

**Examination of the Effects of Sex and Sex Hormones on Monounsaturated Fatty Acid**

**Biosynthesis**

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.

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## Abstract

Monounsaturated fatty acids (MUFA) have been viewed as either beneficial or neutral with respect to health; however, recent evidence suggests that MUFA may be associated with obesity and cardiovascular disease. Sex differences in MUFA composition have been reported in both rats and humans that may be mediated by sex hormones. Therefore, differences in fatty acid and enzyme expression were examined in male and female rats. HepG2 cells treated with  $17\beta$ -estradiol, progesterone and testosterone (0, 10, 30 and 100 nM), and ovariectomized female rats with hormone implants (sham, no treatment, estradiol, progesterone, and estradiol plus progesterone) examined the role of sex hormones in MUFA metabolism. MUFA concentrations were determined by gas chromatography. The mRNA and protein expression of stearoyl-CoA desaturase and elongase 6, key enzymes involved in MUFA biosynthesis, were measured by real-time PCR and immunoblotting, respectively. Elongase 6 protein expression was higher in females as compared with males, increased with estradiol and progesterone treatment of HepG2 cells, and was higher in ovariectomized rats treated with estradiol. Elongase 6 expression was also decreased in HepG2 cells treated with testosterone. In contrast, the expression of stearoyl-CoA desaturase did not appear to be associated with sex or sex hormones in HepG2 cells, although ovariectomized rats treated with estradiol plus progesterone had increased stearoyl-CoA desaturase protein levels compared with sham controls. Sex differences and differences after hormonal treatments were observed in the fatty acid concentrations of MUFA and immediate MUFA substrates including 16:0, 16:1n-7, 18:0, 18:1n-7 and 18:1n-9. These differences in MUFA are consistent with the changes in elongase 6 expression. The effect of sex and hormone status on elongase 6 has not been previously examined, but these results suggest elongase 6 is an important factor in determining MUFA composition. These sex and hormonal differences in

MUFA composition may contribute to sex differences in obesity and cardiovascular disease. Further work examining other factors involved in MUFA composition including oxidation, synthesis of complex lipids and the effect of diet is required.

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## List of Abbreviations

ChREBP	Carbohydrate response element binding protein
DGAT	Acyl-CoA:diacylglycerol acyltransferase
Elovl5	Elongase 5
Elovl6	Elongase 6
FABP	Fatty acid binding protein
FAT/CD36	Fatty acid translocase
FBS	Fetal bovine serum
FDR	False discovery rate
LXR	Liver X receptor
MUFA	Monounsaturated fatty acid
OVX	Ovariectomized rat
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PPAR $\alpha$	Peroxisome proliferator activated receptor $\alpha$
PUFA	Polyunsaturated fatty acid
SCD1	Stearoyl-CoA desaturase 1
SCD2	Stearoyl-CoA desaturase 2
SFA	Saturated fatty acid
SREBP-1c	Sterol regulatory element binding protein-1c
TAG	Triacylglycerol

## Chapter 1

### Introduction

Monounsaturated fatty acids (MUFA) are involved in a wide range of physiological functions, including oxidation for energy, and as components of phospholipids, cholesteryl esters and triacylglycerols (TAG). MUFA can be consumed from the diet, or synthesized within the body by the enzyme stearoyl-CoA desaturase (SCD). Despite the importance of MUFA, the regulation of *de novo* MUFA biosynthesis is not fully understood. One important factor may be sex, as significant differences in MUFA composition have been reported in fingertip prick whole blood of humans (Marangoni et al., 2007) and hepatic phospholipids of rats (Extier et al., 2010; Burdge et al., 2008). These sex differences may be due to the effects of sex hormones on the enzymes involved in MUFA biosynthesis. Estrogen deficiency (following ovariectomy or menopause), for example, has well-established effects on weight gain and lipid accumulation (D'Eon et al., 2005). More specifically, increased mRNA expression of SCD is observed in ovariectomized rats compared with sham-operated controls (Alessandri et al., 2011; Gao et al., 2006; Paquette et al., 2008). This effect is diminished following estradiol supplementation in ovariectomized rats (Alessandri et al., 2011). Changes in MUFA composition occur in menopausal women receiving or not receiving hormone replacement therapy (Stark, Park, and Holub, 2003) and during pregnancy (Stark et al., 2005), suggesting a role for estradiol in the regulation of SCD expression. However, SCD protein and the tissue MUFA composition were either not reported, or not all MUFA were included in the analyses. Furthermore, other enzymes involved in MUFA biosynthesis, such as elongase 6, were not reported in those studies, thereby leaving gaps in the current understanding of sex hormones on MUFA biosynthesis.

Understanding the regulation of MUFA biosynthesis is important as MUFA and SCD have been implicated in chronic diseases and obesity. For example, while MUFA have been traditionally thought of as beneficial, or at least neutral with respect to heart health (Baum et al., 2012), recent reports suggest 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., 2012). Both global and liver-specific SCD-knockout mice show reduced body weight and protection against diet-induced weight gain, as well as increased insulin sensitivity (Ntambi et al., 2002). The activity of SCD is very closely linked to the rate-limiting enzyme in TAG production, acyl-CoA:diacylglycerol acyltransferase (DGAT) (Man et al., 2006). Consequently, MUFA produced by SCD are very easily used as substrates for TAG synthesis, at an even greater rate than dietary MUFA (Man et al., 2006).

Therefore, the goal of this thesis was to assess the effects of sex and sex hormones on MUFA biosynthesis. A comparison of liver, plasma and erythrocyte saturated fatty acid (SFA) and MUFA composition, as well as mRNA and protein expression of enzymes involved in *de novo* synthesis of MUFA were examined in male and female rats. To test the hypothesis that sex hormones are a contributing factor in sex differences, the human cell line HepG2 was cultured with varying concentrations of 17 $\beta$ -estradiol, progesterone or testosterone, to determine their effects on enzyme expression and fatty acid composition. Finally, rats were ovariectomized or sham operated, with some ovariectomized rats being implanted with constant release hormone pellets containing 17 $\beta$ -estradiol, progesterone, or both. Liver and plasma fatty acid composition were determined, and a whole transcriptome analysis comparing sham operated and ovariectomized rats was done to assess gene changes related to MUFA synthesis. Finally, protein analysis was completed on key enzymes involved in MUFA biosynthesis.

## Chapter 2

### Biochemical Foundations

#### 2.1 Overview of Fatty Acids

Fatty acids are a major macronutrient class which not only provide energy, but are involved in cell signalling and long-term energy storage, as well as being crucial components of cell membranes. Fatty acids are present in TAG, phospholipids, and cholesteryl esters, with a small pool of non-esterified fatty acids also being present.

In animals, fatty acids generally contain even numbers of carbons in straight chains. A carboxylic acid (-COOH) is present at one end of the hydrocarbon chain, with the other end being a methyl group (-CH<sub>3</sub>). Fatty acids are classified based on the number of carbon-carbon double bonds in their hydrocarbon structure: saturated fatty acids have no double bonds, monounsaturated fatty acids have one double bond, and polyunsaturated fatty acids have more than one double bond. These double bonds are typically in the *cis* configuration. Fatty acids are named systematically, based on the number of carbon atoms in the hydrocarbon chain, the number of double bonds, and the location of the first double bond relative to the methyl end, such that 18:1n-9 indicates that there are 18 carbons in the chain, with 1 double bond between carbons 9 and 10 relative to the methyl end.

Palmitic acid (16:0) can be produced *de novo* in the body via condensation of successive units of malonyl-CoA (COOH-CH<sub>2</sub>-CO-S-CoA) by the enzyme fatty acid synthase (Schiller and Bensch, 1971). Palmitic acid can be incorporated into complex lipids, or can be further metabolized to longer-chain SFA or unsaturated fatty acids. Fatty acids that can be synthesized within the body are considered non-essential fatty acids. Other fatty acids, such as alpha-

linolenic acid, cannot be synthesized in humans, and are thus considered conditionally essential, as they must be obtained through the diet.

## **2.2 Monounsaturated Fatty Acid Biosynthesis**

MUFA biosynthesis involves both elongation (addition of 2 carbon atoms to the hydrocarbon chain) and desaturation (addition of a double bond) (**Figure 1**). One major site for this metabolism is in the liver (Saleh, Sniderman, and Cianflone, 1999). SCD1 is the main isoform of SCD in the liver of humans and rodents, though several other isoforms have been identified (Miyazaki, Bruggink, and Ntambi, 2006). The preferred substrate of SCD1 is 18:0, which is desaturated to produce 18:1n-9 (Miyazaki, Bruggink, and Ntambi, 2006). Palmitate is also a substrate of SCD1, but the affinity for this fatty acid is not as high (Hudgins et al., 1996). Desaturation of 16:0 by SCD results in palmitoleic acid, 16:1n-7. This product can be elongated, to form *cis*-vaccenic acid, 18:1n-7 (Green et al., 2010). Although these products are less common than 18:1n-9, they can also be incorporated into greater lipids. In addition, fatty acid synthase which synthesizes palmitate *de novo*, and elongase 6 which preferentially elongates SFA and MUFA, are involved in MUFA biosynthesis and elongation (**Figure 1**) (Green et al., 2010). Elongase 5 is less active towards SFA and MUFA compared with polyunsaturated fatty acids (PUFA), but its high expression level in the liver (Wang et al., 2005b) indicates that it is likely involved in hepatic SFA and MUFA metabolism (Green et al., 2010).

## **2.3 Regulation of MUFA Biosynthesis**

The role of sex hormones on regulating SCD1 expression has been studied, with estrogen and testosterone having opposing effects. Estrogen appears to negatively regulate SCD1 expression. Ovariectomized rats show an increase in hepatic SCD1 mRNA levels as compared with sham-operated controls (Alessandri et al., 2011). This effect is diminished following



treatment with an estrogen supplement (Alessandri et al., 2011). The effect of estrogen appears to result from long-term exposure, as ovariectomized mice given an injection of 17 $\beta$ -estradiol and euthanized 2 or 4 hours later did not display any decreases in SCD1 expression as compared with ovariectomized controls (Gao et al., 2006). Testosterone has also been studied with regards to SCD1 activity. Testosterone treatment in both male and female eugonadal rats increased liver SCD1 activity as compared with untreated controls (Marra and de Alaniz, 1989). Cultured Sertoli cells, which express androgen receptors (Shan, Bardin, and Hardy, 1997) and are regulated by testosterone (de Kretser et al., 1998), exhibited an increase in 18:1n-9 and a decrease in 18:0 following treatment with testosterone, compared with control cells (Hurtado de Catalfo and de Gomez Dumm, 2005). However, SCD mRNA and/or protein were not measured in these studies. Fatty acid composition changes were used in these studies to estimate SCD activity, without measuring protein.

SCD1 expression is regulated by nutrients, hormones and transcription factors. Many of these factors exert their effects through 3 major transcription factors, which have been demonstrated to increase transcription of SCD1: sterol regulatory element binding protein-1c (SREBP-1c) (Tabor et al., 1999), carbohydrate response element binding protein (ChREBP) (Iizuka et al., 2004) and liver x receptor (LXR) (Ducheix et al., 2011). Physiological regulators that increase expression or activity of these transcription factors indirectly increase the expression of SCD1, whereas downregulation of these transcription factors leads to a subsequent decrease in SCD1 expression. Both SREBP-1c and LXR have been shown to bind to elements in the promoter region of SCD1 (Mauvoisin and Mounier, 2011).

SREBP-1c is transcribed and translated into an immature peptide, which is anchored on the endoplasmic reticulum. SREBP-cleavage activating protein regulates the formation of the

mature, active form of SREBP. Following cleavage of the immature peptide, the mature SREBP translocates to the nucleus, where it is able to bind to the sterol regulator element located on the promoter of the SCD1 gene, and increase transcription (Ntambi, 1999). PUFA regulate transcription of SCD1 via inhibition of SREBP-1c maturation (Ntambi, 1999). Conversely, increased cellular concentrations of glucose and saturated fatty acids positively regulate SREBP-1c (Hasty et al., 2000). Insulin also activates SREBP-1c transcription (Elam et al., 2010).

ChREBP is required for carbohydrate-induced transcriptional activation of SCD1. Knockout ChREBP mice with normal SREBP-1c had a decrease in mRNA expression of several enzymes in the fatty acid synthesis pathway, indicating that activation of both transcription factors is required for normal expression (Iizuka et al., 2004). ChREBP is regulated at the protein level by reversible phosphorylation: when phosphorylated at Ser-196, Ser-626 and Thr-666, ChREBP is restricted to the cytoplasm. Upon dephosphorylation, ChREBP is translocated from the cytoplasm to the nucleus, where it is able to induce gene transcription. Glucose and fructose both positively regulate ChREBP activity (Grefhorst et al., 2010).

LXR, a member of the nuclear receptor superfamily, is another transcription factor that increases SCD1 transcription. In the absence of ligand (oxysterols, an oxidative derivative of cholesterol) (Janowski et al., 1999), LXR binds to the promoter of target genes and interacts with corepressors, preventing the cellular transcription machinery from interacting with the gene's transcription initiation site (Hu et al., 2003). Upon ligand binding, the corepressors are released, and LXR changes conformation, allowing transcription to begin (Hu et al., 2003).

Other regulators of SCD1 are leptin and peroxisome proliferator activated receptor alpha (PPAR $\alpha$ ). Leptin is a strong repressor of SCD1 expression, through both direct repression of SCD1 transcription (Mauvoisin et al., 2010), and indirectly, via activation of STAT3 (Saxena et

al., 2007), which is a negative regulator of SREBP-1c (Gao et al., 2006). SCD1 expression has also been shown to be dependent on PPAR $\alpha$  (Hellemans et al., 2009).

## **2.4 Sex and Hormone Differences in Tissue SFA and MUFA Composition**

Male rat livers have significantly higher 16:0 in two specific phospholipid classes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas livers of female rats have significantly higher 18:0 in these phospholipids (Burdge et al., 2008). Males also have significantly higher 18:1n-9 in liver PC as compared with females (Burdge et al., 2008). No differences have been reported in other lipid pools. Similarly, the SCD activity index, the ratio of 18:1n-9 to 18:0, is significantly higher in male rat liver PC as compared with female rat liver PC (Extier et al., 2010), suggesting that a greater amount of SCD1 protein may be present in male livers as compared with female livers, leading to the observed increase in 18:1n-9, although this has not been tested. Human studies have confirmed these animal results. As a percentage of total lipids in a fingertip prick blood sample, 18:1n-9 and total MUFA were significantly higher in men as compared with women (Marangoni et al., 2007).

Postmenopausal women not receiving hormone replacement therapy have a significantly lower percentage of 16:0 and 16:1n-7 in serum phospholipids compared with premenopausal women (Stark, Park, and Holub, 2003). Interestingly, the relative percentage of these fatty acids in postmenopausal women taking hormone replacement is even higher than premenopausal women (Stark, Park, and Holub, 2003). Furthermore, the relative percentage of 16:0, 18:0, 16:1n-7 and 18:1n-9 in both plasma and erythrocytes is different at 24 weeks gestation, delivery and 3 months postpartum in women (Stark et al., 2005). Taken together, these results suggest that hormone status plays a major role in MUFA metabolism.

## **2.5 Synthesis of Complex Lipids**

Fatty acids that have been consumed in the diet or synthesized *de novo* are incorporated into complex lipids, including into phospholipids as structural components of cell membranes, and TAG, for energy storage. There are several enzymes and pathways involved in the synthesis of these lipids.

*De novo* synthesis of phospholipids (PC and PE) uses CTP as a cofactor, forming CDP-choline and CDP-ethanolamine as intermediates, respectively (Kennedy and Weiss, 1956). PC can also be synthesized by the action of the enzyme phosphatidylethanolamine methyltransferase, which adds a methyl group to PE. PC synthesis through this pathway has shown to be greater in females (Bjornstad and Bremer, 1966), with expression of phosphatidylethanolamine methyltransferase being higher in females as compared with males (Johnson and Krzysztof Blusztajn, 1998). These sex differences may be associated with sex hormones, as rates of methylation of PE vary during the estrous cycle of rats (Drouva et al., 1987).

Although the fatty acid composition of phospholipids varies greatly, there is a general preference for a saturated fatty acid at the *sn*-1 position and an unsaturated fatty acid at the *sn*-2 position. This specificity results from phospholipid remodelling reactions, where phospholipases remove fatty acids from individual phospholipid species, and acyltransferases selectively place new acyl chains at the free position on the phospholipid (Lands and Hart, 1965).

Like phospholipids, TAG can be synthesized *de novo*, through one of two major pathways, each of which uses fatty acyl CoAs and DGAT to complete the final step (Yen et al., 2008). This *de novo* synthesis occurs via the phosphatidic acid pathway, which involves a stepwise acylation of *sn*-glycero-3-phosphate and/or dihydroxyacetone phosphate, produced during glycolysis, to phosphatidic acid. Phosphatidic acid is hydrolyzed to *sn*-1,2-diacylglycerol,

in a reaction catalyzed by lipin, (Bou et al., 2010), which is used for synthesis of phospholipids or can be acylated further to form a triacylglycerol (Lehner and Kuksis, 1996). TAG can also be synthesized by the monoacylglycerol pathway, whereby two fatty acyl-CoA chains are esterified to a monoacylglycerol backbone (Yen et al., 2008). At low free fatty acid concentrations, fatty acids have been shown to enter the TAG synthesis pathway more readily in female livers as compared with males; however, this sex difference disappears at higher concentrations of fatty acids (Ockner et al., 1979).

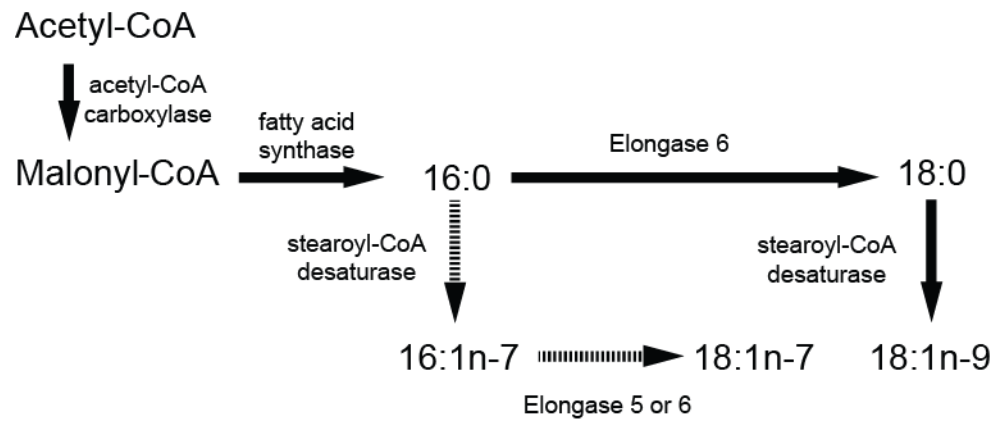
## **2.6 Fatty Acid Uptake and Transport**

The major sites of regulation of fatty acid metabolism are the liver and adipose tissue. The liver derives its fatty acids from both dietary intake and *de novo* lipogenesis. The products of lipid digestion from dietary fatty acids that are absorbed by the intestinal mucosa are re-esterified into TAG in the intestinal mucosa, where they are then packaged into chylomicrons for secretion and transport in the blood (Xiao et al., 2011). TAG synthesized in the liver can be stored or packaged into very low density lipoproteins, which are excreted into the blood for transport to tissues (Xiao et al., 2011). Lipoprotein lipase hydrolyzes these lipoproteins, releasing non-esterified fatty acids that can be taken up by cells (Otarod and Goldberg, 2004). Expression of lipoprotein lipase is higher in muscle of women as compared with men, which appears to be dependent on hormone status (Maher et al., 2009), while expression of hepatic lipase is lower in women (Deeb et al., 2003).

Transport of fatty acids occurs either by simple diffusion across the cell membrane or by the assistance of fatty acid transporters, such as the integral membrane protein fatty acid translocase (FAT/CD36), fatty acid binding proteins (FABP) and fatty acid transport proteins (Stremmel et al., 2001). Both FAT/CD36 and FABP have been shown to have higher expression

in female livers as compared with males (Stahlberg et al., 2004; Ockner et al., 1979). Once inside the cells, fatty acids are first acylated in an ATP-dependent reaction, producing fatty acyl-CoA, thereby preparing the fatty acids for oxidation or incorporation into greater lipids, including TAG, phospholipids and cholesteryl esters.

Non-esterified fatty acids are released from adipose tissue stores during fasting and energy-demanding states, by hydrolysis of adipose tissue TAG. Adipose triglyceride lipase, also known as desnutrin, has been demonstrated to catalyze the initial step in triacylglycerol breakdown (Schoenborn et al., 2006; Zimmermann et al., 2004; Duncan et al., 2007). Hormone sensitive lipase hydrolyzes the second ester bond (Haemmerle et al., 2002; Duncan et al., 2007), and its expression has been demonstrated to be higher in muscle of women as compared with men (Roepstorff et al., 2006). These non-esterified fatty acids can be released into the blood stream, bound to albumin as a transporter (van der Vusse, 2009) to cells for metabolism.



**Figure 1.** Diagram of enzymes involved in *de novo* fatty acid synthesis. Solid arrows indicate dominant pathway while dashed line indicates the alternate pathway.

## Chapter 3

### Methodological Foundations

#### 3.1 Experimental Models

Two commonly used models for studying fatty acid metabolism are animal (rodent) models and cell culture models. Rodents (most often rats or mice) provide information about metabolism at a whole body level. There are many variables that can be manipulated, such as dietary composition, hormone status (through ovariectomization, or implantation of a hormone pellet, for example), and exercise. However, there are many processes that occur within a living organism and interactions occur. Therefore, manipulation of physiological parameters may lead to unknown or undesired side effects, and may make conclusions hard to draw. This drawback of an animal model is somewhat relieved in cell culture models, where exposure to nutrients and hormones can be tightly controlled, and the cells are exposed to only what is provided in their growth medium. Although this provides a model for the effect of a specific target on metabolism, the interaction that may occur in a living system cannot be observed, and therefore the effect seen in cells may not be the same effect as in an animal.

In the present study, HepG2 cells are used to complement experiments in Sprague-Dawley rats. HepG2 is a cell line which originated from the liver of a 15 year old Caucasian male with hepatocellular carcinoma. These cells were chosen because they are regarded as a model system for the liver (van IJzendoorn et al., 1997). However, it is not a perfect model, because they are cancerous cells, which may result in abnormal or uncontrolled growth not seen in a healthy liver. Additionally, cancerous cells often have abnormal response to nutrients, including loss of feedback regulation.

#### 3.2 Separation and Quantification of Fatty Acids



Fatty acids are separated and quantified via the use of chromatography. Chromatography separates a mixture of components based on polarity. There are two phases: the mobile phase, which is a solvent that interacts with and carries the mixture, and a stationary phase that remains in place as the mobile phase continues over it. The two major types of chromatography used in this thesis are gas chromatography and thin layer chromatography.

Gas chromatography uses a carrier gas (such as helium) as the mobile phase, and a liquid as the stationary phase. There are 3 main components to a gas chromatograph: an injector that volatilizes the samples; a column that has a liquid lining, the stationary phase; and a detector that converts the concentration of the components in the gas phase into an electrical signal (James and Martin, 1951). As the carrier gas moves through the column, the components of the sample diffuse in and out of the two phases. The retention time, or the amount of time it takes for the components of the sample to move through the column, depends on factors such as chain length and the number of double bonds, in the case of fatty acids (James and Martin, 1951). The identity of the fatty acids in the sample is confirmed by comparing the retention time of peaks in the sample to those in a known mixture. The concentration of the fatty acids can be determined by adding an internal standard to the samples prior to extraction of the lipids from the sample; the peak area of the fatty acid is then compared to the area of the standard.

In thin layer chromatography, an adsorbent material such as silica gel coating a glass plate, is the stationary phase. Different liquid solvents, depending on the desired separation of the analyte, are used as the mobile phase. The analyte is applied to the bottom of the plate, the origin. The mobile phase moves up the plate through the capillary action and carries the sample with it. The components of the sample are separated based on their solubility in the mobile phase (Kirchner, Miller, and Keller, 1951). Lipids extracted from tissues or cells can be separated into

specific lipid classes including phospholipids, non-esterified fatty acids, TAG and cholesteryl esters. Specific phospholipid classes, PE and PC, among others, can also be separated by varying the solvents of the mobile phase. The specific lipid classes can be isolated and the fatty acid composition of each one can then be determined.

### **3.3 Gene Expression Analysis**

Analysis of gene expression can be accomplished by determining the number of copies of mRNA of a gene of interest, thereby determining whether a gene is expressed at a higher level in one group as compared with another. Reverse-transcriptase real-time PCR and a high-density microarray are the two gene expression analysis methods that were used in this thesis.

Real-time PCR (qPCR) is used to amplify and quantify a target sequence of RNA (Vanguilder, Vrana, and Freeman, 2008). RNA is first isolated from samples, and then reverse-transcribed to cDNA, which is more stable than RNA. Primers are used to amplify a specific sequence, and can be designed specifically for the gene of interest. Cycles of 65°C and 95°C allow the primers to repeatedly amplify the target sequence, followed by dissociation of the double strand, resulting in exponential production of the target gene (Vanguilder, Vrana, and Freeman, 2008). SYBR Green, a highly specific, double-stranded DNA binding dye, is added to the reaction mixture to detect PCR product as it accumulates during PCR cycles. SYBR green binds to double stranded DNA at the end of the elongation step, and it only fluoresces when bound to double stranded DNA. The advantage to this method is that the number of copies of RNA can be quantified. However, few genes can be done at once, making this procedure both time-consuming and costly, depending on the number of samples and the number of genes of interest.

Microarrays provides a high-throughput method for studying gene expression, but often their use is non-targeted and not hypothesis driven. Low-density microarrays are used for a relatively small number of genes (up to several hundred), whereas high-density arrays provide whole transcriptome analysis (Bulera et al., 2001). RNA is extracted the same way as for qPCR, but when it is reverse-transcribed to cDNA, labelled nucleotides are used in the master mix. Samples containing the labelled nucleotides are applied to the array, where they hybridize with the sequences on the array plate, corresponding to the gene sequences in the organism (Schena et al., 1995). The plate is then read, and information is given on relative gene expression (Bulera et al., 2001; Schena et al., 1995). Whereas high-throughput is an advantage to this method, the number of copies of RNA is not quantified. The expression of a gene is relative only and dependent on comparison to another group. The results are then indicated as higher, lower, or no difference in expression, and if different the fold-difference can be determined.

With microarray analysis, the changes in gene expression are assessed on an individual basis that results in a long list of differentially expressed genes with no particular organization. However, in order to understand the biological significance of the results, the gene lists can be organized into metabolic or regulatory pathways that have been compiled from an analysis of the literature (Werner, 2008). This allows for a greater understanding of what changes are occurring between the groups.

### **3.4 Immunoblot Analysis**

Protein is isolated from samples and a sodium dodecyl sulfate buffer is added. The protein/buffer mixture is loaded onto a polyacrylamide gel for electrophoresis that separates proteins based on size, as smaller proteins move through the acrylamide matrix at a faster rate. Following this electrophoresis, the separated proteins are transferred onto a polyvinylidene

fluoride membrane that the proteins adhere to in the same orientation as the gel (Towbin, Staehelin, and Gordon, 1979).

Proteins can then be detected by the use of a primary antibody specific for the protein of interest. Because the membranes bind proteins very easily, they are first “blocked” with a protein solution (such as bovine serum albumin or skim milk powder) prior to the application of the primary antibody, to cover any places on the membrane not already bound with protein, to minimize non-specific binding of the primary antibody (Towbin, Staehelin, and Gordon, 1979). Membranes are washed to remove excess antibody, after which binding of the primary antibody is determined using a horseradish peroxidase-conjugated secondary antibody with enhanced chemiluminescence (Towbin, Staehelin, and Gordon, 1979). Bands are visualized on a flatbed scanner. Luminescence can then be quantified, and the relative amount of specific proteins in each sample is determined.

## Chapter 4

### Rationale and Objectives

#### 4.1 Rationale

MUFA have important roles as a source of energy through oxidation (Bergouignan et al., 2009) and as structural components of complex lipids (Flowers and Ntambi, 2009). However, the role of MUFA and SCD in health and obesity is unclear (Cao et al., 2008; Ntambi et al., 2002; Garcia-Serrano et al., 2011). There is increasing evidence that MUFA and their metabolites have the potential to mediate mammalian physiology through cell signalling and gene expression interactions. As MUFA are endogenously synthesized, they are also explicitly linked to lipogenesis. Therefore, an understanding of the regulation of MUFA biosynthesis and SCD expression is extremely important. There is evidence suggesting sex differences in MUFA composition, in both animal (Burdge et al., 2008; Extier et al., 2010) and human (Marangoni et al., 2007) studies. These differences may be mediated by sex hormones, specifically estrogen, which appears to negatively regulate SCD1 expression (Bryzgalova et al., 2006; Bryzgalova et al., 2008; Gao et al., 2006). The effects of estrogen may be mediated through genomic receptors, such as estrogen receptor alpha or via action on transcription factors. However, studies examining the effect of hormones on MUFA production are scarce, and those that exist are limited to gene expression assessments and not protein content. Therefore, the aim of the current study is to characterize the effect of sex and sex hormones on MUFA metabolism by examining both gene expression and fatty acid composition of primarily hepatic tissue, the major site of fat metabolism.

## **4.2 Objectives**

The first objective of this thesis was to examine the differences in MUFA metabolism between male and female rats. Gene expression at the level of mRNA and protein content of hepatic tissue was examined, and the fatty acid composition of liver and plasma was determined. The specific effects of estradiol, progesterone and testosterone on protein expression and fatty acid composition of hepatic cells was examined in HepG2 cells with increasing concentrations of hormones (0, 10, 30 or 100 nM). Finally, the effects of hormonal manipulations in the whole animal on MUFA metabolism were examined using ovariectomy in a rat model. Ovariectomized females were implanted with time released estrogen, progesterone, or combination estrogen plus progesterone pellets, and compared with ovariectomized without treatment and sham-operated rats. A full transcriptome analysis was used to examine a wide spectrum of metabolic pathways including lipogenesis, fatty acid beta-oxidation, and complex lipid synthesis that can influence MUFA metabolism in the sham-operated and ovariectomized rats. Targeted protein analysis of liver and fatty acid composition analyses of liver and plasma was completed on all groups to confirm transcriptome findings.

## **4.3 Hypotheses**

The hypotheses of the present study are:

1. Expression of SCD1 will be higher in males as compared with females
2. Female rats will have higher concentrations of SFA and lower concentrations of both n-7 and n-9 MUFA in tissues.
3. SCD1 expression in HepG2 cells will decrease with increasing concentrations of estradiol and increase with testosterone. Progesterone will have no effect.

4. Elongase 6 expression in HepG2 cells will increase with increasing concentrations of estradiol and decrease with testosterone. Progesterone will have no effect.
5. Elongase 6 expression will be decreased with ovariectomy and restored with hormone treatment.
6. Microarray analyses will show mRNA of genes of fatty acid synthesis are increased while mRNA of fatty acid oxidation genes are decreased in ovariectomy compared with sham controls.

## Chapter 5

### Analytical Techniques

#### 5.1 RNA Extraction and Reverse-Transcriptase RT-PCR

RNA was extracted from rat livers by homogenization in Trizol® reagent (Invitrogen Co, Frederick, MD). Phases were separated by the addition of chloroform. Integrity of the extracted RNA was verified by agarose gel electrophoresis with ethidium bromide. Purity 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.

Primers for rat sequences of SCD1, SCD2, Elovl5, Elovl6, acetyl-CoA carboxylase, fatty acid synthase and 18S were designed with Primer-BLAST program on the NCBI website and ordered from Sigma-Aldrich. GenBank accession numbers and primer sequences are shown in **Table 1**. Quantitative real-time PCR was performed using SYBR Green qPCR mastermix (Applied Biosystems) on an Applied Biosystems 7500 real time PCR system with software version 1.2.3. The reaction volume was 25 µl. The initial incubation was 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Following 40 cycles, a dissociation curve was produced by increasing the temperature from 60°C to 95°C at a rate of 1°C per minute. Analysis of data was performed by normalizing the threshold cycle number ( $\Delta\text{Ct}$ ) of the gene of interest to the  $\Delta\text{Ct}$  for the housekeeping gene, 18S ribosomal RNA, using the  $2^{-\Delta\Delta\text{Ct}}$  method and expressing male values relative to female values.

#### 5.2 Whole Transcriptome Microarray



RNA was extracted from livers using Trizol reagent as described in section 5.1. Purity of extracted RNA was quantified using the 260/280 ratio on a Nanodrop c2000, and adjusted using autoclaved water for a final concentration of 3.33 ng/ml. Integrity of the RNA was assessed using the Agilent RNA 6000 Nano Kit (Agilent, Mississauga, ON) . Once checked for integrity, 100ng total RNA was used for synthesis of first strand cDNA synthesis, followed by synthesis of the second strand of cDNA. The first cycle of *in vitro* transcription synthesizing cRNA ran for 16 hours. Following this, the cRNA was purified. Second cycle cDNA was synthesized, hydrolyzed and purified. All reagents up until the cDNA purification were from Ambion (Life Technologies, Burlington, ON). The cDNA was then fragmented and labelled with biotin. Labelled cDNA was hybridized to the Rat Gene 1.1 ST Array Strip (part number 901627, Affymetrix, Fremont, CA) containing the whole rat genome, for 20 hours, after which the strips were washed, stained and imaged on the Affymetrix Gene Atlas platform (Affymetrix). All reagents used during fragmentation to the imaging were from Affymetrix. Thermal cyclers information is in **Table 2**.

### **5.3 Immunodetection**

Rat liver lysates were prepared in a lysis buffer containing 20mM HEPES, 5.936mM NaCl, 1.5mM MgCl<sub>2</sub>, 1mM DTT, 20% glycerol (v:v), 0.001% triton X-100 (v:v), and a complete protease inhibitor (Roche Applied Science, Laval, QC, Canada) (Dupont et al., 2001). Protein was extracted from cell pellets in a buffer containing 0.25M sucrose, 0.01M tris-HCl, 0.01M MgCl<sub>2</sub>, 2.5M DTT, and complete protease inhibitor tablets. Protein content was determined by using a bicinchoninic acid procedure. Twenty µg of protein was resolved on a 12.5% polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membranes were blocked with 5% BSA or 5% non-fat milk powder as appropriate in TBS with 0.5% (v:v) Tween-20 (TBS-T) at room temperature for 1 hour.

Membranes were then incubated for 1 hour at room temperature with a primary antibody for SCD1 (Abcam, ab19862, diluted 1:1500 in 5% BSA in TBS-T) or Elongase 6 (Abcam, ab69857, 1:1000 in 5% milk). Membranes were washed with TBS-T, and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit, Santa Cruz Biotechnology) and washed again. Enhanced Chemiluminescence Western Blotting Detection Reagents was applied to the membrane (GE Healthcare, Mississauga, ON) to allow visualization of bands on a Chemigenius2 Bioimaging system (Syngene inc., Frederick, MD). Quantification of luminescence was done using Genesnap software v. 7.07 (Syngene). Values were normalized to female values or vehicle-treated cells, as appropriate. Equal protein loading and transfer was confirmed using a Ponceau S stain (Bioshop, Burlington, ON) (Dam et al., 2012; McMillan and Quadrilatero, 2011; Romero-Calvo et al., 2010).  $\beta$ -actin was used to normalize protein expression from the sham-operated, ovariectomized and hormone treated animals.

#### **5.4 Fatty Acid Analysis**

Plasma and HepG2 cell lipids were extracted using 2:1 chloroform:methanol (v:v) (Folch, Lees, and Sloane Stanley, 1957). Liver and erythrocytes lipids were extracted using 2:2:1.8 chloroform:methanol:water (v:v:v) with cold methanol and overnight freezing to lyse the cells and prevent heme aggregation (Bligh and Dyer, 1959; Metherel et al., 2009; Reed et al., 1960). Butylated hydroxytoluene was included in the extraction reagents to prevent oxidation. For the determination of the fatty acid composition of total lipids, ethyl docosatrienoate (22:3n-3 ethyl ester, Nu-Chek Prep Inc, Elysian, MN) was included as an internal standard. For TAG and phospholipid fatty acid determinations, triheptadecanoate (T-155 Nu-Chek Prep) and 1,2-diheptadecanoyl-*sn*-glycerol-3-phosphocholine (850360P, Avanti Polar Lipids Inc, Alabaster, AL) were included as internal standards. TAG and phospholipids were isolated from total lipid

extracts by thin layer chromatography using 20 x 20 cm plates with a 60Å silica gel layer (Whatman International LTD, Maidstone, England) and 60:40:2 heptane:diethyl ether: acetic acid (v:v:v) as the mobile phase (Christie, 1989). Triacylglycerol and phospholipid bands were visualized under UV light with 2,7-dichlorofluorescein (Sigma-Aldrich, Oakville, ON), identified by comparison to a reference standard and collected by scraping the band from the plate.

Fatty acids in total lipid extracts, TAG and phospholipids were transesterified to fatty acid methyl esters with 14% BF<sub>3</sub> in methanol at 85°C for 1 hour (Morrison and Smith, 1964) with hexane. The hexane containing the fatty acid methyl esters were then separated by fast gas chromatography (Stark and Salem, Jr., 2005). 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 as per gram of liver or erythrocytes, per millilitre of plasma, or per milligram of cellular protein, and qualitatively as relative weight % of total fatty acids.

## 5.5 Statistical Analyses

All statistical analyses were done by using SPSS for Windows, version 15.0. Independent samples t-test was used for comparison between males and females. One-way ANOVA was used for comparison between hormone concentrations in HepG2 cells, and between ovariectomized, hormone treated and sham-operated animals. Tukey's post-hoc test was used following a significant one-way ANOVA result. Significance was inferred when  $p < 0.05$ . Mixed-model ANOVA was used for analysis of microarray data, using a false discovery rate (FDR) of 5% for primary overall analyses. Differences in specific genes of interest determined *a priori* were also examined without FDR.

**Table 1** Rat primer sequences for RT-PCR

Gene	GenBank Accession Number	Forward Sequence	Reverse Sequence
SCD1	NM_139192.2	TGTTCCAGAGGAGGTAACAAGCC	GCAGGAAAGTTTCGCCCCAGC
SCD2	NM_031841	TCACACTGGTCCCCTCCTGCAAG	CTGTGATGCCCAGGGCGCTGATTAC
Elovl5	NM_134382.1	CTCTCGGGTGGCTGTACTTC	AGAGGCCCTTTCTTGTTGT
Elovl6	NM_134383.2	CACAGCCTCGGGCTTGTTTCGT	CTATGGGCCGCCTTCTCGGGA
FASN	NM_017332.1	GGATGTCAACAAGCCCAAGT	CAGAGGAGAAGGCCACAAAG
ACC	NM_022193.1	AGATGCACCTGTACCTTGGG	TGACCAGATCAGAGTGCCTG
18S	M11188	GATCCATTGGAGGGCAAGTCT	AACTGCAGCAACTTTAATATACGCTATT

SCD1: stearyl-CoA desaturase 1, SCD2: stearyl-CoA desaturase 2, elovl5: elongase 5, elovl6: elongase 6, FASN: fatty acid synthase, ACC: acetyl-CoA carboxylase, 18S: 18S ribosomal RNA

**Table 2.** Thermal cycler programs for preparation of total RNA for hybridization to microarray strips.

<b>Program</b>	<b>Heated Lid Temperature</b>	<b>Step 1</b>	<b>Step 2</b>	<b>Step 3</b>	<b>Step 4</b>
First-Strand cDNA Synthesis	50°C	25°C, 60 min	42°C, 60 min	4°C, 2 min	N/A
Second-Strand cDNA Synthesis	RT or disable	16°C, 60 min	65°C, 10 min	4°C, 2 min	N/A
In Vitro Transcription cRNA Synthesis	50°C	40°C, 16 hrs	4°C, Hold	N/A	N/A
2nd-Cycle cRNA Denaturation	75°C	70°C, 5 min	25°C, 5 min	4°C, 2 min	N/A
2nd-Cycle cDNA Synthesis	75°C	25°C, 10 min	42°C, 90 min	70°C, 10 min	4°C, 2 min
RNase H Hydrolysis	75°C	37°C, 45 min	95°C, 5 min	4°C, 2 min	N/A
Fragmentation of ssDNA	N/A	37°C, 60 min	93°C, 2 min	4°C, 2 min	N/A
Labeling of Fragmented ssDNA	N/A	37°C, 60 min	70°C, 10 min	4°C, 2 min	N/A

## Chapter 6

### Differences in Gene Expression and MUFA Composition between Males and Female Rats

#### 6.1 Introduction

Sex differences have been reported in MUFA levels in tissues, with male rats having higher 18:1n-9 in hepatic phospholipids (Burdge et al., 2008; Extier et al., 2010) and female rats having higher 18:0 in hepatic phospholipids (Burdge et al., 2008). Differences have also been observed in humans with men having higher 18:1n-9 in whole blood (Marangoni et al., 2007). 16:0 and 18:0 appear to be influenced by hormonal status in women (Stark, Park, and Holub, 2003). Reports of tissue sex differences in SFA and MUFA composition are limited to these few studies, and it appears that no one has examined sex differences in the n-7 MUFA. Similarly, the mechanism behind the sex differences in tissue fatty acid composition is unclear, as no work has examined sex differences in expression of SCD1 or elongase 6, key enzymes in MUFA biosynthesis.

#### 6.2 Animal Care

All animal experiments were carried out according to guidelines of the University of Waterloo Animal Care Committee and Canadian Council on Animal Care. Ten-week old male and female Sprague-Dawley rats (n = 6 each) (Harlan Laboratories, Mississauga, ON) were housed 3 per cage in the Department of Kinesiology at the University of Waterloo with a reversed 12:12-hour light-dark cycle. The temperature was maintained at  $21 \pm 1$  °C. Rats had *ad libitum* access to food (AIN-93G diet, Harlan Laboratories) and water. At 14 weeks of age, animals were fasted overnight and sacrificed via cardiocentesis after sedation by intraperitoneal sodium pentobarbital injection. Blood was collected by cardiocentesis into an EDTA-containing

syringe, and plasma was isolated by centrifugation at 1500g and stored at -80°C. Tissues were quickly excised, washed in saline (0.9% NaCl, w/v) and frozen in liquid nitrogen prior to storage at -80°C. Fatty acid, RNA and proteins were analyzed as described in **Chapter 5**.

### **6.3 Sex Differences in Enzyme Expression in Rat Livers**

Expression of enzymes involved in hepatic fatty acid synthesis was examined via real-time PCR and western blotting (**Figures 2 and 3**, respectively). Elongase 5 and SCD2 mRNA were 0.6- and 0.7-fold lower in females compared with males. Previous results show no differences in elongase 5 protein (Kitson et al., 2012). In the present study, reliable measurement of SCD2 protein levels was not obtained, as previous studies report low expression levels in liver (Moreau et al., 2006), with transcripts of SCD2 mRNA previously reported to be approximately 1800 times lower than that of SCD1 (Yamazaki et al., 2012). Elongase 6 mRNA and protein expression was 5.9- and 2.0-fold higher, respectively, in females as compared with males. No sex differences were observed in acetyl-CoA carboxylase or fatty acid synthase mRNA. No sex differences were observed in SCD1 mRNA or protein.

### **6.4 Sex Differences in Fatty Acid Composition of Liver, Plasma and Erythrocytes**

Sex differences in the fatty acid composition of plasma and liver were observed mainly in the phospholipids, with no differences in TAG composition. Females had significantly lower concentrations of MUFA in liver and plasma phospholipids but not in erythrocytes (**Table 3**). These results largely corresponded to differences in 18:1n-9 concentrations, but females also exhibited lower 16:1n-7 in plasma phospholipids, lower 18:1n-7 in liver phospholipids and erythrocytes, and lower 20:1n-9 in liver phospholipids. In addition, 16:0 was significantly lower in both liver and plasma phospholipids of females as compared with males. Conversely, 18:0 was significantly higher in female liver phospholipids and erythrocytes.

## 6.5 Discussion

It was hypothesized that males would have higher SCD1 expression and consequently higher liver and blood MUFA concentrations. In the present work, I detected a shift towards lower 18:0 and higher 16:0, 16:1n-7, 18:1n-7 and 18:1n-9 in males as compared with females. While lower 18:0 and higher 18:1n-9 in male liver phospholipids has been reported (Burdge et al., 2008; Extier et al., 2010), my findings report additional SFA and n-7 MUFA sex differences not previously examined. Total fatty acid concentrations did not differ between the male and female rats, and a lack of sex difference in the mRNA expression of acetyl-CoA carboxylase and fatty acid synthase also indicates that sex differences in rat tissue SFA and MUFA are independent of general *de novo* fatty acid synthesis.

Higher concentrations of 16:1n-7, 18:1n-7 and 18:1n-9 were observed in liver, plasma and red blood cells of males as compared with females. Contrary to our hypothesis, SCD1 expression did not differ between males and females. Higher hepatic SCD1 expression in male as compared with female rats has been reported previously (Gustavsson et al., 2010), but the results were generated from a whole genome microarray of mRNA, whereas we presently performed targeted quantitative q-PCR to assess mRNA and immunoblot to assess protein expression. In addition, differences in sexual maturity may contribute to the different results, as the rats in the previous study were 7 weeks of age and first estrous of Sprague-Dawley rats occurs between 36-45 days (Merry and Holehan, 1979). In the present study, the rats were 14 weeks of age.

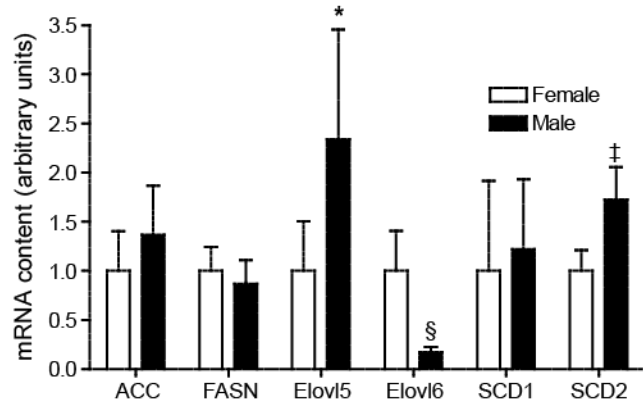
Elongase 6 mRNA and protein expression was significantly higher in female livers, corresponding to the higher concentration of 18:0 in female phospholipids observed in the present study. There has been a report of higher elongase 6 mRNA expression in males as compared with females (Gustavsson et al., 2010), however, this was performed in a non-fasted



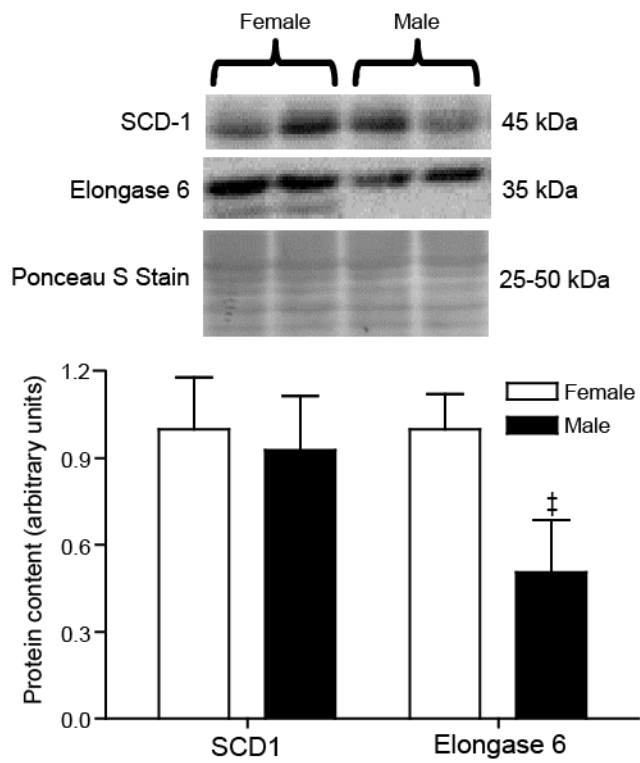
model and elongase 6 is strongly regulated by fasting and refeeding (Matsuzaka et al., 2002; Turyn, Stojek, and Swierczynski, 2010). The higher concentrations of the n-7 family of MUFA in males may also be related to the lower elongase 6 expression in males, as higher 16:0 concentrations and lower 18:0 concentrations could result in more 16:0 being desaturated prior to elongation (Hudgins et al., 1996; Miyazaki, Bruggink, and Ntambi, 2006). Elongase 6 appears to have a larger role in MUFA biosynthesis than previously thought, as these sex differences in SFA and MUFA were independent of differences in SCD1 expression. To our knowledge, this is the first study to report sex differences in elongase 6 together with its relevance to MUFA biosynthesis. This finding is particularly important, because the use of the ratio of 18:1n-9 to 18:0 and 16:1n-7 to 16:0 as indicators of SCD1 expression, without direct measure of SCD1 mRNA or protein levels, is extremely common (Saito et al., 2011; Stefan et al., 2008; Attie et al., 2002; Extier et al., 2010), but this may be ignoring elongase 6 as an important contributor to MUFA synthesis.

Elongase 5 mRNA but not protein was higher in males, suggesting that the mRNA may not be translated into protein, or there is a higher rate of protein turnover in males. Elongase 5 is typically involved in PUFA elongation, but is also known to elongate 16:1n-7 (Wang et al., 2006), and the higher elongase 5 mRNA expression may be in response to the higher levels of n-7 MUFA observed in males. Hepatic SCD2 mRNA expression was higher in males in the present study, but SCD2 protein could not be reliably measured as previous studies report low expression in livers (Moreau et al., 2006; Yamazaki et al., 2012). The higher expression in males suggests that it may be involved in the increased MUFA concentrations observed in males, but its exact role in hepatic MUFA metabolism remains to be elucidated.

To our knowledge, this is the first study to report sex differences in elongase 6 mRNA and protein expression, a novel insight into MUFA metabolism. As sex differences were observed in SFA and MUFA in the present study without sex differences in SCD1 expression, it appears that elongase 6 may have a major influence on MUFA biosynthesis.



**Figure 2** mRNA content of enzymes involved in *de novo* fatty acid biosynthesis in the liver of each sex (n = 6 for each). Significant sex differences are denoted by \*: p<0.05, ‡: p<0.01, §: p<0.005, ACC: acetyl-CoA carboxylase, FASN: fatty acid synthase, Elovl5: elongase 5, Elovl6: elongase 6, SCD1: stearyl-CoA desaturase-1, SCD2: stearyl-CoA desaturase 2.



**Figure 3** Densitometric analysis of protein content and representative immunoblots of enzymes involved in MUFA biosynthesis in rat livers (n = 6 for each sex). Significant sex differences are denoted by ‡: p<0.01. SCD1: stearyl-CoA desaturase-1

**Table 3** Fatty acid composition of liver and plasma phospholipids and erythrocyte total lipids of male and female rats\*

<i>Fatty acid</i>	<b>Liver Phospholipids</b> <i>µg fatty acid/g liver</i>		<b>Plasma Phospholipids</b> <i>µg fatty acid/ml plasma</i>		<b>Erythrocytes</b> <i>µg fatty acid/g erythrocytes</i>	
	<i>Males</i>	<i>Females</i>	<i>Males</i>	<i>Females</i>	<i>Males</i>	<i>Females</i>
14:0	31 ± 8	28 ± 9	N.D.	N.D.	24 ± 9	27 ± 14
16:0	3515 ± 196	2757 ± 616*	90 ± 6	64 ± 11§	672 ± 61	615 ± 83
18:0	3987 ± 325	5943 ± 1429†	85 ± 6	137 ± 62	406 ± 29	548 ± 37§
20:0	16 ± 2	15 ± 2	0.67 ± 0.07	0.67 ± 0.21	4.9 ± 0.9	5.9 ± 1.4
22:0	36 ± 6	38 ± 6	1.8 ± 0.2	2.1 ± 0.4	8.4 ± 1.4	11.1 ± 0.9†
24:0	92 ± 17	110 ± 16	4.9 ± 0.3	5.4 ± 0.9	23 ± 5	27 ± 4
Total SFA	7710 ± 533	8934 ± 3038	184 ± 11	211 ± 72	1231 ± 148	1323 ± 141
14:1n-5	0.4 ± 0.3	0.8 ± 1.1	0.02 ± 0.03	0.01 ± 0.03	0.3 ± 0.2	0.6 ± 0.4
16:1n-7	45 ± 11	34 ± 10	0.66 ± 0.14	0.46 ± 0.14*	7.8 ± 3.2	5.5 ± 1.3
18:1n-7	527 ± 49	293 ± 66§	5.54 ± 2.69	3.44 ± 0.84	61 ± 3	49 ± 5‡
18:1n-9	546 ± 47	390 ± 80†	8.2 ± 1.2	5.6 ± 1.4†	156 ± 14	137 ± 20
20:1n-9	25 ± 5	12 ± 2§	0.42 ± 0.04	0.18 ± 0.05§	3.5 ± 0.6	3.4 ± 0.9
22:1n-9	5.6 ± 2.3	6.2 ± 1.8	0.30 ± 0.06	0.30 ± 0.03	7.7 ± 1.6	9.8 ± 4.6
24:1n-9	26 ± 3	30 ± 4	3.0 ± 0.3	3.0 ± 0.6	14.0 ± 1.7	14.8 ± 1.3
Total MUFA	1177 ± 100	766 ± 159§	18 ± 3	13 ± 3†	251 ± 17	221 ± 24
Total n-6 PUFA	7823 ± 643	7262 ± 1664*	120 ± 10	98 ± 21	1268 ± 182	1141 ± 132
Total n-3 PUFA	1284 ± 134	2096 ± 346§	11 ± 2	14 ± 4	122 ± 11	141 ± 8
Total fatty acids	18406 ± 1183	19391 ± 4242	332 ± 18	335 ± 95	3001 ± 159	2978 ± 166

\*Data is mean ± SD from three determinations of fatty acid concentrations (n = 6 for each sex). Significant sex differences are denoted by \*: p < 0.05, †: p < 0.01, ‡: p < 0.005, §: p < 0.001. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid.

## Chapter 7

### Hormonal Manipulation of HepG2 Cells

#### 7.1 Introduction

Sex differences in MUFA composition and enzyme expression led to the hypothesis that 17 $\beta$ -estradiol and progesterone would decrease SCD1 expression and increase elongase 6 expression. It was also hypothesized that testosterone would result in the opposite expression responses. Previous studies have used hormone manipulated animals to study SCD1 mRNA expression, but the direct effects of hormones on fatty acid composition or protein content has not been examined (Alessandri et al., 2011; Gao et al., 2006; Bryzgalova et al., 2006).

Accordingly, the purpose of this study was to isolate the effects of different concentrations of sex hormones (17 $\beta$ -estradiol, progesterone and testosterone) on MUFA composition and protein expression of SCD1 and elongase 6 in hepatic cells using HepG2 cultures.

#### 7.2 HepG2 Cells Treated with 17 $\beta$ -Estradiol, Progesterone and Testosterone

HepG2 cells were grown in low glucose Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin, in a sterile incubator kept at 37°C and 5% CO<sub>2</sub> (Roh et al., 2012). The medium was replenished every 3 days after rinsing with PBS (Voest et al., 1993). All reagents were from Thermo Scientific (Wilmington, DE) unless specified otherwise. Cells were split when 80% confluence had been reached by treatment with trypsin. Experiments were performed on cells between passage number 10 and 14. Progesterone, 17 $\beta$ -estradiol, (Santa Cruz Biotechnology, Santa Cruz, CA), or testosterone (Sigma-Aldrich, Oakville, ON) were dissolved in ethanol and added to the medium to achieve final concentrations of 0, 10, 30 and 100 nM, with a final volume of ethanol in the medium of 0.05% (v/v). Pilot experiments performed in our laboratory

and others (Portolesi, Powell, and Gibson, 2008; Extier et al., 2009) showed that this volume of ethanol did not affect fatty acid composition. Cells were exposed to medium containing either the vehicle or a hormone for 72 hours (Voest et al., 1993). After 72 hours the medium was aspirated, and the cells were rinsed with phosphate buffered saline and collected following trypsinization. All samples were stored at -80°C until analysis. Fatty acids and proteins were analyzed as described in chapter 5.

### **7.3 Effect of Hormones on Protein Expression in HepG2 cells**

Protein levels of SCD1 in HepG2 cells increased 1.6-fold with 10nM 17 $\beta$ -estradiol and decreased 0.67-fold with 10nM progesterone treatment relative to control (**Figure 4**). Treatment with 30 and 100nM of 17 $\beta$ -estradiol or progesterone had no effect on SCD protein relative to control. Testosterone had no effect on SCD1 at any concentration examined. Elongase 6 protein levels responded to all the hormone treatments. Elongase 6 was increased with 30 and 100 nM 17 $\beta$ -estradiol treatment, and with 100 nM progesterone treatment, relative to controls. In contrast, treatment with 30 and 100 nM testosterone decreased elongase 6 protein relative to control.

### **7.4 Effect of Hormones on HepG2 cell Fatty Acid Composition**

Treatment of HepG2 cells with 10nM 17 $\beta$ -estradiol increased the concentration of MUFA, SFA, PUFA and total fatty acids, specifically in the phospholipids (**Table 4**). Corresponding increases in the concentrations of individual fatty acids were observed, including 16:0, 18:1n-7 and 18:1n-9. Treatment with 30 or 100nM 17 $\beta$ -estradiol had no effect on fatty acid concentration relative to control. Progesterone treatment had no effect on HepG2 fatty acid composition. Treatment with 30nM testosterone resulted in a significant increase of 16:1n-7 in total lipids as compared with 0nM (14 $\pm$ 1.2 vs 10.7 $\pm$ 0.7  $\mu$ g fatty acid/mg cellular protein), with

intermediate levels with 10 and 100nM treatments ( $12\pm 1.2$  and  $12.8\pm 1.5$   $\mu\text{g}$  fatty acid/mg cellular protein, respectively).

## 7.5 Discussion

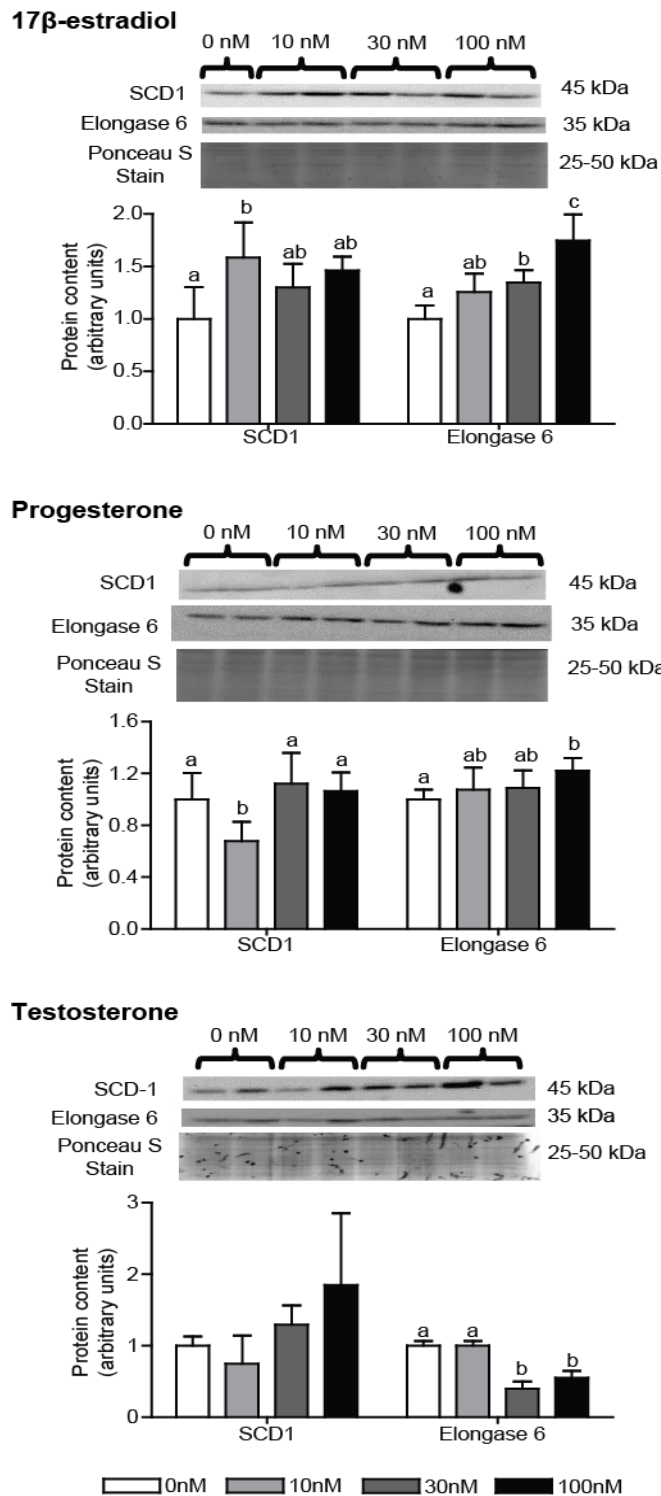
SCD1 protein is positively regulated by 10 nM  $17\beta$ -estradiol and negatively regulated by 10 nM progesterone, while elongase 6 protein is increased following treatment with both these hormones. Testosterone had no effect at any concentration on SCD1, but decreased elongase 6 expression. Treatment with 10 nM  $17\beta$ -estradiol led to an increase in several phospholipid SFA and MUFA species, including 16:0, 16:1n-7, 18:1n-7 and 18:1n-9. However, these results must be interpreted with caution, as the concentration of total fatty acids was also increased with 10 nM treatment, suggesting that this effect may not be specific to MUFA but an overall increase in lipogenesis. These fatty acid differences were not present at 30 and 100 nM  $17\beta$ -estradiol treatments, possibly due to the variable effects of  $17\beta$ -estradiol on fatty acid oxidation (Gower et al., 2002; Toda et al., 2001; Campbell et al., 2003).

The lack of fatty acid differences in cells treated with progesterone and testosterone despite changes in enzyme expression suggests that the incubation time may have been inappropriate to elicit changes. Fatty acid biosynthesis and incorporation into cellular lipids would require time, but too much time may result in feedback inhibition and the loss of differences. Additionally, there may have been hormonal effects on fatty acid incorporation and oxidation which were not measured. While the doses of progesterone and testosterone were physiological (Butcher, Collins, and Fugo, 1974; Salameh et al., 2010), the estradiol concentration was much higher than concentrations that would be observed in humans or rats (Butcher, Collins, and Fugo, 1974). Furthermore, because these hepatocytes are isolated in culture, the metabolic demand on cultured hepatocytes may be dramatically less as compared



with the metabolic demand of a liver *in vivo*. A liver in a living system takes up fatty acids but also synthesizes and exports them for transport depending on the demand of other tissues. The cells in this system do not have the demand from other tissues, and as a result may only synthesize enough fatty acids to meet their own needs, despite an increase in enzyme expression. Additionally, the medium in the present study contained FBS and phenol red. FBS contains a variable amount of hormones (Milo et al., 1976), while phenol red displays approximately 0.001% of the estrogenic activity of 17 $\beta$ -estradiol (Berthois, Katzenellenbogen, and Katzenellenbogen, 1986). Accordingly, use of charcoal-treated medium to remove the steroid hormones in FBS, and phenol-red free medium should be considered for future studies.

Hormonal manipulations of HepG2 cells clearly indicate that sex hormones are influencing SCD1 and elongase 6 expression. The stronger effect appears to be on elongase 6, as it was positively regulated by both estradiol and progesterone, while testosterone decreased protein expression. This strong effect of sex hormones on elongase 6 indicates that it is may be an important contributor to MUFA biosynthesis and observed sex differences in MUFA composition.



**Figure 4** Densitometric analysis of protein content and representative immunoblots of enzymes involved in MUFA biosynthesis in HepG2 cells treated with varying concentrations of 17 $\beta$ -estradiol, progesterone and testosterone (n = 5 for each group). Groups with different superscripts are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SCD1: stearoyl-CoA desaturase 1.

**Table 4** Phospholipid fatty acid composition of HepG2 cells incubated with increasing concentrations of 17 $\beta$ -estradiol for 72 hours\*

<b>Fatty acid</b>	<b>0nM</b>	<b>10nM</b>	<b>30nM</b>	<b>100nM</b>
	<i><math>\mu</math>g fatty acid/mg cellular protein</i>			
12:0	0.12 $\pm$ 0.05	0.18 $\pm$ 0.07	0.11 $\pm$ 0.03	0.15 $\pm$ 0.08
14:0	0.86 $\pm$ 0.15	1.00 $\pm$ 0.08	0.87 $\pm$ 0.17	0.91 $\pm$ 0.18
16:0	12 $\pm$ 1 <sup>ab</sup>	15 $\pm$ 1 <sup>a</sup>	11 $\pm$ 2 <sup>b</sup>	12 $\pm$ 2 <sup>ab</sup>
18:0	5 $\pm$ 1	6 $\pm$ 1	5 $\pm$ 2	5 $\pm$ 1
20:0	0.10 $\pm$ 0.02	0.13 $\pm$ 0.02	0.13 $\pm$ 0.04	0.12 $\pm$ 0.01
22:0	0.16 $\pm$ 0.02	0.16 $\pm$ 0.05	0.15 $\pm$ 0.03	0.15 $\pm$ 0.04
23:0	0.12 $\pm$ 0.01	0.13 $\pm$ 0.04	0.12 $\pm$ 0.03	0.11 $\pm$ 0.02
24:0	0.08 $\pm$ 0.01	0.05 $\pm$ 0.05	0.07 $\pm$ 0.01	0.07 $\pm$ 0.02
Total SFA	18.4 $\pm$ 2.0	22.3 $\pm$ 1.6	18.1 $\pm$ 4.3	18.1 $\pm$ 2.5
14:1n-5	0.19 $\pm$ 0.03 <sup>ab</sup>	0.25 $\pm$ 0.02 <sup>a</sup>	0.17 $\pm$ 0.02 <sup>b</sup>	0.21 $\pm$ 0.04 <sup>ab</sup>
16:1n-7	3.68 $\pm$ 0.42 <sup>ab</sup>	4.63 $\pm$ 0.35 <sup>a</sup>	3.50 $\pm$ 0.61 <sup>b</sup>	3.70 $\pm$ 0.57 <sup>ab</sup>
18:1n-7	6.33 $\pm$ 0.57 <sup>a</sup>	7.87 $\pm$ 0.60 <sup>b</sup>	5.83 $\pm$ 0.86 <sup>a</sup>	6.19 $\pm$ 0.92 <sup>a</sup>
18:1n-9	8.34 $\pm$ 0.72 <sup>a</sup>	10.46 $\pm$ 0.63 <sup>b</sup>	7.60 $\pm$ 0.98 <sup>a</sup>	8.18 $\pm$ 1.43 <sup>a</sup>
20:1n-9	0.18 $\pm$ 0.03	0.21 $\pm$ 0.04	0.16 $\pm$ 0.04	0.16 $\pm$ 0.02
22:1n-9	0.27 $\pm$ 0.20	0.34 $\pm$ 0.20	0.22 $\pm$ 0.20	0.36 $\pm$ 0.28
24:1n-9	0.18 $\pm$ 0.01	0.19 $\pm$ 0.05	0.17 $\pm$ 0.04	0.17 $\pm$ 0.06
Total MUFA	19.2 $\pm$ 1.5 <sup>a</sup>	24.0 $\pm$ 1.4 <sup>b</sup>	17.7 $\pm$ 2.4 <sup>a</sup>	19.0 $\pm$ 2.9 <sup>a</sup>
Total n-6 PUFA	3.3 $\pm$ 0.4 <sup>ab</sup>	4.0 $\pm$ 0.4 <sup>a</sup>	3.0 $\pm$ 0.4 <sup>b</sup>	3.2 $\pm$ 0.5 <sup>ab</sup>
Total n-3 PUFA	1.4 $\pm$ 0.17 <sup>ab</sup>	1.67 $\pm$ 0.13 <sup>a</sup>	1.28 $\pm$ 0.20 <sup>b</sup>	1.33 $\pm$ 0.21 <sup>ab</sup>
Total fatty acids	48.5 $\pm$ 4.9 <sup>ab</sup>	59.3 $\pm$ 3.7 <sup>a</sup>	45.4 $\pm$ 7.7 <sup>b</sup>	47.8 $\pm$ 7.6 <sup>ab</sup>

\* Values are mean  $\pm$  SD, n=5 per group. Values with different superscripts are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid

## Chapter 8

### Ovariectomization and Hormonal Manipulation of Female Rats

#### 8.1 Introduction

Estrogen is known to have effects on lipid metabolism. Declining estrogen status (such as following menopause or ovariectomization) often results in weight gain (D'Eon et al., 2005) accompanied by lipid accumulation including increases in circulating low density lipoproteins (Matthews et al., 1989). Changes in hormone status such as the use of hormone therapy (Stark, Park, and Holub, 2003) and pregnancy (Stark et al., 2005) are also associated with changes in SFA and MUFA in blood fatty acids. The effects of ovariectomization and hormone replacement on SCD1 expression has been examined at the level of mRNA expression but not protein levels (Gao et al., 2006; Alessandri et al., 2011; Paquette et al., 2008). In addition, these previous ovariectomy studies did not examine elongase 6, the fatty acid composition data presented is limited or not presented at all, and only 17 $\beta$ -estradiol treatment was examined.

Results from the previous experiments in Chapters 6 and 7 clearly demonstrate sex differences in MUFA composition and enzyme expression, potentially mediated in part by sex hormones. The purpose of the present study was to manipulate the hormone status of female rats to further understand the role of sex hormones on regulating MUFA metabolism. Hormone manipulation was accomplished by ovariectomization and implantation of constant-release hormone pellets (17 $\beta$ -estradiol, progesterone or a pellet containing both) in ovariectomized rats to assess the effects of these hormones on hepatic enzyme expression and MUFA composition. In addition, a whole transcriptome microarray was performed, comparing sham-operated controls with ovariectomized animals to gain insight on various pathways involved in lipid metabolism.

#### 8.2 Ovariectomized Rats and Hormonal Treatment

Eight week old ovariectomized (n = 25) and sham-operated (n = 6) Sprague-Dawley rats were ordered from Harlan, and housed and fed as described in **Section 6.1**. At ten weeks of age, 19 ovariectomized rats were anesthetized with isoflurane, and the base of each neck was shaved, sterilized with iodine, and a small incision was made. Hormone pellets with a 21 day release period (E-121, HH-115 and P-131, Innovative Research of America, FL) were then implanted subcutaneously, and the incision was closed with staples. The pellets used included 0.5 mg 17 $\beta$ -estradiol /pellet (n = 6), 0.5 mg 17 $\beta$ -estradiol + 15 mg progesterone /pellet (n = 7), and 25 mg progesterone /pellet (n = 6), and were based on previous protocols in the literature (Smith et al., 2009; Kramer and Bellinger, 2009). The staples were removed after one week, and after 2 weeks the rats were fasted overnight, sacrificed and tissues collected as described in Section 6.1.

### **8.3 Plasma Hormone Concentration Analysis**

Plasma 17 $\beta$ -estradiol and progesterone concentrations of all rats were determined using ELISA kits (Estradiol EIA Kit 582251, Progesterone EIA Kit 582601, Cayman Chemical, Ann Arbor, MI). For determination of plasma 17 $\beta$ -estradiol, 1 ml of plasma was concentrated 1:4 as the expected plasma concentration range during the normal estrous cycle of rats is 20-80 pg/ml (Butcher, Collins, and Fugo, 1974), and the range of the assay is 25-3500 pg/ml. Five  $\mu$ l of plasma was diluted 1:100 for determination of plasma progesterone concentration, as the expected concentration during the normal estrous cycle of the rat is 5-45 ng/ml (Butcher, Collins, and Fugo, 1974), and the range of the assay is 25-1000 pg/ml. For each assay, samples and standards were loaded to the 96-well plate in duplicate. Two wells were designated each for blanks, total activity, non-specific binding and maximum binding. Plates were read at a

wavelength of 420 nm, and the plasma concentrations of each hormone were determined by comparison to standard curves.

#### **8.4 Whole Transcriptome Microarray Analysis**

Total RNA (100 ng) was extracted from the livers of 4 ovariectomized (OVX) animals and 4 sham-operated (sham) rats and prepared for microarray analysis as described in **Section 5.2**. Microarray data were Robust Multi-array Averaged as there are several spots on the array for the same gene, and quantile normalized and  $\log_2$  transformed. Principal Component Analysis was used to measure within group variability and ensure clear separation between groups. Variance Component Analysis indicated that the majority of the variance in the data set was due to treatment, with only minor contributions from peg position on the array. The array number had no influence. A mixed-model ANOVA was used to identify differentially expressed genes, with treatment as the fixed effect, and peg position as a random effect to account for the minor influence of this variable (JMP Genomics Version 5, SAS, Cary, NC). To identify the most significant differences in gene expression, multiple testing was accounted for using a false discovery rate (FDR) of 5%, corresponding to a p-value of 0.00027. The FDR is preferred to a standard p-value, because the p-value is used to test individual hypotheses, whereas analysis of gene expression via a microarray involves testing hundreds to thousands of hypotheses simultaneously. The FDR of a test is defined as the expected proportion of false positives among the declared significant results, providing a more directly useful interpretation of results (Pawitan et al., 2005). Several genes of specific interest determined *a priori* were also examined without considering the FDR.

FunNet was used for the analysis of transcriptional interaction networks (Prifti et al., 2008). The list of differentially expressed genes between sham and OVX were characterized into

biological themes using pathways established from the Kyoto Encyclopedia of Genes and Genomes (KEGG). A list of specific biological processes that are differentially expressed between sham and OVX were then identified.

### **8.5 Effect of Hormone Manipulation on Body Weight and Plasma Hormone Concentration**

There were no significant differences in body weight between any of the groups on the day the hormones were implanted (day 0) (**Table 5**). One week after the hormone pellets were implanted, the body weight of the OVX rats was significantly higher than all other groups, and estradiol treated rats had the lowest body weight. Upon sacrifice one week later, the OVX rats had the highest body weight while groups treated with estrogen or estrogen plus progesterone had the lowest body weight.

Ovariectomy resulted in low levels of progesterone and estradiol (**Table 6**), but estradiol levels were not significantly different than the sham controls. In general, the hormone pellets resulted in increased levels of the respective hormones relative to ovariectomized animals, although increases in progesterone were significant only when delivered with estradiol, and never reached sham concentrations. In contrast, estradiol treatments resulted in dramatic increases above sham levels.

### **8.6 Effect of Hormone Status on SCD1, Elongase 6 and SREBP-1c Protein Expression**

SCD1, elongase 6, and SREBP-1c protein expression were not significantly different between OVX and sham (**Figure 5**). SCD1 was 1.4 fold higher in estradiol plus progesterone as compared with sham, while estradiol resulted in a 1.5-fold increase in elongase 6 as compared with sham. SREBP-1c was 1.8-fold higher in progesterone as compared with estradiol plus progesterone treatment. No other differences in these proteins were observed in the other groups.

## **8.7 Effect of Ovariectomy and Hormone Treatment on Hepatic and Plasma Fatty Acid Composition**

Increases in concentration of 16:0, total SFA, 16:1n-7, 18:1n-7, 18:1n-9, 20:1n-9, total MUFA and total fatty acids were observed in the hepatic total lipid extract of OVX as compared with all other groups (**Figure 6**). This appears to be mediated by an increase in liver TAG, where increases in 16:0, 18:0, 16:1n-7, 18:1n-7, 18:1n-9 and total MUFA concentrations was observed in ovariectomized rats, with the total fatty acid concentration in liver TAG being at least twice as high as compared with all other groups. These fatty acid increases appear to be non-specific, and a result of overall higher concentration of all fatty acids rather than specific increases in MUFA only.

Hepatic and plasma phospholipids after estradiol or estradiol plus progesterone treatment appear to be specifically enriched with MUFA that is not due to increases in total phospholipids (**Tables 7 and 8**, respectively). Treatment with estradiol or estradiol plus progesterone resulted in the highest concentration of 16:0, 18:0, 16:1n-7 and 18:1n-7 in hepatic phospholipids, while treatment with estradiol resulted in the highest concentrations of 16:1n-7, 18:1n-7, 18:1n-9 and 20:1n-9 in plasma phospholipids. Similar increases in the relative percentage of these fatty acids were also observed. No significant differences were observed in plasma TAG or non-esterified fatty acids, although progesterone treatment displayed a significant increase in the concentration of 16:0, 18:0, total SFA, 24:1n-9 and total fatty acids in plasma cholesteryl esters.

## **8.8 Differentially Expressed Genes Between Sham-Operated and Ovariectomized Rats**

A total of 120 genes were identified as significantly differentially expressed when analyzed with an FDR of 5%, with 40 being upregulated and 80 genes being downregulated in OVX as compared with sham. Phosphatidic acid phosphatase type 2 domain containing 1A (a



gene involved in TAG synthesis) was upregulated (1.11-fold), while phosphate cytidyltransferase 2, ethanolamine (a gene involved in PE synthesis) was down regulated (0.87-fold) as compared with sham-operated animals.

More than 2000 genes were differentially regulated when considering a p value of <0.05 without FDR. Elongase 6, CD36, fatty acid binding protein 1 (liver), diacylglycerol O-acyltransferase homolog 2, hormone sensitive lipase, apolipoprotein M, lipin 3, and SREBP-2 were all significantly higher in sham as compared with OVX. Genes that were expressed higher in OVX included PPAR $\alpha$ , estrogen receptor  $\alpha$ , phospholipase C, acetyl-Coenzyme A acyltransferase 1 and acyl-Coenzyme A oxidase 1 (palmitoyl). There were no differences observed in the expression of SCD1, LXR, ChREBP, SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, and elongase 5 between sham and OVX in the microarray.

Pathway analysis showed that pyruvate metabolism, arachadonic acid metabolism, fatty acid metabolism, and PPAR signalling pathways were upregulated in OVX, whereas steroid biosynthesis, phosphatidylinositol signalling pathway, and endocytosis were all downregulated in OVX as compared with sham.

## **8.9 Discussion**

Ovariectomization decreases plasma progesterone concentrations as compared with sham, and increases body weight as compared with both sham and ovariectomized rats treated with hormones. Plasma estradiol concentrations were lower, but not significantly in OVX as compared with sham. While the ovaries are a major site of estradiol synthesis in premenopausal women, other sites of biosynthesis are present, and these become important following menopause (Simpson et al., 2000). Extragonadal synthesis, including adipose tissue hormone synthesis, may play a role in maintaining estradiol concentrations (Simpson, 2003). In the current

study, the ovariectomized rats had a significantly greater amount of adipose tissue as compared with sham rats, suggesting that the adipose tissue may have played a role in estradiol synthesis in these animals.

Increased body weight in OVX as compared with sham and hormone treated OVX is consistent with the literature (Paquette et al., 2008; Ribeiro, Jr. et al., 2012; Cao et al., 2012; Xu et al., 2012), as a decline in estradiol status has been shown to result in lipid accumulation and weight gain (D'Eon et al., 2005), in part because ovariectomization increases food intake and fat deposition (Wade, 1975). Specifically, hepatic TAG concentrations were more than two-fold higher in OVX rats as compared with every other group, which agrees with previous reports (Paquette et al., 2008). Not only were total TAG concentrations higher, but concentrations of 16:0, 18:0, total SFA, 16:1n-7, 18:1n-7, 18:1n-9, total MUFA, as well as several PUFA, were also the highest in the OVX rats. This suggests that the increase in MUFA in TAG is likely due to an increase in overall fatty acid content as a result of increased food intake, but may also involve decreased physical activity (Izumo et al., 2012) and different rates of fat oxidation (Gower et al., 2002; Toda et al., 2001), although these were not measured in the present study. Interestingly, OVX rats treated with any hormone pellets were rescued from this accumulation of TAG, and have hepatic TAG concentrations even lower than those in sham rats, suggesting a specific role for estradiol and/or progesterone in hepatic lipid metabolism and food intake.

Results from the microarray provide insights in regards to TAG accumulation with ovariectomy as compared with sham. The expression of phosphatidic acid phosphatase type 2 domain containing 1A that is involved in TAG synthesis was expressed higher in the livers of OVX rats (FDR < 5%) and could be involved in hepatic TAG accumulation. Hormone sensitive lipase, which hydrolyzes TAG to free fatty acids (Holm et al., 2000; Mulder et al., 2003), was

significantly lower ( $p < 0.05$ ) in OVX rats, which has been shown previously (Stubbins et al., 2011). Apolipoprotein M that is involved in lipid transport in blood was also lower in OVX rats ( $p < 0.05$ ). This agrees with previous findings (Wei et al., 2011), and suggests that lipoprotein metabolism may be disrupted with ovariectomy. Dysregulation of lipin (cytosolic phosphatidate phosphatases), which was also lower in OVX rats, has been demonstrated to disrupt fatty acid secretion into lipoproteins as well (Bou et al., 2010). Other fatty acid transport proteins, such as FAT/CD36 and FABP1 were lower in OVX ( $p < 0.05$ ), suggesting that fatty acids may not be as effectively taken up and excreted with ovariectomy. Increased expression of phospholipase C ( $p < 0.05$ ), as seen in OVX rats in the present study, has also been suggested to be a contributing factor to the development of obesity (Hirata et al., 2011). However, the expression of some oxidative genes including acetyl-CoA acyltransferase and acyl-CoA oxidase ( $p < 0.05$ ) was higher in OVX rats. The effect of oxidation, TAG synthesis and incorporation into TAG requires further consideration.

Studying MUFA biosynthesis in the context of differences in lipogenesis, such as with ovariectomy and with obesity, is challenging. Differences in energy intake can affect fatty acid composition of tissues by influencing fatty acid biosynthesis and storage rates and shifting the relationship of fatty acids incorporated from the diet versus fatty acids incorporated from *de novo* synthesis. Although diet intake was not assessed in this study, it is likely that the OVX rats ate more. Future studies involving pair feeding and/or controlled feeding of OVX and sham operated animals may provide novel insight into specific effects of hormone status on MUFA biosynthesis, independent of differences in food intake.

SCD1 was not differentially expressed in livers of OVX and sham rats, as determined by microarray analysis. Western blotting also indicated no differences in SCD1 protein content in

the livers of these two groups. Accordingly, no differences were seen in MUFA composition when comparing sham with OVX rats. No differences in SREBP-1c, ChREBP or LXR were observed in the microarray analysis. The lack of changes in these transcription factors that control SCD1 expression, may partially explain the lack of change in SCD1 expression. No differences in SCD1 expression between sham and OVX rats in this study contradict previous reports that suggest SCD1 expression is increased following ovariectomy and decreased with estradiol treatment with ovariectomy (Paquette et al., 2008; Alessandri et al., 2011). However, these previous increases were based on mRNA expression and not protein content. In addition, these discrepancies related to SCD1 expression with ovariectomy could be due to differences in study protocols. Previously, rats have been ovariectomized at 8 weeks of age and sacrificed at 16 weeks of age (Paquette et al., 2008), or rats have been ovariectomized at approximately 3 weeks of age (well before sexual maturity (Merry and Holehan, 1979)), and sacrificed at 8 weeks of age (Alessandri et al., 2011). There may be effects of hormones on SCD1 expression during sexual maturity, and potentially longer-term effects of hormones on SCD1 expression (Gao et al., 2006). Additionally, plasma hormone concentrations were not reported in the previous studies, so direct comparison to the present observations is difficult.

Hepatic SCD1 was significantly higher with combination estradiol plus progesterone treatment as compared with sham. These results may have relevant implications, as most hormone treatment studies use estradiol only (Paquette et al., 2008; Gao et al., 2006; Alessandri et al., 2011). Treatment with only one of the major ovarian hormones may not accurately reflect physiological hormone status. An increase in concentration of 16:1n-7 and 18:1n-7 accompanied the increase in SCD1 protein, indicating increased desaturation of 16:0 in hepatic and plasma phospholipids of the estradiol plus progesterone group, as compared with sham.

Surprisingly, there were no differences in hepatic elongase 6 protein between OVX and sham rats despite elongase 6 mRNA being 50% lower in OVX as compared with sham. This may be related to a lack of differences in circulating estradiol levels as determined by ELISA. In contrast, the concentration of 18:0 was lower in hepatic phospholipids of OVX rats as compared with sham, possibly indicating a potential decrease in elongase 6 activity. Elongase 6 protein was 1.5-fold higher in OVX with estradiol treatment as compared with sham and OVX with progesterone treated rats, confirming a potential role for estradiol in elongase 6 regulation. Despite the higher levels of elongase 6 in estradiol treated groups, concentrations of 18:0 in hepatic phospholipids in this group were lowest, potentially due to fatty acid oxidation mediated by supraphysiological estradiol concentrations (Toda et al., 2001; Butcher, Collins, and Fugo, 1974).

The final study in this thesis was an attempt at a comprehensive examination on MUFA metabolism in ovariectomized rats. While the results confirm aspects of previous studies examining the effects of ovariectomy on fatty acid metabolism (Paquette et al., 2008; Alessandri et al., 2011), this study is the first to perform a whole genome analysis to identify genes differentially expressed between OVX and sham rats. To our knowledge, this is also the first study examining ovariectomy and MUFA metabolism that has included full fatty acid composition analyses, and examined protein determinations when considering gene expression. Furthermore, estradiol, progesterone, and estradiol plus progesterone treatment were examined in OVX rats. This study provides additional evidence that elongase 6 expression is influenced by sex hormones, but the regulation of both elongase 6 and SCD1 by sex hormones appears to be complex. Sex hormones influence other aspects of lipid metabolism, such as appetite and oxidation of fatty acids that can impact MUFA biosynthesis. In the future, studies controlling

dietary intake are needed in order to examine the potential direct regulation of MUFA biosynthesis by estradiol and progesterone.

**Table 5** Body weight of female rats throughout the study\*

<b>Group</b>	<b>Day 0</b>	<b>Day 7</b>	<b>Day 14</b>
Sham	207.2 ± 6.1	218.0 ± 7.9 <sup>ab</sup>	213.2 ± 6.2 <sup>ab</sup>
OVX	215.4 ± 6.1	238.9 ± 5.6 <sup>c</sup>	241.5 ± 6.7 <sup>c</sup>
OVX + E	212.4 ± 17.1	200.5 ± 12.0 <sup>a</sup>	196.4 ± 11.8 <sup>a</sup>
OVX + P	219.8 ± 6.3	220.3 ± 21.0 <sup>b</sup>	225.7 ± 20.4 <sup>bc</sup>
OVX + EP	212.9 ± 17.2	213.4 ± 7.5 <sup>ab</sup>	201.2 ± 14.3 <sup>a</sup>

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts within a specific day are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Table 6** Plasma progesterone and estradiol concentrations female rats\*

<b>Group</b>	<b>Progesterone</b> <i>ng/ml</i>	<b>Estradiol</b> <i>pg/ml</i>
Sham	70.1 ± 22.8 <sup>a</sup>	26.1 ± 7.1 <sup>ab</sup>
OVX	18.6 ± 13.2 <sup>c</sup>	15.9 ± 2.4 <sup>a</sup>
OVX + E	19.6 ± 5.1 <sup>bc</sup>	173.7 ± 97.6 <sup>c</sup>
OVX + P	34.2 ± 12.5 <sup>bc</sup>	16.4 ± 2.5 <sup>a</sup>
OVX + EP	41.3 ± 7.3 <sup>b</sup>	109.3 ± 62.7 <sup>bc</sup>

\* Values are mean ± standard deviation, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.



**Table 7** Fatty acid composition of liver phospholipids of hormonally manipulated female rats\*

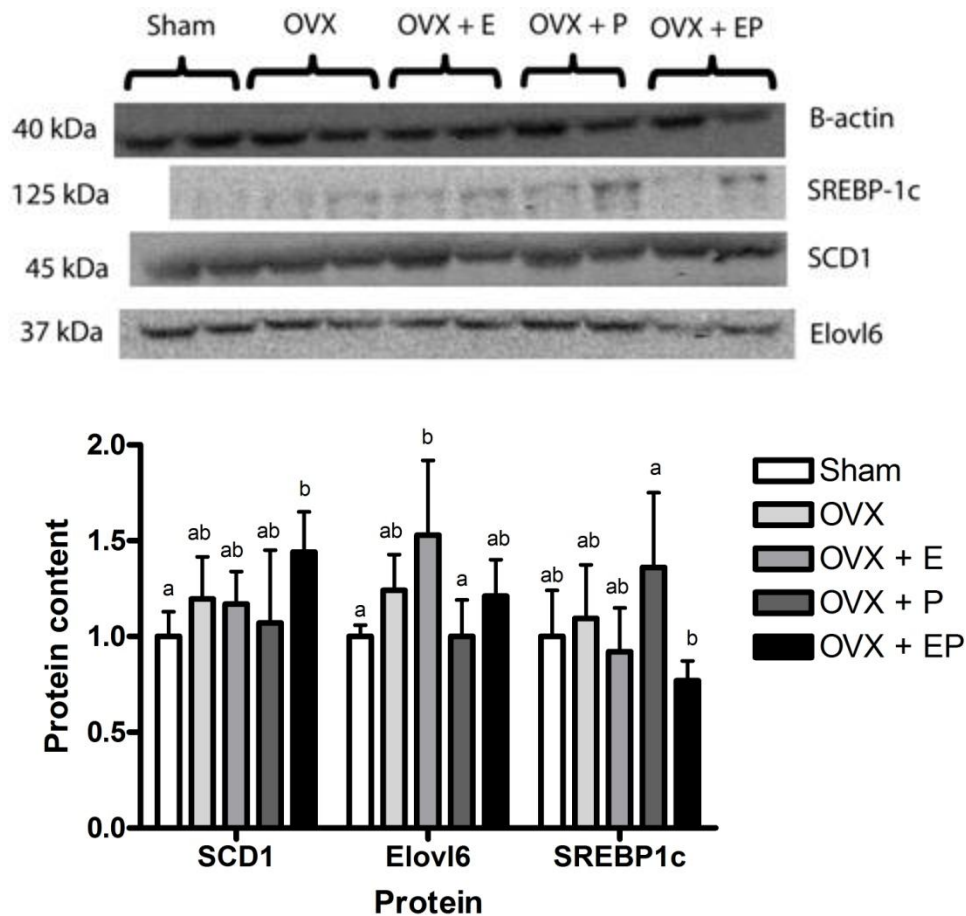
Fatty Acid	Liver Phospholipids				
	<i>µg fatty acid/g liver</i>				
	Sham	OVX	OVX + E	OVX + P	OVX + PE
C 10:0	26 ± 10.31	19.97 ± 4.43	19.44 ± 3.4	29.64 ± 10.98	22.5 ± 8.41
C 12:0	65.43 ± 12.3 <sup>ab</sup>	61.22 ± 11.2 <sup>ab</sup>	52.38 ± 13.14 <sup>a</sup>	84.06 ± 25.53 <sup>b</sup>	63.89 ± 4.03 <sup>ab</sup>
C 14:0	21.08 ± 4.61	20.72 ± 7.51	19.52 ± 4.43	23.4 ± 4.72	21.74 ± 7.12
C 15:0	12.29 ± 2.48 <sup>a</sup>	13.60 ± 3.02 <sup>ab</sup>	17.56 ± 3.67 <sup>b</sup>	13.74 ± 1.12 <sup>ab</sup>	15.16 ± 3.16 <sup>ab</sup>
C 16:0	1320.10 ± 87.19 <sup>ab</sup>	1252.85 ± 123.10 <sup>a</sup>	1478.26 ± 166.98 <sup>b</sup>	1280.84 ± 117.57 <sup>ab</sup>	1690.22 ± 95.67 <sup>c</sup>
C 18:0	3218.82 ± 154.43 <sup>a</sup>	2650.66 ± 239.22 <sup>bc</sup>	2403.38 ± 299.30 <sup>c</sup>	2823.17 ± 300.00 <sup>abc</sup>	2858.94 ± 294.22 <sup>ab</sup>
C 20:0	10.20 ± 2.22	11.37 ± 1.92	9.13 ± 1.54	10.56 ± 2.14	12.62 ± 5.32
C 22:0	22.64 ± 4.00	20.68 ± 3.29	19.36 ± 1.64	21.91 ± 3.8	20.68 ± 5.77
C 23:0	30.34 ± 4.17 <sup>ab</sup>	28.54 ± 5.39 <sup>a</sup>	36.86 ± 5.97 <sup>ab</sup>	28.88 ± 3.25 <sup>a</sup>	37.8 ± 5.56 <sup>b</sup>
C 24:0	64.92 ± 8.87	53.18 ± 6.80	51.57 ± 7.92	55.99 ± 10.83	57.88 ± 10.42
Total SFA	4844.90 ± 141.16 <sup>a</sup>	4168.48 ± 379.53 <sup>bc</sup>	4143.25 ± 442.03 <sup>b</sup>	4429.51 ± 463.58 <sup>ab</sup>	4843.42 ± 355.06 <sup>a</sup>
C 12:1n-3	2.71 ± 0.72	2.69 ± 0.99	5.56 ± 0.68	2.4 ± 0.22	2.71 ± 0.90
C 14:1n-5	5.25 ± 1.61	4.48 ± 0.63	3.38 ± 1.48	5.4 ± 0.96	4.73 ± 2.05
C 16:1n-7	20.81 ± 5.13 <sup>a</sup>	16.61 ± 5.08 <sup>a</sup>	25.67 ± 6.13 <sup>ab</sup>	19.86 ± 3.21 <sup>a</sup>	35.52 ± 9.19 <sup>b</sup>
C 18:1n-7	161.96 ± 15.85 <sup>ab</sup>	135.94 ± 16.13 <sup>a</sup>	193.14 ± 16.26 <sup>bc</sup>	152.52 ± 14.77 <sup>a</sup>	210.71 ± 30.66 <sup>c</sup>
C 18:1n-9	355.56 ± 157.30	308.92 ± 181.99	319.66 ± 73.89	323.96 ± 142.55	277.46 ± 25.35
C 20:1n-9	6.48 ± 0.68	5.5 ± 2.47	6.73 ± 1.08	5.78 ± 1.09	7.10 ± 2.55
C 22:1n-9	9.03 ± 3.19	11.49 ± 4.86	9.65 ± 4.73	13.62 ± 4.03	10.32 ± 5.6
C 24:1n-9	21.31 ± 1.78	22.08 ± 2.92	21.00 ± 5.94	24.18 ± 10.92	24.60 ± 5.47
Total MUFA	589.86 ± 163.72	518.57 ± 187.72	593.97 ± 76.26	554.94 ± 165.62	581.72 ± 51.73
Total N-6 PUFA	3948.85 ± 153.08 <sup>a</sup>	3431.72 ± 290.44 <sup>b</sup>	3250.17 ± 354.33 <sup>b</sup>	3583.82 ± 293.63 <sup>ab</sup>	3616.54 ± 266.29 <sup>ab</sup>
Total N-3 PUFA	1020.58 ± 104.51 <sup>ab</sup>	830.27 ± 126.24 <sup>a</sup>	1169.32 ± 153.31 <sup>bc</sup>	857.47 ± 108.85 <sup>a</sup>	1376.08 ± 135.89 <sup>c</sup>
Total	10632.90 ± 373.68 <sup>a</sup>	9102.06 ± 898.60 <sup>b</sup>	9303.07 ± 909.97 <sup>ab</sup>	9610.58 ± 980.77 <sup>ab</sup>	10566.13 ± 729.98 <sup>a</sup>

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

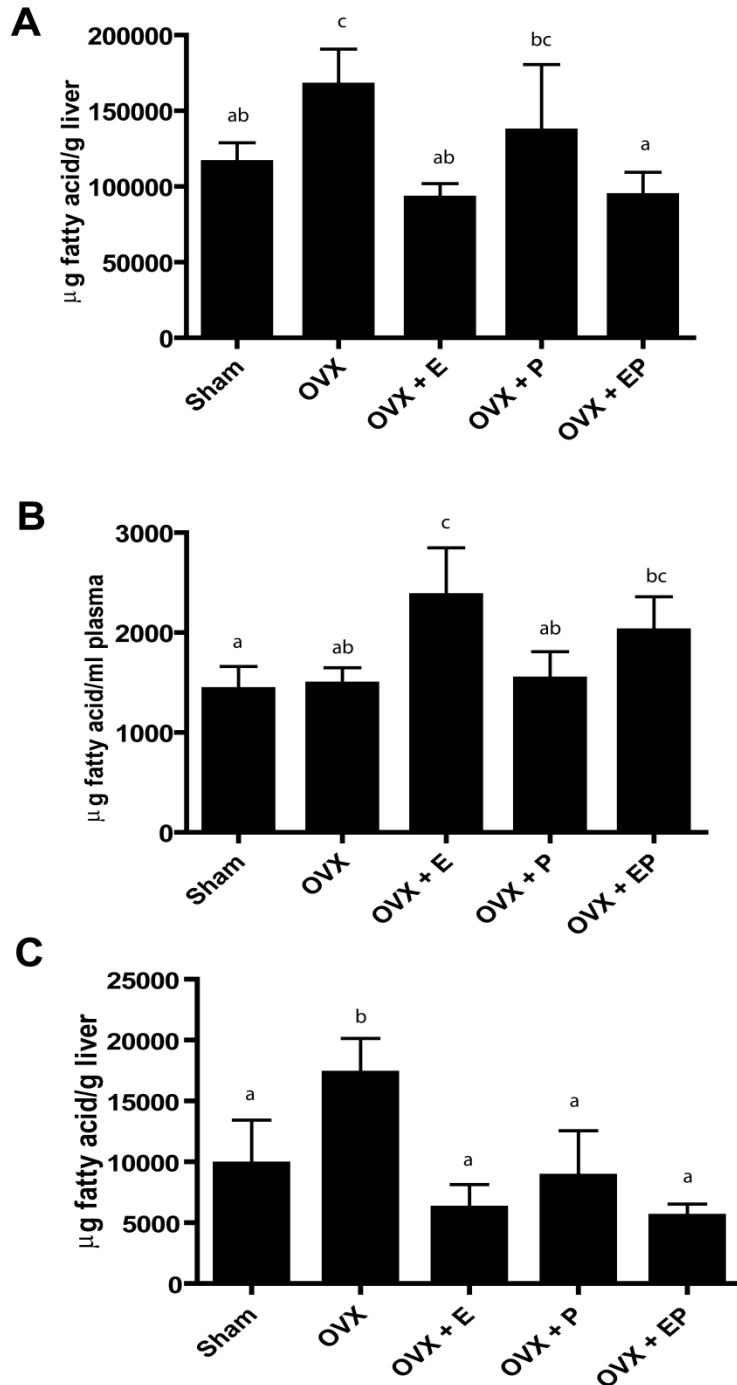
**Table 8** Fatty acid composition of plasma phospholipids of hormonally manipulated female rats\*

Fatty acid	Plasma Phospholipids				
	<i>µg fatty acid/ml plasma</i>				
	Sham	OVX	OVX + E	OVX + P	OVX + EP
C 10:0	0.84 ± 0.57	0.89 ± 0.78	1.03 ± 0.51	1.50 ± 0.67	1.01 ± 0.59
C 12:0	11.82 ± 13.06	18.08 ± 9.92	18.29 ± 8.47	17.98 ± 9.71	17.09 ± 7.93
C 14:0	12.46 ± 3.84	13.58 ± 7.38	13.62 ± 4.33	15.04 ± 3.05	14.68 ± 3.54
C 15:0	2.64 ± 0.49	3.75 ± 1.55	4.25 ± 0.66	3.98 ± 0.81	3.78 ± 0.85
C 16:0	158.54 ± 17.34 <sup>a</sup>	179.26 ± 43.02 <sup>a</sup>	251.6 ± 38.7 <sup>b</sup>	186.42 ± 13.08 <sup>a</sup>	240.01 ± 14.12 <sup>b</sup>
C 18:0	294.27 ± 14.18	271.58 ± 81.78	31.87 ± 38.08	294.56 ± 23.75	361.92 ± 54.94
C 20:0	2.78 ± 0.52	3.57 ± 2.09	3.06 ± 0.88	3.17 ± 0.76	3.48 ± 1.26
C 21:0	0.72 ± 0.8	0.44 ± 0.24	0.33 ± 0.33	0.66 ± 0.21	0.49 ± 0.55
C 22:0	4.11 ± 1.21	4.49 ± 1.64	3.4 ± 0.61	5.07 ± 1.46	4.72 ± 0.68
C 23:0	2.21 ± 0.77 <sup>ab</sup>	1.68 ± 0.71 <sup>a</sup>	3.11 ± 1.01 <sup>ab</sup>	2.44 ± 0.86 <sup>ab</sup>	3.51 ± 0.59 <sup>b</sup>
C 24:0	8.49 ± 1.06	7.58 ± 1.52	6.88 ± 1.59	9.83 ± 2.76	9.11 ± 2.40
Total SFA	497 ± 39 <sup>a</sup>	505 ± 140 <sup>ab</sup>	638 ± 78 <sup>b</sup>	541 ± 31 <sup>ab</sup>	660 ± 60 <sup>b</sup>
C 14:1n-5	0.79 ± 0.58	0.66 ± 0.43	0.78 ± 0.34	0.84 ± 0.53	0.53 ± 0.19
C 16:1n-7	9.29 ± 4.17	9.10 ± 4.12	10.42 ± 2.83	10.18 ± 4.57	10.26 ± 3.72
C 18:1n-7	21.11 ± 4.60 <sup>a</sup>	20.46 ± 6.15 <sup>a</sup>	33.96 ± 3.30 <sup>b</sup>	24.43 ± 2.04 <sup>ac</sup>	32.04 ± 5.69 <sup>bc</sup>
C 18:1n-9	299.67 ± 91.51	309.21 ± 187.67	314.26 ± 82.08	332.51 ± 76.2	291.73 ± 99.16
C 20:1n-9	3.22 ± 0.71	3.44 ± 0.75	3.44 ± 0.83	3.35 ± 0.55	3.43 ± 0.94
C 22:1n-9	8.59 ± 5.92	6.79 ± 2.3	7.86 ± 1.69	8.42 ± 1.32	6.26 ± 1.84
C 24:1n-9	7.64 ± 1.19	5.48 ± 2.71	5.88 ± 2.72	7.87 ± 4.67	6.93 ± 1.85
Total MUFA	353.09 ± 100.31	357.39 ± 198.93	378.55 ± 88.9	391.16 ± 72.94	353.4 ± 102.84
Total N-6 PUFA	291.87 ± 13.95 <sup>a</sup>	276.66 ± 55.74 <sup>a</sup>	430.16 ± 54.6 <sup>b</sup>	329.06 ± 32.27 <sup>a</sup>	416.64 ± 48.52 <sup>b</sup>
Total N-3 PUFA	44.78 ± 1.29 <sup>a</sup>	42.61 ± 9.93 <sup>a</sup>	90.89 ± 16.45 <sup>b</sup>	48.05 ± 8.79 <sup>a</sup>	91.79 ± 15.92 <sup>b</sup>
Total fatty acids	1186.49 ± 141.55	1181.63 ± 380.46	1537.5 ± 183.74	1308.92 ± 52.04	1521.65 ± 170.27

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.



**Figure 5.** Densitometric analysis of protein content and representative immunoblots of enzymes involved in MUFA biosynthesis (n = 5-7 for each group). Groups with different superscripts are significantly different by Tukey's post hoc test ( $p < 0.05$ ) following significant F-value by one-way ANOVA. SCD1: stearoyl-CoA desaturase 1, elovl6: elongase 6, SREBP1c: sterol regulatory element binding protein-1c, Sham: sham-operated, OVX: ovariectomized, + E: with  $17\beta$ -estradiol, + P: with progesterone, + EP: with  $17\beta$ -estradiol plus progesterone.



**Figure 6.** Total fatty acid concentration of A. total lipid extract of liver B. total lipid extract of plasma and C. liver triacylglycerols. Values with different superscripts are significantly different by Tukey's post hoc test ( $p < 0.05$ ) following significant F-value by one-way ANOVA. Sham: sham-operated, OVX: ovariectomized, + E: with  $17\beta$ -estradiol, + P: with progesterone, + EP: with  $17\beta$ -estradiol plus progesterone.

## Chapter 9

### Discussion

#### 9.1 Discussion

Previous studies have reported sex differences in tissue fatty acid compositions of both humans (Marangoni et al., 2007; Crowe et al., 2008) and animals (Burdge et al., 2008; Extier et al., 2010; Kitson et al., 2012) suggesting a role of sex hormones in fatty acid metabolism. Differences in fatty acid compositions have also been observed in ovariectomized and estrogen receptor alpha knockout rodents as compared with intact rodents (Paquette et al., 2008; Alessandri et al., 2011; Bryzgalova et al., 2006; Gao et al., 2006). However, these studies provide incomplete data on differences in MUFA composition and enzyme expression. The goal of this thesis was to characterize sex differences in MUFA composition and enzyme expression, and to further examine the role of sex hormones by using both cell culture and hormonal manipulations of female rats.

I have reported higher concentrations of both n-7 and n-9 MUFA in liver, plasma and erythrocytes of males as compared with females, as initially hypothesized. While higher 18:1n-9 in male liver phospholipids has been previously reported (Burdge et al., 2008), differences in n-7 MUFA have not been reported previously. I hypothesized that the higher concentration of MUFA in males would be due to higher hepatic expression of SCD1, but no sex differences in SCD1 expression were observed. SCD1 activity was not measured. The differences in MUFA composition could be mediated by the significant increased expression of elongase 6 in females. This would affect SCD1 substrate availability. In HepG2 cells, estradiol and progesterone appear to have opposing effects on SCD1, with estradiol increasing, and progesterone decreasing expression while testosterone had no effect. In contrast, elongase 6 expression was increased

with estradiol and progesterone and decreased with testosterone of in culture. Therefore, it was hypothesized that elongase 6 expression would be altered with hormone manipulation, and that elongase 6 would be a stronger determinant of MUFA composition as compared with SCD1 in the ovariectomy study. Elongase 6 expression was 50% lower in OVX rats as compared with sham, whereas SCD1 expression was not different between these two groups as determined by microarray.

The “SCD activity index” is strongly associated with obesity (Chu et al., 2006; Garcia-Serrano et al., 2011) and cardiovascular disease (Warensjo et al., 2008). The use of ratios of fatty acids to indicate enzyme activities involved in fatty acid biosynthesis is common, but it is generally an oversimplification that ignores; the influence of other enzymes involved in biosynthesis, the selective incorporation of fatty acids into greater lipids, and fatty acid oxidation. The findings from the studies in this thesis indicate that this index is not an appropriate estimation of SCD1 activity. When comparing male and female rats in this thesis, an 18:1n-9/18:0-based hepatic SCD activity index in females would be approximately 50% of that of males. This is a result of higher 18:0 concentrations in females that appears to be due to increased conversion of 16:0 to 18:0 through higher expression of elongase 6 in females, and not through different conversion rates of 18:0 to 18:1n-9 by SCD1. Conversely, SCD1 protein is higher in estradiol plus progesterone animals as compared with sham, but using the SCD activity index would suggest that there are no differences in SCD expression between these two groups. Taken together, there is strong evidence that caution and skepticism should be used when making conclusions from the calculation of the SCD activity index, as there is evidence that elongase 6 is also involved that has been overlooked in previous studies.

Hepatic SCD2 mRNA expression was higher in males, suggesting that it may be involved in the increased MUFA concentrations observed in male rats, but this SCD isoform has not been identified in humans (Wang et al., 2005a) and therefore SCD2 was not measured in the HepG2 cell culture experiments. It was not differentially expressed between OVX and sham rats as determined by microarray. Its exact role in MUFA biosynthesis in the liver remains unclear. No other SCD isoforms have been identified in rats (Miyazaki, Bruggink, and Ntambi, 2006).

In the present experiments with HepG2 cells, SCD1 expression increased with 10nM 17 $\beta$ -estradiol treatment, and decreased with 10nM progesterone treatment but higher concentrations had no effect. Treatment with testosterone had no effect on SCD1 at any concentration. The opposing effects of estradiol and progesterone on SCD1 expression may negate each other in female rats, resulting in the lack of sex difference on SCD1 expression observed. However, the effect of these hormones on SCD1 expression may be complex, as treatment with estradiol plus progesterone in OVX rats resulted in a significant increase in SCD1 protein as compared with sham rats. However, the hormone concentrations achieved with the pellets in the present study were not the same as that of sham-operated rats, and these results should be confirmed with physiological hormone doses.

The sex differences and hormonal treatment responses were consistent for elongase 6 expression. Both the mRNA and protein levels were higher in females as compared with males. The higher concentrations of 18:0 and lower concentrations of 16:0 in liver phospholipids of females may be an indirect indicator of elongase 6 activity. Both estrogen and progesterone treatment of HepG2 cells increased elongase 6 expression, while testosterone treatment decreased expression. While elongase 6 protein did not change between OVX and sham rats, plasma estradiol concentrations were not significantly different between these groups. However,

estradiol treatment in ovariectomized rats resulted in an increase in elongase 6 protein, further suggesting a role for estradiol in the regulation of its expression. Examination of the effect of estradiol on elongase 6 at physiological concentrations is required.

The present results from HepG2 cells cannot be directly extrapolated to an animal model, as the cells were treated with individual hormones, whereas a combination of these hormones is present in animals, and the plasma concentration of these hormones fluctuate during the estrous cycle (Butcher, Collins, and Fugo, 1974). Therefore, the effect of hormones on animals may differ from what was presently observed. In addition, hepatic cells in culture do not have to import and export lipids to meet the demands of other tissues, but only fulfill its own lipid metabolism requirements. Therefore, the metabolism that is occurring in HepG2 cells may be different as compared to liver from *in vivo* animal studies.

The sex differences observed in my study are limited to a fasting rodent model and should not be extrapolated to the fed state. Many genes involved in fatty acid biosynthesis including SCD1 and elongase 6 are strongly regulated by fasting and refeeding (Turyn, Stojek, and Swierczynski, 2010; Zhang et al., 2011). While overnight fasting is typically preferred to *ad libitum* food access prior to sacrifice to reduce inter-individual variation, experiments examining fasting and a controlled feeding protocol prior to sacrifice need to be considered for future studies. In addition, OVX results in an increase in appetite, resulting in significant weight gain (Wade, 1975). Because MUFA are a major component of dietary fat in addition to endogenous synthesis, the changes in enzyme expression and MUFA composition are difficult to distinguish between hormonal differences and differences in food consumption. Therefore, a controlled feeding protocol, or a pair-fed design between OVX and sham rats, would provide insight into the effects of hormones on MUFA metabolism without confounding by dietary intake.



In humans, both sex and hormone status appears to be strong determinants of macronutrient preference. While the rats in the present study were fed the same controlled chow diet, women tend to consume higher proportions of carbohydrates and less fat as compared with men (Garriguet, 2007; Metherel et al., 2009). Hormonal shifts associated with the use of hormone replacement therapy by menopausal women (Stark and Holub, 2004), and with pregnancy, delivery and post-partum (Stark et al., 2005) appear to have effects on fat and carbohydrate intake. A shift towards a relatively higher carbohydrate and lower fat consumption can result in an increased influence of endogenous fatty acid synthesis on tissue fatty acid composition. Specifically, with a low fat, high carbohydrate diet, 16:0 biosynthesis is increased resulting in increased availability of 16:0 as a substrate by mass action for tissue incorporation, and desaturation by SCD1 that results in higher levels of the n-7 fatty acids with the potential for decreases in fatty acids derived solely from the diet (Lands, 1995).

This thesis provides evidence from three different types of studies that sex and hormone status affect MUFA composition, specifically in phospholipid fatty acids as no differences were seen in other fatty acid pools. This specific effect on phospholipid fatty acid composition suggests that the observed differences may be due in part to influences on fatty acid incorporation into complex lipids. Changing the fatty acid composition of phospholipids can affect the physical properties of cell membranes, and influence potent lipid mediators involved in cell signalling. A thorough examination of the effect of sex and sex hormones on phospholipid and TAG synthesis and remodelling is warranted. While differences in MUFA composition appear to be selectively different in phospholipids of these studies, the effects of sex hormones are not straightforward, as phospholipid MUFA concentrations were higher in males as compared with females, and highest with estradiol and estradiol plus progesterone treatment as

compared with sham and OVX. This indicates that pathways other than the effect of ovarian hormones on MUFA biosynthesis through SCD1 and elongase 6 may be involved in mediating the sex differences in MUFA composition.

## 9.2 Conclusion

There are significant sex and hormone-based differences in MUFA composition that appear to be phospholipid specific and mediated mainly by differences in elongase 6 expression. This finding is especially important in this field, as differences in MUFA metabolism are largely attributed to SCD1, as the SCD1 activity index is often associated with chronic disease. Presently, three different study designs provide strong evidence that elongase 6 is regulated by estradiol and may play an important role in MUFA metabolism. Further work in MUFA biosynthesis should include assessments of elongase 6 expression and activity.

One difficulty in studying MUFA metabolism is differentiating between exogenous (dietary) and endogenous (biosynthesis) MUFA. A logical next step for continued research examining the effects of hormones on MUFA biosynthesis is comparing enzyme expression and MUFA composition with ovariectomy while using pair-feeding protocols to control diet intake. Limiting the food intake in a group of ovariectomized rats to that of sham controls should prevent the increased body mass and TAG accumulation observed presently. Additionally, stable isotopes could be used for metabolic tracer studies. Labelled glucose would allow *de novo* fatty acid synthesis to be monitored, while the action of SCD1 and elongase 6 could be monitored via labelled 16:0 or 18:0. These stable isotope studies could be conducted in several models, including male and female rats, ovariectomized versus sham operated rats, pair-fed ovariectomized rats versus sham operated rats, as well as cell culture models. Additionally, the expression of genes involved in TAG and phospholipid synthesis, and fatty acid oxidation

requires further exploration. Several genes in these pathways were differentially expressed between sham and OVX rats as determined by microarray.

The preference of elongase 6 for 16:0 as a substrate is well established (Green et al., 2010; Wang et al., 2005b). Elongase 6 knockout mice show increased concentrations of 16:0 and 16:1n-7, and lower concentrations of 18:0 and 18:1n-9, as compared with wild type mice (Matsuzaka et al., 2007). When fed a high-fat diet, elongase 6 knockout mice and wild-type mice both develop hepatosteatosis; however, unlike the wild type mice, the elongase 6 knockout mice have nearly normal insulin sensitivity (Matsuzaka et al., 2007). Conversely, accumulation of 18:0 and 18:1n-9 occur in elongase 6 overexpression (Green et al., 2010). Elongase 6 expression is higher in Zucker obese rats as compared with lean littermates (Fevre et al., 2011), and has been shown to modulate inflammation, oxidative stress, and fibrosis in the liver when fed a high-fat diet, as well as being positively correlated to hepatosteatosis severity (Matsuzaka et al., 2012). These reports highlight the importance of the results of my thesis, as sex differences in elongase 6 expression and the strong evidence that its expression is regulated by estradiol may have implications in human health and the progression of obesity.

This thesis demonstrates that sex and sex hormones are involved in MUFA metabolism, resulting in differences in tissue MUFA composition. Elongase 6 has been identified as a primary target, while SCD1 may be secondary in mediating these differences. Understanding MUFA biosynthesis has implications beyond metabolism, as recent evidence suggests that both MUFA and SCD1 may be associated with cardiovascular disease risk and obesity. This present belief and understanding of the health effects of MUFA and SCD1 is based on work that has been largely completed in men or male rodents, but not females. The results from this thesis have potential impacts on health and interpretation of these present beliefs, as there is strong evidence

that there are sex differences and effects of ovarian hormones on MUFA metabolism. Continued work examining sex differences in fatty acid and lipid metabolism is needed.

**Appendix A. Fatty acid composition of the total lipid extract of livers of hormonally manipulated female rats \***

Fatty acid	Liver Total Lipid Extract mg fatty acid / g liver tissue				
	Sham	OVX	OVX+E	OVX+P	OVX+EP
C 10:0	0.017 ± 0.009	0.017 ± 0.007	0.009 ± 0.004	0.015 ± 0.008	0.013 ± 0.005
C 12:0	0.56 ± 0.07	0.62 ± 0.06	0.52 ± 0.04	0.53 ± 0.11	0.52 ± 0.1
C 14:0	0.23 ± 0.04 <sup>ab</sup>	0.43 ± 0.10 <sup>c</sup>	0.17 ± 0.02 <sup>a</sup>	0.32 ± 0.17 <sup>ab</sup>	0.16 ± 0.03 <sup>a</sup>
C 15:0	17.70 ± 1.89 <sup>a</sup>	27.22 ± 4.00 <sup>a</sup>	15.73 ± 2.01 <sup>b</sup>	22.16 ± 7.62 <sup>ab</sup>	15.91 ± 2.68 <sup>b</sup>
C 16:0	24.64 ± 2.28	26.07 ± 3.69	20.56 ± 2.09	25.07 ± 6.00	21.55 ± 4.10
C 18:0	0.07 ± 0.01 <sup>ab</sup>	0.10 ± 0.02 <sup>a</sup>	0.07 ± 0.01 <sup>b</sup>	0.08 ± 0.02 <sup>ab</sup>	0.06 ± 0.02 <sup>a</sup>
C 20:0	0.22 ± 0.07	0.24 ± 0.11	0.18 ± 0.03	0.20 ± 0.05	0.18 ± 0.03
C 21:0	0.22 ± 0.04	0.21 ± 0.09	0.21 ± 0.05	0.18 ± 0.06	0.20 ± 0.04
C 22:0	0.69 ± 0.05	0.65 ± 0.14	0.62 ± 0.11	0.66 ± 0.13	0.61 ± 0.08
C 23:0	44.74 ± 4.04 <sup>ab</sup>	55.91 ± 6.68 <sup>a</sup>	38.43 ± 4.26 <sup>b</sup>	49.59 ± 14.01 <sup>ab</sup>	39.56 ± 6.82 <sup>ab</sup>
C 24:0	0.004 ± 0.003 <sup>ab</sup>	0.008 ± 0.005 <sup>a</sup>	0.003 ± 0.002 <sup>b</sup>	0.005 ± 0.006 <sup>ab</sup>	0.003 ± 0.003 <sup>ab</sup>
Total SFA	0.55 ± 0.13 <sup>a</sup>	1.14 ± 0.42 <sup>b</sup>	0.43 ± 0.1 <sup>a</sup>	0.80 ± 0.53 <sup>ab</sup>	0.52 ± 0.13 <sup>a</sup>
C 14:1n-5	0.22 ± 0.03	0.21 ± 0.09	0.16 ± 0.04	0.23 ± 0.06	0.17 ± 0.02
C 16:1n-7	1.97 ± 0.21 <sup>a</sup>	2.82 ± 0.53 <sup>b</sup>	2.17 ± 0.22 <sup>ab</sup>	2.39 ± 0.80 <sup>ab</sup>	2.04 ± 0.34 <sup>a</sup>
C 18:1n-7	7.83 ± 1.6 <sup>ab</sup>	15.56 ± 4.14 <sup>c</sup>	4.19 ± 0.64 <sup>a</sup>	10.90 ± 5.47 <sup>bc</sup>	4.17 ± 0.69 <sup>a</sup>
C 18:1n-9	0.08 ± 0.02 <sup>a</sup>	0.15 ± 0.04 <sup>b</sup>	0.07 ± 0.02 <sup>a</sup>	0.11 ± 0.05 <sup>ab</sup>	0.07 ± 0.01 <sup>a</sup>
C 20:1n-9	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.03	0.04 ± 0.01	0.04 ± 0.01
C 22:1n-9	0.17 ± 0.03	0.14 ± 0.03	0.15 ± 0.04	0.15 ± 0.04	0.17 ± 0.04
C 24:1n-9	10.84 ± 1.93 <sup>ab</sup>	20.08 ± 5.00 <sup>c</sup>	7.23 ± 0.88 <sup>a</sup>	14.46 ± 6.87 <sup>bc</sup>	7.20 ± 1.14 <sup>a</sup>
Total MUFA	49.45 ± 6.20 <sup>ab</sup>	76.52 ± 11.62 <sup>c</sup>	34.93 ± 3.27 <sup>a</sup>	60.62 ± 19.91 <sup>bc</sup>	35.32 ± 5.71 <sup>a</sup>
Total N-6 PUFA	10.64 ± 1.11 <sup>a</sup>	14.32 ± 1.84 <sup>b</sup>	11.81 ± 1.73 <sup>ab</sup>	11.85 ± 3.28 <sup>ab</sup>	11.97 ± 2.02 <sup>ab</sup>
Total N-3 PUFA	115.91 ± 12.99 <sup>ab</sup>	167.04 ± 23.64 <sup>c</sup>	92.43 ± 9.52 <sup>a</sup>	136.7 ± 43.84 <sup>bc</sup>	94.06 ± 15.34 <sup>a</sup>
Total Fatty acids	0.017 ± 0.009	0.017 ± 0.007	0.009 ± 0.004	0.015 ± 0.008	0.013 ± 0.005

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix B.** Relative percentage of fatty acids in the total lipid extract of hormonally manipulated female rats \*

<b>Liver Total Lipid Extract</b>					
<i>Percentage of total fatty acids</i>					
<b>Fatty acid</b>	<b>Sham</b>	<b>OVX</b>	<b>OVX+E</b>	<b>OVX+P</b>	<b>OVX+EP</b>
C 10:0	0.014 ± 0.008	0.001 ± 0.004	0.010 ± 0.005	0.011 ± 0.006	0.014 ± 0.005
C 12:0	0.49 ± 0.07 <sup>ab</sup>	0.38 ± 0.04 <sup>c</sup>	0.57 ± 0.06 <sup>b</sup>	0.40 ± 0.06 <sup>ac</sup>	0.55 ± 0.05 <sup>b</sup>
C 14:0	0.20 ± 0.02 <sup>ab</sup>	0.25 ± 0.03 <sup>c</sup>	0.19 ± 0.02 <sup>ab</sup>	0.22 ± 0.05 <sup>bc</sup>	0.17 ± 0.01 <sup>a</sup>
C 15:0	15.28 ± 0.50 <sup>a</sup>	16.26 ± 0.27 <sup>b</sup>	16.96 ± 0.51 <sup>b</sup>	16.07 ± 0.50 <sup>ab</sup>	16.90 ± 0.84 <sup>a</sup>
C 16:0	21.30 ± 1.14 <sup>a</sup>	15.74 ± 2.35 <sup>b</sup>	22.24 ± 0.80 <sup>a</sup>	18.72 ± 2.17 <sup>b</sup>	22.82 ± 1.33 <sup>a</sup>
C 18:0	0.06 ± 0.01 <sup>ab</sup>	0.06 ± 0.01 <sup>ab</sup>	0.07 ± 0.01 <sup>a</sup>	0.06 ± 0.01 <sup>b</sup>	0.06 ± 0.01 <sup>ab</sup>
C 20:0	0.18 ± 0.04	0.14 ± 0.06	0.20 ± 0.03	0.15 ± 0.05	0.19 ± 0.03
C 21:0	0.19 ± 0.03 <sup>ab</sup>	0.12 ± 0.05 <sup>a</sup>	0.23 ± 0.05 <sup>b</sup>	0.14 ± 0.06 <sup>a</sup>	0.22 ± 0.05 <sup>b</sup>
C 22:0	0.60 ± 0.03 <sup>ab</sup>	0.39 ± 0.08 <sup>c</sup>	0.67 ± 0.05 <sup>a</sup>	0.50 ± 0.08 <sup>bc</sup>	0.65 ± 0.08 <sup>a</sup>
C 23:0	38.65 ± 1.10 <sup>ab</sup>	33.57 ± 2.35 <sup>c</sup>	41.51 ± 1.03 <sup>bd</sup>	36.55 ± 2.04 <sup>ac</sup>	41.96 ± 0.92 <sup>d</sup>
C 24:0	0.004 ± 0.002	0.005 ± 0.003	0.004 ± 0.002	0.003 ± 0.003	0.003 ± 0.003
Total SFA	0.47 ± 0.08	0.67 ± 0.18	0.46 ± 0.08	0.54 ± 0.16	0.55 ± 0.09
C 14:1n-5	1.70 ± 0.11 <sup>a</sup>	1.68 ± 0.12 <sup>a</sup>	2.35 ± 0.18 <sup>b</sup>	1.74 ± 0.08 <sup>a</sup>	2.18 ± 0.25 <sup>b</sup>
C 16:1n-7	6.71 ± 0.71 <sup>a</sup>	9.19 ± 1.49 <sup>b</sup>	4.55 ± 0.63 <sup>c</sup>	7.62 ± 1.46 <sup>ab</sup>	4.46 ± 0.49 <sup>c</sup>
C 18:1n-7	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.03	0.08 ± 0.01	0.07 ± 0.01
C 18:1n-9	0.04 ± 0.01 <sup>ab</sup>	0.03 ± 0.01 <sup>a</sup>	0.06 ± 0.03 <sup>b</sup>	0.03 ± 0.02 <sup>a</sup>	0.05 ± 0.01 <sup>ab</sup>
C 20:1n-9	0.15 ± 0.03 <sup>ab</sup>	0.09 ± 0.02 <sup>a</sup>	0.16 ± 0.05 <sup>bc</sup>	0.11 ± 0.01 <sup>ab</sup>	0.19 ± 0.05 <sup>c</sup>
C 22:1n-9	9.30 ± 0.76 <sup>ab</sup>	11.88 ± 1.68 <sup>c</sup>	7.84 ± 0.81 <sup>a</sup>	10.18 ± 1.58 <sup>bc</sup>	7.68 ± 0.73 <sup>a</sup>
C 24:1n-9	42.57 ± 0.75 <sup>a</sup>	45.69 ± 1.03 <sup>b</sup>	37.78 ± 0.76 <sup>c</sup>	44.20 ± 1.00 <sup>b</sup>	37.51 ± 0.58 <sup>c</sup>
Total MUFA	9.19 ± 0.44 <sup>a</sup>	8.58 ± 0.4 <sup>a</sup>	12.72 ± 0.7 <sup>b</sup>	8.75 ± 0.39 <sup>a</sup>	12.71 ± 0.48 <sup>b</sup>
Total N-6 PUFA	0.014 ± 0.008	0.001 ± 0.004	0.010 ± 0.005	0.011 ± 0.006	0.014 ± 0.005
Total N-3 PUFA	0.49 ± 0.07 <sup>ab</sup>	0.38 ± 0.04 <sup>c</sup>	0.57 ± 0.06 <sup>b</sup>	0.40 ± 0.06 <sup>ac</sup>	0.55 ± 0.05 <sup>b</sup>
Total Fatty acids	0.20 ± 0.02 <sup>ab</sup>	0.25 ± 0.03 <sup>c</sup>	0.19 ± 0.02 <sup>ab</sup>	0.22 ± 0.05 <sup>bc</sup>	0.17 ± 0.01 <sup>a</sup>

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix C. Fatty acid composition of liver triacylglycerols of hormonally manipulated female rats \***

Fatty acid	Liver triacylglycerols <i>μg fatty acid/g liver</i>				
	Sham	OVX	OVX+E	OVX+P	OVX+EP
C 10:0	41.21 ± 34.01	47.69 ± 21.31	54.00 ± 31.61	41.40 ± 18.90	64.68 ± 18.21
C 12:0	122.33 ± 29.20	124.33 ± 16.41	134.83 ± 37.16	121.52 ± 33.31	156.18 ± 18.34
C 14:0	65.41 ± 20.51 <sup>ab</sup>	91.34 ± 13.43 <sup>a</sup>	55.06 ± 14.67 <sup>b</sup>	63.16 ± 26.61 <sup>ab</sup>	54.16 ± 13.38 <sup>b</sup>
C 15:0	27.34 ± 7.50 <sup>a</sup>	41.51 ± 3.70 <sup>b</sup>	23.13 ± 2.53 <sup>a</sup>	26.39 ± 10.39 <sup>ab</sup>	23.76 ± 9.11 <sup>a</sup>
C 16:0	1945.03 ± 659.58 <sup>a</sup>	3269.38 ± 431.91 <sup>b</sup>	1264.07 ± 306.67 <sup>a</sup>	1782.98 ± 757.5 <sup>a</sup>	1246.68 ± 254.88 <sup>a</sup>
C 18:0	434.06 ± 120.11 a	592.06 ± 70.28 <sup>b</sup>	415.79 ± 87.65 <sup>a</sup>	376.53 ± 101.05 <sup>a</sup>	417.68 ± 66.07 <sup>a</sup>
C 20:0	7.15 ± 3.89	10.26 ± 1.42	10.71 ± 2.53	5.76 ± 1.79	9.64 ± 4.07
C 21:0	2.78 ± 5.21	1.78 ± 0.63	3.03 ± 3.43	2.57 ± 2.30	2.91 ± 2.60
C 22:0	5.02 ± 3.32	4.23 ± 2.93	6.72 ± 3.71	3.75 ± 3.71	5.05 ± 2.06
C 23:0	49.78 ± 23.24 <sup>a</sup>	120.51 ± 27.89 <sup>b</sup>	45.77 ± 21.73 <sup>a</sup>	52.08 ± 21.89 <sup>a</sup>	36.51 ± 13.31 <sup>a</sup>
C 24:0	8.23 ± 8.98	6.23 ± 2.90	7.50 ± 9.45	2.71 ± 2.25	9.81 ± 5.69
Total SFA	2745.42 ± 846.37 <sup>a</sup>	4360 ± 533.16 <sup>b</sup>	2067.37 ± 315.04 <sup>a</sup>	2524.88 ± 926.16 <sup>a</sup>	2082.84 ± 297.54 <sup>a</sup>
C 14:1n-5	2.03 ± 1.61	3.55 ± 2.26	2.96 ± 2.00	3.74 ± 2.23	3.74 ± 3.67
C 16:1n-7	93.22 ± 37.58 <sup>a</sup>	179.46 ± 55.95 <sup>b</sup>	63.69 ± 13.02 <sup>a</sup>	93.97 ± 61.44 <sup>a</sup>	66.85 ± 22.08 <sup>a</sup>
C 18:1n-7	159.93 ± 60.83 <sup>a</sup>	282.13 ± 55.27 <sup>b</sup>	113.95 ± 26.81 <sup>a</sup>	145.61 ± 66.97 <sup>a</sup>	107.13 ± 19.98 <sup>a</sup>
C 18:1n-9	1946.19 ± 907.66 <sup>ab</sup>	2797.34 ± 593.86 <sup>a</sup>	1156.28 ± 500.02 <sup>bc</sup>	1420.41 ± 706.1 <sup>bc</sup>	905.30 ± 235.86 <sup>c</sup>
C 20:1n-9	11.93 ± 3.27 <sup>a</sup>	20.67 ± 3.52 <sup>b</sup>	14.17 ± 8.14 <sup>ab</sup>	12.60 ± 4.29 <sup>a</sup>	8.23 ± 2.91 <sup>a</sup>
C 22:1n-9	17.50 ± 7.97	21.46 ± 6.87	24.87 ± 11.20	19.53 ± 6.47	25.22 ± 5.87
C 24:1n-9	20.65 ± 35.97	2.15 ± 2.76	4.24 ± 3.56	6.41 ± 6.63	12.87 ± 11.64
Total MUFA	2265.00 ± 981.15 <sup>ab</sup>	3334.82 ± 699.96 <sup>a</sup>	1394.51 ± 521.12 <sup>b</sup>	1715.49 ± 831.14 <sup>bc</sup>	1146.31 ± 243.81 <sup>b</sup>
Total N-6 PUFA	4258.08 ± 1658.78 <sup>a</sup>	8413.34 ± 1460.41 <sup>b</sup>	2396.01 ± 1171.3 <sup>ac</sup>	4004.21 ± 1779.7 <sup>ac</sup>	1977.05 ± 487.13 <sup>c</sup>
Total N-3 PUFA	559.43 ± 208.44 <sup>a</sup>	1174.26 ± 209.68 <sup>b</sup>	334.56 ± 167.8 <sup>a</sup>	577.91 ± 257.37 <sup>a</sup>	316.64 ± 69.82 <sup>a</sup>
Total Fatty acids	9827.94 ± 3596.71 <sup>a</sup>	17282.42 ± 2860.06 <sup>b</sup>	6192.45 ± 1952.58 <sup>a</sup>	8822.50 ± 3738.13 <sup>a</sup>	5522.83 ± 1012.28 <sup>a</sup>

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix D.** Relative percentage of fatty acid composition of liver triacylglycerols of hormonally manipulated female rats \*

Fatty acid	Liver triacylglycerols				
	% total fatty acids				
	Sham	OVX	OVX+E	OVX+P	OVX+EP
C 10:0	0.45 ± 0.41 <sup>a</sup>	0.27 ± 0.09 <sup>a</sup>	0.96 ± 0.67 <sup>ab</sup>	0.55 ± 0.32 <sup>ab</sup>	1.2 ± 0.42 <sup>b</sup>
C 12:0	1.32 ± 0.37 <sup>ab</sup>	0.72 ± 0.18 <sup>a</sup>	2.36 ± 1.03 <sup>bc</sup>	1.54 ± 0.57 <sup>ab</sup>	2.9 ± 0.79 <sup>c</sup>
C 14:0	0.69 ± 0.19 <sup>ab</sup>	0.52 ± 0.04 <sup>a</sup>	0.91 ± 0.24 <sup>b</sup>	0.74 ± 0.21 <sup>ab</sup>	0.98 ± 0.27 <sup>b</sup>
C 15:0	0.29 ± 0.05 <sup>ab</sup>	0.24 ± 0.04 <sup>a</sup>	0.39 ± 0.10 <sup>ab</sup>	0.31 ± 0.08 <sup>ab</sup>	0.44 ± 0.20 <sup>b</sup>
C 16:0	19.86 ± 1.56 <sup>a</sup>	18.54 ± 0.64 <sup>a</sup>	20.2 ± 1.71 <sup>ab</sup>	20.19 ± 1.25 <sup>ab</sup>	22.21 ± 1.53 <sup>b</sup>
C 18:0	4.53 ± 0.64 <sup>a</sup>	3.39 ± 0.42 <sup>a</sup>	6.94 ± 1.86 <sup>b</sup>	4.57 ± 1.06 <sup>a</sup>	7.55 ± 1.08 <sup>b</sup>
C 20:0	0.07 ± 0.03 <sup>a</sup>	0.06 ± 0.01 <sup>a</sup>	0.18 ± 0.06 <sup>b</sup>	0.07 ± 0.02 <sup>a</sup>	0.18 ± 0.07 <sup>b</sup>
C 21:0	0.03 ± 0.06	0.01 ± 0.00	0.06 ± 0.07	0.04 ± 0.05	0.05 ± 0.05
C 22:0	0.05 ± 0.04 <sup>ab</sup>	0.02 ± 0.02 <sup>a</sup>	0.11 ± 0.06 <sup>c</sup>	0.05 ± 0.04 <sup>ab</sup>	0.09 ± 0.03 <sup>bc</sup>
C 23:0	0.50 ± 0.12	0.68 ± 0.07	0.69 ± 0.16	0.60 ± 0.15	0.68 ± 0.36
C 24:0	0.08 ± 0.08	0.03 ± 0.01	0.13 ± 0.15	0.03 ± 0.02	0.17 ± 0.09
Total SFA	28.29 ± 2.64 <sup>ab</sup>	24.77 ± 1.23 <sup>a</sup>	33.77 ± 5.52 <sup>bc</sup>	29.27 ± 3.33 <sup>ab</sup>	7.49 ± 3.11 <sup>c</sup>
C 14:1n-5	0.03 ± 0.02	0.02 ± 0.01	0.05 ± 0.05	0.06 ± 0.05	0.07 ± 0.06
C 16:1n-7	0.94 ± 0.22	1.0 ± 0.21	1.03 ± 0.15	1.0 ± 0.22	1.2 ± 0.31
C 18:1n-7	1.61 ± 0.17 <sup>a</sup>	1.59 ± 0.14 <sup>a</sup>	1.82 ± 0.22 <sup>ab</sup>	1.63 ± 0.07 <sup>ab</sup>	1.93 ± 0.24 <sup>b</sup>
C 18:1n-9	19.59 ± 5.22	15.70 ± 0.99	18.00 ± 5.96	15.06 ± 2.34	16.07 ± 2.52
C 20:1n-9	0.12 ± 0.02	0.12 ± 0.01	0.22 ± 0.13	0.15 ± 0.02	0.15 ± 0.07
C 22:1n-9	0.18 ± 0.07 <sup>a</sup>	0.12 ± 0.04 <sup>a</sup>	0.41 ± 0.18 <sup>bc</sup>	0.23 ± 0.06 <sup>ab</sup>	0.47 ± 0.18 <sup>c</sup>
C 24:1n-9	0.24 ± 0.44	0.01 ± 0.02	0.08 ± 0.09	0.08 ± 0.07	0.25 ± 0.27
Total MUFA	22.86 ± 4.63	18.73 ± 1.12	21.88 ± 5.84	18.91 ± 2.22	20.44 ± 2.17
Total N-6 PUFA	42.78 ± 2.79 <sup>a</sup>	47.44 ± 1.32 <sup>a</sup>	36.36 ± 5.69 <sup>b</sup>	45.02 ± 2.11 <sup>a</sup>	34.96 ± 2.68 <sup>b</sup>
Total N-3 PUFA	5.66 ± 0.08 <sup>ab</sup>	6.62 ± 0.32 <sup>a</sup>	5.11 ± 1.04 <sup>b</sup>	6.48 ± 0.47 <sup>a</sup>	5.64 ± 0.65 <sup>ab</sup>

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.



**Appendix E.** Relative percentage of liver phospholipids of hormonally manipulated female rats \*

Fatty acid	Liver phospholipids % total fatty acids				
	Sham	OVX	OVX+E	OVX+P	OVX+EP
C 10:0	0.24 ± 0.10	0.22 ± 0.05	0.21 ± 0.03	0.31 ± 0.10	0.21 ± 0.07
C 12:0	0.62 ± 0.12 <sup>a</sup>	0.67 ± 0.11 <sup>ab</sup>	0.57 ± 0.14 <sup>a</sup>	0.86 ± 0.18 <sup>b</sup>	0.61 ± 0.06 <sup>a</sup>
C 14:0	0.20 ± 0.04	0.23 ± 0.08	0.21 ± 0.05	0.24 ± 0.04	0.21 ± 0.07
C 15:0	0.12 ± 0.02 <sup>a</sup>	0.15 ± 0.03 <sup>ab</sup>	0.19 ± 0.05 <sup>b</sup>	0.14 ± 0.02 <sup>ab</sup>	0.14 ± 0.03 <sup>ab</sup>
C 16:0	12.41 ± 0.67 <sup>a</sup>	13.77 ± 0.20 <sup>b</sup>	15.88 ± 0.61 <sup>c</sup>	13.34 ± 0.44 <sup>ab</sup>	16.03 ± 0.95 <sup>c</sup>
C 18:0	30.29 ± 1.52 <sup>a</sup>	29.16 ± 0.0 <sup>ab</sup>	25.8 ± 1.22 <sup>c</sup>	29.37 ± 0.97 <sup>a</sup>	27.02 ± 1.61 <sup>bc</sup>
C 20:0	0.10 ± 0.02	0.13 ± 0.02	0.10 ± 0.01	0.11 ± 0.03	0.08 ± 0.02
C 21:0	0.21 ± 0.03	0.23 ± 0.03	0.21 ± 0.01	0.23 ± 0.04	0.19 ± 0.05
C 22:0	0.29 ± 0.04 <sup>a</sup>	0.31 ± 0.03 <sup>ab</sup>	0.40 ± 0.05 <sup>c</sup>	0.30 ± 0.02 <sup>ab</sup>	0.36 ± 0.04 <sup>bc</sup>
C 23:0	0.61 ± 0.07	0.59 ± 0.08	0.55 ± 0.06	0.58 ± 0.06	0.55 ± 0.08
C 24:0	45.58 ± 0.91 <sup>ab</sup>	45.83 ± 0.79 <sup>ab</sup>	44.51 ± 0.58 <sup>a</sup>	46.08 ± 0.98 <sup>b</sup>	45.84 ± 0.9 <sup>ab</sup>
Total SFA	0.03 ± 0.01 <sup>a</sup>	0.03 ± 0.01 <sup>a</sup>	0.06 ± 0.01 <sup>b</sup>	0.03 ± 0.22 <sup>a</sup>	0.03 ± 0.01 <sup>a</sup>
C 14:1n-5	0.05 ± 0.02	0.05 ± 0.01	0.04 ± 0.02	0.06 ± 0.01	0.04 ± 0.02
C 16:1n-7	0.20 ± 0.05 <sup>a</sup>	0.18 ± 0.05 <sup>a</sup>	0.28 ± 0.07 <sup>ab</sup>	0.21 ± 0.04 <sup>a</sup>	0.34 ± 0.09 <sup>b</sup>
C 18:1n-7	1.52 ± 0.12 <sup>a</sup>	1.50 ± 0.13 <sup>a</sup>	2.09 ± 0.27 <sup>b</sup>	1.59 ± 0.14 <sup>a</sup>	2.00 ± 0.33 <sup>b</sup>
C 18:1n-9	3.33 ± 1.42	3.36 ± 1.91	3.42 ± 0.61	3.34 ± 1.33	2.64 ± 0.37
C 20:1n-9	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.02	0.06 ± 0.01	0.07 ± 0.03
C 22:1n-9	0.08 ± 0.03	0.13 ± 0.05	0.1 ± 0.05	0.14 ± 0.03	0.1 ± 0.05
C 24:1n-9	0.2 ± 0.02	0.24 ± 0.04	0.22 ± 0.04	0.25 ± 0.09	0.23 ± 0.04
Total MUFA	5.53 ± 1.44	5.66 ± 1.88	6.39 ± 0.56	5.74 ± 1.43	5.53 ± 0.67
Total N-6 PUFA	37.15 ± 1.06 <sup>a</sup>	37.77 ± 1.22 <sup>a</sup>	34.92 ± 1.16 <sup>b</sup>	37.35 ± 0.9 <sup>a</sup>	34.24 ± 1.26 <sup>b</sup>
Total N-3 PUFA	9.58 ± 0.69 <sup>a</sup>	9.10 ± 0.70 <sup>a</sup>	12.57 ± 1.05 <sup>b</sup>	8.91 ± 0.52 <sup>a</sup>	13.01 ± 0.52 <sup>b</sup>
Total Fatty acids	0.24 ± 0.10	0.22 ± 0.05	0.21 ± 0.03	0.31 ± 0.10	0.21 ± 0.07

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix F. Fatty acid composition of plasma total lipid extract of hormonally manipulated female rats \***

Fatty acid	Plasma total lipid extract				
	<i>µg fatty acid/ml plasma</i>				
	Sham	OVX	OVX+E	OVX+P	OVX+EP
C 10:0	0.45 ± 0.25	0.40 ± 0.32	0.31 ± 0.20	0.30 ± 0.23	0.44 ± 0.30
C 12:0	4.13 ± 0.45	4.64 ± 0.48	4.72 ± 0.65	4.21 ± 0.58	4.56 ± 0.19
C 14:0	1.41 ± 0.21 <sup>a</sup>	1.71 ± 0.19 <sup>ab</sup>	2.10 ± 0.49 <sup>b</sup>	1.56 ± 0.25 <sup>a</sup>	1.79 ± 0.32 <sup>ab</sup>
C 15:0	97.63 ± 14.01 <sup>a</sup>	113.03 ± 9.32 <sup>ab</sup>	198.03 ± 48.11 <sup>c</sup>	110.62 ± 16.15 <sup>a</sup>	156.60 ± 25.38 <sup>bc</sup>
C 16:0	132.70 ± 23.14 <sup>a</sup>	131.73 ± 16.33 <sup>a</sup>	193.15 ± 38.83 <sup>b</sup>	132.87 ± 28.62 <sup>a</sup>	181.41 ± 34.91 <sup>ab</sup>
C 18:0	0.57 ± 0.09 <sup>a</sup>	0.75 ± 0.14 <sup>ab</sup>	0.88 ± 0.09 <sup>b</sup>	0.73 ± 0.13 <sup>ab</sup>	0.72 ± 0.20 <sup>ab</sup>
C 20:0	1.84 ± 0.48	1.99 ± 0.51	1.86 ± 0.46	1.96 ± 0.47	2.01 ± 0.33
C 21:0	1.80 ± 0.39	1.57 ± 0.43	1.98 ± 0.49	1.78 ± 0.52	1.86 ± 0.28
C 22:0	4.46 ± 1.07	4.05 ± 1.23	3.83 ± 0.90	4.72 ± 1.13	5.07 ± 1.45
C 23:0	246.01 ± 36.40 <sup>a</sup>	261.59 ± 25.56 <sup>a</sup>	407.11 ± 85.72 <sup>b</sup>	258.96 ± 44.8 <sup>a</sup>	355.21 ± 52.42 <sup>b</sup>
C 24:0	0.95 ± 0.32	1.01 ± 0.14	1.36 ± 0.57	0.97 ± 0.46	0.92 ± 0.22
Total SFA	3.20 ± 0.79 <sup>a</sup>	4.44 ± 0.93 <sup>ab</sup>	6.13 ± 1.96 <sup>b</sup>	3.64 ± 0.42 <sup>ab</sup>	5.87 ± 2.08 <sup>b</sup>
C 14:1n-5	8.04 ± 1.24 <sup>a</sup>	8.05 ± 1.06 <sup>a</sup>	21.28 ± 3.95 <sup>b</sup>	8.90 ± 1.74 <sup>a</sup>	15.87 ± 3.54 <sup>c</sup>
C 16:1n-7	31.44 ± 7.09 <sup>a</sup>	31.67 ± 4.33 <sup>a</sup>	56.38 ± 16.56 <sup>b</sup>	32.70 ± 8.65 <sup>a</sup>	39.62 ± 11.35 <sup>ab</sup>
C 18:1n-7	1.40 ± 0.21 <sup>a</sup>	1.17 ± 0.22 <sup>a</sup>	2.98 ± 0.88 <sup>b</sup>	1.55 ± 0.25 <sup>ac</sup>	2.31 ± 0.62 <sup>bc</sup>
C 18:1n-9	4.15 ± 4.32	6.57 ± 2.89	5.95 ± 3.14	5.64 ± 2.84	3.46 ± 3.18
C 20:1n-9	3.08 ± 0.82	2.89 ± 0.65	2.66 ± 0.87	2.79 ± 0.84	3.63 ± 1.18
C 22:1n-9	52.27 ± 10.18 <sup>a</sup>	55.81 ± 6.39 <sup>a</sup>	96.73 ± 25.00 <sup>b</sup>	56.19 ± 10.48 <sup>a</sup>	71.68 ± 14.91 <sup>ab</sup>
C 24:1n-9	380.42 ± 65.46 <sup>a</sup>	391.08 ± 53.84 <sup>a</sup>	595.65 ± 112.61 <sup>b</sup>	416.90 ± 74.47 <sup>a</sup>	505.10 ± 95.95 <sup>ab</sup>
Total MUFA	37.12 ± 6.01 <sup>a</sup>	34.66 ± 6.13 <sup>a</sup>	85.61 ± 21.85 <sup>b</sup>	36.00 ± 7.87 <sup>a</sup>	77.24 ± 15.35 <sup>b</sup>
Total N-6 PUFA	715.83 ± 114.79 <sup>a</sup>	743.13 ± 81.53 <sup>ab</sup>	1185.10 ± 239.41 <sup>c</sup>	768.05 ± 136.35 <sup>ab</sup>	1009.23 ± 170.41 <sup>bc</sup>
Total N-3 PUFA	0.45 ± 0.25	0.40 ± 0.32	0.31 ± 0.20	0.30 ± 0.23	0.44 ± 0.30
Total Fatty acids	4.13 ± 0.45	4.64 ± 0.48	4.72 ± 0.65	4.21 ± 0.58	4.56 ± 0.19

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix G.** Relative percentage of plasma total lipid extract of hormonally manipulated female rats \*

Fatty acid	Plasma total lipid extract				
	% total fatty acids				
	Sham	OVX	OVX+E	OVX+P	OVX+EP
C 10:0	0.06 ± 0.03	0.06 ± 0.05	0.03 ± 0.02	0.04 ± 0.04	0.04 ± 0.03
C 12:0	0.58 ± 0.08 <sup>ab</sup>	0.63 ± 0.09 <sup>b</sup>	0.41 ± 0.11 <sup>a</sup>	0.57 ± 0.17 <sup>ab</sup>	0.46 ± 0.09 <sup>ab</sup>
C 14:0	0.20 ± 0.02 <sup>a</sup>	0.23 ± 0.04 <sup>ab</sup>	0.18 ± 0.01 <sup>b</sup>	0.21 ± 0.03 <sup>a</sup>	0.18 ± 0.02 <sup>ab</sup>
C 15:0	13.66 ± 0.73 <sup>a</sup>	15.25 ± 1.27 <sup>ab</sup>	16.59 ± 1.09 <sup>b</sup>	14.48 ± 1.06 <sup>a</sup>	15.56 ± 1.3 <sup>ab</sup>
C 16:0	18.47 ± 1.07	17.70 ± 1.28	16.30 ± 0.82	17.19 ± 1.13	17.98 ± 1.87
C 18:0	0.08 ± 0.01 <sup>ab</sup>	0.10 ± 0.02 <sup>b</sup>	0.08 ± 0.01 <sup>ab</sup>	0.10 ± 0.01 <sup>ab</sup>	0.07 ± 0.02 <sup>a</sup>
C 20:0	0.26 ± 0.05 <sup>a</sup>	0.27 ± 0.06 <sup>a</sup>	0.16 ± 0.01 <sup>b</sup>	0.26 ± 0.04 <sup>a</sup>	0.20 ± 0.02 <sup>ab</sup>
C 21:0	0.26 ± 0.06 <sup>a</sup>	0.21 ± 0.05 <sup>ab</sup>	0.17 ± 0.03 <sup>b</sup>	0.23 ± 0.03 <sup>ab</sup>	0.19 ± 0.04 <sup>ab</sup>
C 22:0	0.63 ± 0.14 <sup>a</sup>	0.54 ± 0.13 <sup>a</sup>	0.32 ± 0.04 <sup>b</sup>	0.61 ± 0.08 <sup>a</sup>	0.50 ± 0.13 <sup>ab</sup>
C 23:0	34.34 ± 0.72	35.21 ± 2.19	34.25 ± 0.34	33.71 ± 0.7	35.27 ± 1.21
C 24:0	0.14 ± 0.05	0.14 ± 0.03	0.12 ± 0.06	0.13 ± 0.08	0.10 ± 0.04
Total SFA	0.48 ± 0.06	0.60 ± 0.15	0.51 ± 0.06	0.48 ± 0.04	0.57 ± 0.16
C 14:1n-5	1.21 ± 0.18 <sup>a</sup>	1.09 ± 0.16 <sup>a</sup>	1.80 ± 0.12 <sup>b</sup>	1.16 ± 0.05 <sup>a</sup>	1.57 ± 0.25 <sup>b</sup>
C 16:1n-7	4.70 ± 0.64	4.26 ± 0.49	4.73 ± 0.84	4.20 ± 0.45	3.91 ± 0.8
C 18:1n-7	0.21 ± 0.03 <sup>ab</sup>	0.16 ± 0.03 <sup>a</sup>	0.25 ± 0.05 <sup>b</sup>	0.20 ± 0.04 <sup>ab</sup>	0.23 ± 0.04 <sup>b</sup>
C 18:1n-9	0.62 ± 0.63	0.88 ± 0.40	0.49 ± 0.22	0.77 ± 0.41	0.38 ± 0.4
C 20:1n-9	0.47 ± 0.15 <sup>a</sup>	0.39 ± 0.09 <sup>a</sup>	0.23 ± 0.06 <sup>b</sup>	0.37 ± 0.09 <sup>ab</sup>	0.36 ± 0.13 <sup>ab</sup>
C 22:1n-9	7.83 ± 0.90	7.52 ± 0.78	8.12 ± 1.07	7.31 ± 0.48	7.13 ± 1.06
C 24:1n-9	52.93 ± 0.82 <sup>a</sup>	52.42 ± 2.25 <sup>ab</sup>	50.32 ± 1.21 <sup>bc</sup>	54.21 ± 0.4a	49.83 ± 1.64 <sup>c</sup>
Total MUFA	5.18 ± 0.26	4.64 ± 0.40	7.16 ± 0.48	4.66 ± 0.27	7.62 ± 0.57
Total N-6 PUFA	0.06 ± 0.03	0.06 ± 0.05	0.03 ± 0.02	0.04 ± 0.04	0.04 ± 0.03
Total N-3 PUFA	0.58 ± 0.08 <sup>ab</sup>	0.63 ± 0.09 <sup>b</sup>	0.41 ± 0.11 <sup>a</sup>	0.57 ± 0.17 <sup>ab</sup>	0.46 ± 0.09 <sup>ab</sup>
Total Fatty acids	0.20 ± 0.02 <sup>a</sup>	0.23 ± 0.04 <sup>ab</sup>	0.18 ± 0.01 <sup>b</sup>	0.21 ± 0.03 <sup>a</sup>	0.18 ± 0.02 <sup>ab</sup>

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix H.** Relative percentage of plasma phospholipids of hormonally manipulated female rats \*

Fatty acid	Plasma phospholipids % total fatty acids				
	Sham	OVX	OVX+E	OVX+P	OVX+EP
C 10:0	0.07 ± 0.04	0.08 ± 0.07	0.07 ± 0.04	0.11 ± 0.05	0.07 ± 0.04
C 12:0	0.95 ± 1.04	1.53 ± 0.84	1.17 ± 0.59	1.35 ± 0.75	1.13 ± 0.53
C 14:0	1.01 ± 0.25	1.07 ± 0.24	0.86 ± 0.23	1.13 ± 0.22	0.95 ± 0.19
C 15:0	0.22 ± 0.03	0.31 ± 0.07	0.27 ± 0.05	0.30 ± 0.06	0.25 ± 0.06
C 16:0	13.00 ± 0.92 <sup>a</sup>	15.09 ± 1.29 <sup>ab</sup>	16.05 ± 1.4 <sup>b</sup>	13.97 ± 0.79 <sup>ab</sup>	15.59 ± 1.34 <sup>b</sup>
C 18:0	24.26 ± 2.21	22.58 ± 1.74	21.22 ± 0.76	22.09 ± 1.88	23.33 ± 1.91
C 20:0	0.23 ± 0.04	0.29 ± 0.08	0.20 ± 0.06	0.24 ± 0.07	0.22 ± 0.07
C 21:0	0.06 ± 0.07	0.04 ± 0.02	0.02 ± 0.02	0.05 ± 0.02	0.03 ± 0.03
C 22:0	0.34 ± 0.10 <sup>ab</sup>	0.37 ± 0.07 <sup>ab</sup>	0.22 ± 0.04 <sup>b</sup>	0.38 ± 0.1 <sup>ab</sup>	0.30 ± 0.03 <sup>ab</sup>
C 23:0	0.18 ± 0.06 <sup>ab</sup>	0.13 ± 0.02 <sup>ab</sup>	0.20 ± 0.05 <sup>ab</sup>	0.18 ± 0.07 <sup>ab</sup>	0.23 ± 0.04 <sup>b</sup>
C 24:0	0.69 ± 0.05 <sup>ab</sup>	0.65 ± 0.14 <sup>ab</sup>	0.44 ± 0.1 <sup>a</sup>	0.74 ± 0.21 <sup>b</sup>	0.59 ± 0.14 <sup>ab</sup>
Total SFA	40.84 ± 2.90	42.14 ± 2.91	40.75 ± 1.43	40.54 ± 2.44	42.68 ± 1.78
C 14:1n-5	0.06 ± 0.04	0.05 ± 0.03	0.05 ± 0.02	0.06 ± 0.04	0.03 ± 0.01
C 16:1n-7	0.74 ± 0.31	0.73 ± 0.27	0.67 ± 0.20	0.76 ± 0.33	0.65 ± 0.2
C 18:1n-7	1.71 ± 0.24 <sup>a</sup>	1.69 ± 0.24 <sup>a</sup>	2.19 ± 0.28 <sup>b</sup>	1.83 ± 0.12 <sup>ab</sup>	2.07 ± 0.31 <sup>ab</sup>
C 18:1n-9	24.12 ± 4.58	23.83 ± 6.55	19.98 ± 3.98	24.89 ± 5.42	18.58 ± 4.95
C 20:1n-9	0.26 ± 0.03 <sup>ab</sup>	0.29 ± 0.05 <sup>a</sup>	0.22 ± 0.03 <sup>b</sup>	0.25 ± 0.03 <sup>ab</sup>	0.22 ± 0.05 <sup>b</sup>
C 22:1n-9	0.70 ± 0.45	0.58 ± 0.20	0.50 ± 0.10	0.63 ± 0.11	0.40 ± 0.11
C 24:1n-9	0.63 ± 0.12	0.47 ± 0.22	0.37 ± 0.14	0.59 ± 0.35	0.45 ± 0.12
Total MUFA	28.45 ± 4.83	27.82 ± 6.49	24.11 ± 4.22	29.28 ± 5.05	22.54 ± 4.87
Total N-6 PUFA	24.09 ± 2.56	23.62 ± 3.7	27.55 ± 2.47	24.66 ± 2.22	27.01 ± 2.73
Total N-3 PUFA	3.70 ± 0.37 <sup>ab</sup>	3.61 ± 0.56 <sup>a</sup>	5.80 ± 0.66 <sup>b</sup>	3.61 ± 0.70 <sup>ab</sup>	5.93 ± 0.78 <sup>ab</sup>
Total Fatty acids	0.07 ± 0.04	0.08 ± 0.07	0.07 ± 0.04	0.11 ± 0.05	0.07 ± 0.04

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix I.** Fatty acid composition of plasma triacylglycerols of hormonally manipulated female rats \*

Fatty acid	Plasma triacylglycerols <i>µg fatty acid/ml plasma</i>				
	Sham	OVX	OVX+E	OVX+P	OVX+EP
C 10:0	0.64 ± 0.37	0.58 ± 0.30	0.78 ± 0.27	0.68 ± 0.44	0.32 ± 0.11
C 12:0	6.61 ± 3.22	6.54 ± 3.71	7.85 ± 1.70	8.77 ± 4.47	3.23 ± 0.67
C 14:0	4.88 ± 2.49	4.85 ± 2.38	3.97 ± 0.35	6.42 ± 2.04	2.90 ± 0.69
C 15:0	26.87 ± 12.92	25.32 ± 6.35	25.87 ± 6.59	27.55 ± 5.36	21.39 ± 3.81
C 16:0	29.89 ± 18.82	28.89 ± 8.96	24.25 ± 5.89	29.74 ± 7.69	20.87 ± 5.41
C 18:0	0.74 ± 0.37	0.79 ± 0.31	0.6 ± 0.13	0.82 ± 0.13	0.39 ± 0.11
C 20:0	0.19 ± 0.06	0.25 ± 0.14	0.24 ± 0.08	0.28 ± 0.07	0.22 ± 0.06
C 21:0	0.32 ± 0.20	0.35 ± 0.16	0.25 ± 0.09	0.35 ± 0.24	0.18 ± 0.05
C 22:0	0.49 ± 0.25	0.68 ± 0.44	0.43 ± 0.22	0.60 ± 0.11	0.25 ± 0.11
C 23:0	71.62 ± 38.88	69.22 ± 19.19	65.13 ± 12.90	75.98 ± 7.97	50.70 ± 9.72
C 24:0	0.55 ± 0.16	0.49 ± 0.21	0.45 ± 0.15	0.46 ± 0.06	0.33 ± 0.09
Total SFA	1.18 ± 0.61	1.07 ± 0.60	0.80 ± 0.43	0.59 ± 0.27	0.71 ± 0.20
C 14:1n-5	1.06 ± 0.44	0.94 ± 0.29	1.18 ± 0.49	0.95 ± 0.01	0.91 ± 0.20
C 16:1n-7	2.90 ± 1.06	2.68 ± 0.80	2.99 ± 0.80	3.24 ± 0.64	2.44 ± 0.16
C 18:1n-7	102.33 ± 39.11	95.76 ± 63.58	81.8 ± 23.41	142.84 ± 72.56	58.99 ± 18.62
C 18:1n-9	0.99 ± 0.36	0.88 ± 0.34	0.86 ± 0.12	1.08 ± 0.20	0.56 ± 0.11
C 20:1n-9	2.05 ± 0.93	2.13 ± 1.07	2.14 ± 0.64	2.03 ± 0.88	0.82 ± 0.27
C 22:1n-9	0.45 ± 0.26	0.37 ± 0.10	0.23 ± 0.06	0.42 ± 0.26	0.34 ± 0.17
C 24:1n-9	111.38 ± 42.37	104.24 ± 64.85	90.45 ± 23.27	151.6 ± 72.55	65.10 ± 19.12
Total MUFA	16.48 ± 6.02	16.40 ± 9.13	23.28 ± 12.72	19.88 ± 10.2	15.15 ± 7.81
Total N-6 PUFA	1.92 ± 0.79	2.16 ± 0.75	3.35 ± 2.76	1.82 ± 0.02	1.98 ± 0.96
Total N-3 PUFA	201.4 ± 83.65	192.02 ± 81.73	182.21 ± 37.44	249.28 ± 85.19	132.92 ± 30.49
Total Fatty acids	0.64 ± 0.37	0.58 ± 0.30	0.78 ± 0.27	0.68 ± 0.44	0.32 ± 0.11

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix J. Fatty acid composition of plasma non-esterified fatty acids of hormonally manipulated female rats \***

<b>Plasma non-esterified fatty acids</b>					
<i>µg fatty acid/ml plasma</i>					
<b>Fatty Acid</b>	<b>Sham</b>	<b>OVX</b>	<b>OVX + E</b>	<b>OVX + P</b>	<b>OVX + EP</b>
C 10:0	0.40 ± 0.21	0.58 ± 0.34	0.50 ± 0.31	0.43 ± 0.06	0.67 ± 0.28
C 12:0	3.57 ± 0.45	4.65 ± 0.93	4.65 ± 0.89	3.73 ± 0.74	4.41 ± 1.05
C 14:0	1.67 ± 0.54	3.34 ± 1.43	2.97 ± 0.56	1.81 ± 0.47	3.16 ± 1.37
C 16:0	13.00 ± 1.7	18.21 ± 3.79	15.16 ± 2.42	12.97 ± 3.74	17.86 ± 3.59
C 18:0	11.92 ± 2.09	15.76 ± 2.5	13.00 ± 2.41	12.44 ± 4.24	14.97 ± 1.71
C 20:0	0.40 ± 0.05	0.49 ± 0.10	0.47 ± 0.03	0.40 ± 0.11	0.44 ± 0.08
C 22:0	0.19 ± 0.01	0.20 ± 0.07	0.19 ± 0.02	0.14 ± 0.07	0.16 ± 0.04
C 24:0	0.32 ± 0.05	0.47 ± 0.17	0.48 ± 0.12	0.24 ± 0.08	0.35 ± 0.13
Total SFA	31.89 ± 4.37	44.35 ± 8.04	38.04 ± 4.35	32.67 ± 9.05	42.59 ± 6.90
C 12:1n-3	0.18 ± 0.04	0.32 ± 0.17	0.26 ± 0.12	0.18 ± 0.04	0.32 ± 0.20
C 14:1n-5	0.34 ± 0.28	0.30 ± 0.21	0.39 ± 0.30	0.15 ± 0.10	0.27 ± 0.20
C 16:1n-7	0.83 ± 0.28	3.44 ± 2.55	2.21 ± 1.90	0.90 ± 0.14	3.52 ± 2.49
C 18:1n-7	1.29 ± 0.26	3.73 ± 2.81	3.00 ± 1.90	1.30 ± 0.44	4.10 ± 2.77
C 18:1n-9	32.32 ± 11.11	66.84 ± 42.22	69.58 ± 12.59	39.67 ± 13.04	70.10 ± 32.86
C 20:1n-9	0.26 ± 0.12	0.64 ± 0.46	0.59 ± 0.21	0.33 ± 0.10	0.72 ± 0.42
C 22:1n-9	0.59 ± 0.34	0.63 ± 0.30	0.61 ± 0.26	0.95 ± 0.28	1.3 ± 0.46
C 24:1n-9	0.12 ± 0.07	0.23 ± 0.09	0.17 ± 0.07	0.23 ± 0.06	0.22 ± 0.10
Total MUFA	35.94 ± 12.39	76.13 ± 48.65	76.77 ± 16.09	43.71 ± 14.1	80.54 ± 39.36
Total N-6 PUFA	11.64 ± 1.80	14.11 ± 2.83	10.34 ± 1.23	10.46 ± 3.21	12.91 ± 2.58
Total N-3 PUFA	1.65 ± 0.20	2.46 ± 1.28	2.03 ± 0.85	1.12 ± 0.35	1.66 ± 0.62
Total fatty acids	81.12 ± 16.40	137.06 ± 58.63	127.18 ± 17.47	87.97 ± 25.33	137.7 ± 47.47

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix K.** Fatty acid composition of plasma cholesteryl esters of hormonally manipulated female rats \*

Fatty acid	Plasma cholesteryl esters <i>μg fatty acid/ml plasma</i>				
	Sham	OVX	OVX + E	OVX + P	OVX + EP
C 10:0	0.55 ± 0.21 <sup>a</sup>	0.32 ± 0.19 <sup>a</sup>	0.54 ± 0.32 <sup>a</sup>	1.24 ± 0.37 <sup>b</sup>	0.74 ± 0.24 <sup>b</sup>
C 12:0	5.68 ± 3.15 <sup>a</sup>	2.55 ± 1.87 <sup>a</sup>	5.19 ± 2.82 <sup>a</sup>	11.32 ± 3.28 <sup>b</sup>	5.99 ± 1.23 <sup>a</sup>
C 14:0	2.34 ± 0.97 <sup>a</sup>	2.76 ± 1.41 <sup>a</sup>	2.61 ± 0.88 <sup>a</sup>	5.91 ± 2.03 <sup>b</sup>	3.58 ± 1.82 <sup>ab</sup>
C 16:0	12.55 ± 2.68 <sup>a</sup>	16.93 ± 5.6 <sup>a</sup>	16.25 ± 3.96 <sup>a</sup>	28.14 ± 6.57 <sup>b</sup>	17.03 ± 5.86 <sup>a</sup>
C 18:0	10.39 ± 3.64 <sup>a</sup>	13.90 ± 6.08 <sup>ab</sup>	12.45 ± 3.36 <sup>a</sup>	25.02 ± 8.39 <sup>b</sup>	11.99 ± 6.38 <sup>a</sup>
C 20:0	0.37 ± 0.02 <sup>a</sup>	0.52 ± 0.26 <sup>ab</sup>	0.42 ± 0.11 <sup>ab</sup>	0.85 ± 0.27 <sup>b</sup>	0.44 ± 0.23 <sup>ab</sup>
C 22:0	0.20 ± 0.11	0.19 ± 0.11	0.16 ± 0.05	0.33 ± 0.08	0.23 ± 0.09
C 24:0	0.28 ± 0.14	0.02 ± 0.14	0.27 ± 0.09	0.42 ± 0.21	0.39 ± 0.08
Total SFA	33.06 ± 10.96 <sup>a</sup>	38.17 ± 14.35 <sup>a</sup>	38.93 ± 10.98 <sup>a</sup>	74.59 ± 19.16 <sup>b</sup>	41.21 ± 15.62 <sup>a</sup>
C 12:1n-3	0.30 ± 0.12 <sup>a</sup>	0.31 ± 0.07 <sup>a</sup>	0.37 ± 0.25 <sup>ab</sup>	0.69 ± 0.28 <sup>b</sup>	0.37 ± 0.16 <sup>ab</sup>
C 14:1n-5	0.39 ± 0.20	0.38 ± 0.19	0.48 ± 0.42	1.16 ± 1.02	0.46 ± 0.56
C 16:1n-7	1.27 ± 0.45	1.84 ± 0.70	1.79 ± 0.58	3.01 ± 1.37	2.63 ± 1.08
C 18:1n-7	1.59 ± 0.51	2.57 ± 1.27	2.06 ± 0.58	3.6 ± 1.41	2.68 ± 1.3
C 18:1n-9	31.85 ± 15.03	64.4 ± 34.87	49.87 ± 24.63	86.36 ± 41.08	72.33 ± 50.11
C 20:1n-9	0.24 ± 0.17	0.58 ± 0.37	0.44 ± 0.18	0.79 ± 0.27	0.49 ± 0.37
C 22:1n-9	1.26 ± 1.58	0.81 ± 0.51	1.49 ± 2.14	3.44 ± 2.31	1.00 ± 0.56
C 24:1n-9	0.15 ± 0.03 <sup>a</sup>	0.19 ± 0.11 <sup>a</sup>	0.28 ± 0.26 <sup>ab</sup>	0.63 ± 0.27 <sup>b</sup>	0.35 ± 0.22 <sup>ab</sup>
Total MUFA	37.05 ± 16.57	71.08 ± 37.62	56.77 ± 24.06	99.68 ± 43.44	80.31 ± 53.28
Total N-6 PUFA	49.56 ± 3.54 <sup>a</sup>	61.32 ± 6.93 <sup>ab</sup>	69.65 ± 11.11 <sup>ab</sup>	64.28 ± 12.69 <sup>ab</sup>	75.2 ± 15.76 <sup>b</sup>
Total N-3 PUFA	2.47 ± 0.25	3.69 ± 1.85	4.12 ± 0.56	4.45 ± 1.13	4.94 ± 1.3
Total fatty acids	122.14 ± 25.07 <sup>a</sup>	174.26 ± 59.04 <sup>ab</sup>	169.47 ± 41.56 <sup>ab</sup>	242.99 ± 50.09 <sup>b</sup>	201.65 ± 67.32 <sup>ab</sup>

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.

**Appendix L. Fatty acid composition of the total lipid extract of livers of of hormonally manipulated female rats \***

<b>Adipose Total Lipid Extract</b>					
<i>mg fatty acid/g adipose tissue</i>					
<b>Fatty acid</b>	<b>Sham</b>	<b>OVX</b>	<b>OVX+E</b>	<b>OVX+P</b>	<b>OVX+EP</b>
C 10:0	0.049 ± 0.025	0.066 ± 0.028	0.059 ± 0.042	0.062 ± 0.025	0.049 ± 0.031
C 12:0	1.62 ± 0.40	1.50 ± 0.36	1.49 ± 0.33	1.52 ± 0.34	1.60 ± 0.55
C 14:0	5.25 ± 1.82	5.55 ± 1.67	5.11 ± 1.11	5.80 ± 1.65	4.81 ± 2.13
C 15:0	109.57 ± 34.23	109.61 ± 30.93	105.22 ± 24.04	117.16 ± 26.98	99.46 ± 42.71
C 16:0	24.27 ± 6.72	23.07 ± 4.43	24.27 ± 6.11	23.09 ± 3.73	21.58 ± 7.52
C 18:0	0.59 ± 0.19	0.42 ± 0.09	0.60 ± 0.14	0.45 ± 0.08	0.53 ± 0.17
C 20:0	0.19 ± 0.08	0.11 ± 0.03	0.18 ± 0.04	0.13 ± 0.03	0.18 ± 0.04
C 21:0	1.74 ± 0.38	1.37 ± 0.34	1.96 ± 0.65	1.49 ± 0.15	1.79 ± 0.49
C 22:0	0.26 ± 0.07 <sup>a</sup>	0.15 ± 0.06 <sup>c</sup>	0.24 ± 0.05 <sup>ab</sup>	0.16 ± 0.03 <sup>bc</sup>	0.21 ± 0.04 <sup>abc</sup>
C 23:0	144.1 ± 43.6	142.4 ± 36.2	139.6 ± 32.0	150.3 ± 32.5	130.6 ± 53.4
C 24:0	0.27 ± 0.13	0.39 ± 0.23	0.25 ± 0.06	0.31 ± 0.13	0.32 ± 0.16
Total SFA	13.35 ± 5.27	16.34 ± 6.65	13.10 ± 2.08	16.83 ± 6.84	14.31 ± 7.58
C 14:1n-5	13.19 ± 3.64	10.41 ± 1.89	13.33 ± 2.86	12.45 ± 2.40	12.78 ± 5.46
C 16:1n-7	137.09 ± 41.632	125.73 ± 33.82	133.59 ± 36.42	135.36 ± 26.19	115.90 ± 56.73
C 18:1n-7	1.31 ± 0.39	0.98 ± 0.26	1.34 ± 0.41	1.12 ± 0.18	1.16 ± 0.45
C 18:1n-9	0.17 ± 0.04	0.19 ± 0.05	0.19 ± 0.04	0.17 ± 0.05	0.17 ± 0.08
C 20:1n-9	0.075 ± 0.038	0.042 ± 0.027	0.073 ± 0.022	0.082 ± 0.024	0.059 ± 0.037
C 22:1n-9	165.5 ± 50.8	154.1 ± 41.9	161.9 ± 41.1	166.3 ± 34.9	143.0 ± 65.2
C 24:1n-9	219.6 ± 66.0	199.0 ± 51.3	211.0 ± 63.2	222.6 ± 31.1	174.6 ± 76.7
Total MUFA	16.7 ± 4.6	16.9 ± 4.5	16.9 ± 4.6	19.4 ± 3.2	15.2 ± 6.7
Total N-6 PUFA	545.9 ± 164.2	512.4 ± 130.6	529.4 ± 139.4	558.6 ± 100.0	464.5 ± 198.5
Total N-3 PUFA	0.049 ± 0.025	0.066 ± 0.028	0.059 ± 0.042	0.062 ± 0.025	0.049 ± 0.031
Total Fatty acids	1.62 ± 0.40	1.50 ± 0.36	1.49 ± 0.33	1.52 ± 0.34	1.60 ± 0.55

\* Values are mean ± SD, n = 5-7 per group. Values with different superscripts for a specific hormone are significantly different by Tukey's post hoc test (p<0.05) following significant F-value by one-way ANOVA. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, Sham: sham-operated, OVX: ovariectomized, + E: with 17β-estradiol, + P: with progesterone, + EP: with 17β-estradiol plus progesterone.



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