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Cardiovascular and renal profiles in rat offspring that do not undergo catch-up growth after exposure to maternal protein restriction

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Original Article

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Abstract

Maternal protein restriction is often associated with structural and functional sequelae in offspring, particularly affecting growth and renal-cardiovascular function. However, there is little understanding as to whether hypertension and kidney disease occur because of a primary nephron deficit or whether controlling postnatal growth can result in normal renal-cardiovascular phenotypes. To investigate this, female Sprague-Dawley rats were fed either a low-protein (LP, 8.4% protein) or normal-protein (NP, 19.4% protein) diet prior to mating and until offspring were weaned at postnatal day (PN) 21. Offspring were then fed a non 'growth' (4.6% fat) which ensured that catch-up growth did not occur. Offspring growth was determined by weight and dual energy X-ray absorptiometry. Nephron number was determined at PN21 using the disector-fractionator method. Kidney function was measured at PN180 and PN360 using clearance methods. Blood pressure was measured at PN360 using radio-telemetry. Body weight was similar at PN1, but by PN21 LP offspring were 39% smaller than controls ($P_{diet} < 0.001$). This difference was due to proportional changes in lean muscle, fat, and bone content. LP offspring remained smaller than NP offspring until PN360. In LP offspring, nephron number was 26% less in males and 17% less in females, than NP controls ($P_{diet} < 0.0004$). Kidney function was similar across dietary groups and sexes at PN180 and PN360. Blood pressure was similar in LP and NP offspring at PN360. These findings suggest that remaining on a slow growth trajectory after exposure to a suboptimal intrauterine environment does not lead to the development of kidney dysfunction and hypertension.

Introduction

Studies from the 20th Century identified nutritional requirements during pregnancy for humans[1,2] and animals[3-6] showing that maternal caloric or macronutrient restriction leads to stunting of offspring growth *in utero* and in early postnatal life. Later studies[7-9] established that adult disease may manifest because of this abnormal fetal development.

Although there are several documented examples of developmental programming in humans,[10-13] the mechanisms underlying the programming of adult disease are best elucidated in animal models. Particular focus has been on the impact of maternal diet on the developmental programming of cardiovascular disease (hypertension and kidney dysfunction). In rodents, consumption of low-protein (LP) diet during pregnancy and suckling, but not in later life, has been reported to lead to low birth weight, high blood pressure, reduced nephron endowment and accelerated postnatal growth (catch-up growth) in offspring.[14-18] The relative contribution of low nephron endowment and subsequent postnatal growth to determination of adult blood pressure remain to be elucidated.

Hypertension is often reported in offspring exposed to a maternal LP diet, however this is not always the case.[19-22] It is unclear whether methodological techniques influence cardiovascular arousal in these models. Some studies provide evidence that the observation of elevated blood pressure under resting conditions may actually be reflective of an elevated cardiovascular arousal or stress response[23] underpinned by elevated basal sympathetic nervous system tone.[24,25] The contributions of cardiovascular arousal, postnatal catch-up growth, obesity and aberrant kidney development to the development of hypertension in offspring of protein-restricted rats remain unclear. Maternal protein restriction may program both renal dysfunction and hypertension,[26,27] but again, outcomes are variable and potentially biased by offspring developing excessive adiposity in later life. In addition to animal studies, studies in humans have provided evidence of adiposity being a known risk factor for cardiovascular and renal disease.[28-31]

In the present study, we utilised a rat model of maternal LP during pregnancy and suckling which results in offspring having reduced nephron endowment but no catch-up growth or excessive adiposity. The aim was to observe whether this primary nephron deficit also resulted in hypertension and cardiovascular arousal in response to both aversive and non-aversive stimuli[32-34] in the absence of a potentially confounding effect of elevated adiposity. To ensure catch-up growth would not occur, after weaning both normal-protein (NP) and LP rats were fed a non 'growth diet' that contained 4.6% fat. Many methods of blood pressure measurement involve restraint and inadvertent cardiovascular arousal, presumably due to activation of the sympathetic nervous system, so radiotelemetric methods were utilised to reduce this potential confounder. We hypothesised that in the absence of catch-up growth a primary nephron deficit does not result in altered renal function or hypertension.

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. Experiments adhered to the ARRIVE guidelines.[35]

Animal husbandry

Nine-week-old female Sprague-Dawley rats were housed in pairs and allowed a 1-week acclimatisation period before being fed *ad libitum* a NP (19.4% wt/wt, Glen Forrest WA, Australia) or a near-isocaloric LP (8.4% wt/wt, Glen Forrest WA, Australia) diet (Table 1). This dietary manipulation continued throughout pregnancy and suckling (21 d postnatal). Then, offspring were weaned to a control diet (20% wt/wt protein, Meat Free Rat and Mouse Diet, Glen Forrest WA, Australia) which was fed *ad libitum* until the time of experimentation. It should be noted that this post-weaning diet is lower in fat (4.8% wt/wt fat), than the so-called 'growth' diet (7% fat wt/wt, AIN93G. Glen Forrest WA, Australia) which many animal facilities provide to stimulate growth in all rodents for several weeks post weaning.

Dams were weighed when a breeder male was introduced for mating and regularly thereafter (three times a week). Pregnancy weight gain was determined based on the difference between the weight when breeder male was introduced and the last recorded weight prior to giving birth (1–3 d before giving birth). Litter size was standardised to between 6 and 12 pups (where possible, equal numbers of male and female pups). When litters consisted of more than 12 pups, additional pups were humanely killed at 1 d after birth at PN1. Litters prior to standardisation of less than 6 pups or more than 20 pups were not included in this study. Offspring were weighed on PN1 and then every 2–3 d (Monday, Wednesday & Friday) until weaning at PN21. During the first week post-partum maternal food and water intake were measured every 2–3 d. Offspring were then weighed every 5 d from PN25 (as animals were able to be individually identified at this time point) until PN180 and then every 15 d until PN360. In order to uphold the 3 Rs of animal ethics (reuse, reduction and refinement) there are variations in the number of offspring used at the various time points, as all animals were used. As there was variability in litter size, not all litters could contribute to every experiment.

Table 1. Nutritional parameters of dietary groups

Nutritional parameters	Diet	
	NP	LP
Digestible energy (MJ/kg)	16.1	16.2
Casein (g/kg)	200	87
DL Methionine (g/kg)	3.0	3.0
Sucrose (g/kg)	100	200
Wheat starch (g/kg)	404	417
Dextrinised starch (g/kg)	132	132
Cellulose (g/kg)	50	50
Canola Oil (g/kg)	70	70
Calcium carbonate (g/kg)	13.1	13.1
Sodium chloride (g/kg)	2.6	2.6
Potassium citrate (g/kg)	2.5	2.5
Potassium dihydrogen phosphate (g/kg)	6.9	6.9
Potassium sulphate (g/kg)	1.6	1.6
AIN93G trace minerals (g/kg)	1.4	1.4
Choline chloride (65%) (g/kg)	2.5	2.5
AIN93G vitamins (g/kg)	10	10

Energy and nutrient composition of normal-protein (NP) and low-protein (LP) maternal diets.

Analysis of body composition, organ weight, food and water intake, and blood glucose level

Body composition was determined under isoflurane anaesthesia by dual X-ray absorptiometry (DEXA) at weaning (PN21), PN180 and PN360. Body fat, lean muscle and bone mineral content were all determined by dual energy X-ray absorptiometry (DXA, QDR-4500 DOS Series, Hologic, USA) specifically calibrated for small animals (such as rodents) using the step phantom as per manufacturer's guidelines.

Organ weight was determined at PN21, PN180 and PN360. These included the kidneys, heart, pancreas, liver, brain, mesenteric fat, peri-renal fat and abdominal fat. At PN360, prior to organ collection, blood glucose concentration was measured using a handheld glucometer. Measurement of food and water intake occurred in PN360 offspring. Food and water were weighed daily over 5 d to determine the average 24-h intake.

Determination of nephron number

Nephron number was estimated in 21-d old offspring using the physical dissector-fractionator method.[36] PN21 was selected as the timepoint for study as nephrogenesis is complete at this age yet there is unlikely to have been nephron loss associated with ageing or secondary to cardiometabolic factors such as obesity or hypertension.[37-39]

The left kidney was fixed in 4% paraformaldehyde,[36,40] transferred to 70% ethanol, processed to paraffin, and exhaustively sectioned at 5 µm.

Total nephron number per kidney was estimated using the equation:

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

where N_{glom} is the total number of peanut agglutinin (PNA)-positive glomeruli in the kidney, SSF (section sampling fraction, 100)

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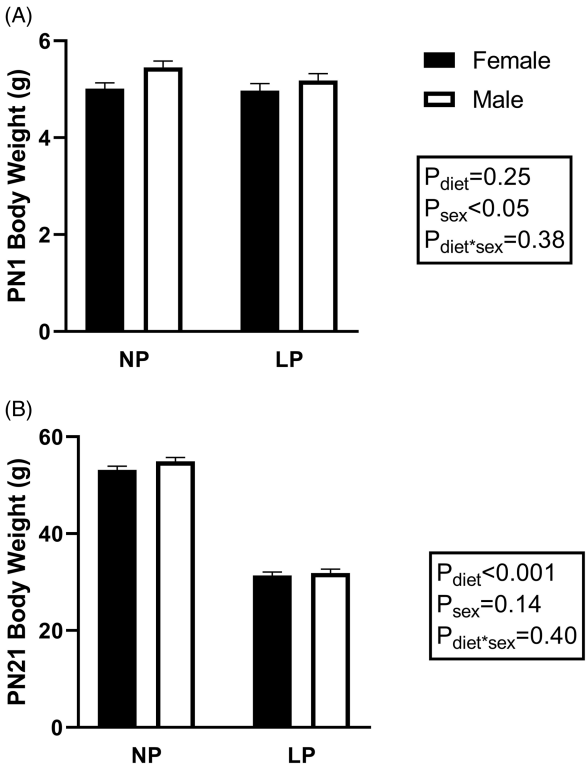

Fig. 1. Body weight at PN1 and PN21 of male and female offspring exposed to either a maternal normal- or low-protein diet. Male and female offspring exposed to a normal- (NP) or low-protein (LP) maternal diet were weighed at postnatal days 1 (**A**) and 21 (**B**). Values are mean ± SEM, analysed by a mixed linear model incorporating least means square regression to account for litter representation (NP PN1 N_{litter} = 11 comprising ♀ = 84, ♂ = 74, PN21 N_{litter} = 8, comprising ♀ = 43, ♂ = 44, LP PN1 N_{litter} = 9, comprising ♀ = 67, ♂ = 65, PN21 N_{litter} = 9, comprising ♀ = 51, ♂ = 36).

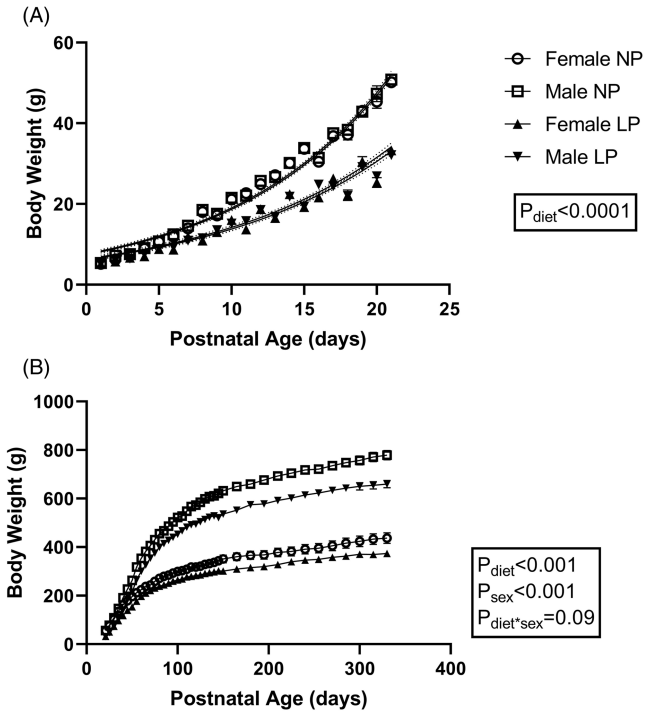

Fig. 2. Postnatal growth curves of male and female offspring exposed to either a maternal normal- or low-protein diet. Postnatal growth curves of male (squares) and female (circles) offspring exposed to either a low- (closed) or normal- (open) protein diet during pregnancy and lactation from PN1 to PN21 (**A**) and PN25 - PN330 (**B**). PN1–21 lines show non-linear growth regression from an exponential growth model for each sex and maternal diet (R squared NP Female = 0.9405, NP Male = 0.9365, LP Female = 0.7750, LP Male = 0.8378; NP N_{litter} = 10, comprising ♀ = 7–76, ♂ = 10–75, LP N_{litter} = 10, comprising ♀ = 5–67, ♂ = 2–65). Values are mean ± SEM, analysed by a mixed linear model incorporating least means square regression to account for litter representation (PN25-330 NP N_{litter} = 6, comprising ♀ = 24–30, ♂ = 25–36, LP N_{litter} = 8, comprising ♀ = 32–47, ♂ = 23–31).

is the reciprocal of the section sampling fraction (the number of sections advanced between section pairs), one fraction (½) accounts for PNA-positive structures that were counted in both directions between the two sections of a disector pair, the other fraction (½) accounts for the disector pair consisting of the n and $n+2$ sections, and Q- is the actual number of PNA-positive glomeruli appearing and disappearing between the reference and look-up sections of the disector.[41] For glomerular counting, kidney sections were observed at a magnification of 150x using a projection microscope.

Measurement of renal function

At PN180 and PN360, under general anaesthesia, glomerular filtration rate (GFR) was determined from the renal clearance of ^{3}H inulin (1μCi/hr ^{3}H-inulin, Perkin-Elmer Life Sciences, Victoria, Australia) while effective renal plasma flow (ERPF) was determined from the renal clearance of ^{14}C- para-aminohippuric acid (0.5 μCi/hr ^{14}C-PAH, Perkin Elmer, Boston, MA).[42] Rats were anaesthetised with thiobutabarbitone sodium (150 mg/kg in saline, Inactin, Sigma-Aldrich Co., St Louis, USA). Catheters were then placed in the carotid artery (for measurement of BP and collection of blood), jugular vein (for infusions) and bladder (for collection of urine).[42,43] To maintain fluid balance during surgery, 2% bovine serum albumin (BSA, Sigma-Aldrich) dissolved in 154 mM NaCl was infused at 1 ml/h/100 g body weight.

^{3}H inulin and ^{14}C PAH, dissolved in 154 mM NaCl, were infused at 0.4 ml/h/100 g body weight for a total of 100 min. After a 60-min stabilisation period, urine was collected over the final 40-min of the infusion. At the end of this 40-min period, blood (approximately 2 ml) was collected. Haematocrit was determined in a small aliquot and the remainder was centrifuged (10 min, 3000 g). Plasma was aspirated and stored at −20 °C prior to subsequent analysis.

Urine and plasma levels of ^{14}C-PAH and ^{3}H-inulin were determined by scintillation counting (Beckman LS6000TA, Beckman Coulter, USA). Each sample was counted for 10 min and the disintegrations per minute (DPM) from each triplicate were averaged. Under general anaesthesia, measurements of GFR, ERPF, filtration fraction (FF), and urine flow (UF) were then calculated as the clearance of ^{3}H-inulin and ^{14}C-PAH, respectively, where:

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

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

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

$$FF = \frac{GFR}{ERBF\ (\textit{effective renal blood flow})}$$

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Table 2. Kidney weight of PN21 offspring

	NP ($N_{litter} = 9$)		LP ($N_{litter} = 8$)		P Values		
	Female (8 pups)	Male (12 pups)	Female (9 pups)	Male (10 pups)	Diet	Sex	Interaction
Left kidney (mg)	307.4 ± 16.7	270.5 ± 8.8	163.6 ± 6.9	166.3 ± 6.8	**<0.001**	0.12	0.07
Left kidney/body weight (mg/g)	5.9 ± 0.4	5.7 ± 0.2	5.4 ± 0.2	5.2 ± 0.2	0.06	0.38	0.81
Right kidney (mg)	328.2 ± 18.8	335.0 ± 16.9	166.0 ± 7.3	174.4 ± 7.3	**<0.001**	0.58	0.96
Right kidney/body weight (mg/g)	6.4 ± 0.4	6.2 ± 0.4	5.5 ± 0.2	5.5 ± 0.2	**0.01**	0.77	0.84
Total kidney (mg)	637.9 ± 33.3	648.3 ± 30.0	329.8 ± 12.9	336.6 ± 12.9	**<0.001**	0.73	0.94
Total kidney/body weight (mg/g)	12.4 ± 0.8	12.0 ± 0.7	10.9 ± 0.3	10.5 ± 0.3	**0.02**	0.53	0.98

Left, right and total kidney weights from male and female offspring exposed to maternal LP or NP diet reported as absolute values and adjusted for body weight. Values are mean ± SEM, N_{litter} represents the number of litters, data analysed by a mixed linear model incorporating least means square regression to account for litter representation.

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

Osmolality

Osmolality of urine and plasma were measured by freezing point depression using an osmometer (Advanced Osmometer 2020; Advanced Instruments, Needham Heights, MA).

Electrolytes

Concentrations of Na^+, Cl^- and K^+ (mmol/l) were measured using a RapidChem 744 Electrolyte Analyser (Bayer Australia Limited, Australia).

Telemetry

Blood pressure and heart rate were measured by radiotelemetry (DataSciences Incorporated, Minnesota, USA) coupled to a customised data acquisition system.[44] Animals were anaesthetised with isoflurane and the probe surgically implanted in the descending aorta and tethered to the peritoneal wall as per the manufacture instructions. Post-operative analgesia and antibiotics were provided and animals recovered for a period of 5 d prior to the commencement of any recording.

Measurement of blood pressure and data collection

Systolic and diastolic blood pressure and heart rate were recorded continuously in unrestrained rats for a minimum of 5 d. The sampling rate was set at 2 s and data pooled to obtain 12 hourly (day and night) average readings over the experimental period.

Cardiovascular arousal tests were also performed to provide a proxy measurement of sympathetic nervous system tone and activation. Animals were exposed to an aversive stimulus of being placed on an oscillating table for 10 min (novel acute stimulus) at 100 rpm. On a different occasion, they were exposed to a novel non-aversive cardiovascular arousal stimulus (presentation of a sultana which was determined to be a favoured treat). For both arousal tests, blood pressure and heart rate were recorded for 5 min prior to and the 10 min during exposure to the stress test, as well as the 30 min after the stress test.

Statistical analysis

Data were analysed using a mixed linear model (maternal diet and offspring sex as independent variables) incorporating least means square regression to account for litter representation (n represents number of litters) (SPSS 21, IBM).[45] This statistical method has been used by various investigators to account for the potential within-litter bias.[46-53] This statistical analysis was used to reduce confounding due to over-representation of a particular litter in the data. Therefore, herein we report findings from an n (litter) of 4-18, which represents between 2 and 79 animals. Throughout the text, figures and tables, n is presented as the number of litters in the analysis (N_{litter}) and also the total number of male and female animals contributing to each observation. Growth curve data from PN1-21 were analysed via non-linear regression (GraphPad Prism v8, GraphPad Software Inc.). An exponential growth equation model was fitted with all parameters unconstrained. Data are expressed as mean ± SEM. Two-tailed $P \leq 0.05$ was considered statistically significant. In the linear model 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 between-subjects factors. When appropriate, repeated measures ANOVA was used, with the Greenhouse-Geisser correction applied to within-subjects factors to account for asphericity.

Results

Postnatal parameters

The weight gain during pregnancy did not differ significantly between groups (NP dam weight 133 ± 6 g $n = 10$, LP dam weight 7 ± 121 g $n = 11$ $p = 0.19$). Litter size was similar in the two groups, with both NP dams and LP dams having 15 ± 1 pups ($p = 0.73$). Offspring PN1 weight did not differ significantly between dietary groups (Fig 1A). Daily maternal water intake (NP 33.6 ± 1.8 ml/24 h $N = 9$, LP 25.8 ± 1.8 ml/24 h $N = 9$, $p < 0.006$), during the first week post-partum was 23% less in LPs compared with NP controls. Daily maternal food (NP 29.1 ± 2.2 g/24 h $N = 9$, LP 24.7 ± 2.2 g/24 h $N = 9$, $p = 0.16$) and energy (NP 470.0 ± 35.0 kJ/g/24 h $N = 9$, LP 400.0 ± 35.0 kJ/g/24 h $N = 9$, $p = 0.18$) intake did not differ between the two dietary groups during the first week post-partum. Postnatal growth was stunted by PN21 (Fig 1). Non-linear regression indicated that there was a significant effect of diet with LP offspring weighing less than NP controls during the suckling stage ($P < 0.0001$, Fig 2). At PN21 both male and female LP offspring

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weighing 39% less than NP controls (Fig 1B). From PN25 onwards (where rats were able to be tracked individually), both female and male LP offspring weighed less than NP controls throughout life (Fig 2), indicating that LP offspring showed no sign of catch-up growth.

Body fat (% body weight) of male and female offspring exposed to maternal protein restriction was not significantly different from that of NP controls at either PN21, PN180 or PN360 (Supplementary Table 1). Similarly, the percentages of lean muscle mass and bone mineral content did not differ significantly between groups at any time point.

Offspring kidney weight was determined at PN21, 180 and 360. At PN21 total kidney weight was 48% less in LP offspring than NP offspring (Table 2). This difference remained statistically significant following adjustment for body weight, although now the difference was only 12%. These effects were similar in both male and female offspring.

No significant differences in the absolute weights of organs were found between the two dietary groups. However, when adjusted relative to body weight, heart weight was 5% ($p = 0.009$) greater and brain weight 9% ($p = 0.014$) greater in LP offspring than NP offspring. Males had larger absolute weights of the kidney, heart, liver, pancreas, mesenteric fat, peri-renal fat and total fat than females. However, following adjustment for body weight, heart, brain and pancreas weights were greater in females, while abdominal fat weight was greater in males (Supplementary Table 2).

At PN360, absolute weights of all organs and fat pads were similar in LP and NP offspring (Supplementary Table 3). Left kidney weight, when adjusted for body weight, was 7% less than NP controls ($P < 0.017$). The significant sex differences observed at PN180 on the absolute weights of the kidney, heart, liver, pancreas, peri-renal fat, abdominal fat and total fat remained at PN360, but the sex effects on the absolute weight of mesenteric fat were no longer observed. At PN360, females had greater mesenteric fat weight relative to body weight.

Over a 24-h period at PN360 there were no significant differences in food, energy or water consumption in offspring exposed to maternal LP or NP diets (Supplementary Table 4). Also, at this age, blood glucose levels were similar in the two dietary groups (Supplementary Table 5).

Nephron number at PN21

At PN21, kidneys of offspring exposed to a maternal LP diet contained fewer nephrons than NP controls, with LP females having 26% fewer nephrons than NP females, and LP males having 17% fewer nephrons than NP males (Fig 3). Nephron number adjusted for body weight was 12% greater in LP female offspring and 22% greater in LP male offspring than NP controls.

Renal physiology at PN180 and PN360

When measured under general anaesthesia, GFR (adjusted for body weight), ERPF (adjusted for body weight) and UF (adjusted for body weight) as well as FF were similar in LP and NP offspring at both PN180 (Fig 4) and PN360 (Fig 5). There were no significant differences in electrolyte excretion between LP and NP offspring at PN180 (Supplementary Table 6). Although, urine chloride concentration was 16% greater in LP females and 44% greater in LP males compared to controls (Supplementary Table 7).

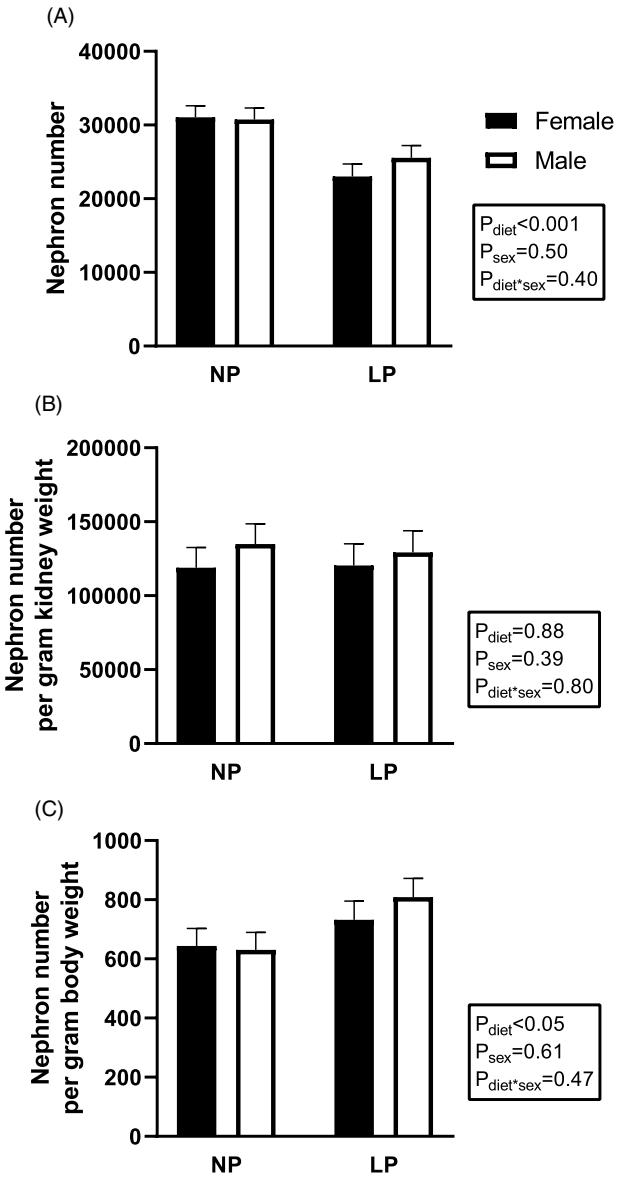

Fig. 3. PN21 nephron number in male and female offspring exposed to maternal low- or normal-protein diet. Total nephron number (**A**), nephron number per gram of kidney weight (**B**), and nephron number per gram of body weight (**C**) at PN21 in male and female offspring exposed to maternal LP or NP diet. Data expressed as mean ± SEM, analysed by a mixed linear model incorporating least means square regression to account for litter representation. (NP: $N_{litter} = 8$, comprising ♀ = 8, ♂ = 8, LP: $N_{litter} = 7$, comprising ♀ = 7, ♂ = 7).

Blood pressure and heart rate

Blood pressure and heart rate were determined in PN360 male and female offspring exposed to maternal LP or NP diet. Both heart rate and MAP were similar in LP and NP offspring during both active and non-active periods (Fig 6).

Cardiovascular responses to an acute stimulus (using an oscillating table) were determined (Fig 7). The response (change in MAP or heart rate from baseline to the average MAP or heart rate during the stress period) to the acute stimulus was similar in LP and NP rats, both for males and females. Similarly, the cardiovascular arousal response to a novel non-aversive stimulus(sultanas) was similar in LP and NP offspring (Fig 7).

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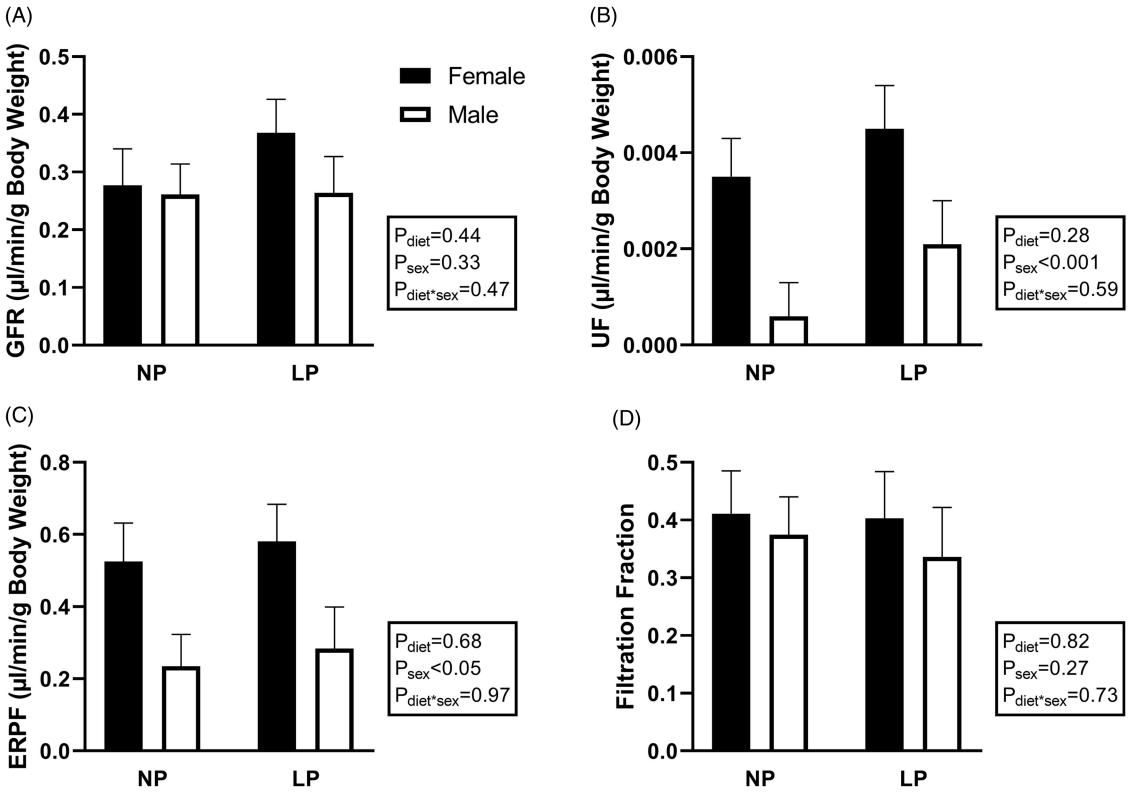

Fig. 4. PN180 renal function while under general anaesthesia in male and female offspring exposed to maternal low- or normal-protein diet. Measurements of glomerular filtration rate (**A**), urine flow (**B**), effective renal plasma flow (**C**) while under general anaesthesia, expressed as µl/min/g of body weight, as well as filtration fraction (**D**) were measured in male and female offspring exposed to maternal LP or NP diet at PN180. Data expressed as mean ± SEM, analysed by a mixed linear model incorporating least means square regression to account for litter representation. (NP: $N_{litter} = 9$, comprising ♀ = 5, ♂ = 7, LP: $N_{litter} = 4$, comprising ♀ = 6, ♂ = 5).

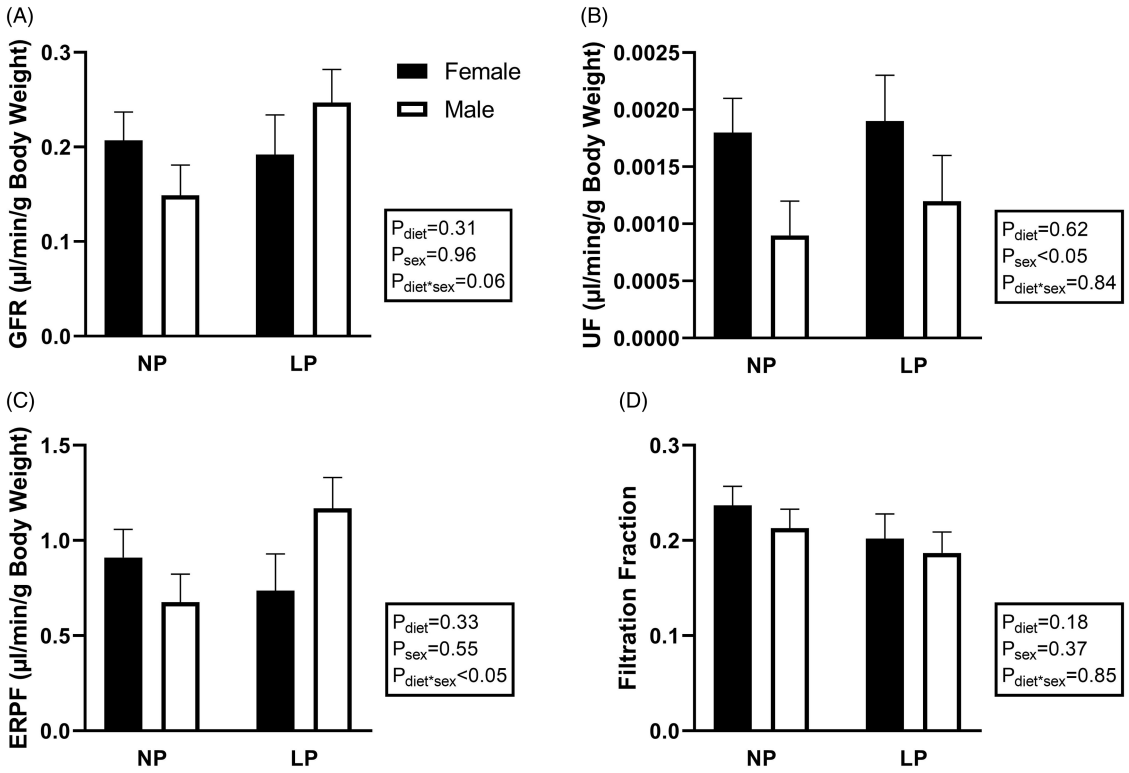

Fig. 5. PN360 renal function while under general anaesthesia in male and female offspring exposed to maternal low- or normal-protein diet. Measurements of glomerular filtration rate (**A**), urine flow (**B**), effective renal plasma flow (**C**) while under general anaesthesia expressed as µl/min/g of body weight as well as filtration fraction (**D**) were determined in male and female offspring exposed to maternal LP or NP diet at PN360. Data expressed as mean ± SEM, analysed by a mixed linear model incorporating least means square regression to account for litter representation. (NP: $N_{litter} = 12$, comprising ♀ = 12, ♂ = 12, LP: $N_{litter} = 10$, comprising ♀ = 7, ♂ = 10).

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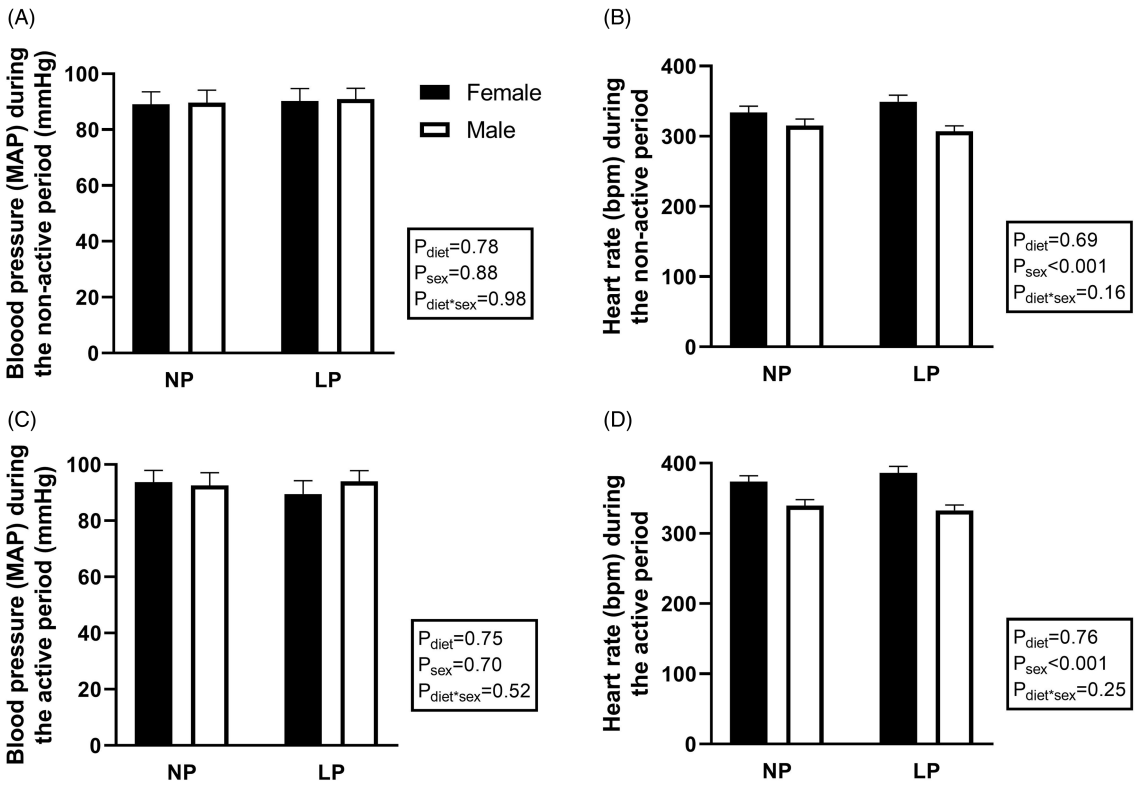

Fig. 6. Mean arterial pressure (MAP) and heart rate in LP and NP offspring at PN360 during non-active and active periods. Mean arterial pressure (MAP) and heart rate during non-active (**A-B**) and active (**C-D**) periods for male and female offspring exposed to LP or NP diet. MAP and heart rate were measured using an indwelling radio-telemetry device. Data are averages of a minimum of four 10-h periods during the active or non-active part of the day. Data expressed as mean ± SEM, analysed by a mixed linear model incorporating least means square regression to account for litter representation. (NP: $N_{litter} = 10$, comprising ♀ = 9, ♂ = 7, LP: $N_{litter} = 8$, comprising ♀ = 7, ♂ = 10).

Discussion

We report the novel outcome that, despite a nephron deficit, offspring exposed to a maternal LP diet and allowed to develop on a low-fat chow did not exhibit adverse renal or cardiovascular health outcomes up to 1 year of age. The LP offspring remained smaller than NP offspring throughout their life suggesting that, despite a poor nutritional plane at the start of life, offspring may not demonstrate abnormalities in blood pressure and renal function when growth is not accelerated.

Exposure to a maternal LP diet followed by feeding of a non 'growth diet' from PN21 to PN360 ensured that these offspring did not undergo catch-up growth after weaning and remained proportionally smaller than control offspring. The offspring of this protocol have a significantly more favourable cardiovascular and renal phenotype than models of material protein restriction followed by catch-up growth.[18,54-65] Without an experimental group exposed to a diet that would promote catch-up growth, we are unable to draw conclusions regarding the specific impact of catch-up growth on adult cardiovascular and renal function. Nevertheless, our current finding complement those of Howie *et al.*[66] They proposed that, in models of maternal undernutrition (caloric restriction), there are crucial periods of development where maternal caloric restriction may have the most significant (and deleterious) effects on offspring health. In support of this proposition, they found that the lack of catch-up growth during the lactation period (achieved by caloric restriction) prevented obesity and other metabolic disorders in later life.[66]

Although maternal diet is a determinant of offspring nephron number, a low nephron endowment is not associated with disease if postnatal factors are favourable. At PN21, kidney weight in LP offspring was almost half that of NP offspring. When adjusted for body weight, kidney weight in LP offspring remained 12% less than in NP offspring. However, at PN180 absolute and relative kidney weights were similar in the two dietary groups. At PN360 absolute kidney weight was similar between the two dietary groups. Although, kidney weight adjusted for body weight was less in LP offspring than in NP offspring. Previously, Habib *et al.*[67] reported in a model that did not show catch-up growth, that male offspring exposed to maternal LP (6% protein) diet had 13% fewer nephrons per gram kidney weight than controls. In contrast, in the present study we found that nephron number per gram of kidney weight was similar across all groups. When considering the impact of nephron number relative to body weight, Vehaskari *et al.*[59] reported a model where offspring demonstrated catch-up growth and in which there were approximately 15–30% fewer nephrons per gram body weight in LP (6% protein) rats (male and female) than in controls. In this study, we found that the kidneys of LP offspring may be lighter and have a smaller organ to body weight ratio compared to controls, however this proportionality is not reflected in nephron number. In fact, the inverse occurs whereby these proportionally (to body weight) small kidneys consist of proportionally (to body weight) more nephrons compared to controls, and this analysis takes into consideration that the LP bodyweight was less than controls. In our study, the LP offspring had more nephrons per gram of body weight than controls. We propose that this is likely due to the controlled growth during the suckling period. Furthermore, in adulthood the LP

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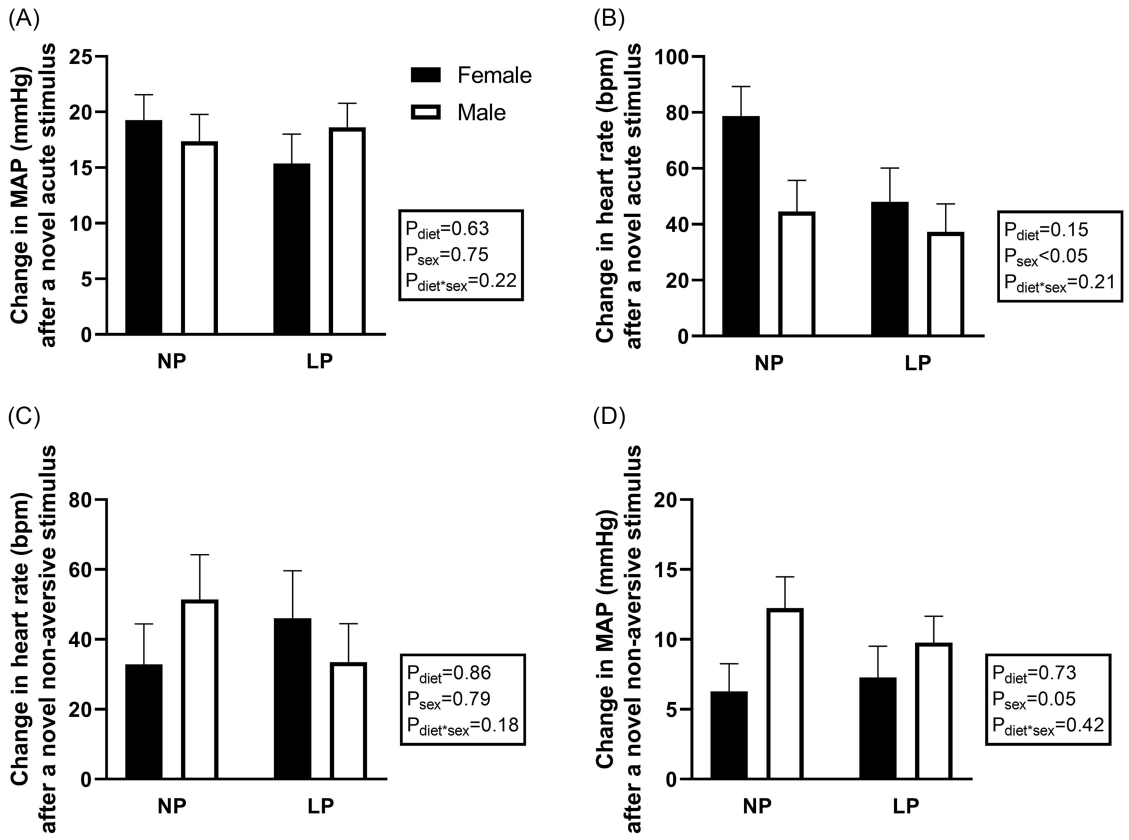

Fig. 7. Change in mean arterial pressure (MAP) and heart rate after exposure to either a novel acute stimulus or non-aversive stimulus in PN360 male and female offspring exposed to either maternal low or normal protein diet. Changes in mean arterial pressure (MAP, **A & C**) and heart rate (**B & D**) following exposure to a novel acute stimulus(**A, B**) or a non-aversive stimulus(**C, D**) in male and female offspring exposed to maternal low protein (LP) or normal protein (NP) diet. The data presented are the differences between averaged MAP and heart rate 5 min before the stress and 10 min during the stress. Data expressed as mean ± SEM, analysed by a mixed linear model incorporating least means square regression to account for litter representation. (NP: $N_{litter} = 10$, comprising ♀ = 9, ♂ = 7, LP: $N_{litter} = 8$, comprising ♀ = 7, ♂ = 10).

offspring had similar cardiovascular and renal function to control offspring.

Maternal protein restriction was not associated with alterations to resting haemodynamic, automatic drive or diurnal rhythms in the offspring. Most reports of haemodynamic status following maternal dietary intervention are based on a single observation epoch,[16,27,55-57,65] and do not consider diurnal variation, nor the potential impact of cardiovascular arousal and autonomic control. Overall, the lack of catch-up growth in the offspring of LP fed rats resulted in a healthy cardiovascular and renal phenotype. This is in contrast to previous reported observations of the effects of maternal LP diet in which offspring demonstrated catch-up growth, greater adiposity, fewer nephrons and higher blood pressure.[15,68-70] Greater adiposity, whether due to programmed obesity or consumption of a high fat diet in adulthood, does cause elevated blood pressure which appears to be driven by excessive sympathetic drive to the kidneys.[24,71-74] Burke *et al.*[74] demonstrated that excessive adiposity can perturb the circadian rhythm of blood pressure and heart rate. The normal cardiovascular function seen in the present study may be a consequence of the animals remaining lean. This hypothesis merits testing in future studies.

Our current experimental model provided an opportunity to investigate the interactions between nephron endowment and the subsequent post-weaning environment in determining adult cardiometabolic and renal phenotype. Several observations suggest that apparent hypertension seen in LP offspring is due to increased cardiovascular arousal induced by indirect techniques to measure blood pressure.[75,76] Our current findings suggest that, without excess adiposity, LP offspring are not hyperreactive to aversive or non-aversive stimuli. Normal blood pressure, in the face of fewer nephrons, is not consistent with the Brenner hypothesis, which predicts that low nephron number results in hyperfiltration, eventual glomerular damage, and ultimately hypertension and chronic kidney disease.[77] Therefore, future studies are required to investigate the role of catch-up growth and consequent adiposity as a critical factor driving hypertension in experimental rodent models of intra-uterine protein restriction.

Lean offspring of LP fed dams maintain normal renal function indices surprisingly, few studies have examined the effects of a maternal LP diet on postnatal kidney function. Nwagwu *et al.*[27] reported reduced GFR (measured using creatinine clearance) in rats at 4 weeks of age following a maternal LP diet, while Hoppe *et al.*[47] in a study of a lifelong (prenatal and postnatal) LP diet in rats observed no differences in GFR or ERPF (24 h clearance of [^{3}H] inulin and PAH) per gram of body weight at any timepoint. While kidney function (though measured under the potentially confounding effects of anaesthesia) and nephron number were determined at different ages there is nothing from the adult functional data to indicate that the nephron deficit at PN21 progresses to significant renal pathology over the life course. Therefore, the apparently well-preserved renal function we observed in LP rats may be due to their relative nephron reserve (per gram body weight), since nephron number adjusted for body weight at PN21 was 12% greater in LP female offspring and 22% greater

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in LP male offspring than NP controls. Nevertheless, it remains to be determined whether these LP offspring have a deficit in renal functional reserve, as can be assessed by administration of a protein or amino acid load.[78]

Limitation of study

The incorporation of additional control groups (rats fed a growth diet during lactation, and another group fed a growth diet during lactation and post-weaning) would have facilitated more definitive conclusions regarding the impact of maternal nutrition and offspring health, particularly the impact of a low protein diet, on adult cardiovascular and renal function. Indeed, in the absence of such data we are unable to determine whether the absence of hypertension or renal dysfunction we observed in the adult offspring of protein restricted dams was due the absence of catch-up growth or to some other unmeasured factor.

An additional limitation of this study is the lack of data on gestational length. These data would have provided extra insight into the impact of maternal protein intake. The experimental design was focused on ensuring breeder females became pregnant, by housing breeder females with breeder males for 1 week. It would have been useful to introduce a grid floor that would have enabled collection of a vaginal plug to accurately determine start of pregnancy. Our previous study exploring the impact of maternal protein intake during gestation revealed that at E20 LP offspring were heavier than control offspring.[53] It is unclear whether this result is indicative of a change in gestational length. Therefore, future studies may wish to further explore the role of maternal protein intake on gestational length.

Conclusion

Our current findings should provide impetus for further research into the effects of a 'postnatal second hit' on susceptibility to kidney and cardiovascular disease. We propose that the normal kidney and cardiovascular function observed in LP offspring in the present study may be due to the LP offspring remaining proportionally smaller than NP offspring, not developing excessive adiposity, and also having proportionally more nephrons than controls in terms of body mass. This hypothesis merits further investigation. Importantly, if there is benefit in maintenance of a stable growth trajectory throughout postnatal life, its demonstration would have important implications for populations transitioning from dietary protein restriction during early life. In such settings, maintenance of a low-fat diet that results in a steady growth trajectory may help compensate for low nephron number. Similarly, in those born small due to poor and/or lack of nutrition, strategies to prevent or minimise catch-up growth might limit the impact of the development of cardiovascular disease due to changes in organs with finite development periods (such as the kidney). Thus, further research to more definitively characterise the impact of catch-up growth after malnutrition in utero could have major implications for the global burden of non-communicable disease.

Supplementary Material. For supplementary material for this article, please visit https://doi.org/10.1017/S2040174422000666

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