EFFECTS OF OMEGA-3 FATTY ACIDS ON MILK PRODUCTION RESPONSES AND MILK AND PLASMA FATTY ACIDS IN DAIRY COWS

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Animal Science – Master of Science

2024

ABSTRACT

The major bioactive omega-3 (n-3) fatty acids (FA) are eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3). These 2 FA are derived from desaturation and elongation of the essential FA alpha-linolenic acid (ALA; C18:3 n-3). Low conversion rates of ALA into EPA and DHA indicate that dairy cows may benefit from direct supplementation of these conditionally essential FA, especially during periods of prolonged inflammation such as the transition period (Pawlosky et al., 2001; dos Santos Neto et al., 2024; Bradford et al., 2015). The first experiment in this thesis evaluated the effect of increasing abomasal infusion doses of DHA on milk and plasma FA as well as milk production responses. Correlations between DHA absorption and milk and plasma DHA were calculated to determine potential biomarkers of DHA absorption. Abomasal infusion of DHA linearly reduced milk somatic cell count (SCC) and increased DHA in milk fat as well as total plasma lipids, phospholipids (PL), triacylglycerols (TAG), and cholesterol esters (CE). DHA absorption was positively correlated with DHA in total plasma lipids, PL, TAG, and milk fat. The second experiment in this thesis evaluated the effect of feeding a calcium salt enriched in EPA and DHA to dairy cows during the 3 weeks before and 3 weeks after parturition on milk production, plasma metabolites, and milk FA during the first 3 weeks of lactation. Carryover effects of n-3 FA on milk production were also evaluated for 6 weeks post-supplementation. The diet containing EPA and DHA reduced milk fat yield for the first 5 weeks of lactation and tended to reduce yields of 3.5% fat-corrected milk (FCM) and energy-corrected milk (ECM). Supplementing EPA and DHA also reduced yields of de novo and mixed FA in milk. The results from our two studies will help to inform dairy farmers, nutritionists, and researchers on strategies to feed EPA and DHA and estimate absorption of these bioactive FA in dairy cows.

This thesis is dedicated to the educators, friends, and family members who encouraged me to attend graduate school. Thank you for all your support and for believing in me.

ACKNOWLEDGEMENTS

I would first like to acknowledge my advisor Dr. Adam Lock. If he hadn't accepted me to work in his lab, I would not have had the opportunity to complete these research projects and write this Master's thesis. I am very appreciative of his feedback, support, and mentoring during my time here at MSU.

I would also like to thank my committee members: Dr. Barry Bradford, Dr. Andres Contreras, and Dr. Miriam Weber. They have all been incredibly important to helping me grow as a graduate student and researcher. Dr. Bradford and Dr. Contreras gave me the opportunity to collaborate with them and their students on planning and executing these projects while Dr. Weber gave me the opportunity to guest lecture in one of her classes to help me grow my teaching skills.

I cannot write this acknowledgement section without mentioning the contributions of our lab manager Lynn Worden. She was so helpful in organizing my studies and data, training me in the lab work, and making sure that we did everything we were supposed to do. I truly would not have gotten through these projects without her support.

I would also like to acknowledge my other lab mates here. I would like to thank Alycia Bales for being so welcoming to me and including me in our lab social events. She also was very helpful in providing me with lots of spreadsheets, inspiration for my presentations, and expertise on running infusion studies. I also want to thank Jair Esteban Parales-Giron for all his help and training in running transition cow studies and for his patience in dealing with all my questions and struggles. I want to acknowledge Dr. Jose Mauricio dos Santos Neto for all his help with statistical analysis and writing about omega-3 fatty acids. I would also like to thank all the other members of the Lock lab past and present for their help with lab analysis and farm work

including Alex Benoit, Viviane Coelho, and the undergraduate students. I am so grateful to have been part of a lab group that is so supportive of one another, and I have enjoyed our many laughs and “off-topic” lab meetings.

I would also like to thank the other graduate students in Anthony Hall. First and foremost, I would like to thank Haylee Reisinger for all her help in designing and running these studies. I am so grateful to have had someone to collaborate with on these studies, and I’m not sure how I would’ve managed on my own. I would also like to acknowledge Sarah Naughton, Morgan Mills, Joe Fehn, Izzy Bernstein, Erika Eckhardt, and the other students from 2240 and 2280 for providing some much-needed distractions from work whenever I needed to take a break.

I would like to thank all the staff at the MSU Dairy farm for all their patience in dealing with me and feeding my cows. They were all so helpful and supportive, and I appreciate their hard work and care for the research projects that take place there.

I must also thank my friends and family for all their encouragement and moral support throughout the ups and downs of grad school. I enjoyed talks on the phone and occasional weekend getaways with my parents Michael and Emily Miller, my siblings Jay and Kortlynn Miller, and my best friends Moriah Quillin, Lauren Lucas, and Leah Roth.

Finally, I would like to thank my fiancé and future husband Jonathan Hoorman for keeping me grounded, believing in me even when I didn’t believe in myself, and for being there for me through every minute of my grad school experience. It hasn’t been easy to be long-distance and to be working late nights, early mornings, and on weekends, but I appreciate the patience and unconditional love and support. I am so grateful for each and every person who made this thesis possible, and I am beyond blessed to have them in my life.

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LIST OF ABBREVIATIONS

ALA	Alpha-linolenic acid
ARA	Arachidonic acid
BCS	Body condition score
BHB	Beta-hydroxybutyrate
BW	Body weight
CE	Cholesterol ester
CLA	Conjugated linoleic acid
CP	Crude protein
DHA	Docosahexaenoic acid
DM	Dry matter
DMI	Dry matter intake
DPA	Docosapentaenoic acid
ECM	Energy-corrected milk
EPA	Eicosapentaenoic acid
FA	Fatty acid
FCM	3.5% fat-corrected milk
HDL	High-density lipoprotein
LA	Linoleic acid
LCAT	Lecithin cholesteryl ester acyl transferase
LCPUFA	Long-chain polyunsaturated fatty acid
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide

MFD	Milk fat depression
n-3	Omega-3
n-6	Omega-6
n-9	Omega-9
NDF	Neutral detergent fiber
NEFA	Nonesterified fatty acid
$PGF2_a$	Prostaglandin F_{2a}
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
SCC	Somatic cell count
SFA	Saturated fatty acid
SPE	Solid phase extraction
TAG	Triacylglycerol
TFA	Total fatty acids
UFA	Unsaturated fatty acid
VLDL	Very low-density lipoprotein

CHAPTER 1

INTRODUCTION

Fatty acids (FA) are nutrients utilized in cell membrane structure, energy production and storage, and cell signaling (Nelson et al., 2021). Each individual FA is comprised of a carboxylic acid head group and a hydrocarbon chain that can vary in length. Saturated FA (SFA) have no double bonds in the hydrocarbon chain, and unsaturated FA (UFA) have one or more double bonds (Nelson et al., 2021). In mammals, there are 2 essential FA that cannot be synthesized by the body and must be supplied in the diet. These are the omega-6 (n-6) FA linoleic acid (LA; 18:2 n-6) and the omega-3 (n-3) FA alpha-linolenic acid (ALA; 18:3 n-3) (Cook & McMaster, 2002). The dietary ratio of n-6:n-3 is of particular interest since reducing this ratio has been linked to reducing the risk and pathogenesis of cardiovascular disease, cancer, and inflammatory diseases in humans (Simopoulos, 2002).

ALA is converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are more bioactive than ALA and exhibit anti-inflammatory effects (Palmquist, 2009). The synthesis of these longer-chain n-3 FA from ALA is inefficient in humans as only ~0.2% of plasma ALA is utilized for the synthesis of EPA (Pawlosky et al., 2001). In dairy cows, abomasal infusion of ALA for 20 d did not increase plasma content of DHA (dos Santos Neto et al., 2024). This may indicate a need to directly supplement EPA and/or DHA to dairy cows. Since EPA and DHA are anti-inflammatory, there is interest in feeding these n-3 FA to dairy cows during the transition from gestation to lactation. Dairy cows experience extensive inflammation during the transition period, which has been linked to increased disease risk and decreased whole-lactation milk production (Bradford et al., 2015). Several studies have indicated that feeding n-3 FA to

dairy cows during early lactation may be beneficial for improving health, reproduction, and milk production (Greco et al., 2015; Sinedino et al., 2017; France et al., 2022).

The primary challenge of feeding EPA and DHA is delivery of these FA to the small intestine for absorption. Unsaturated FA such as EPA and DHA are toxic to rumen microbes (Maia et al., 2007) and rumen bacteria biohydrogenate them into either *trans* unsaturated FA or saturated FA (Palmquist, 2009). This makes it difficult to determine how much n-3 FA are available for absorption by the cow. Therefore, it is hard to determine the impact of specific amounts of n-3 FA on dairy cow metabolism and health. To the best of our knowledge, there have been no studies that have analyzed the effect of increasing abomasal infusion doses of DHA in dairy cows. Previous studies that abomasally infused EPA and/or DHA used non-physiological doses (Rico et al., 2021; Casteñeda-Gutiérrez et al., 2007). Therefore, the objective of Chapter 3 is to evaluate the effect of increasing abomasal infusion doses of DHA in dairy cows on plasma FA, milk FA, and production responses and to determine potential biomarkers for DHA absorption.

Despite the potential for feeding EPA and DHA to dairy cows during the transition period, no studies have fed EPA and DHA during the 3 weeks before and 3 weeks after calving and studied subsequent carryover effects into peak-lactation post-supplementation. In Chapter 4, we fed a calcium salt enriched in EPA and DHA to dairy cows during the 3 weeks before and 3 weeks after calving to evaluate effects on milk production, plasma metabolites, milk FA, and carryover milk production. Our results will help researchers better understand how n-3 FA are transported and utilized in the dairy cow so that well-informed decisions can be made regarding the nutrition of transition dairy cows to support their health and productivity.

CHAPTER 2

LITERATURE REVIEW

Introduction: Essential Fatty Acids

Fatty acids (FA) are important biomolecules that play key roles in cell membrane development, energy production and storage, synthesis of other biomolecules, and cell signaling. FA are typically named and classified by carbon chain length, number of double bonds, and location of their double bonds (Nelson et al., 2021). Table 2.1 describes important FA in biological systems along with their common names and biochemical and nutritional nomenclature.

Of particular interest in nutrition are polyunsaturated fatty acids (PUFA) that contain multiple double bonds in their hydrocarbon chain. Mammals cannot synthesize all of these FA *de novo*, so they are required in the diet and are considered to be essential. The 2 essential FA are linoleic acid (LA; 18:2 n-6) and alpha-linolenic acid (ALA; 18:3 n-3) (Cook & McMaster, 2002). The absolute requirement of these essential FA was first reported in the 1930's when rats were fed diets with or without LA and ALA (Burr and Burr 1930; Burr et al., 1932). LA is an omega-6 (n-6) FA, which means its first double bond on the hydrocarbon chain is 6 carbons away from the methyl end. ALA is an omega-3 (n-3) FA, which means its first double bond is 3 carbons away from the methyl end.

LA and ALA are further desaturated and elongated in the body to produce long chain polyunsaturated fatty acids (LCPUFA); (Cook & McMaster, 2002). Of these LCPUFA, eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) are of particular interest since they are known to have potent biological effects on human health including the reduction of cardiovascular disease, inhibition of carcinogenesis, and the mediation

of chronic inflammation (Palmquist, 2009). EPA and DHA are considered to be conditionally essential since production of these FA may be inadequate under certain conditions (Strijbosch et al., 2012).

Research in the field of human nutrition and medicine has led to interest in investigating EPA and DHA supplementation in dairy cow diets to improve reproduction, mediate inflammatory challenges to improve overall health, and increase yield of milk and milk components. This literature review will explain how EPA and DHA can be provided in the diet, the role of EPA and DHA in the physiology of the dairy cow, the challenges of feeding them, and potential benefits of n-3 supplementation to dairy cows.

Feed Sources of EPA and DHA

The only feed sources that are abundant in EPA and DHA are fish oil or algal oil. Fish oil has been the most frequently investigated source of EPA and DHA in dairy cow diets. Marine fish are an excellent source of n-3 FA, although fish species and processing methods differentially affect fish oil FA profile (Merkle et al., 2017).

Fish meal can also be fed as a source of EPA and DHA. In addition to supplying essential FA, fish meal provides high amounts of rumen undegraded protein and limiting amino acids such as lysine and methionine (NASEM, 2021). One study using early lactation dairy cows supplied ~14 g/d of EPA and 9 g/d of DHA when 5% fish meal was fed in the diet (Heravi Moussavi et al., 2007). Although positive effects have been observed when feeding fish meal to cows, this by-product is seldom used in dairy rations due to its inclusion in pet foods and aquaculture (NASEM, 2021).

In recent years, there has been interest in providing microalgae or seaweed to livestock as an alternative vegetarian source of n-3 FA. Fish do not synthesize n-3. Instead, they obtain these

essential FA by consuming microalgae or other fish. Algal oil is rich in DHA, but it contains minimal EPA (Stamey et al., 2012). Research has shown that feeding microalgae and seaweed to ruminants can provide multiple benefits, but inclusion of these ingredients in feed is uncommon (Makkar et al., 2016; Madeira et al., 2017). Examining effects of DHA on dairy cow physiology is an active area of research interest that could increase focus on algae supplements in dairy cow diets.

Rumen Biohydrogenation and Protection of PUFA

PUFA are toxic to rumen microbes (Maia et al., 2007). To combat the large amount of PUFA in the diet, rumen bacteria biohydrogenate unsaturated fatty acids (UFA) into either *trans* UFA or saturated fatty acids (SFA) (Palmquist, 2009). It is estimated that over 80% of all dietary UFA are either fully or partially biohydrogenated (Palmquist, 2009). This presents a challenge when feeding EPA and DHA to dairy cattle because rumen bypass of these FA is limited. Most dietary LCPUFA are either partially or fully biohydrogenated in the rumen. A meta-analysis by Jenkins and Bridges (2007) suggested that increasing unsaturation increases biohydrogenation. The ruminal disappearance of EPA and DHA happens very fast. A study by Abu Ghazaleh et al. (2002) fed fish oil and examined the rumen digesta of cows. They observed that 73% of the EPA and 77% of the DHA disappeared by 3 hours post-feeding when cows were supplied with 2% fish oil (% diet DM) and observed similar results with 1% fish oil in the diet (Abu Ghazaleh et al., 2002). Doreau and Chilliard (1997) sampled the duodenal contents of cows that were ruminally infused with fish oil and results indicated that over 90% of the EPA and DHA were biohydrogenated in the rumen.

Due to the interest in increasing post-rumen delivery of EPA and DHA, rumen protection technologies have been investigated. The most common commercially available rumen-protected

product that provides EPA and DHA are calcium salts of fish oil. There is limited research on the efficacy of calcium salts protecting EPA and DHA from rumen biohydrogenation. Castañeda-Gutiérrez et al. (2007) observed no differences in n-3 plasma or milk FA content from cows fed calcium salts of fish oil compared to a ruminal infusion of fish oil. However, the calcium salt's slower release of the n-3 FA reduced negative effects in the rumen that were observed with the ruminal infusion of fish oil (Castañeda-Gutiérrez et al., 2007). Other rumen-protection technologies for PUFA are either under development or are not commercially available (Jenkins & Bridges, 2007).

Biohydrogenation makes it difficult to study the effects of EPA and DHA since the delivery of these FA to the small intestine is uncertain in feeding scenarios. Abomasal infusion of these FA can be used to study the effects of known amounts of UFA on the animal. Most of these studies used fish oil (Castañeda-Gutiérrez et al., 2007) or high-ALA flaxseed oil (dos Santos Neto et al., 2024) although one study used algal oil (Rico et al., 2021). More research is needed in this area to better understand the effects of n-3 FA, especially DHA, since there is limited research with this particular FA in dairy cows. In Chapter 3, we delivered known amounts of DHA past the rumen to measure physiological effects on dairy cows.

Absorption and Transport of n-3 FA

The majority of FA are absorbed in the small intestine as nonesterified FA (NEFA) (Noble, 1981). Digestibility of FA varies by degree of saturation and chain length (Pantoja et al., 1996; Boerman et al., 2015), but digestibility of LCPUFA like DHA remains largely unstudied in ruminants. Absorbed FA are re-esterified into triacylglycerols (TAG) and transported in chylomicrons or very low-density lipoproteins (VLDL) (Palmquist, 2009). These FA flow through the lymph and are eventually delivered into the blood (Noble, 1981). Most plasma FA

are transported within lipoprotein particles that include chylomicrons, VLDL, low density lipoproteins (LDL), or high density lipoproteins (HDL). EPA and DHA are mostly transported in high density lipoproteins (HDL) (Offer et al., 2001).

There are four plasma lipid classes present in the blood: cholesterol esters (CE), phospholipids (PL), TAG, and NEFA. These classes are described in more detail in Table 2.2. FA are incorporated differently into each plasma lipid class (Tyburczy et al., 2007; Figure 2.1). DHA is preferentially incorporated into PL while EPA is more commonly incorporated into CE although enrichment of both fractions has been observed with fish oil supplementation (Urrutia et al., 2023; Offer et al., 2001). There is minimal incorporation of these FA in TAG or NEFA (Urrutia et al., 2023). These factors limit uptake of these preformed FA into the mammary gland which obtains most FA from TAG (Moore & Christie, 1981). Future research examining relationships between n-3 intake and enrichment in plasma FA fractions in dairy cows is warranted. Chapter 3 will explore the effects of abomasal infusion of DHA on n-3 enrichment in each plasma lipid class.

Incorporation of n-3 FA into Tissues and Cells

FA are transferred from plasma and incorporated into cell membranes, where hydrocarbon chain length and number of double bonds influence cell membrane fluidity, permeability, and stability (Cook & McMaster, 2002). Therefore, DHA with 6 double bonds is highly prevalent in fluid tissues such as the brain and retina (Arterburn et al., 2006). Bilby et al. (2006) analyzed the FA profile of various tissues of dairy cows and both EPA and DHA were present in the liver, muscle, endometrium, and mammary gland in small amounts. EPA was not detected in adipose tissue, but DHA was found in internal adipose tissue at low levels. Fish oil supplementation increased the incorporation of EPA and DHA into most of these tissues (Bilby et

al., 2006). The low incorporation of EPA and DHA into adipose tissue suggests limited storage of these FA which may indicate a need for dietary supplementation (Arterburn et al., 2006).

Both EPA and DHA are also incorporated into the phospholipid membrane of immune cells, where they play a role in regulating gene expression and modification of protein structure and function (Calder, 2008). In humans, enrichment can occur as soon as 1 day after supplementation if the dose is high enough (Faber et al., 2011). Silvestre et al. (2011b) observed a 5-fold enrichment of EPA and 15-fold enrichment of DHA in neutrophils in cows fed a calcium salt of fish oil for 50 days compared to control cows fed a calcium salt of palm oil.

Conversion of ALA into EPA and DHA

ALA is the most abundant n-3 FA in dairy cow diets, comprising 25-40% of total FA content in most forages (NASEM, 2021). Certain oilseeds such as flaxseed also supply ALA. Chain elongation of FA takes place in the endoplasmic reticulum and mitochondria (Cook & McMaster, 2002). This conversion pathway is outlined in Figure 2.2. The rate-limiting step is the conversion of ALA into stearidonic acid (SDA; 18:4n-3) and is accomplished by the delta-6 desaturase enzyme, which incorporates a new double bond into the hydrocarbon chain (Palmquist, 2009). SDA is further elongated into eicosatetranoic acid (20:4n-3) and is then desaturated by delta-5 desaturase into EPA. Further elongation leads to the production of docosapentanoic acid (DPA; 22:5n-3). Two carbons are added to DPA, and delta-6 desaturase then adds another double bond to the hydrocarbon chain. Finally, the FA is transferred to the peroxisome where beta-oxidation takes place to remove two carbons to yield DHA (Calder, 2013). The rate of this conversion can be influenced by the n-6:n-3 ratio since both LA and ALA compete for the same delta-6 desaturase enzyme (Cook & McMaster, 2002).

As a result of this mechanism, conversion rates of ALA into EPA and DHA are extremely low. Conversion of ALA into DHA in humans is estimated to be less than 1% (Arterburn et al., 2006; Pawlosky et al., 2001). A meta-regression by Arterburn et al. (2006) found that ALA supplementation did not increase plasma phospholipid levels of DHA in humans. These results agree with research conducted in dairy cows where short-term abomasal infusions of ALA from flaxseed oil increased plasma EPA but not DHA, indicating that cows are likely no more efficient than humans when it comes to n-3 conversion (Moallem et al., 2012a; dos Santos Neto et al., 2024).

Although ALA supplementation did not increase plasma levels of DHA in the aforementioned studies, long-term supplementation with ALA may accomplish this goal. Gnott et al. (2020) abomasally infused cows with ALA from flaxseed oil from 9 weeks prepartum to 9 weeks postpartum and analyzed effects on plasma FA. Interestingly, plasma DHA content was increased by the ALA treatment after 9 weeks of supplementation and 1 d after calving, but plasma DHA levels were lower at 28 d postpartum. These results suggest that cows may be more efficient in DHA conversion around calving (Gnott et al., 2020).

Retroconversion of DHA back to EPA also occurs and has been estimated to be ~9% in humans (Conquer & Holub, 1997). A study with dairy cows replaced fish oil in the diet with algae at increasing levels and reported no differences in milk EPA or DHA between the different treatments (Abughazaleh et al., 2009). This confirms that cows are able to convert DHA into EPA and that algae products rich in DHA may be a sustainable vegetarian option to supply long-chain n-3 FA. Due to increased interest in algae as a supplement, more research focusing specifically on DHA is needed in dairy cows. In Chapter 3, we utilize an algae-derived source of DHA.

Biosynthesis of PUFA Derivatives

The n-3 FA, especially EPA and DHA, play important roles in the immune system, which are summarized in Table 2.3. Of particular interest are the synthesis of bioactive lipid mediators for which both n-6 and n-3 FA are precursors (Calder, 2008). These lipid mediators are oxidized byproducts known as oxylipids and are essential in the regulation of the inflammatory response (Contreras et al., 2017). Each oxylipid plays a unique role in the inflammatory process, but their functions are often interrelated and difficult to define (Putman et al., 2022). Figure 2.3 outlines PUFA within phospholipids and summarizes key oxylipid derivatives and their primary functions.

Both LA and arachidonic acid (ARA; 20:4 n-6) are precursors in the production of primarily pro-inflammatory oxylipids, and are the most common FA substrates for oxylipid synthesis (Contreras et al., 2017). During immune activation or lipolysis, FA are released from cellular phospholipids in a reaction catabolized by phospholipase and are enzymatically oxidized through the lipoxygenase pathway, the cyclooxygenase pathway, or the cytochrome P450 pathway (Mavangira & Sordillo, 2013). Inflammatory oxylipids derived from ARA include prostaglandins, thromboxanes, and leukotrienes (Calder, 2012). Although most derivatives of n-6 are considered to be pro-inflammatory, PGE_2 has been shown to have both pro- and anti-inflammatory actions (Calder, 2009). ARA is also a precursor to anti-inflammatory lipoxins (Levy et al., 2001).

During resolution of inflammation, cells begin to produce more anti-inflammatory oxylipids. EPA and DHA are precursors to the production of resolvins, protectins, and maresins (Spite et al., 2014). Resolvins produced from EPA are known as the E-series resolvins, while D-series resolvins are produced from DHA (Spite et al., 2014). These lipid mediators resolve

inflammation and stimulate tissue regeneration (Spite et al., 2014). Enrichment of DHA into phospholipids is of particular interest because there are more oxylipid products produced from DHA than from EPA (Raphael & Sordillo, 2013). Increasing the content of n-3 FA in the phospholipids of cell membranes may contribute to faster resolution of inflammation due to increased production of pro-resolving oxylipids (Calder, 2008).

Effects of EPA and DHA on Inflammation in Cows

An area of growing interest in the dairy industry are strategies to modulate inflammation and improve cow health, especially during the transition period, which is defined as the 3 weeks before and 3 weeks after parturition (Drackley, 1999). Inflammation is a healthy and normal response after parturition, but fast resolution of inflammation is key to ensure a successful transition (Bradford et al., 2015). More than 50% of cows in the fresh period suffer from a subclinical disorder, and strong links have been found between a chronic, heightened inflammatory response and early-lactation metabolic disorders (Bradford et al., 2015; Sordillo & Raphael, 2013). Unresolved inflammation also contributes to reduced DMI, fertility, and productivity (Bradford et al., 2015). Therefore, mitigating inflammation in the early postpartum period may improve cow health as well as other outcomes.

Both EPA and DHA are known to have anti-inflammatory effects. An in vitro study by Contreras et al. (2012) demonstrated that supplementation with n-3 FA altered the FA composition of bovine endothelial cells and reduced the expression of pro-inflammatory cytokines, adhesion molecules, and reactive oxygen species. The cell cultures supplemented with n-3 FA also had increased synthesis of anti-inflammatory eicosanoids (Contreras et al., 2012).

There are limited in vivo studies examining the effects of supplementing EPA and DHA to transition dairy cows on immune function and inflammation. Greco et al. (2015) conducted an

intramammary lipopolysaccharide (LPS) infusion and observed an attenuated immune response in cows fed higher levels of n-3 FA. Another study observed that n-3 FA supplemented cows spent less time in a fevered state post-LPS infusion, indicating a faster resolution of the inflammatory response (Winters, 2023). An intravenous infusion of fish oil administered to cows 12, 24, and 48 hours post-calving reduced plasma haptoglobin and increased DMI and milk yield in the first week after calving (Mezzetti et al., 2022).

Heat stress is another concern in the dairy industry where systemic inflammation plays a key role and contributes to the adverse outcomes observed during heat stress events (Most & Yates, 2021). Ruiz-Gonzalez et al. (2022) abomasally infused fish oil to heat-stressed lactating dairy cows and observed that the n-3 supplemented cows experienced a 2 kg/d recovery in milk yield compared to heat-stressed cows supplemented with corn oil. The n-3 FA supplementation also reduced rectal temperature by 1°C, reduced LPS binding protein, and increased anti-inflammatory oxylipid intermediates in plasma (Ruiz-Gonzalez et al., 2022).

Effects of EPA and DHA on Dry Matter Intake and Milk Yield

Production responses to the supplementation of EPA and DHA have been highly variable. These various effects are likely explained by the amount supplemented, the form of the supplement, diet composition, stage of lactation, and production level of cows. Most positive effects of EPA and DHA on milk yield have been observed when fed during early lactation. Some of these early lactation studies are described in Table 2.4. Greco et al. (2015) fed calcium salts of fish oil, palm oil, and safflower oil to achieve differing dietary ratios of n-6:n-3 from 14 days postpartum. Cows fed higher levels of EPA and DHA with a lower n-6:n-3 ratio had a higher DMI and higher peak milk yield (Greco et al., 2015). A similar result for milk yield was observed when cows were fed 10 g/d of DHA from an algae supplement from 14-120 DIM

(Sinedino et al., 2017) and 44 g/d of DHA from 3 weeks prepartum to 84 DIM (Hostens et al., 2011). A recent study feeding a calcium salt enriched in EPA and DHA during the transition period observed that the n-3 supplement increased yields of energy corrected milk (ECM) in the first 4 weeks postpartum (France & McFadden, 2022). More research is needed to better understand how feeding n-3 FA influences production responses in early lactation, particularly around calving, as most of these studies did not supplement n-3 FA during this critical time period. These studies also did not examine carryover effects of supplementing n-3 FA. In Chapter 4, we study n-3 FA supplementation in cows during the close-up and fresh period and follow cows until peak lactation to examine potential carryover effects.

Feeding unprotected fish oil at over 1% diet DM has resulted in negative effects on DMI (Cant et al., 1997; Whitlock et al., 2002; AbuGhazaleh et al., 2002). A dose-response study of dietary fish oil inclusion by Donovan et al. (2000) showed that feeding fish oil at 1% of the diet did not reduce DMI and increased milk yield compared to the control diet. Both DMI and milk yield were reduced when fish oil was fed at 2% and 3% diet DM (Donovan et al., 2000). The effects of fish oil on DMI are likely a combination of decreased diet palatability (Donovan et al., 2000) and increased gut satiety peptides that are often observed when higher levels of UFA are fed (Relling & Reynolds, 2007).

Fish oil supplementation interacts with other dietary factors including starch and total PUFA to affect milk production results. Pirondini et al. (2015) fed diets containing 0.8% fish oil to mid-lactation cows. Fish oil inclusion interacted with dietary starch level as fish oil increased ECM in low-starch (24% diet DM) diets, but not in high-starch diets (28% diet DM) (Pirondini et al., 2015). Peravian et al. (2022) looked at the interaction between n-3 supplementation and dietary PUFA concentration. When a calcium salt of fish oil was fed at 0.56% diet DM, they

observed that n-3 supplementation reduced milk yield when higher levels of PUFA (2.67% diet DM) were fed compared to lower levels of PUFA (2.12% diet DM; Peravian et al., 2022).

When EPA and DHA positively affect milk yield, it is possible this is an indirect effect due to the anti-inflammatory nature of n-3 FA. Activation of the immune system utilizes a lot of nutrients and energy, and inflammation redirects nutrient partitioning away from milk production (Bradford et al., 2015). Lochmiller and Deerenberg (2000) proposed that immune activation increases energetic costs from 10-40% as a percentage of basal metabolic rate. Kvidera et al. (2017) estimated that the activated immune system of a dairy cow utilizes over 1 kg of glucose within 12 hours. Therefore, mitigating a chronic inflammatory state may restore nutrient partitioning to milk production (Bradford et al., 2015). More research is needed to understand how feeding n-3 impacts cows during the transition period. Chapter 4 will explore the effects of feeding a FA supplement enriched in EPA and DHA to dairy cows during the transition period.

Effects of EPA and DHA on Milk Fat

Effects of EPA and DHA on milk fat in early lactation cows are summarized in Table 2.4. Milk fat depression (MFD) is one of the most common concerns when EPA and DHA are fed. Supplying EPA and DHA can alter biohydrogenation pathways of 18-carbon FA. Donovan et al. (2000) observed that feeding increasing levels of fish oil resulted in a linear reduction in stearic acid (C18:0) and a linear increase in *trans* C18:1 and C18:2 isomers in milk fat. Further *in vitro* work has also observed that both EPA and DHA inhibit the biohydrogenation of LA and ALA to stearic acid and increase biohydrogenation intermediates in both cow and ewe rumen cultures (Toral et al., 2017). Among these biohydrogenation intermediates produced is *trans-10, cis-12* conjugated linoleic acid (CLA). This isomer induces MFD by inhibiting de novo milk fat synthesis and preformed FA uptake (Baumgard et al., 2000; Harvatine & Bauman, 2006).

Although MFD is a risk when feeding EPA and DHA, this may be reduced by feeding a rumen-protected form, by providing adequate levels of fiber, and by limiting the amount of LCPUFA fed. Reducing the negative effects of n-3 on milk fat yield, should continue to be a major focus of n-3 FA research in dairy cows.

To reduce the risk of MFD, EPA and DHA can be fed in the form of a calcium salt of fish oil. First developed at The Ohio State University in the 1980's, calcium salts have been fed to protect UFA and avoid fermentation problems that often occur when feeding additional PUFA (Jenkins & Bridges, 2007). Developed to be insoluble at rumen pH, calcium salts dissociate at the low pH in the abomasum to make FA available for absorption and improve fiber digestibility compared to unprotected UFA (Jenkins & Palmquist, 1984). Castañeda-Gutierrez (2007) observed that feeding calcium salts of fish oil did not negatively affect DMI or milk fat yield, providing rumen inertness not observed with ruminal infusions of fish oil. Feeding calcium salts of fish oil also maintained similar milk fat and plasma concentrations of stearic acid compared to an abomasal infusion of fish oil, indicating that biohydrogenation pathways of 18-carbon FA were not inhibited or altered. Despite this positive aspect, the calcium salt treatments did not deliver any more n-3 past the rumen as indicated by plasma and milk n-3 concentrations (Castañeda-Gutiérrez et al., 2007).

Extensive research has been conducted using EPA and DHA to increase milk fat content of n-3 FA for human consumption, however, there has been little success due to a transfer efficiency of less than 4% (Lock & Bauman, 2004). This is due to high rates of biohydrogenation as well as low incorporation into plasma TAG, which is the main circulating lipid source of preformed FA for the mammary gland (Lock & Bauman, 2004). Feeding LCPUFA does typically alter biohydrogenation pathways in the rumen, which leads to increased secretion of CLA in milk

fat (Donovan et al., 2000). Among these include the *cis-9, trans-11* CLA, which has been shown to have anticarcinogenic and atherogenic effects (Lock & Bauman, 2004).

Effects of EPA and DHA on Reproduction

PUFA are essential for various reproductive functions, and n-3 FA can be fed to improve outcomes (Palmquist, 2009). Sinedino et al. (2017) observed that feeding an algae product as a source of DHA improved pregnancy per artificial insemination (AI) on days 32 and 60 post-AI. They also reported that feeding algae nearly doubled the rate of pregnancy within primiparous cows and tended to increase the rate of pregnancy in multiparous cows (Sinedino et al., 2017). Another study fed a calcium salt of fish oil from 30-160 DIM and observed an overall reduction in pregnancy loss in cows fed fish oil (Silvestre et al., 2011a).

There are several theories as to how n-3 FA supplementation improves reproduction, but a likely mechanism is through altering prostaglandin synthesis. Feeding fish meal has been shown to reduce plasma and uterine prostaglandin F_{2a} (PGF_{2a}) and feeding fish oil increased EPA and DHA in the endometrium of dairy cows (Mattos et al., 2004; Bilby et al., 2006). PGF_{2a} is secreted by the uterus to induce luteolysis. Maternal recognition of the embryo must occur before luteolysis since the corpus luteum is essential for the production of progesterone and continuation of pregnancy (Senger, 2012). A reduction in PGF_{2a} gives the embryo more time to notify the uterus of its presence. There is also evidence to suggest that feeding n-3 FA improves oocyte quality and folliculogenesis although the influence on follicular dynamics is inconsistent and not well understood (Moallem et al., 2013; Moallem, 2018). One theory is that increasing the incorporation of n-3 FA into cell membranes increases membrane fluidity which may improve transfer of nutrients into the cell (Moallem et al., 2013). Oocyte and embryo growth and development rates were enhanced in an *in vitro* study when only 1 μM of DHA was

supplemented, but 100 μM of DHA negatively affected these parameters, indicating a dose-effect (Oseikria et al., 2016).

There is also evidence that EPA and DHA impact reproduction in bulls. DHA comprises around 20% of FA in sperm, and supplementation of fish oil increased concentrations of DHA after 5 weeks of feeding. Supplementation with fish oil, however, negatively impacted sperm motility and morphology compared to bulls fed flaxseed oil (Moallem et al., 2015).

Benefits of EPA and DHA Supplementation During Gestation

DHA is critically important for fetal neurodevelopment during the last trimester of pregnancy, and there is likely an increased requirement for EPA and DHA during pregnancy (Greenburg et al., 2008). EPA and DHA supplementation during gestation may also have epigenetic effects on offspring (Heberden & Maximin, 2017). Although most fetal programming studies with n-3 FA were performed in rodent models, there have been a few studies in ruminants examining the effect of n-3 supplementation during late gestation on offspring. In one study in beef cattle, cows were supplemented with either a control diet rich in palmitic and oleic acid, or a diet enriched in n-6 and n-3 FA during the last 50 days in gestation. Calves born to n-3 and n-6 supplemented cows tended to have improved growth and carcass characteristics (Marques et al., 2017). Another study fed EPA and DHA to sheep in late gestation and observed that maternal supplementation altered gene expression in the hypothalamus of lambs, which may have led to increased growth rate in the offspring (Martin et al., 2018).

One study in late gestation dairy cows observed that calves born to cows fed fish oil had greater plasma DHA concentrations compared to control and flaxseed-fed cows although the results suggested a low placental transfer efficiency of most long-chain FA (Moallem & Zachut, 2012). Another study in dairy cattle observed that n-3 or n-6 FA supplementation to cows during

late gestation increased IgG concentrations in colostrum, and calves born to cows fed fat experienced greater average daily gain than the control group (Jolazadeh et al., 2019a). The cows fed fat also experienced benefits with higher milk yield, lower blood NEFA, and reduced incidence of health disorders, while the n-3 supplement reduced somatic cell count (SCC) (Jolazadeh et al., 2019a, b). The effects of PUFA supplementation on ruminants in late gestation are not well understood, and more studies are needed to better understand these mechanisms.

EPA and DHA for Calves

Calves are the future of the dairy herd, but they are also highly susceptible to increased morbidity and mortality (Karcher et al., 2014). Since EPA and DHA impact immunity and inflammation, there has been interest in supplementing fish oil or algae to calves. Fish oil was shown to attenuate the acute phase response in Jersey calves following an LPS challenge (Ballou et al., 2008). Dairy calves fed fish oil had lower blood lactate, serum haptoglobin, and lower concentrations of inflammatory cytokines compared to calves fed canola oil (Melendez et al., 2022). Flaga et al. (2019) observed that DHA supplementation with algae benefitted the immune system of calves when 9 g/d of DHA was provided. Supplementation of n-3 FA in colostrum increased expression of anti-inflammatory oxylipids in calves in the first week after birth (Oppengorth et al., 2020). Despite the benefits on immune function, supplementation with EPA and DHA may reduce average daily gain and feed efficiency in dairy calves, especially at higher doses (Karcher et al., 2014; Flaga et al., 2019).

Requirements for EPA and DHA

Although there are no established dietary requirements for EPA and DHA, there are recommendations that vary across stage of life. Calder et al. (2020) recommended consuming 250 mg/d of EPA/DHA to support optimal function of a human's immune system. Based on this

recommendation, this would equate to about 2.5 g/d of EPA/DHA to be absorbed by a 700 kg dairy cow if the weight of an average dairy cow is 10 times the weight of an average human.

Due to biohydrogenation rates being challenging to estimate, the ideal feeding rate of EPA and DHA for cows is uncertain. Low levels of EPA and DHA have been shown to have potent biological effects (Sinedino et al., 2017). An *in vitro* dose titration study observed a maximum reduction in secretion of PGF_{2a} in cells treated with only 40 μM of EPA or DHA (Mattos et al., 2003). More research is needed to determine a realistic target of post-ruminal n-3 delivery *in vivo* to optimize bioactive effects.

The feeding studies that observed the most positive effects on milk yield tended to supplement only 10-25 g/d of EPA/DHA in the form of a calcium salt of fish oil or an algae product (Greco et al., 2015; Sinedino et al., 2017; Heravi-Moussavi et al., 2007). When considering a biohydrogenation rate of 70-90% (Palmquist, 2009; Jenkins & Bridges, 2007), only 2.5-7.5 g/d of n-3 would be supplied to the small intestine for absorption if 25 g/d were fed.

Conclusions

Both EPA and DHA are conditionally essential nutrients that play important biological roles. Understanding how dietary n-3 FA impact DMI, milk yield, milk fat synthesis and FA profile, reproduction, and inflammation, in the dairy cow can help scientists find strategies to supply these nutrients and observe optimal responses. The goal of Chapter 3 was to determine an optimal dose of DHA by observing the impacts of abomasal infusions of DHA on production responses and plasma FA profile to find potential biological markers for DHA absorption. In Chapter 4, our goal was to determine immediate and carryover effects of supplementing a calcium salt of fish oil to transition cows during the close-up and fresh period. The results from these two studies will help to improve the understanding of how EPA and DHA impact dairy cow

physiology and performance and will assist in developing practical feeding strategies and recommendations.

CHAPTER 3

EFFECT OF ABOMASAL INFUSIONS OF DOCOSAHEXAENOIC ACID ON PRODUCTION RESPONSES AND PLASMA FATTY ACIDS OF MID-LACTATION DAIRY COWS

Abstract

Our objective was to evaluate the effect of abomasal infusions of increasing doses of docosahexaenoic acid (DHA; C22:6 n-3) on plasma and milk fatty acids and milk production of mid-lactation dairy cows. Eight multiparous ruminally cannulated Holstein cows (97 ± 37 DIM, 49.2 ± 3.3 kg/d milk) were used in a 4x4 Latin Square design. Treatments were abomasal infusions of 0, 2, 4, and 6 g/d of DHA with 11-d treatment periods and 10-d washout periods. DHA was provided via an enriched algal oil (64.5% DHA) and suspended in ethanol (~200 g/d). Samples were collected during the last 4 d of each infusion period. The statistical model included the random effect of cow nested within square and fixed effects of treatment, square, period, and their interactions. Preplanned contrasts tested the linear, quadratic, and cubic effects of increasing doses of DHA. Results are presented in the following order: 0, 2, 4, 6 g/d of DHA. There was no effect of treatment on yields of milk, FCM, ECM, milk fat, or milk protein. Increasing DHA dose linearly decreased SCC (13.3, 12.0, 10.4, 10.8 x 10^3/mL; $P = 0.05$). Increasing DHA dose linearly increased DHA in plasma phospholipids (0.30, 0.50, 0.64, 0.82 g/100g FA; $P < 0.01$), triacylglycerols (0.09, 0.25, 0.40, 0.51 g/100g FA; $P < 0.01$), and cholesterol esters (0.04, 0.04, 0.05, 0.06 g/100g FA; $P < 0.01$). Increasing dose of DHA linearly increased milk fat content of DHA (0.01, 0.04, 0.06, 0.08 g/100g FA; $P < 0.01$), total n-3 fatty acids (0.56, 0.57, 0.63, 0.63 g/100g FA; $P < 0.05$), and the yield of DHA in milk (0.19, 0.58, 0.91, 1.18 g/d; $P < 0.01$). Correlation results are presented as Pearson correlation coefficients.

DHA content in plasma PL and TAG and milk fat were linearly correlated with DHA absorption (0.70, 0.92, 0.93; $P < 0.0001$). In conclusion, increasing abomasal infusions of DHA did not impact short-term production responses of mid-lactation cows, but increased DHA content of key plasma lipids and milk fat, and reduced milk SCC. Plasma or milk fat DHA content may be used to estimate DHA absorption in feeding scenarios.

Introduction

All mammals require essential fatty acids (FA) in their diet because they cannot be synthesized *de novo*. The two essential FA are the omega-6 (n-6) linoleic acid (LA; 18:2 n-6) and omega-3 (n-3) alpha-linolenic acid (ALA; 18:3 n-3). In humans, reducing the dietary ratio of n-6:n-3 FA has been shown to reduce the risk and pathogenesis of many diseases in humans including cardiovascular disease, cancer, and inflammatory diseases (Simopoulos, 2002). Common conventional dairy cow feed ingredients such as corn and soybeans are high in LA (NASEM, 2021). Therefore, there is interest in supplementing additional n-3 FA to provide a better balance of essential polyunsaturated FA (PUFA) in the diet. In dairy cows, decreasing the dietary ratio of n-6:n-3 has increased DMI and the yields of milk and milk components (Greco et al., 2015; France et al., 2022). Feeding more n-3 and less n-6 also attenuated the acute phase response after an intramammary LPS challenge (Greco et al., 2015).

ALA is desaturated and elongated in the body into eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), which are precursors in the biosynthesis of pro-resolving lipid mediators such as resolvins and protectins (Spite et al., 2014; Raphael & Sordillo, 2013). This synthesis pathway is inefficient as only about 0.2% of plasma ALA is utilized for the synthesis of EPA (Pawlosky et al., 2001). Abomasal infusion of ALA in dairy cows for 20 d did

not increase plasma concentrations of DHA (dos Santos Neto et al., 2024). Therefore, there may be a need to directly supplement these bioactive n-3 FA to dairy cows.

The FA profile of cellular phospholipids can impact the production of transcription factors that modulate inflammation, with EPA and DHA playing a critical role in inhibiting pro-inflammatory transcription factors (Calder, 2013). When including EPA and DHA in the diet of dairy cows, immune cell membranes were enriched in these FA (Silvestre et al., 2011b). Therefore, there is emphasis on these long chain derivatives of ALA and how to increase their content in plasma and cell membranes. Fish oil is one of the only dietary sources of EPA and DHA. With increasing concerns about the sustainability of fish oil production, algae may be a promising substitute as a rich source of DHA (Bartek et al., 2021). Providing DHA in the diet by feeding algae increased milk yield and improved reproduction outcomes in dairy cows (Sinedino et al., 2017). DHA is also a potential dietary source of EPA due to the retroconversion of DHA into EPA (Conquer & Holub, 1997).

Increasing the availability of PUFA to the cow is challenging due to the extensive biohydrogenation of UFA in the rumen. Typically, over 80% of all dietary UFA are either fully or partially biohydrogenated (Palmquist, 2009). Because rumen microbes saturate most PUFA, the direct effects of DHA in cows are unknown. To date, there has been no study that has analyzed the effect of increasing abomasal infusion doses of DHA in dairy cows. Therefore, the objective of our study was to evaluate the effect of increasing abomasal infusion doses of DHA on plasma FA, milk FA, and short-term milk production responses in dairy cows using biologically relevant doses. We hypothesized that increasing DHA dose would increase plasma DHA in all lipid fractions, reduce the plasma n-6:n-3 ratio, and increase the content of n-3 FA in milk fat.

Materials and Methods

Study Design and Treatments

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University, East Lansing. Eight ruminally cannulated mid-lactation multiparous Holstein cows from the Michigan State University Dairy Cattle Teaching and Research Center were assigned randomly to a treatment sequence in a 4 x 4 Latin Square design. At the beginning of the study, cows averaged (mean ± SD) 97 ± 37 DIM and 49.2 ± 3.3 kg/d milk yield. Two squares, each with 4 cows, were formed based on lower- [milk yield (mean ± SD) 46.4 ± 1.5 kg/d] and higher-production cows [milk yield (mean ± SD) 52.0 ± 1.5 kg/d]. Cows were then randomly assigned to a treatment sequence. Treatment periods lasted 11 d with a 10 d washout period between each treatment period.

Treatments were 0, 2, 4, and 6 g/d of DHA infused into the abomasum. For the infusions we used algal oil containing 65% DHA (DSM Nutritional Products; Table 3.1). The dose range was selected based on expected DMI, biohydrogenation estimates (Doreau & Chilliard, 1997; AbuGhazaleh et al., 2002), predicted metabolic requirements based on human literature recommendations (Calder et al., 2020), and dose levels used in previous n-3 cow feeding studies (Greco et al., 2015; Sinedino et al., 2017; Heravi-Moussavi et al., 2007). Daily treatments were suspended in ethanol in individual glass jars to equal a total daily infusion amount of 200 g/d. Cows on the 0 g/d treatment received 200 g/d of ethanol. The infusate solution was divided into 4 equal infusions per d that were provided every 6 hours. Abomasal infusion devices were inserted into the abomasum 5 d before the beginning of the study. Infusion lines (0.5-cm diameter polyvinyl chloride tubing) passed through the rumen fistula and sulcus omasi into the abomasum (Lock et al., 2007; Prom et al., 2021). Lines were checked daily throughout the study

to ensure proper placement. Infusate solutions were delivered into infusion lines using 60-mL plastic syringes, and lines were flushed with warm water before and after delivery of treatment (dos Santos Neto et al., 2024)

All cows received a common diet throughout the entire study (Table 3.2) which were formulated to meet nutritional requirements according to NASEM (2021). Dry matter concentration of forages was determined twice weekly, and diets were adjusted when necessary. Cows were housed in individual tie stalls at the Michigan State University Dairy Cattle Teaching and Research Center (East Lansing, MI) throughout the experiment and milked thrice daily (0400, 1200, and 2000 h). Access to feed was blocked from 0600 to 0800 h for collection of orts and offering of new feed. Feed intake was recorded, and cows were offered 115% of expected intake at 0800 h daily. Water was available ad libitum in each stall, and stalls were bedded with sawdust and cleaned thrice daily.

Data and Sample Collection

Production data and samples were collected during the last 4 d of each treatment period (d 8-11). Diet ingredients (~0.5 kg) and orts samples (~12.5%) were collected daily and composited by period and cow for further analysis. Milk yield and intake were recorded daily. Two milk samples were collected at each milking; one aliquot was collected in a tube with preservative (Bronolab W-II liquid, Advanced Instruments, Norwood, MA) and stored at 4ºC for milk component analysis, and the second was collected and stored at -20ºC for milk FA analysis. Blood samples were collected every 9 h and centrifuged at 3,000 x *g* for 15 min at 4ºC for plasma extraction. Fecal samples were collected every 9 h and composited by cow and period. Body weight (BW) was measured twice during the last two days of each treatment period, and body condition score (BCS) was recorded on the last day of each treatment period by 3 trained

investigators according to Wildman et al. (1982). An additional blood sample was taken the day before the start of each treatment period to determine potential carryover effects of treatments on plasma FA.

Sample Analysis and Transfer Efficiency Calculation

Feed ingredients, orts and fecal samples were dried for 72 h at 55ºC in a forced-air oven to determine DM content. Samples were ground in a Wiley mill (1 mm screen; Arthur H. Thomas, Philadelphia, PA), and analyzed for NDF, 240-h indigestible NDF (iNDF), starch, and CP, according to Boerman et al. (2017). Feed ingredients, orts, and fecal samples were analyzed for FA profile according to Bales et al. (2024). Indigestible NDF was used as an internal marker to estimate fecal output to determine apparent total-tract digestibility of nutrients (Cochran et al., 1986). Milk samples were analyzed for fat, true protein, lactose, somatic cell count (SCC), and milk urea nitrogen (MUN) by mid-infrared spectroscopy (AOAC, 1990, method 972.160; Central Star DHI, Grand Ledge, MI). Yields of 3.5% FCM (FCM), energy-corrected milk (ECM), and milk components were calculated from each milking using milk yield and component concentrations, summed for a total daily yield, and averaged for each treatment period. Milk samples for FA analysis were composited by cow and period based on milk fat yield. Milk lipids were extracted, and FA methyl esters prepared and analyzed by gas chromatography as described by Lock et al. (2013). Transfer efficiency of DHA to milk was calculated on an individual cow basis by dividing the mean milk fat yield of DHA by the amount of DHA provided by the infusion treatment or the amount of DHA absorbed. Plasma insulin was determined by ELISA (Bovine Insulin ELISA; Mercodia AB, Uppsala, Sweden) at the Diagnostic Center for Population and Animal Health at Michigan State University (East Lansing, MI).

Plasma was composited by cow and period and lipid extraction was performed using a modified method of Folch et al. (1957) using chloroform and methanol. For FA content of total plasma lipids, sodium methoxide and boron trifluoride in methanol were used for methylation using a modified method of Nuernberg et al. (2007). After drying samples under N2 gas, a 1% hexane solution was prepared, and samples were analyzed using GLC as described by Lock et al. (2013). For FA content of individual plasma lipid fractions, plasma lipids were separated using solid phase extraction (SPE) chromatography (Agren et al., 1992). A vacuum manifold fitted with aminopropyl (N2) SPE columns (1 g / 6 mL) was used to separate cholesterol ester (CE), triacylglycerol (TAG), nonesterified fatty acid (NEFA), and phospholipid (PL) fractions of each sample. The column was loaded with plasma lipidsand flushed with 14.0 mL of hexane to elute the CE fraction. The column was then washed with 8.0 mL of hexane:chloroform:ethyl acetate (100:5:5) to elute the TAG fraction. Columns were washed with 6.0 mL of chloroform:isopropanol (2:1) to remove the waste fraction. Columns were subsequently washed with 8.0 mL of chloroform:methanol:acetic acid (100:2:2) to elute the NEFA fraction and with 10.0 mL of methanol:chloroform:water (10:5:4) to elute the PL fraction. The chloroform layer containing the PL fraction was separated using 5% sodium chloride solution. All fractions were dried under N_2 gas, weighed to determine yield, and reconstituted in 0.5 mL tolulene prior to methylation. Methylation of all fractions was performed using methanolic hydrochloric acid and sodium methoxide according to Bales and Lock (2024). The FAME were reconstituted in hexane to obtain a 1% solution for GLC analysis as described by Lock et al. (2013).

Statistical Analysis

All data were analyzed using the GLIMMIX model procedure of SAS (Version 9.4, SAS Institute Inc.) according to the following model:

$$Y_{ijkl} = \mu + C(S)_{i(j)} + S_j + P_k + T_l + e_{ijkl},$$

where Y_{ijkl} = dependent variable, μ = overall mean, $C(S)_{i(j)}$ = random effect of cow nested within square (i= 1–4), S_j = fixed effect of square (j = 1–2), P_k = fixed effect of period (k = 1–4), T_l = fixed effect of treatment (l = 1–4), and e_{ijkl} = residual error. Interactions between period and treatment, period and square, and square and treatment were initially included in the model and removed when $P > 0.10$ (de Souza et al., 2020). Normality of the residuals was checked with normal probability and box plots, and homogeneity of variances with plots of residuals versus predicted values. Treatment effects were declared significant at $P \leq 0.05$, and tendencies were declared at $0.05 < P \leq 0.10$. Three preplanned orthogonal contrasts were used to determine the linear, quadratic, and cubic effects of increasing doses of DHA. All data are expressed as least squares means and standard error of the means.

Plasma FA profiles were initially tested using the baseline sample from each washout period as a covariate, but it was not significant and was removed from the model. We also had 3 missing samples from the washout period, so we opted not to use it in the final model and instead used treatment by period in the model if significant to adjust for carryover effects. Including the covariate did not alter the interpretation of our results.

Pearson correlation coefficients between absorbed DHA and DHA content in total plasma lipids, plasma TAG, plasma PL, plasma CE, and milk fat were calculated using the CORR model procedure of SAS. Regression analyses were performed using the GLIMMIX model procedure considering the random effects of cow and period.

Results

Treatment effects on nutrient digestibility, milk production, and plasma and milk FA profiles are reported here. Effects of abomasal infusions of DHA on immune responses, oxylipid

profiles, and insulin sensitivity in adipose tissue are discussed in our companion papers (Reisinger et al., unpublished; Contreras et al., unpublished).

Nutrient Digestibility

Digestibility results are reported in Table 3.3. There was no effect of DHA infusion on DMI or digestibility of DM, NDF, or total FA. DHA infusion linearly increased intake, digestibility, and absorption of 22-carbon FA ($P < 0.01$), but intake and absorption of total FA were not affected. There was no effect of treatment on DHA digestibility, but absorption of DHA was increased linearly with increasing DHA dose ($P < 0.01$).

Milk and Milk Components

Production responses are reported in Table 3.4. Increasing abomasal infusions of DHA linearly reduced somatic cell count (SCC) ($P = 0.05$). There was no effect of treatment on the yields of milk, milk fat, milk protein, milk lactose, FCM, or ECM. Infusion of DHA tended to quadratically increase milk fat content ($P = 0.10$) and milk protein content ($P = 0.07$) and linearly increased lactose content ($P < 0.01$). There was no effect of treatment on BCS, BW, or plasma insulin concentration.

Fatty Acid Profile of Plasma Lipids

Total plasma FA results are reported in Table 3.5. Results in the text are reported here as percent increases or decreases in plasma FA content compared to control in the following order: 2, 4, 6 g/d of DHA. Abomasal infusion of DHA linearly increased 22:6 n-3 content (69%, 108%, 131%, $P < 0.01$). Treatment also linearly increased 20:2 n-6 ($P < 0.01$) and 22:5 n-6 ($P = 0.01$). A cubic effect was observed for 18:1 n-9 ($P = 0.03$) and a quadratic effect was observed for 22:0 ($P = 0.02$). There were no effects of treatment on plasma content of total n-3, total n-6, or the ratio of n-6:n-3.

Fatty Acid Profile of Plasma Phospholipids

Plasma PL results are reported in Table 3.6. Within plasma PL, abomasal infusion of DHA linearly increased the content of both C22:6 n-3 (67%, 114%, 174%) and total n-3 FA (5%, 9%, 16%) ($P < 0.01$) and reduced the ratio of n-6:n-3 (6%, 9%, 15%) ($P < 0.01$). Abomasal infusion of DHA quadratically reduced 18:3n-3 ($P = 0.01$). Increasing doses of DHA also linearly increased 22:5 n-6 ($P < 0.01$) and linearly reduced 18:1 n-9 ($P < 0.01$).

Fatty Acid Profile of Plasma Cholesterol Esters

Plasma CE results are reported in Table 3.7. Abomasal infusion of DHA linearly increased C22:6 n-3 (19%, 38%, 54%; $P < 0.01$). DHA infusion also linearly increased the content of 18:2 n-6 ($P = 0.02$) and total n-6 ($P = 0.03$). Increasing DHA linearly reduced the content of 18:0 ($P = 0.01$) and 18:1 n-9 ($P < 0.01$). Abomasal infusion of DHA quadratically affected the content of 18:3 n-3, total n-3, and the ratio of n-6:n-3 ($P < 0.01$). Quadratic effects were also observed for 14:0, 16:1 n-7, and 20:3 n-6 ($P < 0.05$).

Fatty Acid Profile of Plasma Triacylglycerols

Plasma TAG results are reported in Table 3.8. Abomasal infusion of DHA linearly increased C22:6 n-3 (195%, 367%, 503%, $P = 0.001$). Treatment linearly reduced plasma content of 18:1 n-9 ($P = 0.04$) and linearly increased 18:0, 22:5 n-3, and 22:5 n-6 ($P < 0.05$). Increasing abomasal infusions of DHA reduced the ratio of n-6:n-3 ($P < 0.001$).

Fatty Acid Profile of Plasma NEFA

NEFA results are reported in Table 3.9. We only detected 22:6 n-3 in a few samples (n=3). In the majority of samples it was below detectable limits. As a result we were unable to perform statistical analysis of 22:6 n-3 in NEFA. Abomasal infusion of DHA linearly decreased

12:0 ($P = 0.03$) and tended to linearly increase 18:0 ($P = 0.10$). Cubic effects were observed for 16:1 n-7, 20:4 n-6, and total n-6 content in NEFA ($P < 0.05$).

Milk Fatty Acid Contents and Yields

Milk FA contents are reported in Table 3.10. Sources of FA were classified as de novo (< 16 carbons), mixed (16-carbon), and preformed (> 16 carbons). There were no effects of treatment on sources of milk FA. Increasing doses of DHA linearly increased 22:6 n-3 content (182%, 346%, 482%) and total n-3 content of milk fat (2%, 9%, 12%) ($P < 0.01$). The n-6:n-3 ratio was linearly reduced by DHA treatment (2%, 9%, 12%) ($P < 0.01$). Abomasal infusion of DHA linearly increased milk FA content of both 22:5 n-6 and 22:5 n-3 ($P < 0.01$).

Yields of milk FA are reported in Table 3.11. There was no effect of treatment on yields of milk FA by source. Increasing abomasal infusion of DHA linearly increased the yields of 22:6 n-3 (0.39, 0.72, 0.99 g/d; $P < 0.01$) and total n-3 in milk (0.36, 1.13, 1.00 g/d; $P < 0.01$). Treatment also linearly increased the yields of both 22:5 n-6 and 22:5 n-3 ($P < 0.05$). Abomasal infusion of DHA linearly reduced apparent transfer efficiency of DHA into milk from the 2 to 6 g/d treatment based on the amount infused (28.8%, 22.8%, 19.7%; $P < 0.0001$) and on the amount absorbed (30.6%, 24.1%, 20.8%; $P < 0.0001$).

Pearson Correlation Coefficients

The Pearson Square is shown in Table 3.12. DHA in total plasma lipids was positively correlated with DHA in plasma phospholipids (0.72, $P < 0.01$). Milk DHA content was positively correlated with DHA in plasma triacylglycerols (0.95, $P < 0.01$) and plasma phospholipids (0.81, $P < 0.01$). Absorbed DHA was positively correlated with the DHA in total plasma (0.42, $P < 0.05$), plasma PL (0.70, $P < 0.01$), plasma TAG (0.92, $P < 0.01$), and milk fat (0.93, $P < 0.01$).

Regression

Based on results in Table 3.12, we assessed linear relationships between several variables. We observed a positive linear relationship between DHA in plasma and plasma PL (Figure 3.1; $R^2 = 0.52$; $P < 0.0001$), and DHA in plasma TAG and milk fat (Figure 3.2; $R^2 = 0.89$; $P < 0.0001$). Increasing DHA absorption was positively and linearly related to DHA content in milk fat (Figure 3.3; $R^2 = 0.86$; $P < 0.0001$), plasma TAG (Figure 3.3; $R^2 = 0.85$; $P < 0.0001$), and plasma PL (Figure 3.3; $R^2 = 0.49$; $P < 0.0001$).

Discussion

To the best of our knowledge, no previous studies have examined the effect of increasing abomasal infusion doses of long-chain n-3 FA in dairy cows. Abomasal and duodenal infusions of algal and fish oil have been utilized previously (Rico et al., 2021; Castañeda-Gutiérrez et al., 2007; Doreau & Chilliard, 1997), but none used doses as low as those in our current study, and only one of them examined a dose-response effect (Doreau & Chilliard, 1997). The doses of n-3 FA from these studies were non-physiological as they exceed plausible delivery to the small intestine when translated to a feeding scenario. Rico et al. (2021) abomasally infused 143 g/d of DHA and measured effects on FA digestibility, plasma lipids, and milk FA. Castañeda-Gutiérrez et al. (2007) provided ~17 g/d of EPA and ~21 g/d of DHA either infused in the abomasum or rumen or fed as a calcium salt of fish oil and compared transfer efficiency of n-3 FA to milk fat (Castañeda-Gutierrez et al., 2007). Doreau and Chilliard (1997) duodenally infused fish oil to cows at doses of 185 (~30 g/d EPA, ~10 g/d DHA), 276 (~44 g/d EPA, ~15 g/d DHA), and 370 (~59 g/d EPA, ~21 g/d DHA) g/d. Although these studies have provided valuable insights into how n-3 FA impact nutrient digestibility and milk fat, our objective was to determine the impact

of abomasal infusions of increasing doses of DHA to study the impact on cow physiology at amounts we feel could be supplied to dairy cows in a feeding scenario.

The doses we chose were based on previous n-3 cow feeding studies, biohydrogenation estimates, and predicted metabolic requirements based on human literature recommendations. These doses reflect realistic levels that could be supplied to lactating dairy cows when feeding an n-3 FA supplement. Greco et al., (2015) fed 21.3, 14.9, and 10.0 g/d of EPA and DHA to lactating dairy cows in the form of a calcium salt of fish oil. These doses increased EPA and DHA in plasma and milk fat, indicating that some of the EPA and DHA escaped rumen biohydrogenation. There are very few studies examining duodenal flows of EPA and DHA. A meta-analysis by Jenkins and Bridges (2007) suggested that the degree of biohydrogenation of unsaturated FA is increased with increasing levels of unsaturation ruminal losses of ALA are estimated to be ~85% (Jenkins & Bridges, 2007). They also estimated that cattle fed control diets with no added fat only had ~ 5 g/d duodenal flow of ALA (Jenkins & Bridges, 2007). From these estimates, we determined that feeding approximately 20 g/d of EPA/DHA would result in post-ruminal flow of 2-4 g/d of these n-3 FA. We also considered n-3 supplementation recommendations for humans when choosing our doses. While there are no NIH recommendations for EPA and DHA intakes, Calder et al., (2020) recommended 250 mg/d of EPA+DHA to support an optimally functioning immune system. Based on this recommendation, a 700 kg dairy cow would need approximately 2.5 g/d of post-ruminal EPA/DHA if the weight of the average dairy cow is 10 times the weight of the average human. Based on these different approaches, we are confident that our chosen doses of DHA (2, 4, & 6 g/d) provided a sound test of our hypothesis.

FA digestibility was high for all treatments at ~80%. This is likely due to the low FA content of our diet. We chose to provide no extra supplemental fat in the diet in order to avoid potential interactions with our infusion treatments, and our FA intakes were around 600 g/d. A meta-regression by Boerman et al. (2015) demonstrated that as total FA intake decreases, total FA digestibility increases. Control diets without supplemental fat tended to have the highest FA digestibility (Boerman et al., 2015). DHA digestibility was over 90% for all infusion treatments. There are few studies that have examined digestibility of DHA in dairy cows. When Rico et al. (2021) infused DHA at 143 g/d, they observed 22 carbon FA digestibility to be >99% (Rico et al., 2021). We acknowledge that this not directly comparable to our study since the infusion dose was much higher than ours. PUFA are more readily incorporated into micelles in the small intestine, and FA with higher degrees of unsaturation are more digestible (Pantoja et al., 1996; Boerman et al., 2015). Since UFA improve micelle formation, they can also increase digestibility of saturated FA as demonstrated by abomasal infusions of oleic acid (18:1n-9) (Prom et al., 2021). Our DHA infusions did not improve digestibility of total FA, which is likely due to the low FA content of our diet and the fact that digestibility was already very high. Further research is needed to determine potential effects of DHA on digestibility of FA in higher fat diets.

Although we did not observe any treatment effects on milk production responses, our study was not designed with production measures as the primary objective. With only 8 post-peak cows in short term infusion periods, we did not expect DHA to impact the yields of milk or milk components as has been observed in studies that utilized early-lactation cows (Greco et al., 2015; Sinedino et al., 2017). It is, however, important to note that we did observe a linear reduction in SCC, which was likely due to the anti-inflammatory effects of n-3 FA. In humans, supplementation with cod liver oil rich in EPA and DHA reduced neutrophil and monocyte

chemotaxis (Schmidt et al., 1989). Leukotrine B_4 is a major signaling molecule that promotes neutrophil chemotaxis, and its production is reduced by n-3 PUFA supplementation (Sperling et al., 1993). Somatic cells in the mammary gland of a healthy cow include macrophages as the dominant cell type, while neutrophils are the first and predominant responder to an immune challenge during early inflammation due to the production of chemotactic mediators (Riollet et al., 2000). In dairy cows, two studies have supplemented EPA and DHA during the dry period and observed reductions in SCC in the subsequent lactation (Jolazadeh et al., 2019; Badiei et al., 2014). Our results and results from these studies warrant further investigation to the determine the effects of DHA and other n-3 FA on inflammatory responses and resolution of inflammation in dairy cows to maintain cow health and milk quality.

Abomasal infusion of DHA increased the content of DHA in all plasma lipid fractions except for NEFA. DHA content in NEFA was below limits of detection, and we were unable to perform statistical analysis. The PL and CE lipid fractions account for the largest proportion of plasma FA in ruminants and serve as a mobile store for essential FA, while the TAG and NEFA fractions typically supply FA for secondary roles such as energy storage, milk fat secretion, or beta-oxidation (Jenkins et al., 1988; Christie, 1981). Previous studies have indicated that n-3 FA are preferentially incorporated into PL and CE fractions (Urrutia et al., 2023; Offer et al., 2001; dos Santos Neto et al., 2024). It is likely that ruminants do this to conserve essential FA in these low-turnover lipid fractions to prevent deficiency and offset the low availability of n-3 FA from the diet due to biohydrogenation (Caldari-Torres et al., 2016). In our study, we observed a significant linear increase of DHA in plasma PL. Directly supplementing DHA as a source of n-3 FA may be beneficial to cows as abomasal infusions of 43 g/d of ALA for 20 d did not increase DHA content in plasma PL (dos Santos Neto et al., 2024). With 6 double bonds in the

hydrocarbon chain, DHA is used to increase cell membrane fluidity within different tissues and cell populations (Cook & McMaster, 2002; Arterburn et al., 2006). Of particular interest are the PL profiles of immune cell membranes, which influence the expression of pro- and anti-inflammatory genes and proteins as well as synthesis of lipid mediators (Calder, 2008; Raphael & Sordillo, 2013). Higher n-3 content in immune cell PL promotes a more anti-inflammatory phenotype (Raphael & Sordillo, 2013). In humans, total plasma DHA content was positively correlated with DHA content in the cell membranes of peripheral blood mononuclear cells (PBMC) (Grindel et al., 2013). Providing more dietary n-3 FA to dairy cows and enriching the n-3 FA content of PL in cell membranes may be beneficial during times of inflammatory challenges such as the transition period (Bradford et al., 2015). Although our treatments did increase the content of DHA in plasma CE, the content of DHA in this lipid fraction is very low, which is consistent with findings from other studies (Urrutia et al., 2023; Offer et al., 2001). One likely reason for this is that the lecithin cholesteryl ester acyl transferase (LCAT) enzyme responsible for synthesizing CE has a low affinity for DHA (Subbaiah et al., 1993).

Abomasal infusion of DHA increased the content of DHA in plasma TAG. Since TAG is typically the first plasma lipid fraction that FA enter after absorption in the small intestine (Noble, 1981), it is unsurprising that increasing the absorption of DHA increased DHA enrichment in plasma TAG. It is unlikely that DHA remains in this fraction for very long after absorption as a singular bolus infusion of fish oil resulted in peak plasma TAG levels of DHA 6 hours post-infusion, which returned to baseline levels 30 hours post-infusion (Urrutia et al., 2023). Since we were infusing DHA every 6 hours for 11 d, we likely reached a steady state of DHA in TAG for each treatment during infusion periods. Without dietary supplementation of DHA, plasma TAG content of DHA is close to zero (Tyburczy et al., 2008). Our 0 g/d treatment

had 0.09 g DHA /100 g FA in plasma TAG, which is likely higher than most cows that are not supplemented with DHA due to the observed carryover effects and treatment by period interactions in the current study.

The incorporation of DHA into milk fat was also increased with increasing DHA infusion. Although statistically significant, actual increases of DHA in milk fat were numerically small. The mammary gland obtains preformed FA from the blood and exhibits preferential uptake of FA from TAG compared to CE or PL. (Moore & Christie, 1981). The plasma TAG fraction is significantly smaller than the CE and PL fractions (Offer et al., 2001), so the total available DHA in TAG was very low, which likely limited the transfer of DHA into milk fat. Nevertheless, our results are consistent with other studies that reported transfer efficiencies for DHA of 18-25% into milk fat with abomasal infusions of fish oil (Castañeda-Gutierrez et al., 2007; Urrutia et al., 2023). Our 2 g/d dose had a transfer efficiency of ~29% of DHA into milk fat while our 6 g/d dose had a transfer efficiency of ~20%.

Although abomasal infusion of DHA increased DHA content in total plasma lipids, it did not increase total plasma n-3 content or alter the ratio of n-6:n-3. This could partially be due to the fact that the algal oil infusate contained ~13% of the n-6 FA docosapentaenoic acid (DPA; 22:5 n-6). The abomasal infusion treatments linearly increased content of 22:5 n-6 in total plasma, plasma PL, and plasma TAG. Another explanation is that DHA infusion linearly increased LA (18:2 n-6) content in the CE fraction, and quadratically reduced ALA in both CE and PL. LA within CE makes up a substantial proportion of total plasma FA (Tyburczy et al., 2008), so it is unsurprising that the n-6:n-3 ratio in total plasma lipids was unaffected. However, the mechanism of how this occurs is unknown. It is possible that DHA displaced some of the LA within cellular phospholipids and allowed for recycling of LA into circulation within CE. DHA

infusion did reduce the n-6:n-3 ratio in plasma PL by 15% as it increased total n-3 FA content in this fraction. Future research is needed to understand the mechanisms behind these substitutions and relationships between CE and PL in dairy cows.

We assessed linear relationships between several different variables including DHA absorption, total plasma DHA content, DHA content within TAG, PL, and CE, and milk DHA content. The goal of this analysis was to examine the efficacy of potential markers for DHA that could be used to estimate DHA absorption in future feeding studies and in commercial settings. Interestingly, milk DHA content was the variable most highly correlated with DHA absorption (0.93) although DHA in plasma TAG was similar (0.92). Since separating plasma lipid fractions is a time-consuming and difficult process, examining milk DHA content may be a viable method to estimate DHA absorption, especially since milk DHA content is minimal when cows are fed traditional dairy cow diets (Lock & Bauman, 2004). However, it may be easier to collect blood from individual cows on large dairy farms. DHA content in total plasma lipids was not as highly correlated with DHA absorption (0.42). Most plasma DHA was incorporated into the PL lipid fraction, and we analyzed the linear relationship between total plasma lipid DHA content and DHA content in plasma PL (0.72). Analyzing total plasma DHA content could be used to assess plasma PL content; however, milk DHA content had a stronger relationship with plasma PL DHA (0.80).

While our study is novel, we acknowledge it has some limitations. By using mid-lactation cows, we were unable to examine the effect of DHA on dairy cows during an immunologically and metabolically challenging stage of lactation. Since one of the main goals of feeding n-3 FA is to reduce inflammation, we were not able to directly observe those effects since cows were in positive energy balance and not experiencing any major inflammatory challenges. Therefore, we

could not measure the true bioactive effect of DHA on production responses. Despite this limitation, effects on production responses were not our primary objective. We chose to use post-peak cows in a Latin square design to reduce variation and the number of animals used while examining the effects of four different treatments. A major goal of our study was to assess low doses of DHA and determine potential markers for DHA absorption that could be used in future work. We acknowledge that this study design has some limitations since there was a potential for carryover effects. We attempted to minimize these effects with randomized treatment sequences, balancing treatment sequences for carryover effects, adjusting for treatment by period interactions in the statistical model, and using 10 d washout periods. We observed treatment by period interactions for several variables including DHA content in total plasma lipids, PL, TAG, and milk fat. Control cows in periods 2-4 had higher plasma and milk DHA content than the control cows in period 1. In our recent study, abomasal infusion of ALA for 20 d increased plasma PL and CE content of EPA, which had carryover effects that lasted for 20 d following cessation of infusions (dos Santos Neto et al., 2024). Nevertheless, the carryover effects we observed do not alter the interpretations of our study as we still observed strong linear relationships between infusion dose and DHA content in milk and plasma. Future research is warranted to examine effects of long-term abomasal infusions of DHA on enrichment of plasma lipid fractions and subsequent carryover effects.

Conclusion

Our results show that abomasal infusion of DHA in mid-lactation dairy cows at 2, 4, and 6 g/d reduced SCC and increased DHA and total n-3 content in plasma phospholipids, plasma cholesterol esters, plasma triacylglycerols, and milk fat compared to a control treatment. Milk fat and plasma DHA content were highly correlated with DHA absorption and may be used as

markers to estimate DHA absorption in future studies. Our results can be used to better understand how DHA impacts dairy cow physiology to aid in the development of feeding recommendations to optimize cow health and productivity. Further research is needed to develop and assess rumen protection methods to deliver n-3 FA to the small intestine for absorption, and should also focus on developing feeding strategies to supply n-3 FA to cows during nutritionally and immunologically challenging stages such as the transition period.

CHAPTER 4

EFFECT OF FEEDING A CALCIUM SALT ENRICHED IN EPA AND DHA TO DAIRY COWS DURING THE LATE PREPARTUM AND EARLY POSTPARTUM PERIOD ON MILK PRODUCTION RESPONSES AND MILK FATTY ACID PROFILES

Abstract

Our objective was to evaluate the effect of supplemental omega-3 fatty acids (FA) during the close-up and fresh period on production responses and metabolites in early lactation dairy cows. Thirty-five multiparous cows were used in a randomized incomplete block design. Treatments were: 1) a control diet containing a calcium salt of palm oil fed during the close-up and fresh periods at 0.59% and 0.58% of diet DM, respectively (CON); 2) a diet containing a calcium salt enriched in EPA and DHA fed during the close-up and fresh periods at 0.86% and 0.87% of diet DM, respectively (N3). Close-up period treatment diets were fed beginning 21 days prior to due date. After calving, cows were fed the fresh period treatment diets for 23 days. All cows were switched to a common diet after the fresh period to evaluate carryover effects. The statistical model included random effects of block, cow nested within block and treatment, and Julian date and fixed effects of treatment, time, and the interaction of treatment and time. Results are presented in the following order: CON, N3. There was no effect of treatment on DMI, milk yield, BCS, or BW during the fresh period. N3 reduced milk fat yield (2.12, 1.94 kg/d; $P = 0.05$) and tended to reduce milk fat content (4.91, 4.66%; $P = 0.10$) and yields of ECM (52.7, 49.7 kg/d; $P = 0.10$) and FCM (53.1, 49.7 kg/d; $P = 0.09$) during the fresh period. No main effects of treatment were observed during the carryover period on milk production results, but treatment interacted with time for N3 to reduce milk fat yield and milk fat content during weeks 4 and 5 of lactation. There were no effects of treatment on plasma concentrations of insulin,

glucose, NEFA, or BHB. N3 reduced the yields of de novo (416, 375 g/d; $P = 0.01$) and mixed (663, 584 g/d; $P < 0.01$) milk FA sources during the fresh period. N3 also increased milk EPA (0.03, 0.07 g/100 g FA; $P < 0.0001$) and DHA content (0.00, 0.05 g/100 g FA). In conclusion, feeding a calcium salt enriched in EPA and DHA to dairy cows during the 3 weeks before and 3 weeks after parturition did not impact DMI or yields of milk but decreased milk fat yield during the fresh period and first two weeks of the carryover period and tended to decrease ECM and FCM yields.

Introduction

Defined as the 3 weeks before and 3 weeks after parturition, the transition period of dairy cows is marked by negative energy balance where nutrient intake is exceeded by the dramatic increase in demand for energy and nutrients to support milk production (Drackley, 1999). In addition to negative energy balance, periparturient cows also experience systemic inflammation, which has been linked to increased disease risk and decreased whole-lactation milk production (Bradford et al., 2015). Strategies such as the use of non-steroidal anti-inflammatory drugs have been shown to reduce inflammation post-calving and increase whole-lactation milk production (Carpenter et al., 2016). This has led to research focused on nutritional strategies to reduce the negative effects of inflammation in transition dairy cows. Some nutritional strategies that have been investigated in transition cows include methyl donors such as choline and methionine, live yeast and yeast-based products, and bioactive phytoproducts from plants (Lopreiato et al., 2020).

The bioactive omega-3 (n-3) fatty acids (FA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), exhibit anti-inflammatory properties and have been studied extensively in multiple species, particularly in humans and rodent models as well as cell culture (Palmquist, 2009). Supplementing these polyunsaturated FA (PUFA) has been shown to reduce

leukocyte chemotaxis, decrease production of pro-inflammatory eicosanoids and inflammatory cytokines, and increase production of pro-resolving lipid mediators such as resolvins, protectins, and anti-inflammatory endocannabinoids (Calder, 2013; Zaloga, 2021). This has led to interest in examining potential benefits of n-3 FA in dairy cattle. Greco et al., (2015) altered the dietary ratio of n-6:n-3 FA and observed an increase in FCM yield when cows were fed a diet with the highest n-3 content from 14-104 DIM.

Increases in the yields of milk and milk components could be due to an increase in nutrient partitioning to milk production as a result of a reduction in energy and nutrient expenditure for immune activation and maintenance of a proinflammatory state. A review by Lochmiller and Deerenberg (2000) proposed that immune activation increases energetic costs anywhere between 10-40% as a percentage of basal metabolic rate, and over 1 kg of glucose is estimated to be utilized within 12 hours in the activated immune system of the dairy cow (Kvidera et al., 2016). Therefore, immune activation redirects nutrient partitioning away from milk production, and regulating inflammation in the dairy cow could help to alleviate some of these potential limitations (Bradford et al., 2015). For example, feeding 10 g/d of DHA to dairy cows from 27-147 DIM increased milk yield and improved reproductive performance (Sinedino et al., 2017).

Despite the potential of feeding EPA and DHA to transition cows, very few studies investigating these FA have been reported during this period. Most studies started supplementation after 14 DIM (Greco et al., 2015; Silvestre et al., 2011; Sinedino et al., 2017). Some studies investigated n-3 FA supplementation during the dry period (Jolazadeh et al., 2019; Badiei et al., 2014), but did not examine subsequent effects of postpartum supplementation with EPA and DHA. To the best of our knowledge, no studies have investigated whether feeding EPA

and DHA during the transition period has carryover effects into peak lactation. Therefore, the objective of our study was to determine the effect of feeding a calcium salt rich in EPA and DHA to dairy cows during the 3 weeks before and 3 weeks after calving on milk production responses and milk FA and evaluate carryover effects into peak lactation post-supplementation. We hypothesized that feeding EPA and DHA to dairy cows during the transition period would increase yields of milk and milk components in the fresh and carryover period and increase n-3 FA content in milk.

Materials and Methods

Animal Housing and Care

This is the first paper from an experiment that evaluated the effects of feeding a calcium salt enriched in EPA and DHA to dairy cows during the close-up and fresh periods. This paper describes the effect of these diets on DMI, yield of milk and milk components, plasma metabolites, and milk FA profiles. Our companion papers (Reisinger et al., unpublished; Contreras et al., unpublished) describe effects on immune parameters, reproductive measures, oxylipid profiles, and gene expression and insulin sensitivity in adipose tissue.

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University, East Lansing. The study began on September 13, 2023, and ended on April 12, 2024. Cows were housed in individual tie stalls throughout the experiment and milked thrice daily (0400, 1200, and 2000 h). Access to feed was blocked from 0600 to 0800 h for collection of orts and offering of new feed. Feed intake was recorded, and cows were offered 115% of expected intake at 0800 h daily. Water was available ad libitum in each stall, and stalls were bedded with sawdust and cleaned thrice daily. Standard reproduction and herd health protocols were maintained throughout this study.

Study Design and Treatment Diets

Thirty-six Holstein cows from the Michigan State University Dairy Cattle Teaching and Research Center were used in a randomized complete block design. Cows were blocked into 18 blocks by previous lactation 305-d mature-equivalent milk yield (within 1,500 kg), BCS at ~28 d before expected parturition date (up to 0.50-unit difference), and parity (up to 2 lactation difference). We had 3 distinct feeding phases in our study. The close-up (CU) period started 21 days before expected calving date. The fresh (FR) period started after calving and lasted for 23 d. The carryover (CO) period started at 24 DIM and lasted until 63 DIM during which all cows were fed a common diet. Cows within each block were randomly assigned to 1 of 2 treatments fed in the CU and FR periods. The treatments were: (1) control (**CON**) diet containing a calcium salt of palm FA containing no EPA or DHA (0.59% diet DM CU; 0.58% diet DM FR) (n=18) (2) diet supplemented with a calcium salt containing EPA and DHA (0.86% diet DM CU; 0.87% diet DM FR) (**N3**) (n=18). Treatment groups were not different in terms of 305-d mature-equivalent yield ($P = 0.29$; 12564 ± 1881 kg) or BCS ($P = 0.65$; 3.34 ± 0.17) pre-calving. Cows were removed from the study if they were on the CU treatment diet for < 14 d or >28 d. Cows were fed typical CU and FR diets that were supplemented with 2 different commercially available calcium salts which were provided in concentrate mixes for the CU (Table 4.1) and FR diets (Table 4.2). Treatment diets were mixed daily in a tumble-mixer. The ingredient and nutrient composition of all diets are described in Table 4.3. All rations were formulated to meet or exceed predicted animal requirements according to NASEM (2021). Dry matter concentration of forages was determined twice weekly, and diets were adjusted when necessary. From this point forward, the acronyms CON and N3 will be used to refer to the treatment diets, and n-3 will be used to refer to omega-3 FA.

Data and Sample Collection

All samples and body measurements were collected or recorded on the same day of the week, so all collection days are ± 2 d relative to parturition. Colostrum yield was recorded, and samples were taken at first milking within 6 hours after calving. Feed intake and milk yield were recorded daily throughout the entire experiment. Samples of feed ingredients and orts from each cow during the CU and FR periods were collected once per week during the experiment and stored in plastic bags at -20°C until processed. Milk samples were collected twice weekly during the fresh period at each milking. Samples were collected with preservative (Bronolab W-II liquid, Advanced Instruments, Norwood, MA) and stored at 4ºC for milk component analysis. Additional milk samples were collected at 7, 14, and 21 DIM and stored at -20ºC for milk FA analysis. During the carryover period, milk samples were collected once weekly at each milking for milk component analysis. Body weight was recorded at parturition and subsequently recorded 3 times per week postpartum. Body condition was scored weekly after calving (starting at 3 DIM) by 3 trained investigators on a 5-point scale as described by Wildman et al. (1982). Blood samples were collected at 21 d prepartum before treatment diets began, at 10 d prepartum, and at 3, 7, 14, 21, and 42 DIM. After collection, blood samples were centrifuged at 3,000 x *g* for 15 min at 4ºC for plasma extraction. Plasma was stored at -20ºC until further analysis.

Sample Analysis

Feed and orts samples were dried in a 55°C forced-air oven for 72 h, and DM content was calculated. Before drying, feed ingredients were composited by month. Feed samples were ground in a Wiley mill (1 mm screen; Arthur H. Thomas, Philadelphia, PA), and analyzed for NDF, starch, CP, and FA profile according to Boerman et al. (2017). Milk samples were analyzed for fat, true protein, lactose, SCC, and milk urea nitrogen (MUN) by mid-infrared spectroscopy (AOAC, 1990,

method 972.160; Central Star DHI, Grand Ledge, MI). Yields of 3.5% FCM (FCM), energy-corrected milk (ECM), and milk components were calculated from each milking using milk yield and component concentration, summed for a total daily yield, and averaged for each week. Milk samples used for FA analysis were composited by day and cow based on milk fat yield. Milk lipids were extracted and FA methyl esters prepared and analyzed by gas chromatography as described by Lock et al. (2013).

Plasma metabolites were measured at 3, 7, 14, and 21 DIM. Plasma insulin was analyzed using a bovine-specific ELISA (no. 10-1201-01; Mercodia). Plasma glucose (catalog no. 997-03001; Fujifilm), BHB (H7587-01-962; Pointe Scientific, Inc), and NEFA (633-52001; Fujifilm Wako) were analyzed using enzymatic methods.

Statistical Analysis

We removed one cow from analysis due to an early calving date. Data were analyzed separately for the CU, FR, and CO periods. All weekly data were analyzed using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc.) according to the following model:

$$Y_{ijklm} = \mu + T_k + W_j + T_kW_j + B_i + C(B_iT_k)_l + J_m + e_{ijklm}$$

Where μ = overall mean, T_k = fixed effect of treatment, W_j = fixed effect of week, T_kW_j = fixed effect of interaction between treatment and week, B_i = random effect of block, $C(B_iT_k)_l$ = random effect of cow nested within block and treatment, J_m = random effect of Julian date, and e_{ijklm} = residual error. DMI for the close-up period was analyzed using days prepartum as the fixed effect of time rather than the effect of week. The model did not include the effect of time for variables recorded once per period.

The covariance structure used for weekly analysis was the first-order autoregressive structure because it resulted in the lowest Bayesian information criterion for most variables. The

spatial power covariance structure was used for plasma metabolite data because the time between some measurements was not equal. The VC covariance structure was used for DMI during the CU period because it was the only covariance structure that would run for that variable. Pre-calving BCS was used as a covariate for post-calving BCS and BCS change while BW at calving was used as a covariate for post-calving BW and BW change. BW change was calculated using regression analysis of BW recorded 3x per week. BCS change was calculated using regression analysis of average BCS from each week of lactation. Normality of residuals was checked with normal probability and boxplots and homogeneity of variances with box plots with plots of residuals versus predicted values. Effects were declared significant at $P \leq 0.05$ for main effects and $P \leq 0.10$ for interactions while tendencies were declared at $P \leq 0.10$ for main effects and $P \leq 0.15$ for interactions. When interactions were $P \leq 0.15$, the slice option was used to evaluate treatment effects within week.

Results

Close-up Period DMI

There was no effect of treatment for DMI during the CU period ($P = 0.78$). Average DMI for CON was 15.9 kg/d and average DMI for N3 was 15.7 kg/d (SEM = 0.57 kg/d). The effect of time was significant ($P < 0.0001$), but there was no treatment by time interaction ($P = 0.45$).

Fresh Period Production Data

Production results from the FR period are reported in Table 4.5. N3 reduced colostrum yield at first milking (1.2 kg, $P = 0.05$). There was no effect of treatment on milk yield ($P = 0.81$) or DMI ($P = 0.62$). N3 tended to reduce FCM (3.4 kg/d, $P = 0.09$) and ECM yield (3.0 kg/d $P = 0.10$). N3 also reduced milk fat yield (0.18 kg/d, $P = 0.05$, Figure 4.1) and tended to reduce milk fat content (0.25%, $P = 0.10$, Figure 4.2). There was no effect of treatment on milk protein yield, SCC, BW, or BW change. Treatment tended to interact with time for BCS as N3 increased BCS

during the second week of lactation ($P = 0.13$, Figure 4.3), but not during the other weeks. BCS change was not affected by treatment.

Carryover Period Production Data

Production results from the CO period are reported in Table 4.6. A treatment by time interaction was observed for DMI ($P = 0.07$) where N3 tended to reduce DMI in week 9 by 1.0 kg/d, but there were no treatment differences for the other weeks during the CO period. Treatment interacted with time to impact milk fat yield ($P = 0.10$, Figure 4.1) and milk fat content ($P < 0.01$, Figure 4.2). N3 reduced milk fat yield and content during weeks 4 and 5 of lactation. From weeks 6-9, there were no significant differences in milk fat yield and milk fat content between N3 and CON. There was no effect of treatment on the yields of milk, milk protein, FCM, or ECM, BW, BW change, BCS, or BCS change ($P > 0.20$).

Plasma Metabolites

Plasma content of insulin, glucose, NEFA, and BHB are reported in Table 4.7. N3 did not impact plasma concentrations of insulin, NEFA, and BHB during the fresh period. We observed a treatment by time interaction for plasma glucose where N3 reduced plasma glucose concentrations at d 14 ($P < 0.01$).

Milk FA Contents and Yields

Milk FA contents are reported in Table 4.8. Sources of FA were classified as de novo (< 16 carbons), mixed (16-carbon), and preformed (> 16 carbons). Treatment did not affect sources of milk FA. N3 increased milk fat content of most *trans* 18:1 isomers (*trans*-6-8 C18:1, *trans*-9 C18:1, *trans*-11 C18:1 $P < 0.01$) and increased milk fat content of *cis*-9, *trans*-11 C18:2. N3 increased milk fat concentrations of C20:5 n-3 and C22:5 n-3 ($P < 0.0001$). A treatment by time interaction was observed for *trans*-10 C18:1 where N3 increased its concentration during week 3

(Figure 4.4). Milk fat concentrations of C22:6 n-3 were below detectable limits for most cows on the control treatment. Because of this, we were unable to achieve normality when performing our statistical test, so only the means and standard error of the mean are reported for this FA. N3 increased milk fat content of C22:6 n-3 (0.05 g/100 g FA). N3 treatment also increased milk fat content of total n-3 FA and reduced the ratio of n-6:n-3 in milk fat ($P < 0.0001$).

Milk FA yields are reported in Table 4.9. N3 reduced the yields of de novo (41 g/d; $P = 0.01$) and mixed FA (79 g/d; $P < 0.01$). There was no effect of treatment on the yield of preformed FA ($P = 0.14$). N3 increased the yields *trans*-9 C18:1, *trans*-11 C18:1, and *cis*-9, *trans*-11 C18:2 ($P < 0.01$) and tended to increase yields of *trans*-6-8 C18:1 and *trans*-10 C18:1 ($P < 0.10$). N3 reduced milk FA yields of C16:0, *cis*-9 C18:1, C18:2 n-6, C18:3 n-3, and total n-6 ($P < 0.05$) and increased the yields of C20:5 n-3, C22:5 n-3 ($P < 0.0001$), and C22:6 n-3 (0.88 g/d).

Discussion

To the best of our knowledge, our study is the first to feed a calcium salt enriched with EPA and DHA to dairy cows in the 3 weeks before and 3 weeks after calving and subsequently examine carryover effects through peak lactation. Some studies have examined feeding EPA and DHA during the dry period (Jolazadeh et al., 2019; Badiei et al., 2014), and others have fed n-3 FA during early lactation (Heravi-Moussavi et al., 2007; Hostens et al., 2011; Swanepoel & Robinson, 2020) although many did not start until after the first 2 weeks of lactation (Greco et al., 2015; Sinedino et al., 2017; Juchem et al., 2008; Silvestre et al., 2011). One study did feed unprotected fish oil to dairy cows during the same time period that we chose, but their feeding rates were very high (~130 g/d EPA & DHA) and induced milk fat depression (MFD) (Mattos et al., 2004). A recent abstract fed cows a calcium salt of fish oil similar to the supplement we used

during the close-up and immediate postpartum periods to evaluate potential interactions with supplemental rumen-protected methionine, but they did not evaluate subsequent carryover effects and only fed ~1 g/d during the prepartum period and ~6 g/d of EPA and DHA during the fresh period (France et al., 2022). Therefore, we saw a need to examine the effects of feeding a calcium salt enriched in EPA and DHA to cows during the transition period and evaluate potential carryover effects. We formulated our diets to feed ~18 g/d of EPA and DHA during the CU period and ~26 g/d during the FR period.

Our treatment diets were very similar and were typical for close-up and fresh diets fed in the Midwestern United States. The only difference was the inclusion of the 2 different calcium salts. We chose to formulate our diets in a way to balance the FA profile as much as possible. In order to achieve this, we used different inclusion levels for our calcium salts since they were 2 commercially available products and not custom FA blends. We were particularly interested in keeping the ratio of palmitic acid (16:0) to oleic acid (18:1n-9) as similar as possible. Previous work from our lab has shown that the ratio of palmitic to oleic acid has significant impact on cow productivity, particularly in transition cows (de Souza et al., 2021) and high-producing cows (de Souza et al., 2019; Western et al., 2020). The CON FR diet was formulated to provide ~190 g/d of palmitic acid and ~170 g/d of oleic acid while the N3 FR diet was formulated to provide ~170 g/d of palmitc acid and ~165 g/d of oleic acid. This resulted in ratios of palmitic:oleic to be 1.1 and 1.0 for CON and N3, respectively. These ratios were similar for the CU diets, but the FA intakes were lower. After taking the ratio of palmitic to oleic into account, we chose a target feeding rate of EPA and DHA. We chose our feeding rate based on previous feeding studies with n-3 FA (Greco et al., 2015; Heravi-Moussavi et al., 2007) where feeding ~20-25 g/d of EPA and DHA resulted in higher yields of milk and milk fat. Our previous dose-response study with

abomasal infusions of DHA described in Chapter 3 also informed our targeted post-ruminal supply of n-3 FA. For this study, we aimed to feed ~18 g/d and ~26 g/d of EPA and DHA during the CU and FR periods, respectively, to target a post-ruminal supply of 4-5 g/d. This feeding rate assumes an 80% biohydrogenation rate (Palmquist, 2009; Jenkins & Bridges, 2007). These considerations led us to formulate our diets with calcium salt inclusion rates of 0.58% diet DM for the CON diet, and 0.85% diet DM for the N3 treatment diet. Total dietary FA contents were similar between the two treatment groups at ~3.0% for the CU diets and ~3.7% for the FR diets.

A major concern when feeding PUFA is their delivery to the small intestine and subsequent absorption. Since ~70-90% of dietary PUFA are biohydrogenated in the rumen (Palmquist, 2009; Jenkins & Bridges, 2007), this limits the amount of PUFA that can be delivered to the cow. We examined the FA profile of milk from our experimental cows, and our N3 treatment increased milk EPA and DHA content by 0.04 and 0.05 g/100 g FA, respectively, compared to control. These results indicate we were able to successfully deliver some of our EPA and DHA treatments to the cow and are consistent with other studies that examined milk FA profile after feeding a calcium salt of fish oil (Greco et al., 2015; Juchem et al., 2008; Bilby et al., 2006). A challenge in our study - along with most other transition cow studies - was that we were not able to measure how much EPA and DHA were delivered to the small intestine. Since cows on our control diet had below detectable levels of DHA in milk, we can estimate DHA absorption using milk DHA content as a marker of post-ruminal DHA delivery. Cows on our N3 treatment had milk DHA content of 0.05 g/100 g FA. Considering results from our DHA abomasal infusion study where milk DHA content was positively correlated with DHA absorption (0.93), we can estimate that our N3 treatment successfully delivered ~3 g/d of DHA to the small intestine during the FR period. If we assume that biohydrogenation rates of EPA are

similar, we likely also delivered ~3 g/d of EPA post-ruminally. If we assume biohydrogenation rates during the CU period were similar to those in the FR period, we can estimate that we delivered ~4 g/d of EPA and DHA to the small intestine for absorption since inclusion levels of the calcium salts were similar and DMI during the CU period was 70% of DMI during the FR period.

Feeding a calcium salt enriched in EPA and DHA to dairy cows during the CU period reduced colostrum yield by over 1 kg compared to a calcium salt of palm oil. On a percentage basis, this was a dramatic decrease of ~33%. Since limited research has been performed feeding EPA and DHA to dairy cows during the dry period, not much is known about effects on colostrum synthesis. Jolazadeh et al. (2019) did not observe any effects of EPA and DHA on colostrum yield compared to a calcium salt of soybean oil or a non-fat supplemented control. We plan to analyze colostrum composition and FA profile from this study before drawing further conclusions on how EPA and DHA may affect colostrum synthesis in cattle.

We did not observe any treatment effects on milk yield, which is contrary to previous studies that observed increases in milk yield with n-3 FA supplementation (Greco et al., 2015; Heravi-Moussavi et al., 2007; Sinedino et al., 2017). One explanation may be due to our feeding rates of n-3 being different from other studies. Sinedino et al. (2017) provided 10 g/d of DHA while feeding an algae product, and the DHA treatment increased milk yield compared to control. Greco et al. (2015) provided three treatments at differing levels of EPA and DHA that supplied ~21, ~15, and ~10 g/d of EPA and DHA and the treatment providing the most n-3 FA resulted in the highest milk yield. Our target feeding rate was ~26 g/d of EPA and DHA. Another explanation could be timing of supplementation. Greco et al. (2015) did not start feeding n-3 FA until 14 DIM, while Sinedino et al. (2017) started feeding at 27 DIM. Heravi-Moussavi et al. fed

EPA and DHA at ~25 g/d starting at 5 DIM. These studies also did not provide EPA and DHA during the close-up period like we did. France et al. (2022) did provide EPA and DHA during the close-up period, and also did not observe an increase in milk yield, which agrees with our results. A third explanation may relate to different control treatments used. The diets in Greco et al. (2015) all had differing blends of calcium salts of palm oil, safflower oil, and fish oil that altered the dietary FA profile. All of their diets provided EPA and DHA, since their study focused on the dietary ratio of n-6:n-3 (Greco et al., 2015). Sinedino et al. (2017) provided their treatments in a robot milking parlor, so their control treatment was simply a pellet with no algae or FA supplement. Heravi-Moussavi et al. (2007) used a calcium salt of palm oil as their control similar to what we used. The underlying mechanisms of how timing of n-3 FA supplementation and feeding rates affect milk yield are unclear, and more research is needed to better understand this.

Feeding supplemental EPA and DHA to cows during the close-up and fresh period reduced milk fat yield during the fresh period and tended to reduce yields of ECM and FCM. It is not unusual to see a reduction in milk fat when feeding supplemental n-3 FA, especially when unprotected (Donovan et al., 2000; Mattos et al., 2004). Castañeda-Gutiérrez et al. (2007) demonstrated that feeding a calcium salt of fish oil reduced the negative impacts of a ruminal infusion of fish oil on milk fat content and yield. In our study, N3 reduced yields of de novo and mixed FA in milk. Most de novo FA are derived from acetate and butyrate production in the rumen from microbial fiber digestion. Approximately half of mixed FA (C16:0) are also derived from de novo FA synthesis (Mansbridge & Blake, 1997). Providing fish oil to dairy cows has been shown to reduce the yield of de novo and mixed FA in milk (Loor et al., 2005). Although calcium salts are intended to release FA more slowly at rumen pH (Jenkins & Palmquist, 1984), it has been observed that calcium salts of longer chain FA tend to dissociate more easily (Sukhija

& Palmquist, 1990). Therefore, the protection of PUFA is not as effective compared to calcium salts of palm oil high in oleic acid (Jenkins & Bridges, 2007).

Feeding increased levels of PUFA can shift rumen biohydrogenation pathways, which leads to the production of 18-carbon FA *trans* isomers that can induce MFD by inhibiting milk fat synthesis (Griinari et al., 1998; Bauman et al., 2011). EPA and DHA in particular increase production of 18-carbon FA *trans* isomers as they act to inhibit the final biohydrogenation step from *trans* C18:1 to C18:0 (Toral et al., 2017). The most potent inhibitor of milk fat synthesis is *trans*-10, *cis*-12 C18:2 conjugated linoleic acid (CLA) (Baumgard et al., 2000). Although we did not detect this CLA isomer in the milk fat of our treatment cows, we did detect greater levels of several *trans* C18:1 isomers in milk of our treatment cows, indicating shifts in biohydrogenation pathways. This is consistent with Juchem et al. (2008) who fed a calcium salt enriched with EPA and DHA to dairy cows. Swanepoel and Robinson (2020) fed a fish oil supplement providing ~11 g/d of EPA and DHA and observed similar reductions in milk fat yield with increased milk fat content of *trans* C18:1 isomers. Similar to our study, they did not observe changes in milk fat content of *trans*-10, *cis*-12 C18:2 CLA (Swanepoel & Robinson, 2020). *Trans*-10 C18:1 can be used as a marker for mild MFD as reduction in milk fat is highly correlated with milk fat content of this FA (Bauman et al., 2011; Lock et al., 2007). We observed a treatment by time interaction for this isomer where N3 increased its content in milk fat during week 3, which may indicate a build-up of biohydrogenation intermediates over time. N3 also increased the yield of this FA in milk.

To meet increased nutrient demands at the onset of lactation, transition dairy cows mobilize adipose tissue (Contreras & Sordillo, 2011). Some nutritional interventions such as increasing dietary content of oleic acid during the transition period can reduce lipolysis and body

weight loss compared to diets higher in palmitic acid (de Souza et al., 2021) Abomasal infusion of oleic acid also improved insulin sensitivity and reduced lipolysis in adipose tissue of periparturient dairy cows (Abou-Rjeileh et al., 2023). During this period of negative energy balance, cows experience impaired insulin sensitivity in adipose tissue, which reduces plasma insulin concentrations and increases plasma NEFA levels (Herdt, 2000). Supplementing n-3 FA can increase insulin sensitivity and reduce lipolysis and inflammation in adipose tissue (Sinha et al., 2023). Although we did see a tendency for an interaction between treatment and time for BCS at week 2 indicating that our N3 cows may be losing less body condition compared to our control cows, we observed this difference for only one week of the study, and we did not see any differences in plasma NEFA. We also did not see differences in BW, BW change, insulin levels, or preformed milk FA. Based on these results, we do not think that the reduction in milk energy output from feeding EPA and DHA was a result of reducing lipolysis or altering adipose tissue biology. The reduction in milk fat yield is better explained by production of *trans* 18:1 biohydrogenation intermediates in the rumen since the reductions in milk fat yield were de novo and mixed FA sources and not preformed FA.

The decreases in milk fat yield resulted in a tendency for our N3 treatment to reduce ECM and FCM yield during the FR period. Since ECM and FCM are calculated from milk yield and component contents, it is unsurprising that we observed these results. Effects of feeding EPA and/or DHA to early lactation dairy cows on ECM and FCM have varied depending on how milk fat synthesis and milk yield were affected. Greco et al., (2015) observed increases in both milk yield and fat yield, which subsequently increased FCM yield in a diet higher in n-3 content. Sinedino et al. (2017) observed no changes in ECM or FCM when feeding an algae supplement due to a reduction in milk fat content and an increase in milk yield. Hostens et al., (2011) also

fed algae to cows and observed no changes in fat- and protein-corrected milk yield during the first 12 weeks of lactation since algae supplementation increased milk yield but decreased milk fat content and yield.

We observed an interaction of treatment and time during the CO period for milk fat yield and content. At weeks 4 and 5 of lactation, CON continued to maintain higher milk fat yields compared to our N3 treatment despite all cows being fed a common diet. From week 6 onward, there were no treatment differences in milk fat yield and content. Since cows normally recover from MFD 2-3 weeks after induction (Rico & Harvatine, 2013), we expected milk fat yields from the N3 treatment group to increase around week 6 or 7 following the removal of EPA and DHA from the diet. Despite this hypothesis, we did not see this effect. Our CO effects indicate that our N3 treated cows may have synthesized less milk fat than expected due to the removal of the calcium salt. The N3 calcium salt provided palmitic acid, which is beneficial to increasing milk fat production (dos Santos Neto et al., 2021). Our CO results also indicate that the FA profile of our control fat supplement may have been more optimal to support milk fat production than our N3 supplement. FA supplementation during the immediate postpartum can affect subsequent lactation performance as demonstrated by de Souza et al. (2021). Carryover effects on milk production may be explained by increases in mammary cell number (Akers, 2002) or cell secretory activity (Nørgaard et al., 2005). After 2 weeks without the FA supplement, our CON treated cows had lower yields of milk fat and were similar to the milk fat yields of our N3 treated cows, which were consistent over time. This suggests that removing the FA supplement rich in palmitic and oleic acid had detrimental effects on milk fat yield. More research on FA supplementation during the transition period and subsequent carryover effects is needed to better understand these results.

A limitation of our study is that the inclusion levels of our calcium salts were different across treatments. We acknowledge that this could have affected our results. As previously explained in this discussion, we chose to use different inclusion levels in order to balance FA profiles as much as possible. Despite this decision, we did not observe major treatment differences in FA intakes. Total FA content was 3.67% diet DM for CON and 3.75% for N3, and mean DMI was the same for both treatments at 22.3 kg/d. Another limitation of this study is that we did not evaluate digestibility, which would have given us a more accurate idea of FA intake and absorption to better interpret our results. Evaluating nutrient digestibility may have also helped us to better explain our results since our treatment did not impact DMI or BW change but did impact milk energy output.

Our next steps include looking at immune and reproductive effects from this study as well as adipose tissue gene expression and insulin sensitivity, which will be reported in separate papers. Results from previous studies have indicated a need for n-3 FA to reduce inflammation and improve reproductive performance in dairy cows (Greco et al., 2015; Sinedino et al., 2017). If our results did reduce inflammation, the spared nutrients did not go towards supporting milk production. The results from these analyses will help us to determine future work in order to provide blends of FA that can optimize supply of biologically important FA so that we can improve immune function and reproductive performance of dairy cows without sacrificing production of milk and milk components.

Conclusion

In conclusion, feeding a calcium salt enriched in EPA and DHA to dairy cows during the close-up and fresh period did not affect DMI or milk yield, but reduced milk fat yield in the fresh period and early carryover period compared to feeding a calcium salt of palm oil. Our EPA and

DHA treatment also tended to reduce yields of colostrum, ECM, and FCM during the fresh period. Feeding EPA and DHA increased milk n-3 content. Future research should focus on optimal timing of n-3 FA supplementation and feeding rates as well as examining effects of n-3 FA on nutrient digestibility.

CHAPTER 5

CONCLUSION

The long-chain n-3 FA, EPA and DHA, are conditionally essential nutrients that play important roles in the immune and reproductive systems. Supplementing these FA to dairy cows may be beneficial, but also presents several challenges. Understanding optimal feeding and delivery rates of these FA are of primary interest. Other key areas of focus include investigating how n-3 FA are transported in the blood and how they affect digestibility of nutrients and transition cow productivity and health. To our knowledge, no studies have investigated increasing abomasal infusion doses of DHA in dairy cows or potential in vivo markers to estimate DHA absorption. We are also not aware of any studies that have supplemented EPA and DHA to dairy cows during the close-up and fresh periods and evaluated carryover effects through peak lactation. Therefore, the objective of our studies was to evaluate the effects of increasing abomasal infusion doses of DHA on plasma lipids, milk FA, nutrient digestibility, and milk production responses in dairy cows, and to evaluate the effects of feeding a calcium salt enriched in EPA and DHA to dairy cows during the transition period on milk production responses and milk FA.

In Chapter 3, we determined that abomasal infusions of 2, 4, and 6 g/d of DHA linearly increased DHA content in plasma phospholipids, triacylglycerols, and cholesterol esters. Phospholipids had the largest DHA content, but the triacylglycerol fraction had the highest percent increase compared to control. Abomasal infusion of DHA also linearly reduced milk SCC and linearly increased DHA content in milk fat. DHA content in triacylglycerols and milk fat were highly correlated with DHA absorption and may potentially be useful as markers to estimate DHA absorption in future studies as well as in commercial farm settings.

In Chapter 4, we fed a calcium salt enriched in EPA and DHA to dairy cows during the 3 weeks prepartum and 3 weeks postpartum. Feeding EPA and DHA to dairy cows did not affect milk or milk protein yield, but reduced the yield of milk fat, which tended to reduce the yields of ECM and FCM during the fresh period. When feeding a common diet to all cows from weeks 4 to 9 postpartum, we observed carryover effects where our EPA and DHA treatment reduced milk fat yield during weeks 4 and 5. Feeding EPA and DHA reduced the yields of de novo and mixed FA sources in milk fat and increased the yields of n-3 FA and *trans* 18:1 isomer in milk.

Overall, the results from these studies provide insights on how n-3 FA affect dairy cow physiology and how supplementation may be used on-farm. Although results from our abomasal infusion study indicate that DHA may improve milk quality by reducing SCC and cow health by increasing phospholipid content of n-3 FA, caution must be taken when feeding n-3 FA to dairy cows. Results from our transition cow study indicate that feeding a calcium salt of EPA and DHA resulted in a mild milk fat depression that reduced the yields of FCM and ECM. Since dairy farmers are predominantly paid based on the yield of milk components, future research should focus on methods to effectively supply essential n-3 FA to dairy cows during the transition period without compromising milk fat production. Other areas of interest include examining effects of n-3 on immune function in cows, developing more effective rumen-protection technologies to maintain milk fat production and increase n-3 delivery, and determining optimal feeding rates of n-3 to dairy cows to investigate if cows benefit from additional n-3 supplementation. Emphasis should be placed on investigating blends of FA that optimize dairy cow health, reproduction, and productivity to maintain farm profit and sustainability.

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APPENDIX

Table 2.1 Nomenclature of common fatty acids in dairy cattle nutrition

Common Name	Abbreviation	Biochemical Designation	Nutritional Designation	Classification	Major Dietary Sources
Palmitic acid	PA	16:0	16:0	saturated	some fat supplements
Stearic acid	SA	18:0	18:0	saturated	some fat supplements, biohydrogenation of 18:1, 18:2, 18:3
Oleic acid	OA	*cis*-9 18:1	18:1 n-9	monounsaturated, omega-9	corn, high-oleic soybeans, calcium salts
Linoleic acid	LA	*cis*-9, *cis*-12 18:2	18:2 n-6	polyunsaturated, omega-6	corn, soybeans, seed oils
Alpha-linolenic acid	ALA	*cis*-9, *cis*-12, *cis*-15 18:3	18:3 n-3	polyunsaturated, omega-3	leafy forages, flaxseed oil
Arachidonic acid	ARA	*cis*-5, *cis*-8, *cis*-11, *cis*-14	20:4 n-6	polyunsaturated, omega-6	none
Eicosapentaenoic acid	EPA	*cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17	20:5 n-3	polyunsaturated, omega-3	fish oil
Docosahexaenoic acid	DHA	*cis*-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19	22:6 n-3	polyunsaturated, omega-3	fish oil, algae

Table 2.2 Plasma lipid classes in cattle

Item	Lipoprotein Transport[1]	Major FA[1]	Proportion in Plasma (%)[1]	Major source[2]	Biological Role[2]
Cholesterol ester	HDL, LDL	18:2 n-6, 18:3 n-3	57.0	esterification of FA from TAG in liver	cholesterol transport, conservation of essential FA
Phospholipid	HDL, LDL	16:0,18:0,18:1 n-9, 18:2 n-6	24.8	generated from TAG in endoplasmic reticulum	form phospholipid monolayer in lipoprotein particles, cell membrane development
Triacylglycerol	chylomicrons, some VLDL	16:0,18:0,18:1 n-9	4.8	dietary fats	fat storage, energy source, milk fat secretion
NEFA	none (attached to serum albumin)	16:0,18:0,18:1 n-9	2.4	released from adipose tissue	energy source, milk fat secretion

[1]Source: Christie (1981). Reported as % of total lipids in plasma
[2]Source: Nelson et al. (2021)

Table 2.3 Role of EPA and DHA in inflammation and immunity[1]

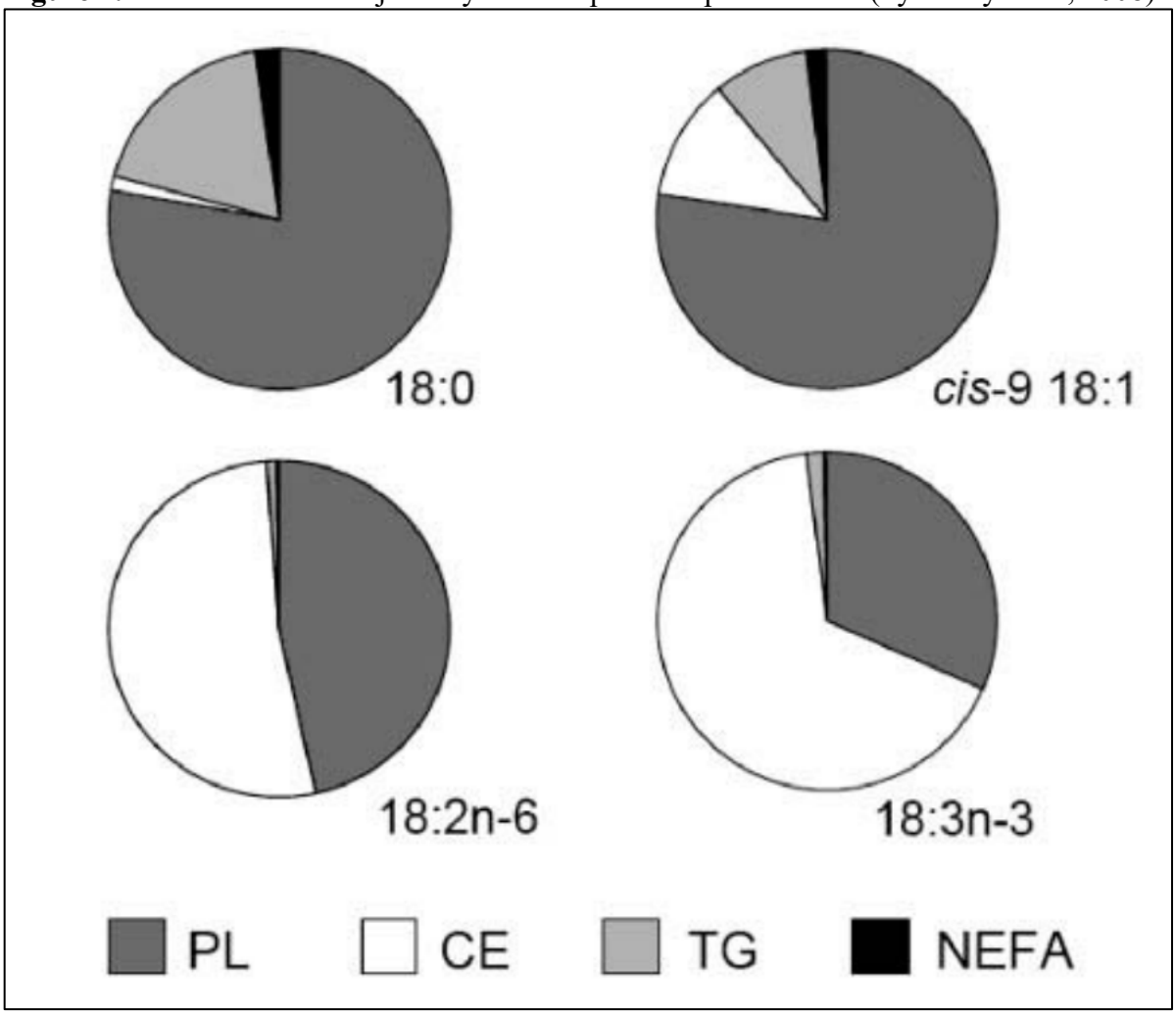
Anti-inflammatory effect	Likely mechanism involved
Reduced leukocyte chemotaxis	Decreased production of chemo-attractants and down-regulated expression of chemo-attractant receptors
Reduced adhesion molecule expression and decreased leucocyte-endothelium interaction	Down-regulated expression of adhesion molecule genes
Decreased production of eicosanoids from arachidonic acid	Lowered membrane content of arachidonic acid; Inhibition of arachidonic acid metabolism
Decreased production of arachidonic acid containing endocannabinoids	Lowered membrane content of arachidonic acid
Increased production of 'weak' eicosanoids from EPA	Increased membrane content of EPA
Increased production of anti-inflammatory EPA and DHA containing endocannabinoids	Increased membrane content of EPA and DHA
Increased production of pro-resolution resolvins and protectins	Increased membrane content of EPA and DHA
Decreased production of inflammatory cytokines	Down-regulated expression of inflammatory cytokine genes
Decreased T-cell reactivity	Disruption of cell membrane rafts due to increased membrane content of EPA and DHA

[1]Source: Calder (2012)

Table 2.4 Summary of effects of EPA and DHA on milk production of cows when supplemented during early lactation

n-3 Supplement	Amount EPA/DHA fed (g/d)	Control	Duration of n-3 Feeding	Milk Yield	Fat Yield	Source
algae	~10 g/d	pellets with no algae	27-147 DIM	+0.9 kg/d	-0.04 kg/d	Sinedino et al. (2017)
calcium salt of fish oil	~25 g/d	calcium salt of palm oil	5-50 DIM	+4.0 kg/d	+0.10 kg/d	Heravi Moussavi et al. (2007)
calcium salt of fish oil	~21 g/d	calcium salt of safflower oil	14-104 DIM	+3.6 kg/d	+0.18 kg/d	Greco et al. (2015)
calcium salt of fish oil	~20 g/d	calcium salt of palm oil	30-160 DIM	unaffected	not measured	Silvestre et al. (2011)
calcium salt of fish oil	~20 g/d	tallow	26-145 DIM	unaffected	unaffected	Juchem et al. (2008)
calcium salt of fish oil	~6 g/d	calcium salt of palm oil	21 d prepartum to 28 DIM	unaffected	+0.13 kg/d	France et al. (2022 ADSA abstract)
fish oil	~128 g/d	olive oil	21 d prepartum to 21 DIM	unaffected	-0.46 kg/d	Mattos et al. (2004)
algae	~44 g/d	concentrate with no algae	21 d prepartum to 84 DIM	+3.0 kg/d	-0.31 kg/d	Hostens et al. (2011)
fish oil-based supplement	~11 g/d	unsupplemented TMR	~14-160 DIM	+2.5 kg/d	-0.09 kg/d	Swanepoel & Robinson (2020)

Figure 2.1 Distribution of major fatty acids in plasma lipid fractions (Tyburczy et al., 2008)[1]



[1] PL = Phospholipids, CE = Cholesterol esters, TG = Triglycerides, NEFA = Nonesterified fatty acids

Figure 2.2 Desaturation and elongation pathway for, ALA, EPA and DHA. (Huang et al., 2020).

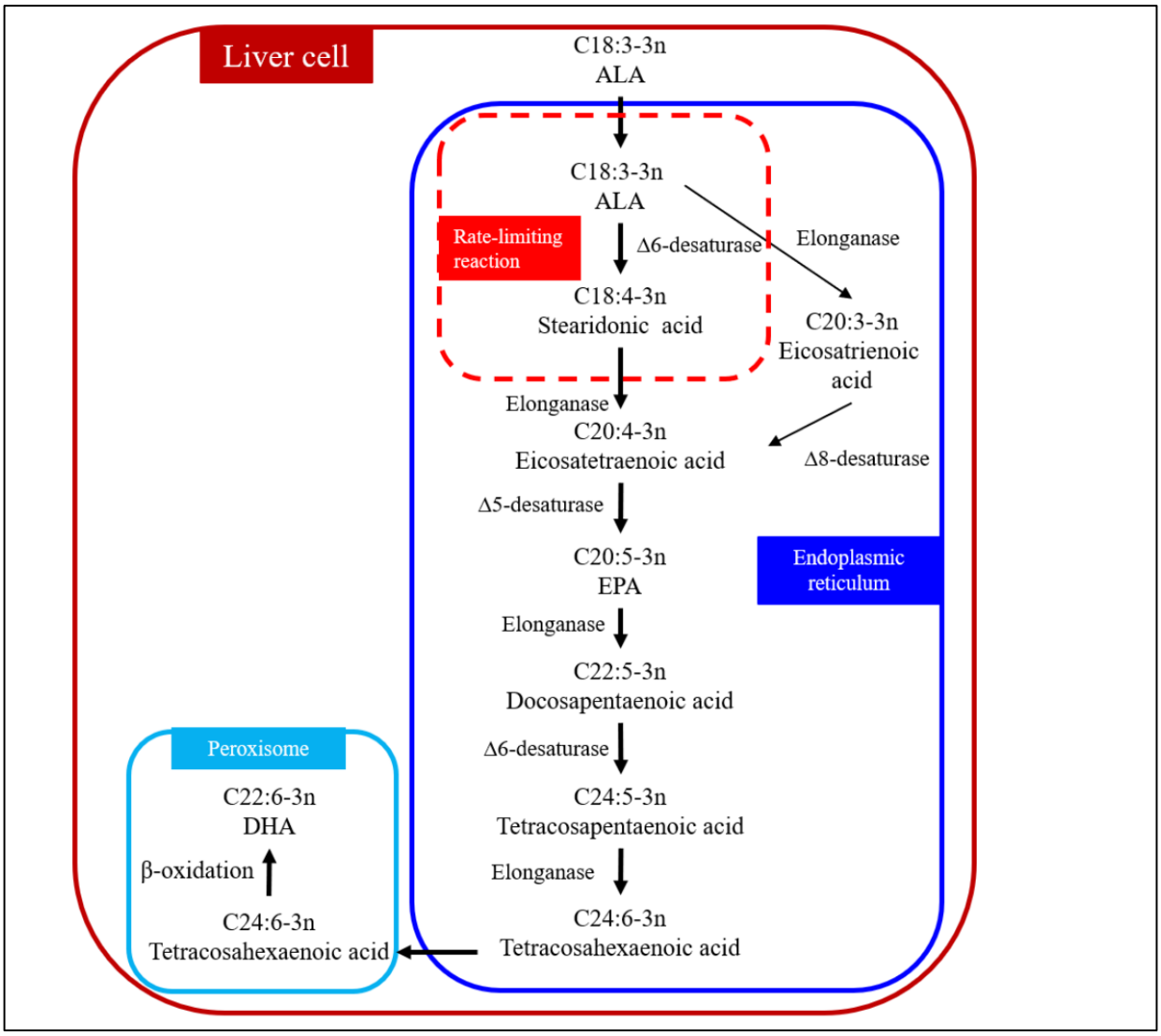
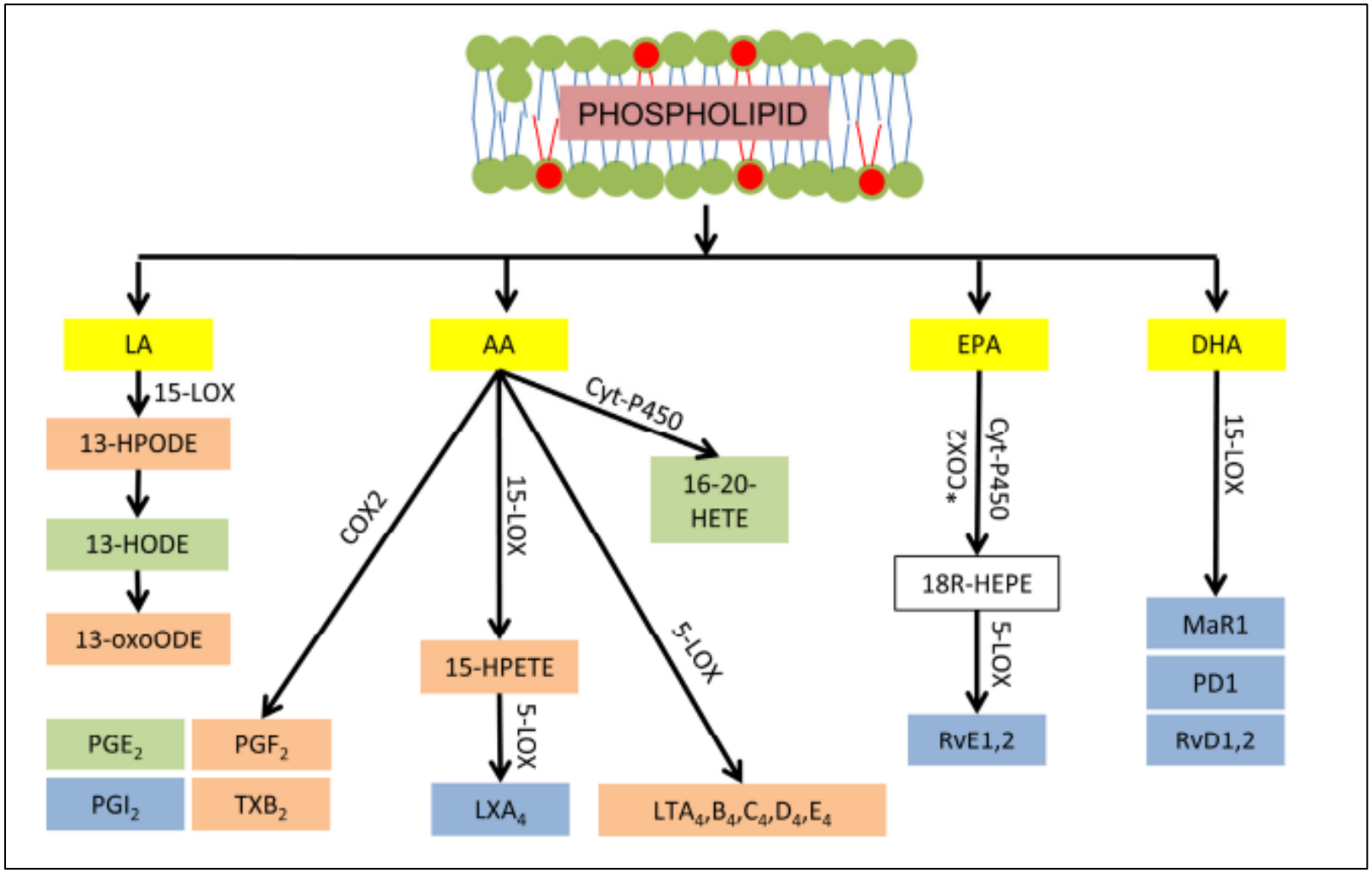


Figure 2.3 Synthesis pathway of major PUFA[1], oxylipid derivatives[2], and functions[3] (Raphael & Sordillo, 2013).



[1] LA = linoleic acid (n-6), AA = arachidonic acid (n-6), EPA = eicosapentaenoic acid (n-3), DHA = docosahexaenoic acid (n-3)
[2] HEPE = hydroxyeicosapentaenoic acid, HETE = hydroxyeicosatetraenoic acid, HODE = hydroxyoctadienoic acid, LT = leukotriene, LX = lipoxin, MaR= maresin, PG = prostaglandin, Rv = resolving, PD = protectin, TX = thromboxane
[3] Oxylipid functions: pro-inflammatory (orange), resolving (blue), variable function (green)

Table 3.1 Fatty acid profile and content of infusate	Algal Oil[1]
Total FA content, % DM	92.9
FA Profile (g/100 g FA)	
16:0	11.5
18:0	0.70
18:1 n-9	6.20
18:2 n-6	0.52
18:3 n-3	0.19
20:5 n-3	0.42
22:5 n-6	12.9
22:6 n-3	65.3

[1] Life's DHA™, DSM-Firmenich (Basel, Switzerland).

Table 3.2 Ingredients and nutrient composition of diet	Diet
Ingredient, % DM	
Corn Silage	41.7
Alfalfa Silage	15.3
Ground Corn	20.2
Soybean Meal	9.21
Soybean Hulls	5.39
Vitamin and Mineral Mix[1]	2.04
Protein Mix[2]	5.76
Potassium Carbonate[3]	0.46
Nutrient Composition, % DM	
NDF	28.7
Forage NDF	21.8
CP	16.6
Starch	29.1
FA	1.86
FA Profile (g/100 g FA)	
16:0	15.0
18:0	2.79
18:1 n-9	18.5
18:2 n-6	52.7
18:3 n-3	9.31

[1]Vitamin and mineral mix contained 20.4% calcium carbonate, 12.5% calcium phosphate, 4.47% sodium sesquinate, 14.1% white salt, 15.7% calcium magnesium carbonate (Min AD, Min ad Inc), 0.03% vitamin A, 0.01% Vitamin 500, 0.34% vitamin E, 14.7 % corn grain, 0.92% Energizer Tallow, 0.51% trace minerals (Micro5, Micronutrients), 2.36% selenium, 0.15% magnesium oxide, and 4.70% magnesium sulfate.
[2]Contains 41.3% heat-treated soybean meal (Amino Plus, Ag Processing Inc), 11.9% calcium carbonate, 18.6% blood meal (Caledonia Pass, Papillon), 10.2% corn grain, 1.04% rumen-protected methionine (Smartamine M, Adisseo), 14.6% sodium sesquinate, and 2.45% urea.
[3]Ion Plus (D&D Ingredients, LLC; Delphos, Ohio)

Table 3.3 Total tract apparent nutrient digestibility of cows abomasally infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
DMI, kg/d	32.5	32.0	32.6	31.4	0.77	0.13	0.31	0.11
Intake, g/d								
Total FA	617	610	624	607	14.8	0.59	0.51	0.14
16-carbon FA	91.3	90.1	91.9	89.0	2.15	0.28	0.43	0.1109
18-carbon FA	515	507	516	499	12.3	0.20	0.53	0.14
22-carbon FA	1.20	3.56	6.03	8.37	0.03	<0.0001	0.59	<0.01
Digestibility, %								
DM	68.4	68.2	68.8	68.5	0.657	0.75	0.91	0.41
NDF	47.3	47.4	48.2	48.3	1.39	0.25	0.96	0.74
Total FA	81.4	79.8	79.2	82.5	2.55	0.70	0.13	0.70
16-carbon FA	78.5	75.4	76.3	77.9	1.86	0.91	0.15	0.63
18-carbon FA	84.5	83.1	82.4	85.8	2.66	0.64	0.14	0.62
22-carbon FA	76.6	88.2	89.9	91.5	0.650	<0.0001	<0.0001	<0.01
DHA	n/a	95.0	93.7	94.6	0.557	0.60	0.13	n/a
Absorbed, g/d								
Total FA	503	490	495	501	22.7	0.95	0.36	0.68
16-carbon FA	71.7	68.4	70.3	69.3	2.57	0.42	0.43	0.24
18-carbon FA	435	424	426	428	19.9	0.60	0.40	0.70
22-carbon FA	0.92	3.14	5.42	7.66	0.03	<0.0001	0.67	0.50
DHA	0.00	1.88	3.77	5.72	0.01	<0.0001	0.03	0.38

[1]Feed ingredients and orts samples were collected on the last four days of each infusion period. Orts samples were composited by cow and period. Fecal samples were collected every 9 hours and composited by cow and period.
[2]Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3]*P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.

Table 3.4 Milk production and plasma insulin of cows abomasally infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
Yields, kg/d								
Milk	45.8	45.7	45.4	45.3	1.37	0.28	1.00	0.87
FCM[4]	44.9	45.3	45.0	44.7	1.59	0.84	0.66	0.81
ECM[5]	44.9	45.2	44.9	44.6	1.48	0.70	0.69	0.86
Fat	1.54	1.57	1.56	1.55	0.06	0.96	0.50	0.75
Protein	1.40	1.39	1.39	1.37	0.04	0.28	0.79	0.88
Lactose	2.26	2.26	2.29	2.26	0.10	0.93	0.63	0.44
Content, %								
Fat	3.42	3.50	3.51	3.45	0.06	0.49	0.10	0.95
Protein	3.08	3.09	3.08	3.03	0.09	0.04	0.07	0.70
Lactose	4.94	4.94	4.95	4.97	0.02	<0.01	0.18	0.46
SCC, 1000/mL	13.3	12.0	10.4	10.8	1.97	0.05	0.38	0.59
BCS[6]	3.06	3.09	3.07	3.05	0.07	0.61	0.31	0.63
BW, kg[7]	731	734	727	729	19.4	0.39	1.00	0.17
Plasma Insulin, uIU/mL	14.5	15.3	15.6	15.6	1.43	0.27	0.61	1.00

[1]Milk yield was recorded and samples were collected on the last 4 days of each infusion period and composited by cow and period.
[2]Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3]*P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.
[4]3.5% FCM = [(0.4324 × kg of milk) + (16.216 × kg milk of fat)] (NRC, 2001).
[5]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.20 × kg of milk protein). This equation corrects milk to a 0.68 Mcal/kg energy basis (Tyrrell and Reid, 1965).
[6]Body condition score was recorded on the last day of each infusion period.
[7]Body weight was measured on the last 2 days of each infusion period.

Table 3.5 Fatty acid (FA) content of plasma lipids of cows abomasally infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
Selected Individual FA[4], g/100 g FA								
14:0	0.54	0.53	0.52	0.55	0.02	0.74	0.04	0.36
16:0	9.51	9.92	9.62	10.2	0.33	0.16	0.71	0.22
16:1 n-7	1.00	1.00	0.98	1.00	0.02	0.95	0.45	0.42
18:0	16.3	16.5	16.3	16.3	0.59	0.88	0.87	0.73
18:1 n-9	4.59	4.60	4.28	4.33	0.11	<0.01	0.67	0.03
20:0	0.026	0.024	0.028	0.033	0.002	0.04	0.12	0.63
22:0	0.020	0.016	0.019	0.023	0.001	0.12	0.02	0.60
∑ n-6	56.5	55.0	55.8	55.5	0.81	0.58	0.45	0.35
18:2 n-6	50.4	49.9	50.6	50.5	0.73	0.78	0.80	0.56
18:3 n-6	0.72	0.71	0.70	0.61	0.10	0.06	0.31	0.71
20:2 n-6	0.069	0.071	0.071	0.076	0.004	<0.01	0.48	0.34
20:3 n-6	2.35	2.36	2.40	2.13	0.10	0.21	0.20	0.47
20:4 n-6	1.58	1.53	1.63	1.46	0.13	0.66	0.66	0.46
22:4 n-6	0.29	0.28	0.30	0.25	0.03	0.27	0.51	0.40
22:5 n-6	0.08	0.10	0.12	0.13	0.01	<0.001	0.31	0.70
∑ n-3	3.94	3.84	4.07	3.74	0.27	0.7	0.59	0.37
18:3 n-3	3.20	3.33	3.44	3.21	0.15	0.84	0.25	0.65
20:5 n-3	0.26	0.23	0.26	0.21	0.03	0.39	0.67	0.15
22:5 n-3	0.35	0.32	0.35	0.28	0.03	0.16	0.40	0.21
22:6 n-3	0.13	0.22	0.27	0.30	0.02	<0.001	0.22	0.88
n-6:n-3	13.9	13.7	13.1	14.0	0.72	0.93	0.40	0.54

[1] Blood collections occurred every 9 hours on the last four days of each infusion period and composited by cow and period.
[2] Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3] *P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.

Table 3.5 (cont'd)

[4]A total of approximately 60 individual fatty acids were quantified. Only select fatty acids are reported in the table.

Table 3.6 Fatty acid (FA) content of plasma phospholipids (PL) of cows abomasally infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
Selected Individual FA[4], g/100 g FA								
14:0	0.16	0.15	0.16	0.16	0.01	0.30	0.34	0.93
16:0	12.4	12.6	12.5	12.6	0.21	0.12	0.63	0.52
16:1 n-7	0.83	0.83	0.85	0.83	0.03	0.70	0.42	0.25
18:0	23.9	23.8	23.9	23.9	0.25	1.00	0.82	0.46
18:1 n-9	5.70	5.60	5.29	5.18	0.21	<0.0001	0.95	0.26
∑ n-6	43.1	43.2	43.3	43.1	0.17	0.88	0.64	0.73
18:2 n-6	36.1	35.9	36.0	36.0	0.25	0.98	0.84	0.70
18:3 n-6	0.03	0.02	0.02	0.02	0.00	0.26	0.05	0.76
20:2 n-6	0.10	0.10	0.11	0.11	0.01	0.10	0.43	0.71
20:3 n-6	4.04	4.08	4.04	3.88	0.14	0.08	0.11	0.84
20:4 n-6	2.24	2.31	2.29	2.32	0.09	0.17	0.59	0.30
22:4 n-6	0.52	0.51	0.52	0.52	0.04	0.68	0.75	0.84
22:5 n-6	0.15	0.20	0.23	0.27	0.01	<0.0001	0.08	0.51
∑ n-3	3.17	3.33	3.45	3.69	0.11	<0.0001	0.41	0.41
18:3 n-3	1.63	1.57	1.57	1.65	0.06	0.61	0.01	0.96
20:5 n-3	0.35	0.35	0.34	0.35	0.02	0.85	0.89	0.67
22:5 n-3	0.89	0.91	0.89	0.87	0.08	0.29	0.13	0.53
22:6 n-3	0.30	0.50	0.64	0.82	0.04	<0.0001	0.52	0.21
n-6:n-3	13.9	13.1	12.6	11.8	0.47	<0.0001	0.99	0.55

[1] Blood collections occurred every 9 hours on the last four days of each infusion period and composited by cow and period.
[2] Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3] *P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.
[4] A total of approximately 50 individual fatty acids were quantified. Only select fatty acids are reported in the table.

Table 3.7 Fatty acid (FA) content of plasma cholesterol esters (CE) of cows abomasally infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
Selected Individual FA[4], g/100 g FA								
14:0	0.92	0.87	0.86	0.88	0.04	0.07	0.03	0.98
16:0	3.30	3.20	3.16	3.20	0.09	0.25	0.31	0.91
16:1 n-7	1.03	0.98	0.92	0.96	0.06	0.01	0.02	0.23
18:0	0.89	0.71	0.60	0.45	0.13	0.01	0.86	0.81
18:1 n-9	1.98	1.92	1.81	1.78	0.07	0.0001	0.58	0.33
∑ n-6	78.4	79.1	79.6	79.3	0.56	0.02	0.13	0.78
18:2 n-6	76.7	77.4	77.8	77.6	0.55	0.03	0.14	0.76
18:3 n-6	0.04	0.04	0.04	0.04	0.00	0.91	0.38	0.82
20:3 n-6	0.49	0.50	0.51	0.49	0.02	0.41	0.02	0.49
20:4 n-6	1.12	1.15	1.15	1.15	0.04	0.19	0.47	0.77
∑ n-3	7.08	6.95	7.00	7.19	0.23	0.14	0.01	0.85
18:3 n-3	6.64	6.50	6.55	6.74	0.23	0.15	0.01	0.88
20:5 n-3	0.38	0.39	0.38	0.38	0.02	0.58	0.35	0.60
22:5 n-3	0.02	0.02	0.02	0.02	0.00	0.59	0.82	0.41
22:6 n-3	0.04	0.04	0.05	0.06	0.01	<0.01	0.86	0.96
n-6:n-3	11.22	11.53	11.49	11.09	0.50	0.38	0.00	0.99

[1] Blood collections occurred every 9 hours on the last four days of each infusion period and composited by cow and period.
[2] Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3] *P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.
[4]A total of approximately 45 individual fatty acids were quantified. Only select fatty acids are reported in the table.

Table 3.8 Fatty acid (FA) content of plasma triacylglycerols (TAG) of cows abomasally infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
Selected Individual FA[4], g/100 g FA								
14:0	1.88	1.81	1.88	1.81	0.11	0.59	0.98	0.36
16:0	15.7	16.4	17.4	16.8	0.73	0.19	0.35	0.54
16:1 n-7	1.28	1.29	1.28	1.22	0.06	0.34	0.50	0.89
18:0	24.9	27.1	29.1	29.1	1.56	0.04	0.44	0.80
18:1 n-9	5.97	5.96	5.89	4.96	0.33	0.04	0.12	0.56
∑ n-6	27.1	23.7	20.2	22.0	2.62	0.12	0.33	0.67
18:2 n-6	25.7	22.3	18.8	20.6	2.58	0.12	0.33	0.64
20:2 n-6	0.01	0.02	0.02	0.01	0.01	0.23	0.10	0.29
20:3 n-6	0.58	0.51	0.63	0.54	0.06	0.99	0.81	0.08
20:4 n-6	0.73	0.67	0.61	0.64	0.06	0.24	0.50	0.74
22:4 n-6	0.10	0.10	0.10	0.11	0.01	0.47	0.37	0.78
22:5 n-6	0.04	0.05	0.07	0.06	0.01	0.02	0.53	0.33
∑ n-3	4.22	3.98	3.69	3.97	0.37	0.53	0.48	0.70
18:3 n-3	3.71	3.31	2.88	3.06	0.35	0.14	0.41	0.69
20:5 n-3	0.27	0.25	0.21	0.22	0.03	0.11	0.53	0.71
22:5 n-3	0.15	0.18	0.19	0.18	0.01	0.03	0.06	0.67
22:6 n-3	0.09	0.25	0.40	0.51	0.01	<0.0001	0.001	0.62
n-6:n-3	6.43	5.90	5.41	5.41	0.21	0.0005	0.17	0.61

[1] Blood collections occurred every 9 hours on the last four days of each infusion period and composited by cow and period.
[2] Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3] *P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.
[4]A total of approximately 50 individual fatty acids were quantified. Only select fatty acids are reported in the table.

Table 3.9 Fatty acid (FA) content of plasma nonesterified fatty acids (NEFA) of cows abomasally infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
Selected Individual FA[4], g/100 g FA								
14:0	2.64	2.72	2.27	2.51	0.15	0.18	0.56	0.06
16:0	33.1	33.7	30.6	33.7	1.13	0.79	0.27	0.07
16:1 n-7	0.81	0.61	0.78	0.66	0.08	0.37	0.55	0.04
18:0	22.3	23.3	24.1	24.7	1.02	0.10	0.88	0.98
18:1 n-9	5.50	5.11	5.24	4.66	0.38	0.18	0.81	0.47
∑ n-6	12.7	11.5	14.4	10.7	1.2	0.56	0.30	0.05
18:2 n-6	10.1	9.06	11.86	8.12	1.19	0.55	0.25	0.06
18:3 n-6	0.73	0.66	0.68	0.74	0.07	0.89	0.37	0.86
20:3 n-6	1.32	1.37	1.35	1.32	0.09	0.96	0.56	0.85
20:4 n-6	0.58	0.40	0.51	0.43	0.06	0.16	0.34	0.05
∑ n-3	1.30	1.19	1.51	1.12	0.14	0.76	0.33	0.08
18:3 n-3	1.28	1.19	1.51	1.10	0.14	0.76	0.26	0.07
n-6:n-3	9.87	9.26	9.84	9.56	0.39	0.81	0.61	0.18

[1] Blood collections occurred every 9 hours on the last four days of each infusion period and composited by cow and period
[2] Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3] *P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.
[4]A total of approximately 40 individual fatty acids were quantified. Only select fatty acids are reported in the table.

Table 3.10 Fatty acid (FA) content of milk from cows infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
Summations by Source, g/100 g FA								
De Novo	28.3	28.2	28.2	28.6	0.43	0.22	0.28	0.60
Mixed	37.9	38.6	38.1	38.2	0.81	0.83	0.32	0.18
Preformed	33.8	33.1	33.7	33.2	0.91	0.36	0.72	0.11
Selected Individual FA[4], g/100 g FA								
4:0	2.67	2.70	2.67	2.68	0.17	0.97	0.82	0.53
6:0	1.92	1.96	1.94	1.97	0.10	0.10	0.94	0.13
8:0	1.18	1.21	1.20	1.22	0.04	<0.01	0.34	0.10
10:0	3.45	3.52	3.50	3.56	0.09	0.06	0.86	0.24
12:0	4.46	4.52	4.52	4.59	0.19	0.22	0.99	0.72
14:0	13.5	13.3	13.4	13.6	0.26	0.68	0.01	0.82
16:0	36.2	36.9	36.4	36.5	0.81	0.65	0.33	0.17
16:1 n-7	1.76	1.71	1.71	1.67	0.10	0.08	0.96	0.64
18:0	6.89	6.97	6.89	7.15	0.33	0.24	0.49	0.40
18:1n-9	15.7	15.6	15.5	15.1	0.77	0.09	0.60	0.91
18:2 n-7[5]	0.36	0.36	0.37	0.34	0.04	0.07	0.21	0.20
∑ n-6	2.54	2.56	2.62	2.51	0.10	0.85	0.11	0.28
18:2 n-6	2.22	2.19	2.28	2.18	0.09	0.79	0.23	0.05
18:3 n-6	0.02	0.02	0.02	0.02	0.00	0.01	0.65	0.84
20:2 n-6	0.02	0.02	0.02	0.02	0.00	0.88	0.75	0.67
20:3 n-6	0.10	0.10	0.10	0.10	0.01	0.83	0.41	0.41
20:4 n-6	0.14	0.15	0.15	0.15	0.005	0.15	0.33	1.00
22:4 n-6	0.03	0.03	0.03	0.03	0.002	1.00	1.00	1.00
22:5 n-6	0.01	0.01	0.02	0.02	0.001	<0.0001	1.00	0.15
∑ n-3	0.56	0.57	0.63	0.63	0.02	<0.0001	0.10	0.00

Table 3.10 (cont'd)

18:3 n-3	0.46	0.45	0.48	0.46	0.02	0.24	0.38	<0.001
20:5 n-3	0.03	0.03	0.03	0.03	0.001	0.33	0.27	0.06
22:5 n-3	0.05	0.05	0.06	0.06	0.003	0.02	0.15	0.20
22:6 n-3	0.01	0.04	0.06	0.08	0.002	<0.0001	0.16	0.90
n-6:n-3	4.57	4.46	4.16	4.01	0.10	<0.0001	0.67	0.10

[1] Milk samples were collected three times per day on the last four days of each infusion period and composited by cow and period.
[2] Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3] *P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.
[4]A total of approximately 60 individual fatty acids were quantified. Only select fatty acids are reported in the table.
[5]Conjugated linoleic acid (CLA) *cis*-9, *trans*-11 18:2.

Table 3.11 Fatty acid (FA) yields of milk from cows infused with increasing amounts of DHA (n=8)[1]

	Treatment[2]					P-value[3]		
Item	0	2	4	6	SEM	Linear	Quadratic	Cubic
Summations by Source, g/d								
De Novo	410	418	413	416	21.4	0.79	0.77	0.66
Mixed	550	557	559	555	28.4	0.75	0.62	1.00
Preformed	487	498	491	480	20.4	0.61	0.35	0.75
Selected Individual FA[4], g/d								
4:0	39.1	40.3	39.2	39.3	3.46	0.94	0.57	0.39
6:0	28.1	29.3	28.5	29.0	2.31	0.59	0.65	0.33
8:0	17.3	18.1	17.7	17.9	1.27	0.46	0.52	0.44
10:0	50.4	52.4	51.4	52.0	3.59	0.57	0.62	0.49
12:0	64.9	66.8	66.3	66.5	4.06	0.61	0.66	0.74
14:0	196	197	196	197	9.75	0.96	0.97	0.81
16:0	524	532	534	531	28.2	0.69	0.62	0.98
16:1 n-7	25.2	24.8	24.8	23.9	1.14	0.16	0.64	0.60
18:0	100	104	101	105	6.94	0.38	0.93	0.27
18:1 n-9	225	228	225	218	9.53	0.28	0.34	0.88
18:2 n-7[5]	5.30	5.33	5.31	4.89	0.69	0.17	0.27	0.68
∑ n-6	37.3	38.3	38.6	36.8	2.37	0.75	0.12	0.73
18:2 n-6	32.3	33.1	33.2	31.6	2.15	0.62	0.14	0.79
18:3 n-6	0.33	0.32	0.30	0.26	0.04	0.02	0.51	0.94
20:2 n-6	0.26	0.25	0.26	0.26	0.04	0.96	0.92	0.89
20:3 n-6	1.45	1.47	1.50	1.40	0.10	0.71	0.34	0.61
20:4 n-6	2.05	2.17	2.19	2.16	0.12	0.09	0.14	0.79
22:4 n-6	0.37	0.37	0.37	0.37	0.03	0.88	0.89	0.99
22:5 n-6	0.17	0.21	0.30	0.33	0.02	<0.0001	0.93	0.09
∑ n-3	8.07	8.43	9.20	9.07	0.24	0.0005	0.21	0.13

Table 3.11 (cont'd)

18:3 n-3	6.66	6.60	6.94	6.64	0.17	0.65	0.35	0.09
20:5 n-3	0.46	0.45	0.48	0.43	0.03	0.53	0.27	0.15
22:5 n-3	0.76	0.80	0.87	0.83	0.05	0.04	0.15	0.30
22:6 n-3	0.19	0.58	0.91	1.18	0.04	<0.0001	0.14	0.91
n-6:n-3	4.63	4.51	4.21	4.05	0.11	<0.0001	0.66	0.12
Apparent Transfer Efficiency, %								
22:6 n-3[6]	n/a	28.8	22.8	19.7	0.81	<0.0001	0.10	n/a
22:6 n-3[7]	n/a	30.6	24.1	20.8	0.88	<0.0001	0.10	n/a

[1] Milk samples were collected three times per day on the last four days of each infusion period and composited by cow and period.
[2] Treatments consisted of abomasal infusions of 0, 2, 4, or 6 g/d of docosahexaenoic acid (DHA).
[3] *P*-values associated with contrasts: (1) the linear effect of increasing DHA; (2) the quadratic effect of increasing DHA; and (3) the cubic effect of increasing DHA.
[5]Conjugated linoleic acid (CLA) *cis*-9, *trans*-11 18:2.
[4]A total of approximately 60 individual fatty acids were quantified. Only select fatty acids are reported in the table.
[6]Calculated as (fatty acid yield in milk / amount of fatty acid provided by infusion dose) x 100.
[7]Calculated as (fatty acid yield in milk / amount of fatty acid absorbed) x 100.

Table 3.12 Pearson correlation coefficients between absorbed DHA, DHA in plasma lipid fractions, and DHA in milk for cows abomasally infused with increasing amounts of DHA (n=8)

Item	DHA Absorbed (g/d)	Total Plasma DHA (g/100 g FA)	Plasma DHA in PL (g/100 g FA)	Plasma DHA in TAG (g/100 g FA)	Plasma DHA in CE (g/100 g FA)	Milk DHA Content (g/100 g FA)
DHA Absorbed	1	0.42[1]	0.70	0.92	0.27	0.93
		(<0.05)[2]	(<0.0001)	(<0.0001)	(0.14)	(<0.0001)
Total Plasma DHA		1	0.72	0.60	0.66	0.52
			(<0.0001)	(<0.001)	(<0.0001)	(<0.01)
Plasma DHA in PL			1	0.89	0.82	0.81
				(<0.0001)	(<0.0001	(<0.0001)
Plasma DHA in TAG				1	0.56	0.95
					(<0.001)	(<0.0001)
Plasma DHA in CE					1	0.51
						(<0.01)
Milk DHA Content						1

[1]The Pearson correlation coefficient of the linear relationship between 2 variables.
[2]The *P*-value associated with the linear relationship between 2 variables.

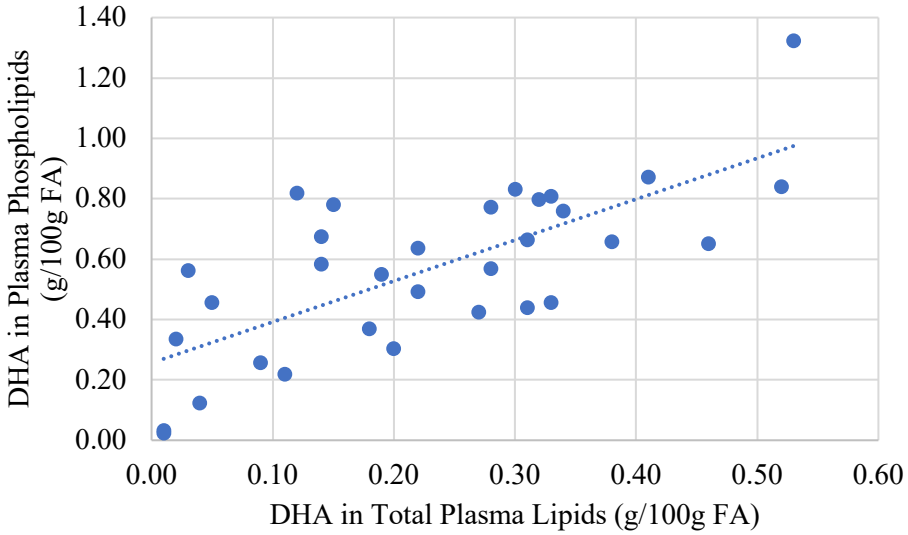


Figure 3.1 Relationship between DHA content of total plasma lipids and plasma phospholipids [DHA in plasma phospholipids (g/100 g FA) = 0.25 + 1.36 x (DHA in total plasma lipids g/100 g FA); $R^2 = 0.52$; $P < 0.0001$].

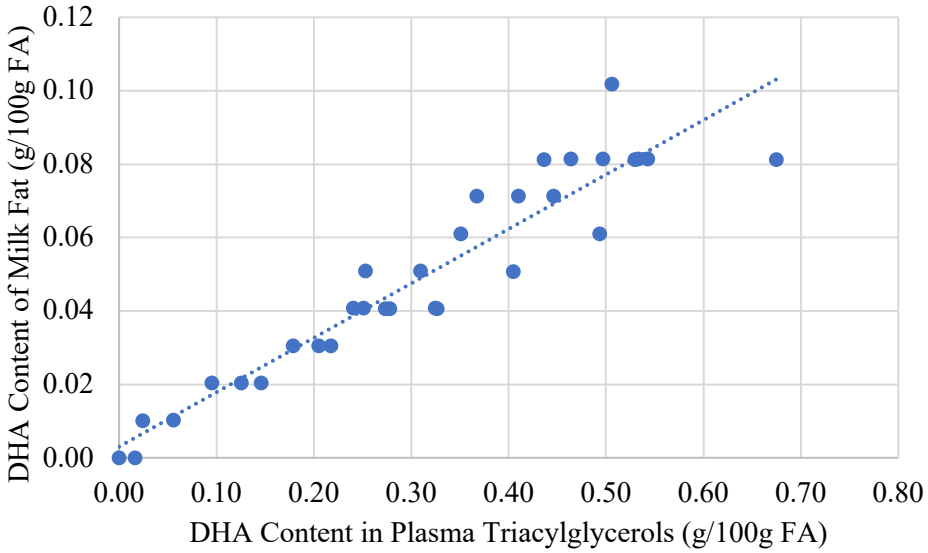


Figure 3.2 Relationship between DHA content of plasma triacylglycerols and milk fat [DHA in milk fat (g/100 g FA) = 0.001 + 0.15 x (DHA in plasma triacylglycerols g/100 g FA); $R^2 = 0.89$; $P < 0.0001$].

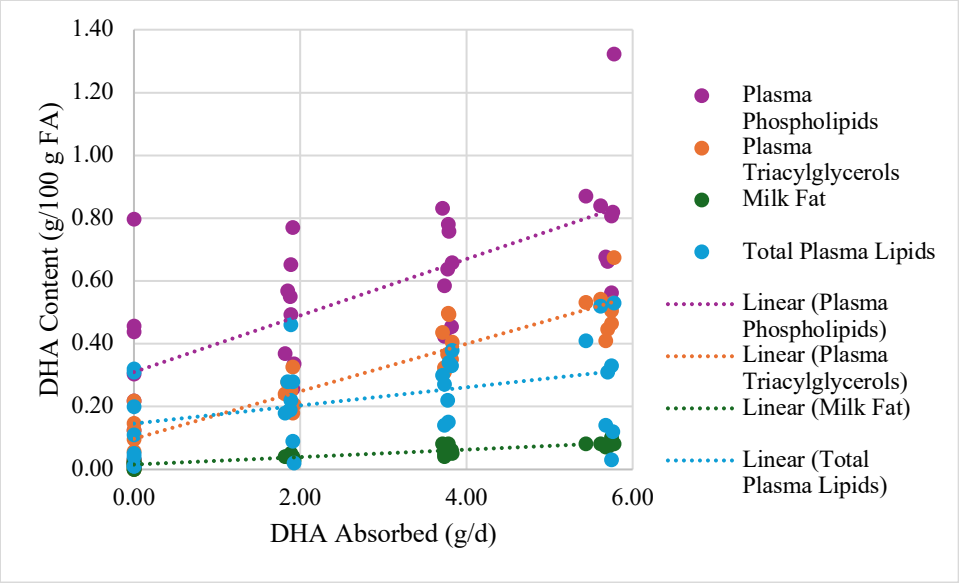


Figure 3.3 Relationship between absorbed DHA and DHA content of plasma phospholipids, plasma triacylglycerols, milk fat, and total plasma lipids.
[DHA in plasma phospholipids (g/100 g FA) = 0.31 + 0.09 x (DHA absorbed (g/d)); $R^2 = 0.49$; $P < 0.0001$].
[DHA in plasma triacylglycerols (g/100 g FA) = 0.10 + 0.08 x (DHA absorbed (g/d)); $R^2 = 0.85$; $P < 0.0001$].
[DHA in milk fat (g/100 g FA) = 0.02 + 0.01 x (DHA absorbed (g/d)); $R^2 = 0.86$; $P < 0.0001$].
[DHA in total plasma lipids (g/100 g FA) = 0.15 + 0.03 x (DHA absorbed (g/d)); $R^2 = 0.18$; $P < 0.01$].

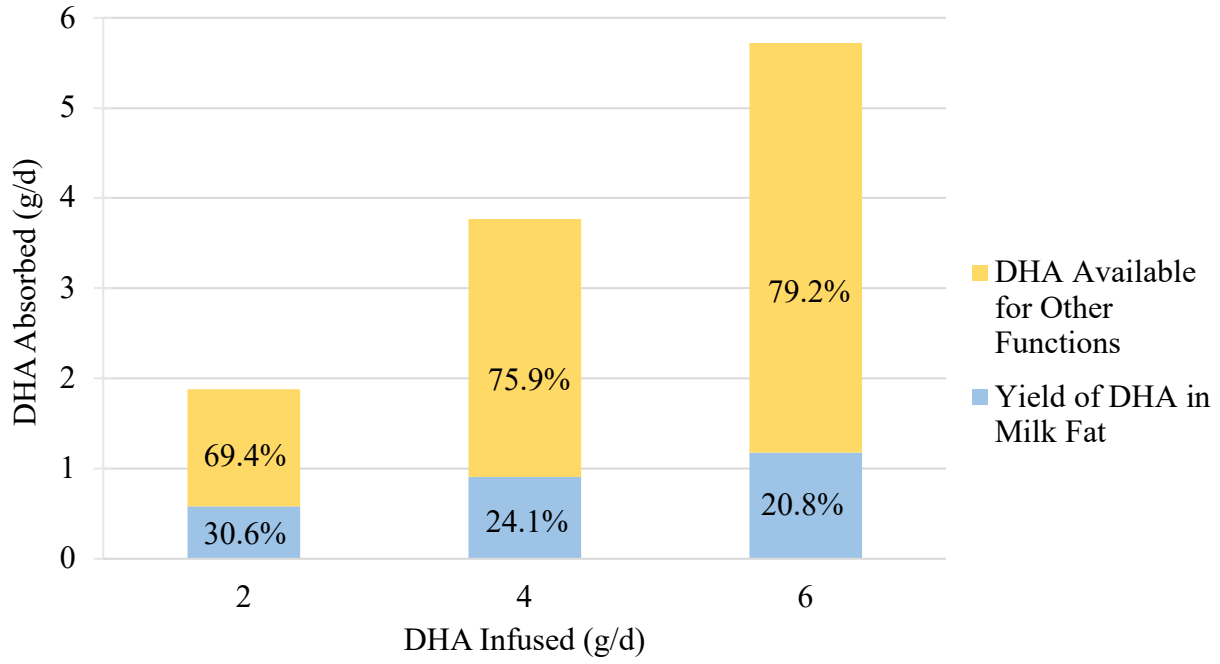


Figure 3.4 Effect of increasing abomasal infusions of DHA on yield of DHA in milk fat (g/d) as a proportion of DHA absorbed.

Table 4.1 Ingredient composition and fatty acid profile of concentrate mixes provided during the close-up period

Item	CU Mix	
	CON	N3
Ingredient, % DM		
Soybean meal	18.8	18.8
Anionic protein supplement[1]	30.2	30.2
Ground corn	3.58	3.58
Vitamin D	0.003	0.003
Vitamin E	0.07	0.07
Tallow	0.76	0.76
Magnesium sulfate	2.51	2.51
White salt	0.56	0.56
Selenium	0.16	0.16
Vitamin A	0.01	0.01
Trace mineral blend[2]	0.05	0.05
Magnesium oxide	0.40	0.40
Soybean hulls	13.4	12.3
By-pass soybean meal[3]	22.9	22.9
Zinc sulfate	0.03	0.03
Blood meal[4]	2.32	2.32
Calcium salt of fish oil[5]	-	3.63
Calcium salt of palm oil[6]	2.47	-
Copper sulfate	0.01	0.01
Rumen protected choline[7]	1.74	1.74
Total Fatty Acids, % DM[8]	4.48	5.11
FA Profile, g/100 g FA		
C16:0	29.2	19.2
C18:0	5.36	25.0
cis-9 C18:1	24.3	12.3
cis-9, cis-12 C18:2	26.6	17.0
cis-9, *cis*-12, *cis*-15 C18:3	3.13	2.35
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 C 20:5	-	2.94
cis-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:5	-	0.59
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:6	-	2.35

[1]SoyChlor (Landus; Des Moines, Iowa).
[2]Micro 5 (Nutreco; Amersfoort, Netherlands).
[3]Amino Plus (Ag Processing; Omaha, Nebraska).

Table 4.1 (cont'd)

[4]Spectrum AgriBlue (Perdue Animal Nutrition; Salisbury, Maryland).
[5]Strata (Virtus Nutrition; Corcoran, California). Supplement (80% total FA content) contained (g/100g FA) 8.9% C14:0, 25% C16:0, 11% *cis*-9 C16:1, 6.7% C18:0, 16% *cis*-9 C18:1, 3% *cis*-11 C18:1, 2.1% *cis-9, cis*-12 C18:2, 3.6% C22:0, 10.5% *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 C 20:5, and 7.6% *cis*-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:6.
[6]EnerGII (Virtus Nutrition; Cocoran, California). Supplement (80% total FA content) contained (g/100g FA) 51% C16:0, 4.1 % C18:0, 37% *cis*-9 C18:1, and 6.6% *cis-9, cis*-12 C18:2.
[7]ReaShure (Balchem Corporation; New Hampton, New York).
[8]Analysis of fatty acid content and profile performed at Eurofins Nutrition Analysis Center (Des Moines, Iowa).

Table 4.2 Ingredient composition and fatty acid profile of concentrate mixes provided during the fresh period

Item	FR Mix	
	CON	N3
Ingredient, % DM		
Distillers grains	25.5	25.5
By-pass soybean meal[1]	14.5	14.5
Calcium carbonate	4.7	4.7
Ground corn	8.2	8.2
Rumen protected methionine[2]	0.4	0.4
Sodium sesquinate	10.2	10.2
Blood meal[3]	20.4	20.4
Dicalcium phosphate	1.0	1.0
Soybean hulls	2.7	-
Potassium carbonate[4]	4.1	4.1
Rumen protected choline[5]	2.7	2.7
Calcium salt of fish oil[6]	-	8.3
Calcium salt of palm oil[7]	5.7	-
Total Fatty Acids, % DM[8]	7.86	11.9
FA Profile, g/100 g FA		
C16:0	31.8	19.7
C18:0	20.5	17.1
cis-9 C18:1	23.9	11.3
cis-9, cis-12 C18:2	18.7	11.5
cis-9, *cis*-12, *cis*-15 C18:3	0.89	1.18
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 C 20:5	-	5.97
cis-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:5	-	1.26
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:6	-	4.96

[1]Amino Plus (Ag Processing; Omaha, Nebraska).
[2]Smartamine (Adisseo; Alpharetta, Georgia).
[3]Spectrum AgriBlue (Perdue Animal Nutrition; Salisbury, Maryland).
[4]Ion Plus (D&D Ingredients, LLC; Delphos, Ohio).
[5]ReaShure (Balchem Corporation; New Hampton, New York).
[6]Strata (Virtus Nutrition; Corcoran, California). Supplement (80% total FA content) contained (g/100g FA) 8.9% C14:0, 25% C16:0, 11% *cis*-9 C16:1, 6.7% C18:0, 16% *cis*-9 C18:1, 3% *cis*-11 C18:1, 2.1% *cis-9, cis*-12 C18:2, 3.6% C22:0, 10.5% *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 C 20:5, and 7.6% *cis*-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:6.
[7]EnerGII (Virtus Nutrition; Cocoran, California). Supplement (80% total FA content) contained (g/100g FA) 51% C16:0, 4.1 % C18:0, 37% *cis*-9 C18:1, and 6.6% *cis-9, cis*-12 C18:2.
[8]Analysis of fatty acid content and profile performed at Eurofins Nutrition Analysis Center (Des Moines, Iowa).

Table 4.3 Ingredient and nutrient composition of close-up, fresh, and carryover diets.

Item	Diet				
	Close-up		Fresh		Carryover
	CON	N3	CON	N3	
Ingredient, % DM					
Corn silage	44.6	44.7	33.2	33.1	38.7
Alfalfa silage	-	-	12.5	12.5	12.8
Alfalfa hay	-	-	5.52	5.58	-
Grass hay	17.5	17.7	-	-	-
Wheat straw	13.8	13.9	-	-	-
Ground corn	-	-	13.9	13.9	19.6
Soybean meal	-	-	10.6	10.5	10.8
Whole cottonseed	-	-	3.28	3.22	9.35
Vitamin and mineral mix[1]	-	-	2.08	2.10	1.95
Soybean hulls	-	-	8.80	8.67	-
Protein supplement[2]	-	-	-	-	6.89
Close-up mix w/o n-3[3]	24.0	-	-	-	-
Close-up mix w/ n-3	-	23.7	-	-	-
Fresh mix w/o n-3[4]	-	-	10.2	-	-
Fresh mix w/ n-3	-	-	-	10.4	-
Nutrient Composition, % DM					
NDF	43.7	43.7	31.1	30.8	27.5
Forage NDF	38.4	38.6	20.1	20.0	19.3
CP	13.2	13.1	18.2	18.2	17.3
Starch	16.1	16.1	22.8	22.8	29.0
FA	2.97	3.02	3.67	3.75	4.12

[1]Contained 20.4% calcium carbonate, 12.5% calcium phosphate, 4.47% sodium sesquinate, 14.1% white salt, 15.7% Min AD, 0.03% vitamin A, 0.01% Vitamin 500, 0.34% vitamin E, 14.7 % corn grain, 0.92% Energizer Tallow, 0.51% Micro5, 2.36% CFE Selenium, 0.15% magnesium oxide, and 4.70% magnesium sulfate.
[2]Contained 67.6% Amino Plus, 9.56% calcium carbonate, 17.9% sodium sesquinate, and 4.93% Ion Plus
[3]Close-up mix formulations outlined in Table 4.1.
[4]Fresh mix formulations outlined in Table 4.2.

Table 4.5 Fresh period (1-23 DIM) dry matter intake, milk yield and composition, body condition score, and body weight of cows fed treatment diets during the close-up (~21 d prepartum) and fresh period (1-23 DIM)

	Treatment[1]			P-values[2]		
	CON	N3	SEM	Treatment	Time	Treatment*Time
Item						
DMI, kg/d	22.3	22.3	0.74	0.96	<0.0001	0.62
Yields, kg/d						
Colostrum	3.64	2.45	0.41	0.05	N/A	N/A
Milk	42.2	41.8	1.10	0.81	<0.0001	0.88
FCM	53.1	49.7	1.34	0.09	<0.0001	0.78
ECM	52.7	49.7	1.29	0.10	<0.0001	0.78
Fat	2.12	1.94	0.06	0.05	<0.0001	0.76
Protein	1.54	1.51	0.04	0.46	<0.0001	0.63
Lactose	2.13	2.03	0.06	0.10	<0.0001	0.54
Content, %						
Fat	4.91	4.66	0.10	0.10	<0.0001	0.33
Protein	3.60	3.65	0.05	0.50	<0.0001	0.12
Lactose	4.84	4.84	0.02	0.73	<0.0001	0.16
logSCC	3.0	3.3	0.17	0.11	<0.0001	0.19
BCS	3.19	3.21	0.03	0.72	<0.0001	0.13
BCS Change /wk	-0.06	-0.06	0.02	0.71	-	-
BW, kg	728	725	5.9	0.63	<0.0001	0.34
BW Change, kg/d	-1.98	-1.93	0.27	0.88	-	-

[1]CON (diet containing a calcium salt of palm fatty acids); N3 (diet containing a calcium salt of fish oil providing EPA and DHA).
[2]P-values refer to the ANOVA results for the main effect of n-3 treatment, the main effect of time, and the interaction of treatment and time.
[3]3.5% FCM = [(0.4324 × kg of milk) + (16.216 × kg milk of fat)] (NRC, 2001).
[4]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.20 × kg of milk protein). This equation corrects milk to a 0.68 Mcal/kg energy basis (Tyrrell and Reid, 1965).

Table 4.6 Dry matter intake, milk yield and composition, body condition score, and body weight of cows fed a common diet during the carryover period (24-63 DIM)

	Treatment[1]			P-values[2]		
	CON	N3	SEM	Treatment	Time	Treatment*Time
Item						
DMI, kg/d	29.4	29.4	0.62	0.91	<0.0001	0.07
Yields, kg/d						
Milk	55.0	53.9	1.57	0.45	<0.0001	0.80
FCM	58.2	56.4	1.46	0.33	0.74	0.37
ECM	57.3	55.9	1.37	0.43	0.76	0.56
Fat	2.13	2.05	0.06	0.29	0.16	0.10
Protein	1.63	1.61	0.04	0.69	<0.001	0.86
Lactose	2.74	2.68	0.07	0.46	<0.01	0.93
Content, %						
Fat	3.92	3.82	0.09	0.45	<0.0001	<0.01
Protein	2.99	3.00	0.04	0.76	<0.001	0.96
Lactose	5.01	5.00	0.02	0.65	0.03	0.40
logSCC	2.0	2.2	0.23	0.29	0.46	0.92
BCS	3.03	3.08	0.03	0.24	<0.01	0.40
BCS Change /wk	-0.02	-0.01	0.01	0.55	-	-
BW, kg	702	702	7.07	0.95	<0.0001	0.64
BW Change, kg/d	-0.14	0.01	0.10	0.27	-	-

[1]CON (diet containing a calcium salt of palm fatty acids); N3 (diet containing a calcium salt of fish oil providing EPA and DHA).
[2]P-values refer to the ANOVA results for the main effect of n-3 treatment, the main effect of time, and the interaction of treatment and time
[3]3.5% FCM = [(0.4324 × kg of milk) + (16.216 × kg milk of fat)] (NRC, 2001).
[4]ECM = (0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.20 × kg of milk protein). This equation corrects milk to a 0.68 Mcal/kg energy basis (Tyrrell and Reid, 1965).

Table 4.7 Plasma metabolites of cows fed treatment diets during the close-up (~21 d prepartum) and fresh period (1-23 DIM)[1]

	Treatment[2]			P-values[3]		
	CON	N3	SEM	Treatment	Time	Treatment*Time
Item						
Insulin, µg/L	0.15	0.14	0.01	0.49	0.13	0.83
Glucose, mg/dL	60.1	59.2	1.51	0.60	0.78	<0.001
NEFA, mEq/L	575	517	44.57	0.33	<0.01	0.29
BHB, mmol/L	0.83	0.83	0.06	0.96	0.29	0.20

[1]Blood samples collected at 3, 7, 14, and 21 DIM.
[2]CON (diet containing a calcium salt of palm fatty acids); N3 (diet containing a calcium salt of fish oil providing EPA and DHA).
[3]P-values refer to the ANOVA results for the main effect of n-3 treatment, the main effect of time, and the interaction of treatment and time.

Table 4.8 Fresh period (1-23DIM) milk fatty acid contents of cows fed treatment diets during the close-up (~21 d prepartum) and fresh period (1-23 DIM)

	Treatment[1]			P-values[2]		
	CON	N3	SEM	Treatment	Time	Treatment*Time
Item						
Summations by Source, g/100 g FA						
De novo	20.3	20.2	0.71	0.91	<0.01	0.27
Mixed	31.9	31.3	0.37	0.21	0.49	0.29
Preformed	47.8	48.4	0.88	0.52	0.03	0.12
Selected Individual FA[3], g/100g FA						
C4:0	3.20	3.35	0.08	0.15	<0.0001	0.60
C6:0	1.94	1.95	0.06	0.87	0.10	0.38
C8:0	1.04	1.03	0.05	0.89	<0.001	0.42
C10:0	2.44	2.33	0.08	0.54	<0.01	0.45
C12:0	2.44	2.35	0.16	0.67	<0.001	0.36
C14:0	8.66	8.72	0.32	0.89	<0.01	0.12
C16:0	30.1	29.6	0.39	0.26	0.17	0.17
cis-9 C16:1	1.75	1.71	0.06	0.69	<0.001	0.10
C18:0	12.1	12.3	0.32	0.73	<0.0001	0.76
trans -6 to -8 C18:1	0.22	0.28	0.01	<0.01	<0.001	0.59
trans-9 C18:1	0.13	0.18	0.01	<0.0001	<0.0001	0.18
trans-10 C18:1	0.34	0.37	0.02	0.33	0.05	0.04
trans-11 C18:1	0.74	1.13	0.05	<0.0001	0.98	0.37
cis-9 C18:1	25.7	24.3	0.75	0.12	0.14	0.03
cis-11 C18:1	0.98	0.99	0.03	0.73	<0.0001	0.04
cis-9, *trans*-11 C18:2 (CLA)	0.25	0.35	0.02	<0.0001	<0.0001	0.01
C20:0	0.10	0.15	0.01	<0.0001	0.08	0.89
C22:0	0.02	0.04	0.002	<0.0001	0.33	0.74

Table 4.8 (cont'd)

∑n-6	2.45	2.46	0.05	0.82	0.57	0.10
cis-9, cis-12 C18:2	2.23	2.23	0.05	0.94	0.35	0.08
cis-5, *cis*-8, cis-11, cis-14 C20:4	0.13	0.14	0.01	0.66	<0.0001	0.20
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16 C22:5	0.02	0.02	0.001	0.47	0.00	0.22
∑ n-3	0.43	0.54	0.02	<0.0001	0.69	0.61
cis-9, *cis*-12, *cis*-15 C18:3	0.33	0.35	0.01	0.43	0.80	0.84
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 C20:5	0.03	0.07	0.003	<0.0001	0.64	0.51
cis-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:5	0.05	0.08	0.004	<0.0001	0.12	0.73
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:6	0.00	0.05	0.003	-	-	-
∑n-6:n-3	6.04	4.61	0.16	<0.0001	0.74	0.63

[1]CON (diet containing a calcium salt of palm fatty acids); N3 (diet containing a calcium salt of fish oil providing EPA and DHA).
[2]P-values refer to the ANOVA results for the main effect of n-3 treatment, the main effect of time, and the interaction of treatment and time.
[3]A total of approximately 60 individual fatty acids were quantified. Only select fatty acids are reported in the table.

Table 4.9 Fresh period (1-23 DIM) milk fatty acid yields of cows fed treatment diets during the close-up (~21 d prepartum) and fresh period (1-23 DIM)

	Treatment[1]			P-values[2]		
	CON	N3	SEM	Treatment	Time	Treatment*Time
Item						
Summations by Source (g/d)						
De Novo	416	375	16.4	0.01	<0.0001	0.40
Mixed	663	584	22.5	<0.01	<0.01	0.92
Preformed	980	908	32.5	0.14	0.03	0.56
Selected Individual FA[3], g/d						
C4:0	66.8	62.8	2.95	0.17	0.13	0.82
C6:0	40.0	36.2	1.73	0.03	<0.001	0.66
C8:0	21.4	19.1	1.03	0.03	<0.0001	0.67
C10:0	49.2	42.9	2.80	0.04	<0.0001	0.22
C12:0	51.4	44.8	3.10	0.06	<0.0001	0.15
C14:0	177	161	6.82	0.04	<0.0001	0.17
C16:0	632	552	21.9	<0.001	0.04	0.79
cis-9 C16:1	35.5	31.7	1.74	0.13	<0.0001	0.31
C18:0	247	230	8.75	0.18	0.04	0.76
trans -6 to -8 C18:1	4.56	5.22	0.29	0.06	0.11	0.15
trans-9 C18:1	2.83	3.46	0.18	<0.01	<0.0001	0.80
trans-10 C18:1	6.54	7.87	0.61	0.10	<0.0001	0.64
trans-11 C18:1	17.9	23.8	2.21	0.01	0.09	0.11
cis-9 C18:1	531	459	22.0	0.03	<0.01	0.35
cis-11 C18:1	20.0	18.2	0.79	0.10	<0.0001	0.15
cis-9, *trans*-11 C18:2 (CLA)	5.33	6.58	0.46	0.03	<0.0001	0.04
C20:0	2.06	2.87	0.10	<0.0001	0.76	0.99
C22:0	0.48	0.71	0.04	<0.0001	0.03	0.63

Table 4.9 (cont'd)

∑n-6	51.4	46.3	1.81	0.05	0.01	0.68
cis-9, cis-12 C18:2	46.9	41.9	1.73	0.04	<0.01	0.63
cis-5, *cis*-8, cis-11, cis-14 C20:4	2.75	2.58	0.11	0.27	<0.0001	0.54
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16 C22:5	0.34	0.30	0.02	0.22	0.0001	0.73
∑ n-3	9.62	9.94	0.53	0.47	0.02	0.60
cis-9, *cis*-12, *cis*-15 C18:3	7.26	6.44	0.30	0.03	0.01	0.92
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 C 20:5	0.71	1.35	0.07	<0.0001	0.36	0.52
cis-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:5	1.09	1.52	0.08	<0.0001	0.26	0.93
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 C22:6	<0.01	0.88	0.05	-	-	-
∑n-6:n-3	5.99	4.57	0.16	<0.0001	0.75	0.62

[1]CON (diet containing a calcium salt of palm fatty acids); N3 (diet containing a calcium salt of fish oil providing EPA and DHA).
[2]P-values refer to the ANOVA results for the main effect of n-3 treatment, the main effect of time, and the interaction of treatment and time.
[3]A total of approximately 60 individual fatty acids were quantified. Only select fatty acids are reported in the table.

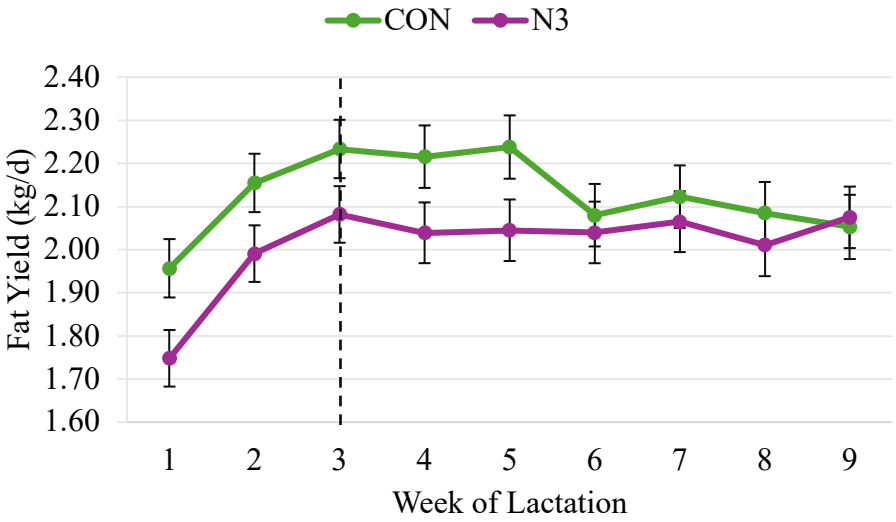


Figure 4.1 Effects of dietary n-3 fatty acid inclusion on yield of milk fat during the fresh (FR) period (1-23 DIM) and carryover (CO) period (24-63 DIM). Diets fed during the FR period were **CON** (diet with a calcium salt of palm oil containing no supplemental EPA or DHA, green line); **N3** (diet with a calcium salt containing supplemental EPA and DHA, purple line). The dash-line on week 3 indicates the start of the CO period, when all cows were fed a common diet with no supplemental calcium salts of fatty acids. N3 reduced yield of milk fat during the FR period (P = 0.05). During the CO period, N3 supplementation interacted with time to reduce milk fat yield during weeks 4 and 5 of lactation compared to CON (P = 0.10). Error bars indicate SEM.

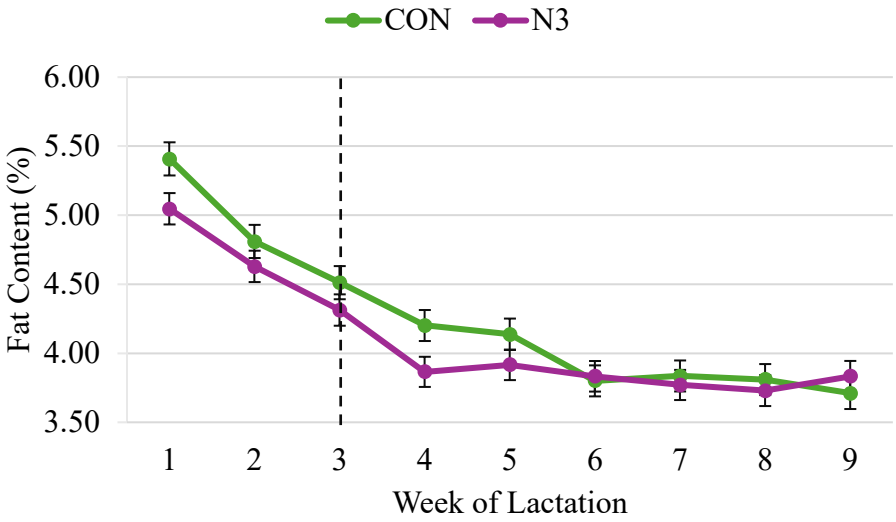


Figure 4.2 Effects of dietary n-3 fatty acid inclusion on milk fat content during the fresh (FR) period (1-23 DIM) and carryover (CO) period (24-63 DIM). Diets fed during the FR period were **CON** (diet with a calcium salt of palm oil containing no supplemental EPA or DHA, green line); **N3** (diet with a calcium salt containing supplemental EPA and DHA, purple line). The dash-line on week 3 indicates the start of the CO period, when all cows were fed a common diet with no supplemental calcium salts of fatty acids. N3 tended to decrease milk fat content during the FR period ($P = 0.10$). During the CO period, N3 supplementation interacted with time to reduce milk fat content during weeks 4 and 5 of lactation compared to CON ($P < 0.01$). Error bars indicate SEM.

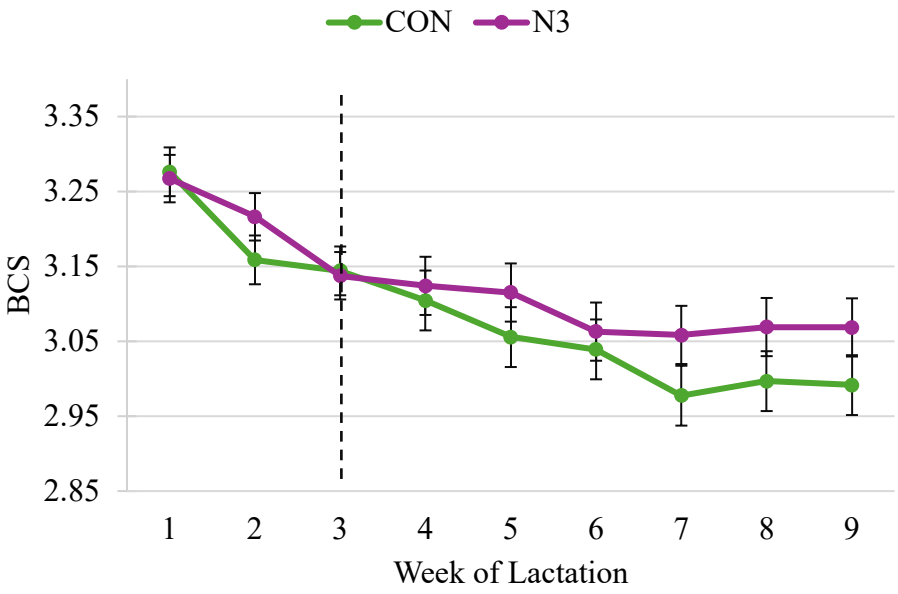


Figure 4.3 Effects of dietary n-3 fatty acid inclusion on body condition score during the fresh (FR) period (1-23 DIM) and carryover (CO) period (24-63 DIM). Diets fed during the FR period were **CON** (diet with a calcium salt of palm oil containing no supplemental EPA or DHA, green line); **N3** (diet with a calcium salt containing supplemental EPA and DHA, purple line). The dash-line on week 3 indicates the start of the CO period, when all cows were fed a common diet with no supplemental calcium salts of fatty acids. A treatment by time interaction was observed during the FR period where N3 tended to increase BCS during week 2 of lactation ($P = 0.13$). No treatment effects or interactions were observed in the CO period. Error bars indicate SEM.

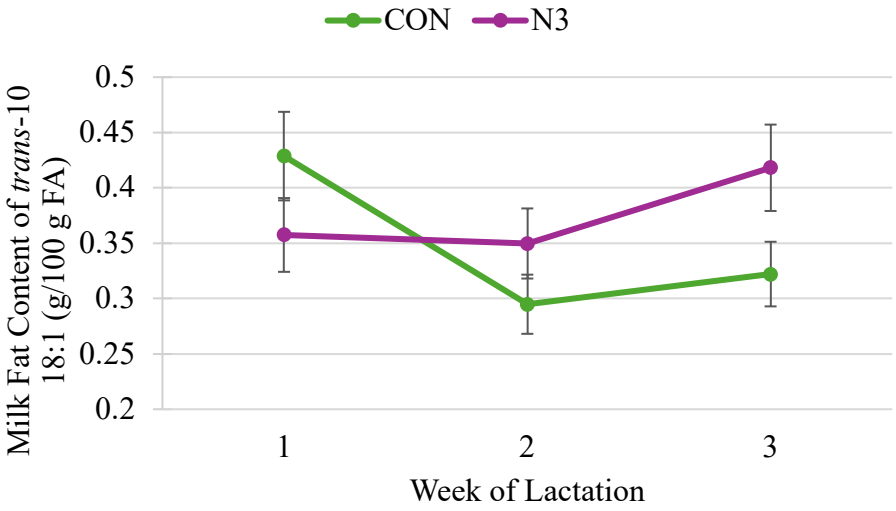


Figure 4.4 Effects of dietary n-3 fatty acid inclusion on milk fat content of *trans*-10 C18:1 during the fresh (FR) period (1-23 DIM). Diets fed during the FR period were **CON** (diet with a calcium salt of palm oil containing no supplemental EPA or DHA, green line); **N3** (diet with a calcium salt containing supplemental EPA and DHA, purple line). Treatment interacted with time where N3 increased milk fat content of *trans*-10 18:1 during week 3 ($P < 0.01$). Error bars indicate SEM.