Characterization of the effect of the menstrual cycle, estradiol and *Fads2* transcript variants on fatty acid composition and enzyme expression

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Estrogen is believed to regulate fatty acid composition based on observations of differences between men and women, and changes associated with altered estrogen levels in women during pregnancy, menopause and hormone treatment. Estrogen is consistently associated with increased levels of 18:0, 16:1n-7 and elongase 6, an enzyme in monounsaturated fatty acids (MUFA) biosynthesis. In some, but not all studies, estrogen has also been associated with increased 22:6n-3 and FADS2 expression. FADS2 is responsible for delta-6 desaturase activity, the rate-limiting step in polyunsaturated fatty acid (PUFA) biosynthesis and is necessary for the synthesis of 22:6n-3, which is critical for cardiovascular health and neural development. In this thesis, the effects of subtle changes in estrogen levels during the menstrual cycle in humans, and the estrous cycle in rats, on fatty acid metabolism was examined. In addition, the response of FADS2 to estrogen was examined using MCF7 cells. The presence of multiple FADS2 transcript variants in MCF7 cells was determined, and the role of two variants in PUFA biosynthesis was examined by transfecting them into HEK 293 cells. During the menstrual cycle in women and estrous cycle in rats, increased blood levels of 16:1n-7 and 22:6n-3, and decreased levels of 22:5n-3 were observed when blood estrogen levels were high. The increased 22:6n-3 and decreased 22:5n-3 suggests an effect of estrogen on FADS2 expression and/or activity. In the rat hepatic expression of *FADS2* mRNA varied over the cycle, but protein levels did not. However, in estrogen sensitive MCF7 cells, *FADS2* mRNA was induced by estrogen. Changes in 22:6n-3 and 22:5n-3 were not observed in the MCF7 cells. Further examination identified multiple transcript variants of *FADS2* in MCF7 cells, and demonstrated that the expression of an alternatively spliced transcript variant (*FADS2_v2*) was considerably higher. Notably, this variant lacked the N-terminal cytochrome-binding domain that is critical for activity, suggesting

that it may not be functional. In transfected HEK 293 cells, indirect evidence of delta-6 desaturase activity was observed for the full length wild type variant (*FADS2_v0/1*) but not for *FADS2_v2*. When the variants were co-transfected, *FADS2_v2* interacted with and inhibited *FADS2_v0/1*. This work helps further understanding of the relationship between estrogen, lipid biosynthetic enzymes and fatty acid status that is important for determination of dietary requirements of 22:6n-3 for women throughout the lifespan.

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Table of Contents

Author's Declaration ii

Abstract iii

Acknowledgements v

Table of Contents vii

List of Figures ix

List of Tables x

List of Abbreviations xii

Chapter 1: General Introduction 1

Chapter 2: Biochemical Foundations 5

Chapter 3: Rationale and Objectives 35
- Rationale 35
- Objectives 37
- Hypotheses 38

Chapter 4: Common Methods 39

Chapter 5: The Effects of Sex, Menstrual Cycle and Oral Contraceptives on Blood Fatty Acids 44
- Introduction 44
- Methods and Materials 45
- Results 47
- Discussion 50
- Conclusion 54

Chapter 6: Effect of Rat Estrous Cycle on Hepatic Expression of Fatty Acid Biosynthesis Enzymes 60
- Introduction 60
- Methods and Materials 61
- Results 63
- Discussion 64
- Conclusion 68

Chapter 7: Effects of Estradiol Treatment on Gene Expression and Fatty Acid Composition in MCF7 Cells 79
- Introduction 79

Methods and Materials 81
Results 82
Discussion 83
Conclusion 85

Chapter 8: Characterization of *FADS2* Novel Transcript Variants 92
Introduction 92
Methods and Materials 92
Results 96
Discussion 97
Conclusion 100

Chapter 9: General Discussion 108

References 115

Appendix 1: Supplementary Forms and Data for Chapter 5 136

Appendix 2: Supplementary Data for Chapter 6 175

Appendix 3: Supplementary Data for Chapter 8 179

List of Figures

Figure 2.1 Monounsaturated fatty acid biosynthesis 31

Figure 2.2 Polyunsaturated fatty acid biosynthesis 32

Figure 2.3 Rat estrous cycle hormone changes 33

Figure 2.4 Human menstrual cycle hormone changes 34

Figure 5.1 Relative proportion of selected fatty acids in erythrocytes of men and women taking or not taking oral contraceptives 58

Figure 6.1 Representative image of stained vaginal smears from rats 71

Figure 6.2 Plasma hormone concentrations in rats over the estrous cycle 72

Figure 6.3 Desaturase gene expression in rats over the estrous cycle 77

Figure 6.4 Elongase gene expression in rats over the estrous cycle 74

Figure 6.5 Desaturase protein content in rats over the estrous cycle 75

Figure 6.6 Elongase protein content in rats over the estrous cycle 76

Figure 6.7 Concentration of 16:1n-7, 22:5n-3 and 22:6n-3 in rats over the estrous cycle 77

Figure 6.8 Proportion of 16:1n-7, 22:5n-3 and 22:6n-3 in rats over the estrous cycle 78

Figure 7.1 Expression of fatty acid elongases in MCF7 cells treated with estradiol 90

Figure 7.2 Expression of desaturases in MCF7 cells treated with estradiol 91

Figure 8.1 Sequence alignment of D6D.1 and D6D.2 103

Figure 8.2 Expression of *Fads2* transcript variants in MCF7 cells treated with estradiol 104

Figure 8.3 Proportion of 20:4n-6 and 22:6n-3 in HEK 293 cells expressing Fads2 variants 105

Figure 8.4 Interaction of *Fads2* transcript variants when co-expressed in HEK 293 cells 106

Figure 8.5 Proportion of 20:4n-6 and 22:6n-3 in HEK 293 cells expressing both *Fads2* variants 107

List of Tables

Table 2.1 Summary of enzymes involved in fatty acid biosynthesis 26

Table 2.2 Summary of sex differences in fatty acid composition 27

Table 2.3 Summary of studies altering hormone status in rats 28

Table 2.4 Summary of studies examining fatty acid composition in humans with altered hormone status 29

Table 5.1 Plasma hormone concentrations in women over the menstrual cycle 56

Table 5.2 Relative proportion of plasma fatty acids in men, women taking and not taking oral contraceptives 57

Table 5.3 Relative proportion of plasma fatty acids in women over the menstrual cycle 59

Table 6.1 Rat primer sequences for real-time PCR 70

Table 7.1 Human primer sequences for real-time PCR 87

Table 7.2 Relative proportion of fatty acids in MCF7 cells treated with estradiol or vehicle 88

Table 7.3 Concentration of fatty acids in MCF7 cells treated with estradiol or vehicle 89

Table 8.1 Human *FADS2* variant summary and primer sequences for real-time PCR 102

Table A1.1 Concentration of plasma fatty acids in men and women taking and not taking oral contraceptives 169

Table A1.2 Concentration of erythrocyte fatty acids in men and women taking and not taking oral contraceptives 170

Table A1.3 Relative proportion of erythrocyte fatty acids in men and women taking and not taking oral contraceptives 171

Table A1.4 Concentration of plasma fatty acids in women over the menstrual cycle 172

Table A1.5 Concentration of erythrocyte fatty acids in women over the menstrual cycle 173

Table A1.6 Relative proportion of erythrocyte fatty acids in women over the menstrual cycle 174

Table A2.1 Concentration of whole blood fatty acids in rats over the estrous cycle and males 176

Table A2.2 Concentration of plasma fatty acids in rats over the estrous cycle and males 177

Table A2.3 Concentration of hepatic fatty acids in rats over the estrous cycle and males 178

Table A3.1 Relative proportion of fatty acids in HEK 293 cells electroporated with *Fads2* variants 180

Table A3.2 Concentration of fatty acids in HEK 293 cells electroporated with *Fads2* variants 181

Table A3.3 Relative proportion of fatty acids in HEK 293 cells electroporated with both *Fads2* variants 182

Table A3.4 Concentration of fatty acids in HEK 293 cells electroporated with both *Fads2* variants 183

List of Abbreviations

ANOVA	analysis of variance
D5D	delta-5 desaturase
D6D	delta-6 desaturase
ER	estrogen receptor
Elovl1	fatty acid elongase 1
Elovl2	fatty acid elongase 2
Elovl5	fatty acid elongase 5
Elovl6	fatty acid elongase 6
Fads1	fatty acid desaturase 1
Fads2	fatty acid desaturase 2
FASN	fatty acid synthase
HUFA	highly unsaturated fatty acid
MUFA	monounsaturated fatty acid
ORF	open reading frame
PUFA	polyunsaturated fatty acid
Scd1	stearoyl-CoA desaturase 1
SFA	saturated fatty acids
TAG	triacylglycerol

Chapter 1

General Introduction

Fatty acids are involved in a wide range of cellular functions, including ATP generation (McCloy et al., 2004), cell signaling (Hong and Lu, 2013), neurotransmission (Garcia and Kim, 1997), energy storage as triacylglycerols (TAG) (Raclot, 2003; Yen et al., 2008), and structural support (Balkova et al., 2009). Sex differences have been reported in both blood and tissue fatty acid composition, which may affect the proper functioning of these processes because different fatty acids have different physiological functions. In women, there tends to be a shift towards having higher levels of 18:0 and 22:6n-3 (Metherel et al., 2009; Patterson et al., 2015; Crowe et al., 2008) while men have higher levels of 16:0 and 22:5n-3 (Patterson et al., 2015; Marangoni et al., 2007).

Differences in the fatty acid composition of body cells and tissues may contribute to sex differences in cardiovascular disease. Premenopausal women are at a lower risk for cardiovascular disease as compared with men (Barrett-Connor, 1997; Bui, Horwich, and Fonarow, 2011). This may be due in part to more favorable fatty acid species profiles in blood and plasma of women. In particular, 16:0 has previously been thought to be associated with an increased risk for cardiovascular disease and diabetes (Yu et al., 2002; Lionetti et al., 2007), although some evidence suggests that there is no relationship (Siri-Tarino et al., 2010; Mozaffarian, Rimm, and Herrington, 2004; Tucker et al., 2005). A diet high in 18:0 have been reported to lower cholesterol levels compared with a diet high in 16:0 (Storm et al., 1997). One particular omega-3 polyunsaturated fatty acid (PUFA), 22:6n-3, has anti-inflammatory (Flock et al., 2014; Dangardt et al., 2010) and anti-arrhythmic properties (GISSI-Prevenzione Investigators, 1999; Xiao et al., 1997). Less is known about the health effects of 22:5n-3, but it

appears to have some protective effects, particularly with platelet aggregation (reviewed in (Kaur et al., 2011)).

These sex differences in fatty acid composition persist even when diet is controlled (Metherel et al., 2009; Patterson et al., 2015; Crowe et al., 2008), suggesting an underlying biological mechanism. Estrogen has been thought to be one such mechanism because circulating estradiol levels are much higher in females compared with males (Eldrup, Lindholm, and Winkel, 1987). Studies have examined conditions associated with changing estrogen concentrations, such as pregnancy, menopause, ovariectomy with or without exogenous estrogen provision, and oral contraceptive use, in both human and rodent models. While not all studies fully agree, in general increasing estrogen concentrations results in higher levels of 16:0, 18:0, 16:1n-7 and 22:6n-3 and lower levels of 20:5n-3, 20:4n-6 and 22:5n-3 (Fehily et al., 1982; Kitson et al., 2013a; Marks et al., 2013a; Stark, Park, and Holub, 2003; Mason et al., 2014; Giltay et al., 2004; Ottosson et al., 1984; Tworek et al., 2000).

Estrogen can regulate gene expression (Gruber et al., 2004) and appears to be involved in the control of enzymes involved in fatty acid elongation and desaturation. Hepatic mRNA and protein expression of elongase 6 has been shown to be positively regulated by estradiol in many models (Marks, Kitson, and Stark, 2013; Marks et al., 2013b). Stearoyl-CoA desaturase appears to be controlled in part by estradiol but not is not consistently responsive to estradiol (Marks, Kitson, and Stark, 2013; Marks et al., 2013b; Paquette et al., 2008). Hepatic Fads2 and elongase 2 mRNA levels are increased following estrogen treatment in ovariectomized rats (Kitson et al., 2013a), but do not increase in HepG2 cells that are treated with estradiol (Sibbons et al., 2014) or decrease in livers of ERα knockout mice (Kitson, 2013). These results suggest that estradiol is involved in mediating the expression of lipid-metabolizing enzymes to affect fatty acid

composition, but also suggest that the mechanism (i.e. nuclear versus non-genomic) may differ in different conditions and systems. Indeed, estrogen receptor α (ERα) knockout mice retain non-genomic estrogen signaling capacity (Couse et al., 1995), HepG2 cells do not express high amounts of ERα (Kelly et al., 2014), ovariectomy does not always result in a decrease in estrogen concentrations compared with sham-operated rats (Marks et al., 2013a), while estrogen treatment in ovariectomized animals can result in supraphysiological concentrations of this hormone (Marks et al., 2013a). Thus, differences across levels of estrogen, as well as differences in mechanisms of estrogen-mediated signalling, are likely responsible for the incongruous nature of changes in lipid-metabolizing hormones, leaving this area poorly understood.

Many of these effects of estrogen on fatty acid composition occur with large changes in estrogen concentrations, such as pregnancy or menopause. However, the effects of estrogen on fatty acid composition have not been looked at during the human menstrual cycle. Hormones change cyclically over the menstrual cycle, but the change is not as dramatic as is observed during menopause and pregnancy. Therefore, the first objective of my thesis was to measure plasma and erythrocyte fatty acid composition in women at two time points of the menstrual cycle when estrogen concentrations should be different from each other, and expand upon findings from other estrogen-based models. The second objective of my thesis was to further explore the effects of cyclical estrogen concentrations by using a rat model. Blood and hepatic fatty acids were measured at all four days of the estrous cycle. Additionally, hepatic enzyme expression was examined at all four time points to determine how cyclical estrogen changes may influence hepatic gene expression and confirm findings from other models. Due to the importance of 22:6n-3 on human health and considerable variability in the changes in this fatty acid in various models examining estrogen, fatty acid desaturase and elongase enzyme mRNA

expression were examined in a breast cancer cell culture line (MCF7 cells) that is highly responsive to estrogen. While the estrogen response and the influence on *FADS2* mRNA expression was intact in the MCF7 cells, in these cells the gene is non-functional and does not produce 20:4n-6 and 22:6n-3. *FADS2* transcripts were examined in detail and a novel truncated *Fads2* transcript variant (*Fads2_v2*) was identified. This transcript variant and the intact variant (*Fads2_v0/1*) were then studied in detail by transfection into HEK 293 cells to determine their individual contributions on fatty acid composition as well as potential interactions between the variants.

Chapter 2.

Biochemical Foundations

2.1 Fatty Acids

Fatty acids are composed of hydrocarbon chains, with a carboxylic acid (-COOH) present at one end, and a methyl group ($-CH_3$) at the other end. Fatty acids are classified based on a variety of chemical properties. The first classification category is based on carbon chain length. Short-chain fatty acids have four carbons or fewer, medium-chain fatty acids have 6 to 10 carbons, long-chain fatty acids have 12 to 18 carbons, and very long-chain fatty acids have >20 carbons. Fatty acids are also classified based on the degree of desaturation of the carbon-carbon bonds. Saturated fats are completely hydrogenated, monounsaturated fatty acids have 1 double bond, and polyunsaturated fatty acids have 2 or more double bonds. Finally, fatty acids are also classified based on the location of their first double bond relative to the methyl (or omega) end. Omega-three fatty acids have a double bond between the third and fourth carbon, counting from the methyl end, and these fatty acids are designated Ω3, or n-3. Omega-six fatty acids have a double bond between carbons six and seven, and omega-nine fatty acids have a double bond between carbon number nine and ten. Taking all these categories together, fatty acids are abbreviated to give information about chain length, degree of desaturation and location of the first double bond. For instance, the fatty acid 22:6n-3 contains 22 carbons, and 6 double bonds. The first double bond is at the n-3 position (between carbons three and four relative to the methyl end). The fatty acid 18:0 contains 18 carbons, and no double bonds.

Fatty acids can be obtained through the diet or through *de novo* biosynthesis. Saturated and monounsaturated fatty acids (SFA and MUFA, respectively) are considered non-essential because they can be synthesized from dietary carbohydrate or amino acid precursors, while

18:2n-6 and 18:3n-3 polyunsaturated fatty acids (PUFA) are essential because they cannot be synthesized and must be obtained from the diet. Very long chain n-6 and n-3 PUFA are often considered "conditionally essential," because longer chain PUFA can be synthesized from essential dietary precursors (Cunnane, 2000), although the extent of this conversion and whether it provides sufficient long chain PUFA has been extensively debated (Domenichiello, Kitson, and Bazinet, 2015; Goyens et al., 2005; Pawlosky et al., 2003b; Talahalli et al., 2010). Fatty acid biosynthesis mainly occurs in the liver and adipocytes, and involves a series of elongation and desaturation steps. Different enzymes are used depending on the substrate, but these desaturation and elongation enzymes are generally found on the endoplasmic reticulum (Nakamura and Nara, 2004; Wang et al., 2005), while fatty acid synthase is cytosolic (Sul and Wang, 1998). The gene and protein names and predominant fatty acid substrates and products are summarized in Table 2.1. Redistribution of fatty acids from the liver to other tissues is mediated through the synthesis and secretion of lipoproteins. The liver is the main site of synthesis of circulating phospholipids (Radding and Steinberg, 1960) and TAG (Rui, 2014; Davis et al., 1979) during fasting. Circulating phospholipid composition is important since there is evidence that the brain derives 22:6n-3 through the selective uptake of this fatty acid as part of a lysophosphatidylcholine (Nguyen et al., 2014) although there is evidence that non-esterified 22:6n-3 is the main pool for brain uptake (Chen et al., 2015). In conditions such as pregnancy, recirculation of fatty acids in blood phospholipids is a major mechanism by which the body transfers n-3 PUFA to the developing fetus (Burdge et al., 2006; Vlaardingerbroek and Hornstra, 2004; Postle et al., 1995). Metabolically active tissues such as heart and muscle hydrolyze plasma TAG using lipoprotein lipase (Karpe et al., 1998) to release non-esterified fatty acids, which can then be taken up into the tissue via several different fatty acid transporters and fatty acid binding proteins (Kiens et al.,

1997; Turcotte, Kiens, and Richter, 1991; Kim and Dyck, 2016; Clarke et al., 2004). The concentration of plasma fatty acids is an important determinant of tissue fatty acid uptake, indicating that passive diffusion is likely an important mechanism for cellular fatty acid transport (Turcotte et al., 1998), likely in part related to activity of fatty acid acyl-CoA synthetases inside the cell influencing the concentration gradient (Coe et al., 1999; Hall, Smith, and Bernlohr, 2003).

2.2 Biosynthesis and Health Effects of Saturated and Monounsaturated Fatty Acids

SFA and MUFA biosynthesis are closely related (Figure 2.1). Carbohydrate catabolism in excess of cellular requirements for ATP results in the elevation of cytosolic levels of acetyl-CoA, which is carboxylated by acetyl-CoA carboxylase to form malonyl-CoA, the major substrate for fatty acid synthase (FAS) (Schiller and Bensch, 1971). The final product of FAS is 16:0, which can be incorporated into complex lipids and stored, or used as a substrate by other synthesis enzymes. Elongase 6 preferentially uses 16:0 as a substrate for elongation (Green et al., 2010), which results in 18:0. Stearoyl-CoA desaturase (SCD) desaturates 18:0 to form 18:1n-9 (Miyazaki, Bruggink, and Ntambi, 2006). SCD can use 16:0 as a substrate for desaturation, although the main SCD isoform expressed in the liver has a greater affinity for 18:0 (Miyazaki, Bruggink, and Ntambi, 2006). If carbohydrate intake is high, however, then SCD may use 16:0 for desaturation instead of 18:0, resulting in an excessive amount of 16:1n-7 flooding the cell (Hudgins et al., 1996), which can be elongated by either elongase 5 or 6 to form 18:1n-7 (Green et al., 2010). Concentrations of n-7 MUFA have been reported to be as low as 10%, or up to 50%, of the 18:1n-9 concentration in plasma from humans and rats (Puri et al., 2009; Marks, Kitson, and Stark, 2013).

Saturated fatty acids (SFA) can be stored in TAG where they can later be released and oxidized for energy, or they can be incorporated into phospholipids as structural components of membranes. SFA also make up a large proportion of the fatty acids in tissues in phospholipids. In rats, approximately 40% of fatty acids in plasma, erythrocytes, brain, heart, liver and muscle are SFA, while approximately 50% of the fatty acids in adipose tissue are SFA (Stark, Lim, and Salem, Jr., 2007).

Ingestion of a diet rich in SFA, and 16:0 in particular, has been associated with an increase in cardiovascular disease risk, disruption of the insulin signaling cascade (Yu et al., 2002; Griffin et al., 1999), and development of insulin resistance (Lionetti et al., 2007) and type II diabetes (Perdomo et al., 2015). In addition, treatment of cultured muscle and adipose cells with 16:0 has been found to increase the expression of several inflammatory cytokines such as IL-6 and TNF-α (Capel et al., 2015; Pillon et al., 2015)and act as a ligand for the proinflammatory toll-like receptor 4 (Huang et al., 2012). However, not all SFA have the same negative health effects. Another SFA, 18:0, has also been found to be positively related to incidence of type II diabetes in a population of older adults (Ma et al., 2015) but is also reported to lower serum total cholesterol concentrations compared with a diet rich in 16:0 (Storm et al., 1997).

As a class, MUFA are more likely to be oxidized or stored in TAG than PUFA (McCloy et al., 2004), possibly because SCD, the rate-limiting enzyme in MUFA biosynthesis, co-localizes with the rate-limiting enzyme in TAG synthesis, acyl-CoA:diacylglycerol acyltransferase 2 (Man et al., 2006). The MUFA composition of tissues varies depending on the tissue. For example, 18:1n-9 is the predominant fatty acid in rat muscle, adipose and liver (Polozova and Salem, Jr., 2007), and the total proportion of MUFA in these tissues is

approximately 30-35% in rats (Stark, Lim, and Salem, Jr., 2007). However, MUFA make up only 11-18% of total fatty acids in plasma, heart and erythrocytes of rats (Stark, Lim, and Salem, Jr., 2007).

Dietary MUFA are generally considered to be healthy and to have beneficial effects on the cardiovascular system, especially when replacing SFA in the diet (Baum et al., 2012; Perdomo et al., 2015) or when co-administered with omega-3 PUFA (Kondreddy, Anikisetty, and Naidu, 2016). Looking at 18:1n-9 specifically, it has been demonstrated in mice to prevent the hepatic inflammation and endoplasmic reticulum stress associated with a high-sucrose low-fat diet (Liu et al., 2016). However, in humans, reports have suggested that high levels of 16:1n-7 and 18:1n-7 in erythrocytes and plasma may be linked to an increased risk for cardiovascular disease (Wu et al., 2011; Djousse et al., 2012a) and MUFA in serum phospholipids has been associated with early increases in fasting glucose levels (Cho et al., 2014).

The enzymes involved in SFA and MUFA biosynthesis have been implicated in obesity and insulin resistance (Ntambi et al., 2002; Matsuzaka et al., 2007). SCD knockout mice are leaner and accumulate less fat than wild type mice. This may be due to the observed increase in expression of lipid oxidation genes and down-regulation of lipid synthesis genes in the knockout mice, compared with the wild type mice (Ntambi et al., 2002). This observation may also be due to inefficient TAG synthesis or storage, since SCD co-localizes with the rate-limiting enzyme in TAG biosynthesis (Man et al., 2006). However, SCD overexpression in muscle cells prevents the inflammatory response associated with 16:0 (Peter et al., 2009), suggesting that part of the reason SCD is induced in obesity may be to help protect the cell against 16:0 toxicity.

Elovl6 knockout animals are susceptible to diet-induced obesity to a similar degree as observed in wild-type animals are, since both groups of animals showed similar increases in

epididymal fat pad weight and total body weight percentage increases over the course of the study (Matsuzaka et al., 2007). However, the *Elovl6* knockout animals retain insulin sensitivity, unlike the wild-type animals which develop insulin resistance (Matsuzaka et al., 2007). Two potential mechanisms for this observation have been identified. *Elovl6* deletion has direct effects on the insulin signaling cascade. In the knockout animals, the IRS-2/Akt signaling pathway, which is critical for transduction of the insulin signal (Hanke and Mann, 2009), retains activity in the liver even with high-fat diet feeding (Matsuzaka et al., 2007). This effect on the insulin signaling pathway was only observed with hepatic signaling and was not evident in adipose or muscle, but was substantial enough to alter whole-body insulin sensitivity (Matsuzaka et al., 2007). Changes in fatty acid composition, such as occur with *Elovl6* gene ablation, change the substrate availability and composition of species of diacylglycerols (DAG) and ceramides, two signaling molecules that have been linked to insulin resistance. Increased DAG results in increased expression of protein kinase C, which impairs phosphorylation of IRS-1 and IRS-2 (Samuel et al., 2007). In *Elovl6* knockout animals, there are lower levels of DAG, and therefore lower expression of protein kinase C, resulting in greater insulin sensitivity (Matsuzaka et al., 2007). Another potential contributing mechanism to *Elovl6* knockout animals being protected from insulin resistance is that loss of *Elovl6* result in a more favorable lipid profile. Because 16:0 is not elongated to 18:0, SCD will use 16:0 as a substrate for desaturation, resulting in the synthesis of 16:1n-7. This fatty acid has been reported to be an adipose tissue-derived lipokine with insulin sensitizing effects on muscle (Cao et al., 2008; Dimopoulos et al., 2006).

These conflicting health effects of SFA, MUFA and the synthesizing enzymes suggest that MUFA may be less toxic than SFA, but the relationship between MUFA and health is not

straightforward, and may be based on the concentration or specific fatty acids species present. This illustrates the importance of furthering our understanding of MUFA metabolism.

2.3 Biosynthesis and Health Effects of n-6 and n-3 Polyunsaturated Fatty Acids

Omega 6 and omega 3 PUFA compete for a common set of enzymes for biosynthesis (Figure 2.2). The essential precursors, 18:2n-6 for omega 6 PUFA and 18:3n-3 for omega 3 PUFA, are first desaturated by delta-6 desaturase (D6D; gene name: *Fads2*) (Melin and Nilsson, 1997). The products of this reaction are next elongated by elongase 5, and then desaturated by delta-5 desaturase (D5D; gene name: *Fads1*) to form 20:4n-6 and 20:5n-3 (Nakamura and Nara, 2004), two physiologically important PUFA. The omega 6 pathway usually ends at 20:4n-6, but 20:5n-3 undergoes two more rounds of elongation by elongase 2 (Pauter et al., 2014), and another round of D6D desaturation (Nakamura and Nara, 2004), before being oxidized in the peroxisome to end with 22:6n-3. There has been a report in MCF7 cells overexpressing *FADS2* that D6D can possess delta-4 desaturation ability and directly desaturate 22:5n-3 to form 22:6n-3 (Park et al., 2015), but this has not been confirmed *in vivo*, and this does not occur in rodents (Pauter et al., 2014). Elongase 5 is the most abundant elongase in the liver (Wang et al., 2005), and is fairly non-specific in substrate preferences since it can use SFA, MUFA or PUFA as substrates (Green et al., 2010). Elongase 2 is crucial in the biosynthesis of 22:6n-3, because when it is knocked out, animals are unable to convert 22:5n-3 to 24:5n-3 and thereby do not make 22:6n-3 (Pauter et al., 2014). This confirms that elongase 2 is the only elongase that can perform this elongation, since it was previously thought that either elongase 2 or elongase 5 could perform this step (Gregory et al., 2011).

Several genetic polymorphisms have been identified in the elongases and desaturases involved in n-6 and n-3 PUFA biosynthesis. These polymorphisms often result in a lower

activity level of the enzyme, resulting in lower product levels from that enzyme. For example, in men, the *Fads1* minor allele rs174550 is associated with lower activity of D5D, and correspondingly results in lower levels of 20:4n-6 and 20:5n-3 in plasma (Takkunen et al., 2016). Some minor alleles in *Fads1* and *Fads2* (rs174546 and rs968567, respectively) are associated with higher levels of 18:3n-3 and lower levels of the longer chain n-3 PUFA products, and some minor alleles in *Elovl2* (rs2236212 and rs12662634) are associated with higher levels of 20:5n-3 and 22:5n-3, but lower levels of 22:6n-3 (Lemaitre et al., 2011). In men, minor allele carriers in both *Fads1* and *Fads2* (rs174537 and rs174576, respectively) show lower levels of 20:5n-3 at baseline, but greater increases in 20:5n-3 content in erythrocytes following fish oil supplementation (Roke and Mutch, 2014), indicating that the response to dietary omega-3 PUFA can be gene dependent.

N-6 and n-3 PUFA are generally found in cell membrane phospholipids. Replacing SFA with either n-3 or n-6 PUFA in cell membranes can negate the effects of 16:0 on inflammatory markers and insulin resistance in muscle cells (Capel et al., 2015) and improve plasma LDL cholesterol levels (Summers et al., 2002). N-6 and n-3 PUFA from membrane phospholipids can be used as substrates for signalling molecules (Fitzpatrick and Soberman, 2001; Bazan, Calandria, and Gordon, 2013). Signalling molecules derived from n-6 PUFA tend to have a pro-inflammatory effect (Khan et al., 2014; Bagga et al., 2003), while n-3 PUFA-derived signaling molecules are anti-inflammatory (Flock et al., 2014; Dangardt et al., 2010; Hong and Lu, 2013; Bagga et al., 2003; Allam-Ndoul et al., 2016). A balance between these classes of PUFA is thus important for maintaining health, with the optimal ratio of intake of n-6/n-3 being between 3:1 and 5:1. Most western diets provide a ratio of approximately 15:1 (Simopoulos, 2002; Simopoulos, 2008; Lands, 2014).

N-3 PUFA have a role in reducing the risk for sudden cardiac death (GISSI-Prevenzione Investigators, 1999; Musa-Veloso et al., 2011), and have antiarrhythmic (Xiao et al., 1997) and TAG lowering effects (Zulyniak et al., 2015). A large amount of n-3 PUFA are not required to see these effects. Intake of as little as 250-500 mg/day of 20:5n-3 plus 22:6n-3, compared with little to no intake, can result in a reduction of coronary heart disease risk by 25% (Mozaffarian and Rimm, 2006). Maximal effects on the anti-arrhythmic properties of 20:5n-3 and 22:6n-3 are seen at intakes of 750 mg/day, but the relative strength of the effect of these fatty acids on lowering blood pressure, heart rate and blood TAG levels continues to increase past this dose (Mozaffarian and Rimm, 2006), indicating that small changes in blood and tissue omega 3 content have large physiological effects.

Omega-3 PUFA are highly concentrated in the brain (Svennerholm, 1968; Breckenridge, Gombos, and Morgan, 1972; Salem, Jr. et al., 1980) and retina (Anderson and Sperling, 1971; Anderson, 1970; van Kuijk and Buck, 1992). In particular, 22:6n-3 comprises 25-40% of total fatty acids in these tissues, likely due to the importance of this specific fatty acid in brain development (Lauritzen et al., 2016), cell signaling (Speizer, Watson, and Brunton, 1991), neurotransmission (Garcia and Kim, 1997) and ocular function (Rotstein et al., 2003; Uauy et al., 2001). The heart also has a relatively high proportion of n-3 PUFA, with ~10% of heart fatty acids being n-3 PUFA. Omega-3 PUFA make up approximately 5% of total fatty acids in tissues such as muscle, liver, plasma and erythrocytes, whereas n-3 PUFA levels are extremely low in adipose tissue, being less than 1.5% of total fatty acids (Stark, Lim, and Salem, Jr., 2007)

Omega-6 PUFA are required for proper growth and development. Omega-6 deficiency is associated with dermatitis (Fujii et al., 2013) and hair loss, and a 15% decrease in body weight (Cunnane and Anderson, 1997). Health effects of n-6 PUFA supplementation are less clear.

Some studies have shown benefits of dietary 18:2n-6 on reducing blood cholesterol levels (Mensink et al., 2003) and n-6 PUFA in reducing the risk for coronary heart disease (Jakobsen et al., 2009; Wu et al., 2014), but not all studies agree (Chowdhury et al., 2014; Ramsden et al., 2013). This is possibly due to the competition between n-6 and n-3 PUFA for synthesis enzymes (Simopoulos, 2008) and the pro-inflammatory properties of n-6 PUFA. In rats, tissue n-6 PUFA composition varies greatly between different tissues. There are low levels of n-6 PUFA in brain and adipose tissue (approximately 10-12% of total fatty acids), intermediate levels in liver and muscle (16-19% of total fatty acids) and greater concentrations in plasma, heart and erythrocytes (29-35% of total fatty acids) (Stark, Lim, and Salem, Jr., 2007).

2.4 Factors that Influence Fatty Acid Composition

Besides synthesis and tissue specificity, several factors influence body fatty acid composition. Diet has a strong effect on tissue and blood fatty acid composition. Increasing the amount of omega-3 PUFA in the diet, such as through eating oily fish or taking a fish oil supplement, results in an increased proportion of omega-3, specifically the biologically important 20:5n-3 and 22:6n-3, in the blood of humans (Dewailly et al., 2001; Patterson et al., 2015; Metherel et al., 2009) and rats (Kim, Jin, and Park, 2015; Tu et al., 2010). In addition to fat composition of the diet, excessive carbohydrate intake can also influence fatty acid composition, by increasing *de novo* fatty acid biosynthesis (Hudgins et al., 1996). Excess carbohydrate intake results in greater amounts of acetyl-CoA, the precursor for *de novo* synthesis of 16:0, thereby increasing levels of this fatty acid and its metabolites in the body.

Vegetarians and vegans of both sexes generally have lower plasma and erythrocyte levels of 20:5n-3 and 22:6n-3, and higher levels of 18:3n-3, compared with omnivores (Kornsteiner, Singer, and Elmadfa, 2008; Rosell et al., 2005; Agren et al., 1995; Sanders, Ellis, and Dickerson,

1978; Melchert et al., 1987), since protein intake is more associated with omega-3 status than fat intake due to n-3 PUFA being present in phospholipids rather than TAG (Stark and Patterson, 2012; Miles et al., 2011). Furthermore, levels of n-3 PUFA are even lower in vegans compared with vegetarians (Kornsteiner, Singer, and Elmadfa, 2008). In vegans and vegetarians, omega-6 levels tend to be higher than in omnivores, while 16:0, 16:1n-7 and 18:1n-9 are lower (Agren et al., 1995; Sanders, Ellis, and Dickerson, 1978); however, these MUFA differences are not always observed (Kornsteiner, Singer, and Elmadfa, 2008), likely due in part to whether there are differences in the proportion of MUFA in the diet between vegans/vegetarians and omnivores or not (Kornsteiner, Singer, and Elmadfa, 2008; Rosell et al., 2005).

Another dietary consideration that affects fatty acid composition is general nourishment. Malnourished children have lower levels of essential fatty acids compared with healthy controls (Holman et al., 1981; Smit et al., 1997; Leichsenring et al., 1995; Koletzko et al., 1986; Wolff et al., 1984), which is likely due to a combination of low overall energy intake and the scarce food source sources that are available are a poor source of essential fatty acids, malabsorption, and oxidation of body lipid stores and what fatty acids are present in the diet (Smit et al., 1997; Koletzko et al., 1986). However, this relationship is complex. Different types of malnutrition show different effects on fatty acid composition. For example, children with marasmus have lower levels of 20:4n-6 compared with children with kwashiorkor (Vajreswari, Narayanareddy, and Rao, 1990; Wolff et al., 1984).

Two unmodifiable factors are also associated with fatty acid composition: age and sex. Age is positively associated with plasma and erythrocyte omega-3s and negatively associated with omega-6s in Japanese and James Bay Cree populations (Ogura et al., 2010; Dewailly et al., 2002). Both omega-3 and omega-6 PUFA are positively associated with age in adipose tissue

(Bolton-Smith, Woodward, and Tavendale, 1997). With sex, the proportion of 22:6n-3 is higher in women compared with men, after controlling for diet in several different populations, (Dewailly et al., 2001; Childs et al., 2014; Giltay et al., 2004; Bakewell, Burdge, and Calder, 2006; Crowe et al., 2008; Marangoni et al., 2007; Metherel et al., 2009; Patterson et al., 2015). The proportion of 16:0, 16:1n-7 and 18:1n-7 is lower, while the proportion of 18:1n-9 and 22:5n-3 is higher, in men compared with women (Giltay et al., 2004; Marangoni et al., 2007; Metherel et al., 2009; Patterson et al., 2015). These observations have been corroborated in animal models. Female rats tend to have higher levels of 18:0, 18:3n-6 and 22:6n-3 (Extier et al., 2010; Kitson et al., 2012; Burdge et al., 2008) and lower levels of 16:0, 18:1n-7, 18:1n-9 and 22:5n-3 compared with males (Burdge et al., 2008; Kitson et al., 2012; Marks, Kitson, and Stark, 2013), when fed the same diet during the experimental period. A summary of sex differences in human and rat fatty acid composition is found in Table 2.2. These results indicate that sex plays an independent role in determining fatty acid composition, since both humans (with diet controlled), and rodents on a standard chow diet, show sex differences in fatty acid composition.

Modifiable factors such as smoking and alcohol consumption are also associated with fatty acid composition. The relative percent mass of 20:5n-3 and 22:6n-3 are lower in smokers compared with non-smokers (Dewailly et al., 2001; Dewailly et al., 2002; Scaglia et al., 2016). Generally, the proportion of 22:6n-3 decreases in plasma of humans as the number of alcoholic drinks per week increases (Dewailly et al., 2002). The same effect of 22:6n-3 depletion is observed in liver, plasma, heart and brain of several different animal models who are chronically fed ethanol, including decreases in activity of synthesis enzymes such as D5D and D6D (Littleton, John, and Grieve, 1979; Nakamura et al., 1994; Pawlosky and Salem, Jr., 1995; Pawlosky and Salem, Jr., 1999), with compensatory increases in 22:5n-6 (Pawlosky and Salem,

Jr., 1995) and 20:3n-9 (Pawlosky and Salem, Jr., 1995), suggesting disrupted essential fatty acid metabolism.

Endurance training is another modifiable factor that also influences tissue fatty acid composition. Levels of 18:2n-6, 18:3n-6 and 18:3n-3 increase in hepatic phospholipids in endurance trained rats compared with untrained animals, while hepatic TAG are not changed in trained animals (Petridou et al., 2005). As expected, the concentration of most fatty acids in TAG, as well as total TAG, decrease in muscle during exercise, but the relative proportion shows a decrease in levels of 16:1n-7 and increases in levels of 18:0, 18:3n-6 and 20:5n-3. Few changes are observed in phospholipids in muscle (Petridou et al., 2005). Concentrations of MUFA increase in adipose TAG, although TAG are enriched with n-6 PUFA rather than MUFA during exercise (Petridou et al., 2005). Conversely, during obesity, total TAG accumulate in muscle, adipose and liver (Donnelly et al., 2005; Puri et al., 2009; Belfiore et al., 1987; Bonen et al., 2004), and these TAG generally contain SFA and MUFA and low levels of PUFA (Borkman et al., 1993). Obese individuals also have higher levels of SFA, MUFA and some n-6 PUFA in plasma phospholipids and cholesteryl esters, and lower levels of 22:6n-3, compared with normal weight individuals (Warensjo, Ohrvall, and Vessby, 2006; Klein-Platat et al., 2005).

2.5 Cell Culture Models and Fatty Acid Composition

Cell culture conditions, such as nutrient composition, hormones, silencing or overexpressing of genes, and more, can be easily manipulated and tightly controlled. As such the specific effects of individual experimental parameters on gene expression or fatty acid composition to be examined, making them a useful complement to human or animal studies. However, there are some considerations when choosing which cell line to use, since there are benefits and limitations to each.

One of the greatest limitations to studying fatty acid metabolism using cell culture is that fatty acid composition of most cell lines is unknown. One study reported the relative proportion of nine fatty acids in several common cancer and normal cell lines (Fermor et al., 1992). To our knowledge, no other reports of fatty acid composition in cell culture models have been reported. HEK 293 cells are commonly used for experiments studying genes involved in fatty acid biosynthesis (Wu, Li, and Gou, 2010; Sinner et al., 2012; Li et al., 2016; Chen et al., 2011), because these cells can be easily transfected to express a gene of interest, but the fatty acid composition of these cells has not been reported. In addition, the media used can drastically alter cellular fatty acid composition (Stoll and Spector, 1984). Levels of 22:6n-3 in fetal bovine serum, which is commonly used to supplement cell culture media, are quite high (van den Elsen et al., 2013), and can inhibit endogenous 22:6n-3 synthesis and mask any subtle effects that might be occurring due to treatment. Cells need the nutrients and hormones in the FBS to grow properly, but these fatty acids may interfere with fatty acid synthesis experiments. Charcoal treating the serum removes hormones and fatty acids, and can be used prior to experiments where those may interfere (Aakvaag et al., 1990). Finally, immortalized hepatic cell lines are useful for studying fatty acid metabolism because they have a long lifespan, they can be genetically stable (Castell et al., 2006), and the liver is one of the major sites of fatty acid metabolism in animals and humans. However, these cell lines also have some disadvantages. Immortalized cell lines are often genetically manipulated which may alter their phenotype, response to experimental treatment and overall normal function (Kaur and Dufour, 2012). Primary liver cell cultures may allow for a more physiological representation of fatty acid metabolism, including hormone studies, but have limitations as they often stop growing after

reaching confluence, they cannot be subdivided easily, and their mRNA expression is disrupted by the collagenase treatment required to prepare the tissue for culturing (Castell et al., 2006).

2.6 Rat Estrous Cycle and Human Menstrual Cycle

Female rats have a four-day estrous cycle. Each stage of the cycle lasts for approximately twenty-four hours. During the cycle, estrogen concentrations fluctuate from 30 pg/ml during the estrus stage, to 40-50 pg/ml during the metestrus and diestrus stages, to highs of 150 pg/ml during proestrus (Flores et al., 2011). Progesterone levels are lowest during estrus, at 5 ng/ml. Levels rise during metestrus and diestrus to approximately 20 ng/ml and peak at 40 ng/ml during proestrus (Smith, Freeman, and Neill, 1975). Typical hormone changes over the rat estrous cycle are shown in Figure 2.3.

During a regular, 28-day human menstrual cycle, hormones change in a predictable pattern, but do not reach the dramatically high or low concentrations observed during pregnancy or menopause, respectively. The first phase of the menstrual cycle is the follicular phase, which occurs from day 1 until ovulation on day 14. At the beginning of the follicular phase, the circulating estrogen concentration is low (approximately 0.1 nmol/L). Then it begins to increase around day 10, and peaks at 0.7 nmol/L prior to ovulation. Following ovulation, the luteal phase occurs. Estrogen concentrations decrease slightly but remain elevated compared with the follicular phase, at 0.5 nmol/L through the duration of the luteal phase (ovulation to day 28) (Sramkova et al., 2015). Progesterone is generally low from menses until ovulation, where it rises sharply and remains elevated throughout the luteal phase (Sramkova et al., 2015). The changes in estrogen, progesterone and leuteinizing hormone over a regular menstrual cycle are shown in Figure 2.4. In general, total triacylglycerols (TAG) and cholesterol in the blood are lower during the luteal phase of the menstrual cycle compared with the follicular phase, in

women with regular menstrual cycles (Pahwa, Seth, and Seth, 1998; Barnett et al., 2004; Kim and Kalkhoff, 1979; Schisterman, Mumford, and Sjaarda, 2014). Plasma non-esterified fatty acids concentrations in blood do not appear to differ based on menstrual cycle phase (Heiling and Jensen, 1992).

Women taking hormonal-based oral contraceptives, which work by either providing a constant dose of hormones over the menstrual cycle (monophasic birth control) or differing doses of hormones depending on the week (bi- and triphasic birth control). Many oral contraceptives have a twenty-one day active period where women take the hormones followed by seven days where no hormones are consumed. Withdrawal bleeding usually occurs approximately four to five days after women stop taking the hormones (Sulak et al., 2000). Some oral contraceptives have different lengths of the active hormone and the off period, but twenty-one days on and seven days off is the most common (Nappi, Kaunitz, and Bitzer, 2016). Hormone dose varies widely in different brands and types of oral contraceptives.

2.7 Estrogen Signaling

Estrogen is a name referring to a group of sex hormones that is synthesized mainly in the ovaries and the testes from cholesterol by the enzyme aromatase (Graham-Lorence et al., 1991). There are 3 main biological forms of estrogen: estradiol, estrone and estriol. Of these, estradiol displays the greatest potency in both breast cancer proliferation and estrogen receptor binding assays (Gutendorf and Westendorf, 2001). Some synthetic forms of estrogen, such as ethinylestradiol and diethylstilbestrol, are actually more potent and bind more strongly to estrogen receptors than estradiol (Gutendorf and Westendorf, 2001). These synthetic forms of estrogen are often used in hormone-based oral contraceptives and hormone therapy.

There are two major isoforms of the estrogen receptor: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα is expressed in uterus, ovaries, pituitary gland, kidney, liver, adrenal gland, heart, testis, prostate gland and the epididymis (Kuiper et al., 1997), and is the primary form involved in mediating female reproduction. ERα is also the major estrogen receptor involved in estrogen signalling in the liver, and therefore is most likely to be the ER form involved in mediating effects of estrogen on hepatic fatty acid biosynthesis. ERβ is more involved in "non-classical" target tissues and in signaling in the male reproductive system, and is expressed in testis, prostate gland, epididymis, pituitary gland, ovaries, bladder, lung, spinal cord and several regions of the brain (Kuiper et al., 1997).

In classic genomic signaling, estrogen crosses the cell membrane and binds to a receptor (ERα or ERβ), which is inactive and surrounded by chaperone proteins. Upon ligand binding, the chaperones dissociate and the receptor dimerizes with another ER, forming a homo- or hetero-dimer (ERα/ERα, ERβ/ERβ or ERα/ERβ) (Cowley et al., 1997), and translocates to the nucleus. This complex can bind to a specific sequence in the promoter region of a gene called the estrogen response element (ERE). The consensus sequence for the ERE is GGTCAnnnTGACC (where n is any base pair) (Gruber et al., 2004). After this binding occurs, transcription of the gene is either increased or decreased. Several co-activators and co-repressors can also interact with the estrogen receptor dimer to regulate its effect on gene expression (Safe and Kim, 2004; Safe and Kim, 2008).

In addition to this classical, genomic signalling pathway, membrane-localized ER have also been reported, which can activate alternate signalling cascades. Three major pathways have been shown to be influenced by membrane ER signalling: Ras-Raf-MEK-MAPK, Src-PI3K-Akt-eNOS, and PLC-PKC-cAMP-PKA (Zhang and Trudeau, 2006). While influencing gene

expression can take hours, membrane ER signaling is very rapid, and occurs within seconds to minutes. In addition, however, these processes can also influence genomic signalling of ER, since these cascades may phosphorylate one of the co-activators or co-repressors of ER action, or phosphorylate ER itself (Zhang and Trudeau, 2006). In this way, the rapid, non-classical membrane-ER pathway can influence the classical, genomic regulatory action of ER by influencing ability of the nuclear form to bind to an ERE.

2.8 Estrogen Effects on Fatty Acid Composition

The higher blood levels of 16:0 and 22:6n-3, and lower levels of 18:1n-9, in women compared with men (Metherel et al., 2009; Marangoni et al., 2007; Crowe et al., 2008) have been hypothesized to be due to effects of estrogen, since estrogen concentrations are much higher in women compared with men (Eldrup, Lindholm, and Winkel, 1987). Several studies in ovariectomized rodents and/or treating rodents with estrogen pellets have been completed (see Table 2.3 for summary of rodent hormonal manipulation studies). In general, ovariectomized animals treated with estrogen have higher blood and hepatic levels of 18:0, 16:1n-7, 18:1n-7, and 22:6n-3, compared with untreated ovariectomized animals (Kitson et al., 2013a; Marks et al., 2013a; Mason et al., 2014). In rodent models, MUFA composition and elongase 6 expression appear to consistently respond to estrogen. Levels of 18:0 are higher and 16:1n-7 and 18:1n-7 are lower in; females compared with males (Marks, Kitson, and Stark, 2013), ovariectomized rats given hormone treatment as compared with untreated ovariectomized rats (Oh, Jin, and Park, 2015; Marks et al., 2013a), and ERα knockout mice compared with wild type mice (Kitson, 2013). The opposite trend is seen in ERα-positive breast cancer cells treated with estrogen. MCF7 and MCF-10F cells have lower levels of 18:0 and higher levels of 16:1n-7 and 18:1n-7 when treated with estrogen (Belkaid et al., 2015), suggesting there may be disrupted MUFA

metabolism in cancer cells that is different from an animal model. This is possibly due to an induction in SCD expression in MCF7 cells (Belkaid et al., 2015), which is usually repressed by estrogen in animals (Paquette et al., 2008). Aromatase knockout mice lack the capacity to synthesize estrogens. Aromatase knockout mice are obese and have hepatic steatosis (Jones et al., 2000), which appears to be due to an increase in fatty acid uptake rather than decreased lipolysis or increased *de novo* biosynthesis (Misso et al., 2003). Aromatase knockout animals are also hyperinsulinemic, hyperleptinemic and hypertriglyceridemic (Jones et al., 2000). These effects are not unique to the aromatase knockout model. Ovariectomized animals are also hyperphagic and accumulate TAG in the liver (Kitson et al., 2015). In both of these models, 17β-estradiol reverses these effects (Chow et al., 2011; Hewitt et al., 2004; Kitson et al., 2013b; Marks et al., 2013b), suggesting that estrogen is an important regulator of lipid metabolism. However, no work has examined fatty acid composition in aromatase knockout mice.

Women tend have higher blood levels of very long chain omega 3 fatty acids as compared with men after controlling for diet (Metherel et al., 2009; Patterson et al., 2015; Marangoni et al., 2007; Crowe et al., 2008). It has been hypothesized that this sexual dimorphism exists to support the fetal demand for 22:6n-3 during pregnancy and is associated with estrogen concentrations (Burdge and Calder, 2005). The omega-3 fatty acid changes after manipulation of estrogen status in rodents by either ovariectomy or knocking out ERα gene expression are not always consistent with this estrogen-based hypothesis from human studies. Female rats have a greater capacity to synthesize 22:6n-3 from dietary precursors compared with males (Burdge and Calder, 2005; Alessandri et al., 2012). Estrogen treatment of ovariectomized rats increases very long chain omega 3 PUFA levels and D6D protein levels (Kitson et al., 2013a; Mason et al., 2014), but ERα knockout mice do not show reduced 22:6n-3 or D6D gene levels (Kitson, 2013).

However, ERα knockout mice have membrane-bound ERα, as it is only the DNA binding domain of the ERα gene that is disrupted (Couse et al., 1995). Therefore it is possible membrane based ERα signalling could be supporting omega-3 PUFA metabolism, which has been proposed previously (Kitson, Stroud, and Stark, 2010). Additionally, in the ERα knockout model estrogen concentrations are ten times higher compared with wild type animals as a compensatory reaction, therefore any membrane based ERα signalling is likely enhanced (Couse et al., 1995).

The effect of sex hormones on fatty acid metabolism in humans can also be examined in models where sex hormones change endogenously such as pregnancy, menopause and over the menstrual cycle or through exogenous use such as oral contraceptives and hormone therapy with menopause (see summary Table 2.4 for summary of studies examining fatty acid composition during hormone changes). In pregnant women estrogen increases more than seven-fold from approximately 3.2 nmol/L during the first trimester to 22.6 nmol/L during the third trimester (Soldin et al., 2005; Meyer et al., 2013). Over the same time course, the plasma proportions of 16:0, 16:1n-7, 22:5n-3 and 22:6n-3 rise, and then decrease back to baseline levels following delivery (Al et al., 1995; Stark et al., 2005; Kuipers et al., 2011; Ghebremeskel et al., 2000), while the proportion of 18:0 and 20:5n-3 remains lower in pregnant women compared with non-pregnant women (Stark et al., 2005). During menopause estradiol falls from an average concentration of 0.43 nmol/L in premenopausal women to approximately 0.08 nmol/L in post-menopausal women (Walsh et al., 1991; Bojar et al., 2015; Fuhrman et al., 2014) and premenopausal women have higher 16:0, 18:1n-9, 18:2n-6 and 22:6n-3 in erythrocytes as compared with postmenopausal women (Tworek et al., 2000). Postmenopausal women taking hormonal therapy have higher levels of 16:0, 16:1n-7, 20:3n-6, and 20:5n-3, and a lower 18:0/16:0 ratio and lower levels of 22:5n-3 than those not taking hormonal therapy (Stark, Park,

and Holub, 2003; Sumino et al., 2003; Ottosson et al., 1984; Stark et al., 2000). Effects of hormone therapy on concentrations of 22:6n-3 in the blood have been mixed (Sumino et al., 2003; Stark, Park, and Holub, 2003). However, the pattern of fatty acid changes with increased estrogen tend to mimic response of increased estrogen during pregnancy. Increased estrogen appears to mediate a response resulting in higher 16:0, 16:1n-7, and 22:6n-3. During the follicular phase of the menstrual cycle, estrogen concentrations are approximately 0.1 nmol/L. These levels peak at 0.7 nmol/L during ovulation and remain at approximately 0.5 nmol/L through the luteal phase (Sramkova et al., 2015). To our knowledge, only one study has examined lipid changes over the menstrual cycle and was completed when determining long chain PUFA such as 22:6n-3 by gas chromatography was more difficult and often not reported. The relative proportion of 16:0, 18:0 and 18:1n-9 in plasma cholesteryl esters is higher during the luteal phase compared with the follicular phase (Mattsson, Silfverstolpe, and Samsioe, 1985) and levels of 18:0 and 18:1n-9 in plasma phospholipids are lower during the luteal phase (Mattsson, Silfverstolpe, and Samsioe, 1985). Full fatty acid composition or quantitative data have not been reported during the menstrual cycle. Studies examining oral contraceptive use have reported higher levels of 16:0, 16:1n-7 and 22:6n-3 in plasma cholesteryl esters (Giltay et al., 2004) and lower concentrations of 20:4n-6, 20:5n-3 and 22:5n-3 in plasma and erythrocytes (Fehily et al., 1982) in women taking oral contraceptives compared with women not taking oral contraceptives.

Table 2.1. Summary of enzymes involved in *de novo* fatty acid biosynthesis.

Common name	Gene name	Protein name	Usual substrates	Usual products
Fatty acid synthase	*Fasn*	FASN	Malonyl-CoA	16:0
Stearoyl-CoA desaturase	*Scd1*	SCD1	18:0, 16:0	18:1n-9, 16:1n-7
Elongase 6	*Elovl6*	ELOVL6	16:0	18:0
Delta-5 desaturase	*Fads1*	D5D	20:4n-3, 20:3n-6	20:5n-3, 20:4n-6
Delta-6 desaturase	*Fads2*	D6D	18:3n-3, 24:5n-3, 18:2n-6, 24:4n-6	18:4n-3, 24:6n-3, 18:3n-6, 24:5n-6
Elongase 2	*Elovl2*	ELOVL2	20:5n-3, 22:5n-3, 20:4n-6, 22:4n-6	22:5n-3, 24:5n-3, 22:4n-6, 24:4n-6
Elongase 5	*Elovl5*	ELOVL5	18:4n-3, 20:5n-3, 18:3n-6, 20:4n-6, 16:1n-7	20:4n-3, 22:5n-3, 20:3n-6, 22:4n-6, 18:1n-7

Table 2.2. Summary of studies reporting sex differences in fatty acid composition in rats and humans.

Study	Subjects	Diet	Results
Rat studies			
Marks et al. 2013	Male and female Sprague-Dawley rats, aged 14 weeks of age	*Ad libitum* access to AIN-93G diet	Females had lower concentrations of 16:0, 18:1n-7 and 18:1n-9, and higher 18:0, in hepatic phospholipids and plasma total lipids compared with males
Extier et al. 2010.	Male and female Wistar rats, aged 8 weeks of age.	*Ad libitum* access to a diet containing sub-optimal amounts of 18:3n-3	Females had higher 22:6n-3 in plasma and liver phospholipids compared with males
Kitson et al. 2012	Male and female Sprague-Dawley rats, aged 14 weeks of age	*Ad libitum* access to AIN-93G diet	Females had lower concentrations of 18:2n-6 and 22:5n-3, and higher 18:3n-6 and 22:6n-3 in hepatic phospholipids and plasma total lipids, compared with males
Burdge et al. 2008	Male and female Wistar rats, aged 15 weeks of age	Mothers were fed four different diets; during lactation and after weaning, rats all received the same diet	The proportion of 16:0, 18:1n-9, 18:2n-6 and 20:5n-3 were lower, while the proportion of 18:0, 20:4n-6 and 22:6n-3 were higher in hepatic phospholipids of females compared with males
Human Studies			
Dewailly et al. 2001	Adult men and women from the Inuit population of Nunavik	Habitual intake, measured by 24 hour food recall, and FFQ in women	Women have a higher proportion of 20:5n-3 and 22:6n-3 in plasma compared with men
Childs et al. 2014	British adult men and women	Subjects received either 1.5g/day or 9.5g/day of 18:3n-3 for 6 months	Women who received 9.5 g/day of 18:3n-3 had higher 20:5n-3 in plasma phospholipids compared with men
Giltay et al. 2004	Men, women who take oral contraceptives and women who do not take oral contraceptives from The Netherlands	Strictly controlled laboratory diet for 3 weeks	Women have higher proportions of 16:0, 16:1, 18:1n-7 and 22:6n-3, and lower proportions of 18:0 and 18:1n-9 in plasma cholesteryl esters compared with men
Bakewell, Burdge and Calder. 2006	Adult British men and women; of the women, half took an oral contraceptive, half did not; blood was taken on day 10 of menstrual cycle	Habitual intake, measured with FFQ	Women have higher 22:6n-3 in plasma compared with men
Crowe et al. 2008	New Zealanders who were at least 15 years of age	Habitual intake, measured with 24 hour recall	Women have lower 20:5n-3 and 22:5n-3, and higher 22:6n-3 in plasma phospholipids compared with men
Marangoni et al. 2007	Italian adults	Habitual intake, measured with 3 day food record	Women have lower 18:1n-9 and 22:5n-3 in whole blood compared with men
Metherel et al. 2009	Canadian men and women	Habitual intake, measured with 3 day diet record	Women have a higher proportion of 16:0 and 22:6n-3, and a lower proportion of 22:5n-3 in blood, compared with men
Patterson et al. 2015	Canadian men and women	Habitual intake, measured with FFQ	Women have a lower proportion of 22:5n-3 and a higher proportion of 22:6n-3 in blood than men

Table 2.3. Summary of studies reporting fatty acid composition and enzyme expression changes during situations of altered hormone status in rodents.

Hormonal manipulation (rodents)			
Alessandri et al. 2011.	Female Wistar rats	Rats were ovariectomized and received either zero, one or two estradiol constant-release pellets, or sham-operated	Levels of 22:6n-3 were higher in hepatic phospholipids of the animals who received estradiol pellets, compared with sham operated and ovariectomized animals
Marks et al. 2013.	Female Sprague-Dawley rats	Rats were ovariectomized or sham-operated. Ovariectomized rats received either no pellet, or a pellet containing estradiol, progesterone, or both, for two weeks	Elongase 6 and stearoyl-CoA desaturase expression and hepatic and plasma phospholipid concentrations of 16:0 and 18:1n-7 were highest in ovariectomized animals receiving estradiol, while 18:0 was lowest in these groups, compared with sham operated, ovariectomized, and animals receiving progesterone.
Jones et al. 2000.	Aromatase knockout mice	No intervention	Aromatase knockout mice are obese and have hepatic steatosis. They are also hyperinsulinemic, hyperleptinemic and hypertriglyceridemic. 17β-estradiol reverses these effects.
Mason et al. 2014.	Athymic female mice (BALB/c nu/nu)	Ovariectomized mice had an estradiol pellet implanted for two weeks, after which time half of the animals had it removed.	18:0 and 22:6n-3 were significantly higher in the animals receiving estradiol, while 18:1n-7 and 18:1n-9 were lower in these animals.
Kitson et al. 2013.	Female Sprague-Dawley rats	Rats were ovariectomized or sham-operated. Ovariectomized rats received either no pellet, or a pellet containing estradiol, progesterone, or both, for two weeks	Delta-6 desaturase expression and hepatic and plasma phospholipid concentrations of 20:5n-3, 22:5n-3, 22:6n-3 and 22:5n-6 were the highest in ovariectomized rats receiving estrogen, compared with the other groups.
Bryzgalova et al. 2006.	ERα knockout mice, wild type controls	No intervention	Hepatic expression of stearoyl-CoA desaturase 1 was twice as high in ERα knockout mice compared with controls
Paquette et al. 2008.	Female Sprague-Dawley rats	Rats were ovariectomized or sham operated. Ovariectomized rats received either no pellet, or a pellet containing estradiol	Stearoyl-CoA desaturase 1 expression was higher in ovariectomized animals compared with sham operated animals, and this effect was not observed in ovariectomized rats who received estrogen pellets.
Kitson et al. 2015.	Female Sprague-Dawley rats	Rats were ovariectomized or sham operated. Ovariectomized rats were either allowed *ad libitum* access to food, or were pair-fed with the sham operated rats.	Ovariectomized rats had higher stearoyl-CoA desaturase 1 expression compared with sham operated rats. This effect was not observed in pair-fed ovariectomized rats.
Pregnancy (Rats)			
Chen, Yang and Cunnane. 1992.	Pregnant Sprague-Dawley rats	Fatty acids were analyzed in non pregnant rats, and at days 13, 15 and 21 of gestation	The proportion of 16:0 and 22:6n-3 increased over gestation, while 18:0 and 20:4n-6 decreased, in serum and hepatic phospholipids.
Burdge, Hunt and Postle. 1994.	Late pregnancy in Wistar rats	Non-pregnant or pregnant rats at 16 and 21 days of gestation were injected with labelled choline or methionine	Phospholipids containing 16:0/22:6n-3 increased during the first 16 days of pregnancy, and continued until term.

Table 2.4. Summary of studies reporting fatty acid composition changes during situations of altered hormone status in humans.

Study	Subjects	Study Design	Results
Pregnancy (Human)			
Stark et al. 2005.	African-American women at 24 weeks gestation, delivery and 3 months postpartum	Comparison of plasma fatty acid composition at 24 weeks gestation, delivery and 3 months postpartum.	In plasma, the proportion 16:0 increased over the course of gestation, while 16:1 and 20:5n-3 decreased. Postpartum, 16:0, 16:1 and 22:6n-3 decreased, while 20:5n-3 increased.
Al et al. 1995.	Pregnant, healthy women	Blood samples were taken at 10, 14, 18, 22, 26, 30, 32, 35, 36 and 40 weeks gestation	22:6n-3 concentrations increased significantly through gestation, although the relative proportion remained stable. Concentration of 22:6n-3 leveled off towards the end of pregnancy in plasma phospholipids. 6 months after delivery, the proportion was lower than during pregnancy.
Kuipers et al. 2011.	Women from 3 different populations, consuming either a low, medium or high fish diet	Comparison of maternal erythrocyte profiles at delivery and 3 months postpartum.	22:6n-3 erythrocyte concentrations were higher at delivery as compared with postpartum erythrocyte concentrations, in all 3 populations.
Otto et al. 2001.	Postpartum fatty acid composition of lactating and non-lactating women	Plasma and erythrocyte fatty acid composition was compared between these 2 groups and over time, beginning at 36 weeks gestation and ending at 64 weeks postpartum.	22:6n-3 percentages declined steadily in both groups postpartum, with a significant difference between groups.
Ghebremeskel et al. 2000.	Non-pregnant Korean women and Korean women in their third trimester	Fatty acid composition of plasma and were compared between these 2 groups.	Pregnant women have a higher proportion of 16:0, 16:1, 18:1n-9 and 22:6n-3, and lower 18:0 and 22:5n-3, compared with non-pregnant women in plasma phospholipids.
Burdge et al. 2006.	Non-pregnant women and pregnant women	Plasma phospholipid composition was measured and compared between non-pregnant women and pregnant women in early (20-22 weeks) and late (30-34) gestation, and postpartum	The concentration of 22:6n-3 in plasma phospholipids increased throughout pregnancy, and was approximately 230% higher in pregnant and post partum women, compared with non pregnant women.
Menopause			
Stark and Holub. 2000.	Postmenopausal women	Algal 22:6n-3 supplementation in postmenopausal women taking and not taking hormone therapy.	Retroconversion of 22:6n-3 to 20:5n-3 was lower in postmenopausal women taking hormone therapy.
Stark et al. 2003.	Premenopausal women and postmenopausal women	Postmenopausal women were receiving or not receiving hormone therapy.	The postmenopausal women receiving hormone therapy had higher concentrations 16:0, 16:1, 20:3n-6 and 22:5n-3 than the women in the other two groups.

Tworek et al. 2000.	Premenopausal and postmenopausal women	No intervention.	Postmenopausal women had lower amounts of 16:0, 18:1n-9, 18:2n-6 and 22:6n-3 in erythrocytes compared with premenopausal women.
Sumino et al. 2003.	Postmenopausal women	Half of the women received hormone therapy and half did not, for one year	Concentration of 20:5n-3 and 22:6n-3 increased at the one year mark compared with baseline, in women who received hormone therapy.
Ottosson et al. 1984.	Postmenopausal women	Women received either ethinyl estradiol or estradiol valerate	Treatment with ethinyl estradiol reduced 16:0 and increased 18:0 levels
Menstrual cycle/oral contraceptives			
Pahwa, Seth and Seth. 1998.	Indian women with regular menstrual cycles	Venous blood was collected at day 10-12 of the menstrual cycle (follicular phase) and day 22-24 (luteal phase)	Serum triacylglycerols were higher during the follicular phase of the menstrual cycle
Barnett et al. 2004.	Caucasian women with regular menstrual cycles	Women consumed a standard laboratory diet. Venous blood was collected at day 3-9 of the menstrual cycle (follicular phase) and approximately 10 days after ovulation (luteal phase)	Total LDL-cholesterol was higher during the follicular phase compared with the luteal phase of the menstrual cycle
Kim and Kalkhoff. 1979.	Women with menstrual cycles 21-35 days in length	Venous blood was collected every 3-5 days for 3 menstrual cycles	Total plasma triacylglycerols were highest at ovulation and fell during the luteal phase, and cholesterol concentrations were the lowest during the luteal phase
Heiling and Jensen. 1992.	Women with regular menstrual cycles, 28-35 days in length	Venous blood was collected at day 4-10 of the menstrual cycle (follicular phase) and day 18-24 (luteal phase)	Plasma non-esterified fatty acid concentrations were not different between the different menstrual cycle phases.
Fehily et al. 1982.	British adult women	Women who use estrogen and progesterone combination oral contraceptives and age-matched controls who do not use oral contraceptives	16:0 was higher, and 18:0, 20:4n-6, 20:5n-3 and 22:5n-3 were lower in plasma phospholipids, in women who use oral contraceptives compared with those who do not
Giltay et al. 2004.	Women from the Netherlands who take or do not take oral contraceptives	Women consumed the same standard laboratory diet for 3 weeks. Blood was collected during the follicular phase of the menstrual cycle	Women taking oral contraceptives had a higher proportion of 16:0, 16:1 and 18:1n-9, and lower 18:0, compared with women not taking oral contraceptives
Mattsson et al. 1985.	Swedish adult women with regular menstrual cycles	Venous blood was collected at day 6-8, 13-15, 20-22 and 27-29 of the menstrual cycle	16:0,18:0 and 18:1n-9 increased in plasma cholesteryl esters during the luteal phase. 18:0 and 18:1n-9 were lower during the luteal phase compared with the follicular phase in plasma phospholipids.

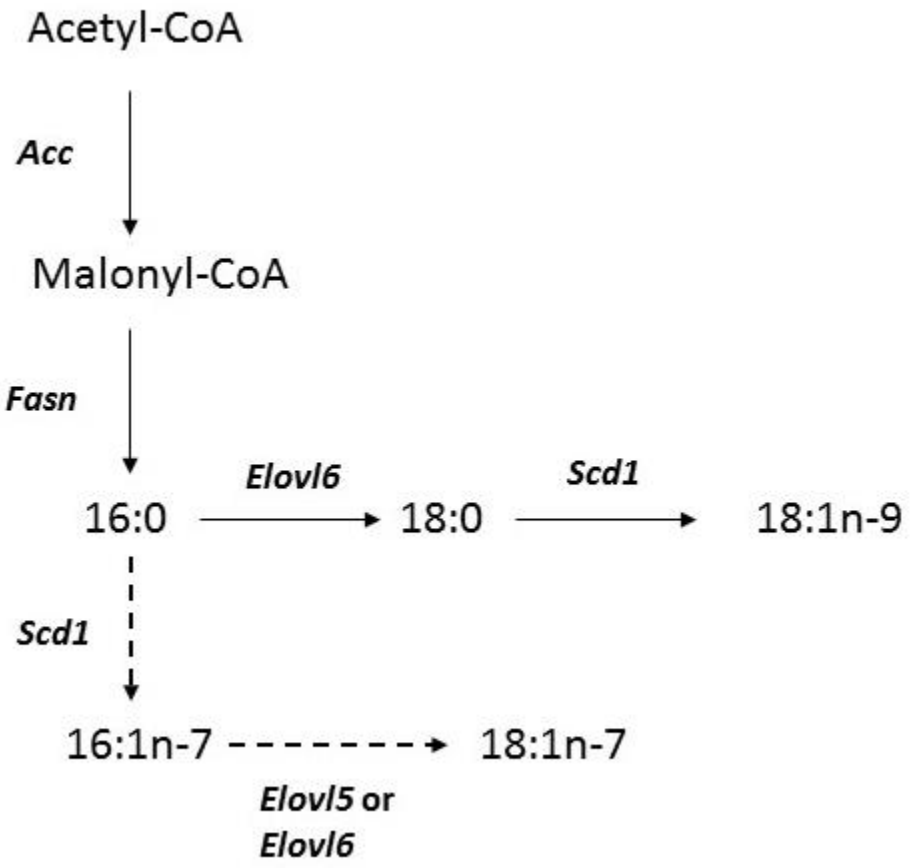


Figure 2.1. Diagram of the *de novo* biosynthesis pathway of saturated and monounsaturated fatty acids. Solid lines represent the usual pathway, while dashed lines represent an alternate pathway that is activated when levels of 16:0 are elevated. *Acc*: acetyl-CoA carboxylase; *Elovl5*: elongation of fatty acids family member 5; *Elovl6*: elongation of fatty acids family member 6; *Fasn*: fatty acid synthase; *Scd1*: stearoyl-CoA desaturase 1.

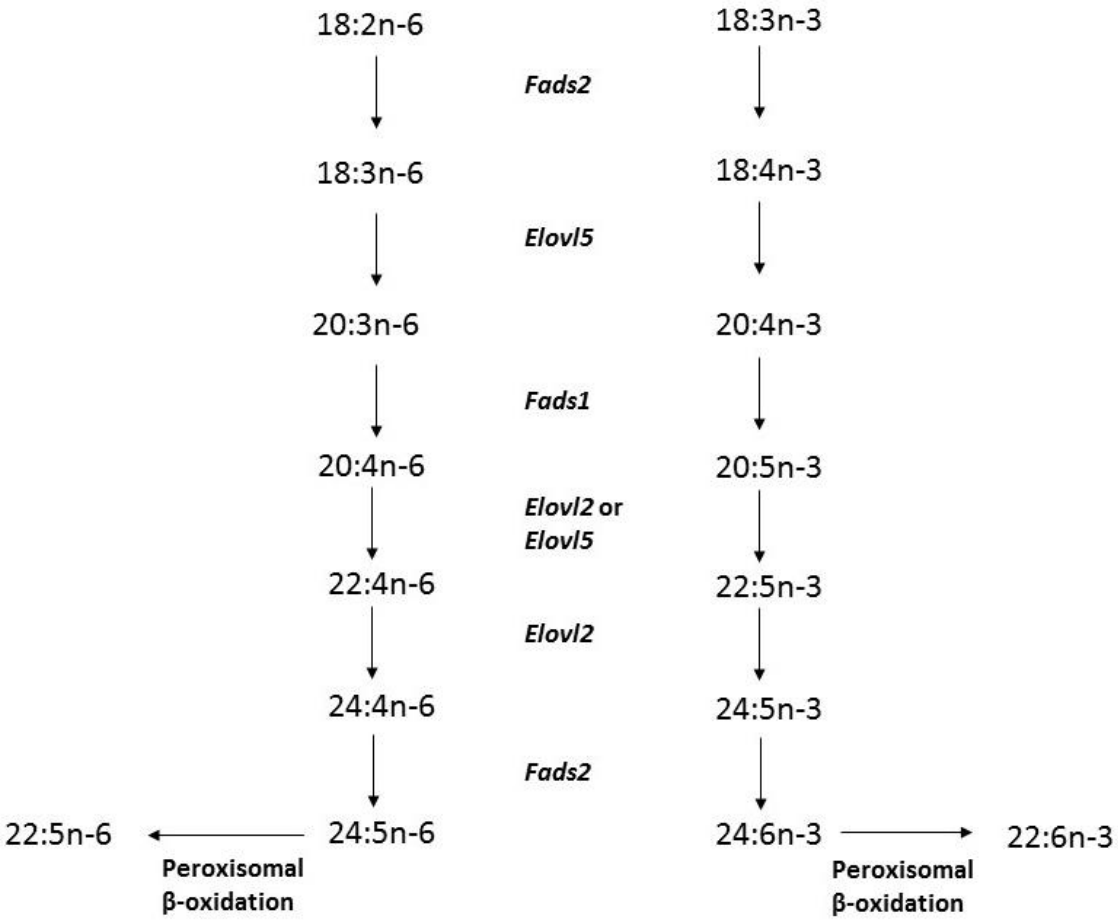


Figure 2.2. Diagram of *de novo* biosynthesis pathway of n-6 and n-3 polyunsaturated fatty acids. *Elovl2*: elongation of fatty acids family member 2; *Elovl5*: elongation of fatty acids family member 5; *Fads1*: fatty acid desaturase 1; *Fads2*: fatty acid desaturase 2.

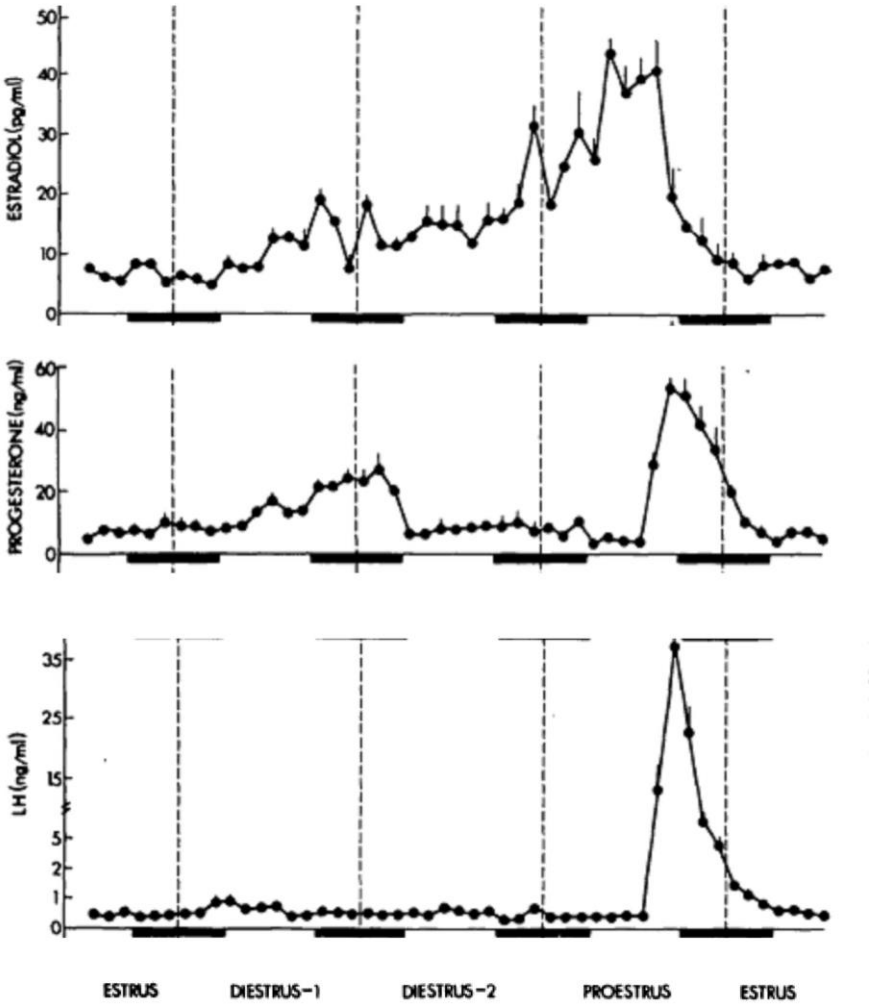


Figure 2.3 Typical hormone pattern of estrogen, progesterone and leuteinizing hormone over the rat estrous cycle. Taken from (Smith, Freeman, and Neill, 1975).

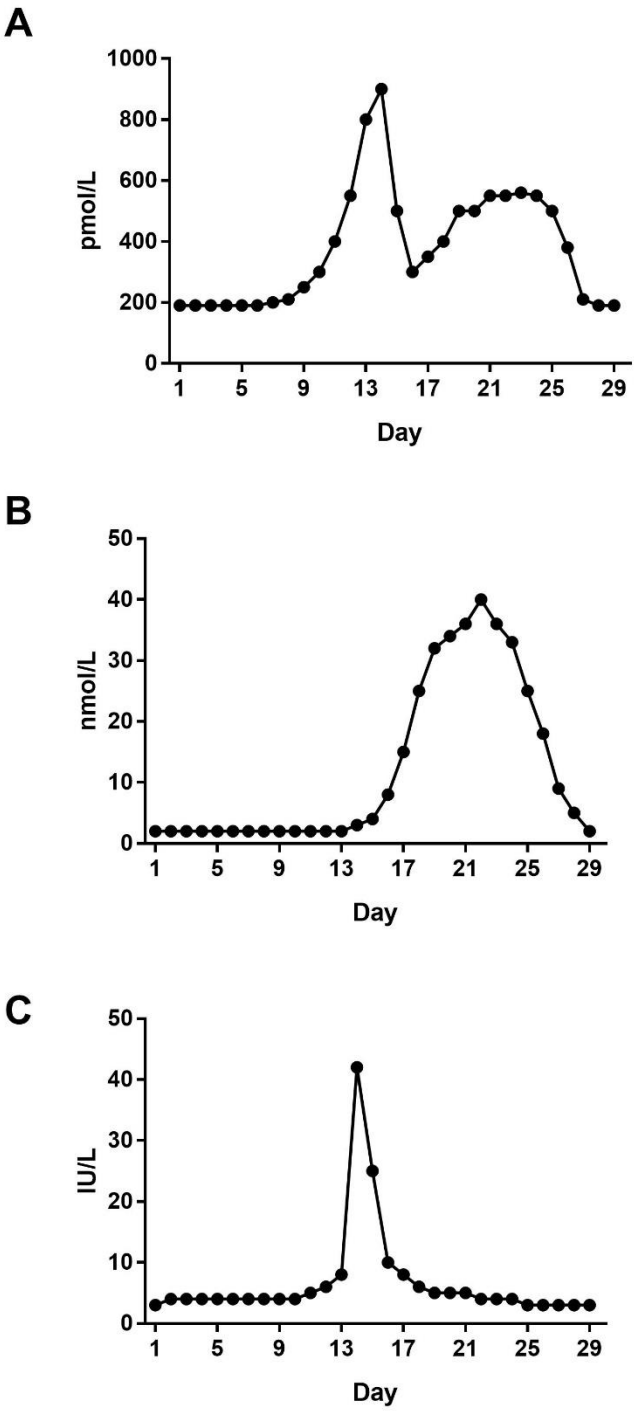


Figure 2.4 Typical hormone pattern of A. estradiol B. progesterone and C. leuteinizing hormone over a regular human menstrual cycle.

Chapter 3.

Rationale and Objectives

3.1 Rationale

Fatty acids are involved in a wide range of cellular functions, including oxidation for energy (McCloy et al., 2004), structural support (Farkas et al., 2000), neurotransmission (Bazan, Musto, and Knott, 2011; Zimmer et al., 2000), cell signaling (Bazan, Musto, and Knott, 2011; Huang et al., 2012) and more. Maintaining adequate n-3 PUFA metabolism is critical during pregnancy because the fetus requires substantial amounts of 22:6n-3 for proper brain development (Burdge et al., 2003; Ghebremeskel et al., 2000; Lauritzen et al., 2016; Roy et al., 2012), which continues during lactation (Koletzko, Cetin, and Brenna, 2007; Otto et al., 2001; Rodriguez-Cruz et al., 2011). N-3 PUFA are also important during later life stages and have been linked to cognition (Carlson, 2001; Luchtman and Song, 2013; McNamara and Carlson, 2006) and a reduction in the risk for cardiovascular disease (GISSI-Prevenzione Investigators, 1999). Blood levels of other fatty acids such as SFA and MUFA have been associated with an increased risk of cardiovascular disease and diabetes (Cho et al., 2014; Dimopoulos et al., 2006; Djousse et al., 2012b; Jakobsen et al., 2009), although this relationship is not entirely clear. Therefore, it is necessary to understand the factors influencing blood and tissue fatty acid composition. Previous work indicates that estrogen is one such factor, because female sex (Crowe et al., 2008; Marangoni et al., 2007), pregnancy (Bascunan et al., 2014; Burdge et al., 2006; Kuipers et al., 2011; Ramos et al., 2003; Vlaardingerbroek and Hornstra, 2004), menopause (Stark and Holub, 2004; Stark et al., 2000; Tworek et al., 2000) and estrogen treatment (Kitson et al., 2013b; Marks

et al., 2013b; Magnusardottir et al., 2009; Giltay et al., 2004) all result in changes in fatty acid composition. However, the mechanism underlying these changes is unknown.

Estrogen may influence fatty acid composition by regulating fatty acid elongase and desaturase mRNA expression, since many reports of estrogen-based changes in fatty acids are accompanied by changes in mRNA levels (Kitson et al., 2013b; Marks et al., 2013b; Childs et al., 2012; Sibbons et al., 2014). Many of these effects occur with large changes in estrogen levels, such as pregnancy or menopause, but it is not known if smaller changes in estrogen levels would have the same effect. Additionally, there are many limitations to hormonal and gene manipulation studies in animals, so it is difficult to make conclusions regarding the role of estrogen in regulating fatty acid elongase and desaturase expression and fatty acid composition because estrogen is not the only factor that is changing.

The effects of small physiological changes in estrogen levels in blood fatty acid composition over the human menstrual cycle and rat estrous cycle were examined. Additionally, hepatic enzyme expression was measured in rats during the estrous cycle. Since the direct effect of estrogen on regulating gene expression and fatty acid composition has not been measured, an estrogen responsive cell line was used to examine the effects of estrogen on gene expression. Lastly, a novel *Fads2* transcript variant was found to be expressed in MCF7 cells, and its activity towards synthesis of 20:4n-6 and 22:6n-3 was examined. These findings will expand our knowledge of the role of sex and estrogen on regulating fatty acid composition and enzyme expression in general, but will also aid in developing guidelines for dietary fat intake over the life cycle.

3.2 Objectives

The first objective of this thesis was to examine if the subtle changes in circulating estrogen concentrations that occur over the menstrual cycle in women would be associated with changes in blood fatty acid composition and if those changes are similar to what is observed during other models of hormone changes. The fatty acid changes suggested that expression and/or activity of Fads2 may be induced by estrogen. Therefore, the second objective was to expand the findings from the first objective by using rats. Blood and hepatic fatty acid levels were measured during the estrous cycle, the rat equivalent of the human menstrual cycle. By using rats, I was also able to examine hepatic enzyme expression and determine if small changes in estrogen mediate changes in fatty acid desaturase and elongase mRNA expression and protein levels. Hepatic fatty acid elongase and desaturase expression did change during the estrous cycle, but not always in accordance with estrogen changes. Thus, the third objective of my thesis was to examine a direct role for estrogen in the regulation of fatty acid biosynthesis by using highly controlled cell culture conditions and examining fatty acid desaturase and elongase expression in MCF7 cells, an estrogen-responsive cell line, when treated with estrogen. Although *FADS2* expression is induced by estrogen in MCF7 cells, levels of 20:4n-6 and 22:6n-3 are not increased. Therefore, the final purpose of my thesis was to examine perturbations in MCF7 fatty acid metabolism. The presence and induction of multiple transcript variants of *FADS2* in MCF7 cells in response to estrogen was determined. The functionality of each transcript variant in the ability to synthesize 20:4n-6 and 22:6n-3 was characterized, in addition to protein interactions between the two variants that may lead to decreased synthesis.

3.3 Hypotheses

1. Blood levels of 18:0, 16:1n-7 and 22:6n-3 will be higher during the luteal phase of the menstrual cycle compared with the follicular phase.

2. Blood levels of 18:0, 16:1n-7 and 22:6n-3 will be higher in women who take oral contraceptives, compared with women who do not take oral contraceptives and men.

3. Hepatic expression of *Elovl6*, *Elovl2* and *Fads2* as mRNA and protein will be highest during proestrus in rats, when circulating estrogen concentrations are highest.

4. Estradiol treatment of MCF-7 cells will increase mRNA expression of *FASN*, *ELOVL6*, *ELOVL2*, and *FADS2* to elevate concentrations of 16:0, 18:0, 16:1n-7, and 22:6n-3 in MCF-7 cells.

5. *FADS2_v2*, which lacks the cytochrome binding domain that is critical for activity, will not synthesize 20:4n-6 or 22:6n-3 when overexpressed in HEK293 cells, while overexpressing *FADS_v0/1* will increase the levels of these two fatty acids.

6. FADS2_v2 will bind to FADS2_v0/1when both are expressed in HEK293 cells to inhibit synthesis of 20:4n-6 and 22:6n-3 by FADS2_v0/1.

Chapter 4.

Common Methods

4.1 Fatty Acid Analyses

Liver, serum, plasma, adipose and cellular total lipids were extracted in 2:1 chloroform:methanol (v:v) according to the method of Folch (Folch, Lees, and Sloane Stanley, 1957) . Red blood cell lipids were extracted using 2:2:1.8 chloroform:methanol:water (v:v:v) with cold methanol and overnight freezing at -80ºC (Bligh and Dyer, 1959; Metherel et al., 2009; Reed et al., 1960). All fatty acid extractions were done in the presence of butylated hydroxytoluene as an antioxidant, and an internal standard: 22:3n-3 ethyl ester (Nu-Chek Prep Inc, Elysian, MN) for total lipids, triheptadecanoate (T-155 Nu-Chek Prep) for triacylglycerols (TAG) and 1,2-diheptadecanoyl-s*n*-glycerol-3-phosphocholine (850360P, Avanti Polar Lipids Inc, Alabaster, AL) for phospholipids, as necessary.

TAG and phospholipids were isolated by thin layer chromatography using 20 x 20 cm plates with a 60Å silica gel layer (Whatman International LTD, Maidstone, England) and a mobile phase of 60:40:2 heptane:diethyl ether: acetic acid (v:v:v) (Christie, 1989). Bands were identified by visualization under UV light with 2,7-dichlorofluorescein (Sigma-Aldrich, Oakville, ON) and comparison to a reference standard. Phospholipids and TAG were collected by scraping the band from the plate and extracting lipids from the silica in 2:1 chloroform:methanol (v:v).

Fatty acids in total lipid extracts, in TAG, and in phospholipids were transesterified to fatty acid methyl esters with 14% BF_3 in methanol at 85°C for 1 hour (Morrison and Smith, 1964), which were then separated by fast gas chromatography (Stark and Salem, Jr., 2005; Metherel, Aristizabal Henao, and Stark, 2013) using a Varian 3900 gas chromatograph (Varian

Inc, Mississauga, ON) with settings as described previously (Metherel, Aristizabal Henao, and Stark, 2013). The column used was a DB-FFAP capillary column with a 15m x 0.10mm inner diameter x 0.10mm film thickness. Hydrogen was used as the carrier gas which had a flow rate of 30 ml/minute. The injector temperature was 250ºC. The flame ionization detector was set at 300ºC. Peaks were identified by comparison to a reference mixture of fatty acids (GLC-569, Nu-Chek Prep Inc) and quantified relative to the internal standard. Fatty acid results are expressed quantitatively per milligram of liver, per 100 microliters of plasma or whole blood and per 200 milligrams of erythrocyte packed cells. Data are expressed qualitatively as relative weight % of total fatty acids.

4.2 Steroid Hormone Analyses

Two millilitres of anhydrous diethyl ether were added to 500µL plasma. Samples were vortexed thoroughly for 1 minute and centrifuged at 1734 × g for 5 min. The supernatant was collected, samples were dried under a stream of N_2 gas and reconstituted into 75uL of 100mM $NaHCO_3$ (pH = 9.0) plus 75uL of 3 mg/mL 1-methylimidazole-2-sulfonylchloride in acetone (Li and Franke, 2015). Samples were heated at 65ºC for 15 minutes. After cooling, samples were transferred to glass vials, spiked with 500 pmol 1,2-diheptadecanoyl-sn-glycero-3-phosphatidylcholine to account for sample-to-sample variability, dried fully under N_2 and reconstituted in 100uL acetonitrile:isopropanol:water +0.1% formic acid. Samples were stored in the dark at 4ºC until analysis by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). Progesterone, estrone, estriol and 17β-estradiol standards were purchased (Sigma-Aldrich, St. Louis, MO, USA) and dilution curves were prepared to quantitate steroids in plasma. UHPLC-MS/MS analyses were performed using a Dionex UltiMate 3000 UHPLC System (Dionex Corporation, Bannockburn, IL, USA) coupled to

a Thermo Q-Exactive Quadrupole-Orbitrap Mass Spectrometer (Thermo-Fisher Scientific, Waltham, MA, USA). The column used was a C 18 Ascentis Express, 15 cm x 2.1 mm x 2.0 um (Sigma-Aldrich, St. Louis, MO, USA). The mobile phase consisted of solutions A (60:40 water:methanol) and B (90:10 isopropanol:acetonitrile +10mM ammonium formate +0.1% formic acid). The gradient protocol used was as follows: from 0 – 2.0 minutes it was 1% solution B, from 2.0 – 6.0 min 20% solution B, 6.0 – 8.0 min 30% solution B, 8.0 – 14.0 min 100% solution B with a hold until 19.5 min. From 19.5 – 20 min solution B was decreased to 1%, and allowed to equilibrate until the 24 minute mark. The flow was set to 260 uL/min, column temperature at 45ºC and tray temperature at 4ºC. The mass spectrometer was operated in positive electrospray ionization mode, spray voltage +3.0kV, 35000 resolution and scan range *m/*z 200-2000.

4.3 RNA Extraction and Reverse-Transcriptase Real-Time PCR

RNA was extracted from animal livers by homogenization in Trizol® reagent (Invitrogen Co, Frederick, MD), or from cells by direct addition of Trizol® to the plate (Marks, Kitson, and Stark, 2013). Phases were separated by the addition of chloroform. Purity of the extracted RNA was determined by using the 260/280 ratio on a Nanodrop c2000 (Thermo Scientific, Wilmington, DE). Samples with a 260/280 ratio of 1.90 or greater were used for cDNA synthesis, using a high capacity cDNA reverse transcription kit (Applied Biosystems, Streetsville, ON) with an MJ mini personal thermal cycler (Biorad Laboratories, Mississauga, ON) with program cycle of 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, and 4°C until storage at -80°C.

The Primer-BLAST program on the NCBI website was used to design primers. Specific primers and sequences are listed in subsequent chapters. Primers were ordered from Sigma-

Aldrich. Reverse transcriptase real-time PCR was performed using SsoFast EvaGreen Supermix (BioRad) on a CFX Connect Real-Time System (BioRad). The program had an initial incubation of 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds and 65°C for 45 seconds. Analysis of data was performed by normalizing the threshold cycle number (Ct) of the gene of interest in a control sample to the Ct for the housekeeping gene, *18S ribosomal RNA*, to produce a ΔCt, which was then normalized to the ΔCt of test sample using the $2^{-\Delta\Delta Ct}$ method. Changes are expressed as relative values.

4.4 Immunodetection

Livers and cells were homogenized in a buffer containing 25 mM Tris, 130 mM NaCl, 2.7 mM KCl, 5 mM EDTA and 1% Triton X-100. Protein content was determined using a bicinchoinic acid procedure (Smith et al., 1985), and an equal amount of protein was separated on a 12% polyacrylamide gel or a 12% TGX Stain-Free fast-cast gel (Bio-Rad), and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk powder or 5% BSA in tris-buffered saline (TBS) with 0.5% (v:v) Tween-20 (TBST) at room temperature for 1 hour, followed by incubation either for 1 hour at room temperature or at 4ºC overnight with primary antibodies in TBST with 1% skim milk powder or 1% BSA. Membranes were washed in TBST for fifteen minutes followed by two five-minute TBST washed, incubated with appropriate secondary antibodies in the same conditions (5% BSA or 5% skim milk in TBST) as the primary antibody for 1 hour at room temperature, washed again with TBST, and detected by chemiluminescence using a BioRad ChemiDoc system. Protein content was normalized to total protein loaded on the gel, as determined either by gel activation and visualization prior to transfer using the ChemiDoc system, or using a Ponceau S stain (Dam et al., 2012).

4.5 Cell Culture Maintenance

MCF7 cells were maintained in 50% Dulbecco's Modified Eagle's Medium (DMEM) with 50% Ham's F12 (DMEM/F12), and HEK 293 cells were grown in DMEM. Medium for both cell lines was supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 mg/mL streptomycin were added. Cells were grown in an incubator at 37°C with 5% CO_2 (Roh et al., 2012). Reagents were from Thermo Scientific (Wilmington, DE) unless otherwise specified. Cells were grown to 80% confluence and then subdivided at an approximate 1:6 ratio following trypsin digest. Specific study protocols using MCF7 or HEK 293 cells are described in individual chapters.

4.6 Statistical Analyses

Statistical analyses were performed using either SPSS for Windows, or GraphPad Prism v4.0. Significance was inferred when $p < 0.05$. Specific statistical tests are described in individual chapters.

Chapter 5.

The Effects of Sex, Menstrual Cycle and Oral Contraceptives on Blood Fatty Acids

5.1 Introduction

Changes in estrogen concentrations are associated with changes in fatty acid composition in humans. This has mainly been observed in comparisons where there are dramatic differences in circulating estrogens and includes pregnancy (Al et al., 1995; Stark et al., 2005; Kuipers et al., 2011; Ghebremeskel et al., 2000), menopause and hormone therapy during menopause (Stark et al., 2005) and hormone therapy in transsexuals (Giltay et al., 2004). It is believed these effects are being mediated by estrogenic effects on the expression of elongase and desaturase enzymes. These effects typically result in shifts in blood fatty acid compositions with increased estrogen being associated with shifts towards increased proportions of 16:0, 16:1n-7 and 22 carbon highly unsaturated fatty acids (HUFA) such as 22:6n-3, and decreased proportions of 18:0, 20:5n-3 but also 22:5n-3. The lack of consistency of an estrogen effect, particularly on n-3 HUFA warrants further investigation. This may be due to differences in the size of the concentration shifts as estrogen increases more than six-fold during pregnancy compared with non-pregnant women (Soldin et al., 2005; Meyer et al., 2013), and decreases approximately five-fold during menopause compared with pre-menopausal women (Walsh et al., 1991; Bojar et al., 2015; Fuhrman et al., 2014).

During regular menstrual cycles, hormones change cyclically but do not reach the dramatically high or low levels as observed during pregnancy and menopause. Studies examining lipid changes over the menstrual cycle are much more limited than pregnancy studies, and to our knowledge, no studies have reported blood fatty acid composition during the menstrual cycle. There have been examinations of women using hormonal-based oral

contraceptives. Oral estrogen was associated with increased 16:0, 16:1n-7 and 22:6n-3 in plasma cholesteryl esters (Giltay et al., 2004) and lower concentrations of 20:4n-6, 20:5n-3 and 22:5n-3 in plasma and erythrocytes (Fehily et al., 1982). Unfortunately, hormone brand and information on monophasic (constant dose of hormones over the menstrual cycle) or triphasic (varying hormone concentrations depending on the week) use was not reported.

Therefore, we wanted to determine if the subtle, physiological changes in sex hormones the resulted in changes in fatty acid composition. Women not taking oral contraceptives and women taking the same brand of a monophasic oral contraceptive were examined at two time points coinciding with high and low blood levels of estrogen. A group of age controlled men were also included as an additional comparison point to confirm previous observations of sex differences in fatty acid composition

5.2 Materials and Methods

Male and female participants between the ages of 17 and 29 were recruited from the University of Waterloo community through posters, email advertisements, oral announcements during lectures, and word-of-mouth (advertisements are shown in Appendix 1). Women were included if they took Alesse brand oral contraceptives (which contains ethinyl estradiol and levonorgestrel at a constant dose over the active period, with a 21 days on, 7 days off protocol), or no form of hormonal based contraceptives within 6 months of the study participation, and had regular menstrual cycles between 28-35 days in length. Individuals with existing cardiovascular disease, diabetes mellitus, lipid disorders or any disorders that affect circulating estrogen were excluded, as were individuals on a vegetarian or vegan diet, or individuals who regularly consumed fatty fish, fish oil or any form of hormonal birth control other than Alesse. Respondents were screened, and filled out prior to their study participation; a food frequency

questionnaire validated for EPA + DHA intake (Patterson et al., 2012) (FFQ found in Appendix 1), a health screening form and a menstrual health questionnaire. All participants signed an informed written consent form (all forms are found in Appendix 1) and all procedures were approved by an Ethics Committee at the University of Waterloo.

Women who were eligible participants attended two blood collections over the menstrual cycle: the first was at day 6-8 of the menstrual cycle, representing the follicular phase of the menstrual cycle, and the second blood collection was completed during day 21-23 of the menstrual cycle, representing the luteal phase. Men attended one blood collection at the beginning of the study. All blood collections were done by a certified phlebotomist in the Laboratory of Nutritional and Nutraceutical Research at the University of Waterloo, after an overnight fast of minimum eight hours. Whole blood was collected by venipuncture into Vacutainers, and EDTA was added as an anticoagulant. Whole blood was centrifuged at 3000 rpm for 15 minutes at 4ºC to separate plasma, buffy coat and erythrocytes. Erythrocytes were washed twice with 0.9% saline prior to storage (Patterson et al., 2015). Aliquots of whole blood, plasma and erythrocytes were stored in a -80ºC freezer prior to fatty acid analyses. Plasma and erythrocyte lipids were extracted and fatty acids analyzed as described in Chapter 4. Plasma hormone concentrations were determined for a randomly selected subset of each group of women (n=6 per group) by UHPLC-MS/MS, as described in Chapter 4.

Statistical analyses were done using SPSS (IBM, Armonok, NY, version 23). Significance was inferred when $p < 0.05$. Data are presented as mean ± standard deviation. In order to examine sex differences in fatty acid composition, each group of women had the fatty acid values averaged over the two time points, to represent an average representative value that incorporates different menstrual cycle stages, resulting in three groups: men, women taking oral

contraceptives, and women not taking oral contraceptives. A one-way ANOVA was used to examine differences between these three groups. Following a significant F-value, Tukey's post hoc test was used to determine differences between groups. The secondary objective was to determine how fatty acids change over the menstrual cycle, in women who took oral contraceptives and women who did not take oral contraceptives, by examining fatty acid composition in these two groups at two different time points of the menstrual cycle. The linear mixed models procedure for repeated measures was used to examine the effect of hormone status (Alesse or no oral contraceptives), time (day 6-8 or 21-23 of the menstrual cycle) and their interaction as fixed factors on blood fatty acid measurements.

5.3 Results

5.3.1 Participant characteristics

Recruitment began in February 2015 and concluded September 2015. A total of 55 individuals were screened after expressing interest in participation (17 men, 38 women). All 17 men were enrolled and completed the study and included in the analyses. Of the 38 women who expressed interest, 32 were enrolled into the study (n=12 for the ethinyl estradiol and levonorgestrel group, herein referred to as oral contraceptive users, and n=20 for the women who were not receiving any oral contraceptives). Two women were not enrolled because they consumed fish oil, and four women were not enrolled because they had irregular menstrual cycles. Of the 12 participants taking oral contraceptives, one participant was excluded from analyses due to hyperlipidemic plasma lipid levels determined during fatty acid analysis, resulting in a final group total of 11. Of the 20 women not taking any oral contraceptives, 2 were excluded for not being able to provide a venous blood sample, and 3 were excluded for not

following the study protocol in regards to maintaining low fish intake throughout the study time period, resulting in a final group total of 15.

The men were 23.7 ± 2.2 years old, the women taking oral contraceptives were 22.5 ± 2.7 years old, and the women not taking any oral contraceptives were 21.9 ± 3.0 years old. The menstrual cycle length was 27.8 ± 0.6 days in length in women taking oral contraceptives and 28.6 ± 1.5 days in length those not taking oral contraceptives. As expected, the women who did not take oral contraceptives had lower estradiol, estrone and progesterone concentrations at day 6-8 (follicular phase), which increased at day 21-23 (luteal phase). This cyclical change in endogenous hormones was not observed in women taking oral contraceptives (Table 5.1). The oral contraceptive provides 100 µg levonorgestrel and 20 µg of ethinyl estradiol per pill. Levonorgestrel was detected in women who took oral contraceptives, but was not found in the women who had not taken oral contraceptives, confirming they did not use oral contraceptives during the experimental time period. Ethinyl estradiol, the other component of the oral contraceptive, was not detectable in the blood samples available using the current analytical procedures.

5.3.2 Sex Differences in Fatty Acid Composition

There were numerous fatty acid differences between men and women taking and not taking oral contraceptives in plasma (proportions in Table 5.2, concentrations in in Appendix 1 - Table A1.1) and erythrocytes (select fatty acids in Figure 5.1, full fatty acid profiles in Appendix 1 - Table A1.2 and Table A1.3). However, there were are distinct identifiable patterns. In general, women taking oral contraceptives had a higher proportion of 16:0 and 16:1n-7, and a lower proportion of 18:0 and 22:5n-3, in both plasma and erythrocytes, compared with men and women not taking oral contraceptives. There were more differences in the plasma as compared

with erythrocytes. Women taking oral contraceptives had a higher proportion of 20:3n-6 in plasma compared with the other groups, and a higher amount of 22:6n-3 compared with men only. Accordingly, the DHA:DPAn-3 ratio was higher in oral contraceptive users compared with men and women not taking oral contraceptives. The DHA:EPA ratio was not different between the groups.

5.3.3 Menstrual Cycle Fatty Acid Composition

A significant oral contraceptive use status x time interaction was observed for the proportions of 20:1n-9, 20:2n-6, 20:3n-6 and the DHA:EPA ratio in plasma fatty acids (Table 5.3, concentration data in Appendix 1, Table A1.4). These fatty acids and the DHA:EPA ratio were higher in the women taking oral contraceptives during high exogenous hormone levels (day 21-23), and did not differ statistically between the other three groups. An effect of hormone status (oral contraceptive use) was observed in the proportion of several fatty acids in plasma (Table 5.3). Women taking oral contraceptives had a higher proportion of 16:0, 16:1n-7 and 20:3n-3, and a lower proportion of 18:0 and 22:5n-3 in plasma than women who did not take oral contraceptives. A higher DHA:DPAn-3 ratio was observed in plasma of women taking oral contraceptives, which appears to be driven by the lower levels of 22:5n-3. Finally, effects of time on plasma fatty acid composition were also observed. In general, at day 6-8 (the follicular phase), women had lower proportions of several SFA, 20:1n-9, 22:1n-9, 24:1n-9, 20:2n-6, 22:5n-6 and 22:6n-3, compared with day 21-23 (the luteal phase). Accordingly, the DHA:DPAn-3 ratio was increased during the luteal phase. During the follicular phase, the proportions of 16:0, 16:1n-7 and 18:3n-6 were higher than during the luteal phase. Fewer changes were observed in erythrocyte fatty acids over the menstrual cycle, and they were mainly observed in the weight

percentage of fatty acids and not concentrations and they tended to reflect fatty acid differences observed in plasma (Appendix 1, Table A1.5 and A1.6).

5.4 Discussion

Previous studies have reported sex differences in fatty acid composition (Crowe et al., 2008; Metherel et al., 2009; Patterson et al., 2015; Marangoni et al., 2007). Sex differences in fatty acid composition have been hypothesized to be partially due to the effects of estrogen (Kitson, Stroud, and Stark, 2010; Giltay et al., 2004; Burdge and Calder, 2005). However, these studies have not controlled for or separated women based on oral contraceptive use or menstrual cycle stage. In the present study we observed that in women taking oral contraceptives containing ethinyl estradiol and levonorgestrel in a constant dose, proportions of 16:0, 16:1n-7, 20:1n-9, 22:1n-9, 24:1n-9, 20:3n-6 and 22:6n-3 were higher and proportions of 18:0 and 22:5n-3 in plasma total lipids were lower, agreeing with previous reports. Interestingly, many of these differences were between women who take oral contraceptives and men. Women with natural cycles had either intermediary values or were similar to men, except for 22:5n-3, which was consistently lower in both groups of women.

There are several reasons why oral contraceptives may have a potent effect on fatty acid metabolism. Ethinyl estradiol has been shown to bind to the estrogen receptors more strongly than estradiol (Kuiper et al., 1997), and have a longer half-life than naturally occurring estrogens (Kapp, 2009). Oral contraceptive use is also associated with a higher hepatic load due to first pass metabolism. The lack of differences between men and women with natural cycleswas somewhat surprising based on previous observations (Metherel et al., 2009). While it is possible that sex differences were largely attributed to women participants taking oral contraceptives

within the women grouping, it is possible that the female participants in the present study had slightly higher levels of fish and omega-3 intake.

Sex differences in blood and tissue content of long chain n-3 PUFA appear to be dependent on low intakes of 20:5n-3 and 22:6n-3 (Pawlosky et al., 2003b), with dietary intake being the strongest factor in determining blood and tissue omega-3 composition (Block, Harris, and Pottala, 2008). Previous studies have reported a higher DHA:EPA and DHA:DPAn-3 ratio in women compared with men that decrease with fish oil supplementation (Metherel et al., 2009; Patterson et al., 2015). Specifically, DHA:EPA in the plasma was reported as 4.82 ± 1.47 for women at baseline (Metherel et al., 2009), while presently plasma DHA:EPA was only 3.30 ± 0.85 in women taking and 2.67 ± 0.85 in women not taking oral contraceptives. The sum of the percentage of EPA+DHA in erythrocytes, an established biomarker of omega-3 PUFA intake (Harris and Von, 2004) was approximately 4.5% for all women in the present study, whereas in the previous studies it was approximately 3.9% for the women (Metherel et al., 2009; Patterson et al., 2015). While all of these studies were done in our laboratory, the previous studies specifically recruited participants with low baseline omega-3 levels and did baseline blood testing prior to admitting participants into the study. Although our exclusion criteria presently included participants with high fish or omega-3 supplementation intake, participants did not have any blood screening done prior to study admittance. Future studies should examine fatty acid composition in men and women taking and not taking oral contraceptives in populations with very low intake. An examination of the effect of omega-3 supplementation may also show different responses when comparing women taking and not taking oral contraceptives, and should be done in future studies.

This differences between the women taking and not taking oral contraceptives is in agreement with a previous report of higher proportions of 16:0, 16:1n-7 and 22:6n-3 in women who take oral contraceptives compared with women who do not (Giltay et al., 2004), and a lower proportion of 22:5n-3 in contraceptive users compared with women who do not use contraceptives (Fehily et al., 1982). Interestingly, the fatty acid changes in women who take oral contraceptives observed presently are similar to observations in postmenopausal women taking hormone therapy (Stark, Park, and Holub, 2003; Ranganath et al., 1996). Both hormone therapy in menopause and monophasic birth control use examined in these studies provided oral ethinyl estradiol at constant doses. Examinations using different dosing paradigms (triphasic birth control), different types of estrogens and different modes of delivery (oral vs. transdermal) may give further insights into the mechanism of estrogens effect on fatty acids.

The higher proportions of 16:0 and 16:1n-7, and lower 18:0 and 22:5n-3 in women taking oral contraceptives was also observed in erythrocyte fatty acids. Erythrocyte fatty acid compositions tend to reflect long-term fatty acid status (Katan et al., 1997; Patterson et al., 2014), thereby suggesting that these changes in fatty acids are chronic and not transient in women taking oral contraceptives. Stronger effects were observed in plasma fatty acids compared with erythrocyte fatty acids. This is likely due to the impact of estrogen on plasma lipoproteins, since estrogen therapy decreases plasma LDL cholesterol levels and increases TAG and TAG-rich VLDL (Walsh et al., 1991; Granfone et al., 1992; Schaefer et al., 1983). Many of the fatty acids that were higher in women, including many SFA and MUFA, are generally found in TAG, which may be one reason that plasma fatty acid composition has more differences between groups than erythrocytes. However, the effects of estrogen on lowering 22:5n-3 levels and increasing 22:6n-3 levels would not be driven by changes in plasma TAG, because these n-3

PUFA are more often found in phospholipids (Marszalek et al., 2005; Ogura et al., 2010). This indicates that estrogen may be influencing plasma n-3 PUFA composition through a different mechanism than for SFA and MUFA. One such mechanism may be through inducing hepatic elongase and desaturase expression, thereby increasing the synthesis of 22:6n-3.

Plasma lipoproteins also change over the menstrual cycle (Pahwa, Seth, and Seth, 1998; Schisterman, Mumford, and Sjaarda, 2014; Barnett et al., 2004; Kim and Kalkhoff, 1979). Total plasma TAG and LDL-cholesterol concentrations are higher during the follicular phase compared with the luteal phase. Our findings indicate that fatty acid composition also changes during the menstrual cycle. In both groups of women, plasma proportions of 16:0, 18:0, 16:1n-7, 18:3n-6 and 22:5n-3 all decrease while 18:1n-7 and 22:6n-3 increase during the luteal phase. The changes in 22:6n-3 and 22:5n-3 were great enough to result in a higher DHA:DPAn-3 ratio during the luteal phase compared with the follicular phase. Overall, these changes corresponded to higher plasma estradiol and progesterone in the luteal phase than in the follicular phase in the present study in the women not taking oral contraceptives. Since SFA and MUFA are often found in TAG, it is possible that the decreased overall plasma TAG levels during the luteal phase may partly explain why these fatty acids decreased. However, changes in plasma lipoproteins would not explain the changes in 22:5n-3 and 22:6n-3, since these fatty acids are not found in high amounts in TAG. Studies using compartmentalized models showing that the main sex difference in 22:6n-3 biosynthesis appears to be the conversion from 22:5n-3 to 22:6n-3 (Burdge and Wootton, 2002; Burdge and Calder, 2005). It appears that small increases in estrogen from the follicular phase to the luteal phase may be associated with a shift towards fatty acids that are produced as a result of increased elongation and desaturation expression and/or activity, specifically increasing at the steps between 22:5n-3 and 22:6n-3. Although progesterone levels

are higher during the luteal phase as well, progesterone has minimal effects on fatty acid composition and elongase and desaturase enzyme expression in rats while estrogen or estrogen plus progesterone have large effects (Kitson et al., 2013b; Marks et al., 2013b; Alessandri et al., 2011; Alessandri et al., 2012), suggesting that estrogen rather than progesterone is likely a greater determinant of fatty acid composition in the present study.

Many of the changes in fatty acid content observed between collection times were observed in both groups of women. Although oral contraceptive users do not have fluctuations in hormone levels during the active time of the contraceptive use because each pill provides the same dose of ethinyl estradiol and levonorgestrel, the day 6-8 blood collection would mainly be at the end of the no hormone time period, while the day 21-23 blood collection was well into the active oral contraceptive use (Sulak et al., 2000). This was confirmed by the five-fold increase in levonorgestrel at day 21-23 compared with day 6-8. Similar changes in fatty acid composition in women taking and not taking oral contraceptives as hormone levels increase further supports the hypothesis that estrogen is one contributing factor in fatty acid composition. However, there are possibly other factors contributing to blood fatty acid composition during the menstrual cycle, since there was a significant hormone x time interaction in plasma levels of several fatty acids.

5.5 Conclusions

This is the first study to report a comprehensive examination of sex differences in fatty acid composition, by separating women based on contraceptive use, and using fatty acid data from two different time points, in addition to examining fatty acid changes over the menstrual cycle. Some changes in SFA and MUFA levels over the menstrual cycle in both groups of women may be due to the effects of estrogen on plasma lipoprotein levels. Plasma TAG levels decrease during the luteal phase of the menstrual cycle, and so do 16:0, 18:0, 16:1n-7 and 18:3n-

6. The decreased levels of 22:5n-3 and increased levels of 22:6n-3, and corresponding increase in DHA:DPAn-3 ratio, are not likely a result of increased plasma TAG because these fatty acids are generally found in low concentrations in TAG. It appears that estrogen is increasing desaturase and/or elongase activity to result in greater conversion from 22:5n-3 to 22:6n-3. This conversion has been shown to be higher in women compared with men previously (Burdge and Wootton, 2002; Burdge and Calder, 2005). The fatty acid data from the present study indicates that this conversion may be further induced during the luteal phase, when estrogen levels are higher. This further supports a role for estrogen in mediating elongase and desaturase expression. Future research should also examine the mechanism of estrogen therapy on fatty acid composition, because women who take oral contraceptives in this study have similar fatty acid profiles to those previously reported in postmenopausal women who take estrogen therapy (Stark, Park, and Holub, 2003; Tworek et al., 2000). Overall, this work shows that there are fatty acid composition differences between women with pharmacological and physiological sources of estrogen, and differences over the menstrual cycle. These factors should be considered in future work examining fatty acid composition and blood lipid levels in women.

Table 5.1: Plasma hormone concentrations in women who take ethinyl estradiol and levonorgestrel and women who do not take oral contraceptives during the follicular phase and luteal phase of the menstrual cycle

	Plasma Hormones pg hormone/ml plasma			
	Women taking oral contraceptives		**Women with natural cycles**	
	Day 6-8	**Day 21-23**	**Day 6-8**	**Day 21-23**
Estrone	23 ± 23^{ab}	7 ± 6^{a}	8 ± 3^{a}	33 ± 15^{b}
Estradiol	51 ± 62^{ab}	3 ± 3^{a}	17 ± 19^{ab}	62 ± 24^{b}
Progesterone	120 ± 133^{a}	123 ± 82^{a}	83 ± 27^{a}	8466 ± 3793^{b}
Levonorgestrel	1865 ± 1173	9399 ± 15502*	n.d.	n.d.

Values are mean ± sd. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for birth control status x time by the linear mixed models procedure. * $p<0.05$ by student's t-test. n.d. not detected.

Table 5.2: Relative proportion of plasma fatty acids in men, women who take oral contraceptives and women who do not take any oral contraceptives.

	Plasma Fatty Acids % weight total fatty acids		
Name	**Men**	**Women taking oral contraceptives**	**Women with natural cycles**
C 12:0	0.12 ± 0.05	0.11 ± 0.03	0.14 ± 0.06
C 14:0	0.94 ± 0.22	1.02 ± 0.28	0.88 ± 0.19
C 16:0	22.92 ± 1.22[a]	25.29 ± 2.74[b]	23.09 ± 2.14[a]
C 18:0	7.10 ± 0.70[a]	6.43 ± 0.73[b]	7.17 ± 0.61[a]
C 20:0	0.18 ± 0.03	0.19 ± 0.03	0.20 ± 0.03
C 22:0	0.40 ± 0.09	0.42 ± 0.08	0.44 ± 0.05
C 24:0	0.37 ± 0.08	0.37 ± 0.07	0.36 ± 0.05
SFAs	32.28 ± 1.62	34.03 ± 2.51	32.44 ± 1.64
C 14:1	0.04 ± 0.02[a]	0.06 ± 0.03[b]	0.06 ± 0.03[b]
C 16:1n-7	1.37 ± 0.43[a]	2.05 ± 0.79[b]	1.42 ± 0.31[a]
C 18:1n-7	1.71 ± 0.21	1.81 ± 0.26	1.66 ± 0.13
C 18:1n-9	18.82 ± 2.29	18.19 ± 1.40	19.32 ± 1.87
C 20:1n-9	0.14 ± 0.03[a]	0.17 ± 0.04[b]	0.14 ± 0.03[ab]
C 22:1n-9	0.07 ± 0.03[a]	0.12 ± 0.03[b]	0.12 ± 0.03[b]
C 24:1n-9	0.55 ± 0.14[a]	0.67 ± 0.13[b]	0.61 ± 0.08[ab]
MUFAs	22.71 ± 2.55	23.07 ± 2.06	23.34 ± 1.95
C 18:2n-6	29.42 ± 2.62	28.03 ± 2.89	29.69 ± 1.97
C 18:3n-6	0.34 ± 0.11	0.29 ± 0.06	0.36 ± 0.13
C 20:2n-6	0.16 ± 0.04[ab]	0.21 ± 0.07[a]	0.15 ± 0.05[b]
C 20:3n-6	1.23 ± 0.30[a]	1.59 ± 0.29[b]	1.29 ± 0.44[ab]
C 20:4n-6	6.99 ± 1.01	6.70 ± 1.32	6.58 ± 1.14
C 22:2n-6	0.02 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
C 22:4n-6	0.17 ± 0.03	0.18 ± 0.05	0.16 ± 0.05
C 22:5n-6	0.12 ± 0.04	0.10 ± 0.08	0.15 ± 0.05
N-6	38.42 ± 2.55	37.13 ± 3.20	38.34 ± 2.05
C 18:3n-3	0.62 ± 0.18	0.67 ± 0.18	0.65 ± 0.14
C 20:3n-3	0.04 ± 0.01[ab]	0.04 ± 0.01[a]	0.03 ± 0.01[b]
C 20:5n-3	0.49 ± 0.14	0.50 ± 0.10	0.51 ± 0.14
C 22:5n-3	0.43 ± 0.07[a]	0.32 ± 0.05[b]	0.40 ± 0.09[a]
C 22:6n-3	1.19 ± 0.24[a]	1.57 ± 0.37[b]	1.33 ± 0.24[ab]
N-3	2.77 ± 0.31[a]	3.10 ± 0.47[b]	2.92 ± 0.28[ab]
EPA+DHA	1.68 ± 0.29[a]	2.07 ± 0.42[b]	1.85 ± 0.26[ab]
DHA:EPA	2.61 ± 0.91	3.30 ± 0.85	2.67 ± 0.85
DHA:DPAn-3	2.89 ± 0.84[a]	5.13 ± 1.43[b]	3.43 ± 1.11[a]
Total	316.58 ± 52.96	352.03 ± 76.52	325.13 ± 46.81

Values are means ± SD. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for one way ANOVA. DHA: 22:6n-3; EPA: 20:5n-3; DPAn-3: 22:5n-3.

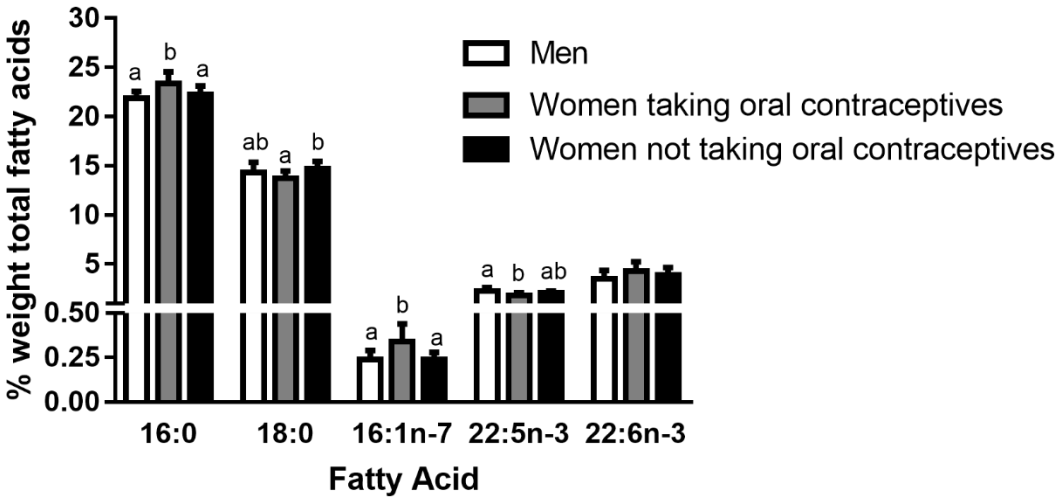


Figure 5.1: Relative proportion of selected fatty acids in erythrocyte in men, women taking oral contraceptives and women not taking oral contraceptives. Values are means ± SD. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for one way ANOVA.

Table 5.3: Plasma fatty acid proportion in women taking and not taking oral contraceptives during the follicular and luteal phase of the menstrual cycle

Plasma Fatty Acids % weight total fatty acids				
	Women taking oral contraceptives		Women with natural cycles	
Name	Day 6-8 (no hormone phase)	Day 21-23 (active hormone phase)	Day 6-8 (follicular phase)	Day 21-23 (luteal phase)
C 16:0[1,2]	26.01 ± 2.76	24.57 ± 2.99	23.52 ± 2.25	22.65 ± 2.45
C 18:0[2]	6.55 ± 0.68	6.32 ± 0.91	7.18 ± 0.59	7.15 ± 0.67
C 20:0[1]	0.18 ± 0.04	0.19 ± 0.03	0.19 ± 0.03	0.20 ± 0.03
C 22:0[1]	0.41 ± 0.10	0.43 ± 0.08	0.42 ± 0.05	0.46 ± 0.08
C 24:0[1]	0.35 ± 0.07	0.39 ± 0.08	0.35 ± 0.06	0.38 ± 0.08
SFAs[1]	34.93 ± 2.90	33.13 ± 2.59	32.93 ± 1.84	31.94 ± 1.89
C 16:1n-7[1,2]	2.21 ± 0.74	1.88 ± 0.90	1.56 ± 0.37	1.28 ± 0.33
C 18:1n-7[1]	1.74 ± 0.29	1.89 ± 0.28	1.65 ± 0.12	1.67 ± 0.18
C 18:1n-9	18.13 ± 1.48	18.25 ± 1.67	19.29 ± 2.08	19.35 ± 1.79
C 20:1n-9	0.14 ± 0.03[a]	0.20 ± 0.04[b]	0.13 ± 0.03[a]	0.15 ± 0.03[a]
C 22:1n-9[1]	0.10 ± 0.03	0.14 ± 0.04	0.11 ± 0.03	0.12 ± 0.03
C 24:1n-9[1]	0.63 ± 0.13	0.70 ± 0.16	0.60 ± 0.10	0.63 ± 0.08
MUFAs	23.02 ± 2.11	23.12 ± 2.35	23.41 ± 2.18	23.27 ± 1.89
C 18:2n-6	27.66 ± 3.09	28.40 ± 3.10	29.40 ± 2.13	29.97 ± 2.02
C 18:3n-6[1]	0.34 ± 0.08	0.25 ± 0.07	0.39 ± 0.17	0.32 ± 0.12
C 20:2n-6	0.17 ± 0.05[ab]	0.24 ± 0.10[a]	0.15 ± 0.05[b]	0.16 ± 0.05[b]
C 20:3n-6	1.50 ± 0.25[ab]	1.68 ± 0.36[a]	1.31 ± 0.43[b]	1.26 ± 0.49[b]
C 20:4n-6	6.64 ± 1.45	6.75 ± 1.34	6.42 ± 1.12	6.74 ± 1.21
C 22:2n-6	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01
C 22:4n-6	0.17 ± 0.04	0.20 ± 0.06	0.16 ± 0.05	0.16 ± 0.07
C 22:5n-6[1]	0.12 ± 0.03	0.16 ± 0.07	0.15 ± 0.04	0.16 ± 0.05
N-6[1]	36.60 ± 3.57	37.67 ± 3.25	37.95 ± 2.15	38.72 ± 2.20
C 18:3n-3	0.64 ± 0.21	0.71 ± 0.19	0.63 ± 0.15	0.67 ± 0.17
C 20:3n-3[2]	0.04 ± 0.02	0.05 ± 0.01	0.03 ± 0.02	0.03 ± 0.01
C 20:5n-3	0.55 ± 0.16	0.46 ± 0.11	0.49 ± 0.14	0.54 ± 0.19
C 22:5n-3[2]	0.32 ± 0.05	0.31 ± 0.06	0.41 ± 0.11	0.39 ± 0.09
C 22:6n-3[1]	1.47 ± 0.36	1.66 ± 0.44	1.30 ± 0.25	1.37 ± 0.29
N-3	3.02 ± 0.45	3.17 ± 0.55	2.86 ± 0.26	2.99 ± 0.47
EPA+DHA	2.02 ± 0.45	2.11 ± 0.50	1.79 ± 0.22	1.91 ± 0.37
DHA:EPA	2.83 ± 0.85[ab]	3.78 ± 1.13[a]	3.00 ± 1.20[ab]	2.81 ± 0.74[b]
DHA:DPAn-3	4.71 ± 1.40	5.55 ± 1.56	3.47 ± 1.26	3.74 ± 1.05
Total[1] (µg fatty acid/100 µl plasma)	362.81 ± 83.68	341.24 ± 75.64	334.87 ± 48.69	315.39 ± 57.37

Values are mean ± sd. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for birth control status x time by the linear mixed models procedure. DHA: 22:6n-3; EPA: 20:5n-3; DPAn-3: 22:5n-3.

[1] significant main effect of time [2] significant main effect of birth control status

Chapter 6.

Effect of Rat Estrous Cycle on Hepatic Expression of Fatty Acid Biosynthesis Enzymes

6.1 Introduction

Sex differences in blood and tissue fatty acid composition in humans are observed even after controlling for diet (Metherel et al., 2009; Patterson et al., 2015; Crowe et al., 2008; Marangoni et al., 2007). Circulating sex hormones has been proposed as a potential mechanism (Kitson, Stroud, and Stark, 2010) and studies in humans during pregnancy, hormone therapy with menopause and in transsexuals support an estrogenic effect on fatty acid metabolism. In the previous chapter, we confirmed that fatty acid differences are also observable in humans during the menstrual cycle, with changes in levels and ratios of the n-3 HUFA being particularly intriguing.

Rat fatty acid metabolism has been demonstrated to be representative of human fatty acid metabolism, making rats a useful model for studying hepatic gene expression changes which are difficult to obtain in humans. Women often have higher levels of 16:0 compared with males, likely due to higher carbohydrate intake (Garriguet, 2007), while female rats tend to have higher levels of 18:0 compared with male rats, because rats are fed the same diet and females have higher levels of elongase 6 (Marks, Kitson, and Stark, 2013). The rat lipoprotein profile is quite different than humans (Yin et al., 2012), and there has been some hypothesis that D6D in humans can possess delta-4 desaturation ability (Park et al., 2015), which is not present in rats (Pauter et al., 2014), but in general hepatic fatty acid metabolism in rats is similar to that of humans. Rat models have been observed to have sex differences in tissue content of fatty acids, but also in hepatic enzyme expression. Females express higher levels of *Elovl6* (Marks, Kitson,

and Stark, 2013), *Elovl2*, *Fads1* and *Fads2* (Kitson et al., 2012; Extier et al., 2010; Burdge et al., 2008), compared with males. Estrogen may be an important mediator of these differences. The expression of *Elovl6* and *Fads2* is decreased in ovariectomized rats compared with sham operated rats, while estradiol treatment of ovariectomized rats increases the expression of these gene products (Kitson et al., 2013a; Marks et al., 2013a; Alessandri et al., 2011). However, in these rodent studies, changes in estrogen concentrations tend to be dramatic withdrawals or treatment that results in supraphysiological levels (Marks et al., 2013a; Strom et al., 2012; Cao and Gregoire, 2016). To the best of our knowledge, the effects of the rat estrous cycle on fatty acid composition has not been examined. During the estrous cycle of the rat, estrogen concentrations fluctuate from 30 pg/ml during the estrus stage, to 40-50 pg/ml during the metestrus and diestrus stages, to highs of 150 pg/ml during proestrus (Flores et al., 2011). Unless rats are pregnant or pseudopregnant, they do not have a real luteal phase (Wilson, Adler, and Le, 1965), thereby limiting how representative the rat estrous cycle may be of the human menstrual cycle. However, using a rodent model allows for the determination of hepatic fatty acid composition and measurements of the hepatic expression of genes involved in fatty acid metabolism. Because of the previously demonstrated effects of estrogen on *Elovl6*, *Fads2* and *Elovl2* (Alessandri et al., 2011; Kitson et al., 2013a; Marks et al., 2013a), we hypothesized the expression of these genes would fluctuate during the estrous cycle in association with estradiol levels. Therefore, female rats were sacrificed daily for four days to collect rats at each stage of the estrous cycle to examine hepatic enzyme expression and fatty acid composition. The stages of the estrous cycle were confirmed using vaginal smears (Hubscher, Brooks, and Johnson, 2005) along with measures of sex hormones in rat plasma. A group of male rats was also included as an additional comparison.

6.2 Materials and Methods

All animal experiments were carried out according to guidelines of the University of Waterloo Animal Care Committee and Canadian Council on Animal Care. Eight-week old male and female Sprague-Dawley rats (Harlan Laboratories, Mississauga, ON) were housed at a temperature of 21 ± 1 °C with *ad libitum* access to AIN-93G standard chow and water, in the Central Animal Housing Facility at the University of Waterloo. Female rats were group housed, in an attempt to synchronize their estrous cycles (McClintock, 1978). All rats were maintained on a cycle of lights on at 6 am and off at 6 pm in order to achieve estrous cycle timing as previously described (Hubscher, Brooks, and Johnson, 2005). At 10 weeks of age, rats were fasted overnight and sacrificed via cardiocentesis after sedation by intraperitoneal sodium pentobarbital injection. Eight or nine female rats were sacrificed each day for four days, to ensure there were enough female rats at each estrous cycle stage. Four male rats were sacrificed on the first day of sacrifice, and four male rats were sacrificed on the last day.

Blood was collected by cardiocentesis into an EDTA-containing syringe. Blood was separated by centrifugation at 3000g to isolate plasma and red blood cells, which were stored at -80°C prior to fatty acid analyses. Livers were quickly excised, washed in saline (0.9% NaCl, w/v) and frozen in liquid nitrogen prior to storage at -80°C. Plasma hormone analysis was performed as described in Chapter 4. Whole blood, plasma and erythrocyte fatty acids were extracted and analyzed as described in Chapter 4.

Hepatic expression of *Elovl6*, *Scd1*, *Elovl2*, *Elovl5*, *Fads1* and *Fads2* was determined in males and females at the four different estrous cycle stages by qPCR, as described in Chapter 4. Primer sequences for these genes are in Table 6.1. Antibodies used for hepatic protein content determination included ELOVL6 (ab69857, Abcam, 1:1000 in milk), SCD1 (ab19862, Abcam,

1:1000 in milk), ELOVL2 (ab176327, Abcam, 1:1000 in milk), ELOVL5 (ARP49667, Aviva Systems Biology, 1:1000 in milk), D5D (Fads1, ab126706, Abcam, 1:1000 in milk) and D6D (Fads2, ab72189, Abcam, 1:1000 in milk).

Estrous cycle stage was determined by obtaining vaginal smears at the time of sacrifice, staining using a modified Papanicolaou stain as described previously (Hubscher, Brooks, and Johnson, 2005) and comparing the stained images to expected cell morphology at each estrous cycle stage. Animals were sorted into groups based on the images obtained from staining for estrous cycle. Data are presented as mean ± s.e for genes and proteins, and mean ± s.d. for fatty acids. Differences in mRNA, protein and fatty acids between the five groups was determined using a one-way ANOVA with Tukey's post hoc test.

6.3 Results

6.3.1 Estrous Cycle Stage Determination

Representative images of stained vaginal smears from each stage of the estrous cycle are presented in Figure 6.1. During proestrus, cells are predominantly large and nucleated while during estrus, some nucleated cells remain, but there are a large proportion of sloughed off, non-nucleated cells. During metestrus, the proportion of leukocytes increases, and during diestrus the leukocytes and nucleated cells are found in similar proportions. As such, the female rats were grouped into 4 groups after staining: diestrus (n= 8), metestrus (n=7), proestrus (n=9) and estrus (n=10) A group of males was included (n=8). Estradiol and progesterone levels of the females tended to reflect expectations for each estrous cycle stage (highest levels at proestrus and lowest levels at estrus and metestrus), but there was large variability in the measurements that resulted in no significant differences (data shown in Figure 6.2).

6.3.2 Sex and Estrous Stage Differences in Hepatic Gene Expression

Hepatic expression of *Fads2* and *Elovl6* mRNA were generally higher during estrus compared with the other stages of and with males (Figure 6.3 and 6.4), while males had highest expression of *Elovl5* (Figure 6.4). *Fads1* mRNA was highest during diestrus and in males. No sex or estrous cycle differences were observed in *Scd1* or *Elovl2* mRNA (Figure 6.3 and 6.4). Protein content of genes involved in fatty acid biosynthesis also reflected differences in sex and estrous cycle stage (Figures 6.5 and 6.6). Content of D5D was higher in males compared with females during metestrus. ELOVL5 protein was lowest in males and females during proestrus, and SCD1 was lowest in males and females during metestrus. ELOVL2 was higher in metestrus than males and females in diestrus. The protein content of FADS2 and ELOVL6 did not differ across any group.

6.3.3 Sex and Estrous Stage Differences in Blood and Liver Fatty Acid Composition

The main fatty acids that demonstrated sex and estrous cycle differences consistently in blood and liver were 16:1n-7, 22:5n-3 and 22:6n-3 (Figure 6.7 and 6.8; full fatty acid composition is shown in Appendix 2, Tables A2.1-2.3). Females during diestrus had lower amounts of 16:1n-7 compared with females during metestrus, in both the relative proportion and absolute amounts in whole blood, and lower amounts compared with females during estrus in liver total lipids. Absolute amounts of 22:5n-3 in whole blood were highest in males compared with females during diestrus and estrus. This pattern was not identical in plasma and liver, but males tended to have higher amounts than females. Concentrations of 22:6n-3 in whole blood were highest in females during proestrus compared with females during diestrus and estrus, and males, but were not different in plasma or liver.

6.4 Discussion

This study demonstrates that hepatic gene expression of fatty acid desaturases and elongases changes depending on estrous cycle stage. This suggests that the small changes in hormones during the estrous cycle, can have an effect on gene expression and fatty acid composition. In general, the gene and protein data presently are showing agreement with each other, although no mRNA changes were observed in *Elovl2* or *Scd1*, but there were protein differences, while mRNA changed in *Fads2* and *Elovl6* but not protein. This is not entirely unusual, as it has been reported that mRNA does not always predict protein levels (Maier, Guell, and Serrano, 2009), and it is possible that there are still sex or estrous cycle differences in enzyme activity. It is also possible that there is a time lag between transcription of a gene and when the mRNA is translated into protein, and increases in protein levels may not be observed until the following stage of the estrous cycle, since the cycle stages are so short.

Changes in hepatic elongase and desaturase gene expression over the four stages of the estrous cycle suggests that estrogen is one contributor to controlling expression of these genes. Presently, the mRNA expression of *Fads1* was highest in males and females during diestrus, rather than females being higher than males (Kitson et al., 2012; Extier et al., 2010) or no sex differences in *Fads1* expression (Childs et al., 2010), as previously reported. Interestingly, pooling all of the females in the present study also resulted in no sex differences in *Fads1* expression (data not shown). *Fads2* expression was highest in estrus and diestrus as compared with males, confirming findings of higher *Fads2* in females (Extier et al., 2010) and regulation by estradiol (Childs et al., 2012; Kitson et al., 2013a) although some reports show no effect of estradiol on *Fads2* expression (Sibbons et al., 2014). We expected *Fads2* expression to be the highest during proestrus as this is when both estrogen and progesterone concentrations are highest in rats, and increasing levels of estrogen or both estrogen and progesterone have been

previously shown to increase levels of D6D in animals (Kitson et al., 2013b). It is possible other mechanisms are regulating *Fads2* expression. High 22:6n-3 intake in females is associated with reduced conversion of 22:5n-3 to 22:6n-3 using stable isotope tracers and compartmental modelling (Pawlosky et al., 2003b). Blood levels and hepatic levels of 22:6n-3 tended to peak at proestrus, so it is possible that feedback inhibition could have occurred although differences in 22:6n-3 were subtle and not always significantly different from other stages.

Estrogen appears to have different effects on elongase mRNA expression that is specific to each individual elongase, rather than affecting all elongases in the same way. *Elovl2* mRNA expression did not show any differences, but was higher in females overall when females were pooled as one group (data not shown), agreeing with previous reports (Kitson et al., 2012). It is possible that only low concentrations of estrogen are required to induce *Elovl2* expression and maximize the effect on mRNA expression, which is why no further increases were seen when estrogen increases. *Elovl6* expression is higher during estrus compared with metestrus. Estrogen levels are similar at these two estrous cycle stages, so it is not known why expression would be higher during estrus compared with metestrus. *Elovl5* expression was highest in males as has been previously reported (Kitson et al., 2012), and estrus had higher expression compared with proestrus, suggesting that estrogen may negatively regulate *Elovl5* expression, since it is higher in males and estrous cycle stages when estrogen is lower. The rats in this study were sacrificed at 10 weeks of age, while other studies have used rats that were 14 weeks old (Kitson et al., 2012). No sex differences have been reported in gene expression at either 3 or 5 weeks of age (Extier et al., 2010), so while 10 and 14 weeks of age are both sexually mature, it would be prudent to characterize the effect of age on expression of enzymes involved in fatty acid biosynthesis

Fewer studies report protein than those that report genes, but females have been reported to have higher protein levels of D5D, D6D and ELOVL6 (Kitson et al., 2012; Marks, Kitson, and Stark, 2013; Extier et al., 2010), suggesting estrogen mediates an increase in protein levels of these specific enzymes. The results from this thesis do not entirely match this hypothesis. There are differences between males and females during metestrus in D5D, but no estrous cycle differences, and no sex or estrous stage differences in D6D. ELOVL2 is higher during metestrus than diestrus and proestrus, but not different from males, confirming previous results of no sex differences (Kitson et al., 2012). The role of estrogen in mediating ELOVL2 protein content requires further examination. ELOVL5 protein is lowest in females during proestrus and males. This is unlikely to be due to estrogen signaling, because these two groups have very different estrogen levels. Overall, it is possible that estrogen is influencing the activity of these enzymes to modulate fatty acid composition rather than absolute protein content, especially since there are differences in hepatic 22:5n-3 and whole blood 22:6n-3 content over the estrous cycle and between sexes. While this observation may be partly due to estrogen-regulated differences in synthesis, since females have been shown to have a greater capacity to convert 22:5n-3 to 22:6n-3 compared with males (Pawlosky et al., 2003b), this does not appear to be the sole factor controlling hepatic and blood fatty acid composition. For example, in the present study, 22:5n-3 is not higher in whole blood and plasma in males compared with all of the female groups. Metestrus appears to be more similar to males in both fatty acid composition and hepatic expression of desaturases and elongases. There may be an estrous cycle effect on hepatic lipoprotein packaging or export of fatty acids, or on mobilization from other tissue stores, that results in higher 22:5n-3 in metestrus compared with the other stages in females. This may be supported by findings from 22:6n-3, since there were no differences observed in liver amounts

when comparing estrous cycle stage or sex, but levels of 22:6n-3 in plasma were highest during proestrus and metestrus, suggesting there may be some sort of regulation of hepatic fatty acids into the blood.

SCD1 is highest during diestrus and proestrus, although content of these proteins have not been previously shown to change based on sex (Kitson et al., 2012; Marks, Kitson, and Stark, 2013), while ELOVL6 surprisingly shows no sex or estrous cycle differences, despite evidence of its regulation by estrogen (Marks, Kitson, and Stark, 2013; Marks et al., 2013b). Amounts of 16:1n-7 were consistently lowest in females during diestrus. The gene data cannot fully explain this finding, since SCD1 protein was highest during diestrus, and there were no changes in ELOVL6. Specific increases in 16:1n-7 occur when hormone levels are highest, such as during pregnancy and in menopausal women taking hormone therapy (Stark, Park, and Holub, 2003; Stark et al., 2005). Hormone levels are lower during diestrus compared with the other estrous cycle stages, which may be one reason that 16:1n-7 is also lower. Future studies should examine the nature of the relationship between 16:1n-7 and estradiol, since the effect seems to be independent of synthesis.

6.5 Conclusions

This is the first study to examine hepatic enzyme expression over the estrous cycle, as well as examine specific fatty acid changes during the estrous cycle, rather than looking at lipid classes as a whole. As such, we have identified that sex differences in hepatic enzyme expression are not uniform across all estrous cycle stages, and are not directly related to estrogen levels. Increasing estrogen levels appear to increase FADS2 mRNA levels, but not protein. Despite no differences in D6D protein content over the estrous cycle, we did observe differences in 22:6n-3

content. Given the importance of 22:6n-3 in human health, a more specific examination of the regulation of 22:6n-3 biosynthesis by estrogen is warranted.

Table 6.1 Rat primer sequences for reverse transcriptase real-time PCR

Gene Name	Accession Code	Sequence (5'-3')
18S	M11188	Forward: CGGACACGGACSGGATTGACAGAT
		Reverse: ATCGCTCCACCAACTAAGAACGGC
Elovl2	AB071986.1	Forward: TGCTTGCCCGTGAGAGCCAC
		Reverse: TGCCACAGGAAGGCGACGAC
Elovl5	NM_134382.1	Forward: CTCTCGGGTGGCTGTACTTC
		Reverse: AGAGGCCCCTTTCTTGTTGT
Elovl6	NM_134383.2	Forward: CACAGCCTCGGGCTTGTTCGT
		Reverse: CTATGGGCCGCCTTCTCGGGA
Fads1	NM_053445	Forward: CCTCTTGTAAAGCACGAGCC
		Reverse: CAAGGGGTCACACTGTTCCT
Fads2	NM_031344	Forward: TCAAAACCAACCACCTGTTCTTC
		Reverse: ACCAGGCGATGCTTTCCA
Scd1	NM_139192.2	Forward: TGTTCCAGAGGAGGTACTACAAGCC
		Reverse: GCAGGAAAGTTTCGCCCCAGC

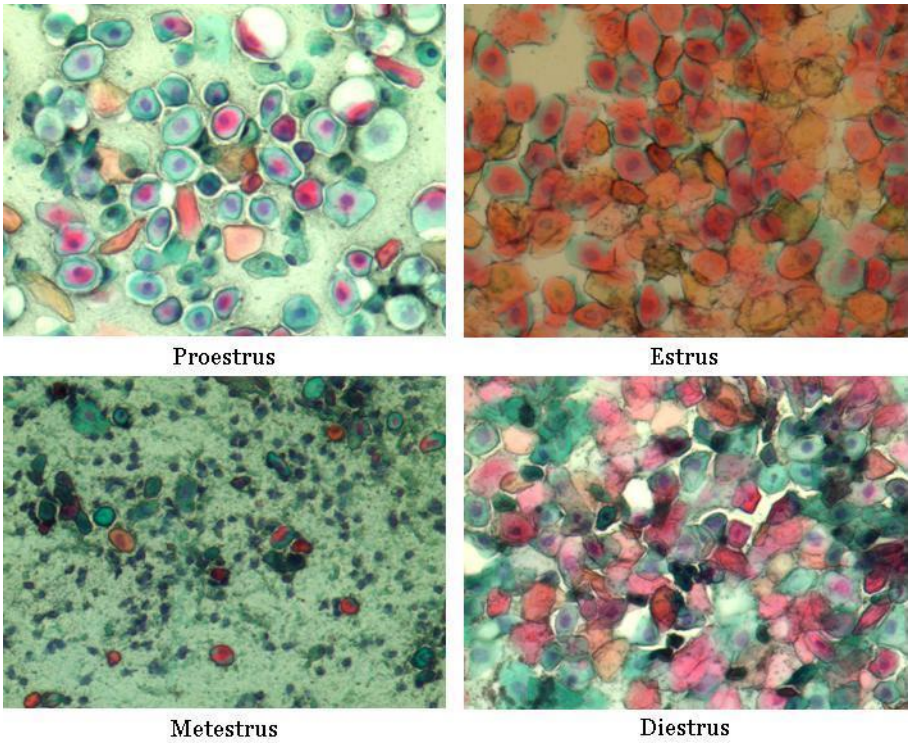


Figure 6.1. Representative figure of stained vaginal smears from 10-week old female rats at the time of sacrifice. Smears were stained using a modified Papanicolaou stain as described previously (Hubscher, Brooks, and Johnson, 2005) and compared to expected cell morphology at each estrous cycle stage.

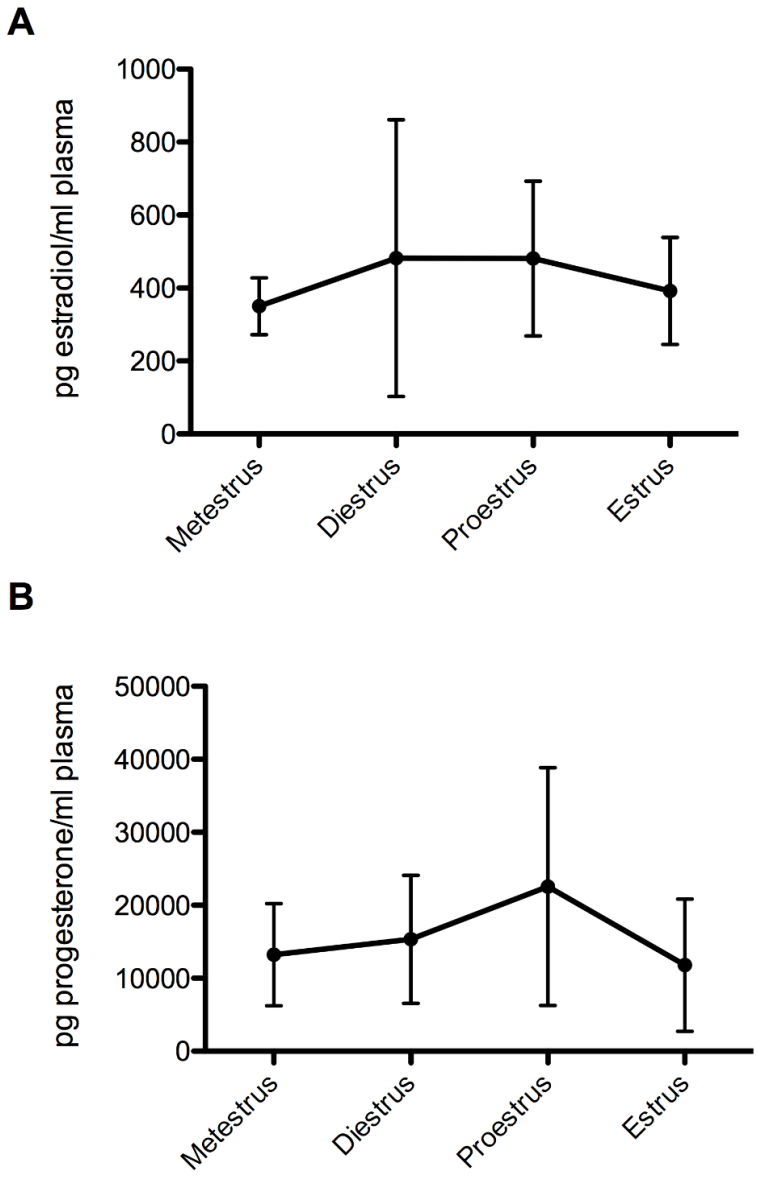


Figure 6.2. Plasma concentrations of A. estrogen and B. progesterone in female rats over the estrous cycle. Data are mean ± s.d.

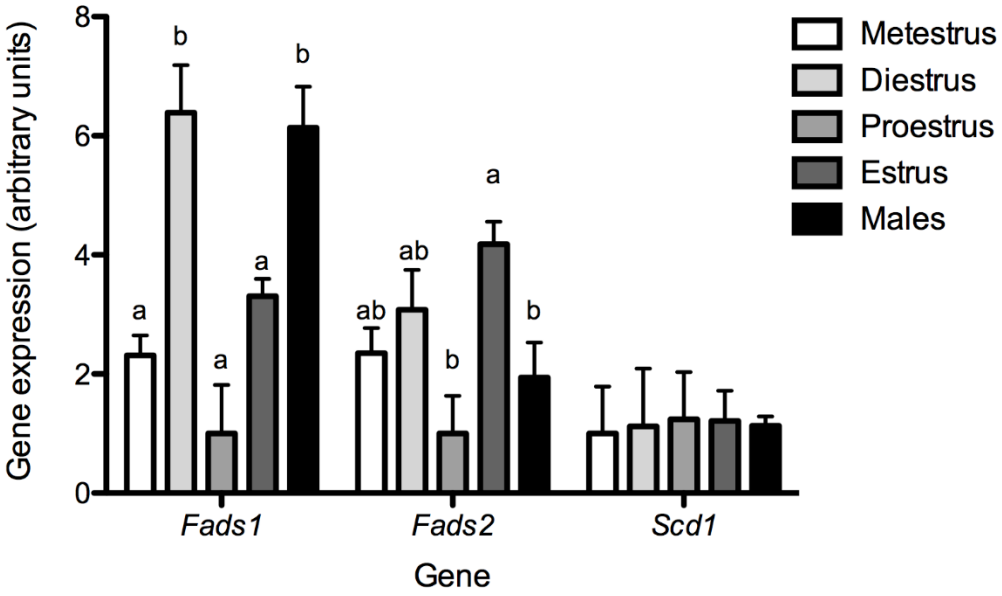


Figure 6.3. mRNA expression of fatty acid desaturases in 10-week old female rats over the estrous cycle and 10-week old male rats. Data are mean ± s.e. Within a gene, means with a different letter are significantly different by Tukey's post hoc test following a significant one-way ANOVA F-value. *Fads1*: fatty acid desaturase 1; *Fads2*: fatty acid desaturase 2; *Scd1*: stearoyl-CoA desaturase 1.

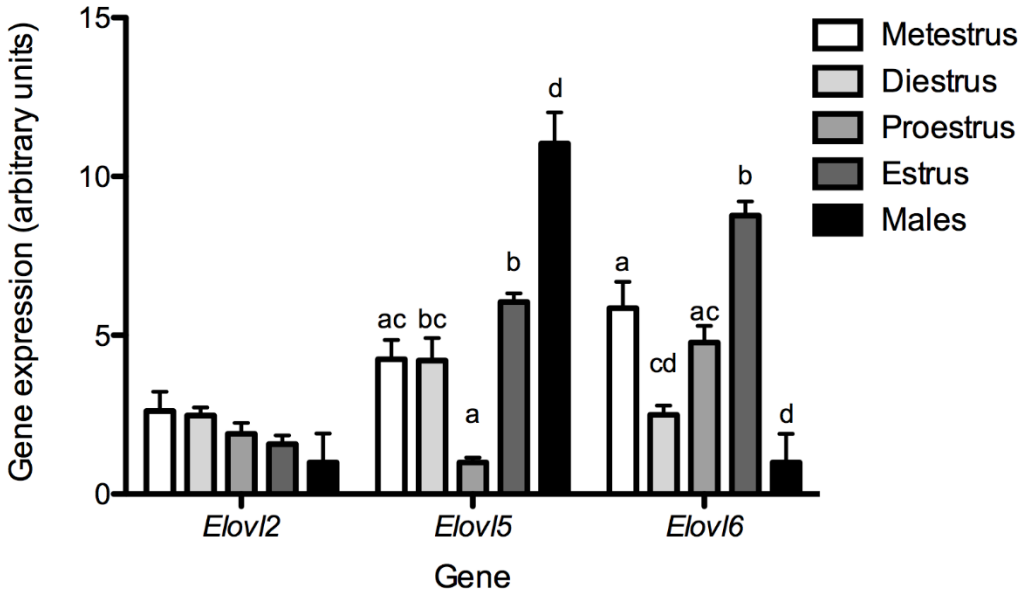


Figure 6.4. mRNA expression of fatty acid elongases in 10-week old female rats over the estrous cycle and 10-week old male rats. Data are mean ± s.e. Within a gene, means with a different letter are significantly different by Tukey's post hoc test following a significant one-way ANOVA F-value. *Elovl2*: fatty acid elongase 2; *Elovl5*: fatty acid elongase 5; *Elovl6*: fatty acid elongase 6.

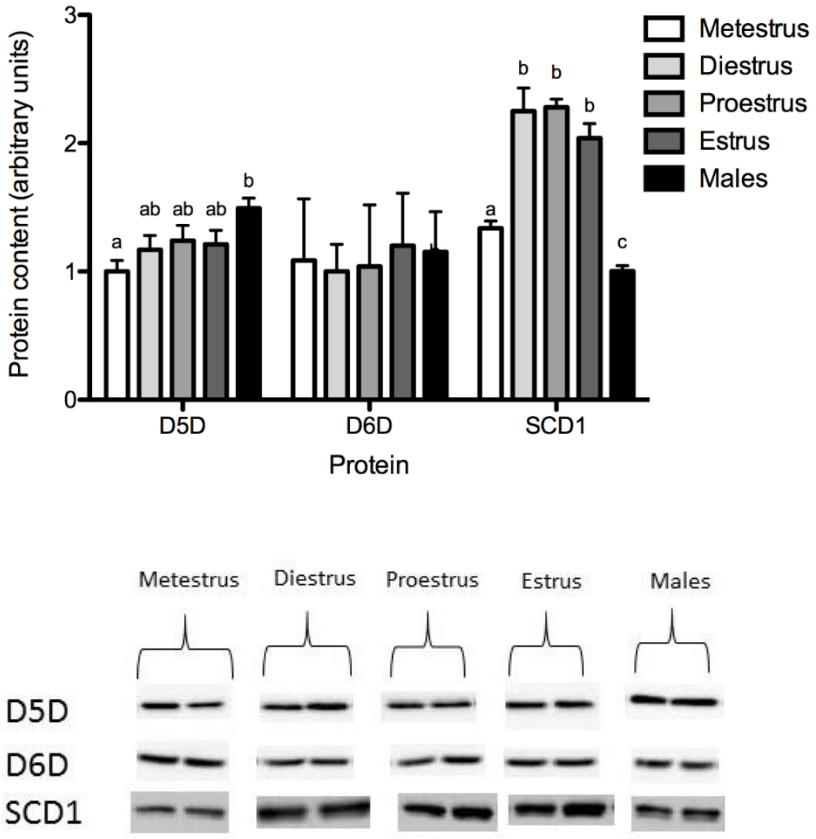


Figure 6.5. Protein content of fatty acid desaturases in 10-week old female rats over the estrous cycle and 10-week old male rats. Data are mean ± s.e. Within a gene, means with a different letter are significantly different by Tukey's post hoc test following a significant one-way ANOVA F-value. D5D: dela-5 desaturase; D6D: delta-6 deasturase; SCD1: stearoyl-CoA desaturase 1.

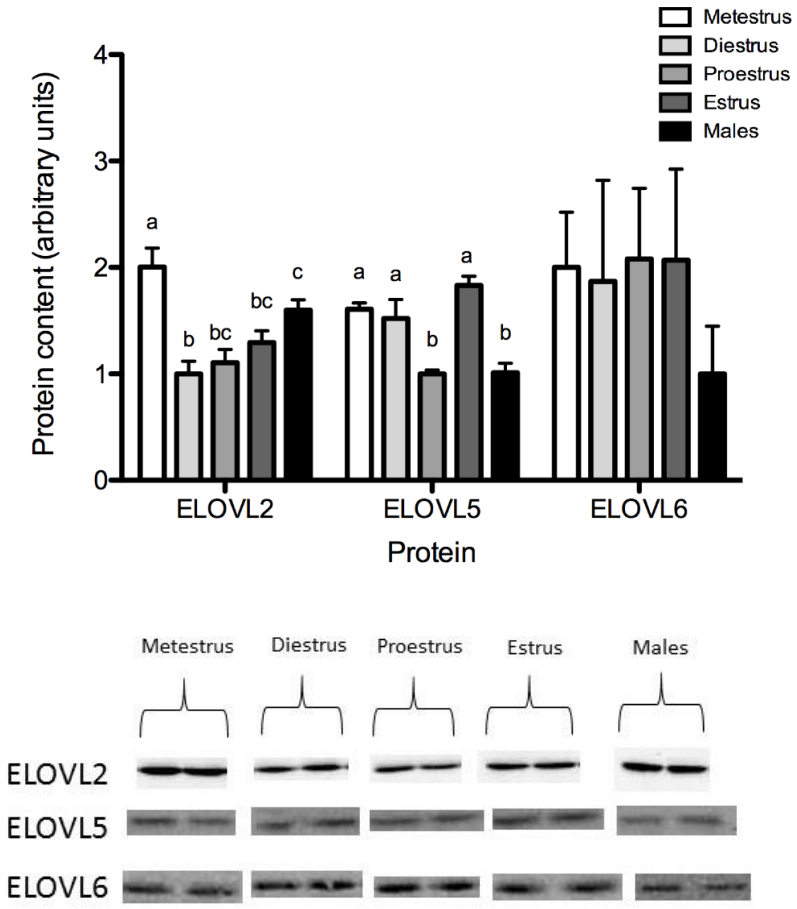


Figure 6.6. Protein content of fatty acid elongases in 10-week old female rats over the estrous cycle and 10-week old male rats. Data are mean ± s.e. Within a gene, means with a different letter are significantly different by Tukey's post hoc test following a significant one-way ANOVA F-value. ELOVL2: fatty acid elongase 2; ELOVL5: fatty acid elongase 5; ELOVL6: fatty acid elongase 6.

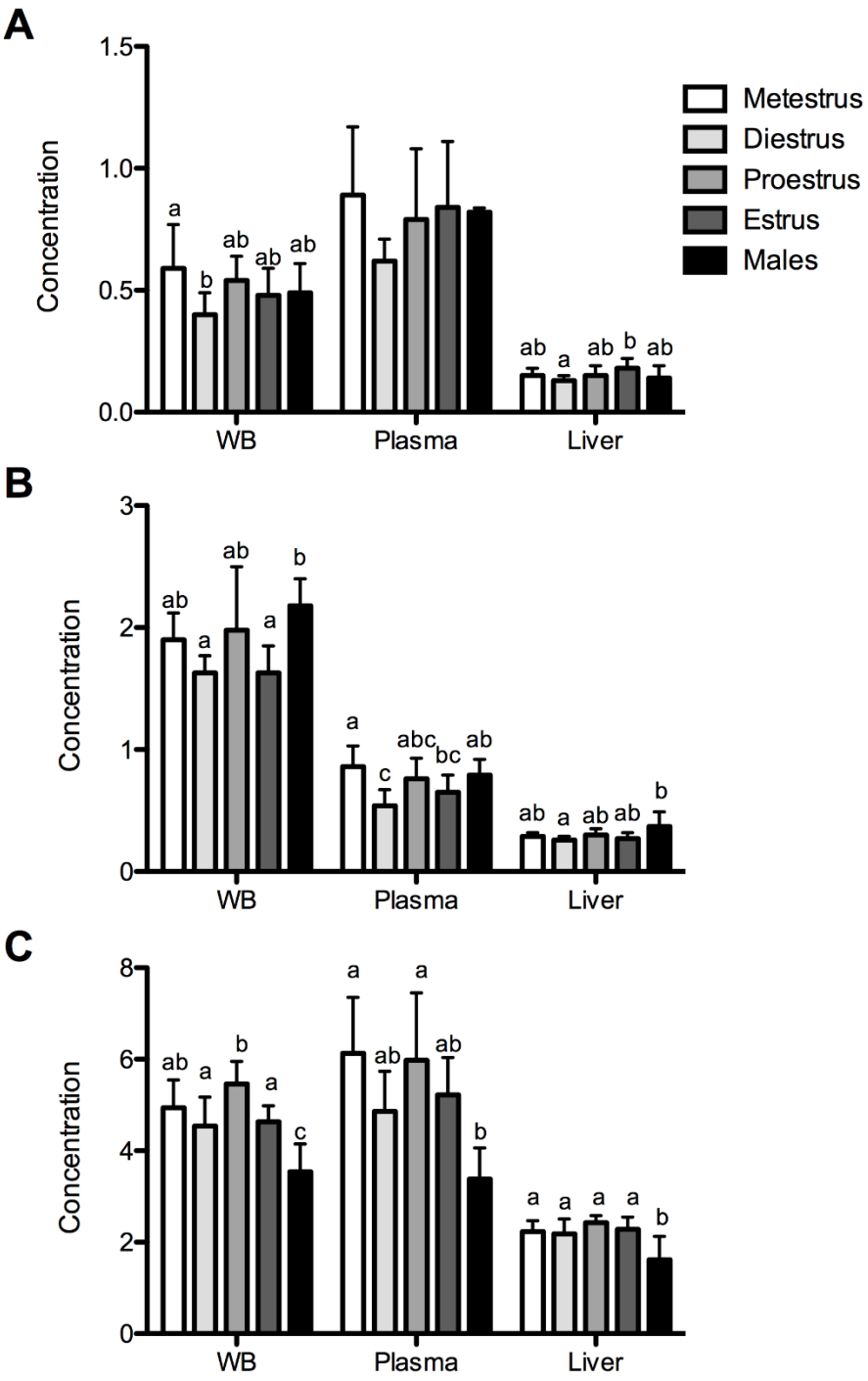


Figure 6.7. Concentration of A. 16:1n-7 B. 22:5n-3 and C. 22:6n-3 in 10-week old female rats over the estrous cycle and 10-week old male rats. Data are presented as µg fatty acid/100 µl whole blood or plasma, or µg fatty acid/mg liver. Data are mean ± s.d. Within a gene, means with a different letter are significantly different by Tukey's post hoc test following a significant one-way ANOVA F-value.

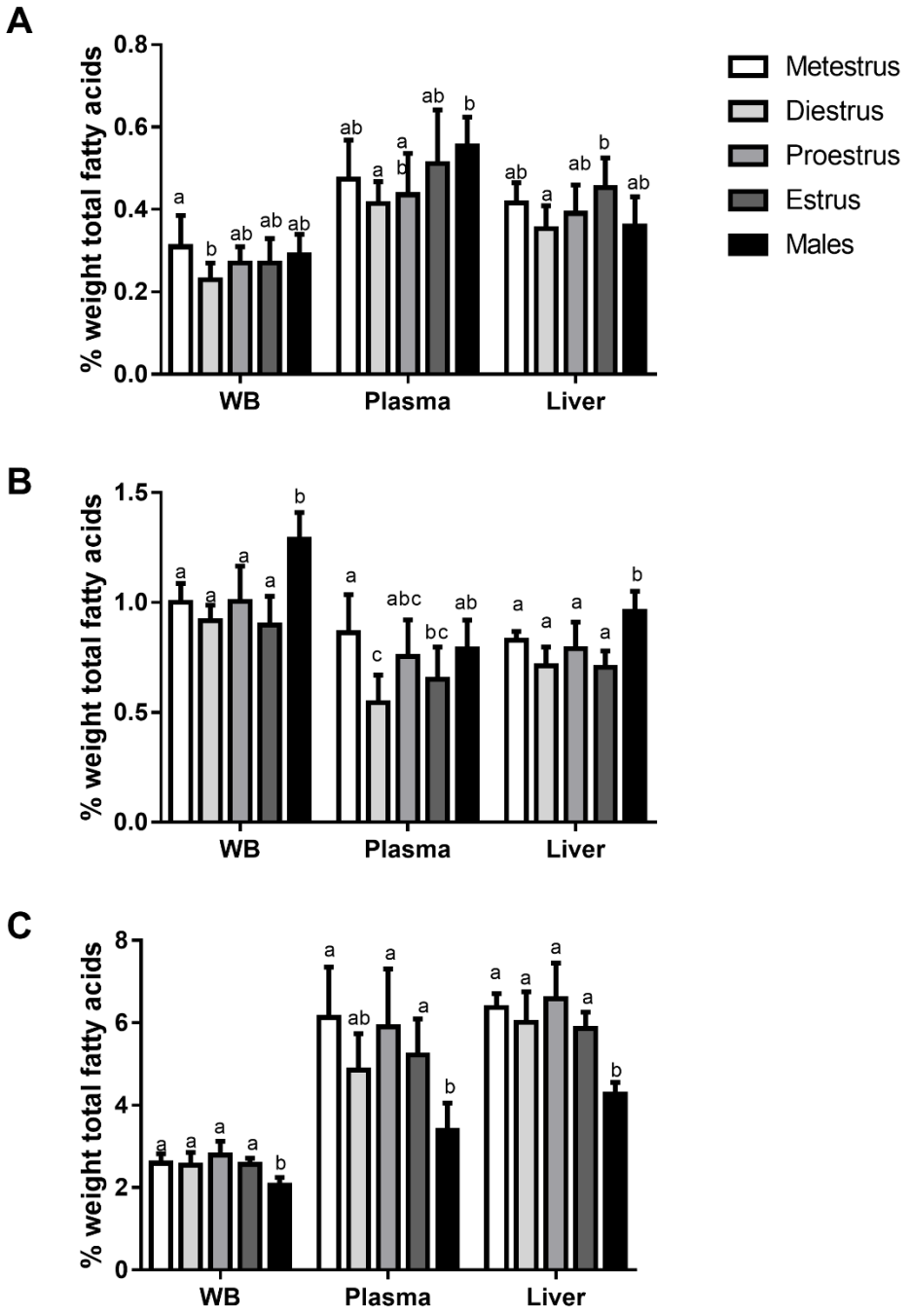


Figure 6.8 Relative percentage of A. 16:1n-7 B. 22:5n-3 and C. 22:6n-3 in 10-week old female rats over the estrous cycle and 10-week old male rats. Data are presented as µg fatty acid/100 µl whole blood or plasma, or µg fatty acid/mg liver. Data are mean ± s.d. Within a gene, means with a different letter are significantly different by Tukey's post hoc test following a significant one-way ANOVA F-value.

Chapter 7.

Effects of Estradiol Treatment on Expression of Fatty Acid Biosynthesis Genes and Fatty Acid Composition in MCF7 Cells

7.1 Introduction

Estrogen has been hypothesized to contribute to previously reported sex differences in both human whole blood (Crowe et al., 2008; Metherel et al., 2009; Giltay et al., 2004; Marangoni et al., 2007) and rodent tissue and blood (Kitson et al., 2012; Marks, Kitson, and Stark, 2013; Burdge et al., 2008) fatty acid composition. Although there are sex differences in progesterone levels as well, few changes are observed in ovariectomized animals treated with progesterone (Kitson et al., 2013b; Marks et al., 2013a), so most research has focused on the effects of estrogen on fatty acid metabolism. Females have higher concentrations of 18:0 and lower concentrations of 16:0 and 16:1n-7 (Marangoni et al., 2007), which may be driven by higher hepatic expression of *Elovl6* (Marks, Kitson, and Stark, 2013) compared with males. Females also have higher concentrations of 22:6n-3 and lower levels of 20:5n-3 and 22:5n-3 compared with men (Metherel et al., 2009; Patterson et al., 2015), possibly as a result of higher hepatic expression of *Fads2* and *Elovl2* (Kitson et al., 2012). Support for this hypothesis comes from the observation of parallel changes in both fatty acid composition and related hepatic gene expression in animal models such as the estrogen receptor α (ERα) knockout mouse model (Bryzgalova et al., 2006), and the rat ovariectomy model with or without estrogen treatment (Kitson et al., 2015; Alessandri et al., 2011; Paquette et al., 2008; Marks et al., 2013a; Kitson et al., 2013a; Mason et al., 2014). However, these animal models have several limitations that confound their use in identifying isolated effects of estrogen on the regulation of fatty acid biosynthesis. For example, the knockout of ERα in mice has been accomplished by disrupting

the DNA binding domain, while leaving the rest of the gene intact. As a result, these animals retain non-genomic ERα-signaling abilities, limiting the conclusions that can be made regarding the regulation of biological processes by estrogen in these animals (Couse et al., 1995). Additionally, ovariectomized animals are generally hyperphagic (Kitson et al., 2015), and estrogen levels are not always decreased relative to the sham-operated animals (Marks et al., 2013a), possibly due to increased synthesis of estrogen in adipose tissue (Simpson, 2003). It is therefore also difficult to make conclusions on the biological role of estrogen based on findings from this model.

Results from Chapters 5 and 6 suggest that changes in estrogen is accompanied by changes in blood fatty acids and hepatic enzyme expression. Cell culture may be a useful model for the study of isolated cellular effects of estrogen, because conditions can be tightly controlled. At least one study has examined effects of 17α-ethinyl estradiol on the regulation of genes involved in fatty acid biosynthesis and demonstrated no effect on mRNA levels of *FADS1*, *FADS2*, *ELOVL2*, *ELOVL5* or on the conversion of 18:3n-3 to 22:6n-3 (Sibbons et al., 2014). In that work, however, HepG2 cells were used. Since this cell line is male and has limited ERα expression (Kelly et al., 2014), it is possible that estrogen signalling is either deficient, or too low to significantly affect gene expression of fatty acid biosynthesis. MCF7 cells, a human breast cancer cell line, are commonly used for studies of the biological effect of estrogen, because these cells are ERα-positive and are responsive to estrogen treatment (Kim et al., 2015; Horwitz, Costlow, and McGuire, 1975). Expression of *Fas* has been demonstrated to be regulated in a similar manner by sterol regulatory element binding protein-1c in MCF7 cells compared with healthy hepatocytes and adipocytes (Yang et al., 2003), suggesting that these cells may be a representative model of fatty acid biosynthesis. Expression and regulation of other genes

involved in fatty acid biosynthesis have not been examined in MCF7 cells, although *Fas*, *Fads1*, *Fads2*, *Elovl5*, and *Elovl6* are expressed in healthy mammary tissue during pregnancy and lactation in rats. Composition of 22:6n-3 is higher in mammary tissue of lactating rats compared with non-pregnant rats, suggesting that mammary tissue can synthesis and accumulate PUFA (Rodriguez-Cruz et al., 2011), likely in conjunction with hepatic synthesis and adipose tissue mobilization. However, no studies have yet examined fatty acid composition or gene expression in MCF-7 cells in response to estrogen treatment.

Therefore, the purpose of this study is to expand upon the findings that estrogen appears to be an important regulator of fatty acid composition and enzyme expression in the human study in Chapter 5 and the rat study in Chapter 6 by examining the effect of isolated estradiol treatment on fatty acid composition and enzyme expression in MCF7 cells, an ERα-positive cell line.

7.2 Methods and Materials

MCF7 cells were grown as described in Chapter 4. Twenty-four hours prior to any experiment with estradiol, the media was changed to media that was free of phenol red, since this compound has weak ER-binding capabilities (Berthois, Katzenellenbogen, and Katzenellenbogen, 1986). The FBS used in the media was also changed to charcoal-stripped FBS, since this process removes all lipids and hormones present from the donor animal, limiting the confounding effects of exogenous fatty acid provision and exogenous hormonal influence (Dang and Lowik, 2005; Aakvaag et al., 1990). At time point 0, 17β-estradiol (Santa Cruz Biotechnology, Santa Cruz, CA) was dissolved in ethanol and added to 100% charcoal-stripped FBS at 10× the maximum concentration (i.e. 1000 nM), where it was allowed to complex with naturally occurring albumin prior to dilution at a 1:10 ratio with phenol red-free DMEM/F12. The final ethanol concentration in the medium was 0.001% (v/v).

Cells were treated with vehicle control (ethanol), or 0.1, 1, 10 or 100 nM estradiol for eight hours, after which time RNA was collected by addition of Trizol directly to the plate, and cDNA was synthesized, as described in Chapter 4. Gene expression of *ELOVL1*, *ELOVL2*, *ELOVL5*, *ELOVL6, FASN*, *SCD1*, *FADS1* and *FADS2* at these different estradiol concentrations was determined by qPCR. Primer sequences for these genes can be found in Table 7.1. Additional plates of cells were treated with vehicle control or 10 nM estradiol, which had the greatest effect on expression of fatty acid biosynthesis genes, for twenty-four hours, and then cells were collected for fatty acid analysis. Total lipid extracts from these cells were analyzed as described in Chapter 4.

One-way ANOVA was used to determine differences in gene expression. Following a significant one-way ANOVA result, Tukey's post hoc test was used to determine differences in gene expression between different estrogen concentrations. Student's t-test was used to determine differences in fatty acid composition between vehicle treated cells, and cells treated with estradiol.

7.3 Results

7.3.1 Effects of Estradiol on Gene Expression in MCF7 Cells

ELOVL2, *ELOVL6* and *FADS2* were all significantly induced in response to estrogen in MCF7 cells (Figures 7.1 and 7.2). A dose-dependent effect of estrogen treatment was evident for *FADS2* and *ELOVL2*, whereas *ELOVL6* was only induced by 10 nM estrogen. *ELOVL1*, *ELOVL5*, *SCD1*, *FADS1* and *FASN* were not significantly induced by treatment with estradiol at any of the concentrations examined (Figures 7.1 and 7.2).

7.3.2 Fatty Acid Composition of Total Lipid Extracts from MCF7 Cells Treated with Estradiol

The relative fatty acid composition of MCF7 cells treated with either vehicle control or 10 nM estradiol for 24 hours is presented in Table 7.2. Changes in the relative proportion of fatty acids were few. The relative percent weight of 10:0 decreased significantly, from 0.65 ± 0.46 to 0.29 ± 0.20. The relative percent weight of 16:1n-7 increased significantly from 9.27 ± 1.34 to 10.22 ±1.26, and the relative percent of 20:5n-3 decreased from 0.33 ± 0.24 to 0.15 ± 0.06 in estradiol treated cells. Importantly, there were no significant changes in any of the very long chain highly unsaturated fatty acids that would be expected to increase with induction of *Fads2* and *Elovl2*, such as 20:4n6 or 22:6n3. When total lipid extracts were examined quantitatively, there was no significant difference in the concentration of total fatty acids between cells treated with vehicle only or with estradiol (31.72 ± 5.62 μg/mg cellular protein versus 32.55 ± 5.52 μg/mg protein, respectively) (Table 7.3). The only fatty acid that remained statistically significantly different when concentrations rather than relative proportions were considered was 10:0. Total cellular levels of 10:0 decreased from 0.33 ± 0.14 to 0.17 ± 0.05 μg/mg protein in cells treated with 10 nM estradiol.

7.4 Discussion

This is the first study to report both changes in the expression of genes involved in fatty acid biosynthesis, and changes in fatty acid composition in MCF7 cells in response to estrogen treatment. Although prior studies have examined these outcomes in HepG2 cells that are male (Aden et al., 1979; Kalthoff et al., 2013), this work is of particular importance since MCF7 cells are an estrogen-responsive, ERα-positive cell line (Kim et al., 2015; Horwitz, Costlow, and McGuire, 1975). A strength of this study is that these changes were measured in a highly controlled manner. Treatment media used in experiments was phenol red-free, which eliminated the weak pseudo-estrogenic binding of phenol red to ERα (Berthois, Katzenellenbogen, and

Katzenellenbogen, 1986; de Faria et al., 2015). FBS used during experiments was treated with charcoal, eliminating confounding exogenous sources of estrogen and other fatty acids and lipids that may be present in variable quantities (de Faria et al., 2015; Simoncini et al., 2005). Thus, the cells were exposed only to the controlled amounts of 17β-estradiol added to the media and its estrogen signaling, so that the effects of estrogen could therefore be examined specifically.

Sex differences and hormonal effects on n-3 polyunsaturated fatty acids (PUFA) have been studied extensively. Specifically, 22:6n-3 is higher in females compared with males (Marangoni et al., 2007; Metherel et al., 2009; Crowe et al., 2008; Kitson et al., 2012; Patterson et al., 2015), in postmenopausal women who take hormone therapy compared with postmenopausal women who do not (Stark, Park, and Holub, 2003), and in male-to-female transsexuals who take oral estrogen therapy (Giltay et al., 2004). Women seem to have a greater capacity to synthesize 22:6n-3 than males (Pawlosky et al., 2003a; Burdge and Wootton, 2002; Burdge, Jones, and Wootton, 2002). The results from the present support this hypothesis. Estradiol treatment increases expression of *ELOVL2* and *FADS2* in the present study, which are involved in the elongation and desaturation of the precursors to 20:4n-6 and 22:6n-3 (Pauter et al., 2014; Melin and Nilsson, 1997). However, although *ELOVL2* and *FADS2* were induced following estradiol treatment of MCF7 cells in the present study, no differences were observed in 20:4n-6 or 22:6n-3 content in MCF7 cells treated with estradiol. It has been proposed that FADS2 is non-functional in MCF7 cells (Park et al., 2011), although it is not known if this lack of function is due to a mutation or deletion in the *Fads2* gene, a post-translational modification or other mechanism. This would explain the lack of a change in 20:4n-6 and 22:6n-3 in our study, but not the presence of 20:4n-6 or 22:6n-3. It is possible that the these fatty acids were taken up from the media during the normal growth phase and retained in the cells, before

charcoal stripped media was used just prior to estradiol treatment. It is also possible that some delta-6 desaturase activity remains in MCF7 cells.

Treatment of MCF7 cells with 10 nM estradiol in the present study significantly increased the relative proportion of 16:1n-7 in total cellular lipids. This fatty acid is higher in livers and plasma of male rats compared with females (Marks, Kitson, and Stark, 2013), but is also increased in ovariectomized rats treated with both estrogen and progesterone compared to ovariectomized rats (Marks et al., 2013a),. The finding of an increase in 16:1n-7 in MCF7 cells treated with estrogen is somewhat surprising, for two reasons. First, *ELOVL6* expression is increased at this estradiol dose. ELOVL6 functions to elongate 16:0 to 18:0, which should reduce levels of 16:0 substrate needed for formation of 16:1n-7. Second, *SCD1*, the enzyme that acts to desaturate 16:0 to 16:1n-7, does not appear to be estrogen responsive. It thus seems unlikely that increased synthesis is the main factor responsible for the observed increase in 16:1n-7. Additional studies will clearly be required to fully understand this phenomenon. Blood and tissue levels of monounsaturated fatty acids like 16:1n-7, and hepatic expression of the genes that synthesize them, have shown to respond similarly in previous studies of hormonal manipulation, including ERα knockout, ovariectomy and estrogen therapy in menopausal women (Paquette et al., 2008; Stark, Park, and Holub, 2003; Marks et al., 2013a; Marks, Kitson, and Stark, 2013; Marangoni et al., 2007). The present results, taken together with previous findings, suggest that estrogen is an important mediator of monounsaturated fatty acid composition, although the mechanism as-of-yet remains unclear.

7.5 Conclusion

In the present work, treatment of MCF7 cells with 17β-estradiol resulted in an increase in expression of the *ELOVL2*, *ELOVL6* and *FADS2* genes. Minimal changes in the relative

proportion of fatty acids in total cellular lipids were observed, with the exception of 16:1n-7, which was higher in cells treated with estradiol, and 10:0 and 20:5n-3, which were both lower in cells treated with estradiol. Multiple studies have demonstrated that MUFA are consistently responsive to estradiol treatment and/or hormonal manipulation involving estrogen, suggesting an important role for this hormone in regulating MUFA composition. While MCF7 cells provide a useful model for studying the effects of estradiol on gene expression, since these cells are ERα-positive, there are limitations to using these cells for fatty acid experiments, since FADS2 has been reported to be non-functional in this cell line (Park et al., 2011). Future studies using an estrogen responsive, non-cancerous cell line, or primary hepatocytes from a female animal, may alleviate some of the limitations of MCF7 cells. However, a better understanding of the perturbations in fatty acid metabolism MCF7 cells is also needed.

Table 7.1 Human primer sequences for reverse transcriptase real-time PCR

Gene Name	Accession Number	Primer Sequence (5'-3')
18S	M11188	Forward: CGGACACGGACSGGATTGACAGAT
		Reverse: ATCGCTCCACCAACTAAGAACGGC
FASN	NM_004104.4	Forward: TTCTGGGACAACCTCATCGGCG
		Reverse: CCTAGACAGGTCCTTCAGCTTGCC
SCD1	NM_005063.4	Forward: ACCGGACACGGTCACCCGTT
		Reverse: AGCTGGTTGTCGGCGCTAGC
ELOVL1	NM_022821.3	F: ACGGAGTCCTTAGCCAGGAT
		R: GGTCATTAGCAAGGGGGACC
ELOVL2	NM_017770.3	Forward: CTCCGCGTACATGCTGGCAGA
		Reverse: CGGATGTCAGCTTCCCCTGCG
ELOVL5	NM_021814.4	Forward: TCCCGGGCCGCATGACATCA
		Reverse: GAACGTGGGCTGACCGTGAC
ELOVL6	NM_024090.2	Forward: CGGTGGTCGGCACCTAATGAAT
		Reverse: GCACCAGTTCGAAGAGCACCGAA
FADS1	NM_013402.4	F: GGCTCGGCCAATGGGAA
		R: GGGCTCCAGGAGTGGATTTG
FADS2	NM_004265.3	F: ACTTTGGCAATGGCTGGATTCCTACCCTC
		R: ACATCGGGATCCTTGTGGAAGATGTTAGG

Table 7.2 Relative proportion of fatty acids in total lipids of MCF7 cells treated with vehicle only or 10 nM estradiol for twenty-four hours.

	MCF7 Cellular Fatty Acids % weight total fatty acids	
Name	Control	10 nM Estradiol
C 10:0	0.65 ± 0.46	0.29 ± 0.20*
C 12:0	0.50 ± 0.18	0.42 ± 0.17
C 14:0	1.96 ± 0.58	2.27 ± 0.51
C 16:0	19.94 ± 3.79	19.70 ± 3.07
C 18:0	13.95 ± 1.58	14.29 ± 1.90
C 20:0	0.30 ± 0.10	0.36 ± 0.11
C 22:0	0.38 ± 0.16	0.33 ± 0.10
C 24:0	0.55 ± 0.12	0.48 ± 0.13
SFAs	40.09 ± 4.38	40.18 ± 2.34
C 12:1	0.10 ± 0.10	0.09 ± 0.05
C 14:1	0.30 ± 0.20	0.29 ± 0.11
C 16:1n-7	9.27 ± 1.34	10.22 ± 1.26*
C 18:1n-7	8.01 ± 1.18	8.48 ± 0.95
C 18:1n-9	29.88 ± 3.40	29.53 ± 1.80
C 20:1n-9	0.89 ± 0.22	0.86 ± 0.09
C 22:1n-9	0.80 ± 0.19	0.79 ± 0.24
C 24:1n-9	0.96 ± 0.22	1.03 ± 0.13
MUFAs	50.15 ± 5.02	51.28 ± 2.95
C 18:2n-6	1.86 ± 0.32	1.73 ± 0.27
C 18:3n-6	0.12 ± 0.06	0.16 ± 0.08
C 20:2n-6	0.23 ± 0.33	0.19 ± 0.09
C 20:3n-6	0.45 ± 0.09	0.43 ± 0.12
C 20:4n-6	3.00 ± 0.48	2.83 ± 0.34
C 22:2n-6	0.46 ± 0.36	0.38 ± 0.28
C 22:4n-6	0.28 ± 0.14	0.25 ± 0.08
C 22:5n-6	0.30 ± 0.21	0.43 ± 0.25
N-6	6.70 ± 1.42	6.37 ± 0.89
C 18:3n-3	0.36 ± 0.21	0.36 ± 0.14
C 20:3n-3	0.12 ± 0.09	0.10 ± 0.05
C 20:5n-3	0.33 ± 0.24	0.15 ± 0.06*
C 22:5n-3	0.47 ± 0.11	0.39 ± 0.14
C 22:6n-3	1.01 ± 0.21	1.01 ± 0.21
N-3	2.26 ± 0.60	2.01 ± 0.29

Results are mean ± sd. Values with a * are significantly different by student's t test.

Table 7.3 Concentration of fatty acids in total lipids of MCF7 cells treated with vehicle only or 10 nM estradiol for twenty-four hours.

MCF7 Cellular Fatty Acids μg fatty acid/mg cellular protein		
Name	Control	10 nM Estradiol
C 10:0	0.33 ± 0.14	0.17 ± 0.05*
C 12:0	0.19 ± 0.06	0.13 ± 0.03
C 14:0	0.40 ± 0.10	0.56 ± 0.16
C 16:0	5.14 ± 1.19	5.28 ± 0.83
C 18:0	4.70 ± 0.94	4.64 ± 0.79
C 20:0	0.09 ± 0.04	0.11 ± 0.04
C 22:0	0.12 ± 0.07	0.12 ± 0.06
C 23:0	0.06 ± 0.04	0.05 ± 0.03
C 24:0	0.17 ± 0.06	0.17 ± 0.05
SFAs	12.64 ± 2.53	13.09 ± 2.93
C 12:1	0.05 ± 0.02	0.04 ± 0.02
C 14:1	0.09 ± 0.04	0.11 ± 0.02
C 16:1n-7	2.85 ± 0.39	3.39 ± 0.86
C 18:1n-7	2.59 ± 0.75	2.62 ± 0.49
C 18:1n-9	10.31 ± 2.48	10.06 ± 1.23
C 20:1n-9	0.33 ± 0.10	0.30 ± 0.06
C 22:1n-9	0.20 ± 0.03	0.24 ± 0.10
C 24:1n-9	0.34 ± 0.11	0.37 ± 0.07
MUFAs	16.72 ± 3.67	17.12 ± 2.66
C 18:2n-6	0.55 ± 0.16	0.53 ± 0.12
C 18:3n-6	0.05 ± 0.03	0.07 ± 0.02
C 20:2n-6	0.04 ± 0.02	0.03 ± 0.02
C 20:3n-6	0.13 ± 0.03	0.11 ± 0.03
C 20:4n-6	0.83 ± 0.18	0.89 ± 0.15
C 22:2n-6	0.07 ± 0.12	0.04 ± 0.02
C 22:4n-6	0.07 ± 0.03	0.06 ± 0.02
C 22:5n-6	0.07 ± 0.06	0.06 ± 0.04
N-6	1.82 ± 0.43	1.79 ± 0.27
C 18:3n-3	0.06 ± 0.01	0.08 ± 0.04
C 20:3n-3	0.03 ± 0.02	0.02 ± 0.01
C 20:5n-3	0.08 ± 0.06	0.05 ± 0.02
C 22:5n-3	0.15 ± 0.03	0.15 ± 0.04
C 22:6n-3	0.26 ± 0.07	0.25 ± 0.04
N-3	0.54 ± 0.10	0.55 ± 0.08
Total	31.72 ± 5.62	32.55 ± 5.52

Results are mean ± sd. Values with a * are significantly different by student's t test.

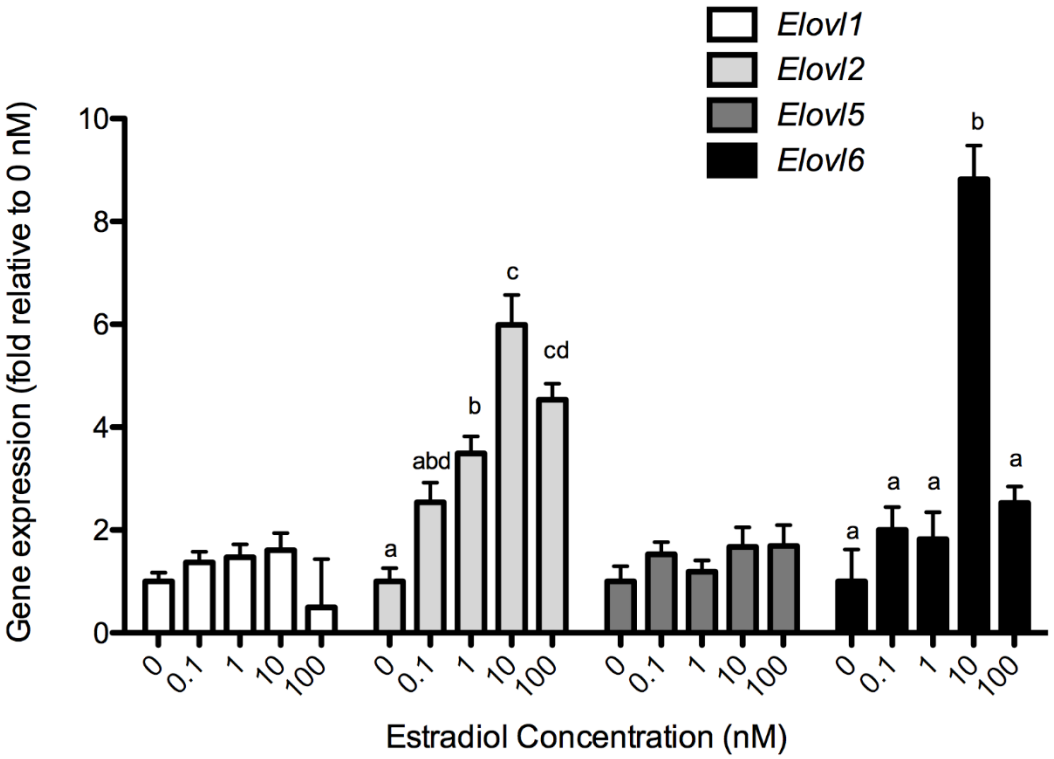


Figure 7.1 Gene expression of fatty acid elongases in response to 0, 0.1, 1, 10 and 100 nM estradiol for 8 hours. Data are mean ± s.e. Within a gene, means with a different letter are significantly different by Tukey's post hoc test following a significant one-way ANOVA F-value. *Elovl1*: fatty acid elongase 1; *Elovl2*: fatty acid elongase 2; *Elovl5*: fatty acid elongase 5; *Elovl6*: fatty acid elongase 6.

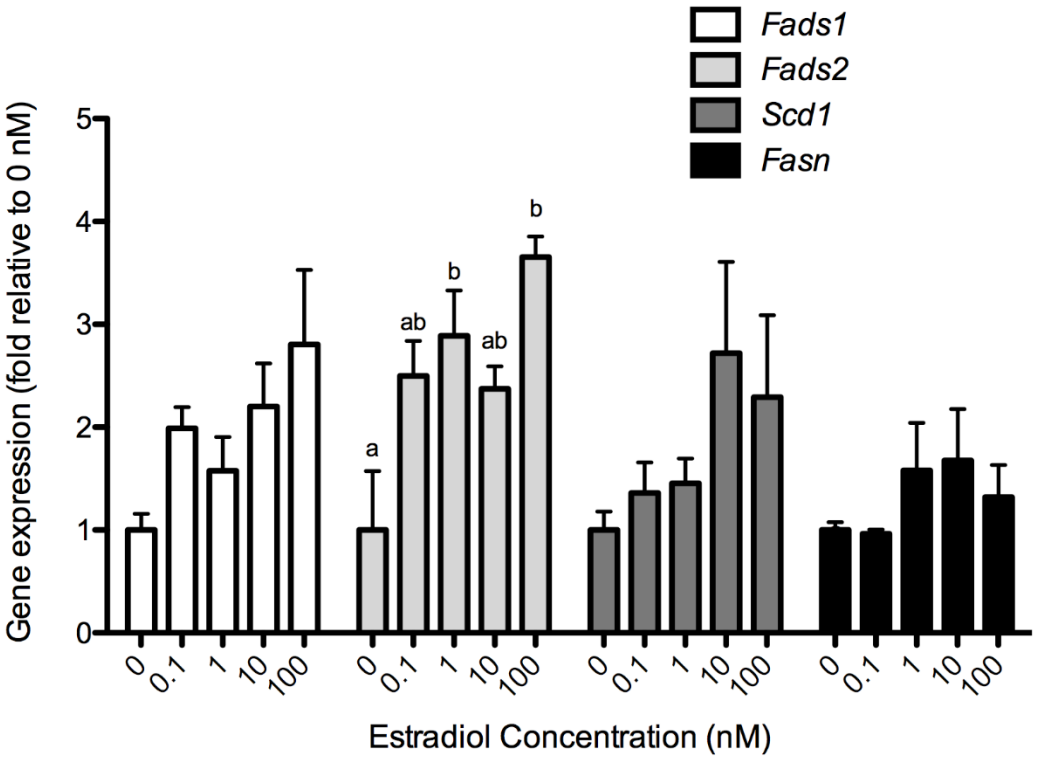


Figure 7.2 Gene expression of fatty acid desaturases in response to 0, 0.1, 1, 10 and 100 nM estradiol for 8 hours. Data are mean ± s.e. Within a gene, means with a different letter are significantly different by Tukey's post hoc test following a significant one-way ANOVA F-value. *Fads1*: fatty acid desaturase 1; *Fads2*: fatty acid desaturase 2; *Scd1*: stearoyl-CoA desaturase 1; *Fasn*: fatty acid synthase.

Chapter 8.

Characterization of *FADS2* Novel Transcript Variants

8.1 Introduction

It has been proposed that *FADS2*, one of the enzymes responsible for synthesizing n-3 and n-6 polyunsaturated fatty acids (PUFA) does not make 20:4n-6 or 22:6n-3 in MCF7 cells (Park et al., 2011) or metabolize shorter-chain omega-6 and omega-3 fatty acids into longer-chain PUFA (Grammatikos et al., 1994). The reason for this observation is not known: we did detect *FADS2* mRNA in MCF7 cells in Chapter 7, but it is unknown if the gene sequence contains a deletion or is mutated and therefore non-functional, or if activity of the protein is repressed because of some other factor. It has also been proposed that *FADS2* is non-functional in HeLa cells (Jaudszus et al., 2014). Both MCF7 (present findings) and HeLa cells (Jaudszus et al., 2014) contain measurable amounts of 20:4n-6 or 22:6n-3. Interestingly, no mutations were identified in the coding sequence of *FADS2* in HeLa cells to explain the proposed lack of function (Jaudszus et al., 2014). Alternate mechanism or mechanisms other than loss of structure-based enzymatic activity, per se, must result in inactivity of this enzyme. Activity of proteins, including PCYT2 and GHRH receptor, can be repressed when a non-active isoform of the enzyme produced from alternative splicing binds to an active form of the same enzyme (Pavlovic, Singh, and Bakovic, 2014; McElvaine and Mayo, 2006; Motomura et al., 1998). This suggests an alternative hypothesis for why D6D, the product of the *FADS2* gene, may be absent in MCF7 cells, even if the *FADS2* gene is not mutated. Therefore, the purpose of the present study was to investigate whether MCF7 cells lack D6D activity because of the presence of, and inhibitory interaction with, a dominant-negative alternate D6D isoform.

8.2 Methods and Materials

8.2.1 Identifying FADS2 Variants

The "Gene" search function on the NCBI website was used to identify two transcripts that coded for identical full-length "*wildtype*" isoforms of D6D (AF084559.1/NM_004265.3 encoding AAG23121.1/NP_004256.1; transcript variants 0/1; *FADS2_v0/1*). This search function was also used to identify a transcript variant produced from alternative splicing, which results in a different coding sequence in the 5' region. As a result, this alternative transcript (NM_001281501.1 encoding NP_0012684301.1; transcript variant 2; *FADS2_v2*) results in production of a protein with a different N-terminal sequence than the wildtype protein. Characterization of protein isoforms arising from alternative splicing is a relatively new field, and naming conventions have not yet been fully developed. According to current best practices, we are therefore naming the wildtype isoform of the D6D protein that is produced from either the *FADS2_v0* or *FADS2_v1* transcripts D6D.1. The isoform produced from the *FADS2_v2* transcript should be called D6D.2.

Based on the protein sequences of D6D.1 and D6D.2, we used UniProt to identify catalytically important domains. Information on these domains, as well as a sequence alignment of these two transcripts, is found in Figure 8.1. Sequence alignment was performed using EMBOSS Needle http://www.ebi.ac.uk/Tools/psa/emboss_needle/ with the default settings (Rice, Longden, and Bleasby, 2000). Primers that could distinguish *FADS2_v0/1* from *FADS2_v2* (sequences in Table 8.1) were designed to determine expression of transcripts encoding for D6D.1 and D6D.2 in MCF7 cells treated with vehicle, 0.1, 1, 10 or 100 nM estradiol for eight hours as described in Chapter 7.

8.2.2 Transfecting FADS2 Variants into HEK 293 Cells

Plasmids encoding the open reading frame (ORF) for the full-length *FADS2_v0/1* transcript (AF084559.1/NM_004265.3), and the alternate *FADS2_v2* transcript, were ordered from GenScript (Piscataway, NJ, USA). The *FADS2_v0/1* ORF was cloned into the pcDNA3.1+C-His vector in-frame with the DNA sequence for a "His-tag", consisting of 6 repeated CAC codons that results in attachment of six histidine residues at the C-terminal end of the protein upon translation. The *FADS2_v2* ORF was cloned into the pcDNA3.1+C-HA vector in-frame with a sequence encoding a hemagglutinin tag, which produces the peptide sequence "YPYDVPDYA" fused to the C-terminal end of the translated protein. Upon arrival, competent *E. Coli* cells were transformed to serve as an ongoing source of the plasmids. The cells were grown on agar plates containing ampicillin as a selection agent for colonies containing the plasmid. Colonies were picked and grown in LB medium containing 100µg/ml ampicillin at 37ºC. Plasmid DNA was mini-prepped from the *E. Coli* cells according to the manufacturer's instructions using a commercially available EZ-10 Spin Column kit (BioBasic, Markham, ON). DNA was stored at -20 ºC prior to use.

HEK 293 cells were collected by trypsinization and transferring to approximately eight ml of DMEM and spinning at 1000 x g for 5 minutes. The DMEM/trypsin supernatant was removed and cells were resuspended in 400 µl of DMEM. Cells were mixed with approximately 10 µg of plasmid DNA containing plasmids encoding for either D6D.1 or D6D.2, isoforms or for pCMV-3-Tag-3a as an empty vector control, and electroporated in 4 mm-gap electroporation cuvettes using an ECM 830 Square Wave Electroporation System (BTX, Holliston, MA) with the following settings, according to the manufacturer's protocol: 5 × four-ms pulses at 230 V). An additional control group of HEK cells was electroporated under identical conditions, except that no vector was used. Cells were immediately transferred to new plates containing fresh media

(DMEM + 10% FBS and antibiotics, as described in Chapter 4), and allowed to grow and adhere to the plate for at least 4 hours. After this time, the media was changed to DMEM with 10% charcoal stripped FBS, which had been depleted of fatty acids. This is an important consideration in studies on PUFA biosynthesis, since there is a particularly high percentage of 22:6n-3 in regular FBS (van den Elsen et al., 2013), and provision of exogenous 22:6n-3 has been shown to inhibit endogenous synthesis of this fatty acid (Domenichiello et al., 2014; Gibson et al., 2013). HEK cells were then cultured for an additional four days after electroporation before harvest.

On day four, cells were collected by centrifuging at 1000 × g for 5 minutes at room temperature. Pellets were lysed by vortexing vigorously in immunoprecipitation (IP) buffer (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA and 1% Triton X-100), and were placed on ice for 15 minutes. Finally, this mixture was centrifuged at 5000 × g for 10 minutes. The cell debris was discarded and the supernatant was stored at -80ºC . An aliquot of the supernatant was used for fatty acid extraction and isolation of phospholipids by thin layer chromatography as described in Chapter 4.

To examine D6D isoform interactions, 5 µg of plasmid encoding the ORF from each of the *FADS2* variants were electroporated together into HEK cells, which were collected four days later. Aliquots of the cells were incubated with either a primary antibody against HA or against His, the unique tags at the end of the gene sequence in the plasmids, or with a secondary antibody, as a negative control. Immunoprecipitations were carried out overnight at 4 ºC with rotation. Antibody/protein complexes were collected by incubation with Protein A/G beads for 2 hours at 4 ºC with rotation. Beads were centrifuged and washed five times using IP buffer. Finally, protein-loading dye was added to the beads, and the entire mixture was boiled to remove the proteins from the beads. This mixture was then ready for analysis by Western blotting, as

described in Chapter 4. Antibodies used for immunodetection were anti-His (2365P, Cell Signaling, 1:1000 in BSA) or anti-HA (2367S, Cell Signaling, 1:1000 in milk).

To determine if the interaction of D6D.1 with D6D.2 resulted in a loss of activity and inhibited 20:4n-6 or 22:6n-3 biosynthesis, cells were electroporated with a constant amount of the *FADS2_v0/1*-expressing plasmid (5 µg), and increasing amounts of *FADS2_v2*-expressing plasmid (0, 2.5, or 5 µg). After electroporation, cells were collected, and phospholipids were isolated and analyzed for fatty acid composition, as described earlier in this chapter.

Student's t-test was used to determine whether expression of alternate *FADS2* transcript variants (encoding for D6D.1 and D6D.2) differed significantly when MCF7 cells were exposed to estradiol compared with controls. One-way ANOVA was used to determine differences in fatty acid composition in HEK 293 cells electroporated with transcript variants. Following a significant one-way ANOVA result, Newman-Keul's post hoc test was used to determine differences in fatty acid levels between groups

8.3 Results

8.3.1 FADS2_v0/1 and FADS2_v2 are Induced by Estradiol in MCF7 Cells

Primers were designed to determine expression of transcripts encoding D6D.1 (*i.e. FADS2_v0/1*) and D6D.2 (*i.e. FADS2_v2*). Both gene variants are detected in MCF7 cells when treated with vehicle only (Figure 8.2). At this baseline, *FADS2_v2* mRNA levels are approximately 6-fold higher than *FADS2_0/1* mRNA levels ($p<0.05$). Expression of *FADS2_v0/1* increases 6-fold when MCF7 cell are treated with 10 nM estradiol, but no other concentration of estradiol results in increased *FADS2_v0/1* expression compared with controls. Expression of *FADS2_v2* significantly increases with 0.1, 1 and 10 nM estradiol (Figure. 8.2),

compared with vehicle treated controls. Expression of *FADS2_v2* remains significantly higher than that of *FADS2_v0/1* at all estradiol concentrations tested.

8.3.2 Fatty Acid Composition of HEK 293 Cells Expressing Control Vector, FADS2_v0/1 or FADS2_v2

Cells electroporated to express *FADS2_v0/1,* encoding for the D6D.1 isoform, have higher relative amounts of one of the major D6D products, 20:4n-6, compared with control cells electroporated with pCMV-3Tag-3a (Figure 8.3; full fatty acid composition is in Appendix 3, Table A3.1 and A3.2). Cells electroporated to express *FADS2_v2,* encoding for the D6D.2 isoform, did not show any differences in the products of D6D activity (i.e. 22:6n-3 or 20:4n-6) compared with controls. As a result, the relative levels of 20:4n-6 were lower in cells transfected with *FADS2_v2* compared with *FADS2_v0/1* (Figure 8.3).

8.3.3 D6D.2 Interacts withD6D.1 and Inhibits Activity

D6D.1 and D6D.2 were found to co-immunoprecipitate when cells were electroporated with plasmids encoding both isoforms (Figure 8.4). Evidence suggested that this interaction allows D6D.2 to negatively regulate the activity of D6D.1. Addition of D6D.1 alone significantly increased cellular levels of 20:4n-6. Electroporation of *FADS2_v2* along with *FADS2_v0/1* in a 1:1 ratio, however, resulted in a significant reduction in the cellular content of 20:4n-6, essentially back to control levels. Notably, transfection of cells with an equimolar ratio of *FADS2_v0/1*:*FADS2_v2* expression plasmid was required to produce this effect. Co-transfection of cells with *FADS2_v0/1*:*FADS2_v2* in a 1:0.5 ratio was insufficient to alter 20:4n-6 levels (Figure 8.5; full fatty acid composition in Appendix 3, Table A3.3 and A3.4).

8.4 Discussion

MCF7 cells express different alternatively spliced transcript variants of the *FADS2* gene. Two reported transcripts (Accession numbers: AF084559.1 (*FADS2_v0*) and NM_004265.3 (*FADS2_v1*)) encode for the same full-length D6D protein, which we refer to as D6D.1 in the current work. These transcripts have highly similar exonal organization, and identical coding regions, differing only by the presence of an additional 207 base pair sequence at the start of the 5' UTR in transcript variant 1 (*FADS2_v1*) that is not present in the originally reported reference sequence for FADS2 (*FADS2_v0*). An alternate transcript, *FADS2_v2* (Accession number NM_001281501.1), encodes for an isoform of the D6D protein (referred to as D6D.2 in the current work) that is 22 amino acids shorter than the full-length isoform. This truncated isoform also has a unique N-terminal sequence that differs from the sequence present at the start of the D6D.1 isoform. D6D.2 is missing the first 68 amino acids that D6D.1 has, and instead has 40 different amino acids (Figure 6.1). The D6D.1 and D6D.2 isoforms are, however, identical throughout the C-terminal 374 bp. The alternate transcript is present at six-fold higher levels than the wild-type transcript. Expression of both transcript variants is induced by estrogen treatment, but levels of *FADS2_v2* remained higher than *FADS2_v0/1* at all levels of estrogen tested.

The full-length D6D.1 isoform has been shown previously to catalyze the desaturation of very long chain fatty acids, and over-expression of this isoform in cells has been found to increase the synthesis of 20:4n-6 and 22:6n-3 in cells (Le et al., 2014). The function of the D6D.2 isoform has not yet been reported. In the wild-type isoform, D6D.1, amino acids 18-95 contain a cytochrome b5-heme binding domain and specifically a 53-His-Pro-Gly-Gly-56 sequence that allows the histidine at amino acid position 53 to bind heme with greater affinity (Cho, Nakamura, and Clarke, 1999). These motifs are essential for catalysis (Cho, Nakamura, and Clarke, 1999), since mutagenesis of His53 to alanine in both the rat D6D and the plant

homologue of D6D abrogates synthesis of the usual D6D products (Sayanova, Shewry, and Napier, 1999; Guillou et al., 2004). Since the D6D.2 isoform lacks these N-terminal sequences (Figure 8.1), we predicted that this alternate form of the enzyme would lack functional desaturase activity. Furthermore, we hypothesized that impaired synthesis of 20:4n-6 and 22:6n-3 in MCF7 cells may occur as a result of a dominant-negative effect of the *FADS2_v2*-encoded D6D.2 isoform. To test this, we transfected HEK 293 cells with plasmids expressing either the wildtype coding region of *FADS2_v0/1* (encoding D6D.1) or the alternate *FADS2_v2* coding region (encoding D6D.2).

As expected, the wild-type D6D.1 was found to have increased activity. Overexpression of plasmid encoding this variant in HEK 293 cells resulted in phospholipids enriched with 15% more 20:4n-6 compared with control cells electroporated with pCMV only. Conversely, levels of 20:4n-6 in cells expressing *FADS2_v2* in the present study were not different from control cells, and were lower than in cells expressing *FADS2_v0/1*. This work demonstrates that the D6D.2 isoform is not catalytically active, and further suggests the necessity of the cytochrome b5- heme binding domain for catalytic activity of human D6D.

PCYT2 (Pavlovic, Singh, and Bakovic, 2014) and GHRH receptor (McElvaine and Mayo, 2006; Motomura et al., 1998) have been demonstrated to have multiple splice variants that interact with each other, with the variant isoforms having a dominant negative effect on the activity of the wild type variant. We hypothesized that a similar effect might occur in MCF7 cells, because *FADS2_v2* is expressed at a higher level than *FADS2_v0/1* in this cell line, and because the D6D.2 isoform appears to be non-functional in HEK 293 cells. Therefore, we directly tested whether D6D.2 could interact with D6D.1 through co-immunoprecipitation, and found that both isoforms associated with each other when expressed in the same cells (Figure

6.4). We hypothesized that this interaction, or at least the presence of both transcript variants, would result in inhibition of 20:4n-6 biosynthesis, since D6D.2 is inactive on its own. Indeed, when HEK cells were electroporated with a constant amount of *FADS2_v0/1* and increasing amounts of *FADS2_v2*, levels of 20:4n-6 were no longer elevated compared with controls, unlike in cells electroporated with *FADS2_v0/1* only. This suggests that D6D.2 is having a dominant negative effect on the ability of D6D.1 to form 20:4n-6. The mechanism behind this effect, and whether it is dependent on the interaction between the two proteins remains unknown, but these results indicate that when D6D.1 and D6D.2 are present in equal amounts, D6D.1 is unable to synthesize 20:4n-6.

Of interest, the proportion of 22:6n-3 was not changed by overexpression of either of the D6D isoforms. Cellular levels of free 18:2n-6, the precursor of 20:4n-6, are typically 5-10 fold higher than cellular levels of free 18:3n-3, the precursor of 22:6n-3. Since D6D can utilize either precursor, competition between the more abundant n-6 PUFA substrate for conversion by ectopically expressed enzyme may account for the lack of effect on 22:6n-3 levels (Harnack, Andersen, and Somoza, 2009). Rats accumulate more 22:6n-3 in plasma and liver lipids when the proportion of 18:3n-3 increases from 0.2% of the total dietary lipids to 1.0%, when 18:2n-6 levels are kept constant at 1% of total dietary lipids (Tu et al., 2010), further suggesting the competition between n-6 and n-3 PUFA for D6D activity. Since we did not presently supplement the media the HEK 293 cells were grown in with additional 18:3n-3, it is possible that 18:2n-6 outcompeted 18:3n-3 for D6D.

8.5 Conclusions

This study is the first to characterize the D6D.2 isoform. This isoform does not synthesize the usual D6D products, 20:4n-6 or 22:6n-3. This suggests that the missing cytochrome b5-heme

binding domain is important in mammalian D6D, which has been shown in the plant (Sayanova, Shewry, and Napier, 1999) and rat (Guillou et al., 2004) form of D6D, but not human D6D. Future studies should target this domain to determine its role in D6D activity and synthesis of 20:4n-6 and 22:6n-3. D6D.2 also appears to be a negative inhibitor of D6D.1 activity, since no increases in 20:4n-6 or 22:6n-3 were observed in HEK cells electroporated with equal amounts of *FADS2_v0/1* and *FADS2_v2* DNA. Since transfecting equal copies of these two transcript variants had an apparent negative effect on D6D activity in HEK cells, I propose that the reason MCF7 cells do not synthesize D6D products in MCF7 cells is because of *FADS2_v2* is expressed at levels 6-fold greater than that of *FADS2_v0/1*, and *FADS2_v2* is negatively inhibiting activity.

Table 8.1 Summary of human FADS2 transcript variant sequence information and primer sequences for reverse transcriptase real-time PCR

Transcript Variant	Transcript Annotation	Transcript Accession Number	Protein Annotation	Protein Accession Number	Protein length	Primer Sequence (5'-3')
0	*FADS2_v0*	AF084559.1	D6D.1	AAG23121.1	432 amino acids	Forward: 5'-CAGCTGGGAGGAGATTCAGA-3' Reverse: 5'-GGAAGGCGCGGAAGGCATCC-3'
1	*FADS2_v1*	NM_004265.3	D6D.1	NP_004256.1	432 amino acids	Forward: 5'-CAGCTGGGAGGAGATTCAGA-3' Reverse: 5'-GGAAGGCGCGGAAGGCATCC-3'
2	*FADS2_v2*	NM_001281501.1	D6D.2	NP_0012684301.1	389 amino acids	Forward: 5'-ATGCACGGCAGGGAGGCGGG-3' Reverse: 5'-GGAAGGCGCGGAAGGCATCC-3'

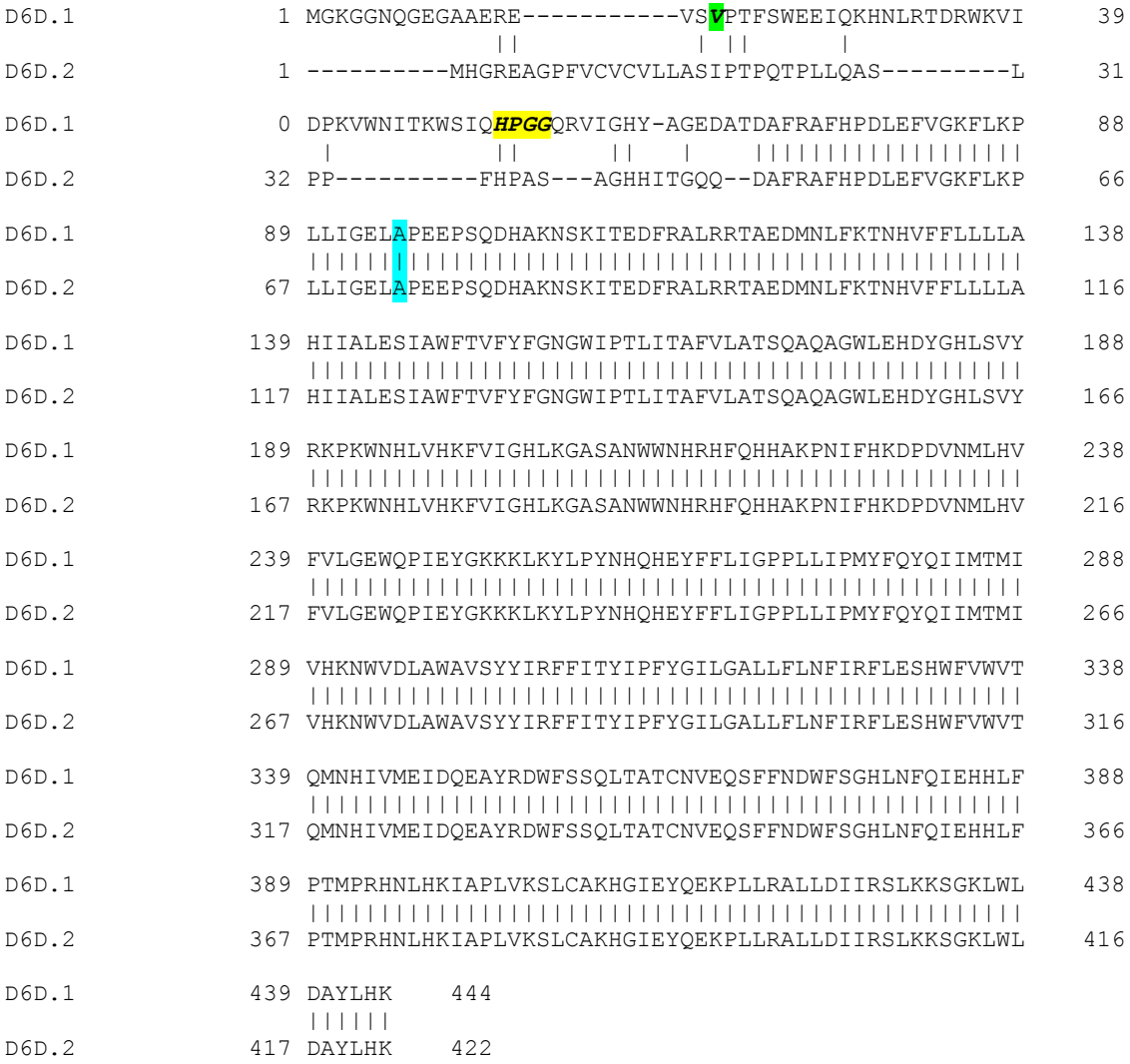


Figure 8.1. Sequence alignment of human D6D.1 and D6D.2 enzymes. Sequence alignment was performed using EMBOSS Needle (Rice, Longden, and Bleasby, 2000). The beginning of the cytochrome b5-heme binding domain is bolded, italicized and highlighted in green. The four-amino acid sequence that is bolded, italicized and highlighted in yellow is required for heme binding and represents a sharp β-turn. The end of the heme binding domain is highlighted in blue.

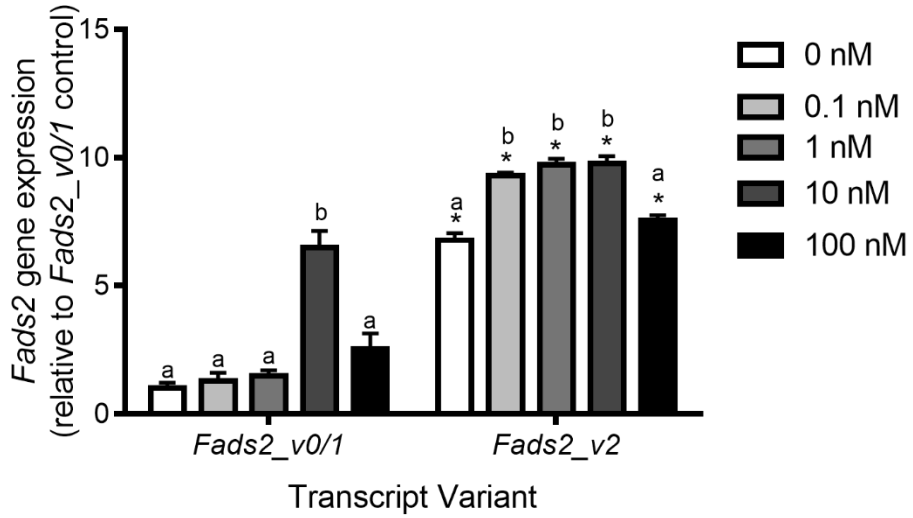


Figure 8.2. Expression of transcripts coding for either wildtype D6D.1 (*FADS2_v0/1*) or D6D.2 (*FADS2_v2*) in MCF7 cells, in response to treatment with increasing concentrations of estradiol for 8 hours. Data are means ± s.e.m.. *p<0.05 versus expression of the *Fads2_v0/1* at the same concentration of estradiol. Within a transcript variant, means with a different letter are significantly different by Newman-Keul's post hoc test following a significant one-way ANOVA F-value.

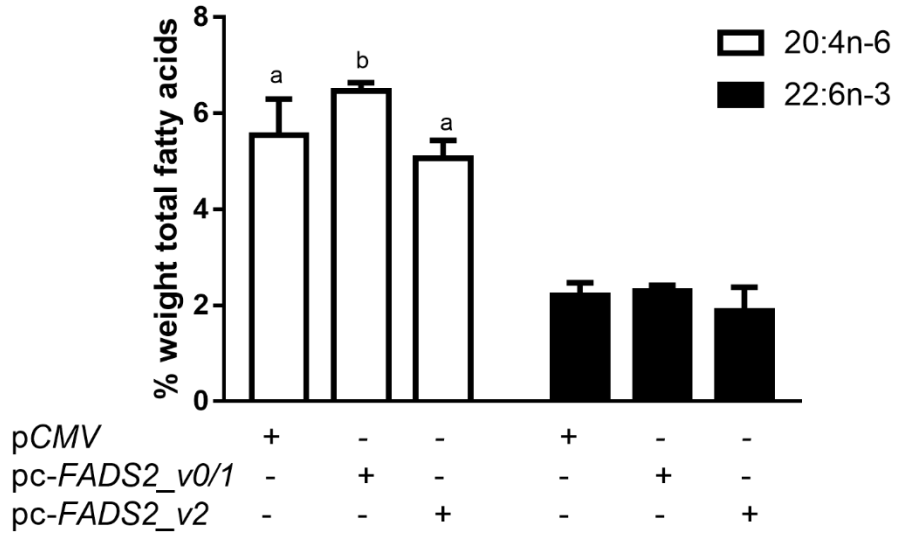


Figure 8.3 Relative proportion of 20:4n-6 and 22:6n-3 in phospholipids from HEK 293 cells electroporated with pCMV (empty vector control), or a plasmid expression vector containing the coding sequence for either D6D.1 (i.e. pc-*FADS2_v0/1*) or. D6D.2 (i.e. pc-*FADS2_v2*). Data are means ± s.d. Within a fatty acid, means with a different letter are significantly different by Newman-Keul's post hoc test following a significant one-way ANOVA F-value.

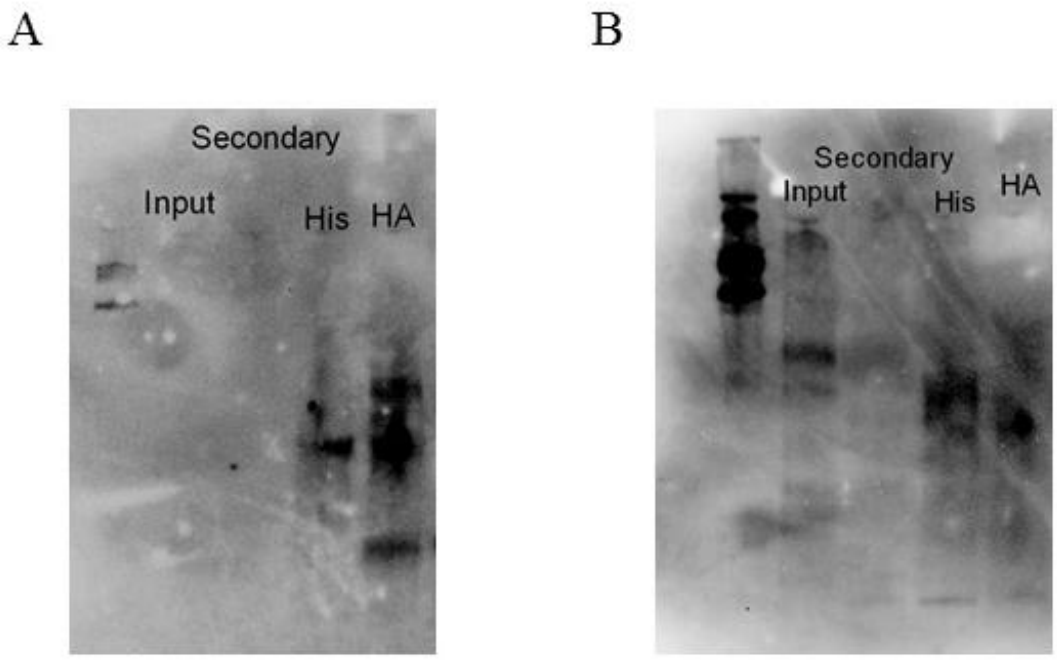


Figure 8.4 D6D.1 and D6D.2 co-immunoprecipitate when expressed together in HEK293 cells. A: Immunoprecipitation using a secondary antibody, an anti-His antibody or a HA antibody, and detected after running on the same gel using an anti-HA antibody. B: Immunoprecipitation using a secondary antibody, anti-His antibody or a HA antibody, and detected after running on the same gel using an anti-His antibody.

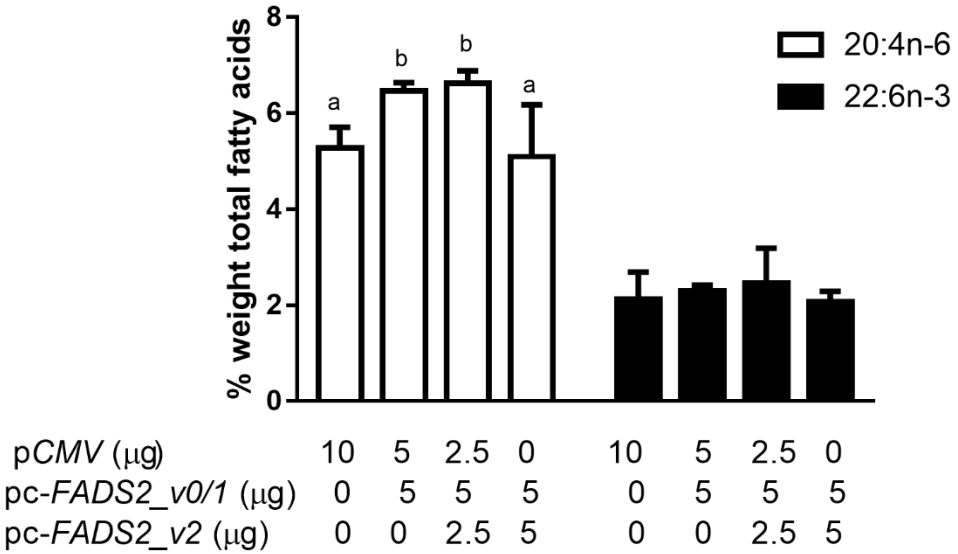


Figure 8.5 Relative proportion of 20:4n-6 and 22:6n-3 in phospholipids from HEK 293 cells electroporated with pCMV (empty vector control), or a plasmid expression vector containing the coding sequence for either D6D.1 (i.e. pc-*FADS2_v0/1*) and increasing amounts of D6D.2 (i.e. pc-*FADS2_v2*). Data are means ± s.d. Within a fatty acid, means with a different letter are significantly different by Newman-Keul's post hoc test following a significant one-way ANOVA F-value.

Chapter 9.

General Discussion

There were four main objectives of this thesis. The first was to confirm if small physiological changes in estrogen over the menstrual cycle are associated with changes in blood fatty acid composition by measuring blood fatty acids at two time points over the menstrual cycle. We hypothesized that levels of 18:0, 16:1n-7 and 22:6n-3 would be higher during the luteal phase compared with the follicular phase. Contrary to our hypotheses, levels of 18:0 did not change with time over the menstrual cycle, and 16:1n-7 was lower during the luteal phase. However, increased levels of 22:6n-3 were observed as expected. We also hypothesized that these three fatty acids would be higher in plasma of oral contraceptive users compared with women not taking oral contraceptives and men. As expected, 16:1n-7 and 22:6n-3 levels were higher in oral contraceptive users, but 18:0 was actually lowest in this group. It appears that increasing levels of estrogen increases 22:6n-3 levels, but the relationship is not as straightforward when examining 16:1n-7. Other models of increasing estrogen increase 18:0 levels (Marks, Kitson, and Stark, 2013; Stark et al., 2005), but the opposite pattern was observed presently, suggesting the relationship between SFA and estrogen is not straightforward.

The second objective in this thesis was to further examine the effects of physiological changes in estrogen concentrations in naturally cycling animals by using a rat model to measure hepatic enzyme expression over the four-day estrous cycle. We hypothesized that mRNA and protein levels of elongase 6, elongase 2 and Fads2 would be highest during proestrus, when estrogen is highest. These hypotheses were all incorrect. Elongase 6 levels were consistently highest in females, but the estrous cycle had no effect on mRNA or protein levels. *Elongase 2* mRNA was not different over the estrous cycle, and protein levels were highest in males and proestrus. Finally, *Fads2* mRNA was highest during estrus, but protein levels were not different at

all. It appears that regulation of these enzymes *in vivo* is controlled by multiple mechanisms. Estrogen may be one of these factors, but is not the only one.

Therefore, the third objective was to confirm the effects of estrogen on gene expression changes in MCF7 cells, an ERα-positive cell line without the additional considerations of diet, other hormone changes, fatty acid feedback regulation and multiple sources of estrogen required for an *in vivo* model. We hypothesized that estrogen treatment would increase mRNA expression of *FASN*, *ELOVL6*, *ELOVL2* and *FADS2*, and levels of 18:0, 16:1n-7 and 22:6n-3. As expected, mRNA levels of *ELOVL6*, *ELOVL2* and *FADS2* increased, confirming that estrogen has a role in regulating mRNA expression of these genes. *FASN* was not increased with estrogen. Levels of 16:1n-7 increased as predicted, but few other changes were observed, unlike our hypotheses. *FADS2* has been previously been predicted to be non-functional in MCF7 cells, so it is likely that this is why no changes in 22:6n-3 levels were observed.

Therefore, the final purpose of this thesis was to examine perturbations in MCF7 fatty acid metabolism. Two transcript variants were identified, and the ability of each to synthesize 20:4n-6 and 22:6n-3 when overexpressed in HEK 293 cells was determined. We hypothesized that overexpressing *FADS2_v0/1* in HEK 293 cells would increase the levels of these two fatty acids, but *FADS2_v2* overexpression would not, compared with controls. These hypotheses were what was observed. We hypothesized that the two variants would interact when both are overexpressed in HEK293 cells to inhibit synthesis of 20:4n-6 and 22:6n-3 by the wild-type variant, which is what was observed, suggesting that *FADS2_v2* acts as a dominant negative inhibitor on *FADS2_v0/1*.

The relationship between estrogen and fatty acid composition was examined in several models, including the human menstrual cycle, rat estrous cycle and in cell lines. Higher levels of 22:6n-3 and lower levels of 22:5n-3 during the menstrual cycle when estrogen is highest suggested estrogen regulation of the expression and/or activity of ELOVL2 or FADS2. Follow up work in

rats confirmed the fatty acid results but hepatic *Elovl2* was not different across the estrous cycle, while *Fads2* was highest at estrus and diestrus stage in rats, but not during proestrus when estrogen is highest. However, *ELOVL2* and *FADS2* did increase with estradiol treatment in MCF7 cells. It is possible that other changes in rats that are not occurring in MCF7 cells contribute to the differences between the two studies. For example, progesterone also increases during the estrous cycle in rats, and has been previously reported to have effects on PUFA biosynthesis and potentially interfering with the effects of estradiol (Childs et al., 2012; Sibbons et al., 2014; Wade, 1975). The estrogen:progesterone ratio is also an important determinant of signaling and estrogenic effects (Sinchak and Wagner, 2012). Progesterone is at its highest during proestrus in the rat, while it is low during estrus when there is a secondary peak in estradiol (Smith, Freeman, and Neill, 1975). Progesterone was not examined in the MCF7 experiments. It may also be possible that when levels of 22:6n-3 are high, feedback regulation occurs to shut off expression of desaturases and elongases, which may have been happening during proestrus in rats, since 22:6n-3 levels were highest. This would not have happened in MCF7 cells, because there were no differences in cellular PUFA levels.

Fads1 and *Elovl5* also changed over the estrous cycle in rats, but did not change with estradiol treatment in cells, suggesting that they are not directly regulated by estrogen, but possibly by additional hormones and gene-regulatory factors present in complex organism systems. It is also possible that eight hours of estradiol treatment was not long enough to elicit changes in expression of these genes in MCF7 cells, because some genes have been demonstrated to require twenty-four hours to show changes in MCF7 cells treated with estradiol (Frasor et al., 2003). Future studies should examine a time course of estrogen treatment, to determine if these genes are induced or repressed by estrogen after a longer time period.

Levels of 20:4n-6 and 22:6n-3 were highest in the blood of proestrus rats and women in the luteal phase, when estrogen peaks. However, levels of these fatty acids were not higher in MCF7

cells treated with estradiol. Expression of *FADS2*, which codes for D6D (the rate-limiting enzyme in the synthesis of these two PUFA species) is induced by estrogen in MCF7 cells. However, this may be explained by the alternate splice variant (*FADS2_v2*) that I characterized in MCF7 cells. This variant codes for a shorter protein that is unable to synthesize 20:4n-6 and 22:6n-3. Expression of *FADS2_v2* was higher in MCF7 cells compared with the wild type *FADS2_v0/1*. Introduction of higher levels of *FADS2_v2* into HEK 293 cells caused an inhibition of 20:4n-6 biosynthesis by co-expressed *FADS2_v0/1*. Since a direct interaction between D6D.1 and D6D.2 was observed, it is possible that D6D.2 directly inhibits the activity of the wildtype enzyme, although additional work will be required to confirm this. Importantly, these studies suggest a mechanism to explain why FADS2 has been reported to be non-functional in MCF7 and potentially other cancer cells. My results suggest D6D activity may still be possible in these cells, but is largely inhibited.

In this thesis, we also report that 16:1n-7 was higher in women who take oral contraceptives compared with both women who do not receive oral contraceptives and men and in total lipid extracts from MCF7 cells treated with estradiol compared with controls, which agrees with prior reports in postmenopausal women taking and not taking hormone therapy (Stark, Park, and Holub, 2003; Tworek et al., 2000). When estrogen concentrations are fluctuating, such as in women who do not receive oral contraceptives and in the estrous cycle in rats, 16:1n-7 is not increased as predictably. The effect of estrogen on 16:1n-7 seems to be reserved for large differences in estrogen exposure that are typically observed with exogenous treatment that is pharmacological versus physiological.

There are several strengths to the studies in this thesis. Firstly, the studies in rats and humans specifically examined estrous or menstrual cycle stage, respectively, as well as birth control use in humans. Often females are under studied because menstrual cycle and estrogen-based changes may complicate analyses, or if females are included, the menstrual cycle is not

considered during analyses. I have shown there are differences in both hepatic expression of fatty acid elongases and desaturases and blood fatty acid composition over the cycle in rats and humans. Researchers should consider these factors when designing experiments in the future, and potentially take multiple experimental measurements in women to account for menstrual cycle to get an accurate representation. Finally, whenever cells were treated with estradiol, this was the only source of estrogen signalling, because the media did not contain phenol red, and charcoal-stripped FBS was used. For plasmid experiments, cells were grown in media containing FBS that had been charcoal-stripped to remove any sources of long chain PUFA, thereby minimizing confounding from exogenous lipids when determining effects of the different *FADS2* transcript variants on fatty acid levels. Additionally, for the plasmid experiments, TLC was performed to isolate phospholipids, the major complex lipid type incorporating 20:4n-6 and 22:6n-3.

However, there are some limitations to these studies as well. While menstrual cycle and birth control use were controlled for and analyzed separately in Chapter 5, data on the timing of when women were taking the oral contraceptives was not collected, so when women may have been exposed to different hormone levels at the time of the blood collections. Future studies should examine the effect of hormone timing on fatty acid composition, in addition to other dosing and delivery methods, such as a triphasic birth control or transdermal patch. Similarly, the timing of the rat estrous cycle was difficult to determine. Both estrogen and progesterone change rapidly over the estrous cycle, particularly during proestrus (Smith, Freeman, and Neill, 1975). We tried to minimize variability by sacrificing rats at the same time of day, but it is possible that the rats were not entirely timed perfectly. This is supported by some of the vaginal stains that appeared to be in between two different cycle stages and the variability in the hormone data, despite measuring steroid hormones by UHPLC-MS/MS. A tighter timeline in rats or allowing them to acclimate longer prior to sacrifice may alleviate some of these limitations. Additionally, the rats were fasted. This minimized the within-experiment variability, but limits the applicability to fed and refed

models since both mRNA expression of *Scd1* and *Elovl6* (Turyn, Stojek, and Swierczynski, 2010) and hepatic fatty acid composition (Marks et al., 2015) have been demonstrated to be regulated by fasting and feeding. Using cell culture allows for specific parameters to be manipulated. However, the FBS in the cell culture media contains factors required for cell growth including fatty acids, specifically HUFA. Although stripped serum was used prior to hormone and plasmid experiments, it is unknown what proportion of the fatty acids measured after treatments were due to the experimental manipulation and what was taken up and incorporated into the cells prior to treatment, when cells were still exposed to regular FBS. It took a lot of time to fully identify the *FADS2* variants and develop a method for examining the effects of the variants on 20:4n-6 and 22:6n-3 levels. As such, many of the conclusions regarding the transfected cells and functionality of the *FADS2* variants are based on fatty acid composition only. Future studies using radiolabeled fatty acids in conjunction with overexpression of the *FADS2* variants and/or an enzyme assay will allow for a more specific measure of the activity of the different variants. Additionally, confirming the role of *FADS2_v2* in dominantly inhibiting *FADS2_v0/1* in MCF7 cells should be completed. This could be achieved by overexpressing *FADS2_v0/1* in these cells to determine if increasing levels of this variant to levels greater than those of *FADS2_v2* can overcome the inhibition, or silencing *FADS2_v2* and determining if activity of *FADS2_v0/1* is no longer repressed.

The work from this thesis expands the current state of knowledge of the effects of sex and estradiol on fatty acid metabolism. 22:6n-3 biosynthesis appears to be upregulated by estrogen, but the expression of enzymes in 22:6n-3 biosynthesis appears to have multiple control factors *in vivo*. Measuring expression levels of multiple transcript variants of *FADS2* is a novel concept employed herein. This is important for future research, as I have characterized D6D.2 as non-functional and inhibitory with regards to the function of the wildtype D6D.1 enzyme. This could be important for understanding the impact of genetics on fatty acid metabolism particularly polymorphisms in populations but also mutations in metabolic disorders and cancer. I have also demonstrated that

some of the inconsistent findings with fatty acid metabolism in sex comparisons may be due to a failure to categorize women and female animal models based on menstrual or estrous cycle stage, as well as exogenous hormone use. Future dietary recommendations for fatty acid intake and requirements for men and women, and women at different life stages (such as monthly recommendations during the menstrual cycle, pregnancy, and menopause) should be considered.

References

Aakvaag, A., Utaaker, E., Thorsen, T. et al. (1990) Growth control of human mammary cancer cells (MCF-7 cells) in culture: effect of estradiol and growth factors in serum-containing medium. Cancer Res. 50: 7806-7810.

Aden, D. P., Fogel, A., Plotkin, S. et al. (1979) Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 282: 615-616.

Agren, J. J., Tormala, M. L., Nenonen, M. T. et al. (1995) Fatty acid composition of erythrocyte, platelet, and serum lipids in strict vegans. Lipids 30: 365-369.

Al, M. D., van Houwelingen, A. C., Kester, A. D. et al. (1995) Maternal essential fatty acid patterns during normal pregnancy and their relationship to the neonatal essential fatty acid status. Br.J.Nutr. 74: 55-68.

Alessandri, J. M., Extier, A., Al-Gubory, K. H. et al. (2012) Influence of gender on DHA synthesis: the response of rat liver to low dietary alpha-linolenic acid evidences higher omega3 4-desaturation index in females. Eur.J.Nutr. 51: 199-209.

Alessandri, J. M., Extier, A., Al-Gubory, K. H. et al. (2011) Ovariectomy and 17beta-estradiol alter transcription of lipid metabolism genes and proportions of neo-formed n-3 and n-6 long-chain polyunsaturated fatty acids differently in brain and liver. J.Nutr.Biochem 22: 820-827.

Allam-Ndoul, B., Guenard, F., Barbier, O. et al. (2016) Effect of n-3 fatty acids on the expression of inflammatory genes in THP-1 macrophages. Lipids Health Dis. 15: 69.

Anderson, R. E. (1970) Lipids of ocular tissues. IV. A comparison of the phospholipids from the retina of six mammalian species. Exp.Eye Res. 10: 339-344.

Anderson, R. E. & Sperling, L. (1971) Lipids of ocular tissues. VII. Positional distribution of the fatty acids in the phospholipids of bovine retina rod outer segments. Arch.Biochem.Biophys. 144: 673-677.

Bagga, D., Wang, L., Farias-Eisner, R. et al. (2003) Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. Proc.Natl.Acad.Sci.U.S.A 100: 1751-1756.

Bakewell, L., Burdge, G. C., & Calder, P. C. (2006) Polyunsaturated fatty acid concentrations in young men and women consuming their habitual diets. Br.J.Nutr. 96: 93-99.

Balkova, P., Jezkova, J., Hlavackova, M. et al. (2009) Dietary polyunsaturated fatty acids and adaptation to chronic hypoxia alter acyl composition of serum and heart lipids. Br.J.Nutr. 102: 1297-1307.

Barnett, J. B., Woods, M. N., Lamon-Fava, S. et al. (2004) Plasma lipid and lipoprotein levels during the follicular and luteal phases of the menstrual cycle. J.Clin.Endocrinol.Metab 89: 776-782.

Barrett-Connor, E. (1997) Sex differences in coronary heart disease. Why are women so superior? The 1995 Ancel Keys Lecture. Circulation 95: 252-264.

Bascunan, K. A., Valenzuela, R., Chamorro, R. et al. (2014) Polyunsaturated Fatty Acid composition of maternal diet and erythrocyte phospholipid status in chilean pregnant women. Nutrients. 6: 4918-4934.

Baum, S. J., Kris-Etherton, P. M., Willett, W. C. et al. (2012) Fatty acids in cardiovascular health and disease: A comprehensive update. J.Clin.Lipidol. 6: 216-234.

Bazan, N. G., Calandria, J. M., & Gordon, W. C. (2013) Docosahexaenoic acid and its derivative neuroprotectin D1 display neuroprotective properties in the retina, brain and central nervous system. Nestle.Nutr.Inst.Workshop Ser. 77: 121-131.

Bazan, N. G., Musto, A. E., & Knott, E. J. (2011) Endogenous signaling by omega-3 docosahexaenoic acid-derived mediators sustains homeostatic synaptic and circuitry integrity. Mol.Neurobiol. 44: 216-222.

Belfiore, F., Iannello, S., Rabuazzo, A. M. et al. (1987) Metabolic effects of short-term fasting in obese hyperglycaemic humans and mice. Int.J.Obes. 11: 631-640.

Belkaid, A., Duguay, S. R., Ouellette, R. J. et al. (2015) 17beta-estradiol induces stearoyl-CoA desaturase-1 expression in estrogen receptor-positive breast cancer cells. BMC.Cancer 15: 440.

Berthois, Y., Katzenellenbogen, J. A., & Katzenellenbogen, B. S. (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc.Natl.Acad.Sci.U.S.A 83: 2496-2500.

Bligh, E. G. & Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can.J.Biochem.Physiol 37: 911-917.

Block, R. C., Harris, W. S., & Pottala, J. V. (2008) Determinants of Blood Cell Omega-3 Fatty Acid Content. Open.Biomark.J. 1: 1-6.

Bojar, I., Gujski, M., Raczkiewicz, D. et al. (2015) Estrogen receptor alpha polymorphisms, estradiol level, and occurrence of atherosclerosis risk factors in healthy postmenopausal women. Med.Sci.Monit. 21: 970-979.

Bolton-Smith, C., Woodward, M., & Tavendale, R. (1997) Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet. Eur.J.Clin.Nutr. 51: 619-624.

Bonen, A., Parolin, M. L., Steinberg, G. R. et al. (2004) Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36. Faseb Journal 18: 1144-+.

Borkman, M., Storlien, L. H., Pan, D. A. et al. (1993) The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. N.Engl.J.Med. 328: 238-244.

Breckenridge, W. C., Gombos, G., & Morgan, I. G. (1972) The lipid composition of adult rat brain synaptosomal plasma membranes. Biochim.Biophys.Acta 266: 695-707.

Bryzgalova, G., Gao, H., Ahren, B. et al. (2006) Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. Diabetologia 49: 588-597.

Bui, A. L., Horwich, T. B., & Fonarow, G. C. (2011) Epidemiology and risk profile of heart failure. Nat.Rev.Cardiol. 8: 30-41.

Burdge, G. C. & Calder, P. C. (2005) Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reprod.Nutr.Dev. 45: 581-597.

Burdge, G. C., Delange, E., Dubois, L. et al. (2003) Effect of reduced maternal protein intake in pregnancy in the rat on the fatty acid composition of brain, liver, plasma, heart and lung phospholipids of the offspring after weaning. Br.J.Nutr. 90: 345-352.

Burdge, G. C., Jones, A. E., & Wootton, S. A. (2002) Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*. Br.J.Nutr. 88: 355-363.

Burdge, G. C., Sherman, R. C., Ali, Z. et al. (2006) Docosahexaenoic acid is selectively enriched in plasma phospholipids during pregnancy in Trinidadian women--results of a pilot study. Reprod.Nutr.Dev. 46: 63-67.

Burdge, G. C., Slater-Jefferies, J. L., Grant, R. A. et al. (2008) Sex, but not maternal protein or folic acid intake, determines the fatty acid composition of hepatic phospholipids, but not of triacylglycerol, in adult rats. Prostaglandins Leukot.Essent.Fatty Acids 78: 73-79.

Burdge, G. C. & Wootton, S. A. (2002) Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br.J.Nutr. 88: 411-420.

Cao, H., Gerhold, K., Mayers, J. R. et al. (2008) Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. Cell 134: 933-944.

Cao, J. J. & Gregoire, B. R. (2016) A high-fat diet increases body weight and circulating estradiol concentrations but does not improve bone structural properties in ovariectomized mice. Nutr.Res. 36: 320-327.

Capel, F., Acquaviva, C., Pitois, E. et al. (2015) DHA at nutritional doses restores insulin sensitivity in skeletal muscle by preventing lipotoxicity and inflammation. J.Nutr.Biochem. 26: 949-959.

Carlson, S. E. (2001) Docosahexaenoic acid and arachidonic acid in infant development. Semin.Neonatol. 6: 437-449.

Castell, J. V., Jover, R., Martinez-Jimenez, C. P. et al. (2006) Hepatocyte cell lines: their use, scope and limitations in drug metabolism studies. Expert.Opin.Drug Metab Toxicol. 2: 183-212.

Chen, C. T., Kitson, A. P., Hopperton, K. E. et al. (2015) Plasma non-esterified docosahexaenoic acid is the major pool supplying the brain. Sci.Rep. 5: 15791.

Chen, Q., Nimal, J., Li, W. et al. (2011) Delta-6 desaturase from borage converts linoleic acid to gamma-linolenic acid in HEK293 cells. Biochem.Biophys.Res.Commun. 410: 484-488.

Childs, C. E., Hoile, S. P., Burdge, G. C. et al. (2012) Changes in rat n-3 and n-6 fatty acid composition during pregnancy are associated with progesterone concentrations and hepatic FADS2 expression. Prostaglandins Leukot.Essent.Fatty Acids 86: 141-147.

Childs, C. E., Kew, S., Finnegan, Y. E. et al. (2014) Increased dietary alpha-linolenic acid has sex-specific effects upon eicosapentaenoic acid status in humans: re-examination of data from a randomised, placebo-controlled, parallel study. Nutr.J. 13: 113.

Childs, C. E., Romeu-Nadal, M., Burdge, G. C. et al. (2010) The polyunsaturated fatty acid composition of hepatic and plasma lipids differ by both sex and dietary fat intake in rats. J.Nutr. 140: 245-250.

Cho, H. P., Nakamura, M. T., & Clarke, S. D. (1999) Cloning, expression, and nutritional regulation of the mammalian Delta-6 desaturase. J.Biol.Chem. 274: 471-477.

Cho, J. S., Baek, S. H., Kim, J. Y. et al. (2014) Serum phospholipid monounsaturated fatty acid composition and Delta-9-desaturase activity are associated with early alteration of fasting glycemic status. Nutr.Res. 34: 733-741.

Chow, J. D., Jones, M. E., Prelle, K. et al. (2011) A selective estrogen receptor alpha agonist ameliorates hepatic steatosis in the male aromatase knockout mouse. J.Endocrinol. 210: 323-334.

Chowdhury, R., Warnakula, S., Kunutsor, S. et al. (2014) Association of dietary, circulating, and supplement fatty acids with coronary risk: a systematic review and meta-analysis. Ann.Intern.Med. 160: 398-406.

Christie, W. W. (1989) Gas chromatography and lipids The Oily Press, Ayr.

Clarke, D. C., Miskovic, D., Han, X. X. et al. (2004) Overexpression of membrane-associated fatty acid binding protein (FABPpm) in vivo increases fatty acid sarcolemmal transport and metabolism. Physiol Genomics 17: 31-37.

Coe, N. R., Smith, A. J., Frohnert, B. I. et al. (1999) The fatty acid transport protein (FATP1) is a very long chain acyl-CoA synthetase. J.Biol.Chem. 274: 36300-36304.

Couse, J. F., Curtis, S. W., Washburn, T. F. et al. (1995) Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. Mol.Endocrinol. 9: 1441-1454.

Cowley, S. M., Hoare, S., Mosselman, S. et al. (1997) Estrogen receptors alpha and beta form heterodimers on DNA. J.Biol.Chem. 272: 19858-19862.

Crowe, F. L., Skeaff, C. M., Green, T. J. et al. (2008) Serum n-3 long-chain PUFA differ by sex and age in a population-based survey of New Zealand adolescents and adults. Br.J.Nutr. 99: 168-174.

Cunnane, S. C. (2000) The conditional nature of the dietary need for polyunsaturates: a proposal to reclassify 'essential fatty acids' as 'conditionally-indispensable' or 'conditionally-dispensable' fatty acids. Br.J.Nutr. 84: 803-812.

Cunnane, S. C. & Anderson, M. J. (1997) Pure linoleate deficiency in the rat: influence on growth, accumulation of n-6 polyunsaturates, and [1-14C]linoleate oxidation. J.Lipid Res. 38: 805-812.

Dam, A. D., Mitchell, A. S., Rush, J. W. et al. (2012) Elevated skeletal muscle apoptotic signaling following glutathione depletion. Apoptosis. 17: 48-60.

Dang, Z. C. & Lowik, C. W. (2005) Removal of serum factors by charcoal treatment promotes adipogenesis via a MAPK-dependent pathway. Mol.Cell Biochem. 268: 159-167.

Dangardt, F., Osika, W., Chen, Y. et al. (2010) Omega-3 fatty acid supplementation improves vascular function and reduces inflammation in obese adolescents. Atherosclerosis 212: 580-585.

Davis, R. A., Engelhorn, S. C., Pangburn, S. H. et al. (1979) Very low density lipoprotein synthesis and secretion by cultured rat hepatocytes. J.Biol.Chem. 254: 2010-2016.

de Faria, A. N., Zancanela, D. C., Ramos, A. P. et al. (2015) Estrogen and phenol red free medium for osteoblast culture: study of the mineralization ability. Cytotechnology.

Dewailly, E., Blanchet, C., Gingras, S. et al. (2002) Cardiovascular disease risk factors and n-3 fatty acid status in the adult population of James Bay Cree. Am.J.Clin.Nutr. 76: 85-92.

Dewailly, E., Blanchet, C., Lemieux, S. et al. (2001) n-3 Fatty acids and cardiovascular disease risk factors among the Inuit of Nunavik. Am.J.Clin.Nutr. 74: 464-473.

Dimopoulos, N., Watson, M., Sakamoto, K. et al. (2006) Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. Biochem J. 399: 473-481.

Djousse, L., Matthan, N. R., Lichtenstein, A. H. et al. (2012a) Red Blood Cell Membrane Concentration of cis-Palmitoleic and cis-Vaccenic Acids and Risk of Coronary Heart Disease. Am.J.Cardiol.

Djousse, L., Matthan, N. R., Lichtenstein, A. H. et al. (2012b) Red blood cell membrane concentration of cis-palmitoleic and cis-vaccenic acids and risk of coronary heart disease. Am.J.Cardiol. 110: 539-544.

Domenichiello, A. F., Chen, C. T., Trepanier, M. O. et al. (2014) Whole body synthesis rates of DHA from alpha-linolenic acid are greater than brain DHA accretion and uptake rates in adult rats. J.Lipid Res. 55: 62-74.

Domenichiello, A. F., Kitson, A. P., & Bazinet, R. P. (2015) Is DHA synthesis from ALA sufficient to supply the adult brain? Prog.Lipid Res.

Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J. et al. (2005) Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J.Clin.Invest 115: 1343-1351.

Eldrup, E., Lindholm, J., & Winkel, P. (1987) Plasma sex hormones and ischemic heart disease. Clin.Biochem. 20: 105-112.

Extier, A., Langelier, B., Perruchot, M. H. et al. (2010) Gender affects liver desaturase expression in a rat model of n-3 fatty acid repletion. J.Nutr.Biochem 21: 180-187.

Farkas, T., Kitajka, K., Fodor, E. et al. (2000) Docosahexaenoic acid-containing phospholipid molecular species in brains of vertebrates. Proc.Natl.Acad.Sci.U.S.A 97: 6362-6366.

Fehily, A. M., Dickerson, J. W., Meade, B. W. et al. (1982) Plasma and erythrocyte membrane fatty acids in oral contraceptive users. Clin.Chim.Acta 120: 41-47.

Fermor, B. F., Masters, J. R., Wood, C. B. et al. (1992) Fatty acid composition of normal and malignant cells and cytotoxicity of stearic, oleic and sterculic acids in vitro. Eur.J.Cancer 28A: 1143-1147.

Fitzpatrick, F. A. & Soberman, R. (2001) Regulated formation of eicosanoids. J.Clin.Invest 107: 1347-1351.

Flock, M. R., Skulas-Ray, A. C., Harris, W. S. et al. (2014) Effects of supplemental long-chain omega-3 fatty acids and erythrocyte membrane fatty acid content on circulating inflammatory markers in a randomized controlled trial of healthy adults. Prostaglandins Leukot.Essent.Fatty Acids 91: 161-168.

Flores, A., Velasco, J., Gallegos, A. I. et al. (2011) Acute effects of unilateral sectioning the superior ovarian nerve of rats with unilateral ovariectomy on ovarian hormones (progesterone, testosterone and estradiol) levels vary during the estrous cycle. Reprod.Biol.Endocrinol. 9: 34.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. J.Biol.Chem. 226: 497-509.

Frasor, J., Danes, J. M., Komm, B. et al. (2003) Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144: 4562-4574.

Fuhrman, B. J., Xu, X., Falk, R. T. et al. (2014) Assay reproducibility and interindividual variation for 15 serum estrogens and estrogen metabolites measured by liquid chromatography-tandem mass spectrometry. Cancer Epidemiol.Biomarkers Prev. 23: 2649-2657.

Fujii, M., Nakashima, H., Tomozawa, J. et al. (2013) Deficiency of n-6 polyunsaturated fatty acids is mainly responsible for atopic dermatitis-like pruritic skin inflammation in special diet-fed hairless mice. Exp.Dermatol. 22: 272-277.

Garcia, M. C. & Kim, H. Y. (1997) Mobilization of arachidonate and docosahexaenoate by stimulation of the 5-HT2A receptor in rat C6 glioma cells. Brain Res. 768: 43-48.

Garriguet, D. (2007) Canadians' eating habits. Health Rep. 18: 17-32.

Ghebremeskel, K., Min, Y., Crawford, M. A. et al. (2000) Blood fatty acid composition of pregnant and nonpregnant Korean women: red cells may act as a reservoir of arachidonic acid and docosahexaenoic acid for utilization by the developing fetus. Lipids 35: 567-574.

Gibson, R. A., Neumann, M. A., Lien, E. L. et al. (2013) Docosahexaenoic acid synthesis from alpha-linolenic acid is inhibited by diets high in polyunsaturated fatty acids. Prostaglandins Leukot.Essent.Fatty Acids 88: 139-146.

Giltay, E. J., Gooren, L. J., Toorians, A. W. et al. (2004) Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. Am.J.Clin.Nutr. 80: 1167-1174.

GISSI-Prevenzione Investigators (1999) Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Lancet 354: 447-455.

Goyens, P. L., Spilker, M. E., Zock, P. L. et al. (2005) Compartmental modeling to quantify alpha-linolenic acid conversion after longer term intake of multiple tracer boluses. J.Lipid Res. 46: 1474-1483.

Graham-Lorence, S., Khalil, M. W., Lorence, M. C. et al. (1991) Structure-function relationships of human aromatase cytochrome P-450 using molecular modeling and site-directed mutagenesis. J.Biol.Chem. 266: 11939-11946.

Grammatikos, S. I., Subbaiah, P. V., Victor, T. A. et al. (1994) n-3 and n-6 fatty acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial cell lines. Br.J.Cancer 70: 219-227.

Granfone, A., Campos, H., McNamara, J. R. et al. (1992) Effects of estrogen replacement on plasma lipoproteins and apolipoproteins in postmenopausal, dyslipidemic women. Metabolism 41: 1193-1198.

Green, C. D., Ozguden-Akkoc, C. G., Wang, Y. et al. (2010) Role of fatty acid elongases in determination of de novo synthesized monounsaturated fatty acid species. J.Lipid Res 51: 1871-1877.

Gregory, M. K., Gibson, R. A., Cook-Johnson, R. J. et al. (2011) Elongase reactions as control points in long-chain polyunsaturated fatty acid synthesis. PLoS.One. 6: e29662.

Griffin, M. E., Marcucci, M. J., Cline, G. W. et al. (1999) Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. Diabetes 48: 1270-1274.

Gruber, C. J., Gruber, D. M., Gruber, I. M. et al. (2004) Anatomy of the estrogen response element. Trends Endocrinol.Metab 15: 73-78.

Guillou, H., D'Andrea, S., Rioux, V. et al. (2004) Distinct roles of endoplasmic reticulum cytochrome b5 and fused cytochrome b5-like domain for rat Delta6-desaturase activity. J.Lipid Res. 45: 32-40.

Gutendorf, B. & Westendorf, J. (2001) Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 166: 79-89.

Hall, A. M., Smith, A. J., & Bernlohr, D. A. (2003) Characterization of the Acyl-CoA synthetase activity of purified murine fatty acid transport protein 1. J.Biol.Chem. 278: 43008-43013.

Hanke, S. & Mann, M. (2009) The phosphotyrosine interactome of the insulin receptor family and its substrates IRS-1 and IRS-2. Mol.Cell Proteomics. 8: 519-534.

Harnack, K., Andersen, G., & Somoza, V. (2009) Quantitation of alpha-linolenic acid elongation to eicosapentaenoic and docosahexaenoic acid as affected by the ratio of n6/n3 fatty acids. Nutr.Metab (Lond) 6: 8.

Harris, W. S. & Von, S. C. (2004) The Omega-3 Index: a new risk factor for death from coronary heart disease? Prev.Med. 39: 212-220.

Heiling, V. J. & Jensen, M. D. (1992) Free fatty acid metabolism in the follicular and luteal phases of the menstrual cycle. J.Clin.Endocrinol.Metab 74: 806-810.

Hewitt, K. N., Pratis, K., Jones, M. E. et al. (2004) Estrogen replacement reverses the hepatic steatosis phenotype in the male aromatase knockout mouse. Endocrinology 145: 1842-1848.

Holman, R. T., Johnson, S. B., Mercuri, O. et al. (1981) Essential fatty acid deficiency in malnourished children. Am.J.Clin.Nutr. 34: 1534-1539.

Hong, S. & Lu, Y. (2013) Omega-3 fatty acid-derived resolvins and protectins in inflammation resolution and leukocyte functions: targeting novel lipid mediator pathways in mitigation of acute kidney injury. Front Immunol. 4: 13.

Horwitz, K. B., Costlow, M. E., & McGuire, W. L. (1975) MCF-7; a human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors. Steroids 26: 785-795.

Huang, S., Rutkowsky, J. M., Snodgrass, R. G. et al. (2012) Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. J.Lipid Res. 53: 2002-2013.

Hubscher, C. H., Brooks, D. L., & Johnson, J. R. (2005) A quantitative method for assessing stages of the rat estrous cycle. Biotech.Histochem. 80: 79-87.

Hudgins, L. C., Hellerstein, M., Seidman, C. et al. (1996) Human fatty acid synthesis is stimulated by a eucaloric low fat, high carbohydrate diet. J.Clin.Invest 97: 2081-2091.

Jakobsen, M. U., O'Reilly, E. J., Heitmann, B. L. et al. (2009) Major types of dietary fat and risk of coronary heart disease: a pooled analysis of 11 cohort studies. Am.J.Clin.Nutr. 89: 1425-1432.

Jaudszus, A., Degen, C., Barth, S. W. et al. (2014) Loss of FADS2 function severely impairs the use of HeLa cells as an in vitro model for host response studies involving fatty acid effects. PLoS.One. 9: e115610.

Jones, M. E., Thorburn, A. W., Britt, K. L. et al. (2000) Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. Proc.Natl.Acad.Sci.U.S.A 97: 12735-12740.

Kalthoff, S., Winkler, A., Freiberg, N. et al. (2013) Gender matters: estrogen receptor alpha (ERalpha) and histone deacetylase (HDAC) 1 and 2 control the gender-specific transcriptional

regulation of human uridine diphosphate glucuronosyltransferases genes (UGT1A). J.Hepatol. 59: 797-804.

Kapp, N. (2009) WHO provider brief on hormonal contraception and liver disease. Contraception 80: 325-326.

Karpe, F., Olivecrona, T., Olivecrona, G. et al. (1998) Lipoprotein lipase transport in plasma: role of muscle and adipose tissues in regulation of plasma lipoprotein lipase concentrations. J.Lipid Res. 39: 2387-2393.

Katan, M. B., Deslypere, J. P., van Birgelen, A. P. et al. (1997) Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: an 18-month controlled study. J.Lipid Res 38: 2012-2022.

Kaur, G., Cameron-Smith, D., Garg, M. et al. (2011) Docosapentaenoic acid (22:5n-3): a review of its biological effects. Prog.Lipid Res. 50: 28-34.

Kaur, G. & Dufour, J. M. (2012) Cell lines: Valuable tools or useless artifacts. Spermatogenesis. 2: 1-5.

Kelly, L. A., Seidlova-Wuttke, D., Wuttke, W. et al. (2014) Estrogen receptor alpha augments changes in hemostatic gene expression in HepG2 cells treated with estradiol and phytoestrogens. Phytomedicine. 21: 155-158.

Khan, S. A., Ali, A., Khan, S. A. et al. (2014) Unraveling the complex relationship triad between lipids, obesity, and inflammation. Mediators.Inflamm. 2014: 502749.

Kiens, B., Kristiansen, S., Jensen, P. et al. (1997) Membrane associated fatty acid binding protein (FABPpm) in human skeletal muscle is increased by endurance training. Biochem.Biophys.Res.Commun. 231: 463-465.

Kim, H. J. & Kalkhoff, R. K. (1979) Changes in lipoprotein composition during the menstrual cycle. Metabolism 28: 663-668.

Kim, K. H., Kim, J. Y., Kwak, J. H. et al. (2015) Different anticancer effects of Saxifragifolin A on estrogen receptor-positive and estrogen receptor-negative breast cancer cells. Phytomedicine. 22: 820-828.

Kim, S., Jin, Y., & Park, Y. (2015) Estrogen and n-3 polyunsaturated fatty acid supplementation have a synergistic hypotriglyceridemic effect in ovariectomized rats. Genes Nutr. 10: 475.

Kim, T. T. & Dyck, J. R. (2016) The role of CD36 in the regulation of myocardial lipid metabolism. Biochim.Biophys.Acta.

Kitson, A. P. Characterization of the effects of sex, pregnancy, and 17ß-estradiol on docosahexaenoic acid biosynthesis. PhD Thesis . 2013.
Ref Type: Generic

Kitson, A. P., Marks, K. A., Aristizabal Henao, J. J. et al. (2015) Prevention of hyperphagia prevents ovariectomy-induced triacylglycerol accumulation in liver, but not plasma. Nutr.Res. 35: 1085-1094.

Kitson, A. P., Marks, K. A., Shaw, B. et al. (2013a) Treatment of ovariectomized rats with 17beta-estradiol increases hepatic delta-6 desaturase enzyme expression and docosahexaenoic acid levels in hepatic and plasma phospholipids. Prostaglandins Leukot.Essent.Fatty Acids Accepted.

Kitson, A. P., Marks, K. A., Shaw, B. et al. (2013b) Treatment of ovariectomized rats with 17beta-estradiol increases hepatic delta-6 desaturase enzyme expression and docosahexaenoic acid levels in hepatic and plasma phospholipids. Prostaglandins Leukot.Essent.Fatty Acids 89: 81-88.

Kitson, A. P., Smith, T. L., Marks, K. A. et al. (2012) Tissue-specific sex differences in docosahexaenoic acid and Delta6-desaturase in rats fed a standard chow diet. Appl.Physiol Nutr.Metab 37: 1200-1211.

Kitson, A. P., Stroud, C. K., & Stark, K. D. (2010) Elevated production of docosahexaenoic acid in females: potential molecular mechanisms. Lipids 45: 209-224.

Klein-Platat, C., Drai, J., Oujaa, M. et al. (2005) Plasma fatty acid composition is associated with the metabolic syndrome and low-grade inflammation in overweight adolescents. Am.J.Clin.Nutr. 82: 1178-1184.

Koletzko, B., Abiodun, P. O., Laryea, M. D. et al. (1986) Fatty acid composition of plasma lipids in Nigerian children with protein-energy malnutrition. Eur.J.Pediatr. 145: 109-115.

Koletzko, B., Cetin, I., & Brenna, J. T. (2007) Dietary fat intakes for pregnant and lactating women. Br.J.Nutr. 98: 873-877.

Kondreddy, V. K., Anikisetty, M., & Naidu, K. A. (2016) Medium-chain triglycerides and monounsaturated fatty acids potentiate the beneficial effects of fish oil on selected cardiovascular risk factors in rats. J.Nutr.Biochem. 28: 91-102.

Kornsteiner, M., Singer, I., & Elmadfa, I. (2008) Very low n-3 long-chain polyunsaturated fatty acid status in Austrian vegetarians and vegans. Ann.Nutr.Metab 52: 37-47.

Kuiper, G. G., Carlsson, B., Grandien, K. et al. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138: 863-870.

Kuipers, R. S., Luxwolda, M. F., Sango, W. S. et al. (2011) Postdelivery changes in maternal and infant erythrocyte fatty acids in 3 populations differing in fresh water fish intakes. Prostaglandins Leukotrienes and Essential Fatty Acids 85: 387-397.

Lands, B. (2014) Historical perspectives on the impact of n-3 and n-6 nutrients on health. Prog.Lipid Res. 55: 17-29.

Lauritzen, L., Brambilla, P., Mazzocchi, A. et al. (2016) DHA Effects in Brain Development and Function. Nutrients. 8.

Le, C. H., Mulligan, C. M., Routh, M. A. et al. (2014) Delta-6-desaturase links polyunsaturated fatty acid metabolism with phospholipid remodeling and disease progression in heart failure. Circ.Heart Fail. 7: 172-183.

Leichsenring, M., Sutterlin, N., Less, S. et al. (1995) Polyunsaturated fatty acids in erythrocyte and plasma lipids of children with severe protein-energy malnutrition. Acta Paediatr. 84: 516-520.

Lemaitre, R. N., Tanaka, T., Tang, W. et al. (2011) Genetic loci associated with plasma phospholipid n-3 fatty acids: a meta-analysis of genome-wide association studies from the CHARGE Consortium. PLoS.Genet. 7: e1002193.

Li, S., Yuan, Y., Wang, T. et al. (2016) Molecular Cloning, Functional Characterization and Nutritional Regulation of the Putative Elongase Elovl5 in the Orange-Spotted Grouper (Epinephelus coioides). PLoS.One. 11: e0150544.

Li, X. & Franke, A. A. (2015) Improved profiling of estrogen metabolites by orbitrap LC/MS. Steroids 99: 84-90.

Lionetti, L., Mollica, M. P., Crescenzo, R. et al. (2007) Skeletal muscle subsarcolemmal mitochondrial dysfunction in high-fat fed rats exhibiting impaired glucose homeostasis. Int.J.Obes.(Lond) 31: 1596-1604.

Littleton, J. M., John, G. R., & Grieve, S. J. (1979) Alterations in phospholipid composition in ethanol tolerance and dependence. Alcohol Clin.Exp.Res. 3: 50-56.

Liu, X., Burhans, M. S., Flowers, M. T. et al. (2016) Hepatic oleate regulates liver stress response partially through PGC-1alpha during high-carbohydrate feeding. J.Hepatol.

Luchtman, D. W. & Song, C. (2013) Cognitive enhancement by omega-3 fatty acids from child-hood to old age: findings from animal and clinical studies. Neuropharmacology 64: 550-565.

Ma, W., Wu, J. H., Wang, Q. et al. (2015) Prospective association of fatty acids in the de novo lipogenesis pathway with risk of type 2 diabetes: the Cardiovascular Health Study. Am.J.Clin.Nutr. 101: 153-163.

Magnusardottir, A. R., Steingrimsdottir, L., Thorgeirsdottir, H. et al. (2009) Docosahexaenoic acid in red blood cells of women of reproductive age is positively associated with oral contraceptive use and physical activity. Prostaglandins Leukot.Essent.Fatty Acids 80: 27-32.

Maier, T., Guell, M., & Serrano, L. (2009) Correlation of mRNA and protein in complex biological samples. FEBS Lett. 583: 3966-3973.

Man, W. C., Miyazaki, M., Chu, K. et al. (2006) Colocalization of SCD1 and DGAT2: implying preference for endogenous monounsaturated fatty acids in triglyceride synthesis. J.Lipid Res 47: 1928-1939.

Marangoni, F., Colombo, C., Martiello, A. et al. (2007) The fatty acid profiles in a drop of blood from a fingertip correlate with physiological, dietary and lifestyle parameters in volunteers. Prostaglandins Leukot.Essent.Fatty Acids 76: 87-92.

Marks, K. A., Kitson, A. P., Shaw, B. et al. (2013a) Stearoyl-CoA desaturase 1, elongase 6 and their fatty acid products and precursors are altered in ovariectomized rats with 17beta-estradiol and progesterone treatment. Prostaglandins Leukot.Essent.Fatty Acids Accepted.

Marks, K. A., Kitson, A. P., Shaw, B. et al. (2013b) Stearoyl-CoA desaturase 1, elongase 6 and their fatty acid products and precursors are altered in ovariectomized rats with 17beta-estradiol and progesterone treatment. Prostaglandins Leukot.Essent.Fatty Acids 89: 89-96.

Marks, K. A., Kitson, A. P., & Stark, K. D. (2013) Hepatic and plasma sex differences in saturated and monounsaturated fatty acids are associated with differences in expression of elongase 6, but not stearoyl-CoA desaturase in Sprague-Dawley rats. Genes Nutr. 8: 317-327.

Marks, K. A., Marvyn, P. M., Henao, J. J. et al. (2015) Fasting enriches liver triacylglycerol with n-3 polyunsaturated fatty acids: implications for understanding the adipose-liver axis in serum docosahexaenoic acid regulation. Genes Nutr. 10: 39.

Marszalek, J. R., Kitidis, C., Dirusso, C. C. et al. (2005) Long-chain acyl-CoA synthetase 6 preferentially promotes DHA metabolism. J.Biol.Chem. 280: 10817-10826.

Mason, J. K., Kharotia, S., Wiggins, A. K. et al. (2014) 17beta-estradiol increases liver and serum docosahexaenoic acid in mice fed varying levels of alpha-linolenic acid. Lipids 49: 745-756.

Matsuzaka, T., Shimano, H., Yahagi, N. et al. (2007) Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. Nat.Med. 13: 1193-1202.

Mattsson, L. A., Silfverstolpe, G., & Samsioe, G. (1985) Fatty acid composition of serum lecithin and cholesterol ester in the normal menstrual cycle. Horm.Metab Res. 17: 414-417.

McClintock, M. K. (1978) Estrous synchrony and its mediation by airborne chemical communication (Rattus norvegicus). Horm.Behav. 10: 264-275.

McCloy, U., Ryan, M. A., Pencharz, P. B. et al. (2004) A comparison of the metabolism of eighteen-carbon 13C-unsaturated fatty acids in healthy women. J.Lipid Res. 45: 474-485.

McElvaine, A. T. & Mayo, K. E. (2006) A dominant-negative human growth hormone-releasing hormone (GHRH) receptor splice variant inhibits GHRH binding. Endocrinology 147: 1884-1894.

McNamara, R. K. & Carlson, S. E. (2006) Role of omega-3 fatty acids in brain development and function: potential implications for the pathogenesis and prevention of psychopathology. Prostaglandins Leukot.Essent.Fatty Acids 75: 329-349.

Melchert, H. U., Limsathayourat, N., Mihajlovic, H. et al. (1987) Fatty acid patterns in triglycerides, diglycerides, free fatty acids, cholesteryl esters and phosphatidylcholine in serum from vegetarians and non-vegetarians. Atherosclerosis 65: 159-166.

Melin, T. & Nilsson, A. (1997) Delta-6-desaturase and delta-5-desaturase in human Hep G2 cells are both fatty acid interconversion rate limiting and are upregulated under essential fatty acid deficient conditions. Prostaglandins Leukot.Essent.Fatty Acids 56: 437-442.

Mensink, R. P., Zock, P. L., Kester, A. D. et al. (2003) Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. Am.J.Clin.Nutr. 77: 1146-1155.

Metherel, A. H., Aristizabal Henao, J. J., & Stark, K. D. (2013) EPA and DHA levels in whole blood decrease more rapidly when stored at -20 degrees C as compared with room temperature, 4 and -75 degrees C. Lipids 48: 1079-1091.

Metherel, A. H., Armstrong, J. M., Patterson, A. C. et al. (2009) Assessment of blood measures of n-3 polyunsaturated fatty acids with acute fish oil supplementation and washout in men and women. Prostaglandins Leukot.Essent.Fatty Acids 81: 23-29.

Meyer, B. J., Stewart, F. M., Brown, E. A. et al. (2013) Maternal obesity is associated with the formation of small dense LDL and hypoadiponectinemia in the third trimester. J.Clin.Endocrinol.Metab 98: 643-652.

Miles, E. A., Noakes, P. S., Kremmyda, L. S. et al. (2011) The Salmon in Pregnancy Study: study design, subject characteristics, maternal fish and marine n-3 fatty acid intake, and marine n-3 fatty acid status in maternal and umbilical cord blood. Am.J.Clin.Nutr. 94: 1986S-1992S.

Misso, M. L., Murata, Y., Boon, W. C. et al. (2003) Cellular and molecular characterization of the adipose phenotype of the aromatase-deficient mouse. Endocrinology 144: 1474-1480.

Miyazaki, M., Bruggink, S. M., & Ntambi, J. M. (2006) Identification of mouse palmitoyl-coenzyme A Delta9-desaturase. J.Lipid Res 47: 700-704.

Morrison, W. R. & Smith, L. M. (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J.Lipid Res. 4: 600-608.

Motomura, T., Hashimoto, K., Koga, M. et al. (1998) Inhibition of signal transduction by a splice variant of the growth hormone-releasing hormone receptor expressed in human pituitary adenomas. Metabolism 47: 804-808.

Mozaffarian, D. & Rimm, E. B. (2006) Fish intake, contaminants, and human health: evaluating the risks and the benefits. JAMA 296: 1885-1899.

Mozaffarian, D., Rimm, E. B., & Herrington, D. M. (2004) Dietary fats, carbohydrate, and progression of coronary atherosclerosis in postmenopausal women. Am.J.Clin.Nutr. 80: 1175-1184.

Musa-Veloso, K., Binns, M. A., Kocenas, A. et al. (2011) Impact of low v. moderate intakes of long-chain n-3 fatty acids on risk of coronary heart disease. Br.J.Nutr. 106: 1129-1141.

Nakamura, M. T. & Nara, T. Y. (2004) Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. Annu.Rev.Nutr. 24: 345-376.

Nakamura, M. T., Tang, A. B., Villanueva, J. et al. (1994) Selective reduction of delta 6 and delta 5 desaturase activities but not delta 9 desaturase in micropigs chronically fed ethanol. J.Clin.Invest 93: 450-454.

Nappi, R. E., Kaunitz, A. M., & Bitzer, J. (2016) Extended regimen combined oral contraception: A review of evolving concepts and acceptance by women and clinicians. Eur.J.Contracept.Reprod.Health Care 21: 106-115.

Nguyen, L. N., Ma, D., Shui, G. et al. (2014) Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. Nature 509: 503-506.

Ntambi, J. M., Miyazaki, M., Stoehr, J. P. et al. (2002) Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. Proc.Natl.Acad.Sci.U.S.A 99: 11482-11486.

Ogura, T., Takada, H., Okuno, M. et al. (2010) Fatty acid composition of plasma, erythrocytes and adipose: their correlations and effects of age and sex. Lipids 45: 137-144.

Oh, Y., Jin, Y., & Park, Y. (2015) Synergic hypocholesterolaemic effect of n-3 PUFA and oestrogen by modulation of hepatic cholesterol metabolism in female rats. Br.J.Nutr. 114: 1766-1773.

Otto, S. J., van Houwelingen, A. C., Badart-Smook, A. et al. (2001) Comparison of the peripartum and postpartum phospholipid polyunsaturated fatty acid profiles of lactating and nonlactating women. Am.J.Clin.Nutr. 73: 1074-1079.

Ottosson, U. B., Lagrelius, A., Rosing, U. et al. (1984) Relative fatty acid composition of lecithin during postmenopausal replacement therapy--a comparison between ethinyl estradiol and estradiol valerate. Gynecol.Obstet.Invest 18: 296-302.

Pahwa, M. B., Seth, S., & Seth, R. K. (1998) Lipid profile in various phases of menstrual cycle and its relationship with percentage plasma volume changes. Clin.Chim.Acta 273: 201-207.

Paquette, A., Wang, D., Jankowski, M. et al. (2008) Effects of ovariectomy on PPAR alpha, SREBP-1c, and SCD-1 gene expression in the rat liver. Menopause. 15: 1169-1175.

Park, H. G., Park, W. J., Kothapalli, K. S. et al. (2015) The fatty acid desaturase 2 (FADS2) gene product catalyzes Delta4 desaturation to yield n-3 docosahexaenoic acid and n-6 docosapentaenoic acid in human cells. FASEB J. 29: 3911-3919.

Park, W. J., Kothapalli, K. S., Lawrence, P. et al. (2011) FADS2 function loss at the cancer hotspot 11q13 locus diverts lipid signaling precursor synthesis to unusual eicosanoid fatty acids. PLoS.One. 6: e28186.

Patterson, A. C., Chalil, A., Aristizabal Henao, J. J. et al. (2015) Omega-3 polyunsaturated fatty acid blood biomarkers increase linearly in men and women after tightly controlled intakes of 0.25, 0.5, and 1 g/d of EPA + DHA. Nutr.Res. 35: 1040-1051.

Patterson, A. C., Hogg, R. C., Kishi, D. M. et al. (2012) Biomarker and dietary validation of a Canadian food frequency questionnaire to measure eicosapentaenoic and docosahexaenoic acid intakes from whole food, functional food, and nutraceutical sources. J.Acad.Nutr.Diet. 112: 1005-1014.

Patterson, A. C., Metherel, A. H., Hanning, R. M. et al. (2014) The percentage of DHA in erythrocytes can detect non-adherence to advice to increase EPA and DHA intakes. Br.J.Nutr. 111: 270-278.

Pauter, A. M., Olsson, P., Asadi, A. et al. (2014) Elovl2 ablation demonstrates that systemic DHA is endogenously produced and is essential for lipid homeostasis in mice. J.Lipid Res. 55: 718-728.

Pavlovic, Z., Singh, R. K., & Bakovic, M. (2014) A novel murine CTP:phosphoethanolamine cytidylyltransferase splice variant is a post-translational repressor and an indicator that both cytidylyltransferase domains are required for activity. Gene 543: 58-68.

Pawlosky, R., Hibbeln, J., Lin, Y. et al. (2003a) n-3 fatty acid metabolism in women. Br.J.Nutr. 90: 993-994.

Pawlosky, R. J., Hibbeln, J. R., Lin, Y. et al. (2003b) Effects of beef- and fish-based diets on the kinetics of n-3 fatty acid metabolism in human subjects. Am.J.Clin.Nutr. 77: 565-572.

Pawlosky, R. J. & Salem, N., Jr. (1995) Ethanol exposure causes a decrease in docosahexaenoic acid and an increase in docosapentaenoic acid in feline brains and retinas. Am.J.Clin.Nutr. 61: 1284-1289.

Pawlosky, R. J. & Salem, N., Jr. (1999) Alcohol consumption in rhesus monkeys depletes tissues of polyunsaturated fatty acids and alters essential fatty acid metabolism. Alcohol Clin.Exp.Res. 23: 311-317.

Perdomo, L., Beneit, N., Otero, Y. F. et al. (2015) Protective role of oleic acid against cardiovascular insulin resistance and in the early and late cellular atherosclerotic process. Cardiovasc.Diabetol. 14: 75.

Peter, A., Weigert, C., Staiger, H. et al. (2009) Individual stearoyl-coa desaturase 1 expression modulates endoplasmic reticulum stress and inflammation in human myotubes and is associated with skeletal muscle lipid storage and insulin sensitivity in vivo. Diabetes 58: 1757-1765.

Petridou, A., Nikolaidis, M. G., Matsakas, A. et al. (2005) Effect of exercise training on the fatty acid composition of lipid classes in rat liver, skeletal muscle, and adipose tissue. Eur.J.Appl.Physiol 94: 84-92.

Pillon, N. J., Azizi, P. M., Li, Y. E. et al. (2015) Palmitate-induced inflammatory pathways in human adipose microvascular endothelial cells promote monocyte adhesion and impair insulin transcytosis. Am.J.Physiol Endocrinol.Metab 309: E35-E44.

Polozova, A. & Salem, N., Jr. (2007) Role of liver and plasma lipoproteins in selective transport of n-3 fatty acids to tissues: a comparative study of 14C-DHA and 3H-oleic acid tracers. J.Mol.Neurosci. 33: 56-66.

Postle, A. D., Al, M. D., Burdge, G. C. et al. (1995) The composition of individual molecular species of plasma phosphatidylcholine in human pregnancy. Early Hum.Dev. 43: 47-58.

Puri, P., Wiest, M. M., Cheung, O. et al. (2009) The plasma lipidomic signature of nonalcoholic steatohepatitis. Hepatology 50: 1827-1838.

Raclot, T. (2003) Selective mobilization of fatty acids from adipose tissue triacylglycerols. Prog.Lipid Res. 42: 257-288.

Radding, CM and Steinberg, D. Studies on the synthesis and secretion of lipoproteins by rat liver slices. Journal of Clinical Investigation 39(10), 1560-1569. 1960.
Ref Type: Generic

Ramos, M. P., Crespo-Solans, M. D., del, C. S. et al. (2003) Fat accumulation in the rat during early pregnancy is modulated by enhanced insulin responsiveness. Am.J.Physiol Endocrinol.Metab 285: E318-E328.

Ramsden, C. E., Zamora, D., Leelarthaepin, B. et al. (2013) Use of dietary linoleic acid for secondary prevention of coronary heart disease and death: evaluation of recovered data from the Sydney Diet Heart Study and updated meta-analysis. BMJ 346: e8707.

Ranganath, L. R., Christofides, J. A., Wright, J. W. et al. (1996) Effects of hormone replacement therapy on platelet membrane fatty acid composition. J.Endocrinol. 148: 207-212.

Reed, C. F., Swisher, S. N., Marinetti, G. V. et al. (1960) Studies of the lipids of the erythrocyte. I. Quantitative analysis of the lipids of normal human red blood cells. J.Lab Clin.Med. 56: 281-289.

Rice, P., Longden, I., & Bleasby, A. (2000) EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet. 16: 276-277.

Rodriguez-Cruz, M., Sanchez, R., Sanchez, A. M. et al. (2011) Participation of mammary gland in long-chain polyunsaturated fatty acid synthesis during pregnancy and lactation in rats. Biochim.Biophys.Acta 1811: 284-293.

Roh, T., Kwak, M. Y., Kwak, E. H. et al. (2012) Chemopreventive mechanisms of methionine on inhibition of benzo(a)pyrene-DNA adducts formation in human hepatocellular carcinoma HepG2 cells. Toxicol.Lett. 208: 232-238.

Roke, K. & Mutch, D. M. (2014) The role of FADS1/2 polymorphisms on cardiometabolic markers and fatty acid profiles in young adults consuming fish oil supplements. Nutrients. 6: 2290-2304.

Rosell, M. S., Lloyd-Wright, Z., Appleby, P. N. et al. (2005) Long-chain n-3 polyunsaturated fatty acids in plasma in British meat-eating, vegetarian, and vegan men. Am.J.Clin.Nutr. 82: 327-334.

Rotstein, N. P., Politi, L. E., German, O. L. et al. (2003) Protective effect of docosahexaenoic acid on oxidative stress-induced apoptosis of retina photoreceptors. Invest Ophthalmol.Vis.Sci. 44: 2252-2259.

Roy, S., Kale, A., Dangat, K. et al. (2012) Maternal micronutrients (folic acid and vitamin B(12)) and omega 3 fatty acids: implications for neurodevelopmental risk in the rat offspring. Brain Dev. 34: 64-71.

Rui, L. (2014) Energy metabolism in the liver. Compr.Physiol 4: 177-197.

Safe, S. & Kim, K. (2004) Nuclear receptor-mediated transactivation through interaction with Sp proteins. Prog.Nucleic Acid Res.Mol.Biol. 77: 1-36.

Safe, S. & Kim, K. (2008) Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. J.Mol.Endocrinol. 41: 263-275.

Salem, N., Jr., Serpentino, P., Puskin, J. S. et al. (1980) Preparation and spectroscopic characterization of molecular species of brain phosphatidylserines. Chem.Phys.Lipids 27: 289-304.

Samuel, V. T., Liu, Z. X., Wang, A. et al. (2007) Inhibition of protein kinase Cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. J.Clin.Invest 117: 739-745.

Sanders, T. A., Ellis, F. R., & Dickerson, J. W. (1978) Studies of vegans: the fatty acid composition of plasma choline phosphoglycerides, erythrocytes, adipose tissue, and breast milk, and some indicators of susceptibility to ischemic heart disease in vegans and omnivore controls. Am.J.Clin.Nutr. 31: 805-813.

Sayanova, O., Shewry, P. R., & Napier, J. A. (1999) Histidine-41 of the cytochrome b5 domain of the borage delta6 fatty acid desaturase is essential for enzyme activity. Plant Physiol 121: 641-646.

Scaglia, N., Chatkin, J., Chapman, K. R. et al. (2016) The relationship between omega-3 and smoking habit: a cross-sectional study. Lipids Health Dis. 15: 61.

Schaefer, E. J., Foster, D. M., Zech, L. A. et al. (1983) The effects of estrogen administration on plasma lipoprotein metabolism in premenopausal females. J.Clin.Endocrinol.Metab 57: 262-267.

Schiller, H. & Bensch, K. (1971) De novo fatty acid synthesis and elongation of fatty acids by subcellular fractions of lung. J.Lipid Res 12: 248-255.

Schisterman, E. F., Mumford, S. L., & Sjaarda, L. A. (2014) Failure to consider the menstrual cycle phase may cause misinterpretation of clinical and research findings of cardiometabolic biomarkers in premenopausal women. Epidemiol.Rev. 36: 71-82.

Sibbons, C. M., Brenna, J. T., Lawrence, P. et al. (2014) Effect of sex hormones on n-3 polyunsaturated fatty acid biosynthesis in HepG2 cells and in human primary hepatocytes. Prostaglandins Leukot.Essent.Fatty Acids 90: 47-54.

Simoncini, T., Fornari, L., Mannella, P. et al. (2005) Activation of nitric oxide synthesis in human endothelial cells by red clover extracts. Menopause. 12: 69-77.

Simopoulos, A. P. (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed.Pharmacother. 56: 365-379.

Simopoulos, A. P. (2008) The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp.Biol.Med.(Maywood.) 233: 674-688.

Simpson, E. R. (2003) Sources of Estrogen and Their Importance. Journal of Steroid Biochemistry and Molecular Biology 86: 225-230.

Sinchak, K. & Wagner, E. J. (2012) Estradiol signaling in the regulation of reproduction and energy balance. Front Neuroendocrinol. 33: 342-363.

Sinner, D. I., Kim, G. J., Henderson, G. C. et al. (2012) StearoylCoA desaturase-5: a novel regulator of neuronal cell proliferation and differentiation. PLoS.One. 7: e39787.

Siri-Tarino, P. W., Sun, Q., Hu, F. B. et al. (2010) Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease. Am.J.Clin.Nutr. 91: 535-546.

Smit, E. N., Dijkstra, J. M., Schnater, T. A. et al. (1997) Effects of malnutrition on the erythrocyte fatty acid composition and plasma vitamin E levels of Pakistani children. Acta Paediatr. 86: 690-695.

Smith, M. S., Freeman, M. E., & Neill, J. D. (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96: 219-226.

Smith, P. K., Krohn, R. I., Hermanson, G. T. et al. (1985) Measurement of protein using bicinchoninic acid. Anal.Biochem. 150: 76-85.

Soldin, O. P., Guo, T., Weiderpass, E. et al. (2005) Steroid hormone levels in pregnancy and 1 year postpartum using isotope dilution tandem mass spectrometry. Fertil.Steril. 84: 701-710.

Speizer, L. A., Watson, M. J., & Brunton, L. L. (1991) Differential effects of omega-3 fish oils on protein kinase activities in vitro. Am.J.Physiol 261: E109-E114.

Sramkova, M., Duskova, M., Vitku, J. et al. (2015) Levels of adipokines and some steroids during the menstrual cycle. Physiol Res. 64 Suppl 2: S147-S154.

Stark, K. D., Beblo, S., Murthy, M. et al. (2005) Comparison of bloodstream fatty acid composition from African-American women at gestation, delivery, and postpartum. J.Lipid Res 46: 516-525.

Stark, K. D. & Holub, B. J. (2004) Differential eicosapentaenoic acid elevations and altered cardiovascular disease risk factor responses after supplementation with docosahexaenoic acid in postmenopausal women receiving and not receiving hormone replacement therapy. Am.J.Clin.Nutr. 79: 765-773.

Stark, K. D., Lim, S. Y., & Salem, N., Jr. (2007) Artificial rearing with docosahexaenoic acid and n-6 docosapentaenoic acid alters rat tissue fatty acid composition. J.Lipid Res 48: 2471-2477.

Stark, K. D., Park, E. J., & Holub, B. J. (2003) Fatty acid composition of serum phospholipid of premenopausal women and postmenopausal women receiving and not receiving hormone replacement therapy. Menopause. 10: 448-455.

Stark, K. D., Park, E. J., Maines, V. A. et al. (2000) Effect of a fish-oil concentrate on serum lipids in postmenopausal women receiving and not receiving hormone replacement therapy in a placebo-controlled, double-blind trial. Am.J.Clin.Nutr. 72: 389-394.

Stark, K. D. & Patterson, A. C. (2012) EPA and DHA--protein, not fat is "where it's at"? Prostaglandins Leukot.Essent.Fatty Acids 87: 49-51.

Stark, K. D. & Salem, N., Jr. (2005) Fast gas chromatography for the identification of fatty acid methyl esters from mammalian samples. Lipid Technol. 17: 181-185.

Stoll, L. L. & Spector, A. A. (1984) Changes in serum influence the fatty acid composition of established cell lines. In Vitro 20: 732-738.

Storm, H., Thomsen, C., Pedersen, E. et al. (1997) Comparison of a carbohydrate-rich diet and diets rich in stearic or palmitic acid in NIDDM patients. Effects on lipids, glycemic control, and diurnal blood pressure. Diabetes Care 20: 1807-1813.

Strom, J. O., Theodorsson, A., Ingberg, E. et al. (2012) Ovariectomy and 17beta-estradiol replacement in rats and mice: a visual demonstration. J.Vis.Exp. e4013.

Sul, H. S. & Wang, D. (1998) Nutritional and hormonal regulation of enzymes in fat synthesis: studies of fatty acid synthase and mitochondrial glycerol-3-phosphate acyltransferase gene transcription. Annu.Rev.Nutr. 18: 331-351.

Sulak, P. J., Scow, R. D., Preece, C. et al. (2000) Hormone withdrawal symptoms in oral contraceptive users. Obstet.Gynecol. 95: 261-266.

Sumino, H., Ichikawa, S., Murakami, M. et al. (2003) Effects of hormone replacement therapy on circulating docosahexaenoic acid and eicosapentaenoic acid levels in postmenopausal women. Endocr.J. 50: 51-59.

Summers, L. K., Fielding, B. A., Bradshaw, H. A. et al. (2002) Substituting dietary saturated fat with polyunsaturated fat changes abdominal fat distribution and improves insulin sensitivity. Diabetologia 45: 369-377.

Svennerholm, L. (1968) Distribution and fatty acid composition of phosphoglycerides in normal human brain. J.Lipid Res. 9: 570-579.

Takkunen, M. J., de Mello, V. D., Schwab, U. S. et al. (2016) Gene-diet interaction of a common FADS1 variant with marine polyunsaturated fatty acids for fatty acid composition in plasma and erythrocytes among men. Mol.Nutr.Food Res. 60: 381-389.

Talahalli, R. R., Vallikannan, B., Sambaiah, K. et al. (2010) Lower efficacy in the utilization of dietary ALA as compared to preformed EPA + DHA on long chain n-3 PUFA levels in rats. Lipids 45: 799-808.

Tu, W. C., Cook-Johnson, R. J., James, M. J. et al. (2010) Omega-3 long chain fatty acid synthesis is regulated more by substrate levels than gene expression. Prostaglandins Leukot.Essent.Fatty Acids 83: 61-68.

Tucker, K. L., Hallfrisch, J., Qiao, N. et al. (2005) The combination of high fruit and vegetable and low saturated fat intakes is more protective against mortality in aging men than is either alone: the Baltimore Longitudinal Study of Aging. J.Nutr. 135: 556-561.

Turcotte, L. P., Kiens, B., & Richter, E. A. (1991) Saturation kinetics of palmitate uptake in perfused skeletal muscle. FEBS Lett. 279: 327-329.

Turcotte, L. P., Petry, C., Kiens, B. et al. (1998) Contraction-induced increase in Vmax of palmitate uptake and oxidation in perfused skeletal muscle. J.Appl.Physiol (1985.) 84: 1788-1794.

Turyn, J., Stojek, M., & Swierczynski, J. (2010) Up-regulation of stearoyl-CoA desaturase 1 and elongase 6 genes expression in rat lipogenic tissues by chronic food restriction and chronic food restriction/refeeding. Mol.Cell Biochem. 345: 181-188.

Tworek, C., Muti, P., Micheli, A. et al. (2000) Fatty acid composition of the red blood cell membrane in relation to menopausal status. Ann.Epidemiol. 10: 477.

Uauy, R., Hoffman, D. R., Peirano, P. et al. (2001) Essential fatty acids in visual and brain development. Lipids 36: 885-895.

Vajreswari, A., Narayanareddy, K., & Rao, P. S. (1990) Fatty acid composition of erythrocyte membrane lipid obtained from children suffering from kwashiorkor and marasmus. Metabolism 39: 779-782.

van den Elsen, L. W., Nusse, Y., Balvers, M. et al. (2013) n-3 Long-chain PUFA reduce allergy-related mediator release by human mast cells in vitro via inhibition of reactive oxygen species. Br.J.Nutr. 109: 1821-1831.

van Kuijk, F. J. & Buck, P. (1992) Fatty acid composition of the human macula and peripheral retina. Invest Ophthalmol.Vis.Sci. 33: 3493-3496.

Vlaardingerbroek, H. & Hornstra, G. (2004) Essential fatty acids in erythrocyte phospholipids during pregnancy and at delivery in mothers and their neonates: comparison with plasma phospholipids. Prostaglandins Leukotrienes and Essential Fatty Acids 71: 363-374.

Wade, G. N. (1975) Some effects of ovarian hormones on food intake and body weight in female rats. J.Comp Physiol Psychol. 88: 183-193.

Walsh, B. W., Schiff, I., Rosner, B. et al. (1991) Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. N.Engl.J.Med. 325: 1196-1204.

Wang, Y., Botolin, D., Christian, B. et al. (2005) Tissue-specific, nutritional, and developmental regulation of rat fatty acid elongases. J.Lipid Res 46: 706-715.

Warensjo, E., Ohrvall, M., & Vessby, B. (2006) Fatty acid composition and estimated desaturase activities are associated with obesity and lifestyle variables in men and women. Nutr.Metab Cardiovasc.Dis. 16: 128-136.

Wilson, J. R., Adler, N., & Le, B. B. (1965) The effects of intromission frequency on successful pregnancy in the female rat. Proc.Natl.Acad.Sci.U.S.A 53: 1392-1395.

Wolff, J. A., Margolis, S., Bujdoso-Wolff, K. et al. (1984) Plasma and red blood cell fatty acid composition in children with protein-calorie malnutrition. Pediatr.Res. 18: 162-167.

Wu, J. H., Lemaitre, R. N., Imamura, F. et al. (2011) Fatty acids in the de novo lipogenesis pathway and risk of coronary heart disease: the Cardiovascular Health Study. Am.J.Clin.Nutr. 94: 431-438.

Wu, J. H., Lemaitre, R. N., King, I. B. et al. (2014) Circulating omega-6 polyunsaturated fatty acids and total and cause-specific mortality: the Cardiovascular Health Study. Circulation 130: 1245-1253.

Wu, Z., Li, D., & Gou, K. (2010) Overexpression of stearoyl-CoA desaturase-1 results in an increase of conjugated linoleic acid (CLA) and n-7 fatty acids in 293 cells. Biochem.Biophys.Res.Commun. 398: 473-476.

Xiao, Y. F., Gomez, A. M., Morgan, J. P. et al. (1997) Suppression of voltage-gated L-type Ca2+ currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. Proc.Natl.Acad.Sci.U.S.A 94: 4182-4187.

Yang, Y., Morin, P. J., Han, W. F. et al. (2003) Regulation of fatty acid synthase expression in breast cancer by sterol regulatory element binding protein-1c. Exp.Cell Res. 282: 132-137.

Yen, C. L., Stone, S. J., Koliwad, S. et al. (2008) Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. J.Lipid Res 49: 2283-2301.

Yin, W., Carballo-Jane, E., McLaren, D. G. et al. (2012) Plasma lipid profiling across species for the identification of optimal animal models of human dyslipidemia. J.Lipid Res. 53: 51-65.

Yu, C., Chen, Y., Cline, G. W. et al. (2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J.Biol.Chem. 277: 50230-50236.

Zhang, D. & Trudeau, V. L. (2006) Integration of membrane and nuclear estrogen receptor signaling. Comp Biochem.Physiol A Mol.Integr.Physiol 144: 306-315.

Zimmer, L., Delion-Vancassel, S., Durand, G. et al. (2000) Modification of dopamine neurotransmission in the nucleus accumbens of rats deficient in n-3 polyunsaturated fatty acids. J.Lipid Res 41: 32-40.

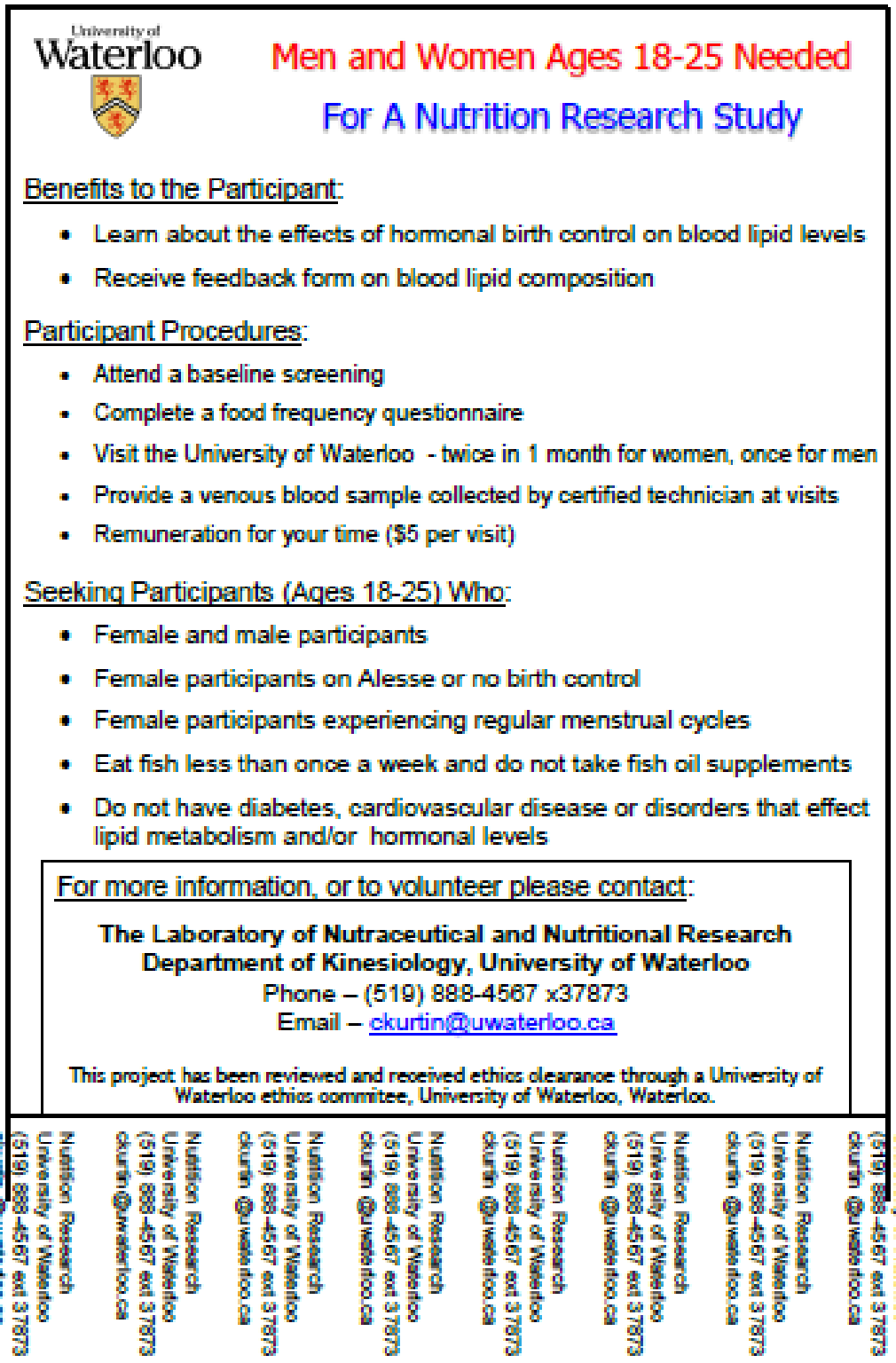
Zulyniak, M. A., Roke, K., Gerling, C. et al. (2015) Fish oil regulates blood fatty acid composition and oxylipin levels in healthy humans: a comparison of young and older men. Mol.Nutr.Food Res.

Appendix 1

Supplementary Forms and Data for Chapter 5

The Effects of Sex, Menstrual Cycle and Oral Contraceptives on Blood Fatty Acids

Appendix 1.1. Advertisements for Chapter 5



University of Waterloo

Men and Women Ages 18-25 Needed

For A Nutrition Research Study

Benefits to the Participant:

- Learn about the effects of hormonal birth control on blood lipid levels
- Receive feedback form on blood lipid composition

Participant Procedures:

- Attend a baseline screening
- Complete a food frequency questionnaire
- Visit the University of Waterloo - twice in 1 month for women, once for men
- Provide a venous blood sample collected by certified technician at visits
- Remuneration for your time ($5 per visit)

Seeking Participants (Ages 18-25) Who:

- Female and male participants
- Female participants on Alesse or no birth control
- Female participants experiencing regular menstrual cycles
- Eat fish less than once a week and do not take fish oil supplements
- Do not have diabetes, cardiovascular disease or disorders that effect lipid metabolism and/or hormonal levels

For more information, or to volunteer please contact:

The Laboratory of Nutraceutical and Nutritional Research
Department of Kinesiology, University of Waterloo
Phone – (519) 888-4567 x37873
Email – ckurtin@uwaterloo.ca

This project has been reviewed and received ethics clearance through a University of Waterloo ethics committee, University of Waterloo, Waterloo.

Nutrition Research
University of Waterloo
(519) 888-4567 ext 37873
ckurtin @uwaterloo.ca

Nutrition Research
University of Waterloo
(519) 888-4567 ext 37873
ckurtin @uwaterloo.ca

Appendix 1.2. Chapter 5 Email Recruitment Script

Mailing Lists Targeted: Applied Health Science Listservs

Email Subject Line: Seeking Participants for a Blood Lipids Research Study

Email Text:

The Laboratory of Nutritional and Nutraceutical Research in the Department of Kinesiology is looking for *male and female* participants between the *ages of 18 and 35* for a blood lipids research study.

Study description:

Sex hormones play a role in lipid levels in the body. It has been shown that there is an increased level of docosahexaenoic acid in females than males. This study is examining the changes in blood lipid levels during the menstrual cycle, which has changing levels of sex hormones. These fluctuating levels of sex hormones result in changes in blood lipid composition. This study will identify these changes.

Participants in this study will be asked to:

- Visit the Department of Kinesiology at the University of Waterloo 2 times over 1 month for female participants and 1 time per month for male participants
- Provide a venous blood sample collected by a certified technician on 2 occasions for female participants and 1 occasion for male participants
- Complete a nutritional questionnaire at baseline

Benefits to the participant:

- Receive up to $10 remuneration

We are seeking participants (ages 18-25) who:

- Eat fish less than once a week and do not take fish oil supplements
- Females with a regular menstrual cycle between 28-35 days for the last 3 months
- Males for comparison
- Women cannot be taking oral medications other than *Alesse* birth control
    - Other oral contraceptives are not allowed
- No conditions that would affect hormone levels

For more information or to volunteer please contact:

The Laboratory of Nutritional and Nutraceutical Research

Department of Kinesiology, University of Waterloo

Phone – (519) 888-4567 ext 37873

Email – k2marks@uwaterloo.ca

Appendix 1.3. Chapter 5 Class Announcement Slide

Women Needed for a Nutrition Research Study

- Seeking Participants Who:
- Female participants on **Alesse** or **no birth control**, and experience regular menstrual cycles
- Eat fish less than once a week and do not take fish oil supplements
- Do not have diabetes, cardiovascular disease or disorders that affect lipid metabolism and/or hormonal levels

- Participant Procedures:
- Visit the University of Waterloo twice in one month to provide a venous blood sample
- Remuneration for your time ($5 per visit)

- For more information, or to volunteer please contact:
- **The Laboratory of Nutraceutical and Nutritional Research, Department of Kinesiology**
- Phone – (519) 888-4567 x37873
- Email – Kristin Marks, k2marks@uwaterloo.ca

- This project has been reviewed and received ethics clearance through a University of Waterloo ethics committee, University of Waterloo, Waterloo.

Appendix 1.4. Information and Consent Forms for Chapter 5

Information and Consent Form

Laboratory of Nutritional and Nutraceutical Research

Department of Kinesiology

University of Waterloo

Title of Project **The Effect of Sex Hormones on Blood Lipidomics**

Principal Investigator: **Professor Ken D. Stark**

University of Waterloo, Department of Kinesiology

(519) 888 – 4567 ext. 37738

Student Investigators: **Kristin Marks, PhD Student**

University of Waterloo, Department of Kinesiology

(519) 888 – 4567 ext. 37873

k2marks@uwaterloo.ca

Chloe Kurtin

University of Waterloo, Department of Kinesiology

(519) 888 – 4567 ext. 37873

ckurtin@uwaterloo.ca

Purpose of Study:

Sex differences in fatty acid levels in the blood have been documented in both humans and rats. These sex differences can have numerous effects on the body. Polyunsaturated fatty acids (PUFA) have shown a cardioprotective effect in regards to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It has been seen in humans that women tend to have higher DHA levels than men. It has been seen in rat models that estradiol contributes strongly to this difference.

The goal of the study is to examine the differences in blood lipid composition during the menstrual cycle. This study is also examining the difference in blood lipid composition between women on hormonal contraceptives and women experiencing normal fluctuating hormone levels. A secondary goal of this study is to examine the sex differences between blood lipid levels. In the study, you will be asked to give two blood samples if you are female and one blood sample if you are male. The first blood sample for females will be taken at day 6-8 of your menstrual cycle and the second blood sample will be taken at day 21-23 of your menstrual cycle. Males will give one blood sample at the beginning of the study.

The results of this study will be helpful in determining the pathway that estrogen modulates to change blood lipid composition. The results will also be used to better understand the effects of birth control and hormone treatments on blood lipid levels. This could help us better understand the change in blood lipid composition in the bodies of women using hormone based birth controls.

Specific details about the study procedures are provided in the following section.

Procedures Involved in this Study:

As a female study participant, you will be asked to do the following:

1. Complete a health status questionnaire, food frequency questionnaire, and menstrual cycle questionnaire to determine study eligibility
2. During the study, do not vary your diet from normal. It is important to continue your diet as normal. Do not make significant changes to your daily exercise.
3. Ensure researchers are told of any changes to medications or oral contraceptives.
4. Arrange for venous blood samples to be taken on day 6-8 of menstrual cycle (from start of the cycle) and days 21-23 of the menstrual cycle.
5. Fast overnight (including no drinking and eating, water is allowed) before the blood sample is taken.
6. Provide venous blood samples two times over the study period for female participants.
7. Allow your blood to be analyzed for whole blood lipid composition and assessing levels of various enzymes involved in lipid synthesis.

As a male study participant, you will be asked to do the following:

1. Complete a health status questionnaire, and food frequency questionnaire to determine study eligibility
2. During the study, do not vary your diet from normal. It is important to continue your diet as normal. Do not make significant changes to your daily exercise.
3. Ensure researchers are told of any changes to medications.
4. Arrange for a venous blood samples to be taken.
5. Fast overnight (including no drinking and eating, water is allowed) before the blood sample is taken.
6. Provide one venous blood sample.

7. Allow your blood to be analyzed for whole blood lipid composition and assessing levels of various enzymes involved in lipid synthesis.

Information is provided on each of the tests. The tests will all be completed at the Laboratory of Nutritional and Nutraceutical research at the University of Waterloo.

Time Commitment:

Each visit to the laboratory should be 15-20 minutes. This will include the initial screening visit to determine eligibility to the study as well as the venous blood collections.

Study Timeline - Females:

Eligibility Screening

- Health status questionnaire
- Menstrual cycle questionnaire
- Food Frequency Questionnaire

Eligible participants will be invited to participate in the following:

Day 6-8 from beginning of menstrual cycle:

- Venous blood sample completed by a trained personnel
- Only one sample will be collected on one of the days. One visit only.

Day 21-23 from beginning of menstrual cycle:

- Venous blood sample completed by a trained personnel
- Only one sample will be collected on one of the days. One visit only.

Study Timeline - Males:

Eligibility Screening

- Health status questionnaire
- Food Frequency Questionnaire

Eligible participants will be invited to participate in the following:

- Venous blood sample completed by a trained personnel
- Only one sample will be collected on one of the days. One visit only.

Personal Benefits of Participation:

You will receive information on your blood lipid composition including the omega-3 status in comparison to current health recommendations. During the study, a food frequency questionnaire will be completed. These results may give you information on your dietary habits.

Detailed Explanation of Procedures and Risks:

Venous Blood Sampling – This is similar to blood samples taken by your physician. Venous blood will be collected under sterile conditions with an 18-21 gauge needle from a vein in your arm and will be collected into sterile glass tubes. For each visit approximately 15mL or 3tsp of blood will be collected. This procedure may result in slight bruising and bleeding. This can be minimized by the application of direct pressure to the point of needle entry into the vein. The use of sterile gauze and alcohol swipes minimizes the risk of infection. Blood fatty acid levels will be determined. Venous blood sampling will be performed by trained and competent laboratory personnel following universal guidelines for handling blood and blood products. This procedure is being done under the delegation of Dr. John Moule.

Special Instructions:

Participants are asked to continue their regular diet and exercise regime. If you make significant changes to your diet or exercise regime, we ask that you inform the researchers of this change.

Any change to your medication, including oral contraceptives, is asked to be reported to the researcher. It is asked that you do not participate in other studies or donate blood during your participation in this study. Participants are asked to fast overnight before the blood tests, including no food or drink other than water. Please refrain from alcohol consumption two days before the scheduled blood draws.

Health Screening Form:

The Health Screening questionnaire asks questions about your current health status. This form guides the researchers with the eligibility into the study and ensures you are a suitable candidate for the study. If you have disorders resulting in abnormal hormone levels, lipid metabolism disorders, or cardiovascular disease, you will not be eligible to participate in the study.

Menstrual Cycle Screening Form (females only):

This questionnaire provides researchers with information regarding the regularity and other aspects of your menstrual cycle. This information is used to guide eligibility into the study. If you have a menstrual cycle outside of 28-35 days, abnormal cycles within the last 3 months, or use hormonal contraceptives other than Alesse, you will not be eligible for this study.

Changing Your Mind about Participation:

Participation is voluntary; therefore, you may withdraw from the study at any point. Please indicate to one of the researchers that you no longer want to participate in this study. If you withdraw, we will ask why you wanted to withdraw, if you agree to provide the information. You do not have to provide us with this information.

Confidentiality:

Confidentiality will be ensured by each participant having an identification code known only by the primary researcher and his research assistants. This will include all data, including questionnaires and blood samples, to be identified by this code. This code will be kept on a paper copy in a locked filing cabinet only accessible by the researchers. The participant codes will be shredded once all data has been entered under anonymous code and feedback provided to participants. The coded participant specimens will be kept in a - 80°C freezer in a locked laboratory in the Department of Kinesiology. Specimens are destroyed during the analytical procedures with any remaining specimens being destroyed at the completion of the study (March 1st, 2016).

Handling of Results and Participant Feedback:

Anonymous data will be analyzed to determine the role of sex hormones on blood lipidomics. The data will be used to make tables and/or graphs and presented in scientific articles and/or at scientific conferences. After the completion of the study, you will be provided with a thank you letter. Also, if you choose to receive your individual results, you will receive a feedback sheet that will include your omega-3 index level (amount of EPA+DHA in your blood) as well as sources of information on how to interpret the number in regards to your health. In this study it is unlikely, but in looking at the fatty acid and lipidomic values of your blood, we may observe an unforeseen or incidental finding that was not the intention of the study. If we observe such a finding, we will send you a letter asking if you want to receive this finding and outlining your options.

Remuneration:

Participants will be provided with $5 per visit to the lab as remuneration. This is to cover the time commitment of the participants. The amount received is taxable. It is your responsibility to report this amount for income tax purposes.

Contact Information:

If you have any questions, contact Professor Ken Stark at (519)–888–4567 ext. 37738, Kristin Marks at k2marks@uwaterloo.ca, or Chloe Kurtin at ckurtin@uwaterloo.ca.

Concerns about Your Participation:

I would like to assure you that this study has been reviewed and received ethics clearance through a University of Waterloo Ethics Committee. However, the final decision about participation is yours. If you have any comments or concerns resulting from your participation in this study, you may contact M. Nummelin, Chief Ethics Officer by calling (519) 888-4567 ext. 36005 or emailing maureen.nummelin@uwaterloo.ca.

Health Screening Form

Present Health Problems

List Current Health Problems:

1.

2.

3.

List medications taken in the last 6 months

1.

2.

3.

Past Health Problems:

Rheumatic Fever	( )	Epilepsy	( )
Heart Murmur	( )	Varicose Veins	( )
High Blood Pressure	( )	Disease of Arteries	( )
High Cholesterol	( )	Emphysema, Pneumonia,	
Congenital Heart Disease	( )	Asthma, Bronchitis	( )
Heart Attack	( )	Back Injuries	( )
Heart Operation	( )	Kidney and liver disease	( )
Diabetes (diet or insulin)	( )	Heartburn	( )
Ulcers	( )	Bleeding disorders	( )
Bleeding from Intestinal Tract	( )	Lipid Metabolism disorders	( )
Enteritis/colitis/diverticulitis	( )	Other (describe overleaf)	( )

Age: _____

Smoking:

Never ( ) Ex-smoker ( ) Regular ( ) Amt/day:_____

Alcohol Intake:

A standard drink is:

-1 can/bottle of beer (355 ml/12 oz)

-1 glass of wine (150 ml/5 oz)

-1 shot of 40% hard liquor or spirits (44 ml/1.5 oz)

How often do you have 10 or more standard drinks on 1 occasion?

( ) Never
( ) Less than Monthly
( ) Monthly
( ) Weekly
( ) Daily or Almost Daily

Dietary Supplements:

Do you take vitamin-mineral or other supplements regularly? Yes ( ) No ( )

If yes, please list:

Type (eg: multivitamin)	**Brand** (eg: Centrum)	**Frequency** (eg: daily)	**Amount** (eg: 1, 500mg capsule)

Do you follow a vegetarian, vegan or restricted diet? Yes ( ) No ( )

If yes, please list all foods restricted from your diet (eg: fish):

Do you have any food allergies/intolerances? Yes ( ) No ( )

If yes, please list:

Have you made any major changes to your diet over the past year? Yes ( ) No ( )

If yes, please list changes and when they took place:

Have you lost or gained weight in the past year? Yes ( ) No ( )

If yes, please describe changes and when they took place:

Current Physical Activity Status:

I consider my physical activity status to be: High ( ), Average ( ), Low ( ).

List the physical activities that you do on a regular basis:

Type (eg: jogging)	**Frequency** (eg: 3x/week)	**Duration** (eg:30 min)

Signature of Participant:_____

Witness:_____

Date:_____

The current study has been identified as requiring medical clearance:

Yes_____ No___X__

Menstrual Health Questionnaire

1. At present, what best describes your menstrual cycle?
   - ☐ I have regular periods
   - ☐ I have irregular periods
   - ☐ My periods have stopped due to menopause, surgery, medications, or other reasons
   - ☐ Other: _____

2. What is the average length of your menstrual cycle? _____

3. What was the date of your last period? _____

4. Have you been pregnant in the last 6 months?
   - ☐ Yes
   - ☐ No

5. Have you taken any form of hormonal birth control in the last 6 months?
   - ☐ Yes
   - ☐ No

   If you said yes to the above question, please indicate the type and brand of the birth control:

   _____

6. Have you ever been diagnosed with the following:
   - ☐ Addison's disease
   - ☐ Polycystic Ovarian Syndrome
   - ☐ Amenorrhea
   - ☐ Turner's Syndrome
   - ☐ Syndromes that cause pituitary to release to much prolactin (i.e. pituitary tumor)
   - ☐ Cancer
   - ☐ Other

CONSENT FORM Participant Code (Office use): _____

I agree to take part in a research study being
conducted by Professor Ken Stark and Chloe Kurtin and Kristin Marks of the Department of Kinesiology, University of Waterloo.

I have made this decision based on the information I have read in the Information letter. All the procedures, any risks and benefits have been explained to me. I have had the opportunity to ask any questions and to receive any additional details I wanted about the study. If I have questions later about the study, I can ask one of the researchers (Professor Ken Stark at his office (519) 888-4567 ext. 37738 or Chloe Kurtin at extension 37873) or by emailing ckurtin@uwaterloo.ca.

I understand that I may withdraw from the study at any time without penalty by telling the researcher.

By signing this consent form the participant does not waive any legal rights that they would otherwise and that any offers of compensation in the event of injury will not limit recourse to other legal remedies.

This project has been reviewed by, and received ethics clearance through a University of Waterloo ethics board at the University of Waterloo. I am aware that I may contact this office (888-4567, ext. 36005) if I have any concerns or questions resulting from my involvement in this study.

Printed Name: _____

Local Address: _____

Please indicate if you would like to receive your individual results:

Yes:_____ No:_____

Signature: _____

_____ _____
Dated at Waterloo, Ontario Witnessed

Appendix 1.5. Food Frequency Questionnaire for Chapter 5

Participant Code (Office Use): _____

Laboratory of Nutritional and Nutraceutical Research

Food Frequency Questionnaire

INSTRUCTIONS: Please answer the following background questions and subsequent 48 questions about your **usual** eating habits over the **past 12 months**.

Date you answered this questionnaire: _____

Age:

( ) Under 19 years ( ) 30-34 years ( ) 45-49 years ( ) 60-64 years

( ) 19-24 years ( ) 35-39 years ( ) 50-54 years ( ) 65 years and over

( ) 25-29 years ( ) 40-44 years ( ) 55-59 years

Gender: ( ) Male ( ) Female

Sociodemographic

1. What is your cultural/racial background?

( ) Caucasian
( ) Chinese
( ) South Asian (ex: East Indian, Pakistani, Sri Lankan)
( ) African
( ) Filipino
( ) Latin American
( ) Southeast Asian (ex: Cambodian, Indonesian, Laotian, Vietnamese)
( ) Arab
( ) West Asian (ex: Afgan, Iranian)
( ) Japanese
( ) Korean
( ) Aboriginal
( ) Other: _____

2. What is the highest level of education you have attained?

( ) Some High School
( ) High School Diploma
( ) Diploma/Certificate from trade, technical or vocational school or business college
( ) Diploma/Certificate from community college, CEGEP or nursing school
( ) Bachelor's undergraduate degree or teacher's college
( ) Master's degree
( ) Degree in Medicine, Dentistry, Veterinary Medicine, Optometry or Law
( ) Earned doctorate (ex: Ph.D.)
( ) Other: _____

3. What is your annual household income?

( ) less than $25,000
( ) $25,000 - $49,999
( ) $50,000 - $74,999
( ) $75,000 - $99,999
( ) more than $100,000

Diet

4. What type of milk do you usually use?

( ) I do not drink milk
( ) Regular cow's milk (non-omega-3)
( ) Regular soy milk (non-omega-3)
( ) *Nielson, Dairy Oh!* 1% or 2% milk
( ) *Nielson, Dairy Oh!* Homogenized milk
( ) Silk Plus, Omega-3 DHA soy milk
( ) So Good, Omega-3 DHA soy milk
( ) Other Omega-3 enriched milk
Please specify Brand:
_____

5. How much **omega-3** milk do you usually use **per day** (including milk added to tea, coffee, cereal, etc.)?

( ) None
( ) 1 tablespoon
( ) 2 tablespoons
( ) ¼ cup (62.5 mL)
( ) ½ cup (125 mL)
( ) 1-2 cups (250-500 mL)
( ) 2-3 cups (500-750 mL)
( ) 3 cups (750 mL) or more

6. What kinds of margarine/spread do you usually use? (ex: On bread, vegetables, in cooking and baking, etc.)

( ) I don't use any margarine/spread
( ) Regular margarine (non-omega-3)
( ) *Becel*, *Omega-3 Plus* Margarine
( ) Other Omega-3 enriched margarine
Please specify brand:
_____

7. How much **omega-3** margarine/spread do you usually use **per day**?

( ) None

( ) 1-2 teaspoons

( ) 1 tablespoon

( ) 2 tablespoons

( ) 3 tablespoons

( ) 4 tablespoons

( ) 5-7 tablespoons

( ) 8 or more tablespoons

8. What kinds of eggs do you usually eat (include eggs in baked goods/cooking)?

( ) *Naturegg Breakfree* omega-3 liquid eggs

( ) *Naturegg* Omega-3 eggs (in shell)

( ) *Naturegg* Omega-3 Pro eggs (in shell)

( ) *Complements* Omega-3 eggs

( ) *President's Choice* Omega-3 eggs

( ) *Gray Ridge* Omega-3 large eggs

( ) *Gray Ridge* Omega-3 extra-large eggs

( ) Regular Chicken eggs

( ) Duck eggs

( ) Other Omega-3 enriched eggs
Please specify brand:

_____

9. On average, how many eggs do you usually eat **per week (**including eggs in baked goods/cooking)?

( ) I don't eat eggs

( ) Less than 1 egg

( ) 1 to 2 eggs

( ) 3 to 5 eggs

( ) 6 or more eggs

10. What kind of juice do you usually drink?

( ) I don't drink juice

( ) Regular juice (non-omega-3)

( ) *Tropicana Essentials*, Omega-3 orange juice

( ) *Minute Maid Fruit Solutions*, Omega-3 mango, orange & passion juice

( ) *President's Choice, Oh Mega J* orange juice

( ) Other Omega-3 enriched juice
Please specify brand:

_____

11. How much **omega-3** juice do you usually drink **per day**?

( ) None

( ) less than one cup (<250 mL)

( ) 1 cup (250 mL)

( ) 1-2 cups (250-500 mL)

( ) 2-3 cups (500-750 mL)

( ) 3 cups (750 mL) or more

12. What kind of yogurt do you usually eat?

( ) I don't eat yogurt

( ) Regular yogurt (non-omega-3)

( ) *Danone Danino* yogurt

( ) *Danone Danino Go* yogurt drink

( ) Other Omega-3 enriched yogurt
Please specify brand:

_____

13. How much **omega-3** yogurt do you usually eat **per week**?

( ) none

( ) less than 1, 100 g pot

( ) 1, 100 g pot

( ) 2 to 3, 100 g pots

( ) 4 to 5, 100 g pots

( ) 6 to 7, 100 g pots

( ) 8 or more 100 g pots

14. What kind of bread do you usually eat?

( ) I don't eat bread

( ) Regular bread (non-omega-3)

( ) *Wonder*+ Omega-3 bread with DHA

( ) Other Omega-3 enriched bread

Please specify brand/type:

_____

15. How much omega-3 bread do you usually eat per day (fresh and toasted)?

( ) none
( ) less than 1 piece

( ) 1 piece

( ) 2 pieces

( ) 3 pieces

( ) 4 pieces

( ) 5-7 pieces

( ) 8 or more pieces

16. What kind of cheese do you usually eat?

( ) I don't eat cheese
( ) Regular cheese (non-omega-3)
( ) Black Diamond, Smart Growth Cheese

Strings with DHA

( ) Other omega-3 enriched cheese
Please specify brand:

_____

17. How much omega-3 cheese do you usually eat **per week**?

( ) None

( ) less than 1oz (~1 cheese string)

( ) 1 oz (~1 cheese string)

( ) 2-3 oz (2-3 cheese strings)

( ) 4-5 oz (4-5 cheese strings)

( ) 6-7 oz (6-7 cheese strings)

( ) 8 or more oz (8+ cheese strings)

18. What kind of nut butter (ex: peanut) do you usually eat?

( ) I don't eat nut butter

( ) Regular nut butter (non-omega-3)

( ) Life Brand, Omega-3 Peanut Butter
( ) Other omega-3 enriched nut butter

Please specify brand:

_____

19. How much omega-3 nut butter do you eat per week?

( ) None

( ) less than 1 tablespoon (tbsp)

( ) 1 tbsp

( ) 2-3 tbsp

( ) 4-5 tbsp

( ) 6-7 tbsp

( ) 8 or more tbsp

20. On average, how often do you eat fish paste? (ex: Surimi, often in Asian dishes)

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Specify the **type** of fish paste, **brand** and **amount** usually eaten (number of teaspoons)

Type of fish paste *Brand* *#tsp*

_____

21. On average, how often do you eat seaweed?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** of seaweed (or **dish** containing seaweed) and **amount** eaten on a typical occasion

Type of seaweed/Dish *Amount*

_____

22. On average, how often do you eat seafood-containing sauce? (ex: fish sauce oyster sauce, etc (often in Asian dishes))?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** of seafood sauce, **brand** and **amount** usually eaten (# tsp)

Type of sauce *Brand* *#tsp*

_____

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **brand** (or 'homemade') and **amount** of fish broth usually eaten

Brand *Amount*

_____

24. How many fish oil supplements (ex:capsules) do you usually take **per day**

( ) None

( ) Number: _____

Please Specify **brand** and **name** of fish oil capsules: (ex: Jamieson, omega-3 Complete)

Brand *Product Name*

_____

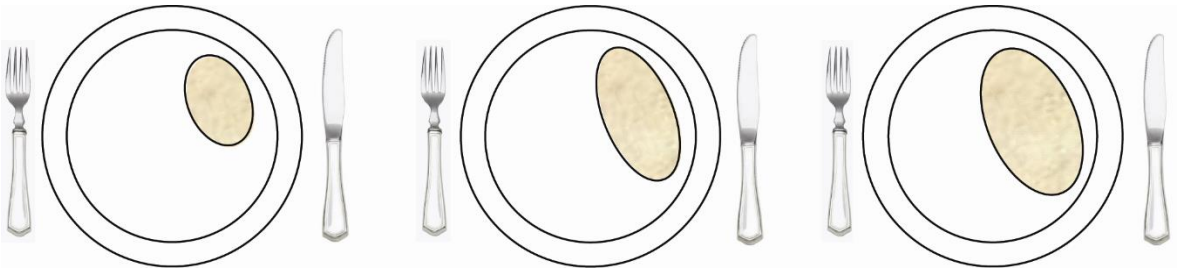
_____

23. On average, how often do you eat fish-based broth (ex: fish broth-based soup)?

( ) Never

( ) less than once per month

25. When you eat meat or poultry, what size serving do you usually eat?



***A**: Deck of cards sized portion = 3oz / 90g*

***B**: 5oz / 150g*

***C**: 7oz / 200g*

( ) Less than **A**

( ) **A**

( ) Between **A** and **B**

( ) **B**

( ) Between **B** and **C**

( ) **C**

( ) More than **C**

26. On average, how often do you eat chicken or turkey?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **types** of chicken/turkey eaten most frequently and whether the **skin** is eaten (ex: Turkey thigh, with skin)

_____

_____

27. On average, how often do you eat duck or goose?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** of duck/goose eaten most frequently and whether the **skin** in eaten. (ex: Duck breast, without skin)

_____

28. How often do you usually eat beef?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** of beef most often eaten (ex: Rump steak):

_____

_____

29. On average, how often do you eat lamb?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** of lamb most often eaten (ex: Loin chop):

_____

30. On average, how often do you eat veal?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** of veal most often eaten (ex: Veal steak):

_____

31. On average, how often do you eat pork? (do not include ham or bacon)

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** of pork most often eaten (ex: Pork chop):

_____

32. On average, how often do you eat bacon?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **number of slices** of bacon usually eaten on each occasion:

_____

33. On average, how often do you eat ham

(not deli/sandwich meat)?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **number of slices** of ham usually eaten on each occasion:

_____

34. On average, how often do you usually eat sliced deli meat (ex: luncheon meats or salami)?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** (ex: lean turkey breast), **brand** and the **number of slices** of sliced deli meat most often eaten:

Type *Brand* *# slices*

_____

_____

35. On average, how often do you eat sausages?

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** (ex: Beef, pork, turkey) and **number** of sausages usually eaten on each occasion:

Type *Number*

_____

36. On average, how often do you eat canned fish? (ex: canned tuna, salmon, sardines, etc.)

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify up to 4 **types** of canned fish

that you regularly eat, including the **brand** and the **amount** typically eaten. (ex: White tuna in water, *Goldseal*, ½ 120g tin)

Type of Fish *Brand* *Amount*

_____
_____
_____
_____

37. On average, how often do you eat fresh or frozen fish? (Include fish meals at home, at a restaurant and take out).

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify up to 4 **types** of fresh or frozen fish that you regularly eat and the **amount** typically eaten

(Include the **brand** for frozen fish. If type of fish is unknown for restaurant fish please write 'restaurant fish').
(ex: Wild Atlantic Salmon, *Highliner*, 150g)

Type of Fish *Brand* *Amount*

_____
_____

38. How often do you eat fresh, frozen or canned shellfish? (ex: shrimp, oysters, scallops, crab, calamari, lobster, etc).

( ) Never

( ) less than once per month

( ) 1 to 3 times a month

( ) once per week

( ) 2 to 3 times per week

( ) 4 to 6 times per week

( ) once per day

( ) 2 or more times per day

Please specify the **type** and **amount** of shellfish eaten on a typical occasion:

Type of Shellfish *Amount*

_____
_____

39. Are there any other meat, poultry or fish products that you eat regularly?

Please specify the **type, amount** and **how often** other meat or fish products are eaten

Type *Amount* *How often*

_____

_____

_____

Food Preparation

40. On average, how many meals prepared outside of your home do you eat? Include restaurant, fast food, take out and cafeteria meals.

( ) None

( ) 1 or 2 a month

( ) 1 per week

( ) 2 per week

( ) 3 to 4 per week

( ) 5 to 6 per week

( ) 1 per day

( ) 2 per day

( ) 3 per day

41. On average, how often do you eat pre-prepared / heat-and-serve meals?

( ) Never

( ) once or twice a month

( ) once a week

( ) twice a week

( ) 3 to 4 times per week

( ) 5 to 6 times per week

( ) once a day

( ) twice a day

( ) three times a day

42. On average, how often do you eat home-prepared meals?

( ) Never

( ) once or twice a month

( ) once a week

( ) twice a week

( ) 3 to 4 times per week

( ) 5 to 6 times per week

( ) once a day

( ) twice a day

( ) three times a day

43. Who usually prepares meals in your home?

( ) Myself

( ) Shared

( ) Spouse/Partner

( ) Other:_____

44. Who usually purchases food?

( ) Myself

( ) Spouse/Partner

( ) Shared

( ) Other:_____

45. On average, how much money do you spend on groceries **per person** in your household **per week**?

( ) less than $15

( ) $15-$25

( ) $25-$35

( ) $35-$50

( ) $50-$75

( ) more than $75

46. On average, how much time do you spend preparing all meals for your household **per day**

( ) less than 15 minutes

( ) 15 to 30 minutes

( ) 30 to 35 minutes

( ) 45 minutes to 1 hour

( ) 1 hour to 1 ½ hours

( ) more than 1 ½ hours

47. How frequently is the food in your home is purchased from the following sources?

Full-service supermarket (ex: Zehrs, Sobeys):

( ) Never

( ) less than once a month

( ) once a month

( ) 2 to 3 times a month

( ) weekly

( ) more than once a week

List full-service supermarkets:

_____

_____

Discount grocery store (ex: Food Basics, Price Chopper, No Frills):

( ) Never

( ) less than once a month

( ) once a month

( ) 2 to 3 times a month

( ) weekly

( ) more than once a week

List discount grocery stores:

_____

_____

Farmer's Market:

( ) Never

( ) less than once a month

( ) once a month

( ) 2 to 3 times a month

( ) weekly

( ) more than once a week

List types of vendors (ex: fruit, fish):

_____

_____

Gourmet stores (including specialty delis and bakeries):

( ) Never

( ) less than once a month

( ) once a month

( ) 2 to 3 times a month

( ) weekly

( ) more than once a week

List gourmet stores:

_____

_____

Natural health food stores:

( ) Never

( ) less than once a month

( ) once a month

( ) 2 to 3 times a month

( ) weekly

( ) more than once a week

List natural health food stores:

_____

_____

Specialty ethnic grocery stores:

( ) Never

( ) less than once a month

( ) once a month

( ) 2 to 3 times a month

( ) weekly

( ) more than once a week

List ethnic grocery stores:

_____

_____

Take out:

( ) Never

( ) less than once a month

( ) once a month

( ) 2 to 3 times a month

( ) weekly

( ) more than once a week

List frequent take out suppliers:

_____

_____

_____

Other Locations:

( ) Never

( ) less than once a month

( ) once a month

( ) 2 to 3 times a month

( ) weekly

( ) more than once a week

List stores:

_____

_____

_____

48. Have you changed your diet in any significant way over the past year?

( ) Yes

( ) No

If yes, please specify the changes:

_____
_____
_____

Thank you for completing this survey

Table A1.1 Plasma fatty acid concentrations in men and women who take or do not take oral contraceptives.

	Plasma Fatty Acids µg fatty acid/100 µl plasma		
Name	Men	Women taking oral contraceptives	Women with natural cycles
C 10:0	0.04 ± 0.02	0.08 ± 0.07	0.07 ± 0.06
C 12:0	0.40 ± 0.21	0.40 ± 0.17	0.47 ± 0.25
C 14:0	3.15 ± 1.14	3.72 ± 1.47	2.94 ± 0.76
C 16:0	75.36 ± 13.38	92.64 ± 28.56	77.73 ± 15.10
C 18:0	23.34 ± 4.45	23.06 ± 4.51	23.83 ± 2.78
C 20:0	0.57 ± 0.09	0.65 ± 0.11	0.65 ± 0.11
C 22:0	1.31 ± 0.30	1.47 ± 0.22	1.45 ± 0.21
C 24:0	1.20 ± 0.25	1.28 ± 0.20	1.20 ± 0.19
SFAs	106.20 ± 18.88	123.90 ± 33.30	108.82 ± 17.26
C 12:1	0.04 ± 0.03	0.02 ± 0.01	0.03 ± 0.01
C 14:1	0.13 ± 0.09[a]	0.23 ± 0.12[b]	0.21 ± 0.09[ab]
C 16:1n-7	4.62 ± 1.93[a]	7.53 ± 3.58[b]	4.76 ± 1.17[a]
C 18:1n-7	5.61 ± 1.15	6.47 ± 1.32	5.58 ± 1.06
C 18:1n-9	62.20 ± 14.26	65.90 ± 15.52	64.61 ± 10.46
C 20:1n-9	0.46 ± 0.11[a]	0.60 ± 0.17[b]	0.48 ± 0.10[a]
C 22:1n-9	0.21 ± 0.07[a]	0.42 ± 0.12[b]	0.38 ± 0.08[b]
C 24:1n-9	1.80 ± 0.39[a]	2.34 ± 0.46[b]	2.05 ± 0.41[ab]
MUFAs	75.04 ± 16.82	83.51 ± 19.61	78.09 ± 12.12
C 18:2n-6	96.39 ± 15.80	100.64 ± 21.62	99.50 ± 16.91
C 18:3n-6	1.17 ± 0.46	1.09 ± 0.42	1.17 ± 0.40
C 20:2n-6	0.54 ± 0.16[a]	0.71 ± 0.23[b]	0.50 ± 0.18[a]
C 20:3n-6	4.12 ± 1.52[a]	5.69 ± 1.40[b]	4.17 ± 1.17[a]
C 20:4n-6	22.79 ± 4.01	24.01 ± 6.96	21.95 ± 4.25
C 22:2n-6	0.07 ± 0.05	0.09 ± 0.03	0.07 ± 0.03
C 22:4n-6	0.57 ± 0.16	0.66 ± 0.20	0.54 ± 0.15
C 22:5n-6	0.40 ± 0.16	0.47 ± 0.19	0.46 ± 0.15
N-6	125.92 ± 19.80	133.23 ± 27.50	128.18 ± 19.12
C 18:3n-3	2.06 ± 0.71	2.47 ± 1.02	2.20 ± 0.68
C 20:3n-3	0.12 ± 0.04[ab]	0.15 ± 0.04[a]	0.09 ± 0.04[b]
C 20:5n-3	1.64 ± 0.63	1.83 ± 0.54	1.73 ± 0.55
C 22:5n-3	1.41 ± 0.38	1.14 ± 0.29	1.32 ± 0.26
C 22:6n-3	3.90 ± 0.91[a]	5.59 ± 1.62[b]	4.52 ± 1.38[ab]
N-3	9.14 ± 1.80[a]	11.18 ± 2.77[b]	9.86 ± 2.13[ab]
EPA+DHA	5.54 ± 1.21[a]	7.42 ± 1.95[b]	6.26 ± 1.62[ab]
Total	316.58 ± 52.96	352.03 ± 76.52	325.13 ± 46.81

Values are means ± SD. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for one way ANOVA.

Table A1.2 Concentration of fatty acids in erythrocytes of men and women taking or not taking oral contraceptives.

Erythrocyte Fatty Acids µg fatty acid/200 mg erythrocytes			
Name	Men	Women taking oral contraceptives	Women with natural cycles
C 12:0	0.33 ± 0.07	0.46 ± 0.18	0.39 ± 0.18
C 14:0	6.85 ± 0.94	6.81 ± 2.04	5.96 ± 1.62
C 16:0	130.54 ± 10.16	134.52 ± 16.96	123.53 ± 13.37
C 18:0	85.21 ± 8.94	79.38 ± 9.10	81.94 ± 9.38
C 20:0	1.86 ± 0.22	1.83 ± 0.37	1.89 ± 0.22
C 22:0	7.56 ± 0.92[a]	6.42 ± 1.30[b]	7.29 ± 1.29[ab]
C 23:0	0.36 ± 0.29	0.41 ± 0.15	0.55 ± 0.39
C 24:0	23.97 ± 3.72[a]	18.78 ± 3.71[b]	21.62 ± 4.19[ab]
SFAs	255.26 ± 20.86	248.68 ± 29.01	243.26 ± 27.88
C 12:1	0.16 ± 0.06	0.31 ± 0.35	0.28 ± 0.30
C 14:1	2.61 ± 0.46	2.36 ± 1.01	1.99 ± 0.74
C 16:1n-7	1.39 ± 0.23[a]	1.92 ± 0.58[b]	1.33 ± 0.26[a]
C 18:1n-7	7.51 ± 0.66	7.31 ± 1.09	6.70 ± 0.92
C 18:1n-9	73.40 ± 5.49[a]	67.61 ± 8.84[ab]	65.55 ± 9.92[b]
C 20:1n-9	1.40 ± 0.18	1.29 ± 0.27	1.23 ± 0.27
C 22:1n-9	0.76 ± 0.12[ab]	0.69 ± 0.12[a]	0.86 ± 0.19[b]
C 24:1n-9	21.86 ± 2.91	20.63 ± 4.16	20.93 ± 3.80
MUFAs	109.09 ± 7.39	102.12 ± 14.01	98.86 ± 13.92
C 18:2n-6	60.31 ± 8.46	55.54 ± 9.10	52.85 ± 6.56
C 18:3n-6	0.27 ± 0.08	0.28 ± 0.17	0.33 ± 0.12
C 20:2n-6	1.58 ± 0.24	1.49 ± 0.36	1.29 ± 0.38
C 20:3n-6	8.61 ± 1.68	8.69 ± 2.11	7.23 ± 2.44
C 20:4n-6	85.35 ± 4.96	77.69 ± 12.40	76.70 ± 10.89
C 22:2n-6	0.40 ± 0.20	0.39 ± 0.18	0.40 ± 0.18
C 22:4n-6	18.39 ± 3.10	18.07 ± 4.79	15.77 ± 4.14
C 22:5n-6	3.26 ± 0.67	2.71 ± 1.69	2.81 ± 0.60
N-6	178.18 ± 14.36[a]	164.84 ± 25.76[ab]	157.00 ± 22.38[b]
C 18:3n-3	0.90 ± 0.21	0.89 ± 0.36	0.84 ± 0.23
C 20:3n-3	0.22 ± 0.17	0.23 ± 0.09	0.25 ± 0.17
C 20:5n-3	3.07 ± 0.84	2.75 ± 0.72	2.63 ± 0.64
C 22:5n-3	13.49 ± 2.13[a]	10.49 ± 1.76[b]	11.05 ± 2.20[b]
C 22:6n-3	20.89 ± 4.85	24.92 ± 6.65	21.02 ± 4.65
N-3	38.57 ± 4.81	39.28 ± 8.03	35.80 ± 6.29
EPA+DHA	23.96 ± 5.32	27.67 ± 6.94	23.65 ± 5.07
Total	581.11 ± 39.26	554.47 ± 72.11	534.93 ± 63.84

Values are means ± SD. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for one way ANOVA.

Table A1.3 Proportion of fatty acids in erythrocytes of men and women taking or not taking oral contraceptives.

	Erythrocyte Fatty Acids % weight total fatty acids		
Name	Men	Women taking oral contraceptives	Women with natural cycles
C 12:0	0.05 ± 0.01[a]	0.08 ± 0.03[b]	0.07 ± 0.03[ab]
C 14:0	1.14 ± 0.16	1.17 ± 0.29	1.07 ± 0.26
C 16:0	21.86 ± 0.72[a]	23.35 ± 1.21[b]	22.23 ± 0.88[a]
C 18:0	14.31 ± 1.03[ab]	13.77 ± 0.69[a]	14.74 ± 0.72[b]
C 20:0	0.31 ± 0.04	0.32 ± 0.06	0.34 ± 0.03
C 22:0	1.26 ± 0.16[ab]	1.12 ± 0.21[a]	1.31 ± 0.19[b]
C 23:0	0.06 ± 0.04	0.07 ± 0.03	0.10 ± 0.07
C 24:0	3.93 ± 0.55[a]	3.25 ± 0.49[b]	3.88 ± 0.62[a]
SFAs	42.70 ± 0.96	43.15 ± 1.24	43.76 ± 1.92
C 12:1	0.03 ± 0.01	0.06 ± 0.07	0.05 ± 0.07
C 14:1	0.43 ± 0.08	0.40 ± 0.16	0.36 ± 0.13
C 16:1n-7	0.24 ± 0.05[a]	0.34 ± 0.10[b]	0.24 ± 0.04[a]
C 18:1n-7	1.25 ± 0.08	1.27 ± 0.13	1.21 ± 0.12
C 18:1n-9	12.27 ± 0.56	11.75 ± 1.03	11.77 ± 1.10
C 20:1n-9	0.23 ± 0.03	0.22 ± 0.04	0.22 ± 0.04
C 22:1n-9	0.13 ± 0.02[a]	0.12 ± 0.02[a]	0.15 ± 0.03[b]
C 24:1n-9	3.61 ± 0.48	3.57 ± 0.56	3.77 ± 0.53
MUFAs	18.18 ± 0.80	17.74 ± 1.54	17.77 ± 1.47
C 18:2n-6	9.93 ± 0.91	9.64 ± 1.17	9.52 ± 0.70
C 18:3n-6	0.05 ± 0.01	0.05 ± 0.04	0.06 ± 0.03
C 20:2n-6	0.26 ± 0.04	0.26 ± 0.05	0.23 ± 0.07
C 20:3n-6	1.47 ± 0.30	1.51 ± 0.34	1.30 ± 0.43
C 20:4n-6	14.33 ± 0.64	13.46 ± 1.25	13.79 ± 1.20
C 22:2n-6	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.03
C 22:4n-6	3.05 ± 0.48	3.12 ± 0.68	2.83 ± 0.65
C 22:5n-6	0.54 ± 0.11	0.45 ± 0.27	0.45 ± 0.16
N-6	29.69 ± 1.10	28.56 ± 2.63	28.34 ± 2.53
C 18:3n-3	0.15 ± 0.03	0.15 ± 0.05	0.15 ± 0.03
C 20:3n-3	0.04 ± 0.03	0.04 ± 0.01	0.04 ± 0.03
C 20:5n-3	0.52 ± 0.14	0.48 ± 0.12	0.48 ± 0.09
C 22:5n-3	2.24 ± 0.37[a]	1.83 ± 0.27[b]	2.05 ± 0.24[ab]
C 22:6n-3	3.53 ± 0.84	4.30 ± 0.93	3.78 ± 0.79
N-3	6.47 ± 0.89	6.80 ± 1.04	6.51 ± 0.92
EPA+DHA	4.04 ± 0.93	4.78 ± 0.97	4.26 ± 0.85

Values are means ± SD. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for one way ANOVA.

Table A1.4 Plasma fatty acid content in women taking and not taking oral contraceptives during the follicular and luteal phase of the menstrual cycle

Plasma Fatty acids µg fatty acid/100 µl plasma				
	Women taking oral contraceptives		Women with natural cycles	
Name	Day 6-8 (no hormone phase)	Day 21-23 (active hormone phase)	Day 6-8 (follicular phase)	Day 21-23 (luteal phase)
C 16:0[1]	97.80 ± 31.22	87.48 ± 27.46	81.36 ± 16.04	74.11 ± 17.73
C 18:0[1]	24.30 ± 5.81	21.83 ± 3.98	24.64 ± 3.30	23.02 ± 3.32
C 20:0	0.65 ± 0.14	0.66 ± 0.10	0.65 ± 0.11	0.65 ± 0.13
C 22:0	1.47 ± 0.19	1.47 ± 0.29	1.43 ± 0.22	1.48 ± 0.25
C 24:0	1.24 ± 0.18	1.32 ± 0.24	1.18 ± 0.21	1.22 ± 0.22
SFAs[1]	130.76 ± 37.86	117.04 ± 30.96	113.64 ± 18.92	104.01 ± 20.54
C 16:1n-7[1,2]	8.25 ± 3.31	6.81 ± 4.05	5.40 ± 1.65	4.12 ± 1.17
C 18:1n-7	6.37 ± 1.44	6.57 ± 1.48	5.70 ± 1.04	5.46 ± 1.30
C 18:1n-9	67.38 ± 16.42	64.42 ± 16.79	66.39 ± 11.52	62.83 ± 12.42
C 20:1n-9[1,2]	0.50 ± 0.16[a]	0.71 ± 0.21[b]	0.46 ± 0.10[a]	0.50 ± 0.13[a]
C 22:1n-9[1]	0.36 ± 0.14[a]	0.48 ± 0.13[b]	0.37 ± 0.10[a]	0.39 ± 0.07[a]
C 24:1n-9	2.27 ± 0.51	2.40 ± 0.47	2.05 ± 0.43	2.05 ± 0.45
MUFAs	85.42 ± 20.20	81.60 ± 21.55	80.59 ± 13.48	75.58 ± 14.54
C 18:2n-6	102.29 ± 24.41	98.99 ± 20.76	101.30 ± 16.67	97.69 ± 19.57
C 18:3n-6[1]	1.28 ± 0.54	0.90 ± 0.37	1.33 ± 0.57	1.01 ± 0.32
C 20:2n-6[1,2]	0.62 ± 0.18	0.81 ± 0.31	0.50 ± 0.19	0.50 ± 0.18
C 20:3n-6[1]	5.56 ± 1.51	5.82 ± 1.48	4.44 ± 1.38	3.91 ± 1.08
C 20:4n-6	24.49 ± 7.86	23.53 ± 6.58	22.09 ± 4.54	21.81 ± 4.68
C 22:2n-6	0.10 ± 0.05	0.08 ± 0.03	0.09 ± 0.05	0.06 ± 0.03
C 22:4n-6	0.63 ± 0.19	0.69 ± 0.24	0.56 ± 0.17	0.52 ± 0.17
C 22:5n-6	0.40 ± 0.12	0.53 ± 0.27	0.51 ± 0.14	0.46 ± 0.14
N-6	135.26 ± 31.46	131.21 ± 25.83	130.61 ± 19.39	125.74 ± 22.29
C 18:3n-3	2.41 ± 1.08	2.52 ± 1.09	2.19 ± 0.63	2.21 ± 0.90
C 20:3n-3[2]	0.13 ± 0.05	0.16 ± 0.05	0.09 ± 0.05	0.09 ± 0.05
C 20:5n-3	2.02 ± 0.64	1.64 ± 0.67	1.68 ± 0.49	1.79 ± 0.85
C 22:5n-3	1.20 ± 0.32	1.08 ± 0.31	1.39 ± 0.35	1.24 ± 0.30
C 22:6n-3	5.41 ± 1.53	5.78 ± 1.85[b]	4.49 ± 1.22	4.56 ± 1.71
N-3	11.18 ± 2.58	11.18 ± 3.22	9.83 ± 1.61	9.89 ± 3.20
EPA+DHA	7.43 ± 1.83	7.42 ± 2.32	6.17 ± 1.23	6.34 ± 2.29
N-6/N-3	12.33 ± 2.11	12.17 ± 2.19	13.37 ± 1.35	13.30 ± 2.45
Total[1]	362.81 ± 83.68	341.24 ± 75.64	334.87 ± 48.69	315.39 ± 57.37

Values are mean ± sd. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for birth control status x time by the linear mixed models procedure.

[1] significant main effect of time

[2] significant main effect of birth control status

Table A1.5 Erythrocyte fatty acid content in women taking and not taking oral contraceptives during the follicular and luteal phase of the menstrual cycle

	Erythrocyte Fatty Acids µg fatty acid/200 mg cells			
	Women taking oral contraceptives		Women with natural cycles	
Name	Day 6-8 (no hormone phase)	Day 21-23 (active hormone phase)	Day 6-8 (follicular phase)	Day 21-23 (luteal phase)
C 14:0	6.54 ± 2.33	7.07 ± 2.16	5.94 ± 1.61	5.98 ± 1.76
C 16:0	130.18 ± 18.61	138.87 ± 19.60	122.07 ± 11.10	125.00 ± 20.35
C 18:0	77.68 ± 11.86	81.07 ± 13.05	80.03 ± 9.69	83.85 ± 14.42
C 20:0	1.73 ± 0.33	1.93 ± 0.47	1.88 ± 0.20	1.89 ± 0.33
C 22:0	6.12 ± 1.27	6.72 ± 1.51	7.30 ± 1.00	7.29 ± 1.71
C 23:0	0.38 ± 0.15	0.44 ± 0.19	0.52 ± 0.42	0.62 ± 0.46
C 24:0	17.96 ± 3.25	19.61 ± 4.62	21.50 ± 3.54	21.74 ± 5.24
SFAs	241.18 ± 32.44	256.19 ± 36.42	239.77 ± 22.76	246.74 ± 41.72
C 16:1n-7[2]	1.99 ± 0.63	1.85 ± 0.59	1.36 ± 0.28	1.29 ± 0.28
C 18:1n-7	7.31 ± 1.44	7.32 ± 1.02	6.71 ± 0.99	6.69 ± 1.06
C 18:1n-9	67.24 ± 9.51	67.99 ± 10.76	65.02 ± 8.55	66.07 ± 12.79
C 20:1n-9	1.27 ± 0.29	1.31 ± 0.29	1.21 ± 0.27	1.25 ± 0.30
C 22:1n-9[2]	0.68 ± 0.16	0.69 ± 0.14	0.82 ± 0.18	0.89 ± 0.27
C 24:1n-9	19.62 ± 3.79	21.64 ± 4.93	21.02 ± 3.58	20.84 ± 4.67
MUFAs	100.60 ± 14.42	103.64 ± 16.37	98.40 ± 12.38	99.32 ± 17.62
C 18:2n-6	54.28 ± 10.00	56.80 ± 9.46	51.99 ± 5.62	53.71 ± 9.27
C 18:3n-6	0.31 ± 0.19	0.28 ± 0.14	0.33 ± 0.11	0.33 ± 0.15
C 20:2n-6	1.48 ± 0.37	1.50 ± 0.36	1.22 ± 0.32	1.36 ± 0.58
C 20:3n-6	8.62 ± 2.47	8.75 ± 1.95	7.19 ± 2.34	7.27 ± 2.65
C 20:4n-6	77.05 ± 14.69	78.33 ± 12.17	75.75 ± 10.12	77.64 ± 14.44
C 22:2n-6	0.34 ± 0.10	0.44 ± 0.29	0.36 ± 0.11	0.45 ± 0.30
C 22:4n-6	18.06 ± 5.32	18.08 ± 4.45	15.52 ± 3.43	16.02 ± 5.11
C 22:5n-6	3.31 ± 1.02	3.30 ± 1.31	2.76 ± 0.65	2.86 ± 0.62
N-6	162.81 ± 30.21	166.87 ± 25.18	154.74 ± 18.58	159.25 ± 30.44
C 18:3n-3	0.90 ± 0.32	0.95 ± 0.40	0.79 ± 0.19	0.89 ± 0.34
C 20:3n-3	0.21 ± 0.10	0.25 ± 0.14	0.22 ± 0.18	0.28 ± 0.21
C 20:5n-3	2.80 ± 0.81	2.69 ± 0.70	2.64 ± 0.58	2.63 ± 0.84
C 22:5n-3	10.57 ± 2.07	10.42 ± 1.79	11.07 ± 1.93	11.04 ± 2.63
C 22:6n-3	24.93 ± 7.39	24.92 ± 6.49	21.00 ± 5.19	21.04 ± 4.73
N-3	39.33 ± 9.13	39.23 ± 8.01	35.73 ± 6.48	35.87 ± 7.24
EPA+DHA	27.73 ± 7.73	27.61 ± 6.82	23.65 ± 5.49	23.66 ± 5.37
N-6/N-3[1]	4.24 ± 0.85	4.37 ± 0.91	4.45 ± 0.86	4.54 ± 0.90
Total	542.91 ± 83.68	566.02 ± 79.56	528.66 ± 52.23	541.21 ± 91.76

Values are mean ± sd.
[1] significant main effect of time
[2] significant main effect of birth control status

Table A1.6 Erythrocyte fatty acid composition in women taking and not taking oral contraceptives during the follicular and luteal phase of the menstrual cycle

Erythrocyte Fatty Acids % weight total fatty acids				
	Women taking oral contraceptives		Women with natural cycles	
Name	Day 6-8 (no hormone phase)	Day 21-23 (active hormone phase)	Day 6-8 (follicular phase)	Day 21-23 (luteal phase)
C 14:0	1.15 ± 0.34	1.20 ± 0.32	1.08 ± 0.29	1.05 ± 0.28
C 16:0	23.01 ± 1.16[ab]	23.68 ± 1.34[a]	22.28 ± 0.81[b]	22.19 ± 1.07[b]
C 18:0[2]	13.73 ± 0.90	13.82 ± 1.10	14.59 ± 1.01	14.89 ± 0.85
C 20:0	0.31 ± 0.06	0.33 ± 0.07	0.35 ± 0.04	0.34 ± 0.04
C 22:0[2]	1.09 ± 0.24	1.15 ± 0.20	1.33 ± 0.17	1.29 ± 0.22
C 23:0	0.07 ± 0.03	0.07 ± 0.03	0.09 ± 0.07	0.11 ± 0.07
C 24:0[2]	3.18 ± 0.48	3.33 ± 0.59	3.93 ± 0.61	3.84 ± 0.69
SFAs	42.65 ± 1.17	43.65 ± 1.57	43.76 ± 1.65	43.77 ± 2.36
C 16:1n-7[1,2]	0.35 ± 0.10	0.32 ± 0.11	0.25 ± 0.05	0.23 ± 0.04
C 18:1n-7	1.29 ± 0.16	1.25 ± 0.12	1.22 ± 0.13	1.19 ± 0.12
C 18:1n-9[1]	11.91 ± 1.06	11.59 ± 1.11	11.86 ± 1.11	11.69 ± 1.14
C 20:1n-9	0.22 ± 0.04	0.22 ± 0.05	0.22 ± 0.05	0.22 ± 0.04
C 22:1n-9[2]	0.12 ± 0.03	0.12 ± 0.02	0.15 ± 0.03	0.16 ± 0.04
C 24:1n-9	3.47 ± 0.56	3.67 ± 0.67	3.83 ± 0.51	3.70 ± 0.64
MUFAs	17.81 ± 1.53	17.66 ± 1.66	17.94 ± 1.49	17.60 ± 1.53
C 18:2n-6	9.60 ± 1.33	9.68 ± 1.06	9.49 ± 0.61	9.55 ± 0.86
C 18:3n-6	0.06 ± 0.04	0.05 ± 0.03	0.06 ± 0.02	0.06 ± 0.03
C 20:2n-6	0.26 ± 0.06	0.26 ± 0.06	0.22 ± 0.06	0.25 ± 0.11
C 20:3n-6	1.52 ± 0.35	1.50 ± 0.34	1.33 ± 0.47	1.28 ± 0.40
C 20:4n-6	13.56 ± 1.37	13.37 ± 1.19	13.81 ± 1.24	13.77 ± 1.28
C 22:2n-6	0.06 ± 0.01	0.07 ± 0.05	0.06 ± 0.02	0.08 ± 0.04
C 22:4n-6[1]	3.16 ± 0.73	3.08 ± 0.65	2.84 ± 0.64	2.81 ± 0.67
C 22:5n-6	0.56 ± 0.14	0.55 ± 0.18	0.50 ± 0.12	0.49 ± 0.10
N-6	28.66 ± 2.95	28.45 ± 2.39	28.79 ± 25.06	28.09 ± 3.14
C 18:3n-3	0.16 ± 0.05	0.16 ± 0.06	0.15 ± 0.03	0.16 ± 0.04
C 20:3n-3	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.03	0.05 ± 0.03
C 20:5n-3	0.50 ± 0.14	0.46 ± 0.11	0.51 ± 0.10	0.48 ± 0.13
C 22:5n-3[1,2]	1.87 ± 0.29	1.78 ± 0.25	2.11 ± 0.22	1.96 ± 0.32
C 22:6n-3	4.37 ± 1.00	4.23 ± 0.88	3.95 ± 0.98	3.76 ± 0.78
N-3[1]	6.92 ± 1.15	6.68 ± 0.96	6.77 ± 1.09	6.42 ± 1.00
EPA+DHA	4.87 ± 1.04	4.70 ± 0.91	4.46 ± 1.03	4.25 ± 0.87

Values are mean ± sd. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for birth control status x time by the linear mixed models procedure.

[1] significant main effect of time

[2] significant main effect of birth control status

Appendix 2

Supplementary Data for Chapter 6

Effect of Rat Estrous Cycle on Hepatic Expression of Fatty Acid Biosynthesis Enzymes

Table A2.1 Concentration of whole blood fatty acids in female rats over all four stages of the estrous cycle, and males.

Whole Blood Fatty Acids µg fatty acid/100 µl whole blood					
Name	**Metestrus**	**Diestrus**	**Proestrus**	**Estrus**	**Males**
C 10:0	0.08 ± 0.08^{ab}	0.13 ± 0.05^{ab}	0.08 ± 0.06^{a}	0.09 ± 0.08^{ab}	0.18 ± 0.03^{b}
C 12:0	0.12 ± 0.04	0.14 ± 0.10	0.10 ± 0.06	0.11 ± 0.04	0.11 ± 0.04
C 14:0	0.82 ± 0.37	0.78 ± 0.35	0.65 ± 0.20	0.69 ± 0.28	0.80 ± 0.26
C 16:0	38.30 ± 3.26	35.44 ± 3.14	39.61 ± 5.41	35.36 ± 2.50	40.00 ± 6.24
C 18:0	37.50 ± 2.66^{a}	35.92 ± 3.29^{a}	40.49 ± 5.12^{a}	35.67 ± 1.45^{a}	25.04 ± 4.74^{b}
C 20:0	0.43 ± 0.07^{ab}	0.43 ± 0.07^{ab}	0.53 ± 0.13^{a}	0.41 ± 0.04^{ab}	0.39 ± 0.06^{b}
C 22:0	0.87 ± 0.09	0.78 ± 0.21	1.01 ± 0.25	0.84 ± 0.13	0.76 ± 0.12
C 23:0	0.39 ± 0.07	0.37 ± 0.13	0.41 ± 0.06	0.36 ± 0.11	0.32 ± 0.12
C 24:0	2.27 ± 0.09	2.08 ± 0.30	2.43 ± 0.39	2.20 ± 0.24	2.13 ± 0.30
SFAs	81.76 ± 5.75^{ab}	76.92 ± 6.50^{ab}	86.30 ± 11.09^{a}	76.62 ± 4.08^{ab}	70.56 ± 11.83^{b}
C 12:1	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
C 14:1	0.14 ± 0.15	0.17 ± 0.14	0.07 ± 0.08	0.08 ± 0.11	0.22 ± 0.11
C 16:1n-7	0.59 ± 0.18^{a}	0.40 ± 0.09^{b}	0.54 ± 0.10^{ab}	0.48 ± 0.11^{ab}	0.49 ± 0.12^{ab}
C 18:1n-7	3.20 ± 0.23^{ab}	2.98 ± 0.35^{a}	3.29 ± 0.36^{ab}	2.96 ± 0.27^{a}	3.68 ± 0.57^{b}
C 18:1n-9	10.60 ± 2.04	9.24 ± 1.28	10.58 ± 1.27	9.85 ± 1.16	10.14 ± 1.70
C 20:1n-9	0.12 ± 0.03^{a}	0.17 ± 0.04^{a}	0.16 ± 0.07^{a}	0.17 ± 0.05^{a}	0.28 ± 0.09^{b}
C 22:1n-9	0.13 ± 0.06	0.14 ± 0.06	0.16 ± 0.06	0.11 ± 0.06	0.15 ± 0.03
C 24:1n-9	1.22 ± 0.13	1.12 ± 0.22	1.19 ± 0.36	1.16 ± 0.19	1.26 ± 0.22
MUFAs	16.03 ± 2.34	14.25 ± 1.75	16.01 ± 1.99	14.83 ± 1.53	16.25 ± 2.65
C 18:2n-6	21.79 ± 3.42^{ab}	19.75 ± 2.34^{a}	21.59 ± 2.99^{ab}	20.50 ± 2.29^{ab}	25.06 ± 4.91^{b}
C 18:3n-6	0.50 ± 0.18^{a}	0.44 ± 0.08^{a}	0.50 ± 0.09^{a}	0.47 ± 0.11^{a}	0.22 ± 0.05^{b}
C 20:2n-6	0.57 ± 0.07^{a}	0.53 ± 0.07^{a}	0.63 ± 0.09^{a}	0.55 ± 0.05^{a}	0.87 ± 0.13^{b}
C 20:3n-6	0.63 ± 0.09	0.60 ± 0.06	0.65 ± 0.07	0.56 ± 0.09	0.61 ± 0.09
C 20:4n-6	51.75 ± 3.81^{a}	50.10 ± 6.15^{a}	53.73 ± 4.28^{a}	50.74 ± 3.05^{a}	42.31 ± 6.94^{b}
C 22:2n-6	0.09 ± 0.06^{a}	0.12 ± 0.06^{a}	0.10 ± 0.08^{a}	0.08 ± 0.07^{a}	0.25 ± 0.04^{b}
C 22:4n-6	2.21 ± 0.14	2.12 ± 0.31	2.14 ± 0.40	1.99 ± 0.26	2.18 ± 0.35
C 22:5n-6	0.66 ± 0.22	0.62 ± 0.10	0.71 ± 0.26	0.57 ± 0.12	0.58 ± 0.15
N-6	78.20 ± 7.46	74.27 ± 8.54	80.05 ± 7.59	75.47 ± 5.10	72.07 ± 12.18
C 18:3n-3	0.36 ± 0.08^{ab}	0.32 ± 0.08^{a}	0.37 ± 0.13^{ab}	0.30 ± 0.07^{a}	0.49 ± 0.17^{b}
C 20:3n-3	0.03 ± 0.01	0.02 ± 0.02	0.05 ± 0.03	0.04 ± 0.04	0.02 ± 0.01
C 20:5n-3	0.69 ± 0.19	0.66 ± 0.16	0.68 ± 0.27	0.59 ± 0.16	0.61 ± 0.16
C 22:5n-3	1.90 ± 0.22^{ab}	1.63 ± 0.14^{a}	1.98 ± 0.52^{ab}	1.63 ± 0.22^{a}	2.18 ± 0.22^{b}
C 22:6n-3	4.94 ± 0.61^{ab}	4.54 ± 0.63^{b}	5.46 ± 0.49^{a}	4.63 ± 0.35^{b}	3.54 ± 0.61^{c}
N-3	7.92 ± 0.95^{ab}	7.17 ± 0.78^{a}	8.55 ± 0.96^{b}	7.19 ± 0.58^{a}	6.85 ± 1.06^{a}
C 20:3n-9	0.03 ± 0.03	0.03 ± 0.02	0.04 ± 0.01	0.03 ± 0.02	0.03 ± 0.02
Total	183.95 ± 12.55	172.65 ± 16.37	190.95 ± 20.39	174.15 ± 9.96	165.76 ± 27.48

Values are means ± SD. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for one way ANOVA

Table A2.2 Concentration of plasma fatty acids in female rats over all four stages of the estrous cycle, and males.

Plasma Fatty Acids µg fatty acid/100 µl plasma					
Name	**Metestrus**	**Diestrus**	**Proestrus**	**Estrus**	**Males**
C 10:0	0.00 ± 0.00	0.02 ± 0.03	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
C 12:0	0.28 ± 0.44	0.39 ± 0.68	0.75 ± 1.72	0.16 ± 0.10	0.36 ± 0.53
C 14:0	1.03 ± 0.72	0.79 ± 0.50	1.23 ± 1.63	0.79 ± 0.32	0.83 ± 0.48
C 16:0	26.75 ± 5.46	21.57 ± 2.90	26.07 ± 5.61	24.38 ± 4.41	26.02 ± 2.79
C 18:0	32.92 ± 5.39^{a}	28.61 ± 3.84^{a}	32.31 ± 6.37^{a}	31.68 ± 3.33^{a}	18.20 ± 2.25^{b}
C 20:0	0.29 ± 0.07	0.25 ± 0.09	0.34 ± 0.17	0.31 ± 0.07	0.24 ± 0.06
C 22:0	0.36 ± 0.09^{ab}	0.28 ± 0.08^{ac}	0.42 ± 0.11^{b}	0.41 ± 0.08^{ab}	0.21 ± 0.04^{c}
C 23:0	0.37 ± 0.14^{ab}	0.25 ± 0.08^{ac}	0.42 ± 0.14^{b}	0.36 ± 0.14^{abc}	0.19 ± 0.06^{c}
C 24:0	0.77 ± 0.20^{a}	0.45 ± 0.17^{b}	0.76 ± 0.19^{a}	0.72 ± 0.20^{a}	0.34 ± 0.05^{b}
SFAs	63.52 ± 10.43^{a}	53.20 ± 6.63^{ab}	62.99 ± 13.86^{a}	59.50 ± 7.98^{ab}	46.93 ± 4.98^{b}
C 12:1	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01
C 14:1	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
C 16:1n-7	0.89 ± 0.28	0.62 ± 0.09	0.79 ± 0.29	0.84 ± 0.27	0.82 ± 0.17
C 18:1n-7	2.21 ± 0.65^{ab}	1.72 ± 0.21^{a}	2.13 ± 0.48^{ab}	2.01 ± 0.40^{a}	2.60 ± 0.35^{b}
C 18:1n-9	12.77 ± 4.38	9.56 ± 1.53	10.89 ± 3.10	11.86 ± 2.98	13.00 ± 3.31
C 20:1n-9	0.13 ± 0.03^{ab}	0.09 ± 0.03^{a}	0.13 ± 0.07^{ab}	0.11 ± 0.04^{ab}	0.18 ± 0.05^{b}
C 22:1n-9	0.25 ± 0.07	0.25 ± 0.09	0.34 ± 0.20	0.25 ± 0.04	0.21 ± 0.04
C 24:1n-9	0.37 ± 0.13^{a}	0.22 ± 0.23^{ab}	0.37 ± 0.20^{a}	0.44 ± 0.18^{a}	0.09 ± 0.04^{b}
MUFAs	16.74 ± 5.43	12.52 ± 1.97	14.64 ± 3.72	15.55 ± 3.59	16.93 ± 3.70
C 18:2n-6	29.72 ± 7.48^{ab}	24.26 ± 3.50^{a}	28.01 ± 5.75^{ab}	27.28 ± 6.30^{ab}	34.86 ± 5.10^{b}
C 18:3n-6	1.08 ± 0.38^{a}	0.74 ± 0.19^{ab}	0.95 ± 0.32^{a}	0.89 ± 0.27^{a}	0.37 ± 0.07^{b}
C 20:2n-6	0.18 ± 0.06^{a}	0.16 ± 0.03^{a}	0.19 ± 0.03^{a}	0.18 ± 0.06^{a}	0.39 ± 0.07^{b}
C 20:3n-6	0.53 ± 0.14^{a}	0.35 ± 0.08^{b}	0.43 ± 0.09^{ab}	0.37 ± 0.10^{b}	0.42 ± 0.06^{ab}
C 20:4n-6	59.35 ± 10.63^{a}	50.97 ± 6.72^{ab}	54.77 ± 8.92^{a}	55.19 ± 8.95^{a}	39.94 ± 5.92^{b}
C 22:2n-6	0.03 ± 0.01	0.04 ± 0.03	0.05 ± 0.04	0.04 ± 0.04	0.06 ± 0.03
C 22:4n-6	0.48 ± 0.11	0.39 ± 0.08	0.49 ± 0.08	0.38 ± 0.08	0.40 ± 0.10
C 22:5n-6	0.22 ± 0.06^{a}	0.19 ± 0.13^{ab}	0.28 ± 0.11^{a}	0.20 ± 0.08^{ab}	0.07 ± 0.03^{b}
N-6	91.58 ± 17.90	77.10 ± 9.49	85.19 ± 14.61	84.53 ± 15.03	76.51 ± 10.45
C 18:3n-3	0.75 ± 0.28^{ab}	0.57 ± 0.16^{a}	0.67 ± 0.33^{ab}	0.65 ± 0.21^{ab}	1.00 ± 0.25^{b}
C 20:3n-3	0.03 ± 0.03	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.02
C 20:5n-3	1.10 ± 0.33	0.85 ± 0.20	1.01 ± 0.35	0.89 ± 0.27	0.93 ± 0.19
C 22:5n-3	0.86 ± 0.17^{a}	0.54 ± 0.13^{c}	0.76 ± 0.17^{abc}	0.65 ± 0.14^{bc}	0.79 ± 0.13^{ab}
C 22:6n-3	6.13 ± 1.22^{a}	4.86 ± 0.88^{ab}	5.98 ± 1.47^{a}	5.22 ± 0.82^{a}	3.38 ± 0.68^{b}
N-3	8.87 ± 1.91^{a}	6.85 ± 1.18^{ab}	8.47 ± 2.01^{a}	7.44 ± 1.27^{ab}	6.14 ± 1.13^{b}
C 20:3n-9	0.06 ± 0.04	0.06 ± 0.03	0.07 ± 0.04	0.08 ± 0.03	0.05 ± 0.02
Total	183.28 ± 32.29	149.73 ± 16.96	176.54 ± 40.10	167.10 ± 26.20	146.56 ± 17.46

Values are means ± SD. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for one way ANOVA

Table A2.3 Concentration of liver fatty acids in female rats over all four stages of the estrous cycle, and males.

	Liver Total Lipids µg fatty acids/mg liver				
Fatty Acid	**Metestrus**	**Diestrus**	**Proestrus**	**Estrus**	**Males**
C 10:0	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
C 12:0	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
C 14:0	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.02	0.09 ± 0.02	0.08 ± 0.02
C 16:0	5.43 ± 0.51	5.49 ± 0.52	5.76 ± 0.80	6.10 ± 1.16	6.76 ± 1.94
C 18:0	8.32 ± 0.58^{ab}	8.80 ± 0.91^{a}	8.65 ± 0.78^{ab}	8.83 ± 0.99^{a}	7.05 ± 1.99^{b}
C 20:0	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.02
C 22:0	0.06 ± 0.02	0.06 ± 0.01	0.05 ± 0.02	0.06 ± 0.01	0.06 ± 0.03
C 23:0	0.07 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.07 ± 0.02	0.06 ± 0.02
C 24:0	0.14 ± 0.03	0.19 ± 0.03	0.14 ± 0.04	0.15 ± 0.02	0.16 ± 0.04
SFAs	14.16 ± 1.05	14.72 ± 1.35	14.8 ± 1.20	15.34 ± 2.01	14.24 ± 3.97
C 12:1	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
C 14:1	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
C 16:1n-7	0.15 ± 0.03^{ab}	0.13 ± 0.02^{a}	0.15 ± 0.04^{ab}	0.18 ± 0.04^{b}	0.14 ± 0.05^{ab}
C 18:1n-7	0.65 ± 0.09	0.62 ± 0.07	0.66 ± 0.07	0.69 ± 0.11	0.97 ± 0.29
C 18:1n-9	2.14 ± 0.37	2.09 ± 0.34	2.26 ± 0.74	2.67 ± 0.79	2.24 ± 0.67
C 20:1n-9	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}	0.06 ± 0.02^{b}
C 22:1n-9	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C 24:1n-9	0.05 ± 0.02	0.05 ± 0.01	0.04 ± 0.02	0.05 ± 0.01	0.06 ± 0.02
MUFAs	3.06 ± 0.48	2.94 ± 0.38	3.17 ± 0.83	3.65 ± 0.95	3.50 ± 1.04
C 18:2n-6	6.17 ± 0.56	6.67 ± 0.82	6.89 ± 2.14	7.76 ± 2.01	8.26 ± 2.41
C 18:3n-6	0.15 ± 0.02^{ab}	0.14 ± 0.03^{a}	0.16 ± 0.04^{ab}	0.19 ± 0.05^{b}	0.08 ± 0.02^{c}
C 20:2n-6	0.10 ± 0.02^{a}	0.10 ± 0.02^{a}	0.10 ± 0.01^{a}	0.11 ± 0.02^{a}	0.24 ± 0.09^{b}
C 20:3n-6	0.16 ± 0.03	0.14 ± 0.01	0.14 ± 0.02	0.15 ± 0.03	0.17 ± 0.07
C 20:4n-6	7.71 ± 0.51	8.08 ± 0.82	8.05 ± 0.59	8.29 ± 0.73	8.29 ± 2.27
C 22:2n-6	0.02 ± 0.01	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.02
C 22:4n-6	0.17 ± 0.04	0.18 ± 0.03	0.18 ± 0.02	0.18 ± 0.04	0.16 ± 0.06
C 22:5n-6	0.10 ± 0.01^{a}	0.09 ± 0.04^{a}	0.10 ± 0.02^{a}	0.09 ± 0.03^{a}	0.04 ± 0.01^{b}
N-6	4.57 ± 1.01	15.41 ± 1.3	15.64 ± 2.44	16.79 ± 2.77	17.25 ± 4.87
C 18:3n-3	0.17 ± 0.03	0.20 ± 0.04	0.20 ± 0.12	0.25 ± 0.10	0.22 ± 0.08
C 20:3n-3	0.02 ± 0.01^{ab}	0.02 ± 0.01^{a}	0.01 ± 0.01^{a}	0.01 ± 0.01^{a}	0.03 ± 0.01^{b}
C 20:5n-3	0.13 ± 0.02	0.14 ± 0.02	0.13 ± 0.04	0.15 ± 0.04	0.14 ± 0.06
C 22:5n-3	0.29 ± 0.03^{ab}	0.26 ± 0.03^{a}	0.30 ± 0.05^{ab}	0.27 ± 0.05^{ab}	0.37 ± 0.12^{b}
C 22:6n-3	2.23 ± 0.24^{a}	2.18 ± 0.33^{a}	2.43 ± 0.15^{a}	2.28 ± 0.27^{a}	1.62 ± 0.51^{b}
N-3	2.84 ± 0.28^{ab}	2.79 ± 0.33^{ab}	3.08 ± 0.20^{a}	2.97 ± 0.43^{ab}	2.38 ± 0.77^{b}
C 20:3n-9	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Total	34.65 ± 2.65	35.88 ± 2.97	36.72 ± 4.33	38.77 ± 5.91	37.39 ± 10.57

Values are means ± SD. Means with different letters are significantly different by Tukey's post-hoc test, following a significant F-value for one way ANOVA

Appendix 3

Supplementary Data for Chapter 8

Characterization of *Fads2* Novel Transcript Variants

Table A3.1 Phospholipid fatty acid composition in HEK 293 cells electroporated with pCMV control, *FADS2_v0/1*or *FADS2_v2*.

	HEK 293 Phospholipids % weight total fatty acids		
	pCMV	***FADS2_v0/1***	***FADS2_v2***
C 10:0	0.14 ± 0.09	0.06 ± 0.04	0.05 ± 0.03
C 16:0	26.21 ± 1.81	26.00 ± 1.80	24.19 ± 1.50
C 18:0	22.29 ± 2.34[a]	25.34 ± 2.16[a]	18.30 ± 1.84[b]
C 20:0	0.43 ± 0.08	0.41 ± 0.08	0.31 ± 0.17
C 22:0	0.36 ± 0.10	0.35 ± 0.15	0.36 ± 0.07
C 24:0	0.68 ± 0.21	0.46 ± 0.15	0.54 ± 0.10
SFAs	50.21 ± 3.33[a]	52.72 ± 3.60[a]	43.87 ± 2.22[b]
C 16:1n-7	3.17 ± 0.79[a]	2.71 ± 1.01[a]	5.90 ± 1.32[b]
C 18:1n-7	4.62 ± 0.26[a]	4.51 ± 0.32[a]	5.21 ± 0.25[b]
C 18:1n-9	22.70 ± 4.37[a]	20.22 ± 3.70[a]	28.87 ± 2.22[b]
C 20:1n-9	0.34 ± 0.03	0.36 ± 0.06	0.41 ± 0.06
C 22:1n-9	1.42 ± 0.76	1.67 ± 0.79	0.97 ± 0.37
C 24:1n-9	0.53 ± 0.20	0.52 ± 0.15	0.38 ± 0.13
MUFAs	32.95 ± 5.04[a]	30.11 ± 4.94[a]	41.94 ± 3.41[b]
C 18:2n-6	1.91 ± 0.31	1.68 ± 0.15	1.60 ± 0.72
C 18:3n-6	0.79 ± 0.72	1.06 ± 0.84	0.40 ± 0.58
C 20:2n-6	0.31 ± 0.35	0.26 ± 0.26	1.16 ± 0.86
C 20:3n-6	0.60 ± 0.16	0.63 ± 0.06	0.49 ± 0.07
C 20:4n-6	5.54 ± 0.74[a]	6.47 ± 0.20[b]	5.06 ± 0.39[a]
C 22:2n-6	0.27 ± 0.25	0.20 ± 0.14	0.20 ± 0.12
C 22:4n-6	1.06 ± 0.13[a]	1.08 ± 0.10[a]	0.82 ± 0.11[b]
N-6	10.47 ± 1.44	11.37 ± 0.72	9.72 ± 0.85
C 18:3n-3	0.41 ± 0.25	0.39 ± 0.20	0.25 ± 0.24
C 20:3n-3	0.42 ± 0.59	0.10 ± 0.09	0.28 ± 0.29
C 20:5n-3	0.51 ± 0.27	0.64 ± 0.41	0.42 ± 0.46
C 22:5n-3	0.91 ± 0.22	0.91 ± 0.18	0.79 ± 0.15
C 22:6n-3	2.21 ± 0.56	2.30 ± 0.12	1.89 ± 0.49
N-3	4.31 ± 1.36	4.33 ± 0.55	3.22 ± 1.14

Values are means ± SD. Means with different letters are significantly different by Newman-Keul's post-hoc test, following a significant F-value for one way ANOVA

Table A3.2. Concentration of phospholipid fatty acids of HEK 293 cells electroporated with pCMV control, *FADS2_v0/1*or *FADS2_v2*.

HEK 293 Phospholipids µg fatty acid/mg cellular protein			
	pCMV	***FADS2_v0/1***	***FADS2_v2***
C 12:0	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C 16:0	1.17 ± 0.27	1.39 ± 0.23	1.56 ± 0.29
C 18:0	0.75 ± 0.26[a]	1.02 ± 0.32[ab]	1.36 ± 0.32[b]
C 20:0	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
C 22:0	0.01 ± 0.01	0.02 ± 0.00	0.02 ± 0.02
C 24:0	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
SFAs	1.98 ± 0.53[a]	2.49 ± 0.55[ab]	3.02 ± 0.60[b]
C 12:1	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
C 14:1	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.01
C 16:1n-7	0.29 ± 0.08	0.36 ± 0.04	0.30 ± 0.07
C 18:1n-7	0.26 ± 0.06	0.29 ± 0.05	0.31 ± 0.05
C 18:1n-9	1.46 ± 0.30	1.64 ± 0.24	1.74 ± 0.37
C 20:1n-9	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01
C 22:1n-9	0.03 ± 0.02	0.05 ± 0.03	0.07 ± 0.05
C 24:1n-9	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.01
MUFAs	2.09 ± 0.46	2.40 ± 0.33	2.50 ± 0.47
C 18:2n-6	0.04 ± 0.04[a]	0.06 ± 0.02[ab]	0.11 ± 0.03[b]
C 18:3n-6	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C 20:2n-6	0.13 ± 0.12	0.06 ± 0.05	0.06 ± 0.05
C 20:3n-6	0.02 ± 0.02	0.02 ± 0.01	0.04 ± 0.01
C 20:4n-6	0.18 ± 0.11	0.25 ± 0.09	0.34 ± 0.10
C 22:2n-6	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
C 22:4n-6	0.02 ± 0.02[a]	0.04 ± 0.02[ab]	0.06 ± 0.02[b]
C 22:5n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
N-6	0.43 ± 0.19	0.47 ± 0.17	0.62 ± 0.15
C 18:3n-3	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C 20:3n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C 20:5n-3	0.02 ± 0.01	0.03 ± 0.04	0.03 ± 0.04
C 22:5n-3	0.02 ± 0.01	0.03 ± 0.02	0.05 ± 0.02
C 22:6n-3	0.06 ± 0.04[a]	0.09 ± 0.03[ab]	0.12 ± 0.04[b]
N-3	0.11 ± 0.05	0.16 ± 0.08	0.21 ± 0.06
PUFAs	0.54 ± 0.23	0.62 ± 0.24	0.83 ± 0.21
HUFAs	0.32 ± 0.19	0.44 ± 0.19	0.63 ± 0.17
Total	4.60 ± 1.15	5.51 ± 1.08	6.35 ± 1.25

Values are means ± SD. Means with different letters are significantly different by Newman-Keul's post-hoc test, following a significant F-value for one way ANOVA

Table A3.3 Phospholipid fatty acid composition in HEK 293 cells electroporated with pCMV control or *Fads2_v0/1* with increasing amounts of *Fads2_v2.*

	HEK 293 Phospholipids % weight total fatty acids			
	10 µg pCMV	**5 µg *FADS2_v0/1***	**5 µg *FADS2_v0/1* + 2.5 µg *FADS2_v2* + 2.5 µg pCMV**	**5 µg *FADS2_v0/1* + 5 µg *FADS2_v2***
C 10:0	0.14 ± 0.09	0.06 ± 0.04	0.04 ± 0.04	0.06 ± 0.01
C 16:0	26.76 ± 1.37	26.00 ± 1.80	26.14 ± 0.79	27.22 ± 0.82
C 18:0	22.18 ± 2.59	25.34 ± 2.16	23.99 ± 4.86	23.07 ± 3.44
C 20:0	0.42 ± 0.08	0.41 ± 0.08	0.47 ± 0.13	0.37 ± 0.14
C 22:0	0.36 ± 0.11	0.35 ± 0.15	0.35 ± 0.00	0.38 ± 0.03
C 24:0	0.71 ± 0.21	0.46 ± 0.15	0.59 ± 0.20	0.61 ± 0.17
SFAs	50.72 ± 3.45	52.72 ± 3.60	51.59 ± 5.49	51.68 ± 4.06
C 16:1n-7	3.30 ± 0.82	2.71 ± 1.01	2.66 ± 0.82	3.95 ± 1.97
C 18:1n-7	4.54 ± 0.19	4.51 ± 0.32	4.75 ± 0.26	4.77 ± 0.38
C 18:1n-9	23.31 ± 4.59	20.22 ± 3.70	21.52 ± 3.63	23.47 ± 3.43
C 20:1n-9	0.34 ± 0.03	0.36 ± 0.06	0.29 ± 0.13	0.36 ± 0.08
C 22:1n-9	1.23 ± 0.68	1.67 ± 0.79	0.89 ± 0.14	0.78 ± 0.11
C 24:1n-9	0.55 ± 0.22	0.52 ± 0.15	0.72 ± 0.10	0.61 ± 0.08
MUFAs	33.47 ± 5.45	30.11 ± 4.94	30.81 ± 4.42	33.94 ± 5.64
C 18:2n-6	1.86 ± 0.33	1.68 ± 0.15	1.98 ± 0.41	1.34 ± 0.55
C 18:3n-6	0.56 ± 0.48	1.06 ± 0.84	0.62 ± 0.59	0.42 ± 0.18
C 20:2n-6	0.36 ± 0.36	0.26 ± 0.26	0.10 ± 0.12	0.07 ± 0.06
C 20:3n-6	0.59 ± 0.17	0.63 ± 0.06	0.62 ± 0.13	0.63 ± 0.16
C 20:4n-6	5.28 ± 0.43[a]	6.47 ± 0.20[b]	6.63 ± 0.26[b]	5.11 ± 1.09[a]
C 22:2n-6	0.30 ± 0.26	0.20 ± 0.14	0.16 ± 0.13	0.05 ± 0.03
C 22:4n-6	1.04 ± 0.14	1.08 ± 0.10	1.12 ± 0.13	0.89 ± 0.10
N-6	9.99 ± 0.94	11.37 ± 0.72	11.24 ± 0.68	8.50 ± 1.86
C 18:3n-3	0.31 ± 0.11	0.39 ± 0.20	0.30 ± 0.12	0.21 ± 0.10
C 20:3n-3	0.42 ± 0.59	0.10 ± 0.09	0.07 ± 0.08	0.08 ± 0.06
C 20:5n-3	0.47 ± 0.28	0.64 ± 0.41	0.60 ± 0.15	0.56 ± 0.13
C 22:5n-3	0.84 ± 0.17	0.91 ± 0.18	0.96 ± 0.12	0.83 ± 0.19
C 22:6n-3	2.12 ± 0.57	2.30 ± 0.12	2.46 ± 0.73	2.07 ± 0.23
N-3	4.03 ± 1.31	4.33 ± 0.55	4.38 ± 0.87	3.75 ± 0.56

Values are means ± SD. Means with different letters are significantly different by Newman-Keul's post-hoc test, following a significant F-value for one way ANOVA

Table A3.4. Concentration of fatty acids in phospholipids of HEK 293 cells electroporated with pCMV control or *Fads2_v0/1* with increasing amounts of *Fads2_v2*.

HEK 293 Phospholipids µg fatty acid/mg cellular protein				
	10 µg pCMV	**5 µg *FADS2_v0/1***	**5 µg *FADS2_v0/1* + 2.5 µg *FADS2_v2* + 2.5 µg pCMV**	**5 µg *FADS2_v0/1* + 5 µg *FADS2_v2***
C 10:0	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.00	0.01 ± 0.00
C 16:0	2.70 ± 0.63	4.08 ± 0.93	2.55 ± 0.90	3.95 ± 1.47
C 18:0	2.19 ± 0.58	4.05 ± 0.88	2.10 ± 0.72	3.58 ± 1.00
C 20:0	0.04 ± 0.01	0.08 ± 0.03	0.04 ± 0.03	0.06 ± 0.01
C 22:0	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.01
C 24:0	0.08 ± 0.01	0.09 ± 0.05	0.07 ± 0.01	0.07 ± 0.01
SFAs	5.06 ± 1.22	8.39 ± 1.89	4.80 ± 1.67	7.72 ± 2.49
C 16:1n-7	0.33 ± 0.10	0.31 ± 0.19	0.31 ± 0.16	0.45 ± 0.26
C 18:1n-7	0.45 ± 0.11	0.65 ± 0.20	0.49 ± 0.17	0.68 ± 0.29
C 18:1n-9	2.11 ± 0.45	2.65 ± 1.06	2.32 ± 0.70	3.09 ± 1.39
C 20:1n-9	0.03 ± 0.00	0.06 ± 0.01	0.04 ± 0.03	0.05 ± 0.02
C 22:1n-9	0.08 ± 0.01	0.18 ± 0.05	0.08 ± 0.04	0.10 ± 0.02
C 24:1n-9	0.07 ± 0.02	0.08 ± 0.05	0.07 ± 0.03	0.09 ± 0.02
MUFAs	3.07 ± 0.69	3.95 ± 1.46	3.30 ± 1.13	4.46 ± 2.00
C 18:2n-6	0.18 ± 0.03	0.25 ± 0.07	0.20 ± 0.02	0.26 ± 0.09
C 18:3n-6	0.08 ± 0.05	0.12 ± 0.07	0.03 ± 0.04	0.08 ± 0.02
C 20:2n-6	0.01 ± 0.01	0.04 ± 0.02	0.00 ± 0.01	0.01 ± 0.00
C 20:3n-6	0.06 ± 0.02	0.08 ± 0.03	0.07 ± 0.03	0.09 ± 0.03
C 20:4n-6	0.52 ± 0.12	0.95 ± 0.24	0.65 ± 0.19	0.88 ± 0.33
C 22:2n-6	0.04 ± 0.02	0.04 ± 0.05	0.02 ± 0.01	0.01 ± 0.01
C 22:4n-6	0.10 ± 0.00	0.18 ± 0.05	0.11 ± 0.02	0.14 ± 0.05
C 22:5n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
N-6	0.98 ± 0.16	1.67 ± 0.32	1.08 ± 0.29	1.47 ± 0.53
C 18:3n-3	0.03 ± 0.01	0.07 ± 0.03	0.02 ± 0.01	0.04 ± 0.01
C 20:3n-3	0.04 ± 0.05	0.05 ± 0.07	0.01 ± 0.00	0.01 ± 0.01
C 20:5n-3	0.06 ± 0.00	0.12 ± 0.01	0.06 ± 0.01	0.10 ± 0.04
C 22:5n-3	0.08 ± 0.01	0.16 ± 0.07	0.09 ± 0.02	0.14 ± 0.08
C 22:6n-3	0.24 ± 0.03	0.30 ± 0.14	0.29 ± 0.09	0.33 ± 0.14
N-3	0.46 ± 0.01	0.70 ± 0.11	0.47 ± 0.13	0.62 ± 0.27
PUFAs	1.44 ± 0.15	2.37 ± 0.44	1.55 ± 0.41	2.08 ± 0.80
HUFAs	1.10 ± 0.11	1.85 ± 0.38	1.28 ± 0.35	1.68 ± 0.67
Total	9.58 ± 2.01	14.71 ± 3.68	9.65 ± 3.21	14.26 ± 5.30

Values are means ± SD. Means with different letters are significantly different by Newman-Keul's post-hoc test, following a significant F-value for one way ANOVA