CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD13133

Transgenerational programming of fetal nephron deficits and sex-specific adult hypertension in rats

Linda A. Gallo[A,E], *Melanie Tran*[A], *Luise A. Cullen-McEwen*[B], *Kate M. Denton*[C], *Andrew J. Jefferies*[A], *Karen M. Moritz*[D] *and Mary E. Wlodek*[A,F]

[A]Department of Physiology, The University of Melbourne, Parkville, Vic. 3010, Australia.
[B]Department of Anatomy and Developmental Biology, Monash University, Clayton, Vic. 3168, Australia.
[C]Department of Physiology, Monash University, Clayton, Vic. 3168, Australia.
[D]School of Biomedical Sciences, The University of Queensland, St Lucia, Qld 4067, Australia.
[E]Present address: Mater Research, Translational Research Institute, Woolloongabba, Qld 4102, Australia.
[F]Corresponding author. Email: m.wlodek@unimelb.edu.au

Abstract. A developmental insult that restricts growth in the first generation has the potential to program disease in subsequent generations. The aim of this study was to ascertain transgenerational growth and cardio–renal effects, via the maternal line, in a rat model of utero–placental insufficiency. Bilateral uterine vessel ligation or sham surgery (offspring termed first generation; F1 Restricted and Control, respectively) was performed in WKY rats. F1 Restricted and Control females were mated with normal males to produce second generation (F2) offspring (Restricted and Control) studied from fetal (embryonic Day 20) to adult (12 months) life. F2 Restricted male and female fetuses had reduced ($P < 0.05$) nephron number (down 15–22%) but this deficit was not sustained postnatally and levels were similar to Controls at Day 35. F2 Restricted males, but not females, developed elevated (+16 mmHg, $P < 0.05$) systolic blood pressure at 6 months of age, which was sustained to 9 months. This was not explained by alterations to intra-renal or plasma components of the renin–angiotensin system. In a rat model of utero–placental insufficiency, we report alterations to F2 kidney development and sex-specific adult hypertension. This study demonstrates that low birthweight can have far-reaching effects that extend into the next generation.

Additional keywords: fetal programming, intrauterine growth, pregnancy, sexual dimorphism.

Received 3 May 2013, accepted 2 July 2013, published online 5 August 2013

Introduction

Perturbations during critical periods of development restrict fetal growth and program adverse cardio–renal outcomes in adulthood (Barker *et al.* 1989; Barker 1995; Hoy *et al.* 1999; Vikse *et al.* 2008). Reduced nephron complement may underlie the development of adult hypertension and renal disease (Hoy *et al.* 2005; Wlodek *et al.* 2007, 2008; Moritz *et al.* 2009; Vehaskari 2010). In the Western world, late gestation utero–placental insufficiency is the most common cause of fetal growth restriction (Henriksen and Clausen 2002). More recently, experimental evidence from both humans and animals suggests that adverse prenatal exposures are not limited to affect the first, directly exposed generation but can have transgenerational effects (Roseboom and Watson 2012).

In humans, poorer health outcomes including neonatal adiposity and autoimmune diseases have been reported in second generation (F2) offspring of mothers, but not fathers, that were prenatally exposed to the Dutch Winter famine. Similarly, Torrens *et al.* (2008) reported the development of hypertension in male and female F2 rat offspring from mothers prenatally exposed to low protein. Elevated blood pressure and reduced nephron number have also been identified in F2 male and female rat offspring, arising from either parent's prenatal exposure to low protein (Harrison and Langley-Evans 2009). Adverse cardiovascular outcomes have been demonstrated across three generations, using a rat model of maternal global nutrient restriction (Ponzio *et al.* 2012) and pregnancy-induced hypertension (reduced uterine perfusion pressure (RUPP); Anderson *et al.* 2006). However, neither of these studies delineated between the parental lines of effect as offspring were derived from same-group male and female crosses.

We and others have utilised a rat model that mimics utero–placental insufficiency, whereby the uterine vessels are bilaterally ligated during late gestation (Simmons *et al.* 2001; Schreuder *et al.* 2007; Wlodek *et al.* 2008). The physical restriction of utero–placental blood flow reflects growth restriction that

occurs in nutrient-abundant societies. It is of similar nature to the RUPP procedure (Anderson *et al.* 2006), albeit dams remain normotensive. Male offspring generally present with more severe cardiovascular outcomes compared with female counterparts (Ojeda *et al.* 2007*a*, 2007*b*; Wlodek *et al.* 2007, 2008; Grigore *et al.* 2008; Moritz *et al.* 2009). Recently, we have shown that when growth-restricted females become pregnant, they exhibit normal uterine artery relaxation (Mazzuca *et al.* 2012) but develop some loss of glucose tolerance (Gallo *et al.* 2012*d*). Growth-restricted females that were not exposed to physiological measurements during late pregnancy carried F2 fetuses of normal weight. Certainly, others have reported transgenerational disease outcomes in the absence of perpetuating growth deficiencies (Painter *et al.* 2008; Torrens *et al.* 2008; Harrison and Langley-Evans 2009; Ponzio *et al.* 2012).

The aim of this study was to determine whether female growth restriction, induced by utero–placental insufficiency, was associated with transgenerational cardio–renal deficits in F2 male and female offspring, in the absence of any further pregnancy challenge. This maternal line effect was investigated using pregnancies that were not exposed to physiological measurements and thus F2 outcomes were not confounded by maternal stress exposure. It was hypothesised that nephron deficits, elevated blood pressure and renal insufficiency would be evident in offspring from growth-restricted mothers compared with those from normal birthweight mothers, and that these effects would be exacerbated by the male gender and ageing.

Materials and methods

Animal procedures

All experiments were approved by The University of Melbourne Animal Ethics Committee before commencement. Wistar Kyoto rats housed in an environmentally controlled room had access to food and tap water *ad libitum*. Rats were mated and utero–placental insufficiency (offspring termed first generation; F1 Restricted) or sham (offspring termed F1 Control) surgery performed at embryonic Day (E) 18 ($n = 17$–19 per group; Wlodek *et al.* 2007). Rats delivered at term (E22). Utero–placental insufficiency reduced total (male and female) litter size (Restricted 5.53 ± 0.37 vs Control 8.53 ± 0.55) but litter size was not equalised between the groups. We have previously shown that reducing litter size from sham-operated dams impairs maternal mammary morphology, lactation and subsequent postnatal growth and health of the offspring (O'Dowd *et al.* 2008; Wadley *et al.* 2008; Wlodek *et al.* 2008). Thus, we do not regard sham-exposed, culled litters as adequate controls. One F1 Control and one Restricted female from each litter was randomly selected and mated with a normal male at 17–23 weeks (offspring termed F2 Control and Restricted, respectively). Pregnant F1 rats were allowed to deliver at term (E22). F2 Control and Restricted male and female offspring were allocated to one of three study ages; postnatal Day (PN) 35 ($n = 10$–11 males; $n = 10$–11 females per group), 6 months ($n = 13$–14 males; $n = 16$–17 females per group) or 12 months ($n = 11$–12 males; $n = 10$–13 females per group). No more than one animal of each sex per F1 litter was allocated to each study age; not all F1 litters had pups allocated to each age. F2 fetuses (E20) from our previously published cohort (Gallo *et al.* 2012*d*) were used for analysis of nephron number only.

Maternal characteristics, bodyweight and growth rates

F1 maternal bodyweights were measured at PN1, PN7, PN14, PN35, 2 and 3 months, and at mating (17–23 weeks) and delivery of F2 offspring. Systolic blood pressure was measured by non-invasive tail-cuff plethysmography (ADInstruments Pty Ltd, Castle Hill, NSW, Australia) before mating in F1 females that were acclimatised to the restraint procedure (Wlodek *et al.* 2007; Moritz *et al.* 2009; Gallo *et al.* 2012*a*, 2012*c*, 2012*d*). F2 male and female bodyweights were measured from E20 to 12 months, and body dimensions (crown-to-rump length, ponderal index, head length and head width) were measured using digital vernier calipers (accurate to 0.01 mm) from PN7 to 12 months. F2 male and female growth rates (g day^{-1}) were calculated from PN1–14, PN14–2 months, 2–3 months and 3–4 months.

Blood pressure

Blood pressure was measured by non-invasive tail-cuff plethysmography in F2 offspring that were acclimatised to the restraint procedure at 2, 3, 4, 6, 9 and 12 months (Wlodek *et al.* 2007; Moritz *et al.* 2009; Gallo *et al.* 2012*a*, 2012*c*, 2012*d*). The final five of 10 acquired traces were recorded and averaged to determine systolic and mean arterial blood pressure. Systolic, diastolic and mean arterial blood pressure and heart rate were also measured using an indwelling tail-artery catheter in F2 Control and Restricted male offspring at 12 months of age, before anaesthetised renal function experiments (Moritz *et al.* 2009; Gallo *et al.* 2012*a*). Rats were anaesthetised with isofluorane and the ventral tail artery isolated. A catheter tip filled with heparinised saline was inserted to a distance of 1 cm. The catheter was secured around the artery and allowed to retract over the incision site. The rat was allowed to recover for 1 h with *ad libitum* access to tap water. Following recovery, the external end of the arterial catheter was attached to a pressure transducer connected to a bridge amplifier. Arterial pressure was acquired for 30 min and the last 15 min were averaged.

Food and water intake, urinary excretions and anaesthetised renal function

F2 animals allocated to 6- and 12-month study ages were weighed and placed individually in metabolic cages for 24 h measurements of food and water intake and urine production (Moritz *et al.* 2009; Gallo *et al.* 2012*a*, 2012*c*, 2012*d*). Rats were acclimatised to metabolic cages by placing them in for short daylight periods of 3 h and 8 h on two separate occasions and 24 h on a third occasion to minimise stress and associated behavioural changes. Measurements began 2 days following the acclimatisation period. Food and water were weighed and rats were allowed *ad libitum* access throughout the experiment. Urine volume was also recorded. Measurements of urine sodium, chloride, creatinine and total protein were performed using commercially available kits according to the manufacturer's instructions (Cobas Integra 400; Roche Diagnostics, Burgess

Table 1. Maternal characteristics

Bodyweights from PN1 to pregnancy and pre-mating systolic blood pressure in F1 Control and Restricted females that gave rise to F2 offspring. Values expressed as mean ± s.e.m., $n = 17$–19 per group. *$P < 0.05$ Restricted vs Control (Student's unpaired *t*-test)

Parameter	Control	Restricted
Bodyweight (g)		
PN1	4.26 ± 0.05	3.34 ± 0.07*
PN7	10.15 ± 0.28	6.99 ± 0.31*
PN14	22.95 ± 0.40	16.94 ± 0.73*
PN35	75.46 ± 1.04	61.28 ± 1.75*
2 months	158 ± 3	147 ± 4*
3 months	209 ± 3	187 ± 3*
Mating	240 ± 3	211 ± 4*
Delivery	276 ± 3	241 ± 4*
Pregnancy weight gain (%)	15.08 ± 0.76	13.94 ± 1.06
Systolic blood pressure (mmHg)	138 ± 3	136 ± 3

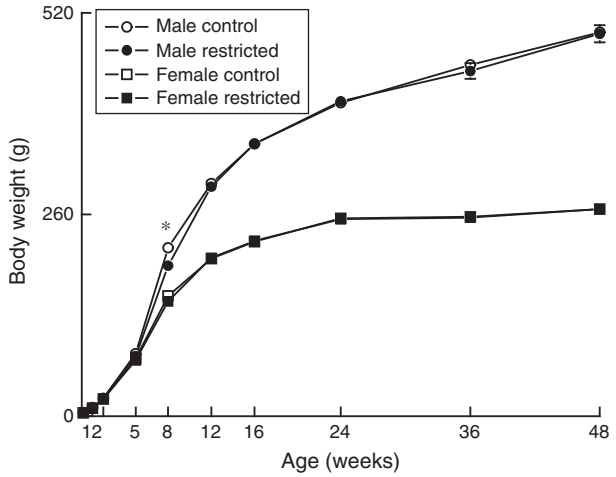

Fig. 1. Bodyweights from PN1 to 12 months in F2 males and females. Values expressed as mean ± s.e.m., $n = 10$–17 per group. *$P < 0.05$ Restricted vs Control males (Student's unpaired *t*-test).

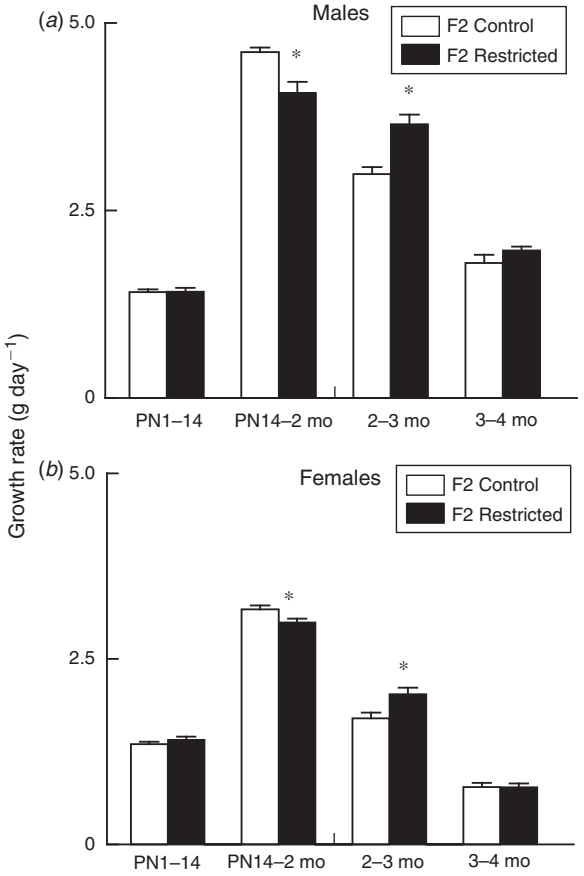

Fig. 2. Absolute growth rates from PN1–14, PN14–2 months, 2–3 months and 3–4 months in F2 (*a*) males and (*b*) females. Values expressed as mean ± s.e.m., $n = 10$–17 per group. *$P < 0.05$ F2 Restricted vs Control (Student's unpaired *t*-test).

Hill, UK). Plasma samples were collected at *post mortem*, immediately upon removal from metabolic cages, and analysed for creatinine and sodium to determine creatinine clearance ((urinary creatinine (μmol L^{-1}) × 24 h urine production (mL))/(plasma creatinine (μmol L^{-1}) × 1440 (min))) and fractional sodium excretion, respectively. Glomerular filtration rate (GFR) and effective renal plasma flow (eRPF) were estimated by renal clearance methods (3H-inulin and para-aminohippuric acid (^{14}C-PAH), respectively) in anaesthetised (150 mg kg^{-1} Inactin; Sigma-Aldrich, Castle Hill, NSW, Australia) F2 Control and Restricted male offspring at 12 months of age followed by *post mortem* (Zimanyi *et al.* 2006; Gallo *et al.* 2012*a*). These were the only groups subjected to the anaesthetised renal function experiment as it was hypothesised that F2 Restricted males would be the most adversely affected compared with females and 6 month old counterparts.

Post mortem tissue collection

Rats were anaesthetised with an intra-peritoneal injection of mixed solution containing ketamine (100 mg kg^{-1}; Parnell Laboratories, Alexandria, NSW, Australia) and Ilium Xylazil-20 (20 mg kg^{-1}; Troy Laboratories, Smithfield, NSW, Australia), unless already anaesthetised with Inactin for renal function studies (males at 12 months of age). F2 Control and Restricted fetal tissues were collected at E20. All rats studied to PN35, 6 months and 12 months of age were exposed to a general *post mortem*, whereby heart and kidneys were excised and weighed. The left kidney was then snap frozen in liquid nitrogen and stored at −80°C until further analyses. An additional cohort of F2 Control and Restricted rats (siblings to those exposed to a general *post mortem*) were perfusion fixed through the abdominal aorta at PN35 (using 4% paraformaldehyde) and kidneys excised for stereological analyses. The stage of the oestrous cycle is known to affect female physiology and thus a vaginal smear was taken at *post mortem* (6 and 12 months) to confirm an even number of animals in proestrus–oestrus and metestrus–diestrus within each group.

Table 2. Organ weights

Heart, kidney and dorsal fat weights at PN35, 6 months and 12 months in F2 males and females. Values expressed as mean ± s.e.m., $n = 10$–17 per group. *$P < 0.05$ Restricted vs Control within sex (Student's unpaired *t*-test). – no data obtained

Parameter	Males		Females	
	Control	Restricted	Control	Restricted
Heart weight[A]				
PN35	0.426 ± 0.015	0.430 ± 0.006	0.443 ± 0.007	0.442 ± 0.008
6 months	0.334 ± 0.006	0.333 ± 0.006	0.379 ± 0.004	0.368 ± 0.004
12 months	0.285 ± 0.005	0.287 ± 0.005	0.381 ± 0.005	0.367 ± 0.004*
Left ventricle weight[A]				
PN35	–	–	–	–
6 months	0.239 ± 0.003	0.240 ± 0.004	0.273 ± 0.003	0.264 ± 0.004*
12 months	0.210 ± 0.005	0.216 ± 0.004	0.283 ± 0.004	0.274 ± 0.004
Kidney weight[A]				
PN35	0.822 ± 0.021	0.858 ± 0.010	0.821 ± 0.013	0.832 ± 0.014
6 months	0.605 ± 0.007	0.624 ± 0.014	0.652 ± 0.009	0.633 ± 0.008
12 months	0.547 ± 0.008	0.556 ± 0.002	0.634 ± 0.013	0.636 ± 0.007
Dorsal fat weight[A]				
PN35	0.430 ± 0.035	0.390 ± 0.030	0.417 ± 0.033	0.406 ± 0.016
6 months	1.277 ± 0.094	1.280 ± 0.090	1.013 ± 0.051	1.050 ± 0.031
12 months	1.942 ± 0.098	2.031 ± 0.144	1.321 ± 0.058	1.363 ± 0.073

[A]Weights are presented as percentage of bodyweight.

Table 3. Body dimensions

Crown-to-rump length, ponderal index, head length and head width from PN7 to 12 months in F2 males and females. Values expressed as mean ± s.e.m., $n = 10$–17 per group. *$P < 0.05$ F2 Restricted vs Control (Student's unpaired *t*-test)

Parameter	Crown-to-rump length (mm)		Ponderal index		Head length (mm)		Head width (mm)	
	Control	Restricted	Control	Restricted	Control	Restricted	Control	Restricted
Males								
PN7	48.18 ± 0.60	46.95 ± 0.36	9.90 ± 0.25	10.47 ± 0.24	18.90 ± 0.20	18.77 ± 0.21	12.53 ± 0.13	12.29 ± 0.17
PN14	58.45 ± 0.45	58.73 ± 0.57	11.52 ± 0.22	11.39 ± 0.31	22.87 ± 0.33	22.79 ± 0.41	14.93 ± 0.19	14.80 ± 0.25
PN35	109 ± 1	107 ± 1	6.23 ± 0.08	6.30 ± 0.08	32.21 ± 0.22	31.41 ± 0.24*	18.65 ± 0.11	18.16 ± 0.08*
2 months	155 ± 1	151 ± 2	5.79 ± 0.12	5.56 ± 0.16	38.27 ± 0.34	37.28 ± 0.27*	23.16 ± 0.13	22.87 ± 0.16
3 months	172 ± 1	169 ± 2	5.95 ± 0.12	6.10 ± 0.11	41.48 ± 0.36	40.35 ± 0.21*	25.11 ± 0.13	24.75 ± 0.20
4 months	175 ± 1	180 ± 3	6.47 ± 0.11	6.09 ± 0.19	41.83 ± 0.31	41.90 ± 0.28	26.06 ± 0.13	26.22 ± 0.28
6 months	198 ± 2	191 ± 2*	5.24 ± 0.14	5.85 ± 0.22*	47.07 ± 0.64	46.33 ± 0.92	27.09 ± 0.25	26.81 ± 0.30
9 months	195 ± 2	188 ± 3	6.21 ± 0.22	6.73 ± 0.28	44.72 ± 0.74	42.86 ± 0.28*	28.17 ± 0.38	27.61 ± 0.36
12 months	190 ± 1	188 ± 2	6.95 ± 0.14	7.17 ± 0.17	45.10 ± 0.71	43.08 ± 0.28*	27.94 ± 0.33	27.78 ± 0.20
Females								
PN7	47.22 ± 0.36	47.07 ± 0.40	9.95 ± 0.19	10.20 ± 0.25	18.71 ± 0.22	18.75 ± 0.15	12.25 ± 0.09	12.20 ± 0.12
PN14	57.27 ± 0.85	59.02 ± 0.54	11.47 ± 0.25	11.03 ± 0.19	22.77 ± 0.41	22.65 ± 0.38	14.93 ± 0.21	14.77 ± 0.18
PN35	105 ± 1	103 ± 1	6.40 ± 0.07	6.51 ± 0.08	31.69 ± 0.15	31.21 ± 0.15*	18.29 ± 0.12	18.08 ± 0.10
2 months	141 ± 1	138 ± 2	5.56 ± 0.09	5.73 ± 0.14	36.77 ± 0.25	35.99 ± 0.27*	22.06 ± 0.15	21.87 ± 0.07
3 months	153 ± 1	153 ± 1	5.67 ± 0.11	5.72 ± 0.14	39.35 ± 0.28	38.66 ± 0.21	23.26 ± 0.15	23.26 ± 0.15
4 months	158 ± 1	158 ± 1	5.77 ± 0.09	5.75 ± 0.11	39.33 ± 0.20	39.60 ± 0.23	23.84 ± 0.14	23.86 ± 0.11
6 months	173 ± 2	165 ± 2*	4.97 ± 0.19	5.70 ± 0.16*	43.84 ± 0.45	41.91 ± 0.42*	24.56 ± 0.26	24.62 ± 0.26
9 months	164 ± 2	163 ± 1	5.88 ± 0.18	5.87 ± 0.09	40.85 ± 0.44	40.92 ± 0.31	25.05 ± 0.20	24.62 ± 0.13
12 months	174 ± 1	169 ± 1*	5.11 ± 0.11	5.59 ± 0.10*	42.98 ± 0.45	43.37 ± 0.47	25.15 ± 0.39	24.69 ± 0.14

Glomerular number and volume

Fetal (E20) glomerular number was determined in the right kidney of F2 Control and Restricted rats as previously described (Cullen-McEwen *et al.* 2011, 2012). Glomerular number and volume and corpuscle volume were determined in the right kidney of F2 Control and Restricted rats that were perfusion fixed at PN35 using the Cavalieri and physical dissector method (Bertram 1995; Wlodek *et al.* 2007; Moritz *et al.* 2009; Gallo *et al.* 2012*d*).

Intra-renal and plasma renin-angiotensin system (RAS) components

In F2 Control and Restricted rats studied to 6 months, renin activity, angiotensin II (AngII) and angiotensin 1–7 (Ang1–7) content were analysed in plasma in frozen renal cortices using radioimmunoassay techniques (Prosearch International, Malvern, Vic., Australia; Singh *et al.* 2011).

Statistical analyses

Values are expressed as mean ± s.e.m., with *n* representing the number of animals per group from different litters. A Student's unpaired *t*-test was used to compare between F1 Control and Restricted maternal characteristics, as well as F2 Control and Restricted offspring at all study ages. For 24-h urinary excretions, a two-way ANOVA was performed to determine main effects of group (F2 Control and Restricted) and age (6 months and 12 months). If significant interactions were observed, individual group means were compared using a Student's unpaired *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Maternal characteristics

Utero–placental insufficiency reduced total (male and female) litter size (Restricted 5.53 ± 0.37 vs Control 8.53 ± 0.55). Male-to-female ratio was not different in Control litters (3.76 ± 0.37 vs 4.76 ± 0.43) but there were fewer ($P < 0.05$) males than females in Restricted litters (2.35 ± 0.26 vs 3.18 ± 0.30). Utero–placental insufficiency surgery reduced ($P < 0.05$) F1 male (−15%; data not shown) and female bodyweight (−21%; Table 1) compared with Controls at postnatal Day (PN) 1. Restricted females remained lighter ($P < 0.05$) than Controls at all ages, including at mating and delivery of F2 offspring (−12.5%), and percentage weight gain was not different (Table 1). Systolic blood pressure was similar between F1 Control and Restricted females before mating (Table 1).

Growth parameters and organ weights

At PN1, total litter size was not different between groups (Control 8.94 ± 0.49 vs Restricted 8.00 ± 0.54). There were no differences in the ratio of F2 male-to-female pups in those from Control dams (3.94 ± 0.39 vs 5.0 ± 0.37) but there were fewer ($P < 0.05$) males than females in those from Restricted (3.27 ± 0.33 vs 4.73 ± 0.44). There were no differences in male and female bodyweight at PN1 to PN35 (Fig. 1), or in growth rate from PN1–14 between Control and Restricted animals (Fig. 2*a*, *b*). From PN14 to 2 months, F2 Restricted animals had slowed ($P < 0.05$) growth rate in both males (−12%) and females (−6%) compared with Controls (Fig. 2*a*, *b*). F2 Restricted bodyweight was reduced (−11%, $P < 0.05$) at 2 months in males only (females −4%, $P = 0.060$; Fig. 1). From 3 months to the end of study at 12 months, bodyweights between F2 Control and Restricted were comparable (Fig. 1), attributed to accelerated (+22%, $P < 0.05$) growth from 2–3 months (Fig. 2*a*, *b*). Thereafter, growth rates were similar between groups (Fig. 2*a*, *b*).

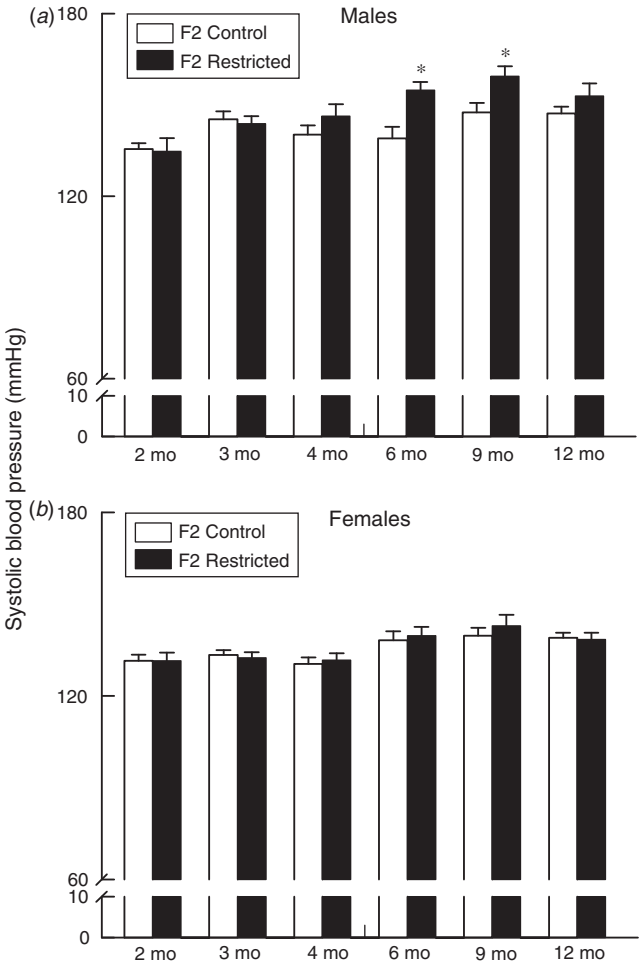

Fig. 3. Systolic blood pressure (tail-cuff plethysmography) at 2, 3, 4, 6, 9 and 12 months in F2 (*a*) males and (*b*) females. Values expressed as mean ± s.e.m., $n = 8$–14 per group. *$P < 0.05$ F2 Restricted vs Control (Student's unpaired *t*-test).

Heart weight (relative to bodyweight) was not different between F2 Control and Restricted animals at PN35 and 6 months but was reduced (−4%, $P < 0.05$) in F2 Restricted females, and not males, at 12 months (Table 2). At 6 months, left ventricular weight (relative to bodyweight) was not different between F2 Control and Restricted males, but was reduced (−3%, $P < 0.05$) in F2 Restricted females (Table 2). There were no differences in left ventricular weight at 12 months of age (Table 2). Kidney weight (right and left pooled; relative to bodyweight) was not different between the F2 Control and Restricted groups at PN35, 6 months or 12 months in males or females (Table 2).

Crown-to-rump length and ponderal index were similar between F2 Control and Restricted animals from PN7 to 4 months of age in both males and females (Table 3). At 6 months, both male and female F2 Restricted animals were shorter ($P < 0.05$) in length compared with the Controls,

Table 4. Food and water intake and urinary excretions

Food and water intake and urinary excretions (24 h) at 6 and 12 months in F2 males and females. Values expressed as mean ± s.e.m., $n = 6$–14 per group. Right column represents results from two-way ANOVA, *$P < 0.05$ vs 6-month counterpart (Student's unpaired *t*-test following observation of significant interaction). – no data obtained; see Fig. 4 for GFR and fractional sodium excretion from renal function experiment at 12 months in males

Parameter	6 Months		12 Months		Two-way ANOVA		
	Control	Restricted	Control	Restricted	Group	Age	Group × age
Males							
Food ($g\,24\,h^{-1}\,kg^{-1}$)	57.93 ± 1.32	57.60 ± 1.72	47.34 ± 1.45	41.75 ± 2.41	NS	$P < 0.05$	NS
Water ($mL\,24\,h^{-1}\,kg^{-1}$)	99.80 ± 6.88	98.11 ± 7.69	93.46 ± 5.05	66.53 ± 3.20	$P < 0.05$	$P < 0.05$	$P = 0.06$
Urine flow ($L\,24\,h^{-1}\,kg^{-1}$)	0.045 ± 0.005	0.050 ± 0.004	0.036 ± 0.004	0.030 ± 0.004	NS	$P < 0.05$	NS
Na^+ ($mmol\,L^{-1}\,(24\,h)^{-1}\,kg^{-1}$)	2.24 ± 0.19	2.37 ± 0.17	2.21 ± 0.20	1.70 ± 0.19	NS	NS	NS
Cl^- ($mmol\,L^{-1}\,(24\,h)^{-1}\,kg^{-1}$)	3.71 ± 0.26	3.98 ± 0.27	3.61 ± 0.34	2.92 ± 0.31	NS	NS	NS
Total protein ($mg\,L^{-1}\,(24\,h)^{-1}\,kg^{-1}$)	46.80 ± 5.49	49.72 ± 5.97	78.89 ± 15.15	49.53 ± 7.92	NS	NS	NS
Creatinine clearance ($mL\,min^{-1}\,kg^{-1}$)	4.12 ± 0.48	4.04 ± 0.35	–	–	NS	NS	–
Fractional sodium excretion (%)	0.311 ± 0.033	0.298 ± 0.028	–	–	NS	NS	–
Females							
Food ($g\,24\,h^{-1}\,kg^{-1}$)	78.48 ± 3.83	71.98 ± 0.91	69.71 ± 2.29	67.25 ± 2.76	NS	$P < 0.05$	NS
Water ($mL\,24\,h^{-1}\,kg^{-1}$)	135 ± 6	146 ± 7	154 ± 5	148 ± 7	NS	NS	NS
Urine flow ($L\,24\,h^{-1}\,kg^{-1}$)	0.068 ± 0.006	0.082 ± 0.004	0.075 ± 0.006	0.062 ± 0.005*	NS	NS	$P < 0.05$
Na^+ ($mmol\,L^{-1}\,(24\,h)^{-1}\,kg^{-1}$)	4.28 ± 0.47	4.16 ± 0.46	4.08 ± 0.39	4.85 ± 0.67	NS	NS	NS
Cl^- ($mmol\,L^{-1}\,(24\,h)^{-1}\,kg^{-1}$)	5.52 ± 0.41	6.19 ± 0.68	6.32 ± 0.54	8.36 ± 1.42	NS	NS	NS
Total protein ($mg\,L^{-1}\,(24\,h)^{-1}\,kg^{-1}$)	10.19 ± 0.93	9.33 ± 1.87	16.84 ± 1.79	21.05 ± 5.52	NS	$P < 0.05$	NS
Creatinine clearance ($mL\,min^{-1}\,kg^{-1}$)	4.18 ± 0.42	3.73 ± 0.60	4.44 ± 0.29	4.85 ± 0.61	NS	NS	NS
Fractional sodium excretion (%)	0.534 ± 0.071	0.509 ± 0.030	0.485 ± 0.046	0.587 ± 0.119	NS	NS	NS

contributing to a larger ($P < 0.05$) ponderal index (Table 3). There were no differences at 9 months but at 12 months of age, F2 Restricted females, but not males, were again shorter ($P < 0.05$) with a greater ($P < 0.05$) ponderal index than Controls (Table 3). Head length was reduced ($P < 0.05$) in F2 Restricted compared with Control males and females at PN35 and 2 months, in males only at 3, 9 and 12 months, and in females only at 6 months (Table 3). Head width was reduced ($P < 0.05$) only at PN35 in F2 Restricted compared with Control males and there were no differences at any age in females (Table 3).

Blood pressure

Systolic blood pressure, measured by tail-cuff, was not different between F2 Control and Restricted male and female offspring at 2, 3 and 4 months of age (Fig. 3*a*, *b*). At 6 months, F2 Restricted males had elevated (+16 mmHg; $P < 0.05$) systolic blood pressure compared with Controls, which persisted ($P < 0.05$) at 9 months but was absent at 12 months (Fig. 3*a*). Mean arterial pressure was similarly elevated ($P < 0.05$) at 6 and 9 months of age in F2 Restricted males compared with Controls (6 months 140.2 ± 2.5 vs 123.2 ± 3.3 mmHg; 9 months 142.9 ± 3.1 vs 132.5 ± 3.2 mmHg) and not different at 12 months (133.7 ± 3.1 vs 134.6 ± 2.4 mmHg). This transient elevation in systolic and mean arterial blood pressure in F2 Restricted males was not evident in females (Fig. 3*b*; mean arterial pressure data not shown). In 12-month-old males only blood pressure was also measured using an indwelling tail-artery catheter. Systolic, diastolic and mean arterial blood pressure and heart rate were not different between F2 Control and Restricted animals (data not shown).

Food and water intake, urinary excretions and anaesthetised renal function

Male and female 12-month-old offspring consumed less ($P < 0.05$, −23% and −9%, respectively) food compared with 6-month-old counterparts, independent of maternal birthweight (Table 4). A tendency ($P = 0.06$) for interaction between age and group revealed that F2 Restricted males, but not females, consumed less (−29%) water than Controls at 12, but not 6, months of age (Table 4).

There were no differences in urinary flow rate, excretions of sodium, chloride and total protein, creatinine clearance and fractional sodium excretion between F2 Control and Restricted groups at 6 and 12 months of age (Table 4). Advanced age was associated with reduced ($P < 0.05$) urine flow rate in F2 Control and Restricted males, and F2 Restricted, but not Control, females (Table 4). In females, but not males, older age was associated with elevated ($P < 0.05$) urinary excretion of total protein regardless of maternal birthweight (Table 4). In 12-month-old males only, renal function was measured using anaesthetised renal clearance techniques. There were no differences in GFR, eRPF, filtration fraction, renal vascular resistance or fractional sodium excretion between F2 Control and Restricted animals (Fig. 4*a*–*e*).

Glomerular number and volume

Glomerular number was reduced ($P < 0.05$) in male (−22%) and female (−15%) F2 Restricted compared with Control fetuses at E20 (Fig. 5*a*, *b*). This deficit did not persist postnatally, after the expected completion of nephrogenesis, such that glomerular number and individual volume were comparable

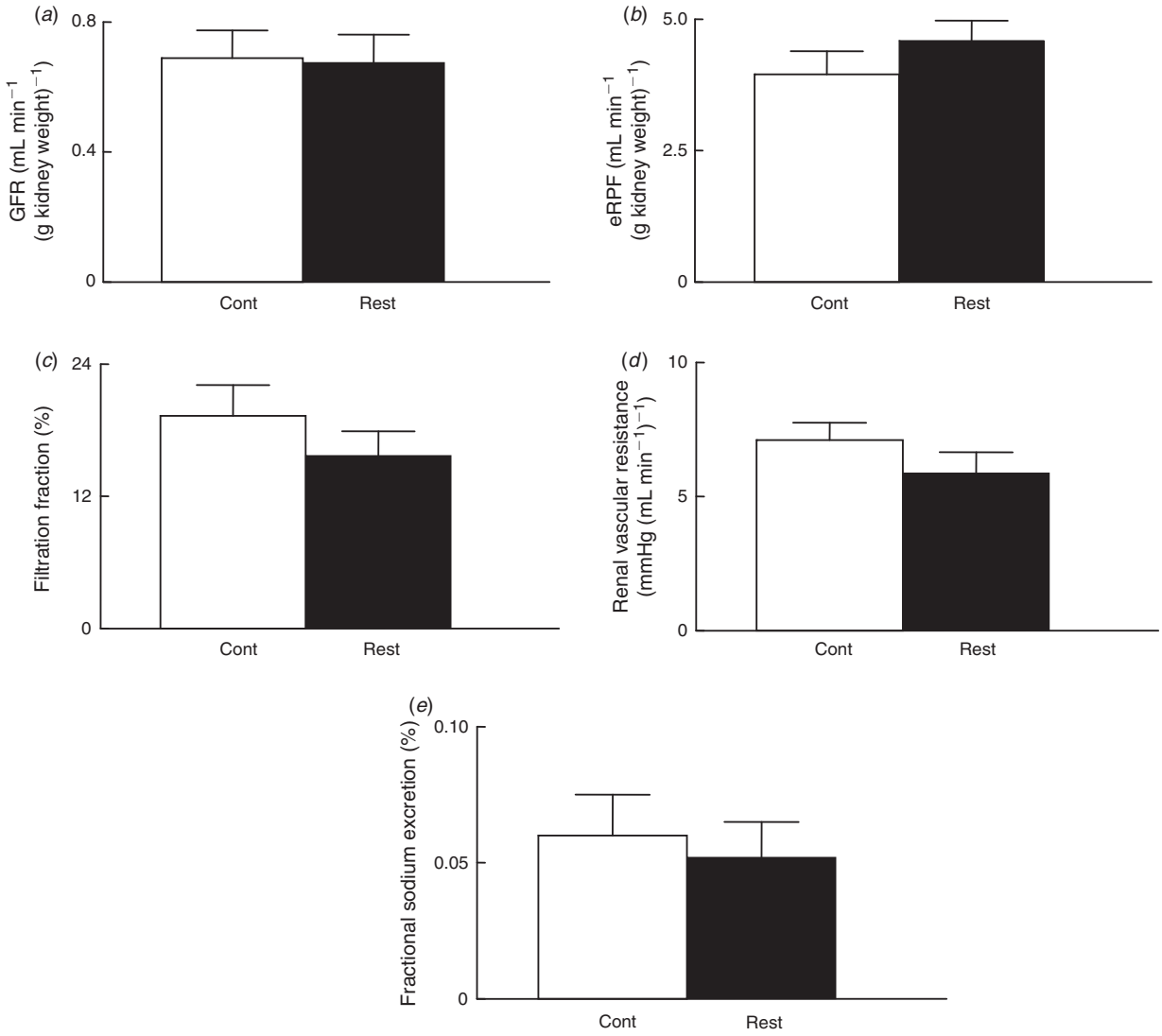

Fig. 4. Renal function. (*a*) GFR, (*b*) eRPF, (*c*) filtration fraction, (*d*) renal vascular resistance and (*e*) fractional sodium excretion at 12 months in F2 males. Values expressed as mean ± s.e.m., $n = 9$–12 per group.

between groups at PN35 (Fig. 5*c–f*). Individual corpuscle volume was also similar between groups at PN35 (Fig. 5*g*, *h*). Total glomerular and corpuscle volumes were not different between F2 Control and Restricted offspring at PN35 (data not shown).

Intra-renal and plasma RAS components

Intra-renal (cortical) renin activity was not different in males, but was elevated ($P < 0.05$) in F2 Restricted females compared with the Control at 6 months of age (Table 5). In F2 Restricted animals, intra-renal AngII content was reduced (−68%, $P < 0.05$) in males but elevated ($P < 0.05$) in females compared with Controls (Table 5). Intra-renal Ang1–7 content was not different between groups in males or females (Table 5). In males and females, there were no differences in plasma renin activity, AngII or Ang1–7 content between F2 Control and Restricted animals (Table 5).

Discussion

Prenatal insults have been linked, in animal models of maternal undernutrition, to deleterious transgenerational effects in the absence of a subsequent insult (Torrens *et al.* 2008; Harrison and Langley-Evans 2009; Ponzio *et al.* 2012). While relevant to developing countries, these models lack translatability to nutrient-rich populations. Therefore, for the first time, we investigated transgenerational growth and cardio–renal outcomes associated with utero–placental insufficiency; the major cause of low birthweight in the Western world. F2 male and female rat offspring from growth-restricted mothers were born of normal weights. However, they experienced slowed (PN14-2 months) followed by accelerated (2–3 months) postnatal growth rates compared with offspring from Control mothers. We report nephron deficits in male and female F2 Restricted fetuses at E20, which were restored to Control levels post-nephrogenesis at

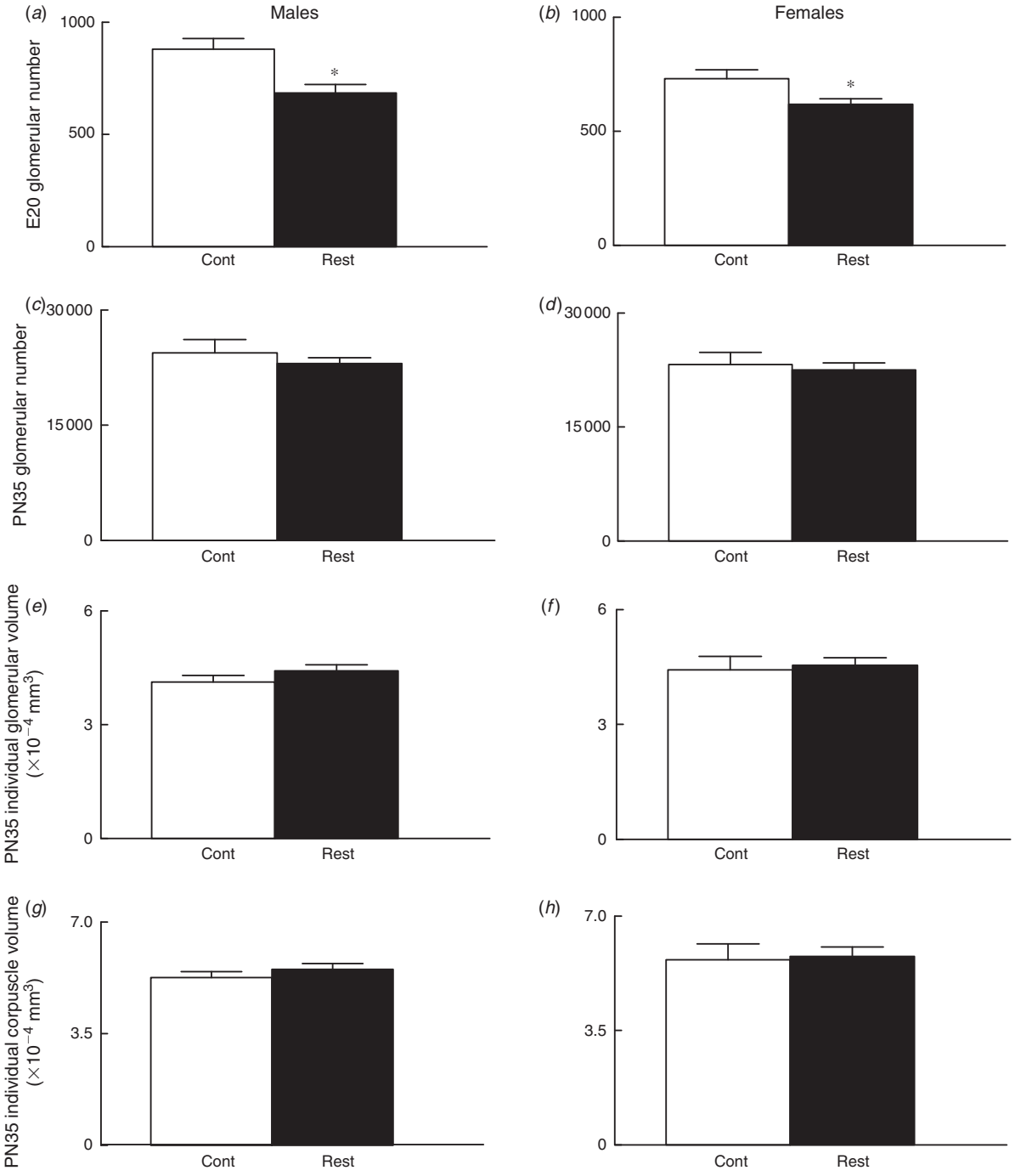

Fig. 5. Glomerular number at (*a*, *b*) E20 and (*c*, *d*) PN35, and individual (*e*, *f*) glomerular and (*g*, *h*) corpuscle volumes at PN35 in F2 (left panel) males and (right panel) females. Values expressed as mean ± s.e.m., $n = 6$ for E20; $n = 9$ for PN35 per group. * $P < 0.05$ F2 Restricted vs Control (Student's unpaired *t*-test).

PN35. As in F1 offspring, systolic blood pressure was elevated at 6 months of age in F2 Restricted males, which persisted at 9 months, and females remained unaffected. The hypertensive phenotype was not associated with increased intra-renal or systemic RAS pressor activity suggesting that other mechanisms are involved. Alterations to nephron development and transient elevations in adult blood pressure may predispose male offspring from growth-restricted mothers to chronic cardio–renal changes when challenged with adverse lifestyle factors.

In the present study, male and female F2 litter size and bodyweights, measured at PN1, were similar between offspring from normal and growth-restricted mothers, consistent with the

Table 5. Intra-renal and plasma RAS components

Intra-renal renin activity, AngII and Ang1–7 content and plasma renin activity, AngII and Ang1–7 content at 6 months in F2 males and females. Values expressed as mean ± s.e.m., $n = 7$–14 per group. *$P < 0.05$ F2 Restricted vs Control (Student's unpaired *t*-test)

Parameter	Control	Restricted
Males		
Intra-renal renin activity (ng mg^{-1} hr^{-1})	63.03 ± 3.20	61.66 ± 5.19
Intra-renal AngII content (ng g^{-1})	11.31 ± 0.96	3.62 ± 0.14*
Intra-renal Ang1–7 content (ng g^{-1})	42.52 ± 2.69	36.50 ± 4.69
Plasma renin activity (ng mL^{-1} hr^{-1})	9.54 ± 1.22	8.77 ± 0.95
Plasma AngII content (pg mL^{-1})	162 ± 11	123 ± 17
Plasma Ang1–7 content (pg mL^{-1})	82.69 ± 9.75	63.82 ± 4.63
Females		
Intra-renal renin activity (ng mg^{-1} hr^{-1})	79.80 ± 8.22	108 ± 8*
Intra-renal AngII content (ng g^{-1})	7.08 ± 0.15	8.36 ± 0.23*
Intra-renal Ang1–7 content (ng g^{-1})	46.27 ± 7.69	44.31 ± 7.66
Plasma renin activity (ng mL^{-1} hr^{-1})	10.37 ± 2.51	9.17 ± 0.99
Plasma AngII content (pg mL^{-1})	100 ± 6	101 ± 11
Plasma Ang1–7 content (pg mL^{-1})	47.77 ± 5.96	34.37 ± 4.20

normal fetal weights previously reported (Gallo *et al.* 2012*d*). It has become increasingly recognised that reduced birthweight may reflect an adaptive strategy that is independent of other phenotypic changes (Hanson and Gluckman 2005). Despite normal fetal and early postnatal bodyweights, male and female F2 offspring from growth-restricted mothers displayed slowed growth during the peri-pubertal period (PN14 to 2 months of age). Together with an accelerated growth phase from 2 to 3 months of age, these data suggest a delay in maturation in offspring born to growth-restricted mothers. Accelerated postnatal growth has been independently (of birthweight) associated with increased risk for later cardiovascular disease (Eriksson *et al.* 2000, 2001; Ben-Shlomo *et al.* 2008). While these early alterations to F2 Restricted male and female growth did not translate into a common cardio–renal phenotype, they may reflect postnatal periods that are vulnerable to challenges (adverse diets, lack of exercise or ageing). Bodyweights never surpassed that of Controls, albeit they exhibited shorter crown-to-rump lengths and thus greater ponderal indices in males at 6 months and in females at 6 and 12 months. Lifestyle challenges during periods of reduced leanness may place these F2 offspring from growth-restricted dams at greater risk of cardiovascular disease.

Males are generally considered more prone to the development of hypertension and renal insufficiency than age-matched, pre-menopausal females (Denton and Baylis 2007; Grigore *et al.* 2008; Mercuro *et al.* 2010). Growth-restricted F1 males, induced by utero–placental insufficiency, had elevated systolic blood pressure at 6 months of age (+9 mmHg; Wlodek *et al.* 2008), while females remained normotensive despite similar reductions in nephron number and individual glomerular hypertrophy (Moritz *et al.* 2009). The present study extends these findings to reflect a similar sexual dimorphism in the next generation. F2 males, but not females, from growth-restricted mothers were hypertensive at 6 and 9 months of age. The exacerbated increase in systolic blood pressure in F2 (+16 mmHg) compared with directly exposed F1 (+9 mmHg) Restricted males at 6 months of age was surprising, given previous reports of similar (Anderson *et al.* 2006; Harrison and Langley-Evans 2009) or 'diluted' transgenerational effects (Brawley *et al.* 2003; Torrens *et al.* 2008; Ponzio *et al.* 2012). Differences in the magnitude of change between generations, and between studies, suggest differences in the signals received by the fetus and interactions with the maternal environment. There appears to be no consistency in the programmed phenotype, challenging the prediction of disease. In the present study, F2 Restricted rats exhibited normal urinary excretions and creatinine clearance, as previously demonstrated in F1 Restricted females (Gallo *et al.* 2012*a*, 2012*c*, 2012*d*). However, F2 Restricted males consumed 29% less water than Controls at 12 months of age, which was somewhat accounted for by the non-significant reduction in urinary output (−17%). The small discrepancy that remained between fluid intake and output may be due, in part, to increased osmotic threshold for water reabsorption. The mechanisms underlying altered drinking behaviour in 12-month-old F2 Restricted males, as well as their physiological relevance, warrant further investigation. It is also worth noting that, relative to bodyweight, females consumed more food than male counterparts, which may be attributed to greater energy expenditure (Lim *et al.* 2011).

RAS inhibition prevents the development of hypertension in adult F1 growth-restricted offspring from prenatally protein-restricted (Vehaskari *et al.* 2004) and RUPP-exposed (Grigore *et al.* 2007) animals. In 6-month-old F2 Restricted rats, we report decreased intra-renal AngII content in hypertensive males and elevated intra-renal renin activity and AngII content in normotensive females. Plasma RAS components were unaltered. In rats prenatally exposed to low protein, others demonstrated enhanced renal angiotensin type 1 (AT1) receptor mRNA and protein expression, in the absence of changes to AngII content (Vehaskari *et al.* 2004). While increased AngII sensitivity may have mediated reduced renal AngII content in F2 Restricted males, previous work in our model reported unaltered expression of renal AT1 receptor mRNA in F1 hypertensive males (Wlodek *et al.* 2008). Certainly, a role for the RAS in the pathogenesis of transgenerational outcomes may be identified in younger, pre-hypertensive animals. Alternatively, decreased renal content of AngII may reflect compensation for elevations in blood pressure that were manifested independently of alterations to the RAS. This may implicate alterations to the hypothalamic-pituitary axis (HPA) axis or vascular function.

Although debatable, a congenital defect in nephron number, as evident in those born small, is often associated with elevations in systemic blood pressure (Brenner *et al.* 1988; Vehaskari *et al.* 2004; Wlodek *et al.* 2008). At E20, both male and female fetuses from Restricted mothers exhibited a 15–22% reduction in nephron number. This nephron deficit did not persist at PN35 (after completion of nephrogenesis), suggesting a developmental delay. An extended period of nephrogenesis in F2 offspring from growth-restricted mothers may represent a critical, early postnatal period where secondary insults have the potential to permanently affect nephron complement. Others have provided evidence for developmentally immature, poorly vascularised

and shrunken glomeruli tufts in preterm baboons with normal nephron endowment (Gubhaju *et al.* 2009). In the present study, however, glomerular and Bowman's size (corpuscle volume) were unaffected in F2 Restricted kidneys at PN35. In rat offspring exposed to prenatal low protein, increased glomerular apoptosis has been demonstrated at 8 weeks of age (Vehaskari *et al.* 2001). Although we cannot discount the possibility of a programmed age-dependent rise in glomerular damage and apoptosis in the present study, this is unlikely given that renal function was unaffected at 6 and 12 months of age. Finally, the lack of postnatal nephron deficit (despite hypertension) differs from previous findings using prenatal protein restriction in rats (Harrison and Langley-Evans 2009). Taken together, these data support the programming of each feature as a distinct, parallel process, such that their individual existence depends on the specific prenatal and postnatal challenges that face the offspring.

At 12 months of age, increased systolic blood pressure observed 3–6 months earlier in F2 Restricted males was attenuated. This was unpredicted given the exacerbated (compared with Controls), age-related hypertension in F1 Restricted males between 5 weeks and 6 months of age (Wlodek *et al.* 2008). Although animals in the present study were considered to be habituated to the restraint tail-cuff procedure, we cannot discount the likelihood of a heightened stress response (Armitage *et al.* 2004) that was, following one year of regular recordings, attenuated at 12 months of age. Noteworthy is that the average group variations of recorded traces within each animal were not different between groups, arguing against a differential stress response. Indeed, radio-telemetry procedures may help to identify whether elevated systolic blood pressure at 6 and 9 months in F2 Restricted males was a basal phenotype. While elevations in systolic blood pressure may contribute to end-organ damage, renal function at 12 months of age was unaffected, suggesting maintenance of glomerular integrity. A protein load to test the renal reserve and other lifestyle challenges may unmask chronic alterations to cardio–renal function in these transiently hypertensive male offspring.

Transgenerational programming of hypertension has been reported in both sexes, via a maternal or combined parental contribution (Anderson *et al.* 2006; Torrens *et al.* 2008; Harrison and Langley-Evans 2009). Inconsistencies in the development of hypertension in F2 females between studies may be attributed to differences in the F1 adaptation to pregnancy. Torrens *et al.* (2003) demonstrated resistance artery dysfunction during pregnancy of the prenatally protein-deprived F1 female and Anderson *et al.* (2006) reported persistence of maternal hypertension in females prenatally exposed to RUPP; both models were associated with hypertension in F2 females (Anderson *et al.* 2006; Torrens *et al.* 2008). In contrast, females born small in the present study exhibited intact vascular adaptations (Mazzuca *et al.* 2012) and normal blood pressure during late pregnancy, albeit some loss of glucose tolerance (Gallo *et al.* 2012*d*). Normal cardiovascular adaptations by the pregnant F1 female, at least during late gestation, may protect against the development of hypertension in female, but not male, F2 progeny in our study; mechanisms of transgenerational effects may differ depending on offspring sex. Certainly, the initial F0 insult in the above-mentioned (Anderson *et al.* 2006; Torrens *et al.* 2008) and present studies may have impacted on developing oocytes, resulting in transgenerational outcomes that are independent of the F1 pregnancy environment (Gallo *et al.* 2012*b*). In order to disrupt the perpetuating cycle of programmed effects, embryo-transfer procedures may help to delineate between adverse maternal influences and embryo-specific effects.

In the present study, utero–placental insufficiency caused a greater loss in the number of male compared with female pups recorded at PN1. Subsequently, F1 females that were born small (Restricted) delivered fewer F2 males than females. Importantly, Control dams from both generations had unaltered sex ratios. Others have reported that exposure to acute hypoxic stress in ewes resulted in a 2-fold greater concentration of plasma cortisol in male compared with female fetuses (Giussani *et al.* 2011). Taken together with our findings, this suggests that males are more sensitive to adverse *in utero* exposure. It is worth noting, however, that in our model of utero–placental insufficiency, the degree of F1 growth restriction and renal morphological deficit, i.e. nephron number, were generally similar between the sexes (Wlodek *et al.* 2007, 2008; Moritz *et al.* 2009). In the present study, Restricted females used to generate the F2 were in fact more growth restricted (−21%) than their male siblings at PN1 (−15%). Although male survival is reduced in response to adverse stimuli, these data suggest that surviving pups are not at any greater disadvantage, at least in early life, compared with their female counterparts. Importantly, this does not rule out the differential effects of adult sex steroids, which may explain the greater propensity for cardiovascular diseases in men.

This study demonstrates, for the first time in a rat model of utero–placental insufficiency, alterations to F2 kidney development and transient hypertension in male adult offspring. Lifestyle challenges during periods of altered postnatal growth rates and elevated blood pressure may predispose offspring of growth-restricted mothers to chronic disease outcomes. Defining the role of the maternal environment compared with embryo-specific effects in mediating transgenerational outcomes may help to minimise the contribution of prenatal insults to adult cardio–renal diseases. Finally, F2 growth and cardio–renal alterations were not wholly consistent with changes observed in directly exposed, F1 offspring (Wlodek *et al.* 2008; Moritz *et al.* 2009; Gallo *et al.* 2012*a*, 2012*c*, 2012*d*). This indicates that adverse signals received by the fetus are established *de novo* in each generation, challenging the forecasting of future disease.

Acknowledgements

The authors acknowledge the technical assistance of Kerryn T Westcott. This work was funded by the National Health and Medical Research Council of Australia (grant #400004), Heart Foundation of Australia (G 08M 3698) and the March of Dimes Birth Defects Foundation, USA (grant #6-FY08–269). L. A. G. was supported by a Heart Foundation Biomedical Scholarship (Australia).

References

Anderson, C. M., Lopez, F., Zimmer, A., and Benoit, J. N. (2006). Placental insufficiency leads to developmental hypertension and mesenteric artery dysfunction in two generations of Sprague–Dawley rat offspring. *Biol. Reprod.* **74**, 538–544. doi:10.1095/BIOLREPROD.105.045807

Armitage, J. A., Khan, I. Y., Taylor, P. D., Nathanielsz, P. W., and Poston, L. (2004). Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *J. Physiol.* **561**, 355–377. doi:10.1113/JPHYSIOL.2004.072009

Barker, D. J. P. (1995). Fetal origins of coronary heart disease. *BMJ* **311**, 171–174. doi:10.1136/BMJ.311.6998.171

Barker, D. J., Winter, P. D., Osmond, C., Margetts, B., and Simmonds, S. J. (1989). Weight in infancy and death from ischaemic heart disease. *Lancet* **334**, 577–580. doi:10.1016/S0140-6736(89)90710-1

Ben-Shlomo, Y., McCarthy, A., Hughes, R., Tilling, K., Davies, D., and Smith, G. D. (2008). Immediate postnatal growth is associated with blood pressure in young adulthood: the Barry Caerphilly Growth Study. *Hypertension* **52**, 638–644. doi:10.1161/HYPERTENSIONAHA.108.114256

Bertram, J. F. (1995). Analysing renal glomeruli with the new stereology. *Int. Rev. Cytol.* **161**, 111–172. doi:10.1016/S0074-7696(08)62497-3

Brawley, L., Itoh, S., Torrens, C., Barker, A., Bertram, C., Poston, L., and Hanson, M. (2003). Dietary protein restriction in pregnancy induces hypertension and vascular defects in rat male offspring. *Pediatr. Res.* **54**, 83–90. doi:10.1203/01.PDR.0000065731.00639.02

Brenner, B. M., Garcia, D. L., and Anderson, S. (1988). Glomeruli and blood pressure. Less of one, more the other? *Am. J. Hypertens.* **1**, 335–347. doi:10.1093/AJH/1.4.335

Cullen-McEwen, L. A., Armitage, J. A., Nyengaard, J. R., and Moritz, K. M. (2011). A design-based method for estimating glomerular number in the developing kidney. *Am. J. Physiol. Renal Physiol.* **300**, F1448–F1453.

Cullen-McEwen, L. A., Armitage, J. A., Nyengaard, J. R., and Bertram, J. F. (2012). Estimating nephron number in the developing kidney using the physical dissector/fractionator combination. *Methods Mol. Biol.* **886**, 109–119. doi:10.1007/978-1-61779-851-1_10

Denton, K., and Baylis, C. (2007). Physiological and molecular mechanisms governing sexual dimorphism of kidney, cardiac and vascular function. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**, R697–R699.

Eriksson, J., Forsen, T., Tuomilehto, J., Osmond, C., and Barker, D. (2000). Fetal and childhood growth and hypertension in adult life. *Hypertension* **36**, 790–794. doi:10.1161/01.HYP.36.5.790

Eriksson, J. G., Forsén, T., Tuomilehto, J., Osmond, C., and Barker, D. J. P. (2001). Early growth and coronary heart disease in later life: longitudinal study. *BMJ* **322**, 949. doi:10.1136/BMJ.322.7292.949

Gallo, L. A., Denton, K. M., Moritz, K. M., Tare, M., Parkington, H. C., Davies, M., Tran, M., Jefferies, A. J., and Wlodek, M. E. (2012*a*). Long-term alteration in maternal blood pressure and renal function after pregnancy in normal and growth-restricted rats. *Hypertension* **60**, 206–213. doi:10.1161/HYPERTENSIONAHA.112.195578

Gallo, L. A., Tran, M., Master, J. S., Mortiz, K. M., and Wlodek, M. E. (2012*b*). Maternal adaptations and inheritance in the transgenerational programming of adult disease. *Cell Tissue Res.* **349**, 863–880. doi:10.1007/S00441-012-1411-Y

Gallo, L. A., Tran, M., Moritz, K. M., Jefferies, A. J., and Wlodek, M. E. (2012*c*). Pregnancy in aged rats that were born small: cardio–renal and metabolic adaptations and second-generation fetal growth. *FASEB J.* **26**, 4337–4347. doi:10.1096/FJ.12-210401

Gallo, L. A., Tran, M., Moritz, K. M., Mazzuca, M. Q., Parry, L. J., Westcott, K. T., Jefferies, A. J., Cullen-McEwen, L. A., and Wlodek, M. E. (2012*d*). Cardio–renal and metabolic adaptations during pregnancy in female rats born small: implications for maternal health and second-generation fetal growth. *J. Physiol.* **590**, 617–630.

Giussani, D. A., Fletcher, A. J., and Gardner, D. S. (2011). Sex differences in the ovine fetal cortisol response to stress. *Pediatr. Res.* **69**, 118–122. doi:10.1203/PDR.0B013E3182042A20

Grigore, D., Ojeda, N. B., Robertson, E. B., Dawson, A. S., Huffman, C. A., Bourassa, E. A., Speth, R. C., Brosnihan, K. B., and Alexander, B. T. (2007). Placental insufficiency results in temporal alterations in the renin–angiotensin system in male hypertensive growth restricted offspring. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**, R804–R811.

Grigore, D., Ojeda, N. B., and Alexander, B. T. (2008). Sex differences in the fetal programming of hypertension. *Gend. Med.* **5**, S121–S132. doi:10.1016/J.GENM.2008.03.012

Gubhaju, L., Sutherland, M. R., Yoder, B. A., Zulli, A., Bertram, J. F., and Black, M. J. (2009). Is nephrogenesis affected by preterm birth? Studies in a non-human primate model. *Am. J. Physiol. Renal Physiol.* **297**, F1668–F1677.

Hanson, M., and Gluckman, P. (2005). Endothelial dysfunction and cardiovascular disease: the role of predictive adaptive responses. *Heart* **91**, 864–866. doi:10.1136/HRT.2004.047381

Harrison, M., and Langley-Evans, S. C. (2009). Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy. *Br. J. Nutr.* **101**, 1020–1030. doi:10.1017/S0007114508057607

Henriksen, T., and Clausen, T. (2002). The fetal origins hypothesis: placental insufficiency and inheritance versus maternal malnutrition in well-nourished populations. *Acta Obstet. Gynecol. Scand.* **81**, 112–114. doi:10.1034/J.1600-0412.2002.810204.X

Hoy, W. E., Rees, M., Kile, E., Mathews, J. D., and Wang, Z. (1999). A new dimension to the Barker hypothesis: low birthweight and susceptibility to renal disease. *Kidney Int.* **56**, 1072–1077. doi:10.1046/J.1523-1755.1999.00633.X

Hoy, W. E., Hughson, M. D., Bertram, J. F., Denton, D. R., and Amann, K. (2005). Nephron number, hypertension, renal disease and renal failure. *J. Am. Soc. Nephrol.* **16**, 2557–2564. doi:10.1681/ASN.2005020172

Lim, K., Armitage, J. A., Stefanidis, A., Oldfield, B. J., and Black, M. J. (2011). IUGR in the absence of postnatal “catch-up” growth leads to improved whole body insulin sensitivity in rat offspring. *Pediatr. Res.* **70**, 339–344. doi:10.1203/PDR.0B013E31822A65A3

Mazzuca, M. Q., Tare, M., Parkington, H. C., Dragomir, N. M., Parry, L. J., and Wlodek, M. E. (2012). Utero–placental insufficiency programmes vascular dysfunction in non-pregnant rats: compensatory adaptations in pregnancy. *J. Physiol.* **590**, 3375–3388. doi:10.1113/JPHYSIOL.2012.230011

Mercuro, G., Deidda, M., Piras, A., Dessalvi, C. C., Maffei, S., and Rosano, G. M. (2010). Gender determinants of cardiovascular risk factors and diseases. *J. Cardiovasc. Med.* **11**, 207–220. doi:10.2459/JCM.0B013E32833178ED

Moritz, K. M., Mazzuca, M. Q., Siebel, A. L., Mibus, A., Arena, D., Tare, M., Owens, J. A., and Wlodek, M. E. (2009). Utero–placental insufficiency causes a nephron deficit, modest renal insufficiency but no hypertension with ageing in female rats. *J. Physiol.* **587**, 2635–2646. doi:10.1113/JPHYSIOL.2009.170407

O’Dowd, R., Kent, J. C., Moseley, J. M., and Wlodek, M. E. (2008). Effects of utero–placental insufficiency and reducing litter size on maternal mammary function and postnatal offspring growth. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R539–R548.

Ojeda, N. B., Grigore, D., Robertson, E. B., and Alexander, B. T. (2007*a*). Oestrogen protects against increased blood pressure in postpubertal female growth-restricted offspring. *Hypertension* **50**, 679–685. doi:10.1161/HYPERTENSIONAHA.107.091785

Ojeda, N. B., Grigore, D., Yanes, L. L., Iliescu, R., Robertson, E. B., Zhang, H., and Alexander, B. T. (2007*b*). Testosterone contributes to marked elevations in mean arterial pressure in adult male intrauterine growth-restricted offspring. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**, R758–R763.

Painter, R. C., Osmond, C., Gluckman, P., Hanson, M., Phillips, D. I., and Roseboom, T. J. (2008). Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. *BJOG* **115**, 1243–1249. doi:10.1111/J.1471-0528.2008.01822.X

Ponzio, B. F., Carvalho, M. H., Fortes, Z. B., and do Carmo, F. M. (2012). Implications of maternal nutrient restriction in transgenerational programming of hypertension and endothelial dysfunction across F1–F3 offspring. *Life Sci.* **90**, 571–577. doi:10.1016/J.LFS.2012.01.017

Roseboom, T. J., and Watson, E. D. (2012). The next generation of disease risk: are the effects of prenatal nutrition transmitted across generations? Evidence from animal and human studies. *Placenta* **33**(Suppl 2), e40–e44. doi:10.1016/J.PLACENTA.2012.07.018

Schreuder, M. F., Van Wijk, J. A., Fodor, M., and Delemarre-van de Waal, H. A. (2007). Influence of intrauterine growth restriction on renal function in the adult rat. *J. Physiol. Biochem.* **63**, 213–219. doi:10.1007/BF03165784

Simmons, R. A., Templeton, L. J., and Gertz, S. J. (2001). Intrauterine growth retardation leads to the development of Type 2 diabetes in the rat. *Diabetes* **50**, 2279–2286. doi:10.2337/DIABETES.50.10.2279

Singh, R. R., Moritz, K. M., Wintour, E. M., Jefferies, A. J., Iqbal, J., Bertram, J. F., and Denton, K. M. (2011). Fetal uninephrectomy in male sheep alters the systemic and renal responses to angiotensin II infusion and AT1R blockade. *Am. J. Physiol. Renal Physiol.* **301**, F319–F326.

Torrens, C., Brawley, L., Barker, A. C., Itoh, S., Poston, L., and Hanson, M. A. (2003). Maternal protein restriction in the rat impairs resistance artery but not conduit artery function in pregnant offspring. *J. Physiol.* **547**, 77–84. doi:10.1113/JPHYSIOL.2002.026120

Torrens, C., Poston, L., and Hanson, M. A. (2008). Transmission of raised blood pressure and endothelial dysfunction to the F2 generation induced by maternal protein restriction in the F0, in the absence of dietary challenge in the F1 generation. *Br. J. Nutr.* **100**, 760–766. doi:10.1017/S0007114508921747

Vehaskari, V. M. (2010). Prenatal programming of kidney disease. *Curr. Opin. Pediatr.* **22**, 176–182. doi:10.1097/MOP.0B013E328336EBC9

Vehaskari, V. M., Aviles, D. H., and Manning, J. (2001). Prenatal programming of adult hypertension in the rat. *Kidney Int.* **59**, 238–245. doi:10.1046/J.1523-1755.2001.00484.X

Vehaskari, V. M., Stewart, T., Lafont, D., Soyez, C., Seth, D., and Manning, J. (2004). Kidney angiotensin and angiotensin receptor expression in prenatally programmed hypertension. *Am. J. Physiol. Renal Physiol.* **287**, F262–F267.

Vikse, B. E., Irgens, L. M., Leivestad, T., Hallan, S., and Iversen, B. M. (2008). Low birthweight increases risk for end-stage renal disease. *J. Am. Soc. Nephrol.* **19**, 151–157. doi:10.1681/ASN.2007020252

Wadley, G. D., Siebel, A. L., Cooney, G. J., McConell, G. K., Wlodek, M. E., and Owens, J. A. (2008). Utero–placental insufficiency and reducing litter size alters skeletal muscle mitochondrial biogenesis in a sex-specific manner in the adult rat. *Am. J. Physiol. Endocrinol. Metab.* **294**, E861–E869.

Wlodek, M. E., Mibus, A., Tan, A., Siebel, A. L., Owens, J. A., and Moritz, K. M. (2007). Normal lactational environment restores nephron endowment and prevents hypertension after placental restriction in the rat. *J. Am. Soc. Nephrol.* **18**, 1688–1696. doi:10.1681/ASN.2007010015

Wlodek, M. E., Westcott, K., Siebel, A. L., Owens, J. A., and Moritz, K. M. (2008). Growth restriction before or after birth reduces nephron number and increases blood pressure in male rats. *Kidney Int.* **74**, 187–195. doi:10.1038/KI.2008.153

Zimanyi, M. A., Denton, K. M., Forbes, J. M., Thallas-Bonke, V., Thomas, M. C., Poon, F., and Black, M. J. (2006). A developmental nephron deficit in rats is associated with increased susceptibility to a secondary renal injury due to advanced glycation end-products. *Diabetologia* **49**, 801–810. doi:10.1007/S00125-006-0175-X