Characterization of the effects of sex, pregnancy, and 17β-estradiol on docosahexaenoic 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

Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid (n-3 PUFA) required for fetal neurodevelopment. Increased DHA levels are associated with 17β-estradiol levels, as DHA is higher in women relative to men and in pregnant relative to non-pregnant women, suggesting a maternal adaptation to supply DHA to the fetus. DHA can be synthesized in the body from shorter n-3 PUFA through sequential elongation-desaturation, with Δ6-desaturase being the rate-limiting enzyme. The goal of the present thesis was to characterize the mechanism underlying higher DHA in situations of altered 17β-estradiol status by examining the expression of DHA synthesis enzymes in rodent models. Fatty acid composition of several lipid classes was measured by gas chromatography and enzyme expression was measured by RT-qPCR and immunoblotting. Hepatic Δ6-desaturase and phospholipid DHA was higher in female relative to male, and in pregnant relative to non-pregnant rats. Similarly, 17β-estradiol supplementation of ovariectomized rats resulted in increased hepatic Δ6-desaturase expression and DHA content, while ovariectomy itself had no effects on DHA levels despite controlling for hyperphagia. Mice deficient in the DNA binding activity of estrogen receptor α (ERα) had no differences in hepatic Δ6-desaturase or DHA levels. These results suggest that 17β-estradiol mediates the higher DHA levels in females and during pregnancy through increasing hepatic Δ6-desaturase expression, although the effects of removing 17β-estradiol signalling through ovariectomy or ERα disruption are less clear. This work helps to explain findings of altered DHA status in response to changes in 17β-estradiol concentrations, possibly resulting in more appropriately tailored dietary DHA recommendations. Also, increased understanding of the regulation of DHA synthesis may improve DHA yields in agri/aquaculture and enable increased content of DHA in the food supply.

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LIST OF ABBREVIATIONS

ALA	α-linolenic acid, 18:3n-3
ANOVA	Analysis of variance
CPT1	Carnitine palmitoyl transferase 1
DHA	Docosahexaenoic acid, 22:6n-3
n-3DPA	n-3 Docosapentaenoic acid, 22:5n-3
n-6 DPA	n-6 Docosapentaenoic acid, 22:5n-6
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
ERα	Estrogen receptor α
ERαKO	Estrogen receptor α knockout
GPER	G-protein coupled estrogen receptor
HUFA	Highly unsaturated fatty acids
LA	Linoleic acid, 18:2n-6
MUFA	Monounsaturated fatty acid
OVX	Ovariectomized rat
OVX+AL	Ovariectomized rat fed *ad libitum*
OVX+E	Ovariectomized rat supplemented with 17β-estradiol
OVX+P	Ovariectomized rat supplemented with progesterone
OVX+PE	Ovariectomized rat supplemented with 17β-estradiol and progesterone
OVX+PF	Ovariectomized rat pair-fed relative to sham-operated rat
PC	Phosphatidylcholine
PDHK	Pyruvate dehydrogenase kinase
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI3K	Phosphoinositide-3 kinase

PL	Phospholipid
PPARα	Peroxisome proliferator activated receptor α
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RER	Respiratory exchange ratio
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SCAP	Sterol response element binding protein cleavage activating protein
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acid
SHAM	Sham-operated rat
SREBP1c	Sterol response element binding protein
TAG	triacylglycerol

CHAPTER 1

GENERAL INTRODUCTION

Intake and blood levels of the omega-3 highly unsaturated fatty acid (n-3 HUFA, ≥ 20 carbons, ≥ 3 double bonds) docosahexaenoic acid (DHA, 22:6n-3) are associated with health benefits to the cardiovascular and neurological systems [reviewed in (Harris et al. 2009)]. Several observational studies have shown that women of reproductive age have higher blood levels of DHA as compared with men, despite no differences in n-3 intake (Bakewell et al. 2006; Crowe et al. 2008; Geppert et al. 2010; Marangoni et al. 2007; Metherel et al. 2009; Sfar et al. 2010). These differences in DHA levels are likely clinically relevant due to the sensitive dose-response effect of n-3 HUFA on sudden cardiac death prevention (Harris et al. 2008; Harris et al. 2009; Mozaffarian et al. 2006), which may contribute to the lower cardiac mortality in reproductive age women as compared to men (Bui et al. 2011). Cardiovascular health is also associated with small differences in blood n-3 HUFA concentration resulting from genetic variation in HUFA synthesis (Li et al. 2013), indicating that small differences in n-3 HUFA synthesis impact health. The importance of DHA supply for fetal neurodevelopment is demonstrated by delayed visual development in pre-term infants not supplemented with DHA (Carlson et al. 1996b). Maternal DHA levels increase significantly during pregnancy (Stark et al. 2005a; Stewart et al. 2007) as an adaptation to provide the fetus with DHA. As DHA intakes during pregnancy are low (Denomme et al. 2005) and tend not to change from non-pregnant intakes (Stark et al. 2005a), the increased maternal blood DHA levels during pregnancy are likely the result of a metabolic adaptation.

The cause of higher DHA in women relative to men and in pregnant relative to non-pregnant women may be 17β-estradiol, which is much higher in women (Eldrup et al. 1987) and

during pregnancy (O'Leary et al. 1991). 17β-estradiol also appears to mediate the higher DHA levels observed in post-menopausal women receiving hormone replacement therapy (Giltay et al. 2004a), in male-to-female transsexuals receiving 17β-estradiol treatment (Giltay et al. 2004b), and women taking oral contraceptives (Giltay et al. 2004b; Magnusardottir et al. 2009). However, the mechanism relating female sex, pregnancy, and 17β-estradiol to higher DHA levels is not known.

DHA can either be obtained from the diet or produced from the essential fatty acid α-linolenic acid (ALA, 18:3n-3) (Voss et al. 1991), with estimates of rates of conversion of ALA to DHA in humans ranging from 0% to 4% [reviewed in (Burdge et al. 2005)]. Women produce a greater proportion of DHA from ALA compared with men (Burdge et al. 2002a; Burdge et al. 2002b) and ALA intake is negatively associated with cardiovascular disease in women (Albert et al. 2005) but not in men (Mozaffarian et al. 2005), suggesting an effect of the sex difference in DHA production on health. This sex difference in DHA biosynthesis occurs only during low n-3 HUFA intakes (Pawlosky et al. 2003a; Pawlosky et al. 2003b), which are typical of North Americans (Ervin et al. 2004). Sex differences in the enzymes that produce DHA from ALA may mediate the increased biosynthesis of DHA observed in females, as well as the higher DHA levels in pregnancy and in response to estrogen.

Studies investigating sex differences in expression of these enzymes have yielded mixed results, with some studies demonstrating increased hepatic expression in females relative to males (Burdge et al. 2008; Extier et al. 2010), while others show no effect (Childs et al. 2010). A small number of studies investigating the effect of pregnancy (Childs et al. 2012) and hormone manipulation (Alessandri et al. 2011) on the expression of these enzymes have been published but report only mRNA expression data of a limited number of enzymes. However, ovariectomy

results in hyperphagia (Eckel 2011) and increased hepatic lipogenesis, which affects DHA metabolism independently of ovarian hormonal status and should be examined. In addition, while estrogen receptor α (ERα) is highly expressed in the liver (Pelletier 2000) and is involved in hepatic lipid metabolism (Matic et al. 2013), the effects of specific estrogen receptors on DHA metabolism are not known.

Accordingly, the role of sex and pregnancy on DHA biosynthesis was assessed by measuring the expression of DHA biosynthesis enzymes and DHA content in male compared with female rats, and in pregnant compared with non-pregnant rats. To explore the mechanism relating 17β-estradiol to DHA biosynthesis, DHA biosynthetic enzyme expression and DHA content was measured in ovariectomized rats supplemented with 17β-estradiol with/without progesterone. To investigate the role of ovariectomy-induced hyperphagia on DHA metabolism, ovariectomized rats were either pair-fed to eugonadal rats or fed *ad-libitum*. To determine the role of estrogen receptor α in DHA metabolism, DHA biosynthetic enzyme expression and DHA levels were assessed in estrogen receptor α-knockout mice compared with wild-type controls.

The results of this research will expand knowledge of the role of sex, pregnancy, and 17β-estradiol on fatty acid metabolism. Changes in DHA levels resulting from altered DHA biosynthesis may have effects on disease risk, indicating the importance of characterizing factors affecting DHA biosynthesis such as sex and 17β-estradiol status. Also, with the exception of pregnancy, current dietary recommendations for DHA do not take into account changes in DHA levels associated with altered DHA biosynthesis. This research may help to inform future DHA intake recommendations tailored to specific populations with altered DHA biosynthetic capacities, such as pre- vs. post-menopausal women (Tworek et al. 2000). An improved understanding of factors regulating DHA synthesis may also help to improve DHA yields in

agriculture or aquaculture, as there is some concern that fish stocks are not sufficient to meet current recommendations for DHA intakes at a population level (Jenkins et al. 2009).

CHAPTER 2

BIOCHEMICAL FOUNDATIONS

Fatty Acids and Lipids

Fatty acids have polar carboxylic acid groups with hydrophobic hydrocarbon chains. Fatty acids are classified based on the number of double bonds in the acyl chain: saturated fatty acids (SFA) have no double bonds, monounsaturated fatty acids (MUFA) have a single double bond, and polyunsaturated fatty acids (PUFA) have greater than or equal to 2 carbon-carbon double bonds. HUFA have greater than or equal to 20 carbons and greater than or equal to 3 carbon-carbon double bonds. Mammals are capable of producing SFAs and MUFAs *de novo* from acetyl-CoA. However, the n-3 and n-6 essential fatty acids ALA and linoleic acid (LA, 18:2n-6) must be obtained from the diet (Widmer and Holman, 1950), as mammals do not possess the enzymology to synthesize these *de novo* or to interconvert between n-3 and n-6 PUFA. From these two 18-carbon PUFA, mammals can synthesize n-6 and n-3 HUFA.

Within cells, fatty acids are typically esterified to either triacylglycerols (TAG) or phospholipids (PL), which are the main energy-storing and structural lipids, respectively, and are found in varying levels in different tissues (**Table 2.1**). The *de novo* synthesis pathway is shared by both TAG and PL, and involves the addition of fatty acids to the sn-1 followed by the sn-2 position of glycerol-3-phosphate. The resulting phosphatidic acid is a substrate for the production of two PL: phosphatidyl inositol (PI) which is involved in cellular signaling, and phosphatidyl glycerol which is the precursor for cardiolipin, a major PL species in mitochondria (Osman et al. 2011). The removal of the sn-3 phosphate from phosphatidic acid results in diacylglycerol, which can be esterified to a third fatty acid to form TAG, or can be esterified to phosphoethanolamine or phosphocholine to form phosphatidyl ethanolamine (PE), or phosphatidyl choline (PC), respectively (Gibellini et al. 2010). A base-exchange reaction forms

phosphatidyl serine (PS) from either PE or PC (Kuge et al. 1997). The relative proportions of each PL species differ by tissue type (**Table 2.2**), however in most tissues PE and PC represent approximately 60-80% of all PL, while in plasma PC represents approximately 85%, with almost no PE. The fatty acid composition of PL is dependent on both the *de novo* incorporation as well as the remodeling process, called the Land's cycle, involving the sequential release of fatty acids from the sn-2 position by phospholipase A_2, followed by re-esterification by a number of different lysophospholipid acyltransferases helps to determine the fatty acid composition of sn-2 fatty acids. HUFA tend to be preferentially esterified in the sn-2 position of PL, with EPA and arachidonic acid (AA, 20:4n-6) enriched in PC, and DHA enriched in PE (Kim et al. 1997). On the other hand, TAG HUFA content is very low (Christie 2003; Wood et al. 1969).

Table 2.1: Lipid class composition of heart, liver, erythrocytes and heart*

	Heart	**Liver**	**Erythrocytes**	**Plasma**
Lipid Class	*Weight % of total lipids*			
Cholesteryl Esters	trace	2	-	16
Triacylglycerols	4	7	-	49
Cholesterol	4	5	30	6
Unesterified Fatty Acids	-	trace	-	2
Phospholipids**	90	86	68	27

*adapted from (Christie 1985), **includes PC, PE, PS, PI, diphosphatidylglycerol, sphingomyelin, and lysophosphatidylcholine

Fatty acids are transported through the bloodstream in lipoproteins, consisting of a PL/cholesterol/apolipoprotein exterior and TAG/cholesteryl-ester interior. Fats derived from digestion are transported in chylomicrons, and lipoprotein lipase on the endothelium hydrolyzes the TAG and the fatty acids are taken up into cells. The TAG-depleted chylomicron remnants are taken up by the liver through receptor-mediated endocytosis. In the fasted state the liver

secretes very low-density lipoproteins, which are converted by lipoprotein lipase to TAG-depleted and cholesterol-rich low-density lipoproteins that are taken up by cells in tissues through receptor-mediated endocytosis.

Non-esterified fatty acids enter cells either through passive diffusion or transporters such as FABPpm or FAT/CD36, or as components of lipoproteins following endocytosis (Chabowski et al. 2007). Retention of fatty acids inside cells is accomplished by fatty acid transport proteins and acyl-CoA synthetases, which provide hydrophobic binding pockets and esterify fatty acids to CoA, respectively (Black et al. 2003; Coe et al. 1999; Watkins et al. 2007). Intracellular fatty acids and fatty acyl-CoA are oxidized for energy and carbon recycling, incorporated into cellular lipids such as TAGs or PLs, or converted into other fatty acids or eicosanoids in the case of HUFA.

Table 2.2: Phospholipid composition of various tissues and blood fractions in the rat

Phospholipid Class	**Brain**	**Heart**	**Liver**	**Erythrocyte**	**Plasma**
	% of total phospholipids				
Phosphatidylethanolamine*	29.5	37	23.5	23	<0.5
Phosphatidylcholine*	40	41	61.5	49	86
Phosphatidylserine	10	1.5	1.7	9.5	0
Phosphatidylinositol	1.5	2	5	4.5	3.4

*includes ether-linked phospholipids and lysophospholipids. Adapted from (Diagne et al. 1984)

Fatty acid β-oxidation occurs in the mitochondria or peroxisomes, with mitochondria being active towards short-, medium-, and long-chain fatty acids, while the peroxisome shows activity towards very long-chain fatty acids. Mitochondrial fatty acid oxidation involves the translocation of acyl-CoA past both mitochondrial membranes via the action of carnitine-palmitoyl transferase (CPT) I and II, with CPTI being the rate-limiting step of mitochondrial fatty aid oxidation (Gropper et al. 2005). Uptake of fatty acids by the peroxisome involves the

ATP-binding cassette transporters (ABC) D1 and ABCD2 (Morita et al. 2012) and peroxisomal β-oxidation involves the activity of both acyl-CoA oxidase (AOX) and multifunctional protein-2 (MFP-2) (Keller et al. 1993).

17β-estradiol

The term "estrogen" generally refers to three steroid hormones, namely estriol, estrone, and 17β-estradiol, with 17β-estradiol being by far the most bioactive, eliciting 10-fold higher potency as compared with estriol, and 100-fold higher potency as compared with estrone in breast cancer cell proliferation and receptor binding assays (Gutendorf et al. 2001). 17β-estradiol is produced by aromatization of testosterone by the enzyme aromatase in the endoplasmic reticulum, primarily in the thecal and granulosa cells of the ovary in the mid-follicular phase in humans and the diestrous phase in rats (Sanders et al. 1997). Aromatase activity is also present in extra-ovarian tissues such as skeletal muscle (Matsumine et al. 1986), adipose tissue (Miller 1991), and Leydig cells of the testes (Brodie et al. 1993), explaining the production of estrogen in males and post-menopausal females.

Once secreted, the actions of 17β-estradiol are mediated by three receptors: estrogen receptor (ER) α, ERβ, or the G-protein coupled estrogen receptor (GPER) (Langer et al. 2010) and are mediated through either genomic or non-genomic actions (**Figure 2.1**). In genomic estrogen signaling, the binding of estrogen to estrogen receptors results in dimerization with another estrogen receptor (ERα or ERβ), followed by binding to an estrogen response element in the promoter of a target gene, which causes altered transcription of that gene. In "non-genomic" estrogen signaling, estrogen binds to GPER or estrogen receptors anchored to the plasma membrane (Acconcia et al. 2004). Dimerization of ERα or ERβ to another estrogen receptor is another non-genomic mechanism (Razandi et al. 2004), and the signal transduction that results

involves a number of second-messenger protein kinases and calcium signaling mechanisms, such as mitogen-activated protein kinases (Migliaccio et al. 1996; Ronda et al. 2007), AMP-activated protein kinase (Rogers et al. 2009), and phosphoinositide-3 kinase – Akt (Marino et al. 2003), rather than direct genomic interaction [reviewed in (Bjornstrom et al. 2005)].

In the liver, ERα, rather than ERβ, appears to mediate the effects of 17β-estradiol on lipid metabolism. In the human and rat liver, ERα is much more highly expressed than ERβ, and expression occurs primarily in the nuclear region (Pelletier 2000). In HepG2 cells, ERα is localized in the nuclei, while ERβ is localized in perinuclear mitochondria (Solakidi et al. 2007). The importance of ERα in hepatic metabolism is demonstrated by increased hepatic expression of genes involved in lipid biosynthesis in whole-body knockout of ERα (Bryzgalova et al. 2006; Heine et al. 2000) which is not seen in a whole-body knockout of ERβ (Ohlsson et al. 2000). Recently, a liver-specific ERα knockout mouse has been produced (Matic et al. 2013) that has similar body weight, insulin sensitivity, and hepatic transcript profiles compared to wild-type controls, suggesting that the effects of whole-body ERα knockout on hepatic lipid metabolism may be mediated by extra-hepatic processes.

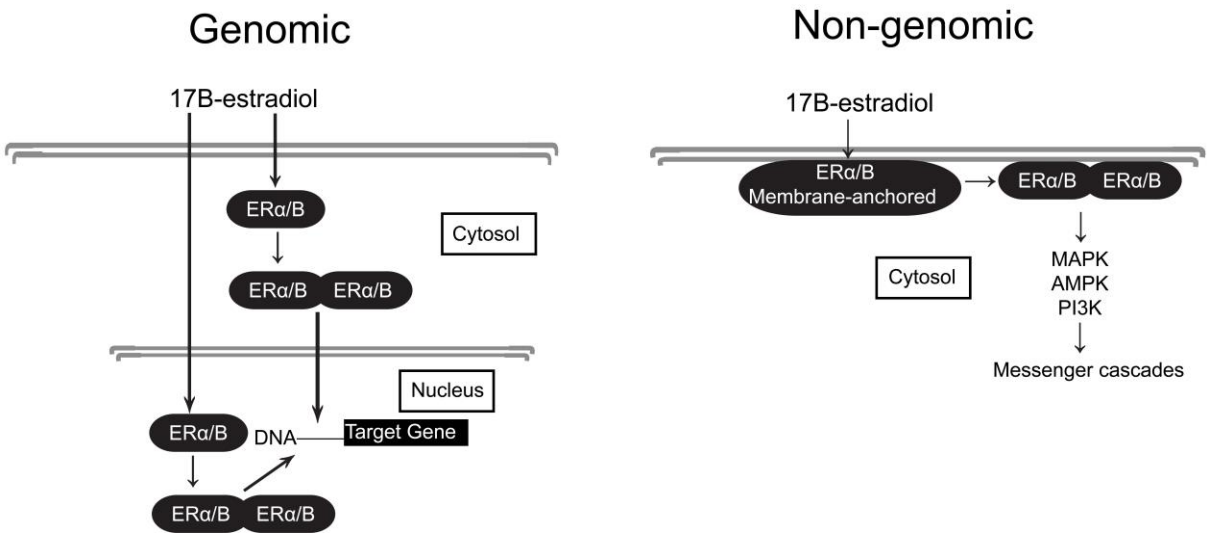

Figure 2.1: Genomic and non-genomic estrogen signalling mechanisms. Genomic mechanism shows both cytosolic and nuclear estrogen receptor activities.

Docosahexaenoic acid (DHA)

DHA is found primarily in the sn-2 position of phospholipids and makes up varying proportions of total fatty acids in the different phospholipid classes, with the order being PE > PS > PC > PI in rodent liver (Holub et al. 2011). However, because of the much higher abundance of PE and PC relative to PS and PI, the majority of DHA is found in PE and PC (Holub et al. 2011; Kim et al. 1997). Once incorporated into phospholipids, DHA assumes a variety of functions including increased membrane fluidity (Yang et al. 2011), second messenger signalling (Jump et al. 2008), and regulation of gene expression (Jump et al. 2008).

DHA is highly concentrated in the retina and brain, particularly in the synaptic regions of neurons (Svennerholm 1968). Supply of DHA to the fetus during pregnancy is essential for neurodevelopment, especially during the third trimester (Kuipers et al. 2012). Dietary DHA deficiency during pregnancy and lactation results in decreased DHA concentrations in neonatal brain and significant deficits in visual and cognitive function in rats and rhesus monkeys [reviewed in (Brenna 2011)]. Similarly, significantly pre-term human infants supplemented with

DHA-rich formulas have transiently improved visual acuity compared to ALA-supplemented groups (Carlson et al. 1996a; Carlson et al. 1996b). In contrast, the evidence for benefits on cognition or for full-term infants is not as strong [reviewed in (Carlson 2009; Cheatham et al. 2006; Gibson et al. 1999)]. Some evidence also indicates that brain DHA is negatively associated with neurological disorders such as clinical depression (Martins 2009) and Alzheimer's disease (Barberger-Gateau et al. 2002; van Gelder et al. 2007).

The cardioprotective effects of dietary DHA, along with eicosapentaenoic acid (EPA, 20:5n-3), have been extensively reviewed (Harris et al. 2009; Kromhout 2012; Mozaffarian et al. 2006), with the most significant effect of EPA and DHA being sudden cardiac death prevention. Meta-analyses of randomized controlled trials and prospective cohort studies of the general population (Harris et al. 2009; Mozaffarian et al. 2006) indicate that the prevention of sudden cardiac death by EPA and DHA is curvilinear with a maximal effect occurring between 250-750 mg/day EPA+DHA intake, beyond which there is limited effect on sudden cardiac death.

Several factors influence DHA levels. The primary determinant of blood n-3 HUFA status is dietary n-3 HUFA intake (Harris et al. 2012). Blood DHA status tends to be positively associated with age (Dewailly et al. 2001; Harris et al. 2012; Ogura et al. 2010) and female sex (Lohner et al. 2013), and negatively associated with smoking (Block et al. 2008; Harris et al. 2012) and alcohol intake (Dewailly et al. 2001; Holub 2002). Some polymorphisms of the n-3 HUFA synthesis pathway have been shown to affect DHA in pregnant women, however the majority of these polymorphisms have been shown to affect levels of n-3 HUFA shorter than DHA [reviewed in (Glaser et al. 2011)]. Feeding of ALA tends to increase levels of EPA and n-3 docosapentaenoic acid (22:5n-3), but typically not DHA (Barcelo-Coblijn et al. 2008; Barcelo-Coblijn et al. 2009).

DHA biosynthesis

The synthesis of DHA from ALA involves sequential desaturation and elongation in the endiplasmic reticulum up to 24:6n-3, followed by perixisomal β-oxidation to DHA (**Figure 2.2**): (Sprecher 2000). N-6 HUFA are derived from this pathway as well via the reaction sequence 18:2n-6 → 18:3n-6 → 20:3n-6 → 20:4n-6 → 22:4n-6 → 24:4n-6 → 24:5n-6 → 22:5n-6. As all enzymes in the pathway are shared by n-3 and n-6 PUFA, substrate competition occurs. This competition is particularly relevant for Δ6-desaurase, which desaturates 18- and 24-carbon PUFA of both the n-3 and n-6 class (D'Andrea et al. 2002; Stroud et al. 2009), and is considered the rate-limiting enzyme in HUFA biosynthesis (Marcel et al. 1968) and limits the accumulation of DHA in a number of experimental models (Hassam et al. 1975; Portolesi et al. 2007). It has been shown that Δ6-desaturase is approximately 2-3 fold more active towards ALA than towards LA (Castuma et al. 1977; Rodriguez et al. 1998). However, because rat and human liver and adipose tissue contains 10-50 times more LA as compared with ALA (Garaulet et al. 2011; Lin et al. 2011; Martinez 1992; Petridou et al. 2005; Stark et al. 2007; Yee et al. 2012) the desaturation/elongation of n-6 PUFA tends to dominate. Δ6-desaturase mRNA is highest in liver (Stoffel et al. 2008), consistent with the highest rates of DHA biosynthesis in the liver relative to other organs (Rapoport et al. 2010).

Several factors have been identified that alter the expression or activity of Δ6-desaturase. One is dietary protein, and it has been shown that substituting carbohydrate for protein isocalorically increases the activity of Δ6-desaturase in rats (Peluffo et al. 1974). Also, ambient temperature is negatively associated with Δ6-desaturase activity (Peluffo et al. 1974). Insulin increases Δ6-desaturase mRNA in primary rat hepatocytes (Wang et al. 2006), and the activity of Δ6-desaturase is decreased with streptozotocin treatment and is restored by insulin treatment

(Eck et al. 1979; Shin et al. 1995). Surprisingly, induction of obesity and insulin resistance by high-fat diet feeding or leptin receptor knockout does not affect hepatic Δ6-desaturase mRNA (Wang et al. 2006). Several single nucleotide polymorphisms in the human Δ6-desaturase gene have been identified that affect blood AA and EPA concentrations, but not DHA concentrations in phospholipids of plasma (Bokor et al. 2010), serum (Schaeffer et al. 2006), and erythrocytes (Rzehak et al. 2009), although analysis of a particular haplotype (with 28 SNP) has shown increased levels of DHA and AA in plasma total lipids in the Northern Swedish Population Health Study (Ameur et al. 2012). Also, certain Δ6-desaturase SNP associated with increased Δ6-desaturase product:precursor ratios have been shown to increase DHA levels in maternal erythrocytes during pregnancy (Koletzko et al. 2011) and colostrum postpartum (Morales et al. 2011), and SNP with lower Δ6-desaturase activity are associated with lower levels of DHA in erythrocytes and breast milk (Xie et al. 2008).

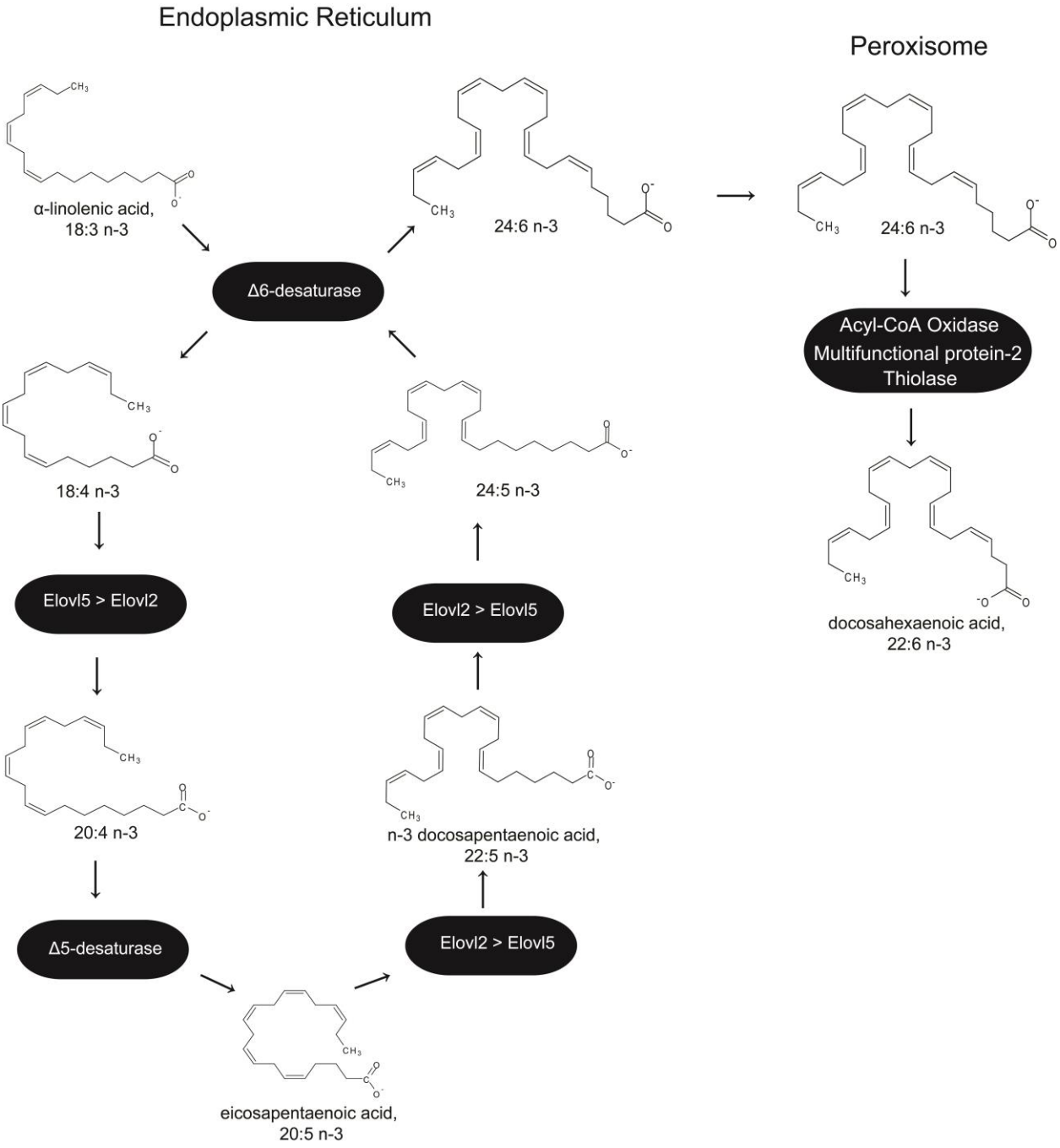

Figure 2.2: The pathway of docosahexaenoic acid biosynthesis from α-linolenic acid. Adapted from (Kitson et al. 2010).

Studies investigating the biosynthesis of n-3 HUFA from ALA in humans have either used stable isotope-labeled fatty acids or increased the dietary intake of ALA. ALA supplementation usually increases eicosapentaenoic acid (EPA, 20:5n-3) and DPAn-3; however no increases in DHA levels are seen [reviewed in (Burdge et al. 2005)]. Stable isotope studies investigating the metabolic fate of ALA suggest that the biosynthesis of n-3 HUFA from ALA is relatively inefficient, with estimates of fractional conversion of ALA to EPA ranging from 0.2% to 21%, and ALA to DHA from 0% to 4% in cohorts of men (Burdge et al. 2005). Also, findings of increased hepatic Δ6-desaturase expression and DHA synthesis secretion in rats on a n-3 PUFA deficient diet for 15 weeks (Igarashi et al. 2007a; Igarashi et al. 2007b), and similar levels of DHA in erythrocytes from vegetarian women compared with omnivores despite negligible DHA consumption in vegetarians suggests that DHA synthesis may be increased to maintain DHA homeostasis when ALA is available (Lakin et al. 1998). Also, evidence from radiotracer infusion studies in unanaesthetized rats shows that the liver synthesis-secretion rate of DHA is 24-times higher than the brain DHA consumption rate (Gao et al. 2009b), suggesting that hepatic DHA synthesis-secretion may be sufficient to meet basic brain DHA requirements.

Transcriptional control of DHA biosynthesis

The expression of Δ5- and Δ6-desaturase is under the control of the transcription factors sterol response element binding factor 1-c (SREBP1-c) (Cho et al. 1999; Nara et al. 2002) and peroxisome proliferator activated receptor α (PPARα) (Song et al. 2002; Tang et al. 2003).

PPARα is a ligand-induced transcription factor that belongs to the nuclear steroid receptor superfamily. Ligands for PPARα include PUFA, monounsaturated fatty acids (MUFA), and several eicosanoids including leukotriene B4 and hydroxyeicosatetraenoic acids (Krey et al. 1997). Ligand binding induces conformational changes that result in dissociation of corepressor

proteins and formation of a dimer with the retinoid-x receptor. The dimer binds to PPARα response elements in the promoter region of selected genes and modulates their transcription (Mandard et al. 2004). The activity of PPARα is also controlled by phosphorylation of serine residues, and several kinase cascades such as ERK-MAPK, p38 MAPK, protein kinase A, and protein kinase C [reviewed in (Burns et al. 2007)]. PPARα increases the expression of genes involved in mitochondrial and peroxisomal β-oxidation, as well as both Δ5- and Δ6-desaturase (Mandard et al. 2004; Tang et al. 2003; Wang et al. 2006). Liver PPARα expression is lower in females relative to males (Ciana et al. 2007; Extier et al. 2010), although estrogen administration increases the expression and activity of mitochondrial and peroxisomal oxidation enzymes [reviewed in (Kitson et al. 2010)], suggesting a complex regulation of PPARα activity by sex and sex hormones.

SREBP1-c is a transcription factor belonging to the basic helix-loop-helix zipper class of transcription factors, and first exists as an immature full-length peptide in the membrane of the endoplasmic reticulum and must be cleaved following transfer to the golgi by SREBP cleavage activating protein (SCAP). The mature form is then translocated to the nucleus by importin-β (Horton et al. 2002) where it interacts with sterol response elements characterized by E-box sequences in promoter regions and modulates gene expression (Amemiya-Kudo et al. 2002). SREBP1-c increases the expression of fatty acid synthesis genes such as fatty acid synthase and acetyl-CoA carboxylase (Amemiya-Kudo et al. 2002), and both Δ5- and Δ6-destaurases (Nara et al. 2002). The activity of SREBP1-c is modulated via transcription (controlled by liver X receptor), by the cleavage of immature SREBP1-c [reviewed in (Jeon et al. 2012)], and by regulation of its mRNA stability by PUFA, especially n-3 PUFA (Xu et al. 1999). This

represents a feedback mechanism whereby n-3 HUFA can regulate the expression of their synthesis enzymes (Jump et al. 2005).

Sex differences in DHA content and metabolism

Several studies have observed sex differences in blood and tissue DHA contents in reproductive age humans and animals [**Table 2.3,** reviewed in (Burdge et al. 2005; Childs et al. 2008; Decsi et al. 2011; Kitson et al. 2010; Lohner et al. 2013)]. Large cohort studies have found higher DHA in serum lipids of women in New Zealand (Crowe et al. 2008) and Tunisia (Sfar et al. 2010), and the Framingham Heart Study (Harris et al. 2012), while a higher DHA:DPAn-3 ratio was observed in whole blood of women in a study of Italians (Marangoni et al. 2007). While following their habitual diets, women were observed to have higher DHA in plasma lipids (Bakewell et al. 2006) and platelet PE and PC (Geppert et al. 2010), and women had higher DHA in erythrocytes and whole blood at baseline in a fish-oil supplementation study (Metherel et al. 2009). Similarly, female rats have higher DHA than males in plasma (Childs et al. 2008; Childs et al. 2010; Extier et al. 2010), liver (Alessandri et al. 2012; Burdge et al. 2008; Childs et al. 2010; Extier et al. 2010), heart (Slater-Jefferies et al. 2010), and erythrocytes (McNamara et al. 2009). Higher DHA in women compared with men appears only to occur in countries in which n-3 HUFA intakes are low, such as in North America (Lohner et al. 2013), suggesting a possible adaptation in women to increase DHA levels when intakes are low.

The increased blood DHA in women is likely mediated by increased synthesis from ALA. In men who had ingested a bolus of [U-^{13}C] ALA the net fractional conversion of ALA to EPA, DPAn-3, and DHA was 7.9%, 8.1%, and 0%, respectively (Burdge et al. 2002a), while women showed fractional conversion rates of 21%, 5.9%, and 9.2% (Burdge et al. 2002b). Another study utilizing stable-isotope ALA demonstrated that women had greater conversion of

DPAn-3 to DHA while consuming a beef-based diet low in n-3 HUFA (providing 59 mg/day EPA+DHA), but not while consuming a high n-3 HUFA fish-based diet (providing 560 mg/d EPA+DHA) (Pawlosky et al. 2003b). The higher DHA biosynthesis from ALA in females also may result from decreased oxidation of ALA (Burdge et al. 2002a; Burdge et al. 2002b; Burdge et al. 2003), suggesting partitioning of this substrate towards n-3 HUFA synthesis.

Increased DHA synthesis may be mediated by increased expression of DHA biosynthesis enzymes; however, studies investigating sex differences in the expression of these enzymes in rats have yielded mixed results. Extier et al. (2010) found increased hepatic mRNA content both of Δ5- and Δ6-desaturase, as well as increased protein content of Δ5-desaturase (Δ6-desaturase protein content was not measured) in female rats as compared with males at 8 weeks of age, but not at 3 weeks or 5 weeks. Burdge et al. (2008) also reported increased hepatic Δ5-desaturase mRNA (no difference in Δ6-desaturase) in livers of older female rats (15-weeks), but Childs et al. (2010) reported no sex differences in hepatic Δ5- or Δ6-desaturase mRNA while varying ALA and LA dietary intakes in rats aged 13 weeks.

Table 2.3: Summary of studies reporting sex differences in DHA content or biosynthesis in rats and humans [adapted from (Kitson et al. 2010)]

Study	Subjects (number)	Dietary fatty acid treatment	Results
Rat Studies			
Burdge et al. 2008	Wistar rats, male and female (n = 24 each)	Maternal = 5.9g ALA/kg diet Lactation/weaning = 0.7g ALA/kg diet	Increased DHA in plasma PC and liver PC and PE (% total fatty acids) in females Higher hepatic D5D expression in females
Extier et al. 2010	Wistar rats, male and female (n = 6 for each sex/time point)	Maternal = 0.05g ALA/kg diet Weanling = 0.2 g ALA/kg diet	Higher DHA in plasma PC and liver PC, PS, and PE in females Higher hepatic expression of D5D and D6D, and lower PPARα and FABP7
Childs et al. 2010	Wistar rats, male and female (n = 6 for each sex/diet group)	Low soybean = 1.6 g ALA/kg diet High fat soybean = 9.1 g ALA/kg diet High fat linseed = 50.2 g ALA/kg diet	Increased DHA in plasma PC, liver PC and PE in females in all diets. No differences in gene expression
Burdge et al. 2008	Wistar rats, male and female (n = 24 each)	Pregnancy = 5.9 g ALA/kg diet Lactation/weaning= 0.7 g ALA/kg diet	Higher DHA in PC and PE, not TAG, in female rats compared with males
Human Studies			
Burdge et al. 2002b	Women (n = 6)	Habitual; 700mg of [U-^{13}C]ALA administered	Fractional appearance of ^{13}C-labelled fatty acids in plasma was: ALA: 63.7%, EPA: 21.1%, DPAn-3: 5.9%, DHA: 9.2%
Burdge et al. 2002a	Men (n = 6)	Habitual; 700mg of [U-^{13}C]ALA administered	Fractional appearance of ^{13}C-labelled fatty acids in plasma was: ALA: 84%, EPA: 7.9%, DPAn-3: 8.1%, DHA: N.D.
Pawlosky et al. 2003a	Men (n = 6) Women (n = 6)	Ad libitum Fish-based Beef-based	Increased conversion of DPAn-3 to DHA in females within beef-based diet
Giltay et al. 2004	Men (n = 72) Women (n = 103)	Controlled diet, free from fish	Increased DHA in serum CE in females
Bakewell et al. 2006	Men (n = 13) Women (n = 23)	Habitual intakes	Increased DHA in plasma TAG, NEFA, PC, and total lipids in females
Crowe et al. 2008	Men (n = 1246) Women (n = 1547)	Habitual intakes	Increased DHA in serum PL and CE in females
Marangoni et al. 2007	Men (n = 47) Women (n = 61)	Habitual intakes	Increased DPAn-3 in whole blood of males
Metherel et al. 2009	Men (n = 10) Women (n = 10)	Baseline habitual diet 4-week supplementation of 4.8g/day EPA+DHA 8-week washout on habitual diet	Increased DHA and decreased DPAn-3 in while blood and erythrocytes of females at baseline Increased DHA:EPA ratio in women in various blood fractions disappeared with supplementation
Geppert et al. 2010	Men (n = 40) Women (n = 34)	Habitual intakes	Higher DHA in platelet PE and PC in females

EPA: eicosapentaenoic acid, DPAn-3: docosapentaenoic acid, DHA: docosahexaenoic acid, PE: phosphatidyl ethanolamine, LA: linoleic acid, ALA: α-linolenic acid, D5D: Δ5-desaturase, D6D: Δ6-desaturase, PPARα: peroxisome proliferator activated receptor α, FABP: fatty acid binding protein, PC: phosphatidyl choline, PS: phosphatidyl serine, PL: phospholipid, CE: cholesteryl esters

Influence of pregnancy on DHA levels and metabolism

The majority of fetal brain and retinal DHA accretion occurs during the third trimester of pregnancy with 2.27 mg DHA /d accreted in the whole body between gestational weeks 0-25, 16.25 mg DHA/d for weeks 25-35, and 41.65mg DHA/d for weeks 35-40 (Kuipers et al. 2012). DHA is provided by the mother through selective placental transfer, as DHA is transferred more efficiently than other fatty acids (Tobin et al. 2009). To meet the demands of the fetus, plasma phospholipid DHA concentration is increased as early as 6 weeks into pregnancy (Otto et al. 2001) and continues to increase up to approximately 30 weeks of pregnancy and then plateaus until birth (Al et al. 1995) with a rapid decrease (< 3 months) after birth (Al et al. 1995; Stark et al. 2005a). Dietary DHA intakes during pregnancy have been shown to be quite stable (Otto et al. 2001; Stark et al. 2005a), indicating a metabolic adaptation is occurring to increase plasma DHA concentration.

Increases in plasma lipids including TAG and PL occur throughout pregnancy (Desoye et al. 1987), suggesting that increased DHA may simply be an effect of increased general hepatic lipid synthesis-secretion. However, this is not the case, as the relative percentage increases in plasma DHA occur at the expense of the relative percentages of 20:4n-6 and EPA (Stark et al. 2005a), indicating a specific enrichment of DHA. Previous work has shown that the DHA content of hepatic *de novo* synthesized diacylglycerol is increased, which is the substrate for TAG, PC, and PE. PC *de novo* synthesis is also increased due to higher activity of cytidine-diphosphate: choline 1,2-diacylglycerol cholinephosphotransferase (Burdge et al. 1994). However, the source of increased DHA for these processes is not known, and may be increased *de novo* DHA synthesis.

Only one study has investigated the effect of pregnancy on hepatic Δ5- and Δ6-desaturase and elovl5 expression, finding increased mRNA of Δ6-desaturase, but not Δ5-desaturase or elovl5 (Childs et al. 2012). The increase in Δ6-desaturase was correlated with the increased concentrations of progesterone, but not estradiol or testosterone, as all three hormones are increased during pregnancy (O'Leary et al. 1991) (**Table 4**).

Table 2.4: Changes in sex hormone concentrations over the course of pregnancy (nmol/L)

	Progesterone	17β-estradiol	Testosterone
Non-pregnant Controls[a]	12-90	0.37-0.77	0.7-3.5
5-weeks pregnancy[b]	26-91	0.69-3.88	0.9-7.4
40-weeks pregnancy[b]	314-1087	22.53-127	2.2-10.8

[a]from the luteal phase of menstrual cycle; [b]ranges represent 95% confidence interval. Adapted from (O'Leary et al. 1991)

Influence of sex hormones on DHA content and metabolism

Various hormonal manipulations are associated with differences in DHA content and metabolism in humans (**Table 2.5**) as well as in cell culture and animal models (**Table 2.6**). In general, estradiol is associated with increased DHA in human, animal and cell culture models.

Human studies - Blood DHA levels are reduced in post- compared with pre-menopausal women (Tworek et al. 2000), corresponding to decreased circulating levels of 17β-estradiol (Ahn et al. 2011; Witt et al. 2010), and hormone replacement therapy either increases (Giltay et al. 2004a; Sumino et al. 2003) or decreases (Stark et al. 2003) circulating DHA. Similarly, women ≥ 60 years old have lower plasma DHA than women < 60 years old (Sfar et al. 2010), and no sex difference is observed in DHA content between older men and women [57-59 years old (Burdge et al. 2007)]. Sex hormones also appear to have effects on reproductive-aged individuals. Male-to-female transsexuals receiving oral ethinyl estradiol had increased cholesteryl ester DHA proportions, while female-to-male transsexuals receiving intramuscular testosterone acetate

injections had decreased cholesteryl ester DHA (Giltay et al. 2004b). Slight increases in DHA concentration have also been observed in women taking oral contraceptives relative to controls (Giltay et al. 2004b).

Animal studies – Administration of testosterone via injection to eugonadal male and female rats results in significantly decreased activity of Δ6-desaturase (Marra et al. 1989). Ovariectomy increased the hepatic mRNA content of Δ5- and Δ6-desaturase with no difference in liver PL DHA, while supplementation of ovariectomized rats with 17β-estradiol returned mRNA content to control values but increased the hepatic PC and PE DHA contents (Alessandri et al. 2011). Ovariectomy also decreases the DHA content of bone marrow (Poulsen et al. 2008a) and erythrocytes (Poulsen et al. 2008b), suggesting that the lower estradiol associated with ovariectomy reduces the synthesis and/or the accretion of DHA.

Cell culture studies –Two studies have utilized the neuroblastoma cell line SH-SY5Y and have found that 17β-estradiol supplementation increases the mRNA of Δ5-desaturase (Extier et al. 2009) and the PE content of EPA and DPAn-3 (Alessandri et al. 2008). Dihydrotestosterone decreased mRNA of Δ5-desaturase (Extier et al. 2009) and progesterone decreases the mRNA of Δ6-desaturase (Extier et al. 2009). In HTC hepatocytes or isolated hepatocytes from female rats, the supplementation of culture medium with 17β-estradiol and testosterone decreased the desaturation of 20:3n-6 to 20:4n-6, while progesterone had no effect (Marra et al. 1988).

Table 2.5: Summary of studies investigating effect of altered hormonal status on highly unsaturated fatty acid metabolism in humans

Study	Cells/Subjects	Treatment / Cohorts	Effects on n-3 HUFA levels
Tworek et al. 2000	Females	Premenopausal (n = 433) Postmenopausal (n = 433)	Higher DHA in erythrocytes in premenopausal women
Stark et al. 2003	Females (43-69 years)	Premenopausal (n = 19) Postmenopausal (n = 34) Postmenopausal receiving hormone therapy (n = 40)	Increased DHA and DPAn-3 in plasma PL in postmenopausal women not receiving hormone therapy
Sumino et al. 2003	Postmenopausal women (43-63 years)	Taking conjugated equine estrogen and medroxyprogesterone acetate (n = 59) Not taking hormone therapy (n = 45)	Increased plasma total lipid DHA and EPA in women taking hormones
Giltay et al. 2004a	Postmenopausal women (47-59 years) Males (60-70 years)	Females 60 mg raloxifene/day (n = 23) Females 150 mg raloxifene/day (n = 20) Females conjugated equine estrogen with medroxyprogesterone acetate (n = 17) Females placebo (n = 23) Males 120 mg raloxifene/day (n = 15) Males placebo (n = 15)	Increased DHA in plasma CE in post-menopausal women taking 150 mg/day raloxifene and in women taking conjugated equine estrogen with medroxyprogesterone acetate at 24 months compared to baseline
Giltay et al. 2004b	Male-to-female transsexuals (eugonadal)	Cyproterone acetate alone (n = 16) Cyproterone acetate with oral ethinyl estradiol (n = 15) Transdermal 17β-estradion	Increased DHA in serum CE following cyproterone acetate with oral ethinyl estradiol
Giltay et al. 2004b	Female-to-male transsexuals (ovariectomized)	Testosterone esters plus anastrozole (n = 16) Placebo (n = 14) Testosterone esters alone (n = 17)	Decreased DHA in serum CE with testosterone esters alone
Giltay et al. 2004b	Women	Using oral contraceptives (n = 32) No oral contraceptive use (n = 71)	Non-significant increase in DHA in serum CE with contraceptive use (p = 0.08)
Stark et al. 2004	Postmenopausal women (45-70 years)	Taking hormone replacement therapy (n = 18) Not taking hormone replacement therapy (n = 14) 2.8g algal DHA vs. placebo in crossover design	Increased estimates of retroconversion of DHA to EPA in plasma phospholipids of women not taking hormone therapy
Burdge et al. 2007	Men (50-65 years) Women (50-65 years)	Men (50-65 years, n = 10) Women (50-65 years, n = 10)	No sex difference in plasma total lipid DHA at this age
Sfar et al. 2010	Women (40-82 years)	Women < 60 years (n = 58) ,Women ≥ 60 years (n = 46)	Higher DHA in plasma total lipids in women < 60 years

EPA: eicosapentaenoic acid, DPAn-3: docosapentaenoic acid, DHA: docosahexaenoic acid, PC: phosphatidyl choline, CE: cholesteryl esters.

Table 2.6: Summary of cell culture and animal studies investigating the effects of hormonal manipulations on highly unsaturated fatty acid metabolism

Study	Cells/Subjects	Treatment / Cohorts	Effects on HUFA
Cell Culture Studies			
Marra et al. 1988	Isolated rat hepatocytes and HTC hepatocarcinoma cells	0.1, 1, 10mM 17β-estradiol 0.1, 1mM progesterone 0.1M testosterone 0.1M estriol	Lower Δ5-desaturation of 20:4n-6 from 20:3n-6 in response to all doses of 17β-estradiol, estriol, and testosterone, but not progesterone
Alessandri et al. 2008	SH-SY5Y neuroblastoma (n = 3 for each condition)	10nM 17β estradiol 30 μM ALA alone 10nM 17β-estradiol with 30 μM ALA	Increased EPA and DPAn-3 in PE with 17β-estradiol
Extier et al. 2009	SH-SY5Y neuroblastoma (n = 4 for each condition)	7μM ALA, LA, or ALA/LA, 10nM 17β-estradiol, dihydrotestosterone, progesterone, or control	Increased EPA, DPAn-3 content and decreased D5D mRNA with 17β-estradiol and ALA treatment Decreased EPA, DHA content and decreased PPARα and D5D expression with dihydrotestosterone and ALA Decreased D6D mRNA with progesterone treatment
Rat Studies			
Marra et al. 1989	Male and female wistar rats	Injection with 260 μg/kg testosterone or vehicle (n = 4 per group for each sex)	Testosterone decreased hepatic D5D and D6D activity
Poulsen et al. 2008a	Female Sprague-Dawley rats	Ovariectomized or sham-operated (n = 10 each)	Lower bone marrow DHA in ovariectomized rats
Poulsen et al. 2008b	Female Sprague-Dawley rats	Ovariectomized (n = 10), ovariectomized with 17β-estradiol n = 12), sham-operated (n = 10)	Lower erythrocyte DHA in ovariectomized group compared with sham-operated and ovariectomiuzed with 17β-estradiol supplementation
Alessandri et al. 2011	Female wistar rat (n = 8 per group)	Ovariectomy Ovariectomy with 8μg/day 17β-estradiol Ovariectomy with 16 μg/day 17β-estradiol Sham operated, no 17β-estradiol	Ovariectomy increased liver D5D and D6D mRNA and decreases cortex PE DHA 17β-estradiol decreased hepatic D5D and D6D and increased liver PE, PC, and PS DHA

EPA: eicosapentaenoic acid, DPAn-3: docosapentaenoic acid, DHA: docosahexaenoic acid, PE: phosphatidyl ethanolamine, LA: linoleic acid, ALA: α-linolenic acid, D5D: Δ5-desaturase, D6D: Δ6-desaturase, PC: phosphatidyl choline, PS: phosphatidyl serine

CHAPTER 3

RATIONALE AND OBJECTIVES

Rationale

The associations between blood levels of DHA and cardiovascular health and neurological development illustrate the importance of understanding factors that regulate DHA levels. Previous work indicates that female sex (Lohner et al. 2013), pregnancy (Stark et al. 2005a), and estrogen (Giltay et al. 2004b) all increase DHA levels; however the underlying mechanism is not characterized.

The higher DHA in women may contribute to increased cardioprotection relative to men (Zheng et al. 2001), as even small changes in DHA status can potentially have significant effects on sudden cardiac death prevention due to the sensitive dose-response of this effect (Mozaffarian et al. 2006). The mechanism underlying higher DHA in women is not known, but may involve increased expression of DHA synthesizing enzymes. Therefore, sex differences in the expression of DHA-synthesizing enzyme and DHA concentrations in liver and plasma should be investigated. In addition, sex differences in organs in which DHA is known to have a protective role, such as in heart or brain, should also be investigated. Measuring sex differences in the expression of transcription factors involved in the regulation of DHA synthesis enzymes will provide insight into differences in the regulation of these enzymes between males and females.

Increased DHA biosynthetic enzyme expression may also underlie the increase in blood DHA in pregnant relative to non-pregnant women, and may reflect an evolutionary adaptation to provide sufficient DHA to a fetus for neurodevelopment. The majority of fetal DHA accretion occurs in the third trimester of pregnancy, and maternal DHA increases steadily until delivery (Stewart et al. 2007), suggesting a time-specific increase in DHA levels and DHA biosynthetic

enzyme expression in pregnancy. Therefore, an analysis of changes in the expression of hepatic DHA concentration, DHA biosynthesis enzymes and related transcription factors over the course of pregnancy is warranted.

Higher levels of circulating 17β-estradiol and higher DHA levels are present in females relative to males and in pregnant relative to non-pregnant females, suggesting that 17β-estradiol may be a causative mechanism. The effects of 17β-estradiol removal or supplementation on expression of DHA biosynthesis enzymes has not been directly investigated, and ovariectomization and hormone supplementation studies can provide insight. However, decreases in 17β-estradiol are associated with hyperphagia (Blaustein et al. 1976; Varma et al. 1999) that can stimulate lipogenesis, making it necessary to control for food intake to estimate the direct effect of 17β-estradiol on DHA metabolism. In addition, although ERα is highly expressed in rat liver tissue (Pelletier 2000), the specific role of ERα in mediating the effect of 17β-estradiol on DHA metabolism is unknown.

Sex differences and the effect of pregnancy on expression of DHA biosynthetic enzymes and DHA levels in rats were examined. To further characterize these effects, the effects of 17β-estradiol supplementation, ovariectomy, and disruption of the ERα gene on DHA biosynthesis were examined. A greater understanding of the effects of these factors on DHA status and metabolism may provide insight of DHA requirements that could enable specific dietary DHA recommendations for men and women, and for women during pregnancy and menopause. These findings will expand our understanding of the role of sex, pregnancy, and ovarian hormones in fatty acid metabolism in general, but also the regulation of DHA synthesis that could be used in efforts to synthesize DHA to increase the content of DHA in the food supply.

Objectives

The primary objective of the current study is to characterize the effects of sex, pregnancy, ovarian hormones and ERα on DHA content and biosynthetic enzyme expression, particularly the rate-limiting enzyme Δ6-desaturase. To investigate the role of sex, the hepatic expression of DHA biosynthesis enzymes and blood and tissue DHA content will be compared between male and female rats. The effect of pregnancy will be investigated by examining virgin, day-15 and -20 pregnant, and day-7 post-partum rats to measure changes in hepatic expression of DHA biosynthesis enzymes and hepatic DHA content. The effects of ovarian hormones will be assessed by comparing the expression of DHA biosynthetic enzymes and hepatic and blood DHA concentrations in ovariectomized rats with/without supplementation with 17β-estradiol and/or progesterone to that of sham-operated rats. To examine the potentially confounding effect of ovariectomy-induced hyperphagia on DHA biosynthesis, a group of ovariectomized rats will be pair-fed relative to sham-operated controls and hepatic and plasma DHA levels and hepatic DHA biosynthetic enzyme expression determined. Finally, ERα-knockout mice and wild-type controls will be used to examine the role of ERα in tissue and blood DHA content and hepatic DHA biosynthetic enzyme expression.

Hypotheses

1. Liver, plasma, heart, brain and erythrocytes of female rats will have higher concentrations of DHA as compared with the same tissues of male rats.
2. Expression of enzymes involved in DHA biosynthesis will be higher in the liver, heart, and brain of female as compared with male rats.
3. Hepatic expression of Δ6-desaturase will be increased at day 15 and 20 of pregnancy compared to virgin and post-partum rats, which will correspond with increases in hepatic DHA concentration.
4. Hepatic expression of Δ6-desaturase and hepatic and plasma DHA content will be lower in ovariectomized rats, and supplementation with estradiol will restore Δ6-desaturase expression and DHA levels to that of sham-operated rats.
5. Controlling for hyperphagia in ovariectomized rats will further reduce hepatic Δ6-desaturase and DHA levels as compared with ovariectomized rats fed *ad libitum*
6. ERα-knockout mice will have lower expression of Δ6-desaturase and lower tissue and blood DHA levels as compared with wild-type mice

CHAPTER 4

MATERIALS AND COMMON METHODS

Animals

All animal procedures were approved by the University of Waterloo Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care. Animals were housed with a temperature of 21 ± 1 °C and a 12:12-h light-dark cycle. Rats were fed a standard laboratory chow diet with fatty acid composition determined by triplicate analysis and reported in **Table 4.1**. Details on animal treatments and characteristics are described in individual studies.

Expression Analysis

Protein determination by western blot **-** Tissues or cells were homogenized by a polytron homogenizer in a buffer containing 0.25mol/L sucrose, 0.01mol/L tris-HCl, 0.01mol/L $MgCl_2$, 2.5mmol/L DTT, and complete protease inhibitor tablets (Roche Applied Science, Laval, QC, Canada). Homogenate protein was measured using the bicinchoninic acid procedure, and 10-20µg of protein were resolved on either a 7.5 or 12.5% polyacrylamide gel and subsequently transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). Membranes were blocked for 1 hour or overnight at 4°C with either 5% skim milk or 5% bovine serum albumin (BSA) in tris-buffered saline with 0.5% (v/v) tween (TBS-T). Membranes were then incubated with primary antibodies for proteins of interest suspended in either BSA or milk for 1 hour at room temperature or overnight at 4°C (specific antibodies used and concentrations are detailed in individual studies). Unbound antibody was then washed off membranes with TBS-T and horseradish peroxidase-conjugated secondary antibody specific to the primary antibody was then incubated for 1 hour at room temperature (Santa Cruz Biotechnology, 1:8000 dilution).

Following TBS-T wash, Enhanced Chemiluminescence Western Blotting Detection Reagents (GE Healthcare, QC, Canada) were added and detection of luminescence was performed using Chemigenius2 Bioimaging System (Syngene inc., Frederick, MD) and analyzed using Genesnap software v 7.07 (Syngene). Molecular weights of proteins were confirmed using Precision Plus Protein WesternC Standards along with Precision Protein Strep-Tactin Horseradish Peroxidase Conjugated secondary antibody (Bio-Rad Laboratories). Equal loading of protein was confirmed using ponceau S stain (Bioshop, Burlington, ON, Canada) or re-probing membranes for β-actin (1:1000 in 5%BSA-TBST, Santa Cruz Biotechnology) following stripping of membranes using a stripping buffer containing 100mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, and 62.5mM tris-HCl.

mRNA extraction and analysis by RT-PCR - Frozen tissue samples were homogenized with a polytron homogenizer in Trizol® Reagent (Invitrogen Co, Frederick, MD). Separation of phases was accomplished by the addition of chloroform, followed by precipitation of RNA from the aqueous phase by the addition of isopropanol. Quantity of extracted RNA was measured spectrophotometrically (Nanodrop 2000c spectrophotometer, Thermo Scientific, Wilmington, DE, USA) and the quality was determined by the appearance of 18s and 28s rRNA bands following agarose gel electrophoresis with ethidium bromide staining. To ensure adequate purity of RNA relative to protein, only samples exhibiting 260nm/280nm absorbance ratios above 1.9 were used. Synthesis of complementary DNA (cDNA) was performed with a high capacity cDNA reverse transcription kit (Applied Biosystems, Streetsville, ON, Canada) with a thermal cycler (MJ mini personal thermal cycler, Biorad Laboratories, Mississauga, ON, Canada) program of 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 seconds, and 4°C until storage at -80 °C.

PCR primers were designed using the Primer-BLAST program on the NCBI webpage (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The accession numbers as sequences for primers used are detailed in individual studies. 18s rRNA was the reference gene, and target gene expression was determined using the $2^{-\Delta\Delta ct}$ method (Livak et al. 2001).

Fatty acid analysis

Lipid extraction **-** Lipids were extracted from plasma and rat chow by a modified version of the method of Folch, Lees, and Sloane Stanley using 2:1 choloroform:methanol (v:v) (Folch et al. 1957; Metherel et al. 2009). Lipids were extracted from tissues using either the method of Bligh and Dyer using 2:2:1.8 chloroform:methanol:water (v:v:v) (Bligh et al. 1959; Metherel et al. 2009; Reed et al. 1960) or Folch, Lees, and Sloane Stanley (Folch et al. 1957), as detailed in individual studies. Internal standards for determination of fatty acid composition of total lipids (Docosatrienoic acid (22:3n-3) ethyl ester, Nu-Check Prep Inc, Elysian, MN), PL (1,2-diheptadecanoyl-sn-glyercol-3-phosphocholine, Avanti Polar Lipids Inc, Alabaster, AL), TAG (triheptadecanoate, Nu-Chek Prep Inc), non-esterified fatty acids (heptadecanoic acid, Avanti Polar Lipids Inc), cholesteryl esters (cholesteryl heptadecanoate, Avanti Polar Lipids Inc), PC (1,2-diheptadecanoyl-sn-glyercol-3-phosphocholine, Avanti Polar Lipids Inc) and PE (1,2-diheptadecanoyl-sn-glyercol-3-phosphoethanolamine, Avanti Polar Lipids Inc) were added as appropriate. Butylated hydroxytoluene (BHT) was present in all lipid extractions as an antioxidant.

Neutral lipid and phospholipid class separation by thin layer chromatography (TLC) **-** Neutral lipids (cholesteryl esters, non-esterified fatty acids, TAGs, and total PLs) were isolated on 20 x 20 cm TLC plates with a 6nm silica gel layer (Whatman Internation LTD, Maidstone,

England) in a mobile phase of heptane:diethyl ether:glacial acetic acid (60:40:2 v/v/v) (Christie 2003). Bands were identified by comparison with a reference standard containing PL, TAG, non-esterified fatty acids and cholesteryl esters. PL classes were isolated on TLC H-plates (Analtech, Newark, DE, USA) using a mobile phase of chloroform:methanol:2-propanol:0.25% KCl:triethylamine (30:9:25:6:18 by volume) (Chen et al. 2011). Bands were visualized by ultraviolet light using 2,7-dichloroflouorescein (Sigma-Aldrich, Oakville, ON, Canada), identified by comparison with reference standards for PC, PE, and PS, and collected into tubes. Lipids were then extracted from silica gel shavings with 2:1 choloroform:methanol (Folch et al. 1957).

Fatty acid composition analysis by fast gas chromatography with flame ionization detection - All lipid extracts were transesterified to form fatty acid methyl esters (FAME) by heating at 85°C for one hour in the presence of 14% boron triflouride in methanol (Morrison et al. 1964). Separation of FAME was accomplished by a Varian 3900 gas chromatograph (Varian Inc, Mississauga, ON, Canada) with settings similar to those described previously (Masood et al. 2005). Briefly, a DB-FFAP capillary column with a 15 m x 0.10 mm inner diameter X 0.10 mm film thickness (J & W Scientific, Agilent Technologies, Palo Alto, CA) was used with H_2 as the carrier gas with a flow rate of 30 ml/min. The injector temperature was 250°C and the flame ionization detector was set at 300°C. Peaks corresponding to fatty acids were identified by comparison to a reference standard (GLC-462 or GLC-569, Nu-Chek Prep Inc) and quantified by comparison to peak area of internal standard. The n-6/n-3 ratio and the % of n-3 HUFA in total HUFA was also calculated.

Statistics

Statistical analysis was performed using SPSS for windows version 15.0. Significant differences were inferred when $p < 0.05$. Specific statistical analyses are detailed in individual studies.

Table 4.1
Measured fatty acid composition of rodent chow

Fatty Acid	**Diet content**
	(µg fatty acid/g of chow)
16:0	5783 ± 447
18:0	1523 ± 166
Total SFA	8058 ± 644
16:1	315 ± 6
18:1n-7	2938 ± 4
18:1n-9	9237 ± 2
20:1n-9	134 ± 9
Total MUFA	9611 ± 1198
18:2n-6	21211 ± 1576
20:4n-6	48 ± 7
Total n-6 PUFA	21364 ± 1547
18:3n-3	2511 ± 143
20:5n-3	126 ± 1
22:5n-3	27 ± 1
22:6n-3	106 ± 7
Total n-3 PUFA	2769 ± 147
Total PUFA	24133 ± 1694
Total Fatty Acids	42759 ± 3233

Values are mean ± SD from triplicate analysis.

CHAPTER 5

TISSUE-SPECIFIC SEX DIFFERENCES IN Δ6-DESATURASE EXPRESSION AND DHA CONTENT IN RATS FED A STANDARD CHOW DIET[1]

INTRODUCTION

Several studies have shown that women synthesize more DHA from ALA as compared with men when n-3 HUFA intakes are low (Burdge et al. 2002a; Burdge et al. 2002b; Pawlosky et al. 2003a), leading to higher blood levels of DHA in women (Bakewell et al. 2006; Crowe et al. 2008; Metherel et al. 2009; Sfar et al. 2010). Higher DHA biosynthesis in women may be associated with higher expression of enzymes involved in the biosynthesis of DHA from ALA. This may be particularly important in the liver, the primary site of *de novo* DHA biosynthesis (Rapoport et al. 2010); however, sex differences in enzymes of DHA biosynthesis may occur in other tissues as well. Previous studies investigating sex differences in enzyme expression in rats have presented mixed results and have only focused on the liver (Burdge et al. 2008; Childs et al. 2010; Extier et al. 2010).

The purpose of this study was to examine sex differences in the tissue mRNA and protein levels of Δ6- and Δ5-desaturase, elovl2, elovl5, and acyl-CoA oxidase in liver, heart, and brain to determine the possible role of these enzymes in sex differences in DHA concentrations. Expression of the transcription factors PPARα and SREBP1c was also measured to determine the transcriptional control of these enzymes. DHA concentration was measured in liver, heart, brain, plasma and erythrocytes to determine the tissue-specificity of sex differences in DHA.

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[1]The contents of this chapter have been previously published and copyright has been assigned to NRC Research Press.
Kitson, A.P., Smith, T.L., Marks, K.A., et al. 2012. Tissue-specific sex differences in docosahexaenoic acid and Δ6-desaturase in rats fed a standard chow diet. *Applied Physiology, Nutrition, and Metabolism* 37(6), 1200-1211.

METHODS

Male and female Sprague-Dawley rats (n = 6 for each sex) were ordered from Harlan (Mississauga, Ontario) and arrived at University of Waterloo at 10 weeks of age. Animals were stored in the animal housing facilities in the department of Kinesiology. After 14 weeks of age, rats were anaesthetized after an overnight fast by intraperitoneal sodium pentobarbital injection (65 mg/kg). Blood was collected into an EDTA containing syringe by cardiocentesis, and plasma and erythrocytes were separated by centrifugation (1500g) and stored at -80°C. Heart, liver, and brain were quickly excised, rinsed with 0.9% (w/v) aqueous NaCl, dried and immediately frozen in liquid nitrogen prior to storage at -80°C for RNA, protein, and fatty acid analysis.

RT-PCR was performed using SYBR Green qPCR master-mix (Applied Biosystems) on an Applied Biosystems 7500 real time PCR system using 25 μl as the reaction volume. Data was expressed relative to female samples. Primers used for RT-PCR were Δ6 desaturase (GenBank accession number NM_031344, 5'-3' TCAAAACCAACCACCTGTTCTTC, 3'-5' ACCAGGCGATGCTTTCCA), Δ5 desaturase (NM_053445, 5'-3' CCTCTTGTAAAGCACGAGCC, 3'-5' CAAGGGGTCACACTGTTCCT), elovl2 (AB071986.1, 5'-3' TGCTTGCCCGTGAGAGCCAC, 3'-5' TGCCACAGGAAGGCGA CGAC), elovl5 (NM_134382.1, 5'-3' CTCTCGGGTGGCTGTACTTC, 3'-5' AGAGGCCCCTT TCTTGTTGT), acyl-CoA oxidase (NM_017340, 5'-3' CTGCTCAGCAGGAGAAATGG, 3'-5' CTCACAGCGCTGTATCGTAT), peroxisome proliferator activated receptor α (NM_013196.1, 5'-3' GCAGACCTCAAATCTCTGGC, 3'-5' GGCCTTGACCTTGTTCATGT), sterol response element binding protein 1c (XM_001075680, 5'-3' CCACCTGTGCAGCTCAGCCC, 3'-5' GGCGTCTGCTGGGTGTTCCC), estrogen receptor α (NM_012689.1, 5'-3' TCCGGCACATG

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AGTAACAAA, 3'-5' TGAAGACGATGAGCATCCAG), and 18s ribosomal RNA (M11188, 5'-3' GATCCATTGGAGGGCAAGTCT, 3'-5' AACTGCAGCAACTTTAATATACGCTATT).

Antibodies used for protein expression analysis were Δ6-desaturase (sc-98480, Santa Cruz Biotechnology, Santa Cruz, CA, 1:400 dilution in BSA), Δ5-desaturase (sc-101953, 1:100 dilution, v/v), elovl2 (sc-54874, 1:250 dilution), elovl5 (sc-54888, 1:250 dilution), PPARα (sc-9000, 1:250 dilution), SREBP1c (sc-8984, 1:500 dilution), or acyl-CoA oxidase (sc-98499, 1:100 dilution). Blocking was performed with BSA, and all antibodies were dissolved in 5% BSA in TBS-T.

The fatty acid composition of plasma, liver, heart, and brain total lipids, PLs, and TAGs was measured. For the liver, fatty acid concentration of PC, PE, PS, and PI was also determined. Quantitation of PL classes was accomplished by adding 22:3n-3 internal standard following TLC. Fatty acid composition of erythrocyte total lipids was also measured.

Comparisons between female and male values were performed by independent samples T-test.

RESULTS

Sex differences in enzyme and transcription factor expression

The mRNA for microsomal and peroxisomal enzymes involved in DHA biosynthesis as well as transcription factors involved in regulation of lipid metabolic enzymes was assessed in liver, brain, and heart (**Figure 5.1**). In the liver, Δ5-desaturase, Δ6 desaturase, and elongase 2 mRNA was 100%, 140%, and 110% higher, respectively, in female rats as compared with males. Conversely, elongase 5 mRNA was 60% lower in female liver. Hepatic mRNA for SREBP1-c and estrogen receptor α (ERα) was 80% and 60% higher, while PPARα mRNA was 50% lower,

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in females compared to males. No differences were observed in hepatic acyl-CoA oxidase mRNA. No sex differences in mRNA for any measured genes were observed in heart or brain.

Hepatic Δ6-desaturase protein expression was 60% higher in females compared with males. PPARα was 30% lower (**Figure 5.2**). No sex differences in any other measured protein were observed in liver. In the heart, no sex differences in protein levels of measured enzymes or transcription factors were observed, and in the brain there was a 10% lower acyl-CoA oxidase protein level and no other sex differences in expression (**Appendix 1 Table 1**).

Sex differences in tissue polyunsaturated fatty acid content

The fatty acid content was also determined for liver, heart, and brain. In liver total lipids (**Table 5.1)**, females had higher DHA concentrations but lower DPAn-3 concentrations as compared with males. In liver total lipid n-6 PUFA, females had higher concentrations of 18:3n-6 and DPAn-6 as compared with males. In liver PL (**Table 5.2**), concentrations of DHA and 18:3n-6 were higher in females as compared with males. DHA and DPAn-6 were also higher in females relative to males in PC, PE, and PS, but not PI (**Figure 5.3**). No sex differences were seen in DHA concentration of hepatic TAG (**Appendix 1 Table 2**).

In heart total lipids (**Table 5.1**), female rats had higher DHA and lower DPAn-3 compared with males. Concentrations of 20:4n-6 and DPAn-6 were higher in females compared with males. In heart PL there were no sex differences in DHA levels; however female heart PL had lower concentrations of DPAn-3, and LA as compared with males (**Table 5.2**). No sex differences in n-3 HUFA concentrations of heart TAG were observed (**Appendix 1 Table 2**).

No statistically significant sex differences in any n-3 PUFA were observed in any lipid fractions in brain, however total n-6 PUFA was lower in females (**Table 5.1**). No sex difference

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was observed in the concentrations of PUFA in brain PL (**Table 5.2**) or TAG (**Appendix 1 Table 2**).

In plasma total lipids, females had higher DHA, 18:3n-6, 22:5n-6, and lower 18:2n-6 and DPAn-3 as compared with males (**Table 5.3**). In plasma PL, females exhibited higher concentrations of DHA as compared with males (**Table 5.3**). No sex difference in plasma TAG n-3 HUFA concentration was observed. In erythrocyte total lipids, females had higher DHA concentration as compared with males (**Table 5.4**).

DISCUSSION

This study demonstrates that female rats have higher hepatic Δ6-desaturase mRNA and protein, which corresponds to higher DHA concentration in liver, plasma, heart, and erythrocytes compared with males. No difference in expression in heart or brain indicates that this sex difference is liver-specific, and suggests that the higher hepatic Δ6-desaturase expression results in higher synthesis of DHA in females relative to males. The tissue-specific sex differences in DHA observed in the present study are in agreement with previous studies that have also reported female rats having higher DHA in PL of plasma (Childs et al. 2008; Childs et al. 2010; Extier et al. 2010), liver (Alessandri et al. 2012; Burdge et al. 2008; Childs et al. 2010; Extier et al. 2010), heart (Slater-Jefferies et al. 2010), and total lipids of erythrocytes (McNamara et al. 2009) but with no differences in brain (Extier et al. 2010) relative to males.

Higher activity of Δ6-desaturase is strongly suggested by the increased hepatic DHA. In addition, higher Δ6-desaturase activity is suggested by higher concentrations of 18:3n-6 and DPAn-6 (the direct n-6 product of Δ6-desaturase and the corresponding n-6 HUFA to DHA, respectively) that were observed in females. Additionally, lower DPAn-3 in females suggests

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increased Δ6-desaturase activity, as Pawlosky et al (2003a) report that the primary sex difference is the conversion of 22:5n-3 to DHA, indicating a "bottleneck" in DHA biosynthesis that is somewhat relieved in females via increased Δ6-desaturase. Higher Δ6-desaturase activity appears to be the enzymatic step in DHA synthesis that is mediating higher DHA in females, as there were no differences observed in the hepatic expression of Δ5-desaturase, elovl2, elovl5, and acyl-CoA oxidase, although factors involved in fatty acid transport or glycerolipid synthesis or remodeling were not measured in the present study.

Previous reports regarding sex differences in hepatic Δ5- and Δ6-desaturase expression in rats have been mixed. Extier et al. (2010) reported increased hepatic Δ5- and Δ6-desaturase mRNA as well as Δ5-desaturase protein content (Δ6-desaturase protein was not reported) in female rats compared with males on an ALA replenishment diet (0.2g ALA/kg diet up from 0.05g ALA/kg diet provided to the mother during pregnancy and lactation). Similarly, Burdge et al. (2008) reported increased hepatic Δ5-desaturase mRNA (no difference in Δ6-desaturase) in female rat liver after manipulation of protein and folic acid supply during pregnancy. Childs et al. (2010) report no sex differences in hepatic Δ5- or Δ6-desaturase mRNA while varying dietary fat, ALA and LA levels. These discrepancies may involve differences in diet, as increased PUFA content of the diet has been shown to decrease Δ6-desaturase expression (Tang et al. 2003) and may limit the ability to detect a sex difference. Differences in the age of rats may alter sex differences in Δ6-desaturase and DHA, if the animals have not fully reached sexual maturity [typically 6.5 weeks (Lau et al. 1996)]. Older age (>9 months) is also associated with significant decreases in Δ6-desaturase activity in mice (Bourre et al. 1992) and rats (Bourre et al. 1990). It is also possible that differences in methodology of mRNA measurements such as different housekeeping genes or quantification techniques may contribute to inconsistent results.

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The mechanism underlying the increased Δ6-desaturase expression in the present study may involve the higher expression of SREBP1c observed in this study and others (Ameen et al. 2004). The overexpression of SREBP1c in isolated rat primary hepatocytes has been shown to increase the mRNA for elongase 2 and Δ5- and Δ6-desaturase, with no difference in elongase 5, similar to the expression pattern observed in females relative to males in the present study (Wang et al. 2006). No differences in SREBP1c protein were observed, possibly because only the endoplasmic reticular peptide was measured. Higher nuclear SREBP1c resulting from higher SCAP expression and/or activity may increase Δ6-desaturase expression. Hepatic SCAP mRNA is elevated in lactating relative to virgin rats (Athippozhy et al. 2011), suggesting that ovarian hormones may influence SCAP expression. Conversely, the lower PPARα in female liver observed in this study and others (Ciana et al. 2007; Extier et al. 2010) suggests that this transcription factor does not contribute to the higher Δ6-desaturase expression in female rats.

We also observed no sex differences in TAG DHA composition in any tissue or blood fraction investigated, despite higher total lipid DHA in liver, heart, and plasma in females compared with males, similar to previous studies (Burdge et al. 2008; Slater-Jefferies et al. 2010). DHA is a minor component of TAG (Christie 2003; Wood et al. 1969) due to poor incorporation (Lemaitre-Delaunay et al. 1999), suggesting that the increased DHA in female tissues is not sufficient to elicit an increase in TAG DHA content.

The higher DHA content observed in female hearts as compared with male hearts is likely due to higher uptake and incorporation rather than increased cardiac synthesis, as no significant differences were found in the cardiac expression of DHA producing enzymes. The sex differences in most heart total lipid HUFA are similar to those in plasma (higher DHA, lower

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22:5n-3, higher 22:5n-6), suggesting that these sex differences are influenced by plasma fatty acid composition.

The sex difference in Δ6-desaturase was found in liver, but not in heart or brain, suggesting a mechanism present only in liver that mediates sex differences in DHA biosynthesis. Higher expression of ERα found in female relative to male liver may mediate this liver-specific effect, as ERα expression was the same between sexes in heart and brain. This would likely result in the total estrogen "signal" to be significantly higher in female as compared to male liver, as both plasma 17β-estradiol and liver ERα were higher.

No significant difference in DHA levels were observed in the brain. This finding is consistent with previous work showing a lack of sex difference in cerebral cortex PL DHA levels (Extier et al. 2010). One study showed higher prefrontal cortex total lipid DHA levels in males (McNamara et al. 2009), suggesting that sex differences in brain DHA content are specific to the brain area investigated. Brain DHA levels are quite stable throughout the lifetime of rats, as DHA-free artificial rearing (Lim et al. 2005a; Ward et al. 1996), and gestational (Greiner et al. 2003) or multi-generational dietary DHA deficiency (Bourre et al. 1984) is required to reduce rat brain DHA stores due to maternal-pup DHA transfer through lactation. Increases in DHA levels in female rat plasma PC relative to males develops after only 8 weeks of age (Extier et al. 2010), suggesting that the sex difference in plasma DHA may be insufficient and/or occurs too late in the animal's development to elicit a significant difference in brain DHA.

CONCLUSION

A greater capacity for hepatic biosynthesis of DHA from ALA is suggested by the higher Δ6-desaturase expression observed in female livers relative to males. The mechanism

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underlying this increased expression is not clear, but may involve differences in transcription factor expression that are isolated to the liver. Identification of the effects of endocrine factors such as sex hormones and cellular signaling mechanisms and transcription factors involved in the sex difference in Δ6-desaturase expression is required.

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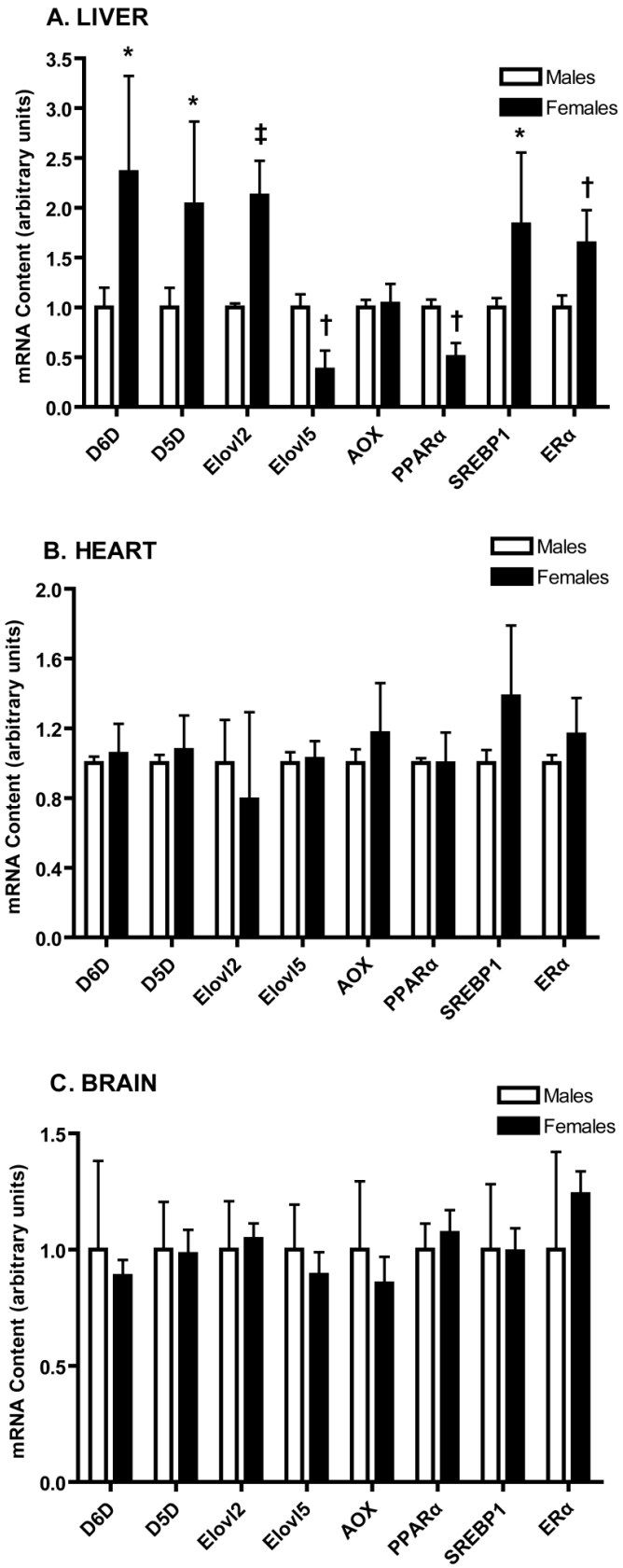

Figure 5.1: mRNA content of enzymes and transcription factors involved in DHA biosynthesis in (A) liver, (B) heart, and (C) brain of male and female rats (n = 6 for each sex). Significance sex differences are denoted by *: $p < 0.05$, †: $p < 0.01$, ‡: $p < 0.005$. D6D: Δ6-desaturase, D5D: Δ5-desaturase, Elovl2: elongase 2, Elovl5: elongase 5, AOX: acyl-CoA oxidase, PPARα: peroxisome proliferator activated receptor α, SREBP1: sterol response element binding protein 1, ERα: estrogen receptor α.

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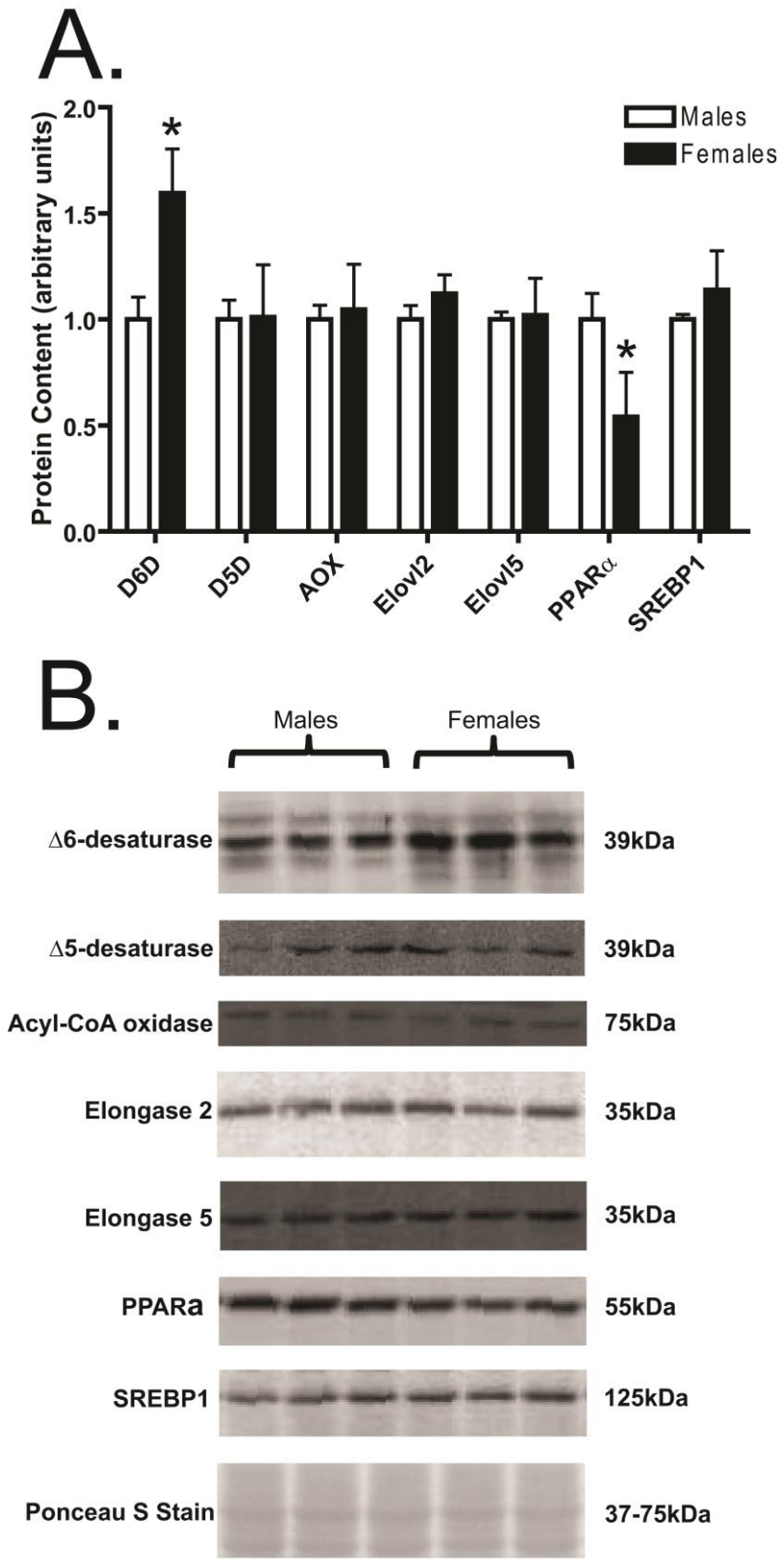

Figure 5.2: (A) densitometric analysis of protein content and (B) representative immunoblots of enzymes and transcription factors involved in DHA synthesis in liver of male and female rats (n = 6 for each sex). Data is representative of three separate blots. Significance sex differences are denoted by *****: $p < 0.05$, ‡: $p < 0.005$. PPARα: peroxisome proliferator activated receptor α, SREBP1: sterol response element binding protein 1.

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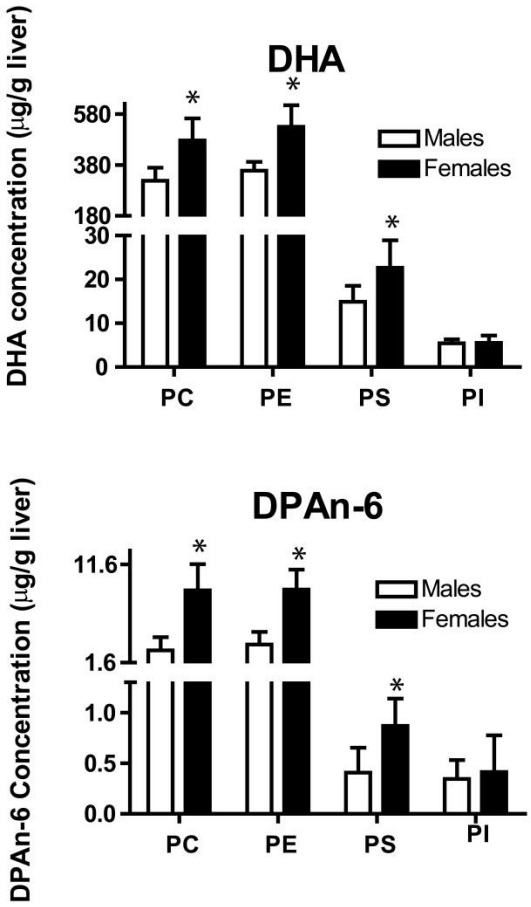

Figure 5.3: Concentrations of docosahexaenoic acid and n-6 docosapentaenoic acid in hepatic phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. *: significantly different from males by independent samples T-test. DHA: docosahexaenoic acid, DPAn-6: n-6 docosapentaenoic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PS: phosphatidylserine, PI: phosphatidylinositol.

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Table 5.1: Fatty acid concentration of total lipid extract from liver, heart, and brain of male and female rats

	Liver		**Heart**		**Brain**	
	μg fatty acid/g organ					
Fatty Acid	***Females***	***Males***	***Females***	***Males***	***Females***	***Males***
SFA[1]	11230 ± 1195	10816 ± 681	8157 ± 224	7495 ± 270*	18111 ± 622	18553 ± 736
MUFA[1]	4546 ± 650	5204 ± 423	5354 ± 905	4771 ± 844	13914 ± 826	14269 ± 2065
18:2n-6	3947 ± 853	4800 ± 637	3088 ± 458	3304 ± 370	774 ± 66	862 ± 78
18:3n-6	91 ± 27	57 ± 19*	30 ± 5	34 ± 2	18 ± 2	18 ± 4
20:2n-6	64 ± 8	143 ± 30*	47 ± 3	61 ± 5*	58 ± 14	68 ± 21
20:3n-6	84 ± 16	87 ± 18	49 ± 5	46 ± 4	135 ± 4	129 ± 19
20:4n-6	6013 ± 747	6699 ± 274	3955 ± 106	3726 ± 112*	3868 ± 160	3949 ± 164
22:4n-6	103 ± 12	93 ± 11	184 ± 12	151 ± 7*	1204 ± 48	1208 ± 77
22:5n-6	51 ± 13	20 ± 8*	162 ± 18	102 ± 16*	170 ± 15	188 ± 24
N-6 PUFA	10356 ± 1370	11902 ± 822*	7515 ± 560	7424 ± 369	6183 ± 138	6425 ± 212*
18:3n-3	160 ± 57	151 ± 26	104 ± 34	82 ± 13	67 ± 5	71 ± 6
20:5n-3	115 ± 34	107 ± 30	22 ± 4	25 ± 4	7 ± 1	7 ± 3
22:5n-3	263 ± 32	316 ± 20*	478 ± 51	560 ± 46*	55 ± 3	58 ± 3
22:6n-3	2221 ± 357	1455 ± 246*	2678 ± 210	2151 ± 399*	4949 ± 150	4723 ± 220
N-3 PUFA	2763 ± 398	2043 ± 257*	3451 ± 351	3085 ± 520	5079 ± 148	4859 ± 217
Total PUFA	13119 ± 1718	13944 ± 851	10966 ± 620	10509 ± 525	11588 ± 792	11284 ± 326
Total Fatty Acids	30121 ± 3400	29807 ± 3306	25892 ± 1425	24250 ± 1557	46267 ± 1263	46663 ± 3311

Data is mean ± SD from three determinations of fatty acid concentrations (n = 6 for each sex). *: significantly different from females ($p < 0.05$). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. [1]Concentration of individual SFA and MUFA can be found in (Marks et al. 2013b).

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Table 5.2: Fatty acid composition of phospholipids from liver, heart, and brain of male and female rats

	Liver		**Heart**		**Brain**	
	μg fatty acid/g organ					
Fatty Acid	***Females***	***Males***	***Females***	***Males***	***Females***	***Males***
Total SFA[1]	8934 ± 2038	7710 ± 533	4903 ± 440	4389 ± 346*	14587 ± 1497	14324 ± 1354
Total MUFA[1]	766 ± 159	1177 ± 100*	598 ± 82	636 ± 61	7661 ± 1328	7700 ± 1601
18:2n-6	1950 ± 511	2501 ± 349	1126 ± 215	1419 ± 177*	198 ± 26	230 ± 31
18:3n-6	43 ± 13	24 ± 6*	21 ± 4	21 ± 3	1 ± 1	1 ± 1
20:2n-6	40 ± 10	100 ± 20*	20 ± 3	28 ± 5*	37 ± 8	51 ± 13
20:3n-6	62 ± 22	56 ± 10	24 ± 3	22 ± 3	95 ± 18	88 ± 15
20:4n-6	5094 ± 1126	5086 ± 323	2276 ± 316	2200 ± 280	3001 ± 478	2974 ± 440
22:4n-6	69 ± 13	51 ± 5*	106 ± 4	89 ± 14*	1012 ± 150	979 ± 116
22:5n-6	2 ± 1	2 ± 1	2 ± 1	1 ± 1	4 ± 3	4 ± 3
Total n-6 PUFA	7262 ± 1664	7823 ± 643	3574 ± 536	3781 ± 441	4353 ± 652	4333 ± 528
18:3n-3	24 ± 9	22 ± 3	14 ± 3	17 ± 3	1 ± 1	1 ± 1
20:5n-3	50 ± 22	35 ± 7	10 ± 1	11 ± 2	3 ± 1	1 ± 1
22:5n-3	194 ± 35	212 ± 18	282 ± 19	334 ± 44*	38 ± 8	46 ± 10
22:6n-3	1825 ± 293	1011 ± 125*	1625 ± 268	1342 ± 242	3786 ± 412	3543 ± 428
Total n-3 PUFA	2096 ± 346	1284 ± 133*	1932 ± 270	1483 ± 610	3831 ± 422	3592 ± 437
Total PUFA	9358 ± 1991	9106 ± 617	5506 ± 782	5264 ± 854	8184 ± 1012	7926 ± 937
Total Fatty Acids	19391 ± 4242	18405 ± 1183	11331 ± 1336	10884 ± 1211	31523 ± 3882	31078 ± 2928

Data is mean ± SD from three determinations of fatty acid concentration (n = 6 for each sex). *: significantly different from females ($p < 0.05$). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. [1]Concentration of individual SFA and MUFA can be found in (Marks et al. 2013b).

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Table 5.3: Fatty acid concentration of total lipids, phospholipids, and triacylglycerol from plasma of male and female rats

	Total Lipids		**Phospholipids**		**Triacylglycerols**	
			µg fatty acid/ml plasma			
Fatty Acid	***Females***	***Males***	***Females***	***Males***	***Females***	***Males***
Total SFA[1]	507 ± 46	462 ± 25	211 ± 72	184 ± 11	194 ± 63	198 ± 89
Total MUFA[1]	85 ± 14	112 ± 7*	13 ± 3	18 ± 3*	56 ± 22	59 ± 17
18:2n-6	202 ± 26	284 ± 27*	30 ± 6	45 ± 4*	79 ± 39	106 ± 39
18:3n-6	6 ± 2	3 ± 1*	0.2 ± 0.1	0.2 ± 0.1	5 ± 2	4 ± 1
20:2n-6	2 ± 0.4	4 ± 1*	0.4 ± 0.1	1 ± 0.2*	2 ± 1	2 ± 1
20:3n-6	5 ± 1	4 ± 1	1 ± 0.3	1 ± 0.1	4 ± 0.5	4 ± 1
20:4n-6	536 ± 82	492 ± 35	65 ± 14	72 ± 7	36 ± 11	32 ± 11
22:4n-6	4 ± 1	4 ± 1	1 ± 0.3	1 ± 0.1	3 ± 1	3 ± 1
22:5n-6	3 ± 0.3	2 ± 0.2*	0.2 ± 0.1	0.2 ± 0.1	1 ± 0.4	1 ± 0.4
Total n-6 PUFA	757 ± 106	795 ± 24	98 ± 21	120 ± 10*	131 ± 53	154 ± 47
18:3n-3	4 ± 2	7 ± 2*	0.3 ± 0.1	0.5 ± 0.5	5 ± 1	6 ± 2
20:5n-3	9 ± 2	8 ± 1	0.4 ± 0.1	0.4 ± 0.1	5 ± 2	6 ± 2
22:5n-3	7 ± 1	9 ± 1*	2 ± 0.5	2 ± 0.3	4 ± 2	3 ± 1
22:6n-3	58 ± 11	33 ± 6*	11 ± 4	8 ± 1*	6 ± 2	6 ± 2
Total n-3 PUFA	80 ± 13	58 ± 4*	14 ± 4	11 ± 2	20 ± 6	21 ± 4
Total PUFA	837 ± 118	852 ± 25	112 ± 25	130 ± 11	151 ± 57	175 ± 49
Total Fatty Acids	1429 ± 173	1427 ± 53	335 ± 95	332 ± 18	401 ± 133	432 ± 133

Data is mean ± SD from three determinations of fatty acid concentration (n = 6 for each sex). *: significantly different from females ($p < 0.05$). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. [1]Concentration of individual SFA and MUFA can be found in (Marks et al. 2013b).

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Table 5.4: Fatty acid concentration of erythrocyte total lipids in male and female rats

Fatty Acid	***Females***	***Males***
Total SFA[1]	1323 ± 141	1231 ± 148
Total MUFA[1]	221 ± 24	251 ± 17
18:2n-6	277 ± 36	405 ± 86*
18:3n-6	4 ± 3	3 ± 1
20:2n-6	9 ± 2	12 ± 2
20:3n-6	10 ± 1	9 ± 1
20:4n-6	785 ± 109	790 ± 118
22:4n-6	40 ± 9	36 ± 8
22:5n-6	15 ± 3	12 ± 2
Total n-6 PUFA	1141 ± 132	1269 ± 182
18:3n-3	3 ± 1	7 ± 4
20:5n-3	11 ± 3	11 ± 2
22:5n-3	40 ± 10	41 ± 6
22:6n-3	87 ± 14	63 ± 11*
Total n-3 PUFA	141 ± 8	122 ± 10*
Total PUFA	1283 ± 139	1391 ± 189
Total Fatty Acids	2978 ± 166	3001 ± 159

Data is mean ± SD from three determinations of fatty acid concentration (n = 6 for each sex). *: significantly different from females ($p < 0.05$). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. [1]Concentration of individual SFA and MUFA can be found in (Marks et al. 2013b).

CHAPTER 6

HEPATIC Δ6-DESATURASE AND DHA IS INCREASED LATE IN PREGNANCY IN RATS

INTRODUCTION

Maternal plasma and erythrocyte DHA is increased during pregnancy (Al et al. 1995; Stark et al. 2005a; Stewart et al. 2007), likely as an adaptation to provide the fetus with the DHA for neurodevelopment. However, the mechanism of this increase in maternal DHA has not been investigated. It is likely that increased synthesis-mobilization of DHA is involved, and pregnancy may increase the expression of DHA biosynthesis enzymes. Also, pregnancy is associated with significantly increased plasma concentrations of 17β-estradiol, which is associated with increased blood DHA levels [reviewed in (Kitson et al. 2010)].

Previous work investigating the effect of pregnancy on hepatic expression of DHA-synthesis enzymes has examined mRNA only in virgin rats and rats at day-12 and -20 of pregnancy (Childs et al. 2012). Adaptations occurring in late-stage pregnancy and elovl5 or peroxisomal oxidation capacity have not been assessed previously. Additionally, hepatic DHA content and biosynthetic enzyme expression have not been measured in post-partum, lactating rats. The present study examines effects of pregnancy at 15- and 20-days post conception and 7-days post-partum on DHA concentrations and DHA biosynthetic enzyme expression relative to virgin controls in rats.

METHODS

Female Sprague Dawley rats were ordered from Harlan (Mississauga, Ontario) and arrived at the University of Waterloo at 6 weeks of age and at 7 weeks of age virgin rats were sacrificed after an overnight fast (n = 6) by removal of the heart following isoflurane anaesthesia.

Eighteen others were mated with proven breeders and pregnancy was confirmed by appearance of a vaginal plug. Pregnant rats were sacrificed after an overnight fast at 15- (n = 6), 20- (n = 5), and 28-days post-conception (n = 6, 7 days post-partum). Livers were removed, washed in saline, and snap frozen in liquid nitrogen.

Livers were pulverized under liquid nitrogen and lipids were extracted (Folch et al. 1957) and total lipid fatty acid composition was determined and the expression of enzymes involved in DHA biosynthesis was also determined. Antibodies used for protein expression analysis were Δ6-desaturase (1:1000 in 5% milk-TBST; from Abcam, Cambridge, Massachusetts), Δ5-desaturase (1:100 in 5% BSA-TBST; from Santa Cruz Biotechnology, Santa Cruz, California), elovl2 (1:250 in 5% BSA-TBST; Santa Cruz Biotechnology), elovl5 (1:250 in 5% BSA-TBST; Santa Cruz Biotechnology), multifunctional protein-2 (MFP2, 1:200 in 5% milk-TBST; Santa Cruz Biotechnology), PPARα, (1:250 in 5% milk-TBST, Santa Cruz Biotechnology), SREBP1c, (1:500 in 5% milk-TBST, Santa Cruz Biotechnology) and ERα (1:1000 in 5% milk-TBST, abcam).

Statistical analysis was by one-way ANOVA with Tukey's post hoc test completed after a significant F value, $P < 0.05$.

RESULTS

The expression of Δ6-desaturase was 27% higher after 15 days of pregnancy and 45% higher after 20 days as compared with virgin rats. Expression returned to baseline levels on day 28 post-conception (**Figure 6.1**). Elovl5 expression was decreased by 30% between post conception days 15 and 20, and returned to baseline levels on day 28. No effects of pregnancy were observed in protein levels of elovl2, MFP2, or Δ5-desaturase. No changes in the protein

expression were seen in ERα, PPARα, or either nuclear or endoplasmic reticulum SREBP1c (**Figure 6.2**).

The concentration of DHA and DPAn-6 in liver increased over the course of pregnancy and reached a peak at 20 days of pregnancy, despite significant decreases in liver total fatty acid concentration at 20 days of pregnancy (**Table 6.1**). The lower liver fatty acid concentration was due to a decrease in MUFA, SFA, and n-6 PUFA concentrations, but n-3 PUFA concentrations increased at 20 days of pregnancy. At 7 days post-partum, DHA levels and total fatty acid concentration returned to baseline, but DPAn-6 remained significantly increased. The higher DHA and DPAn-6 corresponded to a decrease in AA. The concentrations and relative percentages of both LA and ALA were lower at 20 days of pregnancy and at post-partum relative to virgin rats. DPAn-3 and 22:4n-6 were increased at 20 days of pregnancy, and DPAn-3 remained elevated in post-partum. The n-6/n-3 ratio was significantly lower, and the % of HUFA as n-3 HUFA was significantly higher at 20 days of pregnancy relative to virgin controls.

DISCUSSION

This study demonstrates that hepatic Δ6-desaturase expression is increased late in pregnancy, and that this increased expression corresponds to higher levels of DHA in hepatic total lipids. This result suggests that DHA synthesis is increased in pregnancy as a result of increased Δ6-desaturase expression, which may partly underlie the increased plasma DHA concentration observed in pregnancy in humans (Otto et al. 2001; Stark et al. 2005a) and rats (Childs et al. 2012).

The increase in hepatic DHA at 20 but not 15 days of pregnancy is consistent with previous findings showing increased hepatic DHA at 20 days, but not 12 days in rats (Childs et al. 2012). This has been shown to correspond with increases in plasma DHA at 20 days but not

12 days (Childs et al. 2012), which is contrary to findings in humans of increased DHA at 6 weeks or approximately only 15% of the total gestation, indicating a difference between rats and humans in plasma DHA response to pregnancy. Our finding of a gradual increase in Δ6-desaturase protein over the course of pregnancy that peaks at 20 days is contrary to previous work showing a plateau of Δ6-desaturase mRNA maintained between 12- and 20-days gestation (Childs et al. 2012), however the magnitude of the increase in Δ6-desaturase expression is comparable. The discrepancy between a plateau and a continual increase of Δ6-desaturase expression during pregnancy may reflect a possible latency between mRNA expression and the protein response as measured in the present study.

In addition to DHA, the levels of other 22-carbon HUFA such as DPAn-3, DPAn-6 and 22:4n-6 were also increased in liver lipids during pregnancy. This increase in 22-carbon HUFA occurred at the expense of AA, which is consistent with previous findings in humans (Stark et al. 2005a) and rats (Burdge et al. 1994; Childs et al. 2012). The increase in 22-carbon HUFA may reflect increased activity of Δ6-desaturase, as the time-course of the accretion of these fatty acids in liver lipids roughly corresponds to the time-course of increasing hepatic Δ6-desaturase expression. It is also possible that there is selective incorporation of longer-chain HUFA over AA through the selective action of phospholipases or acyltransferases involved in lipid synthesis or remodelling. Increased percentage of total HUFA as n-3 HUFA supports selective incorporation of n-3 HUFA.

Increases in circulating sex hormones may underlie the increase in Δ6-desaturase and DHA in pregnancy. 17β-estradiol, progesterone, and testosterone all increase during pregnancy, and an approximate 10-fold increase in plasma 17β-estradiol concentration was observed in the present study at 20 days which returned to baseline after birth (Chalil 2013). Testosterone

treatment of female-to-male transsexuals decreases serum cholesteryl ester DHA levels, suggesting that testosterone does not mediate the increased DHA seen in pregnancy (Giltay et al. 2004b). On the other hand, 17β-estradiol is associated with increased DHA [reviewed in (Kitson et al. 2010)], and progesterone has been shown to correlate with plasma PC DHA in female rats (Childs et al. 2008). In pregnant rats, significant positive correlations were seen between serum progesterone and both hepatic Δ6-desaturase mRNA and DPAn-6, but not DHA (Childs et al. 2012), suggesting a role of progesterone in HUFA metabolism in pregnancy.

The increase in hepatic DHA and DPAn-6 at day 20 of pregnancy occurred despite significant decreases in the concentration of their 18-carbon precursors, ALA and LA. Previous work suggests that extra-hepatic sources of ALA and LA are utilized to provide substrates for HUFA synthesis. For example, ALA and LA levels in subcutaneous adipose tissue are lowest and HUFA concentration highest at 20-days of pregnancy, suggesting selective mobilization of 18-carbon PUFA (Childs et al. 2012). Accordingly, plasma non-esterified fatty acid concentration doubles during the last half of pregnancy in the rat (Gilbert et al. 1981) and humans (Meneses et al. 2009), corresponding to an increase in the proportion of both LA and ALA relative to SFA and MUFA (Meneses et al. 2009).

Maternal hepatic DHA levels and Δ6-desaturase returned to baseline following birth, which also occurs in erythrocytes and plasma in humans (Al et al. 1995; Stark et al. 2005a), suggesting that no alterations in DHA biosynthesis occur to provide DHA for lactation. However, alternative adaptations occur to provide pups with adequate milk DHA for development. These include increased adipose tissue lipolysis (Naismith et al. 1982) resulting in higher plasma concentration of non-esterified fatty acids (Torres et al. 2004), and a 70-fold increase in mammary Δ6-desaturase expression (Rodriguez-Cruz et al. 2011). Also,

approximately 35% of dietary 18-carbon PUFA are taken up by mammary gland tissue in the lactating rat to provide substrates for HUFA synthesis and to provide the pups with 18-carbon PUFA (Rodriguez-Cruz et al. 2006). It is not clear why mammary Δ6-desaturase is increased while hepatic Δ6-desaturase returns to baseline during lactation, but it is possible that selective DHA transfer is not as effective in mammary tissue as compared with placenta, necessitating increased *in situ* DHA synthesis in mammary tissue.

The decrease in hepatic fatty acids that occurred at 20 days of pregnancy is likely due to a 17β-estradiol mediated increase in the mobilization of hepatic fatty acids and lipids for fetal delivery, rather than increased hepatic fat oxidation. 17β-estradiol supplementation increases hepatic secretion of very low density lipoprotein via increased hepatic expression of microsomal transfer protein in rats (Barsalani et al. 2010). Estradiol treatment of chicken hepatoma cells (Hermann et al. 1997) and primary chicken hepatocytes (Tarlow et al. 1977) increase apolipoprotein CII expression and secretion, and VLDL secretion and synthesis, respectively.

There are several factors that could affect DHA synthesis in pregnancy in addition to changes in sex hormone concentration. Food intake increases 60% during pregnancy, and up to 250% during lactation (Cripps et al. 1975; Rodriguez-Cruz et al. 2006), due to leptin resistance [reviewed in (Ladyman 2008)], which likely provides more substrates for DHA synthesis. Insulin resistance, which occurs during late pregnancy in humans (Endo et al. 2006) and rats (Cacho et al. 2008), may reduce the induction of Δ6-desaturase expression by insulin (Wang et al. 2006), but increases plasma lipid concentrations by increasing hepatic lipid production and secretion and decreasing the uptake of fatty acids by peripheral tissues [reviewed in (Bell 1995; Kuipers et al. 2011)]. However, the selective accretion of 22-carbon PUFA in blood lipids in

pregnancy (Stark et al. 2005a) suggests that synthesis of these fatty acids is specifically increased.

CONCLUSION

The hepatic expression of Δ6-desaturase is increased late in pregnancy in the rat. This may explain the increased hepatic DHA content observed in this study and others, as well as the increased blood DHA observed previously. The underlying mechanism mediating the effect of pregnancy on Δ6-desaturase expression is unclear, but previous findings of increased DHA associated with 17β-estradiol (Kitson et al. 2010) and progesterone (Childs et al. 2008) suggests that the increase in these hormones during pregnancy may be involved.

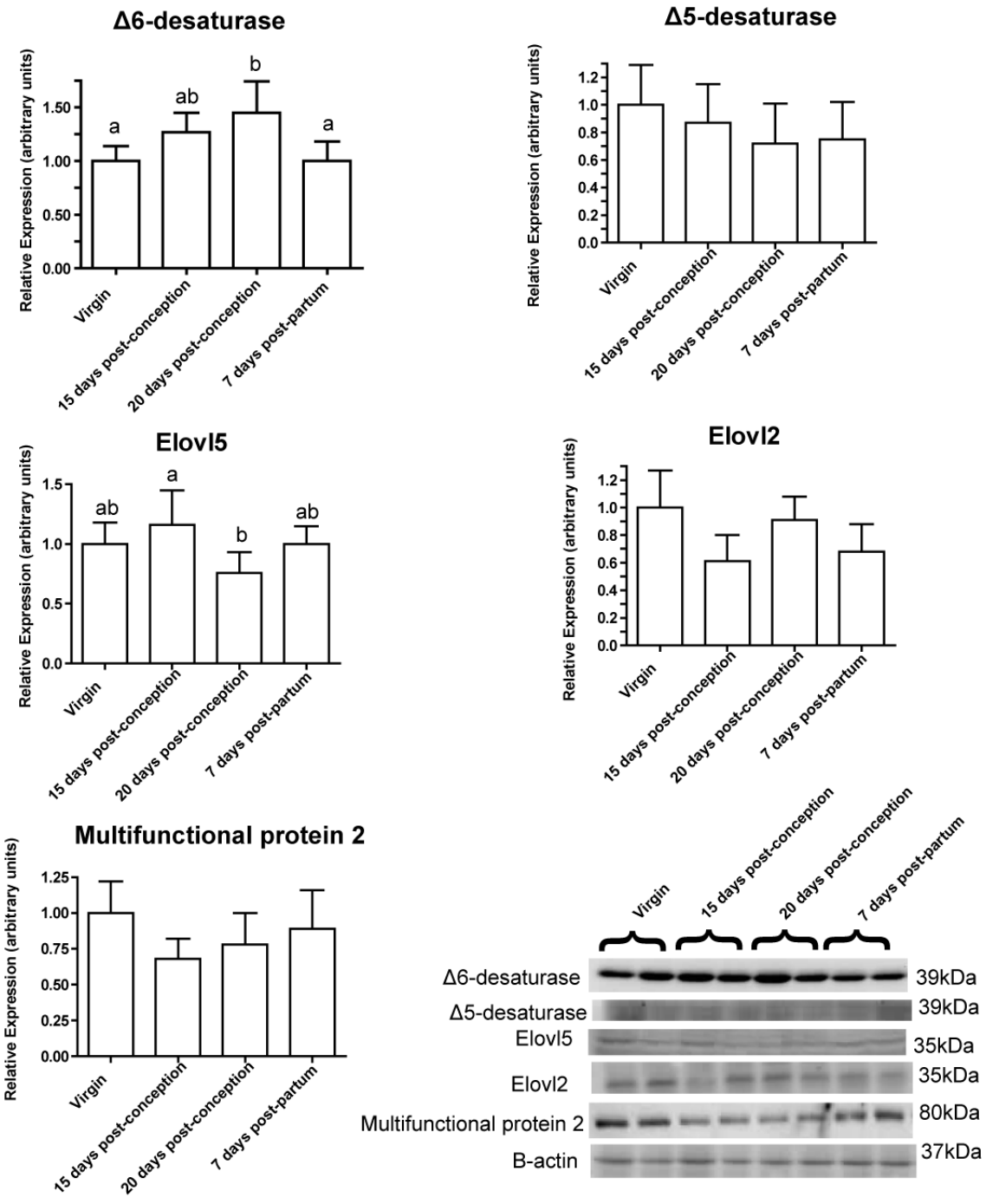

Figure 6.1: Protein expression of DHA biosynthesis enzymes in liver of virgin, pregnant, and post-partum rats. Bars with a different letter are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA.

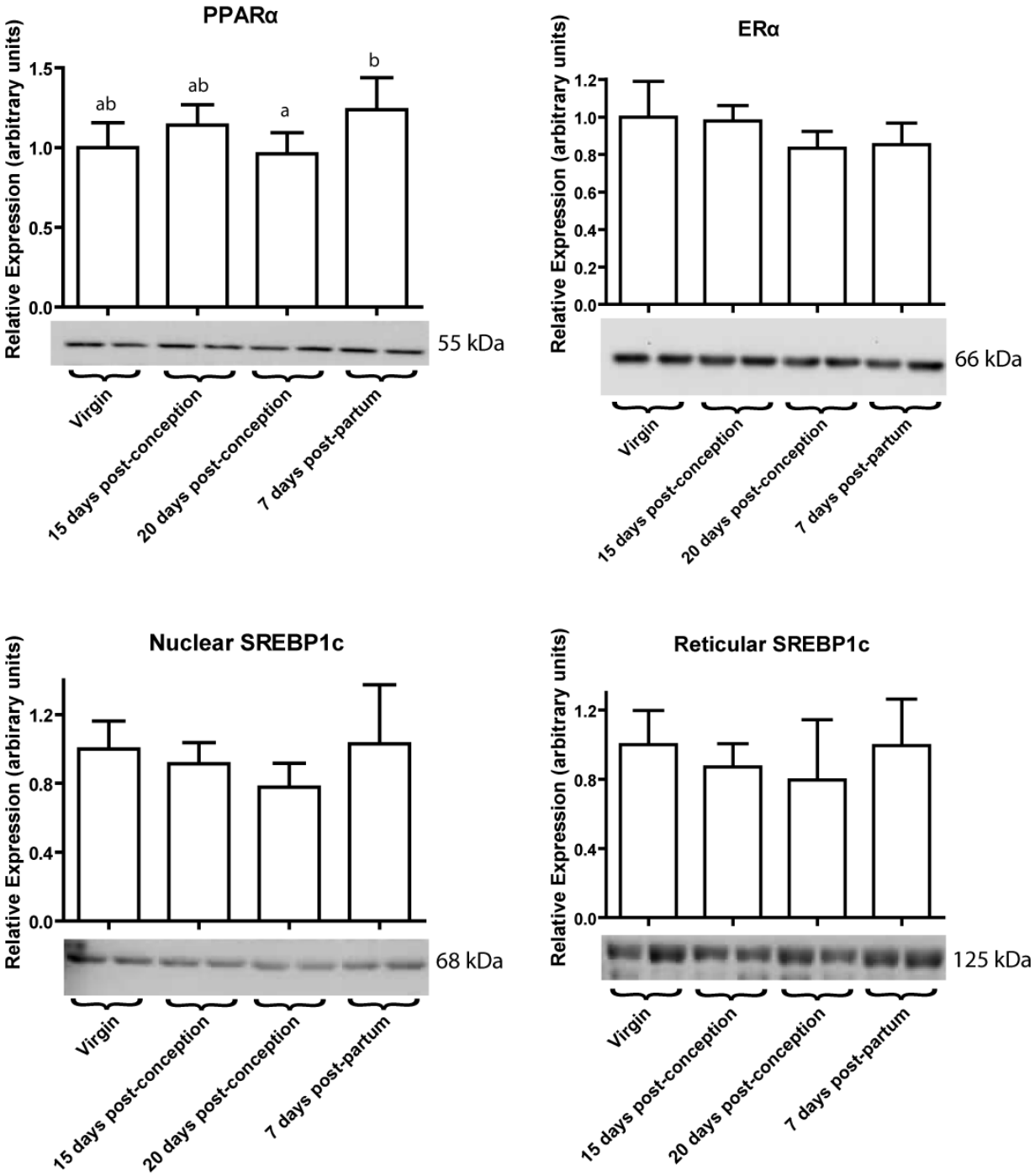

Figure 6.2: Expression of transcription factors and estrogen receptor α in livers of virgin, pregnant, and post-partum rats. Columns with different letter are significantly different by Tukey's post-hoc test following significant F value by one-way ANOVA. SREBP: sterol-response element binding protein, ERα: estrogen receptor α, PPARα: peroxisome proliferator activated receptor α.

Table 6.1: Fatty acid concentration of hepatic total lipids in virgin, pregnant, and post-partum rats

		Pregnant		Post-partum
	Virgin	**15 days**	**20 days**	**7 days**
	μg fatty acid / g liver			
C 16:0	16077 ± 2679	15120 ± 1535	15555 ± 2952	13215 ± 1258
C 18:0	19422 ± 2627[a]	20921 ± 1674[a]	13535 ± 2817[b]	18806 ± 1202[a]
SFA	37210 ± 5902	37457 ± 3072	30076 ± 5904	33248 ± 2430
C 16:1	524 ± 143	454 ± 226	310 ± 128	448 ± 91
C 18:1n-7	1560 ± 256[a]	1369 ± 103[a]	996 ± 226[b]	1415 ± 134[a]
C 18:1n-9	6475 ± 1560	5826 ± 1644	3836 ± 1249	6137 ± 1234
MUFA	8907 ± 1889[a]	7920 ± 1912[ab]	5339 ± 1610[b]	8252 ± 1386[ab]
C 18:2n-6	18555 ± 3953[a]	14616 ± 3489[ab]	9402 ± 2289[b]	12580 ± 1215[b]
C 18:3n-6	316 ± 58[ab]	336 ± 111[ab]	160 ± 98[a]	445 ± 134[b]
C 20:2n-6	217 ± 39	205 ± 16	198 ± 50	236 ± 12
C 20:3n-6	324 ± 53[a]	314 ± 40[a]	157 ± 38[b]	563 ± 107[c]
C 20:4n-6	19343 ± 2543[a]	18893 ± 1670[a]	12530 ± 2686[b]	16036 ± 855[ab]
C 22:4n-6	385 ± 76[a]	350 ± 17[a]	671 ± 116[b]	363 ± 56[a]
C 22:5n-6	235 ± 56[a]	280 ± 125[a]	2020 ± 550[b]	845 ± 225[c]
N-6 PUFA	36172 ± 11246	34949 ± 4936	25149 ± 5284	31085 ± 2049
C 18:3n-3	641 ± 199[a]	415 ± 205[ab]	279 ± 87[b]	213 ± 42[b]
C 20:5n-3	355 ± 111	347 ± 171	161 ± 67	291 ± 65
C 22:5n-3	734 ± 104[a]	734 ± 58[a]	881 ± 210[ab]	1114 ± 153[b]
C 22:6n-3	5650 ± 739[a]	7140 ± 955[ab]	8591 ± 1570[b]	5777 ± 484[a]
N-3 PUFA	7400 ± 1118[a]	8656 ± 993[ab]	9931 ± 1826[b]	7412 ± 639[a]
PUFA	43572 ± 12106	43605 ± 5389	35080 ± 6864	38496 ± 2508
HUFA	23823 ± 8836	28008 ± 2415	25031 ± 4961	25006 ± 1581
N-6/N-3	4.8 ± 1.2[a]	4.1 ± 0.6[a]	2.5 ± 0.3[b]	4.2 ± 0.3[a]
HUFA Score	25.2 ± 0.3[a]	29.4 ± 2.1[b]	38.7 ± 2.5[c]	28.8 ± 1.1[b]
Total	95804 ± 15991[a]	92385 ± 9553[ab]	72337 ± 14456[b]	82141 ± 5870[ab]

Values are mean ± SD. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA. Values with a different letter are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA.

CHAPTER 7

HEPATIC Δ6-DESAURASE EXPRESSION AND DHA ARE INCREASED BY SUPPLEMENTATION OF OVARIECTOMIZED RATS WITH 17β-ESTRADIOL BUT NOT PROGESTERONE[1]

INTRODUCTION

Observations in transsexuals, postmenopausal women receiving hormone replacement therapy, and women taking oral contraceptive pills indicate a positive association between 17β-estradiol and blood DHA levels [reviewed in (Kitson et al. 2010)] suggesting hormonal regulation of the expression of DHA biosynthesis enzymes. In addition to 17β-estradiol, the natural changes in progesterone, such as a 6-10 fold increase during pregnancy in rats (Childs et al. 2012) and humans (O'Leary et al. 1991), and a 2-fold decrease that occurs during menopause (Burger et al. 2002; Eldrup et al. 1987; National Institutes of Health 2011), are also concurrent with changes in blood DHA levels (Stark et al. 2005a; Tworek et al. 2000). The higher DHA levels in females relative to males, and in pregnant relative to virgin rats (Childs et al. 2012) or post-partum women (Stark et al. 2005a) could also be related to circulating17β-estradiol and progesterone. Previous work examining the effect of ovarian hormone supplementation in ovariectomized rats is limited. Characterization of enzyme expression was done at the mRNA level only and did not include elovl enzymes, SREBP1c, or ERα, rats were supplemented with only 17β-estradiol, and fatty acids levels were determined qualitatively (relative fatty acid percentages), but not quantitatively (concentrations in tissues) in only hepatic phospholipids

[1]The contents of this chapter have been previously published and copyright has been assigned to Elsevier.
Kitson, A.P., Marks, K.A., Shaw, B., et al. (2013). Treatment of ovariectomized rats with 17β-estradiol increases hepatic delta-6 desaturase enzyme expression and docosahexaenoic acid levels in hepatic and plasma phospholipids. Prostaglandins, Leukotriene and essential Fatty Acids, 89(2-3), 81-88.

(Alessandri et al. 2011). The mRNA expression of DHA biosynthesis enzymes in neuroblastoma cells treated with 17β-estradiol and progesterone has been examined (Extier et al. 2009), however the results of that study are limited because neuronal cells produce very little DHA [reviewed in (Rapoport et al. 2009)] and do not significantly affect circulating DHA levels. Therefore, the purpose of the present study was to examine the effects of 17β-estradiol and progesterone supplementation on hepatic expression of DHA biosynthesis enzymes at the mRNA and protein level, and hepatic and plasma DHA levels in ovariectomized rats in contrast with sham-operated controls

METHODS

Sprague-Dawley rats were ovariectomized or sham-operated at 8 weeks of age by Harlan technicians. Animals were shipped to and housed in the Animal housing facility in the Department of Kinesiology, and ovariectomized rats were implanted with 21-day constant-release hormone pellets at 10 weeks of age (Innovative Research of America, Sarasota, FL). Pellets were placed in the nape of the neck following isoflurane anaesthesia and provided 0.5 mg 17β-estradiol (E-121 pellet, OVX+E, n = 6), 25 mg progesterone (P-131 pellet, OVX+P, n = 6), 0.5 mg 17β-estradiol plus 15 mg progesterone (HH-115 pellet, OVX+PE, n = 7) or neither (OVX, n = 6). Animals were weighed at baseline and every 7 days during treatment. After 14 days of supplementation, rats were anaesthetized after overnight fast by intraperitoneal sodium pentobarbital injection (65 mg/kg) and sacrificed by removal of the heart following cardiocentesis with a syringe containing EDTA. Liver was collected and quickly washed in saline (0.9% w/v), weighed, and snap-frozen in liquid nitrogen. Liver was pulverized using a mortar and pestle under liquid nitrogen. Plasma was isolated from collected blood by centrifugation at 1500g. All samples were stored at -80°C until analysis.

Antibodies used for protein expression analysis were Δ6-desaturase (1:1000 in 5% milk-TBST; from Abcam), Δ5-desaturase (1:100 in 5% BSA-TBST; from Santa Cruz Biotechnology), elovl2 (1:250 in 5% BSA-TBST; Santa Cruz Biotechnology), elovl5 (1:250 in 5% BSA-TBST; Santa Cruz Biotechnology), MFP-2 (1:200 in 5% milk-TBST; Santa Cruz Biotechnology), PPARα, (1:250 in 5% milk-TBST, Santa Cruz Biotechnology), SREBP1c, (1:500 in 5% milk-TBST, Santa Cruz Biotechnology) and ERα (1:1000 in 5% milk-TBST, abcam). The expression levels of proteins of interest were then quantified relative to sham-operated rats.

Δ6-desaturase mRNA was determined using primers 5'-3' TCAAAACCAACCACCTGTTCTTC, 3'-5' ACCAGGCGATGCTTTCCA on a BioRad CFX Real-Time PCR detection system (Biorad) with PerfeCta SYBR Green FastMix (Quanta Biosciences, Gaithersberg, Maryland), with 18s as housekeeping gene (5'-3' GATCCATTGGAGGGCAAGTCT, 3'-5' AACTGCAGCAACTTTAATATACGCTATT).

Plasma concentrations of 17β-estradiol and progesterone were determined by enzyme-linked immunosorbent assay following manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Briefly, hormones were extracted from plasma using methylene chloride to reduce background noise, and were analyzed in duplicate on 96-well plates and quantified relative to a standard curve.

A high-density microarray was performed on liver RNA samples from sham-operated controls and ovariectomized (unsupplemented) rats (n = 4 each). RNA was extracted as described in **Chapter 4**, except that the integrity of the RNA extract was examined with an Agilent BioAnalyzer (Agilent, Mississauga, ON), and sense cDNA was synthesized from RNA using the Ambion WT expression kit (Life Sciences, Burlington, Ontario). cDNA was fragmented and end-labeled with biotin using GeneChip WT Terminal Labeling and Controls Kit

(Life Sciences), and cDNA was hybridized to the Affymetrix Rat Gene 1.1 ST Array Strip, which comprises > 27,000 unique 25-mer rat sequences and transcripts, and strips were washed, stained and imaged on an Affymetrix Gene Atlas platform (Affymetrix, Fremont, CA). Microarray data was corrected for background noise, quantile normalized using Robust Multi-array Averaging, and summarized by median polish (JMP Genomics Version 5,SAS, Cary, NC, USA). A false discovery rate of 5% (corresponding to a p-value of 0.00027) was used to account for the high type-1 error rate associated with multiple testing. If genes of interest were not significantly affected by treatment at FDR < 0.05, they were interpreted with $p < 0.05$ suggesting a significant effect of treatment.

Fatty acid concentrations of hepatic total lipids and PL and TAG and PC, PE, PS, and PI were determined following TLC separation (Chen et al. 2011) of isolated lipid extracts (Folch et al (1957). Quantitation of PI and PS fatty acid composition was done by adding 22:3n-3 internal standard after TLC. Neutral lipid TLC separation of plasma lipid extracts (Christie 2003) was performed in order to quantitate the fatty acid compositions of PL, TAG, NEFA, and CE fractions.

RESULTS

Enzyme and transcription factor expression

Δ6-desaturase was 31% higher in OVX relative to SHAM rats, and was further increased by 40% and 43% in OVX rats with 17β-estradiol and 17β-estradiol+progesterone treatment, respectively (**Figure 7.1**). Progesterone treatment alone had no effect on Δ6-desaturase expression, which remained higher than SHAM rats and similar to untreated OVX rats. Significantly lower enzyme content of elovl5 was observed in OVX+P rats relative to SHAM, and the enzyme content of MFP-2 was lower in OVX+E compared with OVX+PE rats. No

significant effects of treatments were observed in the enzyme expression of Δ-5 desaturase or elovl2. The mRNA for Δ6-desaturase was higher in OVX+PE relative to SHAM and OVX+P, while OVX+E and OVX were intermediate (**Figure 7.2**). Ovariectomy and hormone treatment did not affect protein expression of PPARα, ERα, and endoplasmic reticulum and nuclear SREBP1c (**Figure 7.3**).

Transcriptome following ovariectomy

No genes of interest were significantly affected by ovariectomy by FDR < 0.05. *A priori* analysis of genes of interest revealed that Δ6-desaturase mRNA was 12% higher in OVX vs. SHAM animals ($p = 0.056$). No significant differences in gene expression of D5D, elovl2, elovl5, or MFP-2 were observed between SHAM and OVX animals (**Table 7.1**).

Polyunsaturated fatty acid concentrations

In liver phospholipids, the concentrations of DHA, DPAn-3, and EPA were significantly higher in OVX+E and OVX+PE compared with OVX, and OVX+P (**Table 7.2**). The relative percentages of these n-3 HUFA were also higher in OVX+E and OVX+PE (**Appendix 2 table 2**), despite OVX+PE having slightly higher total phospholipid fatty acids compared to OVX (Fig. 2). In SHAM animals, the concentration of EPA and DHA was lower than OVX+PE but not OVX+E, while the concentration of DPAn-3 was lower than both groups receiving 17β-estradiol supplementation. Ovariectomy did not result in significant changes in the concentration of any n-3 HUFA in hepatic phospholipids relative to SHAM. With regard to n-6 HUFA in liver phospholipids, concentrations of DPAn-6 were significantly increased in OVX+E and OVX+PE relative to OVX OVX+P and SHAM (**Table 7.2**). No significant effects of hormonal treatment on hepatic phospholipid 20:4n-6 were observed among OVX, OVX+E, OVX+P, and OVX+PE rats, however SHAM rats had significantly higher 20:4n-6 relative to OVX+E. The percentage

of total HUFA as n-3 HUFA in hepatic phospholipids was significantly higher in OVX+E and OVX+PE relative to all other groups (**Table 7.2**). The higher levels of DHA in hepatic phospholipids of the OVX+E and OVX+PE were reflected across the major phospholipid classes, although there were no significant differences across groups in PE DHA (**Figure 7.4, full fatty acid composition in Appendix 2, Tables 2-5**). The increased DHA was most pronounced in PC and was significantly higher in OVX+E and OVX+PE as compared with all other groups. In PS and PI, DHA concentration was the highest in OVX+E and OVX+PE but there were statistical similarities with some of the other groups, particularly with the OVX+PE group. The OVX rats had significantly higher concentrations of fatty acids in liver TAG that was relatively consistent across subclasses of fatty acids (**Table 7.3**).

In plasma phospholipids, concentrations of DHA and DPAn-3 were higher in OVX+E and OVX+PE relative to OVX, OVX+P, and SHAM, while no treatment effect was observed in EPA (**Table 7.4**). In n-6 HUFA, concentrations of 20:4n-6 and DPAn-6 were significantly increased in OVX+E and OVX+PE relative to OVX, OVX+P, and SHAM (**Table 7.4**). Despite increases in both n-6 and n-3 HUFA, the percentage of n-3 HUFA in total HUFA was significantly higher in OVX+E and OVX+PE compared with SHAM, OVX, and OVX+P (**Table 7.4**).

In plasma non-esterified fatty acids, no effect of treatment was observed in n-3 or n-6 HUFA concentrations, however ALA was higher in OVX compared to SHAM, OVX+E, and OVX+P with no difference compared to OVX+PE (**Appendix 2 Table 6**). No effect of treatment on plasma TAG was observed (**Appendix 2 Table 7**). No effect of treatment on HUFA content of plasma cholesteryl esters was observed, although total cholesteryl ester fatty acid concentration was higher in OVX+P compared with SHAM due to higher concentrations of

saturated and monounsaturated fatty acids (**Appendix 2 Table 8**). In adipose tissue TLE, EPA was higher in OVX+P compared with SHAM, and DPAn-3 was higher in OVX+E relative to SHAM (**Appendix 2 Table 9**).

Body weight and hormone concentration

No differences in body weight on day of pellet implantation were observed, however significant differences in body mass occurred over the course of the study (**Figure 7.5**). Body mass of sham rats increased approximately 5%, and no difference in body mass gain was seen between SHAM and OVX+P rats. OVX rats gained 13% of their baseline mass over the course of the study, and OVX+E and OVX+PE both lost approximately 7% of their body mass.

Plasma concentrations of 17β-estradiol and progesterone were lower following ovariectomy, but only the decrease in progesterone was found to be statistically significant (**Figure 7.6**). Treatment with progesterone resulted in significantly higher plasma progesterone in OVX+P and OVX+PE rats relative to OVX rats, but not OVX+E rats, and was lower compared to SHAM rats. Estradiol concentrations were much higher in OVX+E and OVX+PE rats compared with both OVX and OVX+P, but only OVX+E rats had 17β-estradiol concentrations significantly higher than SHAM rats.

DISCUSSION

This study demonstrates a significant increase in the expression of Δ6-desaturase in ovariectomized rats when they are treated with 17β-estradiol or a combination of 17β-estradiol and progesterone, but not progesterone alone. This finding suggests that 17β-estradiol increases the capacity for DHA biosynthesis, which appears to result in increased hepatic synthesis-secretion of DHA. Progesterone, at the dose used in the present study, appears to have no effect on DHA metabolism. The higher hepatic Δ6-desaturase expression following treatment with

17β-estradiol likely mediates the increased expression observed in females relative to males and in pregnant rats relative to virgin and post-partum rats and humans [reviewed in (Kitson et al. 2010)].

Previous work examining the effect of supplementing ovariectomized rats with 17β-estradiol on DHA metabolism demonstrated similar increases in DHA in hepatic PL following treatment of rats with 8 or 16 μg of 17β-estradiol /day (Alessandri et al. 2011). However, in the study by Alessandri et al. (2011) the hepatic mRNA for Δ6-desaturase was lower in ovariectomized rats with 17β-estradiol treatment relative to untreated rats, which is contrary to the findings of the present study at both the protein (increased with 17β-estradiol) and mRNA (increased with combination of 17β-estradiol and progesterone) levels, and does not agree with the fatty acid compositional data. The reason for discordant results may be a slightly higher 17β-estradiol dose used in the present study (20μg/day in OVX+E and OVX+PE), shorter duration of the present study (2 weeks vs. 5 weeks), approximately 12-fold higher dietary n-3 PUFA (mainly ALA) content in the present study, and older rats at ovariectomy and sacrifice. It is also possible that 17β-estradiol increases the mRNA stability of Δ6-desaturase, requiring less of the gene to be transcribed to elicit the desired biological effect. This would explain the lack of agreement between the mRNA data and fatty acid data in Alessandri et al (2011), and mRNA and protein data in the present study. The effects of 17β-estradiol on the stability of Δ6-desaturase mRNA should be assessed in the future.

The results of this study suggest that 17β-estradiol is increasing the synthesis of DHA; however it is only one of several likely factors mediating the increased hepatic and plasma phospholipid DHA levels. For instance, the different relative effect of the treatment on DHA concentration in the individual phospholipid classes suggests a factor influencing DHA acylation

to these phospholipids is also modulated by 17β-estradiol. This might include enzymes involved in phospholipid synthesis or remodelling, as a selective increase in the expression or activity in lysophospholipid acyltransferases or phospholipases can increase the relative accretion of one fatty acid over another. For example, there is some evidence that 17β-estradiol increases the activity of cytosolic phospholipase A2 in MCF-7 cells (Thomas et al. 2006), which may decrease the half-life of 20:4n-6 relative to DHA and may therefore increase the accretion of DHA at the expense of 20:4n-6. Research on the effects of hormones on phospholipid remodelling enzymes is required.

The significantly increased expression of Δ6-desaturase in OVX as compared with SHAM rats has been observed previously (Alessandri et al. 2011), and did not affect phospholipid DHA concentrations. The lack of effect of higher Δ6-desaturase on DHA levels is likely related to the significant accumulation of hepatic TAG in these animals, as it has been shown that hepatic TAG accumulation in hereditary hypertriglyceridemic rats results in significant reduction in Δ6-desaturase activity, with no changes in expression level (Demcakova et al. 2001). Also, non-alcoholic steatohepatitis resulting from high-fat diet feeding increases the mRNA concentrations of Δ6- and Δ5-desaturase but decreases indexes of desaturase activity (Lopez-Vicario et al. 2013), suggesting the TAG accumulation in the present study may have a similar effect on Δ6-desaturase expression. The reason that the increase in Δ6-desaturase expression in untreated OVX rats did not result in increased phospholipid concentrations of DHA, as was the case with 17β-estradiol treatment is unclear, but may involve substrate inhibition of Δ6-desaturase activity resulting from increased hepatic ALA and LA. This type of inhibition has been observed in liver homogenates and isolated microsomes (Blond et al. 1984; Garg et al. 1988).

The mechanism underlying the effect of ovariectomy and 17β-estradiol on hepatic Δ6-desaturase expression and DHA concentration does not appear to involve changes in the expression of PPARα, ERα, or SREBP1c in the present study. This is contrary to previous work demonstrating decreased (Paquette et al. 2008) or increased (Alessandri et al. 2011) mRNA of PPARα and increased mRNA of SREBP1c (Domingos et al. 2012; Paquette et al. 2008) in ovariectomy, which is prevented by supplementation with 17β-estradiol (Alessandri et al. 2011; Paquette et al. 2008). The lack of an effect of treatment on these transcription factors in the present study may be due to the 2 week duration of the present study compared with previous studies of 5 weeks (Alessandri et al. 2011), 8 weeks (Paquette et al. 2008), and 10 weeks (Domingos et al. 2012). Other studies examining hepatic ERα expression show either no effect on mRNA expression following 5 weeks (Alessandri et al. 2011) or an increased protein expression following 12 weeks of ovariectomy (Hao et al. 2010), with no previous findings of an effect of 17β-estradiol supplementation on hepatic ERα expression in ovariectomized rats (Alessandri et al. 2011). The reason for disparate results of hepatic ERα expression may involve differences in the age of the rats at surgery and sacrifice, and the length of time of exposure of rats to ovariectomy. In the present study, increases in Δ6-desaturase do not appear to depend on changes in transcription factor expression, however the time-course of transcription factor response to ovariectomy and 17β-estradiol supplementation should be assessed in the future.

Ovariectomy did not significantly lower 17β-estradiol concentrations compared with SHAM rats in the present study. Increased weight gain and adipose tissue accumulation in ovariectomized rats may have resulted in increased extragonadal 17β-estradiol synthesis, as aromatase activity is present in adipose tissue (Miller 1991). Strategies to prevent the accumulation of adipose tissue with ovariectomy, such as food restriction or exercise, may result

in a greater reduction in circulating 17β-estradiol than observed here, and may provide a better model for studying its effects on DHA metabolism.

The findings of this study suggest a potential mechanism for the increased circulating DHA and DPAn-6 observed in pregnancy relative to pre-pregnancy or post-partum, as concentrations of plasma 17β-estradiol are increased during pregnancy (O'Leary et al. 1991). The results of the present study do not support a role of progesterone in increasing DHA concentrations in pregnancy; however, progesterone treatment did not restore progesterone concentrations to control levels. Future studies that provide greater concentrations of progesterone are required to form conclusions about the role of this hormone in DHA metabolism. Interestingly, hepatic Δ6-desaturase mRNA has been shown to be positively correlated with plasma progesterone in pregnancy (Childs et al. 2012), indicating a potential role of progesterone in DHA synthesis in pregnancy that was not detectable in the present study.

CONCLUSION

17β-estradiol treatment of ovariectomized rats increased hepatic Δ6-desaturase expression and synthesis-mobilization of DHA. This mechanism may explain the higher Δ6-desaturase expression and hepatic DHA concentration observed in females as compared with males and in pregnant relative to virgin and post-partum rats. The mechanism underlying this effect of 17β-estradiol is not clear, but does not appear to involve altered hepatic expression of PPARα, SREBP1c, or ERα.

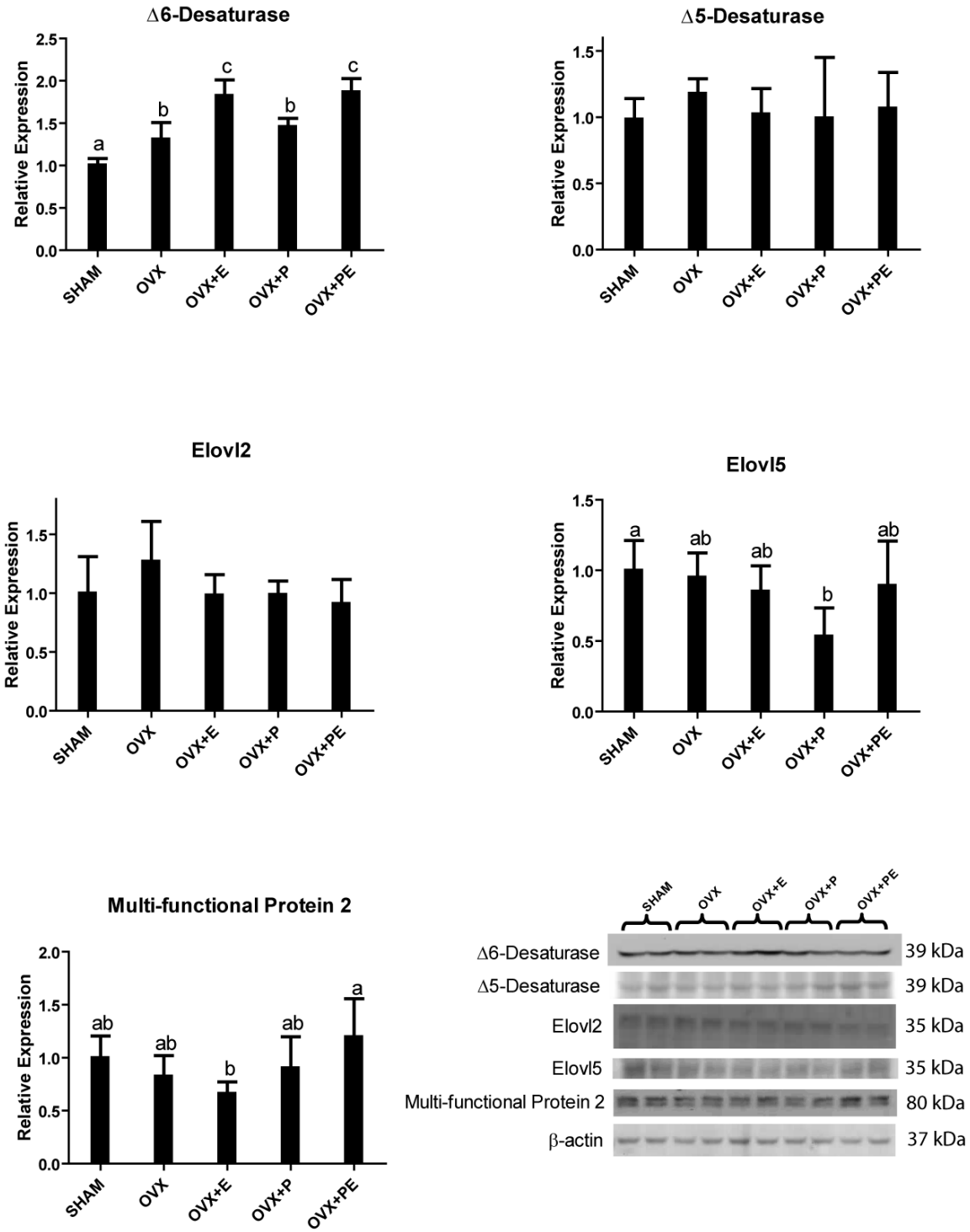

Figure 7.1. Effects of ovariectomy and hormone supplementation on enzyme expression of docosahexaenoic acid biosynthesis enzymes. SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Bars with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA.

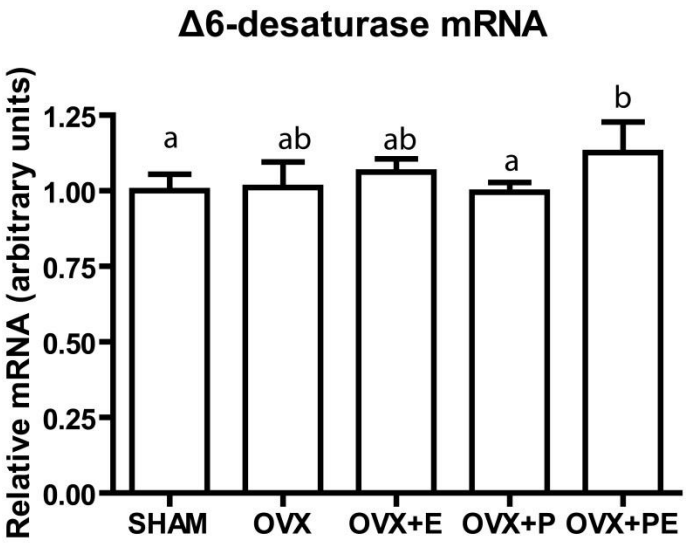

Figure 7.2: Effects of ovariectomy and hormone supplementation on mRNA expression of Δ6-desaturase. SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Bars with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA.

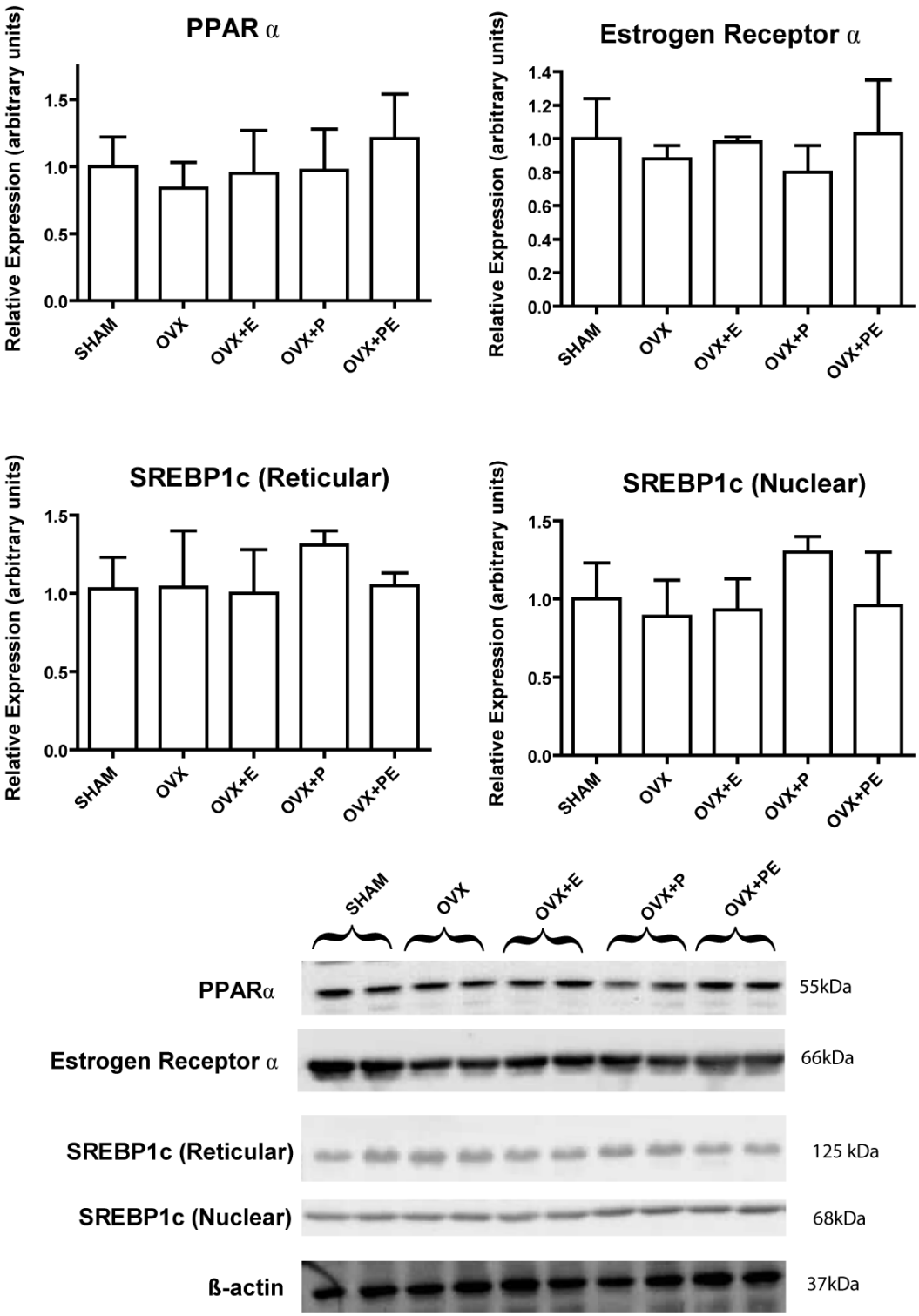

Figure 7.3: Effects of ovariectomy and hormone supplementation on expression of transcription factors. SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Bars with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA.

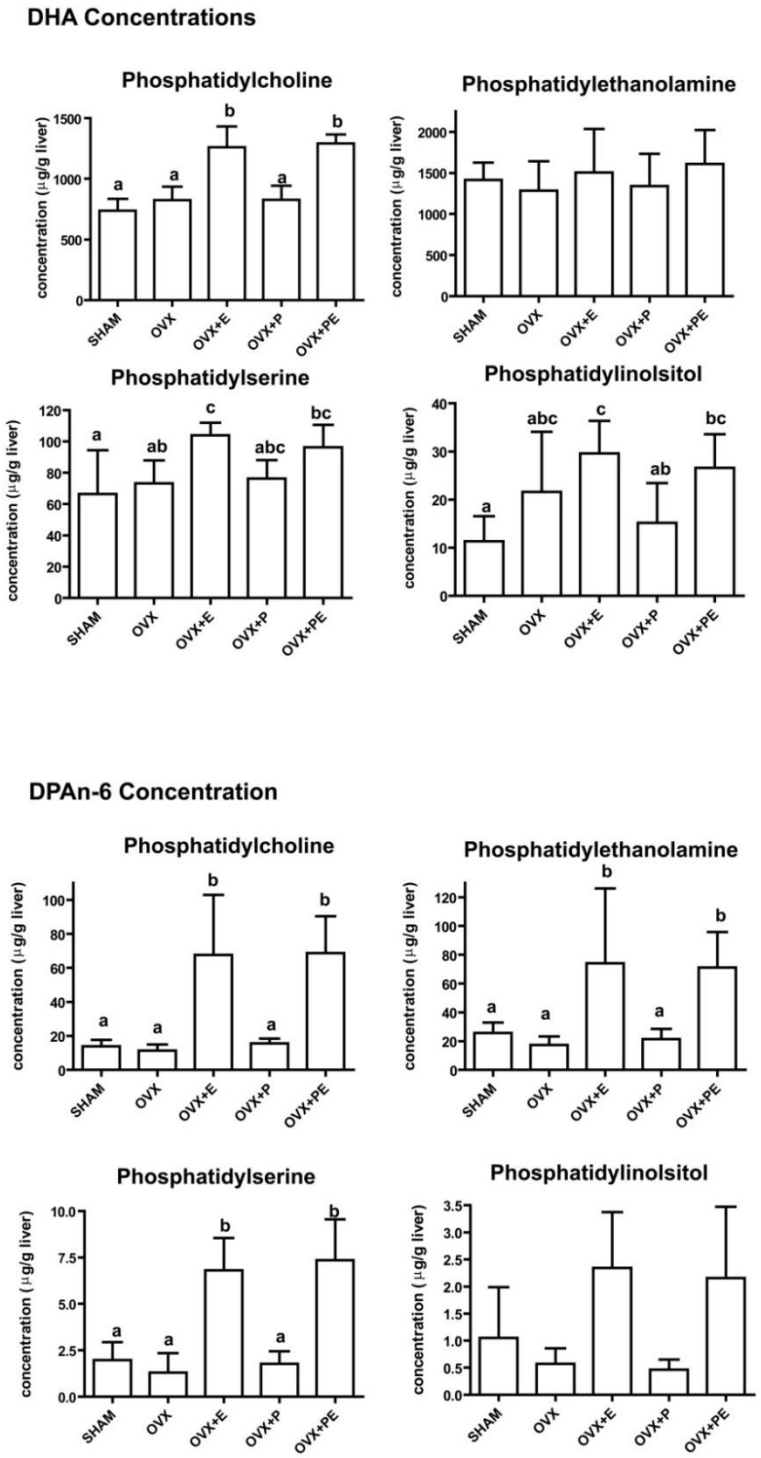

Figure 7.4: Effects of ovariectomy and hormone supplementation on docosahexaenoic acid and n-6 docosapentaenoic acid concentration of hepatic phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Bars with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA.

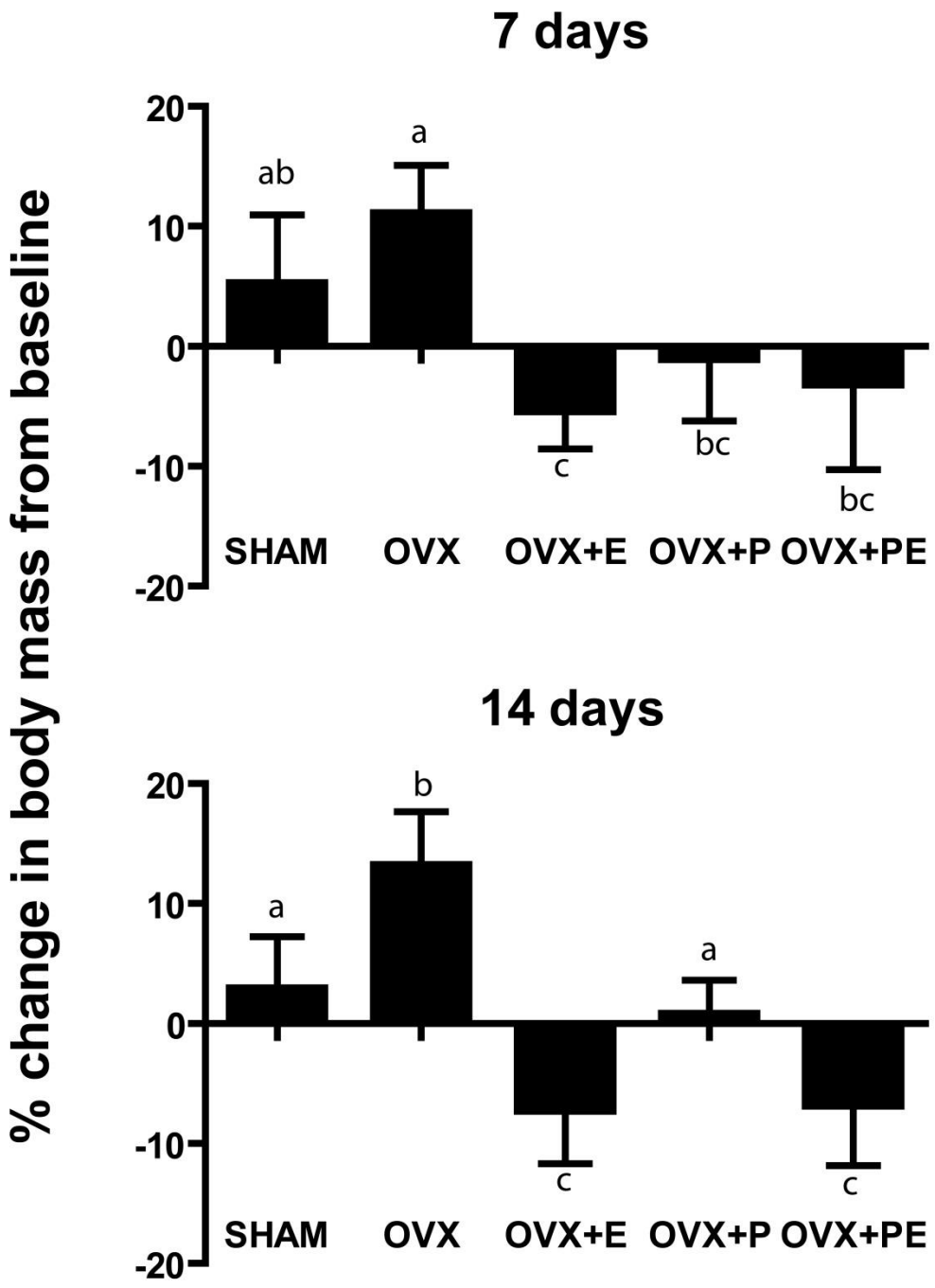

Figure 7.5: Effects of ovariectomy and hormone supplementation on changes in body weight observed at 7- and 14-days. SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Bars with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA.

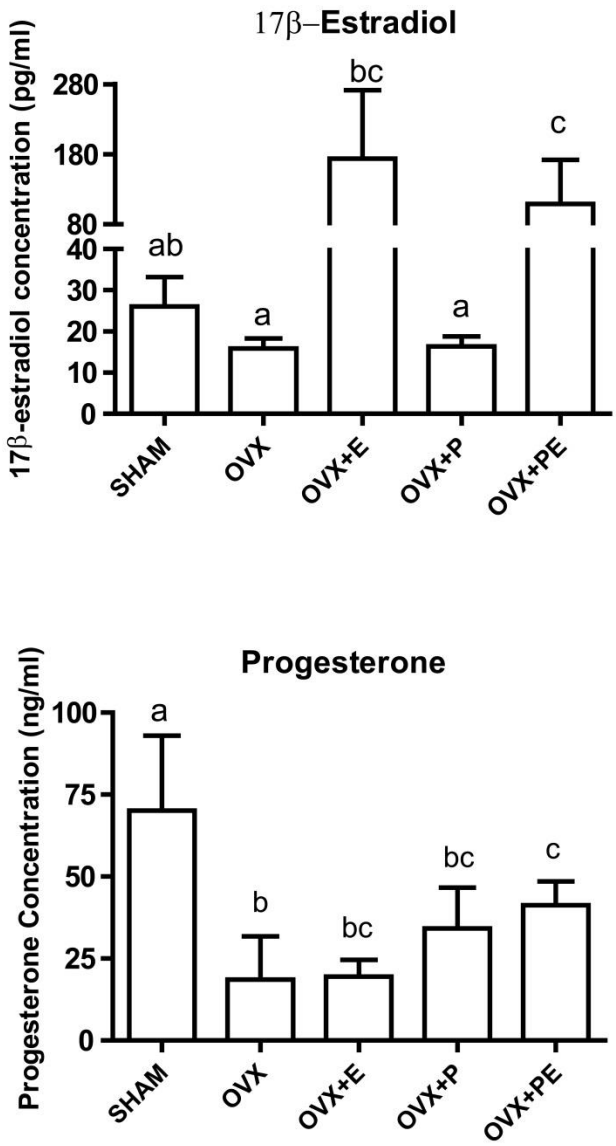

Figure 7.6. Effects of ovariectomy and hormone supplementation on plasma 17β-estradiol and progesterone concentrations. SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Bars with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA.

Table 7.1: Changes in genes involved in DHA production following ovariectomy as determined by high-density microarray. (n = 4 for OVX and SHAM)

Gene Symbol	Enzyme Name	Fold Change (OVX / SHAM)	p-Value
Fads1	Delta-5 desaturase	0.98	0.7754
Fads2	Delta-6 desaturase	1.12	0.0560
Elovl2	Elongase 2	1.06	0.5669
Elovl5	Elongase 5	1.05	0.4467

Table 7.2: Effects of ovariectomy and hormone supplementation on fatty acid concentration of hepatic phospholipids

	SHAM	OVX	OVX+E	OVX+P	OVX+PE
Fatty Acid			*µg/g liver*		
SFA[1]	4845 ± 141[a]	4168 ± 380[bc]	4143 ± 442[b]	4430 ± 464[ab]	4843 ± 355[a]
MUFA[1]	590 ± 164	519 ± 188	594 ± 76	555 ± 166	582 ± 52
C 18:2n-6	1236 ± 64[a]	1081 ± 83[ab]	941 ± 112[b]	1168 ± 120[a]	954 ± 112[b]
C 18:3n-6	38 ± 3	33 ± 3	33 ± 2	31 ± 9	32 ± 6
C 20:2n-6	22 ± 2[ab]	20 ± 2[ab]	24 ± 3[a]	18 ± 3[b]	22 ± 4[ab]
C 20:3n-6	34 ± 4[ab]	30 ± 2[a]	28 ± 7[a]	30 ± 5[a]	39 ± 4[b]
C 20:4n-6	2574 ± 111[a]	2234 ± 223[ab]	2151 ± 275[b]	2299 ± 210[ab]	2486 ± 214[ab]
C 22:4n-6	25 ± 4	20 ± 3	23 ± 4	21 ± 5	24 ± 4
C 22:5n-6	20 ± 5[a]	14 ± 5[a]	50 ± 25[b]	16 ± 4[a]	59 ± 19[b]
N-6 PUFA	3949 ± 154[a]	3432 ± 290[b]	3250 ± 354[b]	3584 ± 294[ab]	3617 ± 266[ab]
C 18:3n-3	12 ± 4[ab]	8 ± 3[a]	12 ± 2[ab]	13 ± 3[ab]	14 ± 3[b]
C 20:5n-3	19 ± 5[ab]	13 ± 3[a]	22 ± 5[bc]	18 ± 8[ab]	29 ± 3[c]
C 22:5n-3	83 ± 13[a]	69 ± 14[a]	123 ± 29[b]	79 ± 15[a]	128 ± 31[b]
C 22:6n-3	907 ± 96[ab]	740 ± 116[a]	1013 ± 149[bc]	749 ± 91[a]	1206 ± 132[c]
N-3 PUFA	1021 ± 105[ab]	830 ± 126[a]	1169 ± 153[bc]	857 ± 109[a]	1376 ± 136[c]
PUFA	4969 ± 249[a]	4262 ± 409[b]	4419 ± 448[ab]	4441 ± 395[ab]	4993 ± 377[a]
HUFA	3662 ± 206[ab]	3120 ± 354[a]	3410 ± 398[a]	3211 ± 319[a]	3970 ± 338[b]
EPA+DHA	925 ± 93[ab]	753 ± 116[a]	1035 ± 149[bc]	766 ± 95[a]	1235 ± 133[c]
n-6/n-3	3.9 ± 0.3[a]	4.2 ± 0.3[a]	2.8 ± 0.3[b]	4.2 ± 0.3[a]	2.6 ± 0.2[b]
HUFA SCORE	27 ± 1[a]	26 ± 1[a]	34 ± 2[b]	26 ± 1[a]	34 ± 2[b]
Total	10633 ± 374[a]	9102 ± 899[b]	9303 ± 910[ab]	9611 ± 981[ab]	10566 ± 730[a]

Values are mean ± SD. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA. SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Values with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA. [1]Concentrations of individual SFA and MUFA can be found in (Marks et al. 2013a).

Table 7.3: Effects of ovariectomy and hormone supplementation on fatty acid concentration of hepatic triacylglycerol

	SHAM	OVX	OVX+E	OVX+P	OVX+PE
			µg/g liver		
SFA[1]	2745 ± 846 [a]	4360 ± 533 [b]	2067 ± 315 [a]	2525 ± 926 [a]	2083 ± 298 [a]
MUFA[1]	2265 ± 981 [ab]	3335 ± 700 [a]	1395 ± 521 [b]	1715 ± 831 [bc]	1146 ± 244 [b]
C 18:2n-6	3349 ± 1372 [a]	6691 ± 1144 [b]	1844 ± 946 [ac]	3139 ± 1394 [ac]	1455 ± 412 [c]
C 18:3n-6	109 ± 38 [a]	220 ± 44 [b]	52 ± 39 [ac]	111 ± 53 [a]	39 ± 15 [c]
C 20:2n-6	18 ± 9 [a]	52 ± 14 [b]	14 ± 9 [a]	20 ± 11 [a]	14 ± 6 [a]
C 20:3n-6	27 ± 11 [a]	58 ± 15 [b]	21 ± 10 [a]	27 ± 13 [a]	17 ± 4 [a]
C 20:4n-6	721 ± 237	1342 ± 276	427 ± 185	681 ± 317	427 ± 98
C 22:4n-6	4.3 ± 4.1	3.2 ± 2.4	9.2 ± 9.5	3.7 ± 3.2	2.8 ± 1.9
C 22:5n-6	24 ± 10 [ab]	41 ± 14 [a]	24 ± 11 [ab]	20 ± 9 [b]	17 ± 5 [b]
N-6 PUFA	4258 ± 1659 [a]	8413 ± 1460 [b]	2396 ± 1171 [ac]	4004 ± 1780 [ac]	1977 ± 487 [c]
C 18:3n-3	198 ± 87 [a]	405 ± 90 [b]	104 ± 49 [a]	197 ± 98 [a]	89 ± 26 [a]
C 20:5n-3	114 ± 49 [a]	210 ± 30 [b]	62 ± 28 [a]	111 ± 48 [a]	64 ± 23 [a]
C 22:5n-3	64 ± 24 [a]	143 ± 31 [b]	54 ± 18 [a]	69 ± 34 [a]	55 ± 17 [a]
C 22:6n-3	176 ± 52 [a]	409 ± 78 [b]	110 ± 79 [a]	195 ± 80 [a]	103 ± 21 [a]
N-3 PUFA	559 ± 208 [a]	1174 ± 210 [b]	335 ± 168 [a]	578 ± 257 [a]	317 ± 70 [a]
PUFA	4818 ± 1862 [a]	9588 ± 1663 [b]	2731 ± 1336 [a]	4582 ± 2037 [a]	2294 ± 550 [a]
HUFA	1138 ± 369 [a]	2213 ± 421 [b]	711 ± 314 [a]	1112 ± 495 [a]	692 ± 151 [a]
EPA+DHA	289 ± 101 [a]	619 ± 106 [b]	172 ± 106 [a]	306 ± 126 [a]	167 ± 34 [a]
N-6/N-3	7.6 ± 0.7 [a]	7.2 ± 0.4 [ab]	7.2 ± 0.8 [ab]	7.0 ± 0.3 [ab]	6.3 ± 0.7 [b]
HUFA Score	31.6 ± 1.9	34.9 ± 1.7	31.9 ± 3.0	34.3 ± 2.2	32.9 ± 1.6
Total	9828 ± 3597 [a]	17282 ± 2860 [b]	6192 ± 1953 [a]	8823 ± 3738 [a]	5523 ± 1012 [a]

Values are mean ± SD. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA, SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Values with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA. [1]Concentration of individual SFA and MUFA can be found in (Marks 2012).

Table 7.4: Effects of ovariectomy and hormone supplementation on fatty acid concentration of plasma phospholipids

	SHAM	OVX	OVX + E	OVX+ P	OVX+PE
			µg/ml plasma		
SFA[1]	497 ± 39 [a]	505 ± 140 [ab]	638 ± 78 [b]	541 ± 31 [ab]	660 ± 60 [b]
MUFA[1]	353 ± 100	357 ± 199	379 ± 89	391 ± 73	353 ± 103
C 18:2n-6	96 ± 12 [a]	101 ± 20 [a]	140 ± 31 [b]	115 ± 10 [ab]	112 ± 9 [ab]
C 18:3n-6	3.2 ± 0.4 [ab]	2.5 ± 0.6 [a]	4.0 ± 0.6 [b]	3.3 ± 0.6 [ab]	3.9 ± 0.7 [b]
C 20:2n-6	1.5 ± 0.3 [a]	1.5 ± 0.6 [ab]	3.0 ± 0.4 [c]	1.7 ± 0.3 [ab]	2.8 ± 1.1 [bc]
C 20:3n-6	2.6 ± 0.3 [a]	2.0 ± 0.7 [a]	4.5 ± 0.8 [b]	2.1 ± 0.5 [a]	4.4 ± 0.7 [b]
C 20:4n-6	183 ± 4 [a]	165 ± 37 [a]	271 ± 40 b	202 ± 29 [a]	284 ± 46 [b]
C 22:2n-6	0.9 ± 0.9	0.7 ± 0.5	0.4 ± 0.3	0.7 ± 0.5	0.8 ± 0.6
C 22:4n-6	3.6 ± 0.9	3.4 ± 1 .0	2.8 ± 0.8	4.4 ± 1.4	3.8 ± 0.3
C 22:5n-6	1.2 ± 0.6 [a]	0.6 ± 0.4 [a]	4.6 ± 2.6 [b]	0.8 ± 0.8 [a]	4.5 ± 1.2 [b]
N-6 PUFA	292 ± 14 [a]	277 ± 56 [a]	430 ± 55 [b]	329 ± 32 [a]	417 ± 49 [b]
C 18:3n-3	2 ± 1	2 ± 1	2 ± 1	4 ± 5	2 ± 1
C 20:5n-3	2 ± 1	2 ± 2	3 ± 1	2 ± 1	3 ± 1
C 22:5n-3	3.7 ± 0.1 [a]	4.4 ± 1.3 [a]	10.5 ± 2.9 [b]	4.9 ± 0.6 [a]	10.5 ± 2.9 [b]
C 22:6n-3	36.3 ± 0.5 [a]	33.0 ± 8.1 [a]	74.4 ± 15.4 [b]	36.8 ± 4.4 [a]	75.3 ± 15.6 [b]
N-3 PUFA	45 ± 1 [a]	43 ± 10 [a]	91 ± 16 [b]	48 ± 9 [a]	92 ± 16 [b]
PUFA	337 ± 14 [a]	319 ± 65 [a]	521 ± 70 [b]	377 ± 38 [a]	508 ± 64 [b]
HUFA	233 ± 4 [a]	212 ± 47 [a]	372 ± 58 [b]	253 ± 35 [a]	387 ± 63 [b]
EPA+DHA	39 ± 1 [a]	35 ± 9 [a]	78 ± 16 [b]	39 ± 5 [a]	79 ± 16 [b]
N-6/N-3	6.5 ± 0.3 [a]	6.5 ± 0.4 [a]	4.8 ± 0.3 [b]	7.0 ± 1.0 [a]	4.6 ± 0.3 [b]
HUFA Score	18.5 ± 0.4 [a]	19.1 ± 1.2 [a]	23.8 ± 1.5 [b]	17.5 ± 1.5 [a]	23.1 ± 0.7 [b]
Total	1186 ± 142	1182 ± 380	1538 ± 184	1309 ± 52	1522 ± 170

Values are mean ± SD. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA, SHAM: sham-operated rats, OVX: ovariectomized rats, OVX+E: OVX supplemented with 17β-estradiol, OVX+P: OVX supplemented with progesterone, OVX+PE: OVX rats supplemented with 17β-estradiol and progesterone. Values with different letter are significantly different by Tukey's post-hoc test following significant F Value by one-way ANOVA. [1]Concentration of individual SFA and MUFA can be found in (Marks et al. 2013a).

CHAPTER 8

OVARIECTOMY WITH OR WITHOUT FOOD RESTRICTION ELICITS NO EFFECT ON HEPATIC AND PLASMA DHA CONCENTRATION OR HEPATIC Δ6-DESATURASE EXPRESSION

INTRODUCTION

Ovariectomy results in increased food intake and hepatic lipid storage (Blaustein et al. 1976; Varma et al. 1999) which can increase the expression of Δ6-desaturase independently of alterations in ovarian hormone status (Demcakova et al. 2001). In addition, the increased storage of LA and ALA in hepatic TAG resulting from ovareictomy-induced hyperphagia can decrease the activity of Δ6-desaturase (Blond et al. 1984; Garg et al. 1988). The increased adipose tissue in ovariectomized rats may also increase 17β-estradiol through extragonadal synthesis. Hyperphagia in ovariectomized rats is therefore a potential confounder when attempting to examine the direct hormonal effects of ovariectomy on Δ6-desaturase expression. Preventing increased adipose depots by limiting the hyperphagia associated with ovariectomy may be a mechanism to achieve low 17β-estradiol levels and control intake stimulated lipogenesis in ovariectomized rats.

Pair-feeding is a method to normalize the intake of food by one group to that of another. In the case of ovariectomized rats, food intake can be normalized to sham-operated controls in an attempt to control for ovariectomy associated hyperphagia. Previous work using food restriction of ovariectomized animals has shown that body weight is comparable to eugonadal rats and is significantly lower compared to ovariectomized rats that have *ad libitum* access to food after 4 weeks (Davidge et al. 2001) and 5 weeks (Shimomura et al. 1989).

The goal of the present study was to investigate the effect of food restriction in ovariectomized rats on hepatic and plasma DHA concentrations and expression of DHA biosynthetic enzymes in rats. Additionally, the effect of pair-feeding on hepatic fatty acid synthase, pyruvate dehydrogenase kinase, (PDHK), carnitine palmitoyl transferase 1 (CPT1), and stearoyl CoA desaturase 1 (SCD1) were assessed to determine hepatic fuel substrate metabolism and muscle CPT1 and PDHK will be measured to estimate fuel substrate oxidation.

METHODS

Animals were ovariectomized or sham-operated by Harlan technicians at 5 weeks of age and arrived at the University of Waterloo at 6 weeks of age. Animals were individually housed in the animal facility in the Department of Kinesiology, and had *ad libitum* access to drinking water. Ovariectomized animals were divided into two groups to receive either *ad libitum* access to standard laboratory chow (OVX+AL) or to be pair-fed (OVX+PF) relative to the sham-operated control group (SHAM) (n = 6 for each group), and feeding protocol lasted for 32 days. Food intake of all animals was measured daily and the OVX+PF animals were provided the mass of food consumed by the SHAM animals the previous day. OVX+PF animals were provided food at the beginning of the dark phase, as it has been shown that this feeding schedule is required to prevent weight gain of pair-fed ovariectomized animals relative to sham-operated controls (Shimomura et al. 1989). Animals were weighed every 4 days.

Whole body metabolism of the rats (n = 4 per group) was determined using a Comprehensive Lab Animal Monitoring System (Oxymax series, Columbus Instruments, Columbus, Ohio, USA). Animals were acclimatized to the monitoring system in two 24-hour sessions. Measurement was performed every 26 minutes over a 24-hour period following a 2-hour acclimation period. Resting metabolic parameters were assessed by monitoring the animals

over a 24 hour period on day 23 of the study. VO_2, VCO_2, body temperature, total cage activity, and energy expenditure for each individual animal was recorded using Oxymax/CLAMS Software (Columbus Instruments) and exported for statistical analysis.

Animals were fasted overnight and sacrificed on day 33 by cardiocentesis with a syringe containing EDTA following sodium pentobarbital injection (65mg/kg). Plasma was isolated by centrifuging whole-blood at 1500g. Liver, adipose posterior hind limb skeletal muscle, and uterus+fallopian tubes were collected, washed in saline (0.9% w/v), weighed, and snap-frozen in liquid nitrogen. Tissues were pulverized using mortar and pestle under liquid nitrogen prior to analysis. All samples were stored at -80°C until analysis.

Antibodies used for protein expression analysis were Δ6-desaturase (Abcam, ab72189, 1:500 v/v skim milk), Δ5-desaturase (Santa Cruz Biotechnology, sc-101953, 1:100 dilution v/v in BSA), elovl2 (Santa Cruz Biotechnology, sc-54874, 1:250 dilution v/v in BSA), elovl5 (Santa Cruz Biotechnology, sc-54888, 1:250 dilution v/v in milk), MFP2 (Santa Cruz Biotechnology sc-135045, 1:200 v/v in milk), PPARα (Santa Cruz Biotechnology, sc-9000, 1:250 dilution v/v in milk), SREBP1c (Santa Cruz Biotechnology, sc-8984, 1:500 v/v in milk), CPT-1α (Cell Signalling Technology, Danvers, Massachusetts, cat no 12252, 1:1000 v/v in BSA, transferred to nitrocellulose membrane), fatty acid synthase (Cell Signalling Technology, 3180, 1:1000 v/v in milk), pyruvate dehydrogenase kinase 1 (Cell Signalling Technology, 3820, 1:1000 v/v in milk), stearoyl-CoA desaturase 1 (Abcam, ab19862, v/v 1:1000 in milk) and ERα (Abcam, ab16460, v/v 1:1000 in milk). Chemiluminescent detection was performed by ECL prime (GE Healthcare).

The fatty acid compositions of plasma PL, TAG, cholesteryl esters and non-esterified fatty acids were determined by gas chromatography (Stark et al. 2005b) after neutral lipid TLC

separation (Christie 2003) following extraction of lipids (Folch et al. 1957). Fatty acid composition of hepatic TAG, total PL, PC, PE, PI, and PS was determined as described in **Chapter 4**. Concentration of PI and PS were determined by addition of 22:3n-3 internal standard after TLC as described in **Chapter 7**.

Measurement of plasma 17β-estradiol was performed by competitive enzyme-linked immunosorbent assay (Cayman Chemical) following extraction of 17β-estradiol from plasma by methylene chloride according to the manufacturer's instructions and as described in **Chapter 7**.

Statistical comparisons between groups were made using one-way ANOVA. Bonferroni post-hoc test was applied to variables in which repeated measurements over time were included in the ANOVA modelling procedure (body weight, food intake). Otherwise, Tukey's post-hoc test was used.

RESULTS

Plasma 17β-estradiol concentration

The plasma concentrations of 17β-estradiol were similar in all three groups, although the uterus+fallopian tube mass of OVX+AL and OVX+PF was lower compared with SHAM (**Figure 8.1**).

Effect of treatment on enzymes and transcription factor expression

Ovariectomy with or without food restriction elicited no effect on the expression of Δ6-desaturase, Δ5-desaturase, elovl2, elovl5, or MFP2 in the liver (**Figure 8.2**). In addition, PPARα, SREBP1c, and ERα protein levels in liver were similar in all three groups (**Figure 8.3**). PDHK1 expression was 20% lower in OVX+AL relative to SHAM, with OVX+PF levels being intermediate in (**Figure 8.4**). Hepatic CPT-1a and fatty acid synthase were similar in all groups, but SCD1 expression in OVX+AL was 63% higher than OVX+PF and 141% higher than

SHAM. In skeletal muscle, CPT-1a was 40% lower in OVX+AL compared with SHAM, while no differences were observed in OVX+PF. No effect on skeletal muscle PDHK1 was observed.

Body mass and resting metabolic parameters

Body weight increased in all animals throughout the study. The body mass of both OVX+AL and OVX+PF was significantly higher than SHAM throughout the study (**Figure 8.5**). OVX+AL and OVX+PF had similar body mass until day 28, after which the mass of OVX+AL was significantly higher. Food intake was approximately 30% lower in SHAM and OVX+PF compared with OVX+AL after the 17th day of the study and remained lower throughout the study (**Figure 8.5**).

The respiratory exchange ratio (RER) was significantly lower in OVX+PF rats compared to OVX+AL, while SHAM was intermediate (**Table 8.1**). The 24-hour average body temperature was higher in OVX+PF compared to SHAM, while OVX+AL was intermediate. No significant differences were observed between groups in VO_2, VCO_2, energy expenditure, total activity, or maximum and minimum body temperature.

Tissue and plasma fatty acid content

No effect of ovariectomy with or without food restriction was observed on the DHA concentration in PL of liver (**Table 8.2**) and plasma (**Table 8.3**). Concentration of 20:3n-6 was higher in OVX+PF relative to SHAM in hepatic total phospholipids, with no difference in plasma PL. DPAn-3 was higher in hepatic PL of both ovariectomized groups relative to SHAM, but this did not translate into differences in plasma PL DPAn-3 levels.

DHA concentrations in hepatic PC, PE, PS, or PI were similar in all groups, but the DHA n-6 equivalent, DPAn-6, was lower in hepatic PC of OVX+AL as compared with SHAM and OVX+AL (**Figure 8.6, full fatty acid composition in Appendix 3, Tables 1-4**). The

concentration of 20:3n-6 PC was also higher in OVX+PF compared with SHAM, while OVX+AL was intermediate (**Appendix 3 Table 1**). In PE, OVX+AL had significantly lower percentage of HUFA as n-3 HUFA and higher n-6/n-3 ratio compared to SHAM and OVX+PF, but no significant differences between individual PUFA (**Appendix 3 Table 2**).

The total fatty acid concentration of hepatic TAG was significantly higher in OVX+AL relative to SHAM and OVX+PF (**Figure 8.7**), due to increases in the concentration of most fatty acids (**Appendix 3 Table 5**). As a result, fatty acid concentrations of liver total lipid extracts were also increased (**Appendix 3 Table 6**). Plasma TAG was significantly increased in both ovariectomized groups relative to SHAM (**Figure 8.7**), with increases in most fatty acids (**Appendix 3 Table 7**). The increase in total fatty acids in plasma and liver were due only to increases in TAG, as no significant effects of treatment were observed in hepatic and plasma PL (**Figure 8.7**). The concentration and relative percentages of fatty acids in plasma CE and NEFA did not differ between groups (**Appendix 3 Tables 8 and 9**, respectively). No differences in the fatty acid composition of adipose or posterior hind limb skeletal muscle were observed (**Appendix 3 Tables 10 and 11**, respectively).

DISCUSSION

This study demonstrates that ovariectomy with or without food restriction does not significantly affect hepatic Δ6-desaturase expression and DHA concentrations in hepatic and plasma PL. An association between withdrawal of ovarian hormones via ovarian removal and DHA synthesis-mobilization is therefore not supported. Additionally, similar expression of Δ6-desturase and DHA concentrations in OVX+AL and OVX+PF rats, despite significantly lower hepatic TAG in OVX+PF, indicates that hyperphagia-induced hepatic TAG accumulation does not affect DHA biosynthetic capacity in rats after 33 days of ovariectomy.

The absence of an effect of ovariectomy with *ad libitum* access to food on Δ6-desaturase expression is contrary to previous research. Hepatic Δ6-desaturase mRNA is increased at 5 weeks after ovariectomy (Alessandri et al. (2011), but this may be due to differences in mRNA and protein expression as discussed previously in **Chapter 7**. We observed increased hepatic Δ6-desaturase protein, but not mRNA, following 4 weeks of ovariectomy (as presented in **Chapter 7**). The lack of effect of ovariectomy on hepatic Δ6-desaturase in our latter study may be due to difference in the age of rats at ovariectomy, as ovariectomy was performed at 8 weeks of age in the study in **Chapter 7** while ovariectomy occurred at 6 weeks of age in the present study which may have been slightly before full sexual maturity was reached. It is possible that ovariectomy after sexual maturity versus ovariectomy during sexual maturation may influence lipid and DHA metabolism afterwards. Similarly, the slightly longer duration of this study compared with the **Chapter 7** study would have resulted in longer exposure of the ovariectomized rats to the higher levels of hepatic TAG. It has been shown that the hepatic transcript profile changes over time in response to hepatic TAG accumulation in mice fed a high-fat diet (Oh et al. 2013; Radonjic et al. 2009), that could explain differences in hepatic transcript profiles of the two ovariectomy studies in this thesis. In addition, the rats in the first study were group-housed (3-4 per cage); while individual housing was used in the second study to accomplish the pair-feeding protocol. Cohabitation has previously been shown to decrease testosterone synthesis in male F344 rats attributable to increased stress-induced corticosteroid levels (Nyska et al. 2002). As some evidence suggests that female rats prefer to be housed in groups, it is likely that individual housing in may increase stress levels, resulting in a decrease in testosterone synthesis, the precursor of 17β-estradiol (Nathan et al. 2001).

Food restriction of ovariectomized rats in the present study reduced body mass relative to those with *ad libitum* food access, however body mass remained significantly higher than sham-operated controls. This has been observed in some studies (Mueller et al. 1980; Roy et al. 1977; Shimomura et al. 1989; Witte et al. 2010) but not others (Liang et al. 2002), and indicates that metabolic adaptations occur in ovariectomy that result in weight gain that are independent of hyperphagia. In ovariectomized *ad libitum*-fed rats, both fat mass and lean mass are increased, while food restriction of ovariectomized rats causes an increase in lean mass only (Chen et al. 2001), indicating greater lean mass relative to total body mass in the OVX+PF groups relative to OVX+AL and SHAM. This change in body composition may be associated with the lower respiratory exchange ratio (RER) in OVX+PF relative to OVX+AL and SHAM, which indicates greater usage of fat for energy rather than carbohydrates. Because fat has approximately 2-fold greater energy density relative to carbohydrate, higher relative fat usage at a given energy expenditure would contribute to body mass gain. Lower RER has been found to be positively associated with body mass in chow or high-fat diet fed mice (Longo et al. 2010), suggesting that switching towards more fat usage for energy may predispose animals to body mass gain. Decreased voluntary locomotor activity as assessed by wheel running has also been associated with ovariectomy in rats (Thomas et al. 1986), but decreased physical activity was not observed during the 24-hour monitoring in our study. Lower skeletal muscle CPT-1 expression in OVX+PF and OVX+AL suggests lower peripheral fatty acid oxidation, which may result in storage of fatty acids as adipose tissue.

The increased hepatic TAG in ovariectomized rats appears to be due to hyperphagia, as food restriction via pair-feeding prevented the increase. This is in agreement with previous work showing reduced hepatic TAG in ovariectomized rats after 13 weeks of 25% caloric restriction

(Pighon et al. 2009a; Pighon et al. 2009b). Ovariectomy has also been shown to decrease the hepatic expression of microsomal triacylglycerol transfer protein (MTP), which is involved in assembly of VLDL for secretion (Barsalani et al. 2010; Cote et al. 2012), suggesting increased storage of hepatic TAG due to impaired lipoprotein secretion. The effects of food restriction on MTP expression are not known, but increased MTP expression may also be involved in decreased hepatic TAG in these animals. The lower hepatic TAG in OVX+PF relative to OVX+AL was associated with lower hepatic expression of SCD-1, which is the rate-limiting enzyme in *de novo* MUFA synthesis and is positively associated with hepatic TAG content (Cohen et al. 2003). Hepatic PDHK was lower in ovariectomized rats regardless of food intake, suggesting an increase in pyruvate decarboxyation and flux of acetyl-CoA into the tricarboxylic acid cycle. Increased acetyl-CoA can be used either for fatty acid synthesis or oxidative phosphorylation, however the lower SCD1 expression in OVX+PF relative to OVX+AL suggests that the acetyl-CoA generated by pyruvate dehydrogenase in OVX+PF is not being used for MUFA or TAG synthesis. Additionally, the lower RER in OVX+PF may indicate greater relative hepatic fatty acid oxidation and less hepatic storage of fatty acids as TAG.

The role of ovariectomy in plasma TAG accumulation is more controversial. In the present study, plasma TAG was increased relative to sham-operated controls following ovariectomy with or without food restriction, which has been observed in another study using ovariectomized Sprague Dawley rats with *ad libitum* access to food (Choi 2008). However, no increase in plasma TAG has been found in *ad libitum*-fed ovariectomized Wistar rats (Kishida et al. 2003), 13-week ovariectomized Sprague Dawley rats (Pighon et al. 2009b), mice (Ludgero-Correia, Jr. et al. 2012), Syrian hamsters (Lucas et al. 2011) and women that have had an ovariectomy (Cheung et al. 1998; Suda et al. 1998). A study in Wistar rats has shown decreased

plasma TAG following 8 weeks of 25% food restriction in ovariectomized rats compared with those fed *ad libitum* (Pighon et al. 2009b). The influence of ovariectomy on plasma TAG may be species or strain specific, as it has been shown that there are significant differences between C57BL/6 mice and Long Evans rats in the response of metabolism and energy expenditure to ovariecomy (Witte et al. 2010). In the present study, the higher plasma TAG in ovariectomized as compared with sham-operated rats may be related to the decreased muscle CPT-1 expression. Peripheral fatty acid oxidation may be decreased and fatty acid uptake by skeletal muscle may be reduced. Previous work has shown no change in white gastrocnemius CPT-1 mRNA expression following ovariectomy in Sprague Dawley rats, which may be explained by different muscle oxidative capacity and mRNA as compared with protein measures. Decreases in lipoprotein lipase expression could also account for higher plasma TAG in ovariectomized animals; however, adipose tissue lipoprotein lipase does not change with 16-days of ovariectomy in Sprague Dawley rats (Toth et al. 2001). The effect of ovariectomy on lipoprotein lipase activity in other tissues has not been examined. The effects of species, strain, and duration of ovariectomy with and without food restriction on plasma TAG levels should be assessed.

Ovariectomy with or without food restriction failed to reduce the circulating concentration of 17β-estradiol in the present study, which is contrary to previous studies (Davidge et al. 2001). It is possible that the eugonadal rats had lower than expected plasma 17β-estradiol concentrations due to sampling during estrous or diestrous phases of the estrous cycle, as serum 17β-estradiol concentrations are 3-fold lower in these phases (Gomes et al. 2005) and are similar between eugonadal and ovariectomized rats (Strom et al. 2008). Also, it has been shown that 17β-estradiol synthesis by muscle, skin, and adipose increases over time in ovarictomized rats, corresponding to increases in serum 17β-estradiol (Zhao et al. 2005). The

finding of similar plasma 17β-estradiol between ovariectomized rats undergoing food restriction or with *ad libitum* access to food is consistent with previous results (Davidge et al. 2001), and suggests that extragonadal 17β-estradiol production is unaffected by feeding status of ovariectomized rats. The immature age of the rats at ovariectomy may also have altered the response of 17β-estradiol to ovariectomy as compared with the response to ovariectomy of a sexually mature rat. However, the uterine atrophy observed in both groups of ovariectomized compared with sham-operated rats suggests that 17β-estradiol withdrawal did occur (Alessandri et al. 2011).

The lack of an effect on Δ6-desaturase expression of ovariectomy with or without food restriction was associated with no differences in the hepatic expression of the transcription factors PPARα, SREBP1c, and ERα. The effect of pair-feeding or food restriction on hepatic expression of these transcription factors has not been previously investigated, but previous studies have shown increased SREBP1c mRNA in rats ovariectomized for 8 (Paquette et al. 2008) and 10 weeks (Domingos et al. 2012). The lack of an effect of ovariectomy on SREBP1c in the present study may be due to shorter duration of ovariectomy and the time-dependent changes in hepatic transcript profiles in response to TAG accumulation (Oh et al. 2013; Radonjic et al. 2009). Similarly, disparate results between this study and others with regards to the effect of ovariectomy on PPARα (Alessandri et al. 2011) and ERα expression (Hao et al. 2010) may be related to differences in the length of time rats are exposed to the hormonal and metabolic milieu of ovariectomy.

CONCLUSION

Prevention of hyperphagia and hepatic TAG accumulation in ovariectomized rats did not result in any differences in hepatic Δ6-desaturase of hepatic phospholipid DHA concentration

relative to *ad libitum*-fed rats, suggesting that this is not a confounding factor in the assessment of the role of ovariectomy in hepatic DHA metabolism. Additionally, the lack of an effect of ovariectomy with or without food restriction on DHA metabolism relative to sham-operated rats suggests that ovarian hormone withdrawal via ovariectomy is not associated with alterations in hepatic DHA metabolism or plasma DHA status.

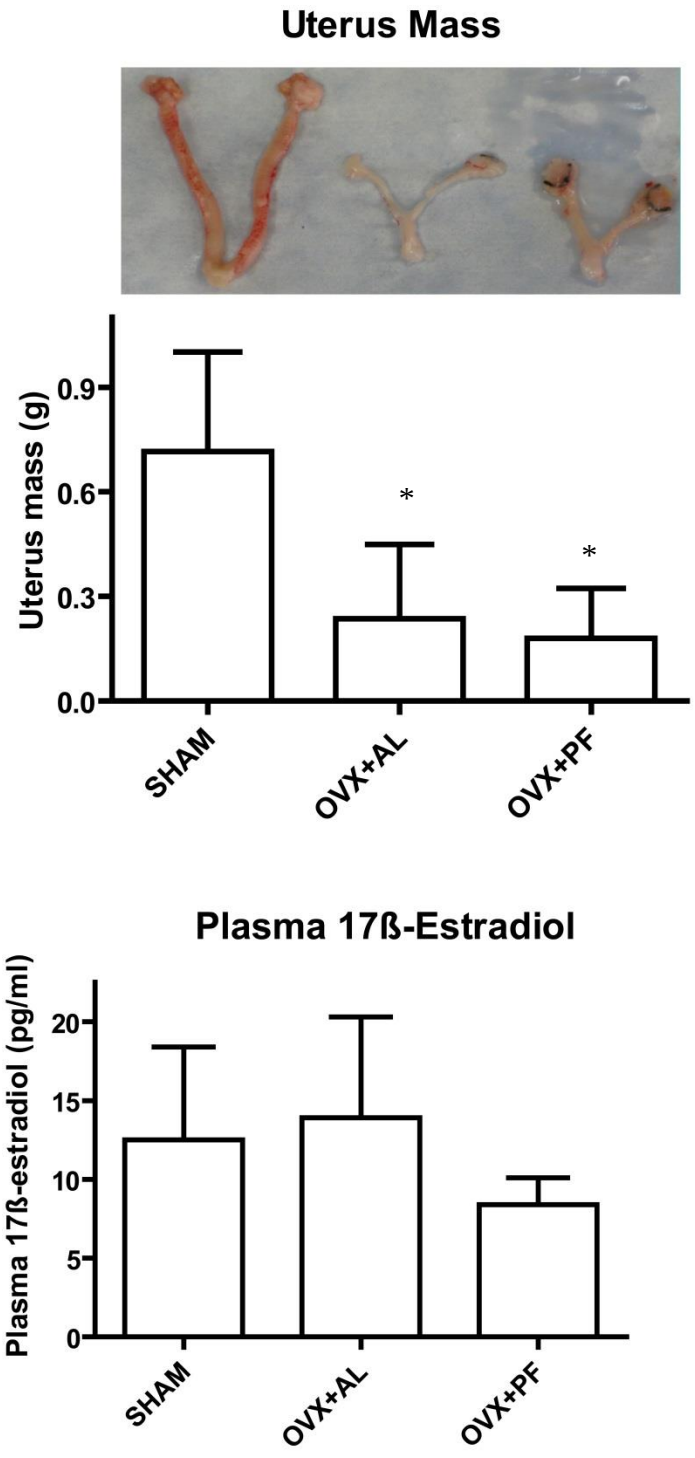

Figure 8.1: Effects of ovariectomy with or without pair-feeding on uterus mass and plasma 17β-estradiol concentration. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. *: significantly lower compared to sham-operated rats.

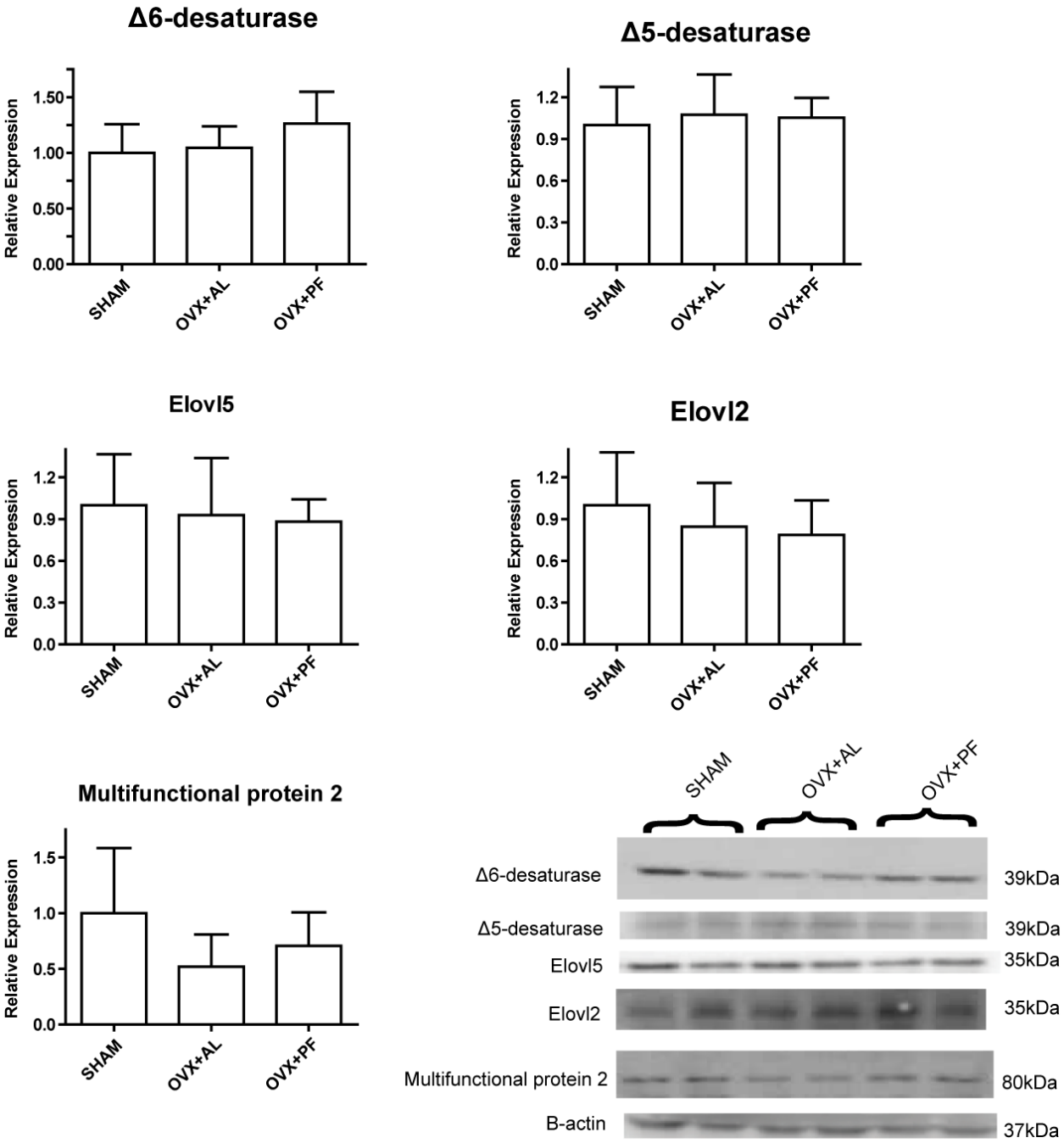

Figure 8.2. Effects of ovariectomy with or without pair-feeding on expression of enzymes of DHA biosynthesis. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats.

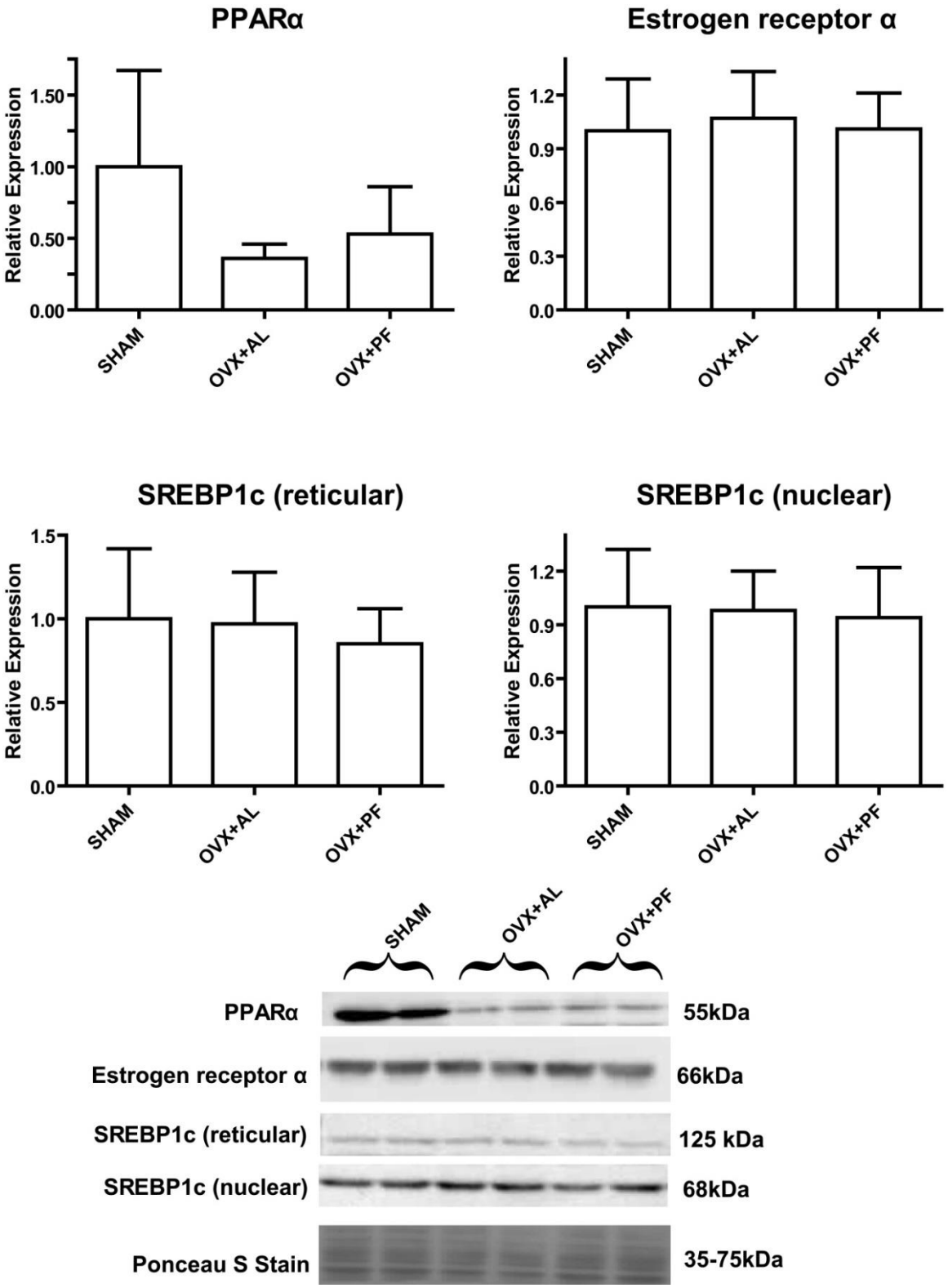

Figure 8.3: Effects of ovariectomy with or without pair-feeding protein expression of transcription factors. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats, PPARα: peroxisome proliferator activated receptor α, SREBP1c: sterol response element binding protein 1c.

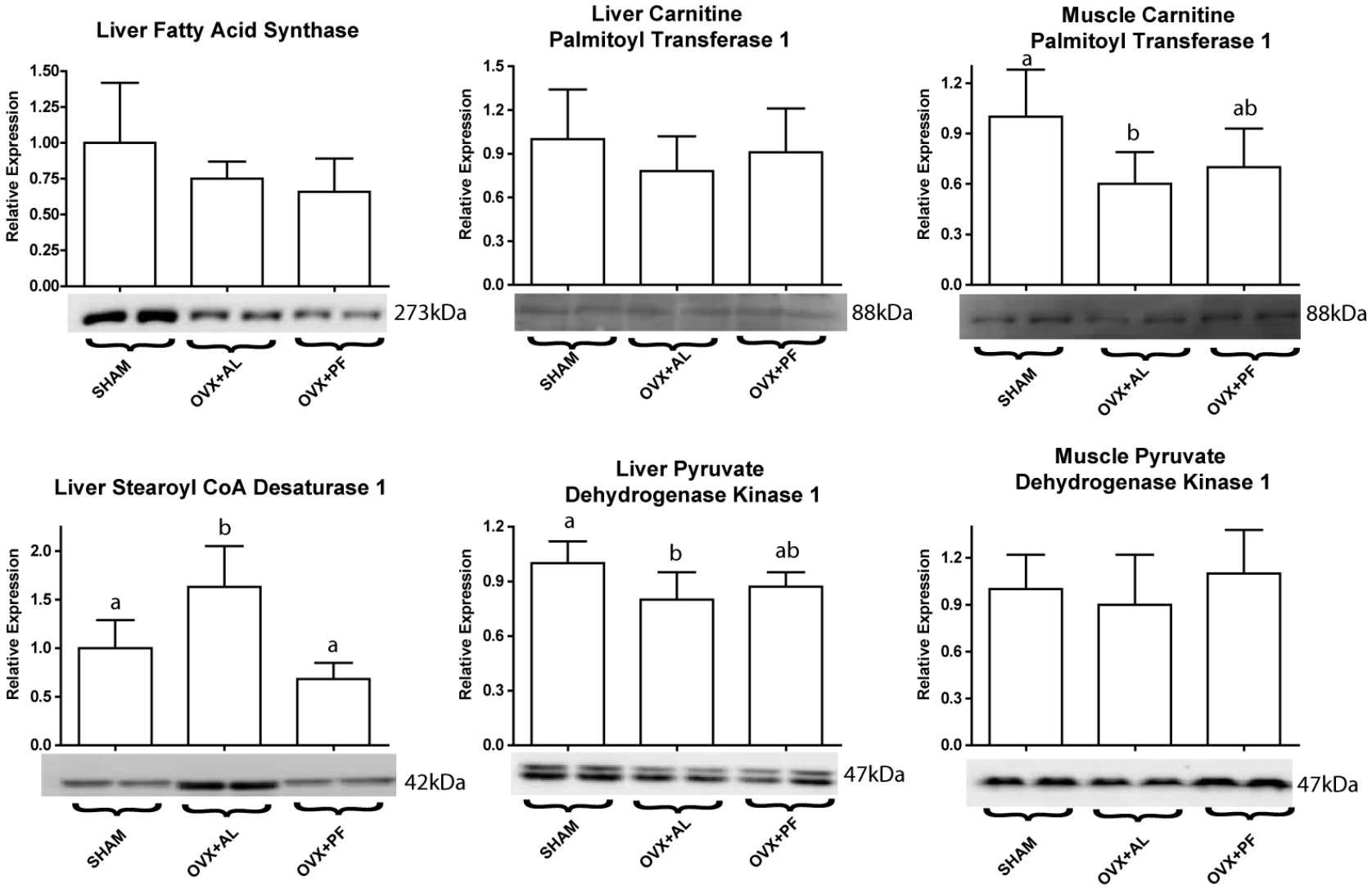

Figure 8.4. Effects of ovariectomy with or without pair-feeding on expression of enzymes involved in fuel substrate metabolism. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. Bars with different letters are significantly different by Tukey's post-hoc test following significant F value by one-way ANOVA.

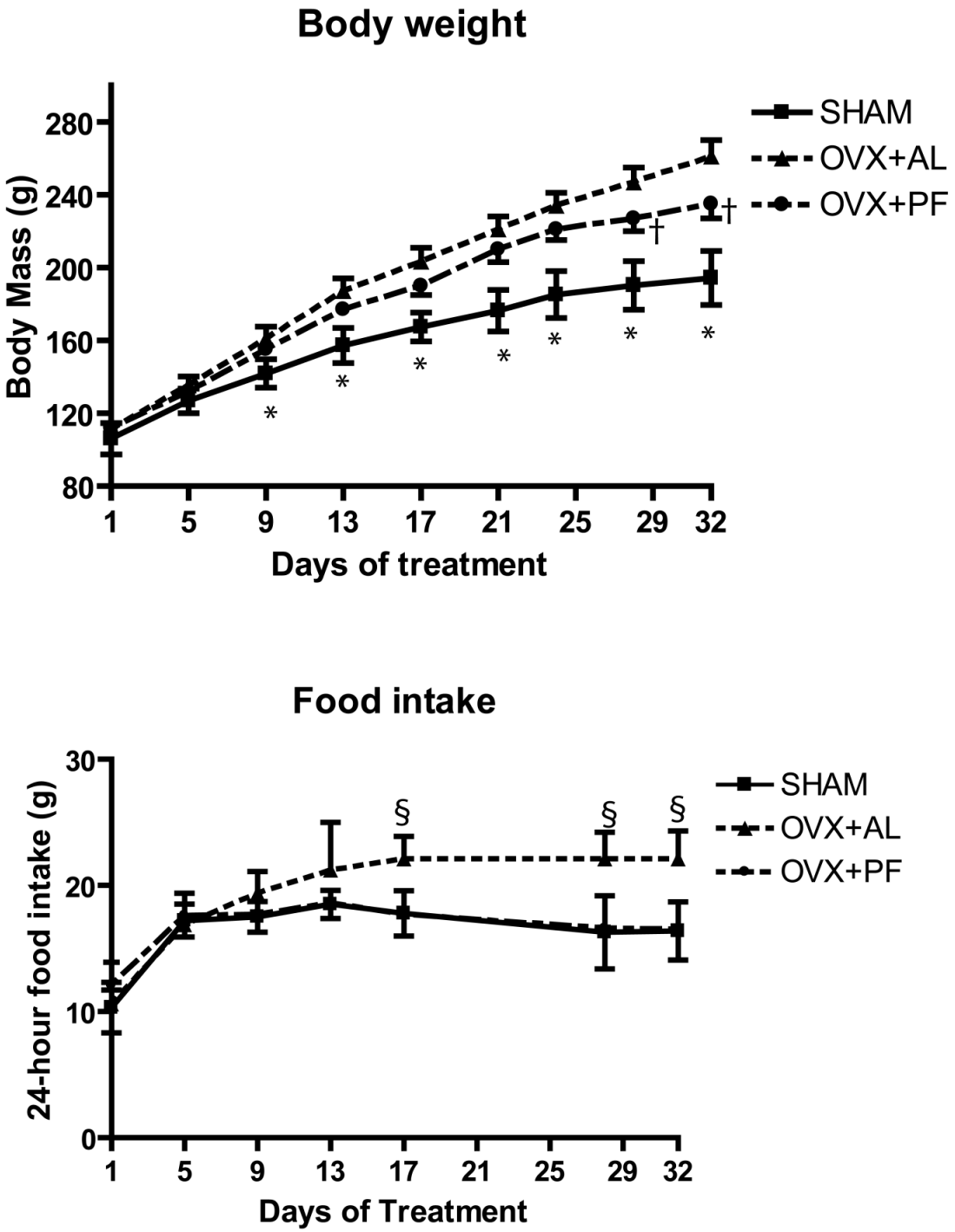

Figure 8.5: Effects of ovariectomy with or without pair-feeding on changes in body weight and food intake. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. *:significantly less than OVX+PF and OVX+AL, †: significantly less than only OVX+AL, §: significantly greater than both SHAM and OVX+PF. Significant differences determined by Bonferroni post-hoc test following significant F value by one-way ANOVA.

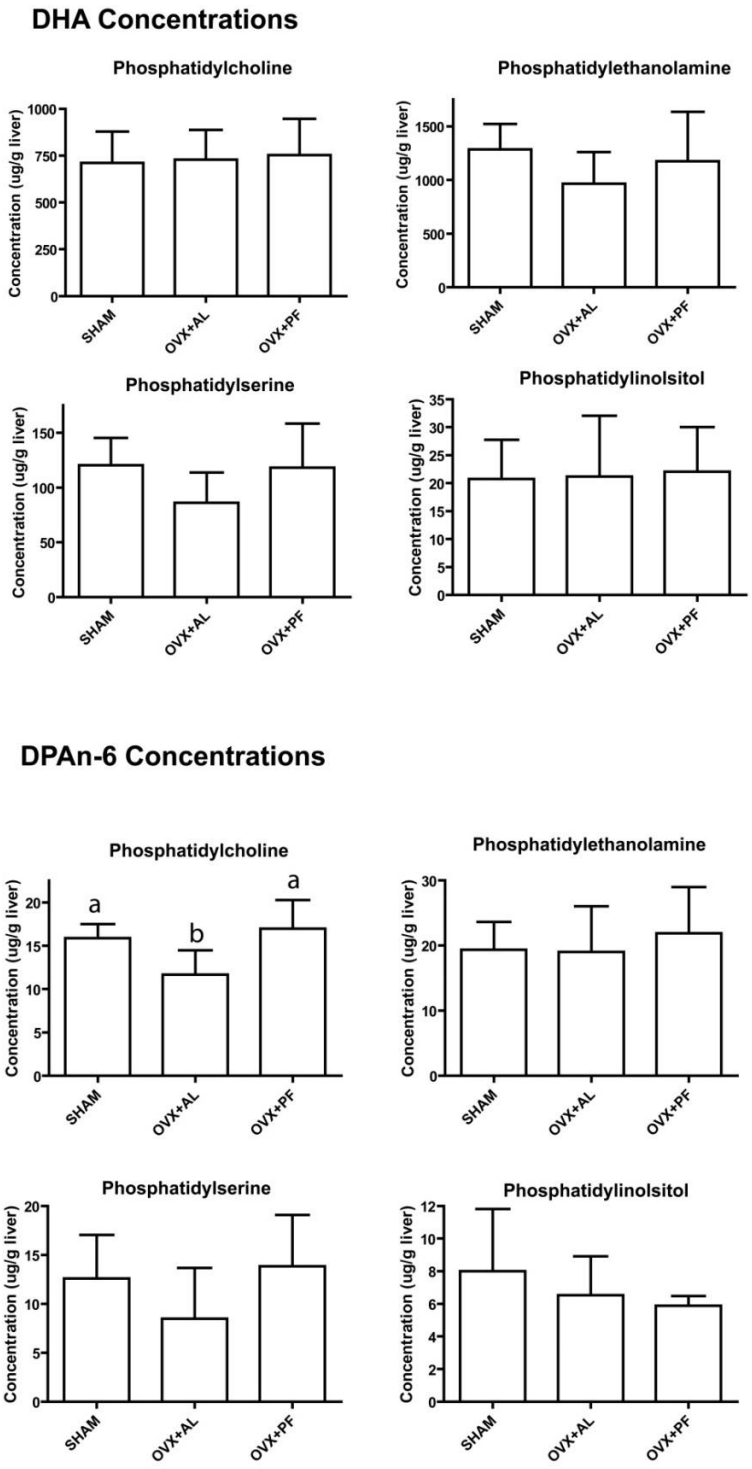

Figure 8.6: Effects of ovariectomy with or without pair-feeding on concentrations of docosahexaenoic acid and n-6 docosapentaenoic acid in hepatic phospholipids fractions. Columns with a different letter are significantly different by tukey's post-hoc test following significant F-value by one-way ANOVA. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats.

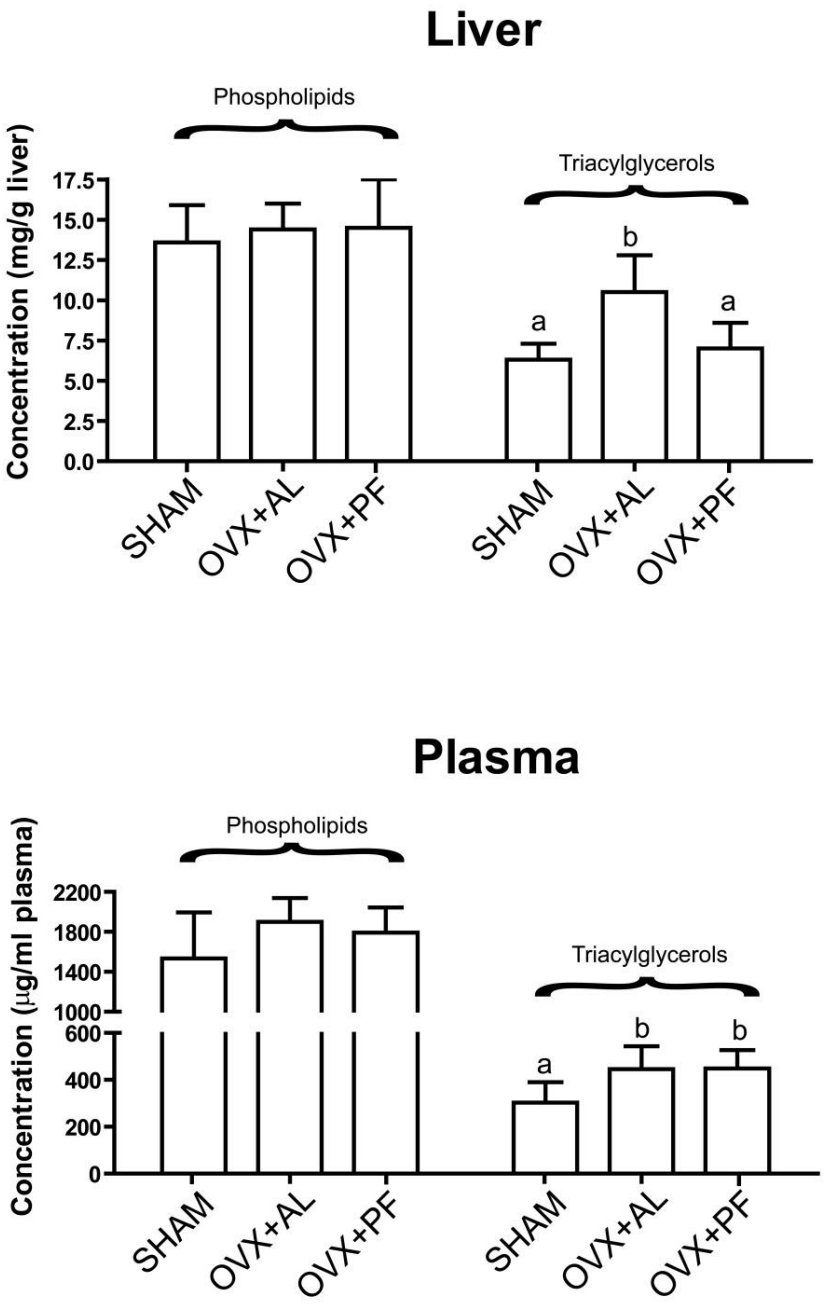

Figure 8.7: Effects of ovariectomy with or without pair-feeding on hepatic and plasma phospholipid and triacylglycerol concentrations. Columns with a different letter are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats.

Table 8.1: Effects of ovariectomy with or without pair-feeding on resting metabolic measurements

Metabolic	**SHAM**	**OVX+AL**	**OVX+PF**
VO_2 (ml/kg/hr)	1697 ± 185	1565 ± 193	1504 ± 117
VCO_2 (ml/kg/hr)	1687 ± 207	1578 ± 175	1448 ± 118
RER	0.992 ± 0.022^{ab}	1.011 ± 0.028^{a}	0.958 ± 0.001^{b}
Energy Expenditure			
kcal/hr	1.5 ± 0.1	1.8 ± 0.2	1.7 ± 0.1
total 24 hr kCal	36 ± 3	44 ± 6	40 ± 2
Activity			
Total activity counts	20758 ± 3615	21160 ± 3903	22000 ± 5050
Body Temperature			
high	24.6 ± 0.3	25.1 ± 0.3	25.4 ± 0.8
low	23.3 ± 0.4	23.6 ± 0.2	23.7 ± 0.3
average	24.2 ± 0.2^{a}	24.5 ± 0.1^{ab}	24.7 ± 0.3^{b}

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats, RER: respiratory exchange ratio. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.

Table 8.2: Effects of ovariectomy with or without pair-feeding on fatty acid concentration of hepatic phospholipids

	SHAM	**OVX+AL**	**OVX+PF**
		µg/g liver	
C 16:0	2092 ± 496	2351 ± 270	2366 ± 354
C 18:0	4021 ± 484	3938 ± 467	3927 ± 982
SFA	6712 ± 1102	6962 ± 811	6954 ± 1472
C 16:1	65 ± 36	70 ± 14	100 ± 41
C 18:1n-7	275 ± 33	312 ± 44	300 ± 51
C 18:1n-9	478 ± 347	392 ± 35	496 ± 143
MUFA	862 ± 420	830 ± 87	947 ± 210
C 18:2n-6	1571 ± 250	1731 ± 183	1696 ± 330
C 18:3n-6	19 ± 7	22 ± 5	23 ± 6
C 20:2n-6	29 ± 9	35 ± 5	32 ± 6
C 20:3n-6	43 ± 9^{a}	47 ± 6^{ab}	56 ± 9^{b}
C 20:4n-6	3095 ± 365	3438 ± 397	3334 ± 722
C 22:4n-6	33 ± 9	39 ± 7	33 ± 9
C 22:5n-6	19 ± 9	14 ± 3	19 ± 8
N-6 PUFA	4825 ± 664	5327 ± 575	5194 ± 1064
C 18:3n-3	16 ± 3	17 ± 4	16 ± 6
C 20:5n-3	26 ± 8	22 ± 4	21 ± 12
C 22:5n-3	69 ± 11^{a}	89 ± 11^{b}	85 ± 13^{ab}
C 22:6n-3	832 ± 153	828 ± 109	856 ± 205
N-3 PUFA	943 ± 165	956 ± 119	978 ± 230
PUFAs	5768 ± 813	6283 ± 681	6172 ± 1267
HUFAs	4117 ± 536	4477 ± 510	4404 ± 946
N-6/N-3	5.2 ± 0.5	5.6 ± 0.4	5.4 ± 0.6
HUFA Score	22 ± 2	21 ± 1	22 ± 2
Total	13655 ± 2387	14355 ± 1596	14486 ± 3032

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.

Table 8.3: Effects of ovariectomy with or without pair-feeding on fatty acid concentration of plasma phospholipids

	SHAM	**OVX+AL**	**OVX+PF**
		μg/ml plasma	
C 16:0	263 ± 50	389 ± 54	405 ± 66
C 18:0	431 ± 122	500 ± 45	453 ± 70
SFA	770 ± 183	980 ± 113	945 ± 122
C 16:1	7 ± 3	9 ± 2	11 ± 5
C 18:1n-7	19 ± 5	25 ± 5	24 ± 4
C 18:1n-9	48 ± 11	60 ± 13	77 ± 19
MUFA	87 ± 20	110 ± 18	128 ± 27
C 18:2n-6	172 ± 49	254 ± 42	278 ± 52
C 18:3n-6	2 ± 1	3 ± 1	2 ± 1
C 20:2n-6	2 ± 1	3 ± 1	3 ± 1
C 20:3n-6	4 ± 1	6 ± 1	7 ± 1
C 20:4n-6	321 ± 108	412 ± 55	362 ± 62
C 22:4n-6	3 ± 1	4 ± 1	4 ± 1
C 22:5n-6	1.2 ± 0.7	1.6 ± 0.4	1.7 ± 0.5
N-6 PUFA	506 ± 160	684 ± 98	659 ± 116
C 18:3n-3	0.6 ± 0.4	1.2 ± 0.5	1.1 ± 0.2
C 20:5n-3	1.7 ± 0.9	1.4 ± 0.5	1.2 ± 0.2
C 22:5n-3	6 ± 2	9 ± 2	8 ± 2
C 22:6n-3	49 ± 15	63 ± 10	58 ± 14
N-3 PUFA	57 ± 18	75 ± 13	69 ± 16
PUFAs	563 ± 177	759 ± 111	728 ± 131
HUFAs	386 ± 128	497 ± 68	443 ± 79
N-6/N-3	9.0 ± 0.9	9.2 ± 0.5	9.7 ± 1
HUFA Score	15 ± 1	15 ± 1	15 ± 1
Total	1466 ± 387	1899 ± 242	1858 ± 272

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.

CHAPTER 9

NO DIFFERENCES IN Δ6-DESATURASE AND HEPATIC DHA IN ESTROGEN RECEPTOR α KNOCKOUT MICE COMPARED WITH WILD-TYPE

INTRODUCTION

Estradiol supplementation of ovariectomized rats increases DHA concentration and Δ6-desaturase expression. It is likely that this effect of 17β-estradiol is mediated by hepatic ERα, as this is the estrogen receptor most highly expressed in the liver (Pelletier 2000) and has been shown to have effects on hepatic fat and carbohydrate metabolism (Dupont et al. 2000; Matic et al. 2013), while estrogen receptor β has no effect (Dupont et al. 2000). DHA tissue content and metabolism in the ERα-knockout mice (ERαKO) (Lubahn et al. 1993) has not been examined previously and can provide insights into the regulation of DHA synthesis by 17β-estradiol. The goal of the present study was to examine the differences in hepatic and plasma DHA and on the hepatic expression of DHA biosynthesis enzymes between ERα-KO and wild-type controls.

METHODS

ERαKO (strain name: B6.129P2-Esr1^{tm1Ksk}/J) and wild-type controls (strain name: B6.129PF2/J) were obtained from Jackson Labs (Bar Harbour, ME, USA) at 5 weeks of age and housed individually in the central animal facility in the University of Waterloo with *ad libitum* access to food and water (n = 6 for each genotype). Mice were sacrificed on post-natal day 65 by cervical dislocation after body weight was assessed, and 24-hour food intake was measured the day prior to sacrifice. Livers were quickly excised and snap-frozen in liquid nitrogen and stored at -80°C until biochemical analysis.

Fatty acid composition of liver total lipids, PL, TAG, PC, PE, PI, and PS were determined by gas chromatography (Stark et al. 2005b) after neutral lipid (Christie 2003) or

phospholipid TLC (Chen et al. 2011) following extraction of lipids from tissue (Folch et al. 1957). 22:3n-3 internal standard was added to PI and PS fractions after TLC.

Antibodies used for western blot analysis were Δ6-desaturase (sc-98480, 1:500 dilution in 1% skim milk), Δ5-desaturase (Santa Cruz Biotechnology, sc-101953, 1:100 dilution v/v in BSA), elovl2 (Santa Cruz Biotechnology, sc-54874, 1:250 dilution v/v in BSA), elovl5 (Santa Cruz Biotechnology, sc-54888, 1:250 dilution v/v in milk), MFP2 (Santa Cruz Biotechnology sc-135045, 1:200 v/v in milk), and ERα (Abcam, ab16460, v/v 1:1000 in milk). Chemiluminescent detection was performed by ECL prime (GE Healthcare).

Statistical comparison between the ERαKO and wild-type mice was done using independent samples T-Test.

RESULTS

No differences were observed in the food intake (3.2 ± 0.6 g/d for ERαKO and 3.0 ± 0.2 g/d for wild-type) or body weight (18.8 ± 0.6 g for ERαKO and 18.0 ± 2.4 g for wild-type) between ERαKO and wild-type mice. ERαKO mice had 40% higher expression of elovl5 compared with wild-type controls, with no differences in Δ6-desaturase, Δ5-desaturase, elovl2, and MFP-2 (**Figure 9.1**). No differences were observed in the content of PPARα and nuclear and reticular forms of SREBP1c (**Figure 9.2**).

The concentration of DHA in liver total lipids, PL, and TAG was not different between ERαKO and wild-type mice (**Table 9.1**). DHA was lower in PE of ERαKO compared to wild-type mice, which corresponded to a decreased n-6/n-3 PUFA ratio and a higher percentage of HUFA as n-3 HUFA (**Table 9.2**). No differences in DHA concentration in PC, PS, and PI were observed between ERαKO and wild-type mice. Higher 20:3n-6 was found in total lipids, PC, and

PE, and PI in ERαKO mice. Higher concentrations of EPA and DPAn-3 were found in total lipids, PL, TAG, PE and PC of ERαKO compared to wild-type mice. Higher concentrations of 16:1n-7 and 18:1n-7 were observed in hepatic total lipids, TAG, PE, and PC

DISCUSSION

This study demonstrates that there are no differences in the hepatic expression of Δ6-desaturase between ERαKO and wild-type mice, corresponding to no differences between these animals in hepatic DHA concentrations, except for a 15% decrease in the PE fraction. ERαKO mice did exhibit higher expression of elovl5, corresponding to higher concentrations of DPAn-3, EPA, and 20:3n-6 in various lipid fractions, suggesting that these mice have altered PUFA metabolism. The higher concentrations of 16:1n-7 and 18:1n-7 indicate that production of n-7 MUFA is higher in ERαKO mice, possibly through higher expression of stearoyl-CoA desaturase 1 (SCD1) that has been seen previously in these mice (Bryzgalova et al. 2006).

The lack of effect of ablation of ERα on Δ6-desaturase and DHA may be due to residual activity of ERα associated with this knockout. This ERαKO mouse is generated by insertion of a neomycin resistance gene 280 base pairs downstream of the transcription start site of exon 2 of the ERα gene (Korach et al. 1996; Lubahn et al. 1993), and alternative splicing has been shown to result in transcription and translation of 2 mutant genes, one of which still contains the hormone-binding domain and binds to endogenous 17β-estradiol (Couse et al. 1995). This variant has approximately 8% of the genomic activity relative to wild-type ERα (Couse et al. 1995), however the possible non-genomic actions of this variant have not been investigated. Exposure of mice that express only the hormone-binding domain of ERα anchored to the cell membrane (Pedram et al. 2009) to an ERα agonist induces lipid catabolism that is absent in a mouse that does not express this domain (Pedram et al. 2013), suggesting that this domain is

sufficient to mediate the non-genomic effects of 17β-estradiol on hepatic lipid metabolism. Importantly, plasma concentrations of 17β-estradiol are increased approximately 4-fold in ERαKO compared to wild-type mice (Couse et al. 1995), suggesting that the non-genomic actions of ERα may be higher in ERαKO mice. However, the degree of hepatic non-genomic ERα signaling present in these animals is not characterized.

Therefore, the ERαKO model utilized presently is likely deficient in genomic ERα signaling only. It can only be concluded that removal of genomic ERα activity does not affect Δ6-desaturase expression and only has minor effects on cellular DHA concentrations, as it is possible that the non-genomic actions of ERα continue to affect these measures. Future work should utilize ERαKO mice that do not express ligand binding domain (Dupont et al. 2000) to estimate the effects of fully functional ERα ablation on Δ6-desaturase expression and DHA concentration. A possible mechanism for non-genomic ERα signalling in regulation of Δ6-desaturase expression and DHA synthesis is detailed in **Figure 9.3**, based on previous work showing increased activation of SREBP1c via phosphoinositide-3 kinase (PI3K) – Akt activity (Krycer et al. 2010), and ERα activates PI3K-Akt by a non-genomic mechanism (Marino et al. 2003). Conversely, the effects on MUFA metabolism observed presently that are likely due to increased SCD1 expression that has been observed previously (Bryzgalova et al. 2006) are due to the genomic effects of ERα, indicating that ERα may have a direct inhibitory role in the expression of this enzyme.

The higher elovl5 expression in ERα-KO relative to wild-type mice is therefore due to the removal of the genomic effects of ERα, indicating that DNA binding of ERα either directly or indirectly regulates elovl5. There is a putative estrogen response element 3.7 kb upstream from the transcription start site of elovl5 (Bajic et al. 2013), suggesting that ERα may inhibit

expression from this site. However, an ERE was also identified 0.6 kb upstream of the Δ6-desaturase promoter and we did not observe significant differences in Δ6-desaturase expression. Characterization of the interaction of ERα with the promoter regions of these genes is required.

The lack of a difference in SREBP1c between ERαKO and wild-type mice is surprising, as reduction of circulating estradiol via ovariectomy increases SREBP1c mRNA, and estradiol supplementation restores it (Paquette et al. 2008). Similarly, SREBP1c cleavage is increased in mice lacking a functional ERα ligand-binding domain (Pedram et al. 2013). This lack of effect in the present study is likely due to the presence of active ligand-binding domain of the estrogen receptor, as it has been shown that this domain alone is sufficient to restore SREBP1c cleavage in mice (Pedram et al. 2013). Similarly, hepatic PPARα expression is typically decreased following ovariectomy (Paquette et al. 2008), indicating a regulatory role of hepatic ERα signaling on PPARα. Though not previously investigated, the presence of the ligand-binding domain may explain the lack of an effect of the ablation of genomic action of ERα on PPARα expression in the present study.

It has been shown previously that these ERαKO mice have increased hepatic mRNA for stearoyl-CoA desaturase 1 and elovl3 (Bryzgalova et al. 2006), however this effect does not appear to translate into increased hepatic lipid storage, as there were no differences in hepatic total lipids or triacylglycerols in the present study. However, higher levels of n-7 MUFA suggest that SCD1 and/or SFA/MUFA elongases such as elongase 3 or elongase 6 (Jakobsson et al. 2006) are more active in the ERαKO mice, and the expression of these enzymes should be assessed.

Previous work has also shown 20% higher body weight in this strain of ERαKO mouse relative to wild-type controls at the same age as the present study (Bryzgalova et al. 2006) which

was not observed presently. This may be due to slightly different genetic backgrounds resulting from different suppliers of mice.

CONCLUSIONS

The elimination of genomic ERα signalling does not affect Δ6-desaturase and exerts mild effects on cellular DHA concentration that are limited to the PE fraction. Non-genomic mechanisms may still be present, and are possibly regulating Δ6-desaturase expression and DHA concentration. However, elovl5 expression was increased, and several n-3 and n-6 elovl5 products were higher in ERαKO compared to wild-types, suggesting that genomic ERα activity regulates this enzyme. Further research using a full ERα ablation is required.

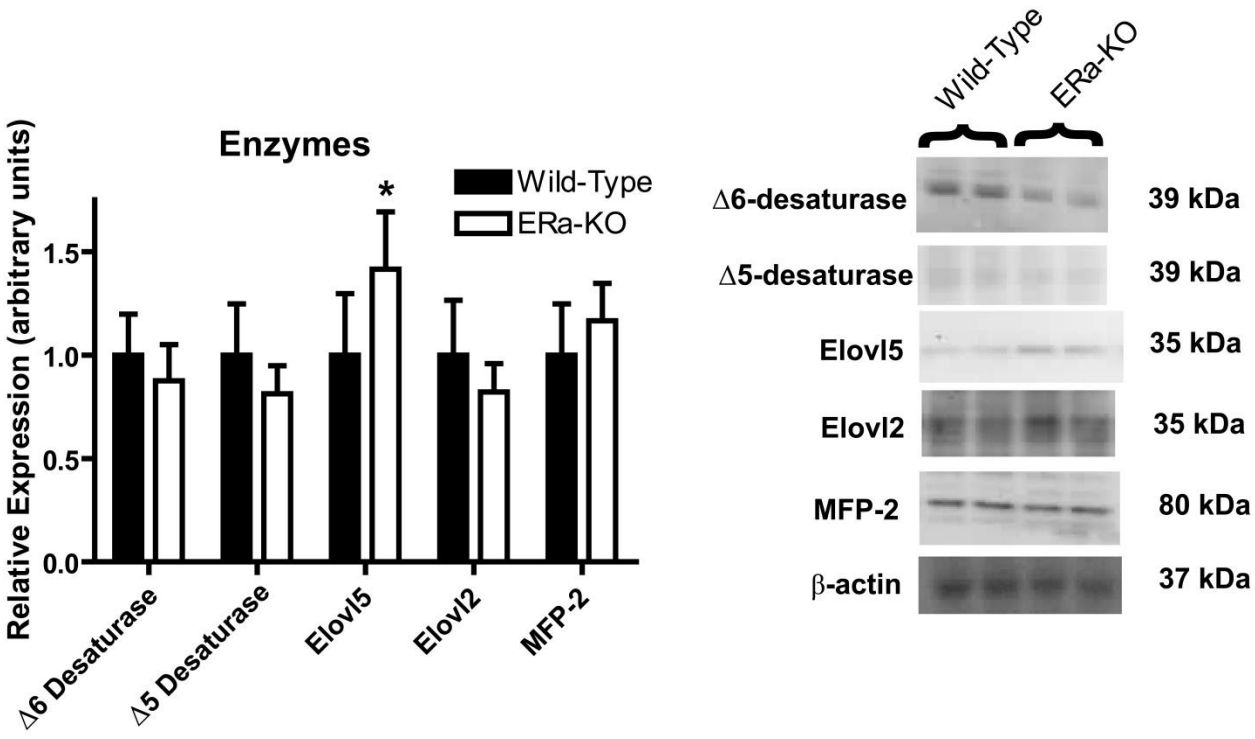

Figure 9.1: Expression of enzymes involved in DHA biosynthesis in wild-type and estrogen receptor α-knockout mice. ERα-KO: estrogen receptor α-knockout, MFP-2: multifunctional protein 2. *Significantly different between groups by independent samples T-test.

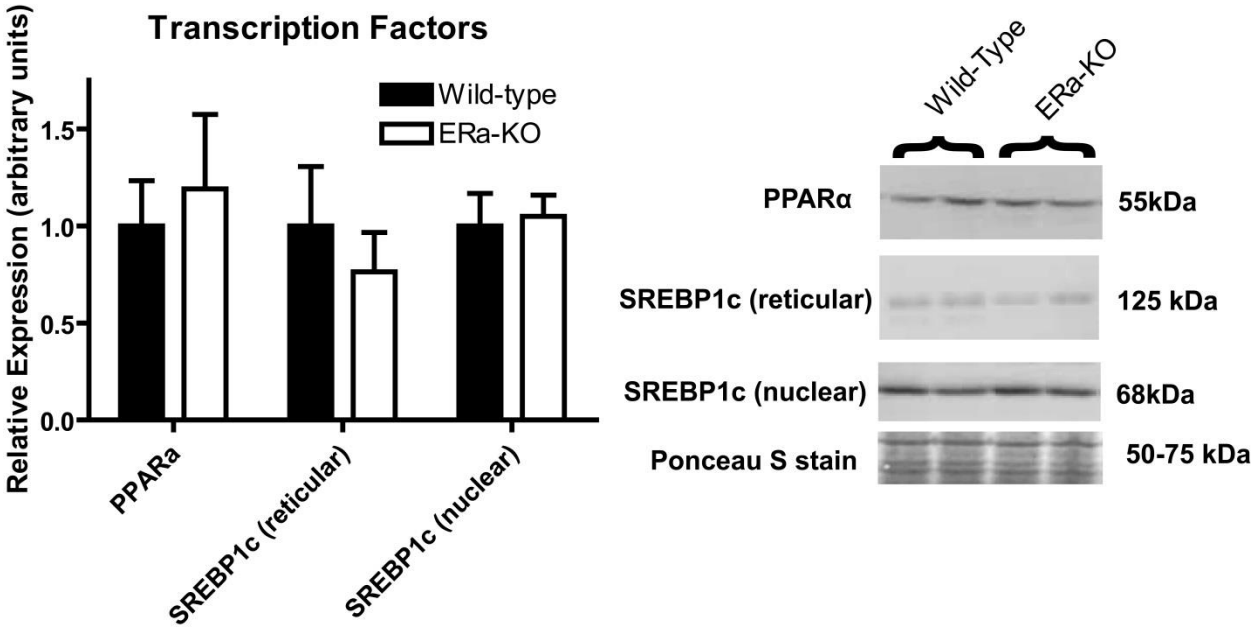

Figure 9.2: Expression of transcription factors affecting expression of enzymes involved in DHA biosynthesis in wild-type and estrogen receptor α-knockout mice. ERα-KO: estrogen receptor α-knockout, PPARα: peroxisome proliferator activated receptor α, SREBP1c: sterol response element binding protein 1c.

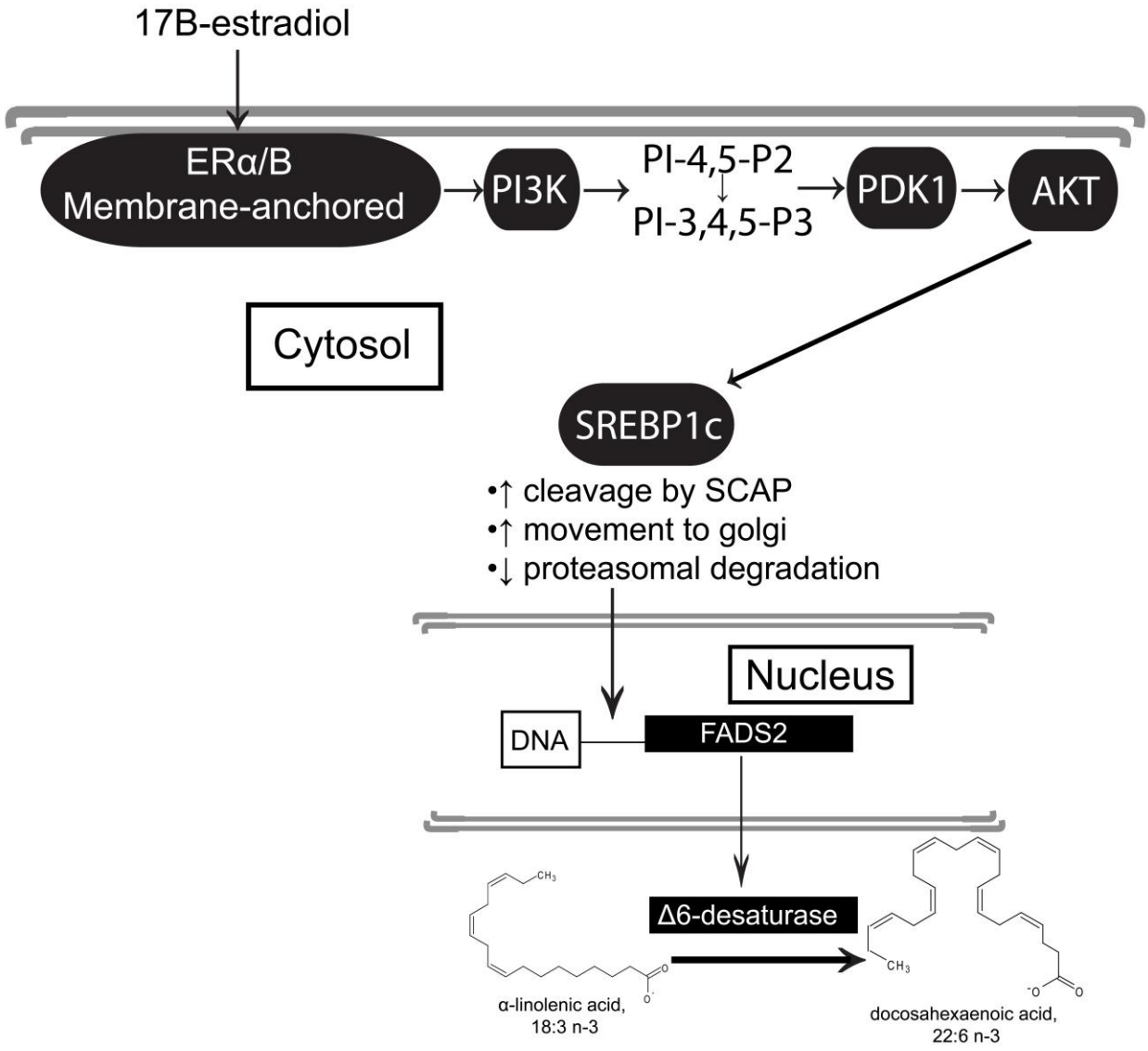

Figure 9.3: Potential non-genomic regulation of DHA synthesis by ERα involving Akt-mediated increase in SREBP1c action. ER: estrogen receptor, PI3K: phosphoinositide-3 kinase, PI: phosphatidylinositol, PDK1: phosphoinositide dependent kinase 1, SREBP1c: sterol response element binding protein 1c, SCAP: SREBP1c cleavage activating protein, FADS2: fatty acid desaturase 2, the Δ6-desaturase gene

Table 9.1: Fatty acid concentration of total lipids, phospholipids, and triacylglcyerols of estrogen receptor α-knockout and wild-type mice.

	Total Lipids		**Phospholipids**		**Triacylglycerols**	
	Wild-Type	***ERα-KO***	***Wild-Type***	***ERα-KO***	***Wild-Type***	***ERα-KO***
Fatty Acid			*μg/g liver*			
C 16:0	28397 ± 3275	28935 ± 7057	6947 ± 1107	6504 ± 2512	3599 ± 1076	3801 ± 844
C 18:0	11796 ± 1276	11019 ± 1285	5753 ± 1047	5174 ± 1654	457 ± 127	389 ± 88
SFA	42868 ± 3618	42408 ± 6571	13216 ± 2230	12185 ± 4354	4272 ± 1237	4412 ± 891
C 16:1	1412 ± 289	2941 ± 1321*	178 ± 28	248 ± 82	267 ± 90	574 ± 120*
C 18:1n-7	1857 ± 309	2710 ± 914	390 ± 60	535 ± 166	264 ± 70	375 ± 117
C 18:1n-9	20208 ± 1476	24176 ± 8494	2050 ± 588	2006 ± 541	3783 ± 889	4771 ± 929
MUFA	20798 ± 8954	30688 ± 10638	2809 ± 610	3143 ± 836	4396 ± 1063	5822 ± 1153
C 18:2n-6	35441 ± 6636	34452 ± 11056	5036 ± 795	4781 ± 1640	5793 ± 1759	5644 ± 1557
C 18:3n-6	659 ± 132	526 ± 198	50 ± 6	44 ± 28	115 ± 34	89 ± 41
C 20:2n-6	347 ± 70	355 ± 73	73 ± 12	64 ± 16	38 ± 20	45 ± 9
C 20:3n-6	895 ± 173	1345 ± 267*	258 ± 71	374 ± 125	66 ± 36	107 ± 19
C 20:4n-6	12224 ± 1627	12038 ± 1038	5071 ± 671	4847 ± 1550	269 ± 80	254 ± 70
C 22:4n-6	302 ± 44	308 ± 62	46 ± 8	50 ± 21	44 ± 15	42 ± 8
C 22:5n-6	200 ± 58	140 ± 22	46 ± 27	31 ± 9	26 ± 9	16 ± 4
N-6 PUFA	50069 ± 7760	49165 ± 10836	10581 ± 1533	10191 ± 3328	6351 ± 1919	6197 ± 1673
C 18:3n-3	1115 ± 319	1571 ± 766	47 ± 4	55 ± 18	217 ± 83	314 ± 82
C 20:5n-3	345 ± 76	657 ± 162*	65 ± 24	107 ± 28*	39 ± 11	83 ± 20*
C 22:5n-3	486 ± 77	775 ± 236*	89 ± 21	120 ± 46	58 ± 16	102 ± 27*
C 22:6n-3	10639 ± 1259	9628 ± 1171	3699 ± 721	3170 ± 1249	434 ± 114	414 ± 103
N-3 PUFA	12585 ± 1402	12631 ± 2185	3900 ± 766	3452 ± 1332	748 ± 215	913 ± 228
PUFA	62654 ± 8544	61796 ± 12935	14481 ± 2188	13643 ± 4638	7099 ± 2124	7110 ± 1897
HUFA	25092 ± 3032	24891 ± 2022	9275 ± 1429	8699 ± 2947	936 ± 248	1018 ± 228
N-6/N-3	4.0 ± 0.6	3.9 ± 0.3	2.7 ± 0.3	3.0 ± 0.3	8.5 ± 0.8	6.8 ± 0.4*
HUFA Score	46 ± 2	44 ± 4	41 ± 3	39 ± 3	57 ± 4	59 ± 3
Total	127521 ± 20779	136511 ± 29700	31355 ± 4754	29433 ± 9828	16169 ± 4514	17823 ± 3705

Data is mean ± SD. *Significantly different from wild-type by independent samples T-test. ERα-KO: estrogen receptor α-knockout mice, SFA: saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA.

Table 9.2: Fatty acid concentration of phospholipid fractions from livers of estrogen receptor α-knockout and wild-type mice.

	Phosphatidylcholine		**Phosphatidylethanolamine**		**Phosphatidylinositol**		**Phosphatidylserine**	
	Wild-Type	***ERα-KO***	***Wild-Type***	***ERα-KO***	***Wild-Type***	***ERα-KO***	***Wild-Type***	***ERα-KO***
Fatty Acid				*μg/g liver*				
C 16:0	4618 ± 264	4715 ± 419	1875 ± 170	1743 ± 63	375 ± 55	339 ± 66	378 ± 59	405 ± 45
C 18:0	3056 ± 327	2892 ± 418	2669 ± 216	2675 ± 105	1543 ± 109	1403 ± 175	1100 ± 81	1101 ± 85
SFA	8350 ± 521	8077 ± 693	4926 ± 370	4700 ± 153	2001 ± 171	1823 ± 249	1692 ± 114	1747 ± 125
C 16:1n-7	88 ± 21	148 ± 32*	33 ± 10	59 ± 11*	12 ± 4	9 ± 6	12 ± 5	17 ± 3
C 18:1n-7	203 ± 25	315 ± 95*	82 ± 16	129 ± 38*	23 ± 22	18 ± 8	13 ± 10	15 ± 6
C 18:1n-9	980 ± 56	1281 ± 200*	859 ± 260	849 ± 118	174 ± 95	223 ± 89	120 ± 57	151 ± 127
MUFA	1390 ± 60	1891 ± 332*	1088 ± 280	1152 ± 149	283 ± 112	322 ± 90	220 ± 70	258 ± 134
C 18:2n-6	2932 ± 221	3097 ± 309	647 ± 69	635 ± 62	91 ± 7	97 ± 10	94 ± 19	106 ± 53
C 18:3n-6	33 ± 17	36 ± 10	6 ± 3	10 ± 9	4 ± 1	5 ± 1	5 ± 2	5 ± 2
C 20:2n-6	25 ± 8	34 ± 10	11 ± 3	11 ± 3	3 ± 1	3 ± 2	3 ± 3	4 ± 2
C 20:3n-6	152 ± 41	246 ± 75*	33 ± 8	48 ± 14*	14 ± 8	24 ± 7*	14 ± 1	15 ± 4
C 20:4n-6	2022 ± 106	1869 ± 303	1437 ± 133	1662 ± 305	574 ± 45	563 ± 58	140 ± 22	131 ± 24
C 22:4n-6	12 ± 6	11 ± 5	22 ± 6	17 ± 7	7 ± 4	8 ± 6	10 ± 2	10 ± 3
C 22:5n-6	35 ± 12	29 ± 10	36 ± 7	29 ± 9	15 ± 7	12 ± 5	20 ± 5	19 ± 7
N-6 PUFA	5239 ± 290	5351 ± 656	2222 ± 169	2439 ± 373	729 ± 51	734 ± 76	306 ± 38	309 ± 71
C 18:3n-3	24 ± 6	33 ± 5*	9 ± 5	10 ± 3	8 ± 3	6 ± 2	8 ± 2	8 ± 2
C 20:5n-3	28 ± 10	39 ± 15	22 ± 8	42 ± 11*	7 ± 4	6 ± 2	4 ± 2	6 ± 2
C 22:5n-3	33 ± 16	52 ± 11*	38 ± 9	43 ± 8	7 ± 3	12 ± 5	8 ± 3	10 ± 3
C 22:6n-3	1476 ± 167	1412 ± 170	1599 ± 152	1368 ± 179*	26 ± 1	24 ± 5	110 ± 7	112 ± 10
N-3 PUFA	1561 ± 185	1536 ± 180	1661 ± 159	1463 ± 189	51 ± 8	47 ± 7	130 ± 9	135 ± 11
PUFA	6800 ± 349	6887 ± 786	3883 ± 242	3903 ± 512	787 ± 49	781 ± 77	436 ± 41	445 ± 68
HUFA	3758 ± 220	3659 ± 495	3180 ± 225	3209 ± 475	656 ± 53	649 ± 69	306 ± 29	303 ± 30
N-6/N-3	3.4 ± 0.5	3.5 ± 0.4	1.3 ± 0.2	1.7 ± 0.2*	15 ± 3	16 ± 2	2.4 ± 0.3	2.3 ± 0.7
HUFA Score	41 ± 3	41 ± 4	52 ± 3	45 ± 3*	6 ± 1	6 ± 1	40 ± 3	42 ± 4
Total	17009 ± 901	17287 ± 1646	10296 ± 705	10103 ± 654	3197 ± 283	2981 ± 265	2533 ± 226	2578 ± 273

Data is mean ± SD. *Significantly different from wild-type by independent samples T-test. ERα-KO: estrogen receptor α-knockout mice, SFA: saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA.

CHAPTER 10

GENERAL DISCUSSION

Due to the very low intakes of DHA in North America, an improved understanding of factors affecting DHA status is required, particularly considering the importance of DHA in neurological development (Brenna 2011) and the sensitive dose-response between DHA status and risk of sudden cardiac death (Mozaffarian et al. 2006). Female sex (Lohner et al. 2013), pregnancy (Stark et al. 2005a), and 17β-estradiol status (Giltay et al. 2004b) are all positively associated with blood DHA levels. This higher DHA is not due to higher dietary n-3 PUFA intake, indicating that higher synthesis of DHA from other n-3 PUFA is likely occurring. Accordingly, the primary purpose of this thesis was to examine differences in the expression of DHA biosynthesis enzymes between sexes and during pregnancy, and to evaluate the potential role of 17β-estradiol in mediating these differences in rodent models.

The higher hepatic Δ6-desaturase expression and DHA concentrations observed presently in female relative to males, in pregnant relative to non-pregnant rats, and in rats supplemented with 17β-estradiol indicates that 17β-estradiol increases the DHA biosynthesis capacity of rats, as hypothesized. Higher Δ6-desaturase expression in pregnancy strongly suggests that this is a mechanism to provide DHA to a fetus, the importance of which is indicated by delayed neural development in pre-term infants not supplemented with DHA (Koletzko et al. 2001) and spatial and cognitive impairments resulting chronic *in utero* deprivation of DHA in rats (Lim et al. 2005b). It is likely that this increase in DHA production is sufficient for fetal neurodevelopment, as several interventions increasing DHA intake of pregnant mothers have failed to result in any gains in cognitive and visual function besides those provided by baseline maternal DHA supply (Makrides et al. 2010; Malcolm et al. 2003; Smithers et al. 2011; Stein et al. 2012). Positive

benefits for fetal/infant neurodevelopment may result from higher dosages of DHA than have previously been assessed (200-400 mg), as the increase in maternal blood DHA resulting from these dosages may be masked by the natural increase in plasma DHA that occurs during pregnancy as a result of increased Δ6-desaturase expression.

The dose-response of DHA synthesis and Δ6-desaturase is not clear. Supplementing rats with 17β-estradiol or 17β-estradiol+progesterone resulted in 620% and 420% higher plasma 17β-estradiol, respectively, associated with significantly higher Δ6-desaturase expression and hepatic and plasma DHA. Surprisingly, ovariectomy in both **Chapter 7** and **Chapter 8** did not reduce DHA levels as expected, and Δ6-desaturase was actually higher in ovarictomized rats in **Chapter 7** which was not observed in **Chapter 8**. It is likely that the effects of ovariectomy on 17β-estradiol concentrations was not large enough to elicit effects on DHA metabolism, as ovariectomy resulted in 17β-estradiol concentrations that were only 40% (**Chapter 7**) and 33% (**Chapter 8**) lower, respectively, compared with controls. Postmenopausal women have plasma 17β-estradiol levels less than 10% those of premenopausal women (Burger 1999), and male 17β-estradiol is 15% and 27% of female estradiol in the follicular and luteal phase, respectively (Eldrup et al. 1987), suggesting that these differences in 17β-estradiol may be sufficient to elicit changes in Δ6-desaturase and DHA status. A dose-response of 17β-estradiol on DHA synthesis should be determined.

It is possible that other sex hormones effect DHA status as well, in addition to 17β-estradiol. For example, testosterone supplementation of female-to-male transsexuals results in decreased serum cholesteryl ester DHA (Giltay et al. 2004b), and progesterone has been shown to correlate with hepatic Δ6-desaturase mRNA in pregnancy (Childs et al. 2012). All three hormones are increased during pregnancy (O'Leary et al. 1991), and males have higher

testosterone and lower 17β-estradiol and progesterone relative to females (Eldrup et al. 1987), making interpretation of the effects of these hormones on DHA metabolism difficult. The effects of all three sex hormones on DHA synthesis should be assessed in male and female animals using hormone supplementation of gonadectomised rats. In addition, hepatocyte cell culture would allow the assessment of these hormones without any extra-hepatic effects, as it has been shown that a liver-specific knockout of ERα displays markedly different hepatic lipid metabolism compared to whole body ERα knockout (Matic et al. 2013).

The mechanism underlying the positive effect of 17β-estradiol on DHA synthesis remains unclear, but does not appear to be mediated by the DHA binding activity of ERα as demonstrated by the lack of an effect of disruption of this activity of ERα on hepatic DHA levels and Δ6-desaturase, contrary to our hypothesis. Also, changes in the expression of PPARα and SREBP1c do not appear to directly mediate the effects of 17β-estradiol on DHA synthesis, particularly considering PPARα expression was lower in female as compared with male liver. Future studies examining the role of non-genomic ERα signalling mechanisms as well as the possible influence of ERβ and GPER on DHA synthesis and Δ6-desaturase expression are required.

Though conducted in animal models, the studies of this thesis can most likely be applied to human populations as well, based on the occurrence of similar changes in DHA status resulting from changes in 17β-estradiol. Differences in DHA production between humans and rats are difficult to determine due to a lack of consensus regarding DHA synthesis rates in humans, with estimates of fractional conversion of ALA to DHA ranging from 0.2-4% (Emken et al. 1989; Pawlosky et al. 2001), roughly corresponding to 0.2-3.1 μmol/d/kg body weight based on daily ALA intake of 1500 mg (Gebauer et al. 2006) in a 70kg individual. On the other hand, rat hepatic DHA synthesis has been directly estimated following infusion of stable isotope-

labelled ALA as 31 μmol/day/kg body weight (Gao et al. 2009a), although this estimate in rats does not account for loss of label during digestion/absorption. Nevertheless, similar changes in DHA status in response to 17β-estradiol suggest that a similar adaptive mechanism exists in both species, although the magnitude of Δ6-desaturase response to 17β-estradiol in humans should be assessed.

Similarly, the PUFA composition of the rodent diet used in this work in relation to North American dietary intakes suggests that these conclusions can be applied to human populations as well. While the amount of total PUFA is higher in this work (approximately 53% of total fatty acids in the rat chow, and 21% in intake estimates of North Americans), the ratio of n-6 to n-3 PUFA is 10:1 for both (Ervin et al. 2004; Kitson et al. 2012). The proportions of EPA and DHA are higher in the present work (0.29 and 0.24% of total fatty acids, respectively) as compared with the North American diet (0.04 and 0.1% of total fatty acids, respectively). However, it is unlikely that this higher n-3 PUFA content would confound the findings of the present thesis as sex differences in hepatic and plasma DHA were found that were similar to a previous study in which n-3 PUFA intakes were much lower [no detectable EPA or DHA, 0.5% of fatty acids as ALA, (Extier et al. 2010)]. In rats, sex differences in DHA synthesis are present when DHA is not consumed or consumed at low levels which is consistent with low intakes in North Americans (Denomme et al. 2005). However, evidence in humans suggests that the sex difference in blood DHA is not present when n-3 HUFA intakes are very high (Lohner et al. 2013), and sex differences in DHA synthesis disappear when subjects are fed a fish-based diet as compared with a beef-based diet {Pawlosky, 2003 5269 /id}. Further study of variable dietary n-3 HUFA intakes on the effects of sex, pregnancy, and 17β-estradiol is warranted to determine the

specific intake where sex difference disappear and the mechanisms involved, as this may be relevant to DHA requirements.

The studies in this thesis have some limitations, including a lack of direct measurement of DHA synthesis. Measures of microsomal Δ6-desaturase activity or whole-body DHA synthesis using isotopically labelled fatty acids would provide clear evidence of changes in DHA metabolism. In addition, it is not known if other mechanisms regulate DHA levels in response to 17β-estradiol besides the expression of biosynthetic enzymes. Full transcriptome microarray may indicate metabolic pathways that are altered in response to the hormonal factors researched in this thesis, and may present alternative hypotheses for the regulation of DHA metabolism by 17β-estradiol. Similarly, the assessment of phospholipid acyl species via HPLC-mass spectrometry would provide insight into phospholipid remodelling activities that may mediate the altered accretion of DHA.

The results of this thesis work expand our understanding of the factors affecting DHA synthesis, particularly in situations of altered 17β-estradiol status. The mechanism underlying higher DHA in women relative to men and in pregnant relative to post-partum women appears to be differences in the circulating concentration of 17β-estradiol, which increases the expression of Δ6-desaturase and the hepatic and plasma concentrations of DHA, likely explaining changes in DHA status in situations of altered 17β-estradiol status. Future DHA dietary recommendations tailored to an individual's ability to synthesize DHA based on their 17β-estradiol status are possible. For example, postmenopausal women may require more DHA than premenopausal women to attain the same health benefits. In addition, a greater understanding of DHA synthesis may lead to greater DHA yields in agri- and aquaculture. Concerns have been raised regarding the environmental sustainability of current dietary DHA recommendations on the basis of

declining fish stocks (Jenkins et al. 2009), and research on factors to increase the synthesis of DHA has the potential to alleviate this limitation. The finding of a biochemical mechanism to provide DHA to a developing fetus further reinforces the importance of this fatty acid in fetal neurodevelopment, and indicates the essentiality of providing DHA to pre-term infants or any other perinatal population sensitive to DHA deficiency.

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APPENDIX 1

Supplementary Data for Chapter 5:

Tissue-specific sex differences in Δ6-desaturase expression and DHA concentration in rats fed a standard chow diet

Table A1.1: Enzyme and transcription factor protein expression in heart and brain of male and female rats

	Heart		Brain	
Enzyme /transcription factor	***Males***	***Females***	***Males***	***Females***
	Arbitrary units			
Acyl-CoA Oxidase	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	0.9 ± 0.1*
Δ6-desaturase	1.0 ± 0.3	0.9 ± 0.3	1.0 ± 0.2	1.0 ± 0.3
Δ5-desaturase	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.3	0.9 ± 0.4
Elovl 2	1.0 ± 0.2	1.1 ± 0.1	1.0 ± 0.4	0.9 ± 0.2
Elovl 5	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	0.8 ± 0.3
Peroxisome proliferator activated receptor	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.1 ± 0.2
Sterol response element binding protein 1c	1.0 ± 0.3	1.2 ± 0.2	1.0 ± 0.2	1.3 ± 0.4

Data is mean ± SD in arbitrary units. *: significantly different from males by independent samples T-test.

Table A1.2: Fatty acid concentration of triacylglycerol from liver, heart, and brain of male and female rats

	Liver		**Heart**		**Brain**	
	μg fatty acid/g organ					
Fatty Acid	***Females***	***Males***	***Females***	***Males***	***Female***	***Male***
16:0	701 ± 166	487 ± 194	544 ± 77	547 ± 153	371 ± 70	490 ± 91*
18:0	117 ± 23	82 ± 16*	324 ± 72	382 ± 78	441 ± 188	443 ± 182
Total SFA	846 ± 194	595 ± 220	934 ± 130	1022 ± 281	936 ± 274	1053 ± 252
16:1	26 ± 11	15 ± 2*	18 ± 11	21 ± 9	23 ± 21	14 ± 15
18:1n-7	49 ± 8	44 ± 15	57 ± 12	69 ± 22	16 ± 4	23 ± 7
18:1n-9	436 ± 90	305 ± 98	272 ± 90	269 ± 79	78 ± 14	129 ± 46*
Total MUFA	521 ± 109	378 ± 117	364 ± 120	382 ± 129	150 ± 10	201 ± 66
18:2n-6	1044 ± 261	911 ± 318	385 ± 122	437 ± 119	40 ± 10	115 ± 85
18:3n-6	29 ± 8	16 ± 2*	7 ± 2	12 ± 9	4 ± 3	5 ± 4
20:2n-6	7 ± 1	11 ± 2*	12 ± 5	23 ± 13	8 ± 10	9 ± 5
20:3n-6	8 ± 2	8 ± 3	14 ± 2	17 ± 14	7 ± 4	13 ± 10
20:4n-6	234 ± 71	133 ± 45*	103 ± 22	104 ± 33	26 ± 3	31 ± 8
22:4n-6	24 ± 7	24 ± 8	23 ± 13	33 ± 16	9 ± 7	12 ± 6
Total n-6 PUFA	1345 ± 314	1106 ± 369	561 ± 145	686 ± 205	112 ± 34	210 ± 97
18:3n-3	55 ± 23	44 ± 12	19 ± 8	28 ± 18	5 ± 3	7 ± 4
20:5n-3	30 ± 14	25 ± 6	15 ± 5	14 ± 10	10 ± 6	10 ± 6
22:5n-3	23 ± 6	30 ± 9	32 ± 7	48 ± 18	9 ± 3	13 ± 1
22:6n-3	57 ± 16	50 ± 18	58 ± 10	68 ± 32	26 ± 11	35 ± 13
Total n-3 PUFA	167 ± 48	151 ± 43	129 ± 26	177 ± 73	56 ± 23	81 ± 26
Total PUFA	1512 ± 359	1257 ± 411	690 ± 156	863 ± 276	168 ± 57	291 ± 113
Total Fatty Acids	2947 ± 667	2340 ± 674	2165 ± 399	2524 ± 587	1364 ± 271	1587 ± 384

Data is mean ± SD. *: significantly different from females ($p < 0.05$). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Table A1.3: Fatty acid concentrations of hepatic phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol in male and female rats.

	Phosphatidylcholine		**Phosphatidylethanolamine**		**Phosphatidylserine**		**Phosphatidylinolsitol**	
	µg fatty acid / g liver							
Fatty Acid	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
C 16:0	1021 ± 85	1706 ± 69*	487 ± 60	624 ± 57*	28 ± 5	35 ± 8	32 ± 4	42 ± 11
C 18:0	1944 ± 277	1552 ± 93*	1006 ± 88	962 ± 106	97 ± 17	93 ± 12	103 ± 16	110 ± 17
SFA	3074 ± 347	3407 ± 182	1546 ± 153	1645 ± 153	145 ± 23	153 ± 18	173 ± 24	183 ± 30
C 16:1	13.4 ± 1.5	21.7 ± 3.6*	4.1 ± 1.1	6.2 ± 1.2*	0.5 ± 0.2	0.9 ± 0.3	0.5 ± 0.3	0.7 ± 0.5
C 18:1n-7	101 ± 17	227 ± 25*	42 ± 7	105 ± 10*	2.3 ± 0.5	3.5 ± 1.3	2.3 ± 0.7	3.8 ± 1.2*
C 18:1n-9	146 ± 11	226 ± 22*	81 ± 10	156 ± 13*	10 ± 5	25 ± 15	27 ± 24	28 ± 41
MUFA	272 ± 25	492 ± 50*	139 ± 17	282 ± 19*	17 ± 6	33 ± 17	34 ± 26	38 ± 43
C 18:2n-6	698 ± 27	1112 ± 154*	216 ± 13	443 ± 80*	11 ± 3	13 ± 6	15 ± 5	23 ± 11
C 18:3n-6	28 ± 5	22 ± 3	10 ± 2	11 ± 1	0.8 ± 0.1	1.1 ± 0.6	0.7 ± 0.1	0.7 ± 0.2
C 20:2n-6	14 ± 2	42 ± 10*	6 ± 1	21 ± 4*	1.2 ± 0.3	1.4 ± 0.2	0.9 ± 0.2	1.5 ± 0.4
C 20:3n-6	21 ± 5	24 ± 5	10 ± 2	11 ± 2	0.9 ± 0.3	0.8 ± 0.1	0.8 ± 0.6	2.2 ± 0.7*
C 20:4n-6	1916 ± 295	2427 ± 101*	872 ± 90	1120 ± 123*	45 ± 11	50 ± 11	68 ± 11	82 ± 14
C 22:4n-6	11 ± 1	10 ± 2	25 ± 2	22 ± 2*	1.6 ± 0.4	1.4 ± 0.4	0.7 ± 0.1	0.8 ± 0.3
C 22:5n-6	9 ± 3	3 ± 1*	9 ± 2	4 ± 1*	0.9 ± 0.3	0.4 ± 0.2*	0.3 ± 0.4	0.3 ± 0.2
N-6	2698 ± 328	3637 ± 240*	1149 ± 103	1632 ± 202*	62 ± 14	68 ± 17	86 ± 15	110 ± 24
C 18:3n-3	7 ± 2	9 ± 1	4 ± 1	5 ± 1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.3	0.6 ± 0.2
C 20:5n-3	15 ± 4	13 ± 3	12 ± 2	11 ± 4	0.5 ± 0.1	0.6 ± 0.2	0.4 ± 0.2	0.3 ± 0.1
C 22:5n-3	48 ± 5	58 ± 4*	58 ± 5	71 ± 23*	2.4 ± 0.3	2.3 ± 0.8	1.2 ± 0.3	2.0 ± 0.6*
C 22:6n-3	477 ± 87	317 ± 52*	530 ± 85	320 ± 111*	23 ± 6	15 ± 4*	6 ± 2	5 ± 1
N-3	546 ± 89	398 ± 51*	605 ± 87	407 ± 135*	26 ± 7	18 ± 4*	8 ± 2	8 ± 2
PUFA	3245 ± 409	4035 ± 219*	1754 ± 181	2039 ± 109*	88 ± 20	86 ± 20	94 ± 16	118 ± 25
HUFA	2497 ± 385	2850 ± 113*	1516 ± 175	1558 ± 60	74 ± 18	70 ± 15	77 ± 12	93 ± 15
N-6/N-3	5.0 ± 0.4	9.3 ± 1.6*	1.9 ± 0.2	5.2 ± 4.4*	2.4 ± 0.4	3.8 ± 0.8*	12 ± 3	13 ± 3
Total	6719 ± 786	8145 ± 435*	3594 ± 350	4173 ± 338*	256 ± 47	280 ± 43	311 ± 50	349 ± 77

Data is mean ± SD. *: significantly different from females ($p < 0.05$). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

APPENDIX 2

Supplementary Data for Chapter 7

Hepatic Δ6-desaturase expression and DHA are increased by supplementation of ovariectomized rats with 17β-estradiol, but not progesterone

Table A2.1: Fatty acid relative percent composition in hepatic phospholipids of hormone-treated rats

	SHAM	OVX	OVX+E	OVX+P	OVX+PE
			Relative area %		
SFA[1]	45.58 ± 0.91 [ab]	45.83 ± 0.79 [ab]	44.51 ± 0.58 [a]	46.08 ± 0.98 [b]	45.84 ± 0.95 [ab]
MUFA[1]	5.53 ± 1.44	5.66 ± 1.88	6.39 ± 0.56	5.74 ± 1.43	5.53 ± 0.67
C 18:2n-6	11.62 ± 0.53 [a]	11.92 ± 0.79 [a]	10.14 ± 1.00 [b]	12.18 ± 0.82 [a]	9.05 ± 1.04 [b]
C 18:3n-6	0.36 ± 0.03	0.36 ± 0.02	0.36 ± 0.05	0.33 ± 0.11	0.30 ± 0.07
C 20:2n-6	0.20 ± 0.01 [ab]	0.22 ± 0.02 [ab]	0.26 ± 0.05 [b]	0.19 ± 0.04 [a]	0.21 ± 0.04 [ab]
C 20:3n-6	0.32 ± 0.04	0.33 ± 0.02	0.31 ± 0.09	0.31 ± 0.06	0.37 ± 0.04
C 20:4n-6	24.21 ± 0.76	24.56 ± 1.01	23.08 ± 0.93	23.94 ± 0.75	23.52 ± 0.98
C 22:4n-6	0.24 ± 0.04	0.22 ± 0.02	0.24 ± 0.04	0.23 ± 0.06	0.23 ± 0.04
C 22:5n-6	0.19 ± 0.04 [a]	0.15 ± 0.04 [a]	0.54 ± 0.24 [b]	0.17 ± 0.02 [a]	0.55 ± 0.16 [a]
N-6 PUFA	37.15 ± 1.06 [a]	37.77 ± 1.22 [a]	34.92 ± 1.16 [b]	37.35 ± 0.90 [a]	34.24 ± 1.26 [b]
C 18:3n-3	0.12 ± 0.04	0.09 ± 0.03	0.13 ± 0.03	0.13 ± 0.03	0.13 ± 0.04
C 20:5n-3	0.18 ± 0.06 [ab]	0.14 ± 0.04 [a]	0.24 ± 0.07 [bc]	0.18 ± 0.08 [ab]	0.27 ± 0.03 [c]
C 22:5n-3	0.78 ± 0.10 [a]	0.75 ± 0.10 [a]	1.33 ± 0.34 [b]	0.82 ± 0.14 [a]	1.21 ± 0.30 [b]
C 22:6n-3	8.51 ± 0.63 [a]	8.11 ± 0.68 [a]	10.87 ± 0.93 [b]	7.79 ± 0.37 [a]	11.39 ± 0.64 [b]
N-3 PUFA	9.58 ± 0.69 [a]	9.10 ± 0.70 [a]	12.57 ± 1.05 [b]	8.91 ± 0.52 [a]	13.01 ± 0.52 [b]
PUFA	46.73 ± 1.39	46.86 ± 1.45	47.49 ± 0.45	46.26 ± 0.98	47.24 ± 1.06
HUFA	34.43 ± 1.13 [a]	34.27 ± 1.54 [a]	36.60 ± 1.17 [b]	33.43 ± 1.25 [a]	37.54 ± 1.02 [b]
N-6/N-3	3.89 ± 0.27 [a]	4.17 ± 0.33 [a]	2.80 ± 0.32 [b]	4.20 ± 0.29 [a]	2.64 ± 0.18 [b]
HUFA Score	27.48 ± 1.35 [a]	26.26 ± 1.21 [a]	33.96 ± 2.12 [b]	26.26 ± 0.80 [a]	34.31 ± 1.59 [b]
Total	97.84 ± 1.04	98.36 ± 0.51	98.39 ± 0.75	98.09 ± 0.18	98.61 ± 0.44

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.
[1]Concentrations of individual SFA and MUFA can be found in (Marks 2012).

Table A2.2: Fatty acid concentration of hepatic phosphatidyl choline of hormone-treated rats

	SHAM	OVX	OVX+E	OVX+P	OVX+PE
			μg/g liver		
C 16:0	2041 ± 476[a]	2272 ± 244[ab]	2699 ± 223[b]	2332 ± 175[ab]	2596 ± 266[b]
C 18:0	4086 ± 785	3626 ± 403	3598 ± 249	3891 ± 271	3757 ± 304
SFA	6749 ± 1388	6501 ± 727	6736 ± 553	6873 ± 648	7096 ± 505
C 16:1	34 ± 6[a]	38 ± 5a[b]	51 ± 4[bc]	36 ± 3[ab]	58 ± 16[c]
C 18:1n-7	171 ± 26[a]	173 ± 15[a]	318 ± 26[b]	195 ± 17[a]	290 ± 59[b]
C 18:1n-9	556 ± 362	617 ± 133	711 ± 186	877 ± 526	600 ± 192
MUFA	782 ± 390	858 ± 144	1109 ± 193	1137 ± 544	967 ± 243
C 18:2n-6	1322 ± 144[ab]	1446 ± 96[a]	1311 ± 139[ab]	1542 ± 135[a]	1198 ± 195[b]
C 18:3n-6	41 ± 5	38 ± 8	40 ± 7	47 ± 12	41 ± 8
C 20:2n-6	23 ± 4[a]	29 ± 4[ab]	39 ± 5[c]	30 ± 4[ab]	36 ± 8[bc]
C 20:3n-6	46 ± 8	44 ± 5	53 ± 9	50 ± 5	55 ± 8
C 20:4n-6	2804 ± 339[a]	3138 ± 382[ab]	3296 ± 221[ab]	3226 ± 260[ab]	3401 ± 264[b]
C 22:4n-6	18 ± 5[a]	22 ± 3[a]	36 ± 7[b]	21 ± 3[a]	33 ± 4[b]
C 22:5n-6	14 ± 4[a]	11 ± 4[a]	67 ± 35[b]	16 ± 3[a]	69 ± 22[b]
N-6 PUFA	4268 ± 469	4729 ± 442	4843 ± 236	3891 ± 2200	4832 ± 363
C 18:3n-3	11 ± 2[a]	9 ± 4[a]	18 ± 4[b]	13 ± 2[ab]	14 ± 3[ab]
C 20:5n-3	16 ± 5[a]	10 ± 2[a]	41 ± 11[b]	13 ± 3[a]	40 ± 6[b]
C 22:5n-3	68 ± 9[a]	78 ± 7[a]	158 ± 45[b]	82 ± 9[a]	139 ± 31[b]
C 22:6n-3	738 ± 97[a]	824 ± 112[a]	1262 ± 171[b]	827 ± 115[a]	1293 ± 73[b]
N-3 PUFA	836 ± 104[a]	923 ± 114[a]	1476 ± 198[b]	936 ± 127[a]	1487 ± 55[b]
PUFA	5104 ± 563[a]	5652 ± 540[ab]	6319 ± 409[b]	4827 ± 2168[ab]	6319 ± 396[b]
HUFA	3706 ± 429[a]	4130 ± 499[a]	4913 ± 442[bc]	4236 ± 387[ab]	5031 ± 323[c]
N-6/N-3	5.1 ± 0.3[a]	5.1 ± 0.4[a]	3.3 ± 0.3[b]	4.3 ± 2.4[a]	3.2 ± 0.2[b]
HUFA Score	22 ± 1[a]	22 ± 1[a]	30 ± 1[b]	22 ± 1[a]	29 ± 1[b]
Total	13076 ± 1736	13415 ± 1357	14695 ± 1057	14219 ± 1619	15266 ± 1667

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.

Table A2.3: Fatty acid concentration of hepatic phosphatidyethanolamine of hormone-treated rats

	SHAM	OVX	OVX+E	OVX+P	OVX+PE
			μg/g liver		
C 16:0	1633 ± 279	1452 ± 402	1971 ± 674	1609 ± 497	1701 ± 408
C 18:0	3758 ± 593	3389 ± 935	3062 ± 1111	3727 ± 1211	2553 ± 652
SFA	6106 ± 1002	5520 ± 1577	5566 ± 1925	5848 ± 1864	4876 ± 1204
C 16:1	29 ± 4	28 ± 9	27 ± 9	22 ± 9	29 ± 12
C 18:1n-7	129 ± 24	113 ± 27	147 ± 55	128 ± 42	125 ± 30
C 18:1n-9	1204 ± 1215	473 ± 105	600 ± 288	567 ± 234	614 ± 393
MUFA	1446 ± 1245	692 ± 155	836 ± 361	797 ± 281	834 ± 418
C 18:2n-6	783 ± 175^{a}	762 ± 196^{a}	400 ± 135^{b}	791 ± 228^{a}	375 ± 85^{b}
C 18:3n-6	24 ± 4^{ab}	22 ± 8^{ab}	16 ± 7^{ab}	28 ± 10^{a}	15 ± 4^{b}
C 20:2n-6	17 ± 5	19 ± 6	20 ± 8	19 ± 7	15 ± 4
C 20:3n-6	40 ± 6	36 ± 12	38 ± 16	44 ± 14	31 ± 5
C 20:4n-6	2281 ± 321	2324 ± 643	1571 ± 461	2464 ± 797	1631 ± 375
C 22:4n-6	61 ± 13	62 ± 16	66 ± 20	64 ± 22	63 ± 15
C 22:5n-6	26 ± 7^{a}	17 ± 6^{a}	74 ± 52^{b}	21 ± 7^{a}	71 ± 25^{b}
N-6 PUFA	3232 ± 517	3243 ± 874	2185 ± 658	3431 ± 1075	2202 ± 486
C 18:3n-3	11 ± 3	7 ± 1	11 ± 5	10 ± 3	9 ± 3
C 20:5n-3	28 ± 8	19 ± 6	26 ± 13	25 ± 9	28 ± 5
C 22:5n-3	139 ± 14	133 ± 31	180 ± 76	150 ± 47	150 ± 33
C 22:6n-3	1414 ± 212	1284 ± 357	1506 ± 530	1339 ± 396	1611 ± 413
N-3 PUFA	1593 ± 224	1444 ± 391	1724 ± 609	1526 ± 447	1797 ± 440
PUFA	4826 ± 720	4687 ± 1259	3909 ± 1255	4957 ± 1509	3999 ± 919
HUFA	3990 ± 546	3877 ± 1060	3462 ± 1130	4109 ± 1268	3584 ± 838
EPA+DHA	1442 ± 213	1303 ± 362	1532 ± 540	1365 ± 404	1638 ± 417
N-6/N-3	2.0 ± 0.2^{a}	2.3 ± 0.1^{a}	1.3 ± 0.1^{b}	2.2 ± 0.2^{a}	1.2 ± 0.1^{b}
HUFA Score	40 ± 2^{a}	37 ± 1^{a}	49 ± 2^{b}	37 ± 2^{a}	50 ± 2^{b}
Total	12885 ± 2729	11380 ± 3113	10807 ± 3538	12240 ± 3871	10269 ± 2525

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.

Table A2.4: Fatty acid concentration of hepatic phosphatidylserine of hormone-treated rats

	SHAM	OVX	OVX+E	OVX+P	OVX+PE
			μg/g liver		
C 16:0	387 ± 184	208 ± 66	356 ± 267	313 ± 75	295 ± 55
C 18:0	1021 ± 477	584 ± 188	959 ± 632	709 ± 176	613 ± 100
SFA	1716 ± 711	1076 ± 285	1529 ± 955	1257 ± 260	1347 ± 153
C 16:1	21 ± 26^{a}	12 ± 5^{a}	18 ± 23^{a}	45 ± 14^{ab}	58 ± 23^{b}
C 18:1n-7	15 ± 10	10 ± 3	16 ± 9	23 ± 10	20 ± 6
C 18:1n-9	162 ± 83	218 ± 118	213 ± 121	201 ± 97	277 ± 185
MUFA	214 ± 89	260 ± 118	268 ± 139	289 ± 89	385 ± 162
C 18:2n-6	43 ± 11^{ab}	42 ± 7^{ab}	38 ± 12^{a}	49 ± 11^{ab}	56 ± 9^{b}
C 18:3n-6	5 ± 2	4 ± 3	5 ± 2	4 ± 1	4 ± 1
C 20:2n-6	11 ± 5	8 ± 1	12 ± 6	9 ± 1	8 ± 1
C 20:3n-6	0.7 ± 0.4	0.4 ± 0.4	0.5 ± 0.5	0.7 ± 0.3	0.8 ± 0.5
C 20:4n-6	115 ± 49	128 ± 25	121 ± 18	151 ± 37	128 ± 25
C 22:4n-6	1.8 ± 0.6	2.2 ± 0.8	2.3 ± 1.6	1.2 ± 0.6	1.4 ± 0.6
C 22:5n-6	2.0 ± 1.0^{a}	1.3 ± 1.0^{a}	6.8 ± 1.7^{b}	1.8 ± 0.7^{a}	7.4 ± 2.2^{b}
N-6 PUFA	179 ± 63	187 ± 30	187 ± 29	218 ± 49	206 ± 25
C 18:3n-3	0.5 ± 0.3	0.4 ± 0.4	0.2 ± 0.2	0.4 ± 0.5	0.5 ± 0.4
C 20:5n-3	8 ± 4	4 ± 1	6 ± 1	7 ± 4	5 ± 1
C 22:5n-3	8 ± 4	4 ± 2	6 ± 5	3 ± 2	3 ± 1
C 22:6n-3	66 ± 28^{a}	73 ± 15^{ab}	104 ± 8^{c}	76 ± 12^{abc}	96 ± 14^{bc}
N-3 PUFA	84 ± 32	83 ± 15	114 ± 8	88 ± 8	105 ± 15
PUFA	262 ± 95	270 ± 40	294 ± 29	307 ± 53	312 ± 35
HUFA	203 ± 81	204 ± 50	241 ± 22	243 ± 42	228 ± 55
EPA+DHA	75 ± 29	67 ± 31	108 ± 7	84 ± 8	87 ± 39
N-6/N-3	2.2 ± 0.2^{a}	2.3 ± 0.4^{a}	1.6 ± 0.2^{b}	2.5 ± 0.5^{a}	2.0 ± 0.3^{b}
HUFA Score	42 ± 3^{ab}	34 ± 10^{a}	47 ± 3^{b}	37 ± 5^{ab}	38 ± 14^{ab}
Total	2271 ± 805	1663 ± 296	1755 ± 353	1921 ± 308	2115 ± 179

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: percentage of total HUFA as n-3 HUFA. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.

Table A2.5: Fatty acid concentration of hepatic phosphatidylinositol of hormone-treated rats

	SHAM	OVX	OVX+E	OVX+P	OVX+PE
			μg/g liver		
C 16:0	259 ± 70^{ab}	324 ± 113^{a}	351 ± 47^{a}	156 ± 35^{b}	261 ± 64^{ab}
C 18:0	1007 ± 193^{a}	1001 ± 195^{a}	1009 ± 93^{a}	739 ± 137^{b}	871 ± 85^{ab}
SFA	1594 ± 263^{a}	1669 ± 361^{a}	1616 ± 122^{a}	1073 ± 214^{b}	1525 ± 157^{a}
C 16:1	9 ± 1	38 ± 38	38 ± 29	4 ± 4	25 ± 14
C 18:1n-7	10 ± 4^{ab}	30 ± 23^{a}	26 ± 9^{ab}	8 ± 3^{b}	23 ± 9^{ab}
C 18:1n-9	108 ± 78	253 ± 142	241 ± 135	111 ± 86	229 ± 81
MUFA	146 ± 86	343 ± 196	327 ± 142	138 ± 93	304 ± 91
C 18:2n-6	61 ± 12	87 ± 24	88 ± 20	68 ± 14	79 ± 22
C 18:3n-6	4 ± 2	6 ± 2	4 ± 1	4 ± 1	4 ± 1
C 20:2n-6	10 ± 1	13 ± 4	15 ± 4	10 ± 2	14 ± 5
C 20:3n-6	0.6 ± 0.3	0.4 ± 0.3	1.1 ± 0.6	0.5 ± 0.5	0.4 ± 0.3
C 20:4n-6	489 ± 81	460 ± 82	498 ± 80	460 ± 127	503 ± 41
C 22:4n-6	2 ± 1	2 ± 1	3 ± 1	2 ± 2	2 ± 1
C 22:5n-6	1.1 ± 0.9	0.6 ± 0.3	2.3 ± 1.0	1.5 ± 2.2	2.2 ± 1.3
N-6 PUFA	569 ± 91	569 ± 102	612 ± 90	546 ± 129	606 ± 59
C 18:3n-3	0.5 ± 1.0	0.3 ± 0.4	0.9 ± 1.0	0.9 ± 0.6	0.3 ± 0.3
C 20:5n-3	6 ± 3^{ab}	6 ± 2^{a}	6 ± 1^{ab}	2 ± 1^{b}	4 ± 1^{ab}
C 22:5n-3	4 ± 1	4 ± 2	3 ± 2	2 ± 1	3 ± 1
C 22:6n-3	11 ± 5^{a}	22 ± 12^{abc}	30 ± 7^{c}	15 ± 8^{ab}	27 ± 7^{bc}
N-3 PUFA	23 ± 6^{a}	33 ± 14^{ab}	41 ± 7^{b}	22 ± 9^{a}	35 ± 6^{ab}
PUFA	592 ± 93	602 ± 110	652 ± 92	568 ± 131	642 ± 64
HUFA	515 ± 84	495 ± 90	544 ± 81	485 ± 128	543 ± 45
N-6/N-3	26 ± 5^{ab}	19 ± 5^{ab}	15 ± 3^{a}	29 ± 13^{b}	17 ± 2^{ab}
HUFA Score	4 ± 1^{a}	7 ± 2^{ab}	7 ± 2^{b}	4 ± 2^{a}	6 ± 1^{ab}
Total	2423 ± 306^{ab}	2710 ± 593^{a}	2690 ± 267^{a}	1847 ± 385^{b}	2565 ± 249^{a}

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.

Table A2.6: fatty acid concentration of plasma non-esterified fatty acids of hormone-treated rats

	SHAM	OVX	OVX + E	OVX + P	OVX+ PE
			µg/ml plasma		
SFA[1]	32 ± 4	44 ± 8	38 ± 4	33 ± 9	43 ± 7
MUFA[1]	36 ± 12	76 ± 49	77 ± 16	44 ± 14	81 ± 39
C 18:2n-6	8 ± 2	11 ± 3	8 ± 1	8 ± 3	10 ± 2
C 18:3n-6	0.18 ± 0.11	0.24 ± 0.1	0.30 ± 0.10	0.27 ± 0.02	0.21 ± 0.11
C 20:2n-6	0.9 ± 0.1	0.8 ± 0.3	0.6 ± 0.3	0.8 ± 0.1	0.8 ± 0.2
C 20:3n-6	0.38 ± 0.04	0.44 ± 0.05	0.36 ± 0.13	0.38 ± 0.08	0.37 ± 0.07
C 20:4n-6	1.6 ± 0.9	0.9 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	1.0 ± 0.5
C 22:2n-6	0.12 ± 0.05	0.12 ± 0.08	0.16 ± 0.02	0.10 ± 0.03	0.09 ± 0.06
C 22:4n-6	0.13 ± 0.01	0.13 ± 0.04	0.14 ± 0.05	0.10 ± 0.03	0.10 ± 0.06
C 22:5n-6	0.10 ± 0.02	0.11 ± 0.08	0.11 ± 0.04	0.13 ± 0.08	0.11 ± 0.03
N-6 PUFA	12 ± 2	14 ± 3	10 ± 1	10 ± 3	13 ± 3
C 18:3n-3	0.4 ± 0.1[a]	0.7 ± 0.2[b]	0.3 ± 0.1[a]	0.3 ± 0.2[a]	0.5 ± 0.1[ab]
C 20:5n-3	0.09 ± 0.03	0.10 ± 0.03	0.10 ± 0.02	0.07 ± 0.03	0.12 ± 0.04
C 22:5n-3	0.36 ± 0.05	0.37 ± 0.04	0.32 ± 0.04	0.35 ± 0.04	0.39 ± 0.07
C 22:6n-3	0.8 ± 0.3	1.2 ± 1.0	1.3 ± 0.9	0.4 ± 0.2	0.7 ± 0.4
N-3 PUFA	1.6 ± 0.2	2.5 ± 1.3	2.0 ± 0.8	1.1 ± 0.4	1.7 ± 0.6
PUFAs	13 ± 2	17 ± 4	12 ± 1	12 ± 3	15 ± 3
HUFAs	3 ± 1	3 ± 1	3 ± 1	2 ± 1	3 ± 1
N-6/N-3	7 ± 2	7 ± 3	6 ± 3	10 ± 3	8 ± 2
HUFA Score	37 ± 6	48 ± 14	54 ± 10	37 ± 4	42 ± 8
Total	81 ± 16	137 ± 59	127 ± 17	88 ± 25	138 ± 47

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA. [1]Concentrations of individual SFA and MUFA can be found in (Marks 2012).

Table A2.7: fatty acid concentration of plasma triacylglycerol of hormone-treated rats

	SHAM	**OVX**	**OVX+E**	**OVX+P**	**OVX+PE**
			µg/ml plasma		
SFA[1]	72 ± 39	69 ± 19	65 ± 13	76 ± 8	51 ± 10
MUFA[1]	111 ± 42	104 ± 65	90 ± 23	152 ± 73	65 ± 19
C 18:2n-6	12 ± 4	12 ± 8	17 ± 9	17 ± 10	11 ± 6
C 18:3n-6	0.3 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
C 20:3n-6	0.4 ± 0.1	0.6 ± 0.4	0.5 ± 0.2	0.4 ± 0.2	0.3 ± 0.1
C 20:4n-6	3 ± 2	3 ± 2	5 ± 3	2 ± 1	3 ± 2
C 22:4n-6	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.3	0.2 ± 0.2	0.6 ± 0.2
C 22:5n-6	0.23 ± 0.12	0.32 ± 0.16	0.28 ± 0.2	0.36 ± 0.05	0.23 ± 0.06
N-6 PUFA	16 ± 6	16 ± 9	23 ± 13	20 ± 10	15 ± 8
C 18:3n-3	0.34 ± 0.18	0.38 ± 0.18	0.47 ± 0.36	0.31 ± 0.03	0.42 ± 0.22
C 20:5n-3	0.2 ± 0.2	0.4 ± 0.2	0.6 ± 0.6	0.3 ± 0.2	0.4 ± 0.4
C 22:5n-3	0.4 ± 0.2	0.7 ± 0.3	0.8 ± 0.4	0.5 ± 0.1	0.5 ± 0.1
C 22:6n-3	0.9 ± 0.3	0.7 ± 0.3	1.5 ± 1.4	0.7 ± 0.2	0.6 ± 0.3
N-3 PUFA	1.92 ± 0.79	2.16 ± 0.75	3.35 ± 2.76	1.82 ± 0.02	1.98 ± 0.96
PUFA	18 ± 7	19 ± 10	27 ± 15	22 ± 10	17 ± 9
HUFA	5.3 ± 3.0	6.2 ± 3.0	9.0 ± 6.2	4.2 ± 0.4	5.8 ± 2.7
N-6/N-3	9 ± 3	8 ± 3	9 ± 3	11 ± 6	8 ± 2
HUFA Score	34 ± 14	30 ± 7	30 ± 6	36 ± 4	27 ± 4
Total	201 ± 84	192 ± 82	182 ± 37	249 ± 85	133 ± 30

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA. [1]Concentration of individual SFA and MUFA can be found in (Marks 2012).

Table A2.8: fatty acid concentration of plasma cholesteryl esters of hormone-treated rats.

	SHAM	OVX	OVX + E	OVX + P	OVX+ PE
Fatty Acid			*µg/ml plasma*		
SFA[1]	33 ± 11 [a]	38 ± 14 [a]	39 ± 11 [a]	75 ± 19 [b]	41 ± 16 [a]
MUFA[1]	37 ± 17	71 ± 38	57 ± 24	100 ± 43	80 ± 53
C 18:2n-6	11 ± 1	16 ± 3	15 ± 4	20 ± 6	17 ± 5
C 18:3n-6	0.5 ± 0.2 [a]	0.4 ± 0.2 [a]	0.6 ± 0.3 [a]	1.4 ± 0.6 [b]	0.9 ± 0.4 [ab]
C 20:2n-6	0.9 ± 0.2 [a]	0.8 ± 0.1 [a]	1.1 ± 0.7 [ab]	2.0 ± 0.8 [b]	0.9 ± 0.3 [a]
C 20:3n-6	0.6 ± 0.2 [a]	0.7 ± 0.2 [a]	0.8 ± 0.5 [a]	1.7 ± 0.7 [b]	0.7 ± 0.2 [a]
C 20:4n-6	36 ± 4 [a]	43 ± 3 [ab]	52 ± 8 [ab]	38 ± 11 [ab]	56 ± 14 [b]
C 22:2n-6	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.3	0.2 ± 0.1
C 22:4n-6	0.15 ± 0.03	0.12 ± 0.06	0.16 ± 0.19	0.24 ± 0.15	0.22 ± 0.22
C 22:5n-6	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.3	0.6 ± 0.6	0.2 ± 0.1
N-6 PUFA	50 ± 4 [a]	61 ± 7 [ab]	70 ± 11 [ab]	64 ± 13 [ab]	75 ± 16 [b]
C 18:3n-3	0.26 ± 0.02	0.32 ± 0.07	0.22 ± 0.06	0.54 ± 0.44	0.69 ± 0.94
C 20:5n-3	0.4 ± 0.1 [a]	0.4 ± 0.1 [a]	0.8 ± 0.4 [bc]	0.5 ± 0.1 [ab]	1.0 ± 0.2 [b]
C 22:5n-3	0.6 ± 0.1 [a]	0.6 ± 0.2 [a]	0.7 ± 0.3 [a]	1.3 ± 0.4 [b]	0.6 ± 0.2 [a]
C 22:6n-3	1.3 ± 0.1	2.4 ± 1.6	2.4 ± 0.4	2.1 ± 1.1	2.7 ± 0.5
N-3 PUFA	2.5 ± 0.3	3.7 ± 1.9	4.1 ± 0.6	4.5 ± 1.1	4.9 ± 1.3
PUFAs	52 ± 4 [a]	65 ± 9 [ab]	74 ± 12 [ab]	69 ± 13 [ab]	80 ± 17 [b]
HUFAs	39 ± 4 [a]	47 ± 5 [ab]	57 ± 8 [ab]	45 ± 10 [ab]	61 ± 14 [b]
N-6/N-3	20 ± 2	19 ± 7	17 ± 1	15 ± 3	15 ± 2
HUFA Score	6 ± 1	7 ± 3	7 ± 1	9 ± 3	7 ± 1
Total	122 ± 25 [a]	174 ± 59 [ab]	169 ± 42 [ab]	243 ± 50 [b]	202 ± 67 [ab]

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA. [1]Concentration of individual SFA and MUFA can be found in (Marks 2012).

Table A2.9: Fatty acid concentration of adipose tissue total lipids of hormone-treated rats

	SHAM	OVX	OVX+E	OVX+P	OVX+PE
			µg/g adipose		
C 16:0	109571 ± 34226	109614 ± 30931	105216 ± 24041	117162 ± 26978	99456 ± 42714
C 18:0	24269 ± 6719	23070 ± 4432	24273 ± 6114	23092 ± 3725	21578 ± 7518
SFA	144064 ± 43595	142405 ± 36219	139593 ± 31981	150343 ± 32546	130572 ± 53477
C 16:1	13353 ± 5268	16343 ± 6646	13097 ± 2081	16832 ± 6841	14309 ± 7580
C 18:1n-7	13187 ± 3644	10405 ± 1885	13334 ± 2858	12451 ± 2391	12776 ± 5461
C 18:1n-9	137092 ± 41632	125726 ± 33821	133588 ± 36422	135364 ± 26186	115904 ± 56734
MUFA	165459 ± 50778	154072 ± 41850	161871 ± 41069	166321 ± 34908	143054 ± 65231
C 18:2n-6	210099 ± 63985	189366 ± 50841	199756 ± 60698	211686 ± 30055	164900 ± 72426
C 18:3n-6	886 ± 237	757 ± 162	806 ± 250	892 ± 89	713 ± 307
C 20:2n-6	1293 ± 425	1158 ± 315	1605 ± 499	1395 ± 194	1150 ± 465
C 20:3n-6	1118 ± 256	893 ± 169	1344 ± 376	1121 ± 164	1157 ± 429
C 20:4n-6	5568 ± 1086	6391 ± 930	6768 ± 1668	6974 ± 977	5789 ± 2840
C 22:4n-6	12 ± 8	12 ± 4	11 ± 7	8 ± 4	15 ± 13
C 22:5n-6	558 ± 200	337 ± 125	608 ± 253	389 ± 91	603 ± 341
N-6 PUFA	219625 ± 65952	199034 ± 51296	210990 ± 63237	222552 ± 31115	174594 ± 76650
C 18:3n-3	12838 ± 3899	13296 ± 3795	12380 ± 3420	14705 ± 2480	10974 ± 4994
C 20:3n-3	113 ± 36	106 ± 29	139 ± 51	127 ± 19	116 ± 58
C 20:5n-3	349 ± 97 [a]	449 ± 126 [ab]	392 ± 130 [ab]	569 ± 102 [b]	436 ± 113 [ab]
C 22:5n-3	1216 ± 188 [ab]	963 ± 245 [a]	1551 ± 402 [b]	1377 ± 237 [ab]	1407 ± 495 [ab]
C 22:6n-3	2213 ± 462	2109 ± 595	2452 ± 708	2581 ± 426	2309 ± 992
N-3 PUFA	16729 ± 4561	16923 ± 4502	16915 ± 4584	19359 ± 3177	15241 ± 6568
PUFA	236354 ± 70440	215957 ± 55598	227905 ± 67696	241911 ± 34167	189835 ± 83170
HUFA	11148 ± 2170	11260 ± 1943	13266 ± 3481	13147 ± 1721	11565 ± 5835
N-6/N-3	13 ± 1 [a]	12 ± 1 [ab]	12 ± 1 [ab]	12 ± 1 [b]	11 ± 1 [b]
HUFA Score	35 ± 1	32 ± 4	34 ± 2	35 ± 2	30 ± 13
Total	545876 ± 164154	512433 ± 130595	529368 ± 139368	558575 ± 99950	464500 ± 198472

Values are mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with ad libitum access to food, OVX+PF: ovariectomized rats pair-fed to SHAM rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids. Values with a different letter superscript are significantly different by Tukey's post hoc test following significant F value by one-way ANOVA.

APPENDIX 3

Supplementary Data for Chapter 8

Ovariectomy with or without food restriction elicits no effect son hepatic and plasma DHA concentration or hepatic Δ6-desaturase expression

Table A3.1: Fatty acid concentration of hepatic phosphatidylcholine of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		μg/g liver	
C 16:0	1185 ± 284	1497 ± 170	1471 ± 306
C 18:0	1968 ± 420	1990 ± 235	1994 ± 649
SFA	6660 ± 1561	7392 ± 847	7322 ± 1903
C 16:1	28 ± 14	37 ± 10	47 ± 21
C 18:1n-7	97 ± 19	115 ± 21	111 ± 22
C 18:1n-9	238 ± 115	256 ± 62	308 ± 100
MUFA	811 ± 320	913 ± 184	1019 ± 305
C 18:2n-6	1642 ± 431	1794 ± 261	1819 ± 468
C 18:3n-6	31 ± 9	27 ± 5	34 ± 11
C 20:2n-6	26 ± 4	32 ± 6	29 ± 4
C 20:3n-6	43 ± 10^{a}	47 ± 8^{ab}	60 ± 12^{b}
C 20:4n-6	3533 ± 696	3893 ± 548	3916 ± 1043
C 22:4n-6	18 ± 5	22 ± 7	22 ± 6
C 22:5n-6	16 ± 2^{a}	12 ± 3^{b}	17 ± 3^{a}
N-6 PUFA	5309 ± 1118	5826 ± 807	5898 ± 1517
C 18:3n-3	14 ± 5	17 ± 3	15 ± 5
C 20:5n-3	26 ± 9	18 ± 4	19 ± 9
C 22:5n-3	77 ± 16	91 ± 15	90 ± 22
C 22:6n-3	711 ± 168	729 ± 159	753 ± 195
N-3 PUFA	828 ± 192	854 ± 173	878 ± 227
PUFA	6137 ± 1303	6680 ± 968	6775 ± 1730
HUFA	4424 ± 889	4811 ± 724	4877 ± 1267
N-6/N-3	6.4 ± 0.4	6.9 ± 0.6	6.7 ± 0.6
HUFA Score	18 ± 1	17 ± 1	18 ± 1
Total	13893 ± 3237	15369 ± 1953	15531 ± 4057

Data is mean ± SD. Values within a fatty acid with a different superscript are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.2: Fatty acid concentration of hepatic phosphatidylethanolamine of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		µg/g liver	
C 16:0	858 ± 86	849 ± 241	929 ± 206
C 16:0 dma	9 ± 1	11 ± 2	12 ± 4
C 18:0	1798 ± 262	1568 ± 430	1649 ± 706
SFA	5859 ± 576	5407 ± 1549	5665 ± 1987
C 16:1	153 ± 115	129 ± 54	109 ± 109
C 18:1 dma	8 ± 0	8 ± 2	7 ± 2
C 18:1n-7	73 ± 19	75 ± 34	71 ± 17
C 18:1n-9	197 ± 31	177 ± 34	262 ± 102
MUFA	887 ± 214	1006 ± 538	948 ± 474
C 18:2n-6	720 ± 121	797 ± 200	707 ± 230
C 18:3n-6	15 ± 7	15 ± 4	15 ± 7
C 20:2n-6	13 ± 4	16 ± 5	15 ± 1
C 20:3n-6	30 ± 4	25 ± 7	32 ± 7
C 20:4n-6	2780 ± 432	2428 ± 681	2518 ± 923
C 22:4n-6	57 ± 3	52 ± 21	52 ± 18
C 22:5n-6	19 ± 4	19 ± 7	22 ± 7
N-6 PUFA	3634 ± 531	3352 ± 906	3361 ± 1184
C 18:3n-3	12 ± 3	13 ± 7	10 ± 5
C 20:5n-3	27 ± 9	19 ± 5	19 ± 15
C 22:5n-3	115 ± 17	110 ± 28	138 ± 28
C 22:6n-3	1284 ± 238	966 ± 292	1174 ± 461
N-3 PUFA	1438 ± 254	1107 ± 329	1341 ± 507
PUFA	5072 ± 752	4459 ± 1232	4702 ± 1689
HUFA	4313 ± 663	3619 ± 1032	3955 ± 1456
N-6/N-3	2.5 ± 0.2^{a}	3.0 ± 0.2^{b}	2.5 ± 0.1^{a}
HUFA Score	33 ± 2^{a}	30 ± 1^{b}	34 ± 1^{a}
Total	12290 ± 1246	11538 ± 3380	12057 ± 4490

Data is mean ± SD. Values within a fatty acid with a different superscript are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.3: Fatty acid concentration of hepatic phosphatidylserine of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		μg/g liver	
C 16:0	203 ± 31	249 ± 122	220 ± 112
C 18:0	720 ± 83	751 ± 277	797 ± 383
SFA	987 ± 113	1065 ± 418	1079 ± 525
C 16:1	8 ± 3	10 ± 5	10 ± 6
C 18:1n-7	23 ± 7	21 ± 8	23 ± 9
C 18:1n-9	110 ± 33	129 ± 56	111 ± 68
MUFA	231 ± 35	349 ± 100	267 ± 107
C 18:2n-6	50 ± 11[a]	31 ± 9[b]	44 ± 14[ab]
C 18:3n-6	4 ± 2	4 ± 1	4 ± 1
C 20:3n-6	5 ± 2	4 ± 1	5 ± 1
C 20:4n-6	223 ± 58	161 ± 105	233 ± 79
C 22:2n-6	11 ± 2	15 ± 4	15 ± 5
C 22:4n-6	8 ± 3	7 ± 3	8 ± 3
C 22:5n-6	13 ± 4	8 ± 5	14 ± 5
N-6 PUFA	328 ± 73	273 ± 67	333 ± 102
C 18:3n-3	1.1 ± 0.6	2.1 ± 2.4	1.9 ± 0.2
C 20:5n-3	4 ± 2	3 ± 1	3 ± 1
C 22:5n-3	12 ± 5	10 ± 3	11 ± 4
C 22:6n-3	120 ± 25	86 ± 28	118 ± 40
N-3 PUFA	138 ± 27	116 ± 45	134 ± 44
PUFA	466 ± 94	389 ± 98	468 ± 144
HUFA	386 ± 84	295 ± 129	390 ± 127
N-6/N-3	2.4 ± 0.5	2.5 ± 0.7	2.5 ± 0.2
HUFA Score	36 ± 5	44 ± 22	34 ± 2
Total	1708 ± 231	1872 ± 640	1840 ± 792

Data is mean ± SD. Values within a fatty acid with a different superscript are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.4: Fatty acid concentration of hepatic phosphatidylinositol of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		μg/g liver	
C 16:0	415 ± 81	358 ± 88	312 ± 53
C 18:0	1183 ± 171	1041 ± 322	1023 ± 233
SFA	1853 ± 301	1620 ± 441	1518 ± 356
C 16:1	44 ± 24	55 ± 30	47 ± 57
C 18:1n-7	31 ± 18	23 ± 10	24 ± 11
C 18:1n-9	174 ± 66	186 ± 131	108 ± 27
MUFA	361 ± 130	356 ± 111	266 ± 70
C 18:2n-6	72 ± 48	95 ± 46	73 ± 56
C 18:3n-6	1.7 ± 0.5	1.6 ± 1.5	1.5 ± 0.4
C 20:2n-6	1.3 ± 0.9	0.8 ± 0.3	1.4 ± 1.1
C 20:3n-6	6 ± 4	6 ± 2	10 ± 6
C 20:4n-6	656 ± 129	556 ± 247	611 ± 183
C 22:4n-6	4 ± 2	4 ± 2	3 ± 1
C 22:5n-6	8 ± 4	7 ± 2	6 ± 1
N-6 PUFA	756 ± 151	684 ± 283	719 ± 213
C 18:3n-3	2 ± 1	2 ± 1	2 ± 1
C 20:5n-3	3 ± 2	1 ± 1	1 ± 1
C 22:5n-3	8 ± 2	6 ± 2	8 ± 4
C 22:6n-3	21 ± 7	21 ± 11	22 ± 8
N-3 PUFA	34 ± 11	30 ± 12	34 ± 12
PUFA	790 ± 153	715 ± 286	752 ± 221
HUFA	705 ± 135	601 ± 251	661 ± 191
N-6/N-3	24 ± 8	24 ± 12	22 ± 5
HUFA Score	5 ± 1	5 ± 2	5 ± 1
Total	3233 ± 460	2905 ± 694	2726 ± 703

Data is mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.5: fatty acid concentration of liver triacylglycerol of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		µg/g liver	
C 16:0	1394 ± 208[a]	2367 ± 456[b]	1612 ± 396[a]
C 18:0	550 ± 66	702 ± 296	553 ± 118
SFA	2336 ± 307[a]	3484 ± 821[b]	2561 ± 509[a]
C 16:1	110 ± 29[a]	201 ± 37[b]	166 ± 73[ab]
C 18:1n-7	161 ± 23[a]	245 ± 72[b]	173 ± 49[ab]
C 18:1n-9	964 ± 203[a]	1796 ± 414[b]	1204 ± 327[a]
MUFA	1259 ± 231[a]	2282 ± 513[b]	1578 ± 398[a]
C 18:2n-6	1787 ± 396[a]	3309 ± 638[b]	1804 ± 523[a]
C 18:3n-6	36 ± 8[a]	61 ± 15[b]	43 ± 13[ab]
C 20:2n-6	12 ± 6[a]	24 ± 7[b]	13 ± 4[a]
C 20:3n-6	14 ± 4[a]	26 ± 6[b]	22 ± 7[ab]
C 20:4n-6	231 ± 36[a]	425 ± 140[b]	237 ± 71[a]
C 22:4n-6	29 ± 15	55 ± 19	47 ± 19
C 22:5n-6	15 ± 8	19 ± 7	16 ± 6
N-6 PUFA	2123 ± 442[a]	3919 ± 804[b]	2182 ± 615[a]
C 18:3n-3	86 ± 21[a]	173 ± 35[b]	78 ± 32[a]
C 20:5n-3	39 ± 10[a]	79 ± 22[b]	33 ± 13[a]
C 22:5n-3	28 ± 8[a]	57 ± 14[b]	37 ± 8[a]
C 22:6n-3	92 ± 18[a]	180 ± 49[b]	98 ± 30[a]
N-3 PUFA	246 ± 51[a]	489 ± 109[b]	247 ± 67[a]
PUFA	2369 ± 488[a]	4409 ± 907[b]	2429 ± 682[a]
HUFA	448 ± 70[a]	841 ± 234[b]	490 ± 131[a]
N-6/N-3	8.7 ± 0.8	8.1 ± 0.7	8.8 ± 0.4
HUFA Score	36 ± 3	38 ± 3	35 ± 1
Total	6281 ± 1026[a]	10533 ± 2250[b]	7063 ± 1614[a]

Data is mean ± SD. Values within a fatty acid with a different superscript are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.6: Fatty acid concentration of hepatic total lipids of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		µg/g liver	
C 16:0	15824 ± 3164[a]	24942 ± 6001[b]	16944 ± 3015[a]
C 18:0	20250 ± 3528[ab]	23787 ± 4680[a]	17045 ± 2504[b]
SFA	39069 ± 6881[ab]	52552 ± 11688[a]	37067 ± 5445[b]
C 16:1	587 ± 168[a]	1061 ± 381[b]	541 ± 106[a]
C 18:1n-7	1612 ± 286[a]	2362 ± 590[b]	1508 ± 160[a]
C 18:1n-9	6886 ± 2303[ab]	11177 ± 3597[a]	6727 ± 1104[b]
MUFA	9491 ± 2658[a]	15039 ± 4629[b]	9107 ± 1390[a]
C 18:2n-6	18032 ± 3566[a]	28829 ± 7209[b]	16206 ± 2731[a]
C 18:3n-6	426 ± 55[ab]	556 ± 165[a]	365 ± 73[b]
C 20:2n-6	194 ± 38	310 ± 182	285 ± 75
C 20:3n-6	298 ± 59[a]	466 ± 136[b]	447 ± 51[ab]
C 20:4n-6	18743 ± 3269[ab]	25294 ± 5327[a]	18165 ± 2655[b]
C 22:4n-6	327 ± 62[a]	551 ± 181[b]	450 ± 85[ab]
C 22:5n-6	150 ± 10	184 ± 46	208 ± 50
N-6 PUFA	38266 ± 6839[a]	56234 ± 12961[b]	36156 ± 5546[a]
C 18:3n-3	644 ± 139[a]	1124 ± 322[b]	417 ± 82[a]
C 20:5n-3	389 ± 118[ab]	594 ± 189[a]	228 ± 35[b]
C 22:5n-3	587 ± 138[a]	928 ± 240[b]	714 ± 165[ab]
C 22:6n-3	5208 ± 814	6692 ± 1685	5191 ± 816
N-3 PUFA	6828 ± 1156[ab]	9338 ± 2280[a]	6550 ± 1075[b]
PUFA	45095 ± 7922[a]	65572 ± 15162[b]	42706 ± 6619[a]
HUFA	25703 ± 4296[a]	34708 ± 7662[b]	25403 ± 3791[a]
N-6/N-3	5.6 ± 0.4	6.0 ± 0.4	5.5 ± 0.1
HUFA Score	24.1 ± 1.6	23.6 ± 1.0	24.1 ± 0.5
Total	97971 ± 17030[a]	137523 ± 32630[b]	91746 ± 13531[a]

Data is mean ± SD. Values within a fatty acid with a different superscript are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.7: Fatty acid concentration of plasma triacylglycerols of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		µg/ml plasma	
C 16:0	77 ± 26	117 ± 34	107 ± 26
C 18:0	44 ± 12	47 ± 11	47 ± 11
SFA	151 ± 53	192 ± 48	181 ± 32
C 16:1	5 ± 4	6 ± 5	6 ± 3
C 18:1n-7	5 ± 2	6 ± 2	7 ± 2
C 18:1n-9	50 ± 17^{a}	85 ± 4^{b}	90 ± 19^{b}
MUFA	64 ± 23^{a}	100 ± 7^{b}	109 ± 19^{b}
C 18:2n-6	50 ± 20^{a}	96 ± 38^{b}	93 ± 20^{b}
C 18:3n-6	1 ± 1	2 ± 1	2 ± 1
C 20:2n-6	0.2 ± 0.1^{a}	0.4 ± 0^{ab}	0.6 ± 0.4^{b}
C 20:3n-6	0.6 ± 0.3	0.4 ± 0.2	0.7 ± 0.5
C 20:4n-6	15 ± 6^{a}	24 ± 8^{ab}	29 ± 9^{b}
C 22:4n-6	0.8 ± 0.2^{a}	1.1 ± 0.3^{ab}	2.7 ± 1.9^{b}
C 22:5n-6	0.6 ± 0.1	0.8 ± 0.2	1.4 ± 0.9
N-6 PUFA	68 ± 26^{a}	124 ± 45^{b}	129 ± 31^{b}
C 18:3n-3	2 ± 1	4 ± 2	4 ± 1
C 20:5n-3	3 ± 1	4 ± 2	4 ± 1
C 22:5n-3	1.4 ± 0.4^{a}	1.9 ± 0.4^{ab}	2.7 ± 1.1^{b}
C 22:6n-3	4 ± 2	6 ± 2	7 ± 2
N-3 PUFA	10 ± 4	16 ± 6	17 ± 4
PUFA	78 ± 30^{a}	140 ± 50^{b}	146 ± 35^{b}
HUFA	25 ± 9^{a}	38 ± 13^{ab}	47 ± 16^{b}
N-6/N-3	7 ± 2	8 ± 1	8 ± 1
HUFA Score	32 ± 4	31 ± 2	29 ± 5
Total	302 ± 87^{a}	445 ± 97^{b}	448 ± 78^{b}

Data is mean ± SD. Values within a fatty acid with a different superscript are significantly different by Tukey's post-hoc test following significant F-value by one-way ANOVA. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.8: Fatty acid concentration of plasma cholesteryl esters of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		μg/ml plasma	
C 16:0	13 ± 4	13 ± 6	12 ± 8
C 18:0	6 ± 1	7 ± 4	7 ± 1
SFA	26 ± 6	30 ± 10	26 ± 8
C 16:1	1.3 ± 0.3	1.7 ± 0.3	1.5 ± 0.5
C 18:1n-7	0.9 ± 0.3	1.2 ± 0.3	1.1 ± 0.4
C 18:1n-9	10 ± 3	16 ± 11	13 ± 1
MUFA	14 ± 4	21 ± 12	17 ± 2
C 18:2n-6	26 ± 12	33 ± 10	31 ± 9
C 18:3n-6	1.1 ± 0.3	1.1 ± 0.2	1.2 ± 0.1
C 20:2n-6	0.4 ± 0.3	0.4 ± 0.3	0.3 ± 0.2
C 20:3n-6	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
C 20:4n-6	85 ± 29	103 ± 28	92 ± 18
C 22:4n-6	0.04 ± 0.01	0.04 ± 0.03	0.05 ± 0.01
C 22:5n-6	0.03 ± 0.02	0.12 ± 0.2	0.77 ± 1.48
N-6 PUFA	112 ± 41	138 ± 38	125 ± 26
C 18:3n-3	0.5 ± 0.2	0.8 ± 0.3	0.5 ± 0.2
C 20:5n-3	1.1 ± 0.4	1.1 ± 0.3	1.0 ± 0.2
C 22:5n-3	0.06 ± 0.05	0.11 ± 0.03	0.06 ± 0.03
C 22:6n-3	2.1 ± 0.8	2.5 ± 0.7	2.3 ± 0.5
N-3 PUFA	4 ± 1	5 ± 1	4 ± 1
PUFA	116 ± 42	143 ± 39	129 ± 27
HUFA	89 ± 30	107 ± 29	96 ± 19
N-6/N-3	28 ± 2	29 ± 3	31 ± 4
HUFA Score	3.9 ± 0.2	3.7 ± 0.4	3.7 ± 0.3
Total	156 ± 51	194 ± 30	173 ± 34

Data is mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.9: Fatty acid concentration of plasma non-esterified fatty acids of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		µg/ml plasma	
C 16:0	11 ± 2	12 ± 2	10 ± 2
C 18:0	8 ± 2	9 ± 2	7 ± 1
SFA	27 ± 7	30 ± 3	25 ± 3
C 16:1	0.6 ± 0.3	1.2 ± 0.4	0.6 ± 0.4
C 18:1n-7	0.5 ± 0.1	0.9 ± 0.6	0.4 ± 0.3
C 18:1n-9	5 ± 1	8 ± 4	6 ± 2
MUFA	7 ± 1	12 ± 5	7 ± 3
C 18:2n-6	5 ± 1	7 ± 2	5 ± 3
C 18:3n-6	0.19 ± 0.03	0.38 ± 0.4	0.27 ± 0.08
C 20:2n-6	0.9 ± 1.2	1.2 ± 1.0	0.3 ± 0.1
C 20:3n-6	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.01
C 20:4n-6	0.8 ± 0.1	1.0 ± 0.3	1.2 ± 0.9
C 22:4n-6	0.09 ± 0.03	0.06 ± 0.04	0.06 ± 0.04
C 22:5n-6	0.02 ± 0.01	0.04 ± 0.03	0.01 ± 0.02
N-6 PUFA	7 ± 2	10 ± 2	7 ± 4
C 18:3n-3	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.2
C 20:5n-3	0.11 ± 0.04	0.20 ± 0.16	0.09 ± 0.04
C 22:5n-3	0.07 ± 0.03	0.08 ± 0.03	0.07 ± 0.03
C 22:6n-3	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
N-3 PUFA	0.8 ± 0.2	1.2 ± 0.4	0.7 ± 0.4
PUFA	8 ± 2	11 ± 3	7 ± 4
HUFA	1.4 ± 0.1	1.8 ± 0.5	1.6 ± 1.0
N-6/N-3	8 ± 1	8 ± 1	9 ± 1
HUFA Score	32 ± 5	34 ± 8	26 ± 7
Total	42 ± 9	52 ± 8	39 ± 6

Data is mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.10: Fatty acid concentration of adipose total lipids of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		μg/g adipose	
C 16:0	188023 ± 61002	165909 ± 40908	167064 ± 42627
C 18:0	42729 ± 24541	35559 ± 14745	33458 ± 7600
SFA	243598 ± 85705	212672 ± 52333	209170 ± 54144
C 16:1	36395 ± 11531	34475 ± 9891	34303 ± 10712
C 18:1n-7	9523 ± 2945	8277 ± 2879	10678 ± 2123
C 18:1n-9	221607 ± 70916	187064 ± 45530	208679 ± 49030
MUFA	269834 ± 84452	231863 ± 55939	255763 ± 61919
C 18:2n-6	286858 ± 103172	258803 ± 79115	224784 ± 70265
C 18:3n-6	1440 ± 620	1172 ± 378	794 ± 635
C 20:2n-6	1742 ± 761	1597 ± 610	1133 ± 510
C 20:3n-6	1199 ± 695	636 ± 356	694 ± 365
C 20:4n-6	7020 ± 2461	7009 ± 1761	4236 ± 1190
C 22:4n-6	1517 ± 889	1332 ± 797	823 ± 449
C 22:5n-6	582 ± 391	487 ± 334	294 ± 186
N-6 PUFA	300576 ± 108018	271692 ± 82730	233013 ± 73157
C 18:3n-3	20437 ± 6885	19656 ± 5982	15105 ± 5068
C 20:5n-3	672 ± 339	745 ± 280	354 ± 136
C 22:5n-3	1349 ± 854	1330 ± 800	611 ± 558
C 22:6n-3	2799 ± 1473	2675 ± 1566	1431 ± 689
N-3 PUFA	25375 ± 9166	24401 ± 7801	17713 ± 6228
PUFA	325951 ± 117039	296092 ± 90500	250725 ± 79256
HUFA	15257 ± 7035	14190 ± 6363	8042 ± 2756
N-6/N-3	11.9 ± 0.7	11.2 ± 0.4	13.4 ± 1.3
HUFA Score	31 ± 4	28 ± 7	24 ± 9
Total	859307 ± 281465	757320 ± 193574	730301 ± 192270

Data is mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.

Table A3.11: Fatty acid concentration of posterior hind limb skeletal muscle total lipids of ovariectomized rats with or without pair-feeding

	SHAM	**OVX+AL**	**OVX+PF**
		µg/g muscle	
C 16:0	3656 ± 954	3444 ± 732	3312 ± 1098
C 18:0	1673 ± 823	1988 ± 148	1896 ± 231
SFA	5643 ± 966	5790 ± 986	5501 ± 1421
C 16:1	249 ± 220	276 ± 148	359 ± 226
C 18:1n-7	1195 ± 1871	442 ± 79	404 ± 128
C 18:1n-9	2142 ± 1018	2451 ± 1156	2330 ± 1558
MUFA	3654 ± 2025	3224 ± 1195	3167 ± 1930
C 18:2n-6	4645 ± 2053	4827 ± 1885	3781 ± 2127
C 18:3n-6	23 ± 11	28 ± 8	24 ± 8
C 20:2n-6	43 ± 23	61 ± 12	44 ± 16
C 20:3n-6	83 ± 10	83 ± 7	78 ± 9
C 20:4n-6	1925 ± 102	1865 ± 67	1820 ± 79
C 22:4n-6	121 ± 16	123 ± 11	113 ± 16
C 22:5n-6	116 ± 28	109 ± 8	116 ± 20
N-6 PUFA	6929 ± 2044	7099 ± 1947	6081 ± 2013
C 18:3n-3	157 ± 118	240 ± 136	174 ± 146
C 20:5n-3	28 ± 14	37 ± 4	27 ± 9
C 22:5n-3	302 ± 26	299 ± 24	279 ± 43
C 22:6n-3	1474 ± 147	1376 ± 92	1368 ± 92
N-3 PUFA	1966 ± 191	1959 ± 122	1853 ± 282
PUFA	8894 ± 1927	9058 ± 2048	7934 ± 2292
HUFA	3734 ± 916	3899 ± 138	3805 ± 224
N-6/N-3	4 ± 1	4 ± 1	3 ± 1
HUFA Score	52 ± 16	44 ± 1	44 ± 1
Total	18595 ± 4378	18720 ± 4459	17085 ± 5722

Data is mean ± SD. SHAM: sham-operated rats, OVX+AL: ovariectomized rats with *ad libitum* access to food, OVX+PF: ovariectomized rats pair-fed to sham-operated rats. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids.