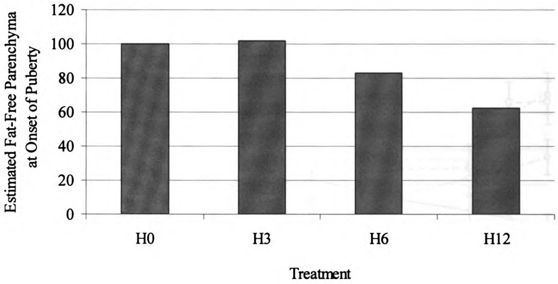

Figure 2. Estimated fat-free parenchymal tissue present at the onset of puberty. Data are presented relative to H0 treatment. The onset of puberty in heifers was assumed to be approximately 275 kg (Capuco et al., 2004; Capuco et al., 1995; Niezen et al., 1996; Whitlock et al., 2002). Data were calculated using daily accretion rates for both body weight and fat-free parenchymal tissue weight.

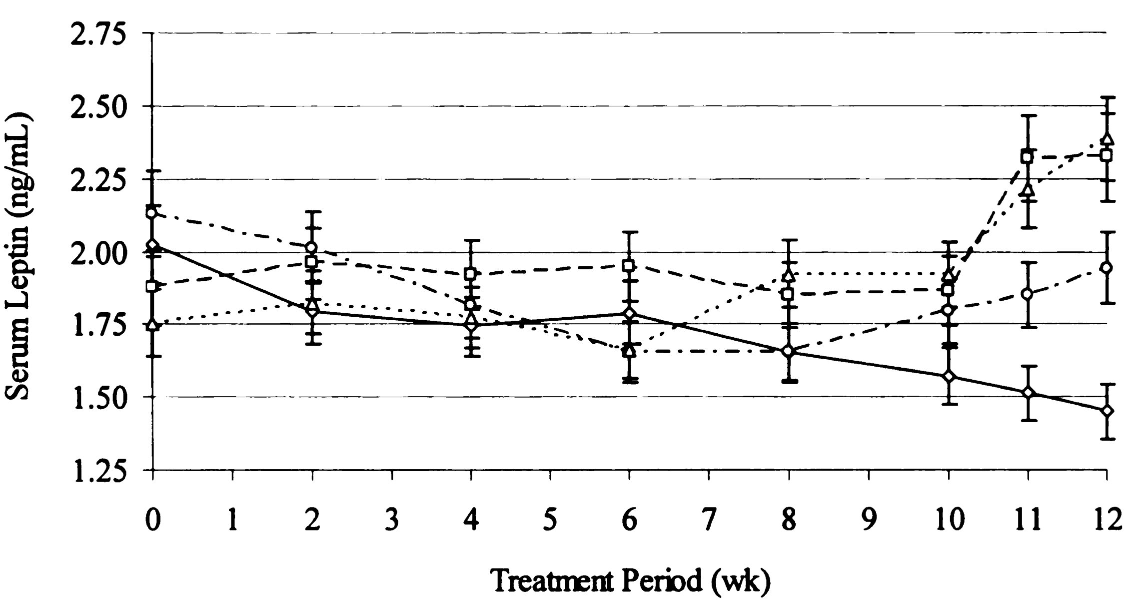

Figure 3. Serum leptin concentrations taken every 2 wk and weekly during the last 3 wk of the treatment period in heifers 11 to 23 wk of age. Heifers (n = 15 or 16/trt) on treatment H0 (——◇——), H3 (- — -○- — -), H6 (- - -Δ- - -), H12 (— —□— —) were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Serum leptin values were log transformed to achieve homogeneous variance. Serum leptin means presented are back transformed. The error term is the average of the lower and upper confidence intervals. Initial serum leptin (wk 0) was used as a covariate. An overall linear effect was evident as heifers were fed the high energy diet for longer durations of time (L: $P < 0.01$).

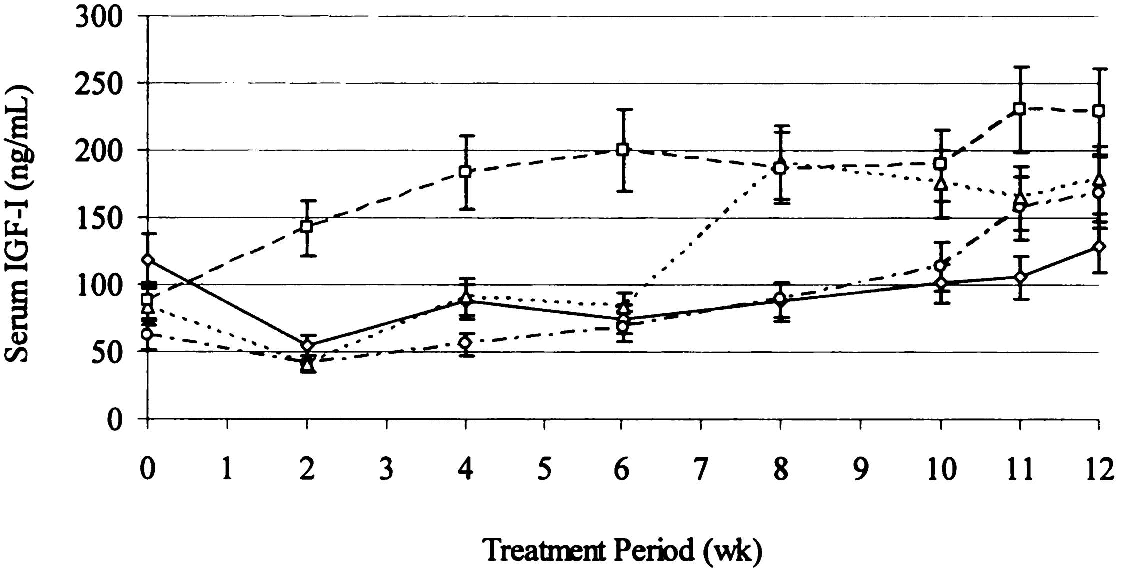

Figure 4. Serum IGF-I concentrations taken every 2 wk and weekly during the last 3 wk of the treatment period in heifers 11 wk to 23 wk of age. Heifers (n = 15 or 16/trt) on treatment H0 (——◇——), H3 (- — -○- — -), H6 (- - -△- - -), H12 (— —□— —) were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Serum IGF-I values were log transformed to achieve homogeneous variance. Serum IGF-I means presented are back transformed. Initial serum IGF-I (wk 0) was used as a covariate. The error term is the average of the lower and upper confidence intervals. Longer durations fed the high energy diet increased serum IGF-I concentration in a linear fashion (L: $P < 0.01$).

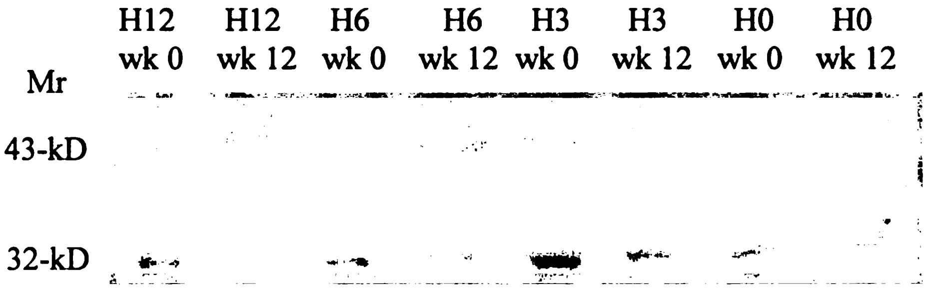

Figure 5. Representative autoradiograph of a western ligand blot showing relative IGFBP-3 (43-kD) and IGFBP-2 (32-kD) abundance in serum samples taken at wk 0 and at slaughter. Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively. Samples were fractionated on a gel, transferred to a membrane and hybridized with [^{125}I]-IGF-I. Mr = relative molecular mass.

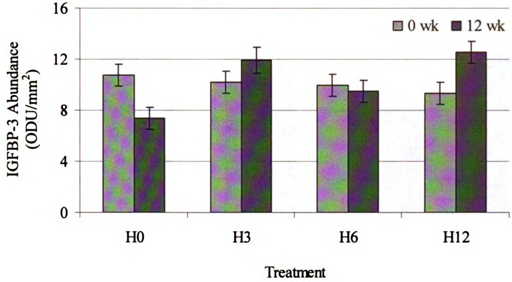

Figure 6. Abundance of IGF-binding protein-3 (IGFBP-3) in serum samples taken at wk 0 and wk 12 of the treatment period. Data represent means for 8 heifers per treatment group. Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively.

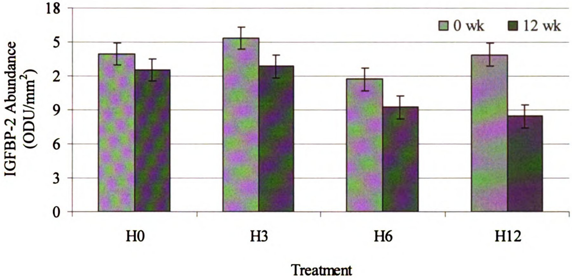

Figure 7. Abundance of IGF-binding protein-2 (IGFBP-2) in serum samples taken at wk 0 and at wk 12 of the treatment period. Data represent means for 8 heifers per treatment group. Heifers on H0, H3, H6, H12 were fed the low energy diet for 12, 9, 6, 0 wk followed by the high energy diet for 0, 3, 6, 12 wk, respectively.