

Sex-Specific Metabolic Outcomes in Offspring of Female Rats Born Small or Exposed to Stress During Pregnancy

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Low birth weight increases adult metabolic disease risk in both the first (F1) and second (F2) generation. Physiological stress during pregnancy in F1 females that were born small induces F2 fetal growth restriction, but the long-term metabolic health of these F2 offspring is unknown. Uteroplacental insufficiency (restricted) or sham (control) surgery was performed in F0 rats. F1 females (control, restricted) were allocated to unstressed or stressed pregnancies. F2 offspring exposed to maternal stress in utero had reduced birth weight. At 6 months, F2 stressed males had elevated fasting glucose. In contrast, F2 restricted males had reduced pancreatic β -cell mass. Interestingly, these metabolic deficits were not present at 12 months. F2 males had increased adrenal mRNA expression of steroidogenic acute regulatory protein and IGF-1 receptor when their mothers were born small or exposed to stress during pregnancy. Stressed control F2 males had increased expression of adrenal genes that regulate androgen signaling at 6 months, whereas expression increased in restricted male and female offspring at 12 months. F2 females from stressed mothers had lower area under the glucose curve during glucose tolerance testing at 12 months compared with unstressed females but were otherwise unaffected. If F1 mothers were either born small or exposed to stress during her pregnancy, F2 offspring had impaired physiological outcomes in a sex- and age-specific manner. Importantly, stress during pregnancy did not exacerbate disease risk in F2 offspring of mothers born small, suggesting that they independently program disease in offspring through different mechanisms. (*Endocrinology* 157: 4104–4120, 2016)

Compromised growth in utero contributes to the development of metabolic disturbances such as diabetes, insulin resistance, and obesity in adult life (1–3). Increasing human and experimental evidence suggests that these disease risks are not limited to the first directly exposed

generation (F1) but may be transmitted to subsequent (F2 and beyond) generations (4, 5). In support of this, studies from the Dutch famine demonstrated that perturbations during pregnancy can have long-lasting effects on offspring health that persists across generations (6, 7). Sim-

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Abbreviations: *Agtr1b*, angiotensin II receptor, type 1b; *Ar*, androgen receptor; AUC, area under the curve; *Cyp21a1*, cytochrome P450, family 21, subfamily a, polypeptide 1; *Cyp11b1*, cytochrome P450, family 11, subfamily b, polypeptide 1; *Cyp11b2*, cytochrome P450, family 11, subfamily b, polypeptide 2; E, embryonic day; GTT, glucose tolerance testing; HOMA-IR, homeostasis model assessment for insulin resistance; HPA, hypothalamic–pituitary–adrenal; *Hsd11b1*, hydroxysteroid dehydrogenase type 1; *Hsd11b2*, hydroxysteroid 11- β dehydrogenase 2; *Igf1r*, IGF-1 receptor; IC, insulin challenge; IPGTT, intraperitoneal GTT; LBW, low birth weight; *Mc2r*, melanocortin 2 receptor; *Nr5a1*, nuclear receptor subfamily 5, group A, member 1; *NrOb1*, nuclear receptor subfamily 0, group B, member 1; *Pdx-1*, pancreatic and duodenal homeobox 1; PN, postnatal day; *Pnmt*, phenylethanolamine-N-methyltransferase; *Srd5a2*, steroid 5 α -reductase 2; *Star*, steroidogenic acute regulatory protein; *Th*, tyrosine hydroxylase.

ilarly, studies in rodents have revealed that a number of pregnancy perturbations can lead to altered glucose and insulin metabolism in next generation offspring (8, 9). Of significant interest, although growth restriction often occurs in the original complicated pregnancy, deficits in birth weight are not always observed in subsequent generations despite offspring presenting with similar physiological outcomes.

Using a well-characterized model of fetal growth restriction (uteroplacental insufficiency) in the rat, we have demonstrated that impaired glucose tolerance and altered first-phase insulin secretion develop in F1 male offspring, whereas females appear to be protected (10–12). Furthermore, these F1 males have a 40%–45% deficit in pancreatic β -cell mass compared with rats of normal birth weight (13), which is consistent with human studies (14). We have recently published that F2 males and females whose F1 mothers were born growth restricted were not themselves born of low birth weight (LBW) (15). However, first-phase insulin responsiveness in F2 male and female offspring was reduced at 6 months in offspring born to F1 mothers that were exposed to uteroplacental insufficiency. We also demonstrated that F2 male offspring of mothers born small had reduced pancreatic β -cell mass, whereas F2 female offspring had increased β -cell mass at the same age. However, with aging to 12 months, deficits were not evident such that all male and female groups had similar insulin responsiveness and β -cell mass (15). Together, this indicates evidence of sex-specific intergenerational transmission of physiological deficits, but with aging, these deficits also develop in control animals.

Sex-specific outcomes have been well established in the field, with studies consistently reporting males and females presenting with different outcomes even when exposed to the same perturbation (16, 17). In order to further understand the mechanisms of disease transmission across generations in our rodent model of growth restriction, we previously characterized maternal adaptations to pregnancy by conducting a series of physiological measurements (consisting of tail cuff blood pressure analysis, glucose tolerance test [GTT], and metabolic cage measurements) on pregnant dams. Although F1 growth-restricted females do not develop metabolic dysfunction under basal conditions, they developed a loss of glucose control while pregnant (18). This may have adverse consequences for the future health of the developing offspring, because a hyperglycaemic environment in utero is associated with a higher risk of developing glucose intolerance and type 2 diabetes later in life (19, 20). Intriguingly, we observed that F1 growth-restricted females that had these physiological measurements performed had F2 fetuses that were lighter during late gestation than those that did

not have these measurements taken, highlighting that maternal stress may act as a second-hit to these females born small to cause fetal growth restriction (18). Furthermore, performing these measurements during pregnancy adversely impacted upon the long-term health of the mothers (21), raising the possibility that the stress of conducting physiological measurements during pregnancy could also have long lasting consequences for the developing offspring.

Although the role of stress during pregnancy on unmasking programmed diseases in LBW individuals has not been previously investigated, stress alone has been well characterized and often programs offspring diseases. Importantly, the nature and severity of programmed diseases are determined by the model, species, and timing of stress exposure (22–24). Human studies clearly demonstrate that maternal stress during pregnancy increases the risk of having a LBW baby (25–27), whereas other studies have demonstrated that maternal stress induces altered glucose and insulin metabolism in offspring (28, 29). Similarly, rats exposed to various stressors during the last week of pregnancy develop postnatal glucose intolerance at 4 months of age when subjected to 3 weeks of high-fat diet before the GTT (30). In contrast, there have been studies demonstrating that stress during pregnancy has no effect on offspring disease outcomes. Specifically, a human study involving 1952 mother-child pairs found no associations between prenatal maternal psychosocial stress and glucose metabolism in children between 5 and 6 years of age (31). Similarly, in the rat, prenatal stress had no effect on basal plasma glucose or insulin concentrations in either sex at 3 months, although female offspring were hyperinsulinaemic following glucose administration (32). In addition, studies have demonstrated that stress exposure during pregnancy can disrupt glucocorticoid production and adrenal steroidogenesis in subsequent generations (33, 34). Prenatal exposure to glucocorticoids is also detrimental to the hypothalamic–pituitary–adrenal (HPA)-axis function in F1 offspring, but importantly, this can persist to the F2 offspring without further glucocorticoid exposure (35).

Although maternal LBW and prenatal stress are both known to be detrimental to offspring health, no studies have investigated the effects of these 2 conditions in combination, and it is unknown whether they would each exacerbate the effects of the other. Predisposition to disease in the next generation is likely mediated by the severity of the stress as well as the maternal vulnerability to the effects of the stress. We hypothesize that rats born small are more likely to be affected by stress when they themselves become pregnant, and thus stress during pregnancy will act as a

second-hit and program a more severe disease phenotype in F2 offspring.

Materials and Methods

Animal procedures

All experiments were approved by The University of Melbourne Animal Ethics Committee (Ethics number 1011865) and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Female Wistar Kyoto rats were housed in an environmentally controlled room (temperature, 22°C) with a 12-hour light, 12-hour dark cycle and had access to food and tap water ad libitum. F0 rats were mated and allocated into a control (sham surgery) or restricted (uteroplacental insufficiency) group. Pregnant dams were anesthetized with 4% isoflurane and 650 mL min⁻¹ oxygen flow (reduced to 3.2% isoflurane and 250 mL min⁻¹ oxygen flow when suturing). Uteroplacental insufficiency surgery was performed by bilateral uterine artery and vein ligation on embryonic day 18 (E18) of pregnancy (term, E22) as previously described (36). Sham surgery was identical except vessels were not ligated. Pregnant rats were allowed to deliver naturally at term. F1 control and restricted female offspring were allocated to an unstressed or stressed (1 per litter per group) pregnancy group and were mated with a normal male between 17 and 23 weeks of age. Those allocated to the stressed group were subjected to a series of physiological measurements as previously described (18), whereas their unstressed counterparts were left unhandled except for general husbandry purposes throughout pregnancy. Briefly, this involved a tail cuff blood pressure measurement (E18), nonfasted ip GTT (IPGTT) (E18) and 24-hour metabolic cage experiment (E19). F2 offspring were delivered naturally at term as per Figure 1.

Postnatal physiological measurements

Body weights of F2 control and restricted offspring that were unstressed or stressed in pregnancy were measured on postnatal day (PN)1, PN7, PN14, and PN35 and 2, 4, 6, 9, and 12 months. At 6 or 12 months of age, F2 male and female offspring were fasted overnight before an insulin challenge (IC). A tail vein blood sample (300 μL) was taken before a sc bolus injection of insulin (1 U kg⁻¹ body weight, Actrapid, Novo Nordisk Phar-

maceuticals). Blood samples were then collected at 20, 30, and 60 minutes after injection. A week later, a fasted IPGTT was performed. Blood was collected before an ip bolus injection of 50% (weight volume⁻¹) glucose (1 g kg⁻¹ body weight; Pharmed) and blood samples collected at 5, 10, 20, 30, 45, 60, 90, 120, 150, and 180 minutes after injection (10, 13).

Plasma glucose concentrations were measured in duplicate using a scaled-down version of the enzymatic fluorometric analysis (10, 12). Inter- and intraassay variability were less than or equal to 5% and less than or equal to 2%, respectively. Plasma insulin concentrations were measured in duplicate using a rat insulin RIA kit (Millipore, Abacus ALS) (10, 12). Inter- and intraassay variability were 8.5%–9.4% and 1.4%–4.6%, respectively. Fasting (basal) plasma glucose and insulin were taken as the average of 2 time points (5 and 10 min before glucose injection). Glucose and insulin area under the curve (AUC) during an IPGTT were calculated as the total AUC from basal to 120 minutes. Glucose AUC during an IC was measured from basal to 90 minutes (37). The insulin to glucose ratio during an IPGTT was calculated by dividing the total insulin AUC by the total glucose AUC. First-phase insulin secretion during an IPGTT was calculated as the incremental area under the insulin curve from basal to 5 minutes, whereas second-phase insulin secretion was calculated as the incremental area under the insulin curve from 5 to 120 minutes (10, 13). Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the following formula: fasting plasma insulin (μU mL⁻¹) × fasting plasma glucose (mmol L⁻¹)/22.5 (10, 13, 38).

Postmortem tissue collection and corticosterone measurement

F2 offspring were anesthetized with an ip injection of ketamine (100 mg/kg body weight⁻¹) and Illium Xylazil-20 (30 mg/kg body weight⁻¹) and a cardiac puncture performed for the rapid collection of plasma for corticosterone analysis. Dorsal white adipose tissue, liver, pancreas, and adrenal glands were excised, weighed, and then snap frozen in liquid nitrogen and stored at -80°C. A piece of pancreatic tissue (~1 cm) from the hepatic end was fixed in 10% neutral buffer formalin for histological analysis. Plasma corticosterone concentrations were measured in 6-month-old offspring as previously described (39). Inter- and intraassay coefficients of variation were 6.5% and 5.5%, respectively.

Pancreatic islet, β-cell morphology, and immunohistochemistry

Pancreatic tissue was processed, embedded in paraffin wax, and exhaustively sectioned at 5 μm. Three sections of equal distance apart were immunostained using a guinea pig polyclonal antiinsulin antibody (1:200 dilution; Dako). Random systematic point counting of 50 fields of view was used to determine relative islet and β-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). Because 1-cm³ tissue weighs approximately 1 g, V_d and pancreatic weight were multiplied to determine absolute islet and β-cell mass, expressed in milligrams (11, 13, 40).

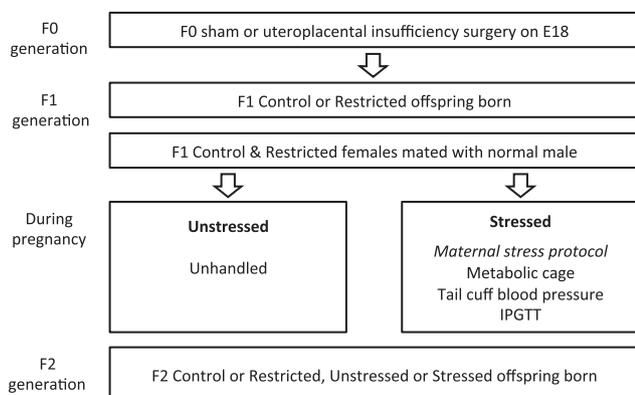


Figure 1. Flow chart illustrating the breeding protocol used to generate F2 offspring.

PCR and Western blotting

Total RNA and protein were extracted from whole adrenal glands and liver. RNA was reverse transcribed using the RT² First Strand cDNA synthesis kit (QIAGEN) as previously described (41). Adrenal mRNA levels of melanocortin 2 receptor (*Mc2r*), steroidogenic acute regulatory protein (*Star*), cytochrome P450, family 21, subfamily a, polypeptide 1 (*Cyp21a1*), cytochrome P450, family 11, subfamily b, polypeptide 1 (*Cyp11b1*), cytochrome P450, family 11, subfamily b, polypeptide 2 (*Cyp11b2*), hydroxysteroid 11- β dehydrogenase 2 (*Hsd11b2*), angiotensin II receptor, type 1b (*Agr1b*), phenylethanolamine-N-methyltransferase (*Pnmt*), IGF-1 receptor (*Igf1r*), and tyrosine hydroxylase (*Th*) were analyzed using quantitative PCR on RT² Profiler Arrays (QIAGEN). In addition, mRNA levels of androgen receptor (*Ar*) (primer pair 1), GATA-binding protein 2 (*Gata2*) (primer pair 1), nuclear receptor subfamily 0, group B, member 1 (*Nr0b1*) (primer pair 1), steroid 5 α -reductase 2 (*Srd5a2*) (primer pair 1), and nuclear receptor subfamily 5, group A, member 1 (*Nr5a1*) (primer pair 1) were analyzed using KiCqStart predesigned primer pairs (Sigma Life Science). Adrenal gland gene expression was normalized to the geometric mean of 3 revalidated endogenous controls (peptidylpropyl isomerase B [*Ppib*], 18S ribosomal RNA [*Rn18S*], and glucuronidase, β [*Gusb*]). Liver mRNA was analyzed using the following TaqMan, primer-probe sets (Life Technologies): hydroxysteroid dehydrogenase type 1 (*Hsd11b1*), Rn00567167_m1; heat shock protein 90- α (cytosolic), class A member 1 (*Hsp90a1*), Rn00822023_g1; nuclear receptor subfamily 3, group C, member 1 (*Nr3c1*), Rn00561369_m1; and the gene that encodes the corticosterone binding protein, serpin peptidase inhibitor, clade A (α -1 antiproteinase, antitrypsin), member 6 (*Serpin6a*), Rn01517119_m1. These assays were performed in multiplex reactions using 18S ribosomal RNA levels as the endogenous control (catalog number 4308329; Life Technologies). Gene expression within the adrenal and liver were both normalized to the average of the unstressed control group and determined using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method.

Twenty micrograms of denatured protein were loaded per sample for analysis of relative protein expression using SDS-PAGE and Western blotting as described previously (42). Membranes were incubated overnight with either rabbit anti-MC2R (1:500, catalog number sc-13107; Santa Cruz Biotechnology), rabbit anti-STAR (1:1000, catalog number ab96637; Abcam), and rabbit anti-IGF1R (1:1000, catalog number sc-712; Santa Cruz Biotechnology) antibody. A mouse anti- β -actin (1:10 000, catalog number A5441; Sigma-Aldrich) antibody was used as a loading control.

Statistical analysis

All data were analyzed using a two-way ANOVA to assess the main effects of being born small due to uteroplacental insufficiency (group) and maternal stress (stress). If a significant interaction was observed, post hoc analysis was performed (Student's unpaired *t* test) to compare individual group means. All data are presented as means \pm SEM, and $P < .05$ was considered statistically significant.

Results

Body and organ weights

Maternal stress during pregnancy reduced F2 male (-5% ; $P_{Stress} = .004$) (Figure 2A) and female (-5% ;

$P_{Stress} = .002$) (Figure 2B) birth weight. F2 birth weight was not affected by the F1 maternal birth weight. F2 male body weights were not affected by maternal birth weight or maternal stress at PN7, PN14, and PN35 and 2, 4, 6, 9, and 12 months at postmortem (Figure 2C). However, at 2 months of age, male offspring of mothers born small were heavier than male offspring of mother of a normal birth weight ($+5\%$; $P_{Group} = .041$) (Figure 2C), irrespective of maternal stress. F2 female body weights were not different at PN7, PN14, and PN35 and 6, 9, and 12 months at postmortem (Figure 2D). However, those exposed to maternal stress were lighter at 2 months (-5% ; $P_{Stress} = .028$) (Figure 2D) compared with unstressed counterparts, whereas offspring of mothers born small were heavier at 4 months ($+7\%$; $P_{Group} = .018$) (Figure 2D) regardless of maternal stress exposure.

Postmortem pancreas, liver, and dorsal white adipose tissue weights were not different across groups in F2 male or F2 female offspring at either 6 or 12 months (Table 1). Adrenal weights were decreased in F2 male offspring of mothers born small (-8% ; $P_{Group} = .021$) (Table 1) at 12 months. Adrenal weights were not affected by maternal stress or maternal birth weight in male offspring at 6 months of age or in female offspring at both 6 and 12 months of age (Table 1).

Metabolic function

At 6 months, maternal stress exposure increased F2 male basal glucose ($+12\%$; $P_{Stress} = .034$) (Figure 3A). Basal glucose was not affected by maternal birth weight or maternal stress in F2 male offspring at 12 months (Figure 3C) or in female offspring at 6 or 12 months (Figure 3, B and D). Glucose AUC in response to IPGTT was not different across groups in F2 male (Figure 3E) and female offspring at 6 months (Figure 3F) and F2 male at 12 months (Figure 3G). However, glucose AUC was reduced in F2 females prenatally exposed to maternal stress (-10% ; $P_{Stress} = .047$) (Figure 3H) at 12 months of age, and this was irrespective of maternal birth weight. Fasting insulin, the ratio of fasting insulin to fasting glucose, HOMA-IR, and first- and second-phase insulin secretion in response to IPGTT were not affected by maternal birth weight or maternal stress exposure in F2 males and females at 6 or 12 months (Table 2). Glucose AUC in response to an IC was not different in F2 male and females at all ages (Table 2).

Pancreatic β -cell mass at 6 months was reduced in F2 males of mothers born small (-31% ; $P_{Group} = .008$) (Figure 4A), although this was not influenced by maternal stress exposure. Pancreatic β -cell mass was not different across all groups in F2 females at 6 and 12 months (Figure 4, B and D) and also in F2 males at 12 months (Figure 4C).

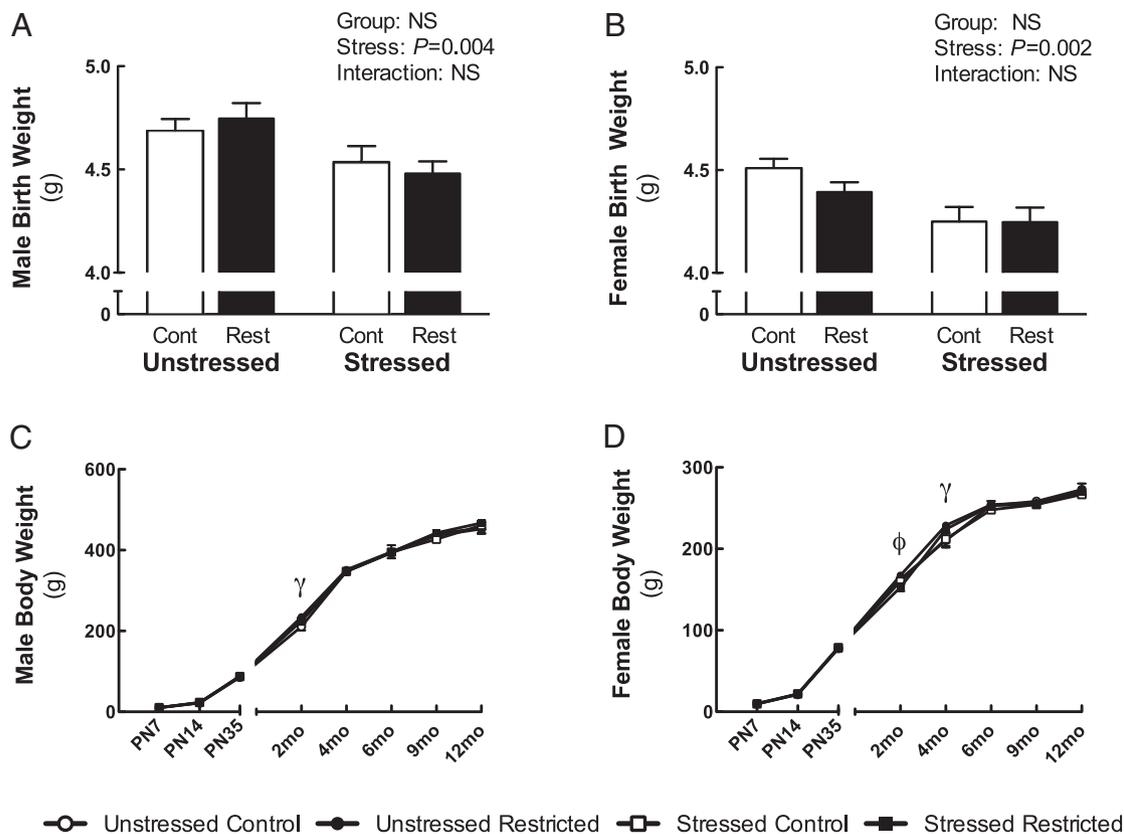


Figure 2. F2 birth weight and postnatal growth profile. Effects of maternal LBW and maternal stress on F2 (A) male birth weight, (B) female birth weight, (C) male postnatal body weights to 12 months, and (D) female postnatal body weights to 12 months (mean ± SEM; n = 10–14 per group). γ , $P < .05$ group effect by two-way ANOVA; ϕ , $P < .05$ stress effect by two-way ANOVA.

Adrenal and liver mRNA and protein expression

At 6 months, adrenal mRNA expression of *Mc2r* was similar in all groups (Figure 5A), but MC2R protein was increased in F2 males that were born to mothers who were small at birth (+15%; $P_{Group} = .03$) (Figure 5B). F2 male

adrenal mRNA expression of *Star* (+18%, $P_{Group} = .04$; +19%, $P_{Stress} = .03$) (Figure 5C) and *Igf1r* (+14%, $P_{Group} = .004$; +18%, $P_{Stress} = .0006$) (Figure 5E) were increased if their mothers were born small or were exposed to maternal stress, although protein levels were not changed

Table 1. Organ Weights Relative to Body Weight (% BW)

	Unstressed		Stressed		Group	Stress	Interaction
	Control	Restricted	Control	Restricted			
Organ weights (% BW)							
Pancreas							
6-month male (n = 8–14)	0.26 ± 0.01	0.26 ± 0.01	0.27 ± 0.02	0.25 ± 0.01	NS	NS	NS
6-month female (n = 9–14)	0.37 ± 0.02	0.36 ± 0.02	0.40 ± 0.02	0.34 ± 0.02	NS	NS	NS
12-month male (n = 4–12)	0.22 ± 0.01	0.23 ± 0.01	0.23 ± 0.02	0.24 ± 0.01	NS	NS	NS
12-month female (n = 7–13)	0.34 ± 0.02	0.30 ± 0.03	0.33 ± 0.02	0.33 ± 0.02	NS	NS	NS
Adrenal							
6-month male (n = 8–14)	0.0115 ± 0.0003	0.0114 ± 0.0009	0.0111 ± 0.0003	0.0114 ± 0.0003	NS	NS	NS
6-month female (n = 9–12)	0.0263 ± 0.0004	0.0267 ± 0.0006	0.0266 ± 0.0004	0.0269 ± 0.0010	NS	NS	NS
12-month male (n = 4–10)	0.0094 ± 0.0004	0.0092 ± 0.0002	0.0100 ± 0.0009	0.0083 ± 0.0003	$P = .021$	NS	NS
12-month female (n = 7–12)	0.0235 ± 0.0009	0.0239 ± 0.0008	0.0224 ± 0.0005	0.0229 ± 0.0007		NS	NS
Liver							
6-month male (n = 8–14)	2.72 ± 0.03	2.74 ± 0.09	2.81 ± 0.06	2.77 ± 0.04	NS	NS	NS
6-month female (n = 11–14)	3.07 ± 0.07	3.14 ± 0.06	3.06 ± 0.04	3.08 ± 0.06	NS	NS	NS
12-month male (n = 4–12)	2.74 ± 0.09	2.64 ± 0.05	2.88 ± 0.22	2.76 ± 0.05	NS	NS	NS
12-month female (n = 7–13)	3.09 ± 0.09	3.01 ± 0.07	2.88 ± 0.07	2.97 ± 0.06	NS	NS	NS
Dorsal white adipose tissue							
6-month male (n = 8–14)	1.20 ± 0.10	1.47 ± 0.10	1.46 ± 0.16	1.55 ± 0.08	NS	NS	NS
6-month female (n = 11–14)	1.07 ± 0.07	1.12 ± 0.08	1.10 ± 0.08	1.13 ± 0.08	NS	NS	NS
12-month male (n = 4–12)	1.92 ± 0.22	1.63 ± 0.17	1.62 ± 0.21	2.03 ± 0.12	NS	NS	NS
12-month female (n = 7–13)	1.37 ± 0.11	1.55 ± 0.16	1.20 ± 0.07	1.42 ± 0.05	NS	NS	NS

Effects of maternal LBW and maternal stress on F2 male and female organ weights relative to body weight at 6 and 12 months (mean ± SEM).

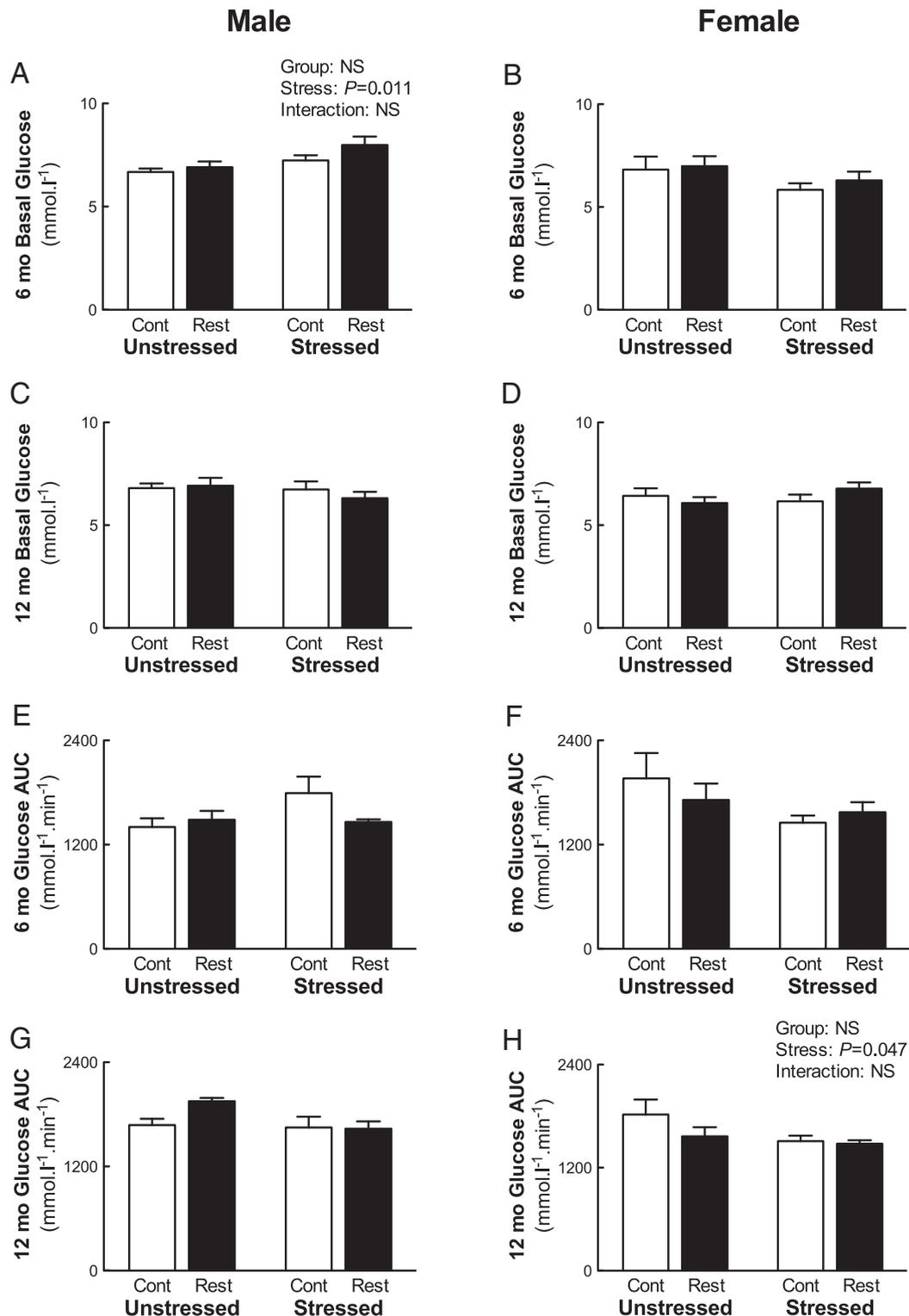


Figure 3. Basal glucose and glucose response to IPGTT. Effects of maternal LBW and maternal stress on basal glucose concentrations of F2 (A) males and (B) females at 6 months, (C) males and (D) females at 12 months, and area under the glucose curve during IPGTT of (E) males and (F) females at 6 months and (G) males and (H) females at 12 months (mean \pm SEM; n = 4–8 per group).

(Figure 5, D and F). *Cyp11b2* mRNA expression was increased if F2 males were exposed to maternal stress during pregnancy (+30%; $P_{Stress} = .002$; Supplemental Table 1). Adrenal mRNA expression of *Cyp21a1*, *Cyp11b1*,

Hsd11b2, *Agtr1b*, *Pnmt*, *Igf1r*, and *Th* were not different across groups at 6 months in F2 males (Supplemental Table 1). Adrenal mRNA expression of *Mc2r*, *Star*, *Cyp21a1*, *Cyp11b1*, *Cyp11b2*, *Hsd11b2*, *Agtr1b*, *Pnmt*, *Igf1r*, and

Table 2. Metabolic Parameters

	Unstressed		Stressed		Group	Stress	Interaction
	Control	Restricted	Control	Restricted			
Male 6 months	n = 6	n = 7	n = 6	n = 6			
Fasting insulin (n = 6–8)	0.71 ± 0.20	0.69 ± 0.19	0.61 ± 0.07	0.67 ± 0.15	NS	NS	NS
Fasting insulin to glucose ratio (n = 6)	0.10 ± 0.03	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	NS	NS	NS
HOMA-IR (n = 6)	5.2 ± 1.5	4.1 ± 1.0	4.7 ± 0.6	5.9 ± 1.6	NS	NS	NS
1st phase insulin AUC (n = 6–7)	3.6 ± 0.9	4.2 ± 0.9	3.5 ± 0.4	4.0 ± 0.8	NS	NS	NS
2nd phase insulin AUC (5–8)	228 ± 38	208 ± 28	216 ± 42	195 ± 22	NS	NS	NS
IC glucose AUC (n = 6–8)	391 ± 27	359 ± 19	360 ± 18	399 ± 25	NS	NS	NS
Female 6 months	n = 6	n = 7	n = 6	n = 6			
Fasting insulin	0.58 ± 0.08	0.53 ± 0.16	0.37 ± 0.06	0.62 ± 0.12	NS	NS	NS
Fasting insulin to glucose ratio	0.09 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	NS	NS	NS
HOMA-IR	4.8 ± 1.0	4.4 ± 1.6	2.6 ± 0.6	3.9 ± 1.1	NS	NS	NS
1st phase insulin AUC	3.7 ± 0.7	3.3 ± 0.9	2.7 ± 0.4	3.3 ± 0.5	NS	NS	NS
2nd phase insulin AUC	99 ± 7	106 ± 10	115 ± 15	112 ± 7	NS	NS	NS
IC glucose AUC	295 ± 11	311 ± 23	320 ± 28	322 ± 40	NS	NS	NS
Male 12 months	n = 8	n = 6	n = 4	n = 7			
Fasting insulin	0.60 ± 0.11	0.51 ± 0.11	0.85 ± 0.41	0.79 ± 0.19	NS	NS	NS
Fasting insulin to glucose ratio	0.09 ± 0.02	0.09 ± 0.02	0.12 ± 0.05	0.12 ± 0.04	NS	NS	NS
HOMA-IR	4.5 ± 1.0	4.3 ± 1.1	6.4 ± 3.4	5.1 ± 1.3	NS	NS	NS
1st phase insulin AUC	2.8 ± 0.6	2.4 ± 0.5	3.6 ± 1.4	3.9 ± 0.5	NS	NS	NS
2nd phase insulin AUC	138 ± 23	107 ± 17	150 ± 26	176 ± 8	NS	NS	NS
IC glucose AUC	338 ± 29	314 ± 35	301 ± 28	333 ± 35	NS	NS	NS
Female 12 months	n = 7	n = 8	n = 6	n = 6			
Fasting insulin	0.74 ± 0.30	0.48 ± 0.08	0.54 ± 0.08	0.51 ± 0.15	NS	NS	NS
Fasting insulin to glucose ratio	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.02	NS	NS	NS
HOMA-IR	3.5 ± 1.3	3.0 ± 0.5	3.8 ± 0.5	3.8 ± 1.3	NS	NS	NS
1st phase insulin AUC	3.1 ± 1.0	2.5 ± 0.5	3.5 ± 0.7	2.8 ± 0.8	NS	NS	NS
2nd phase insulin AUC	122 ± 22	105 ± 15	170 ± 30	120 ± 28	NS	NS	NS
IC glucose AUC	378 ± 32	326 ± 48	267 ± 13	332 ± 41	NS	NS	NS

Effects of maternal LBW and maternal stress on F2 male and female response to IPGTT and IC at 6 and 12 months (mean ± SEM).

Th were not influenced by maternal birth weight or stress during pregnancy in F2 females at 6 months and F2 males at 12 months (Supplemental Table 1). At 12 months, adrenal mRNA expression of *Mc2r*, *Star*, *Cyp21a1*, *Cyp11b2*, *Hsd11b2*, *Agtr1b*, *Pnmt*, *Igf1r*, and *Th* were not different across groups, but *Cyp11b1* was reduced in F2 females exposed to maternal stress (−10%; $P_{Stress} = .0004$) (Supplemental Table 1). Adrenal mRNA expression of *Ar* (+260%; $P = .001$) (Figure 6A), *Nr0b1* (+331%; $P = .01$) (Figure 6C), and *Nr5a1* (+159%; $P = .01$) (Figure 6E) increased in stressed control males at 6 months compared with their unstressed counterparts (*t* test following significant interaction by two-way ANOVA), whereas *Srd5a2* decreased with maternal growth restriction (−42%; $P_{Group} = .01$) (Figure 6G). At 12 months of age, F2 males had increase adrenal expression of *Ar* and *Nr5a1* (+70%, $P_{Group} = .002$ [Figure 6B] and +37%, $P_{Group} = .02$ [Figure 6F], respectively) when born to mothers born small. F2 females had increased adrenal *Ar*, *Nr0b1*, *Nr5a1*, and *Srd5a2* due to maternal LBW at both 6 (+220%, $P_{Group} < .001$ [Figure 7A]; +267%, $P_{Group} < .001$; [Figure 7C]; +147%, $P_{Group} = .005$ [Figure 7E]; +176%, $P_{Group} = .01$ [Figure 7G], respectively) and 12 months (+205%, $P_{Group} < .0001$ [Figure 7B];

+218%, $P_{Group} = .003$ [Figure 7D]; +156%, $P_{Group} = .003$ [Figure 7F]; +150%, $P_{Group} = .02$ [Figure 7H], respectively). In addition, *Nr5a1* expression in these females increased at 6 months (+130%; $P_{Stress} = .048$) (Figure 7E) but decreased at 12 months (−79%; $P_{Stress} = .03$) (Figure 7F) in response to maternal stress exposure. Liver mRNA expression of *Hsd11b1*, *Hsp90*, *Nr3c1*, and *CBG* were not different across groups in males at 6 months (Supplemental Table 1).

Corticosterone concentrations

Given the changes in the adrenal steroidogenic gene expression, plasma corticosterone concentrations were measured at 6 months of age. Plasma corticosterone concentrations at postmortem were not affected by maternal birth weight or maternal stress in F2 male (unstressed control, $182 \pm 16 \text{ ng} \cdot \text{mg}^{(-1)}$; unstressed restricted, $190 \pm 44 \text{ ng} \cdot \text{mg}^{(-1)}$; stressed control, $156 \pm 14 \text{ ng} \cdot \text{mg}^{(-1)}$; and stressed restricted, $196 \pm 19 \text{ ng} \cdot \text{mg}^{(-1)}$) or female (unstressed control, $306 \pm 84 \text{ ng} \cdot \text{mg}^{(-1)}$; unstressed restricted, $474 \pm 185 \text{ ng} \cdot \text{mg}^{(-1)}$; stressed control, $477 \pm$

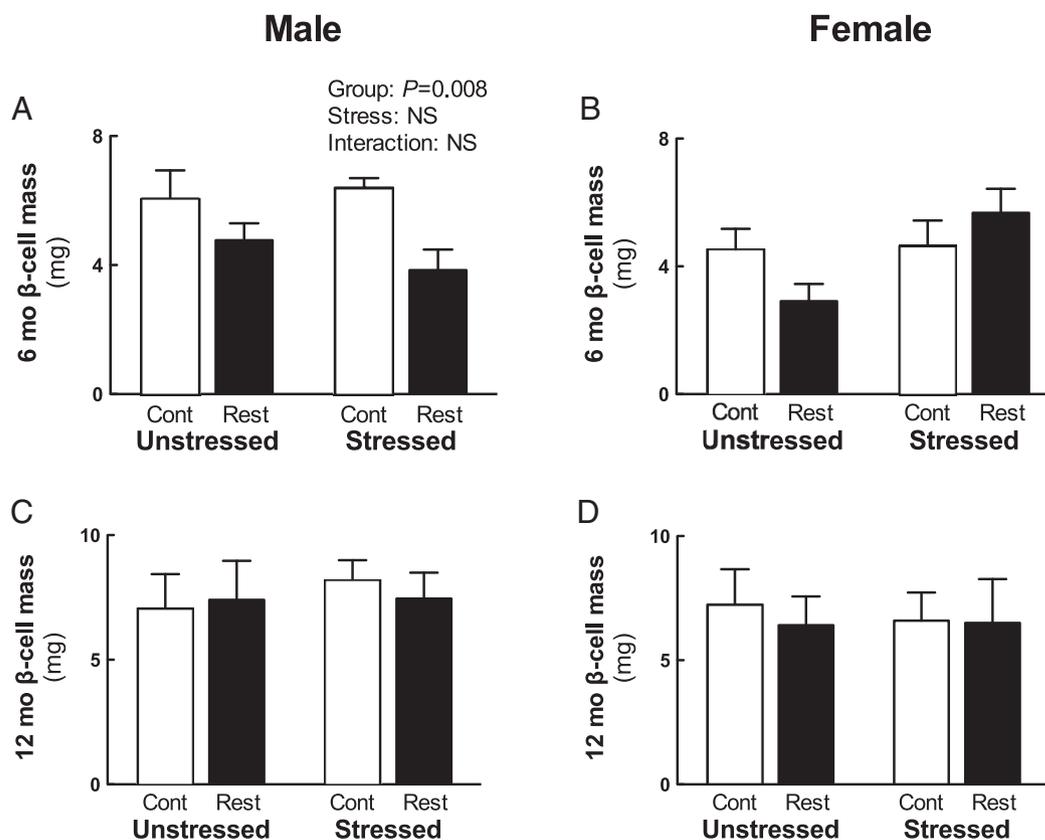


Figure 4. Pancreatic β -cell mass. Effects of maternal LBW and maternal stress on pancreatic β -cell mass of F2 (A) male at 6 months (B) female at 6 months, (C) male at 12 months and (D) female at 12 months (mean \pm SEM; $n = 4$ –7 per group).

237 $\text{ng} \cdot \text{mg}^{-1}$; and stressed restricted, 488 ± 168 $\text{ng} \cdot \text{mg}^{-1}$) offspring.

Discussion

LBW individuals are at an increased risk of developing adult diseases, and these risks can be transmitted to subsequent generations. Females born small are often protected from programmed diseases until they are unmasked by the physiological demands of aging or pregnancy, where programmed outcomes may present as pregnancy complications (18). The risk of developing pregnancy complications in females born small may be further increased by stress during pregnancy, and collectively, this is likely to induce a highly unfavorable in utero environment for the developing fetus (17). We have recently demonstrated in the rat that being born small or being exposed to stress during pregnancy independently programmed long-term adverse health outcomes in the mother after pregnancy (21). The current study expanded on the previous study and aimed to investigate whether the physiological stress encountered during gestation would exacerbate the metabolic disease risks of offspring of females born small, and whether these outcomes would be unmasked or ex-

acerbated with aging. Our results suggest that being born small can induce metabolic effects in F2 male offspring, but that it is not overtly exacerbated if their mother experienced stress during pregnancy and interestingly dissipates with aging. Although these programming outcomes do not progress into overt disease, male offspring develop deficits in both adrenal steroidogenesis and pancreatic β -cell mass. Importantly, F2 females that were subjected to identical prenatal insults did not develop any dysfunction or organ deficits throughout life highlighting sex-specific differences in transgenerational programming. In addition, the current study demonstrated that being born small or being stressed during pregnancy alters the expression of androgen-regulated pathways in the offspring adrenal gland in a sex- and age-dependent manner, suggesting a role for sex steroids in programmed disease outcomes.

We have recently reported that our model of stress during pregnancy increases maternal plasma corticosterone concentrations by approximately 50% at E20 (21). The current study demonstrates that maternal stress reduces birth weight in both male and female offspring, which may be a consequence of the increased maternal corticosterone concentrations. Although the effects of excess maternal

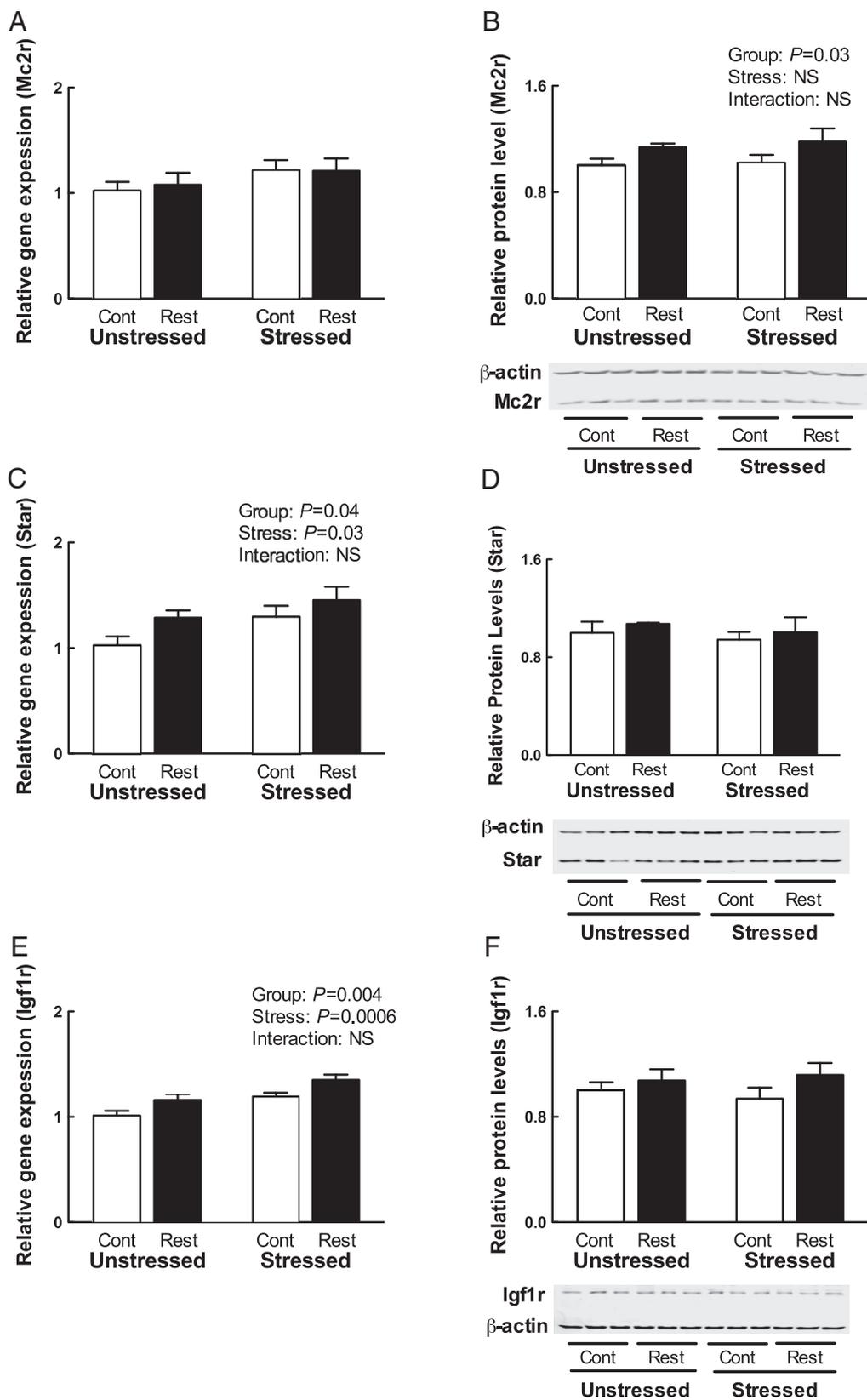


Figure 5. F2 male adrenal gene and protein levels at 6 months. Effects of maternal LBW and maternal stress on adrenal mRNA expression of (A) *Mc2r*, (C) *Star*, and (E) *Igf1r* as well as protein expression of (B) MC2R, (D) STAR, and (F) IGF1R at 6 months of age (mean ± SEM; n = 8 per group for quantitative PCR and n = 6 per group for Western blotting).

Male

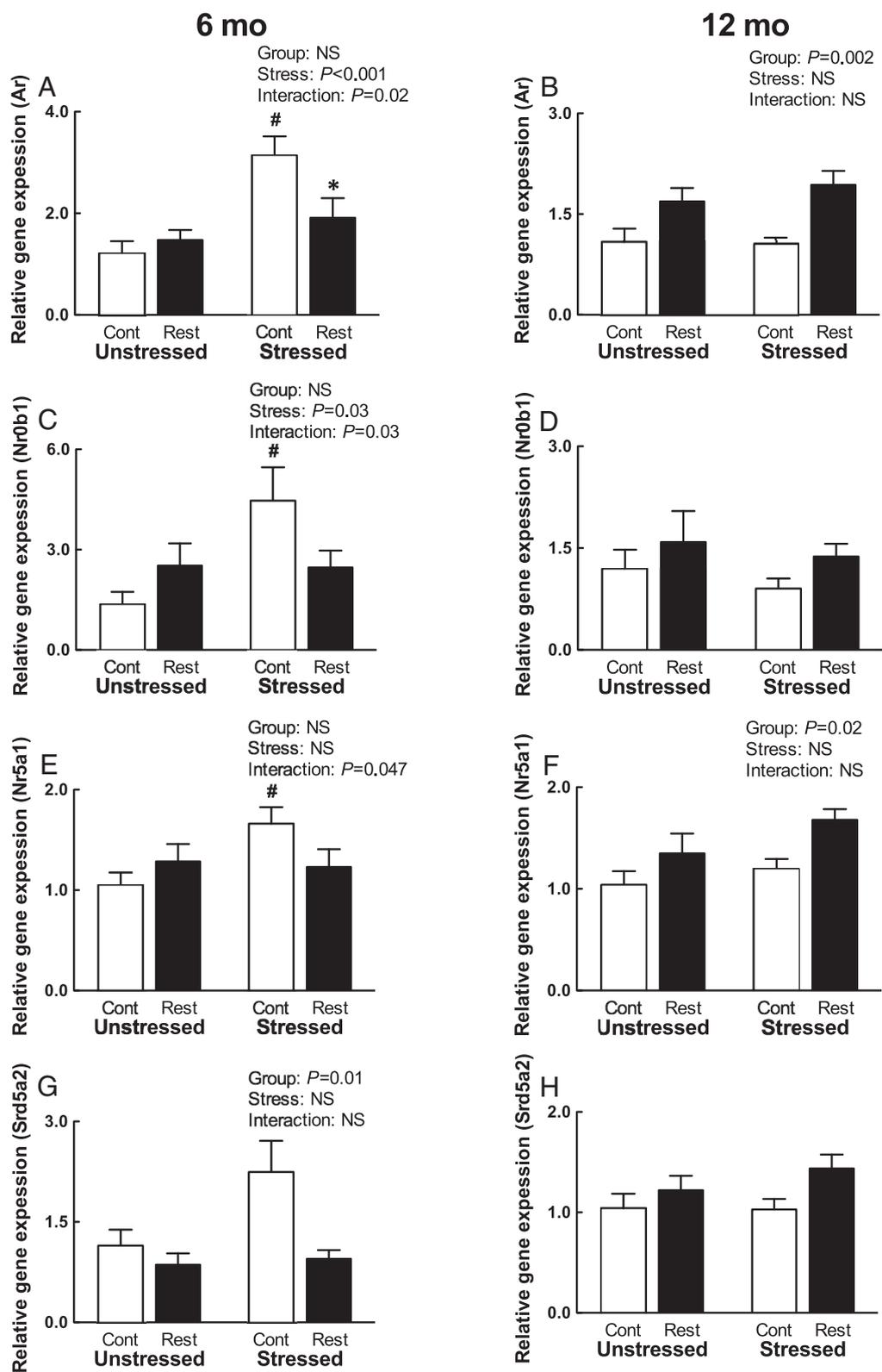
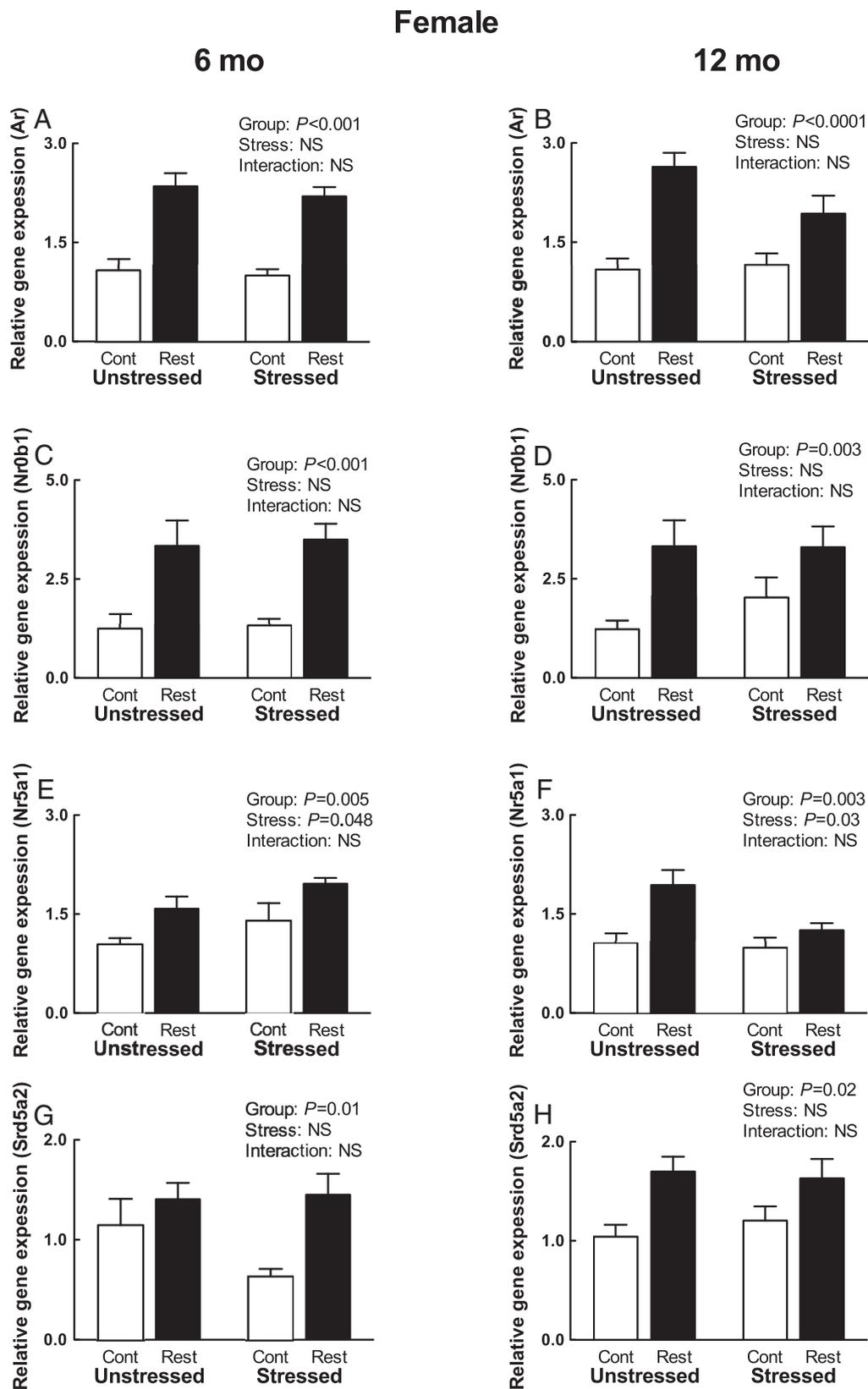


Figure 6. F2 male adrenal androgenic gene expression. Effects of maternal LBW and maternal stress on adrenal mRNA expression of *Ar*, *Nr0b1*, *Nr5a1*, and *Srd5a2* of F2 male at 6 months (A, C, E, and G, respectively) and 12 months (B, D, F, and H, respectively). #, $P < .05$ vs unstressed control (following significant interaction); *, $P < .05$ vs stressed control (following significant interaction).



corticosterone concentrations on fetal development is usually buffered by the placental glucocorticoid barrier, previous studies have demonstrated that this barrier is less effective in late gestation as indicated by reduced *Hsd11b2* mRNA levels and increased placental corticosterone levels at E22 compared with E16 in the rat (43). The late gestation stress exposure in the current study (E18–E20) is likely to have resulted in increased fetal corticosterone exposure during the period of rapid growth that occurs in late gestation. These findings are in agreement with many studies that have demonstrated that sustained prenatal stress or glucocorticoid exposure reduces offspring birth weight and programmed adult diseases (44, 45). Glucocorticoids may directly impair placental function by binding to glucocorticoid-response elements and regulating expression and activity of nutrient transporters and growth factor production that may in turn impair fetal growth (42, 46, 47). In addition, glucocorticoids are known to induce epigenetic changes to key fetal organs and systems, which may have long-term implications throughout life and across generations, although these modifications can continually change in response to postnatal conditions resulting in a variety of physiological outcomes dependent on experiences throughout life (48, 49).

Consistent with our previous findings (15, 50), F2 birth weight was unaffected by maternal birth weight. This was not surprising even though parental birth weight is known to be a strong determinant of offspring birth weight (51, 52), as a multitude of other factors regulate the final birth weight (53–55). Although birth weight is often associated with offspring disease, programmed outcomes often occur in the absence of reduced size at birth (56, 57). Indeed, disease outcomes may be induced through subtle deficits in organ structures or programmed dysregulation of gene expression, each of which can cause unfavorable changes in physiology. Although there were differences between groups in body weights of both F2 males and females at 2 and 4 months of age, overall the growth trajectories of all groups were similar. Importantly, there were no differences in body weight across groups when key measurements were performed at 6 and 12 months of age, and as such, body weight is not likely to play a significant role in programming metabolic disease phenotypes in the current study. At 6 months, F2 male but not female offspring of dams born small had increased adrenal mRNA expression of *Star* and *Igf1r*. Stress during pregnancy also increased the adrenal mRNA expression of both of these factors in male offspring at 6 months, such that the mRNA expression of these 2 factors was highest in male offspring of mothers born small that were also stressed in pregnancy. However, these increases did not translate to significant

changes in protein synthesis. Maternal dexamethasone exposure in the spiny mouse has been shown to program reduced *Star* immunostaining in histological sections of the zona reticularis in the adrenal gland accompanied by reduced plasma levels of Dehydroepiandrosterone in males, but neither *STAR* or Dehydroepiandrosterone was affected in female offspring (58). The current study also demonstrated that F2 male, but not female, offspring of mothers born small had elevated protein expression of *MC2R* in the adrenal gland. This protein is the ACTH receptor responsible for the regulation of overall adrenal corticosterone synthesis. Maternal glucocorticoid exposure in the rat has previously been reported to program elevated *Mc2R* mRNA expression in offspring at 6 months in association with increased plasma corticosterone concentrations (34). Interestingly, no changes in the expression of any adrenal genes or proteins were identified at 12 months of life in this study, suggesting that if the HPA-axis was disturbed at 6 months, the effects did not persist with aging or that the adrenal had made compensatory changes.

Given the increase in the expression of key regulators of corticosterone synthesis in male offspring at 6 months of age, we examined corticosterone concentrations. To our surprise, corticosterone concentrations were not affected by either maternal stress or maternal birth weight. Although these findings were in contrast to the study by Waddell et al (34), they are similar to findings from a model of maternal dexamethasone administration in guinea pigs during gestation, which reported no differences in postnatal basal cortisol concentrations, even when subjected to an ACTH challenge or restraint stress (59). The findings in our current study may be due to the fact our model of stress was relatively mild. Maternal corticosterone concentrations in our rats increased by approximately 50% during late gestation, but this was lower than concentrations previously observed in animals following maternal corticosterone administration (41, 47). In addition, the relatively mild degree of growth restriction (~5%) in our model may not be sufficient to program long-term and permanent HPA-axis impairments.

Importantly, none of the changes in adrenal steroidogenic gene or protein expression observed in our male offspring were identified in female offspring of any group. This is of interest in light of the fact that both male and female offspring of stressed dams were born small. Sex differences in fetal adaptations in response to perturbations are known to increase programmed disease risk in male offspring (60). In utero, males grow at a faster rate than females and continue on this trajectory even in the face of suboptimal conditions, whereas female fetuses are known to alter their growth trajectories and in doing so

adapt to the surrounding environment by investing in long-term survival (61–63). These adaptations can be regulated by the placenta, which itself varies greatly between males and females (64–66). Future studies should investigate the role of the placenta in regulating the sexual dimorphic phenotypes observed, as glucocorticoids have sexually dimorphic consequences on the placenta, although the precise mechanism underlying these differences are not fully understood (41, 61, 67).

Sex-specific adaptations to maternal perturbations may also be mediated by gene dosage effects associated with X chromosome inactivation for genes such as O-GlcNAc transferase or the presence of higher levels of androgens in male fetuses during early life (perinatal androgen imprinting) compared with female fetuses (68, 69). As it is traditionally thought that altered androgen levels underpin many disease states (70–73), we also explored whether there were sex-specific changes to adrenal expression of genes involved in androgen signaling. At 6 months of age, F2 males that were stressed and born to control mothers had increased adrenal *Ar* mRNA expression. Androgens signaling through the adrenal *Ar* is known to regulate the activity of many steroidogenic enzymes (74). This may be due to negative feedback as demonstrated in the *Ar* knockout mouse that has higher levels of ACTH and corticosterone production (75). Interestingly, studies in sheep have demonstrated that prenatal exposure to androgens can program an increase in the mRNA levels of the *Ar* in the ovary (76), suggesting that prenatal events can permanently dysregulate the epigenetic profile of the *Ar*. Indeed, epigenetic dysregulation of the *Ar* may be a potential cause for the sex differences in disease outcomes in the current model and should be investigated in future studies. These changes could potentially be attributed to increased circulating androgens, as testosterone is known to increase adrenal expression of *Ar* (77) such as that observed in the current study. Our current study also demonstrated an increased mRNA expression of *Nr5a1* and *Nr0b1* in F2 males exposed to stress but only if they were born to control mothers. *Nr5a1*, the gene name for steroidogenic factor-1, is an intracellular transcription factor that mediates multiple *Ar*-regulated pathways, including the induction of glucocorticoid production by increasing expression of *Star* (78, 79). However, *Nr0b1*, which encodes dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1, is known to suppress transcription mediated through *Ar* and *Nr5a1* (80). As such, although this pathway is disrupted, the relative contribution to the changes in *Mc2r* and *Star* expression is difficult to ascertain. *Srd5a2*, which is responsible

for the conversion of testosterone to DHT (81), was affected by maternal growth restriction. At 6 months of age, the expression of *Srd5a2* increased in restricted females, which is in direct contrast to the decrease observed in restricted males. In females, the expression of these genes regulating androgen signaling was primarily influenced by maternal birth weight, with these changes again following the pattern of *Ar* expression. This pattern of gene expression was similarly observed in F2 males and females at 12 months, although only *Nr5a1* decreased in response to maternal stress in F2 females. Future studies that measure androgen levels may reveal sex-specific physiological differences that may be driven by the currently measured changes in adrenal gene expression. Consistent with other phenotypes reported in the current study, the dysregulation of genes that regulate androgen signaling does not appear to be exacerbated in the face of both maternal LBW and maternal stress exposure, suggesting once again that the mechanism of disease programming are independent of one another. Quantifying these genes in other organs involved in sex-steroid production such as the testes and ovaries may provide a more comprehensive insight into the way in which maternal growth restriction and maternal stress exposure affects systemic physiological outcomes in a sex-specific manner.

We also investigated the expression of key genes within the liver that are regulated by glucocorticoids to investigate any link between altered corticosterone production or altered glucocorticoid signaling and metabolic dysfunction. Previous studies in sheep have demonstrated that betamethasone exposure during pregnancy resulted in elevated mRNA expression of hepatic *CBG* and *Hsd11b1* in the fetus (82) but did not investigate whether these changes persisted into adult life. In the current study, hepatic *CBG* and *Hsd11b1* in our F2 male offspring were not affected by prenatal stress or maternal birth weight at 6 months despite the fact that these same F2 male offspring had elevated fasting glucose when exposed to maternal stress. This increase in fasting glucose may be attributed to alterations in glucocorticoid and androgen metabolism or signaling that could have resulted from the changes in adrenal gene expression, as changes to either of these pathways are implicated in the development of metabolic diseases (72, 83). Although we did not observe changes to metabolic parameters in F2 females at 6 months, glucose AUC was reduced during IPGTT at 12 months in those exposed to stress, suggesting improved glucose tolerance. This may be due to changes in androgen levels, as

maternal stress exposure similarly resulted in reduced adrenal *Nr5a1* expression, which may in turn result in lower levels of circulating androgens (72).

We have previously demonstrated that F2 male offspring from mothers born small have β -cell deficits at 6 months, which were abolished in later life (15), and similar findings occurred in the present study. Importantly, this effect of maternal growth restriction is in line with the changes we observed in *MC2R*, *Star*, and *Srd5a2*, which are implicated in steroidogenesis and androgen regulation. Although no studies have directly linked changes of these genes in the adrenal gland to morphological changes of the pancreas, it is possible that the pathways mediating these changes in both the adrenal and pancreas are similar. In addition, it may be that these deficits are not directly caused by maternal growth restriction, but rather a consequence of dysregulated glucocorticoid and androgen signaling impacting upon β -cells proliferation or apoptosis. Growth restriction in rats has been shown to permanently reduce expression of pancreatic and duodenal homeobox 1 (*Pdx-1*) in β -cells through epigenetic modifications (84). *Pdx-1* is a transcriptional factor implicated in pancreatic development and normal β -cell function and these epigenetics marks can have long-lasting implications that could be transmitted across generations. Thus, future experiments could determine whether pancreatic *Pdx-1* is altered in our model of growth restriction and stress. It would also be important to determine β -cell proliferation by injecting animals with the proliferative marker bromodeoxyuridine and quantify apoptotic rate to ascertain their relative contribution to the final changes in pancreatic β -cells mass observed. The lack of overt metabolic dysfunction in males born to growth-restricted mothers in the face of this reduction in pancreatic β -cell mass to almost half the amount of control offspring may be explained by the ability of β -cells to adapt according to demand, as evident in a study that reported that pancreatic diabetes manifests only after a reduction in β -cell area of approximately 65% in human patients (85). As individual pancreatic islets were not isolated in the current study, we were unable to determine whether there were changes to insulin secretion of β -cells in response to the deficit. It is possible that hypersecretion could occur as a compensatory mechanism to maintain normal glucose homeostasis. Furthermore, the pancreas is a dynamic organ and β -cells are able to undergo proliferation to meet

increasing metabolic demands (86). This may explain the normalization of β -cells mass to control by 12 months of age observed in these F2 males. However, disruption to normal glucose homeostasis may emerge when insulin demand is chronically increased, resulting in β -cell exhaustion and eventually the failure to compensate (87). Importantly, this normalization is similar to the increase in adrenal gene and protein expression observed in these animals that was subsequently resolved by 12 months. Together, this indicates that male offspring of mothers born small or exposed to mild stress during pregnancy develop structural and molecular organ deficits, which may increase the risk of disease in adulthood. However, in this instance where an organ can adapt, these offspring were able to compensate for these deficits to prevent overt metabolic disease. This highlights that programmed outcomes are not always associated with advanced age but rather that different programmed outcomes can develop over the life course of the animal.

Conclusion

The current study is the first to report offspring sex- and age-specific programming outcome in response to maternal growth restriction and maternal stress during pregnancy. F2 females remained relatively protected from metabolic and adrenal deficits and dysfunction. Contrary to our initial hypothesis, the metabolic, pancreatic, and changes in adrenal genes regulating steroidogenesis observed in F2 males at 6 months were no longer present at 12 months. It is possible that the stressors were not sufficiently severe to cause permanent metabolic diseases in offspring and that a second-hit of obesity or additional lifestyle stressors may reveal overt dysfunction. Importantly, the mild phenotypes induced by maternal stress during pregnancy through commonly used physiological measurement techniques indicate that researchers using these techniques should be aware of the long-term programming effects that may confound results. Critically, stress exposure during pregnancy did not exacerbate the outcomes of offspring of mothers born small, suggesting that they act independently to program diseases. Future studies should elucidate the different mechanisms in which mothers born small and those that are exposed to stress during pregnancy program future diseases in their offspring.

Appendix

Table 3. Antibody Table

Peptide/Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
Insulin		Polyclonal guinea pig antiinsulin	Dako, A0564	Guinea pig; polyclonal	1:200
MC2R		MC2-R (H-70)	Santa Cruz Biotechnology, sc-13107	Rabbit; polyclonal	1:500
STAR		Anti-STAR antibody	Abcam, ab96637	Rabbit; polyclonal	1:1000
IGF-1R		IGF-1R α antibody (N-20)	Santa Cruz Biotechnology, sc-712	Rabbit; polyclonal	1:1000
β -Actin		Monoclonal anti- β -actin clone AC-15	Sigma-Aldrich, A5441	Mouse; monoclonal	1:10 000

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