Effect of Increasing Energy and Protein Intake on Mammary Development in Heifer Calves*

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ABSTRACT

The objective of this study was to determine if increased energy and protein intake from 2 to 14 wk of age would affect mammary development in heifer calves. At 2 wk of age, Holstein heifer calves were assigned to 1 of 4 treatments in a 2 × 2 factorial arrangement with 2 levels of protein and energy intake (moderate, M; high, H) in period 1 (2 to 8 wk of age) and 2 levels of protein and energy intake (low, L; high, H) in period 2 (8 to 14 wk of age), so that mean initial body weights were approximately equal for all 4 treatments (ML, MH, HL, and HH). The M diet in period 1 consisted of a standard milk replacer (21.3% CP, 21.3% fat) fed at 1.1% of BW on a DM basis and a 16.5% CP grain mix fed at restricted intake to promote 400 g of daily gain, whereas the L diet in period 2 consisted only of the grain mix. The H diet in period 1 consisted of a high-protein milk replacer (30.3% CP, 15.9% fat) fed at 2.0% of body weight on a DM basis and a 21.3% CP grain mix available ad libitum. In period 2, the H diet consisted of just the 21.3% grain mix. Calves were gradually weaned from milk replacer by 7 wk and slaughtered at 8 (n = 11) or 14 wk of age (n = 41). Parenchyma from the distal region, midgland, and proximal region relative to the teat from one half of the udder was collected, fixed, and embedded in paraffin. The other half of the gland was used to determine parenchymal mass, protein, fat, DNA, RNA, and extraparenchymal mass. Total parenchymal tissue, parenchymal DNA, parenchymal RNA, and concentrations of DNA and RNA were higher for calves on the H diet during period 1, but were not affected by diet during period 2. Parenchymal fat percentage was increased by the H diet during period 2. The H diet increased extraparenchymal fat during both periods. The area of parenchyma occupied by epithelium was not affected by treatment, but

at the end of period 2, the percentage of proliferating epithelial cells as indicated by Ki67, an marker of cell proliferation, expression was greater for calves on the M diet in period 1 compared with calves on the H diet in period 1. Diets did not influence parenchymal protein percentage or the ratio of RNA to DNA. Higher energy and protein intake from 2 to 8 wk of age increased parenchymal mass and parenchymal DNA and RNA in mammary glands of heifer calves without increasing deposition of parenchymal fat. Diet also influenced histological development of mammary parenchyma and subsequent proliferation of ductal epithelial cells. Implications of these effects for future milk production potential are unknown.

(Key words: calf, heifer, mammary development, nutrition)

Abbreviation key: D = distal, ER = estrogen receptor, H = high protein and energy intake, M = moderate protein and energy intake, MID = mid-gland, L = low protein and energy intake, P = proximal, SUB = subtending ducts, TDU = terminal ductular units.

INTRODUCTION

Little is known about body growth rates during the period between birth and approximately 4 mo of age that encourage maximal mammary development and future milk production potential. Before approximately 9 mo of age, a critical period of heifer mammary growth exists during which mammary growth is allometric (Sinha and Tucker, 1969), and can be negatively affected by high energy intake (Sejrsen and Purup, 1997). However, in calves before weaning, 2 studies have reported that increased intake of whole milk was related to higher future milk production. For example, heifer calves consuming whole milk for BW gains of 1.1 kg/d tended to produce more milk as cows than did heifers fed a restricted amount of whole milk before weaning (Foldager and Krohn, 1994). Bar-Peled et al. (1997) showed that calves suckling cows twice daily tended to produce more milk in their first lactations compared with similar heifers fed a defined amount of milk replacer, although it must

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be noted that the importance of the quantity of whole milk consumed relative to differences in diet composition and feeding method in this effect is unknown. Further evidence of the potential to increase the growth rates of young calves without negatively impacting mammary growth was provided by Sejrsen et al. (1998). This group demonstrated that heifer calves fed increased amounts of whole milk for a high rate of gain from birth until 6 wk of age had amounts of mammary parenchyma mass similar to those of heifers fed for moderate rates of gain when slaughtered at 12 wk of age.

Only 1 study has been reported that addresses the potential for dietary manipulation of mammary development after weaning in calves younger than 3 mo of age. Using calves fed for 2 rates of gain after weaning, Petitclerc et al. (1999) showed that ad libitum feeding increased extraparenchymal tissue volume but reduced the amount of parenchymal tissue when data were adjusted for differences in BW at slaughter, indicating the potential for nutritional impairment of mammary development after the time of weaning around 2 mo of age.

The objective of this experiment was to determine if increasing energy and protein intake using commercial milk replacers and calf starters would affect mammary growth and development in heifer calves younger than 4 mo of age. Results of treatment effects on body growth rate and carcass composition are reported in a companion article (Brown et al., 2005).

MATERIALS AND METHODS

Management of Calves

All procedures were approved by the Animal Use and Care Committee of Michigan State University. Female Holstein calves (44.1 ± 0.9 kg BW) were blocked by date of purchase, as described earlier by Brown et al. (2005). Following the 1-wk adaptation period, calves were randomly assigned to 1 of 4 treatments in a 2×2 factorial arrangement so that mean BW was not different among treatments. Withers height was not different among treatments at the start of the experiment.

In period 1, the moderate (**M**) diet consisted of milk replacer (Calvita Supreme, Milk Specialties Co., Dundee, IL; 21.3% CP, 21.3% fat, ~4.7 kcal of ME/g of DM guaranteed analysis) fed on a DM basis at 1.1% of BW (reconstituted to 11.8% DM), and starter grain (20.5% CP guaranteed analysis; Gold Flake Calf Starter, Nutrena Feeds—Cargill, Inc., Minneapolis, MN) fed at restricted intake to achieve 0.40 kg of average daily gain from 2 to 8 wk of age. The high (**H**) diet consisted of a high-protein milk replacer (Excelerate, Milk Specialties Co.; 30.3% CP, 15.9% fat, approximately 4.4 kcal of ME/g of DM guaranteed analysis) fed on a DM basis at 2.0% of BW (reconstituted to 14.1% DM) and high-protein

starter grain (25.0% CP guaranteed analysis; Herd Builder Calf Starter, Nutrena Feeds—Cargill, Inc.) fed ad libitum. Calves were gradually weaned from milk replacer by 7 wk of age.

From 8 to 14 wk of age, calves on the low (**L**) diet were fed grain at restricted intake to achieve 0.4 kg of average daily gain, whereas calves on the H diet were fed high-protein grain for ad libitum intake. From 8 to 9 wk of age, calves were fed only the respective calf starters, but beginning at 9 wk of age rolled corn was added to both diets. The new diets contained 70% of the respective calf starters and 30% rolled corn. According to laboratory analyses, nutrient values for the new diets were 16.5% CP and 3.8% crude fat for the L diet, and 21.3% CP and 3.7% crude fat for the H diet. Calves had fresh water available at all times.

Tissue Collection

One subset of calves (1 or 2 calves from each block; $n = 5$ calves on M diet; $n = 6$ calves on H diet) was randomly selected and slaughtered at 8 wk of age to assess mammary development at the end of period 1. The remaining 41 calves were slaughtered at 14 wk of age.

Calves were weighed on the afternoon of the day before slaughter. Calves were then fed and allowed 1 h to eat and drink water before being shipped to the Michigan State University Meats Laboratory at 1630 h. Calves were slaughtered approximately 14 to 16 h after last feeding using captive bolt stunning followed by exsanguination. Within 15 min of slaughter, the mammary glands were collected. Mammary glands were bisected into right and left hemiglands and weighed. The left half was frozen flat in liquid nitrogen and stored at -20°C for later analysis. Using the right half, samples of parenchymal tissue from the distal (**D**), midgland (**MID**), or proximal (**P**) region relative to the teat were excised from each calf, fixed for 24 h in buffered formalin, and then transferred to 70% ethanol for later analysis.

The reproductive tracts were examined to confirm that animals were not freemartins and had not reached puberty. One heifer was a freemartin and her data were eliminated from the results.

Mammary Tissue Analysis

The frozen left half of the udder was cut transversely using a band saw into slices 5 to 10 mm thick. Slices from both the anterior and posterior ends that did not contain parenchymal tissue were discarded. Skin, teats, and supramammary lymph nodes were removed. Extraparenchymal fat located outside the border of the parenchyma was weighed. The remaining parenchymal tissue

was weighed and ground into a fine powder using liquid nitrogen in a Waring Blender (Waring Products Division, New Hartford, CT). The powder was mixed and subsampled for subsequent analysis of RNA and DNA (Tucker, 1964), DM, protein, and fat. Dry matter was determined by the difference from wet weight after the sample was placed in a 105°C oven for 24 h. Ash was determined after 5 h of oxidation in a muffle furnace at 500°C. Crude protein was analyzed according to Hach et al. (1987). Fat was determined by Soxhlet ether extraction (AOAC, 1990).

Embedding of Tissue in Paraffin and Preparation of Slides

Multiple pieces of parenchymal tissue from the D, MID, and P regions relative to the teat from one half of the udder were excised and fixed. Replicate tissue samples were embedded in paraffin. One replicate of paraffin-embedded tissue was used to prepare 5- μ m-thick sections, and 4 or 5 serial tissue sections from each sample were placed on a microscope slide.

General Staining Procedures

Hydration of tissue sections began with a deparaffinization step that involved washing slides twice in xylene at 5 min per wash. Slides were then transferred through a series of ethanol washes at 2 min per wash (100, 95, and 70% ethanol). The final step in hydration was a 5-min wash in distilled water. One slide representing each sample was placed in a hematoxylin and eosin solution (Sigma Chemical Co., St. Louis, MO) for 10 min. Following completion of all staining procedures, tissue sections were again dehydrated, adhesive mounting medium (Permount, Fisher Chemicals, Fairlawn, NJ) was applied, cover slips were added to the slides and slides were allowed to dry overnight.

Immunocytochemical Staining Procedures

Identical staining procedures were used for the visualization of estrogen receptor (ER) and Ki67, a marker of cell proliferation. The only variation in procedure involved using different antibodies to identify each protein of interest. In contrast to the general staining procedures, which required that an entire slide receive the stain, multiple proteins were identified on the same microscope slide. The ER and Ki67 staining was performed on serial sections on 1 slide from each sample. Procedures were as described in recent reports (Capuco et al., 2002; Berry et al., 2003).

Determination of Mammary Parenchymal Tissue Composition

Hematoxylin and eosin-stained sections were examined at 40 $\times$ magnification (Olympus BH-2, Opelco, Dulles, VA). Using a glass grid that was fitted onto the eyepiece of the microscope, areas of parenchymal tissue containing epithelium were randomly selected in 2 or more sections from each zone and were focused at 400 $\times$ magnification. A series of images (6 per zone) were captured with a top-mount digital microscope camera (Olympus DP10, Opelco) and transferred to a computer for analysis. Saved images were examined using L-View Pro image analysis software (MMedia Research Corp., Hallandale, FL) by superimposing a 9 $\times$ 8 grid over the image. Each of the 72 intersection points was classified to determine if it was in contact with epithelium, lumen, or stroma within the parenchymal tissue region. Tissue proportions were determined by dividing the number of tissue specific intersection points by the total number of points counted.

Developmental Categorization

The previously described procedure for evaluating tissue composition only examined areas of tissue within lobular areas of apparent parenchymal tissue. Thus, this analysis focused on the relative development of parenchymal tissue compartments rather than overall mammary tissue development (i.e., intralobular areas of adipose and connective tissue were not included). Consequently, to characterize overall tissue development, 3 digital images were obtained from each zone using 40 $\times$ magnification to represent a series of 3 developmental grades (i.e., 1 = least developed to 3 = most developed, based on the amount of parenchymal tissue present within the entire field). Stained tissue sections were subsequently viewed at 40 $\times$ magnification and directly compared with control images simultaneously opened with L-View Pro software. Each sample was assigned a score to allow a semiquantitative evaluation of overall mammary tissue development. Three images per zone were evaluated and averaged for each animal.

Determination of Percentage of ER- and Ki67-positive Epithelial Cells

Slides containing tissue sections stained with primary antibody for ER and Ki67 were first examined under the microscope with the 4 $\times$ objective. Examination under these low-power conditions allowed for epithelial structures to be distinguished as either subtending ducts (SUB) or terminal ductular units (TDU) as described by Capuco et al. (2002) and illustrated in Figure 1. However, abundance of staining was not evident. Subsequently,

Figure 1. Hematoxylin and eosin-stained parenchymal mammary tissue from a prepubertal Holstein heifer. This image illustrates the structure of a developing ductal unit. A terminal ductal unit (TDU) is indicated by a dashed line and the associated subtending duct (SUB) is indicated by brackets. The bar indicates 50 μm .

3 areas of parenchymal tissue containing SUB and 3 containing TDU structures were randomly selected from ER- and Ki67-stained sections from each zone, refocused at higher magnification (40 $\times$ objective), and photographed. These digital images were captured and stored on a computer for subsequent analysis.

For analysis, these images were opened with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) at 50% zoom, and a spatial calibration was made that allowed for the number of epithelial cells to be counted by outlining the epithelial structures using the computer mouse and cursor. The total numbers of ER- and Ki67-positive cells within outlined areas were counted and divided by the total number of epithelial cells to calculate the proportion of either ER- or Ki67-positive cells specifically within SUB or TDU structural elements. Approximately 1200 cells were counted for each variable within each image.

Statistical Analyses

Mammary gland variables for calves slaughtered at the end of period 1 were analyzed as a one-factor ANOVA

with M and H diets as the treatments and date of purchase as the blocks. For the calves slaughtered at the end of period 2, all mammary variables except the histology variables were analyzed as a 2 $\times$ 2 factorial, with diets fed in period 1 (2 to 8 wk of age) $\times$ the diets fed in period 2 (8 to 14 wk of age). The data were analyzed using the GLM procedure of SAS 8.2 (SAS Inst., Inc., Cary, NC), with date of purchase as the block. Least squares means and standard errors of the mean are reported. Significance was declared at $P < 0.05$, and trends at $P < 0.10$.

Data for tissue histological evaluation (% epithelium, % lumen, and % stroma) were also analyzed using the GLM procedure of SAS. However, ER and Ki67 labeling responses within SUB and TDU structures within the mammary gland were evaluated to determine differences between these 2 structures. Comparisons between parenchymal zones (P, MID, or D) were analyzed as the linear contrasts of D vs. MID + P and D vs. P, and are reported as the estimate of the contrast. Because contrasts were nonorthogonal, comparisons were completed using Bonferroni tests of significance.

Table 1. Least squares means for weight and composition of mammary glands of calves fed either the moderate (M) or high (H) diet and slaughtered at the end of period 1 (2 to 8 wk of age).

Item	Moderate (n = 5)	High (n = 6)	SEM	<i>P</i> > <i>F</i>
Total gland, g	106	185	16	0.01
Total gland, g/100 kg of BW	181	255	22	0.05
Parenchyma, g	1.2	4.5	0.9	0.03
Parenchyma, g/100 kg of BW	1.9	6.2	1.1	0.03
Extra-parenchymal fat, g	10.7	36.0	4.4	0.005
Extra-parenchymal fat, g/100 of kg BW	18.1	49.5	6.1	0.009

RESULTS

Mammary Development

Calves fed the H diet during period 1 (2 to 8 wk of age) had 3 times the amount of parenchyma (per 100 kg BW) at 8 wk of age compared with calves fed the M diet ($P = 0.03$; Table 1). Also, calves fed the H diet compared with those fed the M diet from 2 to 8 wk of age had 50% more parenchyma (per kg of BW) at 14 wk, regardless of which diet was consumed from 8 to 14 wk of age ($P = 0.02$; Table 2). Diet during period 2 did not influence mass of mammary parenchyma per 100 kg of BW ($P = 0.76$). Feeding the H diet during either period 1 or 2 increased the mass of mammary extraparenchymal fat per 100 kg of BW ($P < 0.03$; Tables 1 and 2).

Diet during period 1 did not alter the percentage of fat within mammary parenchymal tissue at 14 wk ($P = 0.24$), but the H diet during period 2 increased parenchymal fat on a percentage basis ($P = 0.03$). Protein percentage within the mammary parenchymal tissue was unaffected by diet in either period.

Mammary parenchymal DNA and RNA per 100 kg of BW at 14 wk were higher for calves fed the H diet (HL and HH) compared with the M diet (ML and MH) during period 1 ($P = 0.004$ and $P = 0.009$, respectively; Table

3). The H diet during period 1 increased concentrations of DNA and RNA in parenchymal tissue ($P = 0.002$ and $P = 0.06$, respectively). Diet during period 2 did not affect the amounts or concentrations of mammary parenchymal DNA or RNA. Diet did not affect the RNA:DNA ratio during either period 1 or 2.

Tissue Composition

Feeding level did not significantly affect the percentage of epithelial area within mammary parenchyma. However, the interaction between periods was significant (Table 4, $P = 0.02$). The percentage of lumen was 3.5% greater ($P = 0.01$) for calves on the H diet in period 1 (HH and HL) vs. calves on the M diet (MH and ML). Opposite treatment effects were demonstrated with respect to percentage of stroma, which was 4.0% lower ($P = 0.02$) for calves on the H diet in period 1 (HH and HL) vs. calves on the M diet (MH and ML). Percentages of epithelium in mammary parenchymal tissue did not differ significantly between zones of the gland and averaged 19.6%. There was 3.9% less ($P = 0.01$) lumenal area and 5.3% more ($P = 0.004$) stromal area in zone D vs. MID + P (Table 5), and values were similar for D vs. P.

Table 2. Least squares means for weight and composition of mammary glands of calves fed either the moderate (M) or high (H) diet during period 1 (2 to 8 wk of age) and low (L) or H diet during period 2 (8 to 14 wk of age) and slaughtered at the end of period 2.

Item	ML (n = 11)	MH (n = 10)	HL (n = 9)	HH (n = 11)	SEM	<i>P</i> > <i>F</i>		
						Period 1 diet	Period 2 diet	Period 1 × Period 2
Total gland, g	208	418	242	626	49	0.02	<0.0001	0.07
Total gland, g/100 kg of BW	252	390	274	511	35	0.04	<0.0001	0.14
Parenchyma, g	13	16	22	29	4	0.006	0.21	0.48
Parenchyma, g/100 kg of BW	16	15	24	23	4	0.02	0.76	0.99
Extra-parenchymal fat, g	45	107	59	189	19	0.02	<0.0001	0.06
Extra-parenchymal fat, g/100 kg of BW	52	99	65	153	15	0.03	<0.0001	0.17
Parenchymal fat, ¹ g	1.6	1.4	2.0	4.7	0.9	0.05	0.16	0.11
Parenchymal fat, ¹ %	7.2	8.9	7.0	13.2	1.7	0.24	0.03	0.18
Parenchymal protein, ² g	1.6	1.8	2.4	2.9	0.4	0.02	0.33	0.64
Parenchymal protein, ² %	10.1	9.9	9.8	9.6	0.2	0.18	0.25	0.93

¹Data represent results for 7 heifers on ML, 8 on MH, 7 on HL, and 9 on HH treatments.

²Data represent results for 7 heifers on ML, 8 on MH, 7 on HL, and 10 on HH treatments.

Table 3. Least squares means for nucleic acid content of mammary glands of calves fed either the moderate (M) or high (H) diet during period 1 (2 to 8 wk of age) and low (L) or H diet during period 2 (8 to 14 wk of age) and slaughtered at the end of period 2.

Item	ML (n = 11)	MH (n = 10)	HL (n = 9)	HH (n = 11)	SEM	<i>P</i> > <i>F</i>		
						Period 1 diet	Period 2 diet	Period 1 × Period 2
Parenchymal DNA, mg	39	45	78	108	16	0.002	0.24	0.43
DNA, mg/100 kg of BW	44	42	85	86	14	0.004	0.94	0.93
Concentration DNA, mg/g	2.59	2.61	3.19	3.49	0.23	0.002	0.45	0.51
Parenchymal RNA, mg	54	67	93	136	18	0.003	0.10	0.39
RNA, mg/100 kg of BW	63	63	103	108	16	0.009	0.85	0.86
Concentration RNA, mg/g	4.15	4.12	4.24	4.77	0.19	0.06	0.18	0.14
RNA:DNA	1.67	1.62	1.45	1.43	0.14	0.14	0.78	0.91

Developmental Categorization

Mean developmental categorization score averaged 2.1 across all treatments, but ML calves had the lowest overall developmental score (1.6) (Figure 2). Calves on the H diet in period 2 had a higher ($P = 0.001$) developmental score than calves on the L diet in period 2. For developmental score related to period 1, the contrast of (HH + HL) vs. (MH + ML) was non significant. However, during period 2, the contrast of (HH + MH) vs. (HL + ML) was significant ($P = 0.0006$) for developmental score. Differences between zones of the mammary gland were also seen with regard to level of development. Tissue collected from zone D scored 0.40 and 0.31 units lower ($P = 0.007$ and $P = 0.07$, respectively) than tissue from zones MID and P together or zone P alone (data not shown). The interaction of zone and treatment on developmental score was not significant ($P = 0.17$).

Estrogen Receptor and Ki67

Feeding level did not significantly affect the percentage of ER-positive epithelial cells in SUB or TDU. How-

ever, there was an interaction of diet and period for percentage of ER (SUB) ($P = 0.04$). The Ki67 expression was affected by feeding level (Table 4). In SUB, the percentage of Ki67-positive epithelial cells was lower in calves on the H diet in period 1 vs. calves on the M diet (3.7 vs. 5.1%, $P = 0.05$). Similar dietary effects were noted in TDU, with calves on the H diet in period 1 having fewer Ki67-positive cells than calves on the M diet (4.2 vs. 6.3%, $P = 0.01$).

Neither ER nor Ki67 expression was significantly affected by mammary zone (data not shown). The mean percentages of ER-positive epithelial cells in zones D, MID, and P were 49.5%, 50.5, and 47.5, respectively. The mean percentages of Ki67-positive epithelial cells in zones D, MID, and P were 5.4, 3.9, and 5.0%. However, significant differences existed in epithelial structure with respect to ER expression. Specifically, percent ER-positive epithelial cells averaged 47% in SUB compared with 52% in TDU, respectively ($P = 0.0001$).

DISCUSSION

Our study demonstrates that increased energy and protein intake associated with accelerated calf growth

Table 4. Least squares means for mammary parenchymal tissue composition, and percentage of epithelial cells positive for estrogen receptor (ER) and Ki67 labeling. Tissue was harvested from calves fed either the moderate (M) or high (H) diet during period 1 (2 to 8 wk of age) and low (L) or H diet during period 2 (8 to 14 wk of age) and slaughtered at the end of period 2.

Item (%)	ML	MH	HL	HH	SEM ¹	<i>P</i> > <i>F</i>		
						Period 1 diet	Period 2 diet	Period 1 × Period 2
Epithelium	17.9	20.9	20.5	19.2	1.0	0.64	0.35	0.02
Lumen	5.7	7.0	9.7	10.1	1.5	0.01	0.52	0.74
Stroma	76.4	72.1	69.8	70.7	1.8	0.02	0.31	0.13
ER ⁺ (SUB ²)	49.8	43.1	46.7	48.9	2.5	0.54	0.30	0.04
ER ⁺ (TDU ³)	51.7	50.5	52.1	53.0	2.7	0.53	0.96	0.66
Ki67 ⁺ (SUB ²)	6.1	4.0	3.6	3.7	0.8	0.05	0.18	0.11
Ki67 ⁺ (TDU ³)	6.9	5.6	3.9	4.5	0.9	0.01	0.68	0.24

¹Largest SEM for each variable.

²SUB = Subtending duct.

³TDU = Terminal ductular unit.

Table 5. Least squares means for mammary parenchymal tissue composition for tissue collected from the distal one third of parenchyma relative to the teat (D), mid-gland (MID), or from the proximal one third (P).

Item (%)	Zone			Contrast, <i>P</i> -value (SEM)	
	D	MID	P	D vs. MID+P	D vs. P
Epithelium	18.7 (0.8)	19.8 (0.8)	20.4 (0.9)	-1.4, 0.17 (1.0)	-1.7, 0.15 (1.2)
Lumen	5.5 (1.2)	9.2 (1.2)	9.7 (1.3)	-3.9, 0.01 (1.5)	-4.1, 0.02 (1.7)
Stroma	75.8 (1.4)	71.0 (1.5)	70.0 (1.6)	+5.3, 0.004 (1.8)	+5.8, 0.007 (2.1)

programs increases growth of mammary parenchyma in calves from 2 to 8 wk of age. The extent of development of mammary parenchyma in heifers is important, because the number of mammary cells is a major factor limiting milk production (Tucker, 1981). From about 4 mo of age until puberty, mammary development is negatively affected by rapid body growth rates. Little is known, however, about how growth rates during the period from birth to approximately 4 mo of age are related to mammary development and future milk production potential.

Calves fed the H diet in period 1 in our study had more mammary parenchymal tissue per 100 kg of BW compared with calves on the M diet in period 1. In another study with a treatment period from 5 d to 6 wk of age, Sejrsen et al. (1998) showed that calves on either a high or moderate feeding level had similar amounts of mammary parenchyma when slaughtered at similar ages. Similarly, heifer calves fed a high-energy diet for ad libitum intakes from 6 wk to 4 mo of age had greater mammary volume (1067 cm³) than control heifers (335

cm³) (Petitclerc et al., 1999). Petitclerc et al. (1999) found an increase in parenchymal volume in heifers fed ad libitum, but there was 28% less parenchymal volume after adjusting for BW. However, heifers on their study did not start treatments until 6 wk of age. The older, postweaning age at start of treatment may explain the difference in treatment effects compared with our study.

In period 2, the calves on the H diet had a higher percentage of mammary parenchymal fat, but parenchymal growth was unaffected by diet, similar to the results of Petitclerc et al. (1999). In a study with older but still prepubertal heifers by Capuco et al. (1995), heifers on a corn silage diet gaining 1.0 kg/d from 175 kg of BW until 333 kg of BW had a higher parenchymal fat percentage than did heifers growing at 0.78 kg/d on a corn silage diet or heifers growing at either rate of gain on an alfalfa silage diet. Heifers growing at a high rate of gain on the corn silage diet also had a similar amount of mammary parenchymal tissue compared with heifers in the other 3 groups, but there was an increase in number of adipocytes and a decrease in epithelial cell number. Elongation of the primary ducts was not affected by dietary treatment, but there was decreased branching of the ducts in heifers on the high corn diet. As described by Akers (1990), epithelial cells of the growing ducts pursue stromal growth into the mammary fat pad of heifers, with the absence of epithelial cells adjacent to adipocytes suggesting that mammary adipose tissue might inhibit epithelial cell proliferation.

The H diet in period 1 also increased parenchymal DNA and RNA amount and DNA concentration, and tended to increase RNA concentration. The amount of DNA is an indicator of the number of secretory cell numbers and RNA is an indicator of metabolic activity (Tucker, 1969). The increase in DNA suggests that calves on the H diet in period 1 would produce more milk as cows. In a study by Bar-Peled et al. (1997), heifers with similar BW gains from birth to 6 wk of age as calves on the H diet in our study tended to produce more milk as cows. Foldager and Krohn (1994) determined that heif-

Figure 2. Mammary tissue development score. Data are presented as least squares means and SEM. Tissue was harvested from calves fed either the moderate (M) or high (H) diet during period 1 (2 to 8 wk of age) and low (L) or H diet during period 2 (8 to 14 wk of age) and slaughtered at the end of period 2. For period 1, the contrast of HH + HL vs. MH + ML was not significant; however, during period 2, the contrast of HH + MH vs. HL + ML was highly significant ($P < 0.001$).

ers with higher weight gains (1.1 kg/d) from birth to 8 wk of age tended to produce more milk as cows than did heifers fed for 0.58 kg/d from birth through 6 wk of age. However, in both of these studies, high growth rates were achieved by feeding whole milk. It is possible that other factors, such as hormones and growth factors found in cows' milk, but not in milk replacer, contributed substantially to the observed effects on milk production.

The lack of differences in percentage of epithelium within the parenchymal tissue suggests that none of the dietary treatments markedly affected the overall pattern of ductular tissue development. Regardless, heifers receiving the H diet during the first period had greater percentages of parenchymal lumen area compared with heifers on the M diet. These results may indicate that feeding a diet higher in protein and energy between 2 and 8 wk of age increased development of mammary parenchymal tissue structures, supporting the data regarding parenchymal mass (Table 2), DNA, and RNA concentration (Table 3). However, larger lumens do not necessarily indicate better mammary development in terms of future milk production potential. Larger ducts with increased luminal diameters may represent the appearance of ducts that are developmentally more mature than is normal for the peripubertal stage of development. If these duct structures are less able to support the markedly increased rate of epithelial cell proliferation and the subsequent allometric growth occurring with the onset of estrous cycles and pregnancy, this would clearly be detrimental. It is logical to assume that this increased luminal area would be accompanied by a decrease in stroma, which was the case (Table 4).

Because the procedure for determining tissue composition used intersection points in contact only with parenchyma, this means of data collection would not have detected differences between tissue sections in the amount of area occupied by ducts. Consequently, a semi-quantitative analysis (developmental score) was designed. This assessment reflects the amount of parenchymal tissue present in entire mammary tissue sections, whereas our parenchymal tissue composition analysis evaluates the tissue composition specifically within parenchyma. Heifers fed the H diet during period 2 had increased developmental scores (Figure 2), possibly reflecting the numerically higher average parenchymal weight for heifers on the H diet in period 2.

Heifers fed the M diet during period 1 demonstrated increased epithelial cell proliferation (Ki67) in both SUB and TDU (Table 4) compared with heifers on the H diet, despite also having a lower developmental score. These data support our suggestion that heifers subjected to the H diet in period 1 have larger, more mature ducts (increased luminal area) that subsequently exhibit a reduced rate of epithelial cell proliferation. That is, heif-

ers with the highest proportions of proliferating epithelial cells (MH and ML) also possessed the least amount of luminal space. It is possible that heifers fed for a high rate of gain early are primed for reductions in mammary growth after 14 wk of age, as the reduced percentage of Ki67-labeled epithelial cells in these animals suggests.

Epithelial cell ER expression in SUB and TDU did not differ in response to feeding regimen (Table 4). Although it has been apparent for some time that estrogen plays a crucial role in mammary development (Wallace, 1958), these results suggest that mammary growth differences resulting from dietary treatments are not due to direct interactions between estrogen and its receptor. This does not imply that estrogen had no effect on the mammary developmental variation observed in this study. For example, estrogen is required for the normal function of IGF-I, which is a potent mitogen for prepubertal mammary growth (Ruan et al., 1995). These results also do not imply that the ER was not responsible for mammary growth differences between feeding treatments. It has been postulated that various growth factors, such as IGF-I and epidermal growth factor, might directly stimulate the ER in the absence of its natural ligand (Woodward et al., 2000). Interestingly, calves on the H diet in period 1 exhibited higher plasma IGF-I (Brown et al., 2005) and greater parenchymal growth; however, higher plasma IGF-I concentrations in calves on the H diet in period 2 were not accompanied by greater parenchymal growth than that of calves on the L diet. Dietary control at the local tissue level is clearly complex, with multiple players, and our understanding is minimal particularly of the ruminant. In addition, the interaction of period 1 and period 2 on ER expression in SUB would seem to suggest that dietary effects on mammary tissue growth and morphogenesis are not constant across all stages of development even during the prepubertal period.

Surprisingly, there were no differences in epithelial cell proliferation between zones of the mammary gland. The expectation was that there would be a greater number of proliferating cells at the leading edge of parenchymal penetration into the fat pad (zone D). This suggests that epithelial growth was similar between more mature ductular structures and newer, or more immature, structures at 14 wk of age. Thus, development of side branching appears to be as crucial as TDU expansion in terms of ductular growth early in heifer mammary gland development. Direct support for this comes from the finding that SUB, which would be more prevalent in zones closer to the teat, had a proliferative capacity that was similar to TDU.

When ER and Ki67 expression were categorized by structure, TDU were found to have a significantly greater percentage of ER-positive epithelial cells than

SUB (52.4 vs. 46.6%). In the pubertal mouse model, disruption of certain extracellular proteins led to structure-specific inhibition of ductular growth. For example, blockage of $\beta 1$ integrin, an epithelial cell receptor for the extracellular matrix, reduced terminal end bud number, whereas the remaining gland architecture appeared normal (Klinowska et al., 1999). Our results regarding ER expression in TDU point to a possible structure-specific role for estrogen, or other ER binding proteins, in ductular morphogenesis of the prepubertal heifer mammary gland. The lack of difference between TDU and SUB in Ki67 expression was unexpected. It was anticipated that TDU would have greater cell proliferation numbers due to the fact that these structures comprise the majority of sites where active ductular expansion is occurring. Again, this may be a reflection of the relatively young age of these heifers. Capuco et al. (2002) showed that the percentage of bromodeoxyuridine-labeled epithelial cells increased progressively from the cisternal to the outer regions of the parenchyma in heifers at 3 mo of age. At young ages, it may be that TDU throughout the parenchyma are uniformly active. On the other hand, relative differences in tissue composition in the D compared with the P zones (less luminal space and more stromal tissue) do reflect the expected developmental pattern of progressively more mature tissue near the teat and cistern. This was also reflected in the significantly lower developmental score for D vs. MID+P (1.79 vs. 2.20).

CONCLUSIONS

Increasing energy and protein intake from 2 to 8 wk of age increased mammary parenchymal mass, DNA and RNA of Holstein heifer calves. Increased energy and protein intake from 8 to 14 wk of age did not increase mass of mammary parenchymal tissue or DNA concentration, but encouraged deposition of both intraparenchymal and extraparenchymal fat. We conclude that increasing protein and energy intake in Holstein heifer calves from 2 to 8 wk of age can increase the rate of development of mammary parenchyma. Whether this increase would translate into more milk production later in life is not clear, given the subsequent reduction in rate of cell proliferation in the mammary parenchyma. We suggest that early rapid growth is at least not detrimental if intraparenchymal fat content of the mammary gland is not increased.

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