

Cardio-renal and metabolic adaptations during pregnancy in female rats born small: implications for maternal health and second generation fetal growth

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Non-technical summary Low weight at birth, or being born small for gestational age, is associated with increased risk of a number of adult diseases, including cardiovascular and kidney disease and diabetes. Generally, low birth weight males have a greater risk of developing such diseases but females do present with subtle changes in organ structure and function that might render them susceptible to lifestyle challenges. We show, for the first time, that low birth weight females have largely normal cardiovascular and kidney adaptations to pregnancy but they do develop altered glucose control. We have shown that their own fetuses are growth restricted suggesting that low birth weight and risk of disease development can be passed on to subsequent generations. These results warrant close monitoring of pregnant women who were born small and shape future studies to focus on therapeutic strategies to minimize the transmission of low birth weight and adult disease risk.

Abstract Intrauterine growth restriction caused by uteroplacental insufficiency increases risk of cardiovascular and metabolic disease in offspring. Cardio-renal and metabolic responses to pregnancy are critical determinants of immediate and long-term maternal health. However, no studies to date have investigated the renal and metabolic adaptations in growth restricted offspring when they in turn become pregnant. We hypothesised that the physiological challenge of pregnancy in growth restricted females exacerbates disease outcome and compromises next generation fetal growth. Uteroplacental insufficiency was induced by bilateral uterine vessel ligation (Restricted) or sham surgery (Control) on day 18 of gestation in WKY rats and F1 female offspring birth and postnatal body weights were recorded. F1 Control and Restricted females were mated at 4 months and blood pressure, renal and metabolic parameters were measured in late pregnancy and F2 fetal and placental weights recorded. Age-matched non-pregnant Control and Restricted F1 females were also studied. F1 Restricted females were born 10–15% lighter than Controls. Basal insulin secretion and pancreatic β -cell mass were reduced in non-pregnant Restricted females but restored in pregnancy. Pregnant Restricted females, however, showed impaired glucose tolerance and compensatory glomerular hypertrophy, with a nephron deficit but normal renal function and blood pressure. F2 fetuses from Restricted mothers exposed to physiological measures during pregnancy were lighter than Controls highlighting additive adverse effects when mothers born small experience stress during pregnancy. Female rats born small exhibit mostly normal cardio-renal adaptations but altered glucose control during late pregnancy making them vulnerable to lifestyle challenges.

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Abbreviations AUGC, area under glucose curve; AUCI, area under insulin curve; IPGTT, intraperitoneal glucose tolerance test.

Introduction

The developmental origins of adult health and disease hypothesis proposes that perturbations during critical periods of intrauterine and early postnatal life can programme the developing fetus for later cardiovascular and metabolic diseases in adulthood (Barker, 1995; McMillen & Robinson, 2005). Suboptimal conditions *in utero* alter the development of key fetal organ systems, including reductions in nephron number and pancreatic β -cell mass that may be contributing factors to the common adult phenotypes described (Hoy *et al.* 1999; Simmons *et al.* 2001; Wlodek *et al.* 2007, 2008). Furthermore, early postnatal growth independently predicts adult disease risk, such that catch-up growth in early childhood often provides long-lasting benefits, in contrast to the detrimental effects of a late accelerated growth (Eriksson *et al.* 2001).

Low birth weight, a surrogate marker of intrauterine growth restriction, affects ~8% of pregnancies in the Western world (Martin *et al.* 2007). Uteroplacental insufficiency, rather than maternal malnutrition, is the most common cause (Henriksen & Clausen, 2002), and is characterised by poor placental vascularisation leading to compromised delivery of nutrients and oxygen to the fetus (Wu *et al.* 2006). It becomes most apparent during third trimester when fetal demands are at their greatest and up to 70% of cases occur in the absence of maternal hypertension (Berghella, 2007). We and others have utilised a rat model that mimics this condition, whereby uterine vessels are bilaterally ligated during late gestation resulting in offspring that are born 10–15% lighter than those exposed to sham surgery (Simmons *et al.* 2001; Schreuder *et al.* 2007; Wlodek *et al.* 2007, 2008). Our model is in contrast to the reduced uterine perfusion pressure (RUPP) model of preeclampsia, whereby pregnant dams develop hypertension due to additional clipping of the abdominal aorta (Crews *et al.* 2000; Anderson *et al.* 2006; Gilbert *et al.* 2007). Furthermore, while maternal undernutrition models serve to mimic conditions of the developing world (Zambrano *et al.* 2005; Torrens *et al.* 2008; Harrison & Langley-Evans, 2009), disease outcomes and mechanistic pathways may not be relevant when nutrition is abundant. In our model, uteroplacental insufficiency causes a sexually dimorphic phenotype with males generally having more severe cardiovascular and

metabolic outcomes compared with their female counterparts. Growth restricted male offspring are hypertensive with nephron deficits and glomerular hypertrophy and have impaired metabolic control (glucose intolerance) at 6 months of age (Wlodek *et al.* 2007, 2008; Siebel *et al.* 2008; Wadley *et al.* 2008). Despite a similar reduction in nephron number, our growth restricted females do not become hypertensive (Moritz *et al.* 2009a) and exhibit normal fasting plasma glucose and insulin, and normal glucose tolerance and insulin secretion in response to an intra-arterial glucose tolerance test (Siebel *et al.* 2008; Wadley *et al.* 2008). However, these females have uterine artery-specific endothelial vasodilator dysfunction and increased wall stiffness (Mazucca *et al.* 2010) that may, in turn, compromise their own pregnancy adaptations and the intrauterine environment of the next generation. In addition, female offspring exposed to placental ischaemia in the RUPP model have enhanced vasoconstrictor responsiveness and altered endothelium-dependent and -independent relaxation in mesenteric arteries (Anderson *et al.* 2006).

Pregnancy invokes profound cardiovascular, renal and metabolic adaptations essential to support growth and development of the fetus. By late pregnancy, maternal blood volume expands by up to 50% in humans and 30% in rats, with similar increases in cardiac output (Torgersen & Curran, 2006; Hill & Pickinpaugh, 2008). The hypervolaemic state is secondary to reductions in peripheral vascular tone and together these factors allow for increased uteroplacental blood flow while maintaining maternal blood pressure (Poston *et al.* 1995; Thornburg *et al.* 2000; Torgersen & Curran, 2006). Dilatation of the renal vasculature permits greater blood flow to the maternal kidneys, with glomerular filtration rate reaching peak levels at mid gestation. In turn, increased solute filtration reduces plasma osmolality and viscosity, considered to aid uteroplacental perfusion.

Of interest to the current study, small birth weight women, compared with those born of normal weight, are more likely to develop hypertension during late pregnancy (Klebanoff *et al.* 1999). Indeed, this may be attributed to alterations in maternal vascular remodelling and/or deficits in nephron endowment. Since hypertensive disorders of pregnancy may not allow for normal expansion in maternal blood volume (Torgersen & Curran, 2006), and blood volume increases are linearly correlated

with fetal weight (Duvekot & Peeters, 1994), maternal hypertension may mediate intergenerational transmission of fetal growth restriction. Studies have also shown a strong inverse relationship between a woman's own birth weight and her subsequent risk for gestational diabetes, with underlying mechanisms yet to be elucidated (Seghieri *et al.* 2002).

Given the paucity of information regarding adaptations to pregnancy in small birth weight females, we studied 4-month-old growth restricted rat offspring when they, in turn, become pregnant in the absence of any further uteroplacental insufficiency challenge. We hypothesized that the physiological challenge of pregnancy in growth restricted female rats with uterine artery dysfunction and reduced nephron endowment would unveil adverse cardiovascular, renal and metabolic phenotypes, with adverse consequences for fetal growth in the next generation. Given the potential stress associated with various physiological measurements (Hoppe *et al.* 2009), we also assessed fetal body and placental weights in a cohort of pregnancies where maternal physiological measurements were not made.

Methods

Animal procedures

All experiments were approved by The University of Melbourne Animal Ethics Committee. The authors have read, and the experiments comply with the policies and regulations of *The Journal of Physiology* given by (Drummond, 2009). Wistar-Kyoto rats (9–13 weeks of age) were housed in an environmentally controlled room (22°C) with a 12 h light–dark cycle and access to food and tap water *ad libitum*. Rats were mated and surgery performed on day 18 (Wlodek *et al.* 2007; Siebel *et al.* 2008; Wadley *et al.* 2008; Moritz *et al.* 2009a; Mazzuca *et al.* 2010; Laker *et al.* 2011). Briefly, F0 pregnant rats were randomly allocated to a sham (offspring termed Control) or uteroplacental insufficiency (offspring termed Restricted) group. Restricted group underwent bilateral uterine vessel (artery and vein) ligation surgery. The F0 females delivered naturally at term on day 22 of gestation and birth weights of F1 female offspring (Control and Restricted) were recorded. Uteroplacental insufficiency reduced total (male and female) litter size (5–6 Restricted pups *vs.* 8–11 Control pups) 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 post-natal 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. At 12 weeks of age Control and Restricted females were allocated to either non-pregnant or pregnant groups

(1/litter/group; $n = 12$ /group). Those allocated to the pregnant group were mated (12–19 weeks) with a normal male. Physiological measures (tail cuff blood pressure, intraperitoneal glucose tolerance test (IPGTT) and 24 h metabolic cage) were performed during late gestation (termed pregnant 1) and in age-matched non-pregnant groups. At post-mortem, on embryonic day 20 (E20), F2 fetal and placental weights were recorded. Given the potential stress associated with physiological measures during late gestation, we generated a second cohort of pregnant females (termed pregnant 2), to determine the impact of these measurements on fetal and placental weights; the pregnant rats were not handled (except for animal husbandry purposes) nor were any physiological measures performed.

Body weight, blood pressure and intraperitoneal glucose tolerance test

F1 body weights from Control and Restricted females were measured at postnatal days 1, 6, 14 and 35, and at mating and E20 (post-mortem) in pregnant and at age-matched post-mortem in non-pregnant groups. Systolic blood pressure was measured in the morning by tail-cuff at E18 in pregnant and in age-matched non-pregnant groups that were acclimatized to the restraint procedure (Wlodek *et al.* 2007; Moritz *et al.* 2009a), followed by an IPGTT (Siebel *et al.* 2008; Wadley *et al.* 2008; Laker *et al.* 2011). A non-fasted (fed) IPGTT was performed in pregnant groups to prevent any fetal compromise associated with fasting. To match this, a fed IPGTT was carried out in age-matched non-pregnant females. Blood samples were collected prior to and following an intraperitoneal bolus injection of glucose (Pharmalab, Lane Cove, NSW, Australia; $1 \text{ g (kg body weight)}^{-1}$) and plasma stored at -20°C .

Plasma insulin concentrations were measured in duplicate using a rat insulin radioimmunoassay kit (Millipore, Abacus ALS, Brisbane, Queensland, Australia) (Siebel *et al.* 2008; Wadley *et al.* 2008; Laker *et al.* 2011). Plasma glucose concentrations were measured in triplicate using a scaled-down version of the enzymatic fluorometric analysis (Siebel *et al.* 2008; Wadley *et al.* 2008; Laker *et al.* 2011). Non-fasted basal plasma glucose and insulin were taken as the average of two time points (10 and 5 min before glucose injection). Area under the glucose curve (AUGC) and area under the insulin curve (AUGC) were calculated as the total area-under-curve from basal to 90 min using the trapezoidal model (Matthews *et al.* 1990).

Food and water intake and renal function

At E19, pregnant and age-matched non-pregnant animals were weighed and placed individually in metabolic cages

for 24 h measurements of food and water intake and urine production (Moritz *et al.* 2009a). Rats were acclimatized to the metabolic cages by placing them in for short daylight periods on two separate occasions. Measurements of sodium, creatinine, glucose, uric acid, micro-total protein (Beckman Synchron CX 5, Beckman Coulter Inc., Brea, CA, USA), potassium, chloride (Rapidchem 744, Bayer HealthCare LLC, East Walpole, MA, USA) and osmolality (AdvancedModel 2020 Osmometer, Advanced Instruments, Norwood, MA, USA) were performed. Plasma samples were collected at post-mortem from pregnant and age-matched non-pregnant animals and analysed for creatinine to be used in creatinine clearance calculations (urinary creatinine [$\mu\text{mol l}^{-1}$] \times 24 h urine production [ml]) / (plasma creatinine [$\mu\text{mol l}^{-1}$] \times 1440 [min]).

Post mortem tissue collection

Approximately 2–7 days after IPGTT (when in oestrus, to control for reproductive and endocrine confounders) or at E20, non-pregnant and pregnant female rats, respectively, were anaesthetized with intraperitoneal injection of ketamine (100 mg (kg body weight) $^{-1}$) and Ilium xylazil-20 (30 mg (kg body weight) $^{-1}$). Heart, kidneys, pancreas, adrenals and uterus were excised and weighed. A piece of pancreatic tissue (pregnant and non-pregnant) from the hepatic end and the right kidney (pregnant only) were fixed in 10% neutral buffered formalin for histological analyses. Fetal body weights and dimensions (F2 generation) and placental weights from pregnant groups were measured and presented as an average per litter separated by sex.

Endocrine pancreas morphology

Pancreatic tissue was processed, embedded in paraffin and exhaustively sectioned at 5 μm . Three sections of equal distance apart were selected and immunostained using a guinea pig polyclonal anti-insulin antibody (1:200 dilution, Dako, Kingsgrove, NSW, Australia) (Siebel *et al.* 2010; Laker *et al.* 2011). Pancreatic islet number and area per sectional area (per mm^2) were averaged across the three sections, with islet area arbitrarily divided into small (<5000 μm^2), medium (5000–10,000 μm^2) and large (>10,000 μm^2) (Chamson-Reig *et al.* 2006; Siebel *et al.* 2010). Random systematic point counting of 50 fields of view was used to determine relative islet and β -cell volume density (V_d) using a 700 point grid (700 points/field, V_d equals the number of intercepts on an islet of insulin positive cells as a proportion of intercepts on a pancreas). Given that 1 cm^3 tissue weighs \sim 1 g, V_d and pancreatic weight were multiplied to determine absolute islet and β -cell mass, expressed in milligrams

(Bonner-Weir, 2001; Siebel *et al.* 2010; Laker *et al.* 2011).

Renal stereology

The right kidney was cut into four even pieces and taken for processing and embedding into glycolmethacrylate. Blocks were exhaustively sectioned at 20 μm , with every 10th and 11th section sampled. Total kidney volume (V_{kid}) was estimated using the Cavalieri principle and glomerular number (N_{glom}) and volume (V_{glom}) were estimated using the physical dissector/fractionator method, as previously described (Wlodek *et al.* 2007; Moritz *et al.* 2009a): $V_{\text{kid}} = 1 \times 10 \times t \times a(p) \text{ microfiche} \times P_s$, where 1 is the inverse of slice sampling fraction, 10 is the inverse of the section sampling fraction, t is the section thickness (20 $\mu\text{m} = 0.02 \text{ mm}$), $a(p)$ is the area corresponding to each grid point (grid size/magnification) in mm^2 and P_s is the total number of points overlying all sampled sections; $N_{\text{glom}} = 1 \times 10 \times P_s/P_f \times 1/2f_a \times Q^-$, where 1 is the inverse of slice sampling fraction, 10 is the inverse of section sampling fraction, P_s is the total number of points overlying all tissue, P_f is the total number of points overlying tissue used for estimating glomerular number, $1/2f_a$ is the inverse fraction of the total section area used to count glomeruli; 2 refers to the fact that we used dissectors to count in both directions, Q^- is the number of glomeruli counted; $V_{\text{glom}} = (P_{\text{glom}}/P_{\text{kid}})/(N_{\text{glom}}/V_{\text{kid}})$ and $V_{\text{glom,tot}} = V_{\text{glom}} \times N_{\text{glom}}$.

Statistical analyses

Values are expressed as means \pm SEM with n representing the number of offspring from separate mothers per group. Data were analysed using a two-way ANOVA to determine main effects of uteroplacental insufficiency (Control and Restricted) and pregnancy (non-pregnant and pregnant) or Student's unpaired t test. Two-way ANOVA with repeated measures was performed for IPGTT plasma glucose and insulin concentrations over time. If significant interactions were observed, individual group means were compared using Student's unpaired t test.

Results

Body and organ weights

Uteroplacental insufficiency in F0 females reduced F1 female weight (–10 to –15%) at postnatal day 1 ($P < 0.05$; Table 1). Restricted females remained lighter at postnatal days 6, 14 and 35 ($P < 0.05$), but caught up to Controls at mating (4 months; Table 1). There were no differences in body weight between Control and Restricted

Table 1. F1 female body and organ weights relative to body weight (% BW)

	Non-pregnant		Pregnant	
	Control	Restricted	Control	Restricted
F0 Litter Size	9.0 ± 1.1	5.6 ± 0.3*	10.9 ± 0.5	5.9 ± 0.6*
F1 body weight (g)				
Postnatal day 1	3.9 ± 0.1	3.5 ± 0.1*	4.1 ± 0.1	3.5 ± 0.1*
Postnatal day 6	7.7 ± 0.4	6.2 ± 0.4*	8.7 ± 0.5	7.1 ± 0.7*†
Postnatal day 14	20.2 ± 1.0	17.8 ± 1.1*	21.4 ± 0.9	19.1 ± 2.0*
Postnatal day 35	67 ± 2	62 ± 2*	76 ± 2	65 ± 4*
Mating	—	—	200 ± 4	198 ± 7
Post-mortem	212 ± 6	214 ± 4	285 ± 5	274 ± 8†
Pregnancy weight gain	—	—	85 ± 3	76 ± 3
F1 organ weight (% BW)				
Heart	0.396 ± 0.006	0.396 ± 0.005	0.310 ± 0.006	0.314 ± 0.007†
Kidney	0.332 ± 0.005	0.329 ± 0.009	0.255 ± 0.009	0.254 ± 0.014†
Pancreas	0.270 ± 0.016	0.349 ± 0.050	0.191 ± 0.016	0.200 ± 0.010†
Adrenals	0.028 ± 0.002	0.028 ± 0.003	0.027 ± 0.001	0.022 ± 0.002
Uterus	0.157 ± 0.006	0.154 ± 0.004	0.862 ± 0.035	0.850 ± 0.030†

Measures performed in non-pregnant and pregnant (E20) animals (mean ± SEM; $n = 8-12$ /group).

* $P < 0.05$ vs. Control (main effect); † $P < 0.05$ vs. non-pregnant (main effect). — indicates no measure.

at post-mortem but, body weight was greater in pregnant compared to age-matched non-pregnant animals (+25%, $P < 0.05$; Table 1). Weight gain during pregnancy was not different between Control and Restricted groups (Table 1).

Absolute heart, kidney, pancreas and adrenal weights were not different between groups; however uterus weight was reduced in Restricted females compared with Controls from both age-matched non-pregnant and pregnant groups ($P < 0.05$; data not shown). Relative organ weights were not different between Control and Restricted non-pregnant or pregnant groups (Table 1). In pregnant rats, relative heart, kidney and pancreas weights were reduced compared with non-pregnant rats ($P < 0.05$), but there were no differences in adrenal weight (Table 1). Uterus weight (absolute and relative) was greater in pregnant compared with age-matched non-pregnant rats ($P < 0.05$; Table 1).

Systolic blood pressure and intraperitoneal glucose tolerance test

Systolic blood pressure was not different between Control and Restricted females in non-pregnant and pregnant (E18) groups (Table 2). Similarly, there were no differences in systolic blood pressure between non-pregnant and pregnant groups (Table 2). Non-pregnant Restricted females had reduced fed basal plasma insulin concentrations compared with Control counterparts (−45%; $P < 0.05$; Fig. 1A). Pregnancy in the Restricted group was associated with normal basal plasma insulin concentrations

($P < 0.05$; Fig. 1A). Basal plasma glucose was not different in Restricted compared with Control females in non-pregnant and pregnant (E18) groups (Fig. 1B). Pregnancy was associated with reduced fed basal plasma glucose concentrations compared with non-pregnant levels ($P < 0.05$; Fig. 1B).

In response to IPGTT, pregnant females had greater insulin secretion compared with non-pregnant females ($P < 0.05$), with no difference between Control and Restricted groups (Fig. 1C and E). The increased insulin secretion in pregnant females was associated with lower plasma glucose levels compared with non-pregnant females ($P < 0.05$; Fig. 1D and F). Restricted females, however, did not lower plasma glucose to the same level as Controls during pregnancy, indicating altered glucose tolerance at all time points ($P < 0.05$; Fig. 1D). This was also reflected by increased area under glucose curve in pregnant Restricted females compared with Control counterparts (AUGC; +36%; $P < 0.05$; Fig. 1F).

Food and water intake and renal function

Pregnant females consumed more food and water (+20–25%; E19–20) with reduced urine osmolality and excretion of creatinine and glucose compared with non-pregnant counterparts ($P < 0.05$; Table 2). Restricted females consumed, on average, 10% less food compared with Controls ($P < 0.05$; Table 2). Twenty-four hour urine output was not different between Control and Restricted but was 2-fold greater in pregnant

Table 2. Systolic blood pressure and 24 h renal measurements

	Non-pregnant		Pregnant	
	Control	Restricted	Control	Restricted
Systolic blood pressure (mmHg)	125 ± 3	132 ± 4	127 ± 5	127 ± 3
Food intake (g (24 h) ⁻¹ kg ⁻¹)	78 ± 2	68 ± 4*	94 ± 4	87 ± 4*†
Water intake (ml (24 h) ⁻¹ kg ⁻¹)	148 ± 6	149 ± 7	204 ± 12	198 ± 11†
Urinary excretion				
Urine flow rate (l (24 h) ⁻¹ kg ⁻¹)	0.065 ± 0.004	0.064 ± 0.003	0.129 ± 0.008	0.119 ± 0.008†
Osmolality (mosmol (kg H ₂ O) ⁻¹)	1097 ± 82	911 ± 76	453 ± 59	604 ± 65†
Sodium excretion (mmol l ⁻¹ (24 h) ⁻¹ kg ⁻¹)	2.3 ± 0.2	2.6 ± 0.2	3.2 ± 0.5	1.9 ± 0.2§¶
Potassium excretion (mmol l ⁻¹ (24 h) ⁻¹ kg ⁻¹)	5.0 ± 0.6	5.1 ± 0.6	6.1 ± 0.8	5.9 ± 0.6
Chloride excretion (mmol l ⁻¹ (24 h) ⁻¹ kg ⁻¹)	4.8 ± 0.4	4.66 ± 0.39	7.1 ± 0.9	5.7 ± 0.5†
Creatinine excretion (mmol l ⁻¹ (24 h) ⁻¹ kg ⁻¹)	0.216 ± 0.014	0.216 ± 0.009	0.173 ± 0.015	0.193 ± 0.007†
Uric acid excretion (mmol l ⁻¹ (24 h) ⁻¹ kg ⁻¹)	0.084 ± 0.015	0.073 ± 0.014	0.173 ± 0.053	0.080 ± 0.018
Glucose excretion (mmol l ⁻¹ (24 h) ⁻¹ kg ⁻¹)	0.067 ± 0.009	0.081 ± 0.003	0.047 ± 0.008	0.050 ± 0.007†
Micro-total protein (g (24 h) ⁻¹ kg ⁻¹) × 10 ³	10.4 ± 1.4	10.3 ± 0.7	13.2 ± 2.2	11.8 ± 2.7
Fractional sodium excretion (%)	0.363 ± 0.023	0.434 ± 0.034	0.204 ± 0.032	0.164 ± 0.029†
Creatinine clearance (ml min ⁻¹ kg ⁻¹)	3.1 ± 0.3	3.1 ± 0.2	8.2 ± 1.8	6.5 ± 1.9†

Measurements from in non-pregnant and pregnant animals (systolic blood pressure and renal function at E18 and E19–20, respectively) (means ± SEM; *n* = 8–12/group). **P* < 0.05 vs. Control (main effect); †*P* < 0.05 vs. non-pregnant (main effect); §*P* < 0.05 vs. pregnant Control (following significant interaction); ¶*P* < 0.05 vs. non-pregnant Restricted (following significant interaction).

compared with non-pregnant females (*P* < 0.05; Table 2). Urinary sodium excretion was reduced in pregnant Restricted females, compared with non-pregnant Restricted and pregnant Controls (*P* < 0.05; Table 2). Fractional sodium excretion was not different between Control and Restricted females but was reduced in pregnant compared with non-pregnant rats (–54%; *P* < 0.05; Table 2). Chloride excretion was not different between Control and Restricted groups but was greater during pregnancy compared with non-pregnant females (*P* < 0.05; Table 2). Excretion of potassium, uric acid and micro-total protein were not different between Control and Restricted or between non-pregnant and pregnant groups (Table 2). Creatinine clearance was not different between Control and Restricted but was increased by more than 2-fold during pregnancy compared with non-pregnant females (*P* < 0.05; Table 2).

Pancreatic morphology

Non-pregnant Restricted females had reduced β-cell mass, both absolute and relative, compared with Controls (–36%; *P* < 0.05), which was restored in pregnancy (Fig. 2A and B). Islet proportion per pancreas was also reduced in non-pregnant Restricted compared with Controls (–40%; *P* < 0.05), with no differences during pregnancy (Fig. 2C). Relative number of small

(<5000 μm²), medium (5000–10,000 μm²) or large (>10,000 μm²) islets was not different across groups (data not shown).

Glomerular number and size

Total glomerular number was reduced by 33% in pregnant Restricted females compared with Controls (*P* < 0.05; Fig. 3A). This was associated with increased individual glomerular volume (+37%; *P* < 0.05; Fig. 3B), resulting in similar total glomerular volumes between groups (Fig. 3C).

F2 fetal body and organ weights

Total (male and female) F2 litter size was not different between Control and Restricted (10.5 ± 0.4 vs. 9.8 ± 0.6, respectively) or between Pregnant 1 and Pregnant 2 (10.2 ± 0.5 vs. 9.9 ± 0.6, respectively). F2 sex ratios were also not different between Control and Restricted litters or when compared with the F1 generation (data not shown). Male and female F2 fetuses (E20) from F1 Restricted mothers exposed to physiological measurements during pregnancy (pregnant 1) were 5–6% lighter than Controls (*P* < 0.05; Fig. 4A). However, F2 fetal weight from mothers not exposed to physiological measurements during pregnancy (pregnant 2) was similar between Control and Restricted (Fig. 4B). Interestingly, female fetuses from

pregnant 2 mothers weighed less than male counterparts ($P < 0.05$; Fig. 4B), but sex differences were not observed in those from pregnant 1 mothers (Fig. 4A). Placental weight was not statistically different between Control and Restricted groups from pregnant 1 ($P = 0.07$; Fig. 4C), or pregnant 2 ($P = 0.07$; Fig. 4D). Placental weight was not different between sexes across both cohorts (Fig. 4C

and D). Fetal body weights between cohorts within a sex were not statistically different (Fig. 4A and B), despite heavier placental weights in pregnant 2 compared with pregnant 1 (+7–17%; $P < 0.05$; Fig. 4C and D). This contributed to a greater placental efficiency in pregnant 1 compared with pregnant 2 (+12–15%; $P < 0.05$; data not shown).

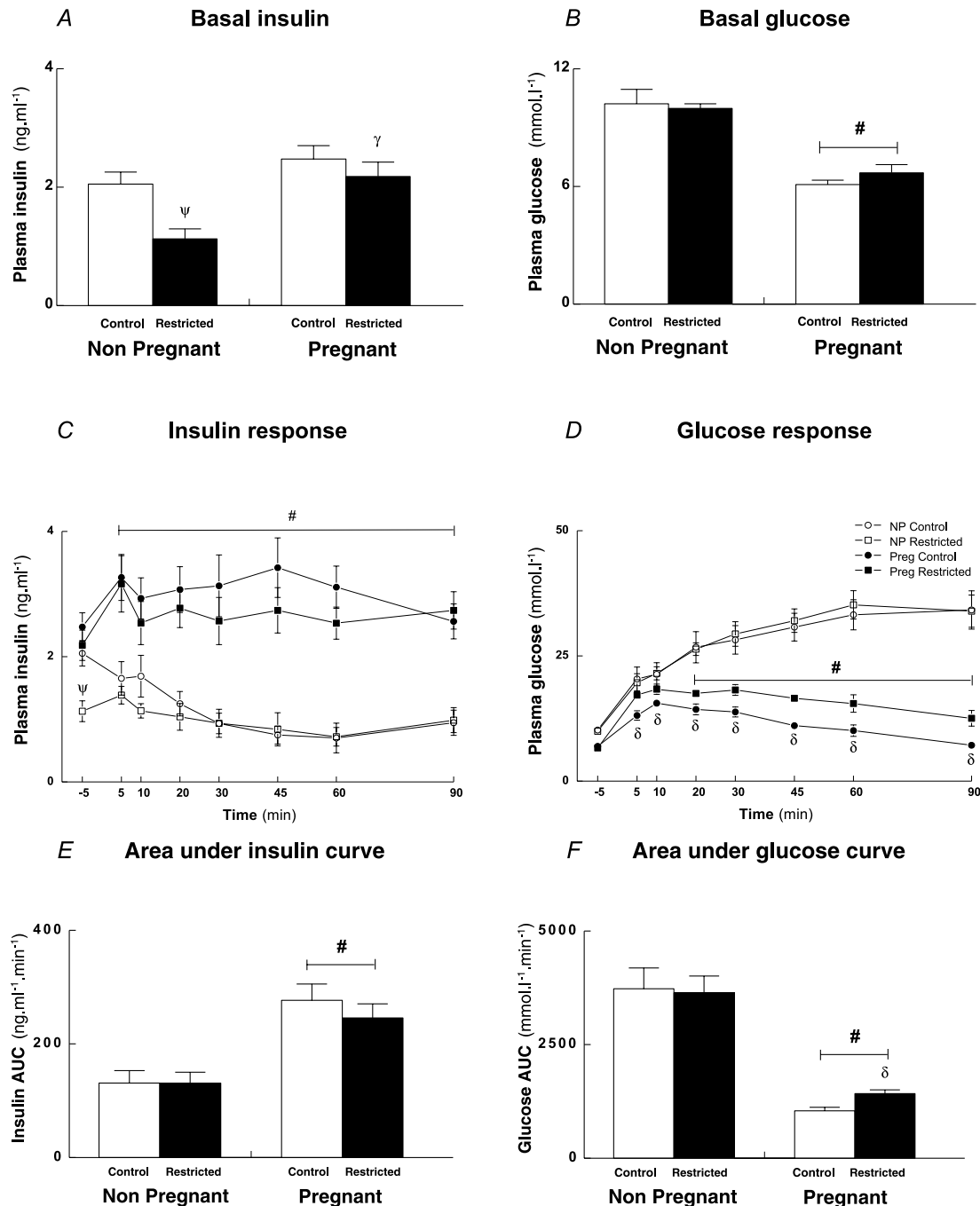


Figure 1. Plasma insulin and glucose prior to and in response to IPGTT
 Measurements from non-pregnant and pregnant (E18) animals (mean ± SEM; $n = 8-10$ /group). † $P < 0.05$ vs. non-pregnant (main effect); ‡ $P < 0.05$ vs. non-pregnant Control; § $P < 0.05$ vs. pregnant Control (following significant interaction); ¶ $P < 0.05$ vs. non-pregnant Restricted (following significant interaction).

Discussion

The present study demonstrates that despite previous reports of vascular dysfunction and nephron deficits in the non-pregnant state (Anderson *et al.* 2006; Moritz *et al.* 2009a; Mazza *et al.* 2010), growth restricted females exposed to uteroplacental insufficiency present with normal blood pressure and no overt renal pathologies in late pregnancy. In addition, basal insulin secretion and pancreatic β -cell mass were reduced in non-pregnant Restricted females but were not different during pregnancy

and first- and second-phase insulin response showed normal elevations. It should be noted, however, that despite reductions in plasma glucose concentration in Restricted females during pregnancy, it did not reach the low levels seen in Controls, indicating some loss of glucose tolerance.

Interestingly, another finding to emerge from our study was that F2 male and female fetuses from Restricted mothers were, on average, 5–6% lighter than those from Control mothers, in pregnancies exposed to physiological measurements (tail cuff blood pressure, IPGTT and 24 h metabolic cage studies) during late gestation (pregnant 1). F2 fetal weight, however, was similar between Control and Restricted in pregnancies from mothers not subjected to physiological measurements (pregnant 2). Thus, our data indicate that growth restricted females exposed to modest stress during their pregnancy programme next generation fetal growth restriction.

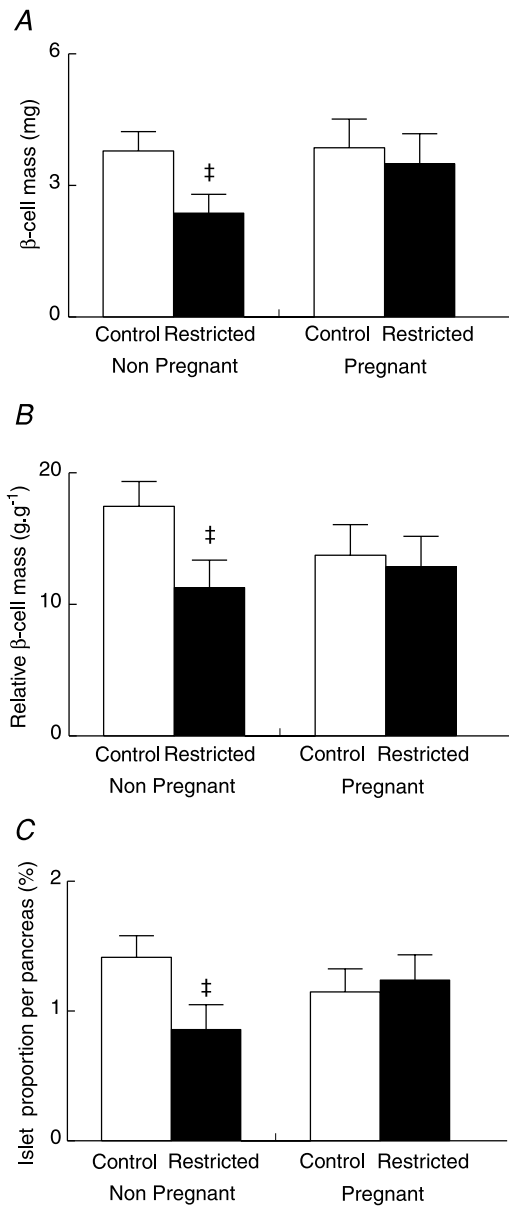


Figure 2. Pancreatic β -cell mass and islet proportion per pancreas

Measurements from non-pregnant and pregnant (E20) animals (mean \pm SEM; $n = 6\text{--}7/\text{group}$). $\ddagger P < 0.05$ vs. non-pregnant Control (following significant interaction).

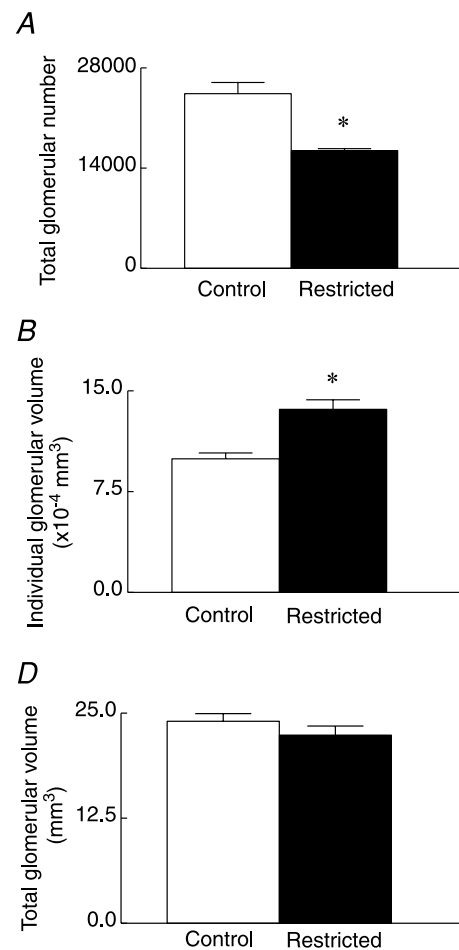


Figure 3. Total glomerular number, and individual and total glomerular volume

Measurements from pregnant (E20) animals (mean \pm SEM; $n = 5/\text{group}$). $*P < 0.05$ vs. Control.

Maternal growth trajectory and organ weights

Female offspring exposed to late gestation uteroplacental insufficiency were smaller from postnatal day 1 and caught up with Controls only at mating (4 months). These present findings are in contrast to recently published work by our group showing that growth restricted females match Control body weight by postnatal day 14 (Mazzuca *et al.* 2010). Differences in growth trajectories between cohorts of the same model, born of similar weights, are intriguing and challenge comparison between studies. It may reflect differences in the genetic pool of the same species across time. Importantly, body weights were not different between groups from commencement of treatment, at mating, and growth restricted female weight did not surpass that of Controls. Thus, our data were not impacted by the confounding effects of maternal weight disparities or obesity. There were no differences in weight gain during pregnancy between Control and Restricted, or between pregnant groups at post-mortem.

Metabolic profile and pancreas morphology

Uteroplacental insufficiency resulted in a 45% reduction in fed basal insulin levels, associated with a 36% deficit in pancreatic β -cell mass in our non-pregnant females. This is consistent with previous studies demonstrating similar reductions in pancreatic β -cell mass and impaired insulin release in growth restricted females (Simmons *et al.* 2001; Styrud *et al.* 2005). These deficits were resolved by late pregnancy and values matched that of pregnant Controls, consistent with a previous study reporting normal islet mass in 4-month-old pregnant rats exposed to perinatal malnutrition (Blondeau *et al.* 1999). This highlights the plasticity of pancreatic β -cells to up-regulate their mass to match increases in insulin demand and, importantly, matched the pregnancy adaptations seen in Controls. However, this did not protect growth restricted females from adverse metabolic control during pregnancy.

Interestingly, despite known increases in β -cell mass by more than 2-fold during mid-pregnancy in rodents (Xue

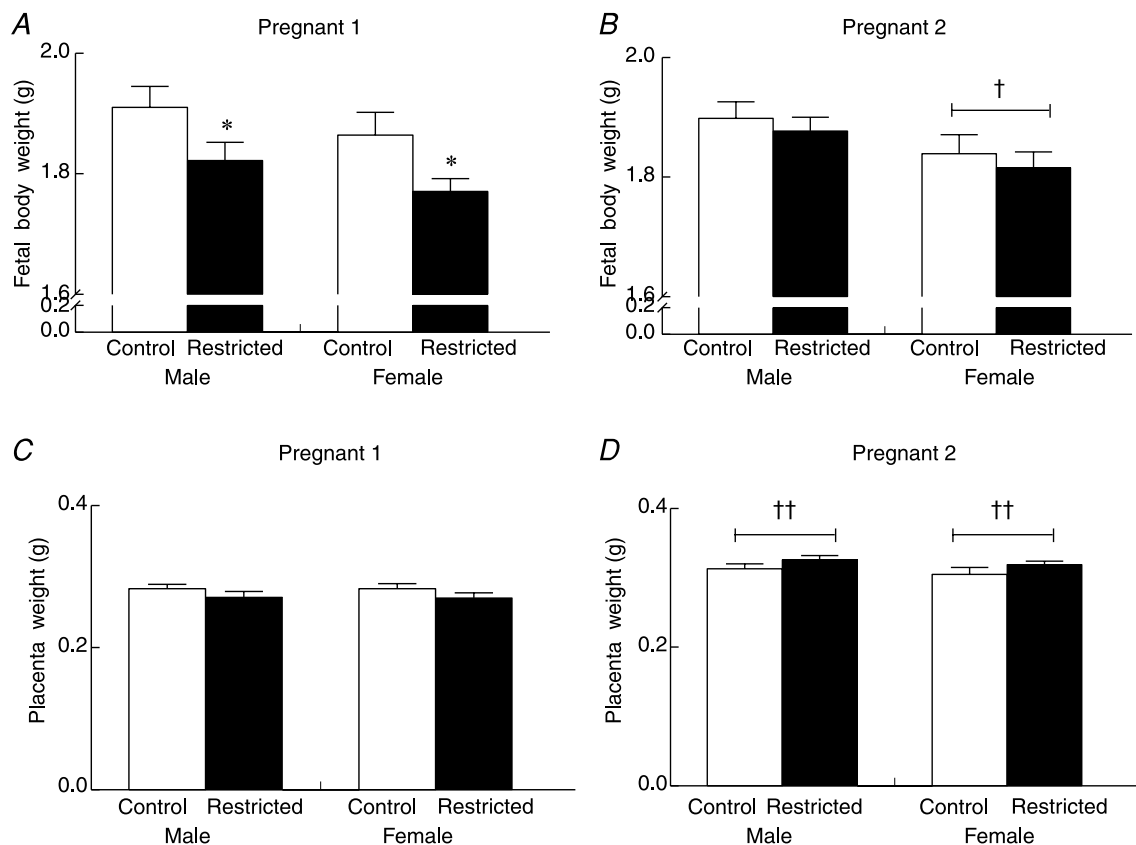


Figure 4. F2 fetal body and placental weights

Measurements in offspring from pregnant 1 (E20) and pregnant 2 (E20) animals and averaged per sex per litter (mean \pm SEM; $n = 9-11$ litters/group). * $P < 0.05$ vs. Control (main effect); † $P < 0.05$ vs. males (main effect); †† $P < 0.05$ vs. pregnant 1 (right vs. left panel within gender; main effect).

et al. 2010), our pregnant females had similar β -cell mass, islet size and number to that of non-pregnant females. Indeed, from late gestation to 1 week post-partum, there is a rapid decline in β -cell mass, accompanied by decreased proliferation and increased apoptosis so that β -cell mass returns to pre-pregnant levels by term (Rieck & Kaestner, 2010; Xue *et al.* 2010). Thus, it is likely that the β -cell mass assessed in the current study, 2 days prior to delivery at gestational day 20, returned to that of pre-pregnant values. If so, the lack of difference observed between Control and Restricted β -cell mass in pregnant females may reflect either a long-term protective effect of pregnancy or rather, a delayed restoration to pre-pregnant values in Restricted animals.

In response to IPGTT during late gestation, we observed an overall reduction in plasma glucose compared with non-pregnant females, but plasma glucose in Restricted females did not decrease to the same level as Controls. Thus, despite normal elevations in insulin secretion, our pregnant Restricted females developed some loss of glucose tolerance that was sustained to at least 90 min post-glucose load compared with Controls. The increased risk of future diabetes and cardiovascular disease is directly proportional to the degree of gestational dysglycaemia, such that women who develop gestational diabetes incur the greatest risk (Carr *et al.* 2008; Retnakaran, 2009). Indeed, plasma glucose concentrations in growth restricted pregnant females remained well below that of non-pregnant values. However, the mild glucose intolerance compared with Controls predicts an increased risk nonetheless. In normal pregnancy, there is increased gluconeogenic activity but glucose is the most abundant substrate able to cross the placenta and is thus largely responsible for the maternal hypoglycaemia observed in pregnant females. This hypoglycaemic state is apparent despite increased insulin resistance by maternal tissues and increased food intake during the latter half of pregnancy (Herrera, 2000). Maternal pancreatic islets adapt to this increased demand for insulin but, as in the current study, this was not sufficient to lower plasma glucose in pregnant Restricted females to that of Controls. Given that the insulin response showed no elevation above Control levels, we suggest insulin sensitivity is normal, and that other mechanisms may be involved. Furthermore, additional life-style insults, including adverse diets (high salt/fat) and obesity, superimposed on pregnancy in these lean growth restricted females may exacerbate this metabolic phenotype with serious implications for future maternal and fetal health.

Blood pressure

Systolic blood pressure was not different between Control and Restricted pregnant (E18) rats, and values were similar to those of non-pregnant females. In contrast, Klebanoff

et al. (1999) reported increased risk of hypertension from mid gestation in 15- to 28-year-old pregnant women, born small for gestational age, which was not associated with hypertension during their mothers' pregnancy. The authors suggested that a reduced number of nephrons, as consistently reported in those born small, may increase a woman's risk of becoming hypertensive during pregnancy. The lengthy duration of human pregnancy may provide greater opportunity for compensatory mechanisms, such as intraglomerular hypertension and glomerular hypertrophy, the latter seen in the current study, to succumb to disease (Moritz & Bertram, 2006). Blood pressure often decreases during early pregnancy attributed to large reductions in systemic vascular tone (Torgersen & Curran, 2006) and measurements at an earlier, more dynamic time-point may have revealed differences in cardiovascular function between Control and Restricted pregnant females.

Kidney function and morphology

In the current study, creatinine clearance, indicative of glomerular filtration rate (GFR), increased by more than 2-fold during pregnancy, as did urine flow rate compared with non-pregnant females. The profound systemic vasodilatation that occurs during pregnancy contributes to increased renal plasma flow and solute filtration, allowing for reduced plasma osmolality and viscosity (Torgersen & Curran, 2006). Marked increases in GFR may result in proteinuria, a gold-standard measure of kidney damage that can be exacerbated in pregnancies complicated by maternal hypertension. We report no differences in maternal blood pressure or urinary micro-total protein excretion in late gestation. Uric acid and potassium concentrations also remained unchanged during pregnancy, but increased urine flow was associated with reduced urinary osmolality, glucose and creatinine concentration compared with non-pregnant females. During the last week of normal rat pregnancy, active sodium reabsorption by renal tubules increases water retention assisting with plasma volume expansion (Atherton *et al.* 1982; Torgersen & Curran, 2006). This was reflected in the 50% reduction in fractional sodium excretion in our pregnant compared with non-pregnant females.

Interestingly, a 45% reduction in urinary sodium concentration was the sole effect of uteroplacental insufficiency on kidney function during pregnancy, compared with Controls and non-pregnant Restricted. This finding cannot be attributed to the 10% reduction in food consumption in Restricted, as we would otherwise observe similar changes in urinary sodium in non-pregnant females. The relevance of altered sodium excretion during pregnancy in growth restricted females is unknown. Novel to this study was the emergence

of individual glomerular hypertrophy in our growth restricted pregnant females, at an earlier age than we previously reported in 18 month old non-pregnant rats with modest renal insufficiency (Moritz *et al.* 2009a). Indeed, the current data appear to reflect 'perfect adaptation' for optimal glomerular filtration given the lack of adverse renal pathology (Kett & Bertram, 2004). However, whether this is merely an acute, protective response during pregnancy that does not progress into the 'vicious cycle' of future renal damage and systemic hypertension (Brenner *et al.* 1988) remains to be determined. Furthermore, additional stressors on already compromised kidneys, such as obesity, high-salt/fat diets or increased maternal age, are likely to challenge the functional renal reserve during and/or after pregnancy in growth restricted females.

F2 fetal parameters

In the present study, fetal body weights were measured for further insight into pregnancy and intergenerational outcomes for growth restricted females. We found that male and female fetuses from mothers who were born small and exposed to physiological measures during late pregnancy (pregnant 1) were on average, 5–6% smaller than those from Control mothers. This fetal growth deficit in offspring whose grandmothers had reduced uteroplacental blood flow during pregnancy was associated with a non-significant ($P = 0.07$) tendency for decreased placental weight. To our knowledge, this is the first reported evidence of intergenerational growth restriction, unmasked by maternal exposure to physiological measures, in a well-established model of uteroplacental insufficiency. Indeed, others have investigated transmitted characteristics from F1 to F2 offspring but these are commonly carried out in maternal protein restriction models and F2 birth weight is largely unaffected (Zambrano *et al.* 2005; Torrens *et al.* 2008; Harrison & Langley-Evans, 2009). Given that offspring from Restricted mothers not exposed to physiological measurements throughout pregnancy (pregnant 2) were not smaller than their Control counterparts, it is likely that elevated maternal glucocorticoids played a role in mediating this intergenerational growth deficit. Findings from animal studies, and indeed humans, have reported that prenatal exposure to excess glucocorticoids is associated with either reduced weight at birth (Kutzler *et al.* 2004; Davis *et al.* 2009) or no change (Moritz *et al.* 2009b). Our data implicate a small maternal birth weight, in combination with environmental stressors, as having an adverse programming influence for next generation fetal growth.

While there were no sex differences in fetal weight from mothers exposed to physiological measures during late pregnancy (pregnant 1), female fetuses from pregnancies

not exposed to such measures (pregnant 2) were, on average, 3% smaller than their male counterparts. This difference between cohorts is likely to be due to the fetal weight reduction in Restricted fetuses from pregnant 1, masking the subtler sexual dimorphism. Finally, when compared to pregnant 2 within a sex, placental weights were 17% lighter in those from mothers exposed to physiological measures during late pregnancy, but no differences in fetal body weights between cohorts indicating a greater placental efficiency. Importantly, however, caution must be exercised when translating such data to humans, given that rats are a litter-bearing species.

Conclusions

Late gestation uteroplacental insufficiency does not, by and large, restrict maternal renal or pancreatic adaptations when growth restricted females in turn become pregnant. The nephron deficit observed in pregnant growth restricted females was accompanied by individual glomerular hypertrophy, allowing for preserved renal function and blood pressure. Pancreatic β -cell mass was over-responsive in Restricted females, matching Control levels during pregnancy, and insulin response to glucose was normal. However, despite the beneficial pancreatic response, the elevated plasma glucose in pregnant Restricted females indicates some loss of glucose tolerance and together with the glomerular hypertrophy, warrants further follow-up in settings of added lifestyle challenges.

Although we have clearly shown mostly beneficial adaptations to late pregnancy in growth restricted females, when and how these adaptations occurred remain to be elucidated. We can speculate that the growth restricted females triggered a predictive adaptive response that allowed them to become 'normal' by late gestation. Understanding the mechanisms by which the kidney (glomerular hypertrophy) and pancreatic (restored pancreatic β -cell mass) changes occur may allow targeted therapeutic strategies for pregnancies that are complicated by pregnancy-induced hypertension, pre-eclampsia, gestational diabetes and intra-uterine growth restriction.

Another consideration is whether the altered pregnancy adaptations invoked to achieve 'normal' renal function instigated an additional stress to the maternal cardiovascular system that may manifest in enhanced disease risk later in life, although preserving immediate function. Similarly, the long term impact on the mothers' metabolic health following the loss of glucose tolerance when Restricted mothers were pregnant remains to be explored. Lastly, we cannot exclude the possibility that the reduced F2 fetal growth was, in part, due to embryonic inheritance passed from previous generations that may be investigated using embryo transfer approaches.

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Author contributions

All authors contributed to the conception and design, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and all authors gave final approval of the version to be published. All experiments were performed at The University of Melbourne, with the exception of renal stereology, which was performed at Monash University.

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