Ultrasonographic Monitoring of Mammary Parenchyma Growth in Preweaned Holstein Heifers

Thesis

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Abstract

Mammary parenchyma (PAR) is present at birth in negligible quantities, undergoes extensive postnatal growth, and will ultimately comprise milk secretory tissue. Over-nutrition in early life may negatively impact future milk yield (MY) by affecting PAR growth. Monitoring in vivo growth of PAR has historically been difficult, necessitating slaughter studies to measure PAR quantity, which precludes measurement of MY. Advances in ultrasound (US) technology warrant revisiting its use as a non-invasive tool to monitor PAR growth in vivo. Holstein heifers (n = 24; 41±1kg initial BW) from a single farm were randomly assigned to 1 of 3 milk replacers (MR) at 2-3d of age. Heifers were fed MR at 660 g/d until weaning at 42d. All MR contained 27% CP (DM basis); fat sources and total fat (DM basis) were as follows: A) only lard, 17% fat, B) animal fat supplemented with 1.25% NeoTect4® (MR, Provimi North America, Brookville, OH), 17% fat, and C) milk fat, 33% fat. Calves had ad libitum access to a common calf starter (20% CP) and water for the duration of the 56d trial. A real time B-mode US with a 7.5MHz convex probe was used to examine two-dimensional (2D) PAR area in all 4 glands of heifers once weekly from 2-3d of age to harvest at 56d. Individual digital images of each gland were saved for further analysis. The left front and left rear glands were also examined by US 24h post-harvest in order to validate final US measurements,
and then bisected to produce a sagittal plane view of PAR for comparison to US images. In all cases, 2D areas of PAR were determined using ImageJ software (NIH). Data were analyzed with a mixed model in SAS that accounted for repeated measures. Additionally, 8wk paired data were analyzed using the correlation procedure. PAR areas grew over time (week, \(P = 0.001\) and were not different due to diet \(P = 0.947\)). Initially, PAR area averaged 0.049±0.020 cm\(^2\)/gland; at day 56 it averaged 0.420±0.015 cm\(^2\)/gland. These values changed over time in a complex pattern (linear, \(P = 0.001\); quadratic, \(P = 0.001\); cubic, \(P = 0.003\)). On average, front glands were smaller than rear glands \(P = 0.002\), while there was no difference between the two front \(P = 0.834\) or the two rear \(P = 0.926\) glands. Positive correlations existed between all paired variables analyzed (Bisected PAR area; PAR area ante-mortem from US; PAR area postmortem from US; all \(r > 0.60\)). As long as dietary nutrients are not limiting, it seems that PAR growth may rely more on initial tissue foundation within a given animal rather than on dietary manipulation prior to 2 mo of age. Therefore, determination of PAR area in vivo may be useful in distinguishing potential high-producing animals at an early age. Future studies will use US to monitor PAR growth during the preweaning period with later comparison to first lactation MY. Lastly, methodology used here demonstrates that US is an effective tool for measuring weekly changes in PAR area in vivo and may be utilized in future experiments.
Dedication

Dedicated to Dad and Mom, all things were done with your love and support. To my amazing brothers, Chris, Nathan, and Justin, and to wee Andrea, who is no less amazing.
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CHAPTER 1: INTRODUCTION

The expansion of knowledge in dairy management has grown tremendously in the past decade, from our knowledge of mastitis, laminitis, and acidosis, to understanding more on the molecular level processes involved with milk synthesis. An important, but often overlooked, aspect of dairy herd management lies in the investment producers make in growing replacement heifers. Replacement heifers are the second largest investment producers make in their dairy operation after feed costs. However, there is a great deal that we do not know about heifer selection. Once heifers are born, there is no perfect method for selection of a heifer that will ensure increased milk production in that animal when compared with herdmates.

As more evidence accumulates surrounding calf growth, it has become clear that future milk production may be more dependent on the amount of established tissue at an early age, rather than any single nutrition program. More simply stated, nutrition cannot overcome poor genetics. Genetic selection is one way to predict lactation potential. Even that has a fair amount of risk involved, because lactation potential is not isolated to one gene. Hundreds of genes dictate quantity of milk produced, through their ability to direct processes such as milk protein, lactose and fat synthesis and secretion. Heritability of genes from parents to progeny is not a guarantee when dairy producers selectively
breed animals to produce offspring that will increase milk production quantity and quality. As a result, producers will not know the quality of the cow until the first lactation, which typically occurs at around 24 mo of age. Two years of feeding, housing, and otherwise caring for a heifer, with no concurrent income generation on the part of that heifer, results in high initial expenditures for dairy producers.

University research aimed at studying mammary growth and development in heifers has largely been isolated to slaughter studies in order to obtain mammary gland information. The tissue of interest in most studies is mammary parenchyma (PAR), which will grow and differentiate into lactating tissue. Recent advances in the understanding of nutrition and its impact on PAR development have been hindered by necessitated slaughter studies performed to obtain mammary gland information. Heifers slaughtered in the preweaning stage never realize lactation, which prevents researchers from studying the long-term implications of the quantity of PAR in early life in relation to lactation capacity in the same animals. Therefore, determining PAR quantity and growth in vivo would be valuable to longevity studies. The results of studies done on PAR growth from birth to lactation could then in turn impact how producers manage replacement heifers.

Advances in ultrasound technology and image resolution have enabled researchers to obtain in vivo information that has not been formerly accessible. In this study, PAR present in heifers from birth until 2 mo of age was observed using a 7.5MHz convex ultrasound probe. The method used here verified that ultrasound is an effective tool in
measuring PAR quantity. Additionally, ultrasound enables observation of PAR growth over time. This is a preliminary step in understanding how established mammary PAR relates to production capacity of that heifer in her first lactation, and perhaps beyond.
CHAPTER 2: LITERATURE REVIEW

*Mammary Growth and Development*

Initial development of mammary epithelial cells begins in utero (reviewed recently in Rowson et al., 2012). When a heifer is born, a rudimentary ductal system is present (reviewed recently in Rowson et al., 2012). The duct system extends from the teat cistern to the gland cistern and ends with the epithelial duct tissue. This epithelial tissue and its surrounding stromal elements are collectively known as mammary parenchyma (PAR). At birth and for several months after, PAR is surrounded by the mammary fat pad (MFP). Meyer et al. (2006b) showed that newborn heifers weighing 46 kg had negligible quantities of PAR, but at 100 kg, PAR weight ranged from 17 to 30 g, depending on diet. Brown et al. (2005a) demonstrated that 8 wk old heifers had 1.2 to 4.5 g of PAR, depending on diet. The MFP is non-epithelial tissue. It contains connective tissue, lymph tissue, nerves and blood vessels (reviewed in Sejrsen, 1994). MFP and PAR grow at the same rate as the rest of the body from birth to roughly 2 to 3 mo of age (Sinha and Tucker, 1969; Meyer et al., 2006b). This type of growth pattern is termed isometric growth. Diet composition and energy density of the diet can both drastically alter MFP growth in dairy heifers (Daniels et al., 2009a), with lesser effects on PAR growth in the
preweaned stage (Brown et al., 2005a; Meyer et al., 2006a; Daniels et al., 2009a). There are drastic differences in growth rates of PAR over the lifetime of a growing heifer. (Sejrsen and Foldager, 1992; Capuco et al., 1995). Important life stages related to mammary growth and development in dairy animals can be broadly described as: pre-weaning (0 to 2 mo), pre-pubertal (2 to 9 mo), peripubertal (9 mo to conception, ~15 mo), pregnancy, and a lactation stage after parturition.

The quantity of milk a cow produces is positively correlated with the number of mammary epithelial cells that are present (Boutinaud et al., 2004). Milk production capacity is therefore reliant on the quantity and function of mammary epithelial cells, rather than the general size of the udder. The amount of milk able to be synthesized by the gland increases with each lactation until the fifth lactation (Larson et al., 1985), but the tissue foundation for all postnatal growth and development is established in early life. Three physiological events known to cause the majority of epithelial cell growth and differentiation within PAR include: the prepubertal allometric growth period, recurring estrous cycles, and gestation (Tucker, 1987). Until lactation occurs, there is no return on the investment of rearing heifers, and growing heifers from birth until first calving averages 22 to 24 mo in U.S. dairy herds (USDA, 2007), with an estimated rearing cost up to $2,150/heifer (Rincker et al., 2011).

Allometric growth of mammary epithelial cells begins after 2 mo of age, at which point PAR grows at a faster rate than the rest of the heifer’s body (Sinha and Tucker,
This allometric growth continues until the onset of puberty at around 9 mo of age or a body weight of 250 to 350 kg for Holsteins (Capuco et al., 1995; Meyer et al., 2006b). Estrous cycles in post-pubertal animals stimulate further PAR growth and development (Sinha and Tucker, 1969). To that point, mammary DNA concentration in post-pubertal heifers follows a regression and growth pattern in conjunction with the estrous cycle; in this manner, mammary gland (MG) growth continues at an isometric rate until gestation (Sinha and Tucker, 1969). It is not until the third trimester of pregnancy that epithelial cells in PAR differentiate to form milk secretory units known as alveoli (Sinha and Tucker, 1969; Swanson and Poffenbarger, 1979). Mammary epithelial cell proliferation and differentiation are nearly complete at parturition (Sejrsen et al., 2000). Following parturition, mammary epithelial cell proliferation continues at an isometric rate, and epithelial cell replacement amounts to approximately 50% of the cells that were in place at calving (Capuco et al., 2001). Programmed cell death, or apoptosis, causes the gradual reduction in epithelial cell numbers as lactation progresses (Capuco et al., 2001), and milk production decreases over time as a result. The research reported by Capuco et al. (2001) demonstrated that by the end of lactation, cells secreting milk were mostly comprised of cells that proliferated after calving in order to replace apoptosed cells.
Management Strategies for Rearing Replacement Heifers

Genetics. Proper management of dairy animals often relies on careful management of genetics within the herd. Milk production traits are heritable and dairy producers can improve the genetic merit of a herd over time. However, genetics is not everything. To elaborate, production characteristics such as milk yield are moderately heritable traits (0.20 to 0.30). Heritability estimates like this describe the ease with which genetic improvement is made, and the phenotypic expression of milk production is only moderately influenced by genotype. Environmental impact can account for as much as 75% of the variation in production, while only 25% may be attributed to genetic merit (Ruen et al., 2010). Milk production characteristics can be altered by the interactions of genes and the environment in which an animal lives.

Genetic analysis is one way dairy producers have been able to make more informed decisions about which heifers are kept as replacement heifers and which are sold. Milk production is controlled by multiple genes, resulting in an incomplete understanding of lactation biology from a genetic standpoint (Suchocki et al., 2010). These genes can control total milk yield, as well as milk protein, milk fat, and lactose synthesis and secretion. Complex interactions arise in the genome of dairy cattle between the epistatic genes that can modify, or be modified, by other genes, and additional variation is added when environmental effects on gene expression are considered (Cohen-Zinder et al., 2011). Knowledge of milk production traits has grown in recent years
through research and mapping of the genome with single nucleotide polymorphisms to identify quantitative trait loci of lactation traits, or long stretches of DNA that are associated with lactation traits (Guillaume et al., 2008; Suchocki et al., 2010; Cohen-Zinder et al., 2011).

These complex research methods are being implemented by breeding companies for genetic selection, as seen in Guillaume et al. (2008). Improvements are being made to increase knowledge regarding which genes impact milk production. Use of this type of genetic information by dairy producers will help dairy producers choose which replacement heifers to keep. Because all of the genes influencing lactation traits have not yet been isolated, lactation traits are polygenic, and subject to environmental impact (Ruen et al., 2010; Suchocki et al., 2010). good management practices are still necessary to optimize milk production in dairy cattle. Chief among environmental factors is likely nutrition, in all stages of the life cycle.

**Nutrition.** Another management strategy for rearing heifers is to achieve milk production at the lowest possible cost. One way to reduce costs is to feed heifers high energy diets to help them reach the body weight required to achieve puberty at a younger age (Schillo et al., 1992; Hoffman et al., 1996; Brown et al., 2005a; Meyer et al., 2006b; Daniels et al., 2009a). Feed cost per unit of body weight gain is lowest in the first 6 mo of life (Kertz et al., 1998), and heifers grow very rapidly at this time. Fifty percent of total, mature height and 25% of total, mature body weight increase occurs at this time.
(Kertz et al., 1998), with a decline in rate of growth until heifers have achieved adult body weight and height. Feeding growing heifers rations with adequate amounts of protein and fat to support or enhance the capacity for growth may be more costly, but it can be beneficial. Noted benefits are that earlier onset of puberty can result in an earlier breeding age, age at first calving, and entry into first lactation, resulting in a faster return on investment for the producer.

A potential downside of this rapid early growth management strategy is that high-energy diets may result in decreased milk production due to negative impacts on prepubertal mammary development (Sejrsen et al., 1982; Petitclerc et al., 1984; Capuco et al., 1995) or pubertal mammary development (Schillo et al., 1992; Rincker et al., 2008). Other cons associated with rapid growth heifer development strategies include: insufficient body weight, reduced pelvic area, and/or excess body condition at calving (Hoffman and Swartz, 1997). If under-developed or over-conditioned replacement heifers calve before 22 to 24 months of age, this may give rise to other physiological obstacles such as dystocia (Abeni et al., 2000), incomplete body growth resulting in reduction of first lactation milk production in favor of body growth (Hoffman et al., 1996), or increased mortalities in offspring due to birthing difficulties.

Rearing heifers too fast can damage PAR development. It has been shown that MG development is affected differently by nutrition when heifers are at different physiological stages (Meyer et al., 2006b). An examination of how growth occurs at
these different stages will help elucidate the effects of diet on the development of PAR and lactation capacity.

**Pre-Weaning Feeding Programs**

*Colostrum.* Maternal alveoli begin secreting milk into milk ducts of the mammary gland around 30 d prior to parturition, which generates a nutrient-dense product, colostrum, that is unique in comparison to milk produced during the rest of the lactation period. Colostrum contains higher concentrations of nutrients essential for the first few hours of life, including protein, fatty acids, vitamins, minerals (Larson, 1958), and most notably, maternal antibodies called immunoglobulins. Mammals with epitheliochorial placentation, as cattle are classified, are unable to transfer maternal antibodies to the fetus during gestation (Salmon, 1999). Neonatal calves are born with a fully developed immune system (Tizard, 1996), but because their immune response is naïve, white blood cells are unprimed and will not recognize foreign antigens. Calves are susceptible to infection by pathogens in the environment into which they are born, and rely on passive transfer of immunity from dam to offspring through ingestion of immunoglobulin-rich colostrum (Salmon, 1999). Ingested immunoglobulins are absorbed intact into the bloodstream to provide a temporary defense mechanism that protects the calf while it builds the ability to mount an immune response (Heinrichs and Elizondo-Salazar, 2009).
Absorption of colostrum is done efficiently via pinocytosis if administered within 4 h postpartum, and less efficiently after that due to a time-dependent reduction in absorption efficiency (Stott et al., 1979). Pinocytosis is nonspecific endocytotic envelopment of molecules by enterocytes lining the small intestine. This can include passage of both immunoglobulins and bacteria. In this way, contaminated colostrum can inoculate the calf with pathogens if precautions are not taken to ensure colostrum and environmental cleanliness (Godden et al., 2003).

At least 2 L of colostrum should be fed in the first 4 h of life, with a total of 4 to 6 L recommended within the first 24 h of life (Godden et al., 2003) to ensure transfer of sufficient antibodies; without transfer of maternal antibodies, morbidity rates increase. Complete gut closure will occur by 24 h postpartum (Stott et al., 1979). Calves that do not ingest adequate amounts of colostrum (10 mg/L IgG) prior to gut closure will experience failure of passive transfer (FPT) (Stott et al., 1979). Calves that ingest less than 2 L of colostrum are most likely to experience FPT (USDA, 2007). Increased morbidity and more severe disease bouts are observed in calves with serum proteins less than 5 g/L (Furman-Fratczak et al., 2011). Serum protein concentration is an indirect measure of IgG concentration. Recent research has shown that 19.2% of all dairy calves have FPT (USDA, 2007; Beam et al., 2009), with a documented link between FPT occurrences and increased morbidity and mortality rates (Beam et al., 2009; Heinrichs and Elizondo-Salazar, 2009).
In addition to immunoglobulins, milk derived from nursing may provide animals with added bioactive factors or hormones to supplement growth and exert effects on development, especially of reproductive tissues (Bagnell et al., 2009; Bartol et al., 2009). Bartol et al. (2009) examined the colostrum-borne hormone relaxin and its effects on uterine development in neonates and concluded that interaction between relaxin and estrogen signaling systems mediate endometrial change. Direct effects of feeding colostrum are not known, but there is a possibility that other factors present in milk may aid in neonatal development; this is known as the lactocrine hypothesis (Bagnell et al., 2009; Bartol et al., 2009). As recently demonstrated, reproductive tissues can be influenced by factors present in milk, and it may follow that the development of PAR, which is also considered reproductive tissue (Daniels, 2010), could possibly be influenced by the same mechanism.

**Post-Colostrum Feeding Programs**

**Whole Milk or Waste Milk.** After initial colostrum feedings, calves are offered a primarily liquid based diet. Improved absorption aids in promoting greater gain to feed ratios (Lee et al., 2009). Whole milk provides benefits over MR, such as reduced mortality, due to differences in the profile of milk constituents and bioavailability of components (Losinger and Heinrichs, 1997; Lee et al., 2009). Whole milk is not frequently fed because it is worth more for dairy producers to sell it rather than feed it to
calves, so a different management practice is to feed waste milk. Waste milk is usually from mastitic cows and is unsalable due to presence of antibiotics or high somatic cell count (Keys et al., 1980), or it can come from transition milk. Transition milk is derived from the 2\textsuperscript{nd} and 3\textsuperscript{rd} milkings, and its composition is between colostrum and whole milk. Neither transition milk nor colostrum is legally saleable out of the bulk tank (Payer and Holmes, 1998). Cows can have up to 68 L of waste milk each year (Blosser, 1979), which is often discarded. With such a quantity of waste milk produced per cow, one can see why 38\% of producers choose to utilize waste milk and use it to feed calves in order to reduce economic losses (Heinrichs et al., 1994). Unfortunately, additional processing and labor are often required before it can safely be fed to calves. Pasteurization reduces the number of pathogens fed through waste milk, thereby reducing morbidity and mortality and improving growth (Jamaluddin et al., 1996). A survey of Wisconsin dairies (Jorgensen and Hoffman, 2005) demonstrated that the nutrient composition of pasteurized waste milk (3.4\% CP, 3.6\% fat) was comparable to saleable whole milk (3.17\% CP, 3.85\% fat) from the bulk tank, on an as-fed basis (NRC, 2001).

A second option for liquid feed is MR, which is discussed in more detail below. The economic study by Godden et al. (2005) estimated that producers could save $34 per calf from birth to weaning if they fed pasteurized waste milk instead of a conventional MR. However, the quantity and quality of waste milk varies daily and may not be readily or continuously available; producers then have to rely on MR for supplementation of the
waste milk feeding regimen. Additionally, pasteurization adds to the amount of time required to care for calves. In contrast to the 38% of US dairy farms feeding waste milk, 59% reportedly rely on MR for pre-weaning nutrition (Heinrichs et al., 1994; USDA, 2007). Much research has gone into the determination of the optimal nutrient density and nutritional profiles for MR to avoid negatively impacting mammary and body growth while maintaining consideration for the economics of raising replacement heifers.

**Milk Replacer.** In nature, calves suckle from their dam and ingest whole milk after the production of colostrum ceases (2-3 d after parturition) until weaning. Milk is produced by the dam, designated for ingestion by her calf, and the calf has unlimited or nearly unlimited access to milk. Whole milk produced by Holstein-Friesian cows can range from 23-27% CP and 26-32% fat, on a dry matter basis (Diaz et al., 2001). In contrast, milk replacer (MR) is typically fed in limited quantities (average 0.45 kg DM/d, or at 1.2% of body weight), and is available in numerous CP:fat ratios. An example of a typical “conventional MR” contains 20% CP and 20% fat (DM basis). When compared to the crude composition of whole milk from Holstein cows mentioned above, even if this product was fed to calves in equal volumes as whole milk, it is evident that total amount of nutrients consumed is much less. Moreover, most calves fed 20:20 MR are fed restricted amounts of this product, further compounding negative growth effects. Newer products are commercially available with nutrient content similar to cows’ milk (28%CP, 20% fat, DM basis).
Digestibility of MR is often reduced when compared with whole milk. The fatty acid profile of whole milk contains high concentrations of saturated, medium chain fatty acids (Jensen, 2002), while MR are commonly made with edible lard in place of milk fat (Davis and Drackley, 1998). Bovine milk contains short-chain, medium-chain, long chain, and some polyunsaturated fatty acids, while animal fat primarily contains long chain fatty acids (Jenkins et al., 1985; Beaulieu and Palmquist, 1995), which are not digested as easily by neonatal calves when compared with medium chain fatty acids. A review of apparent digestibilities of milk, animal, and vegetable fats was reported in a review by Raven (1970). Reported apparent digestibility of animal and vegetable fats are estimated to be 83-93% (Raven, 1970; Jenkins et al., 1985; Diaz et al., 2001), while whole milk digestibility is estimated to be around 95.2 to 97.5% digestible (Raven, 1970). As a result, making changes to the fatty acid (FA) profile of MR to more closely match that of whole milk is a current area of calf research. Two studies by Hill et al. (2007a; 2007b) reported improved average daily gain (ADG) and reduced scouring in bull calves by increasing the concentrations of short-chain, medium-chain, and the essential fatty acids in MR, linoleic and alpha-linolenic acid (Hill et al., 2007b). They also observed increased growth rate when compared to calves fed conventional MR with animal fat as the sole fat source, as well as when pasteurized whole milk was fed (Hill et al., 2007a).
Dried whey, dried whey protein concentrate, and dried skim milk typically comprise the protein in MR. Different fat concentrations and fatty acid profiles can alter bioavailability and reduce growth in calves fed MR compared to those consuming whole milk (Lee et al., 2009; Moallem et al., 2010). By feeding calves MR, the quantity and quality of liquids fed are altered. Therefore, closer considerations regarding milk composition should be made when trying to optimize calf growth.

**Protein and Energy Ratio.** The 20:20 CP to fat ratio is widely used in conventional MR formulation in the United States. Protein and energy requirements of preweaned calves change as the calves grow and age (Diaz et al., 2001). Specifically, protein requirements relative to energy requirements will decrease (Whitlock et al., 2002) with increasing age. Altering the CP to fat ratio has been observed to impact calf growth. (Lassiter et al., 1963; Brown et al., 2005a; Hill et al., 2008).

Brown et al. (2005a) demonstrated that a simultaneous increase in CP and fat content of MR resulted in an increased rate of mammary growth and development up to 8 wk of age, but no effect was observed by providing that type of diet after weaning. An enhanced early-life growth program, such as that used by Brown et al. (2005a) (30.3% CP, 15.9% fat, fed at 2.0% of BW on a DM basis), may maximize lifetime milk yield, as alluded to, but not studied in reports by Brown et al. (2005a), Meyer et al. (2006b), and Daniels et al. (2009b).
First lactation milk yield was studied by Rincker et al. (2011) and Raeth-Knight (2009). Both studies showed no difference in overall costs of rearing when feeding higher protein MR. Calves fed MR with high CP had a greater gain:feed ratio, and increased ADG when compared to conventionally fed calves, but consumed only half the amount of solid starter feed during the preweaning period (Rincker et al., 2011). This may have slowed rumen development to the extent that efficiency of growth and lower gains in the weeks following weaning negated the benefit from improved preweaning growth rates. No difference was seen in milk yield (Rincker et al., 2011). Feeding MR with higher protein and lower fat (30.6% CP, 16.1% fat) had an average cost increase of $1.27 per head per day when compared to conventional MR (21.5% CP, 21.5% fat) (Rincker et al., 2011), but there was no significant difference in overall cost of rearing due to improved feed efficiency and fewer nonproductive days for intensively fed calves. More research on rumen development and the so-called “post-weaning slump” associated with a lag in starter intake and lower ADG will be key in maintaining growth benefits from feeding MR higher in CP and fat.

The focus of a study by Moaellem et al. (2010) was on the long-term benefits of post-weaning supplementation of protein to dairy heifers, but they also observed changes in preweaning growth as a result of increased protein availability. Calves were fed whole milk (25.9% CP, 29.4% fat, as fed) or MR (23.7%CP, 13% fat, as fed). Whole milk fed calves received significantly higher CP compared to calves fed MR. Whole milk fed
calves had greater feed efficiency, overall increased BW, and increased ADG at weaning, and the advantage in BW gained in the preweaning period persisted until calving. The researchers concluded there was added benefit to high feeding rates to calves by feeding whole milk that was only slightly enhanced by increased protein supplementation in the prepubertal period. Moallem et al. (2010) noted an increase in fat deposition in heifers because they were fed greater fat concentrations in whole milk than heifers fed MR. Increased quantity of MFP could result of higher endocrine or paracrine activity in MG, which mitigate increased signaling to PAR to further development (Meyer et al., 2006a; Moallem et al., 2010). Increased PAR development is a possible explanation for the observation that heifers fed whole milk had greater milk production. Other factors associated with whole milk composition on calf growth are discussed below, but an important aspect of this study is shown in the benefits of preweaning nutrition in regard to long-term production.

*Quantity of Milk Replacer.* In 1942, university researchers Savage and McCay introduced the notion that by providing reduced amounts of MR to dairy heifers, producers could save money while still achieving acceptable growth results. Limit feeding calves became the default management recommendation, and led to a stagnated emphasis on the importance of nutrition in early life for nearly 70 years (Savage and McCay, 1942; Appleman and Owen, 1975).
Renewed interest in calf nutrition and growth began with the observation of lower milk production in cows that were limit-fed during development compared with those fed on a higher plane of nutrition (Appleman and Owen, 1975; Diaz et al., 2001; Terre et al., 2009; Moallem et al., 2010; Soberon et al., 2012). The pre-weaning period from birth to 2 mo of age may provide an opportunity to increase growth rates of young calves without negatively impacting subsequent performance by taking advantage of the efficiency of growth during this period (Brown et al., 2005b; Meyer et al., 2006b; Daniels et al., 2009a). This is in contrast to what many researchers previously thought (Sejrsen and Purup, 1997; Lammers and Heinrichs, 2000). Soberon et al. (2012) used two different herds to examine the effects of increased rate of gain on future milk production; one herd had a preweaning ADG of 0.82 ± 0.18 kg/d and the other with a preweaning ADG of 0.66 ± 0.11 kg/d. In both herds, calves were fed until weaning with a MR that was comprised of 28% CP and 15% fat. ADG was positively correlated with increased first-lactation milk yield, with between 850 and 1,113 kg more milk produced per 1 kg gained before weaning (Soberon et al., 2012). Up to 22% of the variation in first lactation milk yield was attributed to preweaning ADG, which reflects the importance of early life nutrition. A second point of consideration with this study is the increased protein content of the MR, as previously discussed. Enhanced feeding is associated with improved feed efficiency (Quigley et al., 2006), resulting in a better cost to gain ratio (Terre et al., 2009).
Alternatively, increased amounts of MR can reduce starter intake, slow ADG in the post-weaning period (Hill et al., 2010; Sweeney et al., 2010), and inhibit rumen development, as reviewed by Baldwin (2004). There is an inverse relationship between milk solids provided and starter consumed by preweaned heifers that lead to slower growth rates during the preweaning period (Brown et al., 2005b; Raeth-Knight et al., 2009). Jasper and Weary (2002) showed that calves allowed ad libitum access to whole milk consumed 89% more milk than calves conventionally fed whole milk at 10% of their BW through bucket feeding, but chose to eat only 16% as much starter, and 17% as much hay as limit-fed calves. Even with less solid feed intake, calves offered feed ad libitum maintained higher BW than limit-fed calves. Despite a lag in solid food consumption before weaning, differences in feed intake and ADG were not maintained post-weaning (Jasper and Weary, 2002); similar results have been seen in other studies (Morrison et al., 2009; Terre et al., 2009).

Elevated starter intake during the pre-weaning period stimulates rumen development (Warner et al., 1956) which is thought to decrease weaning stress by increasing the calf’s ability to digest solid feed (Weary et al., 2008). A concern is that delayed rumen development due to high MR intake will depress growth in calves (Hill et al., 2010). Because milk bypasses the rumen, it cannot contribute to rumen development in the way that starter feed does (Warner et al., 1956). The rumen is anaerobic and provides the appropriate environment for microbial growth and fermentation to promote
the breakdown of ingested plant material. Microbial digestion of starter feed in the rumen leads to the release of soluble chemical products known as volatile fatty acids, which will increase in association with increased dry feed intake (Quigley et al., 1991). Volatile fatty acids interact with the interior surface of the rumen, which is covered with elongated projections of the rumen wall called papillae. Papillae increase the surface area of the rumen in order to increase the absorption of nutrients, and will thicken and elongate as the rumen develops (Arias et al., 1980). Therefore, volatile fatty acid production is limited by the amount of starter feed intake, and management practices that reduce starter intake are inhibiting rumen development through reduced volatile fatty acid production.

Terre et al. (2006) observed increased rumen development and increased starter intake in calves fed conventional MR (12.5% DM dilution rate) compared with calves fed elevated MR (18% DM dilution rate). Urine was evaluated for purine derivatives, and greater purine derivatives in urinary excretion indicated increased rumen microbial activity because of increased microbial nitrogen flow to the duodenum. Rumen development was determined to be greater in calves fed conventional MR because of increased urinary purine compared to calves fed elevated MR. However, calves fed elevated MR maintained a higher BW than conventionally fed calves (Terre et al., 2006). Conversely, Hill et al. (2010) did not demonstrate a post-weaning recovery in starter intake for calves fed MR at an elevated feeding rate (1.09 kg/d, DM basis). However, moderately increased MR feeding (0.66 kg/d) showed no reduction in starter intake or
digestion, and calves had greater ADG than those fed MR at a conventional rate (0.44 kg/d).

As suggested by Moallem et al. (2010), whole milk may impart higher quality nutrition to calves that translates into epigenetic programming, impacting first lactation yields (Moallem et al., 2010). Calves were allowed ad libitum access to whole milk or MR for 30 min twice a day in the preweaning stage, and at 150 d of age, half of the calves from each group were supplemented with additional 2% protein throughout the prepubertal period; this component is discussed in more detail with post-weaning heifer nutrition. A 10.3% increase in milk yield during lactation was observed in calves that received whole milk over MR. Increases in production could be a result of one or more factors, which could include prolactin, leptin, insulin, insulin-like growth factor I, epidermal growth factor, growth hormone (Grosvenor et al., 1993), or relaxin (Bagnell et al., 2009; Bartol et al., 2009). The bioactive components of whole milk, as discussed by Bartol et al. (2010), could influence PAR development.

The promotion of maximal growth by allowing ad libitum access to milk did not have detrimental effects on pre-weaned calves, and may be attributed to calves still undergoing isometric growth before the onset of allometric growth experienced post-weaning (Moallem et al., 2010). Similarly, calves that were allowed to suckle from a cow 3 times daily, until satiety, were observed to have earlier age at first calving and
increased first lactation milk yield compared to cohorts fed 3 L of MR daily (Bar-Peled et al., 1997).

**Nutrition Effects on Mammary Gland: Post-Weaning, Prepubertal**

Holstein heifers will reach puberty at 225-350 kg (Sejrsen et al., 1982) and have an optimal calving weight of 600-650 kg, or about 85% of mature BW (Hoffman and Swartz, 1997). Onset of puberty is dependent on body weight. Whitlock et al. (2002) fed heifers to have an ADG of 1.18 kg/d, which resulted in the onset of puberty as early as 7.4 mo of age at 271 kg BW. In comparison, Capuco et al. (1995) observed puberty in heifers at 245 kg BW, but at 10.4 mo of age. Puberty is generally achieved in US herds by 9 mo of age. In order to attain a pubertal weight by 9 mo of age, heifers should have a rate of gain of 1000 g/d (Whitlock et al., 2002), which can be accomplished with concurrent increases in dietary protein and fat. Protein must be provided in an adequate ratio with fat content to promote structural growth but prevent over-conditioning (Whitlock et al., 2002; Capuco et al., 2004; Brown et al., 2005b). The ratio of dietary protein to energy from birth until 6 mo of age is recommended to be 66 g/Mcal, or 26% of dietary calories derived from protein (VandeHaar, 1998). Growing prepubertal heifers at a maximal rate is done with the hope of capitalizing on entry into lactation at a younger age, but there may be costs associated with rapid rearing. Research shows that the 3 to 9 mo period of postnatal life is a critical period for mammary development. Over-
conditioning heifers by feeding high-energy diets between 3 and 9 mo of age may negatively impact mammary growth more so than elevated energy availability during the pre-weaning period (Sejrsen et al., 1982; Petitclerc et al., 1999). Therefore, examination of diet on mammary growth prior to 3 mo of age may not reveal a significant impact (Brown et al., 2005a; Meyer et al., 2006b; Daniels et al., 2009a).

The role of diet composition and its effect on body fat and MG fat deposition has been examined. Kertz et al. (1987) fed heifers from 3 mo until 12 mo of age with alfalfa hay and either a control heifer grower (13.9% CP) or an accelerated program heifer grower (16.7% CP), formulated for a 10% increase in daily gain over control heifers. Kertz et al. (1987) concluded that there would be no excessive fattening when growth was 1000 g/d, but effects on milk yield were not determined. Capuco et al. (1995) compared heifers with a control (725 g/d) and accelerated (950 g/d) target ADG starting at 7.5 mo of age, and additionally examined the effects of diet composition on fat deposition by providing either alfalfa silage or corn silage in a 2x2 factorial design. High ADG heifers fed corn silage demonstrated increased MG weight and greater mammary fat, but there was no difference in PAR mass among groups. While corn silage diets promoted body and MG fat deposition when compared to heifers fed alfalfa silage diets, no reduction in milk yield was observed between heifers on either of the two diets. The researchers concluded that, since allometric growth begins around 3 mo of age, initiation of diet differences at 7.5 mo of age may have been too late to impact mammary growth
and lead to observable differences in lactation. This implies that early allometric growth is a critical period in which diet can affect mammary growth. Sejrsen et al. (1982) showed that secretory tissue and extraparenchymal fat were inversely related in the prepubertal stage but not postpubertal stage, and concluded that MFP may inhibit secretory tissue development during allometric, but not isometric, growth stages. Ultimately, Capuco et al. (1995) showed that a high rate of gain (1000 g/d) was achievable and did not affect growth in heifers.

Lammers et al. (1999) used 4.5 mo old heifers and fed them similar diets to achieve 700 g/d ADG or 1000 g/d ADG, until puberty. Heifers that gained 1000 g/d achieved puberty 32 d earlier than control heifers (Lammers et al., 1999). After the treatment period ended at 9.5 mo of age, heifers from both groups were fed to have similar BW, age, and body condition score at calving. Accelerated growth heifers demonstrated a 7.5% decrease in total milk yield. A note of important consideration is that both control and accelerated ADG heifers received a total mixed ration with 13.8% CP, which may not have been adequate to promote structural growth and instead promoted body fat deposition. Mammary development will be impacted by rate of gain if diet composition lacks adequate protein.

It appears that increased dietary protein will not enhance mammary development, nor will it impair it (Whitlock et al., 2002). Dietary protein can promote faster skeletal and BW growth (Capuco et al., 2004) without causing excess fat deposition in the MG.
High protein content is required in rapid growth diets, or producers risk increased mammary impairment due to increased fat deposition if protein is limiting (Whitlock et al., 2002), which demonstrates a benefit to feeding higher protein and lower energy diets. Dietary protein:energy ratios are better indicators of possible mammary impairment, where high protein:energy ratios favor lean tissue growth and allow heifers to grow more rapidly than a low protein:energy ratio (Lammers and Heinrichs, 2000).

Moallem et al. (2010) fed weaned heifers identical rations until 150 d of age, at which time half of the calves from each group (MR or whole milk in the preweaning stage) were supplemented with 2% additional protein to their diet. A positive interaction was noted between milk fed in the preweaning stage and the 2% protein added in the prepubertal stage (Moallem et al., 2010). Added protein during the prepubertal stage tended to increase milk production, and a 12.4% increase in milk yield was noted for heifers fed whole milk plus 2% protein in the prepubertal stage (Moallem et al., 2010). This may be attributed more to the whole milk composition because effects on growth and BW gain due to added protein during the prepubertal stage are inconsistent.

Elevated energy intake post-weaning will result in earlier onset of puberty, but may decrease PAR development and promote fat infiltration of established PAR, as mentioned in Rinker et al. (2008). Too much dietary energy can promote weight gains greater than the recommended maximum of 1000 g/d (Sejrsen et al., 1982; Petitclerc et
al., 1999) and promote fat deposition throughout the body. Increased fat deposition in the MG may hinder mammary secretory tissue development. It is important to note that not all fat deposition is detrimental to mammary growth. During mammary growth, epithelial cells in PAR form ducts that branch and elongate into MFP. MFP is therefore required for complete development, as observed in mice (Faulkin and De Ome, 1960). However, amount of MFP required is still uncertain, but it is thought that variations in MFP depot size can inhibit PAR growth or promote PAR growth, depending on the circumstances.

The importance of providing a properly balanced diet is clear; growing heifers require large amounts of both dietary protein and fat. Underfeeding heifers to avoid fat deposition may hinder development potential because the diet is protein-limiting, and overfeeding heifers may likewise hinder development potential; balance between the two extremes is necessary.

**Body Composition**

As discussed, rate of gain can affect mammary growth, but it is also important to consider how rate of gain affects body composition. Body composition can be analyzed by looking at the percentage of total fat in the body cavity, which is determined by evaluation of visceral fat percent from the kidney, pelvic, and heart (KPH). Fat comprises less than 2% of the body weight at birth (Trenkle and Marple, 1983). Body fat depots consist of subcutaneous fat, intermuscular fat, intramuscular fat, and visceral fat. In all
cases, triglycerides are the predominate component of the lipids (Aberle et al., 2001). Fat cells arise from mesenchymal cells (Aberle et al., 2001), which differentiate into adipocytes that act as storage vessels for accumulation of lipid. Adipose tissue grows by both hyperplasia (increase in cell number) and hypertrophy (increase in cell size), usually with hypertrophy dominating an increase in adipose tissue during maturity (Trenkle and Marple, 1983). KPH fat is part of the internal fat depot, and KPH fat combined with other visceral fat deposition will be the first fat deposited when the capacity for utilization of nutrients by muscle or skeletal growth is exceeded. Factors such as breed, gender, and level of nutrition will affect initiation and quantity of fat deposition. Holstein breeds typically have increased KPH fat percentage compared to beef breeds (McKenna et al., 2002). KPH percentage is often examined as a means to assess carcass quality, as it an indicator of body composition. Increasing KPH fat will negatively affect yield grade and decrease the percent of retail product yield (McKenna et al., 2002). Estimated KPH weight divided by hot carcass weight yields the percent KPH fat.

While some researchers will perform body composition analysis on the full body of the animal (Hill et al., 2008), full body composition may also be derived from analysis of the 9th-10th-11th rib-section (Hankins and Howe, 1946; Nour and Thonney, 1994). Evaluation of the 9th-11th rib-section has been an effective mode of analysis in Holsteins (Abdelsamei et al., 2005). Additionally, ribeye area (REA) gives a measure of the total area of the ribeye (longissimus dorsi). Ribeye area can be derived by ultrasound, placing
the probe between the 12th and 13th ribs to obtain a cross-section of the muscle tissue, and can be made in serial measurements to provide a growth curve over time. At slaughter, REA can be measured using a grid overlay on the sagittal cut of the *longissimus dorsi*. REA is mostly used to determine USDA yield grade in cattle slaughtered for beef.

**In vivo Monitoring of Mammary Growth**

There is limited research exploring how pre-weaning nutrition affects mammary growth and long-term milk production because most research culminates in harvesting of the animal (Brown et al., 2005a; Meyer et al., 2006b; Daniels et al., 2009b). There is no verified procedure that allows researchers to accurately assess PAR growth through a quantitative analysis.

However, harvest studies have allowed full quantification and thorough examination of the developmental changes that occur in the udders of pre-weaned heifers. These types of studies have provided scientists with invaluable information about mammary growth and development. Harvesting animals for mammary data has been in practice for at least the past 40 years (Sinha and Tucker, 1969; Meyer et al., 2006b).

Scientists could benefit from obtaining accurate and precise mammary data in vivo because they allow repeated use and continued observation of actual performance of the same animal.
Non-invasive Techniques for Studying Mammary Growth

**Teat Length.** Teat length will increase when exogenous estrogen is administered (Moran et al., 1991; Lammers et al., 1999), and has been used as a de facto bioassay to examine circulating estrogen in heifers during the prepubertal period. Estrogen stimulates an increase cell division, ductal development, and elongation, and is required for prepubertal mammary development (Purup et al., 1993). Recent work by Capuco et al. (2012) indicates that estrogen initiates paracrine signaling to promote mammary stem cell growth. Therefore, the effect that estrogen has on teat length may enable an indirect measurement of mammary epithelial cell development. However, some studies showed no correlation with teat length and amount of secretory tissue present (Whitlock et al., 2002).

**Palpation.** It would be ideal if the MG of prepubertal heifers could be accurately analyzed in vivo to demonstrate if PAR present at a young age has any correlation to future milk production. Palpation is often inaccurate and difficult to systematically assess. Ideas for how to do this started as early as 1955. One example of these explorations is from the doctoral studies of Donoho (1955) at The Ohio State University, where palpations and measuring PAR size with a pair of calipers were attempted to provide a better way of predicting future milk production. It was found to be very difficult to get accurate measurements of secretory tissue because of variation of PAR amount and location among different animals (Donoho, 1955). In a Technical Bulletin
published by the USDA in 1955, a palpation technique was established for heifers 3 to 6 mo of age, along with a standard for palpated PAR score (Swett et al., 1955). A relationship between palpation grade and lactation was established, but it was communicated that the procedure was not likely to be widely implemented because of the subjective evaluation of the technician, the time that it would take for the technician to be trained, and the high probability of error.

**Ultrasound.** Ultrasound technology that was not available in the 1950s has improved to the point where it provides an accurate picture of tissue distinction, is easily transportable to the calf’s pen, and can potentially replace invasive methods that may call for animal harvesting. By utilizing ultrasound, researchers can examine the quantity of PAR tissue *in vivo* in young heifers and perhaps predict lactation performance potential based on physical presence of PAR tissue. A possible relationship could exist between PAR development in early life and actual lactation performance, but this relationship remains undefined.

**The Science of Ultrasonography.** Ultrasonography utilizes high frequency sound waves transmitted into tissue to allow users to “see” internal structures (Rennie, 1997). A probe will emit sound waves at differing frequencies (measured in megahertz, MHz). The probe itself contains crystals that will resonate as sound waves travel back to the probe (Schmidt, 2007). The rate at which resonance of the crystals occurs translates into an image that can be visualized with an ultrasound screen (Rennie, 1997)(Hendee
and Ritenour, 2003). The ultrasound machine can use the echoes to calculate the time it takes for sound waves to travel back to the probe and create a real-time image of structures (Schmidt, 2007). Because different tissues have differing densities, the sound waves travel through and are refracted back to the probe at different rates, and the time difference is crucial to the observation of different tissue densities (Hendee and Ritenour, 2003). In B-mode ultrasonography, the amplitude of returning wavelengths is interpreted by a transducer converted into a degree of brightness (hence “B”-mode) (Rennie, 1997). This allows a grey scale image to be displayed on the ultrasound screen.

**History of in vivo Imaging.** Noninvasive imaging techniques have been explored for years to allow long-term, non-invasive research in animals (Caruolo and Mochrie, 1967; Cartee et al., 1986; Ayadi et al., 2003; Franz et al., 2009). X-ray tomography in heifers (Sorensen et al., 1987) and magnetic resonance imaging (MRI) in goats (Fowler et al., 1990) were two successful options for imaging MG. However, the size of cattle makes these practices cost prohibitive for dairy research, as MRI was an expensive procedure even in goats (Nudda et al., 2000). Three-dimensional imaging has also been tried in lactating cows, although this is currently another cost prohibitive technique for cattle (Franz et al., 2004), and often yields unreliable measurements.

Ultrasound imaging has been practiced on dairy animals beginning in 1967 to explore anatomical components of the udder and to examine changes in milk ducts (Caruolo and Mochrie, 1967) with the use of A-mode, or “amplitude” ultrasonography.
The output generated in early ultrasound studies was limited to a sonogram, which is essentially a line tracing, and interpretation of the results leaves room for error. Carulo and Mochrie (1967) abandoned the use of higher resolution probes in favor of greater exploration depth into the tissue while using A-mode ultrasound, and as a result detection of secretory tissue was not as sensitive.

B-mode ultrasound was invented in 1962 by Joseph Holmes, William Wright, and Ralph Meyerdirk, but to our knowledge the technology was not applied to MG imaging in domestic animals until 1994. Ruberte et al. (1994) developed an imaging technique in lactating goats and verified its use in evaluation of gland morphology. Ultrasound images were compared with post-mortem sagittal sections of the MG from one ewe used in the trial. The probe was positioned at the posterior side of the udder directed downward toward the gland cistern and teat, and the imaging plane localized between the two rear superficial inguinal lymph nodes (Ruberte et al., 1994). The researchers established the effectiveness of ultrasound in examination of morphological changes of the gland in lactating goats. Additionally, they were able to image the glands while full of milk, and noted possible confusion in the identification of fat and milk due to similar anechogenic properties of the two within glands, and noted that milk itself will be more echogenic with increased fat concentrations, as is seen in colostrum (Ruberte et al., 1994).

Ultrasound has also been used to examine tissue thickness in teats that were affected by mastitis or other factors inhibiting milk flow (Franz et al., 2001; Klein et al.,
2005) with differing probes and resonance. Comparison between ultrasound images and histological samples was successfully performed by (2001) to identify morphological structures responsible for different echoic features of the teat canal.

Ayadi et al. (2003) used ultrasound on lactating cows and successfully imaged gland cisterns. In that study, milk secretions were anechogenic next to the more echogenic PAR (Ayadi et al., 2003), meaning that milk appeared dark while PAR was a lighter contrast in an ultrasound image. Exploration of ultrasound utilization as a means of monitoring mammary growth in older (2.2 to 25.2 mo) heifers was performed by Nishimura et al. (2011). Results from the descriptive study showed that PAR can be detected by ultrasound in 2 mo old heifers. In the ultrasound images, PAR was anechogenic next to echogenic MFP. PAR was visualized as a darker mass in the 2 mo old heifers, but as heifers aged, the contrast of PAR decreased due to normal branching morphogenesis of epithelial ducts into surrounding MFP as well as from increased luminal areas, both of which change the echo signature of the tissue. Nishimura et al. (2011) noted the anechoic features of the duct system in older heifers made visualization very difficult. While Nishimura et al. (2011) did not quantify the echoic features of their ultrasound images, they demonstrated that ultrasound was a viable option for obtaining in vivo images that also allowed for the visual differentiation of PAR from surrounding structures.
Quantitative analysis of PAR in ultrasound images would provide scientific validation for using ultrasound imaging as a viable means of monitoring PAR growth in preweaned heifers. Strzetelski et al. (2004) attempted to quantify the area of the PAR present in primiparous cows between 15 and 7 d prior to calving. Post-ultrasound examination of images enabled them to delineate between adipose tissue and secretory tissue by identifying pixel brightness. Their aim was to develop a method to analyze glandular tissue and predict milk yields of cows before parturition based on a correlation between estimated secretory tissue content and actual milk. However, there were no validation measures in their study, such as congruence with dissected tissue, so their results were inconclusive (Strzetelski et al., 2004).

To our knowledge, ultrasound has not been attempted in preweaned heifers as a quantitative means of tracking PAR growth over time and as affected by diet. Ultrasound could negate the necessity for slaughter studies if it were shown successful in tracking changes in PAR growth for heifers fed differing diets. Knowledge of the growth characteristics of PAR in preweaned heifers could be utilized in future studies and correlated to first lactation milk production. Many dairy producers already implement the use of ultrasonography on their farms to verify pregnancies or for estrus detection. If an ultrasound technique could be developed to quantitatively assess the amount of PAR tissue present in preweaned dairy heifers, it could enable dairy producers to accurately
select high-performing replacement heifers with technology already present on their farm.

This highlights a potential need in the dairy industry. If dairy producers had a way to examine mammary growth in young heifers that accurately reflects milk production potential, they could perceivably pick the best replacement heifers to retain with more certainty of a positive return on investment. Dairy producers that have been in the business for a long time have developed their own ways of picking one heifer over another, but these methods are largely subjective.

Specific Objective

The main objective of this study was to develop and validate an ultrasonography technique that will allow for in vivo monitoring of PAR growth in heifers from birth to 2 mo of age. Diets that varied in fat content were included as an experimental factor in an effort to promote change in MG and body composition. A second objective was to develop and validate an ultrasonography technique that will allow for in vivo monitoring of body composition in the same experimental animals.

Hypothesis

The null hypothesis was that excised tissue analysis will not confirm a capacity for the ultrasound technique to yield accurate measurements of PAR area as measured in
vivo for heifers from birth to 8 wk of age. The alternative hypothesis was that ultrasound images allow for accurate observation of changes in PAR growth over time. The second null hypothesis was that diet will not affect MG or body composition, and the alternative hypothesis was that body composition or MG, or both, would be affected by diet.

*Project Synopsis*

Ultrasound was examined as a possible tool in noninvasive evaluation of body composition changes over time. In this project, the growth of PAR in Holstein heifers from birth to 8 wk of age was explored. Ultrasound images were obtained once a week to chart the growth of PAR over time. In the 8th week of life, heifers (n = 24) were harvested and udder samples were collected. In the final week of the study, PAR present in the udders of the heifers was compared morphologically to the images derived from the ultrasound. By ascertaining that the ultrasound images obtained immediately before harvesting were not different from the area of PAR observed from the excised mammary tissue, the ability for ultrasound to confirm PAR size was demonstrated.

Diet did not affect mammary or body composition in this study. Growth curves were plotted for PAR of prepubertal heifers on different diets, and two dimensional area grew over time as the heifers grew. No correlation was found with ultrasound and body composition characteristics. Correlation studies revealed that PAR growth in early life may be an indicator of lactation potential.
CHAPTER 3: MATERIALS AND METHODS

Developing the Ultrasound Technique

Prior to the start of the trial, 2 mo old Jersey calves located at the Waterman Dairy Center (The Ohio State University, Columbus, OH) were utilized to establish scanning methodology, as approved by The Ohio State University Office of Responsible Research Practices (IACUC Protocol # 2011A00000070). Both linear and convex probes of varying frequencies were tested. Use of an Ibex Portable Ultrasound (E.I. Medical Imaging, Loveland, CO) with a 6.0MHz linear probe was tested. The probe was awkward to correctly position because of the cord location on the probe, and it was difficult to maneuver around specific quarters. Use of this US machine and probe combination was abandoned. Next, a 3.5MHz linear probe, 5MHz convex probe, and 7.5MHz convex probe were each tested with an ALOKA 900 Ultrasound (Aloka America, Wallingford, CT). The 3.5MHz linear probe was too large to fit between the legs of the calves, and provided visualization for more depth than necessary. The 5MHz convex probe also was too large and provided more depth resolution than necessary. It was determined that the 7.5MHz convex probe was small enough to easily maneuver and provided optimal resolution for the shallow depth of PAR.

Vegetable oil was initially used as an acoustic aide, but contact between the hide and probe was poor; ultrasound gel was deemed necessary for optimal visualization.
Udder hair removal with clippers was discussed as a potential necessity, but this procedure was not implemented until the last ultrasound of the trial (October 5), mainly to ease udder dissections. Hair did not interfere with any of the previous ultrasound images.

Calves were first made to stand while the probe was positioned underneath of each MG. This was unsuccessful because of an inability to maneuver around the MG, and calves were allowed too much freedom to move. Calves were subsequently laid dorsally to maintain control of the animals, and allow for better visualization of each gland.

**Calves and Management**

Twenty-five Holstein heifers and 25 Holstein bulls (2-3 d old; 41 ± 1 kg initial BW) were purchased from a single dairy farm in Indiana and shipped 3.5 h to the Nurture Research Center (Provimi North America) in New Paris, OH. All calves were provided daily care following acceptable practices as described in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). One of three milk replacer (MR) diets was fed to each calf. All MR contained 27% CP and had varied fat sources: A) only animal fat (17% fat), B) animal fat supplemented with butyrate, medium chain fatty acids, and linoleic acid (17% fat), and C) milk fat (33% fat). The MR were diluted at 15% solids with warm water and fed at 680 g of as-fed powder, which was divided and fed equally at morning and afternoon meals. Whey, whey protein
concentrate, and animal fat encapsulated with whey protein were used in manufacturing milk replacers A and B, as is typical in the US feed industry. Instead of liquid milk, dry, whole milk, selected to be 27% CP and 33% fat, comprised MR C and provided a diet consistent to MR A and B; all powders were blended with a common trace mineral and vitamin premix. All calves were allowed ad libitum access to calf starter (20% CP, DM basis) and fresh water. Calves were housed in 1.2 by 2.4 m individual pens, bedded with long straw, within a curtain sidewall barn with no added heat.

The following procedures were performed by the farm crew employed by Provimi North America. Calves were weighed every 7 d until the end of the trial (d 56). The quantity of dry feed offered and feed refusals were weighed daily. Hip width was measured with calipers, and body condition score (BCS) of calves were measured on d 0 and every 14-d until d 56 by a single experienced technician. A 1 to 5 system using 0.25 unit increments, with 1 being emaciated and 5 being obese, was used for BCS (Wildman et al., 1982). Scores were based on changes around the vertical and transverse processes of the spine, as palpated by one experienced technician, and ranged from 1.5 to 3.5. The 56 d trial was conducted during the months of August through September of 2011. The average ambient temperature inside the barn during the trial was 20°C and ranged from 5 to 38°C based on hourly measurements. Any abnormalities or changes in health or body condition were noted.
Calves received an intranasal, tissue sensitive, respiratory disease vaccine (TSV-2®, Pfizer, Exton, PA) and subcutaneous injections of vitamins A, D, E (Vital E® - A + D, Schering-Plough Animal Health, Union, NJ) and Se (MU-SE®, Schering-Plough Animal Health, Union, NJ) upon arrival. Calves received an intramuscular respiratory disease vaccine (Bovashield® Gold 5, Pfizer, Exton, PA) at d 7 and d 28. All medical treatments were under a protocol of a veterinarian and were based on scouring (fecal scores >2), lethargy, elevated rectal temperatures, and rapid breathing.

Every second bag (22.7 kg) of MR and starter feed was sampled and composited. Composites of feeds were analyzed (AOAC, 2000) for DM (oven method 930.15), CP (Kjeldahl method 988.05), fat (alkaline treatment with Roese-Gottlieb method 932.06 for MR; diethyl ether extraction method 2003.05 for starters and hay), and fatty acids (GC; method 963.22). The procedure of Van Soest et al. (1991) was used to analyze starter NDF, and ADF was analyzed using procedures in Robertson and Van Soest (1981).

Blood samples were collected from the same 4, randomly selected calves per treatment on d 0, 7, 21, and 28; additionally, blood samples were also collected from all heifers on d 0, 49, and 56. Blood sampling was conducted to evaluate potential health benefits from the varied sources of fatty acids; data not shown. At 8 wk of age, female calves were transported to The Ohio State University Meat Lab for harvest (described later).
**Mammary Examination Procedure**

The 24 heifers enrolled in the trial were evaluated for mammary growth by weekly teat length measurements, palpation scoring and mammary ultrasound.

**Teat Length.** Teat lengths were measured weekly by a single person using a 10 cm ruler. All measurements were made prior to ultrasound evaluation.

**Palpation Score.** By 5 wk of age, most heifers developed a palpable quantity of PAR. At 6 wk of age, all heifers had developed palpable PAR, and PAR was palpated at each subsequent week and recorded on a subjective scale of 1 to 6 by a single researcher. The scale is described as follows: 1 being small and threadlike, comparable to a small grain of rice, 2 a larger grain of rice, 3 a small pea, 4 a large pea, 5 as an almond, and 6 as bigger than an almond. Further descriptive notes were made to indicate the overall shape of the PAR, which varied between a rounder feel or an oblong three dimensional shape.

**Mammary Ultrasound.** A real time B-mode ultrasonograph (Aloka 900, AlokaAmerica, Wallingford, CT) with a 7.5MHz convex probe was used to examine PAR area of all four quarters (glands) in calves from 0-2 d of age to 8 wk of age. For the first 2 wk of the trial, still ultrasound images were captured using MediCapture USB170 Software (MediCapture, Inc, Philadelphia, PA). Beginning at 3 wk, ultrasound videos were captured with a DVD recorder connected to the Aloka Ultrasound machine. Still images were later captured from videos using the video processing software Snagit 10 (TechSmith, Okemos, MI). At each exam heifers were laid dorsally with a slight
tendency to the lateral left side. They were manually restrained, and MG were consistently examined in the following order: right front, right rear, left front, and left rear. A single person performed all mammary ultrasound examinations. Ultrasound gel (Chattanooga Group, Inc., Chattanooga, TN) was applied to each quarter to enhance contact with the probe and the hide for optimal visualization. The probe was placed directly on top of the teat, and because applying pressure to the teat caused it to fold, the probe was positioned so that the base of the teat would appear consistently on the left side of the ultrasound screen. Location of PAR varied from calf to calf. The teat was used as a biological marker for aide in identification of PAR.

PAR appeared as a dark contrast compared to surrounding tissue (Figures 1 and 2) and could be tracked in its approximate location dorsal to the teat by palpation and by following the dark contrast with the probe from the teat area. Ultrasound was performed once weekly, including the day before harvest.

**REA Examination Procedure**

REA was measured weekly by ultrasound between the 12th and 13th ribs on the right side of female calves only. Ribeye images were scanned and captured with an Ibex Portable Ultrasound with a 6.0MHz linear probe in order to determine ribeye area (cm²) for the first 5 wk of the trial. After that, REA became too large for this probe, so REA scans were captured with the Aloka 900 and a 3.5MHz linear probe, as seen in Figures 3.
and 4. A standoff pad molded to fit the rib area was used for attachment to the ultrasound probe to aid in ribeye image collection.

**Harvest Procedure**

Heifers left the Nuture Research Center (Provimi North America) in New Paris, OH at 1300 h on October 7, 2011 and arrived at The Ohio State University Meats Lab in Columbus, OH at 1500 h. Udders were clipped as described, and all animals were subjected to mammary and REA procedures described earlier, as this was the final weekly measurement (8 wk). Heifers were fasted overnight and harvested at 0600 h the next day. Heifers were euthanized by captive bolt stunning followed by exsanguination. Rear quarters of the udder were marked with a permanent marker. The entire udder was removed, weighed, and bisected along the medial suspensory ligament. Subsamples of PAR and MFP from the right half of the udder were excised and either immediately snap-frozen in liquid nitrogen and stored at -80°C or fixed in 10% formalin for later analysis (data not shown).

**Mammary Tissue Dissection.** The left udder halves were individually stored in plastic containers and stored at 4°C for 24 h. Then left udder halves were brought to room temperature and a final ultrasound analysis was performed on both the front and rear quarters (Figures 5 and 6). Teat location in relation to PAR on the ultrasound images was confirmed by placing a highly echogenic needle into the teat end until it reached PAR, as
can be seen in Figure 5. Next, the front and rear quarters were bisected by cutting along
the surface at the inserted needle to make a sagittal cut. After the final ultrasound
examination, the central mass of PAR was bisected using the teat as an anatomical
landmark to give a view of the sagittal plane of the PAR in each remaining gland. This
was to correspond with ultrasound images, which also produced a sagittal view of PAR,
similar to the procedure done by Nishimura et al. (2011). Digital images were then taken
with a handheld camera with a ruler included with the udder section. The two-
dimensional areas visualized from the dissection were compared to the two-dimensional
areas measured by ultrasound. After bisection and image capture, the left udder halves
were placed in individually labeled plastic bags and frozen at -20°C for later dissection
and biochemical analysis.

Lab Methods for Mammary Gland Analyses

*Homogenization of Tissue.* At a later date, left udder halves were removed from
storage at -20°C and allowed to thaw for 1 h at 4°C. Semi-frozen udder halves were
weighed and then dissected into PAR, MFP, hide, and teats, by color. Each section was
weighed. PAR and MFP were blended with dry ice to form powdered tissue and stored in
individually labeled plastic bags at -80°C. These samples were used in DNA, lipid, and
protein assays.
Protein and DNA content of MFP and PAR were determined after homogenization of each sample. Homogenates were prepared using a Qiagen TissueLyser LT (Qiagen, UK). Briefly, for DNA homogenates, 2 mL microcentrifuge tubes containing 1 stainless steel bead (7mm) each were placed on dry ice for 15 min. Approximately 30 mg of tissue were added to each tube and placed on dry ice for 15 min. The tubes were then placed in the Qiagen TissueLyser LT Adapter for 2 min at room temperature. Lysis buffer (High Salt Buffer; 0.05 M Na$_2$PO$_4$ + 0.002 M Na$_2$EDTA) was added to MFP and PAR samples, 150µL and 300µL, respectively. Samples were homogenized in the Qiagen TissueLyser LT for 5 min at 50 Hz, then stored at -20°C until analyzed. Protein samples were homogenized as described above with 1X phosphate buffer saline in place of the high salt buffer.

**Protein Assay Miniplate Procedure.** Protein analysis was performed using the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Pierce Chemical Co., Rockford, IL), with bovine serum albumin as the standard. A standard curve was prepared with each 96-well plate. MFP homogenate samples were pipetted into wells at 10µL in duplicate. PAR homogenate samples were pipetted into wells at 2µL in duplicate. Intraassay coefficient of variation (CV) averaged 3.72% for MFP and 5.55% for PAR.

**DNA Assay.** A DNA assay was performed using a DNA quantification kit and fluorescence assay (DNAQF; Sigma-Aldrich, St. Louis, MO). The bisBenzimide Hoechst 33258 dye reagent was used with calf thymus DNA as the standard. Briefly, 2µL of PAR
or 10µL of MFP homogenate samples were added to 2mL of assay solution. The assay solution contained 30µL of bisBenzimide Hoechst 33258 (1mg/mL), 3mL 10X Fluorescent Assay Buffer, and 27mL of molecular grade water. Samples were measure in triplicate on a Turn Quantech Digital Fluorometer (Barnstead/Thermolyne, Dubuque, IA) set on raw fluorescence mode at 360nm excitation (QuantechNB360, Dubuque, IA) and 460nm emission (Quantech SC430, Dubuque, IA) at ambient temperature. Intraassay CV averaged 4.17% for MFP and 4.64% for PAR.

**Lipid Assay.** Lipid content of PAR and MFP was determined by gravimetric analysis essentially according to the method of Hara and Radin (1978) as described recently by Daniels et al. (2009). Extraction of lipid was performed using 9mL of hexane:isopropanol (3:2; vol/vol) added to 500 mg of tissue powder. Butylated hydroxyanisole (Sigma Chemical Co.) was added to prevent fat degradation by acting as an antioxidant. After vortexing tubes, 6 mL of aqueous sodium sulfate (1 g of anhydrous salt per 15 mL of H₂O ) was added. Phases were allowed to separate for 5 min, tubes were vortexed again, then centrifuged for 5 min at 1,000 x g. The upper solvent phase contained the dissolved lipid and was transferred by Pasteur pipette to a clean, pre-weighed tube. 5 mL of hexane:isopropanol were added to the residual lipids left in the original tube. Tubes were vortexed and centrifuged for 5 min at 1,000 x g. The upper solvent layer was again removed and transferred into a pre-weighed tube. The lipid-rich solvent layer was evaporated under N₂ gas in a water bath at 40°C under an analytical
evaporator (N-EVAP model no.112, Organomation Associates Inc., South Berlin, MA). After solvent evaporated, tubes were allowed to sit overnight to allow solidification of lipid residue. Tubes were reweighed, and lipid mass was calculated as the final tube weight minus the initial tube weight. Samples were measured in duplicate. Average intraassay CV was 3.47%.

Lipid analysis as described for MFP required 1 g total tissue, but 19 out of 24 PAR samples had less than 1 g PAR tissue. Assay linearity was checked using 1 g, 500 mg, 250 mg, 125 mg, and 50 mg of starting material in duplicate. It was determined that a minimum of 250 mg of tissue was needed for a single sample for both accuracy and precision, and duplicates would require at least 500 mg of tissue. Fourteen of 24 PAR samples had less than 500 mg of tissue. The decision was made not to complete the lipid analysis in PAR samples.

**Body Composition Approximation**

Kidney, pelvic, and heart fat weight were removed and weighed immediately after harvest to be used in determination of percent KPH of the carcass. Body composition analysis was performed based on procedures as described in Nour and Thonney (1994). The 9-10-11\textsuperscript{th} rib section was collected from the right side of each carcass after they had been shrouded and cooled for 96 h. The section was obtained by measuring 5 cm from the end of the ribeye toward the spine and cutting the rib section away from the carcass.
Each rib section was vacuum sealed, and transported to Wooster, OARDC, and frozen at -20°C for later analysis of water, lipid, and protein content. Equations were derived from Hankins and Howe (1946) with covariates modified by Nour and Thonney (1994).

The 9-11th rib section excluded rib meat between the 8th and 9th rib and included meat between the 11th and 12th section. The section was deboned and spinal cord was discarded. The bone component was weighed then discarded while the lean and fat portion was weighed and ground in a 10-cup Food Processor (Hamilton Beach Brands, Southern Pines, NC) for 1 min on pulse setting. Blended tissue was stored in plastic bags at -20°C. After samples were completely frozen, they were weighed and placed in a vacuum seal chamber for freeze-drying at 100mm Hg for 3 d. Following the freeze-dry procedure, samples were weighed and dry matter was determined. Samples were blended a second time to achieve a powder form. Samples were stored in plastic bags at -20°C until ether extraction and Kjeldahl analysis.

**Ether Extraction.** The percent crude fat was determined by ether extraction. Done in duplicate, 1.000 g of sample was weighed (W) into a solvent resistant bag and heat sealed. Bags were placed in an 100°C oven to remove all moisture, cooled in a dessicator bag, then weighed again (WPD). Samples were placed in the Ankom XT 10 Extractor (Ankom Technology) for 40 min extraction at 90°C. After extraction was complete, extracted bags were placed in a drying oven for 30 min, cooled, and weighed (WF). Percent crude fat was determined with equations [1] and [2]:
Kjeldahl Analysis. The nitrogen concentration was determined (equation [3]) in order to determine percent protein (equation [4]). Nitrogen was determined by block digestion and steam distillation using the Kjeltec 2200 Auto Distillation unit (FOSS Analytical, Hillerod, Denmark). Briefly, 0.1500 g of sample was weighed into 250 mL digestion block tube. Two Kjeldahl catalyst tablets (Kjeltabs Cu, 3.5 g K$_2$SO$_4$ and 0.4 g CuSO$_4$ per tablet) were placed in each tube. 15 mL of H$_2$SO$_4$ was added to each tube, then tubes were placed on digestion block heated to 420° C for one hour. Samples were automatically distilled then manually titrated with H$_2$SO$_4$ to a grey/pink endpoint. Volume of acid required for color change was recorded and used to determine protein content. Samples were done in duplicate.

\[
% \text{Nitrogen} = \frac{(mL \text{acid} - mL \text{blank}) \times 0.001}{\text{gram sample} \times \% \text{Dry Matter}}
\]

\[
% \text{Protein} = % \text{Nitrogen} \times 6.25
\]

The resulting values from the rib section were used to calculate the full body composition of the carcass (equations [5] and [6]), as developed by Nour and Thonney (1994) and as used for body composition analysis in Holstein calves (Abdelsamei et al., 2005):

\[
\text{Fat}_{\text{Carcass}} = 0.79 \times \text{Fat}_{\text{Rib}} + 2.78
\]
\[ Protein_{\text{Carcass}} = 0.7 \ Protein_{\text{Rib}} + 5.29 \]

where \( Fat_{\text{Rib}} \) and \( Protein_{\text{Rib}} \) are the fat and protein concentration of the 9\textsuperscript{th}-11\textsuperscript{th} rib-section.

\textit{Mammary Data Analysis}

\textit{Ultrasound.} DVDs were opened with VLC media player software (VideoLAN Organization) on a personal computer. Ultrasound playback videos were monitored and paused when PAR was fully visualized. Rapid capture of multiple images was possible with Snagit software (TechSmith Corporation) by pressing the “PrntScrn” key on the keyboard. Images were stored by Snagit and were later saved as labeled TIFF files. Images were analyzed to determine PAR area (cm\(^2\)) using the program ImageJ (NIH). The scale of pixels per cm was calibrated in ImageJ using the “straight” tracer. The tick marks on the ALOKA ultrasound images were 2 tick marks per centimeter, and when measured with ImageJ resulted in 150 pixels per centimeter. MediCapture images resulted in 130 pixels per centimeter when calibrated, pertinent for the first 2 wk of the trial. Once the appropriate scale was set, areas were derived by tracing the PAR area with the mouse and cursor using the “freehand” tracer. Pixel area\(^2\) was converted to cm\(^2\).

\textit{Bisected Tissue Images.} As stated previously, left udder halves were bisected to reveal a sagittal plane view of PAR (\textbf{Figures 7} and \textbf{8}). A ruler was included in pictures of bisected tissue to assist in calibrating the scale for ImageJ. PAR area was determined with the same procedure used to determine ultrasound PAR area.
Ribeye Area. The Ibex Portable Ultrasound was utilized until 5 wk of the trial, at which point REA grew beyond the capacity of the Ibex Portable Ultrasound to capture the entire rib section in one image. The Aloka 900 with video recording was used for the remainder of the trial, as described previously. The tick marks on the Ibex images were 1 tick mark per centimeter, and resulted in 58 pixels per cm. REA was determined with ImageJ with the same procedure used to determine ultrasound PAR area.

Statistical Analysis

Heifer was the experimental unit. Data from week 8 ultrasound PAR area and week 8 dissected PAR area were analyzed by two-way ANOVA using PROC MIXED procedure of SAS (Version 9.2, SAS Institute, Inc., Cary, NC). An autoregressive covariance structure [AR(1)] was used throughout the analyses; no denominator degrees of freedom approximation was specified. Fixed effects of diet, week, location (front or rear) and side (left or right) of the gland, all 2-way interactions, and the random term of heifer within diet were represented by the final model:

\[ Y_{ijklm} = \mu + D_i + W_j + L_k + S_l + H_{(ij)m} + E_{ijklm}, \quad E_{ijklm} \sim iid N(0, \delta^2 e) \]

where \( Y \) = dependent variable; \( \mu \) = overall population mean; \( D_i \) = diets 1, 2, 3; \( W_j \) = week 1,…,8; \( L_k \) = location 1, 2; \( S_l \) = side 1, 2; \( H_{(ij)m} \) = random effect of mth heifer within ith diet (1,…,8 per diet); and \( E_{ijklm} \) = residual error term, assumed to be random and
independently distributed. Orthogonal polynomial contrasts were used to test for linear, quadratic, and cubic responses over time.

All 8 wk paired data were analyzed using Pearson’s correlation coefficients with SAS. Additionally, all 8 wk data were analyzed in a separate mixed model analysis that contained fixed effects of diet, quarter (left front or left rear), and mode of PAR area analysis (ante or post-mortem, bisected tissue), and all 2-way interactions. Heifer within diet was the random term. The three way interaction of diet, quarter, and mode was considered as a model term, but was removed from the final model due to lack of significance. The following model was used:

\[ Y_{ijkl} = \mu + D_i + Q_j + M_k + DQ_{(ij)} + DM_{(ik)} + QM_{(jk)} + H_{(i)}l + E_{ijkl}, \]

\[ E_{ijkl} \sim \text{iid} N(0, \delta^2 e) \]

where \( Y \) = dependent variable; \( \mu \) = overall population mean; \( D_i \) = diets 1, 2, 3; and \( Q_j \) = quarter 1, 2; \( M \) = mode of PAR area analysis 1,2,3; \( DQ \) = fixed interaction of diet and quarter; \( DM \) = fixed interaction of diet and mode; \( QM \) = fixed interaction of quarter and mode; \( H_{(i)}l \) = random effect of lth heifer within ith diet (1,…,8 per diet);, and \( E_{ijkl} \) = residual error term, assumed to be random and independently distributed.

When significant, treatment means were separated using preplanned nonorthogonal contrast statements for diet (MR A vs. B, MR A vs. C). Data are reported as least squares means ± standard error of the mean. Significance was declared at \( P \leq 0.05 \) for all procedures.
CHAPTER 4: RESULTS

PAR Growth and Correlation

Data on weekly PAR measurement per diet can be found in Table 1. The diet by week interaction was not significant \( (P = 0.771) \). PAR areas grew over time \( (week, P = 0.001) \) and were not different due to diet \( (P = 0.947) \). Initial PAR area averaged \( 0.049 \pm 0.020 \text{ cm}^2 / \text{gland} \) and increased to \( 0.420 \pm 0.015 \text{ cm}^2 / \text{gland} \) at 56 d ante-mortem. These values changed over time in a complex pattern \( (linear, P = 0.834; \text{quadratic}, P = 0.001; \text{cubic}, P = 0.003) \). Front glands were, on average, smaller than rear glands, with an average front:rear gland ratio of 47:53 \( (P = 0.002) \). There was no difference between the two front \( (P = 0.834) \) or the two rear teats \( (P = 0.926) \). Teat length \( \text{(Table 2)} \) grew weekly \( (P = 0.0001) \) in a complex way \( (linear, P = 0.0001; \text{quadratic}, P = 0.0009) \). There was no correlation between front PAR area \( \text{(ante-mortem)} \) and front teat length \( (r = 0.266, P = 0.2098) \) or rear PAR area \( \text{(ante-mortem)} \) to rear teat length \( (r = 0.265, P = 0.21) \). Correlations of teat length and PAR area ante-mortem by week were not significant \( (r > 0.40, P > 0.05) \). Additionally, front and rear teat lengths were different in length; front teats were longer than rear teats, on average \( (P = 0.0001) \).

Mammary PAR area at 8 wk of age was positively correlated with all other paired variables analyzed, as seen in Table 3. Correlations were performed between PAR area
of bisected sagittal plane view, PAR area ante-mortem from US, PAR area postmortem from US, and PAR weight.

Manual palpation of heifer PAR began at 6 wk of age when PAR became discernible from MFP by feel. Palpation scores showed no difference by diet \( (P = 0.4977) \). The scores increased by week \( (P = 0.0001) \) in a linear \( (P = 0.0001) \) and quadratic \( (P = 0.0295) \) manner. Rear quarter palpation scores were significantly larger than front quarter palpation scores \( (P = 0.0001) \), with no difference between the two front quarters \( (P = 0.8976) \) or the two rear \( (P = 0.9487) \) quarters.

**PAR and MFP Composition**

A summary of PAR and MFP composition can be found in Table 4. Lipid, DNA, and protein content of MFP did not differ by diet. Similarly, DNA and protein content of PAR did not differ by diet. Correlations with PAR composition revealed a significant positive correlation between percent PAR protein and PAR area \( (r > 0.40, P > 0.05) \).

**Body Composition**

Initial and final body weights were not different by diet (Table 5). MR intake and starter intake did not differ by diet, but an increased feed efficiency was observed in calves fed MR B \( (P = 0.01) \) and MR C \( (P = 0.01) \) when contrasted with MR A. This led to increased ADG in heifers fed MR B and C over MR A heifers.
Ribeye area (REA) grew linearly by week \((P = 0.001; \textbf{Table 6})\), with no difference due to diet \((P = 0.5375)\). The exposed REA of the carcass was examined, and a positive correlation was shown between REA ante- and post-mortem at 8 wk of age \((r = 0.74, P = 0.0001)\). No correlation existed between REA and body composition of protein \((r = -0.06, P = 0.7861)\), lipid \((r = -0.32, P = 0.1775)\), or water \((r = -0.37, P = 0.4603)\). Carcass weight and carcass composition, including percent protein, lipid, and water, did not differ by diet \((\textbf{Table 7})\). BCS was evaluated every 2 wk by one technician; measurements were correlated with appropriate weekly REA and body composition data. No correlation was shown between BCS and REA \((r = 0.20, P = 0.372)\) or body composition, including protein \((r = 0.35, P = 0.0876)\), lipid \((r = -0.26, P = 0.2142)\), or water \((r = 0.16, P = 0.4603)\).
CHAPTER 5: DISCUSSION

Basal tissue establishment may be a reliable indicator for developmental potential of PAR in heifers, but there is currently no acceptable way to monitor PAR growth in vivo. Ultrasound is useful for external imaging of mammary glands in dairy heifers (Nishimura et al., 2011). Differences in molecular composition of PAR and MFP allow the two to be visualized as dark and bright contrasts, respectively, with ultrasound. Nishimura et al. (2011) was able to obtain images of dairy heifers at 2 mo of age up to heifers in late pregnancy, with comparison to mammary tissue in postmortem examination. No studies to date have evaluated the growth of PAR with ultrasound in either preweaned heifers or in serial ultrasounds of the same heifer over time. This study was designed to establish ultrasound as an effective method for monitoring PAR growth, with a novel component in examination of preweaned heifers, and to verify the effectiveness of ultrasound through comparison of ultrasound to harvested tissue. It was hypothesized that PAR growth would be measurable with ultrasound, and that weekly analysis of PAR would result in changes of PAR size over time.

First, we wished to know if PAR area could be measured in preweaned heifers soon after birth. Evaluation of the quantity of PAR at birth has previously been evaluated as negligible (Meyer et al., 2006b). Preliminary evaluations on Jersey calves housed at the The Ohio State University dairy farm revealed that some PAR was present, suggesting
that PAR would be detectable in preweaned Holstein calves. Although this experiment suffered from technological difficulties in the first week that resulted in data loss, in data that were analyzed, it was shown that some heifers did not possess any discernible PAR at 2 to 3 d of age. The average PAR area in the first week of life did not exceed 0.05 cm²/gland when analyzed by ultrasound (n = 14 out of 24 heifers). Similarly, in palpation exams performed prior to week 6, PAR was not readily discernible from surrounding MFP. By week 6, PAR mass was palpable in nearly all animals and that is when and why palpation scoring commenced.

Second, we wanted to establish ultrasound as a method for evaluating PAR area in preweaned heifers. Correlations were made between PAR areas derived from ante- and post-mortem ultrasound and bisected tissue, with additional correlation with weight of PAR. Positive correlations of PAR area were shown in all 8 wk PAR area measurements and PAR weight. The correlations demonstrate consistency in the ultrasound method for determining PAR area in vivo and thereby validate the ultrasound method in examination of PAR area in preweaned heifers. While not directly validated, given the strong correlation between PAR area and weight at 8wk, we feel our ultrasound measurements obtained in vivo from weeks 1 through 7 are accurate, precise, and reflect how PAR actually grew over time. To that point, PAR area was plotted by week, with distinction made between front and rear glands; (Figure 9) shows the complex pattern of PAR growth over time, and data within the 95% confidence interval are within the ellipse.
No differences were observed between right and left gland areas (right front vs. left front; right rear vs. left rear). By contrast, the average area of the front glands was significantly smaller than the area of the rear glands. A typical front:rear ratio for gland cistern area in lactating cows is 40:60, as demonstrated by Ayadi et al. (2003) in an ultrasound experiment of cisternal area. The 40:60 ratio was consistent with measured cisternal milk volume. A regression analysis confirmed the accuracy of ultrasound in predicting cisternal milk yield, as correlated with cisternal milk area (Ayadi et al., 2003). Here, rear glands were found to be larger than front glands in preweaned heifers as well (47:53, front:rear). Also, palpation scores were reflective of results obtained from ultrasound. Although palpation is highly subjective, it was shown that one technician could effectively perform palpation exams that later allowed for the detection of weekly changes in PAR size that were consistent with ultrasound findings. Palpation exams were performed to supplement data obtained by ultrasound using a technique similar to that reported in 1955 (Donoho, 1955, Swett et al, 1955). Also it should be noted, palpation exams always took place prior to ultrasound examination; this was helpful in determining approximate PAR structure and location before the ultrasound probe was placed for the exam.

Teat length was not shown to correlate with PAR area ante-mortem by week. Teat length in both front and rear locations did not correlate with PAR area in front and rear
locations, but longer teats were noted for front quarters compared to rear quarters. Teat length was not shown to be a good predictor of PAR area in this experiment.

Heifers were provided one of three MR diets differing in fat source and concentration, while protein concentration was consistent across diets. PAR and MFP of the MG were evaluated for protein and DNA content. Additionally, MFP was evaluated for lipid content (there were insufficient total amounts of PAR tissue to complete the lipid assay in heifers at 8 wk of age). In previous work by Daniels et al. (2009a), no effect of MR composition was seen on PAR size or composition, while total MFP lipid and MFP lipid concentration were influenced by diet. Meyer et al. (2006b) performed an analysis of MFP and PAR content when heifers were harvested at 50-kg increments, and found differences in PAR DNA content; first age of harvest was 2.7 mo. They concluded that nutrient intake did not affect PAR composition, but age at harvest influenced total PAR DNA content. Brown et al. (2005a) also observed increased amount of PAR and PAR DNA due to increased dietary protein and energy in 2 to 8 wk old heifers. In this study, neither PAR nor MFP composition differed due to diet (Table 4). It should be noted that total quantity of MG, as well as quantity of MFP and PAR tissue, was much lower for heifers in this study compared to heifers in the aforementioned studies, at 8 wk of age, despite being of similar final BW (71.73 ± 1.64 kg here; 80.65 ± 1.23 kg in Daniels et al. (2009a); 66.05 ± 1.5 kg in Brown et al. (2005b); average final BW ± SEM). In this study, there was only 18.3% as much PAR (g PAR/ 100 kg EBW) as Daniels et al. (2009a), and
51.9% as much PAR as Brown et al. (2005a). It is possible that genetics are the cause of these differences in PAR tissue presence. At 8 wk of age, it is clear that drastic differences in PAR presence can occur; more research is needed to determine the influence of basal tissue differences on future milk yield.

A second part of this experiment was to examine the utilization of ultrasound in noninvasive evaluation of body composition. First, body growth changes were observed by week (Table 5). There was no difference in body weight due to diet, but increased feed efficiency and ADG were observed in heifers fed MR B and C over MR A. Starter and MR intake were the same across diets. Changes in feed efficiency may be a result of higher bioavailability of fatty acids to heifers. With the same feed intake, heifers on MR B and C had improved weight gain over heifers fed a conventional MR.

REA was measured on a weekly basis to interrogate whether or not ultrasound analysis of ribeye could demonstrate growth over time. REA grew linearly by week, and was not affected by diet (Table 6). While REA grew in a different pattern than PAR, as analyzed by ultrasound, the observation of weekly growth in REA confirms its utility in the establishment of a growth curve for REA. Biological causes for the variation in PAR growth are not well understood. The differences in growth pattern may be attributed to allocation of nutrients for growth. Mammary tissue growth in preweaned heifers is likely a secondary target of nutrient allocation, because it is serving no real function at this point in the heifer’s life. A second interpretation may be that allometric growth of PAR
commences earlier than the documented 3 mo of age, as reported by Sinha and Tucker (1969). Sinha and Tucker’s youngest age group was 3 mo old heifers. It was hypothesized that evaluation of REA may provide information on carcass composition. No positive correlations of REA to body composition were observed in heifers 0 to 8 wks of age. This is not expected biologically, because intramuscular fat deposition does not often exceed 5%, as opposed to body fat. The thickness of body fat is easily measured via ultrasound. Therefore at this time, we cannot say with confidence that weekly US measurement of REA is correlated with body composition in any meaningful way. Every 2 wk, BCS was evaluated. A correlation analysis between BCS and REA was not significant, nor was there any correlation with BCS and body composition. This result was expected: BCS is subjective and estimates fat accumulation, while REA quantitatively measures muscle. The two techniques are assessed to be independent in body composition evaluation. Additionally, the animals used in this study were too young to begin fattening. Neither external evaluation nor in vivo analysis was shown to be an effective predictor of body composition.

Previous studies have used the 9th-11th rib section analysis in Holsteins to estimate full body composition (Abdelsamei et al., 2005). The 9th-11th rib-section from heifers in this study was evaluated for percent protein, lipid, and water. No differences in body composition due to diet were observed. Body composition was approximately 14.3% protein, 5.7% fat, and 72.4% water, which is in agreement with other evaluations of
Holsteins 8 wk of age and at similar BW (Brown et al., 2005b). As an example, this data was in agreement with Brown et al. (2005b), where no difference in carcass composition of Holstein heifers was observed, and body composition was averaged to be 19.9% protein, 4.8% fat, and 71.2% water at 8 wk of age.
CHAPTER 6: CONCLUSIONS

Obtaining PAR measurements via ultrasound is an effective tool for measuring weekly changes in PAR area in vivo. As long as dietary nutrients are not limiting, it seems that PAR growth may rely more on initial tissue foundation within a given animal rather than on dietary manipulation, prior to 2 mo of age. Determination of PAR area by ultrasound examination is quantitative and accurate. Neither ultrasound analysis of REA nor BCS was effective in the determination of body composition. Similarly, ultrasound of PAR was not effective in determination of MG composition. Future studies may incorporate the methods developed here to determine what quantitative evaluation of PAR can reveal about production capacity. Researchers could initiate a longevity study in order to observe the quantity of PAR present at a young age, monitor PAR growth during the preweaning period, and with later comparisons, and discover the implications of early-life epithelial tissue development and timing of nutrient manipulation on future milk yield.
REFERENCES


Moran, C., J. F. Quirke, D. J. Prendiville, S. Bourke and J. F. Roche. 1991. The effect of estradiol, trenbolone acetate, or zeranol on growth-rate, mammary development,


Table 1. Average parenchyma (PAR) area (cm$^2$/gland) from 0-8 weeks of age. Least squares means and interactions of PAR measurements per gland by diet and week.

<table>
<thead>
<tr>
<th>Diet</th>
<th>PAR area$^1$</th>
<th>SEM$^1$</th>
<th>Diet</th>
<th>Week</th>
<th>x week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.056</td>
<td>0.039</td>
<td>0.948</td>
<td>&lt;0.001</td>
<td>0.771</td>
</tr>
<tr>
<td>NeoTec4</td>
<td>0.064</td>
<td>0.039</td>
<td>0.948</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Milk fat</td>
<td>0.028</td>
<td>0.039</td>
<td>0.948</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

$^1$PAR area was determined through ImageJ software, where calibrations standardized the number of pixels per centimeter. Measurements are in square centimeters (cm$^2$).

$^2$Diet: All milk replacer with 27% CP; Control = control milk replacer with animal fat (17% fat); NeoTec4 = animal fat supplemented with butyrate, medium chain fatty acids, and linolenic acid (17% fat); Milk fat = 33% fat. Heifer number for all diets, n = 8.

$^3$SEM = standard error of the mean for diet x week; highest listed.
Table 2. Average teat length (mm) from 0-8 weeks of age. Least squares means and interactions of teat length measurements per gland by diet and week.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Teat Length, mm 0</th>
<th>Teat Length, mm 1</th>
<th>Teat Length, mm 2</th>
<th>Teat Length, mm 3</th>
<th>Teat Length, mm 4</th>
<th>Teat Length, mm 5</th>
<th>Teat Length, mm 6</th>
<th>Teat Length, mm 7</th>
<th>Teat Length, mm 8</th>
<th>SEM²</th>
<th>P-value</th>
<th>Diet x Week</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>10</td>
<td>13</td>
<td>11</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>16</td>
<td>1</td>
<td>0.31</td>
<td>&lt;0.0001</td>
<td>0.195</td>
<td></td>
</tr>
<tr>
<td>NeoTec4</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>12</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>1</td>
<td>0.31</td>
<td>&lt;0.0001</td>
<td>0.195</td>
<td></td>
</tr>
<tr>
<td>Milk fat</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>11</td>
<td>15</td>
<td>16</td>
<td>16</td>
<td>18</td>
<td>18</td>
<td>0.31</td>
<td>&lt;0.0001</td>
<td>0.195</td>
<td></td>
</tr>
</tbody>
</table>

1Diet: All milk replacer with 27% CP; Control = control milk replacer with animal fat (17% fat); NeoTec4 = animal fat supplemented with butyrate, medium chain fatty acids, and linolenic acid (17% fat); Milk fat = 33% fat. Heifer number for all diets, n = 8.

2SEM = Standard error of the mean for diet x week.
Table 3. Correlation of ante- and post-mortem ultrasound, bisected tissue, and tissue weight at 8 wk of age\(^1\).

<table>
<thead>
<tr>
<th>Item</th>
<th>Post-Mortem</th>
<th>Bisected Tissue</th>
<th>Tissue Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ante-Mortem</td>
<td>0.705</td>
<td>0.618</td>
<td>0.741</td>
</tr>
<tr>
<td>Post-Mortem</td>
<td>0.731</td>
<td>0.805</td>
<td></td>
</tr>
<tr>
<td>Bisected Tissue</td>
<td></td>
<td>0.632</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Pearson correlation coefficient. All correlations were \(P = 0.0001\).
Table 4. Mammary parenchyma (PAR) and fat pad (MFP) mass and composition for heifers fed 1 of 3 milk replacer (MR) diets.

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet</th>
<th>SEM²</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A)</td>
<td>B)</td>
<td>C)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>1.11</td>
<td>1.54</td>
<td>1.79</td>
</tr>
<tr>
<td>Weight, g/100 kg of empty BW</td>
<td>1.63</td>
<td>2.50</td>
<td>2.88</td>
</tr>
<tr>
<td>Protein, mg/g of PAR</td>
<td>86.53</td>
<td>91.02</td>
<td>87.67</td>
</tr>
<tr>
<td>Total protein, g</td>
<td>0.10</td>
<td>0.15</td>
<td>0.16</td>
</tr>
<tr>
<td>DNA, mg/g of PAR</td>
<td>4.26</td>
<td>5.04</td>
<td>5.29</td>
</tr>
<tr>
<td>Total DNA, mg</td>
<td>5.19</td>
<td>6.23</td>
<td>10.07</td>
</tr>
<tr>
<td>MFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>66.77</td>
<td>65.34</td>
<td>69.93</td>
</tr>
<tr>
<td>Weight, g/100 kg of empty BW</td>
<td>104.0</td>
<td>104.3</td>
<td>112.3</td>
</tr>
<tr>
<td>Lipids, mg/g of MFP</td>
<td>428.60</td>
<td>424.40</td>
<td>403.50</td>
</tr>
<tr>
<td>Total Lipids, g</td>
<td>32.41</td>
<td>30.32</td>
<td>33.62</td>
</tr>
<tr>
<td>Protein, mg/g of MFP</td>
<td>23.98</td>
<td>31.91</td>
<td>23.11</td>
</tr>
<tr>
<td>Total protein, g</td>
<td>1.52</td>
<td>1.83</td>
<td>1.61</td>
</tr>
<tr>
<td>DNA, mg/g of MFP</td>
<td>1.43</td>
<td>1.36</td>
<td>1.36</td>
</tr>
<tr>
<td>Total DNA, mg</td>
<td>77.35</td>
<td>78.97</td>
<td>73.86</td>
</tr>
<tr>
<td>Total udder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>69.33</td>
<td>67.90</td>
<td>72.49</td>
</tr>
<tr>
<td>Weight, g/100 kg of empty BW</td>
<td>112.96</td>
<td>104.69</td>
<td>115.58</td>
</tr>
</tbody>
</table>

¹Diet: All milk replacer with 27% CP; A.) 17% fat from animal fat B) 17% fat with added fatty acids from NeoTec4, C) 33% fat from milk fat.

²Standard error of the mean for diet (n = 8).
Table 5. Performance of calves fed 3 milk replacers with different fat and fatty acid compositions.\(^1\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet(^2)</th>
<th></th>
<th></th>
<th>Contrast P value(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (n = 8)</td>
<td>NeoTec4 (n = 8)</td>
<td>(C) Milk Fat (n = 8)</td>
</tr>
<tr>
<td>Initial BW, kg</td>
<td></td>
<td>41.2</td>
<td>40</td>
<td>40.8</td>
</tr>
<tr>
<td>Final Weight, kg</td>
<td></td>
<td>69.8</td>
<td>72.9</td>
<td>72.5</td>
</tr>
<tr>
<td>ADG, kg/d</td>
<td></td>
<td>0.51</td>
<td>0.59</td>
<td>0.57</td>
</tr>
<tr>
<td>Milk replacer intake, kg/d</td>
<td></td>
<td>0.635</td>
<td>0.634</td>
<td>0.634</td>
</tr>
<tr>
<td>Starter Intake, kg/d</td>
<td></td>
<td>0.655</td>
<td>0.665</td>
<td>0.63</td>
</tr>
<tr>
<td>Feed Efficiency</td>
<td></td>
<td>0.451</td>
<td>0.516</td>
<td>0.513</td>
</tr>
</tbody>
</table>

\(^1\)Pre-weaning was 0 to 42 d, post-weaning was 43 to 56 d, and overall was 0 to 56 d.
\(^2\)A) 17% fat from animal fat, B) 17% fat from animal fat with added fatty acids from NeoTec4, C) 33% fat from milk fat.
\(^3\)Standard error of the mean for diet (n = 8).
\(^4\)All contrasts are from analysis of 25 Holstein bulls and 25 Holstein heifers. No differences due to gender were found.
<table>
<thead>
<tr>
<th>Diet</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>SEM^3</th>
<th>Diet</th>
<th>Week</th>
<th>Diet x Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.738</td>
<td>6.868</td>
<td>7.453</td>
<td>8.274</td>
<td>8.143</td>
<td>7.890</td>
<td>8.364</td>
<td>9.040</td>
<td>9.095</td>
<td>0.389</td>
<td>0.5375</td>
<td>&lt;0.0001</td>
<td>0.6954</td>
</tr>
<tr>
<td>Milk fat</td>
<td>5.981</td>
<td>6.566</td>
<td>7.413</td>
<td>7.251</td>
<td>7.907</td>
<td>7.529</td>
<td>8.084</td>
<td>8.485</td>
<td>8.976</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^1REA = Ribeye Area; All measurements were made by ImageJ software, where calibrations standardized the number of pixels per centimeter. Measurements are in square centimeters (cm^2).

^2Diet: All milk replacer with 27% CP; Control = control milk replacer with animal fat (17% fat); NeoTec4 = animal fat supplemented with butyrate, medium chain fatty acids, and linolenic acid (17% fat); Milk fat = 33% fat. Heifer number for all diets, n = 8.

^3SEM = Standard error of the mean for diet x week; highest listed.
Table 7. Least squares means for effects of diet on carcass and empty body fat and protein compositions at 8 wk of age.

<table>
<thead>
<tr>
<th>Carcass trait</th>
<th>Diet</th>
<th>A) Control (n = 8)</th>
<th>B) NeoTec4 (n = 8)</th>
<th>C) Milk Fat (n = 8)</th>
<th>SEM³</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td></td>
<td>32.50</td>
<td>31.02</td>
<td>31.53</td>
<td>1.16</td>
<td>0.67</td>
</tr>
<tr>
<td>Weight, % of live weight</td>
<td></td>
<td>50.18</td>
<td>51.15</td>
<td>51.12</td>
<td>0.59</td>
<td>0.42</td>
</tr>
<tr>
<td>Protein², %</td>
<td></td>
<td>14.14</td>
<td>14.97</td>
<td>13.84</td>
<td>0.60</td>
<td>0.40</td>
</tr>
<tr>
<td>Fat², %</td>
<td></td>
<td>5.68</td>
<td>5.76</td>
<td>5.68</td>
<td>0.43</td>
<td>0.99</td>
</tr>
<tr>
<td>Water², %</td>
<td></td>
<td>72.56</td>
<td>72.35</td>
<td>72.32</td>
<td>0.41</td>
<td>0.90</td>
</tr>
</tbody>
</table>

¹Diet: All milk replacer with 27% CP; A) 17% fat from animal fat, B) 17% fat from animal fat with added fatty acids from NeoTec4, C) 33% fat from milk fat.
²Values were derived from the 9-11ᵗʰ rib section to approximate total carcass composition.
³Standard error of the mean for diet (n = 8).
Table 8. Palpation scores from 6 to 8 wk of age.

| Palpation Score\(^1\) | 6 | 7 | 8 | SEM\(^3\) | Test of fixed effects
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diet</td>
</tr>
<tr>
<td>Diet(^2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.7</td>
<td>2.8</td>
<td>3.2</td>
<td>0.44</td>
<td>0.4977</td>
</tr>
<tr>
<td>NeoTec4</td>
<td>2.8</td>
<td>3.1</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk Fat</td>
<td>2.2</td>
<td>2.3</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Palpation scores based on subjective scale from 0 to 6, 0 representing the smallest PAR and 6 representing the largest PAR palpated.

\(^2\)Diet: All milk replacer with 27% CP; Control = control milk replacer with animal fat (17% fat); NeoTec4 = animal fat supplemented with butyrate, medium chain fatty acids, and linolenic acid (17% fat); Milk fat = 33% fat. Heifer number for all diets, n = 8.

\(^3\)SEM = Standard error of the mean for diet x week.
**Figure 1.** Ultrasound image at 8 wk ante-mortem, calf 388, left front quarter.

**Figure 2.** Ultrasound image at 8 wk ante-mortem, calf 388, left front quarter. Parenchyma is circled, area = 0.359 cm².
Figure 3. Ultrasound image of ribeye muscle (Longissimus dorsi) taken between the 12th and 13th rib of calf 387.

Figure 4. Ribeye area is outlined. Ultrasound image of ribeye muscle (Longissimus dorsi) taken between the 12th and 13th rib of calf 387.
Figure 5. Ultrasound image post-mortem, calf 388, left front quarter.

Figure 6. Labeled ultrasound image, post-mortem, calf 388, left front quarter. A) Teat area (blue), B) Needle used for teat area verification post-mortem (red), C) Parenchyma area (green), D) Mammary Fat Pad. Parenchyma area = 0.464 cm².
Figure 7. Bisected left front quarter exposing PAR and teat area, calf 388.

Figure 8. Labeled bisected left front quarter, calf 388. A) Mammary Fat Pad, B) Needle used for teat area verification post-mortem, C) Parenchyma (red outline), D) Teat. Parenchyma Area = 0.632 cm².
Figure 9. Parenchyma Area (cm$^2$) of front and rear quarters, by week (0-8). Areas are from ante-mortem ultrasound analysis.