Effects of Milk Replacer Composition on Measures of Mammary Development in Holstein Heifer Calves

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ABSTRACT

This study was to evaluate effects of milk replacer (MR) composition on: mass and composition of mammary parenchyma (PAR) and fat pad (MFP), growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis gene expression, and putative mammary epithelial stem cells. The hypothesis was that diet during the pre-weaning period alters the development, persistence, or activity of populations of putative mammary epithelial stem cells, possibly through involvement of GH/IGF-I axis molecules. Twenty-four newborn heifers were fed one of four MR diets: CON (20% CP, 21% fat MR fed at 441 g DM/d), HPLF (28% CP, 20% fat MR fed at 951 g DM/d), HPHF (27% CP, 28% fat MR fed at 951 g DM/d), and HPHF+ (27% CP, 28% fat MR fed at 1431 g DM/d). Animals were harvested on d 65 of life and mammary tissue was subjected to biochemical, molecular, and histological examination. By design, the effects of diet were evaluated at a common chronological age, but not necessarily at the same physiological age (body weight). Results from heifers reared on CON were compared to the average results from heifers reared on the other 3 diets. The second comparison evaluated the effect of increased fat in MR when protein content and intake were the same. The final comparison evaluated the effect of increased intake of a high-fat, high-protein MR. Neither diet composition nor nutrient intake in pre-weaned heifers affected PAR weight, PAR composition, GH/IGF-I axis gene expression, or putative mammary epithelial stem cell abundance when assessed at a common chronological age. Changes in MFP size and composition were observed, but no diet effect on GH/IGF-I axis gene expression in MFP was observed. This suggests nutrition is not critical for regulating the expression of local GH/IGF-I axis components or stem cell populations in the developing heifer mammary gland.

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as they shared in my many victories growing up, small, large, or otherwise. The only difference is that now some of them are cheering me on from above.

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Chapter 1: General Introduction

According to the most recent National Animal Health Monitoring System dairy survey, the average age at first calving in the U.S. dairy herd is 25.2 mo (USDA, 2007). This number is down slightly from 25.4 mo in 2002 (USDA, 2002) and 25.8 mo in 1996 (USDA, 1996) and is considerably lower than the "normal age at first calving in large dairy breeds" of 27-30 mo cited in Sejrsen et al. (1982) more than 25 years ago. By reducing age at first calving, dairy producers are able to shorten the period of non-productive life of their animals and therefore receive quicker returns on investments by putting milk in the bulk tank sooner. To achieve a reduced age at first calving, heifers must be bred at a younger age. This is not the whole story though; one must remember that breeding is not possible until puberty is attained. Simply breeding younger heifers will not get the job done. Given that body weight (BW) at puberty has not changed in the past 25 years and puberty is more closely associated with BW than chronological age, it follows that the reason for this national reduction in age at first calving results from management strategies designed to get heifers to a set-BW faster than before. Dairy farmers have attained this by feeding not only more nutrients, but more nutrient-dense diets to their prepubertal heifers. This management strategy however has a downside. Increased nutrient intake in prepubertal heifers has long been blamed as a cause of reduced first lactation milk yields seen in many, but not all, heifers reared on such programs. Therefore the beneficial aspects of a decreased period of non-productive life for heifers fed increased energy diets may be outweighed by the potential loss of lifetime milk production. The research described here further examines the impact of early-life nutrition on heifer mammary development.

GENERAL INTRODUCTION REFERENCES

Sejrsen, K., J. T. Huber, H. A. Tucker, and R. M. Akers. 1982. Influence of nutrition of mammary development in pre- and postpubertal heifers. J Dairy Sci 65:793-800.

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Chapter 2: Review of Literature

Importance of Nutrition with Respect to Whole Body Growth and Organ Growth

"Growth is far more than the increase in the [BW] of an animal from day to day. It involves the changes in the numerous parts of that body. These in turn are the links in the chain that determine the capacity of the body to live and to produce." Those are the words of two pioneering animal scientists, E. S. Savage and C. M. McCay, and they were written nearly 70 years ago (Savage and McCay, 1942). In their comprehensive review of calf nutrition literature, Savage and McCay (1942) highlighted the findings of almost 300 reports dating back to the 1700s, but mainly from the early 20th century. They understood the importance of rearing calves with the main goal of "producing cows with bodies of a type that will permit the optimum production of milk during a long lifetime" (Savage and McCay, 1942). They recognized that optimum lifetime performance probably was not the result of maximum rate of attainment of adult body size, which was contrary to the opinion of most nutrition workers at the time. Savage and McCay (1942) drew attention to the important work of Brody and Kibler (1941) wherein changes in organ weights in relation to BW were discussed. The subject was correctly identified as being of great importance. They recognized "…the relation of growth of parts to the growth of the whole body may be profoundly modified by different states of nutrition. The development of these parts in turn may have great influence upon the ultimate shape of the animal's body, the productive capacity of the body and the resistance of the animal to disease".

Savage and McCay understood that the total increase in BW for an animal consuming one diet may mean different internal conditions than from another animal consuming a different diet although BW increases may be identical. They were aware that "The internal structures of the body determine its productive capacity and only upon the assumption that gross BW always provides a constant internal relationship of organs and the composition of these structures would we expect to find close correlations between the increase of the whole body and of the calf and productive capacity of the cow."

Of most importance to the body of work described in the following pages is that in 1942 Savage and McCay said: "The greatest need today is for establishing the interrelationships between the diet of the calf, the rate of growth, the diet of the cow and the lifetime performance."

The passing of nearly 70 years has not changed the relevance of that statement, despite innumerable advances in the field.

Nutrition and Mammary Development

While discussing calf nutrition, BW growth, organ growth, and eventual milk production quite extensively, Savage and McCay (1942) did not make the obvious link between growth of the body and growth of the udder and its internal structures. Nor did they mention the impact of nutrition on udder growth. In the time since Savage and McCay's report, researchers have been studying such links, interestingly, without mention of the ideas of Savage and McCay. According to an online citation tracker (Web of Science; accessed 3-25-08), Savage and McCay's paper has apparently only been cited 2 times in recent years; neither citation was by lactation physiologists.

Some early pioneers in the general areas of heifer nutrition and mammary development were Herman and Ragsdale (1940s), Swanson (1950s), and Sinha and Tucker (1960s). Through their publications, these scientists and their colleagues developed an audience that was keenly interested in the effects of nutrition on heifer mammary development. As a result, many landmark papers were published in the late 1970s and this trend has carried through to the present (Little and Kay, 1979; Petitclerc et al., 1984; Sejrsen et al., 1982). Past research has for the most part, dealt with post-weaned prepubertal and pubertal heifers. In the late 1990s, research focus shifted to pre-weaned heifers due in part to the interest of many calf milk replacer (**MR**) manufacturers. The previously neglected research area has proven to be hot topic in the industry and much effort has been placed on MR formulation and its effects on calf body growth and on mammary growth.

Today it is known that overfeeding heifers during the allometric period of mammary gland growth (roughly defined as ~2 to 11 mo of age or ~90 to 280 kg BW) can decrease mammary development and first lactation milk yields (Little and Kay, 1979; Radcliff et al., 2000). In contrast, Brown et al. (2005) demonstrated that increased energy and protein intake associated with accelerated calf growth programs increased growth of mammary parenchyma (**PAR**) in calves from 2 to 8 wk of age. These differences did not hold up when heifers were evaluated at 14 wk of age (after weaning), which suggests that the calf is more sensitive to nutrient intake prior to weaning and that the enhancement of mammary development is not maintained after the animal is weaned. The study by Brown et al. (2005) is one of only a handful of studies to evaluate the effects of elevated nutrient intake in pre-weaned heifers. Their results

were quite surprising and have strengthened the notion that a so-called "critical period" for mammary development exists, where PAR is sensitive and responsive to nutrient intake. Further support of this idea comes from a recent serial slaughter study by Meyer et al. (2006a). It was found that when assessed at a common BW of 100 kg, mammary epithelial cell proliferation was 44% higher in heifers fed elevated nutrient intake, as opposed to heifers fed restricted intake. This difference was lost by 150 kg of BW. Meyer et al. (2006a) postulated that an elevated nutrient intake increased epithelial cell proliferation which may have resulted from increased proliferation of mammary stem cells, or their daughters, or both.

Characterization of Mammary Tissue from Pre-weaned Heifers

At birth (~45 kg for Holsteins) PAR is present in negligible quantities, mammary fat pad (**MFP**) is barely palpable, and the udder is close in proximity to the body wall. Meyer et al. (2006b) most recently characterized mammary tissue at this stage and noted: "At 46 kg of BW, [PAR] consisted only of a threadlike mass that extended dorsally above each teat. This structure was not easily excised, so [for analysis] it was left with the MFP and assumed to have a negligible impact on MFP weight and DNA content." In contrast, by 8 wk of age (~90 kg BW) it is not uncommon to palpate a walnut-sized mass of PAR that weighs ~10 g, in addition to a grapefruit-sized mass of MFP that weighs in excess of 90 g (Akers, unpublished data, 2004). So, in a period of time where BW only doubles, growth of PAR far exceeds that, despite little change in outward appearance. In fact, we recently noted a 60-fold increase in PAR weight from birth to 3 mo of age (Akers, unpublished data, 2004). This observation tells us that despite not being fully functional until roughly 24 mo of age, the mammary glands of heifer calves are far from quiescent. These findings, coupled with the wisdom of Savage and McCay (1942) regarding growth of body parts compared to the growth of the whole body, call for further study into the role of nutrition on early-life mammary growth and development.

Physiological vs. Chronological Age in Studies of Mammary Development

As pointed out recently by Meyer et al. (2006a, 2006b), elevated energy intake does not directly impair mammary growth. Decreased PAR weight seen in heifers reared on an elevated energy intake is often just an age effect. This is because many studies are designed to analyze mammary tissue when heifers fed divergent diets reach a common BW. With this design, heifers reared on elevated nutrient intakes are younger than their cohorts at time of evaluation. For

instance, in the study by Meyer et al. (2006b), PAR weight and DNA were lower in heifers fed elevated nutrient intake when compared with heifers fed a restricted energy diet. However, when analyzed with age as a covariate term, diet was no longer a significant term in the model. Furthermore, level of nutrient intake had no effect on lipid or protein composition of PAR. These data collectively demonstrate that PAR is refractory to level of nutrient intake whereas MFP is not.

Brown et al. (2005) showed increased PAR in heifers fed elevated nutrient intake from 2 to 8 wk of age when evaluated at 8 wk (uncommon BW). This seems contradictory to the arguments presented in the previous paragraph, but may not be so. In the Brown et al. (2005) study "age" was arbitrary, because birthdates were not known. Therefore the diet effect could have been confounded with age. Further research is needed to resolve this debate.

Impact of GH/IGF-I Axis Molecules in the Mammary Gland

Nutritive status of the animal affects circulating concentrations of growth hormone (**GH**) and insulin-like growth factor-I (**IGF-I**). These two hormones are implicated in affecting mammary development of the animal. The exact mechanisms of how systemic and locally produced hormones work in concert to increase or decrease rates of mammary development are not fully understood.

GH is essential for pubertal mammary development, as is estrogen. Circulating IGF-I and growth of PAR are both increased by the administration of exogenous GH (Purup et al., 1993) and GH treatment in rats stimulated mammary synthesis of IGF-I (Kleinberg et al., 1990). GH is affected by level of nutrient intake; a "high" nutrient intake will reduce circulating GH concentrations in heifers when compared to a "low" nutrient intake. Based on the above observations, it was postulated that reduced GH concentration in blood might be the basis for impaired mammary development in heifers fed high nutrient intake. The next step in confirming this hypothesis was to determine if the bovine mammary gland has GH receptors (**GH-R**). While the existence of GH-R in PAR is still a topic of debate, the existence of GH-R in MFP is not. GH-R in MFP alone necessitates the indirect action of GH on PAR, which goes along with the somatomedin hypothesis (Salmon and Daughaday, 1957). Indirect actions of GH on PAR are well documented. IGF-I mRNA can be found in MFP (Forsyth et al., 1999), IGF-I receptors (**IGF-IR**) are present in PAR (Forsyth et al., 1999; Purup et al., 1993), IGF-I (but not GH) is stimulatory to bovine epithelial cells (Weber et al., 1999), and the mitogenic effect of serum on

mammary cells in vitro is closely related to the concentration of IGF-I in serum (Weber et al., 2000).

The major site of IGF-I mRNA synthesis is in the liver and there is additional synthesis by peripheral tissues, including the mammary gland (**MG**). The physiological actions of IGF-I are regulated by binding proteins (**IGFBP**), which control the availability of IGF-I to target tissues or cells (Thissen et al., 1994). The effects of IGF-I on DNA synthesis in cultured mammary epithelial cells suggest that local mammary synthesis of IGF-I can modulate growth in vivo.

The above findings concerning IGF-I cannot explain the negative effect of feeding level on mammary development, however, because the concentration of IGF-I in blood is paradoxically increased in animals fed high nutrient intake. The latest explanation is that a change in sensitivity of the mammary tissue to endocrine effects of IGF-I must be present. Weber and others (2000) found that mammary tissue from heifers on a high feeding level responded less to IGF-I in vitro than tissues from moderate-fed heifers, but there were no differences in IGF-IR number or IGF-IR mRNA expression. They suggested that there was an inhibition of IGF-I at the post-receptor level. As it turned out, mammary tissue from high-fed heifers had more locally produced IGFBP-3 than the moderate-fed heifers and the addition of IGFBP-3 to culture inhibited the effect of added IGF-I and mammary gland extracts. Taken together, all of these results suggest that the negative effect of feeding level on mammary growth during the prepubertal period is caused, at least in part, by increased local IGFBP-3 production. Further characterization of the GH/IGF-I axis in pre-weaned animals, especially as affected by nutrition, is therefore warranted.

Impact of Diet on Mammary Stem Cells

Many researchers believe that in early postnatal life, a critical period for mammary development exists where PAR is responsive to elevated nutrient intake. Little is known about this so-called “critical period” and the effect of nutrition on PAR during this stage remains to be fully elucidated. Positive effects of faster rates of gain during the pre-weaning period may alter the development, persistence, or activity of populations of putative mammary epithelial stem cells (very likely via actions initiated or mediated by GH/IGF-I axis molecules) and thereby impact subsequent PAR development.

In support of this idea, mammary epithelial stem cells were suggested as having a participatory role in this phase of postnatal growth (Meyer et al., 2006a). The suggestion came after data from serially slaughtered heifers ranging in BW from 100 to 350 kg and fed either a restricted or elevated nutrient intake were evaluated for divergent rates of mammary epithelial cell proliferation. Meyer et al. (2006a) found that when assessed at a common BW of 100 kg, mammary epithelial cell proliferation was 44% higher in heifers fed elevated nutrient intake, as opposed to heifers fed restricted intake. This difference was not apparent in heifers slaughtered at 150 kg of BW. Meyer et al. (2006a) postulated that an elevated nutrient intake increased epithelial cell proliferation which may have resulted from increased proliferation of mammary stem cells, or their daughters, or both. They noted that in some model organisms, stem cells and their more differentiated daughter cells modulate their proliferation according to nutritional status (Drummond-Barbosa and Spradling, 2001). While no mention of mammary stem cells appeared in the manuscript of Brown et al. (2005), the evidence presented demonstrated that increased energy and protein intake associated with accelerated calf growth programs increased PAR growth in heifers from 2 to 8 wk of age. This growth may have been influenced by stem cells. When the data of Meyer et al. (2006a) Brown et al. (2005) are considered together, the possibility of stem cells being modulated by nutrition seems plausible and demands further study.

Studying Mammary Stem Cells

In mice, one stem cell is enough to completely repopulate an epithelium-cleared MFP (Kordon and Smith, 1998; Stingl et al., 2006). In the bovine, redevelopment of the MG during successive cycles of lactation suggests that a stem cell population exists within PAR. Ellis and Capuco (2002) have even identified and characterized a primary proliferative cell population. So, while the existence of mammary stem cells is not in question, the study of them remains a challenge and is still in its infancy. Markers for differentiated cells are identified and abundant; markers for stem cells are more elusive and there are no known markers unique to all stem cells, let alone mammary stem cells. Despite this, progress has been made in the study of bovine mammary stem cells. Most recently, use of a heritable marker was used for such work (Capuco, 2007) with success.

Remaining Questions

After review of literature, there are several remaining questions about early-life nutrition and its potential influence on heifer mammary development. Chief among these are:

1. Do diet composition and intake affect protein and lipid partitioning in MFP, as suggested by Meyer et al. (2006b)?
2. Is PAR refractory to the level of nutrient intake and MFP not?
3. Is age at harvest, not level of nutrient intake, the single greatest determinant of total PAR DNA content?
4. Do PAR and MFP expression of GH/IGF-I axis genes differ when heifers are fed different diets? If so, are these differences related to mammary development?
5. Is mammary epithelial stem cell activity modulated by diet in young heifers?

Hypothesis

Our working hypothesis is that the positive effects of faster rates of gain during the pre-weaning period alter the development, persistence, or activity of populations of putative mammary epithelial stem cells, possibly through involvement of GH/IGF-I axis molecules. This could thereby impact subsequent mammary development.

Research Synopsis

To address the questions listed above, an experiment was conducted wherein similarly-aged pre-weaned Holstein heifers were fed different diets to achieve various BW gains. Four MR diets were used to make 3 preplanned diet comparisons at the end of the experiment. Heifers were sacrificed at a common age and mammary tissue was subjected to biochemical, molecular, and histological examination. By design, the effects of diet were evaluated at a common chronological age, but not necessarily at the same physiological age (body weight). For the first diet comparison, results from heifers reared on a control MR, were compared to the average results from heifers reared on the other 3 diets. The second comparison allowed for the evaluation of the effect of increased fat in MR when protein content and intake were kept the same. The third comparison allowed for evaluation of the effect of increased intake of a high-fat, high-protein MR.

Rationale and Significance

The experiments outlined in this dissertation will better define the relationship between protein and energy content of MR and the development of PAR and MFP. Specific focus will be on nutritional modulation of GH/IGF-I axis molecules in PAR and MFP. An advantage of the design is that since animals will be evaluated at a common age, any differences seen in development of PAR and MFP can be attributed to diet and not to chronological age differences, as suggested by Meyer et al. (2006a, 2006b). Also, supposing mammary stem cell activity can be manipulated by diet in young animals, there is potential for this work to have a direct impact on the way heifers are managed in the US by influencing productivity (through a shortened period of non-productive life) and lactation efficiency (through increased numbers and activity of mammary secretory cells).

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Chapter 3: Effects of Milk Replacer Composition on Measures of Mammary Development in Holstein Heifers

ABSTRACT

Overfeeding prepubertal heifers may impair mammary parenchymal growth and reduce milk production, but dietary impacts in pre-weaned heifers are largely unknown. This study was to evaluate effects of milk replacer (MR) composition on: mass and composition of mammary parenchyma and fat pad (MFP), growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis gene expression, and putative mammary epithelial stem cells. The hypothesis was that diet during the pre-weaning period alters the development, persistence, or activity of populations of putative mammary epithelial stem cells, possibly through involvement of GH/IGF-I axis molecules. Twenty-four newborn heifers were fed one of four MR diets (n=6/diet): CON (20% CP, 21% fat MR fed at 441 g DM/d), HPLF (28% CP, 20% fat MR fed at 951 g DM/d), HPHF (27% CP, 28% fat MR fed at 951 g DM/d), and HPHF+ (27% CP, 28% fat MR fed at 1431 g DM/d). Water and starter (20% CP, 1.43% fat) were offered ad libitum. Animals were sacrificed on ~ d 65 of age and mammary tissue was subjected to biochemical, molecular, and histological examination. Diet had no effect on mammary parenchyma, but mass and composition of MFP were directly affected by increased nutrient intake. Neither GH/IGF-I axis gene expression nor putative mammary epithelial stem cell abundance were affected by diet when heifers were assessed at a common chronological age. We conclude that diet is not important for regulating the expression of local GH/IGF-I axis components or stem cell populations in the developing heifer mammary gland. Implications with respect to future milk production remain to be determined.

INTRODUCTION

Many researchers believe that in early postnatal life, a critical period for mammary development exists where parenchyma (**PAR**) is responsive to elevated nutrient intake. Little is known about this so-called "critical period" and the effect of nutrition on PAR during this stage remains to be fully elucidated. Traditionally, elevated prepubertal nutrient intake has been implicated in impaired development of PAR (Capuco et al., 1995; Sejrsen et al., 1982). However, recent studies with young heifers suggest a beneficial effect of elevated nutrient intake on PAR (Brown et al., 2005; Meyer et al., 2006a; b). Our goal was to see if such a beneficial role of

nutrition existed when similarly aged heifers were fed different diets and mammary tissue evaluated when heifers were the same age.

Growth hormone (**GH**), IGF-I, their receptors, and IGF binding proteins (**IGFBP**) are components of the GH/IGF-I axis. In some instances, components of this axis have been shown to be affected by nutrition in heifers and have been implicated in inhibited mammary growth in heifers fed elevated nutrient intake (Weber et al., 1999; Weber et al., 2000; Berry et al., 2003c;). In other cases, specific components of the GH/IGF-I axis were not affected by level of nutrient intake (Meyer et al., 2007), nor was development of PAR when age was used as a covariate in the statistical model to analyze the data (Meyer et al., 2006b). These conflicting results demonstrate the need for further study into the role of the GH/IGF-I axis in the bovine mammary gland (**MG**) in both PAR and mammary fat pad (**MFP**). Therefore, another objective was to characterize components of the GH/IGF-I axis in PAR and MFP of young heifers fed different diets.

In addition to the GH/IGF-I axis receiving much research attention, mammary epithelial stem cells may have a participatory role in this so-called critical period of mammary growth (Meyer et al., 2006a). When assessed at a common BW of 100 kg, Meyer et al. (2006a) found that mammary epithelial cell proliferation was 44% higher in heifers fed elevated nutrient intake, as opposed to heifers fed restricted intake. This effect was not observed in heifers slaughtered at 150 kg of BW. Meyer et al. (2006a) postulated that an elevated nutrient intake increased epithelial cell proliferation which may have resulted from increased proliferation of mammary stem cells or their daughters, or both. While no mention of mammary stem cells appeared in the manuscript of Brown et al. (2005), the evidence presented demonstrated that increased energy and protein intake associated with accelerated calf growth programs increased PAR growth in heifers from 2 to 8 wk of age. This growth may have been influenced by stem cells. Further support for the idea that stem cells can be modulated by nutrition come from Drummond-Barbosa and Spradling (2001), who noted that in some model organisms stem cells and their more differentiated daughter cells modulate their proliferation according to nutritional status. When the data of Meyer et al. (2006a) and Brown et al. (2005) are considered together, the possibility of nutritional regulation of stem cell activity seems plausible. Our third main objective therefore was to see if mammary epithelial stem cell activity could be modulated by diet in young heifers.

Our working hypothesis was that the positive effects of faster rates of gain during the pre-weaning period alter the development, persistence, or activity of populations of putative mammary epithelial stem cells, possibly through involvement of GH/IGF-I axis molecules, and thereby impact subsequent mammary development. To address the objectives listed above, an experiment was conducted wherein similarly-aged pre-weaned Holstein heifers were fed different diets to achieve various BW gains. Four milk replacer (**MR**) diets were used to make three diet comparisons at the end of the experiment. Heifers were sacrificed at a common age and mammary tissue was subjected to biochemical, molecular, and histological examination. By design, the effects of diet were evaluated at a common chronological age, but not necessarily at the same physiological age (body weight). Results of diet effects on body growth rate and carcass composition and blood metabolite and hormone concentrations are reported elsewhere (Hill et al., in press and Daniels et al., in press; Appendix A).

MATERIALS AND METHODS

Animals and Treatments

Heifers were managed and fed according to Daniels et al. (in press; Appendix A). Briefly, 24 newborn heifers were fed one of four MR diets (n=6/diet): CON (20% CP, 21% fat MR fed at 441 g DM /d), HPLF (28% CP, 20% fat MR fed at 951 g DM/d), HPHF (27% CP, 28% fat MR fed at 951 g DM/d), and HPHF+ (27% CP, 28% fat MR fed at 1431 g DM/d). Heifers were fed twice daily; water and starter (20% CP, 1.43% fat) were offered free choice. Diets are further characterized in Table 1. At 1 mo of age (32 d ± 1; mean ± standard deviation) heifers were injected intravenously with 5 mg BrdU per kg BW daily for 4 d. The BrdU (Sigma Chemical Co., St. Louis, MO) solution was made in sterile 0.9% NaCl and contained 20 mg BrdU/ml (pH 8.5). One heifer (group 1; HPLF) died unexpectedly at 6 wk of age from acute peritonitis and endotoxemia and was not replaced; data from that animal were excluded.

Mammary Tissue Sampling

Heifers were harvested at 65 d ± 1 (mean ± standard deviation) to evaluate body composition (Hill et al., in press; Appendix A) and mammary development. At the time of slaughter, it was noted that one heifer (group 3; CON) had an irregularly shaped reproductive tract and underdeveloped mammary tissue. This heifer was suspected to be a freemartin; her mammary data were excluded. Heifers underwent a 12 h fast before harvest, were weighed and

then transported to the necropsy lab of the Virginia - Maryland Regional College of Veterinary Medicine for processing the morning of harvest. Heifers were euthanized by phenobarbitol injection (Euthasol, 10 mg/kg BW) and immediately exsanguinated. Udders were removed. The whole udder was weighed and bisected along the median suspensory ligament. The right hemiudder was re-weighed, wrapped in foil, and submerged in liquid nitrogen. Later that day, samples were moved to a -80ºC freezer and stored for later compositional analysis. The left rear quarter was sampled for later RNA analysis (described later) and the left front quarter was sampled for histology (described later).

Right Hemiudder Analysis

Mammary Tissue Dissection. Right hemiudders were removed from the freezer, re-weighed, allowed to partially thaw and were dissected into one of four fractions. These fractions were hide/teats, lymph node, PAR, and MFP; the weight of each fraction was recorded. The hide/teats and lymph node fractions were discarded. At this time, PAR and MFP subsamples were obtained, placed in cryovials, and submerged in liquid nitrogen. Cryovials were shipped to the University of Illinois for microarray analysis of gene expression (Piantoni et al., unpublished data; Appendix A). Remaining tissue portions were refrozen for later analysis.

Biochemical Analyses of PAR and MFP. Dissected PAR and MFP fractions were removed from the freezer, re-weighed, and pulverized to a powder in a freezer mill (6850 Freezer Mill; Spex Sample Prep, Metuchen, NJ). The remaining powder was subsampled and used in separate assays for determination of lipid, protein, and DNA content.

Lipid content was determined gravimetrically according to the method of Hara and Radin (1978). Briefly, 9 ml of hexane:isopropanol (3:2; vol/vol) were added to 500 mg of tissue powder. Butylated hydroxytoluene (Sigma Chemical Company; St. Louis, MO) was added to the hexane:isopropanol mixture at 0.005% and served as an anti-oxidant. Tubes were vortexed and 6 ml of aqueous sodium sulfate (1 g anhydrous salt per 15 ml H_2O) were then added. The suspension was vortexed, phases were allowed to separate, and tubes were vortexed again. Tubes were then spun at 1000 x *g* for 5 min. The upper lipid-rich solvent layer was transferred to a clean pre-weighed tube. Residual lipids were resuspended by adding 5 ml of the hexane:isopropanol mixture to original tubes. Tubes were vortexed then spun at 1000 x *g* for 5 min; the upper solvent layer was removed and decanted into the proper pre-weighed tube. Solvent was evaporated under nitrogen gas at 40°C on an analytical evaporator (N-EVAP model

#112; Organomation Associates, Inc., South Berlin, MA). Tubes were re-weighed after evaporation of solvent and solidification of lipid residue. Lipid mass was calculated as the final tube weight minus the initial tube weight. Samples were run in duplicate in one assay; the intrassay CV averaged 3.95%.

Protein and DNA content of PAR and MFP were determined after homogenization of each fraction in a high-salt buffer. Briefly, 250 mg of tissue were weighed and homogenized (PRO200 Homogenizer; PRO Scientific, Oxford, CT) in 1.5 ml buffer (0.05 *M* Na_2HPO_4 + 2 *M* NaCl + 0.002 *M* Na_2EDTA). Homogenates were spun for 5 min at 1000 x *g* at 4°C to clear samples of lipid and residual connective tissue; the soluble fraction was removed with a Pasteur pipette and transferred to a clean microfuge tube. For the DNA assay, 2 µl of each homogenate fraction were added to 2 ml of assay solution. Assay solution contained 100 µl of 1 mg/ml Hoechst H 33258, 10 ml of 2 *M* NaCl + 100 m*M* Tris + 10 m*M* Na_2EDTA, pH 7.4 buffer, and 90 ml H_2O. DNA was quantified against calf thymus DNA (Sigma Chemical Company; St. Louis, MO); samples were run in triplicate on a Hoefer DQ 300 fluorometer (Hoefer, Inc.; San Francisco, CA) set on the UV fluorescence channel. Intrassay CV averaged 4.90%. Protein in PAR and MFP homogenates was determined via the bicinchoninic acid assay (Pierce; Rockford, IL); BSA was used as the standard. Intrassay CV averaged 2.63% for the protein assay.

For all composition assays, obtained values were multiplied by two to determine total lipid, DNA, and protein content of the udder. Parenchyma and MFP weights are expressed on a raw- and empty body weight (**EBW**) basis. All calculations were made prior to statistical analysis.

Left Rear Quarter Analysis

RNA Isolation and Quantitative Reverse Transcription-PCR Conditions. At slaughter, samples of PAR and MFP from left rear quarters were excised, snap frozen in liquid nitrogen, and stored at -80^{o}C until RNA was isolated. RNA isolation and quantitative reverse transcription-PCR (qPCR) were carried out according to Velayudhan et al. (unpublished data; Appendix A), with slight modifications. Briefly, total RNA was isolated from samples using TRIZOL reagent (Life Technologies; Grand Island, NY) and re-suspended in RNase/DNase–free water. Purity and quantity of RNA was determined using a Nanodrop ND-1000

Spectrophotometer (Nanodrop Technologies Inc.; Wilmington, DE) and integrity was evaluated by denaturing agarose gel electrophoresis. RNA yield data are reported in

Figure 1and were similar to those reported by Piantoni et al. (in press) for PAR and MFP. Single stranded cDNA were reverse transcribed from total RNA by using the High Capacity cDNA Archive kit (Applied Biosystems; Foster City, CA) according to manufacturer's instructions. First, 20 μg of total RNA were reverse transcribed to single stranded cDNA in a final reaction volume of 20 μl using random primers. The cDNA produced was then diluted 15-fold with RNase/DNase-free water. For qPCR reactions, 2 μl of diluted cDNA were used, along with 12.5 μl of SYBR Green dye (Applied Biosystems; Foster City, CA), 9.5 μl of RNase/DNase-free water, 0.5 μl each of 10 μ*M* forward and reverse primers (described below). PCR conditions were: 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. Melting curve analysis suggested the presence of a single product for each qPCR assay. To confirm the presence of a single product, PCR amplicons were analyzed by agarose gel electrophoresis. All qPCR reactions were performed in a 7300 Series Real-Time System and data were analyzed using SDS software (Applied Biosystems; Foster City, CA).

Primers and Data Normalization. In a recent study that utilized mammary tissue from these heifers (Piantoni et al., in press), it was concluded that the geometric average of three housekeeping genes (**HKG**) is ideal for normalization of qPCR data obtained from prepubertal bovine mammary tissue. Specifically, PPP1R11 (plays a role in protein phosphatase-1 inhibition), RPS15A (a component of the 40S ribosomal subunit), and MTG1 (a GTPase), were deemed the most suitable HKG for normalization due to their uniformity in expression between diets and tissue fractions (Piantoni et al., in press). So, for relative quantification of target gene mRNA, the geometric mean of HKG cycles to threshold (**Ct**) values was used to normalize gene expression data. More specifically, relative mRNA abundance (**ΔCt**) of IGF-I, IGF-IR, IGFBP 1-6, and GH-R for each heifer was determined by subtracting the Ct value for the geometric mean of the 3 HKG genes from the target gene Ct (ΔCt method). In qPCR experiments, lower (more negative) ΔCt values correspond to increased mRNA abundance and higher (more positive) ΔCt values correspond to decreased mRNA abundance. Here, qPCR data are presented as $2^{(-\Delta Ct)}$ values. When presented in this manner, larger numbers equate to more mRNA and smaller numbers equate to lesser mRNA. In addition, $2^{(-\Delta Ct)}$ values represent fold increases in expression

relative to the geometric mean of HKG. Furthermore, fold changes in gene expression can also be calculated by comparison of two or more $2^{(-\Delta Ct)}$ values within a gene. If the reader wishes to see ΔCt data, please refer to Appendix B. All primers were ordered from Integrated DNA Technologies (Coralville, IA) and were diluted to 10 μ*M* with RNase/DNase-free water before use in qPCR. Primer sequences used for each gene are shown in Table 2. Primer efficiencies for HKG and each target gene were determined using five dilutions of PAR and MFP cDNA in triplicate according to the equation: % efficiency = $(10^{-1/slope} - 1) \times 100$. Primer efficiencies were approximately equal for target genes and HKG and averaged 98%. Unacceptable primer efficiencies resulted when the IGFBP-6 primers described in Velayudhan et al. (unpublished data) were used, so new IGFBP-6 primers were developed for this experiment using Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Other primer sequences have been described previously (Velayudhan et al., unpublished data; Piantoni et al. unpublished data).

Telomerase Assay. Snap frozen PAR was also used to determine telomerase activity. Telomerase is an enzyme that synthesizes telomeres on chromosome ends; stem cells of renewing tissues express very low levels of telomerase, whereas most other cell types do not express telomerase. Telomerase thus appears to grant stem cells immortality by maintaining telomere length so that the cell never receives a signal to stop dividing. For the assay, a qPCR-based Telomerase Assay Kit (US Biomax, Rockville, MD) was used according to the manufacturer's instructions. Briefly, for each heifer, 40 to 100 mg PAR were homogenized for 20 s in the provided 1x lysis buffer. Samples were incubated on ice for 30 min and then centrifuged for 30 min at 12,000 x *g* at 4ºC. Supernatant was removed and aliquoted. One aliquot was used for protein determination by the BCA assay (described above) and one aliquot was diluted 1:200 with 1x lysis buffer and kept at 4ºC until further processing. A portion of each diluted sample was removed and placed in a new tube, which underwent heat inactivation (85ºC, 10 min). Heat inactivated samples served as negative controls in the assay. Snap frozen bull testes served as an additional positive control in the assay. A standard curve was prepared with eight, 1:5 serial dilutions of the provided control template oligonucleotide (0.5 amoles/μL); a no template control was also used. Master Mix was prepared by mixing 12.5 μL of the provided 2x Quantitative Telomerase Detection Premix with 11.5 μL of PCR Qualified H_2O. For the assay, 1 μL of tissue extract (or standard) was added to 24 μL master mix. Samples were run in duplicate in a 96-well plate. PCR conditions were: 25ºC for 25 min, 95ºC for 10 min, followed by 3-step

cycling (40 cycles) of 30 s at 95ºC, 30 s at 60ºC, and 30 s at 72ºC. A melting curve analysis was included at the end of the assay and yielded a single PCR product that dissociated at approximately 76ºC (data not shown). Telomerase abundance was quantified against a standard curve that had an R^2 of 0.97. For each sample, activity of the heat inactivated aliquot was subtracted prior to statistical analysis and data are presented as amol telomerase per μg PAR protein.

Left Front Quarter Analysis

Microscope Slide Preparation. At slaughter, left front quarters of each udder were "butterflied" open and PAR (~4 mm^2) from 6 regions within the quarter (2 cisternal, 2 middle, and 2 peripheral; Figure 2) were excised and placed into vials of 10% formalin. Tissues were allowed to fix overnight; formalin solution was then removed and replaced with 70% ethanol. Tissues remained in 70% ethanol until embedding in paraffin. At least 2 tissue blocks were prepared for each region; in most cases only one block per region was processed further. Microscope slides were prepared by slicing 5 μm thick sections from the paraffin-embedded tissue blocks with a microtome (Reichert-Jung Model 2040 Autocut; West Germany). Four or 5 serial tissue sections from each sample were mounted onto a Superfrost Plus slide (Fisher Scientific, cat # 12-550-15). Approximately 5 microscope slides were prepared from each tissue block.

BrdU and Ki67 Single Labeling. While not described further here, a BrdU and Ki67 single labeling (brightfield detection) experiment was completed. The decision was made not to include the results in the main body of work because they were slightly redundant. However, results helped determine that processing all six PAR regions was not necessary for fluorescent microscopy; hence only 4 sampling regions are further described below. Details of the BrdU and Ki67 Single Labeling experiment are described in Appendix C.

BrdU-Ki67 Dual Labeling. One microscope slide per region (D, F, G, I; Figure 2) per heifer was subjected to dual immunofluorescent labeling of BrdU and Ki67. The labeling method was carried out essentially according to Capuco (2007) and was designed to identify mammary epithelial stem cells by their retention of labeled DNA strands. Microscope slides were de-paraffinized in xylene (3 x 5 min) and hydrated by passage through a series of graded ethanol washes. Ethanol washes were: 100% (2 x 3 min), 95% (2 x 3 min), and 70% (1 x 3 min). The final step in hydration consisted of soaking slides in distilled water (2 x 2 min). Antigen sites

were retrieved by boiling slides in 500 ml of 10 m*M* citrate buffer (pH 6.0) continuously for 15 min. Slides were allowed to cool completely (~30 min), and were then washed in PBS (pH 7.4; 3 x 2 min). Residual PBS was aspirated with a vacuum and slides were blocked with CAS Block (1-2 drops CAS Block/section; Invitrogen, cat # 00-8120). In the case of positive/reagent control slides (mammary tissue from a BrdU pulse-labeled heifer), individual tissue sections were circled with a PAP barrier pen (Fisher Scientific; cat # NC9720458) before blocking to prevent co-mingling of antibodies in subsequent steps. All slides were incubated for 30 min. The CAS Block was aspirated and 50 µl of combined primary antibody solution were added per section. The primary antibody solution consisted of a mixture of Ki67 rabbit mAb (1:200; clone SP6, Fisher Scientific, cat # RM-9106-SO) and BrdU mouse mAb (1:66.7; clone BMC-9318, Fisher Scientific, cat # MAB3424), diluted in CAS Block. Reagent controls received 50 µl CAS Block instead of primary antibody mixture. Slides were incubated overnight at 4ºC. The next morning, slides were aspirated and washed in PBS (3 x 5 min). Residual PBS was aspirated and 50 µl of combined secondary antibody solution were added per section. The combined secondary antibody solution consisted of 15 µl of Alexa 488 goat anti-rabbit IgG (Invitrogen, cat # A11008) and 15 µl of Alexa 594 goat-anti mouse IgG (Invitrogen, cat # A11005) were added to 3 ml of CAS Block (final dilution of antibodies, 1:200). Prior to use, the secondary antibody mixture was spun at 10,000 x *g* for 10 min to remove aggregates. Slides were incubated with secondary antibody for 60 min in the dark. Afterward, PAP Pen residue was removed from positive/reagent control slides with a xylene soaked cotton swab. All slides were then washed in PBS (3 x 2 min, in the dark, on a rocker platform). Next, individual slides were rinsed by dipping in distilled water; residual water was aspirated and 1-3 drops of Prolong Gold antifade reagent with DAPI (Invitrogen, cat # P36935) was added to each slide. A glass coverslip was then added and slides were allowed to cure 24 h in the dark before viewing by fluorescence microscopy.

Microscopy and Imaging. Slides were visualized using a Nikon Eclipse E600 microscope (Nikon Instruments, Inc.; Melville, NY) fitted with an epifluorescence attachment and photographed with an Olympus QColor 3 digital camera (Olympus America, Inc.; Center Valley, PA). The UV-2E/C DAPI, FITC, and G-2A filter blocks were used for visualization of DAPI, Ki67, and BrdU, respectively. Thirty digital images were obtained per slide; these consisted of 10 sets of BrdU, Ki67, and DAPI images. Each set represented an independent area composed primarily of mammary epithelium. All images obtained were 12 bit monochrome

images taken at 40x magnification. Exposure lengths were 5 to 30, 100, and 100 ms, for DAPI, BrdU, and Ki67, respectively.

BrdU and Ki67 Quantification. Images were analyzed using Image Pro Plus software (version 6.2; Media Cybernetics, Silver Spring, MD). BrdU and Ki67 positive cells, as well as dual-labeled cells, were counted manually and total number of epithelial cells per image determined using image analysis software. Labeling indexes for BrdU and Ki67 were established by dividing the number of positive cells counted per image by the calculated total number of mammary epithelial cells in that image. BrdU positive cells were further categorized as being "heavily stained" or "lightly stained" (

Figure 3). The heavily stained BrdU cells were further characterized by presence or absence of Ki67 stain. Detailed methods for BrdU and Ki67 visualization and quantification are located in Appendices D, E, and F. Total mammary epithelial cells counted did not differ by diet or region (data not shown) and averaged 1680 ± 361 (mean ± standard deviation) cells.

Statistical Analysis

All data were analyzed using the Mixed Procedure of SAS (Version 9.1.3; Cary, NC). Mammary composition and RNA yield data were evaluated with a model that included the main effects of diet and group as well as their interaction. Heifer within diet and group was the random term; no denominator degrees of freedom approximation method was specified, defaulting to Between-Within.

Initial BW was included as a covariate in analysis of BW data; it was not significant (P = 0.760) so it was removed from the final analysis model.

For qPCR data, data submitted to SAS consisted of $2^{(-\Delta Ct)}$ values. Normality of each gene, diet, and tissue fraction combination was checked using the Shapiro-Wilk test in the univariate procedure of SAS (see Appendix B). For this test, the null hypothesis that the data were from a random sample from a normal distribution was rejected when $P < 0.05$. Since some gene, diet, and tissue fraction combinations appeared to be non-normally distributed, and data were not transformed, a more conservative α was selected for analysis of variance; differences were declared significant at $P < 0.01$.

Additionally, prior to analysis of variance, heterogeneity of variance for each gene between diets was inspected with a Hartley F-max test; variances were deemed homogenous so no transformations were made. Four different model statements were considered for these data.

These included a repeated measures analysis and a model that included heifer within diet and group as a random effect, both with and without Satterthwaite's denominator degrees of freedom approximation method. All models yielded the same results (Appendix G), possibly because there were only two fractions. The repeated measures analyses were rejected in favor of a model that included heifer within diet and group as a random effect with no denominator degrees of freedom approximation method. The repeated measure in the rejected model was tissue fraction (PAR and MFP, which were repeated in space in the animal). The $2^{(-\Delta Ct)}$ values for each target gene were tested against a model that included the main effects of diet, group, tissue fraction, as well as all two-way interactions; the three-way interaction was considered, but removed from the final model.

Telomerase assay data were analyzed with a model that included the main effects of diet, group, and their interaction. Heifer within diet and group was used as the random term in the model. Heat inactivation values were used as a covariate.

Stem cell data analysis included region as the repeated measure and the subject used in tests was heifer within diet and group. An autoregressive covariance structure (AR(1)) was used; no denominator degrees of freedom approximation method was specified. Model terms included main effects of diet, group, and region, as well as all two-way interactions; the three-way interaction was considered, but removed from the final model. Regional differences, if present were further examined with a contrast statement that examined peripheral vs. cisternal regions.

All data are reported as least squares means ± standard error of the means; differences were declared significant at $P \leq 0.05$ unless otherwise noted. Non-orthogonal pre-planned contrast statements were used to examine the main effect of diet, if significant. These were: CON vs. all other treatments, HPLF vs. HPHF, and HPHF vs. HPHF+.

RESULTS

Body Growth

Growth data are summarized in **Table 3**. Age and BW at initiation of dietary treatments were not different between diets and averaged 3.5 ± 0.39 d and 40.2 ± 1.93 kg, respectively. Daily BW gains for heifers on each diet averaged 0.47, 0.70, 0.66, and 0.85 kg/d for CON, HPLF, HPHF, and HPHF+, respectively ($P = 0.002$) and are discussed in further detail in Hill et al. (unpublished data). As intended, diet had no effect on age at harvest; average age at harvest

was 64.6 ± 0.38 d. In contrast, BW at harvest was affected by diet ($P = 0.001$). Heifers fed CON weighed less than the average of the other three diets. Heifers fed HPLF weighed more than those fed HPHF at the end of the experiment, despite ADG being statistically equivalent. Heifers fed HPHF+ gained nearly 1.3 times more weight daily than did heifers fed HPHF, and as a result weighed more than HPHF at the end of the experiment (91.6 ± 2.66 kg vs. 75.9 ± 2.66 kg for HPHF+ and HPHF, respectively).

Proportion of Mammary Gland Occupied by PAR and MFP

		Diet Contrasts, *P*		
Item	Diet Effect, *P*	CON vs. the rest	HPLF vs. HPHF	HPHF vs. HPHF+
Total MG, g	0.001	0.006	0.202	0.010
Total MG, g/100 kg of EBW	0.002	0.009	0.047	0.067
PAR, g	0.466	---	---	---
PAR, g/100 kg of EBW	0.683	---	---	---
MFP, g	0.002	0.008	0.219	0.012
MFP, g/100 kg of EBW	0.003	0.012	0.059	0.071

Figure 4 illustrates contribution of PAR and MFP to total MG weight when expressed on a raw-basis and after adjustment for EBW. Total MG and MFP weights were affected by diet on a raw- and EBW-adjusted basis, whereas PAR weights were not. Total MG (skinned right half of the udder including both PAR and MFP, x 2) and MFP weights were lowest in CON when compared to the average of the other 3 diets, both on a raw-basis and after adjustment for final EBW. The addition of fat to an isonitrogenous diet had no effect on total MG and MFP weights on a raw-basis. However, when adjusted for final EBW, HPHF had heavier total MG and MFP than HPLF. Feeding increased volume of a high-fat MR increased total MG and MFP weights on a raw-basis. However, when adjusted for final EBW, total MG and MFP weights were the same for HPHF and HPHF+.

PAR and MFP Composition

Diet did not influence lipid, protein, or DNA composition of PAR (Table 4). In contrast, MFP composition was markedly influenced by diet (Table 4). Lipid concentration and total lipids in MFP were lowest in CON when compared to the average of the other three diets. Heifers fed HPHF had greater lipid concentration and total lipids in MFP than did HPLF. Feeding increased

volume of a high-fat MR did not affect MFP lipid concentration; however, heifers fed HPHF+ had greater total MFP lipids than did HPHF, because HPHF+ had heavier MFP than HPHF.

Concentrations of protein and DNA in MFP were greater than the concentration of lipid in MFP when CON was compared to the other three diets. The same was true when HPLF was compared to HPHF. Protein and DNA concentrations in MFP were not affected by feeding increased volume of a high-fat MR.

GH/IGF-I Axis Genes in PAR and MFP

IGFBP-1 mRNA was not readily detectable in PAR or MFP. To verify the apparent absence of IGFBP-1 in PAR and MFP, IGFBP-1 primers were re-tested in a qPCR assay that included heifer liver cDNA as a positive control in addition to all PAR and MFP cDNA. Liver cDNA amplified and a single PCR product with the expected size was generated while most PAR and MFP samples failed to reach threshold after 40 cycles of PCR. This was taken to mean that IGFBP-1 mRNA is essentially absent in heifers of this age. IGFBP-1 data were not analyzed and are labeled non-detectable. Amplification plots from the 2 qPCR assays are presented in Appendix H.

Diet had no effect on GH/IGF-I axis gene expression in PAR or MFP (Table 5). A few genes were differentially expressed according to tissue fraction (PAR or MFP). IGFBP-2, IGFBP-5, and GH-R mRNA were more abundant in MFP as opposed to PAR while IGF-IR mRNA was more abundant in PAR (Table 5).

Telomerase in PAR

Telomerase abundance in PAR was not affected by diet or any other model effects (for diet, $P = 0.411$; Figure 5).

BrdU and Ki67 in PAR

Diet had no effect on percentage of BrdU- or Ki67-labeled epithelial cells in PAR, but labeling varied by sampling region (Figure 6). Percentage of heavily- and total-BrdU-labeled epithelial cells was largest in cisternal regions of the gland (0.47 ± 0.11% heavy; 2.49 ± 0.48% total) and decreased toward the periphery (0.13 ± 0.11% heavy; 0.79 ± 0.45% total; Figure 6). The opposite was true for Ki67-labeled epithelial cells (9.06 ± 0.82% vs. 10.94 ± 0.78%). Only 4.9% of heavily-labeled BrdU cells were also positive for Ki67 (data not shown), which suggests label-retaining cells proliferate infrequently. Figure 7 and

Figure 8 contain examples of histology images.

DISCUSSION

Meyer et al. (2006b) observed that while increased energy intake directly influenced MFP growth, its influence on PAR development was indirect and time dependent. In their study, heifers were evaluated at the same BW (50 kg increments from 100 to 350 kg), so heifers fed elevated nutrient intake were by default younger than their cohorts fed restricted energy intake. This age effect, not level of nutrient intake, was hypothesized to be the single greatest determinant of total PAR DNA content. Here, we wished to test their ideas by conducting an experiment with similarly aged heifers fed different diets. If the conclusions of Meyer et al. were true, then we should not expect a diet effect on PAR, but MFP should be influenced by diet.

In our experiment, pre-weaned Holstein heifers were fed different MR diets to achieve various BW gains. By design, the effects of diet on mammary growth and development were evaluated at a common chronological age, but not necessarily at the same BW. We set out to answer several questions related to nutrition and mammary growth and development.

First, we wished to know if MR composition and intake affect protein and lipid partitioning in MFP. Total MG weight was affected by diet, but this effect was due to accumulation of MFP since PAR weight was not different between diets (discussed below). This direct effect of diet on MFP is similar to previous studies (Capuco et al., 1995; Meyer et al., 2006b; Sejrsen et al., 1982). As expected, total MG and MFP weights were lowest in CON and increased with increased nutrient intake. Adding fat to an isonitrogenous diet resulted in heifers that weighed less than cohorts fed HPLF at the end of the experiment. However, those fed HPHF had proportionally heavier MFP than HPLF. In addition, per gram of MFP, heifers fed HPHF had more lipids, less protein, and less DNA than their cohorts fed HPLF. This suggests adipocyte hypertrophy in HPHF. The proportionally heavier MFP and more concentrated lipids in MFP did not, however, interfere with development of PAR in HPHF.

Feeding increased volume of a high-fat MR resulted in heifers that weighed more and had heavier total MG and MFP at the end of the experiment, but proportionally, mammary composition did not differ from their cohorts fed HPHF. Composition of MFP was not different between HPHF and HPHF+ per gram of MFP. Since composition of MFP did not differ between HPHF and HPHF+ but MFP weights did, one would intuitively expect total amounts of lipids, protein, and DNA to be different between the diets. We detected no difference in total protein or

total DNA in MFP between HPHF and HPHF+. We observed increased total lipids in HPHF+. DNA concentration in MFP was numerically lower in HPHF+ though, so perhaps with more heifers we would have been able to detect a difference in total DNA. As was the case above, MFP did not interfere with development of PAR in HPHF+.

Secondly, we wished to know if PAR was refractory to the level of nutrient intake and MFP not, as recently suggested by Meyer et al. (2006b). We observed no effect of diet on PAR weight or composition despite marked changes in MFP. This leads us to agree with Meyer et al. (2006b) and conclude that while some tissues, like MFP, are responsive to increased nutrient intake, reproductive organs, like PAR, are not. Furthermore our data lend support to a recent article by Thorn et al. (2008). They reasoned that accumulation of MFP could, in theory, inhibit PAR growth, if MFP expansion triggered the synthesis of proinflammatory cytokines and other proteins associated with inflammation that are known to affect insulin and IGF-I action (Thorn et al., 2008). Ultimately they concluded that while MFP expansion does cause increased production of certain inflammation related peptides, it is unlikely to contribute to reduced PAR growth observed with development or increased nutrition. Our gene expression results further support this theory. Had PAR been hindered by inflammatory cues from MFP that affect IGF-I actions, then we should have observed dietary differences in GH/IGF-I axis component gene expression, either in MFP or PAR. We observed no such differences.

Thirdly, we wished to know if age at harvest, not level of nutrient intake, was the single greatest determinant of total PAR DNA content. We observed no effect of nutrition on total PAR DNA content, despite large differences in diet composition and intake, when heifers were analyzed at a common age, which again leads us to agree with Meyer et al. (2006b). This is an important point because in the past, diminished DNA concentration and total DNA in heifers fed high as opposed to low energy intakes was taken as evidence for an inhibitory effect of nutrient intake and for reduced first lactation milk yields in heifers reared on high nutrient intakes (Petitclerc et al., 1984; Sejrsen et al., 1982). In the end, the effects of elevated nutrient intake on mechanisms that regulate mammary development pale in comparison to the effect of time.

As a fourth objective, we sought to determine if expression of GH/IGF-I axis genes in PAR and MFP differed when heifers were fed different diets. We found no diet effect whatsoever on GH/IGF-I axis gene expression which suggests nutrition is not important for regulating the expression of local GH/IGF-I axis components in developing heifer MG.

While not regulated by diet, several GH/IGF-I axis genes appear to be spatially regulated in bovine MG. For instance, IGF-IR mRNA and GH-R mRNA were inversely related in PAR and MFP, with PAR having relatively more IGF-IR and less GH-R expression than MFP. This is consistent with past findings and supports an indirect mechanism for GH to affect PAR. We observed relatively more IGFBP-5 in MFP as opposed to PAR, which is also consistent with past findings in prepubertal heifers (Berry et al., 2003a).

Overall, the dominant IGFBP was IGFBP-3, which was expressed at high levels in both MFP and PAR, and IGFBP-2 and IGFBP-5 which were preferentially expressed in MFP as opposed to PAR. Plath-Gabler (2001) noted IGFBP-3 and IGFBP-5 as the dominant IGFBP present in bovine PAR evaluated at various stages from puberty through involution. They went on to later conclude that because they found IGFBP-2 expression in PAR to be very weak that it is not regulated and does not have a significant physiological role in bovine MG (Plath-Gabler et al., 2001). Based on our findings, we challenge their conclusion and suggest that IGFBP-2 is at least spatially regulated in the bovine MG of 2 mo old heifers.

mRNA for IGF-I, IGFBP-3, IGFBP-4, IGFBP-6 were found without outstanding fluctuations due to tissue fraction and led us to conclude that they are not nutritionally or spatially regulated in bovine MG of 2 mo old heifers. Berry et al. (2003a) also found IGFBP-3 and IGF-I to be equally expressed in PAR and MFP in intact and ovariectomized heifers. More similar to our findings, Meyer et al. (2007) found IGF-I mRNA abundance to be unaltered by level of nutrient intake in PAR and in MFP in heifers. Recent lines of evidence suggest that locally produced IGF-I plays a larger role in mediating mammary development in prepubertal heifers than does IGF-I from circulation (Berry et al., 2003a; Berry et al., 2001; Weber et al., 1999). Our data are in agreement with this idea. Here we showed that equal PAR development across diets coincides with equal mammary IGF-I mRNA expression. In contrast, serum concentrations of IGF-I were different (Daniels et al., accepted), suggesting that local expression of IGF-I mRNA may be of greater importance to MG development. Furthermore since we detected mRNA for IGF-I in both PAR and MFP we, like others (Berry et al., 2003a; Plath-Gabler et al., 2001), assume that this local production is from adipocytes in PAR and MFP respectively, and not from epithelial cells in PAR.

Almost as interesting as gene expression results obtained, are the gene expression data we were not able to attain. We were unable to detect IGFBP-1 mRNA in PAR or MFP in 2 mo old

heifers; this may be of physiological significance. While our data agree with results from a murine study conducted by Boutinaud et al. (2004), it contradicts a bovine study conducted by Plath-Gabler et al. (2001). In their study IGFBP-1 was most highly expressed in pubertal virgin heifers when compared with all other stages evaluated (lactogenesis, galactopoiesis, and involution) (Plath-Gabler et al., 2001). The heifers used by Plath-Gabler et al. (2001) were 18 mo old and were thus pubertal. Taken together, it appears IGFBP-1 is under ontogenic regulation in bovine MG. Perhaps IGFBP-1, in concert with estrogen and estrogen receptor-α, plays a role in prepubertal allometric or pubertal isometric MG growth by modulating IGF-I actions. While no direct evidence for this is known, Berry et al. (2003a) found that in heifers, ovariectomy prior to 12 wk of age severely affected mammary development, decreased epithelial cell proliferation, decreased IGF-I mRNA in PAR (but not MFP), increased expression of estrogen receptor-α in PAR (Berry et al., 2003b), and tended to decrease expression of a 28 kDa IGFBP in PAR and MFP, which they suggested was either IGFBP-1 or IGFBP-4.

Our final objective was to determine if putative mammary epithelial stem cell activity could be modulated by diet in young heifers. Our working hypothesis was the positive effects of faster rates of gain during the pre-weaning period alter the development, persistence, or activity of populations of putative mammary epithelial stem cells and thereby impact subsequent mammary development. We based our hypothesis on previous work conducted by Brown et al. (2005) and Meyer et al. (2006a; b). To histologically assess putative mammary epithelial stem cells, we successfully used the technique of Capuco (2007). Our results from the histology experiment and telomerase assay collectively provide no evidence that putative mammary epithelial stem cells are affected by protein and fat differences in MR fed to pre-weaned heifers. This statement is further substantiated by our observation that PAR growth and development were not affected by diet and the fact that mammary stem cells are ultimately responsible for the growth and development of PAR. Furthermore, since Ki67-labeling was not affected by diet, we are able to conclude that epithelial proliferation in PAR was not different, which is contrary to the findings of Brown et al. (2005), who witnessed a positive effect of nutrient intake on Ki67-labeling in PAR. Taken together, our data lead us to conclude that the positive effects of elevated nutrient intake observed in young heifers by others (Brown et al., 2005; Meyer et al., 2006b), was an age effect. On the surface, one might not conclude this after reading the study by Brown et al. (2005), because like our study, diets were imposed at a given time and heifers were

evaluated at a set timepoint later. The two studies differ in that diets were imposed after a 2-wk adaptation period in Brown et al. (2005) and shortly after birth in our study. Furthermore, the exact birthdates of heifers was not known in the Brown et al. (2005) study, whereas we knew the birthdates of our animals. This leads us to speculate that the diet difference observed by Brown et al. (2005) was confounded with animal age or in the very least, related to timing of diet introduction.

While no diet effects were measured in our histology experiment, we observed regional differences in BrdU and Ki67 labeling in PAR. This was not surprising given that others (Ellis et al., 2000; Capuco et al., 2002) have previously shown that cells from peripheral PAR zones of the developing bovine mammary gland have greater proliferative and morphogenic potential than cells from the medial PAR mass. These differences are speculated to reflect local tissue regulation necessary for sequential ductular and lobuloalveolar development in vivo. Our results therefore agree with previous findings and are consistent with decreased proliferation and increased cell cycle arrest in cisternal regions of the gland (Capuco, 2007; Capuco et al., 2002; Ellis et al., 2000). Since regional differences in cell proliferation were detectable, this gives us further confidence that had the stem cell population within PAR been affected by diet, our method of detection should have been able to distinguish such differences.

CONCLUSIONS

In conclusion, neither diet composition nor nutrient intake in pre-weaned heifers affected PAR weight, PAR composition, GH/IGF-I axis gene expression, or putative mammary epithelial stem cell abundance when assessed at a common chronological age. Changes in MFP size and composition were observed, but we did not observe a diet effect on GH/IGF-I axis gene expression in MFP. This suggests nutrition is not critical for regulating the expression of local GH/IGF-I axis components in the developing heifer MG. Observed phenotypic differences in MFP are therefore attributed to adipocyte hypertrophy or perhaps a tissue growth response to lipid-sensitive genes, or both.

CONCLUDING REMARKS AND FUTURE RESEARCH

The research presented here answered some important questions concerning heifer mammary development. We re-affirmed the conclusions that: MR composition and intake affect protein and lipid partitioning in MFP; PAR, but not MFP is refractory to the level of nutrient

intake; age at harvest, not level of nutrient intake, is the greatest determinant of total PAR DNA content. Furthermore, we demonstrated that GH/IGF-I axis genes in PAR and MFP are not affected by diet. Perhaps most importantly, we found that putative mammary epithelial stem cell activity is not modulated by diet in young heifers.

Based on our findings, we feel that the HPLF diet was the most beneficial overall diet that we examined. Added fat and increased intake may be beneficial in times of cold stress, but beyond that appear not to positively or negatively affect PAR development. Increased MFP was observed with these diets, but the physiological importance of this to future milk production is unknown.

Growth and development of MFP are directly regulated by diet. Future research should focus on molecules specific to this tissue type, so that basic questions about bovine mammary development may be answered. Lastly, certain GH/IGF-I axis genes appear subject to spatial and ontogenic regulation in bovine MG. This may be of physiological relevance, therefore study of GH/IGF-I axis molecules in bovine MG should continue. This research should focus not only on PAR, but on MFP, as well as the interface between the two tissue fractions. Use of laser capture microscopy would be beneficial in this effort.

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Table 1. Ingredient and nutrient composition of milk replacers varying in protein and fat content fed to Holstein heifers

	Dietary Treatments			
	Milk Replacer			
	CON [1]	HPLF[2]	HPHF[3]	HPHF+[4]
CP, % DM	19.8	28.3	27.1	27.1
Fat, % DM	21.1	19.8	27.6	27.6
Gross Energy, Mcal/kg	4.69	4.90	5.24	5.24
Powder intake, g DM/d	441	951	951	1431
	Starter[5]			
CP, % DM	20.3			
Fat, % DM	1.43			
Gross Energy, Mcal/kg	3.52			
Starter intake, g DM/d	560	449	401	221
	Total Intake[6]			
Gross Energy, Mcal/d	3.61	5.41	5.57	6.94
DMI, g/d	1001	1400	1352	1652

[1] CON= Control milk replacer (MR) with 20% CP and 21% fat, fed at 441 g DM/d.
[2] HPLF= High protein low fat MR with 28% CP and 20% fat, fed at 951 g DM/d.
[3] HPHF= High protein high fat MR with 27% CP and 28% fat, fed at 951 g DM/d.
[4] HPHF+= HPHF fed at 1431 g DM/d.
[5]Starter contained 44.4% ground corn grain, 44.4% soybean meal, 11.2% cottonseed hulls, and 1.0% dried molasses.
[6]Total intake = MR + starter.

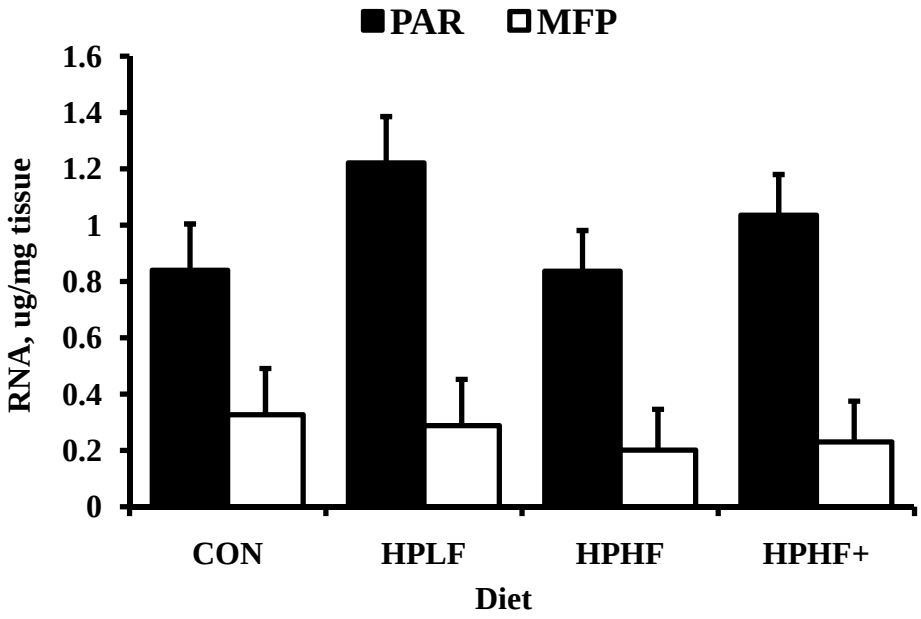


Figure 1. RNA yield data by milk replacer (MR) diet and tissue fraction. Diets were: CON (20% CP, 21% fat MR fed at 441 g DM/d), HPLF (28% CP, 20% fat MR fed at 951 g DM/d), HPHF (27% CP, 28% fat MR fed at 951 g DM/d) and HPHF+ (HPHF fed at 1431 g DM/d). Tissue fraction was either parenchyma (PAR) or mammary fat pad (MFP). RNA concentration was lower in MFP as opposed to PAR ($P = 0.0001$) and was not affected by any other model effects.

Table 2. Sequence of primer pairs used for determining relative abundance of mRNA for somatotropic axis genes in parenchyma and mammary fat pad in prepubertal Holstein heifers

Gene Symbol	Gene name (NCBI Accession number)	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
GH-R *	Growth hormone receptor	CGTCTCTGCTGGTGAAAACA	AACGGGTGGATCTGGTTGTA
IGF-I *	IGF-I	GTTGGTGGATGCTCTCCAGT	CTCCAGCCTCCTCAGATCAC
IGF-IR *	IGF-I Receptor	TCAAGGACGGAGTCTTCACC	GCTCAAACAGCATGTCAGGA
IGFBP-1 *	IGF binding protein 1	ACCAGCCCAGAGAATGTGTC	GTTTGTCTCCTGCCTTCTGC
IGFBP-2 *	IGF binding protein 2	CAAGGGTGGCAAACATCAC	GAGGTTGTACAGGCCATGCT
IGFBP-3 *	IGF binding protein 3	CAGAGCACAGACACCCAGAA	TGCCCCGTACTTATCCACACA
IGFBP-4 *	IGF binding protein 4	GCCGCACACACGTCTATCTA	CGCTTGCATGATTTACACGA
IGFBP-5 *	IGF binding protein 5	GTGCGGCGTCTACACTGAG	TCACGGGAGTCTCTTTTCGAT
IGFBP-6 **	IGF binding protein 6	CGCAGAGACCAACAGAGGAACT	GGGACCCATCTCAGTGTCTTG
PPP1R11 ***	(EH205657)	CCATCAAACTTCGGAAACGG	ACAGCAGCATTTTGATGAGCG
RPS15A ***	(BC108231)	GAATGGTGCGCATGAATGTC	GACTTTGGAGCACGGCCTAA
MTG1 ***	(AK074976)	CTTGGAATCCGAGGAGCCA	CCTGGGATCACCAGAGCTGT

*Described in Velayudhan et al. (2007).
**Described in this article.
***Described in Piantoni et al. (2007).

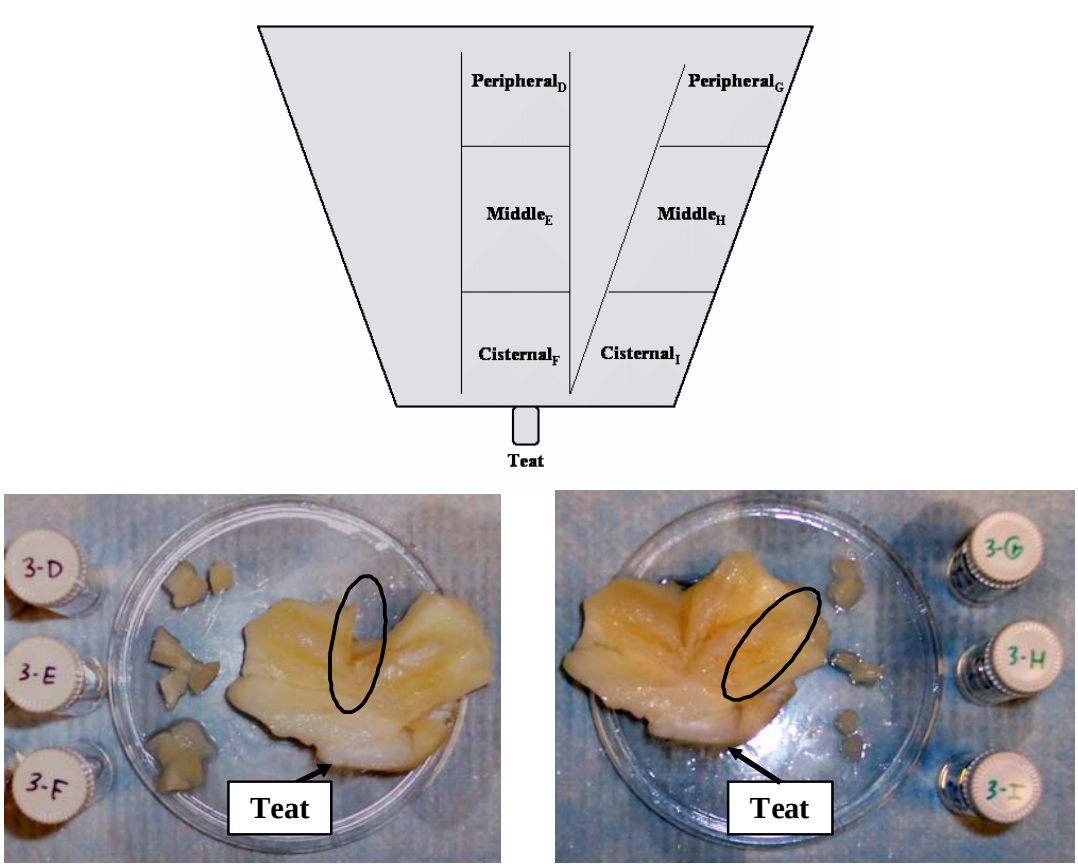


Figure 2. Mammary tissue was sampled from six parenchymal areas (labeled D, E, F, G, H, I) within one udder quarter.

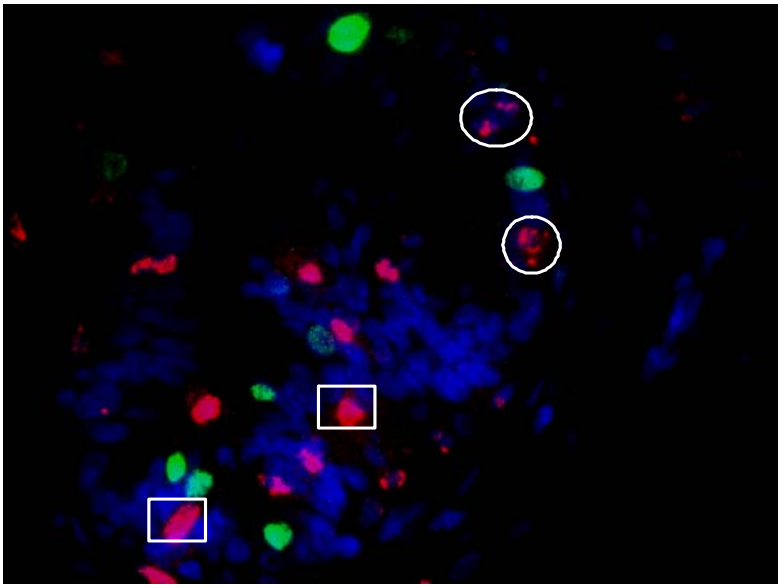
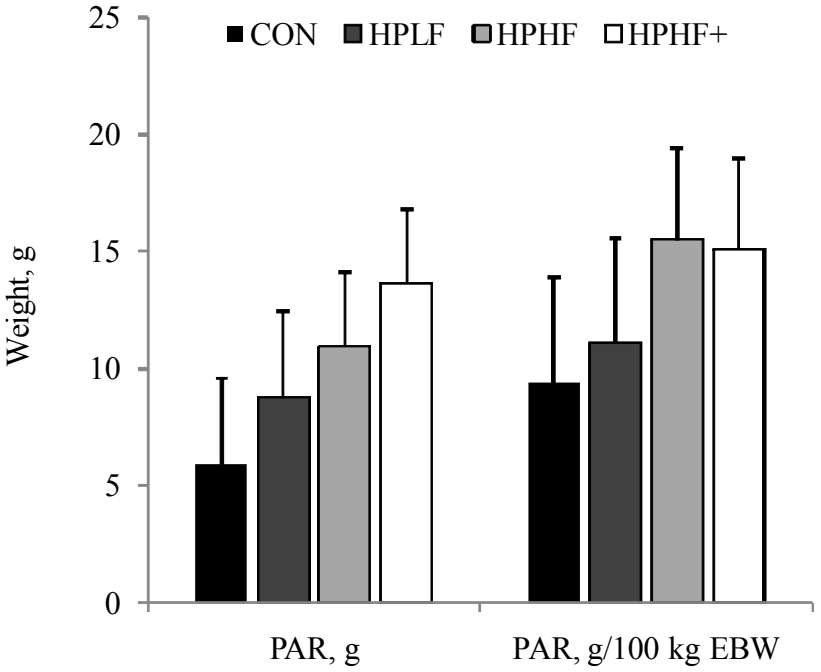


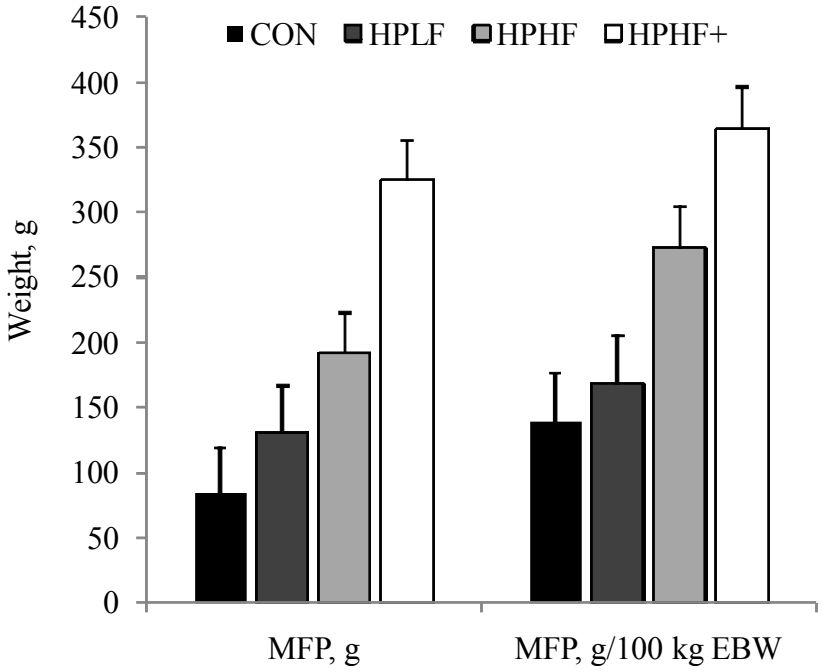
Figure 3. Light (○) vs. dark (□) BrdU staining.

Table 3. Initial and final heifer BW, age, and average daily gains (ADG) on treatment diets

Item	Diet[1]				SEM[2]	Diet, *P*	Diet Contrasts		
	CON n = 5	HPLF n = 5	HPHF n = 6	HPHF+ n = 6			CON vs. the rest	HPLF vs. HPHF	HPHF vs. HPHF+
Initial BW, kg	40.1	44.1	36.3	40.1	1.93	0.075	---	---	---
Initial age, d	3.5	3.3	3.3	3.7	0.39	0.809	---	---	---
Final BW, [3] kg	68.4	86.7	75.9	91.6	2.66	0.001	0.001	0.012	0.001
Final age, d	64.8	64.6	64.5	64.6	0.38	0.921	---	---	---
Lifetime gain, [4] kg/d	0.47	0.70	0.66	0.85	0.05	0.002	0.001	0.532	0.012

[1]Diets were: CON (20% CP, 21% fat MR fed at 441 g DM/d), HPLF (28% CP, 20% fat MR fed at 951 g DM/d), HPHF (27% CP, 28% fat MR fed at 951 g DM/d) and HPHF+ (HPHF fed at 1431 g DM/d).
[2]Standard error of the mean for diet (n = 5).
[3]Data presented here were not analyzed with initial BW as a covariate and inclusion of initial BW as a covariate was not significant ($P = 0.760$).
[4]Calculated energy and protein allowable daily gains (energy/protein, in kg/d) based on actual intake: CON (0.49/0.51), HPLF (0.93/1.12), HPHF (0.99/1.10), HPHF+ (1.44/1.53).


CON
HPLF
HPHF
HPHF+
Weight, g
25
20
15
10
5
0
PAR, g
PAR, g/100 kg EBW


CON
HPLF
HPHF
HPHF+
Weight, g
450
400
350
300
250
200
150
100
50
0
MFP, g
MFP, g/100 kg EBW

Item	Diet Effect, *P*	Diet Contrasts, *P*		
		CON vs. the rest	HPLF vs. HPHF	HPHF vs. HPHF+
Total MG, g	0.001	0.006	0.202	0.010
Total MG, g/100 kg of EBW	0.002	0.009	0.047	0.067
PAR, g	0.466	---	---	---
PAR, g/100 kg of EBW	0.683	---	---	---
MFP, g	0.002	0.008	0.219	0.012
MFP, g/100 kg of EBW	0.003	0.012	0.059	0.071

Figure 4. Contribution of parenchyma (PAR; upper panel) and mammary fat pad (MFP; lower panel) to total mammary gland (MG) weight when expressed on a raw-basis and after adjustment for empty BW for each diet. Diets were: CON (20% CP, 21% fat MR fed at 441 g DM/d), HPLF (28% CP, 20% fat MR fed at 951 g DM/d), HPHF (27% CP, 28% fat MR fed at 951 g DM/d) and HPHF+ (HPHF fed at 1431 g DM/d). Total MG and MFP weights were affected by diet while PAR weights were not (see included table).

Table 4. Mammary parenchyma (PAR) and fat pad (MFP) lipid, protein, and DNA content for heifers fed one of 4 milk replacer (MR) diets

	Diet[1]						Diet Contrasts		
Item	CON (n = 5)	HPLF (n = 5)	HPHF (n = 6)	HPHF+ (n = 6)	SEM[2]	*P* > F[3]	CON vs. the rest Pr > F	HPLF vs. HPHF Pr > F	HPHF vs. HPHF+ Pr > F
PAR									
Lipids, mg/g PAR	113	132	165	161	21.6	0.241	---	---	---
Total Lipids, g	0.9	1.1	1.8	2.2	0.54	0.255	---	---	---
Protein, mg/g PAR	46	38	41	38	2.4	0.085	---	---	---
Total protein, g	0.3	0.4	0.45	0.5	0.16	0.646	---	---	---
DNA, mg/g PAR	2.12	1.79	1.97	2.13	0.233	0.680	---	---	---
Total DNA, mg	11.5	16.9	22.5	32.1	10.41	0.942	---	---	---
MFP									
Lipids, mg/g MFP	455	475	687	706	46.4	0.003	0.010	0.006	0.742
Total lipids, g	39.7	64.6	132.4	233.7	28.25	0.002	0.009	0.100	0.015
Protein, mg/g MFP	20	19	9	6	3.4	0.022	0.053	0.042	0.486
Total protein, g	1.6	2.4	1.6	1.6	0.41	0.477	---	---	---
DNA, mg/g MFP	0.74	0.65	0.23	0.13	0.149	0.026	0.038	0.056	0.601
Total DNA, mg	59.0	79.0	42.5	37.1	14.07	0.177	---	---	---

[1]Diets were: CON (20% CP, 21% fat MR fed at 441 g DM/d), HPLF (28% CP, 20% fat MR fed at 951 g DM/d), HPHF (27% CP, 28% fat MR fed at 951 g DM/d) and HPHF+ (HPHF fed at 1431 g DM/d).
[2]Standard error of the mean for diet (n = 5).
[3]*F*-test *P* value for diet.

Table 5. Relative mRNA abundance* of selected genes in parenchyma (PAR) and mammary fat pad (MFP) in pre-weaned heifers fed one of 4 milk replacer (MR) diets

Gene[2]	Fraction	Diet				SEM[7]	Test of fixed effects, P[1]					
		CON[3]	HPLF[4]	HPHF[5]	HPHF+[6]		Diet	Group	Fraction[8]	Diet x group	Diet x fraction	Group x fraction
		($2^{-\Delta Ct}$ values)										
IGFBP-1	PAR	ND[a]	ND	ND	ND							
	MFP	ND	ND	ND	ND							
IGFBP-2	PAR	0.32	0.24	0.26	1.89	1.20	0.409	0.513	0.001	0.920	0.870	0.831
	MFP	5.51	3.92	5.42	6.58							
IGFBP-3	PAR	13.78	15.13	14.60	19.90	4.99	0.716	0.090	0.030	0.345	0.984	0.142
	MFP	7.82	6.48	7.35	10.53							
IGFBP-4	PAR	0.57	0.51	0.47	0.50	0.09	0.288	0.017	0.594	0.420	0.561	0.011
	MFP	0.70	0.39	0.49	0.59							
IGFBP-5	PAR	1.90	0.96	0.82	1.68	0.96	0.234	0.566	0.010	0.452	0.227	0.514
	MFP	4.80	1.17	3.91	2.33							
IGFBP-6	PAR	1.33	1.10	1.55	2.04	0.83	0.501	0.813	0.071	0.793	0.168	0.435
	MFP	2.54	0.94	3.51	1.96							
GH-R	PAR	0.63	1.06	1.15	1.26	0.40	0.452	0.219	0.002	0.190	0.636	0.350
	MFP	1.85	1.67	2.54	1.89							
IGF-IR	PAR	0.92	0.72	1.07	0.86	0.19	0.548	0.577	0.001	0.815	0.500	0.364
	MFP	0.32	0.16	0.26	0.55							
IGF-I	PAR	1.24	1.05	1.47	1.63	0.34	0.512	0.183	0.104	0.091	0.967	0.017
	MFP	1.78	1.45	1.79	1.87							

*Relative mRNA abundance data are expressed as $2^{-\Delta Ct}$ values; higher values equate to more mRNA.
[1]Significance declared when $P < 0.01$.
[2]IGFBP= IGF binding protein; GH-R = growth hormone receptor; IGF-IR = IGF-I receptor.
[3]CON = Control diet; 20% CP, 21% fat MR fed at 441 g DM/d.
[4]HPLF = High protein, low fat diet; 28% CP, 20% fat MR fed at 951 g DM/d.
[5]HPHF = High protein, high fat diet; 27% CP, 28% fat MR fed at 951 g DM/d.
[6]HPHF+ = High protein, high fat diet fed at increased rate; HPHF fed at 1431 g DM/d.
[7]SEM = Standard error of the mean for diet x fraction; (n = 5).
[8]Fraction was either PAR or MFP.
[a]Non-detectable.

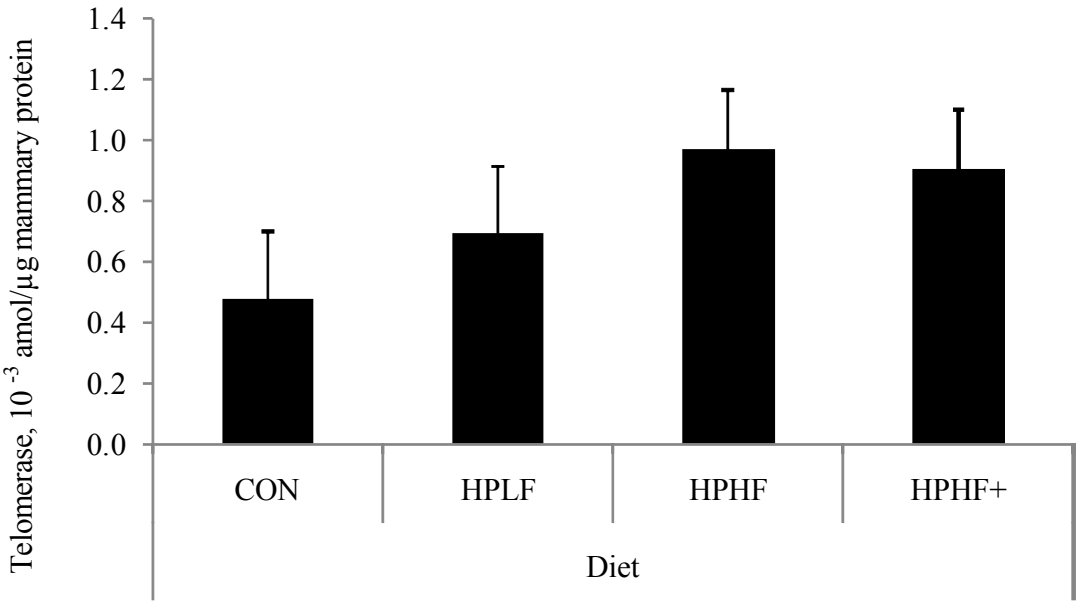
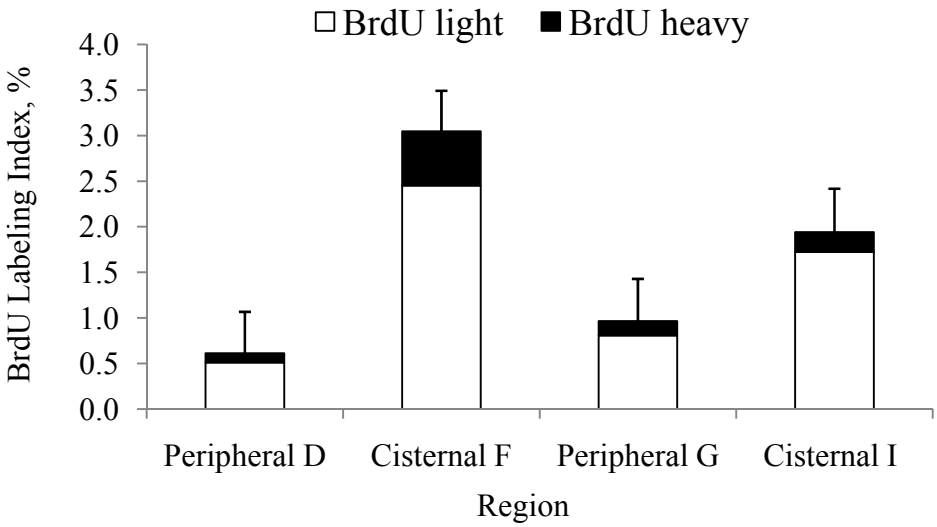
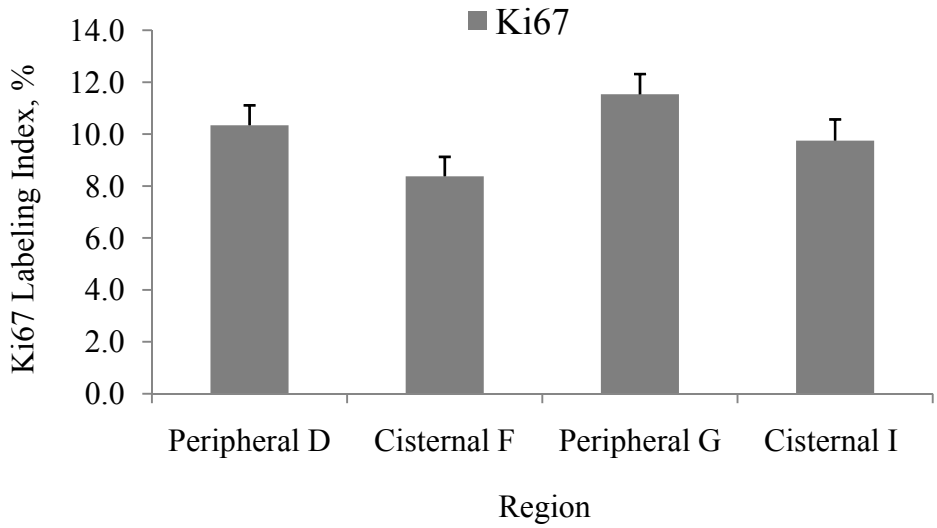


Figure 5. Telomerase abundance in mammary parenchyma was not affected by diet (P = 0.411). Diets were: CON (20% CP, 21% fat MR fed at 441 g DM/d), HPLF (28% CP, 20% fat MR fed at 951 g DM/d), HPHF (27% CP, 28% fat MR fed at 951 g DM/d) and HPHF+ (HPHF fed at 1431 g DM/d).





	Main Effects, *P*		Region Contrast
Item	Diet	Region	Peripheral vs. Cisternal
BrdU labeling index, heavy	0.603	0.002	0.005
BrdU labeling index, total	0.779	0.001	0.001
Ki67 labeling index	0.479	0.010	0.006

Figure 6. Contribution of heavily-labeled BrdU cells to total BrdU-labeled cells by region (upper panel) and Ki67-positive cells by region (lower panel). BrdU and Ki67 labeling indexes were not affected by diet, but were affected by sampling region within the udder (see included table).

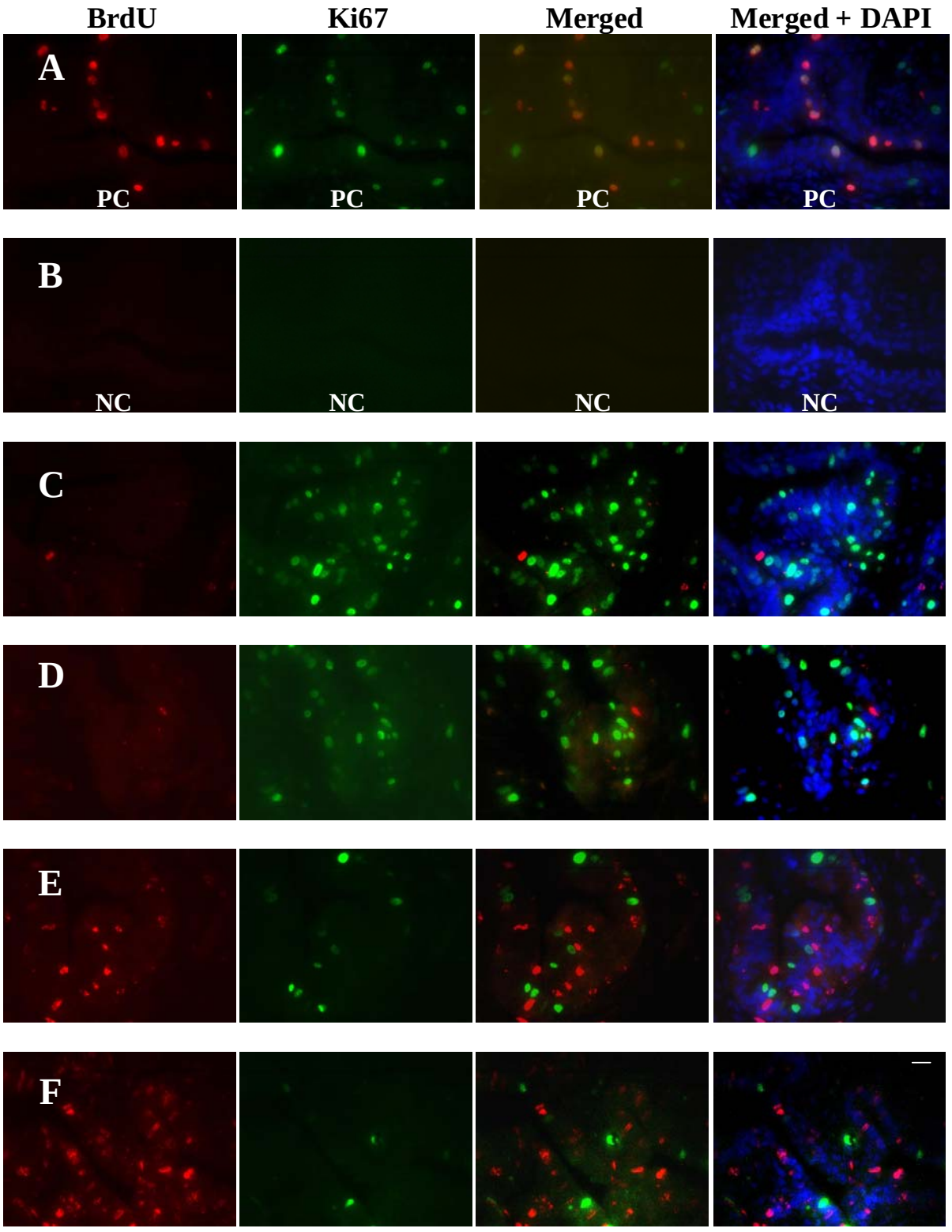

BrdU
Ki67
Merged
Merged + DAPI
A
PC
PC
PC
PC
B
NC
NC
NC
NC
C
D
E
F

Figure 7. Localization of BrdU-labeled and Ki67-labeled epithelial cells in peripheral and cisternal parenchymal regions of the gland. Few BrdU-labeled cells and many Ki67-labeled cells were evident in peripheral regions of the gland; the opposite was true of cisternal regions. (A) Positive control; obtained from a BrdU pulse-labeled heifer. (B) Negative control; serial section of A, primary antibodies were substituted with control sera. (C-D) BrdU and Ki67 staining in two peripheral regions. (E-F) BrdU and Ki67 staining in two cisteral regions. Scale bar = 50 μM.

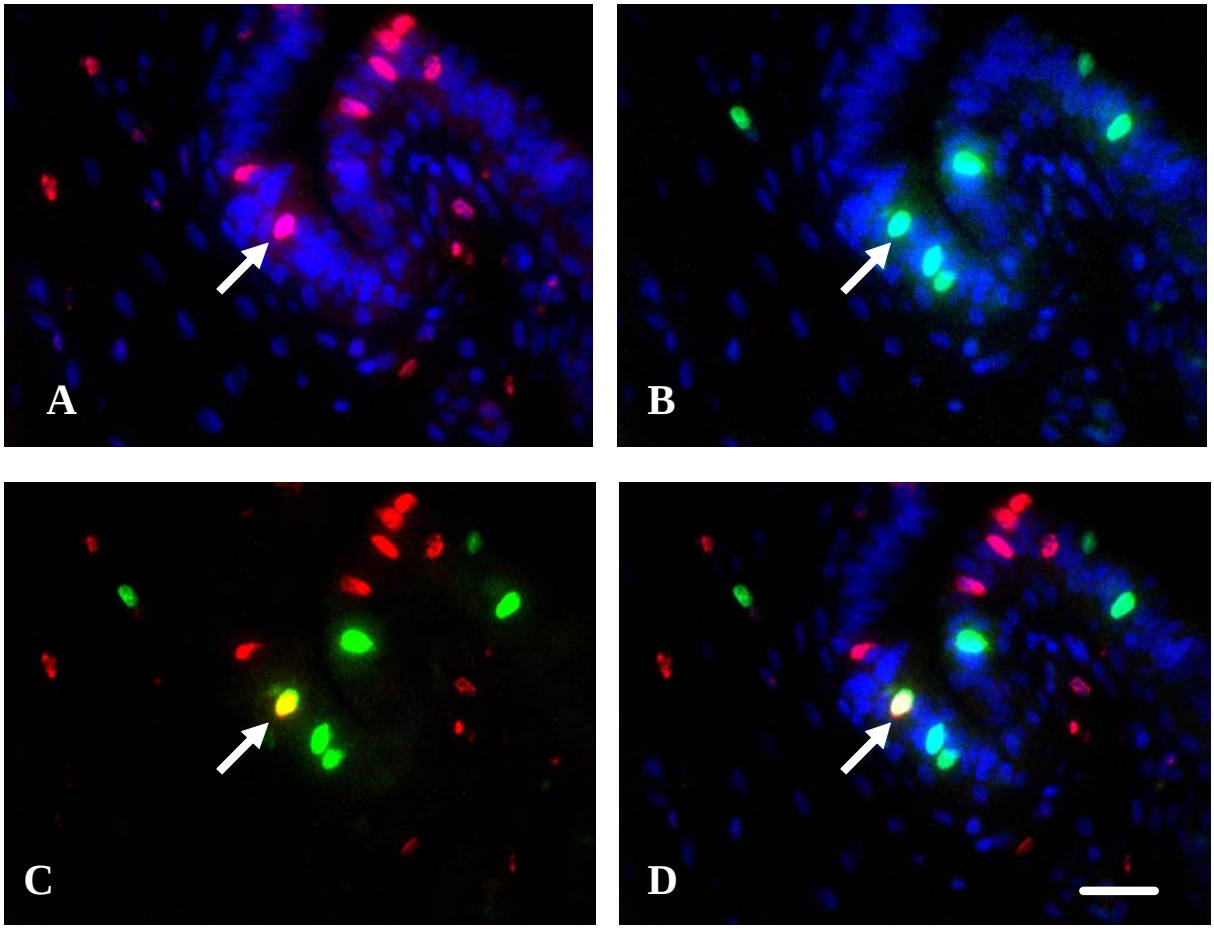


Figure 8. Example of a BrdU/Ki67 dual-labeled mammary epithelial cell (white arrow). A) BrdU + DAPI. B) Ki67 + DAPI. C) BrdU + Ki67. D) BrdU + Ki67 + DAPI. Scale bar = 50 μm.

APPENDICES

Appendix A: Further Reading

For further reading please see:

Velayudhan, B. T., K. M. Daniels, D. P. Horrell, S. R. Hill, M. L. McGilliard, B. A. Corl, H. Jiang, and R. M. Akers. 2007. Developmental histology, segmental expression, and nutritional regulation of somatotropic axis genes in small intestine of prepubertal dairy heifers. J. Dairy Sci. (in review).

Daniels, K. M., S. R. Hill, K. F. Knowlton, R. E. James, M. L. McGilliard, and R. M. Akers. 2007. Effects of milk replacer composition on selected blood metabolites and hormones in pre-weaned Holstein heifers. J. Dairy Sci. (accepted).

Hill, S. R., K. F. Knowlton, K. M. Daniels, R. E. James, R. E. Pearson, A. V. Capuco, and R. M. Akers. 2007. Effects of milk replacer composition on growth, nutrient excretion, and body composition in pre-weaned Holstein heifers. J. Dairy Sci. (accepted).

Piantoni, P., M. Bionaz, D. Graugnard, K. M. Daniels, R. Michael Akers, and J. J. Loor. 2007. Gene expression ratio stability evaluation in prepubertal bovine mammary tissue from calves fed different milk replacers reveals novel internal controls for quantitative PCR. J. Nutr. (accepted).

Campbell, D. E. C. 2007. Identification of tissue distribution and regulation of bovine stearoyl-CoA desaturase by hormones and nutrients. M. S. Thesis. Virginia Polytechnic Institute and State University.

The articles listed above all used tissues from the same heifers described in previous pages of this dissertation.

Appendix B: Side-by-side Comparison of ΔCt and $2^{-\Delta Ct}$ results from qPCR Experiment

With the Shapiro-Wilk test for normality, the null hypothesis that the data are a random sample from a normal distribution was rejected when $P < 0.05$.

Table B1. ΔCt results from Shapiro-Wilk test of normality

			Gene, *P*							
Diet	n =	Fraction	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	GH-R	IGF-IR	IGF-I
CON	5	MFP								
		PAR	0.002					0.013		
HPLF	5	MFP								
		PAR							0.031	
HPHF	6	MFP								
		PAR	0.003			0.016		0.002		0.020
HPHF+	6	MFP			0.040					
		PAR				0.050	0.019			

Table B2. $2^{-\Delta Ct}$ results from Shapiro-Wilk test of normality

			Gene, *P*							
Diet	n =	Fraction	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	GH-R	IGF-IR	IGF-I
CON	5	MFP	0.002							
		PAR						0.013		
HPLF	5	MFP								
		PAR								
HPHF	6	MFP	0.001					0.029		
		PAR						0.043		
HPHF+	6	MFP		0.020			0.012			
		PAR				0.010	0.037			

ANOVA for ΔCt Data (declared different when P < 0.05)

Table B3. Relative mRNA abundance* of selected genes in parenchyma (PAR) and mammary fat pad (MFP) in pre-weaned heifers fed one of 4 milk replacer (MR) diets - ΔCt data

Gene[2]	Fraction	Diet					Test of fixed effects, P[1]					
		CON[3]	HPLF[4]	HPHF[5]	HPHF+[6]	SEM[7]	Diet	Group	Fraction[8]	Diet x group	Diet x fraction	Group x fraction
		(ΔCt values)										
IGFBP-2	PAR	-2.08	-1.42	-2.23	-2.49	1.33	0.171	0.535	0.001 *	0.792	0.225	0.901
	MFP	4.29	7.87	4.42	2.03							
IGFBP-3	PAR	-2.85	-2.31	-2.65	-2.95	0.44	0.891	0.035	0.017 *	0.048 *	0.631	0.064
	MFP	-3.47	-3.47	-3.75	-3.18							
IGFBP-4	PAR	0.91	1.40	1.35	1.32	0.45	0.388	0.057	0.696	0.633	0.755	0.032 *
	MFP	0.54	1.81	1.19	1.02							
IGFBP-5	PAR	-0.58	0.56	0.53	-0.31	0.59	0.279	0.493	0.020 *	0.219	0.316	0.450
	MFP	-1.58	0.00	-1.45	-0.48							
IGFBP-6	PAR	-0.27	0.15	-0.35	-0.80	0.52	0.459	0.586	0.152	0.574	0.152	0.153
	MFP	-1.16	0.19	-1.18	-0.45							
GH-R	PAR	0.50	0.12	0.11	-0.16	0.40	0.674	0.334	0.006 *	0.082	0.347	0.503
	MFP	-0.77	-0.48	-1.21	-0.31							
IGF-IR	PAR	0.18	0.87	0.19	0.46	0.44	0.138	0.326	0.001 *	0.959	0.266	0.373
	MFP	1.70	2.83	2.08	1.01							
IGF-I	PAR	-0.30	0.20	-0.14	-0.54	0.42	0.481	0.345	0.268	0.248	0.943	0.037 *
	MFP	-0.75	-0.07	-0.58	-0.60							

*Relative mRNA abundance data are expressed as ΔCt values; lower values equate to more mRNA.
[1]For ΔCt analysis, significance declared when $P < 0.05$.
[2]IGFBP= IGF binding protein; GH-R = growth hormone receptor; IGF-IR = IGF-I receptor.
[3]CON = Control diet; 20% CP, 21% fat MR fed at 441 g DM/d.
[4]HPLF = High protein, low fat diet; 28% CP, 20% fat MR fed at 951 g DM/d.
[5]HPHF = High protein, high fat diet; 27% CP, 28% fat MR fed at 951 g DM/d.
[6]HPHF+ = High protein, high fat diet fed at increased rate; HPHF fed at 1431 g DM/d.
[7]SEM = standard error of the mean for diet x fraction; (n = 5).
[8]Fraction was either PAR or MFP.

ANOVA for $2^{-\Delta Ct}$ Data (declared different when P < 0.01)

Table B4. Relative mRNA abundance* of selected genes in parenchyma (PAR) and mammary fat pad (MFP) in pre-weaned heifers fed one of 4 milk replacer (MR) diets - $2^{-\Delta Ct}$ data

		Diet					Test of fixed effects, P[1]					
Gene[2]	Fraction	CON[3]	HPLF[4]	HPHF[5]	HPHF+[6]	SEM[7]	Diet	Group	Fraction[8]	Diet x group	Diet x fraction	Group x fraction
		$2^{-\Delta Ct}$ values										
IGFBP-2	PAR	0.32	0.24	0.26	1.89	1.20	0.409	0.513	0.001 *	0.920	0.870	0.831
	MFP	5.51	3.92	5.42	6.58							
IGFBP-3	PAR	13.78	15.13	14.60	19.90	4.99	0.716	0.090	0.030	0.345	0.984	0.142
	MFP	7.82	6.48	7.35	10.53							
IGFBP-4	PAR	0.57	0.51	0.47	0.50	0.09	0.288	0.017	0.594	0.420	0.561	0.011 *
	MFP	0.70	0.39	0.49	0.59							
IGFBP-5	PAR	1.90	0.96	0.82	1.68	0.96	0.234	0.566	0.010 *	0.452	0.227	0.514
	MFP	4.80	1.17	3.91	2.33							
IGFBP-6	PAR	1.33	1.10	1.55	2.04	0.83	0.501	0.813	0.071	0.793	0.168	0.435
	MFP	2.54	0.94	3.51	1.96							
GH-R	PAR	0.63	1.06	1.15	1.26	0.40	0.452	0.219	0.002 *	0.190	0.636	0.350
	MFP	1.85	1.67	2.54	1.89							
IGF-IR	PAR	0.92	0.72	1.07	0.86	0.19	0.548	0.577	0.001 *	0.815	0.500	0.364
	MFP	0.32	0.16	0.26	0.55							
IGF-I	PAR	1.24	1.05	1.47	1.63	0.34	0.512	0.183	0.104	0.091	0.967	0.017
	MFP	1.78	1.45	1.79	1.87							

*Relative mRNA abundance data are expressed as $2^{-\Delta Ct}$ values; higher values equate to more mRNA.
[1]For $2^{-\Delta Ct}$ analysis, significance declared when $P < 0.01$.
[2]IGFBP= IGF binding protein; GH-R = growth hormone receptor; IGF-IR = IGF-I receptor.
[3]CON = Control diet; 20% CP, 21% fat MR fed at 441 g DM/d.
[4]HPLF = High protein, low fat diet; 28% CP, 20% fat MR fed at 951 g DM/d.
[5]HPHF = High protein, high fat diet; 27% CP, 28% fat MR fed at 951 g DM/d.
[6]HPHF+ = High protein, high fat diet fed at increased rate; HPHF fed at 1431 g DM/d.
[7]SEM = Standard error of the mean for diet x fraction; (n = 5).
[8]Fraction was either PAR or MFP.

Appendix C: Immunohistochemical detection of Ki67 and BrdU: Brightfield detection (DAB)

While not included in the main body of the dissertation, in addition to fluorescent detection of BrdU and Ki67, a brightfield detection experiment was also completed. The decision was made not to include the results in the main body of work because it was slightly redundant. This work was important in that results helped determine that processing all six PAR regions was not necessary for fluorescent microscopy, hence only 4 sampling regions were described in the dissertation.

The following is a description of the work that was completed for this experiment.

Materials and Methods

Animals, Treatments, and BrdU Injections. The same animals and therefore the same methods were used here as described in the main dissertation.

Tissue Preparation. The left front quarter was "butterflied" open and samples for histological procedures were collected at slaughter. To prepare samples for paraffin embedding, tissue samples (~4 mm^2) from 6 regions within the quarter (Figure C1) were excised and placed into vials of 10% formalin. Tissues were allowed to fix overnight; the formalin solution was removed and replaced with 70% ethanol, tissue pieces were later embedded in paraffin. Microscope slides were prepared by mounting four 5 μm-thick serial sections of tissue onto superfrost plus microscope slides. Slides were then subjected to the staining protocol (included below).

Microscopy. One slide per heifer per region was analyzed. Six to 10 (depending on epithelium content) independent digital pictures were taken per tissue section. First the 20x objective lens was centered over a random area that contained epithelial tissue in the Ki67-stained tissue section; edges of tissue sections were avoided. The microscope was focused and a digital picture taken. The microscope stage was then moved to the BrdU-stained section (~5 um deep or superficial to the Ki67-stained section); the "serial image" (see Figure C2) was then located and a digital photograph taken. Next, a new region was located in the BrdU-stained section and photographed; the stage was again moved to the Ki67-stained section stage, the corresponding serial image found and a picture taken. The process then started over and

continued until 10 (or as many as possible) independent images were obtained for each region for each heifer. Only Ki67-stained and BrdU-stained serial images were kept for analysis. For instance, if a picture was taken in the Ki67-stained section, but a corresponding serial image was unavailable in the BrdU-stained section due to loss of tissue integrity, then both images were discarded. Images were measured with Image Pro-Plus (version 4.5) software. In each image, both the average number of cells per area of epithelium and the number of BrdU (or Ki67) positive cells per epithelial region were recorded. BrdU and Ki67 labeling indexes were established by dividing number of positive cells by the total number of epithelial cells.

Statistical Analysis. Labeling indexes were submitted for statistical analysis with SAS software. The mixed model procedure was used and the final model included the main effects of diet, region, group, and all two-way interactions. The final model also contained "region" as a repeated measure and heifer within diet and group as the subject for tests of significance. No denominator degrees of freedom method was specified, but Satterthwaite's approximation method was tried; inclusion did not affect overall tests of significance, so it was left out of the final model. Alternative models were also tried that included a random statement (heifer within diet and group; compound symmetry covariance structure) and no repeated statement. Repeated measures analyses yielded the same results as non-repeated measures analyses. The repeated measures analysis was selected as the final model, since the same heifer was sampled 6 times within PAR. Orthogonal polynomial contrast statements were used to further characterize regional differences in labeling index, if present. In all cases, significance was declared when $P < 0.05$.

Figures and Summary of Results.

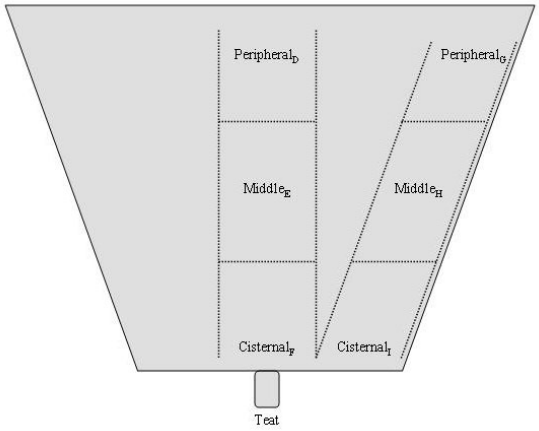


Figure C1. For each heifer, the left front quarter was "butterflied" open and parenchyma was sampled from 6 regions.

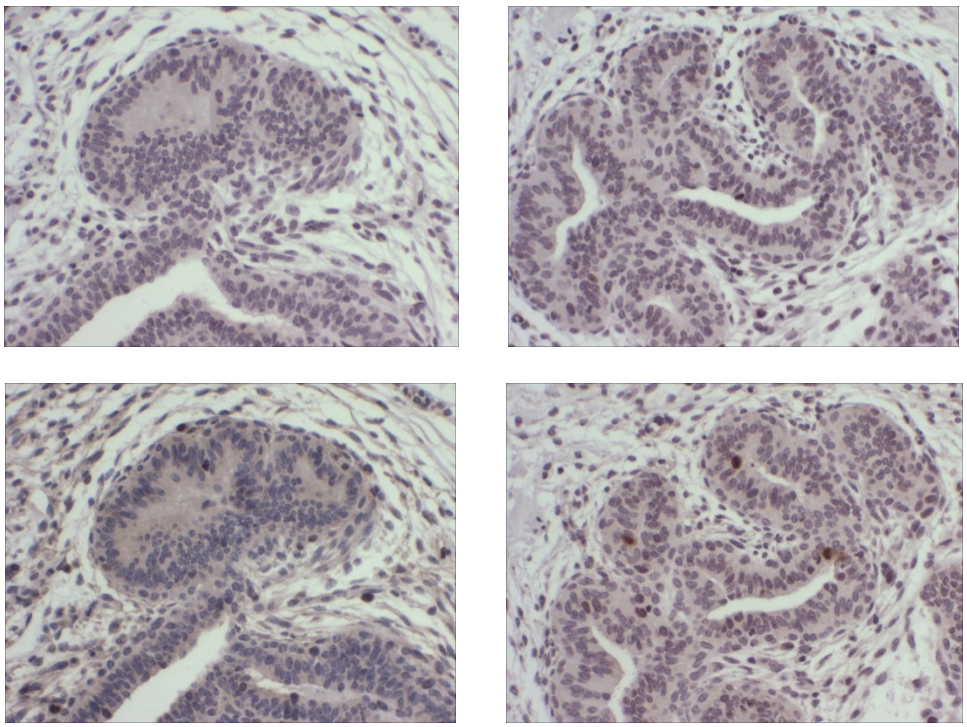
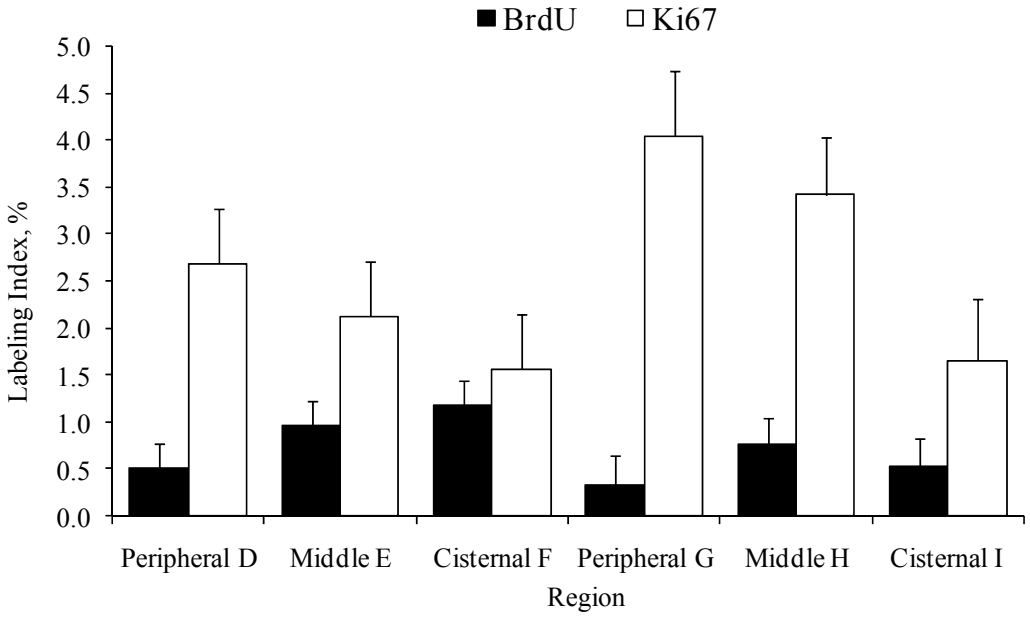


Figure C2. Top row = BrdU stained images. Bottom row = Ki67 "serial images" of BrdU section. Matching image pairs are referred to as "serial images" and are located ~5μm apart from one another.



Effect	df	BrdU labeling Index, %	Ki67 labeling index, %
		--- *P value* ---	
Diet	3	0.597	0.916
Region	5	0.081	0.009[a]
Group	2	0.611	0.377
Diet x region	15	0.543	0.793
Diet x group	6	0.792	0.745
Group x region	10	0.415	0.398

[a]Linear effect of region, $P = 0.002$.

Figure C3. Summary of results from brightfield immunohistochemical detection of Ki67 and BrdU experiment.

Immunohistochemical detection of Ki-67 and BrdU: brightfield detection (DAB)

revised 7/23/07

(Ki67 mouse monoclonal antibody purchased from Invitrogen; Cat # 08-0156)
(BrdU mouse monoclonal antibody (Clone BMC-9318) purchased from Fisher; Cat # MAB3424)
Note:BrdU antibody was formerly Roche and Boehringer Mannheim catalog number 1170376

DAY 1:

1. Deparaffinize and hydrate: ***~275 ml to fill each staining jar***

Xylene	2 x 5 min	RT
100% EtOH	2 x 1 min	RT
95% EtOH	1 x 2 min	RT
70% EtOH	1 x 2 min	RT
Di H2O	1 x 5 min	RT

Turn hotplate on and put beaker of 1x citrate buffer on high to boil

2. Quenching:

0.9% H2O2 in PBS	1x10 min	RT
1xPBS	3 X 2 min	RT

3. Microwave Antigen Retrieval:

Need: glass jar, slider carrier, 500 ml 10 *mM* citrate buffer

Microwave on high for 5 min (boils at 3:30 min)
Sit untouched for 5 min
Microwave on high for 5 min (boils at 30 sec)
Sit untouched for 30 min

Alternative:
boil continuously on hotplate for 15 min; let cool 30 min

4. Wash with 1x PBS:

1x PBS	3 x 2 min	RT

5. Aspirate PBS with vacuum before blocking; one slide at a time

6. Circle individual sections with PAP pen

7. Block with Normal Goat Serum:

1-2 drops 5% goat serum block per section
incubate RT for 30 min; time from first slide

Alternative:
block with reagent 1A of Histostain kit (it's 10% NGS)

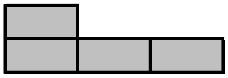
8. Primary antibody incubation:

Aspirate slide (don't wash)
Add 2-3 drops of anti-KI67 primary antibody (use at 100%) to a section
Add 2-3 drops of diluted anti-BrdU primary antibody (use at 100%) to ADJACENT section
diluted anti-BrdU = 20 ul of anti-BrdU + 980 ul of 1% goat serum
Add 2-3 drops of 1% normal goat serum = negative control to another section
Place slides in a humidfying chamber and incubate overnight at 4 C

Alternative:
Incubate 50 min at room temperature

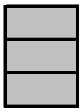
DAY 2:

9. Wash in 1x PBS:



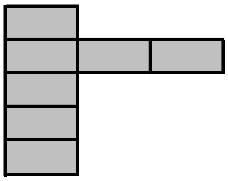
Aspirate slide (don't wash)
2-3 drops 1 x PBS 3 x 2 min

10. Secondary antibody incubation:



Aspirate slide
Add 2-3 drops of biotinylated secondary antibody (Reagent 1B of Histostain kit)
Place slides back in humidifying chamber for 30 min at 4C

Alternative:
Incubate 30 min at room temperature

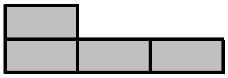


Aspirate slide
2-3 drops 1 x PBS 3 x 2 min
Aspirate slide
Add 2-3 drops of streptavidin-peroxidase (reagent 2 of Histostain kit) ; incubate for 12 min RT

At this point make up DAB solution; it needs to sit for 15 min **IN DARK**

4 drops Reagent A of DAB kit
4 ml of distilled water - mix after adding
4 drops Reagent B of DAB kit
4 drops Reagent C of DAB kit - mix after adding

11. Wash in 1x PBS:



Aspirate slide (don't wash)
1 x PBS 3 x 2 min

12. Antibody detection:

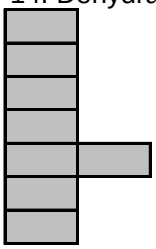
Aspirate slide
Add 2-3 drops of the DAB solution to each section; incubate 8-10 min RT

13. Counterstaining:

Aspirate slide (don't wash)
1 x PBS add to all slides right away
Counterstain with Gill's formulation #2 Hematoxylin for 30 secs
(do one slide at a time to ensure proper counterstaining)

Remove counterstain by gentle squirting of water from a water bottle
Place slide in DiH2O jar until all slides are ready for dehydration

14. Dehydration and coverslipping:



Distilled water 1 x 5 min (2 min after you add last slide to jar)
70% EtOH 1 x 1 min
95% EtOH 1 x 1 min
100% EtOH 1 x 1 min
Xylene 2 x 5 min
Apply 2-3 drops of permount and coverslip
Allow to dry overnight on a tray in hood

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SOLUTIONS / SUPPLIES

10 x Citrate Buffer, pH 6.0 (0.1 *M* = 100 *mM* = 10x)

14. 7 g Sodium citrate tribasic dihydrate (Na3C6H5O7*2H2O; FW = 294.1)
Volume to 500 ml with dd H20
pH to 6.0 with 12 N HCl

use at 1x stength

10 x PBS, pH 7.4 (Dulbecco's PBS - Calcium and Magnesium free)

11.4 g Sodium phosphate dibasic (Na2HPO4; FW = 141.960)
2 g Potassium phosphate monobasic (KH2PO4; FW = 136.10)
80 g Sodium chloride (NaCl; FW = 58.84)
2 g Potassium chloride (KCl; FW = 74.55)
Volume to 1 L
pH to 7.4 wirh 6 N NaOH

use at 1x strength
** if using sodium phosphate dibasic heptahydrate use 21.6 g instead of 11.4 g to make 10x PBS

0.9% H2O2 in PBS (for 10 slides)

9 ml 30% H2O2
267 ml 1 X PBS

5% Goat Serum

5 ml normal goat serum (invitrogen #01-6201)
95 ml 1 x PBS

1% Goat Serum

1 ml normal goat serum (invitrogen #01-6201)
99 ml 1 x PBS

Diaminobenzidine (DAB) Solution (for 10 slides)

4 drops Reagent A of DAB kit
4 ml of distilled water - mix after adding
4 drops Reagent B of DAB kit
4 drops Reagent C of DAB kit - mix after adding; protect from light

Primary antibody for detecting Ki67

(Ki67 mouse monoclonal antibody purchased from Invitrogen; Cat # 08-0156)
Use at 100% strength

Primary antibody for detecting BrdU

(BrdU mouse monoclonal antibody (Clone BMC-9318) purchased from Fisher; Cat # MAB3424)
add 20 ul of anti-BrdU + 980 ul of 1% goat serum

DAB Substrate Kit; Invitrogen; cat #00-2014

Histostain SP Mouse Primary; Invitrogen; cat #95-6543B

Pap Pen; Fisher; cat #NC9720458

Hydrogen peroxide, 30%; 100 ml bottle; Fisher; cat #H325-100

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Appendix D: Detailed Protocol for Operation of Nikon Eclipse Microscope and Olympus Digital Camera

1. Attach Olympus QColor 3 digital camera to fluorescent scope
2. Turn on mercury lamp --- flip power switch --- hold ignition for a few seconds --- release--- lamp ready light should come on soon. There is a power switch on the microscope itself, but it is not necessary to turn it on (it is for a regular lamp)
3. Refer to Nikon instruction manuals next to scope and verify these things
    a. Condenser in proper position
    b. Neutral density filters all out of light path
    c. Polarizer swung out
    d. Analyzer slide pulled out
    e. Shutter (open/close depending)
    f. Light provided to camera and oculars
    g. 40x objective in light path
    h. Proper filter blocks in filter block housing
        i. UV-2E/C DAPI filter for DAPI
        ii. FITC filter for Ki67
        iii. G-2A filter for BrdU
4. Put a slide on the stage, put the DAPI filter block in the light path, open the shutter, focus through oculars --- close the shutter for now
5. Open QCapture Pro software (***for fluorescent picture taking this program is preferred over Image Pro-Plus, for me anyway***)
6. Open the shutter
7. **Acquire --- video/digital** ---at bottom of dialog box click "**more**"
    a. You can change the preview and acquisition resolutions if you would like
    b. **IMPORTANT**: Change the capture depth to "12-bit mono" to capture monochrome images
    c. At the bottom of the dialog box click "**less**"
8. At top of dialog box, click "**preview**" --- a live preview of image should appear
    a. Use fine focus knob to adjust image on screen (it does not match the oculars)
    b. You can play around with the exposure/acquisition time lengths **---for DAPI --- recommend 5 milliseconds (5 -30 milliseconds, depending)**
    c. When ready, click **snap**
    d. Slide filterblock selector over to next position (Ki67)
    e. Change exposure/acquisition time to **100 milliseconds (fine focus not necessary)**
    f. When ready, click **snap**
    g. Slide filterblock selector over to next position (BrdU)
    h. Leave exposure/acquisition time at **100 milliseconds (fine focus not necessary)**
    i. When ready, click **snap**

    j. Slide filterblock selector back over to DAPI position
    k. Change exposure/acquisition time back to 5 milliseconds
    l. Find a new area to take a picture of by moving the stage; repeat step #8 until 10 areas on the slide have been pictured
9. Close the shutter
10. Go back and name/save the 30 untitled images you just captured. Use a naming scheme such as:
    a. 19_01_dapi (19 is animal number, 01 is picture number)
    b. 19_01_ki67
    c. 19_01_brdu
11. Save your images as TIFF images
12. When done for the day, turn mercury lamp off – don't turn it back on for at least 20 minutes (bulb might burst)

Appendix E: Detailed Instructions for Using Image Pro-Plus (v 6.2) to Evaluate Fluorescent DAPI, Ki67, and BrdU Images

Measuring epithelial area in each image using DAPI images

1. On the first day, make sub-folders in the folder with all of your fluorescent pictures
    a. Name one "working_set"
    b. Name the other "done_measuring"
2. Cut and paste about 10-20 DAPI pictures over to the working set folder – pick them at random
3. Open Image Pro --- **file** --- **open** --- browse for your working_set folder --- open a DAPI image
4. **Enhance** --- **equalize** --- **best fit** (chooses best contrast for the image)
5. **Measure** --- **calibration** --- select **spatial** --- choose "40x_NIKON_scope" (or whatever you are using) (VERY IMPORTANT – YOU HAVE TO TURN THE CALIBRATION ON FOR EACH NEW IMAGE THAT YOU OPEN!!!)
6. **Measure** --- **measurements** --- select the polygon tool to measure epithelial area --- make sure the pull-down menu is on "area" and not on something like "length"
7. Export the data to Excel by using features in the "Input/Output" tab in the Measurements window
8. Repeat for each new image

Determination of average area occupied by one epithelial cell (needed for creation of a labeling index)

1. Open Image Pro --- file --- open a DAPI image
2. **Enhance** --- **equalize** --- **best fit**
3. **Measure** --- **calibration** --- select **spatial** --- choose "40x_NIKON_scope" (or whatever calibration you are using)
4. Zoom in on the image so you can see better
5. **Measure** --- **measurements** --- select the RECTANGLE tool ---outline a small area around some epithelial cells--- make sure the pull-down menu is on "area" and not on something like "length"
6. Export the data to Excel
7. Go back to Image Pro and count the number of nuclei present in the box you outlined --- record those data in the Excel sheet too
8. Repeat procedure in several places on an image and use several random images
9. Determine the average area occupied per cell by dividing the rectangular area measured by the number of nuclei counted.
10. Take the average of these measurements and use as your calibrator for your labeling index calculations

Counting positively stained nuclei using BrdU, Ki67, and DAPI images

1. On the first day, make sub-folders in the folder with all of your fluorescent pictures
    a. Name one "working_set"
    b. Name the other "done_counting"
2. Cut and paste about 10-20 complete sets (BrdU, Ki67, DAPI) of your pictures over to the working set folder – pick them at random
3. Open Image Pro --- **file** --- **open** --- browse for your working_set folder --- **open a set of images**
4. For each image:
    a. **Enhance** --- **equalize** --- **best fit**
        i. Use the **contrast enhancement toolbar** (BrdU images; especially gamma correction) to get the best image
        ii. (***optional***) **Process** --- **filters** --- **morphological tab** --- select "**open**" (it will smooth object contours, separate narrowly connected objects, and remove small, dark holes). Suggest using it at the 2x2 square with 2 passes settings.
        iii. (***optional***) Another filter to try is the "**despeckle**" filter located in the enhancement tab. This filter reduces impulse noise; the amount of speckles removed can be adjusted by playing with the "passes" and "sensitivity" settings. This filter seems to work well with 2 passes, and a sensitivity of 10.
    b. Mouse over the BrdU image; right click; tint red; doublecheck contrast again --- adjust with **contrast enhancement toolbar** if needed
    c. Mouse over the DAPI image; right click; tint blue
    d. A composite preview image and a color composite dialog box will then appear. Do counting from the preview image. Use the **annotation toolbar** to help you do this.
        i. Select "Annotate" icon (looks like a pencil drawing a line) from the upper toolbar
        ii. Choose a scheme to keep track of ***darkly and lightly stained epithelial cells***
            1. I used a yellow circle for dark, dense nuclear stain
            2. I used an orange ellipse for light, diffuse nuclear stain
        iii. Zoom and scroll as needed to cover the entire image
        iv. Sometimes BrdU stain is diffuse and sparse and therefore hard to count. One tip is to toggle between the DAPI and BrdU images by clicking the red rectangle in the color composite dialog box – gives you a better idea if the cell in question is really stained or not
        v. When done counting, go to image and count the number of yellow circles and orange ellipses and enter them on a data sheet along with slide and picture number (Hint: It is sometimes easier to count annotations against a black screen; to do this, click on the red and blue rectangles in the color composite dialog box; undo by clicking them again)
    e. Leave current annotations on and mouse over the Ki67 image; right click; tint green

f. A composite image will now be displayed with all 3 color channels visible
    i. Look to see if any dual stained cells are present. If so make a note of it.
    ii. Remove BrdU annotations by clicking the red X in the annotation dialog box
    iii. Use the rectangle in the annotation toolbox to annotate dual labeled cells, if present
    iv. Get rid of the red (BrdU) channel by clicking on the red rectangle in the color composite dialog box
    v. Keep track of Ki67 positive cells by using the annotation tool as in above
    vi. When done, close entire Image Pro Program, it will ask "Save Changes to Composite Preview 1?" Say, no all.
    vii. Repeat steps 3 -4 for all remaining images in working_set folder. When done, transfer them to "done_counting" folder and put more in "working_set" folder

Saving a monochrome TIFF file as a color (or grayscale) JPEG file for presentation purposes

1. Open Image Pro, open a monochrome (TIFF) image
2. **Enhance ---equalize --- best fit** (***if want to save as grayscale, skip to step 4 now***)
3. Mouse over the image; right click
    a. If Ki67, tint image green. ***Two ways to do this***:
        i. Pick "tint green" from list **– *OR* –**
        ii. Pick "apply dye tint" from list ---select "Alexa Fluor **488**" from dye list
    b. If BrdU, tint image red. ***Two ways to do this:***
        i. Pick "tint red" from list **– *OR* –**
        ii. Pick "apply dye tint" from list ---select "Alexa Fluor **594**" from dye list
    c. If DAPI, tint image blue. ***Two ways to do this:***
        i. Pick "tint blue" from list **– *OR* –**
        ii. Pick "apply dye tint" from list --- select "DAPI" from dye list
4. **Edit --- convert to --- RGB 24** --- a new image will appear. You can further play with the contrast by using the contrast enhancement feature under Enhance.
5. **File --- save as** --- name it and confirm that it is a JPEG by looking in the file type box. Your original monochrome TIFF image will still be there too; don't save the changes to it.

Appendix F: General Tips for Use of Image Pro-Plus (v 6.2)

Calibrating Image Pro-Plus for the microscope and objective lenses you use

1. Take digital pictures of a stage micrometer with the microscope and objective lenses of interest
2. Open ImagePro, open a stage micrometer picture to be calibrated
3. **Measure --- calibration --- spatial calibration wizard**
    a. Check the circle for "calibrate active image"
4. "Create spatial calibration" window opens
    a. Name your calibration
    b. Select the spatial reference units --- ums
    c. Check the box for "create a reference calibration"
    d. Draw a reference line
        i. Position the line over your length reference
        ii. It will ask you: "reference calibration represents how many units?"
            1. Fill units in --- OK --- NEXT --- FINISH
5. Verify the calibration
    a. Open up the stage micrometer picture again if it is not still open
    b. **Measure ---calibration** --- select your new spatial calibration from the pull-down menu ---OK
    c. **Measure** --- use some of the tools (line, rectangle, polygon) to confirm your new calibration

Your current calibration will appear in the lower right side of the computer screen when it is on.

Appendix G: Alternative Statistical Models Considered for Analysis of qPCR Data

There was concern that since each heifer was sampled more than once when mammary tissue was taken, that the variable "fraction" (PAR or MFP) was actually a repeated measure in space. The following model statements were submitted to SAS to look in to this. Various combinations of random and repeated statements were tried with and without Satterthwaite's denominator degrees of freedom approximation method.

These models all yield the same results, possibly because of only 2 sampling regions:

```
        model IGFI = trt|group|fraction /ddfm=sat;
        random heifer (trt*group);
AND
        model IGFI = trt|group|fraction;
        random heifer (trt*group);
AND
        model IGFI = trt|group|fraction /ddfm=sat;
        repeated fraction /subject = heifer(trt*group) type =CS;
AND
        model IGFI = trt|group|fraction;
        repeated fraction /subject = heifer(trt*group) type =CS;
```

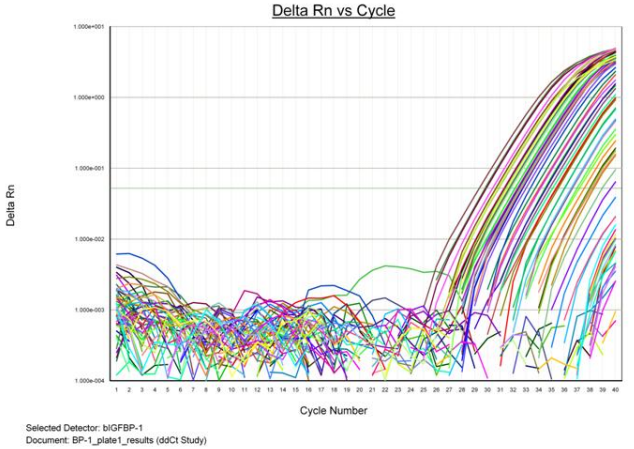
This model ran, but yielded different results than the above 4 models, probably because no "type = " option was included:

```
        model IGFI = trt|group|fraction;
        repeated fraction /subject = heifer(trt*group);
```

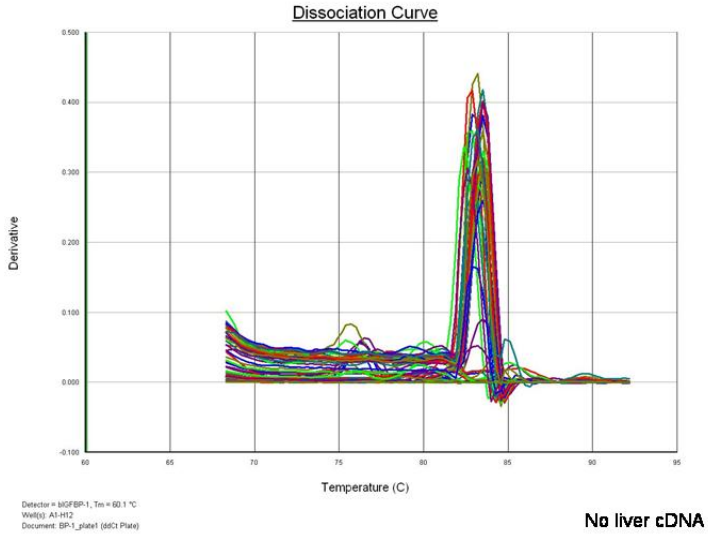
Based on the above results, the decision was made to select the following model statement as the final model for qPCR data:

```
        model IGFI = trt|group|fraction /ddfm=sat;
        random heifer (trt*group);
```

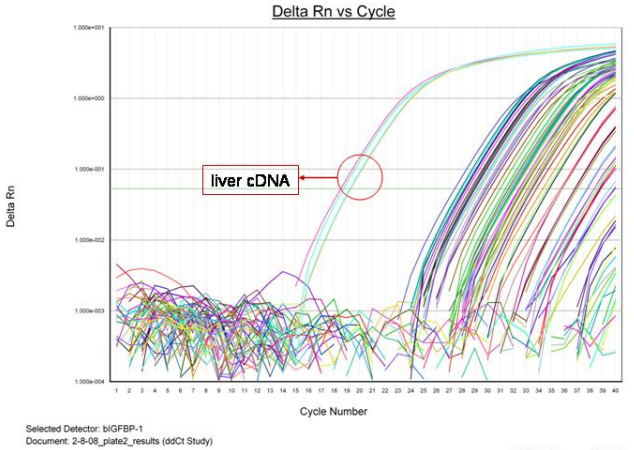
Appendix H: IGFBP-1 qPCR Amplification Plots and Dissociation Curves



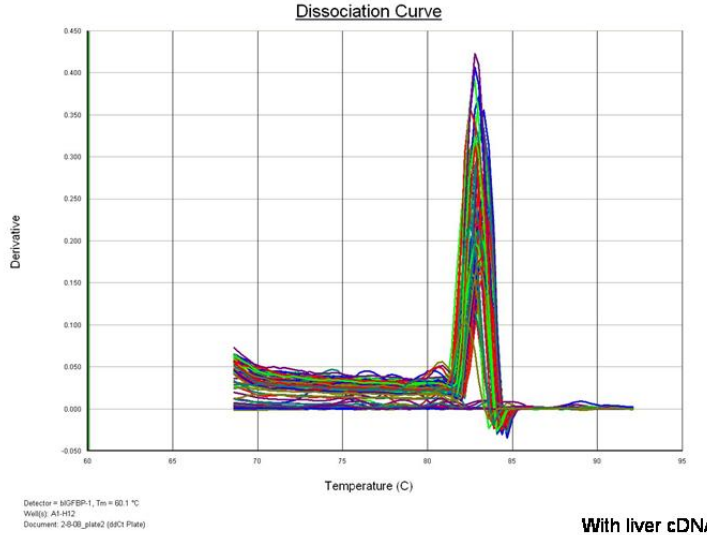
No liver cDNA



No liver cDNA



With liver cDNA



With liver cDNA