Systemic and Intracellular Trafficking of Long-chain Fatty Acids in Lactating Dairy Cattle

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ABSTRACT

Marine oils are used as ration additives to provide omega-3 fatty acids to dairy cows. Supplementing dairy cows with omega-3 fatty acid-rich feeds does not easily increase quantities in milk fat of dairy cows because polyunsaturated fatty acids are biohydrogenated in the rumen. Lipid encapsulation of omega-3 fatty acids provides protection from biohydrogenation in the rumen and allows them to be available for absorption and utilization in the small intestine. Lactating cows were supplemented with rumen protected algae biomass or algal oil in a 4 × 4 Latin Square. Feeding lipid encapsulated algae supplements increased docosahexaenoic acid content in milk fat while not adversely impacting milk fat yield; however, docosahexaenoic acid was preferentially esterified into plasma phospholipid, limiting its incorporation into milk fat. In the second study, triglyceride emulsions of oils enriched in either oleic, linoleic, linolenic, or docosahexaenoic acids were intravenously infused to avoid confounding effects of triglyceride esterification patterns in the small intestine and to compare mammary uptake. Milk transfer of fatty acids delivered as intravenous triglyceride emulsions was reduced with increased chain length and unsaturation. Increased target fatty acids were evident in plasma phospholipid, suggesting re-esterification in the liver. Transfer efficiencies were

37.8, 27.6, and 10.9±5.4% for linoleic, linolenic, and docosahexaenoic acid. Both liver and mammary mechanisms may regulate transfer of long-chain polyunsaturates. Intracellular fatty acid binding proteins (**FABP**) are cytoplasmic proteins that are hypothesized to be essential for fatty acid transport and metabolism by accelerating long-chain fatty acid uptake and targeting to intracellular organelles, such as the endoplasmic reticulum for triglyceride esterification. FABP3 mRNA is highly expressed in bovine mammary and heart tissue, but is not present in MAC-T cells, a bovine mammary epithelial cell line. When overexpressed in MAC-T cells, FABP3 does not appear to be rate-limiting for fatty acid uptake *in vitro* and did not alter lipid metabolism. The function of FABP3 in the mammary gland remains unclear.

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List of Abbreviations

A-V	arterial-venous
ACSL	long-chain acyl-CoA synthetase
AGPAT	acylglycerophosphate acyltransferase
ASP	acid soluble products
CE	cholesterol ester
CLA	conjugated linoleic acid
DHA	docosahexaenoic acid
DMI	dry matter intake
ER	endoplasmic reticulum
EV	empty vector
FA	fatty acids
FABP	fatty acid binding protein
FAME	fatty acid methyl esters
FAO	fatty acid oxidation
FFA	free fatty acid
HDL	high density lipoprotein
LA	linoleic acid
LCFA	long-chain fatty acid
LDL	low density lipoprotein
LNA	alpha-linolenic acid
LPL	lipoprotein lipase

MAC-T	bovine mammary epithelial cell line
MDGI	mammary-derived growth inhibitor
MFD	milk fat depression
n-3	omega-3
PL	phospholipid
PUFA	polyunsaturated fatty acid
RP	rumen-protected
RP Bio	rumen-protected algal biomass
RP Oil	rumen-protected algal oil
OA	oleic acid
PPAR	perixosome proliferator activated receptor
SCD	stearoyl-CoA desaturase
TG	triglyceride
TMR	total mixed ration
UNT	untransfected control
VLDL	very low density lipoprotein

CHAPTER 1: Introduction

Concentrations of milk components determine the value of milk produced. Non-nutritional factors like parity, season, genetics, stage of lactation, and mastitis are known to influence milk composition. Milk fat is the most variable and energy dense component of milk and is easily influenced by nutrition, followed by protein and lactose, respectively, which remain relatively stable (Jenkins and McGuire, 2006). Plant and marine oils, feeding strategy, dietary fiber, concentrate inclusion, and ionophores are known to alter milk fat content and composition. Even though intense genetic selection for milk yield decreases milk fat content, there is minimal effect of selection for milk yield on milk fatty acid (**FA**) profile (Kelm and Freeman, 2000, Kay et al., 2005).

The diet of a lactating dairy cow typically contains 2-5% fat and is enriched in polyunsaturated fatty acids (**PUFA**). Fatty acid composition of both grass and linseed are over 50% alpha-linolenic acid (**LNA**; *cis*-9, *cis*-12, *cis*-15-C18:3), an omega-3 (**n-3**) PUFA, but the proportion of LNA in milk fat is generally less than 1% (Sterk et al., 2012). Similar losses are observed in linoleic acid (**LA**; *cis*-9, *cis*-12-C18:2), an omega-6 PUFA common in grass and oilseeds which make it a predominant FA in diets of lactating dairy cattle. The low milk fat content of PUFA is generally associated with extensive lipolysis and biohydrogenation in the rumen by rumen microorganisms because unsaturated dietary FA are recovered duodenally as saturated FA (Jenkins et al., 2008). Ruminants rely heavily on lipolysis and biohydrogenation by rumen microorganisms, as relatively little hydrolysis occurs in the small intestine. As a result, efforts to maximize transfer of dietary PUFA into ruminant milk and meat most frequently focus on rumen biohydrogenation.

The ability to change milk composition allows for improved processing characteristics, enhanced nutrient composition, and delivery of nutraceuticals for human health. Increasing the level of polyunsaturated fatty acids (**PUFA**) is the typical goal of nutritional modulation of milk fat synthesis, whether for a physical characteristic, such as improving the spreadability of butter, or a nutritional benefit, like decreased milk fat content or enhanced PUFA concentration (Lock and Bauman, 2004, Jenkins and McGuire, 2006). Human health benefits associated with consuming n-3 FA encourage development of functional foods for humans. Biohydrogenation of LA yields conjugated linoleic acid (**CLA**) and vaccenic acid (*trans*-11-C18:1), a substrate for CLA synthesis in tissue, in ruminant products (Griinari et al., 2000). Increased DHA content of fish oil causes greater rumen accumulation of vaccenic acid due to partial hydrogenation of LA (AbuGhazaleh and Jenkins, 2004, Klein and Jenkins, 2011). Conjugated linoleic acids are purported in animal studies to be anticarcinogenic, antiatherogenic, immune-enhancing, and weight-reducing (Kelley et al., 2007, Benjamin and Spener, 2009, McCrorie et al., 2011). Consumer perceptions of milk fat composition and quality may be improved by dietary supplements that increase milk fat content of n-3 FA (Lock and Bauman, 2004).

Additionally, n-3 FA supplements have been purported to improve reproductive efficiency of dairy cattle by inhibiting prostaglandin-$F_{2\alpha}$ synthesis through competition with arachadonic acid as a precursor for prostaglandin synthesis (Staples et al., 1998, Mattos et al., 2000, Mattos et al., 2004). In some studies, feeding n-3 FA during lactation has been found to reduce prostaglandin secretion and improve fertility and embryo survival (Santos et al., 2008). This dissertation will focus on the nutritional manipulation of n-3 PUFA in milk fat.

CHAPTER 2: Review of Literature

RUMINANT LIPID METABOLISM

Biohydrogenation

Supplementing dairy cows with PUFA-rich feeds does not easily increase quantities in milk fat of dairy cows. Transfer of PUFA into milk fat is thought to be primarily limited by biohydrogenation of unprotected supplements (Lock and Bauman, 2004). Digestion of dietary lipid begins in the rumen, where PUFA undergo extensive hydrolysis and biohydrogenation. Lipolytic microorganisms in the rumen hydrolyze most complex dietary lipids, releasing free FA (**FFA**). This is followed by biohydrogenation, a process in which enzymes of rumen microbes add hydrogen, removing double bonds, thereby saturating the fatty acid. Free FA released via hydrogenation undergo varying degrees of biohydrogenation by rumen microorganisms, which primarily produce saturated stearic acid from LA or LNA **(**Figure 2.1). Rumen outflows are predominantly stearic acid with smaller quantities of intermediates of this process, consisting of *trans*-unsaturated monoenes and dienes (Drackley, 2005). Loor et al. (2004) demonstrated the significant PUFA losses due to rumen biohydrogenation (Figure 2.2). Thus, absorbed FA are much more saturated than dietary FA.

Milk Fat Depression

Diet-induced milk fat depression (**MFD**) has been a syndrome of low milk fat yield since first observed by Boussingault the 19th century (cited by Bauman and Griinari, 2003). A variety of diets have been known to induce low milk fat yields and changes in milk fat composition, but

are typically categorized as diets high in readily digestible carbohydrates and low in effective fiber (high grain/low forage) or dietary supplementation of PUFA (Davis and Brown, 1970). These diets modify the rumen environment initiating altered pathways of biohydrogenation and increased rumen outflow of *trans*-isomers (Figure 2.1) and decrease milk fat yield and alter its composition (Figure 2.3). The level of milk fat reduction is dependent upon the interaction of these and other dietary and environmental factors, but can be greater than 50% without changes in milk protein or lactose concentrations (Bauman and Griinari, 2003). Unprotected dietary PUFA alone may adversely impact intake and milk fat yield as a result of altered biohydrogenation pathways (Palmquist et al., 2005).

The biohydrogenation theory of MFD suggests the production of *trans*-intermediates of altered rumen biohydrogenation have increased outflow and negatively impact synthesis of milk fat in the mammary gland. CLA are *trans*-octadecadienoic acids primarily derived from ruminant sources as resulting intermediates of LA biohydrogenation. While CLA are purported to have numerous biological effects, the *trans*-10, *cis*-12-isomer is a potent inhibitor of milk fat synthesis (Baumgard et al., 2000). Peterson et al. (2002) demonstrated a curvilinear relationship between the dose and milk fat yield of *trans*-10, *cis*-12-CLA and the reduction in milk fat yield, and this corresponds with *trans*-10, *cis*-12-CLA milk fat yield in diet-induced MFD. Lock et al. (2007) demonstrated that *trans*-10-C18:1, an intermediate in *trans*-10, *cis*-12-CLA biohydrogenation, has no direct effect on milk fat synthesis and is simply a marker of altered biohydrogenation.

The specific FA present in the unprotected PUFA supplements are the factors that influence pathways of rumen biohydrogenation of dietary FA. Klein and Jenkins (2011) demonstrated docosahexaenoic acid (**DHA**; C22:6, n-3) itself is not converted to *trans*-11-C18:1, but modifies biohydrogenation of other long-chain PUFA present in the rumen, such as LA and

LNA. Docosahexaenoic acid promotes accumulation of vaccenic acid (*trans*-11-C18:1) during LA biohydrogenation, but the presence of other FA in unprotected PUFA supplements interact with dietary LA to increase *trans*-10, *cis*-12-CLA production (Whitlock et al., 2002, AbuGhazaleh and Jenkins, 2004, Whitlock et al., 2006).

Without adequate rumen protection, marine algae supplements increase milk fat DHA content, but decrease dry matter intake (**DMI**), milk yield, and milk fat yield (Franklin et al., 1999, Offer et al., 2001a, Boeckaert et al., 2008). Rumen protection can circumvent interacting factors that influence biohydrogenation and prevent MFD.

Rumen Protection of PUFA

Santos et al. (2008) described a need to develop methods to improve the delivery of specific FA for absorption in their review of the effects of long chain FA on reproduction. Rumen protection guarantees PUFA flow to the duodenum and allows release and absorption by the intestines without impacting fermentation (Jenkins and Bridges, 2007). Technologies promising protection from biohydrogenation include coating PUFA with formaldehyde-treated casein, lipid encapsulation or structural change using calcium salts and FA amides to resist microbial enzymes (Jenkins and Bridges, 2007). Rumen outflow and milk FA transfer are generally inconsistent for rumen-protected (**RP**) PUFA. Whole oilseeds and calcium salts of FA provide consistent duodenal flows, but do not provide a protected source of n-3 PUFA (Castaneda-Gutierrez et al., 2007). Lipid encapsulation is a method that may allow n-3 PUFA to remain protected in the rumen, avoid biohydrogenation, and be available for absorption and utilization (Perfield et al., 2004). Regardless, increasing supply of PUFA to the small intestine by rumen protection does not overcome differences in esterification in the small intestine (Noble,

1978). Rumen-protected fat sources that deliver consistent duodenal flows of n-3 PUFA remain to be elucidated and commercially developed.

Lipid Absorption and Secretion by the Small Intestine

The absorption of long-chain FA (**LCFA**) has been extensively reviewed (Noble, 1978, Bauchart, 1993, Jenkins, 1993). Long-chain FA reach the intestine largely as saturated free FA affixed to the surface of feed particles and bacteria, or as components of microbial phospholipids (**PL**) and sterol esters (Drackley, 2005). Protected FA are delivered to the small intestine as intact triglyceride (**TG**) and digested similarly in monogastrics and preruminants by pancreatic lipase and colipase, releasing free FA and monoacylglycerols (Bauchart, 1993). Bile salts secreted in the duodenum and separate FA from particles by their detergent action, forming micelles via lysolecithin formed by phospholipase A_2. These components are absorbed by diffusion when the micelle moves across the unstirred water layer of the intestinal epithelium (Drackley, 2005). The FA reaching the small intestine are efficiently absorbed due to 1) lysolecithin improving micelle function and FA partitioning, 2) a relatively low intestinal pH which reduces insoluble calcium soap formation, and 3) the increased solubility of FA via taurocholate, the major bile salt of ruminants (Drackley, 2005). Fatty acid digestibility may decline with increased FA intake as micelle formation becomes limiting (Bauchart, 1993).

Volatile FA (2 to 6 carbons) produced by rumen microorganisms are largely absorbed by the rumen and medium-chain FA (less than 12 carbons) are absorbed by the small intestine and secreted into portal blood as non-esterified FA (Hocquette and Bauchart, 1999). In the enterocyte, LCFA are esterified to form cholesterol esters (**CE**), PL, and TG. Micelles form containing a TG and CE core with a surface monolayer of PL and unesterified cholesterol.

Apolipoproteins A and B (characteristically Apo B48; Bauchart, 1993), made in the intestine, are complexed with these lipids into very low density lipoproteins (**VLDL**) and secreted into lymph, entering the bloodstream at the thoracic duct (Palmquist, 1976, Palmquist and Mattos, 1978, Emery, 1979). Other apolipoproteins are synthesized in the liver and transferred from high density lipoproteins (**HDL**) in circulation. Apo CI to CIV are co-factors for lipoprotein lipase and Apo E is required for liver uptake; allowing lipoproteins to control lipid utilization in energy metabolism (Bauchart, 1993). Chylomicrons are the largest and least dense (<0.95 g/mL) of the lipoproteins and are secreted by the enterocyte following a meal; however, VLDL (0.95-1.006 g/mL) secretion is predominant in ruminants due to the low fat content of their diets (Raphael et al., 1973, Bauchart, 1993).

Though TG are the major lipid class found in lymph of monogastrics, the proportion of PL to TG is higher in ruminants (Bauchart, 1993). Increased dietary fat or rumen outflow of PUFA increases chylomicron secretion, but the TG proportion remains unchanged, meaning the surface monolayer components (PL and unesterified cholesterol) are increased during re-esterification in the enterocyte (Noble, 1978). Bolus feeding of lipids is known to increase the TG portion of VLDL (Bauchart, 1993). However, bolus feeding of lipids disturbs rumen function by altering biohydrogenation and reduces DMI. Litherland et al. (2005) demonstrated that reductions in DMI may be more pronounced with unsaturated FFA rather than unsaturated TG reaching the duodenum as a result of feeding increased quantities of supplemental fat.

Systemic Lipid Transport

Unlike monogastrics, synthesis and secretion of VLDL in bovine liver is minimal (Bauchart, 1993, Drackley, 1999). This is related to the continuous flow of digesta in ruminants

as opposed the boluses associated with meals consumed by nonruminants. As TG proportion diminishes, lipoproteins become increasingly dense and contain greater concentrations of CE, PL and apolipoproteins. Intermediate density lipoproteins and low density lipoprotein (**LDL**) are remnants of extensive VLDL TG hydrolysis, but both classes have minimal concentration in the plasma of lactating cows (Bauchart, 1993). High density lipoproteins are the major plasma lipoprotein in ruminants, and are secreted by the liver as a reverse cholesterol transport system, returning excess peripheral cholesterol to the liver for bile secretion (Bauchart, 1993). Rapid metabolism of VLDL (within 5 min) during lactation is due to TG hydrolysis, but the metabolic half-life of LDL and HDL are much greater (Raphael et al., 1973). In fact, the fractional removal rate of VLDL is approximately 10 fold greater than that of LDL (Palmquist and Mattos, 1978). Supporting this rapid turnover, the TG component of VLDL is a precursor for milk fat synthesis in addition to FFA in plasma. Harvatine and Bauman (2011) demonstrated a slight lag in CLA enrichment of and clearance from plasma PL compared to TG with abomasal infusion. This lag in PL clearance supports the decreased turnover rate for HDL compared to VLDL in plasma.

Although feeding supplemental PUFA can overwhelm the normal esterification capacity of the small intestine to allow increased incorporation of PUFA into TG (Bauchart, 1993), partitioning of PUFA into plasma lipid fractions that are less available to the mammary gland is more common (Lock and Bauman, 2004). Previous results have demonstrated that supplemental, very long-chain, n-3 FA are primarily transported in the PL or CE fraction of blood, making them largely unavailable to the mammary gland for enrichment of milk fat (Brumby et al., 1972, Kitessa et al., 2001, Offer et al., 2001b). Christie et al. (1986) hyposthesized selective hydrolysis of VLDL TG at the liver may protect PUFA from nonessential functions. Mammary uptake of FA from plasma is dependent upon the action of mammary lipoprotein lipase (**LPL**) on TG

transported in chylomicrons and VLDL (Palmquist, 1976, Moore and Christie, 1979). Accordingly, FA incorporated into PL or CE at the small intestine or liver are not available for mammary uptake, and this may explain the low transfer of PUFA to milk (Mansbridge and Blake, 1997).

Clearance of PUFA from TG transported in VLDL may not occur via LPL as well. In studies utilizing triglyceride emulsions designed to model VLDL, the rate of clearance is fastest for fish oil emulsion particles, followed by medium-chain TG and last, long-chain TG emulsion particles (Qi et al., 2002). The LPL, apolipoprotein E, LDL receptor, and lactoferrin-sensitive pathways control removal of long-chain TG from emulsions containing primarily omega-6 FA (Qi et al., 2006, Murray-Taylor et al., 2010). Park et al. (2004) proposed increased margination of n-3-rich particles reflected activation of LPL. Margination is particle attachment to endothelium-bound LPL during lipolysis. Qi et al. (2006) then suggested LPL primarily functions as a "bridge" protein to mediate FA uptake through membrane-anchoring. Removal of n-3 particles was less affected by this mechanism and independent of apolipoprotein E, LDL receptor, and lactoferrin. In fact, Murray-Taylor et al. (2010) demonstrated that the uptake of n-3 emulsion particles depends upon cell surface proteoglycans and non-LDL receptor cell surface anchoring. These studies suggest mammary uptake mechanisms present in the lactating dairy cow may conserve essential fatty acids by limiting uptake of n-3 PUFA.

Intracellular Fatty Acid Transport

Following hydrolysis of TG by mammary lipoprotein lipase, locally generated FFA must cross the plasma membrane to enter the cell. There is considerable debate as to whether LCFA, especially PUFA, transfer requires transport proteins or occurs through diffusion through the

lipid bilayer. In nonruminants, rapidly facilitated protein-mediated uptake occurs through the fatty acid translocase/CD36 membrane receptor and fatty acid transport proteins, and CD36 mRNA is found in the milk fat globule membrane (Bionaz and Loor, 2008b). In adipocytes, the fatty acid translocase/CD36 complex is located within cavaeolae, which are flask-shaped lipid rafts rich in sphingolipids and cholesterol that create unique membrane domains (Pohl et al., 2005). The polyunsaturation of DHA and its opposing interaction with sphingolipids and cholesterol cause it to disrupt lipid rafts (Chapkin et al., 2008). It is possible that disruption of the size and distribution of lipid rafts by DHA alters the ability of LPL to anchor VLDL for uptake of FA by fatty acid translocase/CD36 or that the complex itself is disrupted. This might explain why transfer efficiencies for other n-3 PUFA, such as LNA, resemble LA, another 18 carbon FA.

As these LCFA enter the cell, long-chain acyl-CoA synthetases (**ACSL**) catalyze the synthesis of fatty acyl-CoA, which are the substrates for both catabolic and anabolic pathways in the cell. The predominant isoform in bovine mammary tissue is ACSL1, and it may have a role in milk fat synthesis (Bionaz and Loor, 2008b). These ACSL isoforms may have overlapping substrate specificity, but it is possible that tissue specific distribution of each isoform allows for regulation of FA fate within the cell.

Intracellular fatty acid binding proteins (**FABP**) are cytoplasmic proteins that are hypothesized to be essential for fatty acid transport and metabolism by accelerating LCFA uptake and targeting FA to intracellular organelles. Fatty acid binding proteins are over 25 times more effective than albumin in increasing LCFA solubility in the cytoplasm and may target transfer of LCFA to acceptor membranes through direct membrane interaction (McArthur et al., 1999). Intracellular fatty acid trafficking is quite complex, and functional redundancy and cooperation exists among several proteins, including others such as ACSL and fatty acid

transport proteins which also determine metabolic fates of LCFA (Sandoval et al., 2008). There are also distinct differences in the function and binding activity of the nine FABP isoforms identified (Storch and Corsico, 2008).

A 15 kD protein, FABP3 is highly expressed in cardiac and skeletal muscle, and has been linked to fatty acid trafficking, metabolism, and signaling. It was originally isolated in the mammary gland as the primary component of mammary-derived growth inhibitor (**MDGI**) as reviewed by Mather et al. (2000). Characterization of MDGI revealed it to be a complex of FABP3 with some FABP4 (Spener et al., 1990, Yang et al., 1994, Specht et al., 1996). The MDGI complex inhibits cell proliferation in mammary epithelial cell lines *in vitro* and may play a role in the onset of differentiation (Politis et al., 1992, Yang et al., 1994). It has also been shown to suppress tumor formation through a C-terminal-derived 11 amino acid peptide in breast cancer cell lines (Wang and Kurtz, 2000). Breast cancer patients with tumors expressing MDGI had a more favorable prognosis (Nevo et al., 2010). These effects of FABP3 may be independent of its fatty acid ligand binding effects. Clark et al. (2000) determined FABP3 lacks an N-terminal signal peptide, and it is not known if the protein complex is secreted *in vivo*.

According to Bionaz and Loor (2008b, a), temporal patterns of gene expression reveal that FABP3 and FABP4 are highly expressed in bovine mammary tissue during early lactation, which follows the temporal pattern of MDGI expression in lactation observed by Politis et al. (1992). Bionaz and Loor (2008a) proposed a role of FABP3 in channeling LCFA to stearoyl-CoA desaturase (**SCD**) or triacylglycerol synthesis or in LCFA activation of gene expression through peroxisome proliferator activated receptor-γ (**PPAR-γ**). Deletion of the FABP3 gene in mice causes defective fatty acid oxidation (**FAO**) compensated by increased glucose utilization in heart and skeletal muscle (Storch and Corsico, 2008). While Clark et al. (2000) reported no

overt effects of gene deletion on mammary gland phenotype, FABP3 null mice had a lower percentage of total unsaturated fatty acids in milk fat. Additionally, both genetic and chemical inhibition of FABP4 alleviates endoplasmic reticulum (**ER**) stress (Erbay et al., 2007). Further, FABP4/5 knockout mice exhibit protection from high fat diet induced metabolic disorders (Cao et al., 2008). Together, these findings support a potential role for FABP3 and FABP4 in lipid synthesis in the bovine mammary gland though the exact biological function remains to be elucidated.

The human breast cancer cell line MCF7 does not express FABP3 and overexpression of FABP3 in MCF7 cells increased uptake of radioactively labeled palmitate and oleate, but other changes in fatty acid metabolism were not observed (Buhlmann et al., 1999). Other studies have explored the role of overexpression of multiple FABP isoforms on lipid metabolism. Overexpression of FABP3 and FABP4 in L6 myoblasts, and FABP1, FABP3, and FABP4 in Madin-Darby canine kidney cells did not alter palmitate uptake (Prinsen and Veerkamp, 1998, Zimmerman and Veerkamp, 2001). Both L6 myoblasts and Madin-Darby canine kidney cells contain FABP. It is possible that compensation by other cytosolic proteins, even other minimally expressed FABP isoforms, provides FA transport, such that uptake is not rate-limiting. Wolfrum et al. (1999) demonstrated decreased FA uptake in the human hepatoma cell line, HepG2, by knockdown of FABP1. Prior studies reporting a role for FABP3 in FAO reference cardiac or skeletal muscle defects in FAO; however, FAO is very low in mammary epithelial cells (Dimenna and Emery, 1980). This defect in muscle FAO is largely due to inhibition of FA uptake and consequent increased glucose utilization (Binas et al., 1999, Schaap et al., 1999). Kadegowda et al. (2009) reported FABP3 expression increased in MAC-T cells when treated

with rosiglitazone to activate PPAR-γ and palmitate or stearate. These studies support a role for FABP3 in mammary gland lipid metabolism, but the function has not yet been elucidated.

Milk Fat Synthesis

Both preformed FA from plasma and FA synthesized via *de novo* lipogenesis in the mammary epithelial cell contribute to milk fat. Preformed FA in plasma arise from FFA mobilized from adipose tissue or from dietary FA transported in the TG portion of VLDL. Acetate serves as the primary substrate for *de novo* lipogenesis and is converted to malonyl-CoA by acetyl-CoA carboxylase and is chain-elongated by fatty acid synthase catalyzing the addition of volatile FA (Chilliard, 2000). In adipose tissues, FA are elongated to 16 carbons, forming palmitate, but the mammary gland is extremely efficient such that short-chain FA are rapidly released for TG esterification prior to complete elongation by chain-terminating transacylation by fatty acid synthase (Knudsen and Grunnet, 1982). Additionally, LCFA are shuttled to stearoyl-CoA desaturase, which inserts a double bond at the ninth position from the carboxyl end.

Performed, *de novo*, and desaturated FA are all delivered to the endoplasmic reticulum where they are esterified into TG via the glycerol-3-phosphate pathway. As reviewed by Coleman and Mashek (2011), glycerol-3-phosphate is converted to lysophosphatidic acid by glycerol-3-phosphate acyltransferase, adding a fatty acyl-CoA at the *sn*-1 position. Lysophosphatidic acid is converted to phosphatidic acid via acylation of the *sn*-2 position by acylglycerophosphate acyltransferase (**AGPAT**). Multiple isoforms of AGPAT exist, and it is likely each isoform has specificity for incorporation of specific FA in the *sn*-2 position. With the addition of another fatty acyl-CoA, phosphatidic acid is converted to diacylglycerol by

phosphatidic acid phosphohydrolase (or LIPN). Phosphatidic acid is a key intermediate in lipid synthesis and LIPN1 can also function as a co-activator of transcription. The final fatty acyl-CoA, usually a short-chain FA (Breckenridge and Kuksis, 1969), is added by diacylglycerol acyltransferase. A single nucleotide polymorphism in diacylglycerol acyltransferase 1 in the mammary gland allows for genetic selection of cattle with enhanced milk fat yield (Grisart et al., 2004). Triglycerides accumulate within lipid droplets in the cell, which may contain portions of the rough ER, and gradually migrate to the apical plasma membrane of the mammary epithelial cell where they bud off with the plasma membrane forming the milk fat globule membrane (Bauman et al., 2006). The milk fat globule contains numerous proteins associated with triglyceride synthesis, lipid droplet formation, and secretion.

Summary

Supplementation of n-3 FA in lactating dairy cattle requires understanding of the mechanisms of rumen biohydrogenation, digestion and absorption by the small intestine, and uptake by the mammary gland of the lactating dairy cow. These challenges have created an active area of research in the last half-century, increasingly motivated by consumer demand for healthier food choices. The objective of this research was to improve understanding of systemic and intracellular fatty acid transport of LCFA, emphasizing long-chain PUFA.

FIGURES

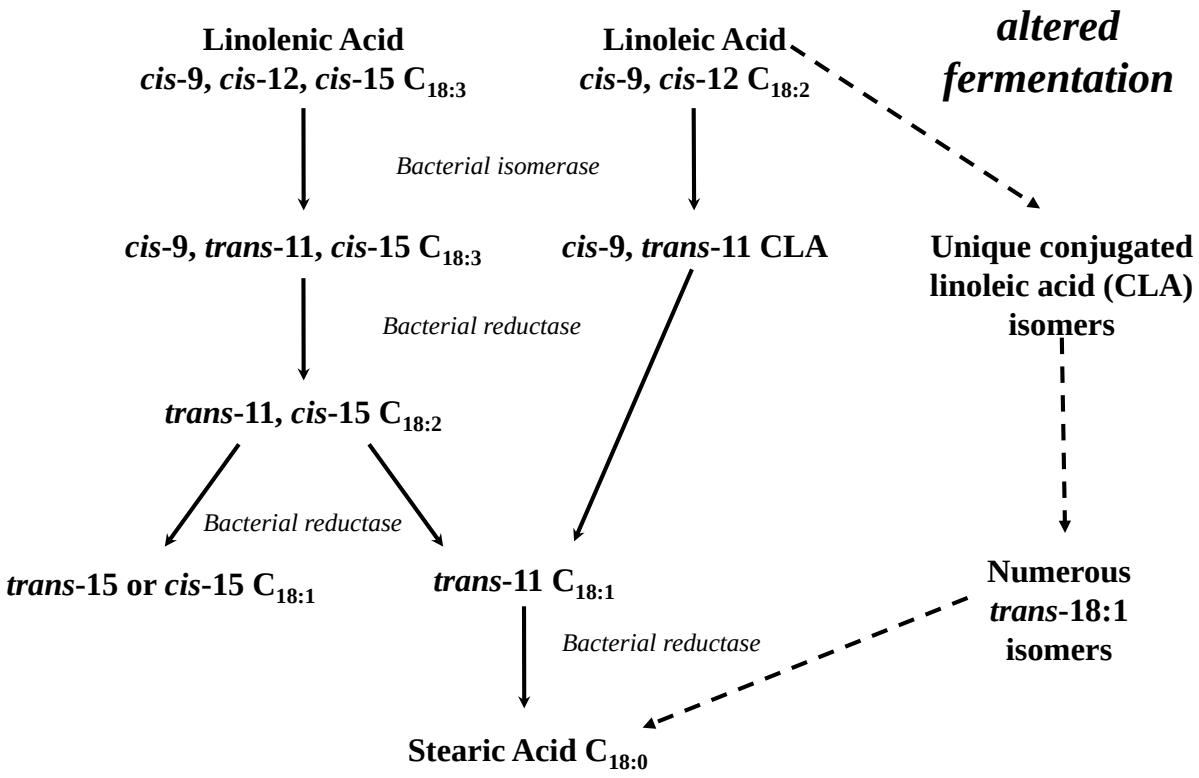

Figure 2.1. Steps in the biohydrogenation of linoleic and linolenic acid by rumen microorganisms. Various proportions of each intermediate are produced depending on rumen conditions. Adapted from Lock and Bauman (2004), Jenkins and McGuire (2006), and Jenkins et al. (2008).

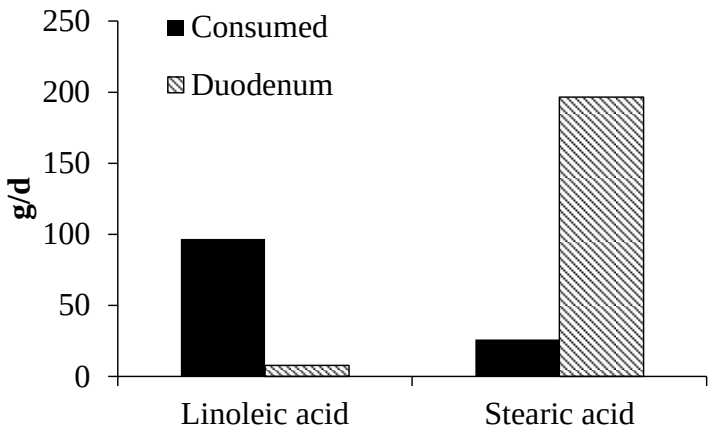

Figure 2.2. Polyunsaturated fatty acid losses due to rumen biohydrogenation as demonstrated by Loor et al. (2004) via linoleic and stearic acid consumption and duodenal flow.

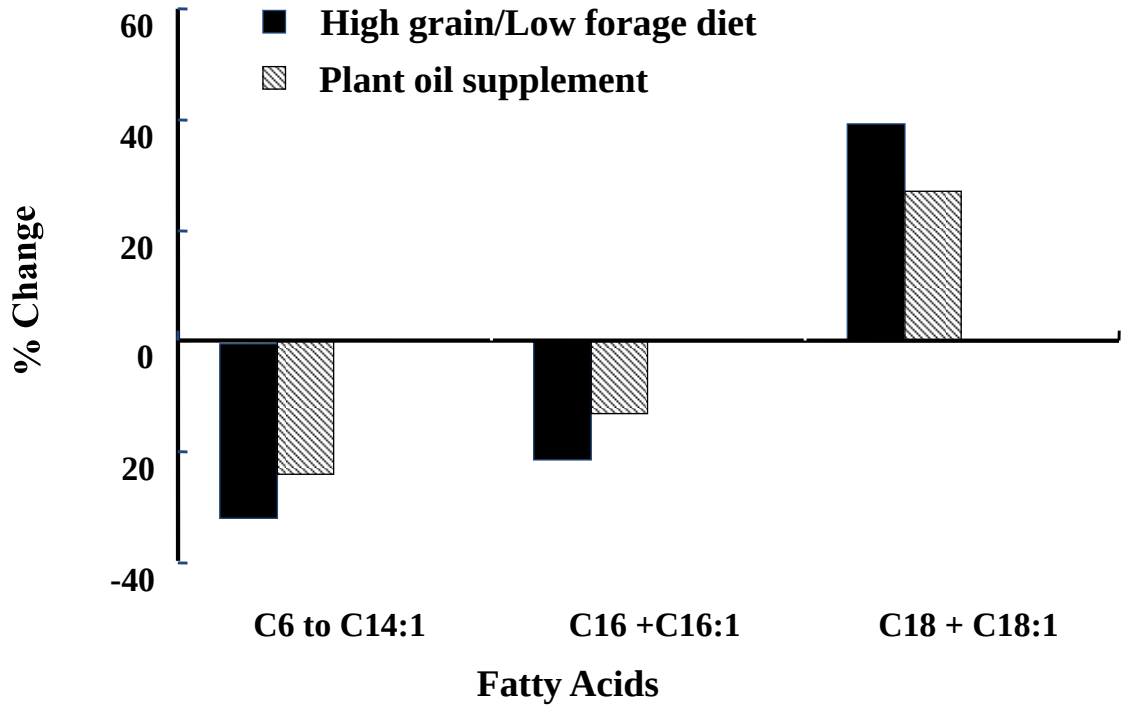

Figure 2.3. Changes in the concentrations of milk fatty acids associated with diet-induced MFD. Both high grain/low forage and plant oil supplementing diets result in reduced milk fat. Adapted from Bauman and Griinari (2001).

CHAPTER 3: Use of Omega-3 Fatty Acid Rich Algae and Their Oil as a Feed Supplement for Dairy Cattle

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INTRODUCTION

Omega-3 fatty acid supplements have been purported to improve reproductive efficiency of dairy cattle by inhibiting prostaglandin-$F_{2\alpha}$ synthesis through competition with arachadonic acid as a precursor for prostaglandin synthesis (Staples et al., 1998, Mattos et al., 2000, Mattos et al., 2004). In some studies feeding n-3 FA during lactation has been found to reduce prostaglandin secretion and improve fertility and embryo survival (Santos et al., 2008). Additionally, health benefits associated with consuming n-3 FA encourage development of functional foods for humans. Therefore, perceptions of milk fat composition and quality may be improved by dietary supplements that increase milk fat content of n-3 FA (Lock and Bauman, 2004).

Fish oil is the most common ration additive used to provide very long-chain n-3 FA to dairy cows. Marine fish do not synthesize n-3 FA; they consume microscopic algae or other algae-consuming fish to obtain n-3 FA. New technology allows for heterotrophic production of algal biomass in bulk fermenters, that do not require the lighting and electricity previously needed for phototrophic algae growth (Harel et al., 2002). Algal biomass produced in these facilities provides a consistent, high quality source of DHA (C22:6 n-3) in TG oils that could be used as a ration supplement for dairy cattle.

Polyunsaturates undergo extensive hydrolysis and biohydrogenation in the rumen, if included directly, which may adversely impact intake and milk fat yield (Palmquist et al., 2005). Increased DHA content of fish oil causes greater rumen accumulation of vaccenic acid due to partial hydrogenation of LA (AbuGhazaleh and Jenkins, 2004, Klein and Jenkins, 2011). Importantly in their review of the effects of LCFA on reproduction, Santos et al. (2008) described how there is a need to develop methods to improve the delivery of specific FA for absorption. Lipid encapsulation is a method that allows PUFA to remain protected in the rumen, avoid biohydrogenation, and be available for absorption and utilization (Perfield et al., 2004). Our objective was to examine use of prototype lipid-encapsulated RP algal biomass or algal oil to enrich milk fat with n-3 FA in lactating dairy cattle.

MATERIALS AND METHODS

Animals and Experimental Design

The Virginia Tech Institutional Animal Care and Use Committee approved all procedures involving animals. Four lactating Holstein cows (193 ± 20 DIM ± SD) were randomly assigned

to treatments in a 4 × 4 Latin square design. Cows were housed in tie stalls and fed individually at the Virginia Tech Dairy Center during each treatment period. Cows were milked in their stalls at 0600 and 1800 h and were allowed exercise for 2 h each day. Cows were acclimated to tie stalls for 24 h prior to supplementation and treatments were supplemented for 7 d. Between each period, cows were returned to freestall housing with the main herd for a washout period of 9 to 14 d. Cows were fed a total mixed ration (**TMR**) formulated according to NRC (2001) recommendations to meet or exceed nutrient requirements (Table 3.1). Fresh feed was mixed daily and cows were fed over half the ration at 0930 h and the remaining portion at 1900 h.

Algal biomass (Gold Value Fat™) and algal oil (Martek DHA™-S) were obtained from Martek Biosciences Corporation (Columbia, MD). Supplements were lipid encapsulated by Balchem Corporation (New Hampton, NY) to provide protection against fatty acid biohydrogenation in the rumen. At each feeding, rations were top dressed with 1X or 0.5X RP algal biomass supplement (**RP-Bio**), 1X RP algal oil supplement (**RP-Oil**), or no supplement. Top dressed supplements were fully consumed when the fresh feed was offered. The 1X supplements provided 29 g/d DHA and 0.5X provided half of this amount (Table 3.2). The total amount of supplemented fat was 112 g/d for 0.5X RP-Bio, 224 g/d for 1X RP-Bio, and 145 g/d for 1X RP-Oil. The total amount of supplement fed was 150, 300, and 194 g/d for 0.5X RP-Bio, 1X RP-Bio, and 1X RP-Oil.

Data Collection, Sampling Procedures, and Analysis

Samples of TMR were collected on alternate days during each period and stored at -20°C until chemical analysis (Table 3.1). Samples were composited by period on an equal weight basis

and submitted to Cumberland Valley Analytical Service Inc. (Hagerstown, MD) for analysis. Intake was recorded daily.

Milk yield was recorded at each milking and samples were collected for 14 consecutive milkings (starting after 12 h of supplementation) on d 1 through d 7 of the treatment period. Milk samples were submitted for analysis of milk fat and true protein content (DHIA, Blacksburg, VA; Foss 4000 Combi North America, Eden Prairie, MN). A second sample was frozen at -20°C until analysis of fatty acid methyl esters (**FAME**).

Blood was sampled from a coccygeal vessel 1 h prior to the morning feeding on d 7. Blood was drawn into evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin and immediately placed on ice. Plasma was collected by centrifugation (1,300 × *g* for 10 min) and stored at -20°C in polypropylene tubes until analysis.

Milk lipids were extracted and methylated according to the method of Kelsey et al. (2003). Lipids were extracted from 3 mL of plasma using the method of Corl et al. (2002) adapted for bovine plasma as described by Perfield et al. (2006). Internal standards (Nu-Check Prep, Inc.) unique to each lipid fraction were added to the plasma lipid extract for method quality control and to determine recoveries. Plasma was fractioned using aminopropyl columns (Bond Elut LRC-NH_2, Varian Inc.), and FAME were prepared from plasma lipid fractions using 1% methanolic sulfuric acid as described by Perfield et al. (2006).

Analysis of FAME was done by gas chromatography (Agilent 6890N GC) using a CP-Sil 88 capillary column (100 m x 0.25 mm i.d. with 0.2 μm thickness; Varian, Inc., Palo Alto, CA). The oven temperature, initially 80°C, was ramped at 2°C/min to 190°C and maintained for 13 min, followed by an increase of 2°C/min to 210°C and held for 14 min. Inlet and detector temperatures were 250°C, and the split ratio was 100:1. The hydrogen carrier gas flow rate was 1

mL/min. Hydrogen flow to the detector was 25 mL/min, airflow was 400 mL/min, and the flow of nitrogen make up gas was 40 mL/min. FA were identified using pure standards (Nu-Check Prep, Inc., Elysian, MN). A butter reference standard (BCR 164; Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was analyzed at regular intervals to determine recoveries and correction factors for individual fatty acyl composition in milk fat. Milk fatty acyl yields were calculated according to Stamey et al. (2010).

Statistical Analysis

Data were analyzed as a 4 × 4 Latin square using the MIXED procedure of SAS (SAS, Inc., Cary, NC). Fixed effects included treatment and period, and the random effect was cow. Plasma data also included the fixed effect of lipid fraction. The REPEATED statement was used for response variables with repeated measurements to account for effect of day and also included fixed effects of day, period by day, and treatment by day. The error term for the REPEATED statement was cow. The covariance structure that yielded the lowest Akaike's information criterion was used (Littell et al., 1998). Orthogonal contrasts were used to separate treatment means: 1) control minus RP supplements, 2) both RP-Bio supplements minus RP-Oil, and 3) 0.5X RP-Bio minus 1X RP-Bio. Significance was declared at $P < 0.05$.

RESULTS

As shown in Table 3.3, DMI and milk yield were not affected by lipid supplementation ($P > 0.05$). Milk fat content and yield and milk true protein content and yield were also not different among treatments.

Feeding RP-Bio or RP-Oil did not alter *de novo* fatty acid synthesis as indicated by short- and medium-chain fatty acyl yields (Table 3.4). Fatty acyl yields of *trans*-C18:1 isomers were affected by lipid supplements. Both *trans*-4 and *trans*-5 were increased ($P < 0.01$) with supplementation compared to control. RP-Bio supplements resulted in greater yields of *trans*-4 and *trans*-5 than RP-Oil. For *trans*-5 yield, 1X RP-Bio (0.7 g/d) was greater ($P < 0.05$) than 0.5X RP-Bio (0.5 g/d). Yields of *trans*-6-8 and *trans*-9 were also greater for cows receiving a lipid supplement compared to control ($P < 0.05$). While yield of *trans*-11-C18:1 was greater ($P < 0.05$) for cows fed lipid supplements and greater for cows receiving 1X RP Bio (27 g/d) than 0.5X RP Bio (20 g/d), it is notable that milk yield of *trans*-10-C18:1 was not changed by lipid supplementation. *Cis*-9, *trans*-11 CLA in milk was also increased for cows receiving a lipid supplement compared to control ($P < 0.05$).

Milk yield of DHA increased ($P < 0.001$) for cows receiving supplements compared to control (Table 3.4). Fatty acyl yield of DHA in milk was also greater for cows receiving RP Bio compared to RP Oil ($P < 0.001$). The temporal pattern of DHA yield in milk (Figure 3.1) shows incorporation of DHA increased daily ($P < 0.05$), but there was no treatment × time interaction ($P = 0.45$). Transfer efficiencies for DHA were 3.4, 2.0, and 1.0±0.22% for 0.5X RP-Bio, 1X RP-Bio, and 1X RP-Oil. Protection of algal biomass rather than extracted algal oil allowed greater transfer of DHA into milk ($P < 0.0001$) and 0.5X RP-Bio allowed greater transfer than 1X RP-Bio ($P = 0.0007$).

Concentration of DHA in d 7 plasma lipid fractions shows that DHA is greatest in plasma PL ($P < 0.0001$; Figure 3.2). Carryover of DHA was noted in plasma PL of control cows despite the washout period, thus the interaction of treatment and plasma lipid fraction was not significant ($P = 0.2$). Docosahexanoic acid was not detected in plasma CE (Table 3.5). Changes in FA

within plasma CE (Table 3.5), FFA (Table 3.6), and PL (Table 3.7) concentration were not observed with lipid supplementation. In plasma TG (Table 3.8), *trans*-6-8-C18:1 was increased with lipid supplementation compared to control ($P < 0.02$).

DISCUSSION

Supplements of RP algal biomass and RP algal oil did not detrimentally influence DMI, milk yield, or milk fat yield though decreases in intake have been observed when unprotected marine algae supplements are fed (Franklin et al., 1999, Offer et al., 2001a, Boeckaert et al., 2008). Unsaturated TG in unprotected supplements are predominantly hydrolyzed in the rumen, and the FFA are biohydrogenated to saturated FFA. The difference in lipids may be influential for responses in DMI. Other studies indicate that reductions in DMI may be more pronounced with unsaturated FFA rather than unsaturated TG reaching the duodenum (Litherland et al., 2005, Castaneda-Gutierrez et al., 2007). While Franklin et al. (1999) offered a xylose-protected marine algae supplement, their results suggest this form of protection was not adequate to prevent rumen outflow of unsaturated FFA and this may have caused the decreased DMI observed in that study. Lipid encapsulation of PUFA provides rumen protection and might allow rumen outflow of unsaturated TG rather than the unsaturated FFA from the unprotected supplements used in previous studies and this possibly explains the lack of effect on DMI with these supplements (Perfield et al., 2004).

Feeding unprotected PUFA can alter rumen biohydrogenation pathways, resulting in production of *trans*-10, *cis*-12-C18:2, a fatty acid known to reduce milk fat synthesis in the mammary gland (Bauman and Griinari, 2003) and reductions in milk fat yield have been

observed when diets were supplemented with unprotected marine algae (Franklin et al., 1999, Offer et al., 2001a, Boeckaert et al., 2008). Also associated with reductions in milk fat yield in these studies were decreases in *de novo* synthesized FA and increased production of *trans*-C18:1 isomers and CLA caused by alterations in rumen biohydrogenation pathways. In the current experiment, lipid encapsulation of both RP-Bio and RP-Oil supplements did not cause reductions in milk fat content and yield while providing a similar amount of dietary DHA. Furthermore, protection of algal biomass rather than extracted algal oil allowed greater transfer of DHA into milk and 0.5X RP-Bio allowed greater transfer than 1X RP-Bio.

The specific FA present in the unprotected PUFA supplements are the specific factors that influence rumen biohydrogenation of dietary FA. Klein and Jenkins (2011) demonstrated DHA itself is not converted to *trans*-11-C18:1, but modifies biohydrogenation of other long-chain PUFA present in the rumen, such as LA and LNA. Docosahexaenoic acid promotes accumulation of vaccenic acid during LA biohydrogenation, but the presence of other FA in unprotected PUFA supplements interact with dietary LA to increase *trans*-10, *cis*-12-CLA production (Whitlock et al., 2002, AbuGhazaleh and Jenkins, 2004, Whitlock et al., 2006). In the current study, milk fat yield of *trans*-C18:1 isomers was increased with supplementation of RP-Bio and RP-Oil. Of the *trans*-C18:1 isomers, production of *trans*-11-C18:1 and, subsequently, *cis*-9, *trans*-11-CLA was increased rather than *trans*-10-C18:1, which is correlated with MFD (Lock et al., 2007). Feeding strategies incorporating supplemental lipids must provide protection to prevent interactions among other long-chain PUFA present in the diet to prevent MFD. In the present study, the increase in milk *trans*-C18:1 FA indicates microalgae supplements influenced rumen biohydrogenation intermediates. The lipid coating also contained *trans*-C18:1 FA (Table 3.2) and might have minimally contributed to changes in *trans*-C18:1 FA. MFD was not

observed because LA biohydrogenation did not shift toward *trans*-10, *cis*-12-CLA production, as evidenced by no change in the isomer's content in milk. The increased variation in yield of *trans*-10-C18:1 in milk suggests a longer feeding period or altered ration formulation might initiate altered rumen biohydrogenation and increased *trans*-10, *cis*-12-CLA outflow.

Other studies using the same source of marine algae observed similar transfer efficiencies with unprotected supplements (Offer et al., 2001a, Boeckaert et al., 2008, Abughazaleh et al., 2009). Toral et al. (2010) also observed DHA transfer efficiencies ranging from 1.6 to 2.5% in sheep supplemented with sunflower oil and incremental levels of marine algae. Using the same algal species (*Schizochytrium*), Franklin et al. (1999) reported DHA transfer efficiencies as high as 8.4 and 16.7% in unprotected and protected algal supplements. Greater availability of preformed FA in early lactation modify milk fat composition (Palmquist et al., 1993), and indeed, lower transfer efficiencies (<5%) were consistently observed for studies starting algal supplementation at mid- to late lactation. Boeckaert et al. (2008) reported experiments in both early and late lactation with transfer efficiencies of 5.9 and 3.1%, respectively. Transfer efficiency in the present experiment may have been improved by supplementing earlier in lactation. It is also possible that lipid encapsulation decreased digestibility of the TG in the RP Bio and RP Oil supplements.

Mammary uptake of FA from plasma is dependent upon the action of mammary LPL on TG transported in chylomicrons and VLDL (Palmquist, 1976, Moore and Christie, 1979). Accordingly, FA incorporated into PL or CE at the small intestine or liver are not available for mammary uptake; however, increased fat feeding can overwhelm normal esterification capacity of the small intestine, allowing increased incorporation of DHA into the TG fraction (Bauchart, 1993). In the present experiment, DHA was preferentially incorporated into plasma PL as it is

normally found in this lipid fraction, preventing mammary uptake and reducing transfer. Offer et al. (2001b) supports this observation, noting that supplemental long-chain PUFA, such as DHA, are not present in plasma TG.

CONCLUSIONS

Feeding lipid encapsulated algae supplements may improve milk fat composition. Supplements did not significantly impact milk production or milk fat yield. Increased milk fat yield of *trans*-C18:1 isomers indicated supplements were altered by rumen biohydrogenation. Preferential esterification of DHA into plasma PL also limited its incorporation into milk fat. It is possible long-chain PUFA like DHA, must be delivered as plasma TG for maximal transfer to milk fat. Long term feeding of lipid encapsulated algae supplements may allow for increased DHA in plasma TG by saturating plasma PL.

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TABLES

Table 3.1. Composition of the TMR.

Item	Amount
Ingredient, % DM[1]	
Corn silage	22.6
Concentrate[2]	18.1
Ground corn	3.5
Alfalfa silage	2.9
Alfalfa hay	2.3
Barley straw	0.5
Chemical composition	
Crude protein, % DM	14.6
Neutral detergent fiber, % DM	34.4
Acid detergent fiber, % DM	20.7
Crude fat, % DM	4.4
Ca, % DM	0.69
P, % DM	0.37
NE_L, Mcal/kg of DM	1.71

[1] Diet DM averaged 46.9%.

[2] Concentrate contained 34.2% soybean meal (48%), 26.4% dehydrated citrus pulp, 6.8% distillers grains, 6.5% wheat midds, 6.6% Pro-Lak (H.J. Baker & Bro., Inc., Westport, CT), 2.9% animal fat, 2.1% Megalac Plus (Church & Dwight Co., Inc., Princeton, NJ), 3.4% dehydrated molasses, 1.7% urea (45%), 3.9% limestone, 2.15% sodium bicarbonate, 0.71% magnesium

oxide, 1.06% Dyna-Mate (Mosaic USA, LLC, Plymouth, MN), 1.08% white salt, 0.20% Availa-4 (Zinpro Corp., Eden Prairie, MN), 0.13% selenium (0.06%), 0.07% Vitamin E-6000, 0.02% Rumensin 90 (Eli Lilly and Company, Indianapolis, IN), 0.17% Vitamin ADE

Table 3.2. Fatty acid composition of lipid supplements.

	Treatments			
Fatty acid, %	RP-Biomass[1]	RP-Oil	Biomass	Oil
14:0	5.1	4.2	9.4	8.4
15:0	0.2	0.2	0.3	0.3
16:0	22.5	22.5	22.7	23.5
16:1	0.0	0.1	0.4	0.1
17:0	0.1	0.1	0.0	0.1
18:0	27.0	29.9	0.6	0.5
18:1, *trans 6-8*	1.0	1.0	0.0	0.0
18:1, *trans 9*	0.9	1.0	0.0	0.0
18:1, *trans 10*	1.0	1.0	0.0	0.0
18:1, *trans 11*	0.8	0.9	0.0	0.0
18:1, *trans 12*	0.9	0.9	0.0	0.0
18:1, *cis 9*	3.5	1.5	4.2	0.2
18:1, *cis 11*	0.4	0.4	0.2	0.1
18:2, *cis 9, cis 12*	0.4	0.2	0.5	0.0
20:0	0.3	0.3	0.1	0.1
18:3, *cis 9, cis 12, cis 15*	0.1	0.0	0.1	0.0
20:4	0.2	0.2	1.2	1.1
20:5	0.6	0.6	1.0	1.4
22:5	7.2	7.4	13.2	15.4

22:6	19.3	18.9	35.3	39.5
Others	8.4	8.6	10.8	9.2

[1] RP = rumen-protected.

Table 3.3. Performance of lactating Holstein cows receiving no supplement (Con), rumen-protected (RP) algal biomass (0.5X RP-Bio or 1X RP-Bio) or RP algal oil (1X RP-Oil) for 7 d. Data presented are least squares means and standard errors.

	Treatments					
Variable	Con	0.5X RP-Bio	1X RP-Bio	1X RP-Oil	SEM[1]	*P*
Dry matter intake, kg/d	20.1	18.4	20.8	19.4	0.65	0.12
Milk yield, kg/d	29.1	27.2	30.9	29.8	0.85	0.07
Milk fat						
kg/d	1.02	1.07	1.01	0.98	0.067	0.83
%	3.5	4.0	3.3	3.3	0.20	0.11
Milk true protein						
kg/d	0.88	0.84	0.95	0.92	0.030	0.11
%	3.0	3.1	3.1	3.1	0.05	0.60

[1] SEM = standard error of the mean.

Table 3.4. Milk fatty acyl yields of lactating Holstein cows receiving no supplement (Con), rumen-protected (RP) algal biomass (0.5X RP-Bio or 1X RP-Bio) or RP algal oil (1X RP-Oil) for 7 d. Data presented are least squares means and standard errors.

	Treatments						Contrasts[1]		
Fatty acyl, g/d	Control	0.5X RP-Bio	1X RP-Bio	1X RP-Oil	SEM[2]	*P*	Con-RP	Bio-Oil	0.5X-1X
4:0 – 12:0	116.0	111.3	102.3	91.4	11.58	0.49			
14:0	90.1	85.5	84.5	75.9	7.02	0.57			
14:1	6.5	6.4	6.7	6.8	1.07	0.99			
15:0	7.2	6.0	7.1	5.9	0.93	0.68			
16:0	262.1	253.9	240.9	228.3	17.21	0.55			
16:1	11.6	12.6	12.0	12.8	1.71	0.95			
17:0	8.0	8.0	7.8	7.3	0.48	0.71			
18:0	102.6	98.0	76.6	86.2	10.98	0.38			
18:1, *trans 4*	0.3	0.5	0.6	0.4	0.05	0.01	**	*	†
18:1, *trans 5*	0.3	0.5	0.7	0.4	0.06	<0.01	**	*	*
18:1, *trans 6-8*	4.6	7.0	10.9	7.2	1.29	0.05	*		†
18:1, *trans 9*	3.1	4.7	7.1	4.8	0.76	0.03	*		†

18:1, *trans 10*	10.2	14.4	33.3	19.6	7.29	0.20			
18:1, *trans 11*	13.0	20.0	27.0	14.6	2.99	0.02	*	*	
18:1, *trans 12*	5.6	8.1	11.5	7.0	0.96	0.01	*	*	*
18:1, *cis 9*	206.9	199.6	169.1	191.1	13.59	0.29			
18:1, *cis 11*	2.8	3.8	4.6	3.1	0.40	0.05			
19:0	3.8	4.8	5.4	4.1	0.39	0.07			
18:2, *cis 9, cis 12*	29.9	30.4	26.6	28.0	2.61	0.73			
20:0	1.2	1.2	1.1	1.1	0.12	0.72			
18:3, *cis 9, cis 12, cis 15*	2.4	2.4	2.1	2.3	0.19	0.72			
18:2, *cis 9, trans 11*	5.4	7.5	10.2	6.4	0.98	0.03	*	†	†
18:2, *trans 10, cis 12*	0.1	0.1	0.4	0.2	0.12	0.36			
20:2	0.3	0.3	0.3	0.2	0.04	0.71			
20:4	1.3	1.4	1.0	1.1	0.16	0.29			
20:5	0.1	0.2	0.3	0.4	0.13	0.37			
22:6	0.1	0.5	0.6	0.3	0.05	<0.001	***	**	
Others	20.6	21.6	23.5	20.0	1.01	0.07			

Glycerol	151.5	150.1	143.3	134.1	9.59	0.58
Total	1067.3	1060.8	1017.4	960.9	63.09	0.63

[1] Orthogonal Contrasts: Control minus RP supplements, 0.5X and 1X RP-Biomass minus RP-Oil, and 0.5X RP-Biomass minus 1X RP-Biomass. †$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

[2] SEM = standard error of the mean

Table 3.5. Cholesterol ester fatty acid concentration in plasma samples from lactating Holstein cows after receiving no supplement (Con), rumen-protected (RP) algal biomass (0.5X RP-Bio or 1X RP-Bio) or RP algal oil (1X RP-Oil) for 7 d. Data presented are least squares means and standard errors for d 7.

	Treatments					
Fatty acid, pmol/mL	Control	0.5X RP-Bio	1X RP-Bio	1X RP-Oil	SEM[1]	*P*
14:0	81.2	80.7	89.3	93.2	10.75	0.81
14:1	71.6	66.9	77.5	80.1	6.92	0.56
16:0	359.2	357.1	365.3	389.3	32.18	0.89
16:1	86.5	97.8	82.6	99.7	19.53	0.90
17:0	4.4	4.3	8.7	12.8	3.80	0.39
18:0	60.5	48.2	46.2	44.2	16.51	0.90
18:1, *cis 9*	229.8	227.3	162.6	255.9	35.73	0.35
18:1, *cis 11*	13.1	15.2	83.6	19.7	36.03	0.48
18:2, *cis 9, cis 12*	6259.9	6274.1	6423.9	6909.0	520.85	0.80
18:3, *cis 6, cis 9, cis 12*	135.8	103.5	99.4	116.3	16.00	0.42
18:3, *cis 9, cis 12, cis 15*	304.0	298.8	316.9	329.5	22.90	0.78
20:2	11.1	<0.1	<0.1	<0.1	5.54	0.44
20:3	33.7	25.3	24.7	26.1	10.80	0.93
20:4	117.1	128.5	126.4	136.0	11.59	0.72
20:5	5.3	12.0	13.0	<0.1	4.20	0.17
22:6	nd[2]	nd	nd	nd		

Others	249.1	212.8	238.5	281.3	44.18	0.74
Total	8026.0	7956.5	8163.3	8793.0	615.22	0.77

[1] SEM = standard error of the mean

[2] nd = not detected

Table 3.6. Free fatty acid concentration in plasma samples from lactating Holstein cows after receiving no supplement (Con), rumen-protected (RP) algal biomass (0.5X RP-Bio or 1X RP-Bio) or RP algal oil (1X RP-Oil) for 7 d. Data presented are least squares means and standard errors for d 7.

	Treatments					
Fatty acid, pmol/mL	Control	0.5X RP-Bio	1X RP-Bio	1X RP-Oil	SEM[1]	*P*
14:0	15.3	13.6	15.4	10.1	1.81	0.24
14:1	3.2	2.6	3.4	2.1	0.51	0.24
15:0	6.2	5.3	5.7	3.5	1.95	0.68
16:0	144.9	150.1	141.0	116.6	18.51	0.47
16:1	3.9	5.0	4.3	3.9	0.97	0.76
17:0	8.5	7.9	6.7	6.9	1.04	0.51
18:0	217.2	212.5	188.8	177.0	27.11	0.58
18:1, *trans 4*	0.4	0.4	1.8	0.7	0.39	0.10
18:1, *trans 5*	0.5	0.4	0.8	0.1	0.35	0.56
18:1, *trans 6-8*	2.6	4.3	5.2	3.4	0.92	0.21
18:1, *trans 9*	3.0	4.0	4.0	3.1	0.67	0.49
18:1, *trans 10*	5.4	12.5	24.6	10.0	5.66	0.15
18:1, *trans 11*	6.1	12.2	9.3	7.5	2.58	0.28
18:1, *trans 12*	3.1	5.0	5.3	3.6	0.93	0.26
18:1, *cis 9*	57.2	80.6	52.4	48.0	15.58	0.36
18:1, *cis 11*	1.1	2.1	2.2	2.1	0.47	0.28
18:2, *cis 9, cis 12*	73.9	61.1	64.4	44.3	35.08	0.91

20:0	1.1	0.4	0.6	0.5	0.73	0.82
18:3, *cis 9, cis 12, cis 15*	3.8	3.3	2.4	2.5	1.59	0.87
18:2, *cis 9, trans 11*	<0.1	0.3	1.1	<0.1	0.56	0.48
18:2, *trans 10, cis 12*	<0.1	<0.1	<0.1	0.2	0.10	0.47
20:2	1.6	2.3	3.9	2.6	0.99	0.42
20:3	2.4	1.6	3.0	2.5	0.72	0.50
20:4	3.4	2.7	4.3	3.1	0.88	0.57
22:6	0.6	1.1	3.0	1.8	0.69	0.13
Others	101.8	88.0	81.0	85.9	12.53	0.60
Total	667.2	678.9	634.2	541.7	97.97	0.64

[1] SEM = standard error of the mean.

Table 3.7. Phospholipid fatty acid concentration in plasma samples from lactating Holstein cows after receiving no supplement (Con), rumen-protected (RP) algal biomass (0.5X RP-Bio or 1X RP-Bio) or RP algal oil (1X RP-Oil) for 7 d. Data presented are least squares means and standard errors for d 7.

	Treatments					
Fatty acid, pmol/mL	Con	0.5X RP-Bio	1X RP-Bio	1X RP-Oil	SEM[1]	*P*
14:0	57.7	84.2	38.5	38.8	24.65	0.54
14:1	25.2	25.0	19.3	13.6	8.85	0.78
15:0	56.1	79.8	41.3	35.4	23.54	0.57
16:0	2865.9	3429.9	1793.6	1852.4	878.87	0.51
16:1	34.3	48.5	21.8	44.3	13.27	0.52
18:0	4321.8	4099.6	2148.7	2543.5	1135.04	0.47
18:1, *trans 6-8*	9.7	38.3	37.6	25.7	13.59	0.45
18:1, *trans 9*	9.5	37.9	38.0	25.5	13.80	0.45
18:1, *trans 10*	30.9	60.0	101.7	48.1	27.00	0.35
18:1, *trans 11*	89.6	265.6	151.7	110.9	89.57	0.54
18:1, *trans 12*	83.1	126.9	86.1	70.5	34.28	0.69

18:1, *cis 9*	1289.2	1472.4	789.9	813.6	375.44	0.51
18:1, *cis 11*	37.8	34.6	19.0	33.0	12.73	0.74
19:0	27.6	41.2	26.0	20.7	10.11	0.55
18:2, *cis 9, cis 12*	5749.9	5888.1	3160.6	3535.1	1609.63	0.53
20:0	9.5	1.9	<0.1	<0.1	3.60	0.26
18:3, *cis 6, cis 9, cis 12*	30.6	24.3	7.0	11.9	11.27	0.46
18:3, *cis 9, cis 12, cis 15*	164.3	179.5	88.3	89.4	54.59	0.53
18:2, *cis 9, trans 11*	<0.1	<0.1	3.7	<0.1	1.84	0.44
20:2	24.8	29.7	16.9	11.0	8.48	0.45
20:3	606.8	696.1	353.6	343.7	212.81	0.57
20:4	456.0	602.3	274.1	304.8	153.09	0.45
20:5	35.1	48.7	23.2	25.4	13.05	0.53
22:6	38.5	128.3	82.7	75.5	29.67	0.27
Others	2382.5	3028.2	1532.7	1471.6	861.18	0.55
Total	18436.5	20471.0	10855.9	11544.1	5469.56	0.53

[1] SEM = standard error of the mean.

Table 3.8. Triglyceride fatty acid concentration in plasma samples from lactating Holstein cows after receiving no supplement (Con), rumen-protected (RP) algal biomass (0.5X RP-Bio or 1X RP-Bio) or RP algal oil (1X RP-Oil) for 7 d. Data presented are least squares means and standard errors for d 7.

	Treatments					
Fatty acid, pmol/mL	Control	0.5X RP-Bio	1X RP-Bio	1X RP-Oil	SEM[1]	*P*
14:0	8.6	17.8	15.0	11.7	3.88	0.35
14:1	4.8	8.1	6.4	6.2	2.26	0.73
15:0	1.0	1.4	0.9	0.4	0.33	0.17
16:0	125.3	210.2	176.8	141.7	53.94	0.62
16:1	3.1	4.6	7.6	3.7	3.16	0.67
17:0	4.6	7.5	5.5	4.2	1.67	0.41
18:0	197.9	284.7	177.4	168.3	95.65	0.73
18:1, *trans 4*	<0.01	1.3	1.2	0.6	0.54	0.29
18:1, *trans 5*	0.1	1.4	1.5	0.7	0.46	0.13
18:1, *trans 6-8*	1.8	10.6	16.5	10.5	3.22	0.05
18:1, *trans 9*	0.7	5.0	7.2	4.4	1.49	0.06

18:1, *trans 10*	<0.01	17.5	48.7	27.0	12.22	0.07
18:1, *trans 11*	7.7	39.7	24.5	18.2	12.55	0.31
18:1, *trans 12*	5.1	12.5	12.4	8.8	3.44	0.36
18:1, *cis 9*	52.7	82.2	88.2	52.7	25.16	0.55
18:1, *cis 11*	6.0	15.7	13.1	9.0	4.61	0.41
18:2, *cis 9, cis 12*	34.8	79.4	78.4	57.8	20.24	0.35
20:0	3.3	4.8	3.9	3.4	1.38	0.81
18:3, *cis 9, cis 12, cis 15*	2.5	6.3	5.8	5.1	1.50	0.30
18:2, *cis 9, trans 11*	<0.1	0.3	<0.1	<0.1	0.21	0.47
20:3	4.8	3.1	6.2	1.2	3.11	0.58
20:4	0.0	2.4	2.1	0.9	0.93	0.26
20:5	<0.01	1.4	<0.1	<0.1	0.87	0.47
22:6	1.9	4.3	4.0	4.7	1.15	0.34
Others	74.0	151.5	138.8	113.6	34.41	0.39
Total	542.9	979.2	846.5	658.2	229.92	0.49

[1] SEM = standard error of the mean.

FIGURES

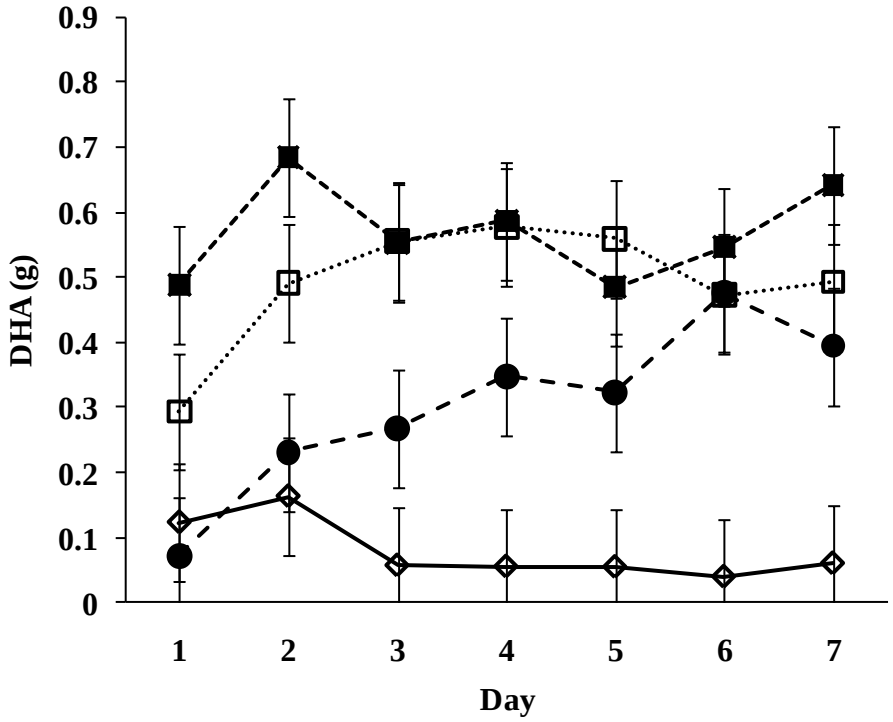

Figure 3.1. Temporal pattern of docosahexaenoate (DHA) yield in milk fat from lactating Holstein cows receiving no supplement (◊), 0.5X rumen-protected (RP) algal biomass (□), 1X RP algal biomass (■) or 1X RP-algal oil (●) for 7 d. Data presented are least squares means and standard errors. A significant main effect of time was detected ($P < 0.05$).

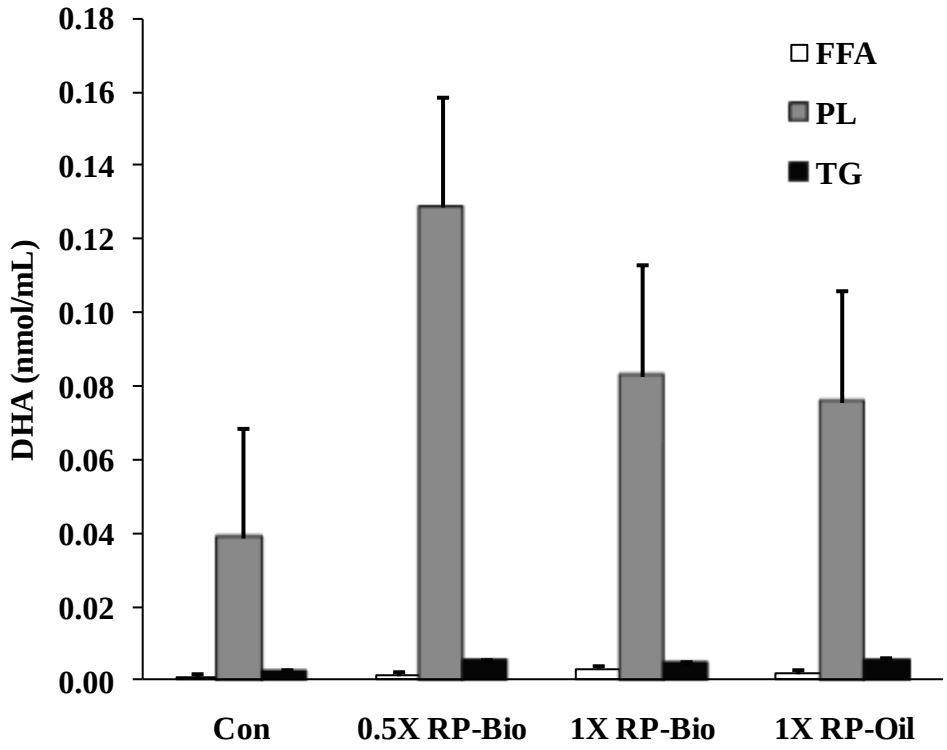

Figure 3.2. Docosahexaenoate (DHA) concentration of plasma from lactating Holstein cows after receiving no supplement (Con), rumen-protected (RP) algal biomass (0.5X RP-Bio or 1X RP-Bio) or RP algal oil (1X RP-Oil) for 7 d by lipid fraction: FFA, phospholipid (PL), and triglyceride (TG). DHA was not detected in CE. Data presented are least squares means and standard errors. A significant main effect of lipid fraction was detected ($P < 0.0001$).

CHAPTER 4: Mammary Uptake of Fatty Acids Varying in Chain Length and Unsaturation Supplied by Intravenous Triglyceride Infusion

INTRODUCTION

Supplementing dairy cows with PUFA-rich feeds does not easily increase quantities of PUFA in milk fat of dairy cows. Transfer of PUFA to milk fat is thought to be limited by biohydrogenation of unprotected supplements, partitioning of PUFA into plasma lipid fractions less available to the mammary gland, and low specificity of mammary LPL for these FA (Offer et al., 2001, Lock and Bauman, 2004). Drackley et al. (1992) increased milk fat yields of FA of increasing chain length and unsaturation with abomasal infusion of supplemental FA, but they were unable to separate effects of chain length and unsaturation. Additionally, increasing supply of PUFA to the small intestine by rumen protection or abomasal infusion does not overcome differences in digestibility and absorption in the small intestine (Noble, 1978, Bauchart, 1993).

Over 90% of plasma lipids in lactating dairy cattle are transported by HDL, predominantly comprised of PL and CE fractions (Moore and Christie, 1979). Very long-chain PUFA in the diet are preferentially incorporated into plasma CE and PL fractions rather than the TG fraction and are subsequently unavailable for mammary uptake by LPL (Brumby et al., 1972, Kitessa et al., 2001, Offer et al., 2001). Though feeding supplemental PUFA can overwhelm the normal esterification capacity of the small intestine and allow increased esterification of PUFA into TG (Bauchart, 1993), supply of PUFA to the small intestine is limited by rumen biohydrogenation. Previous results have demonstrated that supplemented very long-chain n-3 fatty acids are primarily transported in the PL or CE fraction of blood, making them largely

unavailable to the mammary gland for enrichment of milk fat (Brumby et al., 1972, Kitessa et al., 2001, Offer et al., 2001, Stamey et al., *In Press*). Mammary uptake of fatty acids from plasma is dependent upon the action of mammary LPL on TG transported in chylomicrons and VLDL (Palmquist, 1976, Moore and Christie, 1979). *In vitro* experiments by Brumby et al. (1972) demonstrated that HDL are not hydrolyzed by LPL and confirmed TG emulsions of soybean oil had a greater rate of hydrolysis than emulsions of native cod liver oil (Brumby et al., 1972). Accordingly, fatty acids incorporated into PL or CE at the small intestine or liver are not available for mammary uptake.

Ruminal biohydrogenation, digestion, absorption, and esterification all lead to variation in transfer efficiency for dietary FA to milk fat in previous studies. Mashek et al. (2005) successfully systemically infused individual FA as triacylglycerol emulsions avoiding modification of FA in the rumen, and potential differences in absorption and esterification in the small intestine. Using this method, our objective was to compare FA of increasing chain length and unsaturation delivered intravenously as TG emulsions to uncover specific regulation of FA uptake by the mammary gland.

MATERIALS AND METHODS

Animals and Experimental Design

The Virginia Tech Institutional Animal Care and Use Committee approved all procedures involving animals. Twenty-four mid-lactation cows (235 ± 37 d postpartum, mean ± SD) were randomly assigned to one of 4 treatments in a randomized complete block design. Treatments were intravenous infusion of TG emulsions with enrichment of: 1) oleic acid (**OA**), 2) LA, 3) LNA, and 4) DHA. Cows were blocked by breed (purebred Holstein, purebred Jersey, or

crossbred) and milk yield. Emulsions were administered continuously via intravenous jugular infusion pump at 16 mL/h for 72 h. Each cow received 384 mL of emulsion (30 g target FA) per day.

Emulsion Preparation

Emulsions were prepared according to the methods of Mashek et al. (2005) with modifications described. Olive (Kroger, Inc., Cincinnati, OH), grapeseed (DeLallo Co., Jeannette, PA), flax (Flora, Inc. Lynden, WA), and algal (Martek Biosciences Corporation, Columbia, MD) oils were selected for enrichment of target FA in TG: OA, LA, LNA, and DHA. Soy lecithin was purchased from Fisher Scientific (Fair Lawn, NJ), glycerol from Sigma Chemical (St. Louis, MO), and ethoxyquin from MP Biomedicals, LLC (Solon, OH). Quantities of oil (125, 131, 153 and 220 g, respectively) and other ingredients were adjusted to supply 30 g/d of target FA in equal solution volumes (Table 4.1). Coarse emulsions were prepared using a KitchenAid immersion blender (St. Joseph, MI), and the emulsions were passed twice through an APV 15MR homogenizer (SPX Corporation, Delavan, WI) at 500 psi in the first stage and 2000 psi in the second stage. The pH of cooled emulsions was adjusted to 8.3 with 1 *N* NaOH. Emulsions were autoclaved for 25 min at 21 psi and 121°C in volumes not exceeding 2 L. Cooled emulsions were aseptically transferred to 1 L bags and stored in the dark at 4°C. Emulsions were maintained at ambient temperature during infusions and did not separate or coagulate.

Data Collection, Sampling Procedures, and Analysis

Cows were housed in tie stalls at the Virginia Tech Dairy Center and consumed ad libitum a total mixed ration formulated according to NRC (2001) recommendations to meet or exceed their nutrient requirements. Fresh feed was mixed daily and cows were fed over half the ration at 0930 h and the remaining portion at 1900 h. Cows were milked in their stalls at 0600 and 1800 h. There was a 5 d acclimation period prior to infusions and cows were allowed to exercise 2 h/d until infusions began. Samples of the ration were collected during each period and submitted to Cumberland Valley Analytical Services Inc. (Hagerstown, MD) for wet chemistry analysis of nutrient composition (Table 4.2). Intake was recorded daily. Observations were recorded every 4 h during infusions, and included temperature, manure score, and behavior. Manure was scored from 1, diarrhea, to 4, firm and dry. Behavior observations included: standing, lying, ruminating, and eating.

Milk yield was recorded at each milking and samples were collected for 3 d pre-infusion and at each milking during infusions. One aliquot was stored with preservative (bronopol tablet; D&F Control System, San Ramon, CA) at 4°C until submitted for analysis of milk fat and true protein content (DHIA, Blacksburg, VA; Foss 4000 Combi North America, Eden Prairie, MN). A second aliquot was frozen at -20°C until analysis of FAME. Milk lipids were extracted and methylated according to the method of Kelsey et al. (2003).

Blood samples were collected from a coccygeal vessel into Vacutainer tubes containing sodium heparin (Becton Dickinson; Franklin Lakes, NJ) daily prior to the morning feeding and were immediately placed on ice. Plasma was collected by centrifugation (2,300 × *g* for 15 min at 4°C) and stored at -20°C in polypropylene tubes until analysis. Lipids were extracted from 3 mL of plasma using the method of Corl et al. (2002) adapted for bovine plasma as described by Perfield et al. (2006). Plasma lipid fractions were separated by thin-layer chromatography (1500

μm silica G plates; Analtech Inc., Newark, DE) according to the method of Tyburczy et al. (2008). The FAME were prepared from plasma lipid fractions using 1% methanolic sulfuric acid as described by Perfield et al. (2006).

Milk and plasma FAME were analyzed by gas chromatography (Agilent 6890N GC) using a CP-Sil 88 capillary column (100 m × 0.25 mm i.d. with 0.2 μm thickness; Varian, Inc., Palo Alto, CA). For milk FAME, the oven temperature was initially set at 80°C, and was ramped at 2°C/min to 190°C and maintained for 13 min, followed by an increase of 2°C/min to 210°C and held for 14 min. Inlet and flame-ionization detector temperatures were 250°C, and the split ratio was 100:1. For plasma, conditions were as follows: the oven temperature was initially set at 70°C, then increased at 8°C/min to 110°C, then increased at 5°C/min to 170°C and held for 10 min, then increased 4°C/min to 225°C and held for 15 min. The inlet and detector temperatures were 250°C, the split ratio was 100:1, and a 1 μL injection volume was used. The hydrogen carrier gas flow rate was 1 mL/min. Hydrogen flow to the detector was 25 mL/min, airflow was 400 mL/min, and the flow of nitrogen makeup gas was 40 mL/min. FA peaks were identified by using pure methyl ester standards (Nu-Check Prep Inc., Elysian, MN). Milk fatty acyl yields were calculated according to Stamey et al. (2010).

Statistical Analysis

Data were analyzed as a randomized complete block design using the MIXED procedure of SAS (SAS 9.2; SAS Institute, Inc., Cary, NC). Fixed effects included treatment and block, and the random effect was cow within treatment. The REPEATED statement was used for response variables with repeated measures to account for the effect of time and also included fixed effects of time, block by time, and treatment by time. Insignificant effects ($P > 0.05$) were not removed

from the model. The error term for the REPEATED statement was cow. The covariance structure, autoregressive 1, yielding the lowest Akaike's information criterion was used (Littell et al., 1998). Significance of treatment effects was declared at $P \leq 0.05$. Post-hoc analyses were carried out using the Tukey test to test pairwise comparisons. Data are reported as least squares means ± standard error of the means.

RESULTS

Cow Performance and Health

Intravenous infusion of TG emulsions in lactating dairy cattle caused both fever and hypocalcemia during blocks 1 and 2. Hypocalcemia was confirmed via plasma analysis at the recommendation of D. Palmquist (personal communication). Rations were subsequently top-dressed with 75 g limestone to supplement calcium intake. Both cows receiving the LA treatment during blocks 1 and 2 were removed from the study on d 2 of infusions. Phlebitis was not detected. As prophylaxis against fever, cows subsequently received Naxcel® (2.2 mg ceftiofur/kg BW; Pfizer Animal Health, New York, NY) intravenously during 3 d infusions and one day prior according to veterinary prescription. These adjustments prevented incidence of both fever and hypocalcemia during periods 3 through 6.

Milk yield and DMI were not affected by intravenous infusion of TG emulsions (Table 4.3, $P > 0.05$). Milk fat content and yield, and milk true protein content and yield were not different among treatments ($P > 0.05$). There was a significant effect of time ($P < 0.01$) for DMI and milk yield, but the interaction of treatment and time was not significant ($P > 0.05$). Milk true

protein declined initially during infusion, but returned to pre-infusion levels by d 3 (time, $P < 0.0001$; treatment × time, $P < 0.01$).

Milk fat composition

Yield of OA was not significantly increased ($P = 0.82$) in milk fat after 3 d continuous infusion (Table 4.4). Yield of linoleate, linolenate, and docosahexaenoate were all increased ($P < 0.0001$) by infusion of intravenous TG emulsions specifically enriched with LA, LNA, and DHA (Figure 4.1). Yield of eicosapentaenoate also increased ($P < 0.001$) with LNA and DHA infusion. Transfer efficiencies were 37.8, 27.6, and 10.9 ± 5.4% for LA, LNA, and DHA ($P < 0.01$, Figure 4.2). Transfer efficiency of DHA was less than both LA ($P = 0.01$) and LNA ($P = 0.05$). Transfer of LA was not different than LNA ($P = 0.34$).

Yield of *de novo* fatty acyls was 205, 211, 221, and 199 ± 33 g/d for OA, LA, LNA, and DHA and were unaffected by intravenous infusion of TG lipid emulsions ($P = 0.94$; Table 4.4). Palmitate and palmitoleate were similarly unaffected and yields were 261, 280, 293, and 270 ± 46 g/d ($P = 0.93$). Preformed fatty acyl yields were 379, 393, 407, and 343 ± 41 g/d ($P = 0.56$).

Plasma lipid composition

Concentration of FA within plasma lipid classes was determined. Blood volume and plasma content of each lipid class was not measured. No treatment differences were detected for OA or LA in any lipid classes ($P > 0.05$). Plasma CE concentration of target FA was not different ($P > 0.05$) among treatments (Table 4.5). Plasma FFA concentration of DHA was increased ($P = 0.04$) with DHA infusion (Table 4.6). Plasma TG concentrations of DHA also increased ($P < 0.001$) with DHA infusion (Table 4.7). Effect of time ($P = 0.02$) and the

treatment×time interaction ($P = 0.02$) were consistent with DHA increases in the plasma TG fraction. Concentration of eicosapentaenoic acid was altered ($P = 0.04$) in plasma TG by infusion.

In the plasma PL fraction (Table 4.8), n-3 PUFA were increased with infusion. Concentration of LNA was greater than other treatments ($P = 0.0001$) and increased with time ($P = 0.0002$) with LNA infusion. Concentration of eicosapentaenoic acid was greater ($P = 0.03$) for LNA and DHA infusions than OA and LA infusions and increased with time ($P = 0.0002$). Concentration of DHA in plasma PL was increased with DHA infusion ($P < 0.0001$) and with significant effects for time ($P < 0.0001$) and the treatment×time interaction ($P < 0.0001$).

DISCUSSION

Non-lactating, fasted dairy cattle were utilized in previous studies with 4 d intravenous infusion of TG emulsions (Mashek et al., 2005, Lacetera et al., 2007). This may have masked some negative effects of the emulsions observed with lactating cows in this study. In the present study, the pyrogenic response was followed by cessation of DMI and reduction in milk yield. Intervention using prophylactic antibiotic treatment successfully inhibited this negative response, yet DMI and milk yield were reduced with time. It seems unlikely that this response was induced by the limited quantity of FA infused, as the infused amounts were less than is typically consumed in the basal diet of lactating cows and less than 25% of prior studies (Mashek et al., 2005, Lacetera et al., 2007).

Dietary long-chain FA in milk are transported to the mammary gland via chylomicron and VLDL in plasma. In the enterocyte, LCFA are esterified to form CE, PL, and TG. Micelles

form containing a TG and CE core with a surface monolayer of PL and unesterified cholesterol. Apolipoproteins A and B (characteristically Apo B48; Bauchart, 1993), made in the intestine, are complexed with these lipids into VLDL and secreted into lymph, entering the bloodstream at the thoracic duct (Palmquist, 1976, Palmquist and Mattos, 1978, Emery, 1979). Other apolipoproteins are synthesized in the liver and transferred from HDL in circulation. Apo CI to CIV are co-factors for lipoprotein lipase and Apo E is required for liver uptake; allowing lipoproteins to control lipid utilization in energy metabolism (Bauchart, 1993). Chylomicrons are the largest and least dense (<0.95 g/mL) of the lipoproteins and are secreted by the enterocyte following a meal; however, VLDL (0.95-1.006 g/mL) secretion is predominant in ruminants due to the low fat content of their diets (Raphael et al., 1973, Bauchart, 1993).

In the present study, FA were delivered intravenously as emulsions of TG oils. Lipid emulsions were developed to mimic the chylomicron and are more often used to provide parenteral nutrition. Intravenously infused emulsion particles quickly acquire apolipoproteins, such as apolipoprotein CII, through transfer from HDL which allows recognition by LPL (Carpentier and Hacquebard, 2006). Mammary uptake of FA from plasma is dependent upon the action of mammary LPL on TG transported in chylomicrons and VLDL (Palmquist, 1976, Moore and Christie, 1979). Metabolism of lipid emulsions by LPL is similar to the chylomicron, but recent work suggests the uptake mechanism is based on FA composition. Milk transfer efficiencies for LA (38%) and LNA (28%) in this experiment were low compared to transfer efficiencies in the 40–70% range typical for supplemental LA and LNA in studies with mid- to late-lactation cows, though the lower DHA transfer (11%) was similar to studies supplementing marine oils (Chilliard et al., 2000). Milk fat is approximately one quarter OA, thus we were unable to detect transfer.

The TG emulsion particles may not have been hydrolyzed by LPL as efficiently as chylomicrons or VLDL, though a mechanism for this phenomenon has not been proposed (Hultin et al., 1995). The rate of clearance is fastest for fish oil emulsion particles, followed by medium-chain TG and last, long-chain TG emulsion particles (Qi et al., 2002). The LPL, apolipoprotein E, LDL receptor, and lactoferrin-sensitive pathways control removal of long-chain TG from emulsions containing primarily omega-6 FA (Qi et al., 2006, Murray-Taylor et al., 2010). Park et al. (2004) proposed increased margination of n-3-rich particles reflected activation of LPL. Margination is particle attachment to endothelium-bound LPL during lipolysis. Qi et al. (2006) then suggested LPL primarily functions as a "bridge" protein to mediate FA uptake through membrane-anchoring. Removal of n-3 particles was less affected by this mechanism and independent of apolipoprotein E, LDL receptor, and lactoferrin. In fact, Murray-Taylor et al. (2010) demonstrated that the uptake of n-3 emulsion particles depends upon cell surface proteoglycans and non-LDL receptor cell surface anchoring. In the present study, the transfer of DHA was significantly less than LA and LNA. It is possible LPL-rich mammary tissue has substantially less DHA uptake due to these mechanistic differences in membrane-anchoring that determine FA uptake.

Furthermore, it is possible that stereospecific positioning of DHA limited transfer. LPL has stereospecificity for the *sn*-1 and *sn*-3 FA, such that monoglycerides containing PUFA in the sn-2 position may be preferentially diverted to the liver or extra-hepatic tissues with the remnant droplet (Hultin et al., 1995, Couedelo et al., 2012). PUFA tend to be found in the *sn*-2 position of seed oils. Indeed, approximately 45% of the DHA found in algal oil is located at the *sn*-2 position of the TG molecule (Myher et al., 1996). This may have resulted in diversion of PUFA monoglycerides to the liver. Esterification in the small intestine of ruminants may alter

positioning of supplemental LA and LNA, which could explain the lower transfer efficiency of these FA with intravenous infusion of TG emulsions.

In the present study, both LNA and DHA were increased in plasma PL with intravenous infusion of TG emulsions enriched in these target FA. Though LA was not increased, it represents the greatest proportion of plasma PL and re-esterification into this fraction could not be detected using unlabeled FA. A primary benefit of fish oil emulsions in humans is the rapid incorporation of DHA into cellular PL (Carpentier and Hacquebard, 2006), thus, it is also possible that DHA taken up as a result of TG hydrolysis at the mammary gland was incorporated into membrane PL rather than milk fat. In the present study, LNA and DHA were likely re-esterified into PL at the liver. Under normal dietary conditions in ruminants, PUFA are preferentially esterified into PL at the small intestine, and the increased ratio of PL to TG as compared to non-ruminants results in lymph secretion of VLDL rather than larger, chylomicrons (Noble, 1978).

Turnover rates of plasma lipoproteins are dramatically reduced with increasing particle density (Noble, 1978, Palmquist and Mattos, 1978, Moore and Christie, 1979). Harvatine and Bauman (2011) demonstrated a slight lag in CLA enrichment of and clearance from plasma PL compared to TG with abomasal infusion, and plasma PL composition was most affected compared to other lipid classes by abomasal infusion of *trans*-octadecenoic acids (Tyburczy et al., 2008). Mashek et al. (2005) observed increased LNA and DHA in liver following intravenous infusion with minimal effects on peroxisomal oxidation, suggesting these FA may have been taken up as emulsion remnants rather than FFA. These data support the observations that n-3 PUFA are preferentially esterified into PL at both the small intestine and liver and predominantly circulate in plasma within HDL. Yet, the present study suggests fairly rapid re-

esterification of n-3 PUFA into PL in the liver. Further investigations are needed to explore differences between lipoprotein clearance and recycling to improve understanding of lipoprotein turnover and utilization of n-3 PUFA within HDL.

Arterial-venous (**A-V**) differences of individual FA might have allowed for a more accurate representation of the transfer of treatment FA into milk fat and plasma lipid classes. Enjalbert et al. (1998) duodenally infused palmitic, stearic, and OA in lactating dairy cows and used mammary A-V difference to demonstrate mammary uptake of these FA increased more rapidly than arterial concentration. These authors hypothesized increased arterial supply of plasma FFA and TG increased LPL activity regardless of stereospecific positioning, which is not supported by this study.

CONCLUSIONS

Plasma lipid class composition was altered by intravenous infusion of TG emulsions. Preformed and *de novo* milk fatty acyl yields were not influenced by infusions. Re-esterification of treatment FA into plasma PL indicate multiple mechanisms regulate availability of FA for uptake by the mammary gland.

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TABLES

Table 4.1. Fatty acid composition of lipid emulsions.

Fatty acid, %	Treatments				
	OA[1]	LA	LNA	DHA	SEM[2]
18:1, *cis 9*	58.7	13.9	14.7	4.7	1.87
18:2, *cis 9, cis 12*	9.3	59.8	17.1	5.5	0.90
18:3, *cis 9, cis 12, cis 15*	1.1	0.9	51.1	1.5	0.27
22:6	<0.1	<0.1	<0.1	44.9	0.38
Others	30.8	25.4	17.1	43.4	2.08

[1] Olive (Kroger, Inc., Cincinnati, OH), grapeseed (DeLallo Co., Jeannette, PA), flax (Flora, Inc. Lynden, WA), and algal (Martek Biosciences Corporation, Columbia, MD) oils were selected for enrichment of target FA in TG, oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (LNA), and docosahexaenoic acid (DHA).

[2] SEM = standard error of the mean.

Table 4.2. Composition of the TMR[1].

Item	Block 1 and 2	Block 3-6
Dry matter, %	57.7	51.7
Ingredient, % DM		
Corn silage	39.2	49.1
Concentrate[2]	28.9	23.8
Alfalfa silage	9.6	19.5
Ground corn	9.3	
Whole cottonseed	8.8	
Grass hay		7.7
Alfalfa hay	2.5	
Soybean meal (48%)	1.7	
Chemical composition		
Crude protein, % DM	17.5	15.3
Acid detergent fiber, % DM	27.8	24.7
Neutral detergent fiber, % DM	35.7	38.2
Crude fat, % DM	6.9	3.35
Ca, % DM	0.66	1.27
P, % DM	0.43	0.37
NE_L, Mcal/kg of DM	1.70	1.56

[1] Separate rations were fed during blocks 1 and 2 and blocks 3-6.

[2] Concentrate contained 31.1% dehydrated citrus pulp, 21.6% soybean meal (48%), 12.3% wheat midds, 9.3% distillers grains, 9.3% Pro-Lak (H.J. Baker & Bro., Inc., Westport, CT), 3.9%

acidified molasses with fat (9.9% EE; Westway Group, Inc., New Orleans, LA), 3.1% limestone, 1.9% Megalac (Church & Dwight Co., Inc., Princeton, NJ), 1.9% sodium bicarbonate, 1.8% urea (45%), 1.2% Dyna-Mate (Mosaic USA, LLC, Plymouth, MN), 1.2% plain salt, 0.78% magnesium oxide, 0.23% Availa-4 (Zinpro Corp., Eden Prairie, MN), 0.19% Vitamin ADE, 0.12% selenium (0.06%), 0.09% Mepron® M85 (Degussa Corp., Allendale, NJ), 0.07% Vitamin E-60000, 0.02% Rumensin 90 (Eli Lilly and Company, Indianapolis, IN).

Table 4.3. Performance of lactating cows during continuous 3 d infusion of intravenous triglyceride emulsions enriched in oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (LNA), or docosahexaenoic acid (DHA). Data presented are least squares means and standard errors.

	Treatments					*P*[3]		
Variable	OA	LA	LNA	DHA	SEM[1]	Trt[2]	Time	Trt×Time
Dry matter intake, kg/d	18.9	16.6	19.0	16.8	1.29	0.41	0.008	
Milk yield, kg/d	24.6	24.8	26.5	23.4	1.93	0.74	0.009	
Milk fat								
kg/d	1.01	1.09	1.11	1.04	0.092	0.86		
%	4.2	4.7	4.3	4.7	0.16	0.06		
Milk true protein								
kg/d	0.77	0.82	0.87	0.74	0.074	0.62		
%	3.2	3.4	3.3	3.3	0.12	0.80	<0.0001	0.014

[1] SEM = standard error of the mean.

[2] Trt = treatment.

[3] Main effects of time and treatment × time were not reported when insignificant ($P > 0.05$), but remained in the model.

Table 4.4. Milk fatty acyl yield of lactating cows receiving intravenous triglyceride infusions enriched in oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (LNA), or docosahexaenoic acid (DHA) continuously for 3 d. Data presented are least squares means and standard errors for d 3 of infusion.

	Treatments					*P*
Fatty acyl, g/d	OA	LA	LNA	DHA	SEM[1]	Trt[2]
4:0	32.5	35.0	40.2	32.7	6.44	0.68
6:0	18.2	19.6	21.1	18.0	3.26	0.82
8:0	10.8	11.2	11.4	10.1	1.69	0.90
10:0	23.6	24.1	23.8	21.6	3.49	0.92
12:0	26.2	25.9	25.6	23.6	6.39	0.92
13:0	0.7	0.6	0.6	0.6	0.10	0.82
14:0	79.3	81.3	84.5	78.4	13.07	0.97
14:1	7.5	6.9	7.8	8.1	1.32	0.90
15:0	6.4	6.2	6.0	5.7	1.11	0.94
16:0	248	267	281	256	43.8	0.91
16:1	13.4	12.4	12.3	13.4	9.23	1.00
17:0	7.1	7.5	7.5	6.5	0.69	0.54
18:0	90.4	101	102	77.1	13.60	0.39
18:1, *trans 4*	0.3	0.3	0.3	0.2	0.05	0.41
18:1, *trans 5*	0.2	0.3	0.2	0.3	0.14	0.90
18:1, *trans 6-8*	3.3	3.5	4.0	2.9	0.52	0.27
18:1, *trans 9*	2.7	2.7	3.0	2.5	0.39	0.65

18:1, *trans 10*	5.4	6.3	6.3	5.0	1.01	0.60
18:1, *trans 11*	10.3	12.0	14.2	10.4	2.00	0.29
18:1, *trans 12*	5.3	5.2	5.5	4.3	0.92	0.58
18:1, *cis 9*	207	196	206	189	20.3	0.82
18:1, *cis 11*	8.2	7.9	8.4	7.2	1.08	0.74
19:0	4.0	4.0	4.4	3.1	0.72	0.45
18:2, *cis 9, cis 12*	25.3	37.0	26.2	21.7	13.36	<0.0001
20:0	1.1	1.4	1.2	1.0	0.17	0.32
18:3, *cis 9, cis 12, cis 15*	2.4	2.4	10.8	2.4	0.45	<0.0001
18:2, *cis 9, trans 11*	4.3	3.8	4.6	4.6	0.80	0.86
18:2, *trans 10, cis 12*	0.0	0.6	0.6	0.0	0.54	0.57
20:2	0.2	0.2	0.2	0.1	0.05	0.11
20:4	1.5	1.6	1.6	1.6	0.17	0.92
20:5	0.1	0.1	0.4	0.4	0.06	0.0008
22:6	0.0	0.0	0.0	3.3	0.38	<0.0001
Others	25.2	28.9	33.0	26.8	4.94	0.53
Glycerol	144	153	160	140	18.7	0.80
Total	1015	1064	1114	978	143.0	0.84

[1] SEM = standard error of the mean.

[2] Trt = treatment.

Table 4.5. Plasma cholesterol ester concentration (µmol/mL) of lactating cows receiving intravenous TG infusions enriched in oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (LNA), or docosahexaenoic acid (DHA) continuously for 3 d. Data presented are least squares means and standard errors.

	Treatments					*P*[2]		
Fatty acid, µmol/mL	OA	LA	LNA	DHA	SEM[1]	Trt[3]	Time	Trt*Time
14:0	17.3	21.4	23.0	20.7	2.84	0.55		
14:1	12.8	19.4	10.9	15.2	1.95	0.03		
15:0	10.2	11.5	11.6	10.7	0.82	0.59		
16:0	87.2	90.3	88.0	99.3	7.69	0.66		
16:1	27.2	27.5	28.6	38.2	4.36	0.26		
17:0	3.7	5.6	4.9	4.3	0.66	0.26		
18:0	10.0	12.1	12.7	11.4	1.17	0.41		
18:1, *trans 11*	1.2	1.8	2.0	1.6	0.26	0.21		
18:1, *trans 12*	0.8	0.9	0.9	0.7	0.34	0.97		
18:1, *cis 9*	56.7	67.0	71.0	79.5	6.90	0.17		
18:1, *cis 11*	4.8	5.3	5.7	6.7	0.49	0.06		

19:0	1.0	1.5	0.7	0.9	0.23	0.13		
18:2, *cis 9, cis 12*	1673	1667	1763	1677	137.3	0.95		
18:3, *cis 6, cis 9, cis 12*	28.9	33.8	37.4	21.4	5.86	0.28	0.04	0.02
18:3, *cis 9, cis 12, cis 15*	119	126	150	131	12.9	0.37		0.05
18:2, *cis 9, trans 11*	1.1	1.3	1.5	1.6	0.17	0.22		
20:3	12.6	14.1	15.2	16.9	1.66	0.34		
20:4	30.1	37.8	42.4	42.0	4.59	0.22		
20:5	6.8	7.6	10.6	9.5	1.10	0.09		
22:6	16.7	7.2	10.8	7.4	6.98	0.75		
Others	262	263	345	289	17.9	0.01		0.04
Total	2384	2422	2637	2485	186.5	0.78		

[1] SEM = standard error of the mean.

[2] Main effects of time and treatment × time were not reported when insignificant ($P > 0.05$), but remained in the model.

[3] Trt = treatment.

Table 4.6. Plasma free fatty acid concentration (nmol/mL) of lactating cows receiving intravenous TG infusions enriched in oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (LNA), or docosahexaenoic acid (DHA) continuously for 3 d. Data presented are least squares means and standard errors.

	Treatments					*P*[2]
Fatty acid, nmol/mL	OA	LA	LNA	DHA	SEM[1]	Trt[3]
14:0	5.4	9.0	3.1	5.6	1.81	0.19
14:1	0.8	1.1	0.6	1.0	0.18	0.18
15:0	1.0	2.9	1.0	1.2	0.79	0.30
16:0	144	217	57.2	139	64.8	0.42
16:1	6.0	12.8	2.6	5.8	3.60	0.28
17:0	5.0	10.9	2.9	5.6	2.39	0.16
18:0	256	281	98.8	227	89.86	0.50
18:1, *trans 6-8*	1.3	1.1	0.6	0.8	0.36	0.58
18:1, *trans 9*	1.5	1.6	0.7	1.0	0.40	0.39
18:1, *trans 10*	6.4	5.4	2.1	4.9	1.75	0.37
18:1, *trans 11*	8.5	9.9	4.9	11.9	3.66	0.58
18:1, *trans 12*	3.5	4.2	1.6	3.4	1.50	0.66
18:1, *cis 9*	168	249	53.8	149	78.23	0.40
18:1, *cis 11*	8.4	13.1	3.8	9.9	3.99	0.45
19:0	2.3	2.4	1.1	3.2	1.15	0.63
18:2, *cis 9, cis 12*	36.1	47.2	14.3	30.0	13.24	0.38
20:0	2.5	3.2	1.1	2.1	1.27	0.65

18:3, *cis 6, cis 9, cis 12*	8.9	5.8	4.4	4.3	2.66	0.55
18:3, *cis 9, cis 12, cis 15*	1.5	2.2	3.5	1.2	0.66	0.10
18:2, *cis 9, trans 11*	0.4	0.7	0.2	0.6	0.18	0.38
20:2	1.8	0.7	0.8	1.8	0.51	0.25
20:3	9.0	5.0	1.4	4.1	4.06	0.61
20:4	3.0	3.5	1.2	4.1	1.51	0.56
20:5	4.6	4.6	1.5	3.9	2.00	0.64
22:6	1.9	0.0	0.0	15.4	3.90	0.04
Others	40.2	48.5	17.0	61.4	18.36	0.39
Total	728	941	280	699	283.6	0.44

[1] SEM = standard error of the mean.

[2] Main effects of time and treatment × time were not reported when insignificant ($P > 0.05$), but remained in the model.

[3] Trt = treatment.

Table 4.7. Plasma triglyceride concentration (µmol/mL) of lactating cows receiving intravenous triglyceride infusions enriched in oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (LNA), or docosahexaenoic acid (DHA) continuously for 3 d. Data presented are least squares means and standard errors.

	Treatments					*P*[2]		
Fatty acid, µmol/mL	OA	LA	LNA	DHA	SEM[1]	Trt[3]	Time	Trt*Time
14:0	3.8	5.8	2.7	2.6	0.88	0.07		
14:1	2.1	1.4	1.5	1.2	0.26	0.14		
15:0	2.6	2.6	2.0	1.5	0.33	0.08		
16:0	66.4	119	49.4	41.0	28.30	0.26		
16:1	1.5	5.1	1.0	1.3	1.48	0.22		
17:0	2.2	4.8	1.7	1.6	1.13	0.20		
18:0	95.5	132	70.7	55.2	32.10	0.38		
18:1, *trans 6-8*	0.9	0.8	0.9	0.7	0.19	0.79		
18:1, *trans 9*	0.8	0.7	0.6	0.5	0.13	0.44		
18:1, *trans 10*	1.6	1.4	1.4	1.0	0.28	0.48		
18:1, *trans 11*	5.2	6.7	4.8	4.1	1.13	0.44		

18:1, *trans 12*	1.9	2.5	1.4	1.4	0.55	0.43		
18:1, *cis 9*	26.3	66.9	17.3	15.0	18.81	0.23		
18:1, *cis 11*	2.4	3.3	1.7	1.5	0.82	0.40		
19:0	1.1	0.9	1.0	0.8	0.16	0.47		
18:2, *cis 9, cis 12*	7.8	16.7	4.4	4.6	4.12	0.17		
20:0	1.2	2.0	0.9	0.7	0.58	0.48		
18:3, *cis 6, cis 9, cis 12*	3.0	2.0	3.2	3.1	0.88	0.72		
18:3, *cis 9, cis 12, cis 15*	1.1	1.3	1.4	0.5	0.32	0.20		
18:2, *cis 9, trans 11*	0.1	0.1	0.1	0.1	0.05	0.73		
20:3	1.0	3.5	0.8	1.2	0.80	0.10		
20:4	0.6	1.4	0.4	0.6	0.33	0.17		
20:5	0.8	0.3	0.4	0.3	0.13	0.04		
22:6	0.2	0.0	0.0	1.7	0.24	0.0003	0.02	0.02
Others	24.1	45.0	11.4	13.9	10.77	0.16		
Total	254	426	181	156	98.0	0.25		

[1] SEM = standard error of the mean.

[2] Main effects of time and treatment × time were not reported when insignificant ($P > 0.05$), but remained in the model.

[3] Trt = treatment.

Table 4.8. Plasma phospholipid concentration (µmol/mL) of lactating cows receiving intravenous TG infusions enriched in oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (LNA), or docosahexaenoic acid (DHA) continuously for 3 d. Data presented are least squares means and standard errors.

	Treatments					*P*[2]		
Fatty acid, µmol/mL	OA	LA	LNA	DHA	SEM[1]	Trt[3]	Time	Trt*Time
14:0	50.2	46.3	49.2	48.5	5.96	0.44		
14:1	12.1	6.9	10.8	6.5	1.81	0.11		
15:0	10.9	11.5	11.7	11.3	1.23	0.97		
16:0	524	519	565	570	42.8	0.76		0.03
16:1	6.8	6.0	6.6	7.0	0.88	0.87		0.003
17:0	29.7	30.5	28.0	26.4	2.91	0.76		
18:0	742	715	627	644	88.0	0.75		
18:1, *trans 6-8*	1.2	1.1	1.2	1.0	0.52	0.99		
18:1, *trans 9*	2.6	2.0	2.2	1.8	0.50	0.64		
18:1, *trans 10*	3.8	4.4	4.8	3.4	0.79	0.62		
18:1, *trans 11*	18.5	21.4	24.5	20.8	3.35	0.66		

18:1, *trans 12*	14.1	13.3	15.3	12.6	1.68	0.71		0.02
18:1, *cis 9*	252	259	250	281	24.7	0.79		0.003
18:1, *cis 11*	20.3	19.6	20.2	24.0	1.99	0.40		
19:0	3.8	4.4	5.5	4.1	0.81	0.48		
18:2, *cis 9, cis 12*	925	897	959	835	101.1	0.84		0.02
20:0	2.3	2.0	1.7	2.0	0.33	0.66		
18:3, *cis 6, cis 9, cis 12*	5.0	5.0	5.1	3.4	0.99	0.58		
18:3, *cis 9, cis 12, cis 15*	36.4	31.7	69.0	36.3	4.67	0.0001		0.0002
18:2, *cis 9, trans 11*	2.1	2.3	2.6	2.2	0.32	0.80		
20:2	5.5	5.5	4.9	4.1	0.44	0.11		
20:3	104	118	133	122	13.6	0.50		
20:4	95.0	97.0	101	111	8.25	0.54		
20:5	7.5	8.8	12.9	12.8	1.40	0.03	0.04	
22:6	5.1	4.8	6.3	36.3	1.29	<0.0001	<0.0001	<0.0001
Others	433	396	609	472	88.8	0.36		
Total	3312	3223	3526	3298	308.3	0.91		0.03

[1] SEM = standard error of the mean.

[2] Main effects of time and treatment × time were not reported when insignificant ($P > 0.05$), but remained in the model.

[3] Trt = treatment.

FIGURES

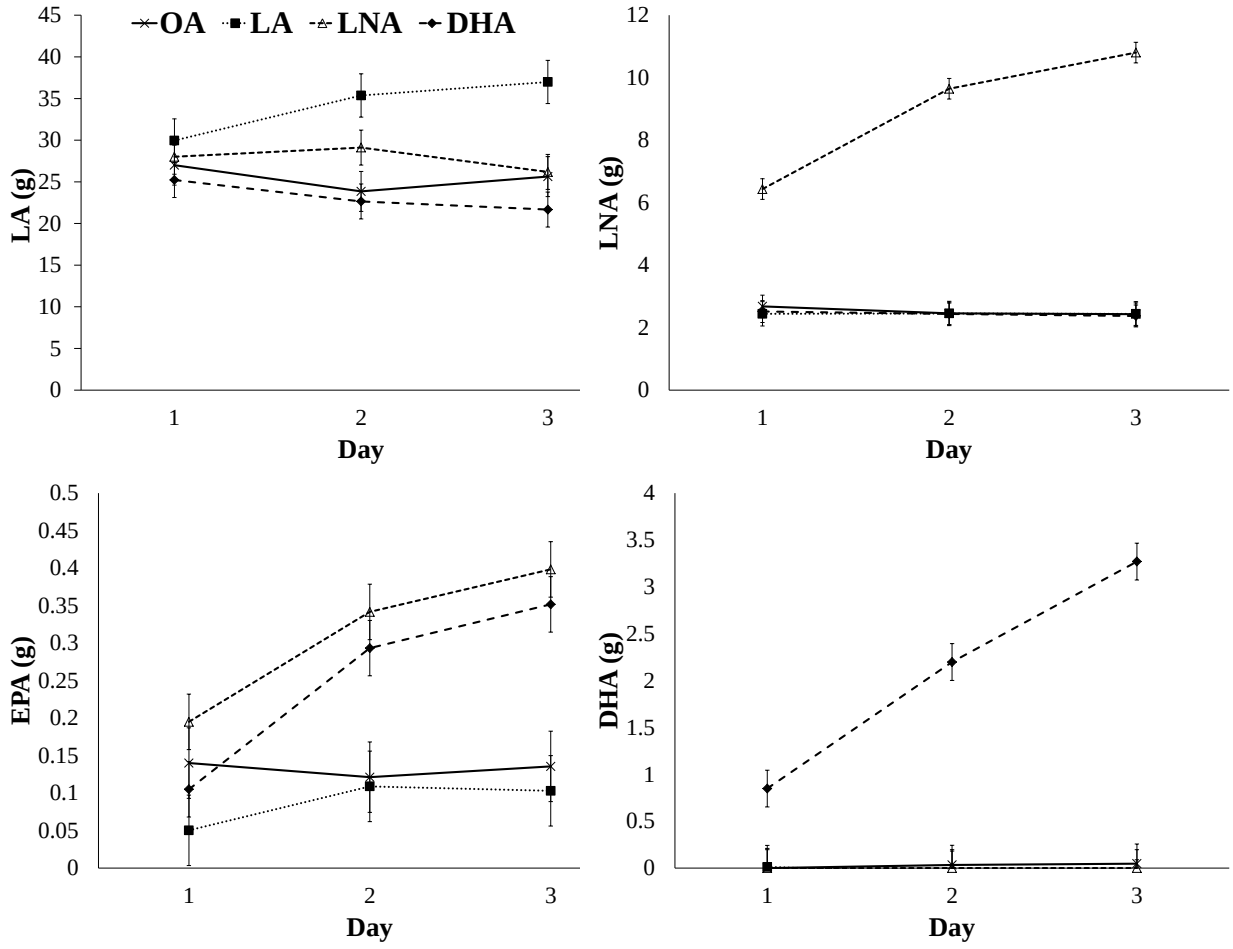

Figure 4.1. Daily milk fatty acyl yield of linoleate (LA), linolenate (LNA), eicosapentaenoate (EPA), and docosahexaenoate (DHA) in cows intravenously infused with triglyceride emulsions enriched in oleic acid (OA), LA, LNA, or DHA. Data are least squares means and standard errors. Main effects of treatment ($P < 0.001$) and treatment × time ($P < 0.05$).

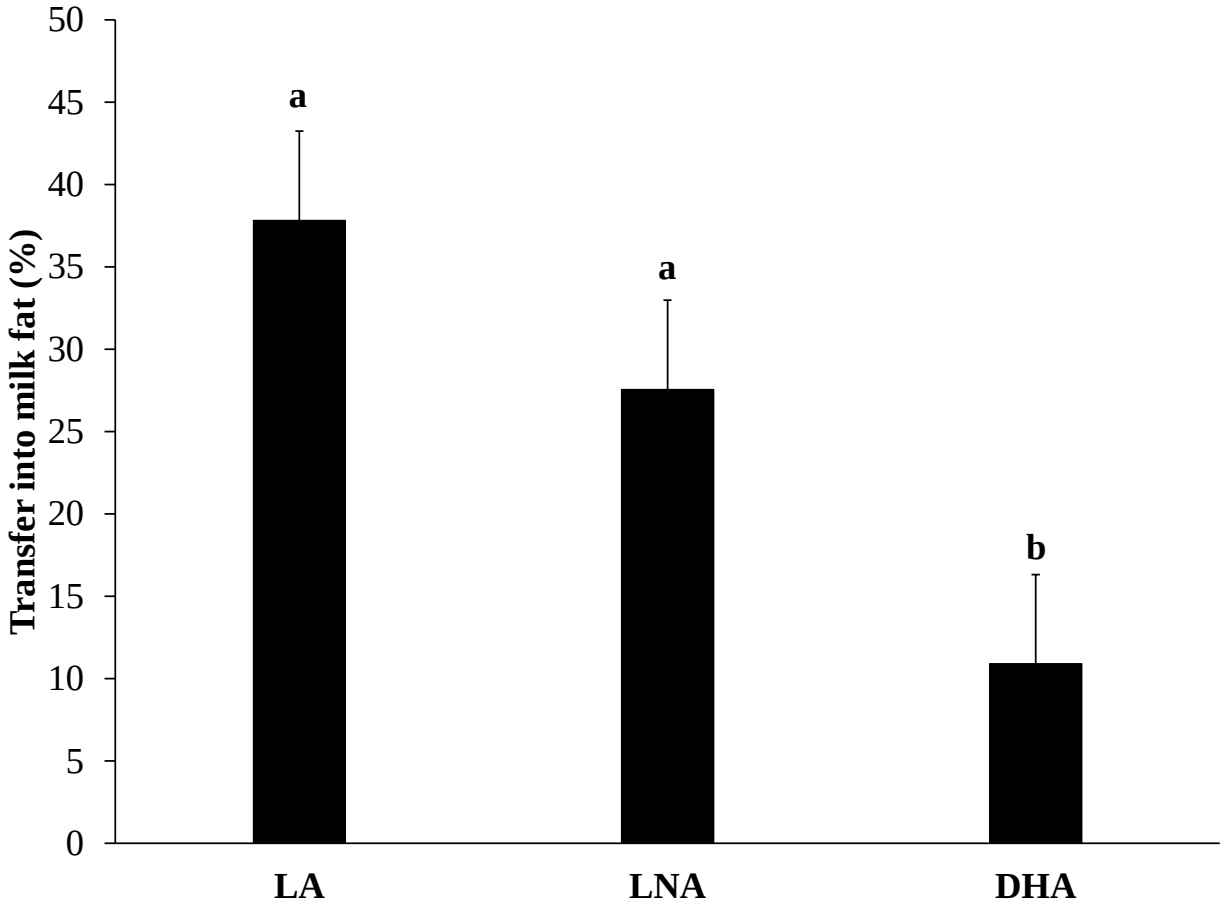

Figure 4.2. Milk transfer efficiency of fatty acids intravenously infused as triglyceride emulsions and enriched in linoleic acid (LA), LNA, or docosahexaenoic acid (DHA). Data are least squares means and standard errors. Means without a common letter differ ($P < 0.05$).

CHAPTER 5: Effects of FABP3 Overexpression in a Bovine Mammary Epithelial Cell Line

INTRODUCTION

Intracellular FABP are cytoplasmic proteins that are hypothesized to be essential for fatty acid transport and metabolism by accelerating LCFA uptake and targeting to intracellular organelles. The objective of the current study was to characterize FABP3 and FABP4 expression in bovine tissues and the role of FABP3 in lipid metabolism of a bovine mammary epithelial cell line (MAC-T). Fatty acid binding proteins are over 25 times more effective than albumin in increasing LCFA solubility in the cytoplasm and may target transfer of LCFA to acceptor membranes through direct membrane interaction (McArthur et al., 1999). Intracellular fatty acid trafficking is quite complex, and functional redundancy and cooperation exists among several proteins, including others such as long-chain acyl-CoA synthases and fatty acid transport proteins which also determine metabolic fates of LCFA (Sandoval et al., 2008). There are also distinct differences in the function and binding activity of the nine FABP isoforms identified (Storch and Corsico, 2008). Therefore, the role of FABP in the mammary gland is not fully understood.

A 15 kD protein, FABP3 is highly expressed in cardiac and skeletal muscle, and has been linked to fatty acid trafficking, metabolism, and signaling. The FABP3 isoform was originally isolated in the mammary gland as the primary component of MDGI as reviewed by Mather et al. (2000). Characterization of MDGI revealed it to be a complex of FABP3 with some FABP4 (Spener et al., 1990, Yang et al., 1994, Specht et al., 1996). The MDGI complex inhibits cell proliferation in mammary epithelial cell lines *in vitro* and may play a role in the onset of

differentiation (Politis et al., 1992, Yang et al., 1994). It has also been shown to suppress tumor formation through a C-terminal-derived 11 amino acid peptide in breast cancer cell lines (Wang and Kurtz, 2000). Breast cancer patients with tumors expressing MDGI had a more favorable prognosis (Nevo et al., 2010). These effects of FABP3 may be independent of its fatty acid ligand. Clark et al. (2000) determined FABP3 lacks an N-terminal signal peptide, and it is not known if the protein complex is secreted *in vivo*.

According to Bionaz and Loor (2008a, b), temporal patterns of gene expression reveal FABP3 and FABP4 are highly expressed in bovine mammary tissue during early lactation, which follows the temporal pattern of MDGI expression in lactation observed by Politis et al. (1992). Bionaz and Loor (2008b) proposed a role of FABP3 in channeling LCFA to SCD or TG synthesis or in LCFA activation of gene expression through PPAR-γ. Deletion of the FABP3 gene in mice causes defective FAO compensated by increased glucose utilization in heart and skeletal muscle (Storch and Corsico, 2008). While Clark et al. (2000) reported no overt effects of gene deletion on mammary gland phenotype, FABP3 null mice had a lower percentage of total unsaturated FA in milk fat. Both genetic and chemical inhibition of FABP4 alleviates ER stress (Erbay et al., 2007). Further, FABP4/5 knockout mice exhibit protection from high fat diet induced metabolic disorders (Cao et al., 2008). Together, these findings support a potential role for FABP3 and FABP4 in lipid synthesis in the bovine mammary gland though the exact biological function remains to be elucidated. The objective of the current study was to characterize FABP3 and FABP4 expression in bovine tissues and the role of FABP3 overexpression in lipid metabolism of MAC-T cells.

MATERIALS AND METHODS

Cloning of bovine FABP3

Bovine FABP3 (NM_174313.2) was amplified by RT-PCR from bovine cDNA using a one-step Master Mix solution (Taqman® One-Step RT-PCR Master Mix, Applied Biosystems, Foster City, CA, USA) and primers as follows: F: gcgttctctgtcgtctttcc; R: ttggctctgctttattgacct. The resulting amplicon was verified by sequencing, gel purified, and cloned into the pCR®4-TOPO® Vector (Invitrogen, Carlsbad, CA). It was then subcloned into the pcDNA3.1(+) vector (Invitrogen) using the *EcoR*I restriction site in the multiple cloning site of pCR4-TOPO and within the insert. The amplicon was subcloned again into the epitope-tagged vector pCMV-Tag 2C using double digests of pcDNA3.1(+) containing the FABP3 amplicon with *Ale*I and *Xho*I and double digests of pCMV-Tag 2C with *EcoR*V and *Xho*I.

Cell culture and treatments

MAC-T cells were routinely cultured at 37°C and 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 100 U penicillin, 0.01 mg streptomycin, and 0.25 µg/mL amphotericin B (1× antibiotic/antimycotic solution; Sigma). Cells were trypsinized and seeded (approximately 1.3×10^5 cells per well) into 6-well plates for 24 h before transfection at approximately 50% confluence. Medium was discarded and replaced with 1 mL per well of fresh growth medium 1 h before transfection. Cells were transfected with 3 µg vector per well, using jetPEI transfection reagent (PolyPlus Transfection, New York, NY). After 24 h cells were

washed with PBS, lysed, and assayed. Treatments included: transfection reagent alone as an untransfected control (**UNT**), empty pCMV-tag2c vector as a negative control (**EV**), pCMV-tag2c vector containing FABP3 sequence (**FLAG-FABP3**), and pcDNA3.1+ vector containing FABP3 sequence (**FABP3**). Experiments were conducted in triplicate and repeated 4 times.

Tissues

Bovine adipose, heart, liver, mammary gland, and small intestine tissues were collected at slaughter to compare expression of FAP3 and FABP4.

Real time Quantitative PCR

Total ribonucleic acid (RNA) was extracted from cells using 1 mL per well and from tissues using 1.5 mL TRI Reagent (Molecular Research Center Inc., Cincinnati, OH) per 100 mg tissue according to the manufacturer's instructions. RNA pellets were resuspended in RNase-free water and concentrations were quantified at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). The Omniscript RT kit (Qiagen, Valencia, CA) was used to reverse transcribe 1 µg RNA per reaction to complementary DNA according to the manufacturer's instructions using oligo-dT (Eurofins MWG/Operon, Huntsvile, AL) as the primer. Real-time quantitative PCR was performed using the Quantitect SYBR Green PCR kit (Qiagen) in an Applied Biosystems 7300 Real-time PCR machine (Foster City, CA). Reactions were as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min. Each reaction was performed in duplicate wells. Serial dilutions of linearized plasmid DNA were used to generate standard curves to determine copy number of FABP3 and FABP4 in bovine tissues. Relative quantification of gene transcripts was determined

using β-actin as the endogenous control gene for cell transfection experiments. Fold change was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Gene-specific primers for the transcripts used in the study are shown in Table 5.1.

Protein Extraction and Immunoblotting

To determine protein abundance, harvested cells (following a wash with PBS) and tissues were homogenized in ice-cold lysis buffer (50 mM Tris pH 7.4, 0.5% Triton X-100, 0.3 M NaCl, 2 mM EDTA pH 8.0, and protease inhibitor cocktail), followed by centrifugation at 16,000 ×g for 15 min at 4°C. Supernatants were collected for protein concentration measurements. Protein concentrations were determined using the Bradford Assay (Bio-Rad, Hercules, CA). Samples were diluted to equal protein concentrations in lysis buffer to ensure equal loading before adding Laemmli sample buffer (Sigma Chemical Co., St. Louis, MO) and heated at 95°C for 10 min. Proteins were separated by electrophoresis using 15% PAGEr Gold PlusPreCast SDS-polyacrylamide gels (Lonza, Rockland, ME) and transferred to a PVDF membrane using a Bio-Rad Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA). Membranes were then blocked in blocking buffer (0.05 M Tris pH 7.4, 0.2 M NaCl, 0.1% Tween, and 5% dried non-fat milk) on a rocker for 1 h at room temperature. Membranes were probed with primary anti-FABP3 (R&D Systems, Inc., Minneapolis, MN) or anti-FLAG (Sigma) at 1:1000 in blocking buffer at 4°C overnight. Membranes were washed in washing buffer (0.05 M Tris pH 7.4, 0.2 M NaCl, and 0.1% Tween) twice for 10 min. Following washing, membranes were respectively incubated with horseradish peroxidase donkey, anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-mouse IgG1secondary antibody (Santa Cruz Biotechnology) at 1:1000 in blocking buffer for 1 h at room temperature. Membranes were washed three times, for 15 min each, and

proteins were detected using ECL-Plus chemiluminescence substrate (GE, Pittsburg, PA) according to manufacturer's instructions.

Fatty Acid Uptake

Fatty acid uptake was measured by separately quantifying the incorporation of [1-^{14}C]-oleate, [1-^{14}C]-linoleate, and [1-^{14}C]-palmitate (MP Biomedicals, Solon, OH) into lipids. Oleate, linoleate, and palmitate (Matreya, LLC, Pleasant Gap, PA) were each weighed, mixed with 0.05 M NaOH to form a sodium salt, and complexed to bovine serum albumin (BSA, Sigma) at a 3:1 molar ratio in 37°C culture media using the method described by Ip and co-workers (1999) with modifications. Cells were transfected, and after 20 h, media was removed and replaced with fresh media containing 100 µM fatty acid and 1 µCi/well [1-^{14}C]-labeled fatty acid and incubated 4h. Following incubation, isotope-containing media was removed and cells were washed with PBS. Cells then were lysed with SDS buffer (0.1% in PBS) and lipids from the lysates were extracted with hexane-isopropanol (3:2). The organic phase was transferred to scintillation vials. Scintillation cocktail (15 mL per vial; Scintisafe 30% Cocktail, Fisher Scientific) was added to scintillation vials. Radioactivity was measured using a LS 6000LL Beckmann scintillation counter (Beckmann Coulter Inc., Brea, CA). Activity was calculated and expressed as nmol of acetate incorporated per h.

Fatty Acid Oxidation

Fatty acid oxidation in cell culture was determined using the method of Consitt et al. (2010) with modifications. Briefly, incubation medium (1mL) was added to each well and plates were covered with parafilm and incubated 3 h. After incubation, 500 µL medium was assayed for

complete FAO using $^{14}CO_2$ and incomplete FAO using ^{14}C-labeled acid-soluble metabolites (ASM) in a Teflon trapping device. Cells were washed once with PBS, harvested in 400 µl of 0.05% SDS lysis buffer, and stored at −80°C for subsequent determination of protein concentration.

Fatty Acid Composition

Fatty acid composition was measured from whole cell lysates using the same harvest method for protein quantification. For each treatment, three wells were pooled and protein concentration was determined. Lysates were extracted with hexane-isopropanol (3:2). FAME were prepared using 1% methanolic sulfuric acid as described by Perfield et al. (2006). FAME were analyzed by gas chromatography (Agilent 6890N GC) using a CP-Sil 88 capillary column (100 m × 0.25 mm i.d. with 0.2 µm thickness; Varian, Inc., Palo Alto, CA). Conditions were as follows: the oven temperature was initially set at 70°C, then increased at 8°C/min to 110°C, then increased at 5°C/min to 170°C and held for 10 min, then increased 4°C/min to 225°C and held for 15 min. The inlet and detector temperatures were 250°C, the split ratio was 100:1, and a 1 µL injection volume was used. The hydrogen carrier gas flow rate was 1 mL/min. Hydrogen flow to the detector was 25 mL/min, airflow was 400 mL/min, and the flow of nitrogen makeup gas was 40 mL/min. Fatty acid peaks were identified by using pure methyl ester standards (Nu-Check Prep Inc., Elysian, MN).

Statistical Analysis

All data were analyzed using the MIXED procedure of SAS (SAS 9.2; SAS Institute, Inc., Cary, NC). The model included the fixed effect of treatment and fatty acid. Replicate was

included as a random effect. The covariance structure that yielded the lowest Akaike's information criterion was used (Littell et al., 1998). Preplanned orthogonal contrasts were used to separate effects of treatment means: 1) UNT minus all vectors, 2) EV minus FLAG-FABP3 and FABP3, and 3) FLAG-FABP3 minus FABP3. Significance of treatment effects was declared at $P \leq 0.05$ and tendency at $P \leq 0.1$. Data are reported as least squares means ± standard error of the means.

RESULTS

The FABP3 isoform was overwhelmingly expressed in bovine heart and mammary gland tissue, followed by small intestine, adipose, MAC-T cells, and liver as revealed by mRNA copy number (Figure 5.1). Protein abundance (data not shown) indicated antibody cross-reactivity with other FABP isoforms, apparently for both liver (FABP1) and small intestine (FABP2), which prevented confidence in FABP3 detection using this antibody. Copy number of FABP4 mRNA indicated predominant expression in bovine adipose tissue, followed by mammary gland, heart, and small intestine, and FABP4 mRNA expression was not detected in bovine liver and MAC-T cells (Figure 5.2).

The absence of detectable FABP3 and FABP4 mRNA in bovine MAC-T cells suggested experiments overexpressing FABP3 may be more conclusive in determining a role in lipid metabolism. We created a FLAG-FABP3 vector to verify expression (Figure 5.3) in each experiment to confidently detect vector expression without cross-reactivity. Mean protein content (three wells were pooled) for MAC-T cells treated with transfection reagent only, empty pCMV-tag2c vector, FLAG-FABP3 vector, or FABP3 vector for 24 h was 2.6, 2.0, 2.2, and 2.3±0.22 mg. While numerically lower, treatment differences were not significant.

Incorporation (Figure 5.4) of radiolabeled palmitate into cellular lipids was significantly reduced by transfection with any vector ($P < 0.0001$). Palmitate incorporation was also calculated during the FAO experiments and followed the same pattern for all treatments (data not shown). Linoleate incorporation also tended to be reduced by transfection (treatment, $P = 0.07$; effect of transfection, $P = 0.01$).

In mammary epithelial cells, FAO is very low and complete oxidation of radiolabeled palmitate to CO_2 was not changed (Figure 5.5). Incomplete FAO to acid soluble products (**ASP**) was influenced by treatments ($P < 0.0001$). Transfected cells yielded more ASP ($P < 0.0001$). Cells treated with FABP3 containing vectors produced less ASP than EV (P = 0.03). Transfection with FABP3 also produced less ($P = 0.002$) ASP than FLAG-FABP3. Total FAO followed the same pattern as ASP. ASP production drove total changes in FAO in MAC-T cells ($P < 0.0001$). Transfected cells had greater total FAO ($P < 0.0001$) compared to UNT. Cells treated with FABP3 containing vectors had lower total FAO than EV ($P = 0.04$). Transfection with FABP3 also oxidized less (P = 0.003) total FAO than FLAG-FABP3 overall.

Differences were not detected in cellular lipid fatty acid composition (Table 5.2). There was a tendency for a lower ratio ($P = 0.1$) of palmitoleate to palmitate (Figure 5.6), which was caused by transfection ($P = 0.02$).

DISCUSSION

Copy number of FABP3 and FABP4 mRNA in bovine mammary tissue, revealed FABP3 to be far more abundant. This follows the pattern of relative FABP3 and FABP4 mRNA expression observed by Bionaz and Loor (2008a). Overall tissue distribution of FABP3 mRNA

also revealed a minimal level of expression in bovine small intestine. Storch et al. (2008) noted FABP3 gene expression in cardiac and skeletal muscle, brain, mammary tissue, kidney, adrenals, ovaries, testis, placenta, lung, and stomach. Additionally, FABP4 gene expression has been noted in adipose tissue and macrophages (Storch and Corsico, 2008). In the present study, FABP4 mRNA was primarily detected in adipose tissue, but was also expressed in bovine heart, mammary gland, and small intestine. The role of FABP3 and FABP4 tissue distribution in the bovine is not well understood, but co-expression of multiple isoforms may allow functional redundancy within the tissue or could indicate independent roles of each isoform.

Because mRNA for neither the FABP3 nor FABP4 genes was detected in bovine MAC-T cells, overexpressing FABP3 could provide conclusive evidence of the role of FABP3 in mammary lipid metabolism. However, fatty acid incorporation was reduced by transfection in the present study, with no FABP3 effect. The human breast cancer cell line MCF7 does not express FABP3 and overexpression of FABP3 in MCF7 cells increased uptake of radioactively labeled palmitate and oleate, but other changes in fatty acid metabolism were not observed (Buhlmann et al., 1999). Other studies have explored the role of overexpression of multiple FABP isoforms on lipid metabolism. Overexpression of FABP3 and FABP4 in L6 myoblasts, and FABP1, FABP3, and FABP4 in Madin-Darby canine kidney cells did not alter palmitate uptake (Prinsen and Veerkamp, 1998, Zimmerman and Veerkamp, 2001). Both L6 myoblasts and Madin-Darby canine kidney cells contain FABP. It is possible that compensation by other cytosolic proteins, even other minimally expressed FABP isoforms, provides FA transport, such that uptake is not rate-limiting. Wolfrum et al. (1999) demonstrated decreased FA uptake in the human hepatoma cell line, HepG2, by knockdown of FABP1. It is possible that the role of

FABP3 in fatty acid uptake may be more easily demonstrated by knockdown in a cell line currently expressing the protein since compensatory mechanisms may be limited.

Most prior studies reporting a role for FABP3 in FAO reference cardiac or skeletal muscle defects because FAO is high in these cells and very low in mammary epithelial cells. This defect in muscle FAO is largely due to inhibition of FA uptake and consequent increased glucose utilization (Binas et al., 1999, Schaap et al., 1999). In the present study, baseline levels of FAO in UNT cells were very low and incomplete. Though FA uptake was not changed by overexpression of FABP3, rate of ASP production increased with transfection, indicating cells may have been stressed.

Fatty acid composition of MAC-T cells in the present study was not altered by FABP3 overexpression. Bionaz and Loor (2008b) suggested FABP3 may channel LCFA to SCD for desaturation. To test this hypothesis, we explored the ratio of palmitoleate to palmitate and oleate to stearate and found no differences. There was a tendency for a decreased ratio of palmitoleate to palmitate induced by transfection, which suggesting SCD activity may have been decreased.

The results of the present study do not point to a functional role of FABP3 in lipid metabolism in mammary epithelial cells. However, FABP3 has also been confirmed as an early marker of acute myocardial infarction (McMahon et al., 2012). The ER functions to regulate cell homeostasis. Nutrient overload stimulates the unfolded protein response and dysregulates ER metabolism inducing ER stress (Gregor and Hotamisligil, 2007). This ER stress may play a role in MFD by regulating lipogenic pathways in mouse mammary epithelial cells (Invernizzi et al., 2010, Bauman et al., 2011). Recent work by Invernizzi et al. (2012) indicates the ER stress pathway components are increased during early lactation in the bovine mammary gland and changes in these pathways are a normal physiological adaption of mammary epithelial tissue to

lactation. Increased FABP3 and FABP4 expression also coincides with early lactation and influx of preformed LCFA to the mammary gland (Politis et al., 1992, Bionaz and Loor, 2008b, a). Thus, in early lactation FABP expression may be an indicator of ER stress. Indeed, deletion of FABP4 and FABP5 in mice is protective against lipotoxicity (Erbay et al., 2007, Cao et al., 2008). Also, macrophages lacking FABP4 have elevated SCD1 expression, increased palmitoleate synthesis, and become resistant to palmitate-induced ER stress (Erbay et al., 2009). Barf et al. (2009) has identified a potent and select class of FABP4 inhibitors which suggests potential for modulating FABP activity. Thus, FABP inhibition may also alleviate ER stress by reducing FA transport to the ER and enhancing lipogenesis.

CONCLUSIONS

Bovine mammary and heart tissue highly express FABP3 mRNA. The FABP3 isoform does not appear to be rate-limiting for fatty acid uptake *in vitro* and overexpression did not alter lipid metabolism in MAC-T cells. The function of FABP3 in the mammary gland remains unclear.

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TABLES

Table 5.1. Primer sequences for transcripts used in real-time quantitative PCR.

Transcript	Accession number		Primers (5'-3')
Actin	AY141970	Forward	ctcttccagccttccttcct
		Reverse	gggcagtgatctctttctgc
FABP3	NM_174313.2	Forward	aagcctaccacaatcatcgaag
		Reverse	ttcaagctgggagtcgagttc
FABP4	NC_007312.3	Forward	catcttgctgaaagctgcac
		Reverse	acccccattcaaactgatga

Table 5.2. Fatty acid composition of MAC-T cells treated with transfection reagent only (UNT), empty pCMV-tag2c vector (EV), FLAG-FABP3 vector (FLAG), or FABP3 (FABP3) vector for 24 h. Data presented are least squares means and standard errors.

	Treatments					*P*
Fatty acid, g/100 g	UNT	EV	FLAG	FABP3	SEM[1]	Trt[2]
14:0	1.2	1.0	1.0	0.9	0.13	0.22
16:0	13.5	10.7	13.7	10.7	1.70	0.38
16:1	1.0	0.7	0.9	0.7	0.12	0.29
18:0	7.3	6.1	8.4	6.4	1.01	0.41
18:1, *cis 9*	9.6	8.2	9.9	7.6	1.40	0.60
18:1, *cis 11*	2.9	2.3	3.0	2.4	0.34	0.34
18:2, *cis 9, cis 12*	2.8	2.1	2.6	2.1	0.29	0.28
18:2, *cis 6, cis 9, cis 12*	0.7	1.5	2.1	0.2	0.77	0.36
18:3, *cis 9, cis 12, cis 15*	0.4	0.3	0.4	0.3	0.06	0.37
18:2, *cis 9, trans 11*	0.7	0.3	0.5	0.3	0.17	0.31
18:2, *trans 10, cis 12*	0.0	0.6	0.6	0.0	0.54	0.57
20:2	0.6	0.7	0.3	0.6	0.21	0.46
20:3	0.4	0.5	0.4	0.3	0.11	0.57
20:4	6.3	5.2	7.2	5.3	0.84	0.36
20:5	0.5	1.2	0.6	0.4	0.41	0.51
22:6	2.8	2.2	3.0	2.2	0.37	0.32
Others	18.5	21.2	27.9	33.0	5.48	0.28

[1] SEM = standard error of the mean.

[2] Trt = treatment.

FIGURES

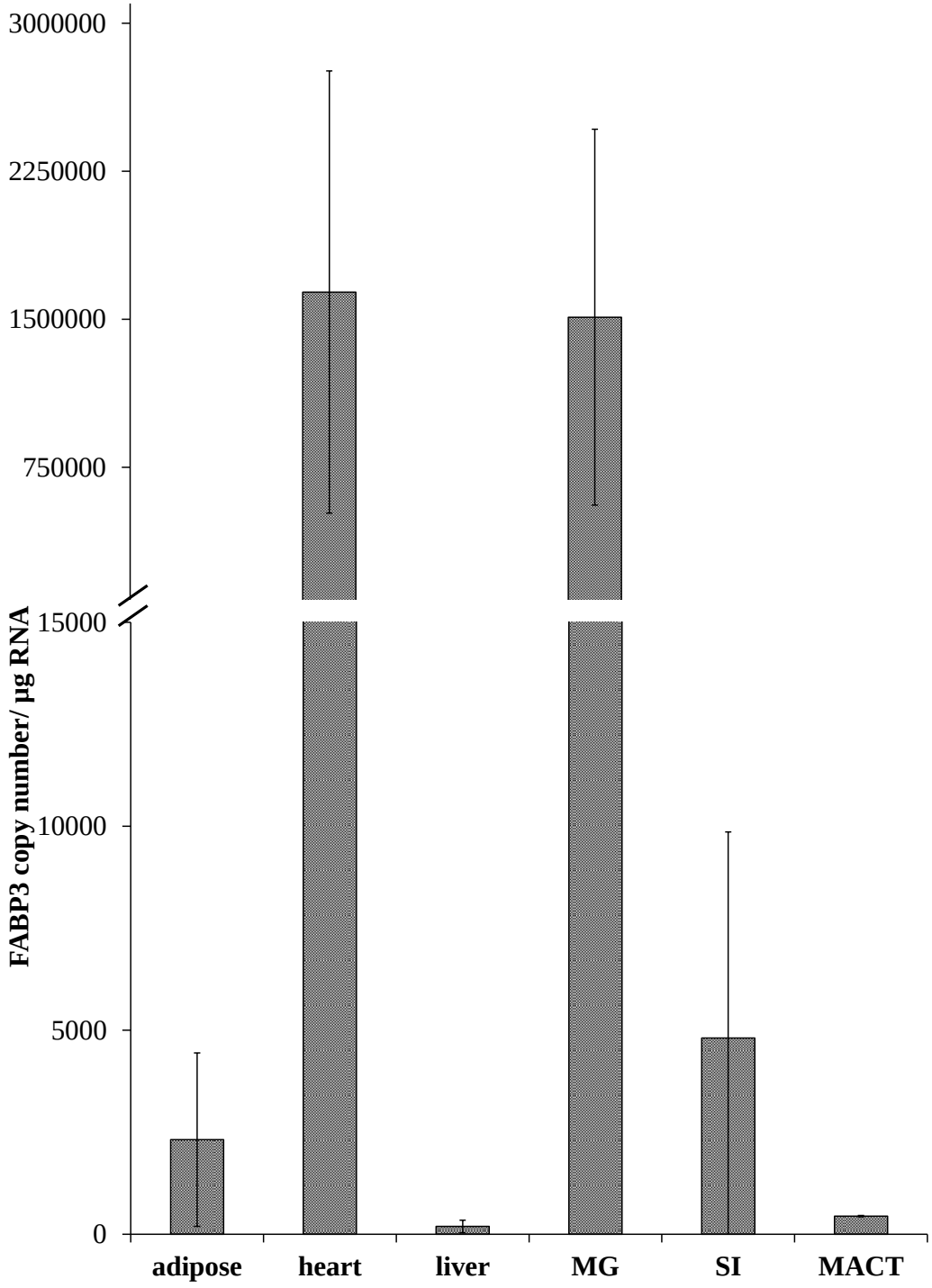

Figure 5.1. Transcript copy number of FABP3 in bovine adipose, heart, liver, mammary gland (MG), and small intestine (SI), and bovine mammary epithelial cell line (MAC-T). Data presented are means ± standard deviation.

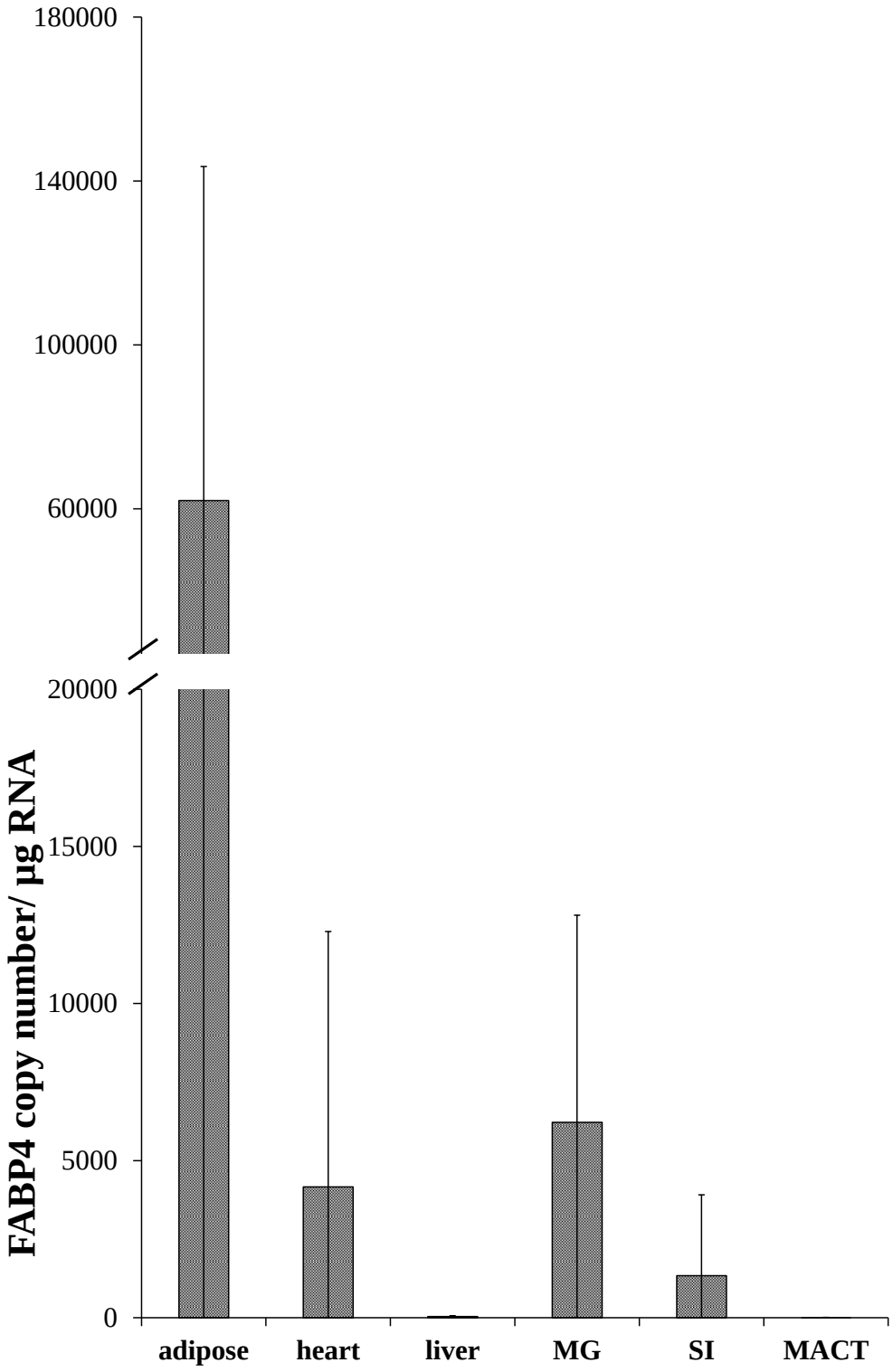
180000
140000
100000
60000
20000
15000
10000
5000
0
FABP4 copy number/ µg RNA
adipose
heart
liver
MG
SI
MACT

Figure 5.2. Transcript copy number of FABP4 in bovine adipose, heart, liver, mammary gland (MG), and small intestine (SI), and bovine mammary epithelial cell line (MAC-T). Data presented are means ± standard deviation.

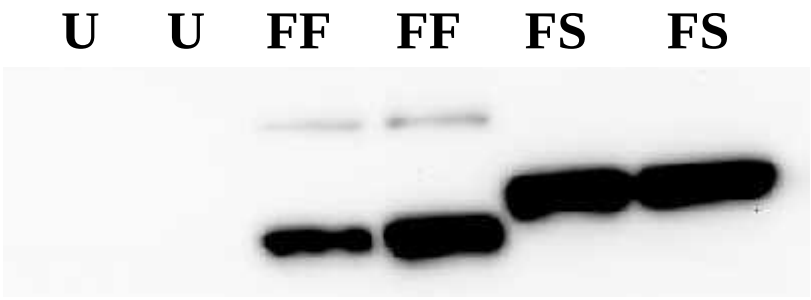

Figure 5.3. Protein abundance of Flag in MAC-T cells treated with transfection reagent only (U), Flag-FABP3 vector (FF), or Flag-SCD vector (FS) for 24 h. Flag-SCD was included as a positive control for the Flag antibody. Protein bands were visualized using Quantity One Software.

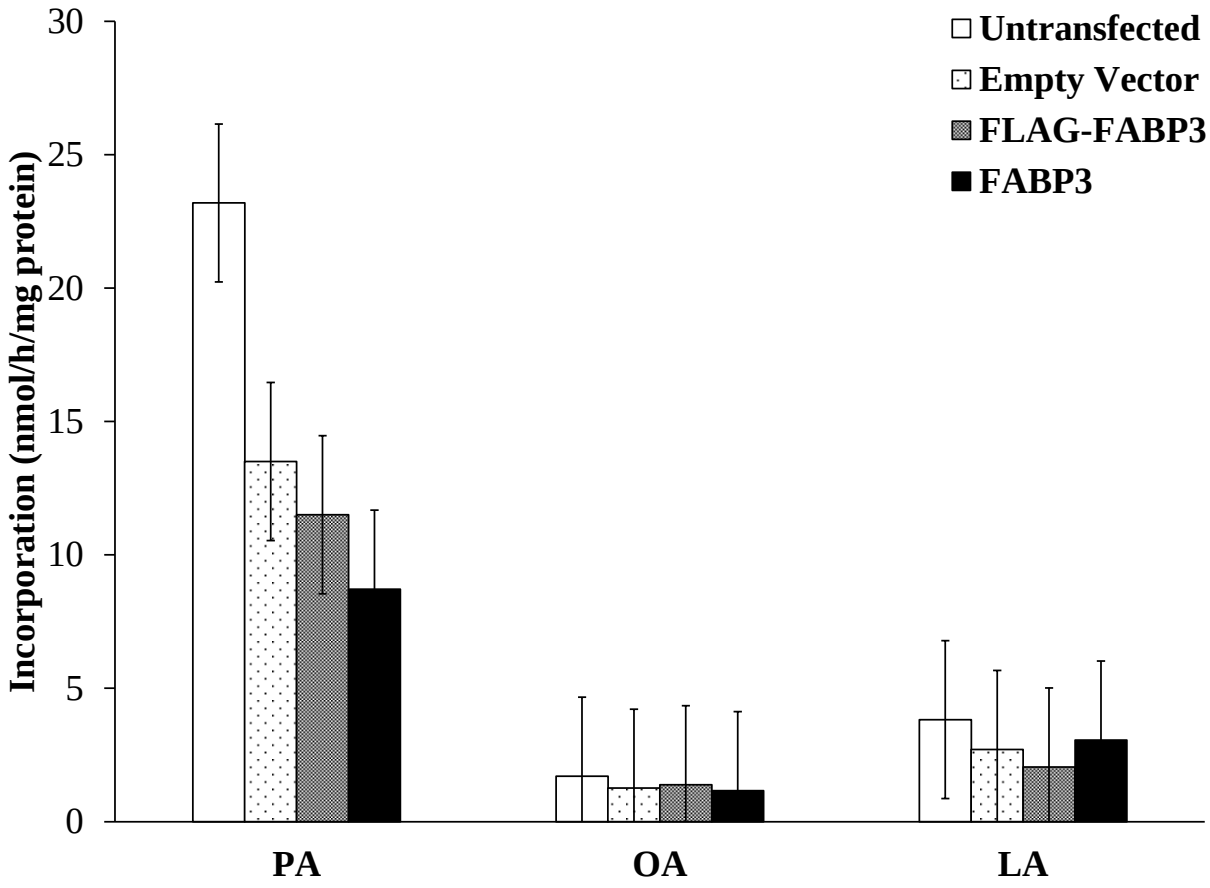

Figure 5.4. Incorporation of [1-^{14}C]-palmitate (PA), [1-^{14}C]-oleate (OA), and [1-^{14}C]-linoleate (LA) in MAC-T cells treated with transfection reagent only (Untransfected), empty pCMV-tag2c vector (Empty Vector), FLAG-FABP3 vector, or FABP3 vector for 24 h. Data presented are least squares means ± standard error.

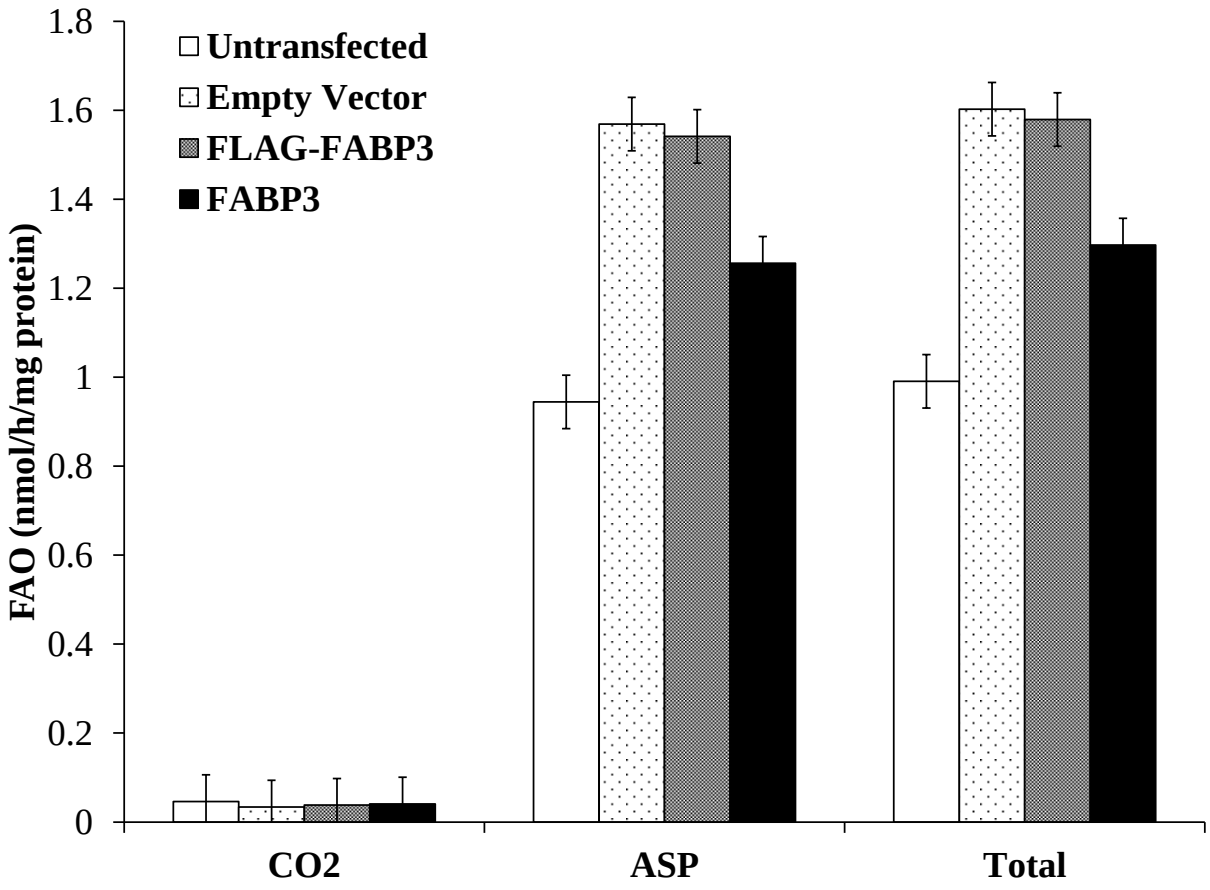

Figure 5.5. [1-^{14}C]-palmitate oxidation to CO_2, acid soluble products (ASP), and total oxidation in MAC-T cells treated with transfection reagent only (Untransfected), empty pCMV-tag2c vector (Empty Vector), FLAG-FABP3 vector, or FABP3 vector for 24 h. Data presented are least squares means ± standard error.

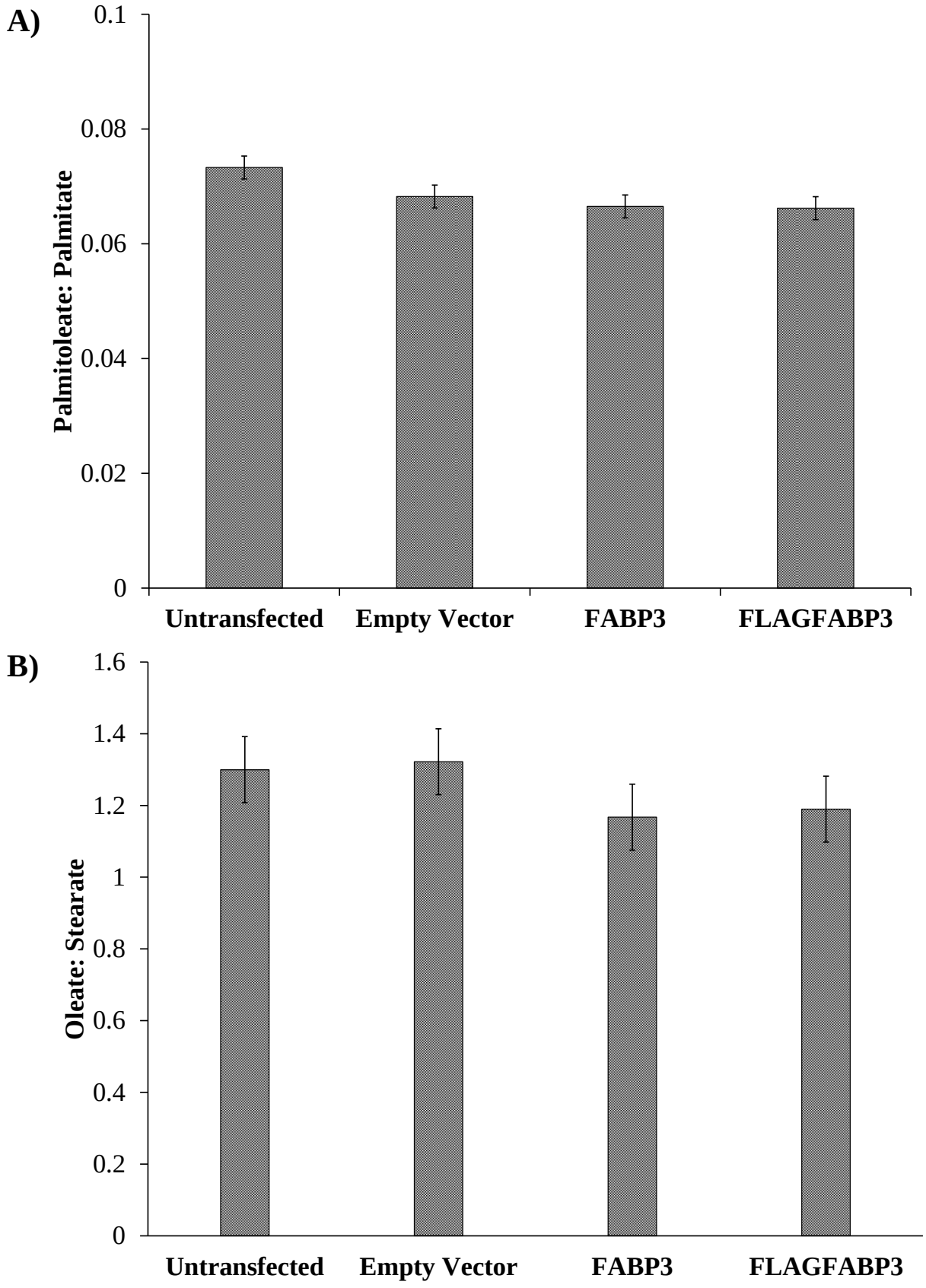
A)
0.1
0.08
0.06
0.04
0.02
0
Palmitoleate: Palmitate
Untransfected
Empty Vector
FABP3
FLAGFABP3
B)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Oleate: Stearate
Untransfected
Empty Vector
FABP3
FLAGFABP3

Figure 5.6. A) Palmitoleate to palmitate ratio and B) oleate to stearate ratio in cellular lipid composition of MAC-T cells treated with transfection reagent only (Untransfected), empty pCMV-tag2c vector (Empty Vector), FLAG-FABP3 vector, or FABP3 vector for 24 h. Data presented are least squares means ± standard error. Differences were not significant.

CHAPTER 6: Conclusions

Increasing PUFA concentration, particularly n-3 FA, in milk fat would improve consumer perception of milk fat quality. Efforts to increase PUFA in milk fat include marine and seed oil supplementation and pasture-based rations. The lactating dairy cow consumes a low-fat diet enriched in PUFA that are almost completely biohydrogenated to saturated FA. Strategies to increase PUFA in milk through rumen-protected supplementation are thwarted by multiple physiological mechanisms to conserve PUFA.

A delicately balanced ration must be maintained to increase dietary fat levels enough to enhance TG esterification of PUFA in the small intestine. Several factors facilitate negative dietary interactions that compromise rumen function and alter biohydrogenation pathways to induce MFD with high fat diets. They include, but are not limited to feeding strategy, high grain rations, low effective fiber, and monensin. It should be noted that supplemental fat in early lactation can also be utilized to increase dietary energy and restore positive energy balance, but the stressors associated with the physiological adaptation to lactation and negative energy balance can also impair rumen biohydrogenation and cause MFD. Well-managed dairy herds maintaining high levels of milk production without compromising fat yield must constantly achieve excellent forage management, consistent feed mixing and delivery, and excellent cow health and body condition.

In the first study, lipid encapsulated algal biomass and oil increased DHA transfer to milk fat. Though *trans*-C18:1 fatty acids were increased with supplementation suggesting biohydrogenation of supplements occurred, the lipid coating must not be overprotective to

optimize digestibility of intact n-3 FA in the small intestine. Lipid encapsulation can protect PUFA from biohydrogenation, but this technique requires further optimization before development of commercially available RP PUFA supplements. It is crucial that the saturated FA coating protect PUFA from rumen microbes, but it must also be highly digestible in the small intestine to allow hydrolysis of TG and maximum PUFA absorption. Additional data reporting duodenal FA flow from the rumen and apparent digestibility of lipid encapsulated PUFA are needed to evaluate the optimal coating technique to maximize FA absorption in the small intestine.

Further, DHA in RP algae supplements was primarily esterified into plasma PL, making it unavailable to the mammary gland for milk fat synthesis. Supposing esterification of dietary PUFA into plasma TG can be maximized at the small intestine, it is still difficult to achieve transfer into milk fat, predominantly for very long-chain n-3 FA, such as DHA. In the second study, triglyceride emulsions of oils enriched in OA, LA, LNA, and DHA were intravenously infused to avoid confounding effects of TG esterification patterns in the small intestine and to compare mammary uptake of fatty acids differing in chain length and unsaturation. Transfer of OA was not detectable. Similar milk fat transfer of LA and LNA were observed, and DHA transfer remained low despite direct intravenous infusion of TG. Re-esterification of n-3 PUFA in the liver was apparent, suggesting multiple mechanisms conserve these FA in lactating cows.

The mammary epithelial cell is rich in LPL, making LCFA uptake extremely efficient, but n-3 PUFA, especially very LCFA like DHA, transported in the TG portion of VLDL are not well-recognized by mammary LPL and use alternate pathways for cellular uptake. Once preformed FA enter the mammary epithelial cell, it is possible PUFA are primarily incorporated into cell membranes rather than TG esterification and export.

Additionally, PUFA are often esterified at the *sn*-2 position of TG, and in rats, TG hydrolysis by LPL results in a PUFA-containing monoglyceride that is recycled to the liver, where it is likely shunted toward phospholipid or cholesterol ester synthesis (Couedelo et al., 2012). Christie et al. (1986) indicate LPL stereospecificity may also occur in ruminants, but evidence of intermediates of triglyceride hydrolysis by LPL in remains unclear. Ruminant LPL activity certainly responds to diet, physiological state, and tissue (Drackley, 2005). This is intriguing because much of the action of PUFA as PPAR ligands occurs as FFA, but these monoglycerides might be engulfed by the liver as remnants of VLDL, rather than by FFA uptake. FFA may also be generated locally by LPL hydrolysis of TG delivered by VLDL. PPAR are viewed as lipid sensors, PPAR-α and PPAR-δ are oxidative and PPAR-γ is adipogenic, but delivery of PUFA as PPAR ligands is not well understood (Ruby et al., 2010).

A benefit of PPAR-α activation in liver is enhanced FAO during early lactation, which can prevent accumulation of liver TG and ketosis. Supplemental PUFA may not result in physiological concentrations of FFA ligands for PPAR-α in the lactating dairy cow if initially esterified as PL rather than TG or if recycled to PL via lipoprotein remnant uptake at the liver. Additional research is needed to understand cellular uptake mechanisms for PUFA delivered as FFA or within lipoprotein remnants in ruminant liver tissue and to clarify the differences between PUFA preference of ruminant liver and mammary gland. This understanding would allow more focus and enlighten exploration of the impact of PUFA delivered as mobilized FFA or dietary TG on activation of PPAR-α in liver. These studies could be conducted both *in vitro,* using isolated hepatocytes and primary mammary epithelial cells, or *in vivo,* via intravenous infusion and subsequent liver and mammary tissue biopsy.

Cellular uptake of FA occurs through multiple mechanisms and FFA uptake across the plasma membrane is facilitated by transport proteins, such as CD36 and fatty acid transport protein. FABP are cytoplasmic proteins that accelerate LCFA uptake by increasing solubility in the cytosol. FABP are hypothesized to target LCFA to specific cellular fates, such as TG esterification at the ER. Though FABP3 causes defective FAO in cardiac and skeletal muscle, no clear role of FABP3 in mammary gland lipid metabolism is apparent. Expression of FABP3 mRNA during lactation is consistent with elevated levels of preformed LCFA uptake in early lactation, but FABP3 does not appear to be rate-limiting for fatty acid uptake *in vitro* and overexpression did not alter lipid metabolism in MAC-T cells.

In mid-lactation dairy cattle, FABP3 and FABP4 expression were altered by diets supplemented with saturated fat or a PUFA blend (Invernizzi et al., 2010). Kadegowda et al. (2009) suggested activation of PPAR-γ by palmitate could be adipogenic in the mammary gland and demonstrated an increase in FABP3 and FABP4 in MAC-T cells treated with rosiglitazone, a PPAR-γ agonist, and palmitate that was reversed with *trans*-10, *cis*-12 CLA. Deiuliis et al. (2010) noted an adipogenic response to flaxseed supplementation in beef cattle, observing increased FABP4 mRNA expression through PPAR-γ activation and TG accumulation in bovine muscle. These data suggest n-3 PUFA may play a role in expression of key proteins involved in lipid trafficking. FABP responses to increased dietary lipid also depend upon FA composition.

While FABP expression can be modified by dietary FA delivered as VLDL, it is unclear whether FFA mobilized from adipose tissue initiate a regulatory response. It is clear that lactating cattle in negative energy balance exhibit ER stress in spleen and mammary tissue (Morris et al., 2009, Invernizzi et al., 2012) and there are significant interrelationships in nutrition and disease in the periparturient dairy cow (Goff, 2006). FABP3 is an early marker of

myocardial infarction, and it is possibly also a marker of increased ER activity and triglyceride esterification in the mammary gland, given the tremendous efficiency of the modern dairy cow.

Understanding of the impact of ER stress on the physiological changes associated with lactation may help identify methods to increase milk component yield. FA-specific lipid transport and intracellular metabolism may allow targeted delivery of n-3 PUFA to benefit the health of lactating dairy cattle by altering expression of key genes in lipid metabolism.

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Appendix A

JOURNAL OF DAIRY SCIENCE

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June 25, 2012

Jennifer A. Stamey
Department of Dairy Science
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Stamey, J. A., D. M. Shepherd, M.J. de Veth, and B. A. Corl. Use of n-3 fatty acid rich algae and their oil as a feed supplement for dairy cattle. J. Dairy Sci. doi:10.3168/jds.2012-5412.

Sincerely,

Susan Pollock
Managing Editor
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