Articles in PresS. Am J Physiol Heart Circ Physiol (March 21, 2014). doi:10.1152/ajpheart.00981.2013

Paternal low protein diet affects adult offspring cardiovascular and metabolic function in mice.

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Abbreviated Running Title: Paternal low protein diet and adult offspring health in mice

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Whilst the association between maternal periconceptional diet and adult offspring health is well characterised, our understanding of the impact of paternal nutrition at the time of conception on offspring phenotype remains poorly defined. Therefore, we determined the effect of a paternal preconception low protein diet (LPD) on adult offspring cardiovascular and metabolic health in mice. Male C57BL/6 mice were fed either normal protein diet (18% casein; NPD) or LPD (9% casein) for 7 weeks prior to mating. At birth, a reduced male:female ratio (P=0.03) and increased male offspring weight (P=0.009) were observed in litters from LPD compared to NPD stud males with no differences in mean litter size. LPD offspring were heavier than NPD offspring at 2 and 3 weeks of age (P<0.02). However, no subsequent differences in body weight were observed. Adult male offspring derived from LPD studs developed relative hypotension (decreased by 9.2 mmHg) and elevated heart rate (P<0.05), whilst both male and female offspring displayed vascular dysfunction and impaired glucose tolerance relative to NPD offspring. At cull (24 weeks), LPD males had elevated adiposity (P=0.04), reduced heart:body weight ratio (P=0.04) and elevated circulating TNF-α levels (P=0.015) when compared to NPD males. Transcript expression in offspring heart and liver tissue was reduced for genes involved in calcium signalling (*Adcy, Plcb, Prkcb)* and metabolism (*Fto*) in LPD offspring (P<0.03). These novel data reveal the impact of sub-optimal paternal nutrition on adult offspring cardiovascular and metabolic homeostasis, and provide some insight into the underlying regulatory mechanisms.

Keywords: Adult offspring health; cardiovascular dysfunction; developmental programming; metabolic homeostasis; paternal diet.

Introduction

Studies in humans and animal models have identified strong associations between adult disease risk and environmental perturbations experienced during early development **(21)**. Gamete maturation and developmental events associated with fertilisation and pre-implantation embryo development appear particularly sensitive to changes in environmental conditions **(50)**. Studies from a diverse range of model species including rat **(24)**, sheep **(40)**, and human populations **(10)** have revealed similar phenotypic changes in offspring growth, cardiovascular and metabolic homeostasis following maternal periconceptional environment manipulation. In the sheep, half maintenance feeding prior to conception induced changes in blastocyst transcript levels for genes associated with metabolic activity **(33)**. In the sheep and cow, global maternal gestational undernutrition has been shown to elevate fetal blood pressure **(14)** impair adult offspring glucose tolerance **(45)** and affect offspring growth and adiposity in a sex specific manner **(26)**. Similarly in rodents, the feeding of a maternal low protein diet (LPD) during gestation induces significant changes in offspring birth weight and growth **(53)**, preferences for high-fat foods **(6)**, insulin resistance **(16)** and hypertension and vascular dysfunction **(37)**.

Whilst our understanding of the developmental consequences of manipulating the maternal environment is well defined, the impact of paternal physiology and nutritional status around conception remains largely under-investigated. Studies in humans and mice have demonstrated that increasing male BMI associates with reduced sperm motility **(19)**, increased incidences of sperm abnormality **(23)** and DNA fragmentation **(11)**, and reduced pregnancy rates **(17)**. In mice, offspring metabolic profiles including hepatic lipid and cholesterol biosynthesis at weaning, serum glucose, IGF-1 and corticosterone levels are altered in response to paternal LPD **(9)** or pre-mating fasting **(2)**. Consumption of a high fat **(27)** or high energy **(36)** diet impacts negatively on sperm motility, DNA integrity and blastocyst developmental rates and impairs offspring pancreatic ß-cell function **(28)**. In men, paternal obesity has been shown to associate with decreased blastocyst development

and live birth rate **(5)** and the DNA methylation status of the *IGF2* differentially methylated region in the cord blood of newborn children **(42)**.

Whilst these studies identify intergenerational transmission of metabolic disorders in young offspring through sperm mediated mechanisms, the impact on adult offspring cardiovascular and metabolic phenotype remains unknown. Therefore, the aim of our current study was to determine the impact of a paternal LPD on well-defined markers of adult offspring cardiovascular and metabolic health, focusing on analysis of adult offspring blood pressure, arterial function, in vivo glucose tolerance and the expression of cardiovascular and metabolic regulatory genes.

Materials and Methods

Animal Treatments

All mice and experimental procedures were conducted using protocols approved by, and in accordance with, the UK Home Office Animal (Scientific Procedures) Act 1986 and local ethics committee at the University of Nottingham. Virgin male (9 week old) and female (5-9 week old) C57BL/6 mice (Harlan Ltd, Belton, Leicestershire, UK) were maintained for 2 weeks at the University of Nottingham's Bio Support Unit on a 07:00–19:00 light-dark cycle at a temperature of 20–22°C with *ad libitum* access to chow (2018 Teklad Global 18% Protein Rodent Diet; Harlan, UK) and water. Weight matched male mice were housed singly and allocated to either a control normal protein diet (NPD; 18 % casein, 42.5% maize starch, 21.3 % sucrose, 10% corn oil, 5% cellulose; n = 8) or isocaloric (calories/gm) low protein diet (LPD; 9% casein, 48.5 maize starch, 24.3% sucrose, 10% corn oil, 5% cellulose; n = 8) offered *ad libitum* (Special Dietary Services Ltd, UK; composition published previously **(24, 52)**) for 7 weeks prior to initiation of mating, and maintained on respective diets until cull at 32 weeks of age (**Figure 1**).

Virgin, chow fed 7-9 week old C57BL/6 females were caged singly with either NPD or LPD studs, with access *ad libitum* to the studs' respective diet. The presence of a vaginal plug the following morning was taken as a sign of mating. Plug positive females were housed singly and maintained on chow until offspring weaning at which time they were culled. At birth, offspring were weighed and the litter male:female ratio determined. At 3 weeks of age all offspring were weaned, the sexes caged separately per litter with access to chow and water *ad libitum* and allocated randomly tail marks with permanent marker for subsequent weekly tracking of individuals. All offspring were weighed weekly from birth till 24 weeks of age. All studs generated 2 litters each, however, during pre-weaning development 2 NPD and 2 LPD litters, each from separate studs, were lost due to maternal infanticide. As such, a total of 14 litters per dietary treatment were analysed.

Blood Pressure Measurement

Blood pressure (systolic and diastolic) and heart rate were measured in stud males at 11 (pre-diet feeding), 17 (pre-mating) and 27 weeks of age, and all generated offspring from all 14 litters per treatment group (n = 42 NPD males, 43 NPD females, 27 LPD males and 42 LPD females in total) at 6, 10, 14 and 18 weeks of age using a computerised, non-invasive, tail-cuff system (Kent Scientific, USA). All mice were acclimatised to the experimental room for at least one hour followed by a minimum of 30 minutes warming at 27-30^{o}C prior to being placed within the restraining and measurement apparatus for 5 minutes prior to measurement.

Glucose Tolerance Test (GTT)

Offspring glucose tolerance was determined in at least one male and female per litter at 22 weeks of age. Offspring were fasted overnight, with access to water *ad libitum,* and weighed immediately prior to GTT. After administration of local anaesthetic (EMLA cream, Eutectic Mixture of Local Anaesthetics, Lidocaine/Prilocaine, AstraZeneca, UK), fasting blood glucose levels were determined in a sample collected from the tail vein using a hand-held glucometer

(Freestyle Optium, UK) prior to an intraperitoneal glucose bolus (2g/kg body weight in PBS). Blood samples were collected from the tail vein at 15, 30, 60 and 90 minutes post-bolus for determination of glucose concentration. All animals were returned to their original cage with accesses to food and water *ad libitum*.

Mesenteric Artery Vasoreactivity

Offspring (n = 8 pairs of male and female offspring per treatment group, each pair from separate litters) vascular function was assessed at 24 weeks of age in isolated small mesenteric artery segments as described previously **(51)** on a wire myograph (Danish Myo Technology A/S, Denmark). Cumulative concentration response curves (CRCs) were measured for the α_1-adrenergic agonist phenylephrine (10^{-9} to 10^{-4} mol/L), and after submaximal (EC_{80}) pre-constriction with the thromboxane mimetic U46619 (10 mmol/L), the vasodilators acetylcholine (ACh; 10^{-9} to 10^{-5} mol/L) and isoprenaline (ISO; 10^{-10} to 10^{-6} mol/L) and the nitric oxide donor sodium nitroprusside (SNP; 10^{-11} to 10^{-5} mol/L) in that order in the same arteries. All drugs were purchased from Sigma (UK).

Tissue Sampling

All mice were culled by cervical dislocation. At 32 weeks of age, stud males were culled and blood samples, taken via heart puncture, were allowed to clot on ice prior to centrifugation at 10,000 rpm, 4^{o}C for 10 minutes, after which serum was aliquoted and stored at -80^{o}C. Liver, kidneys, heart, lungs, testes and retroperitoneal, gonadal, inguinal and interscapular fat (anatomical locations defined previously **(52))** were removed, weighed and stored at -80^{o}C. Left and right caudal epididymi were removed and placed within a pre-warmed 200 µl drop of sperm motility medium (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 30mM HEPES; freshly supplemented with 10 mM lactic acid, 1 mM sodium pyruvate, 20 mg/ml BSA, 25 mM $NaHCO_3$). Epididymi were slashed several times using a 23 gauge needle and left for 15 minutes at 37^{o}C for

sperm to swim out. A sample of sperm was taken for counting using a Neubeur counting chamber prior to assessment of motility. Collected sperm were pipetted under 2 ml of pre-warmed motility medium and left to swim up for one hour at 37^{o}C. Sperm within the top 1.5 ml of medium were collected and counted as above. At 24 weeks of age, offspring were culled for collection of blood and somatic tissues (as described above).

Metabolite and Hormone Measurements

Following cull at 32 weeks of age, stud serum glucose was analysed using a commercial glucose oxidase assay (Sigma, UK) and serum insulin and testosterone levels determined by ELISA (Millipore and R&D Systems respectively, UK). Stud testes were homogenised (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% SDS) prior to protein level determination (DC assay, Bio-Rad Laboratories, CA, USA). Testicular testosterone levels were determined using a commercial ELISA (R&D Systems). Levels of adiponectin and TNF-α were determined in offspring serum at cull by ELISA (R&D Systems). All assays were conducted in accordance with the manufacturer's instructions and measured on a Benchmark microplate reader (Bio-Rad Laboratories).

RNA extraction and transcript expression

RNA was extracted from offspring heart and liver tissues using the RNeasy Mini Kit (QIAGEN, UK) according to manufacturer's instructions. Contaminating genomic DNA was removed by on-column DNase I digestion prior to cDNA synthesis using the ImProm™II kit (Promega, UK) using the included random primers. For Real-Time PCR (RTqPCR), 1 µl (5 ng RNA equivalent) of cDNA was added to a mastermix comprising 10 µl mastermix (2X Precision SYBRgreen Mastermix; PrimerDesign, UK), 0.7 µl primer mix (5 µM forward and reverse primers) and 8.3 µl water per reaction. Water was used in place of cDNA as a no template control. Amplification and detection was performed using a Lightcycler 480 (Roche) and data acquired

using the LightCycler SW 1.5.lnk software. A post-amplification melting curve confirmed the presence of specific products for each primer set. Ct values were converted to relative expression values using the delta-delta Ct method with offspring heart data normalised to the expression of *Ppib* and *Sdha* and liver data normalised to *Pgk1* and *Tbp*. geNorm software **(48)** was used to determine these to be the most stable reference genes. Primer sequences and amplification efficiencies are provided in **Table 1**.

Statistical analyses

Where appropriate, stud male data were analysed using independent samples or repeated measures *t*-tests, following assessment for normality, and Pearson correlation for analysis of correlation between phenotypic measurements (SPSS version 17). Offspring litter sex ratios were analysed using a binomial test (SPSS version 17). Analysis of offspring vascular responsiveness was conducted using GraphPad Prism 6, with the log effective concentration equal to 50% of the maximal response (pEC50) and maximum response for each of the CRCs analysed with an independent samples *t*-test. All other offspring data were analysed using a multilevel random effects regression model (SPSS version 18) **(53)**, accounting for paternal origin of litter, gestational litter size, offspring sex and body weight. Significance was taken at $P<0.05$.

Results

Paternal LPD reduces stud growth

Prior to experimental feeding, there was no difference in mean NPD and LPD stud body weight (**Figure 1**). During the first 3 weeks of feeding, LPD stud males displayed a trend ($P<0.1$) towards a lower body weight (i.e. at 12 -14 weeks of age), becoming lighter ($P<0.05$) during weeks 4 - 9 (i.e. at 15 - 20 weeks of age, mating initiated at 18 weeks of age). Throughout the study, LPD studs grew more slowly ($P=0.004$) (by 12%) than NPD studs. NPD and LPD stud systolic and diastolic blood pressure and heart rate did not differ throughout the entire study (data not shown).

Analysis of stud male organ weights at 32 weeks of age revealed a significantly lighter kidney (NPD 0.171 ± 0.004g, LPD 0.149 ± 0.003g; P=0.0005) and carcass (body weight minus the weight of collected organs and fat pads) (NPD 29.09 ± 0.27g, LPD 27.59 ± 0.47g; P=0.015) weight in LPD males. These differences remained when calculated as a proportion of body weight (P <0.05; data not shown). No difference between NPD and LPD stud testosterone (combined serum and testicular; NPD 9.30 ± 5.24 ng/ml, LPD 11.40 ± 3.92 ng/ml) or serum insulin (NPD 2.26 ± 0.61 ng/ml, LPD 1.44 ± 0.12 ng/ml) were observed. However, serum glucose was higher in LPD studs (NPD 1.32 ± 0.08 mg/ml, LPD 1.55 ± 0.07 mg/ml) at a trend level (P=0.06). No differences in the total number of sperm (NPD $12.90x10^6 \pm 2.30x10^6$/ml, LPD $11.62x10^6 \pm 1.99x10^6$/ml), or number of sperm collected following swim-up (NPD $4.99x10^6 \pm 1.29x10^6$/ml, LPD $4.68x10^6 \pm 0.72x10^6$/ml) were observed between stud groups.

Paternal LPD affects offspring sex ratios, birth weight and adult phenotype

Mean maternal weight prior to conception (16.28 ± 0.11 g), after 2 weeks of pregnancy (23.80 ± 0.29 g) and mean litter size (5.7 ± 0.4) did not differ between treatment groups. However, the proportion of male pups at birth was reduced in the LPD compared to the NPD treatment group (NPD 0.54 ± 0.04, LPD 0.40 ± 0.06; P=0.03), and male offspring birth weight was increased (NPD 1.26 ± 0.02 g, LPD 1.33 ± 0.02 g; P=0.05). LPD offspring were also heavier than NPD offspring at 2 (NPD 6.54 ± 0.07 g, LPD 7.13 ± 0.08 g; P=0.006) and 3 (NPD 7.82 ± 0.10 g, LPD 8.61 ± 0.14 g; P=0.019) weeks of age. At weaning, the sexes were caged separately (mean of 3 NPD males, 2 LPD males, 3 NPD females and 3 LPD females per cage) with no further differences in body weights being observed between NPD and LPD offspring for up to 24 weeks of age (data not shown).

Analysis of correlations between paternal phenotype at the time of mating and offspring early postnatal development revealed significant negative correlations between LPD stud body weight and mean litter male:female ratio (r=-0.444, P<0.0001) and offspring weight at 1 (r =-0.27,

P =0.023) and 2 weeks of age (r =-0.257, P =0.030). In NPD offspring, a positive correlation was observed between stud body weight and litter male:female ratio (r =0.191, P =0.085) and offspring weight at 2 weeks (r =0.210, P =0.06), which was significant at 1 week of age (r =0.224, P =0.046). Additional negative correlations were observed between the number of days studs were on LPD and litter male:female ratio (r =-0.277, P =0.018), offspring birth weight (r =-0.421, P <0.0001), weight at 1 (r =-0.363, P =0.002) and 2 weeks of age (r =-0.348, P =0.003) which were not observed in NPD offspring.

No difference in mean systolic or diastolic blood pressure or heart rates were observed between NPD and LPD offspring at 6, 10 or 14 weeks of age (data not shown). However, at 18 weeks of age, LPD males displayed lower diastolic (NPD 84.80 ± 1.82 mmHg, LPD 75.88 ± 2.16 mmHg), systolic (NPD 113.13 ± 2.19 mmHg, LPD 103.93 ± 2.27 mmHg) and mean (NPD 93.93 ± 1.91 mmHg, LPD 84.86 ± 2.16 mmHg) blood pressure, and elevated mean heart rate (NPD 682 ± 11 beats per minute, LPD 711 ± 16 beats per minute; P<0.05) (**Figure 2**).

At 22 weeks of age, both male and female LPD offspring displayed elevated blood glucose concentrations following an intraperitoneal glucose bolus. At 15 and 60 minutes post injection, LPD males had significantly elevated blood glucose concentrations, with a reduced overall clearance at 60 and 90 minutes (area under the curve, AUC; P=0.034 and 0.029 respectively) (**Figure 3A**). LPD females similarly had elevated blood glucose concentrations at 15 and 30 minutes post injection, and impaired overall clearance at 60 and 90 minutes (AUC, P=0.022 and 0.080 respectively) (**Figure 3B**).

At 24 weeks of age, significantly attenuated vasoconstrictive responses (pEC50) to the α-1 adrenergic agonist phenylephrine (PE) and maximal vasodilatory responses to isoprenaline (ISO) and the nitric oxide donor SNP were observed in arteries from LPD males (**Figure 4A,** P<0.05).

Significantly attenuated pEC50 and maximal responses to SNP were also observed in LPD females (**Figure 4B,** $P<0.05$).

Analysis of offspring organ and fat pad weights at 24 weeks of age revealed significantly increased inguinal (NPD 0.59 ± 0.04g, LPD 0.77 ± 0.07g; P=0.017) and total fat (combined individual fat pads, NPD 2.02 ± 0.01g, LPD 2.46 ± 0.18g; P=0.035) weights in LPD males but not females. When expressed as a percentage of body weight, reduced heart (NPD 0.53 ± 0.01, LPD 0.51 ± 0.02; P=0.04) and elevated inguinal fat (NPD 2.00 ± 0.12, LPD 2.51 ± 0.20; P =0.02) and total fat (NPD 6.81 ± 0.34, LPD 8.06 ± 0.53; P=0.04) proportions were observed in LPD males. As global adiposity levels influence metabolic state and glucose homeostasis, we performed additional retrospective analyses incorporating body weight and adiposity measurements as 'random effects' within our regression analyses of offspring glucose tolerance. We observed a positive interaction between offspring body weight and overall glucose clearance (AUC) such that AUC increased by 40.57 per g increase in body weight (P =0.004), however, no interaction between total fat weight and AUC (P =0.98) was observed. In males, a significant positive interaction between body weight and AUC was observed (AUC increased by 39.11 for each g increase in body weight; P =0.04), but no interaction with total fat and AUC (P =0.41) was present. Female offspring displayed no interaction between body weight and mean AUC (P =0.48), however, a positive interaction with total fat and AUC at a trend level (AUC increased by 219.76 for each g increase in total fat; P =0.07) was observed. No interaction between blood glucose and total adiposity were observed in male or female offspring at each individual time point post glucose bolus. However, body weight was observed to interact positively with mean blood glucose levels in male offspring at 60 (0.621 mmol increase for every g increase in body weight; P =0.017) and 90 minutes (0.682 mmol increase for every g increase in body weight; P =0.008). No such interactions in female offspring were observed. Additional analyses of offspring adiposity and adult health revealed a negative correlation in LPD offspring between BMI (weight g/length from nose to base of tail (cm^2)) and diastolic blood

pressure (r =-0.276, P=0.027) and a positive correlation between total fat (g) and heart rate (r =0.279, P =0.026) at 18 weeks of age which were not present within NPD offspring. Serum adiponectin concentrations were greater (P<0.001) in female than male offspring but were unaffected by paternal diet (**Table 2**). In contrast, there was a paternal-diet by offspring-sex interaction (P = 0.015) for TNF-α which indicated that this cytokine was elevated in LPD male compared to NPD male offspring, with no differences between paternal dietary treatments in female offspring. Analysis of correlation between adiponectin, TNF-α and offspring phenotype revealed significant negative correlations between adiponectin levels and body weight at cull in NPD and LPD offspring (P <0.0001). Additionally, adiponectin levels correlated negatively with body weight at 3 weeks of age (r =-0.384; P =0.033) and TNF-α levels (r =-0.349; P =0.05) in LPD offspring. Finally, a positive correlation between TNF-α levels and body weight at cull (r =0.471; P =0.008) was also observed in LPD offspring.

Gene expression analyses in offspring heart for receptors involved in regulation of cardiac function revealed no differences for adrenergic receptor beta 1 (*Adrb1)*, angiotensin II receptor type 1a (*Agtr1a*), bone morphogenetic protein receptor type II (*Bmpr2)*, cholinergic receptor muscarinic 2 (*Chrm2*) or the solute carrier family 2 (facilitated glucose transporter), member 4 (*Glut4*) between NPD and LPD offspring (data not shown). However, analysis of genes involved in calcium signalling revealed significantly decreased expression of adenylate cyclase 5 (*Adcy5*; P=0.026), phospholipase C beta1 (*Plcb1*; P=0.027) and protein kinase C beta (*Prkcb*; P=0.008) in offspring heart tissue (**Table 3**). As well as having a role in regulation of cardiovascular function, ADCY5, along with FTO (fat mass and obesity associated) have been identified as genes that are altered in type 2 diabetes **(3, 25)**. Therefore, in response to the observations of impaired glucose tolerance and elevated adiposity in LPD offspring, we analysed the expression of *Adcy5* and *Fto* in offspring liver tissue. We observed no change in the expression of *Adcy5,* however, *Fto* was decreased (P=0.006)

316 (**Table 3).** *Fto* expression in offspring cardiac tissue was also reduced (P<0.001) in NPD offspring
317 (**Table 3**).
318
319 **Discussion**
320 To date, the majority of studies detailing the developmental programming of offspring
321 health have focused on manipulation of the maternal environment. As such, our understanding of
322 the impact of paternal nutrition on offspring development and long-term adult health remains poorly
323 defined. In the present study, we have shown that LPD has minimal effects on paternal physiology
324 and fertility (between 18 and 32 weeks of age), but that adult offspring derived from them display
325 significantly impaired cardiovascular and metabolic homeostasis. Our results provide evidence of
326 an intergenerational modification of adult offspring phenotype in response to paternal diet.
327
328 Our results identify a series of offspring growth, cardiovascular and metabolic phenotypes
329 whose regulation is compromised by paternal LPD. At birth, we observed a significantly reduced
330 litter male:female ratio and increased weight of male offspring from LPD studs. In mice, a low
331 calorie diet fed to females results in a selective loss of male embryos during preimplantation
332 development and subsequent skewing of litter sex ratios in favour of males **(38)**. However, no such
333 effects are observed when low calorie diets are fed to males. In contrast, no effects on litter sex ratio
334 have been reported following maternal LPD in the mouse **(53, 54)**. Based on previous reports **(38)**,
335 we do not believe LPD induces a differential production in the number of X and Y bearing sperm in
336 studs. However, differences in capacitation rates or motility between X and Y bearing sperm might
337 explain these effects. Alternatively, LPD semen may induce a uterine environment more favourable
338 to female preimplantation embryos, resulting in a selective loss of male embryos **(38)**. However,
339 additional studies are necessary to determine whether functional differences exist between X and Y
340 bearing sperm, and at what developmental stage offspring sex ratio is established.
341

342 Dysfunctional regulation between constriction and dilatation responses within resistance
343 arteries has been identified in rodent models of cardiovascular programming **(37, 51, 53)**.
344 Augmentation of peripheral vascular function is characterised by the presence of altered
345 endothelium-dependent vasodilation and/or changes in activity of signalling mechanisms regulating
346 vascular smooth muscle function. We identified significant impairments in mesenteric artery
347 responses to the α_1-adrenergic agonist phenylephrine (PE), the ß-adrenoreceptor agonist
348 isoprenaline (ISO) and the nitric oxide donor sodium nitroprusside (SNP) in LPD offspring,
349 however, no impairment in response to endothelial-dependent vasodilator acetylcholine (ACh) was
350 observed. Within the resistance vasculature, endothelium-dependent vasodilatation is mediated
351 predominantly through the action of endothelium-derived hyperpolarizing factor (EDHF) via small
352 and intermediate calcium-activated potassium channels rather than NO **(31)**. Indeed, in conditions
353 of reduced NO bio-availability, up-regulation of EDHF activity has been observed **(39)**. As ACh
354 induces vascular smooth muscle cell hyperpolarization through both eNOS and EDHF pathways, a
355 functional EDHF component would mask any impairment in NO signalling present. This could
356 provide one mechanism through which altered responsiveness to SNP, but not Ach, could be
357 manifest. Calcium homeostasis is central to the regulation of vascular smooth muscle function. PE
358 induces vasoconstriction though the activation of phospholipase C and the mobilisation of calcium
359 from intracellular stores, activating myosin light chain kinase. Conversely, isoprenaline elevates
360 intracellular cAMP levels, activating protein kinase A, which inhibits myosin light chain kinase,
361 causing vasodilatation. Isoprenaline also acts in concert with endothelial nitric oxide synthase,
362 stimulating soluble guanylate cyclase to increase cGMP levels within the vascular smooth muscle,
363 also inhibiting myosin light chain kinase activity. Therefore, impairment in vascular smooth muscle
364 cell calcium signalling would impact negatively on both vaso-constriction and –dilatation function,
365 as observed in LPD offspring. However, from the present study, the exact roles of NO, soluble
366 guanylate cyclase, adenylate cyclase, prostaglandins and calcium-activated potassium channels are
367 uncertain and would require further investigation.

Similarly, modulation of myocardial intracellular calcium signalling through altered sympathetic or parasympathetic innervation, inhibition of calcium entry or AT1 receptor antagonists (angiotensin II) would result in a lowering of blood pressure **(56)**. As no difference in cardiac expression of the ß1-adrenergic, angiotensin II type 1a or the cardiac muscarinic cholinergic receptors were observed in offspring, we assessed the expression profiles of central regulators of intracellular calcium signalling in offspring cardiac tissue. Here, we observed significantly decreased expression of *Adcy5*, *Plcb*, and *Prkcb* in LPD offspring. Adenylate cyclase, in particular Adcy5, plays a central role in modulating cardiac contractility, transducing the signal from the ß-adrenergic receptor, elevating cAMP-protein kinase A signalling and ultimately the influx of calcium ions through voltage-dependent L-type calcium channels. Disruption of cardiac *Adcy5* expression results in decreased in vivo responsiveness to isoprenaline, elevated heart rate through reduced parasympathetic regulation, and reduced hypertrophy and apoptosis **(29, 30)**. Plcb catalyses the hydrolysis of phosphatidylinositol 4,5-bisphophate (PIP2) to generate inositol (1,4,5) triphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 initiates an increase in intracellular calcium, whereas DAG activates protein kinase C beta (Prkcb). In turn Prkcb, a serine and threonine kinase, phosphorylates a wide range of protein targets. Elevated expression of Prkcb isoforms, specifically in the myocardium, is associated with hypertrophy, fibrosis, impairment of left ventricular performance, progressive cardiomyopathy and heart failure **(49, 55)**. In contrast, Prkcb inhibition preserves cardiac contractility by attenuating diastolic dysfunction, myocyte hypertrophy, and collagen deposition **(12)**. Therefore, our observed relative hypotension, tachycardia and reduced heart:body weight ratios observed in LPD offspring may result from impaired parasympathetic stimulation of cardiac tissues providing decreased baroreflex restraint coupled with a reduced rate of age related cardiomyopathy and fibrosis in response to reduced calcium signalling gene expression **(7)**, however, additional studies would be required to verify these conclusions.

In our current study, LPD offspring also exhibited reduced glucose tolerance and elevated adiposity in adulthood. Broad effects on the metabolism of young offspring following paternal nutritional manipulation have been reported. Offspring pancreatic ß-cell function and gene expression are affected by paternal preconception high fat diet in rats **(28)**, with paternal preconception fasting elevating offspring glucose levels in mice **(2)**. Similarly, elevated expression of hepatic lipid and cholesterol biosynthesis genes, with decreased levels of cholesterol esters, at weaning have been observed in offspring mice from LPD fed studs **(9)**, however, adult sex-specific cardiovascular, metabolic and glucogenic phenotype was not assessed. A causal link does exist between cardiovascular and metabolic phenotype, with increased adiposity and altered gene expression profiles, vascular dysfunction and hypertension being observed in female offspring from mouse dams fed a LPD exclusively during preimplantation development **(52, 53)**. Impaired glycaemic homeostasis and increased adiposity are chronic inflammatory conditions associated with elevated levels of adipokines, inflammatory cytokines and oxidised low-density lipopropoteins, all known to impair vascular smooth muscle and cardiac function **(35, 46)**. Therefore, we measured the circulating levels of adiponectin and TNF-α in NPD and LPD offspring. Adiponectin levels correlate negatively with adiposity, with low levels being associating with cardio-metabolic disorders including endothelial dysfunction, type 2 diabetes and blood pressure **(18)**. Conversely, elevated levels of the pro-inflammatory cytokine TNF- α are associated with insulin resistance and impact negatively on vascular function **(4)**. We observed no significant difference in either adiponectin concentration between NPD and LPD males, or between NPD and LPD females. However, female offspring did display significantly higher adiponectin levels than males, reflective of previous reports **(15)**. We also observed a significant paternal-diet by offspring-sex interaction indicating significant differences in the impact of paternal diet on offspring inflammatory responses dependent on sex. As such, the comparatively low levels of adiponectin, coupled with elevated levels of TNF- α, may contribute to the increased vascular dysfunction and impaired glucose homeostasis observed in LPD males. Conversely, in LPD females, the opposite relationship may

provide some protection against developing cardiovascular impairments to the same magnitude as those observed in the LPD males. Interestingly, we observed specific correlations between early postnatal body weight, adiponectin and TNF-α levels and adult body weight in LPD offspring. These data highlight the importance of early development and physiological characteristics (e.g. body weight) and adult markers of metabolic health, a central concept of the Developmental Origins of Health and Disease (DOHaD) hypothesis **(21)**. Indeed, we have demonstrated previously similar associations between early postnatal weight and adult cardiovascular dysfunction in a mouse maternal model of gestational LPD fed exclusively during preimplantation development **(53)**. We also observed a negative correlation between BMI and diastolic blood pressure in LPD offspring, whilst total fat weight correlated positively with heart rate at 18 weeks of age, highlighting additionally the interaction between adult adiposity and cardiovascular regulation within our model.

Human genome-wide associated studies of have identified significant associations between genetic polymorphisms and the risk prediction for type II diabetes including *ADCY5* and Fat Mass and Obesity associated gene (*FTO*) **(1, 3)**. We observed significantly decreased expression of *Fto* in the livers of LPD offspring, with no change in *Adcy5* expression. The *FTO* gene is an AlkB-like, Fe(II)- and 2-oxoglutarate–dependent nucleic acid demethylase, acting on single-stranded DNA and RNA, and has been showed to predispose individuals to diabetes through an effect on body mass index **(43)**. In mice, fasting increases *Fto* expression in the liver within an inverse correlation between *Fto* mRNA and glucose levels **(34)**, whilst knock out of the Fto gene results in reduced lean mass and elevated fat mass **(25).** In mouse models of obesity, reduced hepatic *Fto* expression has been reported **(44).** As such, the reduced hepatic expression of *Fto* observed in NPD mice may disrupt regulation of energy metabolism, predisposing to impaired glucose tolerance and elevated adiposity.

Our observations raise the question as to the underlying developmental mechanisms through which paternal diet modifies adult offspring cardiovascular and metabolic phenotype. Recent studies have identified sperm hypomethylation and histone-enrichment at the promoters of developmental regulatory genes in both mice and men **(8, 20)**. Carone et al., **(9)** also observed significant changes in the epigenome of sperm isolated from stud mice fed a LPD, correlating with weaning offspring metabolic phenotype. These observations highlight the potential that sperm epigenetic status could influence both sperm function and post fertilisation development and gene expression patterns. However, as the sperm epigenome is dramatically remodelled at fertilisation by the cytoplasm of the oocyte, the persistence of sperm epigenetic marks and their effects on offspring phenotype remains unknown. Recently, it has been demonstrated that paternal sensory environment prior to conception, influenced the sensory nervous system structure and function in F1 and F2 generations in mice **(13)**. Interestingly, bisulfite sequencing analysis of paternal sperm and offspring tissue DNA revealed similar patterns of hypomethylation of the olfactory receptor *Olfr151*, proving potential evidence of an epigenetic basis of transgenerational inheritance of phenotype. Secondly, the relative contribution of sperm genomic-mediated programming and that determined by the composition of seminal plasma on long-term development and wellbeing of offspring remains to be established. It is known that seminal plasma cytokines stimulate maternal reproductive tract immunological responses, influencing embryonic, placental and offspring development **(41).** In human assisted reproductive cycles, there is an increasing awareness of the benefits of seminal plasma exposure on appropriate uterine responses and pregnancy outcomes following embryo transfer **(47)**. Whether paternal LPD modifies the composition of the seminal plasma, and the impact this may have on uterine physiology following mating remains to be determined.

Our data extend the concept of developmental programming revealing the role of paternal nutrition in the early origins of adult offspring cardiovascular and metabolic health. These data are timely and relevant to human disease as paternal diet and lifestyle not only contribute to male-factor

infertility **(5)**, but can also influence the cardiovascular and metabolic disease risk in subsequent generations **(22, 32)**. Our observations highlight the need for a greater understanding of the underlying mechanisms through which parental diet and physiology affect gamete maturation, semen quality, and ultimately, long-term offspring health.

Acknowledgements

The authors wish to acknowledge Wing Yee Kwong for her technical assistance and advice. This work was supported by a University of Nottingham Advanced Research Fellowship to Dr. Adam Watkins and a Society for Reproduction and Fertility Academic Scholarship Fund award to Prof. Kevin Sinclair.

Disclosures

The authors declare no conflicts of interest, financial or otherwise.

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660
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662

663 **Figure Legends**

664 **Figure 1.** Mean weekly body weight of NPD (open circles, n = 8) and LPD (closed circles, n = 8)
665 stud males. Experimental feeding began at 11 weeks of age and continued till cull at 32 weeks.
666 Mating to chow fed females began at 18 weeks of age. Error bars are S.E.M, *P<0.05.

667

668 **Figure 2.** Systolic (A), diastolic (B) and mean (C) blood pressure and heart rate (beats per minute;
669 BPM) (D) in NPD (white bars) and LPD (black bars) offspring at 18 weeks of age. n = 34 NPD
670 males, 36 NPD females, 24 LPD males and 42 LPD females. Error bars are S.E.M, *P<0.05.

671

672 **Figure 3.** Mean changes in blood glucose levels following an intraperitoneal glucose bolus (2g/kg
673 body weight) in male (A), and female (B), NPD (open circles) and LPD (closed circles) offspring at
674 22 weeks of age. n = 15 male and 15 female offspring of each diet group representing all litters.
675 Error bars are S.E.M, *P<0.05.

676

677 **Figure 4.** Mean vasoreactivity of isolated mesenteric arteries from male (A), and female (B), NPD
678 (open circles) and LPD (closed circles) offspring at 24 weeks of age. Cumulative additions of
679 phenylephrine (PE) and, after pre-constriction, of the vasodilators acetylcholine (ACh), isoprenaline
680 (ISO) and sodium nitroprusside (SNP). n =8 males and 8 females of each diet group, each pair from
681 separate litters. Error bars are S.E.M. *P<0.05.

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Figure 1

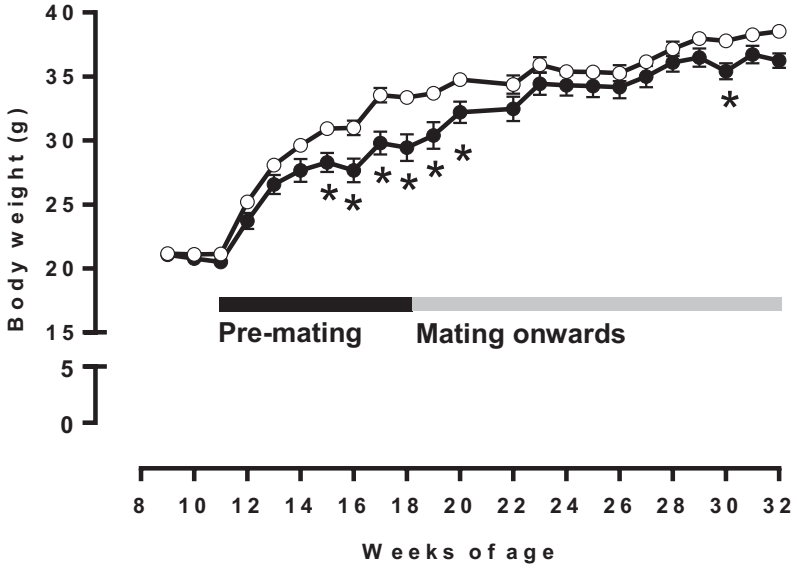
Body weight (g)
40
35
30
25
20
15
5
0
Pre-mating
Mating onwards
8
10
12
14
16
18
20
22
24
26
28
30
32
Weeks of age

Figure 2

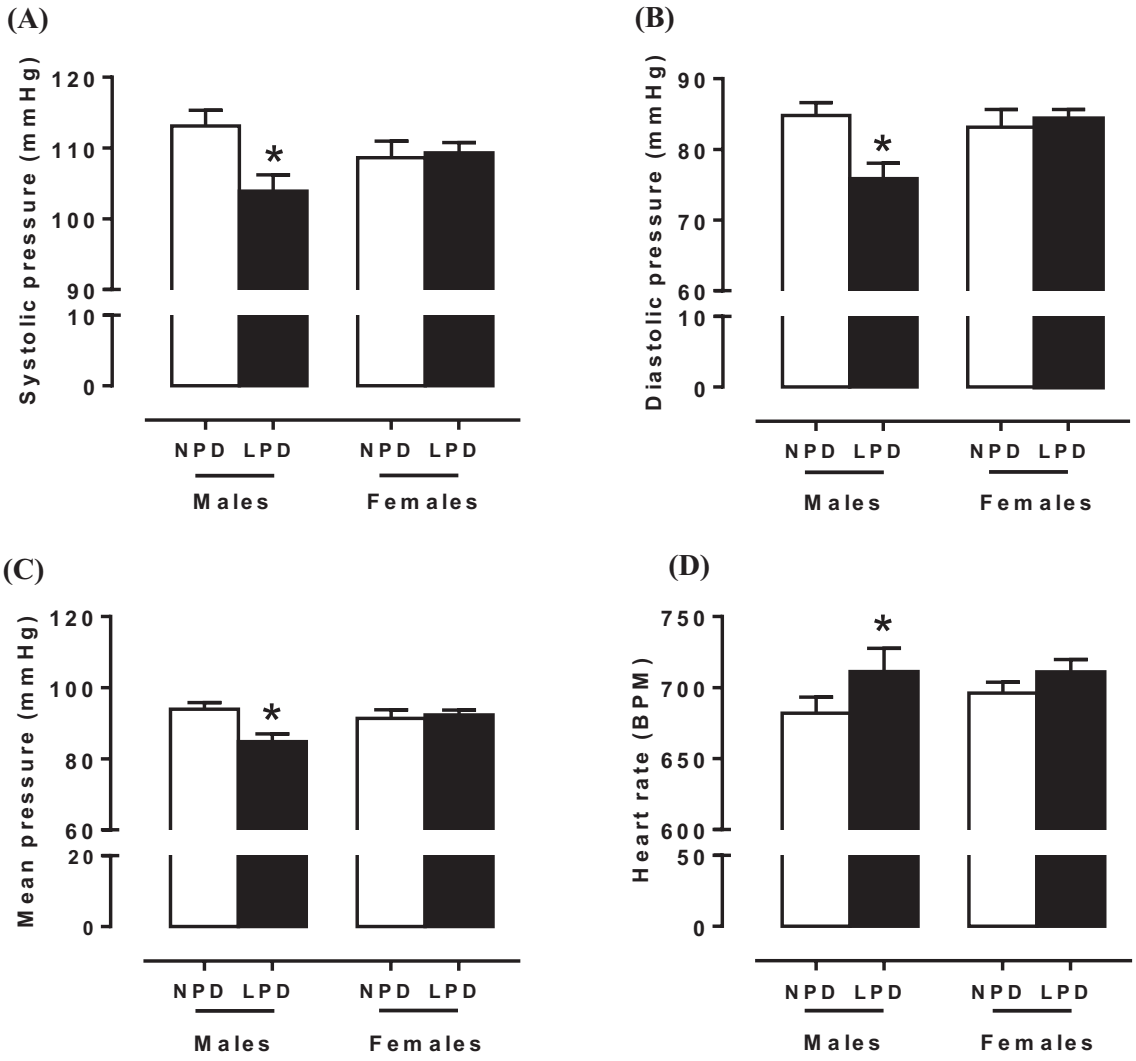
(A)
Systolic pressure (mmHg)
120
110
100
90
10
0
*
NPD LPD NPD LPD
Males
Females
(B)
Diastolic pressure (mmHg)
90
80
70
60
10
0
*
NPD LPD NPD LPD
Males
Females
(C)
Mean pressure (mmHg)
120
100
80
60
20
0
*
NPD LPD NPD LPD
Males
Females
(D)
Heart rate (BPM)
750
700
650
600
50
0
*
NPD LPD NPD LPD
Males
Females

Figure 3

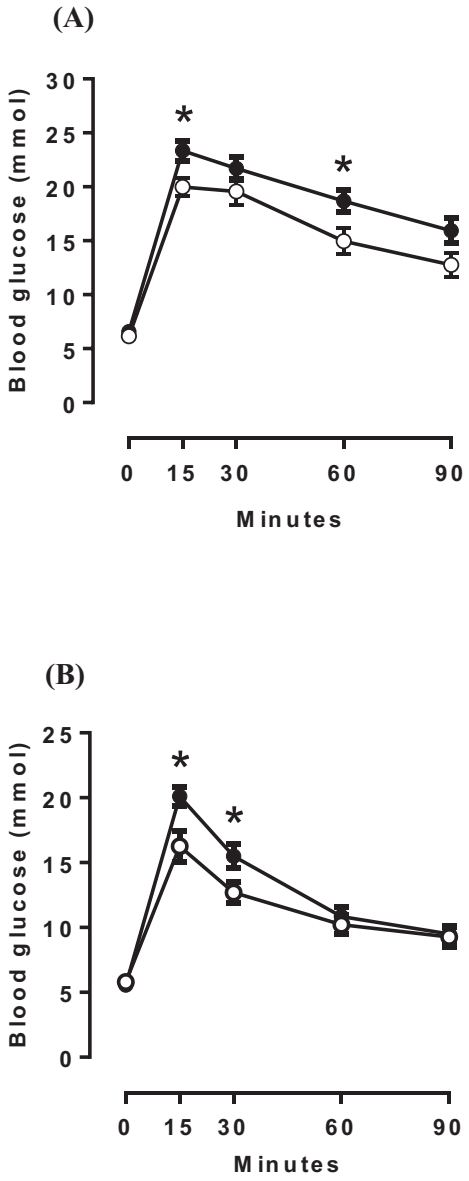
(A)
Blood glucose (mmol)
30
25
20
15
10
5
0
*
*
0
15
30
60
90
Minutes
(B)
Blood glucose (mmol)
25
20
15
10
5
0
*
*
0
15
30
60
90
Minutes

Figure 4

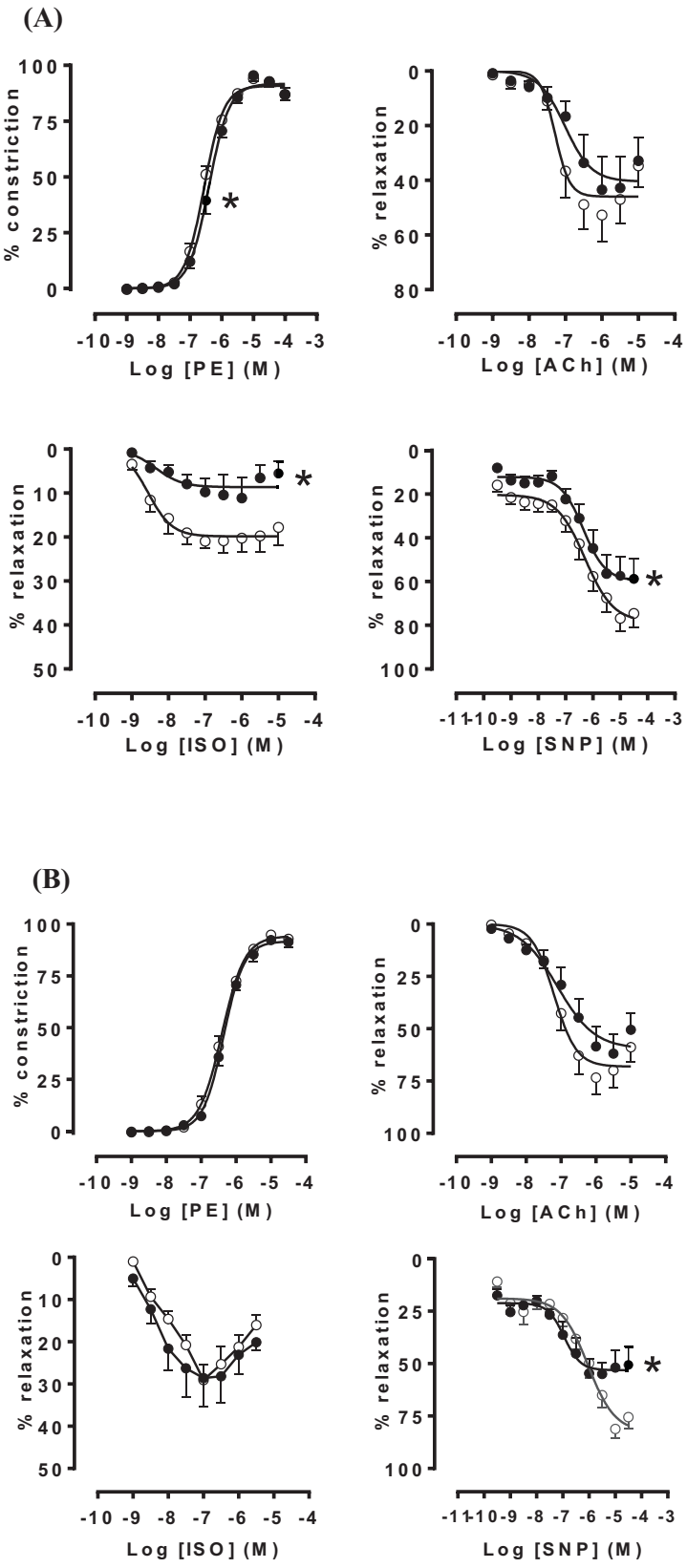
(A)
% constriction
Log [PE] (M)
% relaxation
Log [ACh] (M)
% relaxation
Log [ISO] (M)
% relaxation
Log [SNP] (M)
(B)
% constriction
Log [PE] (M)
% relaxation
Log [ACh] (M)
% relaxation
Log [ISO] (M)
% relaxation
Log [SNP] (M)

Table 1. Real-time qPCR primer details.

Gene Name	Gene Symbol	Accession Number	Primer Sequences		Amplicon Length	Primer Efficiency
			Forward Primer	Reverse Primer		
Adrenergic receptor, beta 1	*Adrb1*	NM_007419.2	ggatcgcctcttcgtcttct	cagtagatgatggggttgaagg	69	1.70
Angiotensin II receptor, type 1 alpha	*Agtr1a*	NM_177322.3	actcacagcaaccctccaag	ctcagacactgttcaaaatgcac	62	1.84
Bone morphogenetic protein receptor, type II	*Bmpr2*	NM_007561.3	gagccctcccttgacctg	gtatcgaccccgtccaatc	60	1.80
Cholinergic receptor, muscarinic 2, cardiac	*Chrm2*	NM_203491.3	tcggtgttaactgtcatcttcc	tcaggttggaccggtttg	109	1.85
Solute carrier family 2, member 4	*Glut4*	NM_009204.2	gacggacactccatctgttg	gccacgatggagacatagc	115	1.83
Adenylate cyclase 5	*Adcy5*	NM_001012765.4	atggaagctggtggcaag	cacctcatagtccccattcag	78	1.70
Protein kinase C beta	*Prkcb*	NM_008855.2	aagcgagggcaatgaaga	cttctggagccttggtacctt	74	1.70
Phospholipase C beta 1	*Plcb1*	NM_001145830.1	tcgatgagaagcccaagc	ggcagccttttgaacttgtc	67	1.75
Fat mass and obesity associated	*Fto*	NM_011936.2	tctgtctgccatcctggtc	tggtaaagtccggacgactc	94	1.72
Phosphoglycerate kinase 1	*Pgk1*	NM_008828	tacctgctggctggatgg	cacagcctcggcatatttct	65	1.70
Peptidylprolyl isomerase B	*Ppib*	NM_011149	ttcttcataaccacagtcaagacc	accttccgtaccacatccat	92	1.80
Succinate dehydrogenase complex, subunit A, flavoprotein	*Sdha*	NM_023281	tgttcagttccaccccaca	tctccacgacaccttctgt	66	1.88
TATA box binding protein	*Tbp*	NM_013684.3	gggagaatcatggaccagaa	gatgggaattccaggagtca	90	1.70

Table 2. Offspring serum adiponectin and TNF-α concentrations.

Sex	Males		Females		Significance (P)		
Diet	NPD	LPD	NPD	LPD	Diet	Sex	Diet x Sex
Adiponectin (ug/ml)	8.82 ± 0.29	8.95 ± 0.30	13.62 ± 0.29	13.20 ± 0.29	-	< 0.001	-
TNF-α (pg/ml)	4.02 ± 1.82	9.38 ± 1.89	5.63 ± 1.86	3.64 ± 1.86	-	-	0.015

Mean serum adiponectin and TNF-α concentrations. n = 16 males and 16 females from each dietary group, with all litters sampled. Values are mean ± S.E.M.

Table 3. Offspring tissue transcript expression.

Tissue	Gene	NPD	LPD	P value
Heart	*Adcy5*	1.00 ± 0.02	0.93 ± 0.02	0.026
	Fto	1.00 ± 0.01	0.88 ± 0.02	<0.001
	Plcb	1.00 ± 0.03	0.89 ± 0.03	0.027
	Prkcb	1.00 ± 0.08	0.78 ± 0.03	0.008
Liver	*Adcy5*	1.00 ± 0.06	0.90 ± 0.06	0.285
	Fto	1.00 ± 0.02	0.90 ± 0.02	0.006

Mean relative transcript expression (± S.E.M.) for selected genes involved in calcium signalling and metabolic regulation from NPD and LPD offspring heart and liver tissue. n = 10 males and 10 females from each dietary group, with each pair from separate litters. Transcript expression normalised to that of *Ppib* and *Sdha* (heart) and *Pgk1* and *Tbp (liver),* and adjusted to NPD values of 1.00.