UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

The Effect of Gender, Pregnancy and Diet upon Rat Tissue Fatty Acid Composition and Immune Function

by

Caroline Elizabeth Childs

Thesis for the degree of Doctor of Philosophy

January 2009

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

SCHOOL OF MEDICINE

Doctor of Philosophy

THE EFFECTS OF GENDER, PREGNANCY AND DIET UPON RAT TISSUE FATTY ACID COMPOSITION AND IMMUNE FUNCTION

by Caroline Elizabeth Childs

This thesis uses a rat model to investigate the effects of gender, pregnancy and maternal dietary fatty acids during pregnancy on adult and fetal tissue fatty acid composition, particularly upon longer-chain (LC) omega-3 (n-3) polyunsaturated fatty acids (PUFA). The effects of pregnancy and of diet during pregnancy upon immune function are also investigated.

Gender differences in fatty acid composition were apparent in the rat with females having higher LC n-3 PUFA contents in plasma, liver and adipose tissue lipids. These differences appeared to result from gender differences in the activities of desaturase and elongase enzymes which generate LC n-3 PUFA rather than from differences in expression of these genes. Increased Δ6 desaturase mRNA expression was observed during pregnancy and correlated to plasma progesterone status.

The immune adaptations of rat pregnancy differed from those reported in human and murine studies. The rat did not demonstrate a reduced Th1:Th2 cytokine ratio during pregnancy, but had reduced expression of CD161 upon the cell surface of maternal blood and spleen NK cells.

Maternal dietary fatty acids during pregnancy significantly affected the fatty acid composition of maternal and fetal tissues, with tissue specific incorporation of LC n-3 PUFA into fetal tissues. Maternal diets rich in n-3 PUFA exerted significant effects upon maternal and fetal immune function, including changes in the expression of CD3 and CD8 upon both maternal and fetal cytotoxic T cells and in the number of $CD161^{+}$ cells (putative NK cells) in the fetal thymus. The maternal diet during pregnancy therefore has the capacity to alter both maternal and fetal immune function, which may have significant effects upon maternal and fetal resistance to infection, and may exert longer-term effects upon offspring health.

TABLE OF CONTENTS

Declaration of Authorship

I, **Caroline Elizabeth Childs** declare that the thesis entitled

The effect of gender, pregnancy and diet upon rat tissue fatty acid composition and immune function

and the works presented in the thesis are both my own and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly while in candidature for a research degree at this University;
- no part of this thesis has previously been submitted for a degree of any other qualification at this University of any other institution;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as:

  Childs, C.E, Romeu-Nadal, M., Burdge, G.C., & Calder, P.C. (2008) Gender differences in the n-3 fatty acid content of tissues. *Proceedings of the Nutrition Society* 67, 19-27.

- parts of this work have been presented as posters and oral presentations at conferences, including:

  7th Congress of the ISSFAL. Cairns Convention Centre, Queensland, 2006

  The Rank Prize Funds: Long Chain Omega-3 Polyunsaturated Fatty Acids - Human Nutritional Requirements and Provision in the Food Chain. Windermere, Cumbria, 2007

  Nutrition Society Summer Meeting: Diet and chronic disease. University of Ulster, Northern Ireland, 2007

  FASEB Summer Research Conference: Nutritional Immunology: Its Role in Health & Disease. Tucson, Arizona, 2007

  8th Congress of the ISSFAL. Kansas City, Missouri, 2008

Signed: _____

Dated: _____

Acknowledgements

I would like to thank my supervisor, Professor Philip Calder for all the valuable encouragement and guidance he has given me throughout my PhD, making this project an enjoyable and rewarding experience. I have especially enjoyed the opportunities to attend conferences, develop collaborations and supervise student projects. I would also like to thank Professor Robert Grimble, Dr Graham Burdge and Professor Alan Jackson who have helped and advised me in the development of my research ideas.

There are numerous people who have helped me with the laboratory methods I have used in this project, including Liz Miles, Jackie Madden, Fred Anthony, John Jackson, Christian Gelouf, Linda Ogilvie-Goddard and Tony Postle. I appreciated the valuable help that Meritxell Romeu-Nadal, Uta Enke, Tessa Romijn and Sam Hoile gave me during their time on student projects. It has been great to collaborate with other projects, especially Eleftheria Diakogiannaki and Michelle Philips. Special thanks go to Ali Fear and Annette West, who have helped me out in numerous ways, sharing the weekend workload, and generally covering for me in the lab when things get hectic!

My wonderful husband Malcolm has given me a huge amount of support throughout this PhD, reminding me to take breaks, enjoy myself and letting me vent off steam when necessary. The rest of my family, especially my parents Reg and Pauline Parsons, and my sister Alexandra Rouse have also been a great help, providing both practical and emotional support. My friends have been great fun over the course of this PhD, and particularly appreciated were those at work, including Paul, Amy, Stella, Maria, Hayati, Paula, Hadi, Bram, and those out in the real world, especially Maz and Lucy.

Abbreviations

11βHSD	11β-hydroxysteroid dehydrogenase
AA	arachidonic acid (20:4n-6)
ADHD	attention deficit/hyperactivity disorder
ALNA	α-linolenic acid (18:3n-3)
APC	antigen presenting cells
AUC	area under curve
BHT	butylated hydroxytoluene
BMI	body mass index
CD	cluster of differentiation
cDNA	copy DNA
CE	cholesteryl ester
CHD	coronary heart disease
CM	chylomicron
Con A	concanavalin A
COX	cyclo-oxygenase
CPM	counts per minute
CVD	cardiovascular disease
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
DGLA	di-homo-γ-linolenic acid (20:3n-6)
DHA	docosahexaenoic acid (22:6n-3)
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid (22:5n-3)
DTH	delayed-type hypersensitivity
EPA	eicosapentaenoic acid (20:5n-3)
ER	endoplasmic reticulum
FADS1	fatty acid desaturase 1 (Δ5 desaturase)
FADS2	fatty acid desaturase 2 (Δ6 desaturase)
FAME	fatty acid methyl ester
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GLA	γ-linolenic acid (18:3n-6)
HDL	high density lipoprotein
HETE	hydroxyeicosatetraenoic acids
HF	high fat (13% fat w/w diet)
HLA	human leukocyte antigens
HPETE	hydroperoxyeicosatetraenoic acids
HRT	hormone replacement therapy
HSL	hormone-sensitive lipase
HUFA	highly-unsaturated fatty acid
ICAM	intracellular adhesion molecules
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
IP_3	inositol trisphosphate
LA	linoleic acid (18:2n-6)
LC	longer-chain
LDL	low density lipoprotein
LF	low fat (3% fat w/w diet)
LOX	lipoxygenase
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LT	leukotriene
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MJ	megajoule
mRNA	messenger RNA
n-3	omega 3
n-6	omega 6
n-9	omega 9
NEFA	non-esterified fatty acid
NK cell	natural killer cell
NKR-P1	natural killer receptor protein 1
NTC	no template control
OA	oleic acid (18:1n-9)
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	prostaglandin
PI	phosphatidylinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
PLA_2	phospholipase A_2
PMA	phorbol myristate acetate
PPAR	peroxisomal proliferators-activated receptors
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
RPE	R-phycoerythrin
RPMI	Roswell Park Memorial Institute (tissue culture medium)
rRNA	ribosomal RNA
RT-PCR	reverse-transcription PCR
SD	standard deviation
SEM	standard error of the mean
SI	stimulation index
SSC	side scatter
STAT	signal transducers and activators of transcription
TAG	triacylglycerol/triglyceride
Th cell	T helper cell
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecules
VLDL	very low density lipoprotein

Definitions - Fatty acid nomenclature

	Trivial name (abbreviation)	Systematic name
12:0	lauric acid	dodecanoic acid
14:0	myristic acid	tetradecanoic acid
16:0	palmitic acid	hexadecanoic acid
18:0	stearic acid	octadecanoic acid
20:0	arachidic acid	eicosanoic acid
22:0	behenic acid	docosanoic acid
16:1n-7	palmitoleic acid	cis-9-hexadecenoic acid
18:1n-9	oleic acid (OA)	cis-9-octadecanoic acid
20:1n-9	gondoic acid	eicosenoic acid
24:1n-9	nervonic acid	tetracosenoic acid
18:2n-6	linoleic acid (LA)	cis-9,cis-12-octadecadienoic acid
18:3n-6	γ-linolenic acid (GLA)	all-cis 6,9,12-octadecatrienoic acid
20:2n-6	eicosadienoic acid	cis-11, cis-14-eicosadienoic acid
20:3n-6	di-homo-γ-linolenic acid (DGLA)	all-cis 8,11,14 eicosatrienoic acid
20:4n-6	arachidonic acid (AA)	all-cis 5,8,14,17 eicoastetraenoic acid
22:5n-6	docosapentaenoic acid	all-cis 4,7,10,13,16-docosapentaenoic acid
18:3n-3	α-linolenic acid (ALNA)	all-cis 9,12,15-octadecatrienoic acid
20:4n-3	eicosatetraenoic acid	all-cis-8,11,14,17-eicosatetraenoic acid
20:5n-3	eicosapentaenoic acid (EPA)	all-cis 5,8,11,14,17 eicosapentaenoic acid
22:5n-3	docosapentaenoic acid (DPA)	all-cis 7,10,13,16,19 docosapentaenoic acid
22:6n-3	docosahexaenoic acid (DHA)	all-cis 4,7,10,13,16,19 docosahexaenoic acid

Chapter 1: Introduction and literature review

1.1 Introduction

The studies described in this thesis use a rat model to investigate the effects of gender, pregnancy and maternal dietary fatty acids during pregnancy upon both adult and fetal tissue fatty acid composition, particularly in relation to omega-3 (n-3) fatty acids. The effects of pregnancy and maternal dietary fatty acids during pregnancy upon immune function are also investigated.

This literature review will therefore provide relevant information on fatty acids, the immune system and the programming hypothesis. The available data on the effects of gender and pregnancy upon tissue fatty acid composition will be reviewed. Literature in support of potential programming of the immune system by maternal diet will also be covered.

1.2 Fatty acids

This section is intended to give a broad overview of fatty acids, including their structure, nomenclature, dietary intakes, endogenous synthesis and physiological functions in order that their interaction with the immune system can later be discussed in detail. Further information upon fatty acids is available elsewhere(1-3).

1.2.1 Structure and nomenclature

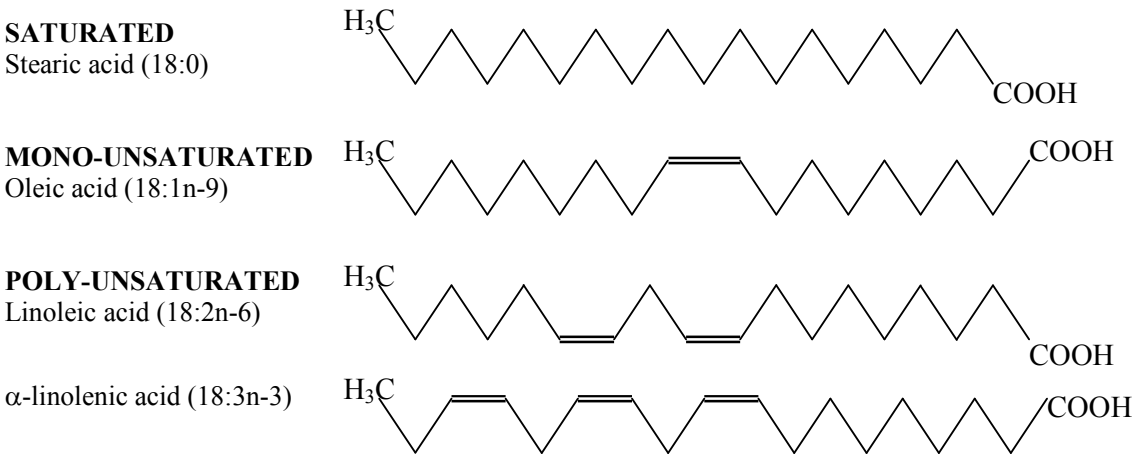
Fatty acids are hydrocarbon chains with a carboxyl (-COOH) group at one end and a methyl ($-CH_3$) group at the other. Biological fatty acids are typically unbranched chains with even numbers of carbon atoms (2-30 or more), with fatty acids in the human diet typically between 8 and 24 carbon atoms in length.

Fatty acids can be named using their trivial or common names, systematic names or shorthand notation. Details of the nomenclature of some common biological fatty acids are found in the definitions section (see page 9). Trivial names are often based on the origins of the fatty acid (e.g. palmitic acid is found in high levels in palm oil). Systematic names are determined by the structural properties of the fatty acid (e.g. the systematic name for palmitic acid is hexadecanoic acid as this fatty acid contains 16 carbons). However, this method of naming becomes complicated when naming complex fatty acids with double bonds and various *cis* vs. *trans* configurations. Shorthand notation can therefore be used to indicate the number of carbons, the number of double bonds, and their position in the fatty acid (counting from methyl terminus to

the position of the first double bond). For example, the shorthand notation for palmitic acid is 16:0 (16 carbons, no double bonds). Shorthand notation for linoleic acid (LA) is 18:2n-6 (18 carbons, 2 double bonds with the first one 6 carbons from the methyl terminus).

Fatty acids without any double bonds in the hydrocarbon chain are termed saturated fatty acids (e.g. stearic acid, 18:0). Fatty acids with double bonds in the hydrocarbon chain are termed unsaturated fatty acids. The number of double bonds in commonly encountered unsaturated fatty acids is between 1 and 6. Unsaturated fatty acids can therefore be further classified into monounsaturated fatty acids (MUFA) with one double bond (e.g. oleic acid, 18:1n-9) or polyunsaturated fatty acids (PUFA) with two or more double bonds (e.g. LA, 18:2n-6; ALNA, α-linolenic acid, 18:3n-3). PUFA can be further classified into omega-3 (n-3), omega-6 (n-6) and omega-9 fatty acids (n-9) based upon the location of their first double bond relative to the methyl terminus. Subsequent *cis*-configuration double bonds in PUFA are separated from one another by a single methylene ($-CH_2-$) group. The structure of some common fatty acids is illustrated in figure 1.1.

Figure 1.1: Structure of some common biological fatty acids



Double bonds in a fatty acid change the shape of the fatty acid, and so can result in different biological properties and functions. The effect of a double bond upon the function of a fatty acid will depend on the number, position and configuration of the double bonds present. Double bonds in biological fatty acids are usually in the *cis* configuration (see figure 1.2). *Trans* configuration double bonds are found in hydrogenated vegetable oils, ruminant fats, plant lipids and seed oils. For example,

conjugated LA (*cis*-9, *trans*-11 18:2) is formed in the rumen and mammary glands of cattle, and so is found in dairy products.

Figure 1.2: *Cis* and *trans* isomers of carbon-carbon double bonds

***Cis* double bond** ***Trans* double bond**

Fatty acids are hydrophobic, and so within biological fluids and tissues are packaged into complex lipid molecules which include, but are not limited to, phospholipids, triacylglycerols (sometimes called triglycerides; TAG) and cholesteryl esters (CE). Free fatty acids are termed non-esterified fatty acids (NEFA), and circulate in the plasma associated non-covalently with albumin. The incorporation of fatty acids into lipid structures is not random, with esterification controlled by enzymes which are specific to the position of the fatty acid. The carboxyl group on fatty acids readily reacts with molecules containing alcohol groups to form ester bonds by condensation (see figure 1.3). For example, fatty acids can form ester bonds with glycerol or cholesterol to form TAG and phospholipids or CE, respectively. The breaking of an ester bond is known as saponification or hydrolysis.

Cell membranes are composed of a phospholipid bilayer. Phospholipids are a glycerol backbone to which two fatty acids and a phosphate head group are attached. Phospholipids contain both hydrophobic regions (e.g. fatty acids) and hydrophilic regions (e.g. phosphate head groups) which allow their interaction with aqueous cellular compartments. The phosphate head group is used to classify the species of phospholipids with common head groups including choline, ethanolamine, serine and inositol. The basic structure of phospholipids can be seen in figure 1.4.

Figure 1.3: Formation of an ester bond between a fatty acid and glycerol

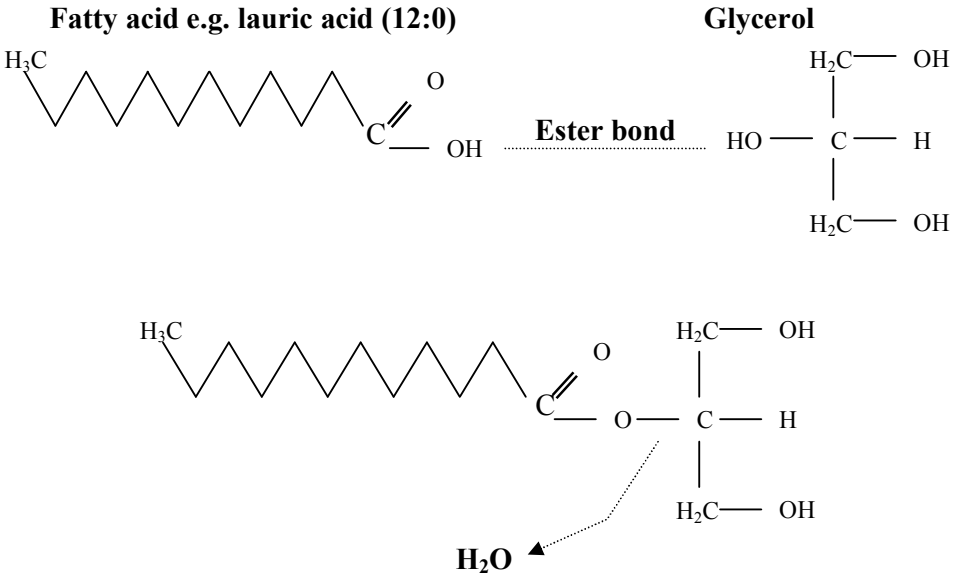
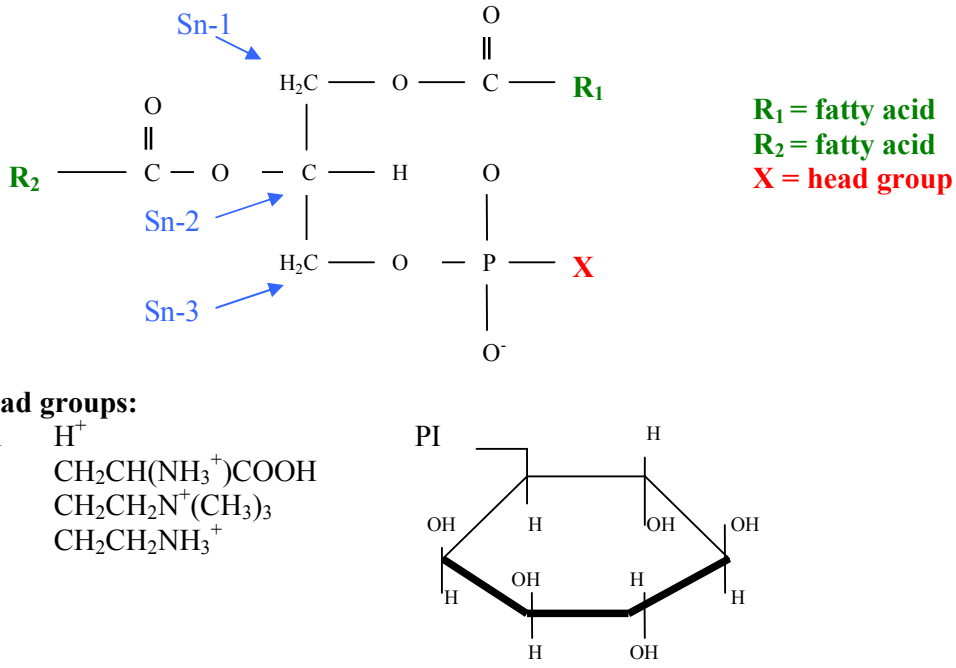


Figure 1.4: Phospholipid structure



Phosphatidylcholine (PC, also known as lecithin) is the major class of phospholipid found within plasma lipoproteins. PC typically has a saturated fatty acid such as palmitic or stearic acid at the sn-1 position and an unsaturated fatty acid such as oleic (OA), linoleic (LA), ALNA or arachidonic acid (AA, 20:4n-6) at sn-2. The cellular

location of phospholipids is often specific to particular phospholipid classes. For example, PC is found in plasma membranes localised on the outer leaflet, and phosphatidylethanolamine (PE) on the inner leaflet.

Acidic phospholipids such as phosphatidylserine (PS), phophatidylinositol (PI) and phosphatidic acid (PA) tend to be found on the inner leaflet of the plasma membrane of cells, and their presence in the outer leaflet triggers apoptosis of the cell. PI makes up around 5% of cell membrane phospholipids and has a sugar (inositol) as its head group. The fatty acids within this phospholipid are almost exclusively stearic acid (18:0) at the sn-1 position with AA at the sn-2 position.

CE and TAG do not contribute to cellular membranes but instead are incorporated into intracellular and circulating lipid particles. CE hydrolase enzymes within cells liberate cholesterol and free fatty acids as required for membrane and lipoprotein formation, and also provide cholesterol for hormone synthesis in adrenal cells. Free cholesterol within cell membranes decreases membrane fluidity.

1.2.2 Dietary fat intake

Most (90-95%) dietary fat is in the form of TAG, a lipid which contains three fatty acids esterified to glycerol. Other forms of dietary fats include phospholipids, glycolipids and cholesterol.

Over last 40 years total fat intake has decreased and the types of fats consumed have changed in Western populations, with declining consumption of saturated fatty acids and increasing intakes of PUFA. For example, table 1.1 shows daily dietary fat and fatty acid intakes among UK adults over time.

Table 1.1: Dietary fat intake among UK adults over the last 50 years (g/day)(3;4)

Year	Total fat	Saturates	MUFA	PUFA
1959	110	53	43	9.2
1969	120	56.7	46.5	11.0
1979	106	47.9	39.7	10.7
1989	90	36.9	33.1	13.6
2003 ♂	86.5	32.5	29.1	15.17 (n-6 = 12.9; n-3 = 2.27)
2003 ♀	61.4	23.3	20.2	11.11 (n-6 = 9.4; n-3 = 1.71)

The 2003 National Diet and Nutrition Survey(4) reported that the average daily fat intake provided approximately 35% of total food energy in both sexes, although there were variations between age groups, with young women most likely to exceed recommendations. Table 1.2 provides a summary of the fats consumed expressed as a percentage of total food energy and also shows the UK dietary reference values (DRV).

Table 1.2: UK dietary fat intake as % of total food energy in comparison with the UK dietary reference values (DRV) (4;5)

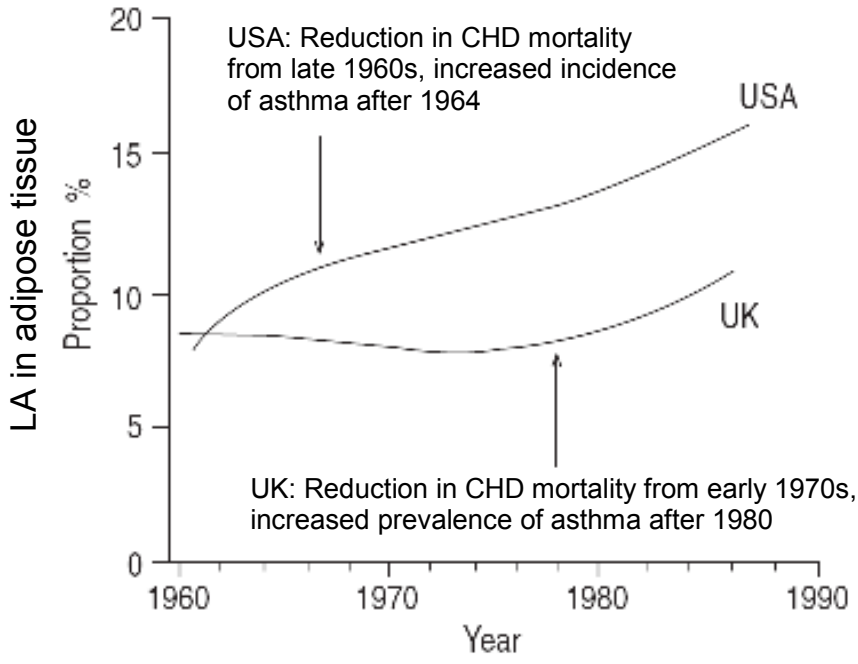
	Men	Women	DRV
Total fat intake	35.8	34.9	< 35
Saturated fats	13.4	13.2	<10
Trans fatty acids	1.2	1.2	< 2
Monounsaturated	12.1	11.5	12
n-3 PUFA	1	1	6-10
n-6 PUFA	5.4	5.3	

The increasing intake of PUFA in the Western diet is largely the result of increased consumption of vegetable-based margarine and cooking oils and decreased use of animal products such as lard and butter. The most common dietary PUFA consumed are the essential fatty acids (EFA) LA (18:2n-6) and ALNA. LA is a fatty acid found in corn, sunflower, safflower and soybean oils. Some nuts and rapeseed and soybean oils are good sources of ALNA, and the richest source of ALNA is linseed oil.

Oily fish are a rich source of the longer-chain (LC) n-3 PUFA eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The LC n-3 PUFA content of fish varies a great deal between species. For example, salmon is a rich source, containing approximately 2.2 g EPA + DPA + DHA per 100 g portion, while white fish such as cod contains around 300 mg of these fatty acids per 120 g portion(6). Though average adult intakes of LC n-3 PUFA in the UK are thought to be approximately 200 mg/day, this average represents a highly skewed distribution as it is estimated that only 27% of UK adults habitually eat oily fish(7). LC n-3 PUFA therefore only make up a small proportion of total n-3 fatty acid intake for the majority of people. The dietary intake of LC n-6 PUFA is also much lower than that of LA, with intake of AA from sources such as meat and eggs estimated to be 50-300 mg/day(8).

The increasing consumption of the n-6 PUFA LA in Western diets is thought to have contributed to decreases in CHD mortality because LA lowers blood cholesterol concentrations, but has been noted to coincide with increased prevalence of asthma. For example, figure 1.5 illustrates the changes in the LA content of adipose tissue over time which is believed to reflect changes in dietary intake. Shortly after the observed increases in LA content of adipose tissue are corresponding reductions in CHD mortality, but increasing prevalence of childhood asthma(9-11).

Figure 1.5: Diagram to illustrate the relationship between the LA content of adipose tissue, reduced rates of CHD mortality and the increased prevalence of asthma. Adapted from (9-11).



1.2.3 Endogenous synthesis of fatty acids

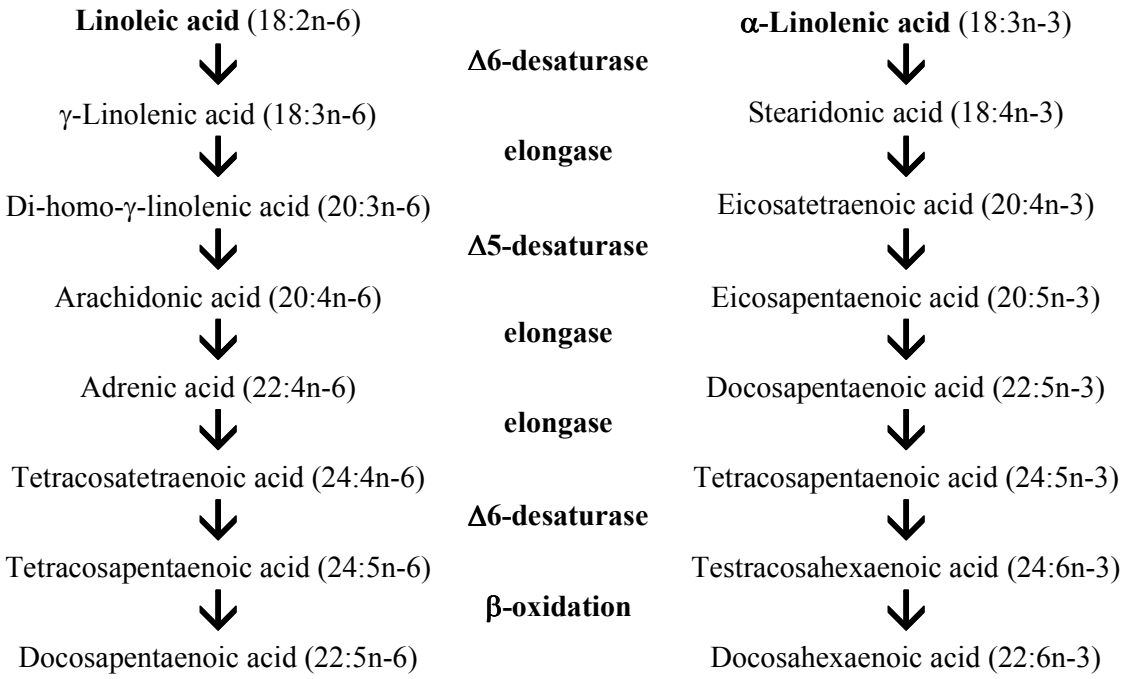
In addition to dietary consumption, fatty acids can be synthesised endogenously *de novo*. Endogenous synthesis of saturated fatty acids occurs by the addition of 2-carbon units (in the form of acetyl coA) to a growing fatty acid chain. This process occurs in the liver, mammary gland and adipose tissue, with the lipids synthesised derived from dietary carbohydrates. The main fatty acid product of endogenous synthesis is palmitic acid (16:0). Further elongation of endogenously synthesised or dietary fatty acids can also generate very long chain saturated fatty acids such as lignoceric acid (24:0).

Fatty acids can also be altered by desaturation or elongation reactions, which mainly occur in the liver. In the endoplasmic reticulum (ER) double bonds are introduced into fatty acids by desaturase enzymes. For example stearic acid (18:0) can be converted to oleic acid (OA, 18:1n-9) by the insertion of a *cis* double bond between carbons 9 and 10. The enzyme involved in this process is Δ9 desaturase, with Δ9 indicating the position of the double bond inserted relative to the carboxyl terminus of the hydrocarbon chain.

Mammalian cells contain Δ5, Δ6 and Δ9 desaturases. Mammals do not have Δ12 and Δ15 desaturases, and therefore cannot insert double bonds into the n-6 or n-3 position, which means that LA and ALNA are fatty acids which can only be obtained via consumption in the diet. Mammals are also unable to interconvert between LA and ALNA. The clinical features of EFA deficiency are reduced brain and body weight, dermatitis, infertility, loss of muscle tone, degenerative changes in the kidney, lung and liver, and increased susceptibility to infection and behavioural changes, but deficiency is rare in humans(12).

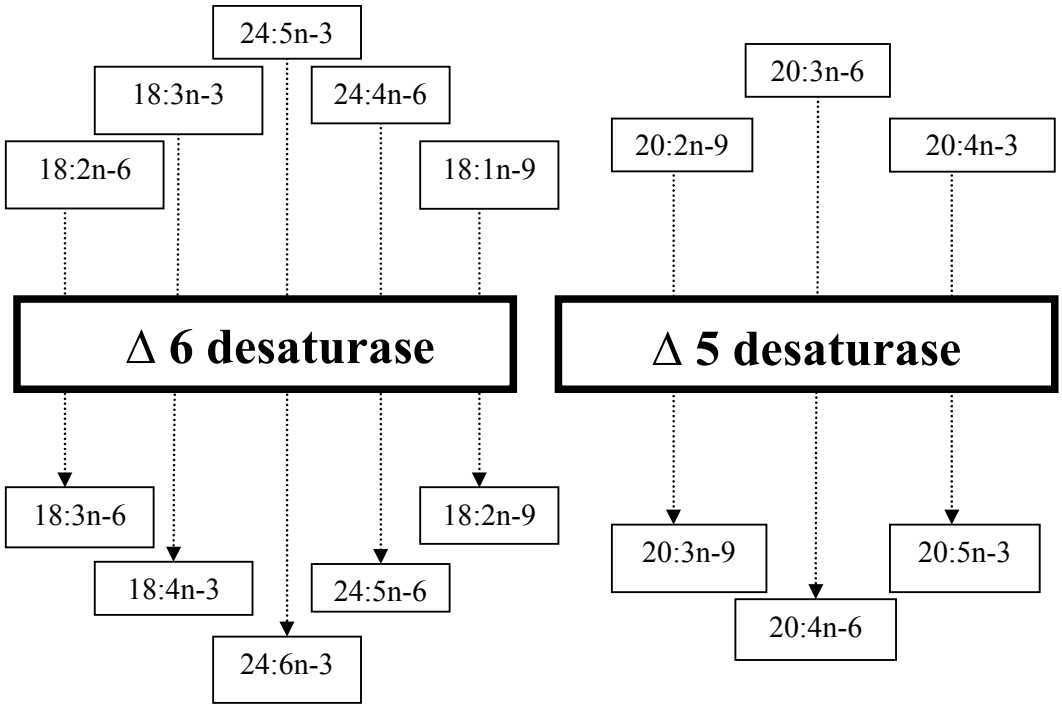
LC PUFA (both n-6 and n-3) can be endogenously synthesised via the action of desaturation and elongation enzymes upon their precursor EFA(13) (see figure 1.6). The elongation and desaturation reactions occur in the ER, and the final β-oxidation step occurs in peroxisomes. This activity of these enzymes is highest in the liver in both humans and rats(14), with desaturase activity also detectable in the adrenal glands, testes and subcutaneous adipose tissue(15). Δ6 desaturase expression has also been identified at a high level in the human brain and lung, and at lower levels in the kidney, pancreas, spleen and skeletal muscle(16). Rat studies have demonstrated that Δ5 and Δ6 desaturase activities are detectable within the fetal liver at days 19 and 22 of gestation, but are not detectable within the placenta(17). Synthesis of LC PUFA has been demonstrated in the human neonate(18).

Figure 1.6: Metabolic pathway of the generation of LC PUFA from their EFA precursors



Δ6 desaturase is the rate-limiting step in this series of reactions, with ALNA the preferred substrate of Δ6 desaturase(19). The use of this series of enzymes for both LC n-6 and n-3 PUFA synthesis results in competition between LA and ALNA for metabolism. However, much more LA is consumed than ALNA in the UK diet, with intake in males identified as 10.5 g/day LA and 1.4 g/day ALNA(4), which suggests that metabolism of the former will predominate. In situations of EFA deficiency the same sequence of enzymes can also act upon OA (18:1n-9) to form mead acid (20:3n-9). There are therefore numerous substrates which compete for the action of Δ6 and Δ5 desaturases, as illustrated in figure 1.7. The competition for Δ6 desaturase is greater due to the involvement of this enzyme in two steps of the synthesis of LC PUFA from EFA(20).

Figure 1.7: Substrates and products of the Δ6 and Δ5 desaturase enzymes



The activity of this series of desaturase and elongase enzymes has been demonstrated to be influenced by hormonal, genetic and dietary variables. A review is available which describes the role of hormones in the regulation of Δ5 and Δ6 desaturase activity(14). In brief, Δ6 desaturase activity is depressed in rat models of diabetes, and restored by the administration of insulin, with corresponding effects upon the LC PUFA content of rat liver lipids. Other hormones which have demonstrated effects upon desaturase activity include glucagon, adrenaline, adrenocorticotrophic hormone, prolactin and steroids which decrease desaturase activity. These effects of hormones were associated with changes in the abundance of Δ6 desaturase mRNA expression.

There are seven different elongase subtypes which have been identified in the mouse, human and rat genomes which vary in their substrate specificity and tissue expression, but are expressed at comparable levels within the liver of all three species. The most abundant elongase subtype in the liver is Elovl 5(21). As was the case for desaturase enzymes, elongase activity is under the influence of hormones, transcription factors and nutrients(22).

Recent studies have identified that there is a significant effect of genotype upon the activity of enzymes involved in the synthesis of LC PUFA in humans. Significant

correlations have been identified between serum phospholipid fatty acids and the haplotype of Δ5 and Δ6 desaturase genes, with haplotype demonstrating significant correlations to the AA content of both serum phospholipids(23) and erythrocyte membranes(24). A deletion in the promoter of the Δ6 desaturase gene has also been demonstrated to associate with significantly lower γ-linolenic acid (GLA, 18:3n-6), EPA and AA contents of subcutaneous adipose tissue and higher 20:2n-6 and 20:3n-3 contents (elongase products of EFA)(25).

The effect of diet upon desaturase and elongase activity was demonstrated in a study where rats received an ALNA-deficient diet over a 15 week period. Those on the deficient diet were found to have significantly higher mRNA expression and activities (assessed using radiolabelled fatty acids *in vitro*) of Δ5, Δ6 desaturase and elongase (elovl 5) enzymes in the liver(26).

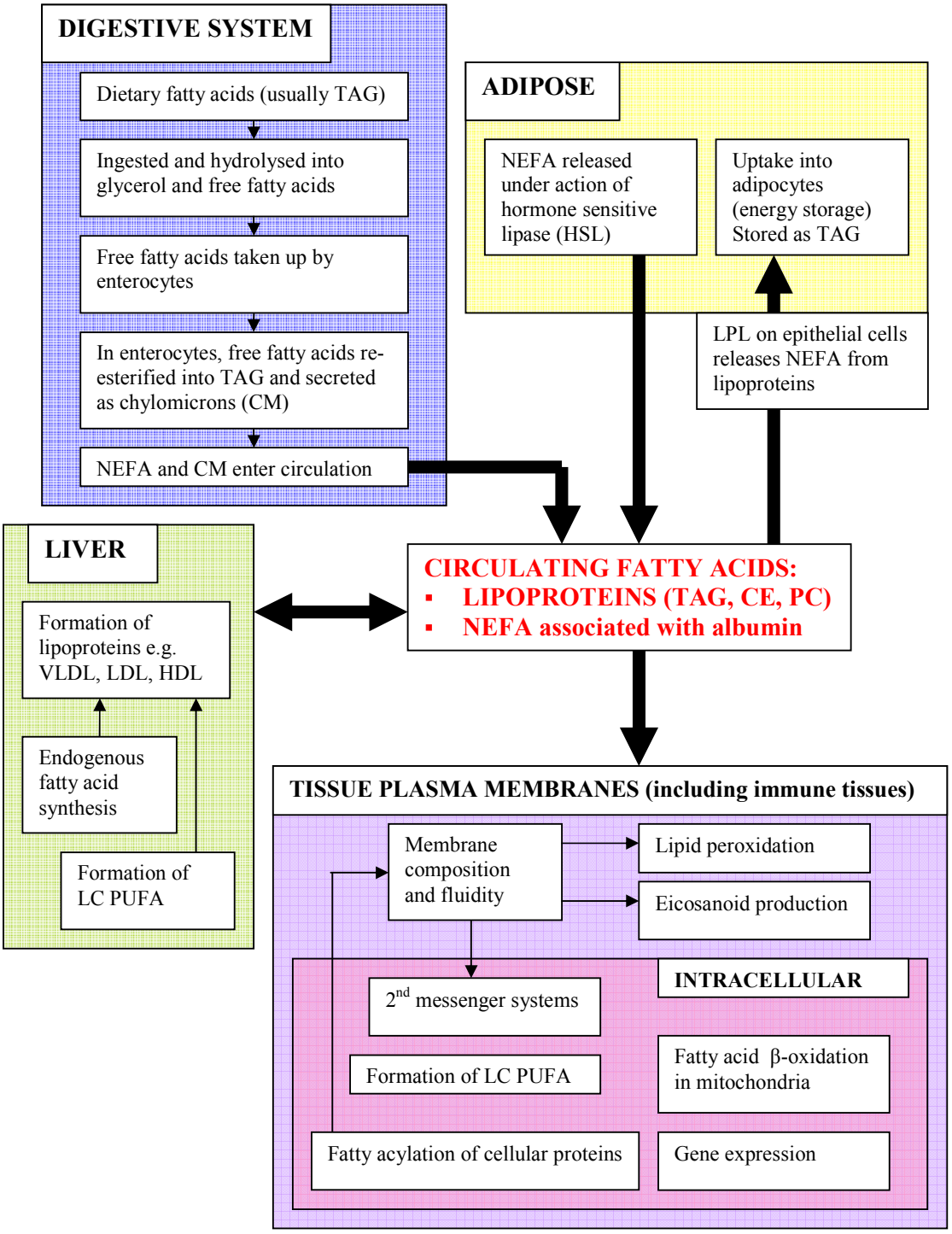
1.2.4 Physiological roles of fatty acids

Fatty acids have numerous physiological roles (see figure 1.8). Fatty acids are substrates for energy generation by β-oxidation and are stored in adipose tissue in situations where energy intake exceeds expenditure. Fatty acids can acylate membrane bound proteins, modulate membrane fluidity(27), interact with intracellular signalling pathways and transcription factors(28;29), and act as substrates for production of signalling molecules(30-35). Unsaturated fatty acids are vulnerable to lipid peroxidation, with susceptibility increasing with the degree of unsaturation(36).

Fatty acids and energy generation

Fatty acid oxidation, also known as β-oxidation, enables the use of fatty acids for energy production. It should be noted that this β-oxidation for energy production takes place in the mitochondria, whereas the β-oxidation step involved in formation of LC PUFA occurs in peroxisomes. Fatty acids in the form of fatty acyl-CoA cross the mitochondrial membrane in a carnitine-dependent process. Fatty acid oxidation involves the progressive removal of 2-carbon units (in the form of acetyl-coA) from the carboxyl end of fatty acids. The acetyl-CoA produced is then oxidised in the Krebs Cycle for energy production.

Figure 1.8: Diagram to illustrate the physiological roles of fatty acids



Abbreviations: CE, cholesteryl ester; CM, chylomicron; HDL, high-density lipoprotein; HSL, hormone sensitive lipase; LC PUFA, longer-chain polyunsaturated fatty acid; LDL, low-density lipoprotein; LPL, lipoprotein lipase; NEFA, non-esterified fatty acid; PC, phosphatidylcholine; TAG, triglycerides; VLDL, very low-density lipoprotein

Fatty acids and energy storage

Adipose tissue is a large physiological store of lipids, with fatty acids stored as intracellular TAG. The release of fatty acids from adipose tissue occurs under the influence of hormones such as adrenaline, noradrenaline, glucagon and insulin upon hormone-sensitive lipase. Incorporation of fatty acids into adipose tissue occurs under the action of lipoprotein lipase on the surface of adipose epithelial cells which liberates NEFA from circulating lipoproteins, which are then esterified into TAG within adipose tissue. The composition of adipose tissue TAG is influenced by past dietary intake and the selective mobilisation of specific fatty acids from adipose tissue. For example, *in vitro* studies have identified that ALNA, EPA and AA are preferentially mobilised from adipose tissue compared to other fatty acids(37).

Fatty acylation of proteins

Fatty acids can covalently bind to proteins resulting in changes to protein-protein interactions, membrane binding affinity and cellular signal transduction. Fatty acylation enables proteins to be anchored to the plasma membrane and plays an important role in signal transduction by enabling relevant signalling components to be localised in close proximity to each other(38). The type of fatty acid involved in acylation confers different effects onto the protein.

Myristic acid (14:0) can form amide linkages to N-terminal glycine residues and is a co-translational modification with a long half-life. Myristoylation enables protein-protein interactions in cytosolic proteins and stabilises protein conformation. Myristoyl switch mechanisms function to control the location of cellular proteins, with the availability of myristic acid for membrane binding under the influence of the conformational shape of the protein. Demyristylase enzymes can remove the myristic acid from proteins, freeing them from cell membranes(38).

Palmitic acid (16:0) can attach to cysteine residues in proteins by thioester bonds. This is a post-translational modification of proteins, and palmitoylated proteins are exclusively membrane bound as palmitic acid has a much higher affinity for membrane binding than myristic acid(38).

Fatty acids and membrane fluidity

The composition of cell membranes can be affected by the availability of dietary fatty acid substrates, though this is not the only determinant of membrane composition. For example, when dietary EFA are consumed, this can also significantly affect the concentrations of downstream LC PUFA which are synthesised from that EFA. For example, provision of dietary ALNA increases the amount of both ALNA and the LC n-3 PUFA EPA within cell phospholipids(39). The structural characteristics of fatty acids (e.g. saturates vs. PUFA) affect how closely the membrane phospholipids can pack together. For example, the six double bonds within DHA result in the fatty acid having a 'helical' conformation which allows a high degree of membrane fluidity. This in turn affects protein movement, and therefore has the potential to impact upon cell functions such as the activity of receptors or adhesion molecules.

Membrane fatty acid composition also affects lipid rafts within cell membranes. Lipid rafts are areas of membrane which are rich in sphingolipids and cholesterol and have PC fractions rich in saturated fatty acids. These membrane areas contain high concentrations of acylated proteins(40). Lipid rafts are associated with trans-membrane proteins, and allows a range of proteins involved in signal transduction to be in close interaction with each other(41).

Fatty acids and intracellular signalling pathways

Fatty acids play several roles in cell signalling mechanisms, including the production of eicosanoids and the inositol lipid cycle. The production of eicosanoids is a key interaction between fatty acids and the immune system, and will be discussed in detail in later sections.

The inositol lipid cycle is a signalling cascade which generates diacylglycerol (DAG) and inositol trisphosphate (IP_3), two important intracellular messengers. The process is initiated when phosphatidylinositol (PI) in the cell membrane is phosphorylated to phosphatidylinositol-4,5-bisphosphate (PIP_2). PIP_2 is positively charged, and attracts phospholipase C (a membrane bound enzyme) which generates DAG and IP_3 by hydrolysis of PIP_2. DAG functions to activate protein kinase C in a calcium dependent

manner. IP_3 increases levels of intracellular calcium by activating the release of calcium ions from the ER.

Fatty acids and gene expression

Fatty acids have the capacity to directly affect gene transcription by binding to transcription factors. Transcription factors bind to areas of genes and act to promote or inhibit transcription. Transcription factors that PUFA have been demonstrated to interact with include: hepatic nuclear factor-4α, liver X receptors (both α and β), peroxisomal proliferators-activated receptors (PPAR)-α, -β/δ and -γ(29;42;43) and sterol regulatory element-binding proteins (SREBP) 1 and 2. By these mechanisms PUFA increase the expression of genes involved in hepatic and skeletal muscle fatty acid β-oxidation, while repressing genes involved in glycolytic, lipogenic and cholesterogenic activities. These gene interactions have the net result of decreasing lipid synthesis and increasing lipid oxidation.

Fatty acids and lipid peroxidation

The incorporation of PUFA into cell membranes increases susceptibility of the cell membrane to lipid peroxidation, as it is the double bonds of fatty acids which are susceptible to oxidation. Lipid peroxidation is induced by hydroxyl radicals, such as those generated from superoxide anions during immune activation. n-3 PUFA are more susceptible to peroxidation than n-6 PUFA as they contain more double bonds. Lipid peroxides are toxic to cells as they have the potential to damage cells by oxidation reactions, including effects on DNA which may cause mutations. The susceptibility of cell membranes to lipid peroxidation can be reduced by the presence of free radical scavengers including α-tocopherol (vitamin E), ascorbic acid (vitamin C) and selenium (essential for activity of catalase). The incorporation of peroxidation susceptible PUFA into membranes may therefore increase the requirements for anti-oxidant nutrients, and should be considered when increasing the dietary intake of PUFA.

1.2.5 n-3 fatty acids and human health

n-3 fatty acids have been studied extensively with regards to their potential benefits upon human health(3;6;44). Benefits identified in adults to date include effects upon cardiovascular disease, inflammatory diseases, and cognitive function, while among infants benefits upon vision and cognitive function have been identified.

Comprehensive reviews of epidemiological studies and human intervention trials of dietary LC n-3 PUFA and cardiovascular disease are available(45-47). There are substantial epidemiological and case-control study data which demonstrate that the risk of cardiovascular disease is lowest among those with the highest fish or LC n-3 PUFA intake or LC n-3 PUFA status. Supplements containing LC n-3 PUFA have been demonstrated to be beneficial in secondary prevention of cardiovascular disease, reducing the risk of mortality related to cardiovascular events in subjects who had previously suffered a myocardial infarction(48).

The clinical applications of LC n-3 PUFA in relation to chronic inflammatory disease, atopic disease and the systemic inflammatory response to surgery and injury have been reviewed(34;49). There is strong evidence from randomized, placebo controlled trials that dietary fish oil supplements are beneficial for patients with the chronic inflammatory disease rheumatoid arthritis(50;51). Benefits to other inflammatory disorders have not been conclusively demonstrated. Evidence for the role of LC n-3 PUFA supplementation in patients receiving enteral formula feeds after surgery in an attempt to reduce sepsis is complicated by the simultaneous provision of other nutrients such as certain amino acids and antioxidant vitamins(49;52).

Meta-analyses have identified statistically significant benefits of supplementation with LC n-3 PUFA among adults with uni-polar and bi-polar depression(53;54) though these benefits are only apparent in populations with an established clinical diagnosis of depressive illness. Epidemiological and post-mortem evidence has generated interest in the potential benefits that LC n-3 PUFA may have in the prevention or treatment of disorders of cognitive function in later life such as dementia and Alzheimer's disease, with further investigation from intervention studies required(44). Research has been conducted to evaluate the potential benefits that LC n-3 PUFA may confer in children with neurodevelopmental disorders such as attention deficit/hyperactivity disorder (ADHD)(55). Studies which evaluated parent/teacher-reported changes in behaviour of children with ADHD have identified benefits of LC n-3 PUFA supplementation, though these studies have used various combinations of EPA and DHA and it is not yet clear which is the most important fatty acid in this setting.

The observation that DHA is found in high concentrations in the retina and accumulates in the brain during early life (from 3 months gestation to 18 months after delivery in humans) suggests that an adequate supply of n-3 PUFA is required for the development

and function of the central nervous system(56;57). Animal studies which have used n-3 PUFA deficient diets identified that this causes visual and cognitive abnormalities. Studies undertaken in pre-term infants have demonstrated that formulas which contain DHA improve visual function early in infancy(58).

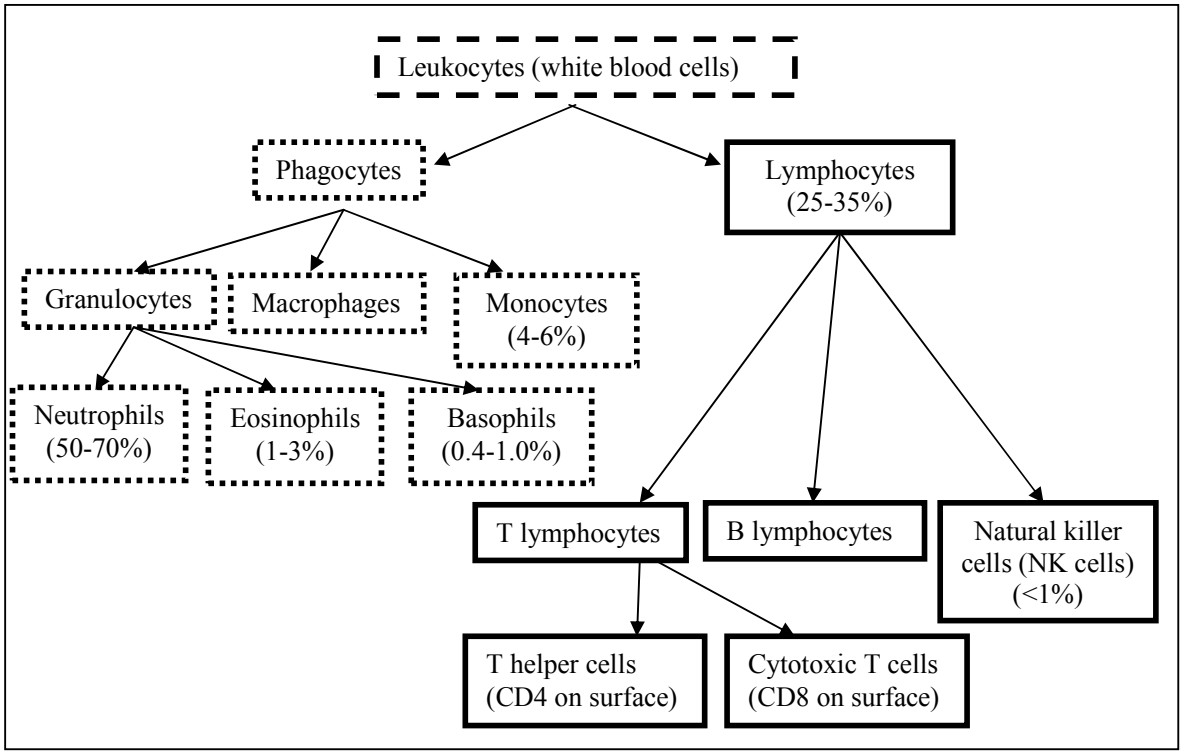
1.3 The immune system

This section is intended to give a broad overview of the immune system, including the cell types and immune organs, innate and acquired immune function and how immune function is regulated by production of signalling molecules such as cytokines. Further detailed information upon the complex and numerous functions of the immune system can be found elsewhere(59;60). When functioning normally, the immune system enables the body to defend itself against potential damage induced by bacteria, parasites, toxins, viruses, foreign tissues and malignant and auto-reactive cells. Immune dysfunction is involved in auto-immune and atopic disease.

1.3.1 Cells of the immune system

Cells of the immune system are leukocytes (also known as white blood cells). All immune cells originate from the bone marrow, as do red blood cells and platelets. The stem cells which give rise to all blood cells are known as pluripotent hematopoietic cells. These stem cells undergo a complex process of maturation and differentiation to generate diverse cell populations. Leukocytes have three main physiological functions: phagocytosis, cytotoxicity and generation of inflammation. The two principal cell populations of circulating leukocytes are lymphocytes and phagocytes. These populations can be further classified into cell subsets, each with their own characteristic properties and functions. The cell types relevant to this thesis which will be discussed in this section are detailed in figure 1.9. There are numerous other important cell types within the immune system such as dendritic cells, which will not be discussed in detail.

Figure 1.9: Lineage of cells types of the immune system and their relative contribution to the circulating leukocyte pool



Granulocytes

Granulocytes include neutrophils, eosinophils and basophils. These cells have roles in phagocytosis, inflammation and antigen presentation. Neutrophils are involved in the acute inflammatory response, adhering to the vessel endothelium, migrating from the bloodstream to sites of infection and phagocytosing foreign particles. Activated neutrophils release granules containing elastase, collagenase, myeloperoxidase and lysozyme, which enable movement through the tissues to the site of inflammation, and produce superoxide radicals, the metabolites of which are important in destroying the invading organism. Eosinophils are abundant at sites of hypersensitivity reactions and contribute to tissue injury and inflammation. They are capable of phagocytosis, but their primary role is in the elimination of multicellular parasitic infections by non-phagocytic mechanisms such as cytotoxic actions. These cytotoxic actions are also involved in the pathophysiology of asthma. Basophils are circulating cells which cause a short lived inflammatory reaction at sites of infection. Mast cells are similar to basophils, but remain localised within connective tissues, rather than circulating in the bloodstream.

Monocytes and macrophages

Monocytes are phagocytic cells which circulate in the bloodstream and are able to migrate to sites of infection, where they develop into resident tissue macrophages, large cells with a long half-life. The movement of cells from the bloodstream to the tissues is known as extravasation, and requires the cell to adhere to the endothelial surface. This occurs via interactions with adhesion molecules such as vascular cell adhesion molecule (VCAM) and intracellular adhesion molecule (ICAM). Metalloproteinases are released enabling monocytes to move through tissues to reach the site of infection where they phagocytose infectious agents, and present antigen from pathogens to other immune cells.

T lymphocytes

T cells are lymphocytes which have matured within the thymus, enabling them to distinguish 'self' antigens from 'non-self'. A failure of T cells to successfully differentiate between self and non-self results in auto-immune disease. Mature T cells in the circulation interact with antigen presenting cells (APC) to initiate and regulate antigen-specific immune responses. Leukocytes are commonly classified based upon their surface membrane proteins, known as clusters of differentiation (CD). For example, a cell surface marker which is unique to T cells is CD3, which is part of the T cell receptor complex. Mature T cells can be further classified into T helper cells (Th cells) or cytotoxic T cells based upon their expression of CD4 or CD8, respectively, though these subsets cannot be distinguished morphologically. Th cells and cytotoxic T cells are the two principal T cell subsets which will be discussed in this thesis, though other T cells subsets have been identified, such as T regulatory cells(61;62).

The interaction between T cells and virally infected cells or APC (including macrophages, B cells and phagocytic cells) is mediated by an interaction between the T cell receptor (TCR) and MHC molecules (major histocompatibility complex, also sometimes referred to as human leukocyte antigens, HLA). MHC I glycoproteins are present on the cell surface of almost every nucleated cell, with exceptions including fetal trophoblasts, hepatocytes and cells of the central nervous system. All proteins within cells are regularly turned over and broken down and fragments of these broken down proteins (typically 6-8 amino acid residues in length) are cycled to the cell membrane on MHC I molecules, enabling the detection of any 'non-self' intracellular

proteins (e.g. of viral origin) by cytotoxic T cells ($CD8^+$). If such proteins are detected the cell is destroyed. MHC II is expressed on a limited range of cell types and is constitutively expressed on B cells, monocytes, macrophages and dendritic cells. MHC II binds peptide fragments which originate from phagocytosed pathogens and presents antigen to Th cells ($CD4^+$ cells). The Th cell will subsequently activate cellular responses that aim to eliminate the pathogen whose antigen was detected.

In addition to the MHC complexes, there are other cell surface markers upon APC which function to present antigen to T cells. While MHC I and II present peptide antigens to T cells, CD1 is a cell surface marker which presents lipids and glycolipids, such as those within the cell membranes of mycobacteria to cytotoxic T cells(63). A potential role of CD1 in self-antigen presentation has also been identified when PE (a phospholipid usually localised within intracellular membranes) is presented via CD1 to T cells. This may suggest a role of CD1 in autoimmune dysfunction(64).

Once T cells have been presented with antigen, they undergo morphological changes, known as blastic formation, when cell diameter more than doubles. T cells function to regulate the intensity and duration of the acquired immune response by both cell to cell interactions and the production of specific soluble mediators such as cytokines. The acquired immune response and cell-specific profiles of cytokine production will be described in detail in later sections.

B lymphocytes

B lymphocytes are classified based upon their ability to produce and secrete immunoglobulins (Ig). The B cell receptor is a membrane bound immunoglobulin (Ig). Ig specific to an antigen are known as antibodies. The structure of membrane bound Ig and antibodies differ only in that Ig expressed on B cell surfaces have an additional hydrophobic sequence at the carboxyl terminus which spans the cell membrane. B cells can be activated by Th cells, or directly stimulated by antigen alone, with the latter known as thymus-independent B cell activation.

Activated B cells are called plasma cells and function to synthesise and secrete large amounts of antibody. Plasma cells have a half-life of just a few days, but the antibody they secrete has a half-life of some months. Some activated B cells will develop into

memory cells, which are morphologically identical to naïve B cells but express antibody on their surface that is already antigen specific. While the MHC induces T cell responses to peptide fragments of antigens, Ig molecules on B cells respond to intact antigens, such as whole native proteins.

There are five classes of Ig: IgM, IgD, IgG, IgA and IgE. Each antibody class has a characteristic function and structure. Naïve B cells have IgM or IgD on their cell surface, and after activation may undergo 'class switching' to produce other Ig isotypes. IgM is produced in the primary response to antigen. IgM can be membrane bound or secreted into blood and lymph, where it forms an unstable pentamer structure of five Ig complexes. IgD is always membrane bound, and is involved in B cell activation. IgG is produced in the secondary response to antigen, persisting in the serum long after initial infection, is the most abundant of the Ig isoforms, and is able to cross the placenta. IgA is a dimerised antibody found within mucous secretions. IgE is involved in allergic reactions and in the response to parasitic infections.

Secreted antibodies function to neutralise pathogens and activate the complement cascade. The complement cascade is an aspect of 'innate' immune function which will be discussed in more detail in a later section. Antibodies neutralise viruses, intracellular bacteria and bacterial toxins by binding to them and preventing their spread from cell to cell. Antibodies with bound antigen are recognised by phagocytic cells, inducing phagocytosis and destruction of the organism. IgE can bind to mast cells, basophils and eosinophils without having bound antigen. This results in mast cells being 'primed' for an allergic response.

NK cells

Natural killer (NK) cells are large granular lymphocytes that provide innate cytotoxic immune responses against virally infected cells. NK cells recognise changes on virally infected cells and destroy them, either by releasing perforin or by binding to the infected cell and destroying it by direct contact. Human NK cells express the surface markers CD16 and CD56. NK cell receptor protein 1 (NKR-P1, also known as CD161) is a receptor expressed upon all rodent NK cells which activates cytotoxic mechanisms(65).

1.3.2 Organs of the immune system

Circulating immune cells represent only a small proportion of the whole body immune cell pool in humans (< 1%), with the majority of immune cells localised within tissues (e.g. resident tissue macrophages) or within organs of the immune system. The major organs of the immune system include the thymus and bone marrow (primary lymphoid organs), spleen and lymph nodes (secondary lymphoid organs) and the mucosal associated lymphoid tissue. Primary lymphoid organs are the site of immune cell generation and maturation. Immune cells within the primary lymphoid organs have not been exposed to antigens. In secondary lymphoid organs lymphocytes come into contact with APC (e.g. dendritic cells). In this section the features of the thymus, spleen and lymph nodes will be outlined. The roles of other immune organs such as the bone marrow and gut-associated lymphoid tissue will not be described.

Thymus

The thymus is located in the thorax, overlying the heart and major blood vessels. The two lobes of the thymus are organised into lobules, and the lobules separated by connective tissue. Each lobule contains lymphoid cells (also known as thymocytes), which are arranged into an outer cortex and an inner medulla. The cortex contains immature cells and the medulla contains the more mature cells. The thymus has a rich vascular supply and a series of lymphatic vessels that drain into the mediastinal lymph nodes. T cells originate in the bone marrow and migrate to the thymus, a site of rapid proliferation and extensive apoptosis. Thymocyte progenitors are 'triple negative', not expressing CD3, CD4 or CD8. Within the thymus these cells undergo maturation to become 'double positive', expressing CD3, and both CD4 and CD8.

Within the cortex of the thymus the TCR on immature thymocytes interacts with MHC-self peptide complexes. It is important that receptors do not become activated in response to 'self' antigens. In order to prevent this, a process of clonal selection, where self-reactive lymphocytes are removed, occurs at the cortico-medullary junction. Lymphocytes which are specific for self antigen are deleted, either by cytotoxic mechanisms or by being made anergic (functionally disabled) at an early stage of lymphoid development. If the interaction between the TCR and self-antigen is of low affinity, the lymphocytes go on to become mature T cells.

Spleen

The spleen in located in the upper left quadrant of the abdomen, behind the stomach and close to the diaphragm. It is surrounded by a collagenous capsule containing smooth muscle that supports the cells within the spleen. There are two main tissue types within the spleen, the red pulp and the white pulp. The macrophages of the red pulp are involved in the destruction of old erythrocytes. The white pulp contains the lymphoid tissues of the spleen. Unlike other secondary lymphoid organs, the spleen is only supplied by blood vessels and not the lymphatic system and therefore reacts to antigens within the bloodstream. The lymphoid tissue of the spleen is arranged around a central arteriole, and this structure is known as the periarteriolar lymphoid sheath (PALS). The PALS has T and B cell areas, with T cells immediately around the central arteriole and B cells organised into follicles. These B cell follicles can be classified as either primary (where B cells have not yet been stimulated by antigen exposure) or secondary (where B cells have been stimulated). Lymphocytes are able to enter and leave the spleen via capillaries leading to the PALS.

Lymph nodes

The role of the lymphatic system is to drain fluid from the tissues past the lymph nodes, screening for antigens. There are approximately 600 lymph nodes located along lymphatic vessels with lymph nodes organised into clusters, such as those found in the axilla and neck. Lymph nodes are surrounded by a capsule and have both T cell and B cell areas. The B cell areas are known as the cortex or follicular areas, and the T cell areas as the paracortex or parafollicular area. Lymph nodes have a blood supply that allows the exchange of immune cells with the circulation. This interaction with circulating blood maximises the chance of memory cells recognising an antigen upon secondary exposure.

1.3.3 Innate and acquired immunity

The actions of the immune system can be classified as either innate (also known as ‘natural’ or ‘non-specific’) or acquired (also known as ‘adaptive’ or ‘specific’). Both systems involve cellular and humoral responses.

Innate immunity

Innate immunity is the first line of defence against pathogens and involves the prevention of entry of infectious agents by physical and biochemical barriers, inflammation, cytotoxic and phagocytosis mechanisms, and activation of the complement cascade. Cells involved in innate immunity have general "antigen" receptors but cannot distinguish between different types of pathogen. Innate immunity evokes similar responses amongst normal individuals, and provides a very rapid but inflexible response to pathogens. Innate immunity forms the early phases of defence for the initial 3-5 days of infection, covering the time required for activation of acquired immunity. Innate immunity is present before exposure to pathogens, and is not enhanced by previous exposure to pathogens.

Physical and biochemical barriers

Physical barriers to pathogens include the skin, mucus, the cilia lining the trachea and the acid pH of the stomach. Biochemical barriers to pathogens include sebaceous gland secretions such as lysozyme, which is secreted by macrophages and acts to degrade bacteria in the interstitial spaces between cells, acting upon petidoglycans in bacterial cell walls. Commensal bacteria within the gut are considered to be a component of innate immunity. Commensal bacteria are tolerated by the body and compete with potential pathogens, effectively providing a physical barrier against pathogen colonisation.

Inflammation

The inflammatory process involves immune cells, adhesion molecules and cytokines and is typified by redness, swelling, heat and pain. These symptoms of inflammation are a result of increased blood flow and increased permeability across blood capillaries, mediated by the release of inflammatory mediators from immune cells close to the site of injury. For example, degranulation of mast cells releases the vaso-active compound histamine. Increased permeability of the endothelium enables leukocytes such as neutrophils and monocytes to undergo extravasation – migration from the capillary lumen into the tissues.

Cytotoxic and phagocytic innate responses

The innate response to viral infection includes cytotoxic targeting of infected cells by NK cells. NK cells act upon virally infected cells by secreting perforin which causes

the target cell to die by apoptosis or membrane damage. Phagocytic cells such as neutrophils (within the circulation) and macrophages (resident in tissues) will be encountered if a pathogen penetrates epithelial surfaces. Phagocytes attach to pathogens, extending pseudopodia to engulf them. The pathogen is then internalised into a phagosome, to which lysosomes fuse, releasing toxic chemicals (such as superoxide radicals and hydrogen peroxide) and resulting in destruction of the pathogen.

The complement cascade

The complement cascade involves serum proteins, which when activated propagate a cascade of signal amplification by activating other complement proteins. The classical pathway of complement activation is mediated by complement proteins binding to antibodies (produced as part of the acquired immune response), but can also be activated directly in response to toxins produced by pathogens. Activated complement proteins act as chemoattractants and induce inflammatory responses. Complement proteins also bind to pathogen membranes and can form a membrane attack complex, a pore in the pathogen cell membrane which induces cell lysis and targeting of the pathogen by phagocytic cells.

An additional function of complement activation is the clearance of antigen-antibody complexes from the blood. Antigen-antibody complexes are very large and can be very damaging, particularly in organs such as the kidneys and so it is important that they are cleared from the circulation quickly. Complement proteins associated with antigen-antibody complexes bind to receptors on red blood cell membranes and enable phagocytic cells (either those in the circulation or resident in tissues such as the spleen and liver) to target these large complexes.

Acquired immunity

Acquired immunity provides highly specific reactions to particular infectious agents in order to eradicate them. Acquired immunity has a component of memory, allowing the infectious agent to be recognised and eliminated rapidly upon secondary exposure. Acquired immunity can be classified into cell mediated immunity and humoral immunity. Acquired humoral immunity involves B cell proliferation and maturation into antigen-specific antibody producing cells (also known as plasma cells), and will not be discussed in detail in this section.

Cell-mediated acquired immunity involves lymphocyte proliferation and differentiation. T and B cells have cell surface receptors specific to a single antigen, often with many copies of this antigen-specific receptor on the cell membrane. A given antigen can stimulate more than one specific lymphocyte, as different fragments of the antigen may be recognised by different lymphocytes. Variation in T and B cell receptor specificity between immune cells is a result of the random assortment and joining of mini-gene sequences when assembling the T or B cell receptor.

The total number of lymphocytes specific for any particular antigen is low, but when an antigen binds with high affinity binding to a lymphocyte receptor this stimulates the lymphocyte to proliferate. Lymphocyte proliferation is also referred to as lymphocyte expansion or clonal expansion. This proliferation results in the genes which enabled specific antigen binding to be passed on to a population of daughter cells. Proliferation responses typically involve thousands of specific lymphocytes generated over 3-5 days, and it is this process of proliferation which is responsible for the lymph node swelling observed with infection. Somatic mutations which occur during the proliferation of lymphocytes may cause minor changes in receptor specificity. If these mutations cause the receptor to interact more strongly with the antigen then these cells will proliferate more effectively and therefore be selected over the other clones generated, increasing the receptor specificity to antigen. As lymphocytes proliferate, a small number will differentiate into memory cells, which are long-lived lymphocytes. These memory cells ensure that when a given antigen is encountered for the second time the immune response generated is stronger and faster than the initial response.

1.3.4 Immune signalling molecules

The diverse cells and tissues involved in the immune system require a series of effective signalling molecules in order to initiate, regulate, and resolve immune responses appropriately during infection. The principal signalling molecules of the immune system are cytokine and eicosanoids.

Cytokines

Cytokines are low molecular weight, highly soluble, stable proteins that regulate the activity of immune cells as well as many other cells and tissues. Cytokine molecules induce their effects by binding to receptors on cell surfaces, causing rapid tyrosine phosphorylation of signal transducers and activators of transcription (STAT).

Phosphorylated STAT dimerise and travel to the cell nucleus, resulting in changes in gene transcription and cell function, resulting in changes in growth, development or activity of the target cell.

Cytokines are important mediators of both innate and acquired immunity and form an important link between these two branches of the immune response. Cytokines can be produced by diverse cell types including non-immune cells, such as the placenta. Cytokines are able to act upon many cell types, and are therefore said to be pleiotropic. Cytokines differ from hormones in that they are produced by dispersed cells rather than specific organs. Various immune cell subsets produce characteristic profiles of cytokines, allowing some immune cells to be classified based upon the range of cytokines they produce, as is the case for T helper cells, which can be characterised as Th1 or Th2 on the basis of their cytokine production profiles.

Th1 and Th2 responses

Th1 and Th2 lymphocytes represent the most polarised subsets of cytokine production among Th cells. Other T cell classes will not be discussed in detail but include Th0 (which express cytokines characteristic of both Th1 and Th2 cells, and may be a precursor cell) and T regulatory cells (also known as Th3(66)) which produce high levels of the cytokine transforming growth factor-β (TGF-β). The cytokines produced by Th1 and Th2 cells promote differentiation towards their own phenotype and inhibit differentiation towards the alternative phenotype. This results in self-amplification and antagonism between the Th1 and Th2 phenotypes. Changes to the Th1/Th2 balance during early life may therefore effect long-term change in the immune response.

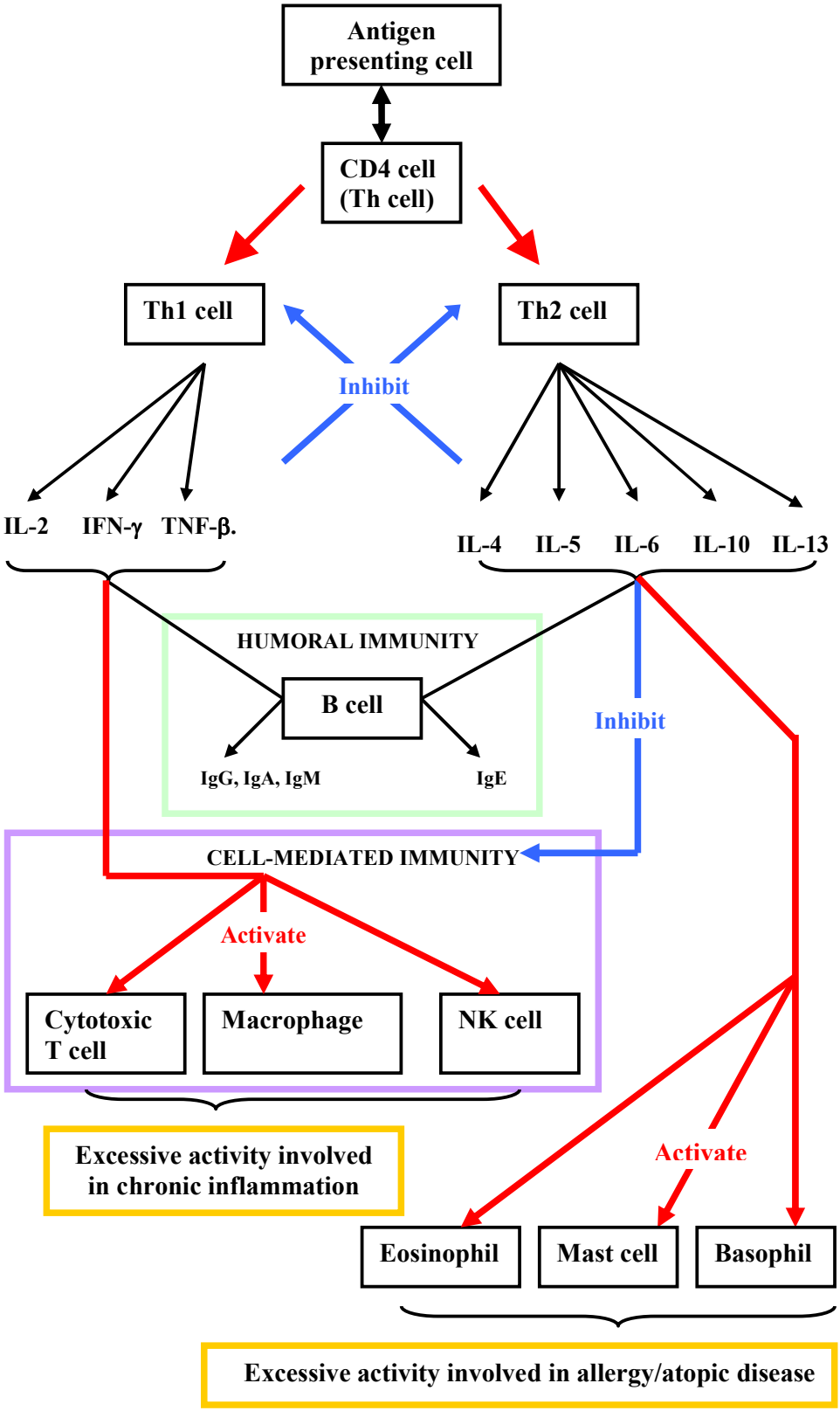
Activated Th1 cells produce the cytokines interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β). Production of IFN-γ is commonly used to identify Th1 cells, as its production is rarely observed by Th2 cells. Th1 cytokines induce activation of macrophages, NK cells and cytotoxic T lymphocytes, and therefore lead to an inflammatory response. Th1 activity is stimulated by interactions with antigens generated from bacteria, fungi and viruses and results in the elimination of these types of organisms. Several autoimmune diseases are associated with inappropriate Th1 responses. Examples of autoimmune diseases (also known as chronic inflammatory diseases) include haemolytic anaemia, type I insulin dependent diabetes, Crohn's disease, rheumatoid arthritis and multiple sclerosis.

Activated Th2 cells produce the cytokines IL-4, IL-5, IL-6, IL-10 and IL-13. IL-4 production is used to identify Th2 cells as its production is rarely observed by Th1 cells. The cytokines generated by Th2 cells induce the differentiation, activation and survival of eosinophils (IL-5 mediated), promote IgE production by B cells (IL-4 and IL-13 mediated) and stimulate the growth of mast cells and basophils. The Th2 response is associated with parasitic infections, and is also generated in individuals with atopic responses in response to common environmental stimuli. Some of the clinical features of atopic disease can be accounted for by an inappropriate Th2 immune response. For example, excessive infiltration of eosinophils into lung tissues is a classic feature of asthma, as are high levels of circulating IgE. Figure 1.10 provides an overview of the interactions between Th1 and Th2 responses, and the down-stream effects they generate.

Atopic responses involve immune cells becoming hypersensitive to food (such as egg protein) or other normal environmental factors (such as pollen). These atopic responses can involve a range of organs and tissues, such as the airways, skin and eyes. Atopic responses to specific stimuli originating from foodstuffs, the environment or animals are commonly referred to as allergies. Atopic responses can result in disease states such as allergic rhinitis, asthma and systemic anaphylaxis. The onset of atopic disease is typically during infancy or early childhood, and so any potential preventative treatments will need to be initiated during early life.

Reviews are available upon the role of the Th1/Th2 balance in atopic disease(67-72), with excessive Th2 responses characteristic features of atopy. Rates of atopic diseases have been increasing in Western populations(72) but vary between subpopulations, indicating that an environmental factor is involved in their development. Environmental factors thought to have a role in the increased rates of allergic diseases include changes in hygiene practices(73), rates of infections(74), levels of air pollution(75), living conditions(76) and dietary changes(9;77). These environmental factors could influence the development of allergy both before and after birth.

Figure 1.10: Overview of Th1 and Th2 cytokine profiles and their downstream effects



Eicosanoids

Eicosanoids are mediators produced by cells including neutrophils, monocytes, macrophages, mast cells and platelets from the PUFA AA, di-homo-γ-linolenic acid (DGLA, 20:3n-6) or EPA within cell membranes. The involvement of fatty acids as a substrate for eicosanoid production means that this aspect of immune function has the capacity to be directly altered by the dietary availability of these fatty acids or their EFA precursors.

Some of the enzymes involved in eicosanoid production, such as cyclooxygenase 1 (COX_1), are constitutively expressed, enabling eicosanoids to be synthesised within minutes of immune cell activation, while COX_2 is induced by proinflammatory or mitogenic agents. PUFA in immune cell membranes are released under the action of the enzyme phospholipase A_2 (PLA_2) in response to a stimulus. PLA_2 responds to Ca^{2+} signalling, translocating from the cytosol to the ER or nuclear membrane where it acts to release fatty acids from the intracellular membranes. The COX and lipoxygenase (LOX) enzymes which are also localised to these intracellular membranes then generate eicosanoid mediators from the fatty acid substrate, which include prostaglandins (PG), leukotrienes and hydroperoxy derivatives (see figure 1.11). This thesis will investigate the effect of pregnancy and diet upon PGE_2 production, and so the functional effects of leukotrienes and hydroperoxy derivatives will not be discussed in detail in this section.

It should be noted that eicosanoids are not the only immune-modulating signalling molecules which derived from fatty acids. Resolvins, docosatrienes and protectins are other molecules which have been demonstrated to be formed during acute inflammation. These molecules are derived from EPA and DHA and exert inflammation resolving effects(33;78). There is also a pathway of AA metabolism by cytochrome P450 enzymes which produces epoxyeicosatrienoic acids (EETs) which are involved in the inflammatory and cardiovascular systems(79;80). Figure 1.11 is therefore a very simplified scheme of the immune mediators produced from AA.

Figure 1.11: Synthesis pathway for prostaglandin, leukotriene and hydroperoxy derivative production from the fatty acid AA

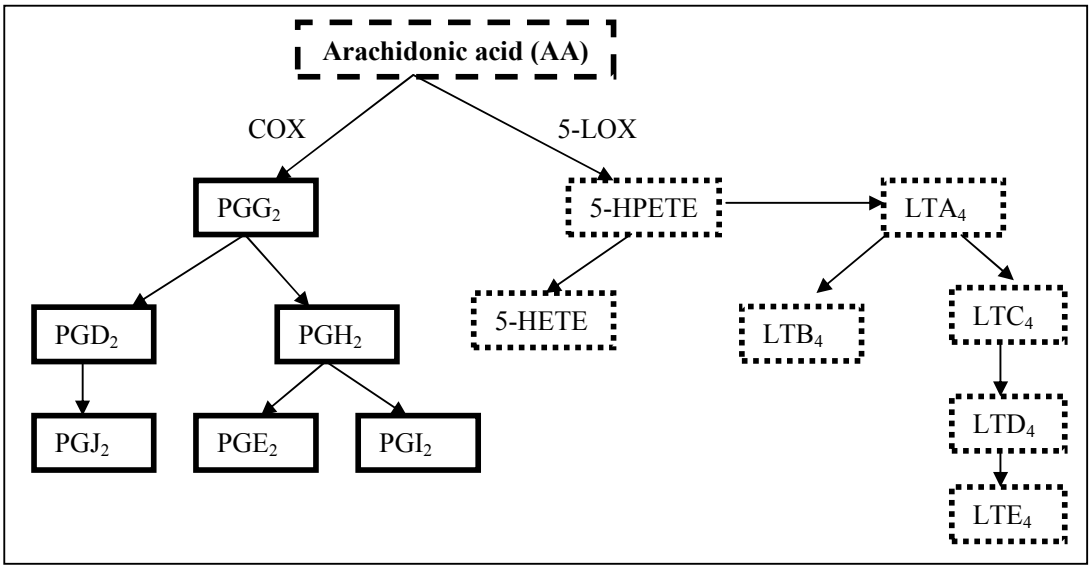
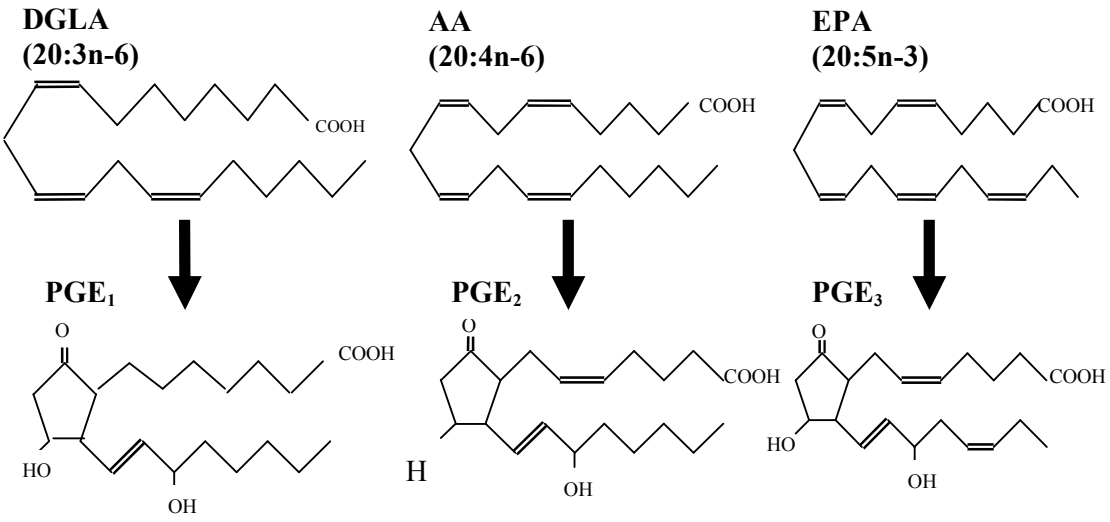


Figure 1.12 shows the PGE isoforms generated from various fatty acids. The differences in their structures affect their potency and immunological signalling properties. Though DGLA, AA and EPA are the most extensively studied prostaglandin substrates, recent studies have identified that there are other fatty acid substrates such as adrenic acid (22:4n-6) which was demonstrated to be a substrate for the formation of di-homo-prostaglandins(81).

Figure 1.12: Illustration of the prostaglandin isoforms which can be generated from fatty acid substrates



The complex, integrated and interactive way in which the immune system functions means that there is no single marker which can be used to draw conclusions about the function of the entire immune system, with the exception of clinical outcomes such as infection. Assessment of immune function therefore requires the measurement of several markers of immune function, ideally a range of *in vivo* and *ex vivo* markers. The markers of immune function which can be feasibly assessed in human trials are detailed elsewhere(82). Markers of immune function which have been rated as being highly useful include vaccination responses and production of mucosal immunoglobulins (IgA), as these can be inferred to represent an *in vivo* co-ordinated immune response to an intervention. Those which scored as being of 'medium' usefulness include *ex vivo* assessments of oxidative burst, NK activity, cytokine production by APC or lymphocytes and lymphocyte proliferation.

1.4 Fatty acids and the immune system

There is now a large body of evidence to suggest that dietary fatty acids can exert effects upon immune function, such as the finding that dietary fish oil supplementation is beneficial to patients with the auto-immune disease rheumatoid arthritis(50;51). There have also been observed associations between changes in dietary fat consumption (particularly increasing LA intake) and changes in the prevalence of atopic disease such as asthma(9). The potential of dietary fatty acids to modulate immune function indicates that changes to dietary intake, or supplementation with specific fatty acids, may provide an effective and minimally-invasive intervention tool in the prevention or management of diseases of immune dysfunction.

The two main mechanisms by which dietary fatty acids may interact with the immune system are via effects upon membrane composition and therefore fluidity, and their role as substrates for eicosanoid synthesis. The potential for PUFA to affect T cell signalling via their effects upon membrane organisation and MHC presentation has been studied *in vitro* and *in vivo*, and detailed reviews are available(83;84). It is possible that there are other mechanisms which link dietary fatty acids to the function of the immune system, including the roles of fatty acids in protein acylation and gene expression, but these have not been fully characterised to date.

1.4.1 Dietary fatty acids and immune cell fatty acid composition

Immune system activation requires increased cell membrane synthesis to meet the demands of increased cell size (e.g. blast formation upon T cell activation), generation of new cells during lymphocyte proliferation and the turnover of membrane phospholipids in phagocytosis. Changes to the fatty acid composition of cell membranes may have effects upon the fluidity of the membrane, affecting membrane-bound protein function, and may also influence the formation of lipid rafts, areas of the membrane which serve to co-localise protein components of signalling cascades. There are human and animal model studies which have demonstrated that the fatty acid composition of immune cell membranes can be significantly altered by manipulation of dietary fatty acid intake.

Human studies have demonstrated that dietary fatty acids have the capacity to significantly alter the fatty acid composition of immune cell membranes. One study provided male volunteers for 12 weeks with up to 9 g/day of tuna oil (LC n-3 PUFA rich) or a placebo (palm oil and soybean oil) or a single dose of linseed oil (ALNA rich), and identified that this could significantly affect the fatty acid composition of neutrophils(85). This study found that the EPA and DHA content of neutrophils increased, and AA content decreased, among those taking capsules which were at least 25% tuna oil, with this change occurring in a dose-dependent fashion, with maximal effects observed after four weeks of supplementation. It was also observed that there were decreased levels of AA in neutrophils among those taking the ALNA-rich linseed oil supplements. It is possible that provision of ALNA increases competition for the action of desaturase and elongase enzymes involved in LC PUFA synthesis, and therefore competitively inhibited the formation of AA from LA. While this study identified significant effects upon immune cell composition, no significant functional effects of supplementation were observed, though only superoxide production and chemotaxis were investigated.

Dietary fatty acids have the potential to alter the fatty acid composition of a wide range of other immune cells. A human study which provided 10-15 g/day fish oil (rich in LC n-3 PUFA) was demonstrated to significantly increase the amount of EPA within platelets, neutrophils, monocytes, T lymphocytes and B lymphocytes phospholipids within two weeks of supplementation(86). This effect upon a wide range of cell types

provides information upon how dietary fatty acids are incorporated into immune cells. Cells with a short half-life (monocytes, 3 days; platelets 8-11 days; neutrophils, 6 hours) may have incorporated these fatty acids into cell membranes during cell formation, or by phospholipid exchange between mature cells and plasma lipoproteins. T and B lymphocytes have a much longer half-life (months to years) and are likely to have predominantly accumulated new fatty acids by exchange with plasma phospholipids(86).

Studies have identified that feeding mice various high fat diets (10% w/w) for four weeks caused significant alterations in macrophage fatty acid composition(87). Mice fed on a fish oil diet had higher levels of EPA, DPA and DHA in macrophage phospholipids compared to other groups, while mice fed on a borage oil or a safflower oil diet (rich in LA acid) had the highest levels of AA in macrophage phospholipids. This indicates that both n-3 and n-6 fatty acid rich diets have the potential to significantly alter immune cell fatty acid composition.

The changes in membrane composition observed as a result of dietary manipulation have the potential to exert effects upon the function of immune cells, via effects on membrane protein function. The interaction between the TCR upon T cells and MHC upon APC has been described as the formation of an immunological synapse. This process requires cytoskeletal rearrangements to maintain a sustained, stable interaction. Both EPA and AA have been demonstrated *in vitro* to inhibit immunological synapse formation by affecting cytoskeleton relocalisation within T cells, and may therefore diminish signalling between T cells and APC(88). Effects of AA and DHA have also been identified upon the expression of MHC I molecules upon APC *in vitro*, with both PUFA reducing expression of this cell surface marker upon B lymphocytes(83). These *in vitro* findings are supported by *in vivo* dietary studies. For example, a study which provided high fat (HF; 20% w/w) fish-oil rich diets to rats demonstrated that MHC II expression upon dendritic cells was significantly lower than seen in HF safflower oil fed or low-fat (2.5% w/w) corn oil fed groups(89).

1.4.2 Fatty acids and prostaglandin synthesis

AA is the fatty acid substrate metabolised by COX to generate PGE_2. It was detailed in earlier sections how alternative fatty acid substrates will generate different PGE isoforms (see figure 1.12), with EPA giving rise to PGE_3, and DGLA giving rise to

PGE_1. Since dietary fatty acid manipulation can affect the membrane composition of immune cells, this will change the availability of fatty acid eicosanoid substrates, which may have significant effects upon immune function due to competitive inhibition of COX activity or differences in potency of the isoforms generated. For example, PGE_2 has been demonstrated to be more proinflammatory, and exerts a stronger positive feedback upon COX_2 expression than PGE_3(90).

The pro-inflammatory effects of PGE_2 include fever, erythema, increased vascular permeability, vasodilation, pain, and oedema. However, the action of PGE_2 is not limited to the induction of inflammation. PGE_2 has also been demonstrated to exert effects upon the Th1/Th2 balance of cytokine production, and so may have an important role in diseases of immune dysfunction such as atopic disease. *In vitro*, PGE_2 enhances the effects of the Th2 cytokine IL-4 upon IgE synthesis by increasing the number of IgE secreting cells, and promotes class switching of B cells expressing IgM to produce IgE(91). PGE_2 suppresses of T- and B-lymphocyte proliferation and activation, NK cell activity and inhibits the production of the Th1 cytokines IL-2 and IFN-γ(92;93). These effects of PGE_2 therefore promote Th2 responses and suppress Th1 responses, and may be involved in the dysregulated Th2 responses which are characteristic of atopic disease.

The n-6 PUFA substrate AA which is used to generate PGE_2 is only consumed at very low levels in the diet, so the majority of AA within cell membranes will be derived from endogenous synthesis of LC PUFA from the EFA precursor LA. This therefore provides a plausible mechanism by which increasing consumption of vegetable oils and margarines, which are rich in LA, may have contributed to increasing prevalence of asthma(9). There is epidemiological evidence which supports this hypothesis, with an Australian study(94) attributing 17% of recent cases of childhood asthma to a high PUFA (predominantly n-6) intake. Additional risk factors identified for childhood asthma were having a parent with a history of asthma and serious respiratory infection in the first two years of life. Factors identified as providing protection against asthma include breastfeeding and having at least three older siblings. This demonstrates clearly that while it is not diet alone that determines the risk of development of atopic disease, dietary fat intake and breastfeeding are two important risk factors with the potential to be modified by behavioural or dietary interventions.

There is an indication from epidemiological studies that regular consumption of fresh oily fish (containing LC n-3 PUFA), but not canned or processed fish, is associated with a reduced risk of current asthma during among children aged 8 – 11 years(95). Human supplementation studies support this hypothesis of a protective effect of n-3 fatty acids, as when healthy male volunteers were provided with supplements containing EPA (1.35 - 4.05 g/day for 12 weeks), inverse correlations were demonstrated between the EPA content of mononuclear cells and PGE_2 production(96).

1.4.3 Dietary fatty acids and immune responses in animal models

Animal studies have clearly demonstrated that dietary fatty acids have the capacity to significantly affect immune function.

A long term feeding study of mice (diets provided for 5 months, including two breeding cycles) identified that those upon high fat (10% w/w) n-3 PUFA rich diets (linseed oil, fish oil) had reduced NK cell activity, which was inversely proportional to the n-3 PUFA content of the diet. Both resting and stimulated superoxide production of macrophages were affected by diet, with those on the n-6 PUFA rich diet having the highest resting production and lowest stimulated production, and those on the fish oil diet demonstrating the opposite relationship. $CD8^+$ cells were lowest among the n-3 PUFA diet groups(97). This demonstrates that dietary fatty acids can significantly affect the proportion of immune cell types and markers of their function. This study also identified significant effects of diet upon the number of successful pregnancies (fish oil > linseed oil > safflower oil > olive oil), an indication that the n-6 PUFA rich diet was growth promoting (though dietary intake was not controlled and may have varied between groups), and significant effects of diet upon brain, spleen and thymus weight.

A study which provided diets to mice that were either low in fat (2.5% w/w) or high in fat (20% w/w) and containing coconut oil (rich in saturated fat), safflower oil (rich in n-6 PUFA) or fish oil (rich in n-3 PUFA) over six weeks looked specifically at the effect of diet upon spleen lymphocytes. The fatty acid composition of spleen lymphocytes was significantly affected by the diet. Proliferation and IL-2 production were greatest for lymphocytes from mice fed coconut oil. IFN-γ production was lowest among the safflower oil and fish oil groups, but no associated effect was seen on IL-4 production. The authors therefore suggested that Th1 cytokines are more sensitive to changes in

dietary fatty acid content than Th2 cytokines(98). This observation is supported by a murine study which provided diets rich (5% w/w) in corn oil or fish oil for two weeks, and then isolated and cultured $CD4^+$ splenocytes. This study identified that fish oil suppresses *in vitro* Th1 development and production of IFN-γ, and the authors speculated that this is responsible for the anti-inflammatory effects of fish oil supplementation(99).

1.4.4 Dietary fatty acids and immune responses among healthy adult humans

In addition to the immune effects which are observed as a result of dietary supplementation with specific fatty acids, it has been identified that variations in both n-3 and n-6 fatty acid composition of immune cells among free-living healthy adults are a significant predictor of markers of immune function. A study of free-living human subjects found that the fatty acid composition of peripheral blood mononuclear cells (PBMC) demonstrated statistically significant correlations to several markers of immune function(100). The proportion of ALNA within PBMC was positively correlated to markers of phagocytosis and the production of both Th1 and Th2 cytokines (IFN-γ and IL-4), and DHA content was positively correlated to markers of phagocytosis and IFN-γ production. In contrast, the LC n-6 PUFA AA content was positively correlated to IL-6 production, a cytokine associated with inflammation.

Interest in the specific interactions between n-3 PUFA and the immune system has resulted in a wide range of available data from *in vitro* studies, *ex vivo* studies and *in vivo* studies, and detailed reviews can be found elsewhere(35;101). Briefly, *in vitro* studies have demonstrated that LC n-3 PUFA inhibit lymphocyte proliferation, decrease IL-2 production, decrease cytotoxic T cell degranulation and inhibit antigen presentation. *Ex vivo* animal and some human studies have supported these findings with LC n-3 PUFA found to inhibit lymphocyte proliferation, decrease NK cell activity, decrease cytotoxic T cell activity and decrease antigen presentation.

1.4.5 Dietary fatty acids and diseases of immune dysfunction

While *in vitro* and *ex vivo* studies can provide useful information upon the potential mechanisms by which fatty acids and the immune system interact, it is of more relevance to human health to investigate the *in vivo* immune responses to dietary fatty

acids, particularly in relation to development or severity of diseases of immune dysfunction. Difficulties in gaining detailed and clear information upon the effect of n-3 PUFA upon immune function in humans are often attributed to the numerous confounding factors which affect immune function, including age, sex, BMI, physical exercise, smoking, stress and vaccination history, factors which can be controlled by the use of animal models.

Clinical trials which have been undertaken in patients with chronic inflammatory diseases have been reviewed elsewhere(101) and have identified robust benefits of LC n-3 PUFA supplementation in rheumatoid arthritis. The results from studies of other autoimmune diseases such as Crohn's disease, ulcerative colitis and psoriasis are mixed, with some studies reporting benefits, and others finding no effect of LC n-3 PUFA supplementation. The effect of n-6 and n-3 PUFA supplementation upon atopic disease has also been reviewed(102). This review identified the modest benefits of DGLA supplementation upon atopic skin, and the mixed and dose-dependent effects of LC n-3 PUFA supplementation of asthmatics. It was suggested in this review that the timing of potential dietary interventions may be important, with intervention before or during the allergic sensitisation phase hypothesised to be of more benefit that supplementation after the onset of established disease.

1.5 Programming

Programming is term used to describe the process by which the environment experienced before birth or during early life exerts long-term effects upon health that are expressed throughout adult life. This process is also known as developmental plasticity(103), the thrifty phenotype hypothesis or developmental origins of adult health and disease(104). Evidence for programming has been identified in both animal and human studies. This section sets out to describe the theoretical basis of programming, evidence from human studies which supports the programming hypothesis, and the potential mechanisms responsible for programming.

1.5.1 Theoretical basis of programming

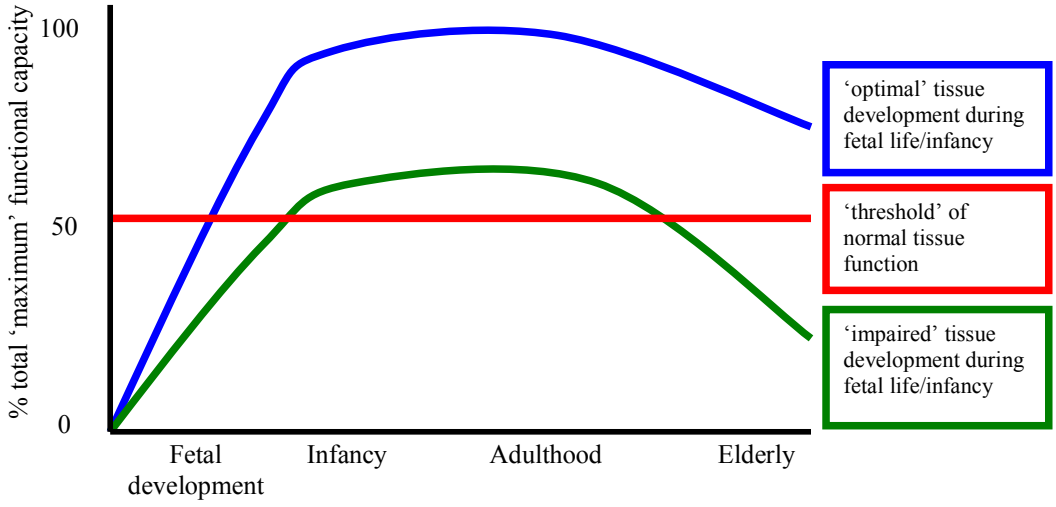
The two concepts which are fundamental to the programming hypothesis are functional capacity, and the importance of the timing of the environmental insult or stimulus to programming.

Functional capacity

The principal that every organ or tissue has a maximum potential functional capacity which is determined by the type and number of cells or functional structures that are present, and a threshold capacity, under which normal tissue function cannot be maintained, is key to the programming hypothesis. A graphical representation of the concept of functional capacity is illustrated in figure 1.13.

As a large proportion of human tissue development occurs *in utero*, it is suggested that a high percentage of functional capacity is established during fetal life. Once a tissue has matured and reached its maximal functional capacity a period of stability follows, before declining in response to stressors such as environmental insults or aging. This decline in functional capacity will eventually reach a threshold under which normal tissue function can no longer be maintained, which may result in the onset of disease symptoms. It has been suggested that programming may modify the maximal functional capacity achieved by a tissue or organ. If programming is beneficial, this may delay or altogether avoid the deterioration of tissue function to a level at which normal functioning is lost. If programming adversely affects tissue development, the maximal functional capacity achieved may be significantly lower, and the threshold of normal tissue function reached earlier or more rapidly in response to environmental stressors.

Figure 1.13: Graphical illustration of the concept of functional capacity



Critical time periods of programming

Periods of rapid cell division within a tissue are critical stages of development, and in humans most rapid cell division occurs during fetal development. Humans undergo rapid growth before birth, with 42 cycles of cell division *in utero* and only 5 after delivery(105). In a less developmentally mature animal, such as the rat, there may also be critical periods after birth. The development of the human immune system is described in detail in a later section (see 1.8.2).

Animal models have clearly demonstrated the importance of timing to programming effects. For example, if female rats are injected with testosterone proprionate before they are 10 days old, ovulation is prevented throughout adulthood, but the same injection given to rats at 20 days old has no effect on later ovarian function(106).

Undernutrition is an environmental insult which is likely to adversely affect tissue development and function. It has been demonstrated that rats undernourished before weaning will never reach the size of their nourished counterparts in adulthood, even with the resumption of full feeding(107). Similarly, rats subjected to undernutrition at 3-6 weeks old (after weaning) or 6-9 weeks old will demonstrate accelerated growth upon refeeding, but will never fully catch up to the growth of controls. In contrast, rats undernourished at 9-12 weeks will demonstrate catch up growth and even exceed the weight of controls(108).

1.5.2 Evidence of programming from human studies

The potential programming effects of the early life environment upon human health were initially identified in research which attempted to understand the mechanisms responsible for the development of non-communicable diseases such as coronary heart disease (CHD). It was identified that the risk of CHD during adulthood was twice as high among those whose siblings were stillborn or had died during early infancy(109), and that the risk of cardiovascular disease (CVD) mortality among adults (aged 40-69 years) was strongly positively correlated with the infant mortality rates of this adult cohort's early years of life(110). Neonatal and infant mortality is strongly influenced by adverse circumstances during pregnancy, birth and infancy, and in these studies was used as an indirect measurement of the health status of the surviving siblings or other children born in the same area. These studies therefore indicated that a poor

environment during fetal development or infancy is a significant risk factor for CVD in adulthood.

Similar studies have since been undertaken using UK cohorts. Corresponding historical infant mortality rates were identified to be significantly correlated to rates of adult non-communicable diseases including CVD, chronic bronchitis, stomach cancer and heart disease(111). This association was seen for both men and women, and this study also indicated that the timing of adverse conditions was likely to be significant. For example, heart disease was closely associated with both neonatal and post-neonatal mortality rates while chronic bronchitis correlated more strongly with post-neonatal mortality than with neonatal mortality. This suggests that environmental factors predisposing to bronchitis in adulthood occur at a later developmental stage.

Studies which used detailed records of birth weight and weight at one year as an indication of the quality of the fetal and infant environment within a cohort of Hertfordshire men(112) identified that men with birth weights under 5.5 lb (1 lb = 0.45 kg) had the highest mortality rates from ischemic heart disease, chronic obstructive lung disease and death from all causes combined. Those with the lowest weights at one year old also had a greater disk of death from ischemic heart disease. This association between birth weight and disease risk was consistent across the full range of birth weights, including those within the normal range.

The growth of the fetus is primarily determined by genetics but can also be modified by maternal size, maternal age and parity, maternal infection during pregnancy, the length of gestation, excessive energy expenditure after mid-pregnancy, pre-eclampsia and the availability of nutrients and oxygen to the fetus. Although nutrition may only play a relatively minor role in determining fetal growth, it does provide the potential for interventions to improve the nutrition of the mother and fetus, in order to reduce the risk of disease in later life. It is also likely that the effects of programming extend beyond simple relationships with fetal growth patterns, and may exert more subtle effects such as modification of tissue structure by altering cell types and cell numbers or alterations in patterns of gene expression or sensitivity to hormonal signals.

A ‘mis-match’ between the fetal and adult environment is also hypothesised to be associated with adverse programming effects. For example, if the fetus is subject to *in*

utero growth retardation as a result of impaired placental function, it may be 'programmed' to survive within an environment which is nutrient-poor. If however, this is an inappropriate adaptation, and the infant is in fact exposed to a nutrient rich environment in early life, this may confer a further disadvantage. There is evidence from human studies that this mis-match occurs. Data from a cohort of adults in Helsinki identified that infants with the lowest birth weight who went on to exhibit later 'compensatory' growth (expressed as high BMI at age 11 years) were at an increased risk of type II diabetes and hypertension(113). Migrant studies have also supported the importance of mis-match to programming. For example, Asian migrants to Britain have high mortality rates from CHD compared with the rest of the UK general population, which cannot be explained by traditional risk factors such as plasma cholesterol, smoking rates or intakes of saturated fats, but are found to relate to the lower birth weights among these populations(114).

Data from human and animal studies which indicate that there are significant programming effects upon immune function will be discussed in detail in a later section (see section 1.9).

1.5.3 Proposed mechanisms of programming

The mechanisms of responsible for programming have not been fully characterised, but potential mechanisms include fetal nutrition, overexposure to glucocorticoids, and epigenetic changes. It is likely that there is significant interaction between the mechanisms.

Fetal nutrition

Nutrient deficiencies, particularly low-protein diets, have been demonstrated to induce programming effects. For example, a study which provided a low protein diet (9% w/w, compared to 18% within a standard rat maintenance diet) to pregnant rats for just the four days following mating demonstrated that this induced significant effects upon offspring birth weight, postnatal growth, blood pressure and organ:body weight ratios which were still apparent up to age 12 weeks(115).

In addition to the effect of nutritional deficiencies, provision of inappropriate excessive nutrients can also exert adverse programming effects. Studies which have provided high protein energy supplements to pregnant mothers have exhibited deleterious effects on neonates including increased rates of pre-term delivery and perinatal death(116-118).

The protein content of the maternal diet during pregnancy has the capacity to alter offspring plasma cortisol concentrations. For example, the adult children of women who had consumed a high-protein (0.45 kg red meat daily) diet during pregnancy under the advice of their doctor were identified to have higher plasma cortisol concentrations and blood pressure than the children of women who did not receive or comply with this advice. It is thought this may be a direct consequence of overexposure of the developing fetus to cortisol, programming the hypothalamic pituitary axis of the offspring to life-long hypercortisolemia(119).

Overexposure to glucocorticoids

It is possible that some of the programming effects which are attributed to maternal diet, particularly a low-protein diet during pregnancy, may be mediated by increased exposure of the fetus to corticosteroids(115). Animal models which provide low-protein diets have identified that this reduces the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) within the placenta. This is an intracellular enzyme which converts active glucocorticoids such as cortisol and corticosterone into inactive metabolites such as cortisone and 11-dehydrocorticosterone(120). The expression of this enzyme within the placenta suggests that it has a role in protecting the fetus from maternal glucocorticoids. Changes in the activity of 11-βHSD in the placenta following dietary restriction is not a result of global reductions in placental enzyme activity as placental enzymes such as maltate dehydrogenase and pyruvate kinase are not significantly affected by maternal protein restriction(121).

Fetal overexposure to maternal glucocorticoids is associated with reduced birth weight(122;123) and later hypertension in both animal models and humans studies(124;125). Elevated glucocorticoid levels cause accelerated fetal maturation, and may therefore be an adaptive response to an adverse fetal environment(104).

Glucocorticoids have immunosuppressive and anti-inflammatory effects, which may affect the development of the fetal immune system. Lymphocytes taken from rhesus monkeys exposed for 2 days prior to parturition to dexamethasone *in utero* had a reduced proliferative response at age 1year and reduced sensitivity to corticosteroids(126). A similar effect has been documented among rhesus monkeys which were stressed during gestation(127). Glucocorticoid hormone receptors are present in the thymus and it has been demonstrated that over-exposure to

glucocorticoids (induced by maternal stress during pregnancy) could induce involution of the fetus rat thymus(128).

Epigenetics

Epigenetic modifications are changes to DNA methylation patterns or chromatin packaging by histone acetylation which influence transcriptional activity and therefore gene expression. These modifications are established early in life and maintained, and allow phenotypic change without changes to the DNA sequence. Epigenetic modifications are involved in the tissue specific expression of genes and the process of imprinting which determines which parental gene allele is expressed. Epigenetic processes are therefore a normal function of fetal development which may have the potential to exert long-term effects upon adult tissue function(124;129).

Maternal protein restriction during pregnancy has been demonstrated to significantly affect the methylation status of specific genes in the offspring, including the glucocorticoid receptor expression and PPAR-α (a gene implicated in cardiovascular and metabolic disorders); with these effects lost if the maternal diet is supplemented with folic acid. The effects of maternal diet were gene-specific, with PPAR-γ methylation status unaffected by maternal protein restriction(129). These data suggest that the effects of maternal nutrition during pregnancy upon glucocorticoid exposure may be mediated by changes in gene methylation status.

1.6 Gender and fatty acid composition

Human studies have identified that men and women differ in their ability to synthesise LC n-3 PUFA from ALNA(130;131), with associated differences in plasma fatty acid composition(132-135). The ability of humans to convert ALNA into LC n-3 PUFA has been studied using two approaches: the provision of ALNA labelled with stable isotopes or provision of additional dietary ALNA. In studies where stable isotope-labelled ALNA is provided, subsequent blood samples are collected to determine the circulating concentrations of stable isotope-labelled metabolites of ALNA such as EPA, DPA and DHA. These studies have the advantage of eliminating any doubt that these products may have come from alternative sources such as the diet or release from adipose tissue. By providing additional dietary ALNA it is possible to assess whether there are

associated increases in circulating concentrations of LC n-3 PUFA in blood, cell and tissue pools, which would be indicative of LC n-3 PUFA synthesis.

1.6.1 Studies of stable isotope labelled ALNA

Data from studies using stable isotope-labelled ALNA gave the first indications that there are gender differences in the ability to synthesis LC n-3 PUFA from ALNA. Studies which provided a dose of ^{13}C-ALNA as part of a meal and subsequently collected blood and breath samples over a three week period indicated that young women (average age 28 y) converted a greater proportion of ALNA into EPA and DHA compared to men (average age 36 y)(130;131).

This finding that women have a higher rate of synthesis of LC n-3 PUFA compared to men is supported by the work of other authors which used ^{2}H-ALNA and mathematical modelling to determine rates of LC n-3 PUFA synthesis in a group of healthy mean and women(136;137). When these data were assessed for gender differences(138) it was found that women receiving a beef-based diet utilised a 3-fold greater amount of DPA to generate DHA compared with men. A similar but non-significant trend was observed in those subjects receiving an *ad libitum* diet.

The studies undertaken using ^{2}H-ALNA indicate that background diet interacts with the gender differences in rates of LC n-3 PUFA synthesis. In these studies subjects were maintained upon controlled diets for two weeks prior to administration of the ^{2}H-ALNA, and remained on this diet as blood samples were collected over a one-week period. The ability of men to generate DHA from DPA was not affected by the diet received, while women had lower DHA synthesis when receiving a fish-based diet, exhibiting a rate of synthesis comparable to that observed in men(138). The synthesis of LC n-3 PUFA in women therefore appears to be sensitive to the dietary availability of these fatty acids. It is possible that diet also affects LC n-3 PUFA synthesis among men, as a study of older men (mean age 52 years) demonstrated that while an ALNA-rich diet did not affect ^{13}C-ALNA conversion to EPA, DPA or DHA, a diet enriched in EPA plus DHA decreased ^{13}C-ALNA conversion to EPA and DPA(139).

A primary limitation of the stable isotope studies is that they do not give insight into the extent to which LC n-3 PUFA might have been incorporated into metabolically relevant tissues such as the liver and adipose tissue. These studies also give little indication of

whether the differences between men and women in the ability to synthesise LC n-3 PUFA relate to differences in the circulating concentrations of those fatty acids.

1.6.2 The effects of dietary ALNA upon LC n-3 PUFA status

Comprehensive reviews of studies which provided increased dietary ALNA and assessed the appearance of EPA and DHA are available(140;141). To date, no study has been undertaken to investigate directly whether the effect of increased dietary ALNA upon LC n-3 PUFA status differs between men and women. Increasing dietary ALNA intake has been found to result in dose-dependent increases in EPA in plasma phospholipids; similar effects are likely for circulating cells such as leukocytes and platelets, although there are fewer studies investigating these. The relationship between increasing dietary ALNA and DHA status is less clear, with some studies reporting a reduced DHA status with increasing dietary ALNA and others reporting no change in DHA status. There is a plausible mechanism by which increasing dietary ALNA intake might reduce DHA synthesis. The action of Δ6 desaturase is required in two stages of the synthesis of LC n-3 PUFA (see figure 1.6). It is possible that increasing dietary ALNA increases substrate competition, inhibiting the Δ6 desaturation of tetracosapentaenoic acid to DHA(20).

The studies which are currently available on the effect of increased dietary ALNA upon concentrations of LC n-3 PUFA vary in their design (see (140) for details). There is wide variation between the studies in terms of duration (2-52 weeks), mode of supplementation (capsules, use of oil mixed into salads, incorporation of ALNA into dietary products such as spreads and muffins), whether LA intake was modified in parallel, and the blood lipid fraction analysed. Studies to date have been conducted in men or mixed groups of men and women, with the exception of one study of lactating women(142). The latter study demonstrated that increased dietary ALNA (20 g/day) increased ALNA, EPA and DPA in plasma, but did not alter plasma or breast milk DHA. There is therefore limited data currently available to describe the effects that increased dietary ALNA may have upon blood and cell LC n-3 PUFA status in women, and no data in non-lactating women.

1.6.3 The effect of gender upon blood and tissue LC n-3 PUFA content

Studies have been undertaken in humans to investigate the effect of gender upon circulating plasma concentrations of LC n-3 PUFA (see table 1.3). While these studies vary in their sample size, degree of dietary control exerted and the range of blood lipids analysed, all have found that women have significantly higher circulating DHA concentrations compared to men and that this is independent of dietary intake(132-135). There is an indication from two of these studies that EPA and DPA are found in lower circulating concentrations in women compared to men which may indicate either a greater rate of conversion of these fatty acids into DHA or their displacement from lipids by DHA.

1.6.4 Potential mechanisms responsible for gender differences

Mechanisms which have been proposed to be responsible for the gender differences observed in LC n-3 PUFA synthesis and tissue content include gender differences in rates of β-oxidation, adipose tissue composition and mobilisation, and the possible influences of sex hormones upon the desaturase and elongase enzymes involved in the synthesis of LC n-3 PUFA(131;133).

Studies using ^{13}C-ALNA which collected breath samples enabled the rate of β-oxidation of ALNA over 24 hours to be calculated via the recovery of $^{13}CO_2$(130;131). These studies estimated the extent of β-oxidation of ^{13}C-ALNA to be 33% in men, and 22% in women. These values and the sex difference observed are similar to those obtained in studies of other fatty acids (e.g. ^{13}C-palmitate(143)) and may therefore reflect the differences in muscle mass between men and women, rather than any specific sparing of ALNA in women. The lower rates of β-oxidation of fatty acids observed in women could potentially leave more ALNA available as a substrate for metabolism into LC n-3 PUFA.

Sex differences in subcutaneous adipose tissue composition have been observed in human studies, with women found to have significantly more DPA and DHA in adipose tissue compared to men, although these fatty acids are relatively minor components of the total fatty acid content (< 0.3%)(144). In the fasting state, plasma NEFA are fatty acids released from adipose tissue under the action of hormone-sensitive lipase. It has

Table 1.3: Summary of human studies which have investigated the effect of gender upon the LC n-3 PUFA composition of blood lipids

Subjects	Country	Age (y)	Dietary control	Lipid fraction	Effects of sex			Reference
					M > F	M = F	F > M	
41 ♀ 41♂	Finland	♀ mean 40 ♂ mean 43	7 day weighed food record	Serum CE Serum TAG Serum phospholipid		EPA DHA EPA	DHA DHA	(132)
103 ♀ 72 ♂	Netherlands	18-67	Controlled diet 3 weeks	Plasma CE		EPA, DPA	DHA	(135)
23 ♀ 13 ♂	UK	18-35	Habitual diet (FFQ)	Plasma TAG Plasma NEFA Plasma PC Plasma CE Total plasma	 DPA 	EPA, DPA EPA, DPA EPA EPA, DPA, DHA DPA	DHA DHA DHA EPA,DHA	(133)
1547 ♀ 1246 ♂	New Zealand	15-65+	Habitual diet (24 h recall)	Serum phospholipid Serum TAG Serum CE	EPA, DPA EPA	 EPA, DPA, DHA 	DHA DHA	(134)

F, female; M, male

been identified that women have higher fasting plasma NEFA concentrations compared to men(133) reflecting the greater proportion of body fat in women compared to men. *Ex vivo* studies have identified that the release of fatty acids from human adipose tissue is selective and dependent upon carbon chain length and the degree of unsaturation, with ALNA and EPA identified as being preferentially released in comparison to other n-3 PUFA(37;145). It is therefore possible that gender differences in adipose tissue composition may directly affect circulating concentrations of LC n-3 PUFA or increase the availability of ALNA for synthesis of LC n-3 PUFA in women compared to men.

Sex hormones and LC n-3 PUFA synthesis

A role for sex hormones in mediating the sex differences in ALNA conversion and in LC n-3 PUFA content of blood (and tissue) lipids has been suggested from studies of women using oral contraceptives or HRT, a study of sex hormone treatment in trans-sexual subjects and *in vitro* and animal studies.

Oral contraceptives

When ^{13}C-ALNA was provided to women it was found that those women using the oral contraceptive pill had higher rates of DHA production over 21 days than women who did not use the pill(131). This finding may indicate upregulation of the desaturase/elongase pathway by female sex hormones such as oestrogen or progesterone. However, the number of female subjects in this study was low (n = 6) and data was not available for circulating sex hormone concentrations in these women.

Data from studies of circulating LC n-3 PUFA concentrations support the suggestion that women using the contraceptive pill have higher rates of DHA synthesis. When 32 women who used oral contraceptives were compared with 72 women who did not use oral contraceptives, a trend was observed ($p = 0.08$) for higher concentrations of DHA in plasma CE among those women using oral contraceptives(135). However, significant differences in age between these two groups of women were mentioned (but not detailed in the paper), which suggests potential differences in menopausal status as study participants were recruited from a wide age range (18-67 y); furthermore data was not collected on sex hormone concentrations in this study. It is possible that the timing of blood sample collection during the menstrual cycle in this study (reported as day 5-9 follicular stage), may be a confounding factor, as significant differences in the fatty acid composition of serum lipids have been identified across the menstrual cycle(146).

Another study which attempted to address the impact of oral contraceptive use indicated that women using oral contraceptives had 60% higher total plasma DHA content, than those who did not use oral contraceptives(133). However, due to the small number of subjects in this study (11 women using oral contraceptives, 12 women not using oral contraceptives) the study lacked statistical power to identify a significant effect of contraceptive pill use. Good control over the stage of the menstrual cycle of subjects was achieved (all samples collected on day 10 of the menstrual cycle) but there was no data available upon circulating sex hormone concentrations, which could have supported the hypothesis that higher plasma sex hormones are responsible for the fatty acid composition differences between women using or not using the contraceptive pill.

Hormone replacement therapy

The effect that use of HRT has upon plasma LC n-3 PUFA status has been assessed. A study of 104 postmenopausal Japanese women, 59 of whom received HRT (0.625 mg oestrogen + 2.5 mg progesterone per day) and 45 of whom received no treatment found that use of HRT for 12 months was associated with increased plasma EPA and DHA(147) which suggests that female sex hormones may up-regulate the synthesis of LC n-3 PUFA from ALNA. However, this study lacked a placebo group (with the subjects themselves deciding whether or not to receive HRT) and did not report any form of dietary assessment or restrictions. It is therefore possible that the differences observed are an effect of differences in dietary LC n-3 PUFA intake between the two study groups.

A placebo controlled study to investigate the effect of HRT or an oestrogen-receptor modulator (Raloxifene) upon the fatty acid composition of plasma CE also found a significant increase in plasma CE DHA content with HRT(148). The potential role of oestrogen rather than progesterone in these observed differences was indicated by the finding that Raloxifene also led to increased DHA content in CE. This study reported a significant inverse relationship between ALNA and DHA content of CE among women using HRT or Raloxifene, which was suggested to be indicative of an enhanced capacity to synthesise DHA from ALNA. An alternative explanation for this finding is that increased dietary ALNA can lead to reductions in DHA status(140). As the women in this study were not controlled for dietary intake, it is possible that this inverse relationship reflects dietary variation in ALNA intake among these subjects rather than an increased conversion of ALNA into DHA.

Sex hormone treatment of transsexual subjects

A study of transsexual subjects was undertaken to assess the influence of cross-sex hormone administration upon fatty acid status(135). This study demonstrated that male to female transsexuals receiving a combination of oral ethyinyl oestradiol and cyproterone acetate had higher DHA concentrations in their plasma CE within 4 months of treatment. Female to male transsexuals receiving intra-muscular testosterone had significantly lower DHA content in plasma CE within 4 months of treatment. This suggests that oestradiol up-regulates, while testosterone down-regulates, the synthesis of DHA from ALNA. The authors suggested that the difference seen in female to male transsexuals was not due to the effect of testosterone treatment itself, but to the associated reductions in circulating oestrogen. However, the effect of testosterone upon circulating oestrogen concentrations was only statistically significant in the subjects who received treatment for 4 months ($p = 0.01$), with the group treated for 12 months not differing significantly in their oestradiol status ($p = 0.09$). The dietary intake of subjects was assessed using the Dutch EPIC food frequency questionnaire (FFQ)(149) but this was only conducted among subjects receiving hormone treatment for 12 months. While the design of this FFQ allows assessment of fish intake, this was not reported.

In vitro and animal studies

There is experimental evidence from animal studies that sex hormones might influence the activity of the desaturase enzymes involved in the conversion of n-3 and n-6 EFA into their longer chain, more unsaturated derivatives. Work to date is limited to the use of n-6 rather than n-3 fatty acids as a substrate for assessment of enzyme activities and has investigated the effect of short-term (less than 2 days) hormone treatment rather than the influence of normal circulating physiological concentrations of sex-hormones.

In vitro studies identified that Δ5 desaturase activity (assessed by production of the n-6 PUFA AA) in liver microsomes isolated from female rats was reduced by 17β-oestradiol and testosterone, and unaffected by progesterone(150). An *ex vivo* study using rat liver microsomes to assess the effect of oestradiol treatment upon Δ6 desaturase activity (assessed by production of the n-6 PUFA γ-linolenic acid, 18:3n-6) identified that treatment of female rats for two days with 17β-oestradiol led to reduced activity(151). The effect of 17β-oestradiol treatment upon serum fatty acid composition

was also assessed, with significant reductions in serum AA content observed, although there was no significant effect upon DPA or DHA content.

A study which assessed the effect of a single interperitoneal testosterone injection in both male and female rats found that 24 hours after testosterone treatment there was increased Δ9 desaturase activity, while Δ6 and Δ5 desaturase activities (assessed using n-6 fatty acid substrates) were inhibited(152). Fatty acid composition analysis performed upon plasma and liver subcellular fractions confirmed that the decreases in Δ6 and Δ5 desaturase activities were associated with reduced DHA content in both the male and female rats. This study also identified that the DHA content of plasma and liver fractions in both testosterone treated and control groups was higher in female rats compared to males, though the statistical significance of these sex differences was not presented.

These studies using rat models support the observations from studies of human transsexual subjects that testosterone treatment may reduce the activity of Δ5 and Δ6 desaturases, reducing the synthesis of LC PUFA, and leading to significant reductions in the tissue content of LC n-3 PUFA such as DHA. Data also suggest that oestrogen may decrease Δ5 and Δ6 desaturase activity, though associated reductions in LC PUFA have only been identified for n-6 PUFA, with no significant effect of oestrogen treatment upon DHA content observed. There are no data currently available upon the effect that circulating sex hormones may have upon the activity of the elongase enzymes involved in the synthesis of LC PUFA.

1.7 Pregnancy and fatty acid composition

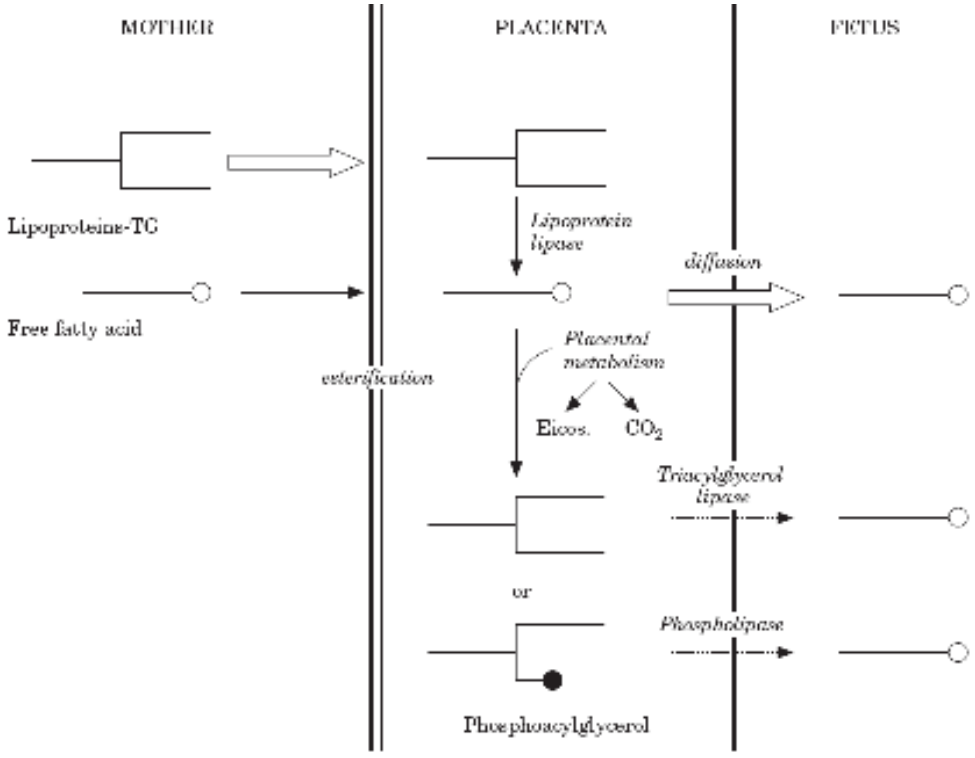
Maternal lipid metabolism is significantly altered during pregnancy in order to meet changing maternal energy requirements and the requirements of the developing fetus. However, the nutrient crossing the placenta in the highest quantities is glucose, followed by amino acids, fatty acids and glycerol(153).

Increased maternal appetite (hyperphagia) is a feature of early pregnancy(154), with fatty acids deposited in adipose tissue(155). Gastrointestinal transit is reduced in later gestation, enabling increased absorption of dietary fat(156). Fatty acids deposited in adipose tissue in early pregnancy become available for placental transfer to the fetus in the final trimester as a result of increased lipolytic activity in adipose tissue, mediated by increased mRNA expression and activity of hormone sensitive lipase and associated

with maternal insulin resistance and reduced LPL activity of adipose tissue(154;155). Hypertriglyeridemia of pregnancy is a result of enhanced VLDL production in the liver, which may be mediated by the effects of oestrogen.

The transport of fatty acids across the placenta releases free fatty acids into the fetal circulation (see figure 1.14). Maternal lipoproteins do not cross the placenta intact; instead maternal TAG from plasma lipoproteins are taken up by the placenta via the action of lipoprotein receptors and lipase activity of the placenta, hydrolysed within the placenta to free fatty acids which are released into the fetal circulation. Free fatty acids are also transported across the placenta in a process involving fatty acid binding proteins (FABP). These transporters are selective, resulting in enrichment of the fetal circulation with LC PUFA(154). The placenta has been demonstrated to preferentially transfer n-3 PUFA in comparison to n-6 PUFA, with perfusion experiments determining the overall selectivity to be DHA > ALNA > LA > AA(157). Free fatty acids which are released into the fetal plasma bind to α-fetoprotein, and are rapidly transported to the fetal liver(155).

Figure 1.14: Transport of fatty acids across the placenta, diagram taken from (153)



1.7.1 Fatty acids and fetal tissue development

Transfer of fatty acids to the fetus is essential to meet both the demands of *de novo* tissue synthesis and to lay down adipose tissue reserves. In humans, 16% of body weight in the newborn is adipose tissue. In contrast, the newborn rat has very low body fat, with white adipose tissue barely detectable at birth, indicating that for the rat there is a significant contribution of lactation to the accumulation of adipose tissue.

Specific maternal dietary fatty acids have been demonstrated to be essential for successful fetal development and later tissue function in both human and animal studies, particularly n-3 PUFA. Transfer of DHA to the developing fetus in human pregnancy predominantly occurs in the last 10 weeks of pregnancy, with the majority of this DHA accumulated within fetal adipose tissue(158). The observation that DHA is found in high concentrations in the retina and accumulates in the fetal brain during early development (3 months gestation – 18 months after delivery) has led to the suggestion that an adequate dietary supply of this fatty acid is required for optimal brain and visual development(159). Animal studies where n-3 fatty acid deficient diets are provided have demonstrated that dietary n-3 fatty acids are essential for normal cognitive and visual function, and a literature review is available(56).

Human studies have investigated the role of LC n-3 PUFA, particularly DHA, when provided in infant milk formula to both preterm and healthy term infants. Meta-analyses indicate that the addition of DHA to pre-term infant formula is beneficial for visual development in early life. Whether these effects persist beyond early life (i.e. after 4 months of age) has not yet been established(58;160). In term infants formula containing DHA was found to improve markers of cognitive function(57). However, the clinical relevance of these reported statistically significant differences and the validity of the neurodevelopmental tests utilised in these studies has been questioned(161).

1.7.2 Human studies of fatty acid status during pregnancy

Human studies have been undertaken to examine the effect of pregnancy upon maternal circulating fatty acid status(162-166). Full details of the findings of these studies are described in table 1.4. These longitudinal human studies have not included pre-conception data, and therefore can only provide information upon the variations in fatty acid status which occur after the first trimester.

Table 1.4: Summary of human studies which have investigated the effect of pregnancy upon circulating fatty acid status (page 1 of 2)

Reference	Study group	Sample type	Effects
Al et al, 1995 (162) The Netherlands	Longitudinal (n=110) 10, 14, 18, 22, 26, 30, 32, 34, 36, 28, 40 weeks	Plasma phospholipids % and quantitative analysis No dietary assessment	% effects: ↑ total saturates throughout gestation ↑ total MUFA throughout gestation ↑ total n-3 and DHA until 18 weeks ↓ total n-6 and AA throughout gestation
Wijendran et al, 1999 (163) USA	Longitudinal n=15 healthy controls n=15 gestational diabetes 27-30 weeks 33-35 weeks 36-39 weeks	Plasma phospholipids % and quantitative analysis Dietary assessment (24hr recall)	% effects in healthy controls: ↑ 16:0 ↓ 18:0 ↓ ALNA ↑ 22:5n-6 ↓ 20:0 ↓ EPA ↓ 20:3n-6 ↓ DPA ↓ AA ↓ DHA ↓ 22:4n-6
Stark et al, 2005 (164) African-American cohort, USA	Longitudinal (n=157) 24 weeks Delivery	Plasma and erythrocyte total lipid extracts % and quantitative analysis Dietary assessment (FFQ)	% effects in plasma: ↑16:0 ↓ 14:0 ↑ 22:0 ↓ 18:0 ↑ 24:0 ↓ 20:0 ↑ 18:1n-9 ↓ LA ↑ 20:1n-9 ↓ GLA ↑ 22:5n-6 ↓ EPA

Table 1.4 (contd.): Summary of human studies which have investigated the effect of pregnancy upon circulating fatty acid status (page 2 of 2)

Reference	Study group	Sample type	Effects		
Burdge et al, 2006 (165) Trinidad	Cross-sectional	Plasma	Quantitative effect in plasma:		
	non pregnant (n=10)	Quantitative analysis	NEFA:		↓ AA
	20-22 weeks (n=6)				
	30-34 weeks (n=13)	No dietary assessment	PC:	↑LA	↓ EPA
				↑ AA	
				↑ DHA	
			TAG:	↑LA	
				↑ALNA	
				↑AA	
Stewart et al, 2007 (166) Scotland	Longitudinal (n=47)	Erythrocyte			
			% effects:	↑ 22:5n-6	↓ 18:0
	12.5 weeks	% and quantitative analysis		↑ ALNA	↓ 20:0
	26.1 weeks			↑ DHA	↓ 22:0
	35.3 weeks	No dietary assessment		↑ 16:1n-7	

The effects of pregnancy upon maternal blood lipid fatty acid status observed in human studies have been mixed, with some studies have identifying a reduction in plasma phospholipid DHA status during prenancy(163), while others report an increased DHA content of plasma phospholipids(165) or red blood cells(166). These differences between studies may be due to variations in sample size, the type of blood lipid sample analysed, and the possible confounding effect of maternal diet, with the potential for differences in mobilisation from maternal adipose stores and the composition of those stores.

The effects observed in human studies have not been limited to LC n-3 PUFA status, with significant effects of pregnancy observed upon saturated fatty acids, MUFA and n-6 PUFA(162-166). No human study to date has determined whether the changes in fatty acid status observed relate to sex hormone status.

1.7.3 Animal studies of fatty acid status during pregnancy

Use of experimental animals provides a clear advantage in the investigation of the effects of pregnancy upon fatty acid composition – enabling control over several of the variables identified as likely confounding factors in human studies, such as variations in dietary intake of the quantity and quality of fats. Animal studies also have the advantage of enabling sampling of fetal tissues to investigate the effects of maternal diet upon fetal tissue composition.

Several studies have been undertaken using rats to investigate the effect of pregnancy upon the fatty acid composition of plasma and liver phospholipids and TAG(167-170). The full details of these studies are described in a later section (see chapter 4), and table 4.1 summarises the results of these studies. Animal studies to date have focused on the effects of pregnancy upon maternal plasma and liver lipid composition. In brief, these studies have all identified that there are significant differences between the fatty acid content of rat tissues at the end of gestation compared to virgin females. Consistent effects of pregnancy observed are higher 16:0 and DHA content, and lower 18:0 and AA content of both plasma and liver phospholipids. The association of 16:0 with DHA status, and 18:0 with AA status, is likely to be due to the pairing of these fatty acids within phospholipid structure.

1.8 Pregnancy and immune function

The developing fetus expresses both maternal and paternal antigens, and so it is essential that adaptations occur to the maternal immune system in order to prevent the rejection of the developing fetus by the maternal immune system. The principal mechanism which is understood to mediate this in humans is the Th2 switch, a process by which maternal immune function shifts away from a predominantly Th1 phenotype, to express a Th2 phenotype. Other changes to the maternal immune system are also likely to be involved in the maintenance of successful pregnancy such as thymic involution.

1.8.1 Maternal immune adaptations to pregnancy

The Th2 switch

The concept that successful pregnancy among both humans and mice is dependent upon regulation of the balance between Th1 and Th2 responses is widely accepted, with several reviews available upon this subject(171-174).

A Th2 response during pregnancy may be advantageous as the Th2 response involves a humoral (antibody-based) rather than cellular immune response. This therefore reduces the activity of maternal immune cells which may target the feto-placental unit, such as cytotoxic T cells or NK cells, while maintaining antibody production. Maintenance of antibody production is beneficial to the fetus, as antibodies such as IgG can be transferred across the placenta and via breast milk, providing protective immunity for the neonate until the infant's own immune system is sufficiently matured(175). There may be disadvantages to the Th2 switch in terms of maternal immune function. An impaired Th1 response may adversely affect maternal responses to pathogens such as bacteria and viral infections and an excessive Th2 response may exacerbate maternal allergies and predispose the infant to atopic disease.

<u>Evidence from human studies for the Th2 switch</u>

In vivo markers of disease status provide strong evidence for a Th2 switch during human pregnancy. Rheumatoid arthritis is an auto-immune disease associated with high levels of Th1 cytokine expression, and symptoms of this inflammatory condition are improved during pregnancy(175). In contrast, systemic lupus erythematosus is an antibody mediated auto-immune disease associated with Th2 cytokine expression, and

primarily affects women during reproductive years(176). The importance of down-regulation of Th1 cytokine production to maintaining a successful pregnancy is apparent from studies which have demonstrated that women with recurrent spontaneous abortions have significantly higher ratios of production of Th1 to Th2 cytokines when compared to women during successful pregnancy(171). There is also evidence to suggest placental deficiencies in IL-10, a Th2 cytokine, in women with recurrent miscarriage(177).

Ex vivo studies also provide supportive evidence for a Th2 switch in human pregnancy. PBMC collected from healthy pregnant women have identified that while the proportions of $CD4^+$ and $CD8^+$ cells within the blood lymphocyte pool remain constant during pregnancy, these cells differ in terms of their cytokine production profile, with a shift towards Th2 producing cells(172).

Evidence from animal studies for the Th2 switch

Murine studies have been used extensively to study the Th1/Th2 balance during pregnancy. *In vivo* disease models in mice have also demonstrated the importance of Th2 responses in the maintenance of successful pregnancy. Infection of mice with the parasite *Leishmania major* induces a strong Th1 response. When pregnant mice were infected with *Leishmania major* there was an increase in spontaneous resorptions which was associated with increased Th1 cytokine production(173). Th1 responses induce NK cell activity which correlates with rates of fetal resorption in the mouse(175). Murine studies have also demonstrated that the injection of Th1 cytokines induces abortion(178).

The rat immune response to pregnancy has been less extensively characterised, with some contradictory findings(179-181). Full details of these studies are provided in chapter 5 (see table 5.1). It is clear that there may be fundamental species differences in the immune response to pregnancy. A study which directly compared human and rat immune responses during pregnancy identified that while the human immune response adapts to have a lower proportion of IFN-γ producing cells, the rat adapts to pregnancy by reducing total lymphocyte numbers(181).

Potential mechanisms of the Th2 switch

It is likely that the placenta is involved in maintaining the maternal Th2 phenotype. For example, the placenta has been demonstrated to produce IL-4, IL-10, PGE_2 and progesterone, which all down-regulate Th1 function and upregulate Th2 function(182).

An *in vitro* study of murine cells investigated whether gene methylation may be involved in determining whether Th cells express a Th1 or Th2 response. The methylation status of the IFN-γ gene in murine cloned Th cells was significantly different between Th1 and Th2 cells, with Th2 cells being methylated in this region, which would act to inhibit transcription of the IFN-γ gene(183). This finding is also supported by a study which used immature Th cells obtained from cord blood, and found that culturing these cells with IL-4 and PGE_2 in order to induce the Th2 phenotype was associated with methylated IFN-γ promoter regions(184). A study which compared the methylation status of IFN-γ and IL-4 promoter regions in adult and cord blood immune cells identified that cord blood, which has lower production of IFN-γ than adult blood samples, exhibited corresponding higher methylation status of the IFN-γ promoter region(185).

However, the findings of these studies on methylation status have been disputed by other authors who suggest that the mutually exclusive pattern of IFN-γ and IL-4 expression in murine Th1 and Th2 cells is not regulated by methylation, and identified that the promoter regions of both IL-4 and IFN-γ had the same patterns of methylation in all Th phenotypes(186). The authors proposed that conflicting results were a result of the long culture times used in other studies, which may cause spontaneous methylation and therefore not be representative of the *in vivo* profile of gene methylation.

Other maternal immune adaptations to pregnancy

Maternal thymus involution is a feature of pregnancy and involves neural, endocrine (e.g. progesterone(177;187), gonadotrophin-releasing hormone and prohibitin(181)) and immune influences(176). Involution is specific to the cortex (the outer structures of the thymus – containing immature T cells), while the medulla (the inner structure of the thymus – containing more mature T cells) undergoes hyperplasia(177). These changes are transient, with the maternal thymus rapidly regenerating after lactation(176).

1.8.2 Development of the fetal immune system

The immune system undergoes complex developmental stages during gestation, with different cell types and organs therefore potentially having different critical periods of development during which specific interventions could be targeted. An adverse condition during these developmental periods therefore has the potential to modify the lifelong immune response.

Intrauterine growth retardation is associated with an increased risk of neonatal and post-neonatal mortality from infectious diseases(188), which suggests impaired immunity. Small for gestational age infants are also at increased risk of infectious morbidity in infancy and early childhood, with impaired thymic development proposed as a key mediator of these processes(189). In situations of nutrient deficiencies, the requirements for successful immune system development in early life may be compromised by the growth of other organs. For example, brain development *in utero* is well conserved in situations of malnutrition and may occur at the expense of other organs such as the thymus.

Organ differentiation occurs in the first trimester of pregnancy, and growth in overall body weight and length occurs in the second and third trimester. A differentiated thymus is apparent by 7-9 weeks gestation, T and B lymphocytes are present in the circulation by the end of the first trimester and reach adult levels in the second, and complement proteins and monocytes are identified after 7 weeks. The fetal immune system is capable of antigen recognition by 12 weeks, although immune system activity remains depressed throughout gestation. Environmental allergens can reach the human fetus *in utero*, via placental transfer (demonstrated for ovalbumin, birch pollen and cat allergen) and transamniotic passage (demonstrated for β-lactoglobulin and ovalbumin)(190). It is therefore possible that allergic sensitisation may occur *in utero*, and indeed infants can be born with allergic hypersensitivity, having IgE present in cord blood, despite the fact that IgE does not cross the placenta, indicating that this was generated by the developing fetus(191).

Infancy is a critical period of immunological development. There is evidence that IFN-γ production is down-regulated in the lymphocytes of neonates(192) and the fetus has been described as Th2 polarised. This results in the fetus/neonate being susceptible to infection, particularly as following birth the neonate enters a microbe dense

environment, and the passive immunity provided by maternal IgG transferred across the placenta will provide only a temporary degree of protection. After delivery, the infant must therefore switch to express a Th1 phenotype in order to cope with the wide range of infections and antigens experienced.

The infant immune system is initially vulnerable due to the immaturity of the range of specific T and B lymphocytes, and as a result the immune system undergoes rapid development in early life. In humans, the thymus reaches its maximum size by age 1 year. The proliferative potential of lymphocytes is maximised in infancy, the volume of functional tissue in the thymus is twice as high in infancy as in adulthood and the number of circulating T and B lymphocytes is three to four times higher(193). The thymus is therefore an energy expensive organ, due both to the numerous cells produced *de novo* and the high rates of cell turnover as part of the mechanism involved in avoiding self-reactivity.

1.9 Programming of immune function

Programming of immune function has not be studied as extensively as the programming of cardiovascular function, but data are available which have identified programming effects of fetal and infant growth, maternal total energy restriction and micronutrient deficiencies upon offspring immune function. To date there is no conclusive evidence to support an effect of maternal dietary fatty acids during pregnancy upon offspring immune function, but available data from animal and human studies does indicate that there is a potential for fatty acids to mediate these effects.

Data from human studies has indicated that programming effects upon immune function are associated with alterations in markers of fetal growth. Raised serum IgE levels are indicative of a Th2-type immune response, and are characteristic of atopic disease. A retrospective study identified that high serum IgE levels in adult men and women (> 50 years old) were associated with larger head circumference at birth(194). Head circumference is largely determined by brain growth, and is often used as a marker of accelerated fetal growth. It is possible that those with accelerated fetal growth will be more sensitive to periods of undernutrition. The authors of this paper suggested that selective 'sparing' of the brain during fetal development, results in reduced availability of blood and nutrients available to other organs, including immune organs, and therefore affects later risk of atopic disease.

This association between fetal growth and immune function was further supported by the data from a prospective cohort which looked at how perinatal factors affected the incidence of atopic disease. This study followed a cohort of 891 children from age 1 to 16 years and monitored the incidences of asthma, eczema and other atopic illnesses such as allergic rhinitis, food allergies and insect bite/sting allergies in relation to measures of perinatal status such as gestational age at birth, birth weight, length and head circumference. Recurrent asthma symptoms were found to associate with a head circumference of 37 cm or greater at birth. Other risk factors which were identified for the development of asthma were gender (with males more likely to develop asthma) and parental asthma or allergic rhinitis(195). The authors of this paper suggested that the effect upon head circumference may be indicative of maternal fatty acid intake, due to the essential role of fatty acids in brain development.

1.9.1 Programming of immune function by total energy restriction

The fact that breast-feeding reduces occurrence of allergic disease, particularly in babies with a family history of atopic disease, is a strong indicator that nutrition in early life has the potential to modulate the risk of atopic disease(105). Both human studies and animal models have demonstrated that total energy restriction during gestation can exert significant effects upon immune function. It would not be ethical to establish human intervention studies of total energy restriction during pregnancy, but data from dietary interventions in developing countries and historical famines can be used as models of total energy restriction.

In The Gambia there is considerable seasonal variation in energy expenditure and energy intake around the harvest seasons. This has profound effect on birth weight resulting in a variation of 200-300 g between those born in the hungry season and those born in the harvest season. Those born during the hungry season were found to have highest mortality rates in adulthood with deaths dominated by infectious and pregnancy-related deaths(196). Supplements providing an additional 1020 kcal/day from week 20 gestation until term delivery among women in The Gambia were found to have a significant positive effect on birth weight, increasing it by an average of 136 g(197). This was also associated with reduced perinatal mortality, which suggests an effect of total energy intake on the development of the immune system.

Animal studies have also identified significant effects of energy restriction during pregnancy upon offspring immune function. A study which provided adult female rats with a calorie restricted diet (25% of the amount given to *ad libitum* fed controls) for 6 weeks prior to conception, and throughout pregnancy and lactation identified that this resulted in diminished antibody responses among both the F_1 and F_2 generations, despite *ad libitum* diet being provided at weaning to the F_1 generation, and throughout F_2 generation development(198).

A rat study of 50% energy restriction during pregnancy identified that this induced severe maternal weight loss and a reduction in offspring birth weight(199). *Ad libitum* feeding was resumed after delivery, and by six weeks of age the undernourished offspring had exhibited catch up growth to reach the same weight as control offspring. Among male undernourished offspring at 4-6 weeks old there were no significant differences in urine volume, concentration of glucose in urine, blood pressure or heart rate in comparison with control. However, several markers of the immune system were found to be altered. In the undernourished offspring there was a reduction in the number of rolling leukocytes, reduced adhesion and migration and increased velocity of rolling. L-selectin (expressed on granulocytes, monocytes and most lymphocytes) and P-selectin (expressed on platelets) were expressed at a lower level in response to TNF-α in the undernourished group. This study suggests that reduced leukocyte migration among undernourished offspring may be the mechanism which increases predisposition to infection after pre-natal undernutrition.

1.9.2 Programming of immune function by micronutrient restriction

Micronutrient restriction during pregnancy has been demonstrated to significantly affect offspring immune function. A study was undertaken which provided pregnant rats with a diet that would induce a moderate zinc deficiency (5 ppm zinc vs. 100 ppm in zinc sufficient diet) from day 7 of gestation to delivery. At delivery pups from zinc-deprived dams were cross-fostered to control dams during lactation, ensuring that zinc deficiency was limited to fetal exposure. This did not significantly affect plasma zinc concentrations, but the zinc deprived pups demonstrated significantly reduced IgM production, and these effects persisted into the F3 generation(200). Zinc deprivation during pregnancy therefore created an immunodeficiency which persisted long after a zinc replete diet was resumed and for more than one generation.

A study provided rats with diets that were either iron adequate (250 ppm during pregnancy and lactation, 35 ppm after weaning), moderately iron deficient (12 ppm) or severely iron deficient (6 ppm) throughout pregnancy and lactation identified significant effects of iron deficiency during pregnancy and lactation upon offspring immune function. Offspring of iron-deficient dams had similar birth weights, but lower spleen weights, total white blood cells counts, and impaired IgG and IgM formation. Iron repletion for three weeks following weaning did not reverse this impairment – this is a short repletion period, and it is unclear whether immune effects would persist into adulthood, which would indicate a true programming effect(201).

1.9.3 Programming of immune function by maternal dietary fatty acids

Little is known about the role of fatty acids in programming. The limited data available from studies using rats indicates that dietary LC n-3 PUFA during pregnancy and early life have the potential to beneficially protect against accumulation of fatty acids within adipose tissue in later life by lowering lipoprotein lipase mRNA expression, but may increase long-term oxidative stress as indicated by higher liver catalase activity(202). n-3 deficient diets provided to rats throughout pregnancy, lactation and in early life have also been demonstrated to result in significantly higher mean arterial blood pressure at age 34 weeks(203).

Animal and human studies to date which investigate the potential effects of dietary fatty acids upon offspring immune function do not demonstrate 'programming' effects, as no study to date has yet investigated whether any effect observed persists into adulthood.

Available data from animal models

When rat pups were fed high fat diets (11.7% w/w) which varied in their EFA content (49.4% vs. 4.7% of total fatty acids) from birth to weaning at 22 days of age it was found that those pups receiving the low EFA diet had lower total body weight, thymus and spleen weights and proliferative responses by cells of the spleen and thymus at 30 and 40 days old. This was associated with changes to the fatty acid composition of the thymus. This demonstrates that EFA deficiency in the postnatal period significantly affects the organ development of the rat and including its immune system(128).

Animal studies of survival rates after infection can be considered to reflect the capacity *in vivo* of immune function to successfully respond to stimuli. Survival rates following exposure to streptococcal sepsis in neonatal rats were assessed after dams were fed a diet that provided 22% energy from fat (either corn oil or menhaden fish oil) throughout pregnancy and lactation. At 7 days old pups were challenged with streptococcal infection. Survival rates from streptococcal challenge were significantly higher in the fish oil group than in the corn oil fed groups(204). Levels of AA and PGE_2 were higher in the lungs of pups from the corn oil fed group than in the fish oil group. This demonstrates that the fatty acid composition of the pre and postnatal diet significantly affects the *in vivo* response to neonatal immune challenge; with the LC n-3 PUFA rich fish oil diet conferring a benefit which is likely to reflect the effects of dietary fatty acids upon eicosanoid production.

Varying the ratios of corn oil (rich in LA) and coconut oil (rich in saturated fatty acids) in high fat (10% w/w) diets provided to rats during pregnancy significantly affected lymphocyte proliferation in fetal lymphocytes, with spleen lymphocytes of the neonates of dams fed coconut oil proliferating more strongly in response to the mitogen concanavalin A (Con A) than those fed corn oil(205). Diets were fed throughout pregnancy and dams were returned to a standard diet at birth. When assessed at 4 weeks old the offspring of dams fed corn oil had a significantly heavier thymus and spleen, although the effect on spleen weight was lost when spleen weight was expressed as proportion of body weight. This indicates that the qualitative supply of fatty acids to the fetus may be important in determining the development of immune organs.

A study which investigated intragastric treatment with 50 mg of a high fat (35% w/w) marine fish extract in addition to a high fat diet (10% w/w, fat source not stated) to rats before and during pregnancy and lactation identified that both the dams and the pups at 3 weeks after birth had significantly higher expression of CD56 (expressed on NK cells), CD25 (IL-2 receptor), CD28 (T cell co-stimulator) and CD62 (selectin adhesion molecule – expressed on most neutrophils and peripheral lymphocytes) in blood(206). However, this study could not determine whether these effects upon immune cell surface marker expression would persist beyond the period of dietary intervention.

Rats provided with high fat (7% w/w) diets with various n-6 to n-3 EFA ratios (linseed, soyabean or sunflower oil) during the last 10 days of gestation, throughout lactation and

after weaning demonstrated that these diets significantly affected offspring development of oral tolerance to later ovalbumin exposure. Those on the n-3 fatty acid rich diet demonstrated a more pronounced oral tolerance response, with significantly lower delayed-type hypersensitivity (DTH) responses and lower production of ovalbumin-specific IgG, IgM and IgE among those exposed to dietary ovalbumin(207). This indicates that the n-3 to n-6 EFA content of the diet can significantly affect development of atopic-type responses, but again does not indicate which time period of dietary intervention was the critical period to effect this change, or whether these effects would persist beyond the period of dietary intervention.

A study which provided diets to sows throughout lactation which varied in their n-6 to n-3 fatty acid ratio and vitamin E content identified that both the fatty acid composition and vitamin E content of the maternal diet significantly affected PGE_2 production by offspring alveolar macrophages, with production lower among offspring from the fish oil dietary group, compared to the sunflower oil fed group. However, the magnitude of this difference was diminished after weaning, when all piglets were returned to a standard feed, and so this does not strongly support a programming effect of dietary fatty acids during lactation in pigs(208).

Available data from human studies

Intervention studies where fatty acid supplements have been provided during gestation or early life have supported the suggestion from epidemiological studies that dietary LC n-3 PUFA are protective against childhood asthma. Several studies have been undertaken which have provided either pregnant women or infants with dietary LC n-3 PUFA and investigated the effect upon atopic disease or cytokine markers of Th1/Th2 balance.

Pregnant women with atopic symptoms were supplemented with 4 g fish oil per day from 20 weeks gestation until birth, and this resulted in significantly lower levels of IL-13 (a Th2 cytokine) in cord blood of their infants compared to the olive oil control group, and demonstrated an inverse correlation with cord red cell membrane DHA status and a positive correlation with the proportion of B cells and NK cells in cord blood(209). Cord blood IL-10 responses to house dust mite or cat hair extract were also significantly lower in the fish oil supplemented group, demonstrating significant inverse

correlations with cord blood red cell membrane EPA(210). However, the clinical significance of these changes in cytokine production is unclear.

Studies have been undertaken in early infancy to assess the potential for dietary n-3 fatty acids to reduce the risk of atopic disease. The Childhood Asthma Prevention Study (CAPS) provided fish oil supplements to infants at risk of atopic disease after 6 months of age or at the onset of bottle-feeding and ALNA-rich spreads and oils as part of a study design which also investigated dust-mite avoidance strategies. Supplementation with fish oil was demonstrated to reduce the prevalence of parent-reported symptoms of wheeze among infants at age 18 months(211). Further analysis indicates that there is a benefit threshold rather than a dose response relationship of infant n-3 fatty acid status, with all quintiles demonstrating similar reductions in the parent-reported wheeze prevalence compared to those within the lowest plasma phospholipid n-3 fatty acid quintile(212). When these children were followed up at age 3 years, there was a significantly lower prevalence of cough symptoms among children in the dietary intervention group(213). At 5 years of age there was no significant effect of dietary intervention upon measures of atopic disease symptoms, indicating that the benefits of the intervention may be limited to early life(214;215).

When fish oil capsules were provided to lactating mothers it was found that infants had significantly higher endotoxin-stimulated IFN-γ production and a significantly higher IFN-γ:IL-10 ratio of whole blood cultures at 2.5 years age when compared to infants whose mothers were supplemented with olive oil during lactation(216). This suggests that fish oil intake in early life promotes infant immune maturation, and that these effects are detectable long after maternal supplementation has ceased. Immune function is not the only infant outcome significantly affected by maternal supplementation during lactation - another study in this cohort of children identified that fish oil supplementation during lactation had a positive effect upon problem solving among girls at 9 months age, but also associated with lower vocabulary comprehension among boys at 1 year old(217).

1.10 Aims of the studies described in this thesis

While human studies have demonstrated clear gender differences in the *de novo* synthesis of LC PUFA and corresponding significant differences in circulating DHA concentrations, investigations of the mechanisms which may be involved in maintaining

these gender differences have not been undertaken. This is in part due to difficulties in controlling for dietary variation between individuals and the limited range of tissues which can be collected in human studies. Similarly, while animal studies have demonstrated that tissue fatty acid composition is significantly altered during pregnancy, there is no available information upon the mechanisms which may be responsible for these effects of pregnancy.

Human and murine pregnancy has been extensively studied in order to investigate the maternal adaptations which allow maintenance of successful pregnancy in the face of potential immune rejection of paternal antigens. However, the data currently available from studies in rats are limited and inconsistent, and indicates that there may be both species and strain variations in the adaptations observed during pregnancy. Studies have been undertaken in rats which identified significant alterations in both maternal and fetal tissue fatty acid composition as a result of changes to the maternal diet during pregnancy. However, there is little data available about the effect of maternal diet during pregnancy upon maternal or fetal immune tissue fatty acid composition, and the implications this may have upon immune function.

This thesis therefore sets out to address these unanswered research questions. It is hypothesised that:

- the rat will demonstrate gender differences in fatty acid composition, which are induced by the effect of sex hormones upon the mRNA expression of desaturase and elongase enzymes. Pregnant rats have higher circulating sex hormone concentrations and will therefore demonstrate an even more marked effect of sex hormones upon desaturase and elongase enzymes.
- increasing the n-3 content of the maternal diet during pregnancy will increase the n-3 content of maternal and fetal immune tissues, reducing the production of PGE_2 from n-6 fatty acid AA. This will therefore reduce the Th2 promoting-effects of PGE_2 and confer benefits to fetal immunity by altering the Th1/Th2 balance of cytokine production, with the potential to alter the development of atopic disease.

Chapter 2: Materials and methods

2.1 Animal husbandry

All animal work was carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986). Experimental procedures were covered by a Home Office Licence, and work undertaken in accordance with Personal Licence specifications. Wistar rats were used throughout. They were aged 10 weeks at the start of experiments and received standard laboratory maintenance chow during a rest/acclimatisation period (5-7 days) after their transport and delivery to the Biomedical Research Facility at the University of Southampton. Animal weight was monitored weekly.

Rats were housed individually while being fed experimental diets to enable accurate monitoring of food intake. Food provided and remaining was weighed every 2-3 days. Diets which were powdery in consistency were sifted to avoid errors in weighed food intake. Uneaten diet was removed to ensure that no food would become unpalatable due to oxidation or rancidity. At the end of the dietary intervention period rats were culled by CO_2 intoxication followed by cervical dislocation.

Where pregnant females were used, mating was undertaken by monogamous breeding (the studs used were not part of any experimental diet group and received standard laboratory maintenance chow). Efforts were made to ensure that a range of males was used for mating within each dietary group. When mating was confirmed by the appearance of a vaginal plug, this was recorded as day 1 of gestation. Females were then housed individually and started on experimental diets. At day 20 of gestation, or for some experiments at day 12 of gestation, the pregnant rats were culled and fetuses collected; normal rat pregnancy is 21-24 days.

The rate of successful pregnancies following mating was high, with few females failing to become pregnant (3 out of 87 females). Day 20 pregnancies with a litter size of less than 6 pups (3 out of 75 pregnant females) were excluded from further data analysis. A summary of successful pregnancies across all cohorts is shown in table 2.1.

Table 2.1: Successful pregnancies achieved over the course of the studies described in this thesis (number of pregnancies successful/number of pregnancies initiated, % success)

		Experimental diet		Chapter			
				4	6	5	7
Day of gestation	12	LF	Soyabean oil	5/6, 83% †		5/6, 83% †	
	20	LF	Soyabean oil	6/6, 100%		6/6, 100%	
		HF	Soyabean oil		6/6, 100%		6/6, 100%
		HF	Linseed oil		6/6, 100%		6/6, 100%
		HF	Salmon oil		6/6, 100%		5/6, 83% *
		HF	Sunflower oil		6/6, 100%		6/9, 67% †**
		HF	Beef tallow		6/6, 100%		6/6, 100%

LF low-fat
HF high-fat
† one female with no visible fetal development
* one litter with 3 pups
** two litters with 3 pups

2.2 Tissue sample collection and storage

Tissues collected from adult animals included blood, liver, adipose tissue (intra-abdominal and subcutaneous), spleen, thymus, mandibular lymph nodes and pancreas. When dissecting pregnant females, the uterus was placed into ice-cold phosphate buffered saline (PBS) until fetal dissection was performed. Placentas were collected from pregnant females. Tissues collected from fetal rats included blood, liver, brain, thymus, spleen, gut and pancreas.

The mandibular lymph nodes were chosen for collection as they were readily identified, and would not be adversely affected during the collection of other organs. Pancreas samples were sent to Peninsula Medical School for histological assessment as part of an ongoing collaboration(218) and no data on the pancreas are reported in this thesis.

Samples collected for *ex vivo* cell culture were stored on ice until processed. Samples collected for fatty acid composition analysis or reverse transcription PCR (RT-PCR) analysis of mRNA expression were immediately snap frozen in liquid nitrogen and stored at -80^{o}C.

2.3 Composition of experimental diets

A low fat (LF) diet with soyabean oil (3% w/w) as the fat source was used as a control diet for virgin and pregnant animals. This is a standard maintenance chow (RM[1]; Special Diet Services, Witham, Essex) which all animals received during their acclimatisation period.

The high fat (HF) experimental diets used were custom made by Special Diet Services, Witham, Essex. These diets were made from standard rat maintenance CRM(1) chow in which 10% of the diet wheat content was replaced with the fat or oil of choice. These diets therefore provide an extra 10% (w/w) dietary fat in addition to the 3% (w/w) soyabean oil contained in the CRM(1). The dietary fats and oils used were soyabean oil, sunflower oil, linseed oil, salmon oil and beef tallow.

2.3.1 Gross energy content

The gross energy contents of the diets used were estimated using the compositional information provided for the base diet by Special Diet Services and adjusting for the removal of wheat and additional lipid content using Atwater factors (see table 2.2).

Table 2.2: Energy and macronutrient content of experimental diets

	LF Soyabean oil	HF Experimental diets
Carbohydrate (g/100 g)	66.4	54.4
(Fibre)	(17.1)	(13.3)
(Sugar)	(4.1)	(3.4)
(Starch)	(45.0)	(37.4)
Ash (g/100 g)	6.00	6.3
Moisture (g/100 g)	9.5-11.5	9.5-11.5
Protein (g/100 g)	14.4	17.1
Fat (g/100 g)	2.7	13.1
Vitamin mix (mg/100 g)	44.9	46.1
(Vitamin E, IU/kg)	(84.1)	(190.2)
Mineral mix (mg/100 g)	10.5	14.9
Energy (MJ/100 g)	1.5	1.71

2.3.2 Vitamin E content

Analysis of the vitamin E content of the component oils used to create the experimental diets by the manufacturer revealed variation between them (see table 2.3). Additional vitamin E was therefore added to the oils used to produce the salmon oil, beef tallow,

soyabean oil and linseed oil diets in order to standardise the vitamin E content to the level found in sunflower oil.

Table 2.3: Vitamin E content of the fats and oils used to prepare the high fat experimental diets, prior to normalisation of vitamin E content

Fat or oil	Vitamin E content (IU/kg)
Salmon oil	117
Sunflower oil	874
Beef Tallow	ND
Soyabean oil	66.2
Linseed oil	9.7

ND indicates not detected

2.3.3 Fatty acid composition

The fatty acid composition of the diets was analysed using samples taken from five pellets per diet, with samples taken from both the exterior and interior of the pellets. The detailed results of the fatty acid analysis can be found in chapter 6. All diets contained a base of 3% soyabean oil and therefore were sufficient in the supply of essential fatty acids (EFA) linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALNA; 18:3n-3).

The principal components of the five HF experimental diets were as follows:

- HF soyabean oil – a high fat diet with comparable fatty acid composition to standard laboratory rat chow (LF soyabean oil diet)
- HF Linseed oil diet – rich in the n-3 polyunsaturated fatty acid (PUFA) ALNA
- HF Salmon oil – rich in longer-chain (LC) n-3 PUFA including eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3)
- HF Sunflower oil – rich in the n-6 PUFA LA
- HF Beef tallow – rich in saturates and monounsaturated fatty acids (MUFA).

2.3.4 Other macro/micronutrients

Data provided by the diet manufacturer indicates that the diets contained a wide range of amino acids including arginine, lysine, methionine, cysteine, tryptophan, histidine, threonine, isoleucine, leucine, phenylalanine, valine, tyrosine, glycine, aspartic acid, glutamine acid, proline, serine and alanine.

Macrominerals incorporated in the diets included calcium, phosphorous, sodium, chloride, potassium and magnesium. Microminerals within the diets included iron, copper, manganese, zinc, cobalt, iodine, selenium and fluorine.

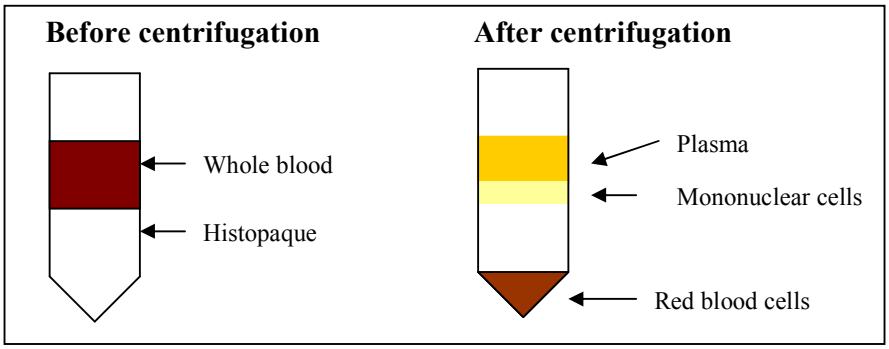
In addition to vitamin E, the diets included vitamins A, B_1, B_2, B_6, B_{12}, C, D, K, folic acid, nicotinic acid, panthothenic acid, choline, inositol and biotin.

2.4 Blood collection and processing

Whole blood was collected from culled adult animals by cardiac puncture, with heparin used as an anti-coagulant. Fetal whole blood was collected by decapitation over a heparinised Petri dish. Typically 2-5 ml of blood was collected from adult animals, and 1-2 ml from each litter. Blood samples were kept on ice until processing to obtain mononuclear cells and plasma, which was undertaken on the day of collection.

To collect peripheral blood mononuclear cells (PBMC) and plasma, whole blood was layered onto histopaque (density 1.077 g/ml; Sigma-Aldrich) in a 15 ml tube. The volume of histopaque used was twice that of the blood sample. Samples were then centrifuged at 2000 rpm for 15 min at 15^{o}C. Figure 2.1 illustrates sample appearance before and after centrifugation.

Figure 2.1: Illustration of method utilised to isolate plasma and mononuclear cells from whole blood samples



After centrifugation plasma was collected and stored at -80^{o}C until analysis. PBMC were collected from the interface, washed in culture medium (RPMI) and pelleted by centrifugation at 1500 rpm for 7 min. Supernatant from pelleted cells was decanted, and PBMC resuspended in 1 ml of RPMI. PBMC were then counted using a Beckman-

coulter counter. After counting, PBMC were either stored as a pellet at -80^{o}C until fatty acid composition analysis, or resuspended for use in *ex vivo* cell cultures.

2.5 Collection of mononuclear cells from lymphoid organs

For tissue rinsing and short-term storage prior to cell culture, RPMI culture medium with additional glutamine (final concentration 2 mM, PAA Labs) and penicillin-streptomycin (final concentration 10 U/ml penicillin + 10 μg/ml streptomycin, PAA Labs) was used. Glutamine supports the growth of cells that have high energy demands and synthesize large amounts of proteins and nucleic acids. Use of both penicillin and streptomycin is effective against both gram-positive and gram-negative bacteria, and is important as the rat organs used for cell culture preparations were collected in a non-sterile environment.

Lymphoid organs (spleen, thymus, lymph nodes) from dams and fetuses were immediately placed into RPMI and kept on ice until processing, which was undertaken on the day of collection. Fetal tissues from each litter were pooled together and handled as one sample. Organs were passed through a wire mesh strainer to produce a cell suspension. This suspension was filtered through lens cleaning tissue (Whatman) to remove debris and centrifuged at 1000 rpm for 10 min to pellet the cells. The cells were resuspended in 5 ml of RPMI, layered over 10 ml of histopaque, and processed as per the protocol for blood samples (section 2.4).

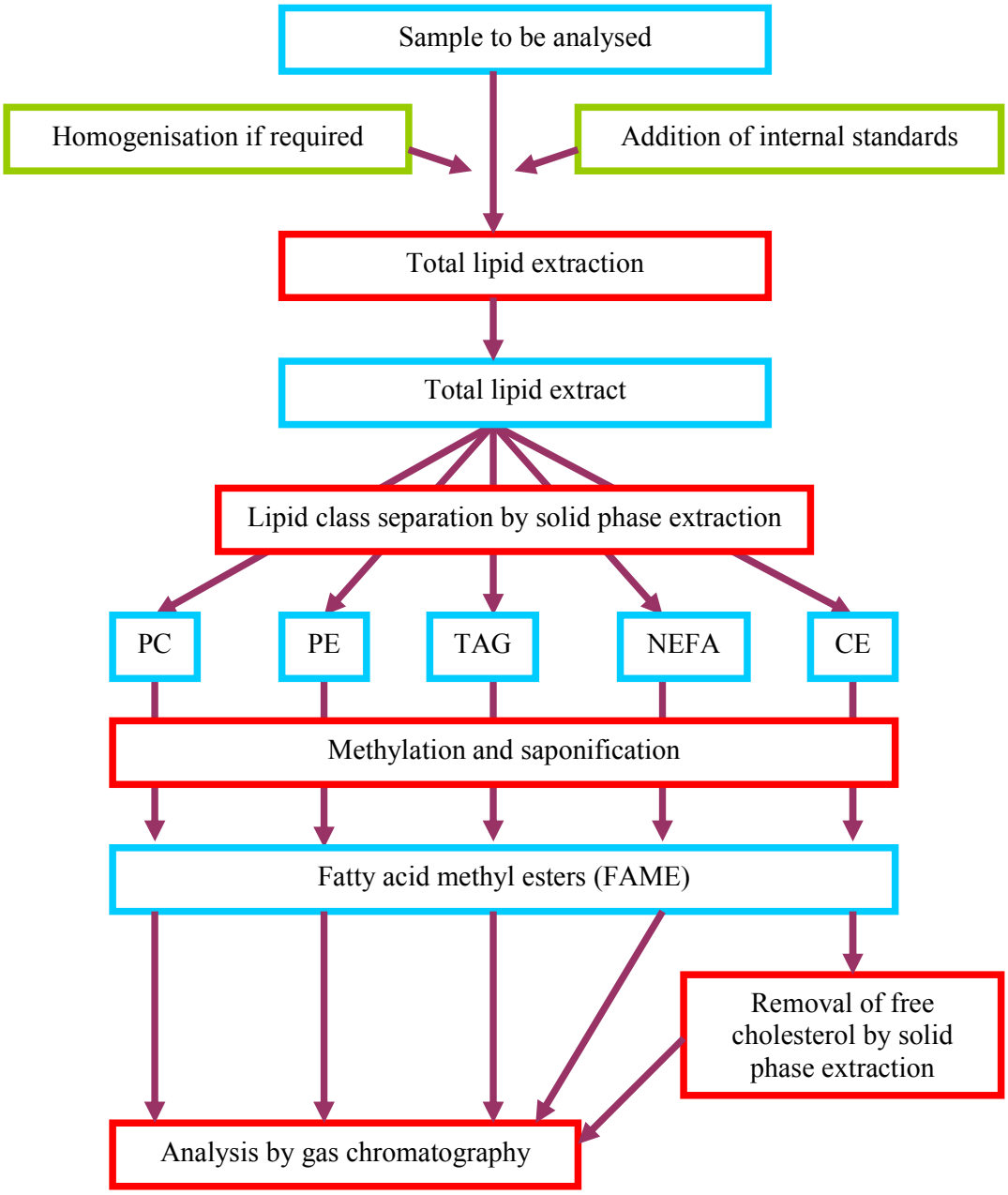
2.6 Fatty acid analysis by gas chromatography

2.6.1 Sample preparation

Before samples are analysed for their fatty acid composition by gas chromatography they must undergo processing to extract their lipid content. The lipid content can then be separated into various polar and non-polar lipid fractions and the resulting lipids must be saponified and methylated prior to analysis by gas chromatography. Figure 2.2 outlines these steps.

The fatty acid composition of plasma was assessed using a 0.2 ml aliquot of plasma diluted with 0.6 ml saline (0.9% NaCl). For analysis of the fatty acid composition of liver, placenta, brain, adipose tissue and experimental diets, a weighed sample (approximately 100 mg) was homogenised in 0.8 ml saline. Mononuclear cells collected from immune tissues were resuspended in 0.8 ml saline prior to analysis.

Figure 2.2: Flow chart of methodologies used in preparation of samples for analysis of fatty acid composition by gas chromatography (PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol; NEFA, non-esterified fatty acid; CE, cholesteryl ester)



Chemicals used for preparation of samples for gas chromatography were obtained from Fisher (chloroform, sodium chloride, methanol, hexane, and toluene), Sigma-Aldrich (butylated hydroxytoluene [BHT], glacial acetic acid, sulphuric acid, potassium bicarbonate, potassium carbonate) and BDH Lab Supplies (ethyl acetate).

To extract lipid, samples were mixed with 5 ml chloroform:methanol (2:1 v/v containing 50 mg/L of the anti-oxidant BHT) and 1 ml 1 M NaCl in a glass screw-cap test tube. Test tubes were vortexed thoroughly and centrifuged at 2,000 rpm for 10 min in order to separate the lower solvent-lipid phase from the upper aqueous phase.

The lower phase containing the lipid was collected, dried down at 40^{o}C under nitrogen gas and redissolved in 1 ml chloroform. Individual lipid classes were separated by solid phase extraction using NH_2-bonded silica cartridges. Full details of this method are found in Burdge et al, 2000 (219). The total lipid extract (dissolved in 1 ml chloroform) was applied to a NH_2-bonded silica column. Lipid fractions were then eluted by the addition of solvent mixtures of varying polarity and acidity. For plasma samples 2 ml chloroform was used to elute CE and TAG from the column. Then 2 ml of chloroform:methanol 60:40(v/v) was used to elute PC from the column. Finally, NEFA were eluted from the column using 2 ml chloroform:methanol:glacial acetic acid (100:2:2 v/v/v). The CE and TAG phase that had been collected was dried down at 40^{o}C under nitrogen gas and re-dissolved 1 ml in hexane. A new NH_2-bonded silica cartridge column was pre-treated with hexane washes before applying the combined CE and TAG fraction. CE was then eluted using 2 ml of hexane followed by TAG using 2 ml hexane:chloroform:ethyl acetate (100:5:5 v/v/v).

A similar approach was used for tissue samples, except that PC was eluted using 1 ml chloroform:methanol 60:40 (v/v) and then PE was eluted using 1 ml methanol. NEFA were not collected from tissue samples, and CE and TAG fractions were not collected from brain or immune cell samples as phospholipids were the primary interest in these tissue types.

The separated lipid classes were saponified and methylated in a simultaneous reaction involving the addition of 0.5 ml toluene and 1 ml of a methanol:sulphuric acid mixture (2% H_2SO_4 v/v) and incubation at 50^{o}C for 2 hr. Samples were then neutralised with a solution containing 0.25 M potassium bicarbonate ($KHCO_3$) and 0.5 M potassium carbonate (K_2CO_3). The fatty acid methyl esters (FAME) produced were recovered using hexane.

FAME were separated and analysed by gas chromatography on a HP6890 Hewlett Packard GC System with a BPX-70 column (Agilent Technologies). Samples were run

under the following conditions: initial temperature: 115^{o}C; hold 2 min; increase temperature at 10^{o}C/min to 200^{o}C; hold 10min; increase temperature at 60^{o}C/min to 245^{o}C; hold 4 min.

2.6.2 Calibration and analysis

Identification of FAME peaks in gas chromatograms was achieved by comparing the retention time of the peaks of interest with the known retention time of FAME standards. To ensure identification of a wide range of FAME, standards were purchased (Sigma-Aldrich, Nuchek) to enable identification and calibration of the FAME within samples of interest:

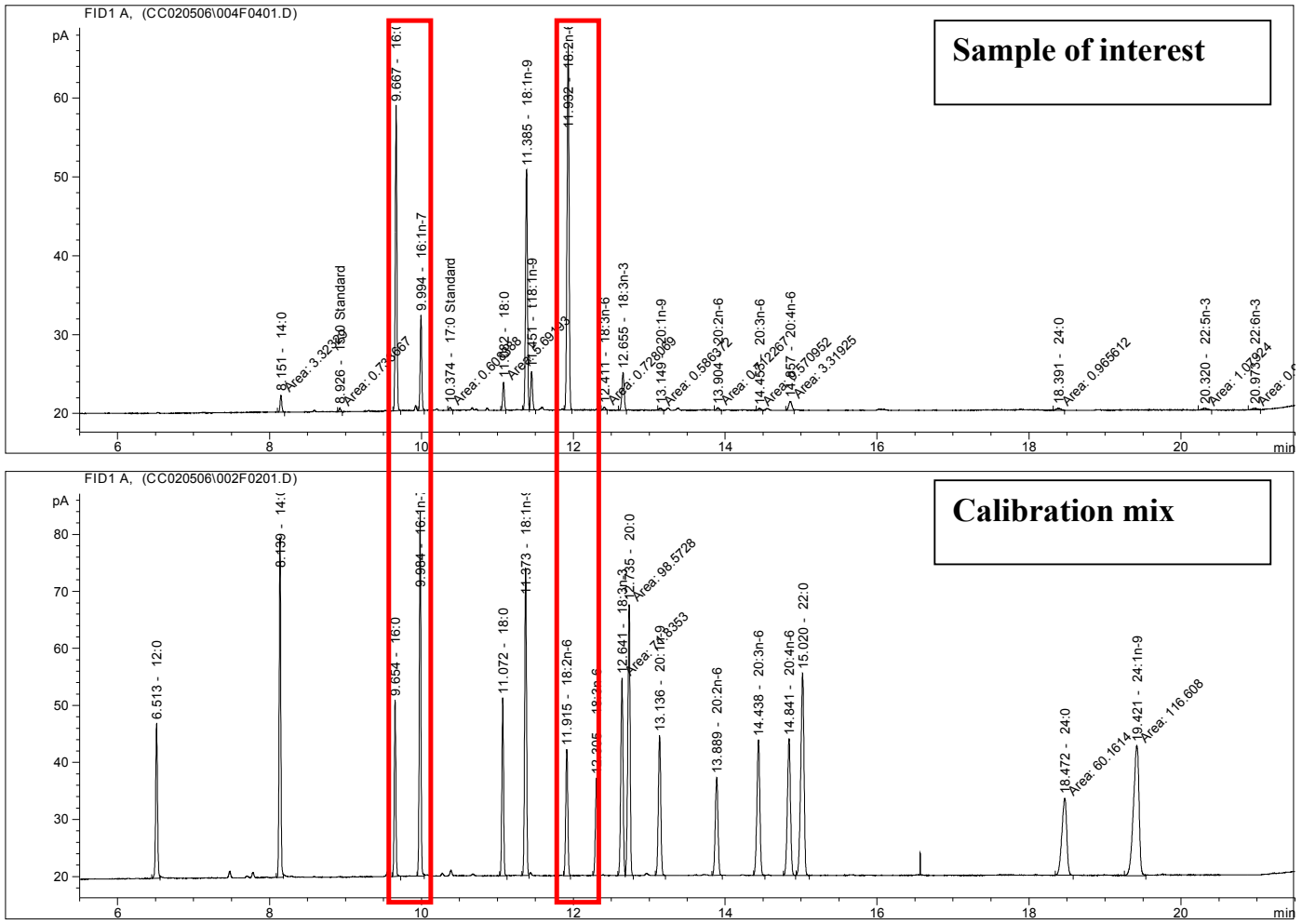
- Saturates – 12:0, 14:0, 16:0, 18:0, 20:0, 22:0
- MUFA – 16:1n-7, 18:1n-9, 20:1n-9, 24:1n-9
- n-6 PUFA – 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:5n-6
- n-3 PUFA – 18:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3

It is possible that two fatty acids may elute at the same time under these run conditions. Selected samples of placenta PE were therefore analysed by mass spectrometry (by Prof Tony Postle, School of Medicine, University of Southampton) to confirm the identity of peaks under question. For example, high levels of what was initially identified as lignoceric acid (24:0) were observed within the tissues of animals on the diets with the lowest n-3 fatty acid content. Mass spectrometry confirmed that this was in fact 22:5n-6, a finding supported by positive correlations of the percentage contribution of this fatty acid with other LC n-6 PUFA such as arachidonic acid (20:4n-6, AA), and inverse correlations with LC n-3 PUFA such as DHA.

Other fatty acids which were found to co-elute under the running conditions used over the course of experiments included 20:3n-9/AA, 22:4n-6/22:3n-9 and 21:0/20:2n-6.

Figure 2.3 demonstrates how sample peaks were identified using standard mixtures of FAME. Data are presented as the mean % contribution of each identified fatty acid (excluding internal standards). Fatty acids with a mean % contribution of < 0.1% are labelled as ND (negligible detected).

Figure 2.3: Example of method used to identify fatty acid methyl esters within samples using prepared calibration mixes



2.6.3 Removal of free cholesterol

Free cholesterol causes problems with the functioning of the gas chromatograph by accumulating on the column, and also causes increased background "noise" on chromatograms, making peak identification more difficult and time consuming. A method was therefore developed to separate FAME from cholesteryl ester (CE) samples after they had been saponified and methylated based upon methods detailed at (220).

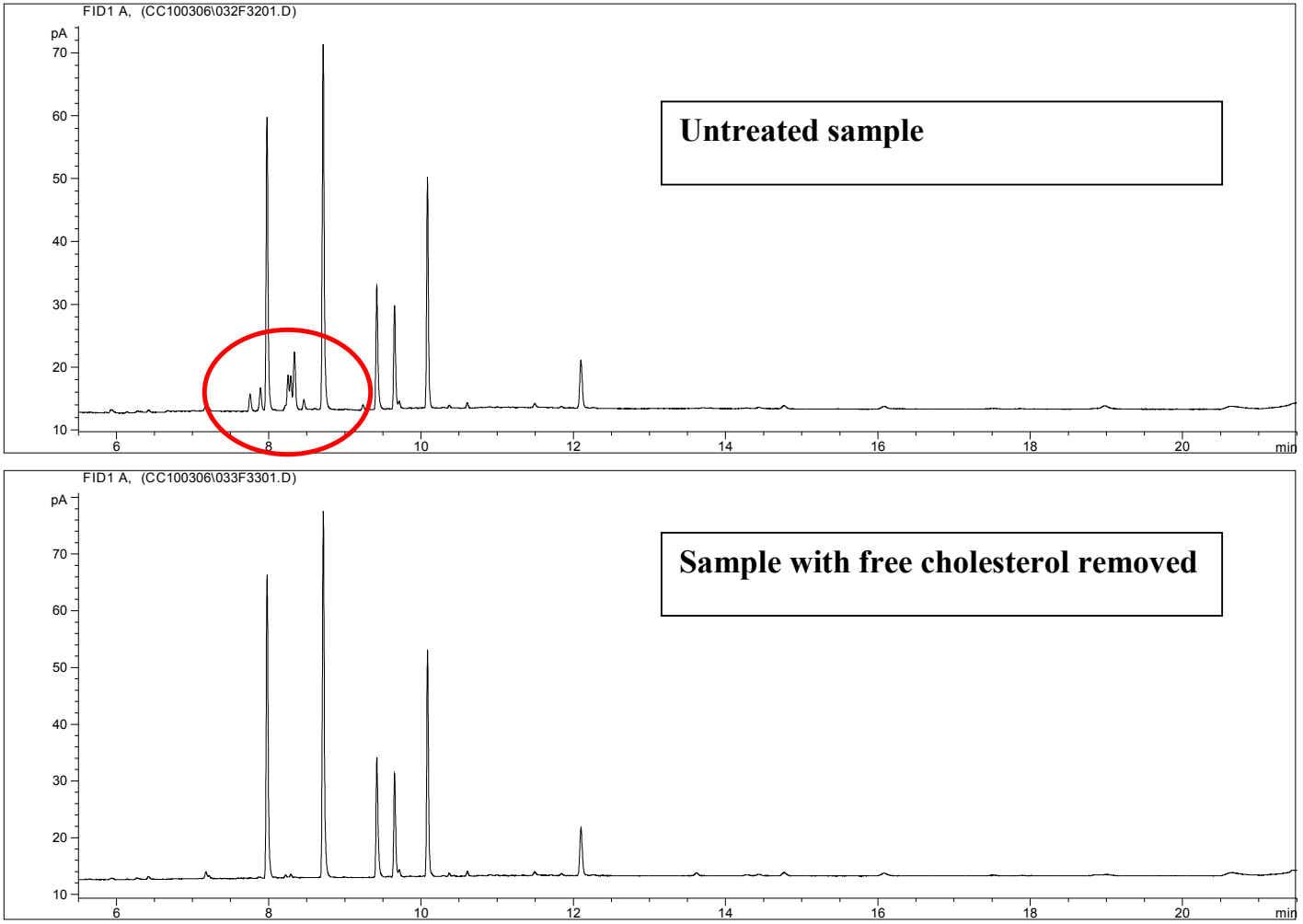
Samples were dissolved in 1 ml hexane and applied to a silica gel column which was pre-treated by washing with hexane. The sample was washed with 3 ml hexane to remove any hydrocarbon contamination. FAME were collected using 2 ml of hexane:diethyl ether (95:5 v/v). Free cholesterol remains within the silica gel column.

This method was developed after testing various hexane:diethyl ether solutions for effectiveness of recovery (99:1, 98:2, 95:5, 90:10 v/v). 95:5 was found to give good recovery of FAME when compared to untreated samples (78 ± 8%). The volume of hexane:diethyl ether (95:5 v/v) required for recovery of FAME was tested using 2 to 5 ml. All volumes gave similar recovery and so 2 ml was used. Attempts were made to reduce the volume of hexane required for pre-treatment washing but this was found to adversely affect the recovery of FAME. Thin layer chromatography (using hexane:chloroform:diethyl ether:acetic acid 80:10:10:1.5 v/v/v/v) was performed which confirmed that this method removed free cholesterol from samples.

The FAME compositions of samples with free cholesterol removed were assessed to ensure there was no FAME specificity associated with this purification step. Treated samples in fact had the advantage of facilitating the detection of trace fatty acids by reducing background interference.

Example gas chromatograph traces illustrating the reduction in background interference achieved using this method can be seen in figure 2.4.

Figure 2.4: Comparative gas chromatograph traces obtained with identical samples to illustrate the benefits of free cholesterol removal



2.7 Plasma lipid and glucose analysis

Samples of maternal and fetal plasma were sent to Dr John Jackson (Institute of Human Nutrition, Southampton General Hospital) for analysis of the concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, TAG, NEFA and glucose.

These assays were performed on a Konelab auto analyser using colorimetric kits from Konelab, Thermo Electron Corporation, and Wako Chemicals. The detection limits for these assays were: glucose, 0.1 mM; HDL-cholesterol, 0.04 mM; LDL-cholesterol, 0.04 mM; TAG, 0.02 mM; cholesterol, 0.1 mM. The detection limit for the NEFA assay was not provided. Maternal and fetal LDL-cholesterol levels and fetal HDL-cholesterol levels were below detection limits for the assays and so these results are not presented.

2.8 Assessment of liver dry weight and lipid content

Dry weights for tissues were assessed by allowing tissues to dry at 80^{o}C until no further change in tissue weight was observed (48 hr). Liver lipid content was assessed by weighing the total lipid extract from a known weight of tissue.

2.9 Assessment of circulating sex hormone concentrations

Plasma samples were sent to Linda Ogilvie-Goddard (Chemical Pathology Division, Southampton General Hospital) for analysis of plasma testosterone, progesterone and oestradiol concentrations. These assays were performed using Beckman Coulter radio-immunoassays with detection ranges of: oestradiol, 36.7-3670 pM; testosterone, 0.35-52.1 nM; progesterone 0.48-90.8 nM.

2.10 Assessment of liver glycogen content

Amyloglucosidase enzyme, rabbit liver glycogen standard, and sodium citrate were obtained from Sigma-Aldrich. A method for liver glycogen assessment was developed based upon available publications(221;222). This involved an enzyme digest of liver glycogen into glucose, analysis of glucose concentrations, then back-calculation to determine original tissue glycogen content, controlling for any basal levels of glucose within the liver.

A weighed sample (approximately 100 mg) of liver was homogenised in 1 ml of 100 mM sodium citrate buffer (pH 5.0) and kept on ice. The homogenised sample was placed into a boiling water bath for 1-5 minutes to inactivate any endogenous glycolytic

enzymes. The homogenate was divided into two equal aliquots. One aliquot was incubated for 2 hr at 55^{o}C with 25 µl of 5 mg/ml amyloglucosidase (sufficient to liberate 200 mg glucose from glycogen over the two hour incubation period). The second aliquot underwent identical incubation conditions but with sodium citrate buffer added in place of the enzyme. After incubation the samples were centrifuged at 13,000 rpm for 10 min, and supernatants collected and sent for analysis of glucose concentration analysis by Dr John Jackson (see section 2.7).

Samples of 1 mg/ml rabbit liver glycogen (with and without enzyme treatment) were run alongside each batch of samples analysed to ensure both the quality of the glycogen standard and enzyme activity. "Blank" samples comprised of sodium citrate buffer (with and without enzyme treatment) were also analysed to ensure there was no contamination with glycogen or free glucose during sample preparation. 1 mg of glycogen generates 1.11 mg glucose when enzyme digested. The glycogen content of liver was calculated as follows:

Glycogen (mg/ml) = [(Glucose reading of enzyme treated aliquot) – (glucose reading of untreated sample)] x [(Expected glucose reading for glycogen standard) / (observed glucose reading for glycogen standard)]

The glycogen concentration (mg/ml) was then used to calculate the % glycogen content of liver wet weight using the known weight of the liver sample which was initially homogenised in 1ml of buffer.

2.11 Measurement of markers of immune function

2.11.1 Flow cytometry

Stains which would allow the identification within lysed whole blood of total T cells ($CD3^+$ cells), T helper cells ($CD3^+/CD4^+$ cells), cytotoxic T cells ($CD3^+/CD8^+$ cells), splenic macrophages ($CD163^+$ cells), natural killer cells ($CD161^+$ cells) and B cells ($CD3^-/CD45RA^+$ cells) in mononuclear cell samples were purchased from AbD Serotec.

Stains were conjugated with fluorescein isothiocyanate (FITC) or R.phycoerythrin (RPE) as follows:

Single stains:	Anti-rat CD163 FITC (IgG_1)
	Anti-rat CD161 FITC (IgG_1)
Dual stains:	Anti-rat CD3 FITC (IgM)/anti-rat CD8 RPE (IgG_1)
	Anti-rat CD3 FITC (IgM)/anti-rat CD4 RPE (IgG_1)
	Anti-rat CD3 FITC (IgM)/anti-rat CD45RA RPE (IgG_1)
Controls:	Mouse anti-human IgG_1 conjugated to FITC
	Mouse anti-human IgM conjugated to FITC
	Mouse anti-human IgG_1 conjugated to RPE

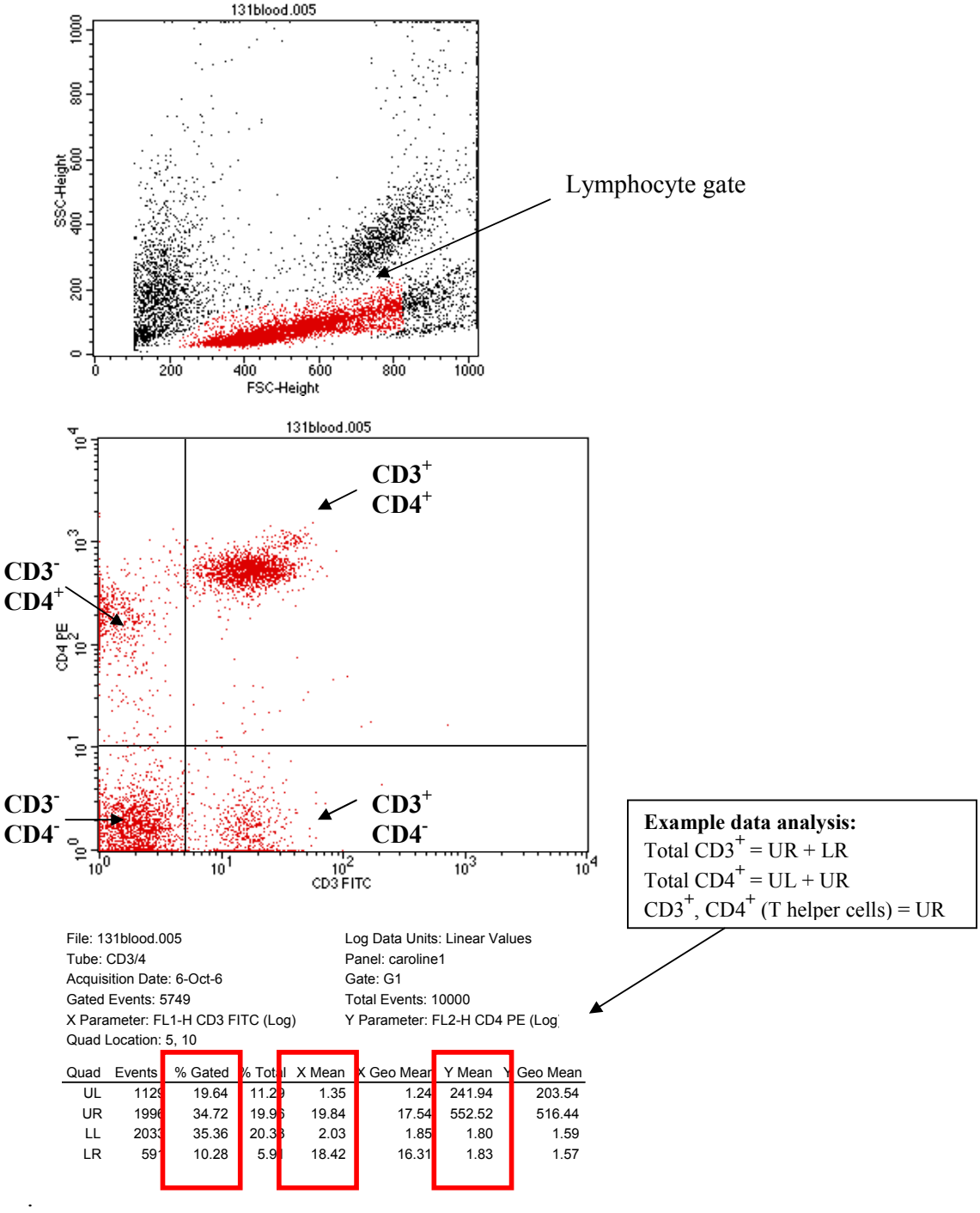
Heparinised whole blood (100 µl per stain) or mononuclear cell suspensions obtained from the thymus, spleen or lymph nodes (1×10^6 cells in 100 µl RPMI per stain) were used for flow cytometry.

Cells were stained for 30 min at room temperature with 10 µl of each of the antibodies detailed above. Whole blood samples were then lysed using 2 ml FACS lysing solution (BD Biosciences) for a further 10 min. All samples were centrifuged to pellet the cells (1000 rpm for 7 min) which were then washed twice in 2 ml PBS containing 1 g/L bovine serum albumin and 0.65 g/L sodium azide. These washing steps serve to remove any unbound antibodies.

Cells were fixed in PBS containing 2% (v/v) formaldehyde and stored at 4^oC until analysis. Flow cytometry was performed on a Becton Dickinson FacsCalibur within 24 hours of sample preparation. 10^5 events were recorded per sample and data analysed using CellQuest software. An example of a forward-scatter, side-scatter plot (FSC, SSC), fluorescence plot and flow cytometry statistics is in figure 2.5.

Controls and unstained samples were used to ensure that there was no significant autofluorescence or non-specific binding of antibody to target cells. Lymphocyte gates were used for analysis of maternal blood, spleen and lymph node data, and the data for these tissues is expressed as a % of gated cells. The mean fluorescence intensity (MFI) of gated cells within each quadrant was used to indicate the relative expression of these markers upon the cell surface. Lymphocyte gating was not required for maternal or fetal thymus, as the cells recovered were a pure lymphocyte population. A threshold of 100FSC was routinely set for all samples. Lymphocyte gates were drawn as appropriate for each sample. Quadrant and region settings for positive staining were kept consistent within each tissue type throughout all sample analysis.

Figure 2.5: Example flow cytometry profiles from a maternal lysed whole blood sample following dual staining for CD3 and CD4



2.11.2 T Lymphocyte proliferation in mononuclear cell cultures

Incorporation of ^{3}H-thymidine into cultured mononuclear cells was used as a measure of the proliferative activity of these cells. 5 x 10^{5} mononuclear cells from the spleen, thymus or blood were cultured in triplicate in RPMI supplemented with glutamine and penicillin/streptomycin (see section 2.5) at 37^{o}C in a 5% CO_2 atmosphere in the

presence of the T cell stimulant Concanavalin A (Con A; final concentrations in the range 0-30 μg/ml; this range was identified in pilot studies). The final culture volume was 200 μl/well and contained 5% fetal calf serum (FCS). After 48 h, 0.2 μCi ^{3}H-thymidine (20 μl) was added to each well and the samples cultured for a further 18 h. Cells were then harvested onto glass fibre filters using a cell harvester. Filters were dried, transferred to scintillation counting vials, 0.1 ml scintillant (Optiphase Hisafe, Perkin Elmer) added and radioactive incorporation measured as counts per minute (CPM) using a liquid scintillation counter. Maximum potential incorporation was verified by direct counting of 0.2μCi ^{3}H-thymidine to ensure a sufficient excess of thymidine was available to proliferating cells.

The CPM achieved by Con A-stimulated cells was expressed relative to the CPM achieved by unstimulated cells, and termed the stimulation index (SI). This SI was then adjusted for the proportion of $CD3^{+}$ cells (T cells, which are responsive to Con A) identified by flow cytometry within the mononuclear cell suspensions.

2.11.3 Cytokine (IFN-γ, IL-4) and PGE_2 production by mononuclear cell cultures

5 x 10^{6} mononuclear cells from the spleen, thymus or blood were cultured at 37^{o}C in a 5% CO_2 atmosphere with/without the T cell stimulant Con A (final concentration 5 μg/ml). The final culture volume was 2 ml/well and cultures contained 5% FCS. After 48 h, culture plates were centrifuged (1000 rpm, 7 min) and supernatants collected and stored at -80^{o}C until analysis.

ELISA kits were used to assess the concentrations of interferon-γ (IFN-γ), interleukin-4 (IL-4) and prostaglandin E_2 (PGE_2) within cell culture supernatants. Kits used for IFN-γ and IL-4 were obtained from Biosource, and kits for PGE_2 from Oxford Biosystems. The sensitivities of the IFN-γ and IL-4 ELISA kits were < 13 pg/ml and < 2 pg/ml, respectively. No sensitivity information was provided with the PGE_2 ELISA kit. No cross-reactivity was reported for IFN-γ or IL-4 ELISA. The PGE_2 ELISA reported 100% cross reactivity with PGA_1, A_2, B_1, B_2, and E_1. This means that prostaglandins detected in cell culture supernatants could be derived from AA or di-homo-γ-linolenic acid (DGLA). Cross reactivity with PGE_3 (derived from EPA) was 17.7%.

Kits were used as per the manufacturer's instructions, with the exception of the IL-4 ELISA, where the following adaptations were made: cell culture supernatant samples were not diluted; IL-4 controls provided to generate standard curve were diluted in RPMI cell culture medium rather than the buffer provided.

2.11.4 Use of fetal calf serum in cell culture

Fetal calf serum (FCS) was used as a medium supplement for cell culture. Autologous plasma was not used because of difficulties in consistently obtaining sufficient plasma from animals to meet the requirements of cell culture and other plasma analysis to be performed such as total lipid and fatty acid composition analysis.

The fatty acid composition of FCS (Sigma-Aldrich) was analysed by quantitative gas chromatography to assess whether the fatty acids present within FCS may affect *ex vivo* cell culture results. Both LC n-6 and n-3 PUFA were detectable in FCS, but the fatty acids found in the greatest amounts in fetal calf serum were 16:0, 18:0 and 18:1n-9 (see Table 2.4).

Table 2.4: Fatty acid composition of a total lipid extract of fetal calf serum used in cell culture preparations (mean ± standard deviation, n = 3)

	% total fatty acids	Final concentration in cell culture* (nM)
16:0	25.9 ± 0.6	19.79 ± 1.02
16:1n-7	2.5 ± 0.1	1.92 ± 0.13
18:0	19.4 ± 0.3	13.35 ± 0.51
18:1n-9	26.0 ± 0.6	18.02 ± 0.21
18:2n-6	8.5 ± 0.3	5.93 ± 0.07
20:3n-6	2.7 ± 0.1	1.71 ± 0.01
20:4n-6	6.5 ± 0.3	4.19 ± 0.06
22:5n-3	3.9 ± 0.8	2.31 ± 0.56
22:6n-3	4.5 ± 0.3	2.68 ± 0.10

*when used at 5% final culture volume

The final working concentrations used in cell culture were calculated based upon the use of 5% FCS in cell cultures. Separation of individual lipid classes demonstrated that the majority of the fatty acids were in the form of PC (~50%) or TAG (~34%). These final culture concentrations are significantly lower than those used in similar proliferation and cytokine production experiments which have identified functional effects of fatty acids upon lymphocytes e.g. 1 – 100 μM(223;224).

2.12 Analysis of hepatic gene expression by two step RT-PCR

2.12.1 RNA extraction

RNA was extracted from 100 mg liver samples by homogenisation in 1 ml Trizol (Sigma). Homogenates were transferred to eppendorf tubes and centrifuged (12,000 g, 5 min, 4^{o}C) to pellet out any fibrous tissue residues/unhomogenised tissue. Supernatants were transferred to new eppendorf tubes, 0.2 ml chloroform added, and tubes shaken for 15 sec. Samples were then incubated at room temperature for 2-3 min and centrifuged (12,000g, 15 min, 4^{o}C).

The upper aqueous phase containing the RNA was transferred to a new eppendorf tube, and RNA precipitated by addition of 0.5 ml isopropyl alcohol and incubation for 10 min. The sample was centrifuged to produce an RNA pellet (12,000g, 15 min, 4^{o}C). Supernatant was removed, and the RNA pellet allowed to air dry for 5-10 min. RNA was resuspended in diethyl pyrocarbonate (DEPC) treated water and stored at -80^{o}C.

2.12.2 RNA quality assessment

Spectophotometry was used to assess the concentration and purity of the RNA sample obtained by measuring absorbance at 260 nm and 280 nm. An absorbance of 1 at 260 nm is equivalent to 40 µg/ml RNA. Therefore if 2 µl of the RNA sample was assessed in 500 µl H_20 in a 1 cm cuvette:

A260 x 40 µg/ml x (0.5 ml) = Total RNA in cuvette

(Total RNA in cuvette) / 2 = RNA in µg/µl in original sample

The ratio of absorbance at 260 nm and 280 nm (A260/A280) is often used to assess the purity of the RNA preparation. Samples diluted in DEPC (RNase deactivator) treated water had a mean ratio of 1.69 and were accepted as sufficiently pure for further analysis(225).

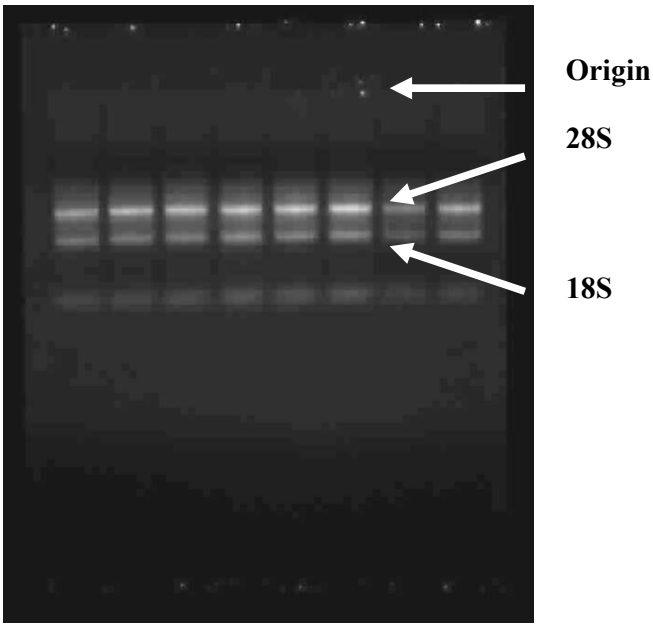
Integrity of the RNA sample was checked by gel electrophoresis. The vast majority of RNA within a sample will be 18S and 28S ribosomal (r) RNA. Visualisation of two clear bands demonstrates that the rRNA has not been degraded (which would be indicated by a 'smeared' appearance), and so the messenger (m) RNA of interest is likely to be intact. The ratio of absorbance of these two rRNA bands can also be used to check sample quality: the 28S band should be approximately twice as intense as the 18S

band. Samples were prepared for running on a 1% agarose gel containing ethidium bromide (1.5 µl of 10mg/ml solution in 50 ml gel) for 30 min at 100V as follows:

x H_20
1 µl Northern loading buffer
2 µl formamide
y 1 µg RNA
(Total volume = 10 µl)

All samples were assessed for rRNA integrity and a mean 28S:18S ratio of 1.67 was achieved. An example gel image achieved is in figure 2.6.

Figure 2.6: Example gel electrophoresis of RNA samples to assess integrity



2.12.3 cDNA production

1 µg RNA was used to synthesise cDNA using M-MLV Reverse Transcriptase (Sigma). RNA was incubated at 70°C for 10 min as below:

1 µl 10 mM dNTP mix (Sigma)
1 µl Random nonamers/primers (Sigma/Promega)
x Doubly distilled H_2O
y 1 µg RNA
(Total volume = 10 µl)

Samples were placed on ice and the reverse transcriptase enzyme was added as below:

6.5 µl Doubly distilled H_2O
2 µl 10 x M-MLV Reverse Transcriptase Buffer
1 µl M-MLV Reverse Transcriptase
0.5 µl RNase inhibitor (40 U/µl, Promega)
(Total volume = 10 µl)

Samples were incubated at room temperature for 10 min, then at 37°C for 50 min. Samples were then heated to 94°C for 10 min to denature the M-MLV reverse transcriptase and stored at -20°C.

Template-free and enzyme-free controls were prepared at the same time as samples of interest with each cDNA production run.

2.12.4 Selection of housekeeping genes

Careful selection of housekeeping genes used to normalize RT-PCR results is essential to ensure that their use as internal controls is appropriate. This was particularly important for this thesis as some commonly used housekeeping genes have demonstrated significant sex differences in expression(226). Housekeeping genes were selected using the 12-gene genNorm rat housekeeping gene kit (PrimerDesign Ltd, Southampton), and results assessed for the most stable gene using geNorm software(227). 12 samples including random samples from each experimental group were assessed by real-time PCR for the 12 housekeeping genes provided in the kit (18S rRNA, ATP synthase, topoisomerase-I, malate dehydrogenase-1, cytochrome C-1, calnexin, ribosomal protein L13, tyrosine-3-monooxygenase, β_2-microglobulin, ubiquitin C, glyceraldehyde-3-phosphate dehydrogenase and β-actin).

Real time PCR was performed under conditions recommended by the genNorm kit with the below reaction mix:

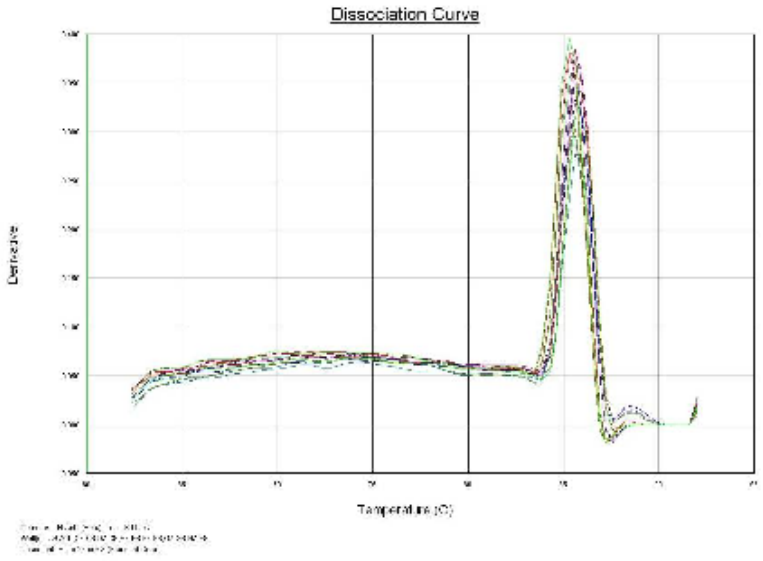
1 µl Resuspended primer mix (300nM working concentration)
10 µl 2 x qPCR Mastermix
4 µl H_20
5 µl cDNA (1 in 10 dilution of cDNA product)
(Total volume = 20 µl)

Based on genNorm analysis results, the housekeeping genes selected as most stable for use were: calnexin, tyrosine-3-monooxygenase and topoisomerase-1 (gender + diet study, chapter 3); calnexin, tyrosine-3-monooxygenase and β_2-microglobulin (pregnancy study, chapter 4). Topoisomerase 1 (NM_022615) is involved in 'uncoiling' the strands of the DNA double helix to enable replication or transcription. Tyrosine-3-monooxygenase (NM_013011) is a protein which mediates signal transduction by binding to phosphoserine-containing proteins. β_2-microglobulin (NM_012512) is a component of major histocompatability class I molecules. Calnexin (NM_172008) retains unfolded or unassembled N-linked glycoproteins within the endoplasmic reticulum.

2.12.5 Dissociation curve analysis

The specificity of all primers was assessed by dissociation curve analysis to determine the melting temperature of the nucleic acid sequences within each sample. If a single product is generated, there will be a single melting temperature achieved after PCR. No primer dimer formation was observed for the any primers at any of the cDNA concentrations used to generate the standard curve. An example dissociation curve achieved for B_2-microglobulin is in figure 2.7.

Figure 2.7: Example dissociation curve (fluorescence as a function of temperature) achieved for B_2-microglobulin, illustrating formation of a single product



2.12.6 PCR using SYBR green

Primers for genes of interest were obtained from Qiagen: FADS1 (Fatty acid desaturase 1, Δ5 desaturase, Rn_FADS1_1_SG); FADS2 (Fatty acid desaturase 2, Δ6 desaturase, Rn_FADS2_1_SG); Elongase 5 (Rn_Elovl5_1_SG).

Real time PCR with SYBR green was undertaken using Applied Biosciences 7500 Fast Real-time PCR System and Applied Biosciences Fast Plates (PN 4346906). PCR reaction mixtures were prepared as below using a 30µl final volume in duplicate and run conditions are as described in table 2.5. NTC (no template controls) were run on every PCR plate for every gene assessed.

2 µl cDNA
25 µl 2 x Universal Master mix (Low Rox, Primer Design)
1.5 µl SYBR green (Primer Design)
5 µl 10 x Qiagen primer
x µl H_2O
50 µl total volume

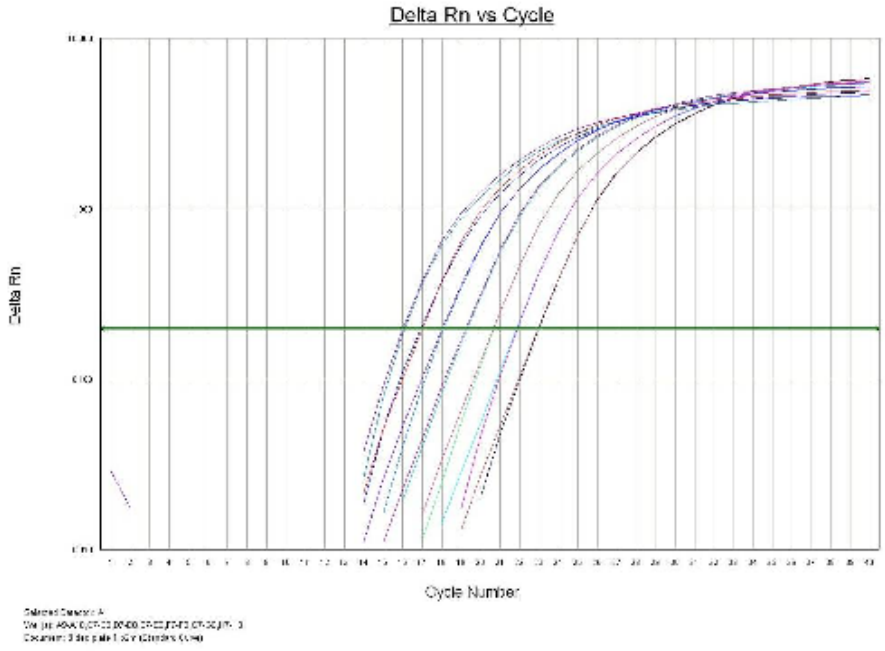
Table 2.5: Reaction conditions used on PCR plates for genes of interest

		Time	Temp (^oC)
	Enzyme activation	2 min 10 min	50 95
Cycling x 40	Denaturation	15 s	95
	Data collection	1 min	60

2.12.7 Data analysis

mRNA content was quantified using the standard curve method. A concentration curve of a random sample of RNA (2 µg, 1 µg, 0.5 µg, 0.25 µg, 0.125 µg, 0.0625 µg, 0.03125 µg) was used to generate a standard curve of cDNA for use in PCR. Standard curves were run for every gene on each PCR plate. An example amplification plot for a standard curve is in figure 2.8.

Figure 2.8: Example amplification plot achieved with real-time PCR (standard curve for B_2-microglobulin)



Arbitrary values were applied to represent cDNA concentration (100, 50, 25, 12.5, 6.25, 3.125, and 1.5625) and a standard curve of Ct values was generated. Samples of interest were then quantified relative to the arbitrary values applied to the standard curve. Genes of interest were expressed relative to the expression of the geometric mean of the three best housekeeping genes. The analysis settings used for each gene are detailed in table 2.6.

Table 2.6: Analysis settings used for PCR data

	Δ Rn threshold	Baseline
β_2-microglobulin	0.2	3-13
Topoisomerase 1	0.2	3-15
Calnexin	0.2	3-15
Tyrosine-3-monooxygenase	0.2	3-15
FADS2 (Δ6 desaturase)	0.28	3-11
FADS1 (Δ5 desaturase)	0.2	3-15
Elovl5 (Elongase 5)	0.248	3-15

2.13 Power calculations and statistics

SPSS version 15.0 was used for statistical analysis. When SPSS boxplots are displayed, the graphs indicate the median, the interquartile range (box length), Tukey's hinges

(12.5 - 82.5th percentile range, 'whiskers' of box), outliers (1.5-3 interquartile ranges from the end of a box, labelled as 'o') and extreme values (more than three inter-quartile ranges from the end of a box, labelled as *).

Data were assessed for differences between groups by one-way or two-way ANOVA with significant differences between groups determined using the Bonferroni correction for multiple comparisons applied to minimise the risk of a type I error (accepting a 'false positive' result). The level of statistical significance required was $p < 0.05$.

Where parametric tests were not appropriate, such as when ordinal values were involved, differences between groups were assessed using the Kruskal-Wallis test. Correlation coefficients (r) were calculated using Pearson or Spearman test as appropriate after data were assessed for normality.

Where stepwise multiple regression analysis was performed, the significant variables and Beta values are provided. Beta values are a measure of how strongly predictor variables influence the variable of interest, and are measured in units of standard deviation. The Beta value indicates the change in standard deviations that will be observed in the variable of interest when the predictor variable changes by one standard deviation.

It was important to consider the number of animals to be studied so that the investigation was suitably powered to identify significant effects, minimize the risk of a type II error (accepting a 'false negative' result) and avoiding the unnecessary use of animals. The primary objective of the studies described in this thesis was to assess the effect of maternal dietary fatty acids during pregnancy upon maternal and fetal immune function. The principal hypothesis is that changes in fetal immune function can be induced by altering the maternal or fetal availability of fatty acids with roles in immune mediator generation such as AA and EPA. One low fat (3%) and five high fat (13%) experiment diets will be used, which vary in their n-6 and n-3 fatty acid contents. Power calculations were therefore determined using available data on the changes in tissue fatty acid composition that can be achieved by maternal diet during pregnancy.

F values indicate the effect size of a statistical test, and are equivalent to the Z score of a standard deviation. This means that the F value specifies how many standard deviations

the mean of one group is from the mean of the other. The sensitivity of a statistical test can be assessed by calculating the minimum effect size which will be found to be statistically significant given the sample size and power required. F values (effect size) were calculated with G*Power (version 3.0.10) using data from a comparable rat study which assessed the changes in maternal and fetal rat tissue fatty acid composition in response to various 10% fat diets during pregnancy(228). F values achieved for variations in mean AA content of maternal and fetal plasma, liver and placenta in this study were in the range 2.90 – 5.70, and for EPA 0.72 - 3.10.

Using these F values *a priori* power tests for ANOVA indicate that for a study with 6 dietary groups, a sample size of 12 (based on lowest AA F value) to 48 (based on lowest EPA F value) would be required for a power of 0.95. A power of 0.80 is a commonly accepted arbitrary value for power, and this would be achieved using a sample size of 12 (based on lowest AA F value) to 36 (based on lowest EPA F value). My studies will therefore use a sample size of n = 6 per group to give a total sample size of 36 when 6 different dietary interventions are used. Table 2.7 summarises the effect size sensitivity of all the study designs which will be undertaken in this thesis.

Table 2.7: F value sensitivity of the studies to be undertaken in this thesis

	Gender study (chapter 3)	Pregnancy study (chapters 4 & 5)	Maternal diet in pregnancy (chapters 6 & 7)
n =	36	18	36
groups	2 genders 3 dietary groups	3 pregnancy time points	6 dietary groups
F value sensitivity (power = 0.80)	0.48 (gender) 0.54 (diet)	0.81	0.65
F value sensitivity (power = 0.95)	0.62 (gender) 0.69 (diet)	1.03	0.81

Chapter 3: Gender and the fatty acid composition of rat tissues

3.1 Introduction

3.1.1 Aims and hypothesis for current investigation

It is the aim of this study to investigate the effect of gender upon tissue n-3 fatty acid composition in rats, and whether any effects are mediated by sex hormones and/or the expression of genes for enzymes involved in endogenous synthesis of longer chain (LC) n-3 polyunsaturated fatty acid (PUFA). In this study rats will therefore be used to examine the hypotheses that:

a) females rats have higher plasma levels of LC n-3 PUFA compared to males
b) gender differences are also apparent within metabolically relevant tissues such as the liver and adipose tissue.
c) plasma docosahexaenoic acid (DHA) content and desaturase and elongase gene expression within the liver are related to plasma sex hormone concentrations.
d) females will show a greater elevation in LC n-3 PUFA in response to an α-linolenic acid (ALNA) rich diet than males as a result of gender differences in desaturase and elongase gene expression.

3.1.2 Dietary sources and typical intakes of n-3 fatty acids

The essential fatty acid (EFA) ALNA (18:3n-3) is found in green leaves, some seeds, nuts and cooking oils, with linseeds and linseed oil having a very high content. The principal dietary source of the LC n-3 PUFA eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) is oily fish.

The average dietary intake of total n-3 PUFA for adults in the UK is 2.27 g/day for males and 1.71 g/day for females(4). For most people the majority of this is in the form of ALNA, as dietary intake of LC n-3 PUFA is highly dependent upon consumption of fish. It is estimated that only 27% of UK adults habitually eat oily fish(7), with an anticipated population average intake of LC n-3 PUFA of about 0.1 to l.2 g/day(6).

3.1.3 Endogenous synthesis of LC n-3 PUFA

LC n-3 PUFA can be generated via endogenous synthesis pathways from the EFA ALNA. This pathway also generates LC n-6 PUFA such as arachidonic acid (AA,

20:4n-6) from the n-6 EFA linoleic acid (LA, 18:2n-6). Endogenous synthesis primarily occurs in the liver, and the pathway is outlined in chapter 1 (figure 1.6).

The ability of humans to convert ALNA into LC n-3 PUFA has been studied using two approaches: provision of substantially increased amounts of ALNA in the diet for a period of weeks to months(140;141) or the provision of ALNA which is labelled with stable isotopes for a shorter period of time(130;131;136-138).

Increasing dietary ALNA intake in humans has been demonstrated to cause dose-dependent increases in the EPA content of plasma phospholipids, indicating that the conversion of ALNA to EPA is reasonably efficient, and subject to the dietary availability of ALNA. The relationship between increasing dietary ALNA and DHA status is less clear, with some studies reporting a reduction in DHA status with increasing dietary ALNA while other studies report no change(140;141). There is limited data currently available to describe the effects that increased dietary ALNA may have upon LC n-3 PUFA status in women(142), and no data from non-lactating women.

Studies using stable isotope-labelled ALNA gave the first indications that there are gender differences in the ability to synthesis LC n-3 PUFA from ALNA. Young women were found to convert a greater proportion of ALNA into EPA and DHA over 21 days compared to men(130;131). However, these studies did not give insight into the extent to which any LC n-3 PUFA generated might have been incorporated into metabolically relevant tissues such as the liver and adipose tissue, as only blood samples were collected. These studies also lacked information on whether the gender differences in the ability to synthesise LC n-3 PUFA had any significant effect upon the total circulating content of these fatty acids.

Other studies have confirmed that there are gender differences in circulating plasma concentrations of LC n-3 PUFA in humans. While these studies vary in sample size, the degree of dietary control exerted and the range of blood lipids analysed, all have found that women have significantly higher circulating DHA concentrations compared to men and that this is independent of dietary intake of n-3 fatty acids(132-135).

Mechanisms which have been proposed to account for the gender differences observed in rates of LC n-3 PUFA synthesis and the circulating concentrations of these fatty acids

include gender differences in rates of β-oxidation(143), adipose tissue composition and mobilisation(133), the influences of sex hormones upon desaturase and elongase enzymes involved in the synthesis of LC n-3 PUFA(131), or gender differences in enzyme specificity during phospholipid biosynthesis(229).

The lower rates of β-oxidation of fatty acids in women may result in more dietary ALNA remaining available for conversion into LC n-3 PUFA. Gender differences in subcutaneous adipose tissue composition have been observed in human studies(144), with women found to have significantly more DPA and DHA in adipose tissue compared to men, although these fatty acids remain relatively minor components of the total fatty acid content (< 0.3%). The higher adiposity of women compared to men and the selective release of fatty acids from human adipose tissue (ALNA and EPA are preferentially released in comparison to other n-3 PUFA(37;145)) may further enhance gender differences in LC n-3 PUFA synthesis and circulating concentrations.

A role for sex hormones in mediating the gender differences in ALNA conversion and the LC n-3 PUFA content of blood (and tissue) lipids has been hypothesised based upon studies of women using oral contraceptives(131;133;135), hormone replacement therapy (HRT)(135;147) and a study of sex hormone treatment in transsexual subjects(135).

Women using oral contraceptives were identified as having a higher rate of conversion of stable isotope labelled ALNA into LC n-3 PUFA than women not using oral contraceptives(131). Women using oral contraceptives have also been identified as tending to have higher circulating concentrations (non-significant) of LC n-3 PUFA(133;135). Similarly, women using hormone replacement therapy (HRT), or male-to-female transsexual subjects receiving synthetic oestrogens were found to have significantly higher circulating concentrations of LC n-3 PUFA(135;147;148).

It has been suggested that the gender differences in LC n-3 PUFA status may relate to the requirement for females of reproductive age to have sufficient capacity to transfer adequate DHA to the developing fetus, while maintaining their own tissue requirements(133).

3.1.4 *In vitro* and animal studies of fatty acid desaturase activities

There is evidence from animal studies that sex hormones influence the activity of the desaturase enzymes involved in the synthesis of LC PUFA. However, work to date has been limited to the use of n-6 rather than n-3 fatty acids as a substrate for assessment of enzyme activities and has investigated the effect of short-term hormone treatment rather than the potential influence of normal circulating physiological concentrations of sex-hormones.

The results of some of these studies have been counter-intuitive to those which would be expected based upon observed human gender differences in fatty acid composition. For example, *in vitro* and *ex vivo* studies demonstrated that 17β-oestradiol reduced Δ5 and Δ6 desaturase activity(150;151). Other studies have been more consistent with the observations from human studies, such as a study of male and female rats which found that testosterone treatment inhibited Δ6 and Δ5 desaturase activities(152), which supports the observation within female-to-male transsexual subjects that the administration of testosterone led to significantly reduced plasma DHA content(135).

3.2 Methods

This study sets out to identify whether there are gender differences in the plasma and tissue LC n-3 PUFA content of rat tissues, whether these differences are affected by the dietary availability of ALNA, and whether sex hormones or expression of desaturase and elongase genes are involved in mediating these gender differences. This will be assessed by determination of the fatty acid composition of tissues including plasma, liver and adipose tissue by gas chromatography, measurement of plasma sex hormone concentrations, and the assessment of liver mRNA expression of desaturase and elongase enzymes by RT-PCR.

All animal work was carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986) (see section 2.1). Experimental diets were provided *ad libitum* over a 20 day period, and tissues collected at the end of the dietary intervention. Fatty acid composition of experimental diets and tissues was determined by gas chromatography (see section 2.6). Plasma circulating sex hormones analysis was provided by Southampton General Hospital (see section 2.9). mRNA expression of desaturase and elongase genes was determined by RT-PCR (see section 2.12).

The effects of diet and gender and diet*gender interactions upon tissue fatty acid composition, plasma sex hormones and mRNA expression were assessed by two-way ANOVA. Correlation coefficients were calculated using Pearson or Spearman test as appropriate after data were assessed for normality.

3.3 Results

3.3.1 Experimental diets

Animals received the experimental diets over a 20 day period. Three diets were used: a low fat (LF, 3% w/w) soyabean oil diet, and two high fat (HF, 13% w/w) diets, one rich in soyabean oil, the other rich in linseed oil (a rich source of ALNA). The fatty acid composition of these diets was analysed (see table 3.1). Further information upon the nutrient composition of these diets is available (see section 2.3).

The LF soyabean oil diet is standard laboratory rat chow, which all animals were maintained on prior to the start of experiments. This diet contains both n-6 and n-3 EFA (LA and ALNA). The HF soyabean oil diet was used to reflect the higher dietary fat intake of a Western human diet, while maintaining the relative contributions of LA and ALNA observed in the LF soyabean oil diet. The HF linseed oil diet is ALNA rich at the expense of LA, and was used to assess whether increased dietary ALNA could modulate gender differences in LC n-3 PUFA status.

Table 3.1: Fatty acid composition of total lipid extracts obtained from experimental diets (% total fatty acids, mean ± standard deviation, n = 5)

Fatty acid	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	ANOVA P value
14:0	0.2 ± 0.2	0.1 ± 0.0	ND	0.683
16:0	19.0 ± 1.1 [a]	12.9 ± 0.5 [b]	8.6 ± 0.3 [c]	**< 0.001**
18:0	4.6 ± 1.0	4.6 ± 0.3	3.7 ± 0.2	0.097
20:0	0.5 ± 0.6	0.3 ± 0.1	0.3 ± 0.1	0.672
22:0	0.2 ± 0.2	0.1 ± 0.2	ND	0.409
Total saturated	24.4 ± 0.8 [a]	18.0 ± 0.5 [b]	12.7 ± 0.2 [c]	**< 0.001**
16:1n-7	0.4 ± 0.4	0.2 ± 0.1	0.1 ± 0.0	0.254
18:1n-9	14.0 ± 1.4 [a]	21.1 ± 0.5 [b]	20.7 ± 0.4 [b]	**< 0.001**
20:1n-9	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.689
Total MUFA	14.6 ± 2.0 [a]	21.5 ± 0.5 [b]	21.1 ± 0.5 [b]	**< 0.001**
18:2n-6	53.7 ± 2.0 [a]	52.8 ± 0.7 [a]	27.7 ± 2.9 [b]	**< 0.001**
18:3n-6	ND [a]	0.2 ± 0.1 [b]	0.2 ± 0.1 [b]	**< 0.001**
20:2n-6	0.4 ± 0.7	ND	ND	0.355
20:4n-6	0.1 ± 0.2	ND	ND	0.505
22:5n-6	0.4 ± 0.4	0.2 ± 0.1	0.1 ± 0.1	0.225
Total n-6 PUFA	54.7 ± 1.7 [a]	53.2 ± 0.4 [a]	27.7 ± 2.7 [b]	**< 0.001**
18:3n-3	5.9 ± 0.5 [a]	6.9 ± 0.2 [a]	38.0 ± 2.8 [b]	**< 0.001**
20:4n-3	0.1 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.530
20:5n-3	0.2 ± 0.2	ND	ND	0.179
22:5n-3	ND	ND	ND	-
22:6n-3	ND	ND	ND	0.397
Total n-3 PUFA	6.3 ± 0.5 [a]	7.3 ± 0.3 [a]	38.5 ± 2.9 [b]	**< 0.001**

ND negligible detected (mean <0.1%)
a,b,c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

3.3.2 Weight gain and food intake

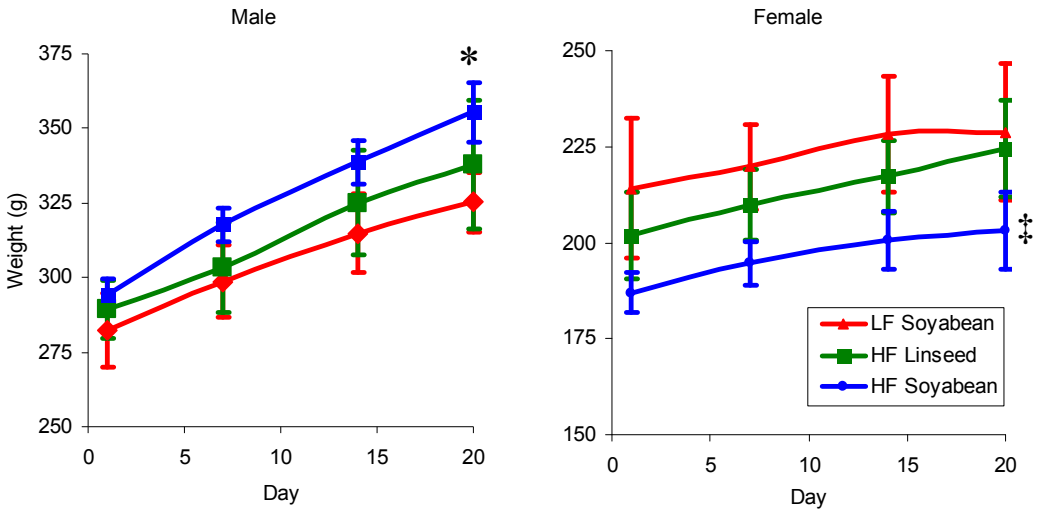
Animal weights were recorded every 7 days over the study period (see figure 3.1). Food intake was monitored by weighing food provided and remaining every 2-3 days in order to calculate energy intake and to ensure that the HF experimental diets were not unpalatable compared to the LF soyabean oil diet.

A clear effect of gender upon percentage weight gain and food (and energy) intake was identified (see table 3.2 and 3.3), with males in all dietary groups gaining significantly more weight and consuming more food and energy over the study period compared to females.

Whilst all groups gained weight, they showed a reduced rate of weight gain with time. This is likely to be an effect of individual caging during the course of the experiments. Prior to the start of experiments animals were housed in groups of 6 or 7, and so their access to food would be limited by social interactions within the group. Individual housing removes this constraint, perhaps causing the initial increase in food consumption and growth, which then stabilises as the animal becomes acclimatised to being housed individually.

There were no significant differences in weighed food intake over the study period between the dietary groups. As would be expected, this led to a significant effect of diet upon the total energy consumed, with the LF soyabean oil group having significantly lower energy intake compared to the HF diet groups.

Figure 3.1: Weight of rats provided with experimental diets over 20 day feeding period (mean ± standard deviation, n=6)



* HF Soyabean males significantly different from LF Soyabean males at day 20 ($p = 0.02$)

‡ HF Soyabean females significantly different from LF Soyabean females at all timepoints ($p < 0.05$). HF Soyabean females significantly different from HF Linseed females on day 7 ($p = 0.029$).

Table 3.2: Weekly % weight gain of rats fed experimental diets (mean ± standard deviation, n = 6 for all groups)

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
Day 7	5.7 ± 0.9	2.8 ± 3.6	8.0 ± 1.6	4.1 ± 1.8	4.8 ± 1.9	4.0 ± 1.9	0.087	**0.001**	0.187
Day 14	5.4 ± 0.7	3.8 ± 2.8	6.6 ± 0.5	3.1 ± 1.5	7.2 ± 0.8	3.5 ± 1.1	0.480	**< 0.001**	0.180
Day 20	3.3 ± 1.6	0.3 ± 3.1	5.0 ± 0.5	1.2 ± 2.1	3.9 ± 1.1	3.3 ± 1.7	0.069	**< 0.001**	0.115

Table 3.3: Food and energy consumption by rats fed experimental diets (mean ± standard deviation, n = 6 for all groups)

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Male	Female	Male	Female	Male	Female	Diet	Gender	**Diet*Gender**
Food consumption (g)	442.4 ± 19.2	321.2 ± 19.1	420.6 ± 12.1	283.8 ± 25.6	400.3 ± 24.9	313.6 ± 23.1	0.077	**< 0.001**	**0.020**
Energy consumption (MJ)	6.2 ± 0.3	4.7 ± 0.3	7.2 ± 0.2	4.9 ± 0.4	6.9 ± 0.4	5.4 ± 0.4	**< 0.001**	**< 0.001**	**0.007**

Diet - Bonferroni post-hoc tests for multiple comparisons:

Energy consumption (MJ) - LF soyabean oil significantly different from HF soyabean oil ($p = 0.002$) and HF Linseed oil ($p < 0.001$)

3.3.3 Plasma fatty acid composition

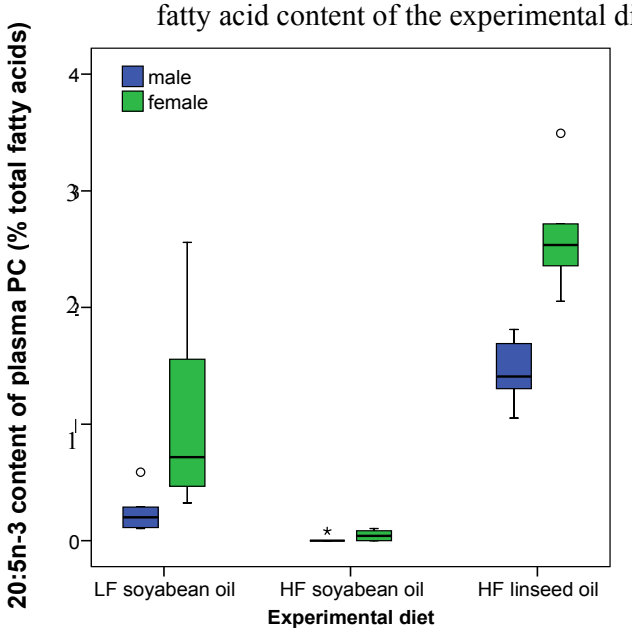
The fatty acid compositions of plasma phosphatidlycholine (PC), triacylglycerol (TAG), cholesteryl esters (CE) and non-esterified fatty acids (NEFA) were assessed by gas chromatography. Full details of the fatty acid composition of these plasma lipids are shown in tables 3.4 to 3.7.

The effect of diet upon plasma fatty acid composition

Diet had a significant effect upon the fatty acid composition of all plasma lipid fractions assessed (see tables 3.4 to 3.7). The HF linseed oil (ALNA-rich) diet group had significantly higher ALNA content of all plasma lipids examined.

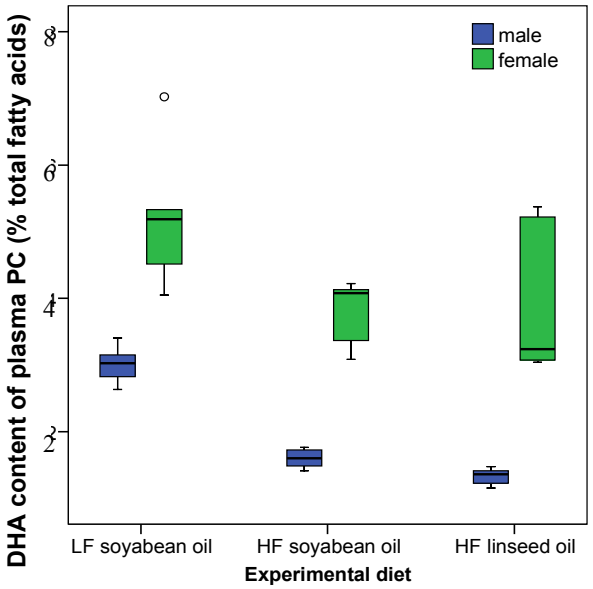
The content of LC n-3 PUFA was also affected by diet. The HF linseed oil diet groups had the highest EPA content in all plasma lipid fractions (see figure 3.2 for PC). This effect was not a simple function of the quantity of ALNA available, as the HF soyabean oil group had the lowest EPA content, suggesting that the synthesis of LC n-3 PUFA may be suppressed in rats by a either a high-fat diet *per se* or a high LA diet, but that this can be overcome by increasing the ALNA content of the diet. The effect of diet upon other LC PUFA in plasma lipids such as DHA was less clear. There was some indication of reduced DHA status in the ALNA-rich diet group (see figure 3.3 for PC). These observations are in accordance with those in human studies, where increased dietary ALNA has been demonstrated to result in significantly increased plasma EPA status, but without an associated increase in plasma DHA status(44;140). It is of interest that the DHA content of plasma PC is maintained in the HF soyabean oil diet, despite the dramatic reductions in EPA content observed. This may indicate that plasma DHA in being maintained at the expense of EPA, and so contributes to the fall in EPA status, or alternatively that DHA is being mobilized from alternative sources such as adipose tissue in order to maintain plasma DHA content in the face of reduced rates of LC PUFA synthesis.

Figure 3.2: Graph to illustrate the relationship between the EPA content (% total fatty acids, n = 6) of plasma PC after a 20 day feeding period and the essential fatty acid content of the experimental diets



ALNA intake (g/100g diet):		
0.16	0.90	4.97
LA intake (g/100g diet):		
1.46	6.91	3.63

Figure 3.3: Graph to illustrate the relationship between the DHA content (% total fatty acids, n = 6) of plasma PC after a 20 day feeding period and the essential fatty acid content of the experimental diets



ALNA intake (g/100g diet):		
0.16	0.90	4.97
LA intake (g/100g diet):		
1.46	6.91	3.63

Table 3.4: Fatty acid composition (% total fatty acids, mean ± standard deviation, n = 6 for all groups) of plasma PC

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
Fatty acid	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
14:0	0.30 ± 0.04	0.19 ± 0.11	0.22 ± 0.03	0.17 ± 0.02	ND	ND	**< 0.001**	**0.034**	0.257
16:0	24.45 ± 0.94	20.71 ± 1.23	21.49 ± 0.68	18.39 ± 1.57	18.78 ± 1.20	15.79 ± 1.09	**< 0.001**	**< 0.001**	0.698
18:0	18.48 ± 0.81	26.72 ± 1.62	21.77 ± 0.82	27.60 ± 1.55	20.93 ± 0.81	27.85 ± 1.59	**0.001**	**< 0.001**	0.081
20:0	0.18 ± 0.12	0.23 ± 0.27	0.21 ± 0.06	0.16 ± 0.04	0.24 ± 0.19	0.18 ± 0.03	0.916	0.639	0.589
22:0	0.11 ± 0.12	0.11 ± 0.18	0.16 ± 0.11	0.12 ± 0.10	0.12 ± 0.29	ND	0.560	0.371	0.747
Total saturated	43.52 ± 1.41	47.96 ± 1.24	43.85 ± 1.21	46.43 ± 0.97	40.15 ± 0.91	43.90 ± 0.99	**< 0.001**	**< 0.001**	0.145
16:1n-7	0.93 ± 0.26	0.80 ± 0.57	0.22 ± 0.06	0.12 ± 0.02	0.47 ± 0.37	0.24 ± 0.11	**< 0.001**	0.140	0.853
18:1n-9	4.83 ± 0.50	4.27 ± 0.90	3.38 ± 0.31	2.85 ± 0.21	5.18 ± 0.56	3.68 ± 0.45	**< 0.001**	**< 0.001**	0.053
20:1n-9	0.26 ± 0.11	0.14 ± 0.16	0.28 ± 0.15	0.17 ± 0.03	0.39 ± 0.14	0.22 ± 0.03	0.085	**0.002**	0.730
24:1n-9	ND	ND	0.10 ± 0.09	ND	ND	ND	0.175	0.227	0.084
Total MUFA	6.04 ± 0.76	5.26 ± 1.33	3.97 ± 0.37	3.14 ± 0.20	6.04 ± 0.80	4.14 ± 0.47	**< 0.001**	**< 0.001**	0.133
18:2n-6	25.23 ± 0.94	20.08 ± 1.79	30.86 ± 1.36	25.61 ± 1.18	35.62 ± 1.57	28.82 ± 2.36	**< 0.001**	**< 0.001**	0.382
18:3n-6	0.49 ± 0.48	1.18 ± 0.86	ND	ND	0.63 ± 0.70	0.26 ± 0.22	**0.002**	0.455	**0.049**
20:2n-6	0.54 ± 0.08	0.45 ± 0.13	0.73 ± 0.41	ND	0.84 ± 0.18	0.68 ± 0.29	**0.001**	**< 0.001**	**0.006**
20:3n-6	0.86 ± 0.26	0.79 ± 0.41	0.47 ± 0.14	0.45 ± 0.07	0.83 ± 0.13	0.84 ± 0.08	**< 0.001**	0.727	0.887
20:4n-6	18.67 ± 2.00	16.14 ± 1.51	17.30 ± 0.73	19.36 ± 0.81	10.10 ± 0.85	11.80 ± 1.53	**< 0.001**	0.360	**< 0.001**
22:5n-6	0.34 ± 0.20	0.72 ± 0.22	0.12 ± 0.07	ND	ND	0.10 ± 0.08	**< 0.001**	**0.003**	**0.001**
Total n-6 PUFA	46.14 ± 1.62	39.36 ± 1.85	49.48 ± 1.12	45.60 ± 1.33	48.01 ± 0.40	42.51 ± 1.56	**< 0.001**	**< 0.001**	0.053
18:3n-3	0.23 ± 0.08	0.20 ± 0.13	0.58 ± 0.25	0.26 ± 0.04	2.05 ± 0.19	1.67 ± 0.35	**< 0.001**	**0.001**	0.100
20:5n-3	0.25 ± 0.18	1.06 ± 0.85	ND	ND	1.45 ± 0.27	2.61 ± 0.49	**< 0.001**	**< 0.001**	**0.009**
22:5n-3	0.76 ± 0.07	0.95 ± 0.11	0.42 ± 0.07	0.61 ± 0.11	0.90 ± 0.07	1.22 ± 0.25	**< 0.001**	**< 0.001**	0.422
22:6n-3	3.01 ± 0.27	5.22 ± 1.02	1.64 ± 0.15	3.91 ± 0.49	1.33 ± 0.12	3.86 ± 1.12	**< 0.001**	**< 0.001**	0.814
Total n-3 PUFA	4.25 ± 0.16	7.41 ± 1.32	2.65 ± 0.36	4.83 ± 0.49	5.73 ± 0.35	9.36 ± 1.51	**< 0.001**	**< 0.001**	0.128

ND negligible detected (mean <0.1%)

Table 3.5: Fatty acid composition (% total fatty acids, mean ± standard deviation, n = 6 for all groups) of plasma TAG

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
Fatty acid	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
14:0	0.83 ± 0.20	0.77 ± 0.27	0.44 ± 0.28	0.33 ± 0.08	0.21 ± 0.10	0.25 ± 0.07	**< 0.001**	0.504	0.611
16:0	26.55 ± 4.22	26.70 ± 2.76	16.47 ± 1.54	17.20 ± 1.85	11.27 ± 0.42	12.40 ± 1.28	**< 0.001**	0.399	0.877
18:0	2.89 ± 0.56	5.26 ± 2.61	4.88 ± 2.64	4.16 ± 0.40	3.67 ± 0.50	3.80 ± 0.46	0.483	0.265	0.059
20:0	0.18 ± 0.08	0.18 ± 0.33	0.23 ± 0.07	0.26 ± 0.05	0.21 ± 0.06	0.14 ± 0.09	0.428	0.825	0.717
22:0	0.16 ± 0.09	0.46 ± 0.66	0.20 ± 0.03	0.15 ± 0.12	0.16 ± 0.08	ND	0.247	0.570	0.207
Total saturated	30.60 ± 4.64	33.37 ± 4.24	22.23 ± 4.00	22.10 ± 1.95	15.52 ± 0.51	16.67 ± 1.00	**< 0.001**	0.243	0.543
16:1n-7	3.73 ± 1.61	3.02 ± 1.17	0.25 ± 0.17	0.32 ± 0.08	0.44 ± 0.21	0.40 ± 0.14	**< 0.001**	0.412	0.462
18:1n-9	20.89 ± 2.04	22.29 ± 3.90	19.12 ± 1.65	17.92 ± 1.24	20.31 ± 0.19	19.65 ± 0.67	**0.003**	0.820	0.258
20:1n-9	0.47 ± 0.11	0.34 ± 0.17	0.25 ± 0.10	0.40 ± 0.14	0.28 ± 0.03	0.26 ± 0.06	**0.018**	0.989	**0.018**
24:1n-9	ND	0.12 ± 0.18	ND	ND	ND	ND	0.187	0.311	0.055
Total MUFA	25.09 ± 3.37	25.77 ± 4.71	19.66 ± 1.52	18.63 ± 1.20	21.03 ± 0.35	20.31 ± 0.64	**< 0.001**	0.674	0.679
18:2n-6	33.53 ± 4.59	29.97 ± 3.76	46.73 ± 3.92	45.32 ± 1.86	26.83 ± 1.25	26.17 ± 1.17	**< 0.001**	0.077	0.496
18:3n-6	0.54 ± 0.26	1.03 ± 0.64	0.16 ± 0.05	0.40 ± 0.04	0.23 ± 0.18	0.27 ± 0.44	**0.001**	**0.031**	0.291
20:2n-6	0.48 ± 0.13	0.40 ± 0.22	0.19 ± 0.21	0.11 ± 0.17	0.23 ± 0.07	ND	**< 0.001**	0.060	0.848
20:3n-6	0.35 ± 0.12	0.38 ± 0.30	0.34 ± 0.08	0.52 ± 0.07	0.22 ± 0.17	0.14 ± 0.13	**0.002**	0.388	0.148
20:4n-6	3.32 ± 1.21	3.85 ± 1.10	2.62 ± 0.64	5.06 ± 1.29	1.19 ± 0.24	1.82 ± 0.59	**< 0.001**	**0.001**	**0.028**
22:5n-6	0.44 ± 0.16	0.45 ± 0.29	ND	0.29 ± 0.16	ND	ND	**< 0.001**	0.084	0.106
Total n-6 PUFA	38.66 ± 6.16	36.07 ± 4.54	50.09 ± 4.25	51.72 ± 1.57	28.69 ± 1.31	28.50 ± 1.17	**< 0.001**	0.756	0.387
18:3n-3	2.64 ± 0.28	2.24 ± 0.38	5.44 ± 0.57	4.91 ± 0.40	29.57 ± 1.74	27.42 ± 3.50	**< 0.001**	0.069	0.353
20:4n-3	0.10 ± 0.09	ND	ND	0.17 ± 0.29	0.18 ± 0.10	0.28 ± 0.21	**0.048**	0.344	0.316
20:5n-3	0.53 ± 0.18	0.81 ± 0.67	0.62 ± 0.25	0.51 ± 0.07	2.37 ± 0.56	3.41 ± 1.35	**< 0.001**	0.083	0.116
22:5n-3	0.80 ± 0.32	0.67 ± 0.33	0.77 ± 0.27	0.67 ± 0.13	1.63 ± 0.51	1.81 ± 1.07	**< 0.001**	0.951	0.731
22:6n-3	1.57 ± 0.57	1.05 ± 0.56	1.15 ± 0.33	1.30 ± 0.23	1.01 ± 0.31	1.59 ± 0.82	0.915	0.696	**0.041**
Total n-3 PUFA	5.65 ± 1.37	4.81 ± 0.86	8.04 ± 0.91	7.56 ± 0.38	34.75 ± 1.46	34.51 ± 1.29	**< 0.001**	0.171	0.801

ND negligible detected (mean <0.1%)

Table 3.6: Fatty acid composition (% total fatty acids, mean ± standard deviation, n = 6 for all groups) of plasma CE

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
Fatty acid	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
14:0	0.28 ± 0.06	0.19 ± 0.21	0.34 ± 0.22	0.23 ± 0.15	0.16 ± 0.17	0.12 ± 0.07	0.114	0.153	0.871
16:0	11.14 ± 1.65	11.82 ± 3.69	9.38 ± 1.77	7.59 ± 1.32	10.28 ± 2.13	7.92 ± 1.48	**0.006**	0.129	0.211
18:0	1.65 ± 0.55	3.05 ± 1.52	2.75 ± 1.74	2.02 ± 0.55	2.81 ± 0.80	2.76 ± 1.25	0.605	0.610	0.102
Total saturated	13.11 ± 2.12	15.09 ± 5.27	12.53 ± 3.74	9.83 ± 1.90	13.29 ± 3.01	10.81 ± 2.46	0.113	0.352	0.175
16:1n-7	0.65 ± 0.16	1.24 ± 0.57	0.61 ± 0.07	0.40 ± 0.06	0.37 ± 0.07	0.42 ± 0.05	**< 0.001**	0.103	**0.002**
18:1n-9	6.70 ± 2.60	5.61 ± 2.42	7.40 ± 1.43	4.58 ± 0.92	9.43 ± 2.19	6.99 ± 2.17	**0.025**	**0.005**	0.574
20:1n-9	ND	ND	ND	0.12 ± 0.27	ND	ND	0.436	0.501	0.653
Total MUFA	7.37 ± 2.63	6.92 ± 2.72	8.06 ± 1.54	5.09 ± 1.21	9.82 ± 2.27	7.41 ± 2.19	0.085	**0.014**	0.359
18:2n-6	26.01 ± 1.83	20.64 ± 2.03	30.46 ± 1.66	24.41 ± 1.50	36.90 ± 1.67	29.60 ± 1.56	**< 0.001**	**< 0.001**	0.390
18:3n-6	0.54 ± 0.18	0.75 ± 0.14	0.29 ± 0.03	0.36 ± 0.04	0.30 ± 0.05	0.44 ± 0.08	**< 0.001**	**< 0.001**	0.327
20:2n-6	ND	ND	ND	ND	ND	0.11 ± 0.13	**0.029**	0.794	0.957
20:3n-6	0.36 ± 0.10	0.26 ± 0.20	0.23 ± 0.02	0.21 ± 0.12	0.34 ± 0.09	0.43 ± 0.04	**0.007**	0.740	0.109
20:4n-6	49.93 ± 6.44	52.88 ± 5.90	45.95 ± 3.99	56.75 ± 3.05	27.68 ± 2.72	35.07 ± 5.99	**< 0.001**	**< 0.001**	0.182
Total n-6 PUFA	76.86 ± 4.76	74.58 ± 7.16	76.92 ± 4.86	81.73 ± 2.95	65.32 ± 3.95	65.65 ± 4.64	**< 0.001**	0.574	0.241
18:3n-3	0.55 ± 0.43	0.29 ± 0.06	1.29 ± 0.20	0.94 ± 0.12	5.79 ± 0.64	4.64 ± 1.49	**< 0.001**	**0.021**	0.254
20:4n-3	0.20 ± 0.14	ND	ND	ND	0.25 ± 0.24	0.27 ± 0.08	**< 0.001**	0.154	0.065
20:5n-3	0.73 ± 0.38	1.26 ± 0.44	0.36 ± 0.07	0.41 ± 0.07	4.74 ± 0.80	9.70 ± 2.27	**< 0.001**	**< 0.001**	**< 0.001**
22:5n-3	ND	ND	ND	ND	ND	0.13 ± 0.07	**0.001**	0.794	0.152
22:6n-3	1.18 ± 0.32	1.86 ± 0.49	0.79 ± 0.15	1.99 ± 0.16	0.72 ± 0.55	1.40 ± 0.29	**0.012**	**< 0.001**	0.162
Total n-3 PUFA	2.66 ± 0.58	3.41 ± 0.76	2.48 ± 0.37	3.33 ± 0.10	11.57 ± 1.07	16.14 ± 2.12	**< 0.001**	**< 0.001**	**< 0.001**

ND negligible detected (mean <0.1%)

Table 3.7: Fatty acid composition (% total fatty acids, mean ± standard deviation) of plasma NEFA

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
Fatty acid	(n=5)	(n=5)	(n=5)	(n=6)	(n=6)	(n=6)			
14:0	0.82 ± 0.24	1.39 ± 0.40	1.02 ± 0.47	1.12 ± 0.35	0.58 ± 0.16	0.45 ± 0.03	**< 0.001**	0.100	**0.037**
16:0	25.66 ± 1.86	28.66 ± 3.38	21.15 ± 2.96	21.92 ± 2.40	16.89 ± 1.67	16.22 ± 2.22	**< 0.001**	0.238	0.234
18:0	30.55 ± 9.69	20.21 ± 5.73	32.30 ± 6.46	30.19 ± 2.99	25.01 ± 4.68	21.99 ± 5.45	**0.014**	**0.021**	0.249
20:0	0.29 ± 0.41	0.35 ± 0.34	0.94 ± 0.15	0.93 ± 0.31	1.50 ± 0.38	0.57 ± 0.17	**< 0.001**	**0.010**	**0.001**
22:0	0.97 ± 0.85	0.12 ± 0.23	0.28 ± 0.09	0.24 ± 0.05	0.71 ± 0.64	ND	0.360	**0.003**	0.106
Total saturated	58.28 ± 10.94	50.73 ± 7.25	55.77 ± 5.40	54.44 ± 2.28	44.69 ± 6.36	39.27 ± 7.33	**< 0.001**	0.060	0.585
16:1n-7	3.52 ± 1.70	2.22 ± 1.61	0.81 ± 0.44	0.18 ± 0.16	1.51 ± 0.30	0.82 ± 0.25	**< 0.001**	**0.013**	0.665
18:1n-9	12.34 ± 3.24	16.32 ± 3.20	9.19 ± 0.64	9.72 ± 0.77	10.97 ± 1.40	11.72 ± 1.77	**< 0.001**	**0.021**	0.118
20:1n-9	0.26 ± 0.25	ND	0.46 ± 0.23	0.29 ± 0.27	0.53 ± 0.17	0.56 ± 0.73	0.076	0.417	0.739
24:1n-9	0.20 ± 0.44	0.10 ± 0.14	ND	ND	0.17 ± 0.28	ND	0.489	0.391	0.456
Total MUFA	16.32 ± 4.63	18.72 ± 3.94	10.45 ± 0.85	10.26 ± 0.74	13.19 ± 1.13	13.11 ± 1.18	**< 0.001**	0.421	0.415
18:2n-6	16.66 ± 5.22	20.14 ± 4.43	21.60 ± 2.81	22.14 ± 3.52	17.35 ± 2.87	18.41 ± 3.69	**0.041**	0.212	0.647
18:3n-6	0.14 ± 0.31	0.56 ± 0.62	0.16 ± 0.14	0.26 ± 0.14	ND	0.21 ± 0.13	0.157	**0.021**	0.443
20:2n-6	ND	ND	0.38 ± 0.12	0.30 ± 0.16	ND	0.12 ± 0.30	**< 0.001**	0.526	0.316
20:3n-6	0.24 ± 0.22	0.11 ± 0.26	0.33 ± 0.18	0.24 ± 0.19	ND	0.31 ± 0.14	0.274	0.734	**0.025**
20:4n-6	5.62 ± 1.05	5.33 ± 1.15	4.31 ± 0.53	4.74 ± 1.24	2.74 ± 0.45	3.21 ± 0.63	**< 0.001**	0.521	0.560
22:5n-6	ND	0.11 ± 0.11	ND	0.39 ± 0.52	ND	ND	0.138	**0.049**	0.138
Total n-6 PUFA	22.65 ± 6.42	26.33 ± 5.63	26.78 ± 3.24	28.06 ± 2.51	20.12 ± 3.22	22.27 ± 3.10	**0.005**	0.114	0.801
18:3n-3	1.20 ± 0.76	1.77 ± 0.44	4.91 ± 1.49	4.88 ± 1.77	19.43 ± 2.34	21.53 ± 4.24	**< 0.001**	0.286	0.531
20:4n-3	0.11 ± 0.18	ND	0.61 ± 0.09	0.72 ± 0.45	0.37 ± 0.44	0.22 ± 0.13	**< 0.001**	0.689	0.550
20:5n-3	0.15 ± 0.25	0.46 ± 0.36	ND	ND	0.82 ± 0.21	1.33 ± 0.33	**< 0.001**	**0.006**	**0.028**
22:5n-3	0.41 ± 0.12	0.39 ± 0.13	0.36 ± 0.23	0.33 ± 0.20	0.84 ± 0.30	1.19 ± 0.32	**< 0.001**	0.238	0.112
22:6n-3	0.88 ± 0.27	1.59 ± 0.67	1.05 ± 0.22	1.31 ± 0.63	0.55 ± 0.32	1.08 ± 0.27	0.061	**0.003**	0.503
Total n-3 PUFA	2.76 ± 1.26	4.22 ± 0.70	6.99 ± 1.89	7.25 ± 2.76	22.01 ± 2.83	25.35 ± 3.60	**< 0.001**	0.064	0.345

ND negligible detected (mean <0.1%)

The effect of gender upon plasma fatty acid composition

Significant effects of gender were observed upon fatty acid composition of all plasma lipid fractions (see tables 3.4 to 3.7). A summary of the significant gender differences observed is given in table 3.8.

Table 3.8: Summary of the significant ($p < 0.05$) effects of gender observed upon fatty acids within plasma lipid fractions

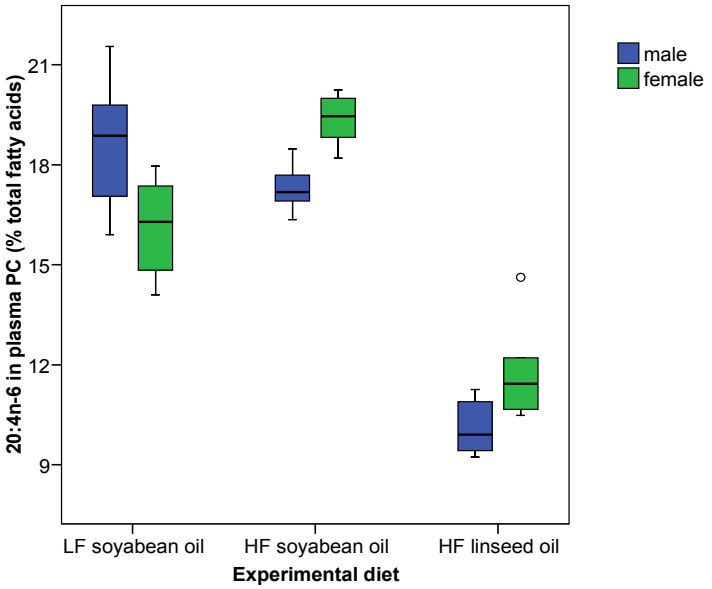
	PC	TAG	CE	NEFA
Males > Females	14:0 16:0 18:1n-9 20:1n-9 18:2n-6 20:2n-6 18:3n-3		 18:1n-9 18:2n-6 18:3n-3	 18:0 22:0 16:1n-7
Females > Males	18:0 22:5n-3 22:6n-3	 18:3n-6	 18:3n-6 20:4n-6 22:6n-3	 18:1n-9 18:3n-6 22:5n-6 22:6n-3
Gender*Diet interactions	 18:3n-6 20:2n-6 20:4n-6 20:5n-3	 20:1n-9 20:4n-6 22:6n-3	 16:1n-7 20:5n-3	14:0 20:0 20:3n-6 20:5n-3

Males were observed to have higher plasma contents of the EFA LA and ALNA across all dietary groups. This may suggest that more EFA are being utilised for LC PUFA synthesis in females than in males. Males also had higher plasma 18:1n-9 content compared to females. This fatty acid can be endogenously synthesised by the action of Δ9 desaturase upon 18:0, and so may indicate greater Δ9 desaturase activity within males compared to females.

Females had higher plasma contents of 18:3n-6 and DHA across all dietary groups. These data correspond well with the gender effects that have been identified in human studies, in particular, that females have significantly higher DHA content of plasma lipids compared to males, independent of diet(132-135).

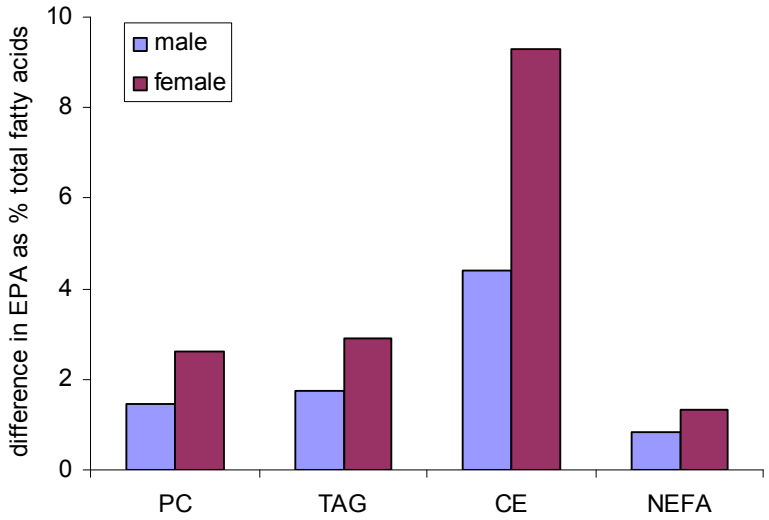
Several plasma fatty acids exhibited gender-diet interactions. The complex relationship between diet and gender upon plasma fatty acid composition is well illustrated by the AA content of plasma PC. In the LF soyabean oil group males had a higher AA content. HF feeding reversed this relationship, and HF linseed oil feeding resulted in significantly lower plasma AA content (see figure 3.4).

Figure 3.4: Graph to illustrate the interaction between gender and diet upon the AA content (% total fatty acids, n = 6) of plasma PC after a 20 day feeding period



It is clear that the effect of dietary ALNA upon LC PUFA status differs between male and female rats. For example, the EPA content of plasma lipids was increased in both male and female rats by the HF linseed oil diet, but the extent of this dietary effect was greater in females, indicating that females may be more responsive to a ALNA rich diet (see figure 3.5). The same trend was also apparent upon DPA status, but not DHA status.

Figure 3.5: The effect of gender upon the EPA content of plasma lipids (% total fatty acids) observed in response to dietary ALNA (mean HF linseed – mean HF soyabean) after a 20 day feeding period



3.3.4 Liver fatty acid composition

The diet and gender relationships which were identified in plasma lipids were also reflected in liver lipid fatty acid composition (see tables 3.9 to 3.12).

The ALNA-rich HF linseed oil diet group demonstrated the highest ALNA and EPA contents in all liver lipid fractions examined. There was also a higher content of DPA in liver lipids of the HF linseed oil group compared to the other dietary groups, and this was more pronounced than was observed within plasma lipids. This may reflect the role of the liver as a primary site of LC PUFA synthesis, and it is possible that a longer feeding intervention might result in this effect of diet upon DPA status also being detectable within plasma lipids. The HF linseed oil diet did not increase the DHA content of liver lipids.

Gender demonstrated numerous significant effects upon liver fatty acid composition, particularly within liver phospholipids (PC and PE). These effects were comparable to those observed within plasma lipids, with males having

higher EFA (LA and ALNA) and 18:1n-9 contents compared to females, and females having higher DHA content.

Males had significantly higher AA content compared to females in liver PC, PE and TAG. This indicates that gender does not exert a simple effect upon the synthesis of all LC PUFA, as LC n-3 and n-6 PUFA demonstrate differential effects of gender.

Strong and statistically significant correlations were identified between plasma and liver lipid fatty acid composition (see table 3.13), which supports a role of the liver in the maintenance of the fatty acid composition of plasma lipids, particularly for LC n-3 PUFA such as DHA (see figure 3.6).

Table 3.9: Fatty acid composition (% total fatty acids, mean ± standard deviation, n = 6 for all groups) of liver PC

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
Fatty acid	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
14:0	0.37 ± 0.06	0.22 ± 0.03	0.17 ± 0.04	0.10 ± 0.01	0.14 ± 0.01	0.11 ± 0.02	**< 0.001**	**< 0.001**	**0.001**
16:0	22.70 ± 1.23	18.39 ± 0.67	17.52 ± 0.73	14.06 ± 1.11	16.31 ± 0.91	12.31 ± 0.62	**< 0.001**	**< 0.001**	0.514
18:0	19.53 ± 0.93	28.62 ± 1.45	23.73 ± 0.93	30.32 ± 1.54	22.04 ± 1.07	29.66 ± 0.74	**< 0.001**	**< 0.001**	**0.040**
20:0	ND	ND	0.22 ± 0.01	0.20 ± 0.06	0.16 ± 0.03	0.14 ± 0.02	**< 0.001**	0.153	0.955
22:0	ND	ND	0.11 ± 0.04	0.08 ± 0.07	0.15 ± 0.02	ND	**< 0.001**	**0.003**	0.458
Total saturated	42.71 ± 1.31	47.26 ± 1.13	41.73 ± 0.74	44.77 ± 0.87	38.80 ± 0.45	42.30 ± 0.29	**< 0.001**	**< 0.001**	0.110
16:1n-7	1.36 ± 0.47	0.61 ± 0.21	0.06 ± 0.01	0.09 ± 0.06	0.20 ± 0.05	0.13 ± 0.04	**< 0.001**	**0.001**	**< 0.001**
18:1n-9	4.20 ± 0.37	3.30 ± 0.48	3.21 ± 0.31	2.25 ± 0.31	4.74 ± 0.51	2.86 ± 0.18	**< 0.001**	**< 0.001**	**0.005**
20:1n-9	0.14 ± 0.04	0.12 ± 0.15	0.30 ± 0.10	0.26 ± 0.10	0.28 ± 0.04	0.27 ± 0.06	**< 0.001**	0.348	0.951
24:1n-9	ND	ND	0.01 ± 0.03	0.03 ± 0.05	ND	ND	0.565	0.655	0.442
Total MUFA	5.71 ± 0.80	4.03 ± 0.72	3.58 ± 0.32	2.62 ± 0.41	5.23 ± 0.51	3.29 ± 0.21	**< 0.001**	**< 0.001**	0.084
18:2n-6	16.47 ± 0.59	12.40 ± 1.22	18.05 ± 1.76	13.26 ± 0.77	23.99 ± 0.71	17.13 ± 1.81	**< 0.001**	**< 0.001**	**0.027**
18:3n-6	0.23 ± 0.06	0.35 ± 0.08	0.17 ± 0.02	0.17 ± 0.05	0.15 ± 0.02	0.17 ± 0.02	**< 0.001**	**0.009**	**0.009**
20:2n-6	0.50 ± 0.12	0.31 ± 0.06	1.62 ± 0.24	1.10 ± 0.21	0.83 ± 0.16	0.47 ± 0.13	**< 0.001**	**< 0.001**	0.067
20:3n-6	1.00 ± 0.28	1.06 ± 0.13	0.68 ± 0.07	0.66 ± 0.08	1.20 ± 0.11	1.19 ± 0.17	**< 0.001**	0.817	0.811
20:4n-6	26.38 ± 2.30	24.33 ± 1.24	28.72 ± 1.31	27.28 ± 1.15	18.69 ± 0.93	18.21 ± 1.63	**< 0.001**	**0.012**	0.441
22:5n-6	0.38 ± 0.20	0.17 ± 0.19	0.17 ± 0.04	0.26 ± 0.11	0.24 ± 0.01	0.27 ± 0.15	0.495	0.494	**0.028**
Total n-6 PUFA	44.96 ± 1.87	38.62 ± 2.02	49.41 ± 1.00	42.73 ± 1.44	45.10 ± 1.00	37.44 ± 2.01	**< 0.001**	**< 0.001**	0.589
18:3n-3	ND	0.13 ± 0.11	0.29 ± 0.07	0.22 ± 0.04	1.66 ± 0.15	1.16 ± 0.17	**< 0.001**	**< 0.001**	**< 0.001**
20:4n-3	ND	ND	0.17 ± 0.10	0.16 ± 0.24	0.19 ± 0.03	0.24 ± 0.04	**0.006**	0.795	0.415
20:5n-3	0.43 ± 0.22	0.71 ± 0.15	0.21 ± 0.05	0.20 ± 0.03	4.05 ± 0.48	5.73 ± 0.80	**< 0.001**	**< 0.001**	**< 0.001**
22:5n-3	1.11 ± 0.17	1.34 ± 0.08	0.93 ± 0.13	1.04 ± 0.13	1.82 ± 0.23	2.12 ± 0.33	**< 0.001**	**0.003**	0.499
22:6n-3	4.90 ± 0.34	7.90 ± 1.12	3.68 ± 0.65	8.26 ± 1.02	3.15 ± 0.15	7.72 ± 1.37	**0.041**	**< 0.001**	0.059
Total n-3 PUFA	6.62 ± 0.54	10.09 ± 1.06	5.28 ± 0.73	9.88 ± 1.33	10.86 ± 0.60	16.97 ± 1.92	**< 0.001**	**< 0.001**	**0.027**

ND negligible detected (mean <0.1%)

Table 3.10: Fatty acid composition (% total fatty acids, mean ± standard deviation, n = 6 for all groups) of liver PE

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
Fatty acid	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
16:0	19.33 ± 1.50	18.41 ± 0.64	14.68 ± 0.53	14.88 ± 1.04	11.90 ± 0.60	11.80 ± 0.32	**< 0.001**	0.339	0.270
18:0	21.52 ± 1.17	26.77 ± 0.59	24.99 ± 0.93	27.49 ± 1.31	24.95 ± 1.25	28.92 ± 0.82	**< 0.001**	**< 0.001**	**0.014**
20:0	ND	ND	0.18 ± 0.04	0.12 ± 0.03	0.13 ± 0.01	0.14 ± 0.06	**< 0.001**	**0.039**	0.164
Total saturated	40.97 ± 0.99	45.20 ± 0.41	39.87 ± 0.93	42.52 ± 1.60	37.04 ± 0.84	40.93 ± 0.82	**< 0.001**	**< 0.001**	0.146
16:1n-7	0.80 ± 0.26	0.33 ± 0.09	0.13 ± 0.02	0.04 ± 0.05	0.17 ± 0.11	0.15 ± 0.06	**< 0.001**	**< 0.001**	**< 0.001**
18:1n-9	3.16 ± 0.52	2.17 ± 0.38	4.92 ± 0.65	2.93 ± 0.41	5.50 ± 0.57	2.96 ± 0.25	**< 0.001**	**< 0.001**	**0.002**
20:1n-9	0.14 ± 0.06	0.22 ± 0.13	0.30 ± 0.04	0.14 ± 0.01	0.25 ± 0.03	0.29 ± 0.08	**0.019**	0.468	**< 0.001**
Total MUFA	4.10 ± 0.46	2.72 ± 0.43	5.35 ± 0.69	3.11 ± 0.42	5.92 ± 0.61	3.40 ± 0.30	**< 0.001**	**< 0.001**	**0.031**
18:2n-6	9.62 ± 1.13	6.40 ± 0.95	14.16 ± 0.93	8.39 ± 0.79	14.12 ± 0.89	8.59 ± 1.11	**< 0.001**	**< 0.001**	**0.007**
18:3n-6	0.15 ± 0.03	0.21 ± 0.08	0.17 ± 0.04	ND	0.12 ± 0.01	0.12 ± 0.03	**0.003**	0.220	**0.001**
20:2n-6	0.34 ± 0.12	0.22 ± 0.03	1.29 ± 0.23	0.73 ± 0.19	0.16 ± 0.04	ND	**< 0.001**	**< 0.001**	**< 0.001**
20:3n-6	0.68 ± 0.11	0.61 ± 0.07	0.57 ± 0.03	0.48 ± 0.08	0.83 ± 0.07	0.71 ± 0.11	**< 0.001**	**0.002**	0.676
20:4n-6	31.14 ± 0.79	25.80 ± 0.68	28.67 ± 0.70	25.66 ± 1.57	23.47 ± 1.54	19.75 ± 1.97	**< 0.001**	**< 0.001**	0.120
22:5n-6	0.35 ± 0.77	ND	ND	ND	0.20 ± 0.02	0.21 ± 0.03	0.196	0.259	0.256
Total n-6 PUFA	42.49 ± 0.93	33.24 ± 1.60	44.90 ± 1.23	35.76 ± 2.48	38.72 ± 1.27	29.26 ± 2.63	**< 0.001**	**< 0.001**	0.977
18:3n-3	0.15 ± 0.01	0.23 ± 0.03	0.39 ± 0.08	0.27 ± 0.02	1.72 ± 0.14	1.44 ± 0.30	**< 0.001**	**0.043**	**0.020**
20:4n-3	0.56 ± 0.12	ND	ND	0.45 ± 0.22	ND	ND	**< 0.001**	0.188	**< 0.001**
20:5n-3	0.47 ± 0.14	0.49 ± 0.09	0.37 ± 0.07	0.25 ± 0.05	6.04 ± 0.63	6.48 ± 0.94	**< 0.001**	0.491	0.340
22:5n-3	2.37 ± 0.22	3.00 ± 0.21	2.09 ± 0.24	2.17 ± 0.24	4.06 ± 0.41	4.20 ± 0.61	**< 0.001**	**0.027**	0.153
22:6n-3	9.10 ± 0.86	15.11 ± 1.86	7.02 ± 0.94	15.93 ± 1.18	6.31 ± 0.36	14.09 ± 1.81	**0.005**	**< 0.001**	**0.040**
Total n-3 PUFA	12.44 ± 0.83	18.84 ± 1.66	9.87 ± 1.02	18.61 ± 1.28	18.32 ± 0.90	26.41 ± 2.22	**< 0.001**	**< 0.001**	0.148

ND negligible detected (mean <0.1%)

Table 3.11: Fatty acid composition (% total fatty acids, mean ± standard deviation) of liver TAG

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
Fatty acid	(n=6)	(n=6)	(n=5)	(n=5)	(n=6)	(n=6)			
14:0	1.37 ± 0.48	1.03 ± 0.23	0.50 ± 0.05	0.44 ± 0.06	0.32 ± 0.05	0.32 ± 0.06	**< 0.001**	0.106	0.159
16:0	31.41 ± 6.18	31.75 ± 3.86	18.29 ± 0.47	20.47 ± 1.76	13.37 ± 0.48	14.98 ± 0.79	**< 0.001**	0.219	0.783
18:0	2.70 ± 0.36	3.88 ± 1.06	1.64 ± 0.14	2.68 ± 0.37	1.90 ± 0.32	2.48 ± 0.49	**< 0.001**	**< 0.001**	0.392
20:0	0.12 ± 0.09	0.17 ± 0.14	0.06 ± 0.01	0.06 ± 0.06	0.60 ± 0.27	0.74 ± 0.15	**< 0.001**	0.243	0.591
22:0	ND	ND	0.06 ± 0.04	0.01 ± 0.02	0.20 ± 0.03	0.10 ± 0.05	**< 0.001**	**< 0.001**	0.570
Total saturated	35.69 ± 6.90	36.83 ± 4.77	20.54 ± 0.53	23.66 ± 1.77	16.39 ± 0.47	18.62 ± 1.10	**< 0.001**	0.096	0.818
16:1n-7	5.28 ± 2.52	3.33 ± 1.22	0.56 ± 0.09	0.57 ± 0.19	0.47 ± 0.14	0.45 ± 0.12	**< 0.001**	0.122	0.094
18:1n-9	21.63 ± 2.21	27.18 ± 3.58	14.89 ± 0.67	15.67 ± 1.34	17.43 ± 1.20	16.55 ± 0.59	**< 0.001**	**0.011**	**0.001**
20:1n-9	0.43 ± 0.07	0.23 ± 0.19	0.19 ± 0.08	0.28 ± 0.05	0.24 ± 0.04	0.18 ± 0.03	**0.009**	0.126	**0.004**
Total MUFA	27.34 ± 4.17	30.74 ± 4.21	15.64 ± 0.58	16.53 ± 1.47	18.14 ± 1.27	17.18 ± 0.60	**< 0.001**	0.232	0.146
18:2n-6	26.03 ± 7.12	24.18 ± 2.88	47.12 ± 0.68	44.16 ± 0.57	28.70 ± 0.90	26.32 ± 1.08	**< 0.001**	**0.045**	0.926
18:3n-6	0.36 ± 0.09	0.56 ± 0.15	0.69 ± 0.07	0.65 ± 0.09	0.29 ± 0.06	0.33 ± 0.07	**< 0.001**	0.056	**0.024**
20:2n-6	0.58 ± 0.14	0.47 ± 0.18	0.64 ± 0.33	0.54 ± 0.28	0.36 ± 0.06	0.27 ± 0.07	**0.005**	0.167	0.989
20:3n-6	0.41 ± 0.16	0.48 ± 0.17	0.92 ± 0.07	1.04 ± 0.26	0.38 ± 0.04	0.43 ± 0.08	**< 0.001**	0.110	0.850
20:4n-6	3.94 ± 1.39	3.41 ± 1.15	3.84 ± 0.36	4.85 ± 0.55	1.29 ± 0.26	1.68 ± 0.43	**< 0.001**	0.317	0.110
22:5n-6	0.47 ± 0.21	ND	0.25 ± 0.07	0.49 ± 0.21	ND	ND	**< 0.001**	0.081	**< 0.001**
Total n-6 PUFA	31.79 ± 8.91	29.11 ± 4.30	53.46 ± 0.54	51.73 ± 1.36	31.01 ± 1.16	29.03 ± 1.04	**< 0.001**	0.159	0.963
18:3n-3	1.61 ± 0.37	1.51 ± 0.29	4.17 ± 0.36	3.74 ± 0.23	21.75 ± 1.71	23.00 ± 2.24	**< 0.001**	0.565	0.236
20:4n-3	0.35 ± 0.85	ND	0.16 ± 0.01	0.12 ± 0.03	0.67 ± 0.10	0.85 ± 0.12	**< 0.001**	0.611	0.222
20:5n-3	0.47 ± 0.19	0.39 ± 0.14	1.21 ± 0.23	0.63 ± 0.16	4.45 ± 0.70	4.21 ± 0.43	**< 0.001**	**0.026**	0.306
22:5n-3	1.06 ± 0.41	0.66 ± 0.20	2.27 ± 0.22	1.47 ± 0.31	4.84 ± 0.73	4.17 ± 0.57	**< 0.001**	**< 0.001**	0.567
22:6n-3	1.69 ± 0.70	0.74 ± 0.13	2.54 ± 0.32	2.11 ± 0.49	2.74 ± 0.58	2.94 ± 0.45	**< 0.001**	**0.027**	**0.026**
Total n-3 PUFA	5.18 ± 1.17	3.31 ± 0.63	10.34 ± 0.62	8.07 ± 1.08	34.36 ± 2.11	35.18 ± 1.09	**< 0.001**	**0.013**	**0.015**

ND negligible detected (mean <0.1%)

Table 3.12: Fatty acid composition (% total fatty acids, mean ± standard deviation) of liver CE

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
Fatty acid	(n=6)	(n=6)	(n=5)	(n=5)	(n=6)	(n=6)			
14:0	0.52 ± 0.35	0.61 ± 0.20	0.40 ± 0.08	0.31 ± 0.18	0.17 ± 0.04	0.23 ± 0.05	**< 0.001**	0.797	0.554
16:0	36.69 ± 4.18	43.69 ± 3.16	31.55 ± 5.06	46.70 ± 4.86	20.11 ± 1.85	23.41 ± 2.21	**< 0.001**	**< 0.001**	**0.003**
18:0	23.27 ± 3.62	21.07 ± 3.48	8.37 ± 3.10	13.78 ± 3.18	16.03 ± 3.10	22.13 ± 3.74	**< 0.001**	**0.013**	**0.010**
20:0	ND	0.26 ± 0.32	ND	ND	ND	ND	0.354	0.100	0.066
Total saturated	60.49 ± 7.03	65.62 ± 3.58	40.35 ± 7.96	60.84 ± 6.51	36.38 ± 4.88	45.84 ± 5.64	**< 0.001**	**< 0.001**	**0.018**
16:1n-7	2.00 ± 0.63	2.04 ± 0.72	0.50 ± 0.38	0.49 ± 0.10	0.51 ± 0.23	0.37 ± 0.09	**< 0.001**	0.807	0.883
18:1n-9	8.73 ± 1.05	7.47 ± 1.98	17.91 ± 2.91	10.88 ± 2.02	14.58 ± 2.02	9.94 ± 1.42	**< 0.001**	**< 0.001**	**0.006**
20:1n-9	0.10 ± 0.11	0.34 ± 0.30	0.18 ± 0.24	ND	0.21 ± 0.06	0.14 ± 0.02	0.304	0.935	**0.026**
Total MUFA	10.83 ± 1.58	9.84 ± 2.56	18.59 ± 2.53	11.40 ± 2.02	15.30 ± 2.14	10.46 ± 1.40	**< 0.001**	**< 0.001**	**0.005**
18:2n-6	14.46 ± 3.25	13.43 ± 1.58	29.68 ± 6.33	17.63 ± 2.87	20.26 ± 1.08	17.30 ± 2.32	**< 0.001**	**< 0.001**	**0.001**
18:3n-6	0.12 ± 0.10	0.37 ± 0.28	0.15 ± 0.15	ND	0.16 ± 0.03	0.17 ± 0.03	0.055	0.385	**0.017**
20:2n-6	0.45 ± 0.24	0.35 ± 0.14	0.10 ± 0.21	ND	0.47 ± 0.15	0.27 ± 0.05	**< 0.001**	**0.022**	0.683
20:3n-6	0.34 ± 0.18	0.21 ± 0.17	0.28 ± 0.16	ND	0.38 ± 0.04	0.38 ± 0.05	**0.002**	0.009	0.113
20:4n-6	10.82 ± 1.84	7.84 ± 1.23	7.43 ± 1.39	7.80 ± 1.70	6.79 ± 0.37	6.65 ± 0.81	**< 0.001**	0.051	**0.010**
Total n-6 PUFA	26.20 ± 5.39	22.19 ± 1.89	37.63 ± 5.38	25.50 ± 4.36	28.05 ± 1.25	24.77 ± 2.33	**< 0.001**	**< 0.001**	**0.019**
18:3n-3	0.72 ± 0.39	0.99 ± 0.24	2.62 ± 0.31	1.88 ± 0.36	14.56 ± 1.78	12.42 ± 2.62	**< 0.001**	0.074	0.111
20:4n-3	ND	0.26 ± 0.63	ND	ND	0.27 ± 0.04	0.33 ± 0.04	**0.046**	0.263	0.495
20:5n-3	0.40 ± 0.21	0.22 ± 0.18	0.34 ± 0.03	ND	3.56 ± 0.63	4.05 ± 0.55	**< 0.001**	0.952	**0.030**
22:5n-3	0.52 ± 0.26	0.20 ± 0.16	0.16 ± 0.25	ND	1.10 ± 0.17	1.07 ± 0.32	**< 0.001**	**0.035**	0.295
22:6n-3	0.85 ± 0.46	0.66 ± 0.40	0.30 ± 0.18	0.38 ± 0.38	0.78 ± 0.10	1.08 ± 0.31	**0.001**	0.596	0.214
Total n-3 PUFA	2.49 ± 1.24	2.34 ± 0.21	3.42 ± 0.20	2.26 ± 0.45	20.27 ± 2.44	18.94 ± 2.37	**< 0.001**	0.107	0.608

ND negligible detected (mean <0.1%)

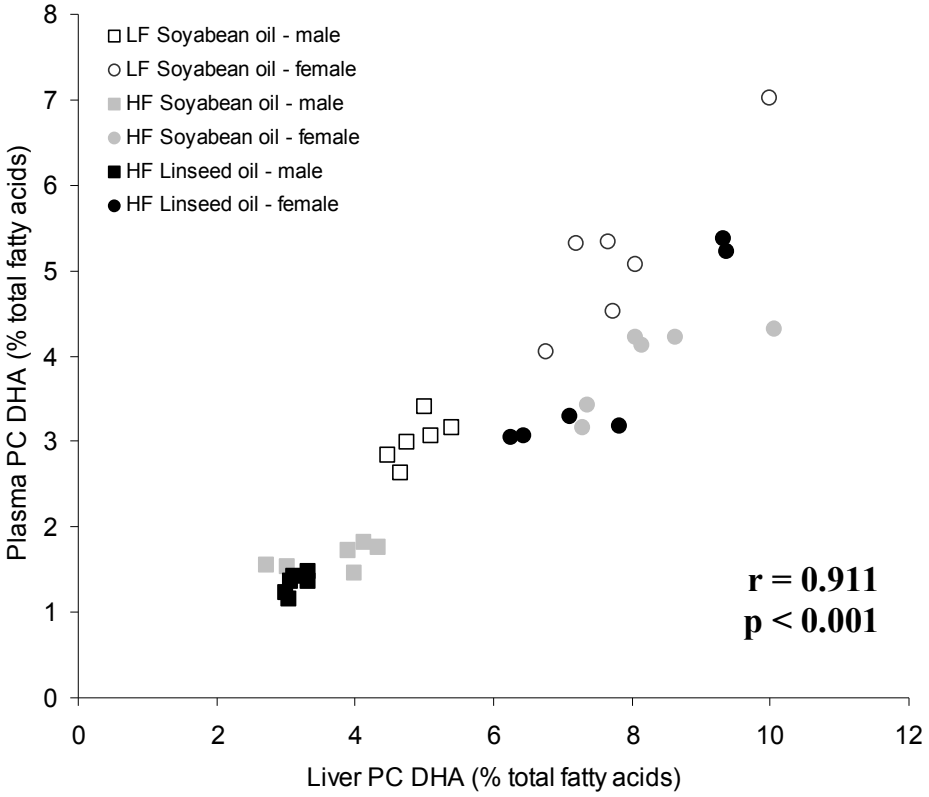
Table 3.13: Correlations observed between plasma and liver lipid fatty acid composition (Pearson r values unless otherwise indicated)

	Plasma PC vs. Liver PC (n = 36)		Plasma PC vs. Liver PE (n = 35)		Plasma TAG vs. Liver TAG (n = 36)		Plasma CE vs. Liver CE (n = 34)	
14:0	0.603	***	0.361†	*	0.862†	***	0.074	
16:0	0.948	***	0.778	***	0.980	***	0.093	
18:0	0.979	***	0.893	***	0.282†		-0.064	
20:0	0.125†		0.211†		-0.226†		-0.249†	
22:0	-0.074†		-0.071†		-0.053†		-	
Total saturated	0.937	***	0.924	***	0.931	***	0.055	
16:1n-7	0.827†	***	0.778†	***	0.822†	***	0.741	***
18:1n-9	0.863	***	0.280		0.631	***	0.486	**
20:1n-9	0.087		0.115		0.409	*	0.145†	
24:1n-9	-0.065†		-		-		-	
Total MUFA	0.862	***	0.331		0.795†	***	0.511	**
18:2n-6	0.911	***	0.859	***	0.729†	***	0.463	**
18:3n-6	0.347†	*	0.239†		0.124		0.535	**
20:2n-6	0.124		-0.032		0.406	*	0.140†	
20:3n-6	0.754	***	0.557	**	0.520	**	0.186	
20:4n-6	0.914	***	0.668	***	0.816	***	0.467†	**
22:5n-6	-0.188		-0.071 †		0.409†	*	-	
Total n-6 PUFA	0.905	***	0.773	***	0.749†	***	0.029	
18:3n-3	0.870†	***	0.868†	***	0.945†	***	0.896†	***
20:4n-3	0.529†	**	0.311†		0.404†	*	0.553†	**
20:5n-3	0.905	***	0.864†	***	0.739†	***	0.724†	***
22:5n-3	0.880	***	0.828	***	0.721	***	0.517†	**
22:6n-3	0.911	***	0.912	***	0.309		0.009	
Total n-3 PUFA	0.902	***	0.908	***	0.932†	***	0.514†	**

† Spearman's correlation r value

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3.6: Illustration of the correlation observed between liver and plasma PC DHA content



3.3.5 Adipose tissue fatty acid composition

The fatty acid composition of total lipid extracts of subcutaneous and intra-abdominal adipose tissue was assessed (see table 3.14 and 3.15). Diet had a significant effect upon the fatty acid composition of adipose tissue, indicating that an experimental dietary period of just 20 days was sufficient to significantly alter the adipose tissue pool of fatty acids in adult rats.

Increasing the ALNA content of the diet significantly increased the ALNA content of adipose tissue. The effect of increased dietary ALNA upon LC n-3 PUFA was complex, as had been observed in plasma and liver lipid analysis. Increasing dietary ALNA increased the EPA content of both subcutaneous and intra-abdominal adipose tissue. However, the effect of diet upon DPA and DHA content of adipose tissue was less clear, with some indication that the high fat diets may inhibit the deposition of these LC n-3 PUFA.

There was a clear trend for females to have significantly more LC PUFA in their adipose tissue than males. This was apparent for both n-6 and n-3 LC PUFA, but these fatty acids remained a relatively minor component of total adipose tissue fatty acids (e.g. DHA < 0.4%, AA < 1.4%).

It is possible that gender differences in plasma lipid composition are related to gender differences in the composition and mobilisation of fatty acids from adipose tissue. Plasma NEFA in the fasted state derive from the release of fatty acids from adipose tissue under the action of hormone-sensitive lipase. In accordance with this, statistically significant correlations were observed between adipose composition and plasma NEFA fatty acid composition (see table 3.16), though it should be noted that blood samples were collected from non-fasted animals.

From these correlations it is observed that while adipose tissue does demonstrate statistically significant correlations with plasma fatty acid composition, these are strongest among saturates, MUFA and EFA, and adipose tissue does not appear to demonstrate a strong relationship with plasma NEFA DHA content (see figure 3.7).

Table 3.14: Fatty acid composition (% total fatty acids, mean ± standard deviation, n = 6 for all groups) of total lipid extracts obtained from subcutaneous adipose tissue

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
Fatty acid	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
12:0	0.25 ± 0.08	0.29 ± 0.04	0.34 ± 0.15	0.15 ± 0.09	0.18 ± 0.07	0.40 ± 0.12	0.512	0.501	**< 0.001**
14:0	1.54 ± 0.15	1.44 ± 0.16	1.18 ± 0.20	0.95 ± 0.11	1.06 ± 0.18	1.30 ± 0.12	**< 0.001**	0.582	**0.002**
16:0	24.45 ± 1.22	22.33 ± 0.87	19.59 ± 1.06	18.56 ± 0.84	18.94 ± 1.26	18.68 ± 1.47	**< 0.001**	**0.006**	0.148
18:0	3.08 ± 0.22	3.37 ± 0.36	3.22 ± 0.22	3.79 ± 0.26	3.27 ± 0.29	3.40 ± 0.48	0.112	**0.004**	0.231
Total saturated	29.40 ± 1.26	27.45 ± 0.97	24.39 ± 0.97	23.50 ± 0.84	23.47 ± 1.47	23.84 ± 1.61	**< 0.001**	**0.052**	0.083
16:1n-7	5.17 ± 0.86	3.86 ± 0.55	3.01 ± 0.88	2.24 ± 0.32	2.47 ± 1.43	3.08 ± 1.03	**< 0.001**	0.177	**0.042**
18:1n-9	24.45 ± 0.89	25.96 ± 0.96	23.38 ± 0.73	26.04 ± 0.70	24.65 ± 1.52	26.52 ± 0.85	0.107	**< 0.001**	0.354
20:1n-9	0.35 ± 0.04	0.49 ± 0.05	0.26 ± 0.05	0.28 ± 0.02	0.19 ± 0.15	0.32 ± 0.05	**< 0.001**	**< 0.001**	0.100
Total MUFA	29.97 ± 1.44	30.31 ± 1.45	26.65 ± 1.36	28.56 ± 0.88	27.31 ± 1.70	29.92 ± 1.77	**0.001**	**0.002**	0.170
18:2n-6	36.55 ± 1.96	37.43 ± 1.84	43.60 ± 1.99	42.84 ± 1.25	35.48 ± 2.82	32.67 ± 2.03	**< 0.001**	0.195	0.101
18:3n-6	0.15 ± 0.04	0.23 ± 0.02	0.15 ± 0.06	0.18 ± 0.02	0.10 ± 0.05	0.16 ± 0.02	**0.001**	**< 0.001**	0.178
20:2n-6	0.31 ± 0.07	0.32 ± 0.02	ND	ND	0.12 ± 0.13	ND	**< 0.001**	0.426	0.698
20:3n-6	0.14 ± 0.03	0.24 ± 0.02	0.14 ± 0.02	0.24 ± 0.06	ND	0.18 ± 0.03	**0.001**	**< 0.001**	0.980
20:4n-6	0.86 ± 0.07	1.30 ± 0.14	0.87 ± 0.25	1.12 ± 0.13	0.56 ± 0.13	0.86 ± 0.10	**< 0.001**	**< 0.001**	0.259
22:5n-6	ND	0.17 ± 0.05	ND	0.10 ± 0.09	ND	ND	**0.016**	**< 0.001**	**0.006**
Total n-6 PUFA	38.00 ± 1.94	39.69 ± 1.98	44.85 ± 1.95	44.47 ± 1.44	36.33 ± 2.76	33.99 ± 2.13	**< 0.001**	0.627	0.074
18:3n-3	2.50 ± 0.18	1.92 ± 0.17	3.75 ± 0.39	2.93 ± 0.23	12.47 ± 3.16	11.22 ± 1.06	**< 0.001**	0.065	0.831
20:5n-3	ND	ND	ND	ND	0.14 ± 0.10	0.29 ± 0.06	**< 0.001**	**0.041**	**< 0.001**
22:5n-3	ND	0.24 ± 0.03	0.16 ± 0.04	0.24 ± 0.04	0.18 ± 0.12	0.39 ± 0.06	**< 0.001**	**< 0.001**	**0.045**
22:6n-3	ND	0.38 ± 0.05	0.15 ± 0.05	0.27 ± 0.09	ND	0.33 ± 0.05	0.785	**< 0.001**	**0.027**
Total n-3 PUFA	2.63 ± 0.31	2.54 ± 0.18	4.12 ± 0.43	3.47 ± 0.31	12.89 ± 3.43	12.25 ± 1.16	**< 0.001**	0.367	0.871

ND negligible detected (mean <0.1%)

Table 3.15: Fatty acid composition (% total fatty acids, mean ± standard deviation, n = 6 for all groups) of total lipid extracts obtained from intra-abdominal adipose tissue

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
Fatty acid	Male	Female	Male	Female	Male	Female	Diet	Gender	Diet*Gender
12:0	0.10 ± 0.05	0.12 ± 0.05	0.13 ± 0.05	ND	ND	0.11 ± 0.03	0.358	0.634	**0.006**
14:0	1.57 ± 0.19	1.18 ± 0.16	1.14 ± 0.09	0.75 ± 0.08	1.03 ± 0.13	0.93 ± 0.05	**< 0.001**	**< 0.001**	**0.011**
16:0	27.23 ± 1.90	23.71 ± 1.32	21.52 ± 0.88	19.06 ± 0.44	20.28 ± 1.18	19.72 ± 1.19	**< 0.001**	**< 0.001**	**0.020**
18:0	2.95 ± 0.17	3.41 ± 0.19	3.18 ± 0.23	3.54 ± 0.19	3.08 ± 0.21	3.41 ± 0.23	0.111	**< 0.001**	0.721
20:0	ND	ND	ND	ND	ND	0.10 ± 0.03	0.598	0.573	0.236
Total saturated	31.91 ± 1.93	28.49 ± 1.49	26.05 ± 1.12	23.49 ± 0.52	24.50 ± 1.23	24.28 ± 1.29	**< 0.001**	**< 0.001**	**0.018**
16:1n-7	6.26 ± 1.12	3.87 ± 0.92	3.58 ± 0.57	2.27 ± 0.32	3.79 ± 1.06	2.95 ± 0.52	**< 0.001**	**< 0.001**	0.072
18:1n-9	23.47 ± 0.63	25.08 ± 0.26	23.00 ± 0.77	24.86 ± 0.90	24.52 ± 0.95	25.63 ± 1.17	**0.006**	**< 0.001**	0.528
20:1n-9	0.33 ± 0.04	0.57 ± 0.09	0.22 ± 0.04	0.36 ± 0.07	0.25 ± 0.07	0.32 ± 0.05	**< 0.001**	**< 0.001**	**0.005**
Total MUFA	30.05 ± 0.85	29.54 ± 1.14	26.81 ± 1.33	27.49 ± 1.11	28.56 ± 1.71	28.90 ± 1.56	**< 0.001**	0.703	0.525
18:2n-6	33.97 ± 2.77	37.45 ± 2.09	41.83 ± 1.93	43.66 ± 0.87	33.47 ± 2.75	32.83 ± 2.15	**< 0.001**	**0.041**	0.083
18:3n-6	0.12 ± 0.06	0.22 ± 0.02	0.13 ± 0.01	0.18 ± 0.02	ND	0.17 ± 0.03	**0.028**	**< 0.001**	0.299
20:2n-6	0.27 ± 0.04	0.29 ± 0.03	0.26 ± 0.04	0.28 ± 0.04	0.19 ± 0.10	0.21 ± 0.03	**0.001**	0.188	0.961
20:3n-6	0.14 ± 0.08	0.22 ± 0.04	0.13 ± 0.02	0.22 ± 0.03	ND	0.17 ± 0.03	**0.035**	**< 0.001**	0.919
20:4n-6	0.77 ± 0.22	0.97 ± 0.10	0.60 ± 0.13	0.85 ± 0.14	0.41 ± 0.13	0.65 ± 0.18	**< 0.001**	**< 0.001**	0.907
22:5n-6	ND	0.10 ± 0.09	ND	ND	ND	ND	0.373	**< 0.001**	0.362
Total n-6 PUFA	35.26 ± 2.65	39.26 ± 2.24	42.95 ± 1.96	45.27 ± 0.94	34.24 ± 2.74	34.08 ± 2.32	**< 0.001**	**0.009**	**0.086**
18:3n-3	2.46 ± 0.13	2.20 ± 0.19	3.86 ± 0.25	3.27 ± 0.27	12.30 ± 1.76	11.83 ± 1.28	**< 0.001**	0.155	0.897
20:5n-3	ND	ND	ND	ND	0.13 ± 0.12	0.26 ± 0.04	**< 0.001**	**0.004**	**0.021**
22:5n-3	0.13 ± 0.08	0.17 ± 0.10	0.16 ± 0.07	0.17 ± 0.06	0.17 ± 0.16	0.36 ± 0.06	**0.012**	**0.017**	0.067
22:6n-3	0.18 ± 0.13	0.29 ± 0.06	0.13 ± 0.05	0.25 ± 0.03	ND	0.24 ± 0.04	**0.044**	**< 0.001**	0.808
Total n-3 PUFA	2.77 ± 0.23	2.71 ± 0.21	4.20 ± 0.33	3.74 ± 0.29	12.70 ± 2.11	12.74 ± 1.31	**< 0.001**	0.649	0.830

ND negligible detected (mean <0.1%)

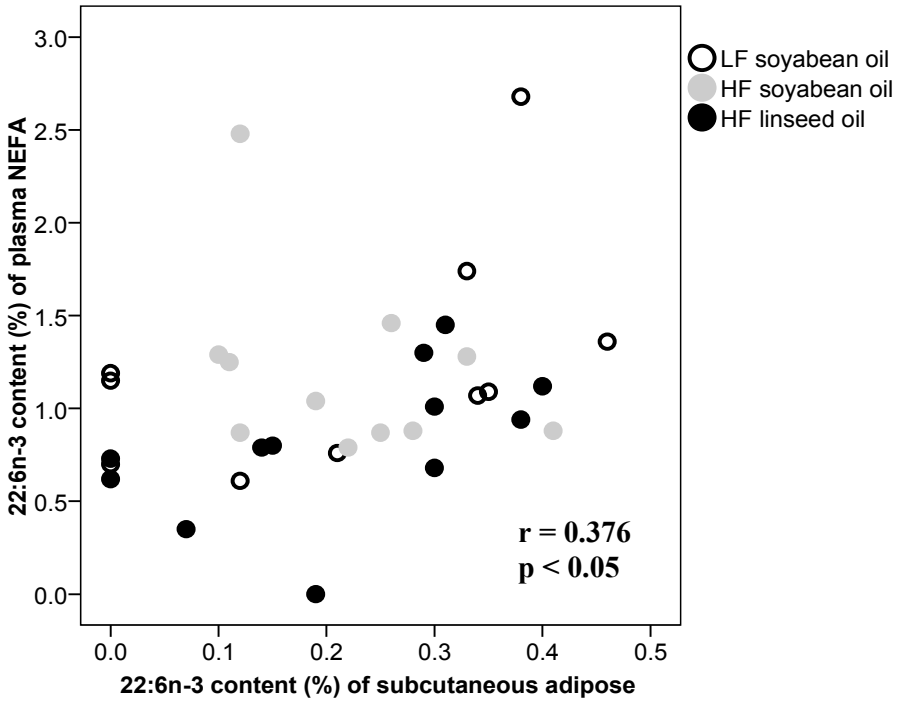
Table 3.16: Correlations observed between adipose tissue fatty acid composition and that of plasma NEFA (Pearson r values unless otherwise indicated)

	Subcutaneous adipose vs. Plasma NEFA (n=33)		Intra-abdominal adipose vs. Plasma NEFA (n=33)	
12:0	-0.058†		0.125†	
14:0	0.090		0.002	
16:0	0.703	***	0.711	***
18:0	-0.052		0.151	
20:0	-0.136		-0.014†	
22:0	-0.360†	*	0.084†	
Total saturated	0.458	**	0.437	*
16:1n-7	0.731	***	0.627	***
18:1n-9	0.169		0.313	
20:1n-9	-0.210		-0.219	
24:1n-9	0.082†		-	
Total MUFA	0.477	**	0.538	**
18:2n-6	0.492	**	0.456	**
18:3n-6	0.375†	*	0.308†	
20:2n-6	0.319†		-0.428†	*
20:3n-6	0.179†		0.128†	
20:4n-6	0.454	**	0.502	**
22:5n-6	0.707†	***	0.417†	*
Total n-6 PUFA	0.568	**	0.544	**
18:3n-3	0.884†	***	0.863†	***
20:4n-3	-0.151†		-0.115†	
20:5n-3	0.540†	**	0.654†	***
22:5n-3	0.559	**	0.497	**
22:6n-3	0.376	*	0.294	
Total n-3 PUFA	0.874†	***	0.862†	***

† Spearman's correlation r value

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3.7: Illustration of the correlation observed between DHA (22:6n-3) content (% total fatty acids) of subcutaneous adipose tissue total lipid extracts and plasma NEFA



3.3.6 Plasma sex hormones

A significant effect of gender was observed upon plasma testosterone and progesterone concentrations (see table 3.17). The lack of a gender effect upon circulating oestradiol is likely due to females not being controlled for their stage of the oestrus cycle, resulting in a high degree of variation.

Animals on the HF diets had significantly higher testosterone and significantly lower oestradiol than those on the LF soyabean oil diet. This effect of high fat diets upon testosterone has been observed in other studies using rat models(230) and has been attributed to high fat diets reducing levels of sex hormone binding globulin, increasing free testosterone(231). However, the assay used to determine testosterone concentrations in this thesis is designed to measure testosterone bound to sex hormone binding globulin (Beckman Coulter, High Wycombe).

Human studies which identified gender differences in fatty acid composition have all identified higher plasma lipid DHA content in females compared to males(132-135).

One of the proposed mechanisms for this gender difference is the involvement of sex hormones, but human studies to date have not determined whether plasma DHA content correlates with circulating sex hormones.

When rat plasma DHA levels were compared with circulating sex hormone concentrations, strong inverse relationships with testosterone, and strong positive relationships with progesterone were seen (see table 3.18 and figures 3.8 and 3.9). When plotted, it was apparent that the correlations achieved may be an artefact of the gender differences in sex hormone concentrations; r values were therefore calculated for each gender (see table 3.19).

Calculation of correlations for each gender identified a significant relationship between oestradiol and DHA status within males. However, when plotted, it is clear that the effect of HF diets upon oestradiol status was significantly involved in this relationship (see figure 3.10). The confounding effects of diet and gender upon sex hormone status therefore make it unlikely that a simple correlation between sex hormone status and DHA content of plasma lipids can be confidently identified.

Table 3.17: Plasma sex hormone concentrations (mean ± standard deviation)

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Males (n=5)	Females (n=6)	Males (n=6)	Females (n=6)	Males (n=5)	Females (n=5)	Diet	Gender	Diet*Gender
Oestradiol (pM)	156.2 ± 21.6	141.5 ± 129.4	42.6 ± 26.0	54.8 ± 47.6	30.0 ± 18.0 †	52.9 ± 59.5	**0.001**	0.777	0.807
Progesterone (nM)	1.7 ± 0.8 †	22.5 ± 25.8	7.7 ± 3.8	54.5 ± 26.4	4.6 ± 1.6	35.9 ± 24.3	0.221	**< 0.001**	0.549
Testosterone (nM)	8.9 ± 4.6	1.8 ± 0.5	17.9 ± 12.7	5.9 ± 0.6	19.6 ± 6.3	4.7 ± 0.6	**0.027**	**< 0.001**	0.359

† (n = 4)

Diet - Bonferroni post-hoc tests for multiple comparisons:

Oestradiol – LF Soyabean oil significantly different from HF Soyabean oil ($p = 0.004$) and HF Linseed oil ($p = 0.005$)
Testosterone – LF Soyabean oil significantly different from HF Soyabean oil ($p = 0.042$) and HF Linseed oil ($p = 0.044$)

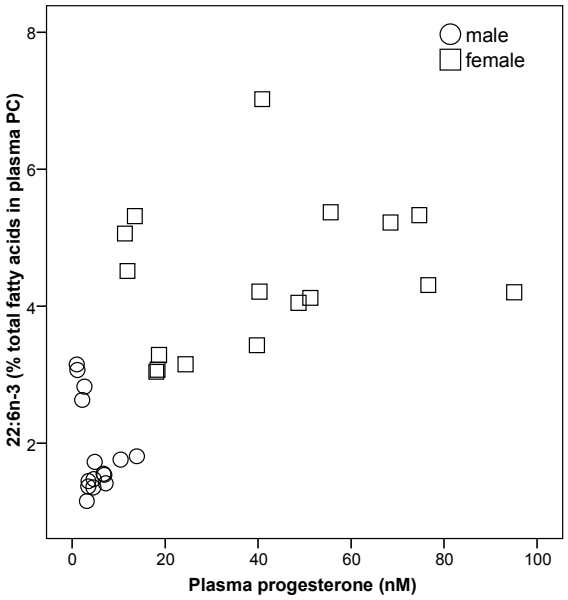
Table 3.18: Correlations between the DHA content (% total fatty acids) and circulating sex hormone concentrations (Pearson r values unless otherwise indicated)

	Testosterone	Oestradiol	Progesterone
PC	**-0.669 ****	0.322	**0.720**** †
	(n = 33)	(n = 32)	(n = 32)
TAG	-0.240	-0.119	0.246 †
	(n = 33)	(n = 32)	(n = 32)
CE	**-0.553****	0.048	**0.608**** †
	(n = 33)	(n = 31)	(n = 31)
NEFA	**-0.474****	0.195	**0.529****
	(n = 30)	(n = 29)	(n = 30)

† Spearman r value

** $p < 0.01$

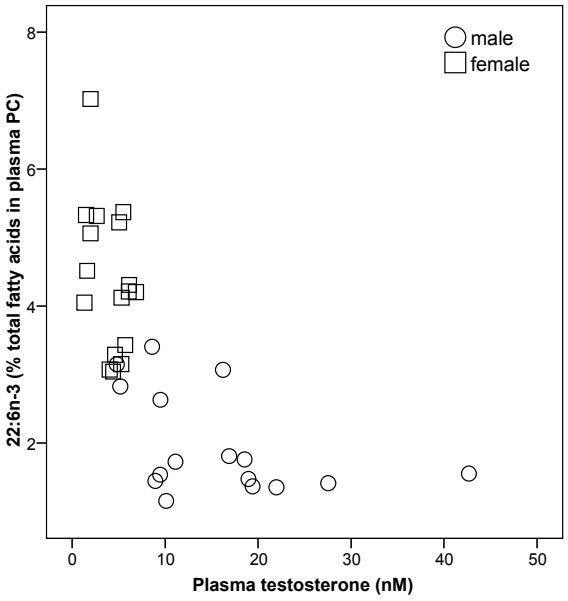
Figure 3.8: Illustration of the correlation observed between plasma progesterone and the DHA content (22:6n-3, % total fatty acids) of plasma PC



All data: r = 0.720, p < 0.01

Males: r = -0.432, p = 0.108
Females: r = 0.245, p = 0.344

Figure 3.9: Illustration of the correlation observed between plasma testosterone and DHA content (22:6n-3, % total fatty acids) of plasma PC



All data: r = -0.669, p < 0.01

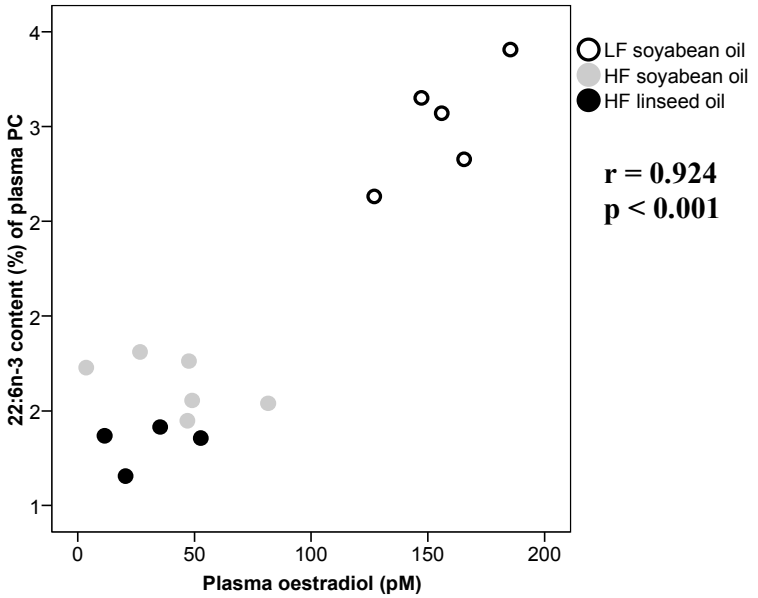
Males: r = -0.457, p = 0.075
Females: r = -0.414, p = 0.099

Table 3.19: Correlations between the DHA content (% total fatty acids) of plasma lipids and circulating sex hormone concentrations for each gender (Pearson r values)

	MALES			**FEMALES**		
	Testosterone	Oestradiol	Progesterone	Testosterone	Oestradiol	Progesterone
PC	-0.457	**0.924*** **	-0.432	-0.414	0.272	0.245
	(n = 16)	(n = 15)	(n = 15)	(n = 17)	(n = 17)	(n = 17)
TAG	-0.416	**0.610 ***	-0.198	0.290	**-0.495***	0.342
	(n = 16)	(n = 15)	(n = 15)	(n = 17)	(n = 17)	(n = 17)
CE	-0.231	0.268	-0.212	0.014	-0.107	0.257
	(n = 16)	(n = 15)	(n = 15)	(n = 16)	(n = 16)	(n = 16)
NEFA	-0.364	0.200	0.178	-0.119	0.167	0.328
	(n = 14)	(n = 13)	(n = 14)	(n = 16)	(n = 16)	(n = 16)

* $p < 0.05$, *** $p < 0.001$

Figure 3.10: Illustration of the correlation observed between plasma oestradiol and plasma PC DHA status (% total fatty acids) in males



3.3.7 Expression of desaturase and elongase enzymes in the liver

Liver samples were assessed for mRNA expression of Δ5, Δ6 and elongase (see table 3.20). No significant effect of gender or diet was identified upon mRNA expression of elongase and Δ5 desaturase. Δ6 desaturase exhibited a significant effect of diet, with those rats fed on the HF linseed oil diet having significantly higher expression of Δ6 desaturase than those on the HF soyabean oil diet.

Liver mRNA expression of desaturases and elongase was correlated to plasma sex hormone concentrations to assess if there was any significant interaction (see table 3.21). Although mRNA expression data did not demonstrate significant gender differences, there are indications that the expression of these genes is influenced by sex hormones, as oestradiol demonstrated an inverse relationship with Δ5 desaturase expression (see figure 3.11).

One way of inferring enzyme activity of desaturase and elongase enzymes within the liver is to calculate the substrate:product ratio of fatty acids found within phospholipids. A high substrate:product ratio would indicate low enzyme activity, and a low substrate:product ratio would indicate high enzyme activity. A positive correlation of a

given variable with the substrate:product ratio would indicate that this variable may be associated with inhibition of enzyme activity, and an inverse relationship would indicate increased enzyme activity. Therefore, correlations between mRNA expression and the substrate:product ratios associated with each enzyme were calculated to investigate whether differences the mRNA expression of these enzymes bear a relationship to tissue fatty acid composition (see table 3.22).

No consistent relationships were identified which would support a relationship between mRNA expression of these enzymes and the fatty acids they act upon within liver phospholipids. This suggests that if there is an effect of sex hormones upon the synthesis of LC PUFA from their EFA precursors, the effect involves mechanisms other than changes in mRNA expression of the genes encoding these enzymes.

Table 3.20: Liver mRNA expression of desaturase and elongase genes in response to diet and gender (arbitrary units of quantitative expression derived from standard curve and adjusted for geometric mean of three housekeeping genes, mean ± standard deviation)

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Male (n=6)	Female (n=6)	Male (n=6)	Female (n=6)	Male (n=6)	Female (n=5)	Gender	Diet	Gender*Diet
Elovl5 (elongase)	0.91 ± 0.18	0.83 ± 0.11	0.72 ± 0.15	0.82 ± 0.11	0.72 ± 0.20	0.80 ± 0.12	0.494	0.159	0.305
FADS1 (Δ5 desaturase)	0.69 ± 0.08	0.61 ± 0.11	0.70 ± 0.09	0.68 ± 0.21	0.72 ± 0.17	0.77 ± 0.15	0.753	0.251	0.579
FADS2 (Δ6 desaturase)	0.64 ± 0.23	0.58 ± 0.17	0.63 ± 0.13	0.56 ± 0.13	0.76 ± 0.20	0.84 ± 0.24	0.793	**0.025**	0.565

Diet - Bonferroni post-hoc tests for multiple comparisons:

FADS2 (Δ6 desaturase) – HF Linseed oil significantly different from HF Soyabean oil (p = 0.046)

Table 3.21: Correlations observed between liver mRNA expression of desaturase and elongase genes and circulating plasma sex hormone concentrations (Pearson R values)

	Testosterone (nM)	Oestradiol (pM)	Progesterone (nM)
Elovl5 (elongase)	-0.265 (n = 32)	0.100 (n = 31)	0.253 (n = 31)
FADS1 (Δ5 desaturase)	0.215 (n = 32)	**-0.387 *** (n = 31)	0.180 (n = 31)
FADS2 (Δ6 desaturase)	0.212 (n = 32)	-0.198 (n = 31)	0.035 (n = 31)

* $p < 0.05$

Figure 3.11: Illustration of the correlation between plasma oestradiol and liver Δ5 desaturase mRNA expression

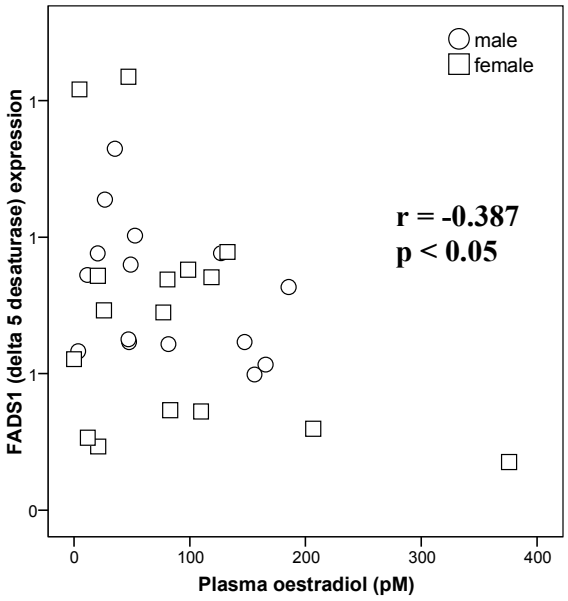


Table 3.22: Correlations observed between liver mRNA expression and their respective substrate and product fatty acid ratios within liver lipids (Pearson r values)

	Liver PC	Liver PE	Liver CE	Liver TAG
Δ6 DESATURASE				
18:2n-6 to 18:3n-6 ratio	0.112 (n = 35)	0.226 (n = 31)	0.122 (n=24)	-0.003 (n = 33)
Δ5 DESATURASE				
20:3n-6 to 20:4n-6 ratio	0.093 (n = 35)	0.099 (n = 34)	-0.182 (n=25)	0.040 (n = 33)
20:4n-3 to 20:5n-3 ratio	0.100 (n = 35)	**0.388*** (n = 34)	0.279 (n=25)	0.331 (n = 33)
ELONGASE				
18:3n-6 to 20:3n-6 ratio	0.113 (n = 35)	0.196 (n = 34)	0.137 (n=25)	0.163 (n = 33)
20:5n-3 to 22:5n-3 ratio	-0.122 (n = 35)	-0.148 (n = 34)	**-0.470*** (n=22)	-0.303 (n = 33)

* $p < 0.05$

3.3.8 Indirect assessment of desaturase and elongase activities in the liver

Although desaturase and elongase mRNA expression was not found to relate to gender or plasma sex hormones, it is possible that the activity of these enzymes may be affected by gender or sex hormones. Substrate:product ratios within liver lipids were therefore calculated to assess the effect of gender or sex hormones upon LC PUFA synthesis.

Substrate:product ratios will be significantly affected by diet, as the fatty acids used to calculate these ratios are provided in different proportions in the three dietary groups, but effects of gender upon substrate:product ratios may indicate that the gender differences in LC PUFA status are mediated by an effect upon enzyme activity.

Δ9 desaturase

Substrate:product ratios for Δ9 desaturase activity were found to be significantly affected by gender in all liver lipid fractions assessed, with females having significantly higher ratios (see table 3.23). This indicates that males have higher Δ9 desaturase activity compared to females.

Testosterone administration has been demonstrated to increase liver Δ9 desaturase activity in rat models(152). Whether this effect can be observed within the physiological range of testosterone had not been determined. The significant inverse relationships between testosterone and the substrate:product ratio observed here support a potential role of testosterone in promoting Δ9 desaturase activity (see table 3.24 and figure 3.12). There was little indication of a relationship between plasma oestradiol and liver Δ9 desaturase activity, while progesterone demonstrated a significant positive correlation with the substrate:product ratio in liver lipids, indicating that circulating progesterone is associated with a reduction in this marker of Δ9 desaturase activity (see figure 3.13).

Table 3.23: The Δ9 desaturase substrate:product ratio within liver lipids (% total fatty acid ratio of 18:0 to 18:1n-9, mean ± standard deviation, n = 6 for all groups unless otherwise indicated)

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Males	Females	Males	Females	Males	Females	Diet	Gender	Diet*Gender
Liver PC	4.7 ± 0.5	8.8 ± 1.5	7.5 ± 1.0	13.9 ± 3.1	4.7 ± 0.4	10.4 ± 0.8	**< 0.001**	**< 0.001**	0.194
Liver PE	7.0 ± 1.1 (n=5)	12.6 ± 2.3	5.2 ± 0.9	9.6 ± 2.0	4.6 ± 0.5	9.8 ± 1.1	**< 0.001**	**< 0.001**	0.566
Liver CE	2.7 ± 0.7	3.0 ± 1.1	0.5 ± 0.2 (n=5)	1.3 ± 0.5 (n=5)	1.1 ± 0.4	2.3 ± 0.7	**< 0.001**	**0.002**	0.279
Liver TAG (x 10^2)	12.6 ± 2.3	14.8 ± 5.5	11.0 ± 0.6 (n=5)	17.1 ± 2.5 (n=5)	10.9 ± 1.7	15.0 ± 2.8	0.685	**< 0.001**	0.322

Diet - Bonferroni post-hoc tests for multiple comparisons:

Liver PC: HF soyabean oil significantly different from LF soyabean oil ($p < 0.001$) and HF linseed oil ($p < 0.001$)
Liver PE: LF soyabean oil significantly different from HF soyabean oil ($p < 0.001$) and HF linseed oil ($p < 0.001$)
Liver CE: All groups significantly different ($p < 0.05$)

Table 3.24: Correlations observed between the ratio of 18:0 to 18:1n-9 content (% total fatty acids) of liver lipids and circulating sex hormones concentrations (Pearson r values unless otherwise indicated)

18:0 to 18:1n-9 ratio	Liver PC	Liver PE	Liver CE	Liver TAG
Testosterone	**-0.401***	**-0.675*****	**-0.495****	**-0.363***
	(n=33)	(n=32)	(n=31)	(n=31)
Oestradiol	-0.181	0.204	0.202	-0.356
	(n=32)	(n=31)	(n=30)	(n=30)
Progesterone	**0.870*****	**0.673****	0.209 †	**0.556****†
	(n=32)†	(n=31)	(n=30)	(n=30)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † Spearman R values

Figure 3.12: Illustration of the correlation observed between plasma testosterone and the ratio of 18:0 to 18:1n-9 within liver PE

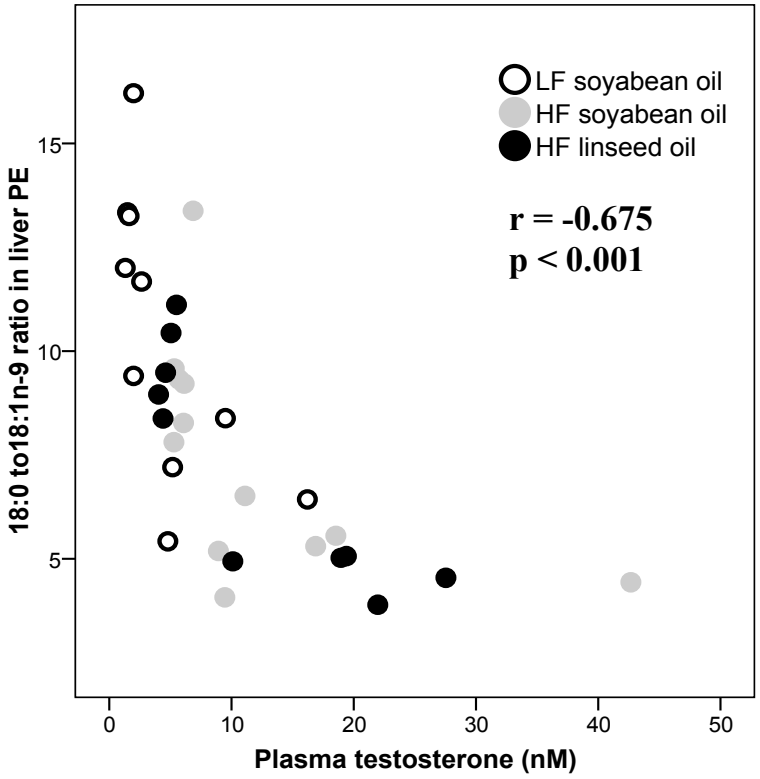
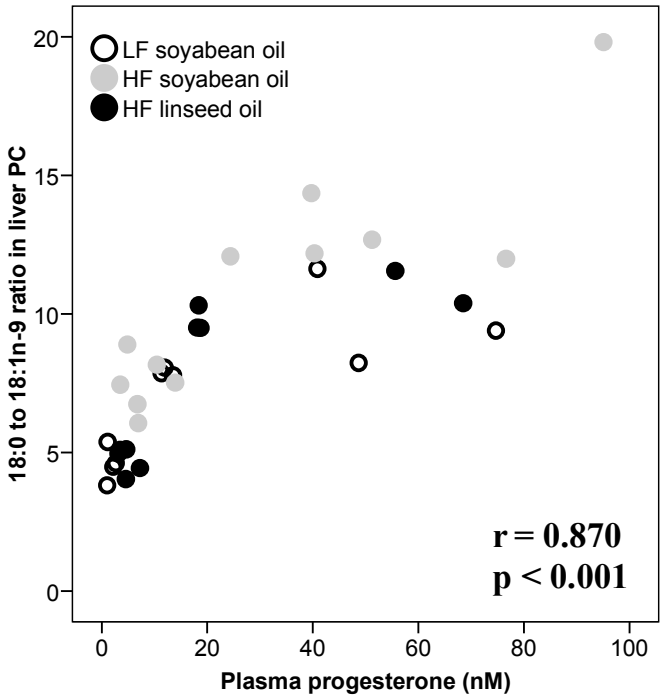


Figure 3.13: Illustration of the correlation observed between plasma progesterone and the ratio of 18:0 to 18:1n-9 within liver PC



Δ6 desaturase

Δ6 desaturase activity can be inferred from by the conversion of the n-6 fatty acid 18:2n-6 to 18:3n-6. Δ6 desaturase also converts 18:3n-3 to 18:4n-3. Here the n-6 fatty acids were used, as 18:4n-3 could not be identified by the gas chromatography method used. Significant gender differences in Δ6 desaturase substrate:product ratios were observed in all liver lipid fractions assessed, with females having significantly lower ratios across all dietary groups (see table 3.25). This suggests that Δ6 activity is significantly greater in females than in males.

Data were assessed for correlations between plasma sex hormone concentrations and fatty acid ratio markers of Δ6 desaturase activity. The significant positive relationship between the 18:2n-6 to 18:3n-6 ratio in liver lipids with plasma testosterone suggests that Δ6 desaturase activity may be inhibited by testosterone (see table 3.26, figure 3.14). Likewise the observed negative correlations with oestradiol concentrations suggest that oestradiol is associated with an increased activity of Δ6 desaturase (see figure 3.15). There was also a non-significant trend for progesterone to relate to this marker of Δ6 desaturase activity (see figure 3.16).

Table 3.25: The Δ6 desaturase substrate:product ratio of fatty acids within liver lipids (18:2n-6 to 18:3n-6, mean ± standard deviation, n = 6 per group unless otherwise indicated)

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Males	Females	Males	Females	Males	Females	Diet	Gender	Diet*Gender
Liver PC	73.7 ± 17.9	36.7 ± 8.6	105.5 ± 5.7	82.5 ± 22.7	158.4 ± 24.9	103.8 ± 19.1	**< 0.001**	**< 0.001**	0.114
Liver PE	65.2 ± 16.0 (n=5)	33.4 ± 10.2	88.1 ± 24.1	83.9 ± 2.8 (n=3)	118.3 ± 8.6	73.0 ± 14.5	**< 0.001**	**< 0.001**	**0.020**
Liver CE	83.4 ± 14.4 (n=4)	38.9 ± 22.2 (n=5)	128.4 ± 17.5 (n=3)	107.4 (n=1)	130.9 ± 26.6	106.3 ± 20.4	**< 0.001**	**0.010**	0.534
Liver TAG	73.0 ± 17.5	44.6 ± 7.1	68.9 ± 7.5 (n=5)	68.6 ± 9.6 (n=5)	104.0 ± 29.9	83.4 ± 19.1	**< 0.001**	**0.011**	0.182

Diet - Bonferroni post-hoc tests for multiple comparisons:
Liver PC – All groups significantly different from each other ($p < 0.001$)
Liver PE, CE – LF soyabean oil significantly different from HF soyabean oil ($p < 0.001$) and HF linseed oil ($p < 0.001$)
Liver TAG – HF linseed oil significantly different from LF soyabean oil ($p < 0.001$) and HF soyabean oil ($p = 0.008$)

Table 3.26: Correlations observed between plasma testosterone concentrations and the ratio of 18:2n-6 to 18:3n-6 within liver lipids (Pearson r values)

18:2n-6 to 18:3n-6 ratio	Liver PC	Liver PE	Liver CE	Liver TAG
Testosterone	**0.545**** (n=33)	**0.660***** (n=29)	**0.534*** (n=22)	0.311 (n=31)
Oestradiol	**-0.478**** (n=32)	**-0.472*** (n=28)	**-0.535*** (n=21)	-0.341 (n=30)
Progesterone	-0.324 (n=32)	-0.338 (n=28)	-0.340 (n=21)	-0.240 (n=30)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3.14: Illustration of the correlation observed between plasma testosterone and the ratio of 18:2n-6 to 18:3n-6 within liver PE

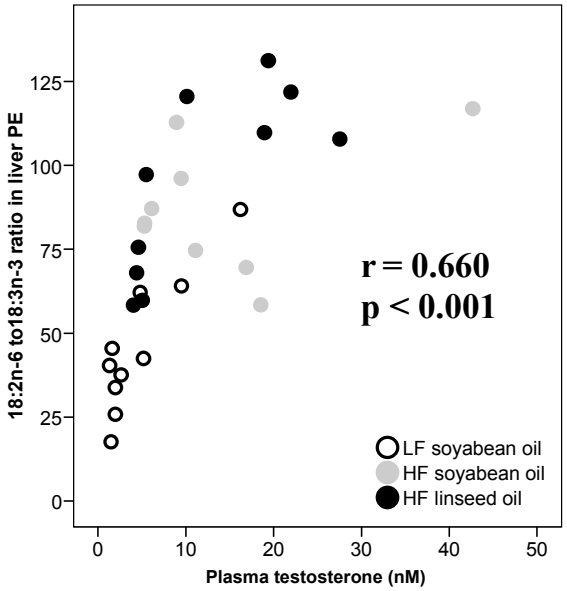


Figure 3.15: Illustration of the correlation observed between plasma oestradiol and the ratio of 18:2n-6 to 18:3n-6 within liver PE

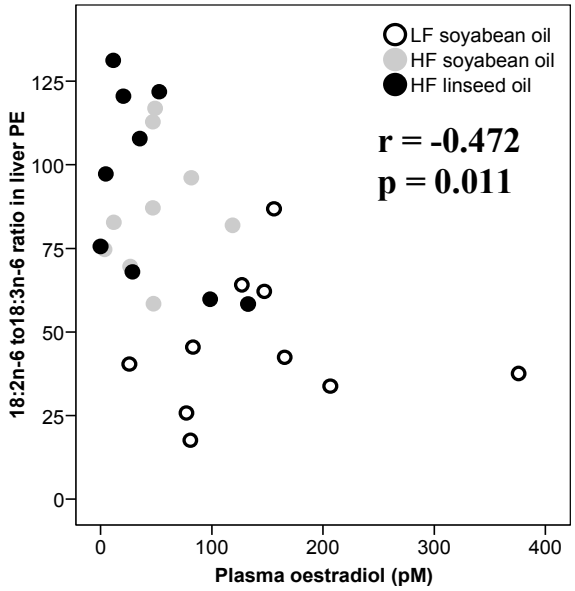
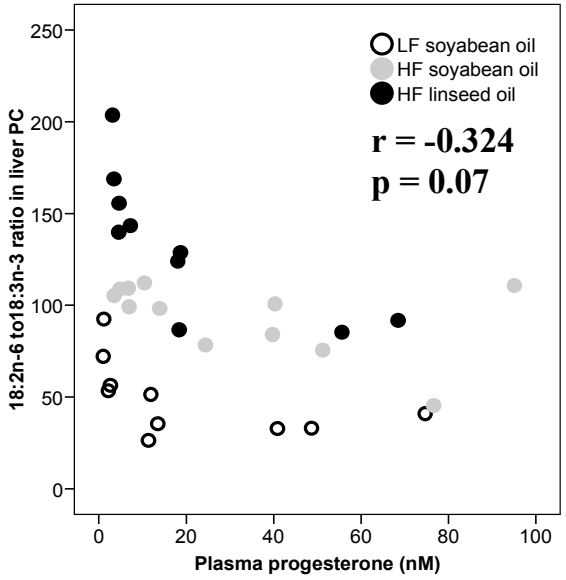


Figure 3.16: Illustration of the correlation observed between plasma progesterone and ratio of 18:2n-6 to 18:3n-6 within liver PC



Δ5 desaturase activity

Δ5 desaturase activity can be assessed by the conversion of 20:3n-6 to AA, or of 20:4n-3 to EPA. There was no indication of an effect of gender upon Δ5 desaturase activity (see table 3.27). Data were assessed for correlations of this marker of Δ5 desaturase activity with circulating sex hormones (see table 3.28). While these correlations supports a relationship between oestradiol and enhanced Δ5 desaturase activity upon n-3 fatty acids, it is clear when plotted (see figure 3.17) that this relationship is a statistical artefact of diet, with 20:4n-3 only detectable in the ALNA-rich HF linseed oil diet.

Elongase

Elongase activity can be assessed by the conversion of 18:3n-6 to di-homo-γ-linolenic acid (DGLA, 20:3n-6), or EPA to DPA. There was some indication that when data for liver PC was used, females have reduced elongase activity compared to males (see table 3.29). No clear pattern of relationships of sex hormones with either n-6 or n-3 fatty acids in liver lipids were observed which would indicate an interaction with elongase activity (see table 3.30). There was some limited indication that oestradiol and progesterone may correlate to increased elongase activity, but this was not observed in the PC fraction, where the significant gender differences in substrate:product ratios were observed.

Table 3.27: The Δ5 desaturase substrate:product ratio of fatty acids within liver lipids (n-6 20:3n-6 to AA; n-3: 20:4n-3 to EPA, mean ± standard deviation, n = 6 per group unless otherwise indicated)

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Males	Females	Males	Females	Males	Females	Diet	Gender	Diet*Gender
n-6 fatty acids									
Liver PC (x 10^3)	3.87 ± 1.30	4.38 ± 0.70	2.36 ± 0.32	2.43 ± 0.24	6.42 ± 0.48	6.58 ± 1.15	**< 0.001**	0.363	0.776
Liver PE (x 10^3)	21.7 ± 3.3 (n=5)	23.8 ± 2.4	20.0 ± 0.9	18.7 ± 2.2	35.6 ± 3.7	36.0 ± 5.0	**< 0.001**	0.730	0.450
Liver CE (x 10^3)	30.7 ± 17.3	29.3 ± 25.5	40.2 ± 27.9 (n=5)	5.8 ± 13.0 (n=5)	56.0 ± 5.1	57.9 ± 11.4	**< 0.001**	0.082	0.056
Liver TAG (x 10^2)	10.2 ± 1.1	14.1 ± 3.3	24.0 ± 2.4 (n=5)	21.4 ± 5.0 (n=5)	30.6 ± 7.6	26.3 ± 5.2	**< 0.001**	0.547	0.093
n-3 fatty acids									
Liver PC (x 10^1)	1.89 ± 3.93	-	7.70 ± 3.63	7.34 ± 1.00	0.46 ± 0.07	0.42 ± 0.08	**< 0.001**	0.624	0.872
Liver PE (x 10^1)	5.67 ± 1.26 (n=5)	-	-	-	0.33 ± 0.03	0.32 ± 0.05	0.309	0.245	0.273
Liver CE (x 10^2)	ND (n=5)	ND (n=4)	ND (n=5)	ND (n=5)	7.59 ± 0.77	8.17 ± 1.39	**< 0.001**	0.404	0.404
Liver TAG (x 10^1)	11.1 ± 27.2	ND	1.33 ± 0.28 (n=5)	2.00 ± 0.29 (n=5)	1.51 ± 0.18	2.06 ± 0.47	0.656	0.413	0.378

Diet - Bonferroni post-hoc tests for multiple comparisons:

n-6 liver PC, PE, TAG: All groups significantly different from each other ($p < 0.001$)
n-6 liver CE: HF linseed oil significantly different from LF soyabean oil ($p = 0.003$) and HF linseed oil ($p < 0.001$)

n-3 liver PC: HF soyabean oil significantly different from LF soyabean oil ($p = 0.005$) and HF linseed oil ($p = 0.002$)
n-3 liver CE: HF linseed oil significantly different from LF soyabean oil ($p < 0.001$) and HF soyabean oil ($p < 0.001$)

Table 3.28: Correlations observed between plasma sex hormone concentrations and the ratio of Δ5 desaturase substrate and product fatty acids within liver lipids (Pearson r values unless otherwise indicated)

20:3n-6 to AA ratio	PC	PE	CE	TAG
Testosterone	0.018	0.131	0.280	**0.430***
	(n=33)	(n=32)	(n=31)	**(n=31)**
Oestradiol	0.067	-0.030	-0.046	-0.347
	(n=32)	(n=31)	(n=30)	(n=30)
Progesterone	-0.090 †	-0.248	-0.188 †	0.070 †
	(n=32)	(n=31)	(n=30)	(n=30)
20:4n-3 to EPA ratio	PC	PE	CE	TAG
Testosterone †	**0.469****	0.259	0.149	0.273
	(n=33)	(n=32)	(n=23)	(n=31)
Oestradiol †	**-0.433***	-0.220	**-0.544****	**-0.398***
	(n=32)	(n=31)	(n=22)	(n=30)
Progesterone †	-0.184	-0.261	0.136	0.280
	(n=32)	(n=31)	(n=22)	(n=30)

† Spearman's r value * $p < 0.05$, ** $p < 0.01$

Figure 3.17: Illustration of the correlation observed between plasma oestradiol and the ratio of 20:4n-3 to 20:5n-3 within liver cholesterol esters

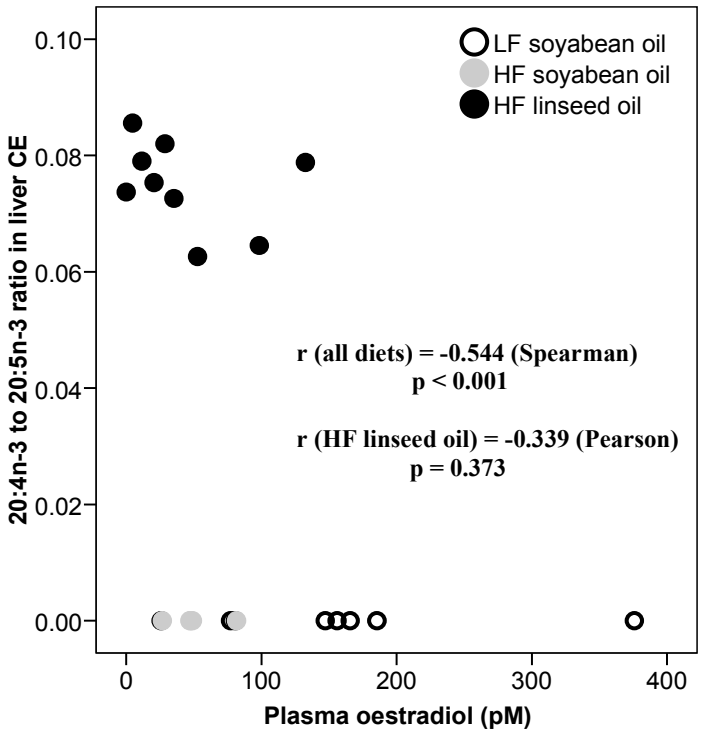


Table 3.29: The elongase substrate:product ratio of fatty acids within liver lipids (n-6: 18:3n-6 to 20:3n-6; n-3: EPA to DPA, mean ± standard deviation, n = 6 unless otherwise indicated)

	LF Soyabean oil		HF Soyabean oil		HF Linseed oil		ANOVA p value		
	Males	Females	Males	Females	Males	Females	Diet	Gender	Diet*Gender
n-6 fatty acids									
Liver PC (x 10^1)	2.39 ± 0.36	3.31 ± 0.63	2.56 ± 0.37	2.60 ± 0.75	1.29 ± 0.17	1.42 ± 0.18	**< 0.001**	**0.023**	0.053
Liver PE (x 10^1)	2.26 ± 0.25 (n=5)	3.44 ± 1.46	2.97 ± 0.74	1.07 ± 1.17	1.45 ± 0.21	1.73 ± 0.39	**0.005**	0.627	**0.001**
Liver CE (x 10^1)	3.6 ± 2.2 (n=5)	15.8 ± 12.4 (n=4)	5.6 ± 3.8 (n=4)	8.0 (n=1)	4.2 ± 0.8	4.4 ± 0.7	0.081	0.056	**0.045**
Liver TAG (x 10^1)	9.7 ± 2.6	12.6 ± 3.9	7.6 ± 1.0 (n=5)	6.5 ± 1.4 (n=5)	7.6 ± 1.4	7.7 ± 1.8	**< 0.001**	0.415	0.131
n-3 fatty acids									
Liver PC (x 10^1)	3.77 ± 1.68	5.34 ± 1.23	2.32 ± 0.53	1.90 ± 0.37	22.5 ± 4.2	27.4 ± 4.2	**< 0.001**	**0.027**	0.055
Liver PE (x 10^1)	1.98 ± 0.65 (n=5)	1.65 ± 0.31	1.79 ± 0.38	1.16 ± 0.32	15.0 ± 2.1	15.6 ± 2.5	**< 0.001**	0.794	0.548
Liver CE (x 10^1)	7.65 ± 2.06 (n=5)	8.39 ± 6.11 (n=4)	10.35 ± 0.66 (n=2)	10.35 ± 0.66 (n=2)	32.8 ± 6.3	41.2 ± 1.5	**< 0.001**	0.263	0.345
Liver TAG (x 10^1)	4.74 ± 1.67	5.87 ± 1.04	5.31 ± 0.82 (n=5)	4.27 ± 0.36 (n=5)	9.24 ± 1.01	10.1 ± 0.8	**< 0.001**	0.373	**0.046**

Diet - Bonferroni post-hoc tests for multiple comparisons:

n-6 liver PC: HF linseed oil significantly different from LF soyabean oil ($p < 0.001$) and HF soyabean oil ($p < 0.001$)
n-6 liver PE: HF linseed and LF soyabean oil significantly different ($p = 0.003$)
n-6 liver TAG: LF soyabean oil significantly different from HF soyabean oil ($p = 0.001$) and HF linseed oil ($p = 0.003$)

n-3 liver PC, PE, TAG: HF linseed oil significantly different from LF soyabean oil ($p < 0.001$) and HF soyabean oil ($p < 0.001$)
n-3 liver CE: HF linseed oil significantly different from LF soyabean oil ($p < 0.001$) and HF soyabean oil ($p = 0.003$)

Table 3.30: Correlations observed between plasma sex hormone concentrations and the substrate:product ratio of fatty acids within liver lipids (Pearson r values unless otherwise indicated)

18:3n-6 to 20:3n-6 ratio	PC	PE	CE	TAG
Testosterone	-0.328 (n=33)	-0.108 (n=32)	-0.142 † (n=23)	-0.289 (n=31)
Oestradiol	0.269 (n=32)	0.150 (n=31)	0.021 † (n=22)	0.273 (n=30)
Progesterone	0.236 † (n=32)	-0.022 (n=31)	0.304 † (n=22)	-0.080 † (n=30)
EPA to DPA ratio				
Testosterone	-0.184 † (n=33)	0.144 † (n=32)	0.101 (n=20)	0.170 (n=31)
Oestradiol	-0.061 † (n=32)	-0.092 † (n=31)	**-0.493* (n=19)**	-0.205 (n=30)
Progesterone †	-0.059 (n=32)	**-0.388*** (n=31)	0.207 (n=19)	-0.083 (n=30)

† Spearman's r value * $p < 0.05$

3.4 Discussion

This aim of this study was to investigate the effect of gender and the dietary availability of ALNA upon the n-3 fatty acid composition of rat tissues, and whether this effect was mediated by sex hormones and/or the expression of genes for enzymes involved in endogenous synthesis of LC PUFA. The hypotheses being tested were:

a) females rats have higher plasma levels of LC n-3 PUFA compared to males
b) gender differences are also apparent within metabolically relevant tissues such as the liver and adipose tissue.
c) plasma docosahexaenoic acid (DHA) content and desaturase and elongase gene expression within the liver are related to plasma sex hormone concentrations.
d) females will show a greater elevation in LC n-3 PUFA in response to an α-linolenic acid (ALNA) rich diet than males as a result of gender differences in desaturase and elongase gene expression.

The data obtained in this study support hypotheses a, b and d; whether the data fully support hypothesis c is not entirely clear but some supportive evidence has been generated. In addition, data collected demonstrated that dietary ALNA itself has a

significant effect upon the expression of Δ6 desaturase, and indicated that individual housing of experimental animals has a significant effect upon patters of growth.

Female rats had higher contents of EPA, DPA and DHA in plasma PC than males, and higher levels of DHA in the other plasma lipids examined. These gender differences are comparable to those observed in human studies, confirming the suitability of the rat as a model for human gender differences in fatty acid composition.

This study expands upon the currently available data from human studies by demonstrating that gender differences in LC n-3 PUFA content which are observed within plasma lipids are also apparent within liver lipids and adipose tissue, and demonstrates significant relationships between the fatty acid compositions of these tissues. For example, the DHA content of plasma phospholipid exhibited strong and statistically significant correlations with the DHA content of liver phospholipids (PC, $r = 0.902$; PE, $r = 0.908$). The DHA content of plasma NEFA exhibited a weaker, yet statistically significant, correlation to the DHA status of subcutaneous adipose tissue ($r = 0.376$). These data indicate that both liver and adipose tissue are likely to have significant metabolic roles in contributing to the gender differences which are apparent plasma DHA content.

Attempts were made to investigate the potential mechanistic basis of these gender differences, with the role of circulating sex hormones and the mRNA expression of enzymes involved in the synthesis of LC PUFA the focus of this study. The role of diet in maintaining gender differences was also investigated, with male and female rats maintained upon one of three experimental diets. These diets varied in their total fat content, and in the availability of ALNA within the diet. The dietary availability of ALNA was under investigation due to its role as a metabolic substrate in the endogenous synthesis of LC n-3 PUFA.

The use of three different experimental diets proved to be a confounding factor when attempts were made to examine the relationship between sex hormone concentrations and the DHA content of plasma lipids. Diet itself exerted a significant effect upon circulating sex hormone concentrations, with both male and female rats on high-fat diets having significantly higher plasma testosterone and lower plasma oestradiol than those maintained on a low fat diet. The potential relationship between plasma sex hormones

and DHA status may therefore be better investigated by examination of a larger group of male and female animals maintained upon a single diet.

mRNA expression of desaturase and elongase enzymes within the liver was investigated in order to assess whether the relative abundance of these enzymes was responsible for the gender differences observed in plasma and tissue fatty acid composition. Data did not support any significant effect of gender or of sex hormones concentrations upon Δ5 desaturase, Δ6 desaturase or elongase mRNA expression in the liver. While investigation of the mRNA expression of enzymes provides a measure of the quantity of these enzymes within the liver, this measure is limited by the lack of insight it allows into the numerous other processes which affect enzyme activity, such as efficiency of translation, post-translational modifications, influence of inhibitors or activators or negative feedback mechanisms. An alternative hypothesis for the mechanisms which underpin gender differences in tissue fatty acid composition which could be proposed is that there are in fact gender differences in the relative activity of desaturase and elongase enzymes within the liver, and not in the relative abundance of these enzymes.

Direct assessment of enzyme activity was not performed during this study, but an indirect measure of enzyme activity was made by calculation of substrate:product ratios of fatty acids within liver lipids. Use of substrate:product ratios are a relatively crude measure of enzyme activity as they cannot take into account the potential influence of specificity in lipid assembly, which is likely to be regulated by more complex processes than the simple availability of intra-cellular fatty acids, and so data obtained from this calculation should be used with caution. However, there were some clear and strong relationships apparent between this marker of enzyme activity and both gender and sex hormone concentrations.

Substrate: product ratios in liver lipids indicated clear gender differences in Δ9 desaturase activity (males > females) and Δ6 desaturase activity (females > males). Data also supports a role of sex hormones in maintaining these gender differences, with ratios indicating a positive correlation between Δ9 desaturase activity and testosterone status, and an inverse relationship with progesterone. This may account for the significantly higher plasma content of 18:1n-9 observed in males compared to females across all dietary groups. Δ6 desaturase substrate:product ratios of activity were inversely related to testosterone status, and demonstrated a positive relationship with

oestradiol status, which may account for the higher plasma content of 18:3n-6 and DHA observed in females compared to males. While these correlations were found to be consistent across numerous liver lipid classes, indicating a close relationship between circulating sex hormone status and this proxy marker of enzyme activity, it must be noted that correlations cannot infer causality, and may be significantly influenced by data which is not normally distributed. Efforts were made to check the nature of the correlations observed by plotting the data, and this did in fact identify 'false positive' correlations, such as was the case for the statistically significant correlation observed between oestradiol status and this fatty acid marker of Δ5 desaturase activity (see figure 3.17).

The fatty acid composition of rat plasma and tissues were found to respond to increased dietary ALNA in a way which was comparable to available data from human studies, demonstrating significant increases in the EPA content of plasma lipids, but without any concurrent increase in DHA content. The increase in EPA was greater for female than male rats, indicating that the LC n-3 PUFA status of females is more responsive to the availability of dietary ALNA. There can be confidence that this effect is not a reflection of simple differences in dietary intake between males and females, as males in fact consumed more diet over the study period, and therefore more ALNA, than their female counterparts. There was a clear indication that the influence of dietary ALNA was lipid-fraction specific, with the effects of the HF linseed oil diet most marked upon the EPA status of CE fractions obtained from plasma and liver lipids. This is likely to reflect a level of cholesterol ester synthase selectivity for fatty acids during lipid assembly.

Diet was observed to exert a significant effect upon Δ6 desaturase mRNA expression, with the HF linseed oil group having the highest expression. As Δ6 desaturase is the rate limiting enzyme in the synthesis of LC PUFA formation, increasing the expression of this single gene may affect the production of down-stream LC PUFA. The specificity of this effect upon n-3 fatty acids may relate to ALNA being the preferred substrate for the action of Δ6 desaturase, with 2-3 times higher maximal enzyme activity compared to LA as a substrate(232).

While using increased dietary ALNA is a relatively inexpensive and non-invasive method of assessing LC PUFA synthesis, one of the principal limitations of using

increased dietary ALNA is the lack of certainty about the metabolic origins of the LC PUFA detected. The findings of this study could be supported further using stable-isotope labelled ALNA to determine the rates of *de novo* synthesis of LC n-3 PUFA in rats, and their flux into other metabolic tissues such as the liver and adipose tissue. This approach would also enable the rates of enzyme activity to be directly assessed within the liver.

Further studies would be required to confirm whether the correlations observed between sex hormone status and the substrate:product ratios used to infer enzyme activity do in fact reflect a causal relationship. One possible approach to this would be the use of isotope labelled fatty acids in hepatocyte cultures in the presence of physiological concentrations of sex hormones. This approach would enable direct measurement of the effect of sex hormones upon enzyme activity. The use of hepatocyte cultures would also be an improvement over the currently available liver microsome data, as this would allow integrated whole cell responses, with sex hormone receptors and their signalling pathways intact.

An additional feature of this study was the effect of individual housing upon weight gain of both male and female rats. Based on this observation, future dietary studies should be conducted in animals which have been allowed to acclimatise to a period of individual housing prior to start of the experimental diet. This would be particularly important in studies of young animals, in order to avoid any confounding influence of changes in the rate of growth which relate to animal housing rather than the experimental diet under investigation.

A 20 day feeing period was arbitrarily used in this study, in part to allow direct comparison with data from studies of experimental diets provided during pregnancy. The data obtained indicate that a more prolonged feeing period may also be worthy of further investigation. This would enable the effects of the HF soyabean oil diet upon LC n-3 PUFA status to be studied further. This is of particular interest, as those rats receiving the HF soyabean oil diet were found to have significantly lower levels of EPA within plasma and liver phospholipids, while maintaining their DHA status (see figure 3.2 and 3.3). It would worthwhile to determine whether more prolonged HF soyabean oil feeding would result in the eventual compromise of plasma and liver DHA status - or

whether there are mechanisms in place which ensure maintenance of DHA status, such as reduced β-oxidation of DHA or its metabolic precursors.

This work undertaken using a rat model also provides some direction for potential future human studies. It would be of interest to determine whether circulating sex hormone concentrations can be correlated to plasma DHA status in humans, though it is likely that rigorous dietary control may be required to prevent a confounding effect of dietary ALNA intake. These studies could be performed in women at various stages of the menstrual cycle, and a study in men would identify whether variation in testosterone status interacts with DHA status. The collection of samples of subcutaneous adipose is possible from human subjects and could be used to determine whether Δ6 desaturase expression or activity within adipose tissue is affected by gender or sex hormones in humans.

Human studies to date have lacked detailed information about the responses of females to increased dietary ALNA. The observation that the EPA status of female rats on an ALNA rich diet reaches much higher levels than is seen in males rats (see figure 3.5) is therefore of relevance to human health, and might indicate that for women, dietary ALNA supplementation can confer some of the health benefits which are associated with LC n-3 PUFA supplementation.

Chapter 4: Pregnancy and the fatty acid composition of rat tissues

4.1 Introduction

4.1.1 Aims and hypothesis for current investigation

This study sets out to address the hypothesis that changes to rat tissue fatty acid composition during pregnancy are mediated by sex hormones and/or the expression of genes for enzymes involved in endogenous synthesis of LC PUFA.

Human studies have identified that men and women differ in their ability to synthesise longer chain (LC) n-3 polyunsaturated fatty acids (PUFA)(130;131;138) and that women have higher circulating concentrations of docosahexaenoic acid (DHA, 22:6n-3) than men(132-135). The study described in chapter 3 has demonstrated that these gender differences are also apparent in the rat. It has been proposed that these gender differences are established in order to ensure an adequate supply of LC PUFA to the developing fetus during pregnancy(133). If this is the case, then it is possible that LC PUFA synthesis may be further upregulated during pregnancy. This may be under the control of sex hormones, and potentially mediated via an effect upon expression of genes involved in the desaturation and elongation of essential fatty acids (EFA) into LC PUFA.

Studies in rats comparing virgin animals with those at the end of pregnancy have shown that the fatty acid composition of phospholipids from plasma and liver is significantly altered in response to pregnancy with higher DHA and lower arachidonic acid (AA) contents(167-170). Human studies also suggest an effect of pregnancy upon blood lipid fatty acid composition, but the effects described are inconsistent. Human studies to date have not included pre-pregnancy data and may be complicated by dietary differences, and the effect of past diet upon adipose tissue composition. It is therefore necessary to use a more controlled setting to evaluate the effect of pregnancy upon tissue fatty acid composition and the potential mechanisms involved. This study is cross-sectional in design to enable sampling of organs such as liver and adipose tissue, which would not be possible in a longitudinal study.

4.1.2 Animal studies of pregnancy and fatty acid composition

Several studies have been undertaken using rats to investigate the effect of pregnancy upon the fatty acid composition of plasma and liver phospholipids and triglycerides. A

summary of the findings of these studies can be found in table 4.1. These studies have all identified that there are significant differences between the fatty acid content of rat tissues at the end of gestation compared to virgin females. Consistent effects of pregnancy observed are higher 16:0 and DHA content, and lower 18:0 and AA content of both plasma and liver phospholipids. The association of 16:0 with DHA status, and 18:0 with AA status, is due to the common pairing of these fatty acids within phospholipid structure. Animal studies to date have not yet identified whether the changes observed in fatty acid composition correlate to changes in circulating sex hormones or in the expression of desaturase and elongase genes in the liver.

4.1.3 Human studies of pregnancy and fatty acid composition

Human studies have been undertaken to examine the effect of pregnancy upon maternal fatty acid status(162-166). Full details of the findings of these studies are described in chapter 1 (see table 1.4). Longitudinal human studies to date have not included pre-conception data, and therefore can only indicate variations in fatty acid status which occur after the first trimester.

The effects observed in human studies have been mixed, which can probably be attributed to variations in sample size, the type of blood lipid sample analysed, potential dilution effects from changes in maternal blood volume during pregnancy, and the confounding effect of maternal diet, with the potential for differences in mobilisation from maternal adipose stores and the composition of those stores. For example, while some studies have identified a reduction in plasma phospholipid DHA status during pregnancy(163), others have reported increased DHA content of plasma phospholipids(165) or red blood cells(166).

The effects observed in human studies have not been limited to LC n-3 PUFA, with significant effects of pregnancy also observed upon saturated fatty acids, monounsaturated fatty acids (MUFA) and n-6 PUFA(162-166). No human study to date has determined whether the changes in fatty acid status observed relate to sex hormone status.

Table 4.1: Summary of rat studies which have investigated the effect of pregnancy upon plasma or liver fatty acid composition

	Study groups	Plasma phospholipids		Liver phospholipids		Liver triglycerides
Smith, 1975 (167) (% content)	virgin day 21 pregnant			↑ 16:0	↓ 18:0 ↓ AA	↑18:2n-6
Cunnane, 1989 (168) (% content)	virgin day 21 pregnant			↑ 16:0 ↑ DHA	↓ 18:0 ↓ 18:2n-6 ↓ AA	↑ AA
Chen et al, 1992 (169) (% content)	virgin day 13 pregnant day 15 pregnant day 21 pregnant †	↑ 16:0 ↑ DHA	↓ 18:0 ↓ 18:2n-6 ↓ AA	↑ 16:0 ↑ DHA	↓ 18:0 ↓ 18:2n-6 ↓ AA	
Burdge et al, 1994 (170) (absolute quantity)	virgin day 16 pregnant day 21 pregnant	↑ 16:0/DHA	↓ 18:0/AA	↑ 16:0/DHA	↓ 18:0/AA *	

↑ Significantly higher content during pregnancy ↓ Significantly lower content during pregnancy

* Exception was liver phosphatidylcholine (PC), where 18:0/AA was significantly higher compared to virgin at day 16 of pregnancy, but significantly lower compared to virgin at day 21 of pregnancy.

† Significant differences were between day 21 of pregnancy and virgin only

4.2 Methods

This study sets out to identify the effects of pregnancy upon plasma and tissue fatty acid composition and to identify whether effects seen are mediated by sex hormones and/or the expression of desaturase and elongase genes. This will be assessed by determination of the fatty acid composition of tissues including plasma, liver and adipose tissue by gas chromatography, measurement of plasma sex hormone concentrations, and the assessment of liver mRNA expression of desaturase and elongase genes by RT-PCR.

All animal work was carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986); for pregnant females, mating was carried out by monogamous breeding (see section 2.1), and maternal tissues collected on either day 12 or day 20 of gestation. Fatty acid composition of experimental diet and tissues was determined by gas chromatography (see section 2.6). Liver lipid, dry weight and glycogen content were assessed (see sections 2.8 and 2.10). Plasma lipid, glucose and circulating sex hormones analysis was provided by Southampton General Hospital (see sections 2.7 and 2.9). mRNA expression of desaturase and elongase genes was determined by RT-PCR (see section 2.12).

The effect of pregnancy upon tissue fatty acid composition, plasma sex hormones and mRNA expression was assessed by one-way ANOVA. Correlation coefficients (r) were calculated using Pearson or Spearman test as appropriate after data were assessed for normality.

4.3 Results

4.3.1 Experimental diets

Animals received a standard maintenance chow throughout the study period. This was a low fat (LF, 3% w/w) soyabean oil based diet (SDS, Witham, UK) which contains adequate n-3 and n-6 EFA. The full details of the nutrient composition of this diet provided by the manufacturer are described in chapter 2 (see section 2.3). The fatty acid composition of the LF soyabean oil diet was assessed by gas chromatography and was described in the previous chapter (see table 3.1). This diet contains little dietary LC PUFA, and so any DHA detected within tissue lipids would derive from endogenous synthesis rather than from direct dietary sources.

4.3.2 Weight gain and food intake

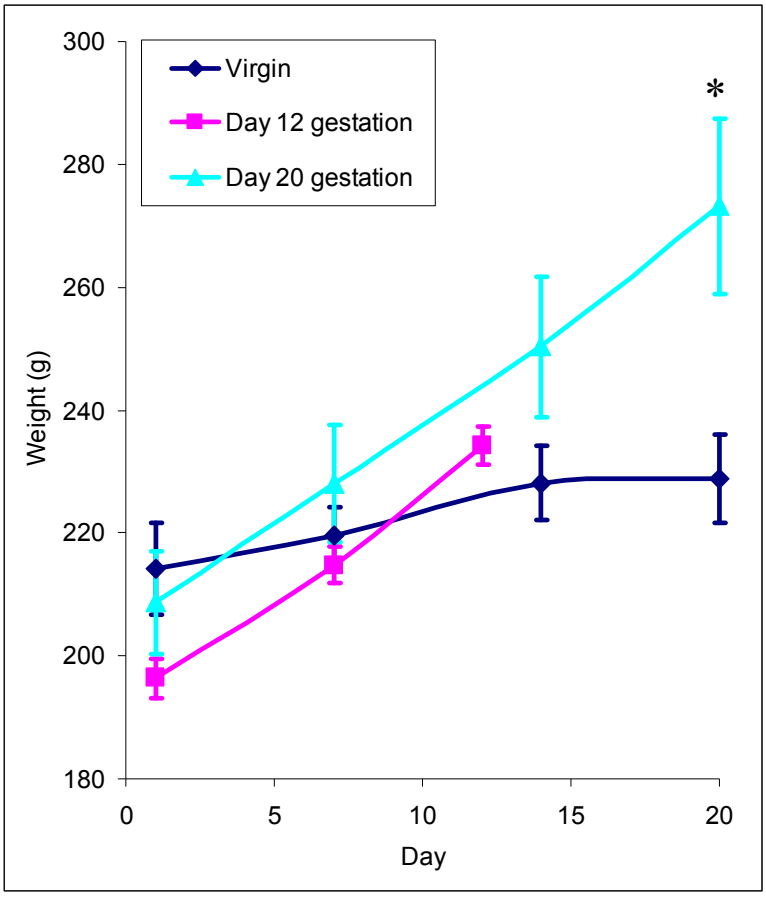
Virgin females had their weight and food intake monitored every 7 days over the study period. Food intake was monitored by weighing food provided and remaining every 2-3 days. Typical rat pregnancy is 21-24 days. Pregnant rats had their weight and food intake monitored over either a 12 or 20 day period from day 1 of gestation (see table 4.2, figure 4.1).

Table 4.2: Weight gain and weighed dietary intake of virgin and pregnant rats over study period (mean ± standard deviation)

	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
Weight gain (g/day)	0.7 ± 0.1 [a]	3.2 ± 0.2 [b]	3.2 ± 1.0 [b]	**< 0.001**
Food intake (g/day)	16.1 ± 1.0	17.3 ± 0.7	17.9 ± 2.2	0.134

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Figure 4.1: The weight of virgin and pregnant rats over study period (mean ± SEM)



* Significantly different from virgin females (T-test, $p = 0.19$)

Pregnant rats gained significantly more weight per day compared to virgin females. There was a non-significant trend for increased food consumption with pregnancy, indicating that pregnancy only induces modest hyperphagia during pregnancy in the rat.

4.3.3 Plasma glucose and lipid concentrations

Plasma glucose concentration was lower and plasma non-esterified fatty acid (NEFA) concentrations higher with pregnancy (table 4.3). Hypertriglyceridemia is a feature of pregnancy which has been observed in both human and rat models(153), yet this was not observed in the current study. However, in this study animals were not fasted prior to collection of blood samples, which may have resulted in a confounding effect of fed status.

Table 4.3: Plasma glucose and lipid concentrations observed in virgin and pregnant rats (mean ± standard deviation)

	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
Plasma glucose (mM)	13.5 ± 2.0 [a]	12.2 ± 3.3 [a]	7.3 ± 2.8 [b]	**0.004**
Total cholesterol (mM)	1.3 ± 0.3	1.4 ± 0.4	1.3 ± 0.4	0.930
HDL-cholesterol (mM)	0.9 ± 0.2	0.9 ± 0.3	0.9 ± 0.2	0.980
TAG (mM)	1.4 ± 0.4	1.3 ± 0.3	1.5 ± 0.7	0.916
NEFA (mM)	0.27 ± 0.18	0.40 ± 0.32	0.68 ± 0.28	0.048 †

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

† no significant differences between groups when Bonferroni post-hoc test applied

4.3.4 Plasma fatty acid composition

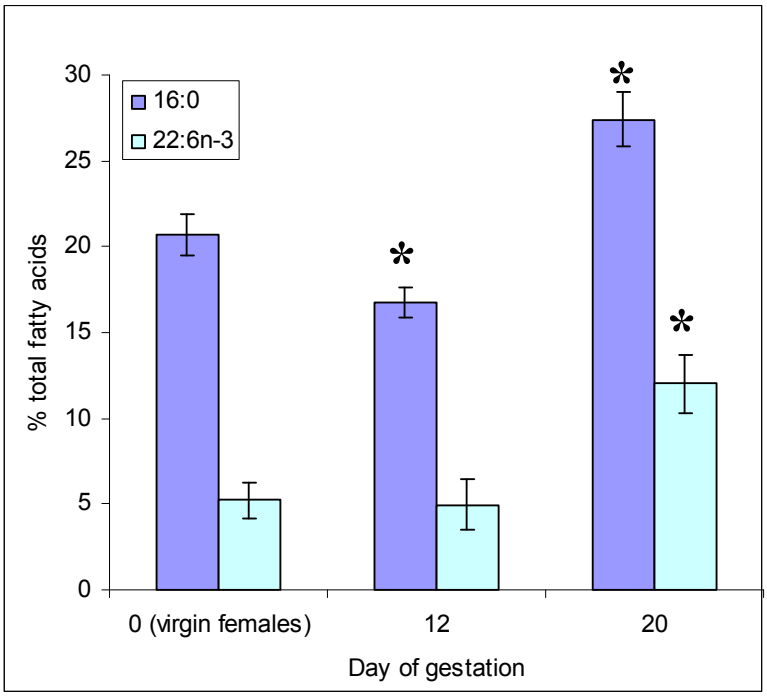
The fatty acid compositions of plasma phosphatidylcholine (PC), TAG, cholesteryl esters (CE) and NEFA were assessed by gas chromatography (see tables 4.4 to 4.7). Other studies using rat models have identified higher 16:0 and DHA and lower 18:0 and AA with pregnancy in rat plasma phospholipids(169;170). These trends were also apparent in the current study (see figures 4.2 and 4.3).

It is clear from the data that the changes in fatty acid content of plasma phospholipids are not simple linear relationships of change over the course of pregnancy. For example, the 18:0 and AA content of plasma phospholipids is significantly higher at day 12 of gestation than in either virgin females or day 20 gestation females.

A summary of the trends observed across all plasma lipid fractions during pregnancy is found in table 4.8. As well as DHA being higher in plasma PC in pregnancy it was also higher in plasma TAG and CE (see figure 4.4). In addition, 22:5n-6 was increased in plasma PC, TAG and CE in pregnancy (see figure 4.5). This indicates that pregnancy exerts complex effects upon endogenous synthesis of n-6 LC PUFA during pregnancy; the lower AA content in plasma PC in pregnancy may indicate increased conversion of AA to 22:5n-6.

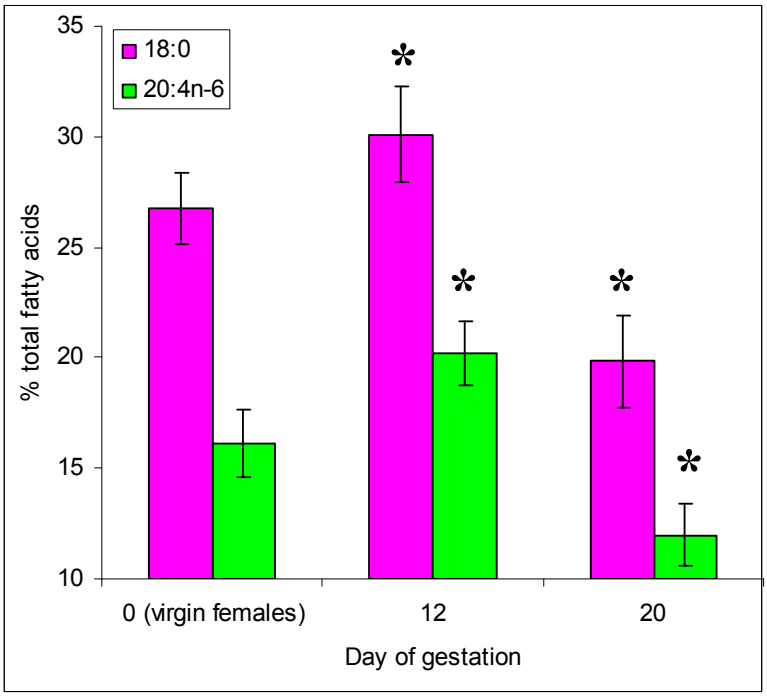
Reduced linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALNA, 18:3n-3) contents of plasma PC, TAG and CE were also observed among pregnant females (see figure 4.6 and 4.7). This indicates that as pregnancy progresses EFA status is reduced, perhaps due to the increased demands of endogenous synthesis of LC PUFA. There was an indication that the ALNA content of some plasma lipids is higher at day 12 gestation, which may indicate either increased mobilisation of ALNA from adipose tissue or lower rates of LC n-3 PUFA synthesis at this point of gestation.

Figure 4.2: The 16:0 and DHA content (% total fatty acids) of plasma PC in virgin and pregnant female rats (mean ± standard deviation)



* significantly different from virgin females ($p < 0.05$)

Figure 4.3: The 18:0 and AA content (% total fatty acids) of plasma PC of virgin and pregnant female rats (mean ± standard deviation)



* significantly different from virgin females ($p < 0.05$)

Table 4.4: Fatty acid composition (% total fatty acids) of plasma PC in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
14:0	0.19 ± 0.11	0.27 ± 0.08	0.24 ± 0.08	0.437
16:0	20.71 ± 1.23 [a]	16.78 ± 0.90 [b]	27.38 ± 1.58 [c]	**< 0.001**
18:0	26.72 ± 1.62 [a]	30.08 ± 2.18 [b]	19.86 ± 2.08 [c]	**< 0.001**
20:0	0.23 ± 0.27	ND	0.24 ± 0.29	0.424
22:0	0.11 ± 0.18	0.04 ± 0.06	0.29 ± 0.43	0.313
Total saturated	47.96 ± 1.24	48.47 ± 1.62	48.02 ± 1.84	0.849
16:1n-7	0.80 ± 0.57	0.40 ± 0.08	1.17 ± 0.80	0.129
18:1n-9	4.27 ± 0.90	4.45 ± 0.79	4.60 ± 0.84	0.802
20:1n-9	0.14 ± 0.16	0.12 ± 0.13	ND	0.125
Total MUFA	5.26 ± 1.33	5.40 ± 1.43	5.78 ± 1.42	0.805
18:2n-6	20.08 ± 1.79 [a]	17.59 ± 2.61 [a]	12.18 ± 1.84 [b]	**< 0.001**
18:3n-6	1.18 ± 0.86	0.13 ± 0.17	1.32 ± 0.93	**0.046**
20:2n-6	0.45 ± 0.13 [a]	0.16 ± 0.22 [b]	0.22 ± 0.18 [ab]	**0.034**
20:3n-6	0.79 ± 0.41 [a]	1.27 ± 0.37 [a]	0.15 ± 0.24 [b]	**< 0.001**
20:4n-6	16.14 ± 1.51 [a]	20.20 ± 1.42 [b]	11.99 ± 1.37 [c]	**< 0.001**
22:5n-6	0.72 ± 0.22 [a]	1.24 ± 0.46 [a]	6.05 ± 0.71 [b]	**< 0.001**
Total n-6 PUFA	39.36 ± 1.85 [a]	39.35 ± 2.32 [a]	31.90 ± 2.67 [b]	**< 0.001**
18:3n-3	0.20 ± 0.13 [ab]	0.84 ± 0.73 [a]	0.13 ± 0.10 [b]	**0.022**
20:4n-3	ND	0.41 ± 0.61	ND	0.097
20:5n-3	1.06 ± 0.85	0.50 ± 0.29	0.88 ± 0.86	0.480
22:5n-3	0.95 ± 0.11 [a]	0.50 ± 0.30 [b]	1.28 ± 0.34 [a]	**0.001**
22:6n-3	5.22 ± 1.02 [a]	4.94 ± 1.48 [a]	12.00 ± 1.73 [b]	**< 0.001**
Total n-3 PUFA	7.42 ± 1.32 [a]	6.78 ± 1.15 [a]	14.30 ± 2.26 [b]	**< 0.001**

ND negligible detected (mean < 0.1%)
a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.5: Fatty acid composition (% total fatty acids) of plasma TAG in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
14:0	0.77 ± 0.27 [a]	1.24 ± 0.07 [b]	0.34 ± 0.19 [c]	**< 0.001**
16:0	26.70 ± 2.76 [ab]	28.27 ± 2.79 [a]	22.74 ± 2.85 [b]	**0.014**
18:0	5.26 ± 2.61	4.10 ± 0.38	5.10 ± 1.58	0.550
20:0	0.18 ± 0.33	0.16 ± 0.09	0.30 ± 0.46	0.758
22:0	0.46 ± 0.66	ND	0.62 ± 0.86	0.315
Total saturated	33.37 ± 4.24	33.80 ± 2.97	29.10 ± 3.05	0.073
16:1n-7	3.02 ± 1.17	3.18 ± 0.56	2.85 ± 1.00	0.850
18:1n-9	22.29 ± 3.90 [ab]	28.02 ± 1.52 [a]	19.27 ± 4.38 [b]	**0.004**
20:1n-9	0.34 ± 0.17	0.26 ± 0.20	0.14 ± 0.22	0.241
24:1n-9	0.12 ± 0.18	ND	ND	0.141
Total MUFA	25.77 ± 4.71 [ab]	31.46 ± 2.04 [a]	22.26 ± 4.93 [b]	**0.010**
18:2n-6	29.97 ± 3.76	25.01 ± 2.35	26.74 ± 4.12	0.096
18:3n-6	1.03 ± 0.64 [ab]	0.39 ± 0.12 [a]	4.00 ± 3.72 [b]	**0.038**
20:2n-6	0.40 ± 0.22	0.27 ± 0.09	0.27 ± 0.29	0.544
20:3n-6	0.38 ± 0.30	0.43 ± 0.24	0.40 ± 0.34	0.951
20:4n-6	3.85 ± 1.10 [a]	3.05 ± 0.94 [a]	8.34 ± 2.56 [b]	**< 0.001**
22:5n-6	0.45 ± 0.29 [a]	0.59 ± 0.19 [a]	1.10 ± 0.18 [b]	**< 0.001**
Total n-6 PUFA	36.07 ± 4.54 [a]	29.75 ± 3.68 [b]	40.85 ± 2.63 [a]	**0.001**
18:3n-3	2.24 ± 0.38 [a]	1.76 ± 0.30 [ab]	1.35 ± 0.40 [b]	**0.003**
20:4n-3	ND	0.13 ± 0.18	ND	0.154
20:5n-3	0.81 ± 0.67	0.45 ± 0.34	2.11 ± 2.29	0.159
22:5n-3	0.67 ± 0.33 [ab]	0.54 ± 0.32 [a]	1.20 ± 0.51 [b]	**0.034**
22:6n-3	1.05 ± 0.56 [a]	2.11 ± 1.03 [ab]	3.14 ± 1.90 [b]	**0.047**
Total n-3 PUFA	4.81 ± 0.86	5.00 ± 2.05	7.80 ± 4.23	0.161

ND negligible detected (mean < 0.1%)
a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.6: Fatty acid composition (% total fatty acids) of plasma CE in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
14:0	0.19 ± 0.20	0.35 ± 0.05	0.21 ± 0.23	0.351
16:0	11.82 ± 3.70	9.72 ± 1.20	14.38 ± 3.33	0.069
18:0	3.05 ± 1.52	2.62 ± 0.94	5.28 ± 2.12	**0.034**
22:0	ND [a]	0.47 ± 0.26 [b]	ND [a]	**< 0.001**
Total saturated	15.08 ± 5.27	13.38 ± 2.27	19.92 ± 5.48	0.086
16:1n-7	1.24 ± 0.57	1.58 ± 0.06	1.40 ± 0.36	0.406
18:1n-9	5.61 ± 2.42	5.76 ± 0.76	6.84 ± 1.34	0.420
20:1n-9	ND	0.63 ± 0.75	ND	0.050
Total MUFA	6.92 ± 2.72	7.99 ± 0.85	8.25 ± 1.61	0.479
18:2n-6	20.65 ± 2.03 [a]	18.69 ± 2.35 [ab]	16.99 ± 2.53 [b]	**0.050**
18:3n-6	0.75 ± 0.14	0.79 ± 0.08	0.90 ± 0.19	0.236
20:2n-6	ND [a]	0.20 ± 0.07 [b]	ND [a]	**< 0.001**
20:3n-6	0.26 ± 0.20	0.44 ± 0.26	0.31 ± 0.76	0.817
20:4n-6	52.88 ± 5.90	53.50 ± 6.22	46.41 ± 3.36	0.070
22:5n-6	ND [a]	0.16 ± 0.16 [a]	1.48 ± 0.22 [b]	**< 0.001**
Total n-6 PUFA	74.58 ± 7.16	73.62 ± 4.20	66.09 ± 5.49	0.050
18:3n-3	0.29 ± 0.06 [a]	1.07 ± 0.54 [b]	0.33 ± 0.09 [a]	**0.001**
20:5n-3	1.26 ± 0.44 [a]	1.69 ± 0.77 [a]	0.44 ± 0.27 [b]	**0.004**
22:6n-3	1.86 ± 0.49 [a]	2.26 ± 0.54 [a]	4.88 ± 1.00 [b]	**< 0.001**
Total n-3 PUFA	3.42 ± 0.75 [a]	5.02 ± 1.36 [ab]	5.76 ± 1.04 [b]	**0.006**

ND negligible detected (mean < 0.1%)
a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.7: Fatty acid composition (% total fatty acids) of plasma NEFA in virgin and pregnant female rats (mean ± standard deviation)

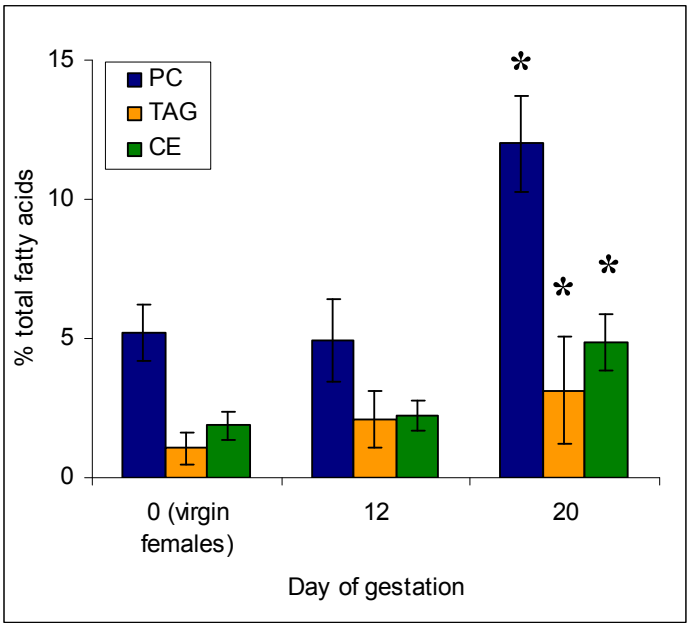
Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
14:0	1.38 ± 0.34	2.11 ± 0.56	1.19 ± 0.74	**0.047**
16:0	27.47 ± 3.14	23.61 ± 1.97	25.22 ± 1.93	0.060
18:0	19.38 ± 5.16 [a]	17.05 ± 2.73 [ab]	11.17 ± 1.81 [b]	**0.004**
20:0	0.33 ± 0.30	0.93 ± 0.35	0.52 ± 0.69	0.164
22:0	0.72 ± 0.97	0.26 ± 0.18	0.45 ± 0.33	0.483
Total saturated	49.29 ± 6.99 [a]	45.12 ± 3.95 [ab]	38.59 ± 3.19 [b]	**0.009**
16:1n-7	2.55 ± 1.64	2.49 ± 0.52	3.13 ± 2.62	0.813
18:1n-9	15.85 ± 2.74 [ab]	13.92 ± 3.77 [a]	19.23 ± 1.13 [b]	**0.016**
20:1n-9	ND [ab]	0.39 ± 0.39 [a]	ND [b]	**0.031**
24:1n-9	0.15 ± 0.17	0.26 ± 0.26	ND	0.154
Total MUFA	18.60 ± 3.51	17.49 ± 3.65	22.41 ± 2.72	0.059
18:2n-6	19.09 ± 4.77 [ab]	12.14 ± 9.36 [a]	23.91 ± 3.03 [b]	**0.021**
18:3n-6	1.84 ± 3.19	0.76 ± 0.83	1.79 ± 1.75	0.677
20:2n-6	0.57 ± 1.24	0.51 ± 0.25	0.27 ± 0.44	0.784
20:3n-6	0.10 ± 0.23 [a]	0.52 ± 0.08 [b]	ND [a]	**0.003**
20:4n-6	5.38 ± 1.12 [a]	11.17 ± 3.08 [b]	3.65 ± 1.25 [a]	**< 0.001**
22:5n-6	0.12 ± 0.10	0.74 ± 0.63	0.68 ± 0.36	**0.038**
Total n-6 PUFA	27.12 ± 4.36	25.10 ± 6.31	30.38 ± 4.79	0.257
18:3n-3	1.66 ± 0.48 [a]	6.66 ± 3.06 [b]	2.20 ± 0.80 [a]	**0.001**
20:4n-3	ND [a]	0.56 ± 0.39 [b]	0.13 ± 0.32 [ab]	**0.018**
20:5n-3	1.20 ± 1.86	3.27 ± 2.90	1.74 ± 1.82	0.308
22:5n-3	0.36 ± 0.13	0.30 ± 0.18	0.93 ± 0.92	0.143
22:6n-3	1.76 ± 0.75	1.92 ± 0.33	3.60 ± 2.64	0.144
Total n-3 PUFA	5.00 ± 2.12	12.29 ± 6.03	8.61 ± 6.12	0.091

ND negligible detected (mean < 0.1%)
a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.8: Summary of the significant differences observed in fatty acid composition between virgin and pregnancy female rats within plasma lipids during the study period

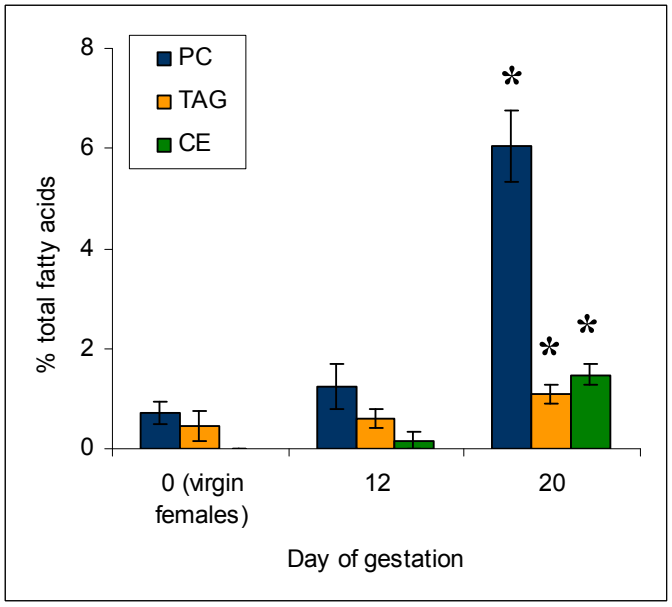
	PC	TAG	CE	NEFA
Day 20 > Day 12 > Virgin	22:5n-6	22:5n-6	22:5n-6	
		22:6n-3	22:6n-3	
Day 20 > Virgin				14:0
	16:0			
			18:0	
				18:1n-9
				18:2n-6
	18:3n-6	18:3n-6		
		20:4n-6		
				22:5n-6
			18:3n-3	18:3n-3
				20:4n-3
	22:5n-3	22:5n-3		
	22:6n-3			
Day 20 < Day 12 < Virgin				18:0
	18:2n-6		18:2n-6	
		18:3n-3		
Day 20 < Virgin		14:0		
		16:0		
	18:0			
		18:1n-9		
	20:2n-6			
	20:3n-6			
	20:4n-6			20:4n-6
	18:3n-3			
			20:5n-3	

Figure 4.4: Graph to illustrate the DHA content (% total fatty acids) of plasma lipids in virgin and pregnant female rats (mean ± standard deviation)



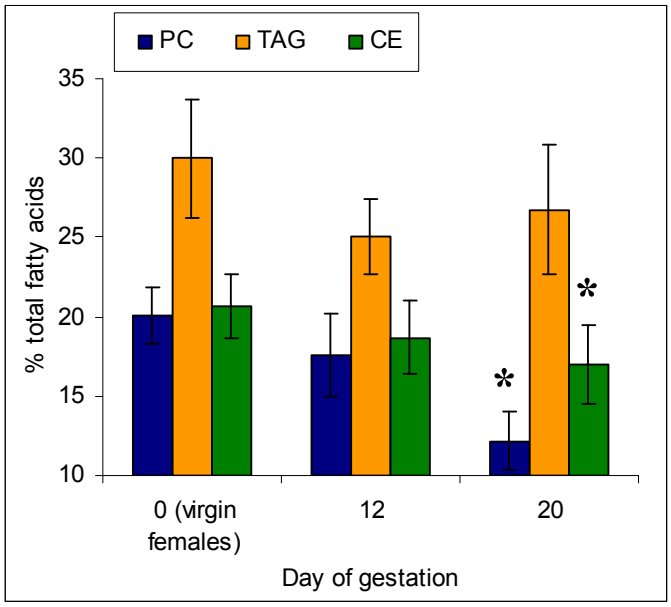
* significantly different from virgin females ($p < 0.05$)

Figure 4.5: Graph to illustrate the 22:5n-6 content (% total fatty acids) of plasma lipids in virgin and pregnant female rats (mean ± standard deviation)



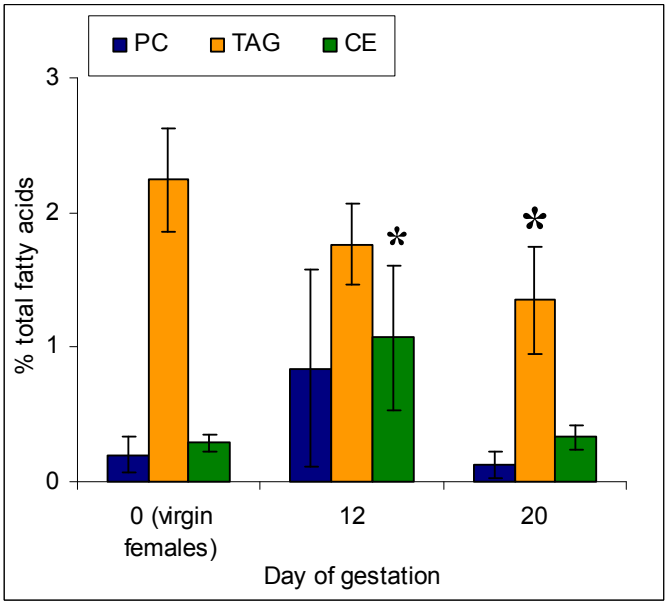
* significantly different from virgin females ($p < 0.05$)

Figure 4.6: Graph to illustrate the LA (18:2n-6) content (% total fatty acids) of plasma lipids in virgin and pregnant female rats (mean ± standard deviation)



* significantly different from virgin females ($p < 0.05$)

Figure 4.7: Graph to illustrate the ALNA (18:3n-3) content (% total fatty acids) of plasma lipids in virgin and pregnant female rates (mean ± standard deviation)



* significantly different from virgin females ($p < 0.05$)

4.3.5 Liver size and composition

Relative liver size and lipid content were highest and liver dry weight lowest in the mid-gestation group (see table 4.9). This suggests that during mid-gestation there is natural accumulation of lipids within the liver. Other studies have also demonstrated higher liver lipid content in pregnant rats than in virgin rats(233). Liver glycogen stores were depleted during pregnancy.

Table 4.9: Liver size and composition in virgin and pregnant female rats (mean ± standard deviation)

	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
Liver (% body weight)	3.7 ± 0.1 [a]	4.3 ± 0.4 [b]	3.6 ± 0.3 [a]	**0.006**
Liver dry weight (% wet weight)	29.4 ± 0.9 [a]	27.2 ± 0.8 [b]	28.4 ± 1.3 [ab]	**0.012**
Liver lipid (% wet weight)	3.2 ± 0.3 [a]	4.5 ± 0.5 [b]	3.4 ± 0.2 [a]	**< 0.001**
Liver glycogen (% wet weight)	5.2 ± 1.0 [a]	4.1 ± 0.7 [a]	1.4 ± 1.6 [b]	**< 0.001**

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

4.3.6 Liver fatty acid composition

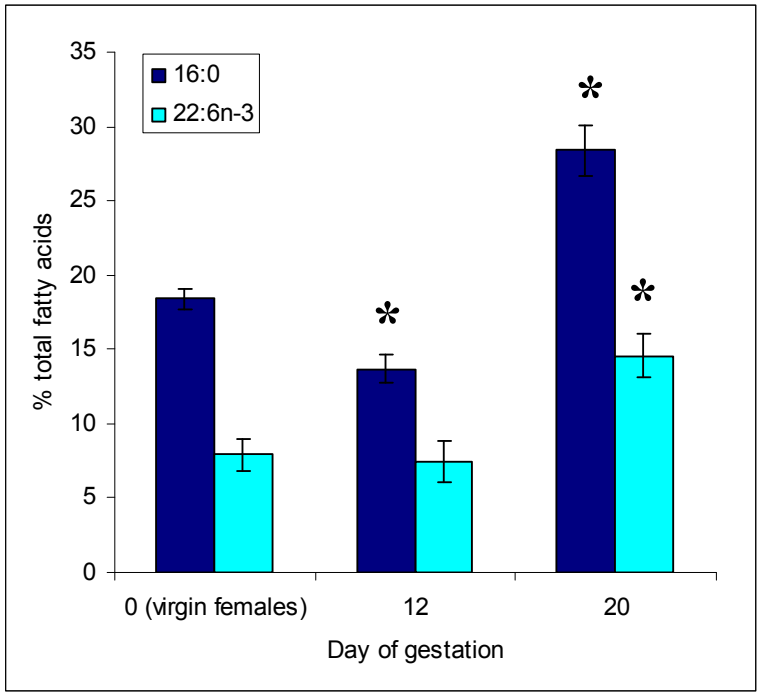
The fatty acid compositions of liver lipids in virgin and pregnant rats are shown in tables 4.10 to 4.13. Previous studies have demonstrated that pregnancy in rats results in higher 16:0 and DHA and lower 18:0, AA and LA content of liver phospholipids(167-170). These features were observed in the current dataset for liver PC and PE (see figures 4.8 to 4.11). As was the case in plasma phospholipids, these effects of pregnancy were not simple linear changes with pregnancy. For example, 18:0 and AA content was significantly higher at day 12 gestation compared to virgin females, but fell to significantly lower levels than those in virgin females by day 20 of gestation.

Liver CE fatty acid composition was also altered in response to pregnancy, with some effects mirroring those observed in liver phospholipids, such as increased DHA and reduced 18:0 at day 20 gestation compared to virgin females. The effects upon other fatty acids differed from those observed in liver phospholipids, with higher LA and AA, and no significant effect on 16:0 content. This indicates that there are lipid fraction specific responses to pregnancy.

Liver TAG fatty acid composition also demonstrated a markedly different response to pregnancy than liver phospholipids, with 18:0 and AA content in liver TAG significantly higher at day 20 gestation than in virgin females, and increased LA content (see figure 4.11), the opposite pattern to that observed within liver phospholipids.

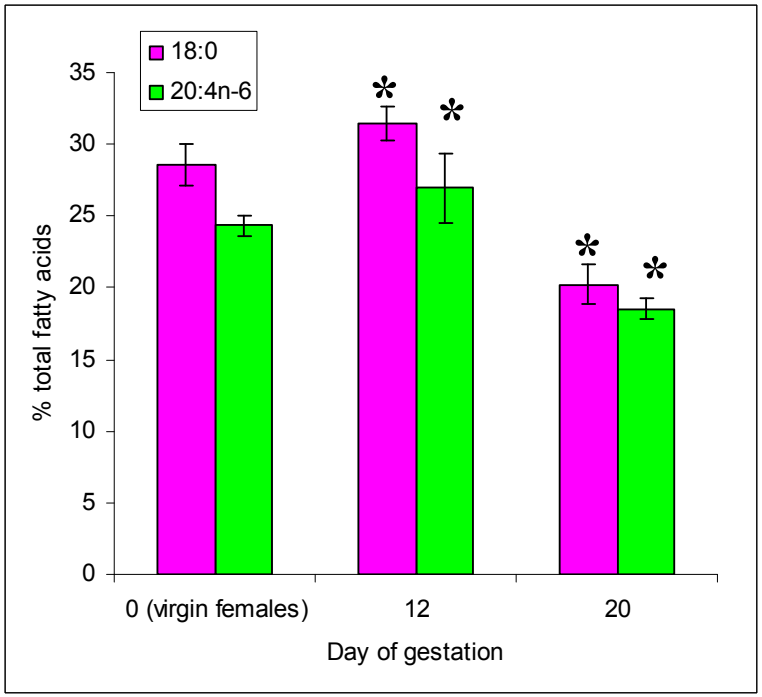
Correlations between the fatty acid composition of liver lipids and plasma lipids were used to determine to what extent plasma lipid composition was related to liver composition (see table 4.14, figure 4.12). Plasma phospholipid and TAG fatty acid composition demonstrated strong and statistically significant correlations with liver phospholipid and TAG fatty acid composition, respectively. These significant correlations were observed across the full range of fatty acids identified, including saturates, MUFA, and n-6 and n-3 PUFA.

Figure 4.8: Graph to illustrate the 16:0 and DHA (22:6n-3) content (% total fatty acids) within liver PC in virgin and female pregnant rats (mean ± standard deviation)



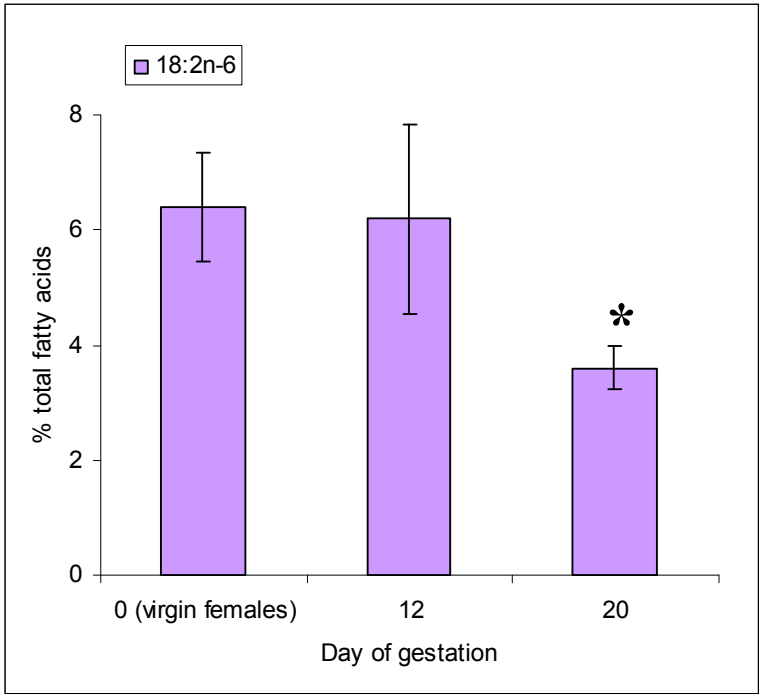
* significantly different from virgin females ($p < 0.05$)

Figure 4.9: Graph to illustrate the 18:0 and AA (20:4n-6) content (% total fatty acids) within liver PC in virgin and female pregnant rats (mean ± standard deviation)



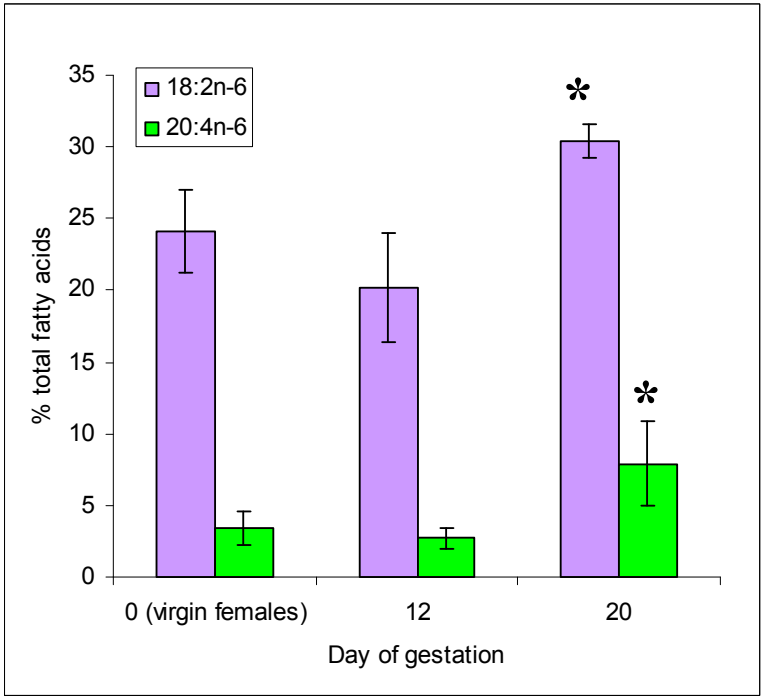
* significantly different from virgin females ($p < 0.05$)

Figure 4.10: Graph to illustrate the LA (18:2n-6) content (% total fatty acids) of liver PE in virgin and pregnant female rats (mean ± standard deviation)



* significantly different from virgin females ($p < 0.05$)

Figure 4.11: Graph to illustrate the LA (18:2n-6) and AA (20:4n-6) content (% total fatty acids) in liver TAG in virgin and pregnant female rats (mean ± standard deviation)



* significantly different from virgin females ($p < 0.05$)

Table 4.10: Fatty acid composition (% total fatty acids) of liver PC in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
14:0	0.22 ± 0.03 [a]	0.19 ± 0.03 [ab]	0.15 ± 0.02 [b]	**0.002**
16:0	18.39 ± 0.67 [a]	13.70 ± 0.98 [b]	28.39 ± 1.73 [c]	**< 0.001**
18:0	28.62 ± 1.45 [a]	31.45 ± 1.14 [b]	20.25 ± 1.42 [c]	**< 0.001**
22:0	ND	0.10 ± 0.03	ND	**0.037**
Total saturated	47.26 ± 1.13 [ab]	46.51 ± 1.68 [a]	48.91 ± 1.36 [b]	**0.033**
16:1n-7	0.61 ± 0.21	0.55 ± 0.04	0.68 ± 0.17	0.434
18:1n-9	3.30 ± 0.48	3.84 ± 0.25	3.63 ± 0.73	0.268
20:1n-9	0.12 ± 0.15	ND	0.11 ± 0.10	0.929
24:1n-9	ND [a]	0.11 ± 0.07 [b]	ND [a]	**0.004**
Total MUFA	4.03 ± 0.71	4.62 ± 0.37	4.35 ± 0.88	0.388
18:2n-6	12.39 ± 1.22	10.96 ± 1.96	10.55 ± 0.91	0.089
18:3n-6	0.35 ± 0.08 [a]	0.24 ± 0.03 [b]	0.38 ± 0.04 [a]	**0.003**
20:2n-6	0.31 ± 0.06 [a]	0.13 ± 0.03 [b]	0.27 ± 0.05 [a]	**< 0.001**
20:3n-6	1.06 ± 0.13 [a]	1.26 ± 0.19 [a]	0.35 ± 0.08 [b]	**< 0.001**
20:4n-6	24.33 ± 1.24 [a]	26.94 ± 1.54 [b]	18.54 ± 0.49 [c]	**< 0.001**
22:5n-6	0.18 ± 0.19 [a]	1.01 ± 0.71 [b]	0.24 ± 0.13 [a]	**0.008**
Total n-6 PUFA	38.62 ± 2.03 [a]	39.54 ± 2.51 [a]	30.33 ± 1.49 [b]	**< 0.001**
18:3n-3	0.13 ± 0.11	0.25 ± 0.20	0.19 ± 0.05	0.327
20:5n-3	0.71 ± 0.15 [a]	0.83 ± 0.40 [a]	0.17 ± 0.11 [b]	**0.001**
22:5n-3	1.34 ± 0.08 [a]	0.80 ± 0.09 [b]	1.37 ± 0.21 [a]	**< 0.001**
22:6n-3	7.90 ± 1.12 [a]	7.45 ± 1.41 [a]	14.59 ± 1.47 [b]	**< 0.001**
Total n-3 PUFA	10.09 ± 1.06 [a]	9.33 ± 1.44 [a]	16.33 ± 1.52 [b]	**< 0.001**

ND negligible detected (mean <0.1%)
a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.11: Fatty acid composition (% total fatty acids) of liver phosphatidylethanolamine (PE) in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
16:0	18.41 ± 0.64 [a]	14.93 ± 0.87 [b]	22.16 ± 1.35 [c]	**< 0.001**
18:0	26.77 ± 0.59 [a]	28.46 ± 0.70 [b]	27.18 ± 1.43 [ab]	**0.037**
Total saturated	45.20 ± 0.41 [a]	45.19 ± 1.83 [a]	49.42 ± 0.99 [b]	**< 0.001**
16:1n-7	0.33 ± 0.09 [a]	0.32 ± 0.03 [a]	0.21 ± 0.05 [b]	**0.010**
18:1n-9	2.17 ± 0.38 [ab]	2.61 ± 0.34 [a]	1.89 ± 0.40 [b]	**0.023**
20:1n-9	0.22 ± 0.13	ND	0.16 ± 0.15	0.220
Total MUFA	2.72 ± 0.44	3.02 ± 0.40	2.26 ± 0.54	0.051
18:2n-6	6.40 ± 0.95 [a]	6.20 ± 1.65 [a]	3.60 ± 0.38 [b]	**0.001**
18:3n-6	0.21 ± 0.08	ND	ND	0.064
20:2n-6	0.22 ± 0.03 [a]	0.13 ± 0.03 [b]	0.21 ± 0.07 [a]	**0.013**
20:3n-6	0.61 ± 0.07 [a]	0.76 ± 0.17 [a]	0.25 ± 0.10 [b]	**< 0.001**
20:4n-6	25.81 ± 0.69 [a]	28.03 ± 2.43 [a]	19.79 ± 0.67 [b]	**< 0.001**
22:5n-6	ND [a]	1.75 ± 1.49 [b]	ND [a]	**0.004**
Total n-6 PUFA	33.24 ± 1.60 [a]	35.20 ± 4.01 [a]	23.91 ± 0.92 [b]	**< 0.001**
18:3n-3	0.23 ± 0.03 [ab]	0.29 ± 0.20 [a]	ND [b]	**0.035**
20:5n-3	0.49 ± 0.09 [a]	0.65 ± 0.31 [a]	0.12 ± 0.08 [b]	**0.001**
22:5n-3	3.00 ± 0.21 [a]	1.66 ± 0.25 [b]	1.83 ± 0.22 [b]	**< 0.001**
22:6n-3	15.11 ± 1.86 [a]	13.99 ± 2.74 [a]	22.38 ± 1.14 [b]	**< 0.001**
Total n-3 PUFA	18.84 ± 1.66 [a]	16.59 ± 2.77 [a]	24.41 ± 1.10 [b]	**< 0.001**

ND negligible detected (mean <0.1%)
a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.12: Fatty acid composition (% total fatty acids) of liver CE in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
14:0	0.61 ± 0.20	0.45 ± 0.08	0.34 ± 0.18	0.058
16:0	43.69 ± 3.16	39.68 ± 7.02	42.95 ± 3.39	0.357
18:0	21.07 ± 3.48 [a]	35.16 ± 9.15 [b]	15.54 ± 2.61 [a]	**< 0.001**
20:0	0.26 ± 0.32	ND	0.42 ± 0.42	0.131
22:0	ND [a]	0.29 ± 0.18 [b]	ND [a]	**< 0.001**
Total saturated	65.62 ± 3.58 [a]	75.58 ± 2.22 [b]	59.26 ± 4.73 [c]	**< 0.001**
16:1n-7	2.04 ± 0.73	1.13 ± 0.23	1.96 ± 0.72	0.061
18:1n-9	7.47 ± 1.98	6.26 ± 0.92	9.06 ± 2.83	0.126
20:1n-9	0.34 ± 0.30	ND	0.44 ± 0.33	0.073
Total MUFA	9.84 ± 2.56	7.43 ± 1.14	11.46 ± 3.37	0.068
18:2n-6	13.43 ± 1.58 [a]	8.33 ± 1.17 [b]	15.01 ± 1.63 [a]	**< 0.001**
18:3n-6	0.37 ± 0.28	ND	0.35 ± 0.35	0.155
20:2n-6	0.35 ± 0.14 [a]	ND [b]	0.38 ± 0.27 [a]	**0.007**
20:3n-6	0.21 ± 0.17	0.32 ± 0.18	0.12 ± 0.15	0.169
20:4n-6	7.84 ± 1.24 [ab]	6.42 ± 2.01 [a]	9.64 ± 1.73 [b]	**0.021**
22:5n-6	ND [a]	ND [a]	0.40 ± 0.07 [b]	**< 0.001**
Total n-6 PUFA	22.19 ± 1.89 [a]	15.13 ± 1.26 [b]	25.90 ± 2.40 [c]	**< 0.001**
18:3n-3	0.99 ± 0.24	0.86 ± 0.34	0.98 ± 0.29	0.725
20:4n-3	0.26 ± 0.63	ND	ND	0.554
20:5n-3	0.22 ± 0.18	0.23 ± 0.15	0.23 ± 0.51	0.999
22:5n-3	0.20 ± 0.16	ND	0.22 ± 0.24	0.498
22:6n-3	0.66 ± 0.40 [a]	0.67 ± 0.39 [a]	1.88 ± 0.39 [b]	**< 0.001**
Total n-3 PUFA	2.34 ± 0.21 [a]	1.85 ± 0.30 [a]	3.38 ± 0.77 [b]	**0.001**

ND negligible detected (mean <0.1%)
a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.13: Fatty acid composition (% total fatty acids) of liver TAG in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
14:0	1.03 ± 0.23 [a]	1.49 ± 0.12 [b]	0.45 ± 0.06 [c]	**< 0.001**
16:0	31.75 ± 3.86 [a]	32.76 ± 3.60 [a]	25.25 ± 1.05 [b]	**0.002**
18:0	3.88 ± 1.06	4.43 ± 0.48	3.53 ± 0.41	0.158
20:0	0.17 ± 0.14 [ab]	ND [a]	0.26 ± 0.13 [b]	**0.031**
Total saturated	36.83 ± 4.77 [a]	38.71 ± 3.97 [a]	29.57 ± 0.98 [b]	**0.002**
16:1n-7	3.33 ± 1.22	3.77 ± 0.76	2.35 ± 0.75	0.065
18:1n-9	27.18 ± 2.58 [ab]	29.70 ± 1.88 [a]	22.65 ± 3.35 [b]	**0.006**
20:1n-9	0.23 ± 0.19	0.30 ± 0.16	0.35 ± 0.05	0.429
Total MUFA	30.74 ± 4.21 [ab]	33.77 ± 2.51 [a]	25.34 ± 3.96 [b]	**0.006**
18:2n-6	24.18 ± 2.88 [a]	20.19 ± 3.84 [a]	30.40 ± 1.20 [b]	**< 0.001**
18:3n-6	0.56 ± 0.15 [a]	0.38 ± 0.13 [a]	0.90 ± 0.15 [b]	**< 0.001**
20:2n-6	0.47 ± 0.18 [a]	0.20 ± 0.07 [b]	0.48 ± 0.09 [a]	**0.004**
20:3n-6	0.48 ± 0.17	0.35 ± 0.14	0.60 ± 0.21	0.108
20:4n-6	3.41 ± 1.15 [a]	2.71 ± 0.74 [a]	7.89 ± 2.97 [b]	**0.001**
22:5n-6	ND [a]	0.50 ± 0.20 [b]	ND [a]	**< 0.001**
Total n-6 PUFA	29.11 ± 4.31 [a]	24.33 ± 4.88 [a]	40.27 ± 3.11 [b]	**< 0.001**
18:3n-3	1.52 ± 0.29 [ab]	1.13 ± 0.34 [a]	1.61 ± 0.22 [b]	**0.035**
20:5n-3	0.39 ± 0.14	0.28 ± 0.17	0.20 ± 0.12	0.098
22:5n-3	0.67 ± 0.20 [a]	0.48 ± 0.28 [a]	1.48 ± 0.73 [b]	**0.007**
22:6n-3	0.74 ± 0.13	1.24 ± 0.63	1.53 ± 0.66	0.059
Total n-3 PUFA	3.31 ± 0.63	3.17 ± 1.38	4.82 ± 1.42	0.062

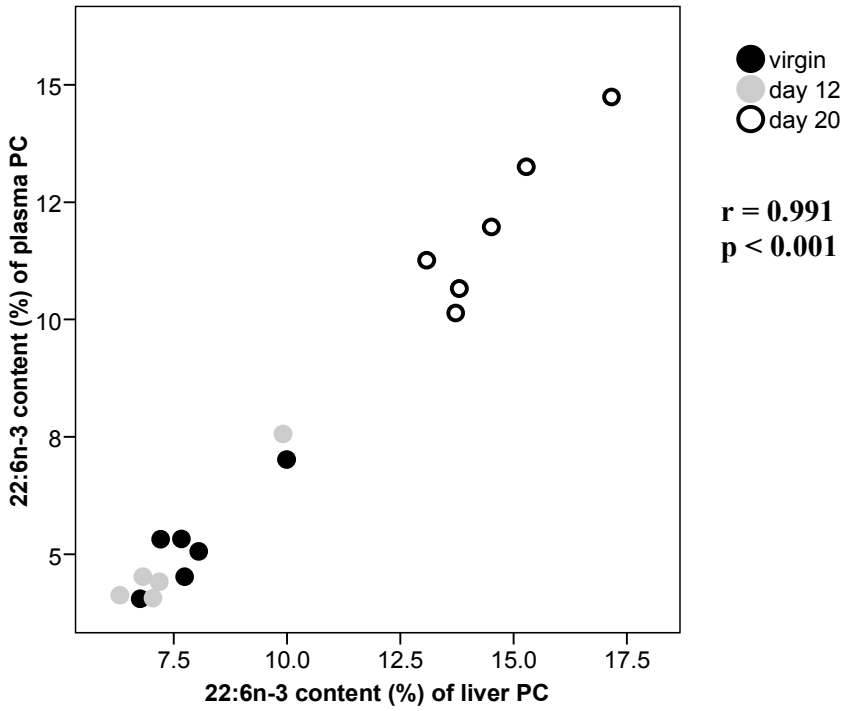
ND negligible detected (mean <0.1%)
a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.14: Correlations observed between liver and plasma fatty acid composition (Pearson r values unless otherwise indicated)

R values	Plasma PC vs. Liver PC (n=17)		Plasma PC vs. Liver PE (n=17)		Plasma TAG vs. Liver TAG (n=17)		Plasma CE vs. Liver CE (n=17)	
14:0	-0.029		-0.193 †		0.860	***	-0.090	
16:0	0.981	***	0.926	***	0.861	***	0.232	
18:0	0.971	***	0.443		0.160		-0.398	
20:0	0.014 †		0.160 †		0.258		0.043 †	
22:0	0.612	**	0.457 †		0.587 †	*	0.925 †	***
Total saturated	0.271		0.102		0.725	**	-0.394	
16:1n-7	0.772	***	0.101		0.733	**	0.474	
18:1n-9	0.741	**	0.571	*	0.815	***	0.575	*
20:1n-9	-0.282 †		-0.101 †		0.039		-0.290	
24:1n-9	0.052 †		-		-		-	
Total MUFA	0.753	***	0.531	*	0.876	***	0.439	
18:2n-6	0.725	**	0.884	***	0.330		-0.038	
18:3n-6	0.555	*	0.385		0.741	**	0.451	
20:2n-6	0.496	*	0.477		0.386		-0.751 †	
20:3n-6	0.861	***	0.811	***	0.558	*	-0.053	
20:4n-6	0.940	***	0.846	***	0.954	***	-0.253	
22:5n-6	-0.208		-0.070 †		-0.175 †		0.856 †	***
Total n-6 PUFA	0.929	***	0.875	***	0.895	***	-0.446	
18:3n-3	-0.107 †		-0.003 †		0.299		-0.338	
20:4n-3	0.784 †	***	-		0.836 †	***	0.247 †	
20:5n-3	0.049		0.106		0.216		0.021	
22:5n-3	0.797	***	0.148		0.882	***	-	
22:6n-3	0.991	***	0.972	***	0.932	***	0.856	***
Total n-3 PUFA	0.964	***	0.910	***	0.893	***	0.402	

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † Spearman r value

Figure 4.12: Graph of the correlation observed between plasma and liver PC DHA contents (% total fatty acids)



4.3.7 Adipose tissue fatty acid composition

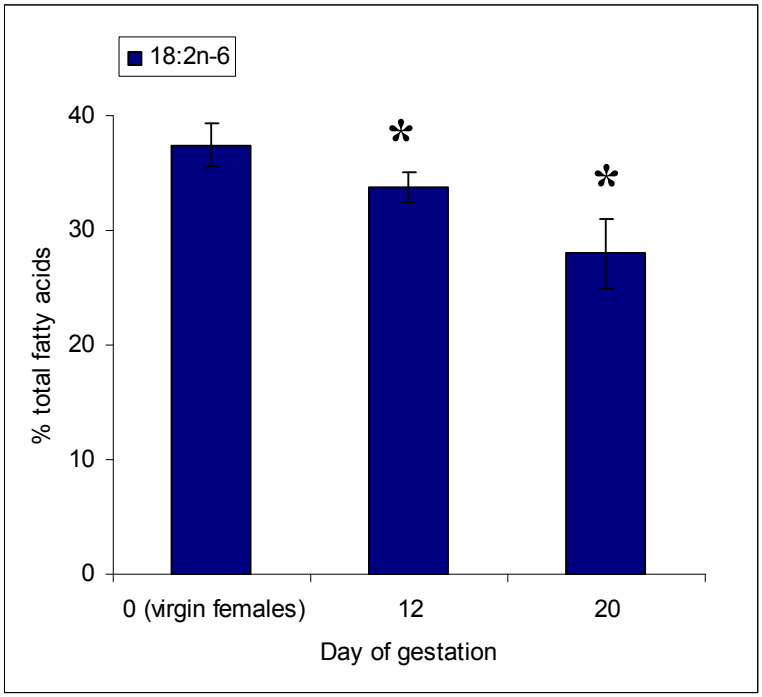
The fatty acid composition of subcutaneous adipose tissue was more affected by pregnancy than intra-abdominal adipose tissue (tables 4.15 and 4.16), indicating that subcutaneous adipose tissue is the more labile store. In subcutaneous adipose tissue, reductions in the content of EFA (see figure 4.13 and 4.14) were observed, supporting the hypothesis that these fatty acids are selectively released from adipose tissue during pregnancy to enable increased LC PUFA synthesis(166).

There was significantly higher AA content of subcutaneous adipose tissue with pregnancy (see figure 4.14). This may indicate that this LC n-6 PUFA is either preferentially deposited in subcutaneous adipose tissue during pregnancy, or that it is not mobilised from adipose tissue as readily as alternative fatty acids. There were low levels of DHA in adipose tissue and these were not significantly affected by pregnancy.

Correlations were performed to assess the strength of the relationship between adipose tissue and plasma NEFA fatty acid composition (see table 4.17). Few significant

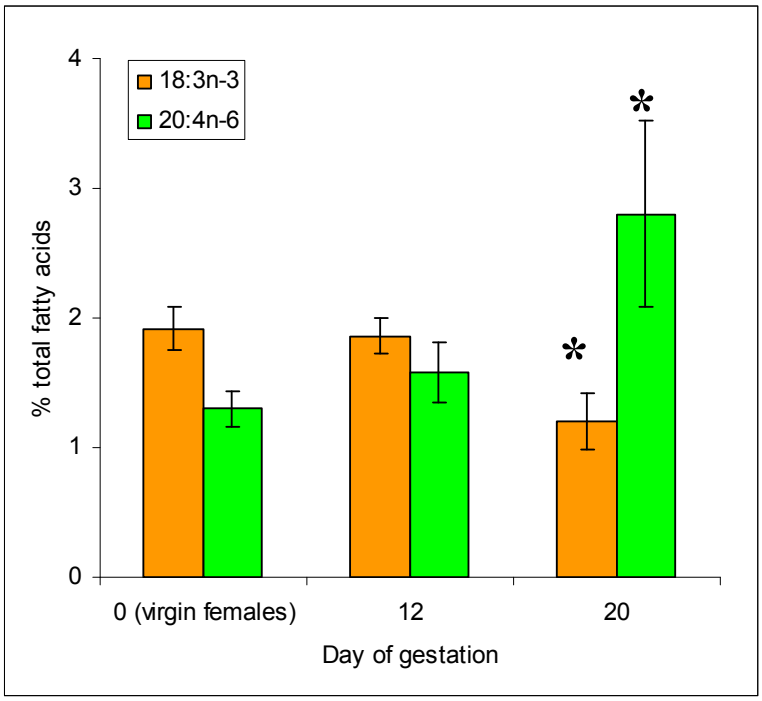
correlations were observed, in contrast to the observations in chapter 3 (see table 3.16), which indicates that changes in diet are a more potent modulator of adipose tissue fatty acid composition than the effects of pregnancy. However, there were significant inverse correlations between EFA in subcutaneous adipose and their contribution to plasma NEFA (see figure 4.15 and 4.16). This may suggest that as pregnancy progresses, more EFA is mobilised from adipose to the circulating NEFA pool, in order to meet the increasing demands of endogenous synthesis of LC PUFA during pregnancy. Higher plasma NEFA concentrations were observed with pregnancy, which may indicate a greater mobilisation of adipose tissue during pregnancy (see table 4.3). However, as animals were not fasted prior to blood sampling, it is possible that these data may be confounded by fed status.

Figure 4.13: The LA (18:2n-6) content (% total fatty acids) of total lipid extracts isolated from subcutaneous adipose tissue in virgin and pregnant female rats (mean ± standard deviation)



* significantly different from virgin females ($p < 0.05$)

Figure 4.14: The ALNA (18:3n-3) and AA (20:4n-6) content (% total fatty acids) of total lipid extracts isolated from subcutaneous adipose tissue of virgin and pregnant female rats (mean ± standard deviation)



* significantly different from virgin females ($p < 0.05$)

Table 4.15: Fatty acid composition (% total fatty acids) of subcutaneous adipose tissue in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
12:0	0.29 ± 0.04	0.41 ± 0.20	0.44 ± 0.16	0.193
14:0	1.44 ± 0.16	1.56 ± 0.11	1.67 ± 0.22	0.112
16:0	22.33 ± 0.87 [a]	24.00 ± 0.85 [b]	25.97 ± 0.93 [c]	**< 0.001**
18:0	3.37 ± 0.35	3.36 ± 0.44	3.93 ± 0.36	**0.036**
Total saturated	27.44 ± 0.97 [a]	29.38 ± 1.09 [a]	32.03 ± 1.50 [b]	**< 0.001**
16:1n-7	3.86 ± 0.55 [a]	4.96 ± 0.59 [ab]	5.15 ± 1.00 [b]	**0.022**
18:1n-9	25.96 ± 0.96 [a]	26.80 ± 1.22 [ab]	28.02 ± 1.47 [b]	**0.037**
20:1n-9	0.49 ± 0.05	0.41 ± 0.15	0.38 ± 0.12	0.250
24:1n-9	ND	ND	ND	-
Total MUFA	30.31 ± 1.45 [a]	32.17 ± 1.64 [ab]	33.56 ± 2.39 [b]	**0.032**
18:2n-6	37.43 ± 1.84 [a]	33.76 ± 1.30 [b]	27.97 ± 3.02 [c]	**< 0.001**
18:3n-6	0.23 ± 0.02 [a]	0.23 ± 0.03 [a]	0.42 ± 0.09 [b]	**< 0.001**
20:2n-6	0.32 ± 0.02 [a]	ND [b]	0.48 ± 0.13 [c]	**< 0.001**
20:3n-6	0.24 ± 0.03 [a]	0.24 ± 0.02 [a]	0.51 ± 0.15 [b]	**< 0.001**
20:4n-6	1.30 ± 0.14 [a]	1.58 ± 0.23 [a]	2.80 ± 0.72 [b]	**< 0.001**
22:5n-6	0.17 ± 0.05	0.24 ± 0.14	0.21 ± 0.06	0.416
Total n-6 PUFA	39.69 ± 1.98 [a]	36.05 ± 1.35 [ab]	32.38 ± 3.88 [b]	**< 0.001**
18:3n-3	1.92 ± 0.17 [a]	1.86 ± 0.14 [a]	1.20 ± 0.22 [b]	**< 0.001**
22:5n-3	0.24 ± 0.03 [a]	0.20 ± 0.06 [a]	0.42 ± 0.12 [b]	**0.001**
22:6n-3	0.38 ± 0.05	0.34 ± 0.12	0.40 ± 0.05	0.531
Total n-3 PUFA	2.55 ± 0.18 [a]	2.40 ± 0.15 [a]	2.03 ± 0.15 [b]	**< 0.001**

ND negligible detected (mean <0.1%)
a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.16: Fatty acid composition (% total fatty acids) of intra-abdominal adipose in virgin and pregnant female rats (mean ± standard deviation)

Fatty acid	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
12:0	0.13 ± 0.05	ND	0.10 ± 0.01	0.569
14:0	1.19 ± 0.16	1.25 ± 0.13	1.17 ± 0.04	0.539
16:0	23.71 ± 1.31	25.09 ± 0.81	24.48 ± 0.66	0.097
18:0	3.41 ± 0.19	3.27 ± 0.34	3.09 ± 0.38	0.237
Total saturated	28.49 ± 1.49	29.75 ± 1.24	28.93 ± 0.86	0.267
16:1n-7	3.87 ± 0.92	4.84 ± 0.24	4.50 ± 0.96	0.165
18:1n-9	25.08 ± 0.26	26.48 ± 1.08	26.08 ± 1.25	0.071
20:1n-9	0.57 ± 0.08	0.44 ± 0.15	0.51 ± 0.16	0.296
Total MUFA	29.54 ± 1.14	31.76 ± 0.92	31.08 ± 2.03	0.066
18:2n-6	37.45 ± 2.08 [a]	34.15 ± 1.48 [b]	35.73 ± 1.31 [ab]	**0.019**
18:3n-6	0.22 ± 0.02	0.23 ± 0.04	0.21 ± 0.02	0.712
20:2n-6	0.30 ± 0.03 [a]	ND [b]	0.30 ± 0.01 [a]	**< 0.001**
20:3n-6	0.22 ± 0.04	0.20 ± 0.03	0.20 ± 0.03	0.761
20:4n-6	0.97 ± 0.10	1.07 ± 0.22	0.86 ± 0.12	0.115
22:5n-6	0.10 ± 0.09	0.14 ± 0.08	0.15 ± 0.05	0.444
Total n-6 PUFA	39.26 ± 2.24 [a]	35.85 ± 1.76 [b]	37.46 ± 1.36 [ab]	**0.026**
18:3n-3	2.21 ± 0.20	2.11 ± 0.11	2.08 ± 0.13	0.353
22:5n-3	0.17 ± 0.10	0.20 ± 0.09	0.20 ± 0.06	0.768
22:6n-3	0.30 ± 0.05	0.34 ± 0.09	0.26 ± 0.07	0.172
Total n-3 PUFA	2.71 ± 0.21	2.65 ± 0.24	2.53 ± 0.13	0.325

ND negligible detected (mean <0.1%)
a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.17: Correlations observed between the fatty acid composition (% total fatty acids) of adipose tissue total lipid extracts and plasma NEFA (Pearson r values unless otherwise indicated)

	Subcutaneous adipose vs. Plasma NEFA (n=17)		Intra-abdominal adipose vs. Plasma NEFA (n=17)	
12:0	-0.061 †		-0.476 †	
14:0	-0.320		-0.311	
16:0	-0.059		-0.329	
18:0	0.088		0.280	
20:0	-0.121 †		0.143	
22:0	-		0.409 †	
Total saturated	-0.173		-0.269	
16:1n-7	0.203		0.193	
18:1n-9	0.320		-0.240	
20:1n-9	0.132 †		0.003†	
24:1n-9	-		0.138 †	
Total MUFA	0.263		-0.174	
18:2n-6	-0.514	*	-0.141	
18:3n-6	-0.114		-0.094	
20:2n-6	0.176		0.060	
20:3n-6	-0.297 †		-0.126 †	
20:4n-6	-0.291 †		0.378 †	
22:5n-6	0.037		0.299	
Total n-6 PUFA	-0.199 †		-0.542 †	*
18:3n-3	-0.531	*	-0.219	
20:4n-3	-		-	
20:5n-3	-0.383 †		-0.276 †	
22:5n-3	0.804	***	0.122	
22:6n-3	0.411		0.153	
Total n-3 PUFA	-0.349		-0.132	

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † Spearman r value

Figure 4.15: Graph to illustrate the relationship observed between the LA (18:2n-6) content (% total fatty acids) of subcutaneous adipose tissue total lipid extracts and plasma NEFA

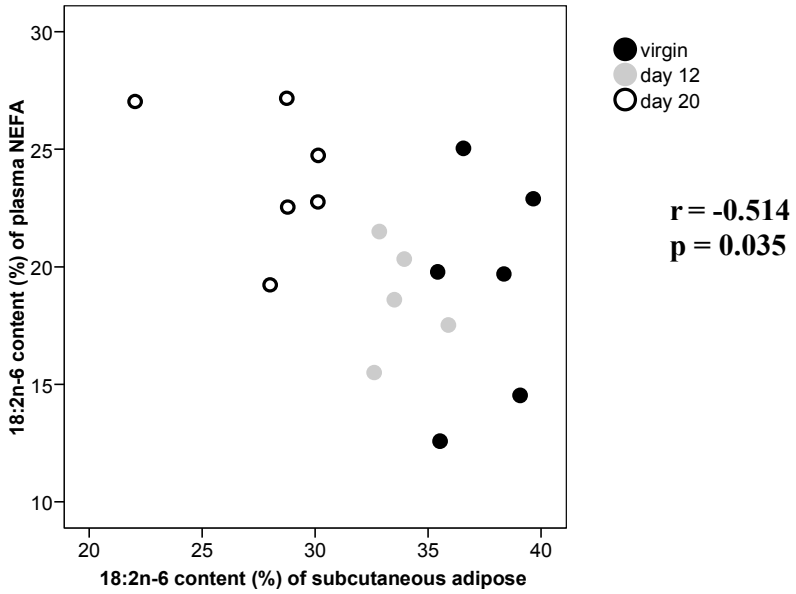
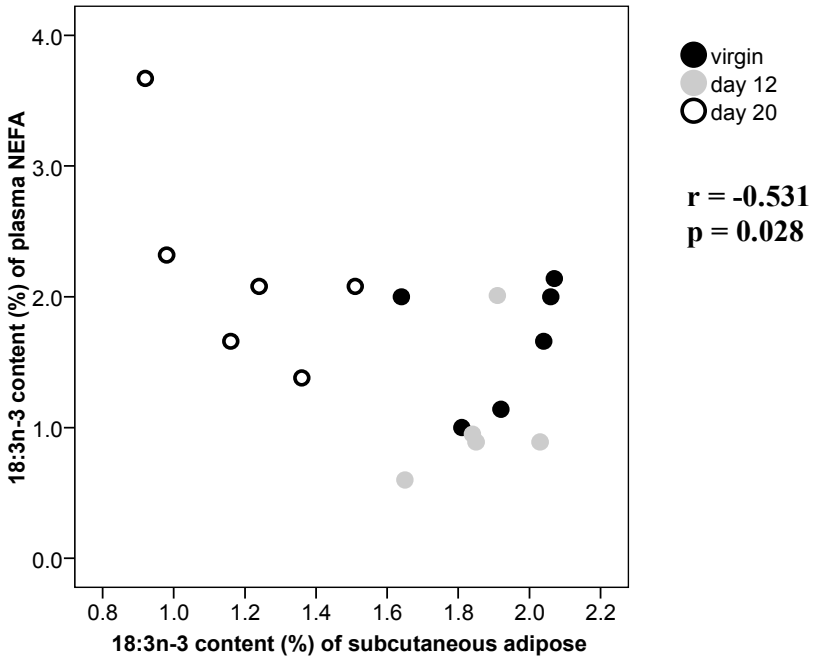


Figure 4.16: Graph to illustrate the relationship observed between the ALNA (18:3n-3) content (% total fatty acids) of subcutaneous adipose tissue total lipid extracts and plasma NEFA



4.3.8 Plasma sex hormones

Plasma concentrations of oestradiol, progesterone and testosterone were measured (see table 4.18). Pregnancy was associated with higher progesterone and testosterone concentrations, but oestradiol was not significantly affected. This is likely to be due to the high variability of oestradiol status among the virgin females, which were not controlled for their stage of the oestrus cycle. Progesterone concentrations were higher mid-pregnancy than at the end of pregnancy. Progesterone and testosterone concentrations demonstrated a significant positive correlation ($r = 0.804$, $p < 0.001$).

Sex hormone concentrations were assessed for correlations with plasma DHA status (see table 4.19). These correlations gave little indication that the trend for increasing DHA status with pregnancy is related to plasma sex hormone concentrations. Where a significant correlation was observed between testosterone status and plasma TAG DHA content, there was a clear difference in the pattern of relationships observed between virgin and pregnant females (see figure 4.17). Plasma testosterone has a significant inverse relationship with plasma TAG DHA status in virgin females ($r = -0.840$, $p = 0.036$), but a trend for a positive relationship among pregnant females ($r = 0.558$, $p = 0.094$).

It is important to consider that the circulating concentrations of fatty acids such as DHA during pregnancy will be affected by the transfer of fatty acids to the developing fetus, which cannot be quantified from the results of this study.

Table 4.18: Plasma sex hormone concentrations in virgin and pregnant female rats (mean ± standard deviation)

	Virgin (n=6)	Day 12 of pregnancy	Day 20 of pregnancy	ANOVA p value
Oestradiol (pM)	141.5 ± 129.4 (n=6)	127.2 ± 41.7 (n=5)	82.8 ± 6.7 (n=4)	0.582
Progesterone (nM)	33.5 ± 25.8 [a] (n=6)	340.4 ± 35.2 [b] (n=4)	180.0 ± 18.6 [c] (n=5)	**< 0.001**
Testosterone (nM)	1.8 ± 0.5 [a] (n=6)	3.9 ± 0.3 [b] (n=5)	3.5 ± 0.9 [b] (n=5)	**< 0.001**

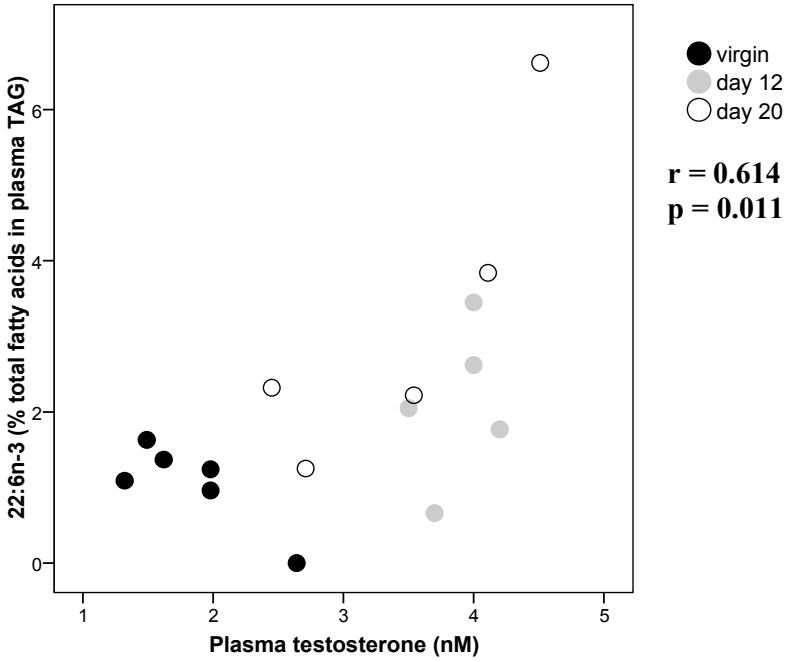
a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.19: Correlations observed between the DHA content (% total fatty acids) of plasma lipids and circulating sex hormone concentrations (Pearson r values)

	Testosterone (n=16)	Oestradiol (n=15)	Progesterone (n=15)
PC	0.379	-0.232	0.094
TAG	**0.614***	-0.368	0.299
CE	0.487	-0.309	0.218
NEFA	0.449	-0.188	0.247

* $p < 0.05$

Figure 4.17: Illustration of the correlation observed between plasma TAG DHA content (% total fatty acids) and plasma testosterone concentrations



4.3.9 Expression of desaturase and elongase genes in the liver

mRNA expression of Δ6 desaturase, Δ5 desaturase and elongase in the liver was assessed to determine whether the expression of the genes for these enzymes changes in response to pregnancy. There were no significant differences between the level of expression of these genes in virgin and pregnant females (see table 4.20).

The mRNA expression of desaturase and elongase genes was correlated to the fatty acid substrates and products of the respective enzymes in liver lipids, to assess whether the

expression of the genes for these enzymes has a direct effect upon their activity within the liver (see table 4.21). High enzyme activity would be indicated by an inverse relationship between mRNA expression and the substrate:product ratio of the fatty acids metabolised by that enzyme. The only significant correlation observed was for the expression of elongase with its n-3 fatty acid substrates and products. However, when plotted, this can be attributed to a single outlier (see figure 4.18). The mRNA expression of desaturase and elongase genes during pregnancy therefore does not appear to exert control over the relative activity of these enzymes.

Expression of desaturase and elongase genes was correlated to plasma sex hormone concentrations (see table 4.22). A significant correlation was identified between plasma progesterone and the expression of the Δ6 desaturase gene (see figure 4.19). This indicates that the expression of the Δ6 desaturase gene, coding for the rate limiting enzyme in the production of LC PUFA, may be influenced by the increasing plasma progesterone concentrations which are a feature of pregnancy.

Table 4.20: Liver fatty acid desaturase and elongase mRNA expression in virgin and pregnant female rats (arbitrary units of expression derived from standard curve and adjusted for geometric mean of three housekeeping genes, mean ± standard deviation)

	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
Elovl5 (elongase)	0.74 ± 0.14	0.85 ± 0.28	0.66 ± 0.13	0.287
FADS1 (Δ5 desaturase)	0.54 ± 0.09	0.60 ± 0.19	0.66 ± 0.23	0.529
FADS2 (Δ6 desaturase)	0.51 ± 0.11	0.91 ± 0.29	0.86 ± 0.59	0.198

Table 4.21: Correlations observed between liver fatty acid desaturase and elongase mRNA expression and their respective substrate and product fatty acid ratios within liver lipids (Pearson r values)

	Liver PC	Liver PE	Liver CE	Liver TAG
Δ6 DESATURASE				
18:2n-6 to 18:3n-6 ratio	-0.007 (n=17)	0.237 (n=12)	-0.269 (n=11)	-0.117 (n=17)
Δ5 DESATURASE				
20:3n-6 to 20:4n-6 ratio	-0.313 (n=17)	-0.275 (n=17)	-0.340 (n=17)	0.020 (n=17)
20:4n-3 to 20:5n-3 ratio	-0.402 † (n=16)	-	-0.536 (n=10)	-0.417 (n=16)
ELONGASE				
18:3n-6 to 20:3n-6 ratio	-0.438 (n=17)	-0.286 (n=17)	-0.106 (n=11)	-0.247 (n=17)
20:5n-3 to 22:5n-3 ratio	**0.638**** (n=17)	**0.595*** (n=17)	**0.680*** (n=9)	0.408 (n=17)

* $p < 0.05$, ** $p < 0.01$

Figure 4.18: Illustration of the correlation observed between liver elongase mRNA expression and the ratio of 20:5n-3 to 22:5n-3 within liver PC

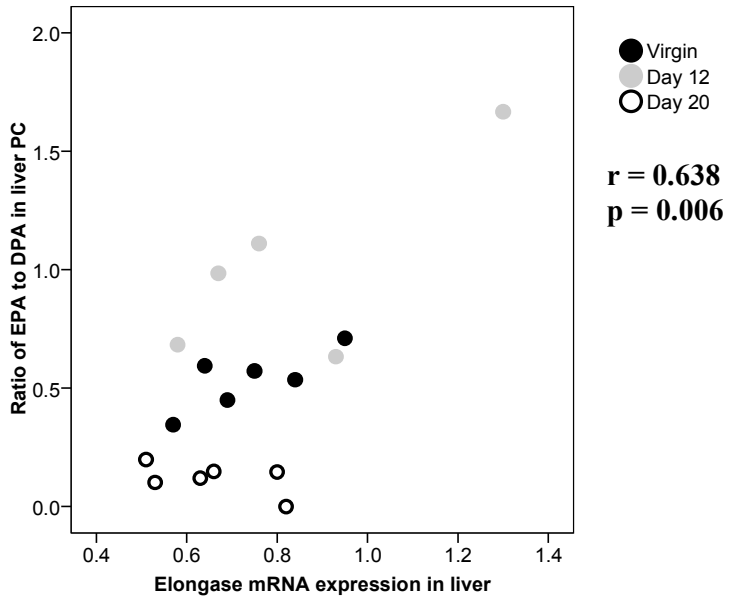
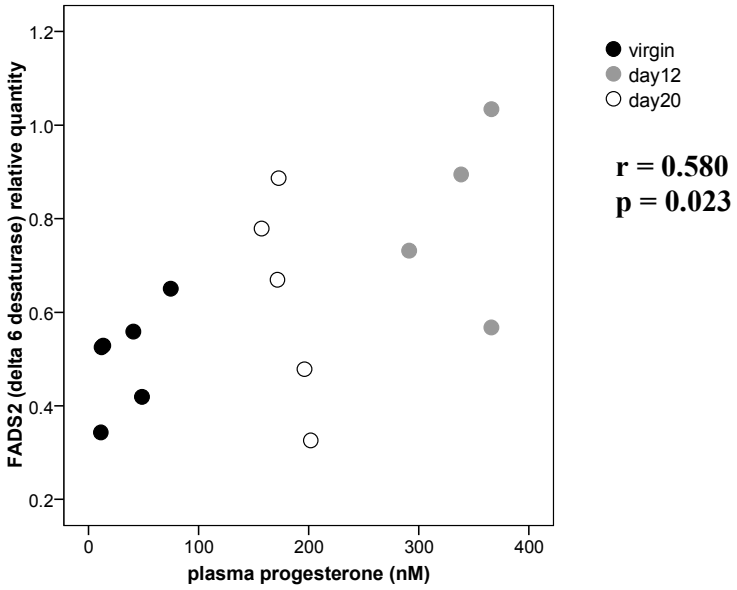


Table 4.22: Correlations observed between plasma sex hormone concentrations and the expression of fatty acid desaturase and elongase mRNA within the liver (Pearson r values)

	Testosterone (nM) (n=16)	Oestradiol (pM) (n=15)	Progesterone (nM) (n=15)
Elovl5 (elongase)	0.027	0.087	0.251
FADS1 (Δ5 desaturase)	0.001	-0.126	0.182
FADS2 (Δ6 desaturase)	0.312	0.066	**0.580 ***

* $p < 0.05$

Figure 4.19: Illustration of the correlation observed between liver Δ6 desaturase mRNA expression and plasma progesterone concentration



4.3.10 Indirect assessment of desaturase and elongase activities in the liver

Substrate:product ratios of fatty acids within liver lipids were calculated as a method of indirectly assessing whether the activities of desaturase and elongase enzymes in the liver change in response to pregnancy. Higher enzyme activity could be inferred by a lower substrate:product ratio.

Δ9 desaturase

Substrate:product ratios were calculated to assess Δ9 desaturase activity (see table 4.23). Data from liver PC and CE suggests that Δ9 desaturase activity is higher in pregnant females than in virgin females, though these effects were mixed. Substrate:product ratios in liver PC indicate that Δ9 desaturase activity is significantly higher in day 20 pregnant females compared with both virgin and day 12 pregnant females. In liver CE, there was also a trend for Δ9 desaturase activity to be higher in day 20 pregnant females compared to virgin females, but these data suggest that Δ9 desaturase activity may be lower at day 12 gestation than in either virgin or day 20 pregnant females.

Correlations were then performed to assess whether there is a relationship between Δ9 desaturase activity and circulating sex hormone concentrations (see table 4.24). There were no consistent trends across lipid fractions which might indicate a role of circulating sex hormones upon the differences observed in the substrate:product ratios of Δ9 desaturase.

Table 4.23: The Δ9 desaturase substrate:product fatty acid ratio within liver lipids in virgin and pregnant female rats (18:0 to 18:1n-9, mean ± standard deviation)

	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
Liver PC	8.8 ± 1.5 [a]	8.2 ± 0.8 [a]	5.8 ± 1.3 [b]	**0.002**
Liver PE	12.7 ± 2.3	11.0 ± 1.4	15.0 ± 3.5	0.072
Liver CE	3.0 ± 1.1 [a]	5.9 ± 2.1 [b]	1.9 ± 1.0 [a]	**0.001**
Liver TAG (x 10^1)	1.5 ± 0.5	1.5 ± 0.1	1.6 ± 0.1	0.878

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.24: Correlations observed between the ratio of 18:0 to 18:1n-9 within liver lipids and circulating sex hormone concentrations (Pearson r values)

18:0 to 18:1n-9 ratio	Liver PC	Liver PE	Liver CE	Liver TAG
Testosterone (n=16)	-0.249	-0.009	0.364	-0.027
Oestradiol (n=15)	0.040	-0.253	-0.158	**-0.735****
Progesterone (n=15)	-0.088	-0.085	**0.548***	0.090

* $p < 0.05$, ** $p < 0.01$

Δ6 desaturase

Substrate:product ratios to assess Δ6 desaturase activity indicated that the activity of this enzyme is lower at day 12 gestation than in virgin or day 20 gestation females (see table 4.25). There were no significant differences between virgin females and day 20 gestation females.

When data for plasma sex hormones was correlated to this marker of Δ6 desaturase activity, there was a trend for reduced Δ6 desaturase activity with increasing progesterone concentrations (see table 4.26). However, when plotted it was clear that this relationship between progesterone and Δ6 desaturase activity is an artefact of the distinct relationships exhibited in virgin females compared to pregnant females, and of the lack of detectable 18:3n-6 in pregnant females (see figure 4.20).

Table 4.25: The Δ6 desaturase substrate:product fatty acid ratio within liver lipids of virgin and pregnant female rats (LA to 18:3n-6, mean ± standard deviation)

	Virgin	Day 12 of pregnancy	Day 20 of pregnancy	ANOVA p value
Liver PC	36.9 ± 9.0 [ab] (n=6)	45.2 ± 5.5 [a] (n=5)	28.0 ± 4.0 [b] (n=6)	**0.003**
Liver PE	33.6 ± 10.6 [a] (n=6)	58.0 ± 8.0 [b] (n=4)	28.1 ± 24.8 [ab] (n=2)	**0.022**
Liver CE	38.9 ± 22.2 (n=5)	65.1 ± 25.9 (n=2)	33.9 ± 15.5 (n=4)	0.253
Liver TAG	44.5 ± 7.1 [ab] (n=6)	54.7 ± 9.9 [a] (n=5)	34.3 ± 5.4 [b] (n=6)	**0.002**

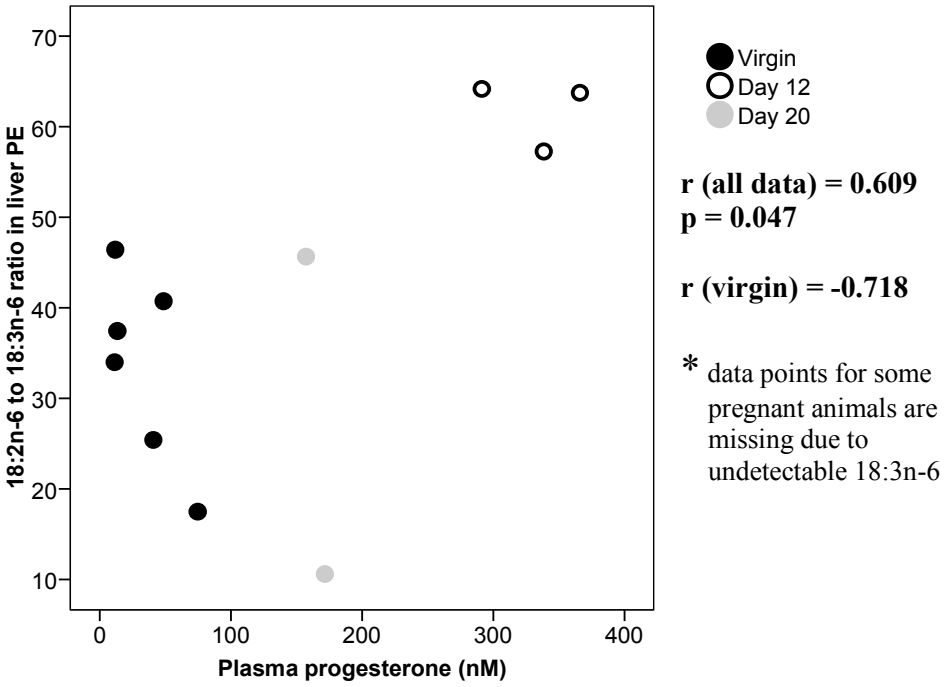
a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.26: Correlations observed between the 18:2n-6 to 18:3n-6 ratio within liver lipids and circulating sex hormone concentrations (Pearson r values)

LA to 18:3n-6 ratio	Liver PC	Liver PE	Liver CE	Liver TAG
Testosterone	0.012 (n=16)	0.330 (n=12)	0.248 (n=10)	0.138 (n=16)
Oestradiol	-0.044 (n=15)	0.015 (n=12)	-0.255 (n=9)	0.189 (n=15)
Progesterone	0.317 (n=15)	**0.609*** (n=11)	0.383 (n=10)	0.446 (n=15)

* $p < 0.05$

Figure 4.20: Illustration of the correlation observed between the ratio of 18:2n-6 to 18:3n-6 in liver PE and circulating progesterone concentration



Δ5 desaturase activity

Substrate:product ratios of n-6 fatty acids indicate that the activity of Δ5 desaturase was higher at day 20 gestation than at day 12 gestation or in virgin females (see table 4.27). The lack of a relationship with n-3 fatty acids is likely to be due to 20:4n-3 often being undetectable in liver lipids. There was no indication that the effect of pregnancy observed upon Δ5 desaturase activity correlated to plasma sex hormone concentrations (see table 4.28).

Table 4.27: The Δ5 desaturase substrate:product fatty acid ratio within liver lipids of virgin and pregnant female rats (n-6: 20:3n-6 to AA; n-3: 20:4n-3 to EPA, mean ± standard deviation)

	Virgin	Day 12 of pregnancy	Day 20 of pregnancy	ANOVA p value
n-6 fatty acids				
Liver PC (x 10^3)	43.9 ± 7.0 [a] (n=6)	46.9 ± 8.8 [a] (n=5)	18.7 ± 3.8 [b] (n=6)	**< 0.001**
Liver PE (x 10^3)	23.8 ± 2.4 [a] (n=6)	26.8 ± 5.4 [a] (n=5)	12.4 ± 4.7 [b] (n=6)	**< 0.001**
Liver CE (x 10^3)	29.3 ± 25.5 [ab] (n=6)	58.8 ± 35.5 [a] (n=5)	14.1 ± 18.1 [b] (n=6)	**0.045**
Liver TAG (x 10^2)	14.1 ± 3.2 (n=6)	12.5 ± 2.5 (n=5)	8.8 ± 4.7 (n=6)	0.066
n-3 fatty acids				
Liver PC (x 10^3)	ND (n=6)	16.9 ± 23.8 (n=5)	ND (n=5)	0.096
Liver PE	ND (n=6)	ND (n=5)	ND (n=5)	-
Liver CE	ND (n=4)	ND (n=4)	1.9 ± 2.7 (n=2)	0.128
Liver TAG (x 10^2)	ND (n=6)	9.6 ± 13.7 (n=5)	ND (n=5)	0.100

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.28: Correlations observed between Δ5 desaturase substrate:product fatty acid ratios within liver lipids and circulating sex hormone concentrations (Pearson r values)

20:3n-6 to AA ratio	Liver PC	Liver PE	Liver CE	Liver TAG
Testosterone	-0.193	-0.179	0.197	-0.470
Oestradiol	0.318	0.286	0.179	0.463
Progesterone	0.020	0.068	0.280	-0.299
20:4n-3 to EPA ratio	Liver PC	Liver PE	Liver CE	Liver TAG
Testosterone	0.328	-	0.407	0.318
Oestradiol	-0.205	-	-	-0.177
Progesterone †	0.459	-	0.137	0.447

Elongase activity

There was limited evidence to support an effect of pregnancy upon elongase activity as assessed using n-6 fatty acid substrates, with only substrate:product ratios in liver PC demonstrating a significant reduction in activity at day 20 gestation compared to day 12 gestation or virgin females. In contrast, there was a clear trend apparent across all liver

lipid fractions for increased elongase activity upon n-3 substrates with pregnancy (see table 4.29). The effects observed upon elongase activity during pregnancy did not correlate to plasma sex hormone concentrations (see table 4.30).

Table 4.29: The ratio of elongase substrate:product fatty acid content of liver lipids in virgin and pregnant female rats (n-6: 18:3n-6 to 20:3n-6; n-3 EPA to 22:5n-3, mean ± standard deviation)

	Virgin	Day 12 of pregnancy	Day 20 of pregnancy	ANOVA p value
n-6 fatty acids				
Liver PC (x 10^1)	3.3 ± 0.6 [a] (n=6)	2.0 ± 0.4 [a] (n=5)	11.7 ± 4.3 [b] (n=6)	**< 0.001**
Liver PE (x 10^1)	3.5 ± 1.5 (n=6)	1.2 ± 0.7 (n=5)	3.4 ± 0.7 (n=6)	0.625
Liver CE	1.6 ± 1.2 (n=4)	0.2 ± 0.2 (n=4)	1.8 ± 0.6 (n=3)	0.060
Liver TAG	1.3 ± 0.4 (n=6)	1.2 ± 0.3 (n=5)	1.7 ± 0.6 (n=6)	0.066
n-3 fatty acids				
Liver PC (x 10^1)	5.3 ± 1.3 [a] (n=6)	10.2 ± 0.4 [b] (n=5)	1.2 ± 0.7 [c] (n=6)	**< 0.001**
Liver PE (x 10^1)	1.6 ± 0.3 [a] (n=6)	3.9 ± 1.6 [b] (n=5)	0.6 ± 0.5 [a] (n=6)	**< 0.001**
Liver CE (x 10^1)	8.4 ± 6.1 (n=4)	10.9 ± 4.0 (n=2)	0.8 ± 1.4 (n=3)	0.102
Liver TAG (x 10^1)	5.9 ± 1.0 [a] (n=6)	6.0 ± 2.0 [a] (n=5)	1.3 ± 0.7 [b] (n=6)	**< 0.001**

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 4.30: Correlations observed between elongase substrate:product fatty acid ratios circulating sex hormone concentrations (Pearson r values)

18:3n-6 to 20:3n-6 ratio	Liver PC	Liver PE	Liver CE	Liver TAG
Testosterone	0.339	0.075	-0.347	0.315
Oestradiol	-0.246	-0.154	0.194	-0.168
Progesterone	-0.034	-0.221	-0.497	0.044
EPA to 22:5n-3 ratio	Liver PC	Liver PE	Liver CE	Liver TAG
Testosterone	0.227	0.340	-0.380	-0.219
Oestradiol	0.087	0.122	-0.372	0.373
Progesterone	0.415	0.506	0.067	-0.035

4.4 Discussion

This study set out to address the hypothesis that changes to rat tissue fatty acid composition during pregnancy are mediated by sex hormones and/or the expression of genes for enzymes involved in endogenous synthesis of LC PUFA. Data was generated in support of this hypothesis, and in addition novel features of the effects of pregnancy upon tissue 22:5n-6 content and the complex nature of the changes in fatty acid composition during pregnancy were identified.

My data confirms the findings of other authors who have identified that the 16:0 and DHA content of both plasma and liver phospholipids is higher at day 20 of pregnancy than in virgin females, and that the 18:0 and AA content is lower. The concurrent effects upon 16:0 with DHA and 18:0 with AA, are most likely to be due to their common pairing within phospholipid structure. The pattern of effects observed upon TAG were markedly different, with the AA content of plasma and liver TAG significantly higher during pregnancy than in virgin females, again confirming the previous findings of other authors(168) and indicating that there are significant functional differences between these lipid pools.

My data highlights additional significant features of the effects of pregnancy upon plasma and tissue fatty acid composition. In particular, the 22:5n-6 content of plasma lipids was found to be significantly higher among pregnant rats at day 20 of gestation compared to virgin females, indicating that the effects of pregnancy upon LC PUFA synthesis are not limited to LC n-3 PUFA. Indeed, the marked increases in the content of 22:5n-6 in plasma and tissues may account for some of the reductions in AA content observed to occur with pregnancy.

The effects of pregnancy on fatty acid composition were also tissue specific. For example, the fatty acid 22:5n-6 was found at the highest levels in plasma lipids at day 20 of pregnancy. In contrast, the content of 22:5n-6 within liver lipids PC, PE and TAG was markedly lower, and peaked at day 12 of gestation, while 22:5n-6 content within adipose tissue was unchanged by pregnancy. The high content of this fatty acid within the maternal plasma, rather than its incorporation into maternal tissues, gives some indication that this fatty acid may in fact be preferentially mobilised into the maternal plasma in order to be available to the fetus.

The mid-gestation (day 12 of pregnancy) group used in this study provides further information upon the nature of the changes in fatty acid composition which occur during pregnancy. There is clearly a complex relationship between fatty acid status and pregnancy, as the day 12 group did not represent a simple 'mid point' of fatty acid composition status when compared to virgin and day 20 gestation animals. In fact, the fatty acid status of this group was often the opposite of what would be expected by simple extrapolation. For example, plasma and liver phospholipid 16:0 and DHA contents were significantly lower in the day 12 group when compared to virgin females, yet these fatty acids were significantly higher than virgin females by day 20 of gestation. Further studies could be conducted to investigate the timing of these extreme changes in fatty acid composition, with a longitudinal study of plasma phospholipids providing a greater potential for insight into these differences than was achieved within the current study design.

Plasma fatty acid composition was observed to have a close relationship with the fatty acid composition of the liver, but a much more limited relationship with adipose tissue composition. This finding is in contrast to the numerous and significant correlations which had been observed between adipose tissue and plasma NEFA fatty acid composition in chapter 3, and indicates that there was a strong influence of dietary fatty acids upon the relationship between adipose tissue and plasma lipids in that study.

Adipose tissue responded to pregnancy in a depot-specific manner, with subcutaneous adipose tissue demonstrating a greater number of changes in fatty acid composition in response to pregnancy than was observed in intra-abdominal adipose tissue. There was a reduction in the EFA content of subcutaneous adipose tissue during pregnancy, indicating that under low-fat feeding conditions, EFA may be preferentially released from subcutaneous adipose tissue during pregnancy in order to meet the requirements for LC PUFA synthesis. However, it would be necessary to demonstrate that these reductions in EFA content of adipose tissue were accompanied by a reduction in total fat mass in order to fully support this suggestion, data which was not available in this study.

As was the case in chapter 3 no significant correlations between plasma DHA content and plasma sex hormones were observed. In chapter 3, this was attributed to the

confounding effects of diet, a variable which was controlled within this study. However, it is possible that within this cohort of pregnant animals, any potential effect of sex hormones upon plasma DHA content may have been obscured by transfer of DHA to the fetus, a variable that could not be assessed in this study, but which could be investigated further using stable-isotope labelled fatty acids.

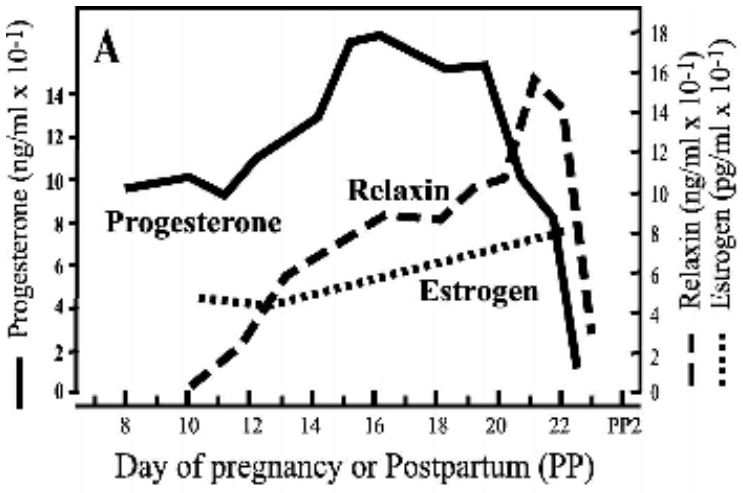
No significant effects of pregnancy were observed upon the mRNA expression of elongase or Δ6 and Δ5 desaturase genes in the liver. When mRNA expression was assessed for potential relationships with sex hormone status, it was found that there was a significant positive correlation between plasma progesterone and the expression of Δ6 desaturase mRNA in the liver. This indicates that the increasing progesterone concentrations which are a feature of pregnancy associate with an increased availability of Δ6 desaturase, a finding of particular importance given that Δ6 desaturase is the rate-limiting step in the synthesis of LC PUFA. This supports the hypothesis that sex hormones and the expression of enzymes involved in LC PUFA synthesis mediate the effect of pregnancy upon tissue fatty acid composition. However, when data from chapter 3 was re-examined, no correlation was identified between mRNA expression of Δ6 desaturase and progesterone status within non-pregnant females (Pearsons $R = 0.257$, $p = 0.337$). This suggests that the relationship between progesterone and mRNA expression of Δ6 desaturase during pregnancy may not be directly causal, but may indicate the influence of an additional variable which associates with changes to progesterone status during pregnancy.

The activities of enzymes involved in LC PUFA synthesis in the liver were indirectly assessed using the substrate:product ratios in liver lipids. Though this marker is a relatively crude index of enzyme activity, as it cannot for example take into account the potential influence of specificity in lipid assembly, it was used to assess whether pregnancy might have an effect upon enzyme activity. These data indicate that there is a complex pattern of changes in enzyme activity with pregnancy. Δ6 desaturase activity as assessed by this marker was lowest at day 12 gestation, and Δ5 and Δ9 desaturase activities higher in day 20 pregnant females than in virgin or day 12 gestation females. There was also a trend for greater elongase activity upon n-3 substrates among day 20 gestation females. Data therefore indicate that pregnancy may induce changes in both the relative abundance, and the activity of the enzymes involved in LC PUFA synthesis.

Few correlations were observed between substrate:product ratios of liver fatty acids and plasma sex hormone status. Of the statistically significant correlations which were observed, there was evidence when plotted that these correlations were in fact artefacts of outliers (see figure 4.18). The statistically significant relationship between testosterone and progesterone status ($r = 0.804$, $p < 0.001$) also make it difficult to say with certainty which of these variables was responsible for any relationship observed between these hormones and markers of enzyme activity. The current data therefore provides some indication of a role of pregnancy upon the activity of desaturase and elongase enzymes, but does not support a relationship between this change in activity and the circulating concentrations of sex hormones assessed.

Testosterone, oestradiol and progesterone are not the only hormones whose concentrations alter in response to pregnancy. It is possible that other hormones which are altered during pregnancy such as relaxin (see figure 4.21), prolactin, aldosterone and corticosterone(234) may also have a role in mediating the effects observed upon fatty acid composition.

Figure 4.21: Levels of ovarian hormones during rat gestation(235)



Based upon the finding of this study, future investigations would benefit from additional time-points during rat pregnancy, particularly given the distinct and non-linear patterns of change during pregnancy in both tissue fatty acid composition, and the substrate:product ratio of fatty acids in liver lipids as an index of desaturase and elongase enzymes. Direct assessment of enzyme activity by use of a method such as provision of dietary stable isotopes would be of use to confirm the indications from the

current data, as well as providing the possibility of gaining quantitative data upon the rates of transfer to the developing fetus. Analysis of additional sex, and other, hormones may provide further information upon the mechanisms controlling fatty acid status during pregnancy.

Among human longitudinal studies of maternal blood lipid fatty acid status during pregnancy, the main information which is currently lacking is pre-conceptional fatty acid status. Given the dramatic differences in the pattern of fatty acid composition between day 12 gestation and virgin data in the rat, this could be of importance. Analysis of plasma samples from large prospective studies of women of child-bearing age such as the Southampton Women's Survey may enable the effects of pregnancy upon fatty acid synthesis, metabolism and status to be investigated further.

Chapter 5: Pregnancy and immune function in the rat

5.1 Introduction

5.1.1 Aims and hypothesis of current study

This study sets out to identify the effects of pregnancy upon markers of maternal immune function in the rat. Pregnancy requires maternal immune adaptations in order to allow tolerance to paternal antigens and avoid rejection of the developing fetus. Adaptations to maternal immunity during pregnancy which have been observed in murine and human studies include thymic involution and the Th1/Th2 switch, but the immune adaptations of the rat during pregnancy are less clear. The effects of pregnancy upon rat immune function will therefore be investigated, in order to later assess whether these normal responses to pregnancy are altered by changing maternal dietary fatty acids during pregnancy.

Immune cell phenotype and selected markers of immune function including lymphocyte proliferation and production of signalling molecules such as prostaglandin (PG) E_2 and the cytokines interferon (IFN)-γ and interleukin (IL)-4, will be assessed. Gross markers of immune adaptations such as lymphoid organ size and cellularity will also be monitored. It is hypothesised that:

a) rat pregnancy causes thymic involution and reduced lymphoid organ cellularity
b) changes in lymphoid organ cellularity are associated with changes to the profile of immune cell subsets present
c) the rat will demonstrate changes in Th1/Th2 balance in response to pregnancy, favouring production of Th2 cytokines such as IL-4.

5.1.2 Thymic involution of pregnancy

Maternal thymic involution is a feature of pregnancy which involves neural, endocrine and immune influences(176). Involution is specific to the cortex (the outer structures of the thymus – containing immature T cells), while the medulla (the inner structure of the thymus – containing more mature T cells) undergoes hyperplasia(177). These changes may therefore prevent the generation of new conceptus-targeted T cells, while maintaining an adequate historical T cell repertoire. Suggested mechanisms involved in thymic involution during pregnancy include the role of mediators such as

progesterone(177;187), gonadotrophin-releasing hormone and prohibitin(181). The maternal thymus then rapidly regenerates after the end of lactation(176).

5.1.3 Th1/Th2 balance during pregnancy

Th1 and Th2 cells are two subsets of $CD4^+$ T helper cells. These cells cannot be distinguished morphologically, but differ according to the cytokines they produce. Th1 cells secrete predominantly pro-inflammatory cytokines such as IFN-γ, tumour necrosis factor (TNF)-β, IL-2 and TNF-α which activate macrophages and cell-mediated immune responses. Th2 cells secrete cytokines including IL-4, IL-5, IL-6, IL-10 and IL-13 which promote antibody or humoral responses. The cytokines of one T helper subset inhibit the activation of the other subset, and so Th1 and Th2 responses are mutually exclusive. The development of Th1 or Th2 dominated responses is affected by the cytokines produced during antigen-presentation, with IFN-γ promoting Th1 responses, and IL-4 promoting Th2 responses. It has been identified that progesterone, a sex hormone associated with pregnancy, can induce production of Th2 type cytokines by human peripheral blood mononuclear cells (PBMC) or established human Th1 clones *in vitro*(174).

A Th2 response during pregnancy is advantageous as the Th2 response involves a humoral (antibody-based) rather than cellular immune response. This prevents maternal immune cells targeting the feto-placental unit, while maintaining antibody production. Maintenance of antibody production is beneficial to the fetus, as antibodies (particularly IgG) can be transferred across the placenta and via breastmilk, providing protective immunity for the neonate until the infant's own immune system is sufficiently matured(175).

5.1.4 Human studies of immune function during pregnancy

Pregnancy associated changes in auto-immune disease symptoms provide evidence for human changes in Th1/Th2 balance during pregnancy. Rheumatoid arthritis is an auto-immune disease associated with high levels of Th1 cytokine expression, which demonstrates improvements during pregnancy(175), while systemic lupus erythematosus is an antibody mediated auto-immune disease associated with Th2 cytokine expression, which primarily affects women during reproductive years(176). These data suggest that a 'switch' towards Th2 cytokine production is a feature of human pregnancy.

Studies which used PBMC collected from healthy pregnant women have identified that in humans the proportions of $CD4^+$ and $CD8^+$ cells within the blood lymphocyte pool remain constant during pregnancy, but differ in terms of their cytokine production profile, with a shift towards Th2 producing cells(172). Women with recurrent spontaneous abortions have demonstrated elevated Th1/Th2 ratios when compared to women who have successful pregnancies(171). Evidence therefore suggests that a Th2 switch is essential for successful maintenance of pregnancy in humans(172).

5.1.5 Animal studies of immune function during pregnancy

Disease models in mice have also demonstrated the importance of Th2 responses in the maintenance of successful pregnancy. Infection of mice with the parasite *Leishmania major* induces a strong Th1 response. When pregnant mice were infected with *Leishmania major* there was an increase in spontaneous resorptions which was associated with increased Th1 cytokine production(173). Murine studies have also demonstrated that the injection of Th1 cytokines induces abortion(178). Th1 responses induce natural killer (NK) cell activity, and the presence of NK cells correlates with fetal resorption in the mouse(175).

Although the murine model has been studied extensively in relation to the immune adaptations of pregnancy, the rat response has been less extensively characterised, with some contradictory findings (see table 5.1). For example, IL-4 producing cells have been identified as being increased(181), decreased(179) or unaffected(180) in rat models during pregnancy.

A study which directly compared human and rat immune responses has identified that there are fundamental species differences in the response to pregnancy, with humans demonstrating reductions in the number of IFN-γ producing cells but without affecting total lymphocyte numbers in the blood, while the rat adapts to pregnancy by reducing total lymphocyte numbers(181).

Table 5.1: Summary of rat studies of immune function during pregnancy

Study	**Model and samples used**	**Response to pregnancy**	
Zhang et al, 2004 (181)	Lewis rat PBMC, spleen and mesenteric lymph node cells	↓ MHC II expression in spleen ↓ CD11c (dendritic cell marker) ↓ antibody production to acetylcholine receptor ↓ antibody production to ovalbumin	↑ IL-10 producing cells in spleen and PBMC ↑ IL-4 producing PBMC ↑ proliferation of mesenteric lymph node cells
Faas et al, 2005 (179)	Wistar rat Whole blood, *in vitro* stimulation with PMA	↓ IL-4 producing Th lymphocytes ↓ IL-4 producing cytotoxic T cells	↑ IFN-γ producing cytotoxic T cells ↑ IFN-γ producing NK cells
Faas et al, 2006 (180)	Wistar rat Whole blood, *in vitro* stimulation with PMA	↓ T lymphocytes ↓ Th lymphocytes ↓ cytotoxic T cells No effect on IL-4 producing cells	↑NK cells ↑ IFN-γ producing Th cells and ↑ IFN-γ producing cytotoxic T cells

5.2 Methods

This study set out to identify whether there are changes in the immune system in normal rat pregnancy. This was assessed by monitoring immune organ weight and cellularity, immune cell subset phenotyping by flow cytometry, lymphocyte proliferation and the production of cytokines and prostaglandins by cultured blood and immune organ mononuclear cells.

All animal work was carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986); for pregnant females, mating was carried out by monogamous breeding (see section 2.1), and maternal tissues collected on either day 12 or day 20 of gestation. Immune cell phenotypes were identified by flow cytometry of lysed whole blood or immune organ mononuclear cell preparations (see section 2.11.1). *Ex vivo* culture was performed to assess lymphocyte proliferation (see section 2.11.2) and the production of IFN-γ, IL-4 and PGE_2 in response to concanavalin A (see section 2.11.3).

The effect of pregnancy upon tissue immune organ weight and cellularity, immune cell subsets, lymphocyte proliferation and the production of cytokines and prostaglandins was assessed by one-way ANOVA. The effect of pregnancy upon the sensitivity of lymphocytes to Concanavalin A (Con A) stimulation was assessed by Kruskal-Wallis test.

5.3 Results

5.3.1 Experimental diets

Animals received a low fat (LF, 3% w/w) soyabean oil based diet (SDS, Witham, UK) which is a standard rat maintenance diet (RM[1]). The nutrient content and fatty acid composition of this diet are described in chapters 2 and 3 (see tables 2.2 and 3.1).

5.3.2 Weight gain and food intake

Virgin females had their weight and food intake monitored over a 20 day period. Pregnant rats had their weight and food intake monitored over either 12 or 20 days of pregnancy (see table 5.2, figure 5.1); typical rat pregnancy is 21-24 days. As would be expected, pregnant females gained significantly more weight per day compared to virgin females. The day 20 gestation group also has significantly higher weight gain per day

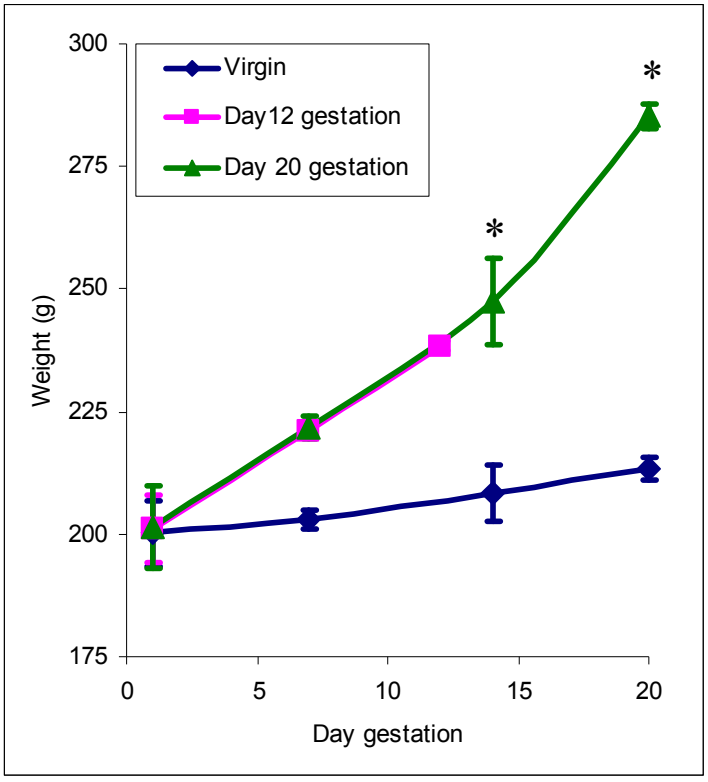
compared with the day 12 gestation group, indicating that further accelerated weight gain was occurring during the 2nd half of gestation. In contrast to the observations in chapter 4 (see table 4.2), this cohort demonstrated a significant increase in food consumption throughout gestation, indicating these rats exhibited significant hyperphagia in response to pregnancy.

Table 5.2: Weight gain and weighed food consumption by virgin and pregnant female rats over the study period (mean ± standard deviation)

	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
Weight gain (g/day)	0.7 ± 0.2 [a]	3.1 ± 0.4 [b]	4.2 ± 0.2 [c]	**< 0.001**
Food consumption (g/day)	14.9 ± 1.1 [a]	17.0 ± 1.3 [b]	19.1 ± 1.2 [c]	**< 0.001**

a, b, c groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Figure 5.1: Weights of virgin and pregnant female rats over the study period (mean ± SEM)



* Significantly different from virgin females (T test, $p < 0.01$)

5.3.3 Immune organ size and cellularity

Immune organs were weighed prior to preparation of cell suspensions, and the number of mononuclear cells recovered counted using a Beckman Coulter counter in order to assess organ cellularity (see table 5.3). Spleen and lymph node weights were maintained throughout pregnancy, while the thymus reduced in absolute weight over gestation. Thymic involution has been reported in other animal models of pregnancy, and is reported to reverse after delivery(176). In the rat, thymic involution was not yet apparent at day 12 of pregnancy. Trends were seen in all immune organs for reduced cellularity with pregnancy, though this only reached significance in the mandibular lymph nodes, which is likely to be due to the high degree of variability in the results obtained for the spleen and thymus.

Table 5.3: Immune organ weight and cellularity of virgin and pregnant female rats (mean ± standard deviation)

	Virgin (n=6)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=6)	ANOVA p value
SPLEEN				
weight (mg)	519.3 ± 36.9	625.5 ± 104.1	523.5 ± 105.4	0.115
% body weight	0.23 ± 0.03 ^{ab}	0.27 ± 0.05 ^{a}	0.19 ± 0.02 ^{b}	**0.007**
total mononuclear cells recovered (x 10^{-6})	63.1 ± 33.0	59.9 ± 28.4	44.3 ± 46.5	0.657
mononuclear cells (x 10^{-3}) per mg	122.7 ± 65.6	93.1 ± 33.7	75.5 ± 68.9	0.408
THYMUS				
weight (mg)	476.1 ± 113.6 ^{a}	492.9 ± 49.2 ^{a}	304.7 ± 67.8 ^{b}	**0.003**
% body weight	0.21 ± 0.04 ^{a}	0.21 ± 0.02 ^{a}	0.11 ± 0.02 ^{b}	**< 0.001**
total mononuclear cells recovered (x 10^{-6})	211.6 ± 128.2	174.5 ± 160.1	64.0 ± 111.2	0.173
mononuclear cells (x 10^{-3}) per mg	469.9 ± 313.6	334.7 ± 268.6	182.4 ± 291.6	0.270
MANDIBULAR LYMPH NODES				
weight (mg)	134.2 ± 80.0	111.9 ± 7.9	113.9 ± 47.9	0.756
% body weight	0.058 ± 0.037	0.048 ± 0.004	0.042 ± 0.012	0.479
total mononuclear cells recovered (x 10^{-6})	4.9 ± 2.6 ^{a}	1.9 ± 0.6 ^{b}	1.1 ± 0.6 ^{b}	**0.004**
mononuclear cells (x 10^{-3}) per mg	46.4 ± 22.6 ^{a}	16.5 ± 5.3 ^{b}	10.4 ± 4.6 ^{b}	**0.001**

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

5.3.4 Cell phenotyping by flow cytometry

Flow cytometry was performed to assess whether the proportions of immune cell subsets or the relative expression of their cell surface markers was altered in response to pregnancy.

T lymphocytes ($CD3^+$) were the predominant cell type within the lymphocyte gate of maternal lysed whole blood (see table 5.4), with the majority of these T cells being T helper cells ($CD3^+CD4^+$). The proportions of T cells, $CD4^+$ cells, $CD4^+$ T cells, B cells and NK cells in the blood lymphocyte population were not significantly altered by pregnancy, although absolute numbers were not determined. Pregnancy was associated with a significantly higher proportion of $CD8^+$ cells in the lymphocyte population. $CD8^+$ cells include cytotoxic T cells ($CD3^+CD8^+$), which also demonstrated a non-significant trend to increase during pregnancy.

The mean fluorescence intensity (MFI) of CD161 on cells in maternal blood was significantly lower in pregnant animals than in virgin females. CD161 is also known as NKR-P1, and is a receptor on NK cells which activates cytotoxicity, and is directly involved in target recognition(65). Decreased expression of this cell surface marker may therefore indicate a reduced capacity for target recognition, which fits with the need to decrease NK cell activity in pregnancy.

The overall proportions of mononuclear cells within the maternal spleen were similar to those observed within maternal lysed blood, with the addition of $CD163^+$ cells, a marker expressed upon mature tissue macrophages. Pregnancy did not affect the proportions of mononuclear cell types isolated from the spleen (table 5.5). Within lymphocytes isolated from the maternal spleen there was significantly lower expression of CD161 upon NK cells among pregnant females.

The predominant cell type within the mononuclear cells isolated from the maternal thymus was T lymphocytes. No significant effects of pregnancy upon relative cell subsets or cell surface marker expression were identified upon mononuclear cells isolated from the maternal thymus (see table 5.6). The lack of an effect of pregnancy within the maternal thymus indicates that all the cell types are equally affected by thymic involution, remaining in constant proportion to each other with pregnancy. The

thymus is a primary lymphoid organ and a site of T cell maturation, and so there will be significant numbers of immature T cells within the thymus. The results obtained here suggest that there are a significant number of $CD4^{+}CD8^{+}$ (double positive) immature T cells (approx. 60 - 70 %), although these could not be directly identified with the staining protocol used. Other studies using rat models have demonstrated that there are significant numbers (> 80%) of double positive immature T cells in the thymus of adult male (age 3 month) rats(236).

The immune cells identified within the maternal lymph nodes were comparable to those within the spleen and PBMC, with significant numbers of T lymphocytes, NK cells and B lymphocytes. No significant effects of pregnancy were observed upon the cell subsets or cell surface marker expression of mononuclear cells isolated from the mandibular lymph nodes (see table 5.7), despite the significant reductions in cellularity that were observed to occur in this tissue with pregnancy. This indicates that all cell types are equally affected by the reductions in cellularity, and remain in the same proportions to each other.

Table 5.4: Immune cell subsets identified in lysed whole blood of virgin and pregnant female rats (% within lymphocyte gate, MFI, mean ± standard deviation)

	Virgin (n = 6)	Day 12 of pregnancy (n = 5)	Day 20 of pregnancy (n = 6)	ANOVA p value
$CD3^+$ (%)	39.3 ± 10.6	47.5 ± 6.8	45.5 ± 7.0	0.268
$CD8^+$ (%)	29.5 ± 3.4 [ab]	25.6 ± 3.8 [a]	33.1 ± 3.5 [b]	**0.019**
$CD4^+$ (%)	45.1 ± 9.0	51.7 ± 5.7	45.1 ± 7.2	0.293
$CD3^+CD8^+$ (%)				
(Cytotoxic T cells)	11.0 ± 3.2	13.8 ± 2.5	14.2 ± 2.0	0.120
mean CD3 intensity	38.3 ± 8.0	34.0 ± 9.7	33.4 ± 5.8	0.522
mean CD8 intensity	71.6 ± 21.6	80.5 ± 32.0	54.3 ± 14.9	0.200
$CD3^+CD4^+$ (%)				
(T helper cells)	27.7 ± 7.1	31.2 ± 8.2	31.1 ± 6.7	0.658
mean CD3 intensity	44.7 ± 9.2	38.7 ± 12.7	41.2 ± 9.8	0.644
mean CD4 intensity	594.8 ± 71.9	589.1 ± 28.3	590.7 ± 28.0	0.979
$CD161^+$ (%)				
(NK cells)	24.2 ± 3.7	19.0 ± 4.9	21.4 ± 2.7	0.104
mean CD161 intensity	303.5 ± 44.9 [a]	209.3 ± 52.0 [b]	247.9 ± 37.9 [ab]	**0.012**
$CD3^-CD45^+$ (%)				
(B cells)	24.9 ± 7.3	21.8 ± 4.7	23.3 ± 6.4	0.729
mean CD45 intensity	316.4 ± 98.1	272.9 ± 56.1	292.6 ± 35.5	0.591

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 5.5: Immune cell subsets identified within the spleen of virgin and pregnant female rats (% within lymphocyte gate, MFI, mean ± standard deviation)

	Virgin (n = 6)	Day 12 of pregnancy (n = 5)	Day 20 of pregnancy (n = 6)	ANOVA p value
$CD3^+$ (%)	48.1 ± 7.3	52.2 ± 8.9	52.8 ± 9.7	0.619
$CD8^+$ (%)	29.0 ± 7.1	25.7 ± 5.9	28.9 ± 5.8	0.625
$CD4^+$ (%)	44.1 ± 6.7	48.2 ± 7.6	49.0 ± 6.7	0.448
$CD3^+CD8^+$ (%) (Cytotoxic T cells)	12.7 ± 3.5	13.4 ± 4.5	14.5 ± 4.6	0.766
mean CD3 intensity	22.4 ± 5.1	22.2 ± 3.4	21.2 ± 2.9	0.840
mean CD8 intensity	29.0 ± 7.0	30.5 ± 5.9	25.2 ± 5.5	0.361
$CD3^+CD4^+$ (%) (T helper cells)	36.3 ± 7.4	39.3 ± 8.1	39.8 ± 7.4	0.706
mean CD3 intensity	37.2 ± 8.5	34.8 ± 7.2	33.4 ± 6.9	0.687
mean CD4 intensity	457.1 ± 51.5	449.8 ± 30.9	439.1 ± 10.2	0.685
$CD161^+$ (%) (NK cells)	18.7 ± 7.7	14.7 ± 4.0	13.1 ± 4.0	0.238
mean CD161 intensity	143.2 ± 42.9 [a]	61.5 ± 18.9 [b]	69.2 ± 25.3 [b]	**0.001**
$CD3^-CD45^+$ (%) (B cells)	29.5 ± 11.2	35.5 ± 6.5	34.0 ± 7.5	0.508
mean CD45 intensity	126.5 ± 27.1	128.6 ± 30.9	125.2 ± 22.0	0.978
$CD163^+$ (%) (splenic macrophages)	20.1 ± 5.9	28.0 ± 5.7	26.7 ± 7.0	0.112
CD163 intensity	33.6 ± 4.9	42.7 ± 9.5	42.0 ± 11.2	0.185

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 5.6: Immune cell subsets identified within the thymus of virgin and pregnant female rats (% total cells, MFI, mean ± standard deviation)

	Virgin (n = 6)	Day 12 of pregnancy (n = 5)	Day 20 of pregnancy (n = 6)	ANOVA p value
$CD3^+$ (%)	30.6 ± 7.7	30.0 ± 11.6	30.7 ± 6.9	0.989
$CD8^+$ (%)	72.7 ± 5.5	72.2 ± 9.6	72.0 ± 4.6	0.983
$CD4^+$ (%)	86.1 ± 4.4	83.2 ± 6.0	84.4 ± 2.3	0.555
$CD3^+CD8^+$ (%) (Cytotoxic T cells)	12.6 ± 4.1	12.3 ± 4.3	12.6 ± 2.3	0.986
mean CD3 intensity	47.7 ± 8.7	48.1 ± 7.7	46.6 ± 2.9	0.933
mean CD8 intensity	36.9 ± 3.8	38.4 ± 7.8	37.9 ± 5.8	0.917
$CD3^+CD4^+$ (%) (T helper cells)	27.6 ± 6.3	26.5 ± 9.3	28.1 ± 6.5	0.932
mean CD3 intensity	59.2 ± 9.0	51.3 ± 5.7	54.8 ± 9.9	0.333
mean CD4 intensity	340.3 ± 19.9	342.6 ± 29.5	348.4 ± 53.0	0.929
$CD161^+$ (%) (NK cells)†	4.6 ± 2.0	3.7 ± 1.5	5.5 ± 2.2	0.327
mean CD161 intensity	105.1 ± 32.1	97.5 ± 46.6	79.2 ± 13.5	0.393
$CD3^-CD45^+$ (%) (B cells)	2.1 ± 0.5	1.9 ± 1.0	2.7 ± 0.9	0.226
mean CD45 intensity	113.7 ± 25.8	86.4 ± 33.3	82.1 ± 13.3	0.095

†These are not likely to be NK cells, but to be another cell type bearing CD161 on the surface

Table 5.7: Immune cell subsets identified within the mandibular lymph nodes of virgin and pregnant female rats (% within lymphocyte gate, MFI, mean ± standard deviation)

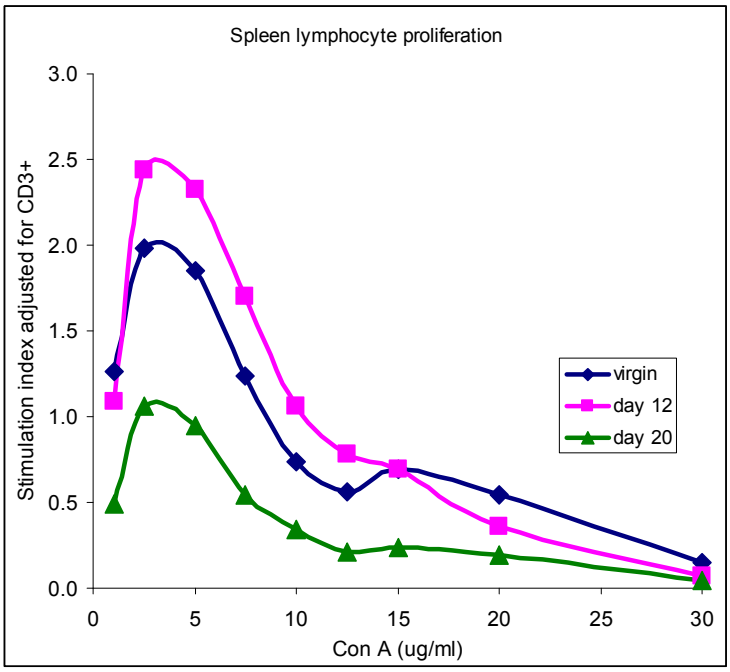
	Virgin (n = 5)	Day 12 of pregnancy (n = 5)	Day 20 of pregnancy (n = 6)	ANOVA p value
$CD3^+$ (%)	50.5 ± 6.1	46.2 ± 5.5	52.9 ± 10.1	0.381
$CD8^+$ (%)	16.8 ± 8.4	13.4 ± 1.7	13.8 ± 2.7	0.072
$CD4^+$ (%)	50.0 ± 4.1	50.3 ± 6.6	56.3 ± 2.7	0.083
$CD3^+CD8^+$ (%)				
(Cytotoxic T cells)	11.5 ± 6.9	8.7 ± 1.8	9.5 ± 3.6	0.617
mean CD3 intensity	16.2 ± 2.7	15.5 ± 1.3	15.6 ± 1.9	0.865
mean CD8 intensity	20.2 ± 6.0	21.0 ± 2.8	17.3 ± 0.7	0.249
$CD3^+CD4^+$ (%)				
(T helper cells)	39.3 ± 6.4	39.1 ± 5.3	43.8 ± 6.6	0.377
mean CD3 intensity	24.0 ± 4.8	24.4 ± 3.6	24.6 ± 4.8	0.975
mean CD4 intensity	438.8 ± 65.2	432.9 ± 34.3	399.4 ± 49.0	0.404
$CD161^+$ (%)				
(NK cells)	10.0 ± 4.5	9.8 ± 2.3	8.8 ± 2.8	0.814
mean CD161 intensity	42.7 ± 12.0	43.2 ± 3.9	41.5 ± 8.0	0.942
$CD3^-CD45^+$ (%)				
(B cells)	33.4 ± 10.2	40.2 ± 5.5	32.2 ± 5.6	0.199
mean CD45 intensity	117.7 ± 29.4	105.4 ± 11.0	102.2 ± 18.7	0.486

5.3.5 Lymphocyte proliferation

Lymphocyte proliferation stimulation index in response to Con A (adjusted for $CD3^+$ cells) was assessed in mononuclear cell suspensions from the maternal spleen, thymus or whole blood samples (see figures 5.2 to 5.4). Proliferative responses were assessed by calculation of area under the curve (AUC) of the Con A dose response (see table 5.8).

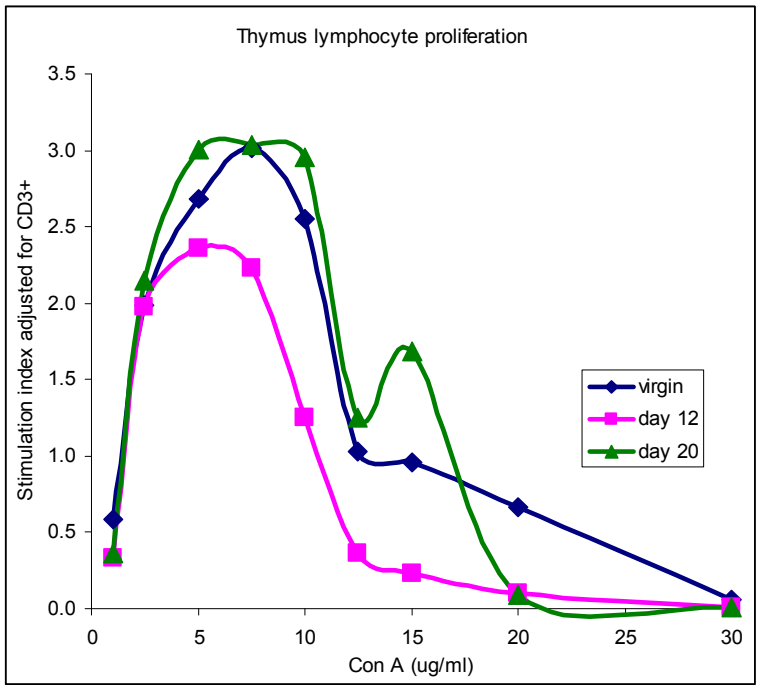
No statistically significant effects upon the proliferative response or changes in sensitivity to Con A stimulation were observed as a result of pregnancy. The lack of significant effect probably reflects the large variability between individual rats, and is a limitation of the cross-sectional design of this study.

Figure 5.2: Mean proliferation of spleen lymphocytes from virgin and pregnant rats (stimulation index, adjusted for $CD3^+$ cell numbers)



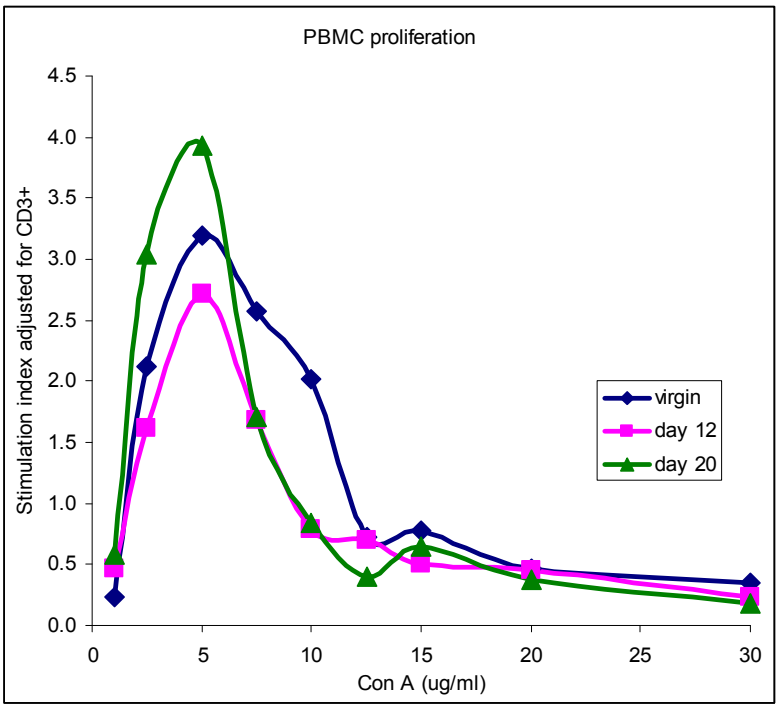
Error bars are omitted for clarity

Figure 5.3: Mean proliferation of thymic lymphocytes from virgin and pregnant rats (stimulation index, adjusted for $CD3^+$ cell numbers)



Error bars are omitted for clarity

Figure 5.4: Mean proliferation of peripheral blood lymphocytes from virgin and pregnant rats (stimulation index, adjusted for $CD3^+$ cell numbers)



Error bars are omitted for clarity

Table 5.8: Proliferation of lymphocytes from virgin and pregnant rats (AUC, adjusted for $CD3^+$ cell numbers, mean ± standard deviation)

	Virgin	Day 12 of pregnancy	Day 20 of pregnancy	ANOVA p value
Spleen	(n=3) 23.3 ±11.5	(n=4) 26.0 ± 14.5	(n=3) 10.1 ± 4.0	0.240
Thymus	(n=6) 36.5 ± 16.3	(n=5) 21.3 ± 14.7	(n=5) 37.3 ± 26.8	0.374
PBMC	(n=3) 33.8 ± 21.6	(n=3) 24.8 ± 24.7	(n=2) 29.9 ± 19.3	0.888

5.3.6 Production of immune signalling molecules

PGE_2

PGE_2 is an eicosanoid mediator produced by monocytes and macrophages from the cell membrane fatty acid arachidonic acid (AA). Significant effects of pregnancy have been observed upon the phospholipid content of AA in tissues including the liver (see chapter 4), and it is possible that these changes with pregnancy also affect the fatty acid composition of immune cells.

No significant effect of pregnancy was observed upon the concentration of PGE_2 in plasma or upon the basal or Con A-stimulated production of PGE_2 by spleen mononuclear cells or PBMC (see table 5.9). Con A is a T cell stimulator, and PGE_2 is not generated within T cells, but from antigen presenting cells such as monocytes and macrophages. Thus PGE_2 measured in the cell cultures is likely to be generated by macrophages in spleen cell cultures and monocytes in PBMC cultures and in turn these cells would have been stimulated by T cell-derived products.

Spleen cell cultures did not respond well to Con A in terms of PGE_2 production suggesting that the T cell - macrophage interaction is not strong in these cultures. In contrast, PBMC cultures produced more PGE_2 when stimulated by Con A, suggesting a functional T cell - monocyte interaction in these cultures. This indicates that circulating monocytes respond to Con A stimulation in a way which differs from the responses of resident macrophages within tissues.

Table 5.9: PGE_2 concentration in plasma and PGE_2 production by spleen and peripheral blood lymphocytes in virgin and pregnant female rats (ng/ml; mean ± standard deviation)

	Virgin	Day 12 of pregnancy	Day 20 of pregnancy	ANOVA p value
Plasma	(n=6)	(n=5)	(n=6)	
	27.0 ± 17.1	21.6 ± 7.7	33.3 ± 32.0	0.688
Spleen	(n=5)	(n=5)	(n=5)	
No Con A	2.5 ± 0.4	3.4 ± 1.7	2.2 ± 1.1	0.315
5 μg/ml Con A	2.5 ± 0.6	2.5 ± 1.1	2.5 ± 0.4	0.998
Mean SI	1.0 ± 0.2	0.8 ± 0.2	1.3 ± 0.7	0.162
PBMC	(n=2)	(n=5)	(n=5)	
No Con A	3.9 ± 3.2	4.2 ± 2.4	3.4 ± 2.7	0.896
5 μg/ml Con A	11.7 ± 13.4	14.7 ± 2.7	11.3 ± 5.9	0.676
Mean SI	2.4 ± 1.5	4.9 ± 3.0	4.5 ± 3.3	0.622

IFN-γ

IFN-γ is a cytokine produced by T helper cells, cytotoxic T cells and NK cells, and is typically used as a marker of Th1 activity. Con A stimulation led to a dramatic increase (several thousand fold) in IFN-γ production by spleen, thymus and peripheral blood lymphocytes. No significant effects of pregnancy were observed upon IFN-γ production by maternal spleen, thymus or peripheral blood cells (see table 5.10).

Table 5.10: IFN-γ production by spleen, thymus and peripheral blood lymphocytes of virgin and pregnant female rats (pg/ml, mean ± standard deviation)

	Virgin	Day 12 of pregnancy	Day 20 of pregnancy	ANOVA p value
Spleen	(n=5)	(n=5)	(n=5)	
No Con A	2.92 ± 6.53	0.52 ± 1.16	0.00 ± 0.00	0.461
5 μg/ml Con A (x 10^{-3})	44.7 ± 22.5	46.9 ± 38.7	38.7 ± 30.9	0.915
Mean SI (x 10^{-3})	33.9 ± 27.1	44.0 ± 41.3	38.7 ± 30.9	0.894
Thymus	(n=6)	(n=5)	(n=5)	
No Con A	8.1 ± 15.1	4.4 ± 3.4	39.5 ± 54.0	0.185
5 μg/ml Con A (x 10^{-3})	14.3 ± 9.6	20.9 ± 12.8	15.1 ± 7.9	0.543
Mean SI (x 10^{-3})	7.1 ± 10.0	13.3 ± 16.9	4.5 ± 4.1	0.476
PBMC	(n=2)	(n=5)	(n=5)	
No Con A	299.9 ± 424.1	76.1 ± 161.3	47.3 ± 31.4	0.272
5 μg/ml Con A (x 10^{-3})	23.5 ± 27.5	13.9 ± 15.7	26.1 ± 25.3	0.676
Mean SI (x 10^{-3})	2.0 ± 2.8	5.0 ± 9.3	0.5 ± 0.3	0.546

SI, stimulation index: ConA stimulated cytokine production / unstimulated cytokine production

IL-4

IL-4 is a Th2 type cytokine generated by T cells. There was no increase in production of IL-4 between unstimulated and stimulated cells. This may in part be due to the antagonistic nature of Th1 and Th2 cytokines, with the dramatically increased production of IFN-γ inhibiting IL-4 production. No significant effects of pregnancy were observed upon IL-4 production by maternal spleen, thymus or peripheral blood lymphocytes (see table 5.11).

Table 5.11: IL-4 production by spleen, thymus and peripheral blood lymphocytes in virgin and pregnant female rats (pg/ml; mean ± standard deviation)

	Virgin	Day 12 of pregnancy	Day 20 of pregnancy	ANOVA p value
Spleen	(n=5)	(n=5)	(n=5)	
No Con A	11.7 ± 12.5	20.2 ± 7.4	11.9 ± 9.1	0.330
5 μg/ml Con A	8.0 ± 6.6	12.6 ± 8.1	7.5 ± 5.3	0.444
Thymus	(n=6)	(n=5)	(n=5)	
No Con A	24.5 ± 21.9	33.4 ± 17.3	16.3 ± 12.9	0.357
5 μg/ml Con A	20.5 ± 21.3	23.8 ± 7.2	6.7 ± 9.0	0.183
PBMC	(n=2)	(n=5)	(n=5)	
No Con A	10.8 ± 2.9	5.1 ± 5.3	6.6 ± 3.2	0.326
5 μg/ml Con A	7.7 ± 3.0	5.2 ± 5.1	5.3 ± 4.6	0.803

Th1 vs. Th2

The relative balance of Th1 and Th2 cytokine production may be more important than absolute concentrations generated. For this reason, the ratio of IFN-γ to IL-4 produced by Con A stimulated cells was calculated (see table 5.12). There was no effect of pregnancy on the ratio of IFN-γ to IL-4 production by stimulated spleen cells or PBMC. However, in the thymus there was a significantly increased Th1:Th2 ratio in late pregnancy.

Table 5.12: IFNγ:IL-4 ratios produced by Con A stimulated spleen, thymus and peripheral blood lymphocytes in virgin and pregnant female rats (mean ± standard deviation)

	Virgin (n=5,6,2)	Day 12 of pregnancy (n=5)	Day 20 of pregnancy (n=5)	ANOVA p value
Spleen IFNγ:IL-4 x 10^{-3}	15.5 ± 17.7	11.3 ± 19.1	6.1 ± 4.0	0.631
Thymus IFNγ:IL-4 x 10^{-3}	1.2 ± 1.1 [a]	0.9 ± 0.5 [a]	6.6 ± 5.2 [b]	**0.015**
PBMC IFNγ:IL-4 x 10^{-3}	2.6 ± 2.6	9.1 ± 16.3	9.1 ± 9.2	0.802

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

5.4 Discussion

The aim of this study was to characterise the normal range of immunological changes in the Wistar rat during pregnancy, in order that the effect of maternal dietary fatty acids upon this process can be evaluated in later studies (chapter 7). The immunological parameters assessed included immune organ weight and cellularity, immune cell subsets, T cell proliferation, and the production of cytokines (IFN-γ and IL-4) and PGE_2. The hypotheses being tested were that:

a) rat pregnancy causes thymic involution and reduced lymphoid organ cellularity
b) changes in lymphoid organ cellularity are associated with changes to the profile of immune cell subsets present
c) the rat will demonstrate changes in Th1/Th2 balance in response to pregnancy, favouring production of Th2 cytokines such as IL-4.

The data from this study fully support hypothesis a. There is some data from immune tissues which is supportive of hypothesis b, though no significant changes in the proportion of cells types were observed within the thymus. Hypothesis c is not supported by the current data, and in fact the Th1:Th2 ratio of cytokines was highest among rats at day 20 of gestation. In addition, data obtained demonstrated that cell surface markers upon maternal NK cells were lower among pregnant females than in virgin female rats.

This study confirms that thymic involution is a feature of Wistar rat pregnancy, as has been observed previously in rats(237), mice and humans(176). Trends for reductions in the absolute numbers of cells recovered from the thymus, spleen and lymph nodes and cellularity of these organs (i.e. number of cells per unit weight) were observed, with this reaching statistical significance within mandibular lymph nodes. This may present an advantage during pregnancy by reducing the number of lymphocytes which may target fetal antigens. However, data was not collected to assess any change within the absolute number of PBMCs during pregnancy and future studies would benefit from this additional information.

There were significant but selected changes in immune cell populations and the expression of cell surface makers in the rat during pregnancy. The changes observed

were increased proportions of $CD8^+$ cells in the PBMC population, and reduced expression of CD161 on NK cells in PBMC and the spleen. No significant effects of pregnancy were observed upon cell populations in the thymus, despite the thymic involution observed. Increasing $CD8^+$ cell numbers suggests that cytotoxic T cell responses are increased in the rat during pregnancy, which may improve the response to viral infection. It has been identified that the presence of NK cells correlates to fetal resorption in the mouse(175). The current data suggests that while the proportion of NK cells in the maternal blood are not affected by pregnancy, the responsiveness of these cells to cytotoxic stimulus may be impaired by the reduced expression of cell surface markers. This would be consistent with the need to reduce NK cell activity during pregnancy. However, data was not collected to assess any change within the absolute number of PBMCs during pregnancy, which may provide further information upon the relevance of these changes in proportions of cell types or their relative cell surface marker expression.

No significant effects of pregnancy were observed upon lymphocyte proliferative responses, or their sensitivity to Con A stimulation. It is possible that any effect of pregnancy upon lymphocyte proliferation was obscured by the degree of biological variation between animals. Some of this variation could have been accounted for if blood samples were taken from the same animal throughout pregnancy rather than comparing different animals. However, it would not have been possible to assess the proliferative response of cells from immune organs such as spleen, thymus and lymph nodes in a longitudinal study.

No significant changes were observed in the absolute production of PGE_2, IFN-γ or IL-4 in response to pregnancy. However, the Th1/Th2 ratio of cytokine production within the thymus was significantly higher among day 20 pregnant rats. Although this is in contradiction to the Th2 shift which has been described in human and murine systems, it is consistent with the findings of other studies of Wistar rats where an increase in IFN-γ producing $CD4^+$ cells was seen(179;180). The rat may therefore not be a suitable animal model for use in investigation of changes to the balance of Th1/Th2 cytokine production which have been observed during human pregnancy. A further timecourse of maternal immune function after delivery and lactation would provide useful information upon the duration of the maternal adaptations observed in the rat.

The cultures used to assess cytokine production in this study were of mixed mononuclear cell populations. It would be advantageous for future studies to investigate the cytokine production of specific cell types, either by purifying specific immune cell subsets prior to culture (e.g. by cell sorting), or use of alternative methods to assess cytokine production, such as flow cytometry techniques which allow simultaneous cell phenotype identification. The use of ELISAs to assess cytokine production limited the number of cytokines which could be assessed within the sample quantity which was available. Use of a multiplex kit which enables the simultaneous analysis of a wider panel of cytokines from a much smaller sample volume would allow further insight into the effect of pregnancy upon cytokine production in the rat, and in particular allow assessment of whether the changes observed reflect a change in the overall capacity for cytokine production, or are limited to specific mediators.

The findings of this study could be taken further by assessing the functional effects of the changes in lymphocyte subsets and cell surface marker expression identified. For example, assays of cytotoxicity or sensitivity to activation could be used to investigate whether the effects of pregnancy which were observed upon the expression of CD8 upon PBMC has a direct functional outcome which may be of relevance to health or disease resistance during pregnancy. Similarly, NK cell activity could be assessed to determine the functional effects of reduced CD161 expression upon NK cell cytotoxicity during pregnancy. A comprehensive understanding of the normal physiological changes in immune function of the rat during pregnancy is essential for future studies to investigate the potential for these processes to be modulated by diet during pregnancy, and whether these changes are likely to be beneficial, or may represent an increased risk of either pregnancy loss or disease risk during pregnancy.

Chapter 6: The effect of maternal dietary fatty acids during pregnancy upon maternal and fetal tissue fatty acid composition

6.1 Introduction

6.1.1 Aims and hypothesis for current investigation

The aim of this study is to investigate the effects of maternal diet during pregnancy upon maternal and fetal plasma and tissue fatty acid composition. In particular, the effect that increasing the α-linolenic acid (ALNA) and longer chain n-3 polyunsaturated fatty acid (LC n-3 PUFA) content of the maternal diet has upon LC n-3 PUFA status of the fetus will be investigated. Data will be used to identify potential predictors of fetal immune tissue fatty acid composition from samples which could feasibly be collected in human studies. These data will also used for later comparison with a parallel cohort in which immune function parameters will be assessed (chapter 7).

This chapter therefore sets out to addresses the following hypotheses:

a) that a change in maternal dietary fatty acid intake during pregnancy will alter maternal plasma fatty acid composition, thereby altering the availability of fatty acids to the developing fetus, including LC n-3 PUFA.
b) that the changes to maternal dietary fatty acid intake during pregnancy will significantly affect maternal tissue composition, including the maternal liver, adipose tissue and immune tissues.
c) that a change in the availability of fatty acids to the developing fetus will alter the fatty acid composition of fetal plasma and developing fetal tissues such as the liver, brain and immune tissues.

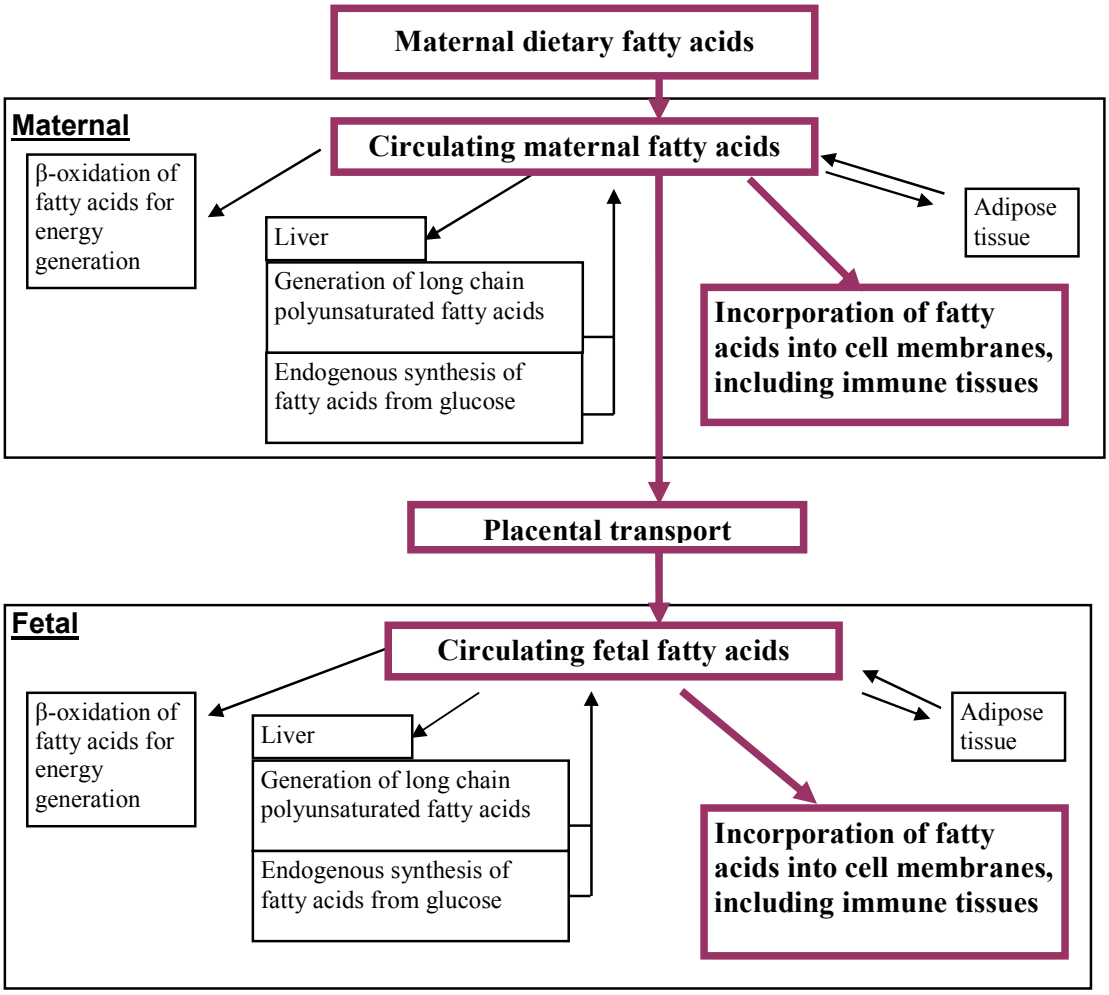
6.1.2 Metabolic fates of maternal dietary fatty acids during pregnancy

The transfer of fatty acids to the developing fetus is unlikely to be a simple reflection of the maternal diet, but will also be influenced by processes such as endogenous maternal lipogenesis, maternal LC PUFA metabolism and the effectiveness and selectivity of placental transfer (see figure 6.1). It is therefore necessary to analyse the fatty acid composition of a wide range of maternal and fetal tissues in order to evaluate the metabolic fate of dietary fatty acids during pregnancy.

Differences in the relative transfer of fatty acids to the developing fetus may have functional effects upon fetal tissues due to the important physiological roles of fatty

acids within those tissues. For example, fatty acids are precursors for the generation of immune signalling molecules; for example, eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6) are used to generate eicosanoids under the action of cyclooxygenase and lipoxygenase enzymes. The relative availability of these fatty acids within immune tissues may therefore result in functional effects.

Figure 6.1: The potential causal pathway by which changes to maternal dietary fatty acids may alter fetal tissue fatty acid composition



6.1.3 Animal studies of diet during pregnancy and the fatty acid composition of tissues

Though there are numerous studies which have demonstrated a significant effect of diet upon tissue fatty acid composition using animal models, there are relatively few studies which have been carried out to date which have demonstrated that fetal rat tissue fatty

acid composition can be altered in response to maternal diet during pregnancy. Those which have been undertaken have often also included feeding experimental diets during the neonatal period, and so it is difficult to identify whether the effects upon tissue composition occurred *in utero*, or during lactation.

One of the more comprehensive studies available to date investigated the effect of feeding diets rich in saturates (palm oil), monounsaturates (olive oil), n-6 PUFA (sunflower oil) and LC n-3 PUFA (fish oil) during pregnancy upon maternal and fetal plasma and tissue fatty acid composition(228). This study identified that maternal diet during pregnancy significantly affected the fatty acid composition of maternal adipose tissue, liver, plasma, and placenta and of fetal plasma and liver. The limitations of this study are the relatively narrow range of fatty acids assessed within tissues (9 fatty acids identified) and the use of total lipid extracts from tissues rather than isolation of specific lipid fractions such as phospholipids and triglycerides from all tissues except the placenta. A study of ALNA and docosahexaenoic acid (DHA, 22:6n-3) rich diets during pregnancy(238) identified that a maternal diet rich in n-3 fatty acids during pregnancy has the capacity to significantly alter the n-3 fatty acid composition of the uterus and placenta. There are no animal studies to date which have investigated the effect of maternal dietary fatty acids during pregnancy upon maternal and fetal immune tissue fatty acid composition.

6.2 Methods

All animal work was carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986); mating was carried out by monogamous breeding (see section 2.1). Experimental diets were provided *ad libitum* over a 20 day period, starting immediately after conception. Maternal tissues were collected at day 20 gestation and included plasma, liver, subcutaneous adipose, intra-abdominal adipose, and mononuclear cells isolated from lymphoid organs and blood samples. Fetal tissues were collected at day 20 gestation and included plasma, liver, brain, and mononuclear cells isolated from the thymus and blood samples. Fatty acid compositions of experimental diets and both maternal and fetal tissues were determined by gas chromatography (see section 2.6). Liver glycogen, lipid and dry weight content were assessed (see section 2.8, 2.10). Plasma lipid and glucose concentration analysis was provided by Southampton General Hospital (see section 2.7, 2.9).

The effects of maternal diet upon variables of interest were determined by one-way ANOVA with Bonferroni post-hoc correction for multiple comparisons. Correlation coefficients (r) were calculated using Pearson or Spearman test as appropriate after data were assessed for normality. Where stepwise multiple regression analysis was performed, the significant variables and Beta values are provided.

6.3 Results

6.3.1 Experimental diets

Pregnant animals received experimental diets from conception to day 20 of gestation. Six diets were used, a low fat (LF, 3% w/w) soyabean oil diet, and five high fat (HF, 13% w/w) diets: HF soyabean oil, HF linseed oil, HF salmon oil; HF sunflower oil, HF beef tallow. The fatty acid composition of these diets was analysed (see table 6.1).

The LF soyabean oil diet is a standard laboratory rat chow, which all animals were maintained upon prior to the start of experiments. This diet contains both n-6 and n-3 essential fatty acids (EFA; linoleic acid (LA) and ALNA). This diet was therefore used as a 'control' diet; with animals receiving this diet not exposed any dietary change during pregnancy.

The HF diets were used in order to represent a % energy from fat which is more representative of that typically consumed in a Western human diet. The HF soyabean oil diet maintained the relative contributions of LA and ALNA as were present in the LF soyabean oil diet, and so allowed the effect of quantitative change in fat intake to be assessed. The remaining HF diets were chosen based upon their characteristic fatty acid compositions, which can be summarised as follows:

- HF linseed oil - rich in the n-3 fatty acid ALNA
- HF salmon oil – rich in monounsaturates (MUFA) and LC n-3 PUFA and containing some LC n-6 PUFA
- HF sunflower oil – rich in LA
- HF beef tallow – rich in saturates and oleic acid (OA, 18:1n-9) at the expense of n-6 PUFA, and therefore representative of the typical UK diet prior to the widespread use of margarine and vegetable oils.

Table 6.1: The fatty acid composition of total lipid extracts obtained from experimental diets (% of total fatty acids, mean ± standard deviation, n=5 per group)

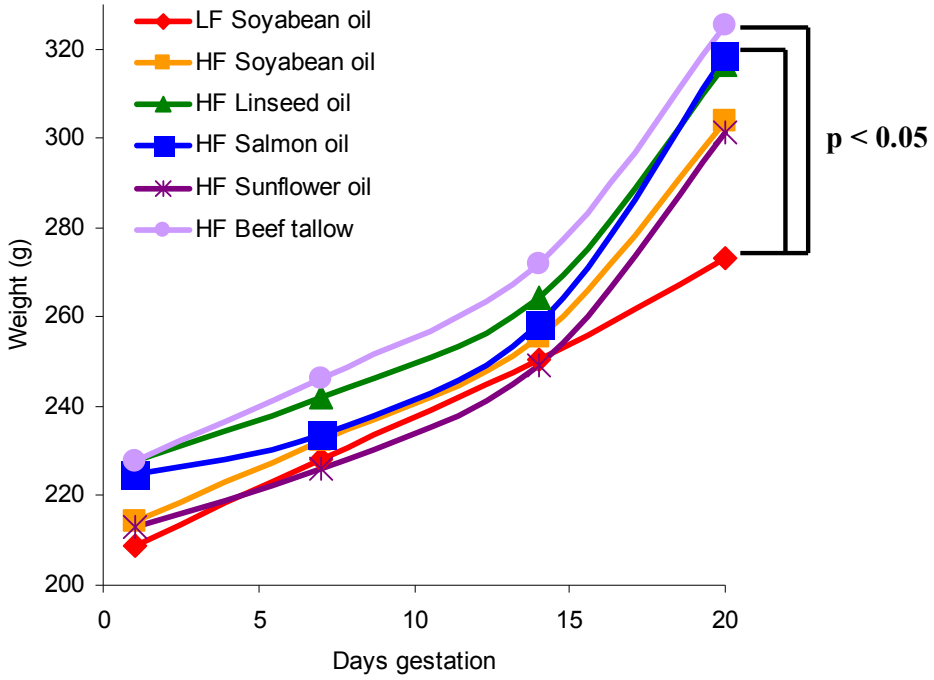
Fatty acids	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef Tallow	ANOVA
14:0	0.2 ± 0.2	0.1 ± 0.0	ND	4.4 ± 0.5 *	0.1 ± 0.0	2.6 ± 0.2 *	**< 0.001**
16:0	19.0 ± 1.1 *	12.9 ± 0.5 *	8.6 ± 0.3	17.1 ± 1.1 *	9.5 ± 0.4	25.2 ± 1.3 *	**< 0.001**
18:0	4.6 ± 1.0	4.6 ± 0.3	3.7 ± 0.2	3.3 ± 0.3	4.0 ± 0.2	18.3 ± 0.6 *	**< 0.001**
20:0	0.5 ± 0.6	0.3 ± 0.1	0.3 ± 0.1	1.0 ± 0.7	0.3 ± 0.1	0.4 ± 0.1	0.067
22:0	0.2 ± 0.2	0.1 ± 0.2	ND	ND	0.2 ± 0.3	ND	0.479
Total saturated	24.4 ± 0.8	18.0 ± 0.5 *	12.7 ± 0.2	25.7 ± 0.9	14.0 ± 0.7	46.6 ± 1.6 *	**< 0.001**
16:1n-7	0.4 ± 0.4	0.2 ± 0.1	0.1 ± 0.0	5.1 ± 0.5 *	0.1 ± 0.0	1.8 ± 0.2 *	**< 0.001**
18:1n-9	14.0 ± 1.4 *	21.1 ± 0.5	20.7 ± 0.4	18.4 ± 0.5 *	21.4 ± 0.6	30.3 ± 1.5 *	**< 0.001**
20:1n-9	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	6.1 ± 0.3 *	0.2 ± 0.1	0.3 ± 0.1	**< 0.001**
24:1n-9	ND	ND	ND	0.2 ± 0.2 *	ND	ND	**< 0.001**
Total MUFA	14.6 ± 2.0 *	21.5 ± 0.5	21.1 ± 0.5	29.8 ± 0.9 *	21.7 ± 0.6	32.4 ± 1.5 *	**< 0.001**
18:2n-6	53.7 ± 2.0	52.8 ± 0.7	27.7 ± 2.9 *	21.1 ± 1.0	61.8 ± 0.6 *	18.4 ± 1.6	**< 0.001**
18:3n-6	ND	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	ND	ND	**< 0.001**
20:2n-6	0.4 ± 0.7	ND	ND	0.3 ± 0.2	ND	ND	0.306
20:3n-6	ND	ND	ND	0.2 ± 0.1 *	ND	ND	**< 0.001**
20:4n-6	0.1 ± 0.2	ND	ND	0.5 ± 0.1 *	ND	ND	**< 0.001**
22:5n-6	0.4 ± 0.4	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	ND	0.189
Total n-6 PUFA	54.7 ± 1.7	53.2 ± 0.4	27.7 ± 2.7 *	22.3 ± 1.0 *	62.0 ± 0.5 *	18.6 ± 1.5 *	**< 0.001**
18:3n-3	5.9 ± 0.5	6.9 ± 0.2	38.0 ± 2.8 *	3.5 ± 0.4	1.7 ± 0.1	2.1 ± 0.2	**< 0.001**
20:4n-3	0.1 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	2.1 ± 1.8 *	0.4 ± 0.4	ND	**0.001**
20:5n-3	0.2 ± 0.2	ND	ND	6.5 ± 1.1 *	ND	ND	**< 0.001**
22:5n-3	ND	ND	ND	2.4 ± 0.5 *	ND	0.1 ± 0.1	**< 0.001**
22:6n-3	ND	ND	ND	7.6 ± 0.4 *	ND	ND	**< 0.001**
Total n-3 PUFA	6.3 ± 0.5	7.3 ± 0.3	38.5 ± 2.9 *	22.2 ± 1.9 *	2.2 ± 0.4	2.4 ± 0.3	**< 0.001**
LA: ALNA ratio	9.1	7.6	0.7 *	6.0 *	36.4 *	8.8	**< 0.001**

ND negligible detected (mean <0.1%) * significantly different ($p < 0.05$) from all other dietary groups

6.3.2 Weight gain and food intake

Once conception was confirmed, dams were housed individually, their weight monitored weekly (see figure 6.2) and food consumption assessed by weighed food intake. Dams on the HF diets exhibited a faster rate of weight gain (see table 6.2), particularly in the final week of gestation. This was not associated with any significant effect of food (g) or energy (MJ) intake or on litter or placental size (see table 6.3). The effects of diet upon maternal weight gain primarily appear to reflect increased maternal organ growth and adiposity. There was no significant effect of maternal diet upon the ratio of fetal:placental weight (an index of placental function, see table 6.4).

Figure 6.2: Mean weight of pregnant females over the study period



Error bars omitted for clarity

6.3.3 Maternal plasma glucose and lipid concentrations

Significant effects of diet were observed upon maternal high density lipoprotein (HDL) cholesterol and triacylglycerol (TAG) concentrations and on the total cholesterol:HDL cholesterol ratio, but not upon plasma glucose, non-esterified fatty acids (NEFA) or total cholesterol (see table 6.5).

Table 6.2: Weight gain and weighed food and energy intake during pregnancy in rats fed experimental diets (mean ± standard deviation, n = 6 per group)

	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
Weight gain (g/day)	3.2 ± 1.0 [a]	4.5 ± 0.7	4.4 ± 0.3	4.7 ± 0.5 [b]	4.4 ± 0.6	4.9 ± 0.8 [b]	**0.004**
Food intake (g/day)	17.9 ± 2.2	17.5 ± 2.3	16.7 ± 1.1	16.2 ± 1.8	17.3 ± 1.3	17.5 ± 2.4	0.695
Energy intake (MJ/day)	0.26 ± 0.03	0.30 ± 0.04	0.29 ± 0.02	0.28 ± 0.03	0.30 ± 0.02	0.30 ± 0.04	0.347

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 6.3: Placenta weight and litter development during pregnancy of rats fed experimental diets (mean ± standard deviation)

	LF Soyabean (n=5)	HF Soyabean (n=6)	HF Linseed (n=5)	HF Salmon (n=6)	HF Sunflower (n=5)	HF Beef tallow (n=6)	ANOVA p value
Placenta weight (g)	5.65 ±1.27	5.63 ± 0.73	5.69 ± 0.86	6.15 ± 0.57	6.06 ± 1.62	6.54 ± 0.76	0.601
Litter weight (g)	27.90 ± 6.69	29.11 ± 7.23	28.46 ± 5.69	28.41 ± 3.22	28.22 ± 10.27	29.65 ± 6.38	0.998
Litter size (range)	8-14	9-14	9-16	12-16	6-14	12-15	
Litter size (n)	12.2 ± 2.1	11.8 ± 1.7	11.7 ± 2.6	13.7 ± 1.6	11.2 ± 3.0	13.3 ± 1.5	0.313
Fetal:placental weight	4.9 ± 0.7	5.1 ± 0.8	5.2 ± 0.8	4.6 ± 0.4	4.8 ± 1.2	4.5 ± 0.6	0.590
Pup weight (g)	2.29 ± 0.40	2.47 ± 0.55	2.57 ± 0.65	2.09 ± 0.23	2.55 ± 0.49	2.24 ± 0.57	0.533

Table 6.4: The placenta and litter as a proportion of maternal weight during pregnancy in rats fed experimental diets (mean ± standard deviation)

	LF Soyabean (n=5)	HF Soyabean (n=6)	HF Linseed (n=5)	HF Salmon (n=6)	HF Sunflower (n=5)	HF Beef tallow (n=6)	ANOVA p value
Placenta (% body weight)	2.07 ± 0.32	1.85 ± 0.24	1.80 ± 0.26	1.94 ± 0.23	2.02 ± 0.56	2.03 ± 0.29	0.719
Litter (% body weight)	10.14 ± 1.80	9.59 ± 2.37	9.00 ± 1.77	8.96 ± 1.21	9.28 ± 2.91	9.23 ± 2.46	0.938
Placenta (% pregnancy weight gain)	9.23 ± 2.45	6.38 ± 1.12	6.40 ± 0.79	6.60 ± 0.56	6.82 ± 1.58	6.84 ± 1.19	0.885
Litter (% pregnancy weight gain)	44.37 ± 10.21	32.76 ± 7.51	26.50 ± 5.23	30.49 ± 3.65	31.60 ± 7.68	31.33 ± 9.36	0.064

Table 6.5: Plasma lipid and glucose concentrations in pregnant rats fed experimental diets (mean ± standard deviation, n=6 per group)

	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
Glucose (mM)	7.3 ± 2.8	7.8 ± 2.3	7.5 ± 1.3	7.4 ± 1.4	7.8 ± 1.3	7.9 ± 1.3	0.993
Total cholesterol (mM)	1.3 ± 0.4	1.7 ± 0.3	1.5 ± 0.2	1.1 ± 0.2	1.5 ± 0.3	1.7 ± 0.4	0.026 †
HDL-cholesterol (mM)	0.9 ± 0.2 [b]	1.0 ± 0.2 [b]	1.0 ± 0.2 [b]	0.8 ± 0.2	0.9 ± 0.2 [b]	0.6 ± 0.2 [a]	**0.001**
Total cholesterol:HDL ratio	1.4 ± 0.26 [a]	1.71 ± 0.18 [a]	1.59 ± 0.18 [a]	1.40 ± 0.15 [a]	1.62 ± 0.26 [a]	3.23 ± 1.48 [b]	**< 0.001**
NEFA (μM)	678.6 ± 280.2	902.7 ± 503.4	717.2 ± 415.6	483.1 ± 339.0	892.5 ± 405.8	951.1 ± 444.7	0.366
TAG (mM)	1.5 ± 0.7 [a]	3.1 ± 1.1 [b]	2.3 ± 0.5	1.7 ± 0.5 [a]	2.1 ± 0.5	3.4 ± 0.9 [b]	**< 0.001**

† no significant differences between groups when Bonferroni post-hoc test applied

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

The HF beef tallow diet group had the lowest plasma HDL cholesterol concentration, and the highest total:HDL cholesterol ratio. The total:HDL cholesterol ratio is commonly used to assess cardiovascular risk in humans, with higher values indicating increased risk(239). This diet during pregnancy may therefore increase maternal cardiovascular risk.

The HF salmon oil group exhibited plasma TAG concentrations which were as low as those observed with the LF soyabean oil diet, which is consistent with the lipid lowering effects of fish oil that have been observed in both rat models(240) and human studies of both normolipidemic and hyperlipidemic subjects(241). However, as animals were not fasted prior to blood collection, it is possible that the effects observed may be confounded by variation in fed status.

6.3.4 Maternal plasma fatty acid composition

The fatty acid composition of phosphatidylcholine (PC), TAG, cholesteryl esters (CE) and NEFA in maternal plasma was determined by gas chromatography (see tables 6.6 to 6.9). Significant effects of maternal diet were observed in all plasma lipid fractions, and across virtually all fatty acids assessed, in line with the properties of the diet consumed.

By comparing the plasma lipids from pregnant rats fed the LF and HF soyabean oil diets, we can infer some of the effects of changing the quantity of dietary fat upon fatty acid composition. The EFA (LA and ALNA) content of plasma lipids was higher in the HF soyabean oil group compared to the LF soyabean oil group. There was also a trend for the LC n-6 PUFA status of the LF soyabean oil group to be greater than the HF soyabean oil group, with 22:5n-6 being significantly higher in plasma PC and CE, and AA significantly higher in plasma TAG. This implies that HF feeding may reduce the endogenous synthesis of LC n-6 PUFA. There was a trend for a similar effect upon LC n-3 PUFA, particularly in plasma TAG and NEFA.

The HF linseed oil diet, which has the highest ALNA content, resulted in the highest ALNA content within plasma lipids, and this was significantly different from all other dietary groups. ALNA is an EFA which can be used for the endogenous synthesis of LC n-3 PUFA. There was a trend for the HF linseed oil group to have higher LC n-3 PUFA status than other HF diet groups (with the exception of the HF salmon oil group), with EPA in the CE fraction in particular being significantly higher than all other

dietary groups (except HF salmon oil). This indicates that endogenous synthesis of LC n-3 PUFA had occurred, as this diet contained no pre-formed LC n-3 PUFA.

The HF salmon oil diet is rich in LC n-3 PUFA, and rats fed this diet had the highest LC n-3 PUFA status, with this observation apparent across all plasma lipids. This higher content of LC n-3 PUFA occurred at the expense of n-6 PUFA, with the HF salmon oil group demonstrating the lowest AA and total n-6 PUFA in the CE fraction. Other fatty acids which were unique to the salmon oil diet such as 20:1n-9 also exhibited levels which were higher than those of any other dietary group.

The HF sunflower oil diet contains the highest LA content of all the diets, and consequently the highest LA content in plasma TAG, CE and NEFA. LA can be used for the endogenous synthesis of LC n-6 PUFA. Despite this, the HF sunflower oil group did not consistently demonstrate the highest LC n-6 PUFA content of plasma lipids. For example, while the AA content of plasma lipids was higher in the HF sunflower oil group than in the HF soyabean, linseed or salmon oil groups, it did not reach levels as high as those observed in the LF soyabean oil or HF beef tallow groups. The 22:5n-6 status of plasma lipids was highest in the sunflower oil group. These changes in n-6 PUFA content were at the expense of LC n-3 PUFA, with the HF sunflower oil group having the lowest DHA content in plasma PC, CE and NEFA.

The HF beef tallow diet contained the highest 16:0, 18:0 and OA content of all the experimental diets. This group had the highest OA content in all plasma lipids when compared to all other dietary groups. This group demonstrated the highest 16:0 and 18:0 content within plasma TAG, but this effect upon saturates was not observed in other plasma lipid fractions. These effects were at the expense of LC n-3 PUFA, with the beef tallow group exhibiting lower levels of DHA than virtually all other dietary groups (with the exception of HF sunflower oil, which had the lowest levels of DHA). Changes to the maternal diet during pregnancy can therefore significantly affect circulating maternal fatty acid composition. This will in turn alter the availability of fatty acids for transport across the placenta and incorporation into maternal tissues.

Table 6.6: Fatty acid composition of plasma PC in pregnant rats fed experimental diets (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acids	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.24 ± 0.08	0.15 ± 0.08	0.24 ± 0.09	0.33 ± 0.08	0.22 ± 0.08	0.24 ± 0.08	0.034
16:0	27.38 ± 1.58	22.99 ± 1.04	22.79 ± 1.32	25.67 ± 1.25	22.64 ± 1.85	24.47 ± 1.56	**< 0.001**
18:0	19.86 ± 2.08	21.28 ± 1.34	21.96 ± 1.42	18.50 ± 1.28	20.10 ± 1.91	20.16 ± 1.05	**0.012**
20:0	0.24 ± 0.29	ND	0.29 ± 0.18	ND	0.19 ± 0.24	0.11 ± 0.13	0.117
22:0	0.29 ± 0.43	ND	0.36 ± 0.15	ND	0.23 ± 0.25	0.10 ± 0.13	0.197
Total saturated	48.03 ± 1.83	44.49 ± 1.60	45.63 ± 1.07	44.66 ± 0.62	43.39 ± 2.29	45.08 ± 1.05	**< 0.001**
16:1n-7	1.17 ± 0.80	0.32 ± 0.24	0.84 ± 0.63	0.67 ± 0.37	0.54 ± 0.34	0.56 ± 0.23	0.080
18:1n-9	4.60 ± 0.84	3.92 ± 0.73	5.32 ± 0.74	5.04 ± 0.68	4.41 ± 0.34	6.88 ± 0.67 *	**< 0.001**
20:1n-9	ND	ND	ND	1.38 ± 0.17 *	ND	ND	**< 0.001**
Total MUFA	5.78 ± 1.42	4.30 ± 0.89	6.15 ± 1.31	7.14 ± 0.49	5.02 ± 0.52	7.44 ± 0.78	**< 0.001**
18:2n-6	12.18 ± 1.84 *	23.73 ± 3.01	21.87 ± 1.40	18.62 ± 0.95	23.11 ± 1.86	20.22 ± 2.54	**< 0.001**
18:3n-6	1.32 ± 0.93	0.77 ± 0.73	1.18 ± 0.68	0.71 ± 0.65	2.12 ± 1.69	0.90 ± 0.68	0.150
20:2n-6	0.22 ± 0.18	0.44 ± 0.26	0.39 ± 0.11	0.40 ± 0.16	0.63 ± 0.11	0.21 ± 0.20	**0.003**
20:3n-6	0.15 ± 0.24	0.36 ± 0.45	0.30 ± 0.33	0.62 ± 0.22	0.12 ± 0.19	0.42 ± 0.25	0.056
20:4n-6	11.99 ± 1.37	10.40 ± 1.80	5.80 ± 0.79	4.32 ± 0.35	9.65 ± 1.48	12.02 ± 1.24	**< 0.001**
22:5n-6	6.05 ± 0.71 *	2.24 ± 0.45 *	ND	ND	8.48 ± 1.18 *	4.23 ± 0.62 *	**< 0.001**
Total n-6 PUFA	31.90 ± 2.68	37.95 ± 2.07	29.55 ± 1.02	24.67 ± 0.86 *	44.11 ± 1.49 *	38.00 ± 0.98	**< 0.001**
18:3n-3	0.13 ± 0.10	0.38 ± 0.11	1.95 ± 0.24 *	0.29 ± 0.08	0.12 ± 0.10	0.23 ± 0.04	**< 0.001**
20:4n-3	ND	ND	ND	0.22 ± 0.11 *	ND	ND	**< 0.001**
20:5n-3	0.88 ± 0.86	0.81 ± 0.90	1.66 ± 0.37	4.86 ± 0.35 *	1.63 ± 1.51	0.64 ± 0.59	**< 0.001**
22:5n-3	1.28 ± 0.34	1.03 ± 0.22	1.91 ± 0.32	1.65 ± 0.17	0.40 ± 0.16	0.78 ± 0.20	**< 0.001**
22:6n-3	12.00 ± 1.73	11.04 ± 1.62	13.14 ± 1.47	16.51 ± 0.86 *	5.33 ± 0.78 *	7.84 ± 1.02 *	**< 0.001**
Total n-3 PUFA	14.30 ± 2.26	13.26 ± 1.75	18.66 ± 1.70 *	23.52 ± 0.91 *	7.48 ± 2.20	9.48 ± 1.35	**< 0.001**

ND negligible detected (mean <0.1%) * significantly different from all other groups (Bonferroni $p < 0.05$)

Table 6.7: Fatty acid composition of plasma TAG in pregnant rats fed experimental diets (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.34 ± 0.19	0.30 ± 0.19	0.20 ± 0.10	1.42 ± 0.41	0.15 ± 0.08	1.25 ± 0.10	**< 0.001**
16:0	22.74 ± 2.85	15.96 ± 1.21	12.97 ± 1.80	17.25 ± 1.09	13.91± 1.13	24.77 ± 1.17	**< 0.001**
18:0	5.10 ± 1.58	4.66 ± 1.32	4.31 ± 0.48	4.15 ± 0.92	4.34 ± 0.58	9.51 ± 1.10 *	**< 0.001**
20:0	0.30 ± 0.46	0.41 ± 0.10	0.23 ± 0.15	0.72 ± 0.38	0.16 ± 0.29	0.63 ± 0.08	**0.009**
22:0	0.62 ± 0.86	0.20 ± 0.14	0.23 ± 0.13	0.09 ± 0.15	0.24 ± 0.19	ND	0.123
Total saturates	29.10 ± 3.06 *	21.53 ± 2.35	17.93 ± 2.15	23.64 ± 1.70	18.80 ± 1.28	36.16 ± 1.83 *	**< 0.001**
16:1n-7	2.85 ± 1.00	0.66 ± 0.36	0.45 ± 0.27	3.11 ± 0.52	0.29 ± 0.27	1.55 ± 0.19	**< 0.001**
18:1n-9	19.27 ± 4.38	20.59 ± 0.69	21.14 ± 1.30	18.43 ± 0.92	20.37 ± 0.50	36.33 ± 1.59 *	**< 0.001**
20:1n-9	0.14 ± 0.22	0.19 ± 0.21	0.33 ± 0.06	4.58 ± 0.59 *	0.34 ± 0.29	0.47 ± 0.24	**< 0.001**
24:1n-9	ND	ND	ND	0.33 ± 0.11 *	ND	ND	**< 0.001**
Total MUFA	22.25 ± 4.92	21.44 ± 0.53	21.92 ± 1.44	26.44 ± 1.67	20.99 ± 0.55	38.39 ± 1.38 *	**< 0.001**
18:2n-6	26.74 ± 4.12	44.42 ± 3.57 *	24.76 ± 1.02	19.63 ± 0.82	49.48 ± 2.80 *	19.40 ± 1.54	**< 0.001**
18:3n-6	4.00 ± 3.72 *	0.99 ± 0.36	0.57 ± 0.32	0.44 ± 0.47	1.06 ± 0.48	0.59 ± 0.23	**0.003**
20:2n-6	0.27 ± 0.29	0.57 ± 0.14	0.26 ± 0.06	0.42 ± 0.04	0.74 ± 0.20	0.37 ± 0.10	**< 0.001**
20:3n-6	0.40 ± 0.34	0.42 ± 0.29	0.12 ± 0.13	0.12 ± 0.14	0.54 ± 0.35	0.28 ± 0.16	0.033 †
20:4n-6	8.34 ± 2.56 *	2.93 ± 1.73	1.15 ± 0.75	0.90 ± 0.15	4.06 ± 0.97	1.47 ± 0.12	**< 0.001**
22:5n-6	1.10 ± 0.18	0.50 ± 0.22	ND	0.28 ± 0.08	1.39 ± 0.34	0.45 ± 0.06	**< 0.001**
Total n-6 PUFA	40.85 ± 2.64 *	49.84 ± 2.09 *	26.87 ± 1.18 *	21.81 ± 0.82	57.28 ± 1.36 *	22.55 ± 1.36	**< 0.001**
18:3n-3	1.35 ± 0.40	4.75 ± 0.78 *	27.44 ± 1.59 *	3.01 ± 0.31	1.26 ± 0.06	1.64 ± 0.22	**< 0.001**
20:4n-3	ND	ND	0.18 ± 0.09 *	0.97 ± 0.07 *	ND	ND	**< 0.001**
20:5n-3	2.11 ± 2.29	0.65 ± 0.39	1.75 ± 0.66	7.70 ± 1.05 *	0.71 ± 0.51	0.40 ± 0.28	**< 0.001**
22:5n-3	1.20 ± 0.51	0.58 ± 0.47	1.56 ± 0.53	4.13 ± 0.54 *	0.25 ± 0.05	0.29 ± 0.13	**< 0.001**
22:6n-3	3.14 ± 1.90	1.21 ± 0.61	2.36 ± 0.40	12.30 ± 1.23 *	0.67 ± 0.28	0.56 ± 0.13	**< 0.001**
Total n-3 PUFA	7.80 ± 4.23	7.19 ± 0.43	33.28 ± 2.06 *	28.11 ± 2.25 *	2.93 ± 0.87	2.89 ± 0.32	**< 0.001**

ND negligible detected (mean <0.1%) * significantly different from all other groups ($p < 0.05$)

† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.8: Fatty acid composition of plasma CE in pregnant rats fed experimental diets (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.21 ± 0.23	0.21 ± 0.17	0.28 ± 0.25	0.33 ± 0.43	ND	0.21 ± 0.19	0.689
16:0	14.38 ± 3.33	12.42 ± 3.37	15.00 ± 2.76	15.56 ± 2.74	11.74 ± 3.25	12.87 ± 2.12	0.188
18:0	5.28 ± 2.12	4.67 ± 2.75	5.91 ± 3.69	5.04 ± 2.73	3.77 ± 0.79	5.97 ± 3.35	0.742
Total saturates	19.91 ± 5.48	17.30 ± 6.21	21.20 ± 6.08	20.93 ± 4.98	15.60 ± 3.86	19.06 ± 5.17	0.435
16:1n-7	1.40 ± 0.36	0.37 ± 0.09	0.49 ± 0.15	1.80 ± 0.29	0.39 ± 0.15	0.94 ± 0.22 *	**< 0.001**
18:1n-9	6.84 ± 1.34	7.40 ± 1.24	8.31 ± 0.76	7.51 ± 1.94	7.15 ± 1.16	11.20 ± 1.90 *	**< 0.001**
Total MUFA	8.25 ± 1.61	7.78 ± 1.27	8.85 ± 0.89	9.39 ± 2.10	7.54 ± 1.30	12.14 ± 2.01	**< 0.001**
18:2n-6	16.99 ± 2.53 *	30.20 ± 2.50	29.07 ± 4.77	22.34 ± 2.18	32.46 ± 1.31	22.73 ± 2.55	**< 0.001**
18:3n-6	0.90 ± 0.19	0.76 ± 0.20	0.38 ± 0.09	0.25 ± 0.22	0.88 ± 0.13	0.60 ± 0.13	**< 0.001**
20:3n-6	0.31 ± 0.76	ND	ND	ND	ND	ND	0.641
20:4n-6	46.41 ± 3.36	37.78 ± 5.54	23.42 ± 2.35 *	16.24 ± 2.52 *	39.11 ± 4.80	40.65 ± 3.76	**< 0.001**
22:5n-6	1.48 ± 0.22 *	0.46 ± 0.24	ND	ND	2.15 ± 0.47 *	0.94 ± 0.21 *	**< 0.001**
Total n-6 PUFA	66.09 ± 5.49	69.27 ± 6.27	52.92 ± 6.33 *	38.91 ± 4.35 *	74.60 ± 4.87	65.04 ± 5.12	**< 0.001**
18:3n-3	0.33 ± 0.08	0.75 ± 0.13	4.64 ± 0.70 *	0.59 ± 0.07	ND	0.28 ± 0.16	**< 0.001**
20:4n-3	0.10 ± 0.11	ND	ND	0.47 ± 0.24 *	ND	ND	**< 0.001**
20:5n-3	0.44 ± 0.27	0.30 ± 0.18	7.07 ± 1.78 *	22.02 ± 2.17 *	ND	0.32 ± 0.17	**< 0.001**
22:5n-3	ND	ND	ND	0.18 ± 0.21	ND	ND	0.022 †
22:6n-3	4.88 ± 1.00	4.57 ± 0.62	5.25 ± 0.91	7.52 ± 1.12 *	2.13 ± 0.41	3.13 ± 0.62	**< 0.001**
Total n-3 PUFA	5.75 ± 1.04	5.66 ± 0.73	17.02 ± 1.98 *	30.77 ± 2.98 *	2.25 ± 0.44	3.79 ± 0.66	**< 0.001**

ND negligible detected (mean <0.1%)
* significantly different from all other groups ($p < 0.05$)
† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.9: Fatty acid composition of plasma NEFA in pregnant rats fed experimental diets (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	1.19 ± 0.74	0.71 ± 0.43	0.55 ± 0.19	1.93 ± 0.42	0.68 ± 0.19	1.05 ± 0.23	**< 0.001**
16:0	25.22 ± 1.93	19.21 ± 2.36	15.70 ± 1.62	23.46 ± 4.77	17.82 ± 2.20	22.97 ± 2.05	**< 0.001**
18:0	11.17 ± 1.81	11.50 ± 2.85	10.05 ± 2.68	15.37 ± 7.05	11.34 ± 2.64	15.38 ± 1.56	0.055
20:0	0.52 ± 0.69	0.30 ± 0.39	0.22 ± 0.29	0.41 ± 0.66	0.33 ± 0.29	0.14 ± 0.22	0.761
22:0	0.45 ± 0.33	0.55 ± 0.44	0.39 ± 0.51	0.64 ± 0.46	0.75 ± 0.19	0.46 ± 0.39	0.624
Total saturates	38.60 ± 3.19	32.31 ± 5.40	26.91 ± 4.06	41.89 ± 11.16	30.99 ± 3.60	39.99 ± 3.60	**< 0.001**
16:1n-7	3.14 ± 2.62	0.96 ± 0.84	1.12 ± 0.58	4.07 ± 2.57	3.12 ± 4.36	3.14 ± 1.12	0.183
18:1n-9	19.24 ± 1.13	17.04 ± 1.31	17.19 ± 1.01	15.40 ± 2.74	16.55 ± 1.97	27.12 ± 3.92 *	**< 0.001**
20:1n-9	ND	ND	ND	1.94 ± 2.24	ND	0.98 ± 2.40	0.085
Total MUFA	22.41 ± 2.72	18.07 ± 0.95	18.31 ± 1.10	21.49 ± 4.92	19.66 ± 2.70	31.30 ± 1.78 *	**< 0.001**
18:2n-6	23.91 ± 3.04	35.98 ± 5.09	20.23 ± 2.97	16.22 ± 4.03	37.48 ± 5.61	17.21 ± 2.59	**< 0.001**
18:3n-6	1.79 ± 1.75	0.92 ± 1.23	1.64 ± 1.53	1.10 ± 2.70	2.96 ± 3.39	0.97 ± 1.95	0.610
20:2n-6	0.27 ± 0.45	0.51 ± 0.59	0.41 ± 0.35	1.07 ± 0.79	0.81 ± 0.43	0.70 ± 0.50	0.152
20:3n-6	0.08 ± 0.20	0.53 ± 0.80	1.15 ± 1.60	1.08 ± 2.44	0.16 ± 0.40	0.77 ± 1.88	0.723
20:4n-6	3.65 ± 1.26	3.09 ± 1.27	1.48 ± 0.16	1.37 ± 0.71	2.91 ± 0.35	3.06 ± 0.50	**< 0.001**
22:5n-6	0.68 ± 0.36	0.43 ± 0.17	ND	ND	1.31 ± 0.26 *	0.60 ± 0.11	**< 0.001**
Total n-6 PUFA	30.38 ± 4.78	41.46 ± 4.52	24.92 ± 1.64	20.87 ± 4.12	45.63 ± 2.71	23.32 ± 2.88	**< 0.001**
18:3n-3	2.20 ± 0.80	4.58 ± 0.62	23.55 ± 3.62 *	2.60 ± 1.15	1.07 ± 0.23	1.61 ± 0.46	**< 0.001**
20:4n-3	0.13 ± 0.32	ND	0.17 ± 0.16	0.61 ± 0.35 *	ND	ND	**< 0.001**
20:5n-3	1.74 ± 1.82	0.54 ± 0.78	1.48 ± 0.98	3.55 ± 1.04	1.14 ± 1.44	0.65 ± 1.58	**0.006**
22:5n-3	0.94 ± 0.92	0.49 ± 0.32	1.30 ± 0.54	2.20 ± 1.12	ND	0.23 ± 0.08	**< 0.001**
22:6n-3	3.60 ± 2.64	2.53 ± 0.59	3.36 ± 1.64	6.79 ± 2.74	1.46 ± 0.32	2.90 ± 1.14	**< 0.001**
Total n-3 PUFA	8.61 ± 6.12	8.16 ± 0.70	29.86 ± 3.92 *	15.75 ± 5.81	3.76 ± 1.58	5.39 ± 1.66	**< 0.001**

ND negligible detected (mean <0.1%) * significantly different from all other groups ($p < 0.05$)

6.3.5 Maternal liver size and composition

Maternal liver size and composition were assessed, with a significant effect of maternal diet observed upon maternal liver weight. The liver weight of dams in the HF linseed, salmon and beef tallow groups was significantly higher than that in the LF soyabean oil group (see table 6.10). However, this significant effect is likely to be related to the differences in maternal weight gain in these groups, as liver weight expressed as a % total body weight was not significantly affected by maternal diet. No significant effect of maternal diet was observed upon maternal liver dry weight, lipid content or glycogen content.

6.3.6 Maternal liver fatty acid composition

The fatty acid compositions of PC, phosphatidylethanolamine (PE), TAG and CE in liver were determined by gas chromatography (see tables 6.11 to 6.14). Significant effects of maternal diet were observed in all liver lipid fractions, and across virtually all fatty acids assessed. The effects observed were in line with the properties of the diets consumed, and comparable, but not identical, to those observed in the maternal plasma.

Within the HF linseed oil group, there was a stronger effect upon LC n-3 PUFA status in the liver than was observed in the plasma, with higher EPA status observed in all liver lipids and higher DHA status in liver phospholipids (PC and PE) in comparison to all other dietary groups (except HF salmon oil). These features were associated with lower AA status of liver phospholipids, and lower LA content of liver TAG. This suggests that accumulation of ALNA within liver TAG is at the expense of LA accumulation, and that synthesis of LC n-3 PUFA occurs at the expense of LC n-6 PUFA synthesis.

The HF sunflower oil diet demonstrated the highest AA content of liver phospholipids out of all dietary groups, a feature which had not been apparent in plasma lipids. This suggests that the synthesis of LC n-6 PUFA is enhanced by a high LA diet. It is possible that this increase is not apparent in plasma lipids due to selective incorporation of AA into other maternal tissues or transfer to the fetus. The effect of the sunflower oil in increasing 22:5n-6 content was only apparent in liver CE, with this fatty acid undetectable in liver PE and TAG.

Table 6.10: Liver weight, lipid and glycogen content in pregnant rats fed experimental diets (mean ± standard deviation, n=6)

	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
Liver wet weight (g)	9.82 ± 1.57 [a]	12.24 ± 1.61	12.72 ± 0.97 [b]	13.24 ± 1.15 [b]	11.71 ± 1.35	13.04 ± 1.80 [b]	**0.003**
Liver wet weight (% body weight)	3.60 ± 0.35	4.01 ± 0.36	4.03 ± 0.36	4.16 ± 0.13	3.88 ± 0.32	4.01 ± 0.39	0.097
Liver dry weight (% of wet weight)	28.4 ± 1.3	29.0 ± 0.9	27.8 ± 1.4	28.0 ± 0.6	28.2 ± 0.9	28.4 ± 1.1	0.552
Liver lipid (g/100 g liver)	3.4 ± 0.2	4.3 ± 1.4	3.9 ± 0.5	3.7 ± 0.3	3.5 ± 0.3	3.5 ± 0.3	0.162
Liver glycogen (g/100 g liver)	1.3 ± 1.6	2.5 ± 1.5	2.7 ± 1.1	2.9 ± 0.9	3.1 ± 0.8	3.1 ± 1.5	0.241

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 6.11: Fatty acid composition of liver PC in pregnant rats fed experimental diets (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.16 ± 0.02	0.14 ± 0.02	0.15 ± 0.02	0.23 ± 0.03	0.19 ± 0.04	0.25 ± 0.04	**< 0.001**
16:0	28.39 ± 1.73	23.88 ± 1.51	22.32 ± 1.40	25.10 ± 0.89	26.30 ± 2.10	25.51 ± 0.93	**< 0.001**
18:0	20.25 ± 1.42	23.19 ± 0.75	21.85 ± 1.41	19.74 ± 1.52	24.50 ± 1.45	23.02 ± 1.29	**< 0.001**
20:0	ND	ND	ND	ND	ND	0.12 ± 0.07	**0.002**
22:0	0.10 ± 0.11	ND	ND	ND	0.12 ± 0.10	ND	0.170
Total saturates	48.91 ± 1.36	47.27 ± 1.24	44.39 ± 1.94	45.06 ± 0.89	51.13 ± 1.72	48.92 ± 0.99	**< 0.001**
16:1n-7	0.68 ± 0.17 *	0.18 ± 0.05	0.19 ± 0.07	0.45 ± 0.10	0.15 ± 0.02	0.38 ± 0.06	**< 0.001**
18:1n-9	3.63 ± 0.73	2.69 ± 0.25	3.12 ± 0.55	2.46 ± 0.38	3.02 ± 0.26	5.20 ± 0.64 *	**< 0.001**
20:1n-9	0.11 ± 0.10	0.10 ± 0.08	0.22 ± 0.49	0.48 ± 0.05	0.11 ± 0.11	0.44 ± 0.27	0.020 †
24:1n-9	ND	ND	ND	0.26 ± 0.07 *	ND	ND	**< 0.001**
Total MUFA	4.44 ± 0.88	2.97 ± 0.22	3.53 ± 0.97	3.65 ± 0.47	3.28 ± 0.28	6.05 ± 0.71 *	**< 0.001**
18:2n-6	10.55 ± 0.91	12.74 ± 1.79	12.43 ± 1.30	9.13 ± 0.23	15.19 ± 0.80 *	9.98 ± 1.46	**< 0.001**
18:3n-6	0.38 ± 0.04	0.27 ± 0.04	0.13 ± 0.07	0.10 ± 0.08	0.37 ± 0.06	0.47 ± 0.17	**< 0.001**
20:2n-6	0.27 ± 0.05	0.46 ± 0.10	0.49 ± 0.62	0.18 ± 0.03	0.67 ± 0.05	0.24 ± 0.05	**0.021**
20:3n-6	0.35 ± 0.08	0.38 ± 0.07	0.57 ± 0.13	0.44 ± 0.07	0.42 ± 0.10	0.51 ± 0.07	**0.001**
20:4n-6	18.54 ± 0.49	16.73 ± 0.87	10.37 ± 0.21 *	6.95 ± 0.19 *	19.43 ± 1.76	17.94 ± 1.56	**< 0.001**
22:5n-6	0.24 ± 0.13	0.10 ± 0.16	0.22 ± 0.19	ND	0.24 ± 0.19	0.10 ± 0.15	0.048 †
Total n-6 PUFA	30.33 ± 1.49	30.69 ± 2.26	24.21 ± 1.32 *	16.80 ± 0.38 *	36.32 ± 1.63 *	29.23 ± 2.79	**< 0.001**
18:3n-3	0.19 ± 0.05	0.23 ± 0.06	1.37 ± 0.21 *	0.14 ± 0.05	0.11 ± 0.07	0.14 ± 0.11	**< 0.001**
20:4n-3	ND	ND	ND	0.16 ± 0.01 *	ND	ND	**< 0.001**
20:5n-3	0.17 ± 0.11	0.17 ± 0.09	3.50 ± 0.95 *	6.30 ± 0.50 *	ND	0.89 ± 0.75	**< 0.001**
22:5n-3	1.37 ± 0.21	1.41 ± 0.18	2.60 ± 0.41	2.26 ± 0.14	0.54 ± 0.06 *	1.38 ± 0.36	**< 0.001**
22:6n-3	14.59 ± 1.47	17.26 ± 1.75	20.40 ± 1.45 *	25.63 ± 1.11 *	8.62 ± 0.48 *	13.39 ± 2.49	**< 0.001**
Total n-3 PUFA	16.32 ± 1.52	19.07 ± 1.66	27.87 ± 1.29 *	34.49 ± 1.15 *	9.27 ± 0.49 *	15.80 ± 3.09	**< 0.001**

ND negligible detected (mean <0.1%) * significantly different from all other groups ($p < 0.05$)

† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.12: Fatty acid composition of liver PE in pregnant rats fed experimental diets (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
16:0	22.16 ± 1.35	17.78 ± 0.61	15.82 ± 0.62 *	18.64 ± 0.47	21.00 ± 1.22	20.13 ± 0.41	**< 0.001**
18:0	27.18 ± 1.43	28.26 ± 0.71	27.87 ± 0.56	25.03 ± 0.66 *	32.80 ± 1.00 *	28.85 ± 0.56	**< 0.001**
20:0	ND	0.11 ± 0.14	ND	ND	ND	ND	0.167
Total saturates	49.42 ± 0.99	46.18 ± 0.35 *	43.69 ± 0.82	43.80 ± 0.65	53.82 ± 2.20 *	49.03 ± 0.37	**< 0.001**
16:1n-7	0.21 ± 0.05	0.12 ± 0.20	ND	0.11 ± 0.11	ND	ND	**0.006**
18:1n-9	1.89 ± 0.40	2.07 ± 0.43	2.05 ± 0.44	1.19 ± 0.29	2.52 ± 0.28	2.36 ± 0.46	**< 0.001**
20:1n-9	0.15 ± 0.15	0.21 ± 0.23	0.17 ± 0.20	0.41 ± 0.21	ND	ND	**0.019**
Total MUFA	2.26 ± 0.54	2.40 ± 0.48	2.26 ± 0.55	1.72 ± 0.26	2.60 ± 0.33	2.44 ± 0.46	**0.037**
18:2n-6	3.60 ± 0.38	4.73 ± 0.66	4.59 ± 0.41	2.86 ± 0.36	6.00 ± 0.51 *	3.65 ± 0.44	**< 0.001**
18:3n-6	ND	0.10 ± 0.17	0.12 ± 0.20	0.14 ± 0.19	0.11 ± 0.12	0.14 ± 0.10	0.969
20:2n-6	0.21 ± 0.07	0.28 ± 0.15	0.21 ± 0.14	0.10 ± 0.05	0.51 ± 0.04 *	ND	**< 0.001**
20:3n-6	0.25 ± 0.10	0.31 ± 0.11	0.27 ± 0.03	0.24 ± 0.10	0.34 ± 0.09	0.31 ± 0.06	0.299
20:4n-6	19.79 ± 0.68	19.91 ± 0.59	13.40 ± 0.57 *	7.32 ± 0.51 *	23.32 ± 0.89	22.98 ± 0.52	**< 0.001**
Total n-6 PUFA	23.91 ± 0.92	25.34 ± 1.00	18.58 ± 0.95 *	10.65 ± 0.88 *	30.28 ± 1.29 *	27.14 ± 0.77 *	**< 0.001**
18:3n-3	ND	0.13 ± 0.08	0.67 ± 0.08 *	ND	ND	ND	**< 0.001**
20:4n-3	ND	ND	ND	0.13 ± 0.07 *	ND	ND	**< 0.001**
20:5n-3	0.12 ± 0.08	0.12 ± 0.10	3.53 ± 0.69 *	5.98 ± 0.57 *	ND	0.17 ± 0.03	**< 0.001**
22:5n-3	1.82 ± 0.22	1.76 ± 0.27	3.24 ± 0.44	2.60 ± 0.17	0.75 ± 0.13 *	1.72 ± 0.59	**< 0.001**
22:6n-3	22.38 ± 1.14	24.07 ± 1.62	28.03 ± 1.50 *	35.07 ± 1.70 *	12.55 ± 1.06 *	19.44 ± 1.28 *	**< 0.001**
Total n-3 PUFA	24.41 ± 1.10	26.09 ± 1.62	35.46 ± 0.84 *	43.83 ± 1.26 *	13.30 ± 1.11 *	21.38 ± 0.81 *	**< 0.001**

ND negligible detected (mean <0.1%)
* significantly different from all other groups ($p < 0.05$)

Table 6.13: Fatty acid composition of liver TAG in pregnant rats fed experimental diets (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.44 ± 0.06	0.38 ± 0.07	0.36 ± 0.08	0.83 ± 0.09	0.40 ± 0.08	0.84 ± 0.12	**< 0.001**
16:0	25.25 ± 1.05 *	19.74 ± 1.33	16.93 ± 1.50	20.97 ± 0.54	18.73 ± 0.92	27.74 ± 1.15 *	**< 0.001**
18:0	3.53 ± 0.41	4.53 ± 0.26	4.84 ± 0.47	3.74 ± 0.50	4.24 ± 0.18	6.83 ± 0.94 *	**< 0.001**
20:0	0.26 ± 0.14	0.21 ± 0.15	0.49 ± 0.1	0.40 ± 0.07	ND	0.49 ± 0.05	**< 0.001**
22:0	ND	ND	0.16 ± 0.20	ND	ND	ND	0.097
Total saturates	29.57 ± 0.98 *	24.86 ± 1.39	22.77 ± 1.33	25.94 ± 0.65	23.48 ± 0.85	35.90 ± 1.87 *	**< 0.001**
16:1n-7	2.35 ± 0.75	0.55 ± 0.20	0.60 ± 0.18	1.71 ± 0.38	0.38 ± 0.10	1.39 ± 0.30	**< 0.001**
18:1n-9	22.65 ± 3.35	21.28 ± 1.12	21.83 ± 2.38	17.90 ± 2.20	20.16 ± 0.55	37.40 ± 2.32 *	**< 0.001**
20:1n-9	0.35 ± 0.04	0.46 ± 0.09	0.31 ± 0.10	1.61 ± 0.35 *	0.42 ± 0.08	0.55 ± 0.10	**< 0.001**
Total MUFA	25.34 ± 3.96	22.28 ± 1.24	22.74 ± 2.43	21.23 ± 2.73	20.96 ± 0.66	39.34 ± 2.49 *	**< 0.001**
18:2n-6	30.40 ± 1.21 *	40.58 ± 2.46 *	24.86 ± 1.99 *	20.75 ± 1.87	45.90 ± 1.40 *	19.46 ± 2.64	**< 0.001**
18:3n-6	0.90 ± 0.15	0.86 ± 0.12	0.36 ± 0.10	0.26 ± 0.09	1.04 ± 0.12	0.57 ± 0.09 *	**< 0.001**
20:2n-6	0.48 ± 0.09	0.79 ± 0.17 *	0.37 ± 0.07	0.31 ± 0.04	1.02 ± 0.12 *	0.32 ± 0.03	**< 0.001**
20:3n-6	0.60 ± 0.21	0.66 ± 0.19	0.33 ± 0.07	0.32 ± 0.04	0.86 ± 0.34	0.22 ± 0.11	**< 0.001**
20:4n-6	7.89 ± 2.97 *	4.47 ± 1.14	1.77 ± 0.22	1.70 ± 0.31	5.07 ± 1.03	2.10 ± 0.43	**< 0.001**
Total n-6 PUFA	40.27 ± 3.11 *	47.36 ± 2.20 *	27.68 ± 1.81 *	23.33 ± 1.94	53.89 ± 0.62 *	22.67 ± 3.09	**< 0.001**
18:3n-3	1.61 ± 0.22	3.07 ± 0.30	20.00 ± 1.46 *	2.77 ± 0.47	0.82 ± 0.08	1.21 ± 0.26	**< 0.001**
20:4n-3	ND	ND	0.39 ± 0.11 *	0.53 ± 0.07 *	ND	ND	**< 0.001**
20:5n-3	0.20 ± 0.12	0.22 ± 0.13	1.54 ± 0.72 *	6.00 ± 0.49 *	ND	ND	**< 0.001**
22:5n-3	1.48 ± 0.73	0.82 ± 0.26	2.32 ± 1.02	6.80 ± 0.64 *	0.30 ± 0.04	0.31 ± 0.21	**< 0.001**
22:6n-3	1.53 ± 0.66	1.38 ± 0.47	2.56 ± 0.73	13.41 ± 1.57 *	0.51 ± 0.09	0.51 ± 0.22	**< 0.001**
Total n-3 PUFA	4.82 ± 1.42	5.49 ± 0.58	26.81 ± 3.16	29.51 ± 1.83	1.66 ± 0.10	2.08 ± 0.63	**< 0.001**

ND negligible detected (mean <0.1%)
* significantly different from all other groups ($p < 0.05$)

Table 6.14: Fatty acid composition of liver CE in pregnant rats fed experimental diets (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.35 ± 0.18	0.34 ± 0.18	0.49 ± 0.26	0.77 ± 0.08	0.33 ± 0.17	0.64 ± 0.09	**< 0.001**
16:0	42.95 ± 3.38	37.72 ± 2.14	35.13 ± 3.10	44.69 ± 2.46	34.67 ± 3.68	42.77 ± 3.18	**< 0.001**
18:0	15.54 ± 2.61	16.60 ± 3.26	15.33 ± 1.97	14.69 ± 2.55	16.74 ± 2.44	17.01 ± 3.77	0.668
20:0	0.41 ± 0.42	0.16 ± 0.19	0.43 ± 0.26	0.40 ± 0.34	0.59 ± 0.36	0.40 ± 0.31	0.363
22:0	ND	ND	ND	ND	0.32 ± 0.52	ND	0.229
Total saturates	59.26 ± 4.73	54.82 ± 4.21	51.47 ± 4.12	60.56 ± 0.85	52.66 ± 5.15	60.90 ± 5.32	**0.001**
16:1n-7	1.96 ± 0.72	0.74 ± 0.40	0.86 ± 0.33	1.81 ± 0.25	1.02 ± 1.48	1.34 ± 0.66	0.041 †
18:1n-9	9.06 ± 2.83	10.09 ± 1.91	10.52 ± 2.13	7.45 ± 1.24	10.26 ± 1.56	13.29 ± 2.44	**0.002**
20:1n-9	0.44 ± 0.34	0.21 ± 0.30	0.30 ± 0.40	0.73 ± 0.06	0.41 ± 0.62	0.43 ± 0.31	0.271
24:1n-9	ND	ND	ND	ND	ND	ND	0.496
Total MUFA	11.46 ± 3.37	11.03 ± 2.40	11.68 ± 2.32	10.01 ± 1.46	11.69 ± 2.68	15.15 ± 3.35	0.050
18:2n-6	15.01 ± 1.64	21.33 ± 1.50 *	15.67 ± 1.61	12.43 ± 0.82	24.15 ± 1.57 *	12.08 ± 1.16	**< 0.001**
18:3n-6	0.35 ± 0.35	0.48 ± 0.23	0.41 ± 0.32	0.12 ± 0.19	0.64 ± 0.44	0.39 ± 0.38	0.190
20:2n-6	0.38 ± 0.27	0.55 ± 0.19	0.35 ± 0.19	0.36 ± 0.11	0.72 ± 0.23	0.43 ± 0.30	0.052
20:3n-6	0.12 ± 0.15	0.31 ± 0.26	0.25 ± 0.30	ND	0.33 ± 0.34	0.16 ± 0.19	0.400
20:4n-6	9.64 ± 1.73	7.48 ± 1.24	4.82 ± 0.69	3.52 ± 0.63	7.12 ± 0.42	8.28 ± 0.98	**< 0.001**
22:5n-6	0.40 ± 0.08	ND	ND	ND	0.71 ± 0.17 *	0.15 ± 0.18	**< 0.001**
Total n-6 PUFA	25.90 ± 2.40 *	30.24 ± 2.08	21.56 ± 1.97	16.50 ± 1.20 *	33.66 ± 2.61	21.49 ± 2.23	**< 0.001**
18:3n-3	0.97 ± 0.29	1.79 ± 0.14	10.55 ± 1.11 *	1.50 ± 0.13	0.66 ± 0.23	0.90 ± 0.34	**< 0.001**
20:4n-3	ND	ND	ND	0.22 ± 0.20	ND	ND	0.102
20:5n-3	0.23 ± 0.51	0.04 ± 0.10	1.97 ± 0.57 *	6.36 ± 0.87 *	0.08 ± 0.20	0.14 ± 0.32	**< 0.001**
22:5n-3	0.22 ± 0.25	0.20 ± 0.17	0.56 ± 0.29	1.11 ± 0.23 *	ND	0.13 ± 0.15	**< 0.001**
22:6n-3	1.88 ± 0.39	1.84 ± 0.40	2.16 ± 0.36	3.75 ± 0.66 *	1.26 ± 0.56	1.25 ± 0.39	**< 0.001**
Total n-3 PUFA	3.38 ± 0.77	3.91 ± 0.25	15.29 ± 1.31 *	12.93 ± 1.30 *	2.00 ± 0.72	2.45 ± 0.68	**< 0.001**

ND negligible detected (mean <0.1%) * significantly different from all other groups ($p < 0.05$)
† no significant differences between groups when Bonferroni post-hoc test applied

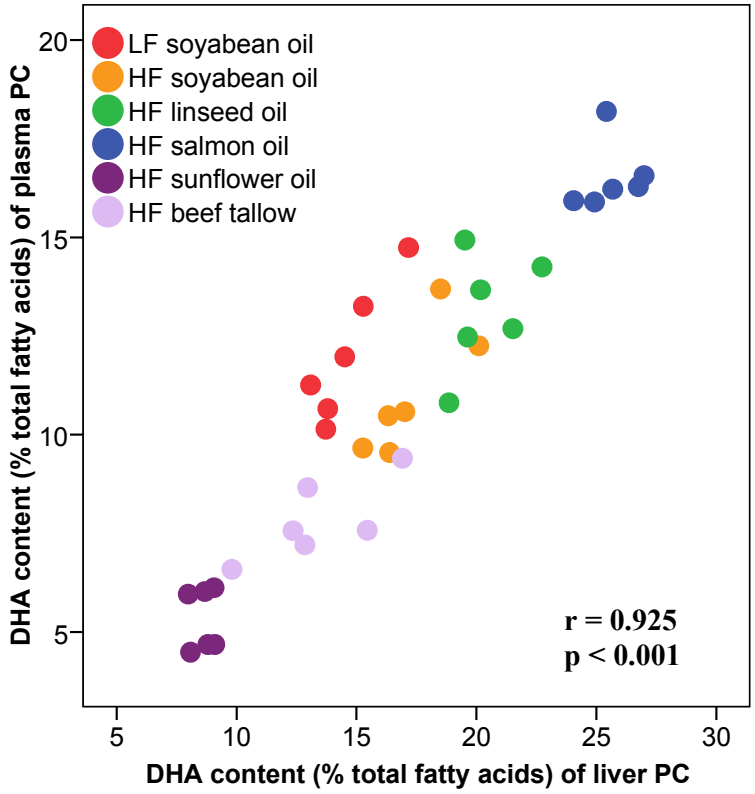
Correlations were used to assess the relationship between fatty acid compositions of maternal liver and plasma lipids (see table 6.15). Plasma and liver TAG fatty acids were strongly correlated across virtually all fatty acids assessed. The relationship between plasma and liver phospholipids was strongest for LC n-3 and n-6 PUFA (see figure 6.3). This reflects the role of the liver as a primary site of LC PUFA synthesis. However, the fatty acid 22:5n-6 was virtually undetectable across all maternal liver lipid fractions with the exception of CE, despite making a significant contribution to maternal plasma composition. This indicates that this fatty acid is either rapidly and selectively exported into the circulation from the liver, or that it is generated by a mechanism which does not involve the liver.

Table 6.15: Correlations observed between the fatty acid composition of liver and plasma lipids in pregnant rats (Pearsons r values unless otherwise indicated, n = 36)

Fatty acid	Plasma PC vs. Liver PC		Plasma PC vs. Liver PE		Plasma TAG vs. Liver TAG		Plasma CE vs. Liver CE	
12:0	-		-		-		-	
14:0	0.384	*	0.101†		0.777†	***	0.226	
16:0	0.645	***	0.455	**	0.953	***	0.326	
18:0	0.522	**	0.227		0.429†	**	0.180	
20:0	0.084†		-0.053†		0.454	**	0.319†	
22:0	0.117†		0.388†	*	0.186†		-	
Total saturated	-0.143		-0.146		0.951	***	0.313	
16:1n-7	0.433	**	0.210†		0.866	***	0.592	***
18:1n-9	0.745	***	0.282		0.829†	***	0.561	***
20:1n-9	0.321†		0.304†		0.660†	***	0.364†	*
24:1n-9	0.391†	*	-		-		-	
Total MUFA	0.669†	***	0.099		0.612†	***	0.484	**
18:2n-6	0.587	***	0.589	***	0.950†	***	0.707	***
18:3n-6	0.175		0.198†		0.697†	***	0.416	*
20:2n-6	0.293		0.541	**	0.723	***	0.049†	
20:3n-6	0.354†	*	-0.017†		0.642	***	-0.114†	
20:4n-6	0.891	***	0.727†	***	0.898†	***	0.896	
22:5n-6	0.343†	*	-		-		0.821†	***
Total n-6 PUFA	0.901	***	0.929	***	0.990	***	0.821	***
18:3n-3	0.613†	***	0.584†	***	0.889†	***	0.915†	***
20:4n-3	0.864†	***	0.740†	***	0.933†	***	0.537†	**
20:5n-3	0.543†	**	0.540†	**	0.793†	***	0.741†	***
22:5n-3	0.915	***	0.920	***	0.944†	***	0.380†	*
22:6n-3	0.925	***	0.938	***	0.931†	***	0.845	***
Total n-3 PUFA	0.938	***	0.948	***	0.929†	***	0.873†	***

† Spearman's r value
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 6.3: Graph to illustrate the observed correlation between the DHA content (% total fatty acids) of plasma and liver PC in pregnant rats



6.3.7 Maternal adipose tissue fatty acid composition

Maternal subcutaneous and intra-abdominal adipose tissue fatty acid compositions were assessed in order to determine how maternal diet might affect accumulation of fatty acids within adipose tissue (see tables 6.16 and 6.17). Both adipose tissue depots demonstrated a significant effect of maternal diet across virtually all fatty acids assessed.

When LF and HF soyabean oil groups are compared, adipose tissue in the former had higher 16:0 and OA content (fatty acids which can be generated by *de novo* synthesis) and lower LA and ALNA content. This indicates that increasing dietary intake of EFA results in these being stored in adipose tissue.

The HF linseed oil group demonstrated a high level of accumulation of ALNA in both subcutaneous and intra-abdominal adipose tissue. There was also a higher content of LC n-3 PUFA content than in other dietary groups (except salmon oil), particularly in subcutaneous adipose tissue where EPA and DPA status achieved levels close to those observed in the salmon oil group. These changes were at the expense of 16:0 and LA content.

The HF salmon oil group had significantly higher LC n-3 PUFA content of adipose tissue than any other dietary group. These fatty acids remained a relatively low proportion of adipose tissue content (e.g. mean DHA in subcutaneous adipose tissue = 3.78 % in salmon oil group), with the total n-3 content of the salmon oil group being significantly lower than that achieved in the linseed oil group.

The sunflower oil group had the highest LA and AA content of adipose tissue. This indicates that AA may be preferentially incorporated into adipose tissue, or that it may be endogenously synthesised within adipose tissue from its EFA precursor.

The HF beef tallow group had the highest adipose tissue content of OA and 18:0 of all the dietary groups, in accordance with the dietary content of these fatty acids.

Correlations between adipose tissue and plasma NEFA fatty acid compositions were used to assess the relationship between adipose tissue and the fatty acid composition of plasma NEFA (see table 6.18). The composition of adipose tissue was found to be closely related to plasma NEFA composition, particularly in relation to the EFA ALNA and LA and some LC PUFA such as docosapentaenoic acid (DPA, both n-6 and n-3). This demonstrates that adipose tissue plays a significant role in determining plasma fatty acid composition during late pregnancy.

The strong positive relationship between ALNA content of adipose tissue and plasma NEFA were not an artefact of the very high ALNA content of adipose tissue achieved in the HF linseed oil diet, and remained highly significant even when this group was excluded (Pearsons $r = 0.790$, $p < 0.001$, see figure 6.4).

Table 6.16: Subcutaneous adipose tissue fatty acid total lipid extract composition in pregnant rats fed experimental diets
(% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
12:0	0.44 ± 0.15	0.23 ± 0.06	0.32 ± 0.06	0.39 ± 0.08	0.24 ± 0.13	0.31 ± 0.07	**0.006**
14:0	1.67 ± 0.22	1.04 ± 0.19	1.16 ± 0.08	2.45 ± 0.24 *	1.02 ± 0.12	1.98 ± 0.15	**< 0.001**
16:0	25.97 ± 0.93 *	19.18 ± 1.18	17.72 ± 0.87	21.62 ± 0.86	17.52 ± 0.78	22.97 ± 1.48	**< 0.001**
18:0	3.94 ± 0.36	4.01 ± 0.31	4.00 ± 0.61	3.97 ± 0.39	3.94 ± 0.21	6.15 ± 0.64 *	**< 0.001**
20:0	ND	ND	0.33 ± 0.19	0.15 ± 0.12	ND	0.17 ± 0.15	**0.001**
Total saturates	32.03 ± 1.50	24.54 ± 1.17	23.54 ± 1.34	28.58 ± 1.25 *	22.78 ± 0.94	31.59 ± 1.85	**< 0.001**
16:1n-7	5.15 ± 1.00	2.51 ± 0.76	2.44 ± 0.37	4.07 ± 0.59	1.93 ± 0.24	3.37 ± 0.98	**< 0.001**
18:1n-9	28.02 ± 1.47 *	24.82 ± 0.76	25.15 ± 0.93	25.10 ± 0.55	24.63 ± 1.03	33.22 ± 0.77 *	**< 0.001**
20:1n-9	0.38 ± 0.12	0.31 ± 0.04	0.28 ± 0.04	0.21 ± 0.24	0.31 ± 0.03	0.27 ± 0.14	0.348
Total MUFA	33.55 ± 2.39 *	27.64 ± 1.13	27.87 ± 1.19	29.38 ± 0.73	26.87 ± 1.12	36.86 ± 1.14 *	**< 0.001**
18:2n-6	27.97 ± 3.03	38.60 ± 2.34	29.71 ± 2.20	28.52 ± 1.35	42.24 ± 1.71	24.91 ± 2.82	**< 0.001**
18:3n-6	0.42 ± 0.08	0.61 ± 0.10	0.36 ± 0.06	0.35 ± 0.07	0.76 ± 0.13	0.46 ± 0.14	**< 0.001**
20:2n-6	0.48 ± 0.13	0.60 ± 0.11	0.42 ± 0.11	0.46 ± 0.09	0.65 ± 0.10	0.39 ± 0.10	**0.001**
20:3n-6	0.51 ± 0.14	0.66 ± 0.14	0.39 ± 0.11	0.48 ± 0.16	0.73 ± 0.11	0.48 ± 0.18	**0.002**
20:4n-6	2.79 ± 0.72	3.15 ± 0.48	1.73 ± 0.40	1.78 ± 0.31	3.88 ± 0.57	2.99 ± 0.95	**< 0.001**
22:5n-6	0.21 ± 0.06	ND	ND	ND	0.30 ± 0.17	ND	**< 0.001**
Total n-6 PUFA	32.38 ± 3.27	43.68 ± 2.17 *	32.61 ± 1.91	31.64 ± 1.81	48.56 ± 1.61 *	29.24 ± 2.73	**< 0.001**
18:3n-3	1.20 ± 0.22	2.78 ± 0.32 *	12.08 ± 0.43 *	2.11 ± 0.17 *	1.24 ± 0.09	1.33 ± 0.26	**< 0.001**
20:4n-3	ND	ND	0.31 ± 0.10	0.68 ± 0.77	ND	ND	**0.003**
20:5n-3	ND	0.30 ± 0.06	1.44 ± 0.43 *	1.84 ± 0.30 *	ND	0.14 ± 0.08	**< 0.001**
22:5n-3	0.42 ± 0.12	0.54 ± 0.17	1.48 ± 0.48 *	1.99 ± 0.14 *	0.20 ± 0.10	0.41 ± 0.15	**< 0.001**
22:6n-3	0.40 ± 0.05	0.51 ± 0.08	0.68 ± 0.15	3.78 ± 0.36 *	0.28 ± 0.16	0.43 ± 0.11	**< 0.001**
Total n-3 PUFA	2.03 ± 0.15	4.13 ± 0.26 *	15.98 ± 1.19 *	10.40 ± 0.97 *	1.79 ± 0.32	2.31 ± 0.18	**< 0.001**

ND negligible detected (mean <0.1%) * significantly different from all other groups ($p < 0.05$)

Table 6.17: Intra-abdominal fatty acid total lipid extract composition in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
12:0	0.11 ± 0.01	ND	ND	0.13 ± 0.06	ND	ND	0.297
14:0	1.17 ± 0.04	0.77 ± 0.12 *	0.80 ± 0.09	2.00 ± 0.24 *	0.83 ± 0.12	1.52 ± 0.21 *	**< 0.001**
16:0	24.48 ± 0.66	19.17 ± 0.97	18.17 ± 0.92	22.45 ± 0.89	18.74 ± 1.52	22.51 ± 1.29	**< 0.001**
18:0	3.09 ± 0.38	3.71 ± 0.44	3.45 ± 0.29	3.50 ± 0.26	3.46 ± 0.17	5.57 ± 0.75 *	**< 0.001**
20:0	ND	ND	ND	ND	ND	0.26 ± 0.06 *	**< 0.001**
Total saturates	28.92 ± 0.86	23.81 ± 1.10	22.53 ± 0.78	28.14 ± 0.87	23.13 ± 1.79	29.94 ± 2.00	**< 0.001**
16:1n-7	4.49 ± 0.96	1.92 ± 0.53	2.26 ± 0.57	4.06 ± 0.57	2.29 ± 0.35	3.07 ± 0.80	**< 0.001**
18:1n-9	26.08 ± 1.26	24.25 ± 0.66	25.29 ± 0.66	25.05 ± 1.20	24.65 ± 0.89	32.23 ± 1.34 *	**< 0.001**
20:1n-9	0.51 ± 0.16	0.34 ± 0.03	0.35 ± 0.07	0.51 ± 0.05	0.34 ± 0.02	0.42 ± 0.06	**0.001**
Total MUFA	31.08 ± 2.03	26.52 ± 0.92	27.90 ± 1.15	29.61 ± 1.50	27.29 ± 1.15	35.72 ± 2.00 *	**< 0.001**
18:2n-6	35.73 ± 1.31	43.65 ± 1.36	33.67 ± 1.06	32.00 ± 1.54	45.28 ± 2.23	30.83 ± 3.04	**< 0.001**
18:3n-6	0.22 ± 0.03	0.20 ± 0.01	0.16 ± 0.02	0.19 ± 0.04	0.23 ± 0.03	0.17 ± 0.03	**< 0.001**
20:2n-6	0.30 ± 0.01	0.29 ± 0.02	0.23 ± 0.04	0.31 ± 0.02	0.29 ± 0.03	0.21 ± 0.03	**< 0.001**
20:3n-6	0.20 ± 0.03	0.24 ± 0.04	0.17 ± 0.03	0.22 ± 0.02	0.28 ± 0.04	0.14 ± 0.08	**< 0.001**
20:4n-6	0.86 ± 0.12	1.06 ± 0.17	0.65 ± 0.15	0.79 ± 0.16	1.24 ± 0.22	0.68 ± 0.13	**< 0.001**
22:5n-6	0.15 ± 0.05	ND	ND	0.12 ± 0.08	0.21 ± 0.10	ND	**< 0.001**
Total n-6 PUFA	37.46 ± 1.36	45.47 ± 1.37	34.89 ± 1.10	33.63 ± 1.68	47.53 ± 2.31	32.07 ± 3.18	**< 0.001**
18:3n-3	2.08 ± 0.13	3.65 ± 0.25 *	13.57 ± 1.10 *	2.61 ± 0.20	1.72 ± 0.12	1.96 ± 0.18	**< 0.001**
20:4n-3	ND	ND	ND	0.55 ± 0.61 *	ND	ND	**0.003**
20:5n-3	ND	ND	0.28 ± 0.08 *	1.30 ± 0.24 *	ND	ND	**< 0.001**
22:5n-3	0.20 ± 0.05	0.22 ± 0.05	0.41 ± 0.17	1.18 ± 0.22 *	0.10 ± 0.13	ND	**< 0.001**
22:6n-3	0.25 ± 0.07	0.31 ± 0.04	0.38 ± 0.12	2.98 ± 0.58 *	0.20 ± 0.14	0.22 ± 0.10	**< 0.001**
Total n-3 PUFA	2.54 ± 0.13	4.21 ± 0.21 *	14.67 ± 1.30 *	8.62 ± 1.42 *	2.04 ± 0.35	2.27 ± 0.26	**< 0.001**

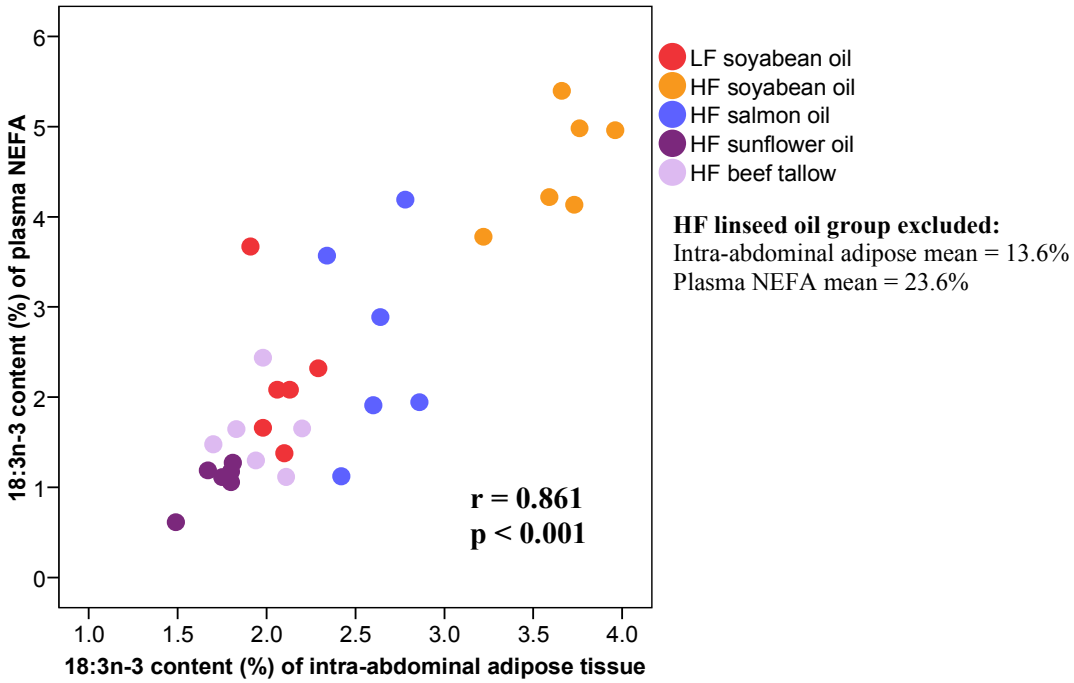
ND negligible detected (mean <0.1%) * significantly different from all other groups ($p < 0.05$)

Table 6.18: Correlations observed between fatty acids in adipose tissue total lipid extracts and plasma NEFA in pregnant rats (Pearsons r values unless otherwise indicated, n=36)

Fatty acid	Subcutaneous adipose vs. Plasma NEFA		Intra-abdominal adipose vs. Plasma NEFA	
12:0	0.156†		0.073†	
14:0	0.666	***	0.696	***
16:0	0.731	***	0.757	***
18:0	0.223†		0.209†	
20:0	-0.207†		-0.112†	
22:0	-0.155†		-	
Total saturated	0.580	***	0.616	***
16:1n-7	0.294		0.421	*
18:1n-9	0.574†	***	0.556†	***
20:1n-9	0.211†		0.283†	
24:1n-9	-		-0.068†	
Total MUFA	0.801	***	0.828	***
18:2n-6	0.833	***	0.837	***
18:3n-6	-0.109†		0.046†	
20:2n-6	0.265		0.176	
20:3n-6	-0.065†		-0.097†	
20:4n-6	0.349	*	0.220	
22:5n-6	0.574†	***	0.415†	*
Total n-6 PUFA	0.726†	***	0.880	***
18:3n-3	0.799†	***	0.877†	***
20:4n-3	0.564†	***	0.524†	**
20:5n-3	0.269†		0.535†	**
22:5n-3	0.712†	***	0.807†	***
22:6n-3	0.586†	***	0.405†	*
Total n-3 PUFA	0.791†	***	0.816†	***

† Spearman's R value * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 6.4: Graph to illustrate the correlation between the ALNA content (% total fatty acids) of intra-abdominal adipose tissue total lipid extracts and plasma NEFA in pregnant rats



6.3.8 Maternal immune cell and tissue fatty acid composition

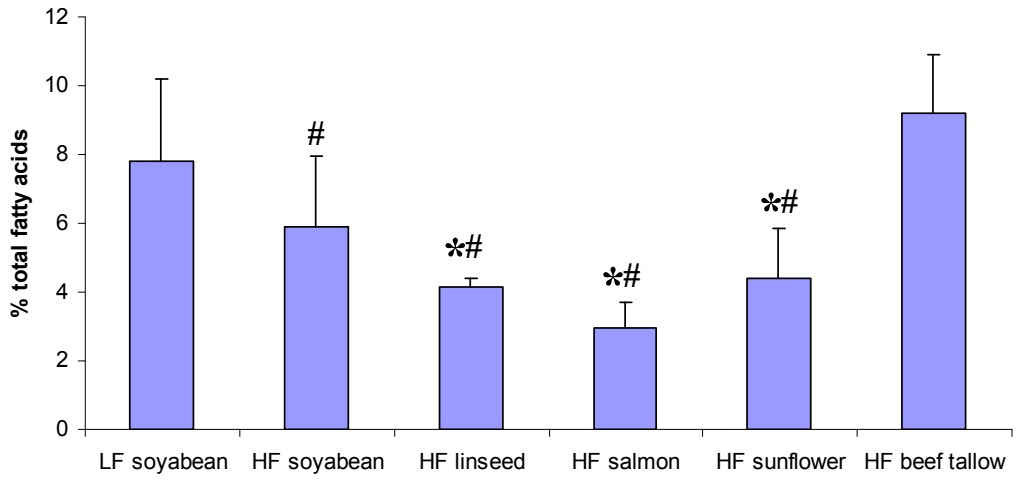
The effect of maternal dietary fatty acid intake upon maternal immune cell and tissue phospholipid (PC and PE) fatty acid composition was assessed. This would indicate whether there was a potential for maternal immune function to be altered via changes in the availability of fatty acids, for example EPA and AA which are utilised for prostaglandin synthesis.

Maternal PBMC PC demonstrated little change in fatty acid composition in response to diet (see table 6.19). In contrast, maternal PBMC PE demonstrated numerous significant effects of diet upon fatty acid status, particularly in relation to LC n-3 PUFA (see table 6.20). The HF salmon oil group had significantly higher 20:4n-3 and EPA content than any other dietary group and higher DPA and DHA content than any of the other dietary groups except the HF linseed oil group. No significant effects of maternal diet upon AA status were observed in maternal PBMC PC or PE.

Within the maternal spleen there were significant effects of diet upon PC and PE fatty acid composition across a wide range of fatty acids assessed, and in particular the n-6 and n-3 fatty acids (see table 6.21 and 6.22). There was significant accumulation of ALNA in maternal spleen phospholipids within the HF linseed oil group. The salmon oil diet also raised levels of DHA, 14:0 and 20:1n-9 to levels higher than any other dietary group in maternal spleen PC and PE.

In terms of AA and EPA content, significant effects of maternal diet were observed within maternal spleen PC and PE (see figures 6.5 and 6.6). The HF salmon oil group had the lowest AA content in maternal spleen PC and PE, though this was not statistically significant in the PE fraction when Bonferroni correction for multiple comparisons was applied due to the wide variation in AA content observed. The EPA content of spleen phospholipids was highest in the salmon oil group, with the linseed oil diet also increasing EPA levels in maternal spleen PE to higher levels than in any other dietary group (except HF salmon oil group).

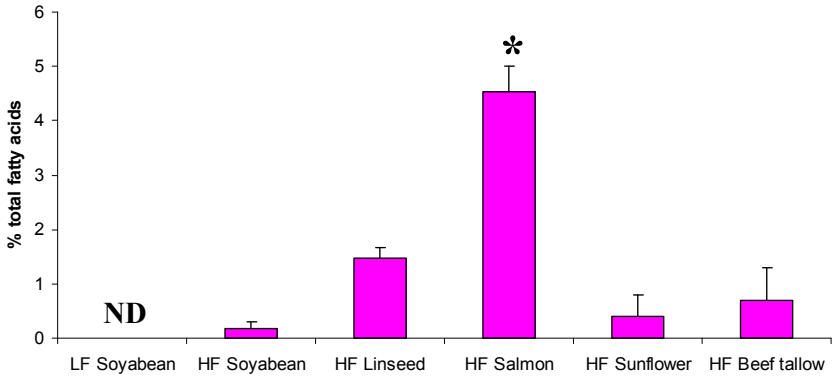
Figure 6.5: The AA content (% total fatty acids) of spleen PC in pregnant rats fed experimental diets (mean ± SEM)



* Significantly different ($p < 0.05$) from LF Soyabean

Significantly different ($p < 0.05$) from HF Beef tallow

Figure 6.6: The EPA content (% total fatty acids) of spleen PE in pregnant rats fed experimental diets (mean ± SEM)



* Significantly different ($p < 0.05$) from all other dietary groups

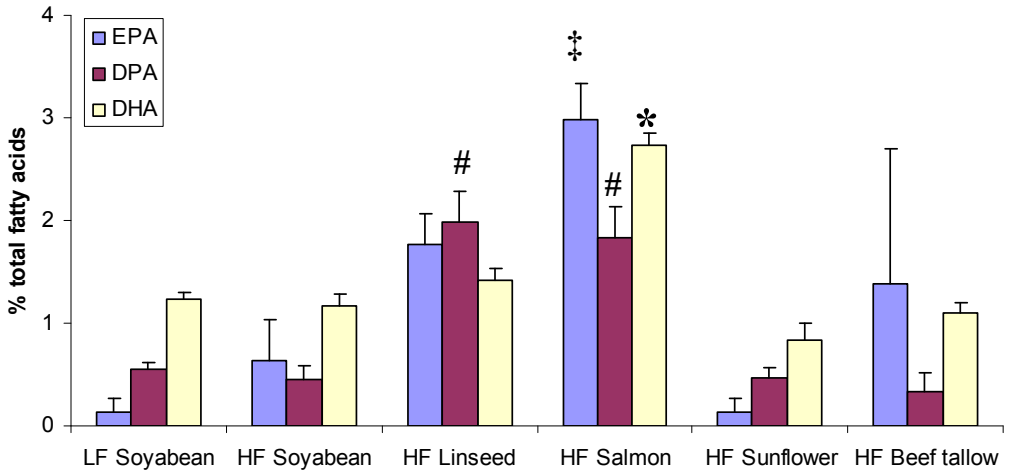
ND Negligible detected (mean < 0.1% total fatty acids)

Within the maternal thymus there were significant effects of diet upon PC and PE fatty acid composition (see tables 6.23 and 6.24). As was the case for maternal PBMC, there were fewer significant effects of diet on maternal thymus PC compared to PE.

Within maternal thymus PC there was significant accumulation of ALNA in the HF linseed oil diet group. Maternal diet significantly affected the AA content of maternal thymus PC, with the HF linseed and HF salmon oil groups exhibiting the lowest content. There was a trend for the HF linseed and HF salmon oil groups to have the highest EPA content, but this was not significant when Bonferroni post-hoc correction was applied due to the degree of variation within these groups.

There was no significant effect of maternal diet upon AA content of maternal thymus PE. Maternal diet had a significant effect upon the LC n-3 PUFA status of the maternal thymus, with EPA, DPA and DHA content highest in the HF linseed and HF salmon oil groups (see figure 6.7).

Figure 6.7: The LC n-3 PUFA content of thymus PE in pregnant rats fed experimental diets (mean ± SEM)



* Significantly different ($p < 0.05$) from all other dietary groups

\# Significantly different ($p < 0.05$) from LF soyabean, HF soyabean, HF sunflower and HF beef tallow

‡ Significantly different ($p < 0.05$) from LF soyabean

These data for maternal immune tissue fatty acid composition demonstrate that the maternal diet during pregnancy has the capacity to alter the fatty acid composition of tissues which are not directly involved in lipid metabolism.

The extent of the effects observed varied with the tissue assessed, with the greatest effect observed upon the spleen, a moderate effect upon the thymus, and few effects on PBMC. This indicates that not all maternal tissues are affected equally by changes to maternal diet, which may reflect the rate of turnover of cells within these tissues, or preferential incorporation of fatty acids into specific cells and tissues. The effects observed upon AA and EPA content in phospholipids will influence substrate availability for eicosanoid synthesis, and so may result in changes in immune function.

Table 6.19: Fatty acid composition of PBMC PC in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation)

	LF Soyabean (n = 6)	HF Soyabean (n = 4)	HF Linseed (n = 4)	HF Salmon (n = 5)	HF Sunflower (n = 6)	HF Beef tallow (n = 4)	ANOVA p value
14:0	0.36 ± 0.29 [b]	ND [b]	0.40 ± 0.29	1.00 ± 0.06 [a]	0.39 ± 0.44	0.35 ± 0.48	**0.004**
16:0	46.33 ± 7.28	42.70 ± 6.30	49.67 ± 5.92	46.59 ± 9.90	40.65 ± 8.17	46.03 ± 12.30	0.639
18:0	20.16 ± 1.82	21.34 ± 0.46	19.83 ± 1.54	15.75 ± 2.11	19.88 ± 5.01	20.16 ± 0.83	0.066
20:0	0.30 ± 0.35	0.52 ± 0.19	0.30 ± 0.20	ND	0.23 ± 0.32	0.45 ± 0.16	0.110
22:0	0.49 ± 0.11 [b]	0.40 ± 0.12 [b]	0.33 ± 0.10	ND [a]	0.24 ± 0.22	0.44 ± 0.04 [b]	**0.001**
Total saturates	67.65 ± 7.81	64.96 ± 6.44	70.53 ± 5.06	63.45 ± 11.16	61.40 ± 6.66	67.42 ± 12.77	0.620
16:1n-7	0.58 ± 0.20	ND	0.36 ± 0.27	0.91 ± 0.79	1.20 ± 1.61	0.31 ± 0.25	0.334
18:1n-9	14.43 ± 4.89	12.69 ± 1.59	9.98 ± 1.64	14.23 ± 9.49	13.03 ± 7.37	10.80 ± 4.07	0.831
20:1n-9	0.25 ± 0.39	0.37 ± 0.73	0.79 ± 0.39	0.81 ± 0.53	1.72 ± 2.85	1.21 ± 0.81	0.556
24:1n-9	0.59 ± 0.56	0.13 ± 0.25	0.44 ± 0.20	0.41 ± 0.48	0.48 ± 0.40	0.38 ± 0.26	0.657
Total MUFA	15.85 ± 5.02	13.27 ± 2.39	11.57 ± 1.92	16.36 ± 9.93	16.44 ± 6.28	12.70 ± 4.44	0.708
18:2n-6	6.45 ± 2.34	11.36 ± 0.84	10.82 ± 1.74	11.35 ± 1.30	10.82 ± 4.06	9.10 ± 4.14	0.052
18:3n-6	0.79 ± 1.50	0.17 ± 0.33	0.47 ± 0.38	0.50 ± 1.00	1.85 ± 2.43	0.16 ± 0.27	0.403
20:2n-6	0.67 ± 0.54	1.01 ± 0.69	0.57 ± 0.66	0.30 ± 0.42	1.04 ± 1.06	0.81 ± 0.20	0.525
20:3n-6	1.40 ± 1.19	1.34 ± 1.04	1.05 ± 0.55	1.19 ± 0.44	1.59 ± 1.21	1.79 ± 1.17	0.907
20:4n-6	3.11 ± 1.10	4.80 ± 3.41	2.53 ± 0.65	2.72 ± 0.62	3.11 ± 1.62	4.63 ± 2.64	0.340
22:5n-6	1.66 ± 3.04	0.09 ± 0.19	0.50 ± 0.33	0.58 ± 0.95	1.21 ± 1.85	0.62 ± 0.14	0.739
Total n-6 PUFA	14.07 ± 4.52	18.78 ± 4.18	15.94 ± 2.96	16.64 ± 3.19	19.63 ± 3.99	17.10 ± 7.48	0.394
18:3n-3	0.29 ± 0.53	0.34 ± 0.12	0.62 ± 0.26	0.10 ± 0.23	0.26 ± 0.35	0.30 ± 0.14	0.385
20:4n-3	ND [a]	0.48 ± 0.55	0.10 ± 0.12	0.72 ± 0.41 [b]	0.22 ± 0.26	0.27 ± 0.35	**0.033**
20:5n-3	0.87 ± 0.51	0.60 ± 0.75	0.62 ± 0.20	0.91 ± 0.36	0.59 ± 0.32	0.95 ± 0.46	0.666
22:5n-3	0.16 ± 0.39	ND	0.21 ± 0.25	0.42 ± 0.61	0.73 ± 1.78	0.20 ± 0.39	0.829
22:6n-3	1.06 ± 0.75	1.58 ± 0.68	0.41 ± 0.32	1.39 ± 0.59	0.73 ± 0.44	1.07 ± 0.89	0.125
Total n-3 PUFA	2.43 ± 1.45	3.00 ± 0.30	1.96 ± 0.70	3.54 ± 1.40	2.53 ± 1.35	2.78 ± 1.30	0.497

ND negligible detected (mean <0.1% total fatty acid content)

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 6.20: Fatty acid composition of PBMC PE in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation)

	LF Soyabean (n = 5)	HF Soyabean (n = 5)	HF Linseed (n = 6)	HF Salmon (n = 5)	HF Sunflower (n = 6)	HF Beef tallow (n = 5)	ANOVA p value
14:0	1.00 ± 0.31	0.70 ± 0.64	1.02 ± 0.72	1.25 ± 0.26	1.13 ± 0.56	1.08 ± 0.39	0.679
16:0	25.31 ± 1.69	26.30 ± 4.26	21.83 ± 4.30	24.34 ± 3.39	27.43 ± 3.65	24.71 ± 2.59	0.155
18:0	20.18 ± 4.59	23.30 ± 3.82	19.30 ± 4.35	17.04 ± 3.02	20.32 ± 3.62	20.98 ± 6.14	0.372
20:0	0.44 ± 0.21	0.68 ± 0.23 [a]	0.31 ± 0.26	0.13 ± 0.19 [b]	0.51 ± 0.21	0.33 ± 0.22	**0.010**
22:0	0.25 ± 0.09	0.36 ± 0.21 [b]	0.36 ± 0.18 [b]	ND [a]	0.36 ± 0.07 [b]	0.33 ± 0.11	**0.011**
Total saturates	47.18 ± 6.41	51.35 ± 4.81	42.80 ± 8.79	42.81 ± 6.33	49.75 ± 7.64	47.44 ± 9.18	0.331
16:1n-7	1.18 ± 0.16	1.19 ± 1.74	0.79 ± 0.98	1.25 ± 0.25	0.52 ± 0.35	0.68 ± 0.28	0.606
18:1n-9	15.61 ± 3.99	16.26 ± 0.80	16.81 ± 1.72	16.17 ± 4.36	15.08 ± 2.92	18.52 ± 2.74	0.529
20:1n-9	0.55 ± 0.36	0.43 ± 0.43	0.54 ± 0.90	3.00 ± 2.37 *	0.67 ± 0.24	0.55 ± 0.54	**0.004**
24:1n-9	0.65 ± 0.39	0.35 ± 0.38	0.31 ± 0.30	0.17 ± 0.24	0.54 ± 0.32	0.41 ± 0.08	0.167
Total MUFA	17.98 ± 4.16	18.22 ± 2.21	18.45 ± 1.67	20.60 ± 6.11	16.82 ± 3.56	20.16 ± 3.29	0.573
18:2n-6	12.53 ± 1.79 [b]	17.61 ± 4.46	15.74 ± 2.70	15.44 ± 2.31	18.82 ± 3.14 [a]	11.92 ± 2.25 [b]	**0.004**
18:3n-6	0.20 ± 0.20	0.25 ± 0.23	0.79 ± 0.93	1.75 ± 1.97	0.18 ± 0.22	0.52 ± 0.52	0.080
20:2n-6	0.80 ± 0.47	1.08 ± 0.27	0.63 ± 0.53	0.35 ± 0.48	0.91 ± 0.75	1.11 ± 0.57	0.237
20:3n-6	1.47 ± 1.00	1.60 ± 0.84	1.30 ± 1.14	1.06 ± 0.32	1.17 ± 0.61	1.10 ± 0.44	0.859
20:4n-6	15.95 ± 7.81	5.56 ± 1.62	10.07 ± 5.21	7.82 ± 3.94	9.28 ± 7.40	14.28 ± 7.72	0.099
22:5n-6	1.08 ± 0.94	0.48 ± 0.85	0.98 ± 1.05	0.59 ± 1.11	0.91 ± 0.77	1.04 ± 0.56	0.853
Total n-6 PUFA	32.02 ± 9.41	26.58 ± 2.39	29.50 ± 6.97	27.02 ± 5.21	31.27 ± 10.07	29.97 ± 9.93	0.845
18:3n-3	0.55 ± 0.24	1.32 ± 0.76	4.86 ± 2.50 *	0.34 ± 0.34	0.49 ± 0.20	0.36 ± 0.25	**< 0.001**
20:4n-3	ND	0.16 ± 0.37	0.34 ± 0.38	1.15 ± 0.76 *	ND	0.14 ± 0.22	**0.001**
20:5n-3	0.39 ± 0.23	0.52 ± 0.10	0.98 ± 0.62	3.13 ± 1.59 *	0.53 ± 0.12	0.53 ± 0.17	**< 0.001**
22:5n-3	0.37 ± 0.54 [b]	0.17 ± 0.37 [b]	1.22 ± 0.85	2.12 ± 1.59 [a]	0.14 ± 0.35 [b]	0.26 ± 0.25 [b]	**0.002**
22:6n-3	1.42 ± 0.52	1.68 ± 0.71	1.84 ± 0.76	2.83 ± 1.15 [a]	0.95 ± 0.41 [b]	1.14 ± 0.49 [b]	**0.004**
Total n-3 PUFA	2.82 ± 0.55 [b]	3.85 ± 1.67 [b]	9.24 ± 3.74 [a]	9.58 ± 3.32 [a]	2.16 ± 0.77 [b]	2.44 ± 0.76 [b]	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * significantly different ($p < 0.05$) from all other groups
a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 6.21: Fatty acid composition of spleen PC in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation)

	LF Soyabean (n=6)	HF Soyabean (n=6)	HF Linseed (n=4)	HF Salmon (n=6)	HF Sunflower (n=5)	HF Beef tallow (n=6)	ANOVA p value
14:0	0.19 ± 0.30	0.26 ± 0.28	0.38 ± 0.27	1.59 ± 0.19 *	0.30 ± 0.28	0.59 ± 0.46	**< 0.001**
16:0	58.73 ± 3.19	53.43 ± 2.21	52.95 ± 0.68	58.06 ± 3.22	55.60 ± 1.96	50.66 ± 2.34	**< 0.001**
18:0	11.55 ± 0.92	13.38 ± 2.91	11.86 ± 2.09	9.44 ± 1.24	13.15 ± 2.65	14.11 ± 2.56	**0.014**
20:0	ND	ND	0.11 ± 0.21	ND	ND	ND	0.342
22:0	ND	0.13 ± 0.14	0.15 ± 0.10	ND	0.23 ± 0.21	ND	0.079
Total saturates	70.51 ± 3.68	67.23 ± 3.10	65.45 ± 2.11	69.20 ± 3.67	69.28 ± 1.84	65.42 ± 3.85	0.067
16:1n-7	0.21 ± 0.33	0.24 ± 0.29	0.27 ± 0.20	0.82 ± 0.26	0.12 ± 0.17	0.90 ± 1.42	0.206
18:1n-9	8.74 ± 2.60	8.39 ± 2.71	9.70 ± 0.43	8.45 ± 0.46	8.79 ± 2.12	10.34 ± 1.03	0.448
20:1n-9	0.75 ± 0.90	0.56 ± 0.48	1.26 ± 0.61	1.78 ± 0.56	0.55 ± 0.50	0.85 ± 0.59	**0.018**
24:1n-9	0.35 ± 0.21	0.28 ± 0.15	0.30 ± 0.04	0.47 ± 0.24	0.30 ± 0.19	0.33 ± 0.08	0.482
Total MUFA	10.06 ± 1.88	9.47 ± 2.18	11.53 ± 0.73	11.52 ± 0.85	9.75 ± 1.67	12.43 ± 1.28	**0.018**
18:2n-6	7.92 ± 0.76	12.27 ± 1.49	12.96 ± 0.88	10.07 ± 1.21 *	12.14 ± 0.67	8.00 ± 0.45	**< 0.001**
18:3n-6	0.26 ± 0.46	ND	ND	0.29 ± 0.27	ND	0.39 ± 0.92	0.571
20:2n-6	1.00 ± 0.38	2.14 ± 0.94	1.44 ± 0.33	0.79 ± 0.15	2.65 ± 0.68	0.91 ± 0.21	**< 0.001**
20:3n-6	0.56 ± 0.29	1.24 ± 1.44	0.55 ± 0.10	0.46 ± 0.12	0.66 ± 0.28	0.95 ± 0.80	0.457
20:4n-6	7.78 ± 2.44	5.92 ± 2.01	4.14 ± 0.27	2.97 ± 0.71	4.38 ± 1.47	9.20 ± 1.72	**< 0.001**
22:5n-6	0.63 ± 0.16	0.50 ± 0.22	0.33 ± 0.07	ND	0.70 ± 0.09	0.61 ± 0.20	**< 0.001**
Total n-6 PUFA	18.16 ± 3.36	22.08 ± 2.87	19.41 ± 1.32	14.65 ± 2.23	20.53 ± 2.57	20.05 ± 2.35	**0.001**
18:3n-3	ND	0.14 ± 0.11	1.53 ± 0.35 *	ND	ND	ND	**< 0.001**
20:4n-3	ND	ND	0.31 ± 0.07	0.73 ± 0.54	0.14 ± 0.19	0.13 ± 0.20	**0.001**
20:5n-3	0.21 ± 0.51	ND	0.35 ± 0.07	1.00 ± 0.35	ND	0.51 ± 0.88	**0.015**
22:5n-3	0.38 ± 0.42	0.30 ± 0.18	0.68 ± 0.12	1.19 ± 0.32	ND	0.63 ± 0.47	**< 0.001**
22:6n-3	0.61 ± 0.38	0.67 ± 0.22	0.74 ± 0.09	1.62 ± 0.48 *	0.17 ± 0.19	0.81 ± 0.22	**< 0.001**
Total n-3 PUFA	1.27 ± 1.21	1.23 ± 0.53	3.61 ± 0.42	4.63 ± 1.14	0.44 ± 0.38	2.10 ± 1.22	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups

Table 6.22: Fatty acid composition of spleen PE in pregnant rats fed experimental diets (% total fatty acid, mean ± standard deviation)

	LF Soyabean (n=5)	HF Soyabean (n=6)	HF Linseed (n=5)	HF Salmon (n=6)	HF Sunflower (n=6)	HF Beef tallow (n=6)	ANOVA p value
14:0	ND	ND	ND	0.52 ± 0.31 *	ND	0.16 ± 0.18	**< 0.001**
16:0	18.07 ± 3.91	17.52 ± 5.81	12.78 ± 1.11	16.35 ± 2.62	19.54 ± 5.92	15.39 ± 3.29	0.173
18:0	21.74 ± 1.55	21.26 ± 1.43	21.16 ± 2.93	17.66 ± 2.79	22.26 ± 2.52	22.48 ± 1.75	**0.011**
Total saturates	39.83 ± 2.67	38.78 ± 6.57	33.99 ± 3.70	34.55 ± 3.64	41.85 ± 8.24	36.82 ± 3.61	0.129
16:1n-7	0.20 ± 0.28	0.14 ± 0.33	0.26 ± 0.16	0.97 ± 0.47	0.43 ± 1.04	0.37 ± 0.39	0.138
18:1n-9	13.10 ± 5.18	14.98 ± 5.33	13.65 ± 2.25	12.20 ± 3.63	15.58 ± 5.33	17.42 ± 4.57	0.432
20:1n-9	0.43 ± 0.27	0.26 ± 0.40	0.70 ± 0.32	1.80 ± 0.83 *	0.28 ± 0.44	0.74 ± 0.48	**< 0.001**
Total MUFA	13.73 ± 4.71	15.38 ± 4.98	14.62 ± 2.48	15.00 ± 3.53	16.34 ± 4.58	17.05 ± 1.79	0.791
18:2n-6	10.97 ± 1.97	20.03 ± 4.08	15.80 ± 1.12	12.95 ± 2.69	19.26 ± 1.98	9.89 ± 1.39	**< 0.001**
18:3n-6	ND	ND	ND	0.47 ± 0.51	0.97 ± 1.85	0.16 ± 0.26	0.359
20:2n-6	1.33 ± 0.27	2.23 ± 0.63	1.41 ± 0.18	0.91 ± 0.27	2.90 ± 0.54	0.88 ± 0.17	**< 0.001**
20:3n-6	1.32 ± 0.23	1.26 ± 0.52	1.16 ± 0.14	1.04 ± 0.14	1.01 ± 0.55	1.86 ± 1.93	0.604
20:4n-6	25.83 ± 6.47	17.90 ± 10.45	19.32 ± 3.87	12.69 ± 2.89	13.76 ± 9.05	24.15 ± 7.28	0.027 †
22:5n-6	1.91 ± 0.64	0.55 ± 0.43	0.10 ± 0.11	0.17 ± 0.16	1.57 ± 0.89	1.61 ± 0.52	**< 0.001**
Total n-6 PUFA	41.44 ± 5.26	42.06 ± 9.80	37.80 ± 3.53	28.22 ± 1.86	39.47 ± 10.76	40.84 ± 4.49	**0.021**
18:3n-3	ND	0.52 ± 0.36	3.14 ± 0.69 *	0.30 ± 0.25	ND	0.16 ± 0.14	**< 0.001**
20:4n-3	ND	ND	0.18 ± 0.13	1.60 ± 0.54 *	0.34 ± 0.73	0.20 ± 0.26	**< 0.001**
20:5n-3	ND	0.18 ± 0.32	1.46 ± 0.47	4.54 ± 1.11 *	0.39 ± 0.97	0.69 ± 1.51	**< 0.001**
22:5n-3	2.05 ± 1.01	1.20 ± 1.13	4.96 ± 1.28	7.23 ± 2.26	0.79 ± 1.01	1.13 ± 0.70	**< 0.001**
22:6n-3	2.79 ± 0.99	1.84 ± 1.79	3.84 ± 0.65	8.55 ± 2.10 *	0.75 ± 0.58	2.63 ± 0.97	**< 0.001**
Total n-3 PUFA	5.01 ± 2.03	3.79 ± 3.10	13.59 ± 2.06 *	22.22 ± 4.45 *	2.34 ± 1.92	5.29 ± 1.26	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content)
* Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.23: Fatty acid composition of thymus PC in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation)

	LF Soyabean (n=5)	HF Soyabean (n=6)	HF Linseed (n=6)	HF Salmon (n=6)	HF Sunflower (n=4)	HF Beef tallow (n=4)	ANOVA p value
14:0	0.33 ± 0.45	ND [b]	ND [b]	0.86 ± 0.70 [a]	0.10 ± 0.21	0.11 ± 0.22	**0.018**
16:0	43.15 ± 8.96	47.76 ± 4.41	44.70 ± 5.81	39.51 ± 7.45	38.74 ± 8.26	39.15 ± 7.17	0.244
18:0	14.99 ± 4.12	11.64 ± 2.12	12.99 ± 4.26	16.27 ± 6.23	19.45 ± 9.14	15.29 ± 3.47	0.272
20:0	0.31 ± 0.45	ND	0.62 ± 1.04	0.56 ± 0.55	0.35 ± 0.71	0.15 ± 0.30	0.536
22:0	0.13 ± 0.18	ND	ND	ND	ND	ND	0.087
Total saturates	58.90 ± 6.09	59.48 ± 2.48	58.42 ± 3.75	57.20 ± 4.85	58.65 ± 4.09	54.70 ± 4.52	0.626
16:1n-7	1.02 ± 0.24	0.51 ± 0.41 [b]	0.72 ± 0.44 [b]	1.78 ± 0.63 [a]	1.01 ± 0.29	1.01 ± 0.40	**0.001**
18:1n-9	13.81 ± 3.72	11.49 ± 0.44	11.83 ± 1.33	16.65 ± 5.38	13.04 ± 1.96	14.48 ± 0.58	0.067
20:1n-9	0.60 ± 0.94	2.10 ± 0.37 [b]	1.67 ± 0.54	0.30 ± 0.74 [a]	1.54 ± 1.06	2.40 ± 1.61 [b]	**0.004**
24:1n-9	0.50 ± 0.35	ND	0.41 ± 0.51	0.48 ± 0.27	0.37 ± 0.27	0.56 ± 0.08	0.236
Total MUFA	15.92 ± 3.16	14.19 ± 0.75	14.62 ± 2.07	19.22 ± 6.20	15.97 ± 1.50	18.44 ± 1.35	0.095
18:2n-6	8.07 ± 1.45	10.15 ± 1.00 [b]	10.72 ± 1.87 [b]	8.99 ± 1.49	10.45 ± 2.16 [b]	6.80 ± 1.04 [a]	**0.004**
18:3n-6	ND	0.32 ± 0.79	0.47 ± 0.64	0.11 ± 0.27	ND	ND	0.448
20:2n-6	1.11 ± 1.10	3.49 ± 0.58	2.55 ± 0.87	1.86 ± 1.29	2.10 ± 2.48	2.17 ± 0.37	0.075
20:3n-6	1.53 ± 0.83	0.97 ± 0.83	1.35 ± 0.70	1.58 ± 0.54	0.81 ± 0.63	1.26 ± 0.19	0.423
20:4n-6	11.76 ± 4.31	10.39 ± 0.76	7.93 ± 2.21 [b]	7.76 ± 1.84 [b]	9.01 ± 2.52	14.46 ± 1.85 [a]	**0.002**
22:5n-6	0.67 ± 0.58	0.35 ± 0.47	0.14 ± 0.16	0.11 ± 0.19	0.67 ± 0.52	0.68 ± 0.18	0.052
Total n-6 PUFA	23.14 ± 6.25	25.68 ± 1.68	23.16 ± 2.79	20.40 ± 1.62	23.04 ± 5.19	25.37 ± 3.06	0.215
18:3n-3	0.13 ± 0.21	ND	1.53 ± 0.27 *	0.10 ± 0.17	0.11 ± 0.21	ND	**< 0.001**
20:4n-3	0.71 ± 0.55	0.11 ± 0.17	0.14 ± 0.24	0.37 ± 0.66	0.34 ± 0.26	0.65 ± 0.18	0.122
20:5n-3	0.10 ± 0.21	0.32 ± 0.41	0.79 ± 0.54	1.51 ± 1.45	0.42 ± 0.44	ND	0.028 †
22:5n-3	0.33 ± 0.46	ND	0.93 ± 0.82	0.23 ± 0.38	0.71 ± 0.95	0.17 ± 0.24	0.116
22:6n-3	0.77 ± 0.71	0.10 ± 0.24	0.39 ± 0.35	0.98 ± 0.58	0.77 ± 0.67	0.56 ± 0.21	0.069
Total n-3 PUFA	2.03 ± 1.57	0.65 ± 0.54 [a]	3.79 ± 1.22 [b]	3.18 ± 2.21	2.35 ± 1.83	1.48 ± 0.46	**0.016**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.24: Fatty acid composition of thymus PE in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation)

	LF Soyabean (n=4)	HF Soyabean (n=5)	HF Linseed (n=5)	HF Salmon (n=3)	HF Sunflower (n=2)	HF Beef tallow (n=3)	ANOVA p value
14:0	0.59 ± 0.46	ND	0.12 ± 0.26	ND	0.52 ± 0.17	0.73 ± 0.57	0.019 †
16:0	16.45 ± 5.99	11.49 ± 3.25	10.47 ± 1.81	11.62 ± 2.27	11.29 ± 0.26	17.43 ± 8.25	0.200
18:0	20.89 ± 5.22	22.36 ± 2.16	21.77 ± 1.60	19.02 ± 2.76	22.18 ± 2.08	15.00 ± 13.85	0.564
20:0	ND	0.18 ± 0.40	ND	ND	ND	0.12 ± 0.20	0.808
Total saturates	38.01 ± 7.33	34.02 ± 5.62	32.36 ± 3.33	30.64 ± 1.96	34.05 ± 2.43	33.28 ± 6.97	0.558
16:1n-7	0.74 ± 0.29	0.90 ± 1.30	0.18 ± 0.40	1.05 ± 0.91	0.46 ± 0.02	0.29 ± 0.50	0.585
18:1n-9	16.76 ± 4.47	14.08 ± 2.34	14.31 ± 1.22	16.95 ± 3.16	10.68 ± 1.31	21.02 ± 2.44	**0.010**
20:1n-9	1.52 ± 0.83	2.40 ± 2.48	1.85 ± 0.66	2.21 ± 0.38	1.67 ± 0.39	1.99 ± 1.84	0.960
24:1n-9	0.31 ± 0.23	ND	ND	ND	0.19 ± 0.05	0.18 ± 0.16	0.110
Total MUFA	19.33 ± 4.15	17.45 ± 4.26	16.38 ± 1.67	20.21 ± 4.25	13.00 ± 1.74	23.49 ± 3.15	0.049 †
18:2n-6	9.38 ± 2.88	10.32 ± 1.44	10.75 ± 1.58	10.13 ± 2.65	10.62 ± 2.51	8.96 ± 2.34	0.855
18:3n-6	0.10 ± 0.12	ND	ND	ND	ND	ND	0.074
20:2n-6	1.19 ± 0.63	2.24 ± 1.33	1.96 ± 0.43	1.11 ± 0.17	3.86 ± 0.88	1.51 ± 0.12	**0.014**
20:3n-6	1.11 ± 0.18	1.75 ± 1.02	1.28 ± 0.16	1.51 ± 0.20	1.20 ± 0.11	2.79 ± 3.03	0.526
20:4n-6	27.71 ± 10.28	30.95 ± 7.21	30.25 ± 4.98	28.54 ± 6.58	33.74 ± 1.90	25.67 ± 1.53	0.783
22:5n-6	0.94 ± 0.27	0.59 ± 0.08	ND	ND	1.74 ± 0.19 *	1.07 ± 0.25	**< 0.001**
Total n-6 PUFA	40.43 ± 8.93	45.85 ± 7.79	44.29 ± 4.98	41.29 ± 4.13	51.16 ± 1.18	40.00 ± 4.20	0.369
18:3n-3	0.12 ± 0.18	0.14 ± 0.31	1.66 ± 0.79 *	ND	ND	ND	**< 0.001**
20:4n-3	0.19 ± 0.22	0.28 ± 0.49	0.14 ± 0.20	0.32 ± 0.28	0.30 ± 0.02	0.34 ± 0.04	0.923
20:5n-3	0.14 ± 0.24	0.64 ± 0.89	1.77 ± 0.68	2.98 ± 0.49	0.13 ± 0.19	1.38 ± 2.28	**0.017**
22:5n-3	0.55 ± 0.12	0.45 ± 0.29	1.98 ± 0.67	1.84 ± 0.41	0.46 ± 0.16	0.33 ± 0.31	**< 0.001**
22:6n-3	1.24 ± 0.12	1.16 ± 0.29	1.42 ± 0.25	2.73 ± 0.16 *	0.83 ± 0.25	1.10 ± 0.18	**< 0.001**
Total n-3 PUFA	2.24 ± 0.31	2.67 ± 1.45	6.98 ± 0.96	7.86 ± 0.59	1.79 ± 0.49	3.23 ± 2.64	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied

6.3.9 Placenta fatty acid composition

The fatty acid compositions of placenta PC, PE, TAG and CE were assessed by gas chromatography (see table 6.25 to 6.28). The placenta is a tissue which is synthesised *de novo* during pregnancy, and so should provide a good indication of how readily maternal dietary fatty acids are incorporated into new tissues during pregnancy. Changes in the fatty acid composition of the placenta may affect the functioning of membrane-bound proteins by inducing changes in membrane fluidity. This may therefore affect the transport of nutrients across the placenta to the developing fetus.

All placenta lipid fractions were significantly affected by maternal diet during pregnancy. The HF linseed oil group had the highest levels of placenta ALNA, with particular accumulation of this fatty acid in placenta TAG and CE. The HF linseed oil group also resulted in high levels of EPA and DPA, but not DHA, within placenta lipids, though levels were not as high as those achieved with salmon oil feeding. Levels of AA in placenta PC were significantly lower than in other dietary groups, but again did not reach the low levels induced by salmon oil diets.

All LC n-3 PUFA were at the highest levels in placenta lipids from the HF salmon oil group. This was associated with the lowest levels of the LC n-6 PUFA AA across all placenta lipids.

The HF sunflower oil group did not have the highest levels of LA within placenta lipids, but did have significantly higher 22:5n-6 content in PC, PE and TAG than any other dietary group. This diet was also associated with the lowest levels of LC n-3 PUFA such as DPA and DHA across all placenta lipid fractions.

The HF beef tallow diet resulted in the highest OA content of placenta lipids, in keeping with the high dietary content of this fatty acid.

These data indicate that the fatty acid composition of the developing placenta during pregnancy is strongly influenced by the dietary availability of fatty acids.

Table 6.25: Fatty acid composition of placenta PC in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.40 ± 0.02	0.36 ± 0.06	0.40 ± 0.09	0.66 ± 0.06 *	0.44 ± 0.09	0.50 ± 0.14	**< 0.001**
16:0	34.14 ± 1.59	29.56 ± 1.24	29.29 ± 1.09	32.77 ± 0.79	30.08 ± 1.21	30.12 ± 1.58	**< 0.001**
18:0	16.97 ± 1.19	19.86 ± 0.75	19.35 ± 0.66	17.05 ± 0.36	19.84 ± 0.94	18.80 ± 1.32	**< 0.001**
20:0	0.18 ± 0.04	0.23 ± 0.06	0.23 ± 0.04	0.23 ± 0.11	0.23 ± 0.05	0.21 ± 0.02	0.631
22:0	0.14 ± 0.07	0.17 ± 0.04	0.22 ± 0.03	0.10 ± 0.05	0.19 ± 0.02	0.18 ± 0.02	**0.001**
Total saturates	51.82 ± 0.50	50.18 ± 0.93	49.49 ± 0.96	50.82 ± 0.68	50.77 ± 0.97	49.82 ± 0.79	**< 0.001**
16:1n-7	1.25 ± 0.08	0.59 ± 0.15	0.77 ± 0.27	1.27 ± 0.17	0.55 ± 0.11	0.89 ± 0.30	**< 0.001**
18:1n-9	7.34 ± 0.26	5.85 ± 0.34	7.30 ± 1.52	7.28 ± 0.76	5.66 ± 0.56	9.91 ± 1.75 *	**< 0.001**
20:1n-9	0.17 ± 0.07	0.18 ± 0.05	0.20 ± 0.04	0.71 ± 0.08 *	0.19 ± 0.04	0.22 ± 0.09	**< 0.001**
24:1n-9	ND	ND	0.11 ± 0.02	0.24 ± 0.04 *	ND	0.10 ± 0.05	**< 0.001**
Total MUFA	8.79 ± 0.25	6.65 ± 0.46	8.38 ± 1.74	9.49 ± 0.84	6.45 ± 0.67	11.11 ± 2.11	**< 0.001**
18:2n-6	14.78 ± 0.69	19.90 ± 1.49	20.76 ± 1.33	18.54 ± 0.92	20.32 ± 1.02	15.08 ± 0.75	**< 0.001**
18:3n-6	0.21 ± 0.14	0.29 ± 0.23	0.28 ± 0.23	0.31± 0.26	0.28 ± 0.15	0.22 ± 0.10	0.937
20:2n-6	0.39 ± 0.04	0.67 ± 0.11	0.50 ± 0.09	0.41 ± 0.10	0.74 ± 0.07	0.39 ± 0.10	**< 0.001**
20:3n-6	0.71 ± 0.09	0.79 ± 0.06	0.93 ± 0.13	0.98 ± 0.07	0.73 ± 0.12	0.77 ± 0.07	**< 0.001**
20:4n-6	15.56 ± 0.83	14.87 ± 0.61	10.61 ± 1.19 *	7.35 ± 0.17 *	14.08 ± 0.89	15.94 ± 0.78	**< 0.001**
22:5n-6	3.20 ± 0.56	1.52 ± 0.62	0.70 ± 0.15	0.52 ± 0.05	4.57 ± 0.69 *	2.85 ± 0.69	**< 0.001**
Total n-6 PUFA	34.87 ± 0.56	38.04 ± 1.07 *	33.78 ± 0.92	28.12 ± 0.97 *	40.72 ± 1.16 *	35.25 ± 1.80	**< 0.001**
18:3n-3	0.10 ± 0.10	0.15 ± 0.07	0.75 ± 0.07 *	0.17 ± 0.08	ND	ND	**< 0.001**
20:4n-3	ND	ND	0.12 ± 0.02 *	0.24 ± 0.02 *	ND	ND	**< 0.001**
20:5n-3	ND	ND	1.02 ± 0.14 *	2.09 ± 0.17 *	ND	0.12 ± 0.15	**< 0.001**
22:5n-3	0.61 ± 0.12	0.61 ± 0.11	1.53 ± 0.17 *	1.90 ± 0.13 *	0.17 ± 0.09 *	0.44 ± 0.11	**< 0.001**
22:6n-3	3.76 ± 0.53	4.30 ± 0.67	4.93 ± 0.86	7.17 ± 0.45 *	1.69 ± 0.12 *	3.20 ± 0.54	**< 0.001**
Total n-3 PUFA	4.52 ± 0.50	5.14 ± 0.80	8.35 ± 0.91 *	11.57 ± 0.54 *	2.06 ± 0.23 *	3.82 ± 0.56	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups

Table 6.26: Fatty acid composition of placenta PE in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.14 ± 0.09	0.08 ± 0.15	0.25 ± 0.39	0.15 ± 0.09	0.13 ± 0.07	0.15 ± 0.08	0.697
16:0	10.41 ± 0.93	9.45 ± 0.62	10.25 ± 3.30	10.78 ± 0.90	9.41 ± 1.15	9.26 ± 0.81	0.457
18:0	18.48 ± 1.68	21.06 ± 0.60	19.77 ± 2.82	17.91 ± 1.36	21.26 ± 1.29	19.82 ± 1.30	**0.007**
20:0	0.66 ± 0.27	0.46 ± 0.29	0.51 ± 0.18	0.56 ± 0.23	0.32 ± 0.21	0.42 ± 0.23	0.239
22:0	0.10 ± 0.16	ND	0.15 ± 0.07	0.15 ± 0.18	ND	ND	0.329
Total saturates	29.78 ± 1.24	31.09 ± 0.80	30.95 ± 1.49	29.55 ± 0.90	31.19 ± 1.96	29.68 ± 1.73	0.141
16:1n-7	1.71 ± 1.89	0.44 ± 0.30	0.70 ± 0.61	0.94 ± 0.32	0.70 ± 0.83	0.58 ± 0.14	0.209
18:1n-9	10.68 ± 0.89	8.66 ± 0.70	10.81 ± 2.55	9.66 ± 1.47	8.50 ± 0.69	11.72 ± 0.75	**0.001**
20:1n-9	0.30 ± 0.09	0.28 ± 0.11	0.50 ± 0.41	1.20 ± 0.15 *	0.24 ± 0.04	0.41 ± 0.09	**< 0.001**
Total MUFA	12.76 ± 2.12	9.39 ± 0.67	12.01 ± 3.56	11.87 ± 1.62	9.43 ± 1.12	12.71 ± 0.71	0.007
18:2n-6	7.74 ± 1.62	10.92 ± 1.19	12.06 ± 2.35	9.67 ± 1.96	10.96 ± 1.31	7.80 ± 0.78	**< 0.001**
18:3n-6	0.35 ± 0.13	0.62 ± 0.51	0.34 ± 0.28	0.80 ± 0.91	0.38 ± 0.33	0.55 ± 0.64	0.622
20:2n-6	8.94 ± 3.22	9.01 ± 2.60	6.67 ± 2.13	7.59 ± 1.69	8.98 ± 2.47	10.17 ± 2.35	0.213
20:3n-6	0.72 ± 0.09	0.86 ± 0.10	0.94 ± 0.16	0.94 ± 0.22	0.78 ± 0.12	0.80 ± 0.08	0.054
20:4n-6	22.87 ± 2.67	23.40 ± 2.11	18.68 ± 3.66	13.88 ± 1.36 *	22.17 ± 1.11	23.11 ± 0.99	**< 0.001**
22:5n-6	6.87 ± 1.14	3.16 ± 0.44 *	0.32 ± 0.06	0.44 ± 0.07	10.67 ± 1.44 *	5.78 ± 1.07	**< 0.001**
Total n-6 PUFA	47.50 ± 2.23	47.97 ± 0.93	39.01 ± 2.52 *	33.32 ± 1.64 *	53.94 ± 1.81 *	48.21 ± 2.38	**< 0.001**
18:3n-3	0.37 ± 0.43	0.19 ± 0.13	0.78 ± 0.16 *	0.24 ± 0.16	0.15 ± 0.15	0.16 ± 0.15	**< 0.001**
20:4n-3	ND	0.11 ± 0.21	0.17 ± 0.04	0.33 ± 0.04	ND	0.12 ± 0.13	**< 0.001**
20:5n-3	0.23 ± 0.18	0.29 ± 0.37	1.39 ± 0.13 *	2.97 ± 0.50 *	0.40 ± 0.63	0.44 ± 0.80	**< 0.001**
22:5n-3	1.52 ± 0.25	1.56 ± 0.32	4.78 ± 0.64 *	5.58 ± 0.56 *	0.63 ± 0.13	1.29 ± 0.16	**< 0.001**
22:6n-3	7.84 ± 0.59	9.40 ± 1.06	10.91 ± 2.03	16.14 ± 1.49 *	4.25 ± 0.34 *	7.39 ± 0.43	**< 0.001**
Total n-3 PUFA	9.97 ± 0.39	11.55 ± 0.79	18.03 ± 2.39 *	25.26 ± 2.16 *	5.43 ± 0.90 *	9.40 ± 1.29	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups

Table 6.27: Fatty acid composition of placenta TAG in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.66 ± 0.11	0.40 ± 0.07	0.65 ± 0.13	1.14 ± 0.16 *	0.49 ± 0.11	0.83 ± 0.07	**< 0.001**
16:0	26.53 ± 1.65	19.73 ± 0.84	20.86 ± 1.04	26.41 ± 2.31	19.16 ± 1.18	23.64 ± 1.29 *	**< 0.001**
18:0	13.89 ± 2.00	16.16 ± 1.88	15.42 ± 2.17	16.74 ± 3.10	16.77 ± 1.74	18.62 ± 2.21	**0.027**
20:0	0.65 ± 0.10	0.77 ± 0.11	0.70 ± 0.12	0.88 ± 0.15	0.81 ± 0.10	0.87 ± 0.07	**0.005**
22:0	0.25 ± 0.03	0.29 ± 0.04	0.19 ± 0.15	0.26 ± 0.17	0.32 ± 0.04	0.28 ± 0.03	0.329
Total saturates	41.98 ± 3.12	37.36 ± 1.39	37.83 ± 2.76	45.43 ± 5.23	37.56 ± 2.30	44.24 ± 2.01	**< 0.001**
16:1n-7	1.84 ± 0.28	0.55 ± 0.21	0.85 ± 0.24	1.62 ± 0.31	0.54 ± 0.11	1.04 ± 0.17	**< 0.001**
18:1n-9	16.98 ± 0.44	12.85 ± 1.66	16.08 ± 1.83	14.90 ± 1.93	12.80 ± 1.05	20.75 ± 1.18 *	**< 0.001**
20:1n-9	0.39 ± 0.11	0.33 ± 0.04	0.29 ± 0.15	2.28 ± 0.33 *	0.37 ± 0.07	0.50 ± 0.08	**< 0.001**
24:1n-9	0.19 ± 0.06	0.12 ± 0.04	ND	0.51 ± 0.41	ND	0.16 ± 0.03	**0.003**
Total MUFA	19.40 ± 0.66	13.86 ± 1.85	17.29 ± 2.08	19.31 ± 2.43	13.78 ± 1.08	22.45 ± 1.23	**< 0.001**
18:2n-6	21.96 ± 1.72	34.17 ± 0.82	25.68 ± 3.48	18.85 ± 2.86	34.05 ± 1.97	19.96 ± 1.45	**< 0.001**
18:3n-6	0.59 ± 0.25	0.78 ± 0.32	0.48 ± 0.27	0.60 ± 0.26	0.62 ± 0.18	0.59 ± 0.24	0.562
20:2n-6	0.59 ± 0.03	0.83 ± 0.12	0.54 ± 0.07	0.62 ± 0.17	1.00 ± 0.22	0.60 ± 0.14	**< 0.001**
20:3n-6	1.09 ± 0.17	0.96 ± 0.09	0.79 ± 0.19	0.77 ± 0.19	1.09 ± 0.17	0.93 ± 0.16	**0.005**
20:4n-6	7.79 ± 1.23	5.83 ± 0.80	3.33 ± 1.24	2.22 ± 0.53	6.65 ± 0.78	6.34 ± 0.88	**< 0.001**
22:5n-6	2.37 ± 0.37	1.42 ± 0.34	0.86 ± 0.15	0.82 ± 0.16	3.49 ± 0.55 *	1.87 ± 0.26	**< 0.001**
Total n-6 PUFA	34.39 ± 2.16	44.00 ± 1.35	31.70 ± 3.04	23.89 ± 3.61 *	46.90 ± 1.97	30.29 ± 1.90	**< 0.001**
18:3n-3	0.97 ± 0.55	1.76 ± 0.56	7.74 ± 1.07 *	1.03 ± 0.28	0.53 ± 0.14	0.81 ± 0.32	**< 0.001**
20:4n-3	ND	ND	0.21 ± 0.18	0.59 ± 0.07 *	ND	0.10 ± 0.09	**< 0.001**
20:5n-3	0.33 ± 0.21	0.43 ± 0.26	1.15 ± 0.20 *	1.97 ± 0.15 *	ND	0.24 ± 0.16	**< 0.001**
22:5n-3	0.77 ± 0.15	0.58 ± 0.13	1.61 ± 0.37 *	2.40 ± 0.16 *	0.20 ± 0.11	0.44 ± 0.13	**< 0.001**
22:6n-3	2.11 ± 0.18	1.99 ± 0.30	2.47 ± 0.68	5.39 ± 0.29 *	0.93 ± 0.18	1.43 ± 0.24	**< 0.001**
Total n-3 PUFA	4.23 ± 0.91	4.79 ± 0.39	13.19 ± 1.92 *	11.37 ± 0.23 *	1.76 ± 0.46	3.02 ± 0.56	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups

Table 6.28: Fatty acid composition of placenta CE in pregnant rats fed experimental diets (% total fatty acids, mean ± standard deviation, n=6 per group)

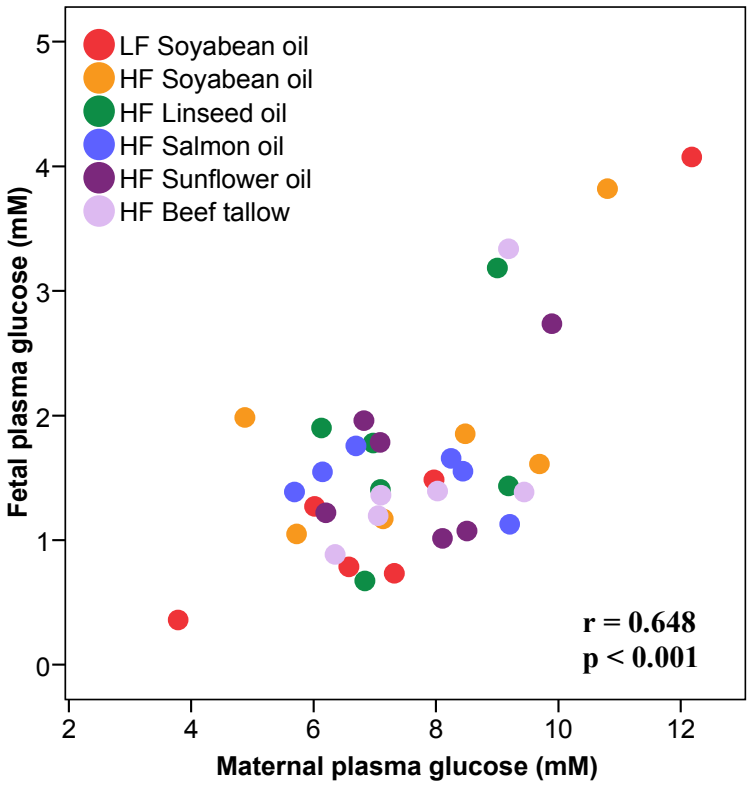
Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.89 ± 0.17	0.79 ± 0.15	0.68 ± 0.24	1.00 ± 0.28	0.73 ± 0.20	0.95 ± 0.33	0.172
16:0	23.84 ± 2.70	19.34 ± 2.83	18.66 ± 1.58	21.58 ± 0.75	18.99 ± 1.32	20.75 ± 3.91	**0.010**
18:0	15.48 ± 2.72	15.49 ± 1.72	17.59 ± 3.89	16.25 ± 2.78	17.80 ± 4.30	19.61 ± 4.49	0.319
20:0	0.90 ± 0.13	0.96 ± 0.21	0.84 ± 0.10	0.88 ± 0.15	0.99 ± 0.16	0.98 ± 0.30	0.690
22:0	0.17 ± 0.26	0.34 ± 0.21	0.37 ± 0.23	0.29 ± 0.16	0.30 ± 0.20	0.31 ± 0.16	0.625
Total saturates	41.28 ± 4.70	36.92 ± 3.21	38.14 ± 5.07	40.00 ± 3.42	38.81 ± 3.71	42.60 ± 2.30	0.181
16:1n-7	2.03 ± 0.28	1.22 ± 0.36	1.11 ± 0.18	1.75 ± 0.18	1.16 ± 0.43	1.61 ± 0.97	**0.017**
18:1n-9	14.38 ± 1.11	13.65 ± 3.04	13.88 ± 2.08	13.38 ± 2.07	12.74 ± 2.82	18.15 ± 7.35	0.200
20:1n-9	0.93 ± 0.14	0.86 ± 0.26	0.89 ± 0.45	2.17 ± 0.30 *	0.86 ± 0.21	0.96 ± 0.30	**< 0.001**
24:1n-9	1.97 ± 0.70	1.46 ± 0.62	1.93 ± 1.65	3.72 ± 1.50	1.28 ± 0.58	1.22 ± 0.54	**0.004**
Total MUFA	19.32 ± 1.77	17.18 ± 3.26	17.80 ± 4.10	21.02 ± 3.41	16.04 ± 3.41	21.95 ± 7.83	0.228
18:2n-6	10.60 ± 1.09	18.22 ± 3.25	14.79 ± 2.51	10.18 ± 0.84	17.72 ± 2.34	11.78 ± 1.06	**< 0.001**
18:3n-6	0.39 ± 0.06	0.37 ± 0.08	0.21 ± 0.16	0.18 ± 0.09	0.42 ± 0.09	0.26 ± 0.05	**< 0.001**
20:2n-6	1.55 ± 0.32	2.69 ± 0.68	1.90 ± 0.52	1.45 ± 0.21	2.86 ± 0.67	1.52 ± 0.76	**< 0.001**
20:3n-6	1.59 ± 0.42	1.84 ± 0.36	1.49 ± 0.25	1.32 ± 0.10	1.98 ± 0.36	1.71 ± 0.79	0.178
20:4n-6	18.78 ± 4.27	16.08 ± 3.53	11.11 ± 1.76	5.87 ± 0.90 *	17.61 ± 3.46	15.38 ± 4.17	**< 0.001**
22:5n-6	1.14 ± 0.69	0.53 ± 0.36	0.52 ± 0.46	0.49 ± 0.41	1.12 ± 1.49	0.42 ± 0.16	0.316
Total n-6 PUFA	34.05 ± 4.23	39.74 ± 3.35	30.02 ± 3.60	19.48 ± 1.15	41.71 ± 3.03	31.08 ± 5.82	**< 0.001**
18:3n-3	0.34 ± 0.05	0.67 ± 0.18	2.94 ± 0.28 *	0.33 ± 0.17	0.26 ± 0.40	0.30 ± 0.09	**< 0.001**
20:4n-3	0.41 ± 0.32	1.18 ± 1.73	0.47 ± 0.27	2.43 ± 0.67	1.11 ± 1.33	0.98 ± 1.09	**0.021**
20:5n-3	0.11 ± 0.19	0.17 ± 0.16	3.36 ± 1.30 *	6.15 ± 1.41 *	0.00 ± 0.00	0.09 ± 0.10	**< 0.001**
22:5n-3	0.51 ± 0.41	0.57 ± 0.39	2.65 ± 1.34	3.33 ± 1.18	0.06 ± 0.14	0.47 ± 0.27	**< 0.001**
22:6n-3	3.98 ± 0.37	3.58 ± 0.52	4.62 ± 0.91	7.27 ± 0.71 *	2.00 ± 0.20 *	2.54 ± 0.92	**< 0.001**
Total n-3 PUFA	5.35 ± 1.10	6.17 ± 1.88	14.04 ± 2.26 *	19.50 ± 1.48 *	3.44 ± 1.52	4.37 ± 1.90	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups

6.3.10 Fetal plasma glucose and lipid concentrations

No significant effects of maternal diet were observed upon fetal plasma total cholesterol, TAG or glucose concentrations (see table 6.29), indicating that the quantitative supply of lipids and glucose to the fetus is well preserved in the face of changes which were observed in maternal lipid concentrations (see table 6.5). There was a significant correlation and concentration gradient between maternal and fetal plasma glucose concentrations (see figure 6.8).

Figure 6.8: Graph to illustrate the correlation between maternal and fetal plasma glucose concentrations



6.3.11 Fetal plasma fatty acid composition

Fetal plasma PC, TAG, CE and NEFA fatty acid compositions were assessed by gas chromatography (see tables 6.30 to 6.33). Significant effects of maternal diet were observed across all fetal plasma lipid fractions, indicating that the maternal diet has the capacity to significantly alter the circulating fatty acids available to the fetus.

Table 6.29: Fetal plasma lipid and glucose concentrations (mean ± standard deviation, n=6 per group)

	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
Total cholesterol (mM)	0.8 ± 0.3	0.8 ± 0.1	0.9 ± 0.4	1.1 ± 0.9	0.8 ± 0.1	0.9 ± 0.2	0.709
Glucose (mM)	1.5 ± 1.4	1.9 ± 1.0	1.7 ± 0.8	1.5 ± 0.2	1.6 ± 0.7	1.6 ± 0.9	0.955
NEFA (μM)	226.7 ± 104.7	302.7 ± 260.6	94.3 ± 62.3	212.8 ± 175.5	102.6 ± 70.2	301.4 ± 223.3	0.153
TAG (mM)	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.540

Table 6.30: Fetal plasma PC fatty acid composition (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.84 ± 0.16	0.85 ± 0.10	0.78 ± 0.14	1.04 ± 0.07	0.81 ± 0.17	0.91 ± 0.14	**0.028**
16:0	27.09± 1.02	25.98 ± 1.96	25.84 ± 1.33	28.82 ± 1.87	27.19 ± 1.79	25.59 ± 1.32	**0.013**
18:0	18.14 ± 1.27	19.06 ± 1.27	19.61 ± 2.45	16.74 ± 0.68	18.82 ± 0.86	16.62 ± 1.31	**0.003**
20:0	0.41 ± 0.26	0.54 ± 0.31	0.52 ± 0.18	0.63 ± 0.37	0.52 ± 0.22	0.75 ± 0.38	0.462
22:0	0.52 ± 0.68	0.32 ± 0.28	0.38 ± 0.24	0.23 ± 0.20	0.21 ± 0.23	0.50 ± 0.21	0.525
Total saturates	47.00 ± 2.01	46.75 ± 2.00	47.12 ± 3.16	47.46 ± 2.09	47.54 ± 2.36	44.38 ± 2.11	0.206
16:1n-7	2.20 ± 0.19	1.60 ± 0.33	1.76 ± 0.39	2.30 ± 0.19	1.71 ± 0.35	2.61 ± 0.70	**0.001**
18:1n-9	10.28 ± 0.95	8.66 ± 0.74	9.66 ± 0.95	11.68 ± 0.46	8.18 ± 2.53	12.92 ± 0.86	**< 0.001**
20:1n-9	0.62 ± 0.49	0.67 ± 0.50	0.75 ± 0.47	0.62 ± 0.20	0.64 ± 0.56	1.10 ± 0.43	0.438
24:1n-9	ND	ND	ND	0.12 ± 0.15	ND	0.10 ± 0.17	0.083
Total MUFA	13.10 ± 1.04	10.93 ± 1.24	12.17 ± 1.22	14.72 ± 0.86	10.53 ± 2.78	16.73 ± 1.06	**< 0.001**
18:2n-6	12.36 ± 1.13	16.02 ± 2.52	16.28 ± 1.51	12.78 ± 0.83	15.95 ± 1.66	11.12 ± 1.20	**< 0.001**
18:3n-6	1.08 ± 0.91	1.35 ± 1.08	1.41 ± 0.88	1.63 ± 1.25	1.12 ± 0.95	2.93 ± 1.28	0.054
20:2n-6	0.37 ± 0.05	0.81 ± 0.80	0.38 ± 0.06	0.43 ± 0.09	0.47 ± 0.12	0.39 ± 0.07	0.229
20:3n-6	1.47 ± 0.48	1.42 ± 0.40	1.47 ± 0.27	1.50 ± 0.48	1.28 ± 0.30	2.08 ± 1.00	0.206
20:4n-6	14.57 ± 1.71	13.36 ± 0.28	7.76 ± 1.06 *	4.40 ± 0.45 *	15.42 ± 0.92	12.73 ± 1.82	**< 0.001**
22:5n-6	1.85 ± 0.43	0.81 ± 0.09 *	0.12 ± 0.14	0.12 ± 0.14	3.26 ± 0.66 *	1.58 ± 0.36	**< 0.001**
Total n-6 PUFA	31.71 ± 1.45	33.77 ± 1.95	27.43 ± 2.38 *	20.86 ± 1.23 *	37.49 ± 1.68 *	30.82 ± 0.74	**< 0.001**
18:3n-3	ND	0.13 ± 0.15	0.81 ± 0.22 *	ND	ND	ND	**< 0.001**
20:4n-3	ND	ND	0.18 ± 0.10	0.10 ± 0.12	ND	ND	**< 0.001**
20:5n-3	0.99 ± 0.80	0.81 ± 0.61	3.30 ± 0.47	4.75 ± 0.92	0.48 ± 0.29	1.90 ± 1.30	**< 0.001**
22:5n-3	0.38 ± 0.08	0.32 ± 0.12	1.01 ± 0.13	0.92 ± 0.11	ND	0.22 ± 0.08	**< 0.001**
22:6n-3	6.83 ± 0.49	7.29 ± 1.09	7.98 ± 0.45	11.16 ± 0.73 *	3.86 ± 0.48 *	5.94 ± 0.71	**< 0.001**
Total n-3 PUFA	8.20 ± 0.80	8.55 ± 1.24	13.28 ± 0.77 *	16.96 ± 1.24 *	4.43 ± 0.40 *	8.07 ± 1.85	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups

Table 6.31: Fetal plasma TAG fatty acid composition (% of total fatty acids, mean ± standard deviation)

Fatty acid	LF Soyabean (n=5)	HF Soyabean (n=6)	HF Linseed (n=6)	HF Salmon (n=6)	HF Sunflower (n=6)	HF Beef tallow (n=6)	ANOVA p value
14:0	0.79 ± 0.31	0.87 ± 0.41	0.52 ± 0.29	1.11 ± 0.57	0.89 ± 0.29	0.72 ± 0.41	0.226
16:0	24.89 ± 1.52	24.62 ± 1.90	23.81 ± 3.76	26.19 ± 2.53	25.27 ± 3.09	25.80 ± 1.07	0.636
18:0	10.28 ± 2.93	8.46 ± 0.65	8.73 ± 1.75	12.25 ± 3.39	8.79 ± 1.76	9.88 ± 1.22	0.042 †
20:0	0.46 ± 0.54	0.41 ± 0.22	0.48 ± 0.27	0.36 ± 0.38	0.31 ± 0.29	0.31 ± 0.28	0.919
22:0	0.39 ± 0.36	0.12 ± 0.29	0.22 ± 0.19	0.23 ± 0.42	0.47 ± 0.27	0.30 ± 0.29	0.463
Total saturates	36.80 ± 2.68	34.49 ± 2.04	33.76 ± 5.21	40.16 ± 6.58	35.73 ± 4.66	37.01 ± 1.04	0.159
16:1n-7	4.26 ± 0.71	2.87 ± 0.76	2.28 ± 0.37	3.21 ± 0.48	2.84 ± 0.33	3.41 ± 0.97	**0.001**
18:1n-9	25.95 ± 1.69	22.30 ± 1.57	21.01 ± 1.06	23.96 ± 2.80	22.99 ± 1.61	33.13 ± 1.46 *	**< 0.001**
20:1n-9	0.60 ± 0.72	0.54 ± 0.47	1.58 ± 1.64	0.85 ± 0.74	1.49 ± 1.80	0.51 ± 0.46	0.382
24:1n-9	ND	ND	ND	0.12 ± 0.18	ND	ND	0.146
Total MUFA	30.82 ± 1.49	25.74 ± 1.72	24.87 ± 2.18	28.14 ± 2.12	27.32 ± 1.15	37.05 ± 1.55 *	**< 0.001**
18:2n-6	14.47 ± 1.36	20.30 ± 1.94	13.36 ± 1.89	7.53 ± 0.97 *	19.45 ± 3.10	11.93 ± 1.51	**< 0.001**
18:3n-6	1.98 ± 0.66	1.56 ± 0.42	1.66 ± 1.35	1.05 ± 0.83	2.05 ± 0.62	1.31 ± 0.39	0.257
20:2n-6	0.44 ± 0.10	0.50 ± 0.11	0.41 ± 0.20	0.23 ± 0.13	0.51 ± 0.11	0.29 ± 0.15	**0.006**
20:3n-6	0.71 ± 0.48	0.90 ± 0.46	1.50 ± 1.65	0.49 ± 0.38	1.46 ± 1.04	0.95 ± 0.60	0.341
20:4n-6	4.49 ± 0.64	4.89 ± 0.94	2.20 ± 0.47	1.01 ± 0.20	5.40 ± 0.96	3.58 ± 0.52	**< 0.001**
22:5n-6	2.69 ± 0.89	1.71 ± 0.39	ND	ND	4.57 ± 0.55 *	2.55 ± 0.56	**< 0.001**
Total n-6 PUFA	24.78 ± 2.45	29.87 ± 2.21	19.13 ± 1.87	10.34 ± 0.48 *	33.44 ± 4.33	20.60 ± 0.99	**< 0.001**
18:3n-3	0.76 ± 0.27	1.21 ± 0.26	6.30 ± 1.78 *	0.59 ± 0.11	0.23 ± 0.13	0.50 ± 0.13	**< 0.001**
20:4n-3	ND	ND	0.23 ± 0.12	0.20 ± 0.23	ND	ND	**< 0.001**
20:5n-3	1.67 ± 1.12	1.14 ± 0.84	3.44 ± 0.60	3.26 ± 0.44	1.55 ± 1.17	0.95 ± 0.42	**< 0.001**
22:5n-3	0.58 ± 0.12	0.62 ± 0.20	2.29 ± 0.58	2.29 ± 0.50	0.17 ± 0.13	0.32 ± 0.08	**< 0.001**
22:6n-3	4.59 ± 0.45	6.93 ± 2.14	9.97 ± 1.85	15.03 ± 3.61 *	1.57 ± 0.29	3.57 ± 0.65	**< 0.001**
Total n-3 PUFA	7.60 ± 1.20	9.90 ± 2.04	22.24 ± 3.18	21.36 ± 4.11	3.51 ± 1.38	5.35 ± 0.79	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.32: Fetal plasma CE fatty acid composition (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	1.13 ± 0.53	1.18 ± 0.42	1.35 ± 0.22	1.58 ± 0.63	1.23 ± 0.54	1.19 ± 1.01	0.811
16:0	18.13 ± 3.22	20.25 ± 1.62	20.09 ± 2.50	22.28 ± 3.14	20.93 ± 2.00	19.00 ± 3.48	0.170
18:0	9.18 ± 1.21	13.16 ± 4.17	10.66 ± 3.49	14.33 ± 3.85	12.60 ± 1.58	11.66 ± 2.46	0.075
20:0	0.22 ± 0.13	0.32 ± 0.05	0.32 ± 0.08	0.38 ± 0.05	0.30 ± 0.06	0.35 ± 0.11	**0.039**
22:0	ND	ND	0.32 ± 0.16 *	ND	ND	ND	**< 0.001**
Total saturates	28.65 ± 3.00	34.91 ± 4.79	32.74 ± 5.82	38.57 ± 6.44	35.06 ± 2.54	32.20 ± 2.32	**0.016**
16:1n-7	6.20 ± 0.73	3.66 ± 0.50	4.01 ± 0.42	5.14 ± 0.80	4.21 ± 0.46	4.80 ± 1.24	**< 0.001**
18:1n-9	22.88 ± 3.65	19.11 ± 2.32	18.76 ± 1.91	20.62 ± 1.77	20.49 ± 2.29	26.19 ± 2.79	**< 0.001**
20:1n-9	0.23 ± 0.13	0.21 ± 0.11	0.28 ± 0.08	0.40 ± 0.09	0.18 ± 0.10	0.31 ± 0.17	0.048 †
24:1n-9	0.28 ± 0.16	0.18 ± 0.14	ND	0.42 ± 0.21	ND	0.21 ± 0.18	**< 0.001**
Total MUFA	29.60 ± 3.91	23.15 ± 2.26	23.08 ± 2.16	26.58 ± 2.30	24.88 ± 2.33	31.51 ± 3.17	**< 0.001**
18:2n-6	10.65 ± 0.48	13.52 ± 2.45	12.57 ± 1.69	8.17 ± 0.72	13.39 ± 1.26	9.12 ± 1.53	**< 0.001**
18:3n-6	0.98 ± 0.09	0.90 ± 0.10	0.73 ± 0.10	0.68 ± 0.11	1.16 ± 0.10	0.96 ± 0.11	**< 0.001**
20:2n-6	0.28 ± 0.23	0.46 ± 0.24	ND	ND	0.65 ± 0.18	0.15 ± 0.18	**< 0.001**
20:3n-6	1.07 ± 0.12	1.29 ± 0.28	0.75 ± 0.15	0.39 ± 0.21	1.35 ± 0.25	0.81 ± 0.14	**< 0.001**
20:4n-6	23.15 ± 1.75	18.88 ± 3.58	11.29 ± 1.60 *	5.99 ± 0.86 *	20.06 ± 0.71	20.25 ± 1.75	**< 0.001**
22:5n-6	1.28 ± 0.21	0.78 ± 0.19	ND	ND	2.00 ± 0.26 *	1.11 ± 0.27	**< 0.001**
Total n-6 PUFA	37.40 ± 1.76	35.84 ± 6.20	25.40 ± 3.42 *	15.27 ± 1.82 *	38.61 ± 1.55	32.40 ± 2.92	**< 0.001**
18:3n-3	0.37 ± 0.19	0.60 ± 0.11	3.69 ± 0.53 *	0.30 ± 0.08	ND	0.23 ± 0.40	**< 0.001**
20:4n-3	0.27 ± 0.67	0.97 ± 1.09	1.53 ± 2.14	0.34 ± 0.29	0.16 ± 0.13	0.87 ± 1.25	0.300
20:5n-3	0.58 ± 0.04	0.64 ± 0.08	6.58 ± 0.92 *	9.22 ± 1.53 *	ND	0.36 ± 0.24	**< 0.001**
22:5n-3	0.36 ± 0.07	0.40 ± 0.10	1.66 ± 0.30	1.80 ± 0.45	ND	0.19 ± 0.16	**< 0.001**
22:6n-3	2.75 ± 0.31	3.48 ± 0.56	5.31 ± 0.65 *	7.92 ± 1.55 *	1.21 ± 0.21	2.23 ± 0.31	**< 0.001**
Total n-3 PUFA	4.34 ± 0.68	6.09 ± 1.01	18.78 ± 2.52	19.58 ± 3.57	1.46 ± 0.16	3.88 ± 1.68	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.33: Fetal plasma NEFA fatty acid composition (% of total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
12:0	0.18 ± 0.17	0.12 ± 0.13	0.11 ± 0.09	ND	0.10 ± 0.13	ND	0.821
14:0	1.61 ± 0.57	1.23 ± 0.38	1.29 ± 0.54	1.63 ± 0.30	1.45 ± 0.39	1.49 ± 0.56	0.608
16:0	24.84 ± 7.35	24.07 ± 0.70	21.83 ± 6.42	25.93 ± 0.96	25.56 ± 2.17	26.09 ± 3.94	0.564
18:0	23.67 ± 6.64	22.88 ± 4.80	20.20 ± 5.45	23.83 ± 2.77	28.13 ± 8.42	26.29 ± 4.48	0.253
20:0	0.20 ± 0.50	ND	ND	ND	0.48 ± 0.59	ND	0.114
22:0	1.24 ± 1.46	0.76 ± 1.01	1.16 ± 0.61	0.63 ± 0.87	0.85 ± 0.95	0.76 ± 0.57	0.851
Total saturates	51.74 ± 14.67	49.12 ± 3.73	44.63 ± 11.85	52.12 ± 3.21	56.57 ± 9.56	54.71 ± 8.13	0.335
18:1n-9	16.28 ± 7.28	14.15 ± 2.10	15.89 ± 2.77	13.08 ± 2.38	14.60 ± 2.96	17.74 ± 4.09	0.409
20:1n-9	1.24 ± 3.05	ND	0.66 ± 1.62	ND	ND	ND	0.552
24:1n-9	ND	ND	0.12 ± 0.30	ND	ND	0.29 ± 0.27	0.158
Total MUFA	17.61 ± 6.86	14.21 ± 2.16	16.68 ± 2.07	13.08 ± 2.38	14.73 ± 3.05	18.03 ± 3.92	0.172
18:2n-6	10.57 ± 4.45 [b]	18.34 ± 3.10 [a]	12.20 ± 4.09	10.78 ± 2.96 [b]	15.11 ± 5.78	10.00 ± 3.14 [b]	**0.008**
20:2n-6	2.37 ± 1.46	1.34 ± 1.37	1.86 ± 0.97	1.24 ± 1.03	1.47 ± 1.16	2.05 ± 1.53	0.605
20:3n-6	0.17 ± 0.41	0.12 ± 0.29	ND	ND	ND	ND	0.781
20:4n-6	4.58 ± 1.97	5.07 ± 1.75	2.89 ± 0.43	2.88 ± 1.64	4.83 ± 1.05	4.80 ± 1.44	0.032 †
22:5n-6	1.63 ± 2.03	0.71 ± 0.30	0.15 ± 0.24	0.43 ± 0.90	1.81 ± 1.09	1.05 ± 0.94	0.081
Total n-6 PUFA	19.32 ± 7.96	25.58 ± 2.90 [a]	17.10 ± 3.62	15.32 ± 4.14 [b]	23.31 ± 7.07	17.96 ± 4.59	**0.020**
18:3n-3	0.77 ± 0.23	1.68 ± 0.58	7.69 ± 3.29 *	0.99 ± 0.39	0.55 ± 0.22	0.89 ± 0.15	**< 0.001**
20:4n-3	ND	ND	ND	0.32 ± 0.48	ND	0.13 ± 0.21	0.252
20:5n-3	1.11 ± 1.67	0.33 ± 0.38	2.20 ± 1.88	1.90 ± 1.89	0.99 ± 0.91	0.33 ± 0.47	0.114
22:5n-3	0.47 ± 0.37 [b]	0.43 ± 0.16 [b]	1.35 ± 0.60 [a]	1.64 ± 0.47 [a]	ND [b]	0.28 ± 0.14 [b]	**< 0.001**
22:6n-3	8.91 ± 2.59 [bc]	8.65 ± 2.64 [bc]	10.27 ± 3.27 [ac]	14.63 ± 5.02 [a]	3.86 ± 0.90 [b]	7.67 ± 1.95 [bc]	**< 0.001**
Total n-3 PUFA	11.34 ± 4.20 [b]	11.09 ± 2.27 [b]	21.60 ± 6.60 [a]	19.47 ± 4.85 [a]	5.40 ± 1.67 [b]	9.30 ± 1.68 [b]	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups
a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts
† no significant differences between groups when Bonferroni post-hoc test applied

The HF linseed oil group had the highest ALNA content within all fetal plasma lipid fractions. This diet was associated with higher LC n-3 PUFA and lower AA content than all other dietary groups (except HF salmon oil).

The HF salmon oil group had the highest LC n-3 PUFA content within fetal plasma lipids. This was associated with the lowest levels of n-6 PUFA (both LA and AA).

Although the HF sunflower oil diet contained the highest dietary level of LA, this group did not demonstrate the highest LA content in fetal plasma. This group demonstrated the highest 22:5n-6 content and the lowest LC n-3 PUFA content of all dietary groups.

The HF beef tallow group exhibited the highest OA content within fetal plasma lipids, but 16:0 and 18:0 contents were not significantly different from other dietary groups, despite this diet having the highest content of these fatty acids.

Maternal TAG and NEFA are transported across the placenta, and the fatty acids released into the fetal circulation as NEFA(153). Data were therefore assessed for correlations between fatty acid compositions of maternal plasma TAG and NEFA and fetal plasma NEFA (see table 6.34).

Significant correlations between fatty acids in maternal and fetal plasma lipids were observed, particularly within n-6 and n-3 PUFA. When plotted, there was evidence of biomagnification of DHA (see figure 6.9) and AA (see figure 6.10) when fetal plasma NEFA was compared to maternal plasma NEFA. The EFA LA and ALNA demonstrated the opposite relationship, with maternal plasma content being significantly greater than fetal plasma (see figure 6.11). The relationship between fetal plasma NEFA and maternal plasma TAG was less clear, with diet having a significant effect upon the relationships observed.

The ratios of LC PUFA to EFA (e.g. AA to LA ratio) have been reported to be higher in fetal than maternal serum(17). These ratios were calculated (see table 6.35), and this feature was also apparent in the current data. This indicates that there is preferential transfer of LC PUFA to the fetus. The high variation in these ratios reflects the effect of maternal diet upon EFA composition. The preferential transfer of LC PUFA rather than

their EFA precursors also indicates that the capacity of the fetus to endogenously synthesise these LC PUFA may be limited.

Table 6.34: Correlations observed between the fatty acid composition of maternal and fetal plasma lipids (Pearsons R values unless otherwise indicated)

	Maternal plasma TAG vs. fetal plasma NEFA (n = 36)		Maternal plasma NEFA vs. fetal plasma NEFA (n = 36)	
12:0	-		0.035†	
14:0	0.132†		0.193	
16:0	0.119		0.108	
18:0	0.136†		0.242	
20:0	-0.369†	*	0.083†	
22:0	0.377†	*	0.376	*
Total saturates	0.145		0.151	
18:1n-9	0.516†	**	0.201†	
20:1n-9	-0.230†		-0.121†	
24:1n-9	†		0.090†	
Total MUFA	0.330	*	0.279	
18:2n-6	0.451†	**	0.532	**
20:2n-6	-0.231		-0.056	
20:3n-6	0.372†	*	0.114†	
20:4n-6	0.434†	**	0.673	***
22:5n-6	0.572	***	0.496	**
Total n-6 PUFA	0.506	**	0.558	***
18:3n-3	0.842†	***	0.721†	***
20:4n-3	0.308†		0.479†	**
20:5n-3	0.246†		0.424†	*
22:5n-3	0.812	***	0.821	***
22:6n-3	0.672†	***	0.445†	**
Total n-3 PUFA	0.813†	***	0.832†	***

† Spearman's R value * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 6.35: The ratio of LC PUFA to EFA within maternal and fetal plasma lipids (mean ± standard deviation)

	Maternal plasma TAG	Maternal plasma NEFA	Fetal plasma NEFA
AA:LA ratio	0.11 ± 0.11	0.11 ± 0.06	0.35 ± 0.14
DHA:ALNA ratio	1.36 ± 1.81	1.40 ± 1.04	9.05 ± 7.01

Figure 6.9: Graph to illustrate the correlation observed between the DHA content (% total fatty acids) of maternal and fetal plasma NEFA

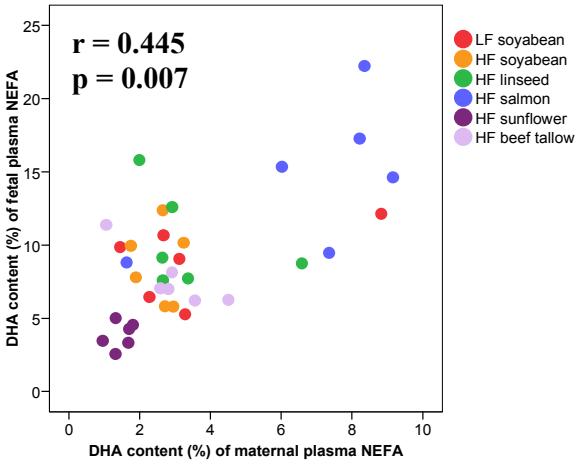


Figure 6.10: Graph to illustrate the correlation observed between the AA content (% total fatty acids) of maternal and fetal plasma NEFA

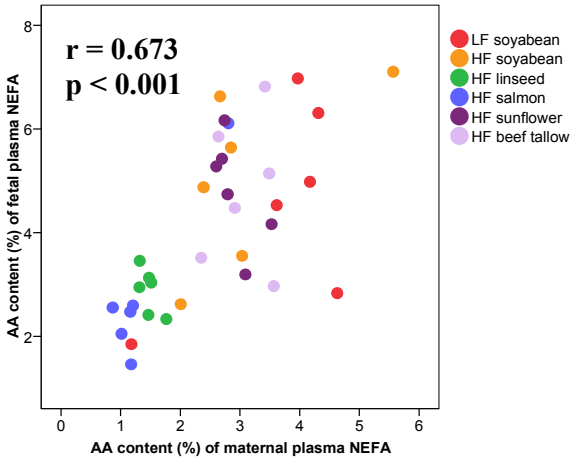
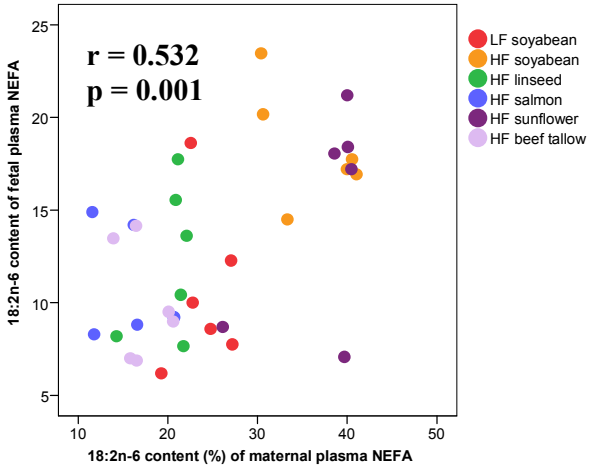


Figure 6.11: Graph to illustrate the correlation observed between the LA content (% total fatty acids) of maternal and fetal plasma NEFA



6.3.12 Fetal liver size and composition

Fetal liver weight was significantly affected by maternal diet during pregnancy (ANOVA p = 0.016), but *post hoc* comparisons lost statistical significance when Bonferroni correction was applied. Maternal diet during pregnancy did not significantly affect fetal liver size as a % body weight, or fetal liver lipid or glycogen content (see table 6.36). There was a significant effect of maternal diet upon fetal liver dry weight. This may indicate an effect of maternal diet upon the protein content of the fetal liver, but this was not assessed.

When fetal liver size and composition data were compared to those of the maternal liver (see table 6.10), several differences were identified. The liver was a greater proportion of body weight in the fetus than in pregnant female rats. The fetal liver also had a lower dry weight and lipid content than observed in maternal liver, but the two had comparable glycogen content.

6.3.13 Fetal liver fatty acid composition

Fatty acid compositions of fetal liver PC, PE, TAG and CE were assessed by gas chromatography (see table 6.37 to 6.40). The data demonstrate that maternal diet has the capacity to significantly alter fetal liver lipid fatty acid composition.

The trends observed in the fetal liver were comparable to those exhibited in fetal plasma. The HF linseed oil group had the highest ALNA content within all fetal liver lipid fractions. This was associated with higher LC n-3 PUFA and lower AA content than all other dietary groups (except HF salmon oil). The HF salmon oil group had the highest LC n-3 PUFA content within fetal liver lipids. This was associated with low levels of n-6 PUFA (both LA and AA).

Although the HF sunflower oil diet contained the highest dietary level of LA, this group did not demonstrate the highest LA content in fetal liver. The AA content of fetal liver lipids was highest in the sunflower oil group, a feature which was not apparent in the maternal liver. In the fetal liver, 22:5n-6 was only detectable in fetal liver CE, where the sunflower oil group demonstrated a significantly higher content than any other dietary group. The sunflower oil group demonstrated the lowest LC n-3 PUFA content of all dietary groups.

The HF beef tallow group exhibited the highest OA content within fetal liver lipids, but the 16:0 and 18:0 contents were not significantly different from other dietary groups, despite this diet having the highest content of these fatty acids.

As was observed in maternal liver lipids, the LC n-6 PUFA 22:5n-6 was almost undetectable in all lipid fractions (with the exception of CE), despite making a significant contribution to fetal plasma fatty acid composition. This may suggest that 22:5n-6 is specifically accumulated within fetal tissues other than the liver.

Table 6.36: Fetal liver weight, lipid and glycogen content (mean ± standard deviation, n=6)

	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
Liver weight (mg)	156.1 ± 28.4	179.8 ± 24.3	197.6 ± 42.9	156.6 ± 16.6	206.4 ± 35.3	163.8 ± 15.4	0.016 †
Liver wet weight (% body weight)	6.8 ± 0.6	7.4 ± 0.9	7.7 ± 0.1	7.6 ± 1.2	8.1 ± 1.1	7.5 ± 1.0	0.315
Liver dry weight (% of wet weight)	20.8 ± 0.7	21.0 ± 0.8	20.7 ± 0.6	19.9 ± 0.4 [a]	21.2 ± 0.7 [b]	20.5 ± 0.9	**0.047**
Liver lipid (g/100 g liver)	2.4 ± 0.3	2.3 ± 0.2	2.4 ± 0.3	2.1 ± 0.2	2.2 ± 0.4	2.6 ± 0.4	0.160
Liver glycogen (g/100 g liver)	2.1 ± 1.1	2.8 ± 1.2	3.0 ± 1.7	1.5 ± 0.4	2.5 ± 1.5	2.3 ± 2.2	0.559

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts
† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.37: Fetal liver PC fatty acid composition (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	1.18 ± 0.11	1.16 ± 0.10	1.07 ± 0.17	1.25 ± 0.22	1.12 ± 0.14	1.23 ± 0.15	0.316
16:0	31.62 ± 0.47	30.46 ± 1.31	29.73 ± 0.63	32.66 ± 0.99	32.18 ± 0.83	31.69 ± 1.07	**< 0.001**
18:0	12.23 ± 0.42	12.78 ± 0.23	13.30 ± 0.46	12.40 ± 0.65	12.64 ± 0.57	12.27 ± 0.44	**0.005**
20:0	ND	0.10 ± 0.16	ND	ND	ND	ND	0.454
22:0	0.45 ± 0.27	0.52 ± 0.12	0.33 ± 0.27	0.31 ± 0.17	0.37 ± 0.16	0.62 ± 0.31	0.190
Total saturates	45.49 ± 0.93	45.03 ± 0.96	44.47 ± 0.88	46.72 ± 0.88	46.31 ± 0.73	45.86 ± 0.53	**< 0.001**
16:1n-7	3.57 ± 0.27 *	2.31 ± 0.19	2.29 ± 0.28	2.90 ± 0.15	2.47 ± 0.33	3.05 ± 0.32	**< 0.001**
18:1n-9	12.56 ± 1.05	10.92 ± 0.84	11.38 ± 1.08	13.37 ± 0.91	11.76 ± 0.73	15.78 ± 1.10 *	**< 0.001**
24:1n-9	0.44 ± 0.34	0.28 ± 0.22	ND	0.20 ± 0.16	ND	0.40 ± 0.21	0.011 †
Total MUFA	16.66 ± 1.10	13.53 ± 0.98	13.77 ± 1.25	16.56 ± 1.03	14.30 ± 0.98	19.31 ± 1.03 *	**< 0.001**
18:2n-6	12.59 ± 1.08 *	15.39 ± 1.16	15.06 ± 0.45	10.62 ± 0.63	15.20 ± 1.26	10.33 ± 0.67	**< 0.001**
18:3n-6	0.63 ± 0.16	0.62 ± 0.07	0.57 ± 0.18	0.37 ± 0.07	0.88 ± 0.24	0.72 ± 0.25	**0.001**
20:2n-6	0.34 ± 0.05	0.41 ± 0.06	0.35 ± 0.05	0.23 ± 0.10	0.45 ± 0.07	0.33 ± 0.08	**< 0.001**
20:3n-6	1.16 ± 0.10	1.30 ± 0.09	1.28 ± 0.14	1.10 ± 0.13	1.33 ± 0.23	1.23 ± 0.19	0.109
20:4n-6	14.46 ± 0.60	14.69 ± 1.09	8.56 ± 0.98 *	5.14 ± 0.36 *	16.80 ± 1.03 *	14.45 ± 1.36	**< 0.001**
22:5n-6	0.19 ± 0.31	0.25 ± 0.39	ND	ND	ND	0.35 ± 0.40	0.182
Total n-6 PUFA	29.37 ± 1.72	32.65 ± 0.96	25.83 ± 1.47	17.46 ± 1.09 *	34.74 ± 1.12	27.41 ± 1.28	**< 0.001**
18:3n-3	0.34 ± 0.11	0.40 ± 0.12	1.67 ± 0.16 *	0.25 ± 0.04	0.15 ± 0.13	0.14 ± 0.12	**< 0.001**
20:4n-3	ND	ND	0.37 ± 0.07 *	0.18 ± 0.15 *	ND	ND	**< 0.001**
20:5n-3	0.55 ± 0.11	0.61 ± 0.16	4.71 ± 0.53 *	6.11 ± 0.58 *	0.14 ± 0.15	0.45 ± 0.27	**< 0.001**
22:5n-3	0.54 ± 0.32	0.50 ± 0.12	0.91 ± 0.07	1.05 ± 0.13	ND	0.39 ± 0.20	**< 0.001**
22:6n-3	7.06 ± 0.35	7.27 ± 0.62	8.26 ± 0.94	11.68 ± 0.48 *	4.29 ± 0.15 *	6.41 ± 0.45	**< 0.001**
Total n-3 PUFA	8.49 ± 0.72	8.79 ± 0.75	15.93 ± 1.13 *	19.27 ± 0.72 *	4.65 ± 0.37 *	7.41 ± 0.64	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.38: Fetal liver PE fatty acid composition (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.15 ± 0.09	0.16 ± 0.08	0.15 ± 0.08	0.17 ± 0.02	0.16 ± 0.08	0.18 ± 0.03	0.990
16:0	17.46 ± 0.80	16.95 ± 0.38	16.79 ± 0.90	17.82 ± 1.48	17.97 ± 0.66	17.49 ± 0.53	0.155
18:0	20.97 ± 0.60	21.59 ± 0.92	21.63 ± 1.17	19.42 ± 0.43 *	23.19 ± 0.57 *	20.99 ± 1.05	**< 0.001**
20:0	0.13 ± 0.15	0.14 ± 0.12	0.02 ± 0.05	0.14 ± 0.13	0.09 ± 0.14	0.11 ± 0.09	0.484
22:0	0.13 ± 0.11	0.17 ± 0.19	0.21 ± 0.04	0.20 ± 0.18	0.10 ± 0.17	0.13 ± 0.11	0.760
Total saturates	38.83 ± 1.23	39.00 ± 1.03	38.80 ± 0.83	37.75 ± 1.40	41.52 ± 0.94 *	38.91 ± 0.88	**< 0.001**
16:1n-7	0.91 ± 0.13	0.61 ± 0.07	0.63 ± 0.15	0.76 ± 0.09	0.81 ± 0.09	0.82 ± 0.09	**< 0.001**
18:1n-9	6.93 ± 0.32	6.36 ± 0.78	6.17 ± 0.73	6.57 ± 0.54	7.21 ± 0.68	8.32 ± 0.51	**< 0.001**
Total MUFA	7.89 ± 0.45	6.99 ± 0.86	6.80 ± 0.86	7.36 ± 0.57	8.05 ± 0.72	9.16 ± 0.62	**< 0.001**
18:2n-6	4.33 ± 0.49	5.04 ± 0.39	5.31 ± 0.32	4.03 ± 0.17	5.63 ± 0.36	4.10 ± 0.40	**< 0.001**
18:3n-6	0.17 ± 0.04	0.20 ± 0.03	0.11 ± 0.09	0.11 ± 0.06	0.23 ± 0.04	0.19 ± 0.03	**0.002**
20:2n-6	0.55 ± 0.50	0.23 ± 0.04	0.17 ± 0.02	0.27 ± 0.20	0.27 ± 0.04	0.53 ± 0.31	0.058
20:3n-6	0.75 ± 0.10	0.82 ± 0.10	0.87 ± 0.12	0.70 ± 0.12	0.89 ± 0.10	0.76 ± 0.11	**0.026**
20:4n-6	25.40 ± 0.67	25.27 ± 0.88	17.59 ± 1.15 *	11.66 ± 0.52 *	28.74 ± 0.66 *	26.63 ± 0.76	**< 0.001**
Total n-6 PUFA	31.20 ± 1.08	31.56 ± 0.97	24.05 ± 1.15 *	16.76 ± 0.47 *	35.76 ± 0.57 *	32.22 ± 0.79	**< 0.001**
18:3n-3	0.25 ± 0.10	0.22 ± 0.07	0.71 ± 0.06 *	0.18 ± 0.04	0.19 ± 0.16	0.15 ± 0.07	**< 0.001**
20:4n-3	ND	ND	0.16 ± 0.09	0.10 ± 0.08	ND	ND	**< 0.001**
20:5n-3	0.43 ± 0.11	0.52 ± 0.11	5.44 ± 0.64 *	8.13 ± 0.61 *	0.11 ± 0.13	0.34 ± 0.12	**< 0.001**
22:5n-3	0.78 ± 0.16	0.79 ± 0.06	2.60 ± 0.26	2.72 ± 0.24	0.48 ± 0.11	0.61 ± 0.07	**< 0.001**
22:6n-3	20.63 ± 0.98	20.92 ± 0.91	21.46 ± 1.59	27.00 ± 0.96 *	13.89 ± 0.79 *	18.61 ± 0.42 *	**< 0.001**
Total n-3 PUFA	22.08 ± 1.11	22.45 ± 1.06	30.36 ± 1.28 *	38.13 ± 1.02 *	14.67 ± 0.75 *	19.71 ± 0.49 *	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content)
* Significantly different ($p < 0.05$) from all other groups

Table 6.39: Fetal liver TAG fatty acid composition (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	2.28 ± 0.29	1.91 ± 0.35	1.64 ± 0.12	1.76 ± 0.30	1.97 ± 0.20	2.05 ± 0.28	**0.005**
16:0	24.28 ± 1.20	21.69 ± 2.32	19.04 ± 0.93	21.49 ± 1.15	23.19 ± 1.26	23.66 ± 1.51	**< 0.001**
18:0	6.55 ± 1.11	7.09 ± 0.94	6.07 ± 1.15	7.48 ± 0.46	7.21 ± 0.66	7.46 ± 0.90	0.071
20:0	0.26 ± 0.09	0.26 ± 0.05	0.76 ± 0.15 *	0.25 ± 0.04	0.24 ± 0.09	0.40 ± 0.14	**< 0.001**
22:0	ND	ND	0.14 ± 0.12	ND	ND	ND	0.055
Total saturates	33.44 ± 2.14	30.98 ± 3.26	27.65 ± 1.92	30.98 ± 1.51	32.61 ± 1.44	33.57 ± 2.32	**< 0.001**
16:1n-7	5.63 ± 0.20 *	2.95 ± 0.64	2.39 ± 0.25	3.07 ± 0.22	3.02 ± 0.38	3.79 ± 0.73	**< 0.001**
18:1n-9	28.97 ± 2.71	25.35 ± 5.05	20.89 ± 2.23	24.24 ± 1.67	26.61 ± 1.46	33.25 ± 5.35	**< 0.001**
20:1n-9	0.26 ± 0.03	0.21 ± 0.03	0.15 ± 0.17	0.27 ± 0.02	0.23 ± 0.13	0.30 ± 0.07	0.169
Total MUFA	34.86 ± 2.88	28.52 ± 5.64	23.44 ± 2.36	28.52 ± 5.64	29.86 ± 1.79	37.40 ± 6.02	**< 0.001**
18:2n-6	17.80 ± 2.66	20.91 ± 6.17	18.39 ± 1.12	13.84 ± 4.53	26.03 ± 1.48	16.78 ± 4.51	**< 0.001**
18:3n-6	1.00 ± 0.16	1.11 ± 0.43	0.69 ± 0.11	0.51 ± 0.28	1.61 ± 0.22 *	1.03 ± 0.12	**< 0.001**
20:2n-6	0.28 ± 0.06	0.35 ± 0.12	0.29 ± 0.04	0.15 ± 0.13	0.47 ± 0.04	0.29 ± 0.11	**< 0.001**
20:3n-6	0.49 ± 0.09	0.54 ± 0.21	0.39 ± 0.08	0.34 ± 0.15	0.66 ± 0.10	0.44 ± 0.15	**0.006**
20:4n-6	3.44 ± 0.57	3.74 ± 1.65	1.56 ± 0.14	1.25 ± 0.94	5.56 ± 1.30	3.20 ± 0.81	**< 0.001**
Total n-6 PUFA	23.01 ± 3.19	26.64 ± 8.42	21.32 ± 1.35	16.09 ± 5.98	34.33 ± 2.75	21.73 ± 5.47	**< 0.001**
18:3n-3	1.11 ± 0.17	1.24 ± 0.48	8.39 ± 0.91 *	1.04 ± 0.32	0.40 ± 0.08	0.68 ± 0.30	**< 0.001**
20:4n-3	ND	ND	0.36 ± 0.04 *	ND	ND	ND	**< 0.001**
20:5n-3	0.33 ± 0.12	0.66 ± 0.72	2.05 ± 0.29	2.01 ± 0.69	ND	0.10 ± 0.15	**< 0.001**
22:5n-3	0.55 ± 0.16	0.97 ± 0.74	2.42 ± 0.18	2.33 ± 0.81	0.33 ± 0.20	1.45 ± 2.61	0.013 †
22:6n-3	6.71 ± 1.60	10.98 ± 4.37	14.37 ± 1.83	19.95 ± 4.15 *	2.45 ± 0.37	5.02 ± 3.42	**< 0.001**
Total n-3 PUFA	8.70 ± 1.94	13.86 ± 5.83	27.59 ± 3.04	25.35 ± 5.42	3.20 ± 0.41	7.29 ± 2.91	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.40: Fetal liver CE fatty acid composition (% total fatty acids, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	1.00 ± 0.28	0.95 ± 0.32	0.81 ± 0.53	0.99 ± 0.30	1.05 ± 0.31	1.33 ± 0.35	0.267
16:0	30.03 ± 1.73	27.98 ± 3.31	27.08 ± 4.71	31.46 ± 2.47	29.39 ± 1.43	31.21 ± 1.80	0.069
18:0	20.09 ± 5.12	22.07 ± 4.21	22.65 ± 5.81	24.46 ± 2.10	21.09 ± 4.15	21.28 ± 2.74	0.577
20:0	0.11 ± 0.12	0.25 ± 0.05	0.12 ± 0.14	ND	0.16 ± 0.13	0.25 ± 0.13	0.095
22:0	ND	ND	0.11 ± 0.15 *	ND	ND	ND	**0.015**
Total saturates	51.23 ± 5.88	51.26 ± 7.14	50.77 ± 9.50	57.00 ± 2.44	51.69 ± 4.85	54.07 ± 3.78	0.457
16:1n-7	6.78 ± 1.27	3.37 ± 0.40	3.00 ± 0.59	3.50 ± 0.49	4.34 ± 0.90	5.26 ± 0.96	**< 0.001**
18:1n-9	21.51 ± 2.52	18.58 ± 2.76	17.48 ± 2.92	16.77 ± 2.49	21.55 ± 1.98	24.79 ± 3.65	**< 0.001**
20:1n-9	0.11 ± 0.12	0.14 ± 0.11	0.21 ± 0.18	ND	ND	0.15 ± 0.17	0.165
Total MUFA	28.39 ± 3.66	22.12 ± 2.94	20.69 ± 3.08	20.31 ± 2.54	25.92 ± 2.87	30.22 ± 4.49	**< 0.001**
18:2n-6	9.19 ± 1.68	13.08 ± 4.47	9.98 ± 3.02	6.19 ± 0.35	12.04 ± 1.42	6.50 ± 0.84	**< 0.001**
18:3n-6	0.13 ± 0.15	0.25 ± 0.13	ND	ND	0.30 ± 0.15	0.14 ± 0.11	**0.001**
20:2n-6	0.31 ± 0.22	0.30 ± 0.16	0.13 ± 0.23	0.13 ± 0.33	0.30 ± 0.25	0.44 ± 0.21	0.244
20:3n-6	0.33 ± 0.17	0.74 ± 0.13	0.33 ± 0.29	0.31 ± 0.49	0.67 ± 0.21	0.28 ± 0.22	0.018 †
20:4n-6	4.52 ± 0.56	4.25 ± 0.58	2.39 ± 0.65	1.69 ± 0.23	5.07 ± 1.05	3.86 ± 0.42	**< 0.001**
22:5n-6	1.25 ± 0.26	0.91 ± 0.29	ND	ND	2.47 ± 0.90 *	1.27 ± 0.42	**< 0.001**
Total n-6 PUFA	15.74 ± 1.78	19.53 ± 4.88	12.90 ± 3.84	8.33 ± 0.65	20.85 ± 3.05	12.49 ± 1.36	**< 0.001**
18:3n-3	0.68 ± 0.17	0.99 ± 0.39	5.44 ± 1.43 *	0.56 ± 0.47	0.15 ± 0.17	0.12 ± 0.14	**< 0.001**
20:4n-3	0.51 ± 0.53	0.26 ± 0.35	0.54 ± 0.66	0.36 ± 0.61	ND	0.30 ± 0.37	0.425
20:5n-3	0.12 ± 0.14	0.20 ± 0.16	1.93 ± 0.40 *	2.81 ± 0.17 *	ND	ND	**< 0.001**
22:5n-3	0.20 ± 0.17	0.44 ± 0.12	1.27 ± 0.25	1.43 ± 0.22	ND	ND	**< 0.001**
22:6n-3	3.14 ± 1.42	5.20 ± 1.92	6.45 ± 2.26	9.19 ± 2.49	1.39 ± 0.49	2.70 ± 0.79	**< 0.001**
Total n-3 PUFA	4.64 ± 1.80	7.09 ± 1.62	15.63 ± 3.64	14.36 ± 2.54	1.55 ± 0.42	3.22 ± 0.88	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied

6.3.14 Fetal brain weight and fatty acid composition

The fatty acid composition of the fetal brain was analysed in order to determine whether there was tissue-specific accumulation of maternal dietary fatty acids, particularly as brain function has been demonstrated to be affected by maternal diet during pregnancy. For example, animals fed n-3 PUFA deficient diets during pregnancy have demonstrated impaired cognitive function among their offspring(56). This indicates the importance of accumulation of LC n-3 PUFA in the fetal brain during development.

Maternal diet did not significantly affect fetal brain weight when expressed as either absolute weight or weight as a % body weight (see table 6.41). The fatty acid compositions of fetal brain phospholipids (PC and PE) were assessed by gas chromatography (see table 6.42 and 6.43). Fetal brain PC demonstrated fewer significant changes in fatty acid composition in response to the maternal diet than was observed in the PE fraction. The PC fraction was saturate-rich, and so may more closely reflect the endogenous production of these fatty acids, rather than the dietary availability of EFA and LC PUFA. This saturate-rich membrane is also suggestive of a lipid-raft membrane domain, so it is possible that the regulation of fetal brain PC is maintained to ensure the formation of lipid rafts. There were some significant effects of maternal diet upon fetal brain PC, particularly upon LC n-6 and n-3 PUFA. Those in the linseed oil and salmon oil dietary groups had the highest EPA and DPA content of brain PC, and were significantly different from all other dietary groups. Those on the salmon oil diet had the lowest AA content of brain PC. The linseed oil and salmon oil groups had the lowest 22:5n-6 content in brain PC. With regard to EPA and DPA content (both n-6 and n-3) of fetal brain PC, there was no significant difference between the linseed oil and salmon oil groups.

In fetal brain PE, all LC n-6 and n-3 PUFA were significantly affected by maternal diet. EPA, DPA and DHA contents were highest in the linseed oil and salmon oil groups. 22:5n-6 content was significantly higher in the sunflower oil group than in any other dietary groups, and lowest in the linseed oil and salmon oil groups. The AA content of fetal brain PE was lowest in the HF salmon oil group, but in the linseed oil group AA was retained at levels comparable to those seen in the other dietary groups.

These data indicate that the linseed oil and salmon oil diet were equally effective at inducing high fetal brain LC n-3 PUFA content when compared to other maternal diets. This suggests that either the products of endogenous synthesis of LC n-3 PUFA are effectively targeted to the fetal brain, or that endogenous synthesis of LC n-3 PUFA from ALNA occurs within the fetal brain. The lack of any detectable ALNA or 20:4n-3 in the fetal brain suggests that there is little endogenous synthesis occurring within the fetal brain. Further studies, perhaps using isotope-labelled ALNA, would be required to fully investigate the source of the LC n-3 PUFA which are incorporated into the fetal brain in the linseed oil group, with possible sources including the products of endogenous synthesis within the maternal or fetal liver.

Table 6.41: Absolute and relative fetal brain weight (mean ± standard deviation)

	Brain weight (mg)	Brain weight (% body weight)
LF Soyabean	115.4 + 26.1	5.2 + 1.5
HF Soyabean	118.5 + 26.9	4.8 + 0.8
HF Linseed	130.1 + 26.1	4.9 + 0.2
HF Salmon	107.8 + 6.4	5.2 + 0.6
HF Sunflower	129.7 + 21.6	4.9 + 1.1
HF Beef tallow	108.8 + 13.7	5.0 + 1.1
ANOVA p value	0.319	0.982

Table 6.42: Fetal brain PC fatty acid composition (% total fatty acid, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	3.37 ± 0.34	3.23 ± 0.25	3.27 ± 0.38	3.41 ± 0.33	3.13 ± 0.52	3.31 ± 0.49	0.856
16:0	52.73 ± 2.54	51.21 ± 3.48	50.35 ± 5.16	50.64 ± 3.72	50.17 ± 3.73	50.32 ± 6.19	0.906
18:0	8.75 ± 0.88	9.81 ± 1.53	9.94 ± 1.97	9.45 ± 1.68	10.04 ± 1.82	9.37 ± 2.57	0.834
Total saturates	64.86 ± 1.79	64.25 ± 2.27	63.57 ± 3.75	63.50 ± 2.38	63.33 ± 2.41	62.99 ± 4.50	0.903
16:1n-7	3.28 ± 0.13	2.91 ± 0.23	3.05 ± 0.25	3.34 ± 0.23	2.90 ± 0.39	2.93 ± 0.31	0.021 †
18:1n-9	18.90 ± 0.57	17.84 ± 0.59	18.13 ± 1.03	19.57 ± 1.09	17.64 ± 1.02	19.06 ± 2.74	0.134
20:1n-9	0.38 ± 0.05	0.35 ± 0.06	0.53 ± 0.56	0.32 ± 0.16	0.27 ± 0.14	0.36 ± 0.04	0.561
Total MUFA	22.56 ± 0.64	21.10 ± 0.79	21.71 ± 1.35	23.23 ± 1.23	20.81 ± 1.31	22.35 ± 2.96	0.097
18:2n-6	1.13 ± 0.17	1.44 ± 0.25	1.78 ± 0.63	1.65 ± 0.35	1.42 ± 0.10	1.44 ± 1.05	0.415
18:3n-6	ND	ND	ND	ND	0.11 ± 0.12 *	ND	**0.002**
20:2n-6	ND	ND	ND	ND	0.18 ± 0.14	ND	0.350
20:3n-6	0.23 ± 0.19	0.43 ± 0.07	0.38 ± 0.19	0.44 ± 0.04	0.34 ± 0.17	0.27 ± 0.14	0.084
20:4n-6	6.97 ± 0.76	7.66 ± 1.12	6.93 ± 1.96	5.30 ± 1.14 ^{a}	8.41 ± 1.68 ^{b}	7.77 ± 2.06	**0.028**
22:5n-6	1.45 ± 0.45 ^{bd}	1.58 ± 0.62 ^{bcd}	0.61 ± 0.51 ^{ad}	0.17 ± 0.21 ^{ad}	2.76 ± 1.07 ^{bc}	1.69 ± 0.80 ^{bcd}	**< 0.001**
Total n-6 PUFA	9.81 ± 1.38	11.19 ± 1.66	9.78 ± 2.26	7.65 ± 1.29 ^{a}	13.21 ± 2.73 ^{b}	11.20 ± 2.96	**0.003**
18:3n-3	ND	ND	ND	ND	ND	0.10 ± 0.16	0.165
20:5n-3	ND ^{b}	ND ^{b}	0.12 ± 0.19 ^{a}	0.29 ± 0.16 ^{a}	ND ^{b}	ND ^{b}	**< 0.001**
22:5n-3	ND ^{b}	ND ^{b}	0.37 ± 0.32 ^{a}	0.43 ± 0.26 ^{a}	ND ^{b}	ND ^{b}	**< 0.001**
22:6n-3	2.77 ± 0.56	3.46 ± 1.17	4.40 ± 2.31	4.90 ± 2.00	2.65 ± 0.92	3.36 ± 1.97	0.138
Total n-3 PUFA	2.77 ± 0.56	3.46 ± 1.17	4.94 ± 2.73	5.62 ± 2.24	2.65 ± 0.92	3.46 ± 1.90	0.034 †

ND negligible detected (mean <0.1% total fatty acid content) * Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied
a, b, c, d groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 6.43: Fetal brain PE fatty acid composition (% total fatty acid, mean ± standard deviation, n=6 per group)

Fatty acid	LF Soyabean	HF Soyabean	HF Linseed	HF Salmon	HF Sunflower	HF Beef tallow	ANOVA p value
14:0	0.64 ± 0.30	0.81 ± 0.31	0.53 ± 0.09	0.78 ± 0.36	0.64 ± 0.17	0.69 ± 0.38	0.572
16:0	18.78 ± 2.93	20.68 ± 4.26	18.13 ± 2.85	20.13 ± 4.44	17.09 ± 1.60	19.13 ± 3.91	0.520
18:0	24.22 ± 1.09	24.11 ± 2.05	24.39 ± 1.39	22.66 ± 1.48	25.96 ± 2.16	22.90 ± 0.95	**0.021**
Total saturates	43.63 ± 2.18	45.60 ± 2.97	43.07 ± 2.38	43.57 ± 4.27	43.69 ± 2.34	42.72 ± 3.85	0.694
16:1n-7	1.27 ± 0.42	1.44 ± 0.35	1.08 ± 0.23	1.73 ± 0.78	1.44 ± 0.53	1.23 ± 0.57	0.355
18:1n-9	11.04 ± 1.39	11.98 ± 2.35	11.18 ± 2.11	13.57 ± 2.91	10.09 ± 1.18	11.64 ± 2.98	0.182
20:1n-9	0.30 ± 0.09	0.31 ± 0.08	0.29 ± 0.13	0.30 ± 0.17	0.14 ± 0.11	0.12 ± 0.18	0.046 †
Total MUFA	12.61 ± 1.59	13.73 ± 2.69	12.55 ± 2.34	15.60 ± 3.63	11.66 ± 1.65	12.99 ± 3.56	0.206
18:2n-6	0.98 ± 0.36	2.08 ± 1.48	1.25 ± 0.48	1.94 ± 1.08	1.49 ± 0.64	1.38 ± 1.15	0.349
20:2n-6	0.45 ± 0.03	0.19 ± 0.18	0.32 ± 0.05	0.59 ± 0.13	0.26 ± 0.16	0.44 ± 0.05	**< 0.001**
20:3n-6	0.43 ± 0.10	0.54 ± 0.06	0.60 ± 0.08	0.74 ± 0.13	0.53 ± 0.12	0.42 ± 0.13	**< 0.001**
20:4n-6	19.87 ± 1.93	19.17 ± 3.21	19.22 ± 1.23	15.06 ± 2.44	21.89 ± 1.51	20.26 ± 3.07	**0.001**
22:5n-6	6.82 ± 1.20	5.20 ± 0.81	2.77 ± 0.22	1.07 ± 0.13	11.01 ± 2.29 *	7.23 ± 1.87	**< 0.001**
Total n-6 PUFA	28.56 ± 2.38	27.19 ± 3.13	24.23 ± 1.45	19.40 ± 1.47 *	35.19 ± 2.64 *	29.73 ± 3.83	**< 0.001**
18:3n-3	ND	ND	ND	ND	ND	0.31 ± 0.48	0.068
20:5n-3	ND	ND	0.34 ± 0.13	0.64 ± 0.35	0.17 ± 0.32	ND	**< 0.001**
22:5n-3	0.33 ± 0.06	0.38 ± 0.09	1.31 ± 0.22	1.58 ± 0.48	0.15 ± 0.16	0.19 ± 0.18	**< 0.001**
22:6n-3	14.87 ± 2.27	13.10 ± 2.50	18.48 ± 3.23	19.22 ± 6.07	9.10 ± 1.80	14.02 ± 3.10	**< 0.001**
Total n-3 PUFA	15.19 ± 2.33	13.48 ± 2.53	20.15 ± 3.49	21.43 ± 6.67	9.46 ± 1.78	14.56 ± 3.49	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content)
* Significantly different ($p < 0.05$) from all other groups
† no significant differences between groups when Bonferroni post-hoc test applied

Potential predictors of fetal brain DHA status

It would not be possible to design a human study in which maternal dietary fatty acids are manipulated during pregnancy, and then samples of the fetal/neonatal brain taken to assess how DHA status was affected. It would be possible to undertake a retrospective analysis of fetal brain fatty acid composition from aborted fetuses, infants which were stillborn or those who died during infancy, but this would likely be subject to limited subject numbers and the findings would be confounded by many factors such as social or health variables. Studies of this type have been undertaken, such as one study which examined the fatty acid composition of brain cortex samples collected from term infants at post-mortem(242). This study identified that breast-fed infants had significantly more DHA and significantly less AA and 22:5n-6 in brain cortex when compared to bottle-fed infants.

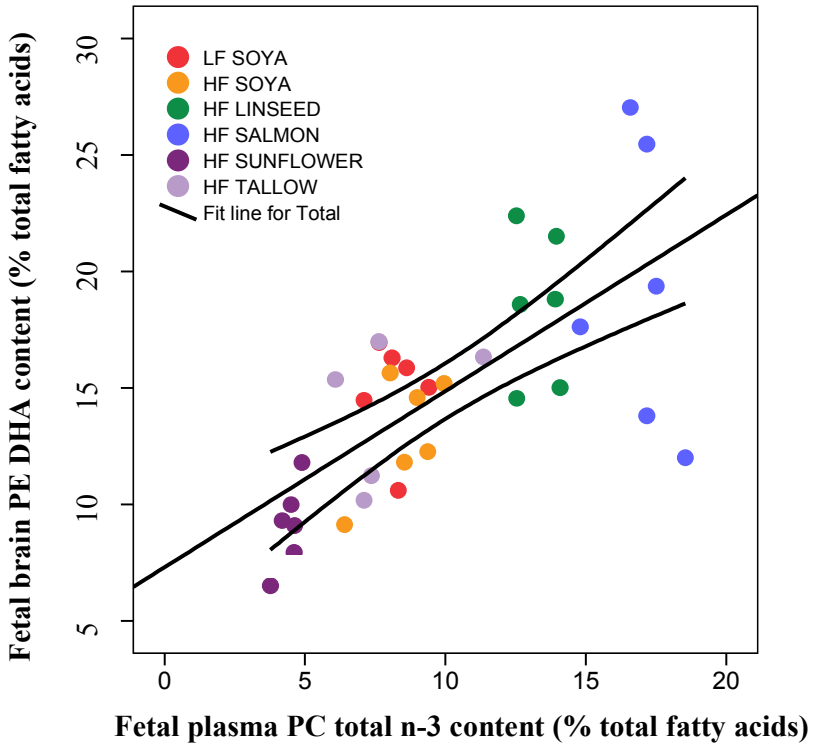
In this study using a rat model, samples which could feasibly be taken from human subjects were therefore assessed for their suitability as a predictor for fetal brain fatty acid status. Maternal plasma, maternal subcutaneous adipose tissue, fetal plasma and placenta were assessed by stepwise multiple regression analysis to determine how their n-3 fatty acid content (expressed as ALNA, 20:4n-3, EPA, DPA, DHA or total n-3 fatty acid content) could be used to predict fetal brain phospholipid DHA content. 78 potential predictive variables were assessed and the model R^2 values and predictor variables used displayed below (table 6.44).

The adjusted R^2 value achieved for fetal brain PE status was stronger than that achieved for PC status. This implies that fetal brain PC DHA content is regulated by a more complex mechanism than by the simple availability of n-3 fatty acids. The relationship between the variables with the greatest Beta value (fetal plasma PC total n-3 content and fetal brain PE DHA status) is illustrated in figure 6.12.

Table 6.44: Stepwise multiple regression analysis results for samples which could feasibly be obtained in a human study which predict fetal brain DHA status

Fetal brain PC DHA content Adjusted $R^2 = 0.555$, $p < 0.001$			**Fetal brain PE DHA content** Adjusted $R^2 = 0.884$, $p < 0.001$		
Predictor variable	**Beta**	**p**	**Predictor variable**	**Beta**	**p**
Fetal plasma NEFA 22:5n-3	0.753	0.001	Placenta CE total n-3 content	0.782	<0.001
Placenta CE 22:5n-3	-0.798	<0.001	Placenta PC 20:4n-3	-0.321	0.017
Fetal plasma PC 20:4n-3	0.512	0.003	Maternal plasma CE 22:5n-3	0.724	<0.001
Fetal plasma NEFA 20:4n-3	0.346	0.035	Maternal plasma NEFA 20:4n-3	-0.848	<0.001
			Fetal plasma PC total n-3 content	1.0009	<0.001
			Fetal plasma TAG 22:6n-3	-0.618	0.001
			Fetal plasma PC 20:4n-3	-0.283	0.006
			Maternal plasma PC 22:5n-3	0.286	0.016

Figure 6.12: Graph to illustrate the correlation observed between fetal plasma PC total n-3 content and fetal brain PE DHA content (95% confidence interval displayed)



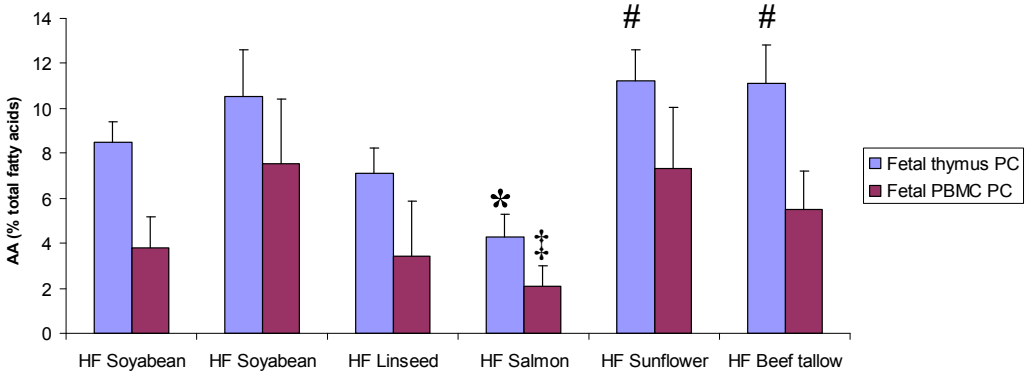
6.3.15 Fetal immune cell and tissue fatty acid composition

The fatty acid compositions of phospholipids in fetal immune tissues were assessed to determine whether maternal diet during pregnancy could significantly affect fatty acid composition, particularly the AA and EPA content of tissues as these fatty acids are precursors for immune signalling molecules such as eicosanoids.

Within fetal thymus PC (see table 6.45), maternal diet had a significant effect upon n-6 fatty acid composition (including AA), and EPA status. Within fetal thymus PE (see table 6.46), maternal diet did not significantly affect n-6 fatty acid status, but had significant effects upon LC n-3 PUFA status, including EPA. Fetal PBMC PC (see table 6.47) demonstrated significant effects of maternal diet, including significant effects upon AA and EPA content.

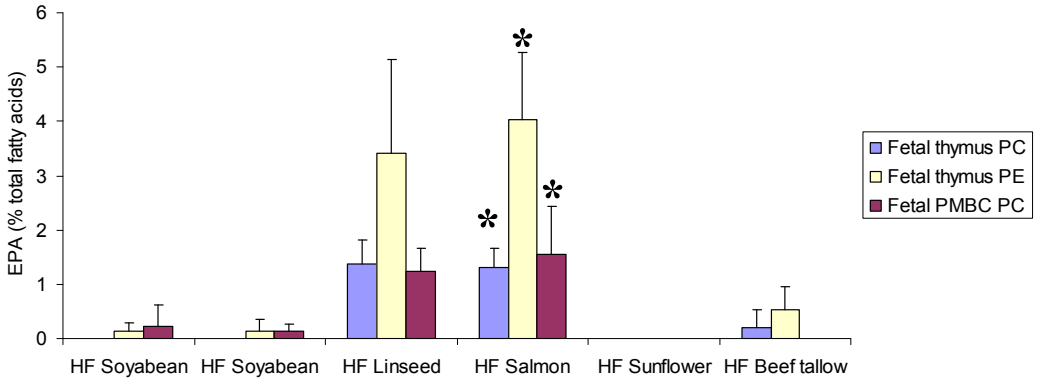
The HF linseed oil and salmon oil diets were associated with the lowest AA content in fetal thymus and PBMC PC (see figure 6.13), and the highest EPA status across all fetal immune tissues assessed (see figure 6.14). The HF linseed oil diet was therefore as effective as the HF salmon oil diet at affecting the EPA content of fetal immune tissues, but without lowering the AA content to the low levels observed in the salmon oil group.

Figure 6.13: The AA content (% total fatty acids) of fetal thymus and PBMC phosphatidylcholine (mean ± standard deviation)



*Significantly different from all other groups ($p < 0.05$) except HF Linseed
Significantly different from HF Linseed and HF Salmon ($p < 0.05$)
‡ Significantly different from HF Soyabean ($p < 0.05$)

Figure 6.14: The EPA content (% total fatty acids) of fetal thymus and PBMC phospholipids (mean ± standard deviation)



* Significantly different from all other groups ($p < 0.05$) except HF Linseed

Table 6.45: Fetal thymus PC fatty acid composition (% total fatty acids, mean ± standard deviation)

Fatty acid	HF Soyabean (n=4)	HF Soyabean (n = 3)	HF Linseed (n = 3)	HF Salmon (n = 6)	HF Sunflower (n = 4)	Beef tallow (n=5)	ANOVA p value
14:0	ND	0.73 ± 1.26	1.99 ± 0.73	0.30 ± 0.75	1.12 ± 1.38	0.68 ± 0.95	0.124
16:0	44.41 ± 2.96	41.06 ± 3.63	40.02 ± 3.31	36.33 ± 6.02	38.31 ± 5.09	36.37 ± 4.97	0.148
18:0	16.48 ± 2.18	16.28 ± 4.18	13.43 ± 2.07	21.10 ± 10.10	17.75 ± 5.89	20.43 ± 9.38	0.686
20:0	0.19 ± 0.22	0.23 ± 0.25	ND	0.34 ± 0.40	0.21 ± 0.43	ND	0.793
Total saturates	61.07 ± 2.75	58.30 ± 1.68	55.51 ± 3.29	58.07 ± 7.65	57.39 ± 2.62	57.58 ± 4.07	0.757
16:1n-7	1.46 ± 1.08	1.65 ± 0.29	2.40 ± 0.32	2.10 ± 0.78	1.58 ± 0.64	1.20 ± 1.12	0.368
18:1n-9	16.22 ± 1.06	13.28 ± 0.64	15.20 ± 0.60	18.30 ± 3.82	13.40 ± 2.79	15.88 ± 3.14	0.097
20:1n-9	1.79 ± 0.49	1.63 ± 0.22	0.89 ± 0.53	1.68 ± 1.44	1.79 ± 1.49	1.10 ± 0.82	0.763
24:1n-9	ND	0.82 ± 0.97	0.23 ± 0.39	0.20 ± 0.16	0.69 ± 1.38	0.94 ± 1.96	0.795
Total MUFA	19.56 ± 2.31	17.37 ± 1.02	18.71 ± 0.59	22.28 ± 5.61	17.47 ± 3.41	19.12 ± 2.58	0.320
18:2n-6	6.17 ± 0.76	8.72 ± 0.64	12.20 ± 1.49 *	7.76 ± 1.42	8.15 ± 1.05	5.97 ± 1.53	**< 0.001**
20:2n-6	0.58 ± 0.06	1.52 ± 0.30	0.45 ± 0.66	0.27 ± 0.21	1.25 ± 0.81	0.38 ± 0.42	**0.005**
20:3n-6	1.79 ± 0.34	0.93 ± 0.81	1.87 ± 0.33	2.63 ± 0.48	2.12 ± 0.90	1.37 ± 0.86	**0.022**
20:4n-6	8.49 ± 0.91	10.51 ± 2.10	7.13 ± 1.12	4.28 ± 1.00	11.23 ± 1.39	11.10 ± 1.74	**< 0.001**
22:5n-6	0.30 ± 0.23	0.31 ± 0.05	0.27 ± 0.24	ND	0.70 ± 0.31	0.50 ± 0.36	**0.006**
Total n-6 PUFA	17.34 ± 1.42	22.03 ± 3.47	21.99 ± 2.11	14.94 ± 1.91	23.50 ± 2.43	19.36 ± 4.04	**0.001**
18:3n-3	ND	0.46 ± 0.63	0.39 ± 0.34	0.43 ± 0.66	0.36 ± 0.73	0.52 ± 1.00	0.906
20:4n-3	0.48 ± 0.31	0.51 ± 0.20	0.30 ± 0.28	0.40 ± 0.55	0.17 ± 0.34	0.80 ± 0.55	0.409
20:5n-3	ND	ND	1.37 ± 0.45	1.30 ± 0.37	ND	0.20 ± 0.34	**< 0.001**
22:5n-3	ND	0.24 ± 0.41	0.65 ± 0.22	0.46 ± 0.26	0.24 ± 0.40	0.32 ± 0.54	0.271
22:6n-3	1.55 ± 0.50	1.07 ± 0.19	1.08 ± 0.12	2.11± 0.79	0.87 ± 0.37	2.10 ± 1.52	0.157
Total n-3 PUFA	2.03 ± 0.67	2.31 ± 1.35	3.78 ± 0.73	4.71 ± 1.14	1.65 ± 1.22	3.93 ± 2.59	0.036 †

ND negligible detected (mean <0.1% total fatty acid content)
† no significant differences between groups when Bonferroni post-hoc test applied
* Significantly different ($p < 0.05$) from all other groups

Table 6.46: Fetal thymus PE fatty acid composition (% total fatty acids, mean ± standard deviation)

Fatty acid	HF Soyabean (n = 4)	HF Soyabean (n = 6)	HF Linseed (n = 4)	HF Salmon (n = 5)	HF Sunflower (n = 5)	HF Beef tallow (n = 4)	ANOVA p value
14:0	ND	0.43 ± 0.68	ND	ND	0.29 ± 0.70	0.59 ± 0.71	0.400
16:0	22.47 ± 3.03	24.65 ± 5.38	15.87 ± 3.49	20.41 ± 3.48	20.19 ± 8.66	19.44 ± 3.71	0.254
18:0	37.03 ± 8.70	36.99 ± 7.31	27.13 ± 6.00	27.16 ± 5.36	32.26 ± 7.68	30.91 ± 8.47	0.163
20:0	0.18 ± 0.36	0.63 ± 0.24	ND	0.30 ± 0.29	0.35 ± 0.33	0.47 ± 0.51	0.139
Total saturates	59.68 ± 10.62	62.75 ± 12.56	43.13 ± 8.62	47.95 ± 6.75	55.72 ± 16.76	51.42 ± 10.83	0.149
16:1n-7	0.59 ± 0.48	0.80 ± 0.46	0.93 ± 0.13	1.49 ± 0.41	0.74 ± 0.15	1.00 ± 0.56	**0.028**
18:1n-9	10.82 ± 6.53	9.80 ± 2.29	13.42 ± 2.30	15.87 ± 2.79	10.47 ± 2.17	12.17 ± 4.09	0.088
20:1n-9	1.04 ± 0.57	0.45 ± 0.42	0.67 ± 0.47	1.20 ± 0.39	0.84 ± 0.53	1.31 ± 0.51	0.080
Total MUFA	12.46 ± 6.74	11.05 ± 2.96	15.11 ± 2.46	18.57 ± 3.10	11.65 ± 2.80	14.48 ± 4.59	0.049 †
18:2n-6	4.62 ± 0.91	5.46 ± 1.26	7.76 ± 0.65	9.02 ± 4.70	5.57 ± 0.62	5.85 ± 2.71	0.067
20:2n-6	0.19 ± 0.22	0.48 ± 0.51	0.53 ± 0.31	0.28 ± 0.16	0.53 ± 0.45	0.43 ± 0.26	0.628
20:3n-6	0.94 ± 1.09	1.04 ± 0.89	1.55 ± 0.51	1.17 ± 1.28	1.76 ± 0.48	1.35 ± 1.10	0.710
20:4n-6	18.24 ± 4.27	14.40 ± 8.90	22.56 ± 4.01	11.95 ± 1.75	22.47 ± 13.57	18.57 ± 8.99	0.308
22:5n-6	0.78 ± 0.53	1.00 ± 1.03	0.55 ± 0.45	0.52 ± 0.63	1.92 ± 1.15	1.59 ± 1.03	0.098
Total n-6 PUFA	24.77 ± 5.80	22.39 ± 10.24	32.95 ± 4.78	22.94 ± 6.67	29.88 ± 15.27	27.84 ± 8.60	0.372
18:3n-3	0.21 ± 0.43	1.09 ± 1.21	0.69 ± 0.80	1.70 ± 2.74	0.77 ± 1.35	2.11 ± 3.26	0.706
20:4n-3	0.29 ± 0.22	0.35 ± 0.32	0.19 ± 0.23	0.15 ± 0.22	0.19 ± 0.23	0.40 ± 0.36	0.677
20:5n-3	0.13 ± 0.16	0.13 ± 0.23	3.41 ± 1.72	4.02 ± 1.24	ND	0.53 ± 0.43	**< 0.001**
22:5n-3	0.16 ± 0.33	0.39 ± 0.09	1.22 ± 0.17	0.96 ± 0.38	0.23 ± 0.31	0.20 ± 0.28	**< 0.001**
22:6n-3	2.30 ± 0.87	1.86 ± 0.66	3.29 ± 0.51	3.71 ± 0.64	1.36 ± 0.22	3.03 ± 0.68	**< 0.001**
Total n-3 PUFA	3.09 ± 1.17	3.81 ± 1.90	8.80 ± 2.14	10.54 ± 2.43	2.75 ± 1.62	6.26 ± 3.09	**< 0.001**

ND negligible detected (mean <0.1% total fatty acid content)
† no significant differences between groups when Bonferroni post-hoc test applied

Table 6.47: Fetal PBMC PC fatty acid composition (% total fatty acids, mean ± standard deviation)

	LF Soyabean (n=3)	HF Soyabean (n=5)	HF Linseed (n=5)	HF Salmon (n=5)	HF Sunflower (n=3)	HF Beef tallow (n=3)	ANOVA p value
14:0	0.94 ± 1.03	0.78 ± 0.45	0.54 ± 0.52	0.83 ± 0.53	1.12 ± 0.34	1.19 ± 0.19	0.610
16:0	50.97 ± 3.00	50.64 ± 3.10	47.60 ± 5.17	47.15 ± 5.47	50.59 ± 4.02	44.57 ± 3.63	0.350
18:0	19.03 ± 5.06	11.77 ± 4.11	19.96 ± 9.94	13.74 ± 4.23	11.61 ± 1.97	13.51 ± 2.04	0.202
20:0	0.32 ± 0.25	0.17 ± 0.12	0.27 ± 0.30	0.29 ± 0.22	0.16 ± 0.14	0.44 ± 0.20	0.578
22:0	0.27 ± 0.15	0.13 ± 0.12	0.42 ± 0.16	0.28 ± 0.23	ND	0.21 ± 0.26	0.146
Total saturates	71.54 ± 1.43	63.49 ± 2.85	68.80 ± 5.52	62.30 ± 2.24	63.57 ± 4.21	59.93 ± 1.48	**0.003**
16:1n-7	1.85 ± 0.61	1.66 ± 0.30	0.86 ± 0.83	1.97 ± 0.26	2.09 ± 0.74	2.06 ± 0.18	0.029 †
18:1n-9	14.45 ± 2.47	12.96 ± 1.31	12.59 ± 1.22	17.54 ± 2.75	14.49 ± 1.47	20.74 ± 1.86	**< 0.001**
20:1n-9	ND	0.23 ± 0.22	0.40 ± 0.56	0.46 ± 0.45	0.14 ± 0.25	ND	0.434
24:1n-9	0.39 ± 0.10	0.17 ± 0.17	0.38 ± 0.27	0.43 ± 0.26	0.22 ± 0.02	0.22 ± 0.21	0.365
Total MUFA	16.70 ± 3.02	15.01 ± 1.21	14.24 ± 1.80	20.39 ± 2.44	16.94 ± 1.83	23.09 ± 1.75	**< 0.001**
18:2n-6	5.31 ± 1.63	9.86 ± 0.48	7.68 ± 2.66	8.75 ± 1.24	8.94 ± 1.68	6.46 ± 1.43	**0.017**
18:3n-6	0.24 ± 0.42	0.31 ± 0.20	0.19 ± 0.31	ND	0.27 ± 0.26	0.10 ± 0.17	0.699
20:2n-6	0.16 ± 0.27	0.29 ± 0.27	0.54 ± 0.73	0.64 ± 0.24	0.38 ± 0.28	1.09 ± 1.05	0.333
20:3n-6	0.28 ± 0.26	1.28 ± 0.72	1.42 ± 0.93	1.70 ± 1.40	0.62 ± 0.54	0.21 ± 0.36	0.146
20:4n-6	3.82 ± 1.34	7.54 ± 2.89	3.44 ± 2.45	2.11 ± 0.87	7.34 ± 2.73	5.50 ± 1.69	**0.007**
22:5n-6	0.91 ± 0.15	0.88 ± 0.29	0.63 ± 0.44	0.96 ± 0.30	1.16 ± 0.26	2.52 ± 2.49	0.135
Total n-6 PUFA	10.72 ± 2.16	20.17 ± 3.07	13.89 ± 4.22	14.25 ± 2.77	18.71 ± 5.54	15.89 ± 0.84	**0.015**
18:3n-3	ND	0.23 ± 0.14	0.61 ± 0.36	0.23 ± 0.21	ND	0.51 ± 0.89	0.246
20:4n-3	ND	0.10 ± 0.13	ND	0.49 ± 0.45	ND	ND	**0.032**
20:5n-3	0.23 ± 0.39	0.13 ± 0.13	1.24 ± 0.43	1.56 ± 0.87	ND	ND	**< 0.001**
22:5n-3	0.19 ± 0.17	ND	0.47 ± 0.32	ND	0.22 ± 0.25	ND	**0.016**
22:6n-3	0.53 ± 0.32	0.79 ± 0.47	0.74 ± 0.40	0.75 ± 0.41	0.34 ± 0.20	0.48 ± 0.42	0.594
Total n-3 PUFA	1.05 ± 0.42	1.33 ± 0.57	3.07 ± 0.85	3.06 ± 1.02	0.78 ± 0.58	1.10 ± 1.14	**0.001**

ND negligible detected (mean <0.1% total fatty acid content)
† no significant differences between groups when Bonferroni post-hoc test applied

Potential predictors of fetal immune tissue EPA status

Maternal plasma, maternal subcutaneous adipose tissue, fetal plasma and placenta were assessed by stepwise multiple regression analysis to determine how their n-3 fatty acid content (expressed as ALNA, 20:4n-3, EPA, DPA, DHA or total n-3 fatty acid content) could be used to predict fetal immune tissue EPA content. 78 potential predictive variables were assessed and the model R^2 values and predictor variables used are detailed in table 6.48.

Although significant relationships between the variables were apparent, the distributions were bimodal, with EPA often only detectable in the immune tissue of animals receiving n-3 PUFA rich diets (linseed and salmon oil). These correlations indicate that the placenta is one of the best predictors of fetal immune tissue EPA status. The relationship between the variable with the greatest Beta value (placenta PE 22:5n-3 content and fetal thymus PC EPA status) is illustrated in figure 6.15.

Figure 6.15: Graph to illustrate the correlation observed between placenta PE 22:5n-3 and fetal thymus PC EPA content (95% confidence interval displayed)

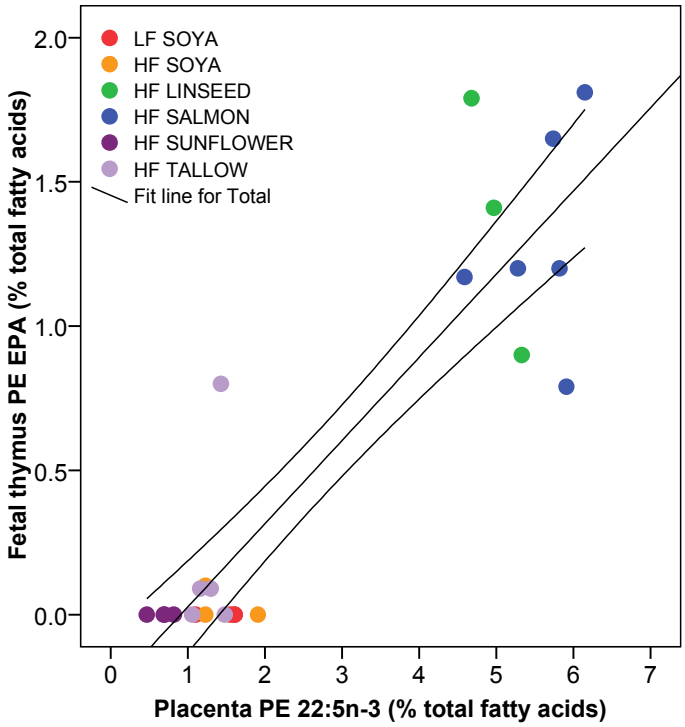


Table 6.48: Stepwise multiple regression analysis results for samples which could feasibly be obtained in a human study which predict fetal immune tissue EPA status

Fetal thymus PC EPA content Adjusted $R^2 = 0.895$, $p < 0.001$			Fetal thymus PE EPA content Adjusted $R^2 = 0.971$, $p < 0.001$			Fetal PBMC PC EPA content Adjusted $R^2 = 0.902$, $p < 0.001$		
Predictor variable	**Beta**	**p**	**Predictor variable**	**Beta**	**p**	**Predictor variable**	**Beta**	**p**
Placenta PE 22:5n-3	0.916	<0.001	Maternal subcut. adipose 20:5n-3	0.439	0.001	Maternal subcut. adipose 20:5n-3	0.769	0.001
Fetal plasma NEFA 22:6n-3	-0.494	<0.001	Fetal plasma NEFA 18:3n-3	0.341	0.001	Fetal plasma NEFA 22:6n-3	-0.558	<0.001
Maternal plasma PC 22:5n-3	0.397	0.016	Maternal plasma CE 22:5n-3	0.304	<0.001	Placenta PE total n-3 content	0.495	0.018
			Placenta CE 18:3n-3	-0.300	0.001			
			Placenta TAG 22:5n-3	0.739	0.001			
			Placenta TAG 22:6n-3	-0.500	0.006			

6.4 Discussion

This chapter had set out to addresses the following hypotheses:

a) that a change in maternal dietary fatty acid intake during pregnancy will alter maternal plasma fatty acid composition, thereby altering the supply of fatty acids to the developing fetus, including the availability of LC n-3 PUFA.
b) that the changes to maternal dietary fatty acid intake during pregnancy will significantly affect maternal tissue composition, including the maternal liver, adipose tissue and immune tissues.
c) that a change in the supply of fatty acids to the developing fetus will alter the fatty acid composition of fetal plasma and developing fetal tissues such as the liver, brain and immune tissues.

The data collected fully support these hypotheses, and in addition generated data which identified novel observations of the effect of maternal diet upon specific incorporation of EPA into immune tissues and DHA/22:5n-6 with the fetal brain, and a significant effect of an ALNA rich diet during pregnancy upon DHA status.

The data from this study supports that of other authors, with maternal plasma, liver and adipose tissues demonstrating a significant effect of maternal diet during pregnancy upon fatty acid composition. As well as increasing the tissue content of fatty acids which were direct components of the experimental diets, there was evidence of maternal endogenous synthesis of LC PUFA from their EFA precursors, particularly within the HF linseed oil group, where higher LC n-3 PUFA contents were observed in the absence of any dietary source of these fatty acids. Clear lipid fraction specificity of EPA incorporation with provision of an ALNA-rich diet was again observed, as was the case in data from chapter 3. EPA is significantly enriched within CE fractions following the provision of an ALNA rich diet, and is incorporated to a much greater extent than is observed in phospholipids or TAG. This feature was pronounced within maternal plasma, placenta and fetal plasma, but was not as marked within maternal and fetal liver, suggesting a level of tissue specificity for lipid fraction incorporation.

The fatty acid composition of maternal plasma demonstrated a close relationship with liver lipids, with statistically significant correlations observed between plasma and liver saturates, MUFA and PUFA. The relationship between maternal adipose tissue composition and plasma NEFA was strongest for the EFA, indicating that EFA are readily incorporated into and mobilised from adipose tissue. This study could not provide information upon total fat mass, only upon the relative proportions of fatty acids within maternal fat depots. It would be of interest to investigate whether the experimental diets had a significant effect upon maternal adiposity, as data indicate that the rate of weight gain during pregnancy is greatest upon the HF beef tallow and salmon oil diet, but without a significant effect of these diets upon litter size. Experimental diets which result in increased maternal body fat might have a greater potential to continue to exert an influence on neonatal tissue fatty acid composition by significantly affecting the fatty acid composition of maternal milk.

Of particular interest to this study was the potential that maternal diet during pregnancy had to alter the fatty acid composition of maternal and fetal immune tissues. EPA and AA are fatty acids with the potential to significantly alter immune function due to their role as substrates for eicosanoid production. The EPA and AA content of both maternal and fetal immune tissues were significantly affected by maternal diet, with the effect of maternal diet tissue and lipid fraction specific. For example, the maternal spleen demonstrated a greater responsiveness to maternal diet in comparison to maternal thymus or PBMC. This may reflect differences in cellular turnover between immune tissue types. Across all immune tissues it was the PE fraction which was more significantly altered by maternal diet than the PC fraction. These differences between the phospholipid classes are likely to be a function of their different cellular locations, and therefore different functional roles. PC tends to be localised on the outer leaflet of plasma membranes, whereas PE is found on the inner leaflet and intracellular membranes. The enzyme which hydrolyses fatty acids from cell membranes for eicosanoid synthesis is phospholipase A_2, an intracellular enzyme. The location of the fatty acid substrates for this enzyme (EPA and AA) within the intracellular environment would therefore alter the availability of substrate to this enzyme. Investigation of both maternal and fetal immune function will be undertaken to assess whether these biochemical changes induced via maternal diet have the capacity to exert functional effects upon the maternal and fetal immune system (see chapter 7).

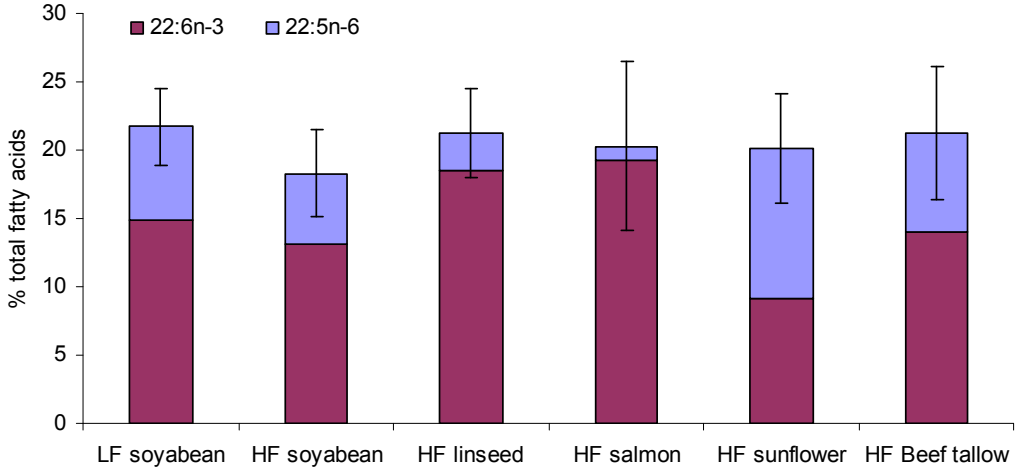
As would be anticipated, the significant differences observed in maternal plasma and tissue fatty acids were mirrored by changes to the fatty acid composition of fetal plasma, liver, brain and immune tissue. When maternal and fetal plasma fatty acid composition data were compared, it was clear that biomagnification of LC PUFA was occurring. Biomagnification is a term which is used to describe the relationship between maternal and fetal plasma fatty acid status, where the LC PUFA such as AA and DHA form a higher proportion of the total fatty acids in the fetal circulation compared to the maternal circulation, which implies a selective transfer of these fatty acids(168).

There was evidence that certain fatty acids, particularly LC n-3 PUFA, are selectively incorporated into specific fetal tissues. A good illustration of this is found by comparing the HF linseed oil and HF salmon oil groups. The HF linseed oil group contains no direct source of LC n-3 PUFA, but these fatty acids can be endogenously synthesised under the action of desaturase and elongase enzymes within the maternal liver. Despite this, the levels of LC n-3 PUFA within maternal plasma, liver, or adipose tissue or within the fetal plasma and liver of the HF linseed oil group were never as high as those within the HF salmon oil group, where dietary LC n-3 PUFA were directly consumed. This would seem to indicate that it is preferable to consume preformed LC n-3 PUFA. However, this was not the case within every fetal tissue assessed. The HF linseed oil and salmon oil diets were equally effective at maximising the DHA content of fetal brain phospholipids and the EPA content of fetal immune tissue phospholipids. This indicates that there is a significant role of maternal and possibly fetal LC n-3 PUFA synthesis in determining fetal tissue LC n-3 PUFA status, with tissue specific incorporation of these fatty acids.

In chapter 4 it was identified that in addition to effects of pregnancy upon in increasing DHA status, there was a significantly higher content of 22:5n-6 among pregnant rats at day 20 of gestation compared to virgin females, indicating that the effects of pregnancy upon LC PUFA synthesis are not limited to LC n-3 PUFA. Data from this chapter also indicated that the effects of pregnancy on 22:5n-6 content were tissue specific with this fatty acid found at high levels in maternal plasma lipids at day 20 of pregnancy, but at much lower levels within maternal liver lipids and adipose tissue, giving an indication that this fatty acid may be preferentially mobilised into the maternal plasma in order to be available to the developing fetus. When data from the current study is assessed, the

22:5n-6 content of tissues was significantly affected by maternal diet, with this fatty acid often undetectable in tissues collected from those on n-3 rich diets (HF linseed and salmon oil) but at much higher levels among the n-6 or saturate rich diets (HF sunflower and beef tallow). The contribution of 22:5n-6 to fetal fatty acid composition was greatest within the fetal plasma and brain, indicating that the brain in particular may have a requirement for these highly unsaturated fatty acids (HUFA). The relationship observed between 22:5n-6 and DHA in fetal brain PE (see figure 6.16) seems to indicate that there is a minimum requirement for the tissue content of HUFA, which will be met by 22:5n-6 in the event of inadequate 22:6n-3 content. For example, there was no significant effect of diet upon fetal brain PE total HUFA content (22:6n-3 + 22:5n-6 as % total fatty acids, one-way ANOVA $p = 0.769$), though the individual contribution of these fatty acids to fetal brain PE content was significantly influenced by maternal diet. These data also imply that maternal plasma 22:5n-6 content may be a simple and useful marker of fetal brain DHA status among pregnant women, with the detection of any 22:5n-6 used as a marker of inadequate dietary n-3 PUFA status.

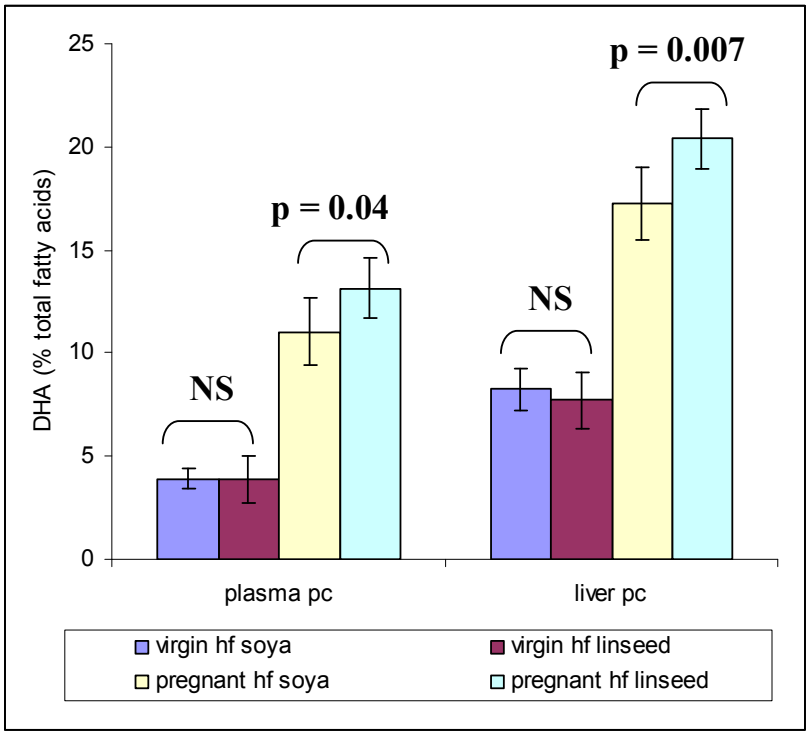
Figure 6.16: Graph to illustrate the relationship between 22:5n-6 and 22:6n-3 within fetal brain PE. Data is mean for each fatty acid ± standard deviation of total HUFA content.



The effects observed upon fetal brain composition could be further investigated to determine whether any behavioural or cognitive changes can be detected in the offspring. Studies undertaken using rat models have demonstrated that n-3 PUFA deficient diets post-weaning resulted in a significant increase in the 22:5n-6 content of brain lipids, and significantly affect behavioural markers, including depression and aggression(243). Whether the same behavioural changes could be induced by less intensive dietary n-3 restriction during pregnancy (e.g. HF sunflower oil group), which also led to increased 22:5n-6 content could be investigated. It would also be of interest to compare the behavioural effects of the HF linseed oil and HF salmon oil diets. Both these groups achieved equivalent DHA content of fetal brain phospholipids, but the HF linseed oil group achieved this without the reductions in AA content which were a feature of the HF salmon oil group. The reductions in AA content which associated with the HF salmon oil diet may be due to inhibition of Δ6 desaturase activity by dietary fish oil, which has been identified in murine models(244). In this respect, the HF linseed oil diet may be advantageous over the HF salmon oil diet.

When data from this study are compared with the earlier data from chapter 3, where virgin females received the same HF linseed oil and HF soyabean oil diet over the same time period (20 days), it is clear that the ALNA rich linseed oil diet has a greater effect upon DHA status during pregnancy than was observed among virgin females (see figure 6.17). This suggests that a maternal diet rich in ALNA can further promote the increases in plasma and tissue DHA status which are a feature of pregnancy. This potential for diet to interact with pregnancy is of relevance to human health, as this could confer the benefits associated with higher DHA status during pregnancy in the absence of direct consumption of LC n-3 PUFA from products such as oily fish.

Figure 6.17: Comparison of the plasma lipid DHA content of virgin females and day 20 gestation females when fed the HF soyabean oil or HF linseed oil diet for 20 days (mean ± standard deviation, effect of diet determined by T-test)



The longevity of the effects of maternal diet upon maternal and fetal tissue composition, and therefore their programming potential, could not be assessed within this study design. However, the significant effects of diet upon maternal weight gain and adipose tissue composition suggest that the maternal diet during pregnancy may continue to significantly affect offspring fatty acid composition (and perhaps tissue function) if the accumulated adipose tissue fatty acids are incorporated in maternal milk. The effects that these maternal diets during pregnancy have upon the maternal and fetal immune system are investigated in chapter 7.

Chapter 7: The effect of maternal diet during pregnancy upon immune function

7.1 Introduction

7.1.1 Aims and hypothesis of current study

This study aims to investigate the effect of maternal dietary fatty acids, particularly n-3 fatty acids, during pregnancy upon maternal and fetal immune function. Data have already demonstrated that pregnancy is associated with alterations in the maternal immune system of the Wistar rat (see chapter 5). The observed effects of pregnancy upon the maternal immune system included thymic involution, reduced lymphoid organ cellularity, an increased proportion of $CD8^+$ cells in blood, reduced expression of CD161 on NK cells in blood and the spleen, and an increased Th1:Th2 ratio of cytokine production by maternal thymocytes.

In chapter 6 it was demonstrated that altering the maternal diet during pregnancy significantly affected both maternal and fetal immune organ fatty acid composition, including the content of EPA and AA, fatty acids which are substrates for eicosanoid production. This chapter therefore sets out to addresses the hypothesis that the type of dietary fatty acids consumed during pregnancy will significantly alter markers of both the maternal and fetal immune function, and these effects correspond to observed changes within the fatty acid composition of immune tissues.

The markers of immune function to be assessed include immune organ weight and cellularity, cell subsets within immune tissues, lymphocyte proliferation, and cytokine and eicosanoid production. Changes observed in immune function will be correlated with the fatty acid composition data obtained from the cohort studied in chapter 6 to determine whether functional changes occur as a direct effect of altered fatty acid composition, and in particular of n-3 PUFA content.

7.1.2 Fatty acids and immune function

Numerous animal studies have been undertaken which have provided various dietary fatty acids and assessed their effects upon immune function. The effects of fatty acids upon lymphocyte functions *in vitro* and *ex vivo* have been reviewed in detail elsewhere(245).

Studies using mouse models which have provided n-3 PUFA rich diets have identified that diets containing EPA or DHA reduced proliferation of splenic T helper cells, and

production of interferon (IFN)-γ(246). Studies have also indicated that the effects of dietary n-3 fatty acids upon immune tissues are organ specific. For example, a murine study demonstrated that a diet with high DHA content resulted in lower expression of CD4 and CD8 on splenocytes, but did not affect thymocytes. Analysis of the fatty acid composition of these cells indicated that DHA was more readily incorporated into splenocytes than thymocytes(247). Reduced CD4 and CD8 expression on T cells in response to dietary DHA has also been observed in murine PBMC(248). Dietary fatty acids have also demonstrated significant effects upon cytokine production, with diets rich in safflower oil (linoleic acid rich) or fish oil (LC n-3 PUFA rich) resulting in significantly lower splenocyte IFN-γ production in comparison to LF or saturate rich diets(98). The potential consequences of these dietary effects during pregnancy have not yet been evaluated.

Studies using rat models have illustrated that the rat and the mouse differ in some of their immune responses to the type of dietary fat provided. In contrast to the observations with mouse models, the quantity and type of dietary fat given to male rats (provided over a 10 week feeding period) did not significantly affect the lymphocyte cell subsets within the spleen or thymus(249). Common between species is the effect of dietary fats upon lymphocyte proliferation. For example, reduced lymph node lymphocyte proliferation was observed in olive oil (monounsaturated rich), evening primrose oil (contains γ-linolenic acid) and menhaden oil (LC n-3 PUFA rich) fed rats when compared to other dietary fats (hydrogenated coconut oil, safflower oil) or low-fat feeding(249) but without any significant effect of the diet upon thymus lymphocyte proliferation. This indicates that dietary fatty acids significantly affect lymphocyte function in the rat, but in a tissue specific manner. Similar results were obtained from a study of male rats which investigated the effect of various PUFA incorporated into high fat (17.8% w/w) diets(250). This study also identified that there was no significant effect of diet upon rat spleen lymphocyte subsets, but did observe lower proliferative responses and PGE_2 production in animals receiving EPA- or DHA-rich diets.

7.1.3 Fatty acids and immune function during pregnancy

The immune system adapts during pregnancy in order to prevent rejection of the fetus via a maternal response to paternal antigens. It is possible that changes to the maternal diet during pregnancy may affect these normal maternal adaptations, or affect the developing fetal immune system, with the potential to affect health and disease risk.

Animal studies to date which have investigated the effect of dietary fatty acids upon immune function during pregnancy have tended to focus upon offspring immune function, and have often also incorporated a period of neonatal feeding, or collected samples after weaning.

One method of assessing immune function in offspring is to assess the incidence of infection, or survival following pathogen challenge. A study which provided rats during pregnancy with a diet rich in either corn oil (linoleic acid rich) or menhaden fish oil(204) identified that when pups were exposed to streptococcal sepsis at age 7 days, survival was significantly better in the fish oil fed group; this was associated with reduced PGE_2 production. This indicates that the fatty acid composition of the pre- and postnatal diet can beneficially affect the response to neonatal immune challenge.

Rat studies have also identified that the maternal diet during pregnancy has the capacity to significantly affect neonatal thymus and spleen weight (both absolute and as a % body weight) and spleen lymphocyte proliferation. Lymphocyte proliferation was higher in the offspring of animals receiving a saturate rich diet (coconut oil based) than a linoleic acid (LA) rich diet (corn oil) during pregnancy. The LA rich diet group also demonstrated significantly higher neonatal thymus and spleen weight (both absolute and as a % body weight)(205).

Following on from observations in epidemiological studies that a high intake of n-6 fatty acids increases the risk of atopic disease in childhood and that n-3 fatty acid intake may be protective(94;95), human studies have been carried out which provide pregnant women or infants with additional dietary n-3 PUFA in an attempt to modulate the infant's susceptibility to atopic diseases such as asthma. Full details of the findings of these studies to date are described in the introduction (see section 1.9.3). In brief, these studies have suggested that providing additional fish oil to pregnant women can alter markers of immune function assessed in cord blood samples, with a tendency for those infants in the fish oil supplemented group to demonstrate lower levels of Th2 type cytokines such as IL-13(209) and IL-4(251). Lower Th2 cytokine levels could reflect a change in the Th1/Th2 balance towards the Th1 phenotype with potential benefits to the infant in the prevention of atopy. Few data are available on the effect that supplementation during pregnancy has upon maternal immune function.

7.2 Methods

The effect of maternal dietary fatty acids upon the maternal and fetal immune system will be assessed by monitoring immune organ weight and cellularity, immune cell subset phenotyping by flow cytometry, lymphocyte proliferation and the production of cytokines and prostaglandins by cultured blood and immune organ mononuclear cells.

All animal work was carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986); for pregnant females, mating was carried out by monogamous breeding (see section 2.1). Animals received experimental diets over a 20 day period from the time of conception. Six diets were used, a low fat (LF) soyabean oil diet, and five high fat (HF) diets: soyabean oil, linseed oil, salmon oil, sunflower oil, beef tallow. The fatty acid composition of these diets is as described in Chapter 6 (see table 6.1). Maternal and fetal tissues were collected on day 20 of gestation. Immune cell phenotypes were identified by flow cytometry of lysed whole blood or immune organ mononuclear cell preparations (see section 2.11.1). *Ex vivo* culture was performed to assess lymphocyte proliferation (see section 2.11.2) and the production of IFN-γ, IL-4 and PGE_2 in response to concanavalin A (see section 2.11.3).

The effect of maternal diet upon the variables of interest was determined by one-way ANOVA with Bonferroni post-hoc correction for multiple comparisons. Correlation coefficients were calculated using Pearson or Spearman test as appropriate after data were assessed for normality. The effect of pregnancy upon the sensitivity of lymphocytes to concanavalin A (Con A) stimulation was assessed by Kruskal-Wallis test.

7.3 Results

7.3.1 Maternal immune organ weights and cellularity

The effects of diet during pregnancy upon maternal immune organ weight and cellularity (see table 7.1) were assessed using tissues from the cohort used in chapter 6, in order that samples of immune tissues from the current cohort could be reserved for histological and gene expression assessment as part of a collaboration with researchers at the University of Utrecht, The Netherlands.

Table 7.1: Immune organ size and cellularity in pregnant rats fed experimental diets (n=6 per group)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
SPLEEN							
Weight (g)	0.52 ± 0.11 [a]	0.73 ± 0.08	0.71 ± 0.07	0.86 ± 0.13 [b]	0.65 ± 0.12	0.75 ± 0.19 [b]	**0.002**
% body weight	0.19 ± 0.02 [a]	0.24 ± 0.03	0.22 ± 0.01	0.27 ± 0.02 [b]	0.21 ± 0.04	0.23 ± 0.04	**0.003**
Total mononuclear cells (x 10^{-6})	44.3 ± 46.5	52.9 ± 42.8	47.37 ± 30.56	113.39 ± 43.03	33.20 ± 33.44	70.85 ± 52.32	0.042 †
Mononuclear cells per mg (x 10^{-3})	75.52 ± 68.85	75.10 ± 70.36	81.66 ± 35.16	137.86 ± 67.47	54.85 ± 43.69	97.18 ± 60.71	0.274
THYMUS							
Weight (g)	0.30 ± 0.07	0.37 ± 0.04	0.34 ± 0.16	0.40 ± 0.08	0.35 ± 0.06	0.41 ± 0.07	0.317
% body weight	0.11 ± 0.02	0.12 ± 0.01	0.11 ± 0.05	0.13 ± 0.02	0.12 ± 0.02	0.13 ± 0.01	0.787
Total mononuclear cells (x 10^{-6})	63.96 ± 111.24	69.51 ± 39.30	61.25 ± 36.01	72.01 ± 75.04	38.75 ± 50.74	62.13 ± 96.23	0.977
Mononuclear cells per mg (x 10^{-3})	182.41 ± 291.57	183.50 ± 89.63	422.10 ± 657.93	186.63 ± 186.19	109.17 ± 134.73	150.37 ± 209.70	0.627
LYMPH NODES							
Weight (g)	0.11 ± 0.05	0.09 ± 0.02	0.11 ± 0.01	0.11 ± 0.04	0.08 ± 0.03	0.11 ± 0.05	0.626
% body weight	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.444
Total mononuclear cells (x 10^{-6})	1.14 ± 0.60	3.02 ± 3.15	1.69 ± 0.41	2.62 ± 1.18	0.96 ± 0.73	3.31 ± 3.15	0.190
Mononuclear cells per mg(x 10^{-3})	10.33 ± 4.65	28.71 ± 21.60	15.84 ± 4.90	25.28 ± 14.09	12.07 ± 8.21	27.13 ± 24.24	0.158

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts
† no significant differences between groups when Bonferroni applied

Maternal diet was found to have a significant effect upon maternal spleen weight (both absolute and relative); with dams on the HF salmon oil and HF beef tallow diets having significantly higher spleen weights that those on the LF soyabean oil diet. This higher spleen weight was associated with higher number of total mononuclear cells recovered from the maternal spleen, though the proportion of mononuclear cells per mg of immune organ tissue was not significantly affected by maternal diet during pregnancy. The reductions in maternal mandibular lymph node cellularity which were observed to occur during pregnancy (see table 5.3), were not significantly affected by the type of fatty acids within the maternal diet during pregnancy.

7.3.2 Maternal immune cell phenotyping by flow cytometry

Maternal immune tissues were analysed by flow cytometry to determine whether dietary fatty acids during pregnancy could alter the relative proportions of immune cells.

There was no significant effect of maternal dietary fatty acids upon the percentage content of total T cells, T helper cells, cytotoxic T cells, NK cells, B cells or macrophages within mononuclear cells isolated from the maternal spleen (see table 7.2). This indicates that the significant effect of diet observed upon spleen size was not the result of an effect upon a particular lymphocyte subset, as the cell types identified maintain their relative contribution to tissue content. There was a significant effect of maternal diet upon the mean fluorescence intensity (MFI) of cytotoxic T cell surface markers, with dams on the HF linseed oil diet (ALNA rich) demonstrating the highest expression of both CD3 and CD8 on their cell surface. This higher expression of these cell surface markers suggests that the sensitivity of cytotoxic T cells to antigen presentation may be greatest in the HF linseed oil group. Effects of n-3 fatty acids upon CD8 expression have been observed upon murine splenocytes, where the expression of some CD8 epitopes was found to be higher when diets containing fish oil are provided(252).

The relative proportions of total T cells, T helper cells, cytotoxic T cells, $CD161^{+}$ cells ("NK" cells) or B cells within mononuclear cells isolated from the maternal thymus were not affected by maternal dietary fatty acids during pregnancy (see table 7.3). There was a significant effect of maternal diet upon the expression of CD8 on cytotoxic T cells, with the HF linseed oil diet group again demonstrating the highest mean expression of this cell surface marker.

The relative proportions of total T cells, T helper cells, cytotoxic T cells, NK cells or B cells within maternal lysed whole blood were not affected by maternal diet during pregnancy (see table 7.4). There was a significant effect of maternal dietary fatty acids upon the number of $CD8^+$ cells. It was observed in an earlier study that pregnant animals at day 20 gestation have higher proportions of $CD8^+$ PBMC when compared to mid gestation females (See chapter 5, table 5.4). The data from the current cohort indicates that maternal diet exerts a significant effect upon $CD8^+$ cells within maternal PBMC, so it is possible that maternal diet during pregnancy interacts with this adaptation to pregnancy.

Mononuclear cells isolated from maternal lymph nodes demonstrated significant effects of maternal dietary fatty acids during pregnancy upon the relative proportions of cytotoxic T cells and T helper cells (see table 7.5). There was a trend for the n-3 PUFA rich diets (HF linseed and HF salmon oil) to have the highest proportion of cytotoxic T cells. The LF soyabean oil diet had a lower proportion of T helper cells than the HF dietary groups. An effect of maternal diet was again observed upon the expression of cell surface markers upon cytotoxic T cells, with the expression of CD3 and CD8 on cytotoxic T cells highest in the HF salmon oil diet group (LC n-3 PUFA rich).

Pregnancy was demonstrated in an earlier study (see chapter 5) to lead to significantly lower expression of CD161 expression on NK cells within lysed whole blood and mononuclear cells isolated from the maternal spleen (see table 5.4 and 5.5). No significant effect of maternal dietary fatty acids was observed upon the expression of this cell surface marker on maternal NK cells in this cohort, indicating that maternal diet does not interact with this adaptation to pregnancy in the Wistar rat.

Table 7.2: Immune cell phenotypes identified within the spleen of pregnant rats fed experimental diets
(% within lymphocyte gate, MFI, mean ± standard deviation)

	LF Soyabean oil (n = 6)	HF Soyabean oil (n = 5)	HF Linseed oil (n = 5)	HF Salmon oil (n = 5)	HF Sunflower oil (n = 6)	HF Beef tallow (n = 6)	ANOVA p value
$CD3^+$ (%)	52.8 ± 9.7	56.7 ± 10.6	63.2 ± 10.2	60.5 ± 10.0	59.6 ± 8.9	62.3 ± 6.7	0.453
$CD8^+$ (%)	28.9 ± 5.8	26.8 ± 6.7	32.3 ± 6.4	27.9 ± 6.5	26.7 ± 2.9	30.0 ± 5.7	0.613
$CD4^+$ (%)	49.0 ± 6.7	47.8 ± 9.1	50.2 ± 4.0	47.3 ± 7.2	52.6 ± 10.9	49.8 ± 6.7	0.893
$CD3^+CD8^+$ (%)							
(Cytotoxic T cells)	14.5 ± 4.6	14.5 ± 2.8	17.5 ± 3.7	17.3 ± 3.3	14.7 ± 1.6	18.9 ± 6.9	0.369
mean CD3 intensity	21.2 ± 2.9 [b]	29.4 ± 4.1	31.7 ± 6.4 [a]	30.0 ± 10.4	24.2 ± 3.0	20.2 ± 2.7 [b]	**0.004**
mean CD8 intensity	25.2 ± 5.5 [b]	37.6 ± 8.0	42.9 ± 12.9 [a]	40.2 ± 9.7	27.9 ± 3.4	25.5 ± 7.2 [b]	**0.002**
$CD3^+CD4^+$ (%)							
(T helper cells)	39.8 ± 7.4	42.7 ± 8.2	45.2 ± 5.5	42.6 ± 7.6	45.8 ± 10.3	43.3 ± 5.6	0.805
mean CD3 intensity	33.4 ± 6.9	34.0 ± 7.4	40.3 ± 10.8	40.0 ± 6.7	40.7 ± 6.8	34.7 ± 5.0	0.316
mean CD4 intensity	439.1 ± 10.2	472.9 ± 28.0	470.0 ± 25.7	472.6 ± 37.5	465.3 ± 24.7	446.6 ± 39.1	0.245
$CD161^+$ (%)							
(NK cells)	13.1 ± 4.0	16.6 ± 11.1	10.9 ± 2.1	11.7 ± 4.0	12.1 ± 2.3	11.8 ± 3.3	0.581
mean CD161 intensity	69.2 ± 25.3	54.5 ± 8.0	52.8 ± 17.8	49.8 ± 6.3	67.2 ± 20.4	53.9 ± 16.5	0.323
$CD3^-CD45^+$ (%)							
(B cells)	34.0 ± 7.4	32.4 ± 10.7	23.2 ± 14.4	25.6 ± 10.2	28.3 ± 10.8	27.7 ± 8.1	0.562
mean CD45 intensity	125.2 ± 22.0	118.5 ± 14.1	138.9 ± 27.4	150.3 ± 45.6	118.8 ± 30.7	145.0 ± 29.5	0.353
Macrophages (%)	26.7 ± 7.0	23.1 ± 8.1	19.3 ± 13.4	24.4 ± 2.9	22.4 ± 5.3	23.9 ± 7.3	0.763
mean CD163 intensity	42.0 ± 11.2	31.6 ± 4.1	44.8 ± 15.6	37.4 ± 8.4	41.5 ± 10.6	43.8 ± 7.6	0.337

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 7.3: Immune cell phenotypes identified within the thymus of pregnant rats fed experimental diets
(% total cells, MFI, mean ± standard deviation)

	LF Soyabean oil (n = 6)	HF Soyabean oil (n = 5)	HF Linseed oil (n = 5)	HF Salmon oil (n = 5)	HF Sunflower oil (n = 6)	HF Beef tallow (n = 6)	ANOVA p value
$CD3^+$ (%)	30.7 ± 6.9	34.1 ± 10.2	31.0 ± 4.7	32.0 ± 6.2	33.4 ± 7.7	31.4 ± 5.9	0.956
$CD8^+$ (%)	72.0 ± 4.6	73.2 ± 6.7	78.7 ± 6.1	75.2 ± 1.1	70.7 ± 8.1	76.5 ± 3.7	0.205
$CD4^+$ (%)	84.4 ± 2.3	83.9 ± 3.6	83.7 ± 4.4	84.5 ± 2.4	82.3 ± 5.9	85.2 ± 3.2	0.865
$CD3^+CD8^+$ (%)							
(Cytotoxic T cells)	12.6 ± 2.3	16.2 ± 3.3	17.9 ± 4.7	16.5 ± 5.0	14.4 ± 4.0	14.7 ± 3.3	0.286
mean CD3 intensity	46.6 ± 2.9	62.9 ± 20.8	56.6 ± 14.0	63.4 ± 15.0	53.2 ± 13.7	46.3 ± 6.4	0.140
mean CD8 intensity	37.9 ± 5.8	60.0 ± 28.2	67.4 ± 33.4	61.7 ± 15.3	39.0 ± 6.1	41.1 ± 8.3	0.038 †
$CD3^+CD4^+$ (%)							
(T helper cells)	28.1 ± 6.5	29.9 ± 8.8	27.4 ± 4.0	28.7 ± 4.9	29.4 ± 5.5	28.2 ± 3.6	0.983
mean CD3 intensity	54.8 ± 9.9	53.0 ± 16.6	59.6 ± 9.9	62.8 ± 3.7	57.9 ± 9.3	58.4 ± 4.5	0.663
mean CD4 intensity	348.4 ± 53.0	365.7 ± 16.6	419.4 ± 82.9	378.6 ± 34.9	339.1 ± 22.7	367.9 ± 45.1	0.128
$CD161^+$ (%)							
(NK cells)†	5.5 ± 2.2	4.9 ± 2.2	4.9 ± 2.2	5.6 ± 1.4	6.6 ± 2.2	4.5 ± 1.9	0.612
mean CD161 intensity	79.2 ± 13.5	78.7 ± 15.5	96.9 ± 32.5	84.7 ± 22.8	82.3 ± 23.4	94.8 ± 16.6	0.598
$CD3^-CD45^+$ (%)							
(B cells)	2.7 ± 0.9	2.2 ± 0.7	2.0 ± 0.6	2.4 ± 0.4	3.3 ± 1.1	2.5 ± 1.7	0.404
mean CD45 intensity	82.1 ± 13.3	93.3 ± 24.8	102.1 ± 44.1	88.2 ± 9.1	102.3 ± 29.2	98.6 ± 29.6	0.771

† no significant differences between groups when Bonferroni applied

†These are not likely to be NK cells, but to be another cell type bearing CD161 on the surface

Table 7.4: Immune cell phenotypes identified within in the peripheral blood of pregnant rats fed experimental diets (% within lymphocyte gate, MFI, mean ± standard deviation)

	LF Soyabean oil (n = 6)	HF Soyabean oil (n = 5)	HF Linseed oil (n = 4)	HF Salmon oil (n = 3)	HF Sunflower oil (n = 5)	HF Beef tallow (n = 6)	ANOVA p value
$CD3^+$ (%)	45.5 ± 7.0	46.3 ± 5.1	51.8 ± 11.5	45.1 ± 9.9	43.7 ± 10.4	48.0 ± 10.5	0.832
$CD8^+$ (%)	33.1 ± 3.5	26.5 ± 3.6	28.1 ± 5.4	29.6 ± 2.1	32.0 ± 1.9	33.5 ± 4.1	0.032 †
$CD4^+$ (%)	45.1 ± 7.2	50.6 ± 4.3	51.3 ± 5.4	51.0 ± 6.2	50.3 ± 6.1	50.1 ± 8.4	0.643
$CD3^+CD8^+$ (%) (Cytotoxic T cells)	14.2 ± 2.0	12.6 ± 1.5	15.2 ± 4.3	14.3 ± 4.2	13.9 ± 4.4	17.9 ± 4.3	0.303
mean CD3 intensity	33.4 ± 5.8	34.8 ± 15.7	40.3 ± 19.8	33.3 ± 17.3	29.9 ± 7.3	27.5 ± 4.5	0.706
mean CD8 intensity	54.3 ± 14.9	72.4 ± 23.7	70.8 ± 7.1	65.6 ± 16.6	50.2 ± 10.9	49.6 ± 15.9	0.116
$CD3^+CD4^+$ (%) (T helper cells)	31.1 ± 6.7	34.5 ± 4.0	38.4 ± 8.2	31.8 ± 7.8	31.1 ± 9.9	32.3 ± 7.1	0.680
mean CD3 intensity	41.2 ± 9.8	27.4 ± 14.6	43.1 ± 10.7	40.4 ± 8.5	39.6 ± 9.5	42.9 ± 9.5	0.220
mean CD4 intensity	590.7 ± 28.0	631.0 ± 44.0	624.8 ± 32.8	614.6 ± 40.0	612.2 ± 40.0	610.7 ± 37.7	0.527
$CD161^+$ (%) (NK cells)	21.4 ± 2.7	18.1 ± 5.3	16.8 ± 3.2	24.9 ± 4.5	21.8 ± 5.6	22.3 ± 4.3	0.147
mean CD161 intensity	247.9 ± 37.9	257.7 ± 66.3	273.2 ± 64.2	251.3 ± 68.3	222.8 ± 42.5	238.5 ± 81.7	0.873
$CD3^-CD45^+$ (%) (B cells)	23.3 ± 6.4	21.4 ± 5.1	17.2 ± 3.4	19.4 ± 5.8	19.7 ± 4.4	18.2 ± 6.4	0.550
mean CD45 intensity	292.6 ± 35.5	273.3 ± 87.1	331.2 ± 48.4	313.6 ± 83.6	298.5 ± 35.1	245.6 ± 51.4	0.285

† no significant differences between groups when Bonferroni applied

Table 7.5: Immune cell phenotypes identified within the mandibular lymph nodes of pregnant rats fed experimental diets (% within lymphocyte gate, MFI, mean ± standard deviation)

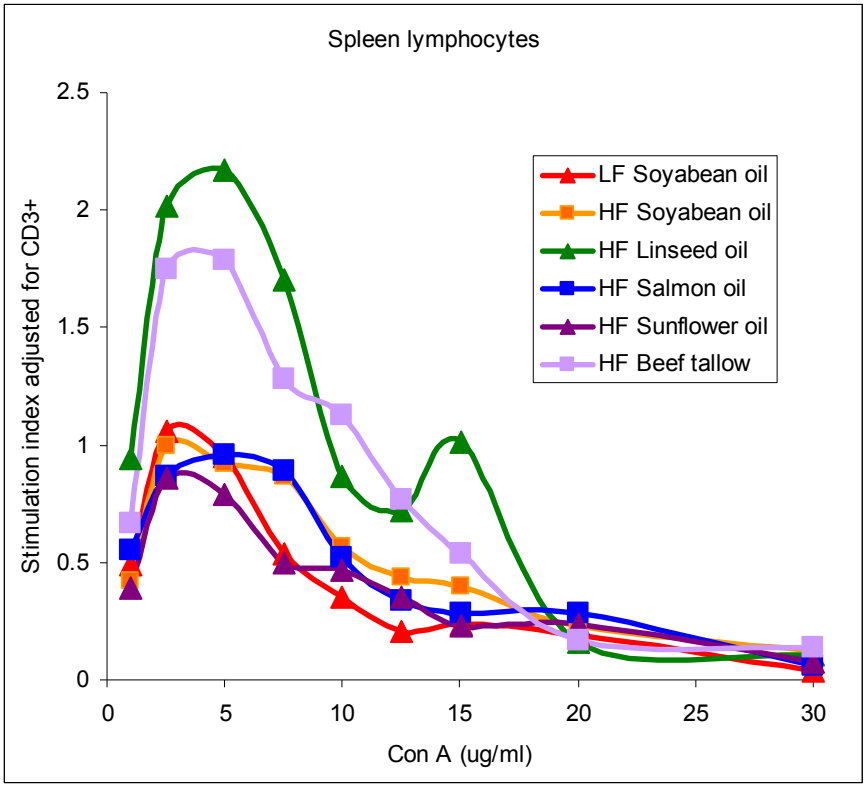
	LF Soyabean oil (n = 6)	HF Soyabean oil (n = 2)	HF Linseed oil (n = 3)	HF Salmon oil (n = 5)	HF Sunflower oil (n = 3)	HF Beef tallow (n = 4)	ANOVA p value
$CD3^+$ (%)	52.9 ± 10.1	71.5 ± 22.0	69.8 ± 10.7	69.0 ± 7.1	64.1 ± 4.0	57.5 ± 2.9	0.054
$CD8^+$ (%)	13.8 ± 2.7	20.6 ± 6.8	21.7 ± 5.1	22.5 ± 5.8	17.1 ± 4.9	17.1 ± 1.6	0.050
$CD4^+$ (%)	56.3 ± 2.7	63.7 ± 11.0	61.2 ± 4.9	58.4 ± 8.5	65.0 ± 4.2	56.3 ± 2.7	0.363
$CD3^+CD8^+$ (%) (Cytotoxic T cells)	9.5 ± 3.6	16.3 ± 7.6	18.5 ± 6.1	18.0 ± 4.2	11.4 ± 3.8	12.0 ± 2.0	0.028 †
mean CD3 intensity	15.6 ± 1.9 [b]	23.1 ± 2.8	18.7 ± 3.1	30.4 ± 11.2 [a]	19.0 ± 4.7	15.7 ± 1.8 [b]	**0.011**
mean CD8 intensity	17.3 ± 0.7 [b]	28.3 ± 5.1	24.5 ± 8.0	36.1 ± 7.6 [a]	20.5 ± 4.0 [b]	19.9 ± 1.4 [b]	**< 0.001**
$CD3^+CD4^+$ (%) (T helper cells)	43.8 ± 6.6	56.9 ± 14.3	54.7 ± 6.2	53.8 ± 4.7	54.6 ± 2.6	47.3 ± 3.3	0.044 †
mean CD3 intensity	24.6 ± 4.8	27.2 ± 15.8	29.5 ± 12.0	33.9 ± 9.7	29.4 ± 6.6	31.2 ± 7.5	0.634
mean CD4 intensity	399.4 ± 49.0	422.8 ± 163.5	457.5 ± 77.0	439.9 ± 43.3	426.9 ± 31.4	453.2 ± 35.2	0.725
$CD161^+$ (%) (NK cells)	8.8 ± 2.8	7.3 ± 1.7	8.6 ± 1.6	8.8 ± 2.4	8.2 ± 0.5	8.7 ± 3.8	0.981
mean CD161 intensity	41.5 ± 8.0	55.3 ± 1.3	32.1 ± 23.0	53.9 ± 16.6	51.3 ± 16.6	36.9 ± 2.1	0.143
$CD3^-CD45^+$ (%) (B cells)	32.2 ± 5.6	21.7 ± 21.7	23.4 ± 10.8	23.4 ± 6.2	24.6 ± 6.7	32.5 ± 5.1	0.303
mean CD45 intensity	102.2 ± 18.7	128.8 ± 8.0	116.6 ± 45.7	145.3 ± 12.4	119.0 ± 22.7	142.1 ± 51.3	0.253

† no significant differences between groups when Bonferroni applied

7.3.3 Maternal lymphocyte proliferation

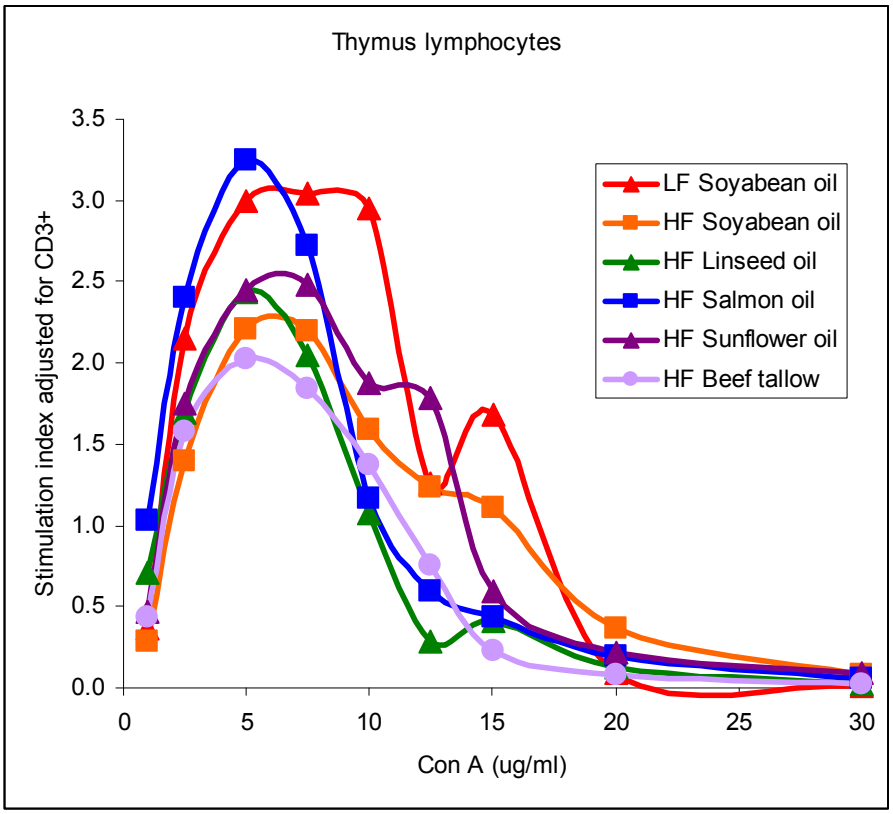
Lymphocyte proliferation in response to Con A (stimulation index, adjusted for $CD3^+$ cells) was assessed in mononuclear cell suspensions derived from the maternal spleen and thymus. Proliferative responses were assessed by calculation of area under the curve (AUC) of the Con A dose response (see figures 7.1 and 7.2, table 7.6). There was little indication of any significant effect of maternal dietary fatty acids upon maternal lymphocyte proliferation.

Figure 7.1: Mean proliferation of spleen lymphocytes from pregnant rats fed different diets (stimulation index, adjusted for $CD3^+$ cell numbers)



Error bars omitted for clarity

Figure 7.2: Mean proliferation of thymic lymphocytes from pregnant rats fed different diets (stimulation index, adjusted for $CD3^+$ cell numbers)



Error bars omitted for clarity

Table 7.6: Proliferative responses of lymphocytes from pregnant rats fed different diets
(AUC, adjusted for $CD3^+$ cell numbers, mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef Tallow	ANOVA p value
Spleen	(n=3) 10.1 ± 4.0	(n=4) 13.1 ± 5.8	(n=3) 24.0 ± 23.9	(n=4) 12.4 ± 7.5	(n=4) 10.2 ± 3.6	(n=6) 20.7 ± 11.3	0.401
Thymus	(n=5) 37.2 ± 26.8	(n=4) 28.4 ± 13.3	(n=5) 21.2 ± 6.1	(n=5) 28.3 ± 21.6	(n=3) 30.2 ± 17.6	(n=6) 20.4 ± 8.9	0.642

7.3.4 Maternal production of immune signalling molecules

PGE_2

PGE_2 is a mediator produced by monocytes and macrophages from AA. Maternal diet altered PGE_2 production by maternal spleen cells, with the diets containing the most n-3 PUFA (HF soyabean, linseed and salmon oil) having the lowest PGE_2 production. No significant effect of maternal dietary fatty acids during pregnancy was observed upon PGE_2 concentrations in maternal plasma, or PGE_2 produced by maternal PBMC (see table 7.7).

IFN-γ

IFN-γ is a Th1-type cytokine produced by T helper cells, cytotoxic T cells and NK cells. Data suggests that maternal dietary fatty acids during pregnancy can alter IFN-γ production by maternal spleen cells, with dams on the HF Beef tallow diet having the highest IFN-γ production by spleen lymphocytes (both stimulated with Con A and unstimulated). No significant effect of maternal diet was observed upon IFN-γ production by the maternal thymus or PBMC (see table 7.8).

IL-4

IL-4 is a Th-2 type cytokine produced by T helper cells, cytotoxic T cells and NK cells. No significant effect of maternal dietary fatty acids during pregnancy was observed upon IL-4 production by cells from the maternal spleen, thymus or blood (see table 7.9).

Th1:Th2 ratio

An increased maternal Th1:Th2 ratio had been observed within thymocytes of pregnant rats when compared to virgin rats (see chapter 5, table 5.12). No significant effect of maternal dietary fatty acids during pregnancy was observed upon the Th1 to Th2 ratio of cytokines produced by cells from the maternal spleen, thymus or blood (see table 7.10).

Table 7.7: PGE_2 concentration in plasma and PGE_2 production by spleen and peripheral blood lymphocytes from pregnant rats fed experimental diets (ng/ml; mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
Plasma	(n=6)	(n=6)	(n=6)	(n=6)	(n=6)	(n=6)	
	33.3 ± 32.0	27.6 ± 19.5	122.5 ± 143.5	34.6 ± 21.1	25.2 ± 6.4	30.7 ± 23.4	0.077
Spleen	(n=5)	(n=5)	(n=6)	(n=6)	(n=6)	(n=6)	
No Con A	2.2 ± 1.1	1.4 ± 1.0	0.6 ± 0.3	0.9 ± 0.5	1.6 ± 0.6	2.8 ± 2.8	0.104
5 μg/ml Con A	2.5 ± 0.4	0.5 ± 0.6	0.7 ± 0.5	0.9 ± 0.4	1.9 ± 0.9	2.9 ± 2.8	0.043 †
Mean SI	1.3 ± 0.7	0.4 ± 0.5	1.1 ± 0.9	1.0 ± 0.4	1.4 ± 0.9	1.2 ± 0.5	0.327
PBMC	(n=5)	(n=4)	(n=6)	(n=4)	(n=6)	(n=4)	
No Con A	3.4 ± 2.7	24.1 ± 43.9	1.5 ± 0.8	21.7 ± 37.6	35.8 ± 79.5	3.7 ± 3.3	0.704
5 μg/ml Con A	11.3 ± 5.9	44.9 ± 73.7	3.7 ± 4.2	23.3 ± 40.3	37.0 ± 71.0	9.8 ± 7.0	0.709
Mean SI	4.5 ± 3.3	1.2 ± 0.2	2.3 ± 1.8	1.3 ± 0.6	2.9 ± 2.3	3.9 ± 3.4	0.304

SI stimulation index
† no significant differences between groups when Bonferroni applied

Table 7.8: IFN-γ production by spleen, thymus and peripheral blood lymphocytes from pregnant rats fed experimental diets (mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
Spleen	(n=5)	(n=6)	(n=6)	(n=5)	(n=6)	(n=6)	
No Con A (pg/ml)	ND	4.4 ± 3.8	3.0 ± 2.8	31.6 ± 62.9	34.5 ± 23.1	50.4 ± 25.3	0.020 †
5 μg/ml Con A (ng/ml)	38.7 ± 30.9	9.7 ± 10.1 [b]	14.5 ± 12.6 [b]	44.3 ± 25.7	23.9 ± 15.6 [b]	80.7 ± 47.8 [a]	**0.006**
Mean SI (x 10^{-3})	38.7 ± 30.9	7.0 ± 11.4	10.1 ± 15.5	35.8 ± 32.9	0.8 ± 0.3	1.8 ± 1.1	0.010 †
Thymus	(n=5)	(n=5)	(n=6)	(n=6)	(n=6)	(n=6)	
No Con A (pg/ml)	39.5 ± 54.0	15.5 ± 26.4	3.5 ± 3.0	48.5 ± 94.7	7.1 ± 7.3	1.6 ± 2.4	0.393
5 μg/ml Con A (ng/ml)	15.1 ± 8.0	7.1 ± 6.2	7.3 ± 4.7	28.9 ± 21.5	24.3 ± 18.3	27.3 ± 35.4	0.421
Mean SI (x 10^{-3})	5.2 ± 4.6	1.5 ± 1.2	3.5 ± 4.6	8.0 ± 13.4	6.2 ± 5.1	12.7 ± 8.1	0.272
PBMC	(n=5)	(n=4)	(n=4)	(n=5)	(n=6)	(n=4)	
No Con A (pg/ml)	47.3 ± 31.4	85.0 ± 126.5	23.7 ± 38.0	115.6 ± 163.5	50.9 ± 109.5	246.4 ± 432.1	0.581
5 μg/ml Con A (ng/ml)	26.1 ± 25.3	2.5 ± 2.2	12.0 ± 13.9	6.9 ± 6.4	8.8 ± 8.7	16.2 ± 3.1	0.207
Mean SI (x 10^{-2})	5.0 ± 2.6	1.1 ± 0.6	3.8 ± 1.5	37.1 ± 56.6	49.8 ± 77.3	45.1 ± 84.1	0.692

SI stimulation index
† no significant differences between groups when Bonferroni applied
a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

Table 7.9: IL-4 production by spleen, thymus and peripheral blood lymphocytes from pregnant rats fed experimental diets (mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
Spleen	(n=5)	(n=6)	(n=6)	(n=6)	(n=6)	(n=6)	
No Con A (pg/ml)	11.9 ± 9.1	17.3 ± 7.1	17.9 ± 10.0	17.0 ± 8.6	21.1 ± 3.8	22.2 ± 10.3	0.430
5 μg/ml Con A (ng/ml)	7.5 ± 5.3	14.8 ± 5.2	21.8 ± 12.8	14.4 ± 12.4	18.5 ± 13.2	19.2 ± 13.4	0.463
Thymus	(n=5)	(n=5)	(n=6)	(n=6)	(n=6)	(n=6)	
No Con A (pg/ml)	16.3 ± 12.9	17.8 ± 7.9	14.5 ± 11.5	13.1 ± 11.7	13.1 ± 5.1	20.0 ± 6.4	0.784
5 μg/ml Con A (ng/ml)	6.7 ± 9.0	14.5 ± 12.7	12.6 ± 8.9	14.3 ± 11.8	18.1 ± 11.6	15.6 ± 8.2	0.621
PBMC	(n=5)	(n=4)	(n=4)	(n=5)	(n=6)	(n=4)	
No Con A (pg/ml)	6.6 ± 3.2	7.9 ± 13.2	13.3 ± 6.5	11.0 ± 15.1	9.7 ± 9.7	10.0 ± 5.5	0.937
5 μg/ml Con A (ng/ml)	5.3 ± 4.6	18.8 ± 9.1	14.6 ± 9.8	17.4 ± 18.1	11.9 ± 9.8	9.0 ± 3.5	0.425

Table 7.10: IFNγ:IL-4 ratios produced by Con A stimulated spleen, thymus and peripheral blood lymphocytes from virgin and pregnant rats (mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
	(n=5)	(n=4)	(n=4)	(n=5)	(n=6)	(n=6)	
Spleen IFNγ:IL-4 x 10^{-3}	6.1 ± 4.0	1.0 ± 1.3	0.9 ± 0.9	9.1 ± 10.2	35.5 ± 83.6	6.0 ± 4.0	0.675
	(n=3)	(n=3)	(n=3)	(n=4)	(n=4)	(n=6)	
Thymus IFNγ:IL-4 x 10^{-3}	5.4 ± 7.0	0.3 ± 0.2	0.3 ± 0.2	2.7 ± 2.2	1.3 ± 1.2	2.1 ± 2.5	0.312
	(n=4)	(n=3)	(n=2)	(n=3)	(n=5)	(n=4)	
Peripheral blood IFNγ:IL-4 x 10^{-3}	9.5 ± 10.7	0.2 ± 0.3	1.5 ± 2.0	0.2 ± 0.2	0.7 ± 0.9	2.2 ± 1.5	0.128

7.3.5 Fetal thymus weight and cellularity

The effects of maternal diet during pregnancy upon fetal thymus weight and cellularity were assessed in tissues collected from the cohort used in chapter 6, in order that samples of immune tissues from this cohort could be reserved for histological and gene expression assessment as part of a collaboration with researchers at the University of Utrecht, The Netherlands.

No significant effects of maternal diet during pregnancy were observed upon fetal thymus weight or cellularity (see table 7.11). The contribution of the fetal thymus towards body weight and its cellularity were comparable to those observed among adult virgin females (virgin female thymus: 0.21 ± 0.04% total body weight, 47.0 x 10^4 cells per mg, see table 5.3).

7.3.6 Fetal immune cell phenotyping by flow cytometry

Mononuclear cells isolated from the fetal thymus were analysed to determine whether maternal dietary fatty acids during pregnancy could alter the proportions of the cell types identified. No significant effects of maternal diet were observed upon the proportion of total T cells, cytotoxic T cells or T helper cells within the fetal thymus. Maternal diet significantly affected the proportion of $CD161^+$ cells ("NK" cells). The HF linseed oil group had the highest proportion of these cells, and the HF sunflower oil group the lowest (see table 7.12). This suggests that the balance of n-3 and n-6 fatty acids in the maternal diet has the capacity to influence the lineage commitment and differentiation of progenitor cells within the thymus.

The expression of CD3 and CD8 upon the cell surface of cytotoxic T cells within the fetal thymus was significantly affected by maternal dietary fatty acids during pregnancy. The HF salmon oil group had a significantly higher expression of CD3 than any other dietary group. Expression of CD8 tended to be highest among the n-3 PUFA rich diet groups (HF linseed and HF salmon oil).

Table 7.11: Fetal thymus weight and cellularity (mean ± standard deviation, n=6 per group)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
Thymus weight (mg/pup)	3.52 + 1.61	4.56 + 0.82	4.62 + 1.50	3.73 + 0.78	4.29 + 1.49	4.02 + 0.89	0.565
Thymus weight (% body weight)	0.15 + 0.06	0.19 + 0.04	0.20 + 0.04	0.18 + 0.04	0.18 + 0.03	0.19 + 0.05	0.598
Total mononuclear cells from whole litter (x 10^{-6})	19.25 ± 6.53	15.65 ± 7.15	16.07 ± 10.29	17.42 ± 6.06	23.77 ± 15.43	17.42 ± 8.93	0.718
Mononuclear cells per mg thymus (x 10^{-4})	97.46 ± 153.33	32.19 ± 19.30	36.28 ± 35.29	37.21 ± 21.02	47.45 ± 18.71	33.90 ± 19.33	0.518

Table 7.12: Immune cell phenotypes identified within the fetal thymus (% within lymphocyte gate, MFI, mean ± standard deviation)

	LF Soyabean oil (n=5)	HF Soyabean oil (n=5)	HF Linseed oil (n=5)	HF Salmon oil (n=5)	HF Sunflower oil (n=6)	HF Beef tallow (n=6)	ANOVA p value
$CD3^+$ (%)	15.5 ± 5.5	16.3 ± 2.8	16.5 ± 4.3	15.1 ± 3.0	13.7 ± 5.4	17.5 ± 5.1	0.789
$CD8^+$ (%)	77.4 ± 9.3	84.8 ± 7.9	84.5 ± 8.3	86.8 ± 2.8	84.1 ± 4.5	73.1 ± 13.3	0.089
$CD4^+$ (%)	82.4 ± 2.4	80.4 ± 7.5	75.7 ± 7.0	78.5 ± 3.8	80.7 ± 5.4	76.7 ± 5.4	0.371
$CD3^+CD8^+$ (%)							
(Cytotoxic T cells)	10.6 ± 4.6	14.8 ± 3.4	16.2 ± 4.3	14.1 ± 2.8	10.9 ± 5.2	11.7 ± 5.1	0.247
mean CD3 intensity	46.5 ± 8.4	54.9 ± 12.7	50.4 ± 10.6	78.1 ± 9.9 *	55.7 ± 12.5	46.3 ± 11.6	**0.001**
mean CD8 intensity	146.2 ± 73.6	198.0 ± 99.0	283.7 ± 80.4	257.0 ± 64.7	164.3 ± 35.8	167.0 ± 93.4	0.042 †
$CD3^+CD4^+$ (%)							
(T helper cells)	16.7 ± 7.1	14.6 ± 3.7	15.3 ± 5.2	15.2 ± 3.5	14.8 ± 5.9	18.5 ± 6.0	0.816
mean CD3 intensity	58.0 ± 11.9	70.4 ± 30.1	70.9 ± 18.0	76.1 ± 25.5	67.1 ± 34.4	49.1 ± 8.0	0.444
mean CD4 intensity	477.4 ± 74.9	477.6 ± 120.1	739.5 ± 210.8	664.7 ± 298.0	499.3 ± 109.3	522.6 ± 144.8	0.097
$CD161^+$ (%)							
(NK cells) †	5.5 ± 1.5	5.3 ± 2.7	9.8 ± 2.7 [a]	7.7 ± 2.4	5.1 ± 2.1 [b]	6.6 ± 2.4	**0.024**
mean CD161 intensity	147.0 ± 53.7	133.9 ± 13.3	101.9 ± 15.7	146.1 ± 32.4	149.5 ± 42.3	139.0 ± 19.1	0.225

† no significant differences between groups when Bonferroni applied

a, b groups which differ significantly from each other (Bonferroni $p < 0.05$) have different superscripts

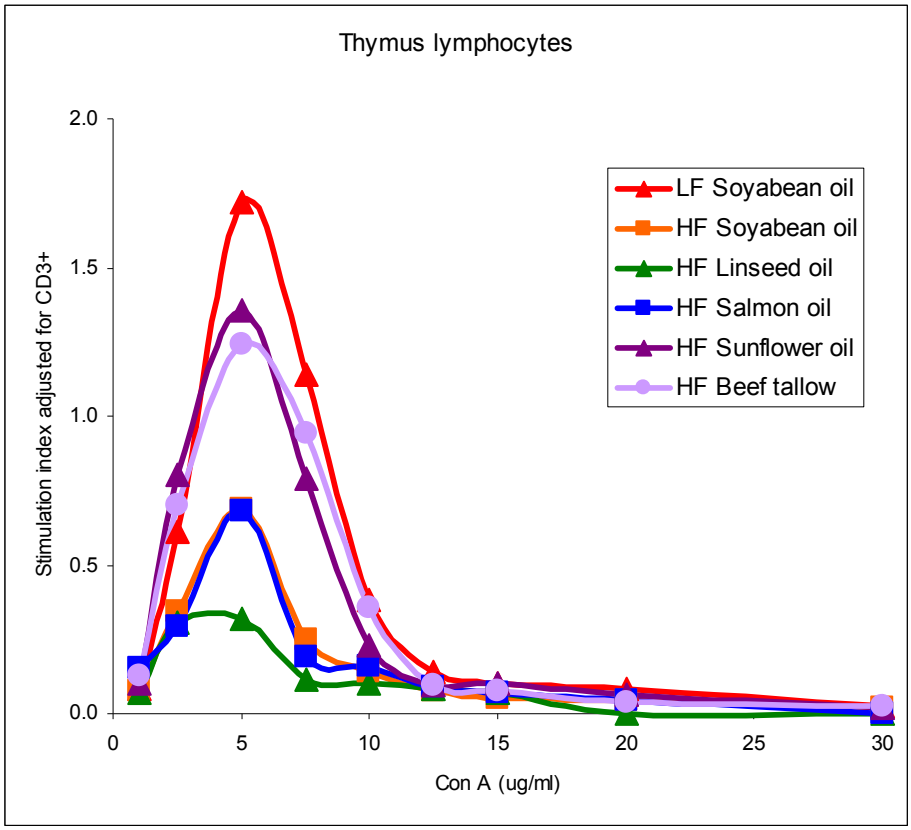
* significantly different from all other groups (Bonferroni $p < 0.05$)

†These are not likely to be NK cells, but to be another cell type bearing CD161 on the surface

7.3.7 Fetal thymocyte proliferation

Proliferation of mononuclear cell isolated from the fetal thymus in response to Con A (stimulation index, adjusted for $CD3^+$ cells) was assessed for the effect of maternal dietary fatty acids during pregnancy by analysis of area under the curve. There was a significant effect of maternal diet during pregnancy upon fetal thymus proliferation, with the n-3 PUFA rich dietary groups (HF soyabean, linseed and salmon oil) tending to have the lowest proliferative responses (see figure 7.3, table 7.13). It should be noted that the data available for fetal thymus proliferation was severely limited by small sample size, due to difficulties in obtaining sufficient cell numbers to perform the analysis.

Figure 7.3: Mean proliferation of fetal thymic lymphocytes (stimulation index, adjusted for $CD3^+$ cell numbers)



Error bars omitted for clarity

Table 7.13: Proliferative responses of fetal thymic lymphocytes (AUC*, adjusted for $CD3^+$ cell numbers, mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef Tallow	ANOVA p value
Fetal thymus	(n=2) 7.0 ± 1.1	(n=2) 2.6 ± 0.8	(n=2) 1.6 ± 0.8	(n=1) 2.63	(n=2) 6.1 ± 0.5	(n=2) 5.8 ± 2.3	**0.004**

* AUC calculated using data points from 1-7.5µg/ml ConA.
NB - Bonferroni not possible as n = 1 in HF salmon oil group.

7.3.8 Fetal production of immune signalling molecules

PGE_2

There was a significant effect of maternal dietary fatty acids upon PGE_2 production by fetal PBMC, with production of PGE_2 being highest in the HF soyabean and HF beef tallow dietary groups. No significant effect of maternal diet was observed upon PGE_2 concentrations in fetal plasma (see table 7.14).

IFN-γ

Fetal immune cells were less responsive to Con A stimulation than was observed in maternal immune cells. No significant effect of maternal dietary fatty acids during pregnancy was observed upon fetal IFN-γ production by thymocytes or PBMC (see table 7.15).

IL-4

Maternal diet during pregnancy exerted a significant effect upon IL-4 production by fetal thymus cells with production being highest in dietary groups rich in n-3 PUFA (see table 7.16).

Th1:Th2 ratio

No significant effect of maternal diet was observed upon the ratio of Th1 to Th2 cytokines produced by fetal thymus cells or PBMC (see table 7.17).

Table 7.14: PGE_2 concentration in fetal plasma and PGE_2 production by fetal peripheral blood lymphocytes (ng/ml; mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
Plasma	(n=6)	(n=6)	(n=6)	(n=6)	(n=6)	(n=6)	
	16.1 ± 7.2	17.8 ± 4.2	14.6 ± 3.8	13.6 ± 4.9	13.3 ± 1.5	13.9 ± 2.7	0.478
PBMC	(n=4)	(n=3)	(n=4)	(n=4)	(n=4)	(n=5)	
No Con A	1.1 ± 0.5	1.2 ± 1.4	1.1 ± 0.9	1.0 ± 0.1	1.1 ± 0.4	1.5 ± 1.3	0.970
5μg/ml Con A	1.0 ± 0.4	5.1 ± 3.9	1.8 ± 0.7	1.0 ± 0.2	0.8 ± 0.3	2.7 ± 2.7	0.084
Mean SI	0.9 ± 0.1	2.7 ± 0.2	1.4 ± 0.7	1.0 ± 0.1	0.9 ± 0.3	1.8 ± 1.2	0.035 †

† no significant differences between groups when Bonferroni applied

Table 7.15: IFN-γ production by fetal thymus and peripheral blood lymphocytes (pg/ml; mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
Thymus	(n=5)	(n=5)	(n=4)	(n=6)	(n=4)	(n=5)	
No Con A	25.9 ± 5.2	3.0 ± 3.0	2.9 ± 3.7	1.6 ± 3.9	0.6 ± 1.1	10.5 ± 23.6	0.636
5μg/ml Con A	45.8 ± 82.0	5.6 ± 2.4	257.6 ± 500.3	15.3 ± 17.1	15.2 ± 15.7	200.3 ± 390.2	0.604
Mean SI	45.6 ± 82.1	4.2 ± 2.9	83.4 ± 154.4	24.9 ± 17.0	15.2 ± 15.7	184.7 ± 397.7	0.746
PBMC	(n=4)	(n=3)	(n=4)	(n=5)	(n=4)	(n=5)	
No Con A	10.4 ± 20.1	4.1 ± 0.5	1.5 ± 1.1	1.9 ± 4.2	32.8 ± 62.6	6.6 ± 9.7	0.557
5μg/ml Con A	6.9 ± 6.0	5.2 ± 2.8	93.6 ± 161.4	197.7 ± 197.5	201.1 ± 219.7	452.4 ± 467.7	0.231
Mean SI	5.5 ± 6.9	1.2 ± 0.6	93.5 ± 161.6	197.7 ± 197.5	91.6 ± 125.6	182.4 ± 232.3	0.485

Table 7.16 IL-4 production by fetal peripheral blood lymphocytes (pg/ml; mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
Thymus	(n=5)	(n=5)	(n=4)	(n=6)	(n=4)	(n=5)	
No Con A	3.5 ± 6.4 [b]	18.5 ± 8.0	22.2 ± 6.2 [a]	15.4 ± 12.0	9.0 ± 7.3	3.4 ± 4.5 [b]	**0.005**
5 μg/ml Con A	6.0 ± 5.6	18.0 ± 13.0	24.2 ± 7.7	14.6 ± 13.2	6.7 ± 3.1	12.7 ± 5.5	0.081
PBMC	(n=4)	(n=3)	(n=5)	(n=5)	(n=4)	(n=5)	
No Con A	8.0 ± 5.9	8.3 ± 7.3	18.8 ± 20.7	15.4 ± 7.1	13.6 ± 7.1	11.9 ± 7.1	0.705
5 μg/ml Con A	7.0 ± 4.7	4.8 ± 6.7	19.9 ± 11.4	13.5 ± 7.6	7.3 ± 5.5	7.7 ± 1.9	0.081

Table 7.17: IFN-γ:IL-4 ratios produced by Con A stimulated thymus and peripheral blood lymphocytes (mean ± standard deviation)

	LF Soyabean oil	HF Soyabean oil	HF Linseed oil	HF Salmon oil	HF Sunflower oil	HF Beef tallow	ANOVA p value
	(n=3)	(n=4)	(n=4)	(n=5)	(n=3)	(n=5)	
THYMUS IFN-γ:IL-4	1.1 ± 1.3	0.8 ± 1.0	19.6 ± 38.6	21.2 ± 30.4	2.7 ± 2.4	15.4 ± 30.0	0.732
	(n=5)	(n=1)	(n=3)	(n=4)	(n=3)	(n=5)	
PBMC IFN-γ:IL-4	4.1 ± 7.4	0.3	7.9 ± 13.7	21.9 ± 21.5	30.0 ± 44.3	69.1 ± 71.1	0.290

7.3.9 Correlations between markers of immune function and tissue fatty acid composition

Maternal and fetal immune tissues from the cohort used in chapter 6 were assessed for fatty acid composition (see tables 6.19 to 6.24; 6.45 to 6.47). These data were correlated with the markers of immune function studied in the current cohort to identify possible relationships between tissue fatty acid composition and function.

Correlations between PGE_2 and fatty acid composition of tissues

PGE_2 is an eicosanoid mediator which is generated from AA. Other authors have demonstrated that human immune cells with the greatest AA content produce the most PGE_2(96). A study which used a rat model identified that feeding n-3 rich diets resulted in significant reductions in PGE_2 production(250). It is hypothesised that by increasing the EPA content of cell membranes the production of PGE_2 is reduced, by either reducing the substrate available for PGE_2 synthesis, or by competitive inhibition of cyclooxygenase by EPA. The current dataset was therefore assessed for correlations between AA or EPA content and PGE_2 production.

A significant positive correlation between the maternal spleen basal PGE_2 production and AA content of the maternal spleen phospholipid PC, but not PE, was observed (see table 7.18, figure 7.4). This suggests that n-3 PUFA rich maternal diets (HF linseed and salmon oil) which are associated with the lowest AA content of spleen phospholipids, will have a direct functional effect on PGE_2 production. Similar but non-significant correlations were also observed between the AA content of spleen PC and Con A stimulated PGE_2 production (see figure 7.5). No significant correlations were observed between the AA content of maternal or fetal PBMC with the PGE_2 produced by these tissues in culture.

There was a clear trend for inverse relationships between the EPA content of tissues and the PGE_2 production by cells from these tissues, though this did not reach statistical significance (see table 7.19, figure 7.6).

Table 7.18: Correlations observed between mean maternal spleen PGE_2 production and phospholipid AA content (% total fatty acids) (n = 6, Pearson R values)

AA as % total fatty acids in	Tissue PGE_2 production	
	No Con A	5 μg/ml Con A
Maternal Spleen PC	**0.916***	0.759
Maternal Spleen PE	0.672	0.618
Maternal PBMC PC	0.048	0.385
Maternal PBMC PE	-0.676	-0.717
Fetal PBMC PC	0.441	0.540

* p = 0.01

Figure 7.4: Graph to illustrate the correlation between maternal spleen cell basal PGE_2 production and spleen cell PC AA content (% total fatty acids) (mean ± standard deviation)

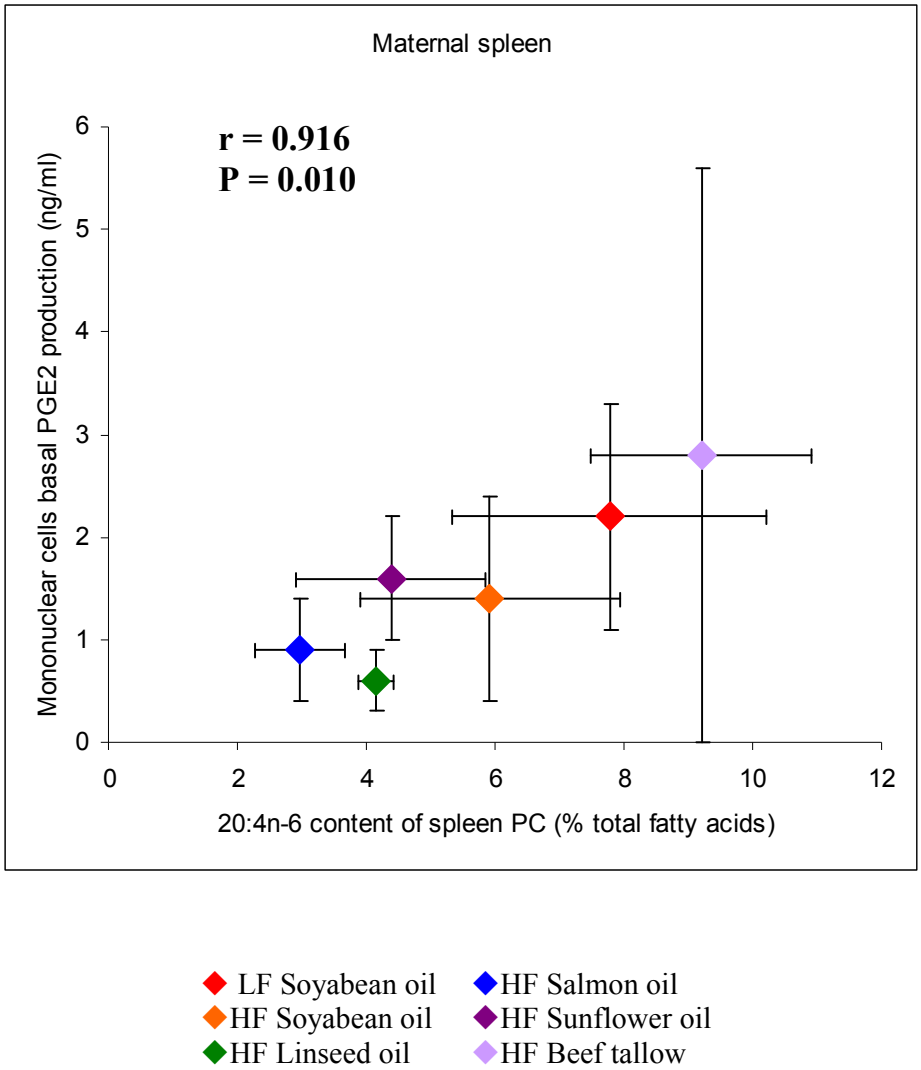


Figure 7.5: Graph to illustrate the correlation between maternal spleen Con A-stimulated PGE_2 production and spleen PC AA content (% total fatty acids) (mean ± standard deviation)

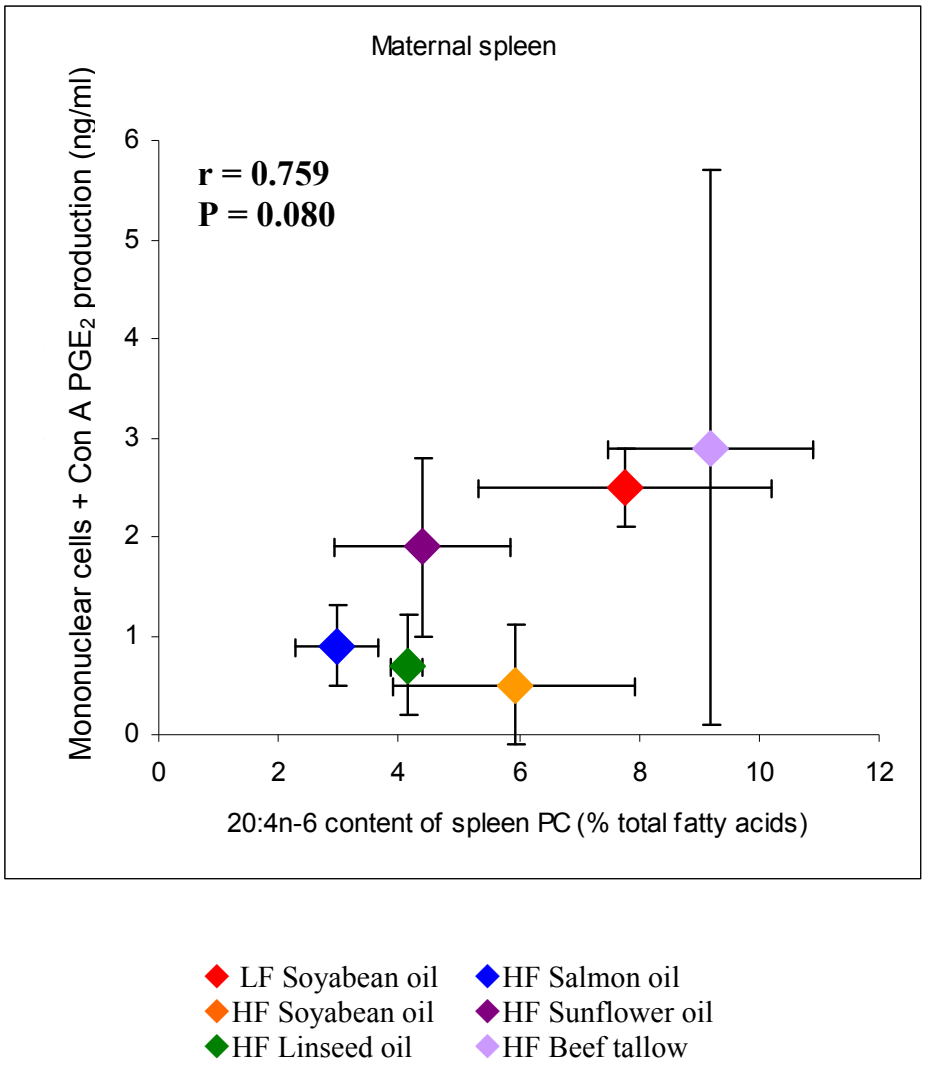
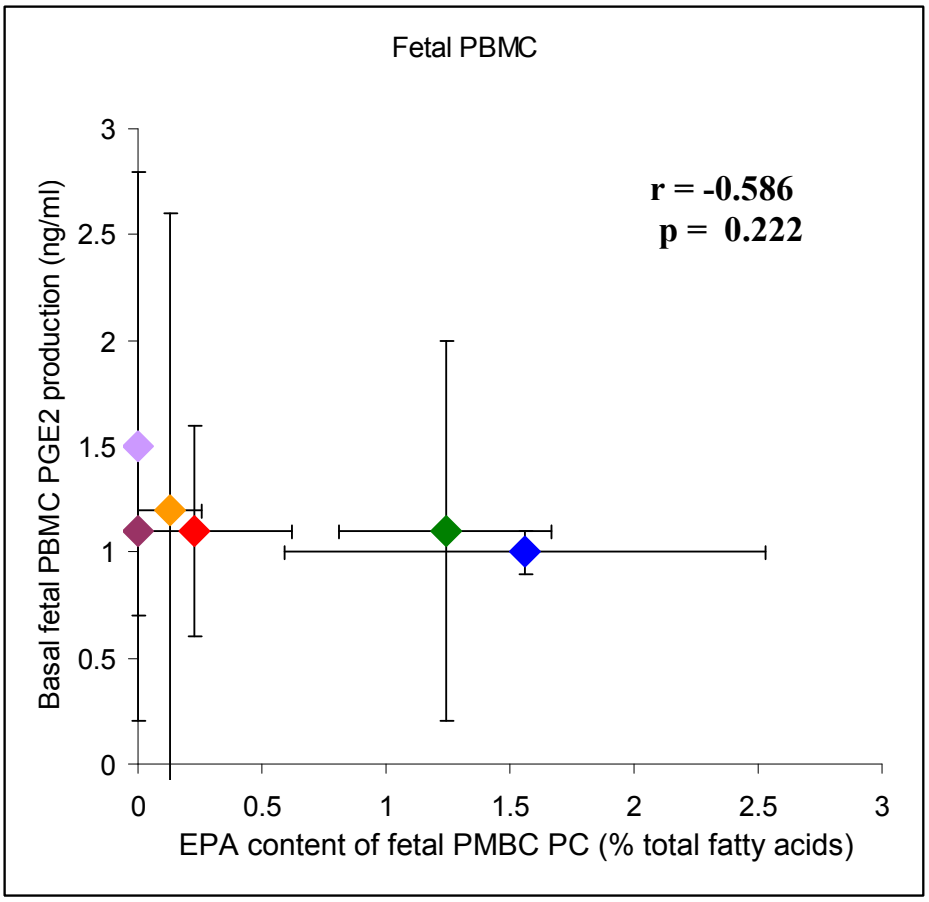


Table 7.19: Correlations observed between maternal spleen PGE_2 production and phospholipid EPA content (% total fatty acids) groups (n = 6, Pearson R values)

EPA as % total fatty acids in	Tissue PGE_2 production	
	No Con A	5 μg/ml Con A
Maternal Spleen PC	-0.180	-0.049
Maternal Spleen PE	-0.534	-0.398
Maternal PBMC PC	-0.458	-0.498
Maternal PBMC PE	0.171	-0.030
Fetal PBMC PC	-0.586	-0.325

Figure 7.6: Graph to illustrate the correlation between fetal PBMC basal PGE_2 production and fetal PBMC PC EPA content (% total fatty acids) (mean ± standard deviation)



Correlations between immune tissue fatty acid composition and cytotoxic T cell surface marker expression

Flow cytometry data identified a clear interaction between maternal diet during pregnancy and the expression of cytotoxic T cell surface markers in both maternal and fetal immune tissues. Studies have demonstrated a significant effect of DHA (when provided *in vitro* or in murine dietary studies) upon the expression of CD8 on cytotoxic T cells(252). It is possible that this is the mechanism responsible for the significantly different CD8 expression of the salmon oil group within lymph nodes (see table 7.5), but does not adequately explain the effects upon CD8 expression observed within the HF linseed oil group in the maternal spleen (see table 7.2). Data were therefore assessed to determine whether the expression of CD3 or CD8 within maternal or fetal immune tissues correlates to the n-3 or n-6 fatty acid composition of the tissues collected from a parallel cohort.

Within the fetal thymus there were positive correlations between LC n-3 PUFA content and CD8 expression (see table 7.20, figure 7.7). No significant correlations between fetal thymus CD3 expression and n-3 PUFA content were observed. The n-6 PUFA content of the fetal thymus was not correlated to the expression of CD3 or CD8 (data not shown).

Within maternal immune tissues inverse correlations were observed between the n-6 PUFA content (particularly 22:5n-6) of immune tissues and the expression of CD3 and CD8 upon cytotoxic T cells (see figure 7.8, table 7.21). No relationships were observed between maternal immune tissue n-3 PUFA composition and the expression of CD3 and CD8 upon cytotoxic T cells (data not shown).

Table 7.20: Correlations observed between the n-3 PUFA content (% total fatty acids) of fetal thymus phospholipids and the MFI of cytotoxic T cell surface marker expression (Pearson r values)

	CD3		CD8	
	PC	PE	PC	PE
18:3n-3	-0.219	0.345	-0.299	0.135
20:4n-3	-0.311	-0.630	-0.311	-0.575
20:5n-3	0.556	0.635	0.986	**0.916***
22:5n-3	0.119	0.471	**0.909***	**0.990****
22:6n-3	0.311	0.383	0.007	0.673
N-3 PUFA	0.480	0.588	0.678	**0.849***

* $p < 0.05$, ** $p < 0.01$

Figure 7.7: Graph to illustrate the correlation observed between the DPA content (% total fatty acids) of fetal thymus PE and the MFI of CD8 on cytotoxic T cells in the fetal thymus (mean ± standard deviation)

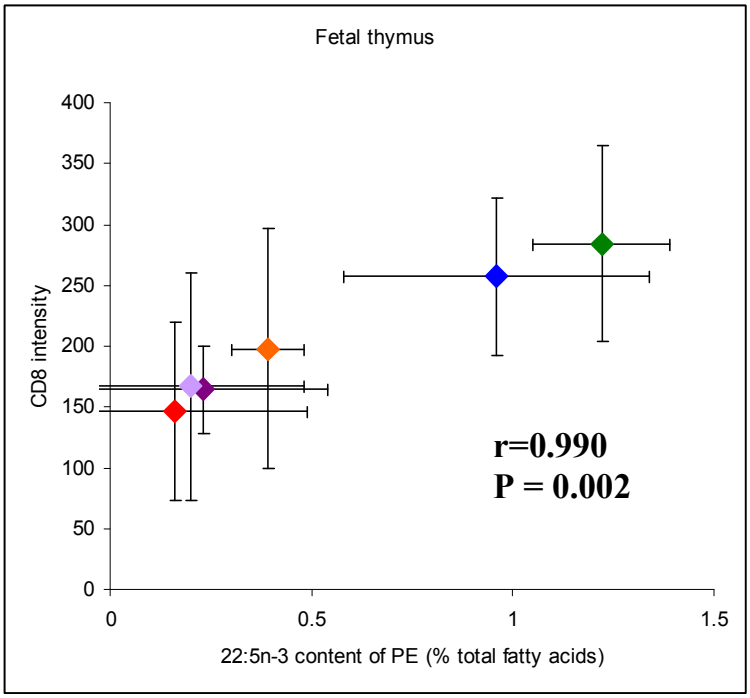


Figure 7.8: Graph to illustrate the correlation between the 22:5n-6 content (% total fatty acids) of maternal spleen PE and the MFI of CD8 on cytotoxic T cells in the maternal spleen (mean ± standard deviation)

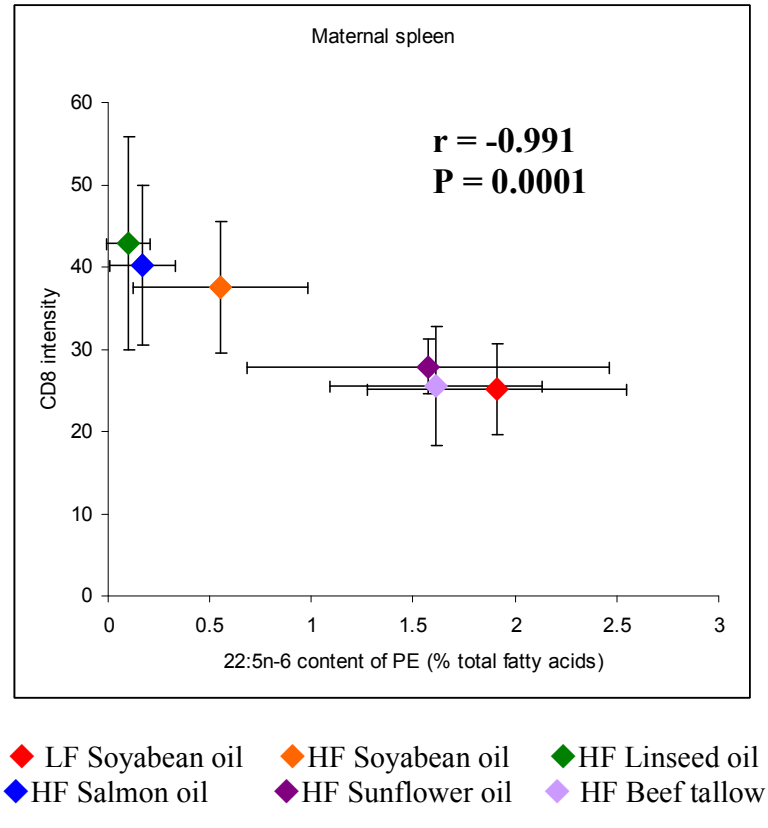


Table 7.21: Correlations observed between the n-6 PUFA content (% total fatty acids) of maternal immune tissue phospholipids and cytotoxic T cell surface marker MFI (Pearson r values)

	Maternal PBMC PC		Maternal thymus PC		Maternal spleen PC		Maternal spleen PE	
	CD3	CD8	CD3	CD8	CD3	CD8	CD3	CD8
18:2n-6	0.234	0.552	0.624	0.537	0.772	0.676	0.527	0.383
18:3n-6	-0.231	-0.497	-0.851	0.667	-0.384	-0.280	0.280	0.018
20:2n-6	-0.360	-0.289	0.589	0.565	0.149	-0.010	0.073	-0.098
20:3n-6	**-0.928****	**-0.836***	-0.080	0.205	-0.097	-0.114	-0.628	-0.511
20:4n-6	-0.469	-0.086	-0.727	-0.645	-0.808	-0.742	-0.616	-0.533
22:5n-6	-0.279	-0.696	**-0.818***	**-0.965****	-0.828	**-0.916***	**-0.961****	**-0.991****
Total n-6 PUFA	-0.332	-0.015	-0.302	-0.216	-0.152	-0.222	-0.492	-0.536

* $p < 0.05$, ** $p < 0.01$

7.4 Discussion

This chapter set out to address the hypothesis that the type of dietary fatty acids consumed during pregnancy will significantly alter markers of both maternal and fetal immune function, and these effects correspond to changes in the fatty acid composition of immune tissues. The data collected supports this hypothesis, with significant effects of maternal diet observed upon both maternal and fetal cytotoxic T cell surface marker expression across a range of immune tissues which in turn demonstrate significant correlations to the n-6 and n-3 content of these immune tissues. In addition, significant effects of maternal diet were observed upon the proportion of $CD161^+$ cells within the fetal thymus.

It was identified in an earlier chapter that the female rat undergoes immune adaptations in response to pregnancy (see chapter 5). The adaptations which had been observed to occur in response to pregnancy were an increased proportion of $CD8^+$ cells in the blood lymphocyte population, reduced expression of CD161 on NK cells in the blood lymphocyte population and in the spleen, and an increased ratio of Th1:Th2 cytokine production by thymocytes. Of these changes associated with pregnancy, maternal diet was found to have a significant effect upon the proportion of $CD8^+$ cells in maternal blood lymphocytes. The diets containing the highest n-3 fatty acid content (HF soyabean, linseed and salmon oil) had the lowest proportion of $CD8^+$ cells. This suggests that maternal diet during pregnancy may exert a significant effect upon maternal immune adaptations to pregnancy. The potential impact of these dietary effects could be further studied by assessment of any changes in the absolute number of CD8+ cells, data which was not collected in this study, or by assessment of the activation or cytotoxic functions of either cytotoxic T cells or NK cells, both of which express CD8.

Additional effects of maternal diet during pregnancy were observed upon markers of maternal immune function, particularly within the spleen. Maternal diets rich in n-3 fatty acids were found to significantly affect spleen weight (highest in HF salmon oil) and expression of CD3 and CD8 on cytotoxic T cells (highest in HF linseed oil group). This suggests that the sensitivity to activation of maternal cytotoxic T cells may be significantly increased by the HF linseed oil diet. This feature could be further assessed

by assays of T cell activation, such as investigating whether there are changes in the expression of activation markers such as CD69 and CD25 upon these cells in response to Con A stimulation. Statistically significant inverse correlations were observed between maternal spleen LC n-6 PUFA content and cytotoxic T cell marker expression, which suggests that the expression of these cell surface markers has a significant relationship with membrane fatty acid composition. While, the data used for this correlation was limited to use of the means for each dietary group as these two variables were assessed in two distinct cohorts, available literature from murine *in vitro* studies does support a causal relationship between fatty acid composition and the expression of CD8(252).

Significant effects of a maternal diet rich in n-3 fatty acids were not limited to the maternal spleen, with significant effects also observed upon expression of cell surface markers of cytotoxic T cells within the maternal thymus and within maternal lymph nodes, which again demonstrated statistically significant inverse relationship with the LC n-6 PUFA content of immune tissue phospholipids. The consistent effects of a n-3 rich maternal diet upon maternal cytotoxic T cells warrants further investigation into the role of cytotoxic T cells within rat pregnancy, their absolute circulating and tissue numbers, sensitivity to activation and markers of cytotoxic function.

Maternal dietary fatty acid intake during pregnancy was also found to significantly affect fetal cytotoxic T cells, with these cells within the fetal thymus having the highest expression of CD3 on their surface within the HF salmon oil group, and the highest expression of CD8 with both the n-3 PUFA rich groups (HF linseed oil, salmon oil). In contrast to the observations where an inverse relationship was observed between CD8 expression and n-6 PUFA, within the fetal thymus there were statistically significant positive correlations between LC n-3 PUFA content of the fetal thymus and CD8 expression, and no significant inverse relationship with n-6 fatty acid content. Taken together both maternal and fetal data provides strong evidence for a significant effect of the n-3 content of the maternal diet during pregnancy upon cytotoxic T cell function. Cytotoxic T cells have important roles in detection of viral infection, and may also have a role in auto-immune disease. Further useful data may therefore be obtained from animal studies among both pregnant and adult animals with auto-immune disease to assess the potential effect of diet upon disregulated cytotoxic immune disease, or upon animal survival in response to viral infection.

In addition to the effects observed upon cytotoxic T cells, a significant effect of maternal diet was observed upon the proportion of $CD161^+$ cells within the fetal thymus, with the highest proportion of these cells observed in the HF linseed oil group. This change to the proportion of cell types within the fetal thymus suggests that the maternal diet has the capacity to influence the lineage and differentiation of cells within the fetal thymus, and is indicative of a potential programming effect of maternal dietary fatty acids during pregnancy. For a true programming effect to be confirmed, data would be required upon the longevity and functional impact of these changes to fetal thymus immune cells, data which was not obtained in this study.

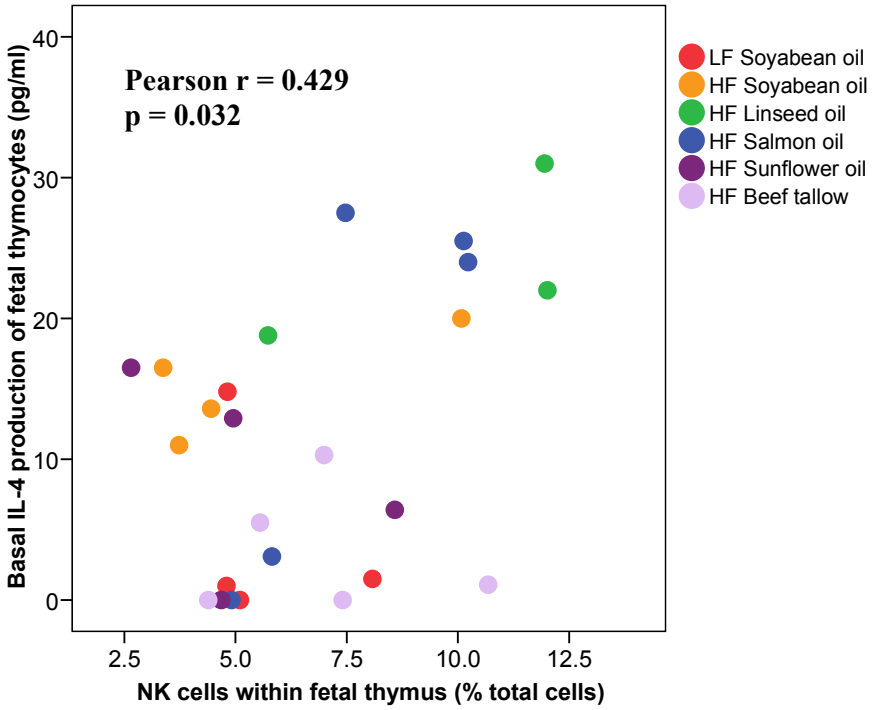
CD161 is typically used as a marker for NK cells since it is a NK cell receptor known as NKR-P1. However, CD161 is also found on other cell types, such as thymus dendritic cells(253) and some T cell subsets. In the fetal mouse, thymic NK cells derive from TNK cells, a progenitor of both T and NK cells(254). It is therefore possible that the maternal diet interacts with a transcription factor which is involved in the divergence of T and NK cells in the thymus. The major sites of NK cell development have not been identified, but the thymus is a known to be a source of NK cells in both the mouse and human fetus. Other potential sources of NK cells include the bone marrow and the liver(255). NK cells were identified in this study using CD161 in a single-staining protocol. It is therefore possible that a subset of the cells identified as NK cells may in fact be NKT cells, i.e. T cells which also express the NK cell surface marker, or dendritic cells. Flow cytometry staining protocols could be developed further in order to categorise more clearly the cells identified within the fetal thymus as NK cells. For example, dual staining of CD161 with CD3, CD4 or CD8 could be used to try and identify the proportion of $CD161^+$ cells which are in fact NKT cells. The samples of immune tissues from this cohort which were reserved for histological and gene expression assessment as part of a collaboration with researchers at the University of Utrecht, The Netherlands, may provide further information on the identity of the cells expressing CD161 within the fetal thymus, and in addition provide an indication of whether the effects of maternal diet during pregnancy result in changes to the structural organisation of the fetal thymus.

NK cells have important roles within innate immunity, and an adequate innate immune response will be essential in neonatal life in response to the antigen exposure which will follow delivery. The work in this thesis could therefore be developed further using

animal models to assess whether the maternal diet during pregnancy can significantly affect neonatal survival of viral infection, or to directly assess fetal NK cell activation and cytotoxic mechanisms.

IL-4 is a cytokine produced by NKT cells(256), and it was observed that the within the HF linseed oil dietary group there was both the highest proportion of $CD161^+$ cells, and the highest basal IL-4 production. Indeed, a significant positive correlation between the proportion of $CD161^+$ cells in the fetal thymus cell population and IL-4 production by those cells was observed (see figure 7.9).

Figure 7.9: Graph to illustrate the correlation between the proportion of fetal thymus $CD161^+$ cells and basal IL-4 production by cultured fetal thymus lymphocytes



IL-4 is a cytokine associated with Th2 immune responses, and excessive Th2 responses are a feature of atopic disease. However, this association should be investigated further before a causal relationship between maternal diet and the risk of later offspring atopic disease is postulated. The cells obtained in this study were from fetal immune tissues, and whether the immune cells of neonatal or weanling rats which have been exposed to environmental antigens would express this same cytokine profile is unclear, and

requires further detailed investigation. The use of ELISAs to assess cytokine production limited the number of cytokines which could be assessed within the sample quantity which was available. Use of a multiplex kit which enables the simultaneous analysis of a wider panel of cytokines from a much smaller sample volume would allow further insight into the effect of maternal diet upon fetal thymus cytokine production, and in particular allow assessment of whether the changes observed reflect a change in the overall capacity for cytokine production, or a true Th2-skewed response.
The longevity of the observed effects of maternal dietary fatty acids upon both maternal and fetal immune function is of interest, and studies could be undertaken to determine whether there is altered susceptibility of both the mother and the offspring to infectious disease, or to development of immune disorders such as atopic responses.

The data obtained in this thesis therefore indicate that maternal diet can exert a significant effect upon markers of both the maternal and fetal immune system, particularly upon cytotoxic T cells, and that the cells types within the fetal thymus can also be significantly affected by maternal diet. A limitation of the data collected in this thesis is the lack of clear functional or clinically-relevant effects of the maternal diet upon the fetal immune system. To gain information into the potential translational value of these data it will be necessary to conduct further detailed studies into the functional effects which are observed in neonatal or early life. This could be achieved by a combination of measurements of alternative markers of immune function such as cytotoxic assays, the use of disease or allergic sensitisation models, or the exposure of neonates to pathogens. Though these investigations are beyond the scope of the current thesis, the data obtained here indicates a strong potential for maternal diet during pregnancy to influence these outcomes, possibly via programming effects.

Chapter 8: Final Discussion and Conclusions

8.1 The effect of gender, pregnancy and diet during pregnancy upon tissue fatty acid composition

8.1.1 Gender

Human studies have demonstrated that gender has a significant effect upon plasma fatty acid composition, with females having significantly higher plasma status of the longer chain n-3 polyunsaturated fatty acid (LC n-3 PUFA) docosahexaenoic acid (DHA) compared to males(132-135) which is independent of dietary intakes of n-3 fatty acids. Studies which have used isotope-labelled fatty acids have identified that this gender difference is likely to be mediated by an increased capacity for LC n-3 PUFA synthesis among women(130;131). Data which was lacking from human studies included the effect of additional dietary α-linolenic acid (ALNA) on LC n-3 PUFA status in women, whether the differences seen in plasma fatty acid composition are also apparent within metabolically relevant organs, and to date have provided little information into the mechanisms which mediate the gender differences in LC PUFA synthesis. While data from human studies which have investigated the effect of oral contraceptives, hormone replacement therapy or sex hormone administration to transsexual subjects suggest that sex hormones are likely to be involved in the gender differences in LC n-3 PUFA synthesis and plasma status(133;135;147;148), no human studies have yet been conducted to investigate whether there are associations between plasma fatty acid composition and hormone status. Animal studies of the effect of sex hormones upon the activity of fatty acid desaturase enzymes in the liver(150-152) have not identified whether these effects are also apparent within the normal physiological range of sex hormone concentrations.

The studies described in this thesis were therefore conducted to address whether the rat is a suitable model for an investigation of the effects of gender upon plasma fatty acid composition, and whether sex hormone status or the expression of fatty acid desaturase and elongase genes in the liver are mechanisms which mediate the gender differences in fatty acid status. The data collected in this study confirmed the suitability of the rat as a model of gender differences in fatty acid composition, and expanded upon data currently available from human studies by demonstrating that these differences are also apparent within the liver and adipose tissue.

This study did not identify any significant relationships between circulating sex hormone status and plasma DHA content, or the mRNA expression of elongase or desaturase enzymes within the liver. The use of mRNA expression as a marker of enzyme activity is limited by the lack of insight it allows into the numerous other processes which affect enzyme activity, such as efficiency of translation, post-translational modifications, influence of inhibitors or activators or negative feedback mechanisms. However, data did provide evidence of an influence of sex hormones upon the activity of desaturase and elongase enzymes within the liver when activity was assessed by the substrate:product ratio of fatty acids within liver lipids. While use of this substrate:product ratio is a crude marker of enzyme activity, as it does not allow any insight into other processes which are involved in the maintenance of membrane composition such as enzyme specificity during lipid assembly, this approach has been demonstrated to be consistent with variations in Δ6 desaturase and Δ5 desaturase activity(257) as assessed by liver microsome experiments. There were clear and strong relationships apparent between this marker and both gender and sex hormone concentrations, including a statistically significant inverse relationship between this marker of Δ6 desaturase activity and testosterone status, and a positive relationship with oestradiol status. It is possible that this effect of sex hormones upon Δ6 desaturase activity may account for the higher plasma content of 18:3n-6 and DHA observed in females compared to males, particularly given that Δ6 desaturase is the rate-limiting enzyme in LC PUFA synthesis. Further investigations will be required to establish with confidence the causal nature of these relationships.

The use of three dietary groups in this study exerted some confounding effects, particularly due to the relationship between dietary fat intake and sex hormone status that was observed. However, use of these dietary groups also identified some novel features of the effect of diet upon LC n-3 PUFA synthesis. A significant effect of diet was observed upon Δ6 desaturase gene expression, with both male and female rats fed a diet high in ALNA having the highest mRNA expression of this enzyme. This supports the observations of other authors that dietary fatty acids can influence transcription factors such as SREPB-1 and PPAR-α(258) which are involved in the regulation of desaturase gene expression(259-262). These data also have important translational value to human health due to the importance of DHA in the development of fetal visual and neural tissues(56;57) and their benefits upon cardiovascular and inflammatory disease among adults. If an increasing dietary intake of ALNA increases the expression

of hepatic Δ6 desaturase in humans, this may have the potential to significantly alter LC n-3 PUFA status in humans by improving access to this rate-limiting enzyme, and due to its substrate specificity for ALNA.

This study therefore addressed several of the research questions which had been unanswered by human and animal studies conducted to date, and indicated clearly that the effect of gender upon fatty acid status is likely to be mediated by an effect of sex hormones upon fatty acid desaturase enzyme activity rather than gene expression. This study provides some direction for future research in this area, particularly into the potential interactions between dietary ALNA and Δ6 desaturase expression. Further research will be required to establish if there is a true causal relationship between sex hormone status and fatty acid desaturase activity, perhaps by either the assessment of hormone status within a human study providing stable isotope fatty acid substrates, or by in vitro assessment of enzyme activity in response to physiological concentrations of sex hormones.

8.1.2 Pregnancy

Existing data indicates that the fatty acid composition of human plasma lipids is significantly altered during pregnancy(162-166). However, the effects observed to date are mixed, and may be complicated by dietary variation between subjects and the lack of pre-pregnancy data in longitudinal studies. Animal models, which enable complete dietary control and collection of comparable data in the non-pregnant state, have identified that there are significant effects of pregnancy upon plasma and liver fatty acid composition, including increases in DHA status(167-170). The mechanisms which may be responsible for the effects of pregnancy have not been investigated in studies to date, and so this study investigated whether sex hormones or fatty acid desaturase and elongase gene expression in the liver are involved.

This study confirmed that pregnancy significantly alters plasma and tissue fatty acid composition, particularly for DHA and AA content, as has been described in previous rat studies. An additional novel observation was that there are marked increases in the 22:5n-6 content of tissues in pregnancy, which indicates that maternal LC n-6 PUFA synthesis is also significantly affected by pregnancy, and may in fact account for the reductions observed in AA content. The effect of pregnancy upon 22:5n-6 status was

tissue-specific, and indicates that this fatty acid may be preferentially mobilised into the maternal plasma in order to be available to the fetus.

There was no significant association between the status of the three sex hormones measured during pregnancy and plasma DHA content. However, it is possible that any potential effect of sex hormone status was obscured by maternal transfer of DHA to the fetus, which could not be quantified in this study. It would be possible to investigate this further in future studies by using isotope-labelled fatty acids provided to animals, and tissue analysis to determine the extent of transfer to the fetus.

A significant positive correlation was observed between circulating progesterone concentrations and Δ6 desaturase mRNA expression in the liver. This indicates that the increasing progesterone concentrations which are a feature of pregnancy associate with an availability of Δ6 desaturase to metabolise essential fatty acids, a finding of particular importance given that the activity of Δ6 desaturase is the rate-limiting step in the synthesis of LC PUFA. However, the lack of this same relationship among non-pregnancy females studied in chapter 3 indicates that further studies will be required to establish whether this relationship is directly causal, or instead under the influence of an additional variable which is associated with changes to progesterone status during pregnancy.

The substrate:product ratio of fatty acids within liver lipids was used as a crude marker of desaturase and elongase activities. The effects of pregnancy upon this index were complex, and did not demonstrate a significant relationship with sex hormone status. There are two possible explanations for this lack of association: it is possible that other hormones which alter during pregnancy (e.g. relaxin, oxytocin) which were not assessed in this study may have been involved in the effects observed; or alternatively that the three timepoints assessed were insufficient to gain an insight into the nature of the changes which occur during pregnancy. The complex pattern of differences in fatty acid composition during pregnancy would support the latter, with the day 12 gestation group demonstrating dramatic differences in fatty acid composition from both virgin and day 20 pregnant females. This indicates that the changes in fatty acid composition during pregnancy and the mechanisms which maintain them are non-linear, and would require the study of numerous additional timepoints, preferably using a longitudinal study design, to evaluate fully.

8.1.3 Diet during pregnancy

Animal studies have demonstrated that the maternal diet during pregnancy has the capacity to significantly alter fetal tissue fatty acid composition, including that of the liver(228) and brain(263). This study set out to investigate whether maternal diet during pregnancy could also significantly alter maternal and fetal immune tissue composition, in order that the potential effect that this may have upon immune function could be investigated. The diets used in this study were chosen to reflect the standard low-fat diet of the laboratory rat (LF soyabean, 3% w/w), and higher fat diets (13% w/w) used to represent human intakes of fat as a proportion of dietary energy. These high fat diets either contained a balance of n-6 and n-3 PUFA representative of that consumed in Western populations (HF soyabean), or were rich in ALNA (HF linseed), LC n-3 PUFA (HF salmon), n-6 PUFA (HF sunflower) or saturates and MUFA (HF beef tallow).

The effect of maternal diet during pregnancy upon maternal and fetal tissue fatty acid composition was studied in detail, in order that any variations in response to diet could later be assessed for their relationship with changes in immune function. This study therefore provided extensive information about the effects of maternal diet during pregnancy upon a wide range of tissues, and demonstrated that maternal diet significantly affects the fatty acid composition of maternal plasma, liver, adipose tissue and immune tissues, placenta and fetal plasma, liver, brain and immune tissues.

It had been anticipated that the HF salmon oil diet would demonstrate the most pronounced effects upon LC n-3 PUFA status, as this diet contains these fatty acids preformed, and would therefore not be subject to the potential limitations of maternal or fetal LC n-3 PUFA synthesis from ALNA. However, it was observed that the HF linseed oil diet resulted in equivalent EPA status in fetal immune tissues and equivalent DHA status in the fetal brain to that achieved within the HF salmon oil diet group. This finding was particularly unexpected as this feature of dietary ALNA was not observed within any other maternal or fetal tissue assessed, and indicates that maternal dietary ALNA during pregnancy may be as effective as preformed LC n-3 PUFA at maximising the DHA content of the fetal brain and EPA status of the fetal immune tissues.

It is well established that sufficient DHA status is required for optimal brain and visual development(56;57). Thus there may be physiological mechanisms that operate to ensure that the brain acquires DHA irrespective of its status in other tissues and that these mechanisms are efficient when DHA is either provided preformed (e.g. in the HF salmon oil group) or is synthesised endogenously when the precursor (ALNA) is abundant (e.g. in the HF linseed oil group). A relationship was also observed between maternal diet and the 22:5n-6 content of the fetal brain. This n-6 highly-unsaturated fatty acid (HUFA) is generated from LA by the action of the same sequence of enzymes which generate DHA from ALNA. It was identified that the total HUFA content of the fetal brain was not significantly affected by maternal diet, and was maintained by either DHA or 22:5n-6 depending upon the dietary availability of fatty acid substrates (see figure 6.16). Whether the balance of DHA and 22:5n-6 in maintaining this HUFA status has functional consequences could potentially be assessed in future studies which include neurological assessments such as effects upon offspring behaviour or memory.

The observation that fetal immune tissues achieve a similar EPA status when the maternal diet is salmon oil or linseed oil rich diet suggests that the immune system may have an optimal EPA requirement for development and that physiological mechanisms operate to achieve this irrespective of the status of other tissues. If so, this is a highly novel observation that is of significant importance in the human context. Thus far the interest in providing LC n-3 PUFA to pregnant and lactating women and to infants in their formula has been driven by a desire to optimise brain and visual function through providing DHA. If the immune system also has a high requirement of LC n-3 PUFA, particularly EPA, then there may be immunologic advantages to those strategies that have been promoted to enhance intake of these fatty acids in women such as consumption of oily fish. Human studies could be feasibly undertaken to determine the effect of dietary ALNA during pregnancy upon the fatty acid composition of neonatal PBMC and any associated changes in disease risk or atopic symptoms.

When data from the HF linseed oil group from this study of diet during pregnancy were compared with the virgin female HF linseed oil group from the gender study conducted in chapter 3, there was a clear indication of a diet-pregnancy interaction, with the production of DHA from ALNA significantly up-regulated during pregnancy when an ALNA rich diet is provided (see figure 6.17). Studies of the mRNA expression of fatty acid desaturase and elongase genes among a cohort of HF linseed fed rats during

pregnancy would therefore be of interest, based upon the observations among non-pregnant rats that dietary ALNA is associated with significant increases in Δ6 desaturase expression. The effect of an ALNA rich diet upon DHA status during pregnancy also has significant implications for human health, and may be of particular relevance among vegetarian or vegan populations to ensure adequate DHA status of the fetus when little LC n-3 PUFA are consumed. It would be possible to assess within human studies the potential effect of dietary ALNA during pregnancy upon markers of cognitive or visual function in infants, particularly those which have been demonstrated to be significantly affected by inclusion of DHA in infant formula(58).

8.2 The effect of pregnancy and diet during pregnancy upon immune function

8.2.1 The effect of pregnancy upon immune function in the rat

The effect of pregnancy upon rat immune function was assessed as available literature had reported conflicting findings(179-181). In order that the effect of maternal diet during pregnancy upon maternal and fetal immune function could be assessed in a later cohort it was important to clearly characterise the effects of pregnancy upon maternal immune function. This study demonstrated that rat pregnancy is associated with significant alterations in immune function. Some of these changes were in agreement with those observed in human and murine studies. For example, thymic involution and reduced lymphoid organ cellularity were observed. These adaptations to pregnancy may reflect a reduction in production of novel T cells within the thymus which may recognise paternal antigens and result in fetal rejection. Other features of rat immune function during pregnancy were in contrast to those described in human and murine studies. While human pregnancy has been demonstrated to involve a Th2 switch, where the maternal immune system produces more Th2-type cytokines(172), in this study the maternal rat demonstrated an increased Th1:Th2 cytokine ratio during pregnancy. These contrasts between human and rat immune adaptations to pregnancy may indicate that the rat is of limited suitability as a model for human pregnancy.

Novel features of rat pregnancy were also identified in this study, such as the lower expression of CD161 upon the cell surface of NK cells within the mononuclear cell populations isolated from maternal blood and spleen, indicating that the responsiveness of NK cells may be reduced during pregnancy. This could be an adaptation to

pregnancy that reduces the risk of fetal rejection, though further information upon the absolute numbers of NK cells and functional assays of activation or cytotoxicity would be useful to confirm this hypothesis. There was an increased proportion of $CD8^+$ cells within the maternal blood lymphocyte population during pregnancy, indicating that cytotoxic immune responses may also be affected by pregnancy. It is possible that this adaptation is in response to the potentially diminished activity of NK cells, and occurs in order maintain maternal resistance to viruses, though further studies would be required to demonstrate that this indeed reflects a change in cytotoxic function.

8.2.2 Maternal diet during pregnancy and maternal and fetal immune function

Provision of diets which varied in the quantity and quality of fatty acids during pregnancy were demonstrated to significantly affect markers of both maternal and fetal immune function. The principal effects observed were changes to the expression of cell surface markers upon both maternal and fetal cytotoxic T cells across a range of tissues including the maternal spleen, PBMCs, lymph nodes, thymus and the fetal thymus. A change in the proportion of $CD161^+$ cells (putative NK cells) within the fetal thymus was also observed, with an association between the proportion of these cells and the production of IL-4, a Th2 cytokine. These effects of maternal diet were pronounced within the n-3 PUFA rich HF linseed and HF salmon oil dietary groups, and were identified to significantly correlate to tissue fatty acid composition. These data indicate that changes to the n-3 and n-6 content of the maternal diet therefore have the capacity to significantly alter both maternal and fetal immune function, which may have significant implications for maternal and fetal resistance to infection, alter the risk of immune dysfunction among offspring, or potentially exert longer-term programming effects upon fetal health if the functional effects are maintained.

While the data obtained in this study informs future research of the potential that maternal diet during pregnancy may have upon both the maternal and fetal immune system, it is not possible to say with certainty what the longer-term or potential programming effects of these changes to markers of the fetal immune system might be. Studies which could take these findings forward could include more detailed assessment of the functional impact of the changes observed within fetal cytotoxic T cells and NK cells, such as assessment of the activation status and cytotoxic activities of these cell types at the same timepoint, or the inclusion of timepoints after delivery to assess the

longevity and influence of environmental antigens upon these processes. Animal studies would be of use to determine whether there are associated changes to neonatal survival following pathogen challenge. Animal models of disease, particularly auto-immune disease and atopic disease models would be of use to determine whether maternal diet may prevent the development, or reduce the severity of these diseases which are associated with immune dysfunction.

Data collected in this study could also inform potential human research. Numerous studies have been undertaken to date where women are provided with addition LC n-3 PUFA supplements or oily fish during pregnancy. Data from my research can be used as a starting point to investigate the potential effect of these interventions upon both maternal and fetal cytotoxic and NK cell activity, and also suggests that maternal dietary ALNA supplementation may significantly alter markers of both the maternal and fetal immune system.

8.3 Future research considerations

The data generated over the course of this study have provided novel information about the mechanisms involved in the maintenance of the gender differences and effects of pregnancy upon fatty acid composition. Data have also been generated which suggest that dietary ALNA may exert stronger effects upon tissue LC n-3 PUFA status in women than has been inferred from human studies of dietary ALNA provision, and may be of particular public health relevance among women and during pregnancy.

The work undertaken to assess the effect of dietary fatty acids during pregnancy has demonstrated that maternal diet has the potential to significantly affect maternal and fetal immune tissue fatty acid composition, and that these changes are associated with significant alterations in markers of both maternal and fetal immune function. These data also demonstrate that maternal dietary fatty acids during pregnancy have the potential to alter other maternal and fetal systems, such as fetal brain development, placental function, and numerous other organ systems which were not directly assessed in this study such as the pancreas and cardiovascular systems.

Data suggest that maternal diet has the capacity to significantly affect fetal immune function, and may therefore exert programming effects. Programming cannot be confirmed in this study, as offspring were not followed beyond day 20 of gestation. It is

possible that the observed effects may only be apparent during either direct dietary intervention, or as a result of indirect fatty acid transfer during lactation, and further detailed studies will be required to investigate whether the significant effects observed have longevity into adulthood. The potential effects that dietary interventions, particularly an increasing intake of n-3 fatty acids during pregnancy may exert upon the fetal immune system, warrant further investigation, particularly against a background of increasing atopic disease prevalence in Western populations.

References

(1) Polyunsaturated fatty acids as nutrients. Stockholm: Almqvist & Wiksells; 1966.

(2) Calder PC, Burdge GC. Fatty Acids. In: Nicolaou A, Kokotos G, editors. Bioactive Lipids.Bridgwater: The Oily Press; 2004.

(3) British Nutrition Foundation. Unsaturated fatty acids : nutritional and physiological significance : the report of the British Nutrition Foundation's task force. London : Chapman & Hall; 1992.

(4) Henderson L, Gregory J, Irving K, Great Britain.Office for National Statistics.Social Survey Division, Medical Research Council.Human Nutrition Research, Great Britain.Food Standards Agency. The national diet & nutrition survey : adults aged 19 to 64 years : volume 2 : energy, protein, carbohydrate, fat and alcohol intake. London : TSO; 2003.

(5) Great Britain.Department Of Health.Committee On Medical Aspects Of, Food P. Fat. Dietary reference values for food energy and nutrients for the United Kingdom : report of the panel on dietary reference values. G.B. : H.M.S.O.; 1991. p. 39-60.

(6) Buttriss J, British Nutrition Foundation [, Buttriss J. n-3 fatty acids and health. British Nutrition Foundation, 1999.

(7) Great Britain SACoN, Great Britain.Committee on Toxicity of Chemicals in Food CPatE. Advice on fish consumption : benefits & risks. London : TSO, 2004.

(8) Calder PC. Dietary arachidonic acid: harmful, harmless or helpful? Br J Nutr 2007 Sep;98(3):451-3.

(9) Black PN, Sharpe S. Dietary fat and asthma: is there a connection? Eur Respir J 1997 Jan;10(1):6-12.

(10) Dwyer T, Hetzel BS. A comparison of trends of coronary heart disease mortality in Australia, USA and England and Wales with reference to three major risk factors-hypertension, cigarette smoking and diet. Int J Epidemiol 1980 Mar;9(1):65-71.

(11) British Heart Foundation Statistics Website: Trends in death rates. Accessed on 18 August 2008;http://www.heartstats.org/datapage.asp?id=722.

(12) Smit EN, Muskiet FA, Boersma ER. The possible role of essential fatty acids in the pathophysiology of malnutrition: a review. Prostaglandins Leukot Essent Fatty Acids 2004 Oct;71(4):241-50.

(13) Leonard AE, Pereira SL, Sprecher H, Huang YS. Elongation of long-chain fatty acids. Prog Lipid Res 2004 Jan;43(1):36-54.

(14) Brenner RR. Hormonal modulation of delta6 and delta5 desaturases: case of diabetes. Prostaglandins Leukot Essent Fatty Acids 2003 Feb;68(2):151-62.

(15) Sjogren P, Sierra-Johnson J, Gertow K, Rosell M, Vessby B, de FU, et al. Fatty acid desaturases in human adipose tissue: relationships between gene expression, desaturation indexes and insulin resistance. Diabetologia 2008 Feb;51(2):328-35.

(16) Cho HP, Nakamura MT, Clarke SD. Cloning, expression, and nutritional regulation of the mammalian Delta-6 desaturase. J Biol Chem 1999 Jan 1;274(1):471-7.

(17) Ravel D, Chambaz J, Pepin D, Manier MC, Bereziat G. Essential fatty acid interconversion during gestation in the rat. Biochim Biophys Acta 1985 Jan 9;833(1):161-4.

(18) Salem N, Jr., Wegher B, Mena P, Uauy R. Arachidonic and docosahexaenoic acids are biosynthesized from their 18-carbon precursors in human infants. Proc Natl Acad Sci U S A 1996 Jan 9;93(1):49-54.

(19) Castuma JC, Brenner RR, Kunau W. Specificity of delta 6 desaturase--effect of chain length and number of double bonds. Adv Exp Med Biol 1977;83:127-34.

(20) Cleland LG, Gibson RA, Pedler J, James MJ. Paradoxical Effect of n-3 Containing Vegetable Oils on Long-Chain n-3 Fatty Acids in Rat Heart. Lipids 2005;40(10):995-8.

(21) Wang Y, Botolin D, Christian B, Busik J, Xu J, Jump DB. Tissue-specific, nutritional, and developmental regulation of rat fatty acid elongases. J Lipid Res 2005 Apr;46(4):706-15.

(22) Wang Y, Botolin D, Xu J, Christian B, Mitchell E, Jayaprakasam B, et al. Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. J Lipid Res 2006 Sep;47(9):2028-41.

(23) Schaeffer L, Gohlke H, Muller M, Heid IM, Palmer LJ, Kompauer I, et al. Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. Hum Mol Genet 2006 Jun 1;15(11):1745-56.

(24) Malerba G, Schaeffer L, Xumerle L, Klopp N, Trabetti E, Biscuola M, et al. SNPs of the FADS gene cluster are associated with polyunsaturated fatty acids in a cohort of patients with cardiovascular disease. Lipids 2008 Apr;43(4):289-99.

(25) Baylin A, Ruiz-Narvaez E, Kraft P, Campos H. alpha-Linolenic acid, Delta6-desaturase gene polymorphism, and the risk of nonfatal myocardial infarction. Am J Clin Nutr 2007 Feb;85(2):554-60.

(26) Igarashi M, Ma K, Chang L, Bell JM, Rapoport SI. Dietary n-3 PUFA deprivation for 15 weeks upregulates elongase and desaturase expression in rat liver but not brain. J Lipid Res 2007 Nov;48(11):2463-70.

(27) Stubbs CD, Smith AD. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 1984 Jan 27;779(1):89-137.

(28) Schoonjans K, Staels B, Auwerx J. The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. Biochim Biophys Acta 1996 Jul 26;1302(2):93-109.

(29) Sampath H, Ntambi JM. Polyunsaturated fatty acid regulation of gene expression. Nutr Rev 2004 Sep;62(9):333-9.

(30) Lewis RA, Austen KF, Soberman RJ. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. N Engl J Med 1990 Sep 6;323(10):645-55.

(31) Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest 2001 Jul;108(1):15-23.

(32) Levy BD, Clish CB, Schmidt B, Gronert K, Serhan CN. Lipid mediator class switching during acute inflammation: signals in resolution. Nat Immunol 2001 Jul;2(7):612-9.

(33) Serhan CN, Arita M, Hong S, Gotlinger K. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. Lipids 2004 Nov;39(11):1125-32.

(34) Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006 Jun;83(6 Suppl):1505S-19S.

(35) Calder PC. N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. Lipids 2003 Apr;38(4):343-52.

(36) Liu J, Yeo HC, Doniger SJ, Ames BN. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. Anal Biochem 1997 Feb 15;245(2):161-6.

(37) Raclot T. Selective mobilization of fatty acids from adipose tissue triacylglycerols. Prog Lipid Res 2003 Jul;42(4):257-88.

(38) Resh MD. Regulation of cellular signalling by fatty acid acylation and prenylation of signal transduction proteins. Cell Signal 1996 Sep;8(6):403-12.

(39) Burdge G. Alpha-linolenic acid metabolism in men and women: nutritional and biological implications. Curr Opin Clin Nutr Metab Care 2004 Mar;7(2):137-44.

(40) Stulnig TM, Zeyda M. Immunomodulation by polyunsaturated fatty acids: impact on T-cell signaling. Lipids 2004 Dec;39(12):1171-5.

(41) Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997 Jun 5;387(6633):569-72.

(42) Combs CK, Bates P, Karlo JC, Landreth GE. Regulation of beta-amyloid stimulated proinflammatory responses by peroxisome proliferator-activated receptor alpha. Neurochem Int 2001 Nov;39(5-6):449-57.

(43) Nakamura MT, Cheon Y, Li Y, Nara TY. Mechanisms of Regulation of Gene Expression by Fatty Acids. Lipids 2004;39(11):1077-83.

(44) Ruxton CHS, Calder PC, Reed SC, Simpson MJA. The impact of long-chain n-3 polyunsaturated fatty acids on human health. Nutr Res Rev 2005;18:113-29.

(45) Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation 2002 Nov 19;106(21):2747-57.

(46) Wang C, Harris WS, Chung M, Lichtenstein AH, Balk EM, Kupelnick B, et al. n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. Am J Clin Nutr 2006 Jul;84(1):5-17.

(47) Calder PC. n-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. Clin Sci (Lond) 2004 Jul;107(1):1-11.

(48) Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Lancet 1999 Aug 7;354(9177):447-55.

(49) Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. Lipids 2001 Sep;36(9):1007-24.

(50) Fortin PR, Lew RA, Liang MH, Wright EA, Beckett LA, Chalmers TC, et al. Validation of a meta-analysis: the effects of fish oil in rheumatoid arthritis. J Clin Epidemiol 1995 Nov;48(11):1379-90.

(51) Goldberg RJ, Katz J. A meta-analysis of the analgesic effects of omega-3 polyunsaturated fatty acid supplementation for inflammatory joint pain. Pain 2007 May;129(1-2):210-23.

(52) Calder PC. Use of fish oil in parenteral nutrition: Rationale and reality. Proc Nutr Soc 2006 Aug;65(3):264-77.

(53) Freeman MP, Hibbeln JR, Wisner KL, Davis JM, Mischoulon D, Peet M, et al. Omega-3 fatty acids: evidence basis for treatment and future research in psychiatry. J Clin Psychiatry 2006 Dec;67(12):1954-67.

(54) Appleton KM, Hayward RC, Gunnell D, Peters TJ, Rogers PJ, Kessler D, et al. Effects of n-3 long-chain polyunsaturated fatty acids on depressed mood: systematic review of published trials. Am J Clin Nutr 2006 Dec;84(6):1308-16.

(55) Richardson AJ. Omega-3 fatty acids in ADHD and related neurodevelopmental disorders. Int Rev Psychiatry 2006 Apr;18(2):155-72.

(56) Lauritzen L, Hansen HS, Jorgensen MH, Michaelsen KF. The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. Prog Lipid Res 2001 Jan;40(1-2):1-94.

(57) Cheatham CL, Colombo J, Carlson SE. N-3 fatty acids and cognitive and visual acuity development: methodologic and conceptual considerations. Am J Clin Nutr 2006 Jun;83(6 Suppl):1458S-66S.

(58) SanGiovanni JP, Parra-Cabrera S, Colditz GA, Berkey CS, Dwyer JT. Meta-analysis of dietary essential fatty acids and long-chain polyunsaturated fatty acids as they relate to visual resolution acuity in healthy preterm infants. Pediatrics 2000 Jun;105(6):1292-8.

(59) Abbas AK, Lichtman AH, Pober JS. Cellular and molecular immunology. 2nd ed ed. Philadelphia : W.B. Saunders; 1994.

(60) Roitt IM, Brostoff J, Male DK, David. Immunology. 2nd ed ed. Edinburgh ; London : Churchill Livingstone with Gower Medical; 1989.

(61) Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997 Oct 16;389(6652):737-42.

(62) Damoiseaux JG, Cautain B, Bernard I, Mas M, Breda Vriesman PJ, Druet P, et al. A dominant role for the thymus and MHC genes in determining the peripheral CD4/CD8 T cell ratio in the rat. J Immunol 1999 Sep 15;163(6):2983-9.

(63) Porcelli SA, Modlin RL. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. Annu Rev Immunol 1999;17:297-329.

(64) Young DC, Moody DB. T-cell recognition of glycolipids presented by CD1 proteins. Glycobiology 2006 Jul;16(7):103R-12R.

(65) Ryan JC, Naper C, Hayashi S, Daws MR. Physiologic functions of activating natural killer (NK) complex-encoded receptors on NK cells. Immunol Rev 2001 Jun;181:126-37.

(66) Fletcher J, Starr R. The role of suppressors of cytokine signalling in thymopoiesis and T cell activation. Int J Biochem Cell Biol 2005 Sep;37(9):1774-86.

(67) Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996 Mar;17(3):138-46.

(68) Romagnani S. The Th1/Th2 paradigm. Immunol Today 1997 Jun;18(6):263-6.

(69) Romagnani S. T-cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol 2000 Jul;85(1):9-18.

(70) Romagnani S. Immunologic influences on allergy and the TH1/TH2 balance. J Allergy Clin Immunol 2004 Mar;113(3):395-400.

(71) Romagnani S. The role of lymphocytes in allergic disease. J Allergy Clin Immunol 2000 Mar;105(3):399-408.

(72) Shamssain MH, Shamsian N. Prevalence and severity of asthma, rhinitis, and atopic eczema: the north east study. Arch Dis Child 1999 Oct;81(4):313-7.

(73) Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. Science 2002 Apr 19;296(5567):490-4.

(74) Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001 Oct 6;358(9288):1129-33.

(75) Great Britain Committee on the Medical Effects of Air Pollutants. HMSO; 1995.

(76) von Mutius E, Weiland SK, Fritzsch C, Duhme H, Keil U. Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. Lancet 1998 Mar 21;351(9106):862-6.

(77) Wijga AH, Smit HA, Kerkhof M, de Jongste JC, Gerritsen J, Neijens HJ, et al. Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: the PIAMA birth cohort study. Thorax 2003 Jul;58(7):567-72.

(78) Schwab JM, Chiang N, Arita M, Serhan CN. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. Nature 2007 Jun 14;447(7146):869-74.

(79) Zeldin DC. Epoxygenase pathways of arachidonic acid metabolism. J Biol Chem 2001 Sep 28;276(39):36059-62.

(80) Spector AA, Norris AW. Action of epoxyeicosatrienoic acids on cellular function. Am J Physiol Cell Physiol 2007 Mar;292(3):C996-1012.

(81) Harkewicz R, Fahy E, Andreyev A, Dennis EA. Arachidonate-derived dihomoprostaglandin production observed in endotoxin-stimulated macrophage-like cells. J Biol Chem 2007 Feb 2;282(5):2899-910.

(82) Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, et al. Markers to measure immunomodulation in human nutrition intervention studies. Br J Nutr 2005 Sep;94(3):452-81.

(83) Shaikh SR, Edidin M. Immunosuppressive effects of polyunsaturated fatty acids on antigen presentation by human leukocyte antigen class I molecules. J Lipid Res 2007 Jan;48(1):127-38.

(84) Yaqoob P, Calder PC. Fatty acids and immune function: new insights into mechanisms. Br J Nutr 2007 Oct;98 Suppl 1:S41-S45.

(85) Healy DA, Wallace FA, Miles EA, Calder PC, Newsholm P. Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. Lipids 2000 Jul;35(7):763-8.

(86) Switzer KC, McMurray DN, Chapkin RS. Effects of dietary n-3 polyunsaturated fatty acids on T-cell membrane composition and function. Lipids 2004 Dec;39(12):1163-70.

(87) Chapkin RS, Somers SD, Schumacher L, Erickson KL. Fatty acid composition of macrophage phospholipids in mice fed fish or borage oil. Lipids 1988 Apr;23(4):380-3.

(88) Geyeregger R, Zeyda M, Zlabinger GJ, Waldhausl W, Stulnig TM. Polyunsaturated fatty acids interfere with formation of the immunological synapse. J Leukoc Biol 2005 May;77(5):680-8.

(89) Sanderson P, MacPherson GG, Jenkins CH, Calder PC. Dietary fish oil diminishes the antigen presentation activity of rat dendritic cells. J Leukoc Biol 1997 Dec;62(6):771-7.

(90) Bagga D, Wang L, Farias-Eisner R, Glaspy JA, Reddy ST. Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. Proc Natl Acad Sci U S A 2003 Feb 18;100(4):1751-6.

(91) Roper RL, Brown DM, Phipps RP. Prostaglandin E2 promotes B lymphocyte Ig isotype switching to IgE. J Immunol 1995 Jan 1;154(1):162-70.

(92) Snijdewint FG, Kalinski P, Wierenga EA, Bos JD, Kapsenberg ML. Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. J Immunol 1993 Jun 15;150(12):5321-9.

(93) Miles EA, Aston L, Calder PC. In vitro effects of eicosanoids derived from different 20-carbon fatty acids on T helper type 1 and T helper type 2 cytokine production in human whole-blood cultures. Clin Exp Allergy 2003 May;33(5):624-32.

(94) Haby MM, Peat JK, Marks GB, Woolcock AJ, Leeder SR. Asthma in preschool children: prevalence and risk factors. Thorax 2001 Aug;56(8):589-95.

(95) Hodge L, Salome CM, Peat JK, Haby MM, Xuan W, Woolcock AJ. Consumption of oily fish and childhood asthma risk. Med J Aust 1996 Feb 5;164(3):137-40.

(96) Rees D, Miles EA, Banerjee T, Wells SJ, Roynette CE, Wahle KW, et al. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. Am J Clin Nutr 2006 Feb;83(2):331-42.

(97) Berger A, German JB, Chiang BL, Ansari AA, Keen CL, Fletcher MP, et al. Influence of feeding unsaturated fats on growth and immune status of mice. J Nutr 1993 Feb;123(2):225-33.

(98) Wallace FA, Miles EA, Evans C, Stock TE, Yaqoob P, Calder PC. Dietary fatty acids influence the production of Th1- but not Th2-type cytokines. J Leukoc Biol 2001 Mar;69(3):449-57.

(99) Zhang P, Smith R, Chapkin RS, McMurray DN. Dietary (n-3) polyunsaturated fatty acids modulate murine Th1/Th2 balance toward the Th2 pole by suppression of Th1 development. J Nutr 2005 Jul;135(7):1745-51.

(100) Kew S, Banerjee T, Minihane AM, Finnegan YE, Williams CM, Calder PC. Relation between the fatty acid composition of peripheral blood mononuclear cells and measures of immune cell function in healthy, free-living subjects aged 25-72 y. Am J Clin Nutr 2003 May;77(5):1278-86.

(101) Calder PC. N-3 polyunsaturated fatty acids, inflammation and immunity: pouring oil on troubled waters or another fishy tale? Nutrition Research 2001;21(1-2):309-41.

(102) Calder PC, Miles EA. Fatty acids and atopic disease. Pediatr Allergy Immunol 2000;11 Suppl 13:29-36.

(103) Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, et al. Developmental plasticity and human health. Nature 2004 Jul 22;430(6998):419-21.

(104) Gluckman PD, Hanson MA. Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. Pediatr Res 2004 Sep;56(3):311-7.

(105) Barker DJP. Mothers, babies, and disease in later life. London : BMJ Publishing Group, 1994; 1994.

(106) Barraclough CA. Production of anovulatory, sterile rats by single injections of testosterone propionate. Endocrinology 1961 Jan;68:62-7.

(107) Widdowson EM, McCance RA. Some effects of accelerating growth. I. General somatic development. Proc R Soc Lond B Biol Sci 1960 May 17;152:188-206.

(108) Widdowson EM, McCance RA. The effect of finite periods of undernutrition at different ages on the composition and subsequent development of the rat. Proc R Soc Lond B Biol Sci 1963 Oct 22;158:329-42.

(109) Rose G. Familial patterns is ischaemic heart disease. Br J Prev Soc Med 1964 Apr;18:75-80.

(110) Forsdahl A. Are poor living conditions in childhood and adolescence an important risk factor for arteriosclerotic heart disease? Br J Prev Soc Med 1977 Jun;31(2):91-5.

(111) Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1986 May 10;1(8489):1077-81.

(112) Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. Lancet 1989 Sep 9;2(8663):577-80.

(113) Barker DJ, Eriksson JG, Forsen T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. Int J Epidemiol 2002 Dec;31(6):1235-9.

(114) McKeigue PM, Marmot MG, Adelstein AM, Hunt SP, Shipley MJ, Butler SM, et al. Diet and risk factors for coronary heart disease in Asians in northwest London. Lancet 1985 Nov 16;2(8464):1086-90.

(115) Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Development 2000 Oct;127(19):4195-202.

(116) Harding JE, Owens JA, Robinson JS. Should we try to supplement the growth retarded fetus? A cautionary tale. Br J Obstet Gynaecol 1992 Sep;99(9):707-9.

(117) Kramer MS. Effects of energy and protein intakes on pregnancy outcome: an overview of the research evidence from controlled clinical trials. Am J Clin Nutr 1993 Nov;58(5):627-35.

(118) Susser M. Maternal weight gain, infant birth weight, and diet: causal sequences. Am J Clin Nutr 1991 Jun;53(6):1384-96.

(119) Herrick K, Phillips DI, Haselden S, Shiell AW, Campbell-Brown M, Godfrey KM. Maternal consumption of a high-meat, low-carbohydrate diet in late pregnancy: relation to adult cortisol concentrations in the offspring. J Clin Endocrinol Metab 2003 Aug;88(8):3554-60.

(120) Benediktsson R, Calder AA, Edwards CR, Seckl JR. Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. Clin Endocrinol (Oxf) 1997 Feb;46(2):161-6.

(121) Langley-Evans SC, Phillips GJ, Benediktsson R, Gardner DS, Edwards CR, Jackson AA, et al. Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. Placenta 1996 Mar;17(2-3):169-72.

(122) Yang K. Placental 11 beta-hydroxysteroid dehydrogenase: barrier to maternal glucocorticoids. Rev Reprod 1997 Sep;2(3):129-32.

(123) Edwards CR, Benediktsson R, Lindsay RS, Seckl JR. 11 beta-Hydroxysteroid dehydrogenases: key enzymes in determining tissue-specific glucocorticoid effects. Steroids 1996 Apr;61(4):263-9.

(124) Drake AJ, Walker BR. The intergenerational effects of fetal programming: non-genomic mechanisms for the inheritance of low birth weight and cardiovascular risk. J Endocrinol 2004 Jan;180(1):1-16.

(125) Langley-Evans S. Fetal programming of immune function and respiratory disease. Clin Exp Allergy 1997 Dec;27(12):1377-9.

(126) Coe CL, Lubach GR. Prenatal influences on neuroimmune set points in infancy. Ann N Y Acad Sci 2000;917:468-77.

(127) Coe CL, Lubach GR. Critical periods of special health relevance for psychoneuroimmunology. Brain Behav Immun 2003 Feb;17(1):3-12.

(128) Dvorak B, Stepankova R. Effects of dietary essential fatty acid deficiency on the development of the rat thymus and immune system. Prostaglandins Leukot Essent Fatty Acids 1992 Jul;46(3):183-90.

(129) Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic Acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J Nutr 2005 Jun;135(6):1382-6.

(130) Burdge GC, Jones AE, Wootton SA. Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*. Br J Nutr 2002 Oct;88(4):355-63.

(131) Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br J Nutr 2002 Oct;88(4):411-20.

(132) Nikkari T, Luukkainen P, Pietinen P, Puska P. Fatty acid composition of serum lipid fractions in relation to gender and quality of dietary fat. Ann Med 1995 Aug;27(4):491-8.

(133) Bakewell L, Burdge GC, Calder PC. Polyunsaturated fatty acid concentrations in young men and women consuming their habitual diets. Br J Nutr 2006 Jul;96(1):93-9.

(134) Crowe FL, Murray Skeaff C, Green TJ, Gray AR. Serum n-3 long-chain PUFA differ by sex and age in a population-based survey of New Zealand adolescents and adults. British Journal of Nutrition 2008;99(01):168-74.

(135) Giltay EJ, Gooren LJ, Toorians AW, Katan MB, Zock PL. Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. Am J Clin Nutr 2004 Nov;80(5):1167-74.

(136) Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N, Jr. Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. J Lipid Res 2001 Aug;42(8):1257-65.

(137) Pawlosky RJ, Hibbeln JR, Lin Y, Goodson S, Riggs P, Sebring N, et al. Effects of beef- and fish-based diets on the kinetics of n-3 fatty acid metabolism in human subjects. Am J Clin Nutr 2003 Mar;77(3):565-72.

(138) Pawlosky R, Hibbeln J, Lin Y, Salem N, Jr. n-3 fatty acid metabolism in women. Br J Nutr 2003 Nov;90(5):993-4.

(139) Burdge GC, Finnegan YE, Minihane AM, Williams CM, Wootton SA. Effect of altered dietary n-3 fatty acid intake upon plasma lipid fatty acid composition, conversion of [13C]alpha-linolenic acid to longer-chain fatty acids and partitioning towards beta-oxidation in older men. Br J Nutr 2003 Aug;90(2):311-21.

(140) Burdge GC, Calder PC. Dietary alpha-linolenic acid and health related outcomes: a metabolic perspective. Nutr Res Rev 2006;19:26-52.

(141) Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n-3 fatty acids in humans. Am J Clin Nutr 2006 Jun;83(6 Suppl):1467S-76S.

(142) Francois CA, Connor SL, Bolewicz LC, Connor WE. Supplementing lactating women with flaxseed oil does not increase docosahexaenoic acid in their milk. Am J Clin Nutr 2003 Jan;77(1):226-33.

(143) Jones AE, Murphy JL, Stolinski M, Wootton SA. The effect of age and gender on the metabolic disposal of [1-13C] palmitic acid. Eur J Clin Nutr 1998 Jan;52(1):22-8.

(144) Tavendale R, Lee AJ, Smith WC, Tunstall-Pedoe H. Adipose tissue fatty acids in Scottish men and women: results from the Scottish Heart Health Study. Atherosclerosis 1992 Jun;94(2-3):161-9.

(145) Raclot T, Langin D, Lafontan M, Groscolas R. Selective release of human adipocyte fatty acids according to molecular structure. Biochem J 1997 Jun 15;324 (Pt 3):911-5.

(146) Mattsson LA, Silfverstolpe G, Samsioe G. Fatty acid composition of serum lecithin and cholesterol ester in the normal menstrual cycle. Horm Metab Res 1985 Aug;17(8):414-7.

(147) Sumino H, Ichikawa S, Murakami M, Nakamura T, Kanda T, Sakamaki T, et al. Effects of hormone replacement therapy on circulating docosahexaenoic acid and eicosapentaenoic acid levels in postmenopausal women. Endocr J 2003 Feb;50(1):51-9.

(148) Giltay EJ, Duschek EJ, Katan MB, Zock PL, Neele SJ, Netelenbos JC. Raloxifene and hormone replacement therapy increase arachidonic acid and docosahexaenoic acid levels in postmenopausal women. J Endocrinol 2004 Sep;182(3):399-408.

(149) Ocke MC, Bueno-de-Mesquita HB, Goddijn HE, Jansen A, Pols MA, Van Staveren WA, et al. The Dutch EPIC food frequency questionnaire. I. Description of the questionnaire, and relative validity and reproducibility for food groups. Int J Epidemiol 1997;26 Suppl 1:S37-S48.

(150) Marra CA, de Alaniz MJ, Brenner RR. Effect of various steroids on the biosynthesis of arachidonic acid in isolated hepatocytes and HTC cells. Lipids 1988 Nov;23(11):1053-8.

(151) Gonzalez S, Nervi AM, Peluffo RO. Effects of estradiol and environmental temperature changes on rat liver delta 6 microsomal desaturase activity. Lipids 1986 Jul;21(7):440-3.

(152) Marra CA, de Alaniz MJ. Influence of testosterone administration on the biosynthesis of unsaturated fatty acids in male and female rats. Lipids 1989 Dec;24(12):1014-9.

(153) Herrera E. Implications of dietary fatty acids during pregnancy on placental, fetal and postnatal development--a review. Placenta 2002 Apr;23 Suppl A:S9-19.

(154) Herrera E, Amusquivar E. Lipid metabolism in the fetus and the newborn. Diabetes Metab Res Rev 2000 May;16(3):202-10.

(155) Herrera E, Amusquivar E, Lopez-Soldado I, Ortega H. Maternal lipid metabolism and placental lipid transfer. Horm Res 2006;65 Suppl 3:59-64.

(156) Herrera E, Gomez-Coronado D, Lasuncion MA. Lipid metabolism in pregnancy. Biol Neonate 1987;51(2):70-7.

(157) Haggarty P, Page K, Abramovich DR, Ashton J, Brown D. Long-chain polyunsaturated fatty acid transport across the perfused human placenta. Placenta 1997 Nov;18(8):635-42.

(158) Haggarty P. Effect of placental function on fatty acid requirements during pregnancy. Eur J Clin Nutr 2004 Dec;58(12):1559-70.

(159) Farquharson J, Jamieson EC, Logan RW, Patrick WJ, Howatson AG, Cockburn F. Age- and dietary-related distributions of hepatic arachidonic and docosahexaenoic acid in early infancy. Pediatr Res 1995 Sep;38(3):361-5.

(160) Uauy R, Hoffman DR, Mena P, Llanos A, Birch EE. Term infant studies of DHA and ARA supplementation on neurodevelopment: results of randomized controlled trials. J Pediatr 2003 Oct;143(4 Suppl):S17-S25.

(161) Koo WW. Efficacy and safety of docosahexaenoic acid and arachidonic acid addition to infant formulas: can one buy better vision and intelligence? J Am Coll Nutr 2003 Apr;22(2):101-7.

(162) Al MD, van Houwelingen AC, Kester AD, Hasaart TH, de Jong AE, Hornstra G. Maternal essential fatty acid patterns during normal pregnancy and their relationship to the neonatal essential fatty acid status. Br J Nutr 1995 Jul;74(1):55-68.

(163) Wijendran V, Bendel RB, Couch SC, Philipson EH, Thomsen K, Zhang X, et al. Maternal plasma phospholipid polyunsaturated fatty acids in pregnancy with and without gestational diabetes mellitus: relations with maternal factors. Am J Clin Nutr 1999 Jul;70(1):53-61.

(164) Stark KD, Beblo S, Murthy M, Buda-Abela M, Janisse J, Rockett H, et al. Comparison of bloodstream fatty acid composition from African-American women at gestation, delivery, and postpartum. J Lipid Res 2005 Mar;46(3):516-25.

(165) Burdge GC, Sherman RC, Ali Z, Wootton SA, Jackson AA. Docosahexaenoic acid is selectively enriched in plasma phospholipids during pregnancy in

Trinidadian women--results of a pilot study. Reprod Nutr Dev 2006 Jan;46(1):63-7.

(166) Stewart F, Rodie VA, Ramsay JE, Greer IA, Freeman DJ, Meyer BJ. Longitudinal assessment of erythrocyte fatty acid composition throughout pregnancy and post partum. Lipids 2007 Apr;42(4):335-44.

(167) Smith RW, Walsh A. Composition of liver lipids of the rat during pregnancy and lactation. Lipids 1975 Oct;10(10):643-5.

(168) Cunnane SC. Changes in essential fatty acid composition during pregnancy: maternal liver, placenta and fetus. Nutrition 1989 Jul;5(4):253-5.

(169) Chen ZY, Yang J, Cunnane SC. Gestational hyperlipidemia in the rat is characterized by accumulation of n - 6 and n - 3 fatty acids, especially docosahexaenoic acid. Biochim Biophys Acta 1992 Aug 19;1127(3):263-9.

(170) Burdge GC, Hunt AN, Postle AD. Mechanisms of hepatic phosphatidylcholine synthesis in adult rat: effects of pregnancy. Biochem J 1994 Nov 1;303 (Pt 3):941-7.

(171) Raghupathy R. Pregnancy: success and failure within the Th1/Th2/Th3 paradigm. Semin Immunol 2001 Aug;13(4):219-27.

(172) Reinhard G, Noll A, Schlebusch H, Mallmann P, Ruecker AV. Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. Biochem Biophys Res Commun 1998 Apr 28;245(3):933-8.

(173) Krishnan L, Guilbert LJ, Wegmann TG, Belosevic M, Mosmann TR. T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. J Immunol 1996 Jan 15;156(2):653-62.

(174) Piccinni MP, Scaletti C, Maggi E, Romagnani S. Role of hormone-controlled Th1- and Th2-type cytokines in successful pregnancy. J Neuroimmunol 2000 Sep 1;109(1):30-3.

(175) Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today 1993 Jul;14(7):353-6.

(176) Clarke AG, Kendall MD. The thymus in pregnancy: the interplay of neural, endocrine and immune influences. Immunol Today 1994 Nov;15(11):545-51.

(177) Tibbetts TA, DeMayo F, Rich S, Conneely OM, O'Malley BW. Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. Proc Natl Acad Sci U S A 1999 Oct 12;96(21):12021-6.

(178) Wilder RL. Hormones, pregnancy, and autoimmune diseases. Ann N Y Acad Sci 1998 May 1;840:45-50.

(179) Faas MM, Bouman A, Veenstra van Nieuwenhoven AL, van der SG, Moes H, Heineman MJ, et al. Species differences in the effect of pregnancy on lymphocyte cytokine production between human and rat. J Leukoc Biol 2005 Oct;78(4):946-53.

(180) Faas MM, Eenling R, van der SG, Moes H, Heineman MJ, Vos P. Cytokine production of in vitro stimulated peripheral lymphocytes during the course of pregnancy and pseudopregnancy in the rat. Am J Reprod Immunol 2006 Apr;55(4):282-90.

(181) Zhang QH, Huang YH, Hu YZ, Wei GZ, Lu SY, Zhao YF. Predominant Th2-type response during normal pregnancy of rats. Sheng Li Xue Bao 2004 Apr 25;56(2):258-62.

(182) Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J Immunol 1993 Nov 1;151(9):4562-73.

(183) Young HA, Ghosh P, Ye J, Lederer J, Lichtman A, Gerard JR, et al. Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. J Immunol 1994 Oct 15;153(8):3603-10.

(184) Katamura K, Fukui T, Kiyomasu T, Iio J, Tai G, Ueno H, et al. IL-4 and prostaglandin E2 inhibit hypomethylation of the 5' regulatory region of IFN-gamma gene during differentiation of naive CD4+ T cells. Mol Immunol 1998 Jan;35(1):39-45.

(185) White GP, Watt PM, Holt BJ, Holt PG. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. J Immunol 2002 Mar 15;168(6):2820-7.

(186) Falek PR, Ben Sasson SZ, Ariel M. Correlation between DNA methylation and murine IFN-gamma and IL-4 expression. Cytokine 2000 Mar;12(3):198-206.

(187) Dixit VD, Sridaran R, Edmonsond MA, Taub D, Thompson WE. Gonadotropin-releasing hormone attenuates pregnancy-associated thymic involution and modulates the expression of antiproliferative gene product prohibitin. Endocrinology 2003 Apr;144(4):1496-505.

(188) Ashworth A. Effects of intrauterine growth retardation on mortality and morbidity in infants and young children. Eur J Clin Nutr 1998 Jan;52 Suppl 1:S34-S41.

(189) Langley-Evans SC, Nutrition Society (. Fetal nutrition and adult disease : programming of chronic disease through fetal exposure to undernutrition. Wallingford : CABI Publishing; 2004.

(190) Jones CA, Vance GH, Power LL, Pender SL, Macdonald TT, Warner JO. Costimulatory molecules in the developing human gastrointestinal tract: a pathway for fetal allergen priming. J Allergy Clin Immunol 2001 Aug;108(2):235-41.

(191) Jarrett E, Hall E. Selective suppression of IgE antibody responsiveness by maternal influence. Nature 1979 Jul 12;280(5718):145-7.

(192) Wilson CB, Westall J, Johnston L, Lewis DB, Dower SK, Alpert AR. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. J Clin Invest 1986 Mar;77(3):860-7.

(193) McDade TW. Life history, maintenance, and the early origins of immune function. Am J Hum Biol 2005 Jan;17(1):81-94.

(194) Godfrey KM, Barker DJ, Osmond C. Disproportionate fetal growth and raised IgE concentration in adult life. Clin Exp Allergy 1994 Jul;24(7):641-8.

(195) Fergusson DM, Crane J, Beasley R, Horwood LJ. Perinatal factors and atopic disease in childhood. Clin Exp Allergy 1997 Dec;27(12):1394-401.

(196) Moore SE. Nutrition, immunity and the fetal and infant origins of disease hypothesis in developing countries. Proc Nutr Soc 1998 May;57(2):241-7.

(197) Ceesay SM, Prentice AM, Cole TJ, Foord F, Weaver LT, Poskitt EM, et al. Effects on birth weight and perinatal mortality of maternal dietary supplements in rural Gambia: 5 year randomised controlled trial. BMJ 1997 Sep 27;315(7111):786-90.

(198) Chandra RK. Antibody formation in first and second generation offspring of nutritionally deprived rats. Science 1975 Oct 17;190(4211):289-90.

(199) Landgraf MA, Martinez LL, Rastelli VM, Franco MC, Soto-Suazo M, Tostes RC, et al. Intrauterine undernutrition in rats interferes with leukocyte migration, decreasing adhesion molecule expression in leukocytes and endothelial cells. J Nutr 2005 Jun;135(6):1480-5.

(200) Beach RS, Gershwin ME, Hurley LS. Gestational zinc deprivation in mice: persistence of immunodeficiency for three generations. Science 1982 Oct 29;218(4571):469-71.

(201) Kochanowski BA, Sherman AR. Decreased antibody formation in iron-deficient rat pups-effect of iron repletion. Am J Clin Nutr 1985 Feb;41(2):278-84.

(202) Chapman C, Morgan LM, Murphy MC. Maternal and early dietary fatty acid intake: changes in lipid metabolism and liver enzymes in adult rats. J Nutr 2000 Feb;130(2):146-51.

(203) Armitage JA, Lakasing L, Taylor PD, Balachandran AA, Jensen RI, Dekou V, et al. Developmental programming of aortic and renal structure in offspring of rats fed fat-rich diets in pregnancy. J Physiol 2005 May 15;565(Pt 1):171-84.

(204) Rayon JI, Carver JD, Wyble LE, Wiener D, Dickey SS, Benford VJ, et al. The fatty acid composition of maternal diet affects lung prostaglandin E2 levels and survival from group B streptococcal sepsis in neonatal rat pups. J Nutr 1997 Oct;127(10):1989-92.

(205) Calder PC, Yaqoob P. The level of protein and type of fat in the diet of pregnant rats both affect lymphocyte function in the offspring. Nutrition Research 2000 Jul;20(7):995-1005.

(206) Lombardi VRM, Cagiao A, Fernandez-Novoa L, Alvarez XA, Corzo MD, Zas R, et al. Short term food supplementation effects of a fish derived extract on the immunological status of pregnant rats and their sucking pups. Nutrition Research 2001 Nov;21(11):1425-34.

(207) Korotkova M, Telemo E, Yamashiro Y, Hanson LA, Strandvik B. The ratio of n-6 to n-3 fatty acids in maternal diet influences the induction of neonatal immunological tolerance to ovalbumin. Clin Exp Immunol 2004 Aug;137(2):237-44.

(208) Lauridsen C, Stagsted J, Jensen SK. n-6 and n-3 fatty acids ratio and vitamin E in porcine maternal diet influence the antioxidant status and immune cell eicosanoid response in the progeny. Prostaglandins Other Lipid Mediat 2007 Aug;84(1-2):66-78.

(209) Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, et al. Maternal fish oil supplementation in pregnancy reduces interleukin-13 levels in cord blood of infants at high risk of atopy. Clin Exp Allergy 2003 Apr;33(4):442-8.

(210) Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, et al. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. J Allergy Clin Immunol 2003 Dec;112(6):1178-84.

(211) Mihrshahi S, Peat JK, Marks GB, Mellis CM, Tovey ER, Webb K, et al. Eighteen-month outcomes of house dust mite avoidance and dietary fatty acid modification in the Childhood Asthma Prevention Study (CAPS). J Allergy Clin Immunol 2003 Jan;111(1):162-8.

(212) Mihrshahi S, Peat JK, Webb K, Oddy W, Marks GB, Mellis CM. Effect of omega-3 fatty acid concentrations in plasma on symptoms of asthma at 18 months of age. Pediatr Allergy Immunol 2004 Dec;15(6):517-22.

(213) Peat JK, Mihrshahi S, Kemp AS, Marks GB, Tovey ER, Webb K, et al. Three-year outcomes of dietary fatty acid modification and house dust mite reduction in the Childhood Asthma Prevention Study. J Allergy Clin Immunol 2004 Oct;114(4):807-13.

(214) Marks GB, Mihrshahi S, Kemp AS, Tovey ER, Webb K, Almqvist C, et al. Prevention of asthma during the first 5 years of life: a randomized controlled trial. J Allergy Clin Immunol 2006 Jul;118(1):53-61.

(215) Almqvist C, Garden F, Xuan W, Mihrshahi S, Leeder SR, Oddy W, et al. Omega-3 and omega-6 fatty acid exposure from early life does not affect atopy and asthma at age 5 years. J Allergy Clin Immunol 2007 Jun;119(6):1438-44.

(216) Lauritzen L, Kjaer TM, Fruekilde MB, Michaelsen KF, Frokiaer H. Fish oil supplementation of lactating mothers affects cytokine production in 2 1/2-year-old children. Lipids 2005 Jul;40(7):669-76.

(217) Lauritzen L, Jorgensen MH, Olsen SF, Straarup EM, Michaelsen KF. Maternal fish oil supplementation in lactation: effect on developmental outcome in breast-fed infants. Reprod Nutr Dev 2005 Sep;45(5):535-47.

(218) Diakogiannaki E, Dhayal S, Childs CE, Calder PC, Welters HJ, Morgan NG. Mechanisms involved in the cytotoxic and cytoprotective actions of saturated versus monounsaturated long-chain fatty acids in pancreatic beta-cells. J Endocrinol 2007 Aug;194(2):283-91.

(219) Burdge GC, Wright P, Jones AE, Wootton SA. A method for separation of phosphatidylcholine, triacylglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction. Br J Nutr 2000 Nov;84(5):781-7.

(220) Low pressure Fractionation of Neutral Lipids: 3rd Procedure. Accessed on 18 August 2008;www.cyberlipid.org/fraction/frac0006.

(221) Carr RS, Neff JM. Quantitative semi-automated enzymatic assay for tissue glycogen. Comp Biochem Physiol B 1984;77(3):447-9.

(222) Serafini MT, Alemany M. A micromethod for the enzymatic estimation of the degree of glycogen ramification. J Biochem Biophys Methods 1987 Oct;15(1):33-9.

(223) Baldie G, Kaimakamis D, Rotondo D. Fatty acid modulation of cytokine release from human monocytic cells. Biochim Biophys Acta 1993 Nov 7;1179(2):125-33.

(224) Rotondo D, Earl CR, Laing KJ, Kaimakamis D. Inhibition of cytokine-stimulated thymic lymphocyte proliferation by fatty acids: the role of eicosanoids. Biochim Biophys Acta 1994 Sep 8;1223(2):185-94.

(225) Wilfinger WW, Mackey K, Chomczynski P. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques 1997 Mar;22(3):474-81.

(226) Verma AS, Shapiro BH. Sex-dependent expression of seven housekeeping genes in rat liver. J Gastroenterol Hepatol 2006 Jun;21(6):1004-8.

(227) Vandesompele J, De PK, Pattyn F, Poppe B, Van RN, De PA, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002 Jun 18;3(7):RESEARCH0034.

(228) Amusquivar E, Herrera E. Influence of changes in dietary fatty acids during pregnancy on placental and fetal fatty acid profile in the rat. Biol Neonate 2003;83(2):136-45.

(229) Burdge GC, Slater-Jefferies JL, Grant RA, Chung WS, West AL, Lillycrop KA, et al. Sex, but not maternal protein or folic acid intake, determines the fatty acid composition of hepatic phospholipids, but not of triacylglycerol, in adult rats. Prostaglandins Leukot Essent Fatty Acids 2008 Jan;78(1):73-9.

(230) Jayapalan S, Saboorian MH, Edmunds JW, Aukema HM. High dietary fat intake increases renal cyst disease progression in Han:SPRD-cy rats. J Nutr 2000 Sep;130(9):2356-60.

(231) Reed MJ, Cheng RW, Simmonds M, Richmond W, James VH. Dietary lipids: an additional regulator of plasma levels of sex hormone binding globulin. J Clin Endocrinol Metab 1987 May;64(5):1083-5.

(232) Rodriguez A, Sarda P, Nessmann C, Boulot P, Leger CL, Descomps B. Delta6- and delta5-desaturase activities in the human fetal liver: kinetic aspects. J Lipid Res 1998 Sep;39(9):1825-32.

(233) Reynolds LK, Ho HP, Taper LJ. Effect of caloric restriction during pregnancy on maternal and fetal body composition in the obese Sprague-Dawley rat. J Nutr 1984 Dec;114(12):2247-55.

(234) Garland HO, Atherton JC, Baylis C, Morgan MR, Milne CM. Hormone profiles for progesterone, oestradiol, prolactin, plasma renin activity, aldosterone and corticosterone during pregnancy and pseudopregnancy in two strains of rat: correlation with renal studies. J Endocrinol 1987 Jun;113(3):435-44.

(235) Lee HY, Sherwood OD. The effects of blocking the actions of estrogen and progesterone on the rates of proliferation and apoptosis of cervical epithelial and stromal cells during the second half of pregnancy in rats. Biol Reprod 2005 Oct;73(4):790-7.

(236) Leposavic G, Pesic V, Kosec D, Radojevic K, rsenovic-Ranin N, Pilipovic I, et al. Age-associated changes in CD90 expression on thymocytes and in TCR-

dependent stages of thymocyte maturation in male rats. Exp Gerontol 2006 Jun;41(6):574-89.

(237) Kendall MD, Atkinson BA, Munoz FJ, de la Riva C., Clarke AG, von Gaudecker B. The noradrenergic innervation of the rat thymus during pregnancy and in the post partum period. J Anat 1994 Dec;185 (Pt 3):617-25.

(238) Perez MA, Hansen RA, Harris MA, Allen KG. Dietary docosahexaenoic acid alters pregnant rat reproductive tissue prostaglandin and matrix metalloproteinase production. J Nutr Biochem 2006 Jul;17(7):446-53.

(239) Wallis EJ, Ramsay LE, Ul H, I, Ghahramani P, Jackson PR, Rowland-Yeo K, et al. Coronary and cardiovascular risk estimation for primary prevention: validation of a new Sheffield table in the 1995 Scottish health survey population. BMJ 2000 Mar 11;320(7236):671-6.

(240) Harris WS. n-3 fatty acids and serum lipoproteins: animal studies. Am J Clin Nutr 1997 May;65(5 Suppl):1611S-6S.

(241) Harris WS. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J Lipid Res 1989 Jun;30(6):785-807.

(242) Makrides M, Neumann MA, Byard RW, Simmer K, Gibson RA. Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. Am J Clin Nutr 1994 Aug;60(2):189-94.

(243) DeMar JC, Jr., Ma K, Bell JM, Igarashi M, Greenstein D, Rapoport SI. One generation of n-3 polyunsaturated fatty acid deprivation increases depression and aggression test scores in rats. J Lipid Res 2006 Jan;47(1):172-80.

(244) Raz A, Kamin-Belsky N, Przedecki F, Obukowicz M. Dietary fish oil inhibits delta-6 desaturase activity in vivo. Journal of the American Oil Chemists' Society 1998;75(2):241-5.

(245) Calder PC, Yaqoob P, Thies F, Wallace FA, Miles EA. Fatty acids and lymphocyte functions. Br J Nutr 2002 Jan;87 Suppl 1:S31-S48.

(246) Ly LH, Smith R, III, Chapkin RS, McMurray DN. Dietary n-3 polyunsaturated fatty acids suppress splenic CD4(+) T cell function in interleukin (IL)-10(-/-) mice. Clin Exp Immunol 2005 Feb;139(2):202-9.

(247) Sasaki T, Kanke Y, Kudoh K, Misawa Y, Shimizu J, Takita T. Effects of dietary docosahexaenoic acid on surface molecules involved in T cell proliferation. Biochim Biophys Acta 1999 Jan 4;1436(3):519-30.

(248) Sasaki T, Kanke Y, Nagahashi M, Toyokawa M, Matsuda M, Shimizu J, et al. Dietary docosahexaenoic acid can alter the surface expression of CD4 and CD8 on T cells in peripheral blood. J Agric Food Chem 2000 Apr;48(4):1047-9.

(249) Yaqoob P, Newsholme EA, Calder PC. The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. Immunology 1994 Aug;82(4):603-10.

(250) Peterson LD, Jeffery NM, Thies F, Sanderson P, Newsholme EA, Calder PC. Eicosapentaenoic and docosahexaenoic acids alter rat spleen leukocyte fatty acid composition and prostaglandin E2 production but have different effects on lymphocyte functions and cell-mediated immunity. Lipids 1998 Feb;33(2):171-80.

(251) Krauss-Etschmann S, Hartl D, Rzehak P, Heinrich J, Shadid R, Del CR-T, et al. Decreased cord blood IL-4, IL-13, and CCR4 and increased TGF-beta levels after fish oil supplementation of pregnant women. J Allergy Clin Immunol 2008 Feb;121(2):464-70.

(252) Jenski LJ, Bowker GM, Johnson MA, Ehringer WD, Fetterhoff T, Stillwell W. Docosahexaenoic acid-induced alteration of Thy-1 and CD8 expression on murine splenocytes. Biochim Biophys Acta 1995 May 24;1236(1):39-50.

(253) Josien R, Heslan M, Soulillou JP, Cuturi MC. Rat spleen dendritic cells express natural killer cell receptor protein 1 (NKR-P1) and have cytotoxic activity to select targets via a Ca2+-dependent mechanism. J Exp Med 1997 Aug 4;186(3):467-72.

(254) Carlyle JR, Zuniga-Pflucker JC. Lineage commitment and differentiation of T and natural killer lymphocytes in the fetal mouse. Immunol Rev 1998 Oct;165:63-74.

(255) Spits H, Blom B, Jaleco AC, Weijer K, Verschuren MC, van Dongen JJ, et al. Early stages in the development of human T, natural killer and thymic dendritic cells. Immunol Rev 1998 Oct;165:75-86.

(256) Leite-De-Moraes MC, Moreau G, Arnould A, Machavoine F, Garcia C, Papiernik M, et al. IL-4-producing NK T cells are biased towards IFN-gamma production by IL-12. Influence of the microenvironment on the functional capacities of NK T cells. Eur J Immunol 1998 May;28(5):1507-15.

(257) Maniongui C, Blond JP, Ulmann L, Durand G, Poisson JP, Bezard J. Age-related changes in delta 6 and delta 5 desaturase activities in rat liver microsomes. Lipids 1993 Apr;28(4):291-7.

(258) Jump DB, Botolin D, Wang Y, Xu J, Christian B, Demeure O. Fatty acid regulation of hepatic gene transcription. J Nutr 2005 Nov;135(11):2503-6.

(259) Tang C, Cho HP, Nakamura MT, Clarke SD. Regulation of human delta-6 desaturase gene transcription: identification of a functional direct repeat-1 element. J Lipid Res 2003 Apr;44(4):686-95.

(260) Li Y, Nara TY, Nakamura MT. Peroxisome proliferator-activated receptor alpha is required for feedback regulation of highly unsaturated fatty acid synthesis. J Lipid Res 2005 Nov;46(11):2432-40.

(261) Rodriguez-Cruz M, Tovar AR, Palacios-Gonzalez B, Del PM, Torres N. Synthesis of long-chain polyunsaturated fatty acids in lactating mammary gland: role of Delta5 and Delta6 desaturases, SREBP-1, PPARalpha, and PGC-1. J Lipid Res 2006 Mar;47(3):553-60.

(262) Matsuzaka T, Shimano H, Yahagi N, memiya-Kudo M, Yoshikawa T, Hasty AH, et al. Dual regulation of mouse Delta(5)- and Delta(6)-desaturase gene expression by SREBP-1 and PPARalpha. J Lipid Res 2002 Jan;43(1):107-14.

(263) Barcelo-Coblijn G, Collison LW, Jolly CA, Murphy EJ. Dietary alpha-linolenic acid increases brain but not heart and liver docosahexaenoic acid levels. Lipids 2005 Aug;40(8):787-98.