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A mouse model of gestational glucose intolerance through exposure to a low protein diet during fetal and neonatal development

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Key points

- Pancreatic β-cell dysfunction is hypothesized to be the significant determinant of gestational diabetes pathogenesis, however pancreatic samples from patients are scarce.
- This study reports a novel mouse model of gestational glucose intolerance in pregnancy, originating from previous nutrition restriction *in utero*, in which glucose intolerance was restricted to late gestation as is seen in human gestational diabetes.
- Glucose intolerance was attributed to reduced β-cell proliferation, leading to impaired gestational β-cell mass expansion in maternal endocrine pancreas, in addition to reduced glucose-stimulated insulin secretion.
- This model reproduces some of the features of gestational diabetes and is suitable for testing safe therapeutic interventions that increase β-cell mass during pregnancy and prevent or reverse gestational glucose intolerance.

Abstract Gestational diabetes mellitus (GDM) is an increasingly prevalent form of diabetes that appears during pregnancy. Pathological studies link a failure to adaptively increase maternal pancreatic β-cell mass (BCM) in pregnancy to GDM. Due to the scarcity of pancreatic samples from GDM patients, we sought to develop a novel mouse model for impaired gestational glucose tolerance. Mature female C57Bl/6 mouse offspring (F1) born to dams fed either a control (C) or low-protein (LP) diet during gestation and lactation were randomly allocated into two subsequent study groups: pregnant (CP, LPP) or non-pregnant (CNP, LPNP). Glucose tolerance tests were performed at gestational day (GD) 9, 12 and 18. Subsequently, pancreata were removed for fluorescence immunohistochemistry to assess α-cell mass (ACM), BCM and β-cell proliferation. An additional group of animals was used to measure insulin secretion from isolated islets at GD18.

Sandra Szlapinski received her Bachelor of Science (Honours) in Biology at Western University in London, Canada. She is now a PhD student in the Department of Physiology and Pharmacology and is also in the collaborative program of Developmental Biology at Western University. She is working with Dr. David Hill at Lawson Health Research Institute within St. Joseph's Health Care. Her thesis aims to elucidate the underlying mechanisms of pancreatic β-cell deficiency in gestational diabetes with the ultimate goal of providing a therapeutic intervention to reverse this deficiency and reduce hyperglycaemia in pregnancy.

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LPP females displayed glucose intolerance compared to CP females at GD18 ($P < 0.001$). BCM increased threefold at GD18 in CP females. However, LPP females had reduced BCM expansion ($P < 0.01$) concurrent with reduced β-cell proliferation at GD12 ($P < 0.05$). LPP females also had reduced ACM expansion at GD18 ($P < 0.01$). LPP islets had impaired glucose-stimulated insulin secretion *in vitro* compared to CP islets ($P < 0.01$). Therefore, impaired glucose tolerance during pregnancy is associated with a failure to adequately adapt BCM, as a result of reduced β-cell proliferation, in addition to lower glucose-stimulated insulin secretion. This model could be used to evaluate novel interventions during pregnancy to increase BCM or function as a strategy to prevent/reverse GDM.

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Introduction

Gestational diabetes mellitus (GDM) is a form of diabetes that develops during pregnancy and regresses postpartum. Between 3 and 20% of women develop GDM, depending on their risk factors (Feig *et al.* 2018). GDM increases the risk of the mother developing subsequent type 2 diabetes mellitus (T2DM) by up to 7-fold compared to euglycaemic pregnancies (Melchior *et al.* 2017). For the offspring, exposure to GDM *in utero* has been linked to an increased risk of childhood obesity and development of T2DM (Sauder *et al.* 2017).

GDM develops due to insufficient insulin secretion during the relatively insulin-resistant state in pregnancy (Barbour *et al.* 2007). The state of peripheral maternal insulin resistance is most prominent during the third trimester when placental growth hormone and placental lactogen levels are highest (Handwerger & Freemark, 2000; Newbern & Freemark, 2011). This ensures normal fetal development by maintaining trans-placental flux of glucose to the fetus. Consequently, maternal euglycaemia is normally maintained through adaptations of β-cell mass (BCM) in maternal pancreas.

Both rodent and human β-cells replicate at a low rate in adulthood (~2% per day) (Finegood *et al.* 1995; Bouwens & Pipeleers, 1998). However, the rise in circulating placental lactogen and prolactin during rodent pregnancy has been shown to trigger proliferation of β-cells around gestational day (GD) 12, which increases BCM and enhances insulin secretion (Parsons *et al.* 1992; Beamish *et al.* 2017). In rodents, BCM increases by β-cell replication and hypertrophy, reaching maximal levels towards late gestation (Rieck & Kaestner, 2010; Beamish *et al.* 2017). Elevated maternal oestrogen levels during pregnancy protect β-cells against apoptosis (Banerjee, 2018). As oestrogen levels drop after parturition, β-cell apoptosis increases (Scaglia *et al.* 1995) while β-cell proliferation decreases (Sorenson & Brelje, 1997; Rieck & Kaestner, 2010) returning BCM to pre-pregnancy levels. The compensatory changes in human BCM remain controversial as the dynamics of BCM expansion are hypothesized to be slightly different from those in mouse (Baeyens *et al.* 2016). Nonetheless, the only two human studies conducted to date have both reported an increase in β-cell area in pancreata of pregnant women at postmortem (Van Assche *et al.* 1978; Butler *et al.* 2010). These data suggest that the pancreas of humans, like rodents, should be able to increase BCM and enhance insulin secretion during pregnancy. Consequently, in situations where BCM expansion is suboptimal, GDM can arise (Xiang *et al.* 2013). Thus, murine GDM models characterized by alterations to BCM may relate to the pathology in humans as both animal models and genome-wide association studies in humans implicate β-cell dysfunction as the largest determinant to GDM pathogenesis (Pasek & Gannon, 2013).

There is currently no reproducibly effective prevention or reversal intervention for GDM. As rates of GDM are on the rise, this poses a threat to both the long- and short-term health of the mother and her offspring. Non-invasive imaging to analyse expansion of BCM in human pregnancy has ethical and technical issues making animal models a desirable alternative for studying the mechanisms leading to GDM. Although there are inevitable differences between rodent and human gestation, these differences are well characterized; consequently, rodents are considered to be valid models for studies of pregnancy pathophysiology. One important similarity between rodent and human gestation is that β-cell dysfunction and insulin resistance both play an important role in determining metabolic dysfunction in human and animal models (Pasek & Gannon, 2013). A useful animal model of GDM would not demonstrate pre-gestational diabetes but show abnormal glucose tolerance as pregnancy progressed. As there are currently few clinically applicable animal models of GDM (Jawerbaum & White, 2010) that meet these criteria, we sought to develop a mouse model relevant to the clinical characteristics of GDM through dietary insult.

It has been established that maternal (F0) dietary protein restriction (low protein (LP) diet) during early

life has long-term effects on the endocrine pancreas of the offspring (F1), which contributes to glucose intolerance in adulthood (Fernandez-Twinn *et al.* 2005). Offspring born to dams fed a LP diet have reduced BCM as neonates resulting from decreased rates of β-cell proliferation and increased apoptosis (Petrik *et al.* 1999). Offspring born to dams fed a LP diet also have impaired β-cell insulin release which further contributes to glucose intolerance in adulthood (Wilson & Hughes, 1997). Using this well-characterized model, we examined whether female offspring (F1) of LP diet-fed dams (F0) would develop glucose intolerance during pregnancy, and whether this was associated with an altered adaptation of BCM in maternal endocrine pancreas and/or insulin secretion in isolated islets of Langerhans. We hypothesized that female offspring (F1) of LP diet-fed dams would be glucose intolerant during pregnancy as a result of impaired β-cell plasticity and reduced insulin secretion.

Table 1. Composition (g/100g of diet) of control *vs.* low protein rodent chow (Bio-Serv, Frenchtown, NJ, USA)

Component	Control	Low protein
Cornstarch	40.0	40.0
Casein (88% protein)	22.3	8.6
Maltodextrin	13.2	13.2
Sucrose	10.0	23.6
Soybean oil	4.5	4.5
Cellulose	5.0	5.0
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
tert-Butylhydroquinone	0.0014	0.0014

Methods

Ethical approval

All animal procedures were approved by the Animal Care Committee of Western University in accordance with the guidelines of the Canadian Council for Animal Care (Approval No. 2018-027). Experiments comply with the policies and regulations of *The Journal of Physiology* and with *The Journal*'s animal ethics checklist.

Animals

Adult (6-week-old) C57Bl/6 male and female mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in a temperature-controlled room with 12 h light/dark cycle at Lawson Health Research Institute, London, ON, Canada. Water and food were given *ad libitum*. Timed pregnancies were accomplished by establishing mouse oestrous cycling (Byers *et al.* 2012). Individual female and male mice were housed together for mating and separated the following morning. Day zero of pregnancy was determined by identification of a vaginal plug. Females were housed individually for the remainder of pregnancy. F0 females were randomly allocated to either a control (C, 20% protein, Bio-Serv, Frenchtown, NJ, USA) or low protein diet (LP, 8%) group (Fig. 1*A*), where an increase in carbohydrate in LP diet (Table 1) yields an isocaloric diet compared to control chow (Snoeck *et al.* 1990). F0 dams were fed either the LP or C diet throughout gestation and lactation. A total of 24 C and 21 LP litters were used for the study. Since the primary objective of our study was to produce a novel rodent model of GDM, we worked only with female offspring. On postnatal day (PND) 21, all female offspring (F1) were weaned onto C diet for the remainder of the study while males were euthanized (Fig. 1*B*). At maturity (PND42), female offspring (F1) born to dams fed either a C or a LP diet were randomly allocated into two subsequent study groups: pregnant (CP, LPP) or non-pregnant (CNP, LPNP, Fig. 1*A*). All pregnant grouped females were time-mated with C diet-fed males, separated the following morning and housed individually for the remainder of the experiment. Dams were euthanized by CO_2 asphyxia following an intraperitoneal glucose tolerance test (IPGTT) on a randomly assigned day of gestation (gestational day (GD) 9, 12 or 18) for comparison to non-pregnant age-matched F1 females. Maternal (F1) blood was collected via cardiac puncture following the IPGTT, and serum insulin and glucagon were quantified using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA) or Mouse Glucagon ELISA kit (Crystal Chem). Pancreata were removed for fixation in 4% paraformaldehyde and sectioned for histology as previously described (Beamish *et al.* 2016). At least three 7 µm-thick cryosections (replicates) were cut from each pancreas with an interval between each section >100 µm.

Glucose tolerance tests

An intraperitoneal glucose tolerance test (IPGTT) was performed on all animals in their home cage prior to euthanasia as previously described (Chamson-Reig *et al.* 2009). For the F0 mice, this was 1 month after parturition while the IPGTTs for the F1 mice were performed at the assigned gestational day or age for the age-matched group. Mice were fasted for 4 h with free access to water, injected intraperitoneally with 5 µl g^{-1} body weight of 40% glucose solution (2g kg^{-1} body weight glucose, Sigma-Aldrich, St Louis, MO, USA), and blood glucose measured from the tail at 0, 5, 15, 30, 60, 90 and 120 min using a One Touch

Ultra2 glucometer. Area under the glucose tolerance curve was analysed using Prism software (version 5.0, GraphPad Software Inc., La Jolla, CA, USA).

Immunohistochemistry

Immunofluorescence immunohistochemistry was performed to co-localize insulin and Ki-67 as a marker for insulin-immunopositive cells undergoing proliferation as previously described (Beamish *et al.* 2017). Slides were viewed by a blinded technician using a Zeiss fluorescence Axioskope microscope and cell counting analysis was performed using ImageJ (Schneider *et al.* 2012). Every insulin-expressing cell was imaged at 20× and counted manually. In this study, an 'islet' was considered to contain >5 β-cells, and an extra-islet 'cluster' as containing 1–5 β-cells.

Immunofluorescence immunohistochemistry was also performed to localize insulin (β-cells) and glucagon (α-cells) for morphometric analysis. Antibodies against insulin (1:200, anti-mouse, Sigma-Aldrich, cat. no. I2018, RRID:AB_260137) and glucagon (1:200, anti-rabbit, Santa Cruz Biotechnology, Dallas, TX, USA, cat. no. NB110-41547, RRID:AB_805593) were applied to cryosections and incubated overnight at 4°C. The following day, secondary antibodies (1:500 Thermo Fisher Scientific, Waltham, MA, USA) were applied against the primary antibody using 555 (cat. no. A-31570, RRID:AB_2536180) and 488 fluorophores (cat. no. A-21206, RRID:AB_141708), respectively, along with 4,6-diamidino-2 phenylindole (DAPI, dihydrochloride, 1:500, Thermo Fisher Scientific, cat. no. D3571, RRID:AB_2307445) to counterstain nuclei. α-cell mass (ACM) and BCM were calculated from at least two sections (replicates) per pancreas (n = 4–7 C and LP animals per time point) as previously described (Beamish *et al.* 2017). β-cell size was calculated by taking the sum of the traced insulin-expressing area and dividing by the total number of β-cells counted for that section. Islets were counted per tissue section and further separated by size into small (less than 5000 μm^2), medium (between 5000 and 10,000 μm^2) and large islets (more than 10,000 μm^2). Tissue represented both the head and tail of the pancreas (Chamson-Reig *et al.* 2006).

Islet isolation and static insulin secretion

Pancreatic islets were isolated from CP (n = 7–9 animals) and LPP (n = 6–8 animals) pancreata on GD18 by collagenase V (Sigma-Aldrich) digestion using a modified sequential dextran gradient protocol (Hehmke *et al.* 1986; Cox *et al.* 2013). Islets were incubated (37°C) overnight in RPMI medium containing 6.5 mmol l^{-1} D-glucose, 10% fetal calf serum, and 1% penicillin–streptomycin.

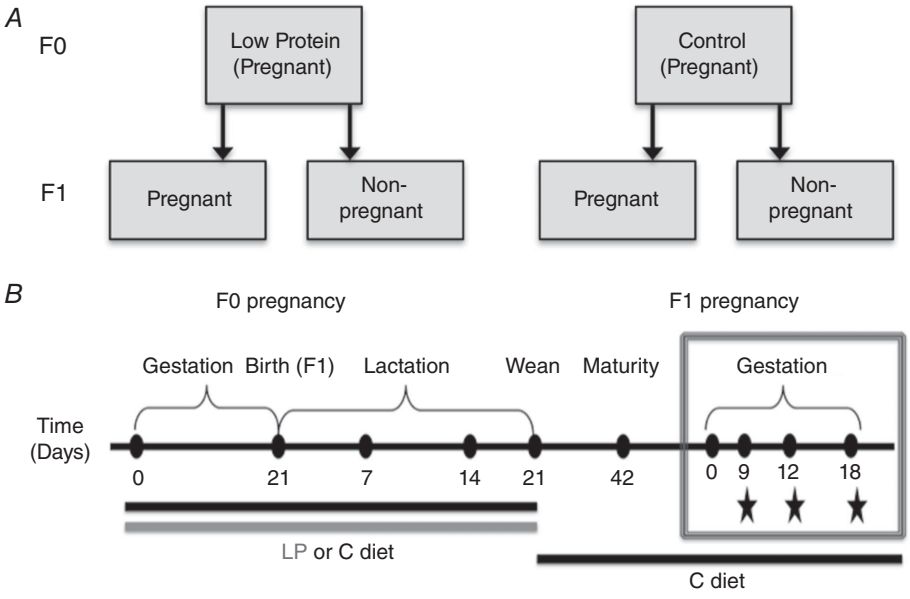

Figure 1. Murine model of gestational glucose intolerance
A, schematic flow chart of experimental groups. F0 females were allocated to LP and C diet groups. F1 female offspring were separated into pregnant, CP and LPP (gestational day 9, 12, 18) and non-pregnant groups (CNP and LPNP). *B*, timeline for treatment and sample collection. The F1 offspring were exposed to the low protein (LP) or control (C) diet during gestation and lactation and weaned onto control diet. At maturity, F1 females were time-mated with control-fed males. Stars demonstrate time points where an intraperitoneal glucose tolerance test was performed prior to euthanasia and removal of the pancreas for histology (n = 4–7 animals for each group). At each time point serum was also collected.

The following day, islets were pre-incubated in Krebs buffer solution (KRB, 119 mmol l^{-1} NaCl, 4.7 mmol l^{-1} KCl, 25 mmol l^{-1} $NaHCO_3$, 2.5 mmol l^{-1} $CaCl_2{\cdot}2H_2O$, 1.2 mmol l^{-1} $MgSO_4{\cdot}7H_2O$, 1.2 mmol l^{-1} KH_2PO_4, 1% bovine serum albumin, 10 mmol l^{-1} Hepes) containing 2.8 mmol l^{-1} glucose for 1 h at 37°C. Groups of 10 islets of similar size were collected into 1 ml of KRB containing either 2.8 or 16.7 mmol l^{-1} glucose (1–3 replicate tubes per animal). Insulin release was determined in the supernatant after 90 min at 37°C using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem).

Statistical analysis

The sample size of four to seven animals per variable in either the LP or C groups was calculated based on achieving a statistically significant difference with an expected standard deviation around mean values for BCM and glucose tolerance of 15% or less based on our previous studies (Beamish *et al.* 2017). Each animal presented as a single unit of analysis (*n*) for the experiments with the F0 C- or LP-fed dams and F1 offspring. For comparisons of litters, each *n* represented an average value for each litter. Data are presented as mean ± SEM, with statistics analysed using GraphPad Prism Version 5.0. An unpaired two-tailed Student's *t* test was used to compare treatment groups (LP *versus* C). A two-way ANOVA followed by Bonferroni's *post hoc* test was used for comparison between treatment groups (LP *versus* C) at each time point during gestation. A repeated measures two-way ANOVA followed by Bonferroni's *post hoc* test was used for comparison of IPGTT curves between treatment groups (LP *versus* C) at each time measurement. Animals with fewer than two fetuses or more than eight were excluded from statistical analyses. Statistical significance was determined as $P < 0.05$.

Results

F0 Animals

No differences were found between consumption of C or LP diet throughout gestation. Maternal weight gain increased steadily in both C- and LP diet-fed dams throughout gestation (Fig. 2*A*). Furthermore, LP diet consumption had no effect on litter size (Fig. 2*B*). There were no differences in the number (Fig. 2*C*) or ratio (Fig. 2*D*) of males to females born to LP *versus* C diet-fed dams. To test for a possible impact of diet on glucose homeostasis, an IPGTT was performed at 1 month post-partum. No differences were found between the IPGTT curves (Fig. 2*E*) or area under the glucose tolerance curves (AUC, Fig. 2*F*) of LP diet-fed females compared to C diet-fed females.

F1 Animals

Offspring born to dams fed a LP diet weighed less at birth (1.25 ± 0.02 *vs.* 1.34 ± 0.03g, $P < 0.05$, unpaired two-tailed

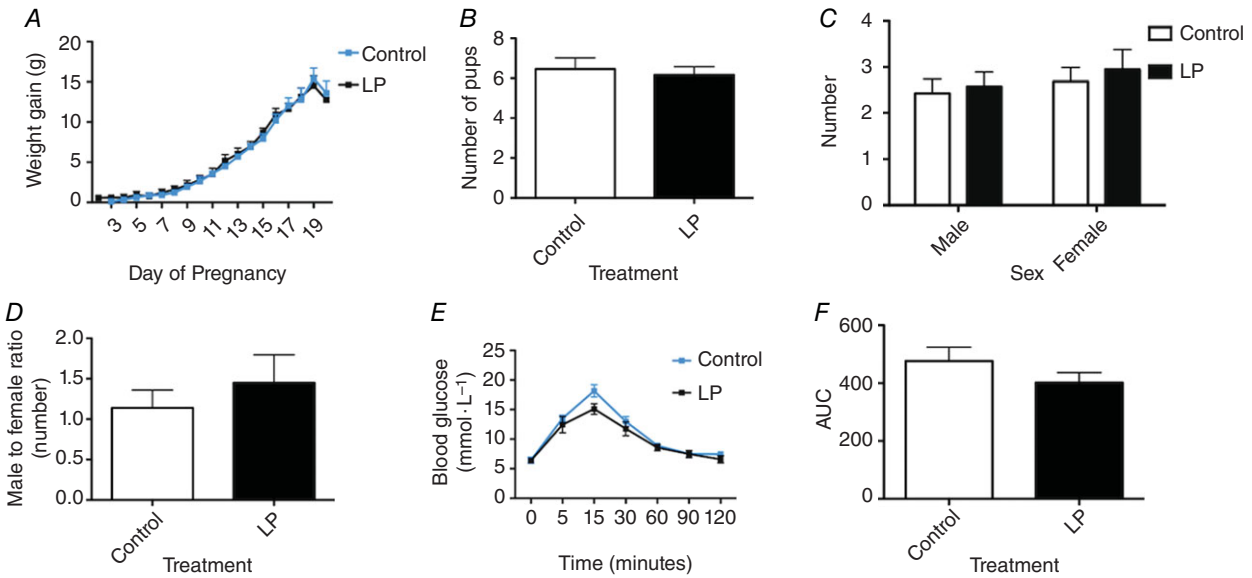

Figure 2. Low protein diet during pregnancy does not alter pregnancy characteristics of F0 dams
A–C, low protein (LP) and control (C) diet-fed F0 dams did not statistically differ in mean values for maternal weight gain (*A*) ($n = 13–14$ animals for each group), litter size (*B*) ($n = 24$ C litters, 21 LP litters), the number of male and female offspring (*C*) ($n = 19$ C litters, 20 LP litters), or the ratio of male to female offspring (*D*) ($n = 19$ C litters, 20 LP litters). *E* and *F*, similarly, glucose tolerance (*E*) and area under the glucose tolerance curve (*F*) did not differ ($n = 8$ C and 7 LP animals for each group). [Colour figure can be viewed at wileyonlinelibrary.com]

Student's *t* test, Fig. 3*A*) and at PND7 (3.56 ± 0.11 *vs.* 4.03 ± 0.15g, $P < 0.05$, unpaired two-tailed Student's *t* test, Fig. 3*A*). Offspring born to dams fed a LP diet continued to weigh less with age, demonstrating significantly reduced body weights compared to controls at weaning, PND21 (8.20 ± 0.34 *vs.* 9.59 ± 0.36g, $P < 0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 3*A*), which persisted until maturity, PND42 (14.81 ± 0.27 *vs.* 16.25 ± 0.19g, $P < 0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 3*A*). The LPP females continued to weigh less than CP females ($P < 0.001$) throughout their own pregnancy (Fig. 3*B*). This was especially apparent during late gestation where the LPP females gained significantly less weight compared to CP females (12.78 ± 1.22 *vs.* 15.24 ± 1.44g, $P < 0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 3*C*). No differences in fetal resorptions were found in LPP females compared to CP females (Fig. 3*D*).

Offspring of low protein-fed mothers are glucose intolerant during their own gestation

There were no significant differences in fasting blood glucose levels between LPP and CP females at any time point during gestation or between CNP and LPNP females. Furthermore, no differences in blood glucose levels or AUC were found for non-pregnant (Fig. 4*A*), GD9 (Fig. 4*B*) or GD12 (Fig. 4*C*) offspring born to dams fed a LP or C diet when subjected to an IPGTT. By GD18, LPP females had significantly higher blood glucose levels compared to CP at 5 min (18.88 ± 2.22 *vs.* 10.73 ± 0.97 mmol l^{-1}, $P < 0.001$, repeated measures two-way ANOVA followed by Bonferroni's *post hoc* test) but no significant difference was found in AUC (Fig. 4*D*).

Offspring of low protein-fed mothers have altered pancreatic morphology during pregnancy compared to controls

Next, we evaluated whether impairments in endocrine pancreas could be contributing to the glucose intolerance that was seen in late gestation of LPP females. Expansion of BCM was maximal on GD18 in CP females (Fig. 5*A*). However, BCM was significantly lower in LPP females compared to CP females on GD18 (0.93 ± 0.16 *vs.* 1.96 ± 0.41g, $P < 0.01$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 5*A*). CP females also showed a maximal peak of ACM on GD18 (Fig. 5*B*). However, LPP females had significantly lower ACM expansion on GD18 compared to CP females (0.17 ± 0.05 *vs.* 0.55 ± 0.17g,

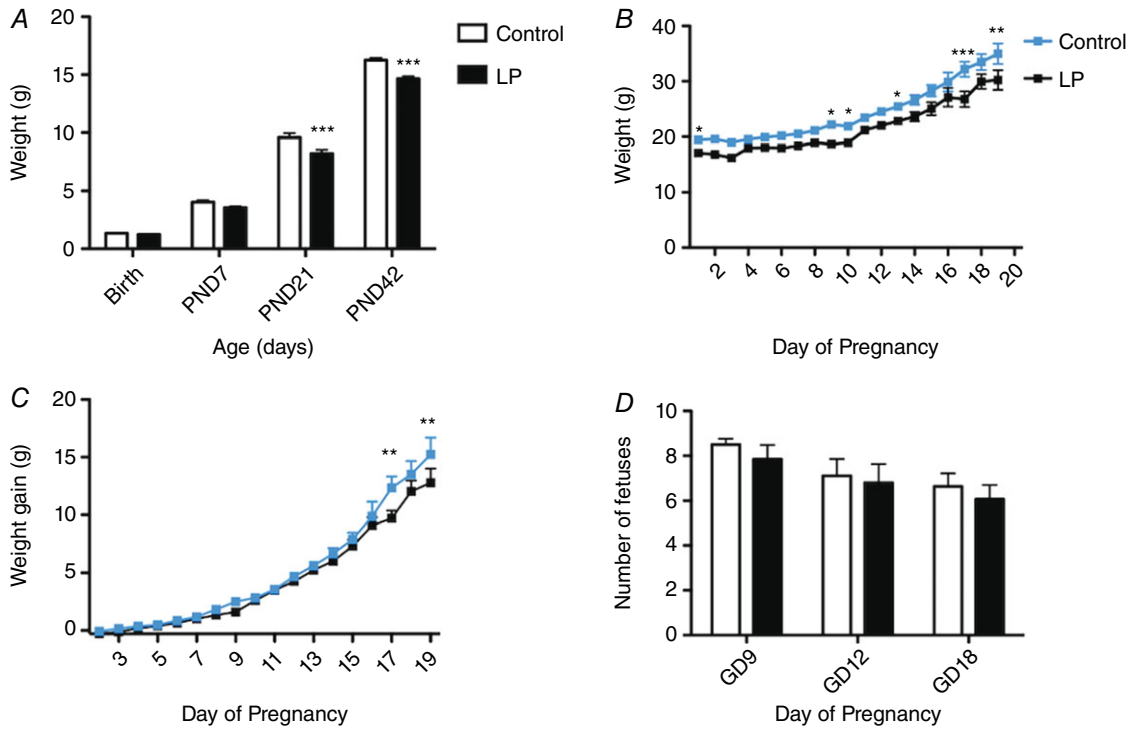

Figure 3. Offspring of LP-fed dams show altered pregnancy characteristics
A, offspring of LP-fed mothers weighed less than controls by weaning (postnatal day (PND) 21, $n = 13–22$ litters for each group). *B* and *C*, LPP females weighed less than CP females throughout gestation (*B*) and put on less weight at late gestation (*C*) compared to CP females ($n = 25$ CP and 24 LPP animals). *D*, the number of fetuses did not differ throughout gestation between CP and LPP females ($n = 7–22$ animals for each group). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, LP *vs.* C. [Colour figure can be viewed at wileyonlinelibrary.com]

$P < 0.01$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 5*B*).

Mechanisms of reduced BCM expansion

To determine whether the reduced BCM was due to decreased β-cell proliferation, we used immunohistochemistry to identify insulin-containing cells co-localized with the DNA synthesis marker Ki-67. Proliferating, insulin-expressing cells were identified in both clusters and islets of offspring born to LP- and C-fed dams. β-cell proliferation increased during pregnancy in CP females but was significantly reduced in LPP females on GD12 (2.11 ± 0.31 *vs.* $3.48 \pm 0.66\%$ $Ki67^{+}Ins^{+}/Ins^{+}$ (total), $P < 0.05$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 5*C*). The reduced β-cell proliferation at GD12 was specific to small β-cell clusters (3.03 ± 1.14 *vs.* $6.47 \pm 1.22\%$ $Ki67^{+}Ins^{+}/Ins^{+}$ (clusters), $P < 0.05$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 5*D*). Representative images of proliferating β-cells (% $Ki67^{+}Ins^{+}$) in non-pregnant, GD12 and GD18 animals are shown in Fig. 6. We found no evidence of co-localized insulin/TUNEL cells (TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling, staining detects apoptosis) during gestation (GD12 and 18) in either the CP or the LPP females. Although there was no effect of treatment on the ratio of α- to β-cells, there was a change with day of pregnancy ($P < 0.001$, two-way ANOVA, Fig. 7*A*). Mean islet size was reduced at GD18 in LPP females (4323 ± 463 *vs.* $7967 \pm 1542\ \mu m^2$, $P < 0.05$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 7*B*); however, there were no differences in β-cell size (Fig. 7*C*). Nonetheless, there was a reduction in the number of small islets in LPP compared to CP females at GD9 (3.80 ± 0.93 *vs.* 12.50 ± 1.43, $P < 0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 7*D*), GD12 (5.92 ± 1.27 *vs.* 11.33 ± 2.32, $P < 0.05$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 7*E*), and GD18 (7.90 ± 1.39 *vs.* 17.88 ± 2.98, $P < 0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 7*F*). No differences in distribution of islet sizes were seen in non-pregnant animals (Fig. 7*G*).

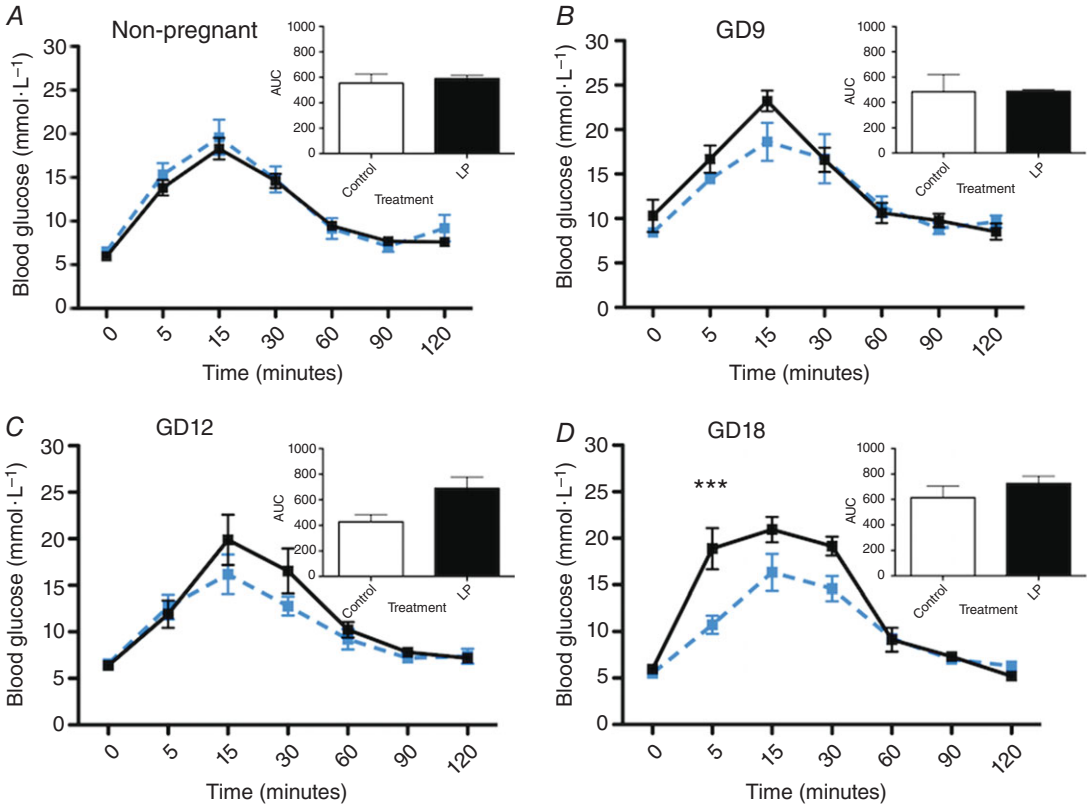

Figure 4. Offspring of low protein-fed mothers are glucose intolerant during their own gestation
Blood glucose (mmol l^{-1}) and area under the glucose tolerance curve (AUC) data from intraperitoneal glucose tolerance tests performed on offspring from low protein (LP, continuous lines) and control-fed (C, dashed lines) mothers. *A–C*, there were no differences between blood glucose and AUC levels of non-pregnant (*A*) ($n = 6$ CNP and 5 LPNP animals), gestational day (GD) 9 (*B*) ($n = 4$ animals for each group), or GD12 (*C*) ($n = 6$ animals for each group) LPP and CP females. *D*, LPP females displayed higher blood glucose levels on GD18 when compared to CP females. However, no differences were found in AUC values ($n = 7$ CP and 4 LPP females). ***$P < 0.001$, LP *vs.* C. [Colour figure can be viewed at wileyonlinelibrary.com]

Offspring of low protein-fed mothers show gestational β-cell dysfunction *in vitro* and *in vivo*

To assess β-cell function, we measured insulin secretion of isolated pancreatic islets from GD18 CP and LPP females. Levels of insulin were similar between LPP and CP females after 90 min in medium containing 2.8 mmol l^{-1} glucose (Fig. 8*A*). However, islets from LPP females secreted less insulin in the presence of 16.7 mmol l^{-1} glucose than CP females (0.22 $\pm$ 0.04 *vs.* 0.49 $\pm$ 0.07 ng ml^{-1} $islet^{-1}$, $P < 0.01$, two-way ANOVA followed by Bonferroni's *post hoc* test, Fig. 8*A*). To confirm these findings *in vivo*, serum insulin was quantified from blood drawn by cardiac puncture following the IPGTT. Confirming the *in vitro* findings, LPP females had lower serum insulin levels compared to CP females on GD18 (0.57 $\pm$ 0.10 *vs.* 1.34 $\pm$ 0.25 ng ml^{-1}, $P < 0.05$, unpaired two-tailed Student's *t* test, Fig. 8*B*). Although serum glucagon levels appeared to be lower in LPP females on GD18, there were no significant differences found when compared to CP females (Fig. 8*C*).

Discussion

This study proposes a novel mouse model of gestational glucose intolerance in which metabolic impairments are restricted to late gestation, as is seen clinically in human GDM. Epidemiological studies in humans have demonstrated strong associations between poor fetal growth, for instance as encountered in times of famine, and susceptibility to metabolic syndrome in adult life (Hales, 1997*a*,*b*). These observations have been replicated in maternal malnutrition studies in animals, resulting in permanent changes in tissue composition and cell size in the offspring during adulthood, ultimately contributing to the metabolic syndrome phenotype (Hales *et al.* 1996). In agreement with the concept of developmental origins of health and disease (Barker & Osmond, 1986), we show in this study that the intrauterine environment influences the risk of metabolic disease in offspring later in life. We previously showed that offspring of LP-fed mothers had reduced BCM and developed glucose intolerance in adulthood (Cox *et al.* 2013). In this study we investigated

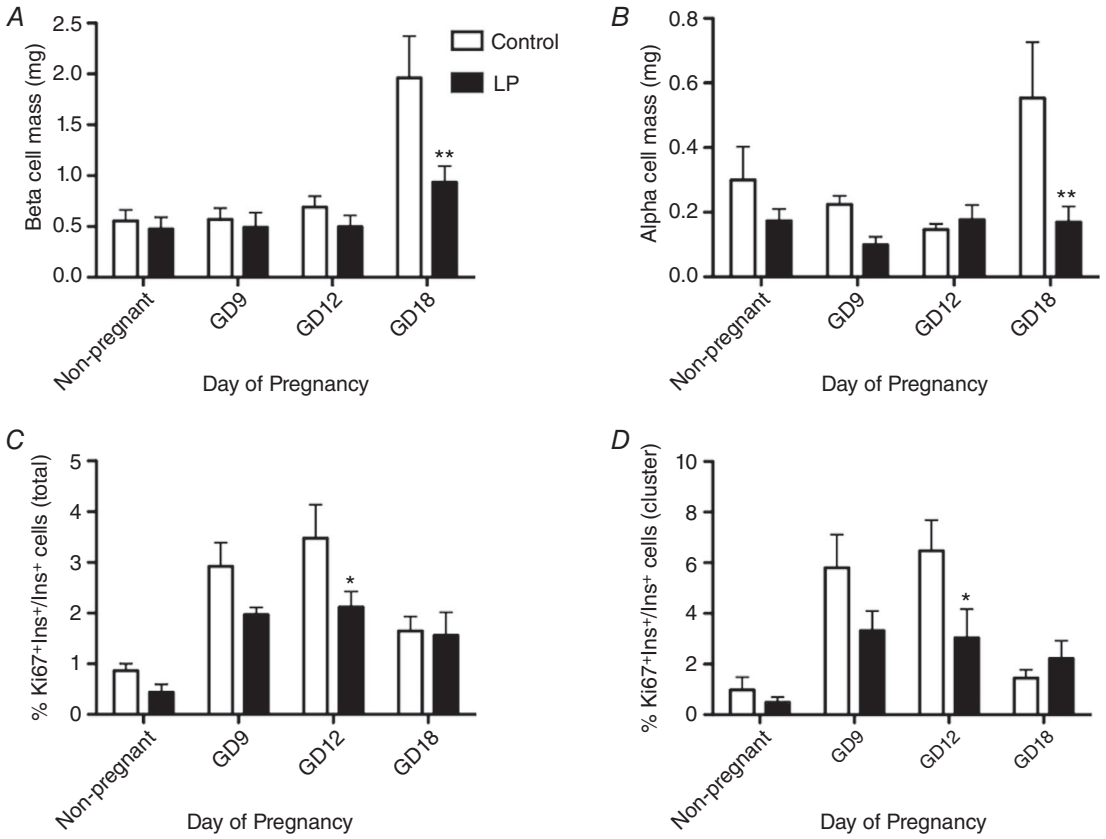

Figure 5. Offspring of LP-fed mothers have altered pancreatic morphology during pregnancy compared to controls
A and *B*, β-cell mass expansion (*A*) and α-cell mass expansion (*B*) were impaired in LPP females compared to CP females on GD18 ($n = 4–6$ animals for each group, 2–3 replicates for each animal). *C*, total β-cell proliferation was reduced during gestation in LPP females on GD12 ($n = 4–7$ animals for each group, at least 2 replicates for each animal). *D*, β-cell proliferation was reduced in clusters during gestation in LPP females on GD12 ($n = 4–7$ animals for each group, at least 2 replicates for each animal). $^{**}P < 0.01$, $^{*}P < 0.05$, LP *vs.* C.

whether offspring of LP-fed mothers would have a predisposition to glycaemic dysfunction during pregnancy and the underlying pancreatic physiology that might contribute towards this phenotype. Overall, LP diet did not impact the pregnancy characteristics of F0 dams as no differences in maternal weight gain, litter size, sex of offspring, or glucose homeostasis were found. Furthermore, it has previously been shown that isolated islets from pregnant LP-fed rats had a similar response to physiological glucose concentrations compared to controls (Milanski *et al.* 2005), while another study concluded that a short period of LP-diet consumption did not alter total area under the glucose and insulin curves during a GTT or basal serum glucose measurements, indicating preservation of pancreatic function (Souza Dde *et al.* 2012). Although this is being extrapolated from rats, we would not anticipate that LP-fed dams in our study would demonstrate gestational glucose intolerance and provide a model for GDM. However, the phenotype was altered in offspring of LP-fed mothers showing a reduced body weight at birth and PND7, which persisted with age and throughout their own pregnancy. Although we do not have data on visceral adipose tissue in our study, a previous study in our laboratory found no differences in visceral adipose tissue between offspring of LP- and C-fed rats at 130 days of age (Chamson-Reig *et al.* 2009). Furthermore, because we are using young, pre-oestropausal mice in our study, we anticipate that there would be no differences in visceral adipose tissue present in our model at this age. In humans, postmenopausal women begin to have increased visceral fat accrual (Camhi *et al.* 2011). Therefore, potential differences in visceral adipose tissue in our model might only be seen at oestropause, which in rodents is at 9–12 months of age (Koebele & Bimonte-Nelson, 2016).

In addition to the physical differences found between offspring of LP- and C-fed mothers, we found maternal glucose intolerance when LPP females were subjected to an IPGTT at GD18. In comparison to our findings, the authors of a similar study using the LP diet model in rats stated that their model did not promote the onset of GDM (Ignácio-Souza *et al.* 2013). However, this claim is made based on AUC data, for which our data are comparable on GD18. Nevertheless, the authors did not include their IPGTT curves, which is where we noted abnormally elevated maternal blood glucose levels in the LPP females. Previous studies found no differences in fasting blood glucose levels in young offspring (PND1, 7, 14 and 30) of LP- and C-fed mothers (Cox *et al.* 2010). Furthermore, LPNP female rodents did not demonstrate glucose intolerance until later in adulthood (Blondeau & Breant, 2005; Chamson-Reig *et al.* 2009; Cox *et al.* 2010),

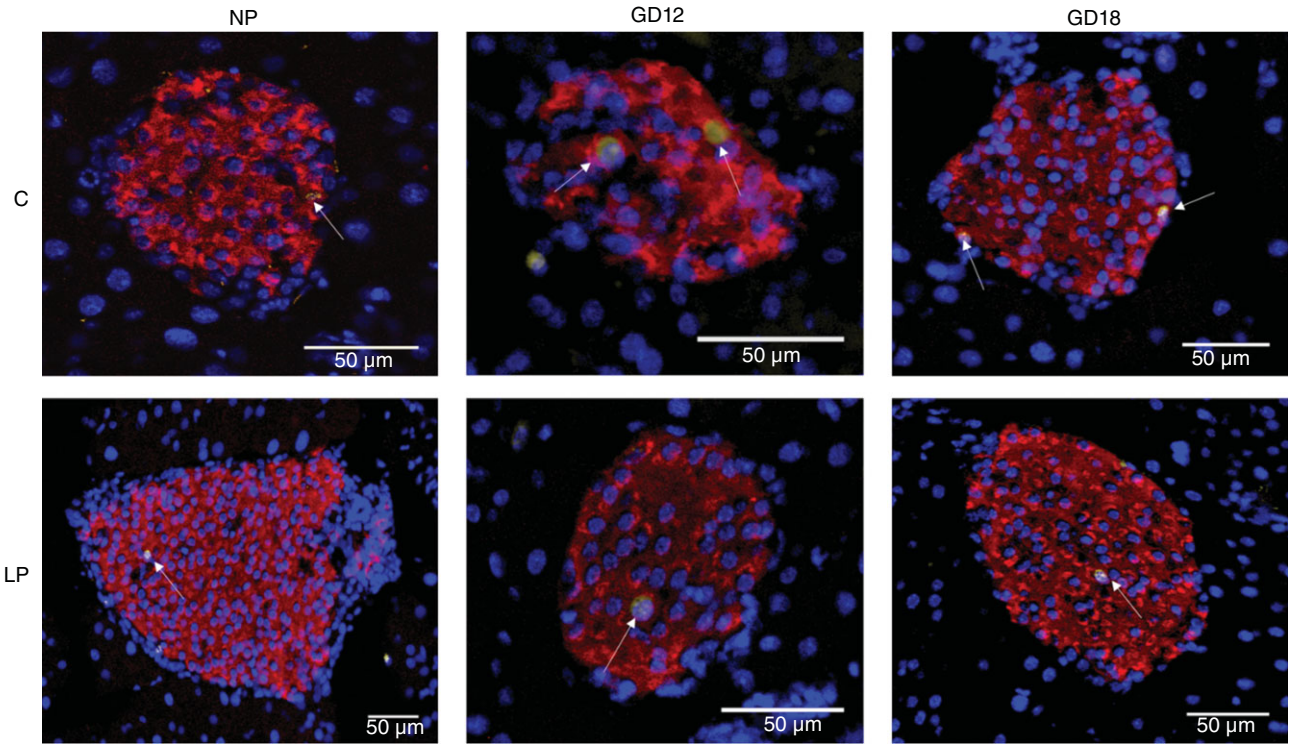

Figure 6. LPP females show reduced β-cell replication at GD12 compared to CP females
Representative immunofluorescence images demonstrating insulin (red), Ki-67 (yellow) and nuclei (DAPI, blue) staining of CNP, LPNP, CP and LPP females (at GD12 and GD18). White arrows demonstrate co-localized insulin and Ki-67 cells as an example of proliferating β-cells within an islet. Scale bar represents 50 µm.

and the onset in young adults in this study is likely to have been precipitated by the metabolic stress of pregnancy since there were no differences in glucose tolerance between CNP and LPNP. Clinically, since prior GDM increases the risk of the mother developing subsequent T2DM (Heida *et al.* 2015), it is plausible that the LPP females could prematurely develop glucose intolerance following pregnancy compared to non-pregnant animals. Future studies investigating metabolic differences and pancreas histology postpartum using our animal model of GDM could prove insightful.

Consistent with previous findings (Xue *et al.* 2010; Beamish *et al.* 2017), we observed that CP females were able to expand BCM to compensate for insulin resistance in pregnancy. However, BCM expansion was impaired in LPP females compared to CP females, as has been postulated to occur in human GDM (Van Assche *et al.* 1979; Huang *et al.* 2009; Rieck & Kaestner, 2010). In

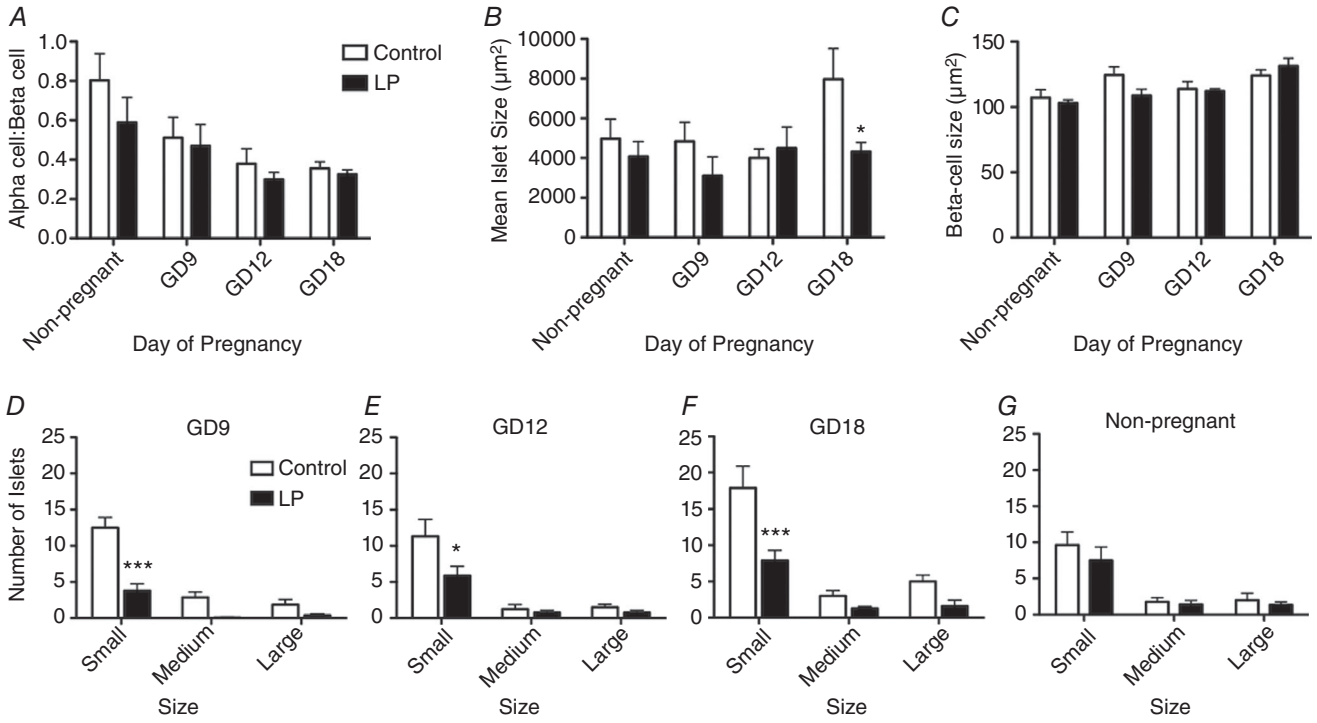

Figure 7. LPP females have an altered distribution of islet sizes, contributing to a reduced mean islet size and BCM expansion at late gestation
A, α- to β-cell ratio varies with day of pregnancy but not between dietary groups. *B*, mean islet size was reduced in LPP females compared to CP females on GD18. *C*, however, this was not due to a change in β-cell size. *D*–*G*, the number of small islets was reduced in LPP females on GD9 (*D*), GD12 (*E*) and GD18 (*F*) but not in LPNP females (*G*) ($n = 4$–6 animals for each group, 2–3 replicates for each animal). $^{***}P < 0.001$, $^{*}P < 0.05$, LP *vs.* C.

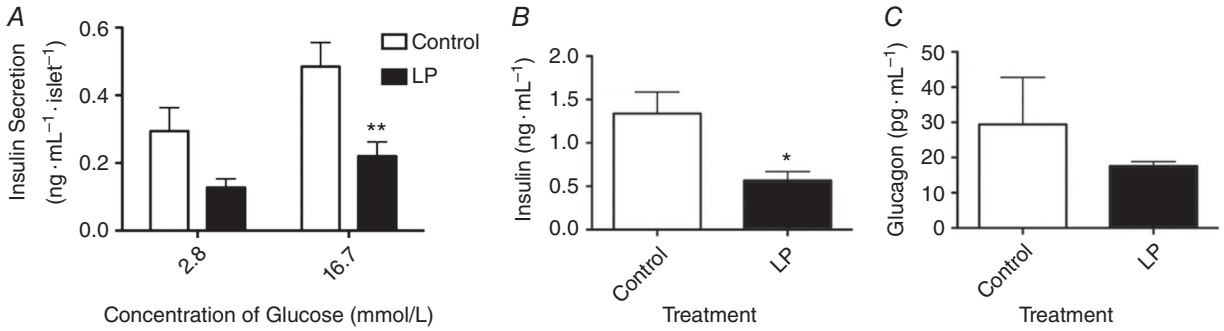

Figure 8. Offspring of low protein-fed mothers show gestational β-cell dysfunction *in vitro* and *in vivo*
A, glucose-stimulated insulin secretion was reduced on GD18 from isolated pancreatic islets from LPP females ($n = 6$–9 animals for each group, 1–3 replicates for each animal). *B*, serum insulin levels of LPP females were reduced on GD18 ($n = 7$ animals for each group). *C*, serum glucagon levels did not differ between CP and LPP females on GD18 ($n = 5$ animals for each group). $^{**}P < 0.01$, $^{*}P < 0.05$, LP *vs.* C.

agreement with our previous work, the expansion of BCM during pregnancy was associated with increased β-cell proliferation (Beamish *et al.* 2017), which was significantly reduced in LPP females. This was particularly apparent within the small extra-islet endocrine clusters which we have previously shown to be a source of β-cell progenitors (Beamish *et al.* 2016). This suggests that the proliferation of progenitors or their differentiation into functional β-cells might be impaired in LPP females. Reduced β-cell proliferation in LPP females contributed to a reduced mean islet size at GD18, consequently contributing to reduced BCM expansion. Although there were no differences in β-cell size in LPP *versus* CP females, there were fewer small islets in LPP females at GD9, 12 and 18 compared to CP females. Since there was a relative increase in large islets of CP females at GD18 compared to GD9, we postulate that β-cell replication within small islets facilitates islet growth into medium and large-sized islets as gestation progresses (GD18). This further contributed to increased BCM expansion in CP females at GD18. However, since LPP females had fewer small islets, there were fewer available to facilitate an adaptive expansion of BCM at GD18. Since there were no differences in islet size distribution in the LPNP *versus* CNP animals, these differences were attributed to the metabolic state of pregnancy. Although increased β-cell apoptosis is seen in offspring of LP-fed mothers (Petrik *et al.* 1999), here we found no evidence of apoptosis within β-cells of LPP or CP females during a subsequent gestation. Thus, this excludes the possibility of β-cell apoptosis contributing to the reduced capacity for BCM expansion in LPP females. These results are in agreement with the findings of another animal model of maternal glucose intolerance during gestation, in which the authors also reported that β-cell apoptosis did not contribute to the impairment of BCM expansion (Xu *et al.* 2015). These results could be attributed to the contribution of the protein survivin, which normally becomes upregulated during gestation and acts as an inhibitor of apoptosis via epidermal growth factor-receptor signalling (Hakonen *et al.* 2014). In addition to the dynamics of BCM investigated in this study, we are the first to report on α-cell dynamics in the pancreas during rodent pregnancy. Although there was no effect of treatment on the ratio of α- to β-cells, there was a change with day of pregnancy. Further to reduced BCM, we found a relative decrease in ACM in CP females throughout gestation when compared to CNP females. While CP females replenished ACM by GD18, this was not found in LPP females. There is evidence through lineage tracing of α-cells that they can replenish β-cells following β-cell loss or during β-cell stress via trans-differentiation (Thorel *et al.* 2010; Ye *et al.* 2015). These findings could implicate α- to β-cell trans-differentiation as a contributor to expanded BCM during pregnancy, which might be impaired in LPP females.

We also examined β-cell functional capacity in our model, since β-cell dysfunction is a key feature of the pathophysiology of GDM (Saisho *et al.* 2010). Although insulin release from isolated islets harvested in late pregnancy in response to basal glucose concentration did not differ between dietary groups, glucose-stimulated insulin secretion (GSIS) was significantly decreased in LPP females. These results are in agreement with other reports in which impaired GSIS was found in islets of offspring of LP-fed mothers as a result of mitochondrial dysfunction in β-cells (Theys *et al.* 2009). Future studies investigating mitochondrial β-cell dysfunction in our model would be insightful as women with GDM demonstrate increased oxidative stress, which has been suggested to contribute to β-cell dysfunction in GDM (Zhu *et al.* 2015). We confirmed our *in vitro* findings *in vivo*, showing that LPP females had reduced serum insulin levels at GD18 compared to CP females. These data supported our hypothesis and implicate β-cell dysfunction both *in vivo* and *in vitro* at late gestation in our model of gestational glucose intolerance. Therefore, in our model a combination of reduced BCM and impaired GSIS most likely contributed to the glucose intolerance seen in LPP females. Since our study was limited to changes in pancreas histology, we cannot eliminate the possibility that insulin resistance at the level of target tissues contributed to glucose intolerance during pregnancy in offspring of LP-fed mothers. However, this has previously been shown to occur only in late adulthood (130 days) and not within the young adult mice used in these studies (Chamson-Reig *et al.* 2009). Nevertheless, a major strength of our study was the ability to reproduce gestational glucose intolerance during pregnancy complications such as GDM in which glucose intolerance is not diagnosed until late gestation. In our study, glucose intolerance was restricted to GD18 and was not seen in the non-pregnant state as has been shown in other models of gestational glucose intolerance (Ignácio-Souza *et al.* 2013). Furthermore, additional animal models of diabetes in pregnancy that demonstrate pre-gestational obesity and diabetes (Holemans *et al.* 2004; Liang *et al.* 2010) display glucose intolerance prior to conception, which is not a true diagnosis of clinical GDM (Kim *et al.* 2010). Animal models utilizing chemical destruction of β-cells are widely used for modelling pre-gestational and gestational diabetes; however, these models more accurately resemble pre-gestational type-1 diabetes as opposed to the characteristics of insulin resistance seen in conditions such as GDM (Caluwaerts *et al.* 2003). Therefore, in comparison to other models, our model of fetal programming of gestational glucose intolerance via dietary insult more accurately demonstrates the hyperglycaemic state of GDM, which occurs only at late gestation. Although our dietary insult involves protein restriction during development, the LP diet is

made isocaloric to the control diet through increased carbohydrate, which prompts the question of whether our findings are due to the effects of reduced protein or increased carbohydrate intake. However, the increase in carbohydrate content represents only a 20% increase compared to the more prominent 60% reduction of protein (20% casein *versus* 8%), which suggests that the glycaemic dysfunction and impairments in pancreas histology and function in our model are more likely the result of reduced protein (Chamson-Reig *et al.* 2009).

Susceptibility to developing GDM arises from a complex combination of both polygenetic and environmental factors. Taking this into account, the developmental programming of adult metabolism utilized in this mouse model of glycaemic imbalance during pregnancy does not reproduce the predominant predisposing causes of human GDM, which include pre-gestational obesity and excessive gestational weight gain (Farrar, 2016; Melchior *et al.* 2017). Nonetheless, there are a number of anatomical and metabolic similarities including a failure to adequately increase BCM during pregnancy and impaired GSIS in late gestation. Postmortem studies of human pancreata obtained from pregnant individuals confirmed an increase in endocrine mass during healthy pregnancies compared to the non-pregnant state (Van Assche *et al.* 1978; Butler *et al.* 2010), and it has been suggested that failure to adaptively increase BCM might contribute to the risk of GDM in humans (Xiang *et al.* 2013). Thus, further research efforts should focus on molecular mechanisms (i.e. signalling via prolactin and/or oestrogen receptors) leading to reduced BCM expansion during gestation so that targeted interventions can be implemented. In conclusion, the model of hyperglycaemia in pregnancy described in this study could prove useful in evaluating pharmacological interventions aimed at safely increasing BCM or GSIS during pregnancy.

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Additional information

Competing interests

The authors have no competing interests to declare.

Author contributions

All experiments were performed in D.H.'s laboratory at Lawson Health Research Institute within St Joseph's Health Care (London, Canada). S.S. – data curation, formal analysis, investigation, methodology, visualization, writing of original draft; R.K. – investigation, methodology; G.R. – investigation, methodology; E.Y. – investigation, methodology; B.S. – investigation, methodology; D.H. – conceptualization, formal analysis, funding acquisition, project administration, resources, supervision, visualization. All authors participated in revision of the manuscript and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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