The effect of increased nutrient intake and exogenous estrogen on mammary gland growth, morphology, histology, and gene expression of Holstein heifer calves

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ABSTRACT

Current data indicates that feeding dairy calves more nutrients in early life allows them to produce more milk in the future. Mechanisms responsible are poorly understood. Thirty-six Holstein heifer calves were fed either a restricted (R; 20.2% crude protein [CP], 19.8% fat, dry matter (DM) basis, fed at 0.44 kg/hd/d, DM basis) or an enhanced (EH; 28.9% CP, 26.2% fat, DM basis, fed at 1.08 kg/hd/day, DM basis) milk replacer (MR) and given either a placebo or estradiol (E2) implant to assess differential responses to E2. Our underlying hypothesis was that calves fed more nutrients are better able to respond to mammmogenic stimuli and will have a more developed mammary gland as a result of imposed treatments. Enhanced-fed calves grew at a faster rate, were heavier at weaning, and had more functional mammary tissue (i.e., parenchyma; PAR) mass in the mammary gland at weaning (7.3-fold). Additionally, biochemical composition of the PAR was not impacted by the dietary treatments imposed. Furthermore, EH-fed calves had an increase in the number of actively dividing cells throughout the mammary PAR as well as increased intensity of estrogen receptor expression in the population of cells expressing the estrogen receptor. Enhanced-fed calves had an up-regulation of genes and pathways in the PAR related to metabolism, cellular signaling, and cellular growth. When given E2, EH-fed calves experienced the greatest overall mammary gland development and had the greatest PAR mass without compromised composition. When comparing EH- and R-fed calves given E2, differential expression of genes and
pathways related to cell growth, cell signaling, and metabolism was observed. In summary, data indicates that enhanced feeding of calves in early life allows increased responsiveness to mammogenic stimuli and a corresponding increase in mammary development. We suggest that this may at least partly explain the improved future milk production in calves fed in this manner.
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Life has a funny way of taking you were you need to go, whether you know it or not. That is most definitely the case for me. If you would have asked me 10... even 5 years ago where I would be today, finishing up a Ph.D. would not have been my go-to answer. I first want to thank my mother and father, Simon and Rhonda Geiger for bringing me up in a household full of love and support. I would not be where I am nor would I be the man I am without the two of you. However, I want to dedicate this document to my wife and son, Abigail and Abram Geiger. Abby, you continually bring the best out in me. You make me want to be the best person I can be and you truly are my best friend. Thank you for your love and support throughout all of this. Abram, you are my little ray of sunshine. You can brighten any day and walking in the door and seeing your beaming face almost brings me to tears. Thanks to both of you for all you do and I hope I’ve made you proud.
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<td>ADF</td>
<td>acid detergent fiber</td>
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<td>ADG</td>
<td>average daily gain</td>
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<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
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<td>bST</td>
<td>bovine somatotropin</td>
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<td>BW</td>
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<td>cd10</td>
<td>cluster of differentiation marker 10</td>
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<td>cm</td>
<td>centimeter</td>
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<td>CP</td>
<td>crude protein</td>
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<td>CPM</td>
<td>count per million</td>
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<td>day</td>
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<td>DEG</td>
<td>differentially expressed genes</td>
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<td>DIA</td>
<td>Dynamic Impact Approach</td>
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<td>ECM</td>
<td>extra-cellular matrix</td>
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<td>EH</td>
<td>enhanced-fed calves</td>
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<td>EH-E2</td>
<td>EH calves given estrogen</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<td>ERE</td>
<td>estrogen response elements</td>
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<td>FDR</td>
<td>false discovery rate</td>
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<td>g</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>gene ontogeny</td>
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<td>HPN</td>
<td>higher plane of nutrition</td>
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<td>IGF-I</td>
<td>insulin-like growth factor-I</td>
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<td>IPA</td>
<td>ingenuity pathway analysis</td>
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<td>kg</td>
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<td>Mcal</td>
<td>mega calorie</td>
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<td>MFP</td>
<td>mammary fat pad</td>
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<td>mg</td>
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<td>μg</td>
<td>microgram</td>
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<td>milliliter</td>
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<td>MR</td>
<td>milk replacer</td>
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<td>MYEC</td>
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<td>NDF</td>
<td>neutral detergent fiber</td>
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<td>ng</td>
<td>nanogram</td>
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<td>p63</td>
<td>Transformation-related protein 63</td>
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<td>PAR</td>
<td>parenchyma</td>
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<tr>
<td>PCA</td>
<td>principal component analysis</td>
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<td>R calves given estrogen</td>
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<td>SMA</td>
<td>smooth muscle actin</td>
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<tr>
<td>TDLU</td>
<td>terminal ductule lobular units</td>
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<td>TDU</td>
<td>terminal ductal units</td>
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<tr>
<td>TEB</td>
<td>terminal end buds</td>
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<td>TED</td>
<td>terminal end ducts</td>
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<tr>
<td>TMM</td>
<td>trimmed mean of M-values</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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CHAPTER I. INTRODUCTION

Younger, more desirable herd mates replace approximately one-third of dairy cows in the United States annually (Capuco et al., 2002). Replacement heifer rearing accounts for approximately 20% of annual farm costs (Heinrichs, 1993, Radcliff et al., 1997, Davis Rincker et al., 2008). Thus, raising replacement heifers is an important aspect of the dairy industry, but is often overlooked by producers as costs are high and returns are low. Costs include feed, housing, and labor, with no return from that animal until first lactation or the heifer is sold (Hoffman and Funk, 1992, Radcliff et al., 1997, Radcliff et al., 2000). Additionally, with the world’s population expected to increase by nearly 2 billion people over the next 40 years, it will be increasingly important to raise productive heifers as demand for milk products increases. With this, potential production cost reductions have been investigated with the two most prominent areas of focus being: 1) decreasing age at breeding and first calving, and 2) increasing mammary development prior to onset of first lactation to increase returns upon initiation of lactation. A dairy survey conducted by the National Animal Health Monitoring System has shown that the average age at first calving for a cow in the United States is approximately 25 mo (USDA, 2007), which has been decreasing slightly over the past few decades (Sejrsen et al., 1982). Decreasing age at first calving seems a logical way to reduce the cost of raising heifers, as reducing age at first calving by one month can decrease heifer-rearing costs by 4.3% (Hill et al., 2008). In other words, a 100-cow dairy in a Mid-Atlantic state (such as Virginia), could decrease annual rearing costs $32,344 by
decreasing age at calving for their herd from 25 to 21 mo (Tozer and Heinrichs, 2001). Whether 21 mo of age is an appropriate calving age is a different topic entirely. By decreasing age at first calving, the producer is essentially shortening the non-productive portion of a heifer's life by encouraging earlier herd entry and earlier productivity. To breed at an earlier age, heifers must reach puberty earlier. Puberty is closely associated with body weight (BW), and pubertal BW is relatively constant, with Holstein heifers reaching puberty between 250 and 280 kg or 40 to 50% of mature weight (Sejrsen, 1994) and optimally calving between 544 and 567 kg (Keown and Everett, 1986). It can be easily understood, then, that the best way to achieve earlier puberty/breeding, is for producers to target heifers to reach a desirable pubertal weight as quickly as possible. This can be achieved by feeding larger than normal amounts of nutrient rich diets. However, it has been well documented that increased nutrient intake during the pre-pubertal period of life is detrimental to mammary development and/or first lactation potential. Whether a cause and effect relationship exists between lower early mammary development and decreased future milk production is yet to be determined. However, it has been hypothesized that deleterious effects of increased nutrient intake and growth during the pre-pubertal period correspond with excess fattening, which may impact milk production due to a reduction in secretory cell number, altered nutrient utilization, or reduced skeletal size (Capuco et al., 2003). Specifically, excess pre-pubertal fattening is correlated with decreased first lactation milk yield by approximately 16% compared with herd mates (Silva et al., 2002). Therefore, it is easy to see that decreasing age at first calving has deleterious effects on first lactation milk yield.
(Ettema and Santos, 2004), and that the negative effects associate with decreasing age at puberty greatly outweigh the positives. Therefore, decreasing age at first calving is not the answer and other strategies should be investigated to increase the efficiency of development in the dairy heifer. According to Sejrsen et al. (2000), three crucial discoveries should be considered when developing these strategies: 1) increased pre-pubertal growth often leads to decreased mammary growth and milk yield potential, 2) the same effect is rarely seen during the post-pubertal period or during pregnancy, and 3) greater BW gain due to increased genetic potential for growth is positively correlated to milk yield (Sejrsen et al., 2000). Therefore, as it appears that post-pubertal manipulation has been relatively ineffective, the focus has shifted towards the pre-pubertal period. The goal of this review is to inform the reader of prior research revolving around this topic, and to offer reasoning behind the approach taken in the current study.
CHAPTER II. REVIEW OF LITERATURE

Mammary Development

Mammary development (mammogenesis) of the ruminant, dairy cattle in particular, has been an area of much focus for many decades. A cow's milk yield is largely dependent on the development of secretory tissue during mammogenesis, the secretory capacity of these cells (lactogenesis), and the cow's ability to maintain that secretory capacity (galactopoiesis). An overview of the literature quickly reveals that a primary focus of research efforts has been the involution and remodeling of the bovine mammary gland during the dry period (pregnancy) in preparation for subsequent lactation (Sejrsen, 1978). Traditionally, mammogenesis has been characterized based on pre-pubertal, post-pubertal, and gestational phases of development. However, the focus of this review is mammary development during the pre-pubertal period, with the pre-pubertal period subdivided into the pre- and post-weaning periods. Although comparatively, less research has been directed towards pre-pubertal mammary development, recent literature has demonstrated its importance. The following text is dedicated to understanding the development that occurs in dairy cattle during this period of life.
Prenatal Ruminant Mammogenesis

The mammary gland is an extraordinary organ. While most other organs are well established and functional at birth, most mammary development occurs postnatally (Knight and Peaker, 1982, Gajewska et al., 2008, Capuco and Akers, 2010). Prenatally, mammary development is first noted by the presence of the mammary streak, or the mammary band (Figure 2.1; Anderson, 1978, Sheffield, 1988). The mammary streak appears at a very early embryonic state at about day (d) 75 of gestation in cattle (Anderson, 1978). The mammary line is the next feature of development, first discovered in the early porcine embryo (Schultze, 1892). The mammary line is distinguishable as an area of stratified cells, and provides an explanation for the linear arrangement of the mammary glands on either side on the mid-line along the ventral body surface (Turner, 1930, Anderson, 1978). The mammary bud formation is the next most prominent and discernable stage in the embryo. Histologically, the mammary bud (hillock) is recognized as a compact cluster of epithelial cells (Anderson, 1978). Two primary cell types exist in cattle mammary epithelium: 1) basal epithelial cells and 2) luminal epithelial cells. The basal epithelium consists of myoepithelial cells (MYEC) that surround the epithelial cells, separating them from the underlying stroma (Sternlicht et al., 1997). The MYEC will eventually be responsible for contraction of the mammary alveoli. The luminal epithelium forms ducts and secretory alveoli and contains populations of cells defined by their hormone receptor status (Deugnier et al., 1995, Macias and Hinck, 2012). These two cell types form the network of ducts and lobules that are involved in milk synthesis/secretion during lactation (Gajewska et al., 2008).
Luminal epithelial cell development appears to be more advanced at the earlier stages of life than the bordering MYEC (Deugnier et al., 2002, Adriance et al., 2005, Gudjonsson et al., 2005). However, MYEC cells eventually become more prominent in pre-pubertal dairy cattle, being round in shape, and forming a continuous ring around the circumference of the ducts and epithelial cell clusters (Williams and Daniel, 1983, Ellis et al., 2012, Safayi et al., 2012). Markers exist that allow for the identification of MYEC (i.e. smooth muscle actin (SMA) and cluster of differentiation marker 10 (CD10), but more recently, markers have been identified (i.e. LTBP1L) to aid the identification of specific luminal epithelial cells (Adriance et al., 2005, Ellis et al., 2012, Chandramouli et al., 2013). It appears that SMA, an early marker of MYEC differentiation, is evident at birth, whereas other markers do not appear until later (Ellis et al., 2012). A recent report (Forster et al., 2014) indicates that Transformation-related protein 63 (p63) is an especially good marker for MYEC nuclei in mammary tissue.

An evident basement membrane separates the mammary bud/hillock from the underlying mesenchymal tissue. The mammary bud is formed by a reorganization of epithelium, not overt epithelial proliferation (Propper, 1978). Finally, upon formation of the mammary bud, cell proliferation increases the epithelial cluster size. The mammary bud then extends into the underlying tissue and the mammary sprout forms (Anderson, 1978). Proliferation toward the ventral surface ultimately results in teat formation in cattle. Upon birth, the mammary gland consists of a short branching ductal network imbedded in a dense stroma (i.e. the mammary fat pad [MFP]) adjacent to the teat (Turner, 1931). This basic mammary
structure will be subject to much change during the course of postnatal life in dairy cattle. Images depicting prenatal mammary development can be found in older literature (Anderson, 1978).

Figure 2.1. Stages of growth of the prenatal mammary gland (Anderson, 1978)
Postnatal Ruminant Mammogenesis

At birth the mammary parenchyma (PAR) consists of a rudimentary set of ducts connected to a cistern cavity that connects to the teat cistern, which ultimately connects to the teat duct (Capuco and Akers, 2010). It is accompanied by a fully developed circulatory system and mammary fat pad (Sejrsen, 1994). It is interesting to note however, that the mammary gland at birth is capable of producing secretions that mimic milk with the correct hormone stimulation, a phenomenon often referred to as “witch’s milk” in humans (Macias and Hinck, 2012).

After birth, the mammary gland of the neonatal heifer begins to grow throughout the pre-pubertal period of life. For this growth and development to occur, mammary epithelial cells must proliferate and ductal growth must advance into the surrounding MFP (Ellis et al., 2000, Akers, 2002). However, the mammary gland is not growing at the same rate as the rest of the body during much of this period of time. Immediately after birth, growth of the mammary epithelium is typically equivalent to the rest of the body (i.e., isometric) (Akers, 2002, Moallem et al., 2010) while growth of the non-epithelial tissue is growing faster (Sejrsen, 1994). Around 2 to 3 mo of age, the rate of mammary growth accelerates. The growth of the mammary gland at this time is allometric, and this period of growth ends after the completion of 1 to 2 estrous cycles after onset of puberty (Davis Rincker et al., 2011). Therefore, traditionally-defined allometric growth occurs during the post-weaning phase of the pre-pubertal period, and much, but not all, work has indicated the negative effects associated with increasing rate of gain during this time. This likely reflects a decrease in the usual length of the allometric phase of development.
Allometric growth generally indicates that the mammary gland is growing at least 3 times faster than the rest of the body (Akers, 2002, Moallem et al., 2010) and is characterized by complex ductal development in both mice and cattle (Macias and Hinck, 2012). During this phase of life, growth of the ductal system is occurring in a highly ordered manner (Figure 2.2; Sheffield, 1988).

Species differences do exist between mice and cattle. In rodents, ductal advancement depends on specialized club-shaped structures at the ends of growing ducts i.e. the “Terminal End Buds (TEB)”. These structures serve the dual function of adding new cells and progressing development forward through the MFP (Williams and Daniel, 1983, Silberstein, 2001). Later, after expansion nears completion and the myoepithelium begins to form, TEB transition into “Terminal End Ducts (TED)” (Williams and Daniel, 1983). At this point in development, cattle differ from mice. In cattle, TEB do not exist, but rather, clusters of ductules arising from large ducts exist and are termed “Terminal Ductule Lobular Units (TDLU)” (Hovey et al., 1999) or “Terminal Ductal Units” (TDU) (Ellis et al., 2012). In addition, proliferating epithelial cells in the bovine mammary gland are located throughout the extending ducts, instead of just near the end (Capuco et al., 2002). Solid cords of proliferating epithelial cells eventually penetrate the MFP to set up the framework for future development. Much attention has been paid to the traditionally-defined allometric phase of development in dairy cattle.
Pre-pubertal mammogenesis has been studied extensively for many decades, with the first experiments utilizing rats and mice. Cowie (1949) performed early experiments using an ovariectomized (OVX) rat model to assess prepubertal mammary development. Cowie found that the mammary gland of rats with intact ovaries grew at a rate greater than the rest of the body, whereas OVX rats had compromised development (Cowie, 1949). Years later, Sinha and Tucker (1969) provided a detailed, quantitative assessment of the allometric period of mammary growth and development in cattle based on measurement of mammary gland DNA content. Near this time, it was also discovered that the number of milk synthesizing cells in the mammary gland accounts for much of the variation in milk production between animals (Tucker, 1969, 1981, Shamay and Gertler, 1986, Tucker, 1987, Capuco et al., 2001, Shamay et al., 2005). However, cell number/population and functionality of those cells together allow the mammary gland to perform at its most efficient capacity.

In general, little lobulo-alveolar mammary development occurs until later stages in dairy cattle (i.e., gestation and into lactation; Akers, 2002). The pre-pubertal phase of mammary development is reserved for penetration and filling of
the MFP by a system of individual ducts with minimal branching in rodents. But in ruminants, this phase of development sets up a scaffolding to support the secretory cells needed for subsequent lactation, and a framework to support lobulo-alveolar during gestation (Hammond, 1927, Sheffield, 1988). Upon establishment of the necessary framework, ductal elongation occurs post-pubertal, during which time the ductal system spreads and extends throughout the mammary fat pad via TDLU (Akers, 2000). Terminal Ductule Lobular Units consist of epithelial cell cords that penetrate the stroma, complete with outgrowths branching out from the solid primary cords. At this time the connective tissue is filled with adipocytes earning it the name of MFP. Pre-pubertal mammary development cannot occur without the presences of the MFP, and it must be noted that the MFP also grows during this period, but at a slower rate than the rest of the mammary gland (Faulkin and Deome, 1960). The secretory and ductal tissues in the mammary gland are epithelial in nature, and referred to as PAR (Capuco and Akers, 2010). The MFP contains cellular and non-cellular (collagen, elastin, etc.) components (Capuco and Akers, 2010). Ductal growth can be quite extensive at this time, although data indicates it is minute in comparison to lactation and pregnancy. However, even minor alterations to the mammary gland or related systems pre-pubertally can have substantial consequences (Sejrsen and Purup, 1997). Thus, it appears pre-pubertal mammary development is essential to the future milk production of the dairy cow.

Mammary mass during the first few weeks (wk) post.birth increases by nearly 20-fold (Akers, 2002). This has been attributed to ovarian hormone activity during the early pre-pubertal phase of life. By d 75 of life, mammary PAR tissue
within each mammary gland has increased to the size of a walnut and is easily noticeable via palpation (Capuco and Akers, 2010). By d 90, this tissue has increased by 60-fold. Thus even though the mass of PAR is only a small fraction of that present at the end of puberty, the relative rate of PAR growth is markedly greater than that for the overall rate of body growth i.e. a doubling of body weight vs. a 60-fold increase in PAR mass between one week and 90 days of age. Therefore, although early mammary development has not been considered a part of the traditionally defined allometric period of development, mammary PAR growth is very much allometric at this time.

Early work to evaluate the significance of pre-pubertal mammary development relied heavily on the effects of treatments on subsequent milk yield and/or physical observations to gauge effects of treatments on mammary development in the dairy heifer. Wallace (1953) was among the first to describe bovine mammary growth from birth until puberty and note a role of the ovary in the pre-pubertal heifer. Following the use of DNA content as a measure of overall mammary development, the DNA content of the PAR was used to gauge the growth and development of the ductal structures vs. the MFP during the pre-pubertal period (Sinha and Tucker, 1969). This technology helped quantify the allometric period of growth, and validated previous, qualitative observations. Average weaning age occurs at roughly 6 to 8 wk of age in dairy calves, indicating that traditional allometric growth initiates shortly after weaning. At the conclusion of the allometric growth phase, mammary development returns to an isometric rate, and remains stable until a short time prior to parturition.
**Ovarian Hormone Impact on Mammary Development**

Mammary development is influenced by a number of hormones, which control the rate of epithelial cell proliferation and PAR development (Neville et al., 2002, Ellis et al., 2012). Regulation of mammogenesis is both systemic and paracrine in nature (Figure 2.3). Following up on earlier research (see above), Silver (1953) was able to successfully restore mammary development in OVX mice with estradiol treatment (1 μg on alternate days). Presl et al (1965) subsequently demonstrated that estradiol synthesis in the ovary began well in advance of puberty. A landmark study by Wallace (1953) demonstrated diminished mammary development and decreased PAR mass by OVX of pre-pubertal heifer calves. Turner et al., (1956) subsequently showed that decreased mammary development after OVX in heifers could be restored by administration of exogenous estradiol. The requirement for estradiol during pre-pubertal mammogenesis has been made very evident and determined to be necessary during the beginning stages of life in not only cattle and mice (Ceriani, 1974, Silberstein et al., 1994, Tucker, 2000, Koos, 2011, Velayudhan et al., 2012), but also swine (Horigan et al., 2009), sheep (Kinder et al., 1995), and various other species (Yamamoto and Turner, 1956). Also, administration of exogenous estradiol has also been shown to stimulate PAR growth in calves with intact ovaries (Li et al., 2006), However, excessive estradiol levels may encourage abnormal mammary growth through the action of various growth factors (Sirbasku, 1978).
Figure 2.3. Schematized view of the events occurring during pubertal development (Macias and Hinck, 2012)

By performing an OVX procedure on heifer calves, ovarian hormonal influence on mammary development can be evaluated. Ballagh et al. (2008) ovariectomized Holstein heifer calves at d 40 of life and assessed mammary cell proliferation. Animals subject to the OVX procedure had precocious MYEC differentiation, indicating a potential decrease in mammary epithelial cell proliferation, as MYEC are thought to limit epithelial cell proliferation (Gudjonsson et al., 2005, Safayi et al., 2012). Ovariectomized heifers also exhibited altered PAR cell populations when compared to intact heifers (Ballagh et al., 2008).

Berry and others (2003b) performed an OVX experiment on 8 Holstein heifer calves between 1 and 3 mo of age. Animals subject to OVX averaged a 174 g reduction in mammary mass and a 10-fold decrease in epithelial cell proliferation (0.25 vs. 2.24% of cells dividing, respectively) (Berry et al., 2003b). Meyer et al.
(Meyer et al., 2006a) ovariectomized dairy heifers at 4.6 mo. of age. Thirty d after surgery, estradiol injections were given at a dose of 0.1 mg/kg BW for three consecutive days. Estradiol administration increased cell proliferation and altered gene expression in the PAR and MFP. Estradiol supplementation also increased insulin-like growth factor I (IGF-I) gene expression by 2-fold, and this increase was greater in the MFP. A more inclusive evaluation of gene expression in the mammary glands of these heifers was also performed (Li et al., 2006), wherein they identified 124 estradiol-responsive genes in the PAR and MFP. Li and Capuco (2008) expanded on these analyses; whereby they identified 2,344 transcripts along with pathways and networks that were influenced by estradiol administration.

Purup et al. (1993) performed OVX procedures on heifer calves at 2.5 mo of age. Ovariectomized calves had decreased total mammary gland weight by 150 g and volume by 141 cm³ when compared to intact heifers at wk 15. Ovariectomized calves had a marked reduction in PAR weight by 239 g, total DNA by 1,143 mg, and total protein by 24 g accompanied by an increase (89 g) in extraparenchymal mammary tissue weight at wk 15. The OVX calves also had decreased epithelial tissue and lumen and increased stroma. Ovariectomy decreased circulating estradiol by 33%, and growth hormone (GH) administration was unable to restore normal mammary development in OVX heifers.

These examples reinforce the need for ovarian hormones during pre-pubertal mammary development. The effects of estradiol on mammary development indicate that estradiol is required primarily for epithelial cell proliferation and ductal development. This concept was further reinforced by two experiments when
tamoxifen, an antiestrogen, was given to either heifers with intact ovaries (Tucker et al., 2016) or murine mammary glands in culture (McGrath, 1983). In both cases mammary development was inhibited. However, estrogens are not the only ovarian hormones necessary for mammary development. Progesterone is necessary to promote lobulo-alveolar development and ductal branching (Rivera, 1964, Hovey et al., 2001), and both estradiol and progesterone are mediated by their respective nuclear hormone receptors (Connor et al., 2007). Research by Woodward et al. (1993) assessed the effects of exogenous estradiol and progesterone on pre-pubertal beef calves weighing between 136 and 161 kg. Treatment with exogenous estradiol at a rate of 0.1 mg/kg BW increased incorporation of [³H]thymidine 11-fold at 96 h post administration whereas progesterone-treated animals (0.25 mg/kg BW) were unresponsive. Supplementation of both hormones provided an intermediate response, and autoradiographic labeling of epithelial cell nuclei was increased for animals given estradiol by 50-fold (5.1 vs 0.1%) at 96 h. This study differentiated the potential importance of estradiol vs. progesterone pre-pubertally, (Woodward et al., 1993). However, the necessity of progesterone for successful mammary development should not be discounted as estradiol and progesterone administration have been shown to stimulate the expression of several growth factors in mice (Bocchinfuso and Korach, 1997), and it is well known that a combination of estradiol and progesterone are absolutely necessary for lobulo-alveolar mammary development to occur during pregnancy (Shyamala, 1997, Yart et al., 2012).
In an effort to better determine the concentrations of estradiol and progesterone needed for mammary development, Sud et al. (1968) ovariectomized Holstein heifers at approximately 14 mo of age and studied the impact of varying amounts and ratios of estradiol and progesterone on mammary development. The amount of estradiol and progesterone needed to produce a histological pattern of normal mammary development were 800 and 200 mg, or 400 and 100 mg, respectively (ratio 4:1). Even more, OVX mice treated with 1 μg estradiol and 1 mg progesterone experienced a longer duration of DNA synthesis in the mammary gland with less variation compared to untreated animals (Bresciani, 1965).
Two main estrogen receptor (ER) isoforms, from separate genes, exist in cattle (Kuiper et al., 1996) and ovine (Colitte and Parillo, 2013). Estrogen receptor-α (ERα) appears to be primarily responsible for ductal growth and morphogenesis. Estrogen receptor-β (ERβ), the other isoform, is undetectable via immunohistochemistry in the bovine mammary gland (Connor et al., 2005). Pre-pubertally, ERα appears as the primary isoform in the bovine mammary gland, and is expressed by a portion of the epithelial cells within the PAR region (Capuco and Akers, 2010) and hepatic cells (Matic et al., 2013). In addition, a percentage of stromal cells between lobules may express ERα. Estrogen receptors act by regulating transcription. When activated by ligand binding, the receptors dimerize, translocate into the nucleus, and bind to specific estrogen response elements (ERE) located in the promoter of target genes. A response then occurs via up-regulation or down-regulation of gene expression (Bjornstrom and Sjoberg, 2005, Marino et al., 2006). Interestingly enough, the mechanism whereby estradiol drives mammary development via its receptor remains somewhat of a mystery due to the fact that proliferating mammary epithelial cells do not express ERα (Zeps et al., 1998, Capuco et al., 2002). Interestingly, however, OVX heifers have been shown to have an increased percentage of ERα positive cells compared to control heifers (Berry et al., 2003b). Although proliferating epithelial cells are overwhelmingly ER-negative, epithelial proliferation response induced by estradiol may be initiated via paracrine mechanisms initiated in ERα positive cells to signal cells lacking a functional ER. Data derived using ERα knockout mice (Mallepell et al., 2006, Capuco and Akers,
2010) indicate ER knockout mice have grossly underdeveloped mammary glands (Bocchinfuso and Korach, 1997), (Korach, 1994). Estrogen receptor-α expression is greatest during the pre-pubertal phase, and decreases with age in both the PAR and MFP (Connor et al., 2005, Meyer et al., 2007).
Other Hormones and Growth Factors Involved in Mammary Development

Since estradiol does not act directly on proliferating epithelial cells, it is logical to hypothesize that intermediary factors must play a role (Sirbasku and Benson, 1980, Forsyth, 1989). Estradiol responsiveness was successfully restored in mixed co-cultures of mammary epithelial cells and fibroblasts, indicating that some stromal-epithelial cell interaction must be necessary for estradiol to encourage mammary cell proliferation (Haslam, 1988). A comprehensive review by Lyons et al. (1958) described multiple experiments in rodents to define the importance of hypophyseal hormones in mammogenesis. Injecting OVX/hypophysectomized rats with extracts from intact pituitaries and estradiol markedly increased mammary development whereas rats treated with estradiol alone failed to respond. These results supported the existence of an interactive mechanism(s) between ovarian and pituitary hormones in control of mammary development. Sejrsen et al. (1986) evaluated the interaction between ovarian hormones and GH in stimulation of mammary growth in cattle. In this study, exogenous GH increased the mass of PAR tissue in pre-pubertal heifers by 18%. Subsequently, Purup et al. (1993), demonstrated that the ovary was necessary for GH to stimulate mammogenesis. Specifically, GH increased PAR tissue in intact animals by 30%, but GH had no effect on mammary development in OVX heifers. Sejrsen et al. (1986) administered bovine somatotropin (bST) to 8 mo old heifers and measured a 45% increase in mammary PAR with no impact on tissue composition. However, Radcliff and others (2000) injected heifers with bST starting at approximately 135 kg BW until pregnancy was confirmed. Injection of bST did not increase milk yield regardless of
dietary treatment (0.8 vs. 1.2 kg/d gain) but no analyses were performed to gauge mammary development (Radcliff et al., 2000).

Growth hormone injections were shown to increase mammary cell proliferation in 6 mo old heifers and increase incorporation of [³H]thymidine into DNA of the PAR in 18 mo old heifers (Berry et al., 2001). Injections of GH did not alter the percentage of epithelial cells expressing ERα. Other recent data has indicated that GH injections given during the pre-pubertal period increase protein and DNA content in the PAR of younger prepubertal heifers (Huderson et al., 2011). The action of GH on the mammary gland remains somewhat of a mystery, as GH receptors are not typically detected via ligand binding assays in the mammary tissue and mammary explants do not respond to added GH in vitro (Keys and Djiane, 1988, Berry et al., 2003b). However, some researchers have demonstrated the presence of GH receptors in bovine mammary tissue immunocytochemically (Feldman et al., 1993, Sinowatz et al., 2000, Plath-Gabler et al., 2001) and via gene expression assays (Glimm et al., 1990, Hauser et al., 1990, Knabel et al., 1998). These complications still leave much to be understood in terms of the role GH plays in mammary development.

Perhaps the effects of GH on bovine mammary development may be both direct and indirect via the capacity of GH to elicit an increase in IGF-I. Indeed, it has long been hypothesized that many of the effects of GH occur through enhanced secretion of IGF-I (Salmon and Daughaday, 1957, Ruan et al., 1995) as IGF-I is increased when exogenous GH is administered (Purup et al., 1993, Umayahara et al., 1994). In addition, treatment with exogenous IGF-I increases mammary epithelial
cell proliferation \textit{in vitro} (Shamay \textit{et al.}, 1988) and IGF-I receptors have been located on mammary epithelial cells (Purup \textit{et al.}, 1995). Insulin-like growth factor-I is an example of one of the many growth factors that may be acting in concert with GH to impact mammary development (McGuire \textit{et al.}, 1992).

Multiple research efforts have been directed toward understanding the likely role of IGF-I in mammary development. Exogenous GH stimulates an increase in circulating IGF-I (Breier \textit{et al.}, 1991, Purup \textit{et al.}, 1995, Walden \textit{et al.}, 1998, Akers \textit{et al.}, 2005). Local production of IGF-I by the fibroblasts in the stroma and MFP has been shown to stimulate mammary development (Shamay \textit{et al.}, 1988, Romagnolo \textit{et al.}, 1992). In fact, in rodents, estradiol has been shown to increase IGF-I production in the MFP (Kleinberg, 1997) by stimulation of ERα positive fibroblasts and adipocytes and altering estradiol status has been shown to impact gene and transcript activity in the bovine mammary epithelium and fat pad (Li \textit{et al.}, 2006, Meyer \textit{et al.}, 2006a, Li and Capuco, 2008). When estradiol was administered to Holstein heifers, MFP expression of IGF-I increased 1.5 fold compared to non-treated heifers (Berry \textit{et al.}, 2001). This local increase in IGF-I likely promotes mammary epithelial cell proliferation in the PAR tissue adjacent to the MFP (Meyer \textit{et al.}, 2007). Others have shown decreased circulating IGF-I concentrations when gains are increased during the pre-pubertal period, which may be associated with decreased mammary development and future milk yields observed (Akers \textit{et al.}, 2000).

The effects of IGF-I are complicated by the presence of at least six insulin-like growth factor binding proteins (IGFBP) (Baxter and Martin, 1989). The most
common IGFBP is IGFBP-3, which has a high affinity for IGF-I binding. These IGFBPs prolong the half-life of IGF-I, transport IGF-I from the circulation, and localize IGF-I to the target cells (Clemmons, 1998). Low concentrations of IGFBP-3 have been shown to be an agonist for IGF-I action, serving as a vehicle to transport IGF-I to its receptor, IGF-IR, whereas high concentrations of IGFBP-3 have been shown to be an antagonist (De Mellow and Baxter, 1988, Blum et al., 1989, Purup et al., 2000, Capuco and Akers, 2010). This is supported by a 50% decrease in IGF-I mitogenic activity when IGFBP-3 was added to tissue explants at a concentration 4 times greater than IGF-I, which resulted in a 26% decrease in DNA synthesis (Weber et al., 1999). It also must be noted that insulin can act in place of IGF-I when present in pharmacological amounts because of the capacity of insulin to bind IGF-1R at high concentrations (Shamay et al., 1988).

IGF-I is primarily synthesized in the liver, but IGF-I mRNA is also detected in the MFP and stroma (Akers et al., 2005). Production of IGF-I mRNA increases during the allometric phase of mammary development (Hovey et al., 1998a). IGF-I mRNA expression is greater in MFP tissue samples compared to PAR tissue samples (Akers, 2002) and IGFBPs have been found to also be locally synthesized in the mammary gland (Clemmons, 1998). To further illustrate the role IGF-I plays in mammary development, OVX performed on pre-pubertal heifers (~4 mo old) decreased mammary PAR growth at 6 mo of age. Ovariectomized heifers had decreased IGF-I expression from MFP tissue in addition to increased IGFBP-3 expression. Increases in IGFBP-3 expression also coincided with a decrease in IGF-I concentration. (Weber et al., 2000). Another study indicated that IGF-I expression did not differ when an
epithelium-free MFP was prepared, providing further evidence that IGF-I expression is influenced by the presence of the adjacent PAR (Berry et al., 2003a). Recently, researchers have tried to determine whether locally produced IGF-I in the mammary gland is more crucial to mammary gland development than systemically produced IGF-I in the liver (Macias and Hinck, 2012). Initial results in the mouse have suggested that the paracrine action of locally produced IGF-I plays a more central role. This was made evident by a hepatic deletion of the IGF-I gene resulting in a 75% reduction in circulating IGF-I concentrations in the blood, while normal mammary gland development was still maintained (Richards et al., 2004).
Role of the Mammary Fat Pad

During development, the MFP begins as a population of stromal cells, in the mesenchyme layer, that remains closely invested with the mammary ducts during postnatal life (Akers, 2002). These cells eventually differentiate and develop into a well-defined MFP, consisting mostly of adipocytes (Hovey et al., 1998b). The MFP also contains other essential elements such as nerves, blood vessels, fibroblasts, various immune cells, and connective tissue (Hovey and Aimo, 2010, Hodson et al., 2013). The bovine MFP is first visible around d 80 in fetus (Oka and Yoshimura, 1986, Sheffield, 1988, Akers, 2000). The MFP facilitates many functions for the rest of the mammary gland including providing a 3-D matrix for physical development of the ductal system, housing vasculature and lymph systems, hormone action, and growth factor synthesis (Oka and Yoshimura, 1986, Hovey et al., 1999). The growth factors synthesized include, but are not limited to: fibroblast growth factors (Wilson et al., 1994), transforming growth factors (Snedeker et al., 1991), hepatocyte growth factors (Rahimi et al., 1994), wnt proteins (Weber-Hall et al., 1994), unsaturated fatty acids (Wicha et al., 1979), and insulin-like growth factors (see above). The presence of the MFP is critical for the growth and differentiation of epithelial cells (Cunha et al., 1995) as early development into the MFP ultimately determines the extent of lobulo-alveolar development later in life (Thibault et al., 2003). According to Ormerod and Rudland (1984), for the PAR tissue to eventually populate the entire MFP, proper linear duct elongation and expansion must occur first. Thus, the MFP is essential for development of the mature 3-D structure of the mammary gland (Hoshino, 1978, Neville et al., 1998, Rowson et al., 2012).
Two main developmental roles surface as pertinent in regards to the MFP: 1) facilitation of proliferation, spreading, and branching of the PAR into the MFP (McFadden et al., 1990, Akers, 2000, Capuco and Akers, 2010) during allometry and 2) the ability of the MFP to produce growth factors in response to sex hormones (Clemmons, 1998, Hovey et al., 1998a, Weber et al., 2000, Akers, 2002, Berry et al., 2003a, Meyer et al., 2006a). Once the MFP has its cohort of mammary ducts after puberty (partially filled in cattle), this framework of ductal elements is adjacent to connective tissue. The framework is then poised to respond with markedly increased cell proliferation during gestation. (Hammond, 1927).

Without the MFP, mammary gland development would be compromised. Hovey et al. (1998b) found that DNA content was increased nearly eight fold after a 7-day culture when the MFP was left intact compared to when it was removed from the epithelium of murine samples. Even further, addition of IGF-I to cultures with an intact MFP increased final DNA content by roughly 1.5-fold. This study confirmed the significance of the MFP in increasing mitogenic activity of the epithelium.

Research has shown that development of the MFP responds to dietary manipulation. Total lipid mass of the mammary gland was increased when target average daily gain (ADG) was doubled in pre-pubertal heifers (Sejrsen et al., 1982). Daniels et al. (2009a) was able to increase MFP weights in calves by 3.8-fold and increase MFP lipid content by 1.5-fold by feeding more nutrient dense milk replacer (MR) pre-weaning. Protein content of the MFP was also altered by dietary treatment. However, all animals were harvested by d 65, so no long-term effects could be ascertained from these findings. McFadden et al. (1990) found increased
MFP weights (as a percentage of the whole mammary gland) and increased MFP lipid content when lambs were given ad libitum access to high energy diets starting at wk 7. Lambs given free access to high-energy diets also had greater total MFP DNA (McFadden et al., 1990). It has been hypothesized that the decreased PAR growth found in heifers raised on a high plane of nutrition is not due to the increased expansion of the MFP experienced by these heifers (Thorn et al., 2008).

The MFP is also highly responsive to estradiol stimulation. A greater IGF-I mRNA response was found in the MFP for estradiol-treated animals when compared to animals not given exogenous estradiol (Meyer et al., 2006a). Additionally, ERα expression does not decrease as BW increases in the MFP like it does in the mammary PAR. Another study found that IGF-I expression was not changed in the MFP when heifers (10 d of age) were reared on diets to achieve either 650 or 950 g/d BW gain and the MFP was found to have the greatest influence on epithelial tissue adjacent to it, as this tissue experienced greater proliferation compared to more centrally located epithelial tissue (Meyer et al., 2007). It appears logical that manipulation of the MFP may influence development of the mammary gland. A study has not been conducted to assess the responsiveness of the MFP to exogenous estradiol and dietary manipulation in the same experiment.
Myoepithelial Cells

Myoepithelial cells are the contractile cells located in the mammary gland, and along with epithelial cells, make up the majority of cells in the mammary epithelium. In mice, MYEC surround the epithelial cell clusters in a single row, and are easily visualized histologically (Williams and Daniel, 1983). However, this is not always true in dairy cattle. Pre-pubertally, MYEC do not usually form a continuous ring around epithelial clumps, as in rodents. Moreover, when calves are OVX pre-pubertally, MYEC morphology is altered. Myoepithelial cells in OVX calves change morphology from a rounded shape (normal) to a more elongated, stellate shape (OVX), which may suggest premature differentiation. This reflects observations in adult cattle where the MYEC form an elongated, interwoven layer surrounding the other mammary cells. It is hypothesized that this layer of MYEC modulate the response of MYEC to the actions of various growth factors (Ballagh et al., 2008). Ovariectomized calves are hypothesized to have decreased mammary gland proliferation due to decreased hormone action as a consequence of altered MYEC morphology (Ballagh et al., 2008, Safayi et al., 2012).
Nutritional Influences on Mammary Development

Rapid rearing of dairy heifers provides potential to increase profitability by decreasing the non-productive period of a heifer’s life (see above). However, it is well documented that rapid, pre-pubertal rearing can have deleterious effects on mammary development and future milk production (Capuco and Akers, 2010). To truly understand the detrimental effects of pre-pubertal nutrient intake on mammary development and future milk production, effects must be distinguished between the pre-weaning and post-weaning phases of the calf’s life. With an increasing amount of research focused on the pre-weaning phase, data has begun to suggest that enhanced feeding during this time is likely beneficial. The following sections are dedicated to describing the effects of nutrition on mammary development and milk potential.
Effects of Nutrition during the Post-pubertal period

In contrast with pre-puberty, increased postpubertal nutrient intake does not appear to have deleterious effects on subsequent milk production (Sejrsen et al., 2000). For example, Sejrsen et al. (1982) utilized 12 post-pubertal heifers (13.1 mo of age, 302 kg BW) fed for a decreased and increased ADG, (588 and 1,164 g/d). No differences were found for total mammary gland or PAR weight, and no excess fat deposition was found in the mammary gland (1,751 vs. 2113 g of total mammary lipid weight). Neither were there differences in PAR DNA or lipids. When assessed as a percent of the whole mammary gland, no differences were found for epithelial cells (10.7 vs. 13.8%), connective tissue (48.6 vs. 53.6%), or fat cells (37.4 vs. 29.9%). Others (Macdonald et al., 2005) reported a slight increase in milk production when ADG was increased starting at puberty until 22 mo of age (Macdonald et al., 2005). These studies illustrate the contrast in response to enhanced feeding pre- vs. post-puberty (Sejrsen et al., 1982, Abeni et al., 2000).
Effects of Nutrition during the Pre-pubertal period

Although it has been generally accepted that excessive pre-pubertal nutrition can have dramatic, negative effects on mammary development in dairy heifers, mechanism involved are poorly understood. Diets dense in nutrients that are fed in an attempt to increase BW gain are appealing as they decrease the age to sexual maturity and calving (Akers, 2000). However, increased pre-pubertal feeding can adversely alter mammary development, lending support to the hypothesis that developmental impairment at this time causes a lifetime decrease in the number of secretory cells in the mammary gland (Tucker, 1981) and ultimately reduced lactation potential (Capuco and Akers, 2010). It has been suggested that over-feeding energy in early life leads to excess fat deposition (Sejrsen et al., 1982) and that this promotes a physiological shift to nutrient partitioning in favor of fat deposition instead of lactation performance (Capuco et al., 1995, Gaynor et al., 1995).

Examples of the detrimental effects of increased pre-pubertal nutrient intake are numerous (Schultz, 1969). Decreased mammary development was first observed as a result of increased feeding level long ago (Herman and Ragsdale, 1946) and was later confirmed in multiple experiments (Swanson, 1960, Gardner et al., 1977, Little and Kay, 1979). In a more recent example, Radcliff et al. (2000) fed 70 Holstein heifers a diet targeting a BW gain of 0.8 or 1.2 kg/d beginning at about 125 kg of BW. Calves fed for greater gain had increased pre-breeding ADG, and were approximately 90 d younger at first insemination. However, heifers fed at a greater rate produced 14% less milk during first lactation compared with controls. This
suggests that the impairments in mammary growth noted in other slaughter studies (Sejrsen et al., 1982) may have reduced/altered mammary development permanently, reducing future milk yield. It is also possible that other factors such as altered metabolism or endocrine effects may also impact future mammary development/function. For example, a companion study also observed decreased GH concentrations when animals were fed for increased ADG (Sejrsen et al., 1983).

Previous research had led researchers to not only look at energy content in the pre-pubertal diet as it effects mammary development, but also energy sources. Capuco et al. (1995) utilized 160 Holstein heifers starting at 175 kg BW to determine the effects of rate of gain (725 vs. 950 g/d) and energy source (alfalfa silage vs. corn silage) on mammary development. Increased weight gain decreased serum GH for both energy sources, whereas mammary PAR DNA and RNA were only reduced for animals fed corn silage at an increased rate. This decrease in PAR DNA and RNA did not translate into decreased lactation performance. Other researchers found that heifers fed greater amounts of energy for longer durations during the pre-pubertal period (starting at 11 wk) experienced increased fat deposition in the MFP resulting in greater mammary mass. However, when adjusted for carcass weight, mammary PAR DNA and RNA decreased linearly as duration of energy intake increased. No lactation data was available for this trial (Davis Rincker et al., 2008). It is hypothesized that decreases in development associated with increased intake during the pre-pubertal period reflect a truncation of allometry. One reason for this is previous work (Meyer et al., 2006b) that found that DNA accretion rate was not impacted by plane of nutrition. Then, as rate of gain increases, the amount
of time the animal spends in the traditional allometric growth period is decreased (Daniels et al., 2006, Thorn et al., 2008, Daniels et al., 2009a), resulting in decreased mammary development. Decreased mammary development associated with increased intake/growth has been found in multiple studies (Sejrsen, 1978, Harrison et al., 1983, Petitclerc et al., 1984, Bettenay, 1985, Sejrsen and Foldager, 1992, Peri et al., 1993, Hohenboken et al., 1995) and reviewed in a meta-analysis (Zanton and Heinrichs, 2005).

One study compared the effect of three separate growth rates (0.6, 0.8, and 1.0 kg/d) on first lactation performance. Holstein heifers (n= 273) on this trial were assigned to one of the three growth rates starting at 90 kg BW and left on trial until 320 kg BW (Van Amburgh et al., 1998). Heifers fed to gain 1.0 kg/d calved about 3 mo earlier than heifers fed to gain 0.6 kg/d and these heifers produced more milk in their first lactation compared to calves gaining 0.6 kg/d (9387 vs. 8558 kg, respectively). However, when pre-pubertal ADG was evaluated on a continuum, little of the variation in milk yield was explained by ADG. This agrees with others (Macdonald et al., 2005) who also found that increased ADG during the pre-pubertal period was unrelated to milk yield (Macdonald et al., 2005). In addition, (Weber et al., 2000b) found no difference in IGF-I or IGFBP-3 mRNA abundance in mammary tissue of animals fed to gain 1.1 compared with 0.55 kg/d.

Similarly, no effect on 1st lactation performance was found when animals were fed to achieve 725 or 395 g/d BW gain on either alfalfa silage or corn silage based diets (Waldo et al., 1998). These data are in agreement with previous work indicating that pre-pubertal energy or protein intake may not negatively impact
mammary development (Valentine et al., 1987, McFadden et al., 1990, Pirlo et al., 1997, Abeni et al., 2000, Whitlock et al., 2002). It is clear that much is yet to be understood when assessing the effects of pre-pubertal nutrition on mammary development and subsequent lactation.

Lammers et al. (1998) utilized 68 Holstein heifers in a 2 x 2 factorial arrangement of treatments starting at 4.5 mo of age to evaluate feeding rate and estradiol treatment. Heifers were targeted to gain either 700 or 1000 g/d BW and half of the animals in each rate of gain were given an exogenous estradiol implant, which was removed at 9.5 mo of age. Feeding for an increased rate of gain reduced age at puberty by 32 d. Animals reared for an increased rate of gain had decreased first lactation performance by 7.1% and estradiol implantation decreased first lactation performance by 5.2%. It is interesting to note that estradiol implantation increased teat length during the treatment period indicating that the implant provided enough estradiol to these pre-pubertal heifers to illicit a response. However, mammary development was only evaluated by non-invasive means, and blood estradiol levels were not quantified in this trial, creating difficulty in drawing any meaningful mechanistic conclusions.
Effects of Nutrition during the Pre-weaning period

The general goal of the pre-weaning period is to attain optimal weight gains, develop a strong and competent immune system, maximize health, and to stimulate rumen development. Recent studies have focused on the effects of enhanced feeding during the pre-weaning period on mammary development. It is well accepted that events occurring, and decisions made pre-weaning can have lasting effects throughout a dairy cow’s life (Heinrichs and Heinrichs, 2011). Milk replacer manipulation has garnered much attention as nearly 70% of calves in the United States consumed MR at some point during the pre-weaning period in 2009 (Raeth-Knight et al., 2009), with that number increasing to well over 85% in 2011 (USDA, 2011). In addition, modifying energy and protein concentrations in the MR is known to be an effective means to increase weaning weight and frame measures in calves (Hammon et al., 2002, Brown et al., 2005b, Bartlett et al., 2006, Cowles et al., 2006, Quigley et al., 2006, Hill et al., 2008, Hill et al., 2009, Raeth-Knight et al., 2009, Geiger et al., 2014). A comprehensive review by Khan et al. (2011) provides the multiple, beneficial effects MR manipulation can provide to calves.

One study utilized 78 heifers starting at 45 kg BW (9.9 d old) reared to gain 650 or 950 g/d. Increased ADG increased MFP weight and DNA content of the PAR. Nutrient intake did not affect lipid or protein content of the PAR (Meyer et al., 2006c). In addition, treatment did not influence mammary epithelial cell proliferation or PAR DNA accretion rate and exit from the allometric growth phase of development did not differ (Meyer et al., 2006b). Moreover, when samples were analyzed for ductal development, ADG did not appear to have a negative effect
during the pre-weaning period on complexity of development (Daniels et al., 2009b). Further analysis determined that the expression of 131 mammary tissue proteins were influenced and over 1,500 genes in both the PAR and MFP had altered expression as a result of differential pre-weaning feeding (Daniels et al., 2006, Piantoni et al., 2012). Other research also found increased expression of genes responsible for cell proliferation when MR with an increased crude protein content was fed (Naeem et al., 2012).

Another study obtained similar results as no difference in PAR mass or composition was found when calves were fed an enhanced MR diet. However, MFP mass was increased and accompanied by an increased lipid content (Daniels et al., 2009a). This study also found that increased nutrient intake increased circulating IGFBP-3 in pre-weaned calves (Daniels et al., 2008). Mammary fat pad data agrees with previous research that found that feeding an enhanced MR diet by adding oil increased MFP mass by 78% at 4 mo of age and 93% at 12 mo of age (Thibault et al., 2003). Another study increased MR intake and found that BW were increased without any adverse effects on first lactation performance (Terre et al., 2009). This agrees with more recent work that found that increasing MR powder intake from 1.2 to 2.1% of BW did not affect first lactation production (Davis Rincker et al., 2011). It appears that when an enhanced diet is fed to young calves, the MFP readily responds, while the PAR may not. Perhaps the reason that the mammary PAR appears unresponsive to nutrient alterations is due to experimental diets not being different enough.
Another study assessed the effects of protein and energy intake in the MR from 2 to 8 wk of age. Calves fed greater nutrients were given a MR with greater CP content at a greater rate. Enhanced fed calves had increased total PAR, PAR DNA and RNA, and concentrations of DNA and RNA in the mammary gland. No differences were found for PAR fat in this study. Calves were not followed through to first lactation to assess milk yield (Brown et al., 2005a). These data may partly explain findings from other research assessing the effects of increased nutrient intake on lactation potential and factors associated with mammary development (Petitclerc et al., 1999, Margerison et al., 2013). If differences remain until lactation, this may explain lactation differences found previously due to feeding calves better pre-weaning.

Other studies have focused on providing increased nutrients during the pre-weaning period through the use of whole milk (WM) instead of MR, which is of additional interest because WM is known to include growth factors such as IGF-I, IGF-II, and IGFBP (Vega et al., 1991, Skaar et al., 1994, Bar-Peled et al., 1997). Moallem et al. (2010) fed MR or WM to 46 Holstein calves from 4 to 60 d of age. Calves fed WM had increased ADG during the pre-weaning period accompanied by a greater weaning BW. Adipose tissue weights were 4% greater for calves fed WM, but calves fed WM produced 10.3% more milk during their first lactation. It was concluded that feeding WM to calves at a young age increased ADG and had long-term positive effects on lactation potential. Bar-Peled et al. (1997) conducted similar research. Forty Holstein heifers were assigned to treatment at birth and were fed either MR or allowed to suckle from their dams three times daily. Calves allowed to
suckle had increased ADG during the pre-weaning period and increased BW at weaning. These calves also tended to produce more milk during first lactation (9624 vs. 9171 kg/300 d). These results may indicate that feeding a liquid feed with greater nutrient content may provide lactation benefits without negatively altering mammary development.

A recent study (Soberon et al., 2012) assessed the relationship between nutrient intake (from either WM or MR) and pre-weaning growth rate on lactation performance. Data from two farms over an extended period of time were used. Initially this study credited the “lactocrine hypothesis” as reason for improved lactation performance as a result of pre-weaning nutrition. The lactocrine hypothesis was originally derived from pig studies that found that WM born factors (primarily relaxin) were needed for full reproductive tract development to occur postnatally (Bartol, 2009). More recently, the lactocrine hypothesis has been applied to cattle in an attempt to credit WM-derived factors (hormones, proteins, IGFs, etc.) for increases in observed milk yield. However, when data was analyzed, ADG gain was significantly correlated with first lactation milk yield regardless of whether WM or MR was fed. On average, heifers produced 850 kg more milk in first lactation for every 1 kg increase in ADG during the pre-weaning period on one dairy, and 1,113 kg more milk for every 1 kg increase in ADG on the second dairy. When energy intake was assessed, cows produced 235 more kg of milk in first lactation for every Mcal of intake of liquid feed over maintenance on the first farm. Pre-weaning ADG accounted for 22% of the variation of first lactation milk yield on this trial for both farms and the authors concluded that liquid feed intake is responsible for some
form of epigenetic programming during the pre-weaning phase of life that sets the heifer up for future lactation success. Whether this will be confirmed in future trials is yet to be determined, but it seems certain that manipulation during the pre-weaning phase offers an opportunity for further research to improve mammary development (Soberon et al., 2012, Kertz and Loften, 2013).
Effects of Fatty Acid Profile on Mammary Development

It appears that certain fatty acids, such as linoleic acid, are essential for epithelial cell proliferation in tissues other than mammary tissue, and thus, may play a role in mammary epithelial cell development (Bandyopadhyay et al., 1987). For example, mice fed linoleic acid-deficient diets had compromised mammary development (Miyamoto-Tiaven et al., 1981). Feeding a protected fat supplement (polyunsaturated fatty acids) to lambs increased the amount of stroma tissue in lambs’ mammary glands, thus providing more potential area for mammary gland expansion and proliferation (McFadden et al., 1990).

Providing heifers with diets rich in soybean oil had no effect on body weight or mammary PAR/MFP weight, but increased 18:1 trans fatty acid concentration in the plasma (Thibault et al., 2003). Despite the fact that treatments ended 6 mo of age, when heifers were assessed at 12 mo of age, heifers fed the high oil diet had a 10% less MFP mass than controls. To complicate matters further, 18:1 trans fatty acids, have been shown to inhibit proteases associated with and required for PAR development. It can be concluded that increased trans fatty acids in the diet may in fact limit MFP and PAR development in dairy calves.

Supplementing Jersey calves with MR diets rich in medium-chain fatty acids (coconut oil) increased frame measures without increasing dry matter intake (Bowen Yoho et al., 2013). Gene expression analysis of liver tissue from the same calves yielded no differentially expressed genes (Swank et al., 2013). However, Holstein calves fed similar diets had increased liver weights (2.43 vs. 2.76 kg, 15%) when compared to their herd mates (Mills et al., 2010). This one example may
indicate that the effects of MR fatty acid composition vary in cattle. When grazing, lactating cows were supplemented with unprotected unsaturated fatty acids, 972 genes in the mammary gland were affected with an absolute fold change of 1.3 (Mach et al., 2011). It appears that fatty acid profile may impact mammary gland development/function, although that effect is still to be fully understood.
Compensatory Growth Programs

Compensatory gain programs utilize alternating periods of nutrient restrictive and nutrient excessive feeding to manipulate mammary growth and subsequent function. These programs were developed to keep growth minimal during the restrictive feeding phase, and accelerated during the excessive stage (Park et al., 1989). The periods of excessive gain are also designed to align with periods of time when the mammary gland is hormone-sensitive/growing (Park et al., 1998).

Ford and Park (2001) assessed the efficacy of a compensatory feeding program in a 3-2-4-3-4-2 mo schedule starting at 6 mo of age. The first 3-month period was a restriction phase, followed by a 2-month excessive stage, alternating restrictive and excessive phases until completion of the trial. It was found that implementing a compensatory feeding program increased first lactation yield and second lactation yield by 21 and 15%. Another similar experiment was conducted to assess mammary development in Holstein heifers as a result of a compensatory feeding regimen. Stair-step feeding increased DNA, RNA, protein, the ratio of RNA to DNA, and the ratio of protein to DNA in the mammary gland of Holstein heifers in middle to late pregnancy. Stair-step feeding also decreased lipid content of the mammary tissue in late pregnancy and increased first lactation milk yield by 9% (Choi et al., 1997). Villeneuve et al. (2010) also reported that feeding restricted nutrients just prior to puberty positively influenced mammary development in ewes.
**Mammary Stem Cells**

The importance of adult stem cells to the biology of both humans and animals cannot be denied. Adult stem cells make up a small percentage of the cells found in mature organ systems, but these cells have the ability to self-renew and generate daughter cells for multiple cell lineages (Figure 2.4; Colitti, 2010, Macias and Hinck, 2012). However, the pluripotent ability of stem cells decreases with age. In mice it has been shown that one stem cell can regenerate an entire mammary gland PAR (Kordon and Smith, 1998; Visvader and Smith, 2011). Other studies have been able to regenerate ductal networks *in vivo* from transplanted mammary stem cells (MSC) and also regenerate mammary glands from MSC transplanted into MFP of virgin mice (Faulkin and Deome, 1960, Young et al., 1971, Ormerod and Rudland, 1986, Kordon and Smith, 1998).

![Figure 2.4. A schematic representation of the stem cell hierarchy within the mammary gland (Macias and Hinck, 2012)](image)

Without the regenerative capabilities of the MSC population, cell recovery would not occur after involution during the dry period in cattle. The MSC population
is therefore responsible for the generation of cells for redevelopment of the mammary epithelium at the end of involution. Because it is believed that there are some common mammary stem cell markers, it may be possible to identify stem cell populations in the bovine mammary gland (Li et al., 2009).

Capuco et al. (2012) have suggested that bovine mammary cells that retain incorporated 5-Bromo-2’-deoxyuridine (BrdU) are a part of the population of MSC. Mammary stem cells can be identified by BrdU incorporation as BrdU is incorporated into the DNA of cells, and the parental strand is retained by the stem cell when it undergoes asymmetric cell divisions. Cross-referencing BrdU incorporation with other cell markers may provide a means to improve MSC identification in bovine samples (Ellis and Capuco, 2002, Capuco et al., 2012).

Mammary stem cell manipulation offers great potential. Logic suggests such manipulation would be applied when the MSC population is most abundant. Ellis and Capuco (2002) noted that the number of pale staining BrdU retaining cells, (believed to include true MSCs), decreases from 2 to 8 mo of age. This suggests that manipulation of the MSC population may be most effective in young animals. However, it should still be noted that even at a young age, this population still makes up less than 0.5% of the total cell population. Evidence exists for the ability to manipulate MSC population in dairy cattle. A recent study conducted by Tucker et al. (2016) found that an estrogen antagonist actually increased the apparent MSC population by about 2.8-fold. This difference was even more pronounced (6.3-fold) in tissue regions that was assumed to be dividing more actively (i.e., near the border of the PAR and MFP). To the best of our knowledge, no research has been done to
assess the MSC population in calves fed differing diets and treated with a mammogenic stimuli; although Daniels et al. (2009a) found that the MSC population was unaffected by liquid diet.
Summary and Rationale

It has been well documented that estradiol is absolutely essential for mammary development in the pre-pubertal heifer. Moreover, ductal elongation and advancement into the MFP during the pre-pubertal period of life establishes the framework for future production. Finally, it is generally understood that the mammary gland can be manipulated to some extent by nutrition during the milk-fed stage of life, and that the most detrimental nutritional impacts on mammary gland development occur when excessive nutrients are fed during the traditional allometric growth phase of life, but not prior to that time.

Therefore it is logical to hypothesize that nutritional manipulation of the mammary gland during pre-weaning may provide an opportunity to promote subsequent PAR growth after puberty and during gestation. It is also logical to hypothesize that selective additional mammogenic stimulation (i.e. an estrogenic stimulus) at weaning, might accelerate ductal development and provide an advantage for subsequent mammary growth.

Therefore, we hypothesize that by feeding a nutrient dense MR during early life, a larger MFP and PAR will be present at weaning. We further hypothesize that providing an estradiol stimulus at weaning through 2 wk post weaning, will result in a mammary gland with greater future milk potential. It is also expected that animals fed a nutrient dense diet pre-weaning will exhibit greater lactation potential, regardless of endocrine treatment.
References


Research Objectives

The objectives of this research are to:

1) Compare mammary gland weight and composition of pre-pubertal Holstein heifer calves fed MR varying in fat and protein content.

2) Compare ductal elongation into the MFP of pre-pubertal Holstein heifer calves fed MR with varying energy and nutritive content with or without an exogenous estradiol stimulant.

3) Compare overall pre-pubertal mammary gland development and histology of Holstein heifer calves fed MR with varying energy and nutritive content with or without an exogenous estradiol stimulant.

4) Determine dose-dependent responses of mammary tissue from pre-pubertal Holstein heifers fed MR with varying energy and nutritive content in-vitro to estradiol stimulation.
CHAPTER III. GROWTH, INTAKE, AND HEALTH OF HOLSTEIN HEIFER CALVES FED AN ENHANCED PRE-WEANING DIET WITH OR WITHOUT POST-WEANING EXOGENOUS ESTROGEN


ABSTRACT

Research has shown that changes in nutrition both before and after weaning can impact mammary development. Additionally, estradiol is known to be a potent mammogenic stimulant. Our objectives were to determine effects of altered pre-wean feeding and exogenous estradiol post-wean on growth, intake, and health. Thirty-six Holstein heifer calves were reared on: 1) a restricted milk replacer (MR) diet fed at 0.44 kg powder dry matter (DM)/day (R; 20.9% crude protein [CP], 19.8% fat, DM basis), or 2) an enhanced MR fed at 1.08 kg powder DM/day (EH; 28.9% CP, 26.2% fat, DM basis). MR feeding was reduced 50% during week 8 to prepare for wean. Starter was offered after week 4 but balanced between treatments. Body weight and frame were measured weekly with intakes and health monitored daily. At wean a subset of calves were harvested (n = 6/diet). Enhanced-fed calves had greater carcass, thymus, liver, spleen and mammary gland (parenchyma and mammary fat pad) weights. EH calves also had greater average daily gain (ADG) starting during week 1 (0.36 vs. vs. -0.06 kg/d) and lasting through week 7 (1.00 vs. 0.41 kg/d). Remaining calves received estradiol implants or placebo and were harvested at the end of week 10 creating four treatments: 1) R, 2) R + estradiol (R-E2), 3) EH, and 4) EH + estradiol (EH-E2). Post-wean ADG was similar between R, EH, and EH-E2 calves, but greater in R-E2 calves than EH calves.
EH-E2 calves had the heaviest mammary glands and R-E2 calves had heavier mammary glands than R calves. EH calves consumed more MR dry matter, CP, and fat, pre-weaning. R-fed calves consumed more starter DM pre-weaning. Fecal score was greater for EH calves (1.74 vs 1.50) pre-weaning, but days medicated did not differ. Fecal scores were lower for R-E2 calves post-weaning. Improved pre-weaning feeding of calves increased body weights and frame measures. Differences in body weights remained post-weaning. Enhanced fed calves showed greater ADG during the pre-weaning period but not post-weaning. Exogenous estradiol may elicit diet dependent growth responses. Analysis of collected samples will allow determination of cellular and molecular processes responsible for the marked differences in mammary development observed.

**Keywords:** mammary gland, milk replacer, estradiol, calf
INTRODUCTION

Some research has shown that greater pre-pubertal average daily gain (ADG) decreases mammary gland development and subsequent milk yield. Radcliff et al. (2000) fed Holstein heifers either a diet targeting a BW gain of 0.8 kg/d or 1.2 kg/d beginning at 125 kg of BW. Calves fed for a greater rate of gain had greater pre-breeding BW gains and were approximately 90 d younger at first insemination. However, these heifers produced 14% less milk during their first lactation. This data corresponds with others (Sejrsen et al., 1982) reporting that mammary parenchymal (PAR) tissue mass and DNA content were decreased by 23 and 32%, respectively, when pre-pubertal heifers were fed for greater weight gain beginning after weaning. However, the mechanisms involved are still not well understood (e.g. failed cell proliferation or cellular differentiation, premature puberty and/or blunting of normal waves of allometric mammary growth with each estrus cycle, etc.; Meyer et al., 2006 a,b).

Recent data (Soberon et al., 2012), however, has indicated that the negative correlation between gain and mammary development may not be the same throughout the entire pre-pubertal period. Specifically, a greater rate of gain during the pre-weaning period may be beneficial to mammary gland development. Historically, it was generally thought that mammary PAR growth was largely quiescent until the onset of allometric mammary growth at approximately 2 – 3 months of age. Recent reports (Capuco and Akers, 2010; Esselburn et al., 2015) show that allometric growth begins much earlier (i.e. a 60-fold increase in
mammary PAR mass from birth until approximately 90 days of age). During this same period there is typically a doubling of body weight (Capuco and Akers, 2010).

A limited number of studies have focused on the impact of pre-weaning MR feeding on mammary development (Daniels et al., 2009; Meyer et al., 2006a, b; Brown et al., 2005). Results indicate that pre-weaning plane of nutrition can alter mammary fat pad (MFP) weight, DNA content of the mammary PAR, total mammary PAR, and total mammary PAR DNA without negatively affecting PAR, lipid or protein content (Meyer et al., 2006a; Brown et al., 2005). Additionally, recent data has shown that increasing pre-weaning ADG by 1 kg/d is associated with an increase of 1,000 kg or more in first lactation milk yield (Soberon et al., 2012). Mechanisms responsible for this increase remain unknown.

We hypothesized that enhanced pre-weaning nutrition positively alters mammary gland development by creating mammary cells that are ‘primed’ to better respond to mammogenic stimuli. The most commonly recognized mammogenic agent at this point in development is estradiol (Akers, 2000). Indeed, removal of estradiol in early life severely impairs mammary gland development, and this impairment can be attributed to lack of estradiol and/or altered estrogen receptor expression (Tucker et al., 2016). We hypothesized that calves fed a higher plane of nutrition pre-weaning would show an enhanced response to exogenous estradiol supplementation. Our first objective was to create two distinct groups of calves via dietary alterations and to assess the impacts of plane of nutrition on body and organ growth and overall mammary development. A second objective was to determine if animals fed a higher plane of nutrition responded differentially to exogenous
estradiol administration. To do this, tissue samples were collected and will be analyzed to discover cellular and molecular mechanisms involved in producing the effects of enhanced pre-weaning nutrition and estradiol treatment on heifer mammary development. In this report, we provide details of animal body and organ growth, performance and health, and general mammary development as influenced by pre-weaning nutrition and treatment with exogenous estradiol post-weaning. Subsequent reports will provide information regarding the mammary gland and its development.
MATERIALS AND METHODS

This experiment was conducted under the review and approval of the Virginia Polytechnic Institute and State University (VT) Institutional Animal Care and Use Committee (#14-045-DASC).

Animal Handling and Experiment Design

Thirty-six (36) Holstein heifer calves were purchased from a single commercial producer (located ~90 miles from campus) and brought to the VT dairy Farm between May and June of 2014. Three batches (n = 12/batch) of heifers were acquired. The heifers were approximately 1 wk old (6.0 ± 2 d) and weighed 39.0 ± 4.4 kg at the time of arrival. Only calves with total serum protein concentrations ≥ 5.5 mg/dL were purchased. Heifers were randomly assigned to treatments, individually housed in outdoor hutches on crushed rock without bedding and quarantined from the Virginia Tech Dairy herd. Heifers were given ad libitum access to water.

Calves were assigned to 1 of 2 experimental MR (n = 18/MR): 1) a restricted MR diet (R; 20.9% crude protein (CP), 19.8% fat, dry matter (DM) basis; Southern States Cooperative, Inc., Richmond, VA) fed at 0.44 kg MR powder/hd/d, DM basis, or 2) an enhanced MR (EH; 28.9% CP, 26.2% fat, DM basis; Land O'Lakes Animal Milk Products Co., Shoreview, MN) fed at 1.08 kg MR powder/hd/d, DM basis. Milk replacers had similar fatty acid profiles due to edible lard being the main fat source, and were fed at 15% solids. Milk replacer was fed in two equal portions twice daily at 0600 h and 1700 h for the first 7 wk of trial. At wk 8 heifers were fed half the usual amount 1x daily at 1700 h to prepare for weaning. Weaning occurred at the
end of wk 8. Starter (25.6% CP, 4.0% fat, 19.8% neutral detergent fiber (NDF), DM basis; Southern States Cooperative, Inc., Richmond, VA) was offered at the end of wk 4 of the trial. In an effort to keep starter intakes similar between treatments, starter was pair fed between R and EH calves. The R-fed calves were offered starter in the amount of what was consumed by EH-fed calves on the previous day. For example, on day 1, all EH-fed calves were offered starter ad libitum. Their starter intake was then averaged, and that average intake amount was offered to R-fed calves the following day. This was done daily as it was assumed R-fed calves would consume more starter than EH-fed calves if offered ad libitum.

A subset of calves (n = 6/diet) was harvested upon weaning to assess dietary effects on organ and tissue development. The remaining calves (n = 24) were either given an estradiol implant (Compudose®, Elanco Animal Health, Greenfield, IN) or a placebo implant on the day of final milk consumption (weaning). The placebo consisted of the silicon material of the estradiol implant without the hormone content. Estradiol implant selection and implantation was conducted similarly to previous research (Lammers et al., 1999). Implants were administered at the base of the left ear. This produced the following treatment groups (n = 6/treatment): 1) calves fed R and given a placebo implant (R), 2) calves fed R and given an estradiol implant (R-E2), 3) calves fed EH and given a placebo implant (EH), and 4) calves fed EH and given an estradiol implant (EH-E2). Calves were supplemented with estradiol for 2 weeks post-weaning, at which point all remaining animals were harvested to assess the effects of estradiol within each diet on body and organ growth, particularly mammary gland growth. Length of estrogen estradiol was
determined based on previous work with older animals (Lammers et al., 1999), although the total length of time of estradiol exposure in the present and reference study differed. Health events were monitored and intakes were also recorded. During post-weaning life, starter intake was kept consistent between diets, regardless of estradiol administration status.

Two calves died within 48 hours of arrival and were not replaced. These were an EH calf scheduled to be harvested at weaning and an EH calf scheduled to be harvested at week 10. In addition, data from one EH calf harvested at week 10 were removed prior to analysis due to chronic illness.

Sample Collection

Milk replacer refusals were weighed at each feeding. Starter orts were collected daily and weighed during the evening feeding. Weekly samples of MR and starter were obtained and analyzed for DM, CP, fat, and ash at the Cumberland Valley Analytical Services Laboratory (Hagerstown, MD). Starter samples were also analyzed for NDF and ADF content.

Fecal and respiration scores were measured daily during the evening feeding. Scales for health measures were adapted from Larson et al. (1977). Respiratory scores were defined as: 1) normal, 2) runny nose, 3) heavy breathing, 4) moist cough, and 5) a heavy dry cough. Fecal scores were defined as: 1) solid, 2) slightly loose, 3) slightly discolored with obvious scours, and 4) discolored with extreme scours. When assessing days with scours, a scour day was defined as a day when a calf had a fecal score ≥ 3. Calves were subjected to weekly growth measures (BW, hip height, withers height, heart girth). Weekly ADG was calculated from BW
for pre- and post-weaning periods. Additionally, at each weekly sampling, a blood sample was collected from the jugular vein. Blood was brought immediately to the lab and centrifuged at 3,000 x g for 20 min. at 4 °C. Serum was stored at -20 °C for subsequent analysis.

*Harvest Procedure*

Heifers were euthanized at Virginia Tech’s Veterinary Facility (approximately 1 mile from their housing). Heifers were harvested using a commercial phenobarbital solution administered intravenously (Fatal-Plus, 10 mg/kg BW, Vortech Pharmaceuticals, Dearborn, MI), exsanguinated, and subjected to organ collection. All animals were treated similarly prior to their scheduled harvest, with all calves receiving their last MR and starter feeding the evening prior to their scheduled harvest (~16 h pre-harvest). Prior to organ collection, exsanguinated BW (carcass weight) was determined.

*Tissue and Organ Collection*

At harvest, the udder was removed (skin intact) from each heifer and weighed as a preliminary indicator of total mammary mass (prior to subsequent analyses). Other organ weights obtained during the course of necropsy included: thymus, liver, whole forestomach (full and empty), reticulorumen (full and empty), combined abomasum and omasum (full), small intestine (full), spleen, pancreas, and kidney (fat removed). The pH of the rumen contents was also measured immediately after full reticulorumen weight was recorded. Throughout necropsy, various frozen and formalin fixed tissue samples were collected for future analysis. The mammary gland was one such tissue and tissue was frozen and formalin fixed
for RNA sequencing, explant culturing, and histological analysis at a later date. This will be covered in future publications.

**Statistical Analysis**

Data were analyzed by ANOVA using the GLIMMIX procedure of SAS (SAS Institute, INC., Cary, NC). Calves were randomly assigned to treatments in a 2 x 2 factorial arrangement of treatments. Main effects included in the model were time (week or day), batch, treatment (R, R-E2, EH, EH-E2), harvest date (when appropriate), and their associated interactions. Data with multiple measures per calf (week and day) were used as repeated measures when applicable. Calf (Calf ID) was treated as a random variable. Batch, treatment, and their interaction were treated as fixed effects whereas residual error was treated as a random effect.

Interactions were tested for weekly and daily effects, but were removed from the model when not significant. Significance was declared at $P < 0.05$ and tendencies are discussed when $0.05 < P < 0.10$. In the instance of significant treatment differences, separation of means were evaluated with the PDIFF procedure of SAS based on Fisher’s protected least significant difference test. Data are given as LS mean ± SEM.
RESULTS AND DISCUSSION

Intake

Diet composition is shown in Table 3.1. Calves fed the EH diet consumed more MR DM (1.02 vs. 0.44 kg/d; P < 0.01; Table 3.2) compared with R calves. The same held true for CP and fat.

R-fed calves consumed more starter DM pre-weaning (286 vs. 237 g/d; P < 0.01; Table 3.2) compared with EH calves. The same was true for CP, fat, NDF, and ADF. However, overall total weekly DM intake was greater during the pre-weaning period for EH-fed calves compared with R-fed calves (8.80 vs. 5.08 kg; P < 0.01). The same pattern was noted for energy and protein intake.

Post-weaning starter intake is shown in Table 3.3. Restricted fed calves given estradiol and R-calves consumed more starter DM, CP, fat, NDF, and ADF during the post-weaning period compared with EH-E2- and EH-calves (See Table 3.3). Differences in starter intake in both the pre- and post-weaning period were not anticipated due to the pair-feeding method employed in this trial. However, even with the employed method, EH and EH-E2 calves would more frequently refuse starter due to their increased milk allowance, resulting in the observed intake differences.

The EH-fed calves consumed more MR DM, CP, fat, and energy during the pre-weaning period. This higher intake provided the calf with greater intake of nutrients to promote greater body growth. It is important to note that greater intake of MR nutrients, aside from CP and fat, occurred due to greater MR intake and not MR composition manipulation. Additionally, fat and protein source were similar
between MR. Therefore, diets used in this trial allowed us to achieve our goal of creating two distinct groups of animals to test our hypothesis without confounding from ingredient differences. In this trial, starter was not offered ad libitum to control for the greater amount of starter consumption typically seen in restricted-fed calves compared with calves fed a higher plane of nutrition in previous trials (de Passille et al., 2011; Daniels et al., 2009; Hill et al., 2008). Instead, we attempted to keep starter intake consistent between treatments. Although the amount of starter offered was paired between dietary treatments, EH-fed calves refused more starter due to increased milk intake, resulting in observed differences.

Growth

Initial body weights were not different between treatments (39.8 vs. 39.4 kg for EH and R calves; P = 1.0; Figure 3.1). Starting at week 2, E-fed calves were heavier throughout the entire pre-weaning period. At weaning, EH-fed calves were 24.5 kg heavier than R-fed calves (P < 0.01). Overall, post-weaning weights were not affected by estradiol, with EH-fed calves remaining heavier than R-fed calves during weeks 9 and 10, regardless of estradiol status (P < 0.01). It is interesting to note the decrease in BW for EH calves compared with EH-E2 calves during week 9. The lack of a decrease in EH-E2 calves may be due to the known influences of exogenous estradiol on growth (discussed below).

Average daily gain was greater for EH-fed calves beginning at week 1 (0.36 vs. -0.06 kg/d; P < 0.01) and lasting through week 7 (1.00 vs. 0.41 kg/d; P < 0.01). However, during week 8 (weaning week), ADG did not differ (0.31 vs. 0.35 kg/d for EH and R calves; P = 1.0), which may be due to compensatory gain experienced by R
calves when milk intake was decreased and grain intake was controlled. The negative ADG associated with the R diet was only observed during week 1 and 2. Overall, a treatment effect was noted for ADG during the post-weaning period. Calves on the R-E2 treatment had greater overall post-weaning ADG compared with EH-calves (0.92 vs. 0.58 kg/d; P = 0.05). However, no other treatment differences existed during the post-weaning period, with all calves gaining at a similar rate during weeks 9 and 10.

During the pre-weaning period, feed:gain efficiency was greater for EH-fed calves compared with R-fed calves (1.42 vs. 1.33 for EH and R calves; P < 0.05). During the post-weaning period, feed:gain was the lowest for EH-fed calves compared with EH-E2, R, and R-E2 calves (0.5 vs. 2.8, 2.2, and 1.8; P < 0.01). Additionally, feed:gain was greater for EH-E2 calves compared with R and R-E2 calves.

It is well known that feeding a higher plane of nutrition leads to greater BW throughout the pre-weaning period, at weaning, and during the immediate post-weaning phase (Cowles et al., 2006; Hill et al., 2008; Geiger et al., 2014). Therefore, the results observed were anticipated, although the extent of the difference was greater than expected. Regardless, this experimental approach produced two groups of animals to test our fundamental hypothesis that pre-weaning diet affects the ability of the mammary gland to respond to mammogenic signals. It is interesting to note that during the post-weaning phase, calves fed R pre-weaning were unable to make up the difference in BW, which can be attributed to the fact that grain intake was controlled. In addition, it was surprising that BW differences did not exist
between EH and EH-E2 fed calves during week 9 and 10. However, the data does indicate that exogenous estradiol might improve the weaning transition process for EH fed calves given EH-E2 calves did not have compromised growth after weaning compared with EH calves, as is evident in Figure 3.1. This may reflect the utility of estradiol implants for use as growth promoters (i.e., improved feed efficiency) in feed lot beef cattle (Preston, 1999).

As anticipated, EH-fed calves had greater ADG during weeks 1 through 7 compared with R-fed calves. During the post-weaning phase, the R-E2 calves had greater ADG compared with the EH and EH-E2 calves. It may be that calves fed R pre-weaning experienced compensatory growth during post-weaning that was enhanced with exogenous estradiol. This should not come as a surprise due to the fact that the larger framed EH-fed calves would need more grain to meet their growth demands and the fact that estradiol implants have long been used in the beef industry to encourage growth and feed efficiency. Average daily gain in this trial was not as great as anticipated for calves fed a higher plane of nutrition. This may be due in part to high ambient temperatures observed during this study. It was apparent that calves were under heat stress during this trial, which may have altered the efficiency with which nutrients were utilized for growth.

Initial hip height did not differ between treatments (80.8 vs. 80.4 cm for EH and R calves; P = 1.0). Starting at week 5, E-fed calves had greater hip heights compared with R-fed calves, and that difference remained throughout the pre-weaning period. At weaning, EH-fed calves were 6.4 cm taller at the hip compared with R-fed calves (94.3 vs. 87.9 cm; P < 0.01). Overall hip heights remained greater
for EH-fed calves during the post-weaning period (P < 0.01). Post-weaning hip height was not affected by estradiol (P < 0.05). Withers height responded similarly to hip height in this study (data not shown).

Initial heart girth did not differ between treatments (77.8 vs. 77.6 cm for EH and R calves; P = 1.0). Starting at week 2, heart girth was greater for EH-fed calves (P < 0.05 for week 2, P < 0.01 for weeks 3 through 8) compared with R-fed calves. At weaning, heart girth measures for EH-fed calves were 10.3 cm greater than that of R-fed calves (P < 0.01). Overall heart girth remained greater for EH-fed calves during the post-weaning period (P < 0.01). During week 10, EH calves no longer had greater heart girth measures compared with R and R-E2 calves, while EH-E2 calves continued to have greater heart girth measures compared with R and R-E2 calves (P < 0.05).

Greater hip and withers height as a result of EH feeding has been shown in previous studies (Hill et al., 2008; Geiger et al., 2014). In addition, heart girth was greater for calves fed EH pre-weaning starting at week 2. Heart girth is more closely associated with BW compared to other frame measures (Heinrichs et al., 1992, Albino et al., 2015), so it is no surprise that the heart girth differences followed a very similar pattern to BW.

Organ Weights

At weaning harvest, EH-fed calves had greater carcass weights compared with R-fed calves (P < 0.01; Table 3.4). In addition, various other organs were heavier for EH-fed calves compared with R-fed calves (See Table 3.4), most notably
for the sake of this trial, the mammary gland. A tendency also existed for EH calves to have greater full reticulorumen weights ($P = 0.07$) compared with R-fed calves.

When adjusted for BW, EH-fed calves had greater whole, untrimmed mammary gland ($P < 0.01$; Table 3.4), thymus ($P < 0.01$), liver ($P < 0.05$), and spleen ($P < 0.05$) weights compared to R-fed calves. Differences no longer existed for full stomach, empty reticulorumen, full omasum and abomasum, small intestines, pancreas, or kidney weights when adjusted for BW.

Data assessing individual organ growth as a result of similar feeding to this study are lacking in the literature. The difference in carcass weights further confirms physiological differences between the two groups of animals and agrees with previous work (Hill et al., 2008). Total udder weight is an incomplete gauge of mammary gland development however these data very strongly suggest that these dietary treatments had an impact on the mammary gland and that the treatments produced glands suitable to test our hypotheses. Additionally, similar experiments have also found that feeding a higher plane of nutrition increases liver (Hill et al., 2008; Kamiya et al., 2009), spleen (Kamiya et al., 2009), and kidney (Kamiya et al., 2009) weights in pre-weaned dairy calves. Conversely, work in goats has demonstrated that feeding a higher plane of nutrition during the milk-fed stage decreases liver weights (Potchoiba et al., 1990). Some organ weight differences would be expected to support a larger calf. After BW adjustment, EH-fed calves had greater amounts of mammary gland, thymus, liver, and spleen tissue. These differences in total udder mass suggest that enhanced nutrition during the pre-weaning period increases mammary development.
While not a part of the present data set, it is relevant to note that when mammary PAR tissue was dissected and weighed there was a 7-fold greater mass in EH fed heifers at the time of weaning. The images given in Figure 3.2 are representative examples of dissected and trimmed mammary tissue from the mammary gland of an R-fed (left) and EH-fed (right) heifer at 8 weeks. In both images the teat appears below and the parenchymal tissue just above the teat and gland cistern. In both cases the parenchymal tissue has been bisected (R) or butterflied into multiple leaflets (EH) as dictated by the size. This was done to allow adequate fixation of the parenchymal tissue. In both cases the parenchymal tissue appears darker and brown to tan in color in comparison to the surrounding mammary fat pad (arrows), much of which has been trimmed way. For most of the R heifers this visually distinctive parenchymal tissue area was very small, thin, and often difficult to dissect in comparison with the EH heifers. When dissected the parenchymal area in R heifers was confined to a small, generally tear shaped region immediately adjacent to the teat. In EH heifers, the parenchymal tissue was easy to identify and the apparent larger size allowed for multiple (3-5) butterflied tissue segments to be prepared. Subsequent reports will provide a detailed analysis of the histological structures within the PAR as well as a biochemical analysis of PAR and mammary fat pad. Our longer-term goal is to evaluate the impact of diet and response of animals on these diets to mammogenic stimulation. These data leave no doubt that these dietary treatments produced very dramatic differences in total udder growth and set the stage for subsequent study. These results, however, are beyond the scope of this report.
At the week 10 harvest (post-estradiol), EH-E2 calves had greater carcass weights than R and R-E2 calves (P < 0.01; Table 3.5). Estradiol affected whole, untrimmed mammary gland weights, with EH-E2 calves having greater weights compared with EH, R, and R-E2 calves (P < 0.01). Additionally, there was a tendency for EH-fed calves to have greater mammary gland weights than R-fed calves (P = 0.06). Thymus weights followed a similar trend to mammary gland weights (P < 0.01). Differences were not found between EH-E2 and EH calves (P = 0.22), but there was a tendency for EH calves to have greater thymus weights than R calves (P = 0.06). Liver weights were not affected by estradiol administrations, with EH-E2 and EH calves having greater liver weights than R-E2 and R calves (P < 0.01). At the week 10 harvest, a tendency was found for EH-E2 calves to have greater full stomach weights compared with R-E2 calves (P = 0.05). Empty reticulorumen weights were greater for EH-E2 calves compared with R-E2 and R calves (P < 0.05). Full omasum and abomasum weights tended to be greater for EH-E2 calves compared with R-E2 calves (P = 0.06). EH-E2 calves had greater spleen weights compared with EH-, R-, and R-E2 -treated calves (P < 0.01). Kidney weights were not affected by estradiol with EH-E2 and EH calves having heavier kidneys compared with R and R-E2 calves (P < 0.01). Additionally, small intestine, full reticulorumen, and pancreas weights were not affected by treatment at the second harvest.

When adjusted for carcass weights mammary gland weights were greater for EH than R calves (Table 3.5). Mammary gland weights were also increased by estradiol administration in both restricted (P ≤ 0.5) and in enhanced fed calves (P ≤ 0.5). Thymus weights were greater for EH-E2 calves compared with R-E2 and R
calves (P < 0.01) and EH calves compared with R calves (P < 0.01). There was a tendency for liver weights to be greater with EH-E2 and EH calves having greater liver weights compared with R-E2 calves (P <0.10). Full stomach weights were greatest for R calves (P < 0.01). Full reticulorumen weights were greater for R calves compared with EH-E2 and EH calves (P < 0.05) and a tendency was found for greater full reticulorumen in R calves compared with R-E2 calves (P < 0.10). Empty reticulorumen weights were greater for R calves compared with EH-E2 and EH calves (P < 0.05). Small intestine weights were greater for R calves compared with EH-E2 calves (P < 0.05). Spleen weights were the greatest for EH-E2 calves compared with EH, R, and R-E2 calves (P < 0.01). Full omasum and abomasum, pancreas, and kidney differences were no longer significant when adjusted for BW.

The week 10 harvest allowed for the assessment of organ differences as a result of estradiol administration to two different groups of calves. The fundamental reason for estradiol administration was to assess how it may differentially affect mammary gland development as a consequence of pre-weaning diet. Estradiol is a classic mammogenic hormone (Capuco and Akers, 2010), and a logical choice as a mammogenic test agent. Additionally, previous work (Lammers et al., 1999) indicated the efficacy of estradiol in this regard and that 2 weeks of estradiol treatment was adequate to elicit a mammary response as these workers noted that teat lengths were significantly increased within 2 weeks. However, it must be noted that in their trial estradiol was administered for a total of 20 weeks. The dose of estradiol provided to these calves was expected to increase circulating estradiol. It is also relevant that ovariectomy in calves caused only a minute decrease in
circulating estradiol but mammary gland development was nonetheless dramatically impaired (Purup et al., 1993). The fact that EH-E2 calves had the greatest mammary gland weights suggests that estradiol differentially affected mammary development in E-fed calves compared with R-fed calves. Future evaluation and analyses will provide details to better understand cellular and molecular processes responsible for diet induced differences in mammary development (R vs. EH) as well as likely differential responses to mammogenic stimulation.

**Health**

Serum protein did not differ between treatments on the day of arrival (P = 0.74; averaging 5.5 ± 0.17 mg/dL; range 5.4 to 5.6 mg/dL. Days medicated averaged 1.8 ± 1.5 and was not impacted by treatment (P = 0.26). Scour days (defined as a day with a fecal score ≥ 3) was affected by treatment (P = 0.02). There was a tendency for calves fed EH to have a greater number of days with scours compared with R and R-E2 calves (10.8 vs. 7.0 and 6.3 d; P < 0.10).

Pre-weaning average fecal score was greater for EH-fed calves compared with R-fed calves (1.7 vs. 1.5; P < 0.01. Pre-weaning respiratory scores were not affected by dietary treatment (P = 0.81). During the post-weaning period, neither fecal scores (P = 0.12) nor respiratory scores (P = 0.18) were affected by treatment averaging 1.07 and 1.01, respectively.

Serum protein on arrival did not differ between treatments because calves were only accepted if they met a serum protein threshold. The fact that days medicated did not differ between treatments or diets agrees with previous work
(Davis-Rincker et al., 2011), but is in direct opposition with data showing that calves fed a higher plane of nutrition were medicated more frequently (Huber et al., 1984) Cowles et al., 2006). However, the number of days with scours was greater for EH calves compared with R and R-E2 calves. Very recent data has indicated that an increase in fecal and/or scour scores does not correlate to an increase in treatment incidents (Sharon et al., 2015), which agrees with data herein. The fact that pre-weaning fecal scores were greater in EH-fed calves confirms previous work showing a greater number of scour days in calves fed a higher plane of nutrition (Raeth-Knight et al., 2009). The fact that fecal scores did not differ post-weaning is not surprising since all calves were fed only grain and post-weaning morbidity was not an issue during this trial. It is important to note that fecal score results in this trial, although different, were within a relatively normal range.
CONCLUSIONS

These data indicate and further confirm that feeding a higher plane of nutrition to dairy calves during the pre-weaning period positively alters intake, body weight, frame measures, and organ growth without compromising calf health. Calves on the EH treatment had greater mammary gland mass. EH-E2 calves had the greatest mammary gland weights compared with all other treatments, whereas R-E2 calves had greater mammary gland weights than R calves. These results indicate a positive mammogenic response to estradiol and possible differential response depending on pre-weaning feeding. Future efforts will allow us to uncover physiological and cellular modifications to classic mammogenic pathways and signaling cascades that may explain these results. The presented data combined with future work may allow for the creation of pre-weaned diets that target these mechanisms to maximize future milk yield potential.
ACKNOWLEDGEMENTS

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Table 3.1. Composition of milk replacer and starter offered to Holstein heifer calves

<table>
<thead>
<tr>
<th>Item (% of DM)</th>
<th>20:20</th>
<th>28:25</th>
<th>Starter</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>95.6</td>
<td>95.7</td>
<td>87.1</td>
</tr>
<tr>
<td>CP</td>
<td>20.9</td>
<td>28.9</td>
<td>25.6</td>
</tr>
<tr>
<td>Fat</td>
<td>19.8</td>
<td>26.2</td>
<td>4.0</td>
</tr>
<tr>
<td>NDF</td>
<td>-</td>
<td>-</td>
<td>19.8</td>
</tr>
<tr>
<td>ADF</td>
<td>-</td>
<td>-</td>
<td>9.2</td>
</tr>
<tr>
<td>Ash</td>
<td>12.3</td>
<td>11.4</td>
<td>8.3</td>
</tr>
<tr>
<td>NE&lt;sub&gt;m&lt;/sub&gt;, Mcal/kg&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.8</td>
<td>3.0</td>
<td>1.8</td>
</tr>
<tr>
<td>NE&lt;sub&gt;g&lt;/sub&gt;, Mcal/kg&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.0</td>
<td>2.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

<sup>1</sup>All nutrient analyses were performed by Cumberland Valley Analytical Services Laboratory (Hagerstown, MD).

<sup>2</sup>20:20 = 20% CP, 20% fat and offered to R and R-E2 calves.

<sup>3</sup>28:25 = 28% CP, 25% fat and offered to EH and EH-E2 calves.

<sup>4</sup>NE<sub>m</sub> = Net Energy for Maintenance.

<sup>5</sup>NE<sub>g</sub> = Net Energy for Gain.
Table 3.2. Pre-weaning milk replacer (MR) and starter intake of Holstein heifer calves fed either a restricted (R) diet or a higher (EH) plane of nutrition

<table>
<thead>
<tr>
<th>Item</th>
<th>R(^1)</th>
<th>EH(^2)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Milk Replacer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM intake, kg/d(^3)</td>
<td>0.44(^a)</td>
<td>1.02(^b)</td>
<td>0.07</td>
</tr>
<tr>
<td>CP intake, kg/d(^4)</td>
<td>0.09(^a)</td>
<td>0.30(^b)</td>
<td>0.02</td>
</tr>
<tr>
<td>Fat intake, kg/d</td>
<td>0.09(^a)</td>
<td>0.27(^b)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Starter</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM intake, g/d</td>
<td>286(^a)</td>
<td>237(^b)</td>
<td>14.5</td>
</tr>
<tr>
<td>CP intake, g/d</td>
<td>73.3(^a)</td>
<td>60.9(^b)</td>
<td>3.7</td>
</tr>
<tr>
<td>Fat intake, g/d</td>
<td>11.5(^a)</td>
<td>9.5(^b)</td>
<td>0.6</td>
</tr>
<tr>
<td>NDF intake, g/d(^5)</td>
<td>56.6(^a)</td>
<td>46.9(^b)</td>
<td>2.1</td>
</tr>
<tr>
<td>ADF intake, g/d(^6)</td>
<td>26.4(^a)</td>
<td>21.9(^b)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\(^1\)R = Calves fed a restricted MR diet (20.9% CP, 19.8% fat) at a rate of 0.44 kg/d, DM basis. n = 6.

\(^2\)EH = Calves fed enhanced MR (28.9% CP, 26.2% fat) at a rate of 1.02 kg/d, DM basis. n = 5.

\(^3\)DM = Dry Matter.

\(^4\)CP = Crude Protein.

\(^5\)NDF = neutral detergent fiber

\(^6\)ADF = acid detergent fiber

\(^a,b\)Differing superscripts within row indicated treatment differences (\(P < 0.01\)).
Table 3.3. Post-weaning starter and starter component intake of Holstein heifer calves fed either a restricted diet or a higher plane of nutrition pre-weaning with or without exogenous estrogen administration immediately post-weaning

<table>
<thead>
<tr>
<th>Item</th>
<th>R\textsuperscript{1}</th>
<th>R-E\textsuperscript{2}</th>
<th>EH\textsuperscript{3}</th>
<th>EH-E\textsuperscript{2}</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM intake, g/d\textsuperscript{5}</td>
<td>1301\textsuperscript{a}</td>
<td>1307\textsuperscript{a}</td>
<td>1228\textsuperscript{b}</td>
<td>1257\textsuperscript{b}</td>
<td>16.4</td>
</tr>
<tr>
<td>CP intake, g/d\textsuperscript{6}</td>
<td>330\textsuperscript{a}</td>
<td>340\textsuperscript{a}</td>
<td>320\textsuperscript{b}</td>
<td>320\textsuperscript{b}</td>
<td>2.5</td>
</tr>
<tr>
<td>Fat intake, g/d</td>
<td>52.0\textsuperscript{a}</td>
<td>52.0\textsuperscript{a}</td>
<td>49.1\textsuperscript{b}</td>
<td>50.0\textsuperscript{b}</td>
<td>0.54</td>
</tr>
<tr>
<td>NDF intake, g/d\textsuperscript{7}</td>
<td>258\textsuperscript{a}</td>
<td>259\textsuperscript{a}</td>
<td>243\textsuperscript{b}</td>
<td>249\textsuperscript{b}</td>
<td>2.9</td>
</tr>
<tr>
<td>ADF intake, g/d\textsuperscript{8}</td>
<td>119\textsuperscript{a}</td>
<td>120\textsuperscript{a}</td>
<td>113\textsuperscript{b}</td>
<td>116\textsuperscript{b}</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\textsuperscript{1}R = Calves fed a restricted MR diet (20.9% CP, 19.8% fat) at a rate of 0.44 kg/d, DM basis. n = 6.
\textsuperscript{2}R-E2 = Calves fed R and given estrogen for 2 weeks post-weaning. n = 6.
\textsuperscript{3}EH = Calves fed enhanced MR (28.9% CP, 26.2% fat) at a rate of 1.02 kg/d, DM basis. n = 4.
\textsuperscript{4}EH-E2 = Calves fed EH and given estrogen for 2 weeks post-weaning. n = 6.
\textsuperscript{5}DM = Dry Matter.
\textsuperscript{6}CP = Crude Protein.
\textsuperscript{7}NDF = neutral detergent fiber
\textsuperscript{8}ADF = acid detergent fiber
\textsuperscript{a,b}Differing superscripts within row indicated treatment differences (P < 0.01)
Table 3.4. Body weight adjustment for organ weights at weaning of Holstein heifer calves fed either a restricted (R) diet or a higher plane (EH) of nutrition

<table>
<thead>
<tr>
<th>Item</th>
<th>R</th>
<th>EH</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcass, kg</td>
<td>48.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.10</td>
</tr>
<tr>
<td>Whole Mammary Gland, g</td>
<td>66.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>255.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.8</td>
</tr>
<tr>
<td>Whole Mammary Gland, g/kg BW</td>
<td>1.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26</td>
</tr>
<tr>
<td>Thymus, g</td>
<td>119.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>440.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.5</td>
</tr>
<tr>
<td>Thymus, g/kg BW</td>
<td>2.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38</td>
</tr>
<tr>
<td>Liver, kg</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05</td>
</tr>
<tr>
<td>Liver, g/kg BW</td>
<td>1.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10</td>
</tr>
<tr>
<td>Full Stomach, kg</td>
<td>3.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.80</td>
</tr>
<tr>
<td>Full Stomach, g/kg BW</td>
<td>6.30</td>
<td>7.50</td>
<td>1.27</td>
</tr>
<tr>
<td>Full Reticulorumen, kg</td>
<td>2.5&lt;sup&gt;y&lt;/sup&gt;</td>
<td>3.2&lt;sup&gt;z&lt;/sup&gt;</td>
<td>0.30</td>
</tr>
<tr>
<td>Full Reticulorumen, g/kg BW</td>
<td>4.87</td>
<td>4.19</td>
<td>0.72</td>
</tr>
<tr>
<td>Empty Reticulorumen, kg</td>
<td>0.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.06</td>
</tr>
<tr>
<td>Empty Reticulorumen, g/kg BW</td>
<td>1.20</td>
<td>1.20</td>
<td>0.08</td>
</tr>
<tr>
<td>Full Omasum and Abomasum, kg</td>
<td>0.70&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05</td>
</tr>
<tr>
<td>Full Omasum and Abomasum, g/kg BW</td>
<td>1.45</td>
<td>1.54</td>
<td>0.09</td>
</tr>
<tr>
<td>Small Intestines, kg</td>
<td>1.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.30</td>
</tr>
<tr>
<td>Small Intestines, g/kg BW</td>
<td>4.06</td>
<td>4.56</td>
<td>0.39</td>
</tr>
<tr>
<td>Spleen, kg</td>
<td>0.30</td>
<td>0.80</td>
<td>0.09</td>
</tr>
<tr>
<td>Spleen, g/kg BW</td>
<td>0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12</td>
</tr>
<tr>
<td>Pancreas, g</td>
<td>25.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.8</td>
</tr>
<tr>
<td>Pancreas, g/kg BW</td>
<td>0.51</td>
<td>0.53</td>
<td>0.06</td>
</tr>
<tr>
<td>Kidney Average, g</td>
<td>17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
<tr>
<td>Kidney Average, g/kg BW</td>
<td>0.35</td>
<td>0.30</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<sup>1</sup>R = Calves fed restricted MR diet (20.9% CP, 19.8% fat) at a rate of 0.44 kg/d, DM basis. n = 6.

<sup>2</sup>EH = Calves fed enhanced MR (28.9% CP, 26.2% fat) at a rate of 1.02 kg/d, DM basis. n = 5.

<sup>3</sup>Body Weight after exsanguination.

<sup>a,b</sup>Differing superscripts within row indicated treatment differences (P < 0.05).

<sup>y,z</sup>Differing superscripts within row indicate treatment tendencies (0.05 > P < 0.10).
Table 3.5. Body weight (BW) adjustment for organ weights collected at week 10 of Holstein heifer calves fed either a restricted diet or a higher plane of nutrition pre-weaning with or without exogenous estrogen administration immediately post-weaning

<table>
<thead>
<tr>
<th>Item</th>
<th>R¹</th>
<th>R-E2²</th>
<th>EH³</th>
<th>EH-E2⁴</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcass, kg</td>
<td>57.2⁵</td>
<td>57.2⁵</td>
<td>69.5ab</td>
<td>74.2a</td>
<td>2.80</td>
</tr>
<tr>
<td>Whole Mammary Gland, g</td>
<td>73₃y</td>
<td>115.9a</td>
<td>132.2az</td>
<td>236.3b</td>
<td>16.4</td>
</tr>
<tr>
<td>Whole Mammary Gland, g/kg BW</td>
<td>1.28a</td>
<td>2.04b</td>
<td>2.22b</td>
<td>3.18c</td>
<td>0.23</td>
</tr>
<tr>
<td>Thymus, g</td>
<td>115.7ay</td>
<td>150.7a</td>
<td>215.6abz</td>
<td>288.5b</td>
<td>26.8</td>
</tr>
<tr>
<td>Thymus, g/kg BW</td>
<td>2.02a</td>
<td>2.66ab</td>
<td>3.47bc</td>
<td>3.88c</td>
<td>0.40</td>
</tr>
<tr>
<td>Liver, kg</td>
<td>1.16a</td>
<td>1.11a</td>
<td>1.59b</td>
<td>1.71b</td>
<td>0.05</td>
</tr>
<tr>
<td>Liver, g/kg BW</td>
<td>7.10</td>
<td>5.78y</td>
<td>6.40</td>
<td>7.45z</td>
<td>0.05</td>
</tr>
<tr>
<td>Full Stomach, kg</td>
<td>12.43a</td>
<td>10.06b</td>
<td>9.56b</td>
<td>10.05b</td>
<td>0.72</td>
</tr>
<tr>
<td>Full Reticulorumen, kg</td>
<td>5.71</td>
<td>4.52</td>
<td>4.91</td>
<td>5.80</td>
<td>0.05</td>
</tr>
<tr>
<td>Full Reticulorumen, g/kg BW</td>
<td>10.05ay</td>
<td>7.88z</td>
<td>7.14byz</td>
<td>7.78byz</td>
<td>0.75</td>
</tr>
<tr>
<td>Empty Reticulorumen, kg</td>
<td>1.23a</td>
<td>1.15a</td>
<td>1.30ab</td>
<td>1.44b</td>
<td>0.01</td>
</tr>
<tr>
<td>Empty Reticulorumen, g/kg BW</td>
<td>2.18a</td>
<td>2.02ab</td>
<td>1.92b</td>
<td>1.94b</td>
<td>0.08</td>
</tr>
<tr>
<td>Full Omasum and Abomasum, kg</td>
<td>1.40</td>
<td>1.37y</td>
<td>1.50</td>
<td>1.68z</td>
<td>0.01</td>
</tr>
<tr>
<td>Full Omasum and Abomasum, g/kg BW</td>
<td>2.38</td>
<td>2.18</td>
<td>2.43</td>
<td>2.27</td>
<td>0.21</td>
</tr>
<tr>
<td>Small Intestines, kg</td>
<td>0.57</td>
<td>0.45</td>
<td>0.59</td>
<td>0.58</td>
<td>0.02</td>
</tr>
<tr>
<td>Small Intestines, g/kg BW</td>
<td>5.62a</td>
<td>5.30ab</td>
<td>5.40ab</td>
<td>4.73b</td>
<td>0.26</td>
</tr>
<tr>
<td>Spleen, kg</td>
<td>0.30a</td>
<td>0.31a</td>
<td>0.45a</td>
<td>0.71b</td>
<td>0.01</td>
</tr>
<tr>
<td>Spleen, g/kg BW</td>
<td>0.65a</td>
<td>0.56a</td>
<td>0.52a</td>
<td>0.92b</td>
<td>0.07</td>
</tr>
<tr>
<td>Pancreas, g</td>
<td>33.5</td>
<td>37.6</td>
<td>37.9</td>
<td>42.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Pancreas, g/kg BW</td>
<td>0.59</td>
<td>0.66</td>
<td>0.59</td>
<td>0.58</td>
<td>0.07</td>
</tr>
<tr>
<td>Kidney Average, g</td>
<td>16.5a</td>
<td>14.5a</td>
<td>24.5b</td>
<td>21.8b</td>
<td>1.0</td>
</tr>
<tr>
<td>Kidney Average, g/kg BW</td>
<td>0.29yz</td>
<td>0.25y</td>
<td>0.35z</td>
<td>0.30yz</td>
<td>0.03</td>
</tr>
</tbody>
</table>

¹R = Calves fed restricted MR diet (20.9% CP, 19.8% fat) at a rate of 0.44 kg/d, DM basis. n = 6.
²R-E2 = Calves fed R and given estrogen for 2 weeks post-weaning. n = 6.
³EH = Calves fed enhanced MR (28.9% CP, 26.2% fat) at a rate of 1.02 kg/d, DM basis. n = 4.
⁴EH-E2 = Calves fed EH and given estrogen for 2 weeks post-weaning. n = 6.
⁵Body Weight after exsanguination.

a,b,c Differing superscripts within row indicated treatment differences (P < 0.05)
y,z Differing superscripts within row indicate treatment tendencies (0.05 > P < 0.10).
Figure 3.1. Weekly body weights (BW) of Holstein heifer calves fed either a restricted diet or a higher plane of nutrition pre-weaning with or without exogenous estrogen administration immediately post-weaning.

* = dietary difference ($P < 0.01$). Treatment numbers are as follows: R (n = 12), EH (n = 9), R-E2 (n = 6), EH-E2 (n = 6).
Figure 3.2. Examples of dissected and trimmed mammary gland parenchyma (PAR; indicated by dashed lines) from calves fed either a restricted (R; left) or enhanced (EH; right) diet pre-weaning. Scale is in centimeters. Pictures were taken following weaning (week 8) harvest. Images are representative of R and EH-fed heifers. Arrows indicated the remnants of the mammary fat pad that was trimmed and removed to prepare the tissue for fixation. The teat (T) appears at the bottom of each sample.
REFERENCES


CHAPTER IV. FEEDING A HIGHER PLANE OF NUTRITION AND PROVIDING EXOGENOUS ESTROGEN INCREASES MAMMARY GLAND DEVELOPMENT IN HOLSTEIN HEIFER CALVES


ABSTRACT

Feeding heifers a high plane of nutrition post-weaning but before puberty can negatively impact mammary gland (MG) development and future milk yield. However, enhanced pre-weaning nutrition may have the opposite effect. Our objectives were to determine the effects of enhanced pre-weaning feeding and exogenous estradiol immediately post weaning on MG development and the composition of the mammary parenchyma (PAR) and mammary fat pad (MFP). Thirty-six Holstein heifer calves (< 1 wk old) were reared on 1 of 2 dietary treatments for 8 weeks. Diets included: 1) a restricted milk replacer (MR) fed at 0.44 kg powder/hd/day, dry matter (DM) basis (R; 20.9% crude protein [CP], 19.8% fat, DM basis), or 2) an enhanced MR fed at 1.08 kg powder/hd/day, DM basis (EH; 28.9% CP, 26.2% fat, DM basis). Upon weaning, calves from each diet (n = 6) were given either a placebo or estradiol implant for 2 weeks creating 4 treatments: 1) R, 2) R + estradiol (R-E2), 3) EH, and 4) EH + estradiol (EH-E2). Calves were housed individually with ad libitum access to water. Starter feeding was initiated at week 5 and balanced between treatments. Udders were evaluated by palpation and selected physical measurements weekly. At weaning, a subset of calves were sacrificed (n = 6/diet) and at the conclusion of the trial (week 10), remaining calves were harvested (n = 6/treatment). Udders were removed, dissected, and weighed. At
week 8, EH calves had longer front and rear teats. Providing estradiol to EH calves increased length of rear teats during week 9 and 10. Enhanced-fed calves had 5.2-fold more trimmed MG mass than R calves. Providing estradiol to EH calves further increased MG weight. Mass of dissected PAR and MFP were markedly greater for EH vs. R calves i.e. 7.3-fold greater PAR tissue. Estradiol increased the mass of both PAR and MFP in EH calves. Feeding a higher plane of nutrition increased total protein, DNA, and fat in the MFP and total protein and DNA in the PAR. Dual-energy x-ray absorptiometry was completed on excised mammary glands and estimates of mammary fat mass were highly correlated with biochemical analyses of fat content. From histological methods, we observed the degree of expansion of epithelium into the adjacent stromal tissue and complexity of ductal development was minimal in R, increased in EH, and increased by estradiol in both dietary treatments. Results provide compelling evidence that pre-weaning nutrition and estradiol administration immediately post-weaning markedly increase MG development in dairy calves. Cellular and molecular mechanisms responsible for these differences are currently under study.

**Keywords:** mammary gland, milk replacer, estrogen, calf
INTRODUCTION

The expense of raising heifers accounts for about 20% of annual dairy farm costs (Heinrichs, 1993). Consequently, producers seek to have heifers calve as early as practically possible to decrease the non-productive period of life for these animals. To achieve earlier calving, heifers must reach puberty at an earlier age. Since puberty and body weight (BW) are highly correlated, early puberty and calving can be achieved by increasing rates of gain (USDA, 2007; Sejrsen et al., 1982).

It is widely reported that an excessive rate of gain during the pre-pubertal period, which can sometimes result from trying to get calves to their pubertal BW earlier, can impair mammary growth and reduce future milk yield (Radcliff et al., 2000; Lammers et al., 1999; Sejrsen et al., 1982). Precise mechanisms responsible for these effects are not completely understood but likely reflect a combination of a reduction in the usual length of peripubertal allometric mammary growth phase and/or altered responses to mammogenic stimuli (e.g., estradiol [E2]; Meyer et al., 2006a, b).

Prior to weaning, the dairy calf is consuming a nutrient dense milk-based diet. During this time feeding for an increased rate of gain was shown to not impair mammary development (Daniels et al., 2009a). In fact, several studies (Meyer et al., 2006b, Brown et al., 2005) suggest that a higher plane of nutrition pre-weaning can stimulate mammary development. Soberon et al. (2012) reported that increasing average daily gain (ADG) by 1 kg/d during the pre-weaning period was correlated with an increase in first lactation milk yield of more than 1,000 kg. Additionally, as
emphasized in the comprehensive review by Khan et al., (2011), improved pre-weaning nutrition benefits not only the growth, but also health and performance of the dairy calf.

Although mechanisms responsible for the positive correlation between pre-weaning gain and future milk potential are unknown, it is clear that mammary gland development during early life is essential to future productivity. Until recently, a general consensus was that mammary PAR growth was minimal during the pre-weaning period, until the onset of allometric mammary growth before puberty (Sinha and Tucker, 1969). Recent data has shown that allometric growth of the mammary parenchyma begins much earlier than once believed. Indeed, there can be a 60-fold increase in PAR mass from 30 until approximately day 90 of life (Esselburn et al., 2015; Capuco and Akers, 2010).

Our hypothesis is that providing heifers with a higher plane of nutrition during the pre-weaning period creates a mammary gland and mammary cells that are ‘primed’ to respond more readily to mammogenic stimuli. Estrogens are classic mammogenic hormones produced primarily by the ovary (Yart et al., 2014). Moreover, mammary tissue of prepubertal ruminants is sensitive to estradiol (E₂; Capuco et al., 2002; Ellis et al. 1998; Woodward et al., 1993). Despite the fact that circulating concentrations of E₂ are very low in prepubertal calves (Purup et al., 1993; Velayudhan et al. 2015) when E₂ influence is removed from the calf via ovariectomy or the use of an E₂ receptor antagonist, mammary gland development is markedly reduced (Berry et al., 2003; Tucker et al., 2016). Perhaps enhanced early
nutrition augments the action of mammogenic stimuli (such as E₂), and for this reason, we selected E₂ to test our hypothesis.

Lammers et al. (1999) assessed the effects of exogenous E₂ and plane of nutrition on mammary gland development in older heifers beginning at 4.5 mo of age over a period of 20 wk. They noted that E₂ treatment increased teat lengths, but E₂ administration and feeding for an increased rate of gain actually decreased future milk yield by 5.2 and 7.1% respectively. An increase in teat length as a result of E₂ treatment or exposure to E₂ in the diet has been used as a non-invasive bio-index for E₂ activity and for example, the effect has been noted in studies with beef heifers (Moran et al., 1991) and lambs (Ellis et al., 1998; Mahgoub et al., 2001).

To test our hypothesis, we fed calves two distinct pre-weaning diets. We have recently reported the effects of these diets on general body growth and development of multiple organs (Geiger et al., 2016). In this report we describe the impacts of these dietary treatments on the udder, mammary PAR, and MFP. We also describe the effects of E₂ (Preston, 1999) administered during the two weeks post-weaning on the same mammary parameters. We hypothesized that calves fed a higher plane of nutrition pre-weaning and given E₂ immediately post-weaning would experience increased mammary gland development compared with controls.
MATERIALS AND METHODS

This experiment was conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (#14-045-DASC).

Animal Handling and Experiment Design

The experimental design and animal handling were as previously described (Geiger et al., 2016). Briefly, calves were assigned to 1 of 2 experimental MR (n = 18/MR): 1) a restricted MR (R; 20.9% CP, 19.8% fat, dry matter (DM) basis; Southern States Cooperative, Inc., Richmond, VA) fed at 0.44 kg/hd/d DM basis, or 2) an enhanced MR (R; 28.9% CP, 26.2% fat, DM basis; Land O’Lakes Animal Milk Products Co., Shoreview, MN) fed at 1.08 kg/hd/d, DM basis. Starter (25.6% CP, 4.0% fat, DM basis, Southern States Cooperative, Inc., Richmond, VA) was offered at the end of wk 4 of the trial. A subset of calves (n = 6/diet) were harvested upon weaning to assess dietary effects on mammary gland development. The remaining calves (n = 24) were either given an E2 implant (Compudose®, Elanco Animal Health, Greenfield, IN) or a placebo implant at weaning. This produced the following treatment groups (n = 6/treatment): 1) calves fed restricted and given a placebo implant (R), 2) calves restricted-fed and given an E2 implant (R-E2), 3) enhanced-fed calves given a placebo implant (EH), and 4) enhanced-fed given an E2 implant (EH-E2). After 2 weeks of E2 treatment all remaining animals were harvested to assess the effect of E2 on calves fed the two different diets.

Sample Collection
Non-invasive mammary gland measures were collected once weekly and included: front and rear teat length, distance from front to rear teats, distance between front teats, distance between rear teats, as well as gland length and width. All mammary measures (i.e., distance between front and rear teats) that resulted in more than one measurement in a given week were averaged for a given calf. Unless presented for a specific weekly period, measurements for each calf represent the average value across the entire period (i.e., pre- or post-weaning) reported. The same researcher collected measurements on a weekly basis.

A dual-energy x-ray absorptiometry (DXA) machine was available for use during this trial. Due to the increased interest in gauging mammary gland development non-invasively (Esselburn et al., 2015), a Lunar Prodigy Advance DXA Machine (PA+130744, GE Healthcare, Little Chalfont, UK) was used to assess mammary tissue composition differences post-harvest. Scanning of each udder half lasted approximately 4 min. At the conclusion of all scans, the software was calibrated to ensure all tissue analyzed was appropriately identified. This was done using the point function to highlight all tissue containing areas of the udder half. The DXA scanning provides values for tissue fat content (%), tissue weight (g), fat weight (g), and lean tissue weight (g). Following the initial DXA scan, skin was removed from each udder half and the process was repeated. Because the pattern of response was essentially identical between udder halves with or without skin, only the scan without skin is reported and correlations between DXA fat determination and biochemical determination of fat content were calculated.

*Animal Harvest and Tissue Collection*
Heifers were euthanized as previously reported (Geiger et al., 2016). The udder was removed within 10 min after exsanguination and bisected medially into left and right halves. The left half was enclosed in plastic wrap and aluminum foil, snap frozen by immersion in liquid nitrogen, and stored at -80 °C. At a later date, the skin was removed from the left hemisphere; the PAR and MFP were dissected, separated, and weighed. Representative PAR and MFP samples were selected and pulverized using a freezer mill (SPEX sample prep freeze/mill® 6850, Metuchen, NJ). The freezer mill was kept cool using liquid nitrogen. Samples were processed using the following sequence: 5 min cool down, 4 min run time, 1 min cool down, 4 min run time. The resulting powder for both the PAR and MFP tissue was stored at -80 °C until subsequent analysis.

At the time of slaughter, the right rear mammary gland was used to collect samples of PAR and MFP tissue that were immediately frozen in liquid nitrogen and stored for future gene expression analysis. Portions of the right rear quarter were also dissected to provide PAR explants for subsequent incubations. The right forequarter was used to collect tissue samples that were fixed for future histological analysis and immunohistochemistry. Briefly, the dissected parenchymal mass with the teat attached was partially bisected or butterflied (depending on size) and immersed in a container of fixative as described in a previous study (Tucker et al. 2016). Examples of dissected, fixed tissues are shown in Geiger et al. (2016). After fixation these tissues were then subsampled to provide PAR from near the teat, midway to the outer region of the parenchymal, and at the margin between the outermost PAR region and the surrounding mammary fat pad. For some of the R
group heifers, the PAR mass was too small to justify subsampling so that the embedded tissues encompassed each of the three tissue regions in individual paraffin blocks.

For measurement of DNA and protein 250 mg samples of pulverized tissue for both MFP and PAR was homogenized using a Pro 200 homogenizer (Pro Scientific, Inc. Oxford, CT). Briefly, pulverized tissue was homogenized in 800 μL of a high salt buffer (0.05 M Na₂HPO₄ + 2 M NaCl + 0.002 M Na₂EDTA, pH 7.4). The homogenizer probe was washed in 700 μL of the same buffer and combined with the initial homogenate. Homogenates were centrifuged 100 x g for 10 min at 4° C and the supernatant was used for subsequent analyses.

**DNA Content**

The DNA content of PAR and MFP tissue was measured as previously described by Daniels et al. (2009b). Samples of the supernatant (2 μL) were transferred to a tube containing 2 mL of assay solution. The assay solution was composed of 100 μL of 1 mg/mL Hoechst H 33258 + 10 mL of 10 mM Tris-Cl + 90 mL of H₂O. Triplicate samples were measured using a Hoefer DQ 300 fluorimeter (Hoefer Inc., San Francisco, CA). Intra-assay CV’s averaged 5.8%.

**Protein Content**

Protein content of PAR and MFP tissue was determined using BCA™ Protein Assay Kit (Pierce, Product #23225, Rockford, IL). After completion of the provided standard curve, 10 μL of each sample supernatant was combined with 200 μL of working reagent in duplicate wells of a 96 well plate. After loading, the plate was gently mixed (30 sec) and incubated at 37° C for 30 min. Absorbency was measured
using a µQuant plate reader (µQuant, Inc., Bio-Tek Instruments, Winooski, VT) at a wavelength of 562 nm. Duplicate readings with a CV ≤ 10.0 % were accepted, samples were repeated as necessary.

**Fat Content**

Fat content of both PAR and MFP tissue was determined using a hexane-isopropanol extraction as described by Daniels et al., (2009b) with the exception that the extraction was initiated using pulverized tissue samples rather than homogenate. Duplicate pulverized tissue samples were assayed for all MFP and PAR samples from each calf. Briefly, acid washed glass tubes with Teflon-lined screw caps were used for the extractions. After recording initial tube weights, 0.5 g of pulverized MFP or 0.25 g of PAR was weighed into a 25 x 150 mm test tube. Each sample-containing tube then received 18 mL hexane-isopropanol (3:2 ratio of hexane to isopropanol) per g of tissue. Tubes were then capped and vortexed for 30 s. Thereafter 12 mL/g of a sodium sulfate solution (1 g Na₂SO₄ in 15 mL H₂O) was added to each tube. Tubes were vortexed for 30 s, left to sit until the phases separated (~5 min.) and vortexed for 30 s. All sample containing tubes were then centrifuged at 1000 x g for 5 min at 4° C. Then, the upper solvent phase was transferred to the small test tube using a glass Pasteur pipette. After the initial lipid extraction, samples were re-extracted and the resulting solvent layers combined in a single tube. Tubes with combined solvent layers were then loaded into an N-Evap (Organomation Associates Inc., Berlin, MA) with a warm water bath at 40° C where the solvent was evaporated under a stream of nitrogen. After evaporation the
difference between initial and final weights of the tubes was used to estimate lipid content of the tissue samples.

*Histological Evaluation*

Samples of fixed parenchymal tissue were embedded in paraffin and tissue sections subsequently prepared, dewaxed, and stained with hematoxylin and eosin for general histology as described in our prior studies (Velayudhan et al., 2012, Tucker et al. 2016).

*Statistical Analysis*

Statistical analysis was conducted as previously described (Geiger et al., 2016) using PROC GLIMMIX in SAS (SAS Institute Inc, Cary, NC). For non-invasive mammary measures, main effects included treatment, time (week), batch (i.e., shipment; calves arrived in 3 shipments for trial; Geiger et al., 2016), and the associated interactions. Data with multiple measures per calf (week) were analyzed as repeated measures when applicable. For slaughter data, main effects included treatment, slaughter date, and the associated interaction. Calf ID was treated as a random variable for all analyses. Batch, treatment, and their interaction were treated as fixed effects whereas residual error was treated as a random effect. Variables that did not contribute significantly to the model were removed. Significance was declared when P < 0.05.
RESULTS AND DISCUSSION

Non-Invasive Mammary Gland Measurements

Overall the non-invasive mammary gland measures we employed to evaluate changes in udder development were highly variable between and within treatments. There were no significant differences for distance between front and rear teats ($P = 0.40$) or distance between front teats ($P = 0.54$). Distance between rear teats was affected by diet with EH calves having greater distance between rear teats than R calves during the pre-weaning period (3.63 vs. 3.27 cm, $P \leq 0.05$). Gland length was greater in EH than in R calves during the pre-weaning period (10.29 vs. 9.21 cm, respectively; $P \leq 0.01$). Pre-weaning gland width was also greater in EH than R calves (11.18 vs. 10.25 cm; $P \leq 0.01$). Data reported for the non-invasive measures above are the average values over the entire pre-weaning period. These data (i.e. larger values for EH calves) correspond with differences measured for biochemical parameters and dissected tissue mass, but alone are of limited value.

Throughout most of the trial there were no detectable differences in the length of front ($P = 0.26$) or rear ($P = 0.58$) teats due to dietary treatment (entire pre-weaning period). However, on week 8 (Figure 4.1), EH-fed calves had longer front and rear teats ($P \leq 0.10$ and $\leq 0.05$). An increase in teat length due to $E_2$ became apparent during the second week of treatment for both front and rear teats (week 10; Figure 4.1). Length of both front and rear teats was increased ($P \leq 0.05$) in $E_2$-treated calves by week 10. These results are similar to reports by Lammers et al. (1999) and Moran et al. (1991) showing calves given $E_2$ had longer teats. The
increase in teat length we observed confirms that the E$_2$ treatment applied to these calves was sufficient to elicit a mammary tissue response.

Non-invasive mammary gland measurements are an attractive approach to gauge mammary gland development since they do not require animal sacrifice or surgery. However, measures such as distance between teats are of limited value i.e. modest correspondence with larger udders. However, to the best of our knowledge, this is the first study indicating that diet can influence teat length of heifers this early in life. It may be that the dietary differences observed reflect the marked increase in MFP mass (see below) and a presumptive increase in adipocytes (i.e. greater MFP DNA). This could allow for enhanced local aromatase activity (To et al., 2015) and therefore a localized increase in E$_2$ in EH calves and thus an effect on teat length.

Figure 4.2 provides results obtained by DXA scanning of udder halves collected at the time of slaughter. It is clear that mammary glands of EH-fed calves had more mammary fat, a result also reflected in biochemical analyses of MFP (see below). Additionally, EH-E2 calves had the greatest amount of mammary fat, among calves sacrificed at 10 weeks, which was also confirmed via biochemical analysis. Perhaps most interestingly, we sought to determine correlations between scanning data, dissection results, and biochemical measures of tissue composition. Correlation data indicated a highly positive correspondence between mammary gland fat determined biochemically and by DXA for all treatments ($r = 0.94$, without skin). However, it is important to note that there was no meaningful correlation between mammary gland lean tissue (as determined by DXA) and either mass of
PAR or DNA content of the PAR. Thus DXA data was not useful in determining the amount of PAR in the developing mammary glands of these young prepubertal calves.

In their recent report, Esselburn et al., (2015) describe some of the history of use of udder measurements and palpation to evaluate mammary development in heifer calves and in particular demonstrate the utility of ultrasound measurements to evaluate udder and mammary development in young heifers. We took advantage of available DXA scanning equipment to determine if this tool could be used to evaluate udder development in these heifers. This methodology has been commonly used in animal experiments (Scholz et al., 2015) and can determine adipose content. Differences in mammary adipose tissue from our animals might be obtainable via DXA.

These data indicate that DXA scanning can be used to determine fat content of the mammary gland. However, mammary glands were removed from the body of the calf before scanning and results presented are from de-skinned mammary glands although results were similar with and without skin intact. It is uncertain if DXA could be used to estimate udder fat content in the intact animal. The goal of ultrasound and similar (i.e., DXA) technologies is to eliminate the need for animal harvest in experiments. If DXA approves an effective means to gauge the developing mammary gland in future experiments, the need for animal harvest may be reduced. In this particular trial, however, animals were harvested at trial conclusion as validating DXA technology was not the primary focus of this trial, but rather an opportunity presented near the time of planned harvest procedures. However, there
is certainly much recent interest in use of a variety of non-invasive techniques including DXA to determine tissue composition in multiple species of animals and in selected organs and tissues (Scholz et al., 2015).

**Gross Mammary Gland Data**

After gross udder weights were recorded (Geiger et al., 2016) the udder was bisected and the left half saved for biochemical analysis. Data for the masses of udder tissue collected at the time of weaning are given in Figure 4.3. Trimmed mammary gland weights were 5.2-fold greater \((P \leq 0.01)\) for EH-fed than R-fed calves. The difference was even more dramatic \((7.3\text{-fold}; P \leq 0.01)\) for the mass of dissected mammary PAR tissue. Similarly, the mass of the MFP was also markedly increased in EH-fed calves compared with R-fed calves \((5.9\text{-fold}; P \leq 0.01)\). Upon adjusting for BW, EH-fed calves had more total mammary tissue compared with R-fed calves \((1.3 \text{ vs. } 0.39 \text{ g/kg BW}; P \leq 0.01)\). Additionally, EH-fed calves had more mammary PAR \((0.07 \text{ vs. } 0.02 \text{ g/kg BW}; P \leq 0.05)\) and MFP \((1.13 \text{ vs. } 0.30 \text{ g/kg BW}; P < 0.01)\) than R-fed calves.

Mammary data describing the effects of E\(_2\) and diet at the week 10 slaughter are given in Figure 4.4. The mass of the trimmed mammary gland was greater \((P < 0.01)\) in EH-E2 heifers than all other groups and there was a progressive increase in R, EH, R-E2, and EH-E2 heifers. Calves in the EH-E2 treatment had more PAR tissue compared to all other calves on trial and more PAR tissue compared with EH-fed calves \((P < 0.05)\). The difference in PAR tissue mass between R-E2 and R calves was non-significant. Calves given EH-E2 also had greater MFP weights compared with all other calves on trial and had greater MFP weights compared with EH-fed calves \((P <
0.01). Upon adjustment for BW, calves in the EH-E2 treatment had more total mammary tissue compared with all other calves on trial, most notably, EH-fed calves (1.26 vs. 0.72 g/kg BW; $P < 0.01$). Additionally, R-E2 calves had more mammary gland tissue than R-fed calves (0.73 vs. 0.41 g/kg BW, respectively; $P < 0.01$). Calves in the EH-E2 treatment had more PAR per unit of BW compared with all other treatments. Amount of MFP tissue per unit of BW responded similarly to PAR tissue.

Our data clearly demonstrate that pre-weaning nutrition can dramatically impact the overall development of the bovine mammary gland. In particular, these data show that enhanced feeding compared with restricted feeding, stimulates growth of both the mammary fat pad and the mammary PAR. Moreover, our hypothesis that mammary tissue derived from enhanced fed calves is better able to respond to mammogenic stimuli is supported by our observations of maximum mammary development (i.e. mass of mammary PAR) in enhanced fed heifers given E$_2$. Restricted-fed heifers were also capable of responding to exogenous E$_2$ but the response was blunted compared with the robust effect of E$_2$ stimulation in enhanced-fed heifers. We hope to uncover underlying mechanisms via gene expression analysis of PAR explants incubated at the time of as well as samples of snap frozen PAR and MFP tissue.

Data herein agrees with previous research indicating that feeding a higher plane of nutrition through MR during the pre-weaning period increases total mammary gland weight (Brown et al., 2005). The most dramatic difference between previous reports and present data was the 7.3-fold increase in mammary parenchyma mass observed between EH- and R-calves. Little data exists indicating
that altering plane of nutrition during the pre-weaning period can lead to the

 dramatic increase observed in the present study. Brown et al., (2005) did find that

 offering a higher plane of nutrition increased mammary PAR mass i.e. 3.8-fold,

 compared to the 7.3-fold difference observed in our trial. This may simply reflect the

 greater difference between treatments employed in this study. The 5.9-fold increase

 in MFP weight observed in this study is consistent with several other trials (Daniels

 et al., 2009b; Meyer et al., 2006b; Thibault et al., 2003).

 Providing E\textsubscript{2} post-weaning to these calves allowed us to test our hypothesis

 and determine if calves fed a higher plane of nutrition are better able to respond to

 mammogenic stimuli. Enhanced-fed calves given E\textsubscript{2} post-weaning did indeed exhibit

 an increase in mammary gland PAR, and MFP tissue weight whereas R-fed calves

 did not. It is important to note, however, that analysis of figure 4.4 appears to

 indicate that change in PAR weights as a result of E\textsubscript{2} appear to be similar for both

 dietary treatments. However, due to the lower PAR weight that resulted from the R

 diet, the difference in PAR weight was not different between R and R-E2 calves

 whereas the difference between EH and EH-E2 calves was statistical. We would

 argue that this alone is worth noting as it is highly unlikely not matter what

 decisions are made regarding R-fed calves, their PAR mass will not be capable of

 reaching values similar to that of EH calves, and especially EH-E2 calves. For this

 reason, we believe these results support our hypothesis (i.e., calves on a higher

 plane of nutrition were better able to respond to mammogenic stimulation). Future

 analyses may help us identify cellular and physiological mechanisms to explain

 these differences in tissue development.
Mammary Gland Composition

Mammary gland composition for both the MFP and PAR of calves slaughtered at weaning are shown in Table 4.1. Mammary fat pad protein was affected by diet with R calves having a greater protein concentration than EH calves \((P \leq 0.01)\). However, EH calves had greater total protein in the MFP than R calves \((P \leq 0.01)\). Mammary gland fat pad DNA concentration was not affected by diet, but MFP total DNA was 5.4-fold greater for EH calves than R calves \((P \leq 0.01)\). Fat concentration of the MFP was greater for EH calves compared with R calves \((P < 0.01)\), as was total fat in the MFP \((P \leq 0.01)\)

Protein concentration of the PAR was unaffected by diet but total protein in the PAR was markedly greater \((6.8\text{-fold}; P \leq 0.01)\) for EH calves compared with R calves. Additionally, PAR DNA concentration was unaffected by diet but total DNA in the PAR was 7.6-fold greater for EH calves \((P \leq 0.05)\). Neither PAR fat concentration nor total fat were effected by dietary treatment.

Biochemical composition of the MFP and PAR of calves given either an E2 or placebo implant immediately post-weaning are shown in Table 4.2. Protein concentration of the MFP was unaffected by post-weaning treatment. However, total protein in the MFP was increased in EH-E2 calves compared with all other calves \((P \leq 0.01)\). DNA concentration of the MFP was also unaffected by post-weaning treatment. However, total DNA in the MFP was greater in EH and EH-E2 calves than R and R-E2 calves \((P < 0.05)\) with R-E2 calves tending to have more total MFP DNA than R-fed calves \((P < 0.10)\). Fat concentration of the MFP was higher \((P \leq 0.05)\) in
EH-E2 than EH, R, and R-E2 calves and EH-E2 calves had greater total fat ($P \leq 0.05$) in the MFP compared with EH, R, and R-E2 calves.

Enhanced fed calves given E$_2$ had more PAR protein than all other calves ($P \leq 0.05$). DNA concentration of the PAR was unaffected by post-weaning treatment. However, total PAR DNA was greater for EH-E2 calves compared with EH, R-E2, and R calves ($P < 0.01$). Fat concentration of the PAR was greatest for R-fed calves compared with all other treatment groups ($P \leq 0.01$).

*Histological Appearance of Parenchyma*

While the general architecture of the epithelial appeared normal in all animals, there were dramatic differences in the degree of development across treatments. As can be seen in the gross anatomy of the PAR shown in Geiger et al (2016), the epithelium of R calves was largely confined in surface folds immediately adjacent to the gland cistern. This is apparent in the simple single bisections needed to expose the entire glandular epithelium to fixative in R calves compared with the multiple butterfly slices that were necessary expose the PAR tissue to fixative in EH and EH-E2 calves. Upon histological examination, in EH calves and especially EH-E2 calves, the mucosal surface of the gland cistern was much larger and more extensively folded. There were also a much greater number of ductal structures that had penetrated into the surrounding stromal tissue in EH and E$_2$ treated calves (Figure 4.5). Major ducts exposed to the luminal space of the gland cistern were usually 1-3 cell layers thick, in addition to a layer of presumptive myoepithelial cells along the basement membrane adjacent to the surrounding stromal tissue. Epithelial structures where invagination and expansion into the surrounding
epithelium had occurred resembled many of the branched ductal structures observed in older prepubertal heifers (Capuco et al., 2002; Rowson et al., 2012). Others have noted differences in the relative epithelial development of the heifer mammary gland due to diet and or body weight (Davis Rincker et al., 2008; Daniels et al., 2009b) but degree of difference between R and EH treatments was markedly greater in this study. This is likely a reflection of the greater difference in the dietary treatments in this experiment. While we did not evaluate a set of calves, sacrificed at the beginning of the trial, the appearance of the tissue of R group heifers was similar to tissues from calves between 2 and 3 weeks of age (Beaudry, et al., 2016). Figure 4.5 illustrates differences in the histological appearance of mammary tissue across treatments.

Previous studies have indicated the ease with which MFP mass and composition may be manipulated as a result of dietary modifications. Mammary fat pad lipid content was increased in EH calves at weaning, leading to an increase in total MFP fat. This agrees with previous work that also noted increases in MFP lipid concentration and lipid mass produced by feeding a higher plane of nutrition (Daniels et al., 2009b). Additionally, R-fed calves had greater protein concentration in the MFP compared with EH calves, although EH calves had an increase in total MFP protein (as a result of MFP size). This contradicts previous work that reported an increase in MFP protein concentration as a result of feeding a higher plane of nutrition (Meyer et al., 2006b).
Of particular interest when assessing the effects of feeding a higher plane of nutrition on mammary development and future milk potential is mammary PAR composition. Mammary PAR composition was unaltered as PAR fat, protein, and DNA concentrations were unaffected by pre-weaning diet. However, total PAR protein and DNA were increased as a result of feeding a higher plane of nutrition. This agrees with Brown et al. (2005) who found that feeding a higher plane of nutrition pre-weaning can lead to an increase in PAR DNA. The fact that protein, fat, and DNA concentrations were unaffected by diet in this trial suggests that the general cellular characteristics of the parenchymal tissue were not impacted. However, it is also evident that the relative appearance of epithelial structures i.e. penetration into the surrounding stromal tissue was nonetheless impacted by diet (Figure 4.5). Given there are also a substantial (and variable number of stromal cells within the parenchymal compartment) it seems clear that the differences in epithelial appearance were not reflected in differences in the DNA concentration of PAR. If future work shows that this type of feeding regimen does not decrease future milk yield potential, this suggested that greater intake and growth rates pre-weaning can be achieved without any negative consequences on mammary development and future performance.

Neither protein nor DNA concentration of the MFP were affected by post-weaning treatment with E2. DNA concentration of the PAR was unaffected by treatment, but total PAR DNA was greatest in EH-E2 calves compared to all other calves on trial. The fact that EH-E2 calves had an increase in total DNA compared with H calves while R-E2 calves did not experience a similar response (albeit a
numerical increase) suggests E₂ was better able to stimulate epithelial cell growth in calves fed a higher plane of nutrition. This idea is reinforced by the fact that calves fed EH and given E₂ post-weaning not only had an increase in total DNA in the PAR, but also had an increase in teat length, mammary gland weight, MFP weight, and PAR weight compared to all other calves on trial.

Demonstration that exogenous E₂ can stimulate mammary development in prepubertal calves is not new but our data showing that pre-weaning feeding appears to markedly alter the responsiveness of the mammary gland to stimulation by E₂ is we believe novel. Thus, our hypothesis that E₂ may act differentially in calves reared on different planes of nutrition is strongly supported. This suggests that nutritional mediation of mammary development is at least partially mediated by changes in the capacity of target cells to respond to mammogenic hormone stimulation.

In subsequent analyses we will seek to determine cellular and molecular mechanisms that would explain observed differences in response to both diet and post-weaning E₂ treatment and specifically if E₂ signaling pathways are impacted by diet.
CONCLUSIONS

Feeding calves differing planes of nutrition resulted in dramatic differences in mammary PAR (7.3-fold) and MFP (5.9-fold) without compromising the biochemical composition of PAR. These results indicate clearly that pre-weaning diet can influence mammary gland development in Holstein heifers. The increase in mammary gland tissue and PAR mass observed when E2 was provided to EH calves, but not R calves, indicates that our fundamental hypothesis was correct. Additional research is underway to determine the molecular/cellular mechanisms and/or pathways responsible for these differences. Dual x-ray absorptiometry proved an effective tool for analysis of mammary fat. However, DXA analysis was unable to effectively quantify mammary PAR and the analysis was performed on removed mammary glands. It is yet to be determined if this technology can effectively measure the mammary fat when the udder is still attached to the body wall of the animal.
ACKNOWLEDGEMENTS

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Table 4.1. Composition of the mammary fat pad and parenchyma at weaning of Holstein heifer calves fed either a control diet or a higher plane of nutrition

<table>
<thead>
<tr>
<th>Item</th>
<th>R</th>
<th>EH</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammary Fat Pad</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein (g)</td>
<td>0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2</td>
</tr>
<tr>
<td>Protein (mg/g)</td>
<td>15.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1</td>
</tr>
<tr>
<td>Total DNA (mg)</td>
<td>4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.18</td>
</tr>
<tr>
<td>DNA (mg/g)</td>
<td>0.14</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>3.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.7</td>
</tr>
<tr>
<td>Fat (mg/g)</td>
<td>112&lt;sup&gt;a&lt;/sup&gt;</td>
<td>667&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.2</td>
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<td><strong>Mammary Parenchyma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein (mg)</td>
<td>0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3</td>
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<tr>
<td>Protein (mg/g)</td>
<td>0.14</td>
<td>0.13</td>
<td>1.8</td>
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<tr>
<td>Total DNA (mg)</td>
<td>2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.33</td>
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<tr>
<td>DNA (mg/g)</td>
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<td>1.95</td>
<td>0.53</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>0.15</td>
<td>2.10</td>
<td>2.18</td>
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<tr>
<td>Fat (mg/g)</td>
<td>106</td>
<td>201</td>
<td>69.8</td>
</tr>
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</table>

<sup>1</sup>R = Calves fed restricted MR (20% CP, 20% fat) at a rate of 0.45 kg/d
<sup>2</sup>EH = Calves fed enhanced MR (28% CP, 25% fat) at a rate of 1.13 kg/d
<sup>a,b</sup>Differing superscripts within row indicated diet differences (P < 0.05)
Table 4.2. Composition of the mammary fat pad and parenchyma at week 10 of Holstein heifer calves fed either a control diet or a higher plane of nutrition pre-weaning with or without exogenous estrogen administration immediately post-weaning

<table>
<thead>
<tr>
<th>Item</th>
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<th>R-E2</th>
<th>EH</th>
<th>EH-E2</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary Fat Pad</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein (mg)</td>
<td>0.7a</td>
<td>1.1ab</td>
<td>1.6b</td>
<td>2.7c</td>
<td>0.3</td>
</tr>
<tr>
<td>Protein (mg/g)</td>
<td>18.7</td>
<td>18.8</td>
<td>17.3</td>
<td>20.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Total DNA (mg)</td>
<td>6.6ay</td>
<td>17.6az</td>
<td>30.8b</td>
<td>29.4b</td>
<td>3.08</td>
</tr>
<tr>
<td>DNA (mg/g)</td>
<td>0.18</td>
<td>0.29</td>
<td>0.35</td>
<td>0.23</td>
<td>0.08</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>8.0a</td>
<td>14.2a</td>
<td>24.4a</td>
<td>64.1b</td>
<td>9.5</td>
</tr>
<tr>
<td>Fat (mg/g)</td>
<td>217a</td>
<td>234a</td>
<td>277a</td>
<td>501b</td>
<td>38.6</td>
</tr>
<tr>
<td>Mammary Parenchyma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein (mg)</td>
<td>0.05a</td>
<td>0.16ab</td>
<td>0.27b</td>
<td>0.82c</td>
<td>0.13</td>
</tr>
<tr>
<td>Protein (mg/g)</td>
<td>22.2yz</td>
<td>30.8z</td>
<td>18.1y</td>
<td>30.1z</td>
<td>3.4</td>
</tr>
<tr>
<td>Total DNA (mg)</td>
<td>3.9a</td>
<td>9.8a</td>
<td>26.1a</td>
<td>59.1b</td>
<td>11.18</td>
</tr>
<tr>
<td>DNA (mg/g)</td>
<td>1.87</td>
<td>1.93</td>
<td>1.73</td>
<td>2.16</td>
<td>0.15</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>0.8ab</td>
<td>0.5a</td>
<td>1.3ab</td>
<td>3.1b</td>
<td>0.71</td>
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<tr>
<td>Fat (mg/g)</td>
<td>385a</td>
<td>98.4b</td>
<td>86.1b</td>
<td>113b</td>
<td>36.8</td>
</tr>
</tbody>
</table>

1R = Calves fed restricted MR (20% CP, 20% fat) at a rate of 0.45 kg/d
2R-E2 = Calves fed restricted and given estrogen for 2 weeks post-weaning
3EH = Calves fed enhanced MR (28% CP, 25% fat) at a rate of 1.13 kg/d
4EH-E2 = Calves fed enhanced EH and given estrogen for 2 weeks post-weaning

a,b,c Differing superscripts within row indicated treatment differences (P < 0.05)

y,z Differing superscripts within row indicate treatment tendencies (0.05 > P < 0.10)
Figure 4.1. Front and rear teat length of Holstein heifer calves fed either a control diet or a higher plane of nutrition pre-weaning with or without exogenous estrogen administration immediately post-weaning. (A) Front teat length during weeks 8, 9, and 10 to assess diet and estradiol effects, and (B) Rear teat length during weeks 8, 9, and 10 to assess diet and estradiol effects. White bar indicates restricted (R), black bar indicates enhanced (EH), bar with diagonal lines indicates R-E2, black bar with white dots indicates EH-E2. 

\textsuperscript{a,b,c}Differing superscripts indicate treatment differences \((P \leq 0.05)\). \textsuperscript{x,z}Differing superscripts indicate treatment tendencies \((P \leq 0.1)\).
Figure 4.2. Dual x-ray absorptiometry data of trimmed mammary glands (without skin) from Holstein heifer calves fed either a restricted diet or a higher plane of nutrition pre-weaning with or without exogenous estrogen administration immediately post-weaning.

(A) Mammary gland fat weight ($P < 0.01$) for calves fed either a restricted or enhanced diet pre-weaning (week 8 harvest), and (B) mammary gland fat weight for calves fed a restricted or enhanced diet pre-weaning with or without exogenous estradiol post-weaning (week 10 harvest; $P < 0.01$). White bar indicates R, black bar indicates EH, bar with diagonal lines indicates R-E2, black bar with white dots indicates EH-E2. Differing superscripts indicate treatment differences ($P \leq 0.01$).
Figure 4.3. Trimmed mammary gland weights of Holstein heifer calves fed either a control diet or a higher plane of nutrition pre-weaning. (A) Trimmed mammary gland weight, (B) Mammary parenchyma (PAR) weight, and (C) Mammary fat pad weight. White bar indicates restricted (R), black bar indicates EH. * = dietary difference ($P \leq 0.01$).
Figure 4.4. Trimmed mammary gland weights of Holstein heifer calves fed either a control diet or a higher plane of nutrition pre-weaning with or without exogenous estrogen administration immediately post-weaning. (A) Trimmed mammary gland weight, (B) Mammary parenchyma (PAR) weight, and (C) Mammary fat pad weight. White bar indicates restricted R, black bar indicates enhanced (EH), bar with diagonal lines indicates R-E2, black bar with white dots indicates EH-E2. Differing superscripts indicate treatment differences ($P \leq 0.05$).
Figure 4.5. Histological depictions of mammary parenchyma of Holstein heifer calves fed either a control diet or a higher plane of nutrition pre-weaning with or without exogenous estrogen administration immediately post-weaning.

The upper panel provides a low power (4x objective lens) view of mammary tissue from an R group calf sampled at 8 weeks of age. This section through a region of the gland cistern shows minimal development of the epithelium beyond the folded epithelial layer of the gland cistern. The middle panel also taken with a 4x objective lens illustrates the much more extensive development of the epithelium of a EH-E2 group heifer at 10 weeks of age. Folding of the surface epithelium is extensive and numerous ductal outgrowths are spreading into the underlying stromal tissue (arrows). The lower panel provides a higher magnification (20x objective lens) of a portion a ductal outgrowth from an EH group heifer at 10 weeks of age. Presumptive myoepithelial cells (arrows) are adjacent to the epithelial cell layer 1-3 cells in thickness. For the upper and middle panels the magnification bar equals 1,000 µM and 50 µM in the lower panel.
REFERENCES


by ultrasound, mammary mass, and composition in Holstein heifers reared on 1 of 3 diets from birth to 2 months of age. J. Dairy Sci. 98:5280-5293.


CHAPTER V. FEEDING AN ENHANCED DIET TO HOLSTEIN HEIFERS DURING THE PRE-WEANING PERIOD IMPACTS STEROID RECEPTOR EXPRESSION AND INCREASES CELLULAR PROLIFERATION

ABSTRACT

Pre-weaning diet and estradiol treatment alters mammary development. Our objectives were to study the effects of diet and estradiol on mammary histology, proliferation, and expression of estrogen (ERα) and progesterone (PR) receptors. Thirty-six Holstein heifer calves were reared on: 1) a control milk replacer (MR) fed at 0.44 kg powder/hd/day, dry matter (DM) basis (R; 20.9% crude protein [CP], 19.8% fat, DM basis), or 2) an enhanced MR fed at 1.08 kg powder/hd/day, DM basis (E; 28.9% CP, 26.2% fat, DM basis). MR was fed for 8 weeks. At weaning a subset of calves were harvested (n = 6/diet). Remaining calves received E₂ implants and were harvested at week 10. Treatments were: 1) R, 2) R + E₂ (R-E₂), 3) EH, and 4) EH + E₂ (EH-E₂). One day prior to harvest calves were given bromodeoxyuridine (BrdU; 5mg/kg BW). At sacrifice, parenchyma (PAR) was removed and fixed. Sections from lower, middle and distal zones were stained with H and E and antibodies to measure expression of ERα, PR, and BrdU. Comparisons with PROC GLIMMIX in SAS on a per area and per cell basis were similar. At week 8, R-fed calves had more (P < 0.01) PR expressing cells in distal PAR. But PR expression intensity was greater (P < 0.01) in EH-fed calves. The proportion of cells expressing ERα was not affected by diet, but expression intensity was increased for EH-fed calves across all zones (62 to 81%; P < 0.01). Percent BrdU positive cells was 2 and 0.5-fold greater (P < 0.01) for EH-fed calves in zone 2 and 3. At week 10, calves treated with estradiol had 3.9-fold greater PR expression intensity. The intensity and percent of cells expressing ERα
was lowest in estradiol-treated calves. Overall, estradiol-treated calves had the most proliferating cells (P < 0.01). Moreover, in zone 3, EH-E2 calves had a higher percentage of proliferating cells than calves on all other treatments (P < 0.01). Results indicate both diet and estradiol administration alter proliferation rates of the mammary epithelium and that changes in expression of ERα and PR are likely at least partially responsible for changes in mammary PAR development associated with enhanced pre-weaning feeding of dairy calves. However, further, more detailed analyses are needed to fully understand mechanisms at play.

**Keywords**: mammary gland, calf, estrogen receptor, progesterone receptor
INTRODUCTION

The goal of the replacement heifer industry is to provide the lactating herd with the most profitable animal possible. With replacement rearing accounting for the second largest on-farm expense (Heinrichs, 1993), the need for the dairy industry to develop the most efficient replacement rearing strategies could not be greater.

Much work has been done over the past decades to assess management of replacement heifers and how this can alter the quality of the replacement generated (i.e., environment, reproduction, etc.). However, the area of most emphasis has by far been nutritional management of the dairy heifer. For some time, the goal of the replacement industry was to quickly convert a replacement to a member of the lactating herd. By doing so, non-productive replacements were quickly becoming productive members of the milking herd and generating income. For this to occur, heifer calves must give birth earlier, and in turn, be bred and reach puberty at an earlier age. With puberty being highly correlated to body weight (BW; Sejrsen, 1994), this means that pubertal BW must be achieved at as early an age as possible. This required feeding a diet that encouraged greater pre-pubertal BW gain. However, it has been documented for some time that greater pre-pubertal gains result in decreased first lactation performance by 15% or more (Radcliff et al., 2000). Therefore, it appears uneconomical to encourage greater BW gain pre-pubertally, and rather, producers should strive to calve replacements in at a more traditional age (i.e., 22-24 months) and focus on the quality of the replacement-rearing program.

Recently, an increasing number of research trials has shown that all portions of the pre-pubertal period are not created equal. In fact, multiple studies have shown that
striving for increased BW gain in replacement heifers during the milk-fed (pre-weaning) stage does not negatively affect first lactation performance as it does during the post-weaning pre-pubertal period as a whole. Many studies have actually reported economical advantages to increasing BW gain during this period of early life (Drackley et al., 2007; Soberon et al., 2012).

Although many studies indicate that striving for a greater rate of gain during the milk-fed stage of life increases first lactation milk yield, mechanisms at play remain largely unknown. Meyer et al. (2006a) found that by feeding a higher plane of nutrition early in life, mammary fat pad (MFP) weights and DNA content of the mammary parenchyma (PAR) can be increased. In this study, however, differences observed within the PAR disappeared when BW was considered. Additionally, Brown et al. (2005) found that providing a higher plane of nutrition to calves in early life resulted in more total PAR tissue, PAR DNA, PAR RNA, and concentrations of DNA and RNA in the mammary gland. These are but two examples of studies that looked to investigate the mechanisms at play behind increased lactation performance observed by feeding a higher plane of nutrition during early life. However, these examples assess the mammary gland on a gross anatomical level. It is logical to believe that if differences exist on this level, functional tissue differences may also exist as a consequence of the diet fed during the pre-weaning phase of life.

Therefore, our lab sought to replicate previous work and assess all levels of mammary gland development. Indeed, we found that by feeding two distinctly different planes of nutrition during the pre-weaning phase of life, we were able to grow a much larger heifer (i.e., roughly 20 kg heavier at weaning; Geiger et al., 2016a). Our results
replicated previous research findings by Brown et al. (2005) in terms of gross tissue differences (PAR and MFP weights) due to feeding a higher plane of nutrition (without compromising PAR quality; Geiger et al., 2016b), but we were also interested in better understanding the mechanisms involved. Data of Brown et al. (2005) likely explains some of the mechanisms (increased mammary development) to account for increased milk yield as a result of enhanced feeding as reported in other studies (Drackley et al., 2007; Soberon et al., 2012), but it is also very likely that other mechanisms are at play. Unfortunately, Brown et al. (2005) did not follow calves through to lactation to confirm or deny this hypothesis.

Histological analyses are effective tools to assess mammary gland development. Daniels et al. (2009b) found that when Holstein heifers were compared at a similar BW, complexity of PAR development was not altered by early-life dietary treatment. Similarly, Brown et al. (2005) found that percentage of epithelium within the mammary PAR was not affected by early life diet. However, percentage of the mammary PAR occupied by lumen was increased for calves fed a more nutrient dense diet in early life, whereas the area of the PAR occupied by stroma was 4% lower for calves fed a higher plane of nutrition during early life. Even further, calves were given a mammary gland tissue development score related to the complexity of mammary gland development observed upon histological examination. No differences were found when mammary gland development score was compared across treatments. A visual assessment of our previous work (Geiger et al., 2016b) indicates that calves fed a higher plane of nutrition experience more complex PAR development during early life. The additive effect of a greater PAR mass (with uncompromised composition) along with more complex
histological development may begin to piece together the story of how early life nutrition can positively alter future milk yield.

The next apparent step after mammary gland composition and histology are assessed is to use immunohistochemical analysis to assess cellular responses. Brown et al. (2005) found that the percentage of estrogen receptor-α (ERα) positive cells was not affected by dietary treatment, but the percentage of proliferating cells (Ki67 labeling) was decreased in both the subtending ducts and terminal ductular units of calves fed a higher plane of nutrition during early life. Another study, however (Meyer et al., 2006b), found that percentage of proliferating cells was increased when calves fed a higher plane of nutrition were compared to control calves at similar body weight. When compared at 100 kg slaughter weight, bromodeoxyuridine (BrdU; indicator of cell proliferation) labeling of mammary epithelial cells was increased by approximately 4% and a tendency existed for increased BrdU labeling when all slaughter weights were compared from that particular trial (slaughtered every 50 kg from 100 to 35 kg BW).

Recent data from our lab (Geiger et al., 2016a) has confirmed results of previously mentioned studies in terms of BW gain and weaning weights when calves are fed a higher plane of nutrition during early life. In addition, we also replicated gross morphological differences on the mammary gland level in terms of mammary PAR weight (Geiger et al., 2016b). In addition, it was shown that this increase in mammary PAR weight as a result of diet during early life did not detrimentally alter mammary gland PAR development or composition, but in fact, improved it. However, our lab did not anticipate the extreme increase in PAR mass that would result from the previously mentioned work. Indeed calves, fed a higher plane of nutrition in our experiment
exhibited a 7.3-fold increase in PAR mass (Geiger et al., 2016b). This is a dramatic difference from previous research that found an increase in mammary gland PAR mass of 3.8-fold (Brown et al., 2005). We believe this may, in part, be due to greater differences in imposed treatments on our trial. This dramatic difference in diet-related PAR developmental is greater than reported in prior studies. This information combined with the fact that calves fed a higher plane of nutrition on our trial experienced more complex PAR development (Geiger et al., 2016b) leads us to believe it is worthwhile and warranted to investigate the mammary gland on a immunohistochemical level.

The objective of this study was to assess the effects of two different pre-weaning diets on expression of receptors for mammogenic steroid hormones and corresponding impacts on proliferation of mammary epithelial cells within the developing mammary gland. A secondary objective was to assess the impact exogenous estradiol might have on the same parameters when provided to these two different groups of calves post-weaning. It is our hypothesis that providing a greater plane of nutrition pre-weaning will alter expression of steroid receptors (ERα and PR) in the mammary epithelial cell population and increase cellular proliferation (greater BrdU expression) within the developing mammary gland. In addition, we further hypothesized that calves fed a higher plane of nutrition and given estradiol immediately post weaning will experience a far greater response in terms of cellular proliferation compared with all other calves on trial as we believe early life nutrition better prepares the mammary gland to respond to mammogenic stimuli (Geiger et al., 2016a,b).
MATERIALS AND METHODS

This experiment was conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (#14-045-DASC).

Animal Handling and Experimental Design

The experimental design and animal handling were as previously described (Geiger et al., 2016a). Briefly, calves were assigned to 1 of 2 experimental MR (n = 18/MR): 1) a restricted MR (R; 20.9% CP, 19.8% fat, dry matter (DM) basis; Southern States Cooperative, Inc., Richmond, VA) fed at 0.44 kg/hd/d, DM basis, or 2) an enhanced MR (R; 28.9% CP, 26.2% fat, DM basis; Land O’Lakes Animal Milk Products Co., Shoreview, MN) fed at 1.08 kg/hd/d, DM basis. Starter (25.6% CP, 4.0% fat, DM basis; Southern States Cooperative, Inc., Richmond, VA) was offered at the end of wk 4 of the trial. A subset of calves (n = 6/diet) was harvested upon weaning to assess dietary effects on mammary gland development. The remaining calves (n = 24) were either given an E2 implant (Compudose®, Elanco Animal Health, Greenfield, IN) or a placebo implant at weaning. This produced the following treatment groups (n = 6/treatment): 1) calves fed restricted and given a placebo implant (R), 2) calves restricted-fed and given an E2 implant (R-E2), 3) enhanced-fed calves given a placebo implant (EH), and 4) enhanced-fed given an E2 implant (EH-E2). After 2 weeks of E2 treatment all remaining animals were harvested to assess the effect of E2 on calves fed the two different diets.

Animal Harvest and Sample Preparation

Animal harvest and sample preparation are outlined in detail in previous work (Geiger et al., 2016a,b). The right fore quarter of the udder was used to collect tissue
samples that were fixed for immunohistochemistry. Briefly, the dissected parenchymal mass with the teat attached was partially bisected or butterflied (depending on size) and immersed in a container of fixative as described in a previous study (Tucker et al. 2016). Examples of dissected, fixed tissues are shown in Geiger et al. 2016a. After fixation these tissues were then subsampled to provide PAR from near the teat, mid-way to the outer region of the parenchymal, and at the margin between the outermost PAR region and the surrounding mammary fat pad. For some of the R group heifers, the PAR mass was too small justify subsampling so that the embedded tissues encompassed each of the three tissue regions in individual paraffin blocks.

*Primary and Secondary Antibody Incubation*

After aspiration of CAS Block, approximately 50 µL of fresh, diluted primary antibody solution was added to each tissue section as appropriate. Slides were incubated with primary antibody overnight at 4° C in a humidified chamber. One or two sections per slide served as a negative control and received CAS Block as a substitute for primary antibody solution. The remaining sections on the slide (n ≥ 2) served as replicates for the desired labeling or staining.

After removal of the primary antibody via vacuum aspiration the slides were rinsed in PBS three times for 5 min. each time. All tissue sections (including negative controls) received 50 µL of the diluted secondary antibody mixture and were incubated for 60 min. at room temperature. Secondary antibody solutions were then aspirated and slides were rinsed in PBS (3x for 5 min. each). Then, PAP pen circles were removed with a cotton swab dipped in xylene substitute. Slides were rinsed again in PBS, aspirated to remove excess liquid, and coverslips were mounted using Slowfade Gold antifade reagent.
containing 4’,6-diamidino-2-phenylindole (DAPI), a counter stain that stains all cell nuclei (Life Technologies Corporations, Grand Island, NY). Completed slides were kept overnight in the dark before imaging.

Immunohistochemical Mammary Analyses

Photomicrographs were taken within 48 h of finishing the staining protocols. Images were acquired using a Nuance FX multispectral imaging system mounted on a Nikon Eclipse 800 epi-fluorescence microscope (Nikon Instruments, Inc., Melville, NY) fitted with Plan Fluor 20x and 40x objectives (Nikon Instruments, Inc., Melville, NY). Excitation light was generated using a mercury lamp light source and standard filter cubes fitted with long pass emission filters. The Nuance system was configured to use multiple customized emission settings for each fluorophore. At least 5 photomicrographs were taken per stained section on each slide.

ImagePro Analysis

ImagePro analyses were conducted similar to that of Tucker et al. (2016). All of the tagged image file format (TIFF) pictures taken by the Nuance System were converted into joint photographic experts groups (JPEGs) for use by the ImagePro software (Media Cybernetics, Inc., Rockville, MD). This allowed for reduction of background necessary to readily identify stained cells. The individual files for each image were pseudo colored; DAPI blue, p40 (MC, p63) green, and ER red, or DAPI blue, PR red, and BrdU green. The individual channel files were used to create composite images for display or evaluation.
Individual areas within each composite image were then outlined for evaluation. Areas were determined and defined by having at least 20 cells that were stained with DAPI that were within the PAR area. There were 3 to 4 areas selected per image.

Areas selected for evaluation were used to determine four different response variables. First the total number of epithelial cells was counted (1). These were the cells whose nuclei were stained with DAPI. Cells that expressed ERα (2) or PR (3) were also counted. Finally, BrdU positive cells (4) were counted. These four response variables were combined with area measures to determine DAPI, ERα, PR, and BrdU positive cells on an area basis and also ERα, PR, and BrdU positive cells on a per cell basis (DAPI positive cells). This was done for each zone within each calf. Additionally, for BrdU, zone 3 was divided into two regions for comparison: 1) Subtending ducts and 2) terminal ductular units (For example see: Brown et al., 2005). Averages were then calculated and treatments compared. All the counts were recorded in a Microsoft Excel Spreadsheet.

**ImagePro Receptor Intensity Analysis**

Photomicrographs were evaluated by identifying at least ten cells in each image that expressed the signal of antibody for either ERα or PR. These cells were selected at random but one criterion was that they did not overlap with another cell. Tissue from all zones was used to evaluate ERα and PR expression intensity. ImagePro software was then used to measure the mean intensity of each selected cell. The same photomicrographs used to count the ERα/PR expressing cells were used for this analysis and all cells selected were within previous identified epithelial clusters.

**Statistical Analysis**
Statistical analysis was conducted as previously described (Geiger et al., 2016a,b) using PROC GLIMMIX in SAS (SAS Institute Inc., Cary, NC). For all analyses, main effects included treatment, mammary gland zone, and the associated interactions. Calf ID was treated as a random variable. Treatment, zone, and their interaction were treated as fixed effects whereas residual error was treated as a random effect. Calf data from slaughter one and two were essentially treated as different experiments and not compared across slaughter dates. Significance was declared at P < 0.05.
RESULTS AND DISCUSSION

Estrogen Receptor-α and Progesterone Receptor Expression

Analysis of both ERα and PR were conducted on a per area (µm²) and per cell basis. Since both estimates were showed essentially treatment-related responses, per cell results are discussed. The importance of estrogens to the mammary gland has been documented on many occasions, including recently (Tucker et al., 2016), making ERα expression in the mammary gland an excellent candidate for immunohistochemical investigation due to the nature of this study. At weaning, percentage of epithelial cells expressing ERα was not affected by diet (Figure 5.1) regardless of zone. Expression of ERα was different depending on zone with zone 3 having the greatest ERα expression (57.5%; P < 0.01) and zone 2 having the lowest (43.4%; P < 0.01). Zone 1 ERα expression was intermediary to zones 1 and 3 and was different from both (51.9%; P < 0.01). Interestingly, at weaning the percentage of epithelial cells expressing PR in the mammary gland was greater for R calves compared with EH calves (Figure 5.1), most likely due to a large difference in PR positive cells in zone 3 of the mammary PAR (17.2 vs 9.9% for R and EH; P < 0.01). Additionally, both zone 2 and 3 had greater PR expression on a per cell basis than zone 1, but were not different from each other (40.3 and 35.0 vs. 28.5% for zones 3, 2, and 1; P < 0.01).

After weaning, half of the remaining calves from each diet received estradiol for two weeks. The presence of estradiol influenced ERα post-weaning while diet did not. Calves receiving estradiol had a decreased percentage of epithelial cells expressing ERα (Table 5.1) across all zones, with the greatest difference present in zone 1. However, the presence of estradiol did not impact PR expression on a per cell basis as PR expression
percentage was not affected by treatment and values covered a very narrow range (Table 5.1).

Previous work assessing the effects of pre-weaning diet on mammary receptor expression is scarce. Brown et al. (2005) fed either a moderate (21.3% CP, 21.3% fat fed at 1.1% BW; 20.5% CP starter) or a high diet (30.3% CP, 15.9% fat fed at 2.0% BW; 25.0% CP starter) to Holstein calves during the pre-weaning period. Results from this study indicated that pre-weaning diet did not influence the percentage of cells expressing ER in either the subtending ducts or terminal ductular units of the mammary PAR. This data agrees with data from the current study. These researchers did not assess PR expression. To the best of our knowledge, PR expression has not been assessed in an experiment involving differential feeding such as this. It is unknown at this time why PR expression would be increased in restricted-fed calves compared with enhanced-fed calves, especially in zone 3. Future analyses involving gene expression data should help us to better understand this particular result.

The effects of exogenous estradiol administration on hormone receptor responses was evaluated in this study. Regardless of diet, calves given estradiol post-weaning experienced a decrease in mammary epithelial cells expressing ERα, possibly due to a receptor saturation and/or down regulation effect. This suggestion is in agreement with a previously conducted study (Meyer et al., 2006c). They found that regardless of ovarian status (calves were either ovariectomized or left intact in this study), ERα expression and ERα transcript abundance were both drastically decreased in the mammary PAR when exogenous estradiol was given to heifer calves (~10 days old to start trial). Interestingly, when the opposite occurs (i.e., removal of estrogens via ovariectomy), a reverse response
is seen and ERα expression increases compared to control animals (Berry et al., 2003). However, other data performing similar procedures has indicated that ERα expression is not altered by ovariectomy (Velayudhan et al., 2015). This is further confirmed by a recent study that removed the influence of estradiol in the young calf by injecting calves with an estrogen antagonist, Tamoxifen (Tucker et al., 2016). This study found that removal of estradiol influence did not alter the percentage of cells expressing ERα.

Data showing the influence of estradiol on PR expression is scarce although it is known that PR is an estradiol-responsive gene (Velayudhan et al., 2015). This alone makes the fact that PR expression was unresponsive to estradiol treatment interesting. However, similar work from an above mentioned study (Meyer et al., 2006c) did not find an influence of estradiol on PR expression in mammary PAR tissue regardless of ovarian status. However, previous work has found a complete absence of PR positive cells when the influence of estradiol is removed via ovariectomy (Velayudhan et al., 2015). A follow up study, however, did not find differences in PR expression in the mammary PAR when calves were treated with an estrogen antagonist (Tucker et al., 2016). It appears that our PR expression data is supported by previous work (Meyer et al., 2006c), but that the influence of estradiol (addition or removal) on PR expression is conflicting. Perhaps by supplying greater amounts of estradiol to these young calves, we may have seen an increase in PR expression levels, as much higher concentrations of estradiol in the blood may have the opposite effect of the ovariectomy procedure performed by Velayudhan et al. (2015).

*Estrogen Receptor-α and Progesterone Receptor Expression Intensity*
Although ERα expression on a per cell basis was unaffected by dietary treatment at weaning, the intensity of ERα expression was impacted. Expression intensity is a measure of the relative number of receptors per positive cell. Calves on the EH treatment had greater ERα expression intensity compared with R-fed calves (Figure 5.2) across all zones. Additionally, ERα expression intensity was impacted by zone with zone 3 having decrease ERα expression intensity compared with zones 1 and 2 (1686 vs. 2205 and 2219 for zones 3, 1, and 2; P < 0.05). Progesterone receptor expression intensity was also impacted by pre-weaning diet. Enhanced-fed calves had increased PR expression intensity compared with R-fed calves (Figure 5.2) across all zones, which is the opposite pattern of that of PR expression percentage. Intensity of PR expression was not different between mammary gland zones.

Estrogen receptor-α expression intensity was impacted by post-weaning treatment. Calves on the EH treatment had greater ERα expression intensity compared with EH-E2 and R-E2 calves (Table 5.2). Interestingly, however, was that EH-treated calves also had greater ERα expression intensity compared with R-fed calves, but R-calves did not have greater ERα expression intensity than EH-E2 and R-RE2 calves. Most important, however, was that calves treated with estradiol had decreased ERα expression across all zones compared with EH calves. Expression intensity of ERα was not affected by zone or a zone by treatment interaction. Progesterone receptor expression intensity was influenced by the presence of estradiol during the post-weaning period. Calves receiving estradiol post-weaning had greater PR expression intensity (3-fold) compared with calves that did not receive estradiol (Table 5.2) with the greatest difference present in zone 1. Post-weaning PR expression intensity did not appear to be
impacted by diet, but was different depending upon mammary PAR zone (P < 0.01). Zone 1 had the greatest PR expression intensity (2177; P < 0.01) followed by zone 3 (1567) and zone 1 (1105).

Recent data indicates that measurement of the percentage of epithelial cells expressing ERα or PR to determine treatment effects on steroid receptors may not tell the whole story. This was first concluded by Velayudhan et al. (2015). These researchers performed an ovariectomy procedure on calves and found that the percentage of cells expressing ERα was not altered by treatment, but that the intensity of expression of ERα was reduced after ovariectomy. To the best of our knowledge, Velayudhan was the first to assess steroid receptor expression intensity in the bovine mammary gland.

We believe our report to be the first to assess steroid receptor expression intensity in the mammary gland of calves fed differing diets. Similar to that of Velayudhan et al. (2015), percentage of cells expressing ERα was unaffected by dietary treatment at weaning. However, expression intensity of ERα was increased by 72% in the mammary PAR of EH-fed calves compared with R-fed calves. This appears to be a dramatic increase, although not as dramatic as seen in previous work (Tucker et al., 2016). Of additional interest is the fact that ERα expression was lowest in mammary PAR zone 3 (furthest from teat) which may be indicative of the fact that ERα is not expressed as readily in proliferating cells (Capuco and Akers, 2010). An interesting observation noted above was the fact that the percentage of calves expressing PR was increased for R-fed calves compared to EH-fed calves by about 5%. However, expression intensity of PR was increased in the mammary PAR of EH-fed calves by 6.8% compared with R-fed calves. This data may begin to piece together the puzzle as to how mammary PAR mass was
increased in EH-calves at weaning (Geiger et al., 2016a) and appears to confirm our working hypothesis that a higher plane of nutrition in early life may prepare the mammary gland to respond more readily to mammogenic stimuli. Mammary PAR mass was increased and composition uncompromised when a higher plane of nutrition was fed in this study (Geiger et al., 2016a,b). Although the proportion of cells expressing ERα was not altered by dietary treatment, functionality as judged by the number of receptors per cell, i.e., expression intensity) was impacted. Knowing the importance of estradiol and its receptor to the mammary gland and its development (Capuco and Akers et al., 2010), this increase in ERα expression intensity may allow the mammary PAR to respond more readily to circulating estradiol. Expression intensity of PR was also increased in EH-fed calves although to a lesser extent than ERα. Although progesterone and its receptor are important to mammary gland development at least after puberty, mammary tissue of pre-pubertal heifers is unresponsive to exogenous progesterone (Capuco and Akers et al., 2010, Woodward et al., 1993)

After weaning and upon estradiol treatment, calves given estradiol had decreased ERα expression intensity compared with EH calves (although not compared with R calves). Previous work has shown that ERα expression intensity in mammary cells of pre-pubertal heifers is reduced after ovariectomy (Velayudhan et al., 2015). Even further, recent research has shown a drastic reduction of over 6-fold in ERα expression intensity when calves are treated with an estrogen antagonist (Tucker et al., 2016). The fact that removal and addition of estradiol responded may in part be due to a receptor saturation effect. The dose of estradiol administered to calves in the present study is much greater than physiological levels, which may be indicated by the decrease in ERα expression
intensity observed when estradiol was given in this study. However, the fact that EH-fed calves had greater ERα expression intensity compared with R-fed suggests greater functionality of those receptors in EH-fed calves. It can be assumed that prior to estradiol supplementation, calves given EH and EH-E2 had similar expression intensity for ERα as they had not been treated differently up to that point. The fact that giving estradiol to those calves resulted in greater PAR mass and more complex PAR histological development (Geiger et al., 2016a,b) may be in part due to that greater ERα expression intensity of EH-fed calves. Restricted-fed calves had ERα expression intensity lower than EH-fed calves, but not different than calves treated with estradiol. Therefore, expression intensity of ERα must have been much lower in EH calves compared with R-fed calves when estradiol was administered.

Although the percentage of cells expressing PR was unaffected by post-weaning treatment, calves given estradiol had an increase in PR expression intensity of 198% (EH-E2 vs. EH) and 271% (R-E2 vs. R). This is not surprising given the fact that PR is a known estrogen-responsive gene (Velayudhan et al., 2015). A previous study (mentioned above) was unable to assess PR expression intensity as the ovariectomized procedure performed complete abolished the presence of PR in the mammary PAR tissue. However, Tucker et al. (2016) found that when the action of ERα was inhibited via Tamoxifen treatment, PR expression intensity was increased by 41%. This is in contradiction to the current study that found a similar response (although much greater in magnitude) with the addition of estradiol. The reasoning behind these results remains unknown and will potentially be clarified by future analyses. However, the discrepancy between the two previously discussed experiments may be due to the fact that the experiment by
Velayudhan et al. (2015) removed the entire ovary and thus the influence of progesterone as well, whereas Tucker et al. (2016) used an estrogen antagonist to selectively remove only the influence of estradiol.

**Mammary Epithelial Cell Proliferation**

Mammary epithelial cell proliferation was determined using BrdU incorporation into the mammary epithelial cell population over a 24-hour period. In theory, all dividing cells should complete at least 1 cell cycle every 24 hours. Since BrdU is incorporated into the cell’s DNA during the S-phase, all dividing cells should pass through the S-phase at least once every 24 hours. Cells that express BrdU should be actively proliferating cells.

At weaning, diet had a significant effect on percentage of cells expressing BrdU. Calves fed the enhanced diet experienced a 1.6x increase in BrdU incorporation compared to R-fed calves (Figure 5.3) across all zones. When considered by zone, BrdU expression was not different between diets in zone one but was 2- and 0.5-fold greater for EH-fed calves compared with R-fed calves for tissue in zone 2 (P < 0.01) and 3, respectively (Figure 5.4). In addition, an overall zone effect was found for BrdU expression. Tissue from zone 3 had greater BrdU expression than tissue from zone 1 (14.0 vs. 10.2%; P < 0.05). However, BrdU expression in zone 2 (12.8%) was not different than zone 1 or 3. To further investigate the differential expression in zone 3, epithelial structures were separated into subtending ducts and terminal ductular units (See: Brown et al., 2005). In both EH- and R- fed calves, BrdU incorporation was greater in the terminal ductular units than in the subtending ducts (Figure 5.4). In addition, the percentage of proliferating cells was greater in the terminal ductular units of EH-fed calves compared with the terminal ductular units of R-fed calves (Figure 5.4).
Estradiol administration post-weaning impacted expression of BrdU in mammary epithelial cells. R-fed calves had the lowest level of BrdU expression (9.8%; P < 0.01; Table 5.3). EH-fed calves had greater BrdU expression at week 10 compared with R-fed calves (13.2%; P < 0.05). Even further, EH-E2 and R-E2 calves had the greatest expression of BrdU in the mammary epithelium when compared to all other calves on trial (19.4 and 17.3% for EH-E2 and R-E2 calves; P < 0.05). BrdU expression was not different between EH-E2 and R-E2 calves. When considered by zone, EH-E2 calves had greater BrdU expression in zone 3 compared to all other calves (13.4% vs. 9.2, 10.6, and 11.2 for EH-E2, R, R-E2, and EH; P < 0.05). A zone effect was also seen at the week 10 slaughter. Expression of BrdU in zone 3 was greater compared with zone 1 and 2 (17.1 vs. 13.3 and 14.4% for zone 3, 1, and 2; P < 0.05). Further analysis of zone 3 yielded interesting results. No treatment differences were seen for BrdU incorporation rate in the subtending duct region of zone 3 (10.4, 10.6, 10.4, and 10.7% for R, R-E2, EH, and EH-E2, respectively). Additionally, for R-fed calves BrdU incorporation rate did not differ between the subtending ducts and the terminal ductular units (10.4 vs. 12.0%). For all other treatments BrdU labeling was greater (P < 0.01) in the terminal ductular units than in the subtending ducts region (26.0 vs. 10.6, 21.2 vs. 10.4, and 35.7 vs. 10.7% for R-E2, EH, and EH-E2, respectively) and BrdU incorporation in the PAR terminal ductular units of EH-E2 treated calves (35.7%) was greater than that of all other calves on trial.

Previous work has assessed the effect of pre-weaning diet on mammary epithelial cell proliferation. Some work has utilized BrdU (similar to this study), while others utilized Ki67. Dual labeling cells with BrdU and Ki67 has indicated that results are comparable (Daniels et al., 2009a). Brown et al. (2005) assessed cell proliferation of
calves fed differing diets via Ki67 labeling. This study found that Ki67 labeling was decreased in both the subtending ducts and terminal ductular units of the mammary PAR when a high diet was fed during the pre-weaning period. The authors of this study attributed this difference to the fact that heifers fed the high diet had mammary ducts and structures that were already mature, and thus, were not proliferating as actively as heifers fed the moderate diet. Another study (Daniels et al., 2009) found that Ki67 labeling was unaffected by pre-weaning diet. Meyer et al. (2006b) fed heifers differing diets (starting at 10 days of age) and harvested heifers in 50 kg increments (i.e., at same physiological, not chronological, age) to look at effects of diet on mammary development. Heifers were fed either an enhanced (29% CP, 19% fat) or a restricted (22% CP, 21% fat) targeting 950 or 650 g/d of BW gain, respectively. In this study, it was found that BrdU labeling was increased for heifers fed the enhanced diet by nearly 2-fold at a common BW of 100 kg. However, differences no longer existed at heavier weights, and BrdU labeling did decrease as BW at harvest increased in this study. It is interesting to note, however, that our study yielded BrdU labeling levels much greater than that of Brown et al. (2005) and Meyer et al. (2006b), at least in terms of the EH calves harvested at weaning. Calves harvested at weaning, did not reach weights of calves on the Meyer et al. (2006b) trial. However, results from the current study are more similar to Meyer et al. (2006b) than to Brown et al. (2005). In fact, increases in proliferative cell as a percentage of epithelial cells was quite similar between the current study (~6%) and the Meyer et al. (2006b) study (~4%). In addition, Brown et al. (2005) did not find a proliferative difference by zone of the mammary gland, whereas the current study found that zone 3 (furthest from teat) had greater BrdU expression than zone 1, which is more similar to results from
Daniels et al. (2009a). Even further, values for cell proliferation (Ki67 in previous study vs. BrdU in this study) in Brown et al. (2005) were very similar regardless of location (i.e., subtending ducts vs. terminal ductular units). In the current study, we found a notable increase in cell proliferation in the terminal ductular units compared to the subtending ducts regardless of treatment and also realized much higher level of BrdU incorporation in our enhanced fed calves in the subtending ducts (~10 vs ~4%) and terminal ductular units (~22.9 vs ~5%) compared with Brown et al. (2005). This may be anticipated as it would be expected tissue currently penetrating the MFP would incorporate more BrdU. Overall, our results appear to indicate that by feeding an enhanced diet in early life, we can increase cellular proliferation of the mammary PAR in dairy heifers. Again, it must noted that harvest in the current study occurred at similar chronological ages, but not physiological ages as in Meyer et al. (2006b). Perhaps the ultimate reason for differences observed herein are due to EH-fed calves advancing to an older physiological state compared to R-fed calves. The authors would argue, however, that results observed, if found to correlate to future milk yield, would make the debate of physiological vs. chronological age moot (i.e., feed more to get more regardless of those factors).

The importance of estradiol to mammary gland development in dairy cattle is evident. Therefore, it may be logical to assume that providing exogenous estradiol to dairy heifers would increase mammary PAR cellular proliferation. Meyer et al. (2006c) found that providing exogenous estradiol to Holstein heifers (regardless of ovarian status) increased incorporation by over 5%. Another study, where an OVX procedure was performed on dairy heifers, found that BrdU incorporation was decreased by 1.5% (Berry
et al., 2003). However, work by Tucker et al. (2016) found an increase in mammary PAR cell proliferation when calves were treated with Tamoxifen. This may serve as yet another reminder of how much we have yet to learn regarding the complex interactions involved in regulating bovine mammary development. Regardless, data herein indicates that estradiol-treated calves experienced the greatest increase in PAR epithelial cell proliferation. Additionally, it appears that the cumulative effect of diet and estradiol yielded the greatest proliferative responses in mammary PAR tissue actively penetrating the MFP. Again, calves in this trial appeared to experience greater BrdU incorporation than that previously reported data indicates. Perhaps of most interest is data from zone 3 comparing the subtending duct regions to that of terminal ductular units. Data from subtending ducts is in relative agreement with previous work (i.e., ~4% greater; Brown et al., 2005), whereas terminal ductular unit proliferation data is dramatically greater. Even some of the more dramatic BrdU/Ki67 incorporation data in existence plateaus at 10-12% (Daniels et al., 2009a; Meyer et al., 2006c). It seems likely that the reason for such high values in the present study (i.e., 35.7% in EH-E2 calves) is most likely due to super-physiological levels of estradiol in circulation. It is unlikely that values this extreme would exist normally. However, much of the research in existence compares by treatment and zone, and does not breakdown zones into subtending ducts and terminal ductular units making it hard to determine if values like this may exist if the zone average was not the only value presented. Even further, the dramatic difference between the terminal ductal units BrdU incorporation values in EH-E2 vs. R-E2 (i.e., 10% difference) perhaps indicate, along with receptor expression data, the incredible difference with which calves reared on these two different diets are able to respond to mammogenic stimuli. Together,
this may indicate the cumulative effect of increased ERα expression intensity in estradiol-treated calves, combined with the increased proliferation rate of these calves (particularly EH-E2 calves in zone 3 and terminal ductular units in zone 3) may be working in concert to explain developmental advantages seen in previous work (Geiger et al. 2016a,b). Additionally, taking into account PAR mass differences (Geiger et al., 2016b), we are left with a mammary PAR region that has a much greater number of dividing cells (especially in currently developing regions) and ERα positive cells that are able to efficiently utilize estradiol in the body and respond to it, resulting in a much more complex histological pattern (Geiger et al., 2016b).
Data herein allow us to further expand upon previous data indicating that feeding a higher plane of nutrition pre-weaning beneficially impacts BW gain, frame size, and mammary gland growth without compromising PAR composition. The purpose of the present work is to understand possible mechanisms involved. Clearly, calves fed an enhanced diet have a mammary epithelium that is more actively proliferating, and these calves are better able to respond to a mammogenic stimuli through a further increase in cellular proliferation, especially in zone 3 of the mammary gland (especially the terminal ductular units). This idea is supported by the observation that although no differences were seen for percentage of cells expressing ERα, expression intensity of ERα-positive cells was improved by enhanced feeding. The exact implications and mechanisms at play causing these changes remain understood and require further investigation.
ACKNOWLEDGEMENTS

The authors would like to acknowledge Land O’ Lakes, Inc. (St. Paul, MN) and Dr. Tom Earleywine for providing milk replacer and support during this trial. We would also like to acknowledge grant support from USDA-NIFA-AFRI, 2016-67015-24575, Impact of Pre-Weaning Nutrition on Endocrine Induction of Mammary Development in Dairy Heifers awarded to R. M. Akers and 2016-67011-24703 (Pre-doctoral Fellowship to A. J. Geiger).
Table 5.1. Steroid receptor expression of Holstein heifer calves fed either an enhanced or restricted diet pre-weaning with or without exogenous estrogen immediately post-weaning.

<table>
<thead>
<tr>
<th>Item</th>
<th>R¹</th>
<th>R-E2²</th>
<th>EH³</th>
<th>EH-E2⁴</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen Receptor-α, %</td>
<td>51.81ᵃ</td>
<td>32.70ᵇ</td>
<td>51.62ᵃ</td>
<td>35.81ᵇ</td>
<td>2.10</td>
</tr>
<tr>
<td>Progesterone Receptor, %</td>
<td>34.10</td>
<td>29.43</td>
<td>32.71</td>
<td>30.30</td>
<td>0.30</td>
</tr>
</tbody>
</table>

¹R = Calves fed restricted MR (20% CP, 20% fat) at a rate of 0.45 kg/d
²R-E2 = Calves fed restricted and given estrogen for 2 weeks post-weaning
³EH = Calves fed enhanced MR (28% CP, 25% fat) at a rate of 1.13 kg/d
⁴EH-E2 = Calves fed enhanced EH and given estrogen for 2 weeks post-weaning
ᵃᵇᶜ Differing superscripts within row indicated treatment differences (P < 0.05)
Table 5.2. Steroid receptor expression intensity of Holstein heifer calves fed either an enhanced or restricted diet pre-weaning with or without exogenous estrogen immediately post-weaning.

<table>
<thead>
<tr>
<th>Item</th>
<th>R(^1)</th>
<th>R-E2(^2)</th>
<th>EH(^3)</th>
<th>EH-E2(^4)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen Receptor-α</td>
<td>1801(^a)</td>
<td>1002(^b)</td>
<td>3446(^c)</td>
<td>1171(^b)</td>
<td>569</td>
</tr>
<tr>
<td>Progesterone Receptor</td>
<td>696(^a)</td>
<td>2584(^b)</td>
<td>801(^a)</td>
<td>2385(^b)</td>
<td>109</td>
</tr>
</tbody>
</table>

\(^1\)R = Calves fed restricted MR (20% CP, 20% fat) at a rate of 0.45 kg/d  
\(^2\)R-E2 = Calves fed restricted and given estrogen for 2 weeks post-weaning  
\(^3\)EH = Calves fed enhanced MR (28% CP, 25% fat) at a rate of 1.13 kg/d  
\(^4\)EH-E2 = Calves fed enhanced EH and given estrogen for 2 weeks post-weaning  
\(^a,b,c\)Differing superscripts within row indicated treatment differences (P < 0.01)
Table 5.3. Percentage of cells proliferating in the mammary gland parenchyma of heifers fed either an enhanced or restricted diet pre-weaning with or without exogenous estrogen immediately post-weaning

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cellular Proliferation, %</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>R²</td>
<td>9.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98</td>
</tr>
<tr>
<td>R-E2&lt;sup&gt;3&lt;/sup&gt;</td>
<td>17.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.92</td>
</tr>
<tr>
<td>EH&lt;sup&gt;4&lt;/sup&gt;</td>
<td>13.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.95</td>
</tr>
<tr>
<td>EH-E2&lt;sup&gt;5&lt;/sup&gt;</td>
<td>19.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.01</td>
</tr>
</tbody>
</table>

<sup>1</sup>Cellular Proliferation determined by BrdU incorporation
<sup>2</sup>R = Calves fed restricted MR (20% CP, 20% fat) at a rate of 0.45 kg/d
<sup>3</sup>R-E2 = Calves fed restricted and given estrogen for 2 weeks post-weaning
<sup>4</sup>EH = Calves fed enhanced MR (28% CP, 25% fat) at a rate of 1.13 kg/d
<sup>5</sup>EH-E2 = Calves fed enhanced EH and given estrogen for 2 weeks post-weaning

<sup>a,b,c</sup>Differing superscripts within column indicated treatment differences (P < 0.01)
Figure 5.1. Estrogen receptor-α (ERα) and progesterone receptor (PR) expression in the mammary parenchyma of Holstein heifer calves fed either a restricted (R) or enhanced (EH) diet pre-weaning. White bar indicates restricted (R), black bar indicates enhanced (EH). * = dietary difference ($P \leq 0.01$).
Figure 5.2. Estrogen receptor-α (ERα) and progesterone receptor (PR) expression intensity in the mammary parenchyma of Holstein heifer calves fed either a restricted (R) or enhanced (EH) diet pre-weaning.
White bar indicates restricted (R), black bar indicates enhanced (EH). * = dietary difference ($P \leq 0.01$).
Figure 5.3. Mammary epithelial cell proliferation in the mammary parenchyma of Holstein heifer calves fed either a restricted (R) or enhanced (EH) diet pre-weaning.
White bar indicates restricted (R), black bar indicates enhanced (EH). * = dietary difference ($P \leq 0.01$).
Figure 5.4. A breakdown of mammary epithelial cell proliferation in the mammary parenchyma and parenchymal regions of Holstein heifer calves fed either a restricted (R) or enhanced (EH) diet pre-weaning.

Bars on left indicate that EH-fed heifers had a greater number of proliferating cells in the mammary parenchyma (See: Figure 5.3). Middle bars indicate that EH-fed heifers had a greater number of proliferating cells in zone 3 of the mammary gland (zone that is actively penetrating stroma). Bars on the right indicate that EH-fed heifers had a greater number of proliferating cells in the proliferative structures (terminal ductile lobular unites; TDLU) within zone 3 of the mammary parenchyma. White bar indicates restricted (R), black bar indicates enhanced (EH). * = dietary difference ($P \leq 0.01$).
REFERENCES


CHAPTER VI. THE IMPACTS OF FEEDING A HIGHER PLANE OF NUTRITION TO PRE-WEANED CALVES ON DIFFERENTIAL EXPRESSION OF GENES AND GENE PATHWAYS IN THE MAMMARY PARENCHYMA AND FAT PAD

ABSTRACT

Pre-weaning diet and estradiol treatment impact mammary growth, proliferation and expression of various steroid receptors within the mammary gland. Our objectives were to study the effects of diet and estradiol on mammary gene expression and impacted pathways within the developing bovine mammary gland. Thirty-six Holstein heifer calves were reared on: 1) a control milk replacer (MR) fed at 0.44 kg powder/hd/d, dry matter (DM) basis (R; 20.9% crude protein [CP], 19.8% fat, DM basis), or 2) an enhanced MR fed at 1.08 kg powder/hd/d, DM basis (E; 28.9% CP, 26.2% fat, DM basis). MR was fed for 8 weeks. At weaning a subset of calves were sacrificed (n = 6/diet). Remaining calves received E2 implants and were sacrificed at week 10. Treatments were: 1) R, 2) R + E2 (R-E2), 3) EH, and 4) EH + E2 (EH-E2). At sacrifice, parenchyma (PAR) and mammary fat pad (MFP) tissue was removed and snap frozen. All tissue was subject to RNA-sequencing. At week 8, 970 differentially expressed genes (DEG) were found in the MFP and 1,561 DEG were found in the PAR as a result of feeding a higher plane of nutrition. In the MFP, pathways impacted were primarily related to organismal systems, metabolism, genetic information processing, environmental information processing, and cellular processes. In the PAR, pathways impacted were primarily related to metabolism, organismal systems, and environmental information processing. At week 10, administration of E2 regardless of diet yielded 4,325 DEG in the MFP and 3,919 DEG in the PAR. In the MFP, pathways impacted were mostly related to
organismal systems, metabolism, and environmental information processing. In the PAR, pathways impacted were primarily related to metabolism and organismal systems. Of most interest was assessing gene expression in EH-E2 compared with R-E2 heifers to see how E2 differentially impacted them. This comparison yielded 4,066 differentially expressed genes primarily involved in pathways responsible for metabolism, organismal systems, cellular processes, environmental information processing, and genetic information processing. Future contrasts and analyses are needed to determine biological relevance of observed results.
INTRODUCTION

It is now widely accepted amongst dairy scientist that management decisions made in early life have significant impacts on future performance and milk yield. In particular, the industry understands that enhanced feeding of dairy calves can increase milk yield potential in first lactation. This is in direct opposition to previous work that has indicated the negative impacts of feeding a higher plane of nutrition (HPN) during the pre-pubertal period of life on first lactation milk yield (Radcliff et al., 2000). With an increasing amount of focus being placed on early life nutrition decisions and the impact they have on subsequent lactation performance, the mechanisms at play remain largely unknown.

The presented work is a continuation of a previously published experiment (Geiger et al., 2016a,b). The fundamental hypothesis of this work has been that feeding a HPN via milk replacer (MR) creates a mammary gland parenchyma (PAR) that is essentially ‘primed’ to better respond to mammogenic stimuli present in the body. This hypothesis is of interest for two primary reasons: 1) the mammary PAR region is growing at a much greater rate in early life than we previously understood (Capuco and Akers, 2010; Esselburn et al., 2015), and 2) concentrations of circulating estradiol (E2) in young dairy calves are quite low (Purup et al., 1993) and an increase in efficiency of E2 utilization could have dramatic implications.

Previously, we have confirmed that by feeding a HPN, body weight (BW), average daily gain (ADG) and frame measure were increased in Holstein heifer calves without compromising animal health (Geiger et al., 2016a). Additionally, by feeding an HPN, the functional tissue mass of the mammary gland (i.e., the PAR
region) was increased significantly (7.3x) without a negative impact on tissue composition (Geiger et al., 2016b). This is a much greater increase than previously observed in similar work (Brown et al., 2005). Therefore, by feeding an enhanced diet, one of our initial objectives of increasing mammary PAR mass without negatively impacting composition was achieved. A secondary objective of creating two physiological different sets of animals to test responsive to E2 was also achieved.

Upon administration of E2 to these two sets of animals, a further increase in mammary PAR mass was seen in calves fed an HPN and given E2 compared to all other calves on trial (Geiger et al., 2016b). This indicated that the fundamental hypothesis of a mammary priming mechanism as a result of feeding a HPN might be true. However, mechanisms responsible were still unknown.

Further analysis of the mammary PAR region indicated that calves fed a HPN experienced a marked increase in complexity of mammary gland development (Geiger et al., 2016b). Even further, by feeding a HPN, the proportion of mammary epithelial cells expressing estrogen receptor-α (ERα) was not impacted (Geiger et al., 2016c), which agrees with previous work (Brown et al., 2005). However, ERα functionality as assessed by expression intensity was increased by nearly 2-fold in heifers fed a HPN. Proliferation of mammary epithelial cells followed a similar pattern (Geiger et al., 2016c). Therefore, it may be that increases in future milk yield as a result of feeding a HPN in early life may be due to an increase of uncompromised PAR mass in concert with an increase in ERα function and mammary epithelial cell proliferation.
When calves fed varying diets were given exogenous E2, ERα expression was not impacted, but ERα function was decreased (Geiger et al., 2016c). However, exogenous E2 increased mammary epithelial cell proliferation significantly in both diets. When further examined, calves fed an enhanced diet and given E2 had the greatest percentage of actively dividing cells in the PAR region that was currently penetrating the MFP. Detailed examination indicated that the proliferative structures within the mammary gland of calves fed HPN and given E2 experienced cellular proliferation at a rate much greater than any previous work has indicated. To an extent, it may be inferred that by feeding an HPN, calves experience a greater increase in PAR mass via greater epithelial cell proliferation and enhanced ERα function, and that the composition of the PAR is not negatively impacting by feeding a HPN. If ERα function were at least partly responsible for observed differences in PAR mass, it would be anticipated that the addition of E2 to calves fed an HPN would result in the greatest PAR development. This was confirmed by maximal, PAR development in calves fed an HPN and given E2 combined with very large increases in cellular proliferation in actively dividing regions. A decrease in ERα presence observed in these calves may indicate an ERα saturation effect (Geiger et al., 2016b,c). A determination of whether feeding HPN induces an enhanced responsiveness of the mammary gland to exogenous E2 requires more detailed analyses to evaluate the effect of nutrition on genes and gene pathways impacted by E2 treatment.

The biological complexity of the calf unavoidably requires a systems biology approach (i.e. a way to systematically study the complex interactions in the animal
using a method of integration instead of reduction; Loor and Cohick, 2009). Important goals of systems biology are to uncover the underlying links (i.e. gene/signaling pathways, regulatory networks, etc.) within and between tissues such as the MFP and/or PAR, and also to discover new emergent properties that may arise from examining the interactions between all components of a particular system. This integrative approach provides the means to arrive at a more complete view of how the organism or system functions. Work in model organisms during the past 15 years has demonstrated the applicability of high-throughput technologies to discern biological networks (Lin and Qian, 2007; Feist and Palsson, 2008). Very few studies have addressed the role of nutrition on molecular adaptations in agricultural species, let alone attempted a more holistic approach using bioinformatics tools. Despite progress in the area of transcriptomics and bioinformatics in livestock research, application of a systems biology approach is still in its infancy (McNamara, 2011; Loor et al., 2013), especially in the calf.

Lemay et al. (2007) were one of the first groups to attempt to apply microarrays, gene network analysis, and bioinformatics (systems biology approach) to understand the molecular basis that underlies that physiology of the murine mammary gland during various stages of development. Tools for bioinformatics analyses included Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA). Among the most salient observations from this approach was the finding that close to 30% of the mammary transcriptome experienced dynamic changes over the lactation cycle, with some crucial elements related to secretion being transcribed prior to lactation and the involution switch being primarily regulated by
transcriptional mechanisms. We also have recently utilized microarrays and bioinformatics, including gene network analysis, to evaluate differences in pre-weaned PAR and MFP tissue from Holstein heifers fed different levels of nutrition (Piantoni et al., 2012). Compared with the control, feeding the high-protein/low-fat MR at 951 g/d resulted in the most dramatic changes in gene expression, with 278 and 588 differentially expressed genes having at least a 1.5-fold change in the PAR and MFP. The most-altered molecular functions in the PAR were associated with metabolism of the cell (molecular transport and lipid metabolism). Most of the genes were down-regulated due to feeding a high-protein/low-fat MR versus a control MR. In the MFP, the most affected genes were primarily associated with metabolism, but changes also occurred in genes linked to cell morphology, cell-to-cell signaling, and immune response. Among the most novel responses observed was the fact that doubling of nutrient intake seemed to inhibit PAR tissue energy metabolism and activity of oxidative pathways that partly serve to protect cells against oxidative stress.

Although the above represents an important application of systems biology concepts in the study of mammary gland biology, the use of GO and IPA for strict analysis of the functional relevance of changes in gene expression has substantial limitations, which have been discussed (Khatri et al., 2012). Although IPA is probably one of the most user-friendly commercial software available for gene network analysis, neither IPA nor GO readily provide a way to evaluate what changes in expression of genes mean in the context of the pathways to which they belong. A recent approach for analyses of transcriptome data provides the user with
a way to rank biological pathways based on their relevance (i.e. “impact” value) and the direction of the change in “flux” based on statistical P-values of affected genes as well as the fold-change between specific comparisons (Bionaz et al., 2012).

The systems biology approach could be used to evaluate differences in gene expression in MFP and PAR tissue from restricted- and enhanced-fed calves. Additionally, differential responses of these two groups of animals to mammogenic hormone stimulation offers unique opportunities to identify molecular, cellular, and biochemical pathways that control neonatal bovine mammary development and growth and further identify observed differences in our lab’s previous work.

Therefore, the objective of this study was to not only assess differential gene expression in the MFP and PAR of calves fed varying diets during the pre-weaning period, but also determine molecular pathways most impacted by these treatments. A further objective was to determine how genes and pathways differentially respond when calves from either a restricted or enhanced diet are given exogenous E2. We hypothesize that calves fed an enhanced diet pre-weaning will experience an up-regulation of genes and gene pathways responsible for cellular proliferation and PAR expansion, and that this up-regulation will be greater in HPN than in calves fed a restricted diet. This then could provide sufficient evidence to support the proposed mechanisms behind previously observed changes as a result of feeding a HPN.
MATERIALS AND METHODS

This experiment was conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (#14-045-DASC).

Animal Handling and Experiment Design

The experimental design and animal handling were as previously described (Geiger et al., 2016). Briefly, calves were assigned to 1 of 2 experimental MR (n = 18/MR): 1) a restricted MR (R; 20.2% CP, 19.8% fat, dry matter (DM) basis) fed at 0.44 kg/hd/d, DM basis, or 2) an enhanced MR (EH; 28.9% CP, 26.2% fat, DM basis) fed at 1.08 kg/hd/d, DM basis. Starter (25.6% CP, 4.0% fat, DM basis) was offered at the end of wk 4 of the trial. A subset of calves (n = 6/diet) were harvested upon weaning to assess dietary effects on mammary gland development. The remaining calves (n = 24) were either given an E2 implant or a placebo implant at weaning. This produced the following treatment groups (n = 6/treatment): 1) calves fed restricted and given a placebo implant (R), 2) calves restricted-fed and given an E2 implant (R-E2), 3) enhanced-fed calves given a placebo implant (EH), and 4) enhanced-fed given an E2 implant (EH-E2). After 2 weeks of E2 treatment all remaining animals were harvested to assess the effect of E2 on calves fed the two different diets.

Sample Collection and Slaughter Procedures

A detailed description of sample collection and animal harvest can be found in previous work (Geiger et al., 2016a,b). Heifers were euthanized as previously reported (Geiger et al., 2016a). Pieces of mammary PAR and MFP (weighing ~13
mg) were removed from the mammary gland upon removal from the body and frozen by immersion in liquid nitrogen, and stored at -80 °C. Frozen tissue samples were delivered to the University of Illinois for further processing.

**Molecular Methods**

Total RNA was extracted from both PAR and MFP samples using QIAzol Lysis Reagent (Qiagen, Valencia, CA). All samples were treated with DNaseI (Qiagen, Valencia, CA) on-column, and quantification was determined using a Nanodrop ND-1000 (Nanodrop Technologies, Rockland, DE).

RNA-Seq cDNA libraries were constructed using total RNA isolated from both MFP and PAR samples at weaning and week 10 slaughter. The Illumina TruSeq v2 RNA Sample Prep kit was used for single-end read library construction following the manufacturer's instructions with mRNA enrichment. Libraries were multiplexed across flow cell lanes of the Illumina HiSeq2500 (Illumina Inc., San Diego, CA) platform.

**Bioinformatics and Statistical Analysis**

Single-end reads were first filtered using Trimmomatic 0.33 (Bolger et al., 2014) using a minimum quality score of 28 leading and trailing with a minimum length of 30 bp long and subsequently checked using FastQC 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were then mapped to the Bos taurus UMD 3.1.1 reference genome (1/29/16 NCBI release) using default settings of STAR 2.5.1b (Dobin et al., 2013) with the quantMode option for gene counts. Further data analysis was conducted on the paired-end reads using R. 3.2.4 (R Core Team, 2016) and the noted packages. Reads uniquely assigned to a
gene were used for subsequent analysis. After accounting for high expression genes and library size differences using trimmed mean of M-values (TMM) normalization in edgeR (Zhou et al., 2014), genes were filtered if 4 samples did not have > 1 count per million (CPM) mapped reads. Normalization of reads was conducted using the voom variance stabilization function in limma (Ritchie et al., 2015). Differential expression analysis was conducted in limma using a mixed model analysis including fixed effects of diet (2 levels), E2 status (2 levels), and batch (3 levels). Calf was treated as a random effect. Raw P-values were adjusted using the false discovery rate (FDR) method (Storey and Tibishirani, 2003). Principal component analysis (PCA) was conducted on voom-transformed values using the R-package affycoretools (MacDonald, 2008) to determine the effect of diet and/or estradiol after removing all other effects in limma.

To select biologically relevant modules for further analysis, module-trait relationships were determined by correlating eigengene values for each model with phenotypic traits of interest (Wilson, 2015) using Pearson correlations in the CORR procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) and visualized in custom heat maps. Genes were selected based on module-trait correlations (P < 0.05) and a suitable number of genes (> 100) for further pathway annotation. The connectivity of a gene to a module parameter ($k_{ME}$) was used to remove genes ($k_{ME} < 0.5$) not well-described by the module. The curated gene list was annotated and analyzed using the Dynamic Impact Approach (DIA; Bionaz et al., 2012). An overview of the complete workflow used for transcriptome data is visualized in Figure 6.1.
All reported means were calculated using the LSMEANS option and mean separation was conducted using the PDIFF statement. Significance was declared when $P < 0.05$. 
RESULTS AND DISCUSSION

Diet and the Mammary Fat Pad

Principal component analysis indicated the MFP transcriptome was impacted by diet at weaning (Figure 6.2). In total, 970 genes were differentially expressed, termed differentially expressed genes (DEG). Of those 970 genes, 506 were up-regulated and 464 genes were down-regulated (at P < 0.05). Within the MFP, pathway categories most impacted by feeding include: Organismal systems, metabolism, genetic information processing, environmental information processing, and cellular processes (Figure 6.3).

The most impacted pathway within the MFP involved ribosome protein synthesis. Within this pathway, 76 genes were either up-regulated or down-regulated. The pathway as a whole was down-regulated (P < 0.05). Of particular interest to the MFP is the fact that both the cytokine-cytokine receptor interaction (16 genes) and the extra-cellular matrix receptor pathways (6 genes) were both up-regulated in the MFP of EH-fed calves (P < 0.05). Additionally, the calcium signaling pathway (15 genes) and the peroxisome proliferator-activator receptor (PPAR) signaling pathway (8 genes) were both up-regulated (P < 0.05) in the MFP of calves fed EH compared with R diets. Interestingly, DNA synthesis pathway was down-regulated in the MFP of EH-fed heifers (8 genes; P < 0.05).

To further gauge responses, highly impacted genes were evaluated. Influenced genes in the MFP as a result of pre-weaning diet were t-cell surface glycoprotein CD1b-2-like (3.7 fold change), leptin (3.4 fold change), glycogen synthase (3.0 fold change), intercellular adhesion molecule 1-like (2.8 fold change),
and Fc fragment of IgG receptor lia (2.7 fold change). Of other relevance is the fact that IGF-I, IGFBP-3, IGF-IR, and ERα were not impacted by diet in the MFP.

Previous work has also assessed the impact of feeding differing diets on gene expression within the MFP and PAR (Piantoni et al., 2012). In that study four different diets were fed. The control diet in both our study and the Piantoni study were similar in regards to protein and fat content and intake of dry matter. The Piantoni study found that greater than 1,500 DEG were identified in the MFP when plane of nutrition was increased. This is a greater observed difference than the current study (970 DEG), which may be due to differences in false discovery rate (FDR; 0.10 in previous study vs. 0.05) or the fact that 3 varying diets were compared to the control whereas only 2 diets were compared in the present work. Similar to the previous work, MFP metabolism was highly impacted by feeding higher plane of nutrition. Additionally, we found that cell signaling was impacted via the calcium signaling pathway and cytokine-cytokine receptor signaling pathways, which agrees with Piantoni et al. (2012). Similar still, previous researchers have found an increase in the FOS gene as a result of feeding a higher plane of nutrition (1.5-fold), which is similar to the nearly 2.5-fold increase observed in the present work. However, one difference of note is the fact that PPAR-gamma was not impacted by diet in the MFP of this trial, whereas it was significantly decreased by greater than 2-fold in previous work (Piantoni et al., 2012). This gene is known to be involved in adipocyte differentiation. This may partly explain the differences observed in MFP mass from our trial compared to previous work (Daniels et al., 2009). Interestingly, ribosome function (translation) was incredibly down-regulated in the MFP by feeding a higher
plane of nutrition. This may explain why our previous work found a decrease in MFP protein content when the EH diet was fed (Geiger et al., 2016b). Furthering this claim may be the fact that fat digestion and absorption was immensely down-regulated in EH-fed calves. Interesting enough, extra-cellular matrix-receptor interaction pathway was increased by greater than 10-fold in EH-fed heifers. Extra-cellular matrix signaling has been shown to be important for mammary gland development (Capuco and Akers, 2010). Perhaps this pathway is in part responsible for previously reported difference in the PAR (Geiger et al., 2016b).

Genes and pathways related to immune function were up-regulated in the MFP of calves fed an enhanced diet. This agrees with previous work (Piantoni et al., 2012). This may indicate an increase in immune function or potential function, or may be indicating that the immune system is involved in mammary development as has been discussed in previous work from our lab (Beaudry et al., 2016).

*Diet and the Mammary Parenchyma*

Principal component analysis also indicates that the mammary PAR transcriptome was impacted by diet at weaning (*Figure 6.2*). In total, 1,561 DEG were found in the mammary PAR as a result of feeding EH versus R. Of those, 895 were up-regulated and 666 were down-regulated (*P* < 0.05). Within the PAR, pathway categories most impacted by feeding include: Metabolism, organismal systems, and environmental information processing (*Figure 6.3*).

The most impacted pathway within the PAR involved pancreatic secretion. Within this pathway, 29 genes were either up-regulated or down regulated. The pathway as a whole was up-regulated (*P* < 0.05). Of particular interest is the fact
that multiple pathways involving the metabolism and/or absorption of micronutrients were impacted. This included over 110 genes and almost all were up-regulated. Of additional interest is the fact that steroid biosynthesis was up-regulated (10 genes) in the PAR of EH-fed calves compared with R fed calves (P < 0.05). Additionally, 25 genes in the vascular endothelial growth factor (VEGF) pathway were impacted and the pathway as a whole was up-regulated (P < 0.05). Even further, 45 genes in the JAK-STAT signaling pathway were impacted by dietary treatment, with the pathway as a whole being up-regulated (P < 0.05).

Of further interest are the genes that are highly impacted in the PAR of EH-fed calves. Highly impacted genes included cathelicidin 4 (4.1 fold change), calcitonin related polypeptide (3.5 fold change), protocadherin 8 (3.5 fold change), DOPA decarboxylase (3.4 fold change), and ADAM metallopeptidase domain 12 (3.4 fold change). Interestingly, IGF-I and ERα were both up-regulated in the PAR of EH-fed calves (0.6 and 1.6 fold change, respectively) while IGFBP-3 and IGF-IR were not impacted.

Compared with previous work (Piantoni et al., 2012), the number of DEG resulting from feeding a higher plane of nutrition were remarkably similar within the PAR (1,561 in present study vs. 1,641 DEG). Similar to this previous work, metabolism was the most impacted pathway in the PAR. In the previous work, lipid metabolism was highly impacted and significantly down-regulated. Herein, lipid metabolism was also impacted but significantly up-regulated. The previous work compared a higher protein, lower fat diet to a control milk replacer (28% protein: 20% fat, vs. 20:20) whereas the current work compared a higher protein, higher fat
to a restricted milk replacer (28:25 vs. 20:20), which may explain this difference. As mentioned, many pathways involving micronutrient metabolism (e.g., linoleic acid, tyrosine, minerals, histidine, sulfur, tryptophan, etc.) were up-regulated in the PAR of EH-fed calves. Observed up-regulations may be responsible for increased efficiency of growth, and thus increased PAR mass without compromised PAR composition as was observed previously (Geiger et al., 2016b). The fact that pancreatic secretions were up-regulated in the PAR of EH-fed calves was puzzling. Many genes associated with a particular pathway are also involved in others. This may be the case here (i.e., random chance that highly up-regulated genes were also involved in pancreatic secretions). Perhaps, more logical, is the possibility that these genes are indicative of genes involved in secretion within the mammary PAR, which may be supported by the observed increase in steroid biosynthesis. Of further interest is the up regulation of VEGF and Jak-STAT signaling pathways along with multiple genes associated with cell signaling and communication in the PAR of EH-fed calves. Multiple genes in both the VEGF (calcium signaling, MAPK signaling, PI3K-Akt signaling) and Jak-STAT (cell cycle, MAPK signaling, PI3K-Akt signaling) pathways ultimately responsible for cellular proliferation were up-regulated by feeding a higher plane of nutrition. An increase in cellular proliferative pathways may in part explain differences in observed cellular proliferation (Geiger et al., 2016c) and increased PAR mass (Geiger et al., 2016b) observed in previous work. Additionally, an up-regulation of IGF-I and ERα may further support this claim, and the up-regulation of ERα is in agreement with expression intensity data from these calves (Geiger et al., 2016c).
An interesting result uncovered by our work was the up-regulation of DOPA decarboxylase in the mammary PAR of heifers fed the EH diet. DOPA decarboxylase is an enzyme involved in the conversion of 5-hydroxyl-L-tryptophan to serotonin. This is of interest given the recent roles uncovered for serotonin in the mammary gland of the transition cow (Moore et al., 2015; Laporta et al., 2013). Five serotonin receptors are present in the bovine mammary gland (Hernandez et al., 2009). Of these, both the 2A (1.4 fold change) and 4 (0.23 fold change) were up-regulated in the PAR of EH-fed calves. No changes were seen for any of the serotonin receptors in the MFP as a result of enhanced feeding. Serotonin research in the bovine mammary gland is still in its infancy and it is clear these results require further investigation.

*Impact of Estrogen in the Mammary Fat Pad of Heifers Fed Differing Diets*

Principal component analysis clearly indicates the MFP transcriptome was impacted by the presence of estradiol at the week 10 harvest (*Figure 6.4*). In the MFP, a total of 4,325 DEG were identified (*P* < 0.05) with 2,047 up-regulated and 2,278 down-regulated. Within the MFP, pathway categories most impacted by E2 include: Organismal systems, metabolism, and environmental information processing (*Figure 6.5*).

The most impacted pathway within the MFP was the renin-angiotensin system, which was heavily down-regulated. Of particular interest is the fact steroid hormone biosynthesis was also down-regulated in the MFP as a result of E2 supplementation (*P* < 0.05). Overall, of the top 25 most impacted pathways, 23 were down-regulated in the MFP as a result of exogenous E2 (*P* < 0.05).
When heifers were treated with E2, some of the most impacted genes included UL 16 binding protein 3 (2.4 fold change), UL 16 binding protein 11 (2.4 fold change), IGFBP-1 (2.3 fold change), Steroid-5 α reductase, α polypeptide 2 (2.3 fold change, and RAR-related orphan receptor-C. (2.2 fold change) Additionally, IGF-I was impacted (1.5 fold change) while IGF-IR, IGFBP-3 and ERα were not.

Of interest within the MFP is the determination of genes and pathways that were differentially impacted when estradiol was provided to either the R or EH diet. When assessed, a total of 102 genes were differentially expressed between diets when E2 was administered (P < 0.05). Of these DEG, 53 were up-regulated and 49 were down regulated. Analysis limitations did not allow for determination of which treatment the up- or down-regulations occurred in, but future contrasts will provide that data. Regardless, a gene set of this size does not provide enough power for further analysis, and thus it has been concluded that in these particular calves, E2 did not have a differential impact between diets.

Previous work assessing the impact of estradiol on gene expression in the bovine MFP is scarce. However, previous work (Li et al., 2006) sought to assess estradiol responsive genes in both the MFP and PAR of intact and ovariectomized heifers. In this study, estradiol administration influenced genes primarily responsible for metabolism and cell signaling. Similarly, in the MFP of E2-treated calves, metabolism pathways were heavily influenced, with the majority being down-regulated. However, cell signaling was also impacted with cell adhesion, ECM-receptor interaction, neuroactive ligand-receptor interactions, and the renin-angiotensin system all being down regulated as a result of E2 treatment.
Additionally, assessment of key genes impacted by E2 in the MFP revealed an impact on immune function (UL 16) and IGF-I. An increase of IGF-I in the MFP due to E2 may coordinate increased PAR growth through its receptor. This could lead to greater mammary development. Due to a lack of response in the MFP when differentially fed calves were treated with E2, inferences regarding whether differences exist as a result of these treatments are hard to make.

*Impact of Estrogen in the Mammary Parenchyma of Heifers Fed Differing Diets*

Principal component analysis clearly indicates the PAR transcriptome was impacted by the presence of estradiol at the week 10 harvest (*Figure 6.4*). In the PAR, a total of 3,919 DEG were identified ($P < 0.05$) with 1,850 up-regulated and 2,069 down-regulated. Within the PAR, pathway categories most impacted by E2 include: Metabolism and organismal systems (*Figure 6.5*). The majority of the top 25 pathways impacted by estradiol in the PAR were involved with metabolism ($n = 22$), primarily vitamin, cofactor, and amino acid metabolism ($P < 0.05$).

The most impacted pathway within the PAR was vitamin B6 metabolism, which was up-regulated. Additionally, the pathway with the most DEG was sphingolipid metabolism (30 genes), which was up-regulated. Of interest in the PAR is the fact that PPAR signaling was down-regulated (21 DEG) as a result of E2 ($P < 0.05$) and that steroid hormone biosynthesis was up-regulated (8 DEG; $P < 0.05$).

Assessment of highly impacted genes revealed that some of the genes most impacted by E2 in the PAR included regulator of G-protein signaling-2 (2.9 fold change), tyrosine hydroxylase (2.1 fold change), tyrosine kinase non-receptor 2 (2.1 fold change), citrate synthase (2.0 fold change), and pyrophosphatase (1.9 fold change).
change). Additionally, IGF-I and IGF-IR were increased (0.9 and 1.2 fold change, respectively) along with ERα (1.5 fold change). IGFBP-3 was not impacted. Serotonin receptor 2A was also up-regulated in the PAR of E2 treated heifers (0.76 fold change).

A central hypothesis to our work is to determine if E2 is able to act with more efficiency within the mammary gland of calves fed an enhanced diet. To do this, transcriptomic responses were assessed within the mammary PAR of calves fed either an enhanced or restricted diet and then given exogenous E2. Overall, 4,066 DEG were impacted as a result of treatment with E2 irrespective of diet. Of these DEG, 2,279 were up-regulated and 1,787 were down-regulated.

At this point, the contrasts employed do not allow for determination as to which diet the specific gene or pathway was up-regulated or down-regulated in when E2 was provided. This data will be available in the future. However, inferences can be made and future follow-up analyses will help us to better determine biological relevance. Within the PAR, pathway categories most differentially impacted by E2 between diets include: Metabolism, organismal systems, cellular processes, environmental information processing, and genetic information processing (Figure 6.6). Moreover, multiple pathways responsible for cell growth and death, cellular communication, and signal transduction were differentially impacted between diets when E2 was given.

The most impacted pathway in the PAR as a result of E2 being given to calves fed differing diets involved the synthesis and degradation of ketone bodies (5 genes). However, the pathway with the largest number of impacted genes was
translation within the ribosome (93 genes; P < 0.05). Especially relevant to the mammary epithelium, the Jak-STAT pathway was differentially impacted when estradiol was given to both diets (37 genes) as was apoptosis (51 genes) and the cell cycle (62 genes). Also relevant to mammary epithelial cells, insulin and ErbB signaling were also differentially impacted by E2 administration depending on diet (65 and 45 genes; P < 0.05).

Additionally, when E2 was given to heifers fed two differing diets, highly impacted genes in the PAR included Ig lambda-like polypeptide A (2.1 fold change), CCR4-NOT trans. complex subunit 6 like (2.0 fold change), gremlin-1 DAN family BMP antagonist (1.9 fold change), arrestin domain containing 3 (1.9 fold change), and neuroligin-1 (1.8 fold change). Even further, IGF-I and ERα were both impacted (1.2 and 1.4 fold change, respectively), and IGFBP-3 and IGF-IR were not affected. Interestingly, serotonin receptor 2A was impacted differentially in the PAR of R-E2 and EH-E2 heifers (1.22 fold change).

Previous work assessing the impacts of E2 on PAR gene expression has found that majority of genes/pathways impacted are involved in protein ubiquitination, cell cycle control, insulin-like growth factor signaling, sterol synthesis, and oxidative phosphorylation (Li et al., 2008). The fact that cell signal pathways were impacted by E2 in the PAR of work by Li et al., (2008) agrees with the current study indicating the E2 treatment up-regulates calcium signaling pathway (6-fold) as well as the regulator of G-protein signaling-2 and tyrosine kinase non receptor 2 genes. However, the majority of signaling pathways in the PAR of E2 treated calves were down-regulated, including: PPAR signaling pathway, ECM-receptor interaction, Jak-
STAT signaling pathway, VEGF signaling, MAPK signaling, and tight and gap junction communication. Of additional interest and in agreement with Li et al. (2008) is the fact that IGF-I signaling appears to have been enhanced through an up-regulation of IGF-I and IGF-IR. This also agrees well with the increase in IGF-I observed in the MFP as a result of E2 treatment and may explain a mode of action for increased PAR mass resulting from E2. Additionally, the up-regulation of ERα may provide evidence of increased utilization of E2 in the PAR when E2 was given.

Perhaps of most interest in the current study is how the PAR of heifers fed differing diets responds to E2 on a gene expression level. Ideally statistical contrasts would be made to determine in which treatment (R-E2 vs. EH-E2) a particular pathway is up-regulated or down-regulated. These contrasts are still in development, but understanding where general differences exist should allow us to better understand gene pathways controlling development of the mammary PAR. Cell growth and death is undoubtedly different between the PAR of R-E2 and EH-E2 heifers. Both cell cycle and apoptosis are regulated differently when E2 was given to these two physiologically different sets of animals. Logically, Jak-STAT pathway and gap junction signaling were both differently impacted by E2 within diet, along with several genes involved with cellular proliferation and G-protein coupled receptor signaling. Additionally, ribosomal translation was highly impacted, which may in part explain differences observed in composition of the PAR as a result of E2 administration (Geiger et al., 2016b). Overall, upon further analysis, we may have a greater understanding of how E2 differentially impacted these animals and their mammary gland development. If pathways and genes related to cell cycle and
growth, proliferation pathways (i.e., Jak-STAT, etc.), and ribosomal translation are up-regulated in EH-E2 heifers compared to R-E2 heifers, this may serve as an explanation for increased mammary PAR growth and cellular proliferation differences observed in previous work (Geiger et al., 2016b,c). Even further, if both IGF-I and ERα are found to be up-regulated in EH-E2 as opposed to R-E2, that would agree with expression intensity data of ERα (Geiger et al., 2016c) and perhaps add a mode of action to explain observed differences from previous published work (Geiger et al., 2016b,c).


CONCLUSIONS

Overall, the focus of the present work was to assess the impact of diet and estradiol, as well as estradiol in heifers fed differing diets on differentially expressed genes and pathways in the MFP and PAR, primarily the PAR. Differences observed in cell signaling pathways, primarily the Jak-Stat and VEGF pathways may serve as explanations for increased cellular proliferation and PAR mass seen as a result of diet (Geiger et al., 2016b,c). After further evaluation of differing contrasts, it may be confirmed that differences observed in cell cycle and growth pathways along with cellular proliferation and ribosomal pathways are positively altered in the PAR of EH-E2 heifers compared with R-E2 heifers, resulting in observed phenotypic differences. It is clear further analysis is needed. Additionally, further analysis of PAR explants subjected to varying concentrations of E2 may provide further light in this subject area, independent of the influence of other organs and tissues.
Figure 6.1. Overview of RNA-Sequencing Analysis Workflow
Flow chart of procedures used ultimately resulting differentially impacted genes and pathways as a result of differential pre-weaning feeding and post-weaning estradiol administration. After RNA isolation and checks for quality, RNA-sequencing data was checked for quality and adapters and barcodes removed from
reads via Trimmomatic and FastQC. Process concluded with cross-referencing between Dynamic Impact Approach results and KEGG Pathways.
Figure 6.2. Effect of plane of nutrition on principal component analysis the mammary fat pad (MFP; top panel) and parenchyma (PAR; bottom panel). Simple depiction of treatment differences between animals. PC1 – principal component; PC2 – principal component analysis 2; Numbers above dots = animal ID number; Enhanced diet (EH) = red diamonds; restricted diet (R) = blue squares. All animals harvested at week 8 slaughter (weaning)
Figure 6.3. Summary of pathways impacted by diet in both the mammary fat pad (MFP; top panel) and parenchyma (PAR; bottom panel) of heifers fed either an enhanced or restricted diet. Differences were calculated to determine impact of feeding a higher plane of nutrition. For exact information up- vs. down-regulation, see text.
Figure 6.4. Effect of plane of nutrition and estrogen treatment on principal component analysis the mammary fat pad (MFP; top panel) and parenchyma (PAR; bottom panel).

Simple depiction of treatment differences between animals. PC1 – principal component; PC2 – principal component analysis 2; Numbers above dots = animal ID number; Enhanced diet (EH) = red diamonds; restricted diet (R) = blue squares; EH
+ estrogen (EH-E2) = green squares; R + estrogen (R-E2) = blue triangles. All animals harvested at week 10 slaughter
Figure 6.5. Summary of pathways impacted by estrogen within diet in both the mammary fat pad (MFP; top panel) and parenchyma (PAR; bottom panel) of heifers fed either an enhanced or restricted diet. Differences were calculated to determine impact of feeding a higher plane of nutrition. For exact information up- vs. down-regulation, see text.
Figure 6.6. Summary of pathways differentially impacted by estrogen in the mammary parenchyma (PAR) of heifers fed either an enhanced or restricted diet.

On going contrasts will help us to determine within which diet a particular gene or pathway is up-regulated or down-regulated as a result of estradiol administration post-weaning.
**REFERENCES**


CHAPTER VII. OVERALL CONCLUSIONS –
EXERPTS FROM: PRE-WEANING MAMMARY GLAND DEVELOPMENT: THE
INFLUENCE OF NUTRITION


SUMMARY TEXT

The goal of the present work was to determine the extent to which improved nutrition impacts the development of the bovine mammary gland. The dairy industry has realized that improved feeding of young dairy calves increases their milk production potential later in life. The results herein indicate that feeding management impacts the development of the calf and particularly mammary growth that may be responsible for increases in future milk yield.
INTRODUCTION

Dairy heifers create no return on investment until they enter into their first lactation or are sold. Thus, methods either reducing the length of the non-productive period of the dairy heifer or increasing the efficiency of early life development resulting in a more profitable first lactation cow are desirable. To promote earlier calving, a heifer must be bred earlier and thus attain puberty at a younger age. Puberty is highly correlated to body weight (BW; as well as composition) and pubertal BW in Holstein heifers has not changed for decades (Sejrsen, 1994). Thus, management schemes have been developed to encourage early attainment of puberty in dairy heifers. This poses a problem for producers as multiple research trials have indicated that accelerating pre-pubertal gains actually decreases first lactation yield (Capuco and Akers, 2010). Several reports have noted that an increased, pre-pubertal average daily gain (ADG) is detrimental not only to first lactation milk yield, but also pre-pubertal mammary gland development (Capuco and Akers, 2010; Radcliff et al., 2000). However, recent data involving enhanced feeding in early life (pre-weaning) has yielded interesting results. These studies have found that by feeding a higher plane of nutrition (HPN) during early life, first lactation milk yield is increased (See Review: Khan et al., 2011). Therefore, it seems clear that the detrimental impacts of accelerated ADG do not pertain to the entire pre-pubertal period. It is also clear that feeding a HPN during the first 2 to 3 months of life is beneficial to future performance (Soberon et al, 2012). Indeed, others have found that feeding a HPN via milk replacer (MR) can increase mammary fat pad (MFP) mass (Daniels et al., 2009a) as well as alter parenchymal (PAR) mass
and/or composition (Meyer et al., 2006a; Brown et al., 2005). The mechanisms responsible for these observed differences in lactation performance are not well understood.

Estradiol (E2) is a key regulator of mammary gland development in the heifer although circulating levels of E2 are low in the blood (Purup et al., 1993). If feeding a HPN increases mammary gland development in the young heifer, we believe it may be a result of altered responsiveness to E2. Perhaps improved feeding of the young calf produces a mammary gland that is ‘primed’ to respond to E2 with increased efficiency.

The mammary gland is unlike many other organs in the sense that the majority of its growth and development occur postnatally. Additionally, the mammary gland is growing at an incredible rate during the first 3 months of life (60-fold; Esselburn et al., 2015; Capuco and Akers, 2010). For some time, it was believed that early life mammary development was relatively quiescent prior to the onset of allometric growth at roughly 3 months of age in Holsteins. Noting that the mammary gland is growing at greater rate in early life, ‘fetal programming-like’ effects may be occurring in the early neonatal period in response to changes in nutrition.

Therefore, the objective of the present work is to determine the impacts of feeding different planes of nutrition to dairy heifer calves on mammary gland development. Assessments will be made on a biochemical, histological, and cellular level. It is our hypothesis that calves fed better will experience an increase in mammary gland development and an increase in various factors associated with
mammary gland development. To test this, pre-weaned Holstein heifer calves were fed a restricted or enhanced MR diet. Post-weaning, half the calves from each diet were given E2 and responsiveness assessed to determine if a HPN responded to E2 with greater efficiency.

**RESULTS**

Calves fed the EH diet consumed more MR DM (1.02 vs. 0.44 kg/d; P < 0.01; Geiger et al., 2016a) regardless of E2 status compared with R calves. The same held true for CP and fat. Initial body weights were not different between treatments (39.8 vs. 39.4 kg for EH and R calves; P = 1.0; Geiger et al., 2016a). Starting at week 2, EH-fed calves were heavier throughout the entire pre-weaning period (data not shown). At weaning, EH-fed calves were 24.5 kg heavier than R-fed calves (P < 0.01). Post-weaning weights were not affected by E2, with EH-fed calves remaining heavier than R-fed calves during weeks 9 and 10, regardless of E2 status (P < 0.01).

It is well known that feeding a HPN leads to greater BW throughout early life (Cowles et al., 2006; Hill et al., 2008; Geiger et al., 2014). This experimental approach produced two groups of animals to test our fundamental hypothesis that pre-weaning diet impacts the ability of the mammary gland to respond to mammogenic signals.

Overall the non-invasive mammary gland measures were highly variable between and within treatments (Geiger et al., 2016b). During week 8, EH-fed calves had longer front and rear teats. An increase in teat length due to E2 became apparent during the second week of treatment for both front and rear teats. Length of both front and rear teats was increased (P ≤ 0.05) in E2-treated calves by week
10. These results are similar to reports by Lammers et al. (1999) and Moran et al. (1991) showing calves given E2 had longer teats. The increase in teat length we observed confirms that the E2 treatment applied to these calves was sufficient to elicit a mammary tissue response.

Trimmed mammary gland weights were 5.2-fold greater ($P \leq 0.01$; Geiger et al., 2016b) for EH-fed than R-fed calves. The difference was even more dramatic (7.3-fold; $P \leq 0.01$) for the mass of dissected mammary PAR and MFP tissue in EH-fed calves compared with R-fed calves (5.9-fold; $P \leq 0.01$).

Mammary data describing the effects of E2 and diet at the week 10 slaughter can be found in Geiger et al. (2016b). The mass of the trimmed mammary gland was greatest ($P < 0.01$) in EH-E2 heifers. Additionally, calves in the EH-E2 treatment had more PAR tissue compared to all other calves on trial ($P < 0.05$). Calves given EH-E2 also had greater MFP weights compared with all other calves on trial and had greater MFP weights compared with EH-fed calves ($P < 0.01$).

Our data clearly demonstrate that pre-weaning nutrition can dramatically impact the overall development of the bovine mammary gland. In particular, these data show that enhanced feeding compared with restricted feeding, stimulates growth of both the MFP and the mammary PAR. Moreover, our hypothesis that mammary tissue derived from enhanced fed calves is better able to respond to mammogenic stimuli is supported by our observations of maximum mammary development (i.e. mass of mammary PAR) in enhanced fed heifers given E2.

Data herein agrees with previous research indicating that feeding a HPN through MR during the pre-weaning period increases total mammary gland weight.
(Brown et al., 2005). The most dramatic difference between previous reports and present data was the 7.3-fold increase in mammary PAR mass observed between EH- and R-calves. Little data exists indicating that altering plane of nutrition during the pre-weaning period can lead to the dramatic increase observed in the present study. Brown et al., (2005) did find that offering a HPN increased mammary PAR mass 3.8-fold, compared to the 7.3-fold difference observed in our trial.

Mammary gland composition for both the MFP and PAR of calves slaughtered at weaning are shown in Geiger et al. (2016b). Mammary fat pad protein was affected by diet with R calves having a greater protein concentration than EH calves ($P \leq 0.01$; Geiger et al., 2016b). Mammary fat pad DNA concentration was not affected by diet. Fat concentration of the MFP was greater for EH calves compared with R calves ($P < 0.01$).

Protein concentration of the PAR was unaffected by diet but total protein in the PAR was markedly greater (6.8-fold; $P \leq 0.01$) for EH calves compared with R calves. Additionally, PAR DNA concentration was unaffected by diet but total DNA in the PAR was 7.6-fold greater for EH calves ($P \leq 0.05$). Neither PAR fat concentration nor total fat were effected by dietary treatment.

While the general architecture of the epithelial appeared normal in all animals, there were dramatic differences in the degree of development across treatments. As can be seen in the gross anatomy of the PAR shown in Geiger et al. (2016a), the epithelium of R calves was largely confined in surface folds immediately adjacent to the gland cistern. This is apparent in the simple single bisections needed to expose the entire glandular epithelium to fixative in R calves.
compared with the multiple butterfly slices that were necessary expose the PAR tissue to fixative in EH and EH-E2 calves. Upon histological examination, in EH calves and especially EH-E2 calves, the mucosal surface of the gland cistern was much larger and more extensively folded. There were also a much greater number of ductal structures that had penetrated into the surrounding stromal tissue in EH and EH-E2 treated calves. Major ducts exposed to the luminal space of the gland cistern were usually 1-3 cell layers thick, in addition to a layer of presumptive myoepithelial cells along the basement membrane adjacent to the surrounding stromal tissue.

At weaning, percentage of epithelial cells expressing ERα was not affected by diet. At weaning the percentage of epithelial cells expressing PR in the mammary gland was greater for R calves compared with EH calves.

Previous work assessing the effects of pre-weaning diet on mammary receptor expression is scarce. Brown et al. (2005) fed either a moderate or a high diet to Holstein calves during the pre-weaning period. Results from this study indicated that pre-weaning diet did not influence ERα expression. This data agrees with data from the current study. Although ERα expression was unaffected by dietary treatment at weaning, the intensity of ERα expression was altered by diet. Calves on the EH treatment had greater ERα expression intensity compared with R-fed calves. Progesterone receptor expression intensity was also increased in EH calves compared with R-fed calves.
Recent data indicates that determining the percentage of epithelial cells expressing ERα or PR to evaluate determine treatment effects may be of limited value (Velayudhan et al. 2015). These researchers noted that ovariectomy of heifer calves did not alter the percentage of cells expressing ERα but expression level per cell was reduced. We believe our report to be the first to assess steroid receptor expression intensity in the mammary gland of calves fed differing diets. Expression intensity of ERα was increased by 72% in the mammary PAR of EH-fed calves compared with R-fed calves. An interesting observation noted above was the fact that the percentage of cells expressing PR was increased for R-fed calves compared to EH-fed calves (~ 5%). However, expression intensity of PR was increased in the mammary PAR of EH-fed calves by 6.8% compared with R-fed calves. This data may begin to piece together the puzzle as to how mammary PAR mass was increased in EH-calfes at weaning (Geiger et al., 2016a).

At weaning, diet had a significant effect on percentage of cells expressing BrdU. Calves fed the enhanced diet experienced a 1.6x increase in BrdU incorporation compared to R-fed calves. To further investigate the differential expression in zone 3, epithelial areas were separated in subtending ducts and terminal ductular units (See: Brown et al., 2005). In both EH- and R- fed calves, BrdU incorporation was greater in the terminal ductular units than in the subtending ducts (data not shown). In addition, the percentage of proliferating cells was greater in the terminal ductular units of EH-fed calves compared with the terminal ductular units of R-fed calves by nearly 20% (P < 0.01).
Brown et al. (2005) assessed cell proliferation of calves fed differing diets via Ki67 labeling. This study found that Ki67 labeling was decreased in both the subtending ducts and terminal ductular units of the mammary PAR when a high diet was fed during the pre-weaning period. Another study (Daniels et al., 2009b) found that Ki67 labeling was unaffected by pre-weaning diet. Meyer et al. (2006b) fed heifers differing diets (starting at 10 days of age) and harvested heifers in 50 kg increments (i.e., at same physiological, not chronological, age) to determine effects of diet on mammary development. Heifers were fed either an enhanced (29% CP, 19% fat) or a restricted (22% CP, 21% fat) targeting 950 or 650 g/d of BW gain, respectively. In this study, it was found that BrdU labeling was increased for heifers fed the enhanced diet by nearly 2-fold at a common BW of 100 kg. It is interesting to note, however, that our study yielded BrdU labeling levels much greater than that of Brown et al. (2005) and Meyer et al. (2006b). Increases in proliferative cell as a percentage of epithelial cells was quite similar between the current study (~6%) and the Meyer et al. (2006b) study (~4%).
OVERALL CONCLUSIONS

Our data clearly indicate that PAR mass can be increased by feeding a HPN without negative impacts on PAR composition. Functional differences in steroid receptor expression and intensity in concert with increased cell proliferation may be responsible for increases in future milk yield observed when a HPN is fed in early life. Additionally, it appears our fundamental hypothesis may be correct as heifers fed HPN experienced exceptional mammary growth when given exogenous E2. Gene and gene pathway analysis appear to indicate that observed changes may be caused by differences in cellular signaling pathways and cellular metabolism. Further investigation into gene and pathway networks at play are needed to fully understand the underlying causes of observed phenotypic results. However, when taken as a whole, the data are clear that if we do the best for our calves, they will return the favor down the road.
REFERENCES


