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UNIVERSITY OF SOUTHAMPTON

ACADEMIC UNIT OF HUMAN DEVELOPMENT & HEALTH

Faculty of Medicine

**The Effect of Maternal Nutrition on the Epigenetic Regulation by DNA  
Methylation of Hepatic Genes Involved in Glucose and Fat Metabolism in the  
Offspring**

by

***Samuel Peter Hoile***

Thesis for the degree of Doctor of Philosophy

December 2011

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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FACULTY OF MEDICINE

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THE EFFECT OF MATERNAL NUTRITION ON THE EPIGENETIC REGULATION  
BY DNA METHYLATION OF HEPATIC GENES INVOLVED IN GLUCOSE AND  
FAT METABOLISM IN THE OFFSPRING

By Samuel Peter Hoile

Altered maternal nutrition during pregnancy induces persistent phenotypic changes in offspring, which can be transmitted between generations and are associated with differences in disease risk. Epigenetic processes have been shown to underlie the effects of the early life environment on phenotype. It is not known if epigenetic marks are transferred directly between generations or established *de novo* in each generation. The extent to which induced changes in phenotype and epigenotype are affected by different nutrients and the timing of nutritional change, and the stability of such effects during the life course are also not known. This thesis describes a series of experiments to address these issues.

To investigate the mechanism by which induced phenotypic and epigenetic traits are transmitted between generations, dietary energy was increased 25% at conception in F0 female rats and maintained at this level to the F3 generation. Body weight, energy intake, glucose and lipid homeostasis and the expression of specific hepatic genes in the pregnant dams differed between generations. In offspring, fasting glucose and fatty acid homeostasis was dysregulated in the F1 generation, but showed progressively improved metabolic control in subsequent generations despite continued exposure to the high energy diet. The effects on plasma glucose concentration were associated with altered mRNA expression and promoter methylation of phosphoenolpyruvate carboxykinase (PEPCK). Altered DNA methylation was associated with changes in the epigenetic regulation of DNA methyltransferase (DNMT) 3a2. Protein restriction of the maternal diet modified the trajectory of changes in maternal and offspring phenotype, gene expression and epigenome between generations. These findings suggest that the changes in signals from mother to the fetus, induced by differences in the interaction between maternal phenotype and nutrition in each generation, provided a stimulus for modification of the phenotype of the offspring. Such effects appeared to produce beneficial adjustments in metabolic control via altered epigenetic control of specific genes.

The effects of different maternal nutritional exposures on the epigenetic regulation of specific genes was investigated by comparing differences in gene regulation following feeding of a protein restricted, 7 and 21% safflower oil and fish oil diets during pregnancy. No offspring showed altered methylation of the PEPCK promoter, whereas feeding altered fat diets lead to significant changes in the promoter methylation of fads2. This suggests that alterations in the epigenetic regulation of transcription of specific genes are related to the nature of the nutritional challenge.

In order to determine whether induced changes in the epigenetic regulation of genes are influenced by the timing of the nutritional challenge during the life course, folic acid supplementation was provided during pregnancy or to the offspring during their juvenile-pubertal (JP) period. Compared to prenatal folic acid supplementation, JP supplementation led to increased PEPCK and DNMT3a2 mRNA expression, and altered promoter methylation contingent on sex. This may be due to specific windows of plasticity in the epigenome. Differences in methylation of PEPCK and DNMT3a2 induced during development were found to change with increasing age. This suggests continued plasticity of the epigenome beyond the immediate developmental period.

Overall, the findings of these studies show that the nature of induced phenotypes involves signals produced by the interaction of the mother and her environment, and the timing and nature of the nutritional challenge acting via the epigenome. Such induced traits may confer harm or benefit both within and between generations according to the nature of the nutritional exposure.

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## DECLARATION OF AUTHORSHIP

I, Samuel Peter Hoile, declare that the thesis entitled

The Effect of Maternal Nutrition on the Epigenetic Regulation by DNA Methylation of  
Hepatic Genes Involved in Glucose and Fat Metabolism in the Offspring

and the work 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 or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- 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;
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- parts of this work have been published as:
  - Hoile, S.P., Lillycrop, K.A., Thomas, N.A., Hanson, M.A. & Burdge G.C. (2011) Dietary protein restriction during F<sub>0</sub> pregnancy in rats induces transgenerational changes in the hepatic transcriptome in female offspring. *PLoS one* 6(7) e21668
  - Hoile S.P., Grenfell, L.R., Lillycrop, K.A., Hanson, M.A. & Burdge G.C. (2011) Increasing the folic acid content of maternal or post-weaning diets induces differential changes in phosphoenolpyruvate carboxykinase mRNA expression and promoter methylation in rats. *British Journal Nutrition* 5:1-6
  - Burdge, G.C., Hoile, S.P., Uller, T., Thomas, N., Gluckman, P.D., Hanson, M.A. & Lillycrop, K.A. Progressive, transgenerational changes in offspring phenotype and epigenotype following nutritional transition. *PLoS one* 6(11) e28282
  - Hoile, S.P., Irvine, N.A., Kelsall, C., Feunteun, A., Lillycrop, K.A., Torrens, C., Calder, P.C., Hanson, M.A. & Burdge, G.C. Maternal fat intake alters 20:4n-6 and 22:6n-3 status, and the epigenetic regulation of *Fads2* in the offspring in rats. *Journal of Nutrition* – Submitted
  -
- parts of this work have been presented as posters and oral presentations at conferences, including:
  - The power of programming conference, Munich, 2010
  - Nutrition society Winter conference, London, 2010
  - Nutrition society annual conference, Reading, 2011
  - DOHAD 7<sup>th</sup> World Congress, Portland, 2011

**Signed:** .....

**Date:**.....

## **Acknowledgements**

I would firstly like to thank my supervisor Dr Graham Burdge for giving me the opportunity to do this PhD. It's been a long journey, but thanks to your constant support and mentoring this has been an enjoyable experience, where I've been able to develop both my expertise in the lab and research ideas. I would also like to thank my supervisor Dr Karen Lillycrop for her guidance and expertise at every stage of my PhD.

I would like to thank all of the members of the Developmental Epigenetics and Epigen groups, in particular Emma, Becky and Abi; even on the days (weeks) when my experiments didn't work, I've enjoyed every minute of my PhD. I've been fortunate to work alongside you all. I would particularly like to thank Emma, as well as your friendship, your wisdom and expertise in the lab have been invaluable.

I would like to thank my family, whose uncompromising faith in me has led me to reach my full potential. My friends, you have kept me sane for last three and a half years. Knowing I have time in the week to relax and have fun has made the stress of writing manageable, thank you.

Most importantly, I would like to thank Lucy. You have been the one most important person in this PhD, and my life. You have put up with all of the moaning and stress associated with this PhD, and yet you've always kept me smiling. Without you to share it with, nothing I accomplish would be worth anything.

## Abbreviations

| Abbreviation    | Full term                                       |
|-----------------|-------------------------------------------------|
| 11 $\beta$ HSD1 | 11 $\beta$ -hydroxysteroid dehydrogenase 1      |
| 11 $\beta$ HSD2 | 11 $\beta$ -hydroxysteroid dehydrogenase 2      |
| ALSPAC          | Avon longitudinal study of parents and children |
| Amy             | Amylase                                         |
| ANOVA           | Analysis of variance                            |
| AOX             | Acetyl oxidase                                  |
| APS             | Ammonium persulfate                             |
| ARA             | Arachidonic acid                                |
| ARTs            | Assisted reproductive technologies              |
| A <sup>vy</sup> | Agouti gene                                     |
| BMI             | Body mass index                                 |
| BRCA            | Breast cancer antigen 1                         |
| BWS             | Beckwith-Weidermann syndrome                    |
| C               | Control                                         |
| C/EBP           | CCAAT/enhancer binding protein                  |
| Camp            | Cyclic adenosine monophosphate                  |
| CBS             | Cystathione $\beta$ -synthase                   |
| cDNA            | Complementary DNA                               |
| CHD             | Coronary heart disease                          |
| ChIP            | Chromatin Immunoprecipitation                   |
| CNS             | Central nervous system                          |
| CPT-1           | Carnitine palmitoyltransferase-1                |
| CRE             | cAMP response element                           |
| CRU             | cAMP response unit                              |
| CVD             | Cardiovascular disease                          |
| Cyc             | Cyclophilin                                     |
| DES             | Diethylstilbestrol                              |
| DHA             | Docosahexanoic acid                             |
| DMEM            | Dulbecco's modified eagle medium                |
| DNA             | Deoxyribonucleic Acid                           |
| DNMTs           | DNA methyltransferases                          |
| dNTPs           | Deoxynucleotide triphosphates                   |
| DOHaD           | Developmental origins of health and disease     |
| EDTA            | Ethylenediaminetetraacetic acid disodium salt   |
| EGF             | Endothelial growth factor                       |
| EGFR            | Endothelial growth response factor              |
| ELISA           | Enzyme linked immunosorbent assay               |
| EMSA            | Electrophoretic mobility shift assay            |
| eNOS            | Endothelial nitric oxide synthase               |
| EPA             | Eicosapentanoic acid                            |
| ETS-1           | E-twenty six-1                                  |
| FAMES           | Fatty acid methyl esters                        |
| FO              | Fish oil                                        |
| FRA             | Folate receptor $\alpha$                        |
| Fru-1,6-BPase   | Fructose-1,6-bisphosphate                       |
| Fru-2,6-BPase   | Fructose-2,6-bisphosphate                       |
| Glc-6-Pase      | Glucose-6-phosphatase                           |
| GR              | Glucocorticoid receptor                         |
| GRE             | Glucocorticoid response element                 |
| GRU             | Glucocorticoid response unit                    |
| HATs            | Histone acetyltransferases                      |
| HDAC            | Histone deacetylase                             |

|               |                                                          |
|---------------|----------------------------------------------------------|
| HDL           | High density lipoprotein                                 |
| HMT           | Histone methyltransferase                                |
| HSP90         | Heat shock protein 90                                    |
| IGF-2         | Insulin-like growth factor 2                             |
| IPTG          | Isopropyl $\beta$ -D-1 thiogalactosidase                 |
| IUGR          | Intra uterine growth restricted                          |
| IVF           | In vitro fertilisation                                   |
| K/O           | Knock out                                                |
| LA            | Linoleic acid                                            |
| LB            | Luria broth                                              |
| LDL           | Low density lipoprotein                                  |
| LP            | Leupeptin                                                |
| MAOA          | Monoamine oxidase A                                      |
| MeCP          | Methyl CpG binding protein 2                             |
| mRNA          | Messenger RNA                                            |
| MMP           | Matrix metalloprotein                                    |
| MTHFR         | Methyltetrahydrofolatereductase                          |
| NDT           | Nucleotide dispensing tip                                |
| NMU           | N-Nitroso-N-methylurea                                   |
| NOS           | Nitric oxide synthase                                    |
| NTDS          | Neural tube defects                                      |
| PAR           | Predictive adaptive response                             |
| PBS           | Phosphate buffered saline                                |
| PC            | Phosphatidylcholine                                      |
| PCR           | Polymerase chain reaction                                |
| PE            | Phosphatidylethanolamine                                 |
| PEP           | Phosphoenolpyruvate                                      |
| PEPCK         | Phosphoenolpyruvate carboxykinase                        |
| PFK-1         | Phosphofructokinase-1                                    |
| PFK-2         | Phosphofructokinase-2                                    |
| PKs           | Protein kinases                                          |
| PLB           | Passive lysis buffer                                     |
| PMSF          | Phenylmethanesulfonylfluoride                            |
| POMC          | Propiomelanocortin                                       |
| PPAR $\alpha$ | Peroxisome proliferator activated receptor $\alpha$      |
| PR            | Protein restricted                                       |
| PRF           | Protein restricted plus folic acid                       |
| PUFA          | Polyunsaturated fatty acid                               |
| RBL           | Retinoblastoma-like protein                              |
| RFC1          | Reduced folate transporter-1                             |
| RM1           | Rat and mouse No.1 maintenance diet                      |
| RNA           | Ribonucleic acid                                         |
| RNA Pol II    | RNA polymerase II                                        |
| RT-PCR        | Reverse transcription-PCR                                |
| RXRA          | Retinoic X receptor $\alpha$                             |
| SAH           | S-adenosyl homocysteine                                  |
| SAM           | S-adenosyl methionine                                    |
| SAO           | Safflower oil                                            |
| SNPS          | Single nucleotide polymorphisms                          |
| SP            | Specificity protein                                      |
| STAT          | Signal transducer and activator of transcription         |
| TAE           | Tris-acetate-EDTA                                        |
| TAG           | Triacylglyceride                                         |
| TEMED         | Tetremethylethylenediamine                               |
| X-Gal         | 5-bromo-4-choloro-3-indoyl- $\beta$ -D-galactopyranoside |



# Chapter 1

## Introduction



## **1. Introduction**

### **1.1 The cardio-metabolic disease pandemic**

There is a worldwide pandemic of heart disease, predominantly coronary heart disease and stroke, and a group of related conditions including type 2 diabetes mellitus and hypertension known collectively as the metabolic syndrome. These diseases are caused primarily by an increase in levels of obesity and represent a major health burden in terms of mortality, morbidity, and financial cost. In Westernised nations, the main factor driving the increasing prevalence of cardio-metabolic disease is the ready availability of cheap, energy-dense foods, together with an increasingly sedentary life style. In developing nations, increased prevalence of cardio-metabolic disease is primarily a feature of migration from rural subsistence culture to an urban Westernised life style. However, developing countries suffer the double health burden of increasing cardio-metabolic disease together with continuing malnutrition and infectious disease in rural communities. In this context, it is of concern that nations with the highest predicted increase in the prevalence of cardio-metabolic disease are those which can least afford it. For example, a meta-analysis of over 30 studies world-wide showed that although the world-wide prevalence of diabetes incidence is predicted to increase from 2.8% to 4.4% from 2000 to 2030, the increase in India alone is predicted to be 151%<sup>[1]</sup>. A similar pattern is also seen in Zimbabwe, where in 1994, 17.4% of 25-49 year olds were classified as obese<sup>[2]</sup>.

In the United Kingdom, one in three deaths are caused by cardiovascular disease (CVD), accounting for around 191,000 deaths per year. The most common causes of death from CVD are coronary heart disease (CHD) and stroke, which account for 46% and 23% of CVD deaths, respectively<sup>[3]</sup>. CVD costs the UK economy around £30.7 billion per year, this constitutes approximately 47% health care costs, 27% loss of production and 26% spent on informal care of people with CVD<sup>[3]</sup>.

Type 2 diabetes mellitus and obesity are traditionally thought of as conditions which occur in adults. For example, a study in Micronesia determined

that diabetes becomes prevalent at 45-55 years old in females and 55-65 years old in males, and obesity presents itself in 15-25 year olds<sup>[4]</sup>. However, there is an increasing concern that children are becoming overweight. It has been estimated that 10% of the world's school children are overweight and one quarter of these are obese<sup>[5]</sup>. In the UK, 29% of girls and 31% of boys aged 2 to 15 years old are classified as over-weight or obese<sup>[6]</sup>. An implication of these findings is that the burden of cardio-metabolic disease in the next generation will be substantially greater than in the current adult population, possibly accompanied by shorter life expectancy and a greater burden on the health care systems

### **1.1.2 Factors which predispose to cardio-metabolic disease**

The risk factors for cardio-metabolic disease are well established, where it is influenced by both genetics and environment. The medical risk factors for the disease are well understood. Increased blood pressure, both systolic and diastolic, are related to an increased risk developing the disease. 45% of heart attacks in Western Europe are due to abnormal blood lipids. As well as being independent risk factors for CVD, being overweight or obese leads to an increase in blood pressure, blood cholesterol and impaired glucose tolerance. Men with type 2 diabetes have a 2-4 fold risk of CVD, whereas for women this is increased to a 3-5 fold risk<sup>[3]</sup>. Alternative explanations for differences between people, in risk of cardio-metabolic disease are, poor diet, in particular salt intake, smoking, alcohol consumption and physical inactivity. Preventions are in place at the nationwide scale to reduce environmental risk factors for CVD, for example campaigns to reduce salt and energy intake in the population. The benefits of understanding the interactive role of the environment on alterations in gene regulation, in terms of disease risk are clear. However, the factors which predispose an individual cause of cardio-metabolic disease are complex.

### **1.1.3 Genetic factors in cardio-metabolic disease**

The role of gene polymorphisms in the cardio-metabolic disease has been studied extensively.

### **1.1.3.1 Evidence from candidate gene studies**

Lusis and Arnett provide a detailed list of risk factors for coronary heart disease, which have significant genetic factors, with the main genes highlighted having roles in determining plasma cholesterol and triglyceride concentrations<sup>[7,8]</sup>. The role of polymorphisms in CVD risk is dependent on environmental factors. This is shown clearly in the epsilon 3 allele of apolipoprotein E, where a two-fold risk of CVD is seen in smokers, compared with non-smokers<sup>[9]</sup>. Hyperhomocysteinemia has been linked to an increased prevalence of cardiovascular disease. The 677C→T mutation of the methyltetrahydrofolate reductase (MTHFR) gene was found to be present in 15% of cardiovascular disease patients and around 5% of control patients<sup>[10]</sup>. The mutation was associated with a three-fold increase in prevalence of cardiovascular disease. In a large meta-analysis, polymorphisms of nitric oxide synthase (NOS3) were tested for links with CVD<sup>[11]</sup>. The study found two polymorphisms, Glu298Asp and 786 T→C, where both were independently associated with cardiovascular disease (odds ratio 1.17 for both).

### **1.1.3.2 Evidence from genome wide association studies**

Genome wide association studies have shown significant correlations between cardiovascular disease risk and specific genes<sup>[12,13]</sup>. In a study of 8,090 African Americans from 5-population based cohorts 6 regions were significantly associated with cardiovascular disease risk, CDKN2A/CDKN2B, HDL-C, FADS1-3, PLTP, LPL and ABCA1<sup>[12]</sup>. All measured regions had previously been shown to be significant in Caucasians also<sup>[12]</sup>. Further advances in genome wide association studies have looked to determine if loci predicted trait levels at all ages. Using participants from the longitudinal Bogalusa Heart Study, Smith et al showed significant effects of CETP with plasma HDL levels and apolipoprotein E with LDL levels<sup>[13]</sup>. Single nucleotide polymorphisms (SNPs) at specific loci were found to have significant age effects for 12 measured phenotypes, such as ESRRB/VASH1 and CDH5/TK5, which were significantly associated with diastolic blood pressure and glucose homeostasis, risk factors for cardiovascular disease, respectively.

Cardiovascular disease represents a major issue in terms of morbidity world-wide. Although progress has been made in understanding the role of genetics and contributing risk factors in the increased risk of the disease, it is not known why individuals with the same genotype and external environment display different disease risk. Risk factors for cardiovascular disease, such as obesity, are now presenting in children, as well as in adulthood. It is important, now more than ever, to understand the link between the early life environment and altered disease risk.

#### **1.1.4 Epidemiology**

Although numerous confounding factors are present, human observations have shown clear examples of early life origins of specific diseases. The following collection of studies aims to give an insight into the data obtained from human observations. The retrospective studies have looked at differences in growth during early life within a population and the prospective studies have looked at outcomes of individuals who differ in certain factors, within a sample cohort.

##### **1.1.4.1 Retrospective observations**

By looking at a large number of subjects, studies have confirmed the relationship between low birth weight and an increased risk of disease in later life<sup>[14,15,16,17,18,19]</sup>. Good record keeping in the early 20<sup>th</sup> century allowed investigation into the relationship between mother's pelvic size, fetal growth and death from stroke in a sample of 13,249 men in Hertfordshire and Sheffield born between 1907 and 1930. Mortality from cardiovascular disease was found to be associated with a small head circumference, thinness or shortness at birth and an altered ratio of placental weight to birth weight<sup>[17,19]</sup>. Measurements of maternal placental weight, birth weight and blood pressure in adults born in Preston between 1935 and 1943 have shown a strong relationship between placental weight and birth weight and systolic and diastolic blood pressure. An increase in birth weight was associated with an increase in mean blood pressure. The relationships between placental weight and blood pressure, and birth weight and

blood pressure were independent of each other, meaning a baby with a large placental weight and small birth weight was found to have the highest blood pressure at age 45-54<sup>[14]</sup>. When considering the similar results shown in these studies it is encouraging to know that all the results use the same guidelines for low birth weight (< 5.5 lb). Studies such as these are limited by the number of confounding variables present, meaning causal relationships cannot be established. They fail to provide potential mechanisms for the relationships seen and due to the numerous environmental differences between each study group it is unwise to generalise to the whole population. The main limiting factor in human studies is that due to ethical reasons they must remain observations, meaning we cannot tell what will occur under more severe circumstances.

During the winter of 1944-1945, the Western Netherlands suffered from a severe reduction in food supplies as a result of Germany's actions in the Second World War. During this period, known as the Dutch hunger winter, calorific intake, which was adequate before the war, fell to below 1000 kcal/day by November 26<sup>th</sup> 1944 and by April 1945 was as low as 500 kcal/day. Following liberation on May 5<sup>th</sup> 1945 food supplies were returned to normal levels. Children's rations were well protected throughout the famine, never falling below 1000 kcal/day<sup>[15,16,20,18]</sup>. The effects of the famine on birth weight and disease risk in later life have been well characterised in numerous studies, each paying close attention to the timing of the famine in regards to the stage of pregnancy (early, mid or late-gestation). A decline in mean birth weight of 300g and an increase in rate of perinatal death was seen in offspring, following famine exposure during late gestation<sup>[15,16]</sup>. Coronary heart disease was measured in 296 adults born during the famine, it was found that an increase in coronary heart disease was only seen in those exposed to famine during early gestation compared to a control group (non-exposed). Those exposed during early gestation tended to have smaller head circumferences and lower birth weights, when compared to the control group<sup>[21]</sup>. At age 50, men and women exposed to famine during early gestation were the only group who had significantly increased BMI, low density lipoprotein (LDL) -high density lipoprotein (HDL) ratio and plasma apolipoprotein B concentrations and significantly reduced plasma HDL

and apolipoprotein A concentrations when compared to non-exposed adults<sup>[22,23,24]</sup>. This resulted in a more atherogenic lipid profile, giving an increased risk of coronary heart disease in these individuals. The Dutch hunger winter highlights the importance of timing of exposure to altered nutrition. It clearly indicates that it is during early development when lifelong alterations in phenotype can be set up. This is useful in two ways, firstly the fact that cardiovascular disease is increased in offspring in mothers exposed to famine during the first trimester tells us those systems which were developing during famine exposure are those at risk of alterations. Secondly it shows that once each stage of development is complete it is no longer at risk of environmentally induced alterations. Although it must be mentioned that more recent findings from Burdge et al, have shown that dietary modification during the juvenile period of rats has the potential to reverse the effects of the maternal diet, indicating the changes caused during early development are sensitive to change<sup>[25]</sup>. Transgenerational transmission of phenotypes following the Dutch Winter Famine will be discussed later.

Human studies in Norway, India and Finland have shown similar results to the UK and Netherlands, in terms of development, disease risk and the role of catch up growth<sup>[26,27,28,29]</sup>. Catch-up growth has been shown to be a risk factor for the development of heart disease, stroke, type II diabetes, and hypertension. Studies agree that slow infant growth followed by catch up growth which leads to an average or above average BMI at aged 7 leads to the highest death rate in Helsinki, Finland<sup>[27,28]</sup>. Although it is clear that different countries will have different nutritional intakes, as well as different environmental exposures, the similar results present in the different populations suggest the mechanisms responsible for altered phenotype are not a result of specific adaptations following migration.

#### **1.1.4.2 Prospective studies**

A number of non-communicable diseases, such as cancer, osteoporosis and diabetes have been linked to a poor early life environment. A focus of the research in the field of DOHaD has been metabolic syndrome. Emerging evidence has shown that lower birth weight and altered placental growth are risk factors for

cardiovascular disease, type 2 diabetes, high blood pressure, high cholesterol and abnormal glucose and insulin metabolism in adulthood<sup>[30,31,32]</sup>. Maternal weight gain during pregnancy, maternal metabolism and signalling across the placenta have also been shown to be factors in metabolic syndrome, with emphasis on the restriction of fetal growth due to the size of the mother<sup>[33]</sup>.

As shown in figure 1.1 disease risk is associated with both low birth weight and high birth weight. High birth weight (defined as  $\geq 4,500$  g) is linked to an increase risk of cancer, in particular breast cancer, hepatoblastoma and childhood myeloid leukaemia<sup>[34]</sup>. Like other predictive adaptive responses leading to stable alterations in genes, these phenotypes are only seen if the disease-inducing environmental cues are encountered later in life.

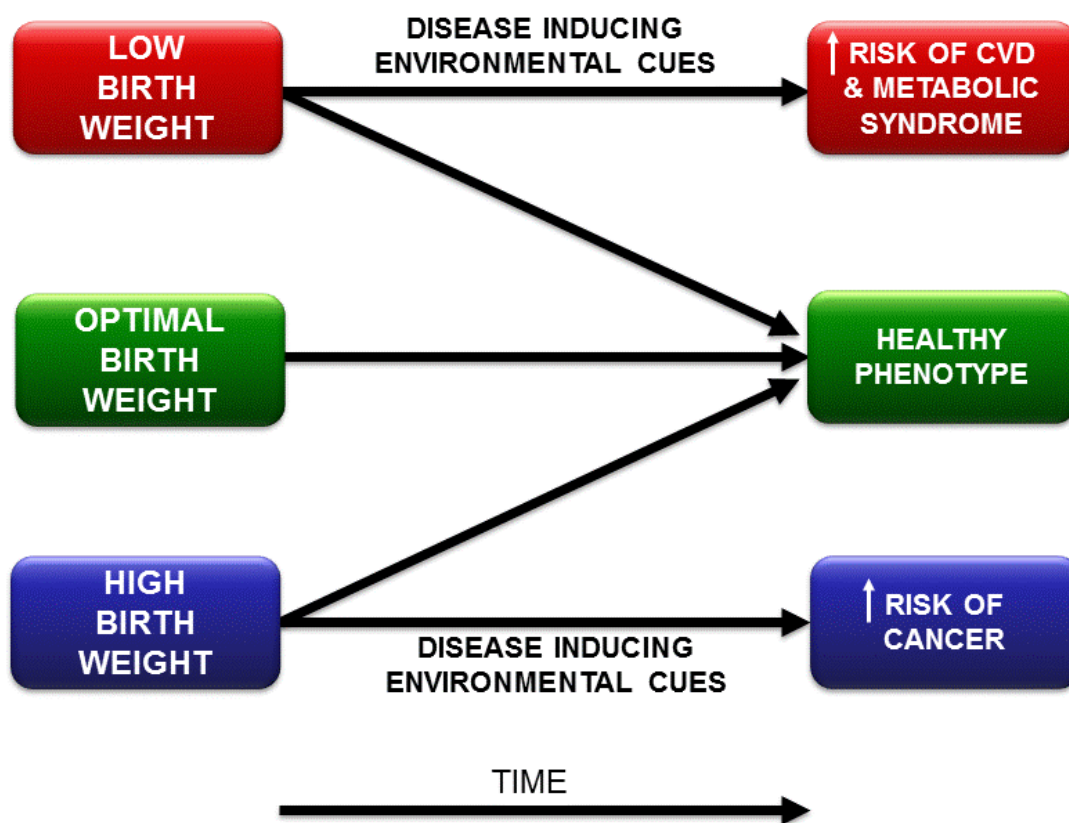


**Figure 1.1 Diagram showing the association between birth weight and later life risk of disease.** In the absence of disease inducing environmental cues, both high and low birth weight will result in a healthy phenotype. In the presence of disease inducing environmental cues throughout life, a low birth weight will result in an increased risk of CVD and metabolic syndrome. In the presence of disease inducing environmental cues throughout life, a high birth weight will result in an increased risk of cancer. An optimal birth weight does not increase the risk of disease.

Infant weight gain and catch-up growth play a vital role in phenotype induction, particularly in development of disease. This is due to the small window of plasticity present in early infancy. Lucas et al have shown that in terms of lung function in adulthood, a combination of both low fetal growth and high infant growth lead to the least beneficial lung development. This sub-optimal development resulted in diminished lung function and an increased risk of chronic obstructive airway disease. Interestingly these findings were not related to birth weight, only growth<sup>[35]</sup>. A review of studies has found that following low birth weight, a high level of catch-up growth in infancy leads to increased blood pressure in adulthood<sup>[32]</sup>. This review looked at a large number of studies encompassing both males and females of all ages and races. Although these studies seem to show the deleterious effects of catch-up growth, a more recent review of similar literature on weight gain in general by Adair et al indicates that any period of weight gain during life is related to an elevated adult blood pressure<sup>[36]</sup>. The review shows that catch-up growth in infancy and young childhood displayed no greater risk compared to other periods of weight gain<sup>[36]</sup>. When considering the evidence it is not clear whether the data on catch-up growth are a result of weight gain alone, or if the window of plasticity in infancy is responsible. In order to confirm this further experiments must be carried out, which distinguish catch-up growth in infancy from growth following excess calorific intake in later life. It is clear however that the concept of weight gain, as a period of change in nutritional intake, provide evidence for the negative result of a predictive adaptive response (PAR) mismatch following birth. Studies into timing of compensatory growth will inevitably lead to benefits on human health, where optimum periods of growth, in terms of reduced detrimental outcomes, will be identified.

Cohort studies have been invaluable in showing the developmental origins of adult disease. The next collection of studies that will be discussed are cohort studies from different areas in the United Kingdom, followed by those worldwide. The Southampton Women's Survey was established to measure the pre-pregnancy characteristics of women aged 20-34 years old living in the city of Southampton. The study followed up the women who became pregnant and



focused on fetal development, post-natal growth and the way in which these are associated with risk of chronic disease later in life. Early data has shown a decrease in maternal liver blood flow at 36 weeks gestation in women who were slim before pregnancy, compared to those who were not<sup>[37]</sup>. Similar to previous findings, babies who were smaller at birth or who had high post-natal weight gain showed a poorer lung function<sup>[37]</sup>. The Nurses' Health study, set up in 1976, provides one of the most significant insights into the health of woman. Although not intended as a cohort to investigate development, the 121,700 participant study has found that a birth weight of over 4.54 kg is associated with a two fold increase in adult onset rheumatoid arthritis in later life<sup>[38]</sup>. A further cohort prospective study which provided links between body size at birth and later health is the Avon Longitudinal Study of Parents and Children (ALSPAC). The study found that an increase in birth weight was associated with an 18% reduction in risk of definite psychosis-like symptoms at age 12<sup>[39]</sup>. The study also looked at the relationship between childhood growth and the same symptoms, and found no association<sup>[39]</sup>. This shows that the direction of phenotype established during prenatal development cannot be changed by patterns of growth in childhood. These cohort studies have used prospective methods to follow the emerging changes in phenotype following different maternal nutrition. As well as the timing of nutritional exposure, it is apparent that the magnitude and duration of the altered phenotype is also dependent on the extent of the nutritional alteration.

Although useful in allowing large populations to be measured and providing relationships between the in utero environments and onset of disease in later life, human observations do not provide the opportunity to explore mechanisms of phenotype induction. As in most observations there are too many variables, such as socio economic status, lifestyle and maternal and paternal background, to allow true causality of results to be determined. Human data is based either on retrospective analysis of past pregnancies or prospective studies covering pre-pregnancy and post-natal life. In retrospective studies, desired data may be lacking. Prospective studies are highly useful in allowing collection of many desired

measurements at various time points, however they require a considerable amount of time to complete, in most cases a lifetime.

Animal studies in controlled environments, whereby interfering variables may be eliminated, must therefore be used in order to establish patterns of developmental origins of adult disease. Due to the shorter lifespan of animals used in these studies, they provide an invaluable method to assess phenotype and genotype in offspring in an appropriate time scale. Transmission of these phenotypes and mechanisms underlying these phenotypes must also be determined.

#### **1.1.4.3 Animal studies**

The most common animal models used in the field of the developmental origins of health and disease are those in rodents, specifically rats. In order to appreciate the benefits of rat models, in terms of understanding human disease development, it is also important to understand their limitations. Although the physiology of the rat is very similar to that of the human, as both have four-chambered hearts and an identical respiratory system, key differences are present. For example, humans have a gall bladder, whereas rats have a bile duct, and where humans have an appendix, rats do not. One key difference in development between humans and rat is seen in the formation of the brain. In humans, development of the brain occurs in utero, whereas in rats, brain development is post-natal. A study by Romijn showed that the rat brain at postnatal day 12-13 is equivalent, to a human baby at term, in terms of development<sup>[40]</sup>.

##### **1.1.4.3.1 Low protein rat model**

The low protein model has been used extensively in rodents to show how a change in protein intake during pregnancy leads to alterations in metabolism and phenotype in the offspring. The following section aims to give an introduction to the model and summarise some of the key findings. The first use of the low protein model, from which all further uses of the model are based, was by Langley-Evans et al<sup>[41]</sup>. The study showed that a maternal low protein diet before and during

pregnancy led to hypertension in 4 week old rat offspring. The finding was independent of sex and was not seen in adult rats fed a low protein diet for a 14 day period, showing the importance of the in utero exposure to the low protein diet<sup>[41]</sup>. Langley-Evans et al went on to show how a low protein diet leads to altered programming by maternally-derived glucocorticoids due to the decrease in 11  $\beta$ -hydroxysteroid dehydrogenase 2 (11 $\beta$ HSD2), a glucocorticoid inactivating placental enzyme. Overexposure to maternally derived glucocorticoids in the low-protein model resulted in altered physiology and endocrine functions in the offspring and as a result disease, caused by loss of homeostatic control<sup>[42,43]</sup>. Experiments by Langley-Evans have used variants of the low protein diet, where protein levels of the diet have stayed constant at 9% casein, but nutrient levels have varied and control diets used have been either 22% or 18% casein. These slight variations in macro and micronutrient intake have led Langley-Evans to describe their critical importance when designing diet protocols<sup>[44]</sup>. Similar to Langley-Evans, a study by Bertram et al also looked at altered glucocorticoid receptor and 11 $\beta$ HSD1 and 11 $\beta$ HSD2 expression in offspring of low-protein diet fed mothers. They found that fetal and neonatal offspring displayed a glucocorticoid dependent rise in systolic blood pressure and a two fold increase in mRNA expression of the glucocorticoid receptor in kidney, liver and brain tissue, compared to control offspring. This increase in gene expression was three fold higher in juvenile and adult rats<sup>[45]</sup>. Similar variants of the low-protein rat model have been used to investigate the developmental origins of disease. These studies have found that the model resulted in a reduced cell number in embryos signifying a slower rate of cellular proliferation<sup>[46]</sup>, smaller birth size of offspring, rapid deterioration of glucose tolerance, increased hepatic PEPCK mRNA expression and decreased glucokinase activity in adulthood, leading to altered metabolism and capacity to deal with glucose<sup>[47,48]</sup>. Ozanne et al, used a perfusion method to assess hepatic glucose output, following a maternal protein restriction<sup>[49]</sup>. The results showed an increased capacity for gluconeogenesis, larger liver lobule size and insulin and glucagon resistance in the reduced protein offspring, when compared to control offspring. As well as changes in liver metabolism caused by

the low protein diet, skeletal muscle and adipose tissue also showed changes in glucose uptake and insulin response<sup>[49]</sup>. As discussed earlier in humans, the relationship between high birth weight and increased cancer incidence has been shown. In rats however, although a relationship between birth weight and cancer risk is present, it appears to be the inverse relationship to that seen in humans. Low birth weight achieved by feeding a low-protein diet (8% casein) was found to correlate to an increased risk of breast cancer, when induced via injection with carcinogen, N-Nitroso-N-methylurea (NMU). High birth weight however, was linked to an increased risk of breast cancer, hyperglycaemia and insulin resistance. When compensatory growth was followed maternal protein restriction levels of early mammary tumour incidence was doubled when compared the control group<sup>[50]</sup>. The forms of cancer investigated in the human and rat studies differ, meaning direct species comparisons cannot be made. The studies do however show the importance of using animal models to look at potential mechanisms present in humans, rather than for direct comparisons.

To further understand mechanisms behind phenotype induction in the low protein rat model, numerous studies have fortified the low-protein diet with extra nutrients, such as folic acid or specific amino acids. In a study where a maternal low-protein diet led to comprised maternal cardiovascular adaptations, folate supplementation improved maternal vascular function and restored offspring cardiovascular function<sup>[51]</sup>. Another study used fortification of a 9% casein low-protein diet with 3% glycine, 3% alanine or 3% urea. The study found that the maternal low-protein diet led to significantly higher blood pressure, when compared to an 18% casein control group<sup>[52]</sup>. The difference was not seen in the low-protein plus glycine group, whereas it was in the other dietary groups. Body weight however was significantly reduced in all low-protein groups compared to the control group, except for the low-protein plus urea group. This study confirms the importance of glycine in normal cardiovascular development<sup>[52]</sup>.

The low protein rat model provides a working example of phenotypic changes caused by altered nutritional intake during pregnancy. The low protein model can be used experimentally to identify possible mechanisms behind the

phenotypic changes seen. By looking closer at systems affected, it will be possible to understand how these differences are brought about, how they are maintained and potentially how they can be avoided.

#### **1.1.4.3.2 Intra uterine growth restriction**

A number of studies have used the intrauterine growth restriction (IUGR) model to investigate the mechanisms behind the developmental origins of disease. The study objective is to cause restriction of growth in the fetus. The most abundant variant of this method involves decreasing maternal nutrition; another cause of growth restriction involves bilateral ligation of the uterine artery. Symptoms of metabolic syndrome have been induced in rats via the IUGR model. These experiments used bilateral ligation of the uterine artery at day 17 of gestation, which led to placental insufficiency and resulted in growth restriction of the offspring. These experiments have found that IUGR leads to obesity, type 2 diabetes and metabolic syndrome<sup>[27,53]</sup>.

Studies using the IUGR model, in rat, have shown an increase in development of hypertension in offspring. IUGR achieved by reduced uterine perfusion in late gestation resulted in low birth weight in offspring. At 4 weeks of age, mean arterial pressure was significantly increased in IUGR offspring, compared to control offspring<sup>[54]</sup>. At 8 weeks of age the elevations in arterial pressure were associated with sex-specific decreases in body weight in male and female offspring, versus control rats. IUGR as a result of chronic maternal under nutrition also led to signs of hypertension in offspring. When dams were fed a 30% *ad libidum* diet during pregnancy, offspring were significantly smaller from term, until postnatal week 12, compared to offspring of *ad libidum* fed dams<sup>[55]</sup>. Blood pressure, measured by tail cuff plethysmography, showed an elevated systolic blood pressure at postnatal 30, 40 and 56 weeks in IUGR offspring. Two studies which used 50% *ad libidum* diet to induce IUGR in offspring, showed significantly altered  $\beta$ -cell development and risk of obesity in offspring<sup>[56,57]</sup>. Feeding a 50% *ad libidum* diet from day 15 of pregnancy led to a significantly decreased body weight and  $\beta$ -cell mass, compared to control offspring, at postnatal day 1<sup>[57]</sup>. Following 21 days of *ad libidum* feeding

there were no significant differences between IUGR and control offspring, in terms of body weight, however a 40% decrease in  $\beta$ -cell mass was retained. The result suggests that IUGR did not result in persistent changes in postnatal growth, but did result in impaired  $\beta$ -cell development. When 50% *ad libidum* was fed to pregnant dams from day 10 of pregnancy, offspring showed a decreased plasma leptin and increased ghrelin concentration<sup>[56]</sup>. The decrease in programming of orexigenic hormones led to an increase in incidence of offspring obesity. The model of intra uterine growth restriction used by Vickers et al involves maternal under nutrition (30% of an *ad libidum* diet) during gestation, followed by a post-weaning control or high fat diet (30% fat). The hyper caloric postnatal diet exacerbated decreased locomotor activities present following the period of under nutrition, which is part of the fetal origin of hyperphagia, obesity and hypertension seen in these animals<sup>[58,59]</sup>. One major problem associated with studies such as these described is the fact that such dramatic nutritional alterations are used to cause a different phenotype. In terms of the Dutch Winter Famine, it could be argued that the 70% reduction in food intake used for the under nutrition group is directly relevant, when using calorific intakes stated for the period in Ravelli 1999<sup>[23]</sup>. However the fact the Dutch Winter famine occurred over 50 years ago in one town in one country implies a lack of relevance to the population in general. These flaws present in animal models reduce the relevance of the findings when considering humans.

A study by Battista et al has shown how a small nutritional change during pregnancy can lead to fetal growth restriction. By feeding a low-sodium diet during the last week of gestation in rats (equivalent in time to the last trimester of human gestation), growth restriction was achieved<sup>[60]</sup>. High blood pressure, elevated plasma urea concentration, decreased urinary sodium and renal dysfunction was seen in adulthood of the IUGR offspring, and creatinine levels were augmented in females only. Although an unlikely comparison to human gestation the study gives a good insight into the consequences of short time nutritional deficiencies and highlights the importance of sex in IUGR rats<sup>[60]</sup>.

IUGR in offspring is not caused by altered nutrition alone. Decreased oxygen supply to dams during pregnancy (hypoxia), has been used experimentally to

induce reduced growth in offspring. In a study by Xu et al, IUGR was caused by hypoxia (12% O<sub>2</sub>) or nutrient restriction (40% *ad libitum*), in Sprague-Dawley rats. In 4-month old offspring, maternal hypoxia resulted in increased collagen I and III mRNA expression, increased left and right ventricular weight/ body weight ratio, decreased MMP-2 activity and decreased functional recovery after ischemia/reperfusion, compared to both control and nutrient restriction induced IUGR offspring groups. There were no differences between nutrient restriction IUGR and control offspring. At 7 months, however, IUGR offspring, regardless of the method of restriction, showed increased left ventricular weight/birth weight ratio, increased collagen I and III mRNA expression, decreased MMP-2 activity and decreased cardiac recovery, compared to control offspring<sup>[61]</sup>. The results suggest that IUGR caused by hypoxia leads to earlier incidence of altered phenotype in offspring, however by 7 months, the method of IUGR had no significant effect on phenotype. Williams et al showed that IGUR, caused by hypoxia, led to altered adult vascular function in offspring<sup>[62]</sup>. Using L-NAME, NO synthase inhibition, in small mesenteric arteries, it was shown that IUGR offspring at 4-months, but not 7-months, had reduced methacholine-induced endothelium dependent relaxation<sup>[62]</sup>.

Although the previous studies do provide insight into the way in which these systems work, they fail to show the minimal level of alteration in nutrition required to bring about these changes. It would be interesting to see if the differences in specific nutrient intake seen across different population groups in humans were sufficient to bring across evident changes in phenotype in experimental animals, if equivalent nutrition was given.

#### **1.1.4.3.3 Maternal high fat feeding**

In the past 50 years the type and amount of fat intake in the Western World has changed dramatically<sup>[63]</sup>. One example of this is the introduction of margarine, which led to an increase in the dietary intake of trans 18:2n-6.

The effects of feeding a high fat diet have been shown experimentally in rodents<sup>[64,65,66,67,68,69,70,71,72]</sup>, where the most common dietary alteration used is

increased supply of saturated fatty acids to dams. Feeding rats a diet containing 20% dietary lard during both pregnancy and lactation led to a defect in endothelium-dependent relaxation in 180 day old offspring, compared to control animals<sup>[71]</sup>. In offspring of Sprague-Dawley rats fed a lard-rich diet during pregnancy and suckling, followed by a control diet fed post-weaning, aortic stiffness was increased and endothelium-dependent relaxation was decreased, compared to control offspring<sup>[65]</sup>. These two studies show a clear effect of maternal fat intake on offspring vascular function. Glucose homeostasis is significantly altered in offspring of high fat fed dams. Increased risk of type-2-diabetes and obesity was shown in male offspring of high fat-fed dams<sup>[64]</sup>. These effects were independent of maternal obesity, birth weight and post-natal macronutrient intake. In a study by Cerf et al, feeding dams a high fat diet during pregnancy and lactation or throughout lactation alone, led to a greater fasting glucose concentration 30 minutes following a meal, which was normalised at 60 minutes<sup>[67]</sup>. Although a high fat diet fed during pregnancy alone did lead to glucose intolerance, this was significantly milder compared to pregnancy and lactation or lactation alone. A further study by the group concluded that 'high fat programming' is induced by maternal saturated fat intake during periods of gestation and/or lactation and programs the physiology and metabolism of the offspring in early life, through failure of pancreatic  $\beta$  cell development and function<sup>[66]</sup>.

Feeding a high fat diet to dams leads to altered regulation of food intake and body weight in offspring. In a multigenerational mouse study, three generations of C57BL/6 dams were fed a chow or high fat diet. The study found that high fat offspring weighed less at weaning, but gained more during the first 8 weeks of life, to achieve a significantly higher body weight at adulthood, compared to control offspring<sup>[68]</sup>. In humans, energy balance, metabolism and behaviour are significantly altered in offspring following a maternal high fat diet<sup>[70]</sup>. The effects, thought to involve altered programming of neural pathways, led to an increased risk of development of mental health disorders, such as anxiety. In rats, altered responses to fat-enriched foods were shown in offspring of high-fat fed dams<sup>[69]</sup>.



The effect was associated with altered dopamine transmission in the nucleus accumbens, and was also related to altered stimulant drug response.

Increased dietary fat intake in rats during the 6 weeks before mating, pregnancy and lactation led to an increase in PPAR $\alpha$ , and a decrease in PPAR $\gamma$  mRNA expression in new-born offspring. This suggests an increase in fatty acid  $\beta$ -oxidation and a decrease in adipose formation in the offspring. The mRNA expression level of PPAR $\alpha$  was negatively correlated ( $r = -0.743$ ,  $p < 0.05$ ) with serum triglyceride concentration<sup>[72]</sup>. The previous studies have all shown that consumption of a high-fat diet during pregnancy and/or lactation, leads to significant changes in the offspring with effects on vascular function, energy intake and body weight and glucose and lipid homeostasis.

Despite the majority of studies into the effects of dietary fat focusing on the detrimental effects of increased saturated fat, as is prevalent in Western society, a number of studies have investigated effects of other forms of fat on phenotypic outcomes. In Sprague-Dawley rats, an n-3 polyunsaturated fatty acid (PUFA) deficient diet during the perinatal period led to an increase in blood pressure in later life<sup>[73]</sup>. The persistence of the effect was confirmed as the effect was still significant, albeit to a lesser extent, following repletion of the fatty acids.

C57BL/6 mice were fed diets identical to Western diets, in terms of cis- and trans-isomers of C18:1, in order to determine whether the maternal transfer of trans-fatty acids, through breast milk, alters offspring body composition and glucose metabolism<sup>[74]</sup>. The study showed that high trans fatty acid feeding led to growth retardation, decreased adiposity and higher fasting glucose levels in offspring, which were not related to insulin insensitivity. Again, these studies have shown effects of maternal fat intake on body weight and glucose homeostasis in offspring. It is clear that the effects of fat are determined by the type and amount of fat altered, however mechanisms may be preserved between fat types.

#### **1.1.4.3.4 Catch up growth**

Catch up growth in humans was discussed previously. An experiment in mice using cross fostering has shown the role catch up growth plays in both health and in longevity. In terms of the gestational diet low-protein diet pups died significantly younger than control diet offspring. Limiting catch up growth was found to be significantly beneficial in terms of lifespan by decreasing the risk of life threatening disease. The biggest increase in life span was seen when comparing postnatal growth restricted mice to catch up mice fed an obesity inducing diet<sup>[75]</sup>. Studies into catch up growth show the importance of matching the diet during embryonic development and early life development. However, they also disagree with the PAR hypothesis, in that the most beneficial diet during life is not always one that mimics the maternal diet during development.

#### **1.1.4.3.5 Maternal overfeeding**

Maternal overfeeding leads to an altered phenotype in offspring similar to under nutrition during pregnancy. Experiments by Kirk et al have shown that offspring of obese rats have an amplified and prolonged neonatal leptin surge<sup>[76]</sup>. This resulted in altered regulation of food intake in the offspring, and therefore obesity risk was increased. In an experiment by Plagemann et al, overfeeding neonatal rats led to rapid early weight gain and an overall metabolic syndrome phenotype. The overfeeding resulted in a higher level of methylation at the promoter of the anorexiogenic gene, proopiomelanocortin (POMC)<sup>[77]</sup>. These studies involving over nutrition during early development have used high fat and high energy diets to result in offspring with increased disease risk. Interestingly the previously discussed studies have shown how severe under and over nutrition can result in phenotypes with similar disease risks, though likely by different mechanisms.

#### **1.1.4.3.6 Endocrine disruptor exposure**

The effect of an altered developmental environment on offspring phenotype in adult life is not limited to dietary modifications or oxygen supply to dams. In rats, exposure of dams to certain phthalate esters led to reproductive toxicity in males<sup>[78]</sup>. Exposure of pregnant dams to endocrine disruptors vinclozin or methoxychlor induced decreased spermatogenic capacity and increased incidence of male infertility in male offspring<sup>[79]</sup>. These studies show that through the maternal circulation, all aspects of the external environment are subject to transmission to the developing offspring, and must therefore be considered, in terms of potential altered disease risk in adulthood.

#### **1.1.4.3.7 Summary of findings from animal models**

Dietary animal models have been vital in widening the understanding of the developmental origins of adult disease, as they allow controlled dietary manipulations and in depth measurements to be taken. These studies have used changes in the maternal nutrition during pregnancy to lead to a phenotypic change in the offspring, in order to increase our understanding. Changes in phenotype must be a result of altered protein synthesis, as a result of altered levels of gene transcription. The next step is to investigate the effect of timing of the nutritional variation and to understand what is happening at the genome level. It is important to find out if it is only during the pregnancy when changes in phenotype can be brought about. It is not known if differences are only seen following extreme nutritional alterations, or if subtle changes in phenotype result from subtle changes in nutritional alterations. Although movement of nutrients across the placenta clearly plays a role in the developing embryo not much is known about other factors, such as hormones, stress and temperature. The limits of these adjustments in developmental trajectory are not fully understood. Whatever mechanisms are responsible for changes in phenotype displayed by the described papers, it is clear that they must be passed during cell division.

It is evident that the maternal effect is vital in transmission of signals from the external environment to the developing offspring, yet no studies to date have

investigated the metabolism and phenotype of the dams exposed to the altered nutrition during pregnancy. An altered metabolism in dams will lead to an altered capability to accurately represent the external environment to the developing offspring. An understanding of changes in maternal metabolism associated with pregnancy will help us to better understand changes caused by nutritional exposures, such as those seen in the previously described studies.

### **1.1.5 Models of the early life origins of disease**

The findings of the studies described above have formed the basis of models to explain the association between the early life environment and later risk of disease.

#### **1.1.5.1 The thrifty gene hypothesis**

The hypothesis has arisen to explain why diabetes, a disease with a strong genetic basis<sup>[80]</sup>, is prevalent when natural selection aims to ensure only genes with a reproductive advantage are transmitted. The theory states that for 99% of man's history we have survived with a 'feast or famine' lifestyle, meaning man utilises all nutrients when nutrition is available, in preparation for the famine which may be ahead<sup>[81]</sup>. Although a great advantage in times of food scarcity, Neel argues that as a result of natural selection modern day man to favour expression of 'thrifty genes', which result in fat accumulation during times of abundant nutrition<sup>[80]</sup>. However, in the current age it seems our metabolism is programmed to store energy, in the form of triglyceride in adipose tissue, for a famine which, due to the current availability of nutrition, never arrives. Over a lifetime, this is leading to an increase in fat accumulation. This has been shown to have links with an increase in obesity and diabetes in our current population<sup>[81]</sup>. However, this hypothesis was proposed in 1962 and to date no 'thrifty genes' have been identified.

#### **1.1.5.2 Fetal programming or the developmental origins of health and disease (DOHaD) hypothesis**

The developmental origins of health and disease (DOHaD) hypothesis suggests that disease risk in later life is influenced by the developmental environment. The hypothesis, which provides a link between genetics and environment, is a progression of Barker's fetal programming hypothesis, which suggests that a malnourished fetus makes adaptations during development that may prove to be detrimental in later life<sup>[82]</sup>. The fetal programming hypothesis was suggested after investigation of the link between ischaemic heart disease and both neonatal and postnatal mortality rates in England and Wales<sup>[82,83]</sup>. Following this finding, Barker further investigated the link between early life and disease risk in adulthood<sup>[82,84,14,85,86,87,88]</sup>. Using samples from the Helsinki cohort, Barker showed that the relationship between development and disease was not restricted to the UK<sup>[87]</sup>. In a study of 3,676 men born between 1934 and 1944 in Helsinki, Finland, hospital admission for or death from coronary heart disease was increased if men were from a low social class, low household income, had a low education, had accelerated weight gain in infancy or were thin at birth. These observations led Barker to conclude that those that grow slow *in utero* maintain an altered metabolism throughout life<sup>[87]</sup>. A key finding of Barker's work has been the introduction of the role of nutrition in the hypothesis of the early life origins of disease<sup>[83]</sup>.

Barker formulated the DOHaD hypothesis, a more general theory that encompasses not only under nutrition, but an altered developmental environment in general, which alters the body's setting of metabolism and hormones as well as the number of cells in key organs<sup>[83]</sup>. Barker concluded that signals from the external environment pass from the mother to the fetus and lead to constant changes in the phenotype of the fetus.

### 1.1.5.3 Predictive-adaptive response and the mismatch hypothesis of the early life origins of cardio-metabolic disease

There is evidence from natural history that parents pass 'developmental cues' to their developing offspring which induce phenotypic changes that predict the environment to which the offspring will be involved. Fetal adaptations during development can be seen in nature. Meadow vole pup (*Microtus pennsylvanicus*) development is affected by hours of light in a day, leading to altered maturation and preparedness for winter, through increased coat thickness<sup>[89]</sup>. Adult desert locust (*Scistocera gregaria*) show density-dependent polymorphisms<sup>[90]</sup>. With obvious survival advantages, overcrowding leads to production of migratory, gregarious offspring, whereas a low population density leads to production of sedentary, isolated locusts<sup>[90]</sup>. Presence of a predator during pregnancy leads to offspring helmet and neck-pedestal formation in *Daphnia cucullata* and *Daphnia pulex*, respectively<sup>[90]</sup>. In *Taeniopygia gutta* (Zebra Finch), male attractiveness can cause mothers to influence the attractiveness of offspring phenotype<sup>[91]</sup>. In a non-genomic mechanism, the size of early stage F2 eggs was significantly greater in response to an experimentally manipulated father's attractiveness (red leg ring compared to pale green leg ring). Gilbert suggests that attractiveness of the father led to a shift in the allocation of resources of the mother, leading to larger eggs. As the effects of paternal attractiveness were present in the F2 generation, the paternal effect may have implications in terms of evolution of the species.

Again, the aim of these adaptations is for offspring to survive to the reproductive age. Based on these observations, Gluckman et al. hypothesised that the embryo adapts to environmental cues in a way which predict the future environment<sup>[92,93]</sup>. If this prediction is correct and the later environment matches that on which the maternal signals were based, this will confer a survival advantage. If however, the environment later in life is significantly different to that predicted then the resulting mismatch may confer a disadvantage. For example, in humans if the signals from the mother predict an energy poor future environment, possibly as a result of placental insufficiency, which would be reflected in lower birth weight, the fetus may develop a phenotype which tends to conserve nutrients.

However, if the infant is born into an energy rich environment then it would tend to store energy to a greater extent than an infant whose phenotype predicted the energy dense environment. Gluckman et al suggest that such metabolic/environmental mismatch may underlie the increased risk of cardio-metabolic disease in infants born at the lower end of the normal birth weight range.

In a study by Cleal et al, pre and post-natal under nutrition led to greater weight gain by weaning in offspring, compared to offspring of control chow fed dams<sup>[94]</sup>. Postnatal under nutrition alone led to altered growth cardiovascular function and basal hypothalamo-pituitary-adrenal axis activity in offspring in adulthood. Offspring that were fed an adequate postnatal diet following prenatal under nutrition exhibited cardia-hypertrophy and altered cardiovascular function in adulthood. The experiment provides evidence for the PAR hypothesis, where the least detrimental outcomes were seen in pre and post-natal under nutrition and adequate maternal and offspring groups, compared to the mismatch offspring groups.

However, the predictive adaptive response hypothesis does not appear to apply in all cases. One example of this has been shown by Bruce et al<sup>[95]</sup>. Female mice were fed either a control (21% kcal fat) or high fat diet (45% kcal fat) before and during gestation. Following birth, offspring were fed either the control or high fat diet. At 15 weeks, offspring of dams fed the high fat diet control diet, which were fed the control diet after weaning, had fatty liver disease. However, the severity of disease was increased if both the mother and offspring were fed a high fat diet<sup>[95]</sup>. According to the PAR hypothesis, the offspring of dams fed the high fat diet should have adapted to the increased fat exposure and have a smaller response to the post-weaning high fat diet than offspring of dams fed the control diet

#### **1.1.5.4 The accelerator hypothesis**

It has been suggested that risk of both type I and type II diabetes mellitus is more dependent on postnatal weight gain than the developmental environment<sup>[96]</sup>. According to the hypothesis weight gain is necessary to cause an accelerated loss of  $\beta$ -cells through apoptosis<sup>[96]</sup>. Although multiple investigations have shown an inverse relationship between age of onset of diabetes and body mass index standard deviation score, it is not the case in all studies<sup>[97]</sup>. Although a useful theory in terms of diabetes susceptibility and prevention, it is limited to the prevalence of a single disease, so is unlikely to benefit the understanding of the link between environment and disease risk.

### **1.2 Maternal adaptations during pregnancy**

Pregnancy and lactation represent a major metabolic demand on the mother, both from the developing fetus and from the need to support the functional changes in reproductive organs. In order to meet these demands there are a number of well-characterised adaptations to maternal nutrient metabolism and to cardiovascular function. Thus the interaction between the diet of the mother, her physiological (for example insulin sensitivity) and physical status (for example relative fat and muscle mass) before conception, and the adaptations to her physiology during pregnancy and lactation, together with the activity of the placenta, provide the 'developmental environment' from which the fetus may derive cues that lead to physiological changes that determine future disease risk.

#### **1.2.1 Adaptations to maternal cardiovascular function during pregnancy**

In humans, cardiac output is increased by the fifth week of pregnancy<sup>[98]</sup>. This increase continues up to 24 weeks, where it is 45% above the level seen in non-pregnant women. The increase in cardiac output is brought about by an increase in heart rate, which occurs from five weeks of gestation and continues until 32 weeks. Following the increase in heart rate, stroke volume is increased at 8 weeks, where it reaches its maximum around 20 weeks<sup>[98]</sup>. By the end of the second trimester, cardiac output is at its maximum; following pregnancy however, it



is reduced by 43%<sup>[99,98]</sup>. The increase in cardiac output is associated with a 40% increase in blood volume<sup>[98]</sup>, which is brought about by both production of several hormones by the fetus and placenta and utero placental circulation acting as an arteriovenous shunt<sup>[100]</sup>. In effect, the increased plasma volume is due to the reabsorption of Na<sup>+</sup> and water retention, as a result of increased aldosterone production. This in turn is a result of placental oestrogen production<sup>[100]</sup>. By acting as a low-resistance circuit, the utero-placental circulation leads to an increase in maternal blood flow, increasing cardiac output and nutrient delivery for further fetal growth<sup>[100]</sup>. The increase in cardiovascular function is dependent on the type of pregnancy, where twin pregnancies show a 15% higher cardiac output compared to singleton pregnancies<sup>[98]</sup>. This further increase is due to an exaggerated increase in heart rate present in twin pregnancies, and an increased left arterial diameter.

#### **1.2.1.2 Adaptations to maternal fat and carbohydrate metabolism during pregnancy and lactation**

Metabolic adaptations in the mother during pregnancy ensure the adequate growth and development of the fetus, provide the fetus with adequate energy substrate that are needed following birth, meet the altered needs of the mother for the increased physiological demands of pregnancy and provide the mother with sufficient energy stores and substrate to cope with the demands of pregnancy as well as those of labour and lactation<sup>[101]</sup>. Changes in the metabolic function of maternal organs facilitate to meet the nutritional demands of the mother and fetus, this involves substantial changes throughout pregnancy from an anabolic state at the start of pregnancy to a catabolic state during late pregnancy<sup>[102,102]</sup>.

During pregnancy, nutrients are transported from the maternal circulation to the developing fetus via the placenta. Regulation of nutrient partitioning during pregnancy involves coordinated control in metabolism between tissues (homeorhetic control)<sup>[103]</sup>. Compared to non-pregnant women, during early pregnancy women are in an anabolic condition, where storage of energy substrate

is a primary goal. In early pregnancy, due to the increased plasma volume, compared to non-pregnant women, there is an apparent decrease in maternal plasma glucose and amino acid concentration, although the total amount remains unaltered. Glucose is the most abundant nutrient crossing the placenta. Despite an increase in gluconeogenesis during early pregnancy, this leads to hypoglycaemia in mothers. In early pregnancy plasma fatty acids become the main energy source<sup>[101]</sup>. Hepatic conversion of glucose to triacylglyceride, for storage in adipose tissue, is increased two-fold, combined with a diminished release of fatty acids from adipose. Combined with hyperphagia<sup>[104]</sup>, the enhanced lipogenesis leads to an increase in storage of energy in the form of triacylglyceride (TAG) in adipose tissue<sup>[105]</sup>.

In late pregnancy, when fetal growth is increased, the effectiveness of insulin decreases, compared to early pregnancy (i.e. insulin resistance). As the glucose utilisation rate by maternal tissue is decreased, this indicates that the mother supplies glucose to the developing fetus at the expense of herself<sup>[106]</sup>. According to Catalano, insulin sensitivity is decreased by 56% at 34-36 weeks gestation<sup>[107]</sup>. The reduction in sensitivity of maternal tissue to insulin is ameliorated shortly after delivery<sup>[108]</sup>. Compared to early pregnancy, late pregnancy is associated with decreased TAG storage in adipose and mobilisation of fat stores as non-esterified fatty acids<sup>[105]</sup>, as well as an increase in levels of fasting gluconeogenesis when compared to early pregnancy<sup>[109]</sup>. This facilitated anabolism ensures sufficient supply of glucose, amino acids and fatty acids to the fetus<sup>[101]</sup>. Further to adaptations in storage and mobilisation of energy substrate during pregnancy, the maternal metabolism is also adapted to provide nutrients to the developing fetus during times of fasting, these will be discussed in the following section.

#### **1.2.1.3 Metabolic adaptations to fasting during pregnancy**

The developing fetus requires oxidisable energy substrate, in the form of fatty acids and glucose. Ketone bodies produced from the  $\beta$ -oxidation of fatty acids provide the primary source<sup>[104]</sup>. When maternal supply of glucose is low, the maternal metabolism must ensure sufficient transport of glucose across the

placenta. It is therefore essential that adaptations in the mother during pregnancy maintain glucose homeostasis in times of fasting, through the process of gluconeogenesis.

During fasting, glucose is taken up by tissues that are not sensitive to insulin control, for example the brain and is transported across the placenta to provide energy substrate for the developing fetus. It is essential that in pregnancy, glucose uptake is precisely matched by the rate of endogenous glucose production, primarily by the liver<sup>[101]</sup>. As in non-pregnant animals, in pregnancy the primary source of glucose is from the breakdown of glycogen. However, these stores are finite, with the average 65kg person storing around 450g glycogen, enough to fuel the brain for 18 hours<sup>[110]</sup>. Therefore glycogen stores do not provide a long term solution for fasting glucose homeostasis. Pregnant animals have a more rapid and exaggerated response to fasting compared to non-pregnant mammals, termed 'accelerated starvation'<sup>[111]</sup>. Fasting in pregnancy is also with 'facilitated anabolism', a maternal adaptation which ensures an adequate supply of nutrients to the fetus<sup>[104]</sup>. Facilitated anabolism involves a more prolonged increase in plasma glucose, greater increase in very low density lipoprotein and a greater fall in plasma glucagon, in the fed state<sup>[112]</sup>. Plasma glucose and the  $\beta$ -hydroxybutyrate, the breakdown product of fatty acid  $\beta$ -oxidation, concentrations are increased in the third trimester of pregnancy, when compared to non-pregnant woman (Figure 1.2).

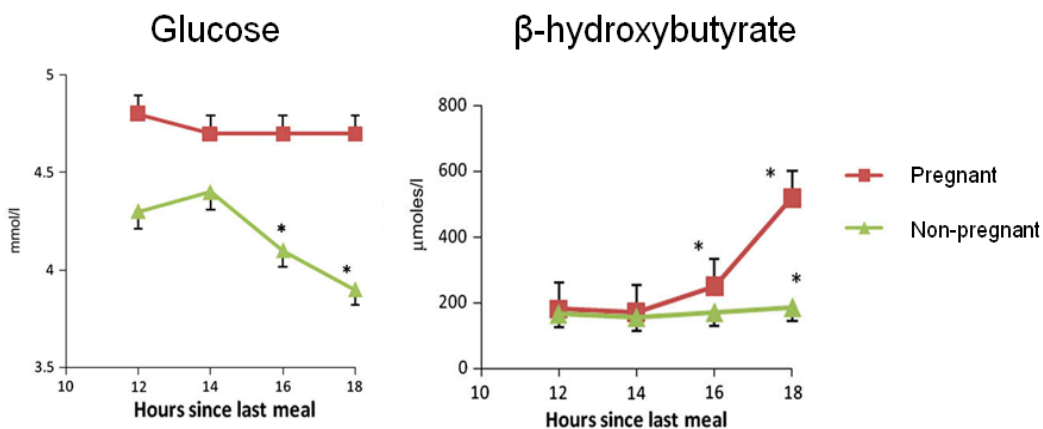


**Figure 1.2 Fasting plasma glucose and  $\beta$ -hydroxybutyrate concentrations in pregnant and non-pregnant women, during the third trimester of pregnancy, from 12 hours to 18 hours following a meal. Adapted from Hadden (2009) *Seminars in Fetal and Neonatal Medicine***

### **1.2.2 Evidence of maternal effects on offspring phenotype**

In order to understand the relevance of changes in maternal physiology it is important to consider the role of the mother in determining offspring development. The maternal effect of physical size, on growth of offspring was first shown when cross-breeding Shire horses and Shetland ponies<sup>[113]</sup>. The experiment was the first to cross different breeds within the same species, to show the maternal effect. It was shown that the size of the mother determined the size of the offspring, such that a foal born from a Shetland sire and Shire dam was larger than a foal born of a Shire sire and Shetland dam. In both large and small breeds, the fetus at birth was approximately proportional to the size of the mother. The study showed that the nutrient supply from the mother plays a role in the size of the fetus, and this in turn may be a function of the size of the mother. Short stature in women leads to altered development of offspring. In a study of 1,180 mothers and 1,511 children (<10 years old) living in the semi-arid region of Alagoes, Brazil, low maternal stature was independently associated with low birth weight, obesity, abnormal adiposity, high systolic blood pressure in offspring<sup>[114]</sup>. Compared to children born of women of normal stature, the offspring showed a higher prevalence of chronic degenerative diseases. In South-central Asia and Sub-Saharan Africa, maternal under nutrition led to short stature and a lower BMI in 10% and 2% of women of reproductive age, respectively<sup>[115]</sup>. In both demographics maternal height was a strong predictor of birth size, independent of pre-pregnancy BMI and weight gain during pregnancy. Maternal smoking, height, weight and education have significant implications in terms of offspring development. Data from the 1980 National Natality Survey shows that women with less than 12 years education are more likely to give birth to offspring with a low birth weight (odds ratio 1.59); this is mainly due to the reluctance to give up smoking in this group, compared to those with a greater education<sup>[116]</sup>. The total effect of smoking was such that every 5 cigarettes smoked a day increased the odds of low birth weight by 26%<sup>[116]</sup>.

Maternal constraint can be defined as either supply limited, such as maternal size, or demand-driven, such as twinning<sup>[33,92]</sup>. It has been shown that maternal constraint is greater in pregnancies where there is small maternal size,

young maternal age and multiple pregnancies<sup>[33,92]</sup>. By affecting nutrient availability and the metabolic hormonal drive to grow, maternal constraint has the potential to affect growth of the fetus, leading to long-term alterations in phenotype and therefore increased disease susceptibility of the offspring<sup>[92]</sup>.

As a common disease in the developed world, numerous studies have investigated the effect of maternal diabetes on alterations in offspring phenotype, in both humans and animal models. A study found that 58% of offspring aged 15-19 years from mothers who had gestational diabetes, weighed 140% or more of their desirable weight, compared with 17% of offspring from the control offspring<sup>[117]</sup>. The study further confirms that maternal diabetes starts the offspring on an altered trajectory of growth in terms of weight gain. However, in a study by Whitaker et al, there was no increase in risk of obesity in offspring of mothers with gestational diabetes<sup>[118]</sup>. Although in this study, the severity of gestational diabetes was mild, and was diet-treated. As well as influencing risk of obesity in offspring, plasma  $\beta$ -hydroxybutyrate concentration during the third trimester of pregnancy in mothers was positively associated with offspring mental development index scores at age 2 years and Stanford-Binet intelligence scale at ages 3, 4 and 5 years<sup>[119]</sup>. In the rat, streptozotocin induced diabetes during the last half of pregnancy, led to an increase in birth weight, pancreatic insulin content, plasma insulin and C-peptide concentration in offspring, compared to control pups<sup>[120]</sup>. Following oral glucose challenge at 10 weeks of age, higher plasma insulin and glucose concentrations were seen, compared to control offspring<sup>[120]</sup>. The results suggest an accelerated growth and abnormal glucose tolerance in offspring of diabetic dams.

The increase in offspring weight, following maternal diabetes, was confirmed in a study by Plagemann et al<sup>[77]</sup>. The offspring were hyperinsulineamic, had an increased risk of obesity and developing diabetes mellitus throughout life. In contrast, lower postnatal body weight gain was seen in offspring of diabetic dams, compared to control offspring, in the rat<sup>[121]</sup>. Gestational diabetes in pregnancy appears to predispose offspring to becoming overweight and developing diabetes throughout life, in both human and animal studies. Although not all results are in

agreement in the direction in change in growth compared to control offspring, in terms of diabetes, there is a clear effect of maternal metabolism on offspring phenotype. There are clear examples of the maternal effect seen in Gambian women. In a longitudinal study ranging from birth to postnatal 18 months, it was shown that offspring growth was significantly associated with maternal parity<sup>[122]</sup>. For a firstborn child, birth weight was significantly lower, but catch-up growth was complete by postnatal 3 months. However, if children were born to mothers of high parity (10 or more) then birth weight was higher than average, but early postnatal growth was poor. The study shows that alterations in physiology, as a result of multiple pregnancy leads to significant differences in the *in utero* environment, which have clear effects on early offspring growth. Although there were clear maternal effects seen in this study set, the maternal effect is not seen in terms of every metabolite. Maternal plasma 25-hydroxyvitamin D concentration during pregnancy had no effect on fetal and infant growth or bone mineral accrual<sup>[123]</sup>. However, one potential reason for the absence of a maternal effect is that due to the population studied (women from the rural area of The Gambia, West Africa), all participants had a high vitamin D status, due to the sun exposure. Perhaps a maternal effect of vitamin D status is only seen across a wider range?

In *Pomacentrus amboinensis* (Coral reef fish) the proportion of maternal energy allocated to the offspring was tested by manipulations in food availability. Although there was no significant effect of maternal condition on number of embryos hatching, or number of hatchlings surviving to a set time, maternal environment influenced the relationship between offspring characteristics and survival, via increased yolk-sac and oil globule size, compared to non-supplemented mothers<sup>[124]</sup>. Avian eggs contain a substantial amount of maternal hormones, such as androgens, which affect behaviour, growth, morphology, immune function and survival of offspring<sup>[125]</sup>. Maternal androgen deposition provides a mechanism for mothers to favour specific offspring for survival, allowing them to adjust their developmental trajectory to the environmental conditions. This was confirmed in a study comparing addition of testosterone to avian eggs to a control egg, where oil was used. Offspring of the androgen supplemented eggs showed increased

survival, which was significant up to postnatal day 23. Following the initial 23 days, the effects of maternal androgen were no longer significantly different to control offspring<sup>[125]</sup>. Duggleby and Jackson showed evidence for altered maternal metabolism on offspring phenotype<sup>[126]</sup>. During late pregnancy, mothers whose amino acid oxidation decreased the most gave birth to heavier infants<sup>[126]</sup>. Following adjustment for length of gestation and sex of infant, it was calculated that maternal amino acid oxidation during pregnancy accounted for 34% of the variation in birth weight.

### 1.2.3 Regulation of gluconeogenesis

In the fasting state, gluconeogenesis provides glucose from non-carbohydrate precursors, including lactate, glycerol and gluconeogenic amino acids, such as glycine, serine and valine. The process occurs primarily in the liver. Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase are key enzymes involved in the pathway. As such, altered regulation of the enzymes will impact plasma glucose levels during fasting. PEPCK converts oxaloacetate to phosphoenolpyruvate (PEP) and carbon dioxide (Figure 1.3). Along with pyruvate carboxylase, PEPCK catalyses the irreversible conversion of PEP to pyruvate which occurs in the glycolytic pathway, by the enzyme pyruvate kinase. As the rate-limiting step of gluconeogenesis, down regulation in PEPCK will result in decreased glucose production.

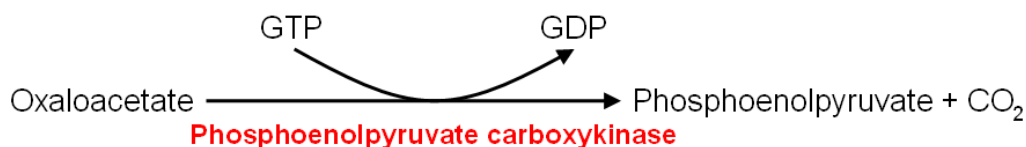


**Figure 1.3 Conversion of oxaloacetate to phosphoenolpyruvate** by the enzyme PEPCK. The reaction requires a phosphate group, provided by GTP.

The final step of gluconeogenesis is the conversion of glucose-6-phosphate to glucose by glucose-6-phosphatase. The reaction is a key determinant of plasma glucose levels during the fasting response (Figure 1.4). In terms of hepatic glucose

production, glucose-6-phosphatase provides the distal step, whereas glucokinase accounts for the proximal step<sup>[127]</sup>. Following this step, glucose enters the blood stream, via the facilitated glucose transporter GLUT2.

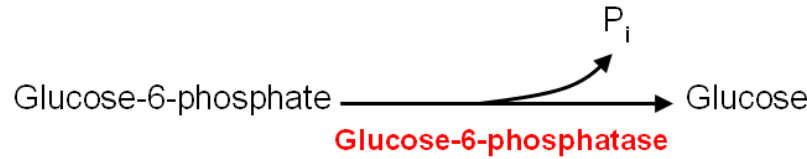


**Figure 1.4 Conversion of glucose-6-phosphate to glucose** by the enzyme glucose-6-phosphatase. The reaction results in phosphate release.

The bi-functional enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase (PFK-2/F-2,6-BPase) works to determine the direction of glucose metabolism, depending on the metabolic state<sup>[128]</sup>. Following a meal, insulin levels are elevated resulting in an increase in PFK-2 activity. This leads to the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate (Figure 1.5 (A)). Fructose-2,6-bisphosphate acts as an allosteric activator of phosphofructokinase-1 (PFK-1) and inhibitor of fructose-1,6-bisphosphatase (F-1,6-BPase). This increases glycolysis and decreases gluconeogenesis. During fasting, glucagon activates F-2,6-BPase, thus reducing fructose-2,6-bisphosphate concentration, and so inhibits PFK-1 (figure 1.5 (B)). Glucagon also up-regulates fructose-1,6-bisphosphatase. Overall, this decreases glycolysis and increases gluconeogenesis. The bi-functional enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase provides the main stage for hormonal regulation of the glycolytic and gluconeogenic pathways.

The three major modes of regulation of the pathways are allosteric, covalent and gene regulation. The allosteric regulation by hormones works mainly through adenylate cyclase and cyclic adenylate monophosphate (cAMP), which leads to phosphorylation of phosphofructokinase-2/fructose-2,6-bisphosphatase by cyclic-AMP-dependent protein kinase when glucagon is high and dephosphorylation of by protein phosphatase-2A when insulin is high<sup>[129]</sup>. Adenine nucleotides (ATP, ADP and AMP) regulate several steps of the pathways when the ATP/ADP ratio is low (when the energy state is low), leading to an increase in glycolysis<sup>[130]</sup>. Other



molecules which regulate the glycolytic and gluconeogenic pathways include citrate, which inhibits PFK and adrenaline and noradrenaline, which play equivalent roles to glucagon and insulin, raising cAMP concentrations via  $\beta$ -adrenergic receptors<sup>[131]</sup>.

As a result of lower plasma glucose levels in fasting, insulin secretion is reduced and glucagon secretion increased, from the  $\beta$  and  $\alpha$ -cells of the pancreatic Islets of Langerhans, respectively. When plasma glucose is low, glucagon acts to maintain blood glucose levels, via interactions with the glucagon receptor in target tissues, such as the liver and muscle. Binding of glucagon to the  $\alpha$ -subunit of the glucagon receptor, a G-protein coupled receptor in the plasma membrane, leads to a conformation change in the receptor which leads to activation of adenylate cyclase<sup>[132]</sup>. In turn, adenylate cyclase leads to an increase in intracellular cAMP. The points of interaction of glucagon in gluconeogenesis are through increasing levels of PEPCK, FBPase-2 and pyruvate kinase, with the net effect being an increase in fasting glucose levels<sup>[132]</sup>. In the early stages of fasting glucagon activates protein kinase A, which in turn leads to activation of glycogen phosphorylase kinase. This enzyme phosphorylates serine 14 residues on glycogen phosphorylase leading to its activation. The result of this is breakdown of glycogen to release glucose-6-phosphate, which is converted to glucose by the enzyme glucose-6-phosphatase.

The 21-carbon steroid hormone corticosterone is the main glucocorticoid present in rodents. Secretion from the cortex of the adrenal glands is increased in situations of stress and fasting. Further to its roles in immune reactions and the stress response, the actions of corticosterone include increased gluconeogenesis, particularly in the liver, mobilization of amino acids from muscle for use as substrate in gluconeogenesis, inhibition of glucose uptake in non-glucose dependent tissue and stimulation of fat breakdown in adipose through lipolysis. In male Wistar rats, administration of the synthetic glucocorticoid, dexamethasone, led to a decrease of insulin binding to the insulin receptor, in adipocytes<sup>[133]</sup>, providing a mechanism for the inhibition of glucose uptake. In a similar mechanism to glucagon, corticosterone leads to increased gluconeogenesis through increased

expression of the PEPCK gene, via increased expression of the glucocorticoid receptor<sup>[134,135]</sup>. Upon activation, the transcription factor, the glucocorticoid receptor (GR) leads to increased expression of genes involved in immunology and metabolism. In terms of fasting, GR has been shown to regulate expression of both PEPCK<sup>[136]</sup> and PPAR $\alpha$ <sup>[137]</sup>. GR exon1<sub>10</sub>, has been shown to be the exon most expressed in liver<sup>[138]</sup>.

The peptide hormone leptin, secreted mainly from white adipose tissue, plays a role in regulation of energy intake, through binding to the leptin receptor in the mediobasal hypothalamus in the brain. This is through three mechanisms, by counteracting the effects of feeding stimulants neuropeptide Y and anandamide and by promoting synthesis of  $\alpha$ -MSH, an appetite suppressant<sup>[139]</sup>. In the metabolic state of fasting, the feeling of satiety is low and therefore levels of plasma leptin are low.

Hormonal regulation of energy metabolism involves multiple hormones acting at both similar and different points to achieve an overall effect. For example both glucagon and corticosterone lead to an increase in gluconeogenesis, through increased expression of PEPCK. However the hormone insulin, which is secreted when plasma glucose is elevated, binds to the insulin receptor to cause a decrease in gluconeogenesis by causing opposite effects to glucagon at the same points of the pathway. One example of this is seen in pyruvate kinase, where insulin maintains the enzyme activity, and therefore levels of glycolysis, by preventing its inhibitory phosphorylation through glucagon and cAMP-dependant kinase<sup>[140]</sup>.

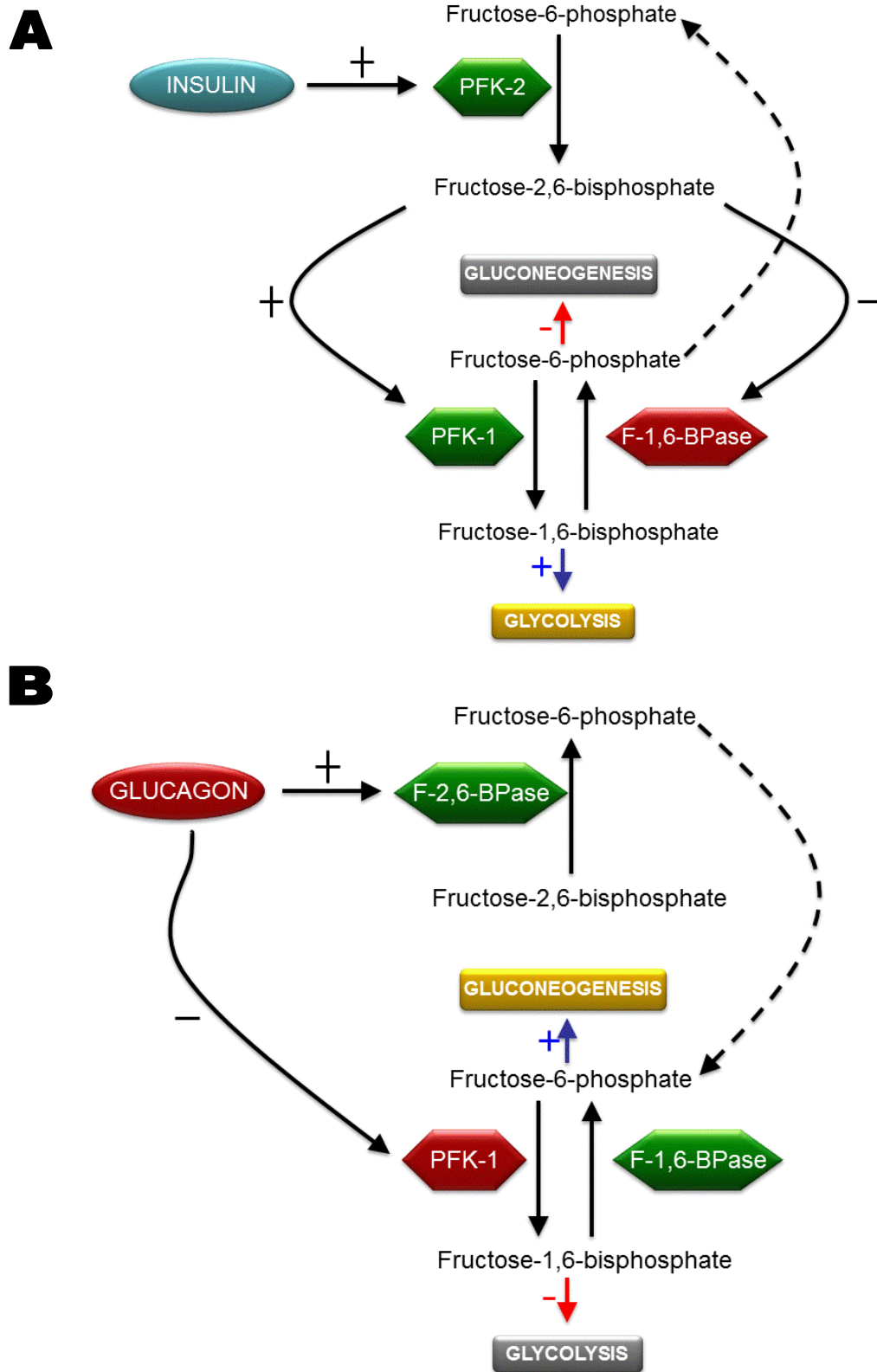


**Figure 1.5** The role of the bi functional enzyme PFK-2/F-2,6-BPase in glucose metabolism in the fed (A) and fasted (B) state. Through the hormonal regulation of insulin and glucagon.

#### 1.2.4 Regulation of fatty acid $\beta$ -oxidation

$\beta$ -oxidation of fatty acids and ketone body production is an essential mechanism to provide energy substrate in the fasted state.  $\beta$ -oxidation and ketogenesis are hormonally induced, where the pancreatic hormones insulin and glucagon play dominant roles. The importance of insulin and glucagon were determined through investigations using anti-insulin serum and glucagon exposure and measuring plasma free fatty acid and ketone body levels over a three hour period, with both leading to a ketogenic profile<sup>[141]</sup>. Although the concentrations of the individual hormones play their roles, it is the glucagon:insulin ratio which has the greater effect<sup>[142]</sup>. The main role of insulin is to control the delivery of free fatty acid substrate to the liver, whereas the main action of glucagon is to enhance the ketogenic machinery of the liver<sup>[141]</sup>. The rate limiting step of  $\beta$ -oxidation is the transport of fatty acids into the mitochondria, the site of  $\beta$ -oxidation, through carnitine-O-palmitoyltransferase-1 (CPT-1)<sup>[110]</sup>. In the fed state an increased plasma insulin concentration leads to a higher level of malonyl-CoA, a potent inhibitor of CPT-1, thereby decreasing the level of  $\beta$ -oxidation. As well as mitochondrial  $\beta$ -oxidation, peroxisomal  $\beta$ -oxidation accounts for 5-30% of the hepatic fatty acid oxidation. The end products of  $\beta$ -oxidation and ketogenesis are the ketone bodies acetoacetate and  $\beta$ -hydroxybutyrate, which are released into the circulation.

The Peroxisome Proliferator Activated Receptor  $\alpha$  (PPAR $\alpha$ ) is a transducer protein, which plays a vital role in lipid metabolism<sup>[143]</sup>. Due to their conserved DNA and ligand-binding domains, PPARs are classed as members of the steroid nuclear hormone family<sup>[144]</sup>. PPAR $\alpha$  is activated by natural fatty acids<sup>[143]</sup>. PPAR $\alpha$  is activated by long to medium (C10-C22) unsaturated fatty acids with unmetabolisable parent molecules being more effective activators, the two best fatty acid activators are linoleic acid (LA) and docosahexaenoic acid (DHA)<sup>[145,146]</sup>. PPAR $\alpha$  activation in the liver leads to activation of fatty acids to acyl-CoA, peroxisomal  $\beta$  oxidation, mitochondrial  $\beta$  oxidation and activation of ketogenesis, through the enzymes acyl-CoA synthetase, acyl-CoA oxidase (AOX), CPT-1, medium chain acyl-CoA synthase and hydroxymethyl glutamyl-CoA synthase<sup>[145]</sup>.

As mentioned previously, CPT-1 is a mitochondrial membrane bound transporter, which transports long chain fatty acids into the mitochondria, for  $\beta$ -oxidation.

### 1.2.5 Polyunsaturated fatty acid metabolism and fetal development

Mammals lack the  $\Delta 12$  and  $\Delta 15$  desaturase enzymes, which are present in plants. These enzymes are required for the synthesis of the polyunsaturated fatty acids  $\alpha$ -linolenic acid (18:3n-3) and linoleic acid (18:2n-6), through the introduction of double bonds<sup>[147]</sup>. As a result these fatty acids are termed essential; therefore they must be consumed in the diet. Linoleic acid is found in vegetable oils, products from vegetable oils (such as margarines) and foodstuffs into which vegetable oils or margarine are incorporated (such as cakes and biscuits).  $\alpha$ -linolenic acid is found within green plants, vegetable oils, such as rapeseed oil and soybean, certain nuts, and linseed.

One of the major roles of the essential fatty acids is to provide substrate for the synthesis of long-chain PUFA, such as arachidonic acid (AA) (20:4n-6), DHA (22:6n-3) and eicosapentanoic acid (EPA) (22:5n-3), through the desaturation-elongation pathway. DHA and EPA can be found in oily fish, where mackerel contains 3g n-3 PUFA/140g, however according to the SACN advice on fish consumption, 74% of people in the UK consume no oily fish. As a result synthesis of long chain n-3 PUFA, through the desaturation-elongation pathway, is essential. The desaturation-elongation pathway occurs within the endoplasmic reticulum of hepatocytes, with the last step occurring in the peroxisome<sup>[147]</sup> (Figure 1.6). The pathway begins with the addition of a double bond, by the enzyme  $\Delta 6$  desaturase, followed by the addition of two carbons to the hydrocarbon chain by elongase. Following this  $\Delta 5$  desaturase adds a further double bond to the fatty acid, and it is subject to two further elongation reactions. The enzyme  $\Delta 6$  desaturase then adds a final double bond to the chain. The membrane bound  $\Delta 5$  and  $\Delta 6$  desaturase enzymes share around 75% homology<sup>[148]</sup> and activity of both enzymes is increased by low PUFA status<sup>[149]</sup>.

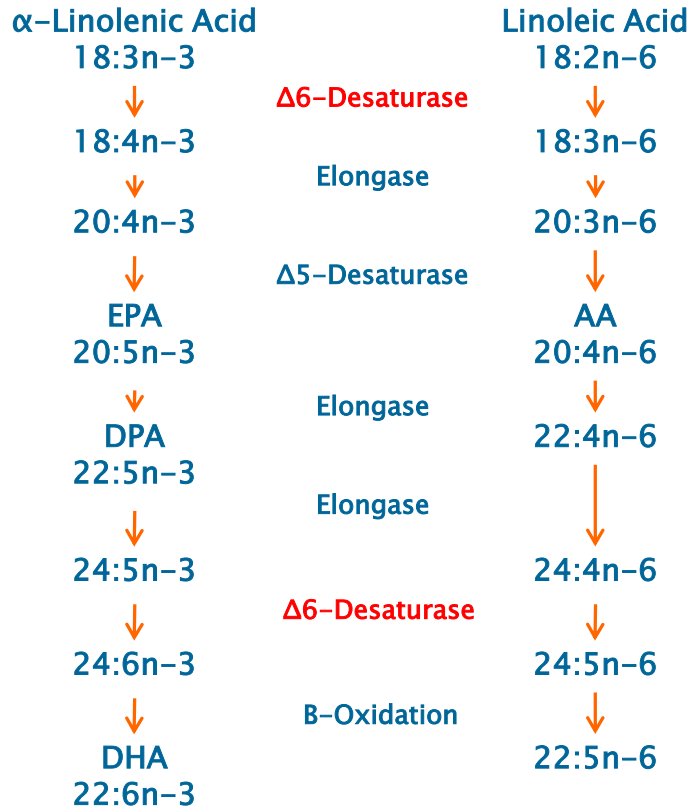


**Figure 1.6 The fatty acid desaturation-elongation pathway** for both n-3 and n-6 fatty acids, where the enzymes for each step are shown in the middle of the pathways.

The fetal liver has limited capacity to carry out the desaturation-elongation pathway<sup>[150]</sup>; therefore the maternal circulation is responsible for providing n-3 PUFA for the developing fetus. In pregnant women, plasma phosphatidylcholine (PC) DHA content increases from 170 $\mu$ mol/l at 16 weeks to 230 $\mu$ mol/l at 40 weeks gestation<sup>[150]</sup>. Numerous studies have investigated the role of n-3 PUFA in fetal development, with focus on visual and cognitive effects. The central nervous system (CNS) appears to be protected from n-3 fatty acid deficiency<sup>[151]</sup>, suggesting it is a priority during development. The most abundant PUFAs found in the brain are DHA and AA<sup>[152]</sup>. Incorporation of PUFA into phospholipids, leads to more fluid cell membranes, ensuring the successful conformational changes of proteins and molecules in the cell membrane and passing of signals from one the external space to within the cytoplasm.

The optimal DHA content of the retina is 19% of total fatty acids<sup>[153]</sup>. One rat study used an electroretinogram to assess the physiological response evoked from the retina by brief flashes of light and showed that a poorer response was present when n-3 deficiency was present<sup>[154]</sup>. In rat, DHA levels in whole brain phospholipids were reduced by 80-90%, in offspring of dams fed safflower oil based diets during pregnancy, compared to soybean oil fed dams. Reflexes and motor abilities were unaltered, and success on the maze task was 60% in the safflower group offspring, compared to 90% in the soybean oil offspring group<sup>[155]</sup>. Maze learning tasks, which involved discrimination between black and white stimuli were performed poorly, although this could be a combination of altered phospholipid composition in the brain and retina. As the rate limiting step in the synthesis of long chain PUFA, the expression and activity of the  $\Delta 6$  desaturase gene, specifically in female offspring (future mothers), following an altered maternal diet, is important in terms of cognitive and visual function in future offspring.

#### **1.2.6 Evidence for effects of altered maternal metabolism on offspring phenotype between generations**

There are few studies which has specifically studied the effect of altered maternal metabolism on the phenotype of the offspring between generations. Alterations in development of the endocrine pancreas, glucose intolerance and insulin resistance in later life have been shown, following an increase in stress situations in the mother<sup>[156,157,158]</sup>. According to Aerts et al, the additional metabolic stress of diabetes during pregnancy and lactation can lead to a diabetogenic tendency in the offspring, potentially leading to gestational diabetes in the offspring in later life<sup>[159]</sup>. If this occurs the cycle will continue and lead to increased risk of gestational diabetes in the subsequent generation. As well as risk of gestational diabetes being passed between generations through altered maternal metabolism during pregnancy, a similar pattern has been shown in terms of obesity<sup>[160]</sup>. Following maternal obesity, offspring are 2-3 times more likely to develop obesity, and this will lead to both an increase in disease risk in later life of the mother and

offspring, as well as continuing the prevalence of obesity between generations. However, whether this effect is purely metabolic, or reflects transition in nutrition in the Western world, is unknown.



### 1.3 Transgenerational transmission of phenotype

*“Non-genomic processes of transmitting environmental information between generations evolved to assist our evolution as we moved across changing environments”*

*Gluckman 2007*

Heritable non-communicable diseases such as type II diabetes, metabolic syndrome, obesity and cardiovascular disease are transmitted between generation, in a manner that does not involve alterations in DNA sequence<sup>[161,162,163]</sup>. Gluckman argues that disease risk has arisen as a ‘maladaptive consequence of an ancestral mechanism of developmental plasticity.’<sup>[164]</sup> It is for this reason that the mechanisms behind the transgenerational transmission of phenotype must be understood.

As discussed by Skinner, true transgenerational transmission requires the effect of environmental exposure to be prevalent in the F3 generation<sup>[165]</sup>. Skinner argues that as the F2 oocyte germ line is developing in the F1 fetus, an altered external environment in the F0 generation pregnancy will directly affect the F0, F1 and F2 generations. As a result, in order for an effect to be truly transgenerational it must be seen in the F3 generation, the first generation not to be directly exposed. This concept is described in Figure 1.7. It is stated that true transgenerational transmission can be seen in the F2 generation if the F0 exposure is postnatal or during adulthood<sup>[165]</sup>. Although not regarded as true transmission of phenotype, alterations in phenotype present in the F2 generation show the magnitude of effect a stimulus in the F0 pregnancy can have. The first selection of studies have shown phenotype transmission to the F2 generation, whereas the second section shows transmission to F3 generation.

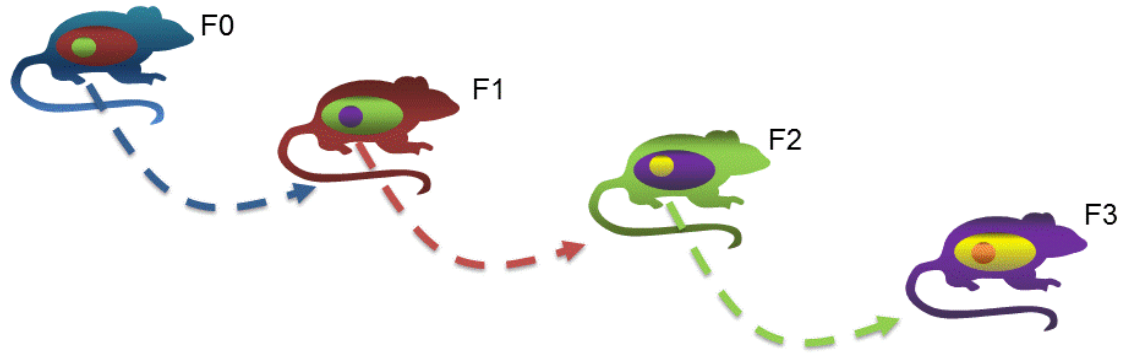


**Figure 1.7** Diagram showing the indirect effect of the F0 generation and direct effect of the F1 generation on the F2 generation phenotype as the F1 fetus and F2 germ cell are present at the time of an altered environmental exposure to the F0 generation.

### 1.3.1 Transmission of an altered phenotype to the F2 generation

By following up periods of altered environment in subsequent generations it has been shown that the changes in phenotype, such as those discussed previously, are transmitted during further reproduction. A recent review by Jablonka provides an in depth view into the developments in the study of transgenerational transmission of phenotype<sup>[166]</sup>. The paper highlights the different ways in which altered phenotypes may be passed on to later generations, such as direct, parallel and somatic induction, as well as parallel induction with non-parallel effects<sup>[166]</sup>. These different mechanisms include the environmental stimulus leading to transmittable changes in the somatic cell line, germ cell line, or both, and the interactions which may be present between them.<sup>[166]</sup> The different methods of induction take into account the fact that an alteration in the somatic cell line has the potential to lead to alterations in phenotype in later generations, by affecting the somatic or germ cell lines in later generations. This seems logical, as an altered F1 phenotype, caused by an environmental stimulus in the F0 pregnancy, may lead to an altered F1 pregnancy. This could be a minimal alteration, which goes unnoticed in the F2 generation, or a greater alteration, such as a difference in metabolism of nutrients, which would be as important as altered diet during the F1 pregnancy.

The following studies provide evidence of transgenerational transmission in humans and rats. In humans, Kaati et al have shown that an increased risk of

cardiovascular disease and diabetes in adulthood is associated with overeating during the slow growth period in the grandparents, during childhood<sup>[162]</sup>. Administration of dexamethasone to pregnant rats, leads to alterations in phenotype of offspring which are transmitted to subsequent generations. Exposure of the fetus to an excess of glucocorticoids, in the form of the synthetic glucocorticoid dexamethasone, resulted in low birth weight leading to later life hyperinsulinaemia and hyperglycaemia. These differences, which are seen in both the F1 and F2 generation are associated with increased activity of hepatic PEPCK.<sup>[167]</sup> The change in phenotype however was not present in the F3 generation. A high fat mouse model used by Dunn et al, showed transmission of an altered phenotype to the second generation<sup>[168]</sup>. Feeding a high fat diet to dams from 4 weeks preconception up to the end of lactation, led to both a significant body length increase and increased insulin resistance in both the first and second generation. Another study which has shown an increased insulin response in the F2 generation following an altered F0 pregnancy is provided by Martin et al. Undernourishment of the developing fetus, provided by feeding dams a low protein diet, followed by a high fat post weaning diet in the F1 offspring was shown to induce an increased insulin response at 30 minutes and 120 minutes following a glucose challenge<sup>[169]</sup>. The findings of this study highlight the susceptibility of the insulin system to transgenerational alteration in phenotype. In the Overkalix cohort study, the slow growth period in young boys and girls was well characterised. Pembrey et al used this data from the Swedish study to see if the slow growth period in children was associated with mortality risk in their grandchildren. The study showed that if their paternal grandfather had experienced a good food supply during their slow growth period then men had an increased mortality risk. Similarly if their maternal grandmother had a good food supply during their slow growth period then women also had an increased mortality risk. If the food availability during this period was poor then an opposite effect was seen<sup>[163]</sup>. The study showed the importance of sex in transmission of disease risk in humans, although the mechanisms are not fully understood. It is likely that it is the role of the X and Y chromosomes which have an effect, rather than gametes; due to the differences in

oocytes and sperm production (oogenesis occurs during fetal life, whereas spermatogenesis is continual following puberty.)

It has been shown by Benyshek et al that in Native Americans levels of type II diabetes in adulthood is associated with fetal malnutrition<sup>[161]</sup>. When considering further generations, the risk of the disease was seen to be propagated through perturbations in the intra uterine environment<sup>[161]</sup>. In 1971 the use of diethylstilbestrol (DES) as a preventative for miscarriages in pregnant women was banned, due to the discovery of its action as a teratogenic agent. In mothers, DES has been shown to lead to almost a two-fold increase in risk of breast cancer<sup>[170]</sup>. In a single case study of a 15 year old girl who developed ovarian carcinoma, the fact that her grandmother had taken DES before giving birth to the girl's mother highlights the potential of DES as an agent capable of transgenerational effects<sup>[171]</sup>. Although this single case is not enough to confirm the role of DES in this cancer development, further investigation into later generations of DES treated women may confirm it.

The following studies have used the low protein rat model to show transgenerational transmission of phenotype up to the F2 generation. As shown by Pinheiro et al, feeding a low protein diet throughout pregnancy and/or lactation led to transgenerational effects in both the F1 and F2 offspring. In the F0 pregnancy dams were fed either a control (C) or protein restriction (PR) diet during pregnancy and lactation. This led to 4 dietary groups; C-C, C-PR, PR-PR and PR-C, where the first diet represents pregnancy and the second lactation. In the F1 offspring, the PR-C group were shown to have a lower birth weight, which following catch up growth matched the C-C in later life. The group also showed an increased glycaemia. Both groups exposed to a PR diet during pregnancy showed impaired growth. Results showed that a PR diet during gestation had beneficial effects on weight at birth, fat mass and growth rates in the F1 generation, but led to insulin resistance in adulthood in both the F1 and F2 generation. A PR diet during lactation also led to insulin resistance, but only in the F2 generation<sup>[172]</sup>. These

results again highlight the consequences of a mismatch between the *in utero* and post natal environment. The study does show that some consequences of a protein restricted diet during pregnancy can be beneficial to health in the offspring, even if a mismatched environment is encountered following birth. It may be that while an altered environment during pregnancy may lead to an increased susceptibility of certain diseases, risk of other diseases may be reduced. It has been hypothesised that feeding of the PR diet during development led to altered control of the leptin and glucose pathways, which are crucial in setting up energy balance circuits during development and maintaining energy balance throughout life<sup>[172]</sup>.

Zambrano et al have shown that feeding a protein restricted diet to rats during pregnancy and/or lactation led to altered body weights, food intake and insulin sensitivity in F1 and F2 offspring. When compared to control animals, male F2 animals, whose mothers were fed a protein restricted diet during lactation alone and female F2 animals, whose mothers were fed a protein restricted diet during pregnancy alone were found to be insulin resistant<sup>[173]</sup>. These results identify the importance of sex in transmission of phenotype in resistance to insulin in terms of the developmental window whereby modifications to the epigenome can take place.

### **1.3.2 Transmission of altered phenotype to the F3 generation**

As discussed previously, an altered phenotype must be transmitted to the F3 generation in order to be termed 'true transmission'. The following studies have all shown 'true transgenerational transmission' in animal models. Although it could be argued that true transmission of phenotype should entail cross fostering of the offspring, in order to be independent from the effects of the maternal physiology, it is widely accepted that studies which cover transmission to the F3 generation are sufficient to display transgenerational transmission of phenotype.

A study by Benyshek et al involved feeding female rats either a control or an isoenergetic protein restricted diet throughout pregnancy and lactation. The F1, F2 and F3 adult offspring were harvested following an overnight hour fast and glucose tolerance test. The study shows that 30 minute plasma glucose levels, following a glucose challenge, were significantly lower in males in the protein restricted offspring, when compared to the control lineage males. As well as this, the male F3 protein restricted offspring also showed significantly higher plasma insulin levels, compared to the control equivalent<sup>[174]</sup>. Although the study clearly shows transmission to F3, it is worth mentioning that the magnitude of result in the F3 generation was less than that of the F2 generation, suggesting that the phenotype may be returning to that of the control animals.

By exposing the developing embryo to the endocrine disruptor vinclozin, at the point of gonadal sex development, a transmittable reprogramming of the male and female germ-lines is created<sup>[175]</sup>. After three generations it was found that in males 92 genes in the hippocampus and 276 genes in the amygdala were altered, and in females 1,301 genes in the hippocampus and 172 genes in the amygdala were altered. These differences were associated with a decreased anxiety-like behaviour in males and an increased anxiety-like behaviour in females<sup>[175]</sup>. These reproducible changes in anxiety behaviour highlight the importance of sex in both the magnitude and direction of transgenerational phenotype induction. The mechanisms behind these sex differences are still unclear. These studies prove that environmental exposures can lead to transmittable alterations in phenotype.

### 1.3.3 The role of transgenerational transmission of phenotype in evolutionary adaptation

The previous studies have shown that a novel stimulus in the F0 generation can lead to an altered phenotype in the offspring in subsequent generations. Although the animal studies have used a severe dietary change to produce a 'wrong' or unhealthy phenotype in order to understand the consequences of an altered developmental environment, studies of this nature have led to the formation of a novel evolutionary hypothesis. In the alternative adaptation hypothesis, West-Eberhard stated that '*novel traits originate and become stable phenotypes within a species, prior to reproductive isolation and speciation*'<sup>[176]</sup>. This concept shows that the phenotype, not the genotype is the object of selection<sup>[176]</sup>. As shown in the transgenerational studies described in the previous sections, provided there is significant environmental variation, an altered phenotype can be present for numerous generations, without genetic variations<sup>[177]</sup>. According to the concept of phenotypic accommodation, a novel input, for example migration resulting in altered nutritional intake, can lead to a novel phenotype. Following this, there will be an initial spread of the phenotype into the community and as a result of 'epigenetic opportunity', genetic accommodation will take place<sup>[178]</sup>. This involves changes in gene frequency. The possible epigenetic processes involved in this accommodation will be discussed in the next section. In a more recent review, Fembery similarly hypothesises that stochastic variation can result from 'soft inheritance', whereby useful traits are passed through generations, which do not require changes in the genome<sup>[179]</sup>.

By investigating the transmission of phenotype through generations, our understanding of the mechanism involved in evolution of species will be increased. Whilst transient exposure to an environmental stimulus may result in an altered phenotype in a number of generations, constant exposure to a new stimulus has the potential to result in a new species, without the need for mutation.

#### **1.3.4 Transgenerational transmission of an altered phenotype following exposure to a constant environmental stimulus**

When considering the data in Section 1.1, it appears that the magnitude of difference in phenotype in the organism is associated with the both strength and duration of the environmental stimulus.

Nutritional transition, such as that seen in migration is associated with an altered risk of disease, leading to an altered survival of the species in the novel environment. Nutritional transition onto a Western diet in China in the 90s, led to an increase intake of fat and complex carbohydrates. There was a causal link between this nutritional transition and increased risk of obesity<sup>[180]</sup>. Due to a lack of studies, the mechanism behind altered disease risk following nutritional transitional is unknown. Past studies have focused on single F0 dietary exposures, rather than effects of sustained alteration.

Due to the expense and time required to undertake, there are few constant exposure studies. Two studies in *Drosophila*, however do show long lasting changes in gene regulation following a constant exposure to an altered nutrition, throughout the life course<sup>[181,182]</sup>. Both studies compared *Drosophila* grown in either a starch or maltose based medium. The first study, carried out by Powell and Andjelkovic, showed how selective changes were present in the amylase gene (*Amy*) in *Drosophila* fed starch, although it must be mentioned that no differences in amylase activity were associated with the changes<sup>[182]</sup>. The second study by Dodd, also showed changes in *Amy* in the *Drosophila* fed starch, where an increase in activity was also present<sup>[168]</sup>. This second study also showed an increased adaptation to the medium in subsequent generations. These studies show that constant exposure to altered nutrition results in constant changes in gene regulation. It would be beneficial to see if these findings are limited to this particular gene, or if the mechanism is present in different genes. These studies have provided evidence for transgenerational adaptation of phenotype following a constant, altered nutrition, which supports the phenotypic accommodation hypothesis. It is now important to investigate the mechanisms responsible for



adaptation following migration to a new environment. As stated in the previous statement it is the phenotype which is responsible for the fitness of the organism, not the genotype. How phenotype can be altered to accommodate a novel environment, without changes in genotype, is the key to alternative adaptation.

## 1.4 Epigenetic processes

It is clear that altered phenotypes can arise following a change in environment. This requires long term, stable alterations to the genome. Changes in the expression of genes have been shown to be involved in the induction of an altered phenotype. As these changes have been shown to be transmitted through generations, the mechanism responsible must also be hereditary. The following section will provide background on processes which can alter expression of genes, without changes in nucleotide sequence. Processes which result in a change of gene expression without altering DNA sequence are termed epigenetic. In the following section the epigenetic processes; DNA methylation (**Section 1.4.1**) and histone modifications (**Section 1.4.2**) will be discussed.

### 1.4.1 DNA methylation

Both X-chromosome inactivation and long term silencing of non-coding DNA, such as introns, repetitive elements and potentially active transposable elements, occur through DNA methylation, by the enzymes DNA methyltransferases (DNMTs)<sup>[183]</sup>. DNMTs transfer methyl groups from S-adenosyl methionine (SAM) to carbon 5 of cytosines in CpG dinucleotides, this chemical modification results in altered DNA-protein interactions<sup>[183]</sup>. In humans 5-methylcytosine represent 1% of all nucleotides in the genome, with 70-80% found within CpG dinucleotides<sup>[184]</sup>. There is a high density of CpG dinucleotides in promoter regions of around 30,000 genes in humans, as well as within introns and exons<sup>[184]</sup>. A 200 base pair section of DNA, with a high GC content and an observed ratio of CpGs greater than 0.6 is termed a CpG island<sup>[185]</sup>. CpG islands are typically unmethylated. CpGs are typically methylated in non-promoter regions and unmethylated in promoter regions, indicating that an absence of methylation is required for expression<sup>[185]</sup>. Although it must be stated that within a population of one cell type, the methylation status at one particular loci will have a level of variability, meaning when measuring DNA methylation it is the overall population that is measured, not single cells.

Methylation of cytosines in a promoter region can result in silencing of gene expression through direct or indirect blocking of transcription. Methylation of cytosines can physically prevent binding of transcriptional machinery, such as RNA Pol II, or indirectly block transcription through the recruitment of methyl cytosine binding proteins. Methyl binding proteins, such as methyl CpG binding protein 2 (MeCP2), can lead to an inhibition of transcription through recruitment of further proteins, such as histone deacetylases (HDAC) and histone methyltransferase (HMT)<sup>[186]</sup>. The silencing of genes via DNA methylation can be seen in Figure 1.8.

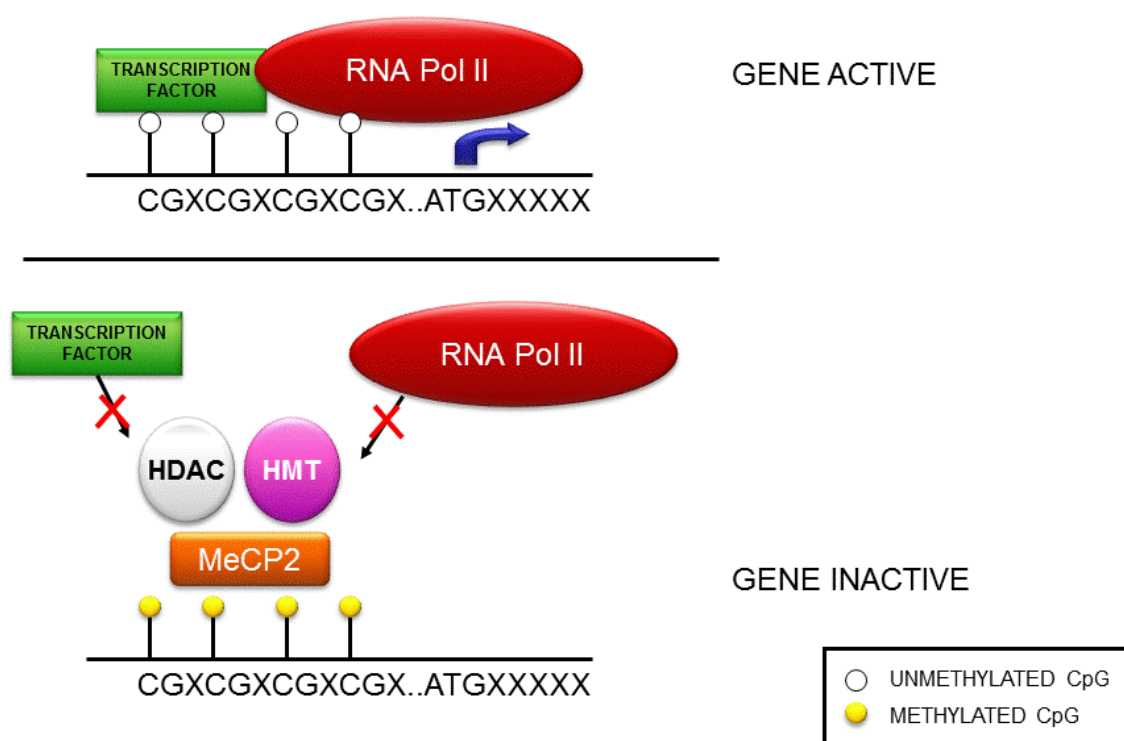


**Figure 1.8 Diagram showing epigenetic silencing of transcription** through the recruitment of MeCP2, HMT and HDAC to methylated cytosine residues within the promoter regions of genes. This creates a physical blocking of transcription factor and RNA Polymerase II (RNA Pol II) binding, to achieve silencing of genes.

There are three main types of DNMTs; DNMT1, DNMT2 and DNMT3. The two DNMT3 subtypes, DNMT3a and DNMT3b are responsible for the *de novo* methylation of the embryo genome, following the extensive demethylation seen in the maternal and paternal genomes following fertilization<sup>[187]</sup>. In somatic cells methylation patterns are maintained by DNMT1 in the S-phase of the cell cycle

during mitosis. Genomes of mature gametes in mammals are highly methylated compared to somatic cells, this is achieved by specific DNMTs, DNMT1o (oocytes) and DNMT1p (sperm)<sup>[184]</sup>. Following the first cell division after fusion of gametes DNMT1s (somatic) maintains methylation status in cells<sup>[184,188]</sup>. DNMT1<sup>-/-</sup> knock out (k/o) mice experiments have shown a loss of both imprinting and x-linked gene expression and embryonic lethality when the enzyme is absent<sup>[189]</sup>. Syzf has hypothesized that the epigenome is a dynamic equilibrium of methylation and demethylation, which is responsive to physiological and environmental signals<sup>[190]</sup>. This hypothesis is founded on the knowledge that genes such as PEPCK show a loss of methylation during development and that post-mitotic cells, such as neurons show a presence of the DNMT1. Although this is supported well by findings, it is still not understood how much of the epigenome is subject to demethylation throughout the life course. If methylation status is determined upon cell division, there would be no need for DNMT1 expression in post-mitotic cells. This methylation equilibrium provides a possible platform for gene-environment interactions<sup>[190]</sup>.

Cellular senescence is associated with a decrease in levels of global methylation. In liver and visceral adipose tissue, the rat genome showed significant differences in DNA methylation with age at >5% of analysed sites<sup>[191]</sup>. Using luminometric methylation analysis to determine global methylation levels and HpaII tiny fragment enrichment to define patterns of CpG methylation, it was shown that the most distinct regions where DNA methylation was decreased with age, was at intergenic sequences and conserved non-coding elements<sup>[191]</sup>. The changes in methylation were consistently found near genes with metabolic function, suggesting a link to pathogenesis of age-related diseases. The study failed to show data on transcription factor binding at sites where methylation was lost, total protein levels and activity. Without these, the overall effect of the findings is unclear. The loss in DNA methylation seen with senescence may be due to the decreased activity of DNMT1 with age<sup>[192]</sup>. As stated earlier, this could have implications on disease risk, such as progression of cancer, when tumour suppressor genes are considered.

This may result in an increase in disease risks associated with aging such as cancer, through improper methylation of tumour suppressor genes and oncogenes. DNA methylation offers a potential long term, heritable mechanism for silencing of genes. Evidence into the role of DNA methylation in alterations of phenotype will be discussed later.

#### **1.4.1.1 Promoter methylation and gene expression**

DNA methylation provides a mechanism by which expression of genes can be modified following an altered environment. The stability of the genome is decreased during embryogenesis, therefore, this window of plasticity allows alterations in DNA methylation to occur. Studies have shown direct associations between promoter methylation and mRNA expression of specific genes.

CpG methylation of the insulin gene promoter was measured in both human (INS) and mouse (Ins2) pancreatic  $\beta$  cells<sup>[193]</sup>. As a ubiquitously expressed gene, it is unsurprisingly that in both species the insulin gene promoter was hypomethylated. Using ChIP assays it was shown that methylation of the promoter suppressed insulin-reporter gene activity by almost 90%, through inhibition of binding at the cAMP response element (CRE)<sup>[193]</sup>. It was further shown in mouse stem cells, that loss of methylation at the Ins2 promoter is associated with differentiation into insulin-expressing cells. In colorectal cells, methylation of the hMLH1 promoter is associated with loss of expression<sup>[194]</sup>. Treatment with methylation inhibitor 5'-azacytidine lead the cells to re-express the gene. In humans, methylation analysis of the POMC promoter in blood mononuclear cells, showed that methylation of a single CpG in the E2F binding site was inversely correlated to expression, however the result was not significant ( $P=0.557$ )<sup>[195]</sup>. However methylation of other CpGs along the promoter may have a greater role in POMC regulation.

Previous studies have presented methylation data as average DNA methylation across the promoter<sup>[196,197,198,187,199,200,201]</sup>. Methylation of gene promoters can regulate mRNA expression through recruitment of methyl CpG binding proteins and by physically blocking recruitment of transcriptional machinery. In terms of physically blocking binding, it is not mean methylation across the whole promoter

that is important, but methylation of specific CpGs. For example methylation of the CG dinucleotide in the CRE (5'-TGACGTCA-3'), results in a loss of transcription factor binding, as well as loss of transcriptional activity of the human glycoprotein hormone  $\alpha$ -subunit in vitro and in vivo<sup>[202]</sup>. The methylation across the whole promoter is therefore irrelevant, in terms of this regulation at this element. The same study however, found that methylation of the CpG situated in the Sp1 binding site had no effect on transcription factor binding<sup>[202]</sup>. CREs are often associated with promoters of cell-type specific genes, whereas Sp1 is mainly associated with regulation of house-keeping genes, this provides a mechanism through which DNA methylation plays a role in cell differentiation. CpG specific methylation also plays a role in transcriptional activity of the Epstein-Barr virus through CBF2 binding<sup>[203]</sup> and expression of the XAF1 gene in human gastric and colon cancers<sup>[204]</sup>. In both examples methylation led to a reduction in mRNA expression. It is therefore essential, when considering promoter methylation to use a sequencing technique to measure methylation of specific CpGs, not average methylation across the promoter.

#### **1.4.1.2 Changes in DNA methylation in aging**

There are few studies, to date, that have investigated methylation across the life course<sup>[205,206]</sup>. In two cohorts in Iceland and Utah, both males and females showed changes in levels of global DNA methylation over time. The average age of individuals was 74.6 years at the time of the first sample, with the second sample taken 11 years later, on average. 29% of an Icelandic cohort of 111 individuals showed significant differences of over 10% methylation change in blood cell DNA methylation, between 1991 and 2002-2005<sup>[205]</sup>. In the Utah cohort, where samples were collected 16 years apart, samples were grouped into families, and those that showed the greatest global methylation loss also demonstrated the greatest loss of gene-specific methylation<sup>[205]</sup>. This suggests that loss of methylation in specific genes may have a genetic component. Differences in DNA methylation between monozygotic twins show variability in methylation across the life course. During early life monozygotic twins are epigenetically indistinguishable,

however during adult life remarkable differences are present in the overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation<sup>[207]</sup>. Using capillary electrophoresis, relative 5-methylcytosine DNA content was measured in 80 pairs of monozygotic twins, with an average age of 30.6 years. A third of twins had epigenetic differences in both DNA methylation and histone modifications, affecting repeat sequences of DNA and single copy genes, which had impacts on gene expression. The differences were more distinct in the older twins, suggesting the role of time, however due to the nature of the study changes in environment could play a part. The study confirms variability in DNA methylation with age. A study by Wong et al, investigated epigenetic variation in twins between the ages of 5 and 10 years old<sup>[208]</sup>. In both monozygotic and dizygotic twins, DNA methylation of the dopamine receptor 4, serotonin transporter and X-linked monoamine oxidase A (MAOA) genes were measured using a sequencing technique. Although differences in variation in DNA methylation were seen in all genes, the extent to which levels changed with time was gene specific. The biggest variation in average methylation was seen in the MAOA promoter, where 74% of children showed at least a 10% change in methylation and 32% of children showed at least a 20% change in methylation. As well as differences in methylation seen between twins, there was a low inter-individual stability over time seen methylation of the MAOA promoter, however this was not significant.

One potential mechanism for the decrease in DNA methylation in age is a decrease in activity of DNMT1<sup>[192]</sup>. In WI-38 fibroblasts activity of DNMT1 decreased steadily with cellular senescence. Over the course of 41 days, maintenance methyltransferase activity of DNMT1 decreased by 69% and *de novo* methyltransferase activity decreased by 83.7%. In immortalised WI-39 fibroblasts however, methyltransferase activity of DNMT1 was elevated. Perhaps variation in promoter methylation with age is a consequence of decreased DNMT1 activity.

One apparent consequence of varied levels of methylation with age is hypermethylation of tumour suppressor genes and hypomethylation of oncogenes, leading to an increased risk of cancer<sup>[209]</sup>. The most widely studied are tumour suppressor genes. Tumour suppressor genes have functions including cell cycle

regulation, DNA repair, apoptosis and drug resistance, inactivation. Increased methylation of the promoter of the breast cancer antigen 1 (BRCA1) gene, which plays a role in DNA repair, is associated with sporadic breast cancer<sup>[210]</sup>. In 2 out of 7 sporadic breast carcinomas, a southern blotting technique revealed hypermethylation of the BRCA1 promoter.

As the methylation status of the genome has been shown to change with time it is important to determine how this global change in methylation affects specific genes.

#### **1.4.1.3 Expression and regulation of DNMT3a2**

All promoters of DNMT3a and DNMT3b lack typical TATA sequences adjacent to their transcription start sites<sup>[211]</sup>. In these promoters Specificity protein-1 (Sp1) binding sites, play a major role in regulation. Using reporter plasmids, over expression of both Sp1 and Sp3 led to up regulation of promoter activity in both DNMT3a and DNMT3b, the effect was confirmed using gel shift assays to confirm Sp1 and Sp3 binding to the promoters<sup>[211]</sup>. DNMT3a2 is a smaller form of DNMT3a, the transcript encoding DNMT3a2 is initiated from a 2kb promoter upstream of exon 7 of the DNMT3a gene<sup>[212]</sup>. In adult liver, DNMT3a2 accounts for around 50% of DNMT3a expression and is the predominant form found in developing tissue. In comparison to DNMT3a, DNMT3a2 lacks the 223 N-terminal amino acids in humans, and 219 N-terminal amino acids in mouse<sup>[212]</sup>. DNMT3a2 is the predominant form found in both embryonic stem cells and embryonic carcinoma cells, and can be detected in testis, ovary, thymus and spleen tissue, where it correlates with high levels of *de novo* methylation<sup>[212]</sup>. In terms of functional differences, it appears that DNMT3a2 localises at chromatin, whereas DNMT3a is concentrated on heterochromatin<sup>[212]</sup>. As well as regulation through Sp1 and Sp3, binding of retinoblastoma-like protein 2 (Rb12) to the DNMT3a2 promoter has also been shown to regulate its expression<sup>[213]</sup>. No evidence to date has shown altered methylation of the DNMT3a2 promoter. However, as the gene is not expressed at detectable levels following *de novo* methylation, it is likely that DNA methylation of



the promoter plays a role. As a result altered methylation at the promoter has the potential to affect *de novo* methylation and DNA methylation in late life.

### 1.4.3 Histone modifications

Histones are proteins found within the cell nucleus. An octamer of 8 histone proteins, together with DNA forms one nucleosome. The main histone proteins are termed H1, H2A, H2B, H3 and H4. Present in the nucleosome is a H3-H4 tetramer, with an H2A-H2B dimer on either side. A chain of nucleosomes is known as chromatin. DNA tightly packed around chromatin is known as heterochromatin, DNA loosely packed around chromatin is known as euchromatin. Euchromatin is associated with a higher rate of expression of DNA, as the DNA is more accessible to transcriptional machinery<sup>[214]</sup>. There are many forms of modification present in histones that result in making genes more or less accessible for expression, this occurs due to the altered charge associated with the added molecules (Figure 1.9). By chemically altering residues on the tails of histones, weaker forces are present between DNA and the histones; this alters the chromatin structure, allowing greater accessibility for transcriptional machinery. The most common modifications that result in activation of genes are acetylation of lysines 9 and 14 and methylation of lysine 4 on H3; these are achieved by histone acetyltransferases (HATs) and HMTs, respectively. The most common chemical modifications that result in a silencing of expression are di- and trimethylation of lysine 9 of H3 by HMTs, G9a and Suv39Hi<sup>[214]</sup>. Methylation of histones is an important method of gene regulation. It is unclear if histone modifications play a role in transmission of altered phenotypes through generations.

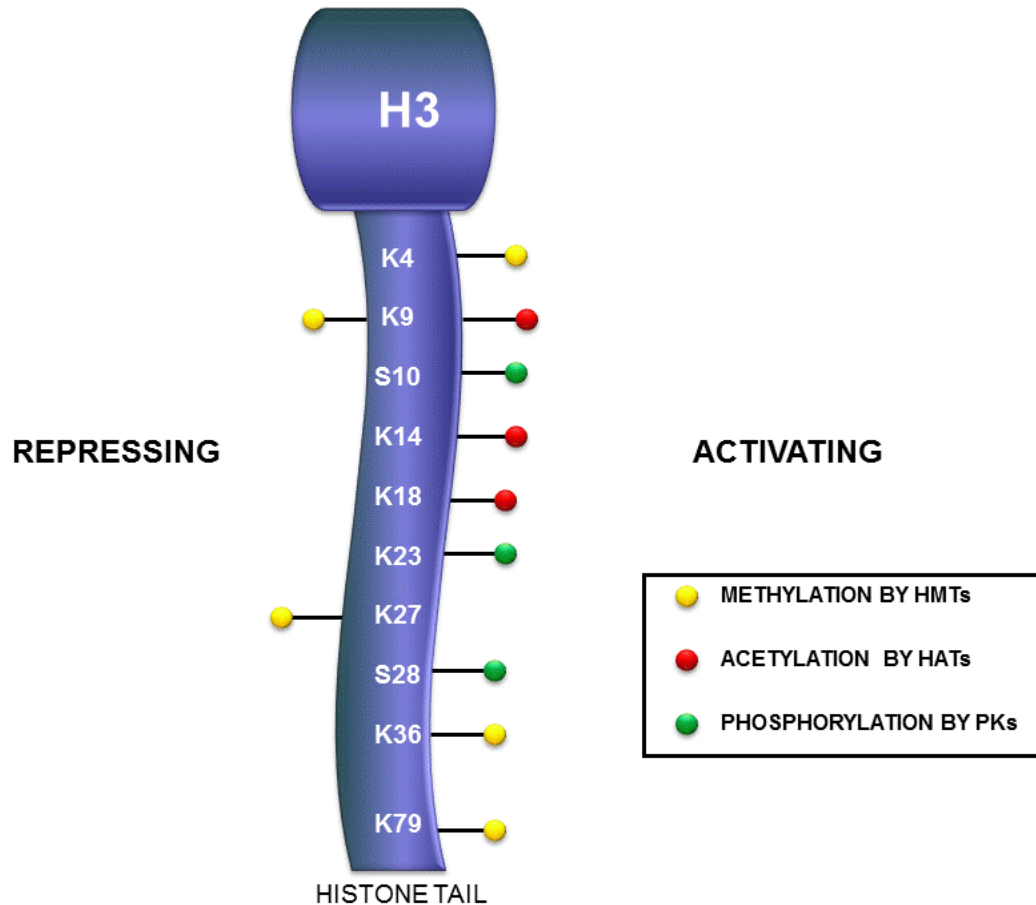


**Figure 1.9** Diagram showing activation and repression of gene activity through covalent modifications of histone H3. Methylation (activating and repressing) is achieved by histone methyltransferases (HMTs). Phosphorylation is catalysed by protein kinases (PKs,) Acetylation is catalysed by histone acetyltransferases (HATs).

#### 1.4.4 Epigenetics and early life origins of disease

The mechanisms behind gene regulation have been discussed with emphasis on those with potential epigenetic modifications. The following human observations and animal studies provide evidence behind epigenetic modifications and change of phenotype, particularly where disease risk results.

As stated previously, the Dutch Winter Famine provides a novel observation into the effects of parental under nutrition and the later life development of disease. Following the recent identification of the role of DNA methylation as a potential mechanism for induction of phenotype, the methylation profiles of adult offspring of

famine parents has been investigated. Heijmans et al measured 5 CpG sites within the downstream methylated region for the Insulin-like growth factor 2 (IGF-2) gene, a maternally imprinted gene that plays a key role in human growth and development. When comparing adults who were subject to periconceptual famine exposure to those who had no famine exposure, it was found that methylation of all 5 measured CpGs was significantly lower in the periconceptually exposed group<sup>[215]</sup>. The result was found to be independent of sex. The group also looked at methylation of the same CpGs in adults exposed to famine during late gestation and found no significant differences to unexposed adults. Interestingly, age also played a factor in methylation status in these CpGs, with a 10 year increase in age significantly associated with a 3.6% reduction in methylation<sup>[215]</sup>. In a similar study by Tobi et al, the methylation status of 15 imprinted and non-imprinted genes involved in growth and metabolic disease was characterised in adult Dutch Winter Famine individuals and their non-famine exposed same-sex siblings. In agreement with Heijmans et al, the study showed that periconceptual famine exposure led to decreased methylation in the gene *INSIGF*, when compared to non-famine exposed same-sex siblings. However this study also found an increase in methylation in the genes *IL10*, *LEP*, *ABCA1*, *GNASAS* and *MEG3*<sup>[201]</sup>, suggesting that famine exposure lead to constant changes in DNA methylation, which is not limited to lower levels of methylation. The study also highlighted the importance of timing of famine exposure, where late-gestation exposure only led to a change in methylation in *GNASAS*, when measuring methylation of 4 non-imprinted and 4 imprinted genes. Again this was comparing famine exposed to non-famine exposed same-sex siblings<sup>[201]</sup>. The study also highlights a role of sex in susceptibility of changes to methylation status in these measured genes, with sex differences in *IGFR*, *LEP*, *IL10* and *APOC1* shown<sup>[201]</sup>. These two studies focus heavily on imprinted genes, in order to show the true consequences of famine exposure during times of development, it would be more beneficial to focus on differences seen in non-imprinted genes. Although these studies show variation in DNA methylation between groups, it must be stated that the level of variation between samples in the methods used is around 5%. The limitations of the

techniques used are such that detection of methylation levels below 5-10% are below that of the stringent background levels of the software, but are subject to acceptance from users. As these findings are based on methylation differences of less than this, we cannot be confident of the results.

Through animal experiments into DNA methylation status in embryos the consequences of In Vitro Fertilisation and Assisted Reproductive Technologies (ARTs) on the epigenome have been investigated. The following studies will give detail into the methylation status of embryos and how implantation results in alterations. The effect of embryo culture and embryo transfer on genomic imprinting was investigated in mice by Rivera et al<sup>[216]</sup>. The results showed that both embryo transfer and culturing followed by transfer resulted in irregular expression of one or more imprinted genes in both the yolk sac and placenta, with culture exacerbating the effects or increasing the number of genes affected<sup>[216]</sup>. A study using the Agouti mouse model by Morgan has investigated 96 hour culturing of zygotes to the blastocyst stage, followed by transfer to a pseudo-pregnant female. A pseudo-pregnant female is one which has received hormonal treatment to prepare the body to accept a blastocyst. Results show a significant increase in pups expressing the active epiallele leading to the yellow coat, showing a decrease in methylation of the A<sup>vy</sup> allele in these mice. Again this result was validated by comparing natural pups to embryonic transfer alone and no difference was observed<sup>[200]</sup>. Further uses of the Agouti mouse in determining epigenetic mechanisms will be explored later in this section. These animal studies clearly show how misexpression of imprinted genes can be caused by *in vitro* techniques. The following group of studies focus on retrospective observations of IVF rates in children with birth defects or higher risks of disease in later life. IVF rates in children with the overgrowth syndrome, Beckwith-Weidemann Syndrome (BWS), or those born with defects, are higher than those conceived naturally<sup>[196,217]</sup>. In the United States of America the incidence of BWS is 0.8%, following IVF treatment this increases to 4.6%<sup>[196]</sup>. This increase is associated with specific epigenetic alterations in LIT1 and H19 in more than 80% of cases<sup>[196]</sup>. In Western Australia, Hansen et al investigated the relationship between intracytoplasmic sperm

injection or IVF and risk of birth defects, when compared to naturally conceived births<sup>[217]</sup>. The group obtained a high sample size by using data from three registries and found that incidence of birth defects increased from 4.2% for naturally conceived births to 8.6% for those born using intracytoplasmic sperm injection and to 9.0% for those born via IVF<sup>[217]</sup>. These findings have been confirmed by experiments by Katari who showed IVF children had a greater risk of low-birth weight, birth defects of any variety and disorders resulting from imprinted genes<sup>[197]</sup>. Methylation of 1,536 CpG sites in more than 700 genes in cord and placenta were measured, and although a high level of variance was seen in results, a lower level of methylation was evident in both imprinted and non-imprinted genes in these tissues<sup>[197]</sup>. These three studies have proved the considerations which must be taken into account when using ARTs, as any altered exposure to the zygotes of developing embryo is likely to result in changes to the epigenome and therefore phenotype of the offspring.

Experiments using the Agouti mouse have shown how fortifying the diet with factors that affect epigenetic machinery resulted in a change of phenotype and physiology of the animal. An experiment by Wolff et al looked at expression of the ectopic agouti gene, which codes for coat colour. In offspring of dams fed a diet fortified with folic acid, choline, betaine and vitamin B<sub>12</sub>, an increase in methylation at the agouti retrotransposon was present. This correlated with a reduced expression in the ectopic Agouti gene (A<sup>vy</sup>), leading to synthesis of eumelanin, which led to the black coated pseudoagouti phenotype<sup>[218]</sup>. As well as change in coat colour it was observed that the wild yellow coat coloured phenotype are typically larger, more obese, hyperinsulinemic, more susceptible to cancer and shorter lived when compared to the pseudoagouti phenotype<sup>[218]</sup>. This experiment shows how folic acid can lead to a changed phenotype through epigenetic modifications. However it is not the only nutritional alteration in the diet, so the results must be taken with caution. It would be beneficial to see the effects of folic acid alone in this model.

As discussed earlier the low protein diet provides a distinct change in metabolism and phenotype in rats which can be utilised to determine mechanisms and pathways which have been altered. Work by Lillycrop et al has shown that feeding a PR diet (9% casein Vs a control of 18% casein) to dams during pregnancy leads to changes in gene expression in the offspring which can in part be explained by alterations in the methylation status of CpGs found within the promoter regions of the genes. In 6 day old PR offspring PPAR $\alpha$  and GR expression was 10.5 fold and 2 fold higher than control offspring, respectively<sup>[198]</sup>. This change was concurrent with a decrease in promoter methylation of 20.6% for PPAR $\alpha$  and 22.8% for GR<sup>[198]</sup>. In later experiments by the group, using the same model, similar patterns in expression and methylation of GR were shown in both 37 and 80 day old offspring<sup>[187,199]</sup>. Expression of the maintenance DNA methyltransferase, DNMT1 was 17% lower in the PR offspring, indicating the role of the enzyme in the changes in expression seen<sup>[187]</sup>. The group also looked at methylation and acetylation of specific lysines in histones, which are associated with an increase in transcription. In the same group of animals they found an increase histone modifications that increase levels of transcription, such as H3K9 methylation, in the PR offspring compared to control, whilst modifications which lead to a suppression of transcription were decreased<sup>[187]</sup>. In a further study by the same group, the methylation status of specific CpG dinucleotides was determined, when using the same animal model<sup>[199]</sup>. In offspring of dams fed the PR during pregnancy methylation of CpGs 2, 3, 4 and 16 of the PPAR $\alpha$  promoter were significantly lower than offspring of dams fed the control diet, giving an overall lower methylation of 26% at the promoter ( $p < 0.05$ )<sup>[199]</sup>. The study also showed that supplementing the PR diet with 5mg/kg folic acid (PRF) led to no differences in methylation at these sites, when comparing the PRF and control offspring. This provides evidence for 'rescuing' the offspring of mothers fed a PR diet, by providing DNA methylation promoting diets.

As well as changes in the epigenome resulting from changes in nutritional exposure, non-nutritional phenotype induction has been shown. Weaver et al showed that an increase in pup licking and grooming and arched backed nursing

by dams altered the epigenome of the glucocorticoid promoter in the hippocampus of the offspring when compared to controls. These differences were seen in the first week of life and were reversed by cross fostering<sup>[206]</sup>. Although the mechanism behind these alterations are unknown, it is likely via a change in plasma levels of specific chemicals, such as endorphins, caused by the grooming. These results show that maternal behaviour can alter the phenotype of the offspring independent of nutrition and that the window of plasticity in the offspring extends beyond birth.

In nature all female honeybees are genetic clones arising from identical larvae, however some become fertile queens and others sterile workers. It has been found that those who are fed with royal jelly, a chemically uncharacterised substance, by young nurse bees become queen bees<sup>[219]</sup>. Those destined to become worker bees are fed a less sophisticated diet. By injecting small interfering RNA, to target DNMT3 in larvae the majority of females (72%) went on to become queens, with fully developed ovaries<sup>[219]</sup>. This study provides evidence that methylation is vital in nature, in the honeybee, in order to determine reproductive status. This provides insight into the role of natural methylation in other species including humans which must be explored.

To date the only study that has shown a role of epigenetic processes in transgenerational transmission of phenotype is by Burdge et al<sup>[220]</sup>. The study found that feeding rats a protein restricted diet during pregnancy led to significantly lower levels of methylation of the promoter regions of both PPAR $\alpha$  and GR in the liver, which were passed to the F2 generation<sup>[220]</sup>. It is not known if differences in epigenetics, which underlie alterations in phenotype between generations are passed between generations unaltered or if changes in the F0 pregnancy lead to variation in the offspring, which have the potential for adaptation.

#### 1.4.5 Folate

The B9 family of water-soluble B-vitamins are known as the folates. Physiologically they have roles in; cell division and repair, nucleotide biosynthesis, 1 carbon metabolism, DNA methylation, phospholipid biosynthesis and homocysteine remethylation<sup>[221]</sup>. Folates are comprised of a pteridine ring, p-amino benzoylglutamate group and polyglutamate tail, where 5 – 7 glutamate residues are normally present. The natural form, which contains the polyglutamate tail, is known as folate, it cannot be synthesised by the body, so is consumed in the diet. Synthetic folate, which only has one glutamate residue (monoglutamate) is known as folic acid.

Folates are transported into cells in the form of 5-methyltetrahydrofolate, this involves the hydrolysis of dietary folate polyglutamates to the monoglutamate forms<sup>[221]</sup>.

Tetrahydrofolates are responsible for one carbon metabolism; this involves the transport of methyl groups ( $\text{CH}_3$ ), which are made available for purine and pyrimidine biosynthesis, amino acid metabolism (serine, glycine and methionine), phospholipid biosynthesis and DNA methylation. The one carbon metabolism pathway takes place in both the mitochondria and cytoplasm. Phospholipid biosynthesis is important in growing tissues for incorporation into the cell membrane, the conversion of phosphatidyl ethanolamine (PE) to phosphatidyl choline (PC) by phosphatidyl ethanolamine methyltransferase, requires the addition of a methyl group. The end point of one carbon metabolism provides methyl groups for either phospholipid biosynthesis or DNA methylation S-Adenosyl-Methionine (SAM) is formed from the methyl groups derived from methionine in the one-carbon pathway<sup>[222]</sup>. As a result of the transfer of a methyl group, SAM is converted to S-Adenosyl-Homocysteine (SAH); this methyl group is available for DNA methylation.



#### **1.4.5.1 Modification of DNA methylation by folic acid**

The result of supplementation of the protein restricted diet during pregnancy, in rats, has been discussed previously. Further experiments involving folic acid supplementation in both rat models and human observations, with particular reference to cancer, have been undertaken.

#### **1.4.5.2 Evidence from animal models**

Folic acid prevented the alterations in gene specific regulation and cardiovascular adaptations, caused by protein restriction alone<sup>[198,51]</sup>. To potentially reverse the effects of protein restriction during pregnancy, the juvenile-pubertal diet has been supplemented with folic acid<sup>[25]</sup>. When feeding offspring of protein restricted dams, additional folic acid for 28 days following weaning, the phenotype was not simply reversed. Offspring that received the folic acid supplemented diet, regardless of the protein content of the prenatal diet showed; increased weight gain, lower plasma  $\beta$ -hydroxybutyrate concentration and increased hepatic and plasma triglyceride concentrations, compared to offspring who received the folic acid adequate diet. The folic acid supplemented offspring also showed changes in the epigenome, where both PPAR $\alpha$  and GR promoter methylation was increased in liver and insulin receptor promoter methylation was decreased in liver and adipose tissue, compared to the folic acid adequate offspring<sup>[25]</sup>.

#### **1.4.5.3 Evidence in humans**

Initiation of cancer can be through altered regulation and expression of both tumour suppressor genes and proto-oncogenes, therefore supplementation of the diet with folic acid has been investigated. Although the majority of studies linking folic acid intake to cancer risk are inconclusive, the relationship between folic acid intake and colorectal cancer has been well investigated. In a controlled placebo trial in patients with a history of colonic dysplasia or neoplasia, folic acid supplementation in adults was shown to improve the DNA methylation status in both colonic mucosa<sup>[223]</sup> and rectal mucosa<sup>[224]</sup> and reduce colonic mucosal cell proliferation<sup>[225]</sup>. A follow-up to the Nurse's Health Study and Health Professionals

study found a 30-40% decrease in risk of colorectal adenoma, when dietary folic acid intake was  $> 700\mu\text{g/day}$ , compared with lower intakes<sup>[226]</sup>. Interestingly a more recent study has shown that both higher and lower intakes of folic acid are associated with a moderate reduction in risk of colorectal cancer<sup>[227]</sup>.

Hyperhomocysteinaemia is a condition where S-adenosyl homocysteine is increased, as a competitive inhibitor for S-adenosyl methionine, this suggests unbalanced methylation<sup>[228]</sup>. DNA methylation, via cytosine extension assay and southern blotting, showed that genome wide DNA hypomethylation was present in men with hyperhomocysteinaemia and uraemia<sup>[228]</sup>. Folate treatment was given to those who were heterozygous for the H19 gene, where it decreased the level of DNA hypomethylation. This clear example of folate restoring DNA methylation levels, however, was only investigated in those heterozygous for the H19 gene, which accounted for around 20% of those with the condition. The study did not comment on the remaining patients.

### **1.5.1 PEPCK**

The enzyme PEPCK is integral to provision of glucose during periods of fasting. An alteration in the regulation of transcription of the gene will therefore lead to a significant difference in the ability to provide glucose for dependent organs during fasting. The following sections provide a review on the current literature detailing the epigenetic regulation of the PEPCK promoter, with particular reference to DNA methylation.

#### **1.5.1.1 Epigenetic regulation of PEPCK**

The PEPCK gene is expressed in high levels in adult liver and kidney. During fetal development PEPCK remains dormant in these gluconeogenic tissues until it is induced in response to changes in the insulin/glucagon ration around fetal day 15-18<sup>[229,230]</sup>. PEPCK gene expression is positively regulated by glucagon<sup>[231,232,233,234]</sup> and glucocorticoids<sup>[167,235,232,236]</sup> and negatively regulated by insulin<sup>[237]</sup>. Regulation by glucagon is via increased levels of cAMP, which leads to activation of PKA<sup>[231]</sup>. cAMP also acts to stabilise PEPCK mRNA, so that it is

degraded after 150 - 240 minutes, compared to its typical degradation time of 30 minutes<sup>[231]</sup>. Administration of the synthetic glucocorticoid, dexamethasone to rats in the last week of pregnancy led to a 60% increase in both expression and activity of PEPCK<sup>[236]</sup>. The effect however, was not seen following dexamethasone administration during the first or second week of pregnancy, suggesting an inability of the gene to respond to altered environment during early development.

The PEPCK promoter has been most extensively studied in the rat; however, there is a high level of homology in the PEPCK gene from -1500 bp to the start site of transcription in human, rat, mouse, cow and dog genomes<sup>[238,136]</sup>. Despite studies providing different definitions of the promoter, there is much agreement into regulation of the promoter. Short et al, state that the region -2088 to + 69 bp of the PEPCK gene is necessary and sufficient to direct hepatocyte specific developmental, hormonal and dietary regulation of the gene<sup>[230]</sup>. However, Chakravarty et al have shown the region -1500 to +73 bp of the PEPCK gene contains all of the regulatory elements required for transcription<sup>[238]</sup>. The initial study into binding at the PEPCK promoter identified 8 protein binding domains, using DNase I footprinting<sup>[234,230]</sup>. The study identified 4 binding sites for cAMP response element (CRE) binding proteins, with the most important functionally at -147 to -130 bp<sup>[234,230]</sup>. Further investigation into the promoter has led to the promoter being divided into three regions, region 1, the closest to the transcription start site, contains one CRE binding site and an NF-1 site, region 2 contains a HNF-1 site, critical for transcription of PEPCK in kidney and region 3, the furthest from the transcription start site, contains the cAMP response unit (CRU) and glucocorticoid response unit (GRU) of the promoter<sup>[238]</sup>. Rather than containing one glucocorticoid response element (GRE), the GRU coordinates the hormonal response of PEPCK to insulin and glucocorticoids and interacts with the CRU to mediate cAMP control of PEPCK transcription<sup>[238]</sup>. Table 1.1 shows a selection of transcription factors which bind at the PEPCK promoter and their role in regulation of the promoter.

| Transcription factor                | Function in PEPCK regulation                                          |
|-------------------------------------|-----------------------------------------------------------------------|
| C/EBP $\alpha$                      | Basal transcription, cAMP-induced gene expression, hepatic expression |
| C/EBP $\beta$                       | Basal transcription, cAMP-induced gene expression, hepatic expression |
| DBP<br>(D-binding protein)          | Basal transcription                                                   |
| NF-1<br>(Nuclear factor-1)          | Inhibits cAMP induced gene expression                                 |
| HNF-1<br>(Hepatic nuclear factor-1) | Renal gene expression                                                 |
| HNF-3                               | Glucocorticoid-induced gene expression                                |
| CREB                                | cAMP-induced gene expression                                          |
| GR                                  | Glucocorticoid-induced gene expression                                |
| THY<br>(Thyroid response element)   | Thyroid-induced gene expression                                       |
| PPAR $\gamma$ 2                     | Adipose tissue specific expression                                    |

**Table 1.1 Transcription factors involved in regulation of PEPCK gene expression**  
modified from Nizielski 96.

The CAAT/enhancer binding protein (C/EBP) is a key regulator of PEPCK gene transcription<sup>[238,231,239,230]</sup>. Deleting the gene for C/EBP $\alpha$  in mouse completely repressed the expression of PEPCK in liver at birth and led to profound hyperglycaemia in 1-2 hour new-born<sup>[230,136]</sup>. Along with a conformational change in chromatin around 19 days of fetal life, the transcription factor plays a vital role in the initial expression of PEPCK in mammals. As well as positive regulation of PEPCK by glucocorticoids, through the GRU and glucagon, through the CRU, PEPCK is also subject to negative regulation, through insulin. In human HepG2 cells, insulin inhibits binding of transcription factors to regulatory sites<sup>[237,230]</sup>, although the direct mechanism is unknown. In a study by Hall et al, ablation of the

insulin-responsive sequence did not prevent the negative regulation by insulin seen in the liver, suggesting the involvement of further elements<sup>[240,230]</sup>. Due to its role in fasting energy metabolism, altered regulation at the PEPCK promoter has the potential to have adverse consequences on health during times of poor nutrition.

#### **1.5.1.2 Methylation at the PEPCK promoter**

There is some evidence that transcription of PEPCK is regulated via DNA methylation. Through use of the methylation inhibitor 5-azacytidine in rat fetuses, it was shown that demethylation of the PEPCK promoter in spleen and liver led to expression of the gene<sup>[241,230]</sup>. Using the methyl sensitive enzymes HpaII and HhaI, it was shown that in tissues which do not express the PEPCK gene, adult spleen, heart muscle and fetal liver, the PEPCK promoter is methylated. However in fetal liver there is a loss of methylation between day 18 and 21 of fetal development, which correlates with expression of the gene<sup>[229,230]</sup>. These changes in methylation are concurrent with changes in the metabolic phenotype of the developing fetus. During in utero development, the maternal circulation provides glucose for the fetus, via the placenta. Following birth and weaning, the offspring must provide their own glucose, from gluconeogenesis, when none is available from the diet. A loss in repressive methylation at the late stages of fetal development therefore appears to prepare the offspring metabolism for the external environment. Although useful in understanding regulation of the gene, these studies do not cover all CG dinucleotides in the PEPCK promoter, only those covered by the methyl sensitive enzymes and the methods used are not as accurate as those currently available. It has been shown that methylation of specific CpGs is sufficient to significantly alter gene expression; therefore the use of a sequencing technique will allow a deeper understanding of regulation of transcription.

## 1.6 Summary of literature and research hypothesis

The cardio-metabolic disease pandemic is an increasing issue in the present population, where although those in developing countries are seeing the greatest risk in the disease<sup>[230,1]</sup>, it is still a major burden on the economy in the UK, with risk factors now presenting in school aged children<sup>[3]</sup>. Although genetic components are evident behind risk factors, there is clear link between environment and genotype.

A plethora of studies have explored the association between the early life risk of disease, with particular focus on the transmission of disease risk between generations<sup>[166]</sup>. In animal studies the mechanisms and extent to which the environment, in particular nutrition of the mother during pregnancy has been fully explored<sup>[242,243,220,244,48,57,41,245,246,247,42,198,187]</sup>. It has become apparent, when considering the altered phenotype of offspring that the duration and magnitude of the altered external plays a fundamental role.

The fasting response requires an orchestrated combination of hormonal effects, to ensure preservation of glucose and provision of energy substrates to key organs. In the state of pregnancy, this response is heightened to ensure the growth of the fetus as well as the survival of the mother<sup>[160,112,102]</sup>. Fatty acid desaturation and elongation is essential in order to produce long chain fatty acids<sup>[147]</sup>. Physiologically, this is important for use in membrane structures, to increase fluidity and transmission of signals through the bilayer.

The optimal functioning of both fasting glucose homeostasis and the fatty acid desaturation-elongation pathway is required for successful survival to reproductive age and beyond. Differences in metabolism have been shown to result from an altered early life environment<sup>[47,244,74,32,70]</sup>. An understanding of the mechanisms behind the changes in phenotype in postnatal life will increase knowledge of this association and could potentially lead to strategies for minimising the deleterious effects of predictive adaptive responses.

Epigenetic processes have been implicated as a potential mechanism behind the early life origins of disease<sup>[184,162,248,216]</sup>. Through inhibition of

transcription DNA methylation can directly or indirectly lead to alterations in phenotype of an organism.

To date no studies have investigated the transmission of DNA methylation marks of specific promoter regions between subsequent generations of different dietary lines.

In order to fully understand the potential for changes in methylation on gene expression and phenotype, it will be necessary to investigate the regulation of gene expression in different dietary exposure groups, in animals of subsequent generations. This will identify if methylation levels of one promoter are subject to variation under different experimental conditions, in a CpG specific manner.

The role of pregnancy in the developmental origins of health and disease is clear, as environmental markers are passed from the maternal circulation to the fetus<sup>[156,157,158]</sup>. However, no studies to date have investigated changes in maternal physiology, gene expression and epigenotype during pregnancy, in a diet specific manner.

The aim of this thesis is to address these unanswered research questions. It is hypothesised that;

-In the rat, increased energy content throughout life, across four generations, will lead to significant differences in weight gain, energy intake and fasting plasma metabolite levels across the generations, as the animals adjust to the high energy content of the diet.

-The trajectory of adjustment towards the high energy diet across the four generations will be significantly altered by a reduced protein content of the F0 diet during pregnancy.

-Underlying differences in gene expression of specific genes involved in fasting carbohydrate and fat metabolism will be related to the significant differences in fasting energy metabolites.

-Differences in DNA methylation of the promoter regions of specific genes will be related to expression levels of the gene, in a CpG specific manner.

-The additional stress of pregnancy will lead to significant differences in the phenotype, gene expression and DNA methylation levels of specific genes.

-The nature of the nutritional exposure will led to gene specific differences in transcriptional regulation.

-Regulation of specific genes will be significantly altered by the form and timing of the altered nutritional environment.

-Where altered promoter methylation is associated with significant differences in gene expression, differences in transcription factor binding will be present.

-Mutation of specific CpGs, which show associations with gene expression, will lead to significantly different levels of gene transcription under basal or activated conditions.





## Chapter 2

### Materials and Methods

## 2. Materials and methods

### 2.1 Materials

**Table 2.1 Reagents used in experiments**

| Reagent/Chemical                                              | Supplier                                    |
|---------------------------------------------------------------|---------------------------------------------|
| 1 kb ladder                                                   | Fermentas, York, UK                         |
| 17 $\alpha$ ethinylestradiol                                  | Sigma-Aldrich, Dorset, UK                   |
| 2018S Teklad Global 18% Protein Diet                          | Harlan, Bicester, UK                        |
| 2-mercaptoethanol                                             | Sigma-Aldrich, Dorset, UK                   |
| 3mm blotting paper                                            | Whatman, Maidstone, UK                      |
| 40% Acrylamide                                                | Fisher, Leicestershire, UK                  |
| 5-bromo-4-chloro-3-indolyl-beta-D-galactosyranoside (X-Gal)   | Fisher, Leicestershire, UK                  |
| 50 X Tris-acetate-EDTA (TAE) buffer                           | Fisher, Leicestershire, UK                  |
| Agarose                                                       | Melford Laboratories, Ipswich, UK           |
| AIN93-G diet                                                  | TestDiet, Indiana, USA                      |
| AIN93-M diet                                                  | TestDiet, Indiana, USA                      |
| Albumin bovine serum                                          | Sigma-Aldrich, Dorset, UK                   |
| Amersham Hybond N+ nylon membrane                             | GE Healthcare, Chalfont, UK                 |
| Aminopropyl silica columns                                    | Varian, California, USA                     |
| Ammonium persulfate (APS)                                     | Fisher, Leicestershire, UK                  |
| Ampicillin                                                    | Sigma-Aldrich, Dorset, UK                   |
| Bromphenol blue                                               | Sigma-Aldrich, Dorset, UK                   |
| Chloroform                                                    | Fisher, Leicestershire, UK                  |
| DNase I                                                       | Sigma-Aldrich, Dorset, UK                   |
| DNase and RNase free water                                    | Fisher, Leicestershire, UK                  |
| Deoxynucleotide Trisphosphates (dNTPs)                        | Sigma-Aldrich, Dorset, UK                   |
| Dulbecco's Modified Eagle Medium, low glucose                 | Sigma-Aldrich, Dorset, UK                   |
| Dual-luciferase reporter assay kit                            | Promega, Southampton, UK                    |
| Ethanol                                                       | Fisher, Leicestershire, UK                  |
| Ethylenediaminetetraacetic acid disodium salt (EDTA)          | Sigma-Aldrich, Dorset, UK                   |
| EZ-DNA Methylation Gold Kit                                   | Cambridge Bioscience, Cambridge, UK         |
| Ficoll 400                                                    | Sigma-Aldrich, Dorset, UK                   |
| fuGENE transfection reagent                                   | Promega, Southampton, UK                    |
| Glucagon DuoSet ELISA                                         | R&D Systems, Abingdon, UK                   |
| Glucagon DuoSet ELISA substrate solution                      | R&D Systems, Abingdon, UK                   |
| Glycine                                                       | Sigma-Aldrich, Dorset, UK                   |
| Goat polyclonal antibody against fads2 sc-109272              | Santa-Cruz Biotechnologies, California, USA |
| Goat polyclonal antibody to rabbit (HRP) ab97051              | Abcam, Cambridge, UK                        |
| HEPES                                                         | Sigma-Aldrich, Dorset, UK                   |
| Hydrochloric acid (HCl)                                       | Sigma-Aldrich, Dorset, UK                   |
| Isopropanol                                                   | Fisher, Leicestershire, UK                  |
| Isoprpyl $\beta$ -D-1 thiogalactopyranoside (IPTG)            | Fisher, Leicestershire, UK                  |
| JM109 competent cells                                         | Promega, Southampton, UK                    |
| Leupeptin                                                     | Sigma-Aldrich, Dorset, UK                   |
| Light-Shift chemiluminescent electro mobility shift assay kit | Fisher, Leicestershire, UK                  |
| Luria broth                                                   | Fisher, Leicestershire, UK                  |

|                                                  |                                    |
|--------------------------------------------------|------------------------------------|
| Marvel powdered milk                             | Sainsburys, Southampton, UK        |
| Methanol                                         | Fisher, Leicestershire, UK         |
| Modified AIN93-G diet                            | TestDiet, Indiana, USA             |
| Molecular weight marker ab1746                   | Abcam, Cambridge, UK               |
| M-MLV reverse transcriptase                      | Sigma-Aldrich, Dorset, UK          |
| NP-40                                            | Sigma-Aldrich, Dorset, UK          |
| Orange G                                         | Sigma-Aldrich, Dorset, UK          |
| Pierce BCA protein assay kit                     | Thermo-Fisher, Illinois, USA       |
| Phenylmethanesulfonylfluoride (PMSF)             | Sigma-Aldrich, Dorset, UK          |
| Phosphate buffered saline (PBS)                  | Sigma-Aldrich, Dorset, UK          |
| Phenol                                           | Fisher, Leicestershire, UK         |
| Pyrogold reagents                                | Qiagen, Crawley, UK                |
| Pyrosequencer annealing buffer                   | Qiagen, Crawley, UK                |
| Pyrosequencer binding buffer                     | Qiagen, Crawley, UK                |
| Pyrosequencer nucleotide tips                    | Qiagen, Crawley, UK                |
| Pyrosequencer reagent tips                       | Qiagen, Crawley, UK                |
| Pyrosequencer wash solution                      | Qiagen, Crawley, UK                |
| PCR primers – biotin labelled                    | Biomers, Söflinger Straße, Germany |
| PCR primers                                      | Invitrogen, Paisley, UK            |
| pGEMT vector                                     | Promega, Southampton, UK           |
| pGI3 vector                                      | Promega, Southampton, UK           |
| Pfu proofreading polymerase                      | Promega, Southampton, UK           |
| Proteinase K                                     | Promega, Southampton, UK           |
| QIAquick gel extraction kit                      | Qiagen, Crawley, UK                |
| QIAquick PCR clean up kit                        | Qiagen, Crawley, UK                |
| Rabbit polyclonal antibody against PEPCK ab28455 | Abcam, Cambridge, UK               |
| Rabbit polyclonal antibody to goat (HRP) ab97100 | Abcam, Cambridge, UK               |
| Random Nonamers                                  | Sigma-Aldrich, Dorset, UK          |
| Rat and Mouse No.1 Maintenance Diet              | SDS, Essex, UK                     |
| Rat leptin ELISA kit                             | Millipore, Missouri, USA           |
| Restriction enzymes                              | Promega, Southampton, UK           |
| ApoI, BglII, Cla, DpnI, HindIII, Xba             |                                    |
| RNase A                                          | Sigma-Aldrich, Dorset, UK          |
| Reference dye for quantitative PCR               | Sigma-Aldrich, Dorset, UK          |
| SequalPrep long polymerase                       | Invitrogen, Paisley, UK            |
| Sodium acetate (NaAc)                            | Sigma-Aldrich, Dorset, UK          |
| Sodium chloride (NaCl)                           | Fisher, Leicestershire, UK         |
| Sodium dodecyl sulphate (SDS)                    | Sigma-Aldrich, Dorset, UK          |
| Sodium deoxycholate                              | Sigma-Aldrich, Dorset, UK          |
| Streptavidin sepharose beads                     | GE healthcare, Chalfont, UK        |
| Sulfuric acid (H <sub>2</sub> SO <sub>4</sub> )  | Sigma-Aldrich, Dorset, UK          |
| SYBR GREEN Master Mix                            | Sigma-Aldrich, Dorset, UK          |
| Taq DNA polymerase Hot start                     | Qiagen, West Sussex, UK            |
| Tetramethylethylenediamine (TEMED)               | Sigma-Aldrich, Dorset, UK          |
| Tri reagent                                      | SDS, Essex, UK                     |
| Tris                                             | Fisher, Leicestershire, UK         |
| Tris-acetate EDTA (TAE)                          | Fisher, Leicestershire, UK         |
| Tris-borate EDTA (TBE)                           | Fisher, Leicestershire, UK         |
| Triton X-100                                     | Fisher, Leicestershire, UK         |
| Tween® 20                                        | Sigma-Aldrich, Dorset, UK          |

## **2.2 Methods**

### **2.2.1 Study design**

#### **2.2.1.1 Animal model**

All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedure) Act (1986). All experimental diets were supplied by TestDiet, Indiana, USA. The composition of the diets is shown in Tables 2.2, 2.3 and 2.4. The energy content of the diets is shown in Figure 2.2. Before arrival to the animal house F0 females were fed the standard breeding colony diet (2018S, Teklad Global 18% Protein Rodent Diet, Harlan, Bicester, UK.) Upon arrival to the animal house, adult female virgin Wistar rats (F0) were fed the standard animal house diet (RM1, Rat and Mouse No.1 Maintenance diet, SDS, Essex, UK) during the 14 day acclimatisation period. Following this, adult females were mated and fed either; a protein sufficient diet (PS) containing 180 g/kg casein (w/w) or an isoenergetic protein restricted diet (PR) containing 90 g/kg casein (w/w), throughout pregnancy (Table 2.2) the diets are based around those used by Burdge et al<sup>[25]</sup>. For the short duration of mating, females were fed the standard animal house diet; this was to ensure all breeding males were not exposed to the altered nutrition of the experiment, in order to avoid male intergenerational effects<sup>[167]</sup>. The experimental design is summarised in Figure 2.1. Sixteen dams were randomly assigned to each diet. Pregnancy was determined by presence of the copulatory plug. On day 8.5 of pregnancy seven females per dietary group were culled for tissue collection. The remainder were allowed to deliver spontaneously at around day 21. Within 24 hours of birth, F1 litters were standardised to eight pups, in order to ensure even nutrition. In order to maximise breeding stock, litters were maximised for females. At delivery, all dams were transferred to the AIN93-G semi-purified breeding diet. After 28 days, three females from each litter were selected at random and weaned onto the AIN-93M, maintenance diet, and placed in group cages, with five rats being placed in each cage. Dams and remaining day 28 old offspring were culled and tissues collected. At 70 days old, one female from each litter was culled, and tissues were collected. The two remaining F1 females from each litter were mated with males who had

received standard chow throughout life. All pregnant females were fed the PS diet throughout pregnancy. Following pregnancy the experimental set up for the F2 offspring was identical to that of the F1 offspring. At 70 days of age F2 females were either killed for sample collection or mated with males who had received standard chow throughout life. All pregnant females were fed the PS diet throughout pregnancy. Following pregnancy the experimental diets used for the F3 offspring were identical to that of the F2 offspring.

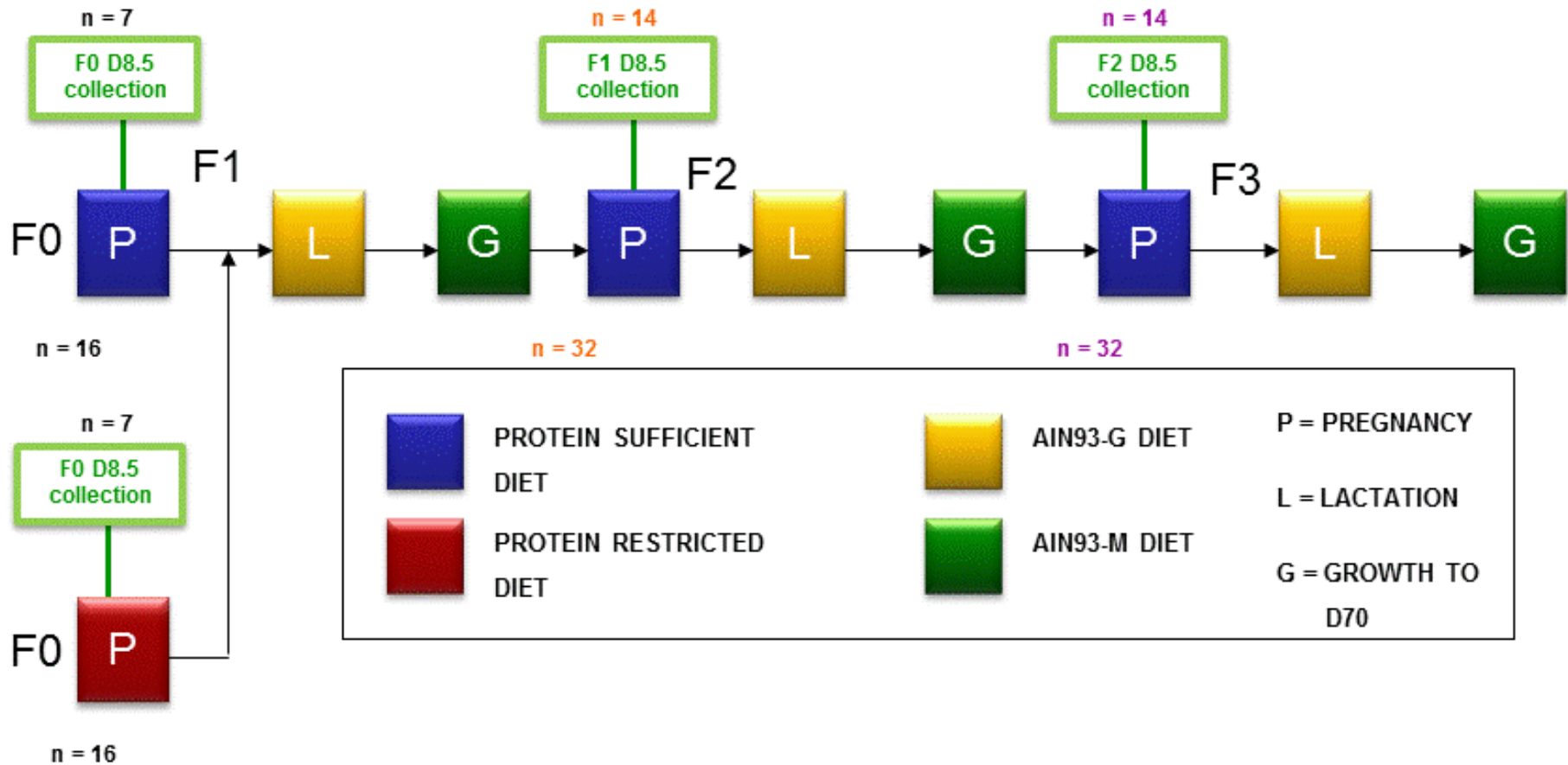


**Figure 2.1 Diagram showing the experimental design of the animal model.** The protein sufficient diet (PS) is shown in blue, the protein restricted diet (PR) is shown in red, the lactation diet is shown in yellow and the maintenance diet is shown in green. Pregnancy is shown by P, lactation by L and growth by G. F0 females were mated and fed either the PS or PR diet throughout pregnancy. Following birth all dams were fed the lactation diet. At 28 days old F1 offspring were weaned onto the maintenance diet. Once 70 days old (adults), F1 females were mated and fed the PS diet throughout pregnancy. Once the F2 offspring were born, F1 dams were fed the lactation diet. At 28 days old F2 females were weaned onto the maintenance diet. Once 70 days old, F2 females were mated and fed the PS diet throughout pregnancy. Following birth all F2 dams were fed the lactation diet. At 28 days old F3 offspring were weaned onto the maintenance diet, they continued on this diet until adulthood. Seven females per dietary group were sacrificed at day 8.5 of pregnancy, in each generation.

|                                          | PS   | PR   | Lactation diet (AIN-93G) | Maintenance diet (AIN-93M) | Animal House diet (RM1) | Breeding colony diet (2018S) |
|------------------------------------------|------|------|--------------------------|----------------------------|-------------------------|------------------------------|
| <i>g/kg</i>                              |      |      |                          |                            |                         |                              |
| Casein                                   | 183  | 92   | 200                      | 140                        | 129                     | 188                          |
| Cornstarch                               | 420  | 482  | 397                      | 466                        | 450                     | 450                          |
| Sucrose                                  | 213  | 243  | 100                      | 100                        | 40.5                    | 50                           |
| Choline                                  | 2.8  | 2.8  | 2.5                      | 2.5                        | 1.1                     | 1.1                          |
| Methionine                               | 9.7  | 7.4  | 5.2                      | 3.6                        | 2.2                     | 4.3                          |
| Cellulose (Crude Fiber)                  | 50   | 50   | 50                       | 50                         | 43.2                    | 38                           |
| Soybean oil                              | 100  | 100  | 70                       | 40                         | 27.1                    | 60                           |
| Total metabolizable energy, <i>MJ/kg</i> | 17.2 | 17.4 | 16.4                     | 15.78                      | 10.74                   | 13.7                         |

**Table 2.2 Table showing the composition of the diets fed to animals during the study.** All nutrients are given as g/kg. Total metabolizable energy is given in MJ/kg. PS, PR, AIN-93G and AIN-93M diets were supplied from TestDiet, Indiana, USA. The animal house diet was supplied by SDS, Essex, England. The 2018S diet was supplied by Harlan, Bicester, England.



|                                           | PS     | PR     | Lactation diet (AIN-93G) | Maintenance diet (AIN-93M) | Animal House diet | Breeding colony diet (2018S) |
|-------------------------------------------|--------|--------|--------------------------|----------------------------|-------------------|------------------------------|
| <i>mg/kg</i>                              |        |        |                          |                            |                   |                              |
| Vitamin A                                 | 0.6    | 0.6    | 1.2                      | 1.2                        | 2.5               | 9.21                         |
| Vitamin D <sub>3</sub> (Cholecalciferol)  | 0.0125 | 0.0125 | 0.025                    | 0.025                      | 0.015             | 0.052                        |
| Vitamin E (α-Tocepherol)                  | 30.55  | 30.55  | 50.25                    | 52.79                      | 56.3              | 128                          |
| Vitamin K (Menadione)                     | 0.14   | 0.14   | 0.29                     | 0.29                       | 10.17             | 102                          |
| Vitamin B <sub>1</sub> (Thiamine)         | 3.1    | 3      | 6                        | 6                          | 8.58              | 117.6                        |
| Vitamin B <sub>2</sub> (Riboflavin)       | 3.6    | 3.3    | 6                        | 6.5                        | 4.33              | 27.2                         |
| Vitamin B <sub>3</sub> (Niacin)           | 15     | 15     | 30                       | 30                         | 61.32             | 87.3                         |
| Vitamin B <sub>5</sub> (Pantothenic Acid) | 8      | 8      | 15                       | 16                         | 20.17             | 141.6                        |
| Vitamin B <sub>6</sub> (Pyridoxine)       | 2.9    | 2.9    | 5.8                      | 5.8                        | 4.81              | 26.8                         |
| Vitamin B <sub>7</sub> (Biotin)           | 0.1    | 0.1    | 0.2                      | 0.1                        | 0.27              | 0.8                          |
| Vitamin B <sub>12</sub> (Cyanocobalamine) | 0.016  | 0.014  | 0.025                    | 0.028                      | 0.0075            | 0.150                        |
| Vitamin C (Ascorbic Acid)                 | -      | -      | -                        | -                          | 259               | -                            |
| Folic acid                                | 1.1    | 1.1    | 2                        | 2.1                        | 0.79              | 8.4*                         |
| Choline Chloride                          | 1960   | 1960   | 1250                     | 1250                       | 1080              | 1120                         |
| Inositol                                  | -      | -      | -                        | -                          | 2300              | 1450                         |
| B-carotene                                | -      | -      | -                        | -                          | 0.16              | 2.5                          |

**Table 2.3 Table showing the vitamin content of the diets fed to animals during the study.** All values are given as mg/kg. PS, PR, AIN-93G and AIN-93M diets were supplied from TestDiet, Indiana, USA. The animal house diet was supplied by SDS, Essex, England. The 2018S diet was supplied by Harlan, Bicester, England. \* As dietary folate.

|                                     | PS   | PR   | Lactation diet (AIN-93G) | Maintenance diet (AIN-93M) | Animal House diet | Breeding colony diet (2018S) |
|-------------------------------------|------|------|--------------------------|----------------------------|-------------------|------------------------------|
| Calcium<br><i>g/kg</i>              | 2.9  | 2.9  | 5                        | 5                          | 7.3               | 10                           |
| Phosphorus (available), <i>g/kg</i> | 1.5  | 0.7  | 1.6                      | 1.1                        | 2.8               | 6.5                          |
| Potassium<br><i>g/kg</i>            | 2.1  | 2.1  | 3.6                      | 3.6                        | 6.7               | 6.8                          |
| Magnesium<br><i>g/kg</i>            | 0.3  | 0.3  | 0.5                      | 0.5                        | 2.3               | 2                            |
| Sodium<br><i>g/kg</i>               | 0.8  | 0.8  | 1                        | 1.3                        | 2.5               | 2.3                          |
| Chloride<br><i>g/kg</i>             | 1.9  | 1.9  | 1.6                      | 2                          | 3.8               | 4                            |
| Fluorine<br><i>mg/kg</i>            | 0.6  | 0.6  | 1                        | 1                          | 10.49             | -                            |
| Iron<br><i>mg/kg</i>                | 21   | 20   | 41                       | 36                         | 159.3             | 225                          |
| Zinc<br><i>mg/kg</i>                | 22   | 19   | 35                       | 35                         | 35.8              | 77                           |
| Manganese<br><i>mg/kg</i>           | 6    | 6    | 11                       | 11                         | 72.4              | 118                          |
| Copper<br><i>mg/kg</i>              | 3.4  | 3.4  | 6                        | 6                          | 11.5              | 15                           |
| Cobalt<br><i>mg/kg</i>              | -    | -    | -                        | -                          | 0.63              | 0.6                          |
| Iodine<br><i>mg/kg</i>              | 0.12 | 0.12 | 0.21                     | 0.21                       | 1.2               | 12                           |
| Chromium<br><i>mg/kg</i>            | 0.6  | 0.6  | 1                        | 1                          | -                 | 0.5                          |
| Molybdenum<br><i>mg/kg</i>          | 0.08 | 0.08 | 0.14                     | 0.14                       | -                 | -                            |
| Selenium<br><i>mg/kg</i>            | 0.1  | 0.1  | -                        | 0.17                       | 0.29              | 0.2                          |

**Table 2.4 Table showing the mineral content of the diets fed to animals during the study.** All minerals are given as either g/kg or mg/kg. PS, PR, AIN-93G and AIN-93M diets were supplied from TestDiet, Indiana, USA. The animal house diet was supplied by SDS, Essex, England. The 2018S diet was supplied by Harlan, Bicester, England.

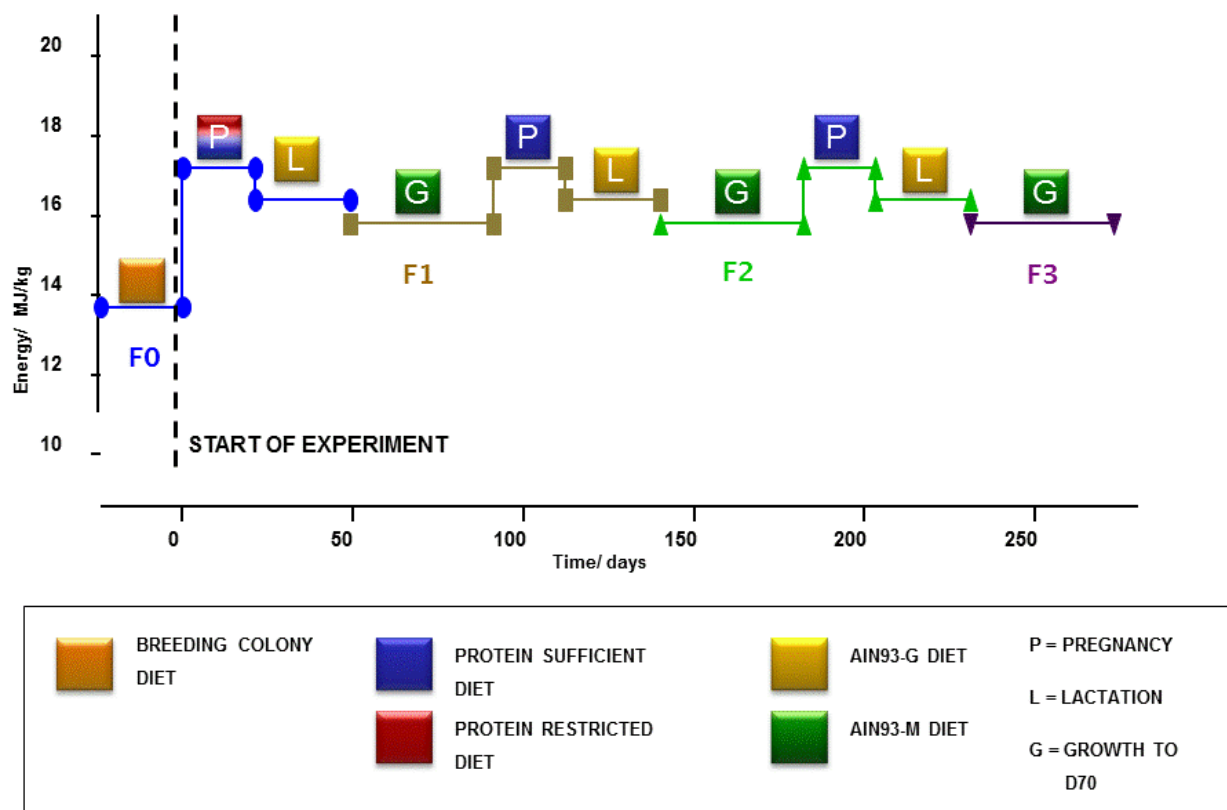


**Figure 2.2** Diagram showing the difference in total metabolizable energy/ MJ/kg between the breeding colony diet and the diets used in the study. The total metabolizable energy for each diet is; 13.7 MJ/kg for the Breeding colony diet, 17.2 MJ/kg for the protein sufficient diet, 17.4 MJ/kg for the protein restricted diet, 16.4 MJ/kg for the AIN-93G diet and 15.8 MJ/kg for the AIN-93M diet. Due to the short exposure of mating females to the animal house diet during mating, it is not shown on the graph.

#### **2.2.1.2 Animal weight and food intake collection.**

Throughout the experiment animal weights and 24 hour food intakes were recorded every 7 days. The 24 hour food intake measurements were achieved by providing each cage a set amount of food (120 -150g for single cages, and 450-550g for group cages), measuring the amount of food remaining 24 hours later and calculating the difference.

#### **2.2.1.3 Tissue collection**

After being culled, liver, heart and blood were collected from each rat. Liver and hearts weights were measured, and tissues were snap frozen in liquid nitrogen upon collection. All tissue was stored at -80°C long term.

#### **2.2.1.4 Plasma analysis**

Blood was collected by cardiac puncture and stored on ice at collection in heparinised tubes, to prevent coagulation. Upon arrival in the lab, blood was centrifuged at 2,000 rpm for 10 minutes, in order to collect plasma. Plasma was stored long term at -80°C. Plasma concentrations of  $\beta$ -hydroxybutyrate, glucose, corticosterone, glucagon and leptin were measured.

For  $\beta$ -hydroxybutyrate and glucose, aliquots of plasma were measured by Dr John Jackson (Institute of Human Nutrition, University of Southampton) using a Konelab 20 autoanalyser. Enzyme-Linked Immunosorbent Assays (ELISAs) were used to measure corticosterone, glucagon and leptin plasma concentrations. Corticosterone concentration was measured by Dr Nicola Thomas.

##### **2.2.1.4.1 Enzyme linked immunosorbent assays (ELISAs)**

Plasma glucagon and leptin was measured using sandwich ELISA kits: Glucagon DuoSet ELISA (R & D Systems, Abingdon, UK) and Rat Leptin ELISA kit (Millipore, Missouri, USA). Both ELISAs were carried out according to manufacturer's instructions. All required reagents were supplied with the Rat Leptin ELISA kit. For the Glucagon DuoSet ELISA, substrate solution (R & D Systems,

Abingdon, UK) was ordered separately and the following solutions were made in the laboratory;

- Phosphate buffered saline – 1 PBS tablet/ 100ml deionized water
- Wash buffer – 0.05% Tween<sup>®</sup>20 in PBS, pH 7.2-7.4
- Reagent diluent – 1% albumin bovine serum in PBS, pH 7.2-7.4
- Stop solution – 2 N H<sub>2</sub>SO<sub>4</sub>

### **2.3.1 Isolation of total RNA**

Tissues were crushed under liquid nitrogen, using a pestle and mortar. Tissue was not allowed to thaw during crushing. Approximately 500mg of each tissue was crushed and placed into each of three 1.5ml eppendorfs. Two eppendorfs contained 100mg, and were reserved for DNA and RNA extraction. The third eppendorf contained 200mg crushed liver, and was used as a reserve supply. Once crushed all tissue was returned to -80°C, for long term storage.

RNA from pulverised tissue was isolated using Trizol. In 1.5ml eppendorfs, 1ml of Trizol was added to 100mg of crushed tissue. Samples were incubated at room temperature for 5 minutes before adding chloroform (200µl). Samples were shaken vigorously by hand for 15 minutes and then left at room temperature for 2-3 minutes. Following centrifugation at 12,000 rpm, at 4°C for 15 minutes, the colourless upper phase was collected and transferred to a fresh 1.5ml eppendorf. RNA was precipitated by addition of 500µl isopropyl alcohol. Samples were then incubated at room temperature for 10 minutes and centrifuged at 12,000 rpm at 4°C for 20 minutes. The supernatant was discarded and the RNA was then washed by addition of 1ml 75% ethanol, vortexing and centrifugation at 7,500rpm at 4°C for 5 minutes. Ethanol was removed and samples were left to dry at room temperature for 5-10 minutes. RNA was dissolved in 20µl DNase and RNase free water by incubation for 10 minutes at 55-60°C. Quality and quantity of RNA was checked using a NanoDrop1000 (Labtech, East Sussex, UK.) spectrophotometer. All RNA had a 260/280 ratio of >1.8. RNA was stored at -80°C.

### **2.3.2 cDNA synthesis**

1µg RNA per sample was treated with DNase before synthesis of cDNA by reverse transcription. 1µl Amplification grade DNase I (1 unit/µl) and 1µl 10x reaction buffer were added to the RNA and the samples were then incubated at room temperature for 15 minutes. 1µl Stop Solution (50mM EDTA) was then added to inactivate the DNase I. Samples were then incubated at 70°C for 10 minutes to denature the DNase I. Stop solution and reaction buffer solutions were supplied with the Amplification Grade DNase I.

Following DNase treatment, 1µg RNA was incubated with 1 µl 10mM dNTP mix and 1 µl 5µM random nanomers in a total volume of 10µl for 10 minutes at 70°C. Following incubation, samples were stored on ice before addition of 1µl M-MLV Reverse Transcriptase (200 units/µl) and 2µl 10x M-MLV Reverse Transcriptase buffer. Samples were incubated at room temperature for 10 minutes, followed by incubation at 37°C for 50 minutes. The reverse transcriptase was then denatured by incubation at 90°C for 10 minutes. cDNA was diluted with DNase and RNase free water to a total volume of 200µl and stored at -80°C.

### 2.3.3 Real time RT-PCR

#### Primers for real time RT-PCR

Real Time RT-PCR primer sequences for cyclophilin, PPAR $\alpha$ , Acyl-CoA oxidase (AOX) and GR were taken from Lillycrop et al<sup>[198]</sup>. Primer sequences for GR<sub>10</sub> and PEPCK were taken from Burdge et al<sup>[220]</sup>. Primer sequences for carnitine-palmitoyltransferase I (CPT-1) and PPAR $\alpha$  were designed and optimised as part of this project. Primers were supplied from Biomers (Söflinger, Germany). All primers were diluted to a concentration of 100 $\mu$ M upon arrival and stored at -20°C. Primer sequences are shown in Table 2.5.

#### Real time RT-PCR

cDNA was diluted to a total volume of 200  $\mu$ l. For each sample, 12.5 $\mu$ l SYBR GREEN, 0.5  $\mu$ l ROX, 5 $\mu$ l of cDNA, 1 $\mu$ l each of 10 $\mu$ M forward and reverse primers and 5.5 $\mu$ l DNase and RNase free water was used, giving a total volume of 25 $\mu$ l. Each cDNA sample was analysed in duplicate. All samples were analysed in 96 well plates using the Step One Plus PCR machine (Applied Bioscience, CA, USA) with two negative controls per plate, where the sample cDNA was replaced with 5 $\mu$ l DNase and RNase free water.

Cycle condition were; holding stage of 95°C for 10 minutes, 40 cycling stages of 95°C for 15 seconds, primer specific annealing temperature for 1 minute and 72°C for 1 minute, a melting curve stage of 95 °C for 15 seconds, 60 °C for 1 minute and a gradual increase to 95°C, which was held for 15 seconds once reached.

For each gene of interest the comparative  $\Delta\Delta$ Ct method was used to measure relative changes in gene expression<sup>[249]</sup>. All genes were normalised to cyclophilin (CYC), a gene which is ubiquitously expressed in a sample groups and is unaffected by the treatments of the studies.

**Table 2.5 Sequence and annealing temperatures of real time RT-PCR primers for analysis of mRNA expression**

| Gene                                   | Forward Primer<br>(5' → 3')    | Reverse Primer<br>(3' → 5') | Annealing<br>temperature<br>/ °C |
|----------------------------------------|--------------------------------|-----------------------------|----------------------------------|
| <b>Cyclophilin</b>                     | TTGGGTCGCGTCTGCTTCGA           | GCCAGGACCTGTATGCTTCA        | 60                               |
| <b>PPAR <math>\alpha</math></b>        | CGGGTCATACTCGCAGGAAAG          | TGGCAGCAGTGGAAGAATCG        | 60                               |
| <b>CPT-1</b>                           | ACCACTGGCCGAATGTCAAG           | AGCGAGTAGCGCATGGTCAT        | 60                               |
| <b>GR1<sub>10</sub></b>                | TGACTTCCTTCTCCGTGACA           | GGAGCCTCCTCTGCTGCTTG        | 60                               |
| <b>PEPCK</b>                           | AGCTGCATAATGGTCTGG             | GAACCTGGCGTTGAATGC          | 55                               |
| <b>Pfkf/Fru-<br/>2,6-P<sub>2</sub></b> | QIAGEN Quantitect primer assay | Q T00185395                 | 60                               |
| <b>Glc-6-Pase</b>                      | QIAGEN Quantitect primer assay | QT00190610                  | 55                               |
| <b>DNMT3a2</b>                         | QIAGEN Quantitect primer assay | QT01783551                  | 55                               |
| <b>Fads2</b>                           | QIAGEN Quantitect primer assay | QT00186739                  | 55                               |

**2.3.3.1 Statistical analysis of real time RT-PCR**

The results of real time RT-PCR analysis were non-parametric and were log<sub>10</sub> transformed before analysis. Comparison between groups was by a general linear model with generation and F0 maternal diet as fixed factors, with Bonferroni's *post hoc* test.



### 2.3.4 DNA extraction

DNA from pulverised tissue was extracted using a high salt method. 100mg of tissue was incubated overnight at 55°C in 500µl TNES buffer (Table 2.6) and 2.5µl proteinase K (>600 mAU/ml). Samples were mixed by inverting the tube several times.

**Table 2.6 The composition and concentrations of chemicals used to produce 50ml TNES buffer**

| STOCK            | Volume added | Final concentration |
|------------------|--------------|---------------------|
| 1M Tris pH 7.5   | 2.5ml        | 50mM                |
| 4M NaCl          | 5ml          | 400mM               |
| 0.5M EDTA        | 10ml         | 100mM               |
| 10% SDS          | 2.5ml        | 0.5%                |
| H <sub>2</sub> O | 31ml         |                     |

Following incubation, 500µl of 3.6M NaCl was added and the samples were shaken vigorously for 20 seconds. Samples were then centrifuged for 10 minutes at 13,000 rpm, at room temperature. Supernatant was then removed and placed into a new 2.2ml eppendorf. 1ml of cold 100% ethanol (kept at -20°C overnight) was then added to the samples. Following inversion, samples were stored at -20°C for 10-30 minutes. DNA was visible in ethanol as a white precipitate. DNA was spooled from ethanol, using glass pipettes, which had been melted under a butane flame to form a hook at the end. Samples were then air dried prior to suspension in 500µl sterile distilled water. 5µl RNase A (10mg/ml) was added to each sample and then incubated at 37°C for 1-2 hours. 500µl phenol: chloroform 1:1 was then added and the samples were mixed by inversion. Samples were centrifuged at 13,000 rpm for 15 minutes, at room temperature. The aqueous phase was transferred to a new 1.5ml eppendorf containing 1ml cold 100% ethanol. 50µl sodium acetate (3M, pH 6.8) was then added. Samples were incubated at -20°C for 10 minutes and then DNA was collected by spooling using a glass hook and the DNA placed into new 1.5ml eppendorfs. Pellets were air dried and suspended in

150µl sterile water. Samples were stored at -20°C. Quality and quantity of DNA was checked using a NanoDrop1000 (Labtech, East Sussex, UK.) spectrophotometer. All DNA had a 260/280 ratio of >1.7. 500ng of each sample was run on an Agarose gel to visualise quality of the DNA.

### **2.3.5 Agarose gel electrophoresis**

DNA and PCR product were run on Agarose gels to determine quantity. 0.8% gels (0.8g Agarose in 100ml 1 X Tris-acetate-EDTA buffer (TAE)) were used for genomic DNA, whereas 1.5% gels (1.5g Agarose in 100ml 1 X TAE) were used for PCR product. Agarose was dissolved in TAE by heating in a microwave and then cooled for 5 minutes before adding 5µl of 10mg/ml Ethidium Bromide. The gel was then poured into the mould, combs inserted, and left to set for 15 minutes. 1 X TAE was used as a running buffer. 3µl of Fermentas 1kb ladder was added to one well in each row used. Samples were mixed with loading dye (4g Ficol 400 and 50mg Orange G in 20ml 1 X TAE) prior to loading onto the gel. All gels were run at 120V until loading dye had reached a suitable distance.

### **2.3.6 Bisulphite conversion of DNA for analysis by pyrosequencing**

All DNA samples used for analysis of methylation were treated with sodium metabisulphite using the EZ-DNA Methylation Gold™ Kit (Cambridge Bioscience, Cambridge, UK) according to the manufacturer's instructions. The kit produced 10µl bisulphite treated DNA for each sample. Bisulphite treated DNA was stored at -20°C, and was used within 14 days.

### **2.3.7 Pre-pyrosequencing PCR of bisulphite converted DNA**

#### **Primers for pre-pyrosequencing PCR**

PCR primer sequences for PEPCK -691bp to -219bp and -215bp to +44bp, DNMT3a2 -428bp to -63bp and -315bp to -110bp and Fads2 -766bp to -473bp, -499bp to -318bp and -139bp to +165bp, where 0bp refers to the transcription start site, can be found in table 2.7. Primer sequences were designed using PSQ Assay Design software (Biotage, Uppsala, Sweden.) Primers were supplied from Biomers

(Söflinger, Germany). All primers were diluted to a concentration of 100µM upon arrival and stored at -20°C.

#### Pre-pyrosequencing PCR

Bisulphite converted DNA was used as a template for amplification by the polymerase chain reaction (PCR.) For each sample, 0.25µl Taq DNA Polymerase Hot Start, 10µl Q solution, 1µl 10mM DNTP mix, 5µl 10 X reaction buffer, 1µl each of 10µM forward and reverse primers, 2µl bisulphite converted DNA and 29.75µl DNase and RNase free water was used, giving a total volume of 50µl for each reaction set up. All samples were measured in 0.5ml eppendorfs using the Thermo Scientific Thermo Hybaid PCR express Thermal Cycler (Fisher Scientific, Leicestershire, UK). Each master mix preparation including a negative control where the sample DNA was replaced with 2µl DNase and RNase free water.

Cycle condition were; holding stage of 95°C for 15 minutes, 45 cycling stages of 94°C for 30 seconds, an amplicon specific annealing temperature (shown in table 2.7) for 30 seconds and 72°C for 1 minute, followed by a final stage of 72°C for 10 minutes. The amplicon specific annealing temperatures are shown in Table 2.7. All PCR products were run on a 1.5% Agarose gel to determine both quality and size of product.

**Table 2.7 Sequences and annealing temperatures of PCR Primers for amplification of PEPCK, DNMT3a2 and Fads2 promoter regions.**

| Bases covered/bp    | Forward Primer (5' → 3')        | Reverse Primer (3' → 5')           | Annealing temperature / °C |
|---------------------|---------------------------------|------------------------------------|----------------------------|
| <b>PEPCK</b>        |                                 |                                    |                            |
| <b>-658 to -405</b> | AGGGGTTAGTATGTATATAGAGT<br>GATT | ATCAAAACACCACAACTATAAA<br>ATATC    | 51                         |
| <b>-417 to -56</b>  | GTGGTGTTTTGATAATTAGTAGT<br>GATT | CCCCTCAACTAAACCTAAAAAC<br>TC       | 51                         |
| <b>-373 to -44</b>  | GTTAGTAGTATATGAAGTTTAAG<br>A    | CCCCTATTAACCAAAAATATAT<br>TCC      | 49                         |
| <b>DNMT3a2</b>      |                                 |                                    |                            |
| <b>-428 to -63</b>  | TTGATGTTTTTTTTTGGTGTGTTT        | CAAAAACCTTCAACCCATCAAT<br>AA       | 50                         |
| <b>-131 to +56</b>  | TGGTGAGTTGTTTGGGTTGTTA          | CCTAACTTTCCTCCACAACATT<br>CA       | 55                         |
| <b>Fads2</b>        |                                 |                                    |                            |
| <b>-766 to -473</b> | TTGTGTATTTTTTGATGTGGTTAG<br>ATT | CCCAACTCAATCCCTAACACCT<br>A        | 53                         |
| <b>-499 to -318</b> | TTTtagGTGTTAGGGATTGAGT          | ACTCCTAAACTAAATATCCTTC<br>AATATACA | 51                         |
| <b>-139 to +165</b> | TTGTTATTGAGGAGTGGGAAGTT         | CCCCTACCATCCAACCTCAT               | 51                         |

### 2.3.8 Pyrosequencing

#### Primers for pyrosequencing

PEPCK, DNMT3a2 and Fads2 primer sequences were designed using PSQ Assay Design software (Biotage, Uppsala, Sweden.) The location of the primers and the area that they cover is shown in Figure 2.8. The positioning of the primers is shown in Figures 2.3, 2.4 and 2.5 for PEPCK, DNMT3a2 and fads2, respectively. Primers were supplied from Biomers (Söflinger, Germany). All primers were diluted to a concentration of 100µM upon arrival and stored at -20°C.

**Table 2.8 Sequences of PEPCK, DNMT3a2 and Fads2 sequencing primers for use in pyrosequencing of the PEPCK, DNMT3a2 and Fads2 promoter regions.**

| Start location | Sequencing Primer (5' → 3') | CpGs covered         | Volume of PCR product added to each pyrosequencing reaction/µl |
|----------------|-----------------------------|----------------------|----------------------------------------------------------------|
| <b>PEPCK</b>   |                             |                      |                                                                |
| -637           | GTGATTATTTTATATTAGGTATTG    | -606                 | 10                                                             |
| -555           | AGAGGATTTAGTAGATATTTAGTG    | -508, -440           | 5                                                              |
| -251           | TAAATATTAACCTCAAACCC        | -248, -218           | 5                                                              |
| -170           | TTATTATTTTTTAAAGTTTATTG     | -129, -100, -90, -81 | 5                                                              |
| <b>DNMT3a2</b> |                             |                      |                                                                |
| -228           | GGTTAGAGGATAGATATTGG        | -207, -190           | 10                                                             |
| -22            | CATCTTAACCTCCC              | -56, -39             | 10                                                             |
| <b>Fads2</b>   |                             |                      |                                                                |
| -748           | GGTTAGATTTGAGTGTG           | -722, -711, -678     | 5                                                              |
| -646           | AAATGAAGTGTAATTTATGTTT      | -623, -608, -585     | 5                                                              |
| -421           | TGAGTTTTATTTTTTATTGATAAT    | -394                 | 10                                                             |
| -112           | ATTTGGTTTTTGAGTAGATA        | -84, -76, -65, -31   | 5                                                              |

Prior to pyrosequencing, both amplicons (-661bp to -219bp and -215 bp +44bp) of the PEPCK promoter were amplified using primers listed in table 2.7. The PCR and sequencing primer binding locations for PEPCK, DNMT3a2 and fads2 are shown in figure 2.3, figure 2.4 and figure 2.5, respectively.

All solutions were removed from -4°C storage, and left on the workbench in order to reach room temperature. Each sequencing primer was initially optimised to

either 5µl or 10µl PCR product per reaction. The volumes of PCR product added are listed in table 2.8. The optimised volume of PCR product was added and made up to 40µl with water in a 96-well PCR plate. 2µl streptavidin sepharose beads and 38µl binding buffer were added to each well, the plate sealed and shaken on a plate shaker for 5 minutes. A master mix containing 0.5µl sequencing primer (10µM) and 11.5µl annealing buffer / sample, was prepared. 12µl of the mix was added to each well of a PSQ HS 96 well plate. The plate was then placed in the holding bay of the PyroMark Q96 Vacuum Prep Workstation (Qiagen, Crawley, UK). The beads from the 96- well PCR well plate were captured onto the vacuum prep tool, ensuring all solution was removed. The vacuum prep tool was then used to wash the beads in 70% ethanol and then separate the strands in denaturation solution. The beads were then washed in wash solution (Qiagen, Crawley, UK), the vacuum switched off and the beads released into a PSQ HS 96 well plate. Sequencing primers were then annealed to samples by placing the PSQ plate on a preheated heating plate and incubated at 80°C for exactly 2 minutes. Following incubation, the plate was removed from the block and left to cool for at least 5 minutes. As instructed by the Pyrosequencer software, the required amount of nucleotide, enzyme and substrate were added to each nucleotide dispensing tip (NDT) ensuring no bubbles were present in tips. Tips were inserted into the holder in the Biotage PyroMark MD Pyrosequencer (Biotage, AB, Kungsgatan, Sweden), and a tip test was performed to check the tips were in working condition. If a tip failed more than 3 times, it was not used. Once all tips were confirmed to be successful, the PSQ plate was inserted into the Pyrosequencer and the samples were then analysed. A quality control was included in the pyrosequencing assay to check 100% bisulphite conversion of DNA.

#### **2.3.8.1 Statistical analysis of methylation data**

Comparison between groups was by a general linear model, with generation and F0 maternal diet as fixed factors, with Bonferroni's *post hoc* test.



**Figure 2.3 Diagram showing the PEPCK promoter with primer and CpG locations.** Diagram shows -830bp to +220bp, where 0bp is the transcription start site. A red highlight represents the transcription start site, a yellow highlight represents CpG dinucleotides, a green highlight represents PCR primers, a dark green highlight represents the overlap region of primer sequences and a blue highlight represents sequencing primers. Blue text represents the area covered by the sequencing primers and underlined text represents exon 1.

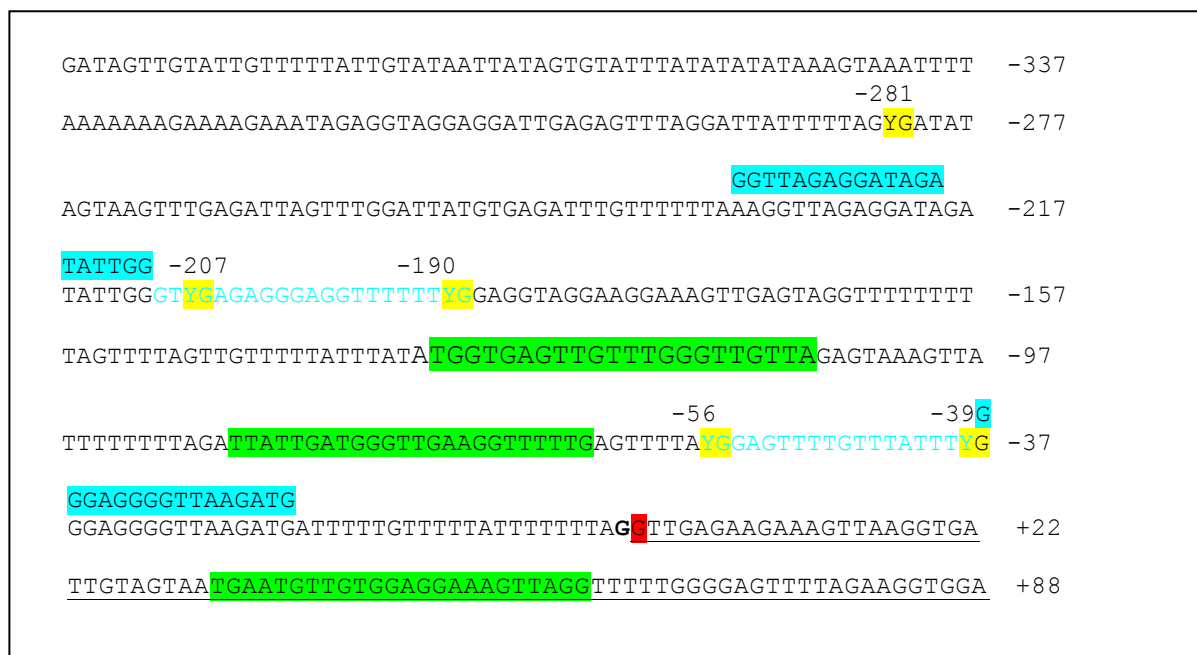


**Figure 2.4 Diagram showing the DNMT3a2 promoter with primer and CpG locations.** Diagram shows -397bp to +88bp, where 0bp is the transcription start site. A red highlight represents the transcription start site, a yellow highlight represents CpG dinucleotides, a green highlight represents PCR primers and a blue highlight represents sequencing primers. Blue text represents the area covered by the sequencing primers and underlined text represents exon 1.



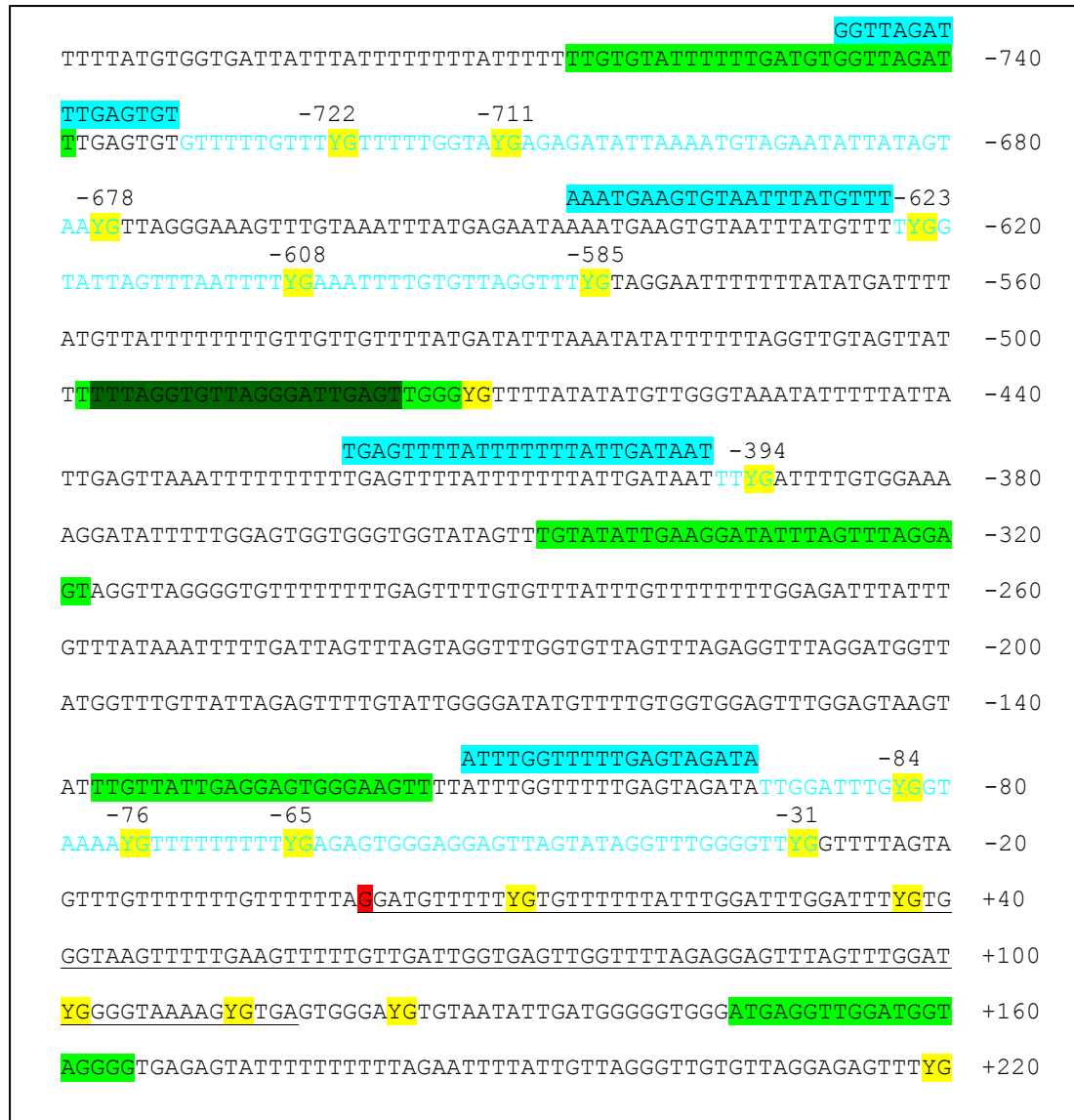


**Figure 2.5 Diagram showing the fads2 promoter with primer and CpG locations.** Diagram shows -800bp to +220bp, where 0bp is the transcription start site. A red highlight represents the transcription start site, a yellow highlight represents CpG dinucleotides, a green highlight represents PCR primers, a dark green highlight represents the overlap region of primer sequences and a blue highlight represents sequencing primers. Blue text represents the area covered by the sequencing primers and underlined text represents exon 1.

### 2.3.9 Promoter cloning

#### 2.3.9.1 Promoter amplification

Promoter regions of the PEPCK, DNMT3a2 and fads2 genes were amplified using RTPCR. Restriction sites for XhoI and HindIII were added to the forward and reverse primers, respectively, in order to provide sticky ends for insertion into the pGI3 vector. The sequences of primers used for each of the amplicons are shown in table 2.9. *Pfu* Proof reading Taq polymerase was used to ensure minimal error when replication of the promoter regions. For each gene, a minimum of 1407bp upstream and a section of the first exon were amplified. Briefly, 0.5µl *Pfu* Taq polymerase 1µl 10mM DNTPs, 5µl 10 x buffer, 1µl each of 10mM forward and reverse primers, 40.5µl DNase free water and 50ng template DNA was used per sample. Samples were run on a thermal cycler G-Storm PCR machine using the following steps. 95°C for 2 minutes, then 40 cycles of 95°C for 1 minute, an amplicon-specific annealing temperature for 30 seconds and 72°C for 1min/kb product. Quality and quantity of PCR product was assessed on an agarose gel. PCR product was gel extracted, to ensure purity, using a QIAquick gel extraction kit (Qiagen, Crawley, West Sussex, UK), according to manufacturer's instructions.

**Table 2.9 Promoter PCR primer sequences**

| Gene                                   | Direction | Sequence                             | Annealing temperature |
|----------------------------------------|-----------|--------------------------------------|-----------------------|
| <b>PEPCK</b><br>-2400bp to<br>+100bp   | Forward   | ATCTCGAGGCGGCTTCACATTCATAAAC<br>A    | 58.9°C                |
|                                        | Reverse   | ATAAGCTTCTGCCTACCTTTCTTCCTC          |                       |
| <b>DNMT3a2</b><br>-1407bp to<br>+172bp | Forward   | ATCTCGAGAACCCCCTCTCCCATCCC           | 58.9°C                |
|                                        | Reverse   | ATAAGCTTCCCCAGCATCAGCCCCTA           |                       |
| <b>Fads2</b><br>-1038bp to<br>+318bp   | Forward   | ATCTCGAGCTCTGGTTCTTTTCCTGGT<br>A     | 58.9°C                |
|                                        | Reverse   | ATAAGCTTAGAGGATGCTGCTAACTATC<br>ACCC |                       |

### **2.3.9.2 Tailing reaction**

Tailing reactions are used to add a string of adenosine nucleotides to the end of *Pfu* Taq PCR product, in order to allow binding of the product to the string of thymines of the P-GEMT vector. The reaction set up is as follows. 8µl of 5 x buffer, 0.4µl of 25 mM DNTPs, 0.5µl GoTaq DNA polymerase and 31.9µl of template DNA and distilled H<sub>2</sub>O were used per sample. The tube was heated to 74°C for 30 minutes, and the product was then cleaned using the QIAquick PCR clean up kit (Qiagen, Crawley, West Sussex, UK).

### **2.3.9.3 Ligation**

pGEM-T or cut pGI3 vectors and PEPCK, DNMT3a2 and fads2 promoter PCR products were ligated using 1µl T4 DNA ligase in a 10µl reaction. The promoter DNA and vector were ligated overnight at 4°C.

### **2.3.9.4 Luria Broth and LB agar plates**

Luria Broth (LB) solution was made by mixing 20g LB broth powder to 1 litre of double distilled H<sub>2</sub>O. The solution was autoclaved before use. LB agar plates were made by adding 1.5g agar powder to 100ml LB broth. The mixture was sterilised using an autoclave, to sterilise the solution and dissolve the agar. Once cooled to around 30°C, filter sterilised ampicillin was added at a final concentration of 100µg/ml, using a sterile technique. If the vector used was pGEM-T, IPTG (isopropyl β-D-1 thiogalactopyranoside), which induces the transcription of β-galactosidase and X-Gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside), an organic compound consisting of galactose linked to a substituted indole, which is broken down by β-galactosidase, giving an insoluble blue-product, were also added to the LB agar, at final concentrations of 0.02% and 400µM, respectively, using a sterile technique. LB agar was poured into 9ml petri dishes, with 25ml used per dish, using a sterile technique and left to set on the work bench. LB agar plates were stored at 4°C prior to use.

### 2.3.9.5 Transformation and screening

The amplified promoter regions were first cloned into the p-GEMT vector to increase the number of copies of the promoter. Once successfully cloned into the pGEMT vector, the promoter region was cut out and ligated into cut pGI3 vector. Diagrams of the pGEMT and pGI3 vector are shown in Figures 2.6 and 2.7, respectively. 2µl of PCR product was added to 50µl JM109 competent cells (Promega, Southampton, UK) in pre-chilled eppendorf tubes and placed on ice for 20 minutes. The DNA/cell mixture was then subjected to heat shock for 45 seconds at 42°C, to allow the cells to take up the DNA. The cells were then placed on ice for 2 minutes. 450µl sterile LB was then added to the cells and incubated at 37°C for 30-60 minutes with shaking. Cells were then centrifuged for 5 minutes at 10,000rpm and 400µl of supernatant discarded. The remaining 100µl and cell pellet were then mixed by pipetting and spread onto an LB Agar plate, containing ampicillin, X-Gal and IPTG. The presence of ampicillin enables transformants to be distinguished from non-transformants, as these are the only cells to contain the Amp<sup>r</sup> gene. Untransformed cells are unable to survive in the presence of ampicillin, suggesting all present colonies represent transformed cells. Plates were incubated at 37°C overnight and then sealed and stored at 4°C.

For pGEMT transformations, presence of X-Gal and IPTG allowed blue-white screening of colonies. The pGEMT vector contains the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide leads to a decrease in the breakdown of β-galactosides into monosaccharides, leading to blue colonies on the LB agar plate. The pGI3 vector does not contain the β-galactosidase gene, so blue-white screening is not utilised, ampicillin screening however, is used.

The pGI3 basic vector contains a modified gene for firefly luciferase, without a functional promoter region. Presence of the Amp<sup>r</sup> gene allows transformants to be identified. Firefly luciferase catalyses the conversion of luciferin to oxyluciferin which generates a light signal. Insertion of the PEPCK, DNMT3a2 or fads2 regulatory DNA into the multiple cloning region of the pGI3 basic vector allows its transcriptional activity to be assessed.

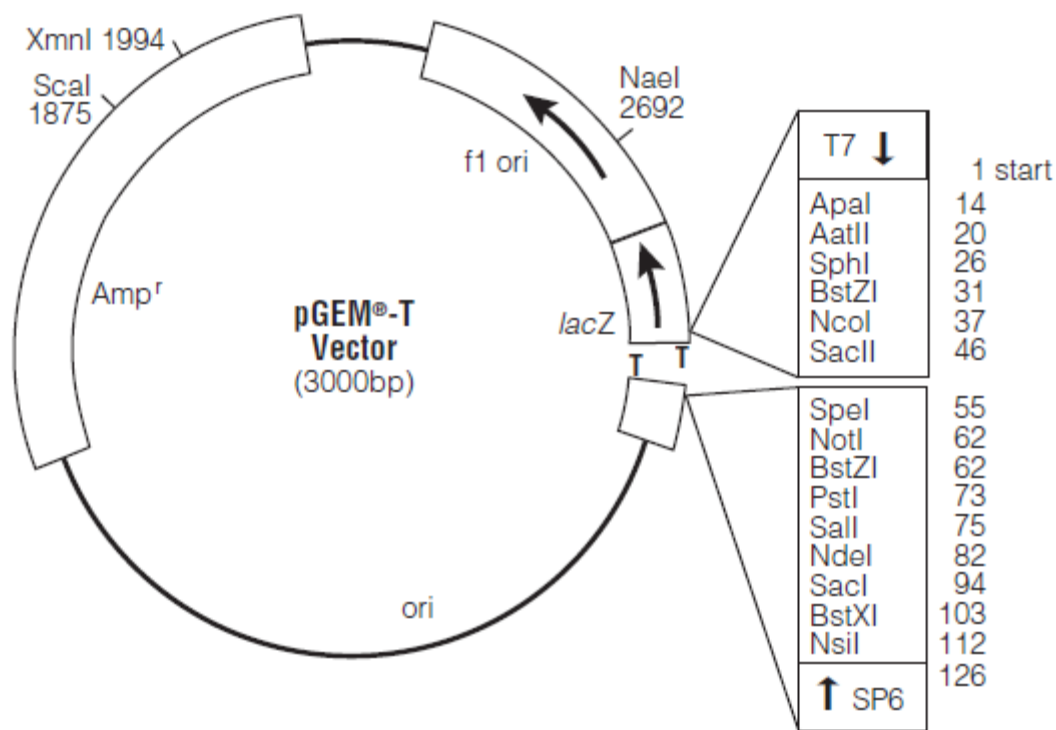


**Figure 2.6** Diagram of the pGEMT vector map and sequence reference points taken from the Promega pGEMT protocol (Promega, Southampton, UK)

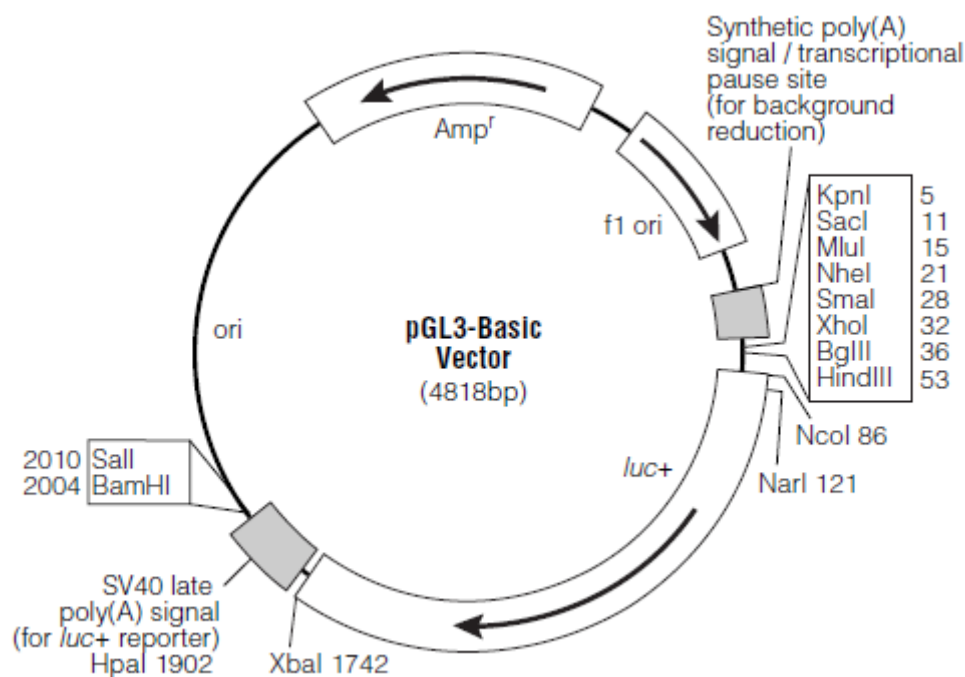


**Figure 2.7** Diagram of the pGL3 vector map and sequence reference points taken from the Promega pGEMT protocol (Promega, Southampton, UK)

#### **2.3.9.6 Restriction digests**

Once sufficient quantity of the transformed pGEMT vector was obtained, the PEPCK, DNMT3a2 and fads2 promoters were cut using XhoI and HindIII (DNMT3a2 and fads2) or XhoI and BglII (PEPCK). The promoter regions were then transformed into pGI3 vectors, which were cut with the same respective restriction enzymes, in order to provide sticky ends for ligation. The vectors were cut using the following technique. 5µg of the transformed pGEMT or stock pGI3 basic vector was digested with 25 units of either XhoI and HindIII or XhoI and BglII in a total volume of 50µl for 60 minutes at 37°C. Completion of the digest was confirmed by running digest product on an agarose gel. The band was cut from the gel and extracted using the QIAquick gel extraction kit (Qiagen, Crawley, West Sussex, UK). Confirmation of transformation was following restriction mapping, which involved digesting transformed vectors with selected restriction enzymes and clarifying promoter presence by size of products. The list of restriction enzymes used is shown in table 2.10.

**Table 2.10 Restriction enzymes used to confirm transfection clones**

| Input           | Restriction Enzymes used | Number of products expected |
|-----------------|--------------------------|-----------------------------|
| PEPCK           | XhoI + BglII             | 1                           |
| PEPCK + pGEMT   | Cla<br>DraI<br>XbaI      | 0<br>5<br>1                 |
| PEPCK + pGI3    |                          |                             |
| DNMT3a2         | XhoI + HindIII           | 1                           |
| DNMT3a2 + pGEMT | BglII<br>Sall<br>NcoI    | 0<br>1<br>2                 |
| DNMT3a2 + pGI3  | NcoI<br>ApoI             | 2<br>6                      |
| Fads2           | XhoI + HindIII           | 1                           |
| Fads2 + pGEMT   | Sall<br>ApoI             | 0<br>5                      |
| Fads2 + pGI3    | ApoI                     | 6                           |

### 2.3.9.7 Sequencing

Samples were sent for external sequencing to GATC biotech, London, UK. In house pGI3 primers shown in table 2.11 were used for forward and reverse reads of sequences.

**Table 2.11 Primer sequences for pGI3 sequencing**

| Direction | Sequence         |
|-----------|------------------|
| Forward   | CTAGCAAAATTGTCCC |
| Reverse   | CTTTATGTTTCTTCCA |

### 2.3.9.8 Single site mutagenesis of the PEPCK, DNMT3a2 and fads2 promoters

In order to understand the role of methylation at specific CpG sites in the PEPCK, DNMT3a2 and fads2 promoters, cytosine to adenosine single point mutations were introduced into the cloned promoters. Only specific CpG sites were mutated, where significant differences in methylation were shown and the region has the potential for a role in transcriptional regulation. The quick-change single point mutagenesis primers were designed using online software from Agilent technologies (Cheshire, UK). Cytosine to adenosine mutations were selected for each primer pair as these were the most energy efficient. The sequences of mutagenesis primers and the mutated cytosines are shown in Table 2.12.

**Table 2.11 Sequences of mutagenesis primers and CpGs targeted**

| <b>Gene –<br/>CpG<br/>targeted</b> | <b>Forward Sequence 5' → 3'</b>                  | <b>Reverse Sequence 5' → 3'</b>                  |
|------------------------------------|--------------------------------------------------|--------------------------------------------------|
| PEPCK<br>CpG -248                  | GTCACGGTCAAAGTTTAGTCAATC<br>AAAAGTTGTGTAAGGACTC  | GAGTCCTTACACAACCTTTTGATTGACT<br>AAACTTTGACCGTGAC |
| PEPCK<br>CpG -90                   | GGCCGGCCCCCTTAAGTCAGAGGC<br>GAG                  | CTCGCCTCTGACTTAAGGGGCCGGCC                       |
| DNMT3a2<br>CpG -56                 | TGAAGGTCCCTGAGTCTCAAGGAG<br>CCTTGC               | GCAAGGCTCCTTGAGACTCAGGGACC<br>TTCA               |
| DNMT3a2<br>CpG -39                 | GGAGCCTTGCTCATTCAAGGAGG<br>GGTCA                 | TGACCCCTCCCTGAATGAGCAAGGCT<br>CC                 |
| Fads2<br>CpG -394                  | TGAGTCTTATCTCTTCACTGATAAT<br>TTAGACCTTGTGGAAAAGG | CCTTTTCCACAAGGTCTAAATTATCAG<br>TGAAGAGATAAGACTCA |

SequalPrep (Invitrogen, Paisley, UK) was used to amplify the mutated pGI3 vector containing the PEPCK, DNMT3a2 or fads2 promoter. In a 20µl reaction, 2µl of 10X reaction buffer. 0.4µl DMSO, 1µl SequalPrep 10X Enhancer A, 15µl distilled H<sub>2</sub>O, 0.5µl 10mM forward and reverse primers (each) and 0.36µl SequalPrep Long Polymerase were used. The reaction condition were as follows, 94°C for 2 minutes, 12 cycles of 94°C for 10 seconds, 55°C for 30 seconds and 68°C for 1 minute/kb, followed by 72°C for 5 minutes. The quantity and quality of the PCR product was



established using an Agarose gel. Samples were incubated with the restriction enzyme DpnI for 1 hour at 37°C, in order to remove the non-mutated supercoiled, input dsDNA. The digested mutated vectors were then transformed into JM109 cells using the previously described technique, in order increase copy number.

#### **2.3.9.9 Transfection**

Rat hepatocyte cells (CC-1) were transfected with the wild type or mutated forms of the fads2 promoter using fuGENE HD Transfection Reagent (Promega, Southampton, UK), according to manufacturer's instructions. Briefly, 2µg of fads2 in pGI3 plasmid DNA, 2µg of Renilla luciferase plasmid DNA and 12µl of transfection reagent were made up in 300µl DMEM, containing 5% serum. After incubation at room temperature for 15 minutes, the media containing the plasmid DNA and transfection reagent was added to washed cells and cells were incubated at 37°C, 5% CO<sub>2</sub> for 4 hours. After this period the media was removed and treatment media was added. The treatment groups used are

- 0pM 17α-ethinylestradiol
- 70pM 17α-ethinylestradiol
- 700pM 17α-ethinylestradiol

Cells were left in the treatment for 24 hours. 6 wells were used per treatment group per promoter type.

17α-ethinylestradiol treatments were made using the following steps.

10mls of 7µM 17α-ethinylestradiol was made up by dissolving 20.74mg 17α-ethinylestradiol in 10ml ethanol. Using one in ten dilutions in upH<sub>2</sub>O the following concentrations of 17α-ethinylestradiol were made, 70nM and 7nM. 100µl of 70nM 17α-ethinylestradiol was added to 9.9ml media (in dish) to get 10mls of 700pM 17α-ethinylestradiol. 100µl of 70nM 17α-ethinylestradiol was added to 9.9ml media (in dish) to get 10mls of 70pM 17α-ethinylestradiol.

#### **2.3.9.10 Luminescence**

Following incubation in the treatment media, media was removed from wells and cells were washed with PBS. The Dual-Luciferase Reporter Assay kit (Promega, Southampton, UK) was used to determine transcriptional activity. 1X Passive Lysis Buffer (PLB) was made by diluting 2.5mls of 5X PLB in 10mls

upH<sub>2</sub>O. 250µl 1X PLB was added to each well and cells were lysed using a rubber policeman. To ensure successful lysis, cells in PLB were subjected to two rounds of freeze thawing using dry ice. 100µl of luciferase assay reagent (LAR II). The TD-20/20 Turner Designs Luminometer (Turner Designs, CA, USA) was then programmed for use. 20µl of lysed cells in PLB was then transferred to the 2.2ml eppendorf containing 100µl LAR II and mixed by pipetting, ensuring no bubbles were created. The firefly luciferase activity across 10 seconds, following a 2 second delay, was then measured and recorded three times. Once all samples had been measured, 100µl of Stop and Glo Reagent was added to each sample and mixed by pipetting. The renilla luciferase activity was then measured and recorded in triplicate for each sample and the tube discarded.

The corrected quantification was measured by dividing the mean firefly luminometer value by the mean renilla luciferase value, for each sample separately.

#### **2.3.9.10.1 Statistical analysis of luminescence data**

The results of transformation luminescence analysis between groups was by a one way analysis of variance (ANOVA) with 17α-ethinylestradiol concentration as a fixed factor, with Bonferroni's *post hoc* test.

#### **2.3.11.1 Preparation of a nuclear extract**

Nuclear extracts were prepared from CC-1 cells. Composition of the buffers used are shown in table 2.12. Cells were suspended in 800µl of buffer A, to which 5µl PMSF (phenylmethanesulfonylfluoride, a serine protease inhibitor) and 2.5µl LP (leupeptin, a cysteine, serine and threonine protease inhibitor) was added prior to use and incubated on ice for 10 minutes. 50µl of 10% (v/v) NP40 (Tergitol-type NP-40, a detergent used to break the cytoplasmic membrane) was added and samples were vortexed for 10 seconds, centrifuged at 12,000rpm for 30 seconds and the supernatant containing the cytoplasm was removed. The pellet was resuspended in 30 -50µl of buffer C, to which 10µl PMSF was added prior to use. Samples were incubated on ice for 15 – 30 minutes, spun at 13,000 rpm at 4°C for 5 minutes. The supernatant, containing the nuclear extract was stored at -80°C.

The nuclear extract was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific, Illinois, USA).

**Table 2.12 Composition of nuclear extract buffers A and C**

| Chemical added   | Volume added | Final concentration |
|------------------|--------------|---------------------|
| Buffer A / 10 ml |              |                     |
| 1M HEPES pH 7.9  | 100µl        | 10mM                |
| 1M KCl           | 100µl        | 10mM                |
| 0.5M EDTA        | 2µl          | 0.1mM               |
| 1M DTT           | 10µl         | 1mM                 |
| H <sub>2</sub> O | 9,773µl      |                     |
| Buffer C / 10ml  |              |                     |
| 1M HEPES pH 7.9  | 200µl        | 20mM                |
| 4M NaCl          | 1ml          | 0.4M                |
| 0.5M EDTA        | 20µl         | 1mM                 |
| 1M DTT           | 10µl         | 1mM                 |

### 2.3.11.2 Annealing oligonucleotides

Oligonucleotides covering selected CpGs were designed to measure transcription factor binding across the promoter regions of the PEPCK, DNMT3a2 and fads2 genes. Table 2.13 shows the sequences of primers used, and the CpGs which they cover. In order to measure the binding of transcription factors to cell extracts from a rat hepatic cell line, CC-1 cells, the complementary oligonucleotides were first annealed, using the following method. Initial denaturation of the oligonucleotides ensured secondary structures were removed and allowed the complimentary oligonucleotides to hybridize.

Oligonucleotides were mixed at a molar ratio of 1:1, in 1.5ml eppendorfs. The oligonucleotides mixture was diluted to a final concentration of 1pmol/µl with 10mM Tris, 1mM EDTA and 50mM NaCl. The samples were heated in a water bath for 5 minutes at 95°C. The water bath was then switched off and left to cool to room

temperature overnight, allowing annealing of the oligonucleotides. The annealed oligonucleotides were stored at -20°C.

**Table 2.13 Sequences of complimentary primers used in EMSAs.**

| <b>Gene/ CpG covered</b> | <b>Forward</b>                       | <b>Reverse</b>                        |
|--------------------------|--------------------------------------|---------------------------------------|
| PEPCK / -248             | AGTCAATCAAACGTTGTGTAAGGAC<br>TCAACTA | TAGTTGAGTCCTTACACAACGTTTGATT<br>GACT  |
| PEPCK / -90              | CCAAAGGCCGGCCCTTACGTCAG<br>AGGCGAGC  | GCTCGCCTCTGACGTAAGGGGCCGGC<br>CTTTGG  |
| Fads2 / -394             | ATCTCTTCACTGATAATTTGACCTT<br>GTGGAAA | TTTCCACAAGGTCGAAATTATCAGTGAA<br>GAGAT |

### 2.3.11.3 Electromobility Shift Assays

Binding of nuclear extract to specific DNA sequences was measured using the LightShift Chemiluminescent Electro Mobility Shift Assay (EMSA) Kit (Fisher, Leicestershire, UK), according to manufacturer's instructions. Gels for EMSA were made using 3.125ml 40% Acrylamide, 1.25ml 10x TBE, 20.6ml ultra-pure H<sub>2</sub>O, 250µl 10% ammonium persulfate (APS) and 25µl Tetramethylethylenediamine (TEMED). Gels were set in electrophoresis tanks and sealed prior to setting. Once set, the inside of the gel electrophoresis tank was filled with 0.5x TBE to cover the gel by a few millimetres and the outside of the gel tank was filled to a level above the bottom of the gel wells, in order to reduce heat during running of the gel. Prior to addition of sample, the wells were flushed and the gel was run at 100V for 30 – 60 minutes. The composition of the binding reactions are shown in table 2.14. Components of the binding reactions were thawed and stored on ice prior to use. Following addition of extract the tubes were placed on ice for 5 minutes, then again for 20 minutes following addition of DNA probes. Before addition of binding reactions to the gel, 5µl of the loading buffer was added to each tube and mixed via pipetting.

**Table 2.14 Composition of binding reactions in EMSA experiments.**

|                                        | <b>1</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> |
|----------------------------------------|----------|----------|----------|----------|----------|
| <b>upH2O</b>                           | 12       | 8.7      | 3.7      | 2        | 0        |
| <b>10X Binding Buffer</b>              | 2        | 2        | 2        | 2        | 2        |
| <b>Poly dccc (1µg/µl)</b>              | 1        | 1        | 1        | 1        | 1        |
| <b>50% Glycerol</b>                    | 1        | 1        | 1        | 1        | 1        |
| <b>100mM MgCl</b>                      | 1        | 1        | 1        | 1        | 1        |
| <b>1% NP40</b>                         | 1        | 1        | 1        | 1        | 1        |
| <b>Unlabelled DNA (1pmol.µl)</b>       | 0        | 0        | 0        | 0        | 2        |
| <b>Nuclear Extract (0.6µg/µl)</b>      | 0        | 3.3      | 8.3      | 10       | 10       |
| <b>Biotin labelled DNA (10fmol/µl)</b> | 2        | 2        | 2        | 2        | 2        |

**Table 2.14 Composition of binding reactions in EMSA experiments** where 1 contains Biotin DNA only, 2 contains nuclear extract and biotin DNA, 3 contains more nuclear extract and biotin DNA, 4 contains the largest quantity of nuclear extract and biotin DNA and 5 contains the largest amount of nuclear extract, biotin DNA and unlabelled DNA, to check specificity of binding.

The gels were run at 100V for about 45 minutes (until the bromophenol blue dye had migrated  $\frac{3}{4}$  down the length of the gel).

In order to transfer binding reactions to nylon membrane, sections of nylon membrane were soaked for at least 10 minutes in 0.5X TBE. The gel was sandwiched with the nylon membrane between 6 pieces of 3mm blotting paper (Whatman, Maidstone, UK), ensuring no air bubbles were present. The binding reactions were transferred at 200mA for 60 minutes, using a Bio-Rad Trans-Blot Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hertfordshire, UK). Following the transfer, the membrane was placed on a dry paper towel, with the bromophenol blue side up, allowing the buffer on the membrane surface to absorb into the membrane.

Transferred DNA was then crosslinked to membrane, at  $120\text{mJ}/\text{cm}^2$  for 1 minute, using a UVP CL-1000 Ultraviolet Crosslinker (UVP, Cambridge, UK).

The biotin-labelled DNA was then detected by chemiluminescence using a VersaDoc Imaging System (Bio-Rad Laboratories, Hertfordshire, UK), using the following procedure. 20ml warm blocking buffer was added to the membrane and

incubated for 15 minutes with gentle shaking. Blocking buffer was then removed from the membrane and conjugate buffer/blocking solution was added, the membrane was then incubated for 15 minutes, with gentle shaking. Conjugate buffer/blocking solution, contained 66.7 $\mu$ l stabilized streptavidin-horseradish peroxidase conjugate and 20ml blocking buffer. The conjugate buffer/blocking solution was then removed and the membrane was washed for 4 times with 20ml 1x wash solution, for 5 minutes each time, with gentle shaking. Following washes, the membrane was dried by touching the edge to a dry paper towel and placed in a fresh container containing 12ml substrate working solution (6ml luminal/enhancer solution and 6ml stable peroxidase solution), the membrane was incubated for 5 minutes, with gentle shaking. The solution was removed from the membrane, and the membrane dried by touching the edge of the membrane to a dry paper towel for 2-5 seconds. The membrane was then wrapped in plastic, avoiding bubbles and wrinkles and chemi-ultrasensitivity was measured using a VersaDoc (Bio-Rad Laboratories, Hertfordshire, UK).

#### 2.3.11.4 Western Blot analysis of protein levels

Total protein levels were measured using Western Blotting. Initially tissue was lysed in order to release all protein from within cells. This was achieved through a two hour incubation of around 30mg tissue in RIPA lysis buffer, containing 1mM PMSF and 2.5 $\mu$ l LP, at 4°C, rotating. The composition of RIPA buffer is shown in Table 2.15.

**Table 2.15 The composition of RIPA buffer**

| Chemical added          | Final concentration |
|-------------------------|---------------------|
| NaCl                    | 150mM               |
| Triton X-100            | 1%                  |
| Sodium deoxycholate     | 0.5%                |
| Sodium dodecyl sulphate | 0.1%                |
| Tris pH 8.0             | 50mM                |

Following incubation the mixture was spun for 20 minutes, at 4°C at 12,000rpm and supernatant, containing protein, removed and placed in a fresh 1.5ml eppendorf. Total protein concentration of samples was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Illinois, USA), where BSA was used as a standard.

Western blot gels were made by casting a three quarters sized resolving gel and topping up with a stacking gel and allowing setting. Water was used on top of the stacking gel to ensure a flat surface for the resolving gel. The composition of the gel mixtures is shown in Table 2.16.

**Table 2.16 Composition of stacking and resolving gels, for two gels**

| Chemical added          | Volume  |
|-------------------------|---------|
| Stacking Gel            |         |
| Stacking gel mix (2X)   | 2.5ml   |
| 30% Acrylamide          | 0.835ml |
| H <sub>2</sub> O        | 1.67ml  |
| 10% Ammonium Persulfate | 50µl    |
| TEMED                   | 5µl     |

| Chemical added          | Volume |
|-------------------------|--------|
| Resolving Gel           |        |
| Resolving gel mix (4X)  | 2.5ml  |
| 30% Acrylamide          | 3.3ml  |
| H <sub>2</sub> O        | 4.1ml  |
| 10% Ammonium Persulfate | 100µl  |
| TEMED                   | 10µl   |

**2X Stacking gel mix** – 6.04g Tris, 180ml H<sub>2</sub>O, pH to 6.8 with concentrated HCl, add 8ml 10% SDS, make up to 200ml, with H<sub>2</sub>O

**4X Resolving gel mix** – 29.04g Tris, 180ml H<sub>2</sub>O, pH to 8.8 with concentrated HCl, add 8ml 10% SDS, make up to 200ml with H<sub>2</sub>O

Gels were pre-run for a minimum of 20 minute before use.

20µg of protein per sample was mixed with an equal amount of 2X Laemmli loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromphenol blue, 0.125M Tris-HCl) and incubated at 95°C for 5 minutes, in order to denature proteins. A Molecular weight marker was used to determine size of detected protein (ab41746, Abcam, Cambridge, UK) Gels were run at 200V for 45 minutes in running buffer (1 litre H<sub>2</sub>O, 3g Tris, 14.4g Glycine, 1g SDS), until the bromphenol blue dye front had moved to the end of the gel.

Gels were transferred onto PVDF membranes, which were pre-soaked in methanol for one minute, sandwiched between 6 layers of 3mm blotting paper, which were pre-soaked in transfer buffer (10% 10X protein running buffer, 25% ethanol, 65% upH<sub>2</sub>O) for 10 minutes prior to transfer. Gels were transferred at 200mA for 60 minutes, using a Bio-Rad Trans-Blot Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hertfordshire, UK).

The nylon membrane was blocked at 4°C overnight, shaking, in PBS with 0.1% Tween-20 containing 5% Marvel, as a source of protein. Following blocking, membranes were washed three times for 5 minutes in PBS with 0.1% Tween-20. Membranes were placed in 50ml falcon tubes and incubated for 1 hour at room temperature, shaking, with 5ml blocking solution containing the appropriate amount of primary antibody. Following incubation with primary antibody, membranes were washed again three times for 5 minutes in PBS with Tween-20. Following the second wash membranes were incubated in 5ml blocking buffer containing the appropriate amount of secondary antibody. Protein levels were detected by placing the membrane on saran wrap and incubating in 1ml West DURA Chemiluminescent substrate Super-Signal (Pierce, Fisher, Leicestershire, UK), for 5



minutes. Following removal of Super-signal solution, chemi-ultrasensitivity was measured using a VersaDoc (Bio-Rad Laboratories, Hertfordshire, UK). The specific primary and secondary antibodies and amounts/concentrations used are shown in Table 2.17.

**Table 2.17 Primary and secondary antibody used for Western blot measurement of Fads2 and PEPCK protein levels.**

| <b>Protein</b> | <b>Primary antibody (Ab) used</b>                 | <b>Amount of primary Ab used</b> | <b>Secondary Ab used</b>                | <b>Amount of secondary Ab used</b> |
|----------------|---------------------------------------------------|----------------------------------|-----------------------------------------|------------------------------------|
| <b>Fads2</b>   | <b>Goat polyAb sc-109272 (SantaCruz, CA, USA)</b> | <b>1µg</b>                       | <b>Rabbit pAb to goat (HRP) ab97100</b> | <b>1/10000 – 1/50000 tested</b>    |
| <b>PEPCK</b>   | <b>Rabbit polyAb ab28455 (Abcam)</b>              | <b>0.5µg/ml</b>                  | <b>Goat pAb to rabbit (HRP) ab97051</b> | <b>0.1µg/ml (1/10000 dilution)</b> |

### **2.3.12 Measurement of fatty acid composition by gas chromatography**

Tissue fatty acid composition measurements were carried by Nicola Irvine, according to the method described by Burdge<sup>[250]</sup>. Briefly, liver (approximately 100mg) was powdered under liquid nitrogen and total lipids extracted with chloroform and methanol<sup>[251]</sup>. Plasma (0.8ml) was extracted with chloroform and methanol<sup>[251]</sup>. PC was isolated from total lipid extracts by solid phase extraction using 100mg aminopropyl silica cartridges<sup>[250]</sup> and converted to fatty acid methyl esters (FAMES) by incubation with methanol containing 2%(v/v) sulphuric acid<sup>[250]</sup>. FAMES were recovered by extraction with hexane<sup>[250]</sup>. The proportions of individual fatty acids were measured by gas chromatography using BPX70 fused silica capillary column (30m x 0.25mm x 0.25µm) on an Aglient 6890 gas

chromatograph equipped with flame ionisation detection<sup>[250]</sup>. FAMES were identified by their retention times relative to standards and quantified using Chemstation software.



## Chapter 3

# The Effect of Dietary Energy and Protein Content on Maternal Phenotype and on the Expression of Genes Involved in Fasting Carbohydrate and Fat Metabolism During Pregnancy

### 3.1 Introduction

Observations in humans and animal models have shown that poor nutrition during pregnancy leads to persistent alterations in the phenotype of the offspring which can be transmitted to subsequent generations<sup>[161,174,171,167,168,170,252,166,169,163,172,175,173]</sup>. The mechanism for such transmission however is unclear.

Despite substantial evidence that abnormal changes in the physiology of the mother, for example gestational diabetes mellitus, induce changes in the phenotype of the offspring over one generation, there has been little investigation about the role of variations in the metabolic adaptations of the mother to pregnancy in the passage of altered phenotype between generations. Therefore, the effect of differences in maternal protein intake during F0 pregnancy and of a persistent increase in energy intake from conception in F0 until the F3 generation on maternal physiology was investigated. We used fasting during pregnancy as a physiological challenge to test the capacity of dams to adapt to pregnancy as the response to fasting is heightened during gestation and so may be vulnerable to the effects of altered nutrition.

### **3.1.2 Aims**

The effect of altered nutrition during pregnancy in the F0 generation on the metabolic response to fasting in pregnant dams was determined in F0, F1 and F2 dams. Body weight and food intake during pregnancy were measured as markers of metabolic partitioning of energy substrates. Plasma glucose concentration was measured as a marker of gluconeogenesis and plasma  $\beta$ -hydroxybutyrate was measured as a marker of fatty acid  $\beta$ -oxidation in dams on day 8.5 of pregnancy. The role of hormonal regulation of fasting metabolism was determined by measuring glucagon, corticosterone and leptin.

Real time RT-PCR was used to test the hypothesis that alterations in the fasting phenotype in pregnancy are associated with changes in the expression of key regulatory genes in fasting energy metabolism: GR, PEPCK, glucose-6-phosphatase (Glc-6-Pase), phosphofructokinase-2/fructose-2-phosphatase (PFK-2), PPAR $\alpha$  and carnitine palmitoyltransferase 1 (CPT-1).

## **3.2 Experimental Design**

### **3.2.1 Animal model**

The experimental design is described in Section 2.2.1. The compositions of all the diets is shown in Tables 2.2, 2.3 and 2.4. The energy content of the diets is shown in Figure 2.2.

### **3.2.2 Methods**

In order to standardise metabolism and provide a state of stress, all rats were fasted for 12 hours, approximately 20:00 to 08:00, prior to sample collection.

#### **3.2.2.1 Molecular biology procedures**

Extraction of RNA and RT-PCR methods are described in section 2.3.5.

### 3.3 Results

#### 3.3.1 Does energy and protein content of the F0 diet during pregnancy alter weight gain and energy intake during pregnancy and lactation?

There was a significant effect of generation and a significant interactive effect of generation and F0 protein intake (both  $P < 0.0001$ ) on weight gain during pregnancy. There was no significant difference in weight gain at day 7, 14 or 28 of pregnancy, between generations or dietary groups (Figure 3.1). F0 dams gained significantly more weight compared to both F1 and F2 dams. At post-conception day 35, F2 dams had a significantly higher weight compared F0 and F1 dams irrespective of F0 protein intake ( $P < 0.0001$ ) (Figure 3.1).

During pregnancy there was no effect of generation or F0 protein intake on energy intake over 24 hours (Figure 3.2). However, there was a significant effect of generation and age on maternal energy intake (interaction  $P = 0.009$ ). F1 PR-PS and F2 dams from both F0 dietary lines consumed more energy during lactation than F1 PS-PS and F0 dams (generation\*time interaction  $P < 0.0001$ ). From 42 days post conception onwards, this was only significantly different from the F0 dams (Figure 3.2). There were significant interaction effects of F0 diet, generation and post-weaning age on litter weight gain ( $P < 0.0001$ ). At post-conception day 28, F2 PR-PS and F3 offspring had a significantly higher weight compared to F2 PS-PS and F1 offspring, this difference remains significant at post-conception day 35. At post-natal day 40 there is no significant difference between litter weights, between groups (Figure 3.3).

Table 3.1 shows the interactive effects of generation and F0 diet on the measured phenotypes.

|                    | Glucose | B-hydroxybutyrate | Corticosterone | Glucagon | Leptin |
|--------------------|---------|-------------------|----------------|----------|--------|
| Generation         | <0.0001 | <0.0001           | 0.008          | <0.0001  | 0.003  |
| F0 diet            | NS      | NS                | NS             | 0.023    | NS     |
| Generation*F0 diet | NS      | 0.036             | 0.005          | 0.023    | NS     |

**Table 3.1 A General Linear Model showing significant interactive effects of generation and F0 diet on measured phenotypes.**

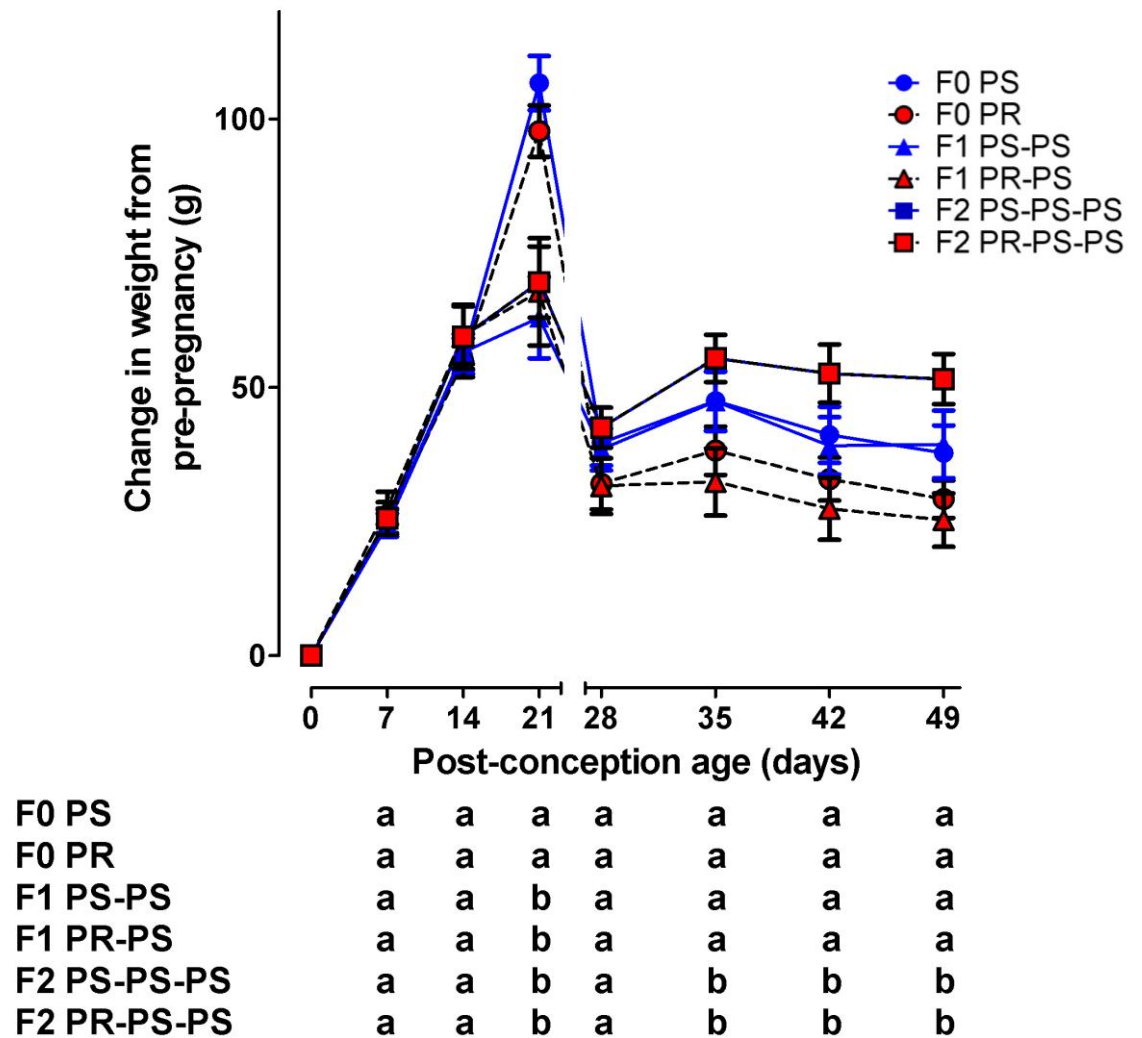


**Figure 3.1 Change in weight from pre-pregnancy in F0, F1 and F2 generation pregnancies.** Values are means with their standard deviations. Where 'a' is significantly different from 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$



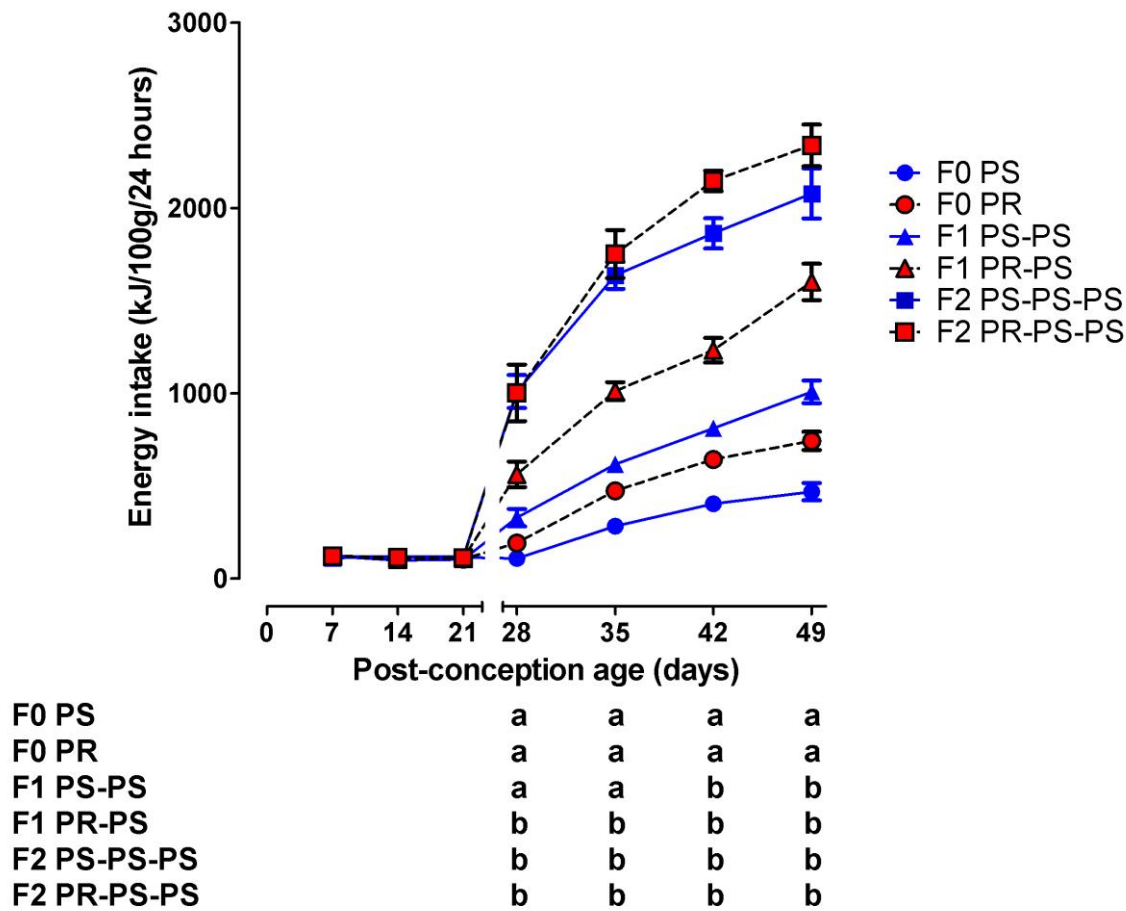


**Figure 3.2 Energy intake during pregnancy and lactation in F0, F1 and F2 generation pregnancies.** Values are means with their standard deviations. Where 'a' is significantly different from 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

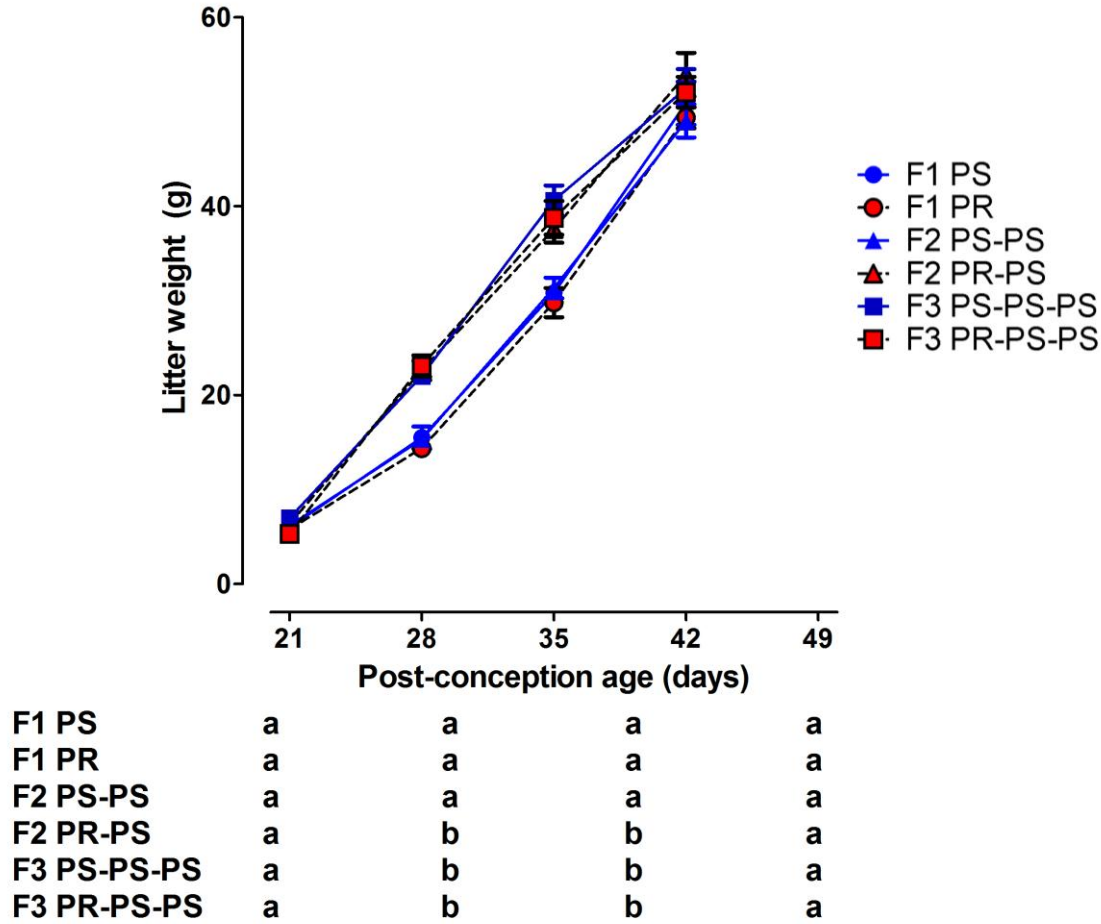


**Figure 3.3 F1, F2 and F3 generation litter weight gain, from delivery until weaning.** Values are means with their standard deviations, where 'a' is significant to 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

### 3.3.2 Does the protein content of the F0 diet during pregnancy alter length of gestation and litter size?

There were no significant effects of energy or protein content of the F0 diet during pregnancy or generation on length of gestation or litter size (Table 3.2).

|                 | Length of Gestation (days) | Litter size (n) |
|-----------------|----------------------------|-----------------|
| F0 PS           | 22 ± 1                     | 11 ± 3          |
| F0 PR           | 23 ± 1                     | 12 ± 3          |
| F1 PS-PS        | 22 ± 1                     | 12 ± 3          |
| F1 PR-PS        | 22 ± 1                     | 12 ± 2          |
| F2 PS-PS-PS     | 22 ± 1                     | 12 ± 3          |
| F2 PR-PS-PS     | 22 ± 1                     | 12 ± 4          |
| Generation (P)  | NS                         | NS              |
| F0 diet (P)     | NS                         | NS              |
| Interaction (P) | NS                         | NS              |

**Table 3.2 Length of gestation and Litter size in F0, F1 and F2 generation pregnancies.** Values are means with their standard deviations. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

### **3.3.3 Does the energy content of the diet and the protein content of the F0 diet during pregnancy alter plasma glucose and $\beta$ -hydroxybutyrate concentrations during pregnancy?**

There was a significant effect of generation ( $P < 0.0001$ ) on fasting plasma glucose concentration (Table 3.1). Both the PS and PR lineages showed lower plasma glucose levels in the F2 pregnancy compared to both the F0 and F1 generations (Figure 3.4). Fasting  $\beta$ -hydroxybutyrate plasma concentrations also showed a significant effect of generation ( $P < 0.0001$ ) irrespective of the protein content of the F0 diet during pregnancy. Both PS and PR lineages showed a higher  $\beta$ -hydroxybutyrate concentration in the F2 pregnancy compared to the F0 and F1 generations (Figure 3.4).

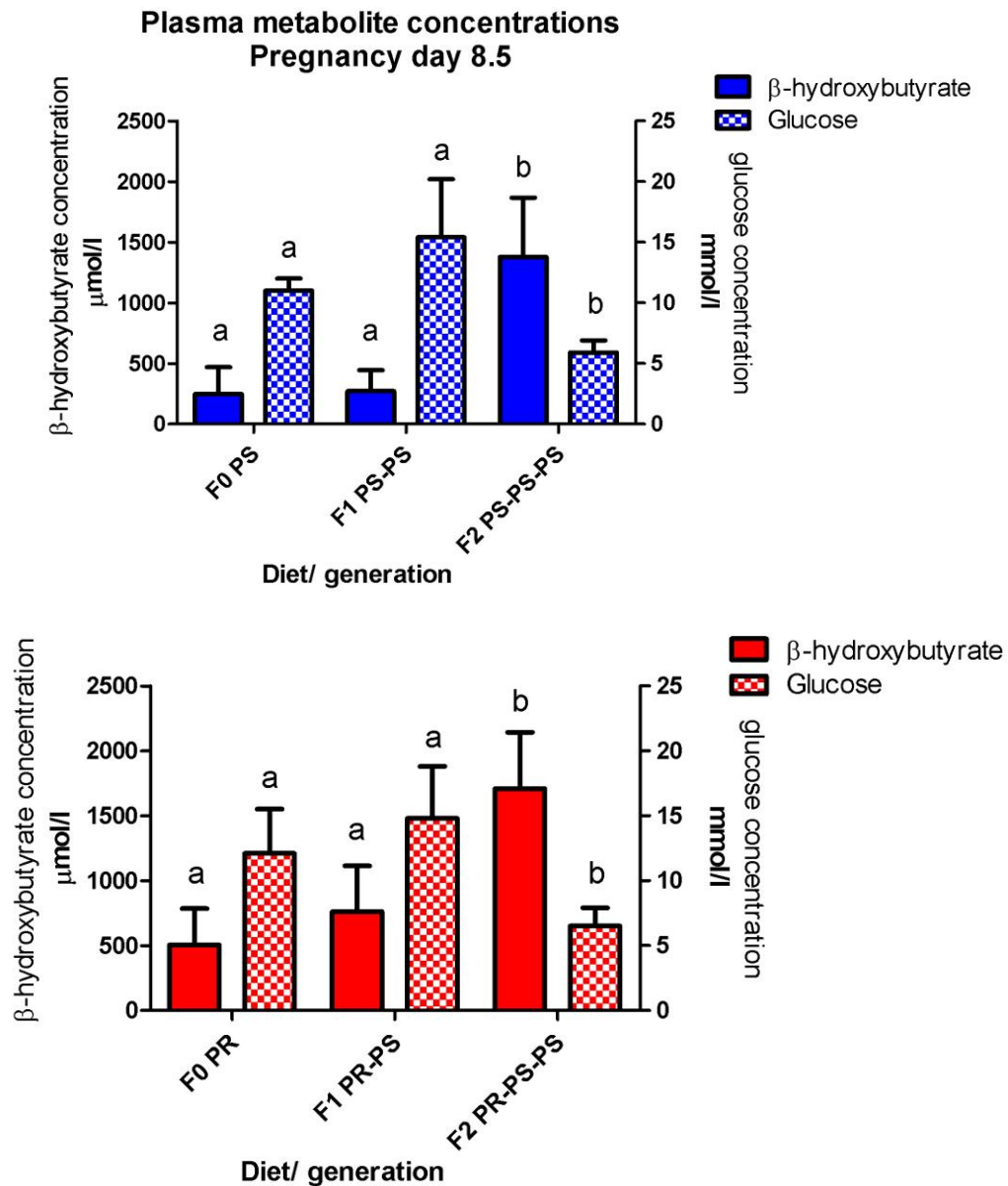


**Figure 3.4 Plasma glucose and  $\beta$ -hydroxybutyrate concentrations in pregnancy day 8.5 in the F0, F1 and F2 generations.** Values are means with their standard deviations. Where 'a' is significantly different from 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.005$ .

### **3.3.4 Does the protein content of the F0 diet during pregnancy alter fasting hormonal regulation of glucose metabolism, appetite and energy expenditure?**

Plasma glucagon showed no significant differences between groups (Table 3.1; Figure 3.5). However, there was a significant interaction effect of generation and F0 diet ( $P=0.023$ ) on plasma corticosterone concentration (Figure 3.5). Plasma corticosterone concentration was significantly higher in F2 dams of both the PS and PR lineage, as well as F1 PR-PS dams, compared to both F0 PS and PR dams. Fasting plasma leptin concentration was significantly higher in the F1 generation ( $P=0.005$ ), compared to both the F0 and F2 generations, irrespective of the protein content of the F0 diet during pregnancy (Figure 3.5).

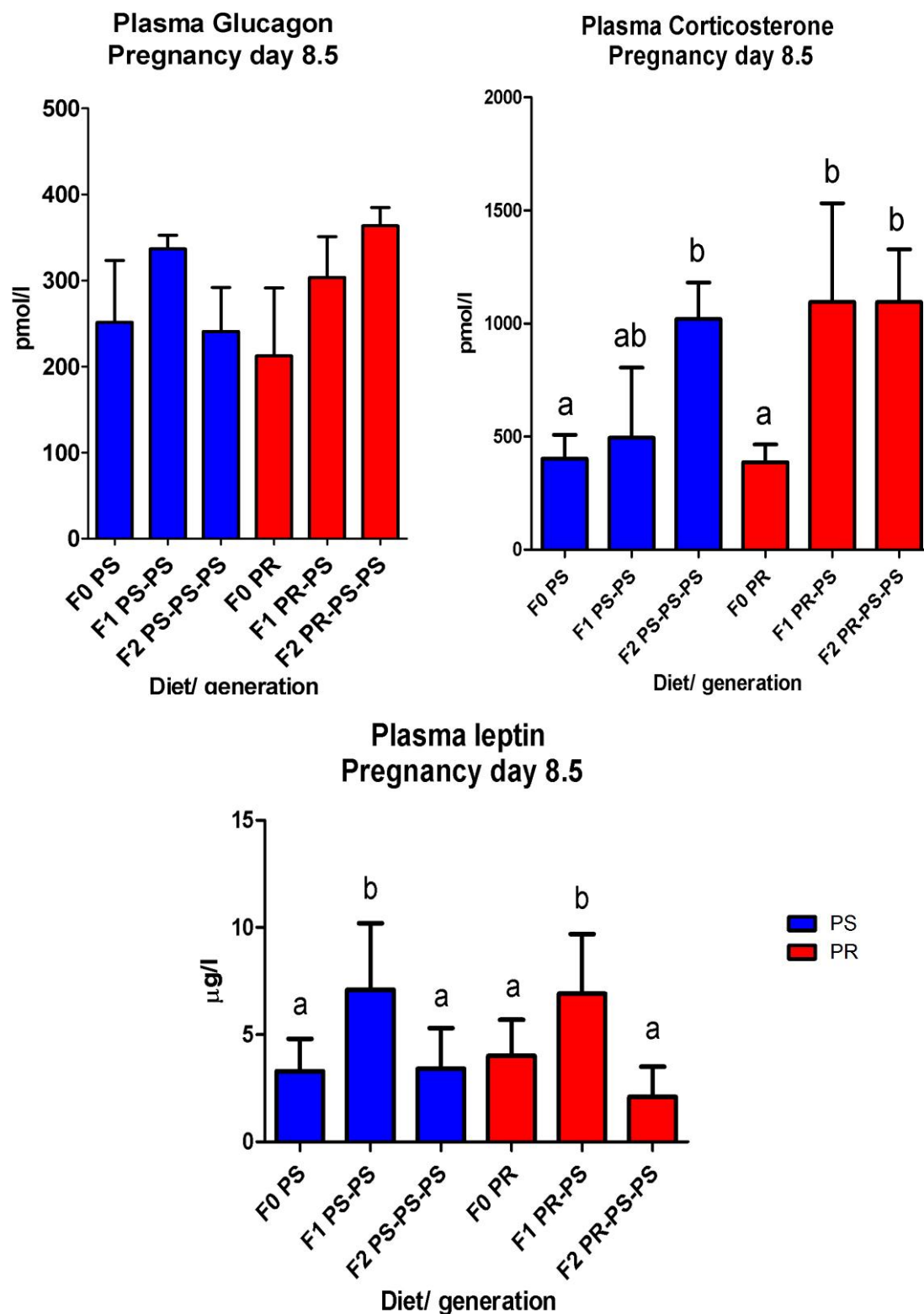


**Figure 3.5 Plasma glucagon, corticosterone and leptin concentrations in pregnancy day 8.5 in the F0, F1 and F2 generations.** Values are means with their standard deviations,. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.

### 3.3.5 Does the protein content of the F0 diet affect expression of hepatic genes involved in glucose metabolism in pregnancy?

There was a significant effect of generation and F0 diet on GR, Glc-6-Pase and PFK-2 ( $P=0.009$ ). There was a significant effect of generation on all genes measured which are involved in glucose metabolism (PEPCK,  $P=0.001$ ; GR, Glc-6-Pase and PFK-2 all  $P<0.0001$ ). There was a significant effect of F0 diet on mRNA expression of Glc-6-Pase ( $P=0.033$ ) and PFK-2 ( $P<0.0001$ ). Expression of GR and Glc-6-Pase was significantly higher in F1 PR-PS and F2 PS-PS dams compared to all other groups (Figure 3.6). Hepatic mRNA expression of both PEPCK and PFK-2 was increased in the F1 PR-PS dams, compared to; F0 dams for PEPCK and all other groups for PFK-2 (Figure 3.6).

Table 3.3 shows the interactive effects of generation and F0 diet on mRNA expression.

|                    | GR      | PEPCK | G6Pase  | PFK-2   | PPAR $\alpha$ | CPT-1   |
|--------------------|---------|-------|---------|---------|---------------|---------|
| Generation         | <0.0001 | 0.001 | <0.0001 | <0.0001 | <0.0001       | <0.0001 |
| F0 diet            | NS      | 0.001 | 0.033   | <0.0001 | NS            | NS      |
| Generation*F0 diet | 0.001   | 0.009 | <0.0001 | <0.0001 | <0.0001       | <0.0001 |

**Table 3.3 A General Linear Model showing significant interactive effects of generation and F0 diet on mRNA expression.**

Linear regression analysis showed that the level of GR mRNA expression was not significantly associated to the plasma level of corticosterone (PS,  $r < 0.0001$ ,  $p = 0.40$ , PR,  $r = 0.1007$ ,  $p = 0.314$ ). There was a significant association between mRNA expression of GR and its target genes PEPCK (PS  $r = 0.305$ ,  $p = 0.02$ , PR,  $r = 0.8803$ ,  $p < 0.0001$ ) PPAR $\alpha$  (PS  $r = 0.733$ ,  $p < 0.0001$ , PR  $r = 0.512$ ,  $p < 0.0001$ ) and CPT-1 (PS  $r = 0.793$ ,  $p < 0.0001$ , PR  $r = 0.805$ ,  $p < 0.0001$ ).



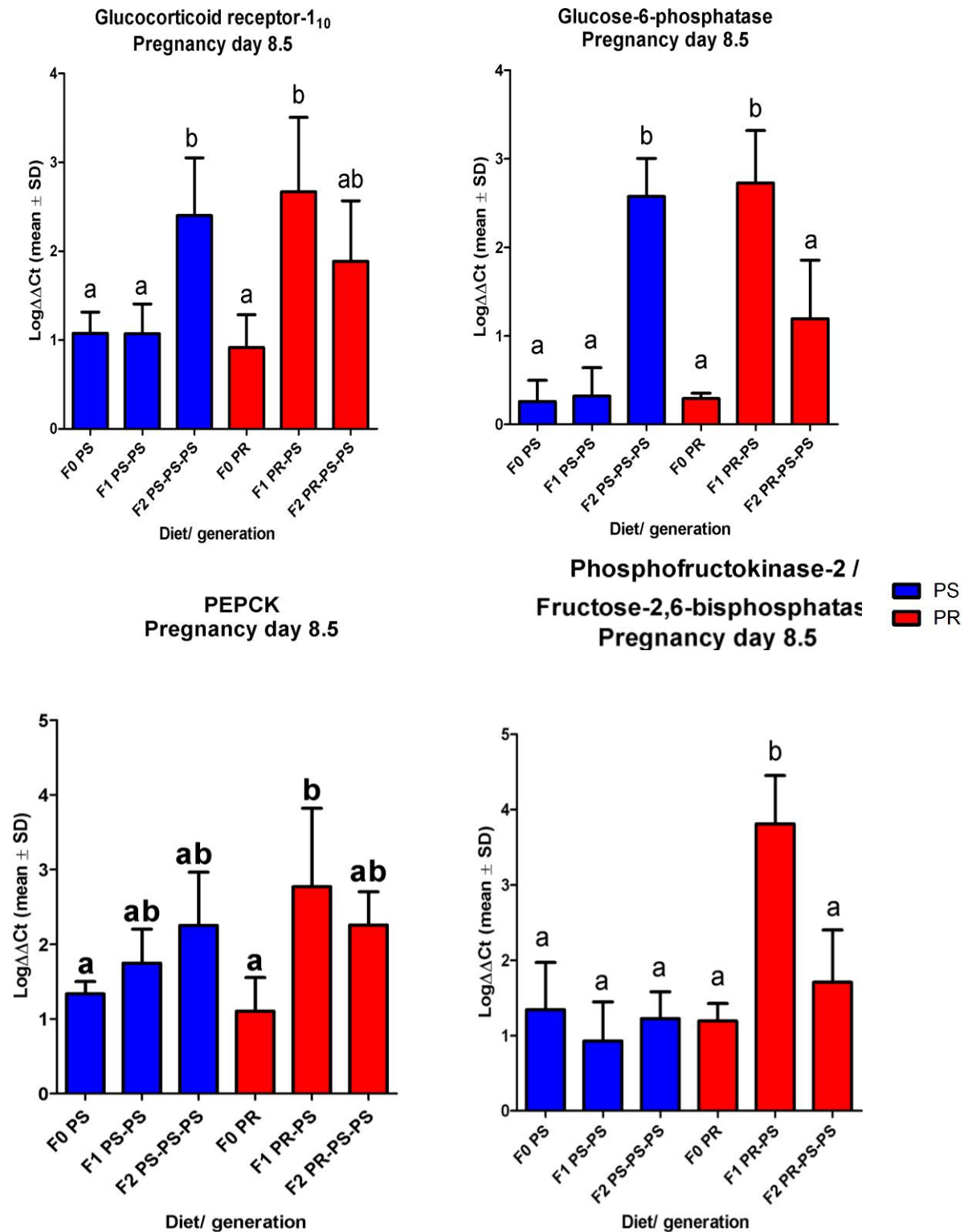


**Figure 3.6** Hepatic GR1<sub>10</sub>, Glucose-6-Phosphatase, PEPCK and PFK-2/F-2,6-BP mRNA expression in pregnancy day 8.5 in the F0, F1 and F2 generations. Values are means with their standard deviations, where 'a' is significantly different from 'b', and 'ab' is not significantly different from either 'a' or 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$ .

### **3.3.6 Does the energy content of diet and F0 pregnancy diet protein content alter expression of hepatic genes involved in fatty acid metabolism in pregnancy?**

There was a significant interaction effect of generation and diet on both PPAR $\alpha$  and CPT-1 mRNA expression ( $P < 0.0001$ ) (Table 3.3). Expression of PPAR $\alpha$  was significantly higher in both the F2 PS-PS-PS and F1 PR-PS groups, compared to all other groups (Figure 3.7). Expression of CPT-1 was significantly higher in both the F2 PS-PS-PS and F1 PR-PS groups, compared to all groups, except for the F2 PR-PS-PS group (Figure 3.7)

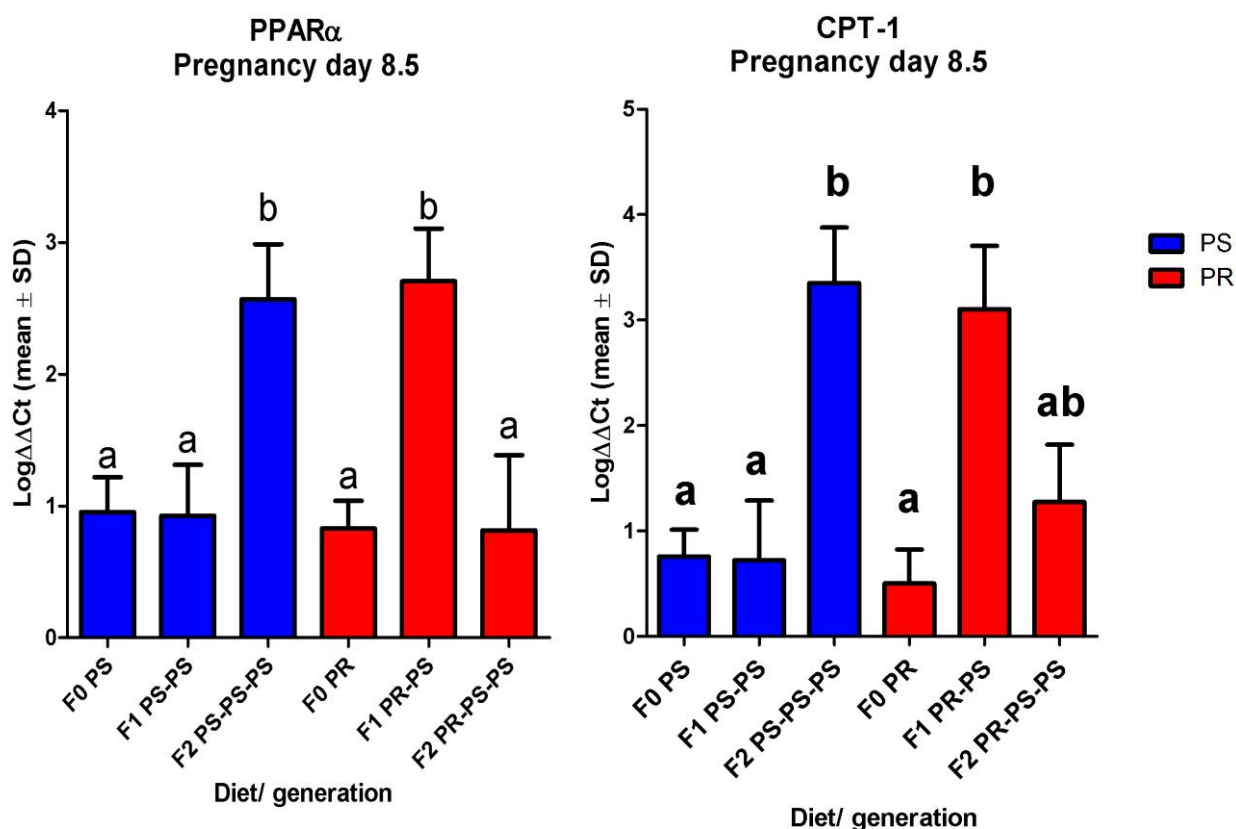


**Figure 3.7 Hepatic PPAR $\alpha$  and CPT-1 mRNA expression in pregnancy day 8.5 in the F0, F1 and F2 generations.** Values are means with their standard deviations, where 'a' is significantly different from 'b', and 'ab' is neither significantly different from 'a' or 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

### **3.4 Discussion**

#### **3.4.1 Maternal phenotype during pregnancy**

The first generation exposed to the high energy diet (both PS and PR F0 dams) gained more weight during pregnancy, specifically in the third trimester (around 40g more than F1 and F2 dams). This not only indicates an effect of the dietary energy content as early as the F0 generation, but also provides possible evidence of adjustment to the high energy content in subsequent generations, as the animals did not gain as much weight during pregnancy after the initial generation. An increase in weight gain could represent a larger litter size, larger offspring weights or an increased storage of energy in the form of adipose. The results show that the litter weights and litter sizes did not account for the increased maternal weight gain during F0 pregnancy. Although F0 dams gained more weight than subsequent generations in pregnancy, F1 PR-PS dams and F2 dams from both F0 diet protein groups showed significantly increased energy intakes, relative to body weight. These findings indicate that although F1 PS-PS and F2 dams were consuming more energy in pregnancy, they were gaining significantly less weight. One possible explanation is that F1 PS-PS and F2 dams partitioned a greater proportion of their diet into energy production compared to the F0 dams.

In order to understand the metabolic phenotype of the dams, the fasting plasma concentrations of glucose and  $\beta$ -hydroxybutyrate were measured. Fasting glucose concentration is increased in early pregnancy and decreased in late pregnancy<sup>[101]</sup>. Throughout pregnancy, plasma levels of non-esterified fatty acids and  $\beta$ -hydroxybutyrate are inversely related to plasma glucose concentration<sup>[111]</sup>.

In both the F0 and F1 pregnant dams, glucose concentration was higher, compared to published concentrations seen in an adult female rat that received no dietary modification<sup>[253,254,48,173]</sup>, irrespective of the protein content of the F0 diet during pregnancy. In the glucose–fatty acid cycle<sup>[255]</sup>, increased plasma glucose concentration increases insulin secretion. Increased insulin increases cellular malonyl CoA concentration, which in turn, will decrease fatty acid  $\beta$ -oxidation by inhibiting CPT-1 activity (Figure 3.8). This suggests F0 dams have lower levels of fatty acid  $\beta$ -oxidation than F1 and F2 dams, potentially leading to increased fat

storage. The plasma glucose concentrations, of over 10mmol/l, in the F0 and F1 dams suggest an exaggerated gluconeogenic response during fasting or impaired glucose uptake, as in the fasted state liver glycogen stores would be depleted. The high level of plasma glucose in the F0 and F1 dams was not associated with an increase in PEPCK, GR or G-6-Pase gene expression, or with increased glucagon concentration. This suggests that gluconeogenesis was not increased in F0 and F1 dams. However, one possible explanation for the increased glucose value in dams is a decreased placental glucose uptake.

In the F2 generation, there was a significantly higher plasma  $\beta$ -hydroxybutyrate concentration, compared to both the F0 and F1 generation, irrespective of F0 diet protein content. The F2 dams showed fasting plasma glucose values are comparable to that for chow fed fasting dams<sup>[253,254,48,173]</sup>. Thus despite the same nutrition, the fasting phenotype of F2 dams differed from that of F0 dams, irrespective of the protein content of the F0 diet during pregnancy. This suggests that after three generations on a diet containing around 16 MJ/kg energy, dams have adjusted and regained homeostatic control during fasting.

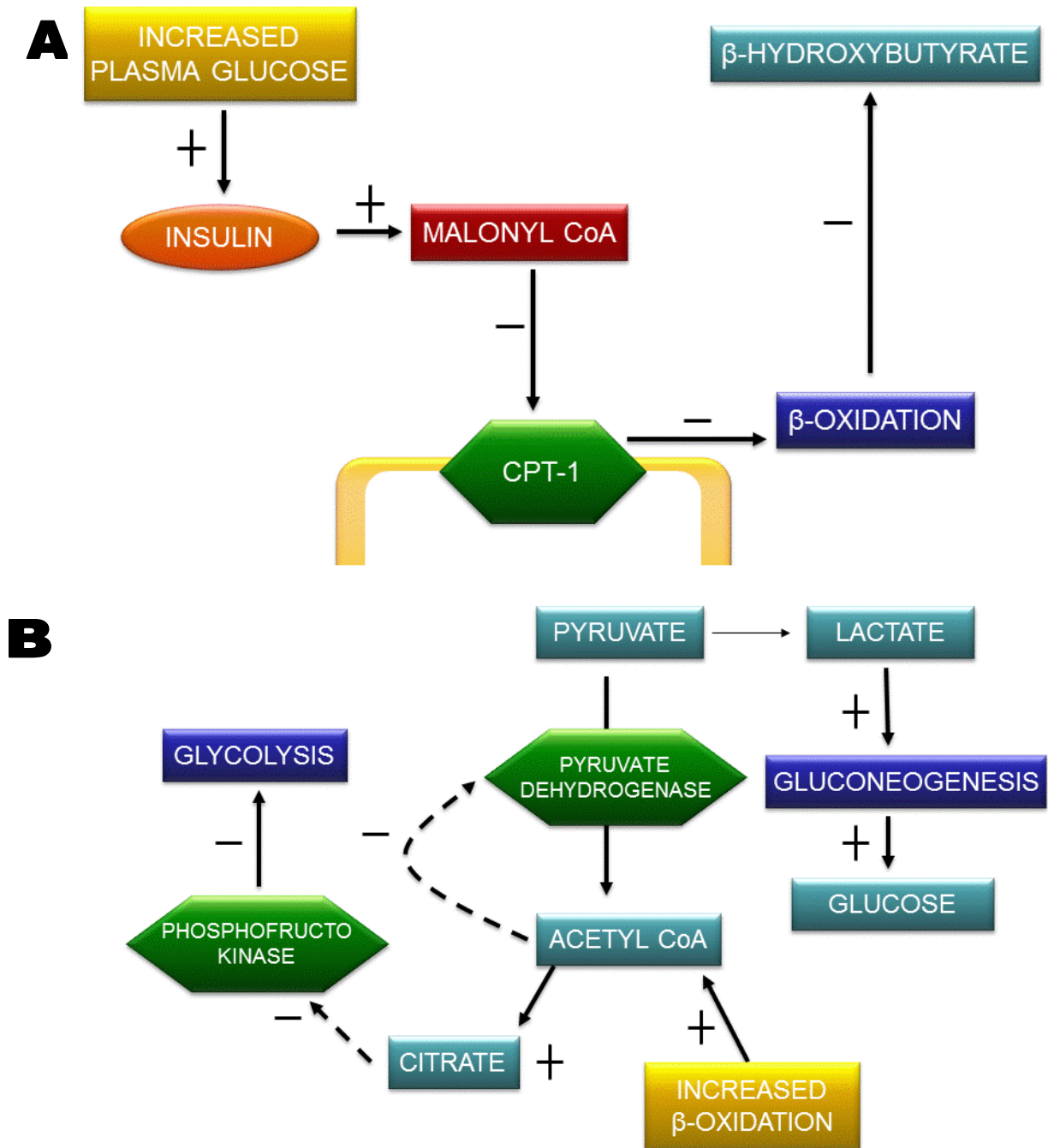


**Figure 3.8 Interactions between glucose and fatty acid metabolism.** Figure **A** shows how high plasma glucose leads to an increase in plasma  $\beta$ -hydroxybutyrate, where an increase in plasma glucose leads to an increase in insulin secretion. This in turn results in an increase in malonyl CoA, which results in an inhibition of CPT-1. The inability of CPT-1 to transport fatty acids across the mitochondrial membrane results in a decrease in  $\beta$ -oxidation of fatty acids, and therefore plasma levels of  $\beta$ -hydroxybutyrate. Figure **B** shows how  $\beta$ -oxidation of fatty acid leads to conservation of glucose by increasing levels of acetyl CoA. Through intermediates, Acetyl CoA leads to inhibition of the glycolytic enzyme phosphofructokinase and increases the level of gluconeogenesis via inhibition of pyruvate dehydrogenase.

There were no differences between generations in fasting plasma glucagon and leptin concentrations. However, plasma corticosterone concentrations were lower in F0 compared to F2 generations. Plasma glucose concentrations decreased from F0 to F2 generations. As plasma corticosterone concentration is increased when glucose levels are low<sup>[101,256]</sup>, these results are in agreement, as the decreased plasma glucose concentration in the F2 generation is concurrent with an increase in plasma corticosterone concentration. Although it was not possible to determine whether there was a direct causal association between plasma corticosterone and glucose concentrations from this experiment.

### **3.4.2 Maternal phenotype during lactation**

During lactation mothers must produce milk for the growing offspring. As a result, dams need to consume enough food to provide for both themselves and the growing pups. F0 dams had significantly higher weight gain during late pregnancy, which was not explained by litter number or weight. As the increased weight was not represented by the F1 offspring, we would have expected mothers to have a significantly higher postnatal body weight. Although it must be stated that dams were weighed up to 7 days following pregnancy, as added stress following pregnancy can lead to dams consuming pups. F0 dams had a significantly lower body weight from day 35 to day 49 of lactation compared to the F2 dams, this suggests that the excess stored energy in the F0 dams was either incorporated into the milk, or oxidised. Both F1 and F2 dams showed a significantly greater energy intake during the period of lactation, compared to the increased energy intake seen in the F0 dams. For F1 dams there is an increased energy intake that is not concurrent with an increased weight gain. For these dams, as energy is not being stored, it is either being oxidised, or it is partitioned into milk synthesis. F2 dams showed a significantly greater weight gain from post conception day 28 to day 35, which remained significant until postnatal day 49. In terms of F2 dams, the increased weight gain can be explained by the increased energy intake, where excess energy is being stored as adipose.

F2 PR-PS and F3 offspring from both protein lineages had significantly higher weight gain during lactation compared to F2 PS-PS and F1 offspring, which is related to an increased energy intake in their respective mothers (F1 PR-PS and F2 dams). Although the increased energy intake during lactation in F1 PS-PS dams is not related to an increased weight gain in F2 PS-PS offspring, all other groups that showed an increased energy intake in the dams, showed increased growth in the offspring. This suggests that the increased energy is being incorporated in the milk. In terms of the F1 PS-PS dams, as the increased energy intake is not related to an increased dam weight or offspring weight, the additional energy must represent a higher level of substrate oxidation. As well as an increased offspring litter weight during lactation, increased energy intake in the F2 dams is also associated with an increased F2 dam weight during lactation. Compared to the F1 PR-PS dams, which do not show an increased body weight, the energy intake of F2 dams is about 25% greater. It seems that the initial increase in energy, present in F1 and F2 dams is reflected in the offspring weight, whereas the additional energy consumed by just the F2 dams, appears to be stored by the dams.

#### **3.4.3 mRNA expression of genes involved in fasting energy metabolism in day 8.5 dams**

Both generation and protein content of the F0 diet during pregnancy had significant effects on genes involved in fasting glucose and fatty acid metabolism. All genes involved in glucose metabolism showed a significant increase in expression in the F1 PR-PS group, with GR and Glc-6-Pase also significantly increased in the F2 PS-PS-PS group. Interestingly, where fasting plasma glucose and  $\beta$ -hydroxybutyrate concentrations appear to return to expected levels by the F2 generation, the mRNA expression of all measured genes was increased in the F1 generation, in the PR group. Although this pattern is not present in the phenotype data, it does suggest an altered capacity for fasting energy homeostasis in the group.



Glc-6-Pase expression is increased 2-3 fold in the state of fasting<sup>[257]</sup>. Administration of the synthetic glucocorticoid dexamethasone to rats led to a 10-fold increase in Glc-6-Pase mRNA expression<sup>[257]</sup>. In the animals of this study the pattern of increase in corticosterone across generations and dietary groups directly matched the pattern of Glc-6-Pase expression. This suggests the increase in Glc-6-Pase may be due to the increased level of plasma corticosterone acting through the GR. During fasting, the bifunctional enzyme PFK2/F-2,6-BPase acts to increase levels of gluconeogenesis. Although this could explain the increased fasting glucose levels in the F1 PR-PS, as increased levels were also seen in both the F0 and F1 dams, other forms of regulation are likely to be involved.

There was an increase in mRNA expression of the genes PPAR $\alpha$  and CPT-1, in the F2 PS-PS-PS and F1 PR-PS groups, compared to all other groups. An increased expression of both PPAR $\alpha$  and CPT-1 would lead to an increase in  $\beta$ -oxidation and therefore an increase in circulating ketone bodies, such as  $\beta$ -hydroxybutyrate. By comparing to phenotype data, we can see that this is in agreement for all PS lineage dams and F0 PR dams. Although gene expression values do not directly match all of the differences seen in circulating glucose and  $\beta$ -hydroxybutyrate in the fasted dams, these genes represent only a sub-set involved in fasting energy metabolism and do not reflect potential changes in enzyme activity or total protein levels.

Increased levels of plasma corticosterone will lead to increased expression of the transcription factor GR (Figure 3.9). In turn, increased GR levels lead to up regulation of PEPCK<sup>[134,136]</sup> and PPAR $\alpha$ <sup>[137]</sup> expression. PPAR $\alpha$  expression will lead to increased CPT-1 expression<sup>[258]</sup>. Linear regression analysis showed that the level of GR mRNA expression was not significantly associated with plasma glucose levels. However, there was a significant positive association between mRNA expression of GR and its target genes PEPCK. In order for mRNA of GR to have an effect on transcription of other genes, the increased expression must represent increased levels of protein synthesis. These relationships suggest that increased gene expression of GR is related to increased protein synthesis of GR, and that the regulation of downstream genes by GR is present.

Overall, these findings suggest that despite being fed a diet with an increased energy content than that fed before conception in F0 dams, there was a progressive adjustment in the regulation of fasting energy metabolism between generations. The pattern of pregnancy and lactation-associated changes in weight and food intake, and in glucose and  $\beta$ -hydroxybutyrate concentration between generations suggests progressive improvement in metabolic control. Furthermore, these findings imply that the interaction between the phenotype of the dam and its environment, specifically diet, differed between generations. This implies that any developmental signals from the dam to the offspring may also differ between generations.

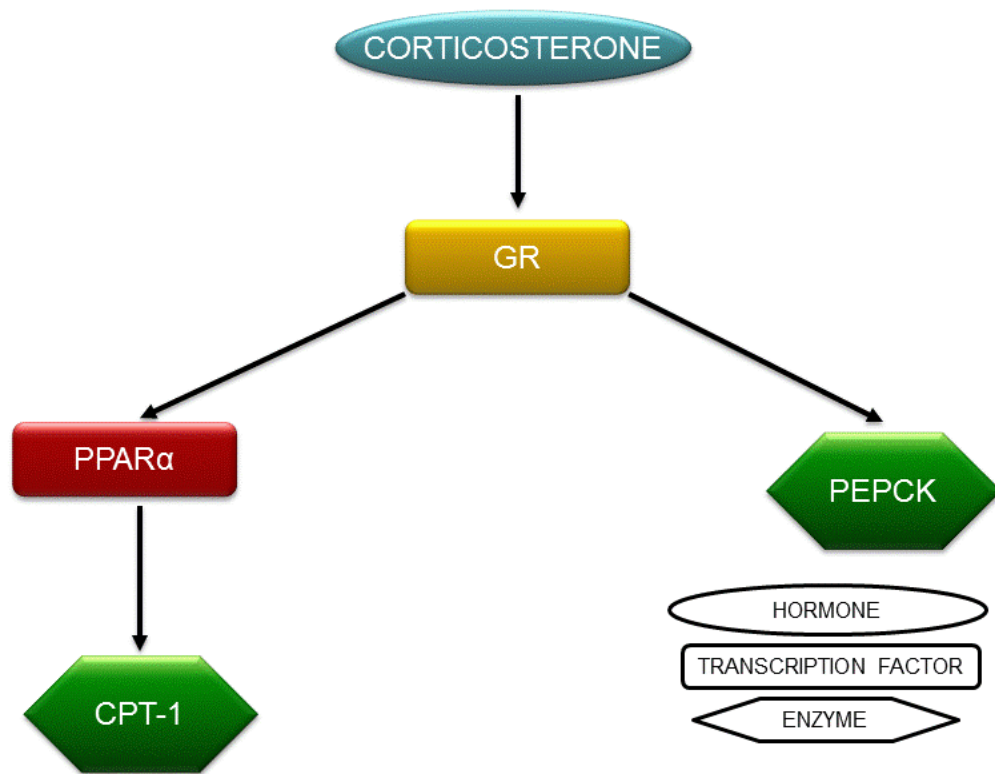


**Figure 3.9 The role of corticosterone in energy metabolism.** Increased levels of corticosterone lead to an increase in GR expression. In turn, GR leads to up regulation of both the gluconeogenic enzyme PEPCK and the key  $\beta$ -oxidation regulatory transcription factor PPAR $\alpha$ .



## Chapter 4

# Phenotypic Characterisation of the Non-Pregnant Offspring in each Generation

#### **4.1 Introduction**

The findings presented in Chapter 3 showed that the fasting phenotype of dams during pregnancy was significantly altered over three generations in a manner which suggested progressive improvement in control of whole body energy balance in response to fasting. The purpose of the experiments described in this chapter is to determine whether the inter-generational effects observed in the dams are also found in the non-pregnant female offspring and also whether there is any direct relationship between the metabolic phenotype of the dams and of their offspring in generation. The growth of female offspring and their food intake after weaning was used to assess regulation of whole body energy balance. As in Chapter 3, capacity to respond to fasting was used as a proxy measure of control of fat and carbohydrate metabolism.

## **4.2 Experimental Design**

### **4.2.1 Animal model**

The experimental design is described in detail in section 2.2.1 and the composition of the diets is summarised in Tables 2.2, 2.3 and 2.4, and in Figure 2.2

### **4.2.2 Methods**

In order to standardise metabolism and provide a state of stress in offspring that may uncover changes in metabolism, all rats were fasted for 12 hours prior to sample collection.

#### **4.2.2.1 Molecular biology procedures**

Extraction of RNA and RT-PCR methods are described in section 2.3.5.

## **4. Results**

### **4.3.1 Does energy content of diet and protein content of the F0 diet during pregnancy alter post-weaning weight gain and energy intake in non-pregnant F1, F2 and F3 offspring?**

There was a significant effect of generation and protein content of the F0 diet during pregnancy on offspring post-weaning weight gain ( $P=0.002$ ). At postnatal day 49, F1 PS offspring and all PR offspring, irrespective of generation, had a significantly lower weight compared to F2 PS-PS and F3 PS-PS-PS ( $P<0.05$ ). On post-natal days 56, 63 and 70, all generations of the PR lineage showed a significantly lower weight gain compared to those of the PS lineage ( $P=0.002$ ) (Figure 4.1). There were significant interaction effects of F0 diet, generation and post-weaning age on offspring energy intake ( $P<0.0001$ ). On post-natal day 28, all F1 and F2 offspring had a significantly higher energy intake, regardless of F0 diet. On post-natal days 35, 42 and 49 this increased energy intake was only seen in the F2 groups. Following this there were no significant differences in energy intake between groups and generations at post-natal days 56 and 63. However, on postnatal day 70, the F2 generation offspring had a significantly higher energy intake compared to both the F1 and F3 offspring (Figure 4.2).

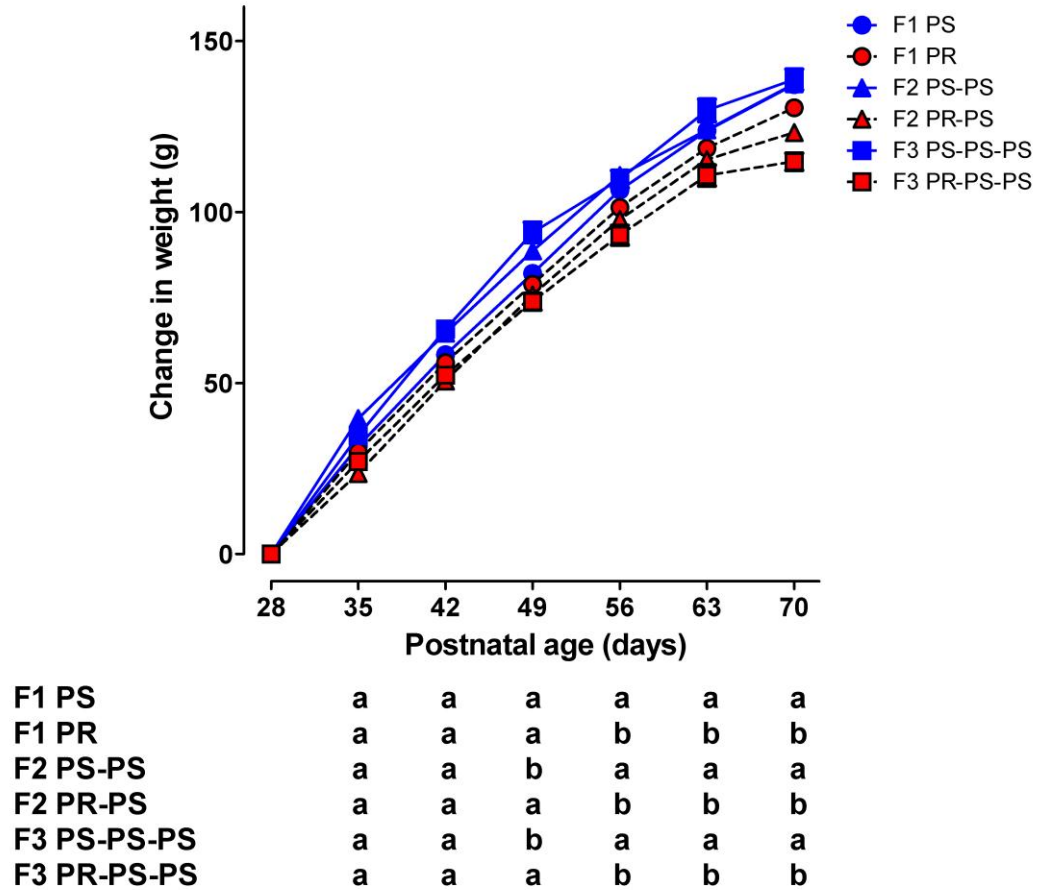


**Figure 4.1 F1, F2 and F3 generation post-weaning weight gain.** Values are means with their standard deviations, where 'a' is significant to 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$



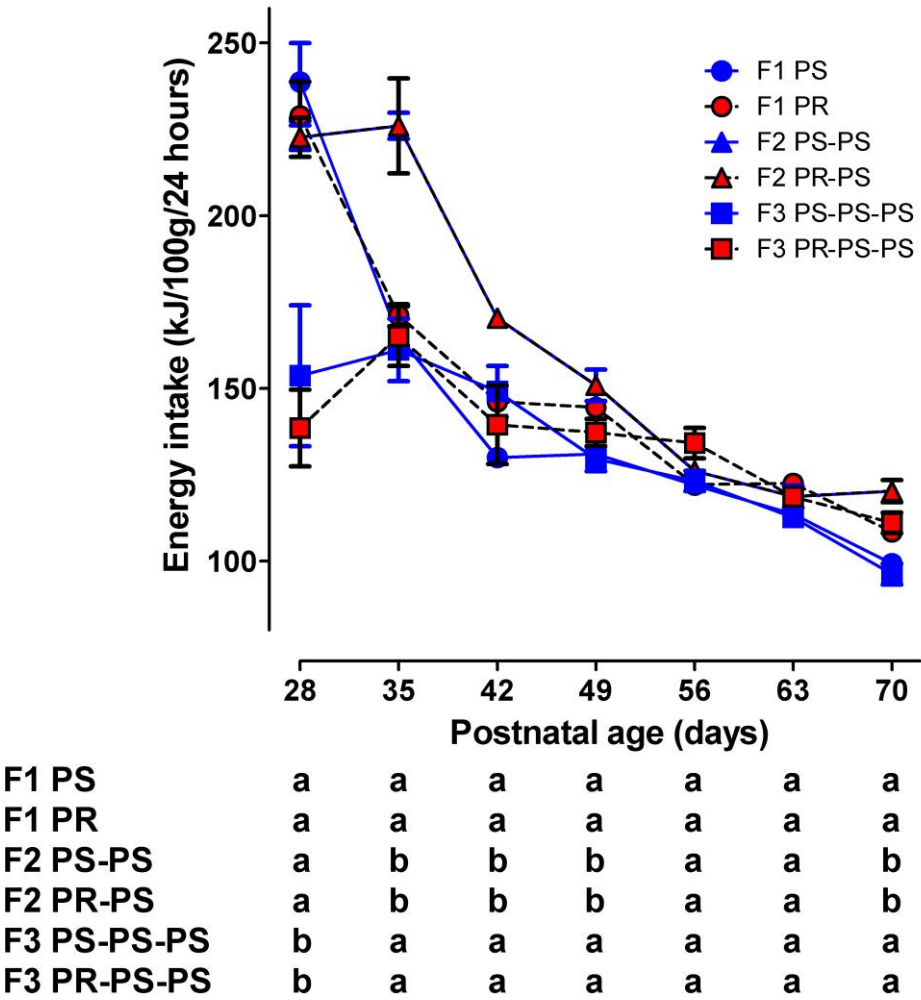


**Figure 4.2 F1, F2 and F3 generation post-weaning energy intake.** Values are means with their standard deviations, where 'a' is significant to 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

#### **4.3.2 Does the energy content of diet and protein content of the F0 diet during pregnancy alter fasting plasma glucose and $\beta$ -hydroxybutyrate concentrations in non-pregnant offspring?**

There was a significant interaction effect of both generation and diet on plasma glucose concentration ( $P < 0.0001$ ) (Table 4.1). Glucose levels were significantly lower in the F3 PR-PS-PS group, compared to all other groups, except for the F2 PR-PS group (Figure 4.3). There were no significant differences in  $\beta$ -hydroxybutyrate concentration between groups (Figure 4.3).

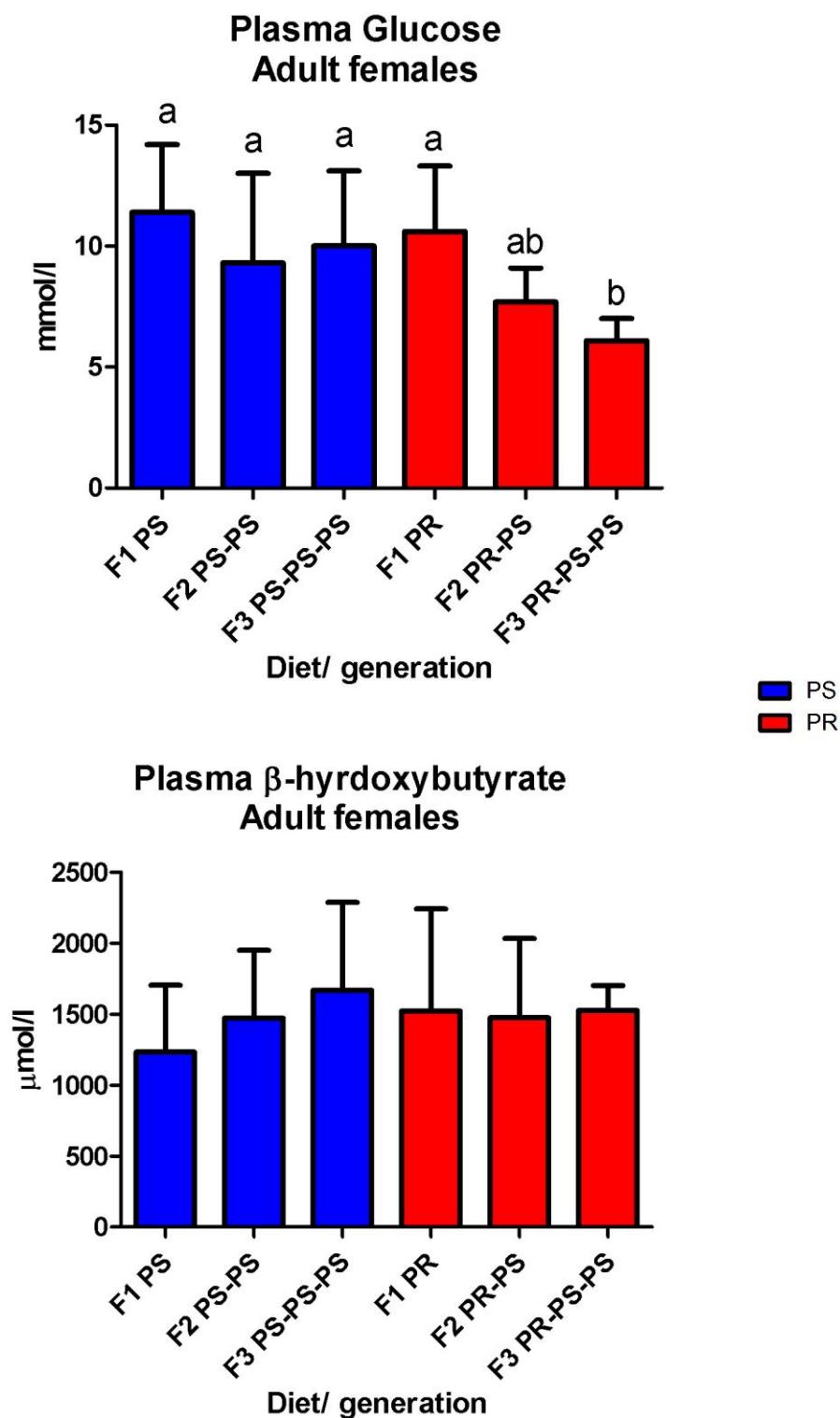


**Figure 4.3 Plasma glucose and  $\beta$ -hydroxybutyrate concentrations in adult females in the F0, F1 and F2 generations.** Values are means with their standard deviations, where 'a' is significantly different from 'b', and 'ab' is neither significantly different from 'a' or 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

#### 4.3.3 Does energy content of diet and protein content of the F0 diet during pregnancy alter fasting plasma concentrations of corticosterone, leptin and glucagon in non-pregnant adult offspring?

In order to investigate whether the plasma corticosterone, leptin and glucagon concentrations in day 70, non-pregnant adult female offspring were affected by increased energy content of diet throughout life and the protein content of the F0 pregnancy diet, the concentrations of these hormones were measured by ELISA (Section 2.2.1.4.1). Measurement of plasma corticosterone was carried out by Dr Nicola Thomas.

There were significant interaction effects for plasma corticosterone concentration for generation and F0 diet ( $P < 0.0001$ ) (Table 4.1). Plasma corticosterone concentration was significantly higher in the F3 generation, irrespective of diet, and the F2 PR-PS group, compared to all other groups (Figure 4.4). Plasma glucagon concentrations showed no significant effect of generation or F0 diet (Figure 4.4). There was a significant effect of generation on plasma leptin concentration in day 70 non-pregnant females ( $P < 0.0001$ ). Plasma leptin levels were significantly lower in the F2 and F3 generation compared to the F1 generation, regardless of F0 diet ( $P < 0.0001$ ) (Figure 4.4).

Table 4.1 shows the interactive effects of generation and F0 diet on the measured phenotypes.

|                    | Glucose | B-hydroxybutyrate | Glucagon | Corticosterone | Leptin  |
|--------------------|---------|-------------------|----------|----------------|---------|
| Generation         | <0.0001 | NS                | NS       | <0.0001        | <0.0001 |
| F0 diet            | <0.0001 | NS                | NS       | 0.004          | NS      |
| Generation*F0 diet | NS      | NS                | NS       | NS             | NS      |

**Table 4.1 The results of analysis of interactive effects between generation and F0 diet using a General Linear Model on fasting concentrations of glucose,  $\beta$ -hydroxybutyrate, corticosterone and leptin.**

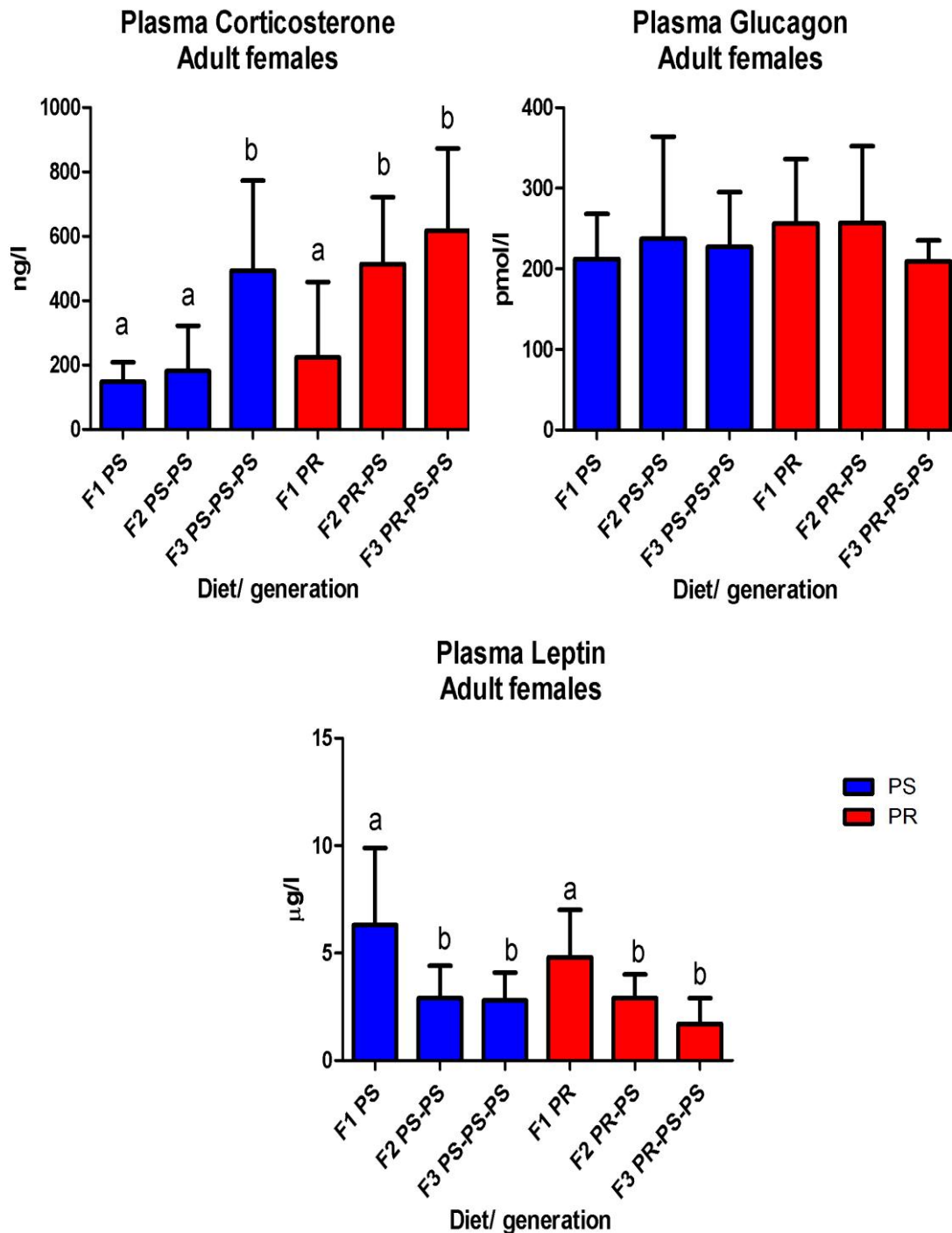


**Figure 4.4 Plasma corticosterone, glucagon and leptin concentrations in adult females in the F0, F1 and F2 generations.** Values are means with their standard deviations, where 'a' is significantly different from 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

#### 4.3.4 Does energy content of diet and protein content of the F0 diet during pregnancy alter expression of genes involved in glucose metabolism in the liver of the adult offspring?

There were significant interaction effects of generation and diet on expression of GR, PEPCK, CPT-1 and PFK-2 in adult non-pregnant female offspring ( $P=0.001$ ). The F1 PR group and F3 adults of both protein lineages showed a significantly higher expression of GR compared to all other PS groups, which was not significantly associated with plasma corticosterone concentration ( $r^2 = 0.0057$ ,  $p=0.633$ ) (Figure 4.5). PEPCK expression was significantly lower in the F2 PS-PS group, compared to all other groups ( $P=0.001$ ) (Figure 4.5). PFK-2 expression was significantly higher in the F2 and F3 PR groups, compared to all other groups ( $P<0.05$ ) (Figure 4.5). There were no significant differences between groups for Glc-6-Pase expression (Figure 4.5). Table 4.2 shows the interactive effects of generation and F0 diet on the measured phenotypes. Linear regression analysis showed no effect of regulation of PEPCK by GR (PS,  $r^2 = 0.056$ ,  $P = 0.22$ , PR  $r^2 = 0.042$ ,  $P = 0.347$ ).

|                    | GR      | PEPCK | G6Pase | PFK-2   | PPAR $\alpha$ | CPT-1   |
|--------------------|---------|-------|--------|---------|---------------|---------|
| Generation         | <0.0001 | 0.001 | NS     | <0.0001 | NS            | <0.0001 |
| F0 diet            | NS      | NS    | NS     | <0.0001 | NS            | NS      |
| Generation*F0 diet | 0.001   | NS    | NS     | <0.0001 | NS            | NS      |

**Table 4.2 A General Linear Model showing significant interactive effects of generation and F0 diet on mRNA expression.**

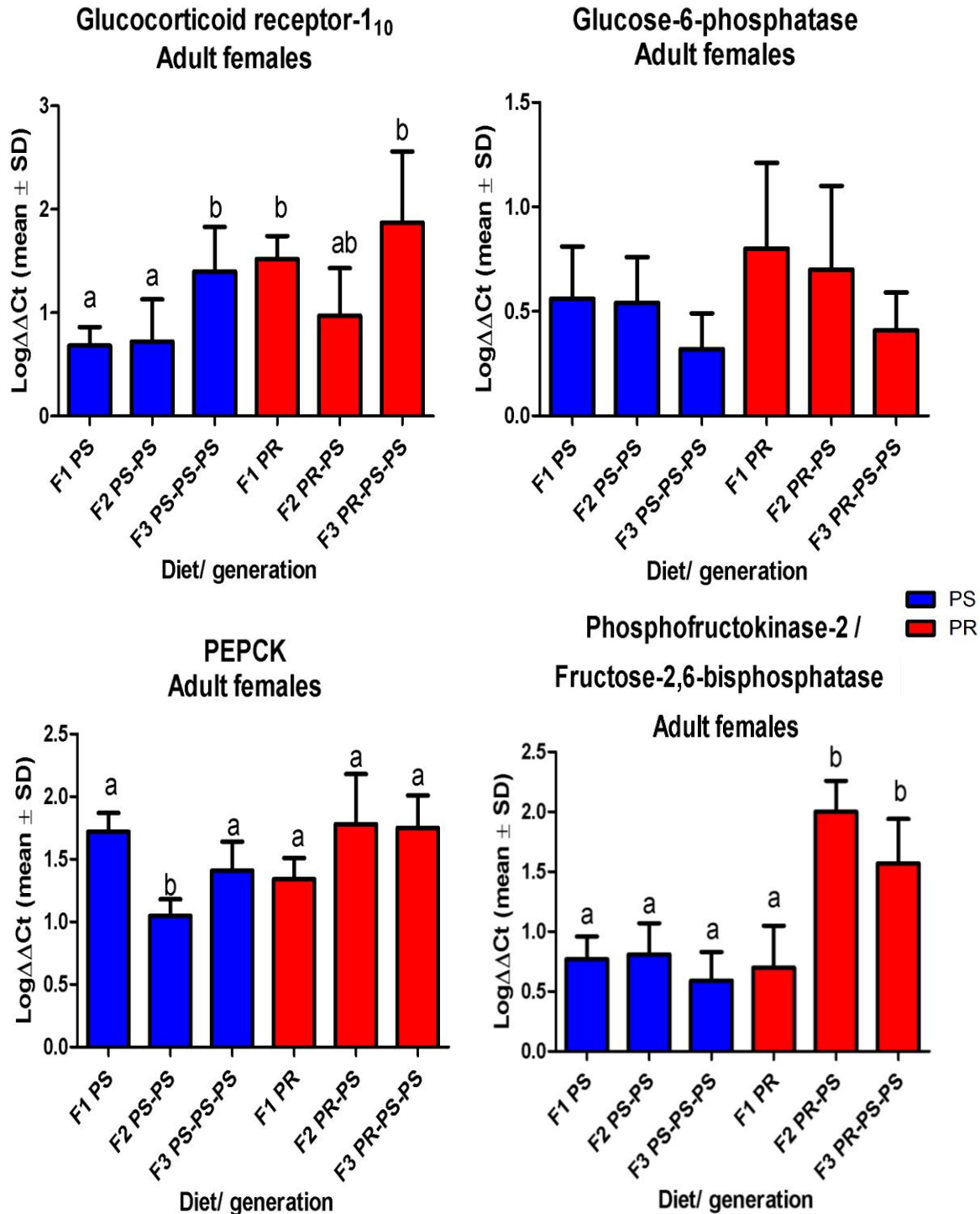


**Figure 4.5 Hepatic GR<sub>10</sub>, Glucose-6-Phosphatase, PEPCK and PFK-2/F-2,6-BP mRNA expression in adult females in the F1, F2 and F3 generations.** Values are means with their standard deviations, where 'a' is significantly different from 'b', and 'ab' is not significant from 'a' or 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

#### **4.3.5 Does energy content of diet and protein content of the F0 diet during pregnancy alter expression of genes involved in fatty acid $\beta$ -oxidation?**

As shown in table 4.2, there were no significant effects of generation or F0 diet on expression of PPAR $\alpha$  in non-pregnant adult female offspring (Figure 4.6). There was a significant effect of generation on CPT-1 expression ( $P < 0.0001$ ), where the F3 offspring had significantly higher CPT-1 expression, compared to all other generations, irrespective of diet (Figure 4.6). There was a significant association between GR mRNA expression and mRNA levels of PPAR $\alpha$  (PS  $r^2 = 0.438$ ,  $P = 0.002$ , PR  $r^2 = 0.587$ ,  $P < 0.001$ ) and CPT-1 (PS  $r^2 = 0.239$ ,  $p = 0.034$ , PR  $r^2 = 0.2177$ ,  $P = 0.025$ ).



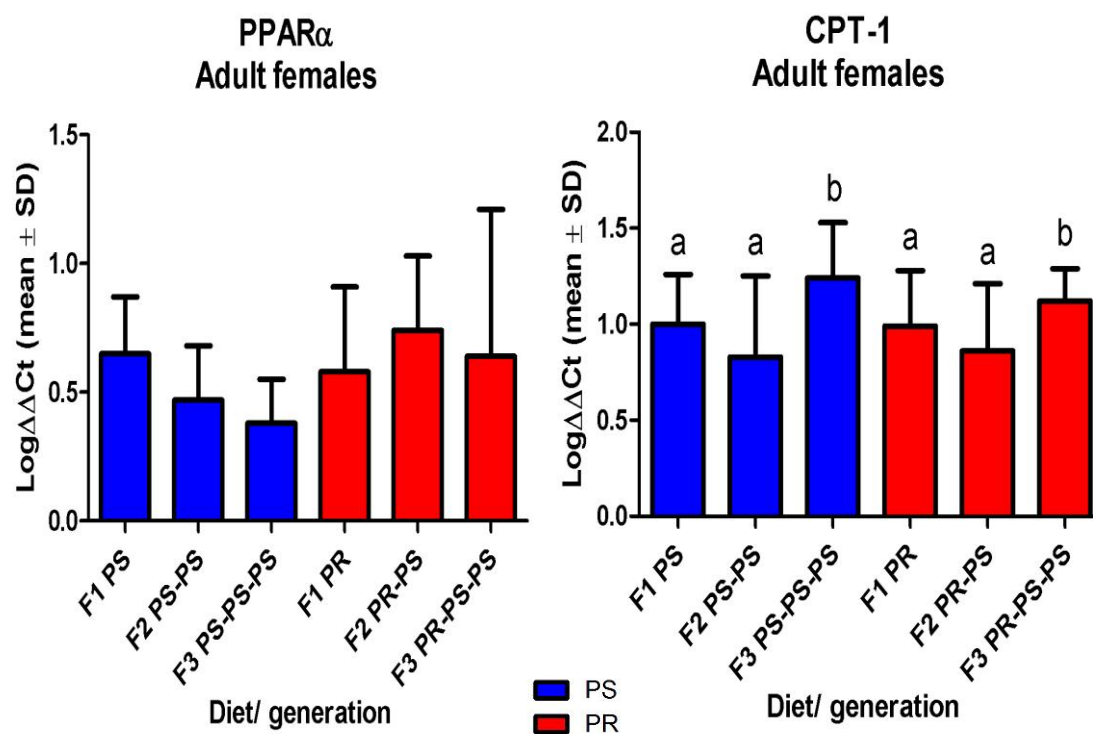


**Figure 4.6 Hepatic PPARα and CPT-1 mRNA expression in adult females in the F1, F2 and F3 generations.** Values are means with their standard deviations, where 'a' is significantly different from 'b'. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

## 4.4 Discussion

### 4.4.1 Non-pregnant offspring phenotype

At every time point, all PR offspring had significantly lower body weights than PS offspring. This implies transmission of a persistent influence of the F0 over three subsequent generations. This may be due to either decreased somatic growth, lower partitioning of dietary energy into adipose tissue or greater physical activity. The metabolic response to fasting in female non-pregnant adult offspring was determined by measuring plasma glucose and  $\beta$ -hydroxybutyrate concentrations. In an adult non-pregnant female rat that received no dietary modification, the fasting plasma glucose concentration is between 4 and 6 mmol/l<sup>[253,254,48,173]</sup>. Total energy and protein content of the F0 diet during pregnancy had significant effects on fasting plasma glucose metabolism. The values are higher than 4 – 6 mmol/l<sup>[254,259]</sup>, the value seen in an adult female rat that received no dietary alteration, in all groups, except for the F3 PR-PS-PS group. There was no significant effect of energy or F0 pregnancy diet protein content on fasting plasma  $\beta$ -hydroxybutyrate concentration, although all offspring had plasma levels higher than that of a standard adult non pregnant female rat that received no dietary modification in any previous generation<sup>[254,259]</sup>. These data suggest that there was an increased level of gluconeogenesis in all offspring, except for the F3 PR-PS-PS group, accompanied by an increase in ketogenesis. One possible explanation for the high fasting glucose concentration is that the constant high energy diet induced an insulin resistant or diabetes-like phenotype.

Circulating plasma levels of corticosterone were shown to be affected by the dietary alterations. F2 PR-PS and F3 dams from both protein lineages had significantly higher plasma corticosterone levels. The F2 PS-PS and F1 groups had fasting plasma corticosterone levels comparable to that shown by Hanson et al<sup>[231]</sup>. An increase in plasma corticosterone would lead to increased expression of GR, and therefore PEPCK. As a result gluconeogenic activity would be increased and plasma glucose levels would be increased. Although fasting plasma corticosterone levels do not agree with plasma glucose levels in the offspring, the role of corticosterone in this phenotype cannot be ruled out. It may be that altered

regulation of the PEPCK gene, such as methylation of specific CpG sites, is preventing an increase in PEPCK expression by corticosterone through inhibition of transcription factor binding.

Generation, but not diet, had a significant effect on plasma leptin concentrations. F1 offspring had significantly increased plasma leptin levels compared to both F2 and F3 offspring. The pattern of changes in plasma leptin concentration did not correspond with the offspring body weight or to food intake. This suggests that leptin secretion and possibly leptin action were dysregulated in all generations, although these changes did not appear to have any effect on the physiology of the offspring.

In summary, the first two generations of offspring exposed to the high energy diet have an increased level of gluconeogenesis and  $\beta$ -oxidation during fasting. The F3 generation, showed a fasting phenotype similar to an animal that received no dietary alteration, suggesting adjustment to the high energy diet. In a similar pattern to the day 8.5 dams, the offspring's adjustment to the high energy diet seems to be associated with an increase in fasting corticosterone concentration, possibly suggesting increased stress during fasting.

#### **4.4.2 mRNA expression of genes involved in fasting energy metabolism in non-pregnant offspring**

The results showed significant changes in expression of key genes involved in fasting glucose and fatty acid metabolism, between generations and F0 diets.

Increased expression of PFK-2/F-2,6-BPase is associated with a high glycolytic flux in the fed state<sup>[128]</sup>. Under the physiological state of fasting, increased levels of glucagon decrease glycolysis and increased gluconeogenesis, by down-regulating the PFK-2 and up-regulating the F-2,6-BPase activities. Transgenic mice over-expressing PEPCK were found to be hyperglycaemic, with higher insulin concentrations and alterations in liver glycogen content<sup>[260]</sup>. Furthermore, primary mouse hepatocytes, which over expressed GR, were found to have increased PEPCK mRNA expression and a type II diabetes phenotype<sup>[261]</sup>.

The differences in mRNA expression seen in the F1 PR and F3 groups of both protein lineages would suggest that these animals have increased levels of gluconeogenesis. If this was the case, then an increased fasting plasma glucose concentration would be expected to be present in these groups compared to all other groups. The pattern of expression of the genes involved in glucose metabolism measured and the fasting plasma glucose values do not correlate completely. This could be due to the involvement of other proteins in the pathway of fasting glucose metabolism, or the protein levels of the measured genes do not match the expression levels. Furthermore, plasma glucose concentration also reflects the rate of glucose uptake. Thus any changes in mRNA expression of gluconeogenic genes may be, at least in part, compensated by alterations in glucose uptake.

Increased mRNA expression of CPT-1 is associated with exaggerated fatty acid  $\beta$ -oxidation and decreased oxidation of glucose<sup>[262]</sup>. Because CPT-1 expression was increased, this would be expected to be accompanied by higher fasting plasma  $\beta$ -hydroxybutyrate concentration in the F3 generation compared to both the F1 and F2 offspring. There is no simple agreement between fasting  $\beta$ -hydroxybutyrate values and mRNA expression of genes involved in fasting fatty acid metabolism. This could be due to the involvement of other enzymes in the pathway of fatty acid oxidation or because the increased gene expression of CPT-1 has not resulted in increased protein levels. Although linear regression analysis showed no effect of regulation of PEPCK by GR, there was a significant effect of GR mRNA expression on mRNA levels of both PPAR $\alpha$  and CPT-1. This suggests the expression levels of these genes are in part a result of increased GR expression.

#### **4.4.3 The relationship between maternal and offspring phenotype**

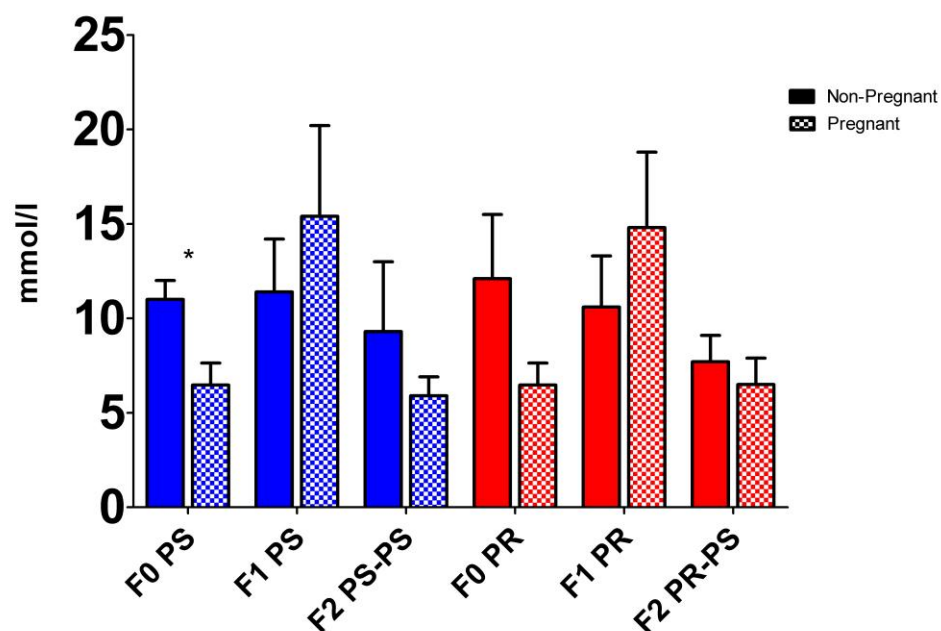
Comparison of the phenotype of dams and offspring allows two questions to be addressed. By comparing non-pregnant and pregnant offspring in the same generation, it is possible to determine whether there was a specific effect of pregnancy on the dietary challenge on metabolism, as a result of fasting. In

addition, comparison of the phenotype of the mother and her offspring may answer the question does the phenotype of the offspring reflect that of the mother.

#### **4.4.4 The effect of pregnancy on fasting metabolite and hormone concentration**

Comparison of litter mates, one of which was mated and the other which was non-pregnant, allows the observation of the effect of pregnancy on fasting energy metabolism and hormone concentration. Pregnancy led to significant differences in fasting plasma glucose values in the F0 generation of the PS lineage (Figure 4.7). This suggests that the pregnancy leads to alterations in fasting glucose. As these differences are not seen in later generations, it appears that the nutritional alteration, of high energy, is sufficient to cover up the effects of pregnancy on fasting glucose levels. Although the effect of pregnancy was not seen in the F0 generation in the PR lineages, this appears to be due to the variability in the F0 PR pregnant group. Pregnancy led to a significantly higher plasma  $\beta$ -hydroxybutyrate level in the F1 PS animals, as shown in Figure 4.7. As this effect was not seen in the F0 generation, or in the PR lineage, it suggests an interactive effect of the generation and F0 maternal diet protein intake. All day 8.5 pregnancy groups have an increased plasma corticosterone level compared to their non-pregnant offspring, as shown in Figure 4.8. Fasting glucagon levels show an identical pattern to glucose, where the effect of pregnancy was seen in the F0 PS group only. Similar fasting leptin concentrations were present for all groups, regardless of pregnancy, shown in Figure 4.8. These results show that for fasting glucose,  $\beta$ -hydroxybutyrate, corticosterone and glucagon concentration, the added physiological stress of pregnancy is sufficient to observe the effects of the dietary alterations. However for fasting leptin concentration the additional stress of pregnancy did not alter the fasting phenotype.

### Plasma Glucose Adult Females



### Plasma $\beta$ -Hydroxybutyrate Adult Females

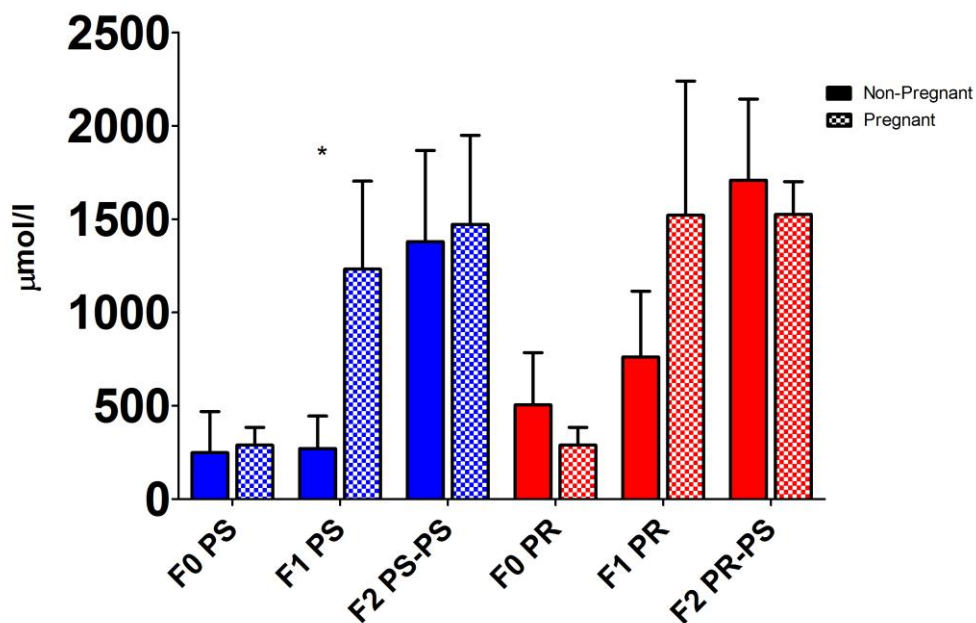


**Figure 4.7 Plasma glucose concentrations in pregnant and non-pregnant adult siblings, females in the F0, F1 and F2 generations.** Values are means with their standard deviations. Significant differences between diets and generation were determined by T-Test.  $P < 0.05$ . \* represents an effect of pregnancy.

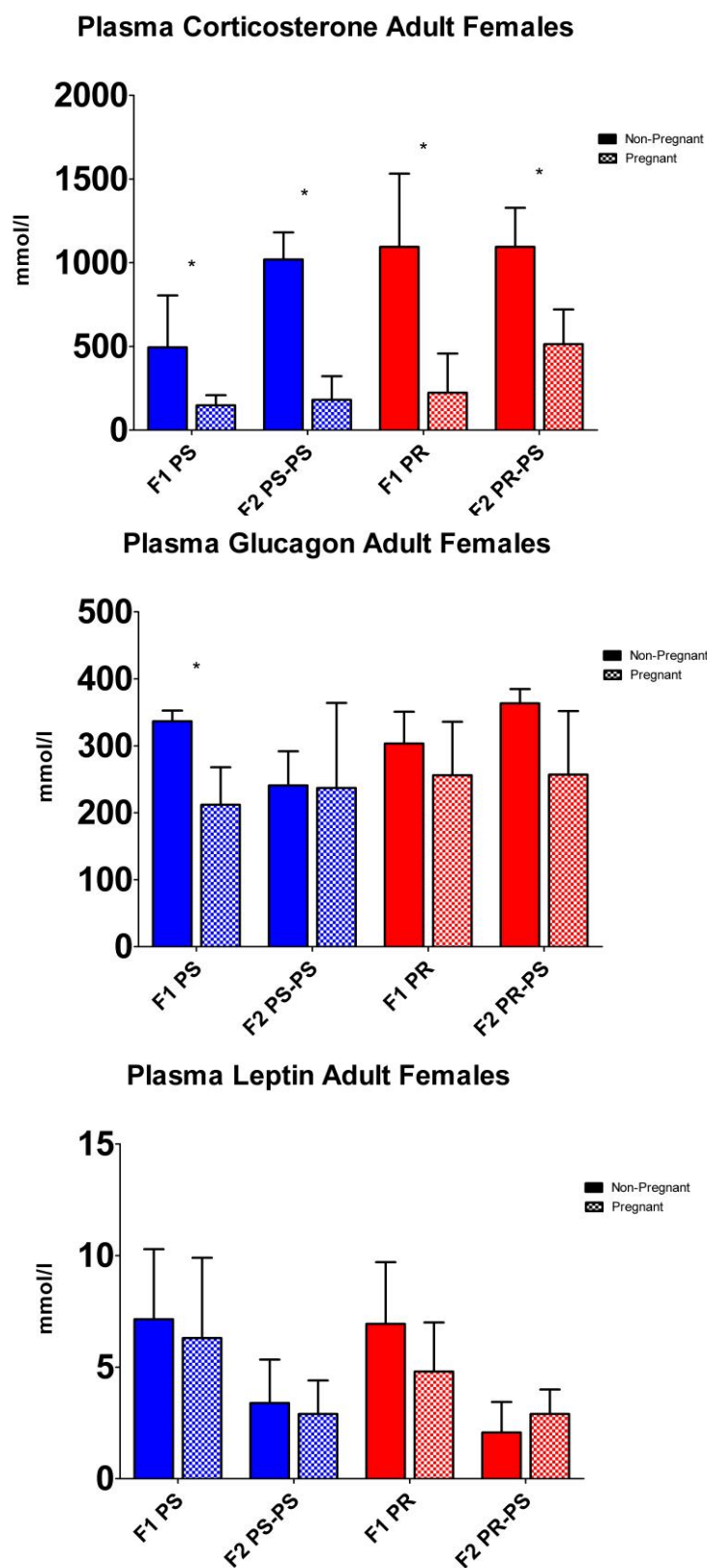


**Figure 4.8 Plasma corticosterone, glucagon and leptin concentrations in pregnant and non-pregnant adult siblings, females in the F0, F1 and F2 generations.** Values are means with their standard deviations. Significant differences between diets and generation were determined by T-Test.  $P < 0.05$ . \* represents an effect of pregnancy.

#### **4.4.5 The relationship between maternal and offspring phenotype**

By comparing data from dams and their offspring, the extent to which the offspring phenotype is based on that of the dams, as suggested in the PAR hypothesis, can be investigated. Alterations in the metabolism of the dam during pregnancy lead to significant changes in the physiological phenotype of the non-pregnant female offspring at day 70. Fasting plasma glucose concentrations were comparable for the F1 offspring (F0 dams), and F2 PS-PS-PS offspring (F1 PS-PS dams), whereas all other groups showed significantly different levels (Figure 4.9). Day 8.5 pregnant dams showed a trend in increased fasting plasma glucose concentration between the F0 and F1 generation; this was not seen in the F1 and F2 generation adult non-pregnant offspring. Unlike their respective mothers, the F3 PS-PS-PS group did not show a fasting glucose level comparable to that of a non-pregnant adult control [253,254,48,173]. This indicates that although their mother's metabolism is adapting to the dietary alterations the F3 PS-PS-PS group have not. This is due to the protein content of the F0 diet during pregnancy (Figure 4.3).  $\beta$ -hydroxybutyrate levels in adult non-pregnant offspring showed no agreement with fasting levels present in dams during pregnancy. In terms of the PAR hypothesis, selected offspring groups match the phenotype of their respective mothers, however this is not seen in all groups, suggesting the PAR hypothesis is insufficient to explain all differences in phenotype.



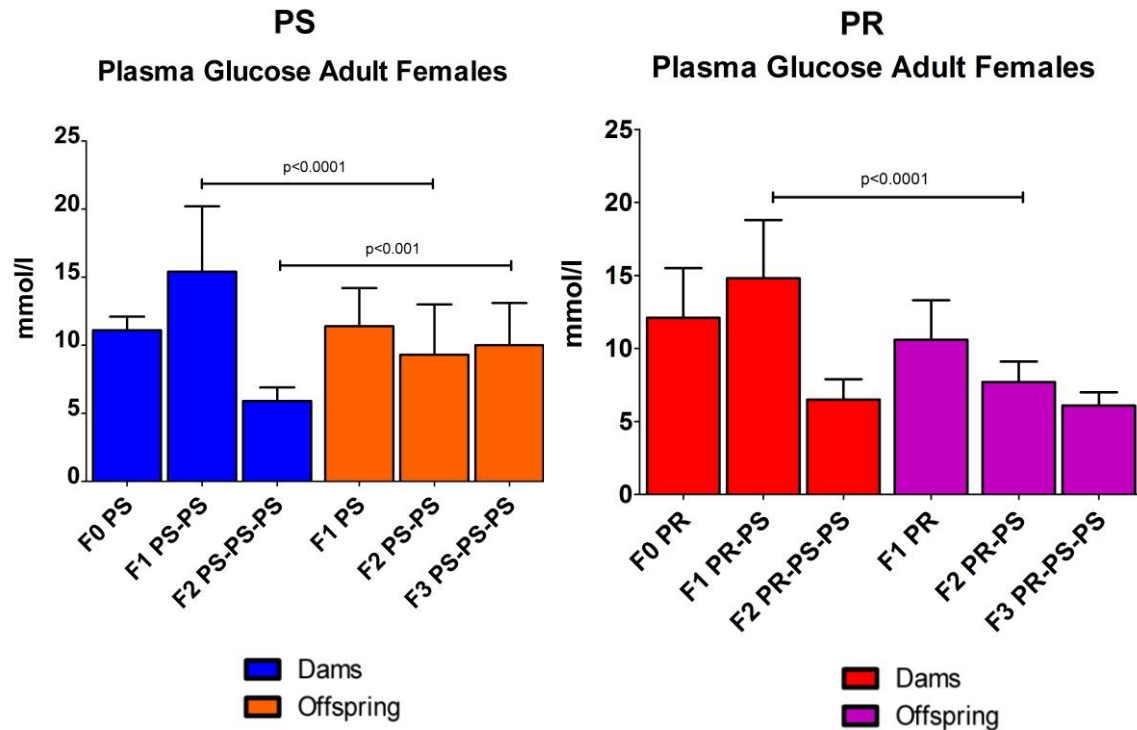


**Figure 4.9 Plasma glucose concentrations in day 8.5 pregnancy in the F0, F1 and F2 generations, and their relative offspring, in both the PS and PR lineages.** Where blue represents pregnant PS, orange represents non-pregnant PS, red represents pregnant PR and purple represents non-pregnant PR. Values are means with their standard deviations. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

In summary, fasting plasma glucose values are increased in the F0 and F1 generation, in dams. Inversely plasma  $\beta$ -hydroxybutyrate values are increased in the F2 generation only. In the F2 generation, where the fasting plasma glucose values are no longer increased, the corticosterone levels are increased, in the PR lineage only. The F2 generation dams show an increased weight gain and energy intake, compared to both the F0 and F1 generations. All offspring groups showed increased fasting plasma glucose and  $\beta$ -hydroxybutyrate concentrations (Figure 4.3), compared to the level seen in a fasting rat that received no dietary alteration, except for the F3 PR group, which had standard glucose values. Fasting corticosterone levels were increased in the F2 PR and both F3 groups, suggesting an increased level of stress in these animals. All PR offspring showed decreased postnatal weight gain, compared to PS groups, in all generations. The postnatal

energy intake, however, was increased in the F2 generation, compared to both the F1 and F3 generations.

Similar plasma concentrations of glucagon and leptin are seen between mother and offspring. In terms of corticosterone, the offspring show a similar pattern of increased fasting corticosterone in the F3 generation, which was also seen in their mothers (F2 generation dams). The increased plasma corticosterone concentration seen in the F1 PR-PS dams was also present in the F2 PR-PS adult offspring (Figure 4.10). Although all values were increased in the dams, possibly representing the additional stress of pregnancy, the pattern of fasting corticosterone concentration in the adult offspring is similar to that seen in their mothers. The results suggest that the role of corticosterone in the fasting response in the offspring showed a similar response to that of its mother during pregnancy, and not that of the first generation exposed to the dietary alteration in utero.

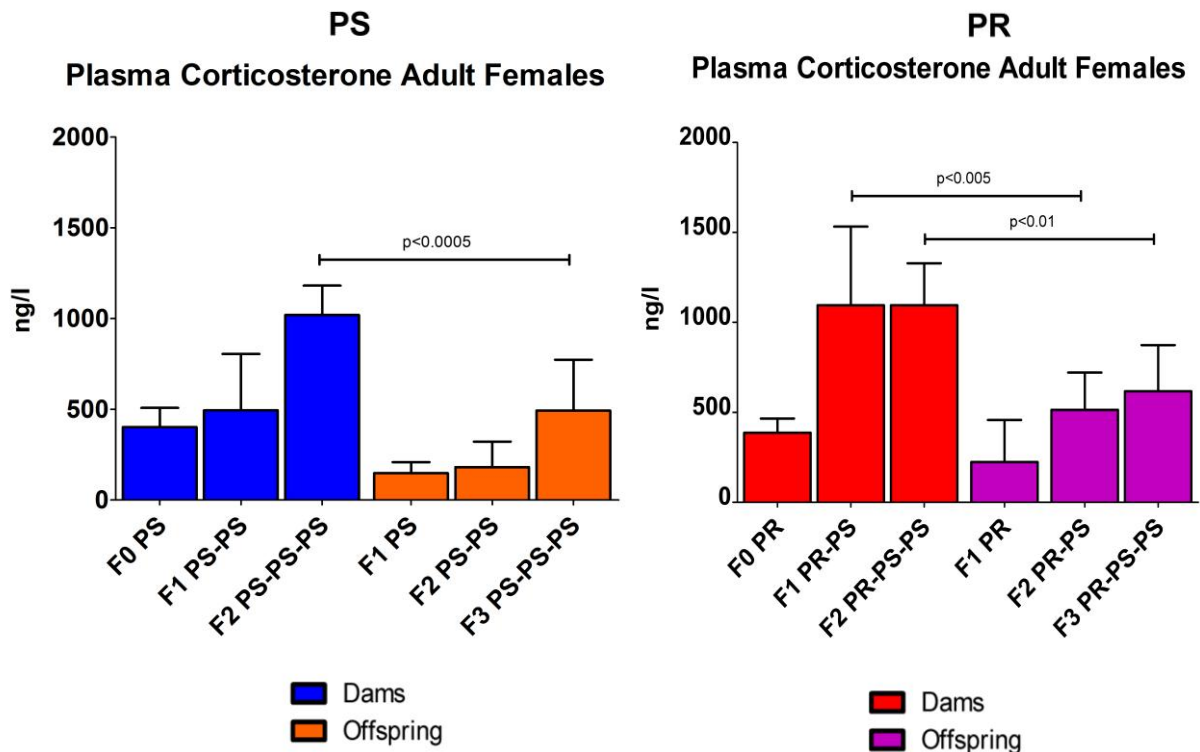


**Figure 4.10 Plasma corticosterone concentrations in day 8.5 pregnancies in the F0, F1 and F2 generations, and their relevant offspring.** Where blue represents pregnant PS, orange represents non-pregnant PS, red represents pregnant PR and purple represents non-pregnant PR. Values are means with their standard deviations. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$

There are differences in fasting metabolites and weight gain between F0 pregnancy diet protein intake lineages for each generation, providing evidence of a transgenerational effect on phenotype. In terms of the core hypothesis, the results confirm that increased energy intake induces an altered phenotype in the offspring, which varies between generations and with the nutrient composition of the F0 diet. As described previously, gene expression in offspring has been shown to be altered following maternal dietary changes during pregnancy <sup>[174,45,187]</sup>. It is now important to determine the expression of genes involved in the pathways of fasting glucose and fatty acid metabolism in the non-pregnant adult offspring, in order to observe the effects of the added stress of pregnancy in fasted adult female rats, in subsequent generations.

According to the PAR hypothesis<sup>[92,93]</sup>, the metabolism of the developing offspring is based on that of its mothers during pregnancy, as the mother's metabolism will be representative of the external environment. The design of the study entails that the nutritional exposure of the offspring was comparable to that of the mothers. In terms of the PAR hypothesis, a match in the developing environment and later life external environment is present; therefore, one would expect the metabolism of the offspring to match that of its mother. Although not all results shown in this section match the expression levels of the day 8.5 dams, there are similarities in the pattern of expression across groups. F1 PR-PS and F2 PS-PS-PS day 8.5 dams and their respective offspring (F2 PR-PS and F3 PS-PS-PS) show similar increases in expression of the genes GR, PEPCK, PFK-2 and CPT-1. This can be seen in terms of PEPCK (Figure 4.11). In terms of the PAR hypothesis, we would expect all gene expression to be similar, this means that the PAR hypothesis does not account for all gene expression levels in offspring.

The primary hypothesis suggests that the fasting phenotype of adult-non pregnant offspring is associated with changes in gene expression, from the data shown it is clear that this is the true. It is now important to determine the mechanisms behind this alteration in mRNA expression between generations.

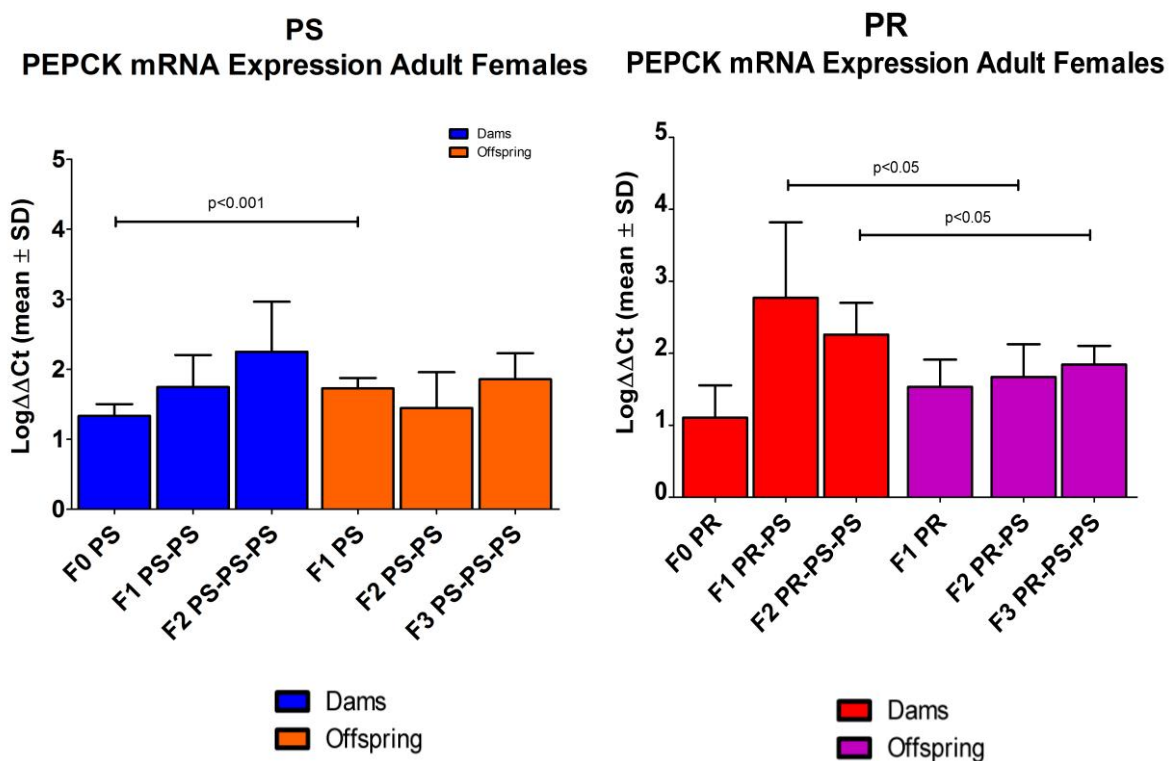


**Figure 4.11 Hepatic PEPCK mRNA expression in day 8.5 pregnancy in the F0, F1 and F2 generations, and their relative offspring.** Where blue represents pregnant PS, orange represents non-pregnant PS, red represents pregnant PR and purple represents non-pregnant PR. Values are means with their standard deviations. Significant differences between diets and generation were determined by two-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$



## Chapter 5

# The Effect of Altered Dietary Energy Content and Protein Content of Maternal Diet on DNA Methylation of the PEPCK and DNMT3a2 Promoters in Dams and Offspring

## 5.1 Introduction

Transition onto a high energy diet led to an altered fasting metabolism in pregnancy across three generations. This resulted in altered regulation of fasting metabolism in offspring, up to the F3 generation. The trajectory of the altered phenotype in both dams and offspring was dependent on the protein content of the maternal diet during pregnancy. Differences in fasting energy homeostasis were associated with significant differences in mRNA of specific genes involved in fasting glucose and fatty acid metabolism, GR, PEPCK, Fru-2,6-BPase, Glc-6-P, PPAR $\alpha$  and CPT-1. One potential mechanism behind the alterations in gene expression and therefore fasting phenotype is epigenetics.

### 5.1.1 The role of DNA methylation in altered phenotype

As discussed in section 1.4, epigenetic processes have the potential to alter expression of genes through the direct or indirect inhibition of transcription. Insulin gene promoter activity was decreased by 90%, in pancreatic  $\beta$ -cells, through increased methylation of the CRE<sup>[193]</sup>. Through Sequenom Mass ARRAY analysis an association was found between methylation status at birth and obesity in childhood<sup>[263]</sup>. Neonatal methylation of the Retinoid X Receptor  $\alpha$  (RXRA) and endothelial nitric oxide synthase (eNos) promoters accounted for over 25% of the variance shown in obesity at age 6 (P=0.009 and 0.002, respectively).

The link between epigenetic marks and an altered phenotype has been shown previously, therefore, it could provide one mechanism for the differences in phenotype seen in the animals of this study.

If DNA methylation is significantly altered in the non-pregnant adult offspring and day 8.5 pregnant dams of the study, as a result of altered nutritional intake, it is not known if these marks will be transmitted between generations unaltered, or if they are established *de novo*.

The methylation status of the PEPCK and DNMT3a2 promoters were measured in both day 8.5 dams and non-pregnant adult females. In order to test the association between promoter methylation and mRNA expression, DNMT3a2 gene expression will be measured in both the day 8.5 dams and non-pregnant adult offspring groups, using RT-PCR.

## **5.2 Experimental Design**

All day 8.5 pregnant dams and non-pregnant adult female offspring samples were from the transgenerational feeding study (Section 2.2).

### **5.2.2 Molecular biology procedures**

Extraction of RNA and RT-PCR methods are described in Sections 2.3.1 to 2.3.3. Extraction of DNA bisulphite treatment of DNA, PCR conditions and pyrosequencing methods are described in Sections 2.3.4 to 2.3.8.



### 5.3 Results

#### 5.3.1 Does energy and protein content of the F0 diet during pregnancy alter methylation of the PEPCK promoter in adult offspring?

There were significant effects of generation on PEPCK promoter methylation at CpGs -508 and -100bp, significant effects of protein content of the F0 maternal diet during pregnancy on methylation at CpGs -248, -129, -100, -90 and -81bp and a significant interactive effect of generation and F0 diet on methylation at CpG -100bp (Figure 5.1). At CpG -508, in the PS lineage, methylation was significantly higher in the F2 and F3 generation, compared to the F1 generation ( $P < 0.005$ ). Methylation at CpG -248 was significantly higher in the F1 generation of the PS lineage, compared to all other groups, except for the F1 PR group; there were no significant differences within the PR lineage. Methylation of CpG -129, in the PS lineage was significantly higher in the F1 generation, compared to both the F2 and F3 generations, in the PR lineage methylation was significantly lower in the F3 generation, compared to both the F1 and F2 generations. At CpG -100, methylation was significantly higher in the F1 generation, regardless of F0 diet, compared to the F2 and F3 generations, in the PR lineage only, methylation in the F3 generation was higher than the F2 generation. In the PS lineage, methylation at CpG -90 was significantly higher in the F1 generation, compared to both the F2 and F3 generation, in the PR lineage, methylation in the F2 generation was significantly higher than both the F1 and F3 generations. Methylation of CpG -81 was significantly lower in the F3 generation of the PS lineage, compared to both the F1 and F2 generations. Table 5.1 shows the interactive effects of generation and F0 diet on PEPCK methylation in adult non-pregnant female offspring.

|               | CpG<br>-606 | CpG<br>-508 | CpG<br>-440 | CpG<br>-248 | CpG<br>-218 | CpG<br>-129 | CpG<br>-100 | CpG<br>-90 | CpG<br>-81 |
|---------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|------------|------------|
| Generation    | NS          | 0.012       | NS          | NS          | NS          | NS          | 0.076       | NS         | NS         |
| F0 diet       | NS          | NS          | NS          | 0.015       | NS          | <0.001      | <0.001      | <0.001     | 0.004      |
| Gen * F0 diet | NS          | 0.057       | NS          | NS          | NS          | NS          | 0.095       | 0.034      | NS         |

**Table 5.1 A General Linear Model showing significant interactive effects of generation and F0 diet on PEPCK promoter methylation in non-pregnant adult female offspring**

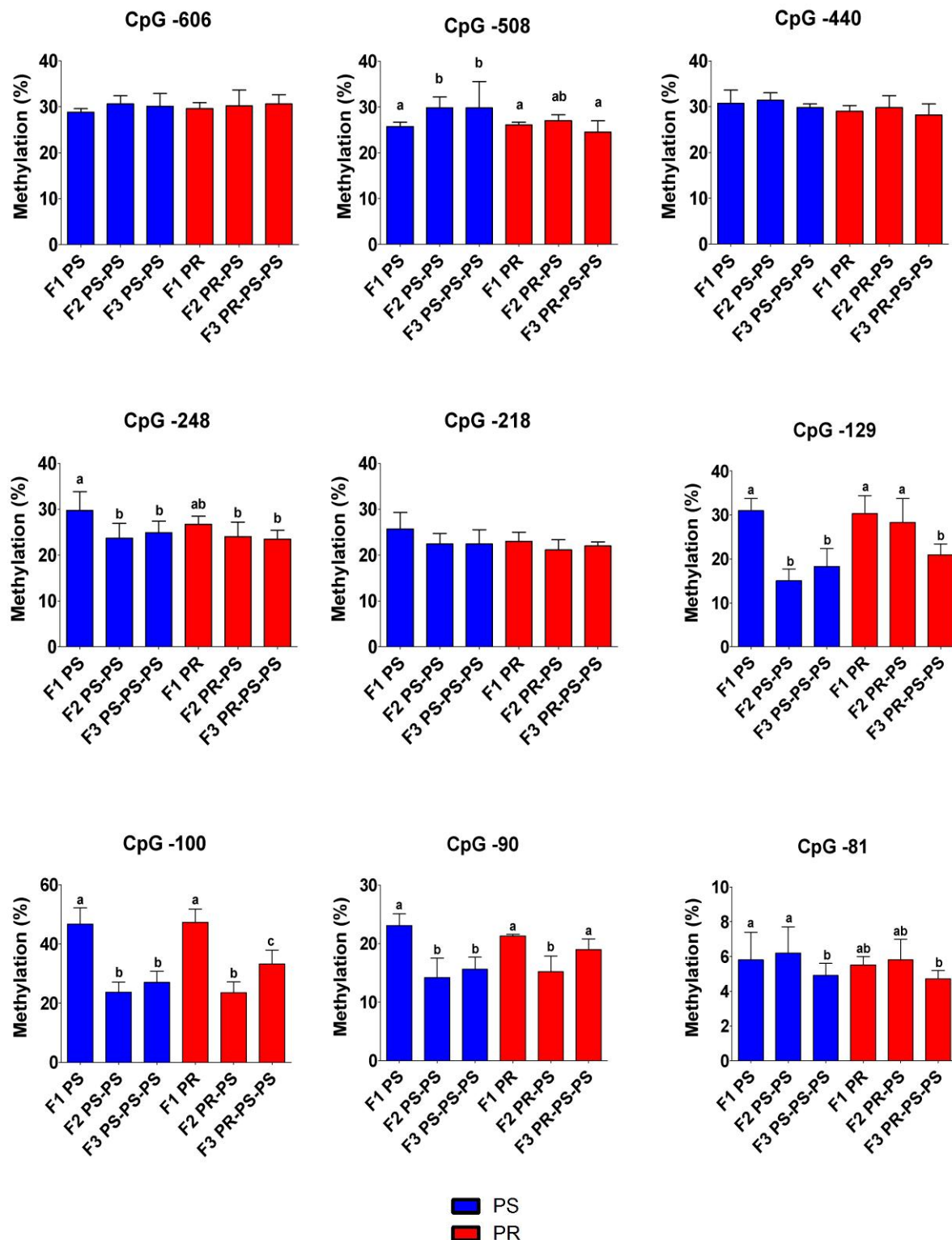


**Figure 5.1 PEPCK promoter methylation in non-pregnant adult female offspring.** Values are means with their standard deviations, where 'a' is significantly different from 'b', and 'ab' is neither significantly different to 'a' or 'b'. Significant differences were by two-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.005$ .

### 5.3.2 Does the energy content of diet and F0 pregnancy diet protein content alter methylation of the PEPCK promoter in pregnancy?

There was a significant effect of generation on PEPCK methylation in day 8.5 dams. Methylation of CpG -129 was significantly lower in the F1 generation compared to the F0 generation, in the PR lineage only (Figure 5.2) There were no further effects of generation on PEPCK methylation. There were no significant effects of diet on PEPCK methylation, at any measured CpG, in day 8.5 dams. Table 5.2 shows the interactive effects of generation and F0 diet on PEPCK methylation in day 8.5 dams.

|               | CpG<br>-606 | CpG<br>-508 | CpG<br>-440 | CpG<br>-129 | CpG<br>-90 | CpG<br>-81 |
|---------------|-------------|-------------|-------------|-------------|------------|------------|
| Generation    | 0.057       | NS          | NS          | 0.031       | NS         | NS         |
| F0 diet       | NS          | NS          | NS          | NS          | NS         | NS         |
| Age * F0 diet | NS          | NS          | NS          | NS          | NS         | NS         |

**Table 5.2 A General Linear Model showing significant interactive effects of generation and F0 diet on PEPCK promoter methylation in day 8.5 dams**

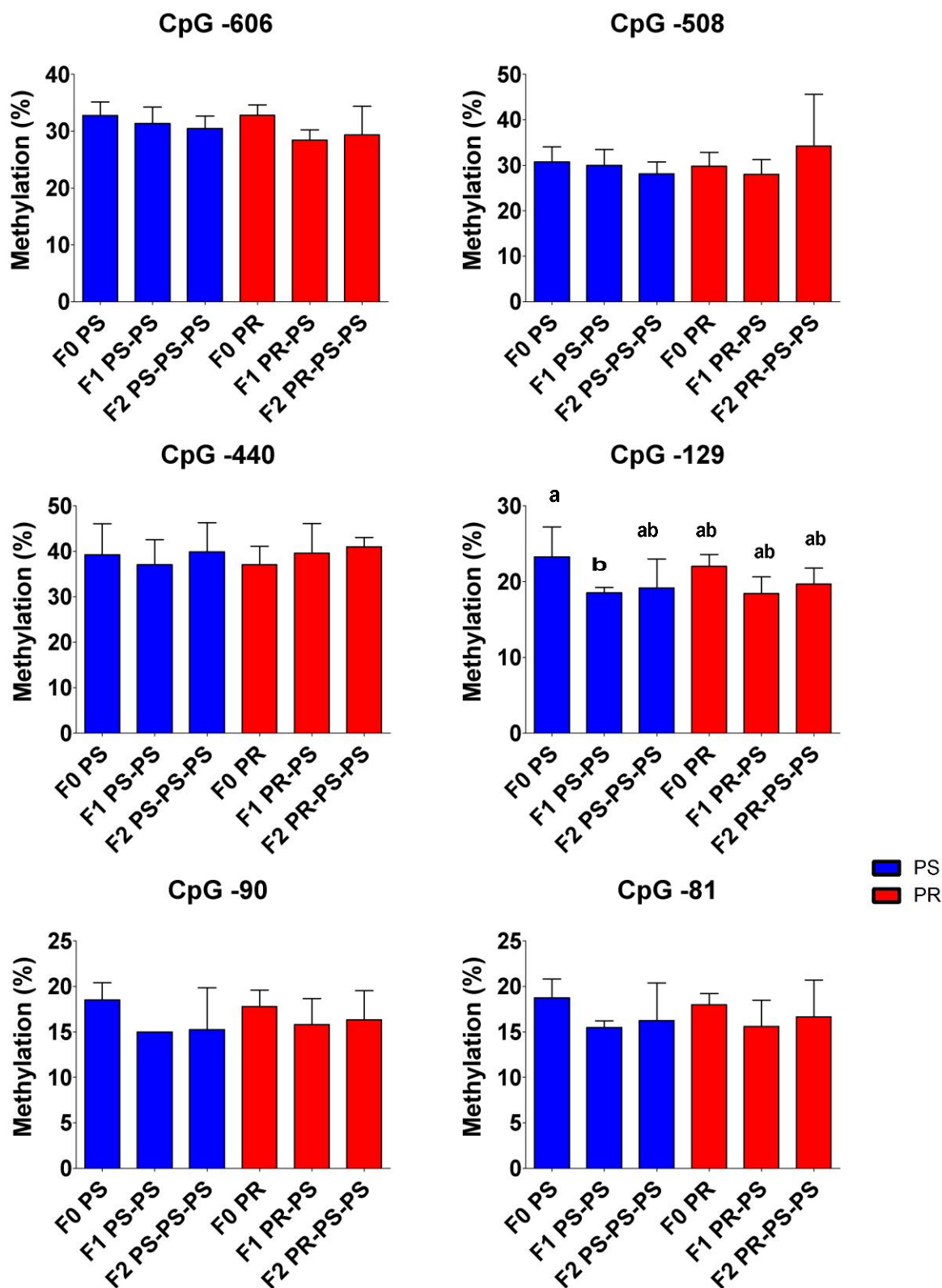


**Figure 5.2 PEPCK promoter methylation in day 8.5 pregnant dams.** Values are means with their standard deviations, where 'a' is significantly different from 'b'. Significant differences were by two-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.005$ .

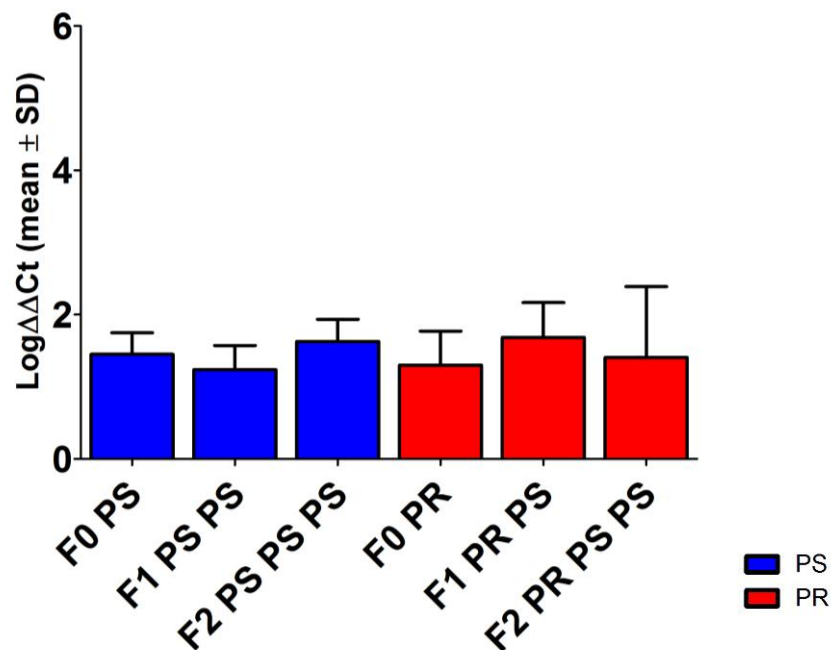
### 5.3.3 Does the energy content of diet and F0 pregnancy diet protein content alter hepatic expression of DNMT3a2 in day8.5 pregnant dams or non-pregnant adult offspring?

There was no effect of generation or diet on DNMT3a2 expression in either day 8.5 pregnant dams or non-pregnant adult female offspring (Figure 5.3). Table 5.3 shows the interactive effects of generation and F0 diet on DNMT3a2 expression. As shown in Table 5.3 there was no significant effects.

|                      | Day 8.5 pregnant | Offspring |
|----------------------|------------------|-----------|
| Generation           | 0.806            | 0.801     |
| F0 diet              | 0.128            | 0.893     |
| Generation * F0 diet | 0.532            | 0.270     |

**Table 5.3 A General Linear Model showing significant interactive effects of generation and F0 diet on DNMT3a mRNA expression in day 8.5 pregnant dams and non-pregnant adult female offspring.**

### DNMT3a2 mRNA expression d8.5 dams



### DNMT3a2 mRNA expression non-pregnant adult female offspring

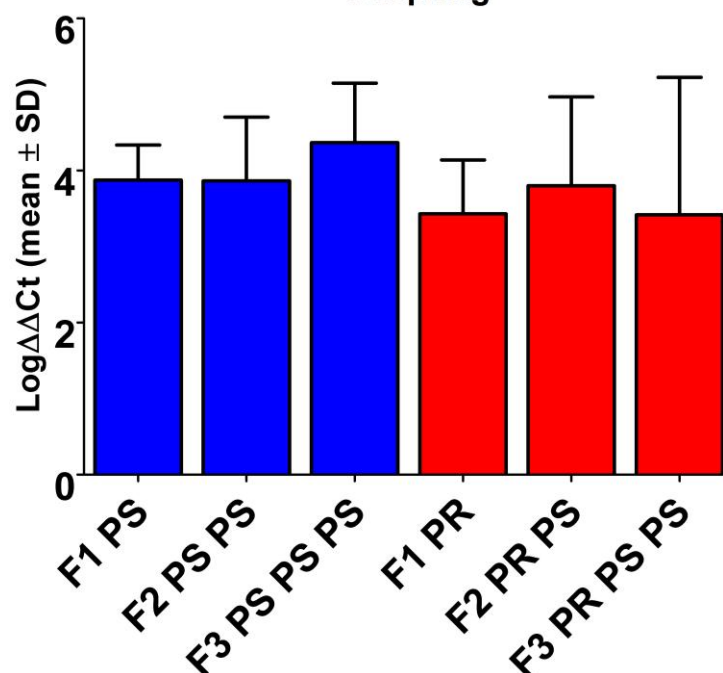


**Figure 5.3 DNMT3a2 mRNA expression in day 8.5 pregnant dams and non-pregnant adult offspring.** Values are means with their standard deviations, where 'a' is significantly different from 'b'. Significant differences were by two-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.005$ .

#### 5.3.4 Does energy and protein content of the F0 diet during pregnancy alter methylation of the DNMT3a2 promoter in adult offspring?

There was a significant effect of both generation and F0 diet on methylation of the DNMT3a2 promoter in non-pregnant adult female offspring ( $P < 0.001$ ) (Figure 5.4). There was a significant interactive effect of generation and F0 diet on methylation at CpGs -56 and -39 ( $P < 0.001$ ). There was no effect of generation or F0 diet on methylation at CpGs -207 and -190. Methylation at both CpG -56 and -39 was significantly higher in the F1 PS group, compared to all other groups. There were no significant effects within the PR lineage for the CpGs. Table 5.4 shows the interactive effects of generation and diet on methylation of the DNMT3a2 promoter in non-pregnant adult-offspring.

|               | CpG<br>-207 | CpG<br>-190 | CpG<br>-56 | CpG<br>-39 |
|---------------|-------------|-------------|------------|------------|
| Generation    | NS          | NS          | <0.001     | <0.001     |
| F0 diet       | NS          | NS          | <0.001     | <0.001     |
| Age * F0 diet | NS          | NS          | <0.001     | <0.001     |

**Table 5.4 A General Linear Model showing significant interactive effects of age and maternal diet on DNMT3a2 promoter methylation in non-pregnant adult female offspring**

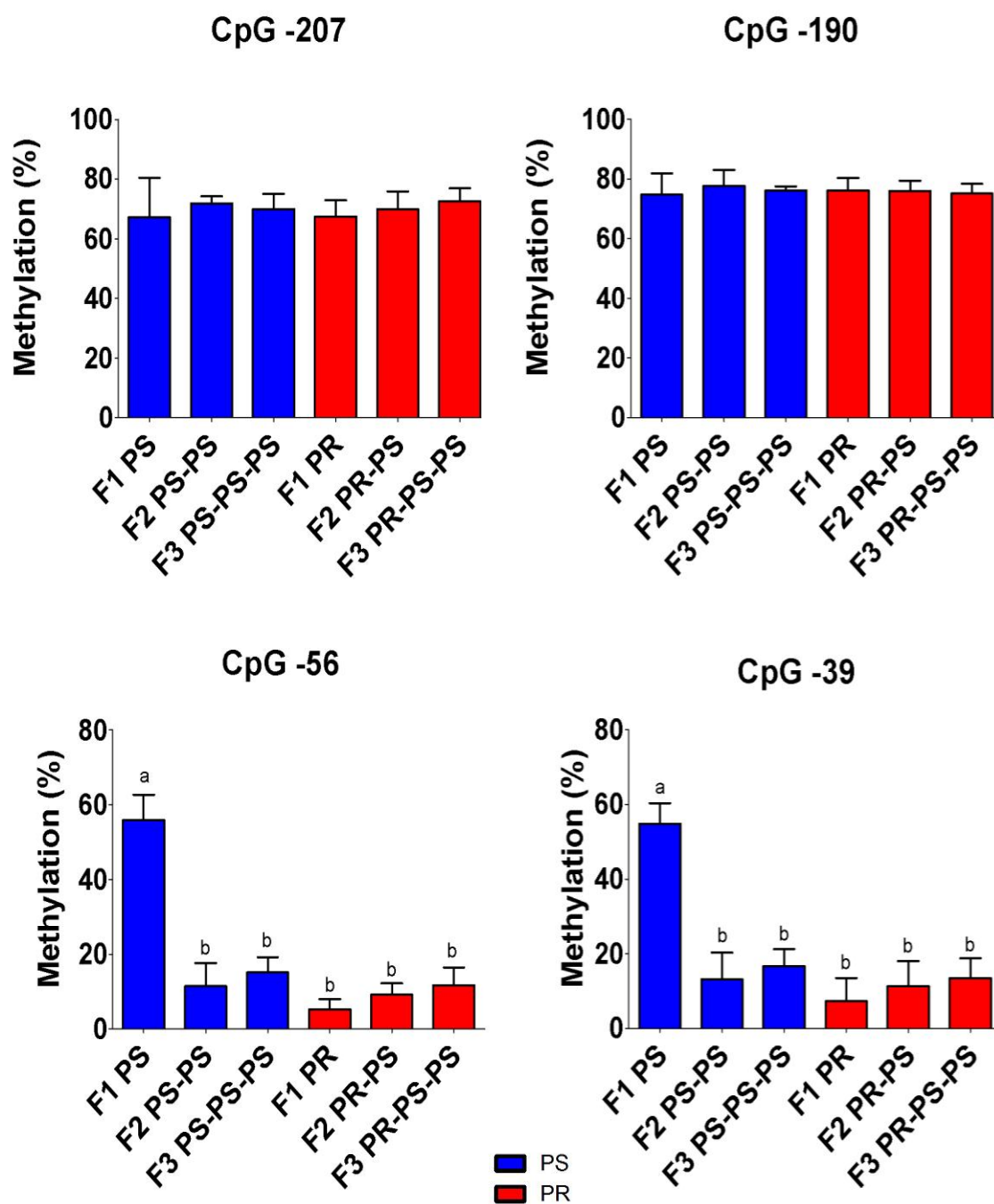


**Figure 5.4 DNMT3a2 promoter methylation in non-pregnant adult female offspring.** Values are means with their standard deviations. Significant differences were by two-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.005$ .



### 5.3.5 Does the energy content of diet and F0 pregnancy diet protein content alter methylation of the DNMT3a2 promoter in pregnancy?

There were no significant effects of energy content of diet or generation on DNMT3a2 promoter methylation, at any CpG, in day 8.5 dams (Figure 5.5). There was a significant interactive effect of generation and F0 diet on methylation at CpGs -56 and -39 (Table 5.5).

|               | CpG<br>-207 | CpG<br>-190 | CpG<br>-56 | CpG<br>-39 |
|---------------|-------------|-------------|------------|------------|
| Generation    | NS          | NS          | NS         | NS         |
| F0 diet       | NS          | NS          | NS         | NS         |
| Age * F0 diet | NS          | NS          | 0.022      | 0.049      |

**Table 5.5 A General Linear Model showing significant interactive effects of age and maternal diet on DNMT3a2 promoter methylation in day 8.5 pregnant dams**

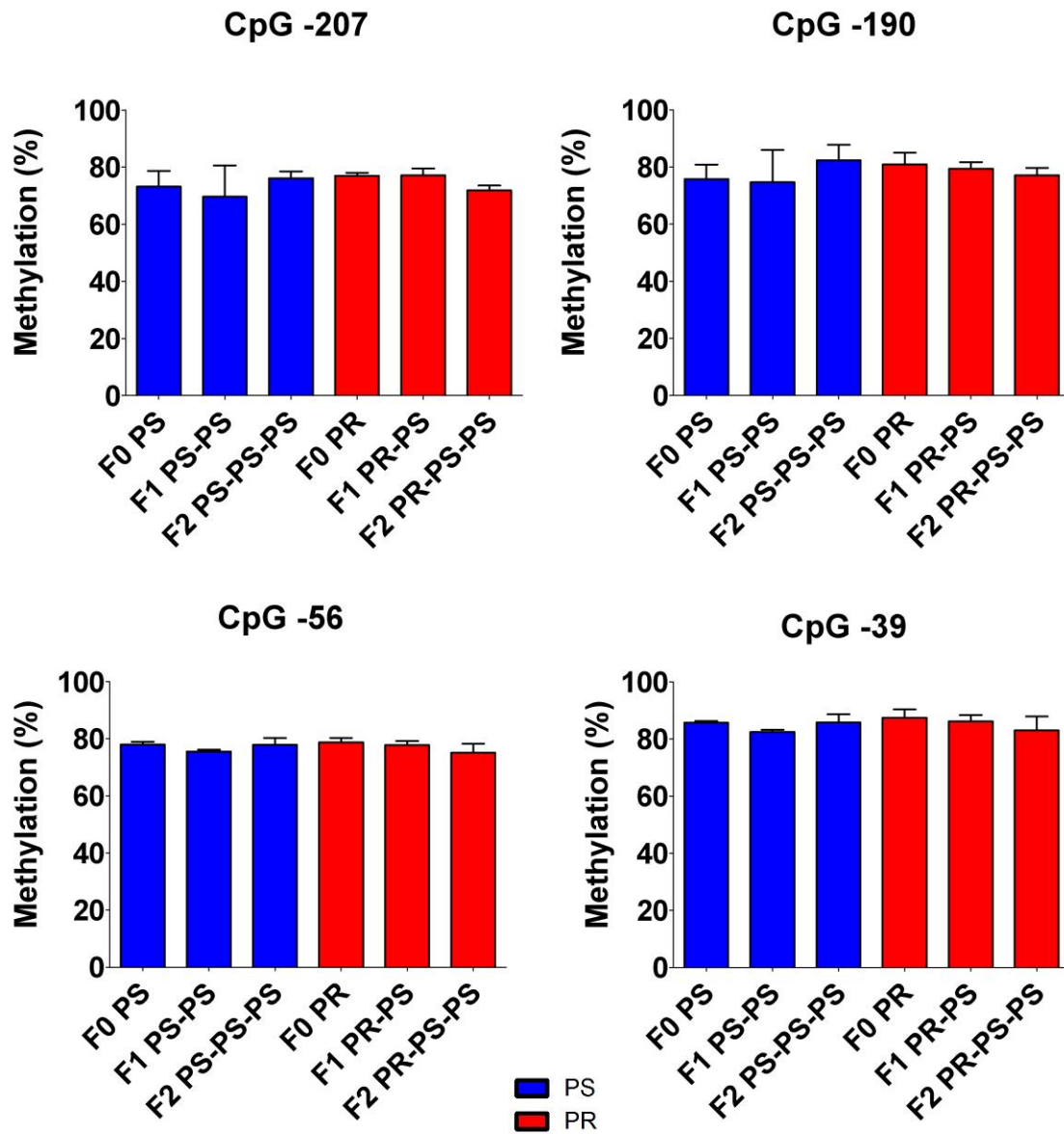


**Figure 5.5 DNMT3a2 promoter methylation in day 8.5 pregnant dams.** Values are means with their standard deviations. Significant differences were by two-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.005$ .

## **5.4 Discussion**

### **5.4.1 PEPCK**

#### **5.4.1.1 CpG specific changes in promoter methylation in non-pregnant adult female offspring**

There was an effect of generation on methylation of CpG-100, where the F1 generation had significantly higher methylation than both the F2 and F3 generations, in both the PS and PR lineages. CpG -129 showed an effect of protein content of the F0 maternal diet during pregnancy, where the PS lineage showed a higher methylation in the F1 group compared to both the F2 and F3 groups, whereas the PR lineage had an higher methylation in both the F1 and F2 groups, compared to the F3 group. According to Skinner's definition of true transgenerational transmission methylation differences present in the F3 generation between the two dietary lines must be heritable, in order to be seen three generations after the initial exposure<sup>[165]</sup>. The importance of measuring methylation of CpGs in a given promoter individually is confirmed when considering this data, where average promoter methylation values may omit interesting findings.

#### **5.4.1.2 The role of promoter methylation in regulation of PEPCK gene expression in offspring**

As stated previously high levels of methylation of the promoter regions of most genes is associated with decreased expression of the gene [196,194,195,197,193,198,187,199,200,201]. Examples include methylation of the Insulin gene promoter in mouse pancreatic cells<sup>[193]</sup> and methylation of the hMLH1 promoter<sup>[194]</sup>. PEPCK expression in the non-pregnant adult female offspring was significantly lower in the F2 PS-PS-PS group, compared to all other groups. In terms of the relationship between methylation and expression of the PEPCK gene, there does not appear to be a clear association. A higher level of methylation is seen in the F2 PS group, compared to the F1 PS group at CpG-508. However at this CpG the F3 PS group also shows a higher level of expression, with no associated decrease in methylation. A common pattern seen in the methylation status of the PEPCK

promoter in the PS lineage is a higher level in CpG specific methylation in the F1 group, compared to both the F2 and F3 groups. This pattern, seen in CpGs -248, -129, -100 and -90, however, is not directly comparable to the pattern of expression seen. Studies by Kuroda and Deng have shown a clear association between levels of promoter methylation and mRNA expression. It may be that under the physiological state of fasting, the effect of the difference in methylation described here remains cryptic. Under alternative situations, whereby PEPCK is regulated by different mechanisms, changes in PEPCK mRNA expression may be present. This could be due to differences in the regulation of the genes studied, where PEPCK is expressed during periods when glucose is low, whereas the insulin gene, measured by Kuroda, is habitually expressed. If genes are expressed under different physiological conditions, it would be expected that the form of regulation and extent to which DNA methylation plays will be significantly different between genes. In order to affect levels of transcription, factors must bind to around the transcription start site. According to Chakravarty the region -1500 to +74 base pairs, relative to the transcription factor start site is necessary and sufficient to direct transcription<sup>[238]</sup>. The most important region, the CRE can be found at -147 to -130 bp<sup>[234]</sup>. It could be that there are further CpGs, which could be having stronger effects on levels of PEPCK transcription. As shown in the murine  $\alpha_1$ -protease inhibitor gene, an enhancer site around 3,000bp upstream of the transcription factor start site has the potential to alter levels of transcription<sup>[264]</sup>, it could be that regions further away from the transcription start site are having effects.

Variation in CpG methylation will reflect altered transcription factor binding and therefore altered levels of transcription, however, this is dependent on the specific form of regulation and the transcription factors involved. Fasting alone may not be sufficient to observe the effects of altered methylation at the PEPCK promoter. The results however, provide conclusive evidence that methylation of the PEPCK promoter is subject to significant variation following an increased energy diet throughout life over three generations, and reduced protein in the F0 maternal diet during pregnancy.

In order to lead to significant differences in the level of gene transcription, alterations in methylation of a promoter region, must directly or indirectly lead to changes in transcription factor binding. The potential transcription factor binding sites covering CG dinucleotides, in the PEPCK promoter, generated using Mat Inspector software, are shown in Figure 5.6. By comparing the transcription factor binding sites to the methylation levels across the groups, it appears that there is altered methylation across the groups at the binding sites for C/EBP, KLFs and CRE. The CRE and C/EBP play a significant role in regulation of transcription of the PEPCK gene<sup>[238,231,239]</sup>. A loss of expression of C/EBP in mice, leads to an absence of expression of PEPCK at birth<sup>[231]</sup>, and activation of the PEPCK gene by cAMP, acting via the cAMP response element leads to a tenfold increase in PEPCK expression within 20 minutes<sup>[239]</sup>. As a result altered methylation of these CpGs is likely to affect binding at these sites, and could contribute to the differences in mRNA expression seen. This suggests that as methylation of the CpGs found within these binding sites is altered between generations, binding of these regulatory may be affected. This altered form of regulation may explain some of the differences in PEPCK gene expression between generations. Functional investigation into the role of specific CpGs in the regulation of transcription of PEPCK and the binding of specific factors to specific regions of the promoter will clarify the specific role of methylation in regulation of transcription of PEPCK.

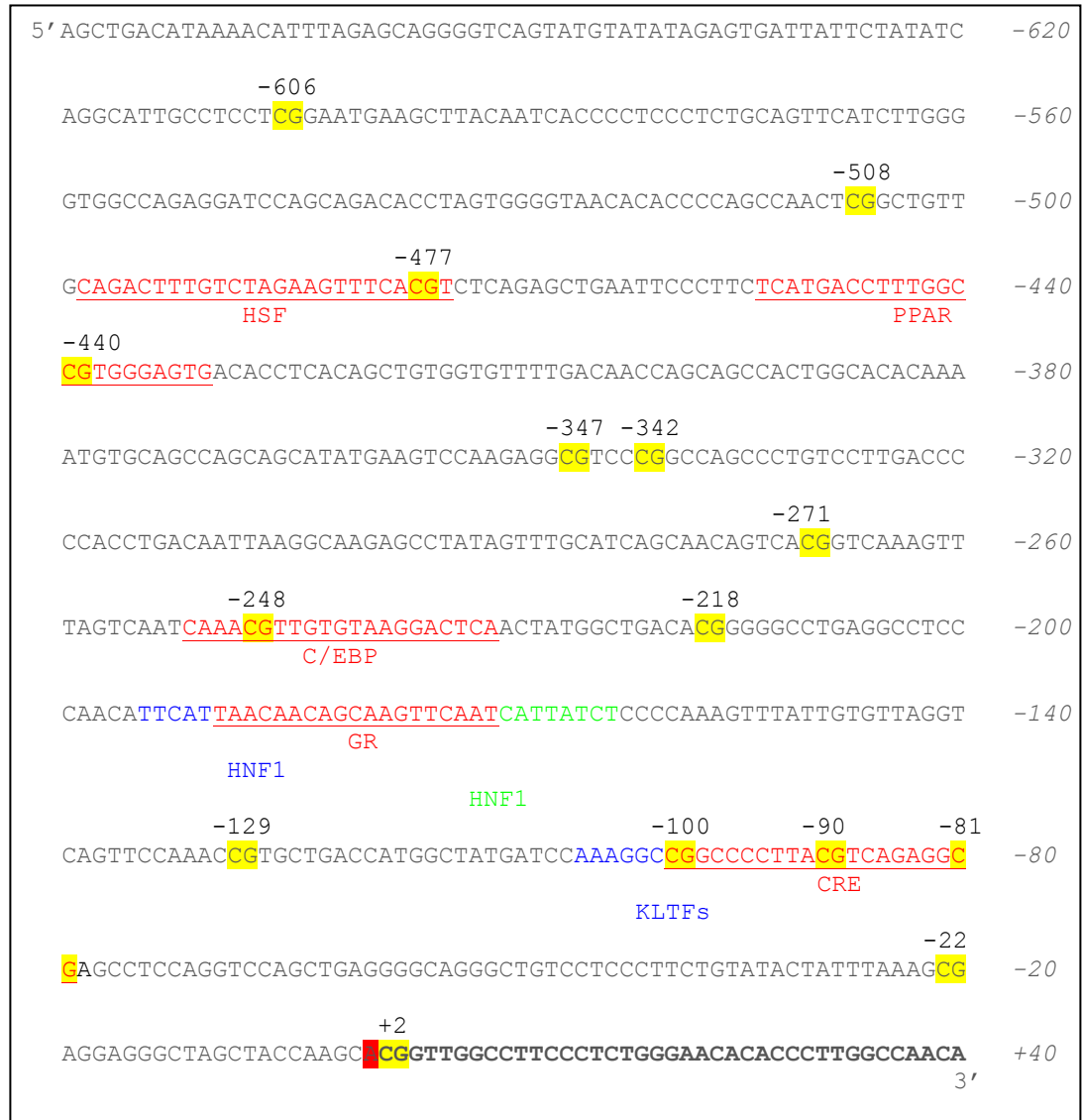


**Figure 5.6 Diagram showing relevant transcription factor binding sites covering CpG dinucleotides, in the PEPCK promoter.** Diagram shows -500bp to +40bp, where 0bp is the transcription start site. Red highlighted represents the transcription start site, yellow highlighted represents CpG dinucleotides, bold text represents exon 1 and underlined represents a transcription factor binding site. Binding sites shown for Heat shock factor (HSF), PPAR, CCAAT enhancer-binding protein (C/EBP), Hepatic nuclear factor 1 (HNF1), GR, Krueppel like transcription factors (KLTFS) and cAMP response element (CRE).

#### **5.4.1.3 The effect of pregnancy on PEPCK promoter methylation**

The differences between both generation and dietary groups were reduced when the litter mates of the postnatal day 70 non-pregnant adult female offspring became pregnant.

Between pregnancy groups there was a significant effect of generation on methylation of the PEPCK promoter in day 8.5 dams at CpG -129 only, where methylation in the F1 PR group was significantly higher than the F2 PR group. There was no effect of the protein content of the F0 maternal diet during pregnancy on PEPCK methylation.

When comparing pregnancy groups to their non-pregnant litter mates, there was a significant effect of the added stress of pregnancy on CpG methylation of the PEPCK promoter at CpGs -440, -129, -90 and -81 bp ( $P < 0.05$ ) (Figure 5.7). The effect was dependant on generation and protein content of the F0 maternal diet during pregnancy in CpGs -440 and -129, and generation only in CpG -90. The effect of the protein content of the maternal diet during pregnancy is seen in CpG -129, where each protein lineage showed different patterns in CpG methylation. The results show that the additional stress of pregnancy leads to a significant difference in methylation of the PEPCK promoter, in a CpG specific manner. However, once pregnant, the significant differences seen between non-pregnant groups are lost. This suggests that regulation of PEPCK may be significantly different in the physiological state of pregnancy.

As discussed in Section 1.2.1.2, the fasting response is significantly altered during pregnancy. As the methylation of the promoter of a key gene involved in fasting is significantly altered in pregnancy this could provide one potential mechanisms for the changes seen.

To date no studies have shown the effect of pregnancy on the epigenetic regulation of transcription of specific gene, therefore it is not known if these effects are replicated in other genes, or if these mechanisms are conserved between species.

#### **5.4.1.4 The role of promoter methylation on regulation of PEPCK transcription in day 8.5 pregnant dams**

In the day 8.5 pregnant dams, there is only one change in methylation of the PEPCK promoter. At CpG -129, the F0 PR group had significantly higher methylation than the F1 PR group. In terms of PEPCK expression in day 8.5 dams, the F1 PR group had an increased expression, compared to both the PS and PR F0 dams. As a result the relationship between the F0 and F1 groups, but not the F1 and F2 groups, can be explained by methylation of the PEPCK promoter at CpG -129. As shown in figure 5.6, this region contains the CRE, suggesting activation of the PEPCK gene by cAMP may be involved. Again methylation of other CpGs may remain cryptic until the animals are under different physiological conditions, where actions of alternative transcription factors may provide further differences between groups.



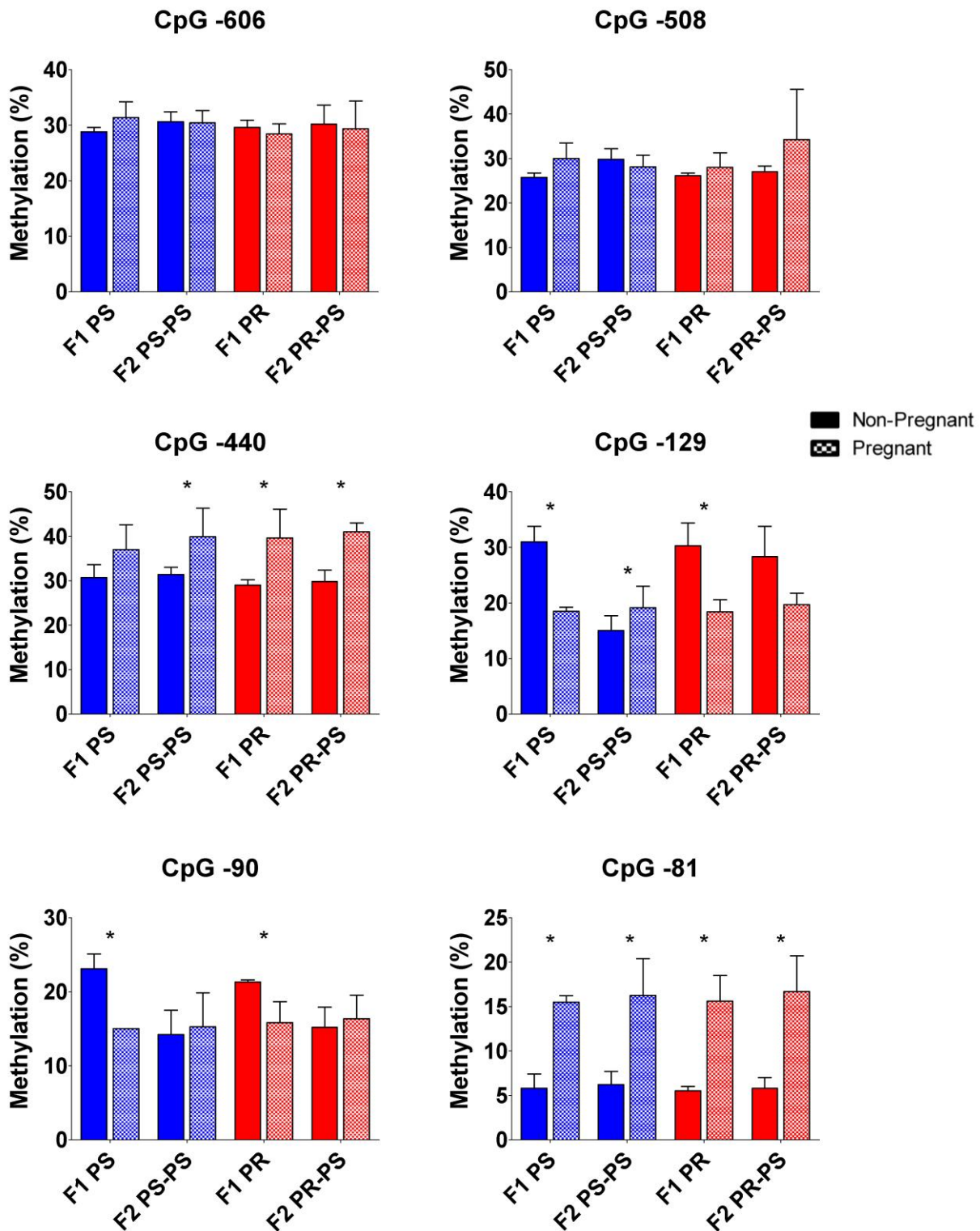


**Figure 5.7 The effect of pregnancy on PEPCK promoter methylation.** Values are means with their standard deviations. Significant differences were by T-TEST ( $P < 0.05$ ), where differences are shown with a '\*'. Solid bars represent non-pregnant adult females. Patterns bars represent day 8.5 pregnant dams. Blue represents the PS lineage and PR represents the PR lineage.

#### **5.4.1.5 The effect of altered maternal physiology on offspring promoter methylation**

There are similarities between the methylation status of the PEPCK promoter in day 8.5 pregnant dams and day 70 non-pregnant adult female offspring, for specific CpGs. The relationship between methylation levels in the PEPCK promoter in the dams and their respective offspring can be seen in figures 5.8 and 5.9 for the PS and PR lineages, respectively. Both dietary lineages show no significant differences between CpG methylation for specific groups, at numerous CpGs. PEPCK methylation in the offspring is significantly different from that of the dams for multiple CpG across all three pregnancies. For the PS lineage, it appears that after multiple pregnancies, the F3 offspring are showing more similar methylation levels to that of the dams (F2 dams), compared to the F1 offspring (F0 dams). Alternatively, in the PR lineage, the smallest difference between dam and offspring methylation is seen in the F2 offspring, rather than the F3. Specific CpGs, such as CpG -81, show a greater difference between dams and offspring, regardless of protein lineage and generation, this can be explained by the large effect of pregnancy, at the CpG. These results suggest that the extent to which offspring methylation is based on maternal methylation levels varies between generations, in a manner dependent on the protein content of the F0 diet during pregnancy.

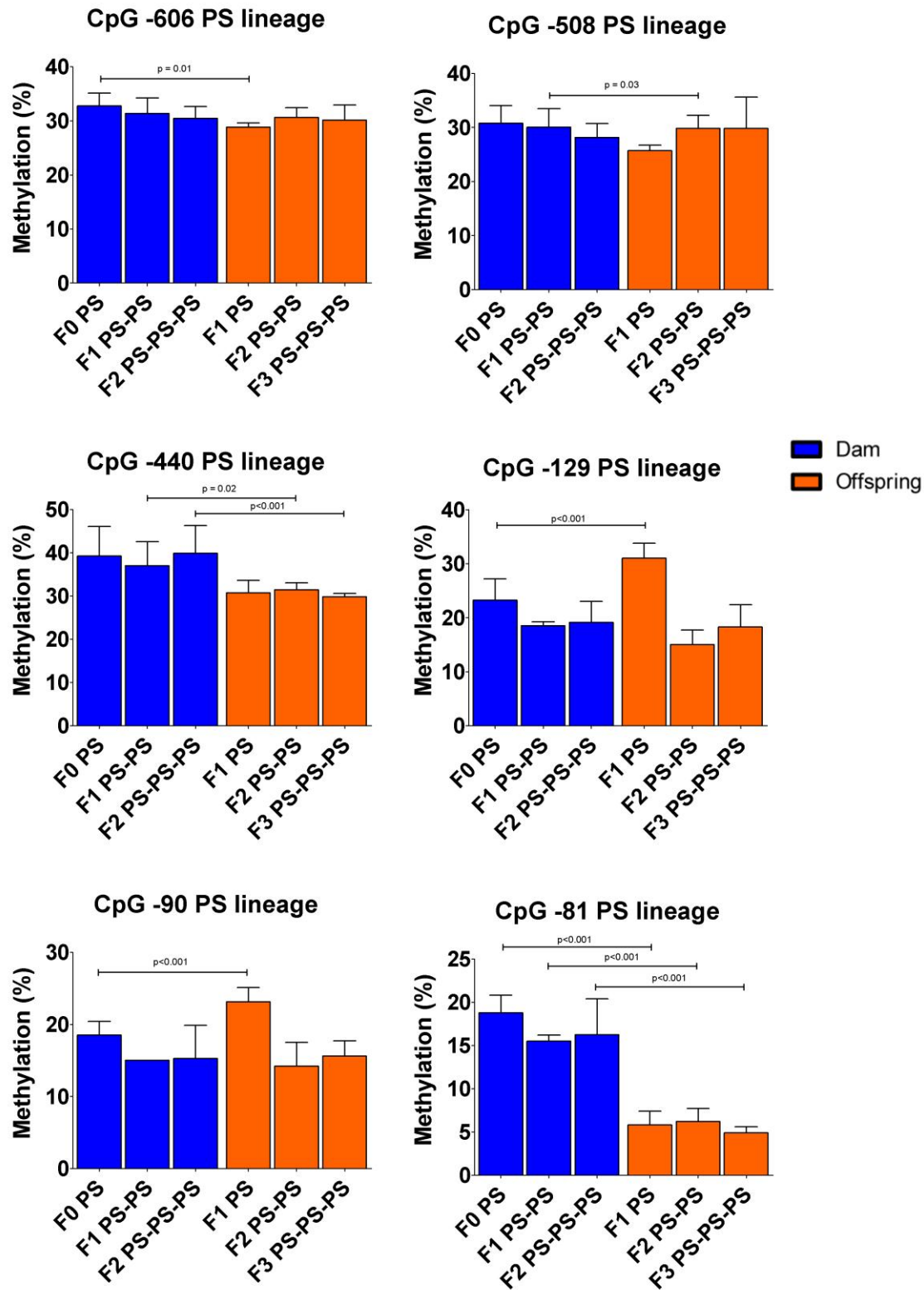


**Figure 5.8 A comparison between maternal methylation and offspring methylation of the PEPCK promoter in the PS lineage.** Values are means with their standard deviations. Significant differences were by T-TEST. Blue bars represent the dam groups, orange bars represent the offspring groups.

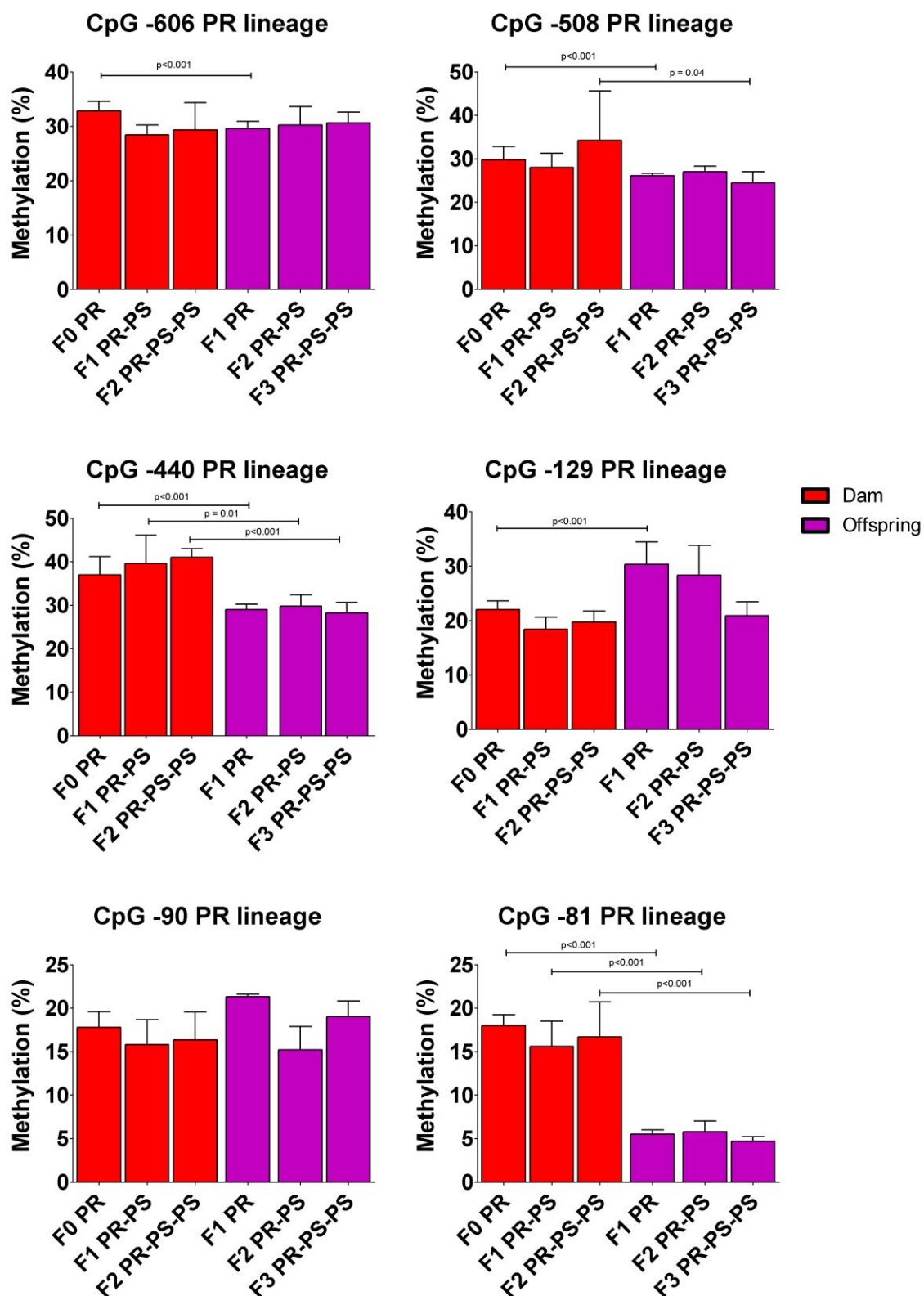


**Figure 5.9 A comparison between maternal methylation and offspring methylation of the PEPCK promoter in the PR lineage.** Values are means with their standard deviations. Significant differences were by T-TEST. Red bars represent the dam groups, purple bars represent the offspring groups.

### 5.4.2 DNMT3a2

No significant differences were present between dietary groups and generation, in terms of DNMT3a2 mRNA expression. However, mRNA expression levels in non-pregnant adult female offspring are around double the level seen in all day 8.5 dam groups. This result suggests that the potential for *de novo* methylation, such as that seen in tissue growth, is greater in non-pregnant females, compared to pregnant females, in the rat. Physiologically, this suggests a compromised ability for tissue regrowth following injury, perhaps as a consequence for the maternal adaptations towards pregnancy. Liver growth during pregnancy, gestational hepatomegaly, occurs in order for the mother to meet the increased metabolic demands of the liver<sup>[265]</sup>. This mechanism, which results from hepatocyte hypertrophy, precedes maternal weight gain and is significantly proportional ( $r^2 = 0.56$ ) to fetal number. A study by Milano has shown that the pregnancy hormones do not play a role in this phenomenon, leaving the responsible mechanism unknown. The results here suggest that increased DNMT3a2 mRNA expression in pregnancy could be a key part in gestational hepatomegaly.

#### 5.4.2.1 Promoter methylation in non-pregnant adult female offspring

Methylation of the DNMT3a2 promoter showed a significant effect of both F0 diet and generation. Methylation of CpGs -56 and -39, in the F1 PS group was significantly higher than all other groups, in both the PS and PR lineages.

#### 5.4.2.2 CpG specific alterations in methylation

The potential transcription factor binding sites, covering CG dinucleotides in the DNMT3a2 promoter are shown in figure 5.10. The only known transcription factors to regulate DNMT3a2 transcription are Sp1, Sp3 and Rb12<sup>[212,211,213]</sup>. As shown in figure 5.10, none of these transcription factors bind across altered CpGs. There is no published data at present, to suggest that altered binding of any of the shown transcription factors will alter expression of DNMT3a2. As a result, it is unknown if binding of any of the described transcription factors are altered by the significant changes in level of methylation.

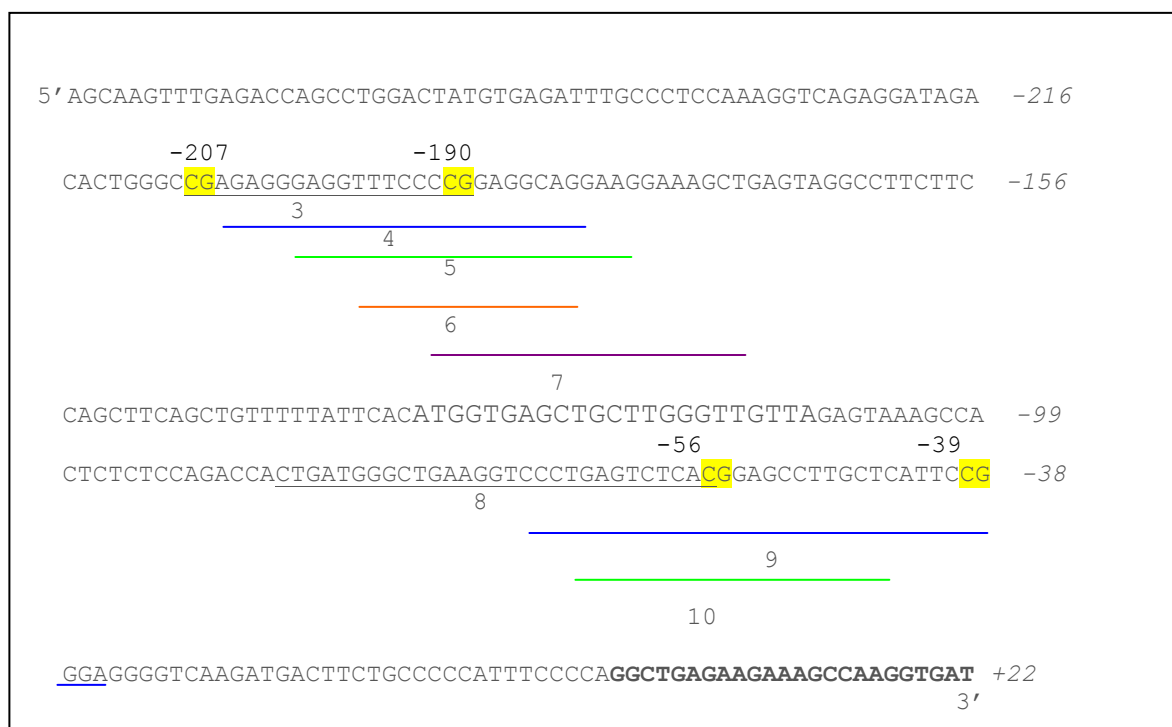


**Figure 5.10 Diagram showing relevant transcription factor binding sites covering CpG dinucleotides, in the DNMT3a2 promoter.** Diagram shows -216bp to +22bp, where 0bp is the transcription start site. Yellow highlighted represents CpG dinucleotides, bold text represents exon 1 and underlined represents a transcription factor binding site. Binding sites shown for neuron-restrictive silencer factor (1 and 9), MAF and AP1 related factors (2), nuclear factor of activated T-cells (3), RXR heterodimers binding sites (4), neuron-specific-olfactory factor (5), activator protein (6), human and murine ETS1 factors (7), PRD1-BF1 and ROZ homologous domain proteins (8) and mouse krueppel like factor (10).

#### **5.4.2.3 The role of promoter methylation in regulation of DNMT3a2 transcription in offspring**

There were no significant changes in DNMT3a2 gene expression across generations or dietary lineages, in the non-pregnant adult female offspring. The level of methylation between groups in CpGs -207 and -190, matches that of the DNMT3a2 expression, such that there is no change across groups. However there are significant changes in methylation of CpGs -56 and -39, which are not represented in expression of the gene. It appears that altered methylation at these CpGs does not play a role in transcription of the gene, but methylation of CpGs -207 and -190 may. Under altered environments, the higher level of methylation present in the F1 PS group may lead to an altered level of transcription of the gene. If this is the case, then these animals may have an altered capacity for *de novo* methylation, such as that involved in differentiation of stem cells, following organ repair,

#### **5.4.2.4 The effect of pregnancy on DNMT3a2 methylation**

Again, with DNMT3a2, the effect of pregnancy on the regulation of transcription may be seen when comparing non-pregnant adult female offspring to their pregnant litter mates.

Between groups, there were no effects of generation or protein content of the maternal diet during pregnancy on methylation of the DNMT3a2 promoter in day 8.5 pregnant dams.

When comparing pregnant to non-pregnant litter mates, pregnancy lead to a significant increase in methylation of specific CpGs at the DNMT3a2 promoter (Figure 5.11). Methylation of CpG -207 was significantly higher in F2 PS-PS and F1 PR pregnant dams, compared to non-pregnant females of the same generation. At CpGs -56 and -39, methylation in all groups was significantly increased in the pregnant animals compared to the non-pregnant groups. This is in agreement with the mRNA expression levels of DNMT3a2, where lower levels are present in the day 8.5 dams. This suggests that through DNA methylation of the DNMT3a2 promoter, mRNA expression is decreased during pregnancy. Again, this could

represent an altered capacity for tissue repair and hepatomegaly during pregnancy, as a consequence of maternal adaptations to pregnancy.

Similar to the PEPCK promoter, the added stress of pregnancy led to a loss of significant differences between groups, in terms of promoter methylation. Again, to date, no one has shown this effect of pregnancy on gene specific methylation.

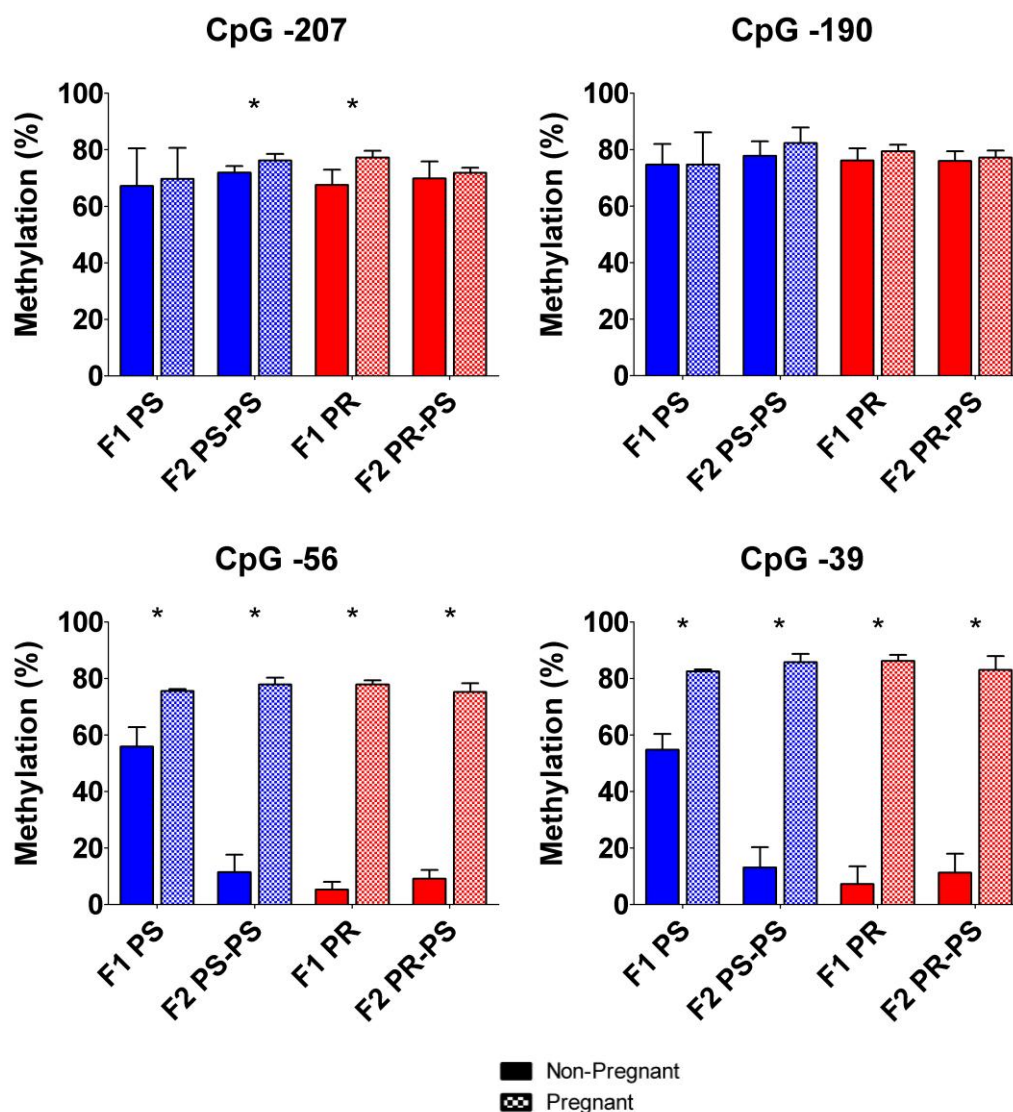


**Figure 5.11 The effect of pregnancy on DNMT3a2 promoter methylation.** Values are means with their standard deviations. Significant differences were by T-TEST ( $P < 0.05$ ), where differences are shown with a "\*". Solid bars represent non-pregnant adult females. Patterned bars represent day 8.5 pregnant dams. Blue represents the PS lineage and PR represents the PR lineage.



#### **5.4.2.5 The role of promoter methylation in regulation of DNMT3a2 transcription in day 8.5 pregnant dams**

There were no significant changes in DNMT3a2 gene expression across generations or dietary lineages, in the day 8.5 pregnant dams.

#### **5.4.2.6 The effect of altered maternal physiology on offspring promoter methylation**

The relationship between methylation levels in the DNMT3a2 promoter in the dams and their relevant offspring can be seen in figures 5.12 and 5.13 for the PS and PR lineages, respectively. CpG methylation at -207 and -190 bp show effects of maternal diet, where F3 offspring in the PS lineage have significantly different levels of methylation, compared to their respective dams (F2) at both CpGs. Whereas in the PR lineage, both F1 and F2 offspring show significantly different methylation at CpG -207, compared to their respective dams (F0 and F1). At the CpGs closest to the transcription start site, -56 and -39, all groups in both lineages show significantly different levels in methylation in offspring, compared to dams. This is likely to be due to the fact that these two CpGs are significantly altered by pregnancy and we are comparing pregnant dams to their non-pregnant offspring.

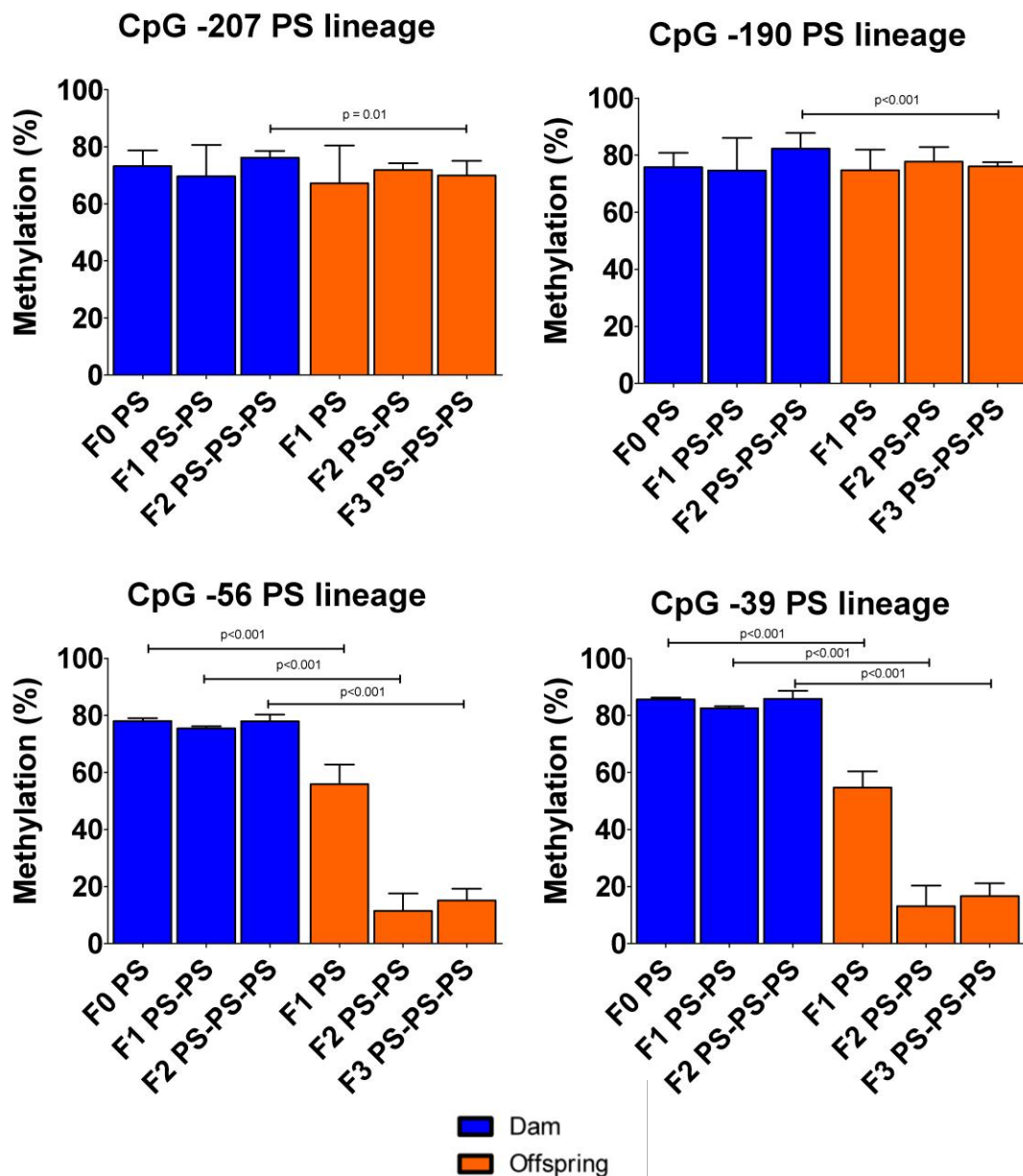


**Figure 5.12 A comparison between maternal methylation and offspring methylation of the DNMT3a2 promoter in the PS lineage** Values are means with their standard deviations. Significant differences were by T-TEST. Blue bars represent the dam groups, orange bars represent the offspring groups.

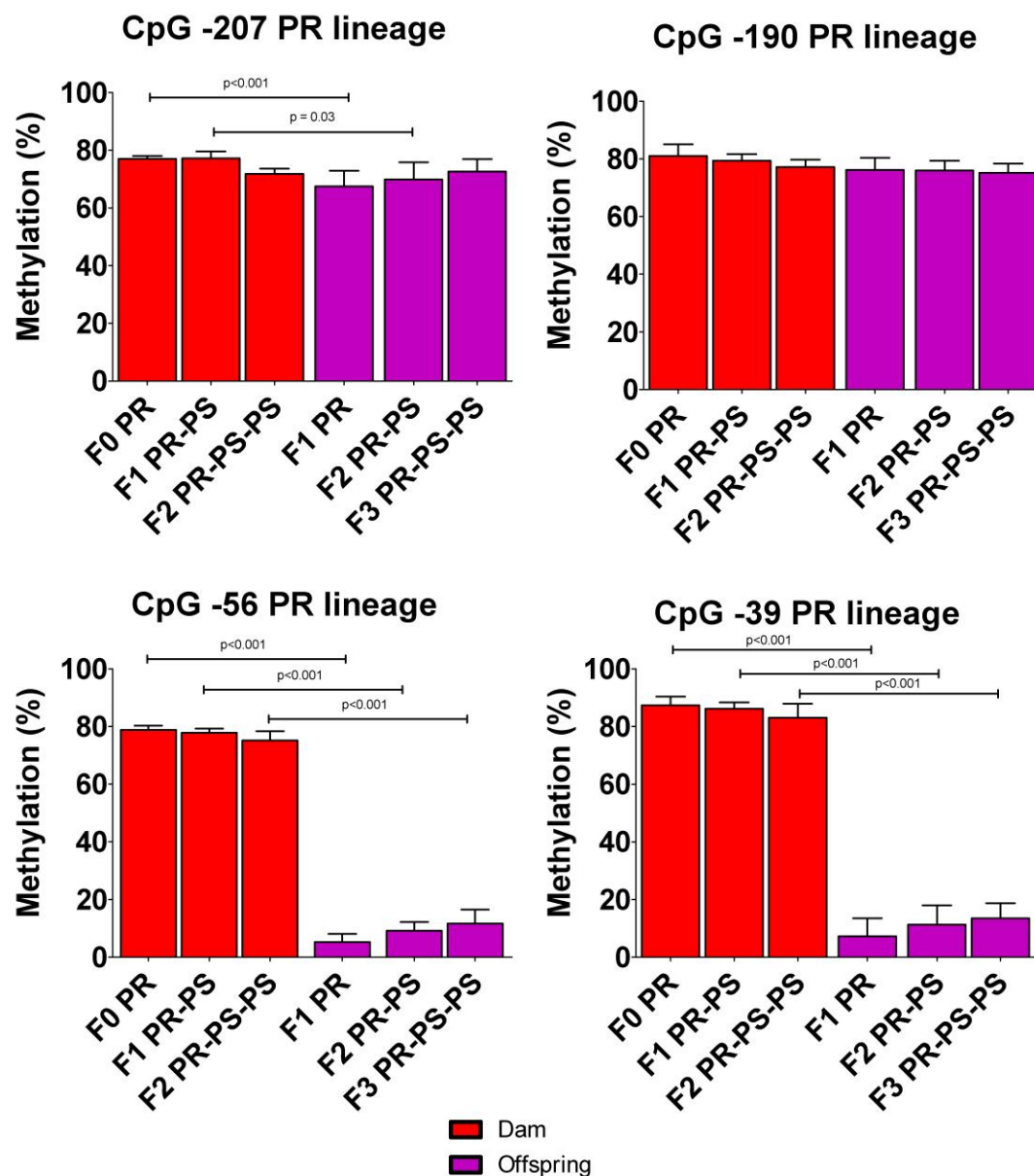


**Figure 5.13 A comparison between maternal methylation and offspring methylation of the DNMT3a2 promoter in the PR lineage.** Values are means with their standard deviations. Significant differences were by T-TEST. Red bars represent the dam groups, purple bars represent the offspring groups.

### 5.4.3 Understanding of PEPCK and DNMT3a2 promoter methylation

From the data, it is evident that for both the PEPCK and DNMT3a2 promoters, methylation levels in the non-pregnant adult female offspring became more variable nearer the transcription start site. This is likely to be associated with a regulatory mechanism, with altered transcription factor binding being involved. This is supported by specific binding sites of transcription factors, with known regulatory functions at the promoters, seen in figures 5.10 and 5.7.

Previous experiments have shown that parental famine exposure led to a decreased in CpG methylation of both the IGF-2<sup>[215]</sup>, and INISIGF promoters<sup>[201]</sup> in offspring, compared to non-famine exposed same-sex offspring. An increase in methylation was seen in the same offspring in the IL-10, LEP, AMCA1, GNASAS and MEG3 genes<sup>[201]</sup>. These findings are in agreement, as dietary alteration has been shown to significantly alter promoter methylation, in a manner which is not restricted to one direction of change. Studies by Burdge and Lillycrop have shown altered methylation of the PPAR $\alpha$  and GR promoters in 34 day old offspring, following maternal protein restriction<sup>[198]</sup>. Although the study did not report CpG specific findings, again the results here have shown the effect of maternal protein restriction on promoter methylation. No studies to date have investigated the effect of dietary modification on PEPCK or DNMT3a2 promoter methylation, therefore it is unknown if the specific findings here are in agreement with published data. Pregnancy and nutritional transition lead to altered methylation of the PEPCK and DNMT3a2 promoters, independently. The effect of pregnancy seems to be so great, that differences between dietary groups are not seen. However differences are present in adult non-pregnant offspring. The differences in promoter methylation are related to significant differences in gene expression.

This data provides initial evidence for methylation changes at the DNMT3a2 promoter, as well as increased evidence for methylation of the PEPCK promoter. Further investigation of other forms of nutritional alteration will further increase knowledge on the regulation of the genes through methylation.

#### **5.4.4 Implications**

If nutritional alterations in one generation lead to significant changes in promoter methylation in subsequent generations, at least up to the F3 generation, then the external environment during pregnancy must be carefully considered. Nutritional alteration throughout pregnancy or sustained alteration in energy intake, leads to significant changes in the epigenome of offspring, which cannot be detected through investigation of the mother, therefore following an altered environment it is vital to follow up in the offspring in later life.

The increased energy intake throughout life in these animals is representative of migration from a low nutritional intake country to a developed country, such as that with a typical Western diet. Significant differences have already been shown in the expression of genes and phenotype of the mothers and offspring exposed to the high energy diet. Now, altered methylation has been shown, in particular in the non-pregnant adult female offspring. This provides a level of concern when considering the impact of a permanent and sometimes severe nutritional change, it appeared that the fasting phenotype began to normalize over the course of the three generations; however changes in DNA methylation may be more persistent. Perhaps a less severe, staggered move onto a new diet may be more beneficial?

#### **5.4.5 Future work**

Data here has provided a further mechanism into the significant alteration in phenotype seen in both the pregnant dams and non-pregnant adult female offspring. The data has shown the additional effect of pregnancy on methylation of the two specific genes. Altered expression of genes was present in the fasting animals, and these, to an extent were related to the altered phenotype. Although no directional relationships are present here, methylation of the promoter regions of genes is involved in their regulation and therefore the observed alterations in expression. However the mechanism here is unclear, it will therefore be necessary to investigate the role of transcription factor binding at the promoter regions covered here and the specific role of single CpGs in the promoters. Luciferase construct will determine the role of specific CpGs on gene transcription and electro

mobility shift assays will show the binding of specific and general transcription factors to the measured CpGs.



## Chapter 6

# The Effect of Aging on Methylation of the PEPCK and DNMT3a2 Promoters



## 6.1 Introduction

The results presented in Chapter 5 showed that methylation of the PEPCK and DNMT3a2 promoters are subject to nutritional alteration. The DOHaD hypothesis is based on the assumption that alterations in phenotype are a result of changes in the epigenome that are stable across the life course. However, it is not known if alterations in DNA methylation, as a result of maternal dietary alteration are maintained with aging.

### 6.1.1 Changes in DNA methylation in aging

As described by Wotton and Jackson, nutrient restriction in early life leads to a series of changes within an organism, which aim to facilitate maximum survival<sup>[266]</sup>. An example of one of these adaptations is reduced energy consumption of the organism, via reduced rates of metabolic processes at both the organ and cellular level. Adaptations such as these however may constrain net deposition of new tissues. Wotton and Jackson hypothesised that the functional consequences of restricted nutrition in early life are readily identified as impaired growth and development. This suggests that alterations in nutrition during development significantly alter the trajectory of growth and development, the mechanism behind this alteration in trajectory is unknown.

As described in section 1.4.1.2, studies have shown that the epigenome is subject to variation throughout the life course<sup>[205,206,208]</sup>. According to Bjornsson, 29% of a male and female Icelandic cohort showed a significant change in blood cell DNA methylation of over 10%, across an 11 year period<sup>[205]</sup>. Cancer is associated with a global decrease in methylation and an increase in methylation of specific genes<sup>[209]</sup>. Therefore the epigenome is subject to specific alterations during the life course. Due to the decrease in activity of DNMT1 with cellular senescence<sup>[192]</sup>, one potential mechanism for the decreased stability of the genome is the decreased maintenance of methylation marks during cell division.

Feeding a protein restriction diet leads to significant changes in the aging process. In a study by Youngman, feeding a 5% or 10% protein diet throughout life inhibited the oxidative damage of proteins seen in the 20% dietary protein control

group<sup>[267]</sup>. The inhibitory effects of protein restriction on the aging process were again seen in a study by Lee<sup>[268]</sup>. In mice, a high-density oligonucleotide array, which measured expression of 6,347 genes in skeletal muscle, showed that protein or calorie restriction, completely or partially prevented changes in gene expression associated with aging. The protein restriction led to a metabolic shift, toward increased protein turnover in the animals. Changes in gene expression have been shown to be related to alterations in the epigenome, in particular DNA methylation. It is not known if the changes in gene expression and decreased protein oxidation seen in aging, following protein restriction are associated with significant alterations in DNA methylation.

Investigation of CpG specific methylation of the PEPCK and DNMT3a2 promoters throughout the life course will show if methylation of the promoters is subject to alteration during periods of development only, or whether such plasticity extends into adulthood. The following question will be addressed in this chapter

-Are changes in PEPCK and DNMT3a2 mRNA expression and promoter methylation, induced by maternal diet maintained across the life course?

## 6.2 Experimental Design

The postnatal day 28, 105, 275 and 475 offspring were from the study described in Section 6.2.1. All day 8.5 dam and non-pregnant adult female offspring samples are from the study previously described, and RT-PCR was carried out as described (Section 2.2 and Section 2.3.5, respectively).

### 6.2.1 Animal model

The experimental design can be seen in Figure 6.1. Experimental diets were supplied by TestDiet, Indiana, USA. The full composition of diets can be seen in Tables 2.2, 2.3 and 2.4, for macronutrients, vitamins and minerals, respectively. Adult females were mated and fed either a protein sufficient diet (PS) containing 180 g/kg casein (w/w) or an isoenergetic protein restricted diet (PR) containing 90 g/kg casein (w/w), throughout pregnancy. All dams were placed on the AIN-76A, lactation diet. After 28 days, offspring were weaned onto the AIN-93M, maintenance diet, and placed in group cages, with five rats being placed in each cage. At postnatal days 28, 105, 275 and 475, six rats from each dietary group were culled, and tissues were collected.

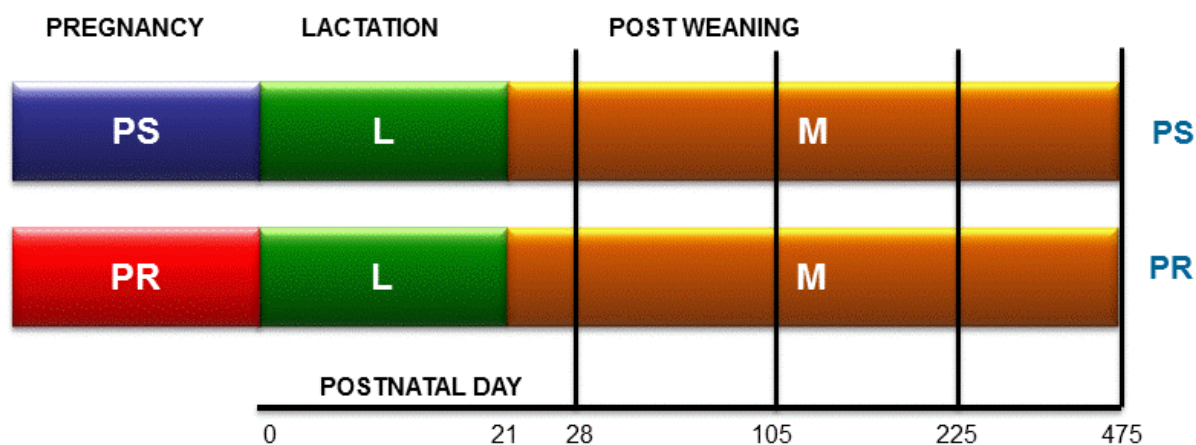


**Figure 6.1 Diagram showing the experimental design of the life course study.** Where the protein sufficient diet is shown by PS, the protein restricted diet by PR, the lactation diet by L and the maintenance diet by M. Dams were fed either the PS or PR diet during pregnancy, during lactation all dams were fed the AIN76A diet. At weaning offspring were moved onto the AIN93-M, maintenance diet.

### **6.2.2 Tissue collection**

After being culled, livers were collected from each rat. Liver weights were measured, and livers were snap frozen in liquid nitrogen upon collection. All tissue was stored at -80°C long term.

### **6.2.3 Molecular biology procedures**

Extraction of DNA and RNA, reverse transcription, bisulphite treatment of DNA, PCR conditions and pyrosequencing methods are described in Section 2.3.

### 6.3 Results

#### 6.3.1 Does mRNA expression of PEPCK or DNMT3a2 alter with age?

There was a significant effect of age on mRNA expression of PEPCK and DNMT3a2 ( $p < 0.0001$  and  $p = 0.001$ , respectively) (Figure 6.2). PEPCK mRNA expression was significantly increased at postnatal day 275, in both the control and protein restricted groups, compared to the day 105 groups.

DNMT3a2 mRNA expression was significantly increased in the control lineage day 275 group, compared to all other time points, in control offspring, and the day 28 and day 475 protein restriction lineage group. There were no significant differences in DNMT3a2 expression within the PR animals. There were no significant effects of diet on mRNA expression of PEPCK or DNMT3a2.

The results of the analysis of variance are shown in Table 6.1

|                     | PEPCK   | DNMT3a2 |
|---------------------|---------|---------|
| Age                 | <0.0001 | 0.001   |
| Maternal diet       | NS      | NS      |
| Age * Maternal diet | NS      | NS      |

**Table 6.1 A General Linear Model showing significant interactive effects of age and maternal diet on PEPCK and DNMT3a2 mRNA expression**

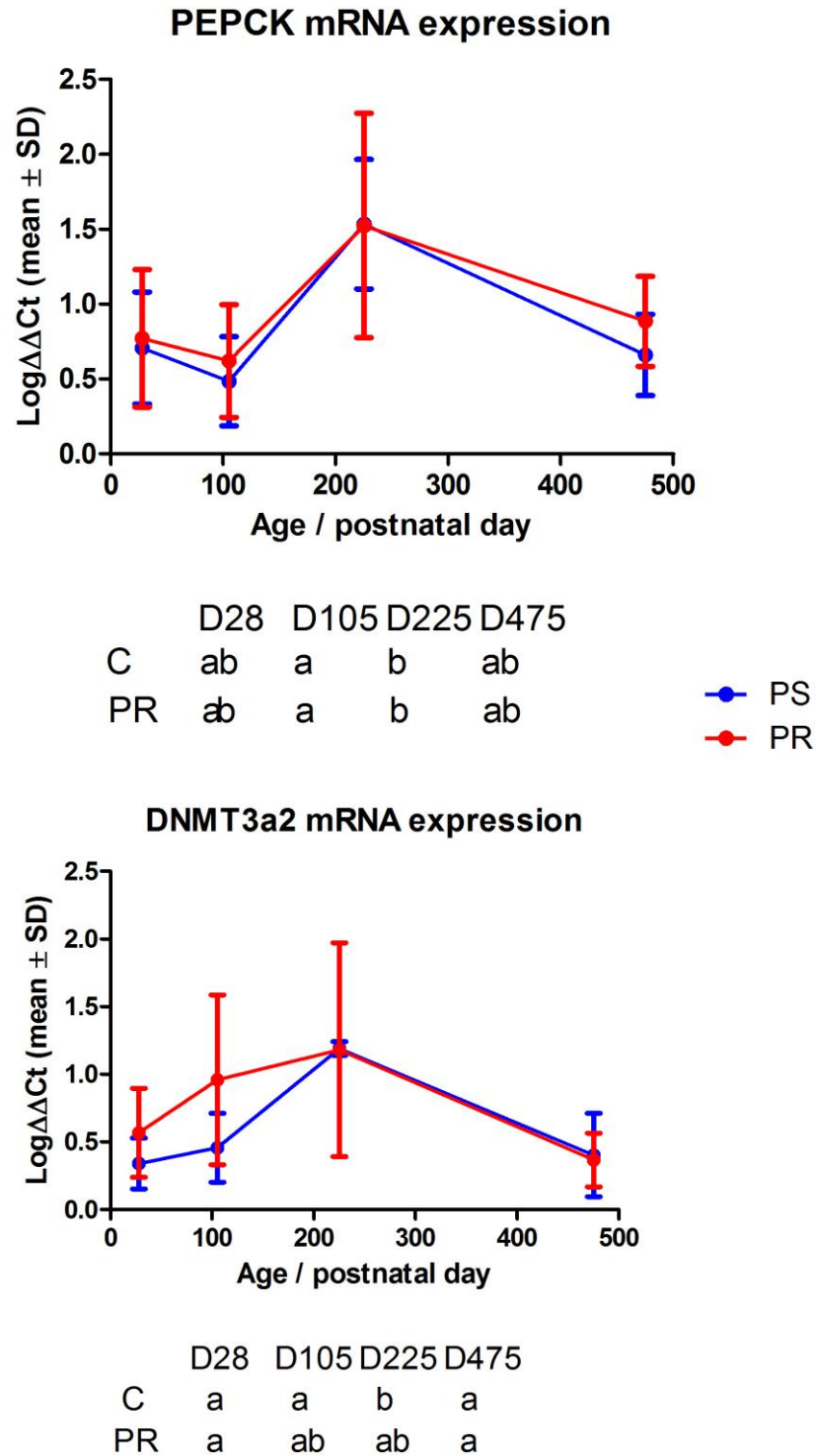


**Figure 6.2 PEPCK and DNMT3a2 mRNA expression in offspring of dams fed either a PS or PR diet during pregnancy.** Values are means with their standard deviations, where 'a' is significantly different from 'b', and 'ab' is neither significantly different from 'a' or 'b'. Significant differences between groups were by one-way ANOVA, with Bonferroni's *post hoc* test. Significant differences between PS and PR groups were determined using an unpaired t-test.  $P < 0.05$

### **6.3.2 Does methylation of the PEPCK promoter alter with age?**

There was a significant effect of age on PEPCK methylation ( $p < 0.001$ ) (Figure 6.3). There was an effect of age of offspring on DNA methylation at CpGs -508, -440, -90 and -81 ( $P < 0.05$ ). In the PS and PR groups, methylation of CpG -508 was significantly higher in the D475 offspring, compared to D28 offspring. Methylation of CpG -440 was significantly increased in the PR day 28 group compared to the day 28 and day 225 PR group, significantly decreased in the PR 225 group, compared to the PR day 105 group and significantly increased in day 475 PR group, compared to the PR 28 group. In the protein restricted lineage, methylation of CpG -129 was significantly increased at day 475 compared to day 28. CpG -90 methylation was significantly lower at 475, compared to day 105, in the PR lineage. At CpG -81, in both the PS and PR lineages, methylation was significantly higher at day 105, compared to all other measured time points. The results of the analysis of variance are shown in Table 6.2.

#### **6.3.2.1 Are changes in PEPCK promoter methylation across the life course altered by maternal diet during pregnancy?**

There was a significant effect of age and maternal diet on PEPCK promoter methylation ( $p < 0.001$ ) (Figure 6.3). At CpG -440, methylation was significantly higher at day 105, following maternal protein restriction. In the protein restriction lineage, methylation of CpG -90 was significantly lower at day 475, compared to the control offspring.

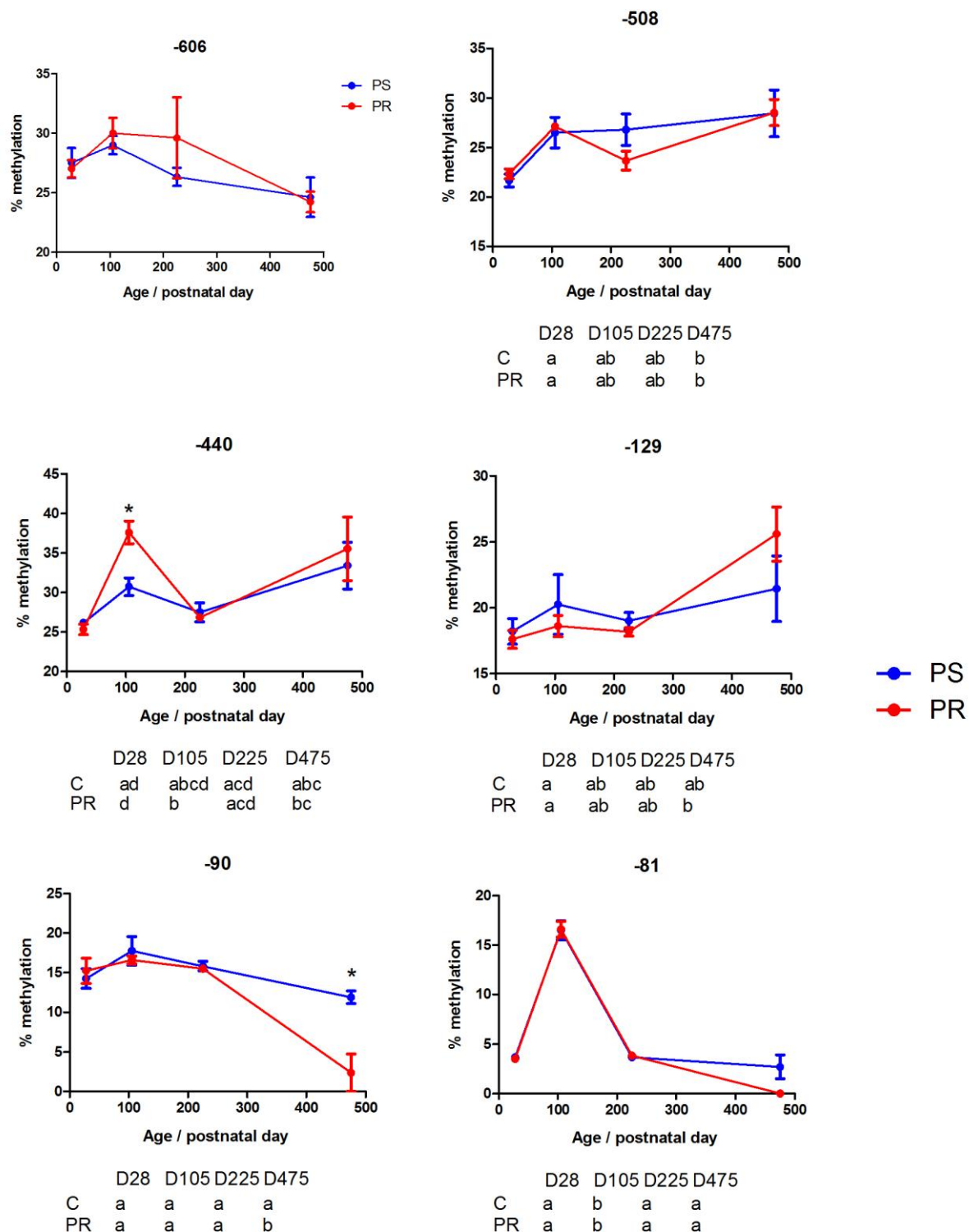


**Figure 6.3 PEPCK promoter methylation in offspring of dams fed either a PS or PR diet during pregnancy.** Values are means with their standard deviations, where 'a' is significantly different from 'b', and 'ab' is neither significantly different from 'a' or 'b'. Significant differences between ages were by one-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.006$ . Significant differences between PS and PR groups are shown by an '\*' and were determined using an unpaired t-test.  $P < 0.05$



|                     | CpG<br>-606 | CpG<br>-508 | CpG<br>-440 | CpG<br>-129 | CpG<br>-90 | CpG<br>-81 |
|---------------------|-------------|-------------|-------------|-------------|------------|------------|
| Age                 | NS          | <0.001      | 0.037       | NS          | <0.001     | <0.001     |
| Maternal diet       | NS          | NS          | <0.001      | NS          | 0.002      | NS         |
| Age * Maternal diet | NS          | NS          | <0.001      | NS          | <0.001     | NS         |

**Table 6.2 A General Linear Model showing significant interactive effects of age and maternal diet on PEPCK promoter methylation**

### **6.3.3 Does methylation of the DNMT3a2 promoter alter with age?**

There was a significant effect of age on methylation at every CpG of the DNMT3a2 promoter (Figure 6.4). At CpG -207, in the PS groups only, methylation was significantly higher at day 105 and 475, compared to the day 28 ( $P < 0.005$ ). At CpG -190, in the PS groups, methylation was significantly lower in the D28 group, compared to the D275 and D475 groups. In the PR lineage, methylation of CpG -190 was significantly increased at day 475, compared to day 105. At CpGs -56 and -39, in both the PS and PR groups, methylation was significantly lower at day 28, compared to all other groups. The results of the analysis of variance are shown in Table 6.3.

#### **6.3.3.1 Are changes in DNMT3a2 promoter methylation across the life course altered by maternal diet during pregnancy?**

There was no effect of maternal diet on methylation at any CpG of the DNMT3a2 promoter (Figure 6.4).

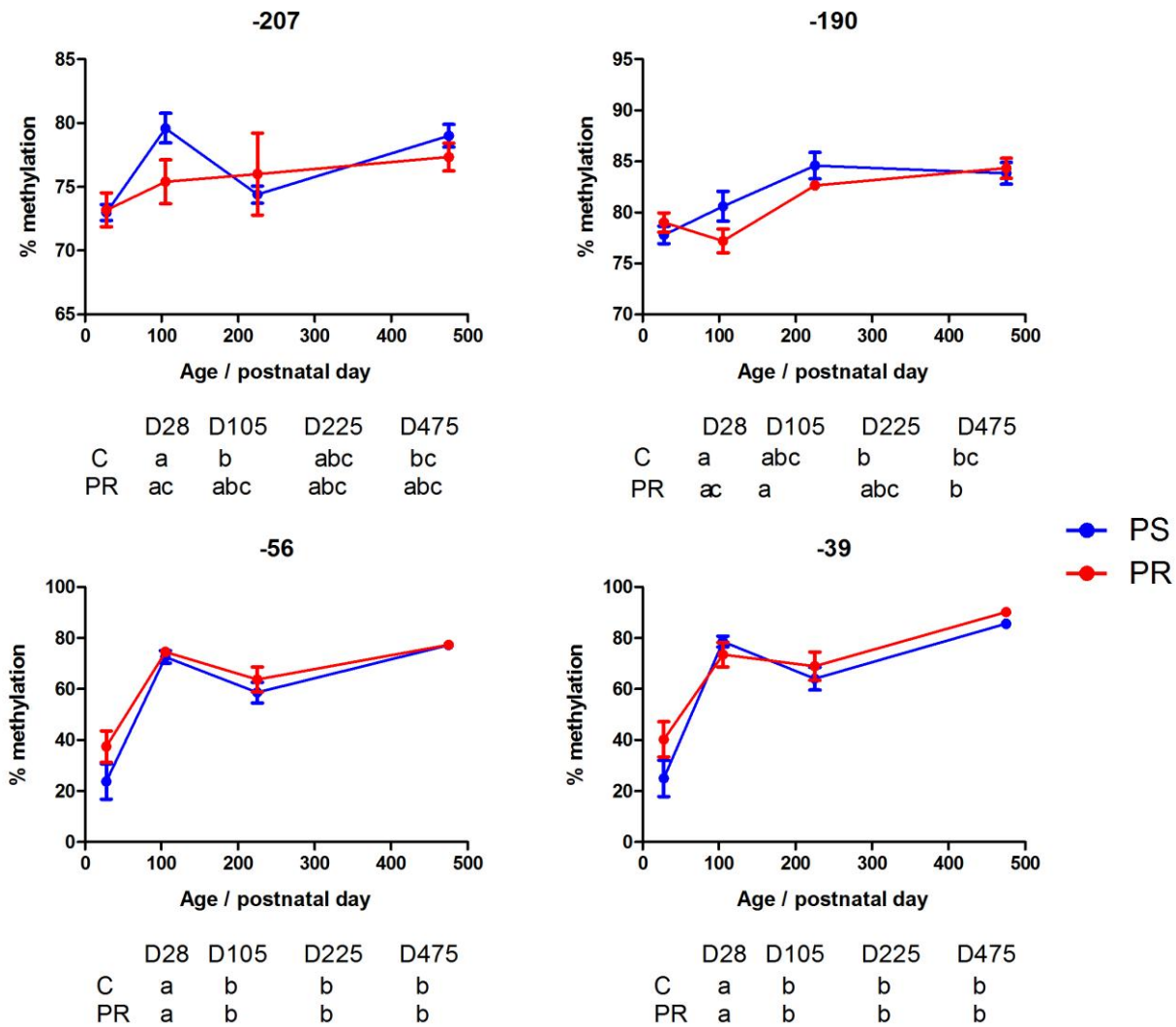


**Figure 6.4 DNMT3a2 promoter methylation in offspring of dams fed either a PS or PR diet during pregnancy.** Values are means with their standard deviations, where 'a' is significantly different from 'b', and 'ab' is neither significantly different from 'a' or 'b'. Significant differences between ages were by one-way ANOVA, with Bonferroni's *post hoc* test. Significant differences between PS and PR groups were determined using an unpaired t-test.  $P < 0.05$

|                     | CpG<br>-207 | CpG<br>-190 | CpG<br>-56 | CpG<br>-39 |
|---------------------|-------------|-------------|------------|------------|
| Age                 | 0.001       | <0.001      | <0.001     | <0.001     |
| Maternal diet       | NS          | NS          | NS         | NS         |
| Age * Maternal diet | NS          | NS          | NS         | NS         |

**Table 6.3 A General Linear Model showing significant interactive effects of age and maternal diet on DNMT3a2 promoter methylation**

## 6.4 Discussion

### 6.4.1 mRNA expression and aging

Although PEPCK expression has been shown to be increased at birth<sup>[229]</sup>, no studies to date have shown variation in fasting PEPCK mRNA expression throughout life. In this chapter it has been shown that PEPCK mRNA expression was significantly altered by aging. At postnatal day 225, PEPCK mRNA expression was significantly higher compared to day 105 offspring, regardless of the protein content of the maternal diet during pregnancy. Physiologically, this suggests that gluconeogenic capacity of the offspring is optimal at day 225 in these rats. This means that during periods of fasting the ability of these animals to provide sufficient glucose for key organs is maximal. Compared to the day 105 group, where PEPCK expression was lowest, this means a decreased requirement for  $\beta$ -oxidation of fatty acids, to provide ketone bodies. As a result the production of free radicals, and therefore free radical damage, as a result of peroxide production, will be lower.

DNMT3a2 mRNA expression was significantly increased in the postnatal day 225 offspring, compared to all other measured ages, in the PS lineage only. This suggests that the capacity for *de novo* methylation is significantly increased at this age. Physiologically there is no clear reason for this increased capacity, as this age is not associated with any obvious changes. Differences in DNMT activity, but not mRNA expression, have been shown to vary with aging<sup>[192]</sup>. In WI-38 human fetal lung fibroblasts, maintenance methyltransferase activity of DNMT1 activity decreased by 19.9% from postnatal day 18 to postnatal day 56, whereas *de novo* methyltransferase activity of DNMT3b activity increased by 317.5%. These results suggest the trajectory of alteration in activity of DNA methyltransferases is significantly different for the individual DNMTs. As mRNA expression was not measured in this study and DNMT3a activity was not measured in the study, it is unknown if these results are expected.

#### **6.4.2 Promoter methylation in aging**

In male rats, methylation of both the PEPCK and DNMT3a2 promoters changes significantly across the life course, in a CpG specific manner. In the PEPCK promoter methylation of CpG -508, -440, -129, -90 and -81 were significantly altered by aging. At CpG -508, methylation was significantly increased at postnatal day 475 compared to day 28, irrespective of maternal protein intake. Methylation of CpG -440 was significantly higher in the PR lineage at days 105 and 475, compared to day 28. In the PR lineage, methylation of CpG -440 and -129 were significantly higher and CpG -90 significantly lower, compared to day 28. At postnatal day 105, methylation of CpG -81 was significantly higher than all other time points. Although the significant differences in DNA methylation are not associated with changes in PEPCK mRNA expression, the results show that methylation of the PEPCK promoter varies with aging in a manner dependant on the protein content of the maternal diet during pregnancy.

In the DNMT3a2 promoter, there was a significant effect of age on CpG methylation. All four measured CpGs showed a similar pattern in change with age; where methylation in the weanlings (postnatal day 28) was significantly lower than all other groups. Again, the methylation status of DNMT3a2 throughout life was not clearly associated with mRNA expression. This could also represent a potential for altered expression of DNMT3a2, under different physiological conditions.

Previous studies into variation of DNA methylation with time have shown significant changes in global methylation with age<sup>[205]</sup> and CpG specific changes in methylation following altered maternal behaviour<sup>[206]</sup>. The data here is not only in agreement with that found previously, but extends upon current knowledge. The data here shows an extensive and specific coverage of two distinct promoter regions, of genes with significantly different expression profiles during the life course. The data clearly shows the effect of nutritional alteration during development extends far beyond adulthood to an age comparable to old age in humans.

Despite DNMT3a2 accounting for 50% of all detected DNMT3a expression, the promoter is primarily active during fetal development, with low levels present in

adult tissue. Whereas the PEPCK promoter is active throughout all postnatal life, therefore differences in variation of methylation throughout the life course are expected. Protein restriction of the maternal diet during pregnancy led to significant alterations in the trajectory of variation in the PEPCK promoter only, leading to methylation levels significantly different between the PS and PR lineages at two time points, in two CpGs. The PEPCK promoter undergoes demethylation during fetal development<sup>[229]</sup>, the time during which the nutritional alteration was present. As fetal development has been shown to provide a window for alterations in epigenetic regulation of gene expression, differences in PEPCK methylation, between PS and PR lineages are not unexpected. In comparison to the PEPCK promoter, methylation of the DNMT3a2 promoter remains high throughout the life course, representing its decreased expression. Szyf hypothesised that the epigenome is a dynamic equilibrium between methylation and demethylation<sup>[190]</sup>. This is brought about by active addition and removal of methyl groups from promoter regions of specific genes. The results here do show a significant alteration in the level of methylation at specific points of the promoter, however they are not in complete agreement with Szyf's hypothesis. Methylation of the PEPCK and DNMT3a2 promoters appear to follow a specific trajectory of change throughout life, rather than a balance between methylation and demethylation. Further experiments investigating changes in methylation of the promoters across a few hours, under an altered state may confirm this. It is logical that variability is more prevalent in genes that have altered levels of expression over minutes or hours. Perhaps genes that are expressed at lower levels, if at all, as a result of DNA methylation are less susceptible to variation over time? Therefore this could represent the fewer changes shown in DNMT3a2, compared to PEPCK.

DNMT1 activity has been shown to decrease with cellular senescence<sup>[192]</sup>. The data here is in agreement with this finding for two CpGs of the PEPCK promoter, CpGs -90 and -81, where methylation is significantly lower in the D475 group, suggesting decreased DNMT1 activity could account for this finding. However this effect is not seen in the other CpGs measured in the PEPCK promoter, or any CpGs in the DNMT3a2 promoter. These promoter regions

represent just two genes from the whole genome, as a result it is impossible to deduce if decreased DNMT1 activity is present here, or if it is having a significant effect on methylation levels.

#### **6.4.3 Methylation and expression**

There are no clear relationships between promoter methylation of both PEPCK and DNMT3a2 and fasting mRNA expression. This does not however rule out the role of DNA methylation in regulation of the genes. The significant differences in methylation of the promoters with aging may remain cryptic until the correct stressor and form of regulation is present. Altered methylation at a specific CpG site, where a specific transcription factor binds may only have an effect on transcription of the gene when the specific form of regulation is present.

#### **6.4.4 Does protein restriction alter trajectory of variation?**

Previous studies have shown that protein restriction throughout life can prevent or inhibit the effects of aging<sup>[268,267]</sup>. Protein restriction of the maternal diet during pregnancy significantly altered the trajectory of change in methylation of the PEPCK promoter across the life course, at specific CpGs. Methylation of CpG -440 is significantly increased at day 105 in protein restricted offspring, compared to control offspring. At CpG -90, methylation was significantly decreased in the PR lineage at day 475, compared to the control offspring. Although these differences in methylation were not associated with change in PEPCK expression, due to potential transcription factor binding at these CpGs, effects may be present under different physiological conditions, where different forms of regulation of the gene are taking place.

Variation in methylation of CpG -207 of the DNMT3a2 promoter and mRNA expression of DNMT3a2 was significantly altered with age in the PS groups. This variation was lost when maternal protein restriction was applied, suggesting an inhibition of the effect of aging in the protein restricted offspring.



#### **6.4.5 Implications**

Aging is associated with significant variation in both mRNA expression and promoter DNA methylation of specific genes. These variations are dependent on the protein content of the maternal diet during pregnancy. These results suggest that the epigenome is not fixed during development, but can be altered during postnatal life, including late stages of adult life. As a result the effect of environmental stimuli in altering disease risk, through changes in the epigenome may be great. Interestingly protein restriction of the diet, which has been shown to lead to a detrimental phenotype when fed to dams during pregnancy and/or lactation, may have beneficial effects in terms of aging, when considering detrimental effects of aging, such as increased cancer risk.

## Chapter 7

# The Effect of Timing of Nutritional Exposure on the Epigenetic Regulation of Phosphoenolpyruvate Carboxykinase and DNA Methyltransferase 3a2

## 7.1 Introduction

DNA methylation is regarded generally as a stable epigenetic mark which is induced during development and then persists throughout the life course<sup>[184,269]</sup>. Furthermore, epigenetic plasticity is considered to be closely linked to developmental plasticity and cell differentiation<sup>[270,271]</sup>, and thus after early development, DNA methylation is essentially resistant to environmental signals. However, data in the previous chapter and a number of studies have challenged this view by showing tissue-specific variation in DNA methylation throughout the life course and in juvenile and adult tissues<sup>[241]</sup>.

### 7.1.1 Evidence for persistence of plasticity in DNA methylation throughout the life course

There are physiological observations of plasticity in DNA methylation throughout the life course. Differentiation of Th1 and Th2 cells involved opposite changes in methylation of the promoter of the IFN $\gamma$  gene promoter. Th1 and Th2 cells act to orchestrate the protective immune response, thorough cytokine production<sup>[272]</sup>. In Th2 cells the IFN $\gamma$  promoter is hypermethylated, whereas in Th1 cells the promoter is hypomethylated<sup>[272]</sup>. The mechanism, which is present in both human and mouse, is also seen in natural killer cells in the mouse<sup>[272]</sup>. Hypermethylation of the IFN $\gamma$  promoter in Th2 cells leads to chromatin condensation and exclusion of CREB proteins from the IFN $\gamma$  promoter<sup>[273]</sup>. In the presence of the methylation inhibitor 5'-azacytidine, Th2 cells increase production of IFN $\gamma$ , through CREB binding<sup>[273]</sup>. The study demonstrates that DNA methylation is highly plastic in the immune system and that this plasticity is critical for function. The role of DNA methylation in the onset of cancer has been shown experimentally<sup>[274,275,276]</sup>. Hypermethylation of tumour suppressor genes, leading to their inactivation has been shown in APC, BRCA1, E-cadherin, LKB1 and MLH1<sup>[275]</sup>. Altered epigenetic regulation of tumour genes affects over half of tumour suppressor genes that cause cancers when mutated in the germ line<sup>[275]</sup>. In ovarian cancer patients, BRCA1 promoter hypermethylation was shown to be an alternative to mutation in causing the inactivation of the BRCA1 tumour suppressor

gene<sup>[274]</sup>. Out of 81 sporadic cases of ovarian cancer, the BRCA1 promoter was hypermethylated in 15%. Out of the cases, BRCA1 hypermethylation was only seen in patients without a family history of breast/ovarian cancer<sup>[274]</sup>. These results suggest that global DNA methylation associated with aging is accompanied by tissue specific gene hypermethylation, such as tumour suppressor genes.

Together these studies show that the period of plasticity of DNA methylation extends throughout the life course. One implication of these findings is that it may be possible to correct changes in promoter methylation induced during development by interventions in later life.

As shown in section 1.4, supplementation of a protein restricted diet with folic acid, in the rat, prevented at least some of the phenotypic and epigenetic changes induced by the PR diet alone. These findings, together with the effect of folic acid supplementation on H19 in men, suggest that dietary interventions with this vitamin may reverse the effects of poor maternal nutrition on DNA methylation. To date, the only study that has investigated post-natal nutritional alteration is by Burdge et al<sup>[25]</sup>. Juvenile pubertal offspring (postnatal day 28) of dams fed a protein restricted diet during pregnancy were fed with either 1mg/kg or 5mg/kg folic acid for 28 days. The effects of the maternal protein restriction however, were not simply reversed, an increased weight gain, lower plasma  $\beta$ -hydroxybutyrate concentration and increased hepatic and triglyceride concentrations were shown, in the 5mg/kg folic acid offspring, compared to the 1mg/kg offspring<sup>[25]</sup>. Changes were also present in the epigenome, where PPAR $\alpha$  and GR promoter methylation was increased in liver, in the folic acid supplemented offspring group<sup>[25]</sup>. This implies that the timing of nutritional exposure may be an important factor which determines its effect on DNA methylation.

To date there have not been any studies which have compared the effects of the same amount of dietary folic acid given during development or in later life on DNA methylation.

Although it is known that folic acid supplementation can induce significant alterations in the epigenome and phenotype, in the protein restricted and IUGR rat

models and in humans, it is not known whether providing the same amount of folic acid at different stages of the life course induces the same changes in DNA methylation.

The purpose of this chapter is to investigate these unanswered questions and to test the hypothesis that

-Timing of nutritional alteration is an important factor in determining the direction and magnitude in alterations in phenotype via changes in DNA methylation.

In order to test the hypothesis, PEPCK and DNMT3a2 gene expression and methylation will be measured in adult offspring, following folic acid supplementation of either the maternal or post-weaning diet.

## **7.2 Experimental design**

### **7.2.1 Animal model**

All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedure) Act (1986). The experimental design can be seen in Figure 7.1. Experiments diets were supplied by SDS, Essex, England (Prenatal study FA and FS and AIN-76A) and PMI Nutrition International, Indiana, USA (Postnatal study FA and FS and AIN-93G). The full composition of all diets can be found in the Tables 7.1, 7.2 and 7.3, for macronutrients, vitamins and minerals, respectively. The energy content of the diets can be seen in Figure 7.2. Before arrival F0 females were fed the standard breeding colony diet (2018S Teklad Global 18% Protein Rodent Diet, Harlan, Bicester, UK.) Upon arrival, adult female virgin Wistar rats (F0) were fed the standard animal house diet (Rat and Mouse No.1 Maintenance diet, SDS, Essex, UK) during the 14 day acclimatisation period. For the short duration of mating, females were fed the standard animal house diet; this was to ensure all breeding males were not exposed to the altered nutrition of the experiment.

For the prenatal supplementation study, six dams were randomly placed on either the folic acid adequate (FA) or folic acid supplemented (FS) diet throughout pregnancy. Pregnancy was determined by presence of the copulatory plug. Within 24 hours of birth litters were standardised to eight pups, in order to ensure even nutrition for pups. All dams were placed on the AIN-76A, lactation diet. After 28 days, offspring were weaned onto the high fat (HF) diet, and placed in group cages, with five rats being placed in each cage. At postnatal day 90 offspring were culled, and tissues were collected. The experimental design can be seen in Figure 7.1.

For the juvenile-pubertal supplementation study, six dams were placed on the folic acid adequate (FA) diet throughout pregnancy. Pregnancy was determined by presence of the copulatory plug. Within 24 hours of birth litters were standardised to eight pups, in order to ensure even nutrition for pups. All dams were placed on the AIN-93G, lactation diet. After 28 days, offspring were weaned onto either the FA or folic acid supplemented (FS) diet, and placed in group cages,

with five rats being placed in each cage. At postnatal day 56, all offspring were moved onto a high fat diet (HF) for 28 days. At postnatal day 84 offspring were culled, and tissues were collected. The experimental design can be seen in Figure 7.1.

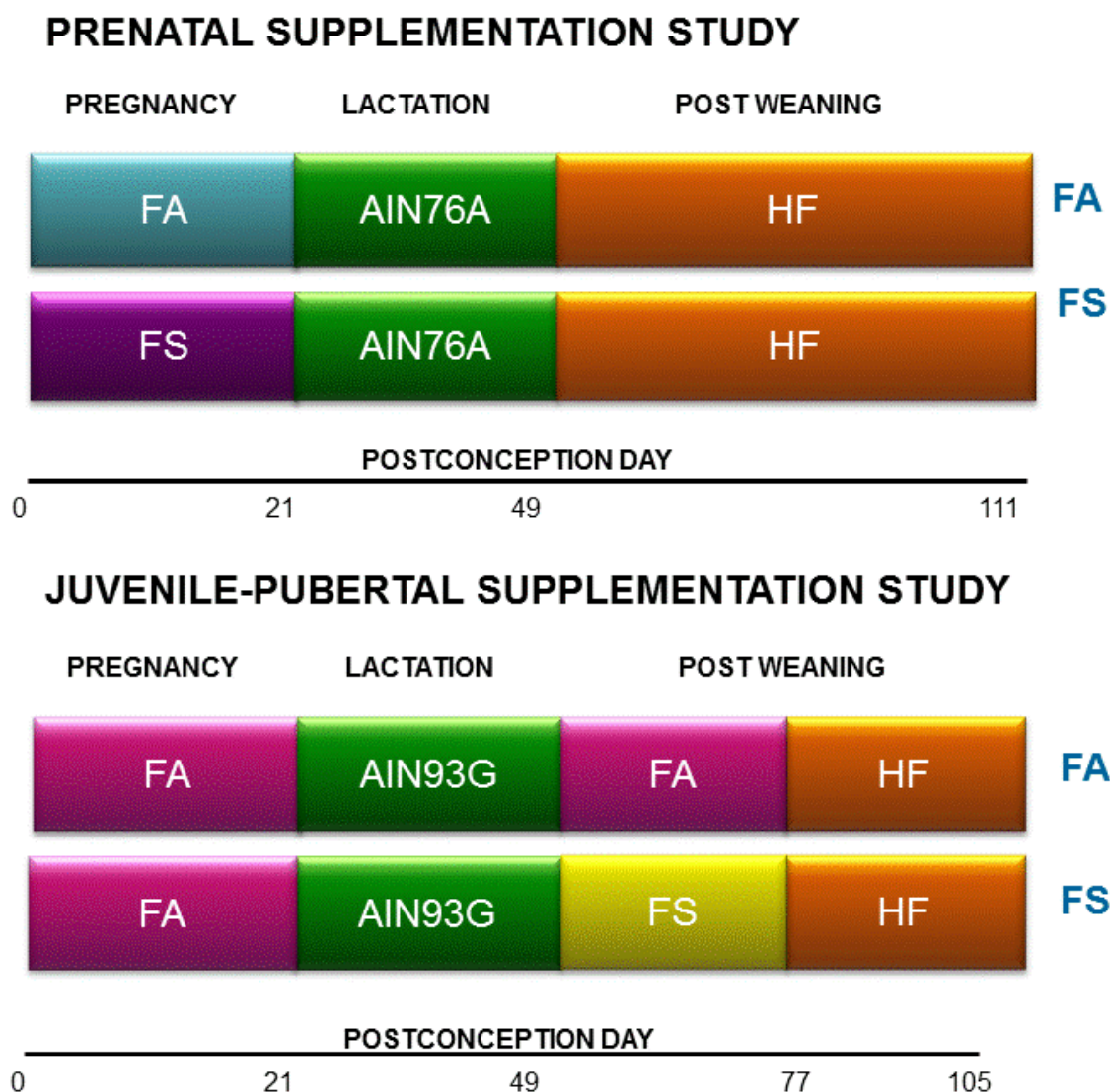


**Figure 7.1** Diagram showing the experimental design of both the prenatal and juvenile-pubertal supplementation studies. Where the folic acid adequate diet is shown by FA, folic acid supplemented diet by FS, the lactation diets by AIN76A and AIN93G and the high fat diet by HF. In the prenatal study, dams were fed either the FA or FS diet during pregnancy, during lactation all dams were fed the AIN76A diet. At weaning offspring were moved onto the high fat diet and continued on this diet until adulthood. In the postnatal supplementation study, all dams were fed the FA diet during pregnancy and the AIN93G diet during lactation. During the Juvenile-pubertal period, offspring were fed either the FA or FS diet. After 28 days, all offspring were moved on to the high fat diet. They continued on this diet until adulthood.

|                                   | Prenatal study |      | Juvenile-Pubertal study |      | AIN-93G | AIN-76A | High fat | Animal House diet | Breeding colony diet (2018S) |
|-----------------------------------|----------------|------|-------------------------|------|---------|---------|----------|-------------------|------------------------------|
| g/kg                              |                |      |                         |      |         |         |          |                   |                              |
|                                   | FA             | FS   | FA                      | FS   |         |         |          |                   |                              |
| Casein                            | 180            | 180  | 170                     | 170  | 200     | 200     | 168      | 129               | 188                          |
| Cornstarch                        | 425            | 425  | 448                     | 448  | 397     | 150     | 318      | 450               | 450                          |
| Sucrose                           | 213            | 213  | 236                     | 236  | 100     | 500     | 206      | 40.5              | 50                           |
| Choline                           | 2              | 2    | 2                       | 2    | 2.5     | 2       | 2        | 1.1               | 1.1                          |
| Methionine                        | 5              | 5    | 5                       | 5    | 5.2     | 3       | 5        | 2.2               | 4.3                          |
| Cellulose (Crude Fiber)           | 50             | 50   | 43                      | 43   | 50      | 50      | 43       | 43.2              | 38                           |
| Soybean oil                       | 100            | 100  | 4                       | 4    | 70      | 50      | 20       | 27.1              | 60                           |
| Lard                              | 0              | 0    | 36                      | 36   | 0       | 0       | 180      | 0                 | 0                            |
| Total metabolizable energy, MJ/kg | 17.3           | 17.3 | 15.9                    | 15.9 | 16.4    | 15.5    | 19.3     | 10.74             | 13.7                         |

**Table 7.1 Table showing the composition of the diets fed to animals during the study.** All nutrients are given as g/kg. Total metabolizable energy is given in MJ/kg. Diets were supplied by SDS, Essex, England (Prenatal study PS and PSF and AIN-76A) and PMI Nutrition International, Indiana, USA (Postnatal study PS and PSF and AIN-93G). The animal house diet was supplied by SDS, Essex, England. The 2018S diet was supplied by Harlan, Bicester, England.



|                  | Prenatal study |     | Juvenile-Pubertal study |       | AIN-93G | AIN-76A | High fat | Animal House diet | Breeding colony diet (2018S) |
|------------------|----------------|-----|-------------------------|-------|---------|---------|----------|-------------------|------------------------------|
| g/kg             |                |     |                         |       |         |         |          |                   |                              |
|                  | FA             | FS  | FA                      | FS    |         |         |          |                   |                              |
| Vitamin A        | 6.4            | 6.4 | 0.013                   | 0.013 | 1.2     | 6.4     | 0.013    | 2.5               | 9.21                         |
| Folic acid       | 1              | 5   | 1                       | 5     | 2       | 2       | 1        | 0.79              | 8.4*                         |
| Choline Chloride |                |     |                         |       | 1.25    |         |          | 1.08              | 1.12                         |
| Inositol         |                |     |                         |       | -       |         |          | 2.30              | 1.45                         |
| B-carotene       |                |     |                         |       | -       |         |          | 0.16              | 2.5                          |

**Table 7.2 Table showing the relevant vitamin content of the diets fed to animals during the study.** All values are given as mg/kg. Diets were supplied by SDS, Essex, England (Prenatal study PS and PSF and AIN-76A) and PMI Nutrition International, Indiana, USA (Postnatal study PS and PSF and AIN-93G). The animal house diet was supplied by SDS, Essex, England. The 2018S diet was supplied by Harlan, Bicester, England.\* As dietary folate.

|                      | Prenatal study |      | Juvenile-Pubertal study |      | AIN-93G | AIN-76A | High fat | Animal House diet | Breeding colony diet (2018S) |
|----------------------|----------------|------|-------------------------|------|---------|---------|----------|-------------------|------------------------------|
| g/kg                 |                |      |                         |      |         |         |          |                   |                              |
|                      | FA             | FS   | FA                      | FS   |         |         |          |                   |                              |
| Calcium g/kg         | 11.3           | 11.3 | 5                       | 5    | 5       | 11.3    | 5        | 7.3               | 10                           |
| Sodium g/kg          |                |      | 1                       | 1    | 1       |         | 1        | 2.5               | 2.3                          |
| Chloride g/kg        |                |      | 2                       | 2    | 1.6     |         | 2        | 3.8               | 4                            |
| Sodium Chloride g/kg | 1.7            | 1.7  |                         |      |         | 1.7     |          |                   |                              |
| Iron mg/kg           | 100            | 100  | 350                     | 350  | 41      | 100     | 350      | 159.3             | 225                          |
| Zinc mg/kg           | 36.2           | 36.2 | 34.0                    | 34.0 | 35      | 36.2    | 34.0     | 35.8              | 77                           |

**Table 7.3 Table showing the relevant mineral content of the diets fed to animals during the study.** All minerals are given as either g/kg or mg/kg. Diets were supplied by SDS, Essex, England (Prenatal study PS and PSF and AIN-76A) and PMI Nutrition International, Indiana, USA (Postnatal study PS and PSF and AIN-93G). The animal house diet was supplied by SDS, Essex, England. The 2018S diet was supplied by Harlan, Bicester, England.

### **7.2.2 Tissue collection**

After being culled livers were collected from each rat. Liver weights were measured, and livers were snap frozen in liquid nitrogen upon collection. All tissue was stored at -80°C long term.

### **7.2.3 Molecular biology procedures**

Extraction of RNA and DNA, bisulphite treatment of DNA, PCR conditions and pyrosequencing methods are described in section 2.3.

### 7.3 Results

#### 7.3.1 Does timing and route of exposure of folic acid supplementation lead to significant differences in PEPCK expression?

Supplementation of the prenatal diet during pregnancy with folic acid, did not lead to significant differences in hepatic PEPCK expression in either sex (Figures 7.2).

There was a significant effect of folic acid supplementation during the juvenile-pubertal period on PEPCK expression, in males only, where supplementation increased expression (Figure 7.2). Folic acid supplementation during the juvenile-pubertal period, did not lead to significant alterations in PEPCK expression in females (Figure 7.2).

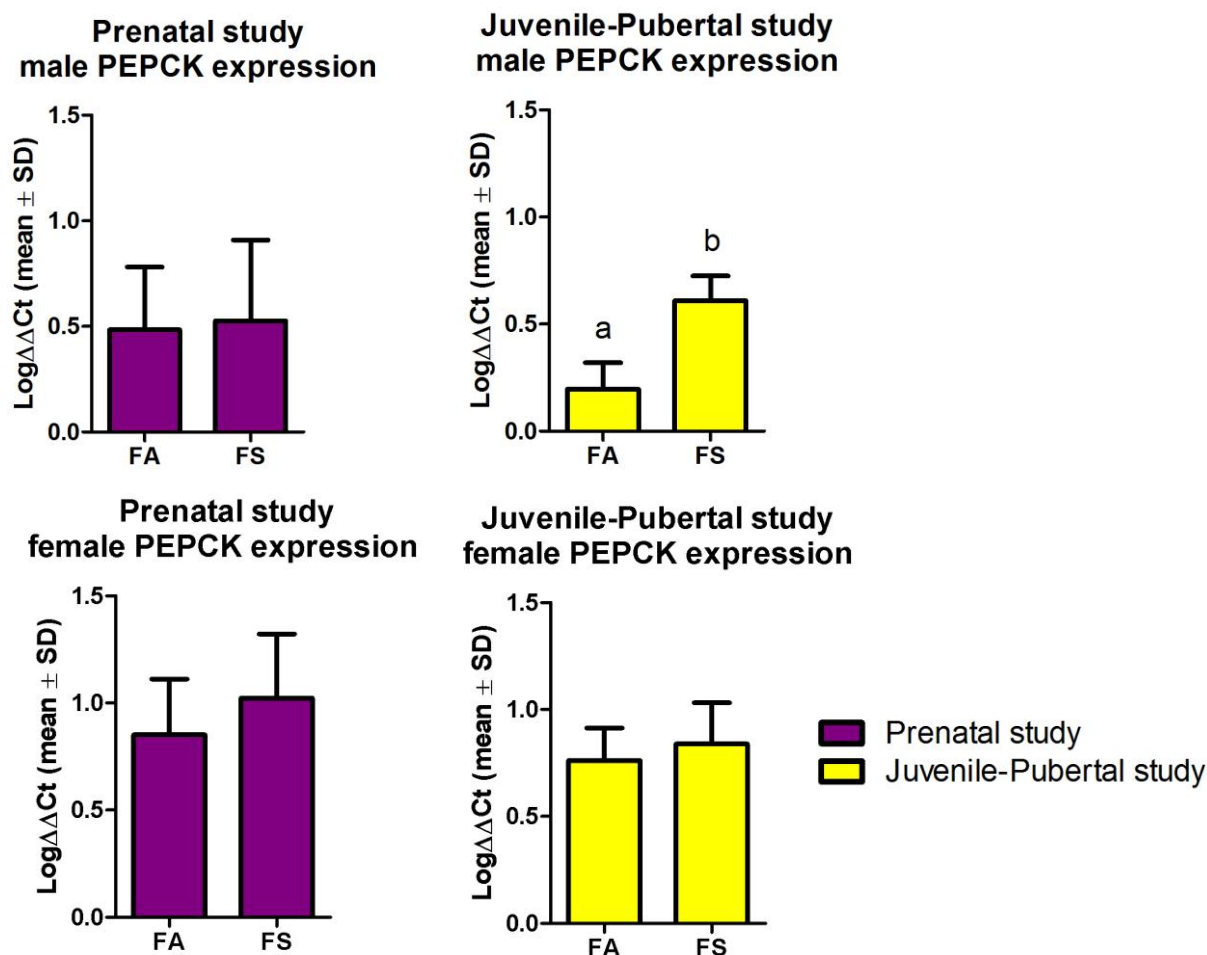


**Figure 7.2** Hepatic PEPCK mRNA expression in male and female offspring from the prenatal and juvenile-pubertal folic acid supplementation studies. Values are means with their standard deviations, where 'a' is significantly different from 'b'. Significant differences between FA and FS groups were determined using an unpaired t-test.  $P < 0.05$

### 7.3.2 Does timing and route of exposure of folic acid supplementation lead to significant differences in PEPCK methylation?

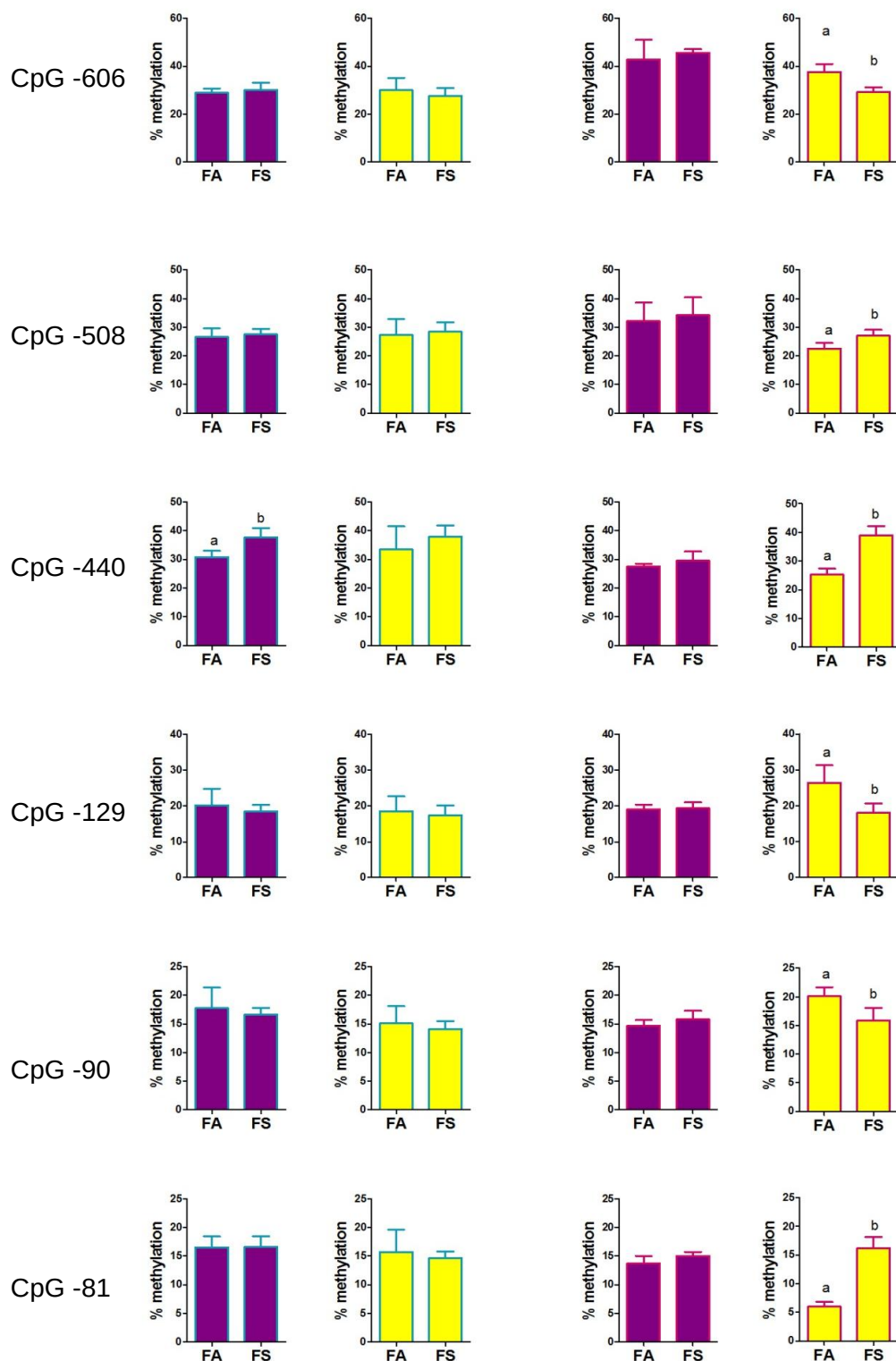
There was a significant effect of folic acid supplementation during the juvenile-pubertal period on PEPCK methylation, in females only (Figures 7.3). Methylation of the PEPCK promoter in female offspring of the juvenile-pubertal supplementation study was increased at CpGs -508, -440 and -81 in the FS group, compared to the FA group. Methylation was decreased at CpGs -606, -129 and -81, in the FS group, compared to the FA group. No differences in methylation of the PEPCK promoter were present in the male offspring, following supplementation of the prenatal diet with folic acid (Figures 7.3). Folic acid supplementation of the prenatal diet led to a significant change in DNA methylation in males only, where methylation of CpG -440 was increased in the FS group, compared to the FA group (Figure 7.3). Table 7.4 shows the interactive effects of sex and folic acid supplementation in both the prenatal and juvenile-pubertal supplementation groups on PEPCK mRNA expression and promoter methylation.

| <b>Prenatal Supplementation</b> | PEPCK Expression | CpG -606 | CpG -508 | CpG -440 | CpG -129 | CpG -90 | CpG -81 |
|---------------------------------|------------------|----------|----------|----------|----------|---------|---------|
| Folic acid supplementation      | NS               | NS       | NS       | 0.02     | NS       | NS      | NS      |
| Sex                             | NS               | NS       | NS       | NS       | NS       | NS      | NS      |
| Folic acid supplementation*Sex  | NS               | NS       | NS       | NS       | NS       | NS      | 0.014   |

| <b>Postnatal Supplementation</b> | PEPCK Expression | CpG -606 | CpG -508 | CpG -440 | CpG -129 | CpG -90 | CpG -81 |
|----------------------------------|------------------|----------|----------|----------|----------|---------|---------|
| Folic acid supplementation       | 0.007            | <0.001   | <0.001   | 0.002    | 0.007    | 0.036   | 0.005   |
| Sex                              | 0.007            | <0.001   | 0.025    | NS       | NS       | NS      | NS      |
| Folic acid supplementation*Sex   | NS               | 0.002    | 0.003    | 0.003    | NS       | NS      | <0.001  |

**Table 7.4 A General Linear Model showing significant interactive effects of folic acid supplementation and sex on PEPCK expression and promoter methylation.**

Prenatal      Juvenile-Pubertal



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### 7.3.3 Does timing and route of exposure of folic acid supplementation lead to significant differences in DNMT3a2 expression?

Supplementation of folic acid during the juvenile-pubertal period leads to a significant decrease in hepatic DNMT3a2 in males (Figure 7.14). The same supplementation did not lead to significant changes in expression in females. Prenatal supplementation of folic acid did not lead to changes in hepatic DNMT3a2 expression, in either sex (Figure 7.4).

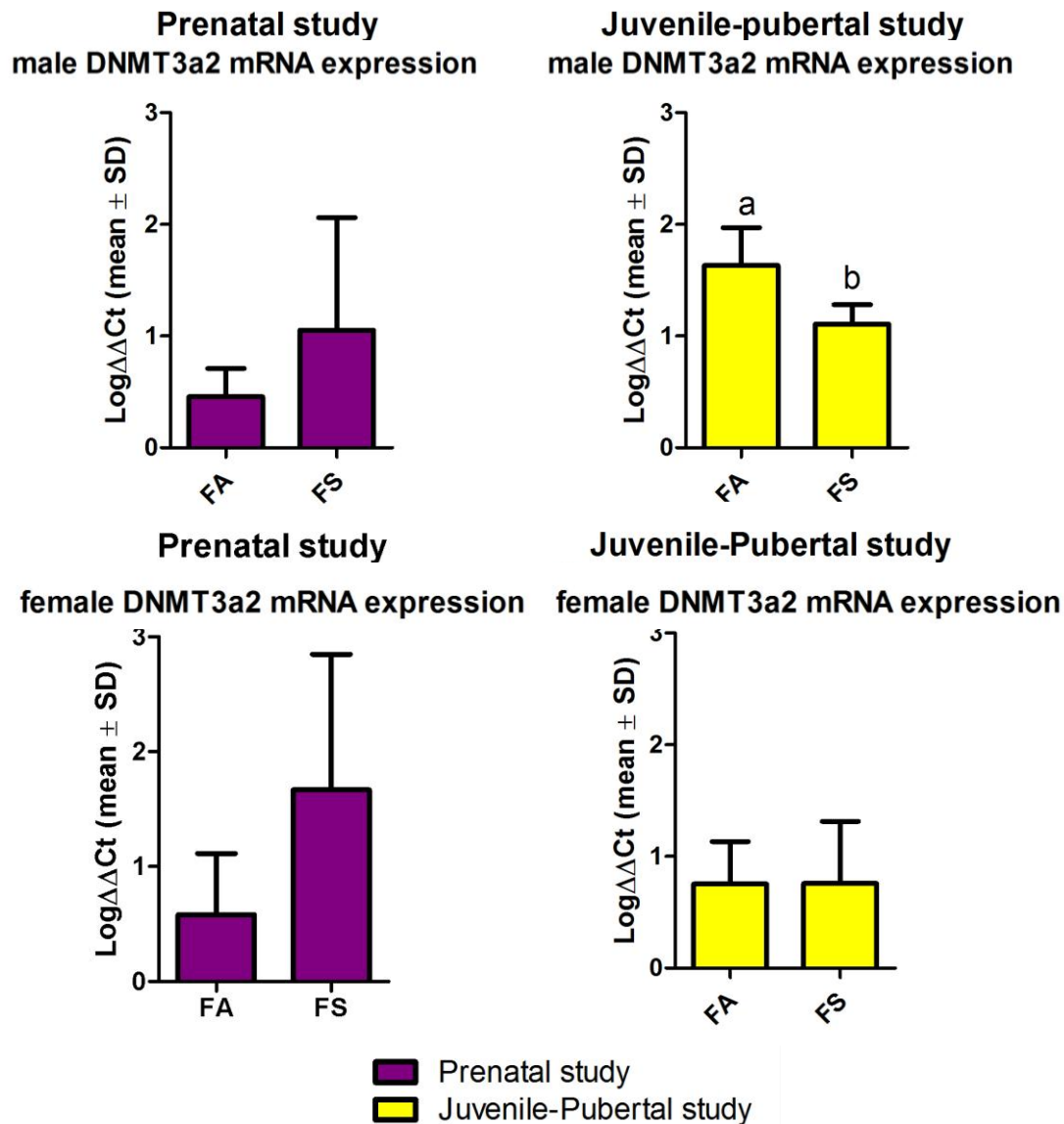


**Figure 7.4** Hepatic DNMT3a2 mRNA expression in male and female offspring from the prenatal and juvenile-pubertal folic acid supplementation studies. Values are means with their standard deviations, where 'a' is significantly different from 'b'. Significant differences between FA and FS groups were determined using an unpaired t-test.  $P < 0.05$

### 7.3.4 Does timing and route of exposure of folic acid supplementation lead to significant differences in DNMT3a2 methylation?

In female offspring of the prenatal supplementation study, folic acid led to a significant increase in methylation of CpGs -190, -56 and -39 (Figures 7.5). There was no change in methylation of CpG -207 (Figure 7.5). There were no differences in methylation of the DNMT3a2 promoter following supplementation with folic acid during the juvenile-pubertal period (Figure 7.5). No differences in DNMT3a2 promoter methylation were present in male offspring, following folic acid supplementation at any time point (Figure 7.5).

Table 7.5 shows the interactive effects of sex and folic acid supplementation in both the prenatal and juvenile-pubertal supplementation groups on DNMT3a2 mRNA expression and promoter methylation.

| <b>Prenatal<br/>Supplementation</b> | DNMT3a2<br>Expression | CpG<br>-207 | CpG<br>-190 | CpG<br>-56 | CpG<br>-39 |
|-------------------------------------|-----------------------|-------------|-------------|------------|------------|
| Folic acid<br>supplementation       | NS                    | NS          | NS          | NS         | NS         |
| Sex                                 | 0.038                 | NS          | NS          | <0.001     | <0.001     |
| Folic acid<br>supplementation*Sex   | NS                    | NS          | NS          | NS         | NS         |

| <b>Postnatal<br/>Supplementation</b> | DNMT3a2<br>Expression | CpG<br>-207 | CpG<br>-190 | CpG<br>-56 | CpG<br>-39 |
|--------------------------------------|-----------------------|-------------|-------------|------------|------------|
| Folic acid<br>supplementation        | <0.001                | NS          | 0.003       | <0.001     | <0.001     |
| Sex                                  | NS                    | 0.038       | <0.001      | <0.001     | <0.001     |
| Folic acid<br>supplementation*Sex    | NS                    | NS          | <0.001      | <0.001     | <0.001     |

**Table 7.5 A General Linear Model showing significant interactive effects of folic acid supplementation and sex on DNMT3a2 expression and promoter methylation.**



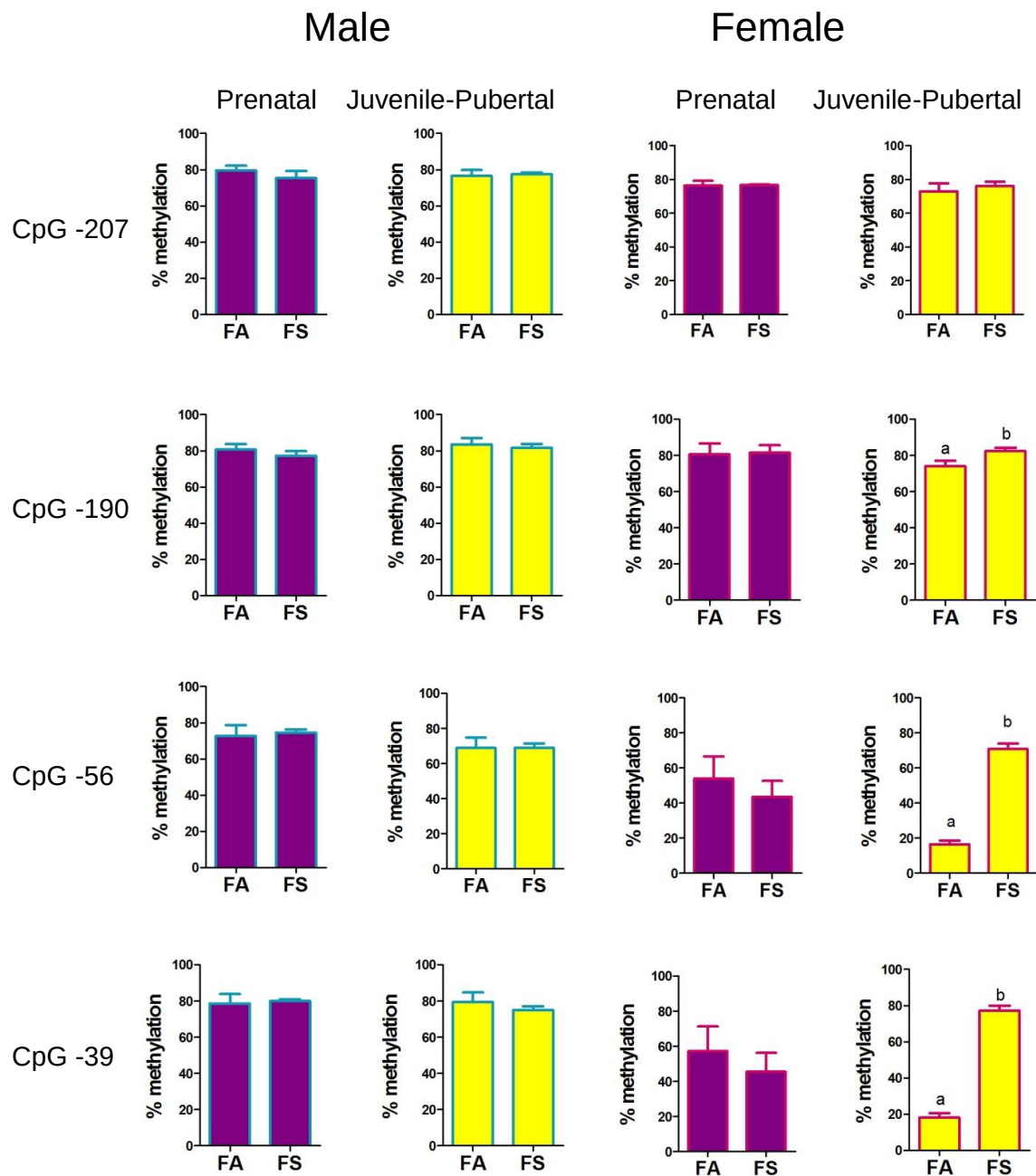


**Figure 7.5 DNMT3a2 promoter methylation in the prenatal and juvenile-pubertal folic acid supplemented offspring.** Values are means with their standard deviations, where 'a' is significantly different from 'b'. Significant differences between FA and FS groups were determined using an unpaired t-test.  $P < 0.05$

## 7.4 Discussion

Both the *in utero* environment and pre-pubescent period provide windows of developmental plasticity<sup>[277]</sup>. These results show that, in terms of regulation of PEPCK and DNMT3a2 genes, folic acid supplementation during the juvenile-pubertal period led to more significant changes in PEPCK and DNMT3a2 expression and promoter methylation, compared to prenatal supplementation. Although other genes may show different findings, in terms of the differences between timing of supplementation, the findings here suggest a different effect when supplementation is provided during the juvenile-pubertal period.

In females, changes in methylation in the PEPCK promoter, following juvenile-pubertal folic acid supplementation, were directionally opposite at specific CpG sites, suggesting the changes to the epigenome are CpG specific. Supplementation led to an increase in methylation of CpGs -508, -440 and -81 and a decrease in methylation of CpGs -606, -129 and -90. In females, no changes in methylation were present following prenatal folic acid supplementation. Supplementation of the prenatal diet during pregnancy in males led to an increase in methylation of CpG -440 in the PEPCK promoter, no other changes in methylation were present. The results suggest that folic acid only leads to differences in mRNA expression and promoter methylation of PEPCK and DNMT3a2 when provided during the juvenile-pubertal postnatal diet.

### 7.4.1 The physiological consequences of folic acid supplementation

PEPCK mRNA was significantly increased in the folic acid supplemented group of the juvenile-pubertal study. As these animals were under the physiological state of fasting, this suggests a greater capacity for gluconeogenesis and a decreased requirement for  $\beta$ -oxidation of fatty acids and creation of free radicals, in the form of peroxide, as a waste product. Unfortunately fasting plasma glucose concentrations were not available to confirm this.

Major differences in PEPCK promoter methylation were only present in the female offspring, supplemented with folic acid during the juvenile-pubertal period. As well

as suggesting timing of exposure as a key determinant in the magnitude of effects seen, it also suggests a role of sex in altered phenotype.

Although the changes in CpG methylation were not in the same direction across the PEPCK promoter, the findings do suggest an altered capacity for gluconeogenesis under different physiological condition. As stated previously, the result of alterations in promoter methylation may remain cryptic unless the correct environment is present.

mRNA expression of DNMT3a2 is higher in males in the FA group of the juvenile-pubertal study, compared to the FS male and female groups. This may suggest that females require a lower level of DNMT3a2, compared to males, representing a lower demand for metabolic plasticity. The results suggest that folic acid supplementation of the male diet during the juvenile-pubertal period leads to gene expression levels similar to that seen in the females. Although it must be stated, that due to the wide range in responses when measuring the mRNA expression of the DNMT3a2 gene, differences between the FA and FS groups of the prenatal study could not be distinguished. This could be due to the lower levels of expression of the DNMT3a2 gene, where greater variance between samples would be present.

Physiologically, the significant differences in the juvenile-pubertal study female offspring, in terms of DNMT3a2 methylation, could represent a significantly altered level of expression of the gene, under different forms of transcriptional regulation. Perhaps the role of DNMT3a2 in tissue growth and repair, in later life, such as in pregnancy, may be significantly altered by altered activating and repressing factors binding to the altered CpGs.

#### **7.4.2 The route of delivery of folic acid supplementation**

The possible explanations as to why folic acid supplementation led to different alterations when provided at the two different time points are; the method of delivery, the level of exposure and the developmental stage at which supplementation was provided. First, the effect seen by postnatal folic acid supplementation may be due to the total available amount of folic acid to the

offspring. When considering the juvenile-pubertal supplementation animals, they received the folic acid supplementation as part of the diet. However, in the prenatal supplementation study, the folic acid is fed to the dams and must first cross the placental barrier, before the fetal circulation is exposed. Transfer of folic acid across the placenta is via the Folate Receptor-a (FRa) and Reduced Folate Transporter 1 (RFC1)<sup>[278]</sup>. Data from Sweiry et al suggests that the rate of transfer of folic acid from the prenatal circulation to the fetal circulation is around 40%<sup>[279]</sup>. The magnitude of effect of folic acid supplementation may be smaller in the prenatal supplementation groups as the amount of folic acid is decreased when crossing the placenta. Alterations in DNA methylation, as a result of increased folic acid intake, are dependent on the level of folic acid. When supplementing the diet of adult rats with 0, 4.5 or 18  $\mu\text{mol}$  folate/kg feed, it was found that global DNA methylation was increased with supplementation, where the highest increase in methylation was found in the highest supplementation group<sup>[280]</sup>. Although, it must be mentioned that the differences seen were very small, with the difference shown being less than 2%, in terms of global methylation levels. This agrees with the data presented here, where the greatest difference in methylation was seen in the juvenile-pubertal supplemented offspring, which were exposed to higher levels of folic acid, through the more efficient route of delivery.

#### **7.4.3 The role of sex in differences in PEPCK and DNMT3a2 promoter methylation**

Females who received folic acid supplementation during the juvenile-pubertal period showed differences in promoter methylation. Similar differences were not present in the males who received the same diets. This is an important and novel finding, which could be explained by involvement of sex hormones, differences in establishment of the epigenome, or the role of the sex chromosomes in establishing methylation marks. Data from the Överkalix data set showed clear differences in transmission of altered phenotype through specific sex lines, where differences in males were dependent on the slow growth period of their paternal grandfather and differences in females were dependent on the slow growth period

of their maternal grandmother<sup>[163]</sup>. In the present study the prenatal folic acid supplementation was provided only to the mother, perhaps this explains why differences were only seen in female offspring? It would be of interest to see the effect of folic acid supplementation in males on the gene regulation of their offspring.

#### **7.4.4 The link between expression and methylation of PEPCK and DNMT3a2 in the offspring groups**

Postnatal folic acid supplementation during the juvenile-pubertal period led to an increase in PEPCK mRNA expression and decrease in DNMT3a2 expression in male offspring. Epigenetics underpins phenotype, however, changes in expression were not related to changes in methylation of the gene promoters, this suggests that in the physiological state of fasting, the mRNA expression of PEPCK and DNMT3a2 is not affected by altered binding at these specific CpGs, or that the changes in methylation shown here are not sufficient to lead to altered binding at the CpG. Other forms of epigenetic regulation, such as histone modifications may be affecting mRNA expression of these genes. Although the relevance of specific CpGs in these genes are unknown, further experiments will investigate this.

Differences in promoter methylation and mRNA expression were not seen between groups following prenatal folic acid supplementation, therefore the genome must be subject to changes in methylation following the establishment of the epigenome during development. As a result, the juvenile-pubertal period may allow a window of intervention, following detrimental adaptations made by offspring during fetal development. Szyf hypothesises that the epigenome is a dynamic equilibrium between methylation and demethylation<sup>[190]</sup>. The hypothesis states that; behaviour, chemical, nutritional and physical modifications can lead to alterations in signaling pathways, which then led to altered activity of DNMTs and DNA methyltransferases (DNMTases). The result is an alteration in DNA methylation levels. The hypothesis originated following the discovery that post mitotic neurons, cells which are fully differentiated and have no mitotic activity, contain DNMTs<sup>[281]</sup>. Szyf interprets this

as the cells having varying levels of methylation over time, to explain the presence of the DNA methylating enzymes, stating that the epigenome is an interface between the dynamic environment and inherited static genome<sup>[190]</sup>.

PEPCK promoter methylation is subject to demethylation during development<sup>[229]</sup>. Postnatal folic acid supplementation can lead to alterations in PEPCK promoter methylation after the methylation status has been determined during development. Potentially, alterations in PEPCK promoter methylation caused by altered exposure during development could be reversed during early postnatal life.

#### **7.4.5 Advances in current understanding**

Previous experiments into folic acid supplementation show greatest effects of folic acid supplementation, when combined with prenatal protein restriction, during pregnancy<sup>[198,51]</sup>. Here results show only one change in DNA methylation caused at a single CpG in the PEPCK promoter, in one sex, following prenatal supplementation. It appears that the effects of folic acid supplementation may be dependent on the protein content of the prenatal diet, during pregnancy. Although the differences between sexes shown here cannot be explained, they are consistent with existing literature. Following altered developmental environments, such as the Dutch hunger winter in humans and the PR and IUGR model in rats, sex-specific differences in specific phenotypic characteristics have been shown<sup>[60,48,23]</sup>.

Women are advised to take folic acid supplements before pregnancy and during the first trimester of pregnancy, in order to decrease the risk of Neural tube defects (NTDs)<sup>[282,283]</sup>. However, the long-lasting effects of folic acid supplementation on the epigenome of offspring in later life must be investigated. Although the risk of NTDs in offspring is clearly reduced following supplementation, it must be investigated if this comes at a cost. Data here has shown that prenatal folic acid supplementation has led to persistent alterations in DNA methylation it is not clear if these changes are detrimental to long term health. In the UK, it is not mandatory to fortify foods, such as bread, with folic acid. If however, this became the case it would be essential to consider the long term health effects of this

fortification, and to determine which level would be most beneficial. It is clear that the epigenome is subject to alterations during the juvenile-pubertal period. As a result it is therefore important to consider which other environmental factors, be they nutritional supplementation, in the form of vitamin supplements for teenagers, or other, may influence phenotype long term.

Folic acid supplementation during the juvenile-pubertal period led to significant changes in promoter methylation of both PEPCK and DNMT3a2, which differed between sexes. These results were not related to changes in expression of the genes. Under different conditions, such as different periods of stress, these changes may lead to significantly different levels in mRNA expression.

## Chapter 8

# The Effect of Dietary Fatty Acid Intake During Pregnancy and Lactation on the Epigenetic Regulation of Phosphoenolpyruvate Carboxykinase and Fads2



## 8.1 Introduction

### 8.1.2 Maternal high fat feeding

As described in section 1.1.3.3, alterations in the fat content of the maternal diet have been shown to lead to significant differences in the phenotype of offspring<sup>[64,65,66,67,68,69,71,72]</sup>. For example, in humans energy balance, metabolism and behaviour were significantly altered in offspring, following a maternal high fat diet<sup>[70]</sup>. In rats, feeding dams a high fat diet during pregnancy and lactation led to a significant increase in fasting glucose concentration 30 minutes after a meal<sup>[67]</sup>. Although the effect of maternal fat intake on offspring phenotype has been well documented, the underlying mechanism is unknown. Alterations in maternal fatty acid intake significantly alter the phenotype of offspring, it is not known if significant differences in regulation of PEPCK transcription also result.

### 8.1.3 $\Delta 6$ desaturase

The discussed studies show clear effects of altered fatty acid intake on offspring phenotype. It is clear that these effects are dependent on the type and amount of fat as well as the window of exposure. n-3 and n-6 PUFA have beneficial roles in terms of tissue function and cell membrane fluidity<sup>[147]</sup>.

In humans and rats, long-chain polyunsaturated fatty acids are produced from essential fatty acids, through the fatty acid desaturation/elongation pathway. The fatty acid desaturation/elongation pathway was described in Section 1.2.5. As discussed, the rate-limiting step in the production of 20:4n-6 and 22:6n-3 is the desaturation of 18:2n-6 and 18:3n-3 by  $\Delta 6$  desaturase<sup>[284]</sup>. Feeding mice a 10% fat diet has been shown to lead to a significant decrease in expression and activity of fads2, the gene which codes for  $\Delta 6$  desaturase, decreased by 50% and 70%, compared to mice fed triolein<sup>[285]</sup>.

A candidate gene study by Lattika et al has showed that polymorphisms in the Fads2 gene accounted for 28.5% of the variance in serum phospholipid levels of 20:4n-6<sup>[286]</sup>.

It is known that *fads2* is significantly altered by alterations in fat intake; however understanding of the role of promoter methylation in understanding of the gene is currently limited to one study, in mouse. In C57BL/6 mice heterozygous for disruption of cystathione  $\beta$ -synthase (*Cbs*<sup>+/-</sup>), feeding of a hyperhomocysternemia inducing diet led to a decrease in expression of the *fads2* gene and a lower activity of  $\Delta 6$  desaturase. Following analysis of a 194bp CpG rich region of the *fads2* promoter, through bisulfite conversion, PCR and pyrosequencing, it was shown that methylation at 8 out of 11 measured sites was significantly increased, compared to control mice<sup>[287]</sup>. Despite a low homology between the mouse and rat *fads2* promoters the study shows plasticity in the methylation of specific CpGs.

Significant alterations in phenotype, as a result of high fat feeding during pregnancy have been shown in terms of fasting energy metabolism and desaturation and elongation of fatty acids. It is not known if the effect of maternal high fat feeding is generalised to a subset of genes or if the effect is more specific. Therefore mRNA expression and promoter methylation of the *fads2* and *PEPCK* genes will be investigated, following feeding of high fat diets to dams during pregnancy and lactation. In order to show the effect of altered *fads2* regulation, liver and plasma phospholipid composition will also be determined.

## **8.2 Experimental design**

All animal procedures and sample collection were undertaken by Dr Christopher Kelsall, University of Southampton.

### **8.2.1 Animal model**

All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedure) Act (1986). The experimental design can be seen in Figure 8.1. Experiments diets were supplied by SDS, Essex, England. The full composition of all diets can be found in the Tables 8.1, 8.2, for macronutrients, and fatty acid composition, respectively. Virgin female Wistar rats weighing 200-250g were fed diets based on the AIN93G formulation containing 7% or 21% (w/w) total fat derived from either safflower oil (SAO) or 20:5n-3 and 22:6n-3 (fish oil, FO) from 14 days before conception and throughout pregnancy and lactation ( $n = 6$  / diet). Diet compositions can be found in Table 8.1. All diets contained the same amount of vitamin E to provide sufficient anti-oxidant capacity at the highest level of FO intake. Female rats were mated with randomly assigned males, day 0 of pregnancy being defined by plug detection. Litters were culled to 8 pups (equal numbers of males and females) within 24 hours of spontaneous birth. Offspring ( $n = 6$  males or females per dietary group) were weaned at 28 days onto AIN93-M diet containing 4% (w/w) soybean oil and maintained on this diet until day 77 when they were fasted for twelve hours and then killed by CO<sub>2</sub> asphyxiation.

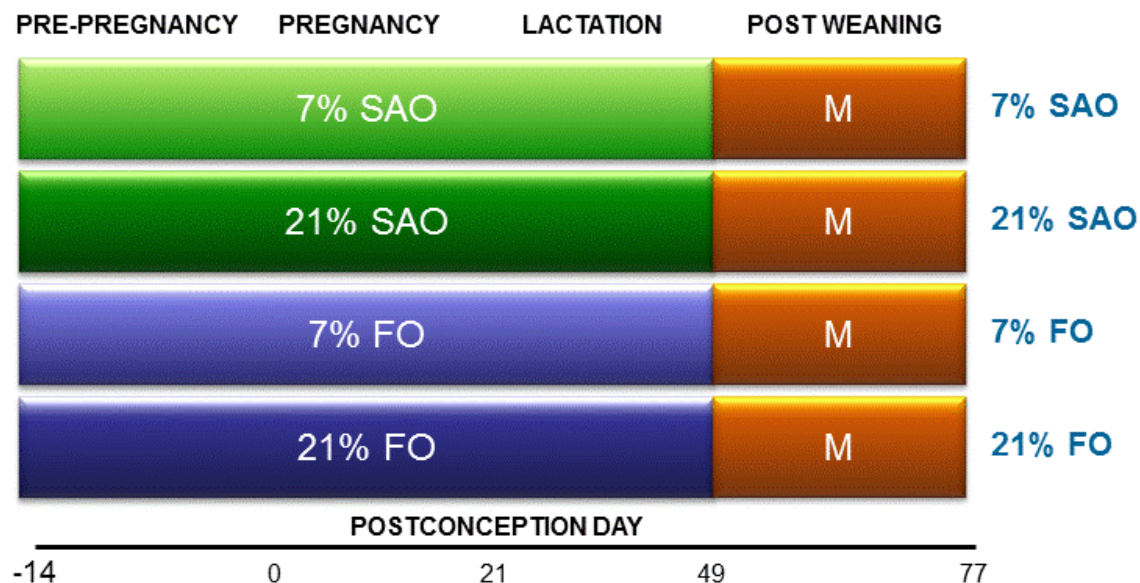


**Figure 8.1 Diagram showing the experimental design of the maternal fatty acid study.** Where the safflower oil diet is shown by SAO and the 20:5n-3 and 22:6n-3 rich fish oil diet is shown by FO. The AIN93M maintenance diet is shown by M. Dams were fed with the relevant fat diet from 14 days prior to conception, throughout pregnancy and lactation. At weaning pups were transferred to the AIN93, maintenance diet. At postnatal day 77, male and female offspring were culled for samples collection.

|                                            | 7% (w/w) |      | 21% (w/w) |      | AIN-93M |
|--------------------------------------------|----------|------|-----------|------|---------|
|                                            | Fat      |      | Fat       |      |         |
| g/kg                                       |          |      |           |      |         |
|                                            | SAO      | FO   | SAO       | FO   |         |
| Casein                                     | 20.0     | 20.0 | 20.0      | 20.0 | 14.0    |
| Cornstarch                                 | 39.7     | 39.7 | 25.7      | 25.7 | 46.6    |
| Sucrose                                    | 10.0     | 10.0 | 10.0      | 10.0 | 10.0    |
| Maltodextrin                               | 13.2     | 13.2 | 13.2      | 13.2 | 15.5    |
| Cellulose                                  | 5.0      | 5.0  | 5.0       | 5.0  | 5.0     |
| AIN-93G<br>Mineral mix                     | 3.5      | 3.5  | 3.5       | 3.5  | 3.5     |
| AIN-93G<br>Vitamin mix                     | 1.0      | 1.0  | 1.0       | 1.0  | 1.0     |
| Choline                                    | 0.25     | 0.25 | 0.25      | 0.25 | 0.25    |
| Total<br>metabolizable<br>energy,<br>MJ/kg | 1.6      | 1.6  | 1.9       | 1.9  | 1.6     |

**Table 8.1 Table showing the composition of the diets fed to animals during the study.** All nutrients are given as g/kg. Total metabolizable energy is given in MJ/kg. Diets were supplied by SDS, Essex, England.

|                                   | 7% (w/w) |     | 21% (w/w) |     | AIN-93M |
|-----------------------------------|----------|-----|-----------|-----|---------|
|                                   | Fat      |     | Fat       |     |         |
| g/kg                              |          |     |           |     |         |
|                                   | SAO      | FO  | SAO       | FO  |         |
| Total saturated fatty acids       | 70       | 213 | 209       | 640 | 110     |
| Total monounsaturated fatty acids | 124      | 202 | 373       | 606 | 165     |
| 18:2n-6                           | 505      | 14  | 1515      | 42  | 376     |
| 18:3n-6                           | N        | 3   | N         | 8   | 2       |
| 20:3n-6                           | N        | 2   | N         | 6   | N       |
| 20:4n-6                           | N        | 10  | N         | 29  | N       |
| 22:4n-6                           | N        | 1   | N         | 4   | N       |
| 22:5n-6                           | N        | 2   | N         | 6   | N       |
| Total n-6 PUFA                    | 505      | 29  | 1515      | 88  | 378     |
| 18:3n-3                           | 1        | 8   | 2         | 23  | 48      |
| 20:5n-3                           | N        | 142 | N         | 427 | N       |
| 22:5n-3                           | N        | 19  | N         | 58  | N       |
| 22:6n-3                           | N        | 83  | N         | 248 | N       |
| Total n-3 PUFA                    | 1        | 252 | 2         | 755 | 48      |
| 18:2n-6 : 18:3n-3                 | 739      | 2   | 739       | 2   | 8       |

**Table 8.2 Table showing the fatty acid composition of the diets fed to animals during the study.** All fatty acids are given as g/kg. Where N represents not detected.

### **8.2.2 Tissue collection**

After being culled livers were collected from each rat and were snap frozen in liquid nitrogen upon collection. All tissue was stored at -80°C long term. Blood was collected by cardiac puncture into tubes containing lithium heparin, and plasma separated from cells by centrifugation. Plasma was stored at -80°C.

### **8.2.3 Molecular biology procedures**

Extraction of RNA and DNA, bisulphite treatment of DNA, PCR conditions and pyrosequencing methods are described in section 2.3.

### **8.2.4 Analysis of phospholipid composition**

Fatty acid composition of liver and plasma phospholipid was determined by gas chromatography, as described in Burdge 2000<sup>[250]</sup>. These analyses were carried out by Nicola Irvine. Briefly, liver (approximately 100mg) was powdered under liquid nitrogen and total lipids extracted with chloroform and methanol<sup>[251]</sup>. Plasma (0.8ml) was extracted with chloroform and methanol<sup>[251]</sup>. PC was isolated from total lipid extracts by solid phase extraction using 100mg aminopropyl silica cartridges<sup>[250]</sup> and converted to fatty acid methyl esters (FAMES) by incubation with methanol containing 2%(v/v) sulphuric acid<sup>[250]</sup>. FAMES were recovered by extraction with hexane. The proportions of individual fatty acids were measured by gas chromatography using BPX70 fused silica capillary column (30m x 0.25mm x 0.25µm) on an Agilent 6890 gas chromatograph equipped with flame ionisation detection. FAMES were identified by their retention times relative to standards and quantified using Chemstation software.

### **8.3 Results**

#### **8.3.1 Does type and amount of maternal dietary fat alter fasting glucose concentration in male and female offspring?**

There were no significant effects of type and amount of maternal dietary fat on fasting glucose concentration in offspring (Figure 8.2) Fasting plasma glucose concentration in female offspring of dams fed a fish oil during pregnancy and lactation were significantly lower than the levels shown in the male offspring.



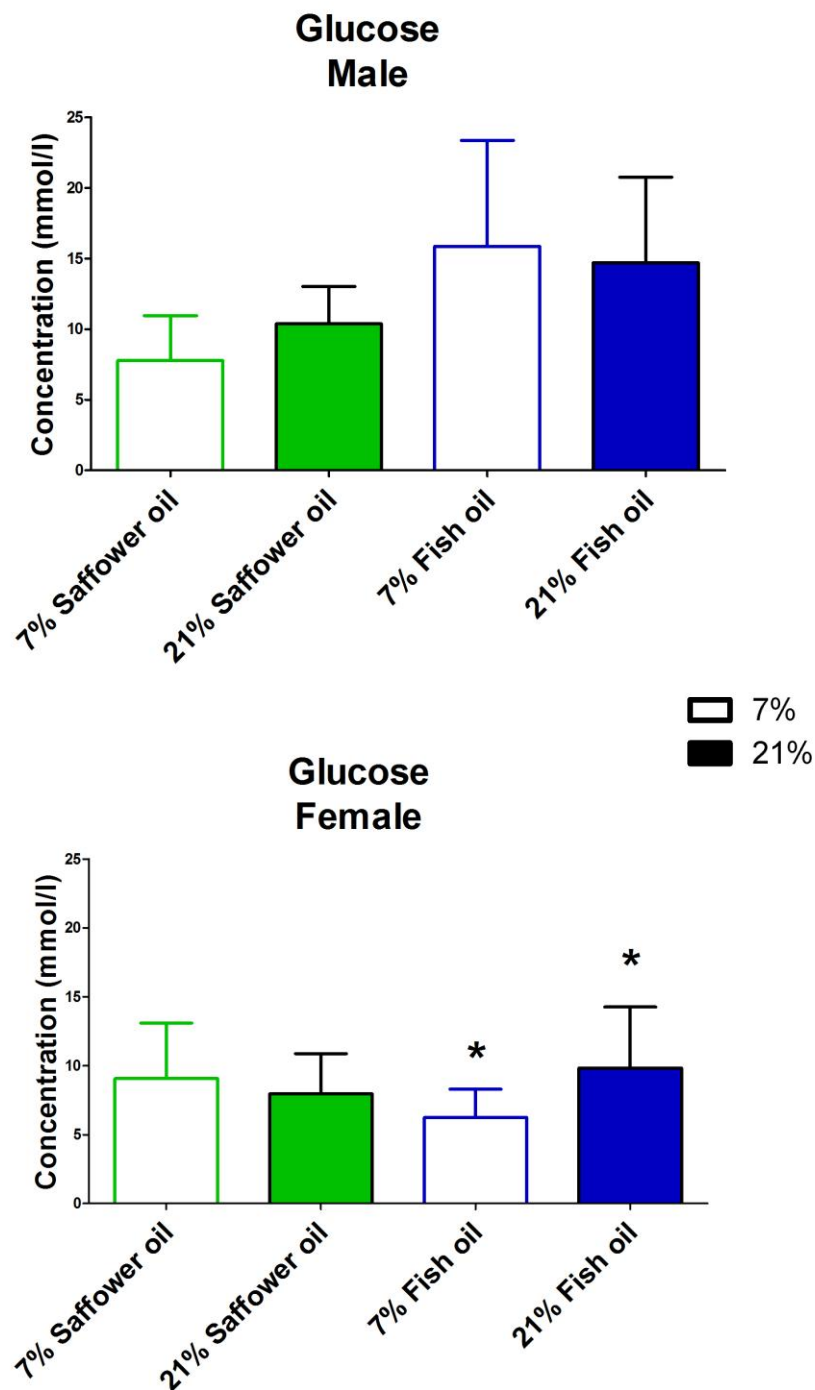


**Figure 8.2 Fasting plasma glucose values in male and female offspring.** Values are means with their standard deviation. \* represents a significant difference between genders, determined by Student's T-test. Significant differences between groups was by one-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$ .

### **8.3.2 Does type and amount of maternal dietary fat alter hepatic PEPCK mRNA expression in male and female offspring?**

There was a significant effect of type of maternal dietary fat on offspring hepatic PEPCK mRNA expression in males only (Figure 8.3).

In male offspring there was a significant effect of amount of dietary fat in the SAO group, where expression was significantly increased in the 21% group, compared to the 7% group.

In female offspring there was no effect of type of maternal dietary fat.

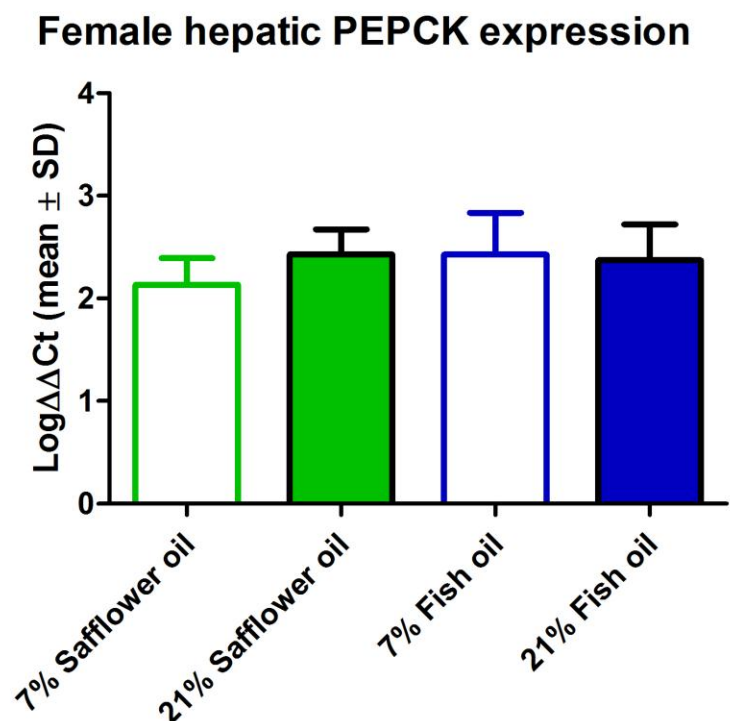
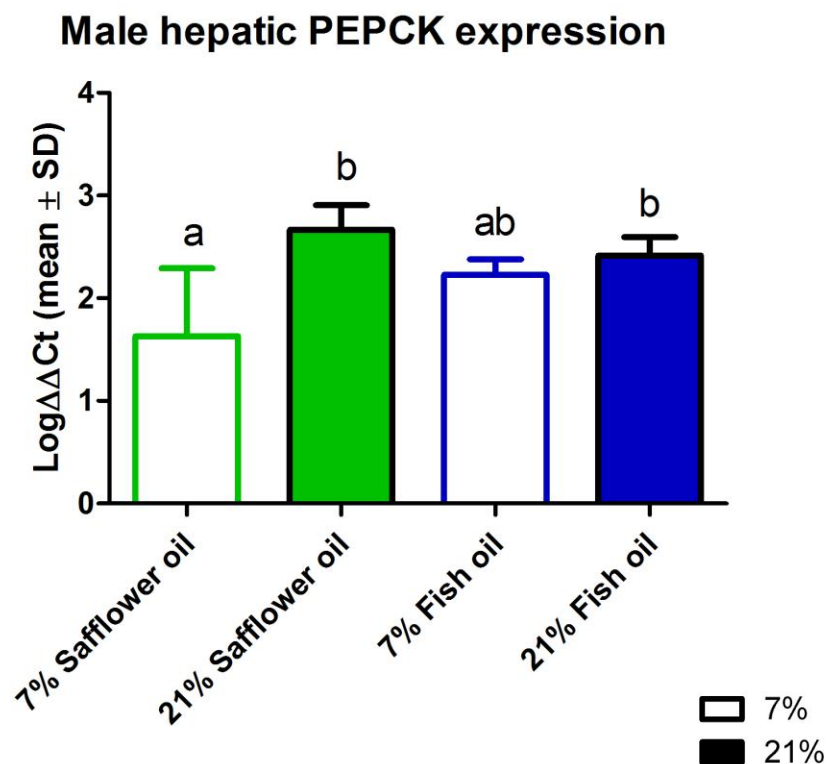


**Figure 8.3 Hepatic PEPCK mRNA expression in male and female offspring.** Values are means with their standard deviations, where 'a' is significantly different from 'b' and 'ab' is neither significantly different from 'a' or 'b'. # represents an effect of amount of maternal dietary fat between 7 and 21% fat. Significant differences between diets was by one-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$ .

### 8.3.3 Does type and amount of maternal dietary fat alter liver PEPCK promoter methylation in male and female offspring?

There were no significant differences between groups in methylation of the PEPCK promoter. CpG -81, in male offspring, appears to show significant differences between groups, where the 21% FO group has no detectable level of methylation (Figure 8.4). However the minimum threshold of the pyrosequencer is 7%, this means no statistical analysis can be performed on results below this level. There were no significant effects of type or amount of maternal dietary fat on liver promoter methylation in female offspring (Figure 8.5).

Table 8.4 shows the interactive effects of type and amount of maternal dietary fat on CpG specific methylation of the PEPCK promoter.

| <b>MALES</b> | CpG<br>-606 | CpG<br>-508 | CpG<br>-440 | CpG<br>-129 | CpG<br>-90 | CpG<br>-81 |
|--------------|-------------|-------------|-------------|-------------|------------|------------|
| % Fat        | 0.526       | 0.169       | 0.509       | 0.701       | 0.053      | 0.195      |
| Fat type     | 0.859       | 0.622       | 0.609       | 0.916       | 0.331      | 0.404      |
| Interaction  | 0.899       | 0.481       | 0.642       | 0.903       | 0.186      | 0.374      |

| <b>FEMALES</b> | CpG<br>-606 | CpG<br>-508 | CpG<br>-440 | CpG<br>-129 | CpG<br>-90 | CpG<br>-81 |
|----------------|-------------|-------------|-------------|-------------|------------|------------|
| % Fat          | 0.569       | 0.438       | 0.817       | 0.738       | 0.371      | 0.316      |
| Fat type       | 0.390       | 0.266       | 0.185       | 0.991       | 0.224      | 0.579      |
| Interaction    | 0.254       | 0.513       | 0.874       | 0.306       | 0.845      | 0.900      |

**Table 8.4 A General Linear Model showing significant interactive effects of type and amount of maternal dietary fat on PEPCK promoter methylation in male and female offspring.**

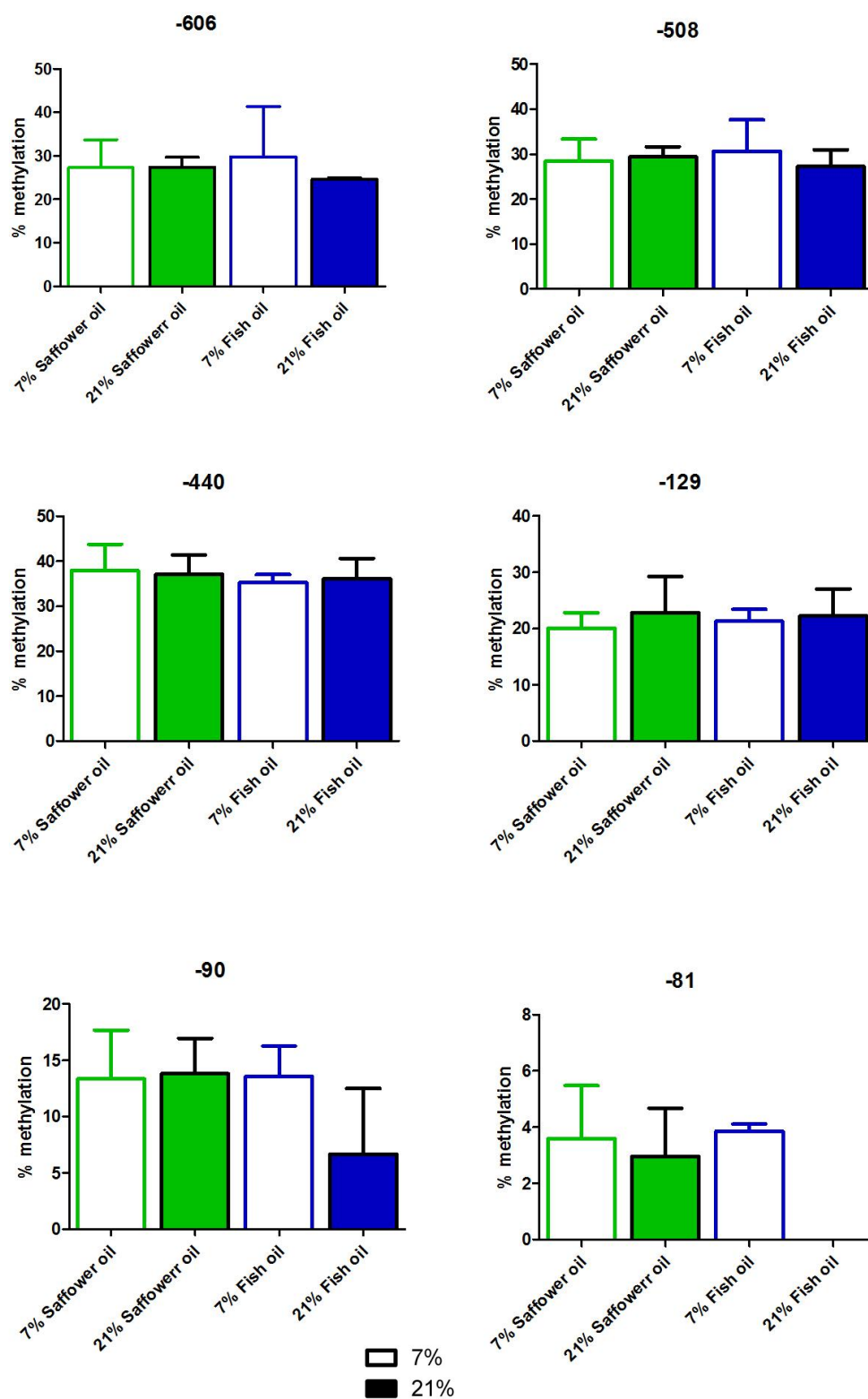


**Figure 8.4 PEPCK promoter methylation in male offspring.** Values are means with their standard deviations, where '#' represents an effect of % maternal fat. Significant differences were by one-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.005$ .

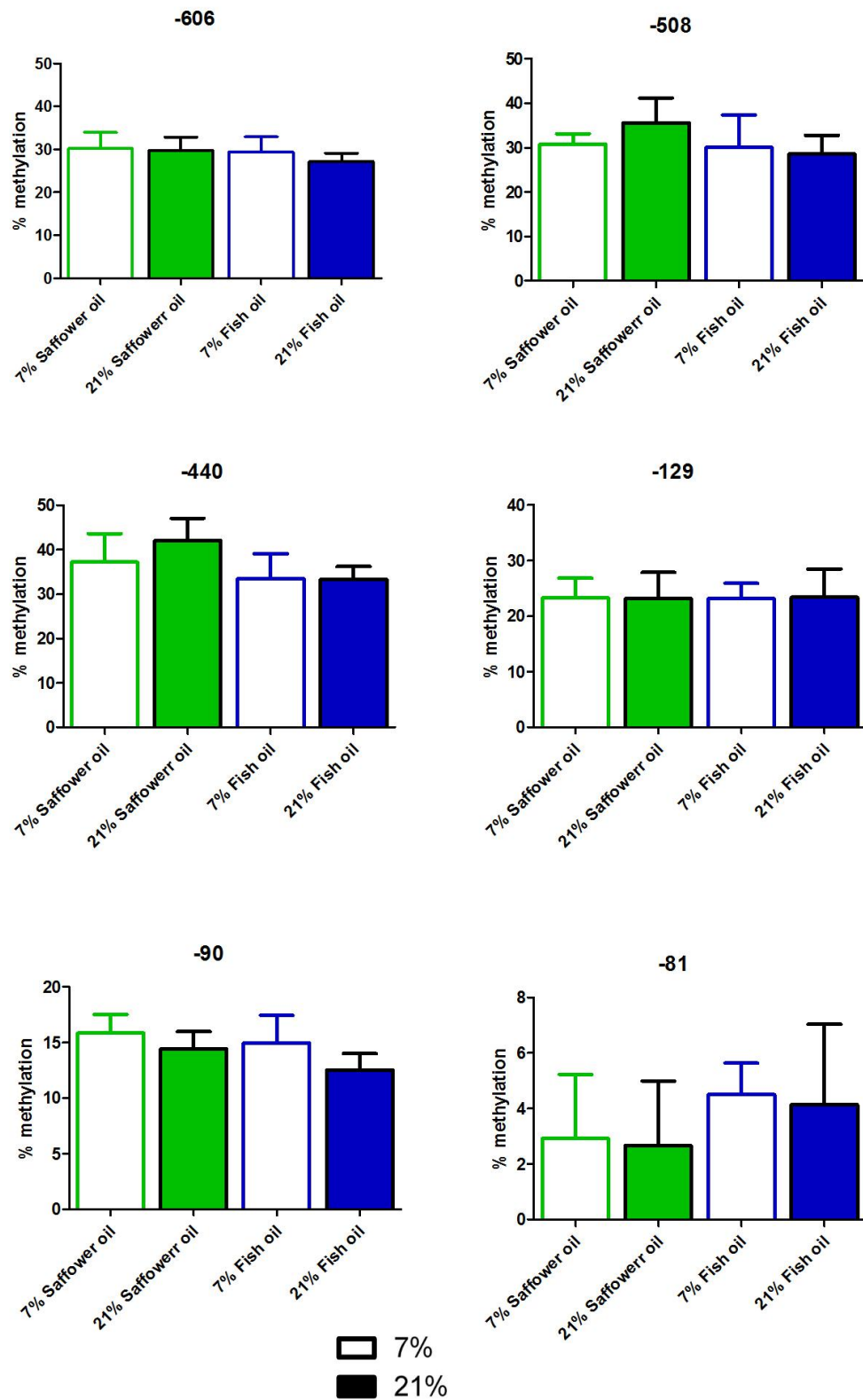


**Figure 8.5 PEPCK promoter methylation in female offspring.** Values are means with their standard deviations. Significant differences were by one-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.005$ .

#### **8.3.4 Does type and amount of maternal dietary fat alter plasma and liver phospholipid composition in male and female offspring?**

There was a significant effect of maternal fat type and amount on the fatty acid composition of liver and plasma phosphatidylcholine (PC), in a manner contingent on sex. As shown in table 8.5, there were no differences in the proportions of 18:2n-6, 18:3n-3 and 18:3n-6 in liver PC. There were relatively small differences between maternal dietary groups and sexes in the proportions of 20:3n-6, 20:5n-3 and 22:5n-3, in liver PC composition. The principal effects of maternal dietary fat content were on the proportions of 20:4n-6 and 22:6n-3 (Figure 8.6).

There were significant single factor effects of sex ( $P < 0.0001$ ), amount of fat ( $P = 0.001$ ) and sex\*fat type ( $P < 0.0001$ ) on the proportion of 20:4n-6 in liver PC. There was no difference between maternal dietary groups in the proportion of 20:4n-6 in male or female offspring of dams fed diets containing 7% fat, in both liver and plasma PC (Figure 8.6) In male offspring, there was an effect of amount of fat for 20:4n-6 content of PC, in liver, in the FO group. In all cases, the 21% groups had a lower 20:4n-6 PC content. In males there was an effect of type of fat on PC 22:6n-3 content, in liver only, where the 21% SAO group had a decreased amount, compared to the 7% group. In females, there was an effect of amount of fat in liver and plasma PC 20:4n-6 content in all groups, except for SAO plasma, where the 21% maternal fat diet led to a decreased 20:4n-6 content. In females, there was an effect of amount of fat in liver and plasma PC 22:6n-3 content in both the SAO and FO groups, where the 21% maternal fat diet led to a decreased 22:6n-3 content

There was a significant effect of sex in PC 22:6n-3 composition in liver for both 7% and 21% SAO groups.

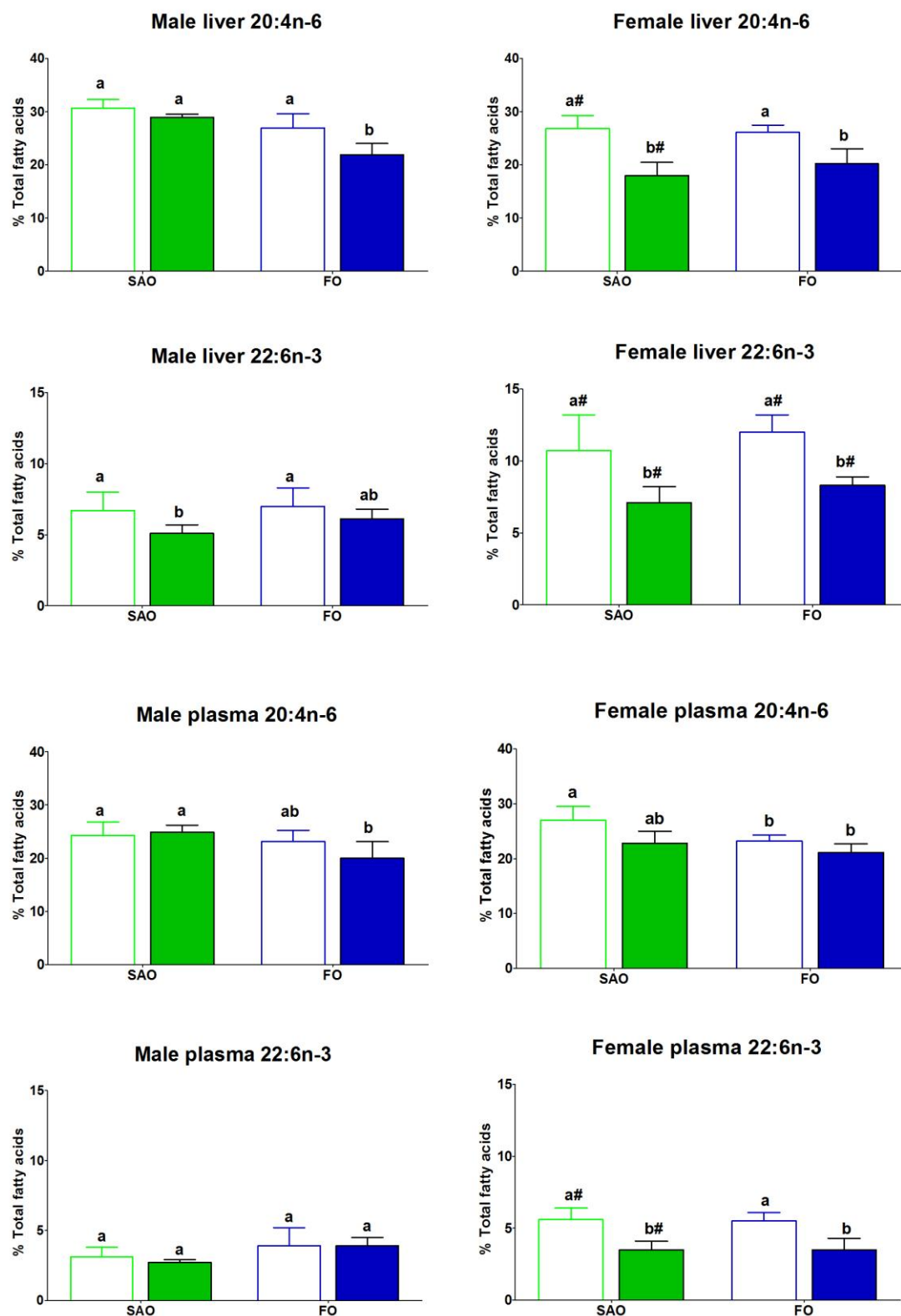


**Figure 8.6 20:4n-6 and 22:6n-3 composition of liver and plasma PC in male and female offspring.** Values are means with their standard deviations. Where 'a' is significantly different from 'b', and 'ab' is neither significantly different from 'a' or 'b'. # represents a significant effect of sex. Significant differences were by one-way ANOVA, with Bonferroni's *post hoc* test  $P < 0.005$ .



|                       | 18:2n-6                  | 18:3n-3   | 20:3n-6                | 18:3n-3   | 20:5n-3                | 22:5n-3   |
|-----------------------|--------------------------|-----------|------------------------|-----------|------------------------|-----------|
|                       | Male 7% fat offspring    |           |                        |           |                        |           |
| SAO                   | 12.1 ± 1.8               | 0.3 ± 0.2 | 0.5 ± 0.2 <sup>a</sup> | 0.2 ± 0.2 | 0.2 ± 0.1 <sup>a</sup> | 0.9 ± 0.3 |
| FO                    | 13.3 ± 1.4               | 0.2 ± 0.1 | 0.8 ± 0.3 <sup>a</sup> | 0.1 ± 0.1 | 0.5 ± 0.3 <sup>a</sup> | 0.9 ± 0.1 |
|                       | Male 21% fat offspring   |           |                        |           |                        |           |
| SAO                   | 12.2 ± 1.6               | 0.1 ± 0.1 | 0.4 ± 0.1 <sup>a</sup> | 0.1 ± 0.1 | 0.1 ± 0.1 <sup>a</sup> | 0.8 ± 0.2 |
| FO                    | 11.4 ± 0.6               | 0.2 ± 0.1 | 1.0 ± 0.4 <sup>b</sup> | 0.1 ± 0.1 | 1.4 ± 0.4 <sup>b</sup> | 1.1 ± 0.4 |
|                       | Female 7% fat offspring  |           |                        |           |                        |           |
| SAO                   | 10.2 ± 1.3               | 0.3 ± 0.1 | 0.4 ± 0.1              | 0.1 ± 0.1 | 0.2 ± 0.2 <sup>a</sup> | 1.0 ± 0.2 |
| FO                    | 9.7 ± 1.1                | 0.2 ± 0.5 | 0.4 ± 0.1              | 0.1 ± 0.1 | 0.5 ± 0.3 <sup>a</sup> | 0.9 ± 0.1 |
|                       | Female 21% fat offspring |           |                        |           |                        |           |
| SAO                   | 10.5 ± 3.2               | 0.2 ± 0.1 | 0.5 ± 0.3              | 0.1 ± 0.1 | 0.3 ± 0.1 <sup>a</sup> | 0.9 ± 0.4 |
| FO                    | 12.5 ± 1.9               | 0.2 ± 0.1 | 0.5 ± 0.2              | 0.3 ± 0.4 | 1.1 ± 0.6 <sup>b</sup> | 0.8 ± 0.2 |
|                       | GLM (p)                  |           |                        |           |                        |           |
| Sex                   | 0.003                    | NS        | NS                     | NS        | NS                     | NS        |
| % fat                 | NS                       | NS        | 0.003                  | NS        | <0.0001                | NS        |
| Type of fat           | NS                       | NS        | <0.0001                | NS        | <0.0001                | NS        |
| Sex*% Fat             | NS                       | NS        | 0.029                  | NS        | NS                     | NS        |
| % fat*type of fat     | NS                       | NS        | 0.001                  | NS        | NS                     | NS        |
| Sex*% fat*type of fat | NS                       | NS        | NS                     | NS        | <0.0001                | NS        |

**Table 8.5 Liver phosphatidylcholine fatty acid composition in male and female offspring**, where 'a' is significantly different from 'b'. Significant differences were determined by one-way ANOVA (p<0.005). General linear model results are also shown.

### **8.3.5 Does type and amount of maternal dietary fat alter hepatic fads2 mRNA expression in male and female offspring?**

There was a significant effect of amount of maternal dietary fatty acid on hepatic fads2 mRNA expression in all groups in the male and female offspring (Figure 8.7). There was no effect of type of fat on hepatic fads mRNA expression in either sex.

Hepatic mRNA expression of the fads2 gene predicted 9% and 26% of 20:4n-6 and 22:6n-3 content of liver PC in males, respectively. In females, fads2 predicted 41% and 15% of 20:4n-6 and 22:6n-3 content of liver PC, respectively.

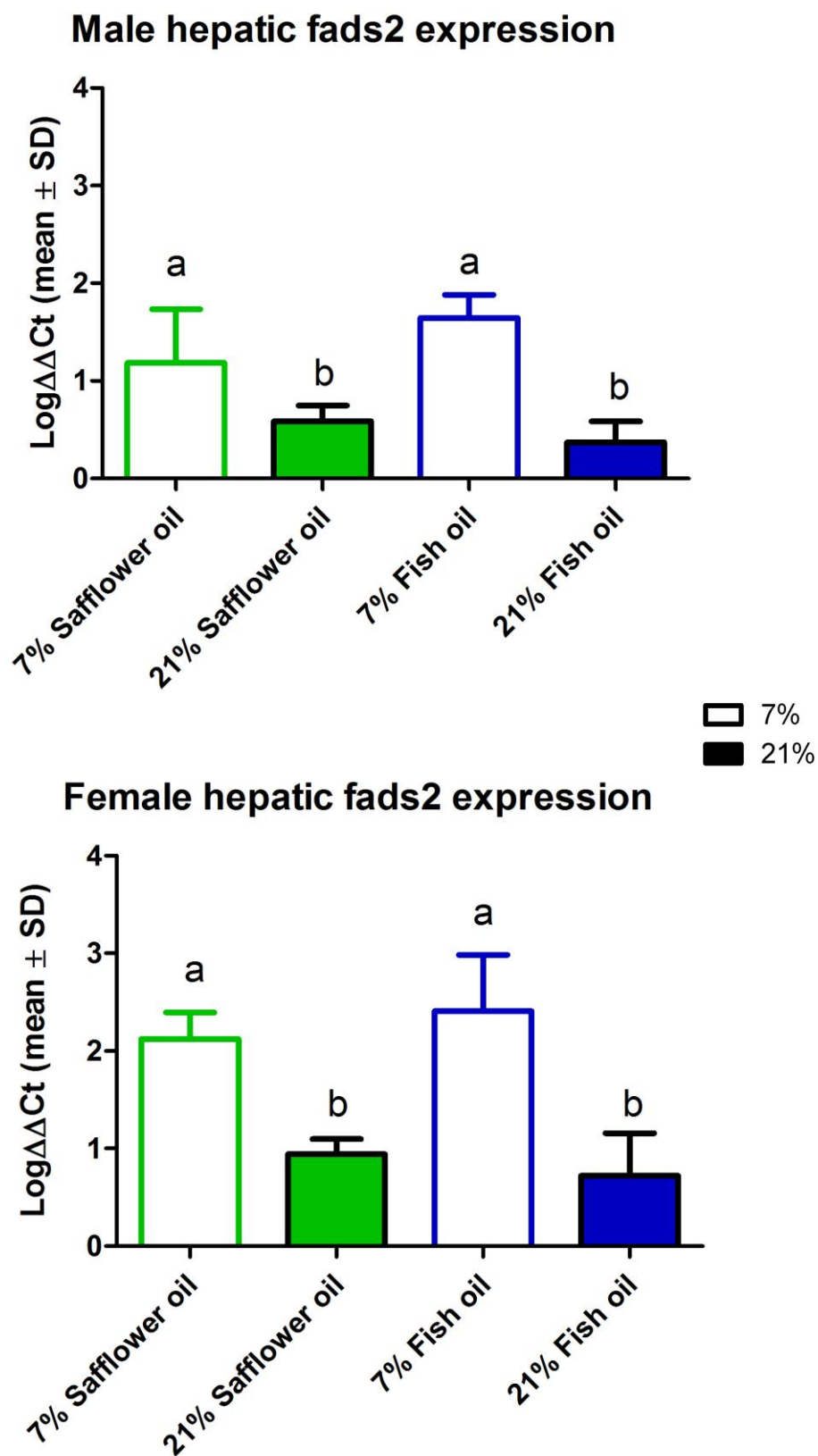


**Figure 8.7 Hepatic fads2 mRNA expression in male and female offspring.** Values are means with their standard deviations. \* represents an effect of amount of maternal dietary fat between 7 and 21% fat. Significant differences between diets was by one-way ANOVA with Bonferroni's *post hoc* test.  $P < 0.05$ .

### **8.3.6 Does type and amount of maternal dietary fat alter liver fads2 promoter methylation in male and female offspring?**

There were significant effects of type of maternal dietary fat on CpG specific methylation of the fads2 promoter, in liver, in male and female offspring (Figures 8.8 and 8.9). There were effects of amount and type of fat in females, in methylation of CpG -394.

In males, methylation of CpG -623 in males was significantly lower in the 7% SAO group, compared to the 7% FO group. In males, methylation of CpG -394 was significantly higher in the 21% FO group, compared to the 21% SAO group. In males methylation of CpG -84 was significantly higher in the 7% SAO group, compared to the 21% SAO group. Methylation of CpG -76 in males was significantly higher in the 7% FO group, compared to the 7% SAO group.

In females, methylation of CpGs -394 was significantly increased in the 21% fat groups, where the 21% FO group was significantly higher than the 21% SAO group. Methylation of CpG -84 was significantly lower in the 7% SAO group, compared to the 7% FO groups. Methylation of CpG -76 was significantly increased in the 7% and 21% FO groups, compared to the 7% and 21% SAO groups. Methylation of CpG -65 was significantly increased in the 7% FO group, compared to both SAO groups.

Table 8.6 shows the interactive effects of type and amount of maternal dietary fat on CpG specific methylation of the fads2 promoter.

| <b>MALES</b> | CpG<br>-722 | CpG<br>-711 | CpG<br>-678 | CpG<br>-623 | CpG<br>-608 | CpG<br>-585 | CpG<br>-394 | CpG<br>-84 | CpG<br>-76 | CpG<br>-65 | CpG<br>-31 |
|--------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|------------|------------|------------|------------|
| % Fat        | 0.32        | 0.09        | 0.001       | 0.24        | 0.63        | 0.94        | 0.32        | 0.001      | 0.01       | 0.25       | 0.22       |
| Fat type     | 0.02        | 0.21        | 0.06        | 0.001       | 0.70        | 0.61        | 0.002       | 0.01       | 0.01       | 0.09       | 0.06       |
| Interaction  | 0.65        | 0.08        | 0.63        | 0.14        | 0.50        | 0.13        | 0.26        | 0.21       | 0.48       | 0.12       | 0.54       |

| <b>FEMALES</b> | CpG<br>-722 | CpG<br>-711 | CpG<br>-678 | CpG<br>-623 | CpG<br>-608 | CpG<br>-585 | CpG<br>-394 | CpG<br>-84 | CpG<br>-76 | CpG<br>-65 | CpG<br>-31 |
|----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|------------|------------|------------|------------|
| % Fat          | 0.33        | 0.08        | 0.96        | 0.96        | 0.47        | 0.56        | 0.04        | 0.05       | 0.07       | 0.68       | 0.15       |
| Fat type       | 0.73        | 0.24        | 0.32        | <0.01       | 0.81        | 0.02        | <0.01       | <0.01      | <0.01      | <0.01      | <0.01      |
| Interaction    | 0.67        | 0.24        | 0.08        | 0.46        | 0.86        | 0.20        | 0.19        | 0.13       | 0.24       | 0.05       | 0.92       |

**Table 8.6 A General Linear Model showing significant interactive effects of type and amount of maternal dietary fat on fads2 promoter methylation in male and female offspring.**

Linear regression analysis was used to show the correlation between promoter methylation and mRNA expression. In males, methylation of CpGs -394, -84 and -76 predicted 6%, 12% and 16% of fads2 expression, respectively. In females the effect was greater where CpGs -394, -84 and -76 predicted 55%, 16% and 28% of fads2 expression, respectively. In both sexes methylation of CpG -394 was negatively associated with fads2 mRNA expression and CpGs -84 and -76 were positively associated with mRNA expression.

## Male offspring *fads2* promoter methylation

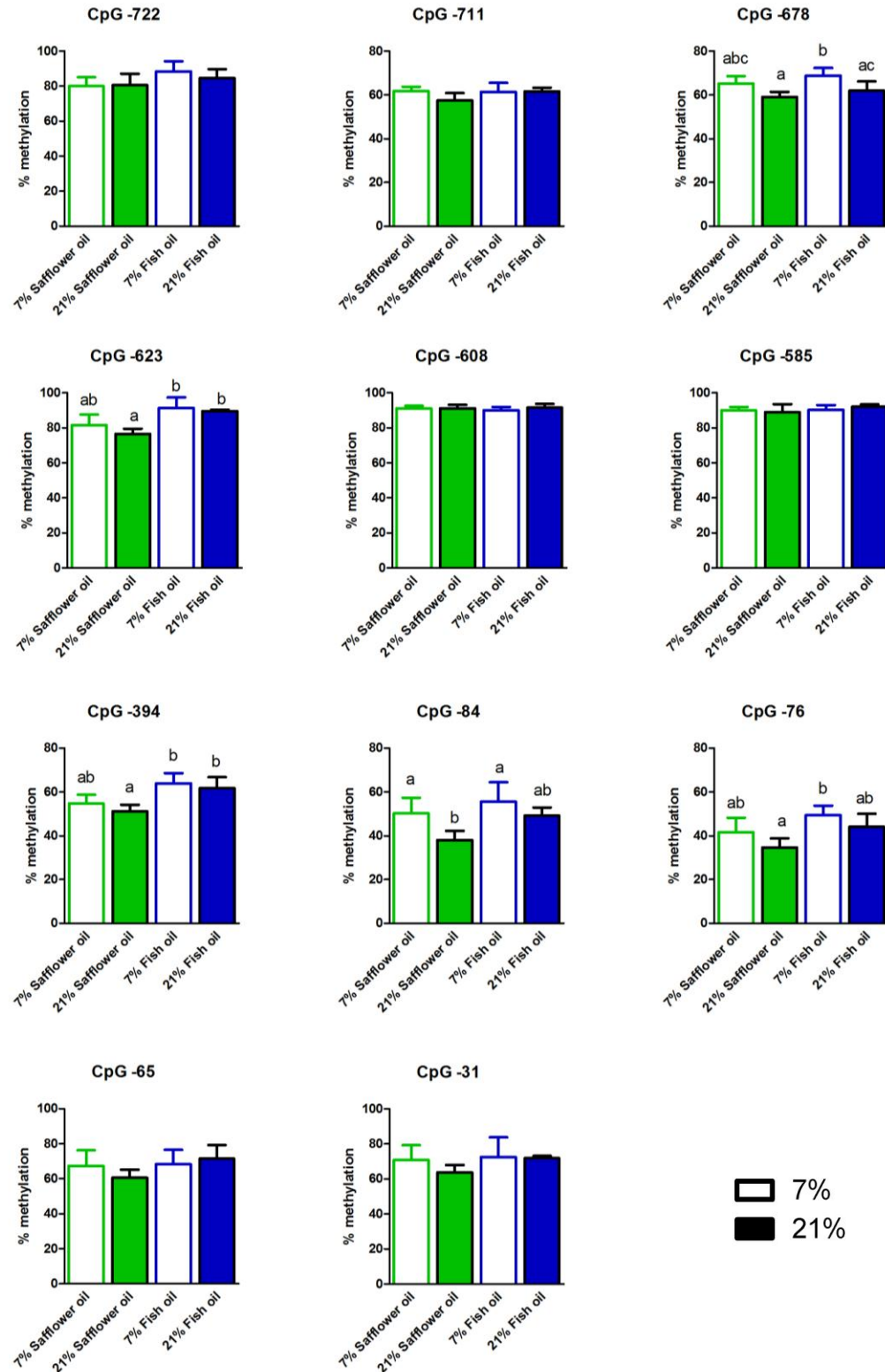


**Figure 8.8 *fads2* promoter methylation in male offspring.** Values are means with their standard deviations. Where ‘\*’ represents a significance of  $P < 0.05$  and ‘\*\*\*’ a significance of  $P < 0.0005$ . ‘#’ represents significantly different from all other dietary groups, with the same maternal % dietary fat. Significant differences were by one-way ANOVA, with Bonferroni’s *post hoc* test  $P < 0.005$ .

## Female offspring fads2 promoter methylation

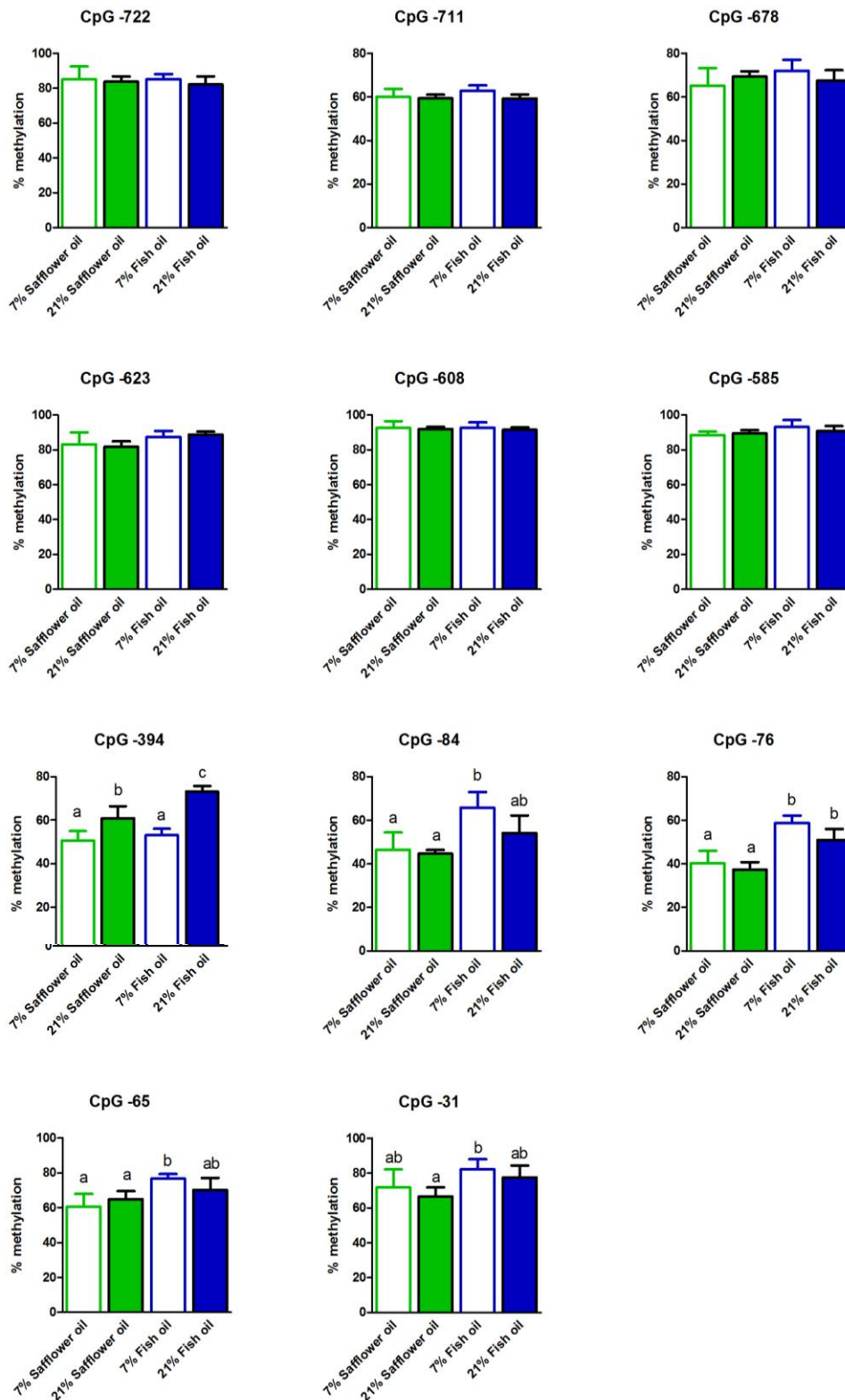


**Figure 8.9 *Fads2* promoter methylation in female offspring.** Values are means with their standard deviations. Where ‘\*’ represents a significance of  $P < 0.05$  and ‘\*\*\*’ a significance of  $P < 0.0005$ . ‘#’ represents significantly different from all other dietary groups, with the same maternal % dietary fat. Significant differences were by one-way ANOVA, with Bonferroni’s *post hoc* test  $P < 0.005$ .

## **8.4 Discussion**

### **8.4.1 Regulation of offspring fasting glucose metabolism**

Differences in hepatic PEPCK mRNA expression do not directly correlate to fasting plasma glucose values in either sex. As PEPCK represents one step in the pathway of fasting glucose homeostasis, differences in fasting glucose may be reflected in mRNA expression of other genes in the pathway.

There were no significant differences in the methylation levels of PEPCK promoter, in either sex. No differences were present in the methylation of CpGs in male or female offspring.

These findings in male and female offspring suggest that altered expression of PEPCK is not through epigenetic regulation of DNA methylation. Altered forms of regulation, such as hormonal, may be responsible for differences seen.

Male FO offspring showed a significantly higher fasting plasma glucose level, compared to female FO offspring. This suggests either an increased level of gluconeogenesis or a decreased uptake of glucose from tissue. Although levels are within the expected range for the species<sup>[254,259]</sup>, the experiment does not allow us to differentiate between the two explanations. As a result it appears that feeding an FO diet to mothers during pregnancy and lactation leads to male offspring which have a greater potential for providing glucose during fasting, compared to their female siblings. Potentially this could result in a selective survival of male offspring over females, in periods of severe fasting.

The 21% fat male offspring show significantly higher levels of fasting PEPCK expression. These results suggest that although altered fat intake during pregnancy does not result in altered fasting glucose levels, effects are seen in terms of PEPCK transcription, suggesting the effect of altered fat is not limited to a subset of genes. Through an increase in the level of PEPCK mRNA expression, the 21% male offspring show a greater capacity for gluconeogenesis, although it appears this is not present at this point in these animals, perhaps as a result of altered regulation of further genes in the gluconeogenic pathway.



#### 8.4.2 Regulation of offspring liver and plasma PC composition

The significant differences in liver and plasma PC composition between groups were mainly evident in 20:4n-6 and 22:6n-3. Hepatic mRNA expression of the *fads2* gene predicted 9% and 26% of 20:4n-6 and 22:6n-3 content of liver PC in males, respectively. In females, *fads2* predicted 41% and 15% of 20:4n-6 and 22:6n-3 content of liver PC, respectively.

Amount and type of maternal dietary fat led to significant differences in CpG specific methylation of the *fads2* promoter. Methylation of CpGs -394, -84 and -76 are associated with expression of the *fads2* gene. In males, methylation of CpGs -394, -84 and -76 predicted 6%, 12% and 16% of *fads2* expression, respectively. In females the effect was greater where CpGs -394, -84 and -76 predicted 55%, 16% and 28% of *fads2* expression, respectively. In both sexes methylation of CpG -394 was negatively associated with *fads2* mRNA expression, suggesting physical blocking of transcriptional machinery or recruitment of methyl CpG binding proteins. CpGs -84 and -76, however, were positively associated with mRNA expression, suggesting blocking of a repressor element to the promoter, following methylation.

Polymorphisms in the *fads2* gene were shown to account for 28.5% of the variation in serum phospholipid 20:4n-6 concentration in humans<sup>[286]</sup>. The results showed here, show that regulation of the *fads2* mRNA expression, via altered DNA methylation is as important as polymorphisms in overall regulation of phospholipid 20:4n-6 and 22:6n-3 composition. In mice, increased methylation of 8 out of 11 measured CpGs was associated with a decreased expression of the promoter<sup>[287]</sup>. Although the results here, show methylation of three of the CpGs measured in rat are associated with expression of the *fads2* gene, and not all negatively associated, the findings are in agreement, in that methylation of the *fads2* gene is an important factor in regulation of gene expression.

Altered plasma membrane PC composition, in terms of PUFA, will lead to significant differences in the fluidity of the lipid bilayer<sup>[147]</sup>. As a result, proteins in the membrane may be unable to change configuration following activation by specific substrate, this will prevent the cell from responding to external stimuli.

Female rats and humans have a higher level of 22:6n-3 in liver and plasma phospholipids, compared to males. Females also have an increased capacity for synthesis of 22:6n-3 in pregnancy, to meet the demands of the developing offspring<sup>[288]</sup>. Variation in the regulation of *fads2* transcription induced during development is therefore an important factor in the future capacity to maintain liver and plasma 20:4n-6 and 22:6n-3 content in offspring.

*Fads2* mRNA expression was significantly decreased in the 21% groups, regardless of the type of fat and sex of offspring. As a result, 20:4n-6 and 22:6n-3 PC content of liver was significantly lower in both male and female 21% groups

Studies have shown the DHA content of phospholipid bilayer in cell membranes is significantly associated with both retinal and brain function<sup>[154,155]</sup>. As offspring are responsible for their own synthesis of long-chain PUFA, from essential dietary precursors, the efficiency of this pathway is vital for optimum function.

In these animals, increasing the fat content of the maternal diet, regardless of the form of fat significantly decreased the DHA content of lipid membranes. As a result, the ability of hepatocytes to respond to external signals may be compromised.

Although more protected from fluctuations in phospholipid content, neurons in the brain of offspring may also show significant changes in DHA content and therefore function, as a result of the altered *fads2* expression. If this is occurring, the high fat offspring may be less responsive to external stimuli.

Although arachidonic acid (ARA) is used as a precursor for both pro and anti-inflammatory molecules, such as prostaglandins, the overall effect of an increase in arachidonic acid is anti-inflammatory<sup>[289]</sup>. As well as DHA, ARA maintains hippocampal cell membrane fluidity, where the combined membrane content of the two fats is around 20%<sup>[290]</sup>. In the brain ARA also aids growth and repair of neurons<sup>[291]</sup>. Therefore, if brain tissue is similar to liver and plasma, in terms of the increased 20:4n-6 content in the 7% offspring, these animals may have an increased capacity for neuron repair, a decreased inflammation and a greater capacity to respond to external stimuli, compared to the 21% groups.

### 8.4.3 Future work

Maternal fat intake may have adverse effects on the capacity of female offspring to synthesise long-chain PUFA during pregnancy and thus reduced ability to provide adequate amounts of fatty acids to their offspring. The results have shown that methylation of the *fads2* promoter is significantly altered by maternal fat intake, and that this has significant effects on both gene expression and the phenotype of the offspring. This shows that *PEPCK* is not the only gene, with metabolic function, that is significantly altered by maternal nutrition at the gene expression and promoter methylation level.

In terms of the DOHaD hypothesis, the results show that feeding a high fat diet has led to a detrimental change in the phenotype of the offspring, in terms of phospholipid composition. However, in order to test if the change in phenotype matches the PAR hypothesis, the offspring would need to also be fed a high fat diet, such as Bruce et al<sup>[95]</sup>.

Maternal high fat feeding led to significant changes in mRNA expression of *PEPCK*, in the 21% SAO male offspring, which were not associated with changes in promoter methylation. This shows that the role of methylation in altered expression of the *PEPCK* gene, following altered maternal nutrition, is dependent on the form of altered nutrition. When a protein restricted diet was used, there was a relationship between *PEPCK* promoter methylation and mRNA expression. However when high fat feeding was used, methylation did not play a role in mRNA expression of *PEPCK*. This is a novel finding.

Altered methylation of the *PEPCK* and *fads2* promoters has been shown to be an important factor in determining mRNA expression of the genes, following certain forms of nutritional alteration. Investigation of transcription factor binding to the promoters, over CpGs which have been shown to be related to mRNA expression will increase understanding of regulation of the *PEPCK* and *fads2* genes.

## Chapter 9

# Validation of the Effect of Altered Methylation of Specific CpG Dinucleotides on the Function of the PEPCK, DNMT3a2 and Fads2 Promoters

## 9.1 Introduction

Significant differences in promoter methylation of CpGs -56 and -39 of the DNMT3a2 promoter, CpGs -248, -100, -90 and -81 of the PEPCK and CpG -394 of the fads2 promoter in offspring of dams fed an altered diet during pregnancy, have been shown in previous chapters. These differences were significantly related to variations in mRNA expression. As stated in Section 1.4.1.2, methylation of single CpG sites in specific promoters is sufficient to affect transcription. For example, methylation of a single CpG site in the CRE in human glycoprotein hormone  $\alpha$ -subunit, both in vitro and in vivo, results in a loss of transcriptional activity, through altered transcription factor binding<sup>[202]</sup>. This relationship is also seen in Epstein-Barr virus, through methylation of the CBF2 binding site<sup>[203]</sup> and the XAF1 gene in human gastric and colon cancers<sup>[204]</sup>. Increased pup licking and grooming and arched backed nursing by dams significantly altered binding of the transcription factor NGFI-A to the glucocorticoid promoter, in the hippocampus of offspring, through altered methylation<sup>[206]</sup>.

In order to investigate whether CpGs that showed altered methylation in the PEPCK, DNMT3a2 and fads2 promoters in the offspring, which were induced by altered maternal diets, are involved in the transcriptional regulation of these genes, the binding of nuclear extract to sequences of the promoter regions of the genes, containing the CpG sites, was investigated.

The positive association between mRNA expression and total protein levels have been shown experimentally in terms of multiple genes<sup>[45,292,50,293,294]</sup>. The purpose of experiments described in this chapter is to determine if this relationship is present in genes measured in this thesis. Although the DNMT3a2 gene has been extensively studied in this thesis, the relationship between mRNA expression and protein levels of PEPCK in non-pregnant adult female offspring and fads2 in offspring of dams fed diets containing FO or SAO during pregnancy, were studied. This is because antibodies against these proteins were available for measurement of protein levels.

PEPCK was selected to investigate the relationship between protein levels and mRNA expression as it has been extensively studied in the experiments described in this thesis and appropriate antibodies for western blot detection of protein were available.

The following experiments aim to answer the following gaps in the knowledge

- Are significant differences in mRNA expression of PEPCK, in non-pregnant adult offspring associated with similar changes in liver protein levels?
- Are transcription factors binding to regions around CpGs -248, -100, -90 and -81 of the PEPCK promoter, CpGs -56 and -39 of the DNMT3a2 promoter and CpG -394 of the fads2 promoter?
- Is the binding of these transcription factors significantly affected by methylation of the CpG sites?
- Does mutation of specific CpG -394 of the fads2 promoter significantly alter levels of gene transcription?

## **9.2 Experimental Design**

Protein extract was prepared from the livers of non-pregnant adult female offspring from the study described in section 2.2, for determination of PEPCK protein levels. Protein extract was prepared from the female offspring of the study described in section 8.2, for determination of fads2 protein levels.

### **9.2.2 Molecular biology procedures**

Analysis of protein levels by Western blotting is described in section 2.3.11.4. Briefly, tissue was lysed in order to release all protein from within cells. 20µg of protein per sample was run on a western blot gel, comprised of both a lower resolving gel and upper stacking gel. Gels were transferred onto PVDF membranes and transferred at 200mA for 60 minutes, The PVDF membrane was blocked with PBS with 0.1% Tween-20 containing 5% Marvel, at 4°C overnight, washed and incubated for 1 hour at room temperature, shaking, with blocking solution containing the appropriate amount of primary antibody. Following

incubation with primary antibody, membranes were washed again and incubated in blocking buffer containing the appropriate amount of secondary antibody. Protein levels were detected incubating the membrane West DURA Chemiluminescent substrate Super-Signal (Pierce, Fisher, Leicestershire, UK), for 5 minutes. Chemi-ultrasensitivity was measured using a VersaDoc.

Transcription factor binding to the promoter sequences within the PEPCK, DNMT3a2 and fads2 promoters were shown using electro-mobility shift assays, as described in section 2.3.11.3. Briefly, components of the binding reaction, such as nuclear extract and labelled or unlabelled oligonucleotides were run on an acrylamide gel. The gel was then transferred for one hour at 200mA and crosslinked. The membrane was then incubated in blocking buffer, conjugate buffer/blocking solution, wash buffer and substrate working solution. Following the incubations, the bands were visualised using a VersaDoc (Bio-Rad Laboratories, Hertfordshire, UK).

Analysis of the role of specific CpGs in transcriptional regulation is described in section 2.3.9. Briefly, the promoter region of fad2 was amplified using a proof-reading PCR reaction, ligated into a pGI3 vector and transfected into rat hepatocyte, CC-1 cells. Quick-change mutagenesis of CpG -394, of the ligated fasd2 promoter-pGI3 complex was used to create the cytosine to adenosine mutation of the fas2 promoter.

### **9.3 Results**

#### **9.3.1 Does mRNA expression of PEPCK in PS lineage F1 and F2 non-pregnant adult female offspring correlate with PEPCK protein levels?**

PEPCK protein levels were measured using western blotting in F1 and F2 PS non-pregnant adult female offspring (n=3/group) (Figure 9.1). Using densitometry values, measured on the VersaDoc Quantity One software, the total levels of protein were determined. F1 and F2 female offspring PEPCK mRNA expression and total protein levels can be seen in Figure 9.2.

Although not significant ( $p=0.16$ ), when considering the densitometry values there appears to be a similarity between RT-PCR and protein levels of PEPCK, between the F1 and F2 offspring. Linear regression analysis of protein densitometry values and mRNA values shows a trend towards a positive correlation ( $p=0.0965$ ,  $r^2=0.656$ ).



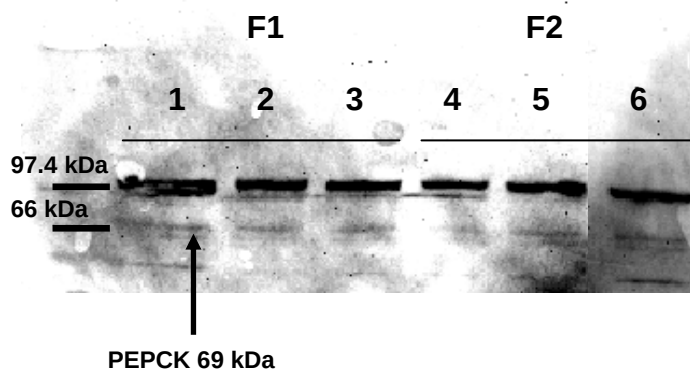


**Figure 9.1** Western Blot gel of F1 and F2 PEPCK non-pregnant adult using Abcam primary antibody ab28455 (Rabbit polyclonal anti-PEPCK) and secondary antibody ab9705 (Goat anti-rabbit horse-radish peroxidase conjugate). The Abcam ab41746 molecular weight marker was used to determine the size of the PEPCK band.

### Non-Pregnant Offspring Hepatic PEPCK mRNA and Protein Levels

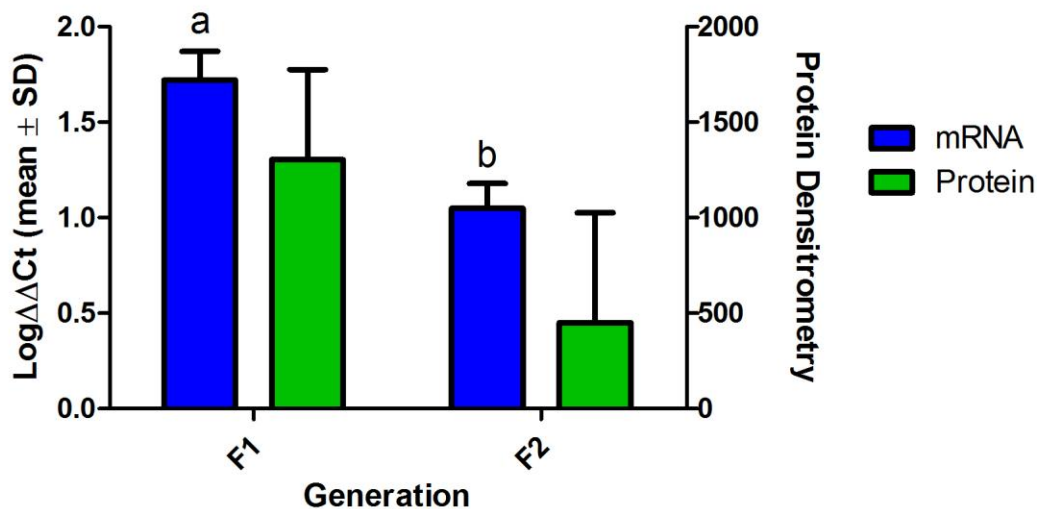


**Figure 9.2** PEPCK mRNA and protein levels in F1 and F2 non-pregnant adult offspring. Although not significant, the decreased PEPCK mRNA expression between the F1 and F2 generations can be seen in the associated protein levels. n = 9/group for RT-PCR analysis, n=3/group for protein analysis.

### 9.3.2 Does mRNA expression of fads2 in offspring of dams fed altered fat diets during pregnancy correlate with fads2 protein levels?

Although attempts were made to investigate the protein level of fads2 in offspring of dams fed diets containing 7% and 21% SAO and FO during pregnancy and lactation, the experiments proved unsuccessful.

Despite using a range of concentrations of both primary and secondary antibody, no bands correlating to detection of fads2 protein could be seen on the western blotting gel (Figure 9.3). The range of concentrations can be seen in table 9.1. As the primary antibody used was the only one available against the fads2 protein in rat, no further progress could be made.

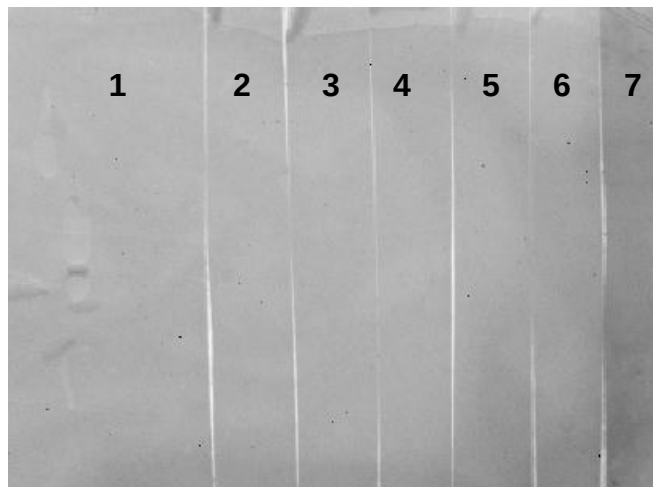


**Figure 9.3 Western Blot gel fads2 optimisation** using goat polyclonal primary anti-fads2, sc-109272 (SantaCruz) and secondary antibody, ab97100 (rabbit anti-goat, horse radish peroxidase conjugate)

| Lane | Dilution of primary Ab | Dilution of secondary Ab |
|------|------------------------|--------------------------|
| 1    | 1/200                  | 1/40000                  |
| 2    | 1/200                  | 1/50000                  |
| 3    | 1/500                  | 1/40000                  |
| 4    | 1/500                  | 1/50000                  |
| 5    | 1/1000                 | 1/40000                  |
| 6    | 1/1000                 | 1/50000                  |
| 7    | 1/200                  | 1/5000                   |

**Table 9.1 Antibody dilutions used in optimisation of fads2 western blot**

### **9.3.3 Does nuclear extract of CC-1 cells bind the region containing CpG -248 of the PEPCK promoter?**

Methylation of CpG -248 was significantly altered between offspring groups. A MatInspector, putative transcription factor binding search showed that the CpG-248 is within a binding site for C/EBP (shown in Figure 5.6), a known regulator of PEPCK transcription<sup>[295]</sup>. As a result the binding of nuclear extract to the region containing CpG -248 was investigated.

Binding of nuclear extract is occurring at the region covering CpG -248 of the PEPCK promoter (lanes 1 and 2 of Figure 9.4). The absence of a band present when an excess of unlabelled probe was added (lane 3 of Figure 9.4) suggests that the unlabelled probe is competing out the labelled probe and the binding is specific. As the addition of a non-specific probe (oligonucleotide for fads2 -394, shown in table 2.13) does not compete out binding of the labelled probe, this again suggests that the binding is specific (lane 5 of Figure 9.4).

#### **9.3.3.1 Is nuclear extract binding to the PEPCK promoter affected by methylation of CpG -248?**

Presence of a band suggests that methylation of CpG -248 does not prevent binding of nuclear extract to the this region of the PEPCK promoter The absence of a band present when an excess of unlabelled probe was added (lane 8 of Figure 9.4) suggests that the unlabelled probe is competing out the labelled probe and the binding is specific. As the addition of a non-specific probe does not compete out binding of the labelled probe, this again suggests that the binding is specific (lane 9 of Figure 9.4).

#### **9.3.3.2 Is the C/EBP binding at CpG -248 of the PEPCK promoter?**

The addition of an antibody against C/EBP  $\beta$  (Abcam ab32358), to the nuclear extract and labelled probe, was used to confirm binding of C/EBP to the PEPCK promoter at CpG -248.

Decreased electromobility of the bands in lanes 5 and 10, compared to lanes 2 and 7, suggests that the transcription factor C/EBP  $\beta$  is binding to CpG -238 of the

PEPCK promoter (lanes 5 and 10 of Figure 9.4). As the band is present in both lanes 5 and 10, this suggests that methylation of the CpG does not alter binding (lanes 5 and 10 of Figure 9.4).

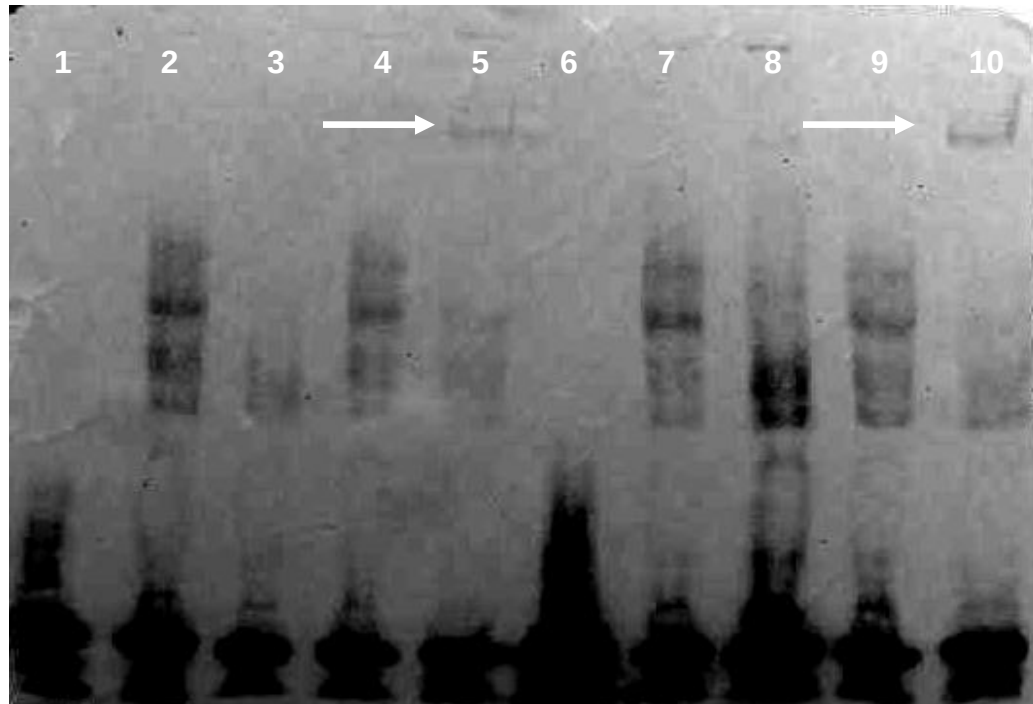


**Figure 9.4 Electromobility Shift Assay gel picture of PEPCK CpG -248 with unmethylated and methylated oligonucleotides.** Lane 1 shows the unmethylated labelled probe only, lane 2 shows the unmethylated labelled probe with nuclear extract, lane 3 shows the unmethylated labelled probe with nuclear extract and an excess of unlabelled probe, lane 4 shows unmethylated labelled probe with nuclear extract and an excess of unlabelled non-specific probe, lane 5 shows unmethylated labelled probe with nuclear extract and antibody against C/EBP. Lane 6 shows the methylated labelled probe only, lane 7 shows the methylated labelled probe with nuclear extract, lane 8 shows the methylated labelled probe with nuclear extract and an excess of unlabelled methylated probe, lane 9 shows methylated labelled probe with nuclear extract and an excess of unlabelled non-specific probe, lane 10 shows methylated labelled probe with nuclear extract and antibody against C/EBP

#### 9.3.4 Does nuclear extract of CC-1 cells bind the region containing CpGs -100, -90 and -81 of the PEPCK promoter?

Methylation of CpGs -100, -90 and -81 was significantly altered between offspring groups. A MatInspector, putative transcription factor binding search showed that the CRE is situated over CpGs -100, -90 and --81 (shown in Figure 5.6), a known regulatory region of PEPCK transcription<sup>[295]</sup>. As a result the binding of nuclear extract to the region containing CpGs -100, -90 and -81 was investigated.

The absence of bands presence in lanes 2, 3, and 4 suggest no nuclear extract is binding to the region covering CpGs -100, -90 and -81 of the PEPCK promoter (Figure 9.5). Lane 1 shows presence of labelled probe only. Lanes 2, 3 and 4 show three amounts of nuclear extract (1 $\mu$ g, 4 $\mu$ g and 5 $\mu$ g, respectively) with labelled probe. Lane 5 shows labelled probe with nuclear extract with an excess of unlabelled probe.

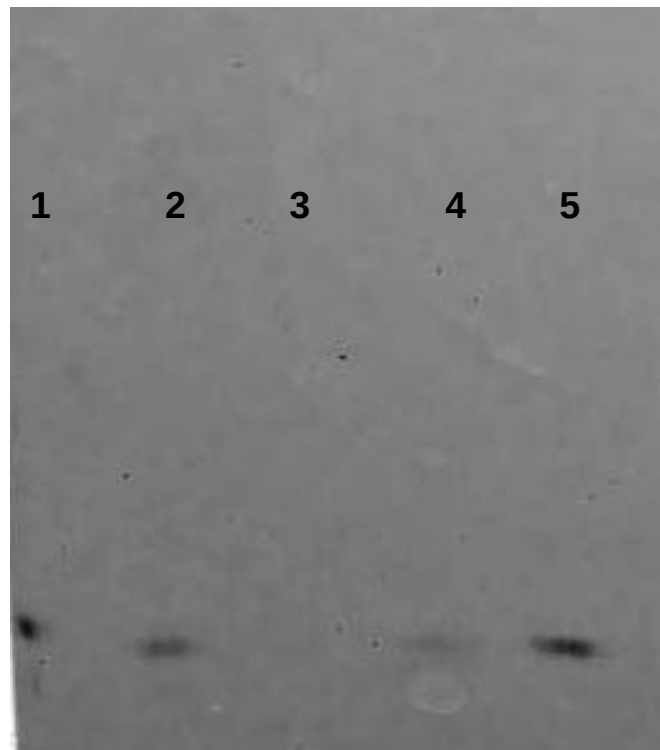


**Figure 9.5 Electromobility Shift Assay gel picture of PEPCK CpGs -100, -90 and -81 with unmethylated oligonucleotides.** Lane 1 shows the unmethylated labelled probe only, lane 2 shows the unmethylated labelled probe with 1  $\mu$ g of nuclear extract, lane 3 shows the unmethylated labelled probe with 4  $\mu$ g nuclear extract, lane 4 shows the unmethylated labelled probe with 5  $\mu$ g nuclear extract and lane 5 shows unmethylated probe with 4  $\mu$ g nuclear extract and an excess of unlabelled probe.

### 9.3.5 Does nuclear extract of CC-1 cells bind the region containing CpG -56 of the DNMT3a2 promoter?

Methylation of CpG -56 was significantly altered between offspring groups. As a result binding of nuclear extract to this region was investigated.

The absence of bands presence in lanes 2, 3, and 4 suggest no nuclear extract is binding to the region covering CpG -56 of the DNMT3a2 promoter (Figure 9.6). Lane 1 shows presence of labelled probe only. Lanes 2, 3 and 4 show three amounts of nuclear extract (1 $\mu$ g, 4 $\mu$ g and 5 $\mu$ g, respectively) with labelled probe. Lane 5 shows labelled probe with nuclear extract with an excess of unlabelled probe.

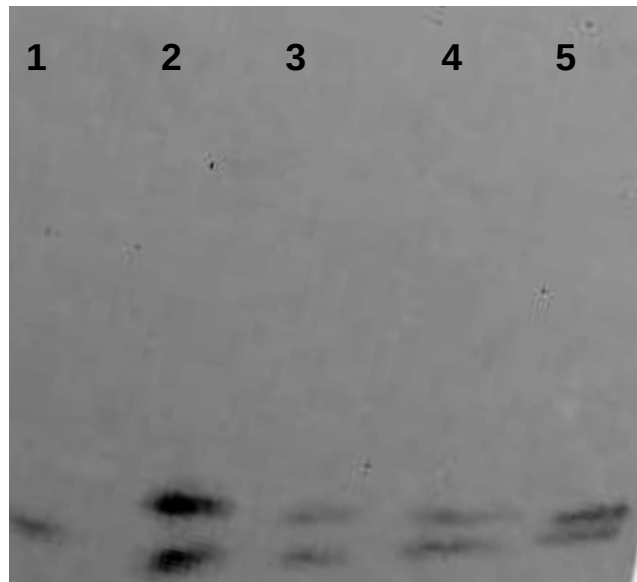


**Figure 9.6 Electromobility Shift Assay gel picture of DNMT3a2 CpG -56 with unmethylated oligonucleotides.** Lane 1 shows the unmethylated labelled probe only, lane 2 shows the unmethylated labelled probe with 1  $\mu$ g of nuclear extract, lane 3 shows the unmethylated labelled probe with 4  $\mu$ g nuclear extract, lane 4 shows the unmethylated labelled probe with 5  $\mu$ g nuclear extract and lane 5 shows unmethylated probe with 4  $\mu$ g nuclear extract and an excess of unlabelled probe.

### **9.3.6 Does nuclear extract of CC-1 cells bind the region containing CpG -39 of the DNMT3a2 promoter?**

Methylation of CpG -39 was significantly altered between offspring groups. A MatInspector, putative transcription factor binding search showed that the CpG -39 is within a binding site for the early growth response factor (EGR), E-twenty six-1 (ETS-1) and signal transducer and activator of transcription 5 (STAT5). As a result the binding of nuclear extract to the region containing CpG -39 was investigated.

Binding of nuclear extract is occurring at the region covering CpG -39 of the DNMT3a2 promoter (lanes 1 and 2 of Figure 9.7). The absence of a band present when an excess of unlabelled probe was added (lane 3 of Figure 9.7) suggests that the unlabelled probe is competing out the labelled probe and the binding is specific. Although a band is present in lane 3, it is not present at the same level as the band in lane 2, suggesting by competing out binding of the factor with the unlabelled probe, a weaker form of binding, by an alternative factor may be occurring. As the addition of a non-specific probe (oligonucleotide for fads2 -394, shown in table 2.13) does not compete out binding of the labelled probe, this again suggests that the binding is specific (lane 5 of Figure 9.7).

#### **9.3.6.1 Are EGR, ETS-1 or STAT5 binding at CpG -39 of the DNMT3a2 promoter?**

The binding of transcription factors was assessed by adding an excess of unlabelled oligonucleotides containing the consensus sequences for early growth response factor (EGR), E-twenty six-1 (ETS-1) and signal transducer and activator of transcription 5 (STAT5) to 4 µg of nuclear extract and labelled probe. In order to confirm that the specific transcription factors are binding to the region of the DNMT3a2 promoter, the addition of the unlabelled probe containing the binding sequences of the factors, must compete out the binding of the extract to the labelled probe. Two independent sources were used to obtain the binding regions for EGR, ETS-1 and STAT5, MatInspector (odd lanes) and Santa Cruz Biotechnologies (even lanes).

The presence of bands in lanes 5 and 6 suggests the EGR factor does not bind to the area covering CpG -39. The presence of bands in lanes 7 and 8 suggests the ETS-1 factor does not bind to the area covering CpG -39. The presence of bands in lanes 9 and 10 suggests the STAT5 factor does not bind to the area covering CpG -39.

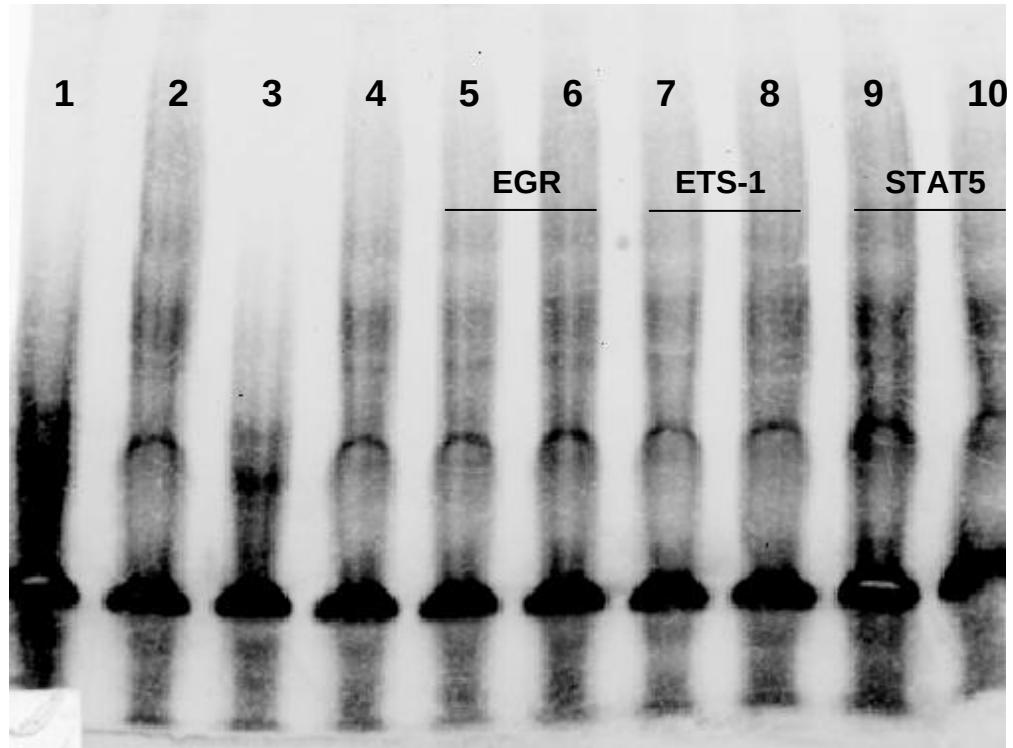


**Figure 9.7 Electromobility Shift Assay gel picture of DNMT3a2 CpG -39 with unmethylated oligonucleotides and consensus sequence oligonucleotides for EGR, ETS-1 and STAT5.** Lane 1 shows the unmethylated labelled probe only, lane 2 shows the unmethylated labelled probe with 4 µg of nuclear extract, lane 3 shows the unmethylated labelled probe with 4 µg nuclear extract and an excess of unlabelled probe, lane 4 shows unmethylated labelled probe with nuclear extract and an excess of unlabelled non-specific probe, lanes 5 and 6 contain the unmethylated labelled probe with 4 µg nuclear extract and an excess of unlabelled consensus sequences of EGR (MatInspector sequence in lane 5, SantaCruz sequence in lane 6), lanes 7 and 8 contain the unmethylated labelled probe with 4 µg nuclear extract and an excess of unlabelled consensus sequences of ETS-1 (MatInspector sequence in lane 7, SantaCruz sequence in lane 8) and lanes 9 and 10 contain the unmethylated labelled probe with 4 µg nuclear extract and an excess of unlabelled consensus sequences of STAT5 (MatInspector sequence in lane 9, SantaCruz sequence in lane 10).



### **9.3.7 Does nuclear extract of CC-1 cells bind the region containing CpG -394 of the fads2 promoter?**

Methylation of CpG -394 was significantly altered between male and female offspring groups. A MatInspector, putative transcription factor binding search showed that the CpG-394 is within a binding site for oestrogen receptor (ER). The role of oestrogen in regulation of fatty acid metabolism has been suggested previously<sup>[296]</sup>. As a result the binding of nuclear extract to the region containing CpG -394 was investigated.

Binding of nuclear extract is occurring at the region covering CpG -394 of the fads2 promoter (lanes 1 and 2 of Figure 9.8). The absence of a band present when an excess of unlabelled probe was added (lane 3 of Figure 9.8) suggests that the unlabelled probe is competing out the labelled probe and the binding is specific. As the addition of a non-specific probe (oligonucleotide for PEPCK -248, shown in table 2.13) does not compete out binding of the labelled probe, this again suggests that the binding is specific (lane 5 of Figure 9.8).

#### **9.3.7.1 Is nuclear extract binding to the fads2 promoter affected by methylation of CpG -394?**

Presence of a band suggests that methylation of CpG -394 does not prevent binding of nuclear extract to the this region of the fads2 promoter. The absence of a band present when an excess of unlabelled probe was added (lane 8 of Figure 9.8) suggests that the unlabelled probe is competing out the labelled probe and the binding is specific. As the addition of a non-specific probe does not compete out binding of the labelled probe, this again suggests that the binding is specific (lane 9 of Figure 9.8). Using densitometry, the intensity of binding of nuclear extract was measured on the VersaDoc Quantity One software (figure 9.9).

#### **9.3.7.2 Is the ER binding at CpG -394 of the fads2 promoter?**

The addition of an antibody against the oestrogen receptor (Abcam ab16660) to the nuclear extract and labelled probe was used to investigate binding of the ER to the fads2 promoter at CpG -394. As shown by the absence of a

SuperShift band in lanes 5 and 10, it cannot be confirmed that the ER transcription factor is binding around CpG -394, whether it is unmethylated (lane 5) or methylated (lane 10) (Figure 9.8)

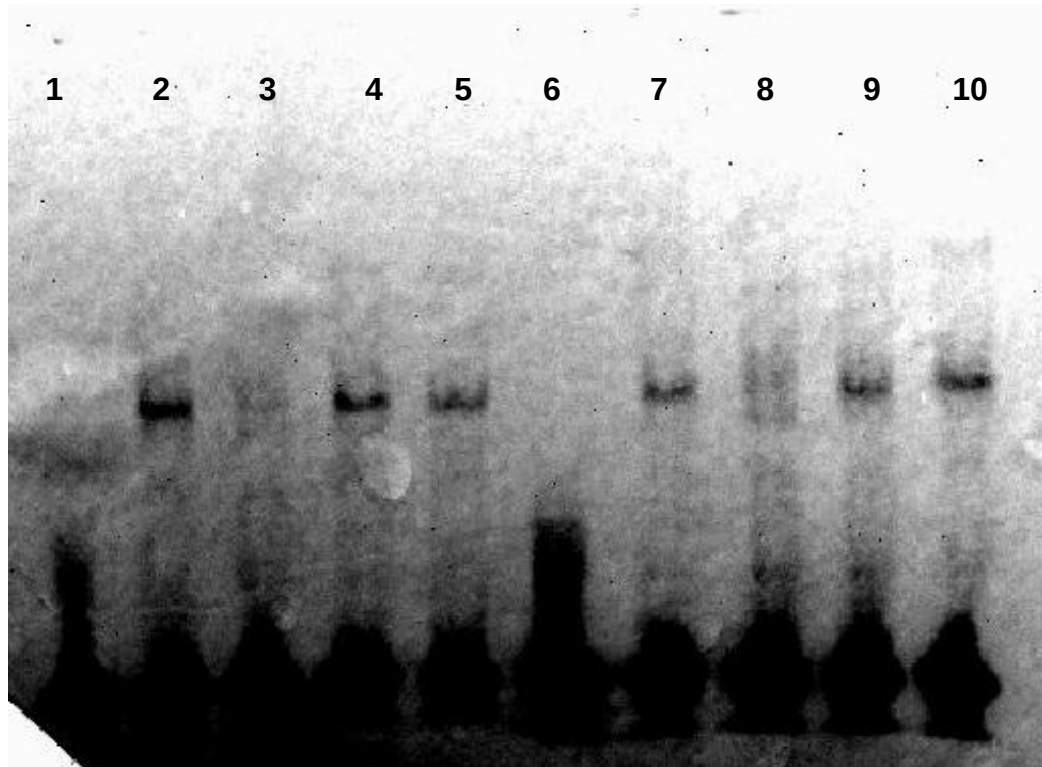


**Figure 9.8 Electromobility Shift Assay gel picture of Fads2 CpG -394 with unmethylated and methylated oligonucleotides.** Lane 1 shows the unmethylated labelled probe only, lane 2 shows the unmethylated labelled probe with nuclear extract, lane 3 shows the unmethylated labelled probe with nuclear extract and an excess of unlabelled probe, lane 4 shows unmethylated labelled probe with nuclear extract and an excess of unlabelled non-specific probe, lane 5 shows unmethylated labelled probe with nuclear extract and antibody against ER. Lane 6 shows the methylated labelled probe only, lane 7 shows the methylated labelled probe with nuclear extract, lane 8 shows the methylated labelled probe with nuclear extract and an excess of unlabelled methylated probe, lane 9 shows methylated labelled probe with nuclear extract and an excess of unlabelled non-specific probe, lane 10 shows methylated labelled probe with nuclear extract and antibody against ER.

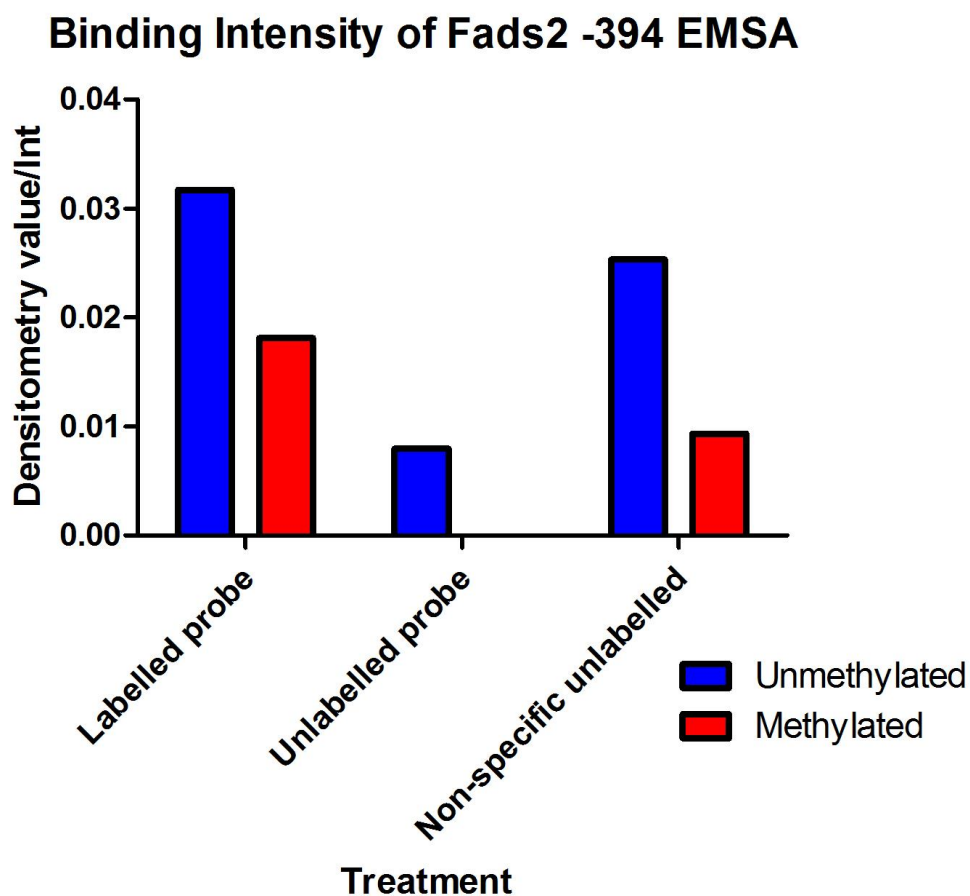


**Figure 9.9 Band intensity of fads2 EMSA** showing the difference in binding of nuclear extract to both unmethylated and methylated probes. Methylation of the labelled probes led to a decreased binding intensity at CpG -394 of the fads2 promoter.

### **9.3.8 Does mutagenesis of CpG -394 of the fads2 promoter alter transcriptional activity of fads2?**

In women, increased exposure to oestrogen is associated with an increased level of the products of linoleic acid metabolism<sup>[297]</sup> and putative transcription factor binding analysis suggests binding of the oestrogen receptor around CpG -394. As a result, the role of CpG -394, in the basal level of fads2 transcription and the response to oestrogen, was investigated. Cloning and mutagenesis was used to determine the effect of mutation of the cytosine of CpG -394 dinucleotide to an adenosine residue.

As shown in Figure 9.10, mutation of CpG -394 had no effect on basal levels of fads2 transcription (shown in the 0pM 17 $\alpha$ -ethinylestradiol bars). In the presence of oestrogen, in the form of 17 $\alpha$ -ethinylestradiol, mutagenesis of CpG -394, from a cytosine to an adenosine, inhibited oestrogen induced activation of fads2 transcription ( $P<0.05$ ).

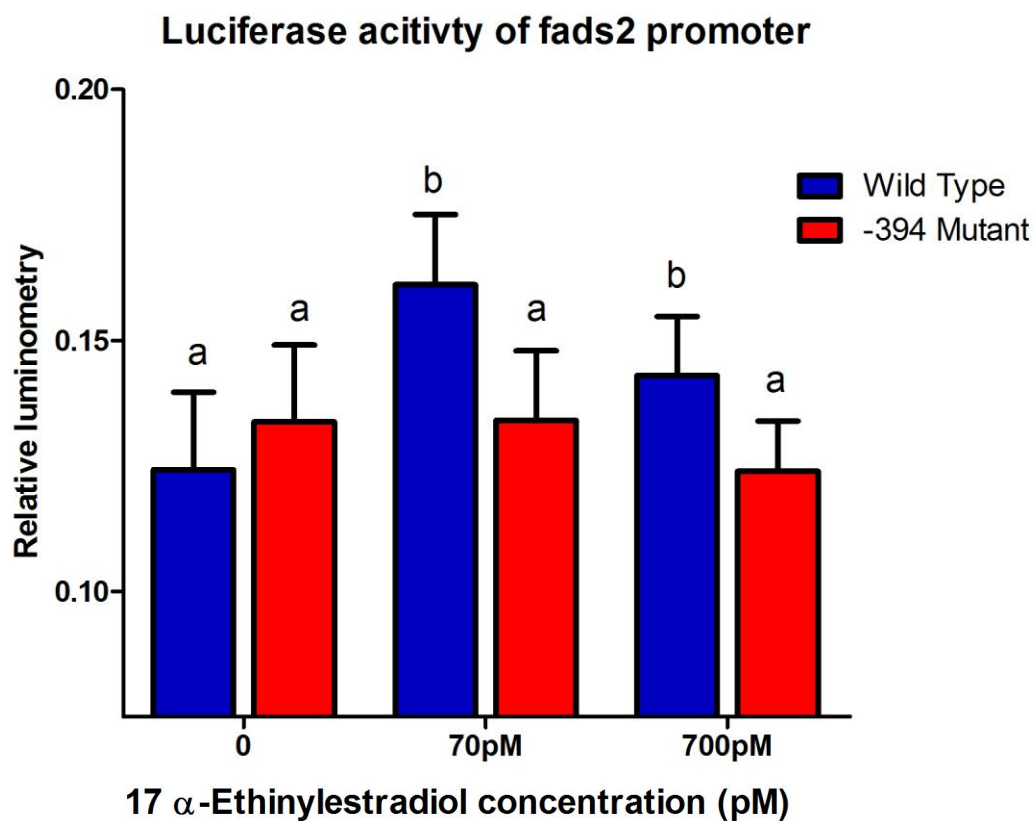


**Figure 9.10** Luciferase activity of the *fads2* promoter in the presence of 0pM, 70pM and 700pM 17 $\alpha$ -ethinylestradiol. Blue bars show the wild-type promoter and red bars show the -394 cytosine to adenosine mutant promoter.

### 9.3.9 Cloning and mutagenesis of DNMT3a2 and PEPCK promoters

Although attempts were made to clone and mutate the CpGs in the DNMT3a2 and PEPCK promoter with known roles in expression of the genes, the experiments were unsuccessful.

In terms of PEPCK, the promoter region was successfully amplified and cloned in pGEM-T, but could not be successfully cloned into the pGI3 vector. The cut plasmid DNA shows presence of the pGI3 vector only, without the presence of the PEPCK promoter (Figure 9.11 (A)). Presence of the PEPCK promoter into pGEM-T was confirmed by sequencing. It is most likely that this is due to the restriction enzymes used for the ligation of the PEPCK PCR product to the digested pGI3 vector.

In terms of DNMT3a2, the promoter region was successfully cloned into the pGI3 vector, however on multiple attempts, sequencing confirmed that the mutagenesis of CpGs -56 and -39 were unsuccessful. The reason for this was unclear. The successful cloning of DNMT3a2 in pGI3 is shown in Figure 9.11 (B), where the digest with NcoI leads to 2 bands and a digest with ApoI leads to multiple bands.

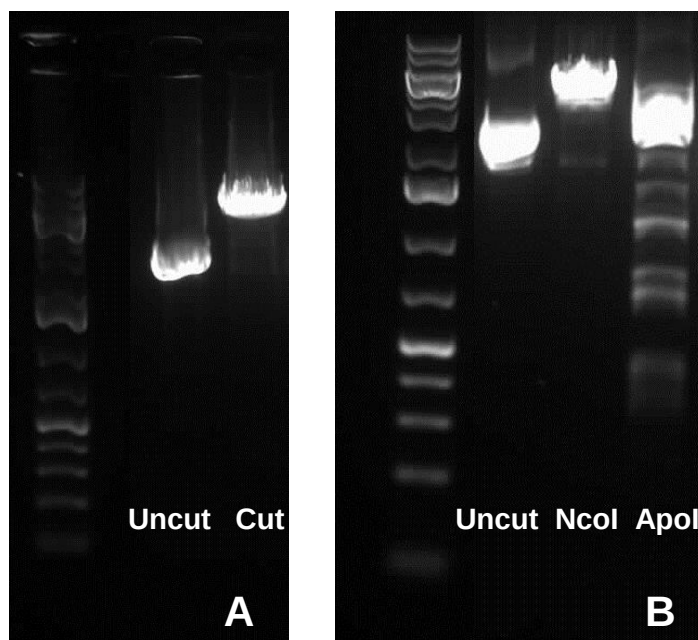


**Figure 9.11** 1% Agarose gels showing the progress of PEPCK and DNMT3a2 cloning where A shows the absence of the PEPCK promoter in pGI3 clones and B shows the restriction map of DNMT3a2 cloned in pGI3, where enzymes NcoI and ApoI were used.

## 9.4 Discussion

Data in this chapter has shown similarities in the pattern of hepatic PEPCK mRNA expression and total protein levels, between the F1 and F2 non-pregnant female offspring, specific binding of the CRE to CpG -248 of the PEPCK promoter, specific binding of nuclear extract to CpG -394 of the *fads2* promoter and CpG -39 of the *dnmt3a2* promoter. The role of CpG -394 in *fads2* transcription, in the presence of oestrogen was also shown.

### 9.4.1 The relationship between mRNA expression and protein levels of PEPCK

The positive correlation between mRNA expression and protein levels has been shown experimentally<sup>[45,292,50,293,294]</sup>. In mIR-29b, acute myeloid leukaemia cells a decreased expression of DNMT3a, DNMT3b and DNMT1 was associated with a decreased level of protein of all three genes<sup>[293]</sup>. As shown by Feng et al, expression of DNMT3a mRNA was related to total protein levels, in mouse CNS cells<sup>[292]</sup>. In rat, expression of GR mRNA was significantly increased in day 20 embryo kidney tissue, following maternal low-protein feeding during pregnancy<sup>[45]</sup>. A similar increase in GR protein levels was seen in the sample group. In another study that used maternal protein restriction to lead to an alteration in offspring phenotype, protein kinase C and p110 $\beta$ -associated p85 $\alpha$  mRNA expression in abdominal fat were significantly reduced ( $p < 0.05$ )<sup>[50]</sup>. The mRNA expression levels were associated with decreased protein levels.

Although the results shown in this chapter show data for PEPCK mRNA and protein levels, a positive relationship is present, which is in agreement with the published literature<sup>[298]</sup>. In mice, injection of retinol binding protein-4 (RBP4) resulted in a 41% increase in hepatic PEPCK expression, compared to control mice ( $P < 0.05$ ). This difference was associated with a 94% increase in liver protein levels of PEPCK<sup>[298]</sup>.

It has been experimentally proven that levels of DNMT3a protein are positively associated with mRNA level<sup>[293]</sup>; therefore it may be assumed that final DNMT3a protein levels are in agreement with measured mRNA expression levels.

Due to post-transcriptional regulation of protein synthesis, these results cannot be extrapolated to suggest that all measured genes correlate with protein levels. However, patterns in mRNA expression of differences between genes, in the same metabolic pathway, such as that shown in Figure 3.13, suggest protein levels may be related to measured mRNA levels for at least a subset of genes.

In terms of implications for the animals, the higher level of PEPCK protein, in liver of F1 PS non-pregnant female offspring, compared to the F2 PS group, suggests a greater capacity for provision of glucose during fasting. As both F1 and F2 groups had high fasting glucose values, compared to an adult female Wistar rat that received no dietary alteration in their life, or the life of the dam suggests that the increase in PEPCK may account for this increase in the F1 generation. As a result, different mechanism may be behind the increased fasting plasma glucose values between generations.

#### **9.4.2 The relationship between promoter methylation and gene expression**

Previous studies have shown a relationship between methylation of gene promoters and mRNA expression, through altered transcription factor binding<sup>[299,300]</sup>. Using a synthetic oligonucleotide, containing a sequence found in the adenovirus major late promoter, it was shown that methylation affects transcription factor binding<sup>[300]</sup>. Using a gel retardation assay, it was found that replacement of a single cytosine residue, with 5-methylcytosine inhibited binding of MCTF 52 base pairs upstream from the transcription factor start site. This relationship however, is not seen in all cases. Methylation of a specific cytosine within synthetic octamers containing Sp1 binding sites did not prevent binding of the factor from HeLa cell nuclear extracts<sup>[301]</sup>. In this study binding of Sp1 showed no significant differences when the octamers were unmethylated or methylated.

The data described in this chapter shows that at CpG -248 of the PEPCK promoter, C/EBP $\beta$  binding can occur and that methylation of the CpG does not prevent binding. Previous studies have shown a role of C/EBP in regulation of PEPCK transcription<sup>[134,302]</sup>. A study by O'Brien, has shown binding of C/EBP specifically regulates PEPCK around -248 base pairs, in regulation by insulin, via



binding to the insulin response element and cAMP, through the CRE, in mice<sup>[302]</sup>. Using C/EBP $\alpha$  -/- and C/EBP $\beta$  -/- mice, it was shown that both forms of C/EBP are involved in regulation of PEPCK<sup>[134]</sup>.

According to Faber et al, methylation off CpG s -90 and -81, inhibit binding of CRE, in the human glycoprotein hormone  $\alpha$  subunit<sup>[299]</sup>. In the studies described in this chapter, EMSAs suggest that no nuclear factor is binding across CpGs -100, -90 and -81 of the rat PEPCK promoter. These experiments were conducted using rat hepatocyte CC-1 cell nuclear extract. In different cell types, the nucleus will contain other factors, which may bind to the region. However, these factors will not be responsible for regulation of gene transcription in liver.

These results confirm that in rat hepatocytes, C/EBP $\beta$  binds across CpG -248 and that methylation of the cytosine residue does not alter binding. In terms of the data in this thesis, it suggests that regardless of methylation at the site, regulation via C/EBP $\beta$  is not altered. This suggests that differences in mRNA expression, which are related to CpG -248 methylation, are not due to altered C/EBP $\beta$  binding. Studies into the PEPCK promoter have shown binding of two specific factors (CREB/ATF and pep A) to the hypersensitive, HS A site of the PEPCK promoter 4,800 base pairs upstream of the promoter, which contains a CRE site<sup>[303]</sup>. Therefore, regulation at this site or others may be significantly altered by increased or decreased methylation.

No studies to date have shown altered binding of transcription factors to specific CpGs in the dnm3a2 or fads2 promoters. Therefore, it is unknown if the results described in this chapter are in agreement with published findings. EMSAs using synthetic oligonucleotides based on regions around CpGs -56 and -39 of the DNMT3a2 promoter, showed presence of binding at CpG -39 only. As the band present was still present after addition of an unlabelled probe the binding of a transcription factor found in rat hepatocytes, which binds at CpG -39 could not be confirmed as EGR, ETS-1 or STAT5. As a result, in these animals, it is a different transcription factor, present in the nucleus of CC-1 cells, which may play a role in regulation of transcription, binding at CpG -39. As the putative transcription factor binding analysis did not highlight any further regulatory factors of interest and the

known regulatory factors do not bind at this region, the identity of this factor is unknown.

Binding of a transcription factor found within the nucleus of CC-1 cells, was confirmed at the region around CpG -394 of the fads2 promoter. This binding is specific and the intensity is decreased by methylation of the CpG. Methylation of CpG -394 did not completely prevent all binding of the factor to the region; this suggests that the CpG -394 may be present in the binding site of the factor, but not in the core site. As homology between the core site of transcription factor binding and the promoter is required for binding, whereas a complete match between the entire binding sequence and promoter sequence will lead to a stronger intensity of binding. The oestrogen receptor is expected to bind across this CpG, as shown in figure 9.12 this could be the case. If the addition of the methyl groups to the CpG is weakening the binding of ER to the site, this may explain the decreased intensity of binding in the presence of the methylated oligonucleotide.



**Figure 9.12 Binding of the oestrogen receptor (ER) to the region around CpG -394 of the fads2 promoter** where the binding site is underlined and core region is in bold. Predicted binding site obtained from MatInspector search.

The addition of an anti-ER antibody to the EMSA reaction set-up did not lead to a shift in the binding of the factor to the synthetic oligonucleotide. Therefore it cannot be confirmed that it is the oestrogen receptor that is binding to the region around CpG -394. However, this does not rule out that the oestrogen receptor is binding here, as the antibody must bind to the factor in a manner that does not prevent the factor from binding to the oligonucleotide. The results suggest that regulation of mRNA expression, through changes in methylation of CpG -394 is likely to be an effect of altered transcription factor binding at CpG -394. As no studies have shown data into binding at the fads2 promoter and the role of promoter methylation, it is not known if this data is in agreement with the literature.

### 9.4.3 Mutagenesis of fads2 and transcriptional activity

Site-directed mutagenesis has shown that the cytosine at position -394 of the fads2 promoter is required for increased transcriptional activity in the presence of oestrogen, in the form of 17 $\alpha$ -ethinylestradiol. Mutagenesis of the cytosine had no impact on basal levels of fads2 transcription (0pM 17 $\alpha$ -ethinylestradiol), suggesting the presence of oestrogen leads to an increase in the level of fads2 transcription, through the binding of a regulatory transcription factor around the cytosine of CpG -394.

Studies have shown a clear effect of oestrogen on ability to synthesise long chain PUFA. In women, increased exposure to oestrogen was associated with an increased level of the products of linolenic acid metabolism, dihomo- $\gamma$ -linolenic acid and ARA, potentially through increased  $\Delta 5$  and  $\Delta 6$  expression<sup>[297]</sup>. Burdge and Wotton have shown that 28 year old women have a 200 times greater ability to synthesise DHA from alpha-linoleic acid, compared to same age males, where undetectable levels were recorded<sup>[304]</sup>. The study used <sup>13</sup>C alpha-linoleic acid consumption and measured the fatty acid content of PC. It was suggested that pregnant and lactating women (women of reproductive age) have an increased synthesis of the long chain PUFA, to provide optimum DHA for the development of the offspring's central nervous system. The administration of 17 $\alpha$ -ethinylestradiol to male to female transsexuals has shown that oestrogen can increase a male's DHA synthesis<sup>[296]</sup>. Plasma cholesteryl ester DHA composition increased more than 40%, following administration of 17 $\alpha$ -ethinylestradiol. The study also investigated the administration of 17 $\alpha$ -ethinylestradiol, in the form of the oral contraceptive pill, to women on DHA status. Women who were taking the pill had a 10% increase in proportion of DHA, compared to those who were not<sup>[296]</sup>. In a separate study which again took into consideration the effect of 17 $\alpha$ -ethinylestradiol, in the form of the contraceptive pill in women, plasma PC DHA content tended to be increased in those using the pill, compared to those who did not<sup>[304]</sup>. These studies have identified a potential mechanism for the increased synthesis of long chain PUFA in the presence of oestrogen, through the increased expression of the fads1 and fads2 genes. Findings in this chapter are in agreement with the literature, into the

increased capacity of long chain PUFA synthesis in females and extend the knowledge of the underlying mechanism, by providing clear evidence of the oestrogen on fads2 transcription and by showing the binding of a specific transcription factor to the region. In the context of the observation studies by Burdge and Wotton, the data presented here suggests that females, or those receiving oestrogen supplements, may have hypomethylation of the oestrogen receptor binding site of the fads2 promoter, which will lead to an increased level of fads2 mRNA expression and increased  $\Delta 6$  protein levels.

Other studies have shown an effect on transcription, following single site mutagenesis. In the BRCA1 COOH terminus, which acts as a transcriptional activation domain, site-directed mutagenesis was used to characterise five known germ lines<sup>[305]</sup>. In dog, mouse and rat, 5 different sites of mutagenesis were associated with an increased level of transcription in four cases and a decreased level of transcription in one case. Although no known studies to date have investigated the effect of site-directed mutagenesis of the fads2 promoter, naturally occurring polymorphisms have been shown to affect levels of transcription of the gene<sup>[286]</sup>. In humans, there are two common polymorphism of the fads2 gene, which increase promoter activity and facilitate transcription factor binding, rs3834458 and rs968567. The activity of rs968567, a C or T base at position -299, was determined, through the use of a luciferase reporter gene assay and EMSA. The allele dependent DNA binding was shown, in terms of two complexes to the region containing the single nucleotide polymorphism. One of these proteins was identified as a member of the ETS family of transcription factors, ELK1. The promoter region, up to -1014 base pairs, was cloned into HepG2 and HeLa cells and the effect of both SNPs was determined<sup>[286]</sup>. It was found that in both cell types promoter activity was increased when the major T allele was present at -942 and the T minor allele was present at -299 base pairs. The greatest increase in promoter activity was caused by the C to T mutation at -299 base pairs (rs968567). Further experimentation by the group has shown that the polymorphism accounts for 28.5% of variance in serum phospholipid composition, in humans<sup>[286]</sup>.

The study by Lattka, along with data in this chapter shows that single bases in the fads2 promoter are sufficient to significantly alter transcription of the gene, in both humans and in the rat. The data provided in this chapter extends this knowledge by showing the importance of the -394 site, on fads2 transcription.

#### **9.4.4 Regulation of long-chain PUFA synthesis by oestrogen**

The composition of both liver and plasma PC content was significantly reduced by the amount of fat in the maternal diet, where an increase in maternal fat led to a decrease in incorporation of ARA and DHA in PC.

Previous studies into altered fat intake of during pregnancy have focused on increase saturated fat intake<sup>[64,65,66,67,68,69,70,71,72]</sup>, there has been little investigation into the role of type and amount of dietary fat on offspring development. A study by Childs et al has shown that feeding of high and low fat soybean oil, high fat linseed oil, high fat salmon oil and high fat sunflower oil diets to dams during gestation, in the rat, led to significant differences in n-6 content of liver and plasma PC of fetal offspring<sup>[306]</sup>. The study found significant differences in linolenic acid content of plasma PC and liver PC and PE between the high and low fat soybean oil groups, where levels were increased in the high fat group. There were no effects of amount of fat, in the soybean groups in terms of liver or plasma PC ARA content. Although this study has looked at the effect of different types of fat in offspring, the data is from fetal tissue, where the supply of long chain PUFA would be from the dams. As a result the findings are not directly comparable to adult offspring.

A maternal high fat diet significantly altered the capacity of offspring to synthesise long chain PUFA. This was reflected in reduced content of ARA and DHA in liver and plasma PC in males and females. A reduced cell membrane content of long chain PUFA, particularly DHA, is associated with decreased cognitive function and retinal response<sup>[154,155]</sup>. As such the ability of hepatocytes to respond to external stimuli may be reduced. As shown in figure 9.13, the proposed mechanism for the decreased ARA and DHA liver and plasma PC content is as follows.

Feeding a maternal high fat diet during pregnancy and lactation led to hypermethylation of CpG -394 of the fads2 promoter, in liver. As a result binding of

the oestrogen receptor was weakened, and therefore transcriptional activity was reduced. Due to decreased *fads2* mRNA production and  $\Delta 6$  desaturase protein, the synthesis of ARA and DHA, through the desaturation, elongation pathway was reduced.

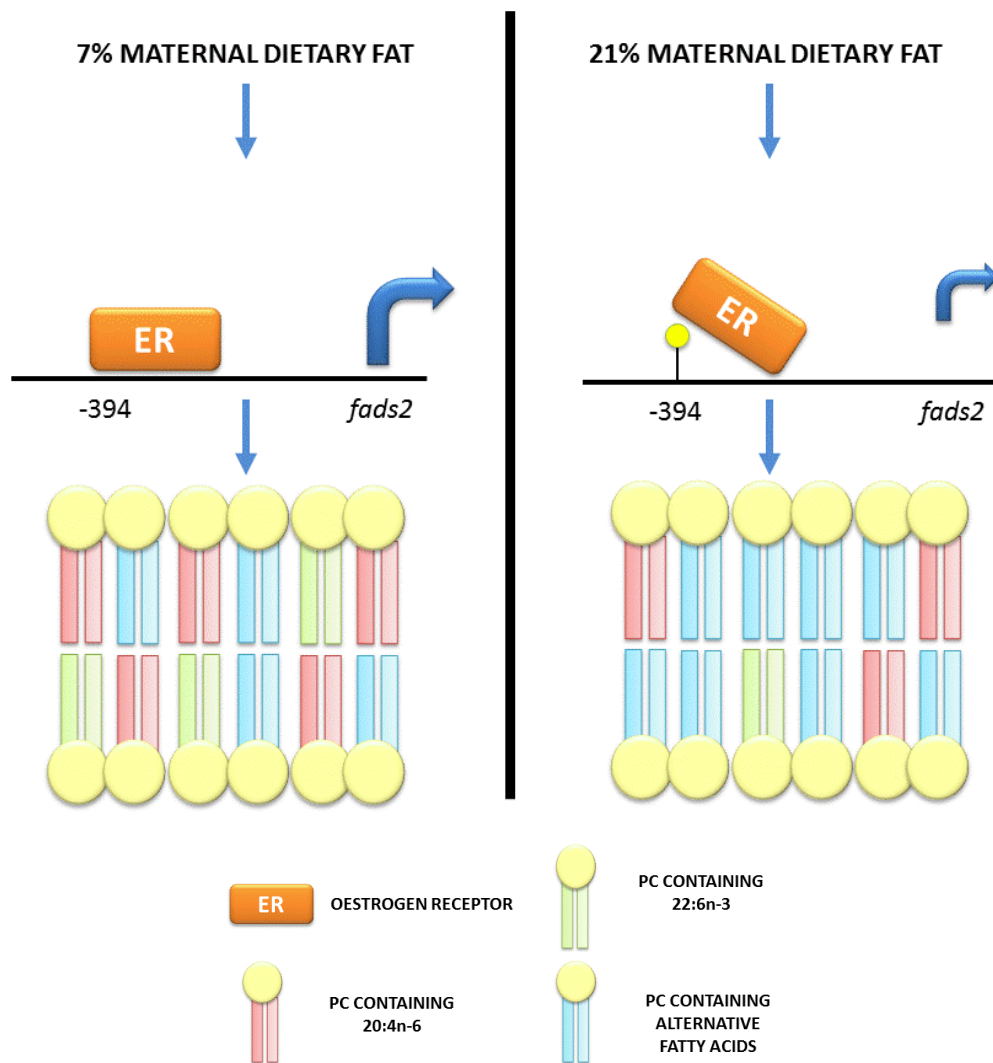


**Figure 9.13 Increased maternal fat intake leads to decreased 20:4n-6 and 22:6n-3 content of liver and plasma phospholipid through reduced ER binding to the *fads2* promoter**

To date, this is the first study to show the role of promoter methylation and transcription factor binding to the *fads2* promoter, so the extent to which these results can be confirmed by the current literature is unclear.

The findings in this chapter have provided a potential mechanism for the altered phenotype, in terms of liver and plasma PC composition, seen in male and female offspring of dams fed an altered fat diet.

## Chapter 10

### Discussion



## 10.1 Discussion

The ability of an organism to respond to changes in its environment is crucial for Darwinian fitness and evolution<sup>[92,33,307,308,309]</sup>. An altered developmental environment *in utero* has the potential to lead to an altered offspring development, which confers altered disease risk in later life<sup>[82,14,86,87,310,311,167,41,312,21]</sup>. In both human and animal models, it has been shown that these altered phenotypes are transmitted between generations, even when the initial stimulus does not persist beyond the F0 generation<sup>[166]</sup>. In humans, nutritional transition is associated with an altered risk of disease<sup>[180]</sup>.

These observations raise questions about the mechanism by which nutrition during development induces changes in phenotype and how such effects may be transmitted between generations. Recent studies in human and animal models have demonstrated that epigenetic processes are involved centrally in the induction of altered phenotypes<sup>[196,197,198,187,199,200,201,215]</sup>. The experiments described in this thesis were designed to investigate three important areas of uncertainty within the field of the early life origins of disease; if and how induced epigenetic marks are transmitted between generations; the degree of specificity with which nutritional exposures in early life alter epigenetic marks; and the stability of induced changes in the epigenome.

## 10.2 Are epigenetic marks transmitted between generations?

The mechanism behind altered phenotype, as a result of long-term nutritional transition has not been widely studied and is limited to two human observation and two studies in drosophila. In humans, there was a causal link in China between migration onto a complex carbohydrate and increased fat content diet, such as that present in the Western world, and risk of obesity<sup>[180]</sup>. However, due to the timing, follow ups in further generations, to test adjustment to the new diet have not been obtained. In India, an increase in obesity in mothers during the 20<sup>th</sup> century, who showed levels of glucose intolerance during pregnancy, lead to a dramatic increase in the presence of low birth weight and thin at birth offspring<sup>[313]</sup>. During adult life, these offspring tended to be insulin resistant and had an

increased risk of developing type II diabetes. Studies in *Drosophila* have shown that constant exposure to altered nutrition leads to persistent changes in gene expression<sup>[181,182]</sup>. These alterations, which led to increased adaptation to medium in subsequent generations, provided primary data on adaptation to altered nutrition across generations.

Animal models have shown that an altered environment during pregnancy can lead to the development of an altered phenotype, which is evident up to the F2 generation. In a study by Benyshek, protein restriction of the maternal diet during pregnancy and lactation led to a significant decrease in fasting glucose level in male offspring, up to the F3 generation<sup>[174]</sup>. However the value in the F3 generation was significantly lower than in the F2 generation and the F3 offspring also showed a significantly higher plasma insulin level. These results show that the altered phenotype is not transmitted unchanged between generations, where the effect of a single period of an altered environment is diminished between generations. This pattern was also seen in a study by Drake, where exposure of dexamethasone during pregnancy in rats, led to an altered phenotype in the offspring, including low birth weight, adult hypertension and increased hepatic PEPCK activity<sup>[167]</sup>. The dexamethasone-programmed phenotype was also seen in F2 generation male offspring, when no altered exposure was present during pregnancy. However, no effects were seen in the F3 offspring, suggesting the consequence of dexamethasone treatment is only seen up until the F2 generation. In humans, it was shown by Giusti et al, that exposure to diethylstilbestrol led to two-fold increase in breast cancer risk in woman<sup>[170]</sup>. In one case, the granddaughter of a diethylstilbestrol exposed woman developed ovarian cancer. This suggests that initial exposure led to a specific alteration which predisposed the granddaughter to development of an alternative form of cancer. Although this is limited to one known case, so must be viewed with caution.

The findings presented here suggest that in rodents, nutritional transition to a high energy diet leads to significant alterations in both the maternal metabolism and offspring phenotype across four generations. Transition onto a high energy diet was associated with significant differences in offspring growth, levels of gene

transcription and promoter methylation. When accompanied by protein restriction during the F0 pregnancy, there were significant changes in the trajectory of alteration in weight gain, hepatic gene expression and promoter methylation. These differences were dependent on generation, such that differences were present in the F3 generation, which were not seen in the F1 or F2 generation offspring.

As suggested by Badyaev, parental effects on development are essential for the transfer of developmental resources needed to reconstruct, maintain and modify genetically inherited components of the phenotype<sup>[314]</sup>. Exposure to the high energy diet led to alterations in maternal metabolism in the F0 generation. This therefore implies an altered developmental environment, and transfer of information, provided from the mother. As a result of the high energy diet during the F0 pregnancy and the maternal affects, F1 offspring had a significantly altered metabolic phenotype. The ability of the F1 dams to interact with the environment and successfully represent and convey information from the external nutrition was altered, this means the F2 developing fetuses were exposed to altered maternal signals. Rather than transmission of an altered phenotype, gene expression levels and epigenotype between generations, it is hypothesised that information which acts as a signal to change, is transmitted between generations, rather than specific methylation marks, which convey an altered gene regulation and therefore phenotype. One possible explanation is that interactions between the maternal metabolism and the environment drive the adjustment of the offspring phenotype, through the establishment of *de novo* methylation marks in each generation. (Figure 10.1).

If this hypothesis is correct, then the loss of an altered phenotype across generations and the presence of a different phenotype generations after an initial exposure, can be explained as follows. The initial alteration led to a significant alteration in the metabolism of the mother, leading to transmission of altered signals during *in utero* development of the F1 offspring. As a result an altered phenotype was present in the F1 generation. This has multiple impacts, as the altered phenotype may portray an altered disease risk in the F1 generation in later

life and an altered metabolism of the F1 generation will lead to significant differences in the ability of F1 dams to transmit information across the placenta. As a result the development of the F2 generation will be altered. If the altered environment, experienced by the F0 dams, during pregnancy, was transient, it is hypothesised that the readjustment of the offspring generation to the original environment will occur within one or two generations. This will depend on the ability of the altered maternal metabolism and physiology in the F1 generation, to convey the appropriate information regarding the external environment, to the developing F2 generation.

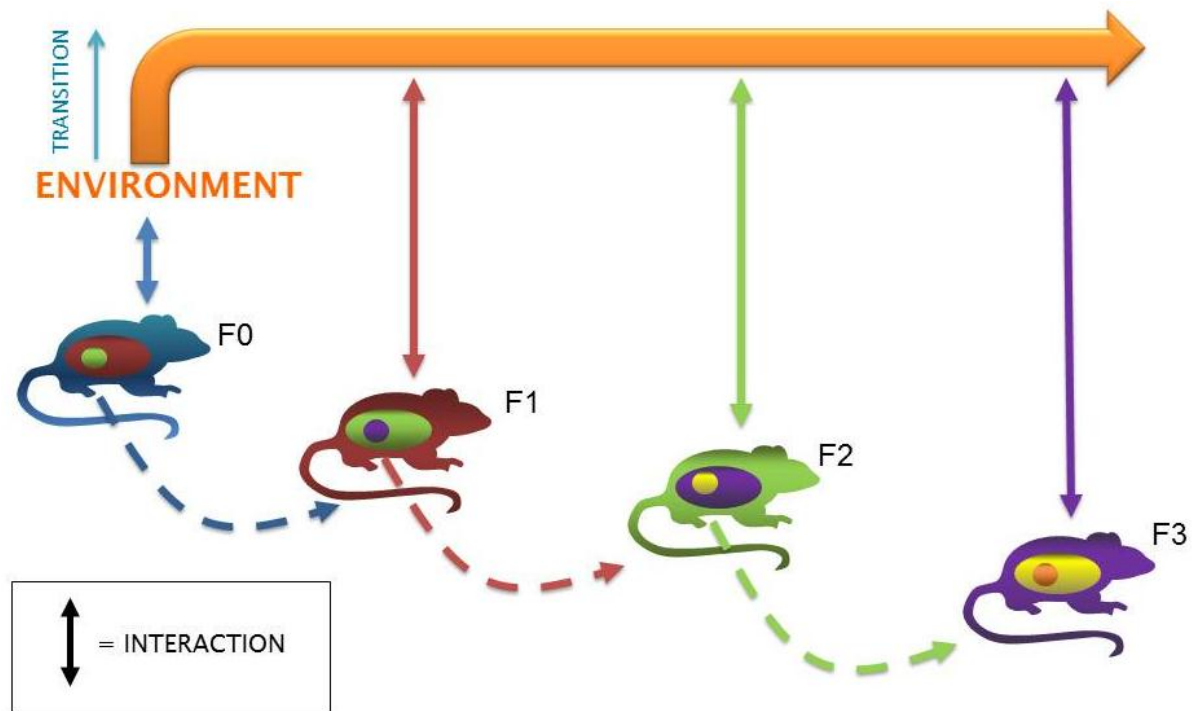


**Figure 10.1 An interpretation of the adjustment towards a novel environment, through altered interactions between maternal phenotype and the environment** where the environment is shown in orange, the F0 generation is shown in blue, the F1 generation is shown in red, the F2 generation is shown in green, the F3 generation is shown in purple and interactions are shown by double-headed arrows. Indirect maternal effects are shown by dashed arrows.

Natural selection is a multi-generation process, which requires opportunistic mutations and altered phenotypes, which confer an increased Darwinian fitness. As a potential mechanism for adjustment to a novel environment, the hypothesis suggested here is in agreement with previous concepts in the field. In the

alternative adaptation hypothesis, West-Eberhard suggests that novel traits originate as stable phenotypes prior to reproductive isolation and speciation<sup>[176]</sup>. One example of this is shown by Slijper, in a two-legged goat<sup>[315]</sup>. The animal, which was born with a congenital defect, could not walk on all fours, and therefore learnt to walk on two legs. Upon dissection, it was found that the bones of the hind leg and leg muscle had undergone dramatic changes. This example shows how the goat has the capacity for two-legged walking, which is utilised only under the conditions that require it. The findings in this study are in agreement with the alternative adaptation hypothesis, in that the metabolism of the rat was able to adjust to the high energy content of the diet, in the apparent absence of genome mutation. Again, in support of the hypothesis stated in this thesis, epigenetic opportunity appeared to be one mechanism behind the alteration in metabolism<sup>[178]</sup>. In our study, it is suggested that protein restriction of the F0 diet during pregnancy, altered the information transmitted from the maternal circulation, to the developing fetus and therefore epigenetic opportunity for adjustment, across generations was altered. It is therefore suggested that *de novo* epigenetic marks are established in each generation, in order to achieve these short time scale alterations in phenotype. In terms of humans, this may suggest that the increased prevalence in cardio-metabolic diseases worldwide may be a consequence of a shift in nutrition, onto a higher fat and complex carbohydrate diet, leading to altered epigenetic marks and an altered phenotype, which conveys a risk towards the disease.

### **10.3 Gene specific effects of altered nutrition**

Candidate gene studies have shown that one form of nutritional alteration or exposure to specific chemicals during pregnancy leads to effects across multiple genes<sup>[316,317,318,175]</sup>. Vinclozin exposure, during embryonic gonadal sex determination led to an epigenetic reprogramming of the male germ line that is associated with transgenerational adult-onset disease states<sup>[316]</sup>. mRNA expression of 196 genes was altered in the F1-F3 generations, where most showed a decreased expression or silencing, following exposure. The effect of

vinclozin exposure during embryonic gonadal sex determination was again shown in a study by Skinner, where in the F3 generation a multitude of genes were altered in the hippocampus and the amygdala. Where effects were sex specific and related to specific behaviour effects in each sex <sup>[175]</sup>.

Lillycrop et al have shown that protein restriction of the maternal diet during pregnancy, in the rat, led to an altered expression of genes in the liver transcriptome in male offspring, where the majority were increased <sup>[318]</sup>. Folic acid supplementation of the protein restricted diet also led to an altered mRNA expression of genes. Interestingly, of all of the genes changed, only a small amount were conserved between the protein restricted and protein restricted plus folic acid diets. In our study, we found that dietary challenge during F0 pregnancy induced altered gene expression in all three generations, but relatively few genes showed transmission of altered expression between generations<sup>[317]</sup>. Differences in the transcriptome between the PS and PR lineages were present for 1,684 genes in the F1 generation, 1,680 genes in the F2 generation and 2,062 genes in the F3 generation. Although differences in the same 113 genes were seen in each generation, 63 of these were directionally opposite. KEGG analysis showed that only the Adherens Junctions pathway was altered in all three generations. These studies suggest that an altered environment during embryonic development leads to an altered expression of multiple genes, that these genes are specific to the nature of the nutrient and that subsequent generations do not necessarily show alterations in the same number or pathways of genes altered.

Data here is in agreement with these findings, showing that specific nutrients induce differential effects on gene regulation. Offspring of dams fed a protein restricted diet had a significantly higher mRNA expression of GR, compared to the PS offspring, but no differences were seen in other genes, such as PPAR $\alpha$  and DNMT3a2. Again, following high fat feeding of dams during pregnancy, offspring showed an increased hepatic expression of PEPCCK, but a decreased mRNA expression of fads2. This suggests that the mechanisms which cause altered regulation of transcription are specific to and within metabolic pathways.

This implies that nutritional alteration in the mother has the potential to alter multiple pathways in different ways. As a result, the mechanism by which altered nutrition leads to an altered expression of genes is highly specific to the specific nature of the altered nutrition, such that addition of folic acid to a protein restricted diet can dramatically alter the trajectory of change. When considering the beneficial aspects of supplementation and fortification of foods, it is important to consider effects on a large number of pathways, which may show different effects in subsequent generations.

#### **10.4 Nutrient specific effects on gene regulation**

It has been shown that different nutrients induce differential effects on gene regulation; however no study to date has compared different natures of nutrition on regulation of the same gene. In terms of PEPCK, it has been shown, in unrelated studies that both a PR diet and exposure to glucocorticoids leads to an increase in mRNA expression. It is not known if different natures of nutritional alteration during pregnancy lead to the same outcome, in terms of regulation of specific genes.

In the current study, the role of type of maternal nutritional alteration on the epigenetic regulation and gene expression of PEPCK was investigated. Protein restriction of the maternal diet, led to significant difference in PEPCK mRNA across the three generations, but no changes between dietary groups in the F1 offspring. These differences were associated with significant differences in methylation of the PEPCK promoter, which were CpG specific. Alterations in folic acid content of the diet had no effect on PEPCK mRNA expression when provided in the prenatal diet; however postnatal supplementation led to a significant increase in PEPCK mRNA expression in males and significant alterations in promoter methylation in females. It was also shown that females have a greater level of PEPCK expression, compared to males, suggesting an increased capacity for provision of glucose during fasting. Altered fatty acid composition of the maternal diet had modest effects on PEPCK mRNA expression and no effects on methylation, in either sex. These findings suggest that alterations in the methylation of the PEPCK promoter are dependent on both the nature of nutritional alteration and the timing.

The results shown here suggest that the mechanism behind the altered gene expression is preserved, regardless of the nature of the nutritional intervention. This is represented by the significant alterations in the methylation status of the genes altered. It appears that the mechanism is highly specific, in that only specific genes are affected by one type of nutritional alteration. Following this finding, the contributions of maternal affects, the nature of the nutritional alteration, duration and timing of exposure are now understood (Figure 10.2).

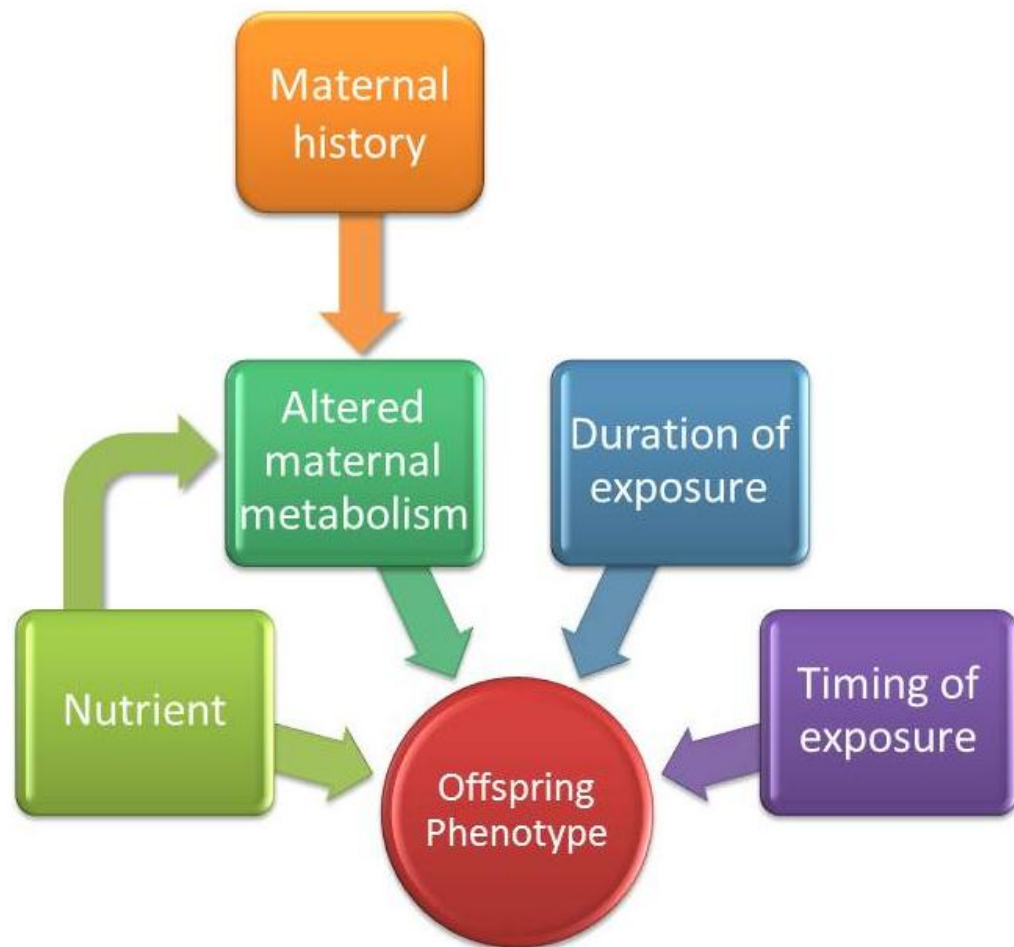


**Figure 10.2 The role of specific factors on offspring phenotype** where altered nutrition and maternal history can significantly alter maternal metabolism



## 10.5 Response to nutrition and implications

The findings in this study have contributed to the understanding of the mechanism behind the altered offspring phenotype as a result of altered nutrition. The current generally accepted view is that DNA methylation provides a stable heritable mechanism by which modifications in the developmental environment can lead to the generation of disease associated phenotypes in later life<sup>[184]</sup>. However, it is known that the methylation status of the epigenome is subject to change with age and that this is associated with prevalence of specific diseases<sup>[192]</sup>. Disease associated phenotypes are also presenting at younger ages<sup>[5]</sup>. These observations suggest that DNA methylation marks may not be as stable as initially hypothesised. Findings here suggest that the level of DNA methylation of specific promoters varies with age, in a manner dependent on the maternal diet during pregnancy. This suggests that DNA methylation is not stable, in the sense that it is unchanged throughout life; rather that the epigenome has plasticity, where promoter methylation is sensitive to fluctuations in the external environment.

Due to the perceived stability of DNA methylation, it is currently hypothesised that transgenerational transmission of phenotype involves stable transmission of specific methylation marks. However, findings by Drake, suggest that changes in the epigenome are only evident up to the F2 generation, following a transient modification in nutrition or chemical exposure<sup>[167]</sup>. My findings suggest that *de novo* methylation is occurring, at least in part, in each generation exposed to a high energy diet. It is this plasticity in the epigenome between generations that allows adjustment of offspring generations towards the novel environment.

The effect of altered nutrition on changes in offspring gene regulation is seen as a fixed mechanism, which does not take into account the nature of the nutritional modification. However, findings here suggest that there is specificity to the plasticity of the epigenome to the altered environment. Although the underlying mechanism may be conserved, findings here suggest that modified regulation of one gene may result from modification in the protein level of the diet, but not when the fat content of the diet is altered. This suggests that the unknown mechanism

linking nutritional change and modification of the epigenome is specific to the nature of the nutritional alteration.

The hypothesised mechanism by which an altered environment leads to a modified phenotype in adulthood is as follows<sup>[184,319,164,320,321]</sup>. An altered external environment, such as modification in nutrition, leads to a change in the parental effects, such as transmission of different information across the placenta from the maternal circulation. This change in maternal signals and altered developmental environment will modify the *de novo* methylation of the epigenome of the developing organism. Through altered regulation of gene transcription, this will lead to a change in the phenotype in the developing organism. If the established phenotype of the developing organism conveys an altered ability to perceive information from the maternal circulation or to process information from the external environment during post-natal development, this may result in further changes to the epigenome. Any change in the external environment during adult life has the potential to lead to changes in the phenotype of the adult organism, as a result of the plasticity of the epigenome. The ability of the epigenome to respond to changes in the environment will depend on the phenotype established during development and previous adult life. The adjustment of the phenotype of the organism to the environment across the life course is possible due to the plasticity of the epigenome. The establishment of an altered phenotype in the adult organism will affect the parental effects on the next generation (Figure 10.3 A). This process underlies the changes in maternal and offspring phenotype and epigenome seen in the findings present here.

When considering this hypothesis in terms of multiple generations, extra effects must be considered. An altered maternal phenotype in the F0 generation will lead to the transmission of altered information to both the F1 developing offspring and F2 germ line, so that the phenotype and epigenome of both are modified. During the F1 pregnancy and F2 *in utero* development, the ability of the F2 offspring to interpret information from the maternal circulation will be somewhat dependent on the F2 germ line phenotype. The adjustment of the offspring phenotype is also dependent on the persistence of the novel environment, where

changes will impact the current and therefore future generations, through changes in the epigenome (Figure 10.3 B).

In mammals, females invest greatly in the production of the next generation, through maternal adaptations to pregnancy such as altered metabolism and storage of nutrients<sup>[322,104]</sup>. As a result, the role of the mother and altered maternal effects has been a focus of investigation here. Studies by Pembrey and Skinner have suggested an importance of paternal effects on the generation of altered phenotypes in offspring<sup>[163],167]</sup>. Although the direct contribution of altered paternal effects, in offspring development, compared to maternal effects, is not clear, the role of the father in the hypothesis suggested here must not be ruled out.

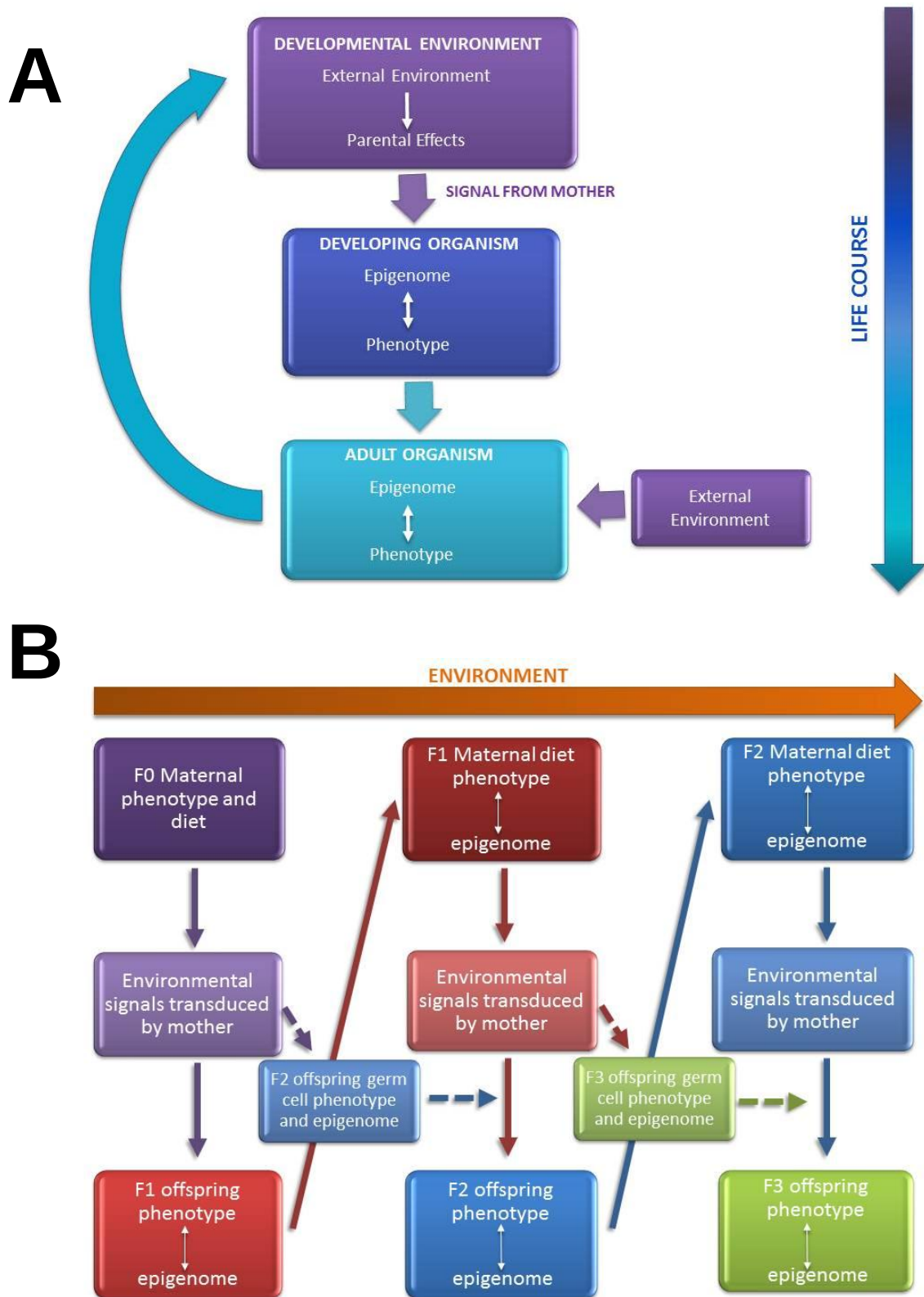


**Figure 10.3** The mechanism by which an altered environment leads to adjustment of the organism to the novel environment through changes to the epigenome in one generation (A) and across multiple generations (B).

## **10.6 Future work**

The findings in this thesis have increased the understanding of mechanisms underlying the generation of altered phenotype in offspring, following altered maternal nutrition; however key questions are now apparent.

It is known that an altered nutrition has the potential to lead to altered epigenetic marks in the offspring; however the intermediate mechanism here still remains unknown. How does a nutritional effect become an epigenetic change?

Different forms of nutritional alteration do not lead to alterations in the same genes, or within genes of the same pathways. Is the mechanism by which nutritional alteration becomes an epigenetic change specific to the form of alteration, or is it preserved?

By answering these questions there is potential to inhibit and perhaps even reverse the establishment of detrimental alterations at the epigenome level. As a result, phenotypes that result in an increased susceptibility to disease may be averted.

## Chapter 11

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