EFFECTS OF FEEDING POLYUNSATURATED OR SATURATED FATTY ACIDS TO PRE-RUMINATING CATTLE ON DIFFERENTIATION AND GROWTH OF BOVINE PREADIPOCYTES FROM INTRAMUSCULAR AND SUBCUTANEOUS FAT DEPOTS

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William Isaac Loucks

Major Professor: Matthew E. Doumit, Ph.D.

Committee Members: Gordon K. Murdoch, Ph.D.; Onesmo Balemba, Ph.D.

Department Chair: Amin Ahmadzadeh, Ph.D.

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AUTHORIZATION TO SUBMIT THESIS

This thesis of William I. Loucks, submitted for the degree of Master of Science with a major in Animal Science and titled "Effects of Feeding Polyunsaturated or Saturated Fatty Acids to Pre-Ruminating Cattle on Differentiation and Growth of Bovine Preadipocytes from Intramuscular and Subcutaneous Fat Depots", has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date: _____
Matthew E. Doumit, Ph.D.

Committee Members: _____ Date: _____
Gordon K. Murdoch, Ph.D.

_____ Date: _____
Onesmo Balemba, Ph.D.

Department Administrator: _____ Date: _____
Amin Ahmadzadeh, Ph.D.

ABSTRACT

The objective was to determine if feeding polyunsaturated fatty acids (PUFAs) *vs*. saturated fatty acids (SFA) to pre-ruminating cattle would preferentially increase preadipocyte development in intramuscular (IM) tissue. At 3 d after birth, 12 Holstein bull calves were divided into 6 cohort groups and fed one of two treatments. The PUFA group (n=6) received whole milk (4 L/d) supplemented with 3% total oil containing Lutalin® (a combination of conjugated linoleic acid (CLA) isomers of cis-9, trans-11 (c9,t11) and trans-10, cis-12 (t10,c12)) and flaxseed oil (FSO) (~54% α–linolenic acid (ALA)) in a ratio of 2:1 FSO:CLA. The saturated fatty acid group (n=6) received 3% supplemental palm oil (~43% palmitic acid). The calves were fed the treatments twice daily for 50 days, after which they were euthanized. Blood samples, perirenal tissue, and the *Longissimus dorsi c*ontaining both SC and IM tissue were taken. Gas chromatography was utilized on prepared samples to determine and quantify the fatty acids present. A growth assay, using clonally derived SC and IM preadipocytes, was performed to evaluate the mitogenic potential of the serum collected and processed from each calf in respective treatment groups. A morphological assessment of adipocyte growth was conducted on the stromal-vascular cells isolated from the calves. Quantitative real-time polymerase chain reaction quantifications of mRNA were conducted on IM, SC, and PR tissue samples harvested from the calves for preadipocyte factor-1, peroxisome proliferator-activated receptor gamma, acetyl CoA carboxylase, fatty acid binding protein, and fatty acid synthase. Stearic, oleic, and eicosenoic acid were higher in IM tissue of PUFA fed calves ($P < 0.05$). Both CLA c9, t11 and CLA t10, c12 tended to be higher in the PUFA group ($P = 0.06$). In SC ($P < 0.01$), PR ($P<0.04$) tissues, and calf serum ($P<0.03$) palmitic acid was greater in SFA fed calves. Eiscosenoic acid, CLA c9, t12, and

CLA t10, c12 were greater in both SC ($P<0.01$) and PR ($P<0.001$) tissue of PUFA fed calves. No significant level of ALA was present in either treatment or any tissue sample. Calf serum from the PUFA group had higher levels of ALA, CLA c9, t11, and CLA t10, c12 ($P<0.0002$). No treatment effect on cell proliferation was seen for IM clonal preadipocytes receiving calf serum. By d4 of growth assay, SC clonal preadipocytes treated with serum from PUFA fed calves grew faster than those treated with SFA ($P=0.03$). The adipogenic differentiation of isolated IM or SC S-Vcells was not affected by depot by treatment. However, there was a treatment by depot effect; the PUFA S-V SC cells had a greater degree of differentiation than the PUFA S-V IM cells ($P=0.01$). There was no treatment effect on relative abundance of any of the genes measured. SC clonal preadipocytes grew faster with calf serum from PUFA treatment, and isolated SC cells from PUFA treated calves differentiated to a greater extent than IM cells. Feeding specific FAs affects both tissue and serum FA profiles, as well as other aspects of adipocyte development.

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA Carboxylase
ALA	Alpha-Linolenic Acid
ARA	Arachidonic Acid
BSA	Bovine Serum Albumin
C/EBP	CCAAT/Enhancer Binding Protein
CLA	Conjugated Linoleic Acid
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
FA	Fatty Acid
FABP	Fatty Acid binding protein
FAS	Fatty Acid Synthase
FBS	Fetal Bovine Serum
GR	Glucocorticoid receptor
GLUT	glucose transporter
GPDH	Glycerol-3-Phosphate Dehydrogenase
IGF-1	Insulin-like growth factor
IM	Intramuscular
LIN	Linoleic Acid
mRNA	Messenger Ribonucleic Acid
OLE	Oleic Acid
ORO	Oil Red O

OM	Omental
PAL	Palmitic Acid
PPAR	Peroxisome Proliferator-Activated Receptor
PR	Perirenal
PBS	Phosphate-Buffered Saline
PUFA	Polyunsaturated fatty acid
PREF	Preadipocyte factor
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RXR	Retinoid X Receptor
SFA	Saturated fatty acid
SAS	Statistical Analysis Systems
S-V	Stromal-vascular
SC	Subcutaneous
TZD	Thiazolidinediones
TRO	Troglitazone
USDA	United States Department of Agriculture

CHAPTER 1

REVIEW OF LITERATURE

Beef Attributes

The physical attributes of beef carcasses are utilized by the United States Department of Agriculture (**USDA**) to assess and assign a Yield and Quality Grade (USDA 1997). These grades give the producer and consumer some indication of the expected percentage of cutability from the carcass and the expected palatability of the cooked beef, respectively. The palatability attributes of beef are characterized by degree of marbling, tenderness, juiciness, and flavor; and are defined as the complex of sensations resulting from the odor, taste, feel, and ease of chewing (Blumer 1963). O'Quinn et al. (2012) found that tenderness, juiciness, flavor liking, and overall liking increased in beef with increased fat content. Higher USDA Quality Grades are associated with higher intramuscular fat content of the meat (USDA 1997) and increased flavor, tenderness, and overall palatability (Smith et al., 1987).

Quality and Yield Grade

The USDA uses eight different Quality Grades for beef carcasses (Prime, Choice, Select, Standard, Commercial, Utility, Cutter, and Canner) to assess eating quality. The three highest grades, Prime, Choice, and Select are usually used for retail cuts of meat, with most of the Prime Grade going to the foodservice industry. Quality Grade is based on carcass maturity and degree of marbling. The physiological maturity of the carcass is determined by ossification patterns of the backbone, lean muscle color, rib bone shape and color, and ribeye texture. There are five classifications for beef carcass maturity: A, B, C, D, and E with A including animals from approximately 9 to 30 months of age and E including animals >96 months of age (USDA 1997). The first four Quality Grades (Prime through Standard) are

applied to young cattle with A or B maturity. Marbling (intramuscular fat (**IM**)) is defined as the intermingling or dispersion of fat within the lean muscle and is evaluated in the ribeye (*Longissimus*) muscle between the 12th and 13th ribs (USDA 1997). Marbling is quantified by nine degrees: abundant, moderately abundant, slightly abundant, moderate, modest, small, slight, trace, and practically devoid. Higher amounts of marbling are associated with higher quality grades (USDA 1997).

Yield Grades indicate the relative amount of lean, edible meat from a carcass, and are defined as the percentage of closely trimmed, boneless cuts from the round, loin, rib, and chuck. External fat thickness at the 12th rib, size of ribeye area, percent of kidney, pelvic, and heart fat, and hot carcass weight are used to assign Yield Grades, which are rated numerically from 1, being the highest expected yielding carcasses, to 5, being the lowest yielding carcasses (USDA 1997).

Higher Quality and Yield Grades are generally more desirable for profitability to the producer and eating quality for the consumer. However, while research and management practices have shown some improvement, the 2011 National Beef Quality Audit (NBQA) continues to identify need for improvement in Quality and Yield Grades as the most significant lost opportunities for beef producers (NBQA 2011). It values loss per head for Quality Grade at $27.83 and Yield Grade at $6.81 totaling $34.64 in lost revenue for these two factors (NBQA 2011). In 2013, 32.5 million cattle were slaughtered in the United States (USDA 2014) when multiplied by $34.64 loss per head; this is a 1.1 billion dollar yearly loss for the beef industry. Clearly, strategies to improve Quality and Yield Grade are economically beneficial to the beef industry.

Marbling

Marbling is the beef industry term to denote the appearance of white flecks or streaks of intramuscular fat between the muscle fibers. Microscopically, marbling is seen as distinct adipose depots with adipocytes embedded in perimysial connective tissue matrix proximal to a blood capillary (Aberle et al., 2001; Hocquette et al., 2010). Triacylglycerols and phospholipids are the main constituents of intramuscular fat (Scollan et al., 2006). Emerson et al. (2013) found that strip loin steaks with increased degrees of marbling were rated by trained sensory panels as having greater juiciness, tenderness, and flavor intensity. There is a direct significant correlation between USDA Quality Grade and eating satisfaction, Prime > Upper 2/3 Choice > Low Choice > Select (Emerson et al., 2013). Clearly, marbling has a positive effect on tenderness, juiciness, flavor, and overall liking (Savell et al., 1987; Lorenzen et al., 1999; Lorenzen et al., 2003, Garmyn et al., 2011; O'Quinn et al., 2012) and is considered the main factor in valuing beef carcasses by both industry and consumers (Harper and Pethick, 2004; Dow et al., 2011). Deposition of marbling (IM tissue) appears to be a life-long event that begins with fetal development, and stimulation of marbling early in life can lead to improvements in Quality Grade at harvest (Funston et al., 2010; Du et al. 2013). Strategies to increase IM fat accretion in cattle would contribute to industry profitability as well as consumer satisfaction.

Tenderness

Numerous studies have shown that consumers rate tenderness as the most important palatability trait (Savell et al. 1987; Miller et al., 1995; Miller et al., 2001). Huffman et al. (1996) found that 51% of consumers considered tenderness the main trait they desired in a steak eaten at home or in a restaurant. Factors that influence meat tenderness are IM fat,

connective tissue content, degree of cross-linking, sarcomere length, and postmortem proteolysis (Koohmaraie et al., 2002; Belew et al., 2003). Several researchers assert that one of the main factors influencing the tenderness of beef is the amount and distribution of IM fat (marbling) (McBee and Wiles 1967; Davis et al., 1979; Tatum et al., 1980; Savell and Cross 1988; Wood et al., 2008). Increased amounts of IM fat are correlated with increased tenderness, juiciness, and overall eating satisfaction in both lamb cuts (Smith and Carpenter 1970) and beef (O'Quinn et al., 2012). However, other research has found that IM fat did not affect tenderness (Parrish eta al., 1973; Garcia-de-Siles et al., 1977, Dow et al., 2011). This was possibly due to differences in age of animal, diet, or the length of post-mortem aging period.

The exact mechanism by which the IM fat contributes to the tenderness of cooked meat is not well established; however, there are four often-cited theories about the effect of IM fat on meat tenderness. Strain theory asserts that as marbling is deposited around perivascular cells inside the perimysium, a thinning of the connective tissue walls takes place and provides for muscle structure expansion (Smith and Carpenter, 1974; Wood, 1999). Wood (1990) found that marbling fat located in the perimysial connective tissue between muscle fiber essentially "opens up" the muscle structure resulting in its ability to be broken down more easily by mastication. Bite theory states that within a bite-size portion of cooked beef, marbling decreases the mass per unit volume and dilutes muscle fibers (Smith and Carpenter, 1974; Jeremiah, 1996). Since fat is less dense with a lower shear force than muscle, meat is able to be chewed more easily, thus being perceived as more tender (Smith and Carpenter, 1974; Savell and Cross, 1988). Lubrication theory maintains that IM fat lubricates the fibers making a more tender and juicy product (Smith and Carpenter, 1974;

Thompson, 2004). Tenderness and juiciness are highly related. IM fat solubilizes when meat is cooked making it easier to chew and release its juices. The meat remains moist even during prolonged mastication, thus appearing more tender (Smith and Carpenter, 1974). Insurance theory propounds that higher levels of IM fat provide insurance if meat is cooked too long, too rapidly, or with high temperatures by allowing it to remain palatable (Briskey and Kauffman, 1971). Fat does not conduct heat as quickly as muscle; therefore, meat with higher IM fat would be able to withstand longer cooking at higher temperatures (Smith and Carpenter, 1974). There is a direct correlation between degree of doneness and decrease in tenderness (Parrish et al., 1973). According to the Beef Customer Satisfaction survey from the National Livestock and Meat Board, 82% of beef consumers cook meat medium to very well done (1995). Behrends et al. (2005) found that consumers in both Chicago and Philadelphia preferred to cook their beef to "medium well and more". Wheeler et al. (1999) found that the palatability of steaks from Top Choice carcasses was less affected by high degrees of doneness than in Low Select carcasses, thus possibly indicating that IM fat helped preserve the palatability of the meat.

Both the 2011 and 2006 National Beef Tenderness survey indicated that USDA Prime beef top loin steaks were given higher tenderness and juiciness ratings than USDA Low Choice and Select steaks (Voges et al., 2007; Guelker et al., 2013). The primary determinant of Quality Grade is the amount of IM fat (NBQA, 2011). Garmyn et al. (2011) found that USDA Quality Grade had a positive linear relationship with both tenderness and juiciness, which led them to conclude that USDA Quality Grade is one of the most valuable tools for predicting palatability characteristics of beef. Conversely, other studies did not identify an effect of either USDA Quality Grade or marbling scores with tenderness (Wheeler, 1994;

Wulf, 1996; Dow et al., 2011). Inconsistencies in tenderness findings could be due to cooking time and temperature, length of post-mortem aging, differences in muscles used in study (Rhee et al., 2004), or of animal-to-animal variations caused by a variety of genetic and management practices.

Juiciness

The identified quality of juiciness is the combined effects of the initial rapid release of meat fluid recognized during the first effects of mastication, and the sustained juiciness resulting from the stimulating effect of fat on the flow of saliva (Blumer, 1963; Savell and Cross, 1988). Increasing levels of IM fat result in greater water retention of the meat during cooking (Wood 1990). This increase in water-holding capacity of meat is possibly due to the lubrication of the muscle fibers during cooking (Smith and Carpenter, 1974). While some studies have found no relationship between IM fat and juiciness (Parrish et al., 1973; Garcia-de-Siles et al., 1977), many researchers have demonstrated a positive correlation between marbling score (Quality Grade) and juiciness (McBee and Wiles, 1967; Tatum et al., 1980; Smith et al., 1987; Wheeler et al., 1994; Wheeler et al., 1999; Muchenje et al., 2009). The total lipid content of IM fat has a positive correlation on the juiciness of cooked meat (Wood et al., 2008). The fatty acids in IM fat melt at or below meat cooking temperatures, which help to maintain juiciness in steaks by coating perimysial connective tissue, thereby reducing loss of moisture (Blumer, 1963; Aberle et al., 2001). Several studies have shown that juiciness increases in a linear manner as marbling levels (Quality Grade) increase (McBee and Wiles, 1967; Tatum et al., 1980; Smith et al., 1984; Smith et al., 1987; Wheeler et al., 1994; Wheeler et al., 1999). When tenderness was held constant, juiciness and flavor became the major attributes that identified consumer satisfaction with beef steaks (Miller et

al., 1995; Miller et al., 2001). The National Beef Tenderness Survey demonstrated that USDA Prime steaks were rated higher for juiciness than USDA Upper 2/3 Choice or USDA Select steaks (Voges et al., 2007). Other researchers concur with this finding, identifying a correlation between juiciness and Quality Grade (Lorenzen et al., 1999; O'Quinn et al., 2012).

Flavor

The flavor of beef is a combination of taste and aroma (Blumer, 1963; Brewer, 2006; Maughan et al. 2012). Fatty acids when oxidized produce carbonyl compounds that contribute to flavor and act as a storage depot for odor producing compounds that are released upon cooking (Hornstein and Crowe 1960; Mottram, 1998). When meat is cooked triglycerides and phospholipids, the major constituents of IM fat, are released and contribute to the ultimate flavor of beef, pork, and lamb (Savell and Cross, 1988; Hocquette, et al. 2010). Increased marbling positively affects the flavor intensity of beef (Blumer 1963; McBee and Wiles 1967; Tatum et al., 1980; Dolezal et al., 1982; Wheeler et al., 1999; Thompson, 2004). A few studies found that the degree of IM fat did not have an effect on flavor (Jones et al., 1991; Parrish et al., 1973; Garcia-de-Siles et al., 1977). However, a majority of researchers found a positive correlation between increased marbling and desirable flavor scores (McBee and Wiles, 1967; Dolezal, 1982; Smith et al., 1984; Goodson et al. 2002). Both Killinger et al. (2004) and Thompson (2004) found that when tenderness was controlled for, flavor scores were positively correlated with IM fat content and were the major attribute in consumer acceptability of the product. O'Quinn et al. (2012) found that in beef strip steaks from USDA Quality Grades from Prime to Standard, increased levels of IM fat positively affected tenderness, juiciness, flavor liking, and overall liking. Interestingly,

they found that flavor liking was more highly correlated with overall liking than either tenderness or juiciness (O'Quinn et al., 2012). Research by Platter et al. (2003) found that even small changes in flavor scores created large changes in overall palatability acceptance by consumers. Corbin et al., (2015) found that IM fat level was the main determinant of beef flavor acceptability in all study samples except those with obvious off-flavors. In contrast with results from previous Quality audits, the 2011 National beef Quality Audit revealed that flavor attributes surpassed tenderness in importance to both consumers and the market sectors closely associated with consumer demand (Igo et al., 2013). It is evident that flavor, which in large part shows a positive relationship with level of IM fat, has a major impact on consumer attitudes and that "taste" is a driving factor in consumer demand for beef products (Savell et al. 1987; O'Quinn et al. 2012; Corbin et al., 2015).

Adipose Tissue Development

The two main functions of adipocytes are to store energy in the form of triacylglycerol when caloric input is greater than required and to break down the stored lipid into free fatty acids when the energy is needed. Adipocytes are formed from pluripotent mensenchymal stem cells (MSCs) found in the vascular stroma of adipose tissue and bone marrow (Lin et al., 2010). For MSCs to become committed to the adipocyte lineage, they must go through a multistep process to become new preadipocytes. The preadipocytes then undergo mitotic clonal expansion where they re-enter the cell cycle as G_1-growth-arrested preadipocytes and usually complete two rounds of division then express several transcription factors that allow differentiation to form mature adipocytes (Tang and Lane, 2012). Some of the transcription factors and enzymes needed for adipocyte development are lipoprotein lipase, CAAT/enhancer binding protein **(C/EBP)** –α,β, and δ, peroxisome proliferator-activated

receptor **(PPAR)**-γ, glycerol-3-phosphate dehydrogenase **(GPDH),** and acetyl -CoA carboxylase **(ACC)-α.** At the conclusion of mitotic clonal expansion, the cells lose their fibroblastic morphology; fill with cytoplasmic triglyceride and gain the morphological and biochemical phenotype of mature adipocytes (Student et al., 1980; Cornelius et al., 1994). Both hyperplasia, the increase in the number of adipocytes, and hypertrophy, the enlargement of existing adipocytes through lipid filling of the unilocular fat droplet, are responsible for adipose tissue accumulation (Cianzio et al., 1985; Shepherd et al., 1993). Preadipocytes and non-differentiated adipocytes, through the process of mitosis, are able to proliferate; however, fully differentiated adipocytes lose the capacity for mitosis and cell proliferation (Ailhaud et al., 1992). Therefore, adipose tissue accretion is accomplished through hyperplasia by preadipocytes and through hypertrophy by differentiated adipocytes. White adipose tissue formation occurs in both pre- and postnatal periods, with a rapid increase in fat cell number and size after birth (Gregoire et al., 1998). There is variability of accretion rates within different adipose tissues as animals continue to grow and fatten through hyperplasia and hypertrophy (May et al., 1994).

Heterogeneity of adipose depots

Research has established that differences exist among adipose tissue depots in sheep (Soret et al., 1999), rats (Broad et al., 1983; DiGirolamo et al., 1998; Bertevello and Seelaender, 2001), humans (Hutley et al., 2003), mice (Sackmann-Sala et al., 2012), pigs(Wang et al., 2013), and cattle (Broad et al., 1983; Cianzio et al., 1985; Grant et al., 2008b). A great deal of research has been conducted on the heterogeneity of human adipose depots with regard to their biochemical properties, responses to hormones, and amount of fat (Pond 1999; Saleh et al., 1999). Human subcutaneous (**SC**) adipocytes have higher rates of

triglyceride synthesis, lipolysis, and lipoprotein lipase activity than intra-abdominal or omental (**OM**) adipocytes (Edens et al., 1993; Boivin et al., 2007). Perilipins are phosphoproteins localized to the surface of triaclglycerol droplets within adipocytes where they regulate the rate of lipolysis. Wang et al. (2003) stated that perilipins expression was higher in SC than OM fat, contributing to the depot differences in lipolysis. Conversely, Arner (1995) found that the rate of lipolysis was lower in SC than OM fat. Cellular glucose uptake differs between adipose depots; OM adipocytes have a greater insulin stimulated glucose uptake and higher numbers of insulin receptors than SC adipocytes (Lefebvre et al., 1998; Stolic et al., 2002; Virtanen et al., 2002). In mice, there are depot-specific differences in enzyme levels, glucose metabolism, plasma levels of insulin, leptin, and adiponectin (Sackmann-Sala, et al., 2012).

There are depot differences in bovine adipose tissue development. Lipid accretion rates in bovine adipose tissue depots occur in the following order; perirenal (**PR**), OM, SC, intermuscular, and IM (Hood, 1983). In PR and SC fat depots most hyperplasia is completed by 8 months of age, whereas cell hyperplasia was still present in IM depots at 15 month of age and brisket fat past 15 months (Hood and Allen, 1973; Cianzio et al. 1985). In the finishing phase of cattle, accretion of fat in SC depots is by hypertrophy while IM depots continue to develop by both hyperplasia and hypertrophy of already recruited adipocytes (May et al., 1994; Sainz and Hasting, 2000). In a measure of adipocyte size, Cianzio et al. (1985) found that IM and brisket fat developed at a slower rate than the other depots based on cell diameter; kidney> mesenteric> SC> intermuscular> IM> brisket. Smaller adipocytes, such as those found in IM and brisket fat, have less lipogenic capacity, thus cell number is essential concerning the quantity of lipid accretion in these depots (Allen et al., 1976; Hood

1983). Mendizabal et al. (1999) found, when comparing adipocytes from PR, OM, and intermuscular depots in growing steers, that PR adipocytes were the largest in size and had the highest lipogenic enzyme activities based on glycderol-3-phosphate dehydrogenase **(GPDH)** and fatty acid synthase **(FAS)** measurements on a per cell basis. Whereas, Rule et al. (1992) found that SC adipose tissue in relation to OM, PR, and intermuscular fat depots has the highest lipid hydrolysis rates. As the above data indicates, the research suggests that the differences in size, lipogenesis and lipolysis rates, as well as metabolic activity are extant in bovine adipose tissue depots.

Depot differences in fatty acid and triacylglyceride synthesis

Depot differences exist in the regulation of *de novo* fatty acid (**FA**) and triacylglyceride synthesis. In ruminants, the main site of de novo FA synthesis is adipose tissue (Dodson, et al., 2010). In monogastrics, glucose from dietary carbohydrates is the main precursor of FA synthesis; however, in ruminants, acetate (a product of rumen metabolism) has been suggested as the main precursor of FA synthesis rather than glucose (Hanson and Ballard, 1967). Smith and Crouse (1984) sought to elucidate the relative contributions of acetate, lactate, and glucose, the three major bovine lipogenic precursors, as substrate in IM and SC adipose tissue. They found that glucose provided 50-75% of the acetyl units in IM adipose tissue and 1-10% in SC adipose tissue; conversely, acetate provided 70-80% of the acetyl units for SC depots and 10-25% in IM depots. Rhoades et al, (2007) found that IM cells incorporated glucose into fatty acid at more than twice the rate as they did with acetate. Bovine IM preadipocytes had an increase in glucose uptake during the first 5-day period of differentiation (Aso et al., 1995). Only glucose transporter **(GLUT)**-1, which is responsible for transporting glucose across the blood-brain barrier independently of

insulin, was detected in clonal bovine IM cells rather than GLUT-4 , which is the insulin dependent glucose transporter protein in muscle and adipose tissue (Aso et al., 1995; Peters et al. 2002). However, after differentiation, higher levels of GLUT-4 expression and higher action of phosphofructokinase and ATP-citrate lyase, which are enzymes needed for conversion of glucose into long-chain fatty acids, were identified in bovine IM adipose tissue compared to SC adipose tissue (Hocquette et al., 2010). The research indicates that bovine IM cells appear to have enhanced ability to utilize glucose for lipogenesis when compared with SC cells, which gives rise to the possibility that manipulation of these differences could influence development of specific adipose depots.

In addition to differences in substrate use between IM and SC depots, differences in triacylglycerol synthesis between depots have also been identified. Fatty acid is incorporated into glycerolipid by both IM and SC adipose tissues at the following rate: palmitate > linolenate > linoleate > stearate. There was increased FA esterification at higher concentrations of FA and the total rate of FA esterification into triglycerides was lower in IM than SC adipose cells (Lin et al., 1992). Thus, IM and SC adipose tissue appear to have different mechanisms of lipid metabolism.

Preadipocyte Proliferation and Differentiation

Preadipocytes are derived from multipotent stem cells that reside in the vascular stroma of adipose tissue and in bone marrow. These stem cells, when stimulated, follow a multistep process to become committed to the adipocyte lineage by becoming preadipocytes, which when exposed to stimulating agents differentiate into mature adipocytes (Ailhaud et al., 1992; Tang and Lane 2012). The amount and rate of preadipocytes proliferation and

differentiation is a factor of species, age, diet, and anatomical site (Kirkland et al., 1990; Soret et al., 1999; Hausman et al., 2001).

Preadipocyte proliferation

Preadipocyte proliferation occurs both pre and post-natally serving a vital role in adipose tissue accumulation (Ailhaud et al., 1992). Several factors have been shown to facilitate or inhibit the conversion of multipotent stem cells to the adipocyte lineage. Members of the Bone Morphogenetic Protein family, specifically BMP-4 and BMP-2 have an activating role (Bowers et al., 2006), while hedgehog signaling inhibits conversion (Spinella-Jaegle et al., 2001). Interestingly, Wnt proteins have been found to activate stem cell commitment to preadipocytes, but inhibit adipocyte differentiation (Ross, et al., 2000; Bowers and Lane 2008). In addition to the effects of gene expression, preadipocyte proliferation is influenced by many other factors such as cytokines, hormones, and growth factors. Wright and Hausman (1995) found that the hormone insulin-like growth factor-1 (**IGF-1**) significantly influence porcine preadipocyte proliferation at all three culture densities studied. Transforming growth factor-β, fibroblast growth factors, and tumor necrosis factor-α are additional paracrine factors that regulate preadipocyte proliferation both negatively, positively, and synergistically (Ailhaud et al., 1992, Butterwith, 1994; Wright and Hausman, 1995; Hausman et al., 2001). Both transforming growth factor-β (Teichert-Kuliszewska et al. 1992) and fibroblast growth factors (Richardson et al., 1992) have been shown to stimulate preadipocyte proliferation. While tumor necrosis factor negatively impacts differentiation (Ron et al., 1992), it acts synergistically with insulin-like growth factor-1 on proliferation in stromal-vascular (**S-V**) primary cultures (Kras et al., 2000).

Preadipocyte differentiation

Preadipocytes undergo a series of events as they differentiate into mature fat cells during which they express genes for transcription factors, enzymes, and other proteins (Ailhaud et al., 1992; Gregoire et al., 1998). The preadipocytes enter the growth arrest (G_1) phase of the cell cycle and enter into mitotic clonal expansion where a transcription factor cascade takes place, changing the preadipocytes into mature adipocytes. The cascade is initiated as differentiation inducers, such as glucocorticoids and methylisobutylxanthine (Yeh et al., 1995) cause the CAMP response element- binding protein to transcriptionally activate the CCAAT (cytosine-cytosine-adenosine-adenosine-thymidine)/enhancer binding proteins (**C/EBP**)-β and C/EBP-δ (Tang and Lane, 2012). Tanaka et al. (1997) found that a single knockout of either (C/EBP)-β or C/EBP-δ had little effect on adipose tissue accumulation, whereas a double knockout of both (C/EBP)-β and C/EBP-δ produced a significant effect, supporting the idea that there is redundancy of function. Both C/EBP-α and peroxisome proliferator-activated receptor (**PPAR**) γ contain C/EBP regulatory elements where C/EBP-β binds to activate their transcription (Yeh et al., 1995; Clarke et al., 1997). Once activated, C/EBP-α and PPARγ work together as pleotropic transcriptional activators of several genes that produce the adipocyte phenotype. Additionally, they have the ability to activate each other, allowing the continued expression of both in mature adipocytes (Wu et al., 1999; Rosen et al. 2002). In the transcriptional cascade sequence, sterol regulatory element-binding protein is activated after the expression of C/EBP-α and PPARγ in the terminal events of differentiation and activates transcription of lipogenic genes that support fatty acid synthesis and triacylglycerol synthesis (Tang and Lane 2012). Stearoyl-CoA desaturase gene, which is SREBP-mediated and is highly expressed during differentiation and expression, was shown

to increase immediately preceding lipid filling in both murine (Casimir and Ntambi, 1996) and bovine (Chung et al., 2006) preadipocytes.

When the cells leave the cell cycle they change in appearance from the fibroblast morphology and accumulate cytoplasmic triglyceride and take on the unilocular appearance and metabolic features of a mature adipocyte (Student et al., 1980; Cornelius et al., 1994). As the adipocytes accumulate triglyceride, they display an increased rate of de novo lipogenesis, expression of FA enzymes, and tricylglycerol biosynthesis, as well as insulin receptors, GLUT4 and leptin (Gregoire et al., 1998; Tang and Lane, 2012).

Depot differences in preadipocytes proliferation and differentiation

Regional differences in preadipocyte proliferation and differentiation are present in adipose tissues of various species. Proliferation and/or differentiation rates, lipogenic enzyme activity, as well as response to endogenous/exogenous stimuli have been indicated. Two separate studies using rats found that at all ages studied, PR cells replicated more rapidly than did epididymal adipose precursors (Djian et al., 1983: Kirkland et al., 1990). Using human S-V cells, Van Harmelen et al. (2004) found that SC cells proliferated faster than those from the OM region. Other studies with human (Anderson et al., 2001) and rat (Dieudonne et al., 2000) S-V cells, found that estrogen triggered a greater increase in proliferation of SC than in OM cells, demonstrating that there are depot differences in response to hormone stimulus. Several studies, with different species, using preadipocytes or S-V cells cultured under identical conditions, demonstrated that differentiation rates varied among adipose depots (Dijan et al., 1985; Adams et al., 1997; Soret et al., 1999; Wu et al., 2000; Grant et al. 2008b) In a study to compare differentiation of bovine S-V cells isolated from IM and SC adipose tissue, Grant et al. (2008b) found that while depots had a similar

percentage of adipogenic colonies, the percentages of differentiated cells in SC colonies was 6.4-fold greater than in IM colonies. Ovine SC S-V cells demonstrated greater differentiation than OM or intermuscular cells (Adams et al., 1996; Soret et al., 1999). Bovine S-V cells treated with triglyceride mixture (Intralipid) displayed greater differentiation in SC cells than OM cells, however when cells were treated with Indomethacin, a known PPARγ agonist, the depot difference was not evident (Wu et al., 2000). This suggests that the differentiation capacity between various bovine depots is dependent upon the ability to use tricylglycerol and/or FA as endogenous PPARγ ligands. Wu et al. (2000), found that cells cultured from bovine SC adipose tissue had a greater response to differentiation media, than cells cultured from OM adipose tissue. In contrast, Grant et al. (2008a) demonstrated that troglitazone (**TRO**) increased differentiation in cultured cells, but had no differential effect on the differentiation of SC or IM cells. In a study by Archibeque et al. (2005), it was found that SC adipose tissue displayed twice the SCD catalytic activity as IM adipose tissue.

Both glucocorticoids and thiazolidinediones (**TZD**) induce depot specific effects on adipogenesis. In a study by Ramsay et al. (1989) in response to the addition of hydrocortisone in treatment media, porcine SC S-V cells differentiated, but PR cells did not. However, in porcine cells, Hausman and Poulos (2004) stated that dexamethasone (**DEX**) increased both SC and IM S-V cells differentiation in equal relative proportions, but no matter what the treatment, SC cells differentiated at a higher rate than IM. In several studies, it has been shown that TZD treatment increased differentiation in human SC S-V cells but not in OM cells (Adams et al., 1997). This is supported by the fact that PPARγ is more highly expressed in SC cells than OM; TZD has been shown to bind to PPARγ, thus

elucidating why SC cells differentiate in the presence of TZD and OM cells do not (Sewter et al., 2002). Caserta et al. (2001), studying the reaction of both rat PR and epididymal cells and human OM and SC cells, found that free FA uptake was greater in rat PR than epididymal cells and human OM cells showed greater uptake than SC cells. They concluded that heterogeneity among depots arose from the distinct intrinsic characteristics of adipose cells and in fact, distinct depots are essentially separate "miniorgans" with preadipocytes from different depots being unique to one another.

Adipogenic Growth Factors and Signaling Agents

The regulation of preadipocyte proliferation and differentiation is influenced by many autocrine, paracrine, and endocrine factors (Poulos et al., 2010a). Insulin, IGF-1, glucocorticoids, fetal bovine serum (**FBS**), TZD, PPARγ, FA, and prostaglandins have all been shown to exert an influence on preadipocytes activity. The role of these factors on adipogenesis is discussed below.

Insulin and IGF-1 have been identified as having an essential role in stimulating adipose cell proliferation, differentiation, as well as accumulation of lipid serving both an autocrine and paracrine function in adipogenesis (Tseng et al., 2004; Bluher et al., 2005). Insulin receptors and IGF-1 receptors are present in preadipocytes and adipocytes, with preadipocytes having more IGF1R and adipocytes having more IR (Entingh-Pearsall and Kahn 2004; Back and Arnqvist 2009; Boucher 2010). Utilizing 3T3-L1 cells, Boney et al. (2000) found that IGF-1 stimulated both cell proliferation and differentiation. Porcine *in vitro* studies have demonstrated that adipose S-V cells express IGF-1 with increased expression during proliferation and differentiation where the IGF1R serves to increase preadipocytes proliferation and differentiation through a mitogen-activated protein kinase cascade (Chen et

al., 1996; Boney et al., 2000; Hausman et al., 2009). Interestingly, the accumulation of IGF binding protein-1 has been demonstrated to significantly suppress adipose cell proliferation in developing porcine adipose tissue by possibly regulating IGF-1 transport and bioavailability (Butterwith, 1994; Chen et al., 1996). In studies by Boucher et al. (2010), it was found that although insulin and IGF-1 preferentially bind to their specific receptors in adipose tissue, they will utilize either IR or IGF1R if the preferential receptor is inactivated. As preadipocytes undergo terminal differentiation to adipocytes, they are more responsive to insulin and the amount of IR increases significantly (Shimizu et al., 1986). In further studies, Boucher et al. (2012), utilizing mice lacking the IR and IGF1R in both white and brown adipose tissue, found that the mice had almost complete absence of all adipose tissue. From this experiment, they concluded that insulin and IGF-1 signaling serve a crucial role in adipose tissue development.

Insulin has been shown to stimulate adipogenesis in several species (Guller et al., 1988; Hausman and Jewell, 1988; Hausman, 1989; Miller et al., 1991; Grant e al., 2008a). Studies demonstrated that insulin stimulated GPDH and LPL activity in rat and porcine S-V cells, thus increasing differentiation (Deslex et al., 1987; Hausman and Jewell, 1988; Hausman, 1989). Guller et al. (1988) found that when insulin was removed from the differentiation media, the GPDH activity was reduced to uninduced levels and decreased differentiation by 97%. Grant et al. (2008a) found that when insulin was removed from the differentiation media, GPDH activity was reduced to 74%. Insulin has been shown to enhance the transcription of several genes involved in adipogenesis such as fatty acid synthetase (**FAS**), GPDH, SCD, and Acyl-CoA Synthetase (Nasrin et al., 1990; Weiner et al., 1991; Moustaid et al., 1994). Therefore, many studies of bovine S-V cells and preadipocytes

included insulin in the differentiation media (Plaas and Cryer, 1980; Aso et al., 1995; Wu et al., 2000). Research has shown that both insulin, IGF-1, as well as, their associated receptors and proteins are essential for preadipocyte proliferation and differentiation.

Glucocorticoids have been shown to have a positive effect on both adipogenic transcription factors and differentiation (Ou et al., 2014). In the cell nucleus, glucocorticoids bind to glucocorticoid receptors (**GR**) forming the glucocorticoid-receptor complex, which then homodimerizes and binds to the glucocorticoid response element on the DNA strand (Ntambi and Kim, 2000). Dexamethasone (**DEX**) is a synthetic glucocorticoid often used in media for cell culture. Chen et al. (1996) found that GR affinity and number increased in *in vitro* pig adipose S-V differentiation; conversely, when glucocorticoids were reduced there was low GR affinity and number. In 3T3-L1 cells, glucocorticoids have been shown to increase the expression of C/EBPδ and C/EBPβ, inducing PPARγ expression and adipogenesis (Wu et al., 1999). However, Hausman (1989) did not find an association between glucocorticoid and C/EBP expression in *in vivo* studies utilizing adipose tissue of late gestation fetal pigs. Ou et al. (2014) demonstrated that GR and apoptosis regulator 1 are required for transcriptional activation of PPARγ and adipocyte differentiation. Specifically, they found that apoptosis regulator 1 was necessary for activation of GR; while depletion suppressed differentiation of mouse mesenchymal stem cells and 3T3-L1 preadipocytes to mature adipocytes, as well as, decreased induction of PPARγ, C/EBPδ and C/EBPβ (Ou et al., 2014). There appears to be an interaction between glucocorticoids and insulin benefiting preadipocytes differentiation. McDonald and Goldfine (1988) found that glucocorticoids increased insulin receptor expression. Insulin has been shown to increase the number and binding affinity of GR in porcine S-V cells *in vitro* (Chen et al., 1996). Research has shown

that intracellular cAMP increases prior to adipocyte differentiation (Ailhaud et al., 1992). Clearly, glucocorticoids foster adipose development.

Thiazolidinediones (**TZD**), known antidiabetic agents, influence adipose conversion through increasing insulin sensitivity, lowering plasma glucose, as well as, serving as PPARγ ligands (Lehmann et al., 1995; Hauner, 2002). Lehmann et al. (1995) found that TZD is a strong and selective activators of PPARγ. Preadipocytes and mesenchymal stem cell lines treated with TZD became differentiation adipocytes, demonstrating the high affinity PPARγ ligand binding and that PPARγ is a molecular target for the adipogenic effects of TZD (Lehmann et al., 1995). In the presence of TZD, insulin sensitivity is increased through the lowering of tissue triglyceride and non-esterified FA, and increases glucose uptake by muscle while it reduces hepatic glucose output (Day, 1999; Kramer et al., 2001; Ye et al., 2001). Cell culture experiments utilizing S-V cells from human (Adams et al., 1997), porcine (Tchoukalova et al., 2000), ovine (Soret et al., 1999), and bovine (Ohyama et al., 1998; Grant et al., 2008b) demonstrate that differentiation is stimulated when cells are treated with TZD in culture. Grant et al. (2008b) utilizing S-V cells, found that treatment with TZD increased the proportion of differentiated cells within colonies by 10-fold. In another experiment, Grant et al. (2008a) utilized clonally-derived bovine SC preadipocytes, found that removing TZD from the media reduced glycerol-3-phosphate dehydrogenase (**GPDH)** activity by 68%. Research clearly indicates that TZD serves as a potent PPARγ ligand and has a direct influence on preadipocytes differentiation.

Peroxisome proliferator-activated receptor gamma, a ligand-activated nuclear receptor, is considered the master regulator of adipogenesis because it is both necessary and sufficient for adipogenesis to occur (Knouff and Auwerx, 2004: Rosen et al., 2006). It

heterodimerizes with retinoid X receptor--α and binds to PPAR response elements in the regulatory regions of target genes to promote transcription of lipogenic genes (Rosen et al., 2000; Knouff and Auwerx, 2004). Chewla et al. (1994) demonstrated that PPARγ is highly expressed in adipose tissue and induced early during adipocyte differentiation.
PPARγ facilitates FA uptake and TAG storage (Tamori et al., 2002). PPARγ helps integrate the control of glucose, energy, and lipid homeostasis (Knouff and Auwerx, 2004). As such, PPARγ can bind to many different ligands, both endogenous and exogenous. Natural PPARγ ligands include medium and long-chain fatty acids such as such as the n-3 PUFAS ALA and n-6 PUFAs LIN and ARA as well as eicosanoids (Escher and Wahli, 2000; Varga et al., 2011; Bionaz et al., 2013). Synthetic ligands for PPARγ include TZD, and some nonsteroidal anti-inflammatory drugs such as indomethacin, ibuprofen, and fenoprofen (Knouff and Auwerx, 2004; Varga et al., 2011).

Two distinct isoforms of PPARγ, transcribed from the same gene, have been identified, PPARγ$_1$ and PPARγ$_2$ (Varga et al., 2011). Sundvold et al. (1997) isolated both PPARγ1 and PPARγ2 isoforms from bovine tissues with the greatest expression in white adipose tissue. PPARγ$_2$ is expressed at high levels almost exclusively in adipose tissue; PPARγ$_1$ is expressed at lower levels in many cell types such as adipose tissue, spleen, liver, and pancreas (Varga et al., 2011). Utilizing PPARγ knockout mice, studies demonstrate that activation and expression of PPARγ, especially PPARγ$_2$, is key to adipose tissue development both *in vivo* and *in vitro* (Rosen et al., 1999; Zhang et al., 2004).

In vitro Examination of Adipogenesis and Signaling Agents

In vitro cell models have proven invaluable in elucidating the mechanisms involved in adipogenesis; however, researchers realize that it is often difficult to extrapolate the findings to live animals since the cells used in the *in vitro* experiments might be quite different from *in vivo* (Poulos et al., 2010b). That being said, the cells used for *in vitro* experiments can, themselves, be significantly different depending on the study design and aim. Due to their easy availability and homogeneity, much research utilizes immortal cell lines, such as 3T3-L1cells, which were developed using clonal expansion of rodent-derived cells and are comprised of a single cell type (Gharbi-Chichi et al., 1984; Fernyhough et al., 2005). However these cell lines do not always replicate the characteristics inherent in the species of interest cells; therefore, many researchers choose to extract and isolate cells from the species studied. Cells cultured directly from the subject are considered primary cells. Primary cell cultures of isolated S-V cells can be obtained following enzymatic digestion and centrifugation of adipose tissue or the tissue explant method. Since S-V cell cultures obtained from enzymatic digestion also contain various cell types, such as fibroblasts, myoblasts, and endothelial cells, they may mimic *in vivo* conditions more closely than single cell types might (Poulos et al. 2010b). Additionally, these derived S-V cells can be collected following various *in vivo* treatments, which allow further levels of research. Clonal cells are derived from one adipogenic precursor cell and are utilized to study intrinsic properties of preadipocytes (Tchkonia et al. 2002; Grant et al. 2008a). Primary and clonal cell cultures seem to have similar preadipocytes developmental patterns. Research by Tchkonai et al. (2002) utilizing primary and clonally-derived cell culture methods showed that there were similar variations in adipogenesis in human adipose depots. In both primary and clonally-

derived cell cultures, differentiation was greatest in SC > mesenteric > OM primary and clonally-derived cell cultures, indicating that the depot differences were inherent within the cell themselves. Both cell culture systems are useful for the study of adipogenesis.

Regardless of the origin of the cells used, most *in vitro* cell culture system demonstrated that is necessary to use some type of serum or growth factor supplement for cell survival and optimal proliferation. Fetal bovine serum (**FBS**) contains binding proteins, several growth factors, hormonal, nutritional, and other less well-defined components that sustain and promote cell growth that mimics the normal *in vivo* cell environment (Barnes and Sato, 1980). It is utilized in many cell culture studies of various cell lines and across species (Djian et al., 1983; Soret et al., 1999; Tchoukalova et al. 2000, Wu et al., 2000). In contrast, research using serum-free media did support ovine S-V cell growth; however, proliferation rates were much less when compared to FBS containing media (Broad and Ham, 1983). Utilizing clonally-derived bovine SC preadipocytes, Grant et al. (2008a) found that the serum lipid supplement Ex-Cyte significantly increased expression of the biochemical indicator of adipogenic differentiation, GPDH. They further demonstrated that bovine differentiation occurred in response to combinations of insulin, Ex-Cyte, DEX, and TRO. Additionally, Grant et al. (2008b) identified the optimum level of TRO supplementation to induce differentiation in bovine primary S-V cultures. Using bovine S-V cells cultured with insulin, IBMX, TRO, and DEX, Hirai et al. (2007) demonstrate successful differentiation. Wu et al., (2000) found that initial differentiation using insulin, IBMX, and DEX was enhanced by the addition of intralipid.

Several studies have demonstrated the mitogenic potential of serum from the animal studied. Weber, et al. (1999) found that in cells cultured from pre-pubertal female calf

mammary tissue the addition of serum prepared from heifers stimulated DNA synthesis by 28% compared with basal medium. They also found that the addition of the prepubertal heifer serum to the culture medium increased cell proliferation in a dose-dependent manner. Caronti et al. (1998) studying the effect of sera from Neurofibromatosis patients and their clinically healthy relatives on glial cell proliferation, found that the maximal serum dilution stimulating glial proliferation was similar in both groups. Balk et al. (1981) utilizing chicken heart mesenchymal cells, found that serum caused greater cell proliferation than plasma.

Dietary Fatty Acids

Fatty acids, identified as PPARγ ligands, influence preadipocytes differentiation by providing a source of cellular energy, encouraging *de nova* FA synthesis, gene expression, and FA uptake (Yu et al., 1995; Kiewer et al., 1997). Fatty acids have been shown to selectively mediate PPARγ transcription activity (Thoennes et al. 1999). Response to different dietary FAs may be species specific with regard to preadipocytes effects. Ding et al. (2000) found that porcine S-V cells showed increased differentiation when exposed to linoleic acid (**LIN**) and both isomers of conjugated linoleic acid (**CLA**), *cis*-9, *trans*-11 (**c9,t11**) -CLA and *cis*-12,trans-10 (**t10,c12**)-CLA. Whereas, 3T3-L1 cells treated with c9, t11-CLA displayed inhibited differentiation as measured morphologically, enzymatically, and by Northern Analysis of gene expression (Brodie et al., 1999). In further research utilizing 18 carbon FA, Ding et al. (2003) found that while all three FA tested increased porcine preadipocytes differentiation, oleic (**OLE)** (18.1) and linoleic (**LIN**) (18.2) had a greater effect than alpha-linolenic (**ALA**) (18.3); additionally, fatty acid binding protein (**FABP**) mRNA was increased several-fold by all three FAs.

Adipocyte determination and differentiation-dependent factor-1 has been shown to be involved in adipocyte gene expression and differentiation, and also participates in regulation of transcription of FAS (Kim and Spiegelman, 1996). Research has shown that treatment with arachidonic acid (**ARA**) and docosahexaenoic acid decreased expression of adipocyte determination and differentiation-dependent factor-1 in porcine adipocyte differentiation (Liu et al. 2005). Utilizing fully differentiated 3T3-L1 adipocytes, Sessler et al. (1996) found that ARA, LIN and ALA acid suppressed SCD enzyme activity as well as SCD1 mRNA; in contrast, OLE and stearic acid (**STE**) did not affect SCD1 mRNA levels. Intralipid, a triacylglycerol mixture, produced greater differentiation in bovine preadipocytes from SC than from OM fat (Wu et al. 2000). Grant et al. (2008a) demonstrated that Ex-Cyte, a serum lipid supplement, increased GPDH activity and protein concentration of clonally-derived bovine SC preadipocytes. Davis (2011) utilizing clonally-derived IM and SC bovine preadipocytes, found that OLE and LIN induced differentiation of SC preadipocytes to a greater extent than IM preadipocytes. Conversely, ARA, and ALA preferentially induced differentiation of IM preadipocytes, while CLA induced differentiation of both SC and IM preadipocytes.

Conjugated linoleic acid **(CLA)** is considered an agonist of PPAR (Meadus et al., 2002). It is a mixture of isomers of linoleic acid, in which the double bonds are *cis* type indicating that within the fatty acid chain the hydrogen atoms attached to the carbon atoms all point the same direction and have a low melting point (Wood et al. 2008). Two isomers, c9, t11 and t10, c12 of CLA occur naturally in food (Brown et al., 2003). Currently there is a great deal of interest in CLA due to its identified positive human health effects on diabetes, cardiovascular disease, cancer, as well as, the immune system, bone health, and body

composition (Schmid et al., 2006). Zhou et al. (2007) demonstrated that t10, c12-CLA reduced the mRNA expression of adipocyte-specific genes in SC adipose tissue S-V cultures, but c9, t11-CLA did not. However, they found that both CLA isomers increased gene expression in IM cultures, indicating that the effect of CLA decreases SC adipose tissue but increase IM adipose tissue due to different regulatory effects on adipocyte-specific genes. Additionally, they found that the CLA-treated IM S-V cells had higher lipid accumulation ($P<0.01$) and that CLA increased the number of Oil Red O-stained cells; together with the upregulation of PPARγ expression, these findings led them to conclude that CLA has the ability to transdifferentiate myoblasts to adipocytes. Dugan et al. (1997) using Large White pigs found that the addition of 2% CLA to feed resulted in increased lean muscle by 2-3%, while reducing SC fat by 6-8%, but interestingly the CLA treatment increased IM fat content. A study by Meadus et al. (2002) found that barrows fed CLA at 11g/kg of feed for 45 days reduced SC fat by 9.2% ,while lean muscle increased by 3.5%, and IM fat increased by 14%. They found that PPARγ levels were significantly increased in the IM tissue of CLA-fed pigs indicating that CLA induced the development of preadipocytes from stem cells to promote IM fat development. The noted differences in IM and SC fat deposits in CLA-fed animals might be caused by the differences in PPARγ expression and adipocyte cellular development between these two depots. Barnes et al. (2012) also found significant increases in marbling scores for barrows supplemented with CLA. Additionally, they stated that the increase in marbling was possibly related to increased size of IM adipocytes. Contrary to these findings, Chung et al. (2006) found that, using a bovine preadipocytes cell line, t10, c12 CLA almost totally abolished SCD gene expression, but c9, t11 CLA had no effect. Zhou et al. (2007) further identified that t10, c12-CLA decreased the number of adipose precursor cells in SC

cultures during proliferation, as well as the total number of cells. Interestingly, they found that t10, c12-CLA treated S-V cells were less rounded and remained fibroblast-like. Kadehowda et al. (2013) found that t10, c12 CLA reduced SCD gene expression in bovine preadipocytes by over 50%. However, Choi et al. (2014) stated that t10, c12 CLA did not significantly affect adipogenic gene expression in either IM or SC bovine adipose tissue, but it did depress *de novo f*atty acid synthesis from acetate in SC adipose tissue. In a study feeding rumen-protected CLA during the early growing period of beef heifers, Schlegel, et al. (2012) found that the treatment changed tissue FA composition, but did not influence beef carcass traits. As a whole, the research demonstrates that CLA differently regulates adipose tissue development in SC and IM depots.

Flaxseed oil is composed of 91% unsaturated fatty acids, 55% of which are ALA (18:3n-3) (Hagemeister et al., 1991). Choi et al. (2014) found that ALA, Oleic acid, and trans-vaccenic acid increased acetate incorporation into lipids in bovine IM adipose tissue i*n vitro*. Many studies have used flaxseed and flaxseed oil to determine its effects on carcass characteristics. Choi, et al. (2016) compared feeding rice bran, flax seed, and sunflower seed on growth performance and carcass characteristics of cattle and found that the flaxseed group had higher scores for flavor, umami, and overall palatability; however, they also had greater fat thickness and a lower Yield Grade. Quinn et al. (2008) found that when crossbred heifers were fed ground flax at 0, 2, 4, and 6% of dry matter intake there was a statistically significant (P=0.03) linear increase in average daily gain as more flax was included in the ration. Feeding flax seed has been shown to increase marbling scores, Yield Grade, and the percentage of steers that grade USDA choice (Droullard et al., 2002; Drouillard et al. 2004). Maddock et al. (2006) found that feeding flaxseed at 8% of diet dry matter increased the

USDA Yield Grade (P=0.01), but did not significantly affect marbling scores; however, the flaxseed-treated group had higher marbling scores than the control. Drouillard et al. (2002) fed weaned calves for 36 to 40 days with tallow or 10% flaxseed; then finished the calves on a common finishing diet. The cattle that had been fed 10% flax had higher marbling scores than those that received tallow. Conversely, porcine studies found that feeding flaxseed produced no differences in animal growth or carcass traits (Romans et al., 1995; Kouba et al., 2003).

Dietary FAs fed to lactating ruminants affect the milk composition and fatty acid profile. Dietary long-chain FA can be incorporated directly into the milk altering the short to long-chain FA ratio (Kennelly 1996). This change in the FA ratio of ruminant milk can be a beneficial for both suckling calves and humans consumers. Several studies have shown that feeding flax can positively alter the fatty acid profile of milk (Kennelly 1996; Goodridge et al., 2001; Petit et al., 2001; Ward et al. 2002) Kennelly (1996) fed whole flax seed at 0, 5, 10, and 15 % dry matter to Holstein cows at mid-lactation. They found that there were linear increases in ALA in the milk as flax seed content increased. Cortes et al. (2010) fed whole flaxseed, calcium salts of flaxseed oil, or a mix of whole flaxseed and calcium salts of flaxseed oil to lactating Holstein cows. They found that while milk yields with regard to protein, fat, lactose, and total solids were similar among all treatments, the concentrations of CLA c9, t11 and ALA were significantly higher in the cows receiving the calcium salts of flaxseed oil and the mix of both whole and calcium salts of flaxseed. Li et al. (2012) fed lactating goats linseed oil, safflower oil or control with no additional oil. Both oil treatments decreased the proportion of short- to medium-chain FA, while increasing CLA c9, t11, CLA

t10, c12, and ALA. In fact, the linseed oil treatment increased both CLA isomers 3-fold and the ALA by 6-fold.

Summary

Taken together, the attributes of tenderness, juiciness, and flavor are all, as demonstrated by research, dependent on the level of IM fat (USDA Quality Grade) of the meat for their positive palatability contributions. Losses in Quality and Yield Grade cost the cattle industry an estimated 1.1 billion dollars yearly. Methodologies that can foster the increase of IM fat, while still preserving a good carcass yield, will be beneficial to both the industry and the consumer.

Fatty acids do stimulate preadipocytes differentiation. The response is dependent upon species, anatomical depot, as well as, the specific FA provided. It has been established that dietary FAs can have an effect on both adipocyte development and the FA profile of muscle. Additionally, the FA content of ruminant milk can be changed by feeding FA. Evidently, there is potential to manipulate adipose development through dietary FA treatments.

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CHAPTER 2

EFFECTS OF FEEDING POLYUNSATURATED OR SATURATED FATTY ACIDS TO PRE-RUMINATING CATTLE ON DIFFERENTIATION AND GROWTH OF BOVINE PREADIPOCYTES FROM INTRAMUSCULAR AND SUBCUTANEOUS FAT DEPOTS

Abstract

The objective was to determine if feeding polyunsaturated fatty acids (PUFAs) *vs.* saturated fatty acids (SFA) to pre-ruminating cattle would preferentially increase preadipocyte development in intramuscular (IM) tissue. At 3 d after birth, 12 Holstein bull calves were divided into 6 cohort groups and fed one of two treatments. The PUFA group (n=6) received whole milk (4 L/d) supplemented with 3% total oil containing Lutalin® (a combination of conjugated linoleic acid (CLA) isomers of cis-9, trans-11 (c9,t11) and trans-10,cis-12 (t10,c12)) and flaxseed oil (FSO) (~54% α–linolenic acid (ALA)) in a ratio of 2:1 FSO:CLA. The saturated fatty acid group (n=6) received 3% supplemental palm oil (~43% palmitic acid). The calves were fed the treatments twice daily for 50 days, after which they were euthanized. Blood samples, perirenal tissue, and the *Longissimus dorsi c*ontaining both SC and IM tissue were taken. Gas chromatography was utilized on prepared samples to determine and quantify the fatty acids present. A growth assay, using clonally derived SC and IM preadipocytes, was performed to evaluate the mitogenic potential of the serum collected and processed from each calf in respective treatment groups. A morphological assessment of adipocyte growth was conducted on the stromal-vascular cells isolated from the calves. Quantitative real-time polymerase chain reaction quantifications of mRNA were conducted on IM, SC, and PR tissue samples harvested from the calves for preadipocyte factor-1, peroxisome proliferator-activated receptor gamma, acetyl CoA carboxylase, fatty acid binding protein, and fatty acid synthase. Stearic, oleic, and eicosenoic acid were higher

in IM tissue of PUFA fed calves ($P < 0.05$). Both CLA c9, t11 and CLA t10, c12 tended to be higher in the PUFA group ($P = 0.06$). In SC ($P < 0.01$), PR ($P<0.04$) tissues, and calf serum ($P<0.03$) palmitic acid was greater in SFA fed calves. Eiscosenoic acid, CLA c9, t12, and CLA t10, c12 were greater in both SC ($P<0.01$) and PR ($P<0.001$) tissue of PUFA fed calves. No significant level of ALA was present in either treatment or any tissue sample. Calf serum from the PUFA group had higher levels of ALA, CLA c9, t11, and CLA t10, c12 ($P<0.0002$). No treatment effect on cell proliferation was seen for IM clonal preadipocytes receiving calf serum. By d4 of growth assay, SC clonal preadipocytes treated with serum from PUFA fed calves grew faster than those treated with SFA ($P=0.03$). The adipogenic differentiation of isolated IM or SC S-Vcells was not affected by depot by treatment. However, there was a treatment by depot effect; the PUFA S-V SC cells had a greater degree of differentiation than the PUFA S-V IM cells ($P=0.01$). There was no treatment effect on relative abundance of any of the genes measured. SC clonal preadipocytes grew faster with calf serum with PUFA treatment, and isolated SC cells from PUFA treated calves differentiated to a greater extent than IM cells. Feeding specific FAs affects both tissue and serum FA profiles, as well as other aspects of adipocyte development.

Introduction

Manipulating adipose tissue development in a depot-specific manner to increase IM fat for consumer palatability, while at the same time reducing wasteful subcutaneous fat, is both the goal and challenge of researchers and industry. Less than optimum Quality and Yield Grades have been identified by the last three National Beef Quality Audits (2000, 2005, 2011) as lost opportunities with regard to production and profitability for the beef industry. For young cattle, the major determiner of Quality Grade is the amount of IM

adipose tissue (marbling); while Yield Grade is affected by muscularity and the amount of adipose tissue (NBQA, 2011). In 2014, the total lost revenue for Quality and Yield Grade was estimated at $34.64 per head/per year totaling a 1.1 billion dollar annual loss to the beef industry (USDA 2014). The positive relationship between marbling and overall palatability has long been recognized (Smith and Carpenter, 1974; O'Quinn et al., 2012; Emerson et al., 2013). Deposition of marbling appears to be a life-long event that begins with fetal development, and stimulation of marbling early in life can lead to improvements in Quality Grade at harvest (Funston et al., 2010; Du et al., 2013). The ability to produce high quality beef that is tender, juicy, and flavorful, while at the same time minimize excess subcutaneous fat would be beneficial to the beef industry.

Bovine adipose tissue depots possess unique characteristics. Adipocyte cell size (Cianzio et al. 1985), lipogenic capabilities (Grant et al. 2008b; Ortiz Colon et al. 2009), and substrate used (Smith and Crouse 1984) differ between bovine adipose depots. In culture, bovine SC preadipocytes differentiate more readily than IM preadipocytes (Grant et al. 2008b; Ortiz Colon et al. 2009). Wu et al. (2000) found that culture with Intralipid caused greater differentiation in preadipocytes from SC than OM tissue. Adipocytes from SC depots are larger and have greater lipogenic capabilities than adipocytes from IM depots (Cianzio et al. 1985). Bovine IM adipose tissue preferentially uses glucose as the substrate for *de novo* fatty acid synthesis, while SC adipose tissue uses acetate (Smith and Crouse 1984). These inherent differences may provide the ability to selectively enhance or inhibit adipogenesis.

The development of adipose tissue occurs through preadipocyte proliferation and differentiation into mature adipocytes that are capable of lipid storage. Adipogenic genes that are influenced by both endogenous and exogenous stimuli regulate preadipocyte

differentiation (Tang and Lane, 2012). Peroxisome proliferator-activated receptor γ is one such gene that is considered the master regulator of adipogenesis. It binds to response elements in the regulatory regions of lipogenic genes to promote transcription (Knouff and Auwerx, 2004). Peroxisome proliferator-activated receptor γ depends on ligands for activation and can bind to many different endogenous and exogenous ligands (Varga et al., 2011).

Preadipocytes from various depots equally express PPARγ, however; depot differences exist in their differentiation and lipid filling capacity. Grant et al. (2000b) and Ortiz-Colon et al. (2009) found that bovine SC preadipocytes differentiated at a higher rate and accumulated more lipid than IM cells. The synthetic PPARγ ligand, rosiglitazone, stimulated adipogenesis in human SC S-V cells but not in OM S-V cells (Adams et al., 1997). Additionally, indomethacin, another synthetic PPARγ ligand, stimulated adipogenesis in bovine OM S-V cells at a higher rate than SC S-V cells (Wu et al., 2000). Endogenous ligands include polyunsaturated fatty acids and eicosanoids which are derived from nutrition or metabolic pathways (Knouff and Auwerx, 2004). These observed adipogenic differences between depots are possibly a reaction to specific PPARγ ligands. Once ligand bound, PPARγ forms a heterodimer with retinoic X receptor, which is bound to corepressor proteins (Rosen et al., 2000; Miard and Fajas, 2005). When a specific ligand binds to PPARγ, it alters the conformation causing the release of corepressors and the recruitment of coactivators such as cAMP response element binding protein (Knouff and Auwerx, 2004). Yu et al. (1995) found that the type of ligand was the factor that determined which coactivators were recruited by the PPARγ-Retinoic X receptor heterodimer and thus determine the target genes activated.

Clearly, the type of ligand available to the cell is crucial in adipogenic regulation.

Natural PPARγ ligands include medium and long-chain FAs (Bionaz et al., 2013). The response to different dietary FA may be species specific with regard to preadipocytes effects. Ding et al. (2000) found that porcine S-V cells showed increased differentiation when exposed to linoleic acid and both isomers of CLA. Whereas, differentiation of 3T3-L1 was inhibited by *cis*-9, *trans*-11-CLA (Brodie et al., 1999). Davis et al. (2011) investigated the role of long-chain fatty acids on differentiation of preadipocytes from bovineIM and SC fat *in vitro*. They found that the polyunsaturated fatty acids, CLA, ARA, and ALA stimulated differentiation of clonally-derived IM preadipocytes to an equal or greater extent than clonally-derived bovine SC preadipocytes. The goal of this present study was to identify if supplementing the diet of pre-ruminating calves with conjugated linoleic acid and α-linolenic acid will preferentially stimulate intramuscular adipogenesis.

Materials and Methods

Experimental Feeding

This experiment utilized twelve pre-ruminating Holstein bull calves from the University of Idaho Dairy herd. Animal care was conducted according to procedures approved by the University of Idaho Animal Care and Use Committee (Appendix A). At birth, calves were weighed and received 4 L of colostrum, vitamin E and selenium injection (MU-SE, Merck Animal Health, Summit, NJ), Bovine Rota-Coronavirus vaccination (Calf-Guard, Pfizer Animal Health, Exton, PA) and Bovine Rhinotracheitis-Parainfluenza$_3$ vaccination (TSV-2, Pfizer Animal Health Exton, PA) as per the standard University of Idaho Dairy operating procedure. At 3 d after birth, the calves were randomly assigned to two groups on a cohort basis (Table 1). The polyunsaturated fatty acid (**PUFA**) group (n=6) received whole milk (4 L/d) supplemented with 3% total oil containing Lutalin®, which is a

combination of conjugated linoleic acid (**CLA**) isomers of cis-9, trans-11 (**c9,t11**) and trans-10,cis-12 (**t10,c12**) (BASF Chemical, Florham Park, NJ) and flaxseed oil (Jedwards, Quincy, MA) in a ratio of 2:1 FSO:CLA. Flaxseed oil contains 70% polyunsaturated fatty acid (PUFA) of which ~54% is α-linolenic acid (**ALA**) (Zambiazi et al., 2007). The saturated fatty acid (**SFA**) group (n=6) received 3% supplemental palm oil (Jedwards, Quincy, MA). Palm oil contains ~43% palmitic acid, 39% oleic acid, and only 11% PUFA (Kennelly 1996). An analysis of the fatty acid composition of the treatment components was performed (Table 2). The calves were housed at the University of Idaho dairy center in separate calf hutches and fed the treatments twice daily for 50 days (Figure 1). All calves received their last feeding 4h prior to euthanasia.

Sample Collection

At the end of the treatment period, calves were euthanized using a captive-bolt and immediately exsanguinated. During exsanguination, a 100 ml blood sample was collected from each animal into two 50 ml centrifuge tubes (ThermoFisher, Waltham, MA) for serum preparation. The blood samples were placed on ice for transport back to the lab. The entire left and right *Longissimus dorsi* containing both SC and IM tissue was taken. Additionally, perirenal (**PR**) tissue was taken at this time. The entire left *Longissimus dorsi* and PR tissues were placed in ice cold Phosphate Buffered Saline (**PBS**) for transport to the laboratory. From the right *Longissimus dorsi* the SC tissue was immediately separated from the muscle (Figure 2). The SC tissue and muscle samples from the right *Longissimus dorsi,* as well as PR tissue, were immediately frozen in liquid nitrogen and maintained at -80^{oC} until processed for RNA extraction and fatty acid quantification.

Fatty Acid Profile Analysis

Lipid extraction was performed using the procedure of Folch et al. (1957) as modified by Yahvah et al. (2015). The sodium methoxide methylation procedure (Christie, 1982) was used to prepare the serum and tissue samples for fatty acid analysis.

Two ml of serum or 1 g of tissue were placed in 50 ml culture tubes with Teflon lined screwcap. To this, 38 ml of a 2:1 chloroform:methanol (Fisher, Pittsburgh, PA) solution was added. This was homogenized with both a hand-held homogenizer (T10 Basic SI Disperser, IKA Works, Wilmington, NC) on 5 setting and a Sonic Dismembrator at 35% amplitude for 40 s (Branson Model 500, Branson, Danbury, CT). The tubes were tightly capped, and centrifuged (Sorvall RT1 model, Thermo Scientific, Waltham, MA) for 10 min at 400 xg. Supernatant fluid was poured through a Buchner funnel under vacuum l with #1 Whatman filter paper into a 125 ml side arm Erlenmeyer flask. The supernatant was then poured into a clean 50 ml glass tube that contained 7.2 ml of 0.58% solution of NaCl in nanopure water, then capped tightly with a Teflon lined screwcap and shaken. This was again centrifuged for 10 min. at 400 xg. The upper aqueous layer was aspirated and discarded. In a 37° C water bath the lower chloroform extract layer was dried down to less than 8 ml under nitrogen gas. Using a glass Pasteur pipette, the remaining extract was transferred into a 12 ml glass vial with a Teflon lined screwcap. The 50 ml tube, which previously contained the extract, was rinsed with 1 ml of chloroform and transferred to the 12 ml vial. This was evaporated with a stream of nitrogen gas in a 37^0 C water bath until dry. The vial was removed from the water bath and capped.

To a 13 X 100 ml glass culture tube containing 1 ml hexane (J.T. Baker, Center Valley, PA), 20-25 mg of sample lipid was added and the mixture briefly vortexed. Next,

40 µl methyl acetate (Sigma Aldrich, St. Lewis, MO) was added and briefly vortexed. Then 40 µl methylation reagent (a 5.58% solution of sodium methoxide (Sigma Aldrich, St. Lewis, MO) in methanol) was added and incubated at room temperature for 10 min. Sixty µl of stop reagent (1 g oxalic acid (Sigma Aldrich, St. Lewis, MO) in ethyl ether (Fisher, Pittsburgh, PA) was added and the tube was recapped tightly and briefly vortexed. It was then centrifuged at 1500 xg For 4 min. The organic phase was transferred to a clean glass tube with a 60 mg of calcium chloride (Sigma Aldrich, St. Lewis, MO) and briefly vortexed. This was incubated at room temperature for 1 hr; then centrifuged at 1500 xg for 4 min. The organic phase was transferred into a Gas Chromatography vial, capped, and analyzed on a Gas Chromatograph (Hewlett-Packard 6890 Series with auto injector: Agilent, Palo Alto, CA).

Growth Assay

This experiment was designed to evaluate the differences in growth stimulating potential of the serum collected and processed from each calf in respective treatment groups. The previously collected blood samples in 50 ml centrifuge tubes were stored at 4° C for 24 hr. The blood was then centrifuged (Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont Instruments, Palo Alto, CA) at 1200 xg and the serum was aspirated with a 25 ml pipette (Corning, Corning, NY). Serum samples (50 ml) were frozen at -80° C until used for this growth assay, as well as the fatty acid profiling. Previously isolated preadipocytes SC and IM clones (Grant et al., 2008a) (Appendix E) were suspended in basal medium (DMEM, 1% antibiotic-antimycotic, 50 µg/ml gentamicin sulfate, 33 µM biotin, 17 µM pantothenate, and 200 µM ascorbate) (Appendix B) and seeded at a density of ~ 8,000 cells per well into ten 24-well plates (16-mm diameter wells) and incubated at 37° C in a humidified

atmosphere of 95% air and 5% CO_2 for 1 d to adhere. At this point, the ten 24-well plates were used to assess the growth after 0, 2, and 4 days treatment. For day 0 treatment, Group 0 (**G0**) plates (1 plate of IM cells and 1 plate of SC cells), which represent the initial cell numbers on day 0, were washed twice with PBS and frozen at -80^{o} C. On day 0 treatment, the remaining two groups of plates, Group 2 (**G2**) and Group 4 (**G4**) (2 plates of IM and 2 plates of SC for each group) had the basal medium (described above) removed and washed twice with DMEM and treatment growth medium applied. Twelve different treatment growth media were prepared each included 95% basal medium and 5% serum from each different animal respectively. G2 and G4 plates were returned to the incubator and incubated for two days under the same conditions as previously described. After two days, G2 plates were removed, washed twice with PBS and frozen at -80. On d 2 treatment media was replaced with fresh treatment media on G4 plates and cells were incubated for two days under the same conditions as previously described. On d 4 the G4 plates were washed and cells frozen as above in preparation for DNA analysis.

Cellular DNA was quantified using a DNA Quantification Fluorescence Assay kit from Sigma Aldrich (St. Louis, MO). A 1 μg/ml working solution of Hoechst 33258 (bisBenzimide) was made by adding 30 μl of the stock 1 mg/ml Heochst 33258 bisBenzimide along with 3 ml of the assay buffer (100 mM Tris HCl, 10 mM EDTA, 2 M NaCl) and 27 ml of molecular biology grade water.

Cells were removed from the freezer and allowed to come to room temperature. Two hundred μl of Assay Buffer were added to each well and, cells were sonicated for 20 seconds using a Sonifier-Cell Disrupter 350 at 19% amplitude (Branson Sonic Power Co., Danbury, CT). Two hundred ml of bisBenzimide working solution was added to each well on a 96-well

plate. To this, samples from each well of the previously prepared 24-well plates were added in duplicate. For IM G0 30 µl were added, for IM G2 20 µl were added, for IM G4 10 µl were added respectively. For all SC samples 30 µl were used. Fluorescence was determined using a BioTek FL600 (BioTek, Winooski, VT) with a 360 nm excitation filter with a 40 nm bandwidth and a 460 nm emission filter with a 40 nm bandwidth. Amount of DNA in each well was determined by comparing the fluorescence to a standard curve created using calf thymus DNA. Each well was considered as a biological replicate. Growth rate was calculated by the exponential growth equation (y=x*e^(kt)) with y being the amount of DNA/well; x being the average ng of DNA per well of four wells at day 0; e being a mathematical constant of 2.718…; k being the growth rate; and t being 2 and 4 days respectively. Unless otherwise stated, media reagents (Appendix B) were of tissue culture grade and purchased from Sigma-Aldrich (St. Louis, MO) and all culture plates were from Corning Inc. (Corning, NY).

Stromal Vascular Isolation

The SC, *Longissimus dorsi*, and PR samples were washed twice in cold PBS. In a biosafety cabinet, each tissue sample was placed on a sterile plate and minced. For each sample, approximately 3 g of tissue were aliquoted into a 50 ml tube. To each tube ~6 ml of collagenase solution was added. SC and PR tissue samples received a 2 mg/ml solution of collagenase, while IM tissue samples received a 12 mg/ml solution of collagenase due to the greater density of connective tissue in muscle.

The collagenase solution was made by first preparing a 41.7 ml solution of 10% bovine serum albumin (**BSA**) (#A8806, Sigma-Aldrich, St. Louis, MO). in Dulbecco's Modified Eagle Medium (**DMEM**; #31600-83, Invitrogen Corp., Carlsbad, CA). Five Hundred mg of type II collagenase derived from *Clostridium histolyticum* (#C6885, Sigma-

Aldrich, St. Louis, MO) was added to form a 12 mg/ml solution of collagenase. This solution was sterile filtered at .2 μm through a syringe-filter (EMD Millipore, Billerica, MA). Eleven ml of the 12 mg/ml solution was mixed with 55 ml of 1% BSA solution in DMEM to make a 2 mg/ml collagenase solution.

After adding the collagenase, the samples were initially mixed by vortexing for 10s and incubated at 37^{o} C in a water bath for 60 min for the SC and PR tissue and 120 min for the IM tissue; all samples were vortexed for 10 s every 5 min throughout the incubation time. Samples were then sequentially filtered through 500 μm and then 53 μm nylon screens (03-500/47; 3- 53/30 Nitex, SEFAR America Inc., Buffalo, NY) screens. The material that passed through the screens was centrifuged in 50 ml tubes at 1000 xg for 10 min. The supernatant fluid was then poured off and the pellet was resuspended in freezing medium containing DMEM with 20% fetal bovine serum (**FBS**). Dimethyl sulfoxide at 10% (Sigma-Aldrich, St Louis, MO) was added dropwise to the cell suspension, which was then aliquoted into 1.8 ml cryovials (Corning, Corning, NY). Vials were incubated overnight at -80^{o}C and then placed in liquid nitrogen to be used for clonal analysis.

Clonal Analysis

Previously isolated S-V cells were plated on 10 cm diameter tissue grade untreated culture dishes (Corning, Corning, NY) at clonal density (400 cells/10-cm plate). The number of cells in the cryo-vial was determined using a hemocytometer (Clay-Adams) and diluted accordingly. Then the cells were incubated in growth medium undisturbed for 8 days. The growth medium consisted of basal medium (DMEM, 1% antibiotic-antimycotic, 50 μg/ml gentamicin sulfate, 33 μM biotin, 17 μM pantothenate, and 200 μM ascorbate), supplemented with 10% FBS. On day 8, plates were washed once with DMEM.

Differentiation medium was applied and left in place for 8 d. The differentiation medium consisted of basal medium, 0.5% FA free bovine serum albumin, 280 nM bovine insulin, 20 mM glucose, 10 μM troglitazone (Caymen Chemical Company, Ann Arbor, MI), and 20 μL/mL Ex-Cyte serum lipid supplement (Appendix C; Millipore, St. Louis, MO). At the end of the 8 d differentiation period, the plates were aspirated of the remaining media and washed twice with PBS. Cells were then fixed overnight with 3.7% formaldehyde, which was prepared by diluting a stock 3.7% formaldehyde solution that contained 10% methanol in PBS. Following this, to morphologically assess adipocyte differentiation by lipid accumulation, the cells were washed twice with PBS and incubated at room temperature with 2.5 ml/plate Oil Red O (ORO) solution for 2 h. The ORO solution was prepared using a protocol adapted from Ramirez-Zacarlas et al. (1992) (Appendix D). Residual ORO solution was removed and the plates were washed twice with PBS. Cell nuclei were stained by adding 2.5 ml Mayer's hematoxylin solution (Sigma-Aldrich, St Louis, MO) to each plate for 15 min. Following this, plates were washed extensively with warm running tap water. Immediately, digital photographs were taken using a Sony cyber-shot digital camera mounted with an adaptor ring (Martin Microscope Col, Easler, SC) to a Nikon inverted microscope (Nikon Diaphot, Nikon Inc., Garden City, NY.) The photomicrographs were used to determine the proportion of differentiated cells within colonies. A differentiated cell was defined as a cell having one or more lipid droplets and a colony with at least one differentiated cell was defined as adipogenic. To determine the proportion of differentiated cells within adipogenic colonies, photomicrographs of 10 randomly selected adipogenic colonies on each plate were taken (Figure 3). Three to seven photomicrographs of each colony were taken across the diameter of the colony to capture a representative cross-section

of cells at 200 X magnification. Evaluators were blinded to depot and treatment to perform the enumerations. The number of nucleated cells per photomicrograph was obtained; subsequently the number of cells showing differentiation and lipid accumulation as described above was ascertained.

Gene Expression Analysis

Samples previously prepared for RNA analysis were removed from the -80° C freezer and shattered with a mortar and pestle under liquid nitrogen. Fifty mg of tissue per sample were placed into 2 ml tubes with 500 µl Trizol (Invitrogen, Carlsbad, CA). The tubes were placed in homogenization blocks, homogenized at 25 Hz for 20 s and this procedure repeated 5 times. Tubes were placed on wet ice for 10 min, and then 100 µl of chloroform was added. Tubes were then inverted gently for 15 s and set at room temperature for 3 min. At this point, samples were centrifuged at 12,000 xg for 15 min at 4 °C. After centrifugation, the aqueous layer was transferred into 1.7 ml tubes containing 1.5 µl of glycogen (Invitrogen, Carlsbad, CA) and 250 µl of 2-propanol were added to each tube. Samples were then stored in a -20 °C freezer overnight to precipitate the RNA.

Samples with precipitated RNA were removed from the freezer and centrifuged at 15,000 xg for 30 min at 4 °C. The supernatant fluid was removed from each tube and discarded. One ml of 75% ethanol solution was added to the tube to wash the pellet. Samples were centrifuged at 12,000 xg for 10 min. The supernatant was again removed and discarded, and the tubes were left open to dry for 3 min. Twenty µl of Nuclease Free water (Invitrogen, Carlsbad, CA) were added to each tube to rehydrate the pellet. Then samples were placed in the -20 °C freezer for at least two hours. At the end of the two hours, to ensure the efficiency

of the extraction, sample RNA was quantified using a nanodrop (Thermo Nanodrop 1000, ThermoFisher Scientific, Waltham, MA).

Since any extraction will have some genomic DNA in the final RNA extract, the samples were treated with DNase using a TURBO DNA-free Kit from Applied Biosystems (Foster City, CA). Into fresh .65 ml tubes, 1.5 µl of 10X Turbo DNase buffer was added (Applied Biosystems, Foster City, CA). The mass of extracted RNA in each tube needed to be 2 µg, therefore varying volumes of the RNA extract prepared in the previously frozen samples were added. Depending on the sample volume, varying amounts of Nuclease Free water were added to make the total volume equal 13.5 µl. One ml of Turbo DNase solution was added to each tube and then the tubes were incubated on a heating block at 37 ºC for 25 min. Two µl of DNase inactivation reagent were added per tube and incubated at room temperature for 5 min, with a brief vortex to mix at 2.5min. Solutions were then centrifuged at 10,000 xg for 1.5 min and stored at -80 ºC.

Following the removal of the extra genomic DNA, as previously described, cDNA was synthesized using a kit from Applied Biosystems (Foster City, CA). A Master Mix was prepared including Reverse Transcriptase reaction buffer, dNTP, Reverse Transcriptase random primers, Reverse Transcriptase enzymes, RNase inhibitors. The Master Mix and 1 µg of DNase treated RNA were placed into a .2 ml tube. Samples were processed in the themocycler (Gene Amp PCR System 9700, Applied Biosystems, Foster City, CA) at 25 ºC for 10 min., 37 ºC for 120 min., 85 ºC for 5s, and then held at 4 ºC until removed. Samples were removed and 60 µl of Nuclease Free water was added. After which, the samples were stored at -20 ºC.

Quantitative real-time polymerase chain reaction (**qRT PCR**) was run on cDNA samples, to determine mRNA abundance for preadipocyte factor-1 (**Pref-1**), PPARγ, acetyl coA carboxylase (**ACC**), Fatty Acid Binding protein (**FABP**), and Fatty Acid Synthase (**FAS**). To make working stock, the forward and reverse primers, purchased from ThermoFisher (Waltham, MA), were suspended in Nuclease Free water to make a 1:10 dilution. Probes purchased from Applied Biosystems (Foster City, CA) were diluted with Nuclease Free water at a 1:40 concentration. A mixture was prepared for each gene, which contained, TaqMan Master Mix, (Applied Biosystems, Foster City, CA), Nuclease Free water, forward and reverse primer, and probes (both from the previously created working stock) (Table 3). Two µl of the cDNA and 13 µl of the prepared mixture per well were dispensed into 96-well plates. Optical adhesive film was placed over the entire plate, which was then centrifuged for 2 minutes at 1000 rpm. The plate was then placed into the PCR machine (7500 Fast Realtime PCR System, Applied Biosystems, Foster City, CA). PCR reactions included a holding time of 20 s at 95 ºC, followed by 40 cycles of melting and extension at 95 ºC for 3 s and 60 ºC for 30 s, respectively. Gene expression analysis of qRT-PCR was performed using the differential Ct (ΔCt) values of each of the candidate genes with that of the matched 18S rRNA values.

Statistical Analysis

Data were analyzed using the T-test procedure (PROC T-TEST) of SAS (SAS Institute, Cary, NC) as appropriate for values obtained from two distinct groups each with independent group data. For clonal analysis of stromal vascular cells, fatty acid profiles, and RNA analysis animal was the experimental unit. For the growth assay, well was the experimental unit. In all experiments main effects are considered significant if the $P \leq 0.05$.

Results and Discussion

Previous work in our laboratory investigating the effects of long chain FA on bovine adipose differentiation and lipid filling, utilizing both clonal derived and tertiary S-V SC and IM cells, found that specific long-chain FAs preferentially induce differentiation in these relative depots. Davis (2011) found that adipogenic differentiation of SC preadipocytes increased in response to OLE and LIN acid; however, differentiation of IM preadipocytes increased in response to ALA and ARA. Further, CLA t10, c12 increased preadipocytes differentiation from both SC and IM preadipocytes to an equal extent. Altering the FA profile of cattle could potentially improve Quality and Yield Grades by stimulating IM adipocyte depot development while downregulating SC adipocyte depot development.

As an extension of this previous *in vitro* work, the current experiment investigated the possible *in vivo* effects a course of specific long-chain PUFA treatment vs SFA treatment would have on various markers of adipogenesis. Funston et al., (2010) found that stimulation of IM tissue development early in life can lead to improvements in Quality Grade at harvest. Du et al. (2013) stated that, since the amount of progenitor cells available to dedicate to adipose tissue declines with animal age, manipulating cell differentiation early in life is more effective to increase IM adipose tissue. Additionally, they found that both nutritional and physiological conditions in the fetal, postnatal, and early post-weaning stages of life can positively affect adipogenesis and the total number of adipocytes. In ruminants, the absorption of free FAs is very efficient in the intestine. However, unsaturated fatty acids fed to ruminating animals typically undergo biohydrogenation by action of the rumen bacteria converting unsaturated FAs to more saturated FAs (Polan, 1964). To avoid concerns with ruminal biohydrogenation, as well as, endeavor to stimulate IM development early in life,

our investigation used milk-fed pre-ruminating calves. The objective was to compare the effects of dietary PUFA vs SFA in pre-ruminating calves on preadipocyte differentiation and adipogenic potential. Subsequent to the feeding course, fatty acid profiles, cell growth, morphological differentiation, lipid accumulation, and expression of adipogenic genes were measured on the cellular level.

Fatty Acid Profile

Gas chromatography was performed on prepared IM, SC, and PR tissue samples and calf serum to determine and quantify the fatty acids present. Lauric acid (12:0), myristic acid (14:0) 14:1, pentadecanoic acid (15.0), palmitic acid (**PAL**) (16:0), stearic acid (**STE**) (18:0), oleic acid (**OLE**) (18:1 c9), 18:2 t9t12, LIN (18:2 c9c12), eicosenoic acid (20:1 c11), ALA (18:3n3), CLA c9,t11 (18:2 c9t11), CLA t10, c12 (18:2 t10c12) were measured for each depot and treatment.

Stearic and eicosenoic acid were higher in the IM tissue of PUFA fed calves than SFA supplemented calves ($P < 0.05$). CLA c9, t11, and CLA t10, c12 tended to be higher ($P=0.06$) in the IM tissue of PUFA fed calves. A trend was also found for LIN, which was greater in the PUFA group than the SFA group ($P=0.09$). Oleic acid was greater in the IM tissue of SFA fed calves ($P=0.01$). For IM tissue samples, there was no difference between dietary treatments for lauric acid, myristic, 14:1, pentadecanoic, PAL, or ALA ($P>0.18$; Table 4).

In SC tissue, lauric, myristic, 14:1 PAL, OLE, and 18:1t12 were greater in the SFA treatment group ($P<0.01$). Eicosenoic acid, LIN, and CLA isomers c9, t11 and t10, c12 were greater in the SC tissue of calves fed PUFA ($P<0.01$). In SC tissue, STE tended to be greater in the SFA group than the PUFA group ($P=0.08$). For SC tissue samples, no difference was

found between treatment groups for pentadecanoic acid, 18:2 t9t12, or ALA (P> 0.12; Table 5).

Myristic acid, OLE, and PAL were all greater in the PR tissue samples of the SFA treatment group (P<0.04). Eicosenoic acid, LIN, and both c9, t11 and t10, c12 isomers of CLA were greater in PR tissue of calves fed PUFA (P<0.04). The SFA treatment group also tended to have higher STE in PR tissue compared with PUFA treatment (P=0.07). In PR tissue samples no difference was found due to dietary FA for lauric, 14:1, pentadecanoic, 18:2 t9t12 , or ALA (P>0.20; Table 6).

In calf serum from the PUFA treatment group ALA, CLA c9, t11, and CLA t10, c12 were greater (P < 0.0002). Lauric, myristic, PAL, and OLE were greater in calf serum from the SFA treatment group (P < 0.03). No treatment difference was found for STE, LIN, or 18:2 t9t12 (Table 7).

Both Choi et al. (2013) and Gang (2012) found that, relative to control, SFA decreased CLA c9, t11 in *Longissimus* tissue, and CLA t10, c12 in SC adipose tissue. We found that both isomers of CLA were almost nonexistent in the SFA treatment group in all depots and serum. Additionally, Choi et al. (2013) found that treatment with SFA increased STE in both *Longissimus* muscle and SC tissue, which is inconsistent with our findings. In studies by Moibi and Chistopherson (2001) and Castro et al. (2004) supplementation with SFA increased the PAL content in internal fat depots (OM, mesenteric, and PR) but did not increase PAL in SC or IM adipose tissue. We found that SFA increased PAL in PR tissue and SC tissue, but not in IM tissue. The reason for this is unclear. However, St. John et al. (1991) found that fatty acid elongase and SCD work together in bovine SC adipose tissue to convert PAL to OLE. We found increased levels of OLE in all depots and serum of SFA

treated calves.

Kennelly (1996) found that feeding flaxseed to lactating Holstein cows reduces the amount of short-chain (C4 to C12) FAs and PAL, while the amount of 18-carbon FAs increased. Baumgard et al. (2002) also found that CLA t10, c12 reduced the amount of milk fat by 42% with the decrease in C4 - C16 (PAL) comprising 63% of the total milk fat decrease. Cinquart et al. (1991) fed steam flaked linseed to growing-fattening bulls and found that this diet increased OLE, LIN, and ALA in PR fat, while myristic, PAL, and STE were reduced. The ALA content doubled compared to control. Gang (2012) identified that palm oil treatment decreased ALA in both muscle tissue and SC adipose tissue. Alvarado-Gilis et al. (2015) found that ALA content in bovine tissue increased in a dose dependent manner when fed flaxseed. Scollan et al. (2001) in a feed trial using Megalac (PAL) as the control and linseed (ALA), or fish oil as the treatment, found that the linseed significantly increased the level of ALA. Additionally, Li et al. (2012) found that linseed oil treatment increased the amount of ALA in goat mammary tissue. Gomez et al. (2015) feeding young Holstein bulls found that linseed or linseed/CLA diets increased the amount of ALA 6-fold in IM tissue. In the current experiment, we did not see a substantial amount of ALA in any of our tissue samples, but the calf serum had a 20-fold higher concentration of ALA in the PUFA treatment group ($P < 0.0001$). The reason for this descrepency is unclear.

Scollan et al. (2001) found that PAL, STE, OLE, LIN were not affected by the linseed treatment. In contrast, we identified that STE was greater in IM tissue in the PUFA treatment group. Palm oil supplemented ram and ewe lambs (Solomon et al., 1992) and bull calves (Partida et al., 2007) had higher IM PAL levels. In the present study, PAL and OLE were greater in SC tissue, PR tissue, and calf serum in the SFA treatment group. However, no

treatment difference was seen for PAL in IM tissue, but OLE was greater in IM tissue of SFA fed calves.

Griinari et al. (2000) found that in ruminants, naturally occurring CLA is produced primarily from ruminal biohydrogenation of PUFAs. Since the calves in our study were pre-ruminating, the greater levels of both treatment CLA isomers present in the calf serum and tissues of the PUFA group, with almost none at all in the SFA group, might indicates that the dietary FA does go into the animal tissue. Rase et al. (2004) found that a higher concentration of CLA in muscle tissue is associated with a higher degree of IM fat. Interestingly, several studies found that the addition of flaxseed to the ration significantly increased both bovine and ovine IM CLA levels (Enser et al. 1999; Wachira et al. 2002; Demirel et al. 2004). Gomez et al. (2015) found that linseed/CLA diets produced a 3-fold increase in CLA c9, t11 of both IM and SC tissue compared to control, which is consistent with our findings. As in our results, Schlegel et al. (2012) found that both *Longissimus* muscle and SC adipose tissue had increased levels of CLA t10,c12 in cattle fed rumen-protected CLA; however, unlike our results, in their study neither depot showed a difference in the amount of CLA c9,t11.

In general, the SFA supplementation tended to increase the shorter chain and more saturated FAs, while the PUFA supplementation increased the PUFAs. This is to be expected since the pre-ruminating calves were essentially monogastric, enabling them to deposit a substantial quantity of the FA as absorbed, without ruminal modification. Additionally, this demonstrates that the PUFA and SFA treatments were effective in inducing FA changes in the calf tissue.

Growth Assay

Several studies have identified the mitogenic potential of serum *in vitro* using porcine (Allen et al., 1982), poultry (Chen and Buchanan 1975; Doumit et al., 1990), human (Caronti et al., 1998), ovine (Olson et al., 1981) and bovine (Weber et al., 1999) cells. Cell growth was enhanced when serum was added to the media. However, there is a paucity of research dealing with using sera from animals given various dietary treatments to elucidate cellular growth potential. The present growth assay evaluated the differences in mitogenic potential of the serum collected and processed from each calf in respective treatment groups (PUFA or SFA). The prepared serum was applied for 0, 2, and 4 days to clonally derived SC and IM preadipocytes isolated from the same steer (Grant et al., 2008a).

On growth d2, IM clonal preadipocytes treated with serum from calves supplemented with PUFA had a 7.18 fold increase in DNA/well, while those treated with SFA had a 6.68 fold increase in DNA/well ($P=0.22$; Table 8). The mean growth rate for IM cells treated with PUFA and SFA was not different ($P>0.20$; Table 8). On d2 no difference was seen in the abundance of DNA/well or mean growth rate for SC preadipocytes from either treatment group ($P>0.6$; Table 8).

On growth d4, IM clonal preadipocytes showed no difference in DNA/well abundance (16.63 fold increase SFA vs 16.57 fold increase PUFA) or mean growth rate when treated with serum from calves supplemented with PUFA or SFA ($P>0.9$; Table 9). To d4 the SC preadipocytes treated with serum from the PUFA treated calves grew faster than those treated with serum from the SFA treatment ($P=0.033$; Table 9). This is reflected by the greater DNA content after 4 days of treatment ($P<0.027$). Conversely, Choi et al. 2013 found

that SC adipocyte volume was greater in palm oil-fed steers as compared to those fed soybean oil.

It is interesting to note that the IM cells despite the treatment had over 3-fold increase in amount of DNA/well than SC cells ($P<0.0001$; Table 10). Consistent with our results, Alberti et al. (2013) found that dietary supplementation with whole linseed and CLA increased IM adipocyte proliferation. However, we also saw an increase in growth with the SFA treatment. The differences observed in the growth assay between IM and SC cells are possibly due to the growth characteristics of the individual preadipocyte clones.

Clonal Analysis

The morphological assessment of adipocyte growth was conducted on the S-V cells isolated from the calves. The S-V cells harvested following the treatments with PUFA and SFA provided the opportunity to study the effects of each treatment on the adipogenic potential of preadipocytes from IM and SC fat depots. Adipocyte differentiation was morphologically assessed by enumerating the number of cells that contained lipid droplets stained with ORO.

There was no depot by treatment difference in the differentiation ability of either IM ($P=0.33$ or SC ($P=0.54$) cells isolated from calves supplemented with PUFA or SFA (Figure 4). Although not statistically different, it is interesting to note that IM cells harvested from SFA treated calves differentiated to a slightly higher percentage than those from PUFA treated calves (Figure 4), since SFA has been shown to not promote differentiation of cultured bovine IM preadipocytes (Davis, 2011). Conversely, there was a treatment by depot effect in that the SC cells harvested from PUFA fed calves had a greater degree of differentiation than the IM cells from PUFA fed calves ($P=0.01$; Figure 5). This is opposite

from the effect we were expecting; however, it is consistent with results from Lin et al. (1992) who found that PAL was incorporated into triacylglycerols at a greater rate in bovine IM cells as compared to SC cells. Conversely, several researchers found that PAL appeared to cause cell apoptosis (Beeharry et al., 2004; Gue et al., 2007, Davis, 2011). Choi et al. (2014) found that the greatest increase in IM volume was in tissue treated with ALA, STE, and *trans*-vaccenic acid. McNeel et al. 2003, utilizing human preadipocytes, and Ding et al. (2000) utilizing porcine SC S-V preadipocytes, both found that isomers of CLA increased differentiation in these cells. Additionally, Zhou et al. (2007) found that both CLA isomers increased lipid accumulation and number of ORO stained cells in IM cultures. However, Ding et al. (2003) found that ALA did not stimulate porcine SC cells to the extent of shorter-chain FA. Davis (2011) found that PAL increased the percentage of ORO positive SC preadipocytes, however not to the extent of OLE or LIN. Additionally, both IM and SC differentiation was stimulated by CLA. We found that the SC cells in both treatment groups displayed greater differentiation than the IM cells. This is consistent with Grant et al. (2008b) and Soret et al. (1999) who found that SC cells differentiated at a greater rate than other depots in bovine and ovine cell cultures, respectively. Wu et al. (2000) found that cells cultured from bovine SC adipose tissue *vs.* OM adipose tissue had a greater response to differentiation media; whereas Grant et al. (2008a) found that the media component TRO increased differentiation in both SC and IM cells without differential effect. Hausman and Poulos (2004) found that the media component DEX increased both SC and IM S-V cell differentiation in equal relative proportions, but no matter what treatment was used, SC cells differentiated at a higher rate than IM. This is consistent with our results.

Gene Expression

Quantitative real-time polymerase chain reaction quantifications of mRNA were conducted on IM, SC, and PR tissue samples harvested from the calves receiving PUFA and SFA treatments. Quantitative real-time polymerase chain reaction (**qRT-PCR**) was used to quantify mRNA abundance of genes involved in adipogenesis, including preadipocyte factor-1 (**Pref-1**), PPARγ, acetyl CoA carboxylase (**ACC**), fatty acid binding protein (**FABP**), and fatty acid synthase (**FAS**). These genes represent a preadipocyte marker (Pref-1), a transcription factor that controls preadipocyte differentiation (PPARγ), and enzymes involved in fatty acid synthesis (ACC, FAS, and FABP) in IM, SC, and PR depots. The 18S rRNA was used as an endogenous reference gene for all RNA analyses**.**

No difference in mean relative abundance of PPARγ mRNA (P=0.44), ACC mRNA (P=0.79), FAS mRNA (P=0.81) as measured by using 7500 Fast QRT-PCR was observed for IM tissue due to dietary fatty acid treatment (Tables 11-13). In SC tissue no difference in mean relative abundance of PPARγ mRNA (P=0.67), ACC mRNA (P=0.99), FAS mRNA (P=0.95; Tables 11-13). No difference in mean relative abundance of PPARγ mRNA (P=0.80), ACC mRNA (P=0.88), FAS mRNA (P=0.46) was observed in PR tissue due to dietary fatty acids (Tables 11-13). However, expression of mRNA for all genes was one to two orders of magnitude greater for PR tissue than from the other depots. Pref-1 and FABP were not expressed in measurable quantities in any of our samples; therefore, no data is extant.

Gene expression in adipose tissue depots was not statistical different due to dietary treatment. However, PPARγ was expressed in varying amount by depot independent of dietary treatment with PR > SC > IM. There were within depot differences in tissue response

to the treatments. In IM tissue twice as much PPARγ was expressed with PUFA treatment compared to SFA. SC tissue showed almost equal expression with each treatment. PR showed slightly more PPARγ expression with the PUFA than with SFA. Interestingly, all three depots had the highest expression of PPARγ in tissue of calves receiving the PUFA treatment. Meadus et al. (2002) found that expression of PPARγ and FABP mRNA were significantly increased by dietary treatment of pigs with CLA. Moya-Camarena et al. (1999), using rat cells, found that CLA c9, t11 is a robust ligand and activator of PPAR. In porcine studies, Zhou et al. (2007) found that both CLA isomers increased expression of PPARγ and FAS in IM tissue; however, in their study CLA t10, c12 decreased gene expression of ADD1, PPARγ, aP2, LPL, and IR in SC S-V cells, but CLA c9, t11 had no effect on expression of these genes in SC S-V cells. Conversely, Thoennes et al. (1999), using MCF-7 human cells found that omega-3 PUFA inhibited the transcriptional activity of PPARγ below controls. Davis (2011) found that PPARγ expression was greater in SC preadipocytes than IM cells when treated with ARA, OLE, ALA, and combinations of these FAs. Expression of PPARγ appears to be influenced by species, depot, and the specific FA ligand.

As we saw with PPARγ, ACC was also expressed in varying amount by depot independent of treatment with PR > SC > IM. Again, SC cells were almost identical in expression between treatments. Choi et al. (2014) using FA as part of their treatment media on freshly harvested Angus steer IM and SC tissue at 12, 14, and 16 month of age, found that CLA t10,c12 did not significantly affect adipogenic gene expression in either IM or SC bovine adipose tissue. Conversely, Kadegowda et al. (2013), utilizing primary bovine preadipocytes, and Baumgard et al. (2002) utilizing bovine milk and mammary tissue, found that treatment with CLA t10,c12 decreased expression of ACC and FAS. However, Davis

(2011) found that ARA, ALA, OLE and combinations of these increased SC preadipocytes ACC mRNA compared to the control.

Fatty Acid Synthase also had a depot response independent of treatment. However PR had greater expression than both SC and IM, which had similar levels of expression unlike PPARγ and ACC. Mendizabal et al. (1999) found that, in Spanish steers, adipocytes from PR were the largest and showed the greatest FAS activity, which is consistent with our results.

There was no statistically significant difference between treatments in expression of the genes measured. Possibly the reason that no significant gene expression was found was the young age of the test subjects. According to the findings of Wang, et al. (2009), qPCR test performed on cattle at 3 months of age showed less gene expression than at 7, 12, 20, and 25 month of age, with most gene expression increasing with age. In fact, their tests showed that while C/EBPβ was expressed in greater amount in an earlier time period, expression of PPARγ did not peak until the last two time periods. However, FABP and FAS were greatly elevated at 7 months of age just prior to weaning. While the gene expression between treatments was not significant, there were depot differences in the current experiment with regard to the amount of gene expression (PR > SC > IM) for both PPARγ and ACC. Hood (1983) determined that lipid accretion rates in bovine adipose tissue depots occur in the following order; PR, OM, SC, intermuscular, and IM. Our results suggest that gene expression would also follow the same order.

Conclusion and Implications

The present study demonstrated that a brief feeding period of SFA or PUFA could effectively alter the FA profile of bovine serum and PR, IM, and SC adipose tissue. In general, the SFA supplementation tended to increase the shorter chain and more saturated

FAs, while the PUFA supplementation increased the longer-chain PUFAs. Treatment with PUFA increased the percentage of CLA c9, t11 and CLA t10, c12 in serum and all tissue samples. The serum also had increased levels of ALA. Treatment with SFA increased the percentages of OLE in serum and all depots, and the percentage of PAL in serum and SC, and PR fat depots. This indicates that it might be possible to alter the FA profile of bovine meat through supplemental feeding. Increasing PUFAs, especially CLA c9, t11 and CLA t10, c12, in human diets has been shown to have several biologically beneficial effects, such as reduced cardiovascular diseases and cancer, improved bone mass, and modulated immune and inflammatory responses (Dilzer and Park, 2012). Isomers of CLA have been shown to foster development of IM adipocytes in porcine, ovine, and bovine tissues (Ding et al., 2000; Zhou et al., 2007; Barnes et al., 2012; Alberti et al. 2013). Therefore, supplemental feeding of PUFA has the potential to improve the amount of CLA isomers in the meat, which would have a dual effect of increasing IM development and contributing to human health.

The results from the growth assay suggest that there are differences in mitogenic potential induced by feeding additional FAs. The serum from PUFA treated calves stimulated SC clonal preadipocytes proliferation to a greater extent than those fed SFA. However, IM clonal preadipocytes proliferated equally, irrespective of dietary treatment of the calf serum applied. Additionally, isolated SC S-V cells from the PUFA treatment differentiated at a higher rate *vs.* IM S-V cells.

Stimulation of IM tissue development early in life is more effective at increasing IM adipose tissue in the finished animal, which can lead to improvements in Quality Grade at harvest (Funston et al., 2010; Du et al., 2013). Nutritional conditions in the fetal, postnatal, and early post-weaning stages of life can positively affect adipogenesis. Drouillard et al.

(2002) fed weaned calves for 36 to 40 days with tallow, high in SFA, or 10% flaxseed, high in PUFA; and then finished all the cattle on a common finishing diet. The cattle that had been fed 10% flax had higher marbling scores (Quality Grade) than those that received tallow. This indicates the potential of an early feeding course with PUFAs may prove both economical and effective in increasing IM adipose tissue. Unfortunately, the current experiment provided indications that SC adipose tissue may be more sensitive to dietary FA treatment than IM adipose tissue in calves under two months of age.

The current study fed pre-weaned calves milk supplemented with FAs. This approach might not be effective for large-scale producers. However, dietary long-chain FAs fed to lactating ruminants can be incorporated directly into the milk, altering the FA profile (Kennelly 1996). Flaxseed oil fed to lactating bovine and caprine animals increased the proportion of ALA, CLA c9, t11, and CLA t10, c12 in the milk (Cortes et al., 2010; Li et al., 2012). Clearly, this provides a method to feed the pre-weaned calf targeted PUFAs in an efficient, economical, and effective manner. This could prove beneficial in altering the carcass characteristics and fatty acid profile of the meat, which would have positive effects for the producer in improved Quality and Yield Grade, as well as the consumer in higher quality and healthier beef.

The results from this study gave some indications that feeding specific FAs may alter the FA profile of tissue and serum of the calves, affect the mitogenic properties of serum, and the propensity of preadipocytes from these calves to differentiate. Further research is needed to see if alterations in FA profile early in a calf's life would have an effect at slaughter on the final FA profile of the meat and the Quality and Yield Grades.

Tables and Figures

Table 1. Calf cohort groups with weights

Cohort Group	Calf #	Treatment	Date of Birth	Birth Wt .	Day 0 Date	Wt D 0	Wt D 25	Wt D 50
1	**108**	SFA	7/22	42	7/28	81	103	139
	109	PUFA	7/22	57	7/28	-	108	142
2	**110**	PUFA	7/24	-	7/29	-	115.5	155.5
	111	SFA	7/26	81	7/29	-	109	146.5
3	**112**	SFA	7/27	88	8/1	93	108	142.5
	113	PUFA	7/29	85	8/1	88	109	141.5
4	**114**	PUFA	7/29	87	8/1	93	112	154
	115	SFA	7/29	93	8/1	105	121.5	152
5	**117**	SFA	8/2	85	8/6	78	106	138.5
	118	PUFA	8/2	99	8/6	101	118	160.5
6	**119**	PUFA	8/2	85	8/6	94	109	144
	120	SFA	8/2	80	8/6	92	111	145.5

Table 2. Fatty acid (percentage of fatty acid methyl ester) composition of dietary treatments

Fatty Acid	Palm Oil	Lutalin® (CLA)	Flax Oil
8:0 Caprylic	0.013618	0	0
10:0 Capric	0.013611	0	0
12:0 Lauric	0.168644	0	0
14:0 Myristic	1.142847	0.0771	0.042087
15:0 Pentadecanoic	0.066336	0	0
16:0 Palmitic	**56.8261**	6.779139	5.27448
16:1n7 c9 Palmitoleic	0.160814	0.085806	0.065951
17:0 Margaric	0.129832	0	0.063401
17:1 c10	0.025178	0	0
18:0 Stearic	4.941934	3.818771	4.095537
18:1 c9 Oleic	34.32076	25.24196	21.38818
18:1 t12	1.134284	1.061405	0.984227
18:2 t9t12	0.126355	0.119678	0
18:2 c9c12	0.397585	1.771385	15.00866
18:3n6y	0.107247	0.258993	0.209313
20:0	0.028358	0	0.139133
20:1 c11	0.023688	0	0
18:3n3	0.106098	0.190866	**52.5815**
18:2 c9t11	0.126154	**29.76881**	0
18:2 t10c12	0	**29.55465**	0
21:0	0	0.215548	0
20:3n6	0.065988	0.656719	0.14753
22:1t13	0	0.167886	0

Greatest % FA in each treatment in bold.

Table 3. Primer and Probe Sequences used in Real-Time PCR

Gene	Accession Number	Primers and TaqMan® Probe Sequences, 5' - 3'
18S	AF243428	FP: CCACGCGAGATTGAGCAAT RP: GCAGCCCCGGACATCTAA TP: ACAGGTCTGTGATGCC
Peroxisome proliferator activated-receptor gamma (PPARγ)	NC_007320.3	FP: GGCGATCTTGACGGGAAA RP: CCCATCATTAAGGAGTTCATGTCAT TP: CAAATCACCGTTTGTTATC
Acetyl-CoA carboxylase alpha (ACCα)	NM_174224.2	FP: CAATGCAGATGACTTCCCTAACC RP: GACAAAGATAGGAGACCCAGGAACT TP: CTTCAGACAGGTTCAAGCT
Preadipocyte Factor 1 (pref-1)	NM _010052.3	FP: AATAGACGTTCGGGCTTGCA RP: GGTCAACGCAAGTTCCATTG TP: CTCAACCCCCTGCGC
Fatty Acid Binding Protein (FABP)	NM _024406.1	FP: CCGCAGACGACAGGAAGGT RP: AGGGCCCCGCCATCT TP: AAGAGCATCATAACCC
Fatty Acid Synthase (FAS)	NM_001012669.1	FP: CAGAAGGTGCTCCAGAGTGA RP: CCCCAGGCCCCATCA TP: CTGGTGATGAATGTCT

Forward primer (FP), reverse primer (RP) and Taqman® probe (TP) sequences were indices along with gene bank accession (GBA) number for the genes analyzed by employing TaqMan ® primer probe system of real-time PCR.

Table 4. Fatty acid (percentage of fatty acid methyl ester) composition of non-esterified fatty acid portion of IM adipose tissue obtained from milk-fed Holstein bull calves receiving either SFA or PUFA oil supplement for 50 days beginning at 3 days of age (n = 6 per treatment)

FATTY ACID	SFA	PUFA	SEM	P-VALUE
LAURIC (12:0)	0.22	0.28	.0394	0.18
MYRISTIC (14:0)	2.56	2.43	.4871	0.80
14:1	0.50	0.54	.0541	0.48
PENTADECANOIC (15:0)	0.57	0.57	.0355	0.95
PAL (16.0)	34.76	34.99	1.3331	0.87
STEARIC (18.0)	23.09	27.17	1.7539	**0.05***
OLEIC (18:1 C9	23.70	16.93	1.9387	**0.01***
18:2 T9T12	0.20	0.32	0.4576	**0.01***
LINOLEIC (18:2 C9C12	0.88	1.77	.0325	0.09
EICOSENOIC (20:1 C11)	0.12	1.11	0.2208	**0.01***
ALA (18:3N3)	0.16	0.20	0.0368	0.26
CLA C9T11 (18.2 C9T11)	0.0	0.28	0.1101	0.06
CLA T10,C12 (18:2 T10C12)	0.0	0.34	0.1277	0.06

Table 5. Fatty acid (percentage of fatty acid methyl ester) composition of non-esterified fatty acid portion of SC adipose tissue obtained from milk-fed Holstein bull calves receiving either SFA or PUFA oil supplement for 50 days beginning at 3 days of age (n = 6 per treatment)

FATTY ACID	SFA	PUFA	SEM	P-VALUE
LAURIC (12:0)	0.38	0.23	0.0348	**0.013***
MYRISTIC (14:0)	3.93	2.70	0.3078	**0.02***
14:1	0.50	0.29	0.0435	**0.007***
PENTADECANOIC (15:0)	0.51	0.38	0.0652	0.13
PAL (16.0)	28.94	24.14	1.0993	**0.01***
STEARIC(18\.0)	15.54	13.79	0.7451	0.08
OLEIC (18:1 C9)	31.91	27.32	0.4487	**0.005***
18:1 T12	1.98	1.73	0.0474	**0.006***
LINOLEIC (18:2 C9C12	4.89	7.22	0.427	**0.005***
EICOSENOIC (20:1 C11)	0.29	7.20	0.7031	**0.01***
ALA (18:3N3)	0.22	0.22	0.0273	0.94
CLA C9T11 (18.2 C9T11)	0.31	2.15	0.1503	**0.006***
CLA T10,C12 (18:2 T10C12)	0.0	1.39	0.0274	**0.004***

Table 6. Fatty acid (percentage of fatty acid methyl ester) composition of non-esterified fatty acid portion of PR adipose tissue obtained from milk-fed Holstein bull calves receiving either SFA or PUFA oil supplement for 50 days beginning at 3 days of age (n = 6 per treatment)

FATTY ACID	SFA	PUFA	SEM	P-VALUE
LAURIC (12:0)	0.22	0.17	.0414	0.23
MYRISTIC (14:0)	3.38	2.86	0.2251	**0.05***
14:1	0.54	0.58	0.0325	0.28
PENTADECANOIC (15:0)	0.27	0.21	0.0377	0.18
PAL (16.0)	32.81	26.51	1.877	**0.01***
STEARIC(18.0)	18.57	14.36	2.2788	0.07
OLEIC (18:1 C9	36.66	32.44	0.8930	**0.002***
LINOLEIC (18:2 C9C12	2.97	3.99	0.4145	**0.04***
EICOSENOIC (20:1 C11)	0.17	6.74	0.3738	**0.0008***
ALA (18:3N3)	0.26	0.20	0.0548	0.31
CLA C9T11 (18.2 C9T11)	0.19	1.74	0.1498	**0.002***
CLA T10,C12 (18:2 $T1^{0C}12$)	0.0	1.50	0.1002	**0.001***

Table 7. Fatty acid (percentage of fatty acid methyl ester) composition of non-esterified fatty acid portion of the serum obtained from milk-fed Holstein bull calves receiving either SFA or PUFA oil supplement for 50 days beginning at 3 days of age (n = 6 per treatment)

FATTY ACID	SFA	PUFA	SEM	P-VALUE
LAURIC (12:0)	0.39	0.20	.0604	**0.0118***
MYRISTIC (14:0)	1.62	0.95	.2482	**0.0334***
PENTADECANOIC (15:0)	0.37	0.30	.0353	0.0866
PAL (16.0)	21.32	16.29	1.04	**0.0007***
STEARIC(18.0)	16.00	17.19	.9152	0.2205
OLEIC (18:1 C9	17.81	10.52	1.0721	**0.0007***
18:2 t9t12	0.22	0.21	.0909	0.8492
LINOLEIC (18:2 C9C12	28.40	27.42	1.5926	0.5629
ALA (18:3N3)	0.74	15.27	.7530	**<0.0001***
CLA C9T11 (18.2 C9T11)	0.17	1.56	.1657	**0.0002***
CLA T10,C12 (18:2 T10C12)	0.00	1.97	.1679	**<0.0001***

Table 8. Day 2 mean ngDNA per well and growth rate on clonally derived IM and SC preadipocytes treated with prepared serum from calves supplemented with either SFA or PUFA

Tissue/Treatment	n	Amount ngDNA/well	p-value	Growth Rate	p-value
IM/ SFA	24	435.8	0.2200	0.9400	0.2283
IM/PUFA	24	468.3		0.9769	
SC/ SFA	20	167.4	0.6743	0.5365	0.6587
SC/PUFA	23	180.4		0.5656	

Table 9. Day 4 mean ngDNA per well and growth rate on clonally derived IM and SC preadipocytes treated with prepared serum from calves supplemented with either SFA or PUFA

Tissue/Treatment	n	Amount ngDNA/well	p-value	Growth Rate	p-value
IM/ SFA	24	1084.8	0.9449	0.6972	0.8990
IM/PUFA	24	1080.6		0.6990	
SC/ SFA	20	266.5	0.0270	0.3891	0.0325
SC/PUFA	23	302.6		0.4212	

Table 10. Growth assay by treatment group, SFA or PUFA, on clonally derived IM and SC preadipocytes with the fold increase over day 0

DAY	TREATMENT	DEPOT	FOLD INCREASE	SEM	p-value
D2	SFA	IM	6.681674	0.3349	>.0001*
		SC	3.123908		
	PUFA	IM	7.180636	0.3479	>.0001*
		SC	3.366014		
D4	SFA	IM	16.63347	0.8531	>.0001*
		SC	4.826806		
	PUFA	IM	16.56822	0.5471	>.0001*
		SC	5.481221		

Table 11. Relative abundance of PPARγ mRNA expression by depot and treatment

Tissue/Treatment	n	Mean Relative Abundance	SEM	p-value
IM/SFA	3	$1.10*10^{-5}$	$2.326 * 10^{-6}$	0.4493
IM /PUFA	4	$2.00*10^{-5}$	0.000011	
SC/SFA	2	$1.01*10^{-4}$	0.000011	0.6743
SC/PUFA	3	$7.70*10^{-5}$	0.000047	
PR/SFA	4	$3.09*10^{-3}$	0.0018	0.8064
PR/PUFA	4	$4.25*10^{-3}$	0.0041	

Calf is the experimental unit. IM is *Longissimus dorsi* tissue, SC is subcutaneous tissue, and PR is perirenal tissue. Relative expression of PPAR mRNA using 7500 Fast qRT-PCR.

Table 12. Relative abundance of ACC mRNA expression by depot and treatment

Tissue/Treatment	n	Mean Relative Abundance	SEM	P-value
IM /SFA	4	$1.91*10^{-4}$	0.000086	0.7994
IM /PUFA	4	$2.27*10^{-4}$	0.000102	
SC/SFA	2	$4.45*10^{-4}$	0.00022	0.9920
SC/PUFA	3	$4.42*10^{-4}$	0.00016	
PR/SFA	4	$5.87*10^{-3}$	0.0030	0.8868
PR/PUFA	4	$6.76*10^{-3}$	0.0052	

Calf is the experimental unit. IM is *Longissimus dorsi* tissue, SC is subcutaneous tissue, and PR is perirenal tissue. Relative expression of ACC mRNA using 7500 Fast qRT-PCR.

Table 13. Relative abundance of FAS mRNA expression by depot and treatment

Tissue/Treatment	n	Mean Relative Abundance	SEM	P-value
IM /SFA	4	$1.21*10^{-4}$	0.000046	0.8163
IM /PUFA	4	$1.08*10^{-4}$	0.000030	
SC/SFA	2	$1.13*10^{-4}$	0.000064	0.9594
SC/PUFA	3	$1.08*10^{-4}$	0.000054	
PR/SFA	4	$5.34*10^{-3}$	0.0015	0.4645
PR/PUFA	4	$1.22*10^{-2}$	0.0081	

Calf is the experimental unit. IM is *Longissimus dorsi* tissue, SC is subcutaneous tissue, and PR is perirenal tissue. Relative expression of Fatty Acid Synthase mRNA using 7500 Fast qRT-PCR.

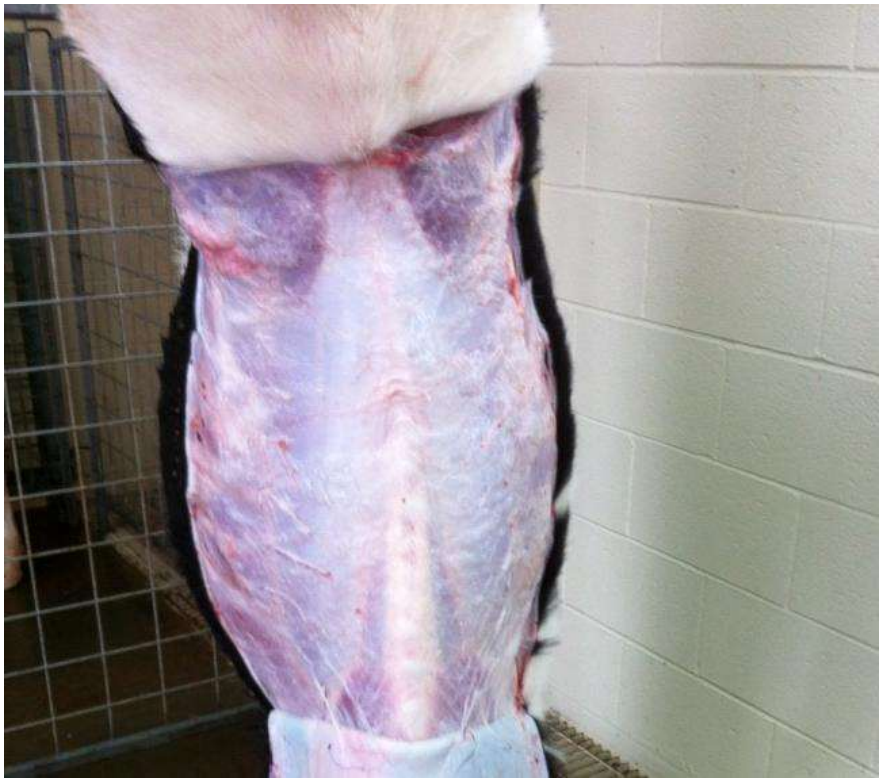
A.

B.

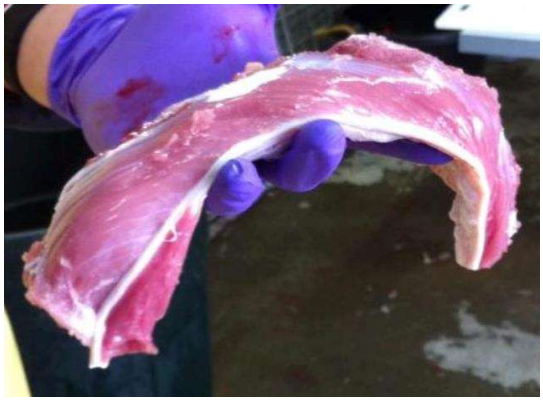
C.

Figure 1. A. Individual calf hutches. B. Preparation of treatments - PUFA treatment = 10 ml Lutalin (CLA) + 20 ml Flaxseed oil per 4 L milk twice per day. SFA treatment = 30 ml palm oil per 4 L milk twice per day. C. Feeding treatments.

A.



B.



C.

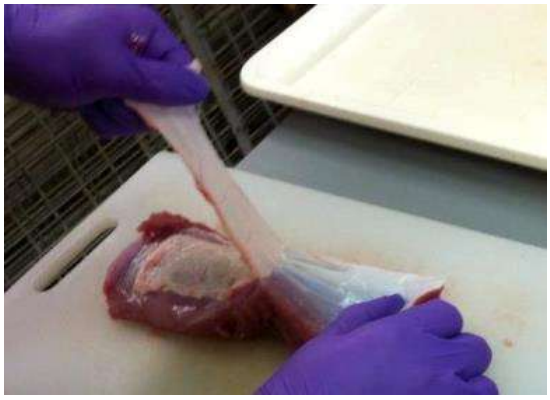


Figure 2. Preparation of samples. A. *Longissimus dorsi* exposed. B. Removal of *Longissimus dorsi.* C. SC separated from *Longissimus dorsi* muscle.

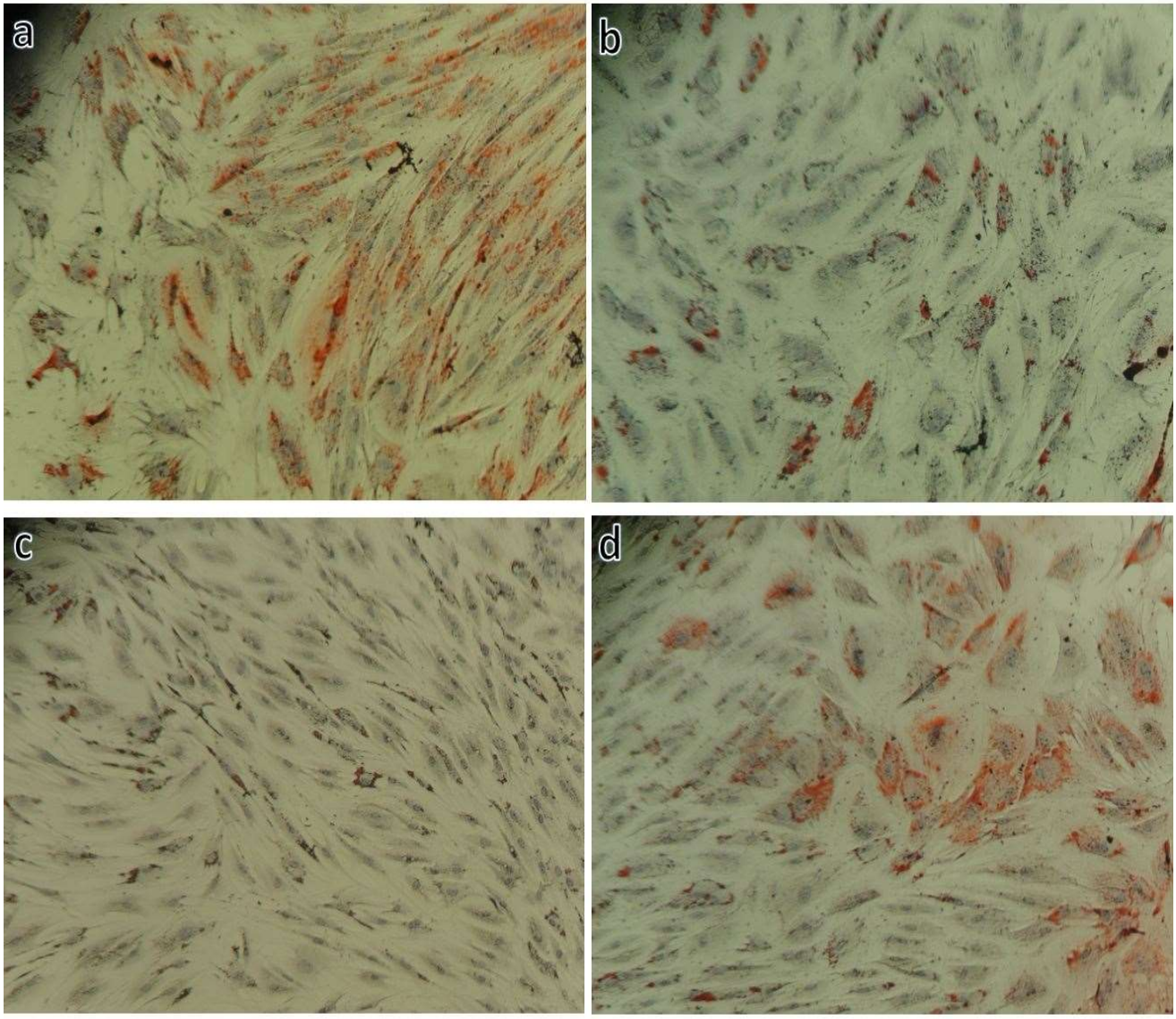


Figure 3: Representative sample of photomicrographs used to determine the proportion of differentiated cells within colonies. Cells were incubated in growth medium undisturbed for 8 days. A differentiated cell was defined as a cell having one or more lipid dropets and a colony with at least one differentiated cell was defined as adipogenic. Photomicrographs of each colony were taken across the diameter of the colony to capture representative cross-section of cells at 200X magnification. photo legend: a = S-V IM calf cells treated with SFA; b = S-V IM calf cells treated with PUFA; c = S-V SC calf cells treated with SFA; d = S-V SC calf cells treated with PUFA.

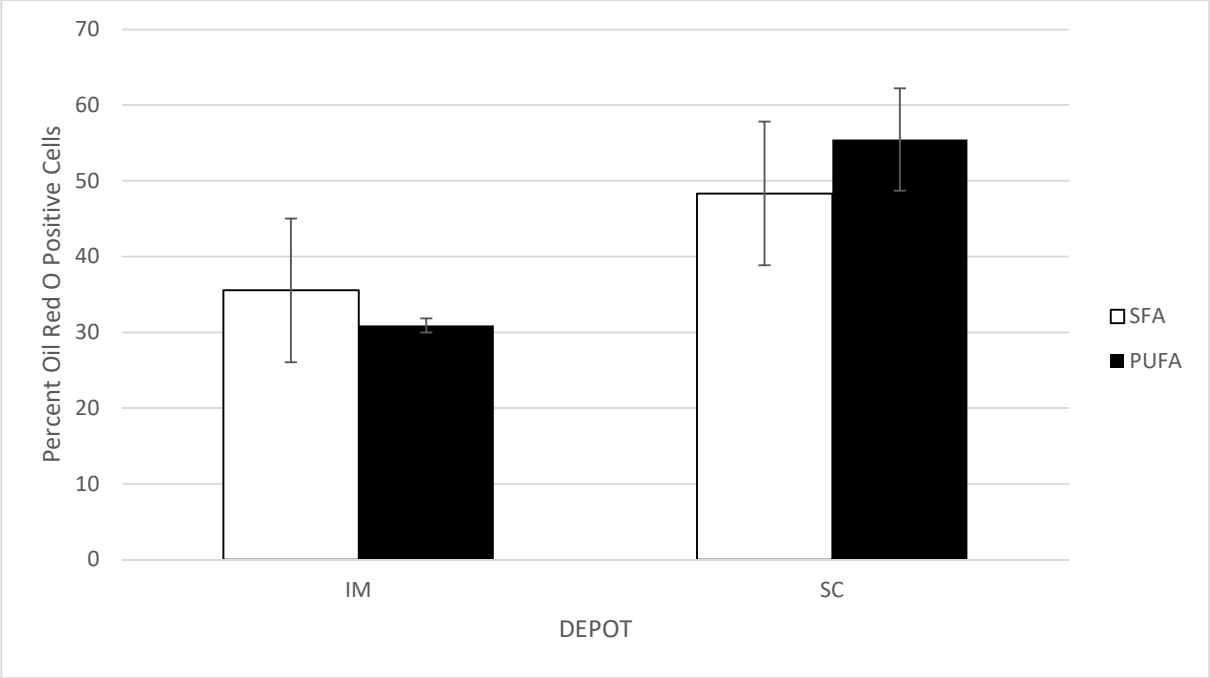


Figure 4. Percent oil red O positive bovine preadipocytes harvested from calves fed either supplemental SFA or PUFA showing the depot effects. Cells were incubated in growth medium undisturbed for 8 day and treated with a common differentiation media for 8d. Percentage of differentiated preadipocytes cells was determined by microscopy. IM (n=5), SC (n=5). Bars represent means ±SEM.

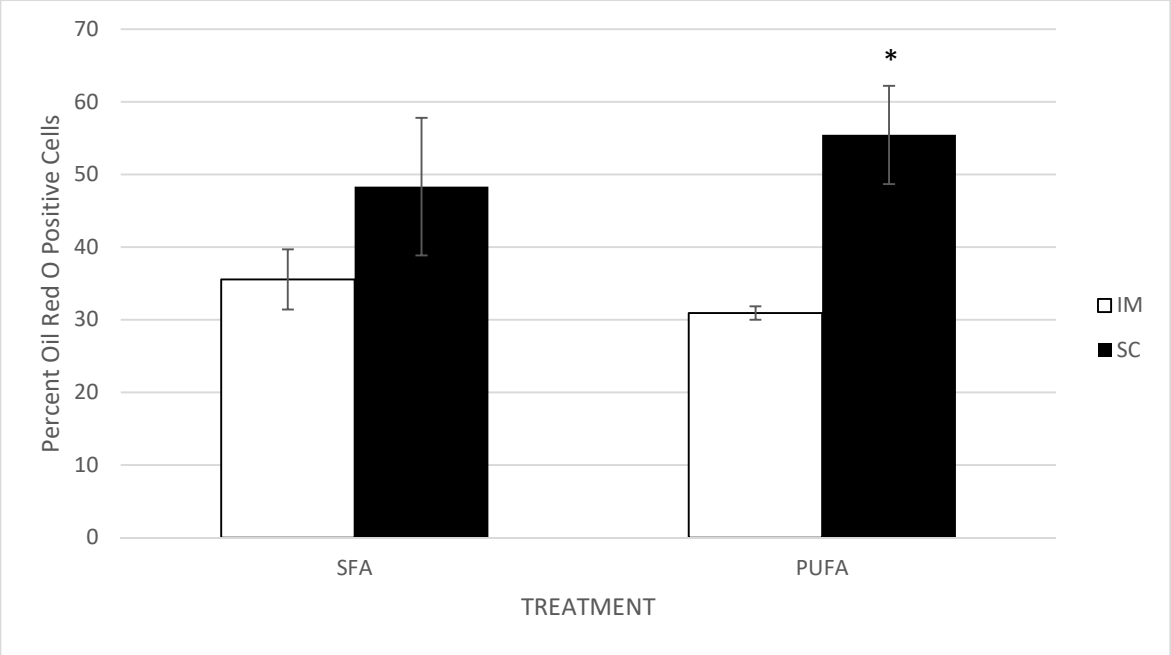


Figure 5. Percent oil red O positive bovine preadipocytes harvested from calves fed either supplemental SFA or PUFA showing the effects of the treatments. Cells were incubated in growth medium undisturbed for 8 days and treated with a common differentiation media for 8 days. Percentage of differentiated preadipocytes cells was determined by microscopy. IM (n=5), SC (n=5). * indicates P=0.01. Bars represent means ±SEM.

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APPENDICES

Appendix A
University of Idaho Institutional Animal Care and Use Approval
Protocol # 2013-77

From: iacuc@uidaho.edu [mailto:iacuc@uidaho.edu]
Sent: Tuesday, July 16, 2013 8:48 AM
To: Doumit, Matt
Subject: Protocol 2013-77 - Will feeding supplemental polyunsaturated fatty acids (PUFAs) to pre-ruminating calves to preferentially stimulate development of marbling?

University of Idaho
Institutional Animal Care and Use Committee

Date: Tuesday, July 16, 2013

To: Matthew Doumit

From: University of Idaho
Institutional Animal Care and Use Committee

Re: Protocol 2013-77
Will feeding supplemental polyunsaturated fatty acids (PUFAs) to pre-ruminating calves to preferentially stimulate development of marbling?

Your animal care and use protocol for the project shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Tuesday, July 16, 2013.

This protocol was originally submitted for review on: Tuesday, June 11, 2013
The original approval date for this protocol is: Tuesday, July 16, 2013
This approval will remain in affect until: Wednesday, July 16, 2014
The protocol may be continued by annual updates until: Saturday, July 16, 2016

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Brad Williams, DVM
Campus Veterinarian
University of Idaho
208-885-8958

Appendix B

Media components

Basal Medium
- DMEM; 5.5 m*M* glucose (Invitrogen 31600-34)
- Antibiotic-antimycotic, 1% (Sigma A5955)
- Gentamicin, 0.1% (Sigma G1397)
- Biotin, 33 µ*M* (Sigma B4639)
- Pantothenate, 17 µ*M* (Sigma P5155)
- Ascorbic Acid, 200 µ*M* (Sigma A4034)

Growth Medium for Clonal Analysis
- DMEM; 5.5 m*M* glucose (Invitrogen 31600-34)
- Antibiotic-antimycotic, 1% (Sigma A5955)
- Gentamicin, 0.1% (Sigma G1397)
- Biotin, 33 µ*M* (Sigma B4639)
- Pantothenate, 17 µ*M* (Sigma P5155)
- Ascorbic Acid, 200 µ*M* (Sigma A4034)
- FBS, 10% (Sigma F2442)

Growth Medium for Serum Growth Assay
- DMEM; 5.5 m*M* glucose (Invitrogen 31600-34)
- Antibiotic-antimycotic, 1% (Sigma A5955)
- Gentamicin, 0.1% (Sigma G1397)
- Biotin, 33 µ*M* (Sigma B4639)
- Pantothenate, 17 µ*M* (Sigma P5155)
- Ascorbic Acid, 200 µ*M* (Sigma A4034)
- Experimental Calf Serum 5%

Freezing Medium
- DMEM; 5.5 m*M* glucose (Invitrogen 31600-34)
- Antibiotic-antimycotic, 1% (Sigma A5955)
- Gentamicin, 0.1% (Sigma G1397)
- FBS, 20% (Sigma F2442)
- Dimethylsulfoxide (Sigma D5879)

Differentiation Medium
- DMEM; 5.5 m*M* glucose (Invitrogen 31600-34)
- Antibiotic-antimycotic, 1% (Sigma A5955)
- Gentamicin, 0.1% (Sigma G1397)
- Biotin, 33 µ*M* (Sigma B4639)
- Pantothenate, 17 µ*M* (Sigma P5155)
- Ascorbic Acid, 200 µ*M* (Sigma A4034)
- Glucose, 20 m*M* (G8769)
- Insulin, 280 n*M* (Sigma I1882)
- Troglitazone, 10 µ*M* (Caymen Chemical Co. 71750)

BSA, 0.5% (Sigma A8806)
Ex-Cyte, 20 μL/mL (Millipore 81-129-2)
Dexamethasone, 250 n*M* (Sigma D2915)

Appendix C

Ex-Cyte media supplement components

Fatty acid composition of Ex-Cyte lipid supplement[a]

Fatty acid	Concentration, mg/mL supplement
Myristic (14:0)	0.067
Palmitic (16:0)	1.13
Palmitoleic (16:1)	0.028
Stearic (18:0)	1.968
Oleic (18:1)	0.797
Linoleic (18:2)	3.01
Linolenic (18:3)	0.086
Homo-2-linolenic (18:3)	0.46
Arachidonic (20:4)	0.47
Eicosapentaenoic (20:5)	0.096
Docosenoic (22:1) or Docosanoic (22:0)	0.048
Docosatetaenoic or Docosatetraenoic (22:4)	0.105
Docosapentaenoic (22:5)	0.096
Docosahexanoic (22:6)	0.029
Others	0.912
Total Fatty acid	9.33
Unsaturated fatty acids	57.5%
Polyunsaturated fatty acids	14.7%

Other Components	Concentration, g/L, supplement
Cholesterol	9.0 – 11.0
Protein	13.0 – 18.0

[a]Data obtained from: Serologicals Corp., Norcross, GA, 1-800-227-9412 in 2005.

Ex-Cyte is an aqueous lipoprotein concentrate containing a mixture of fatty acids, cholesterol, and phospholipids derived from bovine serum.
*Ex-Cyte fatty acid composition is no longer provided by manufacturer.

Appendix D

Oil red-O staining with nuclear counterstain

Adapted from: J. L. RamÍrez-ZacarÍas

1. Make Oil-Red-O (ORO) (Sigma O0625) Working Solution:

<UNDER FUME HOOD>

a. Weigh 0.35 g ORO, add to 100 mL Isopropanol, do not stir.
b. Cover, leave overnight at room temperature.
c. Filter (Whatman filter paper #40) into bottle to remove precipitate.
d. Add 75 mL dd H_2O, do not stir.
e. Cover, leave overnight in 4°C (refrigerator).
f. Filter twice (Whatman filter paper #40).
g. Store at room temperature.

2. Staining Procedure:

<UNSTERILE CONDITIONS! Be sure you are ready to terminate experiment!>

<Formalin must only be used UNDER FUME HOOD>

a. Prepare 10% Formalin (using 3.7% formaldehyde concentration; 10 mL = 1 mL formaldehyde + 9 mL PBS).
b. Remove media from wells and wash wells 3 times with PBS (1 mL/well in 24-well plates).
c. Fix cells: Add 0.5 mL/well 10% Formalin, let fix overnight.
d. Wash wells 2 times with PBS (1 mL/well in 24-well plates).
e. Stain cells with ORO working solution (0.5 mL/well in 24-well plates) for 2 hr at room temperature (Note: will evaporate if left in fume hood)
f. Remove ORO and rinse wells 2 times with dd H_2O.

3. Nuclear Counterstain (Hematoxylin, Sigma MHS16)

a. Stain cells with Mayer's Hematoxylin Solution (0.5 mL/well for 5-15 min)
b. Rinse away stain in running tap water until nuclei are blue (~5-10 min)
c. Photograph cells immediately as ORO may begin to leach from cells

Appendix E

Clone Inventory

Clones isolated from steer #3 (Harvest date 6-30-03)

Clones	
Subcutaneous	Intramuscular
SC3-C21-P7	IM3-C4-P7

Appendix F

Percentage of ORO positive cells

Percent oil red O positive bovine preadipocytes harvested from calves fed either supplemental SFA or PUFA showing effects by depot.. Cells were grown to confluence and treated with a common differentiation media for 8d. Percentage of differentiated preadipocytes cells was determined by microscopy. IM (n=5), SC (n=5).

DEPOT	TREATMENT		SEM	P-VALUE
	SFA	PUFA		
IM	35.55	30.91	4.23	0.3302
SC	48.33	55.46	11.36	0.54559

Percent oil red O positive bovine preadipocytes harvested from calves fed either supplemental SFA or PUFA showing effects by treatment. Cells were grown to confluence and treated with a common differentiation media for 8d. Percentage of differentiated preadipocytes cells was determined by microscopy. IM (n=5), SC (n=5).

TREATMENT	DEPOT	PERCENT OF DIFFERENTIATION	SEM	P-VALUE
SFA	IM	35.54929	10.345	0.251
	SC	30.915125		
PUFA	IM	48.33037	7.515	**0.0146***
	SC	55.45999		

Mean Percent Adipogenic cells of Oil red O positive cells with animal as the experimental unit. IM cells are from *Longissimus dorsi* tissue and SC is subcutaneous tissue, with SFA or PUFA treatment. * indicates that $P<0.05$.

Appendix G

Effects of Fatty Acid Treatments

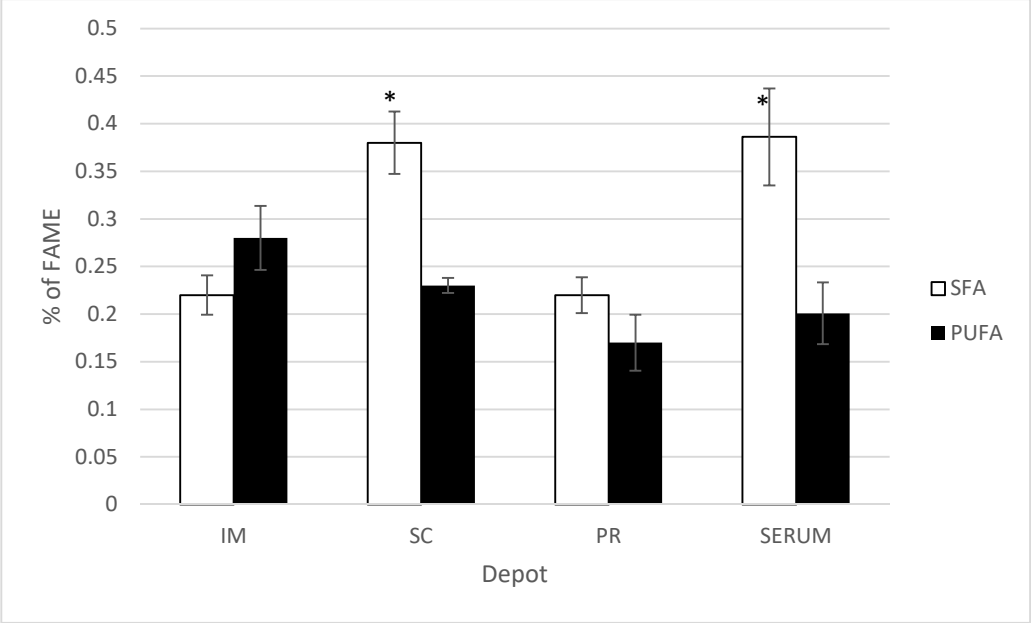

Figure A. Percentage lauric acid (12:0) by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age. * indicates P<0.02.

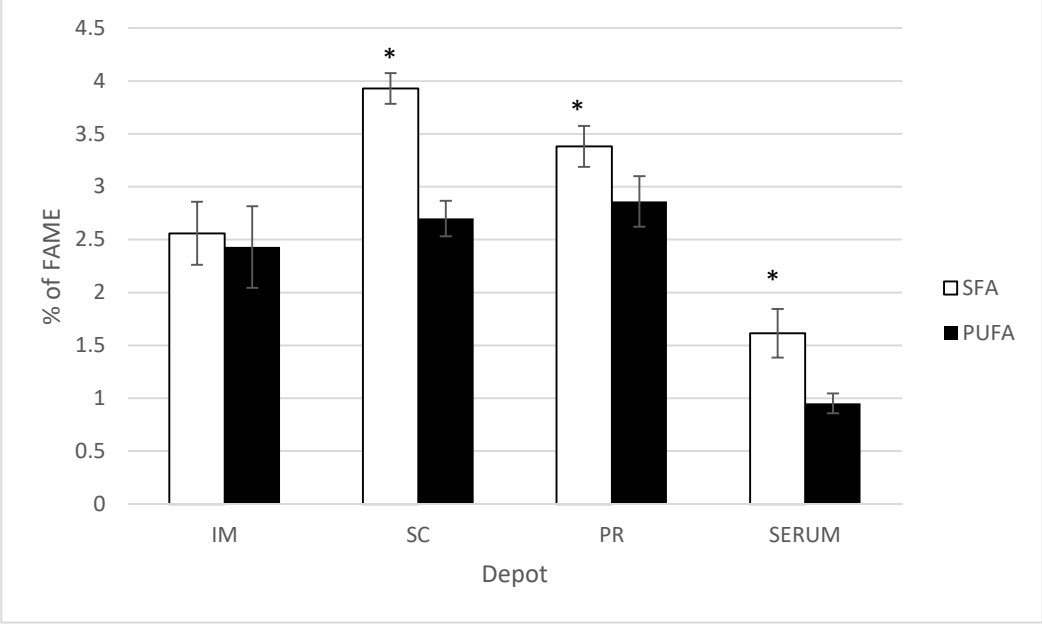

Figure B. Percentage myristic acid (14:0) by depot in Holstein bull calves receiving either SFA or PUFA supplementation for 50 days beginning at 3d of age. * indicates P<0.05.

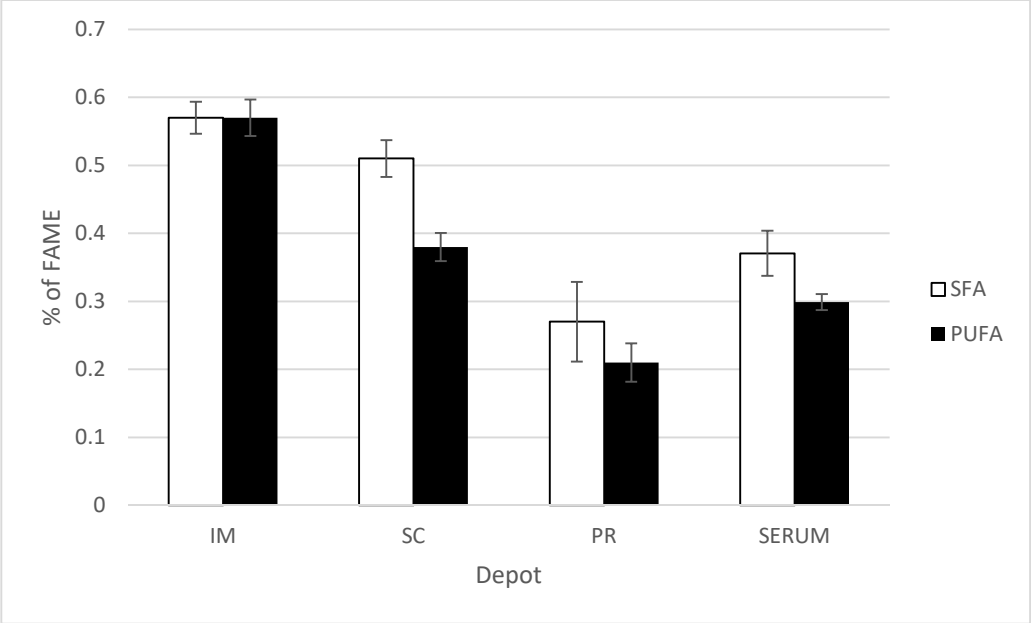

Figure C. Percentage pentadecanoic acid (15:0) by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age.

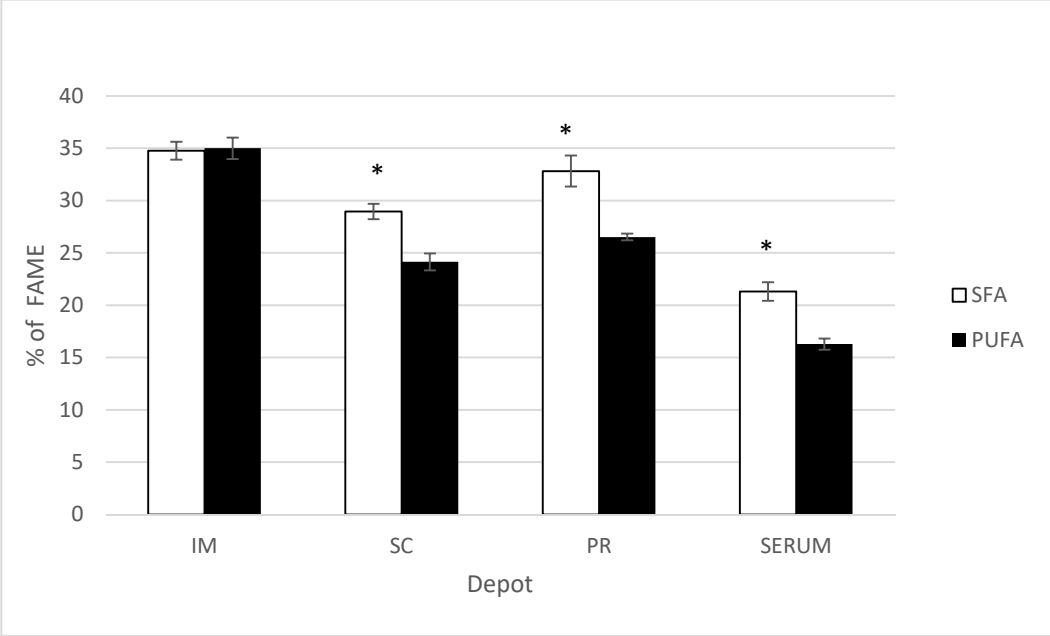

Figure D. Percentage PAL (16:0) by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age. * indicates P<0.01.

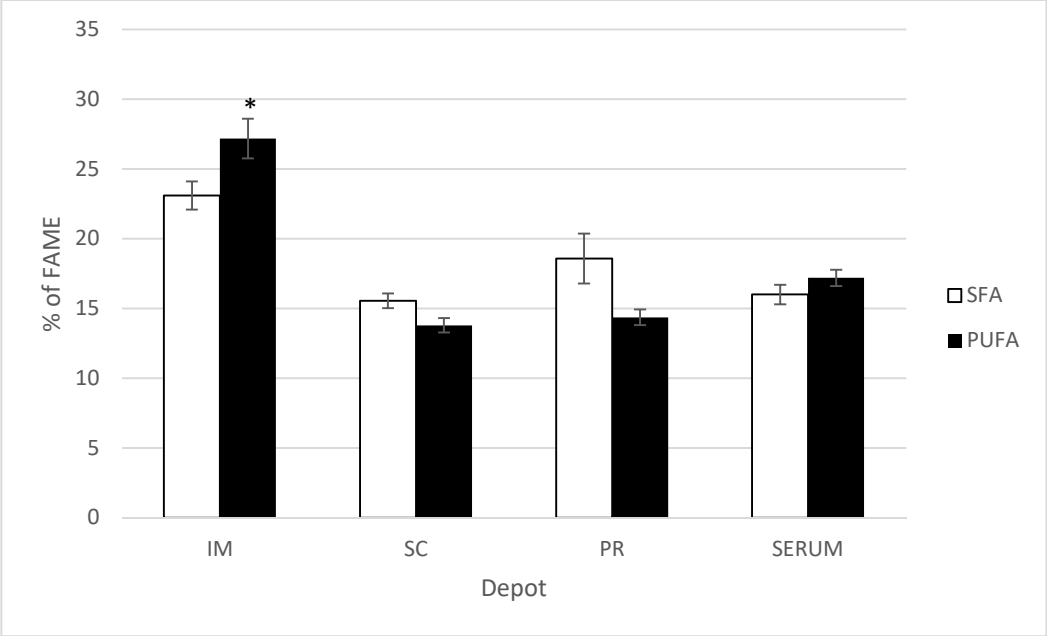

Figure E. Percentage STE by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age. * indicates P<0.05.

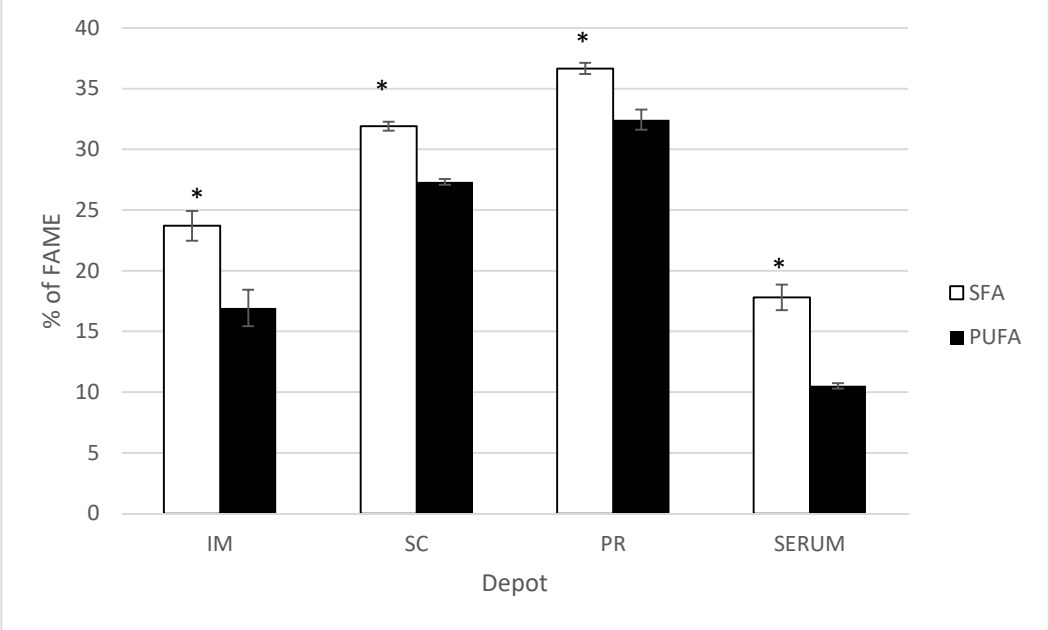

Figure F. Percentage OLE by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age. * indicates P<0.01.

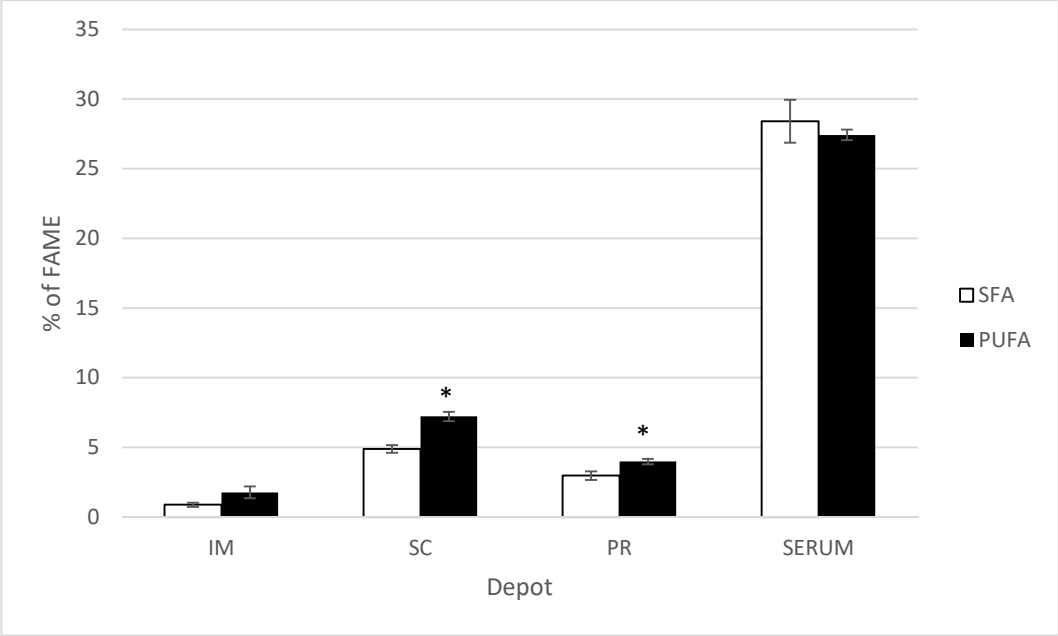

Figure G. Percentage LIN by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age. * indicates P<0.04.

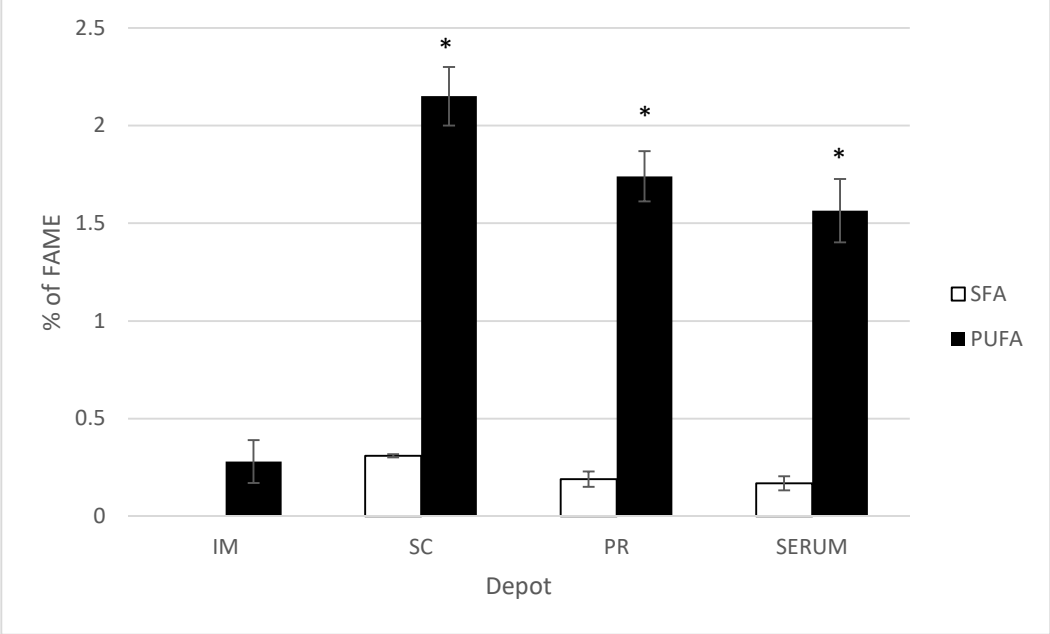

Figure H. Percentage CLA c9,t11 by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age. No bar in a treatment indicated 0% present. * indicates P<0.01.

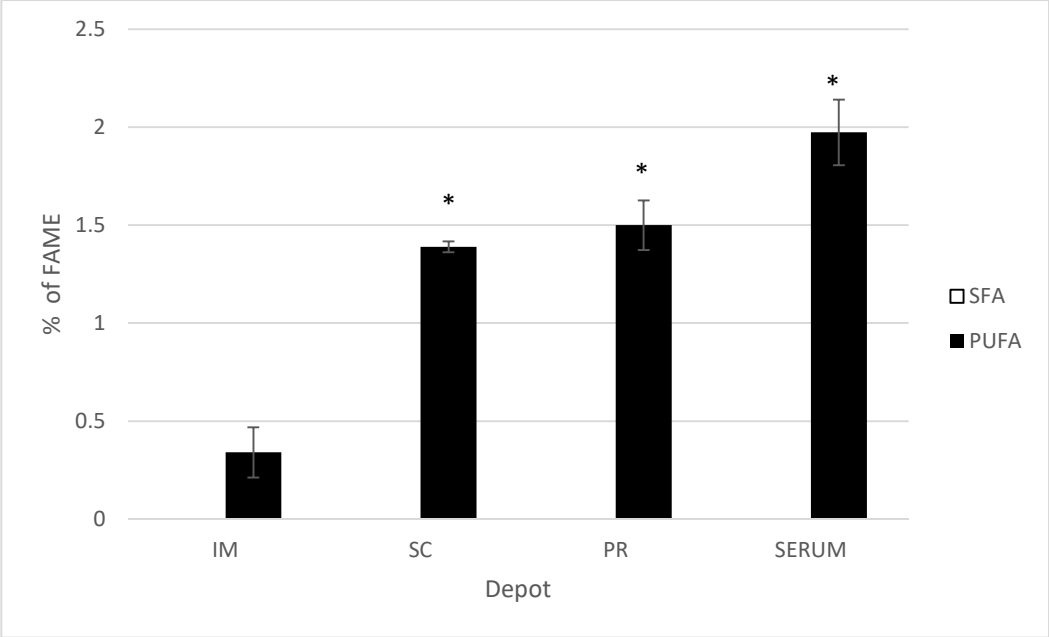

Figure I. Percentage CLA t10c12 by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age. No bar in a treatment indicated 0% present. * indicates P<0.01.

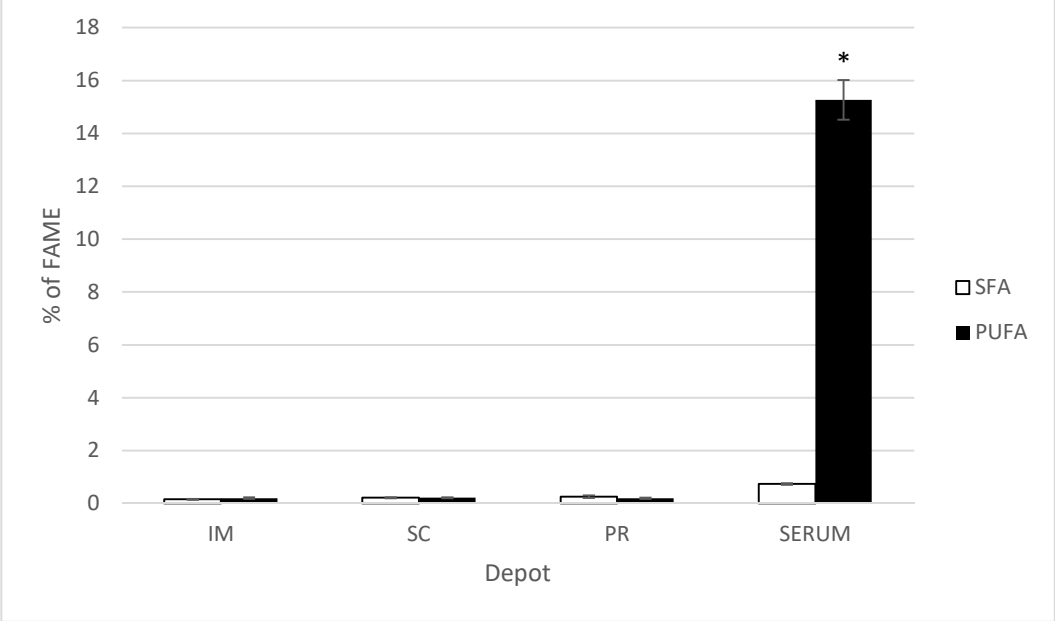

Figure J. Percentage ALA by depot in Holstein bull calves receiving either SFA or PUFA supplementationfor 50 days beginning at 3d of age. * indicates P<0.0001.
*