

Effects of bodyweight and plane of nutrition on mitogenic capacity of mammary extracts in cell culture, mammary growth and development, and protein expression profiles of mammary tissue in Holstein heifers

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ABSTRACT

Mammary gland samples from a large serial-slaughter Holstein heifer nutrition trial were used to determine the effects of stage of development and nutritional management on mitogenic activity of mammary extracts and mammary parenchymal composition. Stage of development and nutritional management of heifers had minimal effects on the mitogenic capacity of mammary extracts, and no significant effects on tissue composition. Two-dimensional proteome maps of heifer mammary extracts were constructed for heifers weighing 200 and 350 kg, respectively at slaughter. Proteins altered by stage of development and/or nutrition were quantified and identified; 820 total protein spots were analyzed. The expression of 131 protein spots differed by dietary treatment only. Stage of development altered the expression of 108 protein spots. Twenty-two protein spots were excised from gels for mass spectrometry analyses. Database searches for proteins with shared primary amino acid sequences were used to identify the proteins. Possible roles of these proteins in mammary development were described. In summary, heifers can be reared on high planes of nutrition without impairing mammary development, but mechanisms governing nutritional and temporal control of mammary development demand further investigation.

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LIST OF ABBREVIATIONS

2D-PAGE; 2-DE	two dimensional polyacrylamide gel electrophoresis
ADG	average daily gain
AIP1	actin interacting protein 1
ALDH1	aldehyde dehydrogenase 1A1
APL	acute promyelocytic leukemia
APS	ammonium persulfate
BCA	bicinchoninic acid
BrdU	bromodeoxyuridine
bST	bovine somatotropin
cpm	counts per minute
DBP	vitamin D-binding protein
DMEM	Dulbecco's modification to Eagle's medium
dpm	disintegrations per minute
DTT	dithiothreitol
ECM	extracellular matrix
ER	endoplasmic reticulum
ER- α	estrogen receptor-alpha
ERp57	protein disulfide isomerase A3 precursor
FBS	fetal bovine serum
<i>g</i>	gravity
GDI-1	Rab GDP dissociation inhibitor alpha
GE	gross energy
GH	growth hormone
GLM	general linear model
GST-mu	glutathione S transferase, class mu
GST-pi	glutathione S transferase, class pi
H	high-gain diet
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
IEF	isoelectric focusing
IGFBP	insulin-like growth factor binding protein (s)
IGF-I	insulin-like growth factor-I
IGF-IR	insulin-like growth factor-I receptor
IPG	immobilized pH gradient
M	moderate-gain diet
MAC-T	mammary alveolar cells transformed with the SV-40 large T-antigen
MGE	mammary gland extracts
Mw	molecular weight
Nas7p	non-ATPase subunit 7

p40.5	26S proteasome regulatory subunit
PDI	protein disulfide isomerase
pI	isoelectric point
protein 12.3	guanine nucleotide binding protein beta subunit like protein 12.3
RUP	rumen-undegradable protein (intestinal protein)
SBP56	selenium binding protein 1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
TCA	trichloric acid
TDU	terminal ductal unit (s)
TEMED	N,N,N,N'-Tetra-methyl-ethylenediamine
TGS	tris, glycine, SDS (buffer)
TMR	total mixed ration

CHAPTER 1: LITERATURE REVIEW

1.1 *Overview of Heifer Mammary Gland Development*

Prepubertal development of the bovine mammary gland is important for future milk production because epithelial cells appearing at this time provide the structural foundation for subsequent development. Near the time of puberty, mammary parenchymal tissue develops into mammary ducts that give rise to alveoli during pregnancy. The extent of development of mammary parenchyma in heifers partially determines milk yield, because numbers of alveoli during lactation are a major factor limiting milk production (Tucker, 1981).

Feeding management practices of heifers from approximately 2 to 11 mo of age can negatively influence mammary development and future milk production. During this so-called “critical period,” high rate of body weight gain (average daily gain (ADG) > 700 g) in Holsteins has led to greater fat deposition in the mammary glands, which impaired development of the mammary epithelium (Sejrsen and Purup, 1997), and also lead to a permanent reduction of the subsequent milk yield potential (Swanson, 1960; Little and Kay, 1979; Sejrsen et al., 2000). However, mechanisms by which milk yield is reduced are still a topic of debate. The general consensus is that during development, mammary epithelial cells are irreparably altered due to the accumulation of fat in the udder; a process mediated in part by local mammary endocrine or growth factor signals.

A key time when heifers are especially sensitive to a high feeding level is the transition from isometric to allometric growth of the mammary gland. Allometric growth of the mammary fat pad and of mammary ducts begins between 2 to 3 mo of age (or roughly 100 kg BW) (Sinha and Tucker, 1969). Allometric growth occurs when a tissue grows at a faster rate than the rest of the body. Once a heifer reaches puberty between 9 and 11 mo of age (250-280 kg BW), the mammary gland returns to an isometric pattern of growth (Sinha and Tucker, 1969), and thereafter there seems to be no effect of feeding level on mammary development (Sejrsen et al., 1982).

It has recently been posited that rapid rates of mammary growth during the prepubertal period may be achieved without undue effects on subsequent milk yields,

as long as rapid growth occurs without excessive fattening (Capuco et al., 1995). Capuco et al. (2004) and Moallem et al. (2004a, 2004b) hypothesized that this, in addition to enhanced skeletal and lean body growth, could be achieved by providing heifers with increased dietary protein in the form of intestinal protein (RUP) in combination with bST administration. Moallem et al. (2004a; 2004b) demonstrated that the combination of dietary RUP and bST administration successfully increased body and skeletal growth rates of heifers and Capuco et al. (2004) concluded that this practice had no adverse effects on mammary growth (examined at 5 and 10 mo of age), or first lactation milk yields.

Another proposed method for efficiently raising heifers without adversely affecting mammary growth is the so-called stair-step compensatory nutrition regimen. This method of feeding developed by Park et al. (1987, 1989) combines periods of alternating dietary energy restriction and realimentation (re-feeding). According to Park (1998) it was developed so as to allow heifer mammary development to be minimal during energy restriction phases, and compensatory during realimentation. Compensatory gain has been defined by Ashworth and Millward (1986) to describe a period of acceleration in growth that occurs after a period of growth restriction, when conditions conducive to growth are restored. Incidentally, the realimentation phases are designed to coincide with times of hormone-sensitive mammary growth (prepuberty, puberty, breeding, and gestation) (Park, 1998). An example of a stair-step compensatory nutrition regimen for dairy heifers used recently by Ford and Park (2001) involved an alternating 3-2-4-3-4-2-mo feeding regimen. That is, it included 3 mo of an energy restriction diet (prepubertal phase) initiated at 6 mo of age (\approx 160 kg BW), followed by energy realimentation for 2 mo. The second step (puberty and breeding phase) included 4 months of energy restriction, followed by 3 months of realimentation. The third and final stair-step (gestation period) included 4 mo of energy restriction, followed by 2 mo of realimentation (Ford and Park, 2001). Heifers reared on this regimen produced 21% (first lactation) and 15% (second lactation) more milk than counterparts reared conventionally (Ford and Park, 2001). Increased milk yields in heifers raised on stair-step compensatory nutrition regimens have been associated with compensatory mammary cell proliferation. This is based on the observation that

pregnant and early lactating rats raised on a stair-step, rather than conventional, regimen had greater mammary cell proliferation, as determined by bromodeoxyuridine incorporation into DNA (Kim et al., 1998). Clearly, the method of how to best raise heifers, in terms of their mammary development, is still undecided. What is clear is that too much fattening, which would result from the overfeeding of high-energy diets, is not desirable.

1.2 Endocrine Control of Mammary Development

Growth Hormone and Insulin-like Growth Factor-I

Mammary glands of pre- and postpubertal heifers grow in response to exogenous bST (Purup et al., 1993; Radcliff et al., 2000). Heifers fed high-energy diets have lower serum concentrations of growth hormone (GH) and this observation suggested the hypothesis that the diet-related inhibition of mammary growth is related to GH secretion. Indeed, the concentration of GH in prepubertal serum is positively correlated to mammary growth (Sejrsen and Foldager, 1992). Prepubertal administration of bST to heifers also increases total amounts of mammary parenchyma (Sejrsen et al., 1986). That circulating concentrations of bST would affect mammary development is interesting, given that mammary tissue parenchyma does not express GH receptors as measured by direct binding assays (Akers, 1985; Capuco et al., 1995). In 1957 Salmon and Daughaday hypothesized that somatotropin's effects are mediated by IGF-I. Research conducted by Purup and others (1993) supports this hypothesis, as they found that bST treatment of prepubertal heifers increased concentrations of IGF-I in serum. Furthermore, bST treatment in rats stimulated mammary synthesis of IGF-I (Kleinberg et al., 1990). Insulin-like growth factor-I is a potent mitogen for bovine mammary epithelial cells *in vitro*, but bST (Purup et al., 1995) is not. The major site of IGF-I mRNA synthesis is in the liver but there is additional synthesis by peripheral tissues, including the mammary gland. Insulin-like growth factor binding proteins (IGFBP) regulate the physiological actions of IGF-I by controlling its availability to cells (Thissen et al., 1994); approximately 95% of circulating IGF-I is bound to IGFBP-3, the predominant IGFBP in serum (Clemmons, 1993). Weber et al. (1999) showed that

IGFBP-3 is up-regulated in overfed heifers and that excess IGFBP-3 can block the mitogenic effects of IGF-I *in vitro*.

Insulin-like growth factor I can act in a paracrine or endocrine manner. Forsyth and others (1999) used *in situ* hybridization to show that in the sheep mammary gland, IGF-I mRNA was expressed in cells of the intralobular connective tissue. The IGF-I receptor mRNA was expressed in the underlying epithelium, supporting a paracrine role for IGF-I to influence mammary epithelial development. In the bovine, mammary epithelial cells are also known to express the IGF-I receptor (Purup et al., 1995), and IGF-I has been shown to stimulate proliferation of mammary epithelial cells from prepubertal heifers *in vitro* (Weber et al., 1999). The effects of IGF-I on DNA synthesis in cultured mammary epithelial cells suggest that local mammary synthesis of IGF-I can modulate growth *in vivo*. However, serum concentrations of IGF-I are generally increased, not decreased like bST concentrations, in heifers reared on a high feeding level (Vestergaard et al., 1995). It is important however, to keep in mind that changes in circulating hormone concentrations do not necessarily explain observed phenotypic changes, or reflect utilization by target tissues. This may be especially relevant regarding IGF-I, given the large family of binding proteins that can modulate its biological activity.

Estrogen

In addition to growing in response to exogenous bST, mammary glands of pre- and postpubertal heifers are capable of growing in response to exogenous estrogen (Berry et al., 2001; Capuco et al., 2002). Furthermore, the apparent secretion of estrogen by the ovary appears to be essential for mammary development. Heifers ovariectomized at 4 mo of age exhibit reduced mammary development at 9 mo of age (Purup et al., 1993); the same is true of heifers ovariectomized at 3 mo of age and evaluated at 6 mo of age (Berry et al., 2003b). Interestingly, normal mammary growth in ovariectomized animals can seemingly be restored by exogenous estrogen (Wallace, 1953), but not bST administration (Purup et al., 1993). The manner in which estrogen positively affects mammary development is presently unclear due to the puzzling observation that proliferating mammary epithelial cells do not express estrogen

receptor- α (ER- α) (Capuco et al., 2002). In 2003, Berry et al. found that bST administration to intact heifers stimulated mammary epithelial cell proliferation, but did not appear to enhance the response to estrogen, despite the increased expression of ER- α (Berry et al., 2003b). These observations further complicate the story of mammary development in heifers, but perhaps demonstrate how little we really know about the basic biology of heifer mammary development and the systemic hormones and locally produced growth factors that are responsible for peripubertal mammary development.

1.3 Rationale and Significance

Despite understanding effects of peripubertal feeding level on whole animal performance, biochemical and cellular mechanisms are poorly understood. This is due largely because of the small abundance of mammary tissue during both the prepubertal and pubertal periods of development, and the invasive nature of mammary tissue collection. This thesis details the use of mammary tissue that has been collected from a large heifer nutrition trial at Cornell University, combined with molecular and cell culture techniques, both of which require only minute amounts of tissue. These approaches were designed to better understand cellular and molecular mechanisms that control mammary development in dairy heifers, especially those reared under moderate and high feeding level situations.

1.4 Cell Culture as a Way to Study Mammary Development

Cell culture is a common way to study factors that impact mammary cell proliferation or function. One approach involves the excision of tissue followed by homogenization to prepare cellular extracts. These extracts can then be used in cell culture experiments to see if they stimulate or inhibit culture cell growth. Waksman et al. (1991) were the first to show that cultured bovine mammary cells proliferate in response to various tissue extracts. Subsequently, Weber et al. (2000) demonstrated the mitogenic response of cultured primary mammary cells to mammary tissue extracts (MGE). More importantly, they showed that extracts from high rate of gain heifers (1.1

kg/d ADG) were less effective at stimulating cell proliferation than extracts from heifers fed for a lower rate of gain (0.55 kg/d ADG). These results, while informative, had some limitations. First, primary cell cultures were used. Primary cell cultures are technically more demanding for consistent standardization than immortalized cell cultures. They are also more difficult to maintain as long-term model systems. The use of an immortalized cell line may alleviate this, since immortalized cell lines have unlimited growth capacity *in vitro*. Bovine mammary alveolar cells produced by stable transfection with SV-40 large T-antigen (MAC-T) are one such cell line (Huynh et al., 1991). Under appropriate culture conditions these cells display a differentiated mammary cell phenotype, but more importantly mimic the response of bovine primary epithelial cells to the addition of MGE (Berry et al., 2003c).

1.5 Evaluation of Histological Features to Categorize Heifer Mammary Development

Evaluation of histological features has been a long-standing technique used by many in the field of mammary biology to study tissue growth and development in both normal and experimental conditions. Recorded histological observations of male and female sheep and cattle mammary development were published in the 1950s (Turner, 1952; Wallace, 1953) and similar techniques have been employed ever since. With the advent of immunochemical staining techniques and advances in microscopy, the breadth of knowledge to be gained from such basic studies is seemingly endless and informative.

1.6 Proteomics Defined

Proteomics is the study of the proteome. The term 'proteome' was coined in 1995 to refer to the complete set of translated proteins in a given biological sample (Wilkins et al., 1995). Unlike the genome, the proteome is subject to changing in response to experimental conditions, such as plane of nutrition and/or stage of development. Changes in the proteome can include such things as altered protein expression, activation, and/or post-translational modifications occurring in response to

signals in the local environment. Proteome analysis currently relies heavily on two-dimensional PAGE (2D-PAGE), a technique first developed in the mid 1970s by Patrick O'Farrel (1975). It is a method that separates proteins first on the basis of their isoelectric point (pI) (charge), and then by their molecular weight (Mw) (size) (Figure 1.1). To separate proteins on the basis of their pI, isoelectric focusing (IEF) is used. This first dimension technique consists of applying an electric field across an immobilized pH gradient (IPG) gel strip containing protein sample. Since proteins have a characteristic pI, when a charge is applied, they will migrate through an electric field until they reach a pH equal to their pI, a point at which the protein will have no net charge. After IEF, IPG strips are equilibrated in buffer that contains SDS; the purpose of equilibration is to uniformly coat all of the proteins in a given sample so that they will all have a uniform negative charge to mass ratio. The second dimension of 2D-PAGE, SDS-PAGE, will then separate proteins perpendicular to the first dimension based on their size only (Figure 1.1). After SDS-PAGE, all gels containing the proteins are fixed and stained. The types of stain available for use in proteomics vary, but a common type of stain to use is SYPRO Ruby, a fluorescent dye, which binds to the basic amino acids in proteins (Nishihara and Champion, 2002) in a proportional fashion. After staining and removal of non-protein bound stain, an imaging device is used to visualize a pattern of protein "spots" the gels. These patterns can be compared with other spot patterns to enable the quantification of proteins that are uniquely or differentially expressed between the samples.

The voluminous data arising from this type of classical proteomic approach are stored in searchable 2D-PAGE databases. The real power of proteomic analysis came about with the development of sophisticated software packages capable of analyzing multiple gels and the many thousands of protein spots that may be present in any one gel. In 2D-PAGE, large numbers of proteins can be separated, but since this is done in a collective fashion, the identity of any individual protein, or "spot", is not known. So, while researchers have known for decades how to separate individual proteins in a given biological sample, they are just now beginning to harness the discovery possibilities with the advent of high-powered computer applications coupled with mass spectrometry. This allows not only the ability to analyze differences in relative

expression but also the ability to identify proteins. This ultimately then allows the detection of differences in gene expression.

1.7 Two-Dimensional Gel Electrophoresis as a Tool to Study Mammary Gland Development

In 1995, Katoh and coworkers extracted cytosolic and nuclear components from mammary tissue of five lactating dairy cows and successfully used them in 2D-PAGE experiments to resolve individual proteins. They clearly demonstrated with immunoblot analysis the presence of annexin proteins on their gels. Wheeler et al. (1997) through the analysis of nuclear extracts from bovine mammary tissue also detected and identified nuclear proteins using 2D-PAGE experiments. They utilized mammary tissue from 14 cows that were at various stages of lactation and were either pregnant or not pregnant at the time of tissue collection. The researchers used visual examination to identify protein spots that appeared to be differentially expressed in their 2D gels, followed by immunoblot analysis or amino acid sequencing of those selected spots. Through their methods, they identified lactoferrin, annexin II, and heavy-chain Ig in nuclear fractions of mammary tissue extracts, and correlated their expression to lactational and gestational states.

1.8 Proteomics as a Tool to Study Mammary Gland Development

Zucchi and Dulbecco (2002) and Zucchi et al. (2002) have published a series of papers wherein they describe the application of proteomic-based technologies to aid in the identification of proteins involved in an early stage of lactogenic differentiation of the mammary gland. In their experiments, they used two cell lines, LA7 (cuboidal-like) and 106 (spindle-like), that were both derived from the RAMA-25 cell line. The RAMA-25 cell line was isolated from mammary adenocarcinoma induced in Sprague-Dawley rats by 7,12-dimethylbenz[a]anthracene. Cells of the LA7 cell line are capable of *in vitro* differentiation into dome structures after exposure to lactogenic hormones, and expression of β -casein and *WDMN1* genes, which are markers of mammary gland functional differentiation *in vivo* (Zucchi and Dulbecco, 2002). Cells of the 106 cell line

on the other hand, are not capable of differentiating into dome structures, even under the direction of inducers (Zucchi and Dulbecco, 2002). The goal of Zucchi and Dulbecco was to characterize proteins differentially expressed in LA7 and 106 cell lines, and to determine their possible roles in dome formation. This project was of interest because the formation of domed structures *in vitro* corresponds, at least in a general sense, to mammary lobular-alveolar development experienced during pregnancy and lactation *in vivo*. Their analyses of 2D gels resulted in the identification of approximately 200 differentially expressed proteins; 50 of these were removed from the gels and were subjected to mass spectrometry analysis. Twenty of the 50 proteins were successfully identified, and their possible roles in dome formation were then investigated. Through a series of cell culture experiments, Zucchi and Dulbecco (2002) were then able to show that tropomyosin-5b, maspin, annexin I, and HSP90- β play roles in the development (or hindrance) of dome formation. Interestingly, Zucchi and Dulbecco (2002), and Katoh et al. (1995) independently identified and implied a role for annexin I in mammary gland differentiation using different animal models and experimental procedures. Moreover, each group listed slightly different Mw and pI estimates for annexin I (Katoh et al. – Mw 36 kDa pI 7.7 and 8.3 (two chains)) (Zucchi and Dulbecco – Mw 43.1 kDa and pI 7.1). This last observation is not really that surprising, as one of the inherent difficulties in proteomic experiments is the non-reproducibility between researchers, and species differences in protein composition.

To date, there have been no published papers specifically dealing with the construction of a 2D-PAGE database of identified bovine mammary gland proteins. This is likely due to inability to perform large-scale and high-throughput data analysis, as well as the fact that the *Bos taurus* genome is incompletely characterized (Talamo et al., 2003; Wait et al., 2002). In addition, published relational maps for human, mouse, and rat proteins are ineffective in the identification of most of their bovine counterparts (Talamo et al., 2003). Aksu et al. (2002) however, have published a paper wherein they detail the creation of a mouse mammary gland 2D-PAGE protein database containing 66 identified protein spots. The relational database that they created is accessible on the World Wide Web under the URL: <http://www.mpiib-berlin.mpg.de/2D-PAGE>.

1.9 Objectives

The objectives of this research were to:

- 1) Compare mitogenic capacity of mammary extracts from Holstein heifers in cell culture as affected by heifer stage of development and nutritional management using an immortalized cell line
- 2) Determine effects of rate of gain and body weight on histological development of the udder with a focus on changes in terminal ductal structures (TDU) in Holstein heifers
- 3) Develop a map of heifer mammary tissue proteins using proteomics and to distinguish populations of proteins likely to explain nutritional control of pre- and post-pubertal mammary development.

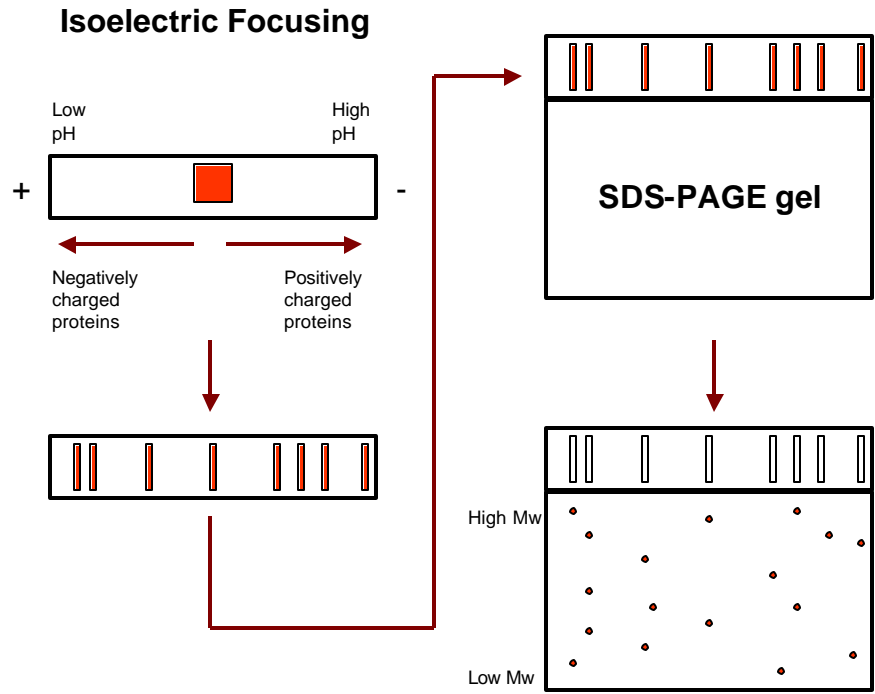


Figure 1.1. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE).

CHAPTER 2: USE OF AN IMMORTALIZED BOVINE MAMMARY EPITHELIAL CELL LINE (MAC-T) TO MEASURE THE MITOGENIC ACTIVITY OF EXTRACTS FROM HEIFER MAMMARY TISSUE: EFFECTS OF NUTRITION AND BODYWEIGHT

2.1 Introduction

Cell culture is a common way to study tissue or other factors that impact mammary cell proliferation or function. One common approach involves the excision of tissue followed by homogenization to prepare cellular extracts. These extracts can then be used in cell culture experiments to determine if they stimulate or inhibit growth of test cells. Waksman et al. (1991) were the first to show that cultured bovine mammary cells proliferate in response to addition of extracts prepared from sample tissues, including the mammary gland. Subsequently, Weber et al. (2000) confirmed the mitogenic response of cultured primary mammary cells to mammary tissue extracts (MGE). More importantly, they showed that extracts prepared from tissues from high rate of gain heifers (1.1 kg/d ADG) were less effective at stimulating cell proliferation than extracts prepared from heifers fed for a lower rate of gain (0.55 kg/d ADG). These results while informative had some limitations. Testing was done with primary cell cultures. Primary cell cultures are technically more demanding for consistent standardization than immortalized cell cultures. They are also more difficult to maintain as long-term model systems. The use of an immortalized cell line may alleviate this problem. MAC-T cells are one such cell line. They were produced from bovine primary mammary alveolar cells by stable transfection with SV-40 large T-antigen (Huynh et al., 1991). Under appropriate culture conditions these cells display a differentiated mammary cell phenotype, but more importantly mimic the response of bovine primary epithelial cells to the addition of MGE (Berry et al., 2003c). Specifically, when MGE used by Weber et al. were tested using MAC-T cells, the response patterns were similar to primary cells. That is, proliferative response to MGE for heifers fed for a high rate of gain was markedly lower than for MGE from lower rate of gain heifers (Berry et al., 2003c).

However, the MGE tested by Weber et al. came from heifers at a single developmental stage (~250 kg BW) rather than a range of developmental stages that

includes tissues representative of specific time points before, during, and after puberty. Thus, the objective of this experiment was to test the mitogenic activity of MGE prepared from tissues collected throughout the peripubertal period, as well as effects of feeding level.

2.2 *Materials and Methods*

Animals and Treatments

The tissue samples used in this study were from animals that were purchased within 1 week of age from commercial dairy farms surrounding Ithaca, New York, and treated at Cornell University, as reported by Meyer et al. (2004). The objective of this study was to evaluate the effect of plane of nutrition from birth to 350 kg on body composition and mammary development in Holstein heifers. Briefly, heifers (n = 78) were randomly assigned to one of two dietary treatments (moderate or high), or a baseline slaughter group (6 heifers, 45 kg BW); within treatments heifers were randomly assigned to one of six slaughter weights (100, 150, 200, 250, 300, or 350 kg BW, respectively). Six heifers per dietary treatment were slaughtered at each time point for a total of 78 heifers (including baseline group). The specific slaughter weights given above were chosen to represent periods when the mammary gland transitions from isometric to allometric growth as well as periods within the allometric growth phase. This was based on the temporal pattern of mammary development reported by Sinha and Tucker (1969).

Heifers on the moderate gain (M) dietary treatment were fed a pre-weaning diet that consisted of a 22% CP and 20% fat (dry matter basis) milk replacer that was fed at 0.20 Mcal gross energy (GE) per kg of BW^{0.75}. Heifers on the high gain (H) dietary treatment were fed a pre-weaning diet that consisted of a 28% CP and 17% fat (dry matter basis) milk replacer that was fed at 0.32 Mcal GE per kg of BW^{0.75}. A 26% CP calf starter was offered to animals beginning at approximately 3 weeks on study. After weaning, which was completed by 7 weeks on treatment, all heifers were fed the 26% CP starter for two additional weeks. From weeks 10 to 13 of treatment, all heifers received TMR 1 (Table 2.4). Thereafter all heifers were fed TMR 2; heifers exceeding

200 kg BW were fed TMR 3 (Table 2.4). Throughout the entire length of the study, heifers were weighed once weekly and the amount of feed offered was adjusted such that heifers on the M treatment could achieve 650 g, and heifers on the H treatment could achieve 950 g of daily gain, respectively. From the initiation of treatment to 150 kg, heifers were housed in individual pens in the Teaching and Research Dairy Center Greenhouse at Cornell University. Upon reaching 150 kg, heifers that were assigned to heavier slaughter weights were grouped in one pen and individually fed via a Calan gate system.

Once heifers assigned to slaughter weights greater than or equal to 250 kg reached 225 kg BW, blood was collected twice weekly via jugular venipuncture, and plasma progesterone concentrations were determined. Progesterone concentrations above 1 ng/mL were interpreted as the heifer having a functional corpus luteum, and therefore the heifer was considered pubertal.

Slaughter Procedure and Mammary Tissue Collection

Dietary treatments were continued until animals obtained their assigned slaughter weight; heifers were weighed before feeding on two consecutive days to monitor target rate of gain. The decision to slaughter at a common bodyweight as opposed to a common age was based on observations that puberty is more closely associated with bodyweight than a specific age (Sejrsen, 1994). Pubertal heifers were slaughtered in the luteal phase of their reproductive cycle.

Humane slaughter of each heifer was performed at the Department of Animal Science Abattoir at Cornell University using a captive bolt stunner followed by exsanguination. At slaughter, the udder of each heifer was bisected along the medial suspensory ligament. One half of the udder was used by Meyer at Cornell University to determine total amounts of parenchymal and extra-parenchymal tissue, and the effect of energy intake on the composition of both. Tissues harvested from the mid-parenchymal region of the second half of the udder were stored in a -20 °C freezer until shipment. Frozen mammary tissue samples were shipped overnight on dry ice to Virginia Tech in one of two batches. Upon arrival samples were placed in an -80 °C freezer.

Preparation of Mammary Tissue Extracts

Mammary tissue extracts were prepared essentially according to Waksman et al. (1991). Briefly, 2 g of frozen mammary tissue were weighed and homogenized in a polytron (Kinematica; Switzerland) for 15 s in a total volume of 10 mL 0.9% NaCl. At this time, 200 μ L of each sample was transferred to a microfuge tube and stored at -80 °C until assayed for DNA content. Homogenates were gently rocked for 90 min at 4 °C on a Speci-Mix platform rocker (Thermolyne Corporation; Dubuque, IA) before being centrifuged at 10,000 x *g* at 4 °C for 20 min to remove cell debris. Supernatant was retained and centrifuged at 100,000 x *g* for 1 h. The resulting supernatant (essentially cytosol) was then filtered through 0.2 μ m Acrodisc[®] syringe filters (Pall Corporation; Ann Arbor, MI) and aliquots were stored in sterile microfuge tubes at -80 °C until bioassay testing. Protein content of all samples was determined via the bicinchoninic acid (BCA) assay (Pierce; Rockford, IL). Bovine serum albumin was used as the standard in the BCA assay.

Mitogenic Testing

To test the mitogenic activity of the MGE, MAC-T cells were plated (2×10^4 cells per well) in Dulbecco's modification of Eagle's medium (DMEM; Mediatech, Inc.; Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Gibco; Rockville, MD), in plastic 24-well tissue culture plates (Falcon, Fisher Scientific; Pittsburgh, PA). Cells were routinely cultured in a CO₂-Auto Zero incubator (Heraeus Inc.; South Plainfield, NJ) set at 37 °C and were supplied with 95% air and 5% CO₂. After an overnight attachment period and 48 h of serum starvation (no FBS), medium was removed and 900 μ L of fresh medium was added along with a dilution of mammary tissue extracts in a total volume of 100 μ L of 0.9% NaCl. Extracts from individual animals were replicated across 4 wells for each extract. Enough mammary extract was added to each well to assure 60 μ g of total protein. The protein amount added was based on preliminary data as well as results reported by Berry et al. (2003). This amount of extract protein added is near the center of MGE proliferation response curves measured for MAC-T cells. Four replicate wells containing 5% FBS were included as a positive control, and 4

replicate wells containing DMEM alone were included as a negative control in the experiment. Cells were allowed to incubate with MGE for 16 hours.

Thereafter, ^3H -thymidine (catalog # 24039, ICN Biomedicals, Inc.; Irvine, CA) diluted in DMEM was added to each well to achieve a final concentration of 1 $\mu\text{Ci}/\text{mL}$. After an additional 2-h incubation period at 37 °C, all medium was removed. The cells in each well were washed with cold Dulbecco's phosphate buffered saline (Gibco; Rockville, MD) (two washes, 1 mL each), ice-cold 10% TCA (two washes, 1 mL each), and finally with ice-cold 100% ethanol (two washes, 1 mL each). Nine hundred μL of 0.3 N NaOH were then added to each well to dissolve the cells, and the plates were incubated for 2-h at room temperature. Next, 100 μL of 3.0 N hydrochloric acid were added to each well to neutralize the cell lysate. Samples were then mixed by gently pipeting up and down, and 200 μL from each well were transferred to a scintillation vial. Five mL of scintillation cocktail (Ecoscint; National Diagnostics; Atlanta, GA) were then added to each vial, and all vials were vortexed then measured using a liquid scintillation counter (1 min/sample) to estimate a cell proliferation index via incorporation of radiolabeled thymidine. Data were recorded in cpm and then mathematically converted to dpm for reporting purposes.

DNA Assay of Mammary Tissue Homogenates

Deoxyribonucleic acid concentration of homogenates was assayed essentially as described by Labarca and Paigen (1980). Briefly, 5 μL of mammary tissue homogenate were placed into a test tube, followed by 1895 μL of DNA assay buffer (see Appendix A), and 100 μL of Hoechst dye (see Appendix A), to yield a total assay volume of 2 mL. Samples were measured in triplicate using a DyNA Quant 200 flurometer (Hoefer Pharmacia Biotech; San Francisco, CA) designed specifically for DNA assays. Calf thymus DNA (catalog # D1501; Sigma Chemical Co.; St. Louis, MO) was used as the standard in the assay.

Statistical Analysis

Main effects of diet and slaughter weight, as well as the interaction between the two were tested with the Mixed procedure of SAS (SAS Institute, Inc.; Cary, NC; Version 8.0) using heifer within the combination of diet and target weight as an error term. Dependent variables were protein/ μg tissue (ng), DNA/mg tissue (ng), dpm/ μg protein, tissue (dpm/ μg), and DNA (dpm/ng). Diet was either moderate or high, and slaughter weights were 150, 200, 250, 300, or 350 kg. The following model statement was used: $Y_{ijkl} = \mu + D_i + W_j + (DW)_{ij} + H_{(ij)k} + E_{(ijk)l}$

where:

Y_{ijkl} = dependent variable(s)

D_i = fixed effect of diet ($i = 1$, moderate, or 2, high)

W_j = fixed effect of target weight ($j = 1, \dots, 5$)

DW_{ij} = effect of interaction of diet and target weight

$H_{(ij)k}$ = random effect of heifer within the combination of diet and target weight ($k = 1, 6$ heifers within subclass),

$E_{(ijk)l}$ = residual (sampling within heifer, $l = 1, 4$).

Orthogonal polynomial contrasts were used to test for trends in dependent variables by heifer bodyweights. Results are presented as LS means \pm SEM. Differences were considered significant at $P < 0.05$; trends were declared for differences that were significant at $P < 0.10$.

2.3 Results

Because tissues were used for multiple purposes and because of limited availability of mammary parenchyma in young animal tissues, all animals were not available for mitogenic assays. For example, tissues from neither the baseline group of heifers, nor the 100 kg group of heifers were available (Table 2.1); the final number of heifers included in analyses was 54. Actual slaughter weights of the animals and actual daily gains of the animals are discussed in detail by Meyer et al. (2004). Summaries of actual slaughter weights and actual daily gains for each of the treatment groups are displayed in Table 2.2 and Table 2.3 respectively.

The intra-assay coefficient of variation for sample replicates in the MAC-T cell proliferation experiment averaged 15.6%. Mammary gland extract protein concentrations ranged from 6.0 to 30.5 ng/ μ g parenchyma. These values were not affected by dietary treatment ($P = 0.9$) (Figure 2.1A), but were affected by slaughter weight ($P < 0.01$) (Figure 2.2A). Specifically, there was a linear decrease in protein concentrations of mammary gland extracts as heifer bodyweight increased ($P < 0.001$). There was also a linear decrease of DNA concentrations of MGE as heifer bodyweight increased ($P < 0.01$) (Figure 2.2B). Heifers reared on the high-gain diet tended to have more DNA present per mg of mammary tissue than did those heifers reared on a moderate-gain diet ($P < 0.1$) (Figure 2.1B).

Regardless of the mode of expression (protein, tissue mass, or DNA basis; Figure 2.4 A, B, C, respectively) there was no consistent pattern of response, and the effect of bodyweight on MAC-T cell proliferation was not significant. However, as shown in Figure 2.3, numerically, extracts from moderate-fed heifers were consistently more effective at stimulating proliferation of MAC-T cells than extracts from high-fed heifers. Expressed on either a protein (Figure 2.3A) or tissue mass basis (Figure 2.3B), these differences were not significant ($P \leq 0.2$). In contrast, when expressed on a cell or DNA basis, the overall effect of diet on test cell proliferation was significant (Figure 2.3C; $P < 0.05$). There was no significant interaction between diet and slaughter weight ($P > 0.5$).

2.4 Discussion

While MAC-T cells are known to proliferate in response to IGF-I (Cohick, 1998), and phenotypically retain many characteristics common to *in vivo* differentiated mammary epithelial cells (Huynh et al., 1991), our data demonstrate that there was only a modest and generally non-significant effect of diet, irrespective of bodyweight from 150 to 350 kg, on the proliferative response of MAC-T cells, irrespective of how the data were expressed i.e. parenchymal mass basis, a protein basis, or a DNA basis (Figure 2.3 and 2.4). These findings are a stark contrast to the report by Berry et al. (2003c), where it was demonstrated that MAC-T cells closely mimicked the response of primary

mammary epithelial cells to mammary tissue extracts from high- or low-fed Danish-Friesian heifers on a parenchymal mass basis.

Our experimental design was essentially the same as that published in 2003, however ours included MGE from heifers slaughtered at a range of bodyweights in addition to being fed a high- or a moderate-gain diet, and also included a greater total number of heifers. We also expressed our data in several ways. Thus, while differences in the proliferative response of MAC-T cells were not significant on a protein basis, there was a small but consistent effect of diet such that differences were significant when expressed on a DNA basis. It was however surprising that the effect of diet was minimal regardless of the mode of expression. Using MGE from Weber et al. (2000), Berry et al. (2003c) noted approximately a doubling of response of extracts from low-fed versus high-fed heifers. This closely mimicked the pattern of response reported by Weber et al. (2000) when the same MGE were tested using primary cells. Interestingly, Weber et al. (2000) did note that for high or low fed heifers also given exogenous bST that the pattern of response was reversed. That is, extracts from high fed heifers were more effective in stimulating the proliferation of primary bovine mammary epithelial cells than extracts from low fed heifers also given bST. For placebo-treated heifers, extracts from low fed heifers were more effective.

Reasons for the differences reported here are unknown, but may reflect genetic differences between animals (Danish-Friesians versus U.S. Holsteins), differences in diet composition, or perhaps differences relating to duration of dietary treatment application and/or bodyweight of animals at time of treatment. As suggested by the bST-modified response, it may be that GH status was different for these heifers. In addition, the heifers used in the experiment by Weber et al. (2000) and Berry et al. (2003c) were fed two different diets to obtain divergent rates of gain. The heifers on the low-gain diet had free access to a diet that was roughage-based, while the heifers on the high-gain diet had free access to a concentrate-based diet and were supplemented with 2 kg/d of the roughage-based diet (Vestergaard et al., 2003). During the pre-weaning phase of the present study, heifers were fed differently comprised milk replacers to achieve divergent bodyweight gains. After weaning however, heifers were fed the same TMR with only the amounts fed adjusted to achieve the desired average

daily bodyweight gains (Table 2.4). Thus the mitogenic effects observed after adding MGE to primary and MAC-T cell cultures documented by Weber et al. (2000) and Berry et al. (2003c) may have ultimately stemmed from differences in diet composition or endocrine status but not specifically plane of nutrition. In support of the notion that diet composition can affect mammary growth, Capuco et al. (1995) found that heifers reared at approximately 1 kg of average daily gain on a corn silage diet exhibited inhibited mammary growth, whereas heifers reared at a comparable rate of bodyweight gain on an alfalfa silage diet did not. In their study, there were no post-treatment differences in parenchymal mass, but there were differences in the area of mammary parenchyma occupied by epithelium, stroma, and adipocytes in those heifers. In particular, heifers reared on the corn silage diet had more parenchymal adipocytes, less stroma, and less epithelium than the heifers reared on the alfalfa silage diet (Capuco et al., 1995).

Furthermore, the experimental period of Weber et al. (2000) and Berry et al. (2003c) was, on average, much shorter than the experimental period in the present study and also started when the animals were at a greater bodyweight than heifers in the present study. The experimental period of Weber et al. (2000) and Berry et al. (2003c) lasted a total of 8 wk, with only five of the 8 wk being a period where animals were fed different diets. Animals fed the low-gain diet in that study weighed 194.3 ± 10.6 kg at the beginning of the experimental period, and 218.7 ± 4.3 kg at the end of the experimental period. Similarly, animals fed the high-gain diet weighed 196.7 ± 10.6 kg at the beginning, and 241.3 ± 4.3 kg at the end of the experimental period (Vestergaard et al., 2003b). The experimental period in the present study started when the heifers were at an average bodyweight of 44.2 kg (10 days old) and continued until each heifer was sacrificed at her pre-determined target bodyweight. The fact that such large differences in the proliferative responses of test cells were seen by Weber et al. (2000) and Berry et al. (2003c) during a relatively short experimental period, and virtually no proliferative differences were seen in the present study, seems to imply that diet composition may affect the cellular/molecular composition of MGE more than plane of nutrition. The major similarities and differences between these studies are summarized in Table 2.5.

There was a linear decrease in protein concentration of MGE with increased heifer bodyweight (Figure 2.2A). This means that for the 300 and 350 kg heifers more MGE, and therefore more mammary tissue, was required for use in the cell proliferation experiment to obtain the same quantity of extract for testing. There was also a tendency for lighter heifers to have larger dpm/ μ g tissue mass values (Figure 2.4B; $P < 0.1$); this suggests that for heifers slaughtered at earlier bodyweights, less mammary tissue is required of them to be able to stimulate MAC-T cell proliferation compared to heifers slaughtered at heavier bodyweights.

An overall treatment difference between heifers reared on a high rather than a moderate diet existed ($P < 0.01$), with heifers reared on a high diet having a lesser average dpm/ng DNA value. This suggests that heifers reared on a high versus moderate diet (irrespective of bodyweight from 150 to 350 kg) had more DNA present in the same amount of mammary tissue (see Figure 2.1B; $P < 0.1$), but did not necessarily have an advantage in their ability to stimulate cell proliferation *in vitro*.

Protein content of MGE from heifers reared on a high diet, irrespective of bodyweight from 150 to 350 kg, averaged 21.9 ± 9.6 ng/ μ g parenchyma; likewise protein content of MGE from heifers reared on a moderate diet averaged 22.0 ± 8.9 ng/ μ g parenchyma. These values are similar to the range of values (24.9 - 46.5 ng extract protein/ μ g parenchyma) reported by Weber et al. (2000) for 24 animals reared on a high or low diet and either treated or not treated with exogenous bST.

Deoxyribonucleic acid content of parenchymal tissue used to prepare MGE from heifers reared on a high or a moderate diet, irrespective of bodyweight from 150 to 350 kg, averaged 771 ± 34 and 705 ± 31 ng DNA/mg parenchyma, respectively. These values are similar to DNA values obtained from the parenchyma of prepubertal lambs, using the same assay protocol (McFadden et al., 1990). Additionally, the DNA data reported here (Figures 2.1B and 2.2B) agree with *in vivo* heifer mammary epithelial cell proliferation data reported by Meyer et al. (2004). In their study, bromodeoxyuridine (BrdU), a thymidine analog, was injected *ante mortem* to assess mammary cell proliferation. Upon immunocytochemical evaluation of tissue *post mortem*, they found that heifers reared on a high- versus moderate-gain diet tended to have more proliferating cells, and that mammary epithelial cell proliferation decreased with

increasing bodyweight and was similar between dietary treatments past 100 kg BW (Figures 2.5 and 2.6).

Our results also support the conclusion of Berry et al. (2003c), that MAC-T cells may not be an accurate model for ovarian-induced changes in mammary growth. A range of mammary tissues, representing physiological states before, during, and after puberty, were included in the MAC-T bioassay. One would assume that pubertal animals have more estrogen-stimulated mammary epithelial cell proliferation that would be reflected in the bioassay results; our bioassay detected no such proliferative changes due to bodyweight, irrespective of plane of nutrition.

While the use of MAC-T cells is attractive due to their widespread availability and their growth properties in culture, they may not accurately reflect changes occurring in the mammary glands of heifers reared on different planes of nutrition, at different stages of physiologic development, or ovarian-induced changes in mammary growth. Further arguments in support of the inability of MAC-T cells to mimic primary cells in culture come from the observations that in MAC-T cells, the addition of the protein hormone leptin to culture media caused a reduction in IGF-I stimulated ^3H -thymidine incorporation (Silva et al., 2002). However, negative effects of leptin on IGF-I mediated proliferation could not be demonstrated in primary epithelial cells isolated from prepubertal heifers (Purup and Sejrsen, 2000). Similarly, Woodward et al. (1995) indicated that the addition of transforming growth factor- β (TGF- β) to the media of MAC-T cells hinders cell proliferation as measured by total DNA and ^3H -thymidine incorporation, but Musters et al. (2004) and Purup et al. (2000) suggested that TGF- β is stimulating both *in vivo* and *in vitro*.

Perhaps proliferative inconsistencies between primary cell cultures and MAC-T cell cultures are due to the origin of MAC-T cells. These cells were developed from primary mammary epithelial cells from lactating cows. The production of an immortalized cell line derived from mammary epithelial cells from prepubertal heifers might prove to be more accurate test cells, due to their less functionally developed phenotype. They may be more sensitive to the constituents of mammary extracts, especially from peripubertal heifers. Unfortunately, to our knowledge such a cell line does not currently exist.

Before the advent of a new test cell line however, there are some other issues that should first be addressed to rule out costly and perhaps unnecessary cell culture work. First, it may prove worthwhile to repeat the experiment reported in this work, but instead of measuring ³H-thymidine incorporation into the DNA of MAC-T cells, total DNA accumulation should be measured. If differences in total DNA accumulation are found to exist, then it would shed doubt on the results obtained here, and may mean that MAC-T cells are useful for testing the mitogenic capacity of mammary gland extracts from prepubertal heifers. Secondly, remaining aliquots of the MGE used in this study should be used in an experiment to test the mitogenic activity of primary cells in culture to see if they respond differently than MAC-T cells. This would involve essentially repeating the work of Weber et al. (2000). Thirdly, it may be worthwhile to repeat the experiments of Weber et al. (1999) using remaining aliquots of the MGE to measure the concentration of IGF-I and the relative abundance of IGFBPs present in samples. It would be interesting to see if the MGE in this study have a larger relative abundance of IGFBPs than the MGE that Weber et al. (1999) and Berry et al. (2003c) used; which could then be attributed to lower mitogenic activity when applied to test cells. Because the effects of IGF-I are mediated by IGFBPs, it also may be of interest to examine potential regulators of IGFBPs in these heifers. In recent years, retinoids (retinoic acid, retinol) have received attention in this role. Retinoids are known inhibitors of epithelial tumor growth and progression in many human mammary cancer cell lines and animal models. For instance, Metz et al. (2002) demonstrated that adolescent vitamin A intake alters mammary gland differentiation in rats, and also indicated that a narrow range of vitamin A intake during adolescence protects against chemically induced mammary tumor formation. Retinoids have also been shown to inhibit IGF-I stimulated bovine mammary epithelial cell proliferation in culture (Woodward et al., 1996; Purup et al., 2001, Cheli et al., 2003). In particular, Woodward et al. (1996) demonstrated that retinoic acid is a potent, but transient inhibitor of MAC-T cellular proliferation and that the growth inhibition is correlated with increased IGFBP-2 accumulation in media and the inhibition of IGF-I stimulated IGFBP-3 protein secretion into media. A novel experiment would be to use ligand blots to correlate the relative abundance of IGFBPs to the relative abundance of retinoid binding proteins in MGE from these heifers. Based

on results of Woodward et al. (1996), it is hypothesized that low relative abundance of retinoid binding proteins (plentiful amounts of active retinoids) correlates with increased relative abundance of IGFBPs (diminished amounts of active IGF-I).

Clearly, the results of the current experiment raise many new research questions. Through our cell culture experiments with MAC-T cells, we have shown that contrary to previous reports and our own hypotheses, maintenance of Holstein dairy heifers on high-gain diets throughout the peripubertal period does not always have deleterious effects on mammary development. Future work proposed by us and other researchers will try to determine the physiological basis for this.

Table 2.1. Summary of samples shipped to Virginia Tech from Cornell University.

Target Slaughter wt (kg)	Number Moderate Diet	Number High Diet	Number of samples available
45	0	0	0
100	0	0	0
150	5	3	8
200	6	6	12
250	6	6	12
300	5	5	10
350	6	6	12
Total	28	26	54

Table 2.2. Summary of actual slaughter weights for heifers.

Target ^a	Trt ^b	Actual ^c	SEM	n ^d
150	H	153.3	1.2	3
150	M	151.8	0.7	5
200	H	202.2	1.8	6
200	M	204.7	2.2	6
250	H	255.0	2.0	6
250	M	255.8	1.5	6
300	H	304.6	2.1	5
300	M	298.2	3.9	5
350	H	359.7	3.1	6
350	M	361.2	2.1	6

^a = Target slaughter weight.

^b = Dietary treatment; H = high (950 g/d ADG); M = moderate (650 g/d ADG).

^c = Actual slaughter weight mean for each group.

^d = number of animals in each group.

Table 2.3. Least-Squares Means of growth data ¹.

Item	Treatment		SEM
	Moderate ²	High ³	
Initial BW, kg	44.4	43.9	0.74
Initial age, d	9.4	10.3	0.70
Initial HH ⁴ , cm	82.9	82.8	0.51
Weight at weaning, kg	75.7 ^a	85.9 ^b	0.66
Length of milk fed phase, d	49.7 ^a	43.3 ^b	1.2
HH at weaning, cm	93.1 ^a	94.3 ^b	0.33
Pre-weaning weight gain, kg/d	0.64 ^a	0.96 ^b	0.015
Pre-weaning HH gain, cm/d	0.27 ^a	0.36 ^b	0.017
Lifetime weight gain, kg/d	0.66 ^a	0.93 ^b	0.009
Lifetime HH gain, cm/d	0.14 ^a	0.18 ^b	0.004

^{a,b} = Means with uncommon superscripts are different at $P < 0.05$.

¹ = Adapted from Meyer et al., 2004.

² = Moderate nutrient intake.

³ = High nutrient intake.

⁴ = Hip height.

Table 2.4. Diet dry matter and nutrient composition ¹.

Item	Diet		
	TMR 1	TMR 2	TMR 3
Ingredient, % of diet DM			
Alfalfa hay	10.3		
Alfalfa silage	15.1	31.8	31.8
Corn silage	13.8	31.8	31.8
Soybean meal			4.2
Heat treated soy bean meal	10.3	18.0	8.4
Starter grain mix ²	50.3		
High moisture corn		15.9	23.1
Rumensin 800		1.6	1.7
Vit. E supplement	0.12	0.10	
Mineral mix	0.12	0.9	1.4
Nutrient, % of diet DM			
CP	24.3	19.0	17.2
NEm, Mcal/kg ³	1.72	1.62	1.65
NEg, Mcal/kg ⁴	1.08	1.03	1.06
ADF	18.1	21.1	20.2
NDF	35.5	34.0	31.4
NFC ⁵	34.3	35.9	40.0
EE ⁶	1.91	3.93	3.45

¹ Reproduced with permission from Meyer et al., 2004.

² 27% CP textured starter.

³ Net energy for maintenance.

⁴ Net energy for gain.

⁵ Nonfiber carbohydrate.

⁶ Ether extract.

Table 2.5. Comparison of a Danish and a U.S. heifer growth study.

Item	<u>Low (Moderate)^a</u>		<u>High^a</u>	
	Danish Study ^b	U.S. Study ^c	Danish Study ^b	U.S. Study ^c
No. of animals	6	36	6	36
Breed of animals	Danish-Friesian	U.S. Holstein	Danish-Friesian	U.S. Holstein
BW at start, kg	194.3 ± 10.3	44.4	196.7 ± 10.3	43.9
BW at slaughter, kg	218.7 ± 4.3	Varied ^d	241.3 ± 4.3	Varied ^d
Planned ADG, g/d	550	650	1100	950
Actual ADG, g/d	476	660	1406	930
Type of diet	Rough. ^{b,e}	22:20 MR ⁱ ; TMR ^{c,k}	Conc. + Rough. ^{b,f}	28:20 MR ⁱ ; TMR ^{c,k}
Experimental period	8 wk ^g	Varied ^{d,h}	8 wk ^g	Varied ^{d,h}

^a = Type of gain diet

^b = Described in detail by Vestergaard et al., 2003.

^c = Described in detail by Meyer et al., 2004.

^d = Slaughter weights were either 150, 200, 250, 300, or 350 kg; selected at random at beginning of trial.

^e = ad libitum access to roughage-based mixture.

^f = ad libitum access to concentrate mixture + 2 kg/d of roughage-based mixture.

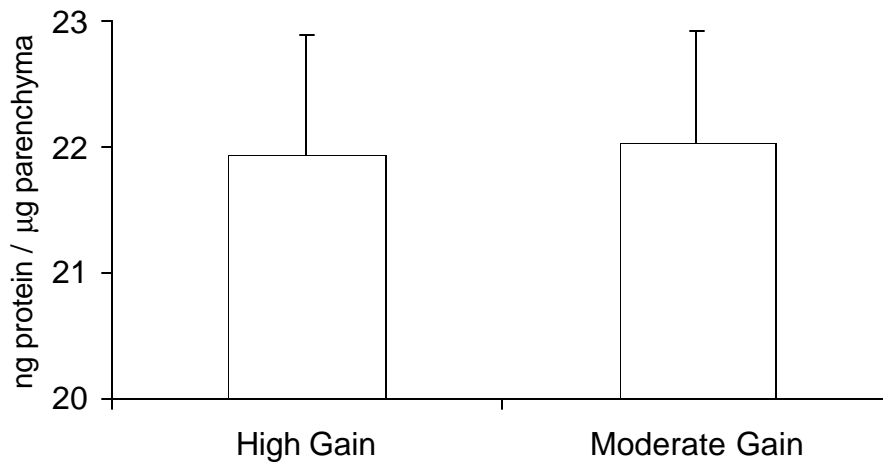
^g = 3 wk of adaptation for all animals (low diet) followed by 5 wk of experimental diet.

^h = Dietary treatments started upon arrival to Cornell University Dairy and ended at slaughter of animal.

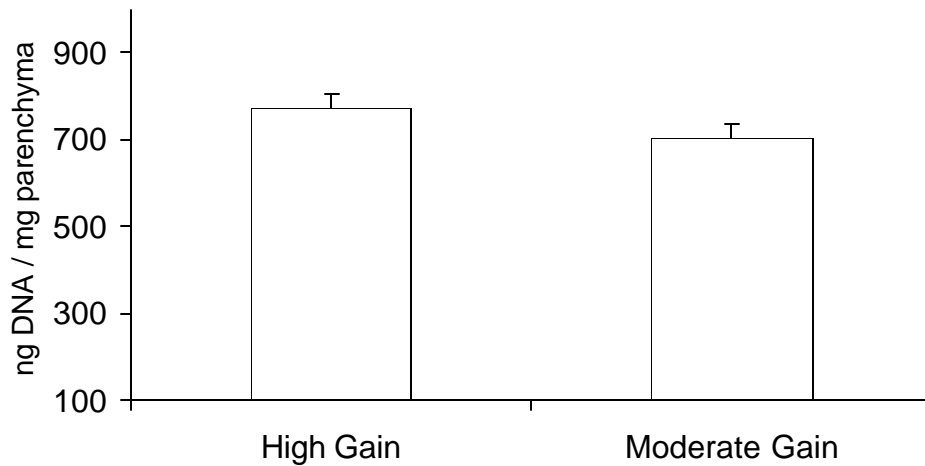
ⁱ = 22% CP, 20% fat milk replacer fed at 0.20 Mcal gross energy (GE) per kg of BW^{0.75}; fed during pre-weaning period.

^j = 28% CP, 20% fat milk replacer fed at 0.32 Mcal GE per kg BW^{0.75}; fed during pre-weaning period.

^k = A series of 3 TMRs was fed post-weaning: TMR1 was fed to all animals in weeks 10-13 of treatment; TMR2 was fed after TMR1 until animals weighed 200 kg; TMR3 was fed to all animals weighing between 200-350 kg. The amount fed was adjusted to achieve average daily gains.

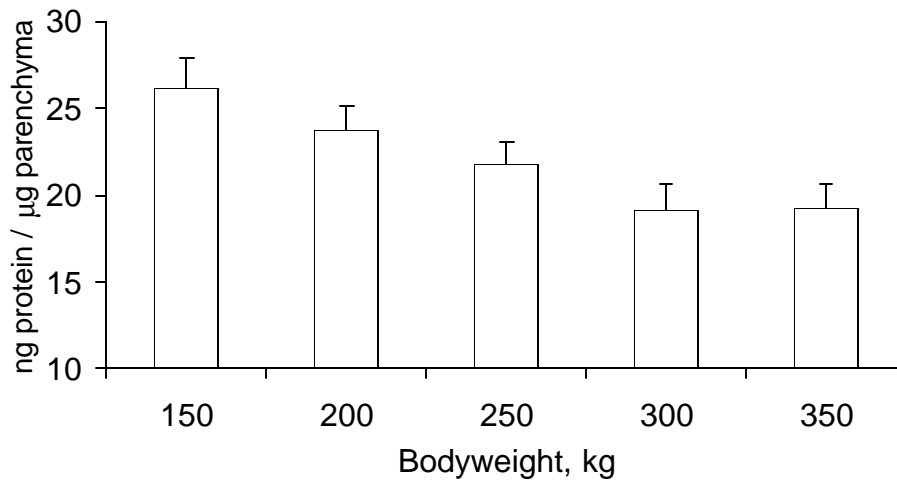


(A)

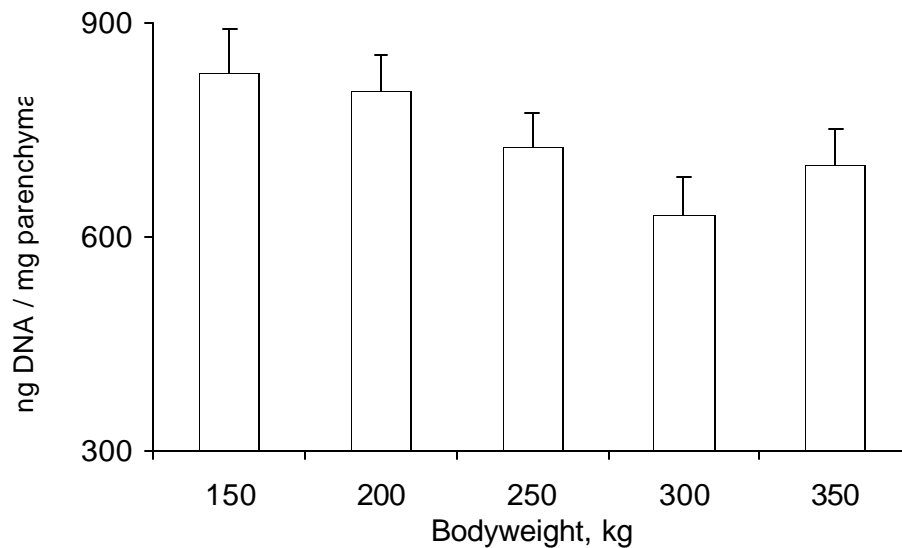


(B)

Figure 2.1. Amount of (A) protein (ng) and (B) DNA (ng) present per μg (A) and mg (B) of mammary parenchyma taken from heifers reared on either a high (950 g/d ADG; n = 26) or a moderate (650 g/d ADG; n = 28) diet, and slaughtered at various bodyweights between 150 and 350 kg. Values are LS means ± SEM for high versus moderate fed heifers across all slaughter weights. Differences were not significant ($P > 0.05$).

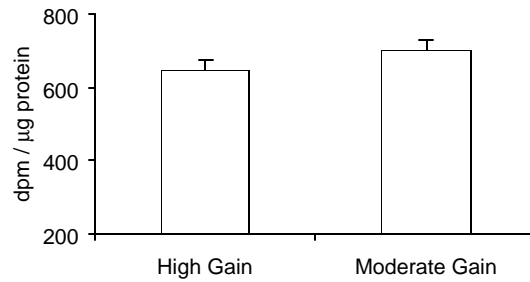


(A)

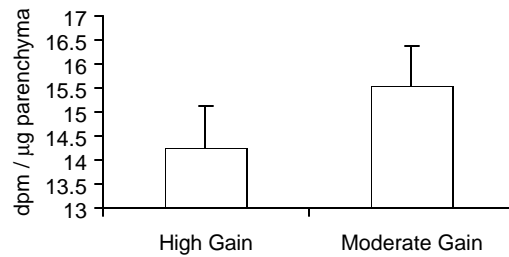


(B)

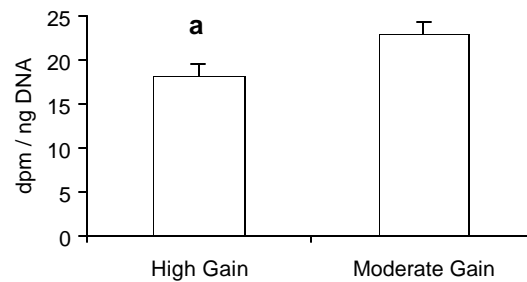
Figure 2.2. Amount of (A) protein (ng) and (B) DNA (ng) present per μg (A) and mg (B) of mammary parenchyma taken from heifers sacrificed at different bodyweights and reared on either a high or a moderate gain diet. Numbers of heifers per slaughter group were as follows: 150, $n = 8$; 200, $n = 12$; 250, $n = 12$; 300, $n = 10$; 350, $n = 12$. Values are LS means \pm SEM for heifers slaughtered at different bodyweights across all dietary treatments. Decreases in protein and DNA content of mammary extracts with increased bodyweight were linear ($P < 0.001$; $P < 0.01$).



(A)

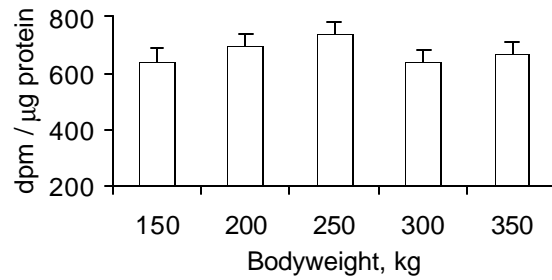


(B)

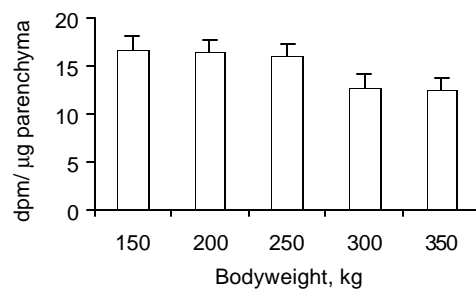


(C)

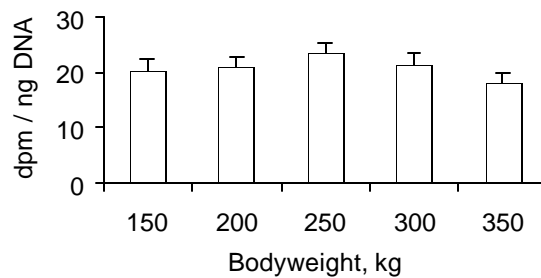
Figure 2.3. Overall proliferative response of MAC-T cells to individual mammary tissue extracts from heifers reared on either a high (950 g/d ADG; n = 26) or a moderate (650 g/d ADG; n = 28) diet, and slaughtered at various bodyweights between 150 and 350 kg. Cell proliferation was measured by incorporation of ^3H -thymidine into DNA (dpm). Data are expressed on a per cell basis by adjusting for the (A) protein content (μg), (B) mass of parenchyma (μg), and (C) DNA content (ng), of tissue used to prepare each extract. Values are LS means \pm SEM for high versus moderate fed heifers across all slaughter weights. Bars with different letters are significantly different ($P < 0.05$).



(A)



(B)



(C)

Figure 2.4. Overall proliferative response of MAC-T cells to individual mammary tissue extracts from heifers sacrificed at different body weights and reared on either a high or a moderate gain diet. Cell proliferation was measured by incorporation of ^3H -thymidine into DNA (dpm). Data are expressed on a per cell basis by adjusting for the (A) protein content (μg), (B) mass of parenchyma (μg), and (C) DNA content (ng), of tissue used to prepare each extract. Numbers of heifers per slaughter group were as follows: 150, $n = 8$; 200, $n = 12$; 250, $n = 12$; 300, $n = 10$; 350, $n = 12$. Values are LS means \pm SEM for heifers slaughtered at different bodyweights across all dietary treatments. No differences were statistically significant ($P > 0.05$).

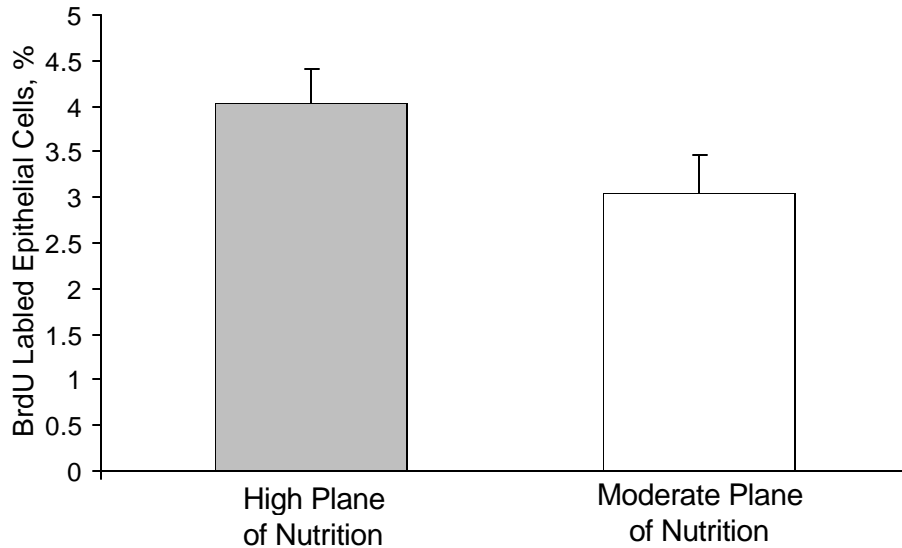


Figure 2.5. Least squares means of bromodeoxyuridine (BrdU) labeling by mammary epithelial cells among prepubertal heifers across six slaughter weights raised on a high (hashed bars) or moderate (open bars) plane of nutrition. Significance of main effects and their interaction: treatment, $P = 0.08$; slaughter weight, $P < 0.01$; interaction, $P = 0.16$ (Reproduced with permission from Meyer et al., 2004).

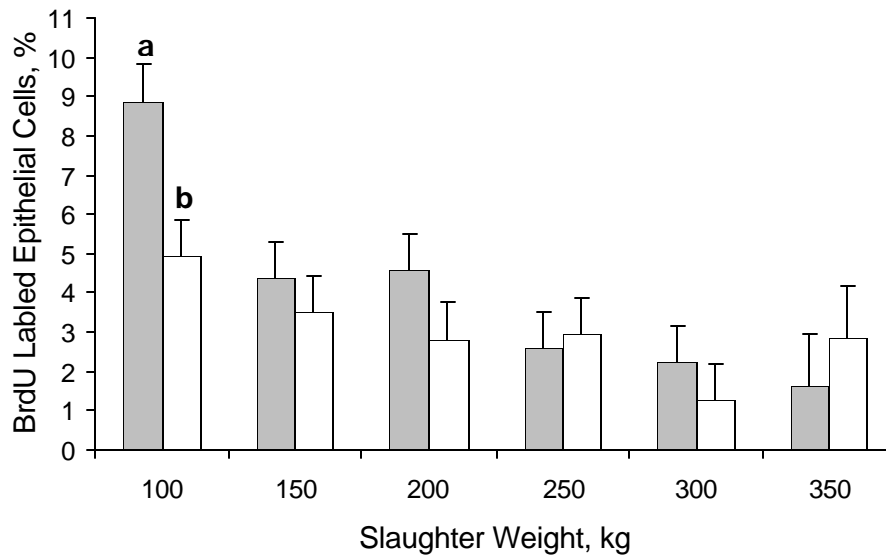


Figure 2.6. Least squares means of bromodeoxyuridine (BrdU) labeling by mammary epithelial cells among prepubertal heifers across six slaughter weights raised on a high (hashed bars) or moderate (open bars) plane of nutrition. Significance of main effects and their interaction: treatment, $P = 0.08$; slaughter weight, $P < 0.01$; interaction, $P = 0.16$. Within slaughter weight, a and b are different at $P < 0.05$ (Reproduced with permission from Meyer et al., 2004).

CHAPTER 3: EFFECTS OF BODYWEIGHT AND PLANE OF NUTRITION ON HISTOLOGICAL DEVELOPMENT OF TERMINAL DUCTAL STRUCTURES IN MAMMARY TISSUE OF HOLSTEIN HEIFERS

3.1 Introduction

Nutrition is an important factor that influences peripubertal heifer mammary growth. Dairy producers, in efforts to lower age at first calving and the period of non-productive life of their replacement animals often maintain their replacement heifers on high-gain diets (bodyweight gains >700 g/d) during the prepubertal phase of life. This practice, while known to increase rates of skeletal and bodyweight growth, has been shown to harm the development of mammary ductular epithelium (Sejrsen and Purup, 1997). With diminished ductular epithelium development there is a corresponding reduction in secretory tissue development and therefore suppressed milk yields (Swanson, 1960; Little and Kay, 1979; Sejrsen et al., 2000). This is because mammary secretory epithelium (alveoli), which develops during gestation, depends on ducts that have developed in earlier life. Thus, the peripubertal period is a critical window in early mammary development for dairy heifers, and is a stage of growth where nutrient management plays a critical role.

From birth to approximately 2 mo of age, Holstein heifer mammary growth consists of the isometric growth of non-epithelial tissues (stroma and blood vessels) (Sejrsen, 1994). Thereafter until puberty, which occurs at about 250 to 280 kg BW (9-11 mo of age), mammary growth is allometric (Sinha and Tucker, 1969), and there is rapid growth of both epithelial ducts and non-epithelial structures (Sejrsen, 1994). At the end of the allometric growth phase, the mammary glands of Holstein heifers weigh about 2 to 3 kg, of which about 0.5 to 1 kg is parenchymal tissue. The parenchyma at this time usually contains 10 to 20% epithelium, 40 to 50% connective tissue, and 30 to 40% adipocytes (Sejrsen et al., 2000). Once a heifer reaches puberty, the mammary gland returns to an isometric rate of growth (Sinha and Tucker, 1969). Thereafter there seems to be little effect of feeding level on mammary development (Sejrsen et al., 1982).

Evaluation of histological features such as those mentioned above has been a long-standing technique used by many in the field of mammary biology to study tissue growth and development in both normal and experimental conditions. Histological observations of male and female sheep and cattle to demonstrate changes in mammary development have a long history (Turner, 1952; Wallace, 1953). However few, if any, of these earlier studies were quantitative. The objective of this experiment was to determine effects of rate of gain and bodyweight on histological development of the udder with a focus on changes in terminal ductal structures (TDU) in Holstein heifers.

3.2 *Materials and Methods*

Animals and Treatments

The tissue samples used in this study were from animals that were purchased within 1 week of age from commercial dairy farms surrounding Ithaca, New York, and treated at Cornell University, as reported by Meyer et al. (2004). The objective of this study was to evaluate the effect of plane of nutrition from birth to 350 kg on body composition and mammary development in Holstein heifers. Briefly, heifers (n = 78) were randomly assigned to one of two dietary treatments (moderate or high), or a baseline slaughter group (6 heifers, 45 kg BW); within treatments heifers were randomly assigned to one of six slaughter weights (100, 150, 200, 250, 300, or 350 kg BW, respectively). Six heifers per dietary treatment were slaughtered at each time point for a total of 78 heifers (including baseline group). The specific slaughter weights given above were chosen to represent periods when the mammary gland transitions from isometric to allometric growth as well as periods within the allometric growth phase. This was based on the temporal pattern of mammary development reported by Sinha and Tucker (1969).

Heifers on the moderate gain (M) dietary treatment were fed a pre-weaning diet that consisted of a 22% CP and 20% fat (dry matter basis) milk replacer that was fed at 0.20 Mcal gross energy (GE) per kg of BW^{0.75}. Heifers on the high gain (H) dietary treatment were fed a pre-weaning diet that consisted of a 28% CP and 17% fat (dry matter basis) milk replacer that was fed at 0.32 Mcal GE per kg of BW^{0.75}. A 26% CP

calf starter was offered to animals beginning at approximately 3 weeks on study. After weaning, which was completed by 7 weeks on treatment, all heifers were fed the 26% CP starter for two additional weeks. From weeks 10 to 13 of treatment, all heifers received TMR 1 (Table 3.4). Thereafter all heifers were fed TMR 2; heifers exceeding 200 kg BW were fed TMR 3 (Table 3.4). Throughout the entire length of the study, heifers were weighed once weekly and the amount of feed offered was adjusted such that heifers on the M treatment could achieve 650 g, and heifers on the H treatment could achieve 950 g of daily gain, respectively. From the initiation of treatment to 150 kg, heifers were housed in individual pens in the Teaching and Research Dairy Center Greenhouse at Cornell University. Upon reaching 150 kg, heifers that were assigned to heavier slaughter weights were grouped in one pen and individually fed via a Calan gate system.

Once heifers assigned to slaughter weights greater than or equal to 250 kg reached 225 kg BW blood was collected twice weekly via jugular venipuncture and plasma progesterone concentrations were determined. Progesterone concentrations above 1 ng/mL were interpreted as the heifer having a functional corpus luteum, and therefore pubertal.

Slaughter Procedure and Mammary Tissue Collection

Dietary treatments were continued until animals obtained their assigned slaughter weight; heifers were weighed before feeding on two consecutive days to monitor target rate of gain. The decision to slaughter at a common bodyweight as opposed to a common age was based on observations that puberty is more closely associated with bodyweight than a specific age (Sejrsen, 1994). Pubertal heifers were slaughtered in the luteal phase of their reproductive cycle.

Humane slaughter of each heifer was performed at the Department of Animal Science Abattoir at Cornell University using a captive bolt stunner followed by exsanguination. At slaughter, the udder of each heifer was bisected along the medial suspensory ligament. One half of the udder was used by Meyer at Cornell University to determine total amounts of parenchymal and extra-parenchymal tissue, and the effect of

energy intake on the composition of both. Tissues harvested from the mid-parenchymal region of the second half of the udder arrived to Virginia Tech via U.S. Mail in one of three forms. Mid-parenchymal mammary tissue samples from some heifers (n = 7) were sent as paraffin-embedded tissue blocks, others (n = 12) were sent as hematoxylin and eosin stained slides, and the remainder (n = 35) were sent as unstained 5 μ m thick sections mounted onto microscope slides. Microscope slides were prepared from those samples sent as tissue blocks, by slicing 5 μ m thick sections from the blocks with a microtome (Reichert-Jung Model 2040 Autocut; West Germany) and mounting them onto microscope slides.

General Staining Procedures for Microscope Slides

Slides that needed to be stained were de-waxed and hydrated in a series of sequential washes. These consisted of two 5-min washes in xylenes, one 2-min wash in 100% ethanol, one 2-min wash in 95% ethanol, one 2-min wash in 70% ethanol, and one 5-min wash in water. After the hydration step, slides were immersed in hematoxylin and eosin stain (Sigma Chemical Co., St. Louis, MO) for 10 min. Excess stain was removed by rinsing slides in running tap water. Slides were then dehydrated by again placing them in a series of sequential washes. These consisted of one 5-min wash in water, one 2-min wash in 70% ethanol, one 2-min wash in 95% ethanol, one 2-min wash in 100% ethanol, and two 5-min washes in xylenes. After the last xylenes wash, 3 drops of Permount[®] (Fisher Scientific; Fair Lawn, NJ) were placed on each slide and a coverslip was placed over the Permount. Slides were allowed to dry overnight on a tray; the next day they were placed in storage boxes.

Digital Image Acquisition

Hematoxylin and eosin stained images were captured with a top-mount digital microscope camera (Olympus DP10, OPELCO, Dulles, VA) under 10x magnification. Each image to be acquired was centered in an area predominately occupied by epithelial (versus stromal) cells. Three different, but representative pictures were taken

at random locations along the microscope slide for each heifer. Digital images were then transferred to a computer, where they were stored.

Area Measurements

All measurements were made using the Image-Pro Plus Version 4.5 for Windows software program (Media Cybernetics, Inc.; Silver Spring, MD). Images of a stage micrometer were taken and used to calibrate the measurement tools used as part of the software package. Each of our 10x magnification images encompassed an area of 871,820 μm^2 . After pilot testing determined that variation between independent images of each tissue sample were minimal, one of the images from each heifer was chosen at random for further analysis. Total areas occupied by interlobular stroma, epithelium, lumen, and intralobular stroma were determined for each image. This was achieved by using the computer mouse to outline desired structural features. The computer program recorded the area, in μm^2 , for each structure that was outlined; these measurements were then exported to a Microsoft Excel spreadsheet where total areas for each mammary parenchymal component were calculated via simple summation. The total area occupied by intralobular stroma was calculated by subtracting the total areas occupied by interlobular stroma, epithelium, and lumen from the total area (871,820 μm^2). The numbers of epithelial and luminal structures in each image measured were also tabulated and recorded.

Statistical Analysis

Main effects of diet and slaughter weight, as well as the interaction between the two were tested for using the GLM procedure of SAS (SAS Institute, Inc.; Cary, NC; Version 8.0). Total area occupied by interlobular stroma, epithelium, lumen, and intralobular stroma, and numbers of epithelial and luminal structures present were used as dependent variables. Diet was either moderate or high, and target slaughter weights were 150, 200, 250, 300, or 350 kg. The following model statement was used:

$$Y_{ijk} = \mu + D_i + W_j + (DW)_{ij} + E_{(ij)k}$$

where:

Y_{ijk} = dependent variables, area or percent of tissue type
 μ = overall mean
 D_i = fixed effect of diet (moderate versus high, $i = 1,2$)
 W_j = fixed effect of target weight ($j = 1, \dots, 5$)
 DW_{ij} = effect of interaction of diet and target weight
 $E_{(ij)k}$ = residual error ($k = 1,6$ heifers within subclass).

Results are presented as LS means \pm SEM. Differences were considered significant at $P < 0.05$; trends were declared for differences that were significant at $P < 0.10$. Orthogonal polynomial contrasts were used to test for linearity in response of dependent variables to bodyweight. A multivariate analysis of variance was conducted to test a vector of all tissue types simultaneously. This was to determine whether the profile of tissue areas (percentages of interlobular stroma, epithelium, lumen, and intralobular stroma) differed by diet or slaughter weight. The dependent variable "total area occupied by lumen" was removed from the model statement for multivariate analysis of variance because all tissue areas totaled 100%. Dietary treatment, heifer slaughter weight, and the interaction of dietary treatment with heifer slaughter weight were tested for all tissues simultaneously.

3.3 Results

Because tissues were used for multiple purposes and because of limited availability of mammary parenchyma in the youngest animal tissues, all animals were not available for examination. For example, tissues from neither the baseline group of heifers, nor the 100 kg group of heifers were available for analysis (Table 3.1); the final number of heifers included in analyses was 54. Actual slaughter weights of the animals and actual daily gains of the animals are discussed in detail by Meyer et al. (2004). Summaries of actual slaughter weights and actual daily gains for each of the treatment groups are displayed in Table 3.2 and Table 3.3 respectively.

The percentages of parenchymal area occupied by interlobular stroma, epithelium, lumen, and intralobular stroma did not significantly differ by bodyweight or dietary treatment, nor was there an interaction between dietary treatment and

bodyweight for these variables (Table 3.5). In the multivariate analysis of variance there were no overall effects of dietary treatment, heifer bodyweight, or the interaction between the two on the array of tissue percentages measured. Overall, the mean percentages of mammary parenchyma occupied by interlobular stroma, epithelium, lumen, and intralobular stroma were 28, 20, 7, and 45%, respectively. These percentages correspond to the area measurements of 244,110 μm^2 , 174,364 μm^2 , 61,027 μm^2 , and 392,319 μm^2 , respectively. Figure 3.3 identifies heifer mammary parenchymal components. Number of epithelial and luminal structures differed by bodyweight, irrespective of dietary treatment. More specifically, numbers of epithelial and luminal structures increased linearly with increased heifer bodyweight ($P < 0.005$, $P < 0.005$, respectively)(Figures 3.1, 3.2, and 3.4).

3.4 Discussion

While there were no statistically significant differences in percent parenchymal composition either over time, as reflected by heifer bodyweight at slaughter, or by dietary treatment, our results agree with Sejrsen et al. (1982) and Forrest (2003). In the study by Sejrsen et al. (1982), pre- and post-pubertal Holstein heifers were fed the same ration, but the total amount eaten differed. Heifers in the restricted group gained on average 613 g/d, and the average daily gain for heifers with ad libitum access to the ration was 1218 g/d. There were no significant dietary treatment differences in the percentage of parenchyma occupied by epithelial cells, connective tissue, adipocytes, or ductular lumen in both pre- and post-pubertal heifers (Sejrsen et al., 1982). Forrest (2003) also concluded that feeding level did not significantly affect the percentage of epithelial tissue in mammary parenchyma (range of 18.0 to 20.9%) from Holstein heifers reared on one of four treatments, corresponding to two levels of energy intake (high and low) and two periods of growth (2 to 8 wk of age, and 8 to 14 wk of age). Across all treatments, Forrest (2003) determined mammary parenchymal composition to be approximately 20% epithelium, 8% lumen, and 72% inter- and intra-lobular stroma. These values are virtually identical to the values reported in this work of approximately 20% epithelium, 7% lumen, and 73% inter- and intra-lobular stroma. It was also

reported by Sejrsen et al. (1982) that at the end of the allometric mammary growth phase (approximately 250 to 280 kg BW), Holstein heifer mammary parenchyma contains approximately 10% epithelial cells, 50% connective tissue, 30 to 40% adipocytes, and 2 to 3% ductular lumen; these approximations tend to agree with the numbers reported here, and by Forrest (2003).

Overall, it appears that the high rate of bodyweight gain imposed in the current experiment had no deleterious effects on parenchymal composition and that the composition of gain for mammary parenchyma did not change over time. Furthermore our observation that there were no differences in mammary parenchymal composition is in agreement with our *in vitro* cell culture findings. In our experiment using the immortalized cell line, MAC-T, we found that the addition of mid-parenchymal mammary gland extracts from the same animals used in the present experiment to culture media had little effect on the mitogenic response of MAC-T cells, as measured by incorporation of the radio-labeled nucleotide, ³H-thymidine, into DNA. Our results reported here also agree with *in vivo* mammary epithelial cell proliferation data reported by Meyer et al. (2004). That is to say, we found no differences in parenchymal composition in these animals due to dietary treatment and/or heifer bodyweight, and Meyer et al. (2004) found no differences in the proportions of mammary epithelial cells actively dividing, due to dietary treatment and/or for heifers with bodyweights greater than 100 kg. Nor did they find any differences in percent fat and percent protein in mammary parenchyma, or parenchymal DNA accretion rates between heifers reared on one of two different planes of nutrition (Meyer et al, 2004).

Based on the experiments conducted thus far with mammary tissues from this specific group of heifers, there seems to be no ill effects nor advantageous biological effects of maintaining heifers on a high lifetime plane of nutrition (target lifetime bodyweight gain of 950 g/d). Thus, any unknown, and therefore untested indicators of inhibited mammary growth in heifers fed the high-gain diets are assumed attributable to the accumulation of extraparenchymal adipose, altered endocrine signaling between the fat pad and parenchyma, and/or altered extracellular matrix (ECM) composition, and not adipose infiltration into mammary parenchyma. Indeed, Meyer et al. (2004) documented that the heifers reared on the high-gain, versus the moderate-gain diet,

had larger whole gland mammary weights, which were attributable to the accumulation of extra-parenchymal adipose. The altered endocrine signaling theorized to coincide with this has not been investigated in these heifers; likewise neither have the constituents of the ECM.

As discussed earlier, the mammary gland is comprised of stromal and epithelial cells, which are known to communicate with each other through the ECM via instructive and permissive cellular signaling (Wiseman and Werb, 2002). More specifically, the mammary stroma helps to direct epithelial proliferation (Wiseman and Werb 2002). The ECM helps to organize cells into tissues and coordinates their cellular functions by activating intracellular signaling pathways that control such things as cell growth, proliferation, and gene expression. The ECM is composed of a variety of proteins and polysaccharides that are secreted into the local environment and are assembled into an organized meshwork that lies in close proximity to the surfaces of the cells that produce them (Alberts et al., 1994). With the use of immunochemical techniques it is possible to examine the abundance of selected proteins that constitute the ECM. In a recent study in our laboratory, Forrest (2003) used such techniques to evaluate the content of the selected ECM proteins laminin, fibronectin, and collagen IV in mammary tissue from Holstein heifers. He found that fibronectin staining intensity adjacent to subtending ducts and terminal ductular structures was increased in heifers fed a high- versus low-plane of nutrition from 2-8 wks of age. This difference is theorized to partially explain the impaired mammary growth observed in high- rather than low-fed heifers and may therefore serve as an indicator of impaired mammary development. That increased fibronectin deposition may be associated with impaired mammary development is supported by work by Berry et al. (2003a), where it was demonstrated that there was greater parenchymal fibronectin deposition in ovariectomized heifers versus intact heifers. Examination of the mammary parenchymal fibronectin deposition of the heifers in this study seems a likely next step to either supporting or refuting this theory. Tissues from this group of heifers would also be of interest in examining the distribution of ECM components for another reason. This is because we observed that the number of epithelial and luminal structures present in mammary parenchyma of the heifers in the current study increased with increasing bodyweight (Figures 3.1 and 3.2); these

measurements are believed to be indicators of mammary parenchymal tissue complexity. That is to say that with increasing bodyweight (maturity), there is undoubtedly more side-branching of mammary ducts to create the “ductular tree” of mammary epithelium that will eventually give rise to alveoli (see Figure 3.4 for representative images of mammary parenchymal complexity). As mammary epithelial tissue expands via ductal branching and elongation from the gland cistern to the more dorsal fat pad, the surrounding stroma (which includes fibroblasts, adipocytes, endothelial cells, and ECM components), must be remodeled. Since the tissues used in the present study came from a wide range of animals sacrificed at different bodyweights, they represent a chronological set of samples. Perhaps changes in ECM component deposition and relative abundance can be documented over time, irrespective of and/or in combination with plane of nutrition by using immunohistochemistry and Western blotting. Indeed, Berry et al. (2003a) implied a role for collagen, (likely collagen I) in regulating morphogenesis of ductular structures, possibly by reorganization to allow penetration of epithelial structures into the fat pad. This idea was based on immunohistochemical results showing that collagen was abundantly expressed in interlobular stroma and large ducts (inactively growing), but minimally expressed near actively growing terminal ductular units and intralobular stroma (Berry et al., 2003a). Berry et al. (2003a) also documented that the deposition of fibronectin, but not laminin, changed with developmental state. They observed that with increasing developmental state (prepubertal, postpubertal, and lactating animals), fibronectin deposition appeared to become increasingly more organized as a basement membrane around epithelial structures.

In summary, we found no histological differences in mammary parenchymal composition in tissues taken from Holstein heifers reared on one of two planes of nutrition and sacrificed at varying bodyweights. These findings suggest that impaired mammary development in heifers reared on high planes of nutrition is more affected by extra-parenchymal adipose accumulation, and/or altered endocrine signaling from the fat pad to the underlying parenchyma. However, further study will be required to clearly understand mechanisms responsible for alterations in peripubertal heifer mammary development.

Table 3.1. Summary of samples shipped to Virginia Tech from Cornell University.

Target Slaughter wt (kg)	Number Moderate Diet	Number High Diet	Number of samples available
45	0	0	0
100	0	0	0
150	5	3	8
200	6	6	12
250	6	6	12
300	5	5	10
350	6	6	12
Total	28	26	54

Table 3.2. Summary of actual slaughter weights for heifers.

Target ^a	Trt ^b	Actual ^c	SEM	n ^d
150	H	153.3	1.2	3
150	M	151.8	0.7	5
200	H	202.2	1.8	6
200	M	204.7	2.2	6
250	H	255.0	2.0	6
250	M	255.8	1.5	6
300	H	304.6	2.1	5
300	M	298.2	3.9	5
350	H	359.7	3.1	6
350	M	361.2	2.1	6

^a = Target slaughter weight.

^b = Dietary treatment; H = high (950 g/d ADG); M = moderate (650 g/d ADG).

^c = Actual slaughter weight mean for each group.

^d = number of animals in each group.

Table 3.3. Least-Squares Means of growth data ¹.

Item	Treatment		SEM
	Moderate ²	High ³	
Initial BW, kg	44.4	43.9	0.74
Initial age, d	9.4	10.3	0.70
Initial HH ⁴ , cm	82.9	82.8	0.51
Weight at weaning, kg	75.7 ^a	85.9 ^b	0.66
Length of milk fed phase, d	49.7 ^a	43.3 ^b	1.2
HH at weaning, cm	93.1 ^a	94.3 ^b	0.33
Pre-weaning weight gain, kg/d	0.64 ^a	0.96 ^b	0.015
Pre-weaning HH gain, cm/d	0.27 ^a	0.36 ^b	0.017
Lifetime weight gain, kg/d	0.66 ^a	0.93 ^b	0.009
Lifetime HH gain, cm/d	0.14 ^a	0.18 ^b	0.004

^{a,b} = Means with uncommon superscripts are different at $P < 0.05$.

¹ = Adapted from Meyer et al., 2004.

² = Moderate nutrient intake.

³ = High nutrient intake.

⁴ = Hip height.

Table 3.4. Diet dry matter and nutrient composition ¹.

Item	Diet		
	TMR 1	TMR 2	TMR 3
Ingredient, % of diet DM			
Alfalfa hay	10.3		
Alfalfa silage	15.1	31.8	31.8
Corn silage	13.8	31.8	31.8
Soybean meal			4.2
Heat treated soy bean meal	10.3	18.0	8.4
Starter grain mix ²	50.3		
High moisture corn		15.9	23.1
Rumensin 800		1.6	1.7
Vit. E supplement	0.12	0.10	
Mineral mix	0.12	0.9	1.4
Nutrient, % of diet DM			
CP	24.3	19.0	17.2
NEm, Mcal/kg ³	1.72	1.62	1.65
NEg, Mcal/kg ⁴	1.08	1.03	1.06
ADF	18.1	21.1	20.2
NDF	35.5	34.0	31.4
NFC ⁵	34.3	35.9	40.0
EE ⁶	1.91	3.93	3.45

¹ From Meyer et al., 2004.

² 27% CP textured starter.

³ Net energy for maintenance.

⁴ Net energy for gain.

⁵ Nonfiber carbohydrate.

⁶ Ether extract.

Table 3.5. Histological components of heifer mammary parenchyma for animals at various bodyweights.

Bodyweight (kg)	% Interlobular stroma		% Epithelium		% Lumen		% Intralobular stroma	
	M ^a	H ^b	M	H	M	H	M	H
150 (n = 5, 3) ^c	33.9 ± 5 ^{d,e}	28.2 ± 7	21.0 ± 3	18.7 ± 3	7.3 ± 1	10.1 ± 2	37.7 ± 4	42.9 ± 5
200 (n = 6, 6)	31.3 ± 5	24.6 ± 5	19.3 ± 2	19.3 ± 2	5.9 ± 1	7.5 ± 1	43.4 ± 4	48.5 ± 4
250 (n = 6, 6)	24.5 ± 5	35.4 ± 5	18.6 ± 2	18.4 ± 2	7.0 ± 1	6.5 ± 1	49.9 ± 4	39.6 ± 4
300 (n = 5, 5)	26.1 ± 5	29.9 ± 5	20.9 ± 3	20.3 ± 3	5.8 ± 1	6.8 ± 1	47.1 ± 4	42.8 ± 4
350 (n = 6, 6)	21.3 ± 5	22.9 ± 5	20.9 ± 2	20.0 ± 2	6.5 ± 1	7.6 ± 1	51.2 ± 4	49.3 ± 4
Diet totals	27.5 ± 2	28.2 ± 2	20.1 ± 1	19.3 ± 1	6.5 ± 1	7.8 ± 1	45.9 ± 2	44.6 ± 2
Grand means ^f	28		20		7		45	

^a = Moderate dietary treatment (650 g/d ADG).

^b = High dietary treatment (950 g/d ADG).

^c = Number of animals per weight group; Moderate, High diets respectively.

^d = Listed values are LS Means ± SEM.

^e = There were no differences in significance of main effects (dietary treatment and bodyweight) nor their interaction for the variables listed.

^f = Grand means for each variable across all dietary treatments and bodyweights.

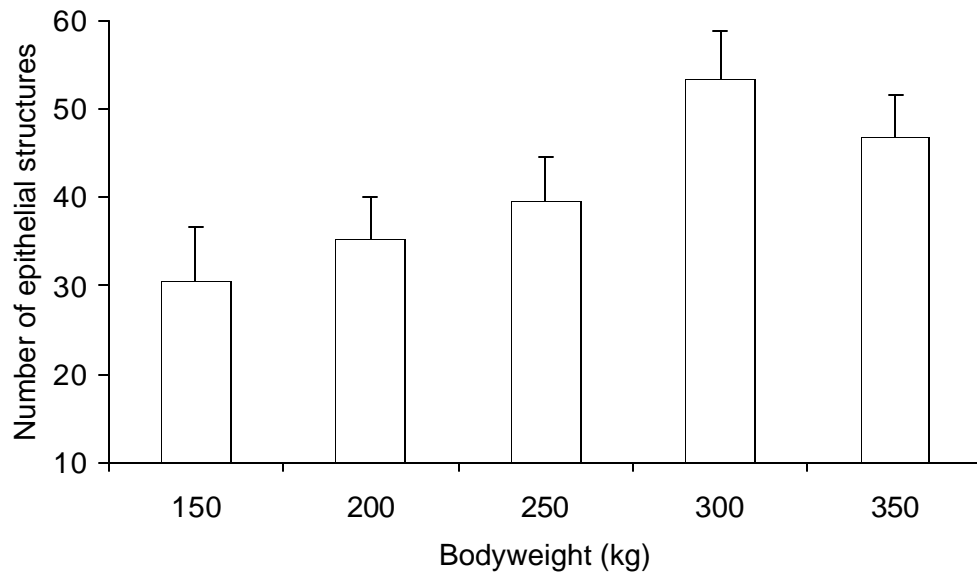


Figure 3.1. Number of epithelial structures present in an 871,820 μm^2 area of mammary parenchyma taken from heifers sacrificed at different bodyweights and reared on either a high or a moderate gain diet. Epithelial structures were counted on microscope slides stained with hematoxylin and eosin. Numbers of heifers per slaughter group were as follows: 150, n = 8; 200, n = 12; 250, n = 12; 300, n = 10; 350, n = 12. Values are LS means \pm SEM for heifers slaughtered at different bodyweights across all dietary treatments. The linear trend in epithelial structures was significant ($P < 0.005$).

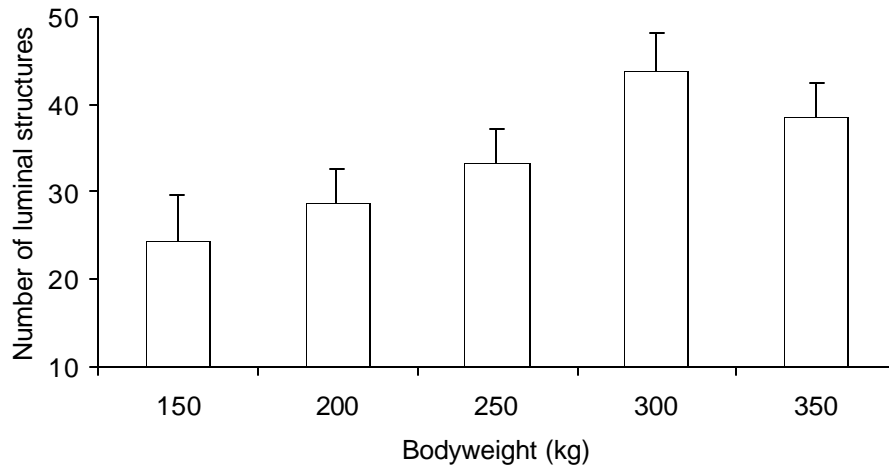


Figure 3.2. Number of luminal spaces present in an 871,820 μm^2 area of mammary parenchyma taken from heifers sacrificed at different bodyweights and reared on either a high or a moderate gain diet. Luminal structures were counted on microscope slides stained with hematoxylin and eosin. Numbers of heifers per slaughter group were as follows: 150, n = 8; 200, n = 12; 250, n = 12; 300, n = 10; 350, n = 12. Values are LS means \pm SEM for heifers slaughtered at different bodyweights across all dietary treatments. The linear trend in luminal structures was significant ($P < 0.005$).

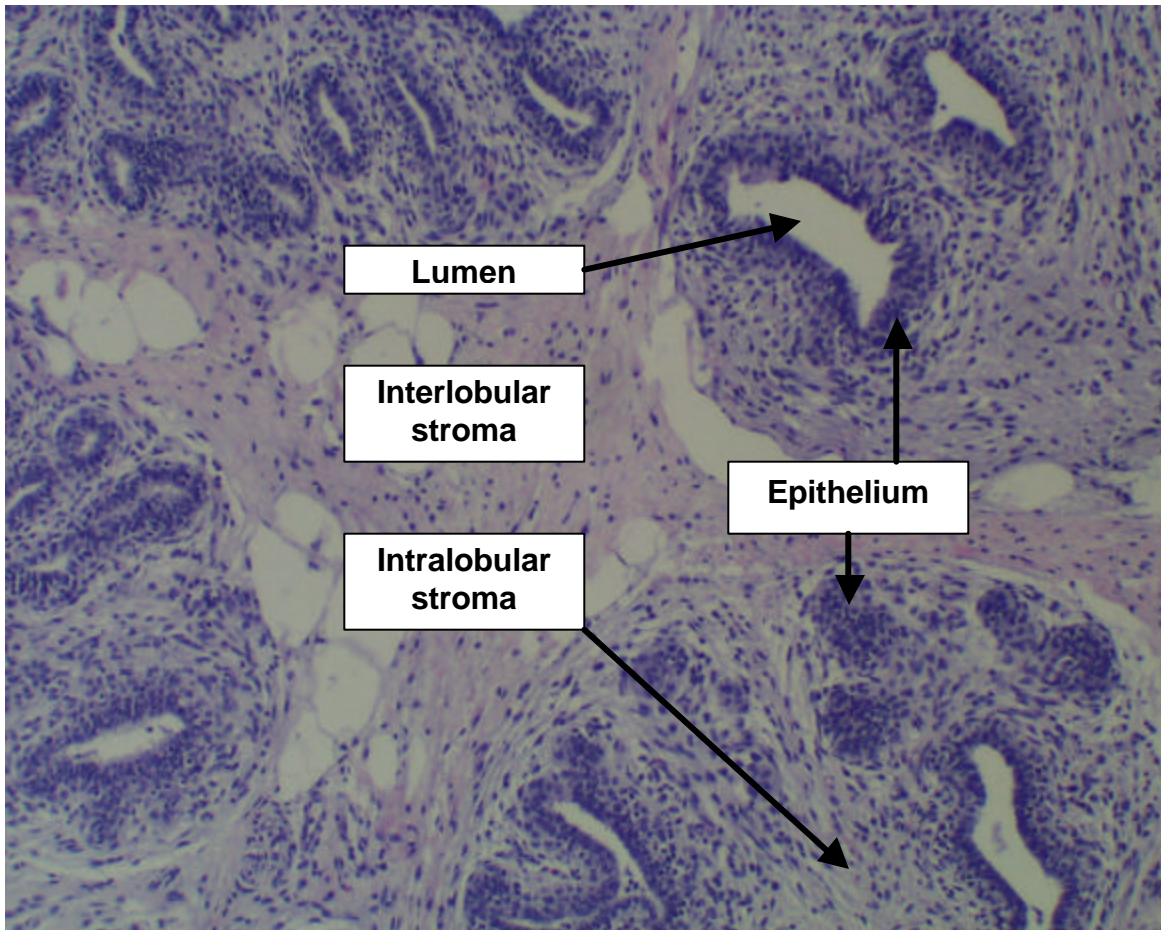
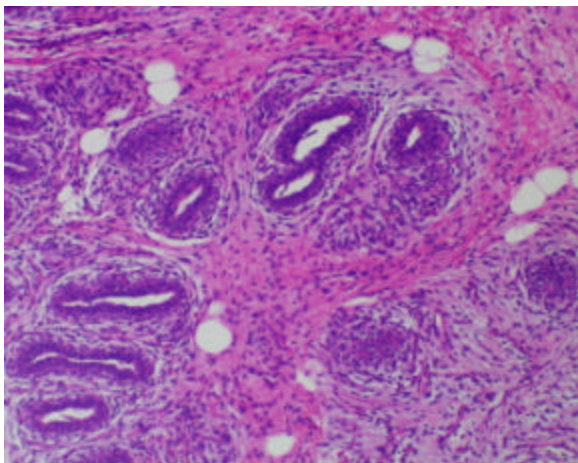
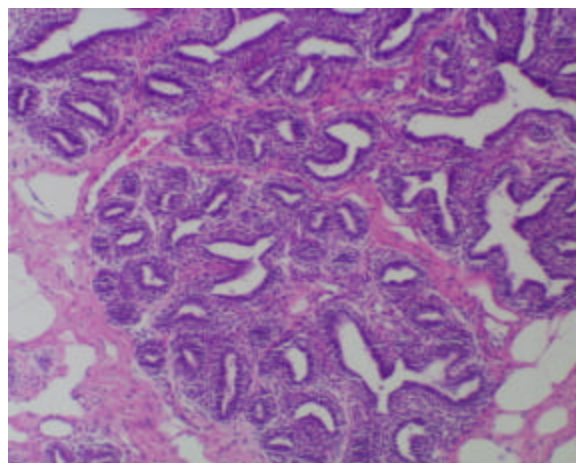


Figure 3.3. Representative image of heifer mammary tissue at 10x magnification stained with hematoxylin and eosin.



150 kg heifer



350 kg heifer

Figure 3.4. Demonstration of mammary gland parenchymal complexity at different heifer bodyweights. Both images are at 10x magnification and are stained with hematoxylin and eosin.

CHAPTER 4: A PROTEOMIC APPROACH TO EVALUATE THE EFFECTS OF BODYWEIGHT AND PLANE OF NUTRITION ON PROTEIN EXPRESSION PROFILES OF CYTOSOLIC MAMMARY GLAND EXTRACTS FROM HOLSTEIN HEIFERS

4.1 Introduction

Mammary development is critical to sustaining the productive life of a dairy cow. Reduced mammary gland development in prepubertal heifers raised on high planes of nutrition coincides with failure of adequate allometric growth of the mammary gland in the peripubertal period. Allometric growth occurs when a tissue grows at a faster rate than the rest of the body. Once a heifer reaches puberty between 9 and 11 mo of age (250 – 280 kg BW), the mammary gland returns to an isometric rate of growth, and negative effects of a high rate of feeding on mammary development are minimized (Sejrsen et al., 1982). Despite understanding effects of peripubertal feeding level on whole animal performance; the biochemical and cellular mechanisms governing alterations in heifer mammary gland development are poorly understood.

An unprecedented and novel approach to studying mammary development in dairy heifers is the use of proteomics, which is the study of the complete set of translated proteins in a given biological sample. The proteome, unlike the genome, is subject to change in response to experimental conditions, such as a high or moderate feeding level, and/or stage of development. To date, there have been no published reports on the construction of a two dimensional-PAGE (2D-PAGE) database of bovine mammary gland proteins from growing heifers. Aksu et al. (2002) however, described creation of a mouse mammary gland 2D-PAGE protein database containing more than 4250 total protein spots and 66 specifically identified protein spots. Regardless, proteomic experiments specific to dairy heifers are essential since relational maps for mouse proteins are ineffective in the identification of most of their bovine counterparts (Talamo et al., 2003).

The objectives of this experiment were 1) to create two-dimensional protein maps of mammary tissue extracts from heifers that were slaughtered at one of two different bodyweights and reared on one of two planes of nutrition and 2) to identify selected

individual proteins whose expression profiles change in response to increased bodyweight and/or plane of nutrition.

4.2 *Materials and Methods*

Animals and Treatments

The mammary tissue samples used in this study were a subset of samples from animals that were purchased at birth from Cayuga County, New York, and treated at Cornell University, as reported by Meyer et al. (2004). The objective of the Cornell study was to evaluate the effect of plane of nutrition from birth to 350 kg on body composition and mammary development in Holstein heifers. Animals (n = 24) were randomly assigned to one of two dietary treatments; within treatments, heifers were assigned to one of 2 slaughter points (200 or 350 kg BW, respectively). The 200 kg slaughter weight encompasses a physiological period when allometric mammary gland growth is predominant, and the 350 kg slaughter weight, a period of post-pubertal isometric growth of the mammary gland. These slaughter weights were based on the temporal pattern of mammary development observed by Sinha and Tucker (1969). Six heifers per dietary treatment were slaughtered at each bodyweight for a total of 24 heifers.

Heifers on the moderate gain (M) dietary treatment were fed a pre-weaning diet that consisted of a 22% CP and 20% fat (dry matter basis) milk replacer that was fed at 0.20 Mcal gross energy (GE) per kg of BW^{0.75}. Heifers on the high gain (H) dietary treatment were fed a pre-weaning diet that consisted of a 28% CP and 17% fat (dry matter basis) milk replacer that was fed at 0.32 Mcal GE per kg of BW^{0.75}. A 26% CP calf starter was offered to animals beginning at approximately 3 weeks on study. After weaning, which was completed by 7 weeks on treatment, all heifers were fed the 26% CP starter for two additional weeks. From weeks 10 to 13 of treatment, all heifers received TMR 1 (Table 4.1). Thereafter all heifers were fed TMR 2; heifers that exceeded 200 kg BW were fed TMR 3 (Table 4.1). Throughout the entire length of the study, heifers were weighed once weekly and the amount of feed offered was adjusted such that heifers on the M treatment could achieve 650 g, and heifers on the H

treatment could achieve 950 g of daily gain, respectively. From the initiation of treatment to 150 kg, heifers were housed in individual pens in the Teaching and Research Dairy Center Greenhouse at Cornell University. Upon reaching 150 kg, heifers were grouped in one pen and individually fed via a Calan gate system. Once heifers assigned to the 350 kg slaughter weight reached a bodyweight of 225 kg, blood was collected twice weekly via jugular venipuncture and plasma progesterone concentrations were determined. Progesterone concentrations above 1 ng/mL were interpreted as the heifer having a functional corpus luteum, and therefore pubertal.

Slaughter Procedure and Mammary Tissue Collection

Heifers were weighed before feeding on two consecutive days to monitor target rate of gain. The approximate age at each slaughter point (for moderate and high diet treatments, respectively) was: 200 kg, 235 and 141 d; 350 kg, 449 and 277 d. The decision to slaughter at a common bodyweight as opposed to a common age was based on observations that puberty is more closely associated with the former than the latter (Sejrsen, 1994).

Humane slaughter of each heifer was performed at the Department of Animal Science Abattoir at Cornell University using a captive bolt stunner followed by exsanguination. At slaughter, the udder of each heifer was bisected along the medial suspensory ligament. One half of the udder was used by Meyer at Cornell University to determine total amounts of parenchymal and extra-parenchymal tissue, and the effect of energy intake on the composition of both. Tissues harvested from the mid-parenchymal region of the second half of the udder were stored in a -20°C freezer until shipment. Frozen mammary tissue samples were shipped overnight on dry ice to Virginia Tech. Upon arrival samples were placed in a -80°C freezer until use.

Preparation of Mammary Gland Extracts

Mammary tissue extracts were prepared essentially according to Waksman et al. (1991). Briefly, 2 g of frozen mammary tissue was weighed and homogenized in a

polytron (Kinematica; Switzerland) for 15 s in a total volume of 10 mL 0.9% NaCl. Homogenates were gently rocked for 90 min at 4 °C on a Speci-Mix platform rocker (Thermolyne Corporation; Dubuque, IA) before being centrifuged at 10,000 x *g* at 4 °C for 20 min to remove cell debris. Supernatant was retained and centrifuged at 100,000 x *g* for 1 h. The resulting supernatant (essentially cytosol) was then filtered through 0.2 μm Acrodisc® syringe filters (Pall Corporation; Ann Arbor, MI) and stored in a series of sterile microfuge tubes at –80 °C for subsequent analyses.

Samples were thawed at room temperature and then desalted using Microcon Y-3 centrifugal filter devices (Millipore Corporation; Bedford MA). Specifically, 300 μL of mammary gland extract were placed in the sample reservoir and then the devices were centrifuged at room temperature at 14,000 x *g* for 50 min. Samples were processed in triplicate. The filter was then washed twice with 100 uL of deionized water (14,000 x *g*, room temperature for 30 min). The retentate was collected into a clean microfuge tube by flipping the reservoir over and centrifuging the device (1020 x *g*; 3 min). Two hundred μL of deionized water were then added to the retentates and triplicate samples were pooled into one final tube. Protein concentrations of the pooled aliquots were determined via the bicinchoninic acid (BCA) assay (Pierce; Rockford IL). Bovine serum albumin was used as the standard in BCA assays. Protein concentrations of extracts ranged from 2.2 to 12.2 μg/μL. Samples were stored at -80 °C.

Pouring Gradient Gels

Eight to 16% (total percentage of acrylamide plus crosslinker) gradient gels were used in proteomic experiments. Gradient gels are cast with acrylamide concentrations that increase from top to bottom so that pore size decreases as proteins migrate further into the gels. Use of gradient gels, as opposed to single percentage gels, allows small proteins to remain in the gels longer than they would in a single concentration gel and thereby allows for both small and large proteins to be resolved more easily in the same gel. Prior to pouring gradient gels, hinged spacer plates (20 x 20.5 (W X L) cm, 1.0 mm; Bio-Rad Laboratories; Hercules, CA) were sealed with sealant (see Appendix B) and placed in a PROTEAN® Plus Multi-Casting Chamber (catalog # 165-4160; Bio-Rad

Laboratories; Hercules, CA). Briefly, all components of the plate sealant gel, except for polymerization initiators Ammonium Persulfate (APS) and N,N,N,N'-Tetra-methylethylenediamine (TEMED) were mixed together in a small flask and aliquoted into a series of 9 microcentrifuge tubes. One tube was prepared at a time by adding APS and TEMED immediately prior to use, thereby preventing premature polymerization. The plate sealant was applied to both the hinged and non-hinged sides of the glass plates. The glass plates (gel cassettes) were placed into a multi-gel casting chamber per the manufacturer's instructions. A small piece of plastic tubing was then used to connect the casting chamber to a peristaltic pump, which itself was connected via tubing to a Model 495 Gradient Former (catalog # 165-4121; Bio-Rad Laboratories; Hercules, CA). Three gel solutions were then made, two that constituted the gradient: a light solution, and a heavy solution, and one, referred to as the syringe gel, that was used to fill the "dead space" in the casting chamber and to prime the pump lines (see Appendix B). The gel solutions were de-gassed for 3 min by stirring under low vacuum, and APS and TEMED were added within 1 min to the syringe gel flask; the pump lines were primed and 40 mL of the gel was sent to the casting chamber. Next, APS and TEMED were added to the light and heavy gel flasks prior to pouring the gel solutions into their respective chambers of the gradient former. After the gels were poured, they were overlaid with 1 mL of purified water, covered with plastic wrap, and allowed to polymerize overnight. The following day, the water overlay was drained from the gradient gels by tipping the entire casting chamber on its side; stacking gels (see Appendix B) were then poured directly on top of the gradient gels. Stacking gels contained a lower concentration of polyacrylamide than gradient gels, which created larger spaces in the gel matrices. The larger spaces allowed the various proteins, regardless of size, to flow through the stacking gels easily and "catch up" with each other. The proteins then began the molecular weight separation (second dimension of 2D-PAGE) in the gradient gels at the same time, from the same starting point, which improved resolution of the proteins.

First Dimension – Isoelectric Focusing

The first dimension of 2D-PAGE, isoelectric focusing (IEF), separates proteins in a sample on the basis of their isoelectric point (pI), which is the pH at which a protein carries no net charge and will not migrate in an electric field. The first dimension separation of proteins was achieved as follows: desalted cytosolic mammary gland extract samples (4 animals per proteomics run; triplicate samples) were thawed at room temperature and then mixed with running buffer (see Appendix B) so that 315 µg of total protein were contained in a final volume of 300 µL, which was then loaded into an IEF tray. An 18 cm 3 -10 nonlinear immobilized pH gradient (IPG) strip (ReadyStrip™; Bio-Rad Laboratories; Hercules, CA) was then placed on top of each sample gel-side down such that the acidic (+) side of the strip was aligned with the anode (red/+) of the IEF cell during rehydration and focusing; each strip was then overlaid with 3 mL of mineral oil to prevent evaporation during focusing. The IEF tray, which can accommodate up to 12 IPG strips at a time, was then placed in a PROTEAN® IEF cell (catalog # 165-4001; Bio-Rad Laboratories; Hercules, CA) and the gels were actively rehydrated for 13 h at 50 V, at a temperature of 20 °C. Focusing parameters for the subsequent electrophoresis run were as follows: start voltage of 0 V, end voltage of 10,000 V, 60,000 total Vh, rapid ramping, 20 °C, run time approximately 11 h. The focusing step allowed all proteins in the samples to migrate to their isoelectric point. Two-D SDS-PAGE Standards (Bio-Rad Laboratories; Hercules, CA) were also included as control samples in some of the IEF runs per the manufacturer's instructions.

Equilibration of IPG Strips

Prior to running the second dimension of 2D-PAGE, it is necessary to equilibrate IPG strips in a buffer that contains SDS to reduce disulfide bonds and alkylates, and also to uniformly coat all proteins so that they will have the same net charge and migrate through the second dimension gel based on size only. To do this, after isoelectric focusing was complete, the mineral oil was decanted, then, each strip was removed one at a time from the IEF tray and gently blotted on both sides with a

Kimwipe (Kimberly Clark Corporation; Roswell, GA) to further remove excess mineral oil. Each strip was then placed gel side up in the appropriate well of an equilibration tray. Strips were subjected to 3 one-half-hour equilibration washes at room temperature. This was achieved by overlaying each strip with the appropriate volume of each equilibration buffer (see Appendix B); the tray was then placed on an orbital shaker (Lab-Line, model 4626) set at low speed. Equilibration buffer was decanted after use by pouring the liquid from the square side of the equilibration tray.

Gel Loading and Second Dimension – SDS-PAGE

While the IPG strips were in their second equilibration wash, the casting chamber was disassembled and the 12 individual gel cassettes were removed and rinsed in reverse osmosis water and placed in an ANYGEL Stand (Bio-Rad Laboratories; Hercules, CA). After the third equilibration wash, IPG strips were rinsed one at a time in 1 x Tris, Glycine, SDS (TGS) buffer (see Appendix B) by holding the basic (-) side of the strip with forceps and dipping it 5 times into a graduated cylinder filled with the buffer. Each strip was then carefully mounted onto the stacking gel of a gel cassette and overlaid with 5 mL of liquefied agarose gel solution that contained bromophenol blue used as a tracking dye (see Appendix B). Molecular weight markers (Precision Plus Protein™ Standard Plugs, Unstained; Bio-Rad Laboratories; Hercules, CA) were added at this time in the space on the left hand side of the IPG strips. The 12 individual gel cassettes were then loaded into a PROTEAN® Plus Dodeca Cell (catalog # 165-4151; Bio-Rad Laboratories; Hercules, CA) containing 24 L of 1 x TGS buffer that was connected to a circulating water bath. Electrophoresis was performed at 20 to 25 °C at a constant voltage of 200 V. The power supply was turned off when the bromophenol blue dye front was within 2 cm of the edge of the gel cassettes (approximately 8 hours after start of run). The gel cassettes were then removed from the tank, and were set in racks. One by one, each gel cassette was cracked open with a small wedge and the used IPG strip, agarose gel, and stacking gel were carefully cut away and discarded. The gradient gel was then gently floated off the glass plate into fixing/washing solution (see Appendix B). After recovery, each gel was then placed into an individual staining/washing box filled with approximately 330 mL fixing/washing solution. The

boxes were then stacked and placed on an orbital shaker (Lab-Line, model 4626). After 30 min of shaking at low speed, the fixing/washing solution was removed and approximately 330 mL of 100% SYPRO[®] Ruby protein gel stain (Bio-Rad Laboratories; Hercules, CA) was poured onto each of the gels. The boxes were again covered and placed back on the orbital shaker for 14 h, after which time the stain was poured off and the gels were subjected to four, 1-h washes with fresh fixing/washing solution. Gels were kept in the 4th wash until imaging, which took place the next day. Protein gel stain was decanted after each use into its original containers, and the same 4 L of stain was used for all 2D-PAGE runs.

Imaging and Gel Storage

A Molecular Imager[®] FX Pro Plus[™] Multimager System (Bio-Rad Laboratories; Hercules, CA) was used to visualize each gel. The imaging device is capable of digitizing images of 2-D gels stained with florescent dyes via direct laser excitation. This imaging device interfaces with Bio-Rad PDQuest[®] software (Bio-Rad Laboratories; Hercules, CA), which was designed to specifically collect, store, interpret, and compare proteomics data. After digitized images of the gels were obtained, gels were individually packaged in thick plastic wrap with fixing/washing solution and stored at room temperature away from ambient light.

Image Analysis

After all raw images of the gels were incorporated into the PDQuest[®] program; they were cropped, filtered, and compiled into a Matchset. After manually landmarking several spots, specific features in the software program were used to perform spot detection, make gel-to-gel comparisons, and complete spot density measurements. Selected automated spot-matching actions were manually verified for accuracy. Matchset data (pI and Mw information, raw spot quantities, and normalized spot quantities) were exported as a file that was accessible through Microsoft Excel.

Statistical Analysis

Matchset data were analyzed in SAS (SAS Institute, Inc.; Cary, NC; Version 8.0). Initially, the GLM procedure was used to evaluate the effects of target slaughter weight and/or dietary treatment of heifers on both raw and normalized protein spot intensities (Appendix C). Subsequently, it was determined that the Mixed procedure (Appendix C) was better suited for the dataset as it handles fixed and random effects, and uses the correct error term without having to enter “test statements”. Although both raw and normalized spot quantities were initially evaluated, we focused on normalized spot quantities. This is justified because the procedure associated with the PDQuest[®] software program corrected for variation in spot size and intensity between gels that was not due to differential protein expression.

In the Mixed procedure of SAS, main effects of diet and slaughter weight, as well as the interaction between the two were tested. Heifer within the combination of diet and target weight was a random term. The dependent variable used in the final analysis was normalized protein spot quantity at each of 820 total protein spots. Diet was either moderate or high, and target slaughter weights were 200, or 350 kg. The following model statement was used: $Y_{ijkl} = \mu + D_i + W_j + (DW)_{ij} + H_{(ij)k} + E_{(ijk)l}$

where:

Y_{ijkl} = dependent variable (normalized protein spot quantity)

μ = overall mean

D_i = fixed effect of diet (moderate versus high, $i = 1,2$)

W_j = fixed effect of target weight (200 versus 350 kg, $j = 1,2$)

DW_{ij} = effect of interaction of diet and target weight

$H_{(ij)k}$ = random effect of heifer within the combination of diet and target weight ($k = 1, 6$ heifers within subclass)

$E_{(ijk)l}$ = residual error (sampling within heifer, $l = 1,3$).

Due to the exploratory nature of this research, differences were considered significant at $P < 0.20$; results are presented as LS means \pm SEM.

A list of all statistically significant protein spots was exported into a Microsoft Excel spreadsheet. Separate spreadsheets were made for spots that differed by diet only, weight only, diet x weight only, and by more than one of the previous effects.

Spots in the spreadsheet that differed by diet x weight only were further categorized by order of ascending LS Means (Table 4.4).

Method for Choosing Protein Spots to Excise for Identification

The spreadsheets that differed by diet and weight only were sorted by ascending *P* values. Spot numbers with the smallest *P* values were deemed the most interesting. The Matchset was then opened with the PDQuest® software program, and spots with the smallest *P* values were located and their presence/absence in member gels was verified manually. A total of 22 spots (*P* values 0.002 to 0.15) were chosen for excision and subsequent mass spectrometry analyses; these 22 spots were processed in one of two batches. The first batch contained 10 excised protein spots and the second batch contained 12 protein spots.

Preparation for Spot Cutting

Prior to spot cutting, it was necessary to re-stain selected gels because some loss of signal intensity occurred during storage of the gels. To do this, selected gels were removed from storage and individually placed into a fixing/staining box filled with approximately 330 mL of 100% SYPRO® Ruby protein gel stain and were left in the dark, overnight, on an orbital shaker (Lab-Line, model 4626) set at low speed. The next morning, the stain was removed, and the gels were washed with fixing/washing solution for 1h; this too was done in the dark and the gels were shaken on the orbital shaker for the allotted time.

On the morning protein spots were to be cut, 96-well non-sterile microtiter plates were rinsed with purified water and air-dried. Plates were then scanned in the Molecular Imager FX Pro Plus device to ensure there was no contamination. One hundred μ L of 0.1% acetic acid was then added to each well that was anticipated to be used in spot cutting, as this aided in the ejection of cored protein spots into the wells of the plate.

Individual protein spots tagged for excision were specified to be cut from manually generated lists that were imported into the PDQuest® software program from

Microsoft Excel. More specifically, arbitrary analysis sets were created in the Matchset of interest for each list of spots imported, using the “Analysis Set Manager” feature of the software program. After arbitrary analysis sets were created, the “Excision Gel Selection” tool was selected and in three steps, the proper analysis set to be used in cutting was selected, the selection mode was verified, and the cuts to be made were also verified. Then, the spot cutter and the camera of the Proteomeworks™ Spot Cutter (Bio-Rad Laboratories; Hercules, CA) were turned on, and the “Integrated Excision Tool” of the PDQuest® program was opened.

Setup for Cutting Gels

Purified water was placed in three of the four wash-cups of the spot cutter, so the tip of the spot cutter could be washed three successive times in between cutting spots. The aperture setting was set at 1.4 for ultraviolet imaging, and the white light was turned off. The proper, previously prepared 96 well plate was placed in its specified location on the platform in the spot cutter. A clean gel cutting sheet was placed on top of the cleaned cutting pad on the platform of the cutter. A line of purified water was placed along the bottom of the gel cutting sheet. With the laboratory lights dimmed to as low as practical, the proper gel to be cut was retrieved from the fixing/washing solution, rinsed by submersion in a container of purified water, and placed on the gel cutting sheet in the spot cutter. Metal bars were placed along the top and bottom edges of the gel to prevent movement during cutting, and a small amount of purified water was added to the surface of the gel. The door to the spot cutter was then closed and the lights in the laboratory turned back on.

Spot Cutting

In the “Integrated Excision Tool” of the PDQuest program, the light source was set to “UV” and the exposure time was set for 7 s. A new image of the gel was acquired and automatically aligned to the previously scanned image of the same gel. An interactive tool was then used that allowed the user to “jump and confirm” that the spots to be cut were indeed the spots that were desired to be cut. Cutting was then initiated

and was totally automated; a 1.5 mm internal diameter excision tip was used for cutting and only one core was taken from each protein spot of interest. On finishing, the 96-well plate containing the first batch of excised protein spots was removed from the cutting apparatus and the 96-well plate for the second batch of protein spots was placed on the apparatus. The plate from the first batch was again scanned in the Molecular Imager[®] FX Pro Plus[™] Multimager System, this time to verify the deposition of protein plugs into the specified wells of the plate. The first plate was then heat sealed and set aside. The spot cutting protocol was then immediately repeated for the second batch of protein spots, which were cut from the same gel as the first batch. After all spot cutting was complete, the gel that was subjected to spot excision was rescanned and its digital image was subsequently compared to the original scanned image of the gel to verify that the cutting program retrieved the correct protein spots. The sealed 96-well plates were then placed in a box, and stored in an -80 °C freezer until mass spectrometry analyses. Spot cutting data, including well locations of the cored protein spots were exported to a Microsoft Excel spreadsheet.

Protein Identification

The sealed 96-well plates were hand-delivered to the Core Laboratory Facility at the Virginia Bioinformatics Institute (VBI) on the campus of Virginia Tech on two separate occasions. On the first trip, the plate containing 10 protein spots was submitted; the plate containing the 12 other protein spots was processed approximately one week later. At VBI, each protein plug in the 96 well plates was subjected to trypsin digestion and mass spectrometry analysis in order to determine primary amino acid sequences of the proteins and then potential protein names. Once protein names and NCBI protein accession numbers were received from VBI, literature searches were conducted. Known functions and characteristics of proteins were documented along with possible roles in heifer mammary development.

4.3 Results

Actual slaughter weights of the animals and actual daily gains of the animals are discussed in detail by Meyer et al. (2004). Summaries of actual slaughter weights and actual daily gains for each of the treatment groups are displayed in Table 4.2 and Table 4.3 respectively.

A total of 7 2D-PAGE runs were conducted for this experiment; 71 total gels were analyzed in the Matchset. Sixty-six of those represented gels from heifers, while 5 represented standard gels. At least 2 replicate gels were required of each heifer for inclusion in the Matchset and gels from all 24 heifers were represented. Some heifers only had duplicate gels (8 heifers), there were triplicate gels for 14 heifers, and 2 heifers were represented in quadruplicate. This was because on the first 2D-PAGE run for these 2 heifers, only one gel from each heifer was suitable for inclusion in the Matchset; therefore triplicate samples for these 2 heifers were electrophoresed in a second 2D-PAGE run and all were included in the Matchset. It was necessary to include one more electrophoresis run than originally planned to include first dimension isoelectric point standards and re-runs. A representative image of a 2-D gel from this experiment is demonstrated in Figure 4.1. A total of 820 individual protein spots was statistically analyzed. Of these, 131 protein spots differed by diet only, 108 differed by heifer bodyweight only, 101 had a significant diet and heifer bodyweight interaction and 135 differed by more than one of these variables. A more detailed listing of the numbers of spots in each of the described groups is available in Table 4.4.

A total of 22 protein spots was excised from 2-D gels and submitted for mass spectrometry analysis (see Figures 4.2, 4.3, and Appendix D). Of the 22 protein spots submitted for mass spectrometry, 15 of them were differentially expressed between heifers reared on different dietary treatments, and 7 of them were differentially expressed according to heifer bodyweight (Table 4.5). Approximate molecular weights and isoelectric points for these particular spots are also listed in Table 4.5. Eighteen of the 22 proteins submitted for mass spectrometry were identified. Protein spots 3303 and 5602 were lost from the first microtiter plate during trypsin digestion. In the second batch, identities for protein spots 2606 and 7502 were inconclusive due to the strong presence of keratin contamination. Protein spot numbers and their corresponding protein names and NCBI protein accession numbers are listed in Table 4.6 for the first

batch, and Table 4.7 for the second batch of protein plugs submitted to VBI. Figure 4.4 is the Guassian Master Image annotated with the identified proteins.

4.4 Discussion

To our knowledge, a systematic proteomic analysis of peripubertal bovine mammary parenchyma cytosol has never been attempted or described before. Through our 2D-PAGE experiments we found that the protein composition of bovine mammary cytosol fluctuated according to the physiological and nutritional state of heifers. Furthermore, through mass spectrometry analyses, we identified several differentially expressed proteins that may one day be implicated in the nutritional and/or temporal control of mammary development. A discussion of protein spots and identified proteins follows.

Protein Spot 6707 - Fascin

Protein spot 6707 was identified as the protein fascin (Figure 4.4). Fascin is a cytoplasmic actin-bundling/crosslinking protein that organizes filamentous actin into bundles. The bundling of actin into polymers, coupled with the action of actin-crosslinking proteins is generally essential for cell migration. Cell migration, especially lateral migration of epithelial cells, is important for mammary development to progress. This is because during normal mammary development, at least in humans, epithelial cells migrate to the tips of growing ducts, and once adulthood is reached, this migration is repressed (Anbazhagan et al., 1998). Fascin expression is typically low in normal epithelial cells, but has been found to be upregulated in metastatic estrogen receptor-negative breast cancer (Grothey et al., 2000). Interestingly, IGF-I receptor (IGF-IR) signaling also contributes to the formation of mammary carcinomas (Resnik et al., 1998), and affects the actin cytoskeleton (Kadowaki et al., 1986). In 2002, Guvakova et al. showed that breast carcinoma cell colonies dispersed in a time-dependent manner upon IGF-IR activation by IGF-I, and that the dissociation involved the formation of fascin-containing lateral cell projections. These observations imply a role for fascin in heifer mammary development, especially in nutritionally directed mammary

development. Heifers reared on the H diet, irrespective of bodyweight, expressed nearly 1.8-fold more fascin protein than heifers reared on the M diet (Table 4.4). Serum IGF-I was generally more concentrated in heifers reared on a high feeding level (Vestergaard et al., 1995). It is therefore postulated that increased IGF-I serum levels in heifers reared on the H diet may have led to increased IGF-IR signaling, and therefore increased fascin protein expression, and ultimately cell migration.

Protein Spot 7704 – Fascin and Serum Albumin Precursor

Protein spot 7704 has been identified as the proteins fascin and serum albumin precursor (Figure 4.4). This protein spot was found to be more highly expressed in heifers on the H rather than M diet. Fascin was described in the previous paragraph and serum albumin precursor is a serum protein. Serum albumin is positively correlated to nutritional status. Because both proteins localized at this location and virtually the same numbers of peptide fragments were identified for each protein (data not shown), it is impossible to say which of these two proteins accounted for the variation in spot densities between diets.

Protein Spot 8301 – Guanine Nucleotide Binding Protein Beta Subunit Like Protein 12.3

Protein spot 8301 was identified as Guanine nucleotide binding protein beta subunit like protein 12.3 (protein 12.3) (Figure 4.4), which is apparently similar to the beta subunit of the trimeric G-protein complex. It is classified as a signal transduction modulator. Trimeric G-proteins (α , β , and γ subunits) are normally found on the cytoplasmic side of cell membranes and are known to couple with seven-transmembrane domain receptors when the receptor binds its natural ligand. The general signal-transduction pathway for G-protein coupled receptors is as follows: on binding of ligand, the receptor activates a nearby G-protein that in turn activates the enzyme adenylate cyclase. Adenylate cyclase generates the cytoplasmic second messenger cyclic adenosine monophosphate (cAMP). The increase in cAMP triggers a chain of reactions regulated by protein kinases, with the ultimate consequence of

evoking some intracellular response to the initial signal (Berg et al., 2002). Depending on the type of G-protein, adenylate cyclase is either stimulated, or inhibited to act. Known effectors of G-protein coupled receptors include many peptide hormones and catecholamines. Notably absent from this list of effectors though are IGF-I, GH, and estrogen, which all evoke cellular responses via other means. Known sub-types of G-protein coupled receptors include α_1 , α_2 , β_1 , β_2 , and β_3 . In the mammary gland of lactating cows, all subtypes are represented in the milk purging system, but hardly any are present in parenchyma (Hammon et al., 1994).

In general, milk removal is inhibited by the stimulation of α -adrenergic receptors (contraction of smooth muscle), and increased by the stimulation of β -adrenergic receptors (relaxation of smooth muscle) (Hammon et al., 1994). In the current study, heifers on the H diet expressed over twice the amount of protein 12.3 as compared to heifers reared on the M diet. Given that adrenergic receptors in general are not present in great abundance in mammary parenchyma of lactating cows, it was interesting to find that a protein similar to the β subunit of the G-protein complex was so differentially expressed in our animals reared on different diets. The β_3 subclass of adrenergic receptors is associated with energy expenditure and adipocyte lipolysis, but is also the least abundant of the β receptors in bovine mammary tissue (Inderwies et al., 2003). Based on our findings, it is interesting to speculate that this subclass of β -adrenergic receptors may be up-regulated in mammary parenchyma in heifers reared on a high, rather than moderate diet. We propose that increased β_3 -adrenergic receptor signaling in parenchymal adipocytes may cause increased lipolysis and thermogenesis in heifers reared on a high, rather than moderate diet. Our observations that mammary parenchyma cytosol from heifers reared on a high versus moderate diet contained differentially expressed levels of several heat-stress related proteins (see later) seems to correspond with our proposition.

Protein Spot 6902 – Transferrin

Protein spot 6902 was identified as transferrin (Figure 4.4). Transferrins can be synthesized by the liver and secreted as serum proteins and can also be locally

produced in, and secreted by the mammary gland as constituents of whey. Lee et al. (1987) documented that in virgin mice, small amounts of transferrin are locally produced by epithelial cells in the mammary gland and that its synthesis increases throughout gestation and lactation. Furthermore, and especially relevant to the current study, it has been documented that milk transferrin levels in lactating rats can be modulated by restricting either total food intake or the protein content of the diet, without altering serum levels of transferrin (Grigor et al., 1988).

Growing evidence suggests that transferrin synthesis is associated with growth and functional differentiation in tissues other than the liver. In *in vitro* studies using the bovine mammary epithelial cell line, MAC-T, Rejman et al. (1992) showed that MAC-T cells proliferate in the presence of transferrin. In primary cell culture experiments using sheep bladder smooth muscle cells, Weinzimer et al. (2001) demonstrated that transferrin and IGFBP-3 were both growth-potentiating, but when the two proteins were co-incubated with the cells, growth was dramatically reduced. This, along with other experiments, helped prove for the first time that transferrin is an IGFBP-3 binding protein (Weinzimer et al., 2001). Weinzimer et al. (2001) explained that the observed effects in cell culture are consistent with transferrin/IGFBP-3 binding with such affinity as to prevent either ligand from interacting with its own natural cell surface receptor (IGF-R and transferrin receptor). Despite these observations, the relationship between transferrin and the IGF/IGFBP-3 axis is still poorly characterized. In humans it is known that serum concentrations of transferrin are positively correlated with IGF-I and IGFBP-3 in pubertal girls (Wilson et al., 1991; Wilson et al., 1992). Also, at the molecular level, IGF-I has been documented to up-regulate the expression of cell-surface transferrin receptors, apparently by redistributing intracellular transferrin receptors to the cell surface (Davis et al., 1986; Davis et al., 1987; Lombardi et al., 1989). This last observation has prompted the speculation that the sequestration or presentation of IGF-I to the cell surface by IGFBP-3 may indirectly influence growth by regulating transferrin receptor density at the cell surface (Weinzimer et al., 2001).

Furthermore, in 1987, Chen et al. observed that transferrin expression in primary mouse mammary epithelial cells was greatly elevated *in vitro* on a reconstituted basement membrane matrix, as compared to plastic surfaces. This observation has

lead researchers to propose that changes in ECM structure and composition may also regulate mammary transferrin levels.

Due to our tissue separation protocol (tissue heterogeneity), and large sequence homology between the two mentioned types of transferrin, we were unable to distinguish if the divergent transferrin levels detected in the current experiment are serum or mammary derived in origin. However, it is likely that the transferrin detected is of mammary origin. This is largely based on our findings that transferrin levels were highest in heifers reared on the H as opposed to M diet. These results tend to agree with Grigor et al., 1988, based on the assumption that transferrin levels in mammary tissue are positively correlated with transferrin levels in milk.

An interesting experiment that comes to mind that would implicate both transferrin and the ECM as having roles in bovine mammary development would be an immunohistochemical experiment with mammary tissues from the heifers in the current study. Serial tissue sections could be stained for transferrin and ECM components, and their relative abundance could be quantified both over time and by heifer dietary treatment.

Protein Spot 8503 – Cytosolic Aspartate Aminotransferase

Protein spot 8503 was identified as cytoplasmic aspartate aminotransferase (Figure 4.4). The rat cytosolic aspartate aminotransferase gene is positively regulated by glucocorticoids and cAMP (Barouki et al., 1989; Aggerbeck et al., 1993), and by the administration of a protein-rich diet (Horio et al., 1988). In the current study, mammary parenchyma cytosolic aspartate aminotransferase levels were nearly 1.5-fold higher in heifers reared on the H diet, versus heifers reared on the M diet. The physiological role of cytosolic aspartate aminotransferase is considered to be to increase the supply of the metabolic intermediate, oxaloacetate, for subsequent use as a substrate for cytosolic phosphoenolpyruvate carboxykinase (Horio et al., 1988).

Protein Spot 1702 – A Wide Range of Proteins

Protein spot 1702 had sequence homologies with many proteins. They included: Rab GDP dissociation inhibitor alpha (GDI-1), serum albumin precursor, alpha-1-antiproteinase precursor, antithrombin-III precursor, vitamin D-binding protein precursor, and protein disulfide isomerase precursor (Figure 4.4). This protein spot was found to be up-regulated in heifers weighing 200, rather than 350 kg. Narrower pH range separations in the first dimension must be employed in future experiments to increase the resolution of individual proteins so that differential expression patterns of proteins may be more fully characterized. Based on the number of peptide sequences identified by mass spectrometry for each of these proteins, the relative abundance of proteins in spot 1702 was as follows: GDI-1 > serum albumin precursor = alpha-1-antiproteinase precursor = antithrombin-III precursor = vitamin D-binding protein precursor > protein disulfide isomerase precursor (data not shown). A brief discussion of each of the proteins in spot 1702 follows.

Rab GDP dissociation inhibitor alpha is a known cytosolic protein that is involved with vesicular transport. It regulates the shuttling of Rab proteins (monomeric GTPases) to and from cytosolic and membrane locations. It does so by acting as a sort of “molecular switch”, by alternating between two distinct conformations, the inactive (GDP-bound) state, and the active (GTP-bound) state. To demonstrate, insulin stimulation of glucose uptake by adipocytes involves the redistribution of glucose transporters from cytosolic organelles to the plasma membrane surface. Shisheva et al. (1994) documented that increases in GDI-1 mRNA and protein abundance were positively correlated with the appearance of plasma membrane glucose transporters, in insulin-treated test cells. Rab GDP dissociation inhibitor alpha is therefore implicated in this, and similar roles, in membrane trafficking.

Serum albumin is the main protein in plasma. Its main function is to regulate colloidal osmotic blood pressure, and is also known to bind water, calcium, sodium and potassium ions, fatty acids, certain hormones, and bilirubin.

Alpha-1-antiproteinase precursor is a precursor to a protein that inhibits the action of the pancreatic protease, trypsin, on a 1:1 molar basis. It is an important protein expressed predominantly during lactation that prevents the degradation of certain milk proteins so that the neonate may ingest them in their most complete form.

Data suggest that alpha-1-antitrypsin is produced locally by the human mammary gland, and that it might play a role in mammary development (Chowanadisai and Lönnnerdal, 2002). In the first high-throughput proteomic analysis of human breast infiltrating ductal carcinoma, Somiari et al. (2003) found alpha-1-antitrypsin to be dysregulated in tissue samples from human patients with this particular form of breast cancer. More specifically, they found expression levels of it to be lower in cancerous tissue than in normal tissue (Somiari et al., 2003).

Antithrombin-III precursor is an important precursor to the plasma protein antithrombin-III, which inactivates the blood-clotting factor, thrombin, by forming an irreversible complex with it (Berg et al., 2002). Antithrombin-III also blocks factors XII_a, XI_a, IX_a, and X_a, which are other proteases in the clotting cascade.

Vitamin D-binding protein precursor is a precursor for vitamin D-binding protein (DBP), which is a plasma protein that belongs to the albumin superfamily of binding proteins. Vitamin D-binding protein is predominantly synthesized in the liver and is secreted into blood plasma. Plasma levels of DBP have been known to increase by as much as 50% in high estrogen states (Haddad et al., 1995). A study conducted by Laing and Fraser (2002) also indicated that a protein- and energy-restricted diet was capable of reducing circulating DBP levels. To date however, no studies implicate DBP as having a role in mammary development of any species, but DBP is multifunctional, so there is still likelihood that it may have a role. Recently DBP has received much attention as a potential therapeutic agent in cancer treatment due to its anti-proliferative and anti-angiogenic properties. These same properties may apply to normal tissue development as well. For example, our heifers slaughtered at 200 kg expressed nearly 2 fold the amount of protein at spot 1702 when compared with heifers slaughtered at 350 kg. Perhaps some of this expression was from the Vitamin D-binding protein precursor and was a signal to slow down mammary cell proliferation and angiogenesis; reasons as to why this would be desired at a time where allometric mammary growth predominates are unclear. As stated previously, refined tissue dissection and protein separation techniques must be employed to further address this issue.

Protein disulfide isomerase precursor was identified elsewhere as being up-regulated in heifers weighing 200 kg (see next), it follows that it may have contributed to

the differences in expression levels for this particular spot as well; its presence in two locations on the 2D-gels may indicate post-translational modifications. The functions of protein disulfide isomerase (PDI) will be discussed in the next section.

Protein Spot 5705 – Protein Disulfide Isomerase A3 Precursor

Protein spot 5705 was identified as protein disulfide isomerase A3 precursor (ERp57) (Figure 4.4), a member of the PDI family. All proteins of the PDI family are present in the endoplasmic reticulum (ER) lumen and ERp57 has also been found in cytosol (Lewis et al., 1986). In the ER, PDIs serve as chaperones, and are therefore involved with the proper folding of proteins, and disulfide bond formation in proteins that are destined to be secreted or incorporated into the cell membrane (Turano et al., 2002). However, in the cytosol their role is less clear; they may maintain the same functions as ER-bound PDIs, but have uniquely been shown to become complexed with STAT3 (protein responsible for signal transduction and transcription regulation) in the cytosol of liver cells (Ndubuisi et al., 1999). A proposed role for mammary cytoplasmic Erp57 has yet to be made, as is the explanation as to why expression levels of it would differ by heifer bodyweight. In the current study we found the expression levels of ERp57 to be approximately 1.5-fold higher in 200 kg, rather than 350 kg heifers. This could possibly be related to temporal gene expression and/or, a stress response due to the accumulation of nascent proteins in the cytosol.

Protein Spot 8108 – A Wide Range of Proteins

Protein spot 8108 had sequence homologies with many proteins. They included: early E2A DNA-binding protein, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), and glutathione S transferase class mu (GST-mu) (Figure 4.4). This protein spot was found to be up-regulated in heifers weighing 200, rather than 350 kg. As previously mentioned though, narrower pH range separations must be employed in future experiments to further separate proteins so that their differential expression patterns may be more fully examined. A brief discussion of each of the proteins found to comprise spot 8108 follows.

Early E2A DNA-binding protein is a single-stranded DNA-binding protein of adenovirus type 4 (Kitchingham, 1985). It is a known nuclear protein in nature that is involved in binding single-stranded DNA in a sequence-independent manner. The reasons for its detection in the cytosol remain unclear.

The cytoplasmic enzyme HGPRT catalyzes one of the first steps in the salvage pathway for the purine bases hypoxanthine and guanine in mammalian cells; it therefore catalyzes the recovery of preformed bases for use in cellular metabolism (Caskey and Kruh, 1979).

Glutathione S transferases (GST) are cytoplasmic proteins known to protect cells from oxidative stress. There are at least 5 classes of GSTs (alpha, mu, pi, theta, and sigma) (Hayes and Pulford, 1995). The biological control of the classes of GSTs is difficult to study and compare, as they demonstrate sex-, age-, tissue-, species-, and tumor-specific patterns of expression (Hayes and Pulford, 1995). The class detected in this protein spot was class mu. Many xenobiotic and endogenous compounds are capable of causing oxidative stress. Glutathione S transferases help protect cells by metabolizing oxidative stressors. The metabolism of many such compounds is biphasic. In the first phase, enzymes metabolically oxidize xenochemicals, and in so doing, form electrophilic intermediates (Guengerich and Shimada, 1991) that are subsequently degraded by phase II enzymes. This process thereby protects cells from oxidative DNA damage. Glutathione S transferase is classified as a phase II detoxification enzyme. In a study by Ansell et al. (2004), it was reported that estrogen exposure down-regulated total GST activity in the uteri, but not mammary glands, of female adult virgin mice. Down-regulation of phase II enzymes promotes increased oxidative DNA damage, and could possibly then, promote cancer. In the current study, the protein expression at spot 8108 was increased in 200 kg (prepubertal) heifers. Perhaps this is a reflection of down-regulation of GST-mu in postpubertal heifers by increased amounts of circulating estrogen.

Protein Spot 7801 – Actin Interacting Protein 1 and Fibrinogen Gamma-B Chain Precursor

Protein spot 7801 was identified as actin interacting protein 1 (AIP1), and fibrinogen gamma-B chain precursor (see discussion on protein spot 2607) (Figure 4.4). Actin interacting protein 1 is a member of the WD repeat family of proteins, and therefore contains highly conserved repeating units usually ending with the amino acids tryptophan (W) and aspartic acid (D) (Ono et al., 2001). Actin interacting protein 1 collaborates with actin-depolymerizing factor/cofilin to enhance filamentous actin disassembly and thus cytoskeleton remodeling (reviewed in Ono, 2003). Cytoskeletal remodeling is required for processes such as cell division, cell motility, endocytosis, and morphogenesis. In our experiment, heifers on the H diet expressed over 2-fold more protein at spot 7801 as compared to heifers reared on the M diet. Perhaps some of the differential expression is attributable to AIP1, which would suggest that heifers reared on high diets undergo more mammary cytoskeletal remodeling, possibly in efforts to proliferate or move, than do heifers reared on moderate diets.

Protein Spot 2607 - Fibrinogen Gamma-B Chain Precursor

Protein spot 2607 was identified as fibrinogen gamma-B chain precursor. The final fibrinogen protein product is composed of many globular subunits and has a molecular weight of 340 kDa. It is a plasma protein involved in the final step of the blood clotting cascade, namely the conversion of it into fibrin by the proteolytic enzyme thrombin (Berg et al, 2002). In our experiment, heifers reared on a high, rather than moderate diet expressed more of this fibrinogen subunit precursor.

Protein Spot 2402 – Multiple Proteins

Protein spot 2402 had sequence homologies to annexin VIII, glandular kallikrein K24 precursor, and elongation factor Tu (Figure 4.4). The regulation of the expression of annexin VIII has been studied in humans with acute promyelocytic leukemia (APL; clonal proliferation of abnormal promyelocytes) (Sarkar et al., 1994). Sarkar et al. (1994) found that annexin VIII has an approximate molecular weight of 36 kDa and that it is mainly located within the plasma membrane and to a much lesser extent, the cytoplasm. Its expression seems to be tissue specific, and is normally expressed in the

placenta and lung, but not blood plasma (mammary tissue was not included in Northern blot analyses) (Sarkar et al., 1994). Interestingly, *all-trans* retinoic acid has been used to treat patients with APL, and it has been shown that complete clinical remission can be achieved through maturation of APL cells (Huang et al., 1988; Chomienne et al., 1990; Castaigne et al., 1990; Warrel et al., 1991). The relevance of this is that annexin VIII mRNA and protein expression in NB4 cells (derived from an APL patient) is diminished upon treatment with *all-trans* retinoic acid (Chang et al., 1992; Sarkar et al., 1994). The true biological function of annexin VIII is unclear, but based on its cellular localization, it is thought to have a role in cell membrane function such as, cell-cell communication, ECM formation, and/or signal transduction. In patients with APL, treatment with *all-trans* retinoic acid seems to exhibit its therapeutic effects by increasing the differentiation rate of undifferentiated cells; low levels of annexin VIII protein coincides with the process.

In our experiment, heifers reared on the high diet expressed nearly 2-fold more protein at spot 2402 than heifers reared on the moderate diet. Furthermore, our approximated molecular weight for this protein spot was 37.6 kDa, which agrees with the estimates for annexin VIII. In this view, low levels of annexin VIII in heifers reared on moderate diets may correspond to increased cellular differentiation as well, and would therefore support the standing hypothesis that heifers reared on high diets have impaired mammary development.

Glandular kallikreins are glycoproteins; due to varying amounts of glycosylation and different patterns of internal cleavage, they have molecular weights of 25-40 kDa. Kallikreins are related to trypsin and other serine proteases (reviewed by Mason et al., 1983). Kallikreins appear to play important roles in processing a wide range of hormone and peptide growth factor precursors (Mason et al., 1983). Kallikrein-24, which was detected in our experiment, was identified due to its shared sequence homology with mouse kallikrein-24. In the mouse, there are 25 known kallikrein genes, all of which encode a pre-proenzyme (zymogen) (reviewed by Clements et al., 2004). The physiological activators and actions of these zymogens are unknown. Kallikrein expression levels in human prostate, ovarian, and breast cancers are currently being studied, where they are emerging as useful prognostic indicators of disease progression

(reviewed by Clements et al., 2004). In our experiment, heifers reared on the high diet expressed nearly 2-fold more protein at spot 2402 than heifers reared on the moderate diet. Furthermore, our approximated molecular weight for this protein spot was 37.6 kDa, which agrees with the estimated range for kallikrein proteins. Heifers reared on a high plane of nutrition that express increased amounts of kallikrein protein when compared to contemporaries reared on a moderate plane of nutrition, may be doing so to activate pools of hormones and growth factors related to nutritional status.

The detection of a peptide with shared sequence homology with bacterial elongation factor Tu is probably due to the fact that for this particular protein spot, not many peptide sequences were encountered with great frequency, or length, as to definitively characterize them. Thus, the relatively uninformative peptide found (-.AGENCGVLLR.-), happens to share some sequence homology with the plant bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC300 (Buell et al., 2003).

Protein Spot 6701 – Aldehyde Dehydrogenase 1A1

Protein spot 6701 was identified as the cytosolic enzyme aldehyde dehydrogenase 1A1 (ALDH1) (Figure 4.4). The enzyme ALDH1 plays a critical role in vitamin A (retinol) metabolism, by aiding in the conversion of vitamin A to its bioactive metabolite, retinoic acid. Retinoic acid has been shown to inhibit IGF-I stimulated bovine mammary epithelial cell proliferation in culture (Woodward et al., 1996; Purup et al., 2001, Cheli et al., 2003). Retinoic acid may inhibit growth by increasing the local secretion of certain IGF-BPs, which could modulate the growth promoting effects of IGF-I. Increased ALDH1 activity is presumed to contribute to the active pool of vitamin A metabolites, and therefore possibly, the inhibition of IGF-I stimulated mammary growth. In the current study, ALDH1 protein expression was increased in heifers reared on the high rather than moderate diet, which is consistent with past hypotheses that increased planes of nutrition impair heifer mammary development. Further studies involving the retinoid/IGF axis are needed.

In the preceding paragraphs retinoic acid was discussed for its abilities to lower cellular annexin VIII protein levels upon administration to patients with APL. Increased ALDH1 expression in heifers reared on high diets is presumed to increase retinoic acid

abundance, retinoic acid in turn is thought to lower levels of annexin VIII protein. We found annexin VIII protein to be increased, not decreased, in heifers reared on high diets. Clearly, the relationship between retinoic acid, ALDH1, and annexin VIII may not be as straightforward as was hoped. The immediate aim of future experiments in this area should be to determine if annexin VIII is truly differentially expressed in heifers reared on different planes of nutrition.

Protein Spot 3505 – 26S Proteasome Regulatory Subunit p40.5

Protein spot 3505 was identified as a regulatory subunit of the 26S proteasome (p40.5) (Figure 4.4). To understand possible roles of this particular regulatory subunit, a brief review of non-lysosomal protein catabolism is in order. In the cytosol, abnormally folded and short-lived proteins are subjected to degradation by a process that requires ATP and ubiquitination of the protein in question. Ubiquitin is a protein that can be thought of as a tag for destruction, and the more ubiquitin molecules attached to a single protein, the more effective the signal for destruction. Ubiquitin covalently binds to the protein destined to be degraded. Upon arrival to the cytosolic 26S proteasome, the ubiquitin-protein complex is subjected to proteolysis. The 26S proteasome is itself composed of two components, a 20S proteasome that contains catalytic activity, and a 19S regulatory subunit that binds to polyubiquitin chains and thus regulates the entry of ubiquitin-tagged proteins into the 20S proteasome (Berg et al., 2002). The protein identified in the current experiment is a subunit of the 19S regulatory subunit of the 26S proteasome. Its function is not fully understood, but the disruption and characterization of its yeast homolog, non-ATPase subunit 7 (Nas7p), has been examined by Hori et al. (1998). Disruption of the Nas7p gene in yeast was not lethal, but prevented proliferation of affected cells at 37 °C, suggesting that the function of the 26S proteasome is impaired under heat stress (Hori et al., 1998). Furthermore, the calculated molecular weight and isoelectric points for human p40.5, 49.2 kDa, and 5.46, respectively, as reported by Hori et al. (1998), are very similar to the approximated values reported here of 45.5 kDa and 5.28.

In the current study, heifers on the moderate diet expressed almost 1.2-fold more of this regulatory subunit than heifers on the high diet. The reasoning for this is

speculative at best, but may reflect a response to heat stress in animals reared on the high diet.

Protein Spot 7102 – Proteasome Subunit Beta Type 2

Protein spot 7102 was identified as proteasome subunit beta type 2 (Figure 4.4). This particular subunit of the 20S component of the 26S proteasome is known to have endopeptidase activity similar to that of chymotrypsin, and a calculated molecular weight of 22.8 kDa (Nothwang et al., 1994). Through our 2D-PAGE experiments, we found this protein to have an approximate molecular weight of 22.9 kDa, and an isoelectric point of 6.97. We also found that heifers on the high diet expressed nearly 1.3-fold more of this subunit than heifers reared on the moderate diet, however the relevance of this to heifer mammary development is completely unknown. As with the previously discussed 26S proteasome regulatory subunit p40.5, expression of this component of the 26S proteasome may correlate to a stress response in animals reared on a high plane of nutrition.

Protein Spot – 7101 Glutathione S Transferase, Class Pi

Protein spot 7101 was identified as glutathione S transferase, class pi (GST-pi) (Figure 4.4). Glutathione s transferases were described earlier in the discussion of protein spot 8108. They help cells diminish oxidative stress by metabolizing harmful compounds. Huang et al. (2003) have recently found that GST-pi-positive infiltrative ductal breast carcinomas are more aggressive and have a poorer prognosis than do corresponding GST-pi-negative tumors in women. This implies the persistent presence of an oxidative stressor in tumors that demonstrate up-regulated GST-pi activity. In this light, GST-pi expression is positively correlated with mammary cell proliferation. In our experiment, heifers reared on the high diet expressed more GST-pi than did heifers on the moderate diet ($P < 0.15$). This may then correlate to increased cell proliferation in heifers on the high diet, which is contrary to past hypotheses and our own *in vitro* findings. More likely, it may reflect that heifers reared on high planes of nutrition are

exposed to more oxidative stressors than heifers reared on moderate planes of nutrition. Further studies in this area are needed.

Protein Spot 2403 – Complement C3 Precursor [Contains: C3a Anaphylatoxin]

Protein Spot 2403 was identified as complement C3 precursor (Figure 4.4). From its amino acid sequence, it was noted that it contains C3a anaphylatoxin. C3a anaphylatoxin is a mediator of local inflammatory processes. C3 helps activate the complement system; synthesis of it is increased during acute inflammation (Alexander et al., 1978). Humans with homozygous C3 deficiency often suffer from recurrent pyogenic infections such as pneumonia and septicemia; the absence of C3 is frequently lethal (Alper et al., 1976; Roord et al., 1983). In our experiment, heifers weighing 200 kg expressed over 2-fold more complement C3 precursor than heifers weighing 350 kg. Perhaps the tissue remodeling that results from allometric mammary growth in 200 kg heifers is also correlated with acute inflammation, and C3 production is increased to combat it.

Protein Spot 5605 - Selenium Binding Protein 1

Protein spot 5605 was identified as the 56 kDa cytosolic protein selenium binding protein 1 (SBP56) (Figure 4.4). Selenium binding protein 1 was originally discovered as a protein that binds exogenously administered radioactive selenium (Bansal et al., 1989; Bansal et al., 1990). The exact physiological role of this protein has not been well characterized. Porat et al. (2000) suggest that SBP56 participates in intra-golgi vesicular transport. Miyaguchi (2004) has suggested that SBP56 may be involved in the initial sequential events in rapid cell growth, such as determining direction of cell outgrowth and recruitment of actin monomers. Observations by Flietakis et al. (2002) suggest that SBP56 expression varies at different developmental stages. This is consistent with our findings that SBP56 protein expression was higher in 350 kg animals, rather than 200 kg animals. Furthermore, Chen and others (2004) reported that low levels of SBP56 expression were present in human lung tumors with high overall proliferation status. This last observation also tends to agree with our findings

that SBP56 protein expression was higher in animals in an isometric (slower growth rate; 350 kg BW), rather than allometric period (faster growth rate; 200 kg BW) of mammary growth. The role of SBP56 in mammary development needs further investigation.

Protein Spot 4601 – Serum Albumin Precursor and Actin-like Protein

Protein spot 4601 was identified as serum albumin precursor and actin-like protein (actin2) (Figure 4.4). Serum albumin was discussed previously so it will not be discussed here. Actin2 was first isolated in 1992 by Tanaka and others. The authors reported that it is distantly related to other known actin cytoskeletal components, sharing 36% identity to mammalian actins, and 60% identity to the yeast actin-like protein. Actin2 is implicated in the control of actin polymerization. E8CASS cells (MCF7 human breast cancer cell variant) respond to estrogens by decreasing their proliferation rate and undergoing cell death (Sonnenschein et al., 1994). In an experiment designed to identify genes that may be involved in estrogen-induced regression, Szalei et al. (2000), conducted a search for genes that turned on in response to estradiol treatment in E8CASS cells. Through their efforts, they identified mRNA that shared 96% homology with the bovine actin2 gene coding region, and 100% identity to the bovine actin2 protein. Therefore, bovine actin2 may respond to estrogen in a similar manner. In our experiment, heifers weighing 200 kg expressed more protein at spot 4601 than did heifers weighing 350 kg. This observation seems to imply that 200 kg heifers exhibit more estrogen-induced depression of cell proliferation, than 350 kg heifers. However, at a time of allometric mammary growth, this idea seems implausible.

General Discussion – Laboratory Methods

Since this was the first large-scale proteomics experiment conducted with bovine peripubertal mammary parenchyma cytosol, we chose to use laboratory products that separate a broad range of proteins. For instance, the immobilized pH gradient strips that we used for our first dimension separation covered a pH range from 3-10; narrower range strips are available and should be used in the future to increase the resolution of

individual protein spots. We also used gradient gels in our second dimension separation; single percentage gels should be considered for use in future experiments.

Cellular heterogeneity seemed to be an issue in our experiment due to the detection of several plasma proteins such as albumin and fibrinogen precursors. In the future, tissue and/or cell separation protocols leading to more homogenous samples should be used. Possible options for this include utilizing centrifugal columns to remove large molecular weight plasma proteins or laser capture microscopy to isolate individual cells of interest.

We detected a total of 820 proteins in our experiment. It has been brought to our attention recently that this is probably an underestimate. The reason for this is the stain that we used, Sypro Ruby, is light-sensitive. We stained our gels and exposed them to ambient light for approximately 2 days before scanning them; there was undoubtedly some loss of signal intensity during this time that may have lead to the omission of several low abundance proteins in our analyses.

The second batch of protein plugs that was processed by VBI contained keratin contamination, which is a major concern with proteomics experiments, due to the sensitivity of mass spectrometry equipment. All possible measures were taken in handling of samples to not introduce contaminants, but it appears some contamination was present. Keratins of various varieties were detected in nearly the entire second batch of samples, but only once in the first. Since both batches of samples submitted to VBI were cut on the same day, from the same gel, and processed in the same manner, we are confident that the keratin was an artifact of gel handling and did not contribute to the differential protein expression of the proteins in the second batch.

General Discussion – Future Research Pursuits

The establishment of a Holstein heifer mammary parenchyma cytosol proteomic map offers virtually unlimited discovery potential and can serve as a launch point for further studies of heifer mammary development. Many proteins identified here can readily be examined further in our laboratory either through Western and/or ligand blot analyses, *in vitro* cell culture experiments, or immunohistochemistry experiments, to name a few. These approaches will help us to better understand the relative

abundance of, ligands for, mitogenic properties of, and cellular localization of proteins of interest, respectively.

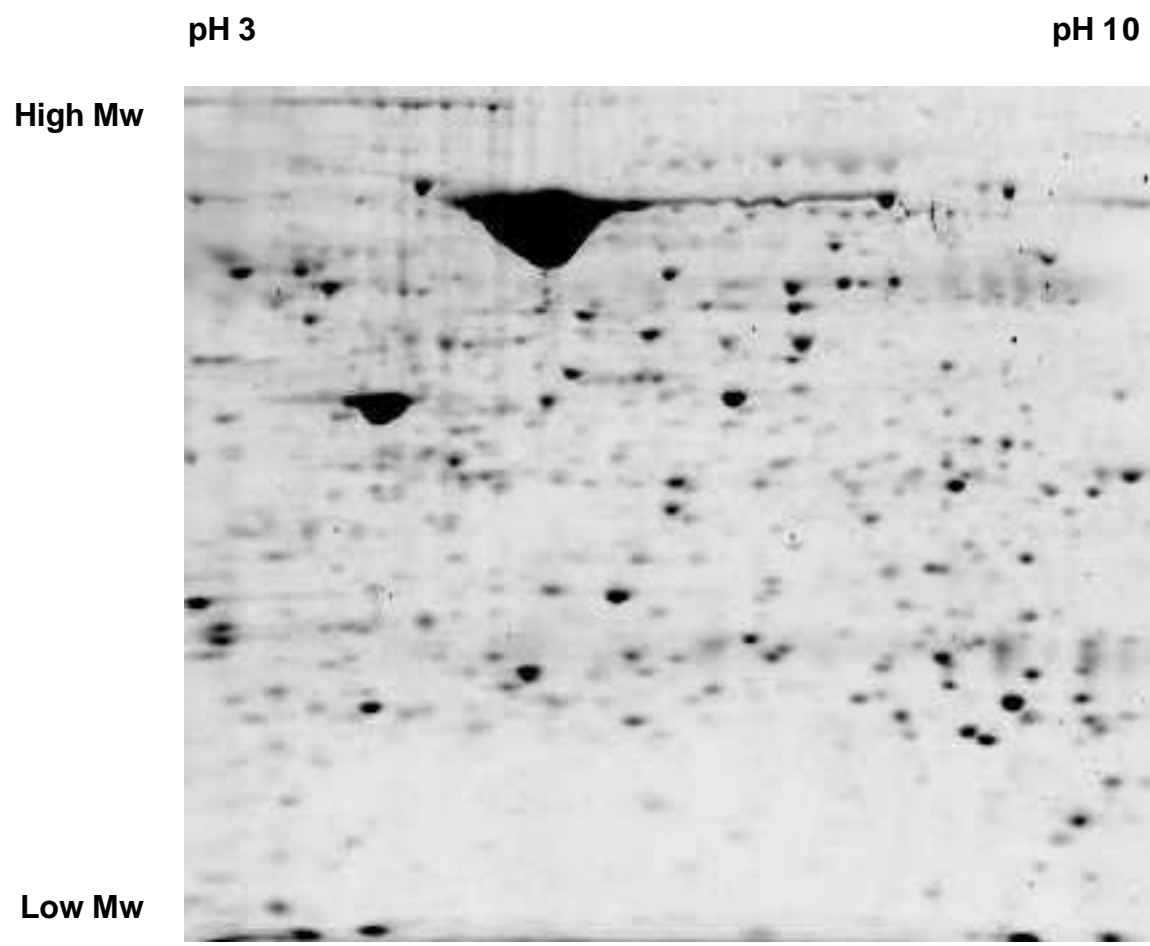


Figure 4.1. Representative image of heifer mammary parenchyma cytosol separated on a 2D gel and stained with Sypro Ruby.

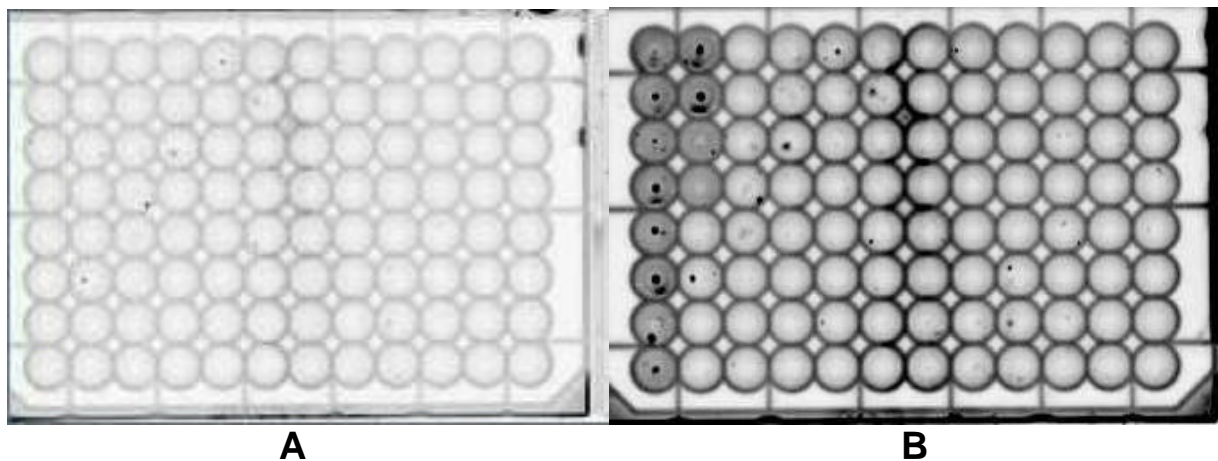


Figure 4.2. Representative scanned images of microtiter plate before (A) and after (B) spot cutting.

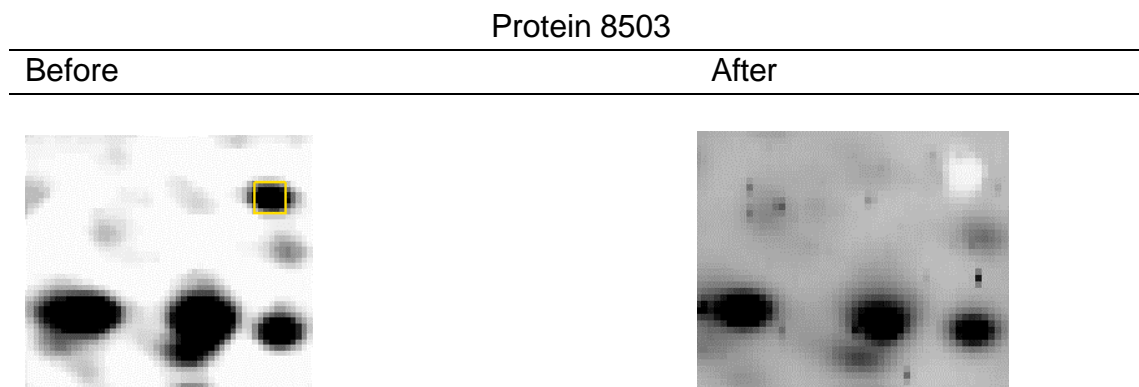


Figure 4.3. Before and after images of a protein spot excised from a 2D gel with an automated spot cutter. The yellow box in the before image is outlining the spot to be excised.

Table 4.1. Diet dry matter and nutrient composition ¹.

Item	Diet		
	TMR 1	TMR 2	TMR 3
Ingredient, % of diet DM			
Alfalfa hay	10.3		
Alfalfa silage	15.1	31.8	31.8
Corn silage	13.8	31.8	31.8
Soybean meal			4.2
Heat treated soy bean meal	10.3	18.0	8.4
Starter grain mix ²	50.3		
High moisture corn		15.9	23.1
Rumensin 800		1.6	1.7
Vit. E supplement	0.12	0.10	
Mineral mix	0.12	0.9	1.4
Nutrient, % of diet DM			
CP	24.3	19.0	17.2
NEm, Mcal/kg ³	1.72	1.62	1.65
NEg, Mcal/kg ⁴	1.08	1.03	1.06
ADF	18.1	21.1	20.2
NDF	35.5	34.0	31.4
NFC ⁵	34.3	35.9	40.0
EE ⁶	1.91	3.93	3.45

¹ From Meyer et al., 2004.

² 27% CP textured starter.

³ Net energy for maintenance.

⁴ Net energy for gain.

⁵ Nonfiber carbohydrate.

⁶ Ether extract.

Table 4.2. Summary of actual slaughter weights for 200 and 350 kg heifers.

Target ^a	Trt ^b	Actual ^c	SEM	n ^d
200	H	202.2	1.8	6
200	M	204.7	2.2	6
350	H	359.7	3.1	6
350	M	361.2	2.1	6

^a = Target slaughter weight.

^b = Dietary treatment; H = high (950 g/d ADG); M = moderate (650 g/d ADG).

^c = Actual slaughter weight mean for each group.

^d = number of animals in each group.

Table 4.3. Least-Squares Means of growth data for 200 and 350 kg slaughter weight heifers¹.

	Treatment		SEM
	Moderate ²	High ³	
Initial BW, kg	98.5	98.2	3.41
Initial Age, d	10.1	10.5	1.33
Initial HH ⁴ , cm	82.8	83.1	1.06
Weight at weaning, kg	75.3 ^a	86.5 ^b	1.17
Length of milk fed phase, d	38.3	32.9	1.96
HH at weaning, cm	93	94.3	0.61
Prewaning wt gain, kg/d	0.64 ^a	0.96 ^b	0.027
Prewaning HH gain, cm/d	0.28	0.35	0.028
Lifetime wt gain, kg/d	0.66 ^a	0.94 ^b	0.017
Lifetime HH gain, cm/d	0.13 ^a	0.17 ^b	0.006

^{a,b} = Means with uncommon superscripts are different at $P < 0.05$.

¹ = Adapted from Meyer et al., 2004.

² = Moderate nutrient intake.

³ = High nutrient intake.

⁴ = Hip height.

Table 4.4. Summary of spots analyzed in proteomics experiments.

820 Total number of spots analyzed

475 Number of spots with $P < 0.20$ differences (diet | weight)

131 differed by diet only

50 expressed more in heifers on moderate-gain diet

81 expressed more in heifers on high-gain diet

108 differed by weight only

67 expressed more in heifers weighing 200 kg

41 expressed more in heifers weighing 350 kg

101 differed by diet X weight interaction only

8	lowest to highest expression:	200M ^a – 350H ^d – 200H ^b – 350M ^c
20"	"	200M – 350H – 350M – 200H
8"	"	200H – 350M – 200M – 350H
15"	"	200H – 350M – 350H – 200M
10"	"	350M – 200H – 200M – 350H
12"	"	350M – 200H – 350H – 200M
11"	"	350H – 200M – 200H – 350M
17"	"	350H – 200M – 350M – 200H

135 differed by more than one of the above

44 diet and weight

23 weight, and diet X weight interaction

32 diet, and diet X weight interaction

36 diet, weight, and diet X weight interaction

^a= 200 kg BW, Moderate-gain diet.

^b= 200 kg BW, High-gain diet.

^c= 350 kg BW, Moderate-gain diet.

^d= 350 kg BW, High-gain diet.

Table 4.5. Characteristics of and differences in normalized quantity of selected bovine mammary parenchyma cytosolic proteins separated by 2D-PAGE and subsequently subjected to mass spectrometry analysis.

Protein	Batch ¹	Est Mw ²	Est pl ³	Main Effect	Norm Qty ± SEM ⁴		P ⁵
					Dietary Trt ⁶		
					M	H	
3303	1	36.4	5.23	Trt	166 ± 29	306 ± 27	0.002
6707	1	72.6	6.32	Trt	1089 ± 174	1872 ± 170	0.004
7704	1	72.9	6.93	Trt	661 ± 140	1297 ± 136	0.004
8301	1	33.4	-1.00 ⁷	Trt	439 ± 119	931 ± 117	0.008
7801	2	103.0	6.69	Trt	187 ± 49	391 ± 43	0.007
6902	1	138.8	5.98	Trt	1057 ± 301	2138 ± 290	0.018
8503	1	46.1	-1.00 ⁷	Trt	154 ± 24	227 ± 23	0.042
2607	2	60.6	5.09	Trt	1169 ± 244	1863 ± 241	0.06
2402	2	37.6	4.88	Trt	358 ± 74	550 ± 73	0.08
6701	2	72.8	5.81	Trt	611 ± 118	913 ± 114	0.08
2606	2	63.3	5.05	Trt	468 ± 100	714 ± 98	0.09
7102	2	22.9	6.97	Trt	758 ± 84	967 ± 80	0.09
7502	2	50.4	-1.00 ⁷	Trt	229 ± 44	331 ± 41	0.11
3505	2	45.5	5.28	Trt	333 ± 25	279 ± 24	0.14
7101	2	24.0	6.73	Trt	202 ± 34	273 ± 33	0.15
					Bodyweight (kg)		
					200	350	
1702	1	76.0	-1.00 ⁷	Wt	1842 ± 173	1086 ± 174	0.006
5602	1	60.6	5.65	Wt	1922 ± 159	1265 ± 160	0.009
5705	1	75.5	5.70	Wt	2813 ± 249	1836 ± 251	0.01
2403	2	42.6	4.93	Wt	441 ± 77	211 ± 77	0.05
8108	1	24.6	-1.00 ⁷	Wt	804 ± 83	565 ± 83	0.05
5605	2	67.4	5.77	Wt	471 ± 132	850 ± 133	0.06
4601	2	60.0	5.41	Wt	706 ± 80	496 ± 81	0.08

¹ = Batch 1 was processed before batch 2.

² = Estimated molecular weight of listed protein, kDa.

³ = Estimated isoelectric point of listed protein, pH.

⁴ = Normalized quantity of protein plus or minus standard error of the means.

⁵ = Significance was declared at $P < 0.20$.

⁶ = Dietary treatment was either moderate (M) or high (H).

⁷ = Computer program unable to return an accurate estimate.

Table 4.6. Components of protein spots determined by mass spectrometry (Batch 1).

Protein spot	Protein components of spot	NCBI Protein Accession Number (s)	Organism (s) matched
3303 ¹	----	----	----
6707	Fascin	2498357, 2498358	Ho ⁵ , Mu ⁸
7704	Fascin	2498358, 2498357	Mu, Ho
	Serum albumin precursor (<i>Allergen Bos d 6</i>) (BSA)	113582, 1351907, 113576	O ⁹ , B ² , Ho
8301	Guanine nucleotide binding protein beta subunit like protein (<i>Receptor of activated protein kinase C</i>) (RACK) (protein 12.3)	3023849, 121027	D ³ , Ho
6902	Transferrin (<i>Siderophilin</i>) (<i>Beta-1-metal binding globulin</i>) (Serotransferrin precursor)	2501351	B
8503	Aspartate aminotransferase, cytoplasmic (<i>Transaminase A</i>) (<i>Glutamate oxaloacetate transaminase-1</i>)	112975, 112973, 416566	Mu, E ⁴ , B
1702	Rab GDP dissociation inhibitor alpha (<i>Rab GDI alpha</i>) (<i>GDI-1</i>)	48428025, 47117855	P ¹⁰ , Mu
	Serum albumin precursor (<i>Allergen Bos d 6</i>) (BSA)	1351907	B
	Alpha-1-antitrypsin precursor (<i>Alpha-1-antitrypsin</i>) (<i>Alpha-1-proteinase inhibitor</i>)	112890	O
	Antithrombin-III precursor (<i>ATIII</i>)	416622	O
	Vitamin D-binding protein precursor (<i>DBP</i>) (<i>Group specific component</i>)	139641	Ho
	Protein disulfide isomerase precursor (<i>PDI</i>) (Prolyl 4-hydroxylase beta subunit) (Cellular thyroid hormone binding protein) (p55)	2507460	Ho
5602 ¹	----	----	----
5705	Protein disulfide isomerase A3 precursor (<i>Disulfide isomerase ER-60</i>) (<i>ERp60</i>) (<i>58 kDa microsomal protein</i>) (p58) (<i>ERp57</i>)	729433, 130232	B, Mu
8108 ^a	Early E2A DNA-binding protein	118735	Ha ⁶
	Hypoxanthine-guanine phosphoribosyltransferase (<i>HGPRT</i>) (<i>HGPRTase</i>) (<i>HPRT B</i>)	1708288	Mu
	Glutathione s transferase (<i>GST CLASS -MU</i>)	232206	Me ⁷

^a = keratin was detected in sample; results not reported.

¹ = protein spot was damaged during preparation for mass spectrometry analysis and was subsequently not included in further analyses.

² = *Bos taurus* (cow).

³ = *Danio rerio* (zebrafish).

⁴ = *Equus caballus* (horse).

⁵ = *Homo sapiens* (human).

⁶ = Human adenovirus type 4.

⁷ = *Mesocricetus auratus* (golden hamster).

⁸ = *Mus musculus* (house mouse).

⁹ = *Ovis aries* (sheep).

¹⁰ = *Pongo pygmaeus* (orangutan).

Table 4.7. Components of protein spots determined by mass spectrometry (Batch 2).

Protein spot	Protein components of spot	NCBI Protein Accession Number (s)	Organism (s) matched
7801 ^a	WD-repeat protein 1 (<i>Actin interacting protein 1</i>) (<i>AIP1</i>)	12230747	Mu ⁷
	Fibrinogen gamma-B chain precursor	120140	B ²
2607 ^a	Fibrinogen gamma-B chain precursor	120140, 1346007	B, R ¹⁰
2402	Annexin A8 (<i>Annexin VIII</i>)	12585137	Mu
	Glandular kalikren K24 precursor (<i>Tissue kallikrein 24</i>) (<i>mGK-24</i>)	48428341	Mu
	Elongation factor Tu (<i>EF-Tu</i>)	37999596	P ⁹
6701 ^a	Aldehyde dehydrogenase 1A1 (<i>Aldehyde dehydrogenase, cytosolic</i>) (<i>ALDH class 1</i>) (<i>ALDHIII</i>) (<i>ALDH-E1</i>)	118494, 42558919, 1706388	E ⁴ , Ma ⁶ , O ⁸
2606 ^{1a}	-----	-----	-----
7102	Proteasome subunit beta type 2 (<i>Macropain subunit C7-1</i>) (<i>Multicatalytic endopeptidase complex subunit C7-1</i>)	9910832	Mu
7502 ^{1a}	-----	-----	-----
3505 ^a	26S proteasome non-ATPase regulatory subunit 13 (<i>26S proteasome regulatory subunit S11</i>) (<i>26S proteasome regulatory subunit p40.5</i>)	20978558	H ⁵
7101	Glutathione s transferase P (<i>GST Class-PI</i>)	121744, 121746	B, H
2403	Complement C3 precursor [Contains: C3a anaphylatoxin]	116594	H
5605 ^a	Selenium binding protein 1 (<i>56kDa selenium-binding protein</i>)	6094240, 134259	H, Mu
4601	Serum albumin precursor	113576, 1351907	H, B
	Actin-like protein (<i>Actin-2</i>)	30581042	D ³

^a = keratin(s) were detected in sample; results not reported.

¹ = major protein identified in sample was keratin; results not reported.

² = *Bos taurus* (cow).

³ = *Drosophila melanogaster* (fruitfly).

⁴ = *Equus caballus* (horse).

⁵ = *Homo sapiens* (human).

⁶ = *Macaca fascicularis* (crab-eating macaque).

⁷ = *Mus musculus* (house mouse).

⁸ = *Ovis aries* (sheep).

⁹ = *Pseudomonas syringae* pv. Tomato.

¹⁰ = *Rattus norvegicus* (Norway rat).

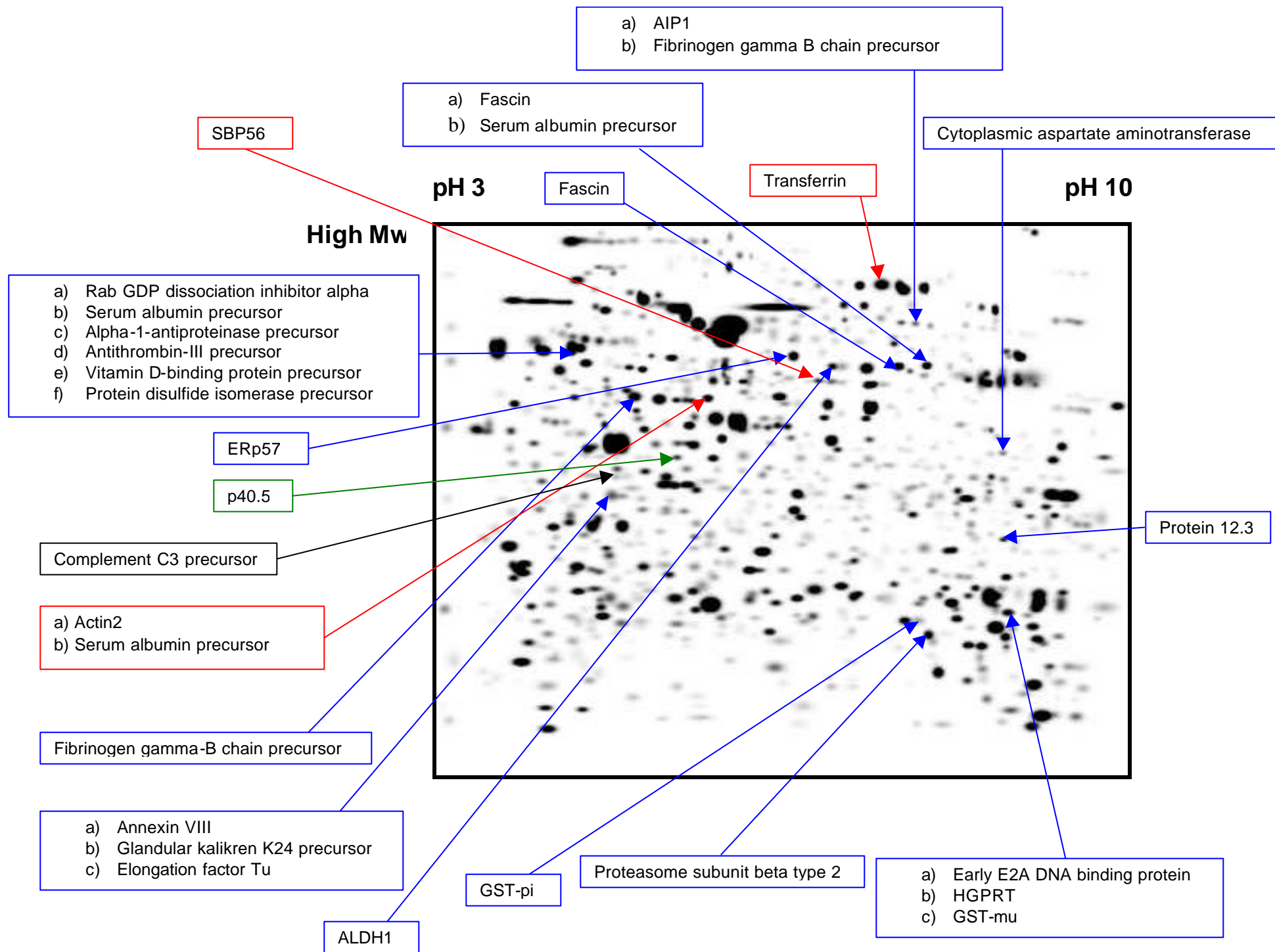


Figure 4.4 Master Image from Matchset with protein annotations.

Conclusions, Implications, and Future Perspectives

The objective of our first experiment was to test the mitogenic activity of MGE prepared from heifer mammary tissues, as affected by plane of nutrition and bodyweight. Through our cell culture experiments with MAC-T cells, we showed that contrary to previous reports, and our own hypotheses, maintaining Holstein dairy heifers on high-gain diets throughout the peripubertal period did not have deleterious effects on mammary development. We concluded this based on a modest and generally non-significant effect of diet, irrespective of bodyweight from 150 to 350 kg, on the proliferative response of MAC-T cells, for all variables i.e. parenchymal mass basis, a protein basis, or a DNA basis.

In the second experiment we sought to determine effects of rate of gain and body weight on histological development of the udder with a focus on changes in terminal ductal structures (TDU) in Holstein heifers. We found that there were no statistically significant differences in percent parenchymal composition either over time, as reflected by heifer bodyweight at slaughter, or by dietary treatment. These results agree with Sejrsen et al. (1982) and Forrest (2003). However, the complexity of mammary development increased with time as reflected in the increase in lumen spaces in older animals.

Based on the experiments conducted thus far with mammary tissues from this specific group of heifers, there seem to be no ill effect nor advantageous biological effects of maintaining heifers on high planes of nutrition (target lifetime bodyweight gain of 950 g/d) throughout the peripubertal period. Thus, any unknown, and therefore untested indicators of inhibited mammary growth in heifers fed the high-gain diets are assumed attributable to the accumulation of extraparenchymal adipose, altered endocrine signaling between the fat pad and parenchyma, and/or altered ECM composition, and not adipose infiltration into mammary parenchyma. These issues need to be addressed further.

In the third experiment we sought to 1) to create two-dimensional protein maps of mammary tissue extracts from heifers that were slaughtered at one of two different bodyweights and raised on one of two planes of nutrition and 2) to identify selected

individual proteins whose expression profiles change in response to increased bodyweight and/or plane of nutrition. Through the use of 2D-PAGE, we were able to isolate and statistically analyze the abundance of 820 total protein spots. Of the 820 spots analyzed, 131 protein spots differed by diet only, 108 differed by heifer bodyweight only, 101 had a significant diet and heifer bodyweight interaction, and 135 differed by more than one of these variables. A total of 22 protein spots was excised from 2-D gels and submitted for mass spectrometry analysis. Of the 22 protein spots submitted for mass spectrometry, 15 of them were differentially expressed between heifers reared on different dietary treatments, and 7 of them were differentially expressed according to heifer bodyweight. Through mass spectrometry analyses, database searches for primary amino acid sequence homology, and literature searches, the identities and biological functions of them were revealed. Their possible roles in mammary development were described. In summary, mechanisms governing nutritional and temporal control of mammary development are complex and require further investigation.

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APPENDIX A

BUFFERS AND SOLUTIONS USED IN MAC-T EXPERIMENTS

Table A1. Components of DNA Buffer used to determine DNA content of mammary homogenates.

10 x DNA Buffer *	
Tris Base	12.1 gm
EDTA-Na ₂	3.7 gm
NaCl	58.4 gm
pH to 7.4 with	Concentrated HCl
Water	to 1 L

* Dilute 1 to 10 to prepare working buffer.

Table A2. Components of dye used to determine DNA content of mammary homogenates.

Concentrated Stock Dye Solution (1 mg/mL in water) *	
Hoechst Dye	10 mg
Water	to 10 mL

* Add 100 μ L Concentrated Stock Dye to 490 μ L water to get working solution.

APPENDIX B

BUFFERS AND GEL SOLUTIONS USED IN PROTEOMIC EXPERIMENTS

Table B1. Components of running buffer used in first dimension electrophoresis of heifer mammary gland extracts.

Urea	12.2 gm
Thiourea	3.8 gm
CHAPS (50%)	2 mL
CA (Biolyte) (3-10)	50 μ l
Purified Water	to 25 mL
TBP (200 mM)	250 μ L*
DTT	0.192 gm*

* add just prior to use.

Table B2. Components of equilibration buffers used between the first and second dimensions of 2D-PAGE.

Equilibration Buffer I *	
Urea	72 gm
20% SDS	40 mL
2 M, pH 8.8 Tris HCl	37 mL
Glycerol	40 mL
Purified Water	to 200 mL
Equilibration Buffer II *	
Equilibration Buffer I	50 mL
DTT	1 gm
Equilibration Buffer III *	
Equilibration Buffer I	50 mL
Iodoacetamide	1.2 gm

* Quantities listed are enough to equilibrate 12 IPG strips.

Table B3. Components of Agarose gel used to mount IPG strips onto stacking gels prior to second dimension electrophoresis.

Agarose	0.5 gm
10 x TGS Buffer	10 mL
Purified Water	to 100 mL

Heat solution (100-150 °C) until it clears
add Bromophenol Blue to desired color

Table B4. Tris, Glycine, SDS (TGS) Buffer used in second dimension electrophoresis.

10 x TGS Buffer (dilute to get 1 x TGS)

Tris, Crystallized Free Base	60.57 gm
Glycine	288.26 gm
SDS	20 gm
Purified Water	to 2 L

Table B5. Components of fixing and washing solution used after second dimension electrophoresis runs.

Methanol	10% *
Glacial Acetic Acid	10% *
Purified Water	80% *

* volume/volume.

Table B6. Components of gels used in proteomic experiments.

Gel Component	Volume (mL), unless noted otherwise ^a				
	Plate Sealant	Syringe	Light	Heavy	Stack
Acrylamide/Bis (30%)	3.34	13.3	68.5	137.1	10
2 M Tris (pH 8.8)	1.8	9.4	48.2	48.2	
2 M Tris (pH 6.8)					4.69
SDS (20%)		5	25.7	25.7	7.5
Purified Water (100%)	4.54	22	113.2	19	50.86
Glycerol (100%)				25.7	
APS (10%) ^b	15 uL ^d	250 uL	1150 uL	1150 uL	250 uL
TEMED (100%) ^c	3 uL ^d	10 uL	60 uL	60 uL	26 uL
Total Volume	10	50	257	257	75

^a Listed quantities are enough to cast 12 gradient gels and 12 stacking gels.

^b Ammonium Persulfate.

^c N,N,N,N'-Tetra-methyl-ethylendiamine.

^d Add this quantity to each milliliter of plate sealant gel aliquoted into a microfuge tube (see Chapter 3).

APPENDIX C

SAS CODE USED IN PROTEOMIC EXPERIMENTS

C1. GLM Procedure with weight statements (rejected in favor of Mixed Procedure).

```
*Proteomics spot analyses;
options ls=100 ps=75 nodate nonumber;
dm 'clear log';
dm 'clear output';
data raw;
infile 'C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\dealing with statistics\NEW cornell heifers for
SAS.prn';
input ssp heifer$ wt$ trt$ qty normqty;
data raw; set raw;
    *the following are MW and pI marker spots;
    if ssp=0014 then delete;
    if ssp=0210 then delete;
    if ssp=0410 then delete;
    if ssp=0606 then delete;
    if ssp=0711 then delete;
    if ssp=0809 then delete;
    if ssp=0810 then delete;
    if ssp=0908 then delete;
    if ssp=0909 then delete;
    if ssp=1111 then delete;
    if ssp=2412 then delete;
    if ssp=4602 then delete;
    if ssp=4604 then delete;
    if ssp=4608 then delete;
    if ssp=6210 then delete;
    if ssp=6708 then delete;
    if ssp=7006 then delete;
    if ssp=7311 then delete;
run;
data raw; set raw;
proc sort; by ssp heifer wt trt;
*proc print; *run;
proc means NOPRINT;
    var qty normqty;
    by ssp heifer wt trt;
    OUTPUT OUT=newspots MEAN=meanqty meannorm VAR= varqty varnorm N= n_qty
n_norm;
    RUN;
*proc print data=newspots; *run;
proc sort data=raw; by ssp;
proc glm data=raw NOPRINT OUTSTAT=var_out;
    by ssp;
    class heifer;
    model qty normqty =heifer/ss3;
run;
```

```

*proc print data=var_out; *run;
data var_qty; set var_out;
  if (_name_='qty') and (_source_ = 'ERROR');
  msqty= ss/df; * = mean square error for qty;
  keep ssp msqty;
  run;
*proc print data=qty_out; *run;
data var_normqty; set var_out;
  if (_name_='normqty') and (_source_ = 'ERROR');
  msnormqty= ss/df;
  keep ssp msnormqty; * = mean square error for normqty;
  run;
*proc print data=normqty_out; *run;
data variances;
merge var_qty var_normqty; by ssp;
*proc print data=variances; *run;
data newspots;
  merge newspots variances;
  by ssp;
*proc print; *run;
data newspots;set newspots;
  wt_qty= (n_qty/msqty); * = n / MSE for individual ssp (qty);
  wt_norm = (n_norm/msnormqty); * = n / MSE for individual ssp
(normqty);
  run;
*proc print data=newspots; *run;
proc sort; by ssp;
proc glm data=newspots NOPRINT OUTSTAT=qty_out; by ssp;
  class ssp wt trt;
  model meanqty = wt trt wt*trt /ss3;
  WEIGHT wt_qty;
  lsmeans trt wt wt*trt/ out=lsmeans_qty stderr pdiff;
  run;
proc print data=lsmeans_qty; run;
*proc print data=qty_out; *run;
data qty_out; set qty_out;
keep ssp _NAME_ _SOURCE_ _TYPE_ PROB;
if PROB < 0.20;
if _source_ = 'ERROR' then delete;
proc print data=qty_out; run;
ODS HTML FILE='C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\lsmeans qty.xls' RS=none;
  Proc print data=lsmeans_qty noobs;
  Run;
ODS HTML CLOSE;
proc glm data=newspots NOPRINT OUTSTAT=norm_out; by ssp;
  class ssp wt trt;
  model meannorm = wt trt wt*trt /ss3;
  WEIGHT wt_norm;
  lsmeans trt wt wt*trt / out=lsmeans_norm stderr pdiff;
  run;
proc print data=lsmeans_norm; run;
*proc print data=norm_out; *run;
data norm_out; set norm_out;
keep ssp _NAME_ _SOURCE_ _TYPE_ PROB;
if PROB < 0.20;
if _source_ = 'ERROR' then delete;

```

```
run;
proc print data=norm_out;run;
ODS HTML FILE='C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\lsmeans norm.xls' RS=none;
  Proc print data=lsmeans_norm noobs;
  Run;
ODS HTML CLOSE;
```

C2. GLM Procedure with test statements and random term (rejected in favor of Mixed Procedure).

```
*Proteomics raw data analysis;
options ls=100 ps=75 nodate nonumber;
dm 'clear log';
dm 'clear output';
data raw;
infile 'C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\dealing with statistics\NEW cornell heifers for
SAS.prn';
input ssp heifer$ wt$ trt$ qty normqty;
data raw;
  set raw;
  *the following are MW and pI marker spots;
  if ssp=0014 then delete;
  if ssp=0210 then delete;
  if ssp=0410 then delete;
  if ssp=0606 then delete;
  if ssp=0711 then delete;
  if ssp=0809 then delete;
  if ssp=0908 then delete;
  if ssp=0909 then delete;
  if ssp=1111 then delete;
  if ssp=2412 then delete;
  if ssp=4602 then delete;
  if ssp=4604 then delete;
  if ssp=4608 then delete;
  if ssp=6210 then delete;
  if ssp=6708 then delete;
  if ssp=7006 then delete;
  if ssp=7311 then delete;
run;
PROC SORT data=raw; by ssp;
proc glm data=raw NOPRINT OUTSTAT= raw_qty; by ssp;
  class heifer trt wt;
  model qty normqty = wt trt wt*trt heifer(wt*trt)/ss3;
    random heifer(wt*trt);
    test H= wt|trt E= heifer(wt*trt) ;
  lsmeans wt|trt/ e=heifer(wt*trt) NOPRINT STDERR out=lsm_raw;
run;
proc print data=lsm_raw; run;
*proc print data=raw_qty; *run;
data raw_qty; set raw_qty;
  keep ssp _NAME_ _SOURCE_ _TYPE_ PROB;
  if PROB < 0.20;
  if _SOURCE_ = 'ERROR' then delete;
*proc print data=raw_qty; *run;
ODS HTML FILE='C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\raw_qty small p values.xls';
  proc print data=raw_qty noobs;
  run;
  ODS HTML CLOSE;
*proc print data=lsm_raw; *run;
```

```
ODS HTML FILE='C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\lsm_raw.xls';
  proc print data=lsm_raw noobs;
  run;
ODS HTML CLOSE;
```

C3. Mixed Procedure.

```
*Proteomics raw data analysis;
options ls=100 ps=75 nodate nonumber;
dm 'clear log';
dm 'clear output';
data raw;
infile 'C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\dealing with statistics\NEW cornell heifers for
SAS.prn';
input ssp heifer$ wt$ trt$ qty normqty;
data raw;
  set raw;
  *the following are MW and pI marker spots;
  if ssp=0014 then delete;
  if ssp=0210 then delete;
  if ssp=0410 then delete;
  if ssp=0606 then delete;
  if ssp=0711 then delete;
  if ssp=0809 then delete;
  if ssp=0908 then delete;
  if ssp=0909 then delete;
  if ssp=1111 then delete;
  if ssp=2412 then delete;
  if ssp=4602 then delete;
  if ssp=4604 then delete;
  if ssp=4608 then delete;
  if ssp=6210 then delete;
  if ssp=6708 then delete;
  if ssp=7006 then delete;
  if ssp=7311 then delete;
run;
PROC SORT data=raw; by ssp;

proc mixed data=raw; by ssp;
  class heifer trt wt;
  model qty = wt|trt / htype=3;
  random heifer(wt*trt);
  lsmeans trt|wt / diff;
  ODS output tests3=testsqty;
  ODS output LSmeans=lsmeansqty;
run;
*proc print data=testsqty;
*proc print data=lsmeansqty;
data testsqty; set testsqty;
  keep ssp Effect FValue ProbF;
  if ProbF < 0.20;
proc print data=testsqty;
ODS HTML FILE='C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\dealing with
statistics\MIXED_raw_lsmeansqty.xls';
  proc print data=lsmeansqty noobs;
  run;

proc mixed data=raw; by ssp;
```

```

class heifer trt wt;
model normqty = wt|trt / htype=3;
random heifer(wt*trt);
lsmeans trt|wt / diff;
ODS output tests3=testsnorm;
ODS output LSmeans=lsmeansnorm;
run;
data testsnorm; set testsnorm;
keep ssp Effect FValue ProbF;
if ProbF < 0.20;
proc print data=testsnorm;
*proc print data=lsmeansnorm;
ODS HTML FILE='C:\Documents and Settings\danielsk\My
Documents\Kristy\Proteomics\dealing with
statistics\MIXED_raw_lsmeansnorm.xls';
proc print data=lsmeansnorm noobs;
run;

quit;

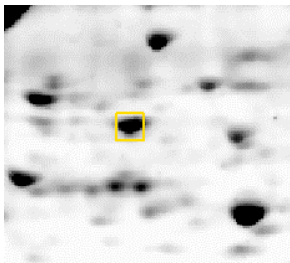
```

APPENDIX D

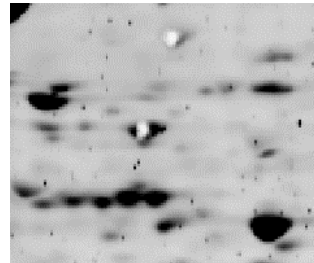
DEMONSTRATIONS OF BEFORE AND AFTER IMAGES OF PROTEIN SPOTS EXCISED FROM A 2D GEL AND SUBMITTED FOR MASS SPECTROMETRY ANALYSIS

Proteins 5602 (boxed) and 5705

Before

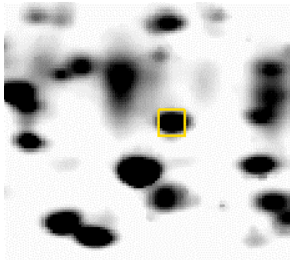


After

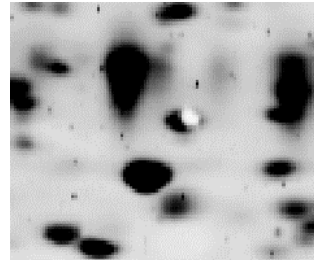


Protein 8108

Before

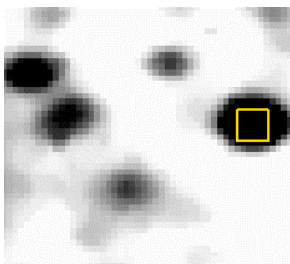


After

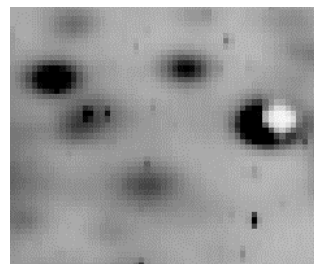


Protein 8301

Before



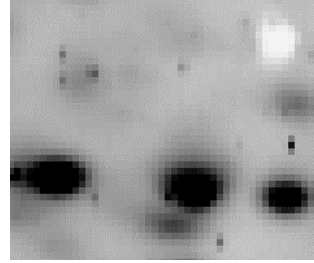
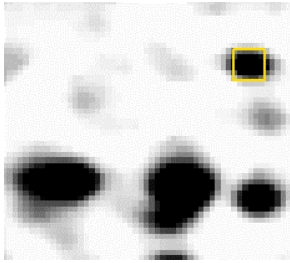
After



Protein 8503

Before

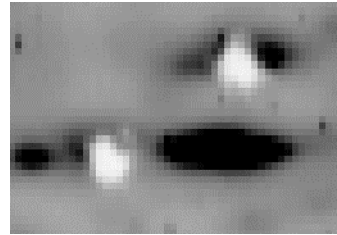
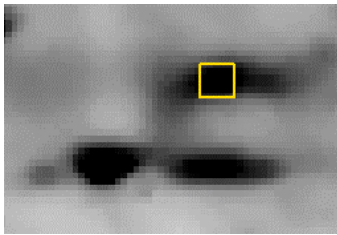
After



Proteins 6701 (boxed) and 5605

Before

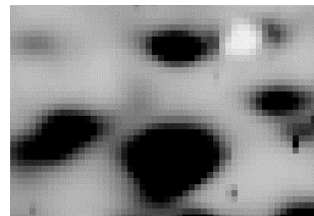
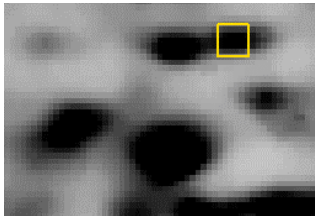
After



Protein 3505

Before

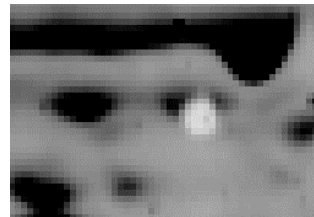
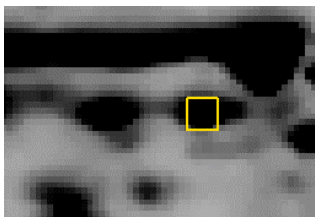
After



Protein 7801

Before

After



VITA

Kristy Marie Daniels was born in West Branch, Michigan on July 27th, 1981. She graduated from Michigan State University in December 2002 with a Bachelor of Science Degree in Animal Science with high honor. In December of 2004 she completed a Master of Science degree program in Dairy Science with the option in Molecular Cell Biology and Biotechnology at Virginia Polytechnic Institute and State University.