THE EFFECT OF MATERNAL PROTEIN AND FOLIC ACID INTAKE ON THE DEVELOPMENTAL PROGRAMMING OF ADULTHOOD DISEASES

Doctor of Philosophy

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2013

When someone you love becomes a memory,

The memory becomes a treasure

- *Unknown author*

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20.12.1926 – 05.07.2011

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"No man is an island entire to itself" John Donne

This adventure started in 2008. It has taken 5 years to get here. It has been the best of times and the worst of times. Upon reflection it has shaped me into a critical observer and someone who is inspired by curiosity. Completing a PhD has taught me to rely on myself and that great rewards come from solid, gruelling hard work. While I have learnt the ability to push myself further than I ever thought possible, it was the environment in which I worked that made this achievable.

I could never have completed this PhD without the supportive environment that Monash University has provided. In particular, I thank the Faculty of Medicine Nursing and Health Sciences for the stipend to undertake my PhD.

First and foremost, I would like to thank Dr James Armitage for the opportunity to undertake my PhD in his laboratory and under his stewardship. James, you have been a fantastic motivator, supporter, supervisor and mentor. You knew when to push me as well as when to allow me find my feet. It always helped knowing your door was open and that you were never too busy for a chat. Your warmth and approachability helped ensure that the lab was fertile ground for growing into a scientist.

Secondly, Dr Luise Cullen-McEwen – you have been a marvellous supervisor. Not only for being responsible for serially sectioning the PN21 kidneys required for nephron determination (Chapter 3 and 6), but some of the best days were working by your side in the laboratory. You have been a fantastic teacher and a comforting source of support. I could not have survived this PhD without you.

Thirdly – Professor John Bertram – you have been a fantastic mentor and overseer of my PhD; your captaincy has been irreplaceable. You helped provide a great environment – as Head of Department you helped build a supportive, friendly, social environment and as a lab head you ensured that there was a balance between work and play. I think the Bertram Lab Meeting and Christmas Party antics will live in infamy.

While my supervisors enabled me to be able to do my PhD, there were also integral people who helped with the research.

I thank Associate Professor Roger Evans for allowing the use of laboratory to conduct renal physiology experiments (Chapter 3 and 6). Your expertise at both renal function and providing double espressos were essential.

Thanks to Dr Marloes Nitert Dekker for completing the methylation studies (Chapter 5) in Queensland. Your expertise and patience were amazing.

Mr Luke Eipper assisted with the nephron number counts (Chapter 4) of E17.25 kidneys that I generated and some of the data were used as part of his Honours Thesis. It was a pleasure having you in the lab Luke, you were dedicated and hard-working.

Ms Nicole Dellios you were a gem and your skills in the lab made sure every animal collection day went smoothly.

To animal house staff at Monash University, particularly Ms Lily Jackanic and Mrs Terry Lane for their wonderful support and taking great care of the animals.

The scientific support I had for completing the research often carried over to psychological support. The Department of Anatomy and Developmental Biology has been an amazing place to work. I have formed some incredible friendships that made it a pleasure to come in to work – Anne O'Connor, Rebecca Douglas-Denton, Jen Lo, Stacey Hokke, Vivian

Nguyen, Anzari Atik, Sheena Bouch and Brooke Huuskes to name but a few of the wonderful people I was fortunate enough to work with. Special mention goes to Dr Chantal Hoppe. Her guidance, support and belief in me were critical for a happy and smooth PhD.

There is no way I would have had the mental and physical strength to survive my PhD without my personal trainer Ms Stef Hanson, and my cycling coaches Mr Conan Daley, Mr Mark Hudson and Ms Nicole Whitburn. Stef helped me find the untapped depths of my determination and lead me on a journey to lose over 25kg and run a marathon, while Mark, Conan and Nicole developed me into a cyclist able to conquer the Pyreness.

While science has dominated my life for the past 5 years – the people outside of the sphere of science helped keep everything in perspective. Ms Shari Aubrey, you have been my ballast, Ms Tina Bond you have kept me smiling, Ms Pip Grindblat you have kept me laughing and Ms Angela Mclaren you kept me moving. You all kept me grounded and centred. AS well as the members of Lead Out Cycling have been the best domestiques for completing a PhD.

The nucleus of my support network is comprised of my family and my closest friends. Sarah Henry, we have worked side-by-side for the last 6 years. Words cannot describe the friendship we have developed over the years – words like ‘supportive’ and ‘loyal’ do not describe the breadth and depth of it. With you by my side, I know nothing can go wrong. Mr Jonathan Bensley, you have been my partner in crime, my touch-stone of reality and the best friend anyone could ask for. Mr Benjamin Barzel, your warmth and generosity are unassailable. I could not have asked for better friends.

To my family; my mother Louise, thank you for understanding my absence and for always been a source of warmth and love. My father, Malcolm, thank you for challenging me to always push for success. My brother, Guy, for understanding what I was going through

and providing unsurpassable IT skills, not to mention the laughter – I could not ask for a better brother. This PhD would have been impossible without such a wonderful supportive family. I would also like to acknowledge the unconditional love and support I received from my Nanna, Una Schumacher, who always believed in me.

DECLARATION

I, Ryan Wood-Bradley, declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution and affirms that to the best of the my knowledge the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Ryan James Wood-Bradley

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ABSTRACT

The leading causes of mortality and morbidity worldwide are cardiovascular disease (high blood pressure, high cholesterol and renal disease), cancer and diabetes. The development of these diseases is related to a complex interaction between adult lifestyle and genetic predisposition. Maternal nutrition can influence the fetal and early life environment and is known to be a risk factor for the future development of adult diseases. A growing body of large-scale human studies suggest that maternal malnutrition may impair organogenesis in the offspring, which can predispose these offspring to high blood pressure and renal dysfunction in adulthood. Studies in experimental animals have further illustrated the significant impact maternal diet has on offspring health. Many studies report changes in kidney structure (namely a reduction in the number of nephrons) in offspring of protein deprived dams. Although the early studies suggested that increased blood pressure was also present in offspring of protein restricted dams, this is not a universal finding and requires clarification. Importantly, to date, the literature offers little to no understanding of *when* in development these changes in kidney development occur, nor are the cellular and molecular mechanisms that drive these changes well characterised. Moreover, the mechanisms linking maternal nutrition and a suboptimal renal phenotype in offspring are yet to be discerned – one potential mechanism involves epigenetics.

Epigenetics can be influenced by maternal diet, particularly the supply of micronutrients involved in methylation – specifically folate or folic acid. When folic acid has been added to protein deficient maternal diets in animal studies it has been shown to reverse the hypertension which may be programmed in offspring of rodents fed a sub-optimal maternal diet. This thesis explores the relationship between maternal protein deprivation and folic acid intake on rat

kidney development, and the development of high blood pressure and renal dysfunction in the offspring.

1.1. Maternal protein restriction – impact on fetal and postnatal growth

Fetuses developing in the face of maternal protein restriction (9% protein versus 20% protein in the control normal diet) showed minimal disruption in their growth trajectory except for an increased placenta:fetal ratio at embryonic day 20 (E20). Analysis of ureteric branching morphogenesis (an important driver of nephrogenesis) at E14.25 using whole metanephric organ culture found no effect of the low protein diet on branching, however a 14% reduction in nephron number was observed *ex vivo* from as early as E17.25 in low protein offspring. Postnatally, offspring of maternal protein deprivation demonstrated slower growth than controls, a 26% (female) and 17% (male) reduction in nephron endowment, but did not show any signs of cardiovascular (blood pressure, heart rate) or renal dysfunction (glomerular filtration rate, effective renal blood flow) in adulthood.

1.2. Maternal folic acid restriction or supplementation – impact on fetal and postnatal growth

When maternal protein restriction was combined with folic acid restriction (<0.05mg/kg folic acid versus 5mg/kg folic acid in controls) the renal phenotype was very similar to that observed following maternal protein restriction alone. Offspring were of a similar weight to that of offspring exposed to maternal protein restriction, and likewise they had a similar nephron endowment, blood pressure and kidney function. Paradoxically, maternal folic acid supplementation (200mg/kg) combined with protein restriction in pregnancy lead to reduced kidney branching morphogenesis in the offspring (13% reduction in branch points and 12% reduction in ureteric tips). This finding was also observed in the metanephric organ culture system when exogenous folic acid was added to the media.

1.3. KIDNEY GENE EXPRESSION AT E14.25

Real-time polymerase chain reaction (RT-PCR) was used to assess expression of key kidney development genes following exposure to a maternal low protein diet with folic acid restriction or supplementation. Genes involved in the regulation of branching morphogenesis were analysed (*Gdnf, Bmp4, Gfrα1*) as were genes involved in mesenchyme to epithelial transition (*Pax2* and *Hnf4α*). Female offspring exposed to maternal protein and folic acid restriction reduced expression at E14.25 of *Pax2, Gdnf, Bmp4* compared with maternal protein restricted controls (no change in males), while male offspring had greater expression of *Gfrα1*. Female and male offspring exposed to maternal low protein and folic acid supplementation had reduced expression of *Pax2*, and females also had reduced expression of *Gdnf* and *Bmp4* compared to controls.

Analysis of genes involved in kidney development for epigenetic changes (altered methylation status) revealed no change in gene methylation despite the changes in levels of expression described above. Although *Gfrα1* expression was negatively correlated with methylation status gene expression and methylation status of *Gfrα1* was most closely correlated in offspring exposed to maternal low protein diet and was not different with the exposure of maternal folic acid intake (either supplementation or restriction).

1.4. OFFSPRING CARDIOVASCULAR FUNCTION – IMPACT OF MATERNAL FOLIC ACID SUPPLEMENTATION OR RESTRICTION

Postnatal bodyweight of offspring exposed to maternal protein restriction with supplementation or restriction of folic acid did not differ compared with low protein controls, however bone mineral content was reduced at postnatal day 21 (PN21), PN180 and PN360 in offspring exposed to maternal low protein and folic acid restriction (LP-FA). Postnatal nephron endowment was significantly greater in LP-FA offspring at PN21. Compared with controls,

neither cardiovascular nor renal function were altered in offspring exposed to maternal folic acid supplementation or restriction.

1.5. Conclusion

The findings from this thesis suggest that maternal diet can have a significant impact on fetal development where a nephron endowment is programmed early, but this does not necessarily lead to adult disease despite the continued presence of a nephron deficit. It is likely that this scenario occurs because offspring did not exhibit catch-up growth, which prevented the development of high blood pressure and renal dysfunction.

Understanding the impact of maternal diet on fetal development and offspring health is an effort targeted at reducing the global burden of non-communicable disease. The particular constituents of the maternal diet that have the greatest impact on offspring health are going to assist in developing effective and health-promoting diets. Folic acid has been shown to have beneficial effects on birth outcomes, but it is not able to compensate for macronutrient deficiencies in the maternal diet. While it has been suggested that folic acid supplementation may lead to an increased risk of developing asthma in childhood, the findings from the current study indicate reduced growth when folic acid supplementation is combined with low protein. There was no indication of disease onset in those offspring. Maternal diet is critical for offspring development, but poor maternal diet may not trigger disease onset without secondary insults.

CHAPTER ONE: INTRODUCTION

1.1 PERSPECTIVES

A global epidemic of cardiovascular and metabolic disease has become apparent in the past 30 years. Although a number of adult and genetic risk factors have been identified, there is now significant evidence to support the hypothesis that the environment encountered during development can contribute to adult disease. This phenomenon, termed the developmental programming of adult health and disease, may help explain the incidence of adult diseases that cannot be solely attributed to known risk factors, such as weight, age, diet and lifestyle (Barker, 2003b). Developmental programming is defined as a *permanent change in the structure or function of an organism due to alterations in development that occur in response to an environmental stimulus during critical periods of organ development* (Lucas, 1991). Such programming may occur in response to an insult impacting upon development of one or more organs, or the entire fetus. Stimuli that are most commonly documented are maternal malnutrition (reviewed by Armitage, et al 2004), maternal behaviour and maternal hormonal status (Brunton and Russell, 2011, Cameron *et al.*, 2008, Cottrell and Seckl, 2009).

This thesis aims to address the following questions.

1.1.1 WHAT IS THE IMPACT OF MATERNAL LOW PROTEIN DIET ON OFFSPRING BLOOD PRESSURE, HAEMODYNAMICS AND RENAL FUNCTION?

Studies in human populations first demonstrated the association between exposure to a poor maternal diet during pregnancy and offspring cardiovascular disease in adulthood (Barker *et al.*, 1993, Barker *et al.*, 1992, Painter *et al.*, 2006b, Painter *et al.*, 2005b), however there is significant controversy as to whether hypertension is programmed by exposure to a maternal low protein diet (see Section 1.2.4). Although these observations in humans are of great importance and provide impetus for research, they are unable to provide insight into the underlying mechanisms due to the difficulty in determining direct cause and effect in human epidemiological studies.

There is a strong link between cardiovascular health and kidney structure and function in humans (Hoy *et al.*, 2008, Hughson *et al.*, 2006, Keller *et al.*, 2003). This relationship has been investigated in animal models of developmental programming (Armitage *et al.*, 2004, Vehaskari and Woods, 2005). Maternal under-nutrition can lead to reduced nephron number in offspring and this may predispose them to poor cardiovascular health (Woods, 2007, Woods *et al.*, 2001, Woods *et al.*, 2005, Nwagwu *et al.*, 2000, Langley-Evans, 1997). However, it remains unclear *when* the maternal diet has its deleterious effect on kidney development. Rodent studies designed to model malnutrition in humans (i.e. using a low protein diet) have reported low birth weight, altered growth trajectory and greater susceptibility to developing cardiovascular disease (see Section 1.2.5 and Table 1.1). An almost ubiquitous finding in offspring rodent studies using a maternal low protein diet has been a reduced nephron number (Langley-Evans *et al.*, 1999b, Vehaskari *et al.*, 2001, Vehaskari and Woods, 2005, Woods *et al.*, 2001, Habib *et al.*, 2011, Harrison and Langley-Evans, 2009, McMullen and Langley-Evans, 2005b, Xie *et al.*, 2012). Notwithstanding this, while many studies have reported a reduction in nephron endowment, few studies have documented the effect on kidney function (Nwagwu *et al.*, 2000, Woods *et al.*, 2005, Lim *et al.*, 2011). Maternal low protein diet is also associated with increased offspring blood pressure in some studies (Langley-Evans, 1997, Langley-Evans *et al.*, 1996a, Langley-Evans and Nwagwu, 1998, Woods *et al.*, 2001, Woods *et al.*, 2004, Langley-Evans *et al.*, 1999b) but the aetiology of high blood pressure is multifactorial and may also involve postnatal growth as well as the embryonic environment (Hales and Barker, 2001).

This thesis will investigate blood pressure and growth using telemetry and dual emission x-ray absorptiometry (DXA) to determine the contribution of postnatal growth and stress on the development of this reported 'programmed' phenotype (Chapter 3).

1.1.2 WHEN DOES A 'PROGRAMMED' NEPHRON DEFICIT OCCUR AND WHAT PROCESS OF KIDNEY DEVELOPMENT IS AFFECTED?

Kidney development has been reported to be sensitive to the intrauterine environment in both humans and rodents (see Section 0). Kidney development occurs over a finite period through stages of branching morphogenesis and nephrogenesis. Nephrogenesis, the formation of nephrons, occurs over a set period of development and at present there are no techniques that enable the process to be re-started in adulthood. Therefore the nephron endowment a mammal possesses at the completion of nephrogenesis is finite. While it is understood that maternal low protein diet leads to a reduced nephron deficit in postnatal life, it is not known which stage or stages of nephrogenesis is/are affected by the maternal diet. Knowledge of specific sensitive periods of kidney development to maternal nutrition would assist in care and identification of at risk pregnancies.

This thesis seeks to investigate the contribution of kidney branching morphogenesis and nephrogenesis (Chapter 4) on the final nephron complement (Chapter 3) in rats exposed to maternal low protein diet and understand how this impacts on later health (Chapter 3). Structural and molecular analyses will be carried out on embryonic day 14.25 (E14.25) rat kidneys and final nephron endowment will be determined at postnatal day 21 (PN21). Kidney function will be determined at PN180 and PN360 and blood pressure will be determined at PN360.

1.1.3 CAN THE PROGRAMMED PHENOTYPE BE RESCUED?

Although much of the research on developmental programming of adult diseases has reported poor outcomes for offspring exposed to a sub-optimal intrauterine environment, there is also evidence that a well-balanced diet combined with micronutrient supplementation leads to improved birth outcomes (Conlisk *et al.*, 2004, Molloy, 2002, Barros *et al.*, 2010). Moreover, there is evidence that the effects of dietary restriction can be ameliorated with supplementation

of micronutrients in the diet (Fernandes *et al.*, 2011, Bhutta *et al.*, 2008, Sebayang *et al.*, 2011). One such micronutrient is folic acid, which has been reported to significantly reduce the occurrence of neural tube defects (NTD) in many populations (Czeizel, 1995b) and to reverse the effects of maternal dietary restriction (protein or caloric) in pregnancy (do Carmo Franco *et al.*, 2009, Torrens *et al.*, 2006, Lillycrop *et al.*, 2010). Folic acid is the synthetic form of folate, a B group vitamin found in abundance in most leafy green vegetables. Folate and folic acid play an important role in DNA synthesis and methylation of biologicals (lipids, proteins and DNA) through their ability to donate a methyl group. This role in methylation, specifically of DNA, has been proposed to be the underpinning mechanism that controls the occurrence of a "programmed" phenotype (Cutfield *et al.*, 2007). Methylation of DNA is capable of altering the phenotype due to effects on levels of gene expression (Doerfler, 2005).

This thesis investigates the potential for folic acid supplementation to prevent the deleterious effects of maternal protein restriction in rats – specifically the impact on kidney development (Chapter 5), nephron number (Chapter 6) and later health (Chapter 6). Structural and molecular analyses will be carried out on E14.25 kidneys and final nephron number determined at PN21. Kidney function will be determined at PN180 and 360 and blood pressure will be determined at PN360.

1.2 DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

In the last century there has been a shift in the cause of mortality. In the early part of the 20th century, other than death in the theatre of war, infectious diseases (or communicable diseases) including tuberculosis and malaria predominated (Beaglehole and Bonita, 2008). The shift in the cause of mortality was driven chiefly by better sanitation and cleaner water supply, as well as better hygiene practices and the availability of anti-microbial agents (Feachem, 1983, Feachem, 1984, Feachem *et al.*, 2010, Bhutta and Salam, 2012, Feachem, 2001). As a result, non-communicable diseases such as cardiovascular diseases and cancers are now the biggest contributors to human morbidity and mortality (Ebrahim and Smeeth, 2005, Beaglehole and Yach, 2003, Beaglehole, 1992). It is well established that lifestyle and genetics play a major role in the development of adult diseases such as cardiovascular disease and diabetes, however over the past 20-30 years a growing body of evidence suggests that insults during critical periods of development may program or alter organ structure and function in a way that predisposes the organism to later disease (Barker, 2008, Barker, 2007).

1.2.1 EARLY EVIDENCE

A number of studies have provided evidence to support the hypothesis that maternal nutritional status plays a critical role on the health and wellbeing of the fetus (van der Spuy, 1985, van der Spuy *et al.*, 1983, Imdad and Bhutta, 2012). Changes to maternal nutritional status may change the ability of the mother to supply the growing fetus with the required nutrients for optimal growth. Compared with women of normal body mass index (BMI) (20-24.9kg/m^2) underweight women (BMI <19.1kg/m^2) have an 18% greater risk of delivering a baby that is small for gestational age (van der Spuy *et al.*, 1988). Notwithstanding the immediate effects on fetal health, moderate changes in maternal diet can also have long-term implications on fetal and postnatal health.

Initial studies in the 20th Century documented the nutritional requirements during pregnancy in humans (Widdowson, 1977, McCance *et al.*, 1938) as well as animals (Widdowson and McCance, 1963, Baird *et al.*, 1971, Dickerson *et al.*, 1971, Widdowson, 1974), indicating the importance of a diet containing sufficient amounts of essential nutrients (proteins, fats, carbohydrates) and micronutrients (iron and calcium) and a sufficient caloric value. These studies reported that protein or micronutrient restriction in the mother lead to poor offspring growth *in utero* and in early postnatal life. It was much later that we began to appreciate the biological consequences of these fetal trade-offs in response to a sub-optimal maternal diet.

Investigations by Forsdahl uncovered a positive correlation between infant mortality in Norwegian counties and cardiovascular diseases, beyond those expected by adult living conditions (Forsdahl, 1977, Forsdahl, 1978, Forsdahl, 1979). These studies formed the foundations of further historical cohort investigations into birth weight, infant growth and the determinants of these parameters.

1.2.1.1 THE BARKER STUDIES

Although a number of studies established evidence that the early life environment might be important in determining the way an organism functions in later life, it is the seminal work by David Barker and colleagues that has popularised and promoted the developmental origins hypothesis.

A critical study by Barker & Osmond (1986b) correlated infant mortality (between years 1921-25) and ischaemic heart disease mortality (between years 1968-78) in a male population in England and Wales. The results of this study indicated an association between infant mortality and ischaemic heart disease – geographic regions with higher rates of infant mortality also had higher rates of death from ischaemic heart disease. Barker and colleagues concluded that a poor start to life may lead to greater susceptibility to adult diseases. Investigating this further, Barker

and colleagues reported an inverse relationship between birth weight and systolic blood pressure at 10 and 36 years of age in both men and women from two UK cohorts (born either in 1946 or 1970). The study also established that individuals at greatest risk of developing disease were often of a lower socioeconomic status. The authors further proposed that the lower standard of living and perhaps a sub-optimal diet during pregnancy had a detrimental effect on the developing fetus. Further epidemiological studies have been performed to examine the hypothesised relationship between birth weight and the incidence of disease. Other studies have documented socioeconomic factors as surrogate markers of diet (Barker, 2003a). These studies have been performed to elucidate important factors that are involved in the incidence of disease and determine whether there are common factors that predispose populations of people to the development of particular adult diseases.

Using low birth weight as a marker for sub-optimal intra-uterine environment, Barker and colleagues have continued to report on different populations and disease states associated with a poor start to life. These studies are discussed below.

1.2.2 DEVELOPMENTAL PROGRAMMING: A THEORETICAL FRAMEWORK

There are few human scenarios in which to study the specifics of developmental programming and, in particular, to identify stimuli that may induce programming of disease in offspring. The early Barker studies (Barker, 1966, Barker and Osmond, 1986b, Barker, 1988, Barker, 1981) focused on birth weight and hypothesised that maternal nutrition in pregnancy was an important factor in determining offspring cardiovascular disease in late adulthood, but this link was based on the assumption that women of lower socioeconomic class had a poor diet in pregnancy. This assumption is certainly open to criticism because it does not take into account the dietary variation in women of a similar socioeconomic class in rural and urban settings. Indeed, in the early part of the 20th Century, women in rural communities may have been poor by standard socioeconomic measures, but those that were involved in farming may have had access to a

more nutritious diet than poor women based in an urban setting (Bygren *et al.*, 2000). In order to establish a strong association between maternal dietary intake and offspring health or disease there is a need to study human populations where discrete periods of malnutrition are documented.

1.2.3 HISTORICAL COHORT STUDIES THAT ESTABLISH THE ROLE OF MATERNAL DIET IN PREGNANCY IN PROGRAMMING OFFSPRING HEALTH AND DISEASE

The role of the maternal diet in programming offspring health and disease has been established by numerous studies of famine in human populations, which are now discussed.

1.2.3.1 THE DUTCH HUNGER WINTER STUDIES

The Dutch Hunger Winter, although a tragic event in human history, provides an opportunity to analyse the effects of human caloric restriction during different periods of gestation. The Dutch Hunger Winter was caused by Nazi blockade of the North-West corner of the Netherlands during World War II, from the winter of 1944 to the spring of 1945. The official ration was reduced to between 400-800 calories a day. Following liberation of the region by the summer of 1945, rations had returned to 2000 calories a day (Roseboom *et al.*, 2001b). Data collected from women in different stages of pregnancy show that calorie restriction had a long-term effect on the offspring. Individuals who were exposed to famine in early gestation alone were not significantly different from those babies that were unexposed to the famine (Roseboom *et al.*, 2001b). Those exposed during mid-gestation demonstrated a greater propensity to develop obstructive airways disease and signs of kidney disease, such as microalbuminuria (Painter *et al.*, 2005b). Individuals who were exposed to famine in late gestation displayed a greater propensity for glucose intolerance in later life (Painter *et al.*, 2005a). These results clearly demonstrate that the maternal environment has lasting and varying effects on offspring (not always on birth weight, however) and that the effect of the maternal environment on offspring health is not discrete or isolated to one organ or organ

system. The cardiovascular, renal systems and metabolism have been altered within the offspring. A more recent finding from those affected by the Dutch Hunger Winter has suggested an epigenetic modification that may have contributed to the change in offspring health (Heijmans *et al.*, 2008). This will be expanded in later sections.

1.2.3.2 The Chinese "Great Leap Forward" studies

In 1959-61 the government of China made major changes to the agricultural system that saw a rapid change in how people lived. This period of time is known as The Great Leap Forward. The transition lead to the costliest (in terms of human wellbeing and life) famine in human history (Cai and Feng, 2005). This famine was brought about by a 25% reduction in grain output due to severe weather conditions and the collectivisation of hundreds of millions of Chinese farms. It is estimated that caloric intake went from 2100 cal/day in 1957 to 1500 cal/day in 1960. The famine had a significant impact on fetal health and wellbeing, with miscarriage rates rising from 3.4% in 1958 to 4.9% in 1961. Huang and colleagues investigated the cardiovascular and metabolic health of adults exposed to the famine during fetal and early life (Huang *et al.*, 2010). Individuals exposed to famine during fetal life were 3-times more likely to develop hypertension in adulthood compared with an unexposed cohort born in 1963. Postnatal exposure (2-3 years of age) lead to reduced height, and underwent catch up growth (increased BMI) (Yang *et al.*, 2008) and developed hypertension, while exposure during pregnancy and infancy resulted in reduced BMI in adulthood. The study of a similar cohort exposed to the famine during different stages of development found that hyperglycaemia was more prevalent in those malnourished during fetal development (Li *et al.*, 2010). This hyperglycaemia was further exacerbated when famine exposed individuals were subject to over-nutrition in later life (Li *et al.*, 2010). However, a study by Song (2010) investigating the long term consequences of the famine on mortality rates found that exposure to famine did not result in an increase in overall mortality or a reduction in lifespan. Although mortality was

unaffected, other parameters of long-term health have been investigated. Song (2009) further reported that being conceived and born during the famine (in rural or urban areas) lead to an increased risk of developing schizophrenia in adulthood. The findings of St Clair (2005) support the work of Song (2009) with respect to the risk of developing schizophrenia. The adult consequences of the famine are only now being established as the famine-exposed individuals are reaching 50 years of age.

1.2.3.3 The Siege of Leningrad

The Siege of Leningrad (1941-4) provides another opportunity to investigate the impact of maternal starvation upon fetal development. Stanner *et al.* (1997) investigated 169 subjects who were exposed to the famine *in utero*. Measuring parameters such as blood pressure, lipid profile, insulin concentration and skin fold thickness, they found no association between intrauterine starvation and cardiovascular health. This is a contrast to the comparable studies of the Dutch Hunger Winter Famine where there was an inverse relationship between intrauterine environment and coronary heart disease, BMI and glucose tolerance. While there may not have been an association between intrauterine exposure to the siege, postnatal (childhood and pubertal) exposure may have impacted upon cardiovascular health. Men and women exposed during years (9-15 and 6-8, respectively) had higher systolic blood pressure and mortality from ischaemic heart disease in adult life (Koupil *et al.*, 2007). This illustrates that programmed phenotypes are not isolated to occur during the fetal period, but may occur during any period an organ or organ system undergoes development or change.

In natural experiments that investigate the impact of human suffering, it is difficult to rule out certain confounding factors. While studies of the Siege of Leningrad, the Dutch Hunger Winter Famine and the Great Leap Forward investigated maternal diet, these periods of human history are also associated with significant levels of stress. Finnish war evacuees have also provided a human model of early life traumatic events and their long-term effects on health. A study by

Alastalo *et al.* (2009) reported that Finnish war evacuees had higher cardiovascular morbidity, prevalence of type 2 diabetes and blood pressure in later life even though they did not experience any reduction in caloric intake during the war.

1.2.4 HUMAN EPIDEMIOLOGICAL STUDIES SUPPORT THE DEVELOPMENTAL PROGRAMMING OF ADULTHOOD DISEASES

1.2.4.1 THE IMPORTANCE OF BIRTH WEIGHT

Epidemiological studies provide compelling evidence linking low birth weight (LBW, <2500g at full term) and disease in later life (Alexander, 2006, Barker and Lackland, 2003, Bonamy *et al.*, 2008, Ligi *et al.*, 2010, Thompson *et al.*, 2001, Wani *et al.*, 2004). LBW can be caused by many factors, including maternal size, age and diet. Low birth weight has been associated with a predisposition to hypertension (Barker, 1993, Barker, 1994, Barker, 2004b), cardiovascular diseases (Norman, 2008), diabetes (Grella, 2007) and stroke (Rinaudo and Lamb, 2008). Particularly implicated is maternal nutrition, which has been shown to have an effect on offspring health, with both over and undernutrition having negative consequences on offspring health (Yajnik *et al.*, 2008, Cleal *et al.*, 2007, Poston, 2007, Pettitt and Jovanovic, 2001).

It is now accepted that a low birth weight is an indication of a suboptimal *in utero* environment and is associated with an increased risk of adult disease. While low birth weight can be used as an important marker of the intrauterine environment, it does not necessarily lead to adult disease, therefore other factors must also make a major contribution to the pathogenesis of these conditions (Armitage *et al.*, 2004). Other markers now being used as an indication of poor fetal growth, or a propensity towards adult disease are placental developmental as well as early childhood growth trajectories (Barker *et al.*, 2010b, Salonen *et al.*, 2009a, Salonen *et al.*, 2009b).

Low birth weight can occur due to genetic and environmental influences. Low birth weight is associated with gene mutations that may affect the maternal environment or placental development (Glanville *et al.*, 2006, Malamitsi-Puchner *et al.*, 2007, McIntyre *et al.*, 2009, Rajakumar *et al.*, 2007, Tzschoppe *et al.*, 2009). Likewise, environmental impacts that reduce placental size will also result in LBW. Another important contribution to fetal size at birth is maternal size, in that small mothers will have small babies (Ounsted, 1986, Ounsted *et al.*, 1986, Rickard *et al.*, 2012). The multifactorial nature of the occurrence of LBW means that it serves as an indication of a sub-optimal intrauterine environment, but does not indicate a cause.

The placenta is the interface between the maternal and fetal environments. Disturbed growth or function of the placenta can lead to significant health problems for mother and baby. Although placental weight is one parameter that correlates with the development of adult diseases (Thame *et al.*, 2000), subtle placental morphological characteristics may also predict the development of developing adulthood diseases. Barker and colleagues demonstrate an association between placental weight and size and hypertension in adulthood, which is highly dependent on maternal size characteristics. Hypertension was observed in 38% of offspring of women of small stature (<160cm) who produced a small placenta (area <200cm^2), whereas this proportion dropped to 21% if the placental area was >320cm^2 (Barker *et al.*, 2010b). These studies further illustrate the importance of monitoring placental development as well as fetal development as they are intimately linked.

Since epidemiological studies have illustrated the importance of maternal diet on birth weight and offspring health, efforts have been made to elucidate the precise component within the maternal diet that has the greatest impact on offspring size and ultimately health. Human studies do not allow the ability to determine this, but a number of human intervention studies combined with animal models have suggested that protein is a major determinant of offspring size and health. The USDA Women, Infant and Children's Food Supplementation initiative

that distributes food vouchers for foods high in protein to eligible pregnant women and families has shown that intervention during pregnancy leads to increased birth weight compared to those that were eligible and did not participate (Metcoff *et al.*, 1985). Other studies have suggested that deficient diets can only be 'rescued' by supplying the deficient element in the diet (i.e. protein or calories) (Lechtig *et al.*, 1975a, Lechtig *et al.*, 1975b, Lechtig *et al.*, 1975c).

1.2.4.2 The importance of postnatal growth

In utero growth is an important marker for health and development of the fetus. Likewise, growth during early infancy has been identified as a critical period of development that is sensitive to insults that can programme the organism to a path of disease. The ability to programme an organism is dependent on its 'plastic' nature. Developmental plasticity refers to an organisms ability to respond to environmental cues, and therefore this definition also includes the host of genes available to alter the phenotype to optimise the organisms potential in particular environments (Barker, 2004a). Organisms can adapt their phenotype in response to changing environments during periods of plasticity; therefore after this period has passed the phenotype is generally set.

1.2.4.3 Catch-up growth; beneficial or detrimental to adult health?

Barker *et al.* (1989) reported that boys weighing less than 18 pounds at 1 year of age had death rates three times higher than boys who were 27 pounds or heavier at 1 year of age. The report concluded that encouraging childhood growth in boys would be beneficial in reducing mortality rates. In the same cohort of subjects, Hales and colleagues reported that reduced growth in early life is linked with impaired glucose tolerance in later life (Hales *et al.*, 1991) – in fact those that were small and remained small until one year of age were three times more likely to have glucose intolerance than those that were heavier (Barker *et al.*, 1989). These findings are not invariant, however.

The ‘thrifty phenotype’ hypothesis (Hales and Barker, 2001) proposes that fetal survival is paramount and that adaptations will be made to optimise growth. This hypothesis explains the occurrence of catch-up growth in light of the early compensation made to enhance growth. These fetal compensations lead to accelerated postnatal growth due to the comparative abundance of nutrients. The thrifty phenotype challenges the practice of over-feeding small babies (intrauterine growth restriction (IUGR) or small for gestational age (SGA) to ensure they follow a higher growth trajectory – which is deemed to be healthier. Instead the thrifty phenotype hypothesis suggests that if small babies were provided nutrition such that they maintained growth at a given percentile they should avoid developing programmed disease (Figure 1.1).

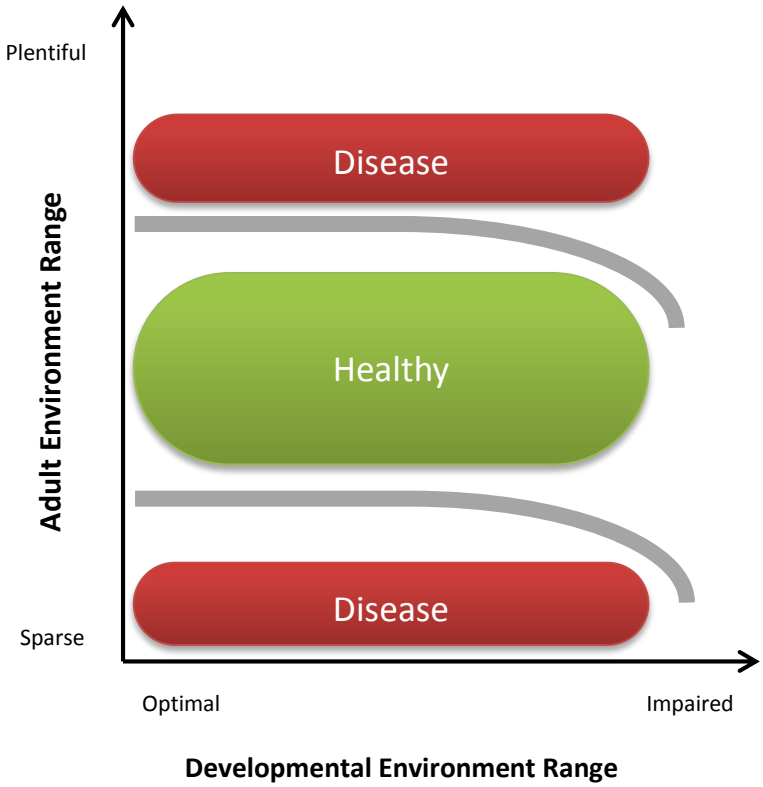

FIGURE 1.1 – THE INTERPLAY BETWEEN THE DEVELOPMENTAL AND ADULT ENVIRONMENTS ON HEALTH

The environmental range in which an organism can be exposed during development or adulthood that may predispose the organism to disease due to a mismatch in environments. In environments of paucity or excess, adaptations can be made in order to optimise growth (healthy), however when these environments no longer match, the physiological outcome can lead to disease. Adapted from (Gluckman and Hanson, 2004).

Infant growth is a major component in the risk of developing adult diseases (Pilling *et al.*, 2008, Barker, 2004c). Maternal under-nutrition leading to reduced offspring birth weight followed by rapid postnatal growth has been shown to be detrimental to adult health. Human cohort studies from Brazil, Guatemala, India, the Philippines and South Africa have documented an association between LBW and rapid childhood growth with an increase in glucose concentration, blood pressure and lipid profiles in adulthood (Victora *et al.*, 2008). The effect of postnatal growth and its detrimental effects on adult health appear to be population specific with infant weight gain in developing countries being associated with increase in lean mass, but in industrial populations it predicts an increase of fat mass and obesity (Wells *et al.*, 2007).

As the epidemiological data mounts and further associations and correlations are identified between birth weight and/or infant growth with disease incidence – so too are the questions about mechanisms. Unfortunately, human studies do not identify potential mechanisms that cause or lead to the phenotypes reported due to intrauterine growth restriction or altered postnatal growth. This is because the ability to determine mechanisms requires intervention to test hypotheses. One strategy is to develop animal models that can allow these mechanisms to be explored. Animal models have identified markers of growth and genetic pathways that are now being used in human epidemiology studies. Through these animal models it is possible to investigate important organ systems to elucidate their role in disease development. Of particular importance to this thesis is the impact on kidney development and future kidney and cardiovascular disease.

1.2.5 DEVELOPMENTAL PROGRAMMING – ANIMAL STUDIES

1.2.5.1 DEVELOPMENTAL PROGRAMMING – THE MATERNAL DIET EFFECT

Models of developmental programming have been established in animal models by altering the maternal diet in order to mimic the human condition (ie. Dutch Hunger Winter Famine). Diets used in animal studies have included low protein, high protein and high salt diets, while some

studies have manipulated levels of specific micronutrients in the diet or caloric restriction (Vehaskari and Woods, 2005). Maternal low protein or caloric restricted diets are often reported to induce low birth weight offspring (do Carmo Franco *et al.*, 2009, Reyes-Castro *et al.*, 2011) which correlate with changes in cardiovascular function and growth (Bertram *et al.*, 2001, Dagan *et al.*, 2009). Changes in kidney structure and function are also reported (Hoppe *et al.*, 2007a, Hoppe *et al.*, 2007b). A majority of studies are descriptive and continue to expand our knowledge of the consequences of a poor maternal diet (Table 1.1), however establishing the underlying mechanisms is elusive. Underlying mechanisms may differ on the basis of the models chosen to induce programming but this remains unclear. It is also unclear under what circumstances a phenotype manifests.

In most studies to date, maternal dietary challenge was imposed only during pregnancy, or until weaning (Ozanne and Hales, 2004). When the diet is changed after weaning, offspring often undergo catch-up growth due to the presence of an optimal growth diet. However, catch-up growth may exacerbate the adverse consequences of developmental programming (Armitage *et al.*, 2004, Ozanne and Hales, 2004, Ozanne and Hales, 2005). Catch-up growth occurs during the normal period of rapid growth and has been identified as a risk factor for development of cardiovascular disease and obesity later in life (Armitage *et al.*, 2004, Ozanne and Hales, 2004). This rapid growth sometimes exacerbates the alterations that occurred *in utero*. The developing fetus has previously adapted to an environment where nutrients (protein, salt, carbohydrates and vitamins) are limited. These adaptations may prove to be maladaptive under metabolically replete conditions. This effective "over-nutrition" (in terms of a replete diet) may cause the offspring to grow beyond its means and predispose offspring to later disease. This is referred to as the Thrifty Phenotype, and is suggested to result in a metabolic syndrome-like phenotype in adulthood due to the sacrifices made *in utero*.

TABLE 1.1 – INVESTIGATIONS OF THE EFFECTS OF MATERNAL PROTEIN RESTRICTION ON OFFSPRING PHENOTYPE

Citation	Animal	Dietary Insult	Results
(Alwasel and Ashton, 2009)	Wistar Rat (male and female) (4 weeks)	Low Protein (LP ,9%) during pregnancy	↑ Mean Arterial Pressure (MAP) in males and females (21 and 23mmHg respectively)
			↔ Glomerular filtration rate (GFR) in males and females
(Barja-Fidalgo *et al.*, 2003)	Wister Rat (male, 60 days old)	Protein free diet during the first 10 days of lactation	↓ 42% plasma insulin ↓ 50% (approx.) inflammation response to an acute inflammatory agent
			↑ 78% plasma corticosterone concentrations
(Dagan *et al.*, 2009)	Sprague-Dawley rat (male, 6 weeks)	6% LP from day 12 pregnancy to birth	↑ 20mmHg systolic blood pressure (SBP) ↑ 2-fold NKCC2 protein abundance in medulla of kidney ↑ Cl^- transport (mmol/l) in kidney
(Coupe *et al.*, 2009)	Sprague-Dawley rat (40 days)	8% LP throughout pregnancy and/or lactation	↑ Hyperphagia 3 weeks after weaning ↑ mRNA expression of the orexigenic pathway
			↓ Birth weight and postnatal growth until 6 weeks of age ↓ 2-3 fold insulin, triglycerides and glucose levels.
(Coupe *et al.*, 2010)	Sprague-Dawley rat (16 days old)	8% LP throughout pregnancy and/or lactation	↑ 1.5 fold plasma leptin levels
			↓ Birth weight with catch-up growth occurring during the first 5 days ↓ Methylation of hypothalamic Pro-opiomelanocortin (POMC)
			↔ Methylation of hypothalamic Cocaine- and amphetamine-regulated transcript (CART) and neauro-peptide Y (NPY)
(Bol *et al.*, 2009)	C57BL6/J mice (male 9 months)	8% LP throughout gestation. After weaning some animals were put on a hyper-caloric diet	↑ Fat pad mass in LP offspring ↑ Plasma cholesterol levels
			↓ Birth weight ↓ LP pancreas weight
(Bertram *et al.*, 2001)	Wistar rats (male and female E20-20 weeks)	8% LP throughout gestation	↑ 25-35mmHG SBP ↑ GR (glucocorticoid receptor) protein and mRNA in kidney
			↓ 8% birth weight ↓ E20 Placenta 11βHSD2 (11 beta hydroxysteriod dehydrogenase 2) mRNA
(Mortensen *et al.*, 2010)	C57Bl/6 mice (day of birth)	8% LP throughout pregnancy	↓ 40% birth weight ↓ PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) mRNA in liver and muscle ↓ Genes involved in energy metabolism in liver and muscle
(Mehta *et al.*, 2002)	Wistar rats (male up to 52 weeks of age)	9% LP throughout pregnancy	↓ 10% bone area ↓ 8% BMC (bone mineral content)
			↔ BMD (bone mineral density)
		9% LP throughout pregnancy	↑ 15-16mmHg SBP (males and females)

(McMullen and Langley-Evans, 2005a)	Wistar rats (male and female at 4 weeks of age)		↔ Kidney $AT_{1A}R$ (angiotensin 1A receptor) and $AT_{1B}R$ (angiotensin mRNA expression ↔ Birth weight
(McMullen *et al.*, 2004)	Wistar rats (male and female at 4 weeks of age)	9% LP throughout pregnancy	↑ ~20mmHg SBP in males and females
			↓ ~40-30% nephron number
			↔ Kidney $AT_{1A}R$ and $AT_{1B}R$ mRNA expression
(Pladys *et al.*, 2004)	Wistar rats (males at 9-12 weeks)	9% LP throughout pregnancy	↑ 16mmHg MABP (mean arterial blood pressure) ↑ Brain AT_1 receptor
			↔ Birth weight ↔ Heart rate
(Ozanne and Hales, 2004)	Mice (male; longevity study)	8% LP during pregnancy and lactation	↑ Longevity for those born small and stayed small
(Ozanne *et al.*, 1996)	Wistar rats (3 months)	8% LP throughout pregnancy and lactation	↑ Glucose transport rate ↑ 2x insulin receptors in the muscle membrane
			↓ Muscle mass and body weight at 3 months
			↔ Plasma insulin ↔ Glut4 (glucose transporter type 4) protein in muscle
(Nwagwu *et al.*, 2000)	Wistar rats (male and female at 4-20 weeks)	9% LP during pregnancy until weaning (4 weeks of age)	↑ 7-21mmHg between 4-20 weeks ↑ BUN (blood urea nitrogern) clearance at 20 weeks ↑ Urine volume and albumin excretion
			↓ Heart rate at 4 and 12 weeks ↓ GFR
			↔ Birth weight
(Rao *et al.*, 2009)	Chickens (E14 and 4 weeks of age)	10% LP for 4 weeks	↑ Growth rate after hatching
			↓ 20-HSD (20-hydroxysteroid dehydrogenase) mRNA in yolk-sac membrane ↓ Yolk leptin at E14
			↔ Hatch weight
(Rees *et al.*, 1999)	Rowett Hooded strain rat (E19 and E21)	90g/kg or 60g/kg LP throughout pregnancy	↑ E19 body weight in the 90g/kg protein group ↑ Maternal serum concentrations of glutamic acid, glutamine and glycine
			↓ E19 and E20 body weight in the 60g/kg protein group ↓ Placental weight at E21 ↓ Maternal threonine levels
			↔ Placenta weight at E19
(Reyes-Castro *et al.*, 2010)	Wistar rats (analysed at 120-150 days)	10% LP throughout pregnancy until weaning	↓ Birth weight ↓ Effect of positive reinforcement on learning
(Roghair *et al.*, 2007)	C57Bl/6J mice (analysed at up to 6 months)	9% LP throughout pregnancy	↓ Growth at 10 days
			↔ SBP (systolic blood pressure) and HR (heart rate) ↔ Fasting glucose levels ↔ Aortic vasoconstriction and vasodilatation

(Torrens *et al.*, 2003)	Wistar rats (E19)	9% LP during pregnancy	↔ Birth weight ↔ Placenta and body weight at E19 ↔ Organ weight (kidney, adrenals, heart, lungs and liver) ↔ Constriction and vasodilatation of the thoracic aorta
(Vehaskari *et al.*, 2001)	Sprague-Dawley rats (up to 8 weeks of age, male and female)	6% LP started at day 10 of pregnancy to birth	↑ Apoptosis in the kidney at 8 weeks of age ↑ 21-25mmHg BP
			↓ 28-29% nephron number
(Langley-Evans *et al.*, 2006)	Wistar rats (analysed at E18 and E20)	9% LP throughout gestation	↑ Liver oxidation damage at E20 ↑ Female DNMT1 (DNA methyltransferase 1) liver expression at E18 and no change at E20
			↓ Male DNMT1 liver expression at E18 and no change at E20 ↓ Liver size at E20 ↓ Placenta size at E18 and 20
			↔ Maternal homocysteine, fetal homocysteine or maternal methionine synthase levels
(Harrison and Langley-Evans, 2009)	Wistar rats (generational study F_1, F_2 and F_3 analysed at 10 weeks of age)	9% LP throughout gestation	↑ SBP 9mmHg-20mmHg in males and females
			↓ 36-40% nephron number
			↔ Plasma glucose or cholesterol
(Maloney *et al.*, 2007)	Rowett Hooded Lister rats (analysed at 25 weeks of age)	9% LP with soya or 9% LP with corn oil 2 weeks prior to pregnancy, throughout pregnancy and lactation until weaning	↑ Female liver mRNA expression of ACC-1 (Acetyl-CoA Carboxylase 1)
			↓ 20% maternal food intake ↓ Maternal blood haematocrit ↓ Birth weight
			↔ Organ weight ↔ Plasma glucose and insulin
(Maloney *et al.*, 2005)	Rowett Hooded rats (analysed at E21)	9% LP 2 weeks prior to pregnancy and throughout pregnancy	↑ Kidney mRNA levels of XXXX at P21, P27 and P57
			↔ Birth weight ↔ Organ weight ↔ Liver mRNA levels of Cdk1 and Gadd153
(Guzman *et al.*, 2006)	Wistar rats (Females 22 months of age)	10% LP throughout pregnancy and lactation	↑ Progesterone in pregnant dams
			↓ Birth weight and abdominal diameter ↓ Corticosterone at PN2 ↓ Onset of puberty ↓ Oestradiol and luteinising hormone
(Habib *et al.*, 2011)	Sprague-Dawley rats (analysed at E20 and 60-80 days postnatally)	6% LP from embryonic day 13 to birth.	↑ E20 serum glucocorticoids ↑ BP 20mmHg
			↓ 14% nephron number in males
(Bellinger *et al.*, 2006)	Wistar rats (18 months, male and female)	8% LP throughout gestation, or E0-7, E8-14, E15-22 or E0-22	↑ Growth rate in all LP treated except late gestation ↑ Food intake at 18 months
			↓ Fat deposits in males
			↔ Birth weight

(Hoppe *et al.*, 2007a)	C57bl6 mice (postnatal day 30 males and females)	9% LP 2 weeks prior to pregnancy, throughout gestation and weaning (21 days)	↓ 22% nephron number ↓ 6-fold AT_{1A} mRNA in females
			↔ Male AT_{1A} mRNA levels
(Hoppe *et al.*, 2007b)	Sprague-Dawley rats (postnatal day 30, males and females)	8% LP 2 weeks prior to pregnancy, throughout pregnancy and postnatal life	↓ Organ weight (all organs except brain) ↓ 31% nephron number ↓ MAP 8mmHg
			↔ GFR ↔ ERBF (effective renal blood flow)
(Joanette *et al.*, 2004)	Wistar rats (E21.5 to PN14)	8% LP throughout gestation and lactation	↓ Growth of pancreas ↓ 7% islet cells
			↔ Blood glucose and plasma insulin
(Khorram *et al.*, 2007)	Sprague-Dawley rats (4 months, male and female)	50% caloric restriction from day 10 to birth	↑ 10 x aorta VEGF (vascular endothelial growth factor) protein at 4 months
			↓ Birth weight ↓ Mesenteric microvascular branches at 1 day old with reduced eNOS ↓ Aorta VEGF protein or mRNA??? at 1 day old
(Fernandez-Gonzalez *et al.*, 2004)	Mouse $B6BAF_1$ (up to 10 weeks)	One-cell embryos were cultured KSOM and 10% FCS or 1g/L BSA (control) until the blastocyst stage	↑ IGF2 mRNA levels in those treated with FCS ↑ Anxiety levels
			↓ 57% embryo implantation ↓ Locomotion
			↔ Neuromotor development
(El Khattabi *et al.*, 2006)	Wistar rats (E20)	8% LP throughout gestation	↓ Hepatocyte levels of IGFII ↓ IGFB1 and IGFBP2
(Bellinger and Langley-Evans, 2005)	Wistar rats (12 weeks of age; males and females)	8% LP during gestation (E0-7 or, E8-14, or E15-22 or E0-22)	↑ Early LP preference for high carbohydrate diet
			↔ Preference for a high-fat diet ↔ Hepatic glycogen content
(Gardner *et al.*, 1997)	Wistar rats (6 weeks of age)	9% LP during gestation	↑ SBP 23mmHg
(Tonkiss *et al.*, 1998)	Sprague-Dawley rats (96 days of age)	6% LP 5 weeks prior to mating and throughout pregnancy	↓ Birth weight
			↑ DBP
(Woods *et al.*, 2005)	Sprague-Dawley rats (female 20 weeks of age)	8.5% LP throughout pregnancy	↓ Birth weight ↑ Renal levels of ANGII and renin
(Woods *et al.*, 2005)	Sprague-Dawley rats (female 20 weeks of age)	8.5% LP throughout pregnancy	↑ MAP
			↓ GFR, ERPF and FF (filtration fraction)
(Langley-Evans *et al.*, 1999c)	Wistar rats (male and female from E20 to 19 weeks age)	9% LP throughout gestation, or d0-7, d8-14 or d15-22	↑ E20 nephron number ↑ SBP 13mmHg at 4 weeks
			↓ Nephron number at 4 weeks
(Manning and Vehaskari, 2001)	Sprague-Dawley rats (4 and 8 weeks, 6-11 months, male and female)	6% LP from gestational day 12 to weaning	↑ Postnatal growth ↑ SBP (41-47mmHg) ↑ Proteinuria (males)
			↓ 15% birth weight

(Elmes *et al.*, 2008)	Wistar rats	8% LP throughout gestation	↔ Cardiac function at baseline ↔ Heart glutathionine levels
			↓ Recovery from ischemic reperfusion injury
(Zimanyi *et al.*, 2004)	Wistar-Kyoto rats (male 4 and 40 weeks)	9% LP throughout pregnancy and lactation	↓ Birth weight (12%) ↓ Reduced average weight gain from birth until weaning ↓ Nephron number (20%)
			↔ SBP
(Bogdarina *et al.*, 2007)	Wistar rats (male and female at weeks 1, 4 and 12)	8% LP throughout gestation and lactation	↑ Female AT_{1A} receptor in the kidney at weeks 1 and 12
			↔ Male AT_{1B} receptor in the adrenal at weeks 1 and 12
			↓ Methylation of the AT_{1B} gene

Maternal protein restriction has been reported to lead to offspring with reduced birth weight, increased blood pressure, altered gene expression, kidney function and immune response (Table 1.1). However, it is also apparent that these findings are not ubiquitous. Discrepancies exist in the reported data due to different diet composition, implementation time period, techniques of measurement (gene expression, kidney function, nephron number and blood pressure), the age of offspring and the species strain and breed differences. What can be resolutely concluded from these studies is that maternal protein restriction influences fetal development and adult health – however what is lacking from these data is information on kidney development (in reference to nephron development), offspring body composition and analysis of offspring kidney function and blood pressure with gold standard techniques. The critical analysis of these studies (Table 1.1) will be discussed throughout this thesis in relevant sections and chapters.

1.2.6 DEVELOPMENTAL PROGRAMMING OF THE KIDNEY

The kidney is particularly susceptible to a suboptimal intrauterine environment. Studies utilizing numerous models of developmental programming have reported altered kidney development, deficits in nephron endowment, altered kidney function, renal pathology and hypertension (see Table 1.1).

1.2.7 HUMAN STUDIES OF DEVELOPMENTAL PROGRAMMING OF THE KIDNEY

Brenner *et al.* (1988) hypothesised that a reduction in renal filtration surface area (due to either lower nephron number or lower filtration surface area per glomerulus) would lead to systemic hypertension and to progressive renal insufficiency. Brenner and colleagues suggested that a deficit in filtration surface area limits the kidneys ability to excrete sodium, ultimately leading to an increase in glomerular capillary pressure which results in glomerular sclerosis (Brenner *et al.*, 1988). This can further lead to a decrease in filtration surface area and an increase in systemic blood pressure (Figure 1.2).

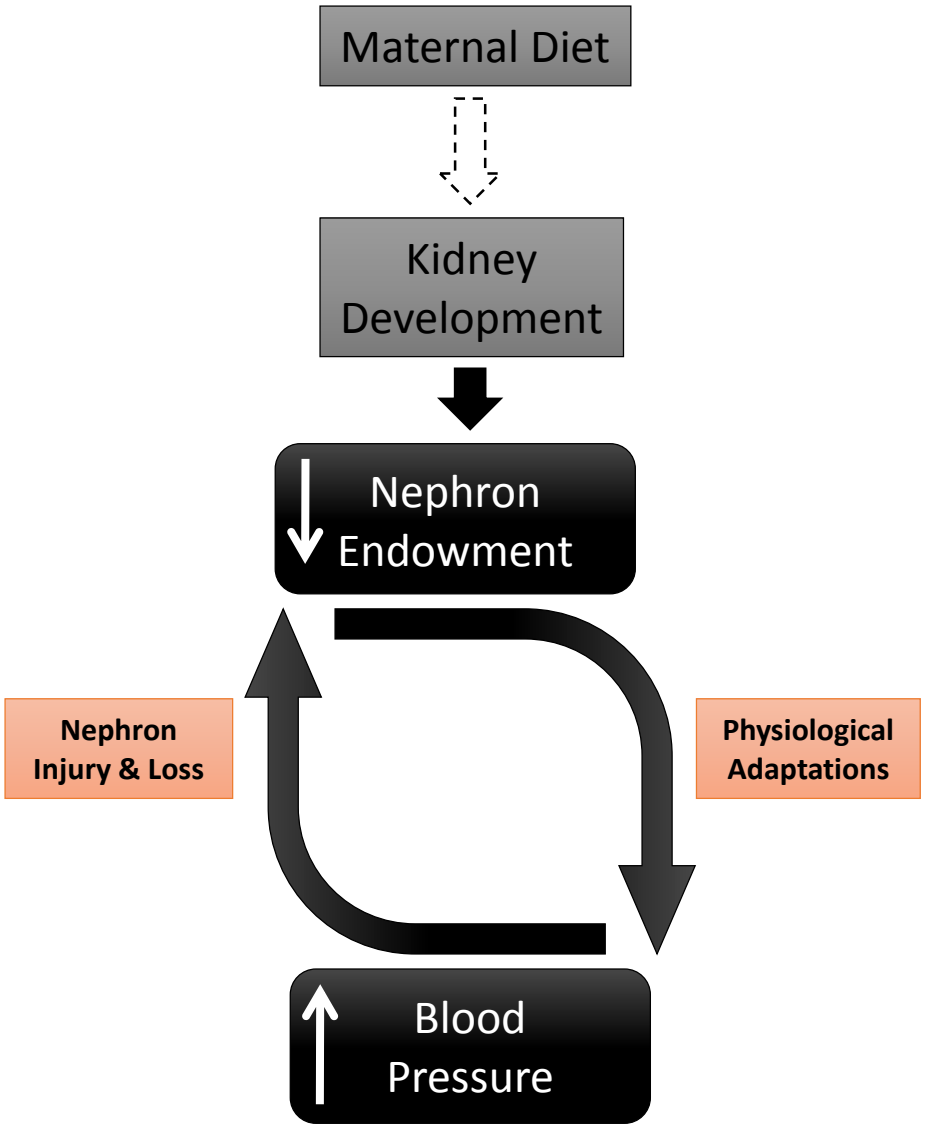

FIGURE 1.2 - THE BRENNER HYPOTHESIS AND THE THEORY OF RENAL DEVELOPMENTAL PROGRAMMING.

It is hypothesised that maternal diet can impact upon offspring kidney development resulting in reduced nephron endowment. Furthermore, it is hypothesised that reduced nephron endowment can cause physiological adaptions within the kidney (including reduced filtration surface area and reduced sodium handling ability) resulting in increased systemic blood pressure. This increase in blood pressure can cause further nephron injury and loss.

Keller *et al.* (2003) reported an inverse association between nephron endowment and hypertension in humans. However, it was unclear whether the lower numbers of nephrons in subjects with a history of hypertension were responsible for hypertension or a consequence of hypertension. Keller *et al.* (2003) analysed kidneys obtained at autopsy from Caucasian subjects (normotensive and hypertensive patients) between the age of 35 and 59 years, and found that 10 normotensive subjects had a median glomerular number of 1,429,200 while 10 hypertensive subjects had a median glomerular number of 702,379. The hypertensive patients, even though they had fewer nephrons, showed very few obsolescent glomeruli which would suggest that the hypertension had not caused the deficit in nephron number. Douglas-Denton *et al.* (2006) also reported that hypertensive subjects have significantly fewer nephrons (746,468 ± 133,240) than normotensive subjects (1,402,360 ± 346,357).

LBW is often used as an indication of intra-uterine growth restriction and has been correlated with the incidence of kidney disease (Lackland, 2005, Lackland *et al.*, 2000). For example, Lackland (2005) provided evidence that kidney failure and end-stage renal disease demonstrate a racial and geographic disparity. This concept is supported by findings of Hoy *et al.* (2006a) that the kidneys of Australian Aboriginals contain 30% fewer nephrons than those of non-indigenous Australians. This phenomenon may explain in part why Australian Aborigines in remote areas have a much greater incidence of renal disease (as well as hypertension and cardiovascular disease) than non-Aboriginal peoples in remote areas (Hoy *et al.*, 2010). A study by Lackland (2004) focused on the incidence of hypertension. This study showed that adults born with LBW had a much higher systolic blood pressure and were more likely to suffer from hypertensive-related end-stage renal disease than those of normal birth weight. However, the association between LBW and nephron number is not always observed in animal studies (Moritz *et al.*, 2009).

1.2.8 ANIMAL STUDIES OF DEVELOPMENTAL PROGRAMMING OF THE KIDNEY

As previously discussed, animal models have been established to mimic the human condition. Many of these experimental models have been based on dietary interventions. Alterations in vitamin A levels, maternal low protein diet and exposure to glucocorticoids can influence nephron endowment, and frequently, but not always, blood pressure (Holemans *et al.*, 1999, Woods *et al.*, 2005, Zimanyi *et al.*, 2006, Gilbert *et al.*, 2005). For example, Langley-Evans *et al.* (1999c) reported a deficit of 13% in nephron number was associated with a 13mmHg elevation in arterial pressure of protein-restricted offspring (Wistar rats exposed to a 9% protein diet, while controls had a normal 18% protein diet). However, nephron deficiency is not always associated with elevated arterial pressure. For example, Zimanyi *et al.* (2006) reported a nephron deficit in Wistar-Kyoto (WKY) rats fed an 8.7% (low) protein diet (while control rats were fed a 20% protein diet). However, there was no alteration in blood pressure or evidence of renal hyperfiltration in adulthood in the protein-restricted group (Zimanyi *et al.*, 2006). The absence of change in mean arterial pressure in adulthood could be a function of the model used (Zimanyi *et al.*, 2006), although it does illustrate that blood pressure is not solely regulated by nephron number.

The relationship between nephron number and blood pressure was investigated by Woods *et al.* (2001) using a model of maternal protein restriction in rats. Protein restricted male offspring had 25% fewer nephrons, 10 mmHg greater mean arterial blood pressure and an 11% deficit in glomerular filtration rate compared with control rats. Calculated single nephron glomerular filtration rate was 20% greater in protein-restricted than in protein-replete offspring. Individual nephron GFR can be an indication of hyperfiltration, which can ultimately lead to further nephron loss, perpetuating this cycle. More recently, Woods *et al.* (2005) reported that female offspring from protein restricted Sprague-Dawley dams (on a 8.5% protein diet, while controls were on a 19% protein diet) had similar mean arterial pressure as control offspring. These

results suggest that sex plays a role in developmental programming, and that it is therefore important that both sexes are considered and investigated independently in studies of developmental programming.

Developmental programming of hypertension can be induced in both sexes. Utilising a maternal protein restriction model in Sprague-Dawley rats, Vehaskari *et al.* (2001) reported that males and females exposed to the protein restricted diets for 4 weeks had a nephron deficit of 28% and 29%, respectively. This deficit in nephron number was accompanied by a 20-25mmHg higher blood pressure in males and females than controls. While Vehaskari has reported similar findings in both male and female offspring, this is not ubiquitous. Woods *et al* (2005) using a low protein diet (8.5%) in Sprague-Dawley rats reported that female offspring had the same blood pressure as controls at 22 weeks of age (Woods *et al.*, 2005) These results show illustrate that both sexes should be investigated as there are subtle differences.

As already illustrated, nephron endowment is affected by the developmental environment. In order to determine the physiological impact of this, it is important to investigate renal function. Nwagwu *et al.* (2000) fed rat dams either 18% (control) or 9% protein diet. After four weeks of postnatal life the offspring were placed on a normal laboratory chow diet. Offspring exposed to the low protein diet *in utero* had an increase in systolic blood pressure varying from 7 mmHg to 21 mmHg between 4 weeks of age and 20 weeks (Nwagwu *et al.*, 2000). Creatinine clearance, a measure of glomerular filtration rate, at 4 weeks of age was less in rats exposed to the low protein diet than controls (Nwagwu *et al.*, 2000).

1.2.9 MECHANISMS OF DEVELOPMENTAL PROGRAMMING

Specific pathways have been linked to the adverse effects of impaired maternal diet on fetal development, including kidney development (Pires *et al.*, 2006). The pathways most commonly associated with the effects of maternal dietary restriction on development of adult

diseases are the renin-angiotensin system (RAS) and glucocorticoid signalling pathways and more recently, epigenetic alterations (Moritz *et al.*, 2003, Waterland and Michels, 2007).

1.2.9.1 Renin-Angiotensin System

The RAS consists of multiple ligands, enzymes and receptors with all components present within the kidney (Figure 1.3) (Kobori *et al.*, 2007). The RAS plays a critical role in the regulation of arterial blood pressure, extracellular fluid volume, and renal hemodynamic and excretory function in adulthood (Cervenka *et al.*, 1999). The RAS is a crucial regulatory factor in homeostasis of glomerular filtration (Moritz *et al.*, 2003, Okubo *et al.*, 1998).

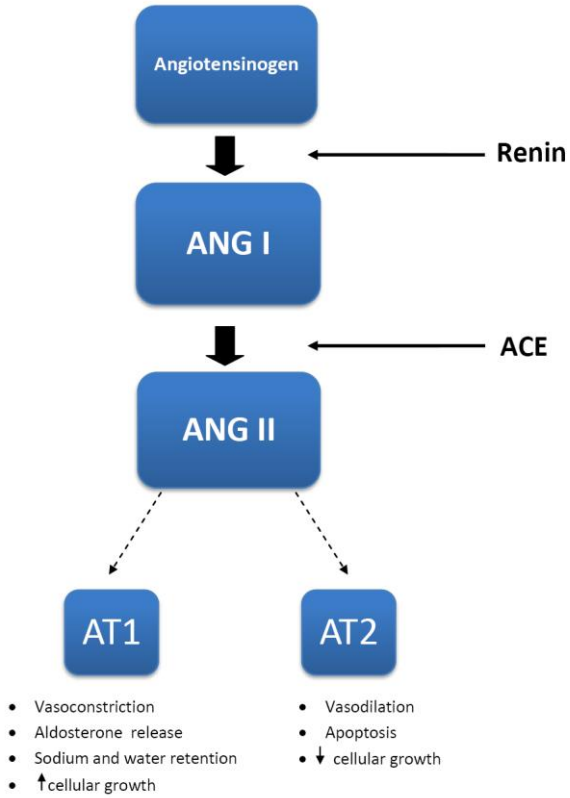

Figure 1.3 - Overview of the classical renin-angiotensin system (RAS).

Circulating angiotensinogen is cleaved by the rate limiting enzyme, renin, to form Angiotensin I (ANG I). Angiotensin Converting Enzyme (ACE) then converts ANG I to Angiotensin II. Many of the effects of ANGII are mediated through two receptors; type 1 angiotensin (AT1) and type 2 angiotensin (AT2) receptors.

The most biologically active component of the RAS, Ang II, constricts vascular smooth muscle cells, enhances myocardial contractility, stimulates aldosterone production, stimulates release of catecholamines from the adrenal medulla and sympathetic nervous system activity, and stimulates thirst and salt appetite (Kobori *et al.*, 2007). When the RAS is inappropriately activated, the intra-renal RAS is a contributor to hypertension and renal injury (Kobori *et al.*, 2007).

Manipulation of the RAS *in vivo* has led to a greater understanding of its role. McMullen *et al.* (2004) investigated the effects of maternal protein restriction on RAS gene expression in offspring. Wistar rat dams were protein restricted during pregnancy and throughout lactation, at which stage all rats were switched to normal laboratory chow. At 4 weeks of age, offspring exposed to a low protein diet had a 23 mmHg higher systolic blood pressure than controls. This was associated with a deficit in renal expression of angiotensin II type 2 (AT_2) receptors. AT_2 receptors are found in cortical tubules, glomeruli and interstitial cells and often have opposing actions to angiotensin II type 1 (AT_1) receptors.

It has been proposed that the nephron deficit of protein-restricted dams is mediated at least partly through inhibition of the RAS (Barker *et al.*, 2006). Angiotensin is essential in forming blood vessels and tubules during nephrogenesis as well as the development of smooth muscle in the ureter (Barker *et al.*, 2006, Burrow, 2000). Vehaskari *et al.* (2004) using Sprague-Dawley rats, showed that hypertension was induced by maternal protein restriction. Rat dams were fed a low protein diet (6% protein) or a normal protein diet (20% protein) during pregnancy and lactation. After weaning pups were placed on normal chow. After dams had given birth and had weaned pups, the pups were put on the normal protein diet. At 28 days or at birth, the rats were humanely killed. Analysis of kidneys using Real-Time PCR showed that at birth, renal expression of the AT_1 receptor was low in those pups exposed to the low protein diet and then

rose above the level in control rats at day 28. This raises the possibility of the RAS being a potential mechanism leading to changes in blood pressure.

1.2.9.2 EPIGENETICS AS A MECHANISM OF DEVELOPMENTAL PROGRAMMING

Epigenetics is the study of heritable changes in gene expression. Heritability requires epigenetic marks to be maintained during cell division and replication. Changes in gene expression – thus not mutations within the genome, but changes in how the genome is expressed - can be controlled by epigenetic 'marks' such as gene methylation, histone modifications and the binding of DNA proteins. These marks can act independently or in concert to modify expression of genes. Epigenetics may play a role in developmental programming of adult disease as it provides a mechanism that explains the change in gene expression, and therefore the change in phenotype. These changes can be brought about through multiple epigenetic pathways that will be discussed in further detail in the following section.

Epigenetic studies have shown that the maternal environment, before, during and after birth can have a significant impact on the genome of the offspring (Dolinoy and Jirtle, 2008, Hales *et al.*, 2011, Hochberg *et al.*, 2011, Ikeda *et al.*, 2012, Romagnolo *et al.*, 2012). Essentially, the epigenetic profile of the organism is malleable during any stage of development – as during this stage the genome is undergoing modifications and specification. Before the genome becomes heritably stable, changes in the epigenetic profile can be incorporated into the phenotypic profile and therefore be inherited over multiple cellular cycles and alter the gene expression of that cell line or organ system. One of the most well studied models of how the maternal environment affects the offspring genome through epigenetic mechanisms, is that of the *agouti* mouse.

The *agouti* viable yellow (*AvY*) mouse strain has been used extensively to demonstrate the effects of a methyl deficient diet and subsequent disease outcome. The agouti gene is under the control of a methylation sensitive promoter and infers a yellow coat colour and an increased preponderance to obesity, diabetes, cancer and a shorter lifespan when expressed (Burdge *et al.*, 2007, Cooney *et al.*, 2002, Wolff *et al.*, 1998). Pregnant *AvY* mice exposed to methyl deficient diets give birth to pups displaying variable coat colours but the majority of offspring present with the agouti phenotype. This phenotype occurs in response to hypomethylation of the agouti locus and an increase in expression. The phenotype is not reversible by later methyl donor supplementation. Where methyl donors are freely available in the maternal diet, the agouti locus remains methylated and the gene repressed, thus the majority of offspring have a brown coat and normal physiology, metabolism and life expectancy.

1.3 EPIGENETICS

The term 'epigenotype' was first used by Waddington to describe the realm that exists between the genotype and phenotype of an organism and the complex developmental processes that occur (Waddington, 1942). The field of epigenetics, studying the heritable changes in gene expression, has been found to have major implications in both developmental biology as well as cancer research. The Human Genome Project which commenced in 1991 had the main objective of sequencing the entire human genetic code, in part, to discover the genetic background to many diseases (Collins *et al.*, 1998, Trent, 2000). After completion of the project in 2006 the ability to track diseases down to single genes (monogenic diseases) remained limited, despite the masses of data collected and analysed. It is now better understood that there is a complex gene-environment interaction that occurs in any biological system that is able to introduce variation and change (Bentley, 2000, Panchal and Brandt, 2001, Frazer, 2012). A greater appreciation for multi-factorial disease has taken place – whereby there is a complex relationship between genes and the environment. It is now understood that the development of

adult diseases such as cancer and cardiovascular disease, are not solely due to a mutation within the genome in most cases but are driven by a change in gene expression. The combined product of the genome and the epigenetic marks, the epigenome, was once thought to be quite stable from cellular generation to generation, but this concept is now being questioned. Evidence suggests that the epigenome is malleable and capable of responding to the environment, not only during the early stages of cell specification and lineage determination. This is evidenced by changes in the epigenome in studies of cancer development (Banerjee and Verma, 2009, Baylin *et al.*, 2001, Jones, 1996, Jones and Baylin, 2002).

1.3.1 EPIGENETIC MODIFICATIONS

The epigenome is maintained by several different molecular systems that regulate the physical structure of the genome, thereby controlling its availability to transcription factors. Transcription factors are critical in gene expression. Chief among the epigenetic marks are methylation, histone modifications and non-coding RNAs. This thesis will focus on methylation, but a brief overview of other epigenetic forms is included below.

1.3.1.1 METHYLATION

The genetic code is comprised of four nucleotides that bind in a systematic and controlled manner. Most importantly for methylation are the cytosine guanine dinucleotides. The chemical bonds between these two nucleotides allows for the enzymatic addition of a methyl group. Cytosine and guanine dinucleotides can occur in high density within the genetic code, referred to as CpG islands. CpG islands are usually found upstream of promoter regions of genes. The methylation of CpG islands commonly leads to gene silencing (Figure 1.4), however partial methylation of a CpG island can lead to altered gene expression (Baker and El-Osta, 2010).

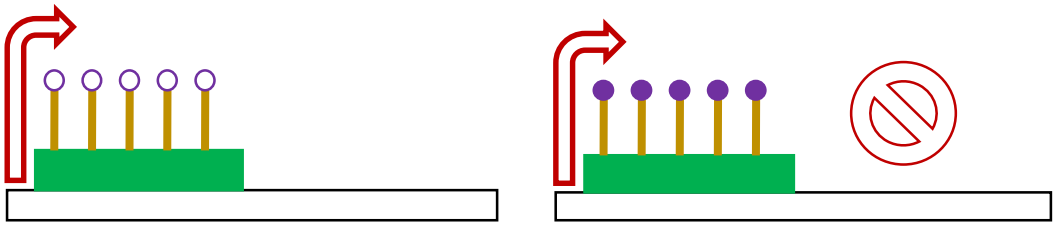

FIGURE 1.4 - GENE METHYLATION AND EXPRESSION.

Gene expression (red arrow) can be altered due to methylation (solid purple markers) of CpG regions (green bar).

Focusing particularly on the role of methylation during development, it is quite clear from numerous studies that paramount in early embryo development (Li *et al.*, 1992, Lyko *et al.*, 1999). Until recently it was believed that the developing embryo undergoes two stages of demethylation in preparation for creating a stable and heritable methylation profile. Initially, at the site of implantation the maternal and paternal genomes are 'wiped' of their methylation marks – via different mechanisms. The maternal genome undergoes a passive demethylation, while the paternal genome undergoes active demethylation. However, it has been shown that there are particular alleles on both the maternal and paternal genomes that undergo demethylation during later stages of development – these allelles are termed 'imprinted'. Two of the major imprinted alleles are Igf2 (insulin growth factor 2) and H19, both of which control the regulation and growth of the developing embryo (Koukoura *et al.*, 2012, Sandovici *et al.*, 2012). The breadth and complexity of epigenetic programming of development will be discussed in the subsequent sections.

The methylation of CpG islands is maintained by a multitude of enzymes, the main ones being the family of DNMTs (DNA methyltransferases, DNMT1, DNMT2a, DNMT2b and DNMT3) (Gicquel *et al.*, 2008). The very early stages of development see significant changes to the genetic code (Geiman and Muegge, 2010). Specifically focusing on the methylation changes, there are currently two theories explaining how the parental genomes undergo methylation

changes to produce the unique profile of the offspring (one that suggests that the methylation patterns in the early embryo are set, while the other suggests that these patterns are more plastic throughout development). Originally it was shown that the paternal genome is actively demethylated at the time of fertilisation (Oswald *et al.*, 2000, Mayer *et al.*, 2000b). This was thought to be due to the fact that the maternal genome was significantly less methylated than the paternal genome at the time of fertilisation (Mayer *et al.*, 2000b). By the 8-cell stage both maternal and paternal genomes have low levels of DNA methylation (Mayer *et al.*, 2000a, Cardoso and Leonhardt, 1999). It is important to note that the entire genome is not completely wiped of methylation marks. Levels of methylation are maintained at imprinted sites such as POI and IAP (intracisternal A particles) repetitive elements (Lane *et al.*, 2003).

During implantation the embryonic genome undergoes *de novo* methylation in order to establish new methylation patterns (Tang and Ho, 2007, Kim *et al.*, 2009). This *de novo* methylation is under the control of DNMT3a and 3B. DNMT3a acts on unmethylated DNA, while DNMT3b can assume its role on both hemimethylated and unmethylated DNA (Vassena *et al.*, 2005). The establishment of methylation is required during development and sets the profile for differentiation of specific cell lineages during the early stages of development.

The establishment of gene methylation status goes through two stages. Initially in the female and male gonads methylation status is erased (Jirtle and Skinner, 2007). Methylation status is then re-established post fertilisation, in a sex-specific manner which is asymmetrical and imperative for normal development (Razin and Shemer, 1995, Bestor, 2000, Santos *et al.*, 1994, Allegrucci *et al.*, 2005). Recent evidence has begun to contradict the original dogma that the methylation status was set for life after conception. Modification of DNA methylation in postnatal life is associated with tumourigenesis (McKenna and Roberts, 2009, Enokida and Nakagawa, 2008, Jones and Baylin, 2002). The hyper- or hypo methylation present within cancer affects a large array of genes that are associated with tumourigenesis, tumour

progression and metastasis (Li, 2007b, Kerr *et al.*, 2007). The most likely cause for this alteration in methylation status is aging and dietary methyl donor consumption (particularly folate intake) which promotes altered expression of cancer genes, such as proto-oncogenes (Suzuki *et al.*, 2006, Richardson, 2002, Jubb *et al.*, 2001). These studies indicate that methylation patterns, and therefore gene expression patterns are not as stable as initially thought. The methylation status of the genome is highly dependent on dietary intake of methyl donors (Friso and Choi, 2005, Friso and Choi, 2002) and this may be the mechanism behind the role of maternal diet in programming adult health or disease in offspring. There is a clear distinction between multigenerational programmed epigenetic changes whereby epigenetic marks are carried through the germline and programmed epigenetic changes within somatic cell lines (Tammen *et al.*, 2013).

Methylation of the genome is highly dependent on the availability of substrates, many of which are a dietary requirement (betaine, vitamin B12, folic acid/folate). The dietary requirements include the availability of methyl (CH_3) groups. One of the principal sources is folate, which will be discussed in later sections.

1.3.1.2 HISTONE MODIFICATIONS

Acetylation and deacetylation of histones can control gene expression. Deacetylation leads to inhibited gene expression due to compaction of the DNA (Lane and Chabner, 2009). These processes are controlled by HATs (histone acetyltransferases) and HDACs (histone deacetylases) (Selvi and Kundu, 2009). Mutations of the genes responsible for encoding HATs and HDACs are associated with various diseases including cancer, diabetes and congenital developmental disorders (Van Beekum and Kalkhoven, 2007).

Investigations of suboptimal intrauterine environments on offspring health have shown, in association with gene expression changes, changes in chromatin structure. Fu *et al.* (2009) investigated the effects of IUGR and the expression of IGF-1 and found that IUGR was

associated with alterations in the histone code, finding changes in both methylation sites and the acetylation of histones of IGF-1, suggesting a possible relationship between intrauterine growth and the methylation of genes. Lillycrop *et al.* (2007) used a low protein model of developmental programming in rats and reported histone modification in the promoter region of the hepatic $GR1_{10}$ (glucocorticoid receptor). This study illustrates the ability for maternal diet to alter offspring gene expression through epigenetic mechanisms. Techniques are now becoming available to investigate the genome with great resolution with less and less substrate required, enabling investigations on single cell types and developing organ systems.

1.3.1.3 NON-CODING RNAS

Previous sections have discussed epigenetic control of gene expression pre-transcription. However, gene expression can also be controlled post-transcriptionally by non-protein coding RNAs (ncRNA). ncRNAs can be broadly grouped into three categories based on size; microRNAs (21-25 nucleotides), small RNAs (100-200 nucleotides) and large RNAs (up to 10,000 nucleotides) (Costa, 2005).

ncRNAs effectively silence and control gene expression via three steps. After being transcribed the ncRNA forms a long double stranded RNA, which is processed into small RNAs by the RNase III enzyme Dicer (Siomi and Siomi, 2009). The small RNA is then unwound and one strand becomes involved with a protein complex (RISC – RNA-Induced Silencing Complex). This complex then searches for the target mRNA and upon binding is able to cleave the target, essentially stopping translation of the protein (Siomi and Siomi, 2009).

Non-protein coding RNAs have been shown to be critical during normal development (Stefani and Slack, 2008), with the identification of specific miRNAs that are capable of reprogramming cells to an embryonic stem cell-like state (Lee *et al.*, 2008, Lin *et al.*, 2008). Investigating the role of Dicer during mammalian kidney development, Nagalakshmi *et al.* (2011) used a transgenic mouse model (*Six2Cre*) where *Dicer* was conditionally ablated from the progenitors

of the nephron epithelium. The removal of *Dicer* lead to elevated apoptosis and premature termination of nephrogenesis. Ablation of *Dicer* from ureteric bud epithelium (using *HoxB7Cre* transgenic mice) resulted in the development of renal cysts and disrupted branching morphogenesis (Nagalakshmi *et al.*, 2011). These results illustrate the importance of miRNA function during kidney development. Imprinting of genes has also been shown to be regulated by ncRNA. A large ncRNA, *Xist* (X inactive specific transcript) is the master regulator of X inactivation in females (Senner and Brockdorff, 2009).

Very few studies of developmental programming have investigated the role of ncRNAs in the propagation of adult disease. Goyal *et al.* (2009b) exposed mice to a protein restricted diet and analysed fetal gene expression of the RAS in the brain. Maternal protein restriction lead to reduced expression of the miRNA mmu-mir-330 which putatively regulates AT2 translation, as well as up-regulation of mmu-mir27A and 27B which regulate ACE-1 mRNA translation. Zhang *et al.* (2009) investigated the consequences of a maternal high fat diet in mice and found changes in the miRNA profile in the high fat exposed offspring when compared to controls.

The phenotypic variability introduced by the ability to modulate levels of gene expression within a generation is evolutionarily clever. It is a Lamarckian quality of the genome that it can adapt efficiently. This malleability of the genome allows the potential to adapt to make the best of any environment, with these changes also able to be inherited by future generations.

1.3.2 Epigenetics and Developmental Programming

Studies that have investigated the consequences of maternal dietary manipulation on offspring phenotype, commonly report changes in gene expression and major function in the offspring (Waterland and Michels, 2007). These changes or modifications are not due to mutations within the genome, but the levels of gene expression, which may feasibly be under the control of epigenetic mechanisms. Epigenetic control of gene expression is maintained and regulated by

three major mechanisms: DNA methylation, histone acetylation and non-protein coding RNA (Waterland and Michels, 2007). This thesis will focus on gene methylation.

Offspring of rats whose mothers were fed protein restricted diets in pregnancy and lactation demonstrate global changes to the methylation status of whole organs, and important gene pathways that play a major role in kidney development and adult function (Lillycrop *et al.*, 2005a, Rees *et al.*, 2000, Bogdarina *et al.*, 2007). These studies have further investigated these effects to show that important enzymes involved in setting the methylation profile of the genome are altered (specifically DNMT1, which is involved in maintaining the methylation status of the genome during replication), as well as receptors involved in protein metabolism (Lillycrop *et al.*, 2008, Lillycrop *et al.*, 2007). Given that methylation is highly dependent on dietary intake of methyl donors, it has now been demonstrated that when either the maternal or early postnatal diet are supplemented with methyl donors (such as glycine and folic acid), the deleterious cardiovascular effects of maternal protein restriction may be at least partially ameliorated or rescued.

1.4 Rescuing the Phenotype Produced by Exposure to a Low Protein Diet

It is well established that maternal nutrition and behaviour can impact upon fetal development and later health. Efforts have been made to provide better care for pregnant women in order to prevent undesirable birth outcomes. During the 20th Century a significant effort was aimed at reducing neural tube defects after it was observed that increasing the amount of folate or folic acid consumed during pregnancy was positively correlated with a reduction in neural tube defects (Czeizel, 1993b, Czeizel, 1993a, Czeizel, 1995c, Czeizel, 1995a, Czeizel, 1998, Czeizel, 2000, Czeizel, 2002, Smithells, 1982, Smithells, 1989, Smithells *et al.*, 2011, Smithells *et al.*, 1985). These studies provided the basis for multiple countries to implement mandatory folic acid fortification of food sources. Evidence is now been collected to suggest

that there is an epigenetic link to some neural tube defects that can be ameliorated by maternal folic acid intake (Ichi *et al.*, 2010).

Given the positive effects of folate during pregnancy and its potential role for impacting upon the epigenome, it is our hypothesis that supplementing the maternal diet with folic acid will ameliorate the deleterious developmental effects of a maternal low protein diet.

1.4.1 FOLATE

1.4.1.1 FOLATE AND 1-CARBON METABOLISM

Methylation of biological molecules (in particular DNA) is a critical process that requires the availability of methyl (CH_3) groups from the diet. This cycle involves numerous enzymes and cofactors that rely heavily on the dietary intake of specific vitamins such as folate, B12 and methionine (Poirier, 2002) (Figure 1.5). This section will focus on the metabolic pathways of 1-carbon metabolism and its end products as well as the role of folate, along with the effect of folate deficiency on the function of the pathway, and finally finishing with a discussion about the role of DNA methylation of genes.

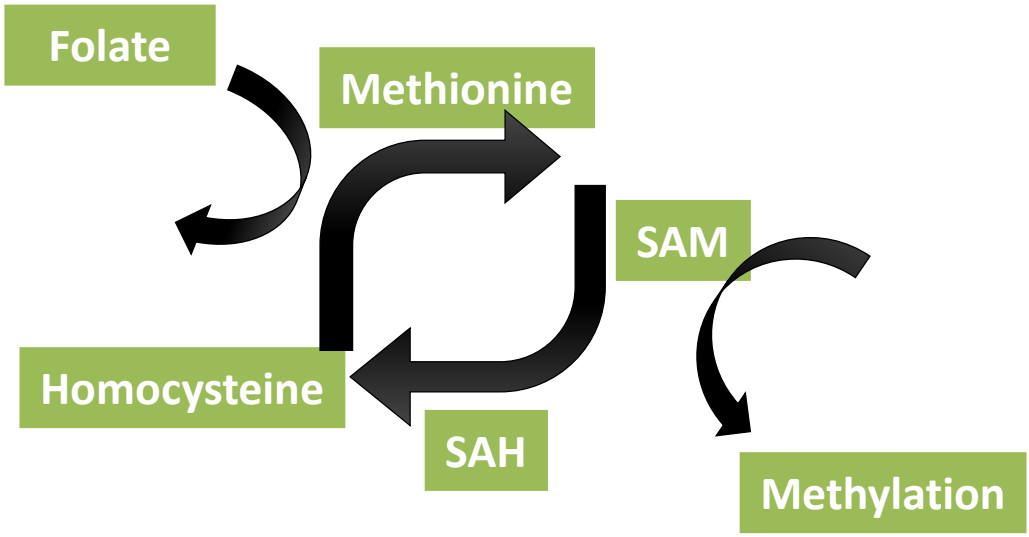

FIGURE 1.5 -THE METHYL CYCLE

The relationship of folate/folic acid in the methylation process, whereby it is able to donate a methyl group to produce SAM (s-adenosyl methionine) which is able to methylate biologicals. SAM loses a methyl group converting to SAH (s-adenosyl homocysteine), leading to homocysteine which is then able to be converted to methionine with the availability of folate or folic acid.

For the 1-carbon cycle to occur, it requires the presence of molecules that are capable of donating 1-carbon groups (methyl groups). There are two principal methyl donors, betaine (from choline) and SAM (S-adenosylmethionine) both of which come from the diet or are derived from dietary constituents, but cannot be produced within the body. The methylation process depends on the abundance of SAM, as the universal methyl donor (Waterland and Jirtle, 2003, Grillo and Colombatto, 2005, Loenen, 2006, Roje, 2006, Grillo and Colombatto, 2008). Upon donating its methyl group, SAM is converted to SAH (S-adenosylhomocysteine). SAH is then further converted to homocysteine and with the presence of N5-methyltetrahydrofolate (a folate derivative) can be re-methylated to form methionine. Methionine is then able to be converted to SAM, maintaining the abundance of SAM. However, there are key steps that occur in an accessory pathway that can impact significantly on the outcome (Figure 1.5).

The re-methylation of homocysteine to form methionine is critical in maintaining low levels of homocysteine and a constant abundance of methionine (and therefore SAM) (van den Vevyer, 2002). The process of re-methylation makes use of folate derivatives (N5-methyltetrahydrofolate as the methyl donor) and requires a coenzyme derived from B12 (cyanocobalamin). An alternative re-methylation step makes use of betaine as the cofactor. The re-methylation of homocysteine relies heavily on dietary intake; therefore deficiencies can impinge the function of the 1-carbon metabolism pathway.

An important focus of the 1-carbon metabolism pathway is that of methylation of the genome (see Section 1.3.1.1).

Folate deficiency can lead to a reduced amount of methionine in the diet due to homocysteine not being re-methylated the re-methylation stage (due to the absence of methyl groups) (Maloney *et al.*, 2007). This can lead to a surplus of homocysteine, which is a risk factor for multiple diseases such as atherosclerosis and neurodegenerative diseases (Forges *et al.*, 2007). This reduction in methylation can lead to the onset of disease due to aberrant gene expression. The *agouti* mouse has been used extensively to demonstrate the effects of a methyl deficient diet and disease outcome (Section 1.2.9.2).

As previously established, derivatives of folate play a key role in the re-methylation of homocysteine to methionine. This pathway involves multiple enzymes and cofactors that can affect the availability of methionine. Briefly, folate once absorbed from the diet is converted to tetrahydrofolate (THF) which, with the use of methylenetetrahydrofolate reductase (MTHFR) is able to donate the methyl group to homocysteine in the presence of cofactors. Much research has been undertaken to understand the role of folate in this cycle. Particular interest has been paid to the role of MTHFR (Bol *et al.*, 2008). Mutations in MTHFR have

been associated with increased levels of homocysteine, low birth weight and congenital abnormalities (Crider *et al.*, 2011b, Glanville *et al.*, 2006, Hobbs *et al.*, 2010).

Deficiencies in the function of MTHFR can lead to a number of consequences. Common mutations (about 12% of the white population) include the point mutation at position 677 from a C to T. This mutation in MTHFR leads to mild elevations of plasma homocysteine. Elevations in plasma homocysteine are associated with increased risk of coronary, cerebral and peripheral vascular disease (Brattstrom *et al.*, 1998).

1.4.2 FOLATE AND DEVELOPMENTAL PROGRAMMING

Investigations into adding one-carbon metabolites (most commonly folate/folic acid) to diets and the reduced risk of developing cardiovascular disease, have reported that the one-carbon pathway has a significant impact on cardiovascular health in the adult (Villa *et al.*, 2007, Moens *et al.*, 2008, Dayal and Lentz, 2008). Very interestingly, the supply of one-carbon metabolites in at-risk pregnancies (such as low birth weight or congenital abnormalities) may have profound and permanent effects upon offspring health.

Jackson *et al.* (2002) reported that addition of the one-carbon metabolite glycine to the maternal diet alleviates hypertension caused by a suboptimal intrauterine environment. Folic acid is a commonly recommended supplement during pregnancy following the overwhelming evidence that a positive correlation exists between folic acid levels and positive birth outcomes, particularly a reduction in neural tube defects (Wilson *et al.*, 2003, Leeda *et al.*, 1998, Feldkamp *et al.*, 2002). Moreover, women planning pregnancy are advised to maintain an intake of folic acid of between 500 and 4000 micrograms per day (Geisel, 2003, Ryan-Harshman and Aldoori, 2008, Wilson *et al.*, 2003). While women are recommended to take 500 to 4000 micrograms of folic acid a day, Tolraova and Harris (1995) report reduced occurrence of cleft lip with or without palate with maternal multivitamin supplementation

(including 10mg/day folic acid) compared to those who were not supplemented during pregnancy. Interestingly, despite the highly encouraged use of folic acid as a pregnancy supplement little is known about the definitive mechanism by which it is protective against fetal defects (Pitkin, 2007, Green, 2002). Given that folic acid is a key dietary requirement throughout pregnancy and lactation, as well as for the development of the fetus, it may be possible that folic acid carries out these functions through an epigenetic mechanism.

1.5 Kidney Development

Maternal diet can influence fetal development, specifically the development of the kidney. Due to the kidneys playing a vital role in health and disease they form the focal point of this thesis. The relationship between maternal diet, kidney development and health outcomes has been previously discussed (Section 0). In the following sections, kidney development (gene expression and the role of epigenetics) will be discussed.

In mammals, the kidney develops through three stages; the pronephros, the mesonephros and the metanephros. The pronephros is a rudimentary structure that develops from the lateral plate and intermediate mesoderm and continues to develop into the mesonephros (Dressler, 2006). The mesonephros is also a transitory organ, although the mesonephric (nephric) duct is important for the development of the metanephros, the permanent mammalian kidney (Dressler, 2006). The metanephros begins to develop at day 35 in human gestation and embryonic day 12 (E12) in the rat (Caruana *et al.*, 2006, Paixao and Alexander, 2013). Nephrogenesis is complete by 36 weeks gestation in humans and by about PN9-10 in rats (Dressler, 2006, Paixao and Alexander, 2013).

1.5.1 URETERIC BRANCHING MORPHOGENESIS

The metanephros begins to develop when the ureteric bud grows out from the Wolffian duct (or mesonephric duct) in response to growth factors secreted by the metanephric mesenchyme (see Dressler 2006 for review). Under the influence of these growth factors, the ureteric bud invades the metanephric mesenchyme and branches.

Branching morphogenesis is the dichotomous arborisation of ureteric epithelium and is responsible for the development of the collecting duct system of the kidney. The process of branching morphogenesis is tightly regulated by inhibitors (BMP4, bone morphogenic protein 4), promoters (GDNF, glial derived neurotrophic factor and c-RET, c-ret tyrosine kinase receptor) and transcription factors (TGFβ, transforming growth factor β superfamily). Branching morphogenesis begins with the interaction between the metanephric mesenchyme and the ureteric bud, whereby inductive signals from both embryonic derivatives (GDNF from the metanephric mesenchyme acts on receptors c-RET and GFRα1 from the ureteric bud) initiate the branching process (Jain, 2009, Little *et al.*, 2010, Michos, 2009, Popsueva *et al.*, 2003).

Tight regulation of branching morphogenesis is essential to both kidney development and function. Utilising the ability to culture whole embryonic kidneys, Cain and Bertram (2006) reported that exogenous BMP4 *in vitro* leads to reduced branching morphogenesis, indicated by fewer branch points, ureteric length and volume.

Branching morphogenesis is intricately involved with nephrogenesis. Induction of nephron development can only occur at the tips of the developing ureteric tree. (Sims-Lucas *et al.*, 2008) reported that *TGFβ2* heterozygous null mutant mice have a 60% augmentation in nephron number at PN30, and *in vitro* studies have shown a 40% increase in total ureteric branching length following 48 hour culture in the presence of exogenous TGFβ2. It is not known whether

maternal protein deprivation influences kidney branching morphogenesis leading to a nephron deficit in adulthood, nor is it known whether the expression of genes involved in branching morphogenesis or nephrogenesis are affected by exposure to protein restriction during development.

1.5.2 NEPHROGENESIS

Metanephric mesenchymal cells adjacent to the ureteric branch tips are induced to condense and undergo epithelialisation. These epithelial vesicles differentiate through various morphological steps (including comma-shaped bodies and S-shaped bodies) to eventually form nephrons (Schmidt-Ott *et al.*, 2006, Shah *et al.*, 2004).

The process of nephron formation occurs at the tips of the developing ureteric tree. These tips induce proliferation and condensation of the surrounding mesenchymal cells – forming the induced mesenchyme. Induced mesenchyme expresses genes involved in extracellular matrix, cell-adhesion molecules and cell survival and proliferation compared to the ureteric tree (Schwab *et al.*, 2003, Stuart *et al.*, 2001, Stuart *et al.*, 2003). The induced mesenchyme epithelialises, under the influence of PAX2, WNT4 and HNF4α (Sariola, 2002, Torban *et al.*, 2006, Kanazawa *et al.*, 2010, Kanazawa *et al.*, 2011) and continues to develop into a comma-shaped body which is characterised by expression of the primitive podocyte marker MnfB and genes involved with BMP signalling (Brunskill *et al.*, 2008). The comma-shaped body develops into an S-shaped body (through FGF8 and PAX2 signalling (Grieshammer *et al.*, 2005, Perantoni *et al.*, 2005, Torban *et al.*, 2006), linking up with the ureteric epithelium. The S-shaped body contains the primitive cells of the nephron with the invasion of angiogenic cells. With capillarisation and the connecting to the tubule (influenced by FGF2 and LIF expression (Plisov *et al.*, 2001) the nephron is complete. However, the nephron continues to undergo maturation in the form of tubule elongation. Notch signalling is essential during nephron development. Knockout of *Rbpj* (a transcription factor required for canonical notch signalling)

in metanephric mesenchyme (using *Rarb2Cre*$^{+}$ mice) resulted in fewer WT1-positive podocyte precursors in the comma- and S-shaped bodies (Bonegio *et al.*, 2011). An integral component of nephrogenesis is the maintenance of the metanephric mesenchyme. This leads to sufficient support of the progression and development of nephrogenesis. An extensive search for genes involved in the maintenance of this cell population has indicated the importance of *Six2*, *Fgf2* and *Bmp7* (Dudley *et al.*, 1999, Kobayashi *et al.*, 2008).

The control of nephrogenesis cessation is a potential critical window during kidney development which may be influenced by maternal diet. The early cessation of nephrogenesis may cause a nephron deficit. One theory states that the cessation of nephrogenesis is dependent on the amount of mesenchyme available and this may account for the large variability in nephron number in mammals (Hughson *et al.*, 2006, Merlet-Benichou, 1999, Rumballe *et al.*, 2011). The metanephric mesenchymal cell population provides the basis from which all the cells of the nephron will differentiate. What controls the cell numbers of this population is unknown and may be influenced by maternal diet manipulation. Nephrogenesis occurs over a finite period. It is not possible to develop new nephrons after the cessation of nephrogenesis. As nephrons are the functional units of the kidney a reduction in number can be detrimental to kidney function.

1.5.3 CONSEQUENCES OF POOR KIDNEY DEVELOPMENT

As described above, the processes of kidney development and nephrogenesis are complex, and are regulated by numerous genes and gene pathways. In addition, teratogens, fetal urinary flow, the fetal/maternal environment and genetics can impinge on nephron development (Welham *et al.*, 2005). It is perhaps not surprising therefore that the number of nephrons in human kidneys varies widely, up to 10-fold (Douglas-Denton *et al.*, 2006, Hoy *et al.*, 2006a, Hughson *et al.*, 2006, Hoy *et al.*, 2005, Hoy *et al.*, 2006b, Hughson *et al.*, 2008, Keller *et al.*, 2003, Nyengaard and Bendsten, 1992). Disruption to kidney development can ultimately influence kidney

function in adult life. While nephron number can vary 10-fold in normal humans, what constitutes as 'low nephron number' in humans is not well understood. Nor is the relationship between low nephron number and kidney function (in humans). Unlike in animal models, where this normal variation does not occur, it is possible to understand the relationship between low nephron endowment and kidney function.

1.5.4 KIDNEY FUNCTION

The kidney is a filtering organ that is involved in salt/water balance, removal of metabolic waste products and toxins. As well as filtering blood plasma, the kidney secretes hormones such as erythropoietin, and 1.23dihydroxyvitamin D_3 which are involved in cardiovascular function and bone health (Bissinger, 1995, Boulter *et al.*, 2001, Braun and Huber, 2002). The focus of this thesis will be on the kidney to function as a filtering organ and this relies on the nephron, the functional unit of the kidney. The nephron includes the glomerulus, proximal convoluted tubule, the loop of Henle and the distal convoluted tubule which connects to a collecting duct, allowing the filtrate to ultimately empty into the bladder via the ureter.

1.5.4.1 FILTRATION

Filtration occurs at the glomerulus, where the leaky capillary and slit diaphragms formed by podocytes allow filtered plasma to cross and enter the Bowman's space. The filtrate then passes through the tubules (proximal, loop of Henle and distal tubules) of the kidneys. The final solution that leaves the kidney as urine is the product of the initial filtration as well as subsequent tubular reabsorption (or absorption) and tubular secretion. Tubular reabsorption involves the reabsorption of water, sodium, glucose and urea. This is a dynamic process, affected by multiple hormones, signalling pathways as well as the structure of the kidney (Di Sole, 2008, Pelayo and Shanley, 1990, Peti-Peterdi and Harris, 2010). The maintenance of absorption and secretion by the tubules is intricately linked to kidney function (Chambrey and Picard, 2011, Eladari and Chambrey, 2010, Eladari *et al.*, 2012). Feedback loops control the

perfusion of the kidney, as well as the glomerulus by altering efferent and afferent arteriole dilation and constriction. The juxtaglomerular apparatus is a collection of cells (macula densa and granular cells) located within the nephron between the distal convoluted tubule and glomerulus (efferent, afferent arterioles that make-up the glomerular tuft) allowing it to monitor the filtrate and plasma. In response to changes in sodium levels, the juxtaglomerular apparatus can release hormones (particularly renin) to counteract these changes, returning sodium concentrations within the blood to normal.

1.5.4.2 GLOMERULAR FILTRATION RATE (GFR)

Glomerular filtration rate is the rate at which plasma is filtered by all nephrons in both kidneys per unit of time. GFR can be used as a measure of kidney health (Levey, 1990). GFR is influenced by renal perfusion, electrolyte levels and glomerular filtration surface area (Brenner *et al.*, 1988, Ritz *et al.*, 2011, Singer, 2001, Singh and Thomson, 2010). In experimental models, GFR is usually estimated by quantifying the clearance (amount of fluid filtered by the kidney) of substances such as creatinine or radiolabelled inulin which are freely filtered by glomeruli but not reabsorbed by the tubules.

Inulin clearance is the 'gold standard' technique to determine GFR. Inulin is a small molecule, which is not bound to plasma proteins, is freely filtered by the glomerulus and not secreted or reabsorbed by the tubules and it is metabolised (Schwartz and Furth, 2007). Radioactive isotopes of inulin (carbon 14, ^{14}C or hydrogen 3, H^3) are often used in laboratory settings (Gaspari *et al.*, 1997).

1.5.4.3 RENAL PERFUSION

The kidney receives approximately 20% of the cardiac output which both supplies the kidney with oxygen and nutrients and is also filtered (Holechek, 2003). Blood flow to the kidney is kept relatively constant via autoregulation. This ability is primarily due to the tubuloglomerular

feedback (TGF) and myogenic response of the vasculature (Just, 2007). Focusing on the nephron and the afferent (arriving) arteriole, capillary loops and efferent (leaving) arterioles, under circumstances of poor perfusion, high blood volume or high plasma NaCl concentration, renal blood flow increases by the dilation of efferent arterioles. Conversely, during periods of greater perfusion, low blood volume or low NaCl concentration, the afferent arterioles constrict to increase blood blow through the capillary loops. Autoregulation of the kidney is tightly regulated within a very narrow physiological window.

The TGF process maintains kidney blood flow and GFR. The TGF regulates a negative feedback loop which monitors sodium concentration in the distal tubule of the kidney. An increase in tubular sodium concentration, detected by the macula densa, leads to a reduction in blood flow, thereby reducing GFR (Blantz *et al.*, 2007). The myogenic response describes the function of smooth muscle to respond to the force of stretching (Just, 2007). This response occurs in vascular tissue upon the rise in blood pressure. Therefore, the myogenic response within the kidney protects against high blood pressure. Under sustained changes to renal blood flow, the baseline of operation of autoregulation is reset (Hall *et al.*, 1990b, Iversen *et al.*, 1998, Iversen *et al.*, 1987, Wang *et al.*, 2012). This resetting phenomenon is driven by changes in sensitivity of the TGF to vasoactive substances such as angiotensin II and nitric oxide (Wang *et al.*, 2012). Likewise, extended activation of the TGF leads to a shifting of the TGF set point. The TGF is able to maintain normal blood flow to the kidney between 80-200mmHg and is therefore able to respond to spikes in blood pressure (Williamson *et al.*, 2008).

1.5.4.4 Renal Structure

While blood supply of the kidney influences GFR, so too does the structure. This is primarily driven by filtration surface area (formed by the capillary loops of the glomeruli). This was well illustrated in a study by Kaufman et al (1975) in which uninephrectomy of male rats lead to reduced GFR and RBF due to the 50% reduction in filtration surface area, even though the

remaining kidney underwent compensatory growth. Glomerular blood flow was increased to a greater extent than GFR leading to a reduced filtration fraction.

1.5.4.5 Importance of Kidney Function

The kidney acts primarily as a filter – removing waste products and maintaining electrolyte levels. Through the maintenance of electrolytes and consequently blood volume, the kidney influences the regulation of blood pressure (Hall *et al.*, 1996, Guyton, 1991a, Guyton, 1991b, Hall *et al.*, 1990a, Guyton *et al.*, 1988, Hall *et al.*, 1986). Abnormal kidney function can be detrimental to health, in particular its ability to clear toxic waste from the body and maintain cardiovascular function.

1.6 Conclusion

This introduction has consolidated the findings from numerous human and animal studies on the phenomenon of developmental programming and considered the implications for postnatal health. Over the last decade, findings from many studies have provided irrefutable evidence of the significant impact of maternal diet and behaviour on adult health and disease. However, there are certain aspects of experimental models and areas of studies that have been over-looked. In particular is the knowledge of *when* a nephron deficit occurs and *which* process or processes of kidney development is/are altered by a suboptimal intrauterine environment. This Introduction has outlined specific processes involved in kidney development that could be affected with some evidence from previous studies. The importance of kidney health has also been addressed. This thesis will investigate the impact of maternal low protein diet on the development of a nephron deficit and the postnatal consequences of a reduced nephron endowment.

Investigations into the effect of a suboptimal intrauterine environment have identified particular insults that induce this suboptimal intrauterine development and lead to poor adult

health. However, few studies have attempted to find an agent that may rescue the phenotype. That is, despite the insult being present, this agent protects the offspring from those deleterious effects. Landmark research has been carried out on the beneficial birth outcomes associated with maternal folate intake. This thesis will explore the possibility that folate/folic acid will ameliorate the negative birth outcomes associated with maternal protein deprivation.

Overall, this thesis will add to the mounting body of work that describes how important maternal health and early postnatal life are for the offspring and community. Identifying early risk factors for the development of adulthood diseases will afford early detection and treatment of individuals at risk and identify a path forward in improving public health through giving the next generation the best start to life medical science can offer.

Developmental programming is emerging as an important factor in the causation of many adult diseases including hypertension, end stage renal disease, type 2 diabetes and cardiovascular disease. It is becoming clear that these diseases cannot be solely caused by lifestyle or genetic factors, but rather a complex interaction between these factors. It is important to understand the effects of the maternal diet on the developmental period and consequently on adult health. Only then can we begin to develop improved screening strategies and optimised therapies for patients.

1.7 Aims and Hypotheses

1.7.1 Chapter 3 – Adult phenotype and cardiovascular profile in offspring exposed to maternal low protein diet

Aim: To determine the cardiovascular and renal physiology phenotype (BP & GFR) of male and female rat offspring exposed to a maternal low protein diet.

Hypotheses:

i) That maternal protein restriction would reduce postnatal growth.

ii) That maternal protein restriction would lead to a nephron deficit in postnatal life.

iii) That maternal protein restriction would not alter postnatal blood pressure.

iv) That maternal protein restriction would reduce postnatal glomerular filtration rate.

1.7.2 Chapter 4 – Consequences of a maternal low protein diet for the developing rat kidney

Aim: To determine when during kidney development a maternal LP diet results in a nephron deficit.

Hypotheses:

i) That maternal protein restriction would reduce fetal and placental growth.

ii) That maternal protein restriction would reduce ureteric branching morphogenesis and that this would be reflected in changes in kidney structure and gene expression.

1.7.3 CHAPTER 5 – MATERNAL FOLIC ACID INTAKE AND KIDNEY DEVELOPMENT

Aim: To determine whether increased levels of folic acid in the maternal diet can prevent the nephron deficit associated with a maternal low protein diet.

Hypotheses:

i) That maternal protein and folic acid restriction would lead to reduced fetal and placental growth, while supplementation with folic acid would ameliorate these effects.

ii) That maternal protein and folic acid restriction would lead to reduced branching morphogenesis in the developing kidney and this would be reflected in a nephron deficit and altered levels of gene expression, while supplementation with folic acid would ameliorate these effects.

iii) That exogenous folic acid exposure to kidneys exposed to a maternal protein and/or folic acid restricted background, would restore normal branching morphogenesis.

iv) That maternal protein and/or folic acid restriction would lead to changes in the methylation of genes specific for kidney development, while maternal supplementation of folic acid would ameliorate these effects.

1.7.4 CHAPTER 6 – THE EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON OFFSPRING CARDIOVASCULAR AND RENAL FUNCTION

Aim: To determine the adult cardiovascular phenotype (BP & GFR) of male and female offspring exposed to a maternal diet deficient in folic acid or supra-supplemented with folic acid.

Hypotheses:

i) That maternal protein and folic acid restriction would lead to reduced growth in postnatal life, while maternal supplementation with folic acid would restore normal growth.

ii) That maternal protein and folic acid restriction would reduce nephron number in postnatal life, while maternal supplementation with folic acid would restore nephron number.

iii) That maternal protein restriction and supplementation with folic acid would not alter adult blood pressure or GFR compared to protein restricted offspring.

CHAPTER TWO: GENERAL METHODS

2.1 Ethics Approval

Experiments were conducted in accordance with the National Health and Medical Research Council of Australia '*Australian Code of Practice for the Care and Use of Animals for Scientific Purposes'* (7th edition, 2004). Approval was granted in advance by the Monash University School of Biomedical Sciences Animal Ethics Committee.

2.2 Experimental design and protocol

Male (non-brother and 8 weeks of age) and female Sprague-Dawley (non-sister and 6-7 weeks of age) rats were obtained from the Animal Resources Centre, Western Australia or Monash Animal Services and housed under standard animal facility conditions (22-23°C, 30-33% humidity, 12-hour light/dark cycle). Water and food were available *ad libitum.* Female breeder rats were fed one of six experimental diets (Speciality Feeds, Glen Forrest, Western Australia) for 3 weeks prior to mating (for experimental overview refer to Figure 2.1):

- normal protein (NP 19.5% casein, 2mg/kg Folic Acid (FA))
- normal protein supplemented with folic acid (NP+FA, 19.4% casein, 200mg/kg FA)
- normal protein restricted folic acid (NP-FA 19.4% casein, <0.05mg/kg FA)
- low protein (LP 8.4% casein, 2mg/kg FA)
- low protein supplemented with folic acid (LP+FA 8.4% protein, 200mg/kg FA)
- or low protein restricted folic acid (LP-FA 8.4% protein, <0.05mg/kg FA)

The LP diet is based on a 50% reduction in total protein (as casein) and has previously been used by this research group to elicit alterations in offspring health, specifically on kidney structure and function (Hoppe *et al.*, 2007a, Hoppe *et al.*, 2007b). This high dose of FA (200mg/kg) approximates a human intake of 1200-1500 micrograms per day, when scaled for body size and metabolic rate. This figure is between 2 and 3 times the dose currently recommended for women who have a previous history of pregnancies affected by neural tube

defects (Obican *et al.*, 2010) but in line with other previous studies that provide 7000-10000micrograms of FA per day (Tolarova, 1982, Tolarova and Harris, 1995).

Dietary components are detailed in full in Tables 2.1, 2.2 and 2.3. Feeding the diets for 3 weeks prior to mating ensured that rats had overcome the initial stress of diet change and the completion of multiple oestrus cycles. Male breeders were maintained on a standard rodent chow containing 19.5% protein and 4.6% fat.

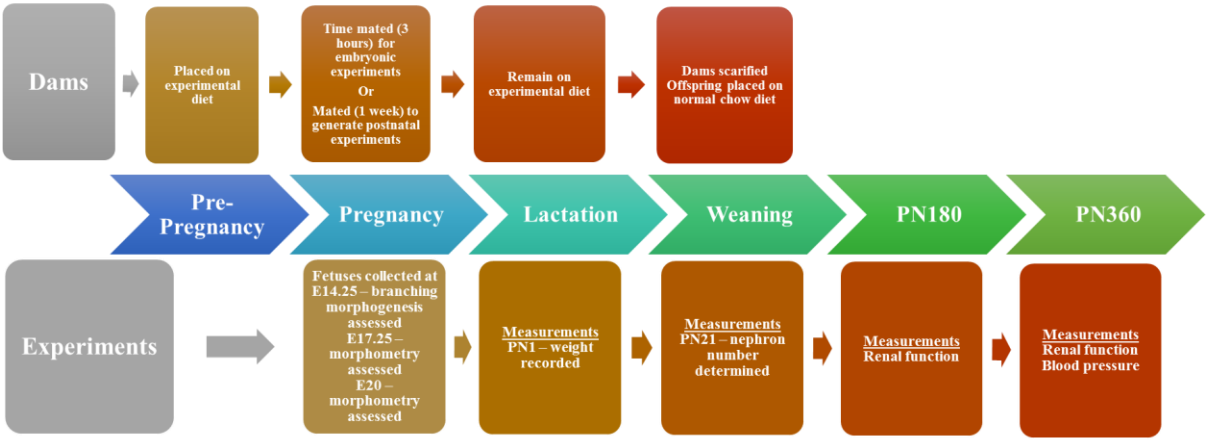

FIGURE 2.1 – EXPERIMENTAL OVERVIEW

Experimental overview covering the experimental diet exposure for dams and offspring, as well ages and stages of offspring experiments.

TABLE 2.1 - OVERVIEW OF NUTRITIONAL PARAMETERS OF THE EXPERIMENTAL DIETARY GROUPS

Nutritional Parameters	Diet					
Ingredients	NP	NP+FA	NP-FA	LP	LP+FA	**LP-FA**
Protein (%)	19.5	19.4	19.4	8.4	8.4	8.4
Total Fat (%)	7	7	7	7	7	7
Digestible Energy (MJ/Kg)	**16.1**	**16.3**	**16.1**	**15.5**	**15.6**	**15.6**

Protein and total fat percentage and energy (MJ/kg) of the control (NP) and experimental diets (NP+FA, NP-FA, LP, LP+FA, LP-FA)

TABLE 2.2 - CALCULATED NUTRITIONAL PARAMETERS OF THE EXPERIMENTAL DIETARY GROUPS

Nutritional Parameters	Diet					
Ingredients	**NP (g/kg)**	**NP+FA (g/kg)**	**NP-FA (g/kg)**	**LP (g/kg)**	**LP+FA (g/kg)**	**LP-FA (g/kg)**
Casein	200	200	200	87	87	87
DL Methionine	3.0	3.0	3.0	3.0	3.0	3.0
Sucrose	100	100	100	200	200	200
Wheat Starch	404	404	404	417	417	417
Dextrinised Starch	132	132	132	132	132	132
Cellulose	50	50	50	50	50	50
Canola Oil	70	70	70	70	70	70
Calcium Carbonate	13.1	13.1	13.1	13.1	13.1	13.1
Sodium Chloride	2.6	2.6	2.6	2.6	2.6	2.6
Potassium Citrate	2.5	2.5	2.5	2.5	2.5	2.5
Potassium Dihydrogen Phosphate	6.9	6.9	6.9	6.9	6.9	6.9
Potassium Sulphate	1.6	1.6	1.6	1.6	1.6	1.6
AIN93G Trace minerals	1.4	1.4	1.4	1.4	1.4	1.4
Choline Chloride (65%)	2.5	2.5	2.5	2.5	2.5	2.5
AIN93G Vitamins	10	10	10	10	10	10

Nutritional dietary content of the control (NP) and experimental diets (NP+FA, NP-FA, LP, LP+FA, LP-FA) expressed as g/Kg. Differences between the diets include casein (protein), folic acid and sucrose, and slight differences in Wheat Starch (LP, LP+FA, LP-FA). Levels of all other ingredients were the same.

TABLE 2.3 - VITAMIN COMPOSITION OF EXPERIMENTAL DIETS.

Vitamin Composition	Diet					
Ingredients	**NP**	**NP+FA**	**NP-FA**	**LP**	**LP+FA**	**LP-FA**
Vitamin A (Retinol)	4000 IU/Kg	4000 IU/Kg	4000 IU/Kg	4000 IU/Kg	4000 IU/Kg	4000 IU/Kg
Vitamin D3 (Cholecalciferol)	1000 IU/Kg	1000 IU/Kg	1000 IU/Kg	1000 IU/Kg	1000 IU/Kg	1000 IU/Kg
Vitamin E (Tocopherol acetate)	75 mg/Kg	78 mg/Kg	75 mg/Kg	75 mg/Kg	75 mg/Kg	75 mg/Kg
Vitamin K (Menadione)	1 mg/Kg	1 mg/Kg	1 mg/Kg	1 mg/Kg	1 mg/Kg	1 mg/Kg
Vitamin C (Ascorbic acid)	NA	NA	NA	NA	NA	NA
Vitamin B1 (Thiamine)	6 mg/Kg	6.1 mg/Kg	6 mg/Kg	6 mg/Kg	6 mg/Kg	6 mg/Kg
Vitamin B2 (Riboflavin)	6 mg/Kg	6.3 mg/kg	6 mg/kg	6 mg/Kg	6 mg/kg	6 mg/kg
Niacin (Nicotinic acid)	30 mg/Kg	30 mg/Kg	30 mg/Kg	30 mg/Kg	30 mg/Kg	30 mg/Kg
Vitamin B6 (Pyridoxine)	7 mg/Kg	7 mg/Kg	7 mg/Kg	7 mg/Kg	7 mg/Kg	7 mg/Kg
Pantothenic acid	16 mg/Kg	16.5 mg/Kg	16 mg/Kg	16 mg/Kg	16 mg/Kg	16 mg/Kg
Biotin	200 µg/Kg	200 µg/Kg	200 µg/Kg	200 µg/Kg	200 µg/Kg	200 µg/Kg
Folic Acid (FA)	2 mg/Kg	200 mg/Kg	<0.05 mg/Kg	2 mg/Kg	200 mg/Kg	<0.05 mg/Kg
Inositol	None added	None added	None added	None added	None added	None added
Vitamin B12 (Cyanocoblamin)	100 mg/Kg	103 mg/Kg	103 mg/Kg	100 mg/Kg	101 mg/Kg	101 mg/Kg
Choline	1600 mg/Kg	1470 mg/Kg	1670 mg/Kg	1600 mg/Kg	1600 mg/Kg	1600 mg/Kg

Vitamin composition of the control (NP) and experimental (NP+FA, NP-FA, LP, LP+FA, LP-FA) diets expressed as mg or µg/Kg. The most important difference in vitamin levels is the amount of FA in the diets. The LP and NP diets had 2mg/Kg, with the +FA dietary groups having 200mg/Kg, and the –FA dietary groups having <0.05mg/Kg. Other differences are choline and B12 amounts, although these were relatively constant across the diets.

2.2.1 BREEDING FOR FETAL EXPERIMENTS

Strict mating protocols were utilised to ensure that all animals were studied at a similar embryonic age. Embryonic weight is often used as an indicator of embryonic age but was not used in these studies given the possibility that maternal diet might alter embryonic growth and therefore weight. Unlike mice, where Theiler staging can identify the stage of embryonic development, it is not possible to accurately stage development in rats. While the number of somites can be used to stage rat embryos up until E13.5 (40-44 somites), after this age external features are used to stage the fetuses. Due to the subjective nature of assessing external features in fetuses, the mating window was tightly controlled to minimise variability in fetal development. Male breeders were placed with two female breeders for 3 hours which was defined as embryonic day (E) 0 of pregnancy. Rats were allowed to mate within a 3 hour time span on day "0" and then again on day "3". They were collected on day 17.25 which meant that if pregnancy occurred on day "0" the fetuses were 17.25 days old and if pregnancy occurred on the second mating, the fetuses would be 14.25 days old. Pregnant rats were anaesthetised with inhaled isoflurane (5% in air) and humanely killed (by exsanguination) at 14.25, 17.25 or 20 days post coitus (also referred to as E14.25, 17.25 and 20), and the fetuses collected and organs dissected. These ages were selected to correlate with stages of kidney development; early branching morphogenesis (E14.25), mid-branching morphogenesis and early nephrogenesis (E17.25) and mid-nephrogenesis and late gestation (E20) (Cullen-McEwen *et al.*, 2011, Cullen-McEwen *et al.*, 2005, Singh *et al.*, 2007). The experimental analyses performed at each fetal age are listed in Table 2.4.

TABLE 2.4 - FETAL TIME POINTS AND ANALYSES CONDUCTED.

	Time		
	E14.25	E17.25	E20
Analyses	Morphometry Metanephric culture Methylation analysis Gene expression	Morphometry Nephron number	Morphometry

Analyses conducted at each fetal age, including gene expression (E14.25), metanephric culture and methylation analysis (E14.25), nephron number (E17.25 – contribution by Honours student Mr Luke Eipper). Morphometry was performed at each age.

2.2.2 BREEDING FOR POSTNATAL EXPERIMENTS

Mating protocols for postnatal experiments were not subject to the stringent time requirements used for the embryonic studies. That is, progeny were required at postnatal ages calculated from the day of birth. As such, one male breeder was placed with one female breeder for a week before being separated. Female breeders were allowed to litter down and deliver naturally with the day after birth denoted as postnatal (PN) day 1.

2.2.2.1 OFFSPRING HUSBANDRY

Offspring were weighed on PN1 and then every 2-3 days until weaning at PN21. During this time offspring were exposed to the same diet as their respective dam. After weaning, offspring were transferred to a chow diet (Table 2.5) which was available *ad libitum*. Offspring were then weighed every 5 days until PN180 and then every 15 days until PN360.

TABLE 2.5 - OFFSPRING DIET SPECIFICATIONS

Ingredient	Amount
Protein	19.6%
Fat	4.6%
Crude Fibre	4.3%
Calcium	0.78%
Phosphorous	0.67%
Sodium Chloride	0.36%
Digestible Energy	14.8 MJ/Kg

Composition of the diet fed to postnatal animals after weaning.

2.2.2.2 Offspring Experimental Time-points

In litters that contained more than 12 pups, additional pups were humanely culled at PN1 to standardise milk availability. In litters of fewer than 12 pups, postnatal growth was assessed to ensure similar growth trajectory with litters of 12 pups. If a litter had less than 8 pups, the litter was not used for postnatal experimental time-points. Even ratios of male to female pups were maintained where possible. Table 2.6 shows the analyses conducted on postnatal animals at the three time-points.

Table 2.6 - Postnatal age at which analyses were conducted.

	Age		
	PN21	**PN180**	**PN360**
Analyses	DXA	DXA	DXA
	Nephron number	Renal Function	Renal Function
			Blood Pressure

Animals were anaesthetised (5% isoflurane in air) and scanned using DXA (Dual X-Ray Absorptiometry) scanning to determine body composition (body fat, lean muscle mass and bone mineral density). This was carried out at all three time-points (PN21, 180 & 360). Renal function experiments were carried at adult time-points PN180 & 360, while blood pressure was only measured at PN360. Nephron number was estimated at PN21, an age when nephrogenesis has finished.

2.3 Fetal Studies

At E14.25, 17.25 and 20, female breeders were euthanized and fetuses were removed and dissected to assess ureteric branching, nephron number and gene expression. Pregnant rats (E14.25, 17.25 or 20) were anaesthetised with inhaled Isoflurane (10 -15 minutes) (Lyppard, Keysborough, VIC, Australia). A midline incision was made and the skin and muscle were retracted. Blood (approximately 3ml) was collected from the inferior vena cava with a 19G needle. Blood was transferred to an EDTA containing tube (Vacuette 4ml K3EDTA Grenier Bio-One, Australia). Maternal blood was centrifuged (3000 x g; Jouan Centrifuge; Thermo Fischer Scientific, MA, USA) for 10 minutes at 4˚C. Plasma was aspirated and stored at -80ºC for subsequent analysis. The anaesthetised rat was euthanized by lethal injection of Pentobarbitone Sodium (100mg/kg with 25 gauge needle, i.p.). Fetuses were removed from the uterine horns and to ensure an RNAse free environment, were placed in a bath of Diethylpyrocarbonate (DEPC, 97% NMR Sigma-Aldrich, Australia) PBS (1:1000). Amniotic fluid was collected by aspirating the fluid from the amniotic sac with a 25G needle. Amniotic fluid was then immediately snap frozen on dry ice in a 1.7ml tube. Fetuses were detached from the placenta, weighed and killed by decapitation.

2.3.1 Study of Branching Morphogenesis by Metanephric Culture

After decapitation, fetuses were placed into a small petri dish which contained Sylgard-silicone elastomer (Dow Corning Corp, MI, USA), and immersed in PBS. Fetuses were then placed on their backs and pinned through the neck and base of the tail. With the use of an Olympus dissecting microscope, arms, legs and tail were gently pulled away from the body. After removing abdominal contents, mesonephroi were located and gently retracted to reveal the metanephric kidneys. Kidneys were aseptically removed and either snap frozen on dry ice in 1.7ml tubes for molecular analysis or assigned to the culture study. Kidneys assigned to the culture study were immediately placed into a petri dish containing serum-free Dulbecco's

modified Eagle's medium (DMEM): Ham's F12 liquid medium (Trace Biosciences, Castle Hill, NSW, Australia) supplemented with 5µg/ml transferrin (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia), 12.9µl/ml L-glutamine (Trace Biosciences, Castle Hill, NSW, Australia), penicillin (100µg/ml) and streptomycin (100U/ml), warmed to 37°C. After kidneys were removed, lower limbs were collected, snap frozen and stored for use in determining fetal sex (see Section 2.3.1.2) (Cullen-McEwen *et al.*, 2002, Singh *et al.*, 2007).

Whole fetal kidneys were placed on 3.0µm pore polycarbonate membranes (Transwell, Corning Star, Cambridge, MA, USA) and cultured in 24-well culture plates at the air-media interface of 500µl serum-free culture media for 48 hours at 5% CO_2 and 37°C. To avoid the confounding effects of a media supplemented with serum, serum-free media was used (Taub and Livingston, 1981).

2.3.1.1 KIDNEY CULTURE IN MEDIA SUPPLEMENTED WITH FOLIC ACID

A number of kidneys were cultured in the presence of FA (Sigma-Aldrich) supplemented media - see Table 2.7 for FA concentration and group allocation. Concentrations of 0.6 and 2mM of FA were selected based on a study by Wentzel and colleagues (Wentzel *et al.*, 2005), which reported beneficial effects (with regard to growth and the absence of embryonic defects) when embryos from diabetic rats were cultured in media supplemented with 0.25 to 2mmol/l FA.

TABLE 2.7 - MATERNAL DIETARY GROUPS AND THEIR RESPECTIVE CULTURE MEDIA GROUP.

	Diet					
Culture environment	**NP**	**NP+FA**	**NP-FA**	**LP**	**LP+FA**	**LP-FA**
	Control media (0mM FA)	Control media (0mM FA)	Control media (0mM FA)	Control media (0mM FA)	Control media (0mM FA)	Control media (0mM FA)
	0.6mM FA	0.6mM FA	0.6mM FA	0.6mM FA	0.6mM FA	0.6mM FA
	2mM FA	2mM FA	2mM FA	2mM FA	2mM FA	2mM FA

Kidneys from fetuses exposed to the experimental diets (NP+FA, NP-FA, LP, LP+FA and LP-FA) were cultured in two different FA concentrations (0.6 and 2mM). All dietary groups were also cultured in control media that did not contain any added FA. The maximum FA concentration was based on the beneficial effects seen in culture by Wentzel *et al.* (2005).

2.3.1.2 Immunohistochemistry for Quantification of Branching Morphogenesis

After 48 hours of organ culture, metanephroi were fixed in methanol at -20°C. For examination with whole-mount immunofluorescence microscopy, the cultured kidneys were permeabolised (0.1% Triton X-100, 15 minutes), washed twice in PBS, and then incubated (2 hours at 37°C) with the primary antibody (1:100 (0.1% Triton-X PBS) Monoclonal Anti-pan Cytokeratin, Sigma-Aldrich, Australia) (Cullen-McEwen *et al.*, 2002, Singh *et al.*, 2007). Kidneys were then washed in 0.1% Triton-X PBS then incubated (2 hours at 37^0C) with a secondary antibody (1:100 (0.1% Triton-X PBS) Alexa-Fluor 488 conjugated goat anti mouse, Invitrogen, Australia). Following this, kidneys were washed twice in PBS for 15 minutes, or until background staining was minimal. Organs were viewed using an Olympus Provis Fluorescent Microscope at 4x and 10x magnification and photomicrographs taken to assist in quantification of branching morphogenesis. Branching morphogenesis was quantified by manually skeletonising the ureteric tree (Singh *et al.*, 2007) (Figure 2.2). A branch point was defined as the intersection of three or more lines. Ureteric tips were defined as terminal branch ends (Figure 2.3).

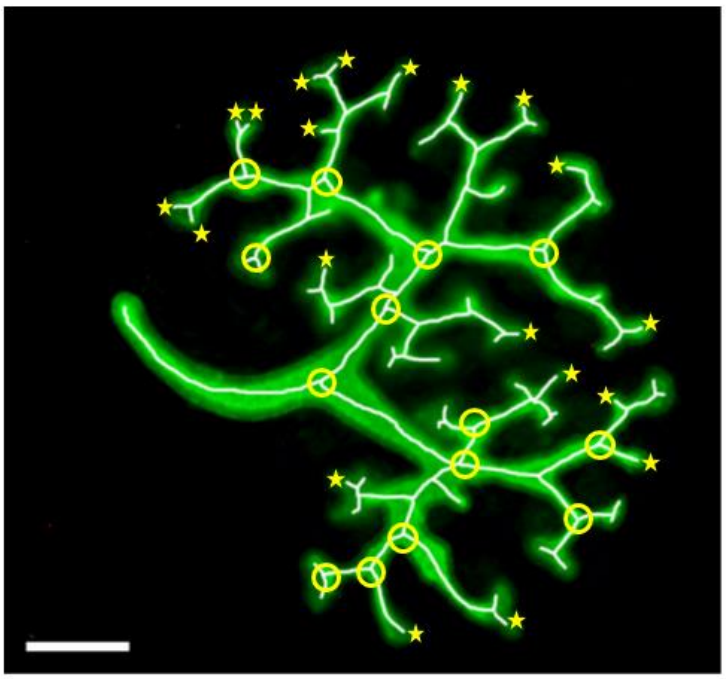

FIGURE 2.2 - SKELETONISED METANEPHROS FOR QUANTIFICATION OF BRANCHING MORPHOGENESIS.

Circles indicate branch points and stars indicate ureteric tips (NB: Not all branch points or ureteric tips are indicated). Scale bar represents 100µm.

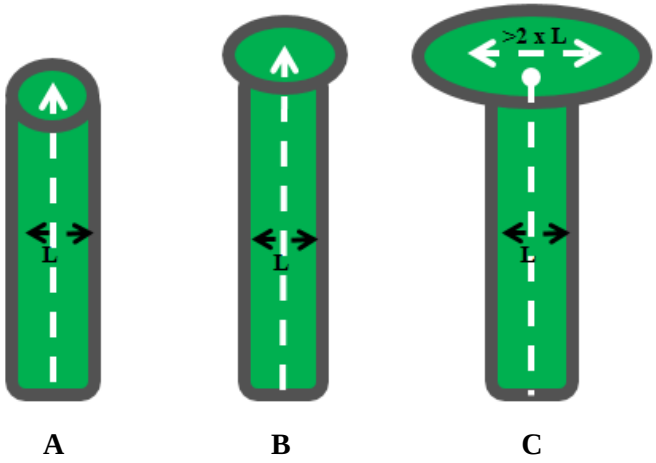

FIGURE 2.3 - DETERMINING BRANCH POINTS VERSUS URETERIC TIPS

To differentiate between a branch point (circle, **C**) and a ureteric tip (arrow head, **A**, **B**, **C**), the rule of 2 x L, where L is the diameter of the epithelial tube, is applied. **A**) denotes a ureteric tip with no thickening or indication of branching, and is therefore counted as a single ureteric tip. **B**) denotes a ureteric tip with some thickening; however this thickening is not greater than 2L and is not counted as a branch point and is therefore a ureteric tip. **C**), shows a branch point leading to two ureteric tips as this distance is greater than 2L.

2.3.1.3 Folic Acid concentration of amniotic fluid and maternal plasma

Folic acid concentration was determined at E14.25 from amniotic fluid and maternal plasma using a chemiluminescense technique. This was carried out commercially by Gribbles Pathology (Victoria, Australia). Amniotic fluid values represent two male and two female samples that were pooled to ensure enough volume (>250µl).

2.3.2 E17.25 Collection

Pregnant Sprague-Dawley rats were humanely killed and dissected as per Section 0. Fetuses were dissected in DEPC PBS. Kidneys collected for stereological analysis were dissected out in ice cold PBS and placed in 4% paraformaldehyde (PFA) (Sigma Aldrich, Castle Hill Australia) for 60 minutes. Tissue was then washed and stored in 70% ethanol at 4ºC.

2.3.3 E20 Collection

Pregnant Sprague-Dawley rats were humanely killed and dissected as per Section 0. Fetal kidneys, adrenal glands, liver, pancreas and heart were removed for future experiments. Fetal and placental weights are reported in this thesis. Sex determination was performed visually (presence of ovaries or testes).

2.3.4 Molecular Techniques

2.3.4.1 DNA extraction and amplification for sex determination

The sex of fetuses at E14.25 was determined by genotyping for the presence of SMC (structural maintenance of chromosomes) using Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, Castle Hill, Australia). Fetal limbs were thawed at room temperature in 1.7ml Eppendorf tubes. Tissue Preparation (100µl per sample) and Extraction Solution (25µl per sample) were added and samples were incubated at room temperature for 10 minutes and then at 95˚C for 3 minutes. This tissue extract was used for PCR amplification (see Table 2.7). A PCR reaction was performed to amplify the SMCX/Y gene (SMCX/Y, Forward and Reverse, Sigma-Genesys,

Australia) (Table 2.8). Samples were run on a 2.5% agarose gel containing GelRed Nucleic Acid Stain (4µl, Biotium, Australia) for 45-60 minutes at 90V. Using gel electrophoresis, it was possible to identify the sex of fetuses from the SMCX/Y gene. The SMCX/Y gene is present on both X and Y chromosome and escapes X inactivation, however the biological role of the gene is unknown (Agulnik *et al.*, 1999). A male fetus displays two SMC gene bands (one from the X chromosome and one from the Y), while one band signifies a female fetus (one band from the two X chromosomes; Figure 2.4). SMC was chosen as a marker of sex over SRY because probing for SRY is prone to false negative results. For example if there was a technical problem with the PCR amplification in a male sample then no SRY band would be present and the animal would be sexed as female with no possibility of realising that the PCR failed.

TABLE 2.7 - SMCX/Y PCR REAGENTS AND VOLUMES.

Reagent	Volume
Water, PCR grade	4.86µl
Red Extract N-Amp PCR reaction mix	10µl
SMC-XY Forward Primer	0.57µl
SMC-XY Reverse Primer	0.57µl
Tissue Extract	4µl
Total Volume	20µl

TABLE 2.8 - SMCX/Y FORWARD AND REVERSE PRIMER SEQUENCE.

Primer	Sequence
SMC XY Forward	5’ TGA AGC TTT TGG CTT TGA G
SMC XY Reverse	5’ CCA CTG CCA AAT TCT TTG G

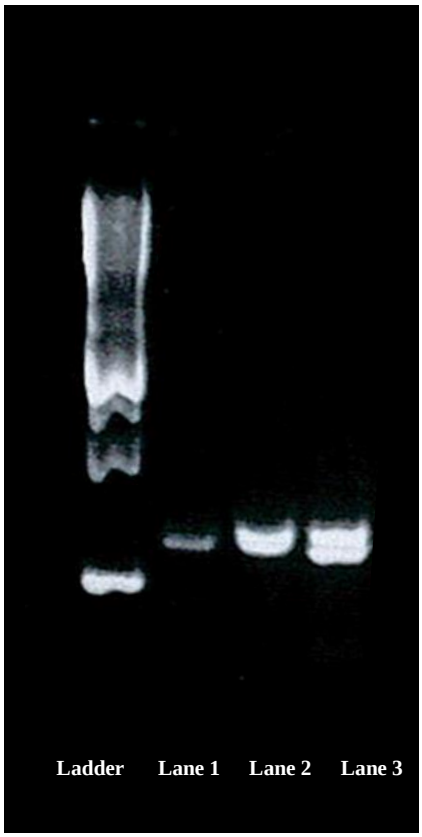

FIGURE 2.4 - GEL ELECTROPHORESIS OF SMCX/Y

Lane 1 is a genomic ladder, and those with one band (Lane 1) depict female samples, and those with two bands represent males (Lanes 2 and 3).

2.3.4.2 - FETAL KIDNEY GENE EXPRESSION

2.3.4.2.1 RNA AND DNA EXTRACTION FROM E14.25 KIDNEYS

RNA and DNA were extracted from both kidneys at E14.25 using a commercially available kit (AllPrep DNA/RNA Mini Kit, Qiagen, Valencia, CA) using the manufacturer's recommended protocol. RNA concentration and quality were determined using a NanoDrop spectrophotometer (ThermoScientific, Australia). The ratio of absorbance at 260/280nm indicated the quality of the sample. For RNA, a ratio between 1.9 and 2.2 was considered sufficient and used in gene expression (Real Time PCR) studies (Mathieson and Thomas, 2012). For DNA, a ratio between 1.6 and 1.9 was considered sufficient and used in bisulphite sequencing experiments (Wang *et al.*, 2011b).

2.3.4.2.2 RNA REVERSE TRANSCRIPTION AND CDNA PRODUCTION AND REAL TIME PCR

A 0.05µg RNA (from a minimum of two male and female kidneys per litter) sample was reverse transcribed (Applied Biosystems Reverse Transcription Reagent Kit, Applied Biosystems, Australia, see Table 2.9). The sample was then run in a thermocycler to produce cDNA (Superscript VILO cDNA synthesis kit, Life Sciences, Australia, Table 2.10).

For Real-Time PCR (RT PCR) SYBR Green was used following the manufacturer's protocol (SensiMix SYBR & Fluorescein Kit, Quantace, Australia). This assay was used to determine relative levels of mRNA expression of genes involved in branching morphogenesis (*Gdnf*, *Gfrα1*, *Bmp4*), nephrogenesis (*Wnt4*, *Pax2*, *Hnf4α*) and maintenance of DNA methylation marks (*Dnmt1*). Genes were compared to 18S as the housekeeper gene. 18S was stabley expressed across all dietary groups for male (Figure 2.5) and females (Figure 2.6) . Tables 2.11 and 2.12 show volumes and conditions required for RT-PCR.

TABLE 2.9 - SUPERSCRIPT VILO CDNA SYNTHESIS KIT.

Reagent	Reaction (x1)
5X VILO™ Reaction Mix	4 µl
10X SuperScript® Enzyme Mix	2 µl
RNA Sample (0.05 µg)	x µl
DEPC-treated water	Up to 20 µl
Total Reaction Volume	20 µl

Reagents and volumes required for reverse transcription of RNA samples, where x denotes the required amount of RNA sample to achieve 0.05µg.

TABLE 2.10 – CDNA PCR SYNTHESIS

Temperature	Time
25°C	10 minutes
42°C	120 minutes
85°C	5 minutes
4°C	Infinity

PCR cycle temperature and time required to produce cDNA from RNA samples.

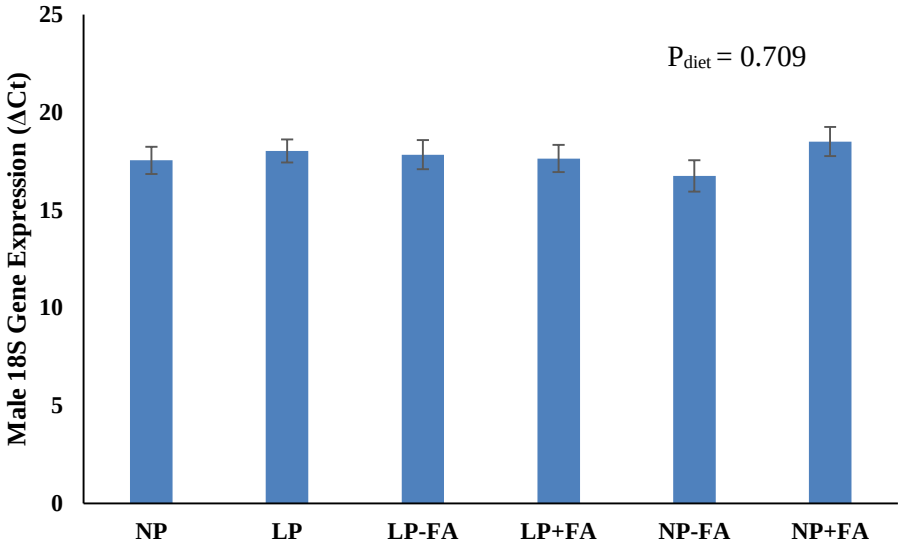

FIGURE 2.5 – MALE 18S GENE EXPRESSION

Male 18S gene expression in E14.25 kidneys after maternal exposure to control (NP) or experimental diets (LP, LP-FA, LP+FA, NP-FA or NP+FA). Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (n=7-15).

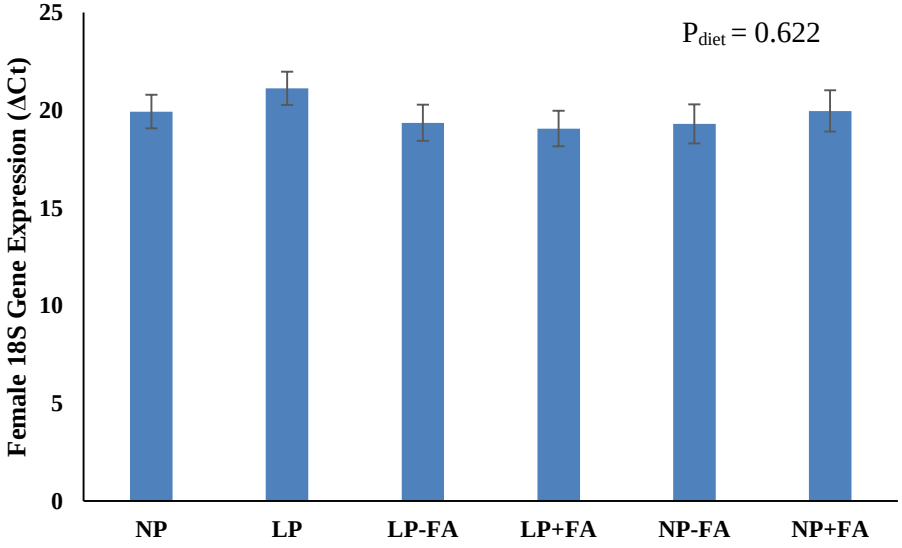

FIGURE 2.6 – FEMALE 18S GENE EXPRESSION

Female 18S gene expression in E14.25 kidneys after maternal exposure to control (NP) or experimental diets (LP, LP-FA, LP+FA, NP-FA or NP+FA). Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (n=7-15).

TABLE 2.11 - RT-PCR VOLUMES.

Reagents	Volume µl (x1)
SensiMix SYBR & Fluorescein	12.5
Water	10.5
cDNA Sample	1
Gene-Specific Primer	1
Total volume	25

TABLE 2.12 - RT-PCR CONDITIONS.

Cycles	Duration	Temperature
1	10 minutes	95°C
40	15 seconds	95°C
	1 minute	60°C

A comparative C_T (cycle of threshold fluorescence) method was used with ribosomal 18S used as an endogenous reference gene. To calculate the relative expression levels in each sample, the C_T value for 18S was subtracted from the C_T value of the gene of interest to give a ΔC_T value (De Medici *et al.*, 2003, Kuchipudi *et al.*, 2012).

2.3.4.3 GENE METHYLATION ANALYSIS

Where real time PCR analysis indicated alterations in gene transcription, the methylation status of the promotor region of these genes was analysed.

2.3.4.3.1 PRIMER DESIGN FOR BISULPHITE SEQUENCING

Using UCSC Genome Browser (http://genome.ucsc.edu/) the DNA sequence of the gene of interest was retrieved (including an extra 1500 bp upstream of the transcription start site as annotated by RefSeq). Methylation-specific primers were designed on the sequence obtained with MethPrimer (http:///www.urogene.org//methprimer/).

Criteria used for amplicon selection include: number of CpGs covered, distance to transcription start site, suggested transcription factor binding sites, and absence of SNPs in primers (Li, 2007a). Primer length was limited to between 20 and 27 bases, optimal amplicon length to 300 to 400 bases. The maximum number of the same nucleotide in a stretch was restricted to four. Methylation specific primers (MSP) were used where there was a SNP in the primer. This

ensured the ability to differentiate between the two primer pairs (ie. one targets the sequence if the C is methylated and remains a C, the other one targets the unmethylated C which is a T). One primer pair specific for methylated cytosine in a CpG dinucleotide in the primer sequence and one primer pair for unmethylated cytosine in the same CpG dinucleotide in the primer sequence were selected for each target gene.

2.3.4.3.2 METHYLATION SPECIFIC PCR

Samples that contained more than 200ng of DNA were bisulfite-converted and concentrated to >40ng/µl by speedvac. DNA was bisulfite-converted using an Epigentek (New York, USA) kit following manufacturer's protocol. Real-time PCR was performed on 20ng bisulfite-converted DNA in duplicate with SYBR green technology in a BioRad iQ5 real-time cycler. The PCR program consisted of three steps: 1) 95°C for 10 min; 2) 40 repeats of 95°C for 30 seconds, 56°C for 40 seconds and 72°C for 60 seconds; and 3) a dissociation curve starting from 56°C with 1°C increments and hold times of 15 seconds. The genes targeted were *Wnt4* in males and *Gfra1* and *Bmp4* in females (Table 2.13). Ratios of amplification of methylated to unmethylated primer products were calculated and compared between the diet groups.

TABLE 2.13 – METHYLATION PRIMERS.

	Bisulphite Sequencing (BS) or Methylation Specific Primer (MSP)	Methylated (M) or Unmethylated (U)	Primer
WNT4	MSP	M	F: GGGATATTTTGGCGGTATTATC
	MSP	M	R: GACCCAACTACTAAATCCCCG
	MSP	U	F: GGGGATATTTTGGTGGTATTATTG
	MSP	U	R: AACCCAACTACTAAATCCCCAC
BMP4	BS		F: GAATTTGGTTAGATGTATTGTATTG
	BS		R: CTATTTTTCTTCCAACCCCTAAAA
	MSP	M	F: TTTCGGTTGTATTTAAGTCGTGTC
	MSP	M	R: GCTAACGAAATTCTCCGTCG
	MSP	U	F: TTTTGGTTGTATTTAAGTTGTGTTG
	MSP	U	R: ACCACTAACAAAATTCTCCATCATA
GFRα1	BS		F: GGGAGGTTTTAGTATTTTGGGTTT
	BS		R: TCCTCTAACCACTCAAAATTCAACT
	MSP	M	F: TTGTTTGGGAAAAGAGGTTAGTTC
	MSP	M	R: CGAAATCGAAACCTTAACGAC
	MSP	U	F: ATTGTTTGGGAAAAGAGGTTAGTTT
	MSP	U	R: CTACCCAAAATCAAAACCTTAACAA

Primers for WNT4, BMP4 and GFRα1. Including primers for bisulphite sequencing, and detecting methylation specific primers that are either methylated (M) or unmethylated (U).

2.3.5 ESTIMATION OF NEPHRON NUMBER IN DEVELOPING KIDNEYS

The gold-standard technique for determining nephron number utilises the disector/fractionator principle and allows for unbiased determination of nephron endowment (Bertram, 1995). This technique has been modified for unbiased counting of glomeruli in developing kidneys but can also be used for determination of nephron number in developed kidneys (Cullen-McEwen *et al.*, 2011).

In the present study, E17.25 kidneys were fixed in 10% neutral buffered formalin, processed to paraffin and exhaustively sectioned at 5µm. Beginning with a random start (n), a minimum of 10 section pairs (n and n+2) were selected for counting nephrons. Sections were histochemically stained with the lectin Peanut Agglutinin (PNA) to identify podocytes which enabled the identification of nephrons from the early S-shaped body stage to the fully developed glomerulus. Sections were de-waxed and rehydrated, and then endogenous peroxidase activity was quenched by immersing sections for 10 minutes in 2% H_2O_2 in

methanol. Sections were then incubated for 30 minutes at 37°C with neuraminidase (0.1 units/ml 1% $CaCl_2$ in PBS) from *vibrio cholerea* (Sigma-Aldrich, Australia) to expose sugar residues in order for the lectin to bind. Sections were blocked for non-specific binding (2% BSA, 0.3% triton in PBS for 30 minutes) then incubated for 2 hours with 20µg/ml biotinylated PNA (Sigma-Aldrich, Australia) diluted in 0.3% triton in PBS, with 1mM $CaCl_2$/$MnCl_2$/$MgCl_2$ then washed in PBS. Biotinylated PNA was visualized (*Elite* streptavidin/biotin amplification ABC kit, Vector Laboratories, USA) and the reaction developed with diaminobenzidine (DAB, Sigma-Aldrich, Australia) and 0.01% H_2O_2 in PBS. Sections were counterstained with haematoxylin.

Total nephron number was estimated using the equation:

$$N_{glom} = SSF * \frac{1}{2} * \frac{1}{2} * Q^{-}$$

Where:

N_{glom} is the total number of PNA-positive developing nephrons in the kidney

SSF is the reciprocal of the section sampling fraction (the number of sections advanced between section pairs)

The fractions (½) account for PNA-positive structures that were counted in both directions between the two sections of a pair, and disector pair of sections consisted of the n and the n+2 sections, and Q^- is the actual number of PNA-positive structures appearing and disappearing between the reference and lookup sections in the disector.

Developing nephron number was quantified by myself and Honours student Mr Luke Eipper.

2.4 POSTNATAL EXPERIMENTS

2.4.1 ESTIMATING TOTAL NEPHRON NUMBER IN ADULT KIDNEYS

Nephron number determination was carried out in kidneys of PN21 offspring. Offspring were anaesthetised with isofluorane (2ml evaporated) and given an overdose of pentobarbital sodium (Lethabarb, 4ml/kg body weight, intraperiotoneal). An abdominal incision was made, and the skin abdominal muscle and peritoneum retracted. Kidneys were located, removed and decapsulated. The right kidney was snap frozen in liquid nitrogen for future experiments. The left kidney was fixed in 4% PFA. The technique (Cullen-McEwen *et al.*, 2011) used was based on the gold standard technique of the physical disector method (Bertram, 1995) and is used in favour of other techniques (such as acid-maceration (Larsson *et al.*, 1980)) as it is the most unbiased method to determine nephron number. Following fixation, kidneys were transferred to 70% ethanol, processed to paraffin, and exhaustively sectioned at 5µm. Beginning with a random start (n), every 50^{th} and 52^{nd} section pairs were collected. Collecting every 50^{th} section pair allowed for the whole kidney to be sampled, ensuring that there were a sufficient number of sections pairs (approximately 10 per kidney) to be counted. Sections were histochemically stained with the lectin Peanut Agglutinin (PNA) to identify podocytes which enabled the identification of nephrons from the early S-shaped body stage to the fully developed glomerulus (Figure 2.7).

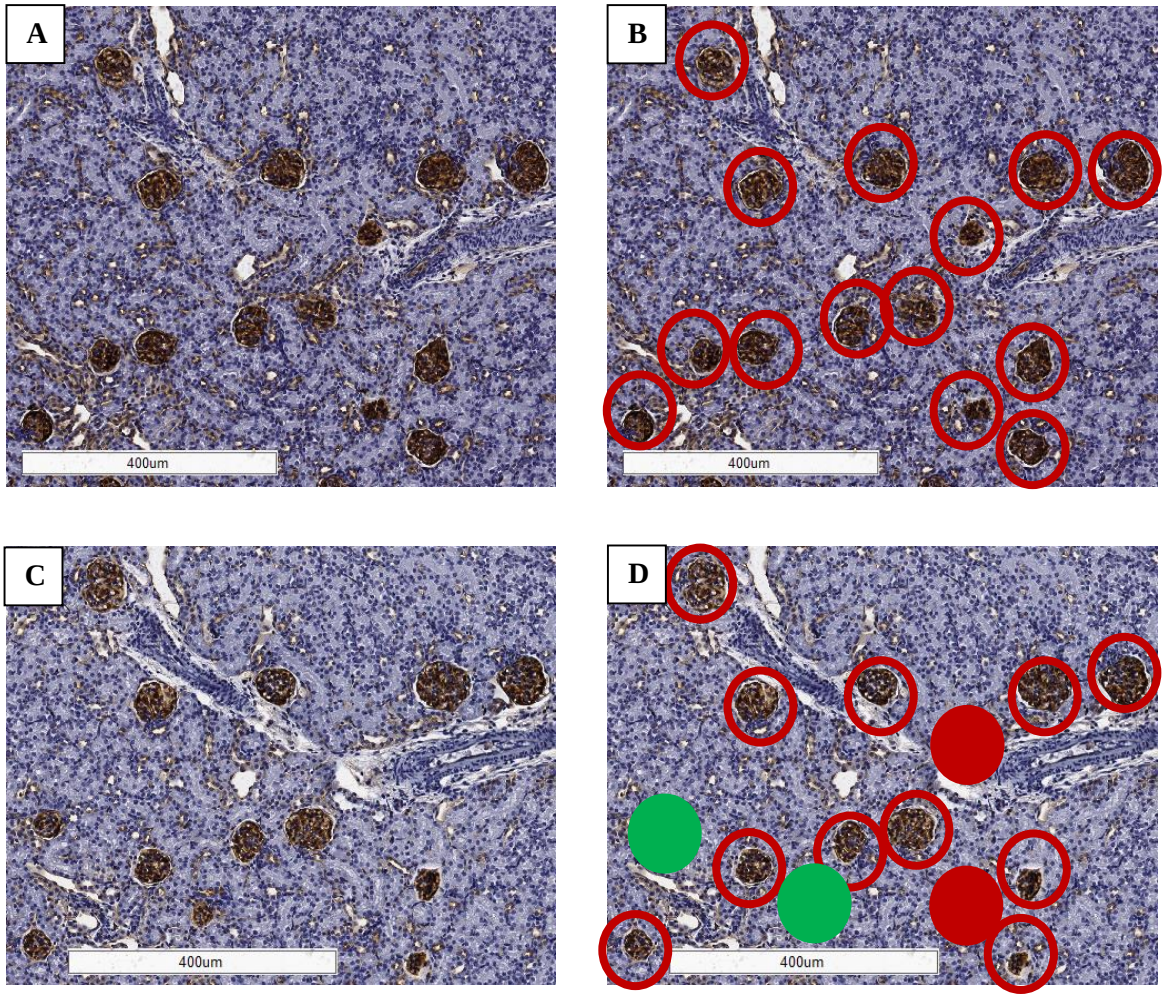

FIGURE 2.7 – ESTIMATING THE NUMBER OF NEPHRONS USING THE PHYSICAL DISECTOR TECHNIQUE.

A) Photomicrograph of a PN21 rat kidney section (nth) stained with peanut agglutinin (PNA) identifying PNA-positive podocytes. **B**) All nephrons with PNA-positive podocytes are marked with open circles. **C**) PNA-stained PN21 rat kidney section (nth +2) stained with PNA. **D**) Glomeruli marked on the nth section are overlayed on the nth+2 section. Glomeruli not visible in the nth+2 section (disappearing glomeruli) are marked by enclosing the red circle (solid red circles). Glomeruli present in the nth+2 section not present in the nth section (appearing glomeruli) are marked by solid green circles. Both appearing and disappearing glomeruli are counted according to the disector principle (Q^-).

2.4.2 RENAL FUNCTION STUDIES

2.4.2.1 GLOMERULAR FILTRATION RATE AND ESTIMATED RENAL PLASMA FLOW

Renal function studies were conducted at PN180 and PN360 for both male and female offspring. Offspring were injected with a long acting anaesthetic (Inactin; thiobutabarbital sodium, Sigma-Aldrich Co, St Louis, USA, intraperiotoneal) at a dose of 150mg/kg in saline (0.9% NaCl; Baxter Healthcare Pty Ltd, NSW, Australia). This anaesthetic was chosen as it has no observable influence on renal function, particularly renal clearance (Walter *et al.*, 1989). The rat was then placed on a heated operating table to maintain body temperature throughout surgery.

Surgery began once full anaesthesia was induced and this was confirmed through the abolition of pedal reflexes. A rectal probe (Cole-Palmer Instrument Company, Chicago, USA) was used during the surgery to monitor body temperature. This was maintained at 37-39°C.

The neck region was shaved and a local anaesthetic (2ml bolus 1% Lignocaine; Xylocaine; AstraZeneca, NSW, Australia) was injected subcutaneously and a midline incision was made. Skin and muscle were retracted and blunt dissection was used to locate the trachea. An incision, one-third the diameter of the trachea was made and a catheter (5cm, 110 polyvinylchloride (PVC) tubing, Microtube Extrusions, NSW, Australia) was inserted to allow unobstructed ventilation. Animals were provided with oxygen (100% low flow, BOC, Australia) during surgery to prevent hypoxia. Blunt dissection was used to expose the right jugular vein (Lim *et al.*, 2011). Ligatures were placed around the jugular vein above and below the site at which a catheter (SV45, Critchely Electrical Products, NSW, Australia) was to be inserted. A small (1mm) incision was made in the jugular vein between the ligatures and the catheter was inserted approximately 2cm toward the heart. The ligatures were then tied off to secure the catheter in place. To replace and maintain fluid balance during surgery, 2% bovine serum albumin in saline (BSA, Sigma-Aldrich Co, St Louis, USA) was infused (1 ml/hr/100g).

Mean arterial pressure (MAP) and heart rate (HR) were measured in unconscious rats throughout the experimental period using a carotid artery catheter (Lim *et al.*, 2011). Blunt dissection was used to expose the left carotid artery. Ligatures (Dysilk 5/0, Dynek Pty Ltd, SA, Australia) and a surgical clamp were placed around the artery above and below the site at which a catheter was to be inserted. The catheter was constructed using SV45 tubing (Critchley Electrical Products, NSW, Australia) with a Teflon tip (812e; Atlantic Tubing Company). A small incision in the artery allowed the insertion of the catheter. The catheter was inserted approximately 1.5 cm toward the heart and secured using ligatures. To ensure proper insertion and to prevent blockages, the catheter was flushed with heparinised saline (100IU/ml). The carotid artery catheter was then connected to a pressure transducer (Cobe Arvada, USA). This analogue signal was amplified (Grass Model 7 polygraphy, Quincy, MA, USA) and the signal relayed to a PC running specialised data acquisition software (Universal Acquisition, University of Auckland, NZ).

An injection of local anaesthetic (1% Xylocaine) was administered subcutaneously into the abdominal region. The area was shaved and a midline incision was made. The skin and muscle were retracted. To prevent tissue damage, blunt dissection was used to locate the bladder. A clamp was used to hold the bladder outside the abdomen. A small (1mm) incision was made in the bladder wall and a bladder catheter (SV10) was inserted. To hold this in place, a ligature (5/0 Dysilk) was then tied around the bladder.

To determine renal function, radiolabelled para-aminohippuric acid (0.5μCi/hr ^{14}C-PAH, Perkin Elmer, Boston, MA) and inulin (1μCi/hr ^{3}H-inulin, Perkin-Elmer Life Sciences, Victoria, Australia) were infused (0.4ml/hr/100g of body weight) via the right jugular vein for 100 minutes (Figure 2.8). The first 60 minutes of PAH/Inulin infusion was allowed for the animal to stabilize after surgery and reach equilibration in the plasma. Radioactive inulin and

PAH have been found to provide the most dependable renal clearance data, when compared to other options such as creatinine (Toto, 1995). Clearance measurements began following the 1 hour PAH/Inulin infusion. Urine produced by left and right kidneys was collected for 40 minutes in pre-weighed tubes.

FIGURE 2.8 - RENAL FUNCTION SURGERY.

Tracheal catheter is placed inside a polyethylene tube connected to an oxygen cylinder. Jugular catheter is connected to infusion pumps for infusion of 2% BSA and the radioactive substances (PAH and tritiated inulin). Bladder catheter is sutured into place and allowed to drain freely into a collection tube (1.7ml Eppendorf tube).

At the end of the 40 minutes, blood (approximately 2ml) was collected from the carotid artery catheter into a heparinised tube. A small sample of this blood was collected into heparinised micro hematocrit tubes (Drummond Scientific Co, Broomall, PA) and centrifuged (2.5 min, 3000g) to allow separation of plasma from erythrocytes. Hematocrit was then determined by calculating the ratio of plasma to red blood cells (Microhematocrit Reader, Hawksley & Sons Ltd, England). The remainder of the blood was centrifuged (3000g; Jouan Centrifuge; Thermo

Fischer Scientific, MA, USA) for 10 minutes and plasma was aspirated and stored at -20ºC prior to subsequent analysis.

To determine urine and plasma levels of ^{14}C-PAH and ^{3}H-inulin, 20µl aliquots of plasma were placed in vials with 2ml scintillation fluid in triplicate. Samples were first allowed to stabilise in the dark for 24 hours. To determine the levels of radioactive tracer, these samples were placed in a scintillation counter (Beckman LS6000TA, Beckman Coulter, USA). Each sample was counted for 10 minutes and the disintegrations per minute (DPM) from each triplicate was averaged. Glomerular Filtration Rate (GFR) and Effective Renal Plasma Flow (ERPF) were then calculated as the clearance of ^{3}H-inulin and ^{14}C-PAH respectively, where:

$$\text{GFR} = \frac{[\text{dpm of Inulin}]\ \text{urine} \times \text{volume (ml)}}{[\text{dpm of Inulin}]\ \text{plasma} \times \text{collection time (mins)}}$$

$$\text{ERPF} = \frac{[\text{dpm of PAH}]\ \text{urine} \times \text{UFR}}{[\text{dpm of PAH}]\ \text{plasma}}$$

$$\text{UFR} = \frac{\text{urine volume (ml)}}{\text{collection time (mins)}}$$

$$\text{Filtration Fraction} = \frac{GFR}{ERBF}$$

$$\text{ERBF} = ERPF \times \frac{1}{1 - \%\, Hematocrit}$$

2.4.2.2 OSMOLALITY

Osmolality of urine and plasma was measured by freezing point depression using an osmometer (Advanced Osmometer 2020; Advanced Instruments, Needham Heights, MA). The osmometer was standardised prior to each run using solutions provided by the manufacturer (100, 900 and 1500mOsm/kg standards). 20µl of each sample was loaded into 1ml tubes to determine osmolality.

2.4.2.3 ELECTROLYTES

Concentrations of Na^+, Cl^- and K^+ (mmol/l) were measured using RapidChem 744 Electrolyte Analyser (Bayer Australia Limited, Australia). Plasma analysis required 55µl of each sample. Urine analysis required a 1:10 dilution of the sample to make a 300µl solution.

2.4.3 MEAN ARTERIAL PRESSURE AND HEART RATE SURGERY

2.4.3.1 SYSTEM SETUP AND PROBE CALIBRATION

Blood pressure, heart rate and locomotor activity were measured using a radiotelemetry system (DataSciences Incorporated, Minnesota, USA).

Data were acquired by interfacing the telemetry setup with a digital acquisition system (Labview). The computer system and program (LabView) was established and set up in coordination with Professor Geoff Head (BakerIDI Heart and Diabetes Institute), Elena Lukoshkova (University of Moscow) and James Armitage (Monash University). The radiotelemetry probe implanted into the aorta transmits on the AM band and this signal is received by a plate at the base of the cage and converted to a voltage signal. An inline analogue pressure adaptor (DSI, Minnesota, USA) was used to allow calibration of the probe prior to insertion into the animal (see below) and this voltage is digitised and stored on the PC (Labview software, National Instruments, Texas, USA). Recording of ambient pressure allows for fluctuations in atmospheric pressure over time to be accounted for when quantifying blood pressure (Kramer *et al.*, 2000, Mills *et al.*, 2000).

Probe calibration was followed according to manufacturer's recommendations. Calibration was confirmed prior to surgery by placing the probe in a sterile vessel and exerting pressures of 0, 100 and 200mmHg. The voltage pressure relationship was determined and a 3 point calibration curve generated. This calibration was confirmed by placing the probe under known pressures between 50-150mmHg and confirming that the pressure shown by the probe matched the

known pressure to within ±2mmHg. Pressure drift in the 2 week period that probes were implanted is less than 1 mmHg according to the manufacturer.

Prior to surgery, rat body weight was recorded. Room temperature was kept between 22-24°C, with humidity ranging between 25-35%. Anaesthesia was induced by placing the rat in an induction box with the gaseous anaesthetic isoflurane (5% v/v isoflurane plus oxygen; Rhodia Australia P/L, Notting Hill, Australia). The anaesthetised rat was taken from the box and placed on a heating pad at 36-37°C to maintain body temperature during surgery. The rat received anaesthetic continuously through a nose cone (2-3% v/v maintenance dose isoflurane plus oxygen 1-2ml/hr) throughout the surgery. When a surgical level of anaesthesia had been reached, as confirmed by abolition of the pedal reflex, surgery commenced. Depth of anaesthesia was regularly monitored throughout the operation.

The abdomen was shaved and a midline incision was made. The skin and muscle were retracted and blunt dissection was used to expose the abdominal aorta below the renal arteries. Arterial clips (Micro serrefin 15g clamping force, FineScience Tools, California, USA) were placed on the aorta above and below the site at which the telemetry probe was to be inserted. With flow occluded in the aorta, a small incision was made in the aorta using a 23G needle and the telemetry probe (TA11-PAC40; Data Sciences International, St Paul, MN, USA) inserted approximately 2cm into the aorta. Commercially available cyanoacyrlate adhesive and mesh fabric were used to seal the aorta and hold the probe-catheter firmly in place. The battery of the probe was placed in the abdomen and anchored to the abdominal wall with the use of non-absorbable suture (Dysilk 4/0, Dynek Pty Ltd, SA, Australia). The wound was then closed with an absorbable suture (Dexon III, Covidien, Ireland) for the muscle wall and skin. At completion of surgery the animals were administered fluid (7ml saline s.c.), an analgesic (0.4ml carprofen i.m., Rimadyl) and an antibiotic (0.3ml Tribactral, i.p.) to aid recovery. The surgery took

approximately 30 minutes to perform. The rats were kept in a heated box until fully recovered from anaesthesia then returned to their home cage and continuous measurement of blood pressure began one week following surgery, in freely moving animals.

2.4.4 MEASUREMENT OF BLOOD PRESSURE AND DATA COLLECTION

Systolic and diastolic blood pressure and heart rate and locomotor activities (amount the animal moves around the cage) were recorded continuously in unrestrained rats via a telemetry system for a minimum of 5 days (Figure 2.9). The signal was relayed to an IBM compatible computer, equipped with an analogue digital converter. The sampling rate was set at 2 seconds. These measurements were pooled to obtain 12 hourly (day and night) average readings over the experimental period.

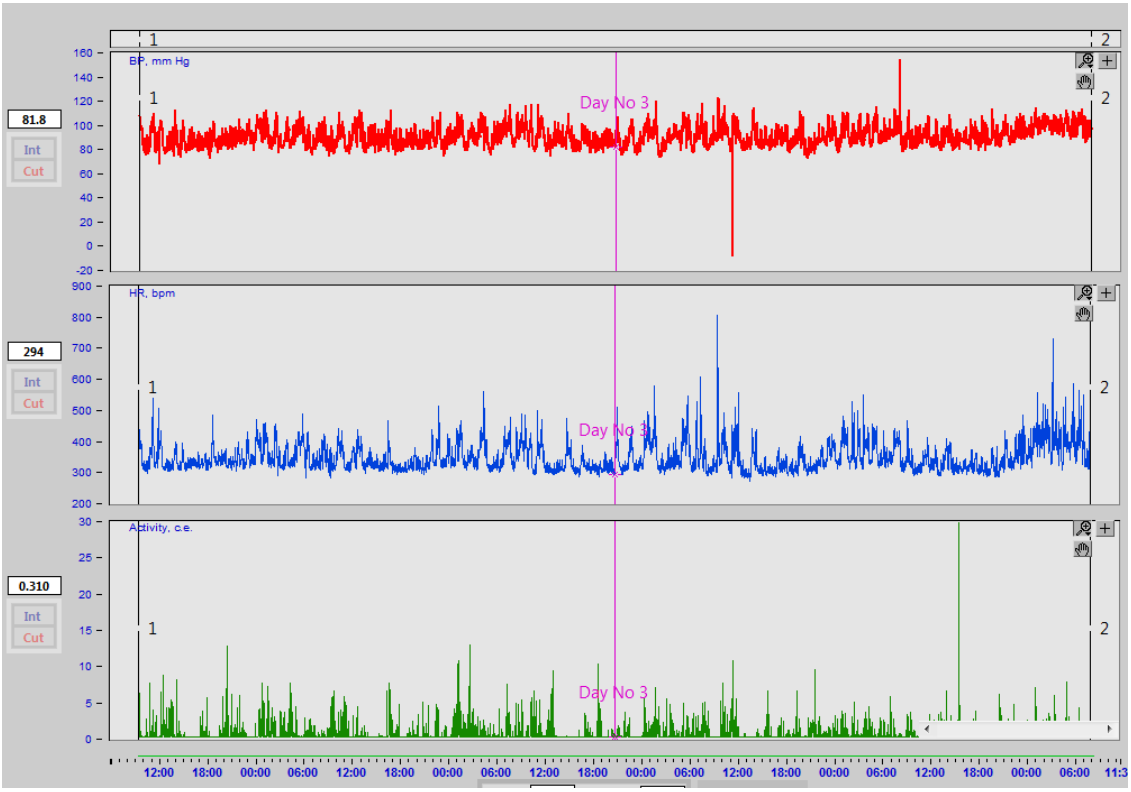

FIGURE 2.9 - TELEMETRY DATA RECORDING.

Red line (trace) indicates blood pressure, blue trace indicates heart rate and green trace indicates activity.

During the continuous recording, animals were subjected to stress tests (novel acute stressor and novel non-aversive stressor) (Davern *et al.*, 2010). These tests were carried out to measure the influence of the limbic system on the control of blood pressure. The novel acute stressor involved strapping the rat box and reading plate to a shaker plate (Ratek, Boronia, Australia). The shaker plate was switched on at 100rpm for 10 minutes, during which heart rate and blood pressure parameters were continuously measured. The novel non-aversive stressor test involved giving rats 4-6 sultanas and heart rate and blood pressure parameters were continuously recorded for 10 minutes after the introduction of the novel food. At completion of the experiment, animals were placed in an induction box with 4% isoflurane to induce anaesthesia. Once anaesthetised, rats were euthanized with an anaesthetic overdose (2ml Lethobarb (pentobarbital sodium, i.p.).

2.4.4.1 Analysis of telemetry recordings

Following the completion of blood pressure recording, the file was opened with Labview (Figure 2.7 above). During the course of an experiment, the signal from the telemetry probe may drop off leading to aberrant peaks and troughs in the recording. To remove the effect of this on data retrieved, a filter was placed over all animal recording files (maximum blood pressure of 160, minimum of 50, maximum heart rate 650 and minimum 200) (Figure 2.10 and 2.11). Locomotor activity was not used due to the inconsistency of the data. Day and night marks were inserted an hour before and after the change in light cycle (Figure 2.12). Therefore, the average night and day recordings were from 10 hours of recording.

The recording was then altered to include marks denoting day and night periods and stress tests carried out. Data were then exported to Microsoft Excel and SPSS for statistical analysis.

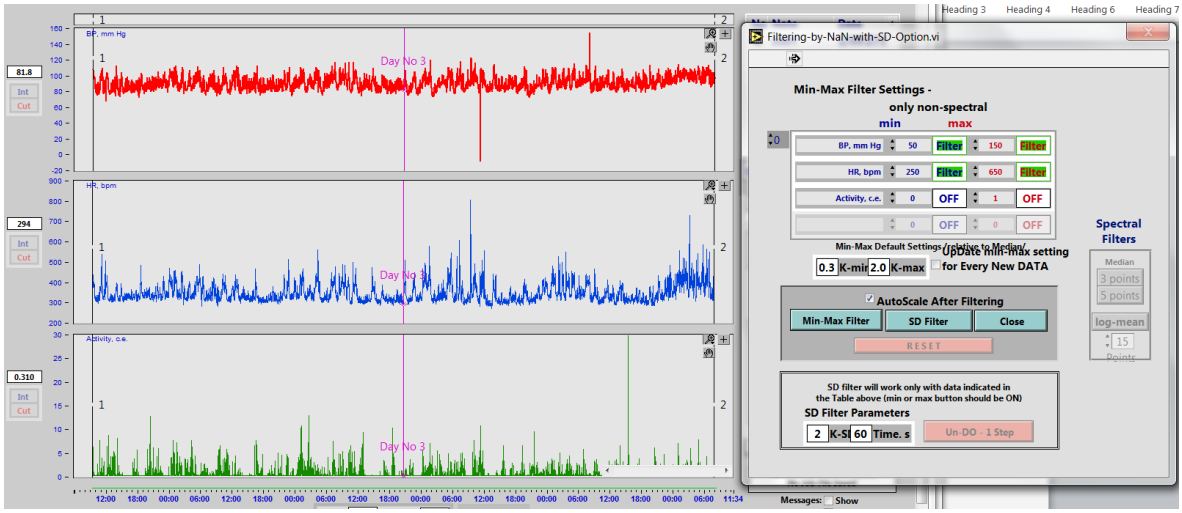

FIGURE 2.10 - TELEMETRY DATA OUTPUT WITH FILTER WINDOW

A filter was applied to the telemetry data to remove incidences where the probe had momentarily dropped out. The same filter was applied across all telemetry traces (blood pressure between 50-160mmHg and heart rate between 200-650bpm).

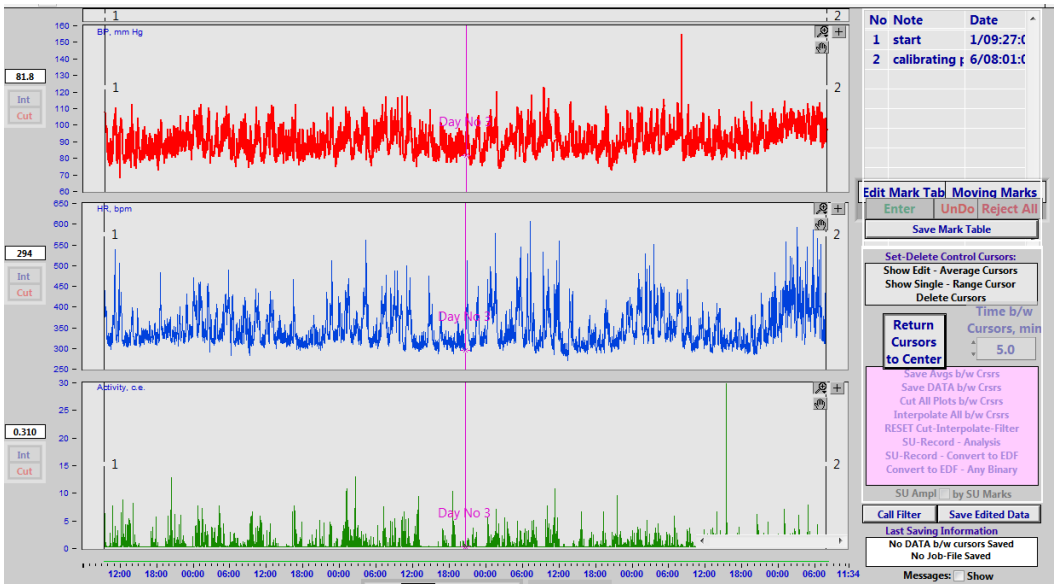

FIGURE 2.11 - TELEMETRY DATA OUTPUT AFTER APPLICATION OF THE FILTER

The application of the filter removes all erroneous data that may have occurred due to the probe signal momentarily dropping out.

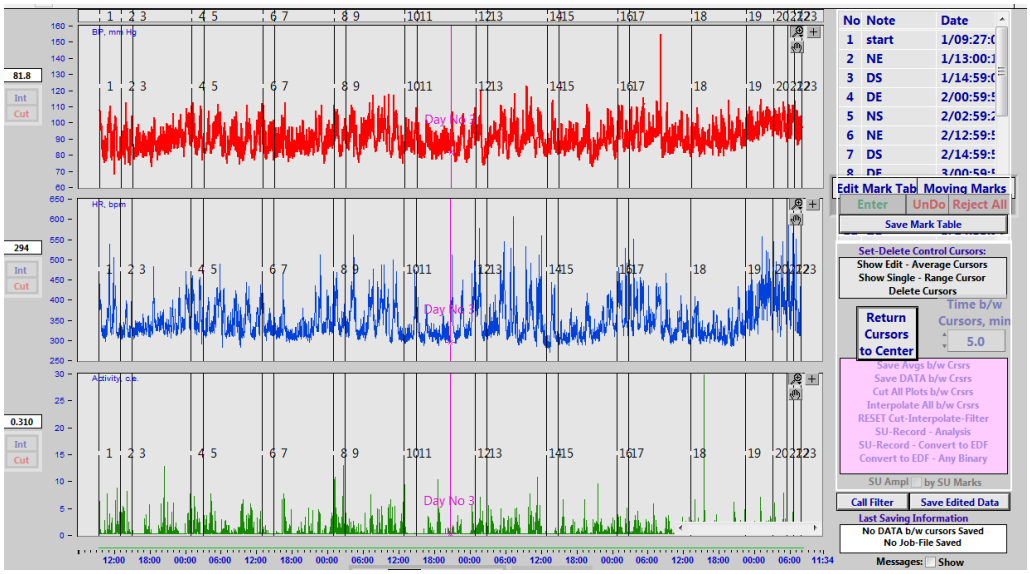

FIGURE 2.12 - TELEMETRY DATA OUTPUT WITH THE INSERTION OF DAY AND NIGHT MARKS.

Day and night marks allowed data to be separated based on whether the rat was active or inactive.

Telemetry data were exported reporting the average values between the day and night marks. This allows for easier analysis of data rather than manually averaging data.

Data were then mined for the average heart rate, mean blood pressure, systolic blood pressure and diastolic blood pressure. Throughout this thesis mean arterial pressure, systolic blood pressure and diastolic blood pressure are reported.

2.4.5 ORGAN COLLECTION

Organs and tissues were collected from male and female rats at PN180 and 360 following surgical anaesthesia (5% v/v isoflurane plus oxygen; Rhodia Australia P/L, Notting Hill, Australia). When a surgical level of anaesthesia was reached, as confirmed by abolition of the pedal reflex, a midline incision was made and the inferior vena cava located. Blood was collected in a 10ml syringe (19G needle) and centrifuged at 3000 x g for 10 minutes. The right kidney was immediately dissected and the renal capsule quickly removed. The kidney was immediately snap frozen in liquid nitrogen for gene expression studies. This was then stored at -80ºC. The left kidney, adipose tissue (abdominal, mesenteric, perirenal fat pads), liver,

pancreas, lung, heart, aorta and brain were collected and weighed. Excess blood was removed by dragging each organ along film (Parafilm M; Pechiney Plastic Packaging, Chicago, IL) for a defined distance. The decapsulated left kidney was immersion fixed in formalin (10% Neutral Buffered Formalin, Chemicon, Australia) for histological studies. The remaining tissue was snap frozen and stored at -80ºC.

2.4.6 DXA ANALYSIS

At PN21, 180 and 360 male and female offspring body composition was analysed by Dual Energy X-ray Absorptiometry (DXA, QDR-4500 DOS Series, Hologic, USA, Figure 2.13). Animals were lightly anaesthetised (Isoflurane, ~3% in air) to prevent movement during the scan.

Prior to scanning, calibration of the machine was required. This was carried out in accordance with manufacturer's recommendations. These included a daily quality control calibration followed by specific calibration for small animals using a step phantom. A test retest for the sensitivity of the machine was performed on mice and shown to have a 95% limit of agree of 5% for body fat and 2% for other parameters (bone mineral content and lean body mass).

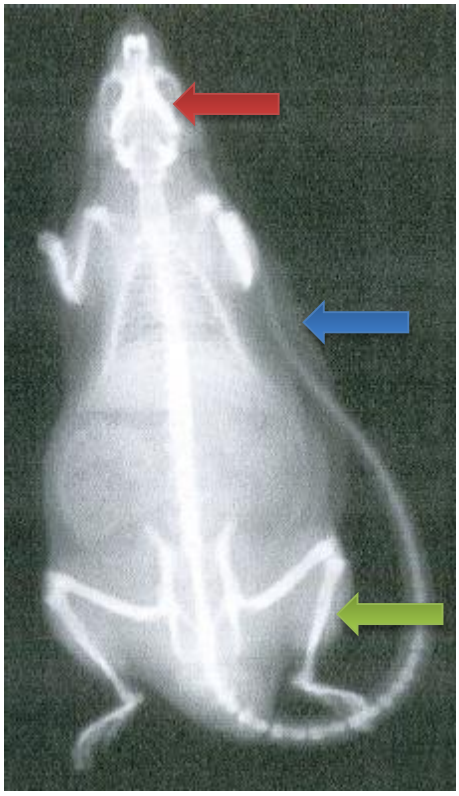

FIGURE 2.13 – DXA SCAN OF MALE RAT

Representative DXA scan of a male rat at PN360. Rats were lightly anaesthetised and placed so that the whole body was scanned. Red arrow indicates bone, blue arrow indicates fat and green indicates muscles.

2.4.7 STATISTICAL ANALYSIS

Data were analysed using a mixed linear model taking into account litter representation (n values represent litter numbers not the total number of rats studied because multiple animals from each litter were chosen for analysis) (SPSS 21, IBM). Data are expressed as mean ± SEM, statistical significance is represented by $P<0.05$, whereby P_{diet} represents the impact of maternal diet, P_{sex} represents the influence of offspring sex, and $P_{diet*sex}$ represents the interaction of these variables. Wherever appropriate, repeated measures ANOVA models were used, as well as linear regressions and comparison of lines of best fit. Graphs were built using Microsoft Office (Microsoft Excel 2013, Microsoft, USA).

CHAPTER THREE: ADULT PHENOTYPE AND CARDIOVASCULAR PROFILE IN OFFSPRING EXPOSED TO MATERNAL LOW PROTEIN DIET

3.1 INTRODUCTION

Studies in the 20th Century documented the nutritional requirements during pregnancy in humans (Widdowson, 1977, McCance *et al.*, 1938) and animals (Widdowson and McCance, 1963, Baird *et al.*, 1971, Dickerson *et al.*, 1971, Widdowson, 1974), highlighting the importance of a diet containing sufficient amounts of macronutrients (proteins, fats, carbohydrates), micronutrients (including iron and calcium) and a sufficient caloric value. These studies showed that caloric or micronutrient restriction in the mother lead to stunting of offspring growth *in utero* and in early postnatal life. Later studies (Barker and Osmond, 1986b, Barker and Osmond, 1986a, Barker and Osmond, 1988) established that adult disease may manifest as a consequence of this abnormal fetal development, and this concept has since been termed "developmental programming of adult health and disease".

Although there are several examples of human developmental programming, for example as consequences of the Dutch Hunger Winter Famine (de Rooij *et al.*, 2007, Painter *et al.*, 2007, Painter *et al.*, 2006b) and the Siege of Leningrad (Bell, 2004, Croft, 2004, Stanner and Yudkin, 2001), the mechanisms underlying the programming of adult disease are best elucidated in animal models. Animal models of developmental programming have been established by altering the maternal diet in order to mimic the dietary insults that may be experienced by humans. Maternal low protein or caloric restricted diets are often reported to induce low birth weight offspring (do Carmo Franco *et al.*, 2009, Reyes-Castro *et al.*, 2011, Woods and Weeks, 2005, Zimanyi *et al.*, 2004). Low birth weight is often associated with increased blood pressure and postnatal weight gain (Coupe *et al.*, 2009, Langley-Evans *et al.*, 1996a, Manning and Vehaskari, 2001, Woods and Weeks, 2005). Maternal protein restriction has also been associated with reduced nephron endowment and higher blood pressure (Langley-Evans *et al.*, 1999c, Nwagwu *et al.*, 2000, Vehaskari *et al.*, 2001, Woods *et al.*, 2001). However, it is not known whether the reported elevation in blood pressure in offspring of protein restricted rats

are directly related to nephron endowment, postnatal growth or merely an artefact induced by techniques used to measure blood pressure.

It has been proposed that exposure to a protein deficient diet *in utero* can programme hypertension in offspring (Langley-Evans, 1997, Langley-Evans *et al.*, 1996a, Langley-Evans *et al.*, 1996c, Langley-Evans *et al.*, 1999b, Vehaskari *et al.*, 2004). However, the finding is not invariant (Black *et al.*, 2004a, Zimanyi *et al.*, 2006). The reasons for the heterogeneity of findings with regard to hypertension are potentially due to the methodology used to determine blood pressure, variability in animal strains and alterations in the micronutrient composition of the low protein diets. Using tail cuff plethysmography and tethered indwelling catheters for the determination of blood pressure can potentially induce a stress response which may be greater in offspring exposed to maternal protein restriction and therefore exhibit higher blood pressure than controls (Kramer *et al.*, 2001, Kramer and Remie, 2005). It is also feasible that different rat strains may respond differently to maternal diet and environment (Adams and Blizard, 1991, Blizard, 1992, Kubisch and Gomez-Sanchez, 1999, Lawler *et al.*, 1993). Specific maternal diet composition has been reported to influence offspring health outcomes, specifically on the origin of the protein and the carbohydrate (Cherala *et al.*, 2006, Langley-Evans, 2000, Jahan-mihan *et al.*, 2011b, Jahan-Mihan *et al.*, 2011a). It is not apparent as to what happens in humans. Evidence from the Dutch Hunger Winter Famine indicate that maternal caloric restriction lead to offspring with microalbuminuria, propensity to type 2 diabetes and coronary heart disease (Painter *et al.*, 2006b, Roseboom *et al.*, 2001a). There is some evidence to suggest that exposure to maternal caloric restriction leads to an altered stress response, in that offspring had a greater systolic blood pressure change due to a psychosocial stressor (Painter *et al.*, 2006a). A similar finding was reported in offspring exposed to the Siege of Leningrad, another natural experiment that allows the investigation of caloric restriction on offspring health.

Pubertal exposure to caloric restriction lead to higher systolic blood pressure and mortality from ischaemic heart disease in adult life (Koupil *et al.*, 2007).

Adult blood pressure may be related to abnormal kidney growth. Brenner *et al.* hypothesised in 1988 that alterations in kidney structure could predispose certain individuals to the development of hypertension (Brenner *et al.*, 1988). The authors proposed that any condition that leads to a reduction of filtration surface area (either fewer glomeruli or glomeruli with smaller capillary filtration surface area) would result in an increase in glomerular pressure and hypertrophy and ultimately glomerulosclerosis, thereby altering kidney function (ability to maintain blood volume) causing a rise in systemic blood pressure. Therefore, it is important that blood pressure is determined in offspring exposed to maternal sub-optimal intrauterine environment using gold standard techniques (radiotelemetry devices) in order to ascertain the relative contributions of the maternal environment, kidney development and postnatal growth.

Maternal low protein diet exposure can influence nephron endowment, and frequently, but not always, blood pressure (Holemans *et al.*, 1999, Woods *et al.*, 2005, Zimanyi *et al.*, 2006, Gilbert *et al.*, 2005). For example, Langley-Evans *et al.* (1999b) reported a deficit of 13% in nephron number was associated with a 13 mmHg elevation in arterial pressure (anaesthetised, indwelling catheter) of protein-restricted offspring (Wistar rats exposed to a 9% protein diet, while controls had a normal 18% protein diet). Offspring exposed to maternal protein restriction had undergone catch-up growth by the time variables were measured.

This relationship between nephron number and blood pressure was investigated by Woods *et al.* (2001) using a model of maternal protein restriction in rats. Protein restricted male offspring had 25% less nephrons, 10mmHggreater mean arterial blood pressure and an 11% deficit in glomerular filtration rate compared with control rats. Protein restricted offspring had also undergone catch-up growth by 4 weeks of age. More recently, Woods *et al.* (2005) reported

that female offspring from protein restricted Sprague-Dawley dams (on a 8.5% protein diet, while controls were on a 19% protein diet) had similar mean arterial pressure (measured using anaesthetised indwelling arterial catheter) as control offspring and no evidence of catch-up growth. Utilising a maternal protein restriction model in Sprague-Dawley rats, Vehaskari *et al.* (2001) reported that males and females exposed to protein restricted diets for 4 weeks had a nephron deficit of 28% and 29%, respectively. This deficit in nephron number was accompanied by a 20-25mmHg higher blood pressure (measured using tail cuff plethysmography) in males and females than controls. Offspring had also undergone catch-up growth by 2 weeks of age. This demonstrates the link between postnatal growth and the observed adult phenotype, whereby early accelerated growth appears to exacerbate accommodations made *in utero* leading to a disease state in adulthood.

Nephron deficiency is not always associated with elevated arterial pressure. For example, Zimanyi *et al.* (2006) reported a nephron deficit in Wistar-Kyoto (WKY) rats fed an 8.7% (low) protein diet (while control rats were fed a 20% protein diet). Zimanyi *et al.* failed to identify any alteration in blood pressure or evidence of renal hyperfiltration in adulthood in the offspring of protein-restricted rats (Zimanyi *et al.*, 2006). The absence of change in blood pressure between the dietary groups may also be a product of offspring exposed to maternal protein restriction failing to demonstrate catch-up growth. Interestingly, reduced nephron endowment may not necessarily lead to systemic high blood pressure. Investigating the spontaneously hypertensive rat (SHR), Black and colleagues (2004a) report no change in nephron number while there was a 29mmHg increase in blood pressure (measured via tail cuff plethysmography). This is in contrast to a previously published paper by Skov *et al.* (1994) who reported reduced nephron number in the SHR compared to normotensive WKY rats. While it is understood that the hypertension that develops in the SHR is multifactorial, the relationship that nephron endowment may play is unclear. The results from these studies

indicate that there relationship between nephron number and blood pressure is not direct and may be influenced by other factors.

Tonkiss and colleagues, using radiotelemetry devices, reported that malnourished rat offspring (6% maternal LP diet) demonstrated a 4mmHg increase in diastolic blood pressure compared with controls, however offspring of protein-restricted rats elicited a greater cardiovascular arousal response (increased systolic pressure) to acute stress compared with controls (Tonkiss *et al.*, 1998). This same study did not report nephron number in malnourished offspring, but did find that malnourished offspring weighed the same as controls at the time of blood pressure measurement. In a rat model of maternal caloric restriction, Brennan and colleagues reported a nephron deficit and no change in adult blood pressure (recorded using radiotelemetry) (Brennan *et al.*, 2008). Therefore, the question still remains about the relative contribution of postnatal growth and nephron number to adult blood pressure.

Jennings and colleagues used a cross-fostering method in rats to compare the contributions of the fetal and postnatal environments on offspring growth (Jennings *et al.*, 1999). Pups that were exposed to protein restriction *in utero* then switched to a control dam post birth underwent catch-up growth and had similar weight to control pups by PN21, while pups that were maintained on a low protein diet postnatally did not. In fact, pups that underwent catch-up growth had reduced longevity. Investigations into catch-up growth also report changes in insulin sensitivity (Petry *et al.*, 1997) and reduced β-cell proliferation and islet size during fetal life (Snoeck *et al.*, 1990). These studies indicate that postnatal nutrition of offspring is critical to the development of disease. Many studies that have linked maternal low protein diet to reduced nephron endowment and hypertension also observed accelerated growth postnatally (Langley-Evans *et al.*, 1994, Langley-Evans *et al.*, 1996c, Woods *et al.*, 2001).

As already mentioned, nephron endowment (number of nephrons that develop within the kidneys) is affected by the developmental environment. In order to determine the physiological impact of this reduction in the number of functional units, an investigation of renal function is necessary. Nwagwu *et al.* (2000) fed rat dams either 18% (control) or 9% protein diet. After four weeks of postnatal life the offspring were weaned onto a normal laboratory chow diet. Offspring exposed to the low protein diet *in utero* had an increased systolic blood pressure varying from 7 to 21mmHg (measured using an indirect tail-cuff method) between 4 weeks of age and 20 weeks (Nwagwu *et al.*, 2000). Creatinine clearance, a measure of glomerular filtration rate (GFR), was lower in the 4-week old rats exposed to the low protein diet compared with controls (Nwagwu *et al.*, 2000). Sahajpal and colleagues used tritiated inulin and PAH clearance to assess kidney function in offspring exposed to maternal protein restriction, with a nephron reduction, and reported no change in GFR or effective renal blood flow (ERBF) (Sahajpal and Ashton, 2003). However, offspring of low protein fed rats did have higher blood pressure (measured using indwelling carotid catheter) and had undergone catch-up growth (Sahajpal and Ashton, 2003). It is well established that there is a link between nephron number, blood pressure and GFR. However, it is possible that the results seen are a product of offspring growth or experimental techniques introducing confounding factors.

In conclusion, the relative impacts of postnatal growth and exposure to a suboptimal intrauterine environment (maternal LP diet) on kidney structure, function and blood pressure remain unclear. The current study aims to assess these variables using maternal protein restriction. More specifically, we hypothesise that, compared with controls, offspring of protein restricted rats will demonstrate:

i) *reduced growth,*

ii) *reduced nephron number,*

iii) *invariant mean arterial pressure and heart rate, and*

iv) *invariant glomerular filtration rate and effective renal plasma flow.*

3.2 METHODS

Experiments were conducted in accordance with the National Health and Medical Research Council of Australia '*Australian Code of Practice for the Care and Use of Animals for Scientific Purposes'* (7th edition, 2004). Approval was granted in advance by the Monash University School of Biomedical Sciences Animal Ethics Committee.

All methods have been fully documented in Chapter 2.

3.2.1 ANIMAL HUSBANDRY

In brief, 9-week old Sprague Dawley rats were fed either a low protein (LP, 8.4% casein) or normal protein (NP, control 19.4% casein) diet for 3 weeks then time-mated. Dietary manipulation continued throughout pregnancy and suckling (21 days postnatal) then offspring were weaned to a control diet (19.4% casein) which was fed *ad libitum* until the time of experimentation. See Sections 2.2 and 2.2.2 for information on the experimental diets used and the rearing of animals.

3.2.2 ANALYSIS OF BODY COMPOSITION AND ORGAN WEIGHT

Body composition was determined by DXA in isoflurane-anaesthetised animals at weaning, PN180 and PN360. Body fat, lean muscle and bone mineral content were all determined with a Hologic DXA scanner specifically calibrated for small animals. See Section 2.4.6 for full details.

Organ weight was determined at PN21, PN180 and PN360. Organs included left kidneys, heart, pancreas, liver, brain, mesenteric fat, peri-renal fat and abdominal fat. See Section 2.4.5 for full details.

3.2.3 DETERMINATION OF NEPHRON NUMBER

Nephron number was estimated in 21 day old offspring using the gold standard physical disector fractionator method (Cullen-McEwen *et al.*, 2011) as per Section 2.4.1. PN21 was selected as the time-point for study as nephrogenesis is complete and is not accompanied by nephron loss associated with aging or as a result of cardiovascular disease such as hypertension.

3.2.4 MEASUREMENT OF RENAL FUNCTION AND BLOOD PRESSURE

GFR and effective renal blood flow were determined in PN180 and PN360 animals using the gold standard of ^{3}H inulin and ^{14}C PAH clearance, respectively. See Section 0 for more details.

Blood pressure (systolic, diastolic and mean arterial pressure) and heart rate were measured in conscious, freely moving animals at PN180 and 360 using an indwelling radio-telemetry device (PA-C40, DSI, USA). Recordings reported in this chapter have been separated into the active and inactive period. An hour at the start and end of each recording period were not included in the analysis to remove any transition effects. For more details on surgical and measuring procedures see Section 2.4.3.

Stress tests were also performed. Animals were exposed to an oscillating table for 10 minutes (novel acute stressor) at 100 rpm, as well as exposed to a novel non-aversive cardiovascular arousal stimulus (presentation of sultanas). For both stress tests, blood pressure and heart rate were recorded for 5 minutes prior, 10 minutes during exposure to the stress test and 30 minutes after the stress test.

3.2.5 STATISTICAL ANALYSIS

Data were analysed using a mixed linear model taking into account litter representation (n values represent litter numbers NOT the total number of animals studied because multiple animals from each litter were chosen for analysis) (SPSS 21, IBM). Data are expressed as mean ± SEM, statistical significance is represented by $P<0.05$, whereby P_{diet} represents the impact of maternal LP diet, P_{sex} represents the influence of offspring sex and $P_{diet*sex}$ represents the interaction between these variables. Wherever appropriate, repeated measures ANOVA models were used. Graphs were built using Microsoft Office (Microsoft Excel 2013, Microsoft, USA).

3.3 RESULTS

3.3.1 POSTNATAL GROWTH AND BODY COMPOSITION

Exposure to maternal protein restriction did not alter offspring birth weight (Figure 3.1); however postnatal growth was stunted by PN21, with both male and female offspring exposed to maternal low protein diet weighing 39% less than controls (NP Female 50.8±1.3g, NP Male 52.2±1.3g, LP Female 32.1±1.2g, LP Male 31.1±1.4g, $P_{diet}<0.001$, $P_{sex}=0.87$, $P_{diet*sex}=0.21$). From PN25 onwards (where rats were able to be tracked individually), both male and female protein restricted offspring weighed less than controls ($P_{diet}<0.001$, Figure 3.2). This pattern continued throughout life, with offspring showing no sign of catch-up growth.

Offspring body composition was determined by DXA (Figure 3.3). Body fat (% body weight) of male and female offspring exposed to maternal protein restriction was not different compared with controls at PN 21, PN180 or PN360 (%Fat PN21 $P_{diet}=0.41$, $P_{sex}=0.32$, $P_{diet*sex}=0.07$, PN180 $P_{diet}=0.14$, $P_{sex}=0.87$, $P_{diet*sex}=0.27$, PN360 $P_{diet}=0.09$, $P_{sex}=0.18$, $P_{diet*sex}=0.43$). Similarly, the percentage of lean muscle mass did not differ between groups at any time point (%Lean Muscle Mass PN21 $P_{diet}=0.47$, $P_{sex}=0.33$, $P_{diet*sex}=0.08$, PN180 $P_{diet}=0.15$, $P_{sex}=0.86$, $P_{diet*sex}=0.27$, PN360 $P_{diet}=0.08$, $P_{sex}=0.13$, $P_{diet*sex}=0.44$). In contrast, bone mineral content was reduced by 25% at PN21 ($P_{diet}<0.05$, $P_{sex}=0.08$, $P_{diet*sex}<0.01$), 14% at PN180 ($P_{diet}<0.05$, $P_{sex}<0.01$, $P_{diet*sex}=0.63$) and 17% at PN360 ($P_{diet}<0.05$, $P_{sex}<0.05$, $P_{diet*sex}=0.66$) in LP offspring compared with controls.

Offspring organ weight was determined at PN21, 180 and 360. Kidney weight at PN21 was higher in males than in females ($P_{sex}<0.05$). Offspring exposed to maternal LP diet had smaller kidneys ($P_{diet}<0.05$, $P_{diet*sex}=0.70$), which were also smaller in proportion to body weight ($P_{diet}<0.05$. $P_{diet*sex}=0.75$, Table 3.1).

At PN180, absolute organ weights were similar in NP and LP offspring (Table 3.2). However, when considered relative to body weight, both heart ($P_{diet}<0.05$, $P_{sex}<0.05$, $P_{diet*sex}=0.72$) and brain ($P_{diet}<0.05$, $P_{sex}<0.05$, $P_{diet*sex}=0.07$) weights were higher in low protein offspring (Table 3.2). Absolute weights of fat pads at PN180 were similar in NP and LP offspring (Mesenteric fat; $P_{diet}=0.38$, $P_{sex}<0.05$, $P_{diet*sex}=0.75$, Peri-renal fat; $P_{diet}=0.14$, $P_{sex}<0.05$, $P_{diet*sex}=0.76$, Abdominal fat; $P_{diet}=0.90$, $P_{sex}=0.54$, $P_{diet*sex}=0.37$), and remained that way when adjusted for body weight.

At PN360, organ weight was similar in offspring exposed to maternal low protein compared to controls (Table 3.3). Fat pad weight was also not different in offspring exposed to maternal low protein compared with controls.

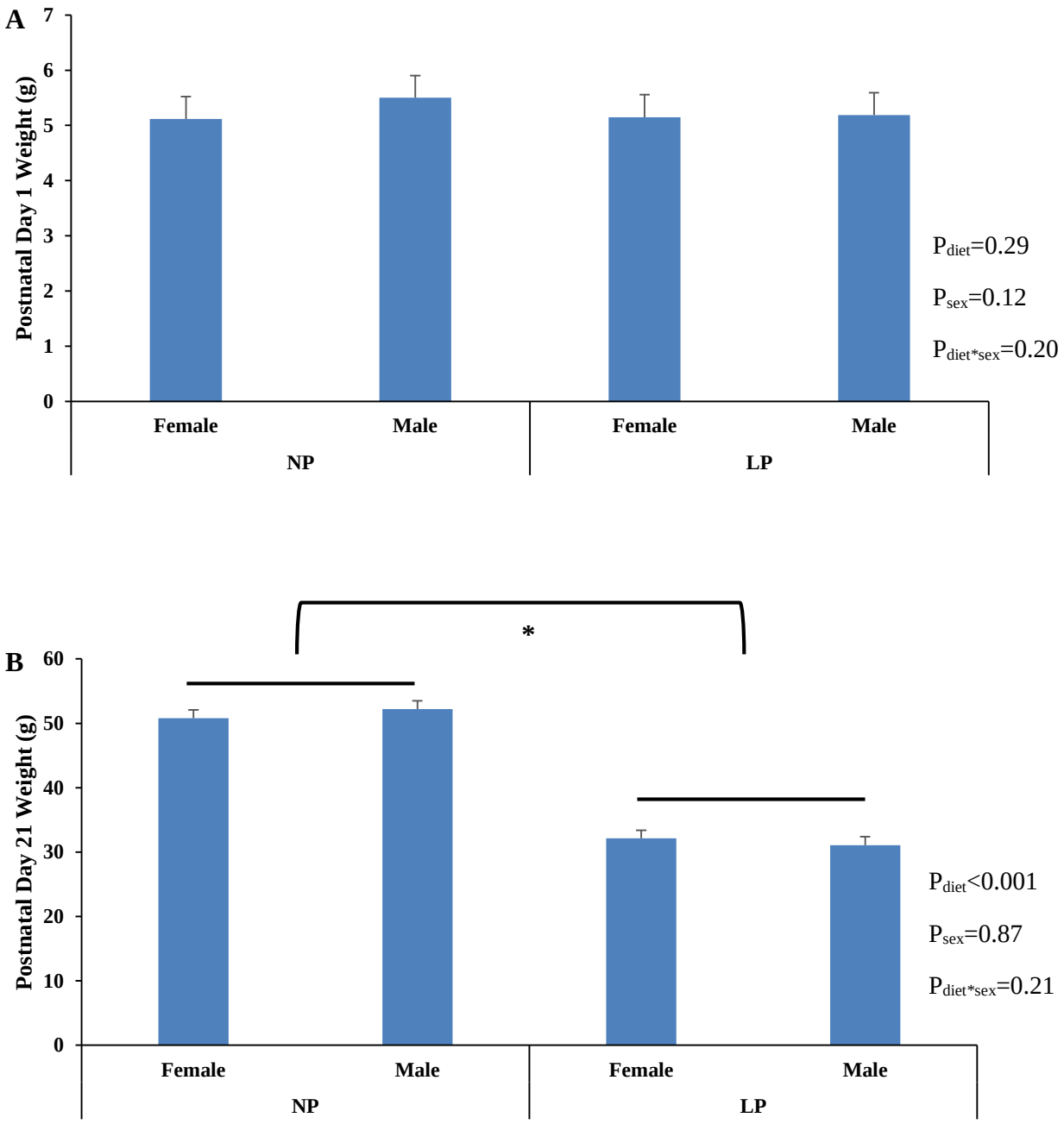

FIGURE 3.1 – POSTNATAL DAY 1 AND 21 BODY WEIGHTS OF MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL NORMAL AND LOW PROTEIN DIET.

Male and female offspring exposed to normal and low protein maternal diet were weighed at postnatal day 1 (**A**) and 21 (**B**). Weight at postnatal day 1 was not different between dietary groups, while at postnatal day 21 offspring exposed to maternal low protein diet weighed less than control animals. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (NP PN1 Male and Female n=10, PN21 n=8, LP PN1 n=9, PN21 n=9), * represented $p<0.001$.

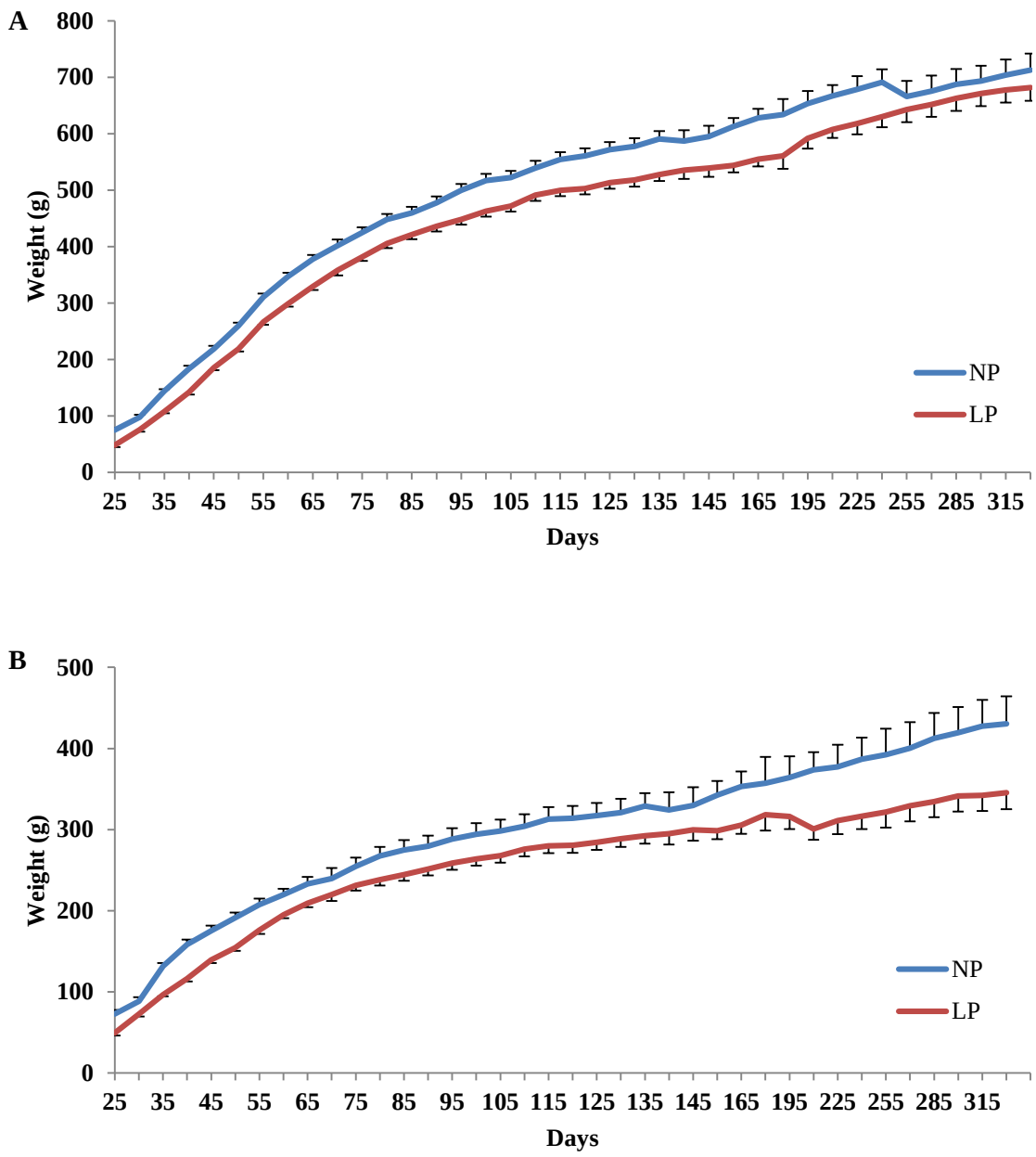

FIGURE 3.2 – GROWTH FROM PN25 TO PN330 OF MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION.

Offspring of male (**A**) and female (**B**) rats exposed to either a low (red line, LP) or normal (blue line, NP) protein diet during pregnancy and lactation demonstrated reduced body weight throughout life (PN25 - PN330). Data expressed as mean ± SEM, analysed by repeated measures ANOVA and weighted for litter (LP n = 5, NP n = 5), $P_{diet}<0.001$, $P_{sex}<0.001$, $P_{diet*sex}=0.09$, $P_{time}<0.001$, $P_{diet*time}<0.001$).

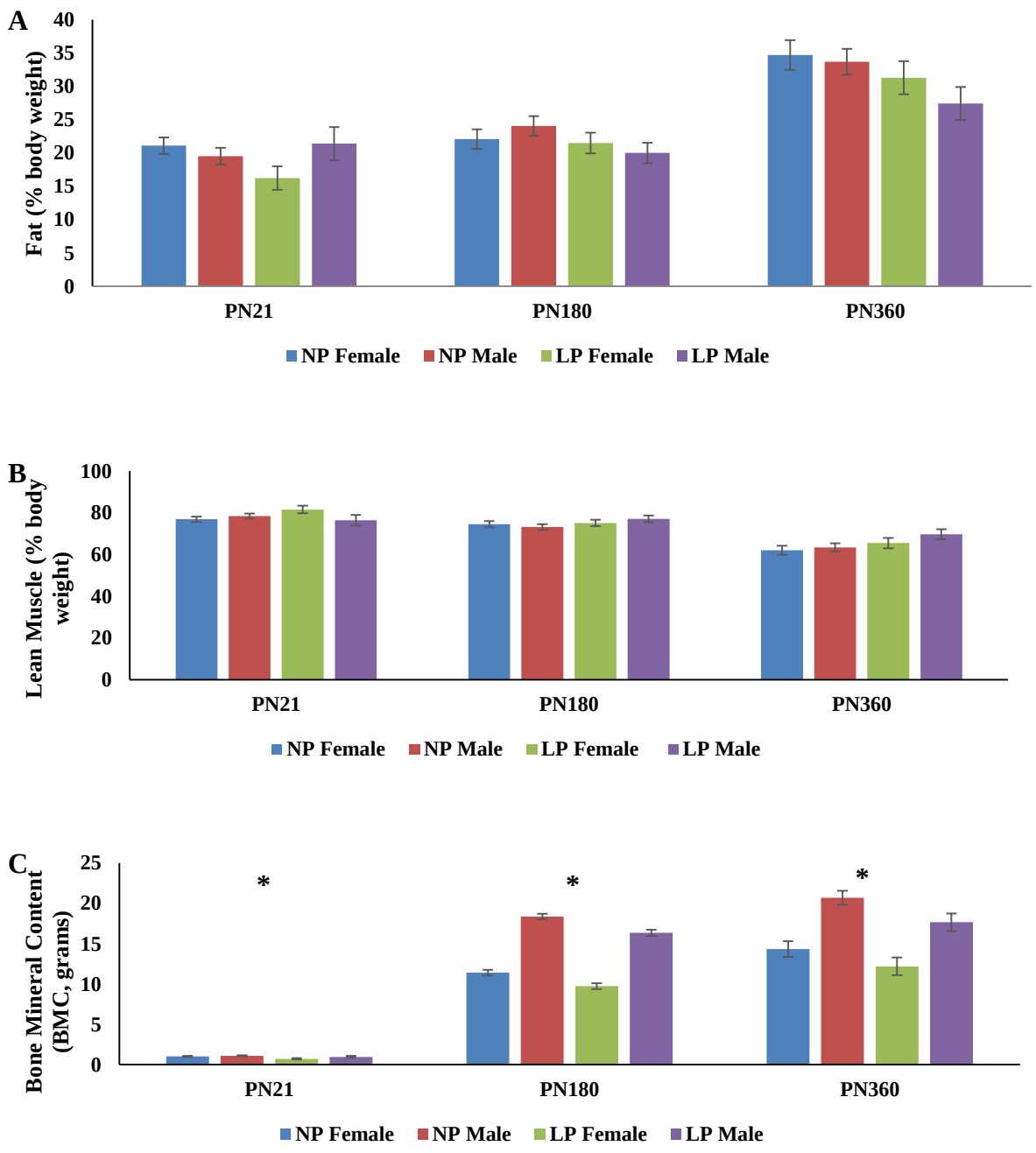

FIGURE 3.3 - PERCENTAGE BODY FAT, LEAN MUSCLE MASS AND BMC AT PN21, PN180 AND PN360 OF OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION.

Offspring exposed to either a low (LP, female green and male purple dashed lines) or normal (NP female blue and male red dashed lines) protein diet during pregnancy and lactation were analysed using a DXA scanner at PN21, PN180 and PN360. The percentage of fat (**A**) lean muscle mass (**B**) and BMC (**C**) were determined. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (PN21, NP ♂ n = 8, ♀ n = 8, LP ♀ n = 4, ♂ n = 2, PN180 NP ♀ n = 10, ♂ n = 10, LP ♀ n = 9, ♂ n = 8, PN360 NP ♀ n = 10, ♂ n = 13, LP ♀ n = 8, ♂ n = 8), * represents $P_{diet}<0.05$.

TABLE 3.1 – KIDNEY WEIGHT OF PN21 OFFSPRING.

Organ	NP		LP		Significance		
	Female (n=8)	Male (n=8)	Female (n=7)	Male (n=7)	Diet	Sex	Interaction
Body weight (g)	50.8 ± 1.3	52.2 ± 1.3	32.1 ± 1.2	31.1 ± 1.4	**<0.001**	0.87	0.21
Left Kidney (g)	0.61 ± 0.07	0.300 ± 0.07	0.162 ± 0.07	0.160 ± 0.07	**<0.05**	0.77	0.92
Left Kidney/body weight (g)	0.006 ± 0.0002	0.006 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	**<0.05**	0.16	0.66
Right Kidney (g)	0.325 ± 0.03	0.315 ± 0.03	0.164 ± 0.03	0.172 ± 0.03	**<0.05**	0.96	0.57
Right Kidney/body weight (g)	0.006 ± 0.0002	0.006 ± 0.0002	0.005 ± 0.0003	0.005 ± 0.0003	**<0.05**	0.37	0.47
Total Kidney (g)	0.630 ± 0.03	0.615 ± 0.02	0.327 ± 0.03	0.332 ± 0.03	**<0.05**	0.86	0.70
Total kidney/body weight (g)	0.012 ± 0.001	0.012 ± 0.001	0.011 ± 0.001	0.010 ± 0.001	**<0.05**	0.19	0.75

TABLE 3.2 – PN180 ORGAN WEIGHTS FROM OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION OR CONTROL DIET.

Organ	NP		LP		Significance		
	Female (n=8)	Male (n=8)	Female (n=6)	Male (n=3)	P_{diet}	P_{sex}	$P_{diet*sex}$
Body weight (g)	366.15 ± 17.05	659.79 ± 14.75	315.19 ± 9.85	574.60 ± 11.78	**<0.001**	**<0.001**	0.09
Left kidney (g)	0.93 ± 0.11	1.46 ± 0.12	0.63 ± 0.14	1.56 ± 0.17	0.49	**<0.05**	0.15
Left kidney/body weight (g)	0.003 ± 0.00009	0.002 ± 0.0001	0.003 ± 0.0001	0.003 ± 0.0001	0.28	0.31	0.80
Heart (g)	1.00 ± 0.06	1.53 ± 0.07	0.89 ± 0.07	1.54 ± 0.09	0.52	**<0.05**	0.41
Heart/body weight (g)	0.003 ± 0.0005	0.002 ± 0.0005	0.003 ± 0.0005	0.003 ± 0.0005	**<0.05**	**<0.05**	0.77
Liver (g)	11.60 ± 2.28	21.44 ± 2.38	9.79 ± 2.46	18.89 ± 2.70	0.19	**<0.05**	0.82
Liver/body weight (g)	0.031 ± 0.001	0.032 ± 0.002	0.032 ± 0.002	0.030 ± 0.002	0.84	0.95	0.47
Brain (g)	1.81 ± 0.06	1.95 ± 0.07	1.86 ± 0.07	1.96 ± 0.09	0.69	0.11	0.74
Brain/body weight (g)	0.005 ± 0.0002	0.003 ± 0.0002	0.006 ± 0.0002	0.003 ± 0.0003	**<0.05**	**<0.05**	0.07
Pancreas (g)	0.72 ± 0.20	0.82 ± 0.20	0.66 ± 0.20	0.85 ± 0.21	0.90	0.08	0.54
Pancreas/body weight (g)	0.001 ± 0.0003	0.001 ± 0.0003	0.002 ± 0.0003	0.001 ± 0.0004	0.49	0.12	0.85
Mesenteric fat (g)	4.91 ± 0.63	7.37 ± 0.77	4.45 ± 0.85	6.39 ± 0.95	0.38	**<0.05**	0.75
Mesenteric fat/body weight (g)	0.013 ± 0.002	0.011 ± 0.002	0.014 ± 0.003	0.010 ± 0.003	0.82	0.06	0.48
Peri-renal fat (g)	7.94 ± 1.71	16.34 ± 1.94	4.52 ± 2.10	14.21 ± 2.56	0.20	**<0.05**	0.76
Peri-renal fat/body weight (g)	0.021 ± 0.016	0.024 ± 0.016	0.014 ± 0.016	0.022 ± 0.016	0.14	**<0.05**	0.42
Abdominal fat (g)	7.13 ± 2.61	6.70 ± 2.67	5.67 ± 2.79	7.82 ± 2.89	0.90	0.54	0.37
Abdominal fat/body weight (g)	0.018 ± 0.002	0.010 ± 0.002	0.017 ± 0.002	0.012 ± 0.003	0.82	**<0.05**	0.57
Total fat (g)	19.98 ± 7.37	31.68 ± 7.69	12.95 ± 7.69	28.42 ± 8.16	0.20	**<0.05**	0.64
Total fat/body weight (g)	0.051 ± 0.005	0.047 ± 0.006	0.040 ± 0.006	0.045 ± 0.007	0.26	0.96	0.47

TABLE 3.3 – PN360 ORGAN WEIGHTS FROM OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION OR CONTROL DIET.

Organ	NP		LP		Significance		
	Female (n=8)	Male (n=8)	Female (n=5)	Male (n=4)	P_{diet}	P_{sex}	$P_{diet*sex}$
Body weight (g)	413.91 ± 23.40	730.44 ± 14.94	460.55 ± 23.40	674.75 ± 38.81	0.87	<**0.001**	0.06
Left Kidney (g)	1.26 ± 0.24	1.83 ± 0.23	1.12 ± 0.25	1.64 ± 0.28	0.18	<**0.05**	0.83
Left kidney/body weight (g)	0.003 ± 0.0002	0.002 ± 0.0001	0.002 ± 0.0002	0.002 ± 0.0003	0.07	0.09	0.17
Heart (g)	1.12 ± 0.06	1.67 ± 0.04	1.23 ± 0.06	1.66 ± 0.12	0.58	<**0.001**	0.45
Heart/body weight (g)	0.003 ± 0.0001	0.002 ± 0.0001	0.003 ± 0.0001	0.002 ± 0.0002	0.66	<**0.05**	0.65
Liver (g)	12.35 ± 1.16	21.33 ± 0.72	13.44 ± 1.23	19.72 ± 2.12	0.85	<**0.001**	0.34
Liver/body weight (g)	0.030 ± 0.002	0.029 ± 0.001	0.028 ± 0.002	0.029 ± 0.003	0.54	0.92	0.70
Brain (g)	2.00 ± 0.08	2.18 ± 0.04	2.02 ± 0.05	2.19 ± 0.14	0.88	0.06	0.96
Brain/body weight (g)	0.005 ± 0.0004	0.003 ± 0.0003	0.004 ± 0.0003	0.003 ± 0.001	0.37	<**0.001**	0.21
Pancreas (g)	0.77 ± 0.07	1.03 ± 0.04	0.85 ± 0.07	1.06 ± 0.22	0.62	0.07	0.85
Pancreas/body weight (g)	0.002 ± 0.0002	0.001 ± 0.0002	0.002 ± 0.0002	0.002 ± 0.0004	0.77	0.06	0.60
Mesenteric fat (g)	6.15 ± 1.15	7.81 ± 2.42	8.47 ± 3.06	7.31 ± 2.90	0.50	0.85	0.29
Mesenteric fat/body weight (g)	0.015 ± 0.001	0.011 ± 0.001	0.018 ± 0.001	0.011 ± 0.003	0.43	<**0.005**	0.51
Peri-renal fat (g)	9.73 ± 2.81	21.92 ± 1.74	11.08 ± 3.0	18.75 ± 8.88	0.86	0.05	0.65
Peri-renal fat/body weight (g)	0.024 ± 0.006	0.029 ± 0.006	0.023 ± 0.006	0.028 ± 0.012	0.82	0.39	0.98
Abdominal fat (g)	8.42 ± 0.91	12.14 ± 0.57	8.99 ± 0.96	15.13 ± 2.89	0.28	<**0.05**	0.46
Abdominal fat/body weight (g)	0.021 ± 0.003	0.017 ± 0.003	0.019 ± 0.003	0.022 ± 0.005	0.37	0.89	0.09
Total fat (g)	24.30 ± 4.07	41.87 ± 2.52	28.53 ± 4.23	41.19 ± 12.85	0.81	<**0.05**	0.73
Total fat/body weight (g)	0.060 ± 0.012	0.056 ± 0.011	0.059 ± 0.012	0.061 ± 0.019	0.81	0.92	0.78

3.3.2 NEPHRON NUMBER AT PN21

Maternal protein restriction lead to a 26% and 17% nephron deficit in female and male offspring of LP dams respectively (NP Female 31044±1575, NP Male 30749±1575, LP Female 23027±1683, LP Male 25545±1683, $P_{diet}<0.05$, $P_{sex}=0.50$, $P_{diet*sex}=0.40$, Figure 3.4) compared with controls. The ratio of nephron number to kidney weight was similar in NP and LP offspring ($P_{diet}=0.88$, $P_{sex}=0.39$, $P_{diet*sex}=0.80$). However, offspring of LP fed dams had a 12% and 22% greater nephron number (when adjusted for body weight) in female and male offspring respectively, compared with controls (NP Female 644±59, NP Male 630±59, LP Female 732±63, LP Male 809±63, $P_{diet}<0.05$, $P_{sex}=0.61$, $P_{diet*sex}=0.47$).

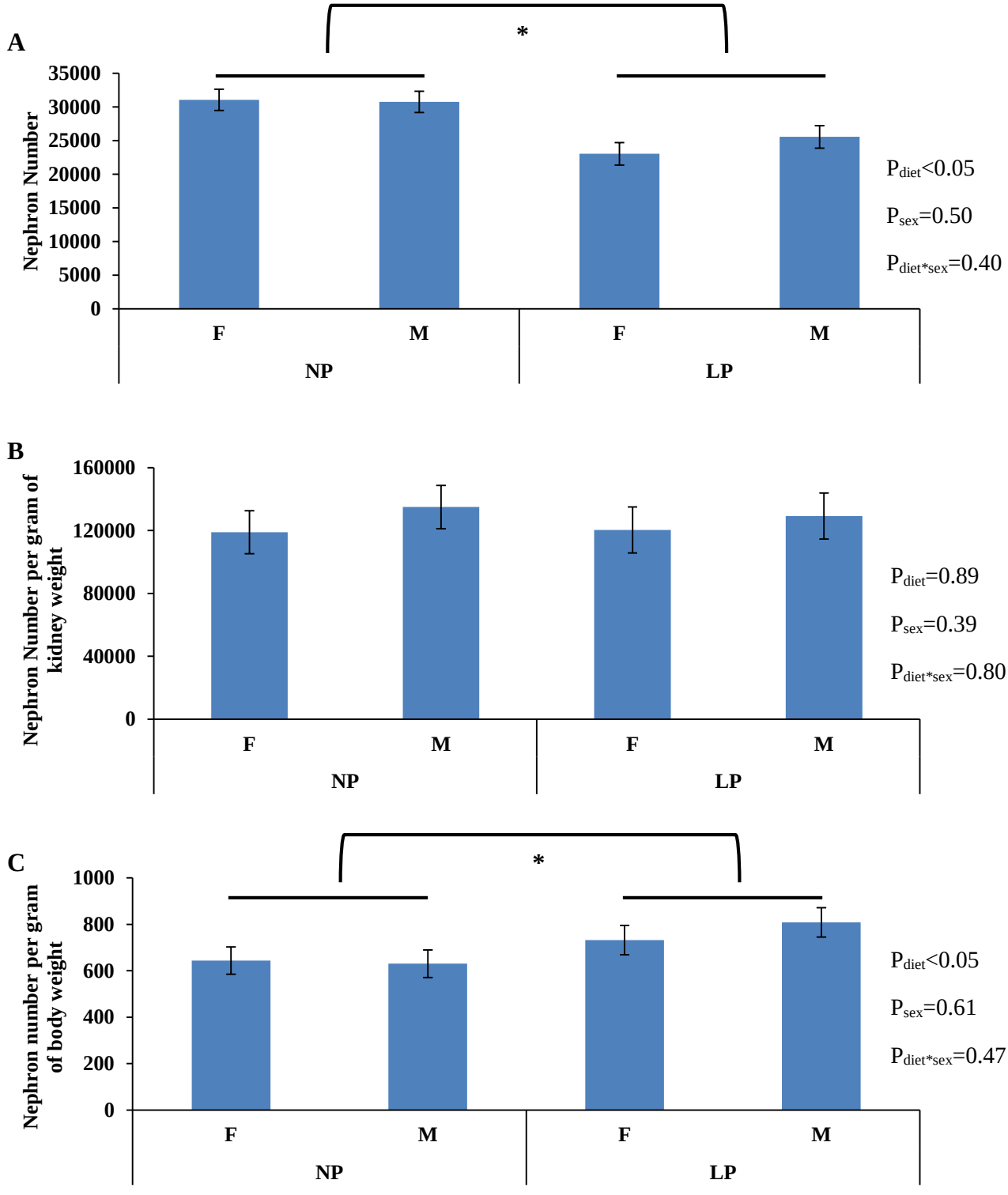

FIGURE 3.4 – PN21 NEPHRON NUMBER FROM MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION.

Nephron number (**A**) expressed as per gram of kidney weight (**B**) and per gram of body weight (**C**) was determined in PN21 male and female offspring that had been exposed to maternal protein restriction. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀ n = 8, ♂ n = 8, LP ♀ n = 7, ♂ n = 7), * represents $P_{diet}<0.05$.

3.3.3 RENAL PHYSIOLOGY IN OFFSPRING EXPOSED TO MATERNAL LOW PROTEIN DIET

Renal physiological function was determined in male and female offspring exposed to either normal (NP) or low (LP) protein maternal diets during pregnancy and lactation. Experiments were carried out when offspring were 180 and 360 days of age.

At PN180, GFR was similar in NP and LP offspring (P_{diet}=0.37, P_{sex}<0.05, $P_{diet*sex}$=0.77). While, ERPF (P_{diet}=0.110, P_{sex}<0.05, $P_{diet*sex}$=0.07) was altered due to sex with ERPF been greater in males in six month old rats (Figure 3.5). UFR was not affected by maternal diet at PN180. Similarly, filtration rate was unaltered by maternal dietary exposure (P_{diet}=0.82 P_{sex}=0.27, $P_{diet*sex}$=0.73).

Electrolyte excretion was also determined in PN180 offspring. Sodium (P_{diet}=0.73, P_{sex}<0.05, $P_{diet*sex}$=0.95), potassium (P_{diet}=0.47, P_{sex}=0.10, $P_{diet*sex}$=0.84) and chloride (P_{diet}=0.78, P_{sex}<0.05, $P_{diet*sex}$=0.45) excretion were all unaltered by maternal protein restriction (Table 3.4).

At PN360 male and female offspring that had been exposed to maternal protein restriction demonstrated no differences in kidney function compared to control offspring (GFR P_{diet}=0.76, P_{sex}<0.05, $P_{diet*sex}$=0.29, ERPF P_{diet}=0.52, P_{sex}<0.05, $P_{diet*sex}$=0.43, UFR P_{diet}=0.33, P_{sex}=0.40. $P_{diet*sex}$=0.86, filtration fraction P_{diet}=0.34, P_{sex}=0.37, $P_{diet*sex}$=0.85).

Electrolyte excretion was also unchanged in male and female offspring exposed to maternal protein restriction compared to controls (Sodium Excretion P_{diet}=0.33 P_{sex}<0.05 $P_{diet*sex}$=0.87, Potassium Excretion P_{diet}=0.10 P_{sex}<0.05 $P_{diet*sex}$=0.17 and Chloride Excretion P_{diet}=0.13, P_{sex}<0.05, $P_{diet*sex}$=0.61, Table 3.4).

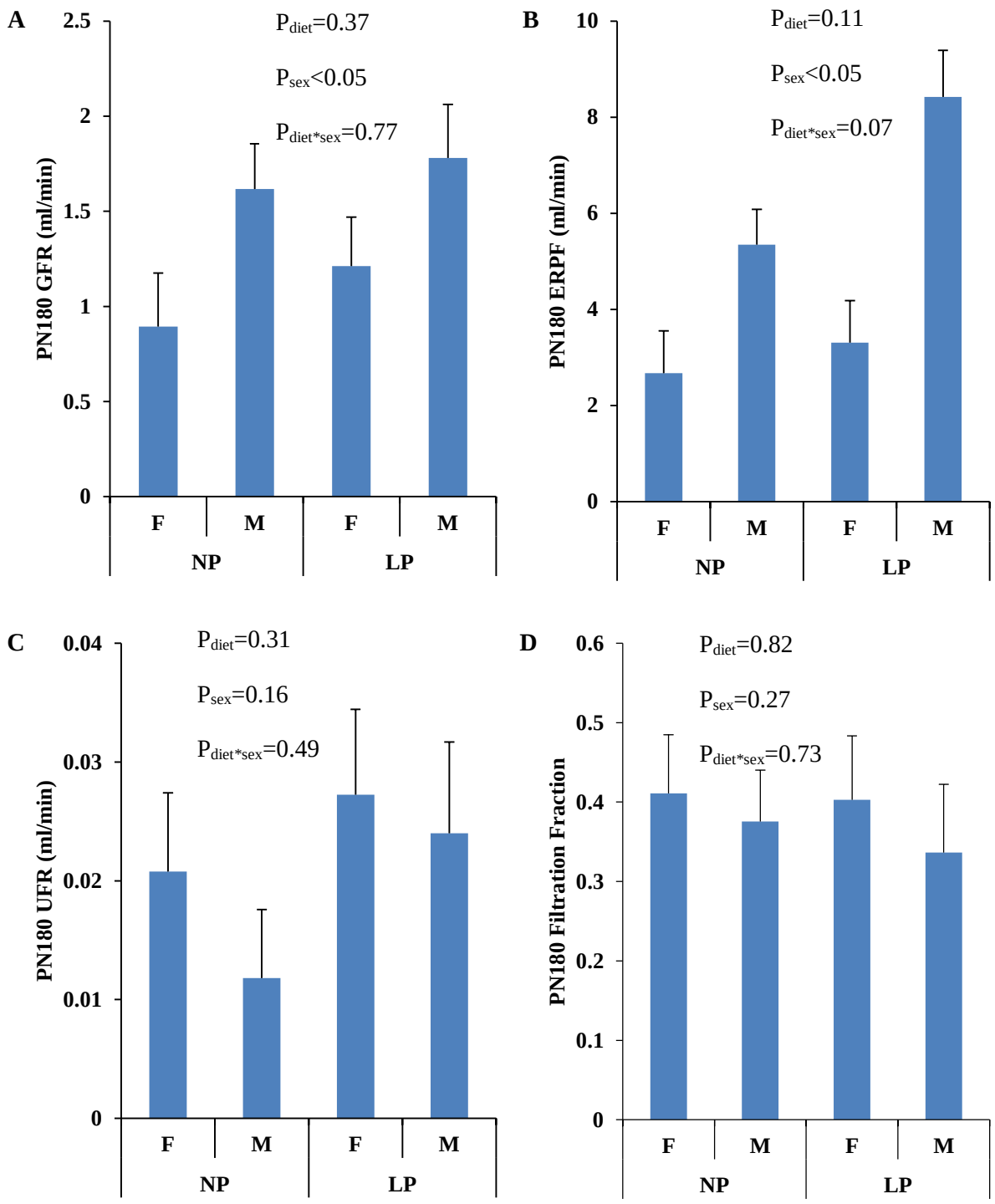

FIGURE 3.5 - PN180 RENAL FUNCTION IN FEMALE AND MALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION.

Glomerular filtration rate (**A**) and effective renal plasma flow (**B**) and urine flow rate (**C**) expressed as ml/min as well as filtration fraction (**D**) were determined in male and female offspring exposed to maternal LP and compared to offspring of NP fed mothers. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀ n = 5, ♂ n = 7, LP ♀ n = 6, ♂ n = 5).

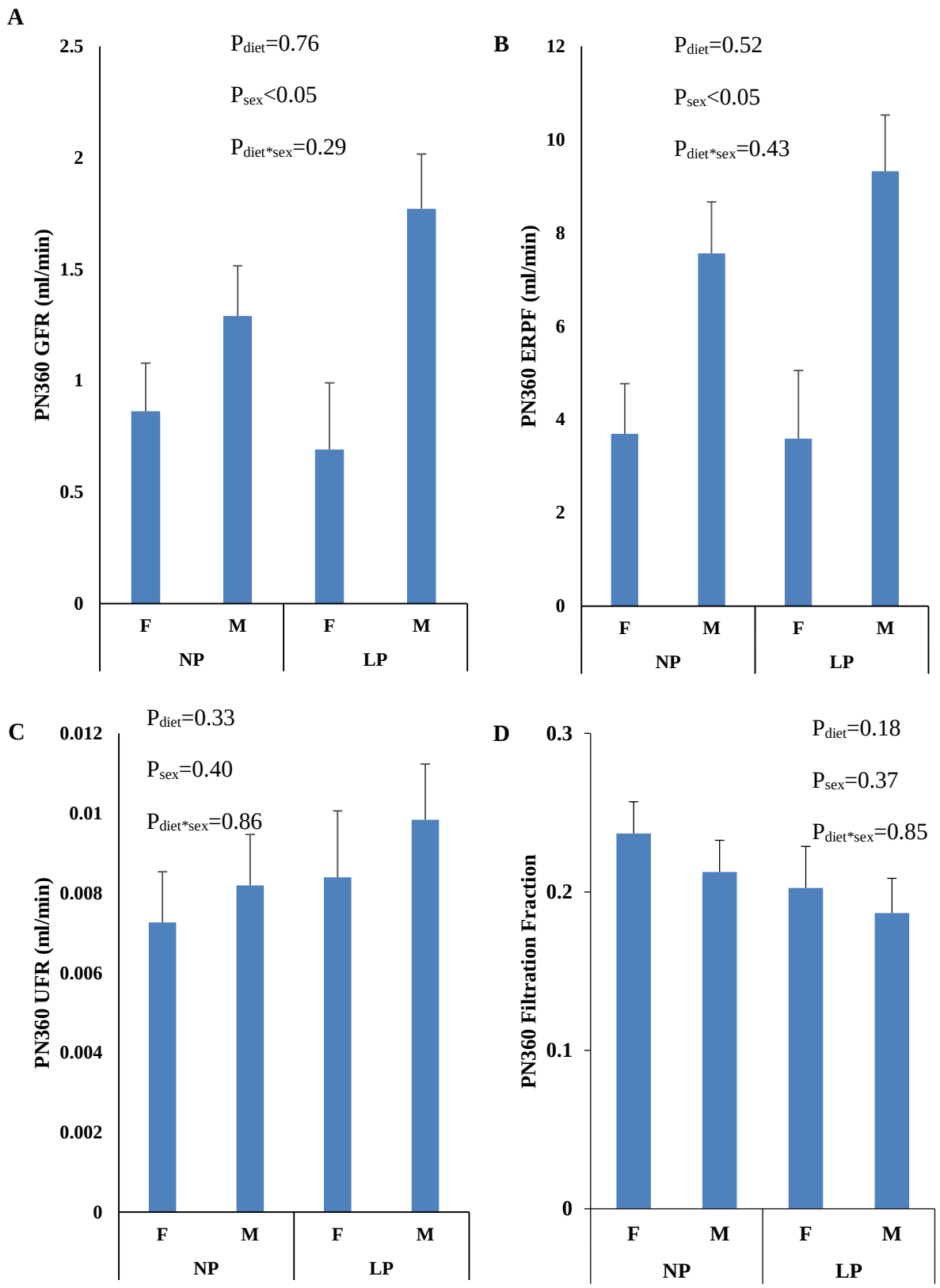

FIGURE 3.6 - PN360 RENAL FUNCTION IN FEMALE AND MALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION.

Glomerular filtration rate (**A**) and effective renal plasma flow (**B**) and urine flow rate (**C**) expressed as ml/min as well as filtration fraction (**D**) were determined in male and female offspring exposed to maternal protein restriction. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀ n = 12, ♂ n = 12, LP ♀ n = 7, ♂ n = 10).

TABLE 3.4 - PN180 ELECTROLYTE PROFILE OF PLASMA AND URINE PRODUCED BY MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION, INCLUDING EXCRETED VALUES.

		Plasma				Urine				Excreted Ealues		
Diet	Sex	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Osmolality (mOsm/kg)	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Osmolality (mOsm/kg)	Na (%)	K (%)	Cl (%)
NP	Female (n=4)	129.39 ± 11.63	3.28 ± 0.33	103.69 ± 7.52	324.25 ± 9.95	76.21 ±19.54	121.96 ± 28.99	151.70 ± 28.27	1323.11 ± 247.05	1.41 ± 0.42	44.24 ± 10.22	2.69 ± 0.58
	Male (n=7)	142.61 ± 8.77	3.67 ± 0.24	112.56 ± 6.00	309.38 ± 7.75	35.48 ± 14.41	143.59 ± 23.69	119.11 ± 20.88	1723.92 ± 213.75	0.27 ± 0.32	30.80 ± 7.72	0.92 ± 0.44
LP	Female (n=6)	156.88 ± 10.25	3.64 ± 0.27	116.18 ± 7.20	321.40 ± 9.22	92.58 ± 16.54	87.27 ± 28.67	136.45 ± 24.01	1070.82 ± 262.85	1.25 ± 0.34	39.54 ± 8.34	2.16 ± 0.47
	Male (n=5)	135.26 ± 11.42	3.45 ± 0.31	105.43 ± 7.78	305.23 ± 10.08	33.17 ± 18.67	94.19 ± 30.65	135.44 ± 27.07	1325.64 ± 274.61	0.17 ± 0.38	22.34 ± 9.14	1.16 ± 0.52
Significance	P_{diet}	0.45	0.84	0.77	0.77	0.75	0.27	0.97	0.35	0.73	0.47	0.78
	P_{sex}	0.60	0.72	0.84	**<0.05**	**<0.05**	0.41	0.42	**<0.05**	**<0.05**	0.10	**<0.05**
	$P_{diet*sex}$	**<0.05**	0.30	0.05	0.92	0.53	0.66	0.45	0.55	0.95	0.84	0.45

Sodium (Na), potassium (K) and chloride (Cl) plasma, sodium and excretory values for male and female offspring exposed to maternal protein restriction.

TABLE 3.5 - PN360 ELECTROLYTE PROFILE OF PLASMA AND URINE PRODUCED BY MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION, INCLUDING EXCRETED VALUES.

		Plasma				Urine				Excreted Values		
Diet	Sex	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Osmolality (mOsm/kg)	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Osmolality (mOsm/kg)	Na (%)	K (%)	Cl (%)
NP	F (n=11)	143.08 ± 6.13	3.55 ± 0.14	110.29 ± 3.39	342.78 ± 12.77	74.54 ± 9.21	66.45 ± 16.22	130.37 ± 17.78	1305.90 ± 171.27	0.61 ± 0.11	20.49 ± 4.13	1.18 ± 0.15
	M (n=10)	139.75 ± 6.43	3.61 ± 0.15	111.86 ± 3.55	334.11 ± 13.59	45.35 ± 9.92	86.07 ± 17.46	87.99 ± 18.62	1544.80 ± 179.63	0.26 ± 0.11	16.27 ± 4.44	0.47 ± 0.16
LP	F (n=6)	127.95 ± 8.30	3.21 ± 0.19	105.68 ± 4.59	313.43 ± 17.54	54.97 ± 13.24	109.45 ± 22.94	142.70 ± 24.04	1458.67 ± 231.90	0.47 ± 0.15	35.79 ± 5.91	1.36 ± 0.21
	M (n=10)	133.56 ± 6.43	3.43 ± 0.15	104.36 ± 3.53	310.58 ± 14.48	31.48 ± 9.97	118.46 ± 18.26	155.59 ± 18.62	1531.40 ± 179.63	0.15 ± 0.11	19.18 ± 4.46	0.82 ± 0.16
Significance	P_{diet}	0.13	0.10	0.12	0.17	0.17	0.12	0.05	0.72	0.33	0.10	0.13
	P_{sex}	0.87	0.37	0.97	0.55	**<0.05**	0.31	0.46	0.42	**<0.05**	**<0.05**	**<0.05**
	$P_{diet*sex}$	0.52	0.61	0.71	0.76	0.77	0.70	0.18	0.67	0.87	0.17	0.61

Sodium (Na), potassium (K) and chloride (Cl) plasma, sodium and excretory values and trans-tubular potassium gradient (TTKG) for male and female offspring exposed to maternal protein restriction.

3.3.4 BLOOD PRESSURE AND HEART RATE ANALYSIS

Blood pressure and heart rate was determined in PN360 male and female offspring exposed to maternal protein restriction using telemetry. Maternal diet did not alter heart rate (Active; P_{diet}=0.76, P_{sex}<0.05, $P_{diet*sex}$=0.25, Non-Active; P_{diet}=0.69, P_{sex}<0.05, $P_{diet*sex}$=0.16), mean arterial pressure (Active; P_{diet}=0.75, P_{sex}=0.70, $P_{diet*sex}$=0.52, Non-Active; P_{diet}=0.78, P_{sex}=0.88, $P_{diet*sex}$=0.98), systolic (Active; P_{diet}=0.76, P_{sex}=0.95, $P_{diet*sex}$=0.53, Non-Active; P_{diet}=0.45, P_{sex}=0.78, $P_{diet*sex}$=0.90) or diastolic blood pressure (Active; P_{diet}=0.37, P_{sex}=0.41, $P_{diet*sex}$=0.63, Non-Active; P_{diet}=0.89, P_{sex}=0.44, $P_{diet*sex}$=0.81) in offspring exposed to maternal LP diet compared with controls (Figures 3.7, 3.8 and Table 3.6).

Cardiovascular responses to an acute stressor (using an oscillating table) was determined (Figure 3.9). The response (change in MAP or heart rate from baseline to the average MAP or heart rate during the stress period) to the acute stressor was similar in male and female offspring exposed to maternal protein restriction or the normal diet (heart rate P_{diet}=0.15, P_{sex}<0.05, $P_{diet*sex}$=0.21, mean arterial pressure P_{diet}=0.63, P_{sex}=0.75, $P_{diet*sex}$=0.22, diastolic blood pressure P_{diet}=0.64, P_{sex}=0.80, $P_{diet*sex}$=0.22, systolic blood pressure P_{diet}=0.58, P_{sex}=0.90, $P_{diet*sex}$=0.33). Similarly, when using a novel non-aversive stressor (sultanas), the cardiovascular response (change in MAP or heart rate from base line to the average MAP or heart rate during the stress period) did not differ between the experimental groups (heart rate P_{diet}=0.86, P_{sex}=0.80, $P_{diet*sex}$=0.18, mean arterial pressure P_{diet}=0.73, P_{sex}<0.05, $P_{diet*sex}$=0.42, diastolic blood pressure P_{diet}=0.64, P_{sex}=0.06, $P_{diet*sex}$=0.56, systolic blood pressure P_{diet}=0.79, P_{sex}<0.05, $P_{diet*sex}$=0.28, Figure 3.10).

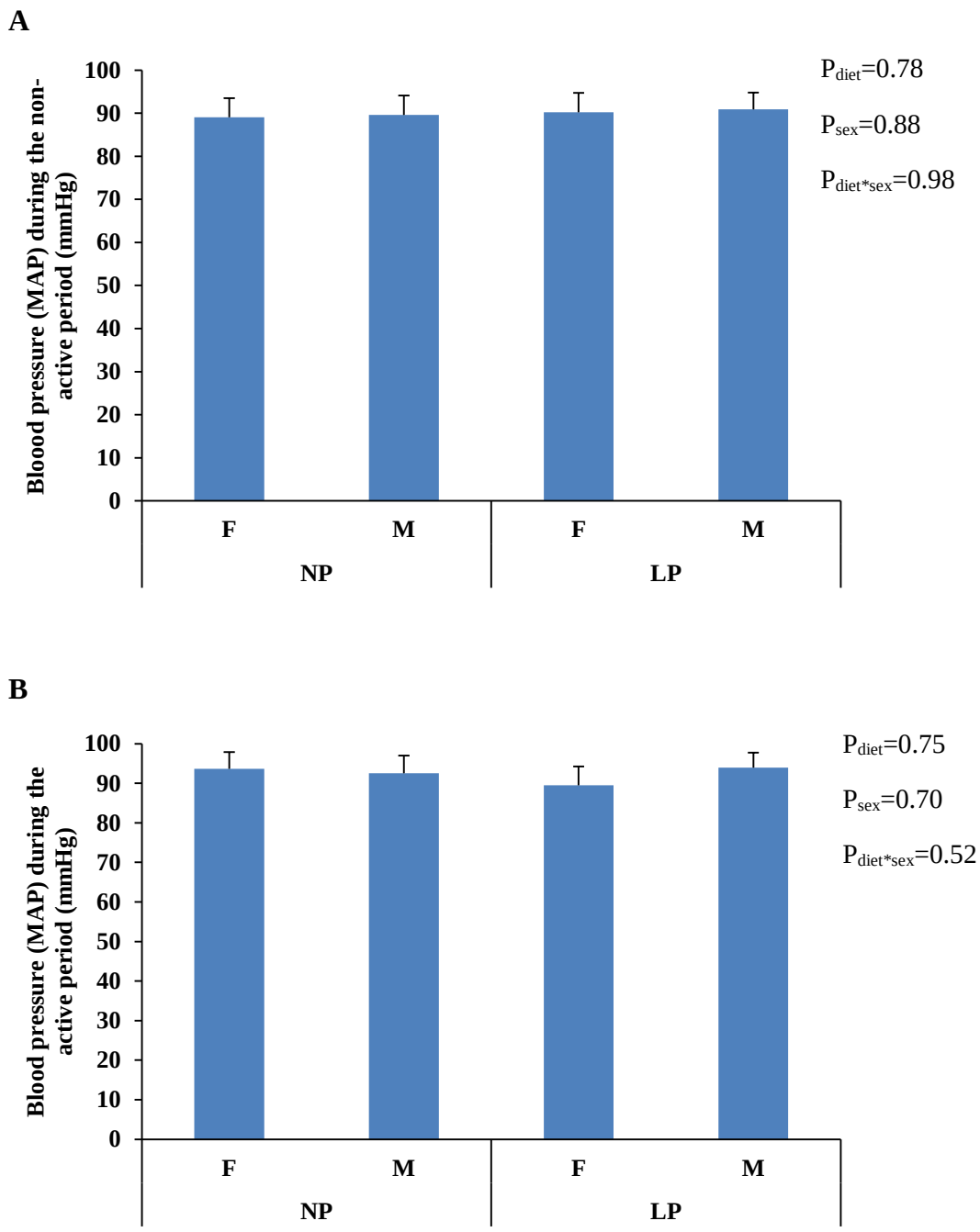

FIGURE 3.7 – PN360 MALE AND FEMALE OFFSPRING MEAN ARTERIAL PRESSURE.

Mean arterial pressure during the non-active (**A**) and active (**B**) period for male and female offspring exposed to maternal protein restriction. Mean arterial pressure was measured using an indwelling radio-telemetry device. The data presented is an average of a minimum of four 10 hour periods during the active part of the day. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀n=8, ♂n=8, LP ♀n=8, ♂n=11).

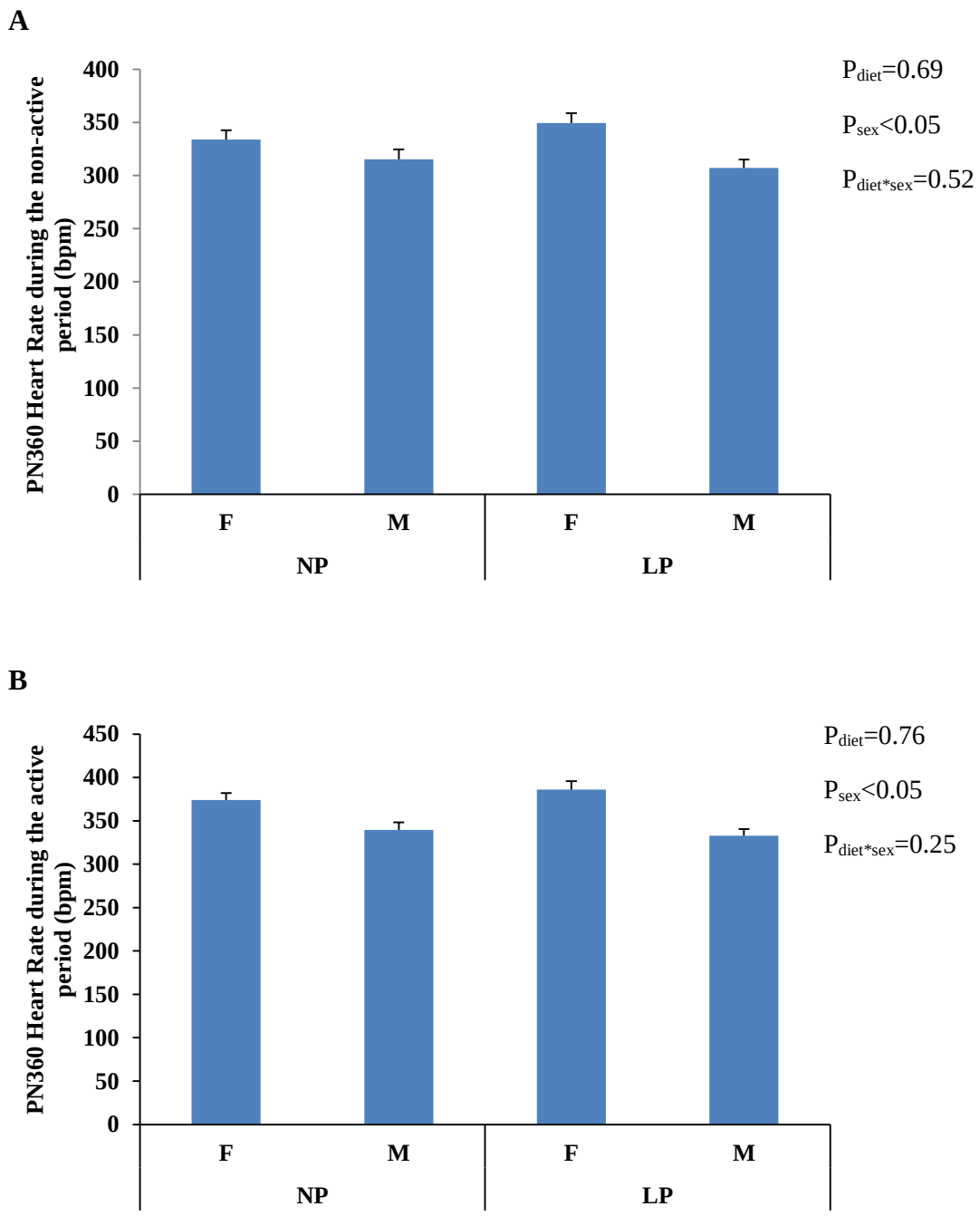

FIGURE 3.8 – PN360 MALE AND FEMALE OFFSPRING HEART RATE.

Heart rate during the non-active (**A**) and active (**B**) period for male and female offspring exposed to maternal protein restriction. Heart rate pressure was measured using an indwelling radio-telemetry device. The data presented is an average of a minimum of four 10 hour periods during the active part of the day. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀n=8, ♂n=8, LP ♀n=8, ♂n=11).

TABLE 3.6 - PN360 BLOOD PRESSURE FOR MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION.

		Non-Active Period			Active Period		
Diet	Sex	MAP (mmHg)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	SBP (mmHg)	DBP (mmHg)
NP	F (n=8)	89.05 ± 4.47	105.89 ± 5.74	73.74 ± 4.19	93.68 ± 4.21	110.46 ± 5.41	79.39 ± 3.94
	M (n=8)	89.62 ± 4.50	103.68 ± 5.82	77.81 ± 4.25	92.55 ± 4.46	106.59 ± 5.74	80.86 ± 4.18
LP	F (n=8)	90.19 ± 4.53	109.64 ± 5.88	74.10 ± 4.29	89.46 ± 4.77	108.66 ± 6.14	73.73 ± 4.47
	M (n=11)	90.93 ± 3.83	108.77 ± 4.95	76.29 ± 3.61	93.95 ± 3.80	111.84 ± 4.90	79.08 ± 3.57
Significance	P_{diet}	0.78	0.45	0.89	0.75	0.76	0.37
	P_{sex}	0.88	0.78	0.44	0.70	0.95	0.41
	$P_{diet*sex}$	0.98	0.90	0.81	0.52	0.53	0.63

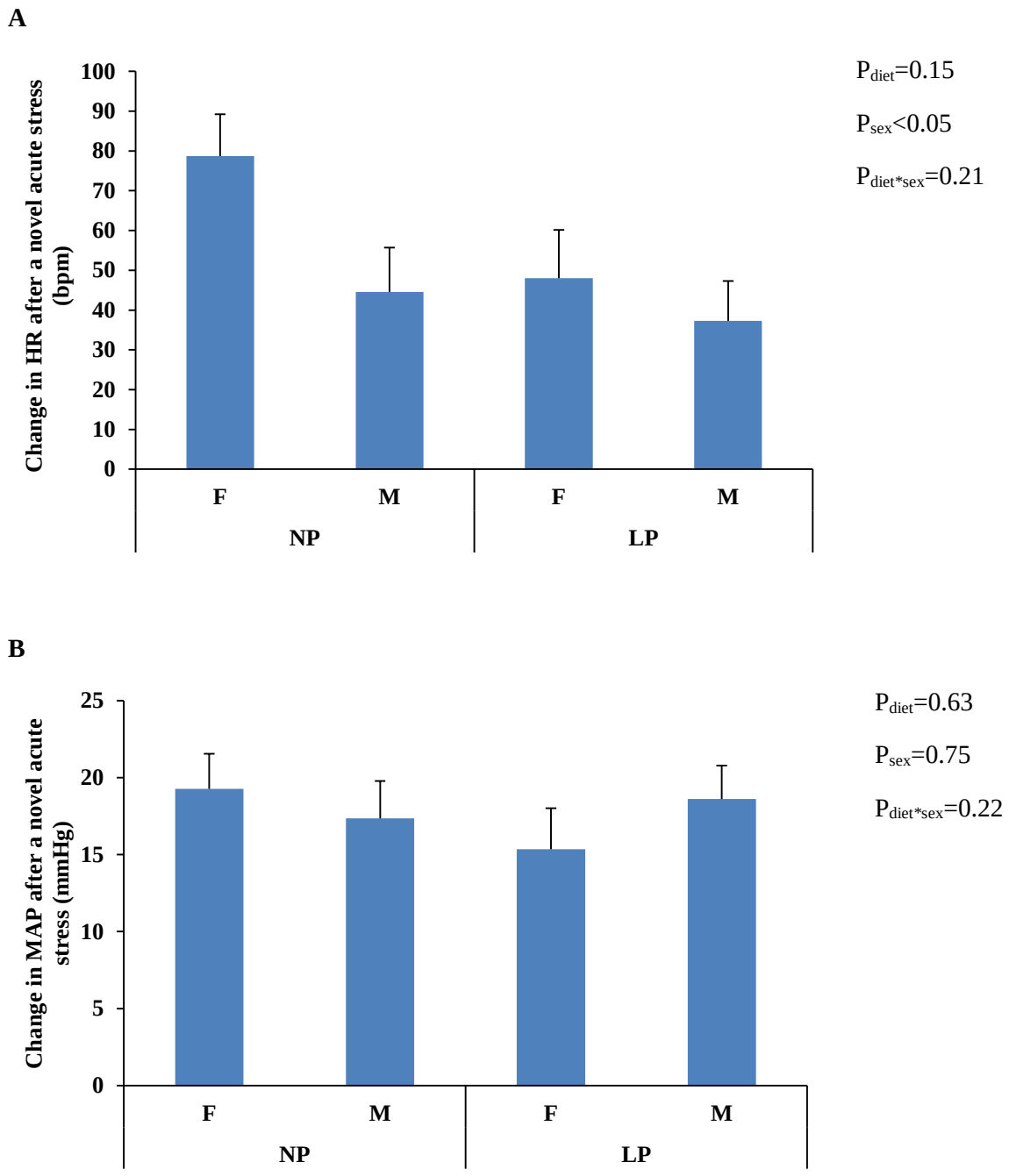

FIGURE 3.9 – PN360 MALE AND FEMALE OFFSPRING CHANGE IN MEAN ARTERIAL PRESSURE AND HEART RATE AFTER EXPOSURE TO A NOVEL ACUTE STRESSOR.

Change in heart rate (**A**) and mean arterial pressure (**B**) after exposure to a novel acute stressor in male and female offspring exposed to maternal protein restriction. Mean arterial pressure was measured using an indwelling radio-telemetry device. The data presented is the difference between averaged mean arterial pressure 5 minutes before the stress and 10 minutes during the stress. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀n=8, ♂n=8, LP ♀n=8, ♂n=11).

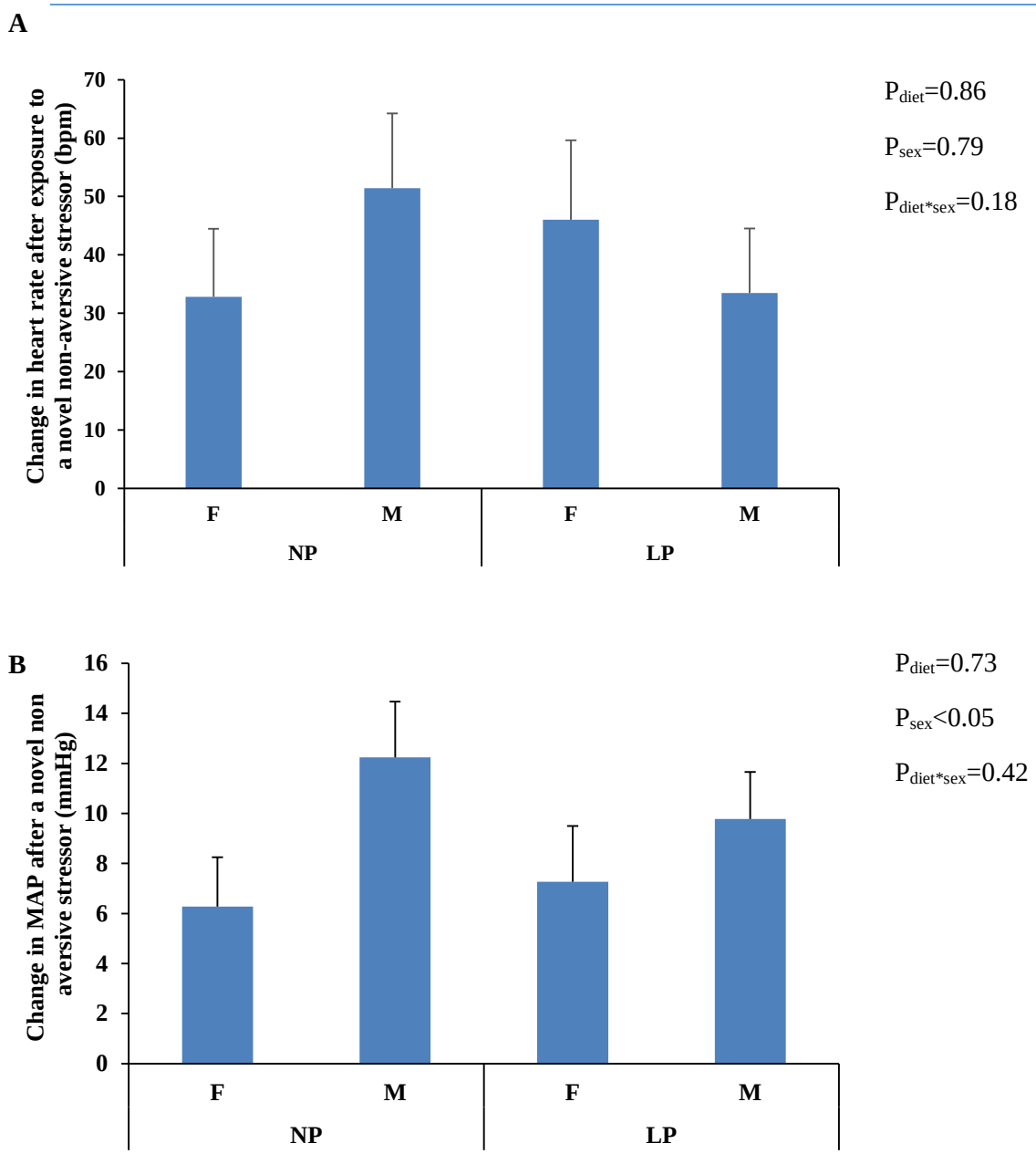

FIGURE 3.10 – PN360 MALE AND FEMALE OFFSPRING CHANGE IN MEAN ARTERIAL PRESSURE AND HEART RATE AFTER EXPOSURE TO A NOVEL NON-AVERSIVE STRESSOR.

Change in heart rate (**A**) and mean arterial pressure (**B**) after exposure to a novel non-aversive stressor in male and female offspring exposed to maternal protein restriction. Mean arterial pressure was measured using an indwelling radio-telemetry device. The data presented is the difference between averaged mean arterial pressure 5 minutes before the stress and 10 minutes during the stress. Data expressed as mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀n=8, ♂n=8, LP ♀n=8, ♂n=11).

3.3 DISCUSSION

The review of the literature in Chapter 1 identified a number of key limitations in current understanding of the role of maternal protein restriction in pregnancy upon offspring cardiovascular and renal function. We hypothesised that, compared with controls, offspring of protein restricted rats would demonstrate:

i) *reduced growth,*

ii) *reduced nephron number,*

iii) *invariant mean arterial pressure and heart rate, and*

iv) *invariant glomerular filtration rate and effective renal plasma flow.*

Maternal protein restriction lead to reduced growth (however proportional to the amount of fat and muscle) after weaning. Analysis of organ weights revealed that at PN21 offspring exposed to maternal low protein diet had small kidneys that remained smaller when adjusted for body weight. At PN180, brain and liver weight per gram of body weight was larger in LP offspring. However, by PN360 there was no difference in organ weight between the dietary groups. These data suggest that animals grow relatively proportionally throughout postnatal life as there is no change in body fat or lean muscle mass percentage. While kidney weight remained significantly lower in offspring exposed to maternal LP diet, these animals also had heavier livers and brains for their body size. This phenomenon has been well described by others (Bol *et al.*, 2008, Hoppe *et al.*, 2007b, Joanette *et al.*, 2004).

Overall, however the growth data suggest a lack of catch-up growth in the offspring of LP fed rats. Catch-up growth can occur in models of programming due to a mis-matching between the *in utero* and postnatal diets (Gluckman and Hanson, 2006, Gluckman *et al.*, 2008). Catch-up growth predominantly results in greater fat deposition without compensatory growth in organ size (Coupe *et al.*, 2012). Therefore, offspring end up obese with proportionally (to body

weight) smaller organs. In this study, exposure to maternal protein restriction led to offspring that did not undergo catch-up growth after weaning and remained proportionally smaller than control offspring.

Nephron number was determined after nephrogenesis had ceased and before animals had been weaned from dams. A 17% and 26% nephron deficit in male and female kidneys exposed to maternal protein restriction is of a similar magnitude to other reports (Habib *et al.*, 2011, Harrison and Langley-Evans, 2009, Hoppe *et al.*, 2007a, Langley-Evans *et al.*, 1999b, Lloyd *et al.*, 2012, McMullen *et al.*, 2004, Vehaskari *et al.*, 2001, Zimanyi *et al.*, 2004). However, we also report the number of nephrons per gram kidney weight and body weight. Nephron number per gram kidney (Habib *et al.*, 2011, Sahajpal and Ashton, 2003) and body (Vehaskari *et al.*, 2001) weight have been previously reported and both studies found that these parameters remain smaller in offspring of LP rats than controls. Contrarily here, nephron number per gram of kidney weight was not different between dietary groups or sex (even though kidneys were smaller in offspring exposed to maternal low protein diet at this age). Most interesting is that protein restricted offspring were found to have more nephrons per gram of body weight. This suggests a greater filtration surface area to body weight ratio. High nephron endowment has been reported to be protective against sodium induced hypertension (Walker *et al.*, 2012). It remains to be determined if the nephron reserve of offspring exposed to maternal LP diet is functional and able to respond to physiological stress.

Blood pressure and heart rate was determined in postnatal offspring at PN360 and were not different between offspring exposed to maternal protein restriction and those exposed to a normal maternal environment. It has been previously hypothesised that there would be higher blood pressure in rats exposed to maternal low protein diet given the overall reduction in nephron number. Previous studies using tail cuff plethysmography report that offspring become hypertensive at 4-6 weeks of age (Langley-Evans *et al.*, 1994). Compared to our study, these

offspring had undergone catch-up growth and blood pressure had been measured using a method known to induce stress. In the present study we observed that offspring of LP dams were smaller (weighed less and had proportionally less muscle and fat) than controls and blood pressure was the same in both dietary groups. To ascertain whether a stress would elucidate a different cardiovascular response in offspring exposed to maternal LP diet, we performed aversive and non-aversive stress tests on offspring. Offspring responded the same way to stress tests. This indicates that even under stress, offspring exposed to maternal LP diet, were not hyper-reactive. The lack of hypertension, combined with the nephron number data suggest that the Brenner hypothesis was not supported. While nephron number is *associated* with blood pressure (Hall *et al.*, 1996, Guyton, 1991c), our data clearly show that low nephron number does not lead to elevated blood pressure in this model. In fact, low nephron number may not be detrimental on its own as it may require a second-hit, such as obesity, a high salt diet or diabetes. The model utilised in this chapter presents the opportunity to further elucidate the associations between nephron number, blood pressure development and postnatal growth.

Nwagwu *et al.* (2000) reported reduced GFR (measured using creatinine clearance) at 4 weeks of age following a maternal low protein diet, while Hoppe *et al.* (2007b) in a study of lifelong low protein exposure reported no change in GFR or ERPF ([^{3}H] inulin and PAH 24 hour clearance) per gram of body weight. Similarly, Sahajpal and Ashton (2003) report that GFR was unchanged in offspring exposed to maternal LP compared to controls (Sahajpal and Ashton, 2003) In this study we determined GFR and ERBF at PN180 and 360 (reported as a proportion of body weight) and found no differences in kidney perfusion or filtering capacity. While it should be noted that due to the short period of equilibrium (1 hour) rats may not have reached equilibrium with the infusion rate. While the finding that maternal protein restriction did not alter kidney perfusion or filtering capacity compared to controls could be a consequence of a nephron reserve, as offspring would have a large filtration surface area for their given body

surface area. Offspring exposed to maternal LP diet appeared to be healthy, showing no diminution in function given the parameters measured. While at PN3600 there was some indication of increased plasma flow in male LP offspring, while it was reduced in female offspring compared to controls. This may indicate that there is age-related change in kidney function after exposure to maternal LP and warrants further investigation.

The findings from this study prompt further research into the effects of a postnatal second hit on susceptibility to renal and cardiovascular disease. It is possible the normal kidney and cardiovascular function observed in LP offspring in the present study were due to the fact that the offspring remained proportionally small and had proportionally more nephrons. This novel finding of a relative nephron reserve may indicate alterations during kidney development (branching morphogenesis or nephrogenesis). Knowledge of when and how maternal diet can alter kidney development will allow better recognition of pregnancies that may be at risk of offspring developing a nephron deficit.

CHAPTER FOUR: CONSEQUENCES OF A MATERNAL LOW PROTEIN DIET FOR THE DEVELOPING RAT KIDNEY

4.1 INTRODUCTION

Exposure to a sub-optimal environment during fetal life is often associated with renal and cardiovascular disease in adulthood. Although cardiovascular disease may arise via several mechanisms, data from both human and animal studies implicate a reduction in nephron number as being central to the disease process (Douglas-Denton *et al.*, 2006, Hoy *et al.*, 2006a). The number of nephrons in normal human kidneys is related to weight at birth - a proxy marker of a sub-optimal intrauterine environment (Hughson *et al.*, 2003, Hughson *et al.*, 2008, Lackland and Barker, 2009). A large number of animal studies indicate that a sub-optimal intrauterine environment induced by maternal low protein (LP) diet, results in offspring kidneys containing 20-30% fewer nephrons than control kidneys (Hoppe *et al.*, 2007a, Sahajpal and Ashton, 2003, Hoppe *et al.*, 2007b, Langley and Jackson, 1994, Langley-Evans, 1997, Langley-Evans *et al.*, 1999a, McMullen and Langley-Evans, 2005a, Woods *et al.*, 2001, Woods *et al.*, 2004), which may contribute to hypertension and chronic kidney disease (Brenner *et al.*, 1988). In support of the literature, the experimental data shown in Chapter 3 indicate that by postnatal day 21 offspring of maternal LP have a nephron deficit. When and how this occurs has not previously been studied in great detail.

In the rat, the first renal vesicles can be histologically detected at approximately E14-15 and the first glomeruli at E16-17. Using tissue collected as part of this PhD thesis, an Honours student in our laboratory (Mr Luke Eipper) demonstrated that the nephron deficit is present as early as E17.25 indicating that kidney development is altered in the earliest stages of nephrogenesis in offspring of maternal LP. This aims to identify some possible underlying mechanisms programming the nephron deficit.

Development of the permanent mammalian kidney, the metanephros, begins when the ureteric bud invades the metanephric mesenchyme (at E13.5 in the rat) and commences the process of

branching morphogenesis, which ultimately forms the ureteric tree. Branching morphogenesis is a critical event in kidney development because it establishes the number of ureteric tips available for nephron induction (Cullen-McEwen *et al.*, 2001, Dressler, 2006, Sakurai *et al.*, 2005). In the present study, whole metanephric organ culture was used to determine if the nephron deficit observed at E17.5 following a maternal LP diet involves a reduction in early branching morphogenesis. Fetal kidneys were removed and cultured at an air/media interface. At the completion of the culture kidneys were fixed and immunohistochemically stained to mark the ureteric epithelium, and the number of ureteric branch points and tips were then counted (Bernstein *et al.*, 1981, Bullock *et al.*, 2001, Singh *et al.*, 2007).

A number of genes have been shown to promote branching morphogenesis in the kidney. Glial cell line-derived neurotrophic factor (*Gdnf*) is a vital growth factor during kidney development (Basson *et al.*, 2006, Costantini and Kopan, 2010, Costantini and Shakya, 2006). Indeed, GDNF knockout mice are born with renal agenesis (Moore *et al.*, 1996). GFRα-1 and Ret are integral receptors of GDNF, and GFRα-1 knockout mice have similar renal defects as GDNF knockout mice (Cacalano *et al.*, 1998). Conversely, bone morphogenetic protein 4 (BMP4) inhibits ectopic and lateral ureteric branching (Cain and Bertram, 2006). To date, expression of these genes has not been analysed during early branching morphogenesis (E14.25) in a model of maternal protein restriction.

The process of nephron formation (nephrogenesis) is dependent on branching morphogenesis because nephrogenesis involves complex molecular interactions between the metanephric mesenchyme and the invading ureteric bud. These reciprocal interactions result in condensation of the mesenchymal cells surrounding the ureteric tips, mesenchyme to epithelial transition (MET), vascularization of the glomerular tuft and formation of renal tubules (Burrow, 2000). A number of genes are involved in nephrogenesis, however *Wnt4* appears to play a key role in MET with *Wnt4* null mice having few, if any nephrons (Davies *et al.*, 2004, Shan *et al.*, 2009).

Likewise, HNF4α is an important factor during MET, as disrupted *Hnf4α* gene expression (caused by siRNA) in mice led to disordered cap mesenchyme (Kanazawa *et al.*, 2011). *Pax2* is a transcription factor involved in nephron determination (Rothenpieler and Dressler, 1993). To date, no studies have analysed the expression of *Gdnf, Wnt4, Pax2, BMP4* or *HNFa* in the kidneys of offspring subject to maternal protein restriction. Indeed the only two studies to have assessed kidney development in the face of a suboptimal intrauterine condition are those by Abdel-Hakeem *et al.* (2008) and Welham *et al.* (2002).

The aims of the experiments described in this Chapter were: (1) to quantify the number of nephrons at E17.25 in male and female offspring and quantify branching morphogenesis (using whole kidney organ culture) in rats subjected to a maternal LP diet; and (2) to compare the expression of genes (*Bmp4*, *Gdnf*, *Gfrα1*, *Wnt4*, *Pax2* and *Hnf4α*) known to play a role in kidney development at E14.25 in normal and LP male and female offspring. We hypothesized that nephrogenesis and branching morphogenesis would be impaired as a result of exposure to the LP diet (evidenced by reduced number of glomeruli at E17.25 and branch points and tips in the ureteric tree after 2 days culture from E14.25), and predicted that expression of branching stimulators such as *Gdnf* and *Gfrα1* would be reduced whereas expression of branching inhibitory genes such as *Bmp4* would be increased. We further hypothesized that exposure to a maternal LP diet would result in reduced expression of the key nephrogenic genes *Wnt4*, *Hnf4α* and *Pax2* at E14.25.

4.2 METHODS

Experiments were conducted in accordance with the National Health and Medical Research Council of Australia '*Australian Code of Practice for the Care and Use of Animals for Scientific Purposes'* (7th edition, 2004). Approval was granted in advance by the Monash University School of Biomedical Sciences Animal Ethics Committee. All methods have been fully documented in Chapter 2.

4.2.1 ANIMAL HUSBANDRY

Nine week old female Sprague-Dawley rats were housed in pairs and allowed a 3 week acclimatization period before being fed *ad libitum* a normal protein (NP, 19.4% wt/wt, Glen Forest WA, Australia) or a near isocaloric low protein (LP, 8.4% wt/wt, Glen Forest WA, Australia) diet. This LP diet has previously been shown to produce a nephron deficit in rodent offspring (Hoppe *et al.*, 2007a, Hoppe *et al.*, 2007b, Zimanyi *et al.*, 2006). Full details of the diets are provided in Sections 2.2 and 2.2.1. These female rats were time-mated (3 hours) and remained on the diet throughout pregnancy until the day of tissue collection (E14.25, E17.25 and E20). Ages for tissue analysis were selected to correlate with stages of kidney development; early branching morphogenesis (E14.25), mid-branching morphogenesis and early nephrogenesis (E17.25), and growth of the fetus and placenta (E20).

4.2.2 QUANTIFICATION OF NEPHRON NUMBER AT E17.25

A technique based on the gold-standard physical disector was used to estimate nephron number at E17.25 (Cullen-McEwen *et al.*, 2011). Briefly, fetuses were collected from pregnant dams 17.25 days after being mated (see Section 2.3.2 for details). Kidneys were removed from fetuses and fixed in 4% PFA before being transferred to 70% ethanol and hand-processed to paraffin.

Kidneys were exhaustively sectioned at 4µm where every 20th and 22nd section was taken and immunohistochemically stained with PNA (see Section 2.3.5). For quantification of nephron number, kidney sections were observed using a microscope at 150x magnification and equipped with a projector arm to enable tracing of the section. Nephron number was determined by counting the number of PNA-positive structures (see Section 2.4.1). These counts were performed by Honours student Luke Eipper and myself.

4.2.3 STUDY OF BRANCHING MORPHOGENESIS BY METANEPHRIC CULTURE AT E14.25

E14.25 kidneys were dissected from NP and LP offspring using aseptic technique and either snap frozen for molecular analysis or assigned to the culture study. Kidneys assigned to the culture study were immediately placed into a petri dish containing serum-free Dulbecco's modified Eagle's medium (DMEM): Ham's F12 liquid medium (Trace Biosciences, Castle Hill, NSW, Australia) supplemented with 5µg/ml transferrin (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) and 12.9µl/ml L-glutamine (Trace Biosciences, Castle Hill, NSW, Australia), warmed to 37°C (Cullen-McEwen *et al.*, 2002, Singh *et al.*, 2007).

Whole kidneys were placed on 3.0µm pore polycarbonate transfilter membranes (Transwell, Corning Star, Cambridge, MA, USA) and cultured in 24-well culture plates in 500µl serum-free culture media for 48 hours at 5% CO_2 and 37°C. To avoid the confounding effects of a media supplemented with serum, a serum-free media was used (Taub and Livingston, 1981). For more information refer to Section 2.3.1.

4.2.4 IMMUNOHISTOCHEMISTRY FOR QUANTIFICATION OF BRANCHING MORPHOGENESIS

After 48 hours of organ culture, metanephroi were fixed in methanol at -20°C. For details on examination with wholemount immunofluorescence microscopy refer to Section 2.3.1.2. Organs were viewed using a fluorescence microscope (Olympus Provis Microscope) at 4x and 10x magnification and photomicrographs taken to assist in quantification of branching

morphogenesis. Branching morphogenesis was quantified by manually skeletonising the ureteric tree (Figure 4.1). A branch point was defined as the intersection of three or more skeleton lines. Ureteric tips were defined as terminal branch ends (Figure 4.2).

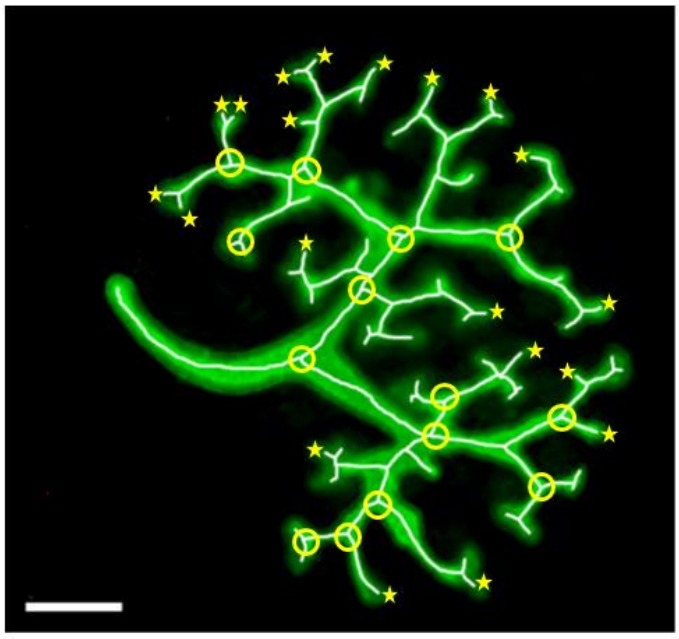

FIGURE 4.1 - SKELETONISED URETERIC TREE USED FOR QUANTIFICATION OF BRANCHING MORPHOGENESIS.

Circles indicate branch points and stars indicate ureteric tips (NB: Not all branch points or ureteric tips are indicated). Scale bar represents 100µm.

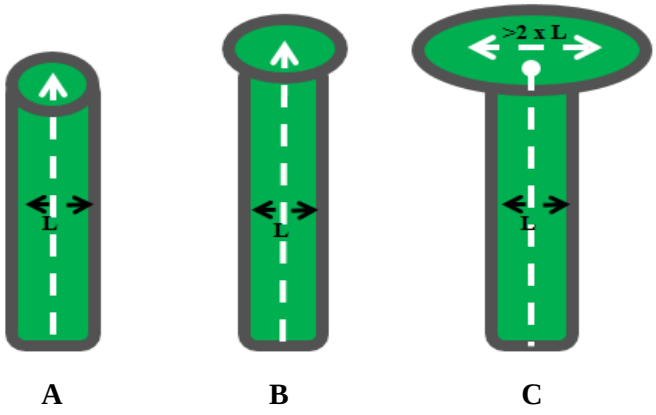

FIGURE 4.2 - DETERMINING BRANCH POINTS VERSUS URETERIC TIPS.

To differentiate between a branch point (circle, **C**) and a ureteric tip (arrow head, **A**, **B**, **C**), the rule of 2 x L, where L is the diameter of the epithelial tube was applied. **A**) denotes a ureteric tip with no thickening or indication of branching, and is therefore counted as a single tip. **B**) denotes a ureteric tip with some thickening; however this thickening is not greater than L, and therefore this is defined as a ureteric tip and not a branch point. **C**) shows a branch point leading to two ureteric tips as this distance is greater than L.

4.2.5 DNA EXTRACTION AND AMPLIFICATION FOR SEX DETERMINATION

The sex of fetuses at E14.25 was determined by genotyping for the presence of SMC (structural maintenance of chromosome) X/Y using Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, Castle Hill, Australia). Please refer to Section 2.3.4.1 for more details.

4.2.6 RNA AND DNA EXTRACTION OF E14.25 KIDNEYS

RNA and DNA were extracted from both kidneys at E14.25 using a commercially available kit (AllPrep DNA/RNA Mini Kit, Qiagen, Valencia, CA) and according to the manufacturer's recommended protocol. RNA concentration and quality were determined using a NanoDrop spectrophotometer (ThermoScientific, Australia). The ratio of absorbance at 260/280nm indicated the quality of the sample. For RNA, a ratio between 1.9 and 2.2 was considered sufficient for gene expression (Real Time PCR) studies (Mathieson and Thomas, 2012). For more details refer to Section 2.3.4.2.1.

4.2.7 RNA REVERSE TRANSCRIPTION, CDNA PRODUCTION AND REAL TIME PCR

A 0.05µg RNA sample (from a minimum of 2 male and female kidneys from each litter) was reverse transcribed (Applied Biosystems Reverse Transcription Reagent Kit, Applied Biosystems, Australia). The sample was then run in a thermocycler to produce cDNA (Superscript VILO cDNA synthesis kit, Life Sciences, Australia,). For Real-Time PCR (RT PCR) SYBR Green was used following the manufacturer's protocol (SensiMix SYBR & Fluorescein Kit, Quantace, Australia). This assay was used to determine relative levels of mRNA expression of genes involved in branching morphogenesis (*Gdnf*, *Gfrα1*, *Bmp4*) and nephrogenesis (*Wnt4*, *Pax2*, *Hnf4α*). Genes were compared with 18S as the housekeeper gene. For more information refer to Section 2.3.4.2.2.

4.2.8 STATISTICAL ANALYSIS

Data were analysed using a mixed linear model taking into account litter representation (n values represent litter numbers NOT the total number of animals studied because multiple animals from each litter were chosen for analysis) (SPSS 21, IBM). Data expressed as mean ± SEM, statistical significance is represented by $P<0.05$, whereby P_{diet} represents the impact of maternal LP diet, P_{sex} represents the influence of offspring sex and $P_{diet*sex}$ represents the interaction of these variables. Wherever appropriate, repeated measures ANOVA models were used. Graphs built using Microsoft Office (Microsoft Excel 2013, Microsoft, USA).

4.3 RESULTS

4.3.1 FETAL GROWTH

Offspring exposed to a maternal LP diet did not demonstrate altered bodyweight in mid-gestation (E14.25; P_{diet}=0.96; E17.25; P_{diet}=0.72), however by E20 there was a trend towards a heavier bodyweight (P_{diet}=0.06) compared with controls. Placental weight was 20% lower in LP offspring at E17.25 (P_{diet}<0.05) and 15% lower at E20 (P_{diet}<0.05). The fetal to placental weight ratio was similar in both groups at E14.25 and 17.25, but was greater at E20 in LP offspring (P_{diet}<0.05, Table 4.1).

TABLE 4.1 - FETAL AND PLACENTAL WEIGHTS AND FETAL TO PLACENTAL WEIGHT RATIO AT E14.25, 17.5 AND 20 IN NP AND LP OFFSPRING.

Age	Parameter	NP (mean ± SEM)	LP (mean ± SEM)	P value
E14.25 (NP n=5, LP n=6)	Fetal weight (mg)	158 ± 5	159 ± 7	P=0.96
	Placenta weight (mg)	157 ± 6	163 ± 8	P=0.54
	Ratio	1.04 ± 0.03	1.01 ± 0.04	P=0.55
E17.5 (NP n=4, LP n=4)	Fetal weight (mg)	738 ± 27	754 ± 30	P=0.72
	Placenta weight (mg)	380 ± 17	305 ± 19	**P<0.05**
	Ratio	2.04 ± 0.15	2.52 ± 0.17	P=0.07
E20 (NP n=9, LP n=4)	Fetal weight (g)	2.83 ± 0.12	3.25 ± 0.15	P=0.06
	Placenta weight (mg)	502 ± 18	422 ± 22	**P<0.05**
	Ratio	5.76 ± 0.39	7.84 ± 0.48	**P<0.05**

4.3.2 NEPHRON NUMBER AT E17.25

Nephron number was 14% lower at E17.25 in fetuses exposed to maternal LP diet compared with NP offspring (NP Female 218±8, Male 235±7, LP Female 193±7, Male 197±12, $P_{diet}<0.05$). The effect of the diet was similar between sexes ($P_{sex}=0.25$, $P_{diet*sex}=0.49$, Figure 4.3).

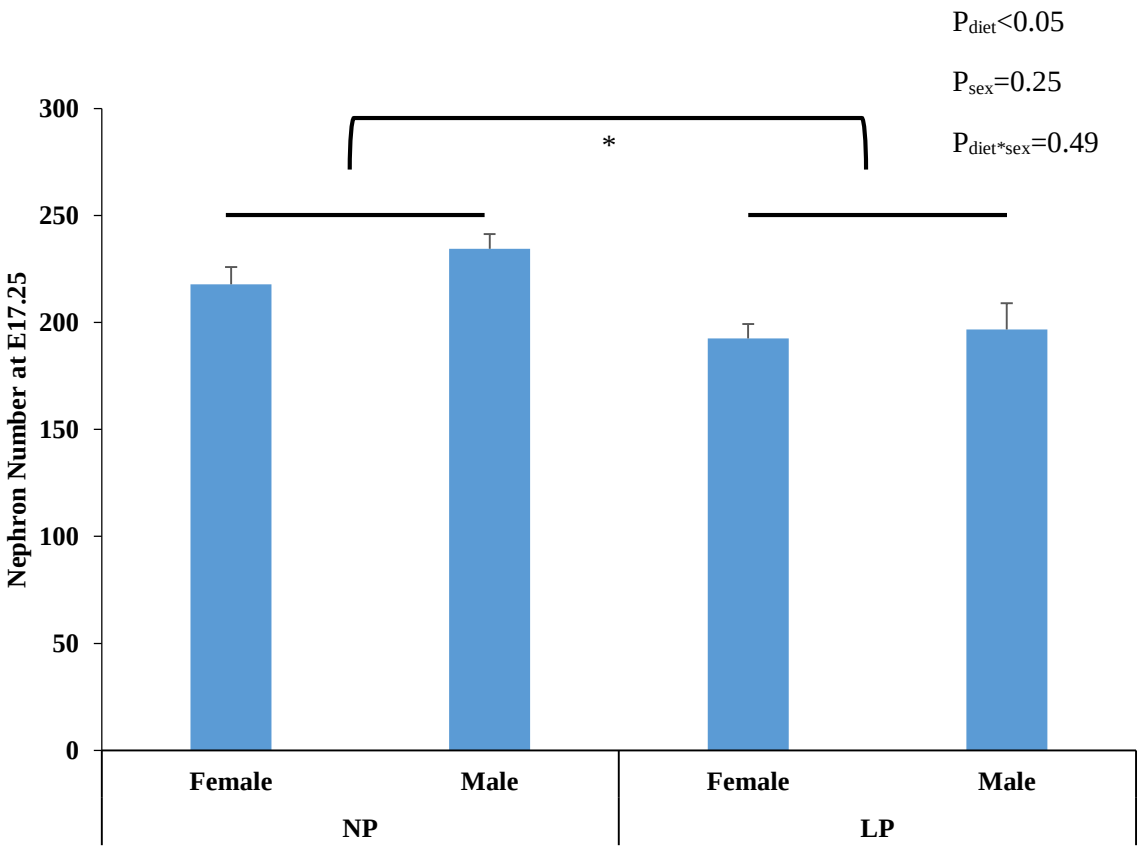

FIGURE 4.3 – NEPHRON NUMBER AT E17.25.

Nephron number was determined in E17.25 female and male kidneys that had been exposed to maternal normal (NP) or low protein (LP) diet. Nephrons were counted using a modified disector/fractionator technique to count PNA-positive structures. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♂ & ♀ n=8, LP ♂ & ♀ n=8), * $P_{diet}<0.05$.

4.3.3 BRANCHING MORPHOGENESIS

Exposure to a maternal LP diet did not appear to alter early branching morphogenesis assessed *in vitro*. The number of branch points did not differ between the sexes and therefore data were pooled to maximise statistical power. The number of branch points did not differ in fetal kidneys as a result of maternal protein restriction (NP; 45.98±4.3, LP 57.3±4.7, P_{diet}=0.49). The number of ureteric tips was also similar in the two groups (NP; 47.69±4.6, LP; 52.69±5.5, P_{diet}=0.49, Figure 4.4).

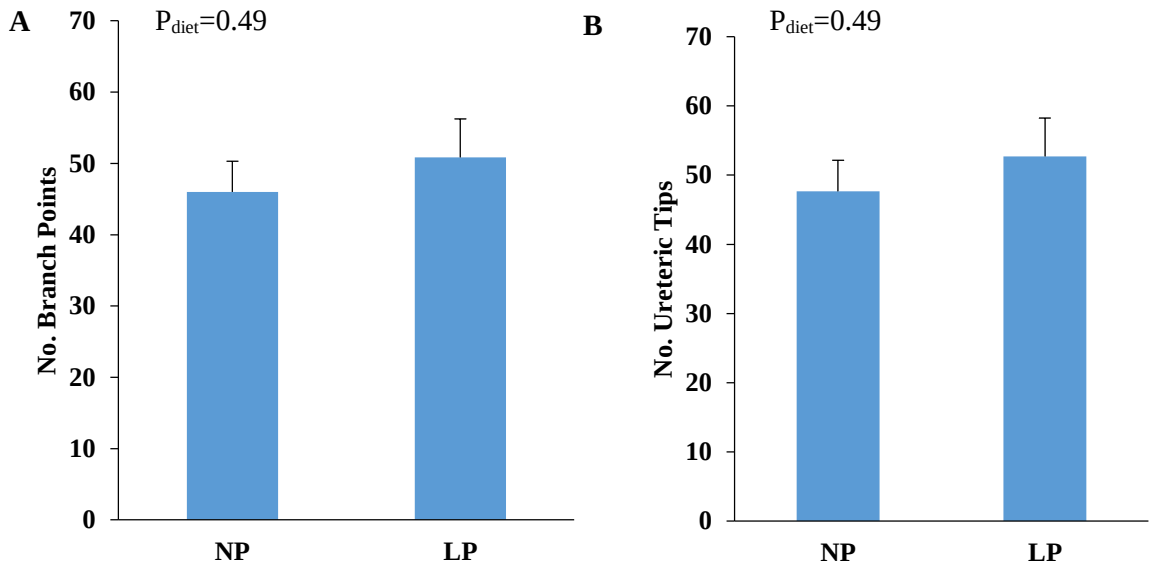

FIGURE 4.4 - NUMBERS OF BRANCH POINTS AND URETERIC TIPS IN CULTURED FETAL KIDNEYS FROM NP AND LP OFFSPRING.

Numbers of branch points (**A**) and ureteric tips (**B**) in fetal kidneys cultured for 48 hours from NP and LP offspring. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (NP n=9, LP n=6).

4.3.4 FETAL KIDNEY GENE EXPRESSION AFTER EXPOSURE TO MATERNAL LOW PROTEIN DIET

Gene expression was determined using reverse transcription (RT) PCR in E14.25 male and female kidneys from fetuses exposed to either maternal NP or LP diets. Genes chosen for analysis fell into two broad categories; those involved in branching morphogenesis (*Gdnf*, *Gfrα1* and *Bmp4*) and those involved in nephrogenesis (*Pax2*, *Wnt4* and *Hnf4α*).

Surprisingly, expression of *Gdnf* was higher in LP offspring than in NP offspring (P_{diet}=0.05, P_{sex}<0.05, $P_{diet*sex}$=0.20), but this effect was only significant in males (Female NP; 0.021±0.017, Female LP 0.060±0.018, P_{diet}=0.13, Male NP; 0.002±0.002, Male LP; 0.006±0.001 P_{diet}=0.05). Levels of *Gfrα1* expression were not affected by maternal diet ($P_{diet=}$0.27, P_{sex}<0.05, $P_{diet*sex}$=0.39) while expression was greater in females compared with males (Female NP; 0.077±0.039, Female LP; 0.140±0.041, P=0.28, Male NP; 0.006±0.005, Male LP; 0.012±0.003, P=0.27). *Bmp4* expression was greater in female kidneys compared with males, but maternal low protein diet did not alter gene expression (P_{diet}=0.24, P_{sex}<0.05, $P_{diet*sex}$=0.23) (Female NP; 0.009±0.005, Female LP; 0.018±0.005, P_{diet}=0.21, Male NP; 0.001±0.001, Male LP; 0.001±0.001, P_{diet}=0.57; Figure 4.5).

There were no effects of either diet or sex on renal expression of *Hnf4α*, *Pax2* and *Wnt4* at E14.25 (*Hnfα*; P_{diet}=0.54, P_{sex}=0.17, $P_{diet*sex}$=0.30, *Pax2*; P_{diet}=0.26, P_{sex}<0.05, $P_{diet*sex}$=0.15, *Wnt4*; P_{diet}=0.26, P_{sex}<0.05, $P_{diet*sex}$=0.33), apart from the fact that expression of *Pax2* and *Wnt4* differed between the sexes (Figure 4.6).

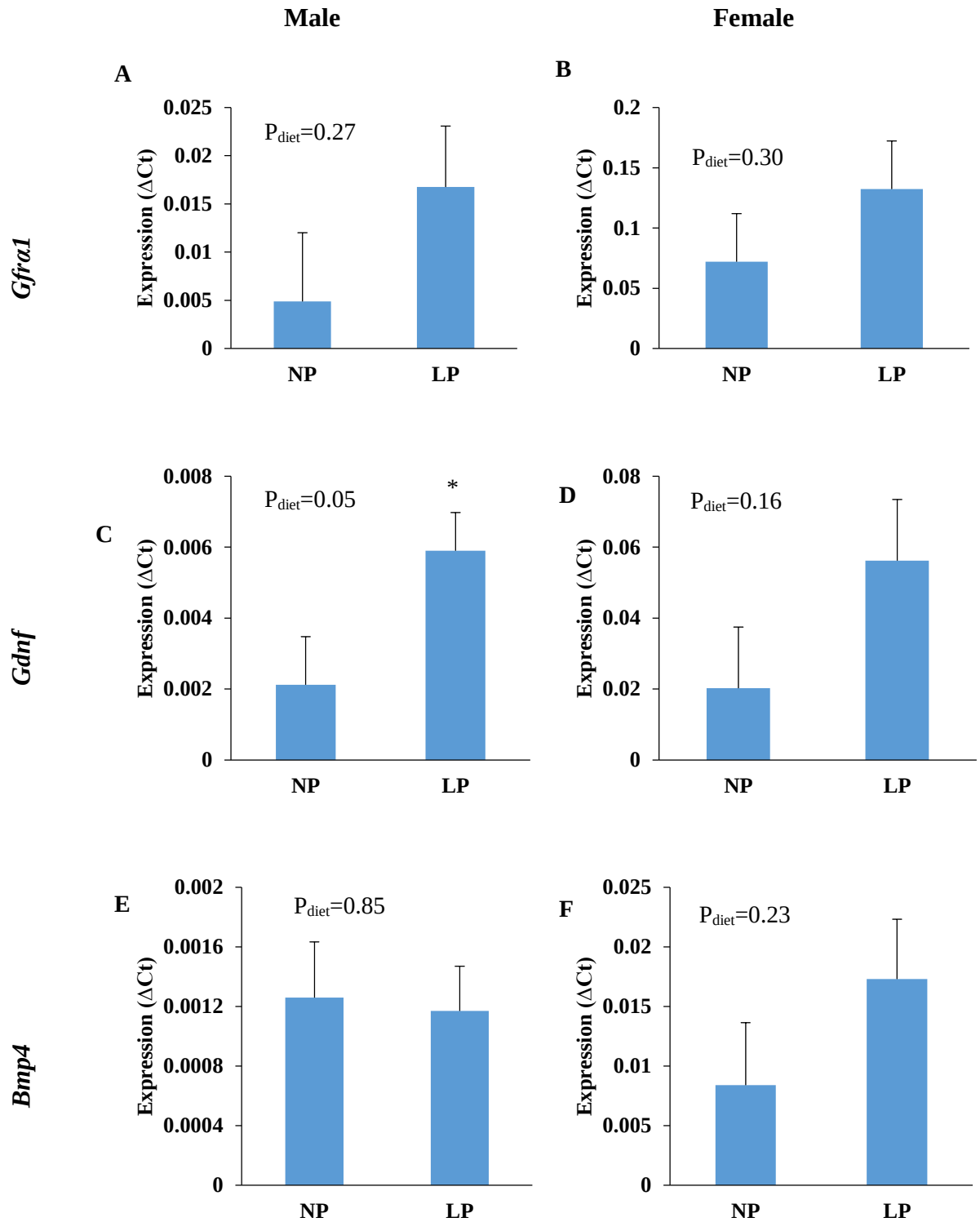

FIGURE 4.5 - GENE EXPRESSION IN MALE AND FEMALE FETAL KIDNEYS AT E14.25 EXPOSED TO EITHER MATERNAL PROTEIN RESTRICTION (LP) OR NORMAL PROTEIN (NP) DIET.

Genes involved in branching morphogenesis (*Gfrα1*; **A** & **B**, *Gdnf*; **C** & **D**, *Bmp4*; **E** & **F**) were assessed using reverse transcription (RT) PCR in male and female offspring exposed to maternal normal or low protein diet. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀n=5, ♂n=5, LP ♀n=5, ♂n=5).

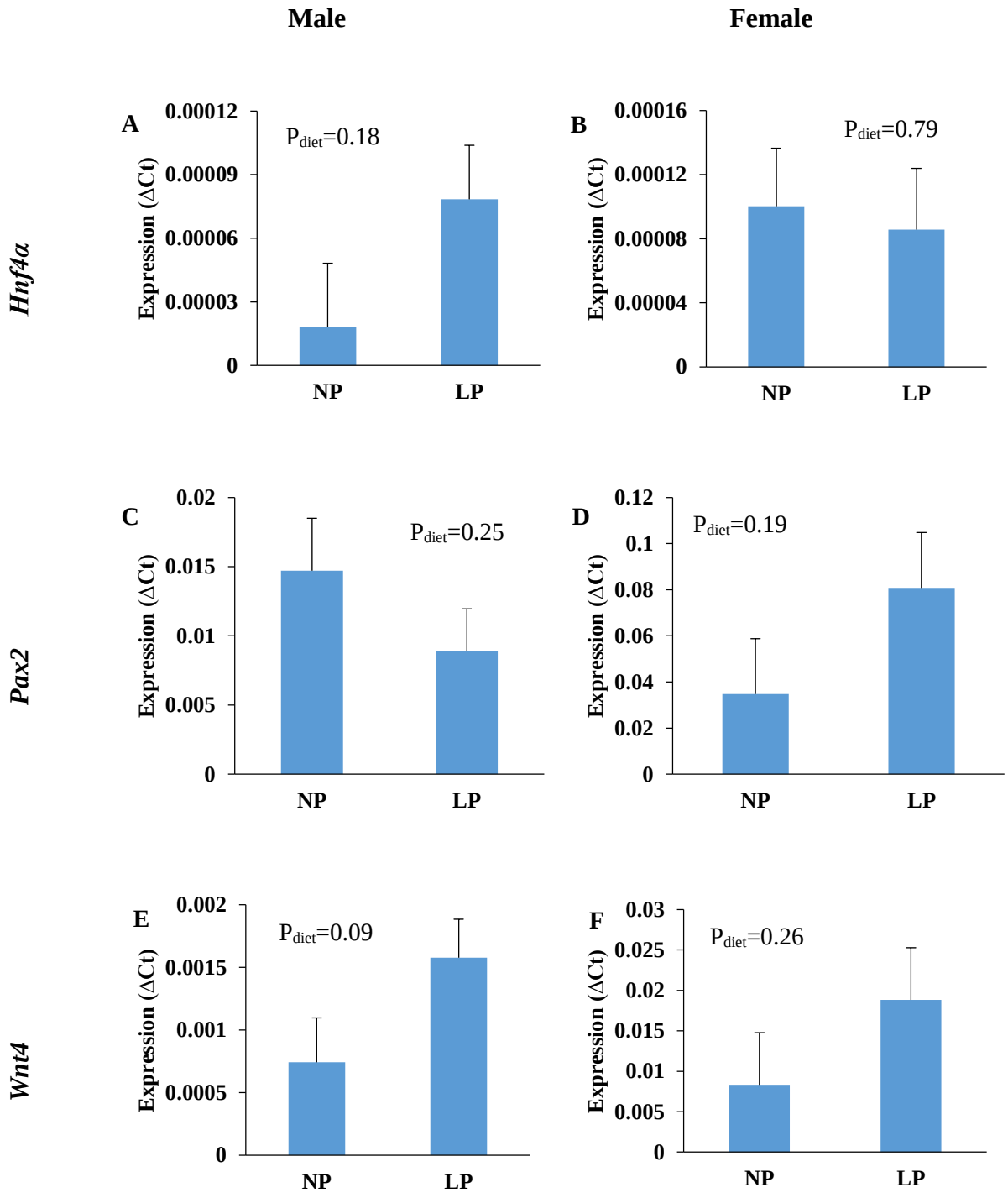

FIGURE 4.6 - GENE EXPRESSION IN MALE AND FEMALE FETAL KIDNEYS AT E14.25 EXPOSED TO EITHER MATERNAL PROTEIN RESTRICTION (LP) OR NORMAL PROTEIN (NP) DIET.

Genes involved in nephrogenesis (*Hnf4α*; **A** & **B**, *Pax2*; **C** & **D**, *Wnt4*; **E** & **F**) were assessed using reverse transcription (RT) PCR in male and female offspring exposed to maternal control or low protein diet. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (NP ♀n=5, ♂n=5, LP ♀n=5, ♂n=5).

4.4 DISCUSSION

It is well known that exposure to dietary protein restriction *in utero* often results in a reduction in nephron number in offspring (Langley and Jackson, 1994, Langley-Evans *et al.*, 1999c, Langley-Evans, 1997, Nwagwu *et al.*, 2000, Woods *et al.*, 2001, Woods and Weeks, 2004), but the timing of this developmental deficit and the molecular mechanisms involved are not known.

This is the first report to show that nephrogenesis in a model of maternal LP diet is reduced as early as E17.25, approximately 2-3 days after the process commences. Furthermore, this nephron deficit (at E17.25) does not appear to be associated with alterations in branching morphogenesis (at E14.25), and surprisingly expression (at E14.25) of GDNF is increased in the LP setting in male offspring.

The reduction in nephrogenesis reported in this study at E17.25 is contrary to the finding by Langley-Evans and colleagues who reported significantly more nephrons in rats exposed to a maternal LP diet at E20 (Langley-Evans *et al.*, 1999c). This difference in findings may be due to the different timepoints analysed in the two studies, or it may be technique dependent, since Langley-Evans *et al.* (1999c) did not use an unbiased stereological method. We have shown that maternal LP diet leads to offspring with a nephron deficit at PN21. Based on our findings of reduced nephron number in LP offspring at both E17.25 and PN21, we conclude that the nephron deficit in this model begins during the early stages of nephron formation. This may be dependent on altered *in utero* growth that results in a slower rate of nephrogenesis, or on reduced numbers of mesenchymal cells available to differentiate into nephrons. Welham and colleagues report that maternal protein restriction lead to offspring with reduced cell number within the metanephros at E15 compared to controls, while at E13 maternal diet had not altered cell number within the metanephros (Welham *et al.*, 2002).

Branching morphogenesis is a vital determinant of final nephron number as nascent nephrons form only at the tips of the ureteric tree (Brophy *et al.*, 2001, Burrow, 2000, Cain and Bertram, 2006). Moreover, the dichotomous branching process means that a modest reduction in branching efficiency could potentially be magnified as a large nephron deficit. In the present study, the efficiency of branching morphogenesis was assessed using an *in vitro* culture system. Whole metanephric organ culture is more often used to determine the effect of exogenous factors (added to the media) on growth and development (Clark *et al.*, 2001, Davies, 2010, Gupta *et al.*, 2003). Kidneys were removed at E14.25 when only 1-2 generations of branches were present, allowing adequate kidney growth to occur *in vitro*. The present data suggest that branching morphogenesis was unaltered by the maternal LP diet. This is a somewhat surprising finding, and may reflect that fact that the kidneys were removed from the adverse environment and cultured for several days in control media before branching was assessed. It should also be noted that a superior method for assessing ureteric branching morphogenesis was recently described, and is known as optical projection tomography (OPT) (Short *et al.*, 2010). OPT is an imaging technique that provides a 3-dimensional reconstruction of the ureteric tree. OPT software is able to measure tree and terminal branch length, tip number, branching iterations, branch angles and inter-tip distances in 3D space. This new technique provides a full 3-dimensional quantitative analysis of ureteric tree development (up to the age of approximately E16 in rats) without the confounding effects of *ex vivo* culture, and thus allows direct assessment of the maternal environment on kidney development. Unfortunately, this technique had not been optimised for use in the rat kidney at the time these *in vitro* studies were conducted.

The renal expression of *Gdnf* was found to be significantly higher in male LP offspring than in male NP offspring at E14.25. This is an unexpected finding given the fact that branching morphogenesis was unaffected at this time-point. It must be remembered, however, that

branching morphogenesis is regulated by many gene products, and that expression of *Gfrα1* (a GDNF receptor) was not affected by the LP diet. It is possible that the increase in *Gdnf* expression in males was a response to promote growth and development (Maswood *et al.*, 2004). Caloric restriction in adult rhesus monkeys lead to increased concetrations of GDNF within the caudate nucleus and this was proposed to be protective against Parkinson-like symptoms induced by a neurotoxin (Maswood *et al.*, 2004). *Gdnf* promotes branching by a process which involves signalling through a receptor tyrosine kinase (*Ret* and *Gfrα1*) (Charlet-Berguerand *et al.*, 2004, Chi *et al.*, 2009, Costantini and Shakya, 2006) and a detailed investigation of this pathway may identify further molecular targets.

RT-PCR identified no differences in level of expression of genes involved in mesenchyme to epithelial transition at E14.25 as a result of the maternal LP diet, despite the fact that nephron number was lower at E17.25. It is possible that changes in expression levels of these genes would have been identified at E17.25, and future studies should investigate this time-point. The GUDMAP database (www.gudmap.org) lists over 3,000 genes known to be involved in nephrogenesis. Therefore a candidate gene approach may lead to oversight on pathways involved in the deficit. The use of next generation sequencing may be sensitive enough to provide the data needed to ascertain these results.

Very few studies have analysed the early gene expression profiles of the kidney exposed to a suboptimal intrauterine environment. Welham and colleagues reported that exposing rats to a maternal LP diet resulted in expression changes of genes involved in apoptosis (*cofilin-1*) and mesenchyme condensation and vasculogenesis (*Prox-1*) at E13, which were associated with a 20% reduction in adult nephron number, determined using the acid maceration technique (Welham *et al.*, 2005). Abdel-Hakkem and colleagues (2008) induced global caloric restriction in Sprague-Dawley rats, and analysed 10 critical genes involved in branching morphogenesis and nephrogenesis at E20. Genes involved in mesenchymal to epithelial transformation (*Wnt4*,

Wt1, *Fgf2* and *Bmp7*) were found to be up-regulated, while genes involved in branching morphogenesis (*Pax2*, *Gdnf*, *Fgf7*, *Bmp4*, and *Wnt11*) were down-regulated (Abdel-Hakeem *et al.*, 2008). These findings suggest that maternal LP diet induces discordant gene expression during kidney development, but it remains to be determined what is causing this aberrant kidney growth. The results from the present study and those of Chapter 3 point to the earliest time points in the nephrogenic period as being sensitive to maternal dietary insult.

The present findings suggest that by E17.25 in offspring of a maternal LP diet there is a nephron deficit which is not associated with altered branching morphogenesis. This suggests that the process of nephrogenesis may be more susceptible to maternal LP diet than branching morphogenesis. Maternal protein restriction may reduce fetal growth towards the end of gestation, or alter cell population numbers, in particular the cap mesenchyme cells responsible for nephron formation (Kobayashi *et al.*, 2008). Nephrogenesis occurs over a large span of mammalian development and is therefore susceptible to the changes in maternal diet.

This knowledge that the earliest period of nephrogenesis is the first time-point where kidney development is slowed in offspring of protein restricted mothers enables future studies to target the nephrogenic period to determine mechanisms and discover potential factors that may protect the kidney, or even ameliorate the negative impact of maternal protein restriction. Future studies may need a fine-grained time-course study analysing kidney development every 6 hours between E14.25 and E17.25, while also determining the mesenchymal precursor population (six2 positive cells).

CHAPTER FIVE: MATERNAL FOLIC ACID INTAKE AND KIDNEY DEVELOPMENT

5.1 INTRODUCTION

It is well established that maternal nutrition and behaviour can impact upon fetal development and later health. In Chapter 3 it was reported that maternal LP in pregnancy lead to a reduction in nephron number in offspring at PN21, and in Chapter 4 this reduction in nephron number was found to be present at E17.25 although kidney branching morphogenesis appeared to be unaltered by maternal dietary restriction. Previous studies have suggested that supplementation of suboptimal maternal diets with folate/folic acid and other one-carbon metabolites can reverse the deleterious effects of a poor maternal diet (Engeham *et al.*, 2009, Jackson *et al.*, 2002, Torrens *et al.*, 2006). It is well established that maternal folate/folic acid intake is important for fetal development and reduces the risk of NTDs (Czeizel, 1993a, Czeizel, 1995a, Abeywardana *et al.*, 2010, Eichholzer *et al.*, 2006, Marks and Potter, 2010, Oakley, 2002). These studies provided the basis for multiple countries to implement mandatory folic acid fortification of food sources (particularly grain-based foods).

Offspring of rats whose mothers were fed LP diets in pregnancy and lactation demonstrate global changes to the methylation status of whole organs, and important gene pathways that play a major role in kidney development and adult function (Bogdarina *et al.*, 2007, Lillycrop *et al.*, 2005b, Rees *et al.*, 2000). Investigations into the impact of a maternal LP diet have reported reduced levels of *Dnmt1* and *Pparα* gene expression. *Dnmt1* is involved in maintaining the methylation status of the genome during replication, while *Pparα* is involved in protein metabolism (Lillycrop *et al.*, 2008, Lillycrop *et al.*, 2007). Jackson *et al.* (2002) showed that addition of glycine, a one-carbon metabolite, to the maternal diet alleviates hypertension caused by a suboptimal intrauterine environment. Folate, or folic acid, is the principal one-carbon metabolite and is essential for production of the universal methyl donor – s-adenosyl methionine (SAM). Folic acid is a commonly recommended supplement during pregnancy following the substantial evidence for a positive correlation between maternal

circulating folic acid concentration and positive birth outcomes (such as reduced malformations and increased birth weight), particularly a reduction in neural tube defects (Wilson *et al.*, 2003, Leeda *et al.*, 1998, Feldkamp *et al.*, 2002). Because of the inverse relationship between neural tube defects and folic acid intake, folic acid fortifications of staple foods such as flour have been carried out in many countries around the world. Moreover, women planning pregnancy are advised to maintain an intake of folic acid of between 500 and 4000 micrograms per day (Geisel, 2003, Ryan-Harshman and Aldoori, 2008, Wilson *et al.*, 2003).

Previous studies have indicated the potential for one-carbon metabolites to ameliorate the negative effects of a suboptimal intrauterine environment. Pregnant rat dams that were exposed to caloric restriction during pregnancy and supplemented with a micronutrient (containing folic acid, selenium, vitamin C and E) did not develop hypertension in adulthood compared to the group that did not receive the micronutrient supplementation (do Carmo Franco *et al.*, 2009). Utilising a maternal LP diet supplemented with 5mg/kg folic acid, Engeham *et al.* (2009) reported that supplementation modulated the preference to high-fat diet in adulthood with no change in genome-wide methylation changes. Torrens *et al.* (2006) reported that folic acid supplementation (+5mg/kg) to a maternal LP diet (9% casein) prevented the elevation of systolic blood pressure. It is yet to be discerned whether these beneficial affects of folic acid supplementation begin during embryonic life and whether they effect the development of particular organs such as the kidney. Surprisingly, no study to date has cultured an organ in the presence of exogenous folic acid to determine the direct effects on organ development.

It has been suggested (Cutfield *et al.*, 2007, Wang *et al.*, 2011a) that the underlying mechanism for the phenomena of the developmental origins of adult health and disease is under epigenetic control. This epigenetic control may rely on specific DNA markers that will alter gene expression, such as methylation. Methylation of biological molecules (in particular DNA) is a critical process that requires the availability of methyl (CH_3; one–carbon metabolite) groups

from the diet. The methyl cycle involves numerous enzymes and cofactors that rely heavily on the dietary intake of specific vitamins such as folate, B12 and methionine in order for SAM to be produced (Poirier, 2002). SAM is required for the methylation of CpG islands on DNA, and altered methylation of CpG islands can lead to altered gene expression (Dolinoy *et al.*, 2007). The addition of methyl groups to the 5' end of cytosine-guanine dinucleotides inhibits the binding of transcription factors and other molecules involved in gene expression leading to gene silencing (Figure 5.1). Gene expression can also be altered due to the methylation of histones; which alters the physical structure of DNA and thereby alters the availability for transcription factors to bind and influence gene expression (Maloney *et al.*, 2005, Maloney and Rees, 2005, Sinclair *et al.*, 2007, Wainfan and Poirier, 1992). When there is aberrant methylation, some genes that are normally silenced become active, and vice versa. Commonly, the CpG islands affected, or under the control of methylation processes, are found in the promoter regions of genes (Section 1.3.1.1).

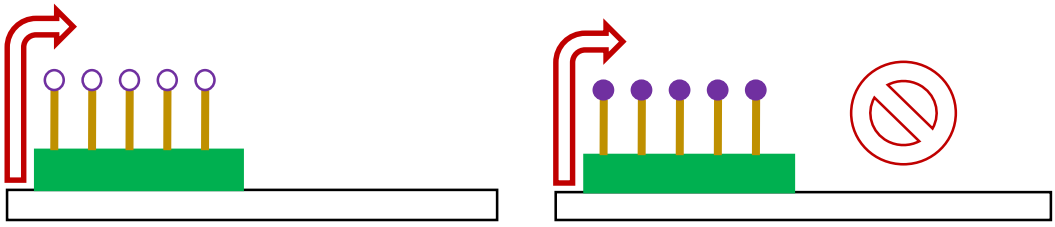

FIGURE 5.1 - SCHEMATIC OF GENE EXPRESSION AND METHYLATION.

Gene expression occurs in methylation sensitive genes (black bar) when CpG dinucleotides (purple sticks) within the promoter region (green bar) are not methylated (clear purple circles). Expression does not occur when the CpG dinucleotides within the promoter region are methylated (solid purple circles).

The impact of maternal folic acid intake on kidney development has not previously been assessed. Wentzel *et al.* (2005) reported beneficial effects of culturing whole embryos, which were previously exposed to maternal diabetes, in the presence of exogenous folic acid (0.25, 1.0 or 2.0mmol/l). They found that the exogenous folic acid lead to development of significantly fewer NTDs. Whether such beneficial effects are also observed with the kidney is unknown. While there is some evidence to suggest histone modifications can influence kidney gene expression (Dressler, 2009b, Dressler, 2009a), there is limited information on potential methylation sensitive genes involved in kidney development (Song *et al.*, 2010).

The rationale for a low protein diet supplemented with folic acid is designed to ascertain the importance of methyl donors in a model of developmental programming. Not to directly emulate the human condition. There is currently no evidence that suggests human maternal folic acid supplementation has an impact on offspring kidney function or *in utero* development. It would be important to determine these parameters, however mandatory fortification has only been around for approximately 20 years in some countries.

The aims of the experiments described in this chapter were to determine:

i) The effects of maternal folic acid intake on fetal and placental growth at E14.25,

ii) The effects of maternal folic acid intake on ureteric branching morphogenesis at E14.25,

iii) The effects of exogenous folic acid on ureteric branching morphogenesis at E14.25,

iv) Expression levels of genes involved in the regulation of ureteric branching morphogenesis and nephrogenesis at E14.25 due to the influence of maternal folic acid intake, and

v) The methylation status of genes involved in ureteric branching morphogenesis and nephrogenesis at E14.25 due to the influence of maternal folic acid intake.

These aims will be carried out to investigate both *in vitro* and *in vivo* effects of folic acid on kidney development by examining early branching morphogenesis and gene expression. *In vitro* studies will involve exogenous exposure to folic acid in a culture environment, while *in vivo* studies will use maternal diet supplementation.

5.2 METHODS

Experiments were conducted in accordance with the National Health and Medical Research Council of Australia 'Australian Code of Practice for the Care and Use of Animals for Scientific Purposes' (7th edition, 2004). Approval was granted in advance by the Monash University School of Biomedical Sciences Animal Ethics Committee. All methods have been fully documented in Chapter 2.

5.2.1 ANIMAL HUSBANDRY

Nine week old female Sprague-Dawley rats were housed in pairs and allowed a 3 week acclimatization period before being fed *ad libitum* one of the following diets (Glen Forest, WA Australia):

- normal protein (NP, 19.5% casein, 2mg/kg folic acid (FA))
- normal protein supplemented with folic acid (NP+FA, 19.4% casein, 200mg/kg FA)
- normal protein and restricted folic acid (NP-FA, 19.4% casein, <0.05mg/kg FA)
- low protein (LP, 8.4% casein, 2mg/kg FA)
- low protein supplemented with folic acid (LP+FA, 8.4% casein, 200mg/kg FA), or
- low protein and restricted folic acid (LP-FA, 8.4% casein, <0.05mg/kg FA).

Female Sprague-Dawley rats were time-mated (3 hours) and remained on the diet throughout pregnancy until the day of tissue collection (E14.25, 17.25 and 20). The LP diet has previously been shown to produce a nephron deficit in offspring (Hoppe *et al.*, 2007a, Hoppe *et al.*, 2007b, Zimanyi *et al.*, 2006) and we confirmed this in the present study (Chapter 3). The dose of folic acid in the diet was determined on the basis of an investigation into the efficacy of 10mg per woman per day on reducing birth defects (Tolarova and Harris, 1995). Although these are high

doses, the literature does not offer any evidence that high concentration of FA is deleterious because the vitamin is water-soluble and is cleared by the kidney.

Refer to Section 2.2 for full details of the experimental diets used.

Ages were selected to correlate with stages of kidney development; early branching morphogenesis (E14.25), mid-branching morphogenesis and early nephrogenesis (E17.25) and growth of the fetus and placenta were determined just prior to birth (E20).

Fetuses were removed from the uterine horns and to ensure an RNAse free environment, were placed in a bath of Diethylpyrocarbonate (DEPC) (97% NMR Sigma-Aldrich, Australia) PBS (1:1000). Fetuses were detached from the placenta, weighed and killed by decapitation.

Kidneys were dissected using aseptic technique and either snap frozen for molecular analysis or assigned to the culture study. Kidneys assigned to the culture study were immediately placed into a petri dish containing serum-free Dulbecco's modified Eagle's medium (DMEM): Ham's F12 liquid medium (Trace Biosciences, Castle Hill, NSW, Australia) supplemented with 5µg/ml transferrin (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) and 12.9µl/ml L-glutamine (Trace Biosciences, Castle Hill, NSW, Australia), warmed to 37°C (Cullen-McEwen *et al.*, 2002, Singh *et al.*, 2007).

Whole kidneys were placed on 3.0µm pore polycarbonate transfilter membranes (Transwell, Corning Star, Cambridge, MA, USA) and cultured in 24-well culture plates in 500µl serum-free culture media for 48 hours at 5% CO_2 and 37°C. To avoid the confounding effects of a media supplemented with serum, a serum-free media was used (Taub and Livingston, 1981). For more information refer to Section 2.3.1.

5.2.2 IMMUNOHISTOCHEMISTRY FOR QUANTIFICATION OF BRANCHING MORPHOGENESIS

After 48 hours of organ culture, metanephroi were fixed in methanol at -20°C. For details on examination with wholemount immunofluorescence microscopy refer to Section 2.3.1.2. Organs were viewed using a fluorescence microscope (Olympus Provis Microscope) at 4x and 10x magnification and photomicrographs taken to assist in quantification of branching morphogenesis. Branching morphogenesis was quantified by manually skeletonising the ureteric tree (Figure 5.2). A branch point was defined as the intersection of three or more skeleton lines. Ureteric tips were defined as terminal branch ends (Section 2.3.1.2)

5.2.3 DNA EXTRACTION AND AMPLIFICATION FOR SEX DETERMINATION

The sex of fetuses at E14.25 was determined by genotyping for the presence of SMC (structural maintenance of chromosome) X/Y using Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, Castle Hill, Australia). See Section 2.3.4.1 for more details.

5.2.4 RNA AND DNA EXTRACTION FROM E14.25 KIDNEYS

RNA and DNA were extracted from both kidneys at E14.25 using a commercially available kit (AllPrep DNA/RNA Mini Kit, Qiagen, Valencia, CA) using the manufacturer's recommended protocol. RNA concentration and quality were determined using a NanoDrop spectrophotometer (ThermoScientific, Australia). The ratio of absorbance at 260/280nm indicated the quality of the sample. For RNA, a ratio between 1.9 and 2.2 was considered sufficient and used in gene expression (Real Time PCR) studies (Mathieson and Thomas, 2012). For more details see Section 2.3.4.2.1.

5.2.5 RNA REVERSE TRANSCRIPTION, CDNA PRODUCTION AND REAL TIME PCR

A 0.05µg RNA sample (from a minimum of 2 male and female kidneys from each litter) was reverse transcribed (Applied Biosystems Reverse Transcription Reagent Kit, Applied Biosystems, Australia). The sample was then run in a thermocycler to produce cDNA

(Superscript VILO cDNA synthesis kit, Life Sciences, Australia,). For Real-Time PCR (RT PCR) SYBR Green was used following the manufacturer's protocol (SensiMix SYBR & Fluorescein Kit, Quantace, Australia). This assay was used to determine relative levels of mRNA expression of genes involved in branching morphogenesis (*Gdnf*, *Gfrα1*, *Bmp4*) and nephrogenesis (*Wnt4*, *Pax2*, *Hnf4α*). *Dnmt1* expression was also determined in male and female kidneys at E14.25. *18S* served as the housekeeper gene. For more information see Section 2.3.4.2.

5.2.6 GENE METHYLATION ANALYSIS

Methylation-specific primers were designed on the sequence obtained with MethPrimer (http:///www.urogene.org//methprimer/). One primer pair specific for methylated cytosine in a CpG dinucleotide in the primer sequence and one primer pair for unmethylated cytosine in the same CpG dinucleotide in the primer sequence were selected for each target gene.

Samples that contained more than 200ng of DNA were bisulfite-converted and concentrated to >40ng/ul by speedvac. DNA was bisulfite-converted using an Epigentek kit (Sapphire Biosciences, Australia) following manufacturer's protocol. Real-time PCR was performed on 20 ng bisulfite-converted DNA in duplicate with SYBR green technology in a BioRad iQ5 real-time cycler. The genes targeted were *Wnt4* in males and *Gfrα1* and *Bmp4* in females. Ratios of amplification of methylated to unmethylated primer products were calculated and compared between the diet groups. For more details see Section 2.3.4.3.

5.2.7 DETERMINATION OF FOLIC ACID CONCENTRATION

Folic acid concentration was determined by Gribbles Pathology using a chemiluminescence technique. Folic acid concentration was determined in maternal plasma and fetal amniotic fluid (2 male and 2 female samples that were pooled per litter). For more information see Section 0.

5.2.8 STATISTICAL ANALYSIS

Data were analysed using a mixed linear model taking into account litter representation (n values represent litter numbers NOT the total number of animals studied because multiple animals from each litter were chosen for analysis) (SPSS 21, IBM). Values are expressed as mean ± SEM, and statistical significance is represented by $P<0.05$, whereby P_{diet} represents the impact of maternal diet, P_{sex} represents the influence of offspring sex, and $P_{diet*sex}$ represents the interaction of these variables. Wherever appropriate, repeated measures ANOVA models were used. LSD post-hoc analysis was also performed to identify differences between dietary groups. Graphs were built using Microsoft Office (Microsoft Excel 2013, Microsoft, USA).

5.3 RESULTS

5.3.1 FETAL GROWTH

Fetal and placental weights were measured at E14.25, E17.25 and E20 (Table 5.1) from dams exposed to maternal LP and folic acid supplementation or restriction.

5.3.1.1.BODYWEIGHT

Maternal folic acid supplementation (LP+FA) did not change fetal weight at E14.25 in comparison with LP controls. However, LP+FA fetuses were approximately 10% heavier than LP fetuses at E17.25 and E20 ($P<0.05$). Maternal folic acid restriction (LP-FA) did not alter E14.25 or E20 fetal weight, but significantly increased E17.25 weight compared to LP controls ($P<0.05$).

5.3.1.2 PLACENTAL WEIGHT

Placental weights at E14.25 were similar in the three dietary groups (Pdiet=0.970). However, E17.25 LP-FA and LP+FA was associated with a 15% and 12% increase (respectively) in placental weight compared to LP fetuses ($P_{diet}<0.05$). At E20, placental weights were again similar in the three dietary groups ($P_{diet}=0.838$, Table 5.1).

5.3.1.3 FETUS TO PLACENTAL WEIGHT RATIO

The fetus to placental weight ratio was similar in all three dietary groups at E14.25 and E20 (Table 5.1). However, at E17.25, while there was no difference in the fetus to placental weight ratio between LP-FA and LP+FA and LP controls, the ratio was significantly smaller in LP+FA than in LP-FA ($P_{diet}<0.05$).

5.3.1.4 MATERNAL NP, NP-FA AND NP+FA FETAL, PLACENTAL AND RATIO RESULTS

Maternal folic acid supplementation or restriction of a NP diet did not impact upon fetal or placental weights at E14.25, or upon the fetus to placental weight ratio (Table 5.2).

TABLE 5.1 – FETUS AND PLACENTA WEIGHTS AND FETAL:PLACENTA WEIGHT RATIO AT E14.25, E17.25 AND E20 AFTER EXPOSURE TO MATERNAL LP AND FOLIC ACID SUPPLEMENTATION OR RESTRICTION.

Age	Parameter	LP (mean ± SEM)	LP-FA (mean ± SEM)	LP+FA (mean ± SEM)	P_{diet}	Posthoc (LP vs LP-FA)	Posthoc (LP vs LP+FA)	Posthoc (LP-FA Vs LP+FA)
E14.25 (LP n=6, LP-FA n=5, LP+FA n=5)	Fetus (mg)	159 ± 7	159 ± 7	154 ± 7	P=0.79	NA	NA	NA
	Placenta (mg)	163 ± 8	162 ± 9	165 ± 9	P=0.97	NA	NA	NA
	Ratio	1.01 ± 0.05	0.96 ± 0.05	0.97 ± 0.05	P=0.77	NA	NA	NA
E17.25 (LP n=4, LP-FA n=5, LP+FA n=3)	Fetus (mg)	750 ± 22	903 ± 16	830 ± 23	**P<0.05**	**P<0.05**	**P<0.05**	**P<0.05**
	Placenta (mg)	303 ± 8	343 ± 6	356 ± 9	**P<0.05**	**P<0.05**	**P<0.05**	P=0.24
	Ratio	2.52 ± 0.09	2.70 ± 0.07	2.36 ± 0.09	**P<0.05**	P=0.10	P=0.20	**P<0.05**
E20 (LP n=4, LP-FA n=6, LP+FA n=5)	Fetus (g)	3.25 ± 0.34	2.73 ± 0.28	3.65 ± 0.28	P=0.10	NA	NA	NA
	Placenta (mg)	422 ± 26	442 ± 21	431 ± 21	P=0.84	NA	NA	NA
	Ratio	7.84 ± 0.87	6.28 ± 0.71	8.77 ± 0.71	P=0.08	NA	NA	NA

TABLE 5.2 – FETUS AND PLACENTA WEIGHTS AND FETAL TO PLACENTAL WEIGHT RATIO AT E14.25 AFTER EXPOSURE TO A MATERNAL NORMAL PROTEIN DIET AND FOLIC ACID SUPPLEMENTATION OR RESTRICTION.

Age	Parameter	NP (mean ± SEM)	NP-FA (mean ± SEM)	NP+FA (mean ± SEM)	P_{diet}
E14.25 (NP n=11, NP-FA n=5, NP+FA n=4)	Fetus (mg)	158 ± 6	162 ± 9	147 ± 10	P=0.51
	Placenta (mg)	157 ± 6	159 ± 9	149 ± 11	P=0.76
	Ratio	1.04 ± 0.04	1.05 ± 0.05	1.03 ± 0.06	P=0.98

5.3.2 MATERNAL PLASMA AND FETAL AMNIOTIC FLUID FOLIC ACID CONCENTRATIONS

5.3.2.1 MATERNAL PLASMA FOLIC ACID LEVELS

Maternal protein intake (low or normal) was not associated with alterations in folic acid concentration in the maternal plasma at E14.25 (Figure 5.2A). However, plasma folic acid concentrations were reduced by 38% in LP-FA and were 70% greater in LP+FA ($P<0.05$) compared with LP controls (Figure 5.2B). Maternal NP+FA lead to an 84% increase in plasma folic acid concentration compared to controls ($P<0.05$, Figure 5.2C). There was no difference in maternal plasma folic acid concentrations between rats fed NP and NP-FA maternal diets ($P=0.23$).

5.3.2.2 AMNIOTIC FLUID FOLIC ACID LEVELS

Folic acid concentrations within amniotic fluid did not differ between NP and LP exposed fetuses at E14.25 (Figure 5.3A). However, folic acid supplementation of a maternal LP diet increased the amniotic fluid concentration of folic acid by 489% compared to LP (Figure 5.3B). LP-FA did not alter the amniotic fluid folic acid concentration. Similar results were seen for fetsues exposed to folic acid supplementation of a NP diet with a 70% increase in amniotic fluid concentration compared to controls, while no change in the folic acid concentration of amniotic fluid was observed in fetuses exposed to NP-FA (Figure 5.3C).

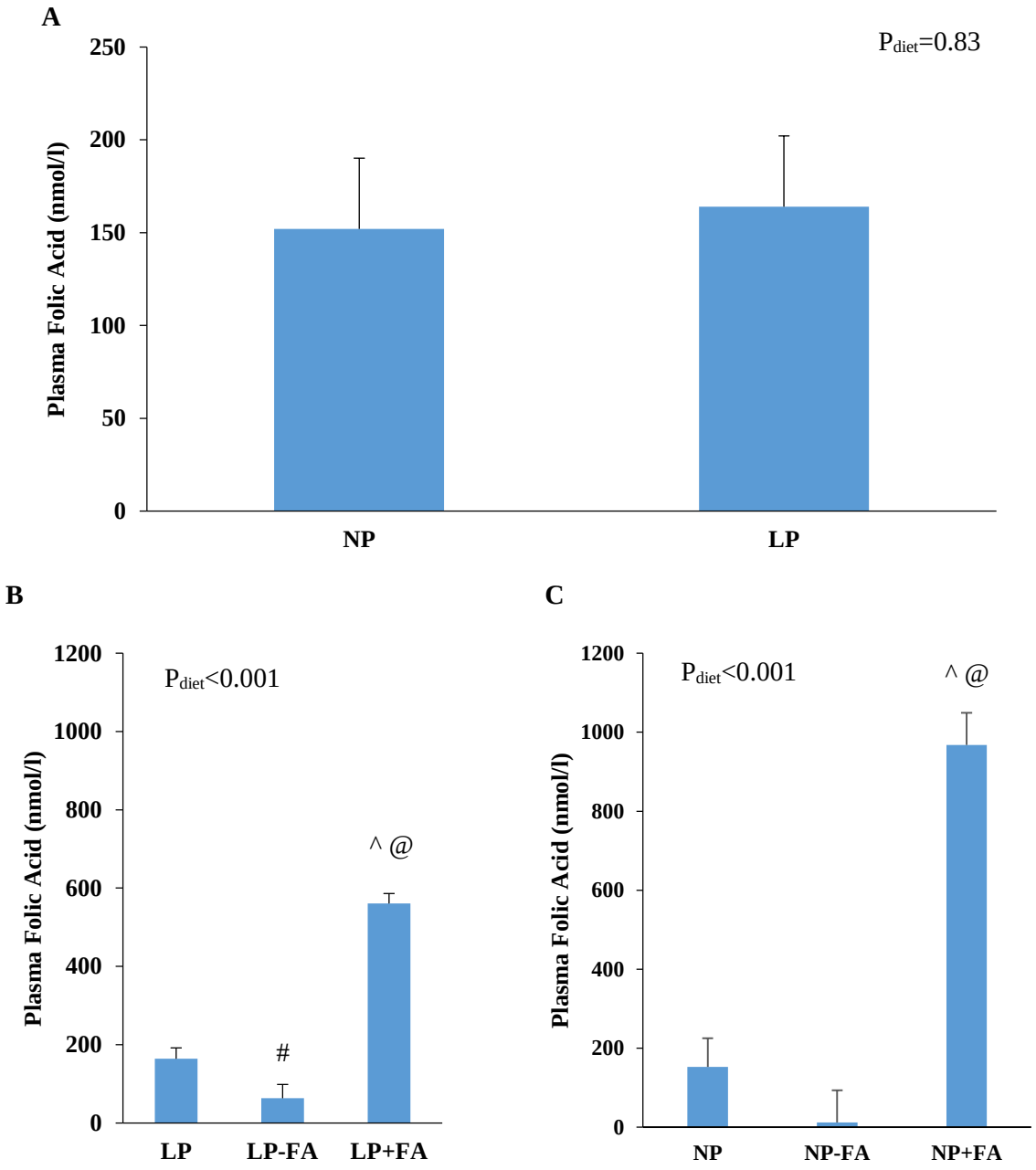

FIGURE 5.2 - MATERNAL PLASMA FOLIC ACID CONCENTRATIONS.

Maternal plasma folic acid concentration at E14.25 after exposure to a normal protein or low protein diet with folic acid supplementation or restriction. Graphs displayed with NP vs LP (A), LP vs LP-FA & LP+FA (B) and NP vs NP-FA and NP+FA (C). Values are mean ± SEM, analysed using least means squares analysis and a LSD post-hoc analysis was performed, symbols denote P<0.05, # NP/LP Vs –FA P<0.05, ^ NP/LP Vs +FA P<0.05, @ -FA Vs +FA P<0.05. NP n=5, LP n=5, NP-FA n=4, NP+FA n=4, LP-FA n=3, LP+FA n=6.

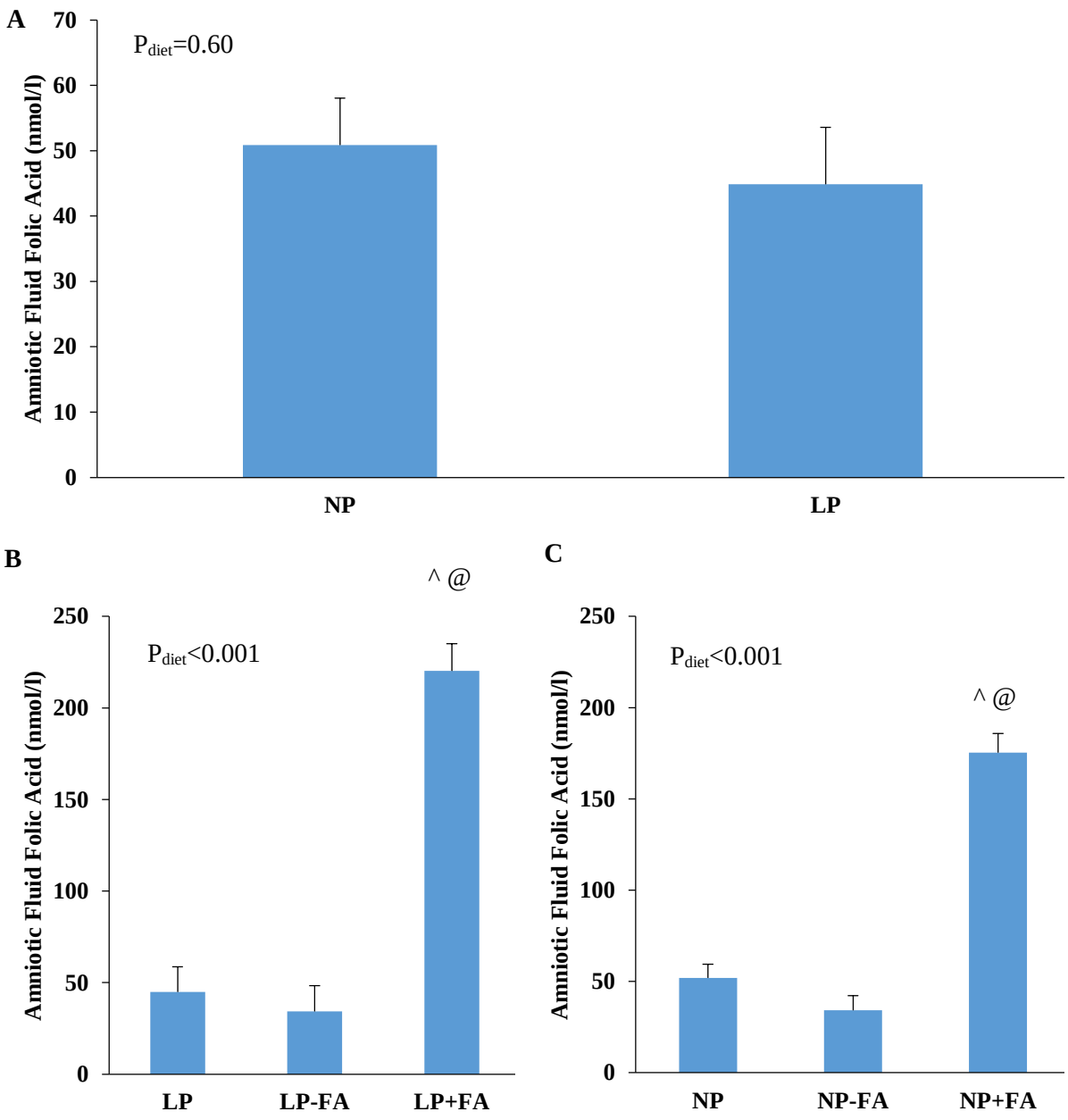

FIGURE 5.3 - AMNIOTIC FLUID CONCENTRATION OF FOLIC ACID AT E14.25 AFTER EXPOSURE TO MATERNAL DIETARY MANIPULATION.

Amniotic fluid folic acid concentration at E14.25 after exposure to a normal protein or low protein diet with folic acid supplementation or restriction. Graphs displayed with NP vs LP (A), LP vs LP-FA & LP+FA (B) and NP vs NP-FA and NP+FA (C). Male and female data have been pooled as there was no sex effect. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed, LSD post-hoc analysis was also performed. ^ NP/LP Vs +FA P<0.05, @ -FA Vs +FA P<0.05. NP n=6, LP n=6, NP-FA n=5, NP+FA n=3, LP-FA n=5, LP+FA n=4.

5.3.3 BRANCHING MORPHOGENESIS OF KIDNEYS AT E14.25

5.3.3.1 THE EFFECT OF MATERNAL FOLIC ACID INTAKE ON KIDNEY BRANCHING MORPHOGENESIS

Branching morphogenesis was determined in E14.25 kidneys after 48 hours in culture (Figure 5.4). Culturing kidneys from fetuses exposed to NP+FA trended towards 18% fewer branch points (P=0.06) and 19% fewer ureteric tips (P=0.06) compared with controls. NP-FA did not alter branch point or ureteric tip number (Figure 5.4). LP+FA did not alter branch point or ureteric tip number. However, LP-FA lead to 13% (P<0.05) and 12% (P<0.05) reductions in numbers of branch points and tips respectively.

5.3.3.2 THE EFFECT OF EXOGENOUS FOLIC ACID ON KIDNEY BRANCHING MORPHOGENESIS

Kidneys exposed to the maternal NP diet and cultured in the presence of 0.6mM exogenous folic acid for 48 hours had similar numbers of branch points and tips as those cultured in control media (0mM FA) for 48 hours. However, following culture in 2mM folic acid the numbers of branch points and tips were reduced by 28% (P<0.05) and 18% (P<0.05) respectively (Figures 5.5A and B). Kidneys exposed to NP-FA and then cultured in 0.6 and 2mM of folic acid displayed a trend towards reduced numbers of branch points (P=0.06) and tips (P=0.05) compared to 0mM folic acid (Figure 5.5 C and D). Kidneys from fetuses exposed to maternal NP+FA diet had greater numbers of branch points (P<0.05) and tips (P<0.05) following culture in the presence of 0.6mM of exogenous folic acid, while there was no difference following culture with 2mM folic acid compared to controls (0mM FA, Figure 5.5E and F).

Kidneys exposed to maternal LP diet and cultured in the presence of 0.6 or 2mM of folic acid displayed no change in numbers of branch points and tips compared to controls (0mM FA, Figure 5.6A and B). Similarly, kidneys exposed to LP-FA and cultured in 0.6mM folic acid demonstrated no change in branch points or tips. However, culture of LP-FA kidneys in the presence of 2mM of folic acid lead to 13% and 14% fewer branch points (P<0.05) and tips

respectively ($P<0.05$) compared to controls (0mM FA, Figure 5.6C and D). Kidneys from fetuses exposed to LP+FA were not altered following culture in 0.06mM FA. However, there was a trend to a reduced number of branch points and tips following culture in 2mM folic acid compared to controls (0mM FA, $P_{culture}=0.06$, Figure 5.6E and F).

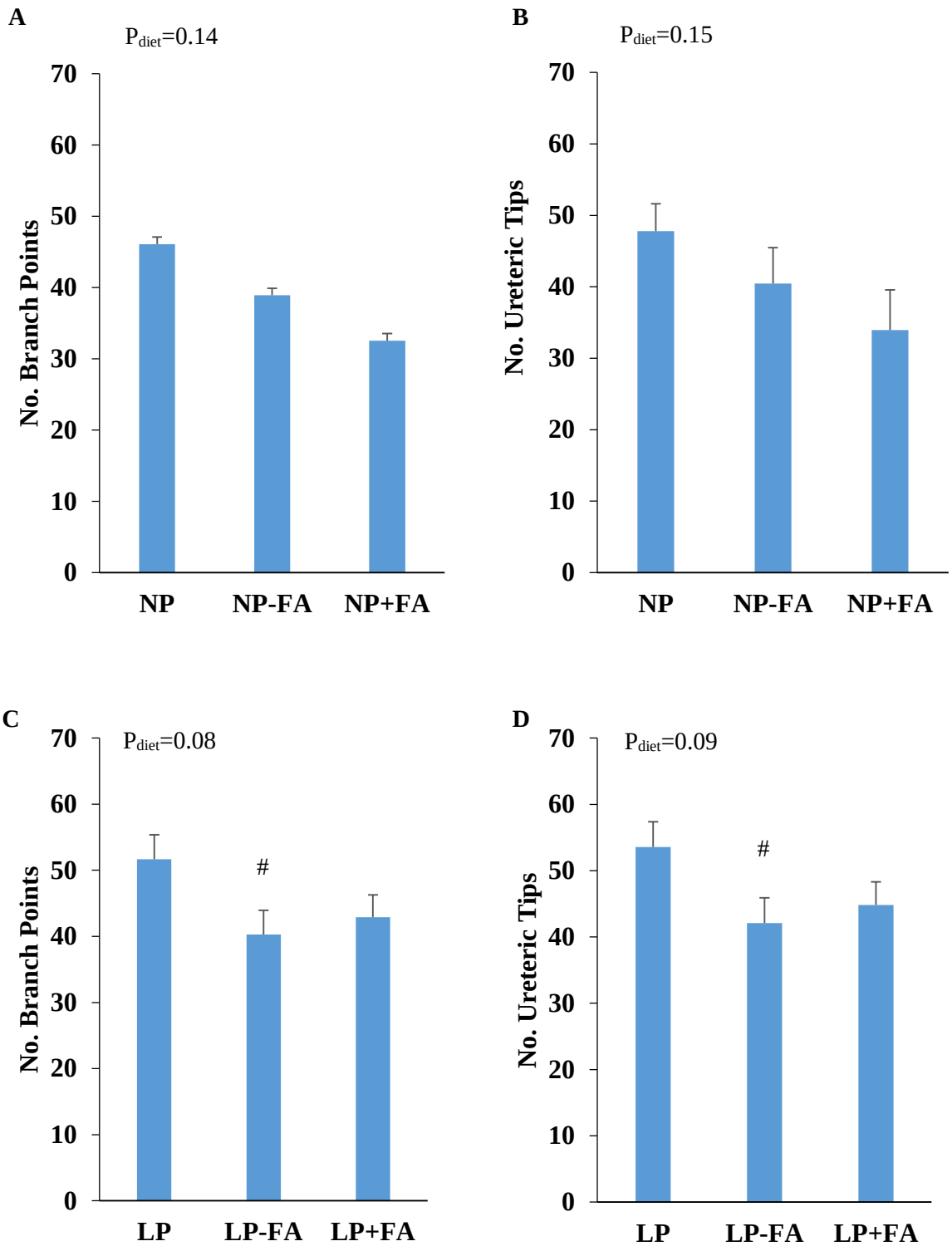

FIGURE 5.4 – NUMBERS OF URETERIC BRANCH POINTS AND TIPS IN KIDNEYS CULTURED FROM FETSUES EXPOSED TO MATERNAL DIET MANIPULATION.

Numbers of ureteric branch points and tips in kidneys cultured for 48 hours in serum-free media. Kidneys are from fetuses exposed to maternal LP (**C**, **D**) or NP (**A**, **B**) with –FA or +FA. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed, LSD post-hoc analysis was also performed. # NP/LP Vs –FA $P<0.05$, NP n=9, LP n=6, NP-FA n=5, NP+FA n=4, LP-FA n=5, LP+FA n=5.

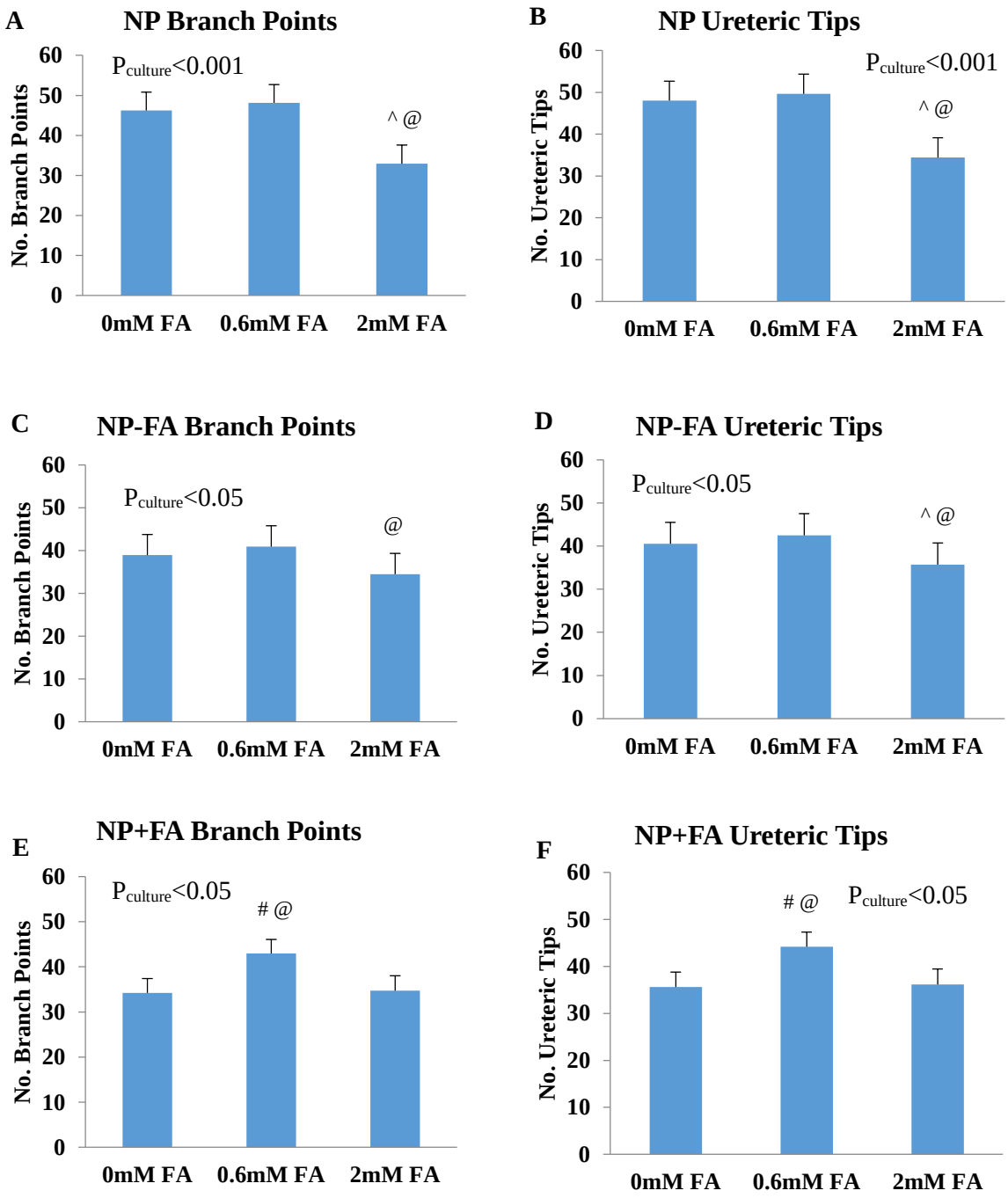

FIGURE 5.5 – NUMBERS OF URETERIC BRANCH POINTS AND TIPS IN KIDNEYS CULTURED IN VARYING CONCENTRATIONS OF FOLIC ACID FROM FETUSES EXPOSED TO NP, NP-FA OR NP+FA DIETS.

Numbers of ureteric branch points (**A**, **C**, **E**) and ureteric tips (**C**, **D**, **F**) in kidneys cultured for 48 hours in serum-free media supplemented with 0mM, 0.6mM or 2mM folic acid. Kidneys are from fetuses exposed to maternal normal or low protein diets with folic acid supplementation or restriction. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed, symbols denote LSD post-hoc analysis P<0.05 # 0mM FA Vs 0.6mM FA P<0.05, ^ 0mM FA Vs 2mM FA P<0.05, @ 0.6mM FA Vs 2mM FA P<0.05. NP n=9, NP-FA n=5, NP+FA n=4.

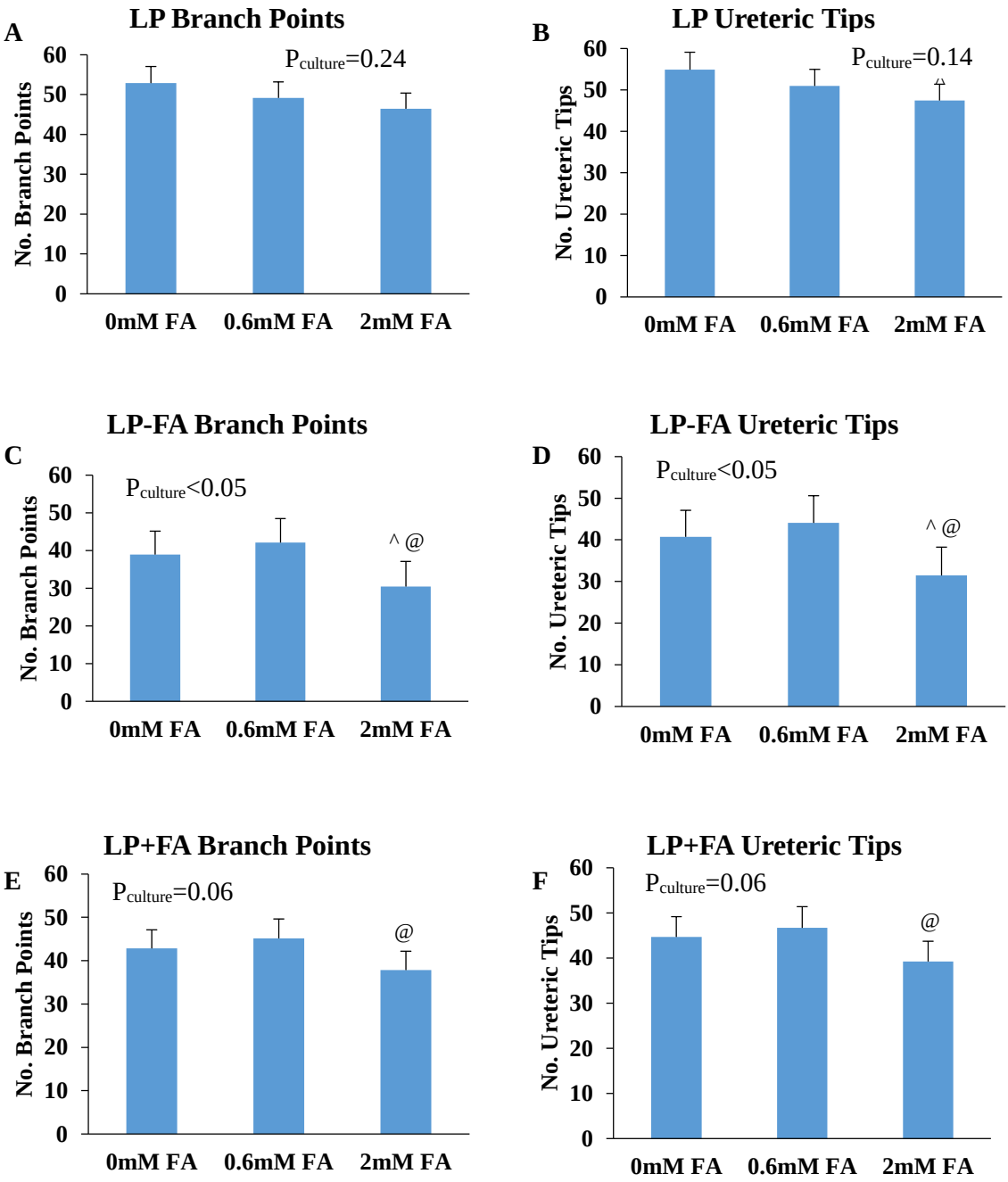

FIGURE 5.6 - BRANCH POINTS AND URETERIC TIPS FROM KIDNEYS CULTURED IN VARYING CONCENTRATIONS OF FA FROM FETUSES EXPOSED TO LP, LP-FA OR LP+FA.

Numbers of ureteric branch points (**A**, **C**, **E**) and tips (**B**, **D**, **F**) in kidneys cultured for 48 hours in serum-free media supplemented with 0mM, 0.6mM and 2mM folic acid. Kidneys are from fetuses exposed to LP, LP-FA or LP+FA. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed, symbols denote LSD post-hoc analysis ^ 0mM Vs 2mM FA $P<0.05$, @ 0.6mM Vs 2mM FA $P<0.05$. LP n=6, LP-FA n=5, LP+FA n=5.

5.3.4 KIDNEY GENE EXPRESSION AT E14.25

Gene expression was determined at E14.25 in kidneys from fetuses exposed to maternal normal or low protein with supplemented or restricted folic acid using reverse transcription PCR.

5.3.4.1 EXPRESSION OF SELECT GENES INVOLVED IN BRANCHING MORPHOGENESIS

Analysis of genes involved in ureteric branching morphogenesis revealed changes in levels of expression associated with the maternal diet (Figure 5.7 and 5.8). LP-FA or LP+FA did not alter *Gdnf* expression in male kidneys compared with LP kidneys, while in contrast female kidneys demonstrated a significant reduction in *Gdnf* expression in both LP-FA ($P<0.05$) and LP+FA ($P<0.05$) exposed kidneys compared to LP female kidneys ($P_{sex}<0.05$, $P_{diet}<0.05$, $P_{diet*sex}=0.078$, Figure 5.7A and B).

Maternal LP+FA lead to reduced *Gfrα1* expression in female kidneys compared to LP kidneys ($P_{diet}=0.14$,$P_{sex}<0.05$, $P_{diet*sex}<0.05$). However, *Gfrα1* expression was not altered in male kidneys exposed to maternal NP+FA or NP-FA ($P_{diet}=0.67$, $P_{sex}=0.16$, $P_{diet*sex}<0.05$, Figure 5.7C and D).

Bmp4 expression was not altered in male kidneys exposed to LP-FA or LP+FA, however female kidneys exposed to LP-FA or LP+FA demonstrated less Bmp4 expression than female LP kidneys ($P_{diet}=0.07$, $P_{sex}<0.05$, $P_{diet*sex}=0.06$, Figure 5.7E and F).

Maternal NP+FA and NP-FA did not alter *Gdnf* expression in male or female kidneys ($P_{diet}=0.39$, $P_{sex}<0.05$, $P_{diet*sex}=0.31$, Figure 5.8A and B) compared to NP controls. However, female kidneys exposed to NP-FA had significantly less expression of *Gfrα1* compared to NP female kidneys ($P<0.05$, Figure 5.8C and D). Maternal NP-FA lead to greater *Bmp4* expression in male kidneys compared to NP male kidneys ($P<0.05$), while there was no change in female gene expression (Figure 5.8E and F).

5.3.4.2 EXPRESSION OF SELECT GENES INVOLVED IN NEPHROGENESIS

Genes involved in nephrogenesis (*Pax2*, *Wnt4* and *Hnf4α*) varied in expression levels between sex and dietary groups (Figures 5.9 and 5.10). *Pax2* expression was reduced in male LP+FA versus LP-FA kidneys but was not altered by other maternal dietary interventions (P_{diet}=0.07, P_{sex}<0.05, $P_{diet*sex}$<0.05). Neither LP-FA nor LP+FA altered *Wnt4* expression in male or female kidneys compared to LP controls (P_{diet}=0.14, P_{sex}<0.05, $P_{diet*sex}$=0.19). *Hnf4α* expression was not altered by LP-FA or LP+FA in female kidneys compared to LP controls (P_{diet}<0.05, P_{sex}=0.23, $P_{diet*sex}$=0.72), but the LP+FA diet in males lead to a significant reduction of *Hnf4α* compared to LP males (P<0.05).

Neither NP-FA nor NP+FA altered *Wnt4* expression in female kidneys (P_{diet}=0.60, P_{sex}=0.54, $P_{diet*sex}$<0.05), however NP+FA male kidneys had greater Wnt4 expression (P<0.05) than NP control kidneys. Finally, neither NP-FA nor NP+FA altered male or female kidney expression of *Hnf4α* compared with NP controls (P_{diet}=0.36, P_{sex}<0.05, $P_{diet*sex}$=0.37).

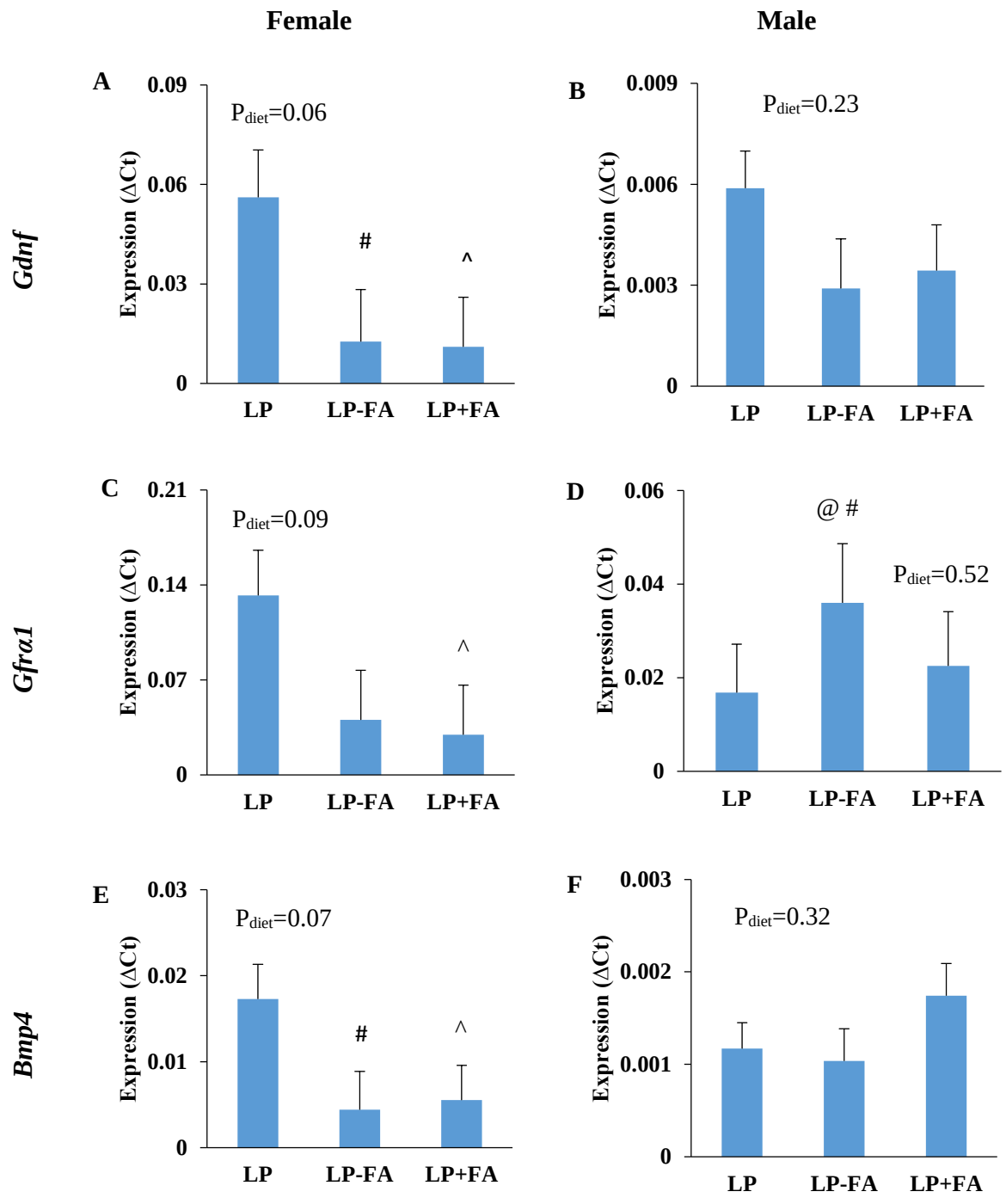

FIGURE 5.7 – EXPRESSION OF GENES INVOLVED IN BRANCHING MORPHOGENESIS IN MALE AND FEMALE KIDNEYS EXPOSED TO MATERNAL LOW PROTEIN AND FOLIC ACID RESTRICTION OR SUPPLEMENTATION.

Gene expression in male and female kidneys exposed to maternal low protein diet with folic acid supplementation or restriction. Expression of genes involved in branching morphogenesis (*Gdnf*; **A** & **B**, *Gfrα1*; **C** & **D** and *Bmp4*; **E** & **F**). Expression determined using Real Time PCR. Housekeeping gene used was 18s. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed, LSD post-hoc analysis was also performed # LP Vs –FA P<0.05, ^ LP Vs +FA P<0.05, @ -FA Vs +FA P<0.05. LP ♂n=6 ♀n=5, LP-FA ♂n=5 ♀n=5, LP+FA ♂n=5 ♀n=4.

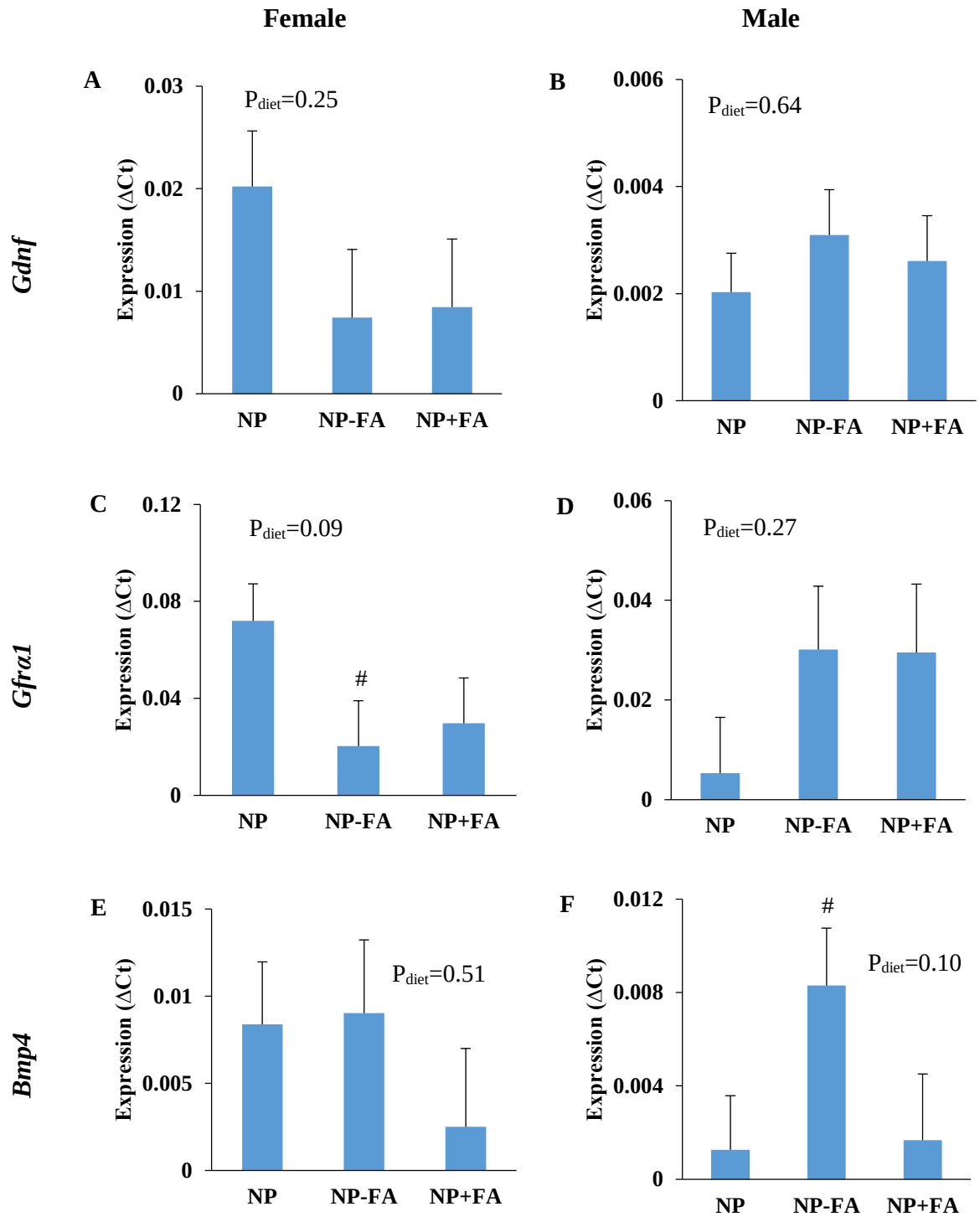

FIGURE 5.8 – EXPRESSION OF GENES INVOLVED IN BRANCHING MORPHOGENESIS IN MALE AND FEMALE KIDNEYS EXPOSED TO MATERNAL NORMAL PROTEIN AND FOLIC ACID RESTRICTION OR SUPPLEMENTATION.

Gene expression levels in male and female kidneys exposed to maternal normal protein diet with folic acid supplementation or restriction. Expression of genes involved in branching morphogenesis (*Gdnf*; **A** & **B**, *Gfrα1*; **C** & **D** and *Bmp4*; **E** & **F**). Expression determined using Real Time PCR. Housekeeping gene used was 18s. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed, LSD post-hoc analysis was also performed # NP Vs –FA P<0.05. NP ♂n=5 ♀n= 5, NP-FA ♂n=4 ♀n=5, NP+FA ♂n=4 ♀n=4.

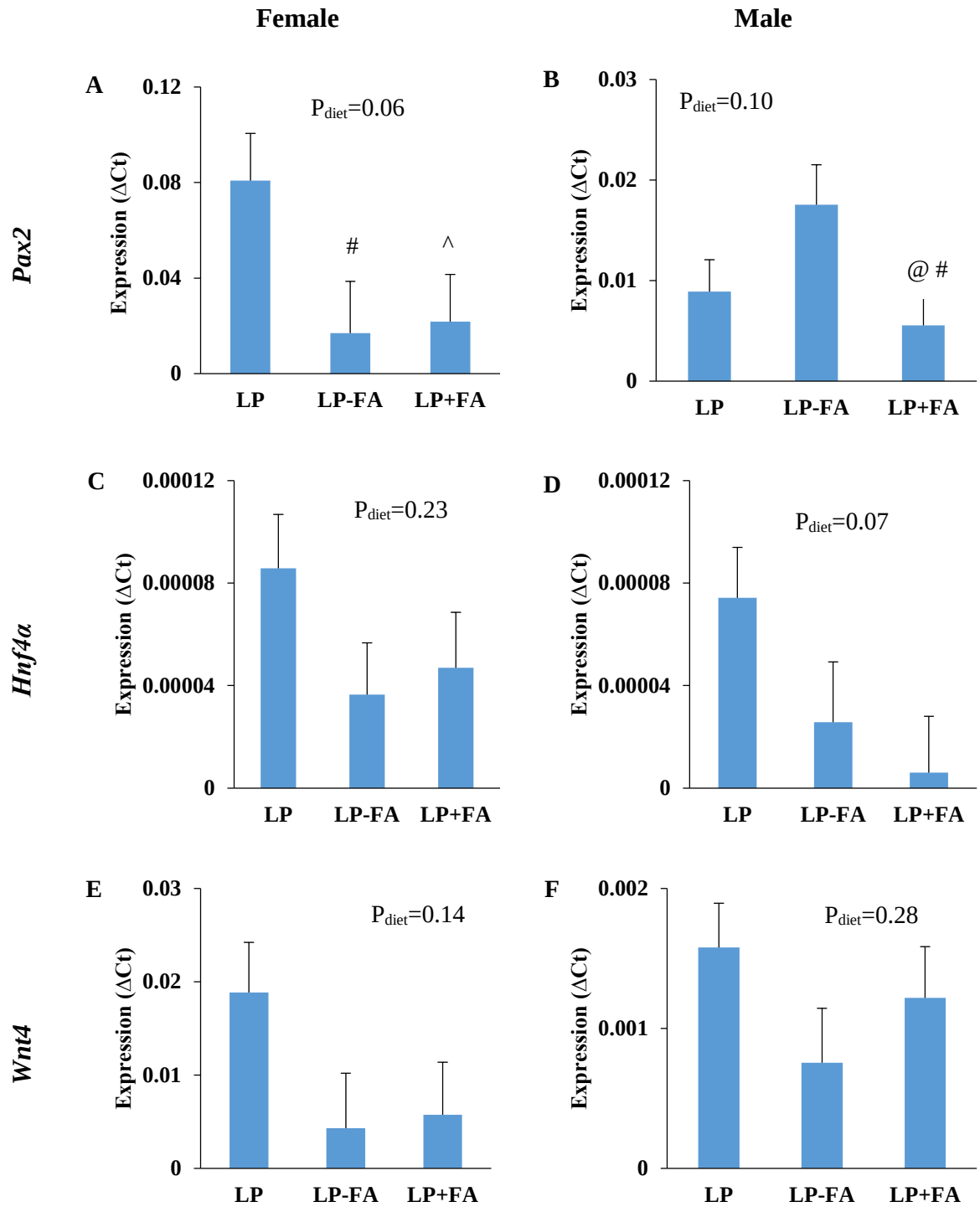

FIGURE 5.9 – EXPRESSION OF GENES INVOLVED IN NEPHROGENESIS IN MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL LOW PROTEIN DIET AND FOLIC ACID RESTRICTION OR SUPPLEMENTATION.

Gene expression levels in male and female kidneys exposed to maternal low protein diet with folic acid supplementation or restriction. Expression of genes involved in branching morphogenesis (*Pax2*; **A** & **B**, *Hnf4α*; **C** & **D** and *Wnt4*; **E** & **F**). Expression determined using Real Time PCR. Housekeeping gene used was 18s. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed, symbols represent LSD post-hoc analysis, # LP Vs –FA P<0.05, ^ LP Vs +FA P<0.05, @ -FA Vs +FA P<0.05. LP ♂n=6 ♀n=5, LP-FA ♂n=5 ♀n=5, LP+FA ♂n=5 ♀n=4.

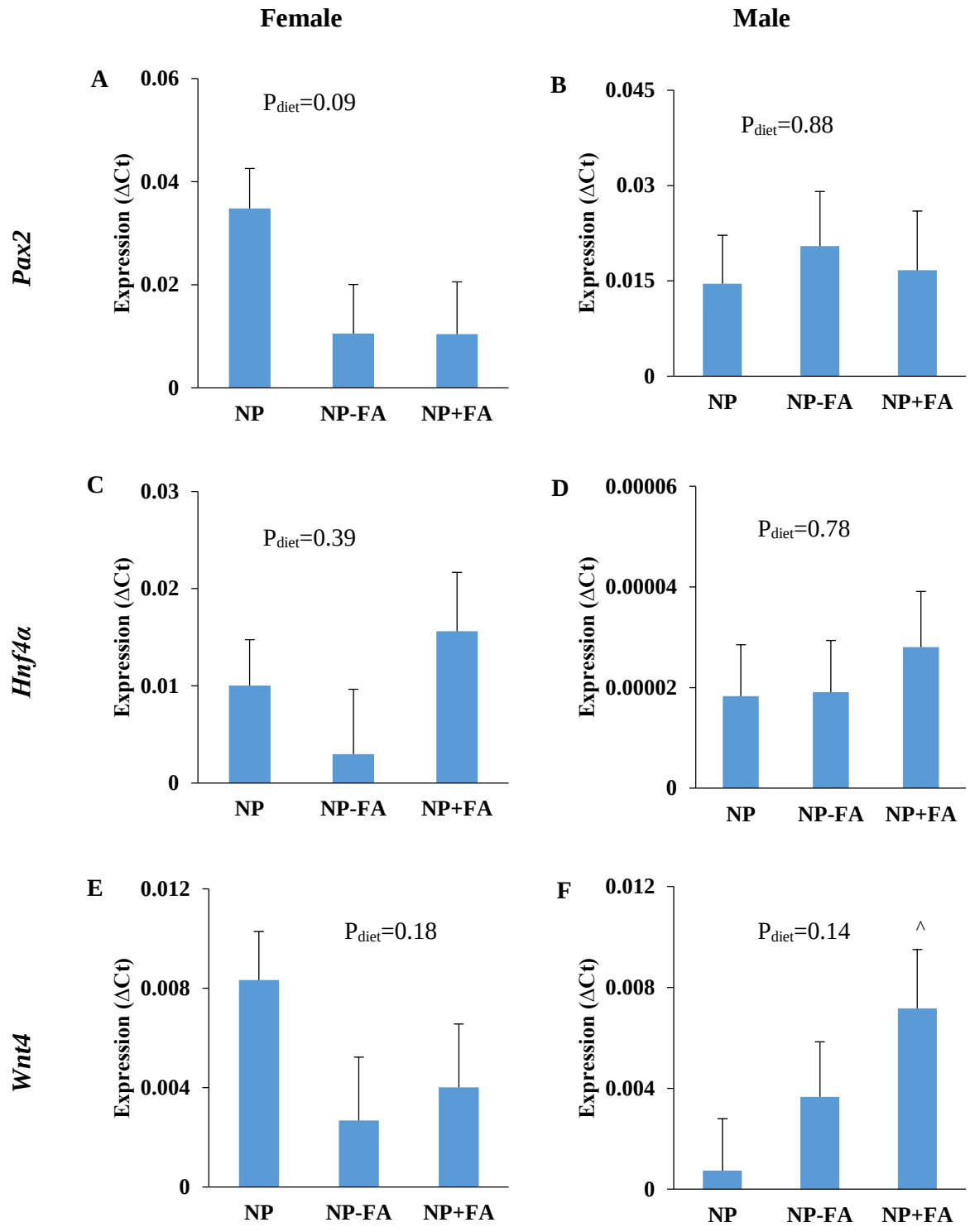

FIGURE 5.10 – EXPRESSION OF GENES INVOLVED IN NEPHROGENESIS IN MALE AND FEMALE OFFSPRING EXPOSED TO NP, NP-FA AND NP+FA.

Gene expression in male and female kidneys exposed to maternal normal protein diet with folic acid supplementation or restriction. Expression of genes involved in branching morphogenesis (*Pax2*; **A** & **B**, *Hnf4α*; **C** & **D** and *Wnt4*; **E** & **F**). Expression determined using Real Time PCR. Housekeeping gene used was 18s. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed. No significant differences were found. NP ♂n=5 ♀n= 5, NP-FA ♂n=4 ♀n=5, NP+FA ♂n=4 ♀n=4.

5.3.5 KIDNEY GENE METHYLATION STATUS

Methylation status was determined for *Gfrα1*, *Bmp4* and *Wnt4* genes from kidneys at E14.25. These genes were selected as they had changes in levels of expression as well as CpG islands upstream of the promoter regions (Section 0).

Female *Gfrα1* methylation status was not different in offspring exposed to NP, LP, LP-FA or LP+FA. There was, however, a negative correlation between the level of gene expression and methylation, suggesting that *Gfrα1* expression is indeed under the control of methylation (Figure 5.11). When the linear relationship was compared between samples, LP females had a stronger correlation between gene expression and methylation than compared to NP females ($P<0.05$, Figure 11C).

Male *Wnt4* methylation was not different in NP and LP+FA offspring. There was no relationship between gene expression and methylation level (Figure 5.12).

Female *Bmp4* was unmethylated, with no amplification of the methylated primers in the PCR (data not shown).

Dnmt1 expression was analysed using RT-PCR in male and female kidneys (Figures 5.13 and 5.14). *Dnmt1* expression within the kidney was not altered by maternal LP compared to NP controls, however females had greater levels of expression than males ($P_{diet}=0.32$, $P_{sex}<0.05$, $P_{diet*sex}=0.14$). Expression of *Dnmt1* in LP-FA and LP+FA female kidneys appeared to be reduced compared to LP controls, although the probability (p) value just failed to reach statistical significance. There was no change in male kidney *Dnmt1* gene expression ($P_{diet}<0.05$, $P_{sex}<0.05$, $P_{diet*sex}=0.057$). NP-FA or NP+FA did not alter the expression of *Dnmt1* in males or females compared to controls ($P_{diet}=0.22$, $P_{sex}=0.13$, $P_{diet*sex}=0.37$).

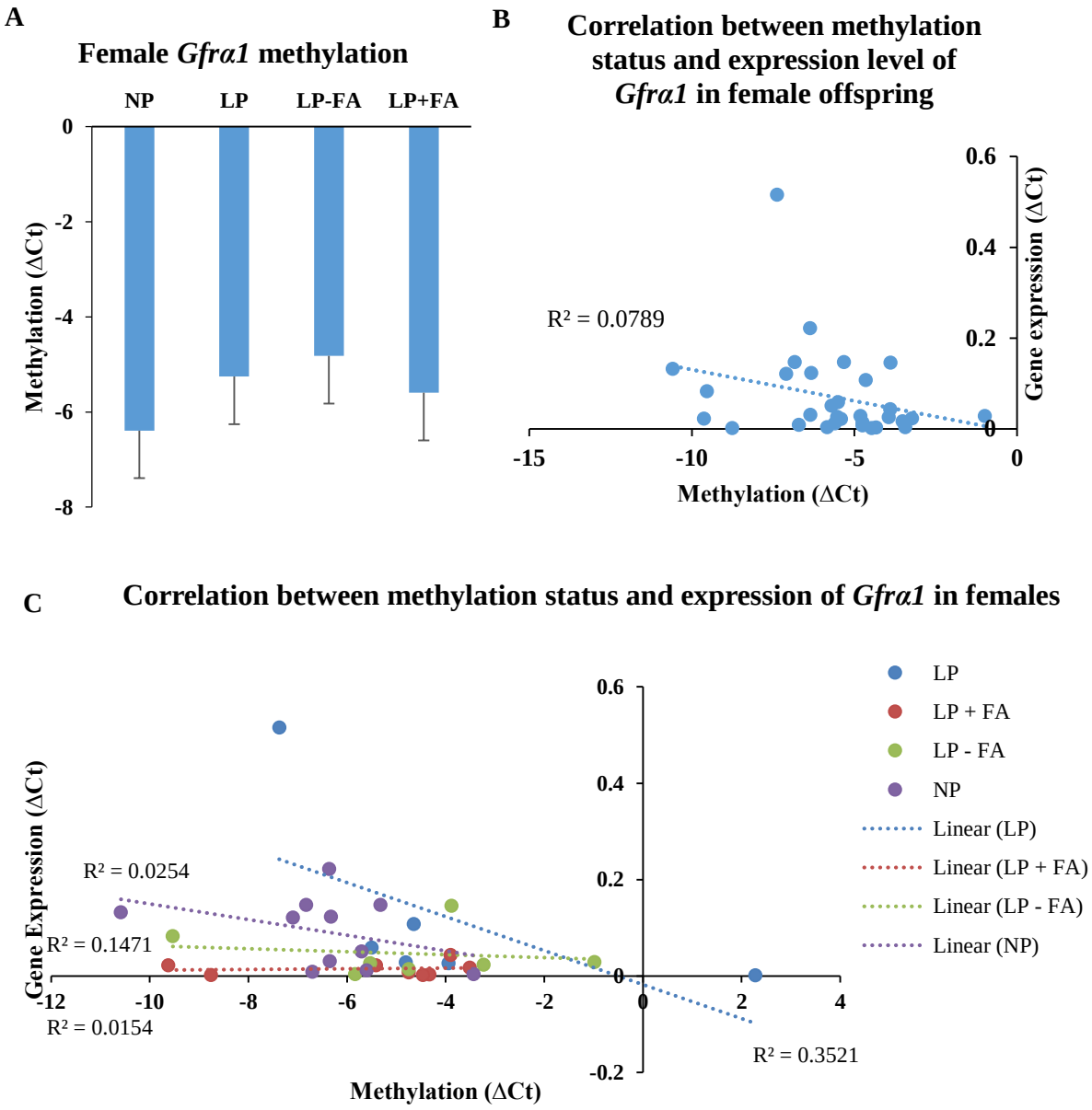

FIGURE 5.11 - METHYLATION OF *Gfrα1* IN E14.25 KIDNEYS FROM OFFSPRING EXPOSED TO MATERNAL NORMAL OR LOW PROTEIN DIET AND FOLATE SUPPLEMENTATION OR RESTRICTION.

The level of methylation within the promoter region of *Gfrα1* (**A**) was determined in E14.25 male kidneys from offspring exposed to maternal normal protein, low protein, low protein low FA and low protein high FA. Methylation level was compared to the level of expression of *Gfrα1* (**B**) and per diet basis (**C**). Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed. Regressions represent all samples analysed. NP ♂n=4, LP ♂n=4, LP-FA ♂n=5, LP+FA ♂n=5.

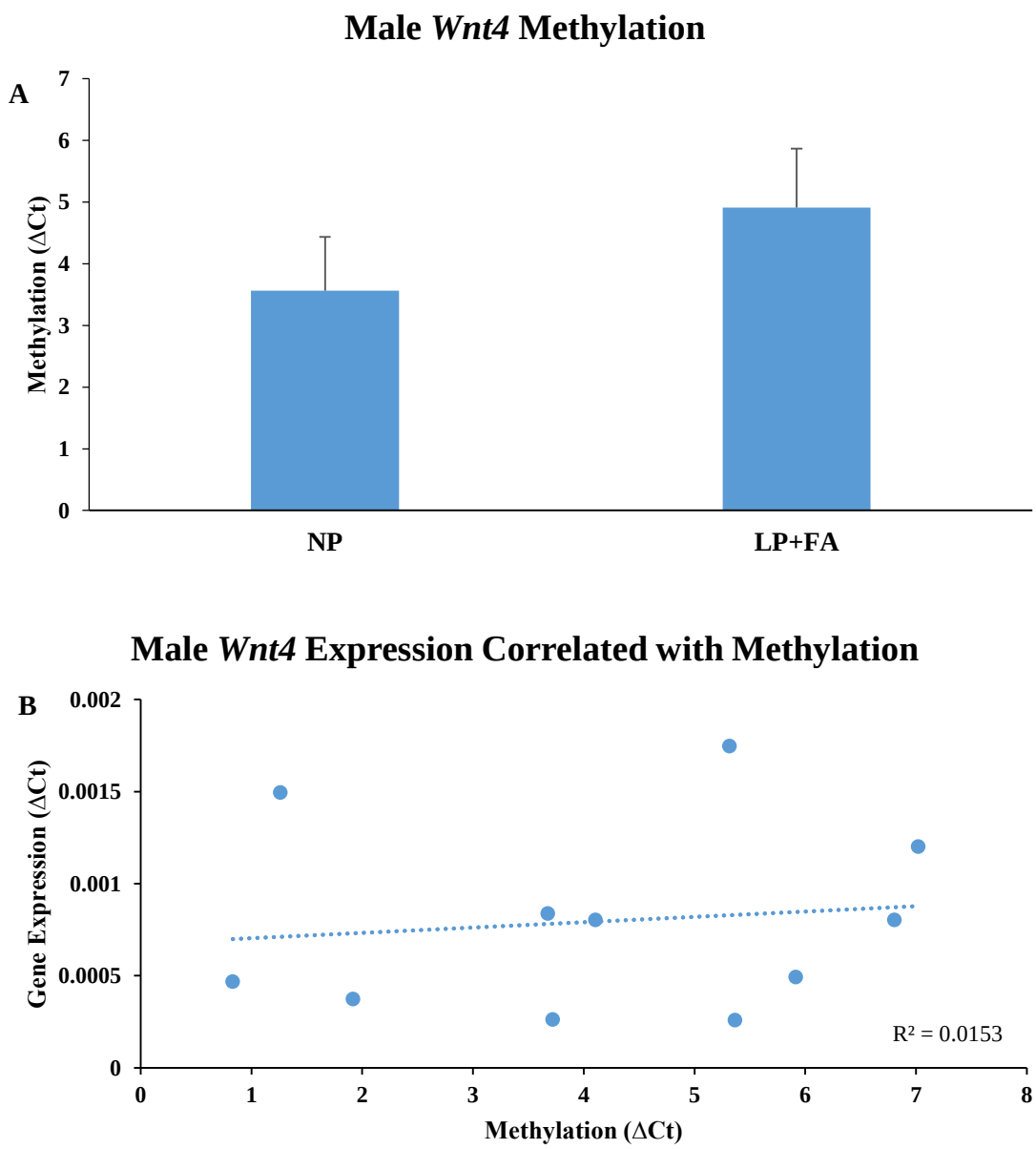

FIGURE 5.12 - METHYLATION OF *WNT4* IN E14.25 KIDNEYS FROM MALE OFFSPRING EXPOSED TO MATERNAL FOLATE SUPPLEMENTATION.

The level of methylation within the promoter region of *Wnt4* (**A**) was determined in E14.25 male kidneys from offspring exposed to maternal normal protein or low protein high FA. The methylation level was also compared to the level of expression of *Wnt4* (**B**). Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed. Correlation represents all samples analysed. NP ♂n=4, LP+FA ♂n=5.

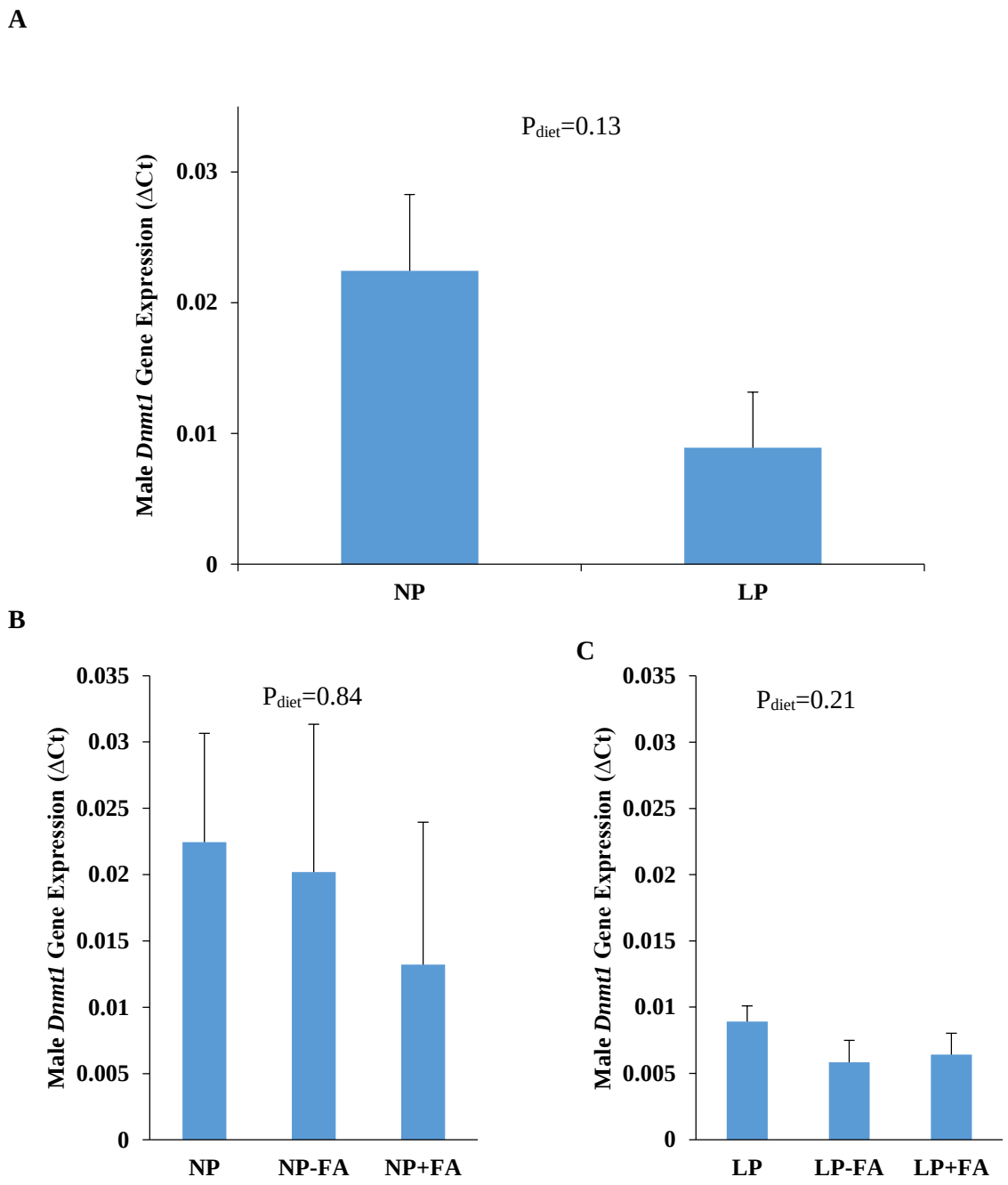

FIGURE 5.13 - MALE *DNMT1* KIDNEY GENE EXPRESSION AT E14.25.

Expression levels of *Dnmt1* in male kidneys exposed to maternal normal or low protein (**A**) with folic acid supplementation or restriction (**B** & **C**). Gene expression determined using Real Time PCR. Housekeeping gene used was 18s. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed, and symbols represent statistical significance determined by LSD post-hoc analysis. No significant differences were identified. NP ♂n=5 ♀n= 5, NP-FA ♂n=4 ♀n=5, NP+FA ♂n=4 ♀n=4, LP ♂n=6 ♀n=5, LP-FA ♂n=5 ♀n=5, LP+FA ♂n=5 ♀n=4.

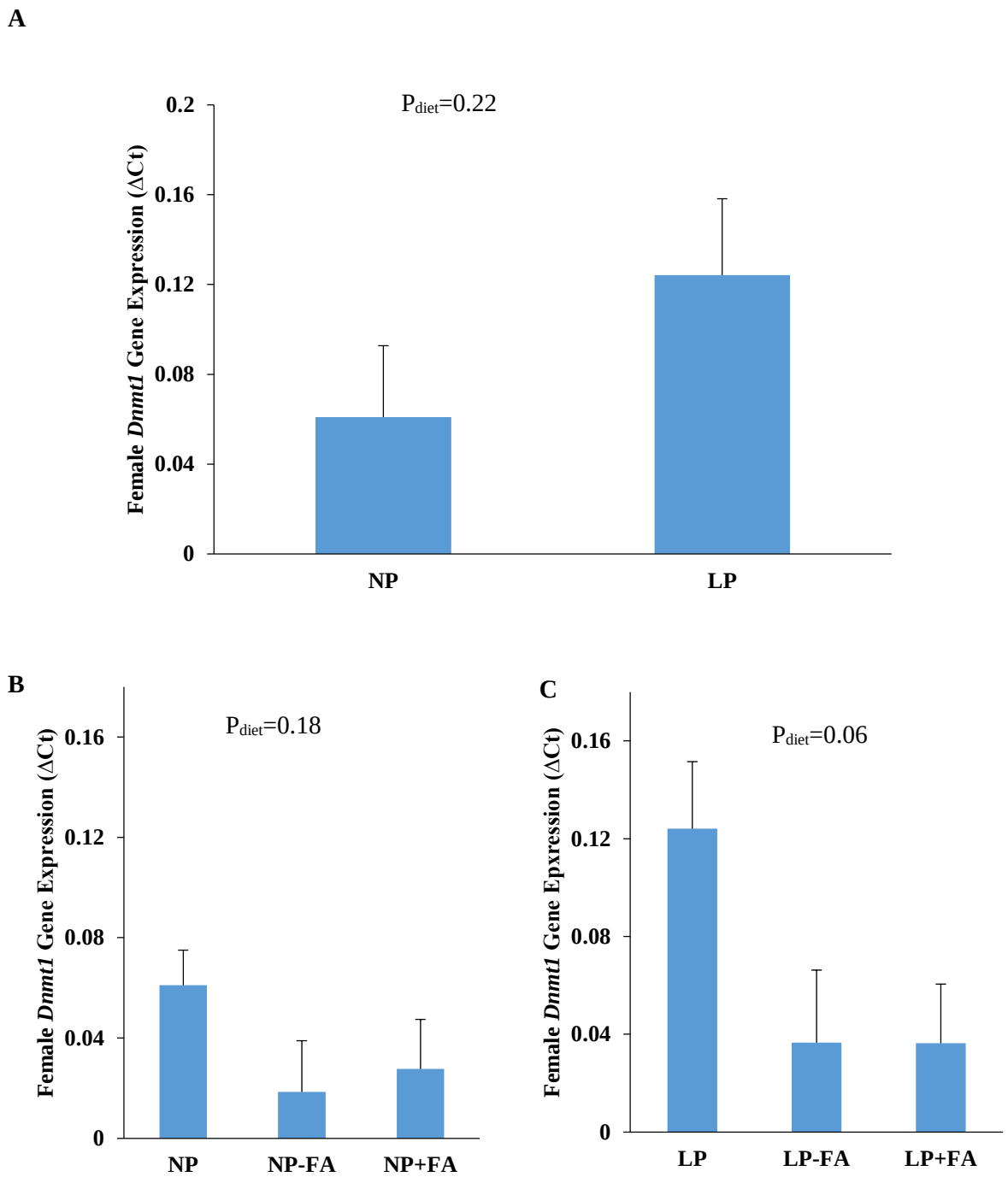

FIGURE 5.14 - FEMALE *DNMT1* KIDNEY GENE EXPRESSION AT E14. 25.

Expression levels of *Dnmt1* in female kidneys exposed to maternal normal or low protein diets (**A**) with folic acid supplementation or restriction (**B** & **C**). Gene expression determined using Real Time PCR. Housekeeping gene used was 18s. Values are mean ± SEM, analysed using least means squares analysis where n represents the number of litters analysed. No significant differences were found. NP ♂n=5 ♀n= 5, NP-FA ♂n=4 ♀n=5, NP+FA ♂n=4 ♀n=4, LP ♂n=6 ♀n=5, LP-FA ♂n=5 ♀n=5, LP+FA ♂n=5 ♀n=4.

5.4 DISCUSSION

The role of folic acid during development has been correlated with positive birth outcomes (Czeizel, 1995c, Czeizel, 1996, Czeizel, 2004). So strong is the evidence in reducing NTDs that many countries around the world have mandated that flour be fortified with folic acid in an effort to increase the folic acid intake in women in their reproductive years (Bar-Oz *et al.*, 2008, Crider *et al.*, 2011a). The fortification of flour products has been a success with up to a 70% reduction in NTDs after fortification (Eichholzer *et al.*, 2006). The success of folic acid fortification programmes illustrates the ability for population-wide improvements to be made on pregnancy by the intake of specific vitamins. The experiments described in this chapter aimed to assess the potential of folic acid intake to ameliorate the deleterious effects on kidney development reported in Chapters 3. Surprisingly, increased folic acid intake was associated with a reduction in branching morphogenesis; a finding that was not induced in LP animals alone.

As expected, maternal folic acid supplementation substantially increased maternal folic acid plasma concentration. Interestingly, there was no observed reduction in plasma folic acid concentration in dams fed a NP-FA diet although consumption of a LP-FA diet was associated with reduced plasma folic acid concentration. This suggests there may be an interplay between protein and folic acid intake. Interestingly, folic acid absorption in the gut can be affected by gut bacteria, which in turn can be affected by diet (Camilo *et al.*, 1996, Scott *et al.*, 2013). The findings also suggest there may be another source of folic acid other than the maternal diet, and this is supported by studies showing that some gut bacteria produce folate and are able to restore folate concentrations in deficient environments (LeBlanc *et al.*, 2010, Pompei *et al.*, 2007a, Pompei *et al.*, 2007b, Wegkamp *et al.*, 2007).

Maternal folic acid supplementation also leads to an increase in folic acid concentration in the amniotic fluid while maternal folic acid restriction did not alter amniotic fluid levels. Interestingly, while maternal low protein and folic acid restriction resulted in lower concentrations of folic acid in maternal plasma, the amniotic fluid concentration was unchanged. This may be related to polarised folate transporters within the placenta (folate receptor α and reduced folate carrier and proton-coupled, high-affinity folate transporter) which can lead to a preferential shunt of folic acid/folate across the placenta (Cherukad *et al.*, 2012).

Fetal growth at E14.25 was not affected by maternal protein or folic acid intake. However, by E17.25, both maternal folic acid supplementation and restriction lead to increases in fetal and placental weight. Maternal folic acid supplementation has previously been reported to increase birth weight in humans (Iyengar and Rajalakshmi, 1975, Rolschau *et al.*, 1979) and this may be due to the role of folate/folic acid in cell proliferation and nucleotide synthesis (Strickland *et al.*, 2013). However, the increase in fetal weight gain was not maintained throughout gestation in the present study, suggesting that maternal folic acid intake may have specific effects during stages of development.

Kidney branching morphogenesis was assessed using an *in vitro* culture system. Exposure to maternal low protein and folic acid restriction was associated with reduced branching morphogenesis, suggesting that folic acid deficiency may negatively impact kidney development. Kidneys exposed to maternal supplementation of folic acid showed no change in branching morphogenesis compared to low protein controls.

Previous studies indicate that exogenous folic acid (0.6-2.0mmol) in a culture setting can have beneficial effects on embryonic malformations caused by gestational hyperglycaemia (Wentzel *et al.*, 2005). It is unclear whether folic acid intake could be affecting the dam, placenta or both and thereby affecting fetal size and development. Therefore, there might be secondary effects

of maternal folic acid intake and we aimed to determine whether folic acid had a direct effect on kidney development using the explant culture system. Branching morphogenesis in kidneys exposed to maternal protein restriction alone was not altered when cultured in the presence of exogenous folic acid. Unlike kidneys exposed to maternal low protein and folic acid supplementation or restriction when cultured with 2mM exogenous folic acid which had significantly reduced branching morphogenesis. These results suggest that high doses of folic acid in a culture setting may have a toxic effect on kidney development. It is known that acute folic acid administration (<250mg/kg body weight) can induce acute renal failure in adult offspring due to acute tubular necrosis (Szczypka *et al.*, 2005), and although the fetal kidneys in the present study were not exposed to anything like this concentration it is feasible that the folic acid supplementation may have affected kidney development by a number of processes including oxidative stress (Gupta *et al.*, 2012). Oxidative stress during development can lead to an increase in apoptosis (Takahashi, 2012). Future studies may aim to assess the role of reactive oxygen species and oxidative stress on kidney development, particularly determining the role of folic acid in producing superoxides.

Kidney gene expression was investigated at E14.25 with a focus on candidate genes involved in branching morphogenesis or the mesenchyme to epithelial transition that are so vital for the process of nephrogenesis. With regards to genes involved in the regulation of branching morphogenesis, the findings revealed that males were relatively protected from maternal low protein and folic acid supplementation or restriction. However, females showed reduced expression of *Gdnf* and *Bmp4* when exposed to maternal low protein and folic acid restriction or supplementation, while *Gfrα1* was only reduced by maternal low protein and folic acid supplementation. To date no study has investigated the role of maternal folic acid intake on renal gene expression. These data support the *in vivo* finding that branching morphogenesis was reduced by maternal low protein and folic acid restriction, suggesting that maternal folic

acid intake is important for kidney development. The molecular and culture data of kidneys exposed to maternal low protein and folic acid supplementation are not complementary in that the genes analysed do not reflect a reduced branching morphogenesis phenotype. Future studies could investigate the role of folate metabolism genes and their influence on kidney development. Folate receptors are expressed in the kidney and may provide an avenue whereby folate/folic acid influence kidney development (Kamen and Smith, 2004).

Pax2 (a transcription factor that regulates epithelial differentiation during nephrogenesis) expression was reduced in females exposed to the maternal low protein diet and folic acid restriction or supplementation. Maternal low protein and folic acid restriction also lead to a reduction in *Pax2* expression in males, while maternal low protein and folic acid supplementation reduced Hnf4α expression. Interestingly, *Hnf4α* regulates expression of folate receptor 1 (Salbaum *et al.*, 2009). This may indicate that reduced *Hnf4α* levels within kidneys exposed to maternal folic acid supplementation may be a mechanism by which the intracellular intake of folic acid is limited.

Maternal normal protein and folic acid supplementation significantly reduced branching morphogenesis after 48 hours in culture. To determine the direct effect of folic acid, kidneys were cultured with exogenous folic acid. Control kidneys exposed to exogenous folic acid showed reduced branching morphogenesis at high folic acid concentrations (2mM), with no change observed at lower levels (0.6mM). There was a trend for similar results with kidneys exposed to maternal normal protein and folic acid restriction. However, kidneys exposed to normal protein and folic acid supplementation had increased branching morphogenesis with 0.6mM folic acid and no change at 2mM. The beneficial effect of exogenous folic acid in a culture setting reported by Wentzel *et al.* (2005) was replicated in the kidney, though it was dependent on maternal diet exposure. Exposure through the maternal diet may alter

biochemical pathways that may alter the methyl pathway – possibly through the activity of essential enzymes (MTHFR) or the receptors (FOLR1).

In males, kidney levels of *Wnt4* expression were reduced following exposure to maternal normal protein and folic acid supplementation, while females demonstrated reduced expression of *Pax2* when exposed to maternal normal protein and folic acid restriction. Maternal normal protein diet and folic acid restriction lead to reduced *Gfrα1* expression in females, but increased levels of *Bmp4* in males. While taken together these findings would suggest reduced branching morphogenesis, this was not seen in the culture study. This is perhaps most likely due to the fact that only a handful of genes were assessed. Future studies should aim to perform a global analysis of renal gene expression in the setting of altered maternal diet and folic acid supplementation or restriction. It is also possible that the fold changes in levels of expression observed for the genes currently examined were not great enough to induce a change in renal phenotype.

Methylation status was investigated in *Bmp4*, *Wnt4* and *Gfrα1* as these genes were considered good candidates as they contain large CpG islands upstream of the promoter region. Comparing gene expression and methylation status it is assumed there is a negative correlation (ie. when gene expression is high, methylation is low) (Boyes and Bird, 1991). However, Wnt4 expression and methylation status did not follow this model. It is possible that the regions of Wnt4 being investigated were not related to gene regulation. While Yu *et al.* (2009) suggest that the up-stream promoter of Wnt4 is methylation sensitive due to CpG , the present finding suggests that maternal diet manipulation did not alter methylation of these regions. *Gfrα1* followed the anticipated relationship between expression level and gene methylation, where high concentrations of methylation were associated with low concentrations of gene expression. Comparing the lines of best fit between dietary groups, protein restriction was found to result in a stronger relationship between methylation and gene expression. This

suggests that maternal folic acid intake may alter the expression of *Gfrα1*. This was hypothesised by Godmann *et al.* (2010) who reported that *Gfrα1* expression in sperm is controlled by the methylation and acetylation of histone H3. In the current study Bmp4 was not methylated at all. While primers were designed to target potentially methylation-sensitive regions of genes, it is possible that other regions may be responsible for controlling gene expression. Dnmt1 expression and methylation were analysed at E14.25. The findings of Esfandiari *et al.* (2003) and Ghoshal *et al.* (2006) suggest that methyl deficient diets (ie low folic acid intake) can lead to reduced *Dnmt1* expression in rat livers. We report no change in *Dnmt1* expression in the current study which may reflect that the methyl pool is not significantly reduced in fetuses exposed to folic acid intake (supplemented or restricted), and likewise *Dnmt1* expression was not up-regulated due to more methyl groups present in the diet. Therefore, while some gene expression may be related to the methylation status of the gene, there was no overall change in gene methylation due to sufficiency within the methyl pool. It is unknown if this is specific to the kidney or whether *Dnmt1* sensitivity to methyl groups is organ specific.

The findings from the experiments described in this Chapter suggest that supplementing or restricting a normal protein diet with folic acid does not improve kidney development, and may in some situations negatively impact upon kidney development. Similarly, supplementation or restriction of folic acid levels in the setting of a maternal low protein diet did not reverse the deleterious effects of the low protein diet on kidney development. Therefore, the beneficial effect of folic acid may be dependent on particular other factors.

CHAPTER SIX: THE EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON OFFSPRING CARDIOVASCULAR AND RENAL FUNCTIONS

6.1 INTRODUCTION

It is well established that maternal nutrition and behaviour can impact upon fetal development and later offspring health. Efforts have been made to provide better care for pregnant women in order to prevent undesirable birth outcomes. During the 20th Century, significant research effort was aimed at reducing neural tube defects – it was found that increasing the amount of folate or folic acid consumed during pregnancy was positively correlated with a reduction in neural tube defects (Czeizel, 1993b, Czeizel, 1995c, Czeizel, 1995b, Schorah and Smithells, 1993, Wild *et al.*, 1994, Smithells *et al.*, 2011). Despite the correlation between maternal folic acid intake and the reduction in the incidence of neural tube defects, no definitive mechanism has yet been identified.

Understanding the phenomenon of developmental programming has focused on the role of maternal diet and data suggests that caloric and protein restriction can be deleterious to offspring health (Langley-Evans, 2013). It remains unclear whether there are particular micronutrients, amino acids or vitamins in the maternal diet that play a role in models of developmental programming. Studies described in Chapter 5 showed that exposing rat fetuses to maternal LP-FA lead to a 13% reduction in ureteric branching morphogenesis compared to LP controls, while LP+FA did not alter ureteric branching morphogenesis. It is unknown whether reduced branching morphogenesis results in deleterious effects on postnatal health, although reductions in kidney branching or nephrogenesis might be hypothesised to result in abnormal kidney growth and renal disease in adulthood.

Folic acid is involved in the one-carbon metabolism cycle, and is the major methyl donor for the biologically vital processes of lipid and DNA methylation as well as the synthesis of nucleotides (Ohrvik and Witthoft, 2011). During periods of high cell proliferation, such as embryonic and early fetal life, there is greater demand for one-carbon metabolites (Greenberg

et al., 2011). Folic acid has been used as a maternal diet supplement to ameliorate the disease phenotype, such as hypertension, low birth weight and altered gene expression, induced in offspring born to protein restricted rats (Burdge *et al.*, 2009, Lillycrop *et al.*, 2005a, Lillycrop *et al.*, 2010, Torrens *et al.*, 2006). It is hypothesised that folic acid supplementation protects the developing fetus from the consequences of maternal protein restriction by maintaining levels of methionine and low levels of homocysteine (Fetoui *et al.*, 2009, Petrie *et al.*, 2002, Rees *et al.*, 2006). It has also been suggested that additional folate during pregnancy maintains the integrity of the methylation of the genetic code due to its essential role in the methionine pathway and impact on methyl production (Haggarty *et al.*, 2013). Folate, or folic acid, is the principal one-carbon metabolite and plays an essential role in the production of the universal methyl donor – s-adenosyl methionine. Studies in humans and rodents have shown that the addition of one-carbon metabolites (most commonly folate/folic acid) to the diet lowers the risk of cardiovascular disease (Villa *et al.*, 2007, Moens *et al.*, 2008, Dayal and Lentz, 2008). In addition to the effect in the adult, the supply of one-carbon metabolites in pregnancy may have profound and permanent effects upon offspring health.

No study to date has analysed folic acid supplementation to a protein restricted maternal diet on offspring growth and nephron number and the impact of these parameters on blood pressure and renal function While there is some evidence to suggest that supplementation of the maternal diet with folic acid or 1-carbon metabolities is beneficial to offspring health. .Jackson *et al.* (2002) showed that addition of glycine, a one- carbon metabolite, to the maternal diet alleviates hypertension caused by a suboptimal intrauterine environment induced by maternal protein restriction. Likewise, Torrens *et al.* (2006) used a maternal low protein diet supplemented with folic acid (5mg/kg) and analysed systolic blood pressure in adult male offspring and reported that folic acid ameliorated the rise in systolic blood pressure attributed to maternal protein restriction.

Results described thus far in this thesis have demonstrated that a maternal low protein can lead to a nephron deficit at E17.25 and PN21, with no subsequent alteration in adult renal function or blood pressure. This nephron deficit was not associated with a change in branching morphogenesis at E14.25. The aim of the present study was to determine the adult phenotype of offspring exposed to a maternal diet deficient in folic acid or supra-supplemented with folic acid. It was originally hypothesised that folic acid supplementation would prevent the nephron deficit observed in offspring of maternal low protein dams, and likewise there would be no change cardiovascular function as measured by blood pressure, renal blood flow and glomerular filtration rate. However, the results from Chapter 5 may suggest that adult cardiovascular function may actually be worse after exposure to maternal low protein and folic acid restriction. In the present study, adult kidney nephron number, glomerular function and blood pressure were quantified.

6.2 METHODS

Experiments were conducted in accordance with the National Health and Medical Research Council of Australia '*Australian Code of Practice for the Care and Use of Animals for Scientific Purposes'* (7th edition, 2004). Approval was granted in advance by the Monash University School of Biomedical Sciences Animal Ethics Committee. All methods were described in detail in Chapter 2.

6.2.1 ANIMAL HUSBANDRY

Nine week old female Sprague-Dawley rats were housed in pairs and allowed a 3 week acclimatization period before being fed *ad libitum* one of the following diets (Glen Forest, WA Australia):

- low protein (LP 8.4% casein, 2mg/kg FA)
- low protein supplemented with folic acid (LP+FA 8.4% protein, 200mg/kg FA)
- or low protein restricted folic acid (LP-FA 8.4% protein, <0.05mg/kg FA).

Dietary manipulation continued throughout pregnancy and suckling (21 days postnatal) then offspring were weaned to a control diet (19.4% casein) which was fed *ad libitum* until the time of experimentation (PN21, 180 and 360). See Sections 2.2 and 2.2.2 for information on the experimental diets used and the rearing of animals.

6.2.2 BODY WEIGHT AND BODY COMPOSITION ANALYSIS

Anaesthetised animals were scanned with a DXA machine (QDR-4500 DOS Series, Hologic, USA) to determine body fat, lean muscle and bone mineral content at PN21, PN180 and PN360. Refer to Section 2.4.6 for full details.

6.2.3 STEREOLOGICAL ANALYSIS

Nephron number was determined in PN21 offspring using the gold standard physical disector/fractionator (Cullen-McEwen *et al.*, 2011) as per Section 2.4.1.

6.2.4 PHYSIOLOGICAL EXPERIMENTS

Glomerular filtration and effective renal blood flow were determined in PN180 and PN360 offspring using the gold standard of ^{3}H inulin and ^{14}C PAH clearance, respectively. Refer to Section 0 for more details.

Blood pressure (systolic, diastolic and mean arterial pressure) and heart rate were measured in conscious, freely moving animals at PN360 using an indwelling radio-telemetry device (PA-C40, DSI, USA). Recordings reported in this chapter have been separated into the active and inactive period. An hour at the start and end of each period were not included in the analysis to remove any transition effects. For more details on surgical and measuring procedures refer to Section 2.4.3.

Stress tests were also performed in order to quantify the cardiovascular arousal response. Animals were placed on an oscillating table for 10 minutes (novel acute stressor) at 100 rpm, as well as given a novel non-aversive stressor (fed a sultana) (Davern and Head, 2011). For both stress tests, blood pressure and heart rate were recorded for 5 minutes prior, 10 minutes during exposure to the stress test and 30 minutes after the stress test.

6.2.5 STATISTICAL ANALYSIS

Data were analysed using a mixed linear model taking into account litter representation (n values represent litter numbers not the total number of animals studied because multiple animals from each litter were chosen for analysis) (SPSS 21, IBM). Data are expressed as mean ± SEM, and statistical significance is represented by $P<0.05$, whereby P_{diet} represents the

impact of maternal diet, P_{sex} represents the influence of offspring sex and $P_{diet*sex}$ represents the interaction of these variables. Wherever appropriate, repeated measures ANOVA models were used. Graphs were built using Microsoft Office (Microsoft Excel 2013, Microsoft, USA).

6.3 RESULTS

6.3.1 POSTNATAL GROWTH

6.3.1.1 BODY WEIGHT

Restriction or supplementation of a maternal low protein diet with folic acid did not alter PN1 body weight (P_{diet}=0.14, P_{sex}=0.66, $P_{diet*sex}$=0.92; Figure 6.1A). Notwithstanding this, LP-FA offspring demonstrated an 18% reduction in body weight at PN21 compared with LP controls (P_{diet}<0.001, P_{sex}=0.61, $P_{diet*sex}$<0.43; post hoc analysis $P_{LP\ vs\ LP\text{-}FA}$<0.05, Figure 6.1B). Postnatal growth (from PN25) was not altered by maternal diet (Figure 6.1).

6.3.1.2 BODY COMPOSITION

Offspring body composition was determined by DXA. Body fat (%) of male and female offspring exposed to LP-FA or LP+FA were not different compared with LP controls at any of the ages examined (PN21 P_{diet}=0.41, PN180 P_{diet}=0.14, PN360 P_{diet}=0.44). Similarly, lean muscle (expressed as a % of bodyweight) was not affected by maternal diet (PN21 P_{diet}=0.47, PN180 P_{diet}=0.15, PN360 P_{diet}=0.08, Figure 6.3).

Bone mineral content was altered by maternal diet manipulation at PN21 (P_{diet}<0.05, P_{sex}<0.001, $P_{diet*sex}$=0.26), and PN360 P_{diet}<0.05, P_{sex}<0.001, $P_{diet*sex}$<0.05), but not PN180 (P_{diet}=0.12, P_{sex}<0.05, $P_{diet*ses}$=0.15). Bone mineral content was reduced by 38% in offspring exposed to LP-FA compared with LP at PN21 (LP; Female 0.705±0.07 Male 0.960±0.09, LP-FA; Female 0.501±0.05, Male 0.535±0.07, LP+FA; Female 0.535±0.07, Male 0.593±0.05 (post hoc analysis $P_{LP\ vs\ LP\text{-}FA}$<0.05) and 8% at PN180 (LP; Female 9.728±0.490, Male 16.351±0.519, LP-FA; Female 9.639±0.555, Male 14.274±0.519, LP+FA; Female 10.116±0.555, Male 15.321±0.490 (post hoc analysis $P_{LP\ vs\ LP\text{-}FA}$<0.05). Bone mineral content was 12% greater in offspring exposed to LP+FA at PN360 compared with LP (LP+FA, post-hoc analysis $P_{LP\ VS\ LP+FA}$<0.05; Figure 6.3).

6.3.1.3 ORGAN WEIGHT

At PN21, offspring of both LP+FA and LP-FA fed rats demonstrated a 11% and 17% reduction in kidney weight, respectively compared with LP offspring ($P_{diet}<0.05$, $P_{sex}=0.97$, $P_{diet*sex}=0.73$; Table 6.1). At 180 days of age, offspring of LP+FA demonstrated reduced kidney weight relative to body weight compared with LP controls ($P_{diet}<0.05$) that was not affected by offspring sex ($P_{sex}=0.41$, $P_{diet*sex}=0.41$; Table 6.2). At PN360, offspring of LP+FA dams demonstrated greater kidney to body weight ratio than LP controls. There was no evidence for heart, liver, brain, pancreas or fat pad weights being altered by maternal diet (Table 6.3).

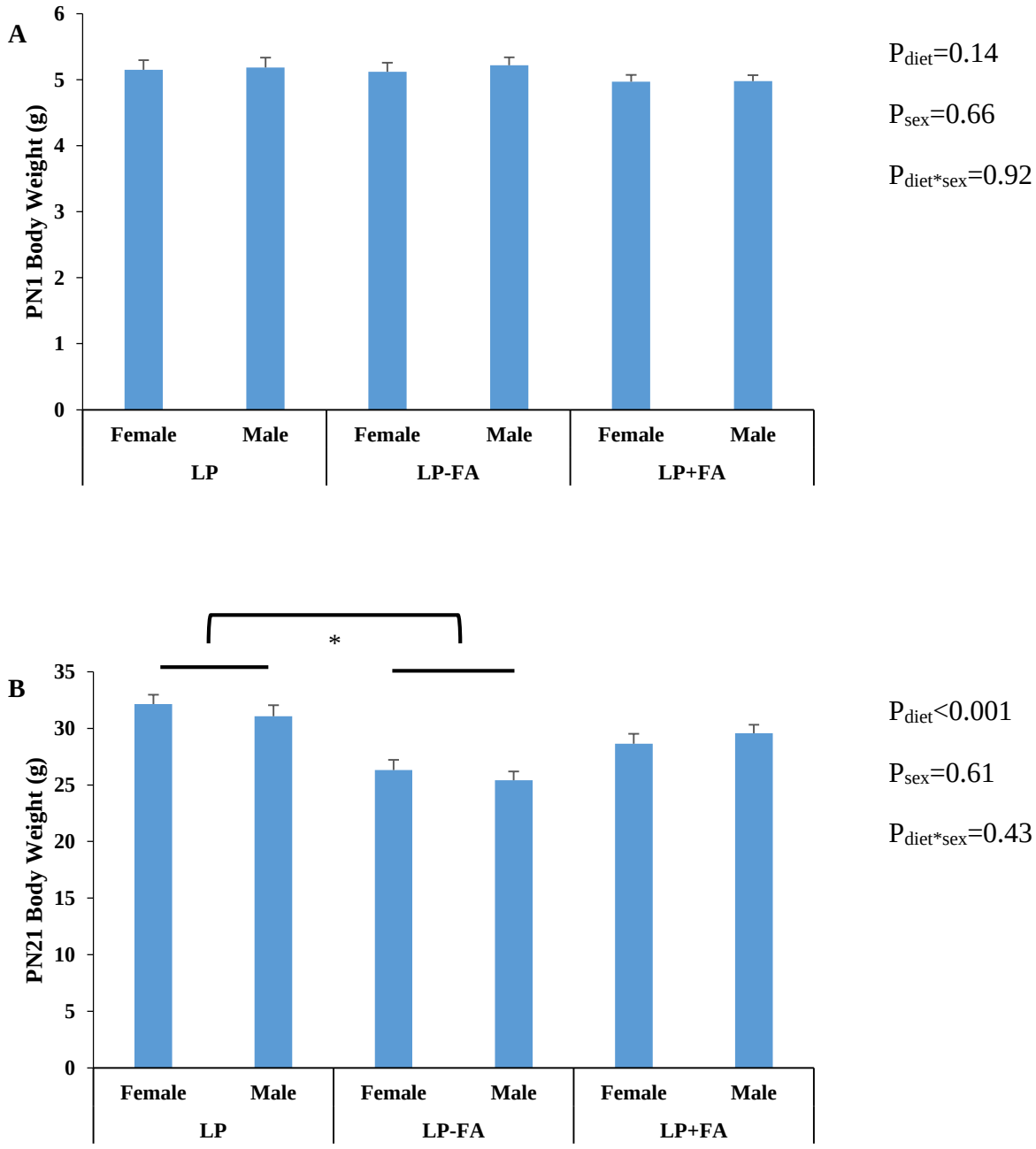

FIGURE 6.1 – POSTNATAL DAY 1 AND 21 BODY WEIGHTS OF MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL LOW PROTEIN DIET AND FOLIC ACID SUPPLEMENTATION OR RESTRICTION.

Male and female offspring exposed to low protein and folic acid supplemented or restricted maternal diets were weighed at postnatal day 1 (**A**) and 21 (**B**). Weight at postnatal day 1was not different between maternal dietary groups, while at PN21 there was a difference between dietary groups. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP PN1 n=9, PN21 n=9, LP-FA PN1 n=11, PN21 n=8, LP+FA PN1 n=11, PN21 n=10), * represents P<0.001.

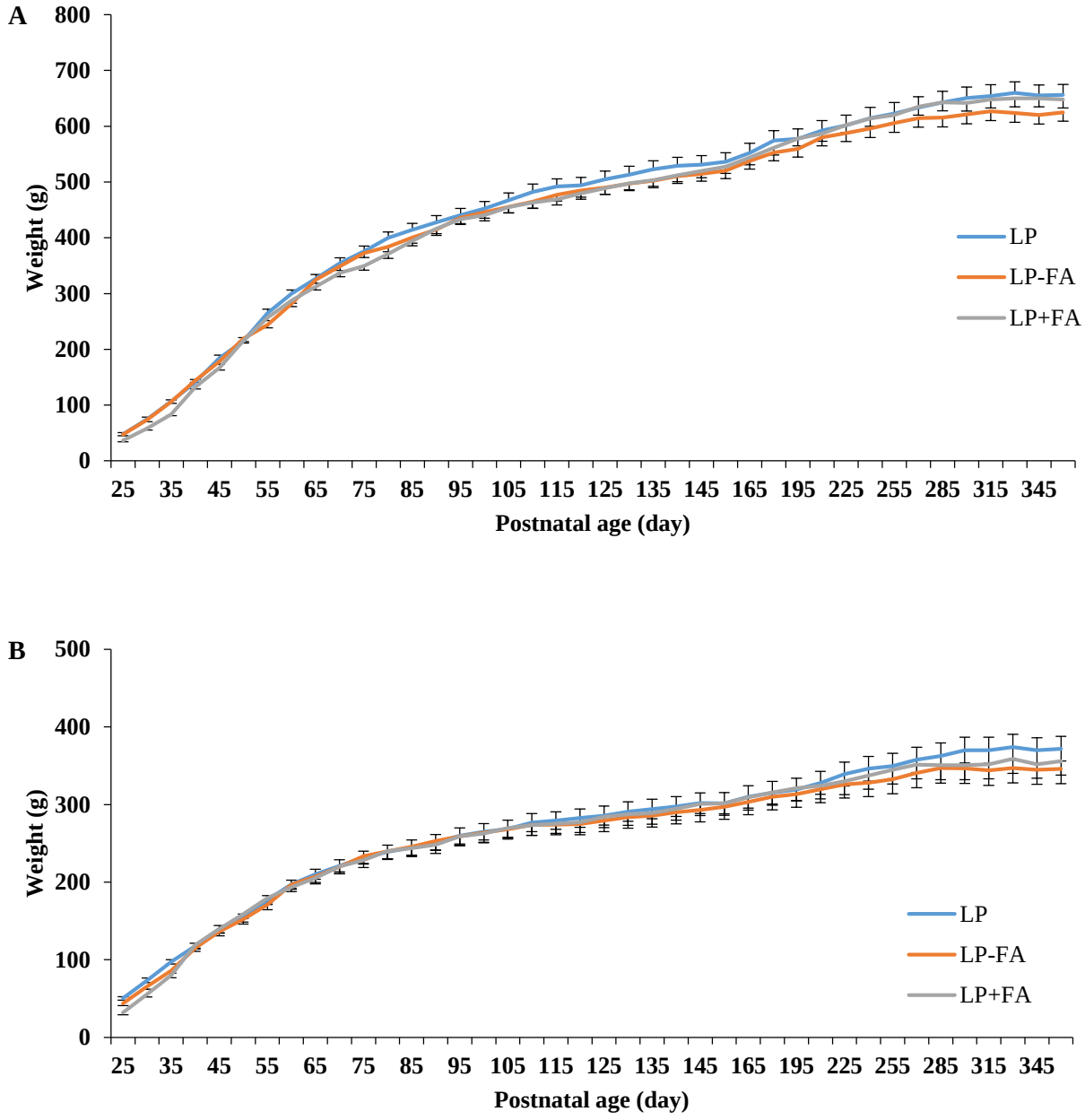

FIGURE 6.2 – GROWTH FROM PN25 TO PN360 OF MALE AND FEMALE OFFSPRING.

Male (**A**) and female (**B**) offspring of rats exposed to either a low protein (blue line, LP) or LP low folic acid (red line, LP-FA) or LP high folic acid (green line, LP+FA) diet during pregnancy and lactation were weighed regularly throughout postnatal life (until PN360). Values are mean ± SEM, analysed by repeated measures ANOVA that was weighted for litter (LP n = 5, LP+FA n = 8, LP-FA n = 6), P_{diet}=0.61, P_{time}<0.001, $P_{diet*time}$=0.29.

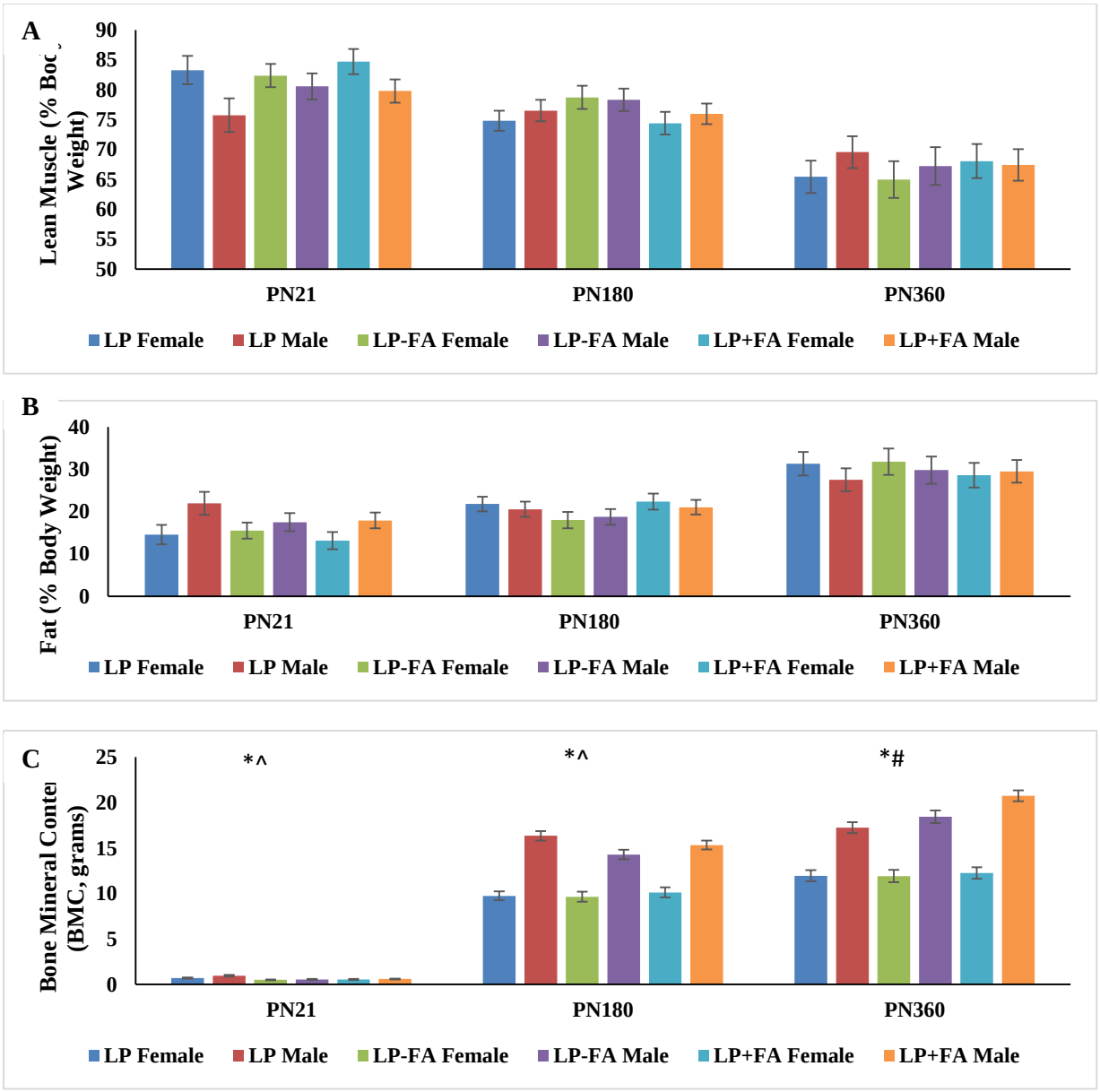

FIGURE 6.3 – POSTNATAL LEAN MUSCLE, FAT AND BONE MINERAL CONTENT (BMC) AT PN21, PN180 AND PN360 OF OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION OR FOLIC ACID RESTRICTION OR SUPPLEMENTATION.

Offspring of female rats exposed to either a low protein (LP), low protein low folate (LP-FA) or low protein high folate (LP+FA) diet during pregnancy and lactation were analysed using DXA at PN21, PN180 and PN360. Lean muscle percentage (**A**), fat percentage (**B**) and bone mineral content (**C**) were determined. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (PN21, LP ♂n=2, ♀n=4, LP-FA ♀n=7, ♂n=4, LP+FA ♀n=4, ♂n=7, PN180 LP ♀n=9, ♂n=8, LP-FA ♀n=7, ♂n=8, LP+FA ♀n=7, ♂n=9, PN360 LP ♀n=8, ♂n=8, LP-FA ♀n=7, ♂n=6, LP+FA ♀n=7, ♂n=9). LSD post hoc test was performed comparing dietary groups. Significant differences between maternal dietary groups represented by * (compared with LP), ^ (compared with LP-FA) and # (compared with LP+FA).

TABLE 6.1 – PN21 OFFSPRING ABSOLUTE AND RELATIVE KIDNEY WEIGHT.

Organ	LP		LP-FA		LP+FA		Significance			Post-hoc		
	Female (n=4)	Male (n=4)	Female (n=6)	Male (n=6)	Female (n=4)	Male (n=4)	P_{Diet}	P_{Sex}	$P_{diet*sex}$	LP Vs LP-FA	LP Vs LP+FA	LP-FA Vs LP+FA
Body weight (g)	30.17 ± 4.35	32.00 ± 4.35	25.83 ± 4.35	25.00 ± 4.35	30.50 ± 4.35	31.33 ± 4.35	0.12	0.81	0.91	NA	NA	NA
Left Kidney (g)	0.162 ± 0.03	0.167 ± 0.03	0.140 ± 0.03	0.132 ± 0.03	0.145 ± 0.03	0.149 ± 0.03	**<0.05**	0.97	0.73	**<0.05**	**<0.05**	0.22
Left Kidney/body weight (g)	0.005 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	**<0.05**	0.31	0.36	0.52	**<0.05**	0.05
Right Kidney (g)	0.164 ± 0.03	0.172 ± 0.03	0.134 ± 0.03	0.130 ± 0.03	0.149 ± 0.03	0.158 ± 0.03	**<0.05**	0.70	0.86	**<0.05**	0.30	0.14
Right Kidney/body weight (g)	0.005 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	0.005 ± 0.0002	0.06	0.81	0.83	NA	NA	NA

Body weight and kidneys weights for PN21 offspring that had been exposed to maternal LP, LP-FA or LP+FA diets. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀=4, ♂=4, LP-FA ♀n=6, ♂n=6, LP+FA ♀n=4, ♂n=4). LSD post hoc test was performed comparing dietary groups.

TABLE 6.2 – PN180 ORGAN AND FAT PAD WEIGHTS FOR OFFSPRING EXPOSED TO MATERNAL LP, LP-FA OR LP+FA DIETS.

Organ	LP		LP-FA		LP+FA		Significance			Post-hoc		
	Female (n=5)	Male (n=4)	Female (n=2)	Male (n=5)	Female (n=4)	Male (n=6)	P_{diet}	P_{sex}	$P_{diet*sex}$	LP Vs LP-FA	LP Vs LP+FA	LP-FA Vs LP+FA
Body weight	309.50 ± 29.69	603.60 ± 37.55	347.63 ± 29.69	631.92 ± 23.29	336.08 ± 23.29	613.58 ± 24.24	0.55	**<0.001**	0.96	NA	NA	NA
Left Kidney (g)	0.633 ± 0.22	1.562 ± 0.23	0.848 ± 0.22	1.552 ± 0.21	0.752 ± 0.21	1.433 ± 0.21	0.39	**<0.05**	0.41	NA	NA	NA
Left kidney/body weight (g)	0.003 ± 0.0001	0.003 ± 0.0001	0.002 ± 0.0001	0.002 ± 0.00007	0.002 ± 0.00007	0.002 ± 0.00007	**<0.05**	0.41	0.79	0.06	**<0.05**	0.38
Heart (g)	0.886 ± 0.06	1.540 ± 0.07	0.965 ± 0.06	1.516 ± 0.06	0.989 ± 0.05	1.541 ± 0.05	0.67	**<0.05**	0.64	NA	NA	NA
Heart/body weight (g)	0.002 ± 0.001	0.002 ± 0.001	0.001 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.21	0.22	0.72	NA	NA	NA
Liver (g)	9.786 ± 3.23	18.895 ± 3.35	10.222 ± 3.278	24.383 ± 3.166	11.299 ± 3.144	20.330 ± 3.166	0.10	**<0.05**	0.07	**<0.05**	0.25	0.21
Liver/ body weight (g)	0.032 ± 0.003	0.030 ± 0.004	0.029 ± 0.003	0.034 ± 0.003	0.032 ± 0.003	0.032 ± 0.003	0.81	0.38	0.06	NA	NA	NA
Brain (g)	1.859 ± 0.141	1.957 ± 0.150	1.897 ± 0.145	2.050 ± 0.137	1.8886 ± 0.135	2.132 ± 0.137	0.355	**<0.05**	0.54	NA	NA	NA
Brain/body weight (g)	0.005 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.002 ± 0.001	0.004 ± 0.001	0.002 ± 0.001	0.50	**<0.05**	0.98	NA	NA	NA
Pancreas (g)	0.656 ± 0.068	0.854 ± 0.083	0.724 ± 0.083	1.035 ± 0.059	0.843 ± 0.055	0.953 ± 0.059	0.11	**<0.05**	0.31	NA	NA	NA
Pancreas/body weight (g)	0.002 ± 0.0004	0.001 ± 0.0005	0.001 ± 0.0004	0.001 ± 0.0003	0.002 ± 0.003	0.001 ± 0.0003	0.38	0.12	0.65	NA	NA	NA
Mesenteric fat (g)	4.452 ± 0.831	6.388 ± 0.929	3.235 ± 0.831	8.458 ± 0.657	4.898 ± 0.619	7.314 ± 0.657	0.68	**<0.05**	0.08	NA	NA	NA
Mesenteric fat/body weight (g)	0.014 ± 0.001	0.010 ± 0.002	0.009 ± 0.001	0.012 ± 0.001	0.014 ± 0.001	0.011 ± 0..001	0.21	0.33	0.05	NA	NA	NA
Peri-renal fat (g)	4.516 ± 2.232	14.208 ± 2.734	4.500 ± 2.446	17.832 ± 1.933	5.766 ± 1.823	13.864 ± 1.933	0.71	**<0.05**	0.45	NA	NA	NA
Peri-renal fat/body weight (g)	0.014 ± 0.006	0.022 ± 0.006	0.012 ± 0.006	0.024 ± 0.006	0.016 ± 0.006	0.021 ± 0.006	0.96	**<0.05**	0.41	NA	NA	NA
Abdominal fat (g)	5.667 ± 1.703	7.822 ± 1.796	5.133 ± 1.703	10.477 ± 1.554	6.953 ± 1.525	7.992 ± 1.554	0.64	**<0.05**	0.09	NA	NA	NA
Abdominal fat/body weight (g)	0.017 ± 0.002	0.012 ± 0.003	0.015 ± 0.002	0.015 ± 0.002	0.020 ± 0.002	0.012 ± 0.002	0.06	**<0.05**	0.08	NA	NA	NA
Total fat (g)	12.948 ± 3.840	28.418 ± 4.596	12.869 ± 4.159	36.767 ± 3.400	17.617 ± 3.240	29.170 ± 3.400	0.55	**<0.05**	0.18	NA	NA	NA
Total fat/body weight (g)	0.040 ± 0.005	0.045 ± 0.007	0.036 ± 0.006	0.051 ± 0.005	0.050 ± 0.004	0.045 ± 0.005	0.57	0.29	0.14	NA	NA	NA

Body, organ and fat pad weights for PN360 offspring exposed to maternal LP, LP-FA or LP+FA diets. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=5, ♂n=4, LP-FA ♀n=2, ♂n=5, LP+FA ♀n=4, ♂n=6). LSD post hoc test was performed comparing dietary groups, NA denotes not applicable.

TABLE 6.3 – PN360 ORGAN AND FAT PAD WEIGHTS FOR OFFSPRING EXPOSED TO MATERNAL LP, LP-FA OR LP+FA DIETS.

Organ	LP		LP-FA		LP+FA		Significance		
	Female (n=5)	Male (n=2)	Female (n=4)	Male (n=5)	Female (n=4)	Male (n=3)	P_{diet}	P_{sex}	$P_{diet*sex}$
Body weight (g)	460.55 ± 29.85	674.75 ± 43.74	379.33 ± 49.49	678.10 ± 30.82	385.40 ± 39.90	679.33 ± 27.12	0.44	**<0.001**	0.37
Left Kidney (g)	1.123 ± 0.25	1.638 ± 0.27	1.437 ± 0.290	1.630 ± 0.24	1.188 ± 0.259	1.733 ± 0.237	0.54	**<0.05**	0.37
Left kidney/body weight (g)	0.002 ± 0.0003	0.002 ± 0.0004	0.003 ± 0.0004	0.002 ± 0.0003	0.003 ± 0.0004	0.003 ± 0.0003	0.10	**<0.05**	0.33
Heart (g)	1.227 ± 0.239	1.659 ± 0.261	1.056 ± 0.261	1.479 ± 0.244	1.144 ± 0.253	1.759 ± 0.239	0.15	**<0.05**	0.40
Heart/body weight (g)	0.003 ± 0.0001	0.002 ± 0.0002	0.003 ± 0.0002	0.002 ± 0.0001	0.003 ± 0.0002	0.003 ± 0.0001	0.60	**<0.05**	0.30
Liver (g)	13.440 ± 2.661	19.719 ± 3.108	9.340 ± 3.108	21.136 ± 2.692	10.628 ± 2.948	20.252 ± 2.600	0.66	**<0.05**	0.24
Liver/body weight (g)	0.028 ± 0.001	0.029 ± 0.002	0.025 ± 0.002	0.031 ± 0.001	0.029 ± 0.002	0.030 ± 0.001	0.84	0.07	0.20
Brain (g)	2.021 ± 0.098	2.185 ± 0.134	1.854 ± 0.108	2.195 ± 0.098	1.911 ± 0.104	2.207 ± 0.098	0.43	**<0.05**	0.36
Brain/body weight (g)	0.004 ± 0.001	0.003 ± 0.001	0.005 ± 0.001	0.003 ± 0.001	0.005 ± 0.001	0.003 ± 0.001	0.58	**<0.05**	0.68
Pancreas (g)	0.852 ± 0.077	1.064 ± 0.230	0.653 ± 0.133	0.976 ± 0.081	0.726 ± 0.115	1.041 ± 0.077	0.60	**<0.05**	0.92
Pancreas/body weight (g)	0.002 ± 0.0002	0.002 ± 0.0004	0.002 ± 0.0003	0.001 ± 0.0002	0.002 ± 0.0003	0.002 ± 0.0002	0.90	**<0.05**	0.93
Mesenteric fat (g)	8.467 ± 1.059	7.308 ± 3.176	7.534 ± 1.833	8.281 ± 1.123	7.166 ± 1.588	7.302 ± 1.059	0.88	0.95	0.89
Mesenteric fat/body weight (g)	0.018 ± 0.002	0.011 ± 0.005	0.018 ± 0.003	0.012 ± 0.002	0.019 ± 0.002	0.011 ± 0.002	0.98	**<0.05**	0.79
Peri-renal fat (g)	11.077 ± 4.196	18.750 ± 12.588	10.725 ± 7.268	30.534 ± 4.451	8.996 ± 6.294	19.977 ± 4.196	0.54	**<0.05**	0.66
Peri-renal fat/body weight (g)	0.023 ± 0.008	0.028 ± 0.016	0.025 ± 0.011	0.042 ± 0.008	0.024 ± 0.010	0.028 ± 0.008	0.47	0.21	0.60
Abdominal fat (g)	8.990 ± 1.112	15.128 ± 3.335	7.770 ± 1.926	10.502 ± 1.179	8.728 ± 1.668	10.679 ± 1.112	0.38	**<0.05**	0.59
Abdominal fat/body weight (g)	0.019 ± 0.002	0.022 ± 0.005	0.018 ± 0.003	0.015 ± 0.002	0.023 ± 0.002	0.016 ± 0.002	0.37	0.28	0.17
Total fat (g)	28.533 ± 5.818	41.186 ± 17.453	26.029 ± 10.076	49.317 ± 6.170	24.890 ± 8.726	37.958 ± 5.818	0.73	0.05	0.79
Total fat/body weight (g)	0.059 ± 0.008	0.061 ± 0.022	0.061 ± 0.013	0.069 ± 0.008	0.067 ± 0.011	0.055 ± 0.008	0.87	0.92	0.50

Organ and fat pad weights for PN360 offspring that had been exposed to maternal LP, LP-FA or LP+FA diets. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=5, ♂n=2, LP-FA ♀n=4, ♂n=5, LP+FA ♀n=4, ♂n=3).

6.3.2 POSTNATAL NEPHRON NUMBER

Offspring of LP-FA fed rats demonstrated an increased nephron endowment (18%) compared with offspring of LP at PN21, while offspring of LP+FA did not demonstrate a difference in nephron number ($P_{diet}<0.05$, $P_{sex}=0.58$, $P_{diet*sex}=0.12$, Figure 6.4A). The number of nephrons per gram of kidney tissue was also greater (41%) in LP-FA offspring compared to LP offspring ($P_{diet}<0.05$, $P_{sex}=0.36$, $P_{diet*sex}=0.57$, post hoc analysis $P_{LP \text{ vs } LP\text{-}FA}<0.001$, Figure 6.4B).

The ratio of nephron number to body weight was 38% greater in LP-FA offspring than in LP offspring ($P_{diet}<0.05$, Figure 6.4C). Nephron number relative to body weight was not sex dependent ($P_{sex}=0.97$), but there was evidence for a maternal diet*sex interaction ($P_{diet*sex}<0.05$) which suggested that males exposed to LP-FA during development had more nephrons than females, and that the inverse occurred in LP+FA offspring. Offspring of LP+FA did not demonstrate a difference in nephron number to body weight ratio compared to LP control offspring, while in post-hoc analysis there was a 24% reduction compared to offspring of LP-FA.

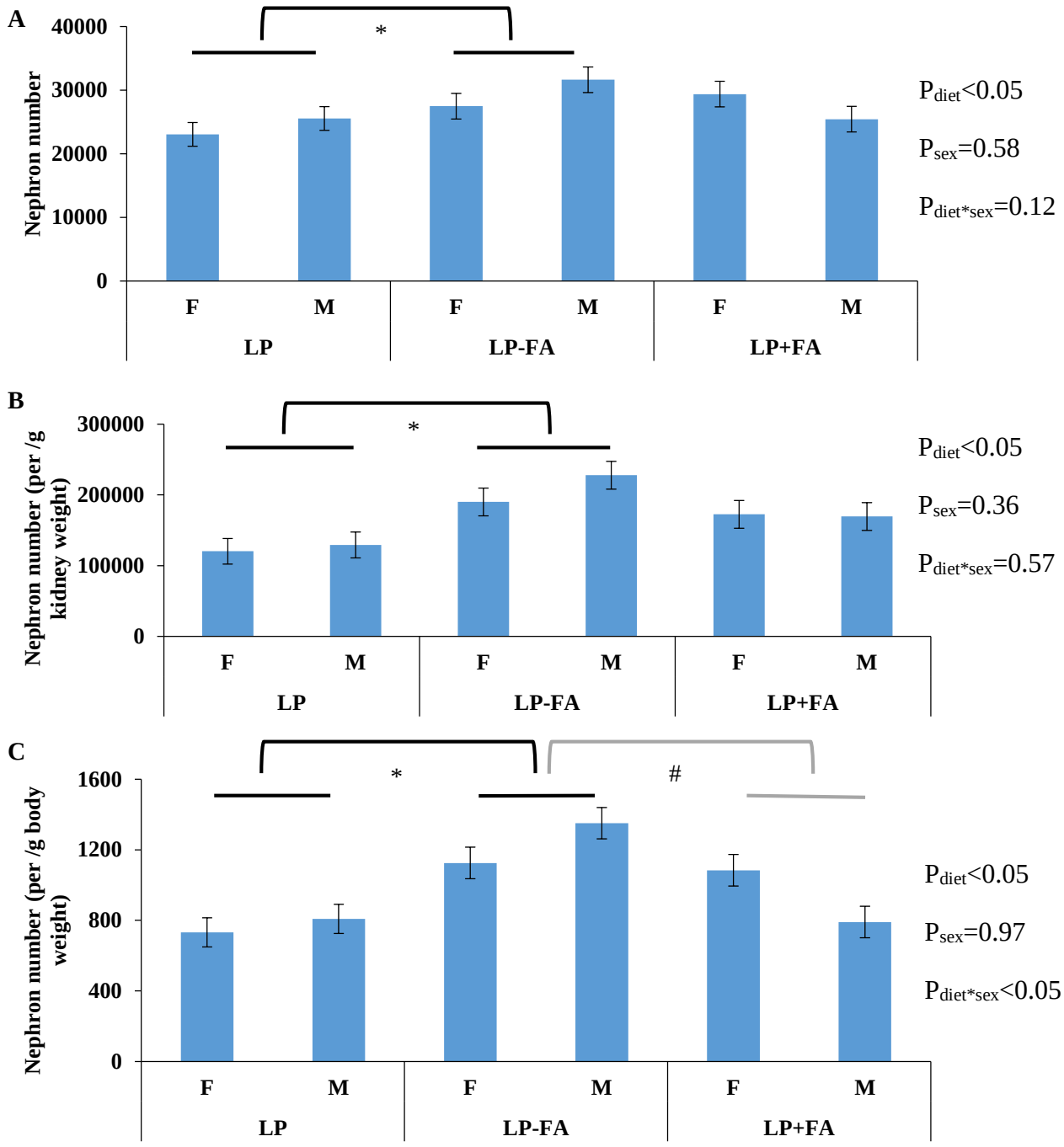

FIGURE 6.4 – PN21 NEPHRON NUMBER FOR FEMALE AND MALE OFFSPRING EXPOSED TO MATERNAL LP OR LP WITH FOLIC ACID RESTRICTION/SUPPLEMENTATION.

Nephron number (**A**), nephron number per gram of kidney tissue (**B**), and nephron number per gram of bodyweight (**C**) was determined in PN21 male and female offspring that had been exposed to maternal LP, LP with folic acid restriction or LP with folic acid supplementation. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=7, ♂n=7, LP-FA ♀n=6, ♂n=6, LP+FA ♀n=6, ♂n=6), $P_{diet}<0.05$, $P_{sex}<0.05$, $P_{diet*sex}<0.05$. LSD Post hoc analysis significant differences between maternal dietary groups represented by * (compared with LP) and # (compared with LP+FA).

6.3.3 RENAL PHYSIOLOGY IN OFFSPRING EXPOSED TO MATERNAL LOW PROTEIN WITH FOLIC ACID SUPPLEMENTATION OR RESTRICTION

Renal function was determined in male and female offspring at 180 days and 360 days of age (Figures 6.5 and 6.6).

6.3.3.1 GLOMERULAR FILTRATION RATE (GFR)

GFR was not altered by maternal dietary exposure (GFR: PN180 $P_{diet}=0.36$, $P_{sex}<0.05$, $P_{diet*sex}=0.67$ Figure 6.5A, PN360 $P_{diet}=0.76$, $P_{sex}<0.05$, $P_{diet*sex}=0.29$, Figure 6.6A).

6.3.3.2 EFFECTIVE RENAL PLASMA FLOW (ERPF)

ERPF was unaltered at PN180 by maternal dietary manipulation (ERPF: $P_{diet}=0.06$, $P_{sex}<0.05$, $P_{diet*sex}=0.20$, Figure 6.5B).

Maternal dietary exposure did not alter ERPF at PN360 (ERPF: $P_{diet}=0.88$, $P_{sex}<0.05$, $P_{diet*sex}=0.31$; Figure 6.6B). Similar to data at PN180, females had significantly greater plasma flow than males.

6.3.3.3 URINE FLOW RATE

Urine flow rate at PN180 and PN360 was not altered by maternal dietary exposure (PN180: $P_{diet}=0.60$, $P_{sex}=0.55$, $P_{diet*sex}=0.83$, PN360: $P_{diet}=0.50$, $P_{sex}=0.54$, $P_{diet*sex}=0.91$).

6.3.3.4 FILTRATION FRACTION

Filtration fraction in male and female offspring at 180 and 360 days was not influenced by maternal diet exposure (PN180 $P_{diet}=0.49$, $P_{sex}=0.12$, $P_{diet*sex}=0.55$ and PN360 $P_{diet}=0.90$, $P_{sex}<0.05$, $P_{diet*sex}=0.10$).

6.3.3.5 URINARY ELECTROLYTES

The urinary and plasma electrolyte profile of offspring was assessed at PN180 and 360 (Table 6.4 and 6.5). Urine sodium and chloride concentration was altered by maternal dietary exposure, with LP-FA diet leading to offspring with greater chloride concentration at PN360

only. However, maternal diet did not alter the concentration of sodium or potassium in either plasma or urine at PN180 or PN360.

The fractional excretion of sodium was not different at PN180 or 360 in male or female offspring exposed to the three maternal diets (PN180 $P_{diet}=0.57$, $P_{sex}=0.09$, $P_{diet*sex}=0.45$ and PN360 $P_{diet}=0.88$, $P_{sex}<0.05$, $P_{diet*sex}=0.41$). The fractional excretion of potassium and chloride was not altered due to maternal folic acid intake.

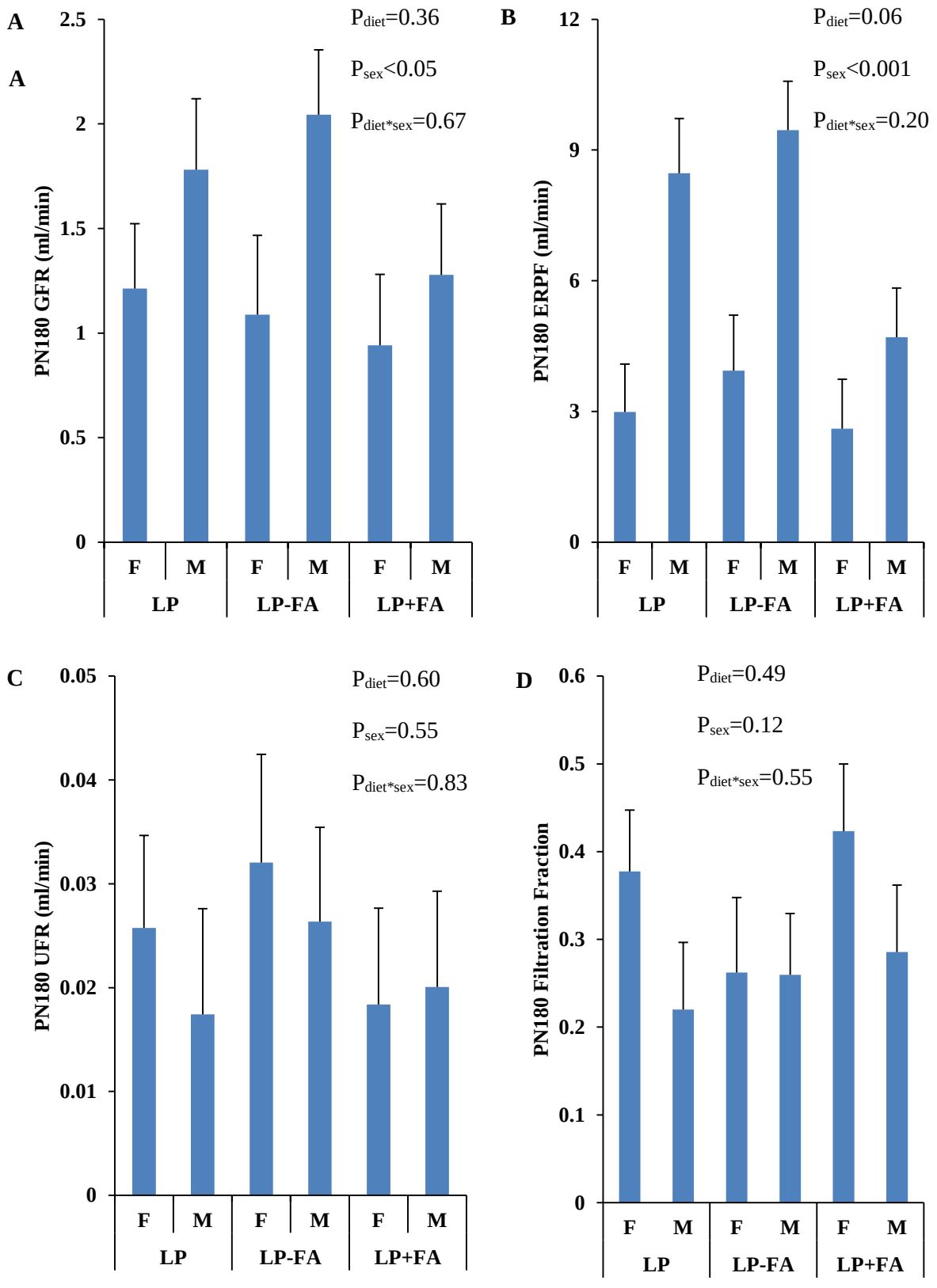

FIGURE 6.5 - RENAL FUNCTION OF PN180 OFFSPRING EXPOSED TO EITHER MATERNAL LOW PROTEIN OR FOLIC ACID SUPPLEMENTATION/RESTRICTION

GFR (ml/min) (**A**), ERPF (ml/min) (**B**), UFR (ml/min) (**C**) and filtration fraction (**D**) in male and female offspring exposed to maternal LP and/or folic acid supplementation/restriction. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=6, ♂n=5, LP-FA ♀n=4, ♂n=6, LP+FA ♀n=5, ♂n=5).

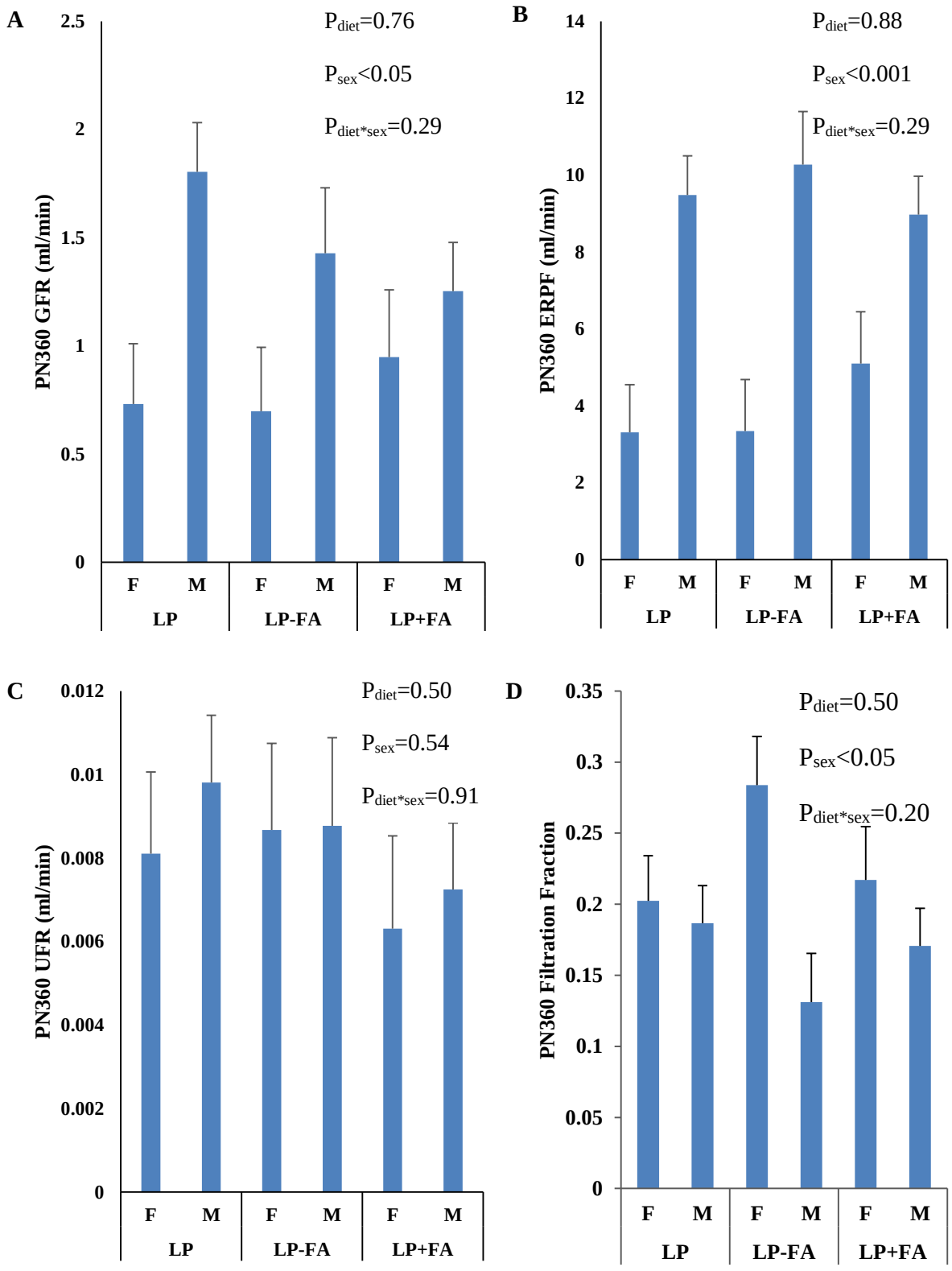

FIGURE 6.6 - RENAL FUNCTION AT PN360 IN FEMALE AND MALE OFFSPRING EXPOSED TO EITHER MATERNAL LOW PROTEIN WITH FOLIC ACID RESTRICTION/SUPPLEMENTATION

Glomerular filtration rate (ml/min) (**A**), effective renal plasma flow (ml/min) (**B**), urine flow rate (ml/min) (**C**) and filtration fraction (**D**) in male and female offspring exposed to maternal LP and/or folic acid supplementation/restriction. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=7, ♂n=10, LP-FA ♀n=6, ♂n=6, LP+FA ♀n=5, ♂n=10).

TABLE 6.4 – ELECTROLYTE PROFILE OF PN180 OFFSPRING EXPOSED TO MATERNAL DIETARY MANIPULATION.

		Plasma				Urine				Excretion values		
Diet	**Sex**	**Na (mmol/l)**	**K (mmoll)**	**Cl (mmol/l)**	**Osmolality (mOsm/kg)**	**Na (mmol/l)**	**K (mmol/l)**	**Cl (mmol/l)**	**Osmolality (mOsm/kg)**	**Na (%)**	**K (%)**	**Cl (%)**
LP	Female (n=6)	156.95 ± 9.87	3.64 ± 0.26	116.20 ± 6.92	321.50 ± 67.80	92.50 ± 18.63	87.27 ± 27.34	136.42 ± 23.55	1071.10 ± 256.96	1.24 ± 0.34	40.47 ± 8.87	2.38 ± 0.83
	Male (n=5)	135.63 ± 11.03	3.46 ± 0.29	105.54 ± 7.52	304.96 ± 67.92	35.08 ± 20.59	94.32 ± 29.40	135.28 ± 26.55	1327.32 ± 269.02	0.24 ± 0.38	24.25 ± 10.05	1.50 ± 0.94
LP-FA	Female (n=4)	126.13 ± 9.22	2.87 ± 0.32	100.53 ± 5.43	292.98 ± 11.47	51.46 ± 17.69	57.66 ± 17.98	118.17 ± 30.28	650.64 ± 250.65	1.21 ± 0.41	36.14 ± 10.52	2.32 ± 0.90
	Male (n=6)	141.25 ± 7.53	3.67 ± 0.27	108.21 ± 4.43	296.69 ± 11.10	55.53 ± 14.44	89.48 ± 16.13	152.88 ± 26.19	1397.02 ± 209.60	0.78 ± 0.35	23.93 ± 9.04	1.50 ± 0.83
LP+FA	Female (n=5)	144.80 ± 8.25	3.61 ± 0.28	113.38 ± 4.86	285.17 ± 10.83	89.36 ± 15.82	102.13 ± 15.98	144.64 ± 26.98	1187.64 ± 224.04	1.22 ± 0.37	59.92 ± 9.38	2.90 ± 0.81
	Male (n=5)	143.00 ± 8.25	3.77 ± 0.28	113.20 ± 4.86	281.00 ± 10.25	58.38 ± 15.82	97.23 ± 15.67	122.58 ± 26.85	1377.06 ± 223.91	1.09 ± 0.37	50.52 ± 9.35	2.90 ± 0.78
Significance	P_{diet}	0.11	0.37	0.11	0.12	0.45	0.32	0.99	0.38	0.57	0.06	0.40
	P_{sex}	0.96	0.24	0.92	0.17	**<0.05**	0.29	0.78	**<0.05**	0.09	0.10	0.28
	$P_{diet*sex}$	0.22	0.21	0.36	0.09	0.11	0.39	0.54	0.46	0.45	0.93	0.76

Sodium (Na), potassium (K) and chloride (Cl) plasma and urine values as well as excretory values for male and female offspring exposed to maternal LP and/or folic acid supplementation or restriction. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=6, ♂n=5, LP-FA ♀n=4, ♂n=6, LP+FA ♀n=5, ♂n=5).

TABLE 6.5 – ELECTROLYTE PROFILE OF PN360 OFFSPRING EXPOSED TO MATERNAL DIETARY MANIPULATION.

		Plasma				Urine				Fractional Excretion values		
Diet	Sex	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Osmolality (mOsm/kg)	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Osmolality (mOsm/kg)	Na (mmol/l /min)	K (mmol/l /min)	Cl (mmol/l /min)
LP	Female (n=7)	127.95 ± 7.12	3.23 ± 0.16	105.68 ± 3.93	256.18 ± 34.41	52.37 ± 14.09	108.90 ± 24.99	146.08 ± 31.57	1477.50 ± 213.40	0.48 ± 0.14	36.33 ± 8.07	1.50 ± 0.31
	Male (n=10)	133.56 ± 5.52	3.43 ± 0.12	104.36 ± 3.04	312.72 ± 26.44	33.43 ± 10.91	115.38 ± 19.00	156.59 ± 24.05	1547.38 ± 163.31	0.15 ± 0.11	19.19 ± 6.11	0.86 ± 0.24
LP-FA	Female (n=6)	155.20 ± 7.12	3.61 ± 0.16	115.93 ± 3.93	324.45 ± 34.11	76.82 ± 14.09	101.88 ± 25.04	173.88 ± 31.01	809.10 ± 210.61	0.58 ± 0.14	37.53 ± 7.88	1.77 ± 0.31
	Male (n=6)	148.18 ± 7.12	3.65 ± 0.13	113.20 ± 3.93	388.94 ± 34.27	42.60 ± 14.09	134.91 ± 25.93	134.49 ± 31.34	1714.87 ± 212.17	0.18 ± 0.14	26.20 ± 8.00	0.84 ± 0.32
LP+FA	Female (n=5)	141.52 ± 7.80	3.46 ± 0.17	109.90 ± 4.30	309.71 ± 37.17	81.36 ± 15.43	146.84 ± 24.96	157.00 ± 33.56	1653.89 ± 228.87	0.39 ± 0.15	29.27 ± 8.47	0.97 ± 0.31
	Male (n=10)	146.86 ± 5.52	3.83 ± 0.12	113.00 ± 3.04	323.09 ± 26.37	62.60 ± 10.91	136.81 ± 18.68	129.26 ± 23.90	1864.48 ± 162.61	0.33 ± 0.11	28.02 ± 6.06	0.92 ± 0.23
Significance	P_{diet}	**<0.05**	0.09	**<0.05**	0.12	0.09	0.47	0.93	0.09	0.88	0.87	0.54
	P_{sex}	0.81	0.10	0.92	0.10	**<0.05**	0.53	0.42	**<0.05**	**<0.05**	0.12	**<0.05**
	$P_{diet*sex}$	0.59	0.56	0.72	0.70	0.81	0.52	0.65	0.10	0.41	0.65	0.20
Post-hoc analysis	LP vs LP-FA	**<0.05**	NA	**<0.05**	NA	NA	NA	NA	NA	NA	NA	NA
	LP vs LP+FA	**<0.05**	NA	0.08	NA	NA	NA	NA	NA	NA	NA	NA
	LP-FA vs LP+FA	0.29	NA	0.42	NA	NA	NA	NA	NA	NA	NA	NA

Sodium (Na), potassium (K) and chloride (Cl) plasma and urine values as well as excretory values for male and female offspring exposed to maternal LP and/or folic acid supplementation or restriction. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=7, ♂n=10, LP-FA ♀n=6, ♂n=6, LP+FA ♀n=5, ♂n=10). LSD post hoc test was performed comparing dietary groups (NA denotes 'not applicable').

6.3.4 CARDIOVASCULAR HAEMODYNAMICS

Cardiovascular haemodynamic function was determined by radiotelemetry in 12 month-old male and female offspring exposed to the maternal LP diet or the LP diet with folic acid supplementation/restriction. There was no significant effect of maternal dietary intake on offspring mean arterial pressure (Figure 6.7), heart rate (Figure 6.8), systolic or mean arterial pressure during either the active or inactive periods (Table 6.6).

The cardiovascular arousal response to acute aversive stress was not altered by maternal diet (P_{diet}=0.11). There was no effect of sex on heart rate due to stress (P_{sex}=0.74), but but there was an interaction effect whereby males exposed to folic acid supplementation had a higher heart rate than low protein controls (, $P_{diet*sex}$<0.05). The change in mean arterial pressure (P_{diet}=0.33, P_{sex}=0.13, $P_{diet*sex}$=0.70), systolic (P_{diet}=0.44, P_{sex}=0.11, $P_{diet*sex}$=0.79) and diastolic (P_{diet}=0.23, P_{sex}=0.20, $P_{diet*sex}$=0.58) blood pressure due to the acute aversive stressor did not differ in offspring of LP+FA or LP-FA rats compared with LP offspring (Figure 6.7).

The haemodynamic response to a non-aversive stimulus was not altered by maternal diet or offspring sex. Heart rate, mean arterial pressure, and diastolic and systolic blood pressure were similar in the three dietary groups following the non-aversive stimulus (heart rate P_{diet}=0.78, P_{sex}=0.68, $P_{diet*sex}$=0.30, mean arterial pressure P_{diet}=0.47, P_{sex}=0.20, $P_{diet*sex}$=0.64, systolic blood pressure P_{diet}=0.48, P_{sex}=0.25, $P_{diet*sex}$=0.70, diastolic blood pressure P_{diet}=0.41, P_{sex}=0.16, $P_{diet*sex}$=0.53) (Figure 6.8).

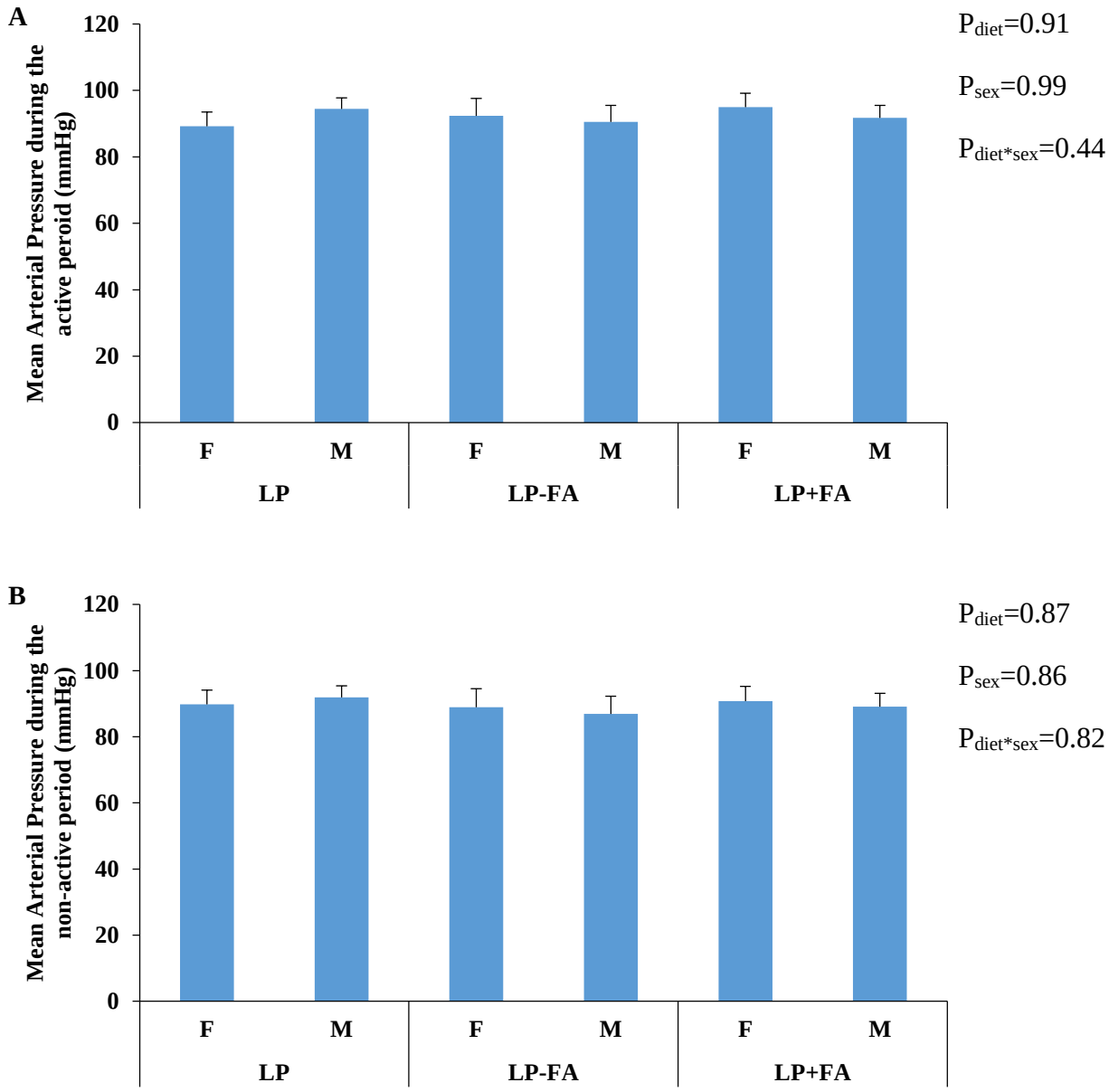

FIGURE 6.7 – MEAN ARTERIAL PRESSURE IN MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION AND/OR FOLIC ACID SUPPLEMENTATION RESTRICTION.

Mean arterial pressure during the active (**A**) and non-active (**B**) period for male and female offspring exposed to maternal protein restriction or LP with folic acid supplementation or restriction. Mean arterial pressure was measured using an indwelling radio-telemetry device. The data presented are an average of four 10 hour periods during the non-active part of the day. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=7, ♂n=11, LP-FA ♀n=5, ♂n=6, LP+FA ♀n=7, ♂n=9).

TABLE 6.6. SYSTOLIC AND DIASTOLIC BLOOD PRESSURE FROM OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION AND/OR FOLIC ACID SUPPLEMENTATION OR RESTRICTION DURING THE NON-ACTIVE AND ACTIVE PERIOD.

		Non-Active Period		Active Period	
Diet	Sex	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)
LP	Female (n=7)	109.64 ± 5.88	74.10 ± 4.29	108.66 ± 6.14	73.73 ± 4.47
	Male (n=11)	108.77 ± 4.95	76.29 ± 3.61	111.84 ± 4.90	79.08 ± 3.57
LP-FA	Female (n=5)	102.63 ± 6.69	77.53 ± 6.08	106.31 ± 6.29	80.92 ± 5.79
	Male (n=6)	105.76 ± 6.43	71.69 ± 5.84	109.25 ± 6.02	75.61 ± 5.54
LP+FA	Female (n=7)	109.94 ± 5.26	74.89 ± 4.78	114.38 ± 4.98	79.08 ± 4.60
	Male (n=9)	105.71 ± 4.80	75.74 ± 4.36	108.40 ± 4.52	78.49 ± 4.17
Significance	P_{diet}	0.77	0.99	0.85	0.92
	P_{sex}	0.97	0.85	0.84	0.98
	$P_{diet*sex}$	0.67	0.56	0.35	0.48

Systolic and diastolic pressure was measured using an indwelling radio-telemetry device. The data presented are an average of four 10 hour periods during the non-active or active part of the day. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=7, ♂n=11, LP-FA ♀n=5, ♂n=6, LP+FA ♀n=7, ♂n=9).

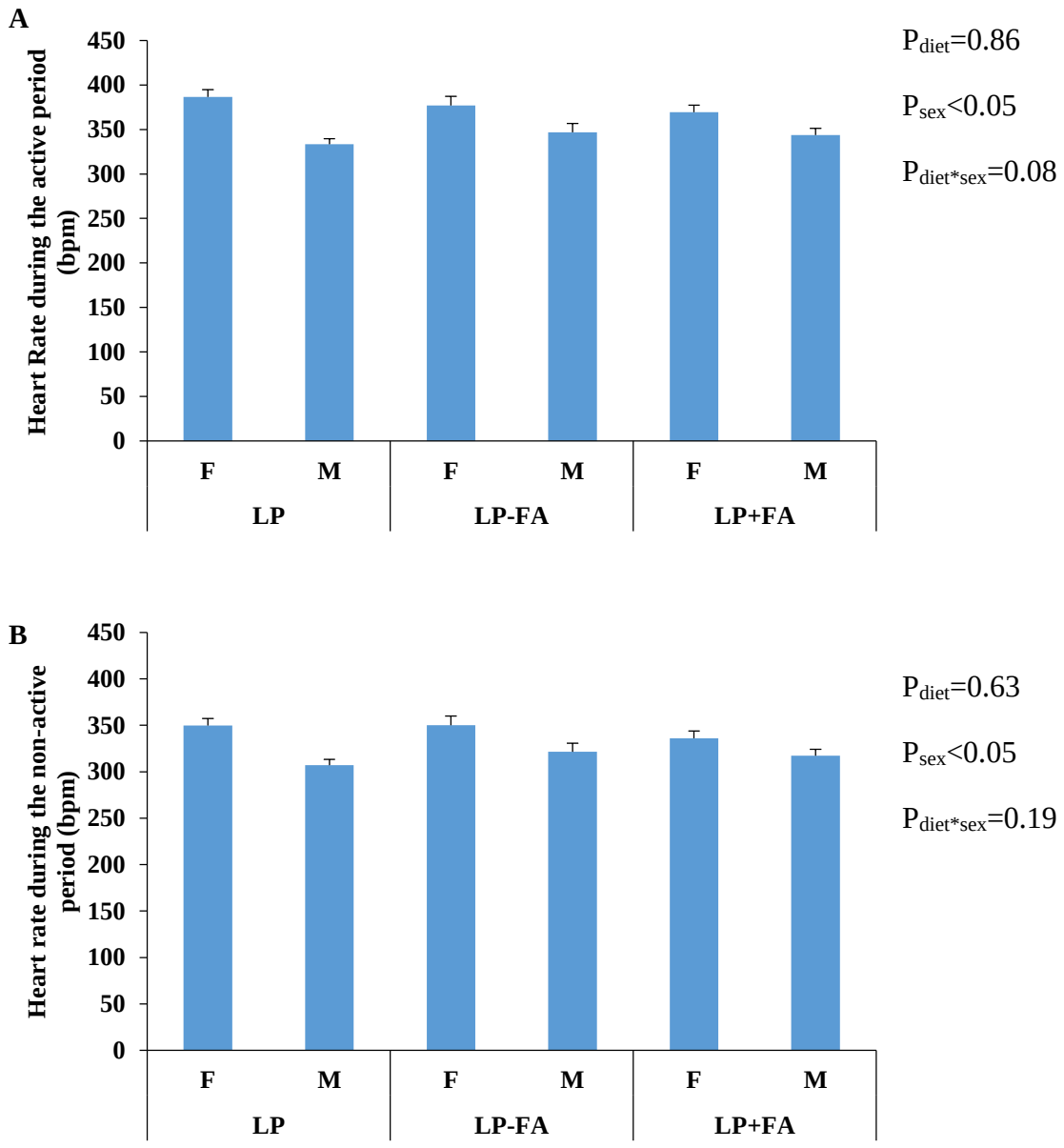

FIGURE 6.8 – HEART RATE IN MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION AND/OR FOLIC ACID SUPPLEMENTATION RESTRICTION.

Heart rate during the active (**A**) and non-active (**B**) period of male and female offspring exposed to maternal LP or LP and folic acid supplementation or restriction. Heart rate was measured using an indwelling radio-telemetry device. The data presented are an average of four 10 hour periods during the non-active part of the day. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=7, ♂n=11, LP-FA ♀n=5, ♂n=6, LP+FA ♀n=7, ♂n=9).

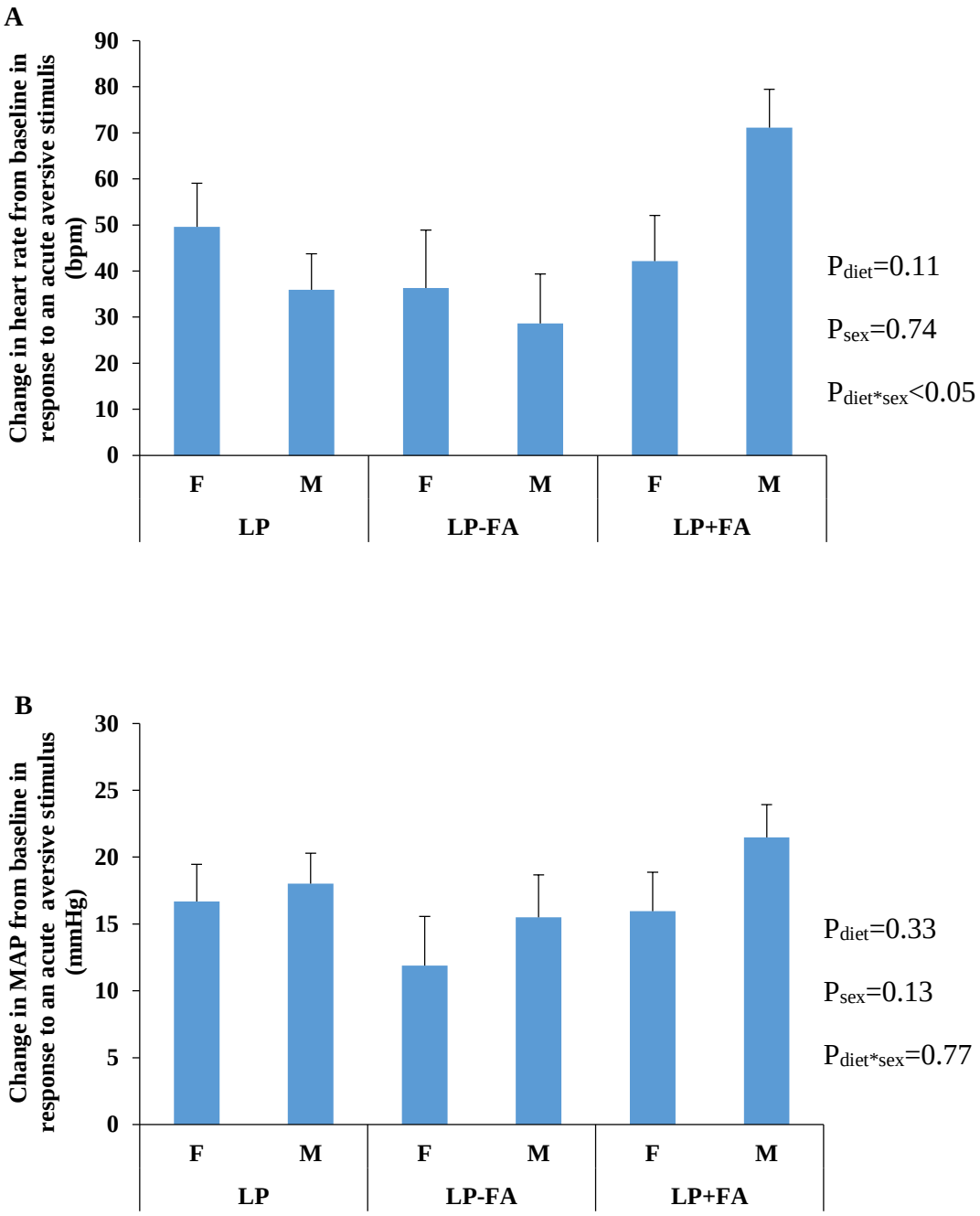

FIGURE 6.9 – RESPONSE TO AN ACUTE AVERSIVE STIMULUS IN MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION AND/OR FOLIC ACID SUPPLEMENTATION/RESTRICTION.

Change in heart rate (**A**) and mean arterial pressure (**B**) in response to an aversive stimulus in male and female offspring exposed to maternal LP or LP and folic acid supplementation or restriction. Heart rate and blood pressure were measured using an indwelling radio-telemetry device. The data presented are the difference between averaged mean arterial pressure 5 minutes before the stress and 10 minutes during the stress. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=7, ♂n=10, LP-FA ♀n=4, ♂n=6, LP+FA ♀n=6, ♂n=9).

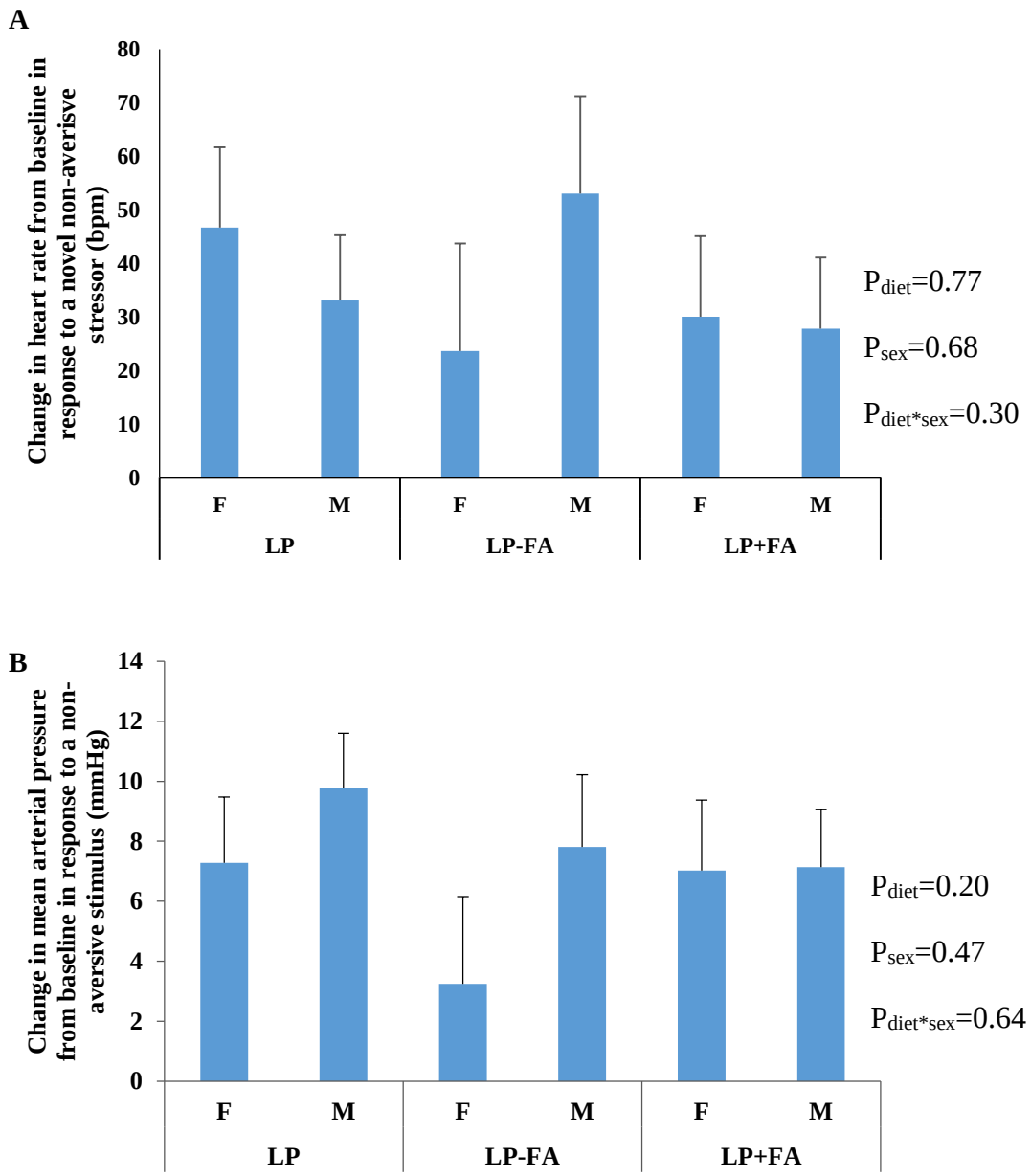

FIGURE 6.10 – RESPONSE TO A NON-AVERSIVE STIMULUS IN MALE AND FEMALE OFFSPRING EXPOSED TO MATERNAL PROTEIN RESTRICTION AND/OR FOLIC ACID SUPPLEMENTATION/RESTRICTION.

Change in heart rate (**A**) and mean arterial pressure (**B**) in response to a non-aversive stimulus in male and female offspring exposed to maternal LP or LP and folic acid supplementation or restriction. Heart rate and blood pressure were measured using an indwelling radio-telemetry device. The data presented are the difference between averaged mean arterial pressure 5 minutes before the stress and 10 minutes during the stress. Values are mean ± SEM, analysed by least means square regression taking into account litter representation (LP ♀n=7, ♂n=10, LP-FA ♀n=4, ♂n=6, LP+FA ♀n=6, ♂n=9).

6.4 DISCUSSION

The aim of the experiments described in this Chapter was to determine whether maternal folic acid intake during pregnancy and suckling had any influence on offspring growth, nephron number and cardiovascular haemodynamics. It was hypothesised that a maternal diet deficient in protein and supplemented with folic acid would result in a greater nephron number compared with LP offspring without folic acid supplementation as well as superior renal and cardiovascular function as measured by blood pressure and kidney function (GFR and ERBF). Interestingly, the data did not support this hypothesis. Rather, the findings indicate that maternal LP and folic acid restriction lead to an 18% *increase* in postnatal nephron number compared with LP controls. Moreover, the data indicate that folic acid supplementation of the maternal LP diet did not alter nephron number from that of maternal LP control animals.

Previous studies had suggested the increase in blood pressure that was caused by exposure to a maternal LP diet during development could be ameliorated when the maternal diet was supplemented with folic acid or other one-carbon metabolites (glycine) (Jackson *et al.*, 2002, Torrens *et al.*, 2006). In the present study, maternal protein restriction did not give rise to offspring with high blood pressure (see Chapter 3). The mechanism underlying this maintenance of normal blood pressure is not known but we hypothesise that it results from offspring of LP rats failing to show catch-up growth and remaining small throughout life. This suggests that maternal diet lead to stunted postnatal growth, with protein restricted offspring being proportionally (with regard to fat and lean muscle) smaller than controls. There was also a deleterious effect on kidney development with reduced glomerular number at both E17.25 and PN21. LP offspring did not develop elevated blood pressure or kidney disease in later life.

Torrens *et al.* (2006) used a maternal protein-restricted diet supplemented with folic acid (5mg/kg) in rats and reported attenuation of high blood pressure (measured using tail cuff

plethysmography) compared with offspring who did not receive folic acid supplementation. In the present study we did not observe a change in postnatal blood pressure due to maternal folic acid intake. While there were significant changes in nephron number, there was no impact on cardiovascular function in adulthood. Neither PN180 nor 360 offspring showed evidence of compromised renal function. Similarly, at PN360 offspring did not demonstrate altered blood pressure or heart rate whether measured under basal or stressed situations.

Maternal LP together with folic acid restriction led to greater nephron endowment at PN21. This finding is surprising given that our previous studies (Chapter 5) showed that folic acid reduces branching morphogenesis in the early-developing kidney. There is a relationship between kidney branching morphogenesis and later nephron number (Hokke *et al.*, 2013, Walker *et al.*, 2011), whereby an increase in branching morphogenesis leads to greater nephron endowment, and the inverse is also true. Here we report that early branching morphogenesis is reduced (Chapter 5) in LP offspring with folic acid restriction but a greater nephron endowment occurs in postnatal life. Without more knowledge on kidney development (such as staged counting of developing glomeruli, analysis of *in vivo* branching morphogenesis and progenitor cell numbers) it is not possible to conclude how the maternal LP and folic acid restriction impacts kidney development. Nonetheless, this finding warrants further attention as it appears that low folic acid may facilitate nephrogenesis perhaps by speeding up the rate of nephrogenesis or extending the developmental window when this process may occur.

Previous studies have suggested a beneficial effect of folic acid in the setting of a sub optimal intrauterine environment, particularly those induced by a maternal LP diet (Engeham *et al.*, 2009, Lillycrop *et al.*, 2010, Torrens *et al.*, 2006). We hypothesised that this beneficial effect may protect the kidney from the deleterious effects of a LP diet, however, while exposure to a LP diet during development resulted in a reduction in nephron number (Chapter 3), folic acid supplementation saw no impact on nephron number. In fact, the opposite effect was observed,

with folic acid restriction associated with an 18% increase in nephron endowment The mechanism by which reduced folic acid intake could lead to a greater nephron endowment is unknown. Dietary folic acid restriction is not reported to have beneficial or positive health outcomes. However, Virk *et al.* (2012) recently reported that blocking folate production by *E. coli* in the gut of *C. Elegans* reduced the aging process of *C. Elegans* and increased longevity. There is a strong relationship between the gut microbiome and digestion and the availability of nutrients to the host (Tilg and Kaser, 2011). It may be possible that the beneficial role of the LP-FA diet is contributed by changes in commensural gut bacteria. Given the beneficial effects of maternal folic acid intake on the reduction of neural tube defect (NTD) occurrence, it is not suggested that FA be removed from the diet, but rather supra-supplementation avoided.

An interesting secondary finding of the present study was that while maternal folic acid intake in pregnancy and suckling did not alter offspring growth trajectory compared with maternal LP, there were changes in BMC at PN21 and PN180, with LP-FA offspring having lower BMC than LP offspring. A reduction in BMC may indicate altered bone metabolism (Heaney, 2003) which may manifest as a result of impaired calcium handling by the kidneys or changes in molecular and cellular mechanisms within bone. The nephron expresses specific cellular receptors responsible for monitoring extracellular calcium levels (Geibel, 2010). A study by Ashton *et al.* (2007) reported that maternal protein restriction lead to offspring with altered kidney function including calcium handling, which they hypothesied lead to reduced trabecular bone within the head of offspring femurs (Ashton *et al.*, 2007)It is unknown if a greater nephron endowment in offspring exposed to LP-FA could negatively alter calcium handling by the kidneys.

At PN360 LP+FA offspring had significantly greater BMC than LP controls. It is possible that this altered BMC could be due to changes in homocysteine (Wolters *et al.*, 2005). Increased homocysteine has been identified as a risk factor for osteoporosis, however the mechanism has

not been discerned (Herrmann *et al.*, 2005b, Herrmann *et al.*, 2005a). Chapter 5 results indicate that during fetal life, folic acid restriction leads to reduced folic acid concentration in the amniotic fluid. Given that the maternal diet continued until offspring were weaned, it is likely that offspring of folic acid deplete dams continued to have reduced plasma folic acid concentrations until the post-weaning period. Reduced folic acid supply can lead to hyperhomocysteinemia due to alterations in the methylation pathway (Yamamoto *et al.*, 2012). Increased homocysteine levels may promote collagen accumulation within bone, thereby reducing bone mineral content and strength. Investigating this link, Tyagi *et al.* (2011) used a mouse model (genetically programmed to develop hyperhomocysteinemia-associated bone remodelling) and demonstrated that supplementation with folic acid ameliorated the reduced blood flow and reduced bone density associated with high levels of homocysteine. Whether or not early folic acid depletion or excess results in permanent down regulation of the methyl pathway is yet to be determined, however it may be possible that reduced folate/folic acid levels during development lead to high levels of homocysteine, potentially perturbing bone blood flow early leading to reduced BMC and also inversely increasing BMC in the folic acid supplemented group at PN360.

Methotrexate is an inhibitor of dihydrotetrahydrofolate reductase and is used during chemotherapy for arthritis. Methotrexate treatment has been shown to lead to bone defects (Elliot *et al.*, 2004). To further illustrate this relationship, Fan *et al.* (2009) treated juvenile rats with low-dose methotrexate and supplemented with folic acid which maintained primary spongiosa bone volume as compared with rats treated with methotrexate alone. This study, in conjunction with others, may suggest that reduced folate/folic acid during development may lead to changes in homocysteine, which could reduce bone mineralisation (Levasseur, 2009). This effect may not last throughout adulthood, as animals exposed to maternal LP and folic acid restriction did not have changes in BMC at PN360. Offspring exposed to maternal LP and

folic acid supplementation had significantly greater BMC than LP controls at PN360. This may be a long term beneficial effect of maternal folic acid supplementation during development leading to greater blood flow of bones and function of resident bone cells.

Although the dose of folic acid used in the present experiment was high, it is not unfeasible for women living in a country with folic acid fortification in wheat products, who eat predominantly green leafy vegetables, and have a previous history of pregnancies effected by NTD (and are therefore encouraged to take up to 4mg folic acid a day), to be having large doses of folic acid every day. Fortification of food has led to children taking large doses of folic acid (Sacco *et al.*, 2013). There are also suggestions for the USA to fortify corn flour in addition to wheat flour in order to target at risk populations that do not regularly consume wheat products; Hispanic women commonly consume less folic acid than non-Hispanic women and are at a greater risk of NTD-affected pregnancies (Canfield *et al.*, 2009, Hamner *et al.*, 2011). Hamner *et al.* (2013) suggested that fortification of corn flour would lead to 2.6% of the adult US population consuming over 1000µg/day of folic acid, which when combined with high dose folic acid of approximately 10000µg as suggested by Tolarova, *et al.* (1982) would be very close to the equivalent dose consumed by the rats in the present study. No study to date has used the level of folic acid supplementation used in the current study. A recently published study in humans does suggest a link to the development of childhood asthma with maternal folic acid intake (Whitrow *et al.*, 2009). The link between maternal over-supplementation and poor fetal outcomes has not been addressed and warrants further research given the current findings.

Mechanisms involved in how folic acid may have deleterious effects on the developing fetus have not been fully elucidated; however epigenetics may be involved. Excessive consumption of folic acid may result in an oversupply of methyl donors and increase the prevalence of gene methylation, thereby resulting in altered levels of expression of critical genes. Folic acid plays

a significant role in development and warrants careful experimentation to ascertain the impact of high levels during pregnancy. It will also be imperative to monitor countries that have mandatory fortification measures of folic acid in place for excessive intake and potential adverse outcomes (Cordero *et al.*, 2008, de Lourdes Samaniego-Vaesken *et al.*, 2012).

Moderation of folic acid intake during pregnancy may result in the best outcomes for both mother and child. Future studies may target the postnatal growth period as a potential critical window in which dietary perturbations may have long-lasting effects on adult health. It may be the protective element in the studies reported in this chapter that offspring remained small into adulthood.

CHAPTER SEVEN: GENERAL DISCUSSION

Well-acknowledged is the impact of maternal diet (particularly the role of low protein) on offspring growth, development and future health (Lackland and Barker, 2009, Langley-Evans, 1997, Langley-Evans *et al.*, 1996b, Langley-Evans *et al.*, 1996c, Vehaskari *et al.*, 2001, Woods *et al.*, 2001, Woods *et al.*, 2004). The phenomena of developmental programming of adult cardiovascular and renal disease in response to exposure to a protein restricted diet during development has been of significant interest to researchers in recent years. The literature provides strong evidence that maternal low protein leads to reduced nephron endowment in offspring (Langley-Evans *et al.*, 1994, Langley-Evans *et al.*, 1996c, Langley-Evans *et al.*, 1999b, Woods *et al.*, 2001). There is also evidence for a relationship between maternal protein intake and the occurrence of high blood pressure and reduced nephron endowment in adulthood (Langley-Evans, 1997, Langley-Evans *et al.*, 1996a, Nwagwu *et al.*, 2000, Tonkiss *et al.*, 1998, Vehaskari *et al.*, 2004, Woods *et al.*, 2001, Sahajpal and Ashton, 2003, Sahajpal and Ashton, 2005). However, this finding is not invariant; multiple studies show a reduction in nephron endowment but no evidence of hypertension (Lim *et al.*, 2011, Zimanyi *et al.*, 2004). The mechanisms that link maternal low protein and the resultant adult phenotype in her offspring are yet to be elucidated, however it is hypothesised that there is a role for the placenta, hormones and epigenetic changes in the fetal genome (Avagliano *et al.*, 2012, Barker *et al.*, 2010a, Bertram *et al.*, 2001, Bressan *et al.*, 2009, Burton and Waddell, 1999, Doherty *et al.*, 2003, Fernandez-Twinn *et al.*, 2003, Fowden *et al.*, 2011, Fowden and Forhead, 2009, Gao *et al.*, 2011, Gardner *et al.*, 1997, Goyal *et al.*, 2009a, Rosario *et al.*, 2011, Slater-Jefferies *et al.*, 2011, Zeng *et al.*, 2012).

What is less well characterised, and what this thesis aimed to identify, was:

1) the timing of perturbed kidney development in offspring of protein deprived rats

2) which aspects of kidney development appear to be most affected by exposure to maternal LP diet

3) the impact of suboptimal kidney development on renal function and blood pressure after exposure to maternal LP diet, and

4) the potential for folic acid supplementation to protect or rescue the deleterious phenotype induced by a maternal LP diet.

7.1 STUDIES ON KIDNEY DEVELOPMENT IN OFFSPRING OF PROTEIN RESTRICTED DAMS

Maternal LP diet has been reported to result in reduced nephron number in adulthood (Hoppe *et al.*, 2007a, Langley-Evans *et al.*, 1994, Woods *et al.*, 2001), however the path to this phenotype has not been analysed during development. Nephrogenesis occurs during fetal development and is complete before birth in humans, it is therefore an oversight that the element of the maternal environment that impacts on kidney development has not been studied during development. The final number of nephrons in the adult kidney is determined by a well-controlled pattern of gene expression, suppression and the interaction between the metanephric mesenchyme and ureteric epithelium (Dressler, 1999, Dressler, 2006). In broad terms, the major processes controlling kidney function are vascular function, blood pressure and the filtration surface area of the kidney. The findings from this thesis indicate that the process of branching morphogenesis, at least in the rat, is not influenced by maternal LP diet. Although large numbers of studies have documented a reduction in final nephron number in offspring exposed to a maternal LP diet, no studies have investigated whether this reduction in nephron number occurs due to one of the earliest processes of kidney development; branching morphogenesis. Similarly, there are sound data to show that gene expression and protein levels are up or down regulated in adult offspring of LP dams (Alwasel *et al.*, 2010, Woods and Weeks, 2004, Zimanyi *et al.*, 2004), but there is very little understanding of how maternal LP affects the developing kidney. Due to the major influence of branching morphogenesis on final nephron endowment it is surprising that the gene expression of the developing kidney has not been heavily studied in models of developmental programming, given that branching morphogenesis occurs solely during fetal development in both humans and animals. We used a combination of techniques to assess the developing kidney, including organ culture to assess branching morphogenesis and real time PCR to assess gene expression in a semi-quantitative

manner. We showed that maternal protein restriction does not appear to impact branching morphogenesis of the kidney and this morphological analysis was supported by outcomes of the gene expression analysis. This was a surprising finding given the well accepted relationship between branching morphogenesis and adult nephron number (Nigam and Shah, 2009).

We do appreciate that this conclusion is drawn from a cross-sectional study of kidney development which was analysed in an *in vitro* setting, therefore it is important to consider that a maternal LP diet may have a gradual impact on kidney development throughout the fetal period– rather than an immediately apparent one. While there are caveats with a culture environment (ie. the kidney is no longer in a protein restricted environment and grows flat, avascular and aneural), it does allow the ability to assess branching morphogenesis of the kidney and the direct impact of factors on branching morphogenesis. It is important that this study be replicated using technology that can measure the kidney in a 3D environment. OPT would allow for data to be obtained on branch points and ureteric tips as well as the lengths and angles of branches as well as the ability to analyse the kidney at different ages in a 3D environment (Short *et al.*, 2010). This type of analysis has just been completed in growth-restricted offspring of streptozotocin diabetic mothers. Hokke *et al.* (2013) reported reduced ureteric tip number and ureteric tree length in E14.5 embryos exposed to maternal hyperglycaemia compared to controls. It would be important to confirm that branching morphogenesis is not affected in a similar fashion in offspring of LP dams.

Nonetheless, the current *in vitro* findings are supported by the gene expression studies in that there was no change in levels of expression of genes responsible for branching morphogenesis (*Gdnf, Gfrα1* and *Bmp4*) in the setting of a LP maternal diet. Very few studies have analysed the expression profile of the early developing kidney after exposure to maternal diet manipulation (Abdel-Hakeem *et al.*, 2008, Welham *et al.*, 2005, Welham *et al.*, 2002). While ultimately the most powerful study would have been to investigate global gene expression in

the kidney, the resources were not available to us. While there was no change in branching morphogenesis at the time-point chosen for study (in culture or indicated by changes in gene expression of *Bmp4*, *Gdnf* or *Gfrα1*), similarly no change was detected in the expression levels of genes involved in nephrogenesis (*Pax2*, *Wnt4* and *Hnf4α*). Notwithstanding the lack of effect of maternal protein restriction upon branching morphogenesis, there are changes in nephron endowment after the cessation of nephrogenesis. Therefore future studies would benefit from assaying the kidney during different periods of development in order to determine which genes and gene pathways are influenced by maternal LP diet.

At E14.25, the timepoint when kidneys were placed in culture, the expression levels of six key kidney development genes were analysed (Stuart *et al.*, 2001). Future studies may wish to utilise Next Generation Sequencing (NGS) techniques in order to get a global picture of how the kidney is developing at a given time point. The development of an organ is dictated by the genome and the environment – where genes and their environment overlap is an area in which epigenetics plays a significant role. NGS would provide some of this information (Hou *et al.*, 2013). A proteomic and small RNA approach would also be required to garner a full picture in order to fully understand the impact on development (Ho and Kreidberg, 2013, Ho and Kreidberg, 2012, Saxena *et al.*, 2011). While no change in branching morphogenesis was observed at E14.25 (just after kidney development has begun in the rat), there was a significant reduction in the number of nephrons present at E17.25. This suggests three possible avenues may have contributed to the adult phenotype: (1) branching morphogenesis was not affected and the resultant phenotype was completely reliant on later steps in nephrogenesis; (2) the LP maternal diet leads to reduced global fetal development which leads to fewer nephrons.; and (3) the maternal LP diet reduces the number of cap mesenchyme progenitor cells that give rise to all cells of the nephron. This third option may provide the most plausible explanation for why there was no change in branching morphogenesis but there was a change in nephron

number. The nephron progenitor cells of the cap mesenchyme are Six2 positive and may present an avenue of future research, whereby the impact of maternal LP diet on the cell population is determined by the number of Six2 positive cells during stages of kidney development (Kobayashi *et al.*, 2008).

7.2 Folic Acid and Kidney Development

The whole metanephric organ culture system allowed us to assess whether folic acid had a direct effect on kidney development. A previous study had reported a beneficial effect of culturing whole embryos in media supplemented with 2mM folic acid (Wentzel *et al.*, 2005). Wentzel *et al.* (2005) used a whole rat embryo (E9) culture system to assess whether 2mM folic acid fortification of the media reduced developmental malformations caused by maternal diabetes. The present study revealed a complex relationship between exogenous folic acid supplementation in the media and maternal supplementation. Maternal supplementation of folic acid was deleterious to kidney branching morphogenesis, however maternal folic acid restriction and low exogenous supplementation (0.6mM) increased branching morphogenesis. The findings from the addition of exogenous folic acid indicate that folic acid has a direct effect on kidney branching morphogenesis, independent of any effect on maternal factors such as placentation and the maternal hormonal milieu, or systemic fetal factors such as vascular development and systemic insulin or other growth factors. The relationship between folic acid exposure and branching morphogenesis was recapitulated by the experiment of folic acid intake in vivo (ie. maternal diet supplementation), and the resultant reduction in nephron number seen in adulthood. Evidence of a deleterious effect of maternal folic acid intake was reported in a human epidemiological study which showed that folic acid fortification has led to a greater susceptibility to asthma (Whitrow *et al.*, 2009). It is unknown whether the findings reported in the current thesis and the study by Whitrow *et al.* (2009) are linked, although branching morphogenesis plays a key role in both kidney and lung development. However, it is unknown if lungs of asthmatics have reduced alveolar number or bronchial/bronchiolar branching. It is also possible that folic acid may be impacting on cell specialisation and differentiation (Yuan *et al.*, 2012). Future studies may wish to address these apparent deleterious effects of fortified levels of folic acid, since with large doses of folic acid recommended to women with a previous

history of pregnancies affected by NTD there is potential for these women to expose the developing fetus to potentially dangerous levels of folic acid. While it is appreciated that in certain circumstances large doses of folic acid are required to overcome other physiological hurdles (genetic variants in key enzymes involved in the methylation process or medication interfering with folate metabolism), it remains unknown what the optimal dose of folic acid is (ie. the dose which reduces the number of NTDs with the minimal amount of developmental defects).

7.3 MATERNAL LOW PROTEIN DIET AND OFFSPRING HEALTH OUTCOMES

Although multiple studies have reported low nephron number in offspring of LP diet dams, the renal physiological effects of this reduction in nephron number have received scant attention and findings have been contradictory. Alwasel and Ashton (2009) reported no change in GFR in male or female rat offspring exposed to maternal LP diet (no nephron number reported), while Nwagwu *et al.* (2000) reported reduced GFR in rat offspring exposed to maternal LP diet (no nephron number reported). Hoppe *et al.* (2007a, 2007b) using a model of life long protein restriction in rats reported no change in GFR with a 31% reduction in nephron number. In the present study we determined that offspring exposed to maternal LP diet did not display any changes in GFR or effective renal blood flow. GFR can be influenced by body size and the capacity of the kidney to respond to haemodynamic changes (vascular tone, nephron number) (Fesler and Mimran, 2011). Offspring exposed to maternal protein restriction remained smaller throughout postnatal life and combined with a relative increase in nephron number to body weight, there exists a potential nephron reserve. Future studies could aim to determine the size of glomeruli, and thereby determine if this nephron reserve involves a significant increase in FSA and the functionality of this nephron reserve.

A nephron reserve provides a buffer to disease processes and the impact of nephron loss associated with aging (Goyal, 1982, McLachlan, 1978). This is the first study to report greater nephron number per gram of body weight in a study of maternal dietary manipulation. While total nephron number provides information on the state of the kidney, it does not give an indication of kidney function. Nephron number is responsible for forming the filtration surface area of the body which is instrumental in maintaining whole body homeostasis through salt/water balance. The ability for the kidney to maintain whole body homeostasis relies upon multiple pathways and physiological responses (vessel tone,

renin-angiotensin system, filtration surface area), however there does not appear to be a direct relationship between filtration surface area and disease (Black *et al.*, 2004b). In this thesis we report an 18% nephron reserve in offspring exposed to a maternal LP diet. This may suggest that maternal LP can lead to slow fetal growth without an apparent detriment to organ development. Without having conducted a comprehensive series of studies in adult offspring to assess the robustness of this phenotype we cannot conclude that a maternal LP diet was positive *per se*. However, we can conclude that animals born small, that stay small, retain normal renal function.

In this thesis, gold standard techniques for measuring GFR and MAP were utilised. However, while we used radio-labelled tracers, this limits the ability to re-assess animals' long term (for ethical reasons). Future studies may wish to utilise a new approach for the measurement of GFR in conscious unrestrained animals, which detects the plasma clearance of FITC-labelled sinistrin without the need for catheterisation or injection of radioactive tracers (Schock-Kusch *et al.*, 2011, Schock-Kusch *et al.*, 2009, Schreiber *et al.*, 2012). This approach enables the long term monitoring of filtration function in unanaesthetised animals. Such a system allows probing physiological experiments that could investigate the robustness of a kidney with a nephron reserve – such as high salt diets, impact of diuretics and antidiuretics.

7.4 Maternal folic Acid intake and offspring health

The use of folic acid in the current studies was based on the findings from previous studies in which a maternal LP diet was supplemented with folic acid (Burdge *et al.*, 2009, Chmurzynska *et al.*, 2012, Engeham *et al.*, 2010, Lillycrop *et al.*, 2005a, Lillycrop *et al.*, 2010, Rao *et al.*, 2006, Torrens *et al.*, 2006). These studies generally reported beneficial effects of the folic acid supplementation, such as the amelioration of hypertension and restored gene expression. In the studies described in this thesis we used a diet fortified with 200mg/kg of folic acid. This level of folic acid is significantly higher than most previous studies, but consistent with the levels of folic acid supplementation used by Tolarova (Tolarova, 1982). Folic acid is a water soluble B vitamin that needs to be taken daily for optimal health (Obican *et al.*, 2010). While the level of folic acid used in this thesis is higher than the recommended dose for women with a history of pregnancies affected by NTDs (Czeizel, 1995a, Czeizel, 2005), it is achievable in this age of fortified food, health conscious and self-medicating women. We report that this high dose of folic acid had a negative impact on kidney development, and lead to reduced somatic growth in postnatal life. These findings suggest that supplementation of a low protein diet with high doses of folic acid is not beneficial, and does not present an avenue in which the low nephron number phenotype could be prevented.

Among the justification for the using a folic acid supplemented diet were reports of changes to gene methylation due to maternal LP diet (Gong *et al.*, 2010, Slater-Jefferies *et al.*, 2011, Zeng *et al.*, 2012) and the reversal of aberrant methylation with a diet supplemented with folic acid (Burdge *et al.*, 2009, Lillycrop *et al.*, 2010, Torrens *et al.*, 2006). Methylation analysis was performed on genes that displayed changes in levels of expression (*Wnt4*, *Bmp4* and *Gfrα1*) due to the LP diet, and this enabled us to assess correlations between gene expression levels and methylation status. The level of *Gfrα1*

expression was strong correlated with methylation status, suggesting that *Gfrα1* expression is epigenetically controlled and influenced by maternal diet. The data for the other genes (*Bmp4* and *Wnt4*) suggest they are not methylation sensitive, or that their expression is controlled by other transcription factors. They may indicate whether epigenetic control over gene expression (Godmann *et al.*, 2010, Ivanova *et al.*, 2012, Yu *et al.*, 2009). *Wnt4* has been shown to contain highly conserved CpG islands within the promoter region (Yu *et al.*, 2009), however in our experiment a correlation between gene expression level and methylation status was not observed. Investigating cisplatin resistant gastric tumours, *Bmp4* expression was identified as being methylation sensitive and related to drug resistant gastric cancers (Ivanova *et al.*, 2012). No data has been published on *Gfrα1* and its epigenetic control within the kidney, but in the male germ cell line, *Gfrα1* expression has been shown to be sensitive to the methylation of H3 histone through HDAC activation (Godmann *et al.*, 2010). While we have identified one potential gene involved in kidney development to be sensitive to the methylation of its promoter region, it may indicate that other genes warrant investigation to determine how much of kidney development is epigenetically controlled.

7.5 CONCLUDING REMARKS

The dietary intake and nutritional status of pregnant and breastfeeding mothers is a major public health concern given the robust evidence from human and animal studies which indicates that a healthy pregnancy (normal birth weight and absence of congenital defects) predicts a healthy start to life that will continue into the offspring's adult life. However, maternal nutrition is still a major health concern world-wide, in both third world nations (malnutrition) and first world nations (overnutrition). The consequence of poor maternal diets can potentially affect future generations, leading to them developing hypertension, diabetes, obesity or the metabolic syndrome. There is strong evidence to suggest the mechanism behind the phenotype induced by a sub-optimal intrauterine environment is of epigenetic origins. Studies have indicated changes in methylation of key genes such as IGF2 to alter fetal growth. However, what is yet to be discerned is if it is possible to reverse or protect the developing fetus from aberrant epigenetic changes. While the current generation's *in utero* experience cannot be altered, there are protective measures that can be encouraged to minimise the potential for the in utero environment to be deleterious to future health. The lack of catch-up growth reported in this thesis may be a contributing factor to the reason why the LP offspring did not develop disease in postnatal life.

The phenomena of developmental programming is not restricted to the nine months of *in utero* life, the lactational environment or even puberty – it can impact upon successive generations through germ line alterations. To protect future generations against the potential deleterious impact of a poor maternal diet on offspring health, it is important that scientific research continues to investigate the sensitive periods of development and the factors that regulate development, and disseminate this information to the public so that individuals can make informed decisions.

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