## ABSTRACT

Title of Document: **REGULATION OF MILK FAT SYNTHESIS BY DIETARY FATTY ACIDS**

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The objectives of this thesis research were to determine the molecular mechanisms by which dietary fatty acids (FA) regulate lipogenic gene expression and milk fat synthesis. Principal component and multivariate analyses were conducted to establish the relationship between milk fat and FA concentrations in lactating cows fed milk fat depressing (MFD) diets. This analysis showed that in addition to the established inhibitory effect of t10c12 CLA, t7-18:1 and t7c9-CLA isomers might be involved in MFD. Lactating mice were used to test the effects of several individual trans-18:1 isomers and t10c12-CLA on milk fat synthesis, lipogenic genes in liver and mammary tissues. Both MFD and extensive conversion of t7-18:1 to t7c9-CLA in mammary and liver tissues were shown in mice fed the t7-18:1. As expected, t10c12-CLA feeding caused MFD and reduced the expression of lipogenic transcription factor (TF) *SREBP-1C.* Potential roles of the TF *ChREBP*, *PPARG,* and *INSIG1* were also established.

A subsequent study aimed to establish whether these mechanisms operated in lactating dairy cows. Compared with uninfused controls and a t10c12 CLA negative control, post-ruminal infusion of butterfat which contains all fatty acids in the same proportion to those found in milk fat to a mixture of fats containing only the long chain fatty acids were examined. Milk fat content, milk yield and mammary lipogenic gene expression were increased by butterfat but not by the long chain fatty acid mixture. This suggested that rates of short and medium chain fatty acid synthesis might be limiting for milk fat production. Unlike the results from the mouse study, *SREPB-1C* expression was not altered by FA treatments including t10c12-CLA.

The effects of individual FA effects and a PPARγ-specific agonist (Rosiglitazone) on mRNA expression via qPCR of 19 genes with roles in *de novo* synthesis, FA uptake and transport, desaturation, triacylglycerol synthesis, transcriptional regulation, and nuclear receptor signaling in a MACT cell culture system were examined. The FA regulated mammary lipogenic gene expression to different extents. PPAR-γ activation of *de novo* lipogenesis coupled with exogenous FA availability might play a role in regulating milk fat synthesis. These experiments demonstrate the role of FA in regulating mammary lipogenic pathways, highlighting the complexity and multiple transcriptional factor involvement in milk fat synthesis.

# REGULATION OF MILK FAT SYNTHESIS BY DIETARY FATTY ACIDS

By

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# Dedication

**……. to the unflinching love and support of**

**my parents,**

**sisters**

**&**

**teachers.**

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# Table of Contents

# List of Tables

# List of Figures

# List of Abbreviations

| | |
|---|---|
| *ACACA* | acetyl-Coenzyme A carboxylase alpha; |
| *AGPAT* | 1-acylglycerol-3-phosphate O-acyltransferase |
| *ChREBP* | carbohydrate response element binding protein |
| CLA | conjugated linoleic acid |
| FA | fatty acid |
| *FASN* | fatty acid synthase |
| *INSIG1* | *i*nsulin induced gene 1 |
| LCFA | long chain fatty acid |
| *LPL* | lipoprotein lipase |
| *LXRA* | liver X receptor alpha |
| MCFA | medium-chain fatty acid |
| MFD | milk fat depression |
| *Mlx* | MAX-like protein X |
| PHVO | partially hydrogenated vegetable oil |
| *PPARA* | peroxisome proliferator activated receptor alpha |
| *PPARG* | peroxisome proliferator activated receptor gamma |
| *RXR* | retinoid X receptor |
| *SCAP* | SREBP cleavage activating protein |
| *SCD1* | stearoyl-Coenzyme A desaturase 1 |
| *SCD2* | stearoyl-Coenzyme A desaturase 2 |
| *SREBP1* | sterol regulatory element binding transcription factor 1 |
| *THRSP* | thyroid hormone responsive SPOT14 homolog. |

# Chapter 1: INTRODUCTION

The current milk component pricing system (**MCP**) was introduced by the Federal Milk Marketing Administration in 2000. Accordingly, there has been a shift in the producer payment from the historic system based on the volume of milk (adjusted for fat content) to one based primarily on the amounts of milk fat and protein produced. The MCP system provides a powerful economic incentive for dairy producers to produce high value milk components, namely fat and protein while there is little or no incentive to produce components directly associated with milk volume (lactose and minerals) that have little or no value (Erdman, 2008). Milk component yields are driven by both milk volume and component concentration. The dairy cow's diet has not been shown to consistently affect milk lactose and mineral content (Sutton, 1989). Compared to milk fat responses, only modest effects of diet on milk protein concentration have been reported (Sutton, 1989). Milk fat is the milk component most easily manipulated by diet (Sutton, 1989). Reports in the literature have shown that milk fat percentage and yield can be reduced up to 46% (Piperova et al., 2000; Peterson et al., 2003) by milk fat-depressing diets containing high levels of grain and polyunsaturated fatty acids (**PUFA**).

It has been demonstrated that conjugated linoleic acid **(CLA)** and trans-18:1 fatty acids **(FA)** arising from incomplete rumen biohydrogenation of dietary PUFA can markedly alter milk fat synthesis. Numerous studies have shown that concentrations of trans-18:1 FA or CLA can be increased in milk by dietary means (Griinari et al., 1998, Piperova et al., 2000, Peterson et al., 2003) or abomasal infusion of trans-18:1 FA or CLA mixture (Gaynor et al., 1994; Romo et al., 1996;

Chouinard et al., 1999) and this results in reduction of milk fat concentration. Examination of the isomer profile of trans-18:1 FA (Griinari et al., 1998) and CLA (Griinari and Bauman., 1999) indicated that increases in ruminally-derived trans-10-containing 18:1 and 18:2 isomers in milk were more closely associated with milk fat depression **(MFD)** than the general increase in total trans-18:1 FA or CLA. Abomasal infusion of partially hydrogenated vegetable oil **(PHVO) (**Gaynor et al., 1994; Romo et al 1996), or diets supplemented with Ca-salts of trans-18:1 both containing isomers from t6 to t16 (Piperova et al., 2004) can cause MFD. However, when individual trans-18:1 isomers, including t9-, (Rindzig and Schultz, 1974), t11- and t12- (Griinari et al., 2000) were post-ruminally infused, milk fat was not affected. Although high concentration of t10-18:1 are typically observed in milk fat of lactating cows fed MFD diets (Piperova et al.,2000; Peterson et al., 2003; Loor et al., 2004), abomasal infusion (40 g/d) t10-18:1 was not effective in reducing milk fat percentage in lactating cows ( Lock et al., 2007). Baumgard et al. (2000) provided convincing evidence that abomasal infusion of t10c12 CLA inhibits milk fat synthesis. In an earlier study, Chouinard et al. (1999) observed MFD in lactating cows abomasally infused with a CLA mixture lacking t10c12 CLA.

Feeding experiments have also shown that the concentration of t10c12 CLA in milk does not always account for the degree of MFD (Peterson et al., 2003) and that reduction in milk fat concentration can occur without increases in t10c12 CLA in milk (Piperova et al., 2004; Loor et al., 2005a). The t8c10 CLA and c11t13 CLA isomers, usually present in lower concentrations in commercial CLA mixtures, were examined by Perfield et al. (2004) and they found no effect of these isomers on milk

fat synthesis. These collective results suggest that there must be other biohydrogenation intermediates besides t10c12 CLA that are involved in MFD.

The mechanisms causing MFD are not well understood. The classical dietary-induced MFD is characterized by a predominant decreases in the short **(SCFA)** and medium chain fatty acids **(MCFA)** of milk fat by decreasing *de novo* FA synthesis (Bauman and Griinari, 2003). Similar effects on *de novo* FA synthesis were observed when milk fat yield was reduced by t10c12-CLA (Loor and Herbein, 1998). Feeding a MFD diet (Piperova et al., 2000; Peterson et al., 2004) to lactating dairy cows, or feeding t10c12-CLA to rats (Ringesis et al., 2004) and mice (Lin et al., 2004) lead to decrease in mRNA abundance and enzymatic activity of several mammary lipogenic enzymes. Transcriptome profiling of lactating mammary glands of mice demonstrated that substantial regulation of lipid synthesis occurred at the level of mRNA expression and implicated the transcription factor SREBP-1c as one of the regulators (Rudolph et al., 2006). Studies with MAC-T cells (Peterson et al., 2004) and lactating dairy cows (Harvatine and Bauman, 2006) have shown that the effect of t10c12-CLA on fat synthesis could be regulated via the SREBP-1c which may act as a global regulator of milk fat synthesis. However, SREBP-1c knock out mice exhibit 50% lower rate of FA synthesis without fully suppressing lipogenic gene expression (Liang et al., 2002). This suggested that other transcription regulators may be involved in FA synthesis. Studies with cultured primary hepatocytes (Dentin et al., 2004) and *SREBP-1c* knock out mice (Lizuki et al., 2004) have reported a role of carbohydrate response element binding protein (*ChREBP*) in the coordinated control of glucose metabolism, FA synthesis, and TAG synthesis in liver.

Recently, Bionaz and Loor (2008) demonstrated that PPARγ nuclear receptor was up-regulated by more than three-fold at the onset of lactation and this was maintained throughout lactation in dairy cows. Simultaneous increases in expression of genes related to FA uptake, transport, synthesis and desaturation (Bionaz and Loor, 2008) were also observed, suggesting that part of the long chain fatty acids **(LCFA)** effects could be mediated through PPARγ.

Understanding the mechanism by which FA regulate milk fat synthesis would enable us to develop feeding strategies that can alter milk fat yield depending on the needs of the dairy industry. The present study was based on the premise that: "*Fatty acids regulate milk fat synthesis by altering lipogenic gene expression in the mammary gland*" and my hypothesis was: "**Fatty acids affect mammary gene expression by altering multiple transcription regulators."**

**Specific Study Objectives:**

1. To determine the relationship between milk FA and diet-induced MFD employing principal component and multivariate analyses as a means to potentially identify individual FA effects.
2. To determine the effects of several individual trans-18:1 isomers and t10c12-CLA on fat synthesis, and expression of lipogenic genes and transcription regulators in mammary and liver tissues of lactating mice.

3. To compare the effects of abomasal infusion of butterfat containing all FA present in milk, including the short and medium chain FA, with infusion of only the LCFA present in milk, on the milk FA composition, milk fat yield and mammary lipogenic gene expression in lactating dairy cows.
4. To determine the effects of individual FA on mammary lipogenic gene expression and to ascertain the role of *PPARγ* in regulation of lipogenic genes.

# Chapter 2: LITERATURE REVIEW

Milk fat is the most variable component of the milk constituents (Sutton, 1989). Milk fat content and composition varies with the animal breed/type, physiological and dietary conditions (Sutton et al., 1989; Schutz et al., 1990). Typically, milk fat consists of 97-98% triacylglycerols, the remaining percentages being phospholipids, esterified and unesterified sterols and other components associated with the milk fat globular membrane surrounding the milk fat droplets. Milk fat triacylglycerols of the cows consist of 19% short (C4 to C8), 19% medium (C10 to C14,) and 62% long chain (>C16) fatty acids (Jensen, 2002). The short and medium chain fatty acids and part of palmitic acid (C16) are synthesized *de novo* in the mammary gland. The long chain (>C16) fatty acids are derived from the uptake of circulating fatty acids that originate from either absorption of dietary lipids or mobilization of body fat (Dils, 1983).

## Biosynthesis of milk fat

*De novo milk fatty acid synthesis*

The *de novo* FA synthesis involves the synthesis of acetyl CoA and malonyl CoA, and the step wise elongation of 'primed' malonyl CoA by the enzyme fatty acid synthetase. The process requires the provision of NADPH for the reductive steps in the pathway (Dils, 1983).

Acetate and beta-hydroxybutyrate are the predominant precursors for mammary fatty acid synthesis in ruminants (Moore and Christie, 1979). Acetate and butyrate, the major volatile fatty acids produced in the rumen are absorbed into the portal circulation across the rumen wall. In the rumen wall, butyrate is extensively metabolized into beta-hydroxybutyrate while a small amount of acetate is metabolized before entering the blood circulation (Bergaman and Wolf, 1971). Considerable amounts of endogenous acetate from acetyl CoA are produced in the liver (Costa et al., 1976) and the lactating mammary gland (Annison and Linzell, 1964). Beta-hydroxybutyrate contributes about 8% of the carbon in the total milk fatty acids (Palmquist et al., 1969). About 50% of the four-carbon atoms at the methyl terminal end of the *de novo* synthesized fatty acids arise from beta-hydroxybutyrate, while the remaining carbon atoms are derived from acetate (Palmquist et al., 1969). Beta-hydroxybutyrate is oxidized in the mitochondrian by the enzyme beta - hydroxybutyrate dehydrogenase, producing acetyl CoA. However, the acetyl CoA produced in the mitochondria can not be used for lipogenesis which occurs in the cytosol (Bauman and Davis, 1974). Therefore, the contribution of butyrate to fatty acid synthesis is limited mostly to synthesis of the initial four carbons of the fatty acid carbon chain.

In ruminants, glucose is not a precursor for fatty acid synthesis as the activities of the enzymes ATP citrate lyase and NADP malate dehydrogenase are extremely low in the mammary tissue (Bauman and Davis, 1974). Synthesis of fatty acids involves reductive steps requiring the reducing equivalents NADPH. In ruminant mammary gland, NADPH is produced predominantly by isocitrate cycle

involving NADP isocitrate dehydrogenase and to some extent by pentose phosphate pathway involving glucose-6-phosphate dehydrogenase and 6-phospho gluconate dehydrogenase (Bauman et al., 1970). About 50 % of NADPH produced in the ruminants is by the pentose phosphate pathway using glucose (Bauman et al., 1973).

*Synthesis of fatty acid chain*

Acetate taken up by the mammary cells is converted to acetyl CoA in the presence of the enzyme 'Acetyl CoA Synthetase'. Acetate and beta-hydroxybutyrate can also be converted to acetyl CoA and butyryl CoA by Fatty Acid Thiokinases on the surface of the rough endoplasmic reticulum.

Synthesis of malonyl CoA from acetyl CoA is the first 'committed step' in fatty acid synthesis and the reaction is catalyzed by the enzyme 'Acetyl CoA Carboxylase (ACC) (Dils, 1983). This step is considered rate limiting for fatty acid biosynthesis as the activity of ACC is lower than the activities of other enzymes involved in fatty acid synthesis (Ganguly, 1960). A high correlation between ACC and lipogenic activity vs. low correlation between lipogenic activity and other enzymes involved in fatty acid synthesis in the mammary tissue at different stages of lactation cycle indicates its importance in mammary lipogenesis (Mellenberger et al., 1973).

Acetyl CoA Carboxylase is an enzyme complex of two polypeptide subunits with molecular weight of 230kD. Each subunit contains catalytic domains of biotin carboxylase, transcarboxylase, biotin carboxyl carrier protein and the regulatory allosteric site (Wakil et al, 1983). ACC occurs both in catalytically inactive protomeric form and active polymeric form (Moore and Christie, 1979). The activity

of ACC is regulated by interconversion between these two forms. The active polymeric form is promoted by citrate while inactive polymeric form is promoted by malonyl CoA and other long chain fatty acids (Moore and Christie, 1979). Covalent modifications can also regulate ACC activity. Phosphorylation of ACC by cyclic AMP-dependent protein kinase lowers the specific activity, while dephosphorylation reverses it to high specific activity (Dils, 1983). The phosphorylation of ACC is accompanied by depolymerisation to its protomeric form (Lent et al., 1978). Citrate seems to have allosteric control over ACC through covalent modification as physiological concentrations of citrate have been shown to activate the dephosphorylated ACC (Witters et al., 1979).

*Elongation of fatty acid chain*

The second step in the *de novo* fatty acid synthesis involves fatty acid synthetase (FAS), which catalyses elongation of acetyl/butyryl CoA to long chain fatty acids by malonyl CoA. FAS is the largest known multifunctional enzyme composed of two identical polypeptide subunits of molecular weight 263kD. Each peptide unit is a complex of seven enzyme units, including acetyl transacylase, malonyl transacylase, beta-ketoacyl synthetase, beta-ketoacyl reductase, dehydratase, enoyl reductase and thioesterase I, and an acyl carrier protein (ACP) site (mol wt. 10kD) with it's 4' phosphopantetheine group (Wakil et al., 1983).

The first step, referred to as the priming reaction, is catalyzed by acetyl transacylase, where acetyl CoA / butyryl CoA condenses on to a cystein residue of beta- ketoacylsynthetase. Though both acetyl CoA and butyryl CoA can be used as

the primers, butyryl CoA is the preferred primer in ruminants (Lin and Kumar, 1972). The priming reaction is followed by esterification of the 4' phosphopantetheine residue of the ACP by malonyl transacylase. The acetyl group is then transferred from the cystein residue to the ACP. A condensation reaction between acyl and malonyl groups of acetyl CoA and malonyl CoA form the acetoacetyl group bound to ACP. The acetoacetyl ACP undergoes reduction, dehydration, and reduction by the enzymes beta–ketoacyl reductase, dehydratase and enoyl reductase, respectively and give rise to butyryl ACP. The butyryl group transfers to the cysteine group of beta-ketoacyl synthetase enzyme and the vacant 4' phosphopantetheine residue of the ACP is esterified by malonyl CoA. The butyryl group is transferred back to ACP and the process of condensation followed by other steps is repeated. Each cycle lengthens the fatty acyl ACP chain by the addition of two carbons at the carboxyl end of the fatty acid. Finally the chain elongation is terminated by reaction catalyzed by the enzyme thioesterase I.

The fatty acid termination process leading to the synthesis of the short- and medium-chain fatty acids in milk of ruminants are not completely elucidated (Bauman and Davis, 1974). However, based on *in vitro* studies it is proposed that the pattern of fatty acids synthesized in the mammary tissue can be changed by altering the concentrations of acetyl CoA and malonyl CoA. Increasing the ratio of acetyl CoA:malonyl CoA increases the proportion of small- and medium-chain fatty acids, while a decrease in the ratio increases the proportion of the long-chain fatty acids (Bauman and Davis, 1974). Similarly, altering the levels of ACC and FAS can affect the pattern of milk fatty acids (Bauman and Davis, 1974). Increasing the amounts of

FAS relative to ACC resulted in higher short and medium milk fatty acids, while increased ACC reversed the pattern. The shifts in the pattern of milk fatty acids were attributed to the competitive nature of acetyl CoA and malonyl CoA to bind to ACP. The binding of acetyl CoA to ACP instead of malonyl CoA led to the termination of the growing fatty acyl chain (Bauman and Davis, 1974).

Due to rumen biohydrogenation, very small amounts of unsaturated long chain fatty acids are absorbed from the intestine in ruminants. Unsaturated long chain fatty acids with one double bond are produced in the mammary gland. The enzyme fatty acyl CoA desaturase can desaturate myristic (C14:0), palmitic (C16:0), and stearic acid (C18:0) to the respective C14:1, C16:1, and C18:1 monounsaturated fatty acids (Dils, 1983).

*Uptake of preformed blood lipids by the mammary gland*

The serum lipids of the lactating cow are predominantly present as high-density lipoproteins (HDL) (92%), low-density lipoproteins (LDL) (7%) and very low density lipoprotein (VLDL) (1%) (Raphael et al., 1973). About 2% of the serum lipids are associated with chylomicrons (Wendlant and Davis, 1973). The serum VLDL of lactating cows contains 57% triacylglycerol, while LDL contains 12% triacylglycerol. Under normal conditions, about 90% of the fatty acids in plasma triacylglycerol or non esterified fatty acids (NEFA) consist of 16:0, 16:1, 18:0 and 18:1 (Moore and Christie, 1979). Most esterified fatty acids taken up by the mammary gland are from chylomicrons and VLDL (Moore and Christie, 1979). The enzyme, lipoprotein lipase (LPL) bound to the capillary endothelial surface plays a

major role in this process (Moore and Christie, 1979). Triacylglycerols in the chylomicrons adhering to the luminal surface of the endothelium are hydrolyzed by LPL. It is presumed that LPL is involved in the attachment of chylomicrons to the endothelium, as LPL can adsorb chylomicrons as shown by *in vitro* studies (Moore and Christie, 1979). Hydrolysis of triacylglycerols by LPL is seen only in chylomicrons attached to the capillary endothelium and is initiated by the cleavage of an acyl ester bond in position *sn*-1. A part of the diacylglycerols may be further hydrolyzed at the luminal surface. The resulting monoacylglycerols and diacylglycerols are transported across the endothelial cells through microvesicles (Moore and Christie, 1979). Hydrolysis of the diacylglycerols continues in the microvesicles and the products are released at the sub endothelial space where the monoacylglycerols are hydrolyzed into free fatty acids and glycerol. The free fatty acids, monoacylglyceols and glycerol entering the mammary alveolar cell can be used for milk fat synthesis. A part of the glycerol, the magnitude of which depends on the glycerokinase activity in the mammary tissue and the free fatty acids produced at the endothelial surface, also enters the blood flow (Moore and Christie, 1979).

*Synthesis of triacylglycerol*

Fatty acids and *sn*2 monoacylglycerol/glycerol 3-phosphate are required for the biosynthesis of triacylglycerol. The fatty acids arise either from *de novo* synthesis or are derived from the circulating blood lipids (Moore and Christie, 1979). The fatty acids are activated to CoA thioester by ATP-dependent acyl CoA synthestases. Glycerol-3-phosphate originates either from triose phosphate through glycolysis or

through phosphorylation of glycerol originating from hydrolysis of triacylglycerol (Bauman and Davis, 1974). About 70% of the glyceride glycerol in the mammary gland of the cow is derived from glucose through glycolysis (Luick and Kleiber, 1961). The remaining arises from the phosphorylation of free glycerol by the enzyme glycerokinase (Bauman and Davis, 1974).

The triacylglycerol biosynthesis in the mammary gland can occur through three different pathways - *sn*-glycerol 3-phosphate pathway, monoglyceride pathway and dihydroxyacetone pathway. Of the three, the *sn*-glycerol 3-phosphate pathway is the principal route for triacylglycerol synthesis in bovine mammary gland (Dils, 1983). In the *sn*-glycerol 3-phosphate pathway, the glycerol 3-phosphate is acylated sequentially on positions *sn*1 and *sn*2 to form phosphatidic acid. The enzyme phosphatidate phosphatase hydrolyses the phosphate at the *sn*-3 position resulting in the formation of diacylglycerol. The diacylglycerol is acylated by specific acyltransferases to form triacylglycerol (Moore and Christie, 1979). The *sn*3 position is acylated last in the biosynthesis of triacylglycerol.

The positional distribution of fatty acids on glyceride glycerol of milk fat in ruminants shows that the short chain fatty acids (C4-C6) are almost exclusively (95%) esterified to the *sn*-3 position. All of the C4:0, 93% of C6:0, and 63% of the C8:0 are esterified to *sn*-3 position (Jensen et al., 1991). The distributions of other fatty acids in the triacylglycerol appear to vary depending on the molecular weight of the triacylglycerol (Parodi, 1982). The C10:0, C12:0 and C14:0 fatty acids are predominantly found in the *sn*-2 position. Palmitate (C16:0) is almost equally distributed between the *sn*-1 and *sn*-2 positions. Oleate (C18:1) is preferentially

distributed in the *sn*-3 position in high molecular weight triacylglycerols and at *sn*-1, in the low molecular weight triacylglycerol. Stearate (C18:0) selectively esterifies at *sn*-1 position (Parodi, 1982). The concentration of palmitate increases at the position *sn*-2 with the increase in the molecular weight (Bauman and Davis, 1974). The unsaturated fatty acids are preferentially esterified at *sn*-3 position in higher molecular weight triacylglycerols. Though the exact mechanisms involved in the positioning of the fatty acids in milk fat triacylglycerol in ruminants is not clearly understood, specific mammary acyltransferases may be involved (Parodi, 1982).

## Lipid metabolism in liver in relation to milk fat synthesis

In ruminants, liver does not play a major role in fatty acid synthesis and *de novo* fatty acid synthesis is very low (Hanson and Ballard, 1967; Hood et al., 1980). Also, liver has low levels of LPL and hepatic triacylglycerol lipase (Emery et al., 1992) and therefore takes up fatty acids mostly through endocytosis of triacylglycerols (Emery et al., 1992). The uptake of fatty acids depends on the plasma concentration of NEFA and the rate of blood flow to the liver (Bell, 1979). The uptake of NEFA could occur by a simple diffusion which is directly related to the concentration of NEFA in the blood or through facilitated transport by the liver-fatty acid binding protein (L-FABP), fatty acid translocase and fatty acid transporter protein (FTP) (Abumrad et al., 1998).

In liver, fatty acids may be stored as trigacylycerols, secreted in bile or as lipoproteins, or oxidized to carbon dioxide, acetic acid or ketone bodies (Emery et al., 1992). Hepatic fatty acid oxidation in ruminants is regulated by the rumen

fermentation products acetate and propionate. Their conversion to malonyl CoA, inhibits carnithine acyl transferase enzyme, essential for the transport of fatty acids into mitochondria, for β oxidation (Jesse et al., 1986; Reid and Husbands, 1985). Propionate, when metabolized into glycerol, diverts the fatty acids to storage by esterification rather than oxidation (Jesse et al., 1986).

Due to increased fatty acid mobilization, there is an increased influx of NEFA into the liver during late gestation and early lactation. Hypergluconemia accompanied by hypoinsulinemia can decrease liver concentrations of malonyl CoA leading to increased oxidation of fatty acids, producing ketone bodies (Zammit, 1990). In ruminants, the release of triacylglycerol from the liver is slower than in monogastrics due to insufficient rates of apolipoprotein B synthesis (Grummer, 1993). The slower rate of triacylglycerol release and more rapid rate of synthesis lead to the accumulation of triacylglycerol in the liver (Emery et al., 1992) in the transition period.

## Lipid metabolism in adipose tissue in relation to milk fat synthesis

In contrast to monogastrics where liver is an active site of fatty acid synthesis, adipose tissue is the major site of fatty acid synthesis in non-lactating ruminants. Specific metabolic alterations in lipid synthesis and mobilization occur during pregnancy and lactation. Adipose lipid synthesis is decreased during middle to late pregnancy, while lipolysis is stimulated to meet the energy needs of the mammary gland during lactogenesis. In an *in vitro* study with bovine adipose tissue obtained at

different stages of pregnancy and lactation, McNamara and Hillers (1986b) observed a 95% decrease in lipogenesis and a 50% decrease in fatty acid esterification at one month postpartum compared to one month prepartum cows. Lipogenesis and esterification rebounded back to higher rates during mid-lactation (McNamara and Hillers, 1986a,b). Rates of lipogenesis and lipolysis in the adipose tissue depended on the production potential and the dietary energy level of the animal (McNamara, 1991).

A decrease in adipose tissue lipogenesis reflects a decrease in FAS, fatty acid dehydrogenases and LPL enzyme activities (Smith and Walsh, 1988). A large decrease in LPL activity during early lactation was observed in animals with a negative energy balance (Smith and Walsh, 1988, Chilliard, 1993) but not when the animals were in positive energy balance (McNamara et al., 1987).

Lipolysis in adipose tissue is regulated by hormone sensitive lipase (HSL), which catalyses the release of free fatty acids and glycerol from stored triacylglycerides. McNamara (1991) reported that the activity of HSL increased during lactation and was highest in the high production group fed low-energy diets and remained elevated even after the energy balance returned to a positive state. Glycerol can not be recycled by the adipose tissue; therefore, glycerol release and blood glycerol concentrations have been used as indirect indicators of HSL activity (McNamara, 1991). Rates of lipolysis depend on the amount of the enzyme and the proportion of its active form. Relative rate of release of individual fatty acids from adipose tissue during lipolysis is not necessarily associated with their relative proportion in adipose tissue triacylglycerols for reasons not yet clear (McNamara,

1991). Smith and McNamara (1990) reported that the HSL activity peaked at approximately 60 days postpartum and then declined as the animals began to regain reserves. The HSL activity is regulated by changes in the balance of insulin and glucagons, catecholamines, adenosine and prostaglandin E isoforms in response to dietary energy supply in relation to the needs of the animal (McNamara, 1991).

In adipose tissue, lipolysis and fatty acid esterification proceed continuously. The balance of the two processes is determined by the energy states and by short and long term hormonal control (Vernon, 1980). During early and mid pregnancy, the animal is in positive energy balance and there is an increased esterification in the adipose tissue. During late pregnancy and early lactation the animal moves into a negative energy balance due to increased demand by the fetus and onset of lactation resulting in increased lipolysis (Vernon, 1980). Adipose tissue switches from a primarily anabolic state in early and mid pregnancy to a catabolic state in late pregnancy and early lactation (McNamara, 1991). The majority of these adaptations could be accounted for primarily by insulin and glucagon while sex steroids, growth hormone and prolactin are also important as they are involved in metabolism of pregnancy and lactation (McNamara, 1991). In addition, nor-epinephrine released from the sympathetic nervous system regulates lipolysis directly by activating HSL (McNamara, 1991).

## Dietary factors affecting milk fat synthesis and composition

Dietary factors have a profound influence on milk fat synthesis and composition by providing long-chain fatty acids or indirect supply of precursors for

milk fatty acid synthesis. Milk fat content and composition are affected by dietary factors including amounts, sources and fatty acid profiles of the diet, forage physical form and forage to concentrate ratio.

*Fatty acid profile of the diet*

Generally, forages contain small amounts of fat (1-3%) containing primarily unsaturated fatty acids such as linoleic acid (C18:2; 13 g/100 g FA) and linolenic acid (C18:3; 60 g/100 g FA) (Christie, 1981). Supplemental oil seeds are sources of oleic (C18:1) and linoleic acids, while fish oil is the only source of eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6).

Fat supplements derived from cottonseed, soybean and sunflower seed have greater than 50% polyunsaturated fatty acids (PUFA) , whereas tallow, canola meal, sunflower and safflower are relatively high in monounsaturated fatty acids (Grummer, 1991). In addition to the fatty acid composition, the form of supplemented fat affects milk fat percentage. Feeding free oil depressed milk fat yield, while fat supplemented as part of cottonseed and soybean seeds had no effect (Mohamed et al., 1988). In contrast, ground oil seeds seem to have more effect in decreasing milk fat compared to ungrounded oil seeds, possibly due to quicker release of oil in ground seeds due to loss of protection in the rumen (Grummer, 1991).

*Physical form of the forage*

Quality and quantity of forage has an effect on the milk fat concentration. Woodford et al. (1986) reported a linear increase in milk fat percentage from 3.5 to

4.0% when forage was increased from 28 to 53% of diet dry matter. Depression of milk fat percentage was prevented when forage mean particle size was greater than 0.64 cm possibly due to increased chewing time, leading to higher saliva production that helped to maintain rumen pH (Woodford et al., 1986).

*Forage to concentrate ratio*

Reducing the forage to concentrate ratio in diets generally results in decreased milk fat percentage if the forage level falls below 50% of the total diet. The decrease in milk fat accelerates when the proportion of concentrate in the diet is more than 60% and the depression in milk fat percentage can be as much as 50% of normal with diets containing more than 70% concentrate (Doreau et al., 1999). Sutton (1989) reported a decrease of 0.17 % in milk fat per 1% decrease in dietary ADF concentration in the dry matter. Earlier, Erdman (1988) reported an increase of .056% in milk fat for each percentage increase in ADF.

Replacing high-forage diet with low-forage diet decreased yields of all fatty acids with exception of C18:2(Gaynor et al., 1995; Griinari et al., 1998). The C6:0 to C16:0 content of milk fat is typically reduced and proportions of C18:1 and C18:2 are increased when low roughage diets are fed (Grummer, 1991). Gaynor et al. (1995) reported increases in the total trans 18:1 fatty acids and proportions of C18:1 and C18:2 in milk fat when the diet constituted 80% concentrate. Similar results for total trans 18:1 were reported by Kalscheur et al. (1997) but other milk fatty acid did not vary significantly between cows fed diets containing 40 or 75% concentrate.

*Fat source and level of fat supplementation*

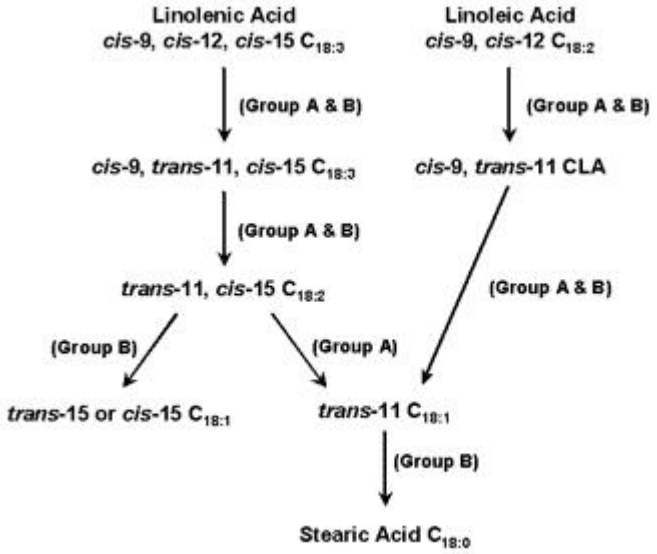
Supplementation of vegetable oils, oilseeds and marine oils decreases the milk fat content, while there appears to be only a moderate effect when animal fat sources are fed (Chilliard et al., 2001). Meta-analysis of data from studies with protected and unprotected tallow showed increased milk fat yield with protected tallow. This was mainly due to a substantial increase in stearic and oleic acids coupled with a decrease in medium-chain fatty acids (Chilliard et al., 2001). In the same study, using unprotected tallow caused similar changes in medium-chain fatty acids but smaller increases in the long-chain fatty acids (Chilliard et al, 2001). Use of animal-vegetable oil blends, calcium soaps, hydrogenated vegetable fat and saturated fatty acids as supplements decreases C8:0 to C15:0 fatty acids. Changes in C16:0, C18:0 and C18:1 were dependent on C16:0 and C18:0 fatty acid contents of the respective supplements (Palmquist et al., 1993). In addition, interactions between fat source and forages in the basal diet may influence the animal response (Onetti and Grummer, 2004) possibly by their combined effect on rumen biohydrogenation.

The level of fat supplementation seems to have a direct effect on the milk fatty acid composition. Grummer (1991) reported an inverse relationship between level of fat supplementation and the proportion of C4: 0 to C14:0 milk fatty acids, synthesized *de novo* in the mammary gland. The decrease in short- and medium-chain fatty acids was compensated by the increase in proportions of C18:0 and C18:1 in milk fat.

## Rumen biohydrogenation

Most ruminant diets contain at least of some amounts (2%) of unsaturated fatty acids, the composition of which depends on the type of feed ingredients. A forage-based diet would have linolenic acid as the predominant fatty acids while diets with oil seeds or oil supplements would have higher concentrations of oleic and linoleic acids. In the rumen, dietary lipids are hydrolysed to free fatty acids by microbial lipases and the unsaturated free fatty acids are biohydrogenated to saturated fatty acids by rumen microbes (Harfoot and Hazlewood, 1988). Rumen bacteria are predominantly involved in biohydrogenation and the process involves several steps (Fig 2.1).

Figure 2.1. Rumen biohydrogenation pathway




(Harfoot and Hazlewood, 1997)

The first step in biohydrogenation of both linoleic (18:2, c9, c12) and linolenic acid (18:3, c9, c12, c15) involves the isomerization of *cis*12 double bond to *trans* 11

double bond to form conjugated cis/trans double bond intermediates. Further reduction of the conjugated dienoic acids to monoenoic acids is accompanied with considerable double bond migration and formation of a variety of positional trans -18:1 isomers. (Griinari and Bauman, 1999; Mosley et al., 2002). *Trans* 11-18:1 is the major end product in this first step of biohydrogenation. The second step of biohydrogenation involving hydrogenation of *trans* 11-18:1 to stearic acid occurs very slowly and is considered rate limiting in the complete biohydrogenation sequence of PUFA (Kemp and Lander, 1984). Both steps of biohydrogenation involve separate groups of rumen bacteria (Harfoot and Hazlewood, 1988).

Changes in ruminal pH can result in a shift in microbial populations and related . changes in the profile of biohydrogenation end products (Griinari and Bauman, 1999). Diets with low roughage and high concentrate and/or containing higher PUFA supplements reduce the pH in the rumen, affecting the ruminal microflora. The altered ruminal microflora affects the rumen biohydrogenation process, leading to increased accumulation of biohydrogenation intermediates, predominantly consisting of *trans*-18:1 isomers (Kalscheur et al., 1997). The pattern of *trans*-18:1 and CLA isomers formed during biohydrogenation, depends largely on the dietary profile of unsaturated fatty acids (Loor et al., 2002). Using modeling analysis, Harvantine and Allen (2006) reported that increasing dietary unsaturated fatty acids stimulated the extent of 18:2 and 18:3 biohydrogenation, but inhibited the extent of 18:1 and *trans* 18:1 fatty acid biohydrogenation, leading to accumulation of the latter in the rumen.

In the rumen, the *trans*-18:1 fatty acids and the CLA isomers are adsorbed to ruminal digesta and pass to the duodenum, the site of nutrient absorption. About 80% of *trans*-18:1 fatty acids in the duodenal flow enter the lymphatic system without any selective absorption of individual isomers (Bickerstaffe et al., 1972). The intestinal epithelium has small amounts of stearoyl-CoA desaturase activity which is evident from increased levels of cis-octadeca-9-enoate (18:1) in the lymph compared to the levels in the duodenum (Bickerstaffe et al., 1972).

Similarly, there appears to be no selectivity in the uptake of different *trans*-18:1 fatty acids and CLA isomers by the mammary tissue. In the mammary tissue, some of the *trans*-18:1 fatty acids (especially *trans*-11) are further desaturated by the enzyme stearoyl-CoA desaturase to give rise to c9t11-CLA. Most of c9t11-CLA originates from desaturation of *trans*-11 18:1 fatty acid, making it the predominant CLA in cow's milk fat. The contribution of endogenously synthesized CLA to milk fat is much greater than the CLA originating from the rumen (Piperova et al., 2002).

## Trans-18:1 fatty acids and CLA in milk fat depression

Experiments (Gaynor et al., 1994; Romo et al., 1996; Baumgard et al., 2000; Piperova et al., 2004) have shown the involvement of either *trans*-18:1 and/or CLA isomers in milk fat depression. Teter et al. (1990) reported decreased milk fat in lactating mice fed *trans*-18:1 fatty acids regardless of the level of a fat or essential fatty acid content in the diet. In dairy cows, abomasal infusion of fat mixture containing *trans*-18:1 fatty acid significantly reduced milk fat content (Gaynor et al., 1994; Romo et al., 1996). The involvement of *trans*-18:1 fatty acids in MFD was

further investigated in feeding studies with dairy cows fed high grain diets with or without buffers (Kalscheur et al., 1997). Milk fat depression was observed with increased *trans*-18:1 fatty acid flow to the duodenum and increased *trans*-18:1 fatty acids in milk fat, irrespective of the origin of the isomers - either from diet or from incomplete biohydrogenation of unsaturated fatty acids (Wonsil et al., 1994). Griinari et al., (1998) reported a close relationship between MFD and increased *trans*-10 18:1 fatty acid in milk fat of dairy cows supplemented with different levels of concentrate with either corn oil or dry fat product. However, they did not find any correlation between total *trans*-18:1 fatty acids and fat percentage in milk. Feeding a MFD diet consisting of 70% concentrate and 5% soybean oil to lactating Holstein cows increased the total milk *trans* fatty acid content by seven fold predominantly due to an increase in *trans*-10 18:1 fatty acid (Piperova et al., 2000). A small increase in other *trans*-18:1 fatty acid isomers was also seen in addition to significant ($P<0.01$) increases in t10c12-CLA and t7c9-CLA (Piperova et al., 2000).

Loor et al. (2005a) reported a five-fold increase in *trans*-10 18:1 fatty acid in milk fat-depressed Holstein cows fed high-concentrate diets with or without linseed oil, confirming the possible role of this particular 18:1 fatty acid isomer in MFD. During MFD, *trans*-10 18:1 fatty acid replaces *trans*-11 18:1 fatty acid as the predominant *trans* FA (Griinari et al., 1998; Piperova et al., 2000). While these experiments suggest *trans*-10 18:1 isomer a potential inhibitor of fat synthesis, abomasal infusion of pure *trans*-10 18:1 does not support such an effect in lactating cows (Lock et al., 2007) . Further, an increase in milk concentrations of other *trans*-18:1 isomers during MFD suggests possible involvement of these isomers in MFD.

For example, Pretch et al. (2002) reported a significant increase in trans 6+7+8-, trans 13+14-18:1 fatty acids in high-yielding Holstein Friesian cows supplemented with rumen-protected fatty acids. However, they did not report the changes in milk fat percentages in the study. Significant ($P$<0.001) negative correlation was seen between trans 6+7+8-, trans 9-, trans 10- and trans-11 18: 1 and milk fat percentage in Holstein cows fed dry ground corn grain or high-moisture corn (Bradford and Allen, 2004).

Among the CLA isomers, the role of t10c12-CLA in MFD has been well established. Using abomasal infusion of purified t10c12-CLA in Holstein cows Baumgard et al. (2000) identified t10c12-CLA as the isomer responsible for MFD. A curvilinear response was observed between levels of t10c12-CLA and MFD in abomasally-infused lactating animals (Peterson et al., 2002). Abomasal infusion of c9t11-CLA had no effect on milk fat in lactating Holstein cows (Baumgard et al., 2000) suggesting that this major CLA isomer is not involved in MFD. Similarly, abomasal infusion of fat mixtures enriched in t8c10-CLA and c11t13-CLA failed to depress milk fat content in lactating cows (Perfield et al., 2004). Although t10c12-CLA has been shown to directly depress milk fat, changes in concentrations of milk t10c12-CLA during diet-induced MFD have been insufficient to account for the reduction in milk fat content. Piperova et al. (2004) reported decreased milk fat in lactating dairy cows without increase in t10c12-CLA when the animals were fed Ca salts of *trans* 18:1 fatty acids and CLAs. Similarly, other studies (Bradford and Allen, 2004; Loor et al. 2005a) reported the absence of any correlation between t10c12-CLA and milk fat content of diet-induced MFD. These findings suggested possible

involvement of other fatty acid isomers in MFD. Recently, at least two other CLA isomers t7c9-CLA (Piperova et al., 2002) and t9c11-CLA (Perfield et al., 2005) were shown to increase in milk fat during MFD and were strongly negatively correlated to milk fat content These combined results suggest that further studies are needed to determine the fatty acid isomers involved in MFD.

## Molecular mechanisms involved in milk fat depression

During MFD, the decrease in milk fat has been attributed predominantly to inhibition of *de novo* fatty acid synthesis. The decrease in short- (C4 to C8) and medium-chain (C10 to C16) fatty acids in milk fat could affect the formation of mammary triacylglycerol synthesis and, in part, could explain the decrease in milk fat content (Loor and Herbein, 1998; Baumgard et al., 2002). Our previous experiments and studies by others have provided evidence that mRNA abundance and gene expression of key lipogenic enzymes were inhibited during dietary MFD (Piperova et al., 2000; Peterson et al., 2003). Similarly, addition of fish oil to dairy cow diets decreased the mRNA abundance of *ACC, FAS* and *SCD* in mammary tissue (Ahnadi et al., 2002). The reduction observed in mRNA abundance correlated with the degree of MFD (Ahnadi et al., 2002; Peterson et al., 2003). Peterson et al.(2003) reported that in addition to *ACC* and *FAS*, dietary-induced MFD down regulated the expression of *GPAT*, *AGPAT, SCD* and *LPL* in lactating mammary gland. Abomasal infusion of t10c12 CLA reduced mRNA abundance for lipogenic enzymes involved in *de novo* fatty acid synthesis, fatty acid uptake and transport, desaturation of fatty acids and triacylglycerol synthesis in the mammary tissue explants obtained from the

infused animals (Baumgard et al., 2002). In the bovine mammary cell line (MAC-T), mRNA abundance for *ACC, FAS* and *SCD* was decreased within 48 hrs of incubation with t10c12 CLA, but not with c9t11 CLA (Peterson et al., 2004). Similarly, *ACC, FAS* and *SCD* mRNA abundance was reduced by feeding t10c12 CLA, c9t11 CLA and t11-18:1 to lactating mice (Lin et al., 2004).

Each of these MFD studies suggests that the lipogenic enzymes are possibly regulated through a common pathway or mediator at the level of gene expression. The role of the transcription factors, sterol response element binding protein-1 (SREBP-1) and thyroid hormone response protein (THRSP) have been suggested (Peterson et al., 2004; Harvatine and Bauman, 2006). However, Bionaz and Loor (2008) have questioned the proposal that SREBP1 is the central regulator of milk fat synthesis and have suggested a pivotal role for PPARγ and INSIG1.

*Sterol response element binding protein (SREBP)*

Sterol response element binding proteins (SREBPs) are the transcriptional factors associated with regulation of cholesterol and lipid metabolism. They activate the expression of at least 30 enzymes involved in the synthesis of cholesterol and lipids (Horton et al., 2003). There are three isoforms of SREBP- 1a, 1c and 2. The isoform SREBP-1a is involved in regulation of both fatty acid and cholesterol metabolism while SREBP-1c and SREBP-2 are involved in fatty acid metabolism and cholesterol metabolism, respectively. The isoform SREBP-1c is found predominantly *in vivo* while SREBP-1a is predominantly found in cell lines (Shimomura et al., 1997).

SREBP is a membrane-bound precursor protein found in endoplasmic reticulum with a molecular weight of 125kDa comprising 1150 amino acids present in three domains. The first, $NH_2$ – terminal domain (~ 480 amino acids) contains a basic helix-loop-helix leucine zipper domain that functions as a transcription factor. The second domain (~80 amino acids), consists of two trans-membrane helices linked by a short (~31 amino acid) loop protruding into the lumen of the endoplasmic reticulum. The third domain (~590 amino acids), consists of the COOH- terminal segment (Brown and Goldstein, 1997). The positioning of the three domains gives a hairpin shape to the molecule with the first and the last domains extending into the cytosol. SREBP binds to SREBP cleavage activating protein (SCAP) at the third domain and is anchored to the endoplasmic reticulum membrane through INSIG proteins (Horton et al., 2002; Yang et al., 2002). SCAP contains a sterol sensory domain which senses the concentration of sterols in the cell. Whenever the sterol concentrations in the cells are low, the SREBP-SCAP complex is detached from INSIG proteins, and SCAP escorts it to golgi. It is sequentially cleaved by two proteases, site1 protease (S1P) and site 2 protease (S2P) to release a 68-kDa mature SREBP. The mature protein translocates to the nucleus and binds to sterol response elements (SRE) in the promoter region of the lipogenic genes, activating the transcription process. Additional recruitment of co-activator proteins is necessary for complete transcriptional activation by SREBP-1a and SREBP-2. However, no reports are available on the recruitment of co activator proteins by SREBP-1c (Edwards et al., 2000). Though, SREBP-1a and SREBP-1c have similar action on regulation of lipogenic genes, SREBP-1a is 5 to 12 times more potent than SREBP-1c in activating

the target genes, possibly due to differences in the acidic transcription activating domains at the $NH_2$ – terminal domain ( Pai et al., 1998).

Dietary polyunsaturated fatty acids are known to decrease the production of fatty acids in the liver by down regulating genes encoding lipogenic enzymes and up regulating genes encoding proteins, and/or enzymes involved in fatty acid oxidation (Jump et al., 1994; Xu et al., 1999; Clarke, 2001). Feeding PUFA to mice decreased mature SREBP-1 while mRNA or the precursor SREBP bound to membranes were only slightly reduced, indicating that the effect of PUFA is mediated by a decrease in the mature form of SREBP-1 in liver (Yahagi et al., 1999). The decrease in mRNA levels of lipogenic genes corresponded to the pattern of suppression in mature SREBP-1. Dietary PUFA did not affect the lipogenic gene expression in transgenic mice in which high amounts of the mature form of SREBP-1 in the liver were over expressed. The transgenic mice had transgene-encoded amino acids 1-436 of human SREBP-1c under the control of the PEPCK promoter and the protein terminated before the transmembrane segment, which enabled it to enter the nucleus directly without a requirement of proteolysis and over expression of SREBP-1. This confirmed the role of mature SREBP-1 in the regulation of lipogenic genes (Yahagi et al., 1999).

Feeding fish oil to mice reduced precursor SREBP-1, precursor SREBP-2, *SREBP-1c* mRNA and mature SREBP-1 but did not alter *SERBP-1a* mRNA suggesting that the mature form was reduced due to down regulation of SREBP-1c mRNA (Kim et al., 1999). Hepatic (HepG2) cells treated with PUFA significantly decreased precursor SREBP-1 (Xu et al., 1999) and hepatic abundance of *SREBP-1*

mRNA (Mater et al., 1999; Xu et al., 1999) along with mature SREBP-1. Similarly, HEK-293 cells treated with different unsaturated fatty acids decreased mRNA levels of *SREBP-1a* and *SREBP-1c* in addition to the respective mature proteins (Hannah et al., 2001). Increases in chain length and degree of unsaturation increased the effectiveness of fatty acids to reduce the mature SREBP-1 (Hannah et al., 2001). In the same study, production of SREBP-1a by a cDNA expressed from an independent promoter decreased mature SREBP-1a without changing the concentrations of mRNA. Also, when mature SREBP-1a was expressed from an independent promoter engineered to express the truncated form of SREBP-1a that enters the nucleus with out a requirement for proteolysis, fatty acids did not change the concentrations of mature SREBP1. The authors concluded that the fatty acids could down regulate mature SREBP by two mechanisms: one at the level of mRNA expression and the other at the proteolytic processing of the precursor protein. At the mRNA level, the regulation may occur either at transcriptional or post-transcriptional levels. The post-transcriptional regulation, in addition to suppression of proteolytic cleavage of SREBP-1, occurs through accelerating the decay of mRNA as demonstrated in rat hepatocytes in monolayer culture (Xu et al., 2001). The transcriptional regulation of *SREBP-1* mRNA involves two factors - a feed-forward regulation by mature SREBP-1 and liver X- activated receptors (LXRs) (Horton et al., 2002).

LXRs are nuclear receptors activated by oxysterols, the intermediates of cholesterol synthesis, and form active heterodimers with RXRs (retinoid X receptors). LXR enhances transcription of *SREBP-1c* by binding to LXREs (LXR response elements) in the promoter region. PUFA antagonizes the activation of LXR by its

endogenous ligands (Ou et al., 2001) and inhibits the binding of LXR/RXR heterodimer to the LXREs in the *SREBP-1c* promoter (Yoshikawa et al., 2002) thereby reducing the activation of SREBP-1c expression.

The *SREBP-1c* promoter region in mouse has one putative nuclear factor Y (NF-Y) binding site, in addition to two LXR-response elements and one sterol regulatory element (SRE). The mature SREBP binds to SREs in the promoter region to activate the gene transcription and further requires the binding of NF-Y and/or SP-1 binding in the nearby sites (Amemiya-Kudo et al., 2000).

These studies indicate the involvement of multiple steps in the regulation of lipogenic gene expression and confirm that the regulation occurs through altering the content of mature nuclear SREBP-1c. However, deletion of SREBP-1c resulted in ~50% reduction in fatty acid synthesis and expression of lipogenic genes was not completely suppressed in the *SREBP-1c* knockout mouse (Liang et al., 2002). This indicated the possible involvement of other transcriptional factors like ChREBP in regulation of lipogenic genes.A series of *in vitro* studies in primary culture hepatocytes has shown that glycolytic and lipogenic gene expression is synergistically regulated by SREBP-1c and ChREBP (Dentin et al., 2004).

*Carbohydrate response element-binding protein*

The transcription factor, ChREBP is activated in response to high glucose concentrations in the liver and up regulates the lipogenic genes upon binding to glucose response elements (Uyeda et al., 2002). Studies with knockout mice for *SREBP1c*$^{-/-}$ have confirmed the role of ChREBP in coordinated control of glucose

metabolism, fatty acid synthesis and triacylglycerol synthesis (Lizuku et al., 2004).

ChREBP has a molecular weight of 94.6 kDa with 864 amino acids and contains several domains – nuclear localization signal (NLS) near the N-terminus, polypropylene domains, a basic helix-loop-helix leucine zipper (b/HLH/Zip), and a leucine-zipper-like domain. It also contains several potential phosphorylation sites for cAMP-dependent protein kinase (PKA) and AMP-activated protein kinase (AMPK) (Uyeda et al., 2002).

ChREBP is regulated reciprocally by the actions of glucose and cAMP (Kawaguchi et al., 2002). ChREBP is translocated into the nucleus by a high glucose concentration in the cell. The movement of ChREBP from cytosol to nucleus is regulated by dephosphorylation of the phosphor-Ser 196 site present closer to the NLS site. In the nucleus, the transcription factor binds to DNA upon dephoshphorylation of Ser 568 and Thr 666 residues. The dephosphorylation of the residues in both the cytosol and the nucleus is mediated by protein phosphatase 2A (PP2A) which is activated by xylulose 5-phosphate (X5P), a pentose phosphate pathway intermediate (Dentin et al., 2005a). On dephosphorylation of the residues, ChREBP interacts with another transcription factor Max-like protein X (Mlx), and forms a heterodimeric complex. This complex helps to recognize and bind to carbohydrate response elements (ChRE) that are glucose responsive (Stoeckman et al., 2004).

On the other hand, phosphorylation of the Ser and Thr residues of ChREBP by cAMP prevents nuclear translocation and DNA binding. Similarly, AMP activated protein kinase (AMPK) prevents DNA binding. This was confirmed by using 5-

amino-imidazolecarboxamide ribotide, a specific activator of AMPK in the cell. In the same study, a large increase in AMPK activity was seen due to increased cytosolic AMP concentration in response to acetate, octanoate and palmitate in hepatocytes (Kawaguchi et al., 2002). Similarly, PUFA suppressed ChREBP activity by altering its translocation from cytosol to nucleus as a result of decreased xylulose-5 phosphate concentration (Dentin et al., 2005b).

***Peroxisomal proliferator active receptors (PPARs)***

PPARs are members of the nuclear hormone receptor family which consists of three isoforms - PPARα, PPARβ and PPARγ, which are activated upon binding to ligands such as fatty acids and fatty acid derivatives. PPARs heterodimerize with retinoid-R-receptor (RXR) and modulate the expression of genes containing peroxisone proliferative response elements (PPRE) (Berger and Moller, 2002). PPARα is present mostly in liver and is involved in regulation of genes related to FA oxidation, while PPARγ is involved in lipogenesis and differentiation of cells in adipose tissue (Auwerex, 1999; Smith, 2002). Some of the PPARγ target genes in adipose include *LPL, CD36, FABP, ACACA, FAS, SCD, ACSL* and *AGPAT* (Way et al., 2001). Although, the role of PPARγ in lipid metabolism in adipose is well established, its role in the mammary tissue is not completely explored (Wan et al., 2007). Bionaz and Loor (2008) reported increased PPARγ expression with the onset of lactation in bovine mammary tissue. A simultaneous increase in genes related to FA uptake, transport, synthesis and desaturation (Bionaz and Loor, 2008) was also observed suggesting that part of the LCFA effects could be mediated through PPARγ.

PPARγ has been shown to be important in maintaining the quality of maternal milk (Wan et al., 2008). A targeted deletion of PPARγ in the mouse mammary gland led to increased oxidizing enzymes and the production of inflammatory lipids, which affected the health of the nursing pups (Wan et al., 2008). This suggests that PPARγ could play an important role in regulation of mammary lipid synthesis.

## Possible regulatory mechanism in the mammary gland

Relatively little work has been done on the molecular mechanisms involved in regulation of lipid synthesis in bovine mammary cells. Peterson et al.(2004) reported decreased abundance of the mature SREBP-1, whereas no reduction was observed for precursor SREBP-1 and mRNA of *SREBP-1* in mammary cell lines treated with t10c12 CLA. Loor et al. (2005) measured the gene expression profiling in the mammary gland of cows fed a MFD diet using a bovine oligonucleotide microarray. While eighteen genes associated with fatty acid metabolism were down regulated, the expression of *SREBP-1* was not altered (Loor et al., 2005c). However, Harvatine and Bauman (2006) suggested a role of SREBP-1 and THRSP in regulation of milk fat synthesis in lactating dairy cows. Nevertheless, in a comprehensive study on gene networks driving milk fat synthesis during lactation in dairy cows, Bionaz and Loor (2008) suggested an important role for PPARγ and INSIG1 in regulation of milk fat synthesis.

The results from earlier studies suggest that the regulation of lipogenic genes in the mammary gland might occur as a result of changes in transcriptional regulators (Havativen and Bauman, 2006; Bionaz and Loor, 2008). MFD induced either by altering the diets or by infusion has generally decreased milk fat content by 25 to

50%. These dietary interventions were not able to reduce milk fat beyond a certain point or completely eliminate the milk fat content. This suggests that, in addition to SREBP-1c, mammary lipogenic genes could be regulated by another major transcription factor, possibly ChREBP or PPARγ. To my knowledge, the role of ChREBP in regulation of milk fat synthesis has not been reported any where in the literature.

Based on the previous literature review, it is hypothesized that the depression in milk fat synthesis due to increased concentrations of trans-18:1 and/or CLA isomers in milk could be mediated through their action on the expression of transcriptional regulators. In addition to the established role of SREBP1 and THRSP, transcriptional regulators such as ChREBP, PPARγ, LXR and RXR could also play important roles.

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# Chapter 3: EXPERIMENT 1

## Principal component and multivariate analysis of milk long chain fatty acid composition during diet-induced milk fat depression.[1]

[1] A. K. G. Kadegowda, L. S. Piperova, and R. A. Erdman. 2008. J. Dairy Sci. 91: 749-759.

## ABSTRACT

The objective of this study was to assess the relationship between individual milk fatty acids and diet induced milk fat depression using principal component (PCA) and multivariate analysis (MA). Cow treatment observations (n=63) from 3 published feeding experiments with lactating dairy cows (Piperova et al., 2000; Piperova et al., 2002; and Piperova et al., 2004) were used in the analyses. In the PCA, principal component loading plots 1 (PC1) and 2 (PC2) described 55.9% of the total variation in milk FA and fat concentrations. Saturated FA (14:0, 16:0, and 17:0) and milk fat percentage showed negative loading for PC1. Trans-18:1 isomers (t6+7+8 to t15), t7c9 CLA, and t10c12 CLA showed positive (opposite) loading suggesting a negative relationship between these isomers and milk fat percentage. Cis11t13 CLA and c9t11 CLA were associated with the PC2 axes (neutral), indicating that they were not associated with MFD. Multivariate analysis with milk fat percentage as dependent variable and individual PC1 positive loading variables showed a break point relationship for t6+7+8-, t9-, t10-, and t13+14 -18:1 and a linear relationship for t11-, t12-, t15-18:1, t10c12 CLA and t7c9 CLA. Subsequent MA was conducted on 41 treatment means from 12 independent experiments from the literature where concentrations of t6+7+8-, t9-, t10-, and t11-18:1, and c9t11, and t10c12 CLA were reported. Significant negative effects of t9-18:1, t10-18:1, and t10c12 CLA, on milk fat percentage were observed. In this study, the PCA and MA analysis showed that among trans-18:1 isomers, t10-18:1 was most negatively correlated to milk fat percentage. However, the threshold concentration related to maximum MFD indicated that the relative potency was greatest for t6+7+8- and least

for t10-18:1. These results suggested that t6+7+8-18:1 might be more important than t10-18:1 in MFD. Principal component analysis also showed that t10c12, and t7c9 CLA were the isomers most negatively correlated to milk fat percentage, implying a possible role of t7c9 CLA in MFD. Additional experiments are needed to establish whether t7-18:1 is involved in MFD or that its effects are mediated via the endogenously synthesized t7c9 CLA.

**Key words:** milk fat depression, fatty acids, principal component analysis, multivariate analysis.

# INTRODUCTION

It has been demonstrated that conjugated linoleic acid **(CLA)** and trans-18:1 fatty acids **(FA)** arising from incomplete rumen biohydrogenation of dietary poly-unsatureated FA **(PUFA)** can markedly alter milk fat synthesis. Numerous studies have shown that concentrations of trans-18:1 FA or CLA can be increased in milk via dietary means (Griinari et al., 1998; Piperova et al., 2000; Peterson et al., 2003) or abomasal infusion (Gaynor et al., 1994; Romo et al., 1996; Chouinard et al., 1999) and can cause reduction of milk fat concentration. Examination of the isomer profile of trans-18:1 FA (Griinari et al., 1998) and CLA (Griinari and Bauman. 1999) indicated that an increase in ruminally-derived trans-10-containing 18:1 and 18:2 isomers in milk was more closely associated with milk fat depression **(MFD)** than the general increase in total trans-18:1 FA or CLA. It was demonstrated that abomasal infusion of partially hydrogenated vegetable oil **(PHVO) (**Gaynor et al., 1994; Romo et al., 1996), or diets supplemented with Ca-salts of trans-18:1 both containing isomers from t6 to t16 (Piperova et al., 2004) can cause MFD. However, when individual trans-18:1 isomers, including t9-, (Rindzig and Schultz, 1974), t11-, t12- (Griinari et al., 2000) were post-ruminally infused, milk fat was not affected. Although high concentrations of t10-18:1 are typically observed in milk fat of lactating cows fed MFD diets (Piperova et al.,2000; Peterson et al., 2003; Loor et al., 2004), abomasal infusion of 40 g/d t10-18:1 was not effective in reducing milk fat percentage in lactating cows (Lock et al., 2007). Baumgard et al. (2000) provided convincing evidence that abomasal infusion of t10c12 CLA inhibits milk fat

synthesis. Nevertheless, in an earlier study, Chouinard et al.(1999) observed MFD in lactating cows abomasally infused with a CLA mixture lacking t10c12 CLA.

Feeding experiments have also shown that concentration of t10c12 CLA in milk does not always account for the degree of MFD (Peterson et al., 2003) and that reduction in milk fat concentration can occur without increases in t10c12 CLA in milk (Piperova et al., 2004; Loor et al., 2005a). The t8c10 CLA and c11t13 CLA isomers usually present in lower concentrations in commercial CLA mixtures were examined by Perfield et al. (2004). They found no effect of these isomers on milk fat synthesis. These collective results suggest that there must be other biohydrogenation intermediates besides t10c12 CLA involved with MFD.

Multivariate analysis **(MA)** can be applied to visualize the multidimensionality of the data to identify underlying variables that may contribute to dietary MFD. Principal component analysis **(PCA)** is a multivariate technique that reduces the dimensionality of data by transforming a number of related variables into a set of uncorrelated variables, while retaining as much variation as possible. The transformed new variables, referred to as principal components **(PC)** are the linear combinations of the original variables. The first PC (PC1) accounts for the maximum variability while the remaining PC (PC2, PC3…PCn, n= number of variables) account for the remaining variability in the data. Each PC is independent and orthogonal to the other. Generally, only the first few PC describe the majority of total variation in the data as indicated by the Eigen values (Jolliffe, 2002; Kent and Coker, 1992). As a means to potentially identify individual FA effects, the objective of this

study was to assess the relationship between milk FA and diet induced MFD using PCA and MA analysis.

# MATERIALS AND METHODS

### *Experimental Design and Diets*

Individual cow within treatment observations (n=63) from 3 published feeding experiments (Piperova et al., 2000; Piperova et al., 2002, and Piperova et al., 2004) with lactating dairy cows conducted at the University of Maryland were used in this analysis. The studies were comprised of diets with different forage to concentrate ratios (with or without vegetable oil or buffer addition) and diets supplemented with Ca- salts of trans 18:1 fatty acids or CLA. Diets were formulated to meet the NRC (2001) requirements for milk production at 40 kg/d and 3.5% fat. Details of the experiments used in the study are presented in Table 3.1. In each experiment, cows were housed in individual stalls and fed individually. Forage and concentrate dry matter (DM) was measured weekly, and the TMR was adjusted accordingly to maintain constant forage to concentrate ratio on a DM basis during the experiment. Milk production was recorded at each milking. At the end of each experimental period, milk samples from 6 consecutive milking were collected and composited for FA analysis.

***Analytical Methods***

The FA composition of milk samples and Ca-tFA and Ca-CLA supplements was determined using FA methyl esters **(FAME)** prepared by mild trans-esterification with 0.04 M $H_2SO_4$ in methanol, at room temperature, using GLC conditions previously described (Piperova et al., 2000). Trans-18:1 concentration in milk and isomer distribution were determined by a combination of preparative Ag+-thin layer chromatography and GLC analysis (Piperova et al., 2000). Argentation-high performance liquid chromatography was used to determine CLA isomer distribution patterns (Piperova et al., 2000).

***Statistical Analysis***

Relationships between milk FA were evaluated from PCA loading plots, based on the correlation matrix, using XLSTAT software (XLSTAT- Pro 7.5.2 for windows, Addinsoft, New York, NY). Relationships between individual milk FA concentrations (% of total FAME) and fat percentage on individual cow treatment observations were analyzed using NLmixed procedure (SAS, 2000) with a break point or simple linear regression model analysis depending on the best fit for each individual FA. FA were analyzed only if they were present in all experiments. Data for short chain FA were available only in one of the three experiments used and were not included in the analysis.

The break point analysis used the following model:

$$Y = b_0 + (b_1 * X) + E_n$$

If $X \leq$ break point, then:

$Y=b_2+ E_n$

Where:

Y= Milk fat%

$b_0$ & $b_2$ = Intercept values for Regression Lines 1 and 2 respectively.

$b_1$ = Slope of Regression Line 1.

$E_n$= Experimental effect.

The break point in this analysis is the concentration of FA where regression Lines 1 and Line 2 intercept.

Subsequent MA were conducted on treatment means (n = 41) from 12 independently published experiments (Donovan et al., 2000; Loor et al., 2002; Peterson et al., 2002b; AbuGhazaleh et al., 2003; 2004; Peterson et al., 2003; Shingfield et al., 2003; Loor et al., 2003; 2005a, 2005b; Loor and Herbein, 2003; Selberg et al., 2004) that reported concentrations of t6+7+8-, t9-, t10-, t11-18:1 and, c9t11 and t10c12 CLA isomers in milk of lactating cows.

## RESULTS

Correlation coefficients between the variables are presented in Table 3.2. The 14:0, 16:0, 17:0 and 18:0 saturated FA were positively correlated ($P < 0.001$; $P < 0.001$; $P < 0.03$; and $P < 0.02$, respectively) to milk fat percentage. Conversely, 14:0, 16:0 and 17:0 FA were negatively correlated to trans-18:1 and CLA isomers, with the exception of c9t11 CLA, c11t13 CLA , t16-18:1 and t15-18:1(for 17:0). The 18:0 FA was associated negatively to trans-18:1, t6+7+8-, t10-18:1 FA and t10c12 CLA. Total trans-18:1 and individual trans-18:1 isomers (except t16-18:1) were negatively

correlated to milk fat concentration ($P < 0.001$). The t7c9 CLA, and t10c12 CLA isomers were significantly ($P < 0.001$) negatively correlated to milk fat concentration, contributing to the negative correlation observed for the total CLA. Linoleic acid and non-conjugated 18:2 isomers (18:2i) were negatively correlated to milk fat and were positively correlated to CLA and trans-18:1 isomers with the exception of c9t11 CLA, c11t13 CLA and t16-18:1. Among the CLA, t7c9 CLA and t10c12 CLA isomers were highly correlated (r = 0.831; $P < 0.001$) to each other and both were positively correlated ($P < 0.001$) to trans-18:1 isomers (t6+7+8 to t-15). Cis9t11 CLA was positively correlated to t7c9 CLA (r = 0.365, $P < 0.03$), t11-18:1 (r = 0.236, $P < 0.06$) and t12-18:1 (r = 0.355, $P < 0.01$), while c11t13 CLA was positively correlated to 16:0 (r = 0.18). Trans-18:1 isomers, with the exception of t-16, were highly correlated ($P < 0.001$) to each other and to t7c9 CLA and t10c12 CLA.

In the PCA, the PC1 and PC2 described 55.93% of the total variation in milk FA and fat concentration across the three experiments (Table 3.3). Principal Components 1 and 2 had Eigen vectors of 10.12 and 4.42 describing 38.9% and 17% of the total variance, respectively. Eigen vectors are the coefficients of loadings that explain whether all variables in the data are described by the PCA. The Eigen vectors for PC1 and PC2 (Table 3.4) showed that except for 18:3, all other FA were described by either PC1 or PC2, or by both.

The position of individual FA in the loading plot defined their relationship to milk fat percentage (Figure 3.1). Four clusters of FA were identified on the loading plot: Cluster 1, consisting of 14:0, 16:0, and 17:0 was positively correlated to milk fat ($P < 0.05$, Table 3.3); Cluster 2, consisting of trans-18:1 isomers (t6+7+8 to t15),

total CLA, t7c9, and t10c12 CLA loaded opposite to milk fat percentage, showed a strong negative correlation (Table 3.2); Cluster 3, including 15:0 and 16:1; and Cluster 4, consisting of 18:0, c18:1, t16-18:1, and c9t11, c11t13 CLA, were both associated with the PC2 neutral axis, suggesting no relationship, either positive or negative, with milk fat percentage. Finally, linolenic acid (18:3), positioned close to the origin of the plot, was not related to any of the PC.

To further elucidate the relative importance of each CLA isomer to MFD, individual CLA isomers were subtracted from the total CLA concentration to determine their effects on the position of the remaining CLA on the loading plot (Figures not included). Total CLA, t10c12 and t7c9 CLA isomers were inversely correlated to milk fat percentage emphasizing their involvement in MFD (Figure 3.1, Table 3.2). Subtraction of either t7c9 CLA or t10c12 CLA from total CLA did not change the position of the remaining CLA isomers relative to the PC1 axis (Figure not included). However, when both t7c9 CLA and t10c12 CLA isomers were subtracted from total CLA, the remaining CLA isomers were positioned closer to the neutral PC2 axis (Figure 3.2). The c9t11 CLA and c11t13 CLA were not associated with changes in milk fat percentage. Due to the positive correlation between all trans-18:1 isomers, subtractions of individual isomers from total trans-18:1 did not change their position on the loading plot (Figure not included).

Multivariate analysis on PC1 positively-loaded variables, showed a break point relationship for t6+7+8-, t9-, t10- and t13+14-18:1 FA and a linear relationship for t11-, t12- t15-18:1, t10c12 CLA and t7c9 CLA with milk fat percentage (Figure 3.3, Panels 1.A to 9.A). Subsequent MA of the independently gathered literature

means showed a break point relationship only for t10-18:1, and a linear relationship between t6+7+8-, t9-, t11-18:1 and t10c12 CLA, and milk fat percentage (Figure 3.3, Panels 1.B, 2.B, 3.B, 4.B and 8.B).

## DISCUSSION

The PCA analysis and results from the correlation matrix confirmed previous reports (Bradford and Allen, 2004; Loor et al., 2005a) that trans-18:1 and CLA isomers were negatively correlated to milk fat percentage. Although, the results showed negative correlation of C18:2 to milk fat percentage, there is no evidence that 18:2 that escaps rumen biohydrogenation is directly involved in MFD. Studies (Gaynor et al., 1994; Romo et al., 1996) in lactating cows post-ruminally infused with fat mixtures containing similar amounts of C18:2 showed MFD only when the infusion mixture contained trans-18:1 FA. However, there is uncertainty as to the potential for individual trans-18:1 isomers to reduce milk fat concentration (Offer et al., 1999). Earlier experiments (Newbold et al., 1998) have found that change in milk fat concentration was related negatively to the change in t10-18:1 of milk but was not related to changes in t9- and t11-18:1. Offer et al. (1999) reported significant negative correlations between concentration of fat in milk and proportions of t9-, t10- and t11-18:1 of milk FA. Loor et al. (2005a) reported that total trans-18:1, t6+7+8-, t9-, t11-, and t12-18:1 were negatively correlated to concentrations of *de novo* FA in milk from lactating cows fed low- or high-concentrate diets with or without linseed oil. Increased concentration of t6+7+8-18:1 in milk, was observed by others (Griinari. et al., 1998; Piperova et al., 2000, Peterson et al., 2003) in cows fed MFD diets.

Previously, Precht et al. (2002) suggested that t6+7+8-18:1 might be a key isomer in dietary MFD.

In the current study, trans-10-18:1 had the greatest negative correlation with milk fat percentage (r = 0.633; $P < 0.01$; Table 3) among all trans-18:1 isomers. Griinari et al. (1998) first suggested that the degree of MFD may be related to the proportions of individual trans-18:1 isomers in milk fat and noted that t10-18:1 replaced t11-18:1 as the predominant trans-18:1 isomer in milk during MFD. High t10-18:1 concentration was consistently observed in lactating cows during dietary MFD (Piperova et al., 2000; Peterson et al., 2003; Loor et al., 2004). However, during dietary-induced MFD, both t10-18:1 and t10c12 CLA increased simultaneously, making it difficult to determine if t10-18:1 had an effect on milk fat synthesis that was independent of t10c12 CLA effects. Shingfield et al. (2006) showed that 0.74 of the variation in milk fat concentration of lactating cows fed corn silage-based diets supplemented with fish and sunflower oil was due to changes in milk t10-18:1 concentration. However, they did not suggest that t10-18:1 was the causative factor for the decrease in milk fat concentration. Rather, it was concluded that high concentration of t10-18:1 could be an indicator of rumen conditions favorable for MFD to occur. Furthermore, Lock et al.(2007) recently reported that abomasal infusion of 43g/d t10-18:1 for 3 d did not reduce milk fat synthesis in dairy cows.

Break point analysis was used in this study, as a means to determine the threshold concentration of the respective FA isomer associated with maximum MFD (Figure 3.3). The underlying assumption was that there is a limit to the degree of

MFD regardless of the amount of inhibitory rumen biohydrogenation products taken up by the mammary gland. Indeed, titration studies with t10c12 CLA (Baumgard et al., 2001) and CLA mixtures (Chouinard et al., 1999) suggested a maximal milk fat depression of about 50% compared to the controls.

In this study, the relationship between individual isomers (t6+7+8- to t15-18:1, t7c9 CLA and t10c12) and milk fat percentage (Figure 3.3; 1.A-9.A) displayed similar patterns. There was a cluster of data points, especially at low isomer concentrations, where milk fat was variable in relation to isomer concentration. As isomer concentration increased, a linear decrease in milk fat was observed until concentrations of trans-18:1 or CLA reached the break point threshold level. As milk trans-18:1 or CLA isomer concentrations increased beyond the break point, no further depression in milk fat concentration was apparent regardless of isomer concentration. Using this approach, maximal reduction in milk fat percentage was observed at 0.69, 0.86, 2.37 and 1.94% (% of FAME) for t6+7+8-, t9-, t10- and t13+14-18:1, respectively (Figure 3.3). If these isomers caused MFD, then their estimated relative potencies would be greatest for t6+t7+t8- and least for t10-18:1. However, the potencies could be related to the preferential synthesis of these trans-18:1 FA in the rumen during MFD.

Individual (Figure 3.3, Panel 3A) and literature-derived (Figure 3.3, Panel 3.B) data for t10-18:1 were consistent where the threshold concentrations were similar, 2.37% and 2.52%, respectively. Further, there was greater variation in milk fat responses with t10-18:1 concentration less than 1.3%. Recently, Lock et al.(2007) reported no change in milk fat with post-ruminal infusion of 43g/d t10-18:1 for 3 d in

lactating cows. However, milk t10-18:1 was only 1.11%, well below the suggested threshold concentration and also within the concentration range where milk fat responses are variable and would be more difficult to detect. Applying the 0.6 percentage unit increase in milk t10-18:1 observed by Lock et al.(2007) to equations shown in Figure 3.3, Panels 3.A and 3.B, the expected decline in fat percentage would have been 0.4 - 0.5 percentage units. Inconsistent milk fat responses that have been observed at low t10-18:1 concentration could be an alternative conclusion versus a total lack of response to t10-18:1 suggested by Lock et al. (2007).

Post-ruminal infusion studies with increasing amounts of t10c12 CLA (Baumgard et al., 2000; Baumgard et al., 2001; Peterson et al., 2002a) showed a curvilinear decline in milk fat, eventually approaching a plateau at maximum MFD. Using literature data obtained from experiments with dietary-induced MFD, it was showed that the relationships between t6+7+8-, t9-, t11-18:1 and t10c12 CLA and milk fat percentage were linear only. However, maximal t10c12 CLA concentration was much lower than that observed in post-ruminal infusion studies. Therefore, a possible reason for the lack of a defined break point for t10c12 CLA and other isomers was the inadequate number of observations at high concentrations of these isomers to establish a plateau in milk fat concentration.

As indicated by the slope (Figure 3.3), the unit decline in milk fat percentage per unit increase in milk trans-18:1 isomers, was highest for t6+7+8-18:1 (-2.59) and lowest for t10 (-0.42) suggesting that t6+7+8-18:1 might be more important than t10-18:1 in MFD. Among the CLA isomers, t10c12 CLA was most closely associated with milk fat synthesis (slope, -15.51; Figure 3.3. 8.A), followed by the t7c9 CLA

(slope, -6.55; Figure 3.3. 9.A), implying a possible role of t7c9 CLA in MFD. The slopes for these CLA isomers were much greater than those observed for t10-18:1 and other trans-18:1 isomers.

Principal component analysis also showed that t7c9 CLA and t10c12 CLA were the CLA isomers most negatively correlated to milk fat percentage. The importance of t10c12 CLA in MFD has already been established (Baumgard et al., 2000), and Bauman and Griinari (2001) have reported a negative relationship between t10c12 CLA and milk fat concentration. Trans-10, cis12 CLA is also the biohydrogenation precursor for t10-18:1 in the rumen (Griinari and Bauman, 1999). Earlier studies (Piperova et al., 2000; Peterson et al.,. 2003; Loor et al., 2004) have shown that high-concentrate diets supplemented with PUFA can shift ruminal biohydrogenation towards formation of t10c12 CLA and t10-18:1 causing MFD.

Possible effects of t7c9 CLA on milk fat synthesis have not been investigated mainly because of a lack of a pure source for experimental use. Feeding (Piperova et al., 2002) and abomasal infusion (Corl et al., 2002) experiments have provided evidence that t7c9 CLA is almost exclusively produced in the mammary gland via $\Delta^9$-desaturation of ruminally-derived t7-18:1. However, it is very difficult to analytically separate t6-, t7-, and t8-18:1, which prevents quantitation of t7-18:1 conversion to t7c9 CLA.

Shingfield et al. (2003) provided evidence that the proportions of t5-, t10-, t11- t12-18:1, and t7c9 CLA were simultaneously and substantially increased in milk of lactating cows fed diets containing fish oil. Pottier et al. (2006) recently reported that concentrations of both t10-18:1 and t7c9 CLA were reduced in milk fat when

dietary vitamin E supplementation successfully alleviated MFD. These observations were consistent with the highly positive correlation found between t7c9 CLA and t10-18:1 (r=0.61; $P < 0.001$) in this study (Table 3.3; Figure 3.1).

The PCA confirmed earlier reports that the c9t11 CLA (Baumgard et al., 2000) and c11t13 CLA isomers (Perfield et al., 2004) were not associated with changes in milk fat (Figure 3.1; Table 3.2). However, in some cases of dietary-induced MFD, an increase in t9c11 CLA has been observed. Abomasal infusion of CLA mixture enriched in t9c11 CLA suggested that this isomer may be responsible for at least part of the decrease in milk fat production during MFD (Perfield et al., 2005). Examination of milk FA composition responses to fish- and sunflower oil-supplemented diets for dairy cows showed a strong correlation between milk fat and t9c11 CLA (Shingfield et al., 2006). Using a dietary strategy to reduce milk fat yield in lactating cows, Roy et al. (2006) found that decreases in fat yield were accompanied by increases in milk t10-18:1 and t9c11-CLA. The authors suggested that these isomers could be directly or indirectly related to anti-lipogenic activities in the mammary gland.

It could be speculated that configuration of the conjugated double bonds may define specific effects of individual CLA isomers on milk fat synthesis. Perfield et al. (2006) compared abomasal infusion of t10t12 CLA to that of t10c12 CLA and reported that t10t12 CLA did not reduce milk fat yield but significantly altered the desaturase index and milk FA composition in lactating cows. It appears that at least some CLA isomers with trans/cis conjugated double bonds have the ability to

decrease milk fat (i.e., t10c12, t9c11, and possibly t7c9,) whereas, the major cis/trans CLA were not effective (c9t11, c11t13).

In summary, the PCA and MA analysis showed that among trans-18:1 isomers, t10-18:1 was most negatively correlated to milk fat percentage. However, the threshold concentration related to maximum MFD indicated that the relative potency was greatest for t6+7+8- and least for t10-18:1. These results suggested that t6+7+8-18:1 might be more important than t10-18:1 in MFD. Principal component analysis also showed that t10c12 and t7c9 CLA were the isomers most negatively correlated to milk fat percentage, implying a possible role of t7c9 CLA in MFD. Additional experiments are needed to establish whether t7-18:1 is involved in MFD or that its effects are mediated via the endogenously synthesized t7c9 CLA.

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Table 3.1. Details of the experimental data used in the principal components analysis

| Experiment | Observations /Treatment | Total Observations | Treatments |
|---|---|---|---|
| Piperova et al., 2000 | 11 | 22 | Control diet (Forage/concentrate 60/40); MFD[1] diet (25 % forage and 70 % concentrate with 5 % soybean oil); |
| Piperova et al., 2002 | 4 | 16 | High-forage (60% Forage) with or without buffer addition; Low-forage (25% Forage) with or without buffer addition; |
| Piperova et al., 2004 | 5 | 25 | Control; 100g Ca-tFA[2] supplement; 200g Ca-tFA supplement; 400g Ca-tFA supplement; 100g Ca-CLA supplement; |

**[1]Milk Fat Depression**
**[2] Calcium salts of trans fatty acids**

Table 3.2. Correlation coefficients for the milk fatty acids (% of total fatty acids) from individual cow observations reported by Piperova et al., (2000, 2002, and 2004)

| | Fat % | 14:0 | 15:0 | 16:0 | 16:1c | 17:0 | 18:0 | 18:1t | 18:1c | 18:2 | 18:2i | 18:3 | CLA | t7c9 | c9t11 | t10c12 | c11t13 | t6+7+8 | t9 | t10 | t11 | t12 | t13 | t15 | t16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fat % | 1 | | | | | | | | | | | | | | | | | | | | | | | | |
| milk, kg | -0.271* | | | | | | | | | | | | | | | | | | | | | | | | |
| 14:0 | 0.420** | 1 | | | | | | | | | | | | | | | | | | | | | | | |
| 15:0 | 0.019 | 0.465** | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 16:0 | 0.531** | 0.449** | -0.198 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 16:1c | 0.232 | -0.065 | 0.159 | 0.106 | 1 | | | | | | | | | | | | | | | | | | | | |
| 17:0 | 0.369** | 0.289* | 0.218 | 0.363** | 0.023 | 1 | | | | | | | | | | | | | | | | | | | |
| 18:0 | 0.288* | -0.186 | -0.390** | 0.182 | -0.436** | -0.282* | 1 | | | | | | | | | | | | | | | | | | |
| 18:1t | -0.646** | -0.483** | 0.031 | -0.789* | -0.069 | -0.545** | -0.327** | 1 | | | | | | | | | | | | | | | | | |
| 18:1c | 0.063 | -0.420** | -0.651** | 0.214 | -0.235 | 0.043 | -0.662** | -0.265** | 1 | | | | | | | | | | | | | | | | |
| 18:2 | -0.614** | -0.312* | 0.127 | -0.656** | -0.063 | -0.463 | -0.407** | 0.780** | -0.133 | 1 | | | | | | | | | | | | | | | |
| 18:2i | -0.573** | -0.358** | 0.279* | -0.794** | 0.086 | -0.449** | -0.480** | 0.764** | -0.453** | 0.636** | 1 | | | | | | | | | | | | | | |
| 18:3 | 0.044 | 0.137 | 0.114 | -0.179 | -0.050 | 0.090 | -0.081 | -0.016 | -0.034 | 0.230 | 0.153 | 1 | | | | | | | | | | | | | |
| CLA | -0.431** | -0.332* | 0.037 | -0.427** | -0.089 | -0.154 | -0.018 | 0.426* | 0.187 | 0.507* | 0.295* | 0.222 | 1 | | | | | | | | | | | | |
| t7c9 | -0.483** | -0.345** | -0.091 | -0.494** | -0.044 | -0.392** | -0.215 | 0.623* | 0.023 | 0.601** | 0.388** | 0.020 | 0.462** | 1 | | | | | | | | | | | |
| c9t11 | 0.102 | 0.059 | -0.006 | 0.117 | -0.051 | 0.188 | 0.204 | -0.192 | 0.398** | 0.044 | -0.230 | 0.228 | 0.464** | 0.365** | 1 | | | | | | | | | | |
| t10c12 | -0.577** | -0.304* | 0.096 | -0.588** | -0.060 | -0.400** | -0.426** | 0.707* | -0.276* | 0.687** | -0.559** | -0.009 | 0.340** | 0.831** | 0.051 | 1 | | | | | | | | | |
| c11t13 | -0.092 | -0.111 | 0.395** | 0.301* | -0.284* | 0.047 | 0.210 | -0.202 | 0.431** | -0.059 | 0.331** | 0.036 | 0.190 | -0.020 | 0.229 | 0.019 | 1 | | | | | | | | |
| t6+7+8 | -0.602** | -0.409** | 0.013 | -0.721** | -0.025 | -0.525** | -0.389** | 0.895** | -0.265* | 0.714** | 0.702** | 0.007 | 0.389** | 0.738** | -0.140 | 0.790** | -0.176 | 1 | | | | | | | |
| t9 | -0.558** | -0.451** | -0.054 | -0.681** | -0.010 | -0.477** | -0.282* | 0.847** | -0.136 | 0.668** | 0.656** | 0.035 | 0.422** | 0.707** | -0.043 | 0.648** | -0.186 | 0.929** | 1 | | | | | | |
| t10 | -0.633** | -0.426** | 0.073 | -0.778** | -0.028 | -0.507** | -0.416** | 0.964** | -0.334** | 0.775** | 0.766** | 0.039 | 0.378** | 0.607** | -0.183 | 0.716** | -0.205 | 0.888** | 0.816** | 1 | | | | | |
| t11 | -0.520** | -0.390** | -0.065 | -0.503** | -0.199 | -0.296** | 0.019 | 0.698** | 0.102 | 0.616** | 0.437** | -0.049 | 0.394** | 0.592** | 0.236 | 0.479** | -0.049 | 0.612** | 0.622** | 0.691** | 1 | | | | |
| t12 | -0.457** | -0.482** | -0.276* | -0.398** | -0.214 | -0.247* | 0.221 | 0.504** | 0.363** | 0.488** | 0.221 | -0.076 | 0.404** | 0.561** | 0.355** | 0.379** | 0.049 | 0.413** | 0.455** | 0.479** | 0.844** | 1 | | | |
| t13 | -0.486** | -0.418** | -0.177 | -0.509** | -0.225 | -0.314* | -0.005 | 0.700** | 0.097 | 0.641** | 0.398** | -0.021 | 0.403** | 0.638** | 0.232 | 0.567** | 0.007 | 0.658** | 0.734** | 0.659** | 0.762** | 0.777** | 1 | | |
| t15 | -0.342** | -0.250** | -0.151 | -0.393** | -0.262 | -0.207 | 0.062 | 0.583** | 0.096 | 0.454** | 0.295* | -0.010 | 0.291** | 0.538** | 0.209 | 0.465** | 0.031 | 0.607** | 0.706** | 0.529** | 0.660** | 0.625** | 0.890** | 1 | |
| t16 | 0.047 | -0.117 | -0.177 | 0.044 | -0.290 | 0.090 | 0.502** | -0.023 | 0.475** | 0.037 | -0.162 | -0.077 | 0.094 | 0.128 | 0.476* | -0.066 | 0.180 | -0.076 | 0.061 | -0.080 | 0.503** | 0.660** | 0.539** | 0.567** | 1 |

*$P < 0.05$ **$P < 0.01$.

Table 3.3. Principal component Eigen values for Principal Component 1 and Principal Component 2.

| Principal Component | Eigen value | Proportion of total variance (%) | Cumulative variance proportion (%) |
|---|---|---|---|
| PC1 | 10.12 | 38.93 | 38.93 |
| PC2 | 4.42 | 17.00 | 55.93 |
| ∑ PC3-PC26 | 11.46 | 44.07 | 100.00 |

Table 3.4. Coefficients of the loadings (Eigen vectors) for PC1 and PC2

| Response variable | PC1 | % Contribution for PC1 | PC2 | % Contribution for PC2 |
|---|---|---|---|---|
| Fat % | -0.224 | -9.73 | 0.003 | 0.19 |
| Milk, kg | 0.007 | 0.30 | 0.204 | 12.60 |
| FA | | | | |
| 14:0 | -0.160 | -6.90 | -0.136 | -8.40 |
| 15:0 | -0.003 | -0.10 | -0.281 | -17.36 |
| 16:0 | -0.248 | -10.80 | 0.109 | 6.73 |
| 16:1c | -0.034 | -1.50 | -0.203 | -12.54 |
| 17:0 | -0.168 | -7.50 | -0.059 | -3.64 |
| 18:0 | -0.093 | -4.00 | 0.342 | 21.12 |
| 18:1t | 0.295 | 12.81 | -0.083 | -5.13 |
| 18:1c | -0.040 | -1.74 | 0.399 | 24.64 |
| 18:2 | 0.262 | 11.38 | -0.049 | -3.02 |
| 18:2i | 0.232 | 10.08 | -0.223 | -13.77 |
| 18:3 | 0.015 | 0.65 | -0.050 | -3.27 |
| t7c9 | 0.244 | 10.60 | 0.066 | 4.08 |
| C9t11 | 0.011 | 0.48 | 0.251 | 15.50 |
| t10c12 | 0.249 | 10.82 | -0.070 | -4.32 |
| C11t13 | -0.037 | 1.60 | 0.238 | 14.70 |
| tFA | | | | |
| t6+7+8 | 0.288 | 12.51 | -0.088 | -5.44 |
| t9 | 0.281 | 12.21 | -0.030 | -1.85 |
| t10 | 0.289 | 12.55 | -0.113 | -6.98 |
| t11 | 0.246 | 10.69 | 0.163 | 10.07 |
| t12 | 0.206 | 8.95 | 0.285 | 17.60 |
| t13 | 0.257 | 11.16 | 0.179 | 11.06 |
| t15 | 0.217 | 9.43 | 0.185 | 11.43 |
| t16 | 0.045 | 1.95 | 0.363 | 22.42 |
| Total loadings (absolute) | 2.302 | 100.00 | 1.619 | 100.00 |

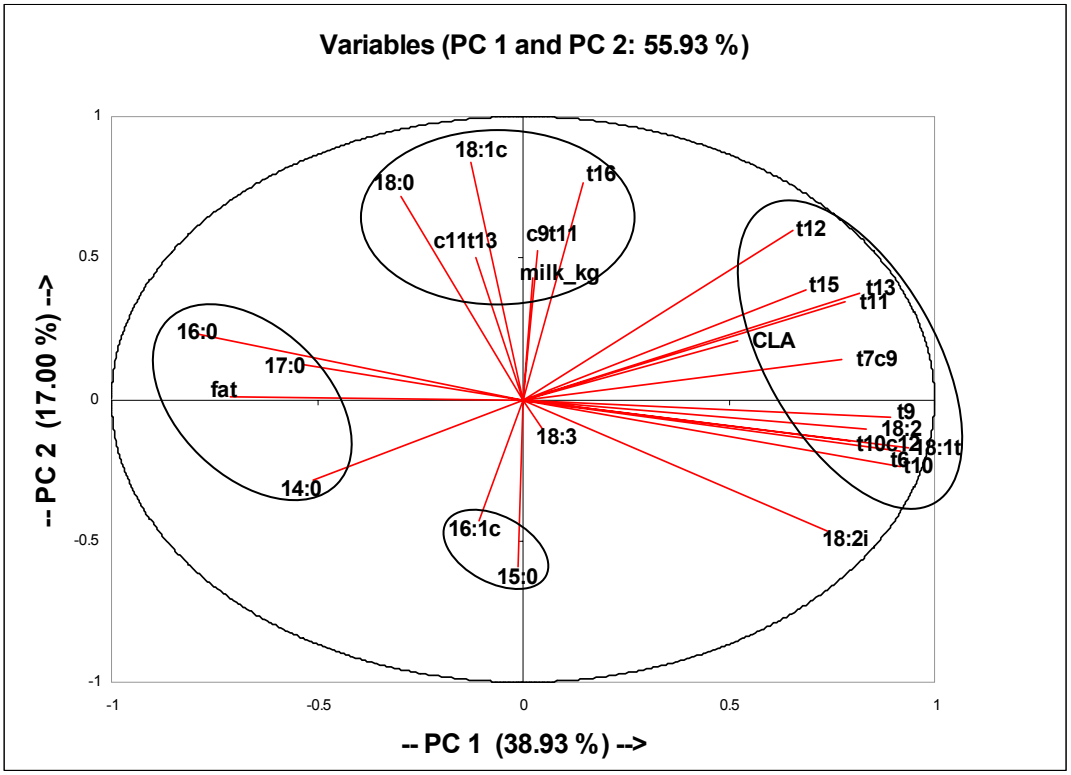



Figure 3.1. Loading plot describing the relationship among milk fatty acids derived from a principal component analysis based on proportions (% of total fatty acids) for C14 to C18 FA in milk from 3 experiments (n=63). Four clusters of FA were distinguished. Trans-18:1 isomers (except t16-18:1), total CLA, t10c12 CLA and t7c9 CLA were loaded opposite (negatively related) to milk fat percentage, while saturated FA (14:0, 16:0 and 17:0) were loaded with milk fat percentage.

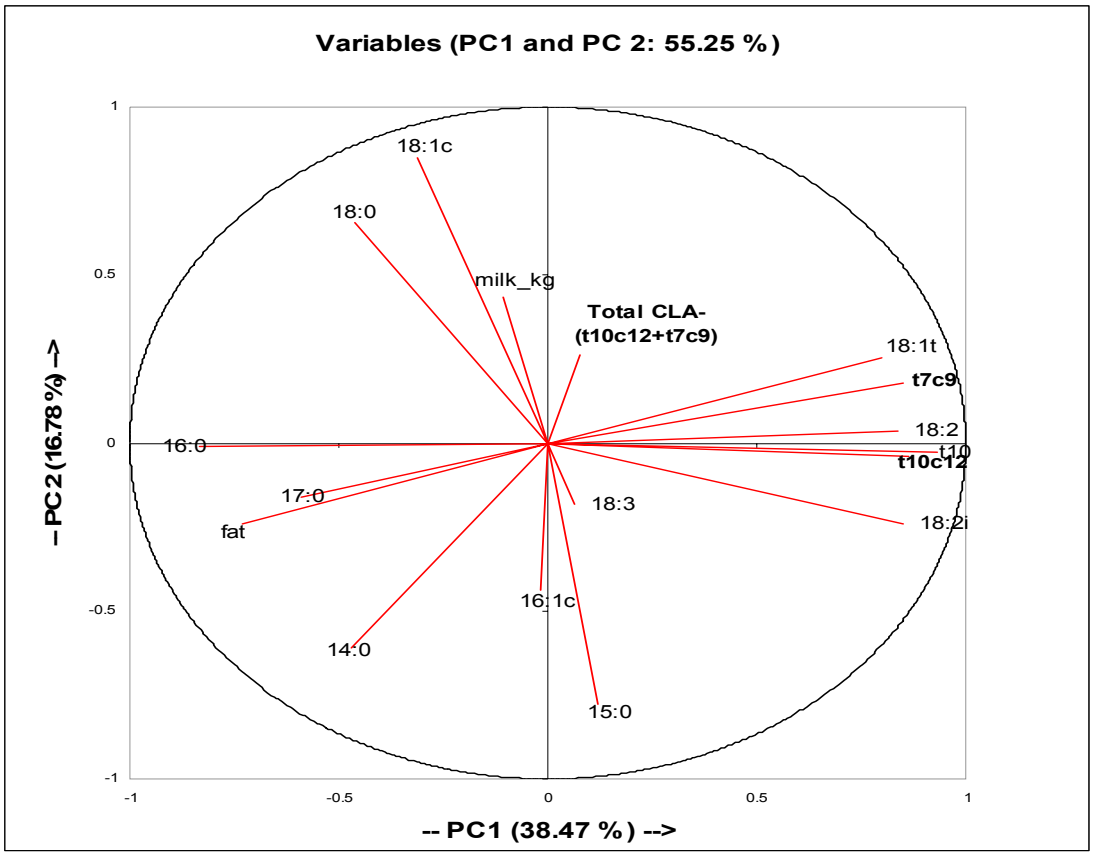
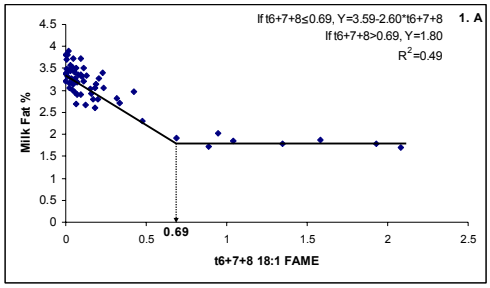


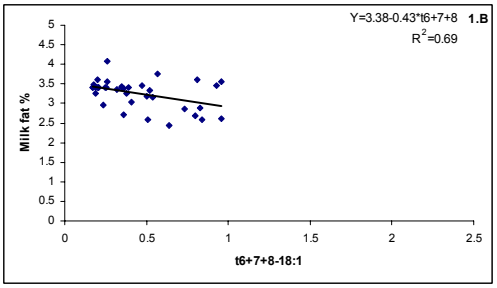

Figure 3.2. Loading plot describing the relationship among milk CLA isomers derived from a principal component analysis, based on the proportions (% of total fatty acids) for CLA isomers. The position of total CLA shifted closer to PC2 (neutral) axis when t10c12 CLA and t7c9 CLA were subtracted.

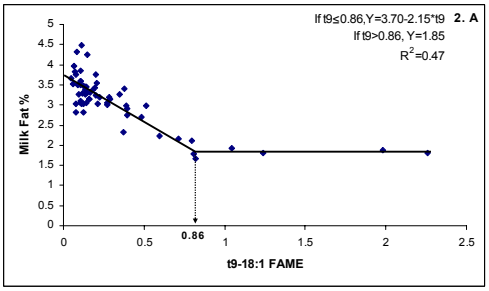
Figure 3.3. Relationship between milk fat percentage and fatty acid isomers, as explained by multivariate analysis.

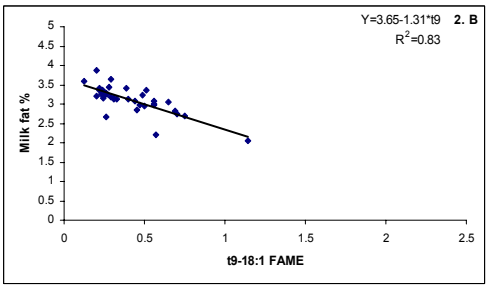
**Panels 1.A to 9.A.** The data points are individual observations from the experiments reported by Piperova et al., (2000; 2002; and 2004). The break point analysis fitted for t6+7+8-, t9-, t10-, and t13+14-18:1 (Panels 1.A, 2.A, 3.A and 6.A, respectively). A linear relationship was observed between the other FA isomers and milk fat percentage.

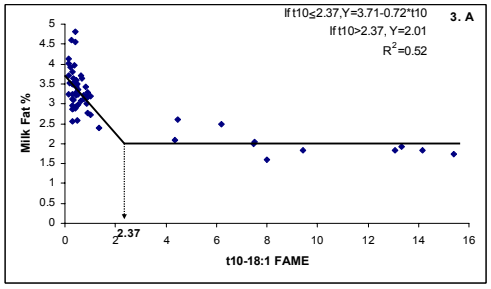
**Panels 1.B to 8.B.** The data points are reported means from the literature studies used. Only t10-18:1 (3.B) fitted the breakpoint analysis. A linear relationship was observed between the other FA isomers and milk fat percentage.

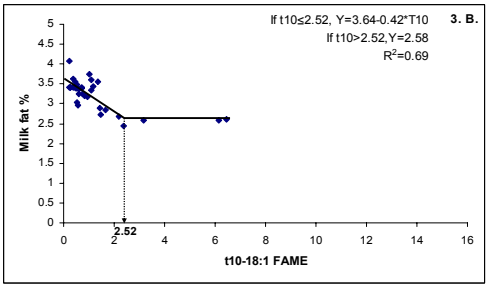


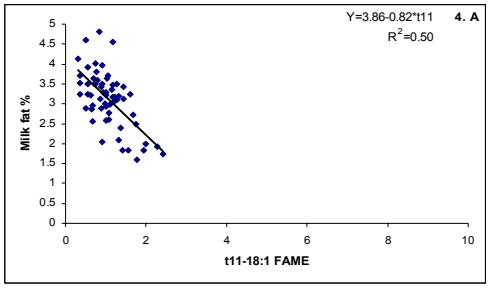



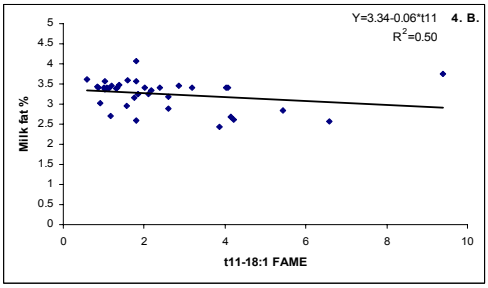



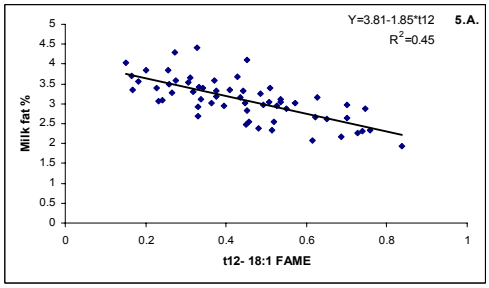



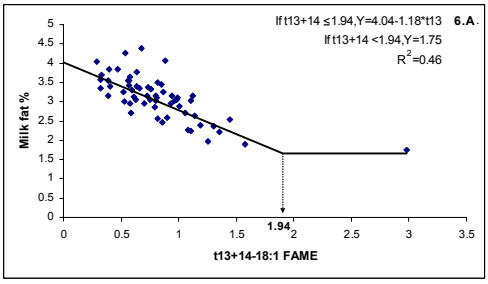



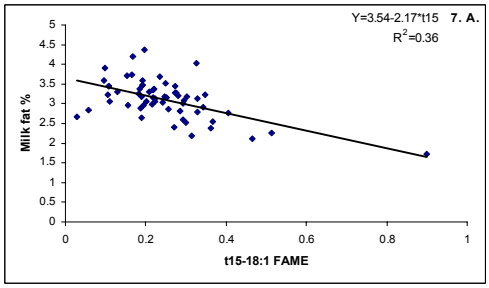



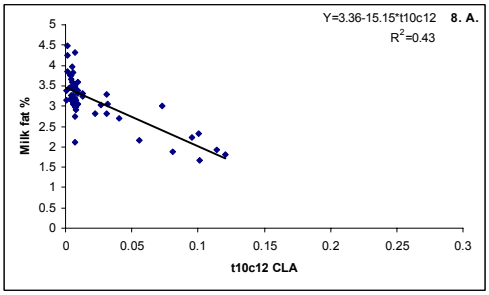

Y=3.86-0.82*t11 4. A
R2=0.50
Milk fat %
t11-18:1 FAME

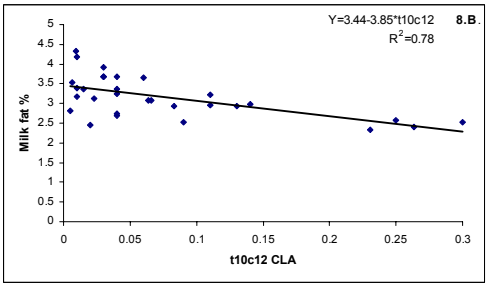

Y=3.34-0.06*t11 4. B.
R2=0.50
Milk fat %
t11-18:1 FAME

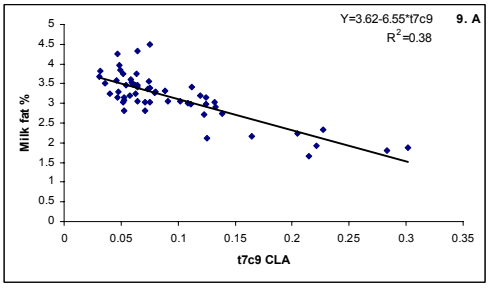

Y=3.81-1.85*t12 5.A.
R2=0.45
Milk fat %
t12- 18:1 FAME


If t13+14 ≤1.94,Y=4.04-1.18*t13 6.A.
If t13+14 <1.94,Y=1.75
R2=0.46
1.94
Milk fat %
t13+14-18:1 FAME


Y=3.54-2.17*t15 7. A.
R2=0.36
Milk fat %
t15-18:1 FAME


Y=3.36-15.15*t10c12 8. A.
R2=0.43
Milk fat %
t10c12 CLA


Y=3.44-3.85*t10c12 8.B.
R2=0.78
Milk fat %
t10c12 CLA


Y=3.62-6.55*t7c9 9. A
R2=0.38
Milk fat %
t7c9 CLA

# Chapter 4: EXPERIMENT 2

## Milk fat depression induced by trans-18:1 isomers or t10c12-CLA feeding in murine mammary gland may be mediated by multiple transcription regulators.[1]

[1] Submitted to J. of Nutrition.
[1] Presented in part at the ADSA-ASAS annual meetings-2007 and 2008.

A. K. G. Kadegowda, B. B. Teter, J. Sampugna, P. Delmonte, L. S. Piperova and R. A. Erdman. 2007. Trans-7-octadecenoic acid decreased milk fat and altered CLA composition in milk of lactating mice. J. Dairy Sci. 90. Suppl. 1. 113.

A. K. G. Kadegowda, E. E. Connor, B. B. Teter, J. Sampugna, L. S. Piperova and R. A. Erdman. 2008. Mammary and liver lipogenic gene expression in lactating mice fed diets supplemented with trans 18:1 isomers or t10c12-CLA. J. Dairy Sci. 91. Suppl. 1. 146.

## ABSTRACT

The objectives were to determine the effects of several individual trans-18:1 isomers and t10c12-CLA on fat synthesis, and expression of lipogenic genes and transcription factors in liver and mammary tissues in lactating mice. Thirty lactating C57Bl6J mice were randomly assigned to 6 diets supplemented with one of the following isomers t7-, t9-, t11-18:1, t10c12-CLA or PHVO (partially hydrogenated vegetable oil) from day 6 to day 10 postpartum. Milk fat percentage was decreased by t10c12-CLA (44%; $P$<0.001), t7-18:1 (27%; $P$< 0.001) and PHVO (23%; $P$<0.001), compared to Control. Amounts of total CLA were greatest in milk of mice fed t7-18:1 (2.4%, $P$ <0.01), followed by t10c12-CLA (1.9%, $P$ <0.01) and t11-18:1 (1.1%, $P$ <0.01). The increased CLA found in milk of mice fed t7-18:1 was a result of substantial endogenous synthesis of t7c9-CLA, representing 87% of total CLA. In the mammary gland, t10c12-CLA decreased gene expression related to *de novo* FA synthesis (*FASN*, $P$<0.01; *ACACA* $P$<0.01), triacylglycerol formation (*AGPAT,* $P$<0.01), desaturation (*SCD1*, $P$<0.05; *SCD2* $P$<0.05) and transcriptional regulation (*SREBP-1c*, $P$<0.01; *ChREBP*, $P$<0.01; *THRSP*, $P$<0.01; *PPARA* $P$<0.05; *PPARG*, $P$<0.05). PHVO and t7-18:1 decreased the expression of *AGPAT*, *SCD1* and *THRSP* ($P$<0.05). Mammary gene expression was not altered by t-9- or t-11-18:1. In liver, expression of *AGPAT* was significantly decreased by diets supplemented with t10c12-CLA ($P$<0.01), PHVO ($P$<0.01) and t7-18:1($P$<0.01). In contrast to mammary gland, CLA increased gene expression of hepatic *PPARA* ($P$<0.05) and decreased *PPARG* ($P$<0.05). There was a substantial increase in total FA(per g of dry tissue) in liver of dams fed the t10c12-CLA (81%, $P$<0.01), PHVO (35%, $P$<0.05), t7-18:1 (38%,

*P*<0.05), and t11 18:1- (26%, *P*<0.05) supplemented diets. The results demonstrated extensive conversion of t7-18:1 to t7c9-CLA in mammary and liver tissues of mice fed the t7-18:1 diet. The observed milk fat depression suggests potential involvement of t7c9-CLA in regulation of milk fat synthesis. In addition to *SREBP-1C,* the study establishes a role of *ChREBP*, *PPARG* and *INSIG1* in regulation of milk fat synthesis.

**Key Words:** trans-18:1 FA, milk fat depression, transcriptional regulation, gene expression

# INTRODUCTION

Effects of transoctadecenoic acids (trans-18:1 FA) on milk fat synthesis in lactating mice fed partially hydrogenated vegetable oil **(PHVO)** were first reported by Teter et al. (1990). Partially hydrogenated vegetable oils contain a mixture of positional trans-18:1 isomers ranging from t4-(trans-4) to t16-18:1. Since trans-18:1 FA are also intermediates in rumen biohydrogenation, it was hypothesized that trans-18:1 FA are involved in decreased milk fat synthesis **(MFD)** in lactating dairy cows (Gaynor et al., 1995). Studies showed that abomasal infusion of PHVO (Gaynor et al., 1994; Romo et al., 1996) or dietary supplementation of Ca salts of trans-18:1 FA containing all trans-18:1 isomers (Piperova et al., 2004) reduced milk fat content in lactating cows. Concentrations of trans-18:1 FA in milk generally correlates with MFD. However, in some studies increases in milk trans-18:1 FA do not correspond to reductions in milk fat yield (Kalscheur et al., 1997). Griinari et al. (1998) reported that MFD in lactating cows may be associated with changes in trans-18:1 FA isomer profile and an increase in t10-18:1 isomer rather than with an increase in total trans-18:1 content. This suggested a possible involvement of specific isomers in MFD. Individual t9- (Rindsig and Schultz., 1974), t10- (Lock et al., 2007), t11- and t12-18:1 (Baumgard et al., 2000) isomers were examined but no effects on milk fat synthesis were observed. However, meta analysis of published data obtained from lactating cows fed MFD diets suggested a possible role of t7-18:1 or t7c9-CLA in MFD (Kadegowda et al., 2008).

In contrast to trans-18:1 isomers, the potential role of CLA in MFD is better established. T10c12-CLA was identified as a CLA isomer that inhibits milk fat

synthesis (Baumgard et al., 2000). However, concentration of t10c12-CLA in milk does not always account for the degree of MFD (Peterson et al., 2003), and MFD without changes in t10c12-CLA have been reported by others (Piperova et al., 2004; Loor et al., 2005). This implies that other CLA or trans-18:1 isomers may have a role in regulating milk fat synthesis, and at least two other CLA isomers, t9c11- (Schingfield et al., 2006) and t7c9-CLA (Kadegowda et al., 2008), have shown a strong negative correlation to milk fat content.

The mechanisms involved in MFD are not well understood. Bauman and Griinari (2003) reported that diet-induced MFD alters the milk FA profile by decreasing *de novo* FA synthesis. Similar effects on *de novo* FA synthesis were observed when milk fat yield was reduced by t10c12-CLA (Looret al., 1998). Feeding a MFD-diet (Peterson et al., 2003,Piperova et al., 2000) to lactating dairy cows, or t10c12-CLA to rats (Ringeseis et al., 2004) and mice (Lin et al., 2004), caused a decrease in mRNA abundance and enzymatic activity of mammary lipogenic enzymes. Transcriptome profiling of mammary gland of lactating mice demonstrated that substantial regulation of lipid synthesis occurred at the level of mRNA expression and the transcription factor SREBP-1c was implicated as one of the regulators (Rudolph et al., 2007). Studies with MACT cells (Peterson et al., 2004) and lactating cows (Harvatine and Bauman, 2006) have shown that the effect of t10c12-CLA on fat synthesis could be regulated via the *SREBP-1c* transcription factor acting as a possible global regulator for milk fat synthesis. However, *SREBP-1c* knockout mice exhibited a 50% decrease in FA synthesis without fully suppressing lipogenic gene expression (Liang et al., 2002). This suggested that other transcription

regulators may be involved in FA synthesis. Studies with cultured primary hepatocytes (Dentin et al., 2004) and *SREBP-1c* knockout mice (Lizuka et al., 2004) have reported a role of the carbohydrate response element binding protein (ChREBP) in the coordinated control of glucose metabolism, FA synthesis and triacylglycerol **(TAG)** synthesis in liver. Based on these findings, it was hypothesized that effects of dietary FA on milk fat synthesis could be regulated through multiple transcriptional regulators in the mammary gland.

The objectives were to determine the effects of several individual trans-18:1 isomers and t10c12-CLA on fat synthesis, and expression of lipogenic genes and transcription factors in mammary and liver tissues of lactating mice.

## MATERIALS AND METHODS

### *Animals, diets and treatments*

The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland. Timed pregnant multiparous female C56Bl/6J mice used in this study were obtained from the Department of Animal and Avian Sciences, University of Maryland. Dams and their pups were housed in shoebox cages and provided with *ad libitum* food and water. Pregnant mice were fed a commercial rodent diet (5001 Rodent Lab Diet ®, Purina, Richmond, IN, USA) consisting of 23% CP, 4.5% fat and 6.0% fiber until 2 days prepartum. From day 2 prepartum until day 6 postpartum, dams were fed a Control diet. On day 6 postpartum, they were randomly assigned to one of the six treatment diets (Control, PHVO, t10c12-CLA, t7-18:1, t9-18:1 and t11-18:1). The Control diet consisted of

sucrose 590 g/kg, vitamin free casein 200 g/kg, alphacel 50 g/kg, fat 100 g/kg (cocoa butter 14.3 g/kg, corn oil 30.9 g/kg, olive oil 34.8 g/kg, oleic acid 20 g/kg), AIN 76 Mineral Mixture 40 g/kg, AIN 76 vitamin mixture 15 g/kg, DL-Methionine 3 g/kg and Choline bitartarate 2 g/kg. The oleic acid was replaced completely with the respective fatty acids in treatments t7-18:1, t9-18:1 and t11-18:1, but was replaced at 30% in the t10c12-CLA treatment consistent with the documented potency of t10c12-CLA. The fat mixture in the PHVO treatment consisted of vegetable shortening 45.73 g/kg, corn oil 26.6 g/kg, soybean oil 7.6 g/kg and oleic acid 20 g/kg, and the FA composition was similar to that of the Control diet. The fatty acid composition of the diets fed to lactating mice is presented in Table 4.1.

Except for the Control (n=7) and t-7 18:1 (n=3), treatment groups consisted of 5 lactating mice. Mice and their pups were randomly assigned to treatments. Litter size was adjusted to 6 or 7 pups to maintain uniform milk synthesis among dams. Lactating mice were fed the experimental diets from day 6 to day 10 postpartum. Daily feed intake was recorded during the experimental period. On day 6 and day 10 postpartum, milk samples were collected by suction and milk fat percentages measured as described by Teter et al. (1990). Milk fat percentages estimated by crematocrit as volume percentages were converted to weight percentages using calibration factors developed by Teter et al. (1990). The milk samples were stored at -20°C for FA analyses. Body weights of dams and pups were recorded before milking. On day 10 postpartum, the animals were sacrificed using isoflurane and individual liver and mammary tissues were collected from dams and pooled livers

from pups from each diet. The livers were fast-frozen in liquid nitrogen, and stored at -80°C until RNA or lipid extraction.

***Fatty acid analyses***

Fats in the diets and milk samples were saponified with 0.5 N NaOH in methanol for 30 min at 50°C and transesterified to FA methyl esters **(FAME)** as previously described (Kadegowda et al., 2008). Trinonadecenoin (Nu-Chek Prep, Inc. Elysian, MN, USA) was included as an internal standard. The FAME were analyzed using a Hewlett Packard (HP 5890) gas chromatograph (Hewlett Packard, Sunnyvale, CA, USA) equipped with a 30 m x 0.25 mm fused silica capillary column coated with SP-2380 (Supelco Inc., Bellefonte, PA, USA) and FID detector. The injector and detector temperature were maintained at 250°C. The initial oven temperature was 155°C (held for 5 min) and was programmed to increase at a rate of 1.4°C per min, to a final temperature of 195°C (held for 10 min). Peak identification was based on relative retention times of commercial standards including GLC-68B and GLC-463 (Nu-Check Prep Inc.)

Individual CLA isomers in milk were analyzed as methyl esters in a Waters 2690 HPLC separation module (Waters Associates, Milford, MA, USA), equipped with a waters 2996 PDA detector, and 3 Chromsphere 5 lipids (250 x 4.6 mm, 5 μM, Varian) HPLC columns in series, using conditions described previously (Kadegowda et al., 2008). A CLA standard mixture (Nu-Chek Prep Inc.), spiked with t7c9-CLA standard, was used to identify individual isomers.

Liver tissues were lyophilized and FAME were prepared as described for diet and milk samples. FAME were analyzed using an Agilent 6890 gas chromatograph

(Agilent Technologies, Santa Clara, CA, USA) equipped with a SP 2560 capillary column (100 m x 0.25 mm, 0.2 mm, Supelco, Belefonte, CA, USA) and a FID detector. Gas chromatograph conditions were as described by Kadegowda et al. (2008). The individual FA and 18:1 isomers were identified using GLC-463 standard mixture (Nu-Chek Prep Inc.).

***Extraction of total RNA***

Total RNA was isolated from ~1g of tissue using Qiazol lysis reagent (Qiagen Inc., Valencia, CA, USA) and RNeasy isolation kits with on-column DNAse digestion (Qiagen Inc.). The RNA quality was evaluated using the Agilent 2100 Bioanalyzer with RNA 6000 Nano Labchip kits (Agilent Technologies) while concentrations were determined using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

***Quantitative Real-Time RT-PCR***

Purified RNA, diluted to a concentration of 75 ng/µl, was used for the first-strand cDNA synthesis by reverse transcription using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Seven-hundred and fifty nanograms of RNA were used per 30-µl reaction volume. A parallel control reaction was performed in the absence of reverse transcriptase for each first-strand synthesis. Incubation conditions were: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min. A 2-µl aliquot of cDNA reaction product and 10 *p*mol of each primer were used per 25-µl real-time PCR reaction with 12.5 µl of iQ SYBR Supermix (Bio-Rad Laboratories). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/) based on murine

mRNA sequences available in GenBank. Primer sequences and annealing temperature used for each gene target are presented in Table 4.2. Real-time PCR was performed in the Bio-Rad iCycler (Bio-Rad Laboratories) with the following conditions: 95°C for 3 min, followed by 40 cycles of 94°C for 15 sec, annealing temperature for 30 sec, and 72°C for 30 sec, with fluorescence detected during the extension step.

Standards for each gene target were prepared from PCR amplicons using each primer pair, purified using the QIAquick gel extraction kit (Qiagen Inc.). Product concentrations were determined using the Agilent 2100 BioAnalyzer and DNA 500 kits (Agilent Technologies). The PCR amplicons were sequenced using a CEQ8000 automated DNA sequencer and DTCS Quickstart Chemistry (Beckman Coulter, Fullerton, CA, USA) to confirm amplification of the expected murine nucleotide sequence. The quantity of cDNA in unknown samples was calculated from the appropriate external standard curve ranging from 1 x $10^2$ to 1 x $10^7$ molecules/µl run simultaneously with samples as described by Connor et al. (Connor et al., 2005). Samples and standards were assayed in duplicate. Assay performance for each gene is reported in Table 4.2.

***Genes examined***

***Lipogenic genes:*** Fatty acid synthase (*FASN*), acetyl-Coenzyme A carboxylase alpha (*ACACA*), 1-acylglycerol-3-phosphate O-acyltransferase (*AGPAT*), stearoyl-Coenzyme A desaturase 1 (*SCD1*), stearoyl-Coenzyme A desaturase 2 (*SCD2*), and lipoprotein lipase (*LPL*); ***Transcription regulators:*** thyroid hormone responsive SPOT14 homolog (*THRSP*), sterol regulatory element binding

transcription factor 1 (*SREBP1c*), carbohydrate response element binding protein (*ChREBP*), peroxisome proliferator activated receptor alpha (*PPARA*), peroxisome proliferator activated receptor gamma (*PPARG*), liver X receptor alpha (*LXRA*), retinoid X receptor (*RXR*); ***Co-activators***: SREBP cleavage activating protein (*SCAP*), insulin induced gene 1 (*INSIG1*), and MAX-like protein X (*Mlx*).

***Statistical Analysis***

Data were analyzed using the Mixed Procedure in the Statistical Analysis System (Version 8.2, 2004, SAS Institute, Cary, NC, USA). The fixed effects were the treatments, while the random portion of the model included the individual animals. Data from day 6 postpartum were used as a covariate for the analyses of milk fat percentage and milk FA composition. Gene expression data were analyzed as described by Connor et al. (2005). A probability of ($P$<0.05) was considered statistically significant.

## RESULTS

**Feed intake, body and organ weights and pup growth rate.**

Daily feed intake, body and organ weights and pup growth rate are presented in Table 4.3. Compared to Controls, dietary supplementation of t10c12-CLA decreased the average feed intake in dams by 23% (6.98 vs. 5.35 g/d, $P$<0.03). The average dam body weight was not affected by FA supplements on day 10 postpartum. Liver weights were increased by 30% with t10c12-CLA and by 20% with t7-18:1 treatments ($P$<0.05) but were decreased by 11% in mice fed t9-18:1 diet ($P$<0.10).

The pup growth rates were significantly reduced with t10c12-CLA ($P<0.01$), t7- ($P<0.05$), and t9-18:1 ($P<0.05$) FA-containing diets.

**Milk fat percentage and milk volume.**

Milk fat percentage (Table 4.3) was decreased by diets supplemented with t10c12-CLA (41%, $P<0.001$), t7-18:1 (26%, $P<0.05$) and PHVO (23%, $P<0.05$). The t9- or t11- 18:1 diets did not affect milk fat percentages. Milk volume, calculated based on the average litter body weight (29), tended to be lower with t10c12-CLA, t7- and t9-18:1 treatments. These data must be interpreted with caution as they may be influenced by the lower litter body weight and the decrease in milk fat content observed in mice fed the t10c12-CLA and t7-18:1 diets.

**Milk FA composition.**

The decrease in milk fat content observed in mice fed t10c12-CLA, PHVO and t7-18:1 diets was predominantly due to reduction in the proportion of medium-chain (<16:0) FA (MCFA) (Table 4.4). Total MCFA was decreased by 41%, 21%, and 16% with the t10c12-CLA, PHVO and t7-18:1 diets, respectively. Compared to Control, t10c12-CLA decreased C10:0 (40%, P <0.1), C12:0 (34%, $P<0.01$) and C14:0 (45%, $P<0.01$), while the decrease of individual MCFA was in the range of 15 to 20% with PHVO and t7-18:1 supplementation (Table 4.4). Concentrations of 16:0 were reduced by the t10c12-CLA (20%, $P<0.01$) and PHVO (13%, $P<0.05$) treatments. The decrease in MCFA and 16:0 in milk of mice fed t10c12-CLA led to a proportional increase in 18:0, 18:1cis9, 18:2 and 20:4 long-chain FA (LCFA).

**Milk trans-18:1 and CLA isomer profile.**

The trans-18:1 and CLA isomers provided by the diets were taken up by the mammary gland and significant proportions ($P<0.01$) were incorporated to milk fat (Table 4.5). Total trans-18:1 FA was markedly increased with the trans-18:1-supplemented diets (Table 4.5). The highest amount of trans-18:1 was observed with PHVO (3.7%), followed by t9- (3.1%), t11-(1.8%) and t7-18:1(1.6%). Trans-18:1 isomers from t6- to t14-18:1 were increased with the PHVO diet ($P< 0.05$). Trans-18:1 FA were not detected in the milk of mice fed t10c12-CLA and only trace amounts of t9-18:1 were found in milk of Control mice.

Amounts of total CLA were greatest in milk of mice fed t7-18:1 (2.4%), followed by t10c12-CLA (1.9%) and t11-18:1 (1.1%) (Table 4.5). The predominant CLA was t7c9-CLA in the milk of mice fed t7-18:1 FA. Small amounts of t7c9-CLA (0.08%) were also found in the milk of mice fed PHVO. The t10c12-CLA was the major milk FA isomer of mice fed t10c12-CLA and was not detected in any other treatments. The predominant isomer in milk of mice fed the Control, PHVO, t11- and t9-18:1-supplemented diets was c9t11-CLA.

**Fatty acid composition of dam liver.**

The total FAME (per g of dry tissue) in dam liver was substantially increased (Table 4.6) by diets supplemented with t10c12-CLA (81%, $P<0.01$), PHVO (35%, $P<0.05$), t7-18:1 (38%, $P<0.05$), and t11-18:1 (26%, $P<0.05$). The proportion of 16:0 was greater in t10c12-CLA-, t7- ($P<0.01$) and t9-18:1- ($P<0.05$) fed mice. Compared to Control, the proportion of 18:0 was reduced ($P<0.01$) in liver of mice fed all diets except t9-18:1 where only a tendency ($P=0.07$) was observed. The 20:4

and 22:0 LCFA were also higher ($P<0.01$) in liver of mice fed the t10c12-CLA-, PHVO-, and t7-18:1-supplemented diets (Table 4.6). Total trans-18:1 FA was increased ($P<0.01$), reflecting the uptake of dietary-supplemented isomers (Table 4.6). The amount of total CLA was greatest for t7-18:1 (1.5%) followed by t11-18:1 (1.35%), mainly due to the endogenous CLA synthesis from the respective trans-18:1 isomers (Table 4.7). T7c9-CLA was found in liver of mice fed t7-18:1 and PHVO. Cis9t11-CLA was present in all animals along with smaller amounts of other CLA isomers (c11t13, t11t13, and cis / cis CLA). Trans 10cis12-CLA was observed only with t10c12-CLA supplementation (Table 4.7).

**Fatty acid composition of pup liver.**

Compared to dam liver, proportions of 16:0, 18:0 and 20:4 were 2 to 3 fold higher in pup liver, while c9-18:1 was greatly reduced (~50%) (Table 4.8). Total trans-18:1 FA was increased ($P<0.05$) in pup livers nursing dams fed the trans-18:1 isomers (Table 4.9). The CLA isomer profile reflected the respective milk CLA composition. T7c9-CLA represented 76% and 6.5% of the total CLA in liver of pups nursing from mice fed the t7-18:1 and PHVO diets, respectively. The c9t11-CLA was 75% and 53% of the total CLA in pups nursing from mice fed the t11-18:1 or PHVO diets, respectively.

**Gene expression.**

Potential regulatory mechanisms involved in milk fat synthesis were examined by measuring the expression of key lipogenic genes, transcription

regulators and nuclear receptors in mammary and liver tissues using quantitative RT-PCR.

***Expression of mammary lipogenic genes.*** Compared to Controls, the t10c12-CLA treatment decreased the expression of genes involved in FA synthesis (*FASN* (-55%, $P<0.01$), *ACACA* (-45%, $P<0.01$)), triacylglycerol (TAG) formation (*AGPAT* (-34%, $P<0.01$)) and desaturation (*SCD1* (-35%, $P<0.05$), *SCD2* (-35%, $P<0.05$))(Fig 4.1A). The PHVO and t7-18:1 treatments decreased mRNA abundance for *AGPAT* ($P<0.05$), *SCD1* ($P<0.05$) and tended to decrease *FASN* ($P=0.2$) (Fig 4.1A). The *SCD2* was increased by t7-18:1 (53%, $P<0.05$). Mammary lipogenic genes were not affected by the t9- and t11-18:1 FA-supplemented diets, consistent with a lack of change in milk fat content.

***Expression of mammary transcription regulators, nuclear receptors and transcription co-activators*** The t10c12-CLA diet decreased the mRNA abundance of *SREBP-1c* ($P<0.01$), *ChREBP* ($P<0.01$), *THRSP* ($P<0.01$), *PPARA* ($P<0.05$) and *PPARG* ($P<0.05$) (Fig 4.1A and B). PHVO supplementation decreased the expression of *SREBF1* ($P<0.05$), *THRSP* ($P<0.05$) and *PPARA* ($P<0.05$) (Fig 4.1A). Trans 7-18:1 decreased *THRSP* ($P<0.05$) (Fig 4.1A), increased *Mlx* ($P<0.05$) (Fig 4.1A) and tended to increase *INSIG1* (30% $P<0.10$) (Fig 4.1A)and *LXRA* (22%, $P<0.16$) (Fig 4.1A). Expression of *SCAP* was reduced ($P<0.15$) by the t10c12 diet (20%), t7-18:1- (20%) and PHVO- (12%) supplemented diets and was increased ($P<0.05$) by t9-18:1 (Fig 4.1C).

***Expression of hepatic lipogenic genes, transcription regulators, and nuclear receptors.***

Of the lipogenic genes examined in the liver, only *AGPAT* expression was significantly decreased by the supplementation of t10c12-CLA ($P<0.01$), PHVO ($P<0.01$) and t7-18:1 ($P<0.05$) (Fig 4.2A; 4.2B). All diets supplemented with individual (t7-, t9- and t11-) trans-18:1 FA up regulated the expression of *SCD2* ($P<0.05$) and *LPL* ($P<0.05$). In contrast to the mammary gland, the t10c12-CLA-supplemented diet increased *SREBP1* ($P<0.01$) and *PPARA* ($P<0.05$) hepatic gene expression and reduced the expression of *PPARG* ($P<0.01$), and *RXR* ($P<0.05$) (Fig 4.2C). The *PPARA* expression tended to increase with the supplementation of PHVO and t9-18:1 (P=0.23). The t7-18:1 FA increased gene expression of *SREBP1c* ($P<0.01$), *LXRA* ($P<0.01$), and *INSIG1* ($P<0.05$) (Fig 4.2C, 4.2D). Partially hydrogenated vegetable oil decreased the expression of *SCAP* ($P<0.05$).

## DISCUSSION

Analysis of the relationship between individual trans-18:1 isomers and milk fat percentage has shown differences in their relative potency to reduce milk fat content (Kadegowda et al., 2008). Consistent with previous reports (Teter et al., 1990; Gaynor et al., 1995; Romo et al., 1996), it was observed that dietary addition of PHVO, a source of t4 to t16-18:1 isomers, decreased milk fat synthesis in this study. Dietary supplementation of t9- or t11-18:1 did not alter milk fat content and was consistent with earlier reports (Teter et al., 2003; Loor et al., 2003). Using principal component analysis of data from lactating cows fed MFD-diets (Kadegowda et al.,

2008), we hypothesized the potential involvement of trans-7 18:1 isomer in MFD. This is the first study to demonstrate that dietary supplementation of t7-18:1 can indeed reduce milk fat content in mice.

The supplemented trans-18:1 and t10c12-CLA were transferred to milk, causing a concomitant increase in the concentrations of these isomers. The amount of apparent milk trans-18:1 FA was greater in mice fed PHVO or t9-18:1 than that observed in mice fed the t7- or t11-18:1 diets. This was due to the substantial conversion of these isomers to t7c9- and c9t11-CLA via $\Delta^9$ desaturation of the respective trans-18:1 precursors. Experiments with lactating cows where c9t11- and t7c9-CLA were measured in rumen fluid (Corl et al., 2002) or duodenal flow (Piperova et al., 2002) have shown that these isomers were almost exclusively synthesized de novo by the action of $\Delta^9$ desaturase in the mammary gland. To our knowledge, this is the first study to show direct conversion of dietary t7-18:1 to milk t7c9-CLA. The proportion of t7c9-CLA (2.4% FAME) was higher in milk from mice fed t7-18:1 compared to that of c9t11-CLA (1.13% FAME) in mice fed t11-18:1. Considering the proportions of the trans-18:1 precursors and the CLA produced, we estimated that 58% of t7-18:1 and 36% of t11-18:1 were converted to t7c9-CLA and c9t11-CLA, respectively. Conversion efficiencies between 29 and 31% were previously reported for t11-18:1 in lactating cows (Griinari et al., 2000; Shingfiled et al., 2007). The results suggest that, compared to t11-18:1, t7-18:1 might be a preferred $\Delta^9$-desaturase substrate in mammary tissue. Due to the significant synthesis of t7c9-CLA observed in mice fed t7-18:1, we speculated that the effect of t7-18:1 on milk fat synthesis might be mediated through t7c9-CLA. It was previously reported

that the t7c9-CLA and t10c12-CLA were the isomers most negatively correlated to milk fat percentage (Kadegowda et al., 2008).

It is established that MFD is characterized by a reduction of *de novo* synthesized FA (Loor and Herbein, 1998). This reduction in *de novo* FA synthesis is associated with a decrease in mRNA abundance of key lipogenic enzymes in the mammary gland (Baumgard et al., 2001). In the present study, changes in FA <16:0 were observed in milk fat of mice fed t10c12-CLA, PHVO and t7-18:1 diets. The expression of *ACACA* and *FAS* was reduced with the t10c12-CLA diet, and expression of *FAS* tended to decrease in mice fed the PHVO and t7-18:1 diets. The mRNA abundance of genes regulating desaturation (*SCD1*) and TAG formation (*AGPAT*) were also reduced in mice with MFD. *SCD* expression is important for TAG synthesis (Ntambi et al., 2004) and desaturation is reported to be essential for initiation of lipogenesis or TAG synthesis (Sampath et al., 2007). Two isoforms (*SCD1, SCD2*) of *SCD* exist that can desaturate 16:0 and 18:0. Although their physiological functions are not completely elucidated, it is known that *SCD1* is predominantly expressed in liver, while the expression of *SCD2* increases by several folds in the mammary tissue at the onset of lactation (Rudolph et al., 2007).

In this study, the decrease in mRNA abundance of *SCD1* and *SCD2* in mice fed t10c12-CLA and PHVO resulted in reduced 18:1c9 in milk, compared to Control. Yield of 18:1c9 was also reduced by t7-18:1 but not by the other trans-18:1 treatments. Supplementation of t7-18:1 decreased *SCD1* but not *SCD2*, suggesting that the desaturation of t7-18:1 to t7c9-CLA may have been regulated by the *SCD2* gene isoform.The expression of *LPL*, controlls the uptake of LCFA for milk fat

synthesis, was not affected by treatment, suggesting that the changes in milk fat occurred at the level of *de novo* FA synthesis and TAG formation rather than FA uptake. The lack of an effect of diets containing t-9-18:1 or t11-18:1 on the mammary lipogenic gene expression is consistent with their inability to alter milk fat content.

Harvatine and Bauman (2006) suggested that the coordinated suppression of lipogenic genes in mammary tissue during MFD is controlled via a common regulatory mechanism involving the transcription factor SREBP-1c. SREBP-1c is known to regulate at least 30 genes involved in FA and cholesterol metabolism (Horton et al., 2003) including *ACACA*, *FAS*, *SCD* and *LPL* (Shimomura et al., 1998). SREBP-1c, synthesized as a precursor protein, is associated with SCAP and is anchored to the endoplasmic reticulum membrane through INSIG proteins (Horton et al., 2002; Liang et al., 2002). Upon detaching from INSIG proteins, SREBP-1c is escorted by SCAP to golgi and is processed to an active fragment, before translocation to the nucleus to activate the lipogenic genes (Rawson, 2003).

Formation of the active nuclear form of SREBP-1c is predominantly regulated at the level of transcription by insulin (43), LXRA (44), and SREBP-1c (feed-forward regulation) (Horton et al., 2002) and at the level of proteolytic processing of the precursor SREBP protein by co-regulators such as INSIG1 (Engelking et al.,2004; Takaishi et al., 2004) and SCAP (Matsuda et al., 2001). In this study, *SREBP-1c* mRNA abundance was significantly decreased by t10c12-CLA and PHVO. However, these effects were probably not mediated through LXRA as the treatments did not affect the *LXRA* mRNA expression. Decreased *SCAP* mRNA expression in response to feeding t10c12-CLA and PHVO indicated post-transcriptional secondary

regulation of SREBP. Conversely, t7-18:1 treatment tended to increase *SREBP-1c* expression, which correlated with higher *LXRA* mRNA. There was a tendency of the t7-18:1 diet to decrease (20%) *SCAP* mRNA and to increase (30%) *INSIG1* mRNA expression, possibly leading to a reduced synthesis of active *SREBP-1c* form (Takaishi et al., 2004; Matsuda et al., 2001). Consequently, the MFD effects of t7-18:1 might be regulated through the SREBP co-activators SCAP and INSIG1.

Milk fat depression in dairy cows is generally characterized by a 20 to 50% reduction in milk fat content (Baumgard et al., 2001). Post-ruminal infusion of t10c12-CLA (Baumgard et al., 2001) or feeding MFD-diets (Piperova et al., 2000) have not reduced milk fat by more than 50%. Similarly, ~50% reduction in FA synthesis was observed in liver of *SREBP-1c* knockout mice (Liang et al., 2002), indicating that lipogenic gene expression was incompletely suppressed. This suggests a role for other transcription regulators in liver lipogenesis. Recently, a series of studies *in vitro* (Liang et al., 2002) using primary hepatocytes demonstrated that glycolytic and lipogenic gene expression is synergistically regulated by *SREBP-1c* and *ChREBP*. A study with *SREBP1c*$^{-/-}$ knockout mice has confirmed the role of *ChREBP* in coordinated control of glucose metabolism, FA, and TAG synthesis (Lizyka et al., 2004). However, *ChREBP* has to form a heterodimeric complex with another transcription factor, *Mlx,* for induction of the target genes (Stoeckman et al., 2004; Ma et al., 2005). In this study, mammary mRNA expression of *ChREBP* was decreased with the t10c12-CLA but not with the other treatments. This effect on *ChREBP* could partially explain the differences in the degree of MFD observed between t10c12-CLA and the other FA treatments.

The expression of *PPARA* and *PPARG*, known to regulate expression of genes involved in liver and adipose tissue lipid metabolism (Smith, 2002) were measured. *PPARA* is also associated with up regulation of genes involved in FA oxidation (Smith, 2002). In this study, the expression of *PPARA* was decreased with PHVO and t10c12-CLA, indicating that FA oxidation is not a contributor to MFD. The *PPARG* is mostly expressed in adipose tissue and its role in mammary gland is not well understood (Rudolph et al., 2007). It was observed a decrease in *PPARG* mRNA abundance in the mammary tissue with t10c12-CLA but not with the other treatments. Similar effects of t10c12-CLA on *PPARG* gene expression were reported in mature adipocytes (Granlund et al., 2003). These results suggest that the t10c12-CLA effects on FA synthesis could be regulated via similar mechanisms in both mammary and adipose tissues.

*THRSP* is a nuclear protein associated with regulation of *de novo* lipid synthesis in lipogenic tissues (Kinlaw et al., 1995). Although, the exact function of *THRSP* is unknown, it is speculated that it functions as an allosteric regulator of lipogenic genes (Lafave et al., 2006). Zhu et al. (2005) demonstrated that milk TAG content in *THRSP*-null mice was significantly reduced due to a decrease in *de novo* lipid synthesis. Similarly, Harvatine and Bauman (2006) reported that diet-induced MFD and t10c12-CLA treatment down regulated the expression of *THRSP* in mammary gland of lactating cows. It was observed that a decrease in *THRSP* mRNA expression reduction was proportional to the degree of MFD in mice fed t10c12-CLA, PHVO and t7-18:1.

Dietary effects of FA on hepatic lipogenesis during lactation are not well understood. We measured the FA profile and lipogenic gene expression in liver to determine the effects of trans-18:1 FA and t10c12-CLA on hepatic lipid metabolism during lactation. Compared to the proportions found in milk, only 40 to 60% of dietary trans-18:1 and 12% of t10c12-CLA isomers were transferred to liver, suggesting preferential uptake by the lactating mammary tissue. Liver FA content (total FAME per g of liver) was significantly increased with supplementation of t10c12-CLA, PHVO, t7- and t11-18:1 and was associated with an increase in liver weight. Vishwanadha et al. (2006) reported increased liver weight in growing mice fed t10c12-CLA due to accumulation of fat in a dose-dependendent manner. Studies by Takahashi et al. (2003) found that accumulation of TAG in the liver of growing mice fed t10c12-CLA was accompanied by up regulation of *FAS*, *SCD*, *THRSP* and *SREBP-1* gene expression. Others have reported that effects of FA on hepatic lipogenesis depend on the specific CLA isomer (Clement et al., 2002), duration of feeding (Wang et al., 2005) and physiological condition (Lin et al., 2004). Trans 10c12-CLA is known to cause hepatic steatosis, while feeding c9t11-CLA has no effect on liver FA content (Clement et al., 2002). Hepatic steatosis in growing mice was observed during long-term (Vishwanatha et al., 2006, Clement et al., 2002) but not during short-term feeding (Wang et al., 2005) of t10c12-CLA. Dietary t10c12-CLA did not affect liver lipid content or hepatic mRNA abundance of *ACACA*, *FAS* or *SCD* in lactating mice (Lin et al., 2004). We observed increased accumulation of fat without changes in the mRNA expression of major hepatic lipogenic genes, *FASN*, *ACACA*, *SCD1*, and *THRSP* in mice exhibiting MFD. However, *AGPAT* mRNA

abundance was reduced by the PHVO, t10c12-CLA and t7-18:1 treatments, indicating decreased hepatic TAG synthesis. This suggests that the FA delivered by the blood TAG-rich lipoproteins were mostly responsible for the hepatic FA accumulation as reported by Degrace et al. (2003) in mice fed t10c12-CLA. There was a shift in the proportions of major FA with a substantial increase in 16:0 and decrease in 18:0 in mice with expressed MFD. Similar changes in FA composition due to increased uptake of plasma NEFA were reported in postpartum cows with fatty liver (Rukkwamsuk et al., 1999). The decrease in liver 18:0 content could be related to an increased FA oxidation, due to the up regulation of *PPARA* expression.

All diets containing trans-18:1 FA increased expression of liver *LPL* controlling the FA uptake. About 80% of t7-18:1 and 45% of t11-18:1 transferred to liver was converted to t7c9-CLA and c9t11-CLA, respectively. To our knowledge, this is the first study to demonstrate direct conversion of t7-18:1 to t7c9-CLA in hepatic tissue. Compared to mammary gland, the conversion efficiency of the trans-18:1 precursors to CLA isomers was greater in liver, most likely due to the higher expression of hepatic *SCD1* or increased *SCD2* mRNA abundance in mice fed the trans-18:1.

In contrast to the lipogenic genes, *SREBP-1c* expression was significantly up regulated in liver of mice fed t10c12-CLA and t7-18:1 diets. However, the *INSIG1* expression was concomitantly increased, which could lead to decreased synthesis of active *SREBP-1c,* and lack of response in the hepatic lipogenic genes. Hence, the FA effects on the hepatic lipogenesis could be mediated through *INSIG1*.

Development of offspring is affected by the maternal diet. Analysis of pup liver fat content and FA composition in this study showed that the supplemented FA were taken up by the nursing pups. The growth rate of pups nursing mothers with MFD was reduced presumably due to decreased energy intake. Decrease in growth rate was reported by others (Ringseis et al., 2004; Lin et al, 2004) for pups nursing from mothers fed t10c12-CLA. Loor et al. (2003) reported a 28% decrease in body weight of pup litters nursing from dams fed t10c12-CLA.

In conclusion, MFD was observed in mice fed t10c12-CLA, PHVO and t7-18:1 diets. The t-7-18:1 isomer was extensively converted to t7c9-CLA in mammary and liver tissues of mice receiving the t7-18:1 treatment. Our data suggests that the MFD effects of t7-18:1 could have been mediated through t7c9-CLA. All MFD treatments altered milk fat at the level of *de novo* FA synthesis and TAG formation. The differences in the milk fat responses due to dietary FA could be related to their effects on multiple transcriptional regulators in the mammary gland. In addition to *SREBP-1C*, this study established involvement of *ChREBP*, *THRSP*, and *PPARG*, supporting involvement of multiple transcriptional regulators in the regulation of milk fat synthesis. Lastly, the effects of FA on lipogenic gene expression were more pronounced in the mammary tissue than in liver.

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Table 4.1. Fatty acid composition of the diets fed to lactating mice

| | TREATMENT | | | | | |
|---|---|---|---|---|---|---|
| Fatty Acid | Control | PHVO | t10c12 CLA | t7 18:1 | t9-18:1 | t11-18:1 |
| | ----------g/100g FAME---------- | | | | | |
| 14:0 | 0.4 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 |
| 16:0 | 11.9 | 8.8 | 12.0 | 11.3 | 11.6 | 11.4 |
| 16:1 cis | 0.4 | 0.1 | 0.4 | 0.4 | 0.4 | 0.4 |
| 18:0 | 7.3 | 6.8 | 6.8 | 7.1 | 7.6 | 7.1 |
| 18:1 cis-9 | 57.7 | 47.6 | 51.9 | 38.0 | 38.2 | 37.5 |
| 18:1 cis-11 | 0.9 | 1.8 | 1.0 | 1.0 | 0.9 | 1.0 |
| Trans-18:1 isomer: | | | | | | |
| 6+7+8 | ND[1] | 0.9 | 0.1 | 21.1 | ND | ND |
| 9 | 0.1 | 7.6 | 0.1 | ND | 19.9 | ND |
| 10 | ND | 1.5 | ND | ND | ND | ND |
| 11 | ND | 1.4 | ND | ND | ND | 20.8 |
| 12 | ND | 0.8 | ND | ND | ND | ND |
| 13+14 | ND | 0.9 | ND | ND | ND | ND |
| 15 | ND | 0.5 | ND | ND | ND | ND |
| 16 | ND | TR[2] | ND | ND | ND | ND |
| 18:2n-6 | 18.7 | 18.0 | 19.5 | 19.0 | 19.1 | 19.3 |
| 18:2i | 0.1 | 0.9 | 0.1 | 0.1 | 0.1 | 0.1 |
| 18:3n-3 | 0.6 | 0.9 | 0.3 | 0.6 | 0.6 | 0.5 |
| 20:0 | 0.3 | 0.3 | 0.5 | 0.5 | 0.0 | 0.5 |
| 20:1cis-11 | 0.2 | 0.1 | 0.3 | 0.2 | 0.2 | 0.2 |
| 22:0 | 0.2 | 0.3 | 0.2 | 0.0 | 0.2 | 0.1 |
| cis-9,trans-11CLA | 0.1 | TR | 0.2 | TR | TR | 0.1 |
| Trans-10, cis-12 CLA | TR | TR | 6.1 | TR | TR | TR |
| Other | 1.2 | 0.4 | 0.4 | 0.4 | 0.9 | 0.4 |

[1] ND – not detected

[2] TR – traces <0.1 g/100g FAME

Table 4.2. Summary of gene targets evaluated by quantitative real-time RT-PCR

| Gene | Fragment Size (bp) | Sense Primer (5′→3′) | Antisense Primer (5′→3′) | Annealing temperature (°C) | PCR Efficiency (%) | | Correlation Coefficient ( r ) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Mam[1] | Liver | Mam | Liver |
| *FASN* | 106 | ACCTCTCCCAGGTGTGTGAC | TGGATGATGTTGATGATGGA | 58.9 | 90.8 | 91.3 | 0.999 | 0.996 |
| *ACACA* | 106 | GAAAATCCACAATGCCAACC | GTCCCAGACGTAAGCCTTCA | 58.9 | 97.7 | 101.5 | 0.999 | 0.995 |
| *AGPAT* | 125 | TTGTCTGTTGCGTTCTGAGG | GCCTGTGAGGAGTGTCAGGT | 63.3 | 88.0 | 85.5 | 1.000 | 0.999 |
| *SCD1* | 124 | TCCAGTGAGGTGGTGTGAAA | TTATCTCTGGGGTGGGTTTG | 63.3 | 97.1 | 84.8 | 0.999 | 0.999 |
| *SCD2* | 109 | TGTGCCTATGTCCCAGATGA | GAAACGGAGTCCTGCATGTT | 63.3 | 90.8 | 84.8 | 0.999 | 0.998 |
| *LPL* | 119 | AGCCCTTGCTAGGAGAAAGC | GGGATGCCGGTAACAAATTA | 58.9 | 84.6 | 85.1 | 1.000 | 1.000 |
| *THRSP* | 116 | AGCAAACACGGAATCCAAAG | CTTTCCCCAGGGATAAAAGC | 58.9 | 98.0 | 88.1 | 0.995 | 0.998 |
| *SREBP1* | 103 | GTGAGCCTGACAAGCAATCA | GGTGCCTACAGAGCAAGAGG | 63.3 | 85.7 | 85.5 | 0.994 | 1.000 |
| *ChREBP* | 119 | TCCTGCACTGTTGCTAATGC | TCCTGTTTAGGTCCCCAGTG | 63.3 | 85.9 | 86.2 | 0.997 | 0.999 |
| *PPARA* | 117 | ACTTCGCTATCCAGGCAGAA | CAGACCAACCAAGTGTTGTGA | 58.9 | 85.0 | 92.6 | 1.000 | 0.996 |
| *PPARG* | 94 | TGCAGCTCAAGCTGAATCAC | ACGTGCTCTGTGACGATCTG | 58.9 | 93.2 | 94.1 | 0.999 | 0.999 |
| *LXRA* | 108 | GATCTGGGATGTCCACGAGT | GTCTGCCCTTCTCAGTCTGC | 63.3 | 97.0 | 85.1 | 1.000 | 0.999 |
| *RXR* | 107 | TCCTTGGGAGGGTCTTCTCT | GGGCAGGTAGCAACACAGAT | 63.3 | 98.2 | 81.4 | 1.000 | 1.000 |
| *SCAP* | 106 | TCAGCCAAACATTTGCTCAC | CTGCGGTCCCAGATACTGAT | 58.9 | 89.7 | 85.1 | 0.998 | 1.000 |
| *INSIG1* | 105 | TGAGTCGCTGTCTGCTGTTT | TCACAGATTGCAAGCTCCAC | 63.3 | 89.2 | 86.0 | 0.999 | 1.000 |
| *Mlx* | 116 | AGTCTGTTCAGGGAGCCTCA | GAGACCAAGCAGAGGCAAAC | 63.3 | 85.4 | 90.0 | 0.999 | 0.999 |

[1]Mam - Mammary tissue

Table 4.3. Effects of fatty acid supplementation on milk fat, milk volume, feed intake, body and tissue weight

| | TREATMENT | | | | | | |
|---|---|---|---|---|---|---|---|
| Item | Control | PHVO | t10c12 CLA | t7-18:1 | t9-18:1 | t11-18:1 | SEM |
| Dam | | | | | | | |
| Milk fat (wt %) | 22.55 | 17.43** | 13.28** | 16.70* | 22.70 | 20.45 | 0.96 |
| Milk production[1] (g) | 3.51 | 3.23 | 2.12 | 2.69 | 2.73 | 3.60 | 0.53 |
| Body wt (g) | 26.00 | 27.24 | 25.86 | 26.93 | 26.75 | 25.94 | 0.44 |
| Feed intake (g) | 6.98 | 6.53 | 5.35* | 7.54 | 6.77 | 6.81 | 0.55 |
| Liver wt (% body wt) | 6.30 | 7.03 | 8.99** | 7.49 | 5.62 | 6.54 | 0.58 |
| Mammary tissue (g) | 1.23 | 1.63 | 1.41 | 2.00** | 1.38 | 1.35 | 0.18 |
| Pups | | | | | | | |
| n/litter | 6 | 6 | 7 | 7 | 7 | 6 | |
| Avg. pup wt (g) | 4.14 | 4.24 | 4.14 | 4.42 | 3.83 | 4.39 | 0.28 |
| Pup growth rate (g/d) | 0.31 | 0.25 | 0.16** | 0.21* | 0.21* | 0.27 | 0.03 |
| Litter liver tissue (g) | 0.27 | 0.40 | 0.43 | 0.42 | 0.32 | 0.47 | 0.09 |

[1] Calculated value

* * $P<0.01$

* $P<0.05$

Table 4.4. Concentrations of fatty acids in milk of mice fed trans-18:1 FA or t10c12-CLA

| | TREATMENT | | | | | | |
|---|---|---|---|---|---|---|---|
| Fatty Acid | Control | PHVO | t10c12 CLA | t7-18:1 | t9-18:1 | t11-18:1 | SEM |
| | g/100g FAME[1] | | | | | | |
| 10:0 | 2.69 | 1.44 | 1.64 | 2.26 | 2.21 | 2.06 | 0.60 |
| 11:0 | 0.15 | 0.08 | 0.04 | 0.16 | 0.13 | 0.17 | 0.04 |
| 12:0 | 8.30 | 6.72 | 5.48** | 6.98 | 7.74 | 8.34 | 0.61 |
| 12:1 | 0.10 | 0.09 | 0.01** | 0.09 | 0.10 | 0.12 | 0.03 |
| 14:0 | 12.69 | 10.54 | 6.99** | 10.71 | 12.37 | 13.58 | 0.65 |
| 14:1 | 0.23 | 0.23 | 0.08** | 0.21 | 0.23 | 0.17 | 0.03 |
| 16:0 | 22.43 | 19.58* | 17.99** | 22.45 | 23.60 | 23.67 | 0.64 |
| 16:1 trans | 0.48 | 0.54 | 0.76** | 0.35 | 0.44 | 0.41 | 0.05 |
| 16:1 | 2.54 | 3.23 | 2.24 | 3.16 | 2.93 | 2.96 | 0.37 |
| 18:0 | 1.84 | 1.90 | 3.08** | 1.94 | 1.85 | 1.76 | 0.18 |
| 18:1 cis-9 | 35.38 | 36.04 | 3.94** | 32.28 | 31.67 | 29.08* | 1.36 |
| 18: 1cis-11 | 2.03 | 2.84** | 2.03 | 2.57 | 2.24 | 2.29 | 0.17 |
| Total trans-18:1 | 0.02 | 3.67** | ND[2] | 1.56 | 3.07** | 1.78 | 0.46 |
| 18:2 n-6 | 7.16 | 7.76 | 9.88** | 8.73 | 7.88 | 7.83 | 0.37 |
| Total CLA | 0.23 | 0.65 | 1.82** | 2.41** | 0.22 | 1.13** | 0.12 |
| 18:3 n-3 | 0.05 | 0.04 | 0.06 | 0.01 | 0.02 | 0.04 | 0.03 |
| 20:0 | 0.02 | ND | 0.13 | ND | ND | ND | 0.03 |
| 20:1 | 1.48 | 1.51 | 1.41 | 1.73 | 1.30 | 1.54 | 0.18 |
| 20:2 | 0.58 | 0.72 | 0.63 | 1.04 | 0.71 | 0.92 | 0.11 |
| 20:3 | 0.59 | 0.51 | 0.38 | 0.29 | 0.43 | 0.57 | 0.08 |
| 20:4 | 0.54 | 0.78 | 1.32 | 0.53 | 0.40 | 0.68 | 0.10 |
| 22:1 | 0.19 | 0.19 | 0.15 | 0.27 | 0.13 | 0.18 | 0.04 |
| 22:4 | 0.27 | 0.29 | 0.26 | 0.10 | 0.10 | 0.70 | 0.16 |
| Other | 0.13 | 0.11 | 0.27 | 0.04 | 0.13 | 0.08 | 0.06 |
| Summary: | | | | | | | |
| <16:0 | 24.18 | 19.16 | 14.26** | 20.36 | 22.74 | 24.39 | 1.58 |
| 16:0 | 22.42 | 19.57* | 17.99** | 22.44 | 23.60 | 23.62 | 0.62 |
| >16:0 | 50.48 | 57.74* | 66.68** | 53.41 | 50.18 | 48.00 | 1.67 |
| MUFA[3] | 40.26 | 42.53 | 48.86** | 38.21 | 37.08 | 34.52 | 1.54 |

[1] FAME - Fatty acid methyl esters
[2] ND - Not detected
[3] MUFA - Mono unsaturated fatty acids
* * $P<0.01$
* $P<0.05$

Table 4.5. Concentrations of trans-18:1 and CLA isomers in milk fat of lactating mice fed trans-18:1 FA or t10c12-CLA

| | TREATMENT | | | | | | |
|---|---|---|---|---|---|---|---|
| Fatty Acid | Control | PHVO | t10c12 CLA | t7-18:1 | t9-18:1 | t11-18:1 | SEM |
| | g/100g FAME[1] | | | | | | |
| Total 18:1 trans | 0.02 | 3.68** | ND[2] | 1.56* | 3.07** | 1.78* | 0.46 |
| Trans 18:1 isomer: | | | | | | | |
| 6+7+8 | ND | 0.47* | ND | 1.56** | ND | ND | 0.12 |
| 9 | 0.02 | 0.85** | ND | ND | 3.07** | ND | 0.13 |
| 10 | ND | 1.13** | ND | ND | ND | ND | 0.12 |
| 11 | ND | 0.35* | ND | ND | ND | 1.78** | 0.15 |
| 12 | ND | 0.48** | ND | ND | ND | ND | 0.05 |
| 13+14 | ND | 0.40** | ND | ND | ND | ND | 0.06 |
| Total CLA | 0.24 | 0.78** | 1.91** | 2.38** | 0.22 | 1.13** | 0.12 |
| CLA isomer: | | | | | | | |
| cis-11, trans-13 | 0.02 | 0.10 | 0.01 | 0.02 | 0.01 | 0.11 | 0.02 |
| trans-10, cis-12 | ND | ND | 1.42 | ND | ND | ND | 0.09 |
| cis-9, trans-11 | 0.19 | 0.48** | 0.21 | 0.16 | 0.19 | 1.01** | 0.06 |
| trans-8, cis-10 | TR[3] | 0.01 | 0.08* | ND | 0.01 | 0.01 | 0.01 |
| trans-7, cis-9 | ND | 0.08 | ND | 2.17** | ND | ND | 0.02 |
| trans, trans CLA | 0.01 | 0.04 | 0.16* | 0.01 | 0.06 | 0.04 | 0.02 |
| cis, cis CLA | TR | 0.02 | 0.03* | 0.03 | TR | 0.01 | 0.01 |

[1] FAME – Fatty acid methyl ester
[2] ND - Not detected
[3] TR- traces (<0.01)
* * $P<0.01$
* $P<0.05$

Table 4.6. Concentrations of fatty acids in liver of mice fed trans-18:1 FA or t10c12-CLA

| | TREATMENT | | | | | | |
|---|---|---|---|---|---|---|---|
| Fatty Acid | Control | PHVO | t10c12 CLA | t7-18:1 | t9-18:1 | t11-18:1 | SEM |
| Total FAME/liver, dry, (g) | 0.26 | 0.35** | 0.47** | 0.36* | 0.30 | 0.33* | 0.03 |
| | ---------- g/100g FAME[1] ---------- | | | | | | |
| 14:0 | 0.54 | 0.55 | 0.74** | 0.64* | 0.62* | 0.55 | 0.03 |
| 16:0 | 12.88 | 13.44 | 21.51** | 17.01** | 15.27* | 13.87 | 0.68 |
| 16:1 | 1.47 | 1.92 | 2.80** | 2.10 | 2.05 | 1.85 | 0.23 |
| 16:1t | 0.02 | 0.01 | 0.02 | 0.03 | 0.02 | 0.02 | 0.00 |
| 17:0 | 0.08 | 0.06 | 0.06 | 0.01 | 0.09 | 0.09 | 0.01 |
| 17:1 | 0.13 | 0.12 | 0.11 | 0.14 | 0.12 | 0.10 | 0.01 |
| 18:0 | 6.54 | 5.04** | 3.67** | 4.57** | 5.63 | 5.10** | 0.37 |
| 18:1 cis-9 | 55.15 | 55.37 | 54.01 | 54.00 | 51.18 | 54.32 | 1.74 |
| 18:1 cis-11 | 3.90 | 4.07 | 3.72 | 4.27 | 3.79 | 3.79 | 0.27 |
| Total trans-18:1 | 0.28 | 1.56** | 0.28 | 0.45 | 2.09** | 0.95** | 0.14 |
| 18:2 | 7.14 | 6.53 | 6.00 | 7.87 | 8.68 | 8.29 | 0.85 |
| Total CLA | 0.56 | 0.57 | 0.54 | 1.50** | 0.60 | 1.35** | 0.06 |
| GLA[2] | 0.20 | 0.16 | 0.15 | 0.09 | 0.24 | 0.26 | 0.04 |
| 18:3 | 1.51 | 1.36 | 1.13* | 1.58 | 1.37 | 1.39 | 0.07 |
| 20:0 | 0.06 | 0.04 | 0.04 | 0.04 | 0.06 | 0.03 | 0.01 |
| 20:2 | 0.19 | 0.07 | 0.08* | 0.11 | 0.21 | 0.16 | 0.04 |
| 20:4 | 5.11 | 3.61* | 1.91** | 2.63** | 4.30 | 4.01 | 0.42 |
| 22:0 | 0.77 | 0.48* | 0.26** | 0.33** | 0.83 | 0.66 | 0.09 |
| 24:0 | 0.11 | 0.11 | 0.06 | 0.08 | 0.17 | 0.14 | 0.04 |
| Other | 3.40 | 4.73* | 3.00 | 2.75 | 2.81 | 3.25 | 0.27 |

[1] FAME - Fatty acid methyl ester
[2] GLA - Gamma linoleic acid
* * $P<0.01$
* $P<0.05$

Table 4.7. Concentrations of trans-18:1 and CLA isomers in liver fatty acids of lactating mice fed trans-18:1 or t10c12-CLA.

| | TREATMENT | | | | | | |
|---|---|---|---|---|---|---|---|
| Fatty Acid | Control | PHVO | t10c12-CLA | t7 18:1 | t9 18:1 | t11-18:1 | SEM |
| | g/100g FAME[1] | | | | | | |
| Total trans-18:1 | 0.28 | 1.56** | 0.28 | 0.45 | 2.09** | 0.95** | 0.14 |
| Trans 18:1 isomer: | | | | | | | |
| 6+7+8 | ND[2] | 0.05 | | 0.27 | | | |
| 9 | 0.22 | 0.28 | 0.13 | 0.08 | 1.94** | 0.11 | 0.09 |
| 10 | | 0.62 | | | | | 0.05 |
| 11 | 0.03 | 0.14** | 0.12 | 0.02 | 0.14 | 0.80** | 0.14 |
| 12 | | 0.21 | | | | | |
| 13+14 | | 0.13 | | | | | |
| 16 | 0.02 | 0.13* | 0.02 | 0.08 | 0.02 | 0.04 | 0.03 |
| Total CLA | 0.56 | 0.57 | 0.54 | 1.50** | 0.60 | 1.35** | 0.06 |
| CLA isomer: | | | | | | | |
| trans-7, cis-9 | | 0.02 | | 1.00 | | | 0.15 |
| cis-9, trans-11 | 0.04 | 0.25** | 0.07 | 0.08 | 0.03 | 0.64** | 0.04 |
| trans-10, cis-12 | | | 0.20** | | | | 0.01 |
| cis-11, trans-13 | 0.07 | 0.04 | 0.06 | 0.15 | 0.03* | 0.06 | 0.02 |
| trans-11, trans-13 | 0.08 | 0.01 | 0.12 | 0.05 | 0.26 | 0.15 | 0.06 |
| cis, cis CLA | 0.36 | 0.24 | 0.12** | 0.24 | 0.27 | 0.36 | 0.07 |

[1] FAME - Fatty acid methyl ester
[2] ND – Not detected
* * $P<0.01$
* $P<0.05$

Table 4.8. Concentrations of fatty acids in liver tissues of pups nursing dams fed trans-18:1 FA or t10c12-CLA

| | TREATMENT | | | | | | |
|---|---|---|---|---|---|---|---|
| Fatty Acid | Control | PHVO | t10c12 CLA | t7-18:1 | t9-18:1 | t11-18:1 | SEM |
| | ---------- g/100g FAME[1] ---------- | | | | | | |
| Total FAME/liver (g), dry | 0.17 | 0.14 | 0.11 | 0.13 | 0.18 | 0.14 | 0.02 |
| 14:0 | 2.99 | 2.17 | 0.93** | 2.09 | 2.68 | 2.28 | 0.33 |
| 16:0 | 23.30 | 21.72 | 22.02 | 24.04 | 23.31 | 24.59 | 0.90 |
| 16:1 | 0.13 | 0.98 | 0.75* | 1.08 | 1.19 | 1.02 | 0.12 |
| 16:1t | 0.08 | 0.03 | 0.07 | 0.06 | 0.07 | 0.06 | 0.02 |
| 17:0 | 0.09 | 0.09 | 0.17** | 0.12 | 0.10 | 0.12 | 0.01 |
| 17:1 | 0.08 | 0.10 | 0.16* | 0.09 | 0.11 | 0.09 | 0.02 |
| 18:0 | 11.25 | 10.87 | 15.76* | 12.93 | 10.90 | 12.86 | 1.38 |
| 18:1 cis-9 | 27.94 | 24.71 | 21.36 | 22.15 | 24.16 | 19.91 | 3.21 |
| 18:1 cis-11 | 2.15 | 2.15 | 1.85* | 2.17 | 2.25 | 2.08 | 0.10 |
| Total trans-18:1 | 0.10 | 3.47** | 0.23 | 0.67* | 2.37** | 1.42** | 0.16 |
| 18:2 | 11.89 | 12.91 | 12.77 | 14.51** | 13.04* | 13.06* | 0.32 |
| Total CLA | 0.73 | 0.30 | 0.71 | 0.52 | 0.59 | 0.53 | 0.09 |
| GLA[2] | 0.31 | 0.32 | 0.21 | 0.26 | 0.36 | 0.34 | 0.05 |
| 18:3 | 0.53 | 0.47 | 0.46 | 0.54 | 0.51 | 0.48 | 0.05 |
| 20:0 | 0.25 | 0.05** | 0.56* | 0.03** | 0.02** | 0.02** | 0.04 |
| 20:2 | 0.48 | 0.57 | 0.40 | 0.61 | 0.44 | 0.54 | 0.08 |
| 20:4 | 13.93 | 14.66 | 19.27 | 14.92 | 14.42 | 16.57 | 1.81 |
| 22:0 | 0.32 | 0.39 | 0.41 | 0.27 | 0.32 | 0.22 | 0.07 |
| 24:0 | 0.35 | 0.53 | 0.29 | 0.09 | 0.26 | 0.55 | 0.23 |
| Others | 2.72 | 4.03 | 3.06 | 2.88 | 3.06 | 3.27 | 0.35 |

[1] FAME - Fatty acid methyl ester

[2] GLA - Gamma linoleic acid

* * $P<0.01$

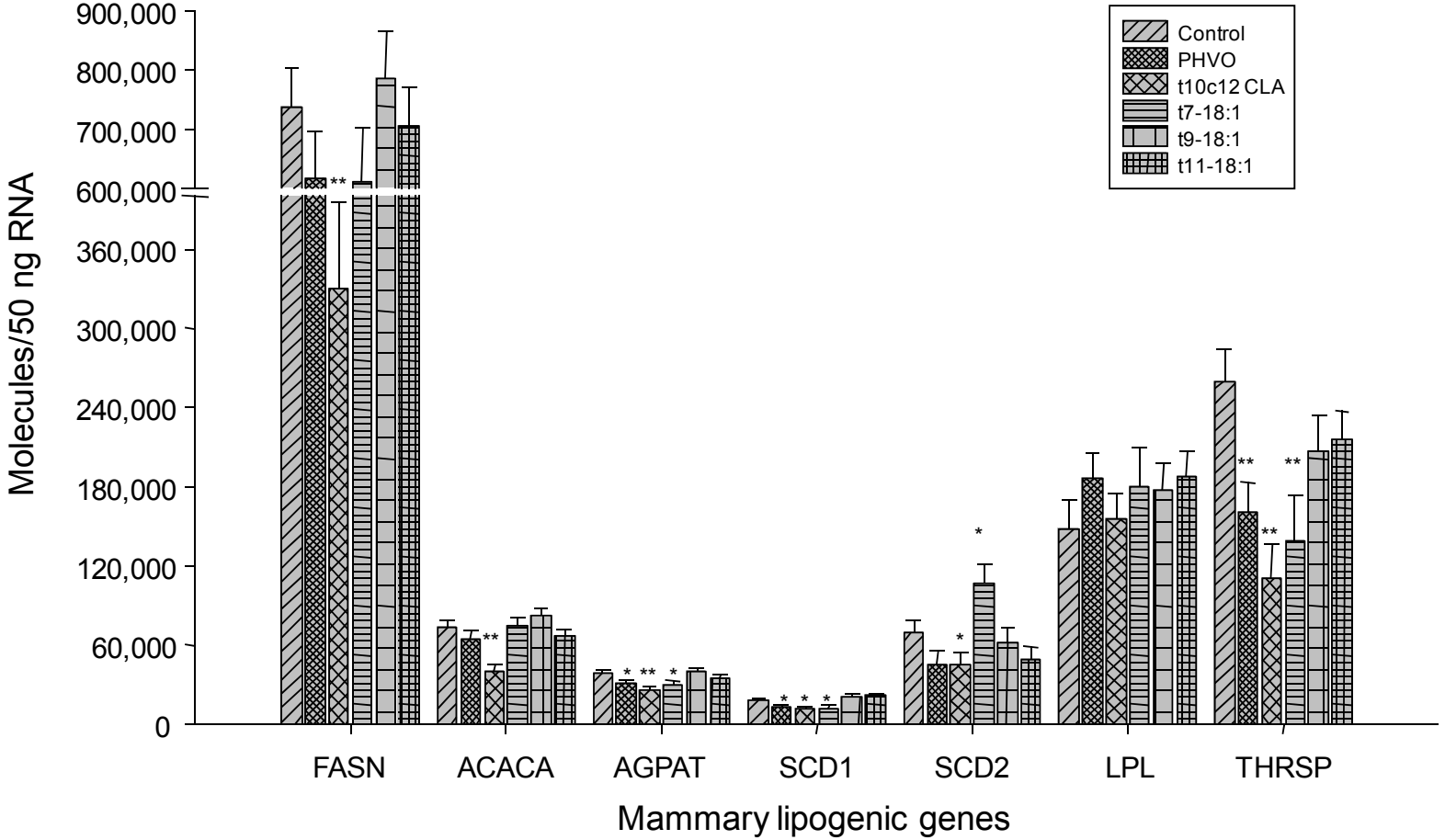
* $P<0.05$

Table 4.9. Concentrations of trans-18:1 and CLA isomers in liver of pups nursing dams fed trans-18:1 FA or t10c12-CLA.

| | TREATMENTS | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | PHVO | t10c12-CLA | t7-18:1 | t9-18:1 | t11-18:1 | SEM |
| | g/100g FAME[1] | | | | | | |
| Total trans-18:1 | 0.10 | 3.47** | 0.23 | 0.67* | 2.37** | 1.42** | 0.16 |
| Trans isomer: | | | | | | | |
| 5 | ND[2] | 0.03 | ND | ND | ND | ND | 0.01 |
| 6+7+8 | ND | 0.15** | ND | 0.49** | ND | ND | 0.06 |
| 9 | 0.07 | 0.63** | 0.12 | 0.09 | 2.30** | 0.08 | 0.11 |
| 10 | ND | 0.73 | 0.03 | ND | ND | ND | 0.06 |
| 11 | 0.03 | 0.59** | 0.05 | 0.05 | 0.04 | 1.33** | 0.05 |
| 12 | ND | 0.69 | ND | ND | ND | ND | 0.07 |
| 13+14 | ND | 0.58 | ND | ND | ND | ND | 0.08 |
| 16 | ND | 0.08 | 0.03 | 0.04 | 0.04 | 0.02 | 0.01 |
| Total CLA | 0.73 | 0.30 | 0.71 | 0.52 | 0.59 | 0.53 | 0.09 |
| CLA isomer: | | | | | | | |
| trans-7, cis 9 | ND | 0.02** | ND | 0.40** | ND | ND | 0.14 |
| cis-9, trans-11 | 0.04 | 0.16** | 0.05 | 0.06 | 0.03 | 0.40** | 0.03 |
| trans-10, cis-12 | ND | ND | 0.24** | ND | ND | ND | 0.04 |
| cis-11, trans-13 | ND | ND | 0.24** | ND | 0.03 | 0.04 | 0.04 |
| trans-11, trans-13 | 0.11 | 0.09 | 0.06 | 0.05 | 0.12 | 0.09 | 0.02 |
| cis ,cis CLA | 0.54 | 0.03* | 0.11 | 0.02 | 0.36 | 0.03 | 0.12 |

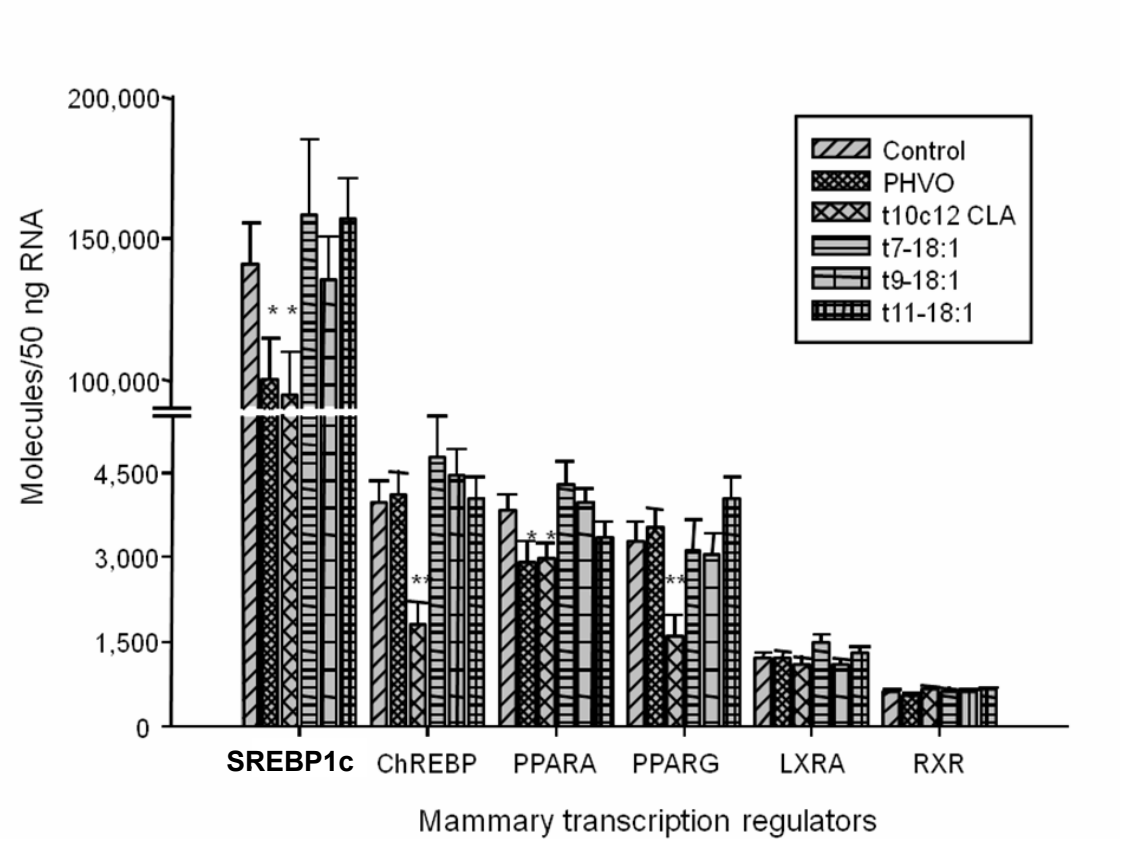
[1] FAME - Fatty acid methyl ester
[2] ND – Not detected
* * $P<0.01$
* $P<0.05$

Figure 4.1A. Effects of trans18:1 FA and t10c12 CLA on the expression of mammary *FASN*, *ACACA*, *AGPAT*, *SCD1*, *SCD2*, *LPL* and *THRSP*. Values are means±S.E, n=7 for control, 3 for t7-18:1 and 5 for PHVO, t9- and t11-18:1 treatments. Values are molecules/50ng of RNA. Asterisks indicate differences from Control. * $P<0.05$ and ** $P<0.01$.



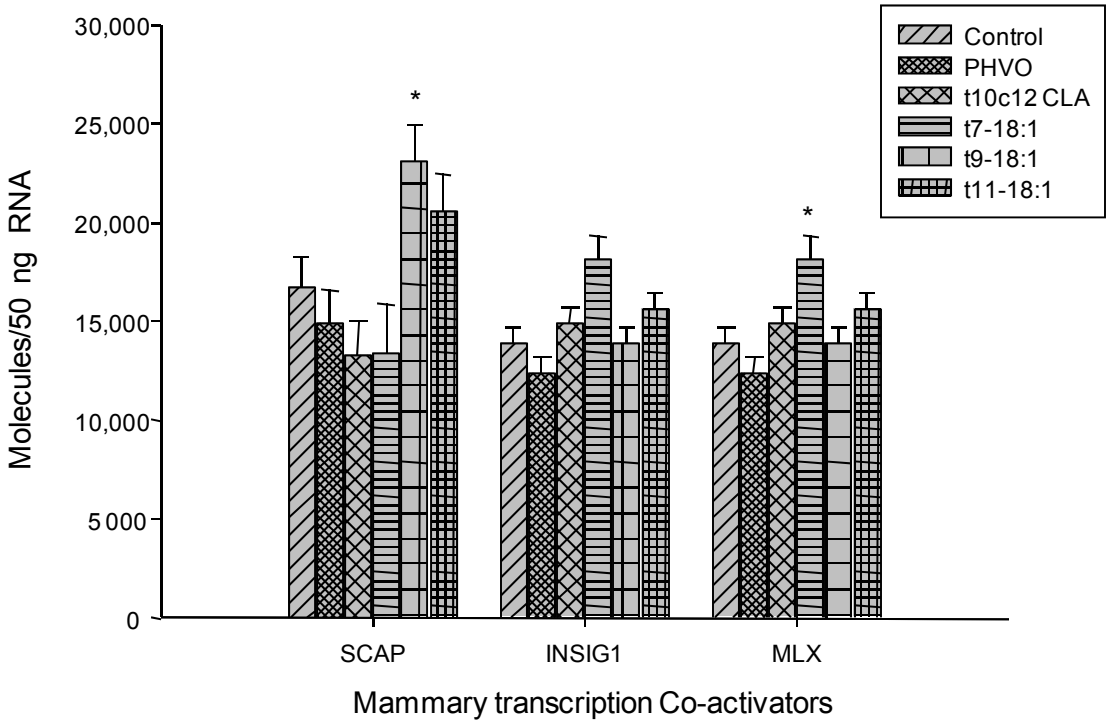

* * $P<0.01$
* $P<0.05$

Figure 4.1B. Effects of trans18:1 FA and t10c12 CLA on the expression of mammary mammary transcriptional regulators *SREBP1*, *ChREBP*, *PPARA*, *PPARG*, *LXRA* and *RXR* . Values are means±S.E, n=7 for control, 3 for t7-18:1 and 5 for PHVO, t9- and t11-18:1 treatments. Values are molecules/50ng of RNA. Asterisks indicate differences from Control. * $P<0.05$, and ** $P<0.01$.




* * $P<0.01$
*$P<0.05$

Figure 4.1C. Effects of trans18:1 FA and t10c12 CLA on the expression of mammary and transcriptional co-activators *SCAP*, *INSIG1* and *Mlx*. Values are means±S.E, n=7 for control, 3 for t7-18:1 and 5 for PHVO, t9- and t11-18:1 treatments. Values are molecules/50ng of RNA. Asterisks indicate differences from Control. * $P<0.05$, and ** $P<0.01$.




* $P<0.05$

Figure 4.2A. Effects of trans18:1 FA and t10c12 CLA on the expression of hepatic *FASN*, *ACACA*, *SCD1*,and *THRSP*. Values are means±S.E, Control n=7, t7-18:1 n=3 and PHVO, t9- and t11-18:1 n=5. Values are molecules/50ng of RNA. Asterisks indicate differences from Control. **P*<0.05, and ***P*<0.01.

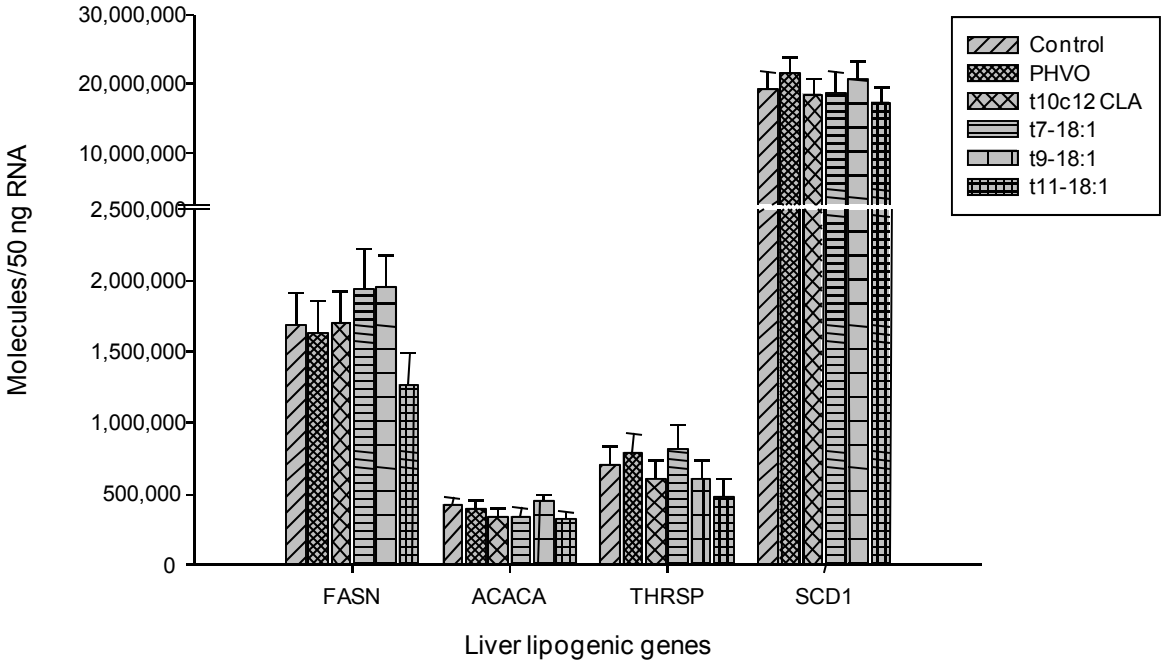
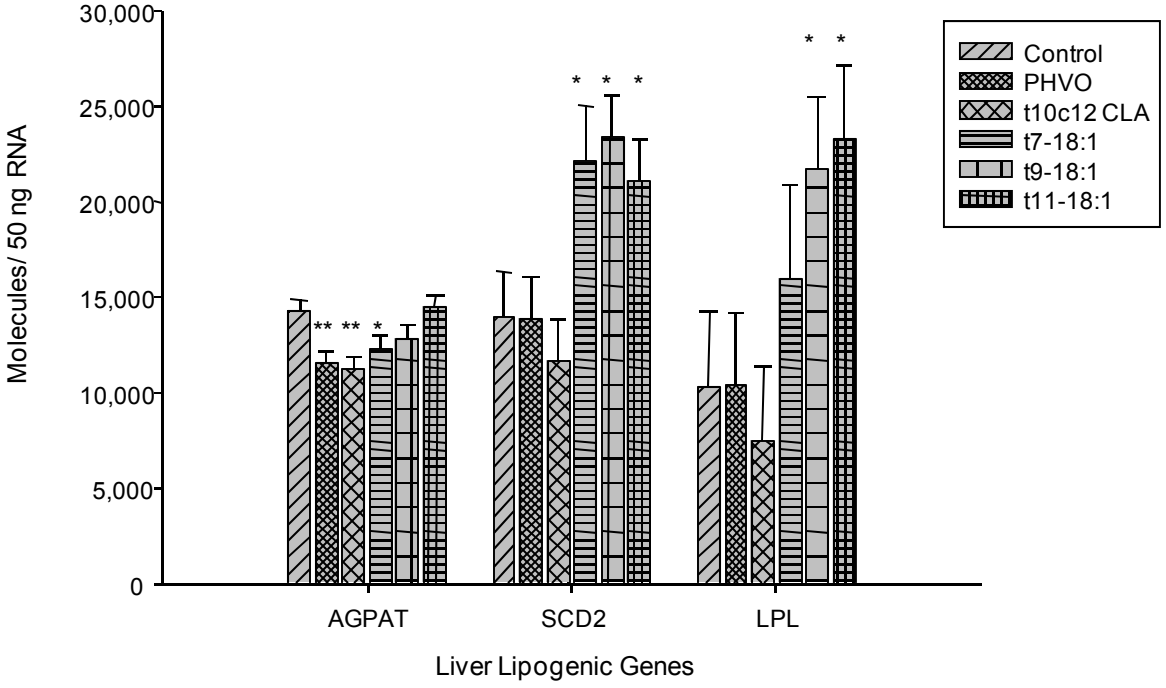
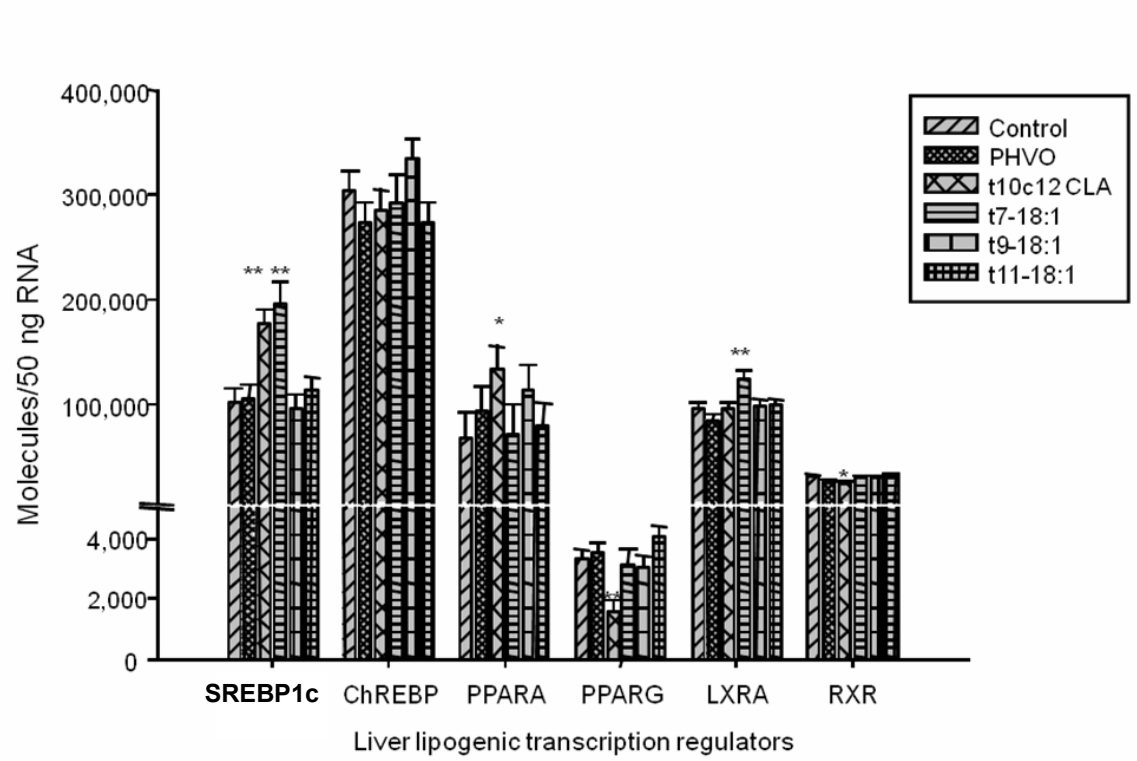


Figure 4.2B. Effects of trans18:1 FA and t10c12 CLA on the expression of *AGPAT*, *SCD2* and *LPL* in liver. Values are means±S.E, Control n=7, t7-18:1 n=3 and PHVO, t9- and t11-18:1 n=5. Values are molecules/50ng of RNA. Asterisks indicate differences from Control. **P*<0.05, and ***P*<0.01.



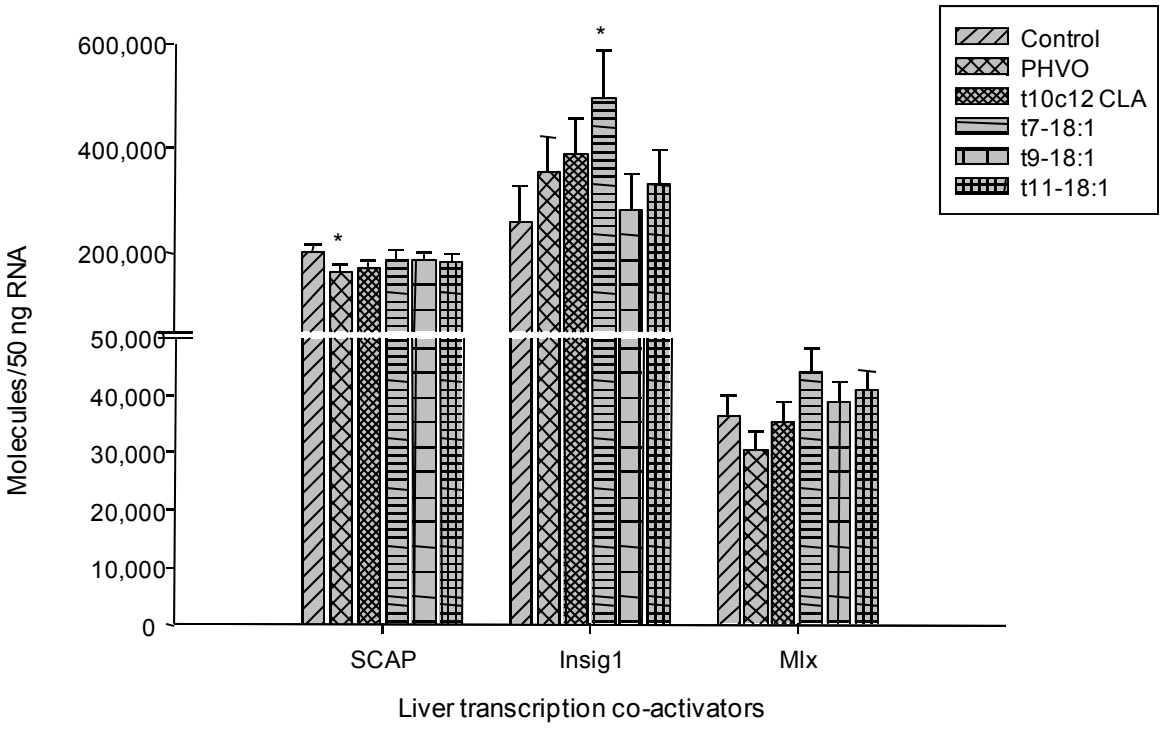

* * *P*<0.01
* *P*<0.05

Figure 4.2C. Effects of trans18:1 FA and t10c12 CLA on the expression of hepatic transcriptional regulators- *SREBP1*, *ChREBP*, *PPARA*, *PPARG*, *LXRA* and *RXR* . Values are means±S.E, Control n=7, t7-18:1 n=3 and PHVO, t9- and t11-18:1 n=5. Values are molecules/50ng of RNA. Asterisks indicate differences from Control. **P*<0.05, and ***P*<0.01.




* * *P*<0.01
**P*<0.05

Figure 4.2D. Effects of trans18:1 FA and t10c12 CLA on hepatic expression of transcriptional co-activators *SCAP*, *INSIG1*, *Mlx*. Values are means±S.E, Control n=7, t7-18:1 n=3 and PHVO, t9- and t11-18:1 n=5. Values are molecules/50ng of RNA. Asterisks indicate differences from Control. **P*<0.05, and ***P*<0.01.




* *P*<0.05

# Chapter 5: EXPERIMENT 3

## Abomasal infusion of butterfat increases milk fat in lactating dairy cows.[1]

[1]A. K. G. Kadegowda, L. S. Piperova, P. Delmonte, and R. A. Erdman . J. Dairy Sci. 2008. 91. 2370-2379.

## ABSTRACT

The objective of this study was to compare the effects of abomasal infusion of butterfat containing all fatty acids (FA) present in milk, including the short and medium chain FA, with infusion of only the long chain FA (LCFA) present in milk, on the FA composition and milk fat yield in lactating dairy cows. Eight rumen fistulated Holstein cows, in early lactation (49±20 DIM) were used in a replicated 4x4 Latin square design. Treatments were abomasal infusion of: 1) no infusion (Control); 2) 400 g/d butterfat (Butterfat); 3) 245 g/d LCFA (blend of 59% cocoa butter, 36% olive oil, and 5% palm oil) providing 50% of the 16:0 and equivalent amounts of C18 FA as found in 400 g of butterfat; and 4) 100 g/d conjugated linoleic acid (CLA, negative control), providing 10 g of t10c12 CLA. Fat supplements were infused in equal portions 3 times daily at 0800, 1400, and 1800 h during the last 2 wk of each 3 wk experimental period. Daily dry matter intake (DMI) and milk production were unaffected by the infusion treatments. Butterfat infusion increased milk fat percentage by 14% ($P < 0.03$) to 4.26% and milk fat yield by 21% ($P < 0.02$) to 1421 g/day compared with Controls (3.74 % and 1178 g/day). Milk fat percentage and fat yield were decreased by 43% ($P < 0.001$) by CLA. Milk protein percentage was higher (3.70%; $P < 0.01$) in CLA infused cows than in Control (3.30%), Butterfat (3.28%) or LCFA (3.27%) treatments. While LCFA had no effect on fat synthesis, abomasal infusion of butterfat increased milk fat percentage and yield suggesting that the availability of short and medium chain FA may be a limiting factor for milk fat synthesis. **Key words:** lactating dairy cows, milk fat synthesis, de novo fatty acids

# INTRODUCTION

The current milk component pricing system (**MCP**) was introduced by the Federal Milk Marketing Administration in 2000. Accordingly, there has been a shift in the producer payment from the historic system based on the volume of milk (adjusted for fat content) to one based primarily on the amounts of milk fat and protein produced. The MCP system provides a powerful economic incentive for dairy producers to produce high value milk components, namely fat and protein. Milk component yields are driven by both milk volume and component concentration. The dairy cow's diet has no effect on milk lactose and mineral content (Sutton, 1989). Compared to milk fat responses, only modest effects of diet have been reported on milk protein concentration (Sutton, 1989). Milk fat is the milk component most easily manipulated by diet (Sutton, 1989). Reports in the literature have shown that milk fat percentage and yield can be reduced up to 46% (Piperova et al., 2000; Peterson et al., 2003) by milk fat depressing diets (**MFD**) containing high levels of grain and polyunsaturated fatty acids (**PUFA**).

In contrast, very few studies have demonstrated consistent ways to increase milk fat concentration. Abomasal infusion of mostly saturated long chain fatty acids (**LCFA**) tended to increase milk fat yield compared with infusion of mostly unsaturated fatty acids (**FA**) or mixtures of both (Drackley et al., 1992). Abomasal infusion of canola, soybean or sunflower oil did not significantly affect milk fat percentage in lactating cows, but changed milk FA profile reflecting the FA composition of the infused oils (Christensen et al., 1994). Inclusion of fats and oils in

the diet of lactating cows usually decreases the proportion of *de novo* FA produced by the mammary gland (Clapperton and Banks, 1985; LaCount et al., 1994)

Short and medium chain FA (6:0 to 14:0, plus 50% of 16:0), constitute 50% of total milk FA and originate from *de novo* FA synthesis in the mammary gland (Palmquist and Jenkins, 1980). These FA are essential for the formation of milk triacylglycerols (Moore and Christie, 1979) and for maintaining the fluidity of milk fat (Barbano and Sherbon, 1980). With exception of oleic acid, which is produced from stearic acid by the delta-9 desaturase system in the mammary gland, the LCFA in milk are derived from dietary sources (Palmquist and Jenkins, 1980). Experiments with MFD in lactating cows have demonstrated that yields of short and medium chain FA synthesized *de novo* were reduced to a greater extent than LCFA yields (Loor and Herbein, 1998; Chouinard et al., 1999; Baumgard et al., 2002). These observations suggest that provision of short and medium chain FA via dietary means might enhance milk fat content, reducing the need for *de novo* synthesis.

One could reason that a fat containing the FA composition identical to milk fat would provide the "Ideal" fat supplement for milk fat production. Alternatively, if short and medium chain FA are not limiting, then a fat supplement containing only LCFA with a composition identical to that found in milk fat would be "Ideal" for meeting the needs of FA that are typically absorbed from the diet. These conceptual approaches were used as means to potentially increase milk fat synthesis. The objective of this study was to compare the effects of abomasal infusion of butterfat containing all FA present in milk, including the short and medium chain FA, with

infusion of only the long chain FA present in milk, on the FA composition and milk fat yield in lactating dairy cows.

## MATERIALS AND METHODS

*Animals, Experimental Design, Treatment and Sampling*

All procedures for this experiment were conducted under a protocol approved by the University of Maryland Institutional Animal Care and Use Committee. Eight rumen fistulated multiparous Holstein cows in early lactation (49±20 DIM) were used in a replicated 4x4 Latin square design balanced for carryover effects. Treatments were abomasal infusion of: **1)** no infusion (**Control**); 2) 400 g/d butterfat as a source of short and long chain FA **(Butterfat)**; 3) 245 g/d of a LCFA mixture providing 50% of the 16:0 and equivalent amounts of C18 FA as found in 400 g of butterfat (**LCFA**); and 4) 100 g/d of commercial conjugated linoleic acid (**CLA**) mixture providing 10 g of trans (t)10, cis(c)12 CLA/day which served as a negative control.

In the LCFA treatment, only 50% of the palmitic acid found in the butterfat was included, as 50% of palmitic acid is thought to be synthesized *de novo* (Palmquist and Jenkins, 1980). The LCFA mixture was a blend of 59% cocoa butter, 36% olive oil (Unilever, Englewood Cliffs, NJ), and 5% palm oil (GloryBee Foods Inc., Eugene, OR, USA). In the Butterfat treatment, butter oil was prepared from commercially available unsalted butter (Wellsley Farms, USA) melted at 37°C, and separated from the protein coagulate by filtering. The CLA mixture was provided by Vitrus Nutrition (Corcoran, CA). Amounts of post-ruminally infused individual FA in the

LCFA mixture and Butterfat are shown in Figure 5.1. The FA composition of the fat supplements is presented in Table 5.1.

Experimental periods were 3 wk. The first wk of each period was without fat infusion to reduce carryover effects. This was followed by 2 wk of abomasal infusion. The fat was infused via tygon® tubing (0.48 cm i.d, 0.64 cm o.d; VWR Scientific, Bridgeport, NJ) that passed through the ruminal cannula, the rumen, the omasum and into the abomasum, where the line was maintained using a 10 cm circular plastisol flange. The fat mixtures were solubilized at 50°C in air oven and mixed well before infusion. The amount of each FA mixture was divided into equal portions and manually infused three times a day (133.33 g of butterfat, 81.6 g of LCFA and 33.33 g of CLA at 0800, 1400, and 1900 h). Actual amounts of infused fat were recorded each day. Patency and location of the infusion line inside the cow were checked on alternate days.

Cows were housed in individual tie stalls and were fed a basal diet containing 55% forage and 45% concentrate (DM basis) to meet NRC (2001) nutrient specifications for a 650 kg cow producing 40 kg milk containing 3.7% milk fat and 3.1% milk protein. Ingredient and chemical composition of the basal diet is given in Table 5.2. Diets were fed as TMR once daily at 0800 h. Forage and ingredient DM were measured weekly, and the TMR was adjusted accordingly to maintain a constant forage-to-concentrate ratio on a DM basis. Amounts of feed offered and refused were recorded once daily. Cows were milked twice-a-day at 0600 and 1600 h and milk production was recorded electronically at each milking. Samples for milk composition and FA analysis were collected from the last 6 consecutive milkings of

wk 3 of each experimental period. Milk fat, protein, and SCC were determined by infrared analysis (Foss Milk-O-Scan, Foss Food Technology Corp., Eden Prairie, MN) on fresh samples from individual milkings. A subset of samples from each milking was composited and frozen at -20$^{o}$C for subsequent analysis of individual FA.

*Fatty acid composition*

Milk fat was extracted using a modified Folch procedure (Christie, 1982) from composited wk 3 samples. The FA methyl esters **(FAME)** were prepared by mild transesterification with 0.4 mol/L $H_2SO_4$ in methanol (Christie, 1982) at room temperature, using trinonadecenoin (Nu-Chek Prep, Inc. Elysian, MN, USA) as internal standard (Piperova et al., 2002). The FAME were analyzed using Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a Supelco 2560 capillary column (100 m x 0.25 mm, 0.2 mm, Supelco) and a FID detector. The column was maintained at 173°C isothermal. Hydrogen was used as carrier gas with a linear velocity of 26 cm/s, and split ratio 1:100. The injection port and detector were maintained at 250°C. Detector air flow was 400 ml/min, and hydrogen flow was 30 ml/min. Helium make-up gas was used at 30 ml/min. Individual FA and 18:1 isomers were identified using GLC-463 standard mixture (Nu-Chek Prep Inc., Elysian, MN).

Although GC conditions can be adjusted to quantify fatty acids methyl esters from 8:0 to 12:0 with some accuracy, the very short fatty acid methyl esters are volatile, water soluble and require correction factors (Krammer et al., 1997). To avoid

these difficulties, short and medium chain fatty acids were analyzed as butyl esters (FABE), which were mathematically converted to FAME and normalized to the FAME chromatogram. The original FABE procedure of Gander et al., (1963) was modified as previously described (Piperova et al., 2000). Milk samples (200 μL), in screw capped glass tubes were heated at 100°C for 1h in the presence of 1 mL of butyl alcohol and 200 μL of acetyl chloride. Aliquots of the upper layer, containing FABE were analyzed on a short 25 m x 0.2 mm fused silica capillary column coated with HP1 (Hewlett Packard; Avondale, PA) using a Hewlett-Packard 5880 GLC (Avondale, PA) equipped with a split injector and flame ionization detector. Helium was used as the carrier gas at a flow rate of 2mL/min with a split ratio of 45:1. After 5 min at 90°C, the column temperature was raised (4°C /min) to 106°C and at 10 minutes, programmed at 5°C/min to a final temperature of 250°C. Standard mixtures, including GLC-60 (Nu-Chek Prep Inc., Elysian, MN), were converted to FABE to aid in the identification and quantification of components.

Individual CLA were analyzed as methyl esters using a Waters 2690 HPLC (Waters Associates, Milford, MA) separations module, equipped with a waters 2996 PDA detector, and 3 Chromspher 5 lipids (250x 4.6 mm, 5 μM, Varian) HPLC columns in series. The mobile phase was 0.1% acetonitrile in hexane, at 1 ml/min, kept in a sealed bottle at -15$^{o}$C. The PDA detector was operated between 190 nm and 300 nm, and HPLC chromatograms read at 233 nm were used for quantitative analysis. Columns were maintained at 20°C. Typical injection volume was 1 μL,

containing 10-25µg of FAME. A CLA standard mixture (Nu-Chek Prep Inc., Elysian, MN) was used to identify individual isomers. Details on the identification and quantification of the CLA isomers by HPLC analysis have been reported elsewhere (Eulitz et al., 1999; Sehat et al., 1998).

*Statistical analysis*

Milk production, milk components, and FA composition data were analyzed as a replicated 4 x 4 Latin square using the Mixed Procedure in the Statistical Analysis System (Version 8.2, 2004, SAS Institute, Cary, NC). The fixed effects were the treatments while the random portion of the model included cow, square, and period. Probability of $P \leq 0.05$ was considered statistically significant. One cow was diagnosed with displaced abomasum at the end of Period 1 that was surgically repaired. To allow for recovery from surgery, data from this cow were not included for experimental Periods 1 and 2 (Control and LCFA treatments, respectively). Because of missing data for this cow, the results are presented as least squares means. The largest standard error was reported in the treatments with unequal replication. Fisher's protected least significance differences were used for pair wise treatment mean comparisons.

## RESULTS

The FA profile of the infused mixtures is presented in Table 5.1. Butterfat infusion provided short and medium chain FA (C < 16), in addition to palmitic acid and C > 18 LCFA. Also, the Butterfat treatment contained small amounts of CLA (0.63 %) with c9t11 CLA being the predominant isomer. The LCFA mixture was

formulated to provide equivalent amounts of LCFA of dietary origin, as in the infused butterfat, assuming that 50% of 16:0 in milk fat is derived from the diet. The CLA supplement contained 55% of CLA with c9t11 (14.59%), c11t13 (9.44%) and t10c12 (9.66%) being the major isomers.

Average daily DMI, milk production, and milk composition are presented in Table 5.3. The daily DMI and milk production was not affected by the infusions. However, Butterfat infusion significantly ($P < 0.05$) increased FCM (3.5%) compared to the Control. Milk production efficiency ($FCM/NE_LI$) was lower with CLA infusions but did not differ due to butterfat or LCFA infusions from the Control.

Butterfat infusion increased milk fat percentage by 14% ($P < 0.03$) to 4.26% and milk fat yield by 21% ($P < 0.02$) to 1421 g/day compared to controls (3.74 % and 1178 g/day). Infusion of LCFA had no effect on either milk fat percentage or milk fat yield although milk fat yield was numerically (8.5%) higher compared to Controls. Infusion of CLA decreased milk fat percentage and fat yield by 43% ($P < 0.001$), while, milk protein percentage was 0.40 to 0.43 percentage units greater (3.70%; $P < 0.01$) compared to the other treatments.

Concentrations (g/100g FAME) and yield (g/d) of individual FA in milk are shown in Tables 5.4 and 5.5, respectively. Fatty acid concentrations of individual short and medium chain FA that are synthesized *de novo* in the mammary gland did not differ between Control and Butterfat or LCFA infused cows. Compared to the Controls, concentrations of C < 16:0 (total short and medium chain) FA, palmitic acid or C > 16:0 FA were not altered due to butterfat infusion while LCFA infusion decreased C<16:0 and palmitic acid but increased C > 16:0 FA. Concentrations of the

total monounsaturated FA (**MUFA**) were significantly greater ($P < 0.05$) in cows infused with Butterfat and LCFA compared to the Controls (Table 5.4). The yield of short and medium chain FA was increased by 21% with Butterfat ($P < 0.05$). Cows infused with butterfat produced milk with numerically the highest yield of 16:0 and significantly greater yields of 14:0, 15:0 and 17:0 (Table 5.5, $P < 0.02$, 0.01, and 0.02, respectively). Milk of cows infused with Butterfat was enriched with 14:1, 16:1, 17:1 and 18:1 cis, causing a 37% ($P < 0.001$) increase in the yield of total MUFA. Similar changes were observed in the yield of PUFA (18:2n-3, $P < 0.004$; 18:2i, $P < 0.001$; and 18:3n-3, $P < 0.001$) with the Butterfat treatment. Yields of 18:1 cis-9 (26% increase; $P < 0.005$) and 18:2n-6 ($P < 0.002$) FA were most affected by the LCFA infusion. Total saturated FA (**SFA**) were marginally increased with Butterfat and LCFA infusion.

Except for 4:0 concentrations, CLA infusion reduced concentrations (20%, $P < 0.001$) and yield (56%, $P < 0.0001$) of all short and medium FA and 16:0. CLA had similar effect on the yield of FA C > 16:0 (38% decrease; $P < 0.001$) and proportionally decreased all SFA (Table 5.5). Compared with the Controls, the concentration (489%, $P < 0.001$; Table 6) and yield (336%, $P < 0.001$; Table 7) of total CLA increased in cows infused with CLA. This reflected the increase in the percentage and yield of individual CLA isomers (Table 5.6 and 5.7). The c9t11 CLA isomer was the predominant CLA isomer in milk fat of cows receiving all treatments. Milk fat percentage of trans-18:1 was increased in cows infused either with Butterfat or CLA (Table 5.6), compared to the Control or LCFA infused cows. However, the yield of trans-18:1 was lowest in cows receiving CLA due to the reduction of milk fat

(Table 5.7). Trans-11-18:1 was the predominant isomer in milk of cows receiving all treatments. Trans-18:1 and CLA isomer profile in cows infused with LCFA was similar to that in the Control.

## DISCUSSION

This is the first study to examine the effects of post ruminal infusion of de novo (short and medium-chain FA) versus dietary derived (LCFA) FA on milk fat synthesis in lactating cows. As our LCFA treatment contained nearly identical amounts of LCFA (C 18 plus 50% of 16:0) as in butterfat, inferences about the role of short and medium chain FA on milk fat synthesis can be made by comparing the Butterfat and LCFA treatments. In contrast, CLA infusion was used as a negative control to demonstrate responsiveness of cows to changes in FA infusion. The CLA mixture provided 10 g/day of t10c12 CLA and decreased milk fat yield by 43%. Consistent with other CLA infusion experiments (Chouinard et al., 1999, Baumgard. et al., 2000), *de novo* FA synthesis and FA desaturation were predominantly inhibited.

Generally, DMI is affected by abomasal infusion of highly unsaturated FA (Bremmer et al., 1998; Benson et al., 2001) and to a lesser degree of saturated LCFA (Bremmer et al., 1998). The infusion mixtures in this study contained both saturated and unsaturated FA (Table 1), and the maximum amount of FA infused was limited to 400 g/d to avoid possible adverse effects on DMI (Drackley et al., 1992).

Infusion of FA did not affect DMI and resulted in a marginal increase in milk production in cows infused with Butterfat and LCFA treatments. Milk fat percentage and yield were significantly increased with the Butterfat but not with

LCFA infusion treatments. The increase in total milk fat was more apparent when yields of individual FA were compared. The differences in the effects of Butterfat and LCFA were associated with the short and medium FA provided by the butterfat. Earlier experiments (Storry et al., 1969) did not show changes in milk fat yield when synthetic tripropionin, tributyrin, tricaproin, tricaprylin, tricaprin containing short chain FA (3:0, 4:0, 6:0, 8:0 and 10:0) were intravenously infused for 2 days in lactating dairy cows. However, milk fat yield was increased by 16% during intravenous infusion of trilaurin (12:0) and trimyristin (14:0) (Storry et al., 1969). Similarly, a 12% increase in milk fat yield was reported in lactating cows fed diets supplemented with coconut oil, containing predominantly 12:0 and 14:0 FA (Astrup et al., 1974; Storry et al., 1971). Pure saturated FA (12:0, 14:0, 16:0 or 18:0) fed to lactating cows (Steele and Moore, 1968b) produced variable changes in milk fat content but increased these FA in milk, suggesting that the proportions of FA from treatment supplements were increased in blood triacylglycerols. Comparable results were reported (Enjalbert et al., 2000) in lactating cows receiving duodenal infusion of palmitic, stearic or oleic acids.

In this study, infusion of butterfat significantly increased the yield of FA with C $\leq$ 16:0, raising the overall milk fat yield compared to the other treatments. The increase was predominantly due to greater yield of 12:0 to 14:0 medium chain FA. Short and medium chains FA are better absorbed than LCFA and are transported via the portal venous blood system (Grummer and Socha, 1989). Nevertheless, their transfer to milk fat may be reduced due to extensive metabolism by the extra-mammary tissues (Grummer and Socha, 1989).

The apparent transfer efficiency of the abomasally infused FA was calculated (amount of FA excreted in milk fat as a % of the amount infused) by subtracting the FA yield in the Control treatment. It should be recognized that these apparent values reflect changes not only in uptake but also in the net synthesis of FA. The overall transfer efficiency for C ≤ 16:0 in milk of cows infused with Butterfat was 63% with transfer efficiencies of 44% (4:0), 0% (6:0), 0% (8:0), 33% (10:0), 67% (12:0), 83% (14:0), and 58% (16:0), respectively. While 6:0 and 8:0 were most likely metabolized by other tissues, the transfer efficiencies of 10:0, 12:0 and 14:0 FA increased with the increase in chain length. Storry et al., (1974) reported a net transfer of 42% and 48% for 12:0 and 14:0, respectively to milk when protected coconut oil was fed to lactating cows. Compared to the Control or LCFA infused cows, yields of 10:0 and 12:0, tended to be higher, and yield of 14:0 was significantly greater in milk of cows infused with butterfat. The potential of 12:0 and 14:0 to increase is greater than that of shorter chain length FA probably because the ratio of lymphatic to hepatic portal vein uptake (greater lymphatic absorption) is positively correlated to FA chain length (Leveille et al., 1967). These FA might be expected to be metabolized to a lesser degree by the liver and be more available for the mammary gland (Grummer and Socha, 1989).

About 60% of 16:0 present in the Butterfat was transferred in milk, and yield of 16:0 tended to be higher compared to the control or LCFA infused cows. Feeding studies (Steele and Moore, 1968) have shown that dietary addition of pure palmitic acid caused the greatest increase in milk fat yield in dairy cows, compared to addition of myristic or stearic acid. Addition of palmitic acid increased percentages and yields

of 16:0 and 16:1 FA in milk fat and decreased 10:0, 12:0 and 14:0 as well as 18:0 and 18:1. Similarly, yield of 16:0 was increased and total FA in milk were significantly higher during duodenal infusion of palmitic acid (Enjalbert et al., 2000).

Palmitic acid is an important FA for the synthesis of triacylglycerol in the mammary gland (Hansen and Knudsen, 1987). Initiation of acylation of the sn-1 position is a prerequisite for triacylglycerol synthesis and palmitic acid is the most preferred substrate for the initial acylation of L-α glycerolphosphate by acyltransferase to form sn-1-lysophasphatidic acid (Kinsella and Gross, 1973). It has been shown (Kinsella and Gross 1973) that myristyl, stearyl and oleyl CoA were rapidly acylated when sn-1-lysophasphatidic acid was used as substrate but were poorly acylated without the latter indicating that these FA are taken up mostly in the second step of triacylglycerol synthesis.

Infusion of LCFA did not significantly alter milk fat yield. In contrast with other reports (Grummer, 1991; LaCount et al., 1994), yield of FA C < 16:0, including *de novo* FA was not significantly reduced by LCFA infusion but rather it was maintained as in the Controls. Butterfat and LCFA infusion mixtures contained equal amounts of LCFA (except for 16:0) resulting in a similar increase in milk FA C > 16:0. However, compared to the Butterfat treatment, the proportion of C18 FA was considerably greater than the proportion of palmitic acid in the LCFA infusion mixture. Steele and Moore (1968a) reported that addition of cottonseed oil (containing 21% palmitic acid and 76% total C18 FA) or tallow (containing 30% palmitic acid and 60 % total C18 FA) to the diet of lactating cows increased stearic and oleic acids in milk fat but did not alter yield of 16:0. In contrast, yields of 16:0

and 18:1 were increased when a mixture of 64% palmitic acid and 34% total C18 FA was added to the diet of lactating cows (Steele and Moore, 1968b). In our study, yield of 16:0 in cows infused with LCFA, was maintained at a level similar to that observed in the Controls, while the yield of 16:0 was increased with Butterfat treatment. The difference between these treatments may have been influenced by the C18 to 16:0 ratios in the infusion mixtures. It has been suggested (Steele and Moore, 1968a; Grummer,1991), that if *de novo* synthesis of 16:0 is inhibited by increased uptake of LCFA from the blood, the net yield of 16:0 in milk fat will depend on whether dietary transfer of 16:0 to milk fat is sufficient to compensate for the decrease in *de novo* synthesis.

In conclusion, the abomasal infusion of butterfat compared with LCFA demonstrated that milk fat synthesis could be regulated by the supply of short and medium chain FA in lactating cows. In addition, the increased availability of palmitic acid, the preferred substrate for the initial acylation of L-α- glycerophosphate may have also stimulated triacylglycerol synthesis in the mammary gland. Compared to the Controls, cows infused with butterfat produced milk fat with greater amounts of 12:0 and 14:0, MUFA, and PUFA. An increase in the proportions of unsaturated FA in milk fat is desirable because of the potential health benefits when included in human diet. However, further studies are needed to test and refine the role of short and medium chain FA on milk fat synthesis.

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**Table 5.1.** Fatty acid composition of the Butterfat, LCFA[1] and CLA[2] supplements

| Fatty acid | Butterfat | LCFA | CLA |
|---|---|---|---|
| | g/100g FAME[3] | | |
| 4:0 | 3.34 | | |
| 6:0 | 2.22 | | |
| 8:0 | 1.06 | | |
| 10:0 | 1.93 | | |
| 12:0 | 3.00 | | |
| 14:0 | 12.09 | 0.18 | 0.15 |
| 14:1 | 0.66 | | |
| 15:0 | 0.89 | | |
| 15:1 | 0.01 | | |
| 16:0 | 26.73 | 24.41 | 5.79 |
| 16:1 | 1.17 | 0.55 | 0.12 |
| T16:1 | 0.02 | | |
| 17:0 | 0.61 | | |
| 17:1 | 0.18 | | |
| 18:0 | 11.45 | 21.17 | 2.54 |
| 18:1 cis-9 | 26.79 | 45.67 | 29.02 |
| 18:1 cis-11 | 0.54 | | 0.72 |
| 18:1 cis-12 | 0.55 | | 0.02 |
| 18:1 cis-13 | 0.09 | | 0.06 |
| 18:1cis-14+19:0 | 0.18 | | 0.62 |
| 18:1 trans total | 2.09 | | 0.23 |
| 18:2 n-6 | 2.80 | 6.37 | 0.88 |
| 18:3 | 0.39 | 0.44 | 0.29 |
| 22:0 | 0.04 | | 0.14 |
| Others | 0.56 | 1.21 | 3.21 |
| [4]c8[5]t10 18:2 | 0.00 | | 0.68 |
| t8c10 18:2 | 0.00 | | 0.75 |
| c9t11 18:2 | 0.55 | | 14.59 |
| c9t13 18:2 | 0.00 | | 0.74 |
| c13t12 18:2 | 0.00 | | 1.09 |
| c11t13 18:2 | 0.01 | | 9.44 |
| 10c12 18:2 | 0.01 | | 9.66 |
| t11c13 18:2 | 0.03 | | 1.00 |
| t11t13 18:2 | 0.00 | | 1.17 |
| tt18:2 | 0.02 | | 10.71 |
| cc18:2 | | | 6.21 |

[1] Long chain Fatty Acids
[2]Conjugated linoleic acids.
[3]Fatty acid methyl esters
[4] c=cis
[5] t=trans

**Table 5.2.** Ingredient and chemical composition of the basal diet

| Ingredients | % DM |
|---|---|
| Corn silage | 30.13 |
| Alfalfa haylage | 23.00 |
| Corn grain, ground | 21.71 |
| Citrus pulp | 8.05 |
| Soybean meal | 14.35 |
| Limestone | 0.26 |
| Salt | 0.48 |
| Magnesium oxide | 0.32 |
| Sodium bicarbonate | 1.04 |
| Potassium magnesium sulfate | 0.17 |
| Dicalcium phosphate | 0.39 |
| Trace minerals and vitamins[1] | 0.10 |
| | |
| Chemical composition | |
| DM % | 66.71 |
| ---------- % of DM ---------- | |
| CP | 17.43 |
| RUP[2] | 33.34 |
| ADF | 19.88 |
| NDF | 29.80 |
| $NE_L$ [2], Mcal/kg | 1.75 |
| Ca | 0.69 |
| P | 0.38 |
| Na | 0.54 |
| Mg | 0.36 |
| S | 0.22 |
| K | 1.20 |
| C | 0.50 |

[1] Trace mineral and vitamin mix combined (per kg mix) 15mg Mn, 50mg Zn, 10 mg Cu, 0.60mg I, 0.20 mg Co, 0.30 mg Se, 0.12 g retinyl acetate, 0.40g cholecalciferol and 0.05 mg dl-tocopheryl acetate.
[2] Calculated value.

**Table 5.3.** Least squares means for dry matter intake (DMI), milk production, and milk composition from cows fed the Control diet or the Control diet plus abomasal infusionof CLA[1], Butterfat, or LCFA[2] supplements

| | Treatment | | | | |
|---|---|---|---|---|---|
| Item | Control | CLA | Butterfat | LCFA | SEM |
| DMI, kg/d | 23.7 | 24.1 | 24.2 | 25.7 | 1.1 |
| Milk | | | | | |
| kg/d | 31.8$^{ab}$ | 29.9$^{b}$ | 33.7$^{a}$ | 33.1$^{ab}$ | 2.5 |
| FCM, 3.5% | 32.87$^{b}$ | 23.65$^{c}$ | 37.72$^{a}$ | 35.04$^{ab}$ | 2.71 |
| Efficiency, FCM/$NE_L$ | 0.78$^{a}$ | 0.55$^{b}$ | 0.83$^{a}$ | 0.77$^{a}$ | 0.44 |
| Efficiency, FCM/DM | 1.37$^{b}$ | 0.98$^{c}$ | 1.51$^{a}$ | 1.38$^{b}$ | 0.07 |
| Milk NE/ NE Intake | 0.53$^{a}$ | 0.41$^{b}$ | 0.56$^{a}$ | 0.52$^{a}$ | 0.02 |
| Fat, % | 3.74$^{b}$ | 2.16$^{c}$ | 4.26$^{a}$ | 3.79$^{ab}$ | 0.19 |
| Fat yield, g/d | 1178$^{b}$ | 661$^{c}$ | 1421$^{a}$ | 1279$^{ab}$ | 107.7 |
| Protein,% | 3.30$^{b}$ | 3.70$^{a}$ | 3.28$^{b}$ | 3.27$^{b}$ | 0.12 |
| Protein, g/d | 1033 | 1086 | 1061 | 1085 | 62.6 |
| Lactose, % | 4.63$^{a}$ | 4.45$^{b}$ | 4.59$^{ab}$ | 4.70$^{a}$ | 0.08 |
| SCC, ($10^3$/ml) | 102 | 174 | 205 | 97 | 82.8 |

[1]Conjugated linoleic acids.
[2]Long chain fatty acids.
[abc] Least square means within a row with different superscripts differ ($P < 0.05$)

**Table 5.4.** Least squares means for fatty acid composition of milk from cows fed the Control diet or the Control diet plus abomasal infusion of CLA[1], Butterfat, or LCFA[2] supplements

| | Treatment | | | | |
|---|---|---|---|---|---|
| Fatty acid | Control | CLA | Butterfat | LCFA | SEM |
| | ----------g/100g of FAME[3] ---------- | | | | |
| 4:0 | 3.45 | 3.04 | 3.52 | 3.47 | 0.36 |
| 6:0 | 1.81[a] | 0.83[b] | 1.54[a] | 1.71[a] | 0.10 |
| 8:0 | 1.24[a] | 0.55[b] | 1.06[a] | 1.17[a] | 0.08 |
| 10:0 | 3.22[a] | 1.55[b] | 2.83[a] | 2.96[a] | 0.19 |
| 12:0 | 4.01[a] | 2.41[b] | 3.91[a] | 3.73[a] | 0.25 |
| 14:0 | 12.31[a] | 10.96[b] | 12.65[a] | 11.78[ab] | 0.37 |
| 14:1 | 1.14[ab] | 1.19[ab] | 1.38[a] | 1.05[b] | 0.08 |
| 15:0 (iso) | 0.23[a] | 0.19[b] | 0.22[a] | 0.23[a] | 0.02 |
| 15:0 (anteiso) | 0.51 | 0.47 | 0.51 | 0.50 | 0.03 |
| 15:0 | 1.22 | 1.25 | 1.27 | 1.13 | 0.04 |
| 15:1 | 0.07 | 0.03 | 0.02 | 0.01 | 0.02 |
| 16:0 | 35.08[a] | 35.41[a] | 33.10[ab] | 31.53[b] | 1.33 |
| 16:0 (iso) | 0.36 | 0.31 | 0.35 | 0.33 | 0.03 |
| 16:1 | 1.63 | 1.98 | 1.76 | 1.22 | 0.16 |
| t16:1 | 0.04 | 0.03 | 0.03 | 0.03 | 0.01 |
| 17:0 | 0.79[a] | 0.71[b] | 0.78[a] | 0.74[ab] | 0.03 |
| 17:0 (iso) | 0.06 | 0.06 | 0.06 | 0.06 | 0.00 |
| 17:1 (iso) | 0.15[b] | 0.18[a] | 0.19[a] | 0.20[a] | 0.01 |
| 17:1 (anteiso) | 0.43 | 0.39 | 0.41 | 0.38 | 0.03 |
| 17:1 | 0.21[a] | 0.23[a] | 0.21[a] | 0.18[b] | 0.01 |
| 18:0 | 8.08[ab] | 8.91[a] | 7.31[b] | 8.47[ab] | 0.42 |
| 18:1 cis-9 | 16.50[b] | 18.01[b] | 18.13[b] | 20.84[a] | 0.84 |
| 18:2i[4] | 0.47[b] | 0.58[a] | 0.60[a] | 0.44[b] | 0.03 |
| 18:2n-6 | 2.40[b] | 2.89[a] | 2.52[b] | 2.93[a] | 0.14 |
| 18:3i[5] | 0.14[b] | 0.09[c] | 0.11[c] | 0.17[a] | 0.01 |
| 18:3n-3 | 0.40[b] | 0.46[a] | 0.40[b] | 0.43[ab] | 0.03 |
| 20:0 | 0.10[ab] | 0.11[a] | 0.09[b] | 0.12[a] | 0.01 |
| GLA[6] | 0.03 | 0.03 | 0.03 | 0.03 | 0.00 |
| Other | 0.55[ab] | 0.73[a] | 0.59[ab] | 0.49[b] | 0.07 |
| Summations | | | | | |
| <16:0 | 29.21[a] | 22.47[b] | 28.91[a] | 27.74[a] | 0.89 |
| >16:0 | 31.40[b] | 34.63[ab] | 32.60[b] | 36.21[a] | 1.35 |
| >20:0 | 0.16[b] | 0.20[a] | 0.20[a] | 0.20[a] | 0.01 |
| MUFA[7] | 20.17[b] | 22.04[ab] | 22.13[a] | 23.91[a] | 1.19 |
| SFA[8] | 72.47[a] | 66.76[c] | 69.20[ab] | 67.93[bc] | 1.38 |
| PUFA[9] | 3.04[c] | 3.59[a] | 3.26[bc] | 3.57[ab] | 0.16 |

[1]Conjugated linoleic acids. [2]Long chain fatty acids. [3]Fatty acid methyl esters.
[4] Non-conjugated 18:2 isomers. [5] Non-conjugated 18:3 isomers. [6] Gamma linoleic acid. [7] Mono unsaturated fatty acids. [8] Saturated fatty acids. [9]Polyunsaturated fatty acids.[abc] Least square means within a row with different superscripts differ ($P < 0.05$)

**Table 5.5.** Least squares means for yield of fatty acids in milk from cows fed the Control diet or the Control diet plus abomasal infusion of CLA[1], Butterfat, or LCFA[2] supplements

| | Treatment | | | | |
|---|---|---|---|---|---|
| Fatty acid | Control | CLA | Butterfat | LCFA | SEM |
| | ----------g FAME[3]/day ---------- | | | | |
| 4:0 | 39.19[a] | 18.48[b] | 49.30[a] | 45.43[a] | 5.75 |
| 6:0 | 21.02[a] | 5.98[b] | 21.02[a] | 26.76[a] | 2.50 |
| 8:0 | 14.13[a] | 3.93[b] | 14.56[a] | 15.53[a] | 1.59 |
| 10:0 | 36.52[a] | 10.96[b] | 39.12[a] | 31.40[a] | 3.78 |
| 12:0 | 46.07[a] | 16.07[b] | 54.58[a] | 48.99[a] | 4.75 |
| 14:0 | 144.31[b] | 73.24[c] | 179.34[a] | 149.56[ab] | 14.51 |
| 14:1 | 13.30[b] | 7.59[c] | 19.70[a] | 13.75[b] | 1.65 |
| 15:0 (iso) | 2.76[a] | 1.31[b] | 3.14[a] | 2.81[a] | 0.26 |
| 15:0 (anteiso) | 6.08[a] | 3.30[b] | 6.96[a] | 6.29[a] | 0.61 |
| 15:0 | 14.24[b] | 8.27[c] | 18.33[a] | 14.45[b] | 1.60 |
| 15:1 | 0.69 | 0.05 | 0.15 | 0.12 | 0.29 |
| 16:0 | 417.36[a] | 234.29[b] | 476.21[a] | 415.79[a] | 46.61 |
| 16:0 (iso) | 3.47[a] | 2.14[b] | 4.67[a] | 4.24[a] | 0.55 |
| 16:1 | 18.87[b] | 11.32[c] | 25.10[a] | 14.80[bc] | 2.11 |
| t16:1 | 0.31[ab] | 0.14[b] | 0.42[a] | 0.24[ab] | 0.10 |
| 17:0 | 9.24[b] | 4.78[c] | 11.21[a] | 9.42[b] | 0.90 |
| 17:0 (iso) | 0.75[a] | 0.41[b] | 0.80[a] | 0.72[a] | 0.08 |
| 17:1 (iso) | 1.44[b] | 1.18[b] | 2.58[a] | 2.56[a] | 0.28 |
| 17:1 (anteiso) | 5.16[ab] | 2.76[c] | 5.72[a] | 4.53[b] | 0.54 |
| 17:1 | 2.41[b] | 1.45[c] | 3.08[a] | 2.29[b] | 0.42 |
| 18:0 | 90.10[a] | 60.54[b] | 102.17[a] | 105.14[a] | 8.85 |
| 18.1 cis-9 | 193.42[b] | 117.59[c] | 256.41[a] | 258.40[a] | 15.77 |
| 18:2i[4] | 5.68[b] | 3.84[c] | 8.34[a] | 5.68[b] | 0.58 |
| 18:2n-6 | 28.26[b] | 18.59[c] | 35.62[a] | 36.49[a] | 2.23 |
| 18:3i[5] | 1.12[bc] | 0.62[c] | 1.40[a] | 1.23[ab] | 0.17 |
| 18:3n-3 | 4.73[a] | 3.07[b] | 5.55[a] | 5.31[a] | 0.45 |
| 20:0 | 0.94[bc] | 0.77[c] | 1.26[ab] | 1.52[a] | 0.15 |
| GLA[6] | 0.37[a] | 0.15[b] | 0.34[a] | 0.38[a] | 0.06 |
| Other | 6.46[b] | 4.73[c] | 8.29[a] | 5.97[bc] | 0.68 |
| Summations | | | | | |
| <16:0 | 338.31[b] | 149.18[c] | 406.20[a] | 355.09[ab] | 30.42 |
| >16:0 | 361.86[b] | 226.44[c] | 458.74[a] | 447.19[a] | 33.11 |
| >20:0 | 1.78[bc] | 1.32[c] | 2.45[ab] | 2.49[a] | 0.13 |
| MUFA[7] | 235.60[b] | 142.08[c] | 313.16[a] | 296.36[a] | 20.13 |
| SFA[8] | 849.97[a] | 446.77[b] | 986.96[a] | 881.84[a] | 78.03 |
| PUFA[9] | 36.37[b] | 23.97[c] | 46.96[a] | 45.30[a] | 3.27 |

[1]Conjugated linoleic acids.[2]Long chain fatty acids.[3]Fatty acid methyl esters.
[4] Non-conjugated 18:2 isomers.[5] Non-conjugated 18:3 isomers.[6] Gamma linoleic acid.[7] Mono unsaturated fatty acids.[8] Saturated fatty acids.[9]Polyunsaturated fatty acids.[abc] Least square means within a row with different superscripts differ ($P < 0.05$)

**Table 5.6.** Least squares means for CLA[1], trans-18:1, and cis-18:1 isomers in milk fat of cows fed the Control diet or the Control diet plus abomasal infusion of CLA, Butterfat, or LCFA[2] supplements

| | Treatment | | | | |
|---|---|---|---|---|---|
| Fatty acid | Control | CLA | Butterfat | LCFA | SEM |
| | ----------g/100g of FAME[3] ---------- | | | | |
| CLA | | | | | |
| c8t10 | trace | 0.04[a] | trace | trace | 0.01 |
| c9t11 | 0.40[b] | 0.83[a] | 0.51[b] | 0.38[b] | 0.07 |
| t8c10 | trace | 0.28[a] | trace | trace | 0.03 |
| c10t12 | trace | 0.02[a] | trace | trace | 0.00 |
| t9c11 | trace | 0.06[a] | trace | trace | 0.01 |
| c11t13 | trace | 0.44[a] | 0.01[b] | 0.02[b] | 0.12 |
| t10c12 | trace | 0.24[a] | trace | 0.01[b] | 0.06 |
| t11c13 +21:0 | 0.04[b] | 0.05[a] | 0.03[b] | 0.03[b] | 0.01 |
| cis/cis CLA | trace | 0.28[a] | trace | trace | 0.03 |
| trans/trans CLA | 0.09[b] | 0.35[a] | trace | trace | 0.04 |
| Total CLA | 0.53[b] | 2.59[a] | 0.55[b] | 0.44[b] | 0.21 |
| trans 18:1 | | | | | |
| 4 | 0.01 | 0.01 | 0.01 | 0.01 | 0.00 |
| 5 | 0.01 | 0.01 | 0.01 | 0.01 | 0.00 |
| 6+7+8 | 0.17[c] | 0.22[a] | 0.20[ab] | 0.17[bc] | 0.04 |
| 9 | 0.20[b] | 0.25[a] | 0.24[a] | 0.21[b] | 0.01 |
| 10 | 0.27[c] | 0.40[a] | 0.36[ab] | 0.28[bc] | 0.10 |
| 11 | 0.80 | 0.84 | 0.84 | 0.81 | 0.07 |
| 12 | 0.21[b] | 0.26[a] | 0.26[a] | 0.22[b] | 0.02 |
| 13+14 | 0.47[c] | 0.56[a] | 0.52[b] | 0.41[d] | 0.02 |
| 16 | 0.20[b] | 0.26[a] | 0.23[b] | 0.20[b] | 0.01 |
| Total trans 18:1 | 2.34[b] | 2.81[a] | 2.67[a] | 2.32[b] | 0.10 |
| cis 18:1 | | | | | |
| 11 | 0.47[b] | 0.64[a] | 0.50[ab] | 0.52[ab] | 0.04 |
| 12 | 0.27[b] | 0.29[ab] | 0.32[a] | 0.27[b] | 0.01 |
| 13 | 0.05[c] | 0.08[a] | 0.07[b] | 0.04[d] | 0.00 |
| cis 14 + 19:0 | 0.13[b] | 0.15[a] | 0.14[ab] | 0.13[b] | 0.01 |

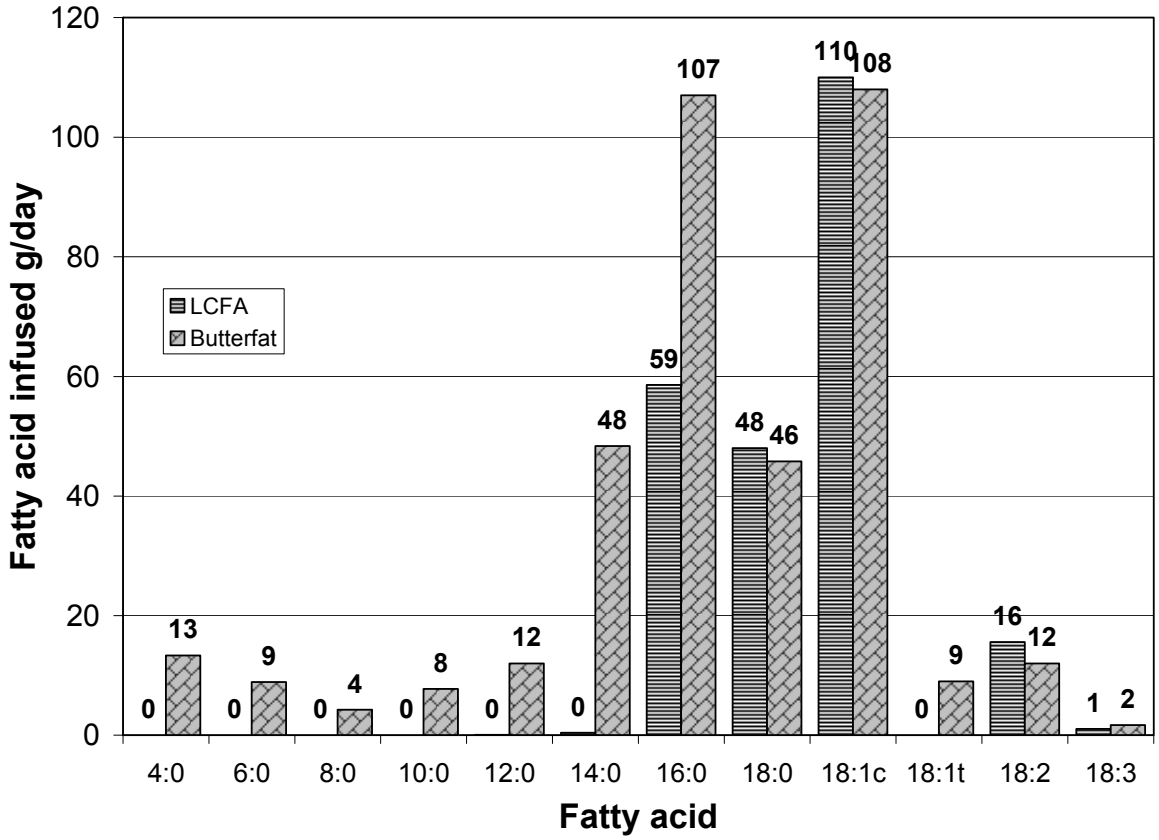
[1]Conjugated linoleic acids.
[2]Long chain fatty acids.
[3]Fatty acid methyl esters.
[4] c=cis
[5] t=trans
[abc] Least square means within a row with different superscripts differ ($P < 0.05$)

**Table 5.7.** Least squares means for yields of CLA[1], trans-18:1, and cis-18:1 isomers in milk of cows fed the Control diet or the Control diet plus abomasal infusion of CLA, Butterfat, or LCFA[2] supplements

| | Treatment | | | | |
|---|---|---|---|---|---|
| Fatty acid | Control | CLA | Butterfat | LCFA | SEM |
| | ----------g FAME[3]/day ---------- | | | | |
| CLA | | | | | |
| [4]c8[5]t10 | | 0.25 | | | |
| c9t11 | 4.72[b] | 5.24[b] | 7.28[a] | 4.83[b] | 0.63 |
| t8c10 | 0.05[b] | 1.27[a] | 0.02[b] | | 0.24 |
| c10t12 | | 0.06 | | | |
| t9c11 | 0.01[b] | 0.32[a] | | | 0.09 |
| c11t13 | 0.04[b] | 2.34[a] | 0.04[b] | 0.04[b] | 0.29 |
| t10c12 | 0.02[b] | 1.50[a] | 0.01[b] | 0.02[b] | 0.13 |
| t11c13 +21:0 | 0.35 | 0.31 | 0.30 | 0.35 | 0.05 |
| cis/cis CLA | 0.02[b] | 1.81[a] | 0.01[b] | 0.00[b] | 0.17 |
| trans/trans CLA | 0.15[b] | 1.65[a] | 0.09[b] | 0.08[b] | 0.22 |
| Total CLA | 5.63[b] | 16.77[a] | 7.87[b] | 5.40[b] | 1.51 |
| trans 18:1 | | | | | |
| 4 | 0.14 | 0.08 | 0.12 | 0.14 | 0.03 |
| 5 | 0.13 | 0.09 | 0.13 | 0.13 | 0.03 |
| 6+7+8 | 2.03[bc] | 1.50[c] | 2.82[a] | 2.17[b] | 0.23 |
| 9 | 2.42[b] | 1.63[c] | 3.44[a] | 2.61[b] | 0.25 |
| 10 | 3.22[bc] | 2.63[c] | 5.08[a] | 3.55[b] | 0.40 |
| 11 | 9.50[b] | 5.72[c] | 11.76[a] | 10.31[ab] | 1.17 |
| 12 | 2.34[b] | 1.73[b] | 3.38[a] | 2.39[b] | 0.36 |
| 13+14 | 5.58[b] | 3.77[c] | 7.31[a] | 5.16[b] | 0.67 |
| 16 | 2.42[b] | 1.72[c] | 3.14[a] | 2.53[b] | 0.24 |
| Total trans 18:1 | 27.78[b] | 18.87[c] | 37.18[a] | 28.99[b] | 2.82 |
| cis 18:1 | | | | | |
| 11 | 5.42[bc] | 4.07[c] | 7.31[a] | 6.57[ab] | 0.68 |
| 12 | 3.19[b] | 1.97[c] | 4.47[a] | 3.54[b] | 0.39 |
| 13 | 0.64[b] | 0.48[c] | 0.93[a] | 0.57[b] | 0.06 |
| cis 14 + 19:0 | 1.60[b] | 1.02[c] | 2.03[a] | 1.61[b] | 0.16 |

[1]Conjugated linoleic acids.
[2]Long chain fatty acids.
[3]Fatty acid methyl esters.
[4] c=cis
[5] t=trans
[abc] Least square means within a row with different superscripts differ ($P < 0.05$)

**Figure 5.1.** Fatty acid composition of post-ruminally infused Butterfat and LCFA mixture



# Chapter 6: EXPERIMENT 4

## Effect of abomasal infusion of butterfat, long chain fatty acids or CLA on milk fatty acid composition and mammary tissue lipogenic gene expression in lactating cows.[1]

[1] **Presented in part at the annual ADSA-ASAS conference, 2008.**

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**ABSTRACT**

Mammary tissue lipogenic gene expression and milk fatty acid composition were studied during abomasal infusion of butterfat, long chain fatty acids or CLA mixture in lactating cows. Eight rumen fistulated Holstein cows (49±20 DIM) were used in a replicated 4x4 Latin square design. Treatments were: 1) Control (no infusion); or abomasal infusion of; 2) 400 g/d Butterfat; 3) 245 g/d LCFA (blend of 59% cocoa butter, 36% olive oil, and 5% palm oil) providing 50% of the 16:0 and the amounts of C18 FA, equivalent to that found in 400 g of butterfat (LCFA); and 4) 100 g/d conjugated linoleic acid (CLA, negative control), providing 10 g of t10c12 CLA. Lipid supplements were infused in equal portions 3X daily during the last 2 wk of each 3 wk experimental period. Compared with Controls, Butterfat infusion increased milk fat yield by 21% ($P < 0.02$), CLA decreased milk fat yield by 43% ($P < 0.001$), and LCFA had no effect on fat yield. Infusion of Butterfat increased (21%, $P<0.05$) the yield of FA with ≤16:0-carbons. Milk yield of mono- and poly-unsaturated FA was greater in cows infused with Butterfat (by 33% and 29%) or LCFA (by 25% and 24%), compared to Control. Infusion of CLA reduced yield of all FA synthesized *de novo* (56%, $P<0.001$) and reduced FA desaturation index (40%, $P<0.001$). Expression of genes involved in FA uptake (*LPL, CD36*), intracellular FA activation and transport (*FABP3, ACSS2, ACSL1*), de novo FA synthesis (*ACACA, FASN*), desaturation (*SCD*), and trigacylglycerol synthesis (*AGPAT6, GPAM*) tended to increase by 30% to 40% due to Butterfat, while LCFA showed opposite effects. The expression of lipogenic genes were not modified by CLA. Results suggest that supply

of short and medium chain FA with Butterfat infusion might potentially up regulate mammary lipogenic gene expression and increase milk fat yield in lactating cows.

**Key Words:** butterfat, LCFA, t10c12-CLA, mammary lipogenic gene expression

# INTRODUCTION

The current federal milk pricing system **(MCP)** provides powerful economic incentives for dairy producers to produce milk solids that have high commercial value (fat and protein) while there is little or no incentive to produce components directly associated with milk volume (lactose and minerals) that have little or no value (Erdman, 2008). The ability to manipulate yield of milk components according to the prevailing market trends would be economically advantageous for dairy producers. Among major milk components, milk fat concentration can be easily manipulated by dietary interventions (Sutton, 1989).

One of the strategies used to alter milk fat content and fatty acid **(FA)** composition in lactating dairy cows is the use of dietary lipid supplements. Supplementation of polyunsaturated FA (**PUFA**) to a high concentrate diet can reduce milk fat content up to 46% (Piperova et al., 2000). The phenomenon of decreased milk fat synthesis due to supplementation of highly digestible carbohydrates and/or vegetable/marine oils is known as "Milk Fat Depression" (**MFD**). Significant research has been done on MFD in lactating dairy cows. Feeding and abomasal infusion experiments have established the role of trans-18:1 FA and CLA in development of MFD. Specifically, t10c12 CLA has been identified as the isomer responsible for MFD (Baumgard et al., 2000). Recently other CLA isomers – t9c11 (Shingfield et al., 2006) and t7c9 CLA (Kadegowda et al., 2008) have been negatively correlated to changes in milk fat content and their role has been investigated. The classical dietary-induced MFD is characterized by predominant decrease in the short (**SCFA**) and medium chain fatty acids (**MCFA**) and to a lesser

extent, the long chain FA **(LCFA)**. The decrease in *de novo* synthesized FA is associated with inhibition of lipogenic enzymes activity and mRNA abundance (Piperova et al., 2000). Peterson et al. (2003) reported that dietary-induced MFD affects expression of mammary genes involved in *de novo* FA synthesis, FA uptake and transport, FA desaturation and triacylglycerol **(TAG)** synthesis. The magnitude of decrease corresponded with the intensity of MFD (Piperova et al., 2000, Peterson et al. 2003).

Conversely, very few studies have shown that lipid supplementation can consistently increase milk fat synthesis (Banks et al., 1990; Drackley et al., 1992; Harrison et al., 1995; Enjalbert et al., 2000). Recently, using abomasal infusion of butterfat compared to long chain FA, it was demonstrated that milk fat yield can be increased by supply of SCFA and MCFA (Kadegowda et al., 2008). The mechanisms regulating these alterations of milk fat synthesis are not completely understood.

A large-scale transcript analysis of lipogenic genes in lactating mice have shown that regulation of mammary lipid synthesis occurs at the level of mRNA expression (Rudolph et al., 2007). Gene transcription exerts long-term regulation of metabolism (Desvergne et al., 2006), and as such, represents an important control point. In lactating cows, the effects of dietary saturated and unsaturated FA on mammary lipid metabolism occur partly through changes in lipogenic enzyme abundance or activity (Shingfield and Griinari, 2007). Although, the molecular mechanisms by which fatty acids regulate bovine mammary lipogenic gene expression are not completely established, there is evidence that transcriptional regulators are involved (Peterson et al., 2004; Harvatine and Bauman, 2006).

The transcription factor SREBP-1c is one of the regulators with a role in mammary lipid metabolism (Rudolph et al., 2007). Studies with MACT cells (Peterson et al., 2004) and lactating cows (Harvatine and Bauman, 2006) have shown that the effect of t10c12-CLA on fat synthesis could be regulated via the SREBP-1c transcription factor acting as a possible global regulator for milk fat synthesis. In addition, MFD was associated with decrease in *THRSP* expression (Harvatine and Bauman, 2006). We hypothesize that the regulation of mammary lipid synthesis could occur at multiple regulatory points. An increase in milk fat synthesis could be a result of up-regulated lipogenic gene expression as opposed to down-regulated lipogenic gene expression observed during MFD. The objective of the study was to determine the effects of abomasal infusion of butterfat, a source of short and medium chain fatty acids versus the LCFA on bovine mammary lipogenic gene expression.

## MATERIALS AND METHODS

*Animals, Experimental Design and Treatment*

Details of the animals and experimental design were reported previously (Kadegowda et al., 2008). Briefly, eight rumen-fistulated multiparous Holstein cows in early lactation (49±20 DIM) were used in a replicated 4x4 Latin square design. The treatments were: 1) Control (No infusion); 2) Butterfat (Post-ruminal infusion of 400 g/d butterfat as a source of short and medium chain FA); 3; LCFA (Post-ruminal infusion of 245 g/d long chain FA mixture delivering equivalent amount of long chain FA as found in 400 g of milk fat); and 4) CLA (Post-ruminal infusion of 100 g of

CLA mix providing 10 g of t10c12 CLA/d). Details on the composition of fat mixtures, infusion technique, and ingredient and nutrient composition of the diet have been previously described (Kadegowda et al., 2008). All procedures for this experiment were conducted under protocol R-06-41 approved by the University of Maryland Institutional Animal Care and Use Committee.

*Mammary Tissue Biopsy*

Mammary tissue samples were collected at the end of each period using a modified procedure described by Farr et al (1996). Briefly, a 1-2 cm scalpel (sterile) incision was made through the skin, and the mammary gland capsule punctured. A core of mammary tissue was taken from the gland using the biopsy tool, rotated by a slow speed electric motor. The core was cut, the retractable blade extended and the core was severed with a few revolutions of cutting blade of the biopsy tool. The mammary biopsy samples were immediately frozen in liquid $N_2$, and preserved at -80° C for RNA extraction.

Immediately after removal of the biopsy tool, pressure was applied until the visual signs of bleeding were absent. To reduce bleeding and swelling, a plug of Surgicel (Ethicon Ltd, Somerville, NJ), a cellulose absorbable hemostat, was inserted into the wound and closed with stitches. Antibiotic powder and single intramuscular injection of 100 mg of the systemic antibiotic Oxytetracycline was applied. Incision sites were inspected daily, kept clean and treated with topical antiseptic and/or aerosol bandage spray as needed. The cows were hand-milked for 3 days to thoroughly remove the blood clots lodged in the gland. The animals were monitored

for signs of site effects, fever, changes in defecation, feed intake and refusals, daily milk production and daily temperature.

*RNA Extraction*

Total RNA was extracted from ~0.5 g tissue using ice-cold TRIZOL reagent (Invitrogen, Carlsbad, CA). The RNA concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality was evaluated using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples (n=24) with an RNA integrity number of 6 to 9 were used for further analysis (Control (n=7), Butterfat (n=5), CLA (n=7), LCFA (n=5). Genomic DNA was removed with DNase using RNeasy Mini Kit columns (Qiagen, Valencia, CA). A portion of the assessed RNA was diluted to 100 mg/L using DNase-RNase free water prior to reverse transcription.

*Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)*

Sufficient cDNA was prepared to run all selected genes- *ACACA, FASN, SCD, AGPAT6, GPAT, DGAT1, LPL, ACSL1, ACSS2, CD36, SREBP1, SREBP2, LPIN1, SCAP, INSIG1, PPARγ, THRSP*. Each cDNA was synthesized by RT-PCR and cDNA used for qPCR previously described (Bionaz and Loor, 2007). Briefly, each cDNA was synthesized by RT using 100 ng RNA, 1 μL dT18 (Operon Biotechnologies, AL), 1 μL 10 mM dNTP mix (Invitrogen Corp.), 1 μL Random Primers (Invitrogen Corp., CA), and 7 μL DNase/RNase free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 9 μL of master mix composed of 4.5 μL 5X first-strand buffer, 1 μL 0.1 M DDT, 0.25 μL (100 U) of SuperScriptTM III

RT (Invitrogen Corp.), and 0.25 μL of RNase Inhibitor (Promega, WI) and 3 mL DNase/RNase free water were added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25°C for 5 min, 50°C for 60 min and 70°C for 15 min. The cDNA was then diluted 1:3 with DNase/RNase free water.

For qPCR analysis, 4 μL of diluted cDNA were combined with 6 μL of a mixture composed of 5 μL 1x SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 μL each of 10μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate and a 4-point relative standard curve (4-fold dilution) plus the non-template control were used (User Bulletin #2, Applied Biosystems). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) using the following conditions: 2 min at 50 °C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 sec plus 65°C for 15 sec. Complete details regarding qPCR protocol can be found at http://docs.appliedbiosystems.com/pebiodocs/04364014.pdf. Data were analyzed with the 7900 HT Sequence Detection Systems Software (version 2.2.3, Applied Biosystems).

***Primer Design and Testing***

Primer Express 3.0 software (Applied Biosystems), optimized for use with Applied Biosystems qPCR Systems, was used for primers design using default features, except for the amplicon length, which was fixed at minimum of 100 bp .

Primers were designed across exon junctions when possible to avoid amplification of genomic DNA. The exon junctions were uncovered blasting the sequence against bovine genome(Genome Browser Gateway, 2008). Primers were aligned against publicly available sequences in NCBI (National Center for Biotechnology Information, 2008) and UCSC(Genome Browser Gateway, 2008). The primer sequences are presented in Appendix 1. Prior to qPCR, primers were tested using the same protocol as for qPCR without the dissociation step in a 20μL reaction. Part of the PCR product was analyzed in a 2% agarose gel stained with ethidium bromide to assess presence of the product to an expected size and the presence of primer-dimer, with the remainder being purified using Qiaquick PCR purification kit (Qiagen) and sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana. Only primers with high specificity evaluated by a single band on agarose gel, absence of primer-dimer, amplification of the target cDNA verified by sequencing, and a unique peak in the dissociation curve after qPCR reaction were used.

*Statistical Analysis*

A normalization factor calculated as a geometric mean of 4 internal control genes (*EIF3K, GPR175, MRPL39* and *UXT*) using geNorm was used to normalize the qPCR data. The Mixed procedure in the Statistical Analysis System (Version 8.2, 2004, SAS Institute, Cary, NC) was used to evaluate the treatment effects on the normalized mRNA abundance. Model fixed effects were the treatments while the random effects included cow, square and period. Probability of ($P \leq 0.05$) was considered as statistically significant.

The qPCR data are presented as percentage change relative to the Control group. The standard error for each treatment was estimated as described by Bionaz and Loor (2008a). Briefly, normalized data were transformed to obtain a perfect mean of 1.0 for Control, leaving the proportional difference between the biological replicate. The same proportional change was calculated in all the treatments to obtain a fold change relative to Control.

## RESULTS

The relative mRNA abundance of 20 genes related to FA uptake, transport, FA and triacylglycerol (**TAG**) synthesis and transcriptional regulation are presented in Table 6.1, Table 6.2 and Figure 6.1. The greatest relative mRNA abundance were observed for *SCD* (29.99%), *FASN* (23.57%), *FABP3* (18.22%) and *CD36* (10.39%) which accounted for 82% of the overall mRNA abundance. The genes *ACACA* (0.94%), *ACSS2* (0.9%), *DGAT1* (0.08%), *PPARG* (0.08%), *SCAP* (0.09%), *THRSP* (0.02%) and *ChREBP* (0.001%) had lower expression. This low abundance is reflected in the median threshold cycle (Ct), which is the cycle number at which the sequence detector was able to amplify the product (Figure 6.2).

The mRNA abundance of *LPL*, a key enzyme involved in the hydrolysis of plasma triacylglycerol and *CD36*, a plasma membrane-bound protein involved in cellular FA entry were affected by the FA infusions. Compared to Control, butterfat infusion significantly increased *LPL* expression (47%, $P < 0.05$) and tended to increase *CD36* (47%, $P = 0.25$, Figure 6.5). Conversely, LCFA treatments tended ($P<0.3$) to decrease the expression of *LPL* (-187%, Figure 6.3) and *CD36* (-61%,

Figure 6.5) while CLA did not affect *LPL* expression but tended to increase the expression of *CD36* (35%, $P < 0.2$, Figure 6.5).

Compared to Control, the expression of *ACSL1* and *ACSS2*, genes related to intra-cellular activation of fatty acids, were up regulated with Butterfat (47.87% and 62.66%, respectively) but were not affected by other treatments (Figure 6.5). The expression of *FABP3*, a gene regulating intra-cellular FA transport was significantly (44%, $P < 0.05$) up-regulated with Butterfat, but was down-regulated with LCFA (-47%, Figure 6.5). The expression of *FABP4* was down regulated ($P<0.3$) with LCFA and t10c12-CLA, but was not affected with Butterfat infusion when compared to Control.

Butterfat infusion increased the mRNA abundance of *ACACA* (35%, $P < 0.12$) and *FASN* (40%, $P < 0.13$), genes related to FA synthesis; *SCD* (40%, $P < 0.27$, Figure 6.3), a gene related to desaturation; *AGPAT6* (43%, $P < 0.12$, Figure 6.4), *DGAT1* (13%, $P < 0.13$) and *GPAM* (43%, $P < 0.05$), genes related to triacylglycerol synthesis (Figure 6.3 and Figure 6.4). LCFA infusion decreased the expression of *FASN* (-101%, $P < 0.07$) while CLA infusion did not affect the expression of any genes related to FA and TAG synthesis or desaturation (Figure 6.3 and Figure 6.4).

The mRNA abundance of the transcriptional regulators *SREBF1, SREBF2, THRSP, ChREBP* and *LPIN1,* the nuclear receptor *PPARG*, and the transcriptional co-activators *INSIG1* and *SCAP* were measured to elucidate the mechanisms involved in regulation of lipogenic genes (Figure 6.6). Butterfat infusion tended to increase the expression of *SREBF1* (30%), *SREBF2* (25%), *THRSP* (23%), *LPIN1* (46%) and *INSIG1* (30%) but tended to decrease *SCAP* (-84%, $P<0.2$). However, LCFA

infusions tended to decrease the expression of *PPARG* (-109%, $P < 0.2$), *SREBF1* (-47%), *THRSP* (-52%) and *SCAP* (-77%). The infusion of CLA tended to decrease the expression of *SREBF1* (-36%) and *SCAP* (-77%).

## DISCUSSION

The studies on the regulation of milk fat synthesis due to dietary FA in lactating dairy cows have largely focused on the effects of feeding or abomasal infusions of CLA, particularly t10c12-CLA (Piperova et al., 2000; Harvatine and Bauman, 2006), and/or LCFA (Banks et al., 1990; Drackley et al., 1992; Enjalbert et al., 2000). Some of the studies with CLA have examined the changes in mammary lipogenic gene expression or activity, in addition to changes in the milk FA profile (Piperova et al., 2000; Baumgard et al., 2002; Peterson et al., 2003; Harvatine and Bauman, 2006). Effects of LCFA on mammary lipogenesis have focused on changes in milk FA profile (Enjalbert et al., 2000) and not gene expression. To my knowledge other data describing the effect of SCFA and MCFA in regulation of mammary lipid synthesis in dairy cows are not available.

Abomasal infusion of butterfat, a source of short- and medium-chain FA increased the mRNA abundance of *LPL* and *CD36*, while an opposite effect was observed with the LCFA treatment. The differences in responses could be attributed to the short- and medium-chain FA as both Butterfat and LCFA treatments contained equal amounts of LCFA. The enzyme LPL plays a role in the hydrolysis of plasma triacylglycerols and is critical for the delivery of dietary FA to the mammary gland (Fielding and Frayn, 1998). In turn, CD36 is a plasma membrane-bound protein involved in cellular FA entry. Butterfat treatment increased *LPL* and *CD36*

expression most likely due to increased availability of substrates (TAG), which determines their utilization by the mammary gland (Annison et al., 1968). Abomasal (Enjelbert et al., 2000) or duodenal (Gangliostro et al., 1991) infusion of lipid supplements could trigger increased apparent mammary FA uptake. Mammary uptake of LCFA could also be affected by the type and means of lipid supplementation. Ahnadi et al. (2002) reported decreased milk 18:0 and 18:1 concentrations and decreased *LPL* expression in lactating cows due to dietary fish oil supplementation compared to un-supplemented control. In this study, LCFA infusion decreased *LPL* and *CD36* expression but increased milk LCFA concentration and yield (Kadegowda et al., 2008) suggesting that some of the infused FA were taken up by the mammary gland.

Mammary FA uptake is driven by intra-cellular FA activation, FA binding to intracellular fatty acid binding proteins (**FABP**) and intracellular metabolism (TAG synthesis) (Mashek and Coleman, 2006). Fatty acid activation is important to prevent the FA efflux out of the mammary cells (Mashek and Coleman, 2006) and would aid FA binding to FABP. The genes *ACSL1* and *FABP3* are the dominant genes related to FA activation and transport in lactating dairy cows (Bionaz and Loor, 2008). The protein ACSLI is located on the plasma membrane (Doege and Stahl, 2006) while FABP is shown to interact with *CD36* (Spitsberg et al., 1995). The location of these proteins enables the mammary cells to trap the FA inside the cells. In this study, Butterfat infusion increased the expression of *ACSL1*, *ACSS2, FABP3 and FABP4*, suggesting that the FA were immediately activated and transported for TAG synthesis in the mammary cells. Increased *ACSL1* expression could have enhanced the uptake

of infused FA in this treatment. Marszalek et al. (2004) reported that over-expression of *ACSL1* and *ACSL2* isoforms in differentiated neurons increased uptake rates of oleic, arachidonic and docosahexaenoic acids by 25 to 115%. The increased expression of *ACSS2,* a gene related to activation of acetate to acetyl CoA, could be a consequence of higher palmitic acid provided with the Butterfat treatment. It was observed significant increase in *ACSS2* expression when MAC-T cells were exposed to 100 μM of 16:0 but not to 18:0, 18:1, t10-18:1, t10c12-CLA or 20:5 (Kadegowda et al., 2008). In this study, the LCFA treatment did not affect *ACSS2* expression. However, the LCFA mixture contained only 50% of the palmitic acid provided with the Butterfat treatment suggesting that concentration of palmitic acid could be a factor in regulation. LCFA treatment decreased the expression of *FABP3* and *FABP4,* which correlated with the decreased expression of genes related to FA uptake.

Butterfat infusion up regulated the expression of genes related to FA synthesis (*ACACA, FASN*), FA desaturation (*SCD*) and TAG synthesis (*GPAM, DGAT1, AGPAT6*). These changes were correlated with increase in milk FA yield and significantly higher concentrations of MCFA (14:0, 14:1, 15:0), c9-18:1 and 18:2 (Kadegowda et al., 2008). This suggested that the incorporation of SCFA and MCFA provided with the infused butterfat into milk fat could have activated the mammary FA-synthesizing machinery. The increased availability of acetyl CoA due to up regulation of *ACSS2* expression could have increased expression of *ACACA,* the gene responsible for conversion of acetyl CoA to malonyl CoA. The increased availability of malonyl CoA in addition to other substrates (SCFA and MCFA) provided by the butterfat correlated with up-regulated *FASN* gene expression. In ruminants, *FASN*

contains a loading acyltransferase with wider substrate specificity and is capable of loading and releasing short- and medium-chain FA (Barber et al., 1997). Therefore, the increase in milk c9-18:1 in the Butterfat treatment could be a consequence of increased *FASN* which would elongate the short and medium chain FA and *SCD* expression which would desaturate the elongated FA.

Butterfat infusion significantly increased the expression of *GPAM* gene that catalyzes the first step in TAG formation, and *AGPAT* and *DGAT* genes involved in the subsequent steps of TAG synthesis. Butterfat contains substantial amounts of palmitic acid, an important FA for TAG synthesis in the mammary gland (Hansen and Knudsen, 1987). Initiation of acylation of *sn*-1 position is prerequisite for TAG synthesis and palmitic acid is the preferred substrate for the initial acylation of L-α glycerol phosphate by *GPAM* to form *sn*-1-lysophasphatidic acid (Kinsella and Gross, 1973). We can speculate that the increase observed in *GPAM* expression coupled with greater availability of palmitic acid could have stimulated the synthesis of phosphatidic acid. Again, increased *AGPAT6* expression and the availability of MCFA would increase the diacylglycerol synthesis. Kinsella and Gross (1973) have showed that myrystyl, stearyl and oleyl CoA were used in the second step of TAG synthesis and are rapidly acylated when *sn*-1-lysophasphatidic acid was used as substrate. Finally, an increase in *DGAT1* with the higher availability of SCFA, the preferred substrates for the *sn*-3 position would increase milk TAG output.

In contrast to Butterfat, the LCFA treatment decreased *FASN* expression which was reflected in the reduced concentrations of <16:0 FA in milk. Long-chain FA (Drackley et al., 2007; Palmquist et al., 1993) and PUFA (Ahnadi et al., 2002) are

known to decrease concentrations of FA synthesized *de novo* suggesting down-regulation of lipogenic genes or decreased enzyme activity. Ahnadi et al. (2002) reported decreased expression of *ACACA* and *FASN* leading to 60% reduction in *de novo*-synthesized FA in dairy cows supplemented with fish oil.

***Possible Molecular Mechanisms Involved In Regulation Of Lipid Synthesis.***

The coordinated increase in all genes related to FA synthesis, uptake, transport and TAG synthesis due to Butterfat infusion and the opposite effect observed with LCFA treatment suggested that the FA effects could be mediated through a common regulatory mechanism at the transcriptional level. Although, molecular mechanisms regulating lipid synthesis in lactating dairy cows are not well established (Bernard et al., 2006; Shingfield and Griinari, 2007), a role for *SREBP1* in regulation of mammary FA synthesis has been suggested (Peterson et al., 2004; Harvatine and Bauman, 2006).

The transcription factor SREBP1 is synthesized as a precursor protein, is associated with SCAP and is anchored to the endoplasmic reticulum membrane through INSIG proteins (Horton et al., 2002; Yang et al., 2002). Upon detaching from INSIG proteins, SREBP1 is escorted by SCAP to golgi and is processed to an active fragment, before translocation to the nucleus to activate the lipogenic genes (Rawson, 2003). The tendency of SREBP1 to increase with Butterfat infusion but to decrease with LCFA suggested that at least part of the effects of the infused FA could have been mediated through this transcriptional regulator. In addition, the co-activators INSIG1 and SCAP involved in the formation of SREBP1 nuclear active fragment, could play a role in the differences observed. The decreased SCAP expression with

LCFA suggested reduced synthesis of active nuclear fragment, which in turn would decrease the overall lipogenic gene expression. On the other hand, Butterfat treatment up regulated *INSIG1* and down regulated *SCAP* expression, both negatively correlated to SREBF1 activity, in contrast to the overall (increased) lipogenic gene expression. Bionaz and Loor (2008b) have suggested an important role for *INSIG1* in milk fat synthesis in bovine mammary gland. The expression of *INSIG1 and SREBF1* increased during peak lactation and the mRNA abundance of *INSIG1* was higher than that of *SREBF1* (Bionaz and Loor 2008b). Similar results for *INSIG1* mRNA abundance were observed in this study (Figure 1.). Bionaz and Loor (2008b) have suggested that the increased *INSIG1* expression could be due to increased *SREBF1* and *SREBF2* expression or a consequence of its short half-life.

It is possible that the amounts of active nuclear *SREBP* produced with Butterfat treatment could have been enough to increase the overall lipogenic gene expression. Butterfat infusate contained a mixture of FA which could have also expressed individual effects on different transcriptional regulators. It was demonstrated (Kadegowda et al., 2008) that feeding t10c12-CLA decreased the mRNA abundance of *SREBP1, ChREBP* and *PPARG* while PHVO containing a mixture of trans-18:1 FA affected only *SREBP1* expression in lactating mice.

*PPARG* is a nuclear receptor involved in regulation of genes related to FA uptake and transport (Desvergne, 2006). Bionaz and Loor (2008b) reported up regulation of *PPARG* expression in lactating mammary tissue of dairy cows and have suggested a key role of this gene in milk fat synthesis. In this study, infusion of LCFA

decreased *PPARG* expression (-109%) which correlated with reduced expression of genes related to FA uptake and transport.

The LCFA treatment decreased the expression of *THRSP,* a nuclear protein associated with regulation of *de novo* lipid synthesis in lipogenic tissues (Kinlaw et al., 1995). Although, the exact function of *THRSP* is unknown, it was speculated that it functions as an allosteric regulator of lipogenic genes (LaFave et al., 2006). Zhu et al. (2005) demonstrated that milk TAG content in *THRSP*-null mice was significantly reduced due to a decrease in *de novo* lipid synthesis. Similarly, Harvatine and Bauman (2006) reported that diet-induced MFD and t10c12-CLA treatment down regulated the expression of *THRSP* in mammary gland of lactating cows. Kadegowda et al (20008) observed a decrease in *THRSP* mRNA expression, proportional to the degree of MFD in mice fed t10c12-CLA, PHVO and t7-18:1.

The *LPIN1*, a gene related to diacylglycerol synthesis is expressed in the bovine mammary tissue (Bionaz and Loor, 2008b). Bionaz and Loor (2008b) have reported an increase in *LPIN1* expression during lactation and have suggested a possible regulatory role in milk fat synthesis. In this study, the *LPIN1* gene expression was up regulated by three folds with the fat infusions suggesting possible association between the availability of FA substrates and gene expression.

Data from this study reported earlier (Kadegowda et al., 2008) showed that the CLA infusion decreased milk fat percentage and yield up to 45%. However, the CLA infusion providing 10g of t10c12-CLA/d did not alter mammary lipogenic gene expression.

The role of t10c12-CLA in decreasing *de novo* FA synthesis is established in lactating dairy cows (Baumgard et al., 2000). The degree of MFD depends on the quantity of t10c12-CLA (Peterson et al., 2003). Infusion of t10c12-CLA around 10g/d has decreased milk fat up to 45% in addition to decreased mRNA expression of genes related to FA uptake, synthesis, desaturation and TAG synthesis (Baumgard et al., 2002; Harvatine and Bauman, 2006). Although, it was observed that the degree of MFD was similar to these studies, the mRNA expression of lipogenic genes was not affected. The major difference between the studies was the duration of infusion 3 to 5 days in earlier studies vs. 14 days in the present experiment. This could be due to a possible effect of time on the lipogenic gene response to t10c12-CLA infusion, where the animal would try to maintain physiological (lactational) homeostasis. However, dietary supplementation of Ca-salts of t1012-CLA consistently reduced milk fat yield for 20 wks (Perfield et al., 2002; Bernal-Santos et al., 2003), but the lipogenic gene response was not examined in these studies.

Lack of lipogenic response to lipid supplementation have been reported earlier in lactating beef cows (Murrieta et al., 2006) and lactating goats (Bernard et al., 2005). Dietary high linoleate safflower seed supplementation in lactating beef cows significantly decreased the MCFA but did not affect the levels of *ACACA, FASN* and *SCD* mRNA(Murrieta et al., 2006). Similarly, Bernard et al., (2005) reported decreased C10:0- C17:0 FA without any effects on the *ACC* and *FAS* expression in lactating goats supplemented with oleic sunflower oil.

In conclusion, the differences observed in the lipogenic gene response between the Butterfat and LCFA treatments suggest that milk fat synthesis is

regulated differently when SCFA and MCFA are provided. Availability of FA substrates coupled with increased lipogenic gene expression lead to increased milk fat synthesis in lactating dairy cows infused with butterfat. Although, LCFA infusion decreased lipogenic gene expression, milk fat content was maintained due to an increase in milk yield. Infusion of CLA decreased milk fat but did not affect mammary lipogenic gene expression, suggesting possible involvement of other regulatory mechanisms in milk fat synthesis during long term CLA infusion.

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Table 6.1. Slope, coefficient of determination of standard curve (R2), efficiency (E), median Ct, and mRNA abundance of measured transcripts.

| Gene | Median Ct[1] | Median ΔCt[2] | Slope | $(R^2)$[3] | Efficiency [4] | mRNA % abundance[5] |
|---|---|---|---|---|---|---|
| *ACACA* | 21.5 | -0.3 | -2.99 | 0.998 | 2.16 | 0.94 |
| *ACSL1* | 20.4 | -1.3 | -3.30 | 0.997 | 2.01 | 1.80 |
| *ACSS2* | 21.3 | -0.3 | -3.37 | 0.997 | 1.98 | 0.90 |
| *AGPAT6* | 21.0 | -0.7 | -3.27 | 0.996 | 2.02 | 1.21 |
| *CD36* | 18.6 | -3.4 | -3.02 | 0.998 | 2.14 | 10.18 |
| *ChREBP* | 32.5 | 11.0 | -2.83 | 0.997 | 2.26 | 0.00 |
| *DGAT1* | 24.6 | 3.1 | -3.15 | 0.999 | 2.08 | 0.08 |
| *FABP3* | 16.9 | -4.7 | -3.40 | 0.996 | 1.97 | 18.22 |
| *FABP4* | 20.3 | -1.3 | -3.09 | 0.998 | 2.11 | 1.99 |
| *FASN* | 17.0 | -4.9 | -3.24 | 0.998 | 2.04 | 23.56 |
| *GPAM* | 19.8 | -2.2 | -3.31 | 0.997 | 2.01 | 3.35 |
| *INSIG1* | 21.5 | -0.1 | -3.11 | 0.998 | 2.10 | 0.79 |
| *LPIN1* | 22.2 | 0.6 | -3.22 | 0.996 | 2.04 | 0.47 |
| *LPL* | 19.5 | -2.6 | -3.17 | 0.997 | 2.07 | 4.74 |
| *PPARG* | 24.7 | 3.0 | -3.18 | 0.998 | 2.06 | 0.08 |
| *SCAP* | 24.8 | 3.2 | -3.40 | 0.997 | 1.97 | 0.09 |
| *SCD* | 16.9 | -5.2 | -3.24 | 0.995 | 2.03 | 29.98 |
| *SREBF1* | 22.4 | 0.9 | -3.31 | 0.997 | 2.00 | 0.39 |
| *SREBF2* | 23.2 | -0.6 | -2.91 | 0.995 | 2.21 | 1.22 |
| *THRSP* | 27.3 | 5.5 | -3.67 | 0.998 | 1.87 | 0.02 |

[1] The median is calculated considering all time points and all cows.
[2] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each cow.
[3] $R^2$ stands for the coefficient of determination of the standard curve.
[4] Efficiency is calculated as $[10^{(-1 / \text{Slope})}]$.
[5] mRNA abundance = $1/E^{(\text{Median}\Delta\text{ Ct})}$.

Table 6.2. Abundance of genes measured**.** Percent relative mRNA abundance and median Ct of genes measured by qPCR.

| Gene | Cellular Location | Type | mRNA abundance | Median Ct |
|---|---|---|---|---|
| *ACACA* | Cytoplasm | enzyme | 1.2741 | 21.5 |
| *ACSL1* | Cytoplasm | enzyme | 2.4404 | 20.4 |
| *ACSS2* | Cytoplasm | enzyme | 1.2287 | 21.3 |
| *AGPAT6* | Cytoplasm | enzyme | 1.6469 | 21.0 |
| *CD36* | Plasma Membrane | other | 13.8382 | 18.6 |
| *DGAT1* | Cytoplasm | enzyme | 0.1024 | 24.6 |
| *FABP3* | Cytoplasm | transporter | 24.7526 | 16.9 |
| *FABP4* | Cytoplasm | transporter | 2.6973 | 20.3 |
| *FASN* | Cytoplasm | enzyme | 32.0157 | 17.0 |
| *GPAM* | Cytoplasm | enzyme | 4.5521 | 19.8 |
| *INSIG1* | Cytoplasm | other | 1.0710 | 21.5 |
| *LPIN1* | Nucleus | other | 0.6424 | 22.2 |
| *LPL* | Cytoplasm | enzyme | 6.4355 | 19.5 |
| *MLXIPL* | Nucleus | transcription regulator | 0.0001 | 32.5 |
| *PPARG* | Nucleus | ligand-dependent nuclear receptor | 0.1107 | 24.7 |
| *SCAP* | Cytoplasm | other | 0.1164 | 24.8 |
| *SCD* | Cytoplasm | enzyme | 40.7415 | 16.9 |
| *SREBF1* | Nucleus | transcription regulator | 0.5265 | 22.4 |
| *SREBF2* | Nucleus | transcription regulator | 1.0117 | 23.2 |
| *THRSP* | Nucleus | other | 0.0322 | 27.3 |

Figure 6.1. Percent relative mRNA abundance of measured lipogenic genes in bovine mammary gland (n=24)

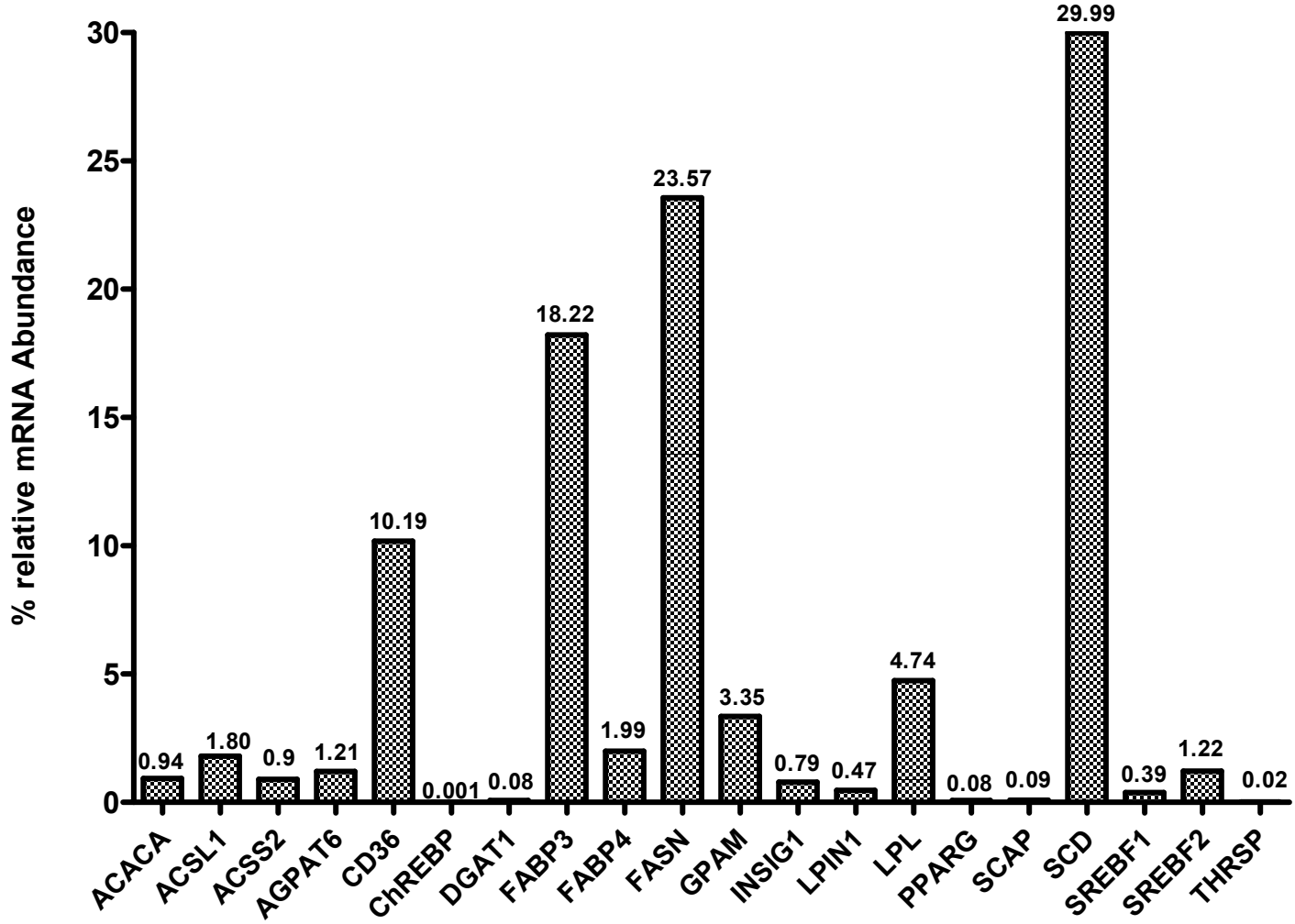


Figure 6.2. Median Ct values of measured lipogenic genes in bovine mammary tissue (n=24).

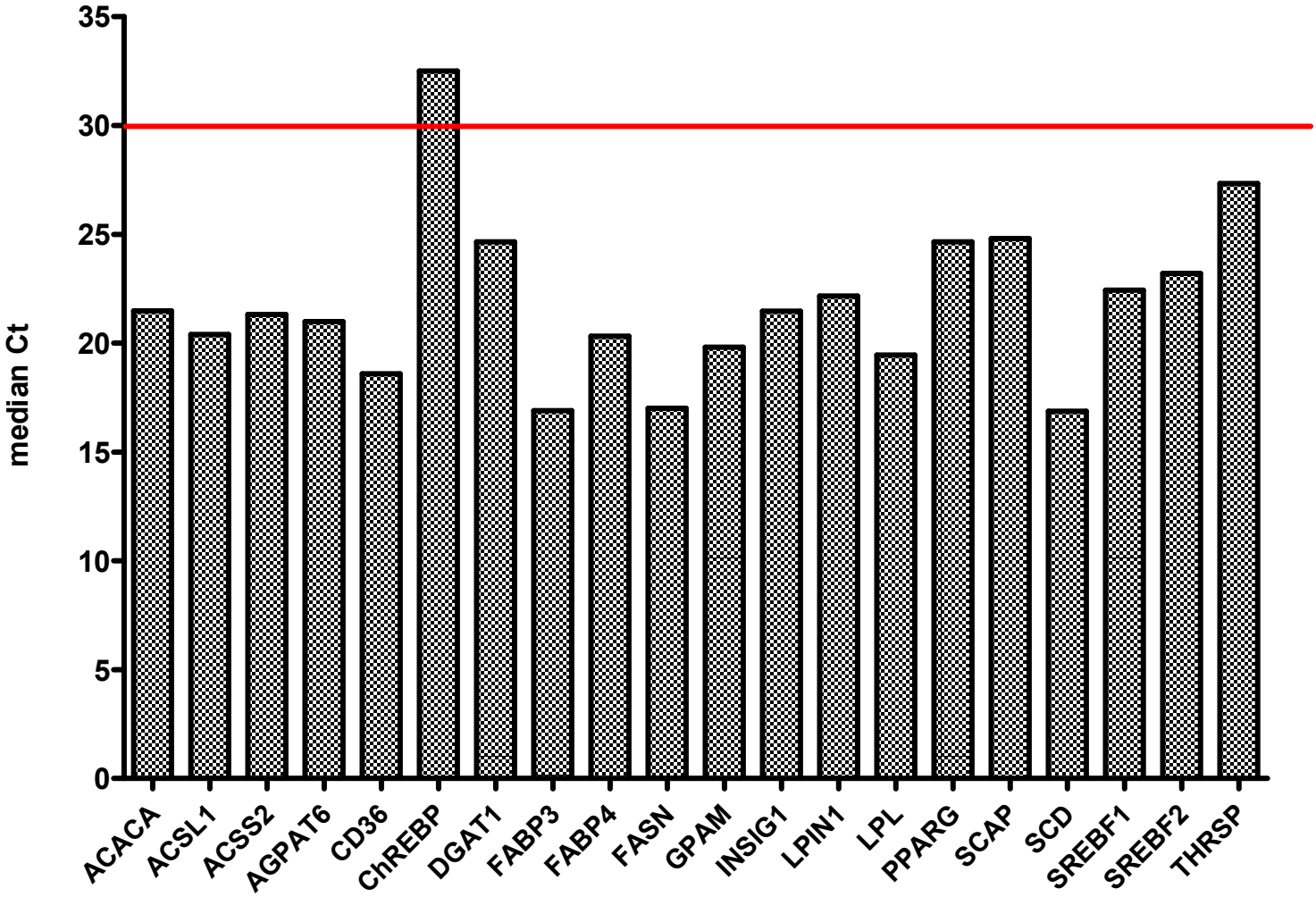
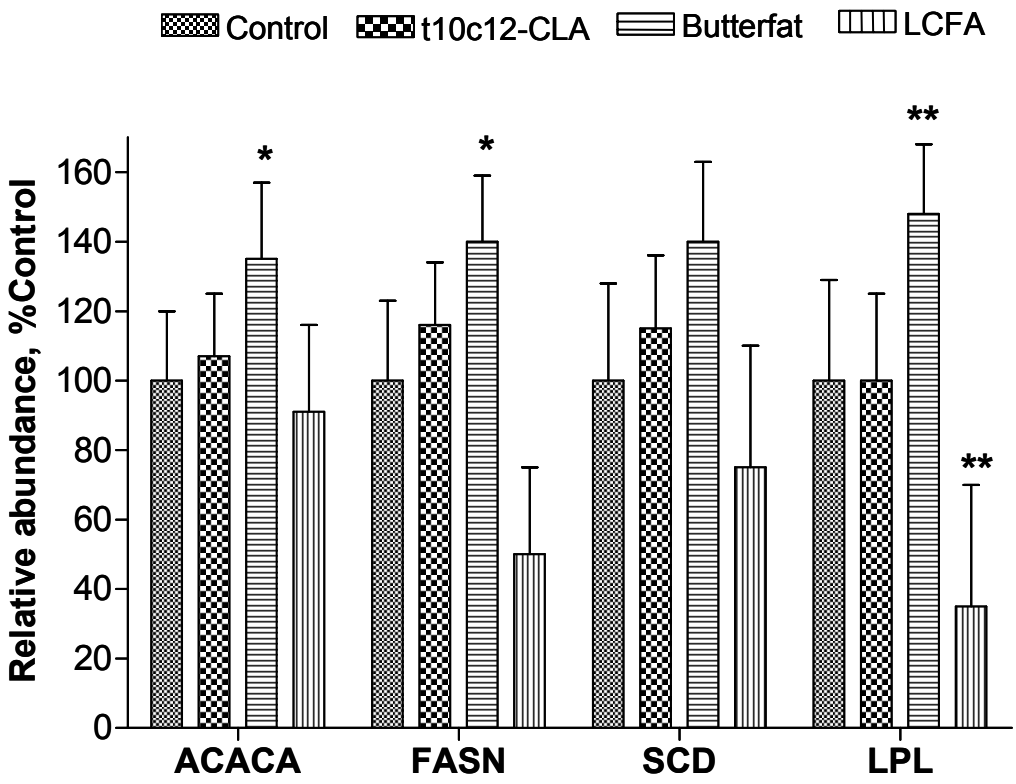
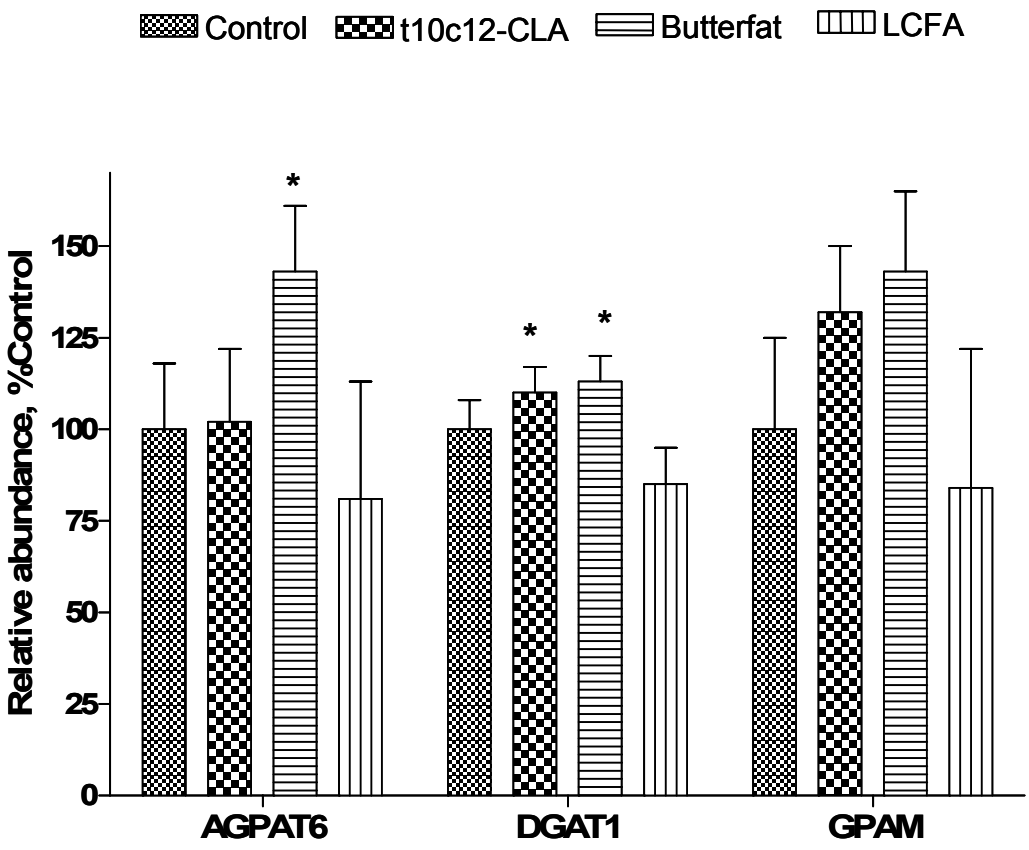


Figure 6.3. Treatment effects on genes related to fatty acid synthesis, desaturationa and fatty acid uptake in bovine mammary samples (n=24).




** $P < 0.05$
* $P<0.15$

Figure 6.4. Treatment effects on genes related to triacylglycerol synthesis in bovine mammary samples (n=24)




* $P < 0.15$

Figure 6.5. Treatment effects on genes related to fatty acid transport and activation in bovine mammary samples (n=24).

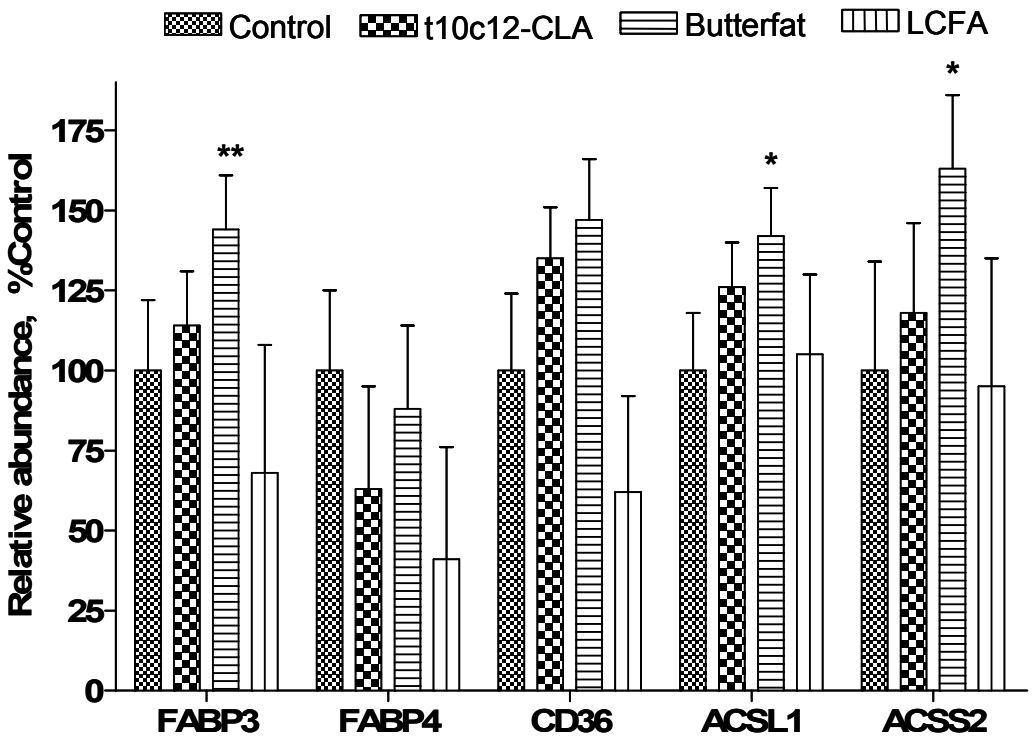
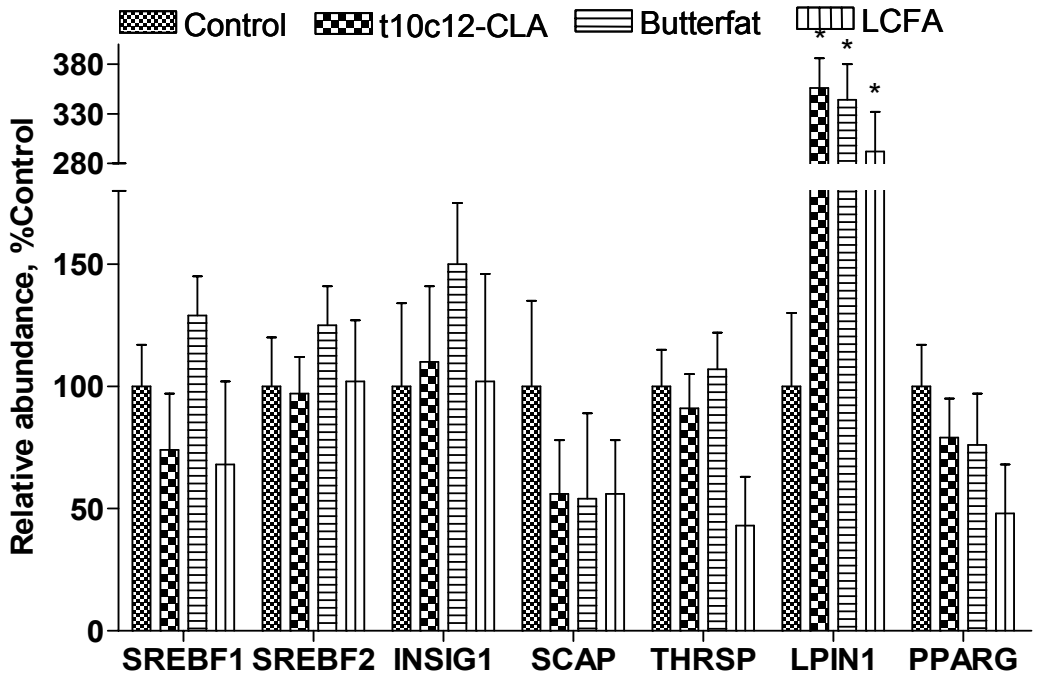
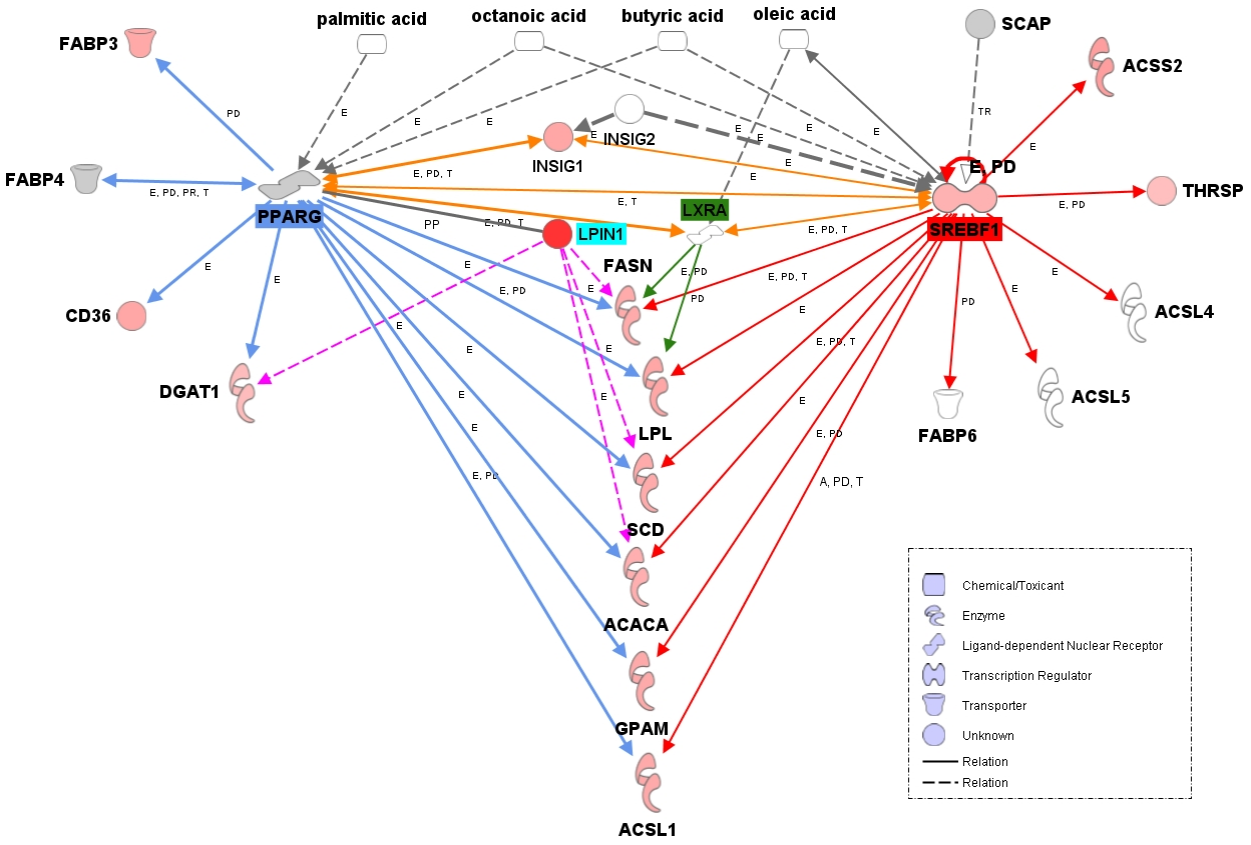


Figure 6.6. Treatment effects on mammary transcriptional regulators in bovine mammary samples.




* $P < 0.05$

Figure 6.7. Gene networks developed using IPA showing potential regulatory mechanisms involved in lipogenesis.



# Chapter 7: EXPERIMENT 5

## Lipogenic gene expression in MAC-T cells is affected differently by fatty acids and enhanced by PPAR-gamma activation[1]

[1]**Preseneted in part at the Annual ADSA-ASAS meeting, 2008.**

**A.K.G. Kadegowda, M. Bionaz, L. S. Piperova, R. A. Erdman and J. J. Loor. 2008. J. Dairy Sci. Suppl. 1. 566.**

## ABSTRACT

Recent work has focused on trans10,cis12-CLA (t10c12CLA) and its effect on mammary lipogenic gene expression, while the effects of other fatty acids (FA) remain ill-defined. Objectives were to test individual FA effects on mRNA expression via qPCR of 19 genes with roles in *de novo* synthesis (*ACACA, FASN*), FA uptake (*LPL, CD36*), intracellular FA transport (*FABP3, FABP4*), desaturation (*SCD*), triacylglycerol (TAG) synthesis (*AGPAT6, DGAT1, GPAM, LPIN1*), transcriptional regulation (*SREBF1, SREBF2, INSIG1, THRSP*), and nuclear receptor signaling (*PPARγ*). A PPARγ-specific agonist (Rosiglitazone, ROSI) was used to assess the role of this nuclear receptor on mammary lipogenesis. Lipid droplet (LD) formation was quantified with Oil Red O staining. MAC-T cells were cultured in triplicate for 12 h with 50 μM ROSI or 100 μM t10-18:1, t10c12CLA, 16:0, 18:0, c9-18:1, 20:5, or ethanol (control). Except for 16:0 and 18:0, all other FA increased ($P < 0.05$) *CD36* expression (~770%) and, decreased *LPL* (~150%) and *FABP3* (~200%) relative to control. 16:0 and 18:0 elicited greater mRNA of *FABP3* (+500%) and *FABP4* (+160%) over control. Responses common to 16:0 and 18:0 included greater *THRSP* (~90%), *INSIG1* (~200%), *AGPAT6* (~150%), *DGAT1* (~60%), and *LPIN1* (~90%), coupled with greater LD formation with 16:0. Trans10-18:1 and t10c12CLA reduced expression of *ACACA* (~60%), *FASN* (~50%), *SCD* (~240%), and *LPIN1* (~30%). *SREBF1* was lower with t10c12-CLA (~200%), c9-18:1 (150%), and EPA (~140%) over control. Cis9-18:1 and EPA also decreased *ACACA* (~40%) and *SCD* (~300%). No effects were observed for *PPARγ* but ROSI up regulated by >40%

*ACACA*, *FASN, SCD, LPIN1, AGPAT6, DGAT1, SREBF1, SREBF2*, and *INSIG1* without changes in LD formation over control. Results showed that FA regulate mammary lipogenic gene expression to different extents. Further, PPAR-gamma activation of *de novo* lipogenesis coupled with exogenous FA availability might play a role in regulating milk fat synthesis.

**Key words**: fatty acids, lipogenic gene expression, PPARγ , Rosiglitazone

# INTRODUCTION

Lactating mammary tissue synthesizes short and medium chain fatty acids while long chain FA (LCFA) are supplied from the plasma (Dils, 1983). Plasma non-esterified fatty acids (NEFA) originate mainly from the diet and also from adipose tissue mobilization (Dils, 1983). Mammary uptake of LCFA depends on the plasma NEFA concentrations (McArthur et al., 1999). Once taken up by the mammary cells, LCFA are incorporated into triacylglycerols (TAG). It is known that LCFA affect *de novo* syntheses of short and medium chain FA (Hansen and Knudsen, 1987) and they also have been shown to regulate gene transcription in bovine kidney epithelial cells (MDBK) (Thering et al., 2007; Bionaz et al., 2008).

In non-ruminants, most LCFA, and specifically polyunsaturated fatty acid (PUFA) are known to affect lipogenic gene expression through peroxisomal proliferator activator receptors (PPARs). However, data showing similar effects in bovine mammary epithelial cells are lacking. Recently, Bionaz and Loor (2008b) demonstrated that PPARγ nuclear receptor was up-regulated by more than three-fold at the onset of lactation and maintained throughout lactation in dairy cows. Simultaneously, increases in genes related to FA uptake, transport, synthesis and desaturation (Bionaz and Loor, 2008a) were also observed suggesting that part of the LCFA effects could be mediated through PPARγ. It has been reported (Harvatine and Bauman, 2006), , FA could regulate lipogenic gene expression through *SREBP-1c* and *THRSP* in mammary cells. However, these are most likely indirect effects as FA can not bind directly to these transcriptional regulators. On the other hand, FA are

natural ligands of PPARγ and can directly bind to the nuclear receptor and affect its target genes (Berger and Moller,2002).

We hypothesized that FA regulate mammary lipogenic gene expression through multiple transcriptional regulators, where in addition to *SREBF1* and *THRSP*, *PPARγ* could play an important role. The objective of this experiment was to determine the effects of individual FA on mammary lipogenic gene expression and to ascertain the role of *PPARγ* in lipogenic gene regulation.

## MATERIALS AND METHODS

### *Cell Culture and Treatments*

Bovine mammary epithelial cells (MAC-T) (Provided by Dr. Dan Peterson, Cal Poly San Luis Obispo) were cultured in minimum essential medium/Earle's Balanced Salts HyQ (MEM/EBSS, HyClone, Logan, UT) with insulin (5mg/L, Sigma, I6634, St Lois, MO), hydrocortisone (1mg/L, Sigma, H0888), transferrin (5 mg/L, Sigma, T1428), ascorbic acid (5μM/L, Sigma,A4544), sodium acetate(5mM/L, Sigma, S5636, St Lois, MO), fetal bovine serum (10%, GIBCO®, Invitrogen, Carlsbad, CA), progesterone(1mg/L, Sigma P8783), lactalbumin (0.05%, Sigma, L5385), α-lactose (0.05% ,Sigma,47287-U,) and Penicillin/Streptomycin (10ml/L, HyClone, sv30010, Logan, UT) in sterile cell culture flasks. Cell cultures were maintained in a water-jacketed incubator (Forma Scientific, Model 3158, Marietta, OH) with 5% $CO_2$ at 37 °C. The medium was changed every 24 h. The cells were transferred to a lactogenic medium consisting of a high glucose

Dulebecco's Modified Eagle's Medium (HG-DMEM, HyClone, Logan, UT) with bovine serum albumin (1g/L), prolactin (2.5 mg/L) when the cells were ~70% confluent. The cells were cultured for 24h (~100% confluent) before initiating the treatments.

Treatments were Control (NaOH +EtOH), palmitic acid (16:0; N-16-A, Nu-Chek Prep, Inc., Elysian, MN), stearic acid (18:0; N-18-A, Nu-Chek Prep, Inc., Elysian, MN), oleic acid (*cis*9-18:1; 1022, Matreya, Pleasant Gap, PA), *trans*10-18:1 (kindly provided by Dr. M. Pete Yurawecz, FDA), *trans*10, *cis*12 CLA (#1249, Matreya, Pleasant Gap, PA), eicosapentaenoic acid (20:5n-3, EPA; #N-20-A, NuChek Prep, Inc., Elysian, MN), and Rosiglitazone (Cayman Chemical, Ann Harbor, MI).

In the FA treatments, treatment FA were saponified with 95:5 ethanol:NaOH solution to obtain a stock concentration of 30 m*M* fatty acid/L that was used to provide a final cell culture concentration of 100uM. Cells were treated for a period of 12h and the treatments were done in triplicate. Total cell counts (concentration/ml) were recorded with Coulter Particulate Counter (Beckman Coulter). The intra-cytoplasmic lipid droplets in cells were visualized with Oil O Red staining. Amounts of intra-cytoplasmic triglyceride content were determined by extracting and quantifying Oil O Red stain at 510 nm as suggested by Ramriez-Zacarias et al.(1992). Cells were harvested in 1 mL TRIzol® reagent (Invitrogen, Carlsbad, CA) and stored at -80 °C until RNA extraction.

***RNA Extraction and Quantitative RT-PCR***

Total RNA was extracted from the cells using ice-cold TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) as explained by Loor et al.,(2005). The RNA

concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Genomic DNA was removed with DNase using RNeasy Mini Kit columns (Qiagen, Valencia, CA). A portion of the assessed RNA was diluted to 100 mg/L using DNase-RNase free water prior to RT.

Sufficient cDNA was prepared to run all selected genes. Each cDNA was synthesized by RT-PCR and cDNA was used for qPCR as previously described (Bionaz and Loor, 2007). Briefly, each cDNA was synthesized by RT using 100 ng RNA, 1 μL dT18 (Operon Biotechnologies, AL), 1 μL 10 mM dNTP mix (Invitrogen Corp., CA), 1 μL Random Primers (Invitrogen Corp., CA), and 7 μL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 9 μL of Master Mix composed of 4.5 μL 5X First-Strand Buffer, 1 μL 0.1 M DDT, 0.25 μL (100 U) of SuperScriptTM III RT (Invitrogen Corp., CA), and 0.25 μL of RNase Inhibitor (Promega, WI) and 3 mL DNase/RNase free water was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:3 with DNase/RNase free water.

For qPCR analysis, 4 μL of diluted cDNA were combined with 6 μL of a mixture composed of 5 μL 1x SYBR Green master mix (Applied Biosystems, CA), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA). Each sample was run in triplicate and a 4 point relative standard curve (4-fold dilution) plus the non-template control were used (User Bulletin #2, Applied Biosystems, CA). The reactions were performed in an ABI Prism 7900 HT SDS

instrument (Applied Biosystems, CA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Complete details regarding qPCR protocol can be found at http://docs.appliedbiosystems.com/pebiodocs/04364014.pdf. Data were analyzed with the 7900 HT Sequence Detection Systems Software (version 2.2.3, Applied Biosystems, CA).

***Primer Designing and testing*** Primer Express 3.0 software (Applied Biosystems, Foster City, CA), optimized for use with Applied Biosystems qPCR Systems, was used for primer design using default features, except for the amplicon length, which was fixed at a mininum of 100 bp . Primers were designed across exon junctions when possible to avoid amplification of genomic DNA. The exon junctions were uncovered blasting the sequence against bovine genome (Genome Browser Gateway, 2008). Primers were aligned against publicly available sequences in NCBI(National Center of Biotechnology Information, 2008) and UCSC(Genome Browser Gateway, 2008). Prior qPCR, primers were tested using the same protocol as for qPCR without the dissociation step in a 20 µL reaction. Part of the PCR product was run in a 2% agarose gel stained with ethidium bromide to assess presence of the product to a expected size and presence of primer-dimer, the rest was purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA) and sent to sequence at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana. Only primers with high specificity evaluated by a single band on agarose gel, absence of primer-dimer, amplification of the right cDNA verified by

sequencing, and a unique peak in the dissociation curve after qPCR reaction were used.

***Statistical Analysis***

A normalization factor calculated as a geometric mean of 3 internal control genes (*MRPL39, RP9* and *UXT*) using geNorm was used to normalize the qPCR data. The Mixed procedure in the Statistical Analysis System (Version 8.2, 2004, SAS Institute, Cary, NC) was used to evaluate the treatment effects on the normalized mRNA abundance. Fixed effects were treatment while the random portion of the model included the sample/replicates (cell culture flasks). Probability of ($P \leq 0.05$) was considered as statistically significant.

The qPCR data is presented as percentage change relative to Control. The standard error for each treatment was estimated as described by Bionaz and Loor (2008a). Briefly, normalized data were transformed to obtain a perfect mean of 1.0 for Control, leaving the proportional difference between the biological replicates. The same proportional change was calculated in all the treatments to obtain a fold change relative to Control.

***Networks Development by Ingenuity Pathway Analysis***

Networks among measured genes were developed by using Ingenuity Pathway Analysis® (IPA, www.ingenuity.com; Redwood City, CA, USA). This allows for the examination of gene networks within large gene expression datasets based on published relationships across several organisms. The gene expression data set comprising of gene identifiers, corresponding expression values (logarithm

transformed percentage of expression relative to ethanol control), and pair-wise comparison *P*-values were uploaded into IPA. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. A cutoff of ($P \leq 0.05$) was set to identify genes whose expression was significantly and differentially regulated by treatments. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. A network is a graphical representation of the molecular relationships between gene products. Gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges generated by IPA are supported by at least one reference from published literature, or from canonical information stored in the Ingenuity Pathways Knowledge Base. To summarize results from our data, we generated networks where custom edges were added to account for new findings and edges not demonstrated by our data were removed. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with various labels that describe the nature of the relationship between the nodes (e.g., A for activation, E for effect on expression).

## RESULTS

The MAC-T cells showed increased accumulation of TAG with palmitic acid (240%), stearic acid (180%), oleic acid (190%) and t10c12-CLA (350%), compared to control (Figure 7.1). The maximum accumulation of lipid droplets, visualized by the Oil- O-red staining was observed in the presence of t10c12-CLA followed by oleic acid (Figure 7.2 ).

The relative mRNA abundance of lipogenic genes related to FA uptake, activation, transport, TAG synthesis and transcriptional regulators is presented in Table 7.1 and Figure 7.3. the qPCR assay conditions and results of lipogenic genes are presented in tables 7.2 and 7.3, respectively. Maximum mRNA abundance was observed for *SCD* (36%) and *FASN* (18%) which constituted 54% of the overall mRNA of the examined lipogenic genes. The relative abundance of the transcriptional regulators, *SREBF1* (9%) and *SREBF2* (8%) represented 17% of the total mRNA abundance. The lowest mRNA abundance (< 0.1%) was observed for *CD36* (0.02%), *LPL* (0.02%) and *THRSP* (0.04%) and which was reflected with higher median threshold (**Ct**) values, the cycle number at which the sequence detector amplifies the product (Figure 7.4). The Ct value of *ChREBP* transcriptional regulator was over 37 and therefore was not detectable.

The expression of *LPL* and *CD36*, genes regulating FA uptake (Figure 7.5) were affected to different extents by individual treatment. Compared to the Control, *LPL* expression was significantly ($P < 0.01$) down regulated with the unsaturated FA, 18:1(-123%), 20:5 (-172%), t10-18:1(-93%) and t10c12 CLA (-125%). Conversely, the expression of *CD36*, the membrane bound protein involved in FA uptake into mammary cells was increased with all treatments compared to Control. Maximum *CD36* response was observed with 18:0 (1400%, $P < 0.001$), 20:5 (1450%, $P < 0.001$), and t10c12-CLA (1330%, $P < 0.002$). Expression of *ACSS2*, one of the genes associated with activation of FA, was up regulated with 16:0 (168%, $P < 0.001$) but was not affected by the other treatments. Similarly, expression of *ACSL1* was also not affected by the FA (Fig. 7.6).

*FABP* genes are involved in regulation of intra-cellular FA transport. The mRNA abundance of FABP3 and FABP4 was significantly increased with the saturated FA, 16:0 (755%, $P < 0.001$ and 170 %, $P < 0.001$) and 18:0 (338%, $P < 0.001$and 157, $P < 0.001$). 18:1 increased the expression of *FABP4* (69%, $P < 0.001$) but tended to decrease the *FABP3* (-161%, $P < 0.17$) expression. Trans10c12-CLA (-162%) and t10-18:1(-92%) also tended to decrease *FABP3* expression.

ROSI increased the expression of genes related to FA synthesis (*ACACA*, 85%, $P < 0.001$ and *FASN*, 54%, $P < 0.003$) and desaturation (*SCD*, 54%, $P < 0.001$) (Figure 7.7). The expression of *ACACA* and *FASN* was significantly ($P < 0.001$) reduced by 18:0 (-55 and -66%), t10-18:1 (-38 and -61%) and t10c12-CLA (-82 and -23%), compared to Control. While 18:1 and 20:5 reduced the *ACACA* expression by -36% ($P < 0.05$) and -42% ($P < 0.01$), *FASN* expression was not affected. The mRNA abundance of *SCD*, gene related to desaturation of FA was significantly ($P < 0.001$) up-regulated with 16:0 (93%, $P < 0.001$) but was down-regulated by the presence of FA containing one or more double bonds: 18:1(-422%), t10-18:1(-98%), t10c12-CLA (-355%) and 20:5 (-206%).

The mRNA abundance of *GPAM*, *AGPAT6* and *DGAT1* (Figure 7.8) were measured to test the effect of treatments on the different steps of TAG synthesis from FA and glycerol substrates. While none of the treatments affected *GPAM* expression, *DGAT1* was increased with 18:0 (87%, $P < 0.001$) and ROSI (74%, $P < 0.001$). *AGPAT6* expression was significantly ($P < 0.05$) increased by all the treatments (except t10-18:1) - 16:0 (131%), 18:0 (162%), 18:1 (153%), t10c12-CLA (98%), 20:5 (184%) and ROSI (263%).

The mRNA abundance of transcriptional regulators *SREBF1, SREBF2, THRSP, LPIN1;* nuclear receptor *PPARG* and transcriptional co-activators *INSIG1* and *SCAP* were measured to elucidate the mechanisms involved in regulation of lipogenic genes due to individual FA treatments (Figures 7.9 and 7.10). *SREBF1* expression was significantly ($P < 0.001$) decreased with 18:1 (-138%), t10c12-CLA (-187%) and 20:5 (-120%) but was increased with ROSI (100%). Similarly, SREBF2 expression was significantly ($P < 0.05$) decreased with 18:1 (-82%), t10-18:1 (-19%), t10c12CLA (-71%) and 20:5 (-30%) but was increased with 16:0 (53%) and ROSI (74%). Of the Co-activators *SCAP* and *INSIG1* that are known to regulate the SREBP activity, the *SCAP* expression was decreased with 20:5 (-57%, $P < 0.02$) but was increased with ROSI (32%, $P < 0.05$). *INSIG1* expression was increased with 16:0 (297%, $P < 0.001$), 18:0 (91%, $P < 0.001$), 20:5 (83%, $P < 0.001$) and ROSI (142%, $P < 0.001$) but was decreased with 18:1 (-89%, $P < 0.05$) and t10c12-CLA (-89%, $P < 0.05$).

The 16:0 and 18:0 saturated FA significantly ($P < 0.05$) increased *THRSP* expression (Figure 7.10) by 88% and 98%, respectively. None of the treatments affected *PPARG* expression (Figure 7.10). Conversely, the expression of *LPIN1* (Figure 7.10) was affected by all the treatments. *LPIN1* expression was increased with saturated FA (16:0, 140%, $P < 0.001$; 18:0, 29%, $P < 0.05$) and ROSI (117%, $P < 0.001$) but decreased with unsaturated FA (18:1, -58%, $P < 0.01$; t10-18:1, -30%, $P < 0.08$; t10c12-CLA, -33%, $P < 0.07$; 20:5, -37, $P < 0.04$).

## DISCUSSION

The molecular mechanisms regulating milk fat synthesis in lactating ruminants have not been completely elucidated (Bernard et al., 2008). A coordinated suppression of lipogenic genes during milk fat depression lead to the speculation that the mechanism of milk fat synthesis could be controlled at a common regulatory point. Studies have largely focused on the role of SREBP1 and THRSP in regulating milk fat synthesis (Peterson et al., 2004; Harvatine and Bauman, 2006). Recently, Bionaz and Loor (2008b) reported up-regulation of *PPARγ* during lactation in dairy cows suggesting its pivotal role in regulation of milk fat synthesis. The results from our study support the view of Bionaz and Loor (2008b) on possible role for *PPARγ* in milk fat synthesis. We observed that treatment of MACT cells with ROSI, a specific PPARγ agonist increased the expression of genes related to FA uptake (*CD36*), FA synthesis (*ACACA, FASN*), desaturation (*SCD*), TAG synthesis (*AGPAT6*, *DGAT1, LPIN1*), transcriptional regulators of fat synthesis (*SREBF1, SREBF2, THRSP*) and co-activators (*SCAP, INSIG1*).

Genes related to FA uptake (*CD36, LPL*) and activation (*ACSL1*) are known *PPARγ* target genes (Desvergne et al., 2006). In addition, genes related to FA synthesis (*ACACA, FASN*), desaturation (*SCD*), and TAG synthesis (*AGPAT*) were up- regulated with *PPARγ* agonist (GW1929) treatment in the adipose tissues of male ZDF rats (Way et al., 2001). In ruminants, a coordinated increase in the expression of *LPL, ACACA, FASN* and *PPARγ* expression in ovine adipose tissue due to propionate infusion suggested a possible regulatory role for *PPARγ* (Lee and Hossner, 2002). In the present study, ROSI did not affect the expression of *LPL* and *ACSL1*, which are

known *PPARγ* target genes in adipose. This indicated that the regulation of lipogenic gene expression through *PPARγ* could be different between adipose tissue and mammary epithelial cells. In addition to the established target genes, *PPARγ* affected the mRNA abundance of other transcriptional regulators (*SREBF1, SREBF2, LPIN1, THRSP*) and co-activators (*SCAP and INSIG1*). Based on these observations, we could speculate that increased SREBF expression could increase the expression of other down-stream lipogenic genes. Nevertheless, increased *INSIG1* expression would decrease the formation of active nuclear SREBF protein and *INSIG1* is regulated by *PPARγ* (Kast-Woelbern et al., 2004). This suggests that many of the lipogenic genes could be directly regulated by *PPARγ.*

We tested the effects of saturated (16:0, 18:0), unsaturated FA (18:1, 20:5) and rumen biohydrogenation intermediates (t10-18:1, t10c12-CLA) on mammary lipogenic genes to determine if some of their effects were regulated through *PPARγ* as reflected in the expression of *PPARγ* target genes. Compared to Control, the treatments with saturated FA (16:0 and 18:0) increased intra cytoplasmic TAG contents (Figure 7.1) but did not form visible lipid droplets (Figure 7.2). However, increased TAG content was correlated with lipid droplet formation with oleic acid (18:1) treatment. Similar observations were reported by Yonezawa et al., (2004) in bovine mammary epithelial cells (bMEC) treated with palmitic acid, stearic acid and oleic acid. The increased intra cellular TAG content due to 16:0, 18:0 and 18:1 correlated with increased expression of *CD36,* gene related to FA translocation, in both this study and the study of Yonezawa et al., (2004). In this study *CD36* expression increased from 2.5 (ROSI) to 15 fold (18:0, 20:5 and t10c12 CLA).

Increased *CD36* with all treatments suggests that the exogenous FA concentrations could play a role in FA uptake by the mammary cells.

Mammary FA uptake is also driven by intra-cellular FA activation, FA binding to intracellular proteins (FABP) and intracellular metabolism (TAG synthesis) (Mashek and Coleman, 2006). *ACSL1,* one of the predominant genes related to FA activation, (Bionaz and Loor, 2008a) is present on the plasma membrane (Doege and Stahl, 2006). In this study, none of the treatments affected *ACSL1* expression but palmitic acid increased *ACSS2* expression, the gene related to acetate activation. Hansen and Knudsen (1987) have reported increased synthesis and incorporation of butyrate (C4:0) in TAG when bovine and ovine dispersed mammary epithelial cells were incubated with palmitic acid. Their observations correspond to the increased *ACSS2* expression observed in our study.

FABPs are proteins involved in intra cellular transport and are shown to directly interact with *CD36* (Spitsberg et al., 1995). The proteins *FABP3* and *FABP4* are the predominantly expressed FABP isoforms in bovine mammary epithelial cells (Bionaz and Loor, 2008a). In this study, the saturated FA (16:0 and 18:0) significantly increased the expression of *FABP3* and *FABP4* while oleic acid (18:1) increased *FABP4* expression suggesting the involvement of different FABP isoforms for transport of different FA.

Long-chain FA are known to affect mammary FA synthesis in lactating dairy cows (Chilliard et al., 2001) as feeding or infusion of LCFA supplements is known to decreases *de novo* FA synthesis in the mammary cells. However, these effects depend on the type of LCFA where unsaturated FA supplements result in greater decreases

(Drackley et al., 1992). In this study, oleic acid and EPA decreased the mRNA expression of *ACACA* but not *FASN*. ACACA is reported to control 63% of FA synthesis while FASN controls the remaining 37%, as measured by metabolic flux control in bovine mammary homogenates (Wright et al., 2006). The biohydrogenation intermediates, t10-18:1 and t10c12-CLA decreased expression of both genes related to FA synthesis. The role of t10c12-CLA in decreasing the expression of lipogenic genes is well established (Peterson et al., 2003). However, to our knowledge this is the first report showing a direct effect of t10-18:1 on mammary lipogenic gene expression. Recently, Lock et al (2007) reported a lack of effect of trans-10-18:1 on milk fat synthesis in lactating cows. However, milk t10-18:1 was only 1.11%, well below the concentration of 2.5% required for maximal MFD suggested by (Kadegowda et al., 2008). This study suggests that a higher concentration of t10-18:1 could potentially induce milk fat depression by decreasing lipogenic gene expression.

Of the saturated FA, stearic acid is known to decrease *de novo* FA synthesis FA while palmitic acid (16:0) stimulates TAG formation (Hansen and Knudsen, 1987). Expression of *ACACA* and *FASN* were reduced with stearic acid but were not affected by palmitic acid in the present study. Nevertheless, increased TAG synthesis due to palmitic acid could be possibly due to the fact that palmitic acid is the most preferred substrate for the initial acylation of L-α glycerolphosphate by acyltransferase to form sn-1-lysophasphatidic acid. Initiation of acylation of the sn-1 position is a prerequisite for TAG synthesis in the mammary gland (Kinsella and Gross, 1973).

The mRNA abundance of *SCD* was increased with palmitic acid but was not affected with stearic acid. Both, palmitic acid and stearic acids are the preferred substrates for desaturation by *SCD*. Nevertheless, increased *SCD* expression with palmitic acid suggests involvement of other regulatory mechanisms. Expression of *SCD* was decreased with the presence of unsaturated FA possibly due to a feed back inhibition. Of the genes related to TAG synthesis, AGPAT6 expression was significantly increased with all treatments (except trans-10-18:1), indicating that most of the FA were diverted towards TAG synthesis in the mammary epithelial cells.

Mammary lipogenic gene expression is regulated through transcriptional regulators *SREBF1, SREBF2, INSIG1, PPARγ, THRSP* and *LPIN1* (Bionaz and Loor, 2008b). In this study, the unsaturated FA decreased the expression of *SREBF1* and *SREBF2*, the transcriptional regulators known to control the lipogenic gene expression. All the unsaturated FA except EPA decreased *INSIG1* expression. *INSIG*1 is shown to be under the control of *SREBP1* (Kast-Woelbern et al., 2004). Therefore, reduced *SREBP1* expression could have reduced *INSIG1* expression with these treatments. Decreased mRNA abundance of *SCAP* and increased abundance of *INSIG1* indicated that EPA could regulate the synthesis of active nuclear *SREBP* through both transcriptional and post-translational mechanisms. Similarly, increased mRNA abundance of *INSIG1* due to saturated FA suggested a post-translational decrease in *SREBP* proteins which in turn could potentially down regulate the mammary lipogenic genes. However, palmitic and stearic acid increased *LIPN1* and *THRSP* expression; the transcriptional regulators involved in TAG (Coleman and

Lee, 2004) and *de novo* FA (LaFave et al., 2006) synthesis, respectively. This could explain the increased TAG synthesis by these treatments.

Summarizing the results (Figure 7.12), ROSI, *a PPARγ* agonist identified that the down-stream genes controlled by *PPARγ* in mammary epithelial cells, were genes related to FA synthesis, FA desaturation, TAG synthesis and mammary transcriptional regulators. The results from the study suggested that part of the effects due to palmitic acid could be mediated through *PPARγ* activation. However, *SREBF* could also play a major role in lipogenic gene regulation when unsaturated FA- 18:1, 20:5, t10-18:1 and t10c12-CLA were present. We demonstrated that the effects of t10-18:1 were similar to that of t10c12-CLA in decreasing mammary lipogenic gene expression at the levels of FA used in this study. However, keeping in view the limitations of cell culture work, *in vivo* studies with lactating dairy cows would be essential to validate the results from our study.

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Table 7.1. Percent relative mRNA abundance and median threshold cycle (Ct) of genes measured by quantitative PCR.

| Gene | Cellular Location | Type | mRNA abundance | Median Ct |
|---|---|---|---|---|
| *ACACA* | Cytoplasm | enzyme | 0.06 | 24.61 |
| *ACSL1* | Cytoplasm | enzyme | 0.11 | 23.84 |
| *ACSS2* | Cytoplasm | enzyme | 0.23 | 22.68 |
| *AGPAT6* | Cytoplasm | enzyme | 0.23 | 22.50 |
| *CD36* | Plasma Membrane | other | 0.00 | 30.93 |
| *DGAT1* | Cytoplasm | enzyme | 0.09 | 23.76 |
| *FABP3* | Cytoplasm | transporter | 0.21 | 22.54 |
| *FABP4* | Cytoplasm | transporter | 0.01 | 27.53 |
| *FASN* | Cytoplasm | enzyme | 0.87 | 20.48 |
| *GPAM* | Cytoplasm | enzyme | 0.01 | 27.06 |
| *INSIG1* | Cytoplasm | other | 0.13 | 23.51 |
| *LPIN1* | Nucleus | other | 0.13 | 23.21 |
| *LPL* | Cytoplasm | enzyme | 0.00 | 32.24 |
| *MLXIPL* | Nucleus | transcription regulator | | 39.06 |
| *PPARG* | Nucleus | ligand-dependent nuclear receptor | 0.01 | 26.18 |
| *SCAP* | Cytoplasm | other | 0.20 | 22.55 |
| *SCD* | Cytoplasm | enzyme | 1.78 | 19.07 |
| *SREBF1* | Nucleus | transcription regulator | 0.44 | 21.64 |
| *SREBF2* | Nucleus | transcription regulator | 0.42 | 21.61 |
| *THRSP* | Nucleus | other | 0.00 | 30.11 |

Table 7.2. Slope, coefficient of determination of standard curve ($R^2$), efficiency (E), median threshold cycle (Ct), and mRNA abundance of measured transcripts.

| Gene | Slope | $(R^2)$[1] | Efficiency(E)[2] | Median Ct[3] | Median $dCt_4$ | Relative mRNA abundance[5] |
|---|---|---|---|---|---|---|
| *ACACA* | -3.45 | 0.997 | 1.95 | 24.6 | 4.3 | 1.12 |
| *ACSL1* | -3.73 | 0.998 | 1.85 | 23.8 | 3.6 | 2.16 |
| *ACSS2* | -3.77 | 0.997 | 1.84 | 22.7 | 2.4 | 4.59 |
| *AGPAT6* | -3.54 | 0.999 | 1.92 | 22.5 | 2.2 | 4.71 |
| *CD36* | -3.63 | 0.998 | 1.89 | 30.9 | 10.6 | 0.02 |
| *ChREBP* | -2.56 | 0.959 | 2.46 | 39.1 | | 0.00 |
| *DGAT1* | -3.38 | 0.996 | 1.98 | 23.8 | 3.5 | 1.91 |
| *FABP3* | -3.48 | 0.997 | 1.94 | 22.5 | 2.4 | 4.28 |
| *FABP4* | -3.99 | 0.998 | 1.78 | 27.5 | 7.5 | 0.26 |
| *FASN* | -3.56 | 0.998 | 1.91 | 20.5 | 0.2 | 17.65 |
| *GPAM* | -3.57 | 0.999 | 1.91 | 27.1 | 6.9 | 0.23 |
| *INSIG1* | -3.69 | 0.998 | 1.87 | 23.5 | 3.3 | 2.54 |
| *LPIN1* | -3.49 | 0.999 | 1.94 | 23.2 | 3.1 | 2.69 |
| *LPL* | -3.85 | 0.994 | 1.82 | 32.2 | 12.0 | 0.02 |
| *PPARG* | -3.15 | 0.999 | 2.08 | 26.2 | 6.0 | 0.26 |
| *SCAP* | -3.33 | 0.997 | 1.99 | 22.5 | 2.3 | 4.01 |
| *SCD* | -3.58 | 0.998 | 1.90 | 19.1 | -0.9 | 36.13 |
| *SREBF1* | -3.81 | 0.993 | 1.83 | 21.6 | 1.3 | 8.96 |
| *SREBF2* | -3.56 | 0.998 | 1.91 | 21.6 | 1.3 | 8.46 |
| *THRSP* | -3.60 | 0.996 | 1.90 | 30.1 | 9.8 | 0.04 |

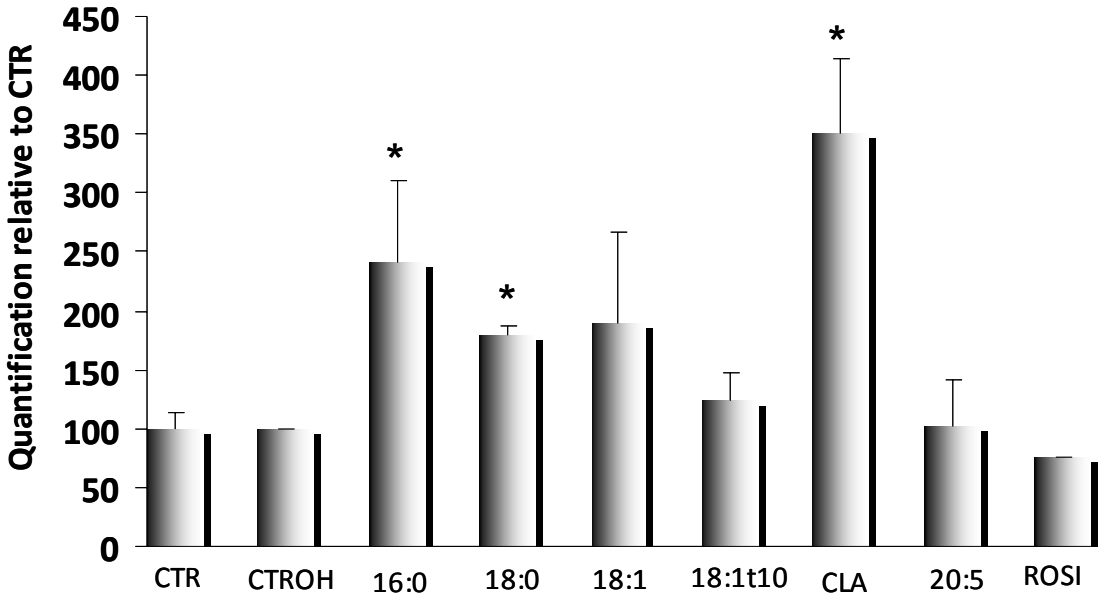
[1] $R^2$ stands for the coefficient of determination of the standard curve.
[2] Efficiency is calculated as $[10^{(-1 / \text{Slope})}]$.
[3] The median is calculated considering all time points and all cows.
[4] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each cow.
[5] mRNA abundance = $1/E^{(\text{Median}\Delta\ Ct)}$.

Table 7.3. Effect of long-chain fatty acids and Rosiglitazone (ROSI) on lipogenic gene expression relative to the Control treatment.

| Gene | Control | C16:0 | C18:0 | C18:1 | t10-18:1 | t10c12-CLA | EPA | ROSI | *P value* |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | % Change, relative to Control[1] | | | | |
| ***ACACA*** | $0^{b}$ | $-4.94^{bc}$ | $-55.46^{cd}$ | $-35.84^{bc}$ | $-38.08^{bc}$ | $-81.84^{d}$ | $-42.12^{bcd}$ | $84.73^{a}$ | 0.001 |
| ***ACSL1*** | 0 | -7.47 | 24.12 | 9.41 | -2.67 | 3.47 | 15.17 | 15.85 | 0.41 |
| ***ACSS2*** | $0^{bcd}$ | $167.54^{a}$ | $3.46^{bc}$ | $-25.90^{cd}$ | $-16.64^{cde}$ | $-31.05^{e}$ | $3.14^{bc}$ | $18.26^{b}$ | 0.001 |
| ***AGPAT6*** | $0^{d}$ | $130.99^{bc}$ | $162.14^{b}$ | $152.84^{b}$ | $40.26^{cd}$ | $97.75^{bc}$ | $184.36^{ab}$ | $262.82^{a}$ | 0.001 |
| ***CD36*** | $0^{b}$ | $539.10^{b}$ | $1407.09^{a}$ | $315.65^{b}$ | $477.25^{b}$ | $1331.13^{a}$ | $1453.46^{a}$ | $140.00^{b}$ | 0.003 |
| ***DGAT1*** | $0^{cd}$ | $41.02^{abc}$ | $86.91^{a}$ | $42.19^{abc}$ | $24.78^{bc}$ | $38.50^{abc}$ | $-17.03^{d}$ | $73.86^{ab}$ | 0.01 |
| ***FABP3*** | $0^{cd}$ | $754.65^{a}$ | $338.40^{e}$ | $-162.07^{e}$ | $-92.08^{de}$ | $-161.31^{e}$ | $12.81^{c}$ | $39.53^{c}$ | 0.001 |
| ***FABP4*** | $0^{c}$ | $170.90^{a}$ | $157.19^{a}$ | $68.97^{b}$ | $0.40^{c}$ | $0.77^{c}$ | $-1.88^{c}$ | $36.32^{bc}$ | 0.001 |
| ***FASN*** | $0^{b}$ | $29.84^{bc}$ | $-68.26^{e}$ | $57.77^{bc}$ | $-61.13^{de}$ | $22.75^{cd}$ | $-3.77^{bc}$ | $53.73^{a}$ | 0.001 |
| ***GPAM*** | 0 | -74.45 | -0.99 | -0.63 | -0.37 | 14.67 | -30.13 | -25.40 | 0.36 |
| ***INSIG1*** | $0^{d}$ | $297.49^{a}$ | $90.95^{bc}$ | $-89.29^{e}$ | $-13.14^{d}$ | $-89.18^{e}$ | $83.39^{c}$ | $141.56^{b}$ | 0.001 |
| ***LPIN1*** | $0^{bc}$ | $140.03^{a}$ | $29.21^{b}$ | $-57.55^{d}$ | $-29.70^{cd}$ | $-32.58^{cd}$ | $-37.12^{cd}$ | $116.67^{a}$ | 0.001 |
| ***LPL*** | 0 | -27.02 | 1.55 | -122.63 | -93.40 | -124.97 | -172.31 | -48.34 | 0.157 |
| ***PPARG*** | 0 | 24.10 | 19.26 | -12.96 | -15.28 | 10.72 | 0.33 | 5.67 | 0.56 |
| ***SCAP*** | $0^{a}$ | $-13.82^{a}$ | $-8.92^{a}$ | $17.02^{a}$ | $8.97^{a}$ | $12.42^{a}$ | $-56.77^{b}$ | $31.54^{a}$ | 0.01 |
| ***SCD*** | $0^{b}$ | $93.30^{a}$ | $3.25^{b}$ | $-421.89^{f}$ | $-97.63^{c}$ | $-354.95^{e}$ | $-205.53^{d}$ | $53.77^{ab}$ | 0.001 |
| ***SREBF1*** | $0^{b}$ | $6.27^{b}$ | $-4.95^{b}$ | $-137.66^{d}$ | $-46.79^{c}$ | $-186.82^{e}$ | $-119.70^{d}$ | $99.71^{a}$ | 0.001 |
| ***SREBF2*** | $0^{b}$ | $52.90^{a}$ | $2.45^{b}$ | $-82.30^{d}$ | $-18.66^{bc}$ | $-71.43^{d}$ | $-29.94^{c}$ | $35.33^{a}$ | 0.001 |
| ***THRSP*** | $0^{abc}$ | $88.48^{ab}$ | $96.80^{a}$ | $-19.34^{abc}$ | $-31.36^{bc}$ | $-58.53^{c}$ | $-19.39^{c}$ | $43.52^{abc}$ | 0.07 |

[1]Superscripts with unlike letters differ.

Figure 7.1. Effect of long-chain fatty acids and Rosiglitazone (ROSI) on triacylglycerol accumulation in MAC-T cells relative to Controls (CTR).




* $P < 0.05$

Figure 7.2. Effect of long-chain fatty acids and Rosiglitazone (ROSI) on lipid formation. Intra-cellular triacylglycerol were stained by Oil-Red-O-staining.

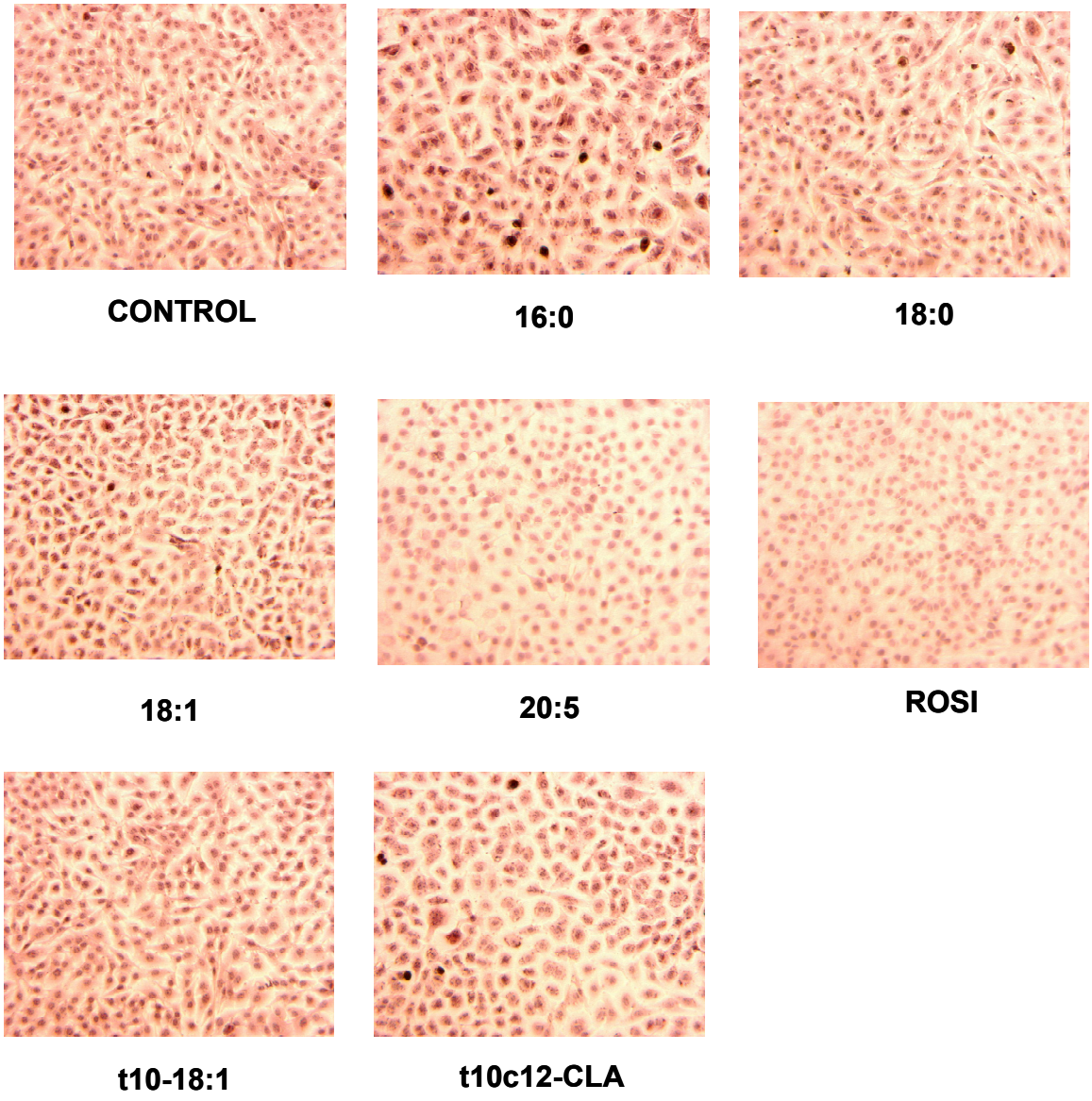


Figure 7.3. Percent relative mRNA abundance of measured lipogenic genes.

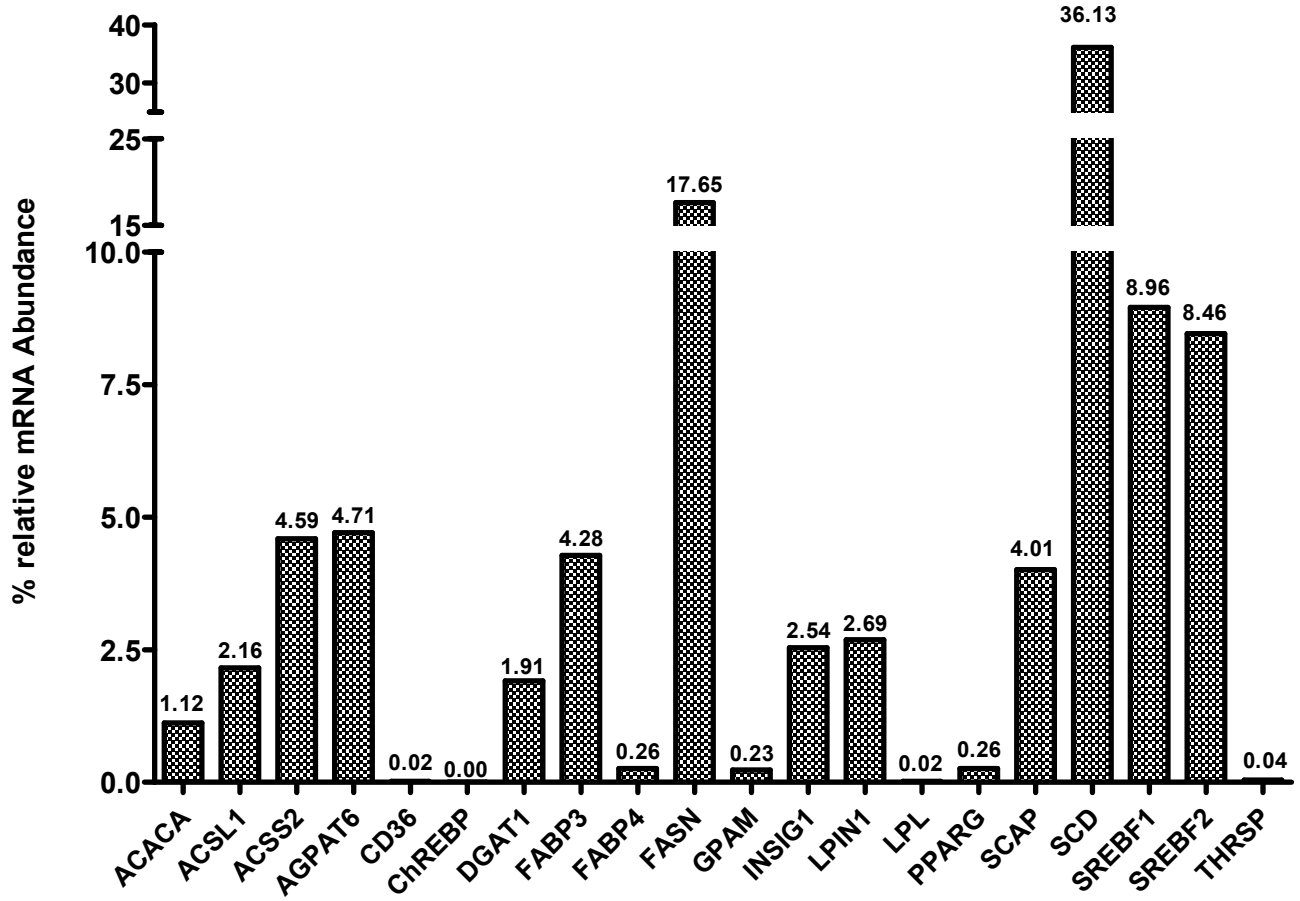


Figure 7.4. Median threshold cycle (Ct) values of measured lipogenic genes.

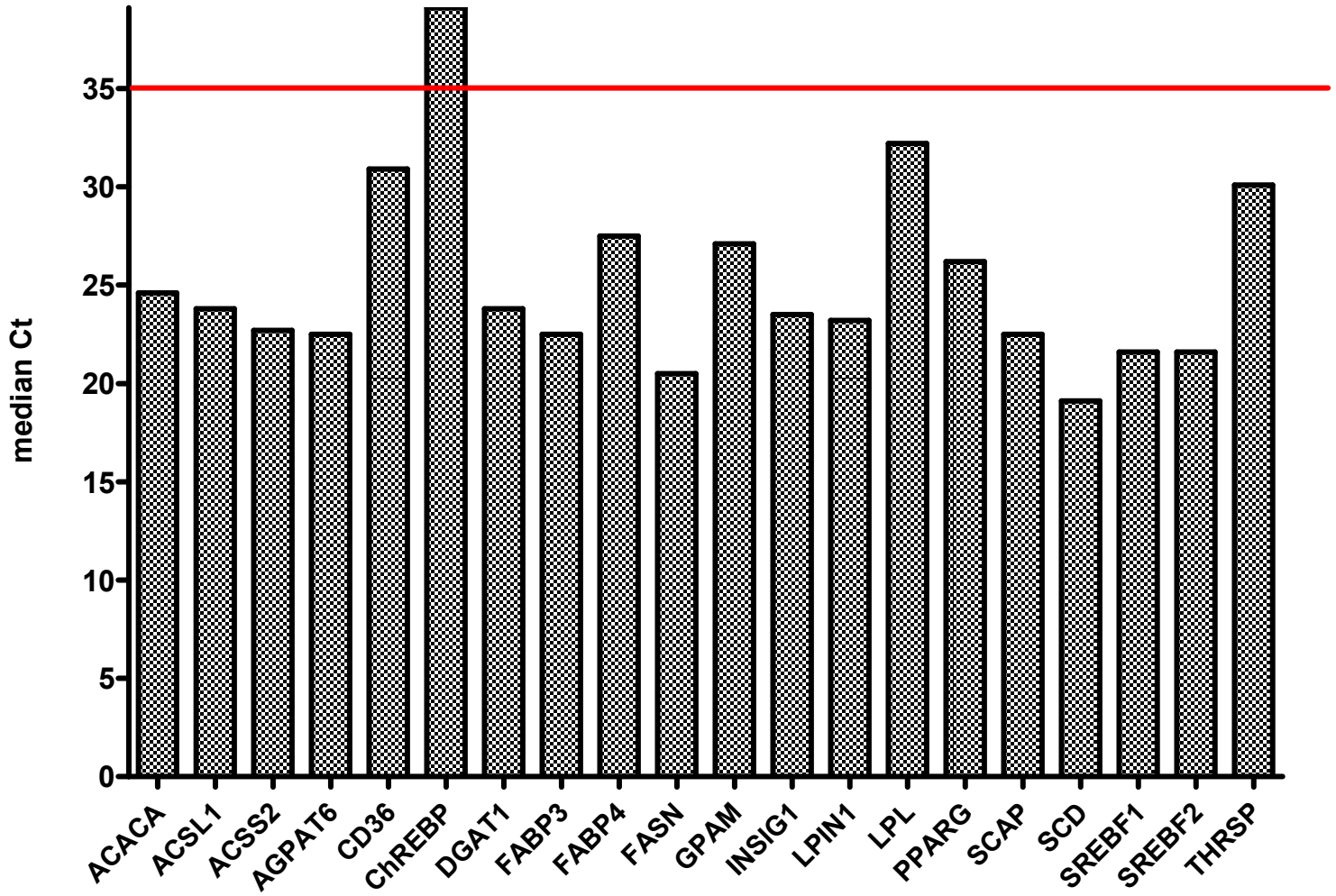
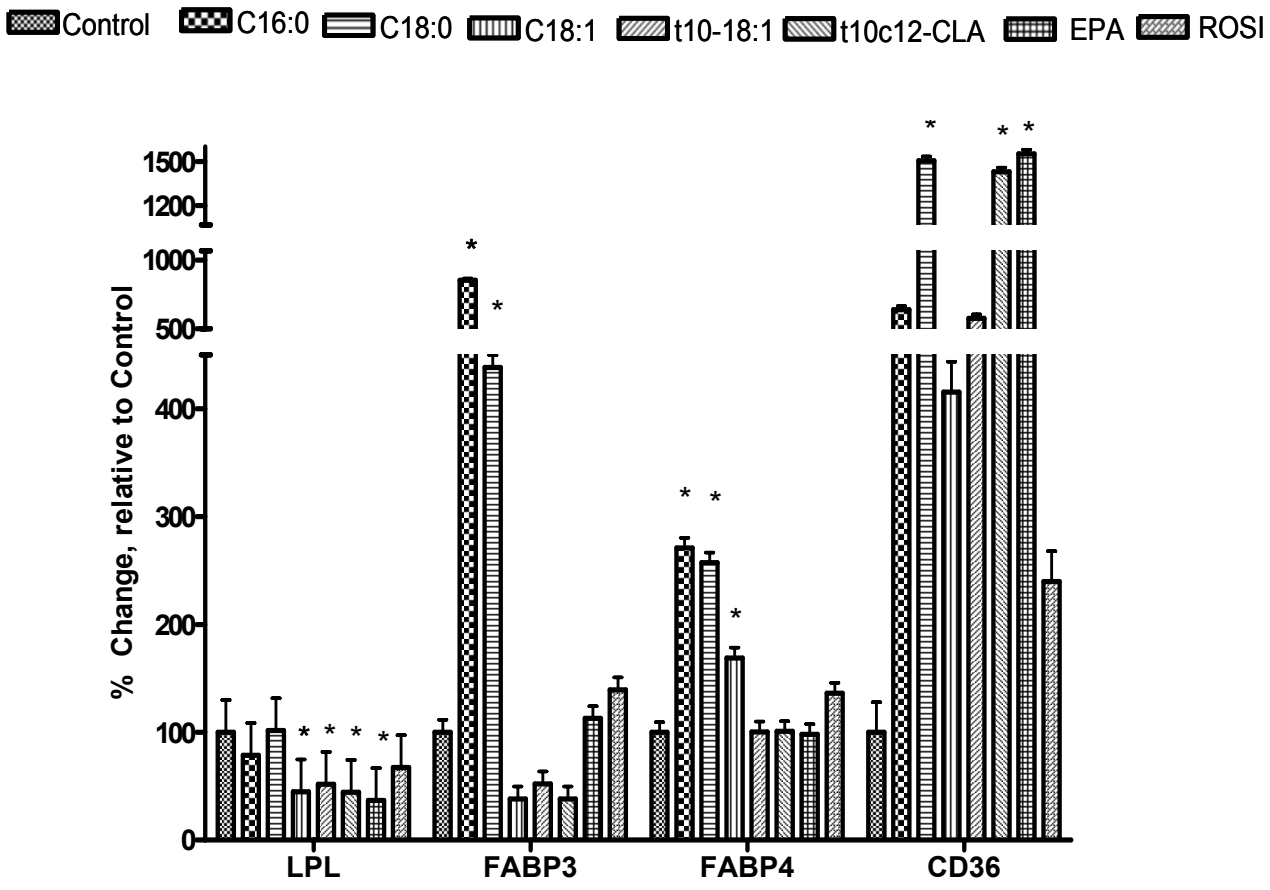
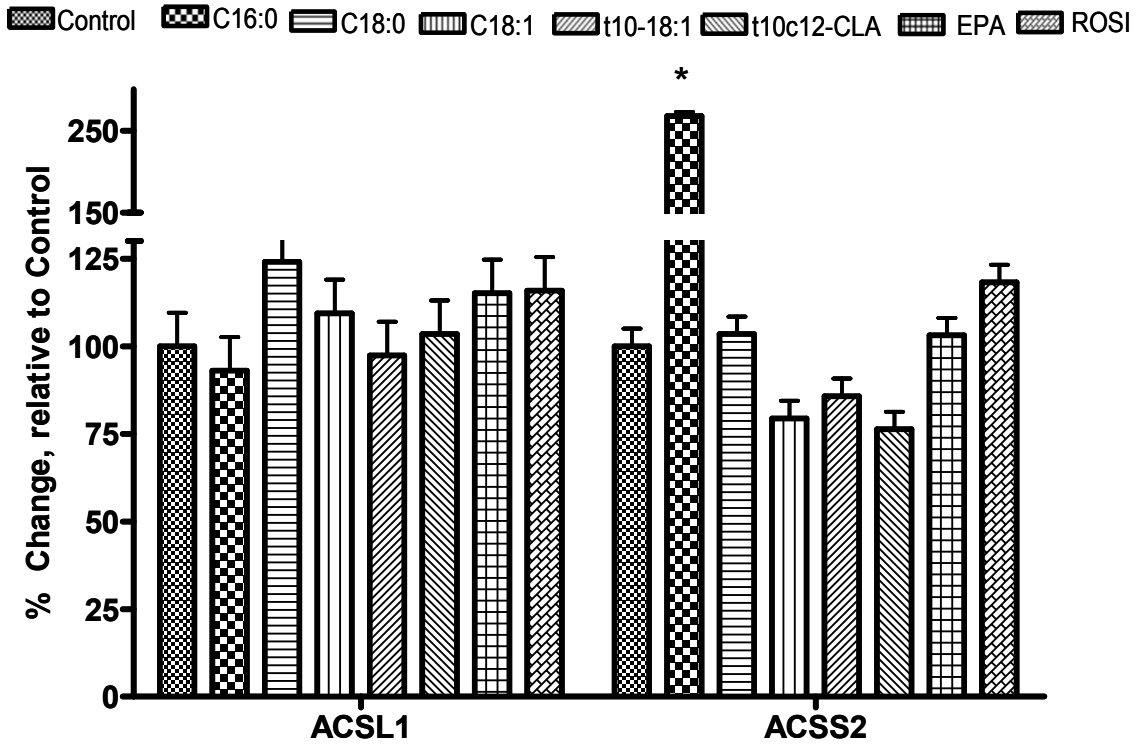


Figure 7.5. Effect of long-chain fatty acids and Rosiglitazone (ROSI) on mRNA abundance relative to the Controls of genes related to FA uptake and transport.



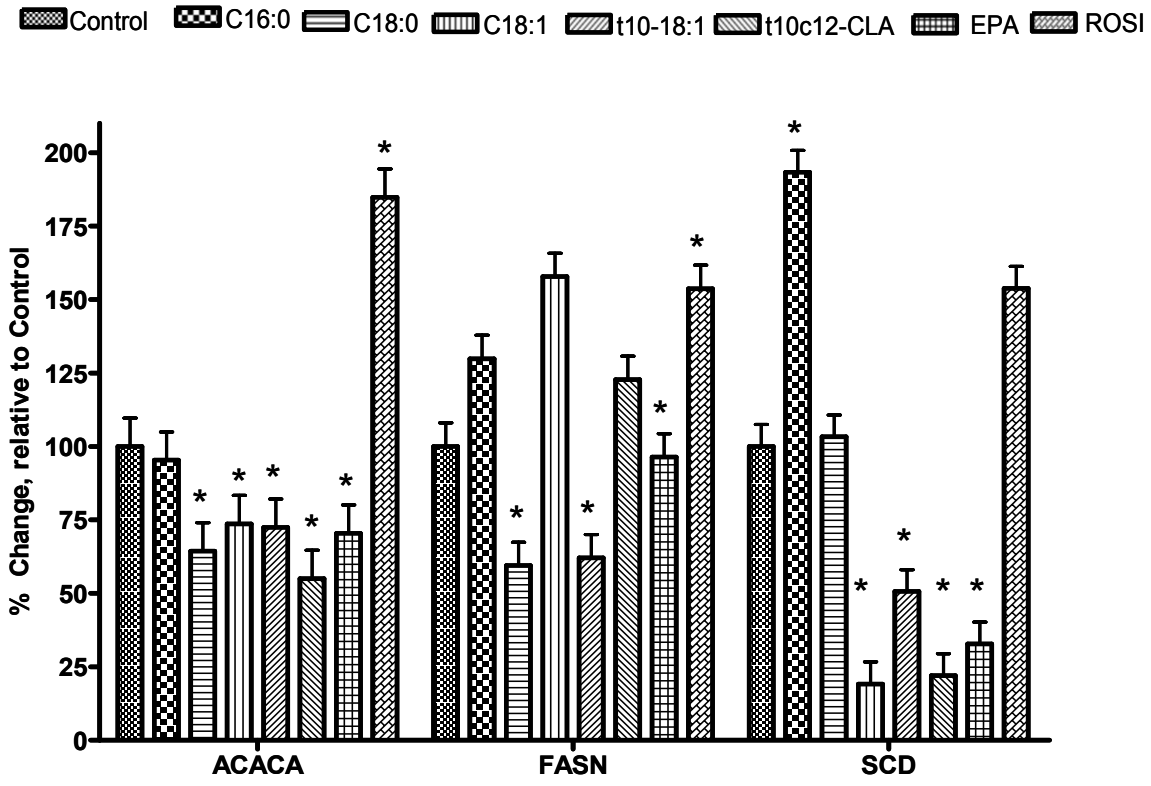

* $P < 0.05$

Figure 7.6. Effect of long-chain fatty acids and Rosiglitazone (ROSI)on mRNA abundance relative to the Controls of genes related to fatty acid activation.



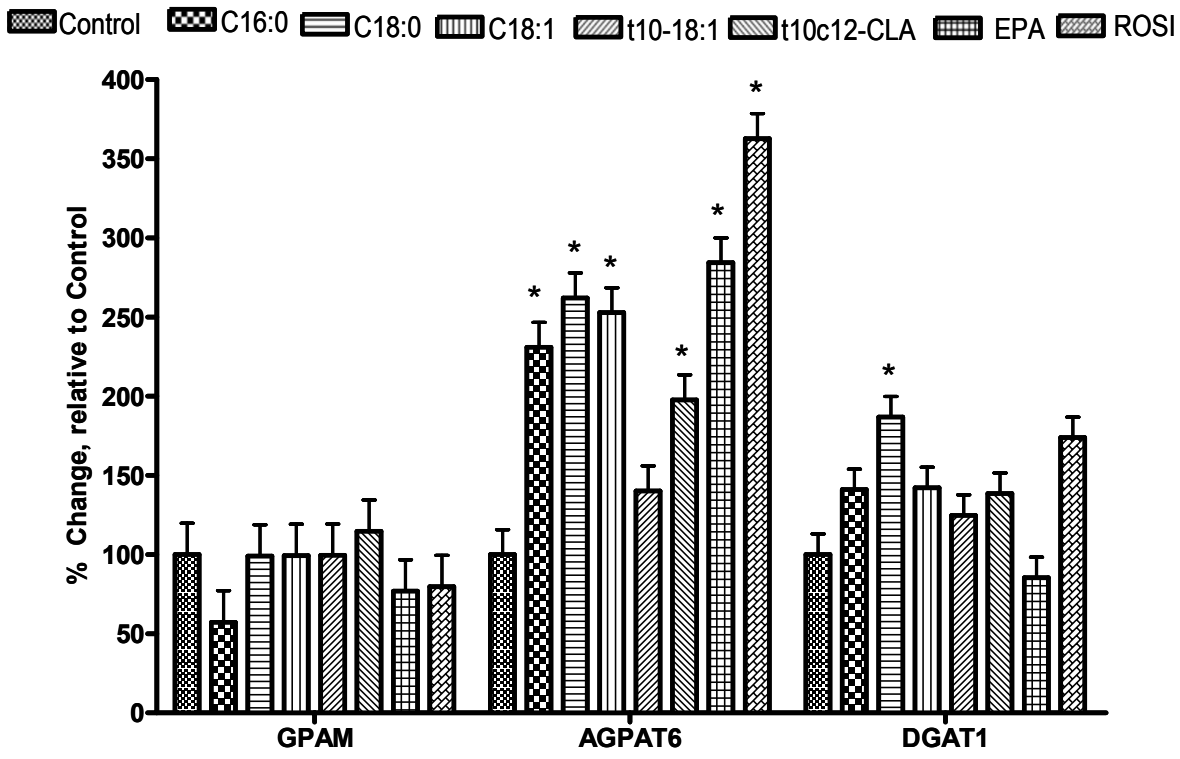

* $P < 0.05$

Figure 7.7. Effect of long-chain fatty acids and Rosiglitazone (ROSI) on mRNA abundance relative to the Controls of genes related to FA synthesis and desaturation.



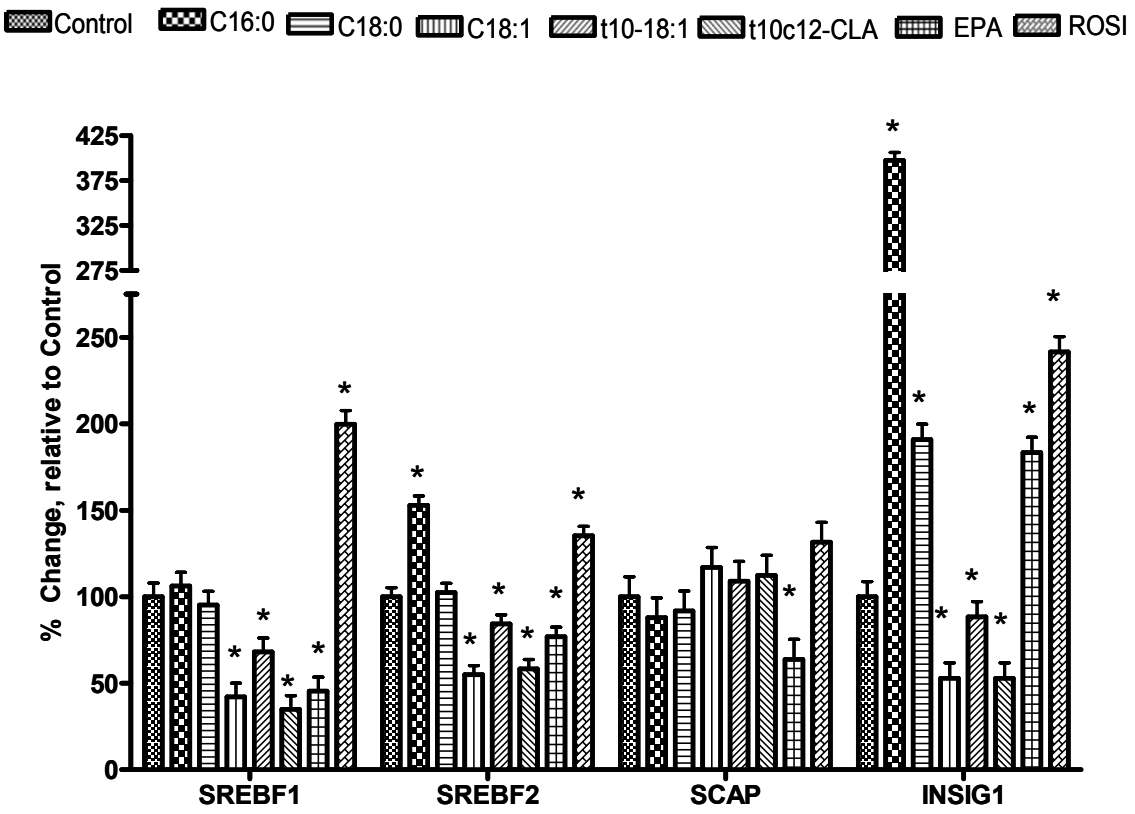

* $P < 0.05$

Figure 7.8. Effect of long-chain fatty acids and Rosiglitazone (ROSI) on mRNA abundance relative to the Controls of genes related to TAG synthesis.



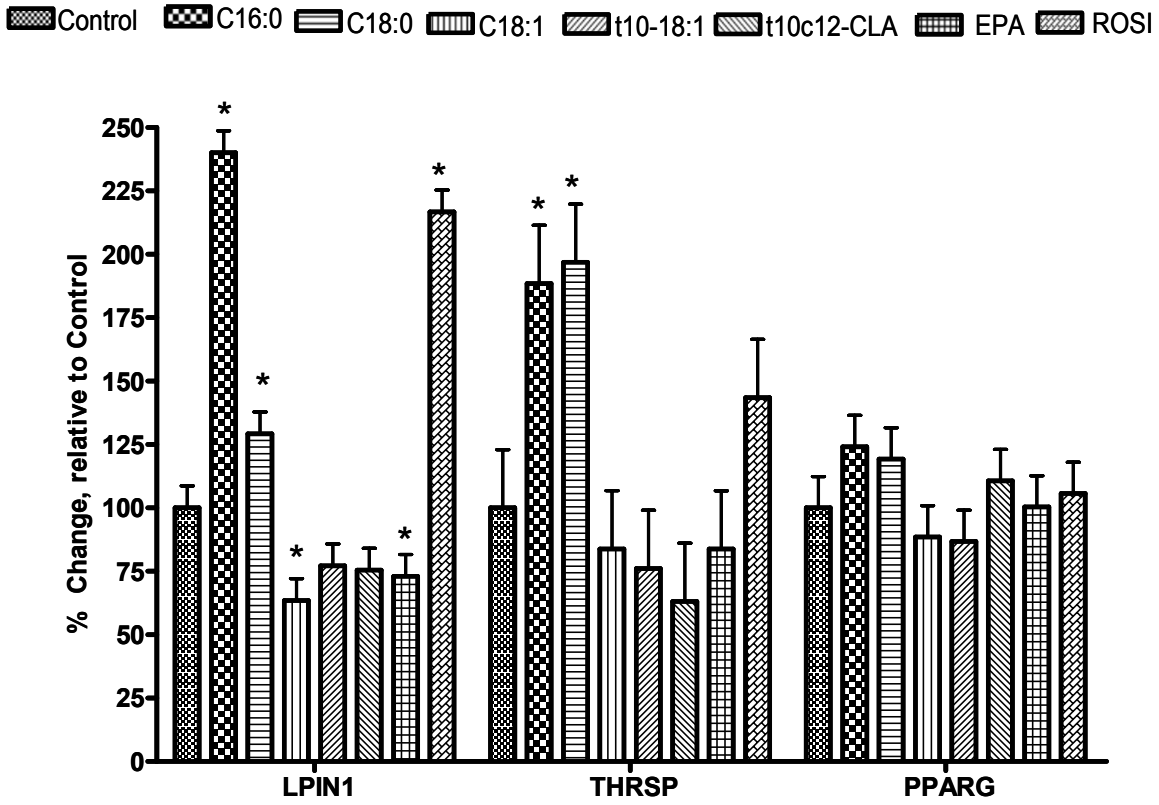

* $P < 0.05$

Figure 7.9. Effect of long-chain fatty acids and Rosiglitazone (ROSI) on mRNA abundance relative to the Controls of genes related to transcriptional regulation.




* $P < 0.05$

Figure 7.10. Effect of long-chain fatty acids and Rosiglitazone (ROSI) on mRNA abundance relative to the Controls of genes related to transcriptional regulation.




* $P < 0.05$

Figure 7.11. Effect of long-chain fatty acids and Rosiglitazone (ROSI)on cell counts.

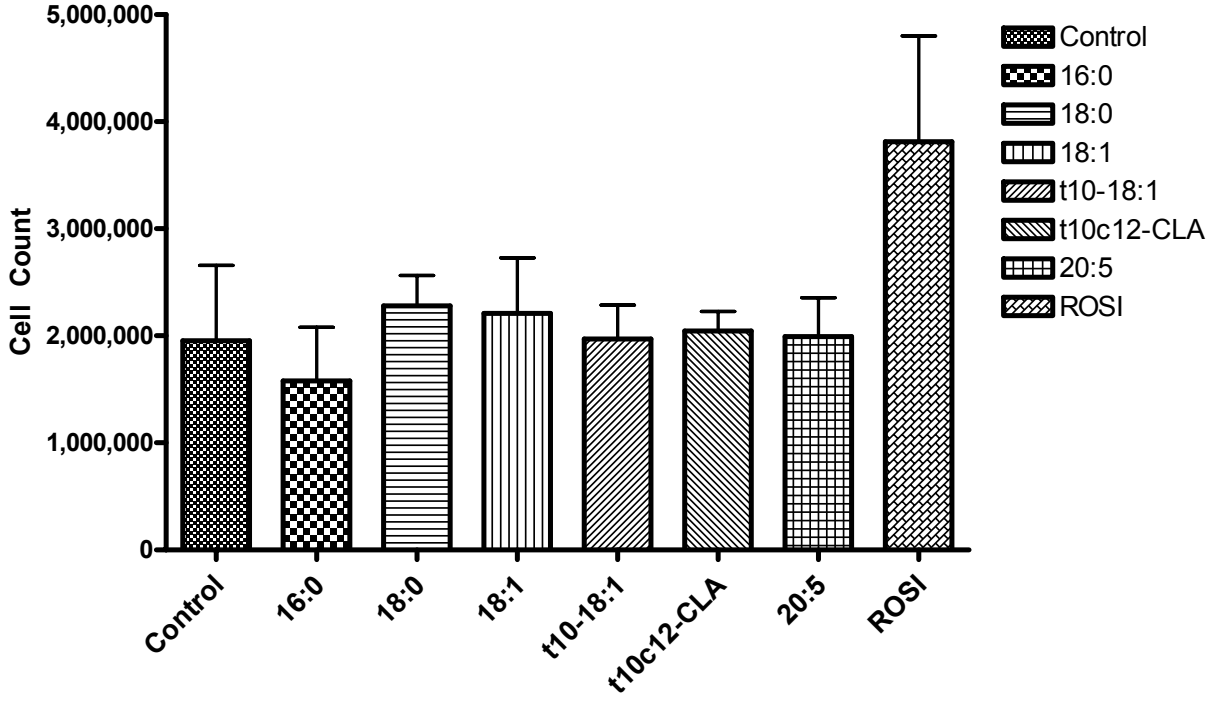
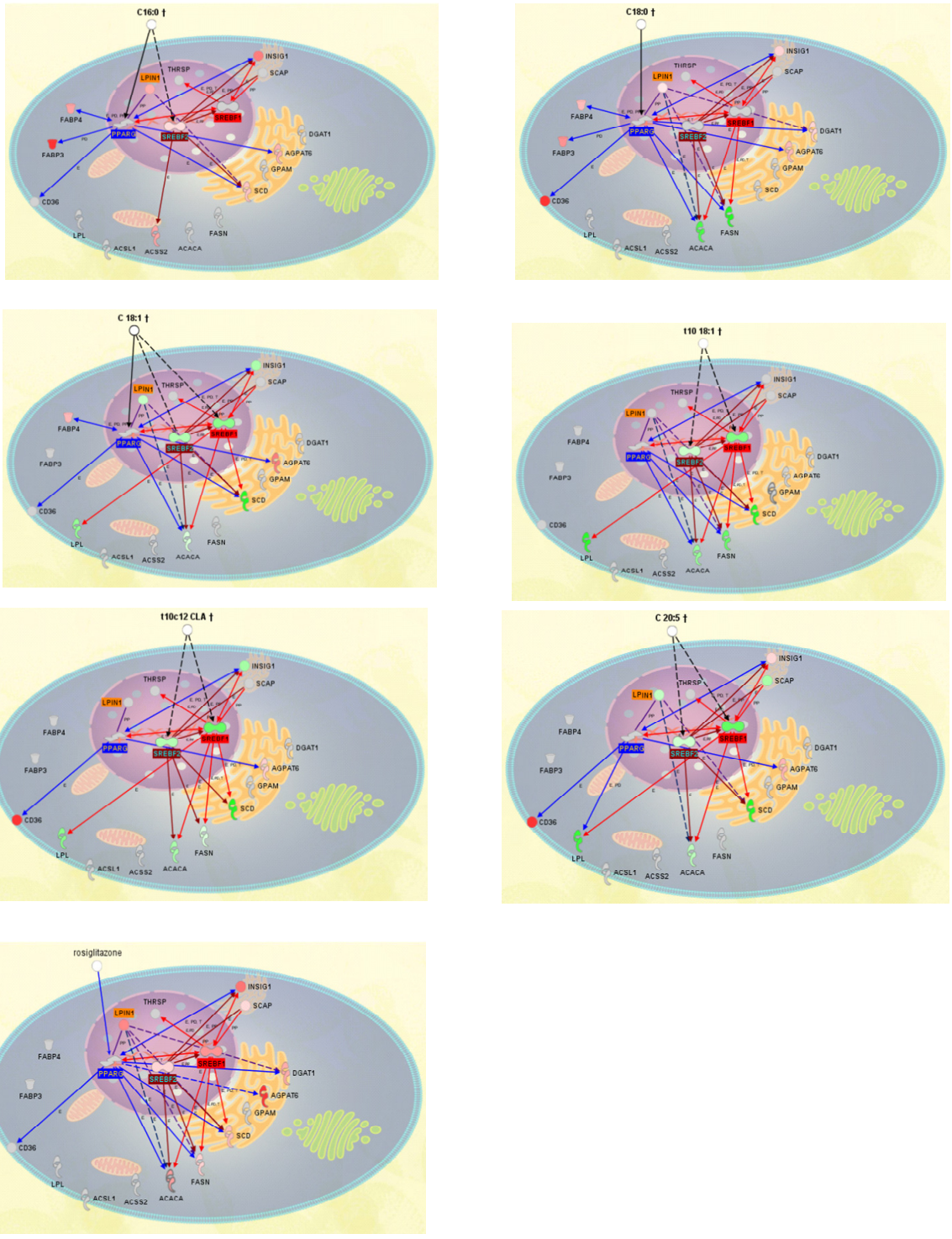


Figure 7.12. Networks developed using IPA showing potential regulatory mechanisms involved in each FA.



# Chapter 8: EXPERIMENT 6

## Identification of internal controls for quantitative PCR in mammary tissue from lactating cows receiving various lipid supplements[1].

[1]**Presented in part at the annual ADSA-ASAS meeting, 2008.**

**Kadegowda. A. K. G., M. Bionaz, B. Thering, L. Piperova, R. A. Erdman, and J. J. Loor. 2008. J. Dairy Sci. Suppl. 1. 437.**

## ABSTRACT

Dietary lipid supplements affect mammary lipid metabolism through changes in lipogenic gene expression. Quantitative PCR (qPCR) is one of the most sensitive, reliable, and accurate techniques available to date for gene expression analysis. However, variation introduced in qPCR data by analytical/technical errors needs to be accounted for via normalization using appropriate internal control genes (ICG). Objectives were to mine individual bovine mammary micro array data on >13,000 genes across 66 cows from two independent studies to identify the most suitable ICG for normalization of qPCR. In addition to unsupplemented control diets, cows were fed saturated or unsaturated lipids (fish oil, Energy Booster) for 21 d, or were infused with supplements (Butterfat, CLA mixture, long-chain fatty acids) into the abomasum to modify milk fat synthesis and fatty acid profiles. GeneSpring® GX identified 49 genes that did not vary in expression across the 66 samples. Subsequent gene network analysis revealed that 22 of those genes were not co-regulated. Among those, *COPS7A*, *CORO1B*, *DNAJC19*, *EIF3K*, *EMD*, *GOLGA5*, *MTG1*, *UXT*, *MRPL39*, *GPR175*, and *MARVELD1* (sample/reference expression ratio = 1 ± 0.1) were selected for qPCR analysis upon verification of goodness of BLAT/BLAST sequence and primer design. Relative expression of *B2M*, *GAPDH*, and *ACTB*, previously used as ICG in bovine mammary, was highly unstable (0.9 ± 0.6) across studies. Gene stability analysis, via geNorm software, uncovered *EMD*, *MARVELD1*, *MRPL39*, *GPR175*, *UXT*, and *EIF3K* as the most stable genes and, thus, suitable as ICG. geNorm also indicated that use of 3 to 5 ICG was most appropriate for calculating a normalization factor. Overall, results showed that the geometrical

average of at least three among *EMD*, *MARVELD1*, *MRPL39*, *GPR175*, *UXT*, and *EIF3K* is ideal for normalization of mammary qPCR data in studies involving lipid supplementation of dairy cows.

**Key words:** internal control genes, qPCR, lipid supplements, lactating dairy cows

# INTRODUCTION

Regulation of bovine mammary lipid synthesis remains a very active field of investigation (Bauman et al., 2006). Large-scale transcript analysis of lipogenic genes in lactating mice has shown that regulation of mammary lipid synthesis occurs at the level of mRNA expression (Rudolph et al., 2007). While, the molecular mechanisms by which dietary fatty acids regulate bovine mammary lipogenic gene expression are not completely established, there is evidence of a role for transcriptional regulators (Peterson et al., 2004; Harvatine and Bauman, 2006).

The advent of bovine microarray tools (Loor et al., 2007), has enabled comprehensive large-scale evaluation of mRNA expression in tissues due to nutrition and/or physiological state (Loor et al., 2006; 2007). Use of this technology promises to substantially increase our knowledge of transcriptional adaptations in bovine tissues. Despite the advantages of microarrays, however, quantitative RT-PCR (qPCR) remains the method of choice for evaluation of mRNA expression. Quantitative RT-PCR is one of the most sensitive, reliable, and accurate techniques available for gene expression analysis. Central to the applicability of qPCR is the fact that variation introduced due to sampling, analytical, and technical errors during the procedure should be accounted for via normalization. Use of appropriate internal control genes (ICG) is the most reliable method currently used for data normalization (Vandesompele et al., 2002). Use of ICG allows normalization of differences due to initial quantity of RNA, RNA handling and variation in kinetics of reverse-transcription reaction. The selected ICG should not vary due to the type of cells/tissues or respond differently to treatments (Vandesompele et al., 2002). Thus,

proper selection and evaluation of ICG is critical to avoid additional variation in the data.

Most recent studies assessing the effects of lipid supplements on bovine mammary lipid synthesis regulation at the level of mRNA expression have relied on commonly-used ”housekeeping genes” such as *actin-β* (Peterson et al., 2003; Peterson et al., 2004; Harvatine and Bauman, 2006), *GAPDH* (Baumgard et al., 2002), *B2M* (Harvatine and Bauman, 2006), and/or 18S rRNA (Harvatine and Bauman, 2006). Among these, *actin-β* and *GAPDH* have been shown to be inappropriate as ICG in bovine mammary due to the presence of co-regulation (Bionaz and Loor, 2007; Piantoni et al., 2008). Further, due to its high abundance, the use of 18S rRNA for normalization is questionable (Vandesompele et al., 2002). In the present study, we mined individual cow microarray data from two independent studies with lactating dairy cows supplemented with different lipids to identify potential ICG. The specific objective of the study was to use pair-wise analysis of expression ratios (Vandesompele et al., 2002) among several genes to identify potential ICG for qPCR normalization from mammary tissue of lactating dairy cows supplemented with lipids.

## MATERIALS AND METHODS

*RNA extraction, qPCR, and Primer Design and Testing*

Mammary tissue samples (n = 66) from lactating dairy cows fed saturated or unsaturated lipid supplements for 21 days (Thering et al., 2007) or infused with

Butterfat [source of short and long-chain fatty acids (LCFA)], LCFA or a CLA mixture for 14 days (Kadegowda et al., 2008) in addition to unsupplemented controls were used in this study. Mammary tissue was collected via percutaneous biopsy, immediately frozen in liquid $N_2$, and preserved at -80° C for RNA extraction as described by Bionaz and Loor (2007).

Total RNA was extracted from ~0.5 g tissue using ice-cold TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality was evaluated using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples (n=66) with an RNA integrity number of 6 to 9 were used for further analysis. Genomic DNA was removed with DNase using RNeasy Mini Kit columns (Qiagen, Valencia, CA). A portion of the assessed RNA was diluted to 100 mg/L using DNase-RNase free water prior to RT. Sufficient cDNA was prepared to run all selected genes. Each cDNA was synthesized by RT-PCR and cDNA used for qPCR previously described (Bionaz and Loor, 2007). Each sample was run in triplicate and a six-point relative standard curve plus the non-template control were used. The 4-fold-dilution standard curve was made using cDNA from a pool RNA of all the samples. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, Foster City, CA) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C.

The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Data were

calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, Foster City, CA). Primer Express 3.0 software (Applied Biosystems, Foster City, CA), optimized for use with Applied Biosystems qPCR Systems, was used for primers design using default features, except for the amplicon length, that was fixed at min of 100 bp (Table 8.2). Primers were designed across exon junctions when possible to avoid amplification of genomic DNA. The exon junctions were uncovered blasting the sequence against bovine genome (Genome Browser Gateway, 2008). Primers were aligned against publicly available sequences in NCBI(National Center of Biotechnology Information, 2008) and UCSC (Genome Browser Gateway, 2008). Prior qPCR, primers were tested using the same protocol as for qPCR without the dissociation step in a 20 μL reaction. Part of the PCR product was run in a 2% agarose gel stained with ethidium bromide to assess presence of the product to a expected size and presence of primer-dimer, the rest was purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA) and sent to sequence at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana. Only primers with high specificity evaluated by a single band on agarose gel, absence of primer-dimer, amplification of the right cDNA verified by sequencing, and a unique peak in the dissociation curve after qPCR reaction were used.

*Selection and Evaluation of ICG*

Sixty-six bovine microarrays from Thering et al. (2007) and Kadegowda et al. (2008) were used to identify potential ICG. The selection criterion was as described by Piantoni et al. (2008) and Tramontana et al. (2008) with modifications. Briefly,

genes with 3 SD above background were uploaded to GeneSpring GX software (Agilent Technologies, Santa Clara, CA) and normalized using Lowess. Only genes with medium-to-large mRNA abundance (≥100 relative florescent units) and expression ratio ~1.0 ± 0.2 between sample and reference in at least 60 of 66 samples were evaluated as potential ICG. In addition to the genes meeting these criteria (n = 39), 9 previously-tested ICG (*ACTB, GAPDH, RPS23, RPS9, MTG1, ITGB4BP, MRPL39, RPS15A,* and *UXT)* in bovine mammary tissue (Bionaz and Loor, 2007) were used to assess co-regulation using Ingenuity Pathway Analysis® (IPA, Redwood City, CA). Subsequently, the selected genes were verified for goodness of BLAT and BLAST sequences and primers were designed. The mRNA expression of the selected genes was assessed via qPCR as described by Bionaz and Loor (2007). Analysis of expression ratio stability and optimal number of ICG was conducted with geNorm software (Vandesompele et al., 2002). The applicability of the most stable ICG was tested by conducting qPCR on genes associated with fatty acid metabolism, e.g. acetyl-CoA carboxylase-α (*ACACA*), fatty acid synthetase (*FAS*), stearoyl-CoA desaturase (*SCD*), fatty acid binding protein 3 (*FABP3*), and glycerol-3-phosphate acyl-transfrease, mitochondrial (*GPAM*).

*Statistical Analysis*

Normalized data of ICG and lipid-metabolism genes were analyzed using the MIXED procedure of SAS (SAS Inst., Cary, NC). For the latter, separate statistical analysis were conducted for each experiment. Fixed effects were treatments while cow was the random effect.

## RESULTS AND DISCUSSION

### Selection of Potential ICG

The first criterion for selecting ICG that we applied was identification of genes with stable expression across all 66 microarrays encompassing 6 different lipid supplements and two controls. Among >13,000 annotated transcripts using this initial filtering step we identified 49 transcripts with expression ratio of ~1.0 ± 0.2. Of these, 10 were excluded from the subsequent steps as the identity and function of the transcripts could not be verified. The genes *ACTB, GAPDH,* and *B2M* are among the most commonly used ICG in studies of bovine mammary lipid synthesis regulation (Peterson et al., 2003; Harvatine and Bauman, 2006), were not evaluated further because they were highly unstable across the entire set of microarrays, i.e. expression ratio averaged ~0.9 ± 0.6 (Fig. 8.1) confirming earlier observations in non-ruminant tissues (Glare et al., 2002; Vandesompele et al., 2002).

### Co-Regulation Analysis

The second criterion applied was testing for absence of co-regulation, i.e., the selected genes should not be regulated through common upstream effectors (e.g., transcription factors). The co-regulation among potential ICG was assessed by using Ingenuity Pathway Analysis® (IPA, www.ingenuity.com; Redwood City, CA, USA). The IPA allows the examination of gene networks within large gene expression datasets based on published relationships across several organisms. Network analysis of the 39 transcripts selected in the first step plus 9 ICG previously tested (Bionaz and

Loor, 2007) identified 21 with no known co-regulation (Figure 8.2). IPA revealed co-regulation between commonly used ICG such as GAPDH, B2M, and ACTB through the transcription factors MYC, MYCN, TP53, EPO, and KITLG (Figure 2). Among the relationships uncovered, the expression of GAPDH, ACTB, RPS9, and UXT is regulated by MYC (Grandori et al., 2005), while expression of GAPDH, ACTB, B2M, RPS9, and RPS29 is regulated by MYCN (Boon et al., 2001; Valentijn et al, 2005). In addition, ACTB and B2M also are regulated by EPO (Kolbus et al., 2003) and KITLG (Kolbus et al., 2003). TP53 also regulates GAPDH and ACTB (Ginsberg et al., 1991).

The 21 transcripts with no known co-regulation, in addition to *UXT*, were used for further analysis. Despite the apparent co-regulation of UXT by MYC, this gene was previously found as a suitable ICG for use in bovine mammary tissue (Bionaz and Loor, 2007; Piantoni et al., 2008). We verified the annotation of sequences on the microarray (Loor et al., 2007) for all the selected genes through BLAST and BLAT searches, and excluded *SLC35E2* from further analysis. In addition, we were unable to design appropriate primers for *DND1, FAM57A, PCF11, PPFIA3,* and *SMUG1*. Highly-specific primers were designed for the remaining transcripts and these were subsequently tested by sequencing (data not shown). Among these, 11 were selected for qPCR analysis: *COPS7A, CORO1B, DNAJC19, EIF3K, EMD, GOLGA5, GPR175, MARVELD1, MTG1, MRPL39,* and *UXT* (Table 8.1).

**ICG Expression Profile and Stability Evaluation**

Bionaz and Loor (2007) suggested that changes in mRNA expression of ICG could be due to a potential dilution effect on constantly expressed genes driven by increased expression of milk component synthesis genes as a consequence of equal amounts of RNA used for qPCR analysis. In essence, a simple statistical analysis on the expression of ICG as selection criteria would not take this dilution effect into account. Dilution effects, in our experience, are most evident in experiments where changes in physiological state of the tissue are of interest [e.g., non-lactating vs. lactating; Bionaz and Loor (2007); Tramontana et al. (2008)]. However, in the case of lipid supplementation it is well-known that the nature of dietary fat can elicit changes in mRNA of lipogenic genes (Shingifeld and Griinari, 2007). Thus, to examine potential dilution effects on selected ICG we evaluated relative mRNA abundance as % change from MRPL39 (Table 8.2). The relative percentage abundance was calculated as $1/E^{(Ct)}$ (where E = efficiency [10-1/slope] and Ct = cycle determined by the threshold applied to the maximum amplification of the standard curve). Among the ICG, the mRNA abundance varied from 0.91 (UXT) to 47.62 (EIF3K). However, the mRNA abundance of most of the transcripts was closer (<2.5 fold differences). Only, MARVELD1 (16.23), EIF3K (47.62), EMD (4.91) and DNAJC19 (3.54) had higher expression (>2.5 folds) compared with MRPL39. On the contrary, the relative mRNA abundance of most the genes related to lipid synthesis were substantially higher than measured ICG. Thus, it could be possible that decreased mRNA expression of ICG occur due to a dilution effect. This was overcome, however, by use of pair wise comparison method (Bionaz and Loor, 2007),

which determines the expression ratio across samples. The ratios would remain constant when all genes with stable expression are affected to a similar extent by errors during the steps from RNA extraction through qPCR analysis (Vandesompele et al., 2002). The pairwise comparison of ICG was conducted using the geNorm software (http://medgen.ugent.be/~jvdesomp/geNorm/). Briefly, stability (M = gene-stability measure) refers to the constancy of the expression ratio between two non-co-regulated genes among all samples tested. The more stable the expression ratio among two genes, the more likely that the genes are appropriate internal controls, i.e. two ideal control genes should have an identical expression ratio in all samples regardless of experimental conditions, cell, and/or tissue type. The lower the M value, the higher the stability. geNorm also performs an analysis to determine the utility of including more than 2 genes for normalization by calculating the pairwise variation (V) between the normalization factor (NF) obtained using n genes (best references) ($NF_n$) and the NF obtained using n+1 genes (addition of an extra less stable reference gene) ($NF_{n+1}$). A large decrease in the pairwise variation indicates that addition of the subsequent more stable gene (i.e. with lowest M value) has a significant effect and should be included for calculation of the NF. Once the most stable internal reference genes are selected, the NF is calculated using the geometrical average between them to normalize qPCR data.

The genes *EIF3K, UXT, GPR175, MRPL39, MARVELD1*, and *EMD* were the most stable while *MTG1* and *COPS7A* were the least stable transcripts (Figure 8.4). The largest change in pairwise expression ratio stability, from 0.20 (Figure 8.4, Panel 1) to 0.077 (Figure 8.4, Panel 2), would occur if *GPR175* was selected as a third ICG

in combination with *EIF3K* and *UXT*, shown to be the most stable genes. The second largest change in variation, from 0.059 (V3/4) to 0.039 (V5/6), would be observed when using 5 ICG instead of 3. There were smaller changes in the variation due to subsequent addition of genes. These results suggested that use of 3 to 5 genes as ICG would be appropriate to normalize gene expression data from bovine mammary tissue in lipid-supplementation studies. An important result from our analysis is the fact that ICG selected for one physiological or experimental condition may or may not be suitable for a widely-different condition. For example, Bionaz and Loor (2007) found *UXT* as a suitable ICG for studies with bovine mammary gland during lactation cycle, which we confirmed. In contrast, *MTG1,* which was one of the most stable ICG in studies of prepubertal bovine mammary development (Piantoni et al., 2008) had lower stability value in the present study. Our analysis uncovered novel ICG that could be used in similar studies as alternatives to the less-reliable *ACTB*, *GAPDH*, 18S, or *B2M* for normalization.

**mRNA Expression of ICG and Lipid Metabolism Genes**

The mRNA expression of selected ICG in mammary tissue of lactating cows fed different lipid supplements are presented in Figure 8.3. Compared with controls, ICG expression in response to lipid supplements did not differ significantly and had similar patterns across all treatments from both experiments. Vandesompele et al. (2002) originally suggested the use of at least 3 appropriate ICG for normalization. On practical grounds, it is important to establish an optimum number of ICG for normalization. To assess this, we studied mRNA expression patterns of *ACACA*, *FASN*, *SCD*, *GPAM*, and *FABP3* independently in both experiments prior to and after

normalization using NF calculated with the geometrical mean of 3 or 6 most stable genes (Figure 8.5). Changes due to use of 6 vs. 3 ICG were minimal and, thus, not justified. Therefore, we propose that the geometric mean of *EIF3K, UXT*, and *GPR175* is appropriate to normalize data arising from lipid-supplementation studies of could be optimum for normalizing the data from mammary tissue in lactating cows.

## CONCLUSIONS

The results from the study showed that the relative expression of *B2M*, *GAPDH*, and *ACTB*, previously used as ICG in bovine mammary tissue from lipid-supplemented cows, was highly unstable across two studies representing 66 individual cow observations and 6 different lipid supplements. Gene stability analysis, via geNorm software, uncovered *EMD*, *MARVELD1*, *MRPL39*, *GPR175*, *UXT*, and *EIF3K* as the most stable genes and, thus, suitable as ICG. Analysis also indicated that use of 3-to-5 ICG was most appropriate for calculating a normalization factor. Overall, geometrical average of at least three among *EMD*, *MARVELD1*, *MRPL39*, *GPR175*, *UXT*, and *EIF3K* is ideal for normalization of mammary qPCR data in studies involving lipid supplementation of dairy cows.

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Table 8.1. Accession number, symbol, sequence, amplicon size and molecular functions of genes tested with qPCR.

| Accession # | Gene Symbol | Primer | Primers (5' --3') | Amplicon Size (bp) | Molecular Function |
|---|---|---|---|---|---|
| NM_001105331 | *COPS7A* | F.267 | AGGCCCCTGGTGTCTACGT | 143 | protein binding |
| | | R.407 | CCGGGCTTCGGCTAAGTAGT | | |
| NM_001024510 | *CORO1B* | F.1043 | GAGATTGCCCGGTTCTACAAACT | 100 | actin binding |
| | | R.1142 | CGGGATACAGATCATCCTGGAA | | |
| NM_001034458 | *DNAJC19* | F.124 | GTGGTAGCAGTTGGACTGACCAT | 125 | heat shock protein binding |
| | | R.248 | CTGAAGGCAGTTTTTGGTAGACTTT | | |
| NM_001034489 | *EIF3K* | F.345 | CCAGGCCCACCAAGAAGAA | 125 | translation initiation factor activity |
| | | R.469 | TTATACCTTCCAGGAGGTCCATGT | | |
| NM_203361 | *EMD* | F.490 | GCCCTCAGCTTCACTCTCAGA | 100 | protein binding |
| | | R.589 | GAGGCGTTCCCGATCCTT | | |
| NM_001098007 | *GOLGA5* | F.1370 | GAGCTACAGCAGCAAGTCAAAGTG | 103 | protein-tyrosine kinase activity;protein binding;ATP binding; |
| | | R.1472 | CTTTAGACTGGAGTATTCGAGTAGCTTTT | | Rab GTPase binding;protein homodimerization activity |
| NM_001102314 | *GPR175* | F.388 | TGCTGCTCATCCCCAATGT | 125 | G-protein coupled receptor activity |
| | | R.512 | CACCACGAACACCAGGATATAAAA | | |
| NM_001101262 | *MARVELD1* | F.2262 | GGCCAGCTGTAAGATCATCACA | 100 | |
| | | R.2361 | TCTGATCACAGACAGAGCACCAT | | |
| NM_001025327 | *MTG1* | F.277 | GATCTGAAGGAGCAGCAGAAAATT | 110 | |
| | | R.386 | GTTGGGATGACCTGCTTGACA | | |
| NM017446 | *MRPL39* | F.493 | AGGTTCTCTTTTGTTGGCATCC | 101 | |
| | | R.593 | TTGGTCAGAGCCCCAGAAGT | | |
| NM_001037471 | *UXT* | F.134 | CAGCTGGCCAAATACCTTCAA | 125 | protein binding;microtubule binding;beta-tubulin binding; |
| | | R.288 | GTGTCTGGGACCACTGTGTCAA | | actin filament binding |

Table 8. 2. Slope, coefficient of determination of standard curve (R2), efficiency (E), median Ct, and mRNA abundance of measured transcripts.

| Gene | Slope | R^2 | E | Median Ct | mRNA abundance |
|---|---|---|---|---|---|
| *MRPL39* | -3.137 | 0.993 | 2.08 | 23.49 | 1.00 |
| *UXT* | -3.179 | 0.998 | 2.06 | 23.94 | 0.91 |
| *CORO1B* | -3.154 | 0.997 | 2.08 | 22.75 | 1.88 |
| *DNAJC19* | -3.323 | 0.989 | 2.00 | 23.06 | 3.54 |
| *COPS7A* | -3.137 | 0.998 | 2.08 | 23.22 | 1.22 |
| *EMD* | -3.157 | 0.994 | 2.07 | 21.46 | 4.91 |
| *GOLGA5* | -3.202 | 0.995 | 2.05 | 22.73 | 2.46 |
| *MARVELD1* | -3.431 | 0.995 | 1.96 | 21.54 | 16.23 |
| *MTG1* | -3.302 | 0.996 | 2.01 | 23.56 | 2.25 |
| *EIF3K* | -3.396 | 0.996 | 1.97 | 19.73 | 47.62 |
| *GPR175* | -3.106 | 0.998 | 2.10 | 23.32 | 0.95 |
| *ACACA* | -2.993 | 0.998 | 2.16 | 21.48 | 2.05 |
| *FASN* | -3.239 | 0.998 | 2.04 | 17.01 | 172.91 |
| *FABP3* | -3.397 | 0.996 | 1.97 | 16.90 | 326.17 |
| *GPAM* | -3.309 | 0.997 | 2.01 | 19.81 | 31.72 |
| *SCD* | -3.243 | 0.995 | 2.03 | 16.87 | 193.62 |

Figure 8.1. Expression pattern of potential ICG in addition to commonly-used ICG (*ACTB, GAPD, B2M*).

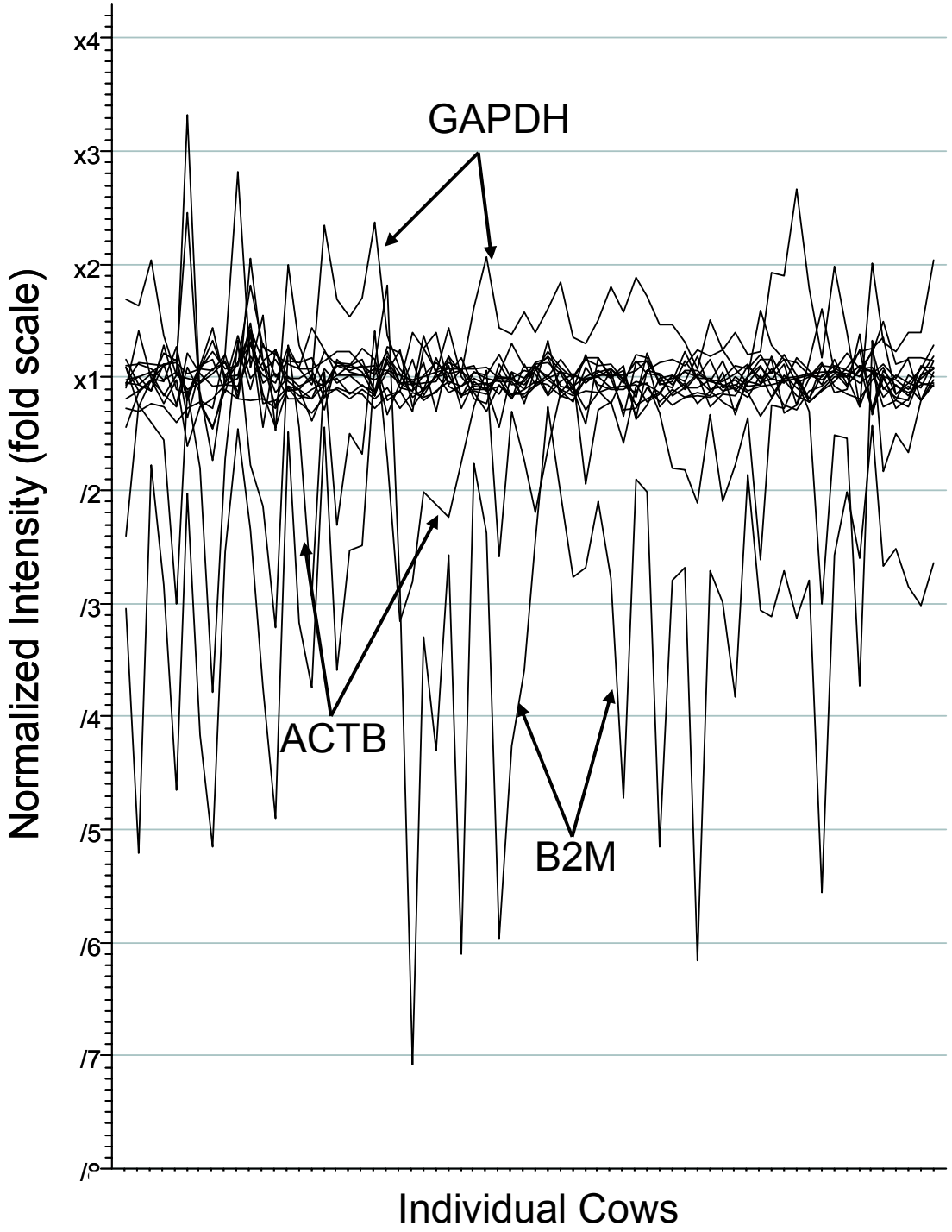
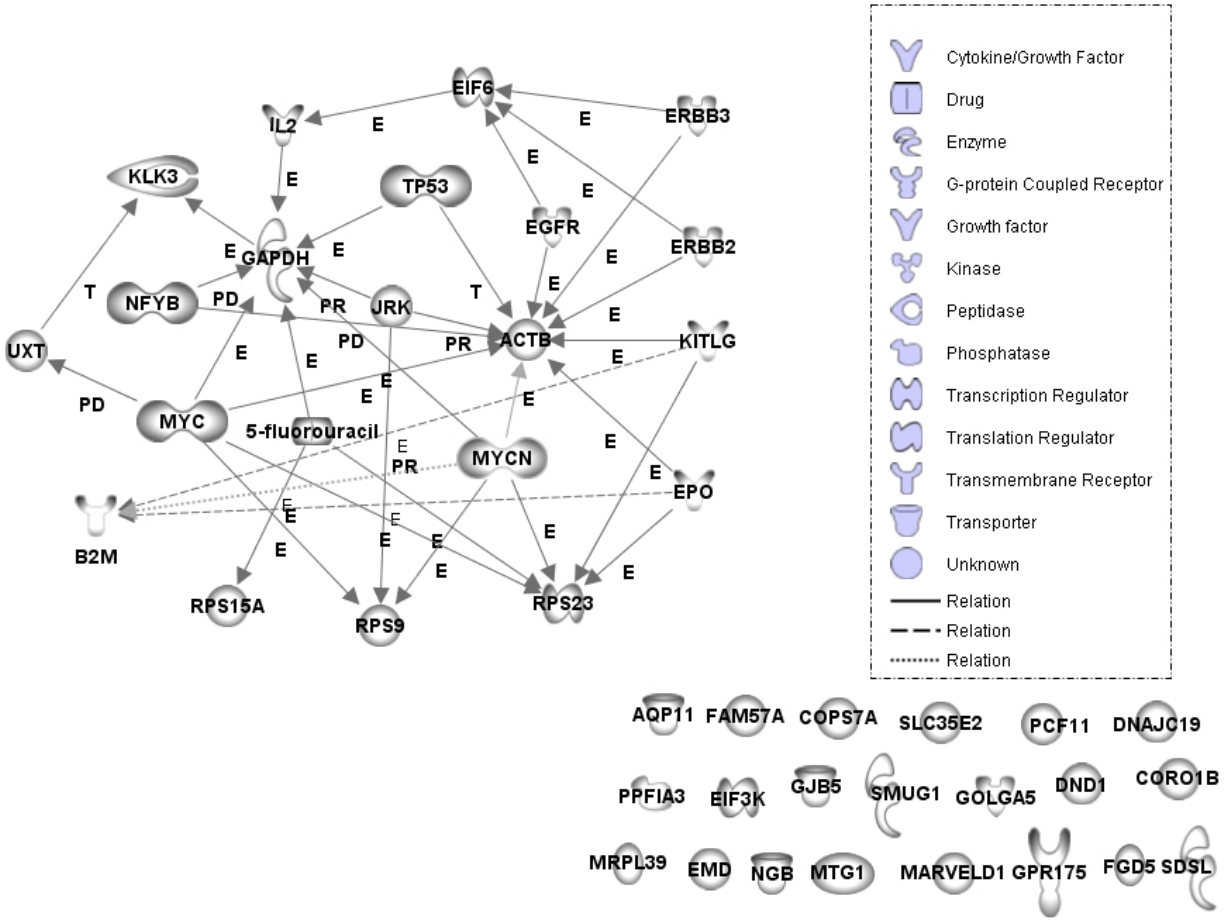


Figure 8.2. Interactions to determine co-regulation among potential ICG.



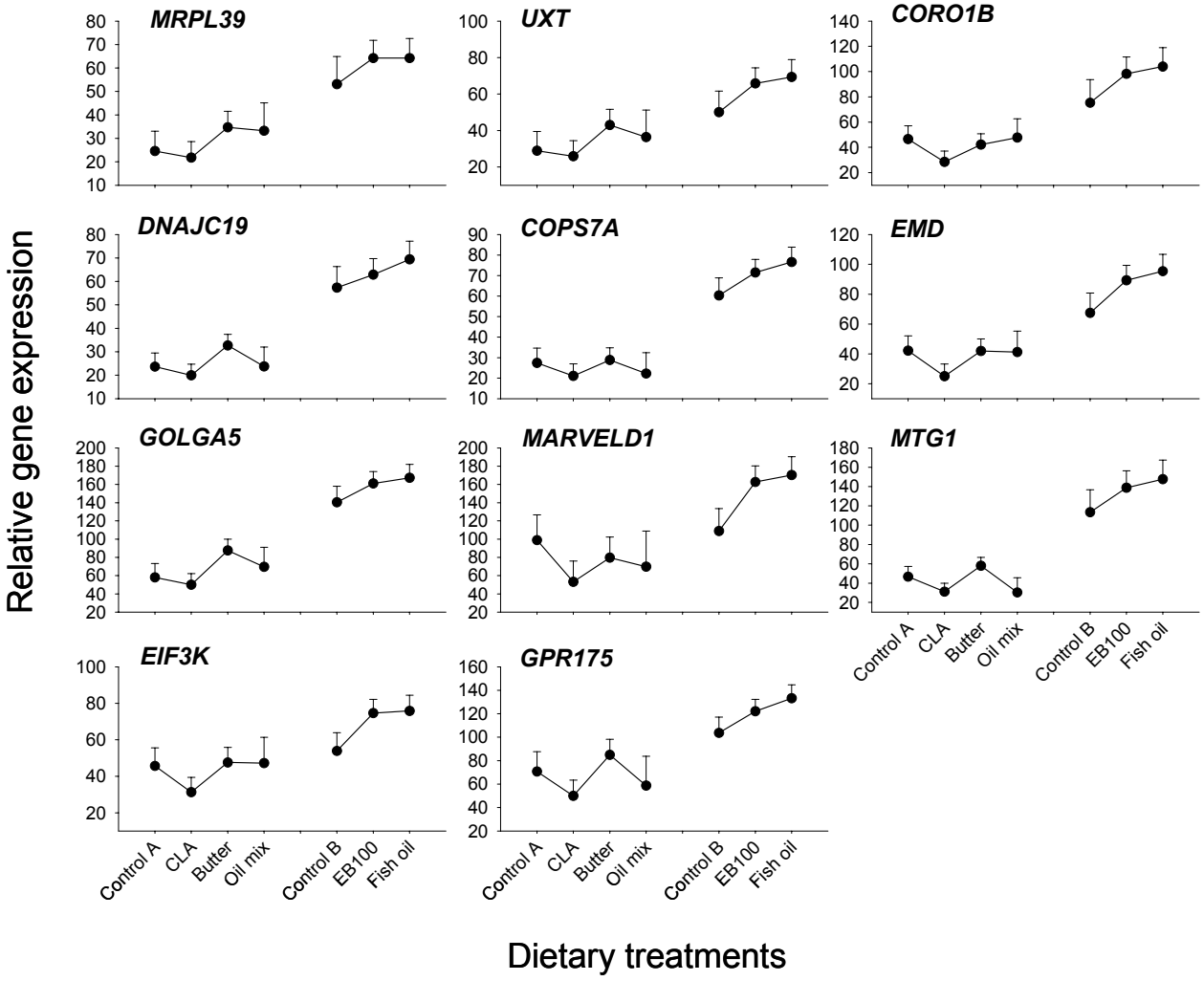
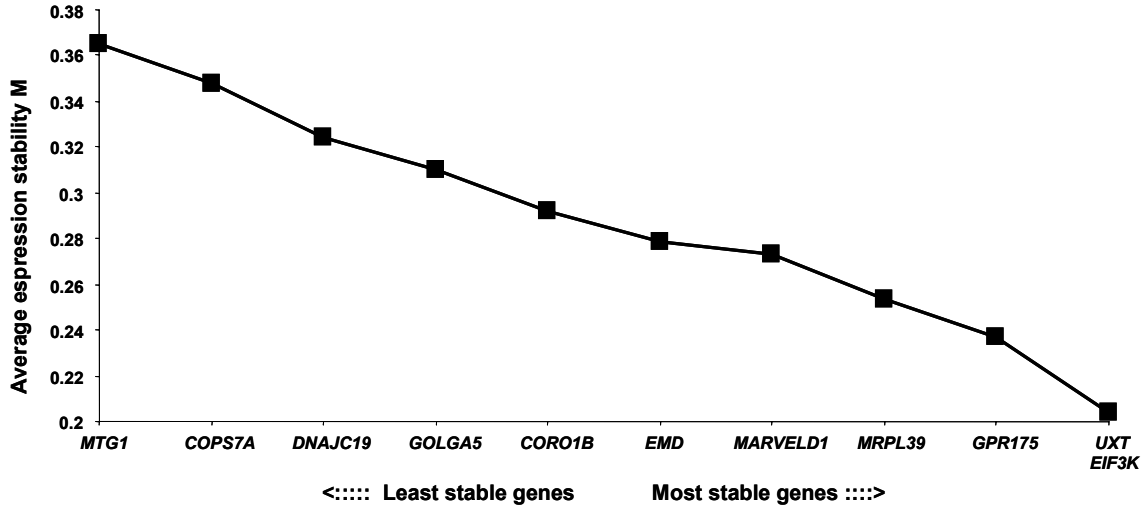
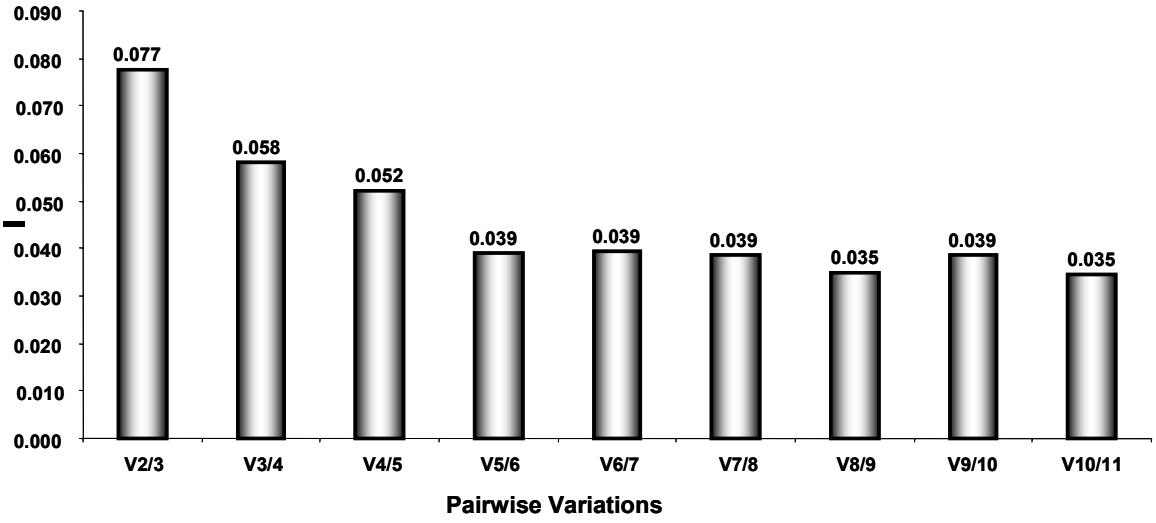



Figure 8.3. Mammary tissue mRNA expression patterns (mean ± SEM) of potential ICG tested in cows receiving control, lipid supplements into the abomasum (experiment A; control A, CLA, butter, and oil mix), or lipid supplements in a TMR (experiment B; control B, EB100, and fish oil). Data shown are from transformed Ct values with a standard curve. There were no statistically significant effects due to treatments.

Figure 8.4. geNorm results from the average expression stability of potential ICG calculated via pairwise comparisons and determination of optimal ICG number for normalization



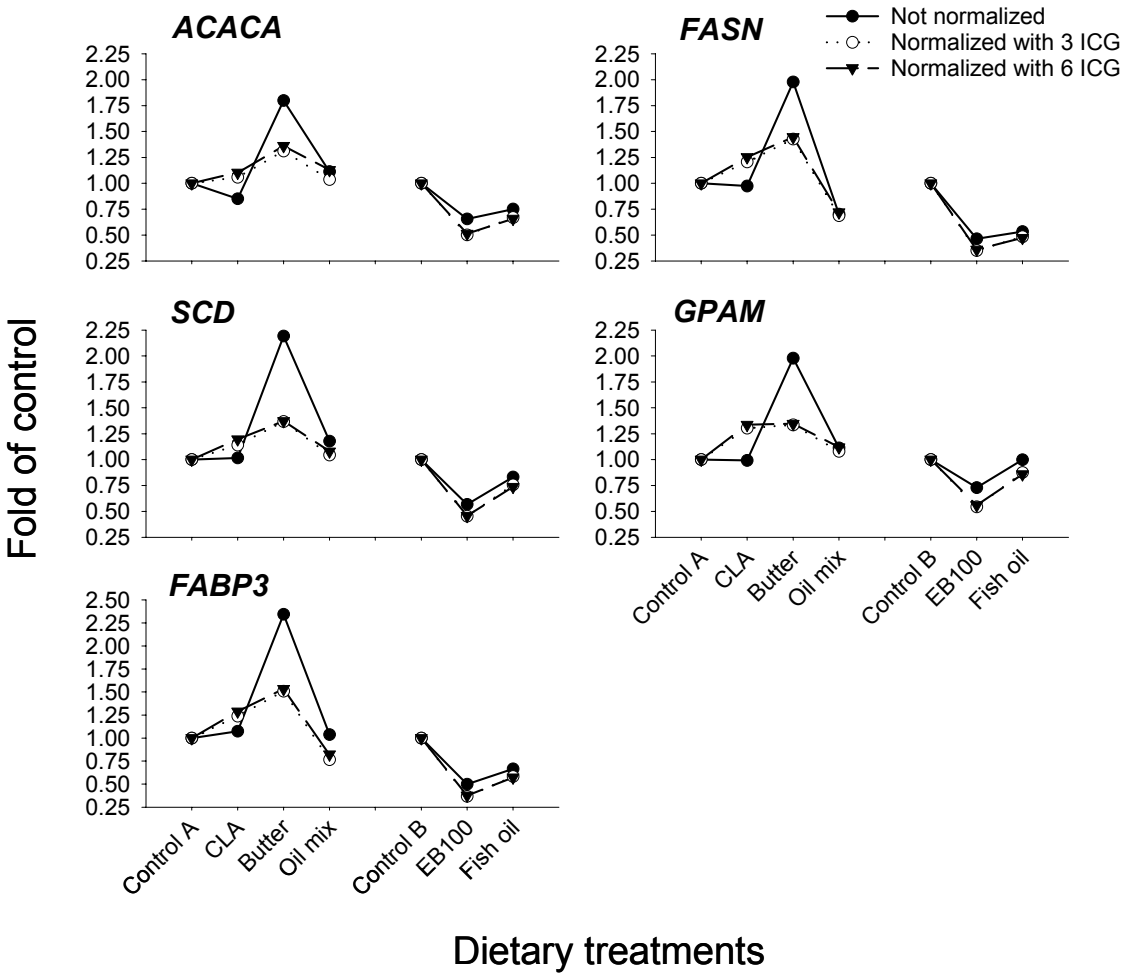



Figure 8.5. Patterns of mammary tissue *ACACA*, *FASN*, *SCD*, *GPAM*, and *FABP3* prior to and after normalization using NF calculated with the geometrical mean of 3 or 6 most stable genes from geNorm analysis. All data from experiment A (control A, CLA, butter, and oil mix) tended ($P < 0.15$) to be significant only when not normalized. In experiment B (control B, EB100, and fish oil) *ACACA*, *FASN*, and *FABP3* data were significant ($P < 0.07$) when normalized with 3 or 6 ICG.

# Appendix 1A

GenBank accession number, hybridization position, sequence, amplicon size, and source of primers for *Bos taurus* used to analyze gene expression by qPCR.

| Accession # | Gene | Primers[1] | Primers (5'-3')[2] | bp[3] | Source |
|---|---|---|---|---|---|
| AJ132890 | *ACACA* | F. 3709 | CATCTTGTCCGAAACGTCGAT | 101 | Bionaz and Loor, 2008b |
| | | R. 3809 | CCCTTCGAACATACACCTCCA | | |
| BC119914 | *ACSL1* | F. 1929 | GTGGGCTCCTTTGAAGAACTGT | 120 | Bionaz and Loor, 2008a |
| | | R. 2047 | ATAGATGCCTTTGACCTGTTCAAAT | | |
| BC134532 | *ACSS2* | F. 1881 | GGCGAATGCCTCTACTGCTT | 100 | Bionaz and Loor, 2008b |
| | | R. 1970 | GGCCAATCTTTTCTCTAATCTGCTT | | |
| DY208485 | *AGPAT6* | F. 171 | AAGCAAGTTGCCCATCCTCA | 101 | Bionaz and Loor, 2008a |
| | | R. 271 | AAACTGTGGCTCCAATTTCGA | | |
| X91503 | *CD36* | F. 743 | GTACAGATGCAGCCTCATTTCC | 81 | Bionaz and Loor, 2008b |
| | | R. 823 | TGGACCTGCAAATATCAGAGGA | | |
| NM_174693 | *DGAT1* | F. 177 | CCACTGGGACCTGAGGTGTC | 101 | Bionaz and Loor, 2008b |
| | | R. 277 | GCATCACCACACACCAATTCA | | |
| DN518905 | *FABP3* | F. 458 | GAACTCGACTCCCAGCTTGAA | 102 | Bionaz and Loor, 2008a |
| | | R. 559 | AAGCCTACCACAATCATCGAAG | | |
| DV778074 | *FABP4* | F. 402 | TGGTGCTGGAATGTGTCATGA | 101 | Bionaz and Loor, 2008a |
| | | R. 502 | TGGAGTTCGATGCAAACGTC | | |
| CR552737 | *FASN* | F. 6383 | ACCTCGTGAAGGCTGTGACTCA | 92 | Bionaz and Loor, 2008b |
| | | R. 6474 | TGAGTCGAGGCCAAGGTCTGAA | | |
| AY515690 | *GPAM* | F. 1963 | GCAGGTTTATCCAGTATGGCATT | 63 | Bionaz and Loor, 2008b |
| | | R. 2026 | GGACTGATATCTTCCTGATCATCTTG | | |
| XM_589325 | *INSIG1* | F. 438 | AAAGTTAGCAGTCGCGTCGTC | 120 | Bionaz and Loor, 2008b |
| | | R. 557 | TTGTGTGGCTCTCCAAGGTGA | | |
| DV797268 | *LPIN1* | F. 147 | TGGCCACCAGAATAAAGCATG | 101 | Bionaz and Loor, 2008a |
| | | R. 247 | GCTGACGCTGGACAACAGG | | |
| BC118091 | *LPL* | F. 327 | ACACAGCTGAGGACACTTGCC | 101 | Bionaz and Loor, 2008b |
| | | R. 427 | GCCATGGATCACCACAAAGG | | |
| NM_181024 | *PPARG* | F. 135 | CCAAATATCGGTGGGAGTCG | 101 | Bionaz and Loor, 2008b |
| | | R. 235 | ACAGCGAAGGGCTCACTCTC | | |
| DV935188 | *SCAP* | F. 990 | CCATGTGCACTTCAAGGAGGA | 108 | Bionaz and Loor, 2008b |
| | | R. 1097 | ATGTCGATCTTGCGTGTGGAG | | |
| AY241933 | *SCD* | F. 665 | TCCTGTTGTTGTGCTTCATCC | 101 | Bionaz and Loor, 2008b |
| | | R. 765 | GGCATAACGGAATAAGGTGGC | | |
| TC263657 | *SREBF1* | F. 143 | CCAGCTGACAGCTCCATTGA | 67 | Bionaz and Loor, 2008b |
| | | R. 209 | TGCGCGCCACAAGGA | | |
| DV921555 | *SREBF2* | F. 4134 | AGGTCTCTGGGCACCATGC | 101 | Bionaz and Loor, 2008b |
| | | R. 4234 | CATCACCGCAACCCCAAG | | |
| AY656814 | *THRSP* | F. 631 | CTACCTTCCTCTGAGCACCAGTTC | 151 | Bionaz and Loor, 2008b |
| | | R. 781 | ACACACTGACCAGGTGACAGACA | | |

1 Primer direction (F – forward; R – reverse) and hybridization position on the sequence

2 Exon-exon junctions are underlined

3 Amplicon size in base pair (bp)

# Appendix 1B

Sequencing results of genes using BLASTN from NCBI against nucleotide collection.
( Bionaz and Loor, 2008a, b)

| Gene | Sequence |
|---|---|
| *ACACA* | TCTCGACTGGTTGCTGTGATAGAAGAAGTTTGGGTAGGACAGTCAAAAATCGACGTTTCGGACAAGA TGGA |
| *ACSL1* | GCAGATGTCAAGGTATTCTAGAAGACATGGTGAGGCTCGGGAAGGAGTCTGGCCTAAAACCATTTG AACAGGTCAAAGGCATCTATAG |
| *ACSS2* | CTGTGCGATGGCCACATCTTCAGCCCAGCTCTCACTGAGGAGCTCAAGAAGCAGATTAGAGAAAAGA TTGGCCATCGCCCTG |
| *AGPAT6* | CTGAGGACCTGCATCATAATACATCGGGTGGATGGATGGTTCAAAAAGGGGAAGTTTCGAAATTGG GAGCCCAACAGTTATTACGAA |
| *CD36* | AGTTGAAGACAAGGGTATTGCAATTTTTCTCCTCTGATATTTGCAGGTCCA |
| *DGAT1* | CGCAGCGATCCCTGTTCAGTTCTGACAGTGGCTTCAGCAACTACCGTGGCATCCTGAATTGGTGTGT GGTGATGCACA |
| *FABP3* | GACTGTGTTCTGAGGGTCTTGTTGTTTTTATGATGACTGTGTCCCCATTCACTTCGATGATTGTGGTA GGGCTATTAAG |
| *FABP4* | CCCGAGTTATGAGAGAGCGTAGCCAAGGGATATTGAAATGGATGACGTTTGCATCGAACCTCCAAA |
| *FASN* | GCGAGACGTCTAGGTGTACGGGTGCCAGTCACGGATGCCCAGGATGTGAGTCACAGCCTTCAACGA GGTAAG |
| *INSIG1* | CAGGTCAGCGCGAGTGGGCCAGCGTGATGCGCTGCGTGGCCGTCTTCGTTGGCATCAACCACGCTA GTGCATAAACTGGGAA |
| *LPIN1* | GATCATCTAGTGATGAGGAGCATGCAGCCGCCAAGCCGTCCAGCACAAGCCACCTCCCCCTGTTGTCC AGCAGTCAGCAA |
| *LPL* | GCACTGTGAAGGTCTCTGCCTGAGCTGCAGAAAGAACCGTTGCAACAACATGGGCTACGAGATCAAC AAGGATT |
| *PPARG* | TAACAGTACTCTCCTAAAATACGGCGTGCACGAGATCATTTACACGATGCTGGCCTCCTTGATGAA |
| *SCAP* | GAGTATGGCGACAGGGAGTATGTAGGGTGGGTCACCAGGGGGGGGATGAGCTCAGCGATGCCAATCTCCTCC TTGAAGTGCACATGGGA |
| *SCD* | GACGGCAGGATCTGTGGATGAACGTTTCAAAACAGCCTGTTTTTTTGCCACCTTATTCCGTATATAGCCAG |
| *SREBF2* | ACAGACTCGAGGGCCAGTGGCCACTTGTGGAGCAGCCTCAACGTTAGTGGGGCCACCTGTGACCCCAACCTTAA CCATGCCTGGTCCAGCAA |
| *THRSP* | CGTCATCTGCTCCGTTAGGCTGCCTGCTGCTGTTCACAACTCCTCACTCCTCTTACTAGCTTGGGGTCGGAAGCC AGTGATTCATGAGGGACCACATGTCTGTCACCTGGTCAGTGTGTA |

# Appendix 1C

Sequencing results obtained from PCR product of *Bos taurus* specific primers for isoforms under investigation.

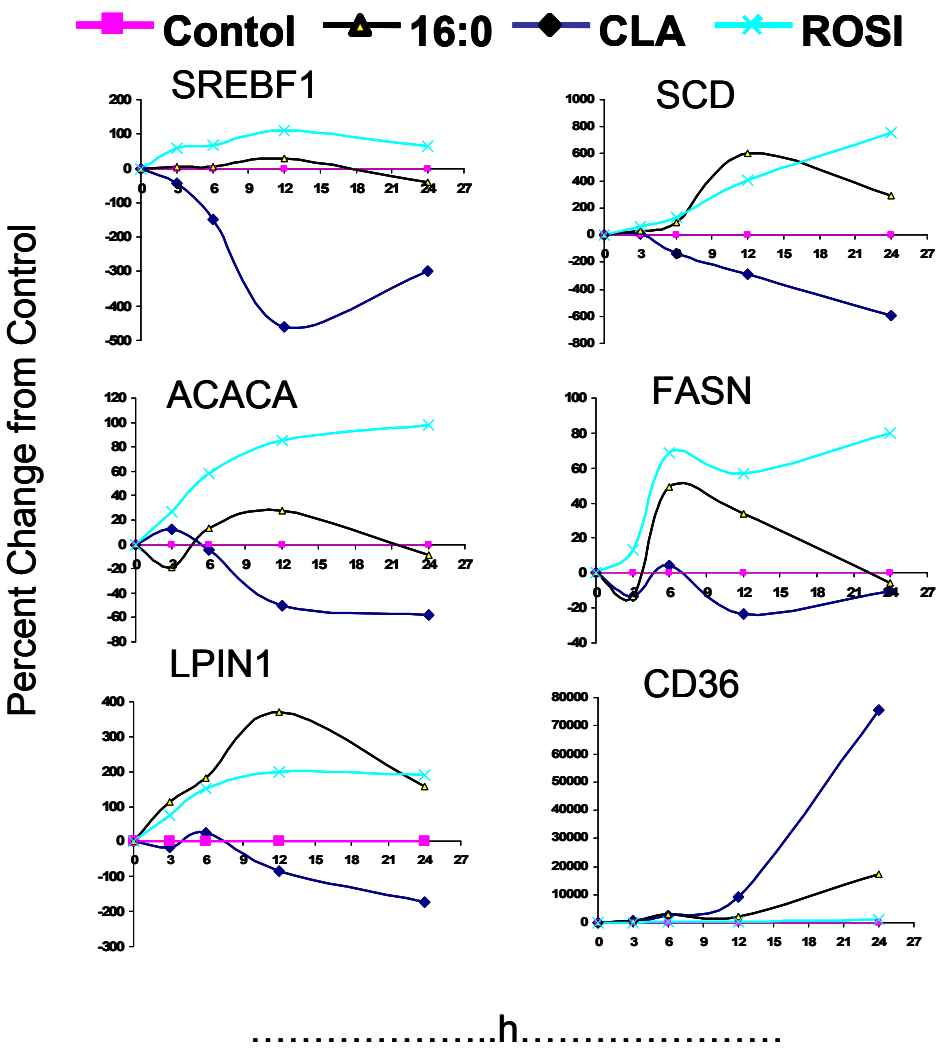
( Bionaz and Loor, 2008a, b)

| Gene | Best hit in NCBI | Score |
|---|---|---|
| *ACACA* | Bos taurus acetyl-coenzyme A carboxylase alpha (ACACA) | 93.7 |
| *ACSL1* | Bos taurus similar to acyl-CoA synthetase long-chain family member 1 | 153 |
| *ACSS2* | Bos taurus acetyl-CoA synthetase 2 | 134.0 |
| *AGPAT6* | Bos taurus 1-acylglycerol-3-phosphate O-acyl*trans*ferase 6 (AGPAT6) | 81.8 |
| *CD36* | Bos taurus CD36 molecule (thrombospondin receptor) | 86.0 |
| *DGAT1* | Bos taurus diacylglycerol O-acyltransferase homolog 1 (mouse) | 122.0 |
| *FABP3* | Bos taurus heart fatty acid-binding protein (FABP-3) | 95.6 |
| *FABP4* | Bos taurus fatty acid binding protein 4, adipocyte (FABP4) | 86 |
| *FASN* | Bos taurus fatty acid synthase (FASN) | 71.9 |
| *INSIG1* | PREDICTED: Bos taurus similar to insulin induced gene 1, transcript variant 2 | 123.0 |
| *LPIN1* | PREDICTED: Bos taurus similar to KIAA0188, *trans*cript variant 4 (alias of LPIN1) | 113 |
| *LPL* | Bos taurus lipoprotein lipase, mRNA | 123.0 |
| *PPARG* | Bos taurus peroxisome proliferator-activated receptor gamma | 107.0 |
| *SCAP* | Predicted: Similar to sterol response element binding protein cleavage-activating protein | 83.8 |
| *SCD* | Bos taurus stearoyl-CoA desaturase (delta-9-desaturase) | 79.8 |
| *SREBF2* | Predicted: Bos taurus similar to sterol regulatory element-binding transcription factor 2 | 136.0 |
| *THRSP* | Bos taurus thyroid hormone-responsive protein | 187.0 |

GPAM and SREBF1 were not sequenced due to the short PCR product (<70 bp), which requires cloning.

# Appendix 2

**Preliminary MAC-T study to determine the optimal time point (Chapter 7)**




Temporal pattern of lipogenic genes subjected to treatments: Control, Palmitate (16:0), t10c12-CLA, ROSIGLITAZONE (Time Points studied: 0, 3, 6, 12, 24 h). The maximum response for most of the genes was observed at 12 h.

# REFERENCES

Bionaz, M. and J. J. Loor. 2008a. *ACSL1, AGPAT6, FABP3, LPIN1*, and *SLC27A6* are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation. J. Nutr. 138: 1019–1024.

Bionaz, M and J.J. Loor. 2008b. Gene network driving bovine milk fat synthesis during the lactation cycle. BMC Genomics. 9:366.