

# Transgenerational metabolic outcomes associated with uteroplacental insufficiency

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## Abstract

Intrauterine growth restriction increases adult metabolic disease risk with evidence to suggest that suboptimal conditions *in utero* can have transgenerational effects. We determined whether impaired glucose tolerance, reduced insulin secretion, and pancreatic deficits are evident in second-generation (F2) male and female offspring from growth-restricted mothers, in a rat model of uteroplacental insufficiency. Late gestation uteroplacental insufficiency was induced by bilateral uterine vessel ligation (restricted) or sham surgery (control) in Wistar-Kyoto rats. First-generation (F1) control and restricted females were mated with normal males and F2 offspring studied at postnatal day 35 and at 6 and 12 months. F2 glucose tolerance, insulin secretion, and sensitivity were assessed at 6 and 12 months and pancreatic morphology was quantified at all study ages. At 6 months, F2 restricted male offspring exhibited blunted first-phase insulin response (–35%), which was associated with reduced pancreatic  $\beta$ -cell mass (–29%). By contrast, F2 restricted females had increased  $\beta$ -cell mass despite reduced first-phase insulin response (–38%). This was not associated with any changes in plasma estradiol concentrations. Regardless of maternal birth weight, F2 control and restricted males had reduced homeostatic model assessment of insulin resistance and elevated plasma triglyceride concentrations at 6 months and reduced whole-body insulin sensitivity at 6 and 12 months compared with females. We report that low maternal birth weight is associated with reduced first-phase insulin response and gender-specific differences in pancreatic morphology in the F2. Further studies will define the mode(s) of disease transmission, including direct insults to developing gametes, adverse maternal responses to pregnancy, or inherited mechanisms.

## Key Words

- ▶ pancreas
- ▶ insulin secretion
- ▶ glucose metabolism
- ▶ gender
- ▶ ageing

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## Introduction

Numerous studies have reported a strong link between low weight at birth and increased susceptibility to diabetes, insulin resistance, and obesity in adult life (Hales *et al.* 1991, Barker *et al.* 1993, Simmons *et al.* 2001, Styrod *et al.* 2005). This association has been conceptualized by the developmental origins of health and disease hypothesis,

whereby alterations in the fetal and postnatal environments influence the development of key metabolic organs involved, leading to reduced insulin secretion and sensitivity and impaired glucose tolerance in adult life. We have previously shown that maternal uteroplacental insufficiency was associated with impaired glucose

tolerance, reduced insulin secretion, and a 40–45% reduction in  $\beta$ -cell mass in adult first-generation (F1) growth-restricted male offspring (Siebel *et al.* 2008, Wadley *et al.* 2008, Laker *et al.* 2011). By contrast, F1 growth-restricted females displayed normal glucose tolerance despite reduced basal insulin secretion and a 36% decrease in  $\beta$ -cell mass (Wadley *et al.* 2008, Gallo *et al.* 2012b).

Evidence is now emerging that suboptimal conditions *in utero* are not limited to affect the first, directly exposed generation but can have transgenerational effects. Studies from the Dutch hunger winter provide evidence that maternal exposures can have detrimental effects on offspring health that are transmitted across generations (Heijmans *et al.* 2008). Women who were undernourished *in utero* during the famine delivered second-generation (F2) babies that had increased neonatal adiposity suggestive of increased obesity and diabetes risk in later life (Painter *et al.* 2008). Transgenerational effects on glucose and insulin metabolism have also been reported in numerous maternal low-protein animal studies (Hoet & Hanson 1999, Zambrano *et al.* 2005, Benyshek *et al.* 2006, Pinheiro *et al.* 2008). Insulin resistance was evident in F2 male offspring from mothers exposed to low protein during lactation and F2 females from mothers exposed to low protein during gestation (Zambrano *et al.* 2005), demonstrating sex-specific differences according to the developmental time window to which the F1 fetus was exposed. However, no effects were reported on F2 offspring when their mothers were fed a low-protein diet throughout pregnancy and lactation (Hoet & Hanson 1999, Zambrano *et al.* 2005, Benyshek *et al.* 2006, Pinheiro *et al.* 2008), highlighting the importance of pre- and postnatal nutrition on disease transmission. In a maternal global caloric restriction study, reduced birth weight was transmitted to the F2 and these offspring developed obesity via the maternal line (Jimenez-Chillaron *et al.* 2009), suggesting that maternal metabolic dysfunction during pregnancy may be a key contributor.

Despite greater relevance to Western societies, considerably less is known regarding the effects of uteroplacental insufficiency on transgenerational programming. In female rats born small due to uteroplacental insufficiency, although confounded by obesity, pregnancy unmasked maternal diabetes that led to macrosomic F2 offspring with increased adiposity, impaired glucose tolerance, and reduced insulin sensitivity (Boloker *et al.* 2002). In this study, however, F1 growth-restricted females were fostered onto un-operated mothers following birth, which may have impacted on the F1 females' own

pregnancy adaptations and subsequent F2 metabolic health. In our rat model of uteroplacental insufficiency, growth-restricted female rats developed loss of glucose control on day 20 of pregnancy in the absence of obesity (Gallo *et al.* 2012b). Therefore, in this study, we determined whether metabolic disease risk (impaired glucose tolerance and pancreatic morphological deficits) was evident in F2 male and female offspring, via the maternal line, in the absence of any further pregnancy interventions.

## Materials and methods

### Animal procedures

All experiments were approved by The University of Melbourne Animal Ethics Committee before commencement and conducted in accord with accepted standards of humane care. Wistar-Kyoto rats (12 weeks of age) were housed in an environmentally controlled room (22 °C) with a 12 h light:12 h darkness cycle and access to food and tap water *ad libitum*. Rats were mated and surgery was performed on day 18 of pregnancy (Wlodek *et al.* 2005). Briefly, F0 pregnant rats were randomly allocated to a control (sham surgery) or restricted (uteroplacental insufficiency) group (20 pregnant dams/group). Restricted group underwent bilateral uterine vessel (artery and vein) ligation surgery while surgery for control was the same except that vessels were not ligated (Wlodek *et al.* 2005). The F0 females delivered naturally at term on day 22 of pregnancy and pups remained with their original mothers after birth and throughout lactation to eliminate confounding factors associated with cross-fostering. Litter size was not equalized across generations because we have previously reported 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). Thus, we do not regard sham-exposed, culled litters as adequate controls. F1 body weights were measured at postnatal day (PN) 1, 7, 14, and 35 and at months 2, 3, and 4 (mating). At ~17–23 weeks of age, F1 control and restricted females (one randomly allocated female per litter;  $n=17$ –19 females/group) were mated with normal males and three F2 cohorts were generated: PN35 and 6 and 12 months (one male and one female per litter allocated to each cohort,  $n=8$ –17/group). Pups remained with their mothers after birth and throughout lactation. Maternal pregnancy weight gain was calculated in F1 control and restricted females and F2 offspring body

weights were measured at PN1, 7, 14, and 35 and at 2, 4, 6, 9 and 12 months of age. Absolute and fractional growth rates were calculated in F2 offspring between weeks 2–5 to assess lactation growth rates, weeks 5–8 to assess growth rates around puberty, and weeks 8–12 to assess juvenile growth rates.

### Insulin challenge and intraperitoneal glucose tolerance test

Adult F2 male and female offspring (6 and 12 months) were fasted overnight before an insulin challenge (IC) the following morning. A tail vein blood sample (300  $\mu$ l) was taken before and following a s.c. bolus injection of insulin and blood samples taken at 20, 40, and 60 min (Actrapid, Novo Nordisk Pharmaceuticals, North Rocks, NSW, Australia; 1 U/kg body weight; Siebel *et al.* 2008, Tran *et al.* 2012). Two weeks after the IC, F2 male and female offspring were anesthetized with 4% isoflurane and 650 ml/min oxygen flow (reduced to 3.2% isoflurane and 250 ml/min oxygen flow when suturing). The right carotid artery was catheterized and catheter patency was maintained by flushing daily with 0.6 ml heparinized saline (units). An intraperitoneal glucose tolerance test (IPGTT) was performed 4 days after the catheterization surgery following an overnight fast. Arterial blood samples (250  $\mu$ l) were taken (10 and 5 min) before and after an i.p. bolus injection of glucose (1 g/kg body weight; Pharmed, Lane Cove, NSW, Australia) and blood samples were taken at 5, 10, 20, 30, 45, 60, 90, and 120 min. The blood removed was subsequently replaced with a similar volume of heparinized saline (Siebel *et al.* 2008, Wadley *et al.* 2008). Animals remained conscious and unrestrained in their cage throughout the IPGTT experiment. At completion of the IC or IPGTT experiment, animals were allowed access to food and water *ad libitum*.

### Postmortem blood and tissue collection

Nonfasted F2 rats were anesthetized with an i.p. injection of ketamine (100 mg/kg body weight) and Ilium Xylazil-20 (30 mg/kg body weight) and blood collected for determination of plasma glucose, insulin, triglyceride, estradiol ( $E_2$ ), adiponectin and leptin. Pancreas, liver, dorsal fat, and hindlimb leg muscles (soleus, extensor digitorum long, gastrocnemius, plantaris, and tibialis cranialis) were excised and weighed. A piece of pancreatic tissue from the hepatic end was fixed in 10% neutral buffer formalin for histological analysis.

### Plasma metabolite analyses

Plasma glucose concentrations were measured in duplicate using a scaled-down version of the enzymatic fluorometric analysis (Laker *et al.* 2011, Gallo *et al.* 2012b). Plasma insulin concentrations were measured in duplicate using a commercially available rat insulin RIA kit (Millipore, Abacus ALS, Brisbane, QLD, Australia; Siebel *et al.* 2008, Wadley *et al.* 2008, Laker *et al.* 2011, Gallo *et al.* 2012b). Plasma triglyceride concentrations were measured in duplicate on an automated centrifugal analyzer (Cobas Integra 400, Roche Diagnostics). Plasma triglyceride concentrations were not measured in male offspring at 12 months. Plasma adiponectin and  $E_2$  (in females only) were measured in duplicate using an ELISA kit (Merck Millipore Corporation) and EIA kit (Cayman Chemical, Ann Arbor, MI, USA) respectively. Plasma leptin was measured in duplicate using a rat adipokine multiplex kit (Merck Millipore Corporation). Fasting plasma glucose and insulin was taken as the average of two time points (10 and 5 min before glucose injection). Glucose and insulin area under curve (AUC) were calculated as the total AUC from basal to 120 min for IPGTT and glucose AUC from basal to 60 min for IC. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin values obtained before the glucose bolus:  $HOMA-IR = \text{fasting plasma insulin } (\mu\text{U/ml}) \times \text{fasting plasma glucose (mmol/l)} / 22.5$  (Matthews *et al.* 1985, Siebel *et al.* 2008, Laker *et al.* 2011).

### Pancreatic immunohistochemistry

Pancreatic tissue was processed, embedded in paraffin wax, and exhaustively sectioned at 5  $\mu$ m for all cohorts. Three sections of equal distance apart were immunostained for insulin-positive  $\beta$ -cells using a guinea pig polyclonal anti-insulin antibody (1:200 dilution, Dako, Kingsgrove, NSW, Australia;  $n=5-8$ /group; Siebel *et al.* 2010, Laker *et al.* 2011, Tran *et al.* 2012). Pancreatic islet number and area per sectional area (per  $\text{mm}^2$ ) were averaged across the three sections, with islet area arbitrarily divided into small (<5000  $\mu\text{m}^2$ ), medium (5000–10 000  $\mu\text{m}^2$ ), and large (>10 000  $\mu\text{m}^2$ ) (Chamson-Reig *et al.* 2006). A random systematic point counting of 50 fields of view were used to determine relative islet and  $\beta$ -cell volume density 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  $\text{cm}^3$  tissue weighs  $\sim 1$  g,  $V_d$  and pancreatic weight are multiplied to determine absolute islet and  $\beta$ -cell mass,

**Table 1** F1 litter size and body weights. All data are expressed as mean  $\pm$  s.e.m.;  $n = 17$ – $19$ /group

	Control	Restricted
Total litter size	7.6 $\pm$ 0.6	5.8 $\pm$ 0.6*
Body weight (g)		
PN1	4.26 $\pm$ 0.05	3.34 $\pm$ 0.07*
PN7	10.2 $\pm$ 0.3	7.0 $\pm$ 0.3*
PN14	23.0 $\pm$ 0.4	16.9 $\pm$ 0.7*
PN35	75 $\pm$ 1	61 $\pm$ 2*
2 months	158 $\pm$ 3	147 $\pm$ 4*
3 months	209 $\pm$ 3	187 $\pm$ 3*
Mating (4 months)	240 $\pm$ 3	211 $\pm$ 4*
Delivery	276 $\pm$ 3	241 $\pm$ 4*
Pregnancy weight gain (%)	15.1 $\pm$ 0.8	13.9 $\pm$ 1.1

\* $P < 0.05$  vs control (Student's  $t$ -test between control and restricted).

expressed in milligrams per gram (Bonner-Weir 2001, Siebel *et al.* 2010, Tran *et al.* 2012).

### Statistical analyses

Values were expressed as mean  $\pm$  s.e.m. with  $n$  representing the number of offspring from separate mothers per group. For F1 maternal body weights, a Student's unpaired  $t$ -test was used for comparisons between control and restricted. A three-way ANOVA was performed to determine main effects of group (control and restricted), gender (male and female), and age (PN35 and 6 and 12 months). Where appropriate, data were split when main effects of age or gender were observed and a two-way ANOVA was performed with group and gender or group and age. If significant interactions were observed, individual group means were compared using Student's unpaired  $t$ -test. Statistical significance was set at  $P < 0.05$ .

## Results

### F1 maternal body weights

F1 females exposed to uteroplacental insufficiency were born lighter ( $-23\%$ ) than sham-exposed surgery counterparts and remained lighter at all ages studied ( $P < 0.05$ , Table 1). F1 restricted females were lighter at mating ( $-12\%$ ) and at delivery ( $-13\%$ ,  $P < 0.05$ ), but overall percentage pregnancy weight gain was not different between F1 control and restricted females (Table 1).

### F2 body and organ weights

F2 litter total (male and female) size at PN1 was not different between groups (control:  $8.94 \pm 0.49$  vs

restricted:  $8.00 \pm 0.54$ ) across all cohorts. When separated by sex, average litter size was not different between control and restricted in F2 male and female offspring (Table 2). F2 body weight was similar between control and restricted at all time points, except at 2 months of age where F2 restricted males, but not females, were lighter ( $-11\%$ ) than control counterparts ( $P < 0.05$ , Table 2). F2 restricted males slowed their growth during lactation and puberty as indicated by reduced absolute and fractional growth rates between 2–5 and 2–8 weeks ( $P < 0.05$ , Fig. 1A, B, C and D), but growth rate was only reduced in F2 restricted females during lactation between 2–5 weeks ( $P < 0.05$ , Fig. 1A and B). Between 8–12 weeks, F2 restricted offspring accelerated their growth compared with controls ( $P < 0.05$ , Fig. 1E and F), such that they were of similar body weight by 3 months of age (Table 2). At PN1, and from weaning at PN35 until postmortem, F2 females remained lighter than males ( $P < 0.05$ , Table 2).

Absolute and relative (to body weight) weights of liver, dorsal fat, and total hind limb leg muscles were not different between F2 control and restricted at 6 and 12 months of age (Table 3). Absolute and relative pancreas weight was reduced in F2 restricted groups at 6 months ( $P < 0.05$ ) but was not different at PN35 or at 12 months (Table 3). Absolute liver, dorsal fat, and total hind limb leg muscle weights were reduced in F2 females compared with F2 males at 6 and 12 months while relative dorsal fat was reduced at 6 and 12 months in F2 females compared with F2 males ( $P < 0.05$ , Table 3). Relative liver weight was increased in F2 females compared with F2

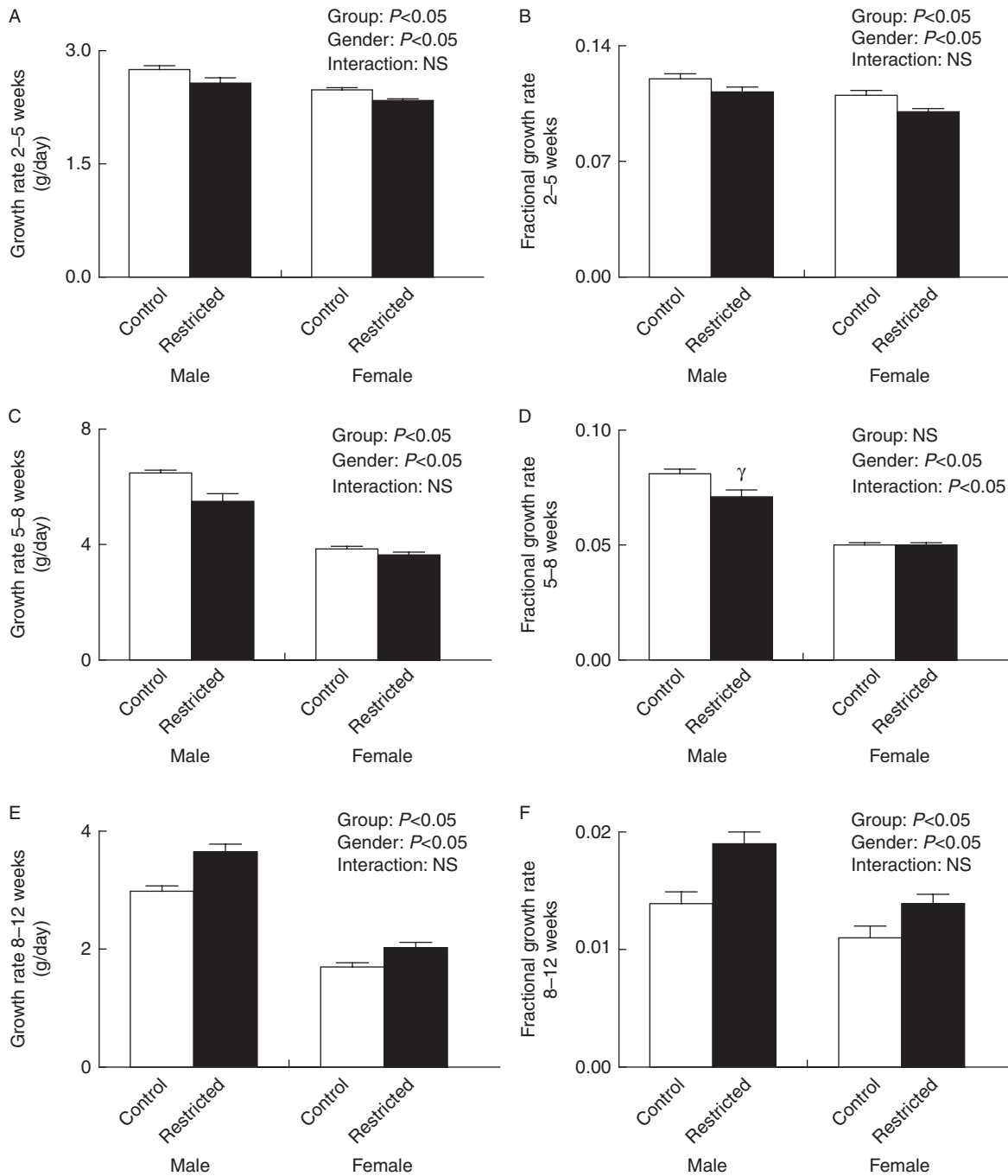
**Table 2** F2 male and female offspring body weights. All data are expressed as mean  $\pm$  s.e.m.;  $n = 12$ – $17$ /group per sex

	Male		Female	
	Control	Restricted	Control	Restricted
Average litter size	4.13 $\pm$ 0.36	3.36 $\pm$ 0.80	5.00 $\pm$ 0.37	4.73 $\pm$ 0.44
Body weight (g)				
PN1	4.63 $\pm$ 0.05	4.64 $\pm$ 0.05	4.39 $\pm$ 0.06	4.41 $\pm$ 0.07*
PN7	11.0 $\pm$ 0.2	10.8 $\pm$ 0.3	10.3 $\pm$ 0.2	10.6 $\pm$ 0.2
PN14	23.0 $\pm$ 0.5	23.2 $\pm$ 0.6	22.0 $\pm$ 0.4	22.7 $\pm$ 0.5
PN35	81 $\pm$ 1	77 $\pm$ 2	74 $\pm$ 1	72 $\pm$ 1*
2 months	217 $\pm$ 3	194 $\pm$ 6 <sup>†</sup>	155 $\pm$ 2	148 $\pm$ 2*
3 months	300 $\pm$ 2	296 $\pm$ 6	203 $\pm$ 3	204 $\pm$ 2*
4 months	351 $\pm$ 2	351 $\pm$ 7	225 $\pm$ 2	226 $\pm$ 4*
6 months	414 $\pm$ 6	413 $\pm$ 8	248 $\pm$ 3	248 $\pm$ 3*
9 months	453 $\pm$ 7	445 $\pm$ 10	257 $\pm$ 4	256 $\pm$ 3*
12 months	495 $\pm$ 8	493 $\pm$ 11	267 $\pm$ 5	267 $\pm$ 3*

\* $P < 0.05$  vs male (main effect by two-way ANOVA) and <sup>†</sup> $P < 0.05$  vs male control (Student's  $t$ -test following observation of significant interaction).

males at 6 and 12 months while relative pancreas and total hind limb leg muscle weights increased at 6 and 12 months of age respectively ( $P < 0.05$ , Table 3). Absolute pancreas weight increased with age in F2 males but only

increased from PN35 to 6 months in F2 females ( $P < 0.05$ , Table 3). Absolute and relative dorsal fat weights were increased at 12 months compared with 6 months ( $P < 0.05$ , Table 3).



**Figure 1**

Absolute and fractional growth rates in F2 male and female offspring. Growth rates measured between 2–5 weeks (A and B), 5–8 weeks (C and D), and 8–12 weeks (E and F). All data are expressed as mean  $\pm$  S.E.M.;

$n = 10\text{--}12/\text{group per sex}$ .  $^{\gamma}P < 0.05$  vs male control (Student's *t*-test following observation of significant interaction).



**Table 3** F2 male and female offspring organ weights. All data are expressed as mean  $\pm$  s.e.m.;  $n = 12\text{--}17/\text{group per sex per age}$ 

	Male		Female	
	Control	Restricted	Control	Restricted
<b>PN35</b>				
Pancreas (g)	0.360 $\pm$ 0.030 <sup>a</sup>	0.403 $\pm$ 0.029 <sup>a</sup>	0.350 $\pm$ 0.023 <sup>a</sup>	0.363 $\pm$ 0.027 <sup>a</sup>
Body weight (%)	0.452 $\pm$ 0.041 <sup>a</sup>	0.512 $\pm$ 0.029 <sup>a</sup>	0.482 $\pm$ 0.031 <sup>a</sup>	0.517 $\pm$ 0.030 <sup>a</sup>
<b>6 months</b>				
Pancreas (g)	0.864 $\pm$ 0.041 <sup>b</sup>	0.738 $\pm$ 0.033 <sup>b,†</sup>	0.803 $\pm$ 0.035 <sup>b</sup>	0.705 $\pm$ 0.041 <sup>b,†</sup>
Body weight (%)	0.215 $\pm$ 0.011 <sup>b</sup>	0.182 $\pm$ 0.008 <sup>b,†</sup>	0.315 $\pm$ 0.012 <sup>b</sup>	0.277 $\pm$ 0.016 <sup>b,†,‡</sup>
Liver (g)	11.4 $\pm$ 0.2	11.3 $\pm$ 0.3	8.45 $\pm$ 0.2	8.05 $\pm$ 0.12 <sup>‡</sup>
Body weight (%)	2.81 $\pm$ 0.03	2.79 $\pm$ 0.02	3.30 $\pm$ 0.07	3.16 $\pm$ 0.03 <sup>‡</sup>
Dorsal fat (g)	5.14 $\pm$ 0.36	5.22 $\pm$ 0.41	2.71 $\pm$ 0.18	2.67 $\pm$ 0.08 <sup>‡</sup>
Body weight (%)	1.28 $\pm$ 0.09	1.28 $\pm$ 0.09	1.06 $\pm$ 0.07	1.05 $\pm$ 0.03 <sup>‡</sup>
Total hindlimb leg muscles (g)	3.05 $\pm$ 0.07	3.12 $\pm$ 0.07	2.03 $\pm$ 0.08	2.03 $\pm$ 0.03 <sup>‡</sup>
Body weight (%)	0.754 $\pm$ 0.013	0.769 $\pm$ 0.012	0.800 $\pm$ 0.032	0.801 $\pm$ 0.011
<b>12 months</b>				
Pancreas	1.17 $\pm$ 0.07 <sup>c</sup>	1.22 $\pm$ 0.12 <sup>c</sup>	0.728 $\pm$ 0.029 <sup>b</sup>	0.730 $\pm$ 0.027 <sup>b,‡</sup>
Body weight (%)	0.237 $\pm$ 0.013 <sup>c</sup>	0.254 $\pm$ 0.027 <sup>c</sup>	0.273 $\pm$ 0.010 <sup>b</sup>	0.274 $\pm$ 0.009 <sup>b</sup>
Liver (g)	12.6 $\pm$ 0.4	12.4 $\pm$ 0.6	8.05 $\pm$ 0.17	7.92 $\pm$ 0.11 <sup>‡</sup>
Body weight (%)	2.56 $\pm$ 0.07	2.54 $\pm$ 0.07	3.02 $\pm$ 0.06	2.97 $\pm$ 0.03 <sup>‡</sup>
Dorsal fat (g)	9.63 $\pm$ 0.64 <sup>*</sup>	9.92 $\pm$ 0.73 <sup>*</sup>	3.53 $\pm$ 0.18 <sup>*</sup>	3.59 $\pm$ 0.20 <sup>*,‡</sup>
Body weight (%)	1.94 $\pm$ 0.10 <sup>*</sup>	1.99 $\pm$ 0.12 <sup>*</sup>	1.32 $\pm$ 0.06 <sup>*</sup>	1.34 $\pm$ 0.07 <sup>*,‡</sup>
Total hindlimb leg muscles (g)	3.22 $\pm$ 0.09	3.17 $\pm$ 0.09	2.09 $\pm$ 0.03	2.11 $\pm$ 0.04 <sup>‡</sup>
Body weight (%)	0.651 $\pm$ 0.017	0.654 $\pm$ 0.018	0.787 $\pm$ 0.011	0.790 $\pm$ 0.008 <sup>‡</sup>

\* $P < 0.05$  vs 6 months (main effect by three-way ANOVA), <sup>†</sup> $P < 0.05$  vs control (main effect by two-way ANOVA), <sup>‡</sup> $P < 0.05$  vs male (main effect by two-way ANOVA), and significant differences for pancreas weight across age are indicated by different letters, 'a' is different from 'b' and 'c' (one-way ANOVA).

### Basal metabolic parameters and plasma analyses

Nonfasted plasma glucose and insulin levels were not different between F2 control and restricted or between genders at weaning on PN35 (Table 4). Fasting plasma glucose and insulin concentrations, fasting insulin to glucose ratio, and HOMA-IR were not different between F2 control and restricted at 6 and 12 months of age (Table 4). Nonfasted plasma adiponectin and leptin concentrations were not different across groups at PN35 or at 6 months (Table 4). Nonfasted plasma triglyceride concentrations were increased in F2 restricted males at 6 months (+34%) and F2 females at 12 months (+47%) compared with controls ( $P < 0.05$ , Table 4). Nonfasted plasma  $E_2$  was not different between F2 control and restricted females at 6 or 12 months of age (Table 4).

Fasting plasma glucose (+10%) and insulin (+40%) concentrations, fasting insulin to glucose ratio (+30%), HOMA-IR (+47%), nonfasted plasma triglycerides (+35%), and leptin (+50%) concentrations were higher in F2 males compared with their F2 female counterparts at 6 months ( $P < 0.05$ ) but were not different between genders at 12 months (Table 4). Plasma adiponectin concentrations were lower in F2 males compared with F2 female counterparts ( $P < 0.05$ , Table 4).

F2 restricted females at 12 months had increased (+33%) plasma triglyceride compared with their 6-month counterparts ( $P < 0.05$ , Table 4). Basal plasma insulin concentrations and fasting insulin to glucose ratio were increased with age from 6 to 12 months in F2 females ( $P < 0.05$ , Table 4). HOMA-IR was increased with age from 6 to 12 months in both F2 males and females ( $P < 0.05$ , Table 4). Plasma adiponectin concentrations were reduced in F2 male and female offspring at 6 months compared with PN35, but plasma leptin concentrations were increased with age in both F2 male and female offspring ( $P < 0.05$ , Table 4).

### Glucose tolerance, insulin secretion, and whole-body insulin sensitivity

In response to a glucose load, plasma glucose profile was not different across groups at 6 and 12 months (Fig. 2A, B, E and F), as reflected by the area under glucose curve (AUGC) (Fig. 3A and B). Glucose-stimulated insulin secretion was not different between F2 control and restricted (Fig. 2C, D, G and H) but increased with age at 12 months in F2 males and females ( $P < 0.05$ , Fig. 3C and D). The insulin secretory response to glucose, expressed as the ratio of area under insulin curve (AUI) to AUGC, was

**Table 4** Metabolic parameters measured in F2 male and female offspring. All data are expressed as mean  $\pm$  S.E.M.;  $n=8-10$ /group per sex per age

	Male		Female	
	Control	Restricted	Control	Restricted
<b>PN35</b>				
Nonfasted glucose (mmol/l)	9.30 $\pm$ 0.64	8.57 $\pm$ 0.48	9.99 $\pm$ 0.78	10.21 $\pm$ 0.66
Nonfasted insulin (ng/ml)	0.21 $\pm$ 0.03	0.20 $\pm$ 0.02	0.17 $\pm$ 0.02	0.22 $\pm$ 0.01
Adiponectin ( $\mu$ g/ml)	54 $\pm$ 6	48 $\pm$ 7	48 $\pm$ 7	47 $\pm$ 4
Leptin (pg/ml)	1239 $\pm$ 188	1550 $\pm$ 189	1441 $\pm$ 222	1581 $\pm$ 241
<b>6 months</b>				
Fasting glucose (mmol/l)	7.58 $\pm$ 0.39	7.12 $\pm$ 0.25	6.70 $\pm$ 0.28	6.48 $\pm$ 0.28 <sup>†</sup>
Fasting insulin (ng/ml)	0.48 $\pm$ 0.11	0.38 $\pm$ 0.07	0.24 $\pm$ 0.04	0.25 $\pm$ 0.03 <sup>†</sup>
Fasting insulin:glucose ratio	0.063 $\pm$ 0.015	0.052 $\pm$ 0.010	0.034 $\pm$ 0.005	0.041 $\pm$ 0.007 <sup>†</sup>
HOMA-IR	3.84 $\pm$ 0.94	2.97 $\pm$ 0.58	1.75 $\pm$ 0.36	1.72 $\pm$ 0.18 <sup>†</sup>
Triglyceride (mmol/l)	0.792 $\pm$ 0.057	1.063 $\pm$ 0.086 <sup>  </sup>	0.607 $\pm$ 0.057	0.563 $\pm$ 0.044 <sup>†</sup>
Adiponectin ( $\mu$ g/ml)	20 $\pm$ 2 <sup>†</sup>	21 $\pm$ 2 <sup>†</sup>	34 $\pm$ 2 <sup>†</sup>	37 $\pm$ 3 <sup>†,‡</sup>
Leptin (pg/ml)	7541 $\pm$ 789 <sup>†</sup>	6331 $\pm$ 397 <sup>†</sup>	3738 $\pm$ 441 <sup>†</sup>	3510 $\pm$ 418 <sup>†,‡</sup>
Estradiol (pg/ml)	–	–	108 $\pm$ 12	125 $\pm$ 11
<b>12 months</b>				
Fasting glucose (mmol/l)	7.56 $\pm$ 0.53	7.42 $\pm$ 0.31	7.06 $\pm$ 0.27	7.60 $\pm$ 0.39
Fasting insulin (ng/ml)	0.49 $\pm$ 0.06	0.67 $\pm$ 0.14	0.56 $\pm$ 0.09 <sup>§</sup>	0.60 $\pm$ 0.07 <sup>§</sup>
Fasting insulin:glucose ratio	0.065 $\pm$ 0.006	0.093 $\pm$ 0.020	0.074 $\pm$ 0.013 <sup>§</sup>	0.078 $\pm$ 0.011 <sup>§</sup>
HOMA-IR	4.09 $\pm$ 0.87 <sup>§</sup>	5.25 $\pm$ 1.07 <sup>§</sup>	4.14 $\pm$ 0.83 <sup>§</sup>	4.35 $\pm$ 0.55 <sup>§</sup>
Triglyceride (mmol/l)	–	–	0.519 $\pm$ 0.035	0.850 $\pm$ 0.118 <sup>*,§</sup>
Estradiol (pg/ml)	–	–	134 $\pm$ 17	97 $\pm$ 12

\* $P < 0.05$  vs control, <sup>†</sup> $P < 0.05$  vs male (main effect by two-way ANOVA), <sup>‡</sup> $P < 0.05$  vs PN35 (main effect by two-way ANOVA), <sup>§</sup> $P < 0.05$  vs 6 months (main effect by two-way ANOVA), <sup>||</sup> $P < 0.05$  vs male control (Student's *t*-test following observation of significant interaction), and – indicates no measure.

not different between F2 control and restricted or between genders but increased with age at 12 months compared with 6 months ( $P < 0.05$ , Fig. 3E and F).

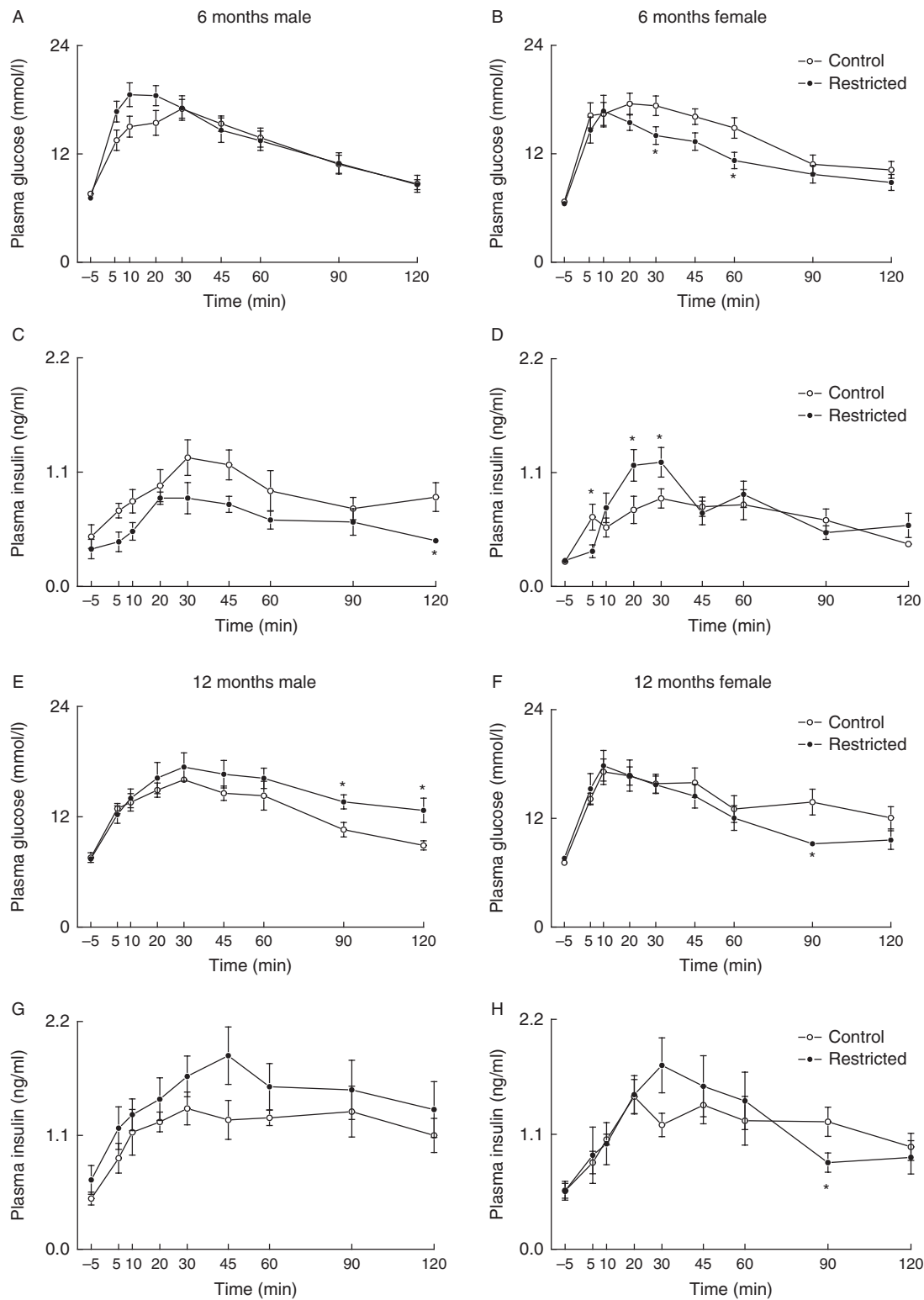
First-phase insulin response, represented as AUC from basal to 5 min, is an indication of  $\beta$ -cell response to glucose during an IPGTT. At 6 months of age, F2 restricted had a significantly reduced ( $-35$  to  $38\%$ ) first-phase insulin response compared with their control counterparts ( $P < 0.05$ , Fig. 4A). First-phase insulin response was higher ( $+28\%$ ) in F2 males compared with F2 females ( $P < 0.05$ , Fig. 4A). At 12 months, first-phase insulin response was not different between F2 control and restricted or between genders but was overall greater compared with 6-month counterparts ( $P < 0.05$ , Fig. 4B). Second-phase insulin response (AUC from 5 to 120 min), an indirect measure of peripheral insulin sensitivity, was not different between F2 control and restricted or between genders at both 6 and 12 months but also increased with age ( $P < 0.05$ , Fig. 4C and D).

Whole-body insulin sensitivity, assessed by the glucose AUC in response to an IC, was not different between F2 control and restricted at both 6 and 12 months but F2 females were more sensitive as indicated by the lower glucose AUC compared with their F2 male counterparts ( $P < 0.05$ , Fig. 4E and F). When compared with 6 months, insulin

sensitivity was reduced as indicated by the greater glucose AUC in both F2 male and female offspring ( $P < 0.05$ , Fig. 4F).

### Pancreatic morphology

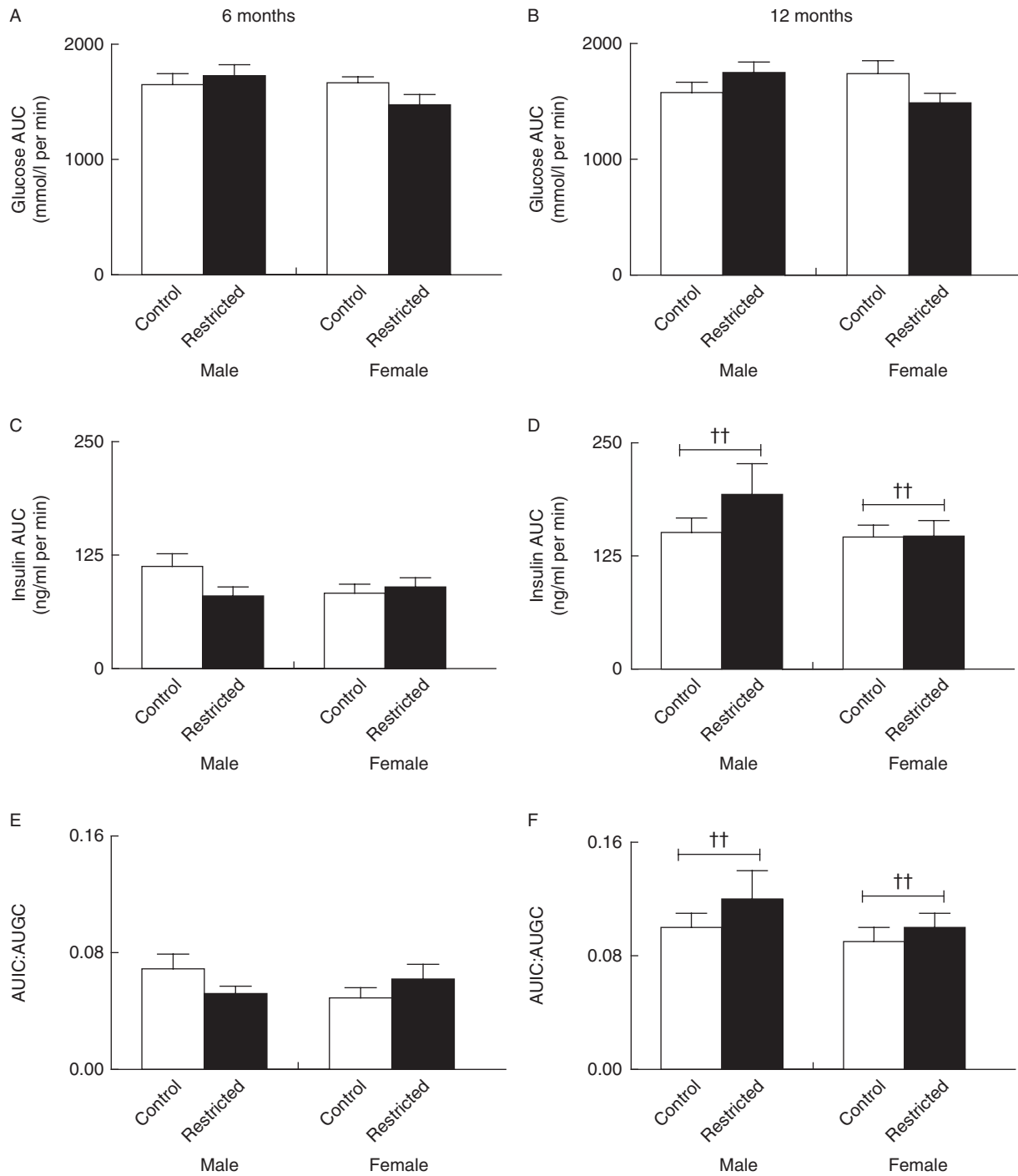
The proportion of  $\beta$ -cells per islet was not different between F2 control and restricted at all ages studied (data not shown). In males,  $\beta$ -cell mass was not different between F2 control and restricted at PN35 or at 12 months but was reduced ( $-29\%$ ) at 6 months ( $P < 0.05$ , Fig. 5A). By contrast, F2 restricted females had elevated  $\beta$ -cell mass at PN35 and 6 months (twofold,  $P < 0.05$ ) compared with controls but was not different at 12 months (Fig. 5B). Islet mass was reduced ( $-40\%$ ) in F2 restricted males and increased ( $+33\%$ ) in restricted females compared with control counterparts at PN35 ( $P < 0.05$ ) but was not different at 6 and 12 months of age (Fig. 5C and D). Average islet size distribution was not different between F2 control and restricted males (Fig. 5E) but the increase in  $\beta$ -cell and islet mass in F2 restricted females may partly be due to increased number medium and large islets ( $P < 0.05$ , Fig. 5F). Pancreatic  $\beta$ -cell and islet mass increased with age in F2 males but only increased from PN35 to 6 months in F2 females ( $P < 0.05$ , Fig. 5C and F), consistent with the observations in pancreatic weight over time (Table 2).

**Figure 2**

Plasma glucose and insulin profile during IPGTT. Plasma glucose profile (A and B) and insulin profile (C and D) at 6 months and at 12 months (E, F, G and H). All data are expressed as mean  $\pm$  S.E.M.;  $n=8-10$ /group per

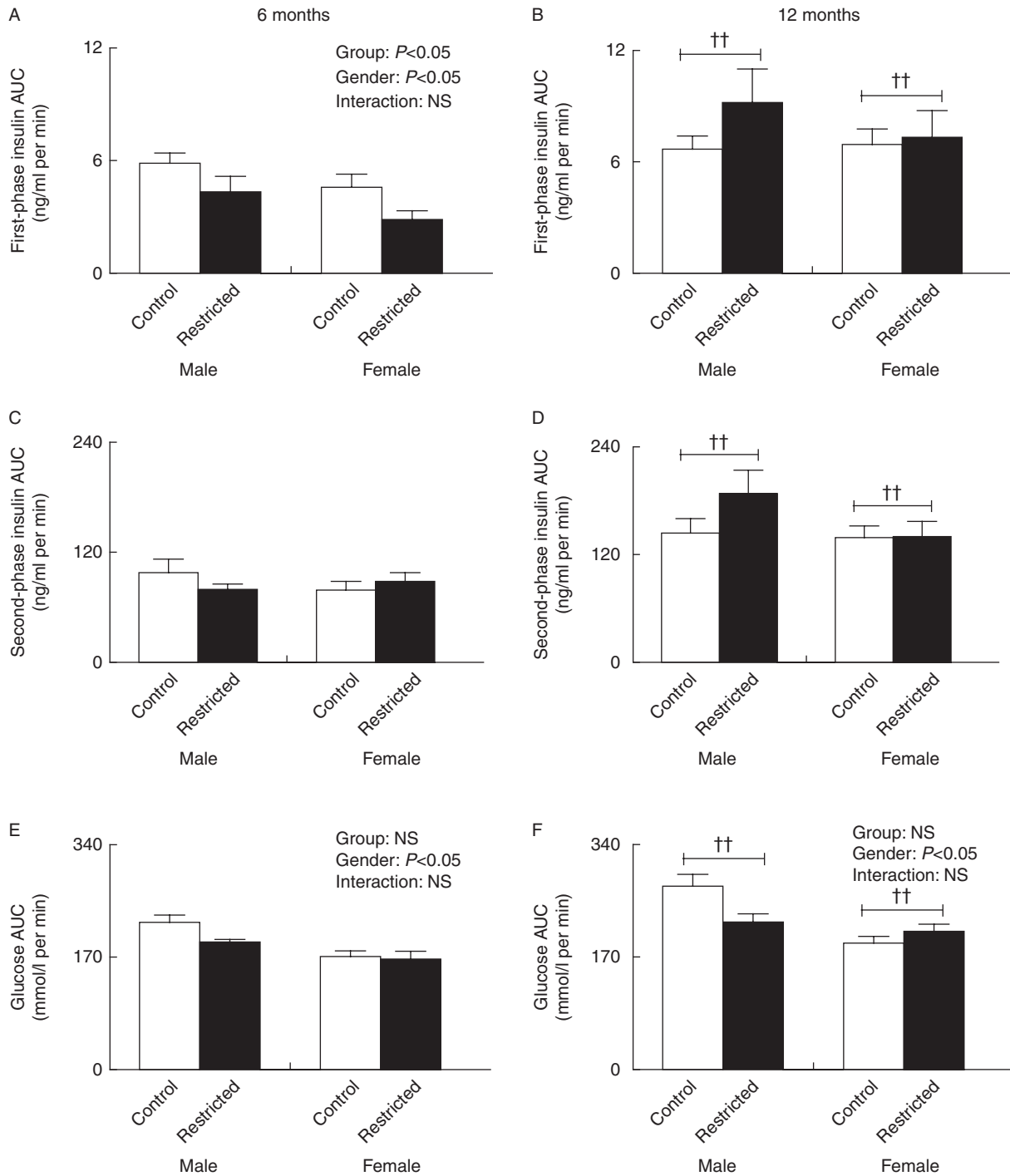
sex per age. \* $P < 0.05$  vs control (Student's unpaired  $t$ -test between control and restricted).



**Figure 3**

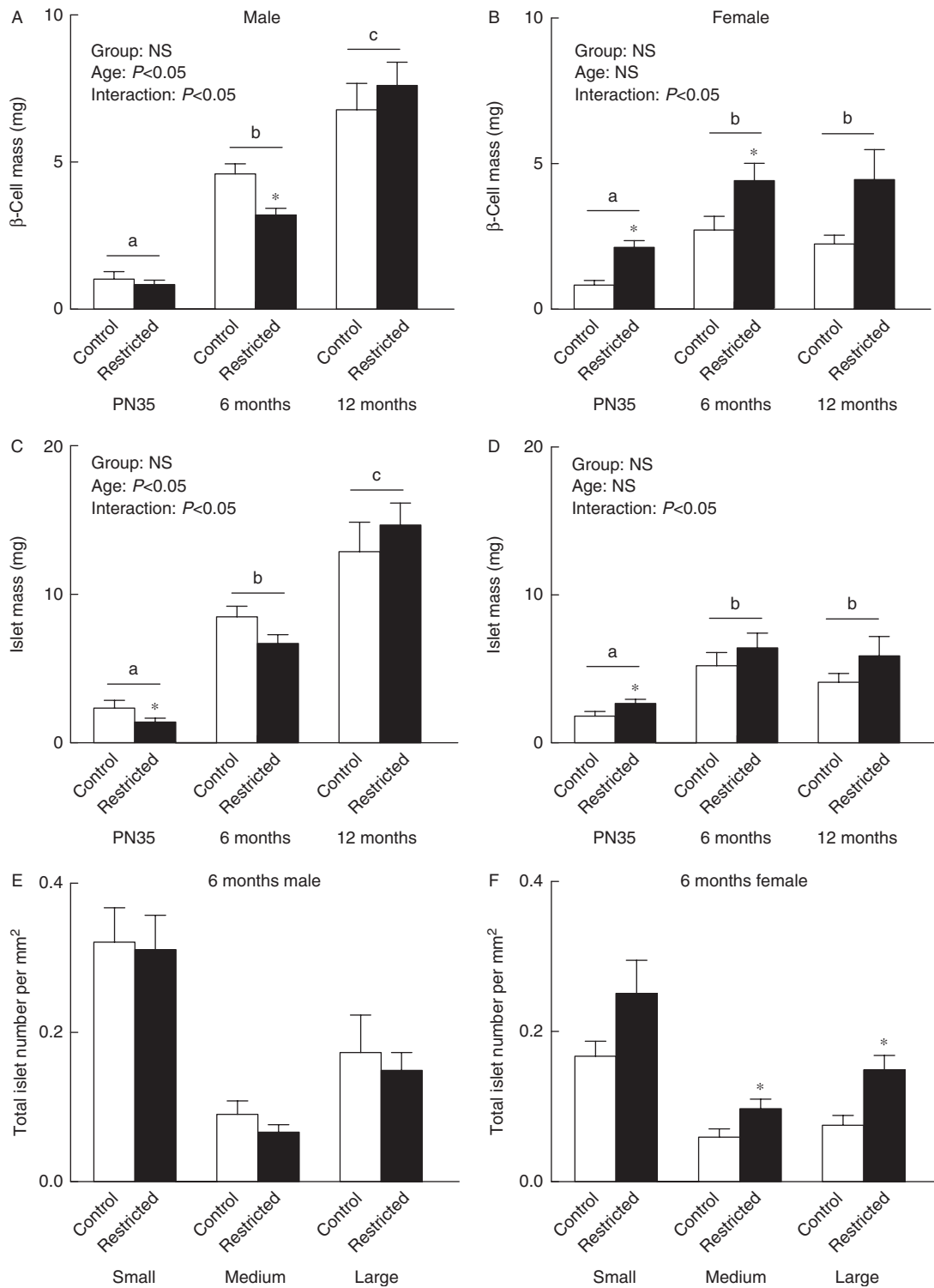
Plasma glucose and insulin response during IPGTT. Plasma glucose AUC (A and B), plasma insulin AUC (C and D), and ratio of AUC:AUGC (E and F). All

data are expressed as mean  $\pm$  S.E.M.;  $n=8-10$ /group per sex per age.  $\dagger\dagger P < 0.05$  vs 6 months (main effect by three-way ANOVA).

**Figure 4**

Insulin secretion and whole-body insulin sensitivity. First-phase insulin secretion (A and B), second-phase insulin secretion (C and D), and whole-body insulin sensitivity (E and F). All data are expressed as mean  $\pm$  S.E.M.;

$n = 8-10$ /group per sex per age. <sup>††</sup> $P < 0.05$  vs 6 months (main effect by three-way ANOVA).

**Figure 5**

Pancreatic morphology.  $\beta$ -Cell mass (A and B), islet mass (C and D), and total islet number per mm<sup>2</sup> (E and F). All data are expressed as mean  $\pm$  s.e.m.;  $n = 5-8$ /group per sex per age. \* $P < 0.05$  vs control (Student's unpaired  $t$ -test

between control and restricted for each age studied) and significant differences across age are indicated by different letters, 'a' is different from 'b' and 'c' (one-way ANOVA).

## Discussion

In this study, we report reduced first-phase insulin response in F2 male and female offspring at 6 months from F1 mothers who were exposed to uteroplacental insufficiency and born small. This was associated with reduced pancreatic  $\beta$ -cell mass in F2 restricted males but increased  $\beta$ -cell mass in F2 restricted females. Regardless of maternal birth weight, F2 control and restricted male offspring had reduced whole-body insulin sensitivity and elevated plasma triglyceride concentrations, compared with female counterparts at 6 months. Furthermore, ageing to 12 months in F2 restricted male and females restored  $\beta$ -cell mass and first-phase insulin response to control levels leading to normal glucose homeostasis with age. These data provide compelling evidence for transmission of pancreatic morphological deficits and blunted first-phase insulin response to F2 restricted male via the maternal line at 6 months. While these deficits were absent with ageing to 12 months, additional lifestyle challenges may predispose offspring from growth-restricted mothers to chronic metabolic disease phenotypes.

Late gestation uteroplacental insufficiency reduced total litter size and body weights at PN1 in F1 offspring compared with those exposed to sham surgery. When these F1 growth-restricted females became pregnant, F2 body weights at PN1 were similar to those born from a normal birth weight mother, consistent with our previous study reporting no differences in F2 fetal weights (Gallo *et al.* 2012b). Interestingly, however, F2 restricted males were 11% lighter than controls at 2 months of age, as a result of slowed growth rate during lactation and around puberty onset. Indeed, we have previously reported that the uterine vessel ligation surgery adversely affects maternal lactogenesis, compromises milk quality and quantity, and reduces calcium transport into milk that restrains F1 postnatal growth (O'Dowd *et al.* 2008). Thus, the compromised early postnatal feeding may subsequently impact on the F1 females' own pregnancy adaptations, regardless of their prenatal exposure to uterine vessel ligation surgery and influencing F2 postnatal growth. Certainly, future studies using cross-fostering may distinguish between prenatal and lactational influences on F1 development and subsequent F2 health. Nevertheless, the slowed growth during lactation and puberty in F2 restricted males highlight a transgenerational programming effect on postnatal growth trajectory (Romano *et al.* 2010), which may have contributed to the blunted insulin profile. From 2 months, F2 restricted male

and female offspring accelerated their growth such that by 3 months they were of similar body weight to controls. While catch-up growth in early childhood has proven beneficial, late accelerated growth may independently increase the risk of metabolic diseases in later life (Eriksson *et al.* 2001).

Previously, we have reported reduced  $\beta$ -cell mass at 6 months of age in F1 restricted males (–40 to 45%) and females (–36%) (Siebel *et al.* 2010, Laker *et al.* 2011, Gallo *et al.* 2012b). Here, we report a similar  $\beta$ -cell mass deficit (–29%) with a trend for reduced islet mass (–21%) in F2 restricted males at 6 months via the maternal line, but not at PN35 or 12 months. Pancreatic  $\beta$ -cell mass positively correlates with glucose-stimulated insulin secretion (Larsen *et al.* 2006, Ackermann & Gannon 2007), and indeed, these morphological deficits observed in F2 restricted males at 6 months of age were associated with reduced first-phase insulin secretory response. The absence of reduced  $\beta$ -cell mass and first-phase insulin response at 12 months suggests that F2 restricted males retain the capacity to adapt to age-related increases in insulin demand and subsequently restore  $\beta$ -cell mass similar to control levels. Furthermore, as  $\beta$ -cell mass was not explored beyond 12 months of age, it is possible that these compensatory mechanisms may not be sufficient as rats age and that the combination of diminished  $\beta$ -cell function and increased insulin resistance may lead to frank decompensation of glucose tolerance. By contrast, our intriguing and novel finding of increased  $\beta$ -cell mass in F2 restricted females occurs from PN35 and was sustained to 6, but not 12 months of age. The morphological changes in F2 restricted females at 6 months were partly attributed to increased mean number of medium and large islets suggesting individual  $\beta$ -cell hypertrophy compared with their control counterparts. The significant reduction in  $\beta$ -cell mass in F2 restricted males and increased in F2 restricted females at 6 months paralleled with trends in islet mass with no differences in the proportion of  $\beta$ -cells per islet between control and restricted of either sex. This argues against transdifferentiation of endocrine cell types; all cell types were likely to have been equally affected during development. Future studies using a separate cohort of animals for islet isolation will assist in defining the intrinsic cell function and mechanisms responsible for the increased  $\beta$ -cell mass in restricted females at PN35 and 6 months of age. It is possible that *Pdx1* expression is elevated in restricted females compared with controls or that the  $\beta$ -cell response to estrogen is heightened leading to increased rates of proliferation.

Gender differences in response to programming influences on glucose and insulin metabolism have been extensively studied with males at greater risk of developing diabetes and insulin resistance (Sugden & Holness 2002, Maloney *et al.* 2011). In our studies, growth-restricted F1 males developed impaired glucose tolerance and reduced insulin secretion at 6 months while females were unaffected (Siebel *et al.* 2008, Wadley *et al.* 2008). During pregnancy at 4 months, however, our F1 growth-restricted females developed loss of glucose tolerance (Gallo *et al.* 2012b), which may have adverse health consequences for the next generation. Indeed, studies have reported that exposure of the fetus to a hyperglycemic environment *in utero* increases risk of diabetes (Nishizawa *et al.* 2002). In this study, however, F2 male and female offspring from growth-restricted, mildly glucose intolerant pregnant mothers exhibited normal glucose tolerance and adequate insulin secretion at 6 and 12 months of age and no evidence of increased adiposity. Given the relatively young age of study (6–12 months), it is likely that insulin demand had not peaked; however, ageing and/or postnatal ‘second hits’ may certainly reveal a clinically relevant metabolic phenotype. Our study is in contrast with previous studies reporting transgenerational persistence of dysregulated glucose and insulin homeostasis in F2 offspring (Boloker *et al.* 2002, Zambrano *et al.* 2005, Benyshek *et al.* 2006). Differences between studies suggest that variables including obesity or adverse diets (high fat/salt) in the F1 mother and F2 offspring may unmask a metabolic phenotype that was not apparent in our model. Additionally, it is possible that the uterine vessel ligation surgery had a direct impact on F1 gametes that gave rise to the F2, which could be confirmed by investigating the paternal line or using an embryo transfer procedure to separate out maternal influences vs embryo-specific effects. The prenatal and lactational environment may also impact on F1 development and subsequent F2 health; therefore, future studies using cross-fostering techniques will distinguish the mechanisms responsible for these changes in the F2 offspring.

Abnormal pregnancy adaptations may be associated with transgenerational effects (Oh *et al.* 1991, Chen *et al.* 2010, Gallo *et al.* 2012a), but inheritance might also contribute to the relationship between birth size and diabetes risk in offspring. In this study, we determined whether metabolic disease risk is evident in F2 male and female offspring in a rat model of uteroplacental insufficiency via the maternal line. We report that low maternal birth weight is associated with gender-specific metabolic alterations in the F2, with reduced first-phase insulin

response and pancreatic morphological deficits in F2 restricted male offspring at 6 but not 12 months of age. In females, the reduced first-phase insulin response was associated with increased rather than reduced  $\beta$ -cell mass at 6 months. Additional lifestyle challenges may predispose these offspring to more severe metabolic dysfunction. The loss of glucose control reported during late pregnancy in our F1 growth-restricted females (Gallo *et al.* 2012b) may have contributed to the F2 metabolic effects reported in this study. However, it is difficult to determine the extent of intrauterine alterations in the F1 mother vs inheritance in the transmission of a diabetic phenotype. Therefore, further studies should distinguish between direct exposure of F1 germ cells to the initial insult, inheritance, and/or an adverse maternal adaptation that leads to disease in subsequent generations.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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