

## INTEGRATIVE

**Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring.**

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Early embryonic development is known to be susceptible to maternal undernutrition, leading to a disease-related postnatal phenotype. To determine whether this sensitivity extended into oocyte development, we examined the effect of maternal normal protein diet (18% casein; NPD) or isocaloric low protein diet (9% casein; LPD) restricted to one ovulatory cycle (3.5 days) prior to natural mating in female MF-1 mice. After mating, all females received NPD for the remainder of gestation and all offspring were litter size adjusted and fed standard chow. No difference in gestation length, litter size, gender ratio or postnatal growth was observed between treatments. Maternal LPD did, however, induce abnormal anxiety-related behaviour in open field activities in male and female offspring ( $P < 0.05$ ). Maternal LPD offspring also exhibited elevated systolic blood pressure (SBP) in males at 9 and 15 weeks and in both genders at 21 weeks ( $P < 0.05$ ). Male LPD offspring hypertension was accompanied by attenuated arterial responsiveness in vitro to vasodilators, acetylcholine and isoprenaline ( $P < 0.05$ ). LPD female offspring adult kidneys were also smaller, but had increased nephron numbers ( $P < 0.05$ ). Moreover, the relationship between SBP and kidney or heart size or nephron number was altered by diet treatment ( $P < 0.05$ ). These data demonstrate the sensitivity of mouse maturing oocytes in vivo to maternal protein undernutrition and identify both behavioural and cardiovascular postnatal outcomes, indicative of adult disease. These outcomes likely derive from a direct effect of protein restriction, although indirect stress mechanisms may also be contributory. Similar and distinct postnatal outcomes were observed here compared with maternal LPD treatment during post-fertilisation preimplantation development which may reflect the relative contribution of the paternal genome.

## Introduction

Recent studies have shown preimplantation mammalian development to be sensitive to both *in vivo* and *in vitro* environmental conditions resulting in altered blastocyst potential and long term changes in fetal and postnatal health and physiology (Thompson *et al.* 1995; Sinclair *et al.* 1999, 2007; Kwong *et al.* 2000; Ecker *et al.* 2004; Fernandez-Gonzalez *et al.* 2004; Fleming *et al.* 2004; Sinclair and Singh, 2007; Watkins *et al.* 2007, 2008). This sensitivity *in vivo* may be explained by the ‘Developmental Origins of Health and Disease’ (DOHaD) hypothesis which proposes that changes in the uterine environment mediated by maternal dietary quality induce responses within the embryo or fetus to match growth and metabolic phenotype to predicted postnatal nutrient availability. However, such responses may become maladaptive if predicted and actual nutrient levels are inconsistent, leading to adult onset cardiovascular and metabolic diseases (Gluckman & Hanson, 2004; McMillen & Robinson, 2005). Effects on developmental plasticity could also be induced in the periconceptual period, affecting oocyte maturation as well as embryo development, and this is the subject of the current study.

In mammals, oocyte maturation is characterised by the resumption of meiosis, extrusion of the first polar body and re-organization of cytoplasmic constituents for fertilization. Acquisition of oocyte developmental competence does not occur in isolation, but is dependent upon the surrounding somatic cumulus cells with which there is a complex exchange of developmental signals mediated via gap junctions and diverse pathways including Kit, GDF (growth differentiation factor) and BMP (bone morphogenetic protein) components (Eppig, 2001; Amleh & Dean, 2002; Kidder & Mhawi, 2002; Richards *et al.* 2002; Thomas & Vanderhyden, 2006). Several studies have shown oocyte maturation to be sensitive to environmental conditions which may also have more long term developmental consequences. *In vitro* maturation of mouse, sheep and human oocytes reduces their viability and developmental competence (Eppig & Schroeder, 1989; Barnes *et al.* 1996; Mikkelsen *et al.* 1999; Bertolini *et al.* 2002), resulting in altered fetal growth (Bertolini *et al.* 2002). *In vivo*, follicular growth and oocyte quality are affected by a range of dietary manipulations in domestic animals (Armstrong *et al.* 2001). Thus, dietary energy levels influence both follicle morphology and number and the composition of follicular fluid in ewes and heifers (Boland *et al.* 200; O’Callaghan *et al.* 2003), while increased protein and urea

intake and associated elevation in ammonia in follicular fluid result in reduced blastocyst development (McEvoy *et al.* 1997; Sinclair *et al.* 2000; Adamiak *et al.* 2005; Powell *et al.* 2006). However, it is unclear from *in vivo* studies to date what the relative sensitivity of oocyte or embryo environments might be to periconceptual dietary challenge maintained both before and after mating (Edwards & McMillen, 2002; Sinclair *et al.* 2007). In contrast, the embryo environment *in vivo* has been clearly demonstrated to be sensitive to maternal diet restriction (Kwong *et al.* 2000; Watkins *et al.* 2008).

Using a mouse model with large sample size, we have investigated the effect of maternal dietary protein restriction during oocyte maturation on postnatal growth, behaviour and cardiovascular physiology. We have found that maternal low protein diet (LPD) provided exclusively during oocyte maturation has minimal effects on postnatal growth but results in abnormal anxiety-related behaviour, elevated systolic blood pressure, reduced vascular responsiveness and altered kidney allometry and organization. Our results indicate firstly that oocyte maturation is a sensitive period with respect to maternal diet and onset of adult disease, and secondly that the oocyte and early embryo exhibit common and distinct environmental susceptibilities that may derive from the presence or absence of the paternal genome.

## **Methods**

**Animal treatments.** These were conducted using protocols approved by UK Home Office and local ethics committee. Female MF-1 mice were bred in-house (University of Southampton Biomedical Research Facility) and maintained on 0700-1900 hour light cycle with controlled temperature and standard chow and water *ad libitum*. Vaginal smears were taken from virgin females (7-8.5 weeks) to determine metoestrus at which point females were singly caged and randomly assigned either normal protein diet (NPD; 18% casein) or isocaloric low protein diet (LPD; 9% casein; Kwong *et al.* 2000; Watkins *et al.* 2008). After 3.5 days, females were mated overnight with MF-1 males (Harlan). Plug positive females were placed on NPD and individually housed for the remainder of gestation whilst plug negative females were removed from the study. In total, nineteen females of each dietary treatment (NPD and LPD) were allowed to develop their pregnancies to term, at which time all animals were returned to standard chow. Offspring were weighed on day of birth and

weekly for up to 28 weeks. Where possible, litter size was adjusted at birth to six (three males and three females). At 3 weeks, offspring were weaned and the sexes caged separately. Animal coding allowed for all outcome analyses to be conducted 'blind' to ensure objectivity.

**Behaviour.** At 4, 5, 6, 8, 11, 14, 17 and 20 weeks, offspring were subjected to species-specific locomotory, anxiety-related (open field) and affective (burrowing, nesting, glucose solution consumption) activity tests as previously described (Guenther *et al.* 2001; Cunningham *et al.* 2003; Cunningham *et al.* 2007; Watkins *et al.* 2008). Weeks 4-6 were taken as acclimatory and weeks 8-20 as assay tests. The illuminated open field (white PVC arena, 30 cm<sup>2</sup>) test comprised 3 minute observations with automated recording of total distance travelled, number of hind rears, jumps, time spent resting and average velocity.

**Systolic blood pressure (SBP).** SBP was determined at 9, 15 and 21 weeks by tail-cuff plethysmography as previously described (Watkins *et al.* 2006). Briefly, mice were allowed to acclimatize to 27-30°C before four recordings were taken per mouse; heart rate was monitored as an indicator of stress (Langley-Evans *et al.* 1996).

**Mesenteric vasoreactivity.** Vascular function in male offspring was assessed at 22 weeks in isolated small mesenteric arteries following cervical dislocation. The mesenteric arcade was excised and immersed in physiological salt solution (PSS; Torrens *et al.* 2003) at 4°C. Small mesenteric arteries (luminal diameter ca. 100-300 µm) were dissected free of connective tissue and mounted in PSS on a wire myograph (Multi Myograph Model 610M; JP Trading, Aarhus, Denmark) for analysis of isometric tension as described previously (Brawley *et al.* 2003; Torrens *et al.* 2003; Torrens *et al.* 2006). Briefly, following normalisation, cumulative concentration response curves (CRCs) were measured for the  $\alpha_1$ -adrenergic agonist, phenylephrine (PE; 10<sup>-9</sup> to 10<sup>-4</sup> M). Vessels were then pre-constricted with a submaximal dose of PE (EC<sub>80</sub>) and CRCs to the endothelium-dependent vasodilator, acetylcholine (ACh; 10<sup>-9</sup> to 10<sup>-5</sup> M), the  $\beta$ -adrenoceptor agonist, isoprenaline (ISO; 10<sup>-10</sup> to 10<sup>-6</sup> M) and the endothelium-independent vasodilator, sodium nitroprusside (SNP; 10<sup>-11</sup> to 10<sup>-5</sup> M)

were conducted in that order. All drugs were purchased from Sigma (Poole, Dorset, UK).

**Kidney nephron counting.** At 28 weeks, female mice were terminally anaesthetized by an intraperitoneal injection of a ketamine (Fort Dodge) acepromazine (C-Vet) anaesthetic followed by thoracotomy. A butterfly needle (Venisystems, Butterfly -23 ST) was inserted into the left ventricle and the vena cava was cut. Mice were perfused with ~15 ml heparinized saline (0.5 units/ml saline, Sigma) at a mean pressure of 100 mm Hg to prevent clot formation in the kidney, followed by ~15 ml neutral buffered formalin (Gurr). Left kidneys were removed and immersion fixed in formalin for 48 hours, followed by overnight dehydration through graded alcohols (Leica TP 1020 tissue processor). Each kidney was cut into eight pieces (left/right, anterior/posterior and dorsal/ventral portions) and processed into glycol methacrylate (JB-4 Embedding Kit; Polysciences Europe GmbH, Germany). In preliminary studies, the two blocks comprising anterior, dorsal, left and right kidney pieces were found not to differ significantly in nephron number from the remaining six blocks and were subsequently used as representative of the entire kidney. These blocks were exhaustively sectioned at 2  $\mu\text{m}$  using an ultramicrotome (R. Jung Heidelberg). Every 100th and 110th section was mounted and stained with toluidine blue (1% toluidine blue w/v in 1% sodium tetra borate w/v in distilled water) for approximately 5-10 seconds, dried and cover slipped with DPX (Gurr). Unbiased stereological counting was carried out using Stereo Investigator (Microbrightfield, Williston, VT) software connected to a Dialux 22 microscope (Leica) with a motorized x-y-z stage (Ludl, Hawthorne, NY) and a DEI-750D video camera (Optronics, Goleta, CA). An unbiased counting frame (500  $\mu\text{m}$  square) was randomly placed over the 100th section and glomeruli within or touching the inclusion lines were counted. The trace was then overlaid on the 110th section and glomeruli present here but absent from the 100th section were counted. On average, 10-15 pairs of sections were analysed per block and an estimate of kidney glomerular number was obtained using the fractionator principle (Bertram, 2005).

**Angiotensin converting enzyme (ACE) activity.** Serum and lung ACE activity were analysed as previously described (Watkins *et al.* 2006; Watkins *et al.* 2007). Briefly, serum samples were incubated in the presence of hippuryl-L-histidine-L-leucine

acetate salt (HHLA; Sigma) solution in phosphate buffer at 37°C followed by addition of cyanuric chloride (Sigma, Poole, Dorset, UK) in 1,4 dioxane (Sigma, Poole, Dorset, UK). Four replicates per sample were analysed on a Dynex MRX2 plate reader at 380 nm. Samples containing only serum and buffer were used as negative controls. Sample readings were analysed against a standard curve prepared in buffer and treated exactly as the samples. Serum ACE activity was expressed as nanomoles of hippurate formed per ml<sup>-1</sup> serum per minute; 15-17 samples per treatment group were analysed. The intra- and inter-assay variation was 3.88% and 17.1% respectively.

Lung tissue (approximately 50 mg) was homogenized in buffer, centrifuged and the supernatant removed and stored. The pellets were homogenised a second time and the supernatants removed after centrifugation as before. Samples of each extract were mixed with buffer, water and hippuryl-L-histidine-L-leucine acetate salt (Sigma, Poole, Dorset, UK). Negative controls included the addition of HCl prior to addition of the hippuryl-L-histidine-L-leucine. Samples and blanks were incubated at 37°C prior to the addition of HCl, NaOH, KH<sub>2</sub>PO<sub>4</sub> and cyanuric chloride. Sample readings were analysed against a standard curve prepared in buffer and treated exactly as the samples. Samples were analysed in duplicate on a Dynex MRX2 plate reader at 380 nm, and an average activity taken. Tissue protein content was measured using the Bio-Rad protein assay kit. Tissue ACE activity was expressed as nanomoles hippurate formed per mg of protein per minute. The intra- and inter-assay variation was 16.9% and 22% respectively.

**Organ allometry.** At 28 weeks, following cervical dislocation, blood samples were removed via heart puncture and liver, left and right kidneys, heart, lungs and brain were dissected, weighed, and snap frozen in liquid nitrogen and stored at -80°C.

**Statistical analyses.** Student's *t*-test was used to analyse mean gestational litter size and male:female ratio (SigmaStat statistical software, version 3.5). Vascular responsiveness data (cumulative concentration response curves) were analysed using 2-way ANOVA (Prism 3.0, GraphPad Software Inc) as previously (Torrens *et al.* 2003; Torrens *et al.* 2006). All other postnatal data (birth weight, postnatal weights, behavioural data, SBP, organ weights and ratios, kidney glomerular number and

correlations) were analysed using a multilevel random effects regression model (SPSS version 14) which takes into account the hierarchical nature of the data with between-mother and within-mother variation and different parameters measured from individual animals (Kwong *et al.* 2004; Watkins *et al.* 2006). Thus, differences identified between treatment groups throughout the study are independent of maternal origin of litter, gestational litter size, body weight, unless otherwise stated. Week of testing was included into the analysis of the behavioural data.

## Results

**Litter size, birth weight and postnatal growth.** Gestation length, gestational litter size and offspring gender ratio were not significantly different between dietary groups (Table 1). LPD males displayed a trend towards elevated mean birth weight when compared to NPD males (Fig. 1;  $P = 0.073$ ) independent of gestational litter size and gender ratio. No difference in birth weight was observed between female groups. No difference in mean weekly weight was evident between treatment groups up to 28 weeks (Fig. 2 A, B).

**Behaviour.** When examined on an individual week basis, LPD males displayed reduced distance travelled at 14, 17 and 20 weeks (reduced by 37.4, 54 and 40.5% respectively vs NPD; all  $P < 0.03$ ), and increased time resting at 17 weeks (by 9.1 % vs NPD;  $P = 0.039$ ) compared to NPD males. LPD females also displayed reduced distance travelled at 20 weeks (by 38.4%;  $P = 0.037$ ), reduced numbers of rears at 8, 11, 14 and 20 weeks (by 36.7, 35.94, 41.4 and 49.8% vs NPD;  $P < 0.035$ ), reduced numbers of jumps at 8, 14 and 20 weeks (by 48.3, 69.74, 89.3% vs NPD;  $P < 0.05$ ), increased time resting at 14, 17 and 20 weeks (by 5.0, 2.8, 8.4% vs NPD;  $P = < 0.05$ ) and increased velocity at 11 weeks (by 41.2%;  $P = 0.042$ ). All differences were independent of maternal origin and gestational litter size. Integration of all weekly data into mean open field activities, taking into account week of testing, showed that the male LPD offspring displayed reduced mean distance travelled, number of rears and jumps, increased velocity ( $P < 0.005$ ), and a trend towards an increased time spent resting (Fig 3A;  $P = 0.071$ ). LPD females also displayed reduced distance travelled, mean number of rears and jumps, increased time resting and increased velocity compared to NPD females (Fig. 3B;  $P < 0.01$ ). Analysis of affective behaviour showed LPD females displayed a trend towards increased burrowing activity after 2 hours and



overnight (by 16.5 and 5.7% respectively vs NPD;  $P = 0.096$  and  $0.087$ ), however, no differences were observed between treatment groups for either glucose solution consumption or in nest construction (data not shown).

**Systolic blood pressure.** Mean systolic blood pressure (SBP) of offspring was determined at 9, 15 and 21 weeks, with the mean of these values recorded as ‘Life SBP’ (Fig. 4 A, B). LPD males displayed elevated SBP at all assay times (4.23 % higher Life SBP than NPD;  $P = 0.002$ ; Fig. 4A) whilst LPD females had a trend towards a higher SBP at 15 weeks ( $P = 0.09$ ) and significantly higher SBP at 21 weeks and for Life SBP (2.88 % higher Life SBP than NPD;  $P = 0.01$ ; Fig. 4B). Whilst these data were independent of maternal origin and body weight, gestational litter size positively influenced 21 week SBP in males (+1.11 mm Hg per value increase in litter size,  $P = 0.034$ ), and Life SBP in females (+0.68 mm Hg per value increase in litter size,  $P = 0.014$ ).

#### **Mesenteric artery vasoreactivity**

The  $\alpha_1$ -adrenergic agonist, PE, produced a concentration-dependent vasoconstriction which was similar in LPD and NPD males (Fig. 5A). Vasodilatation to the endothelium-dependent vasodilator, ACh, and the  $\beta$ -adrenoceptor agonist, ISO, in PE-precontracted arteries was attenuated in the LPD group (Fig. 5B, C;  $P = 0.001$  and  $0.014$  respectively). Dilatation to the endothelium-independent vasodilator, SNP, was similar between the two groups (Fig. 5D).

**Organ allometry.** No differences in organ weights (liver, kidney, heart, lung, brain and cerebellum) or organ:body weight ratios were evident between LPD and NPD males at 28 weeks (Table 2A). However, LPD females had reduced right (Table 2B;  $P = 0.031$ ) and combined (data not shown;  $P = 0.044$ ) kidney:body weight ratios compared to NPD females. A positive correlation was present between both left and right kidney:body weight ratios and Life SBP in LPD males (Fig. 6A, B;  $P < 0.007$ ), different ( $P < 0.015$ ) to NPD males which showed no correlation between these factors. LPD males also displayed a positive correlation between heart:body weight ratio and Life SBP (Fig. 6C;  $P = 0.004$ ), different from NPD males ( $P = 0.05$ ) which

showed no correlation between these factors. All differences were independent of maternal origin and gestational litter size.

**Kidney nephron counts.** Morphometric analysis of left kidney glomerular number in female offspring at 28 weeks revealed increased numbers within the LPD group (28.1% higher than NPD;  $P = 0.03$ ; Fig. 7A). When mean glomerular number was expressed as per gm or  $\text{cm}^3$  of kidney tissue, LPD kidneys demonstrated a significant increase and a trend towards an increased glomerular number respectively (32.8 and 35.1% higher than NPD;  $P = 0.031$  and  $0.091$  respectively; Fig. 7A). Whilst these data were independent of gestational litter size and kidney weight effects, when mean glomerular number/ $\text{cm}^3$  was analysed independently of litter size, a significant increase was observed for the LPD group ( $P = 0.036$ ). Analysis of glomerular number:Life SBP correlation revealed the LPD group to have a negative correlation ( $r^2 = -0.902$ ,  $P = 0.007$ ), whilst the NPD group displayed no correlation ( $r^2 = 0.382$ ,  $P = 0.228$ ; Fig. 7B).

**Angiotensin converting enzyme (ACE) activity.** No difference in serum ACE activity was observed between NPD and LPD males ( $126.22 \pm 14.30$  and  $146.53 \pm 14.19$  nanomoles hippurate respectively,  $P > 0.05$ ), or females ( $81.14 \pm 8.00$  and  $105.17 \pm 12.53$  nanomoles hippurate respectively,  $P > 0.05$ , data not shown). No difference in lung ACE activity was observed between NPD and LPD males ( $3.25 \pm 0.52$  and  $3.11 \pm 0.48$  nanomoles hippurate respectively,  $P > 0.05$ ) or females ( $3.30 \pm 0.38$  and  $3.45 \pm 0.67$  nanomoles hippurate respectively,  $P < 0.05$ , data not shown).

## Discussion

Collectively, our data demonstrate that maternal LPD treatment exclusively during mouse oocyte maturation (3.5 days prior to conception) is sufficient to alter postnatal phenotype resulting in behavioural and cardiovascular changes indicative of adult disease. These findings, together with those previously identified by maternal protein restriction exclusively during preimplantation development (Kwong *et al.* 2000; Watkins *et al.* 2008), or during both pre- and post fertilisation periods (Edwards & McMillen, 2002; Sinclair *et al.* 2007), provide strong support for the DOHaD hypothesis and the vulnerability of the periconceptual period, including that of oocyte maturation, to environmental conditions.

Analysis of postnatal behavioural outcomes revealed predominant effects induced by LPD treatment during oocyte maturation, on both male and female offspring. These included altered open field activity, notably reduced exploratory behaviour (distance travelled, rearing, jumping), increased time spent resting, and increased velocity. Behavioural testing for species-typical spontaneous activities as used here provide sensitive assays of well-being, devised to identify a mild sickness phenotype in murine prion and related disease models prior to the detection of clinical biochemical symptoms (Guenther *et al.* 2001; Cunningham *et al.* 2003; Cunningham *et al.* 2007). The reduced exploratory activity and longer rest periods are difficult to interpret in isolation but these deficits signify reduced responsiveness to environmental stimuli which may associate with increased anxiety and/or a change in affective behaviour (Guenther *et al.* 2001; Cunningham *et al.* 2003; Cunningham *et al.* 2007). Altered postnatal open field activity has also been identified, especially in females, in response to maternal LPD restricted to the preimplantation period, further indicating the importance of periconceptual dietary environment on adult behaviour (Watkins *et al.* 2008).

The second major change in phenotype detected in offspring following maternal LPD treatment during oocyte maturation is associated with the cardiovascular system. Male and female LPD offspring developed relative hypertension with females showing delay in its inception but, in both sexes, Life SBP was elevated significantly. We believe this is the first report to demonstrate hypertension associated with maternal protein restriction during the period of oocyte maturation alone, although LPD treatment during pregnancy or limited to the preimplantation period has been shown previously to lead to raised SBP in rodent models (Langley & Jackson, 1994; Kwong *et al.* 2000; Fleming *et al.* 2004; Gluckman & and Hanson, 2004; McMillen & Robinson, 2005; Sinclair & Singh, 2007; Watkins *et al.* 2008). The origins of hypertension using these models for gestational diet restriction, together with domestic animal models, appear complex and may involve alteration to the hypothalamic-pituitary-adrenal (HPA) axis and the renin-angiotensin system during fetal development leading to loss of normal cardiovascular control and peripheral vessel dysfunction (Brawley *et al.* 2003; Torrens *et al.* 2003; Fleming *et al.* 2004; Gluckman & and Hanson, 2004; McMillen & Robinson, 2005; Torrens *et al.* 2006).

To interpret the potential contributory components of hypertension in LPD offspring, we examined arterial vessel responsiveness to both vasoconstrictors and endothelium-dependent and –independent vasodilators in male offspring. We found that mesenteric arteries from LPD animals displayed attenuated dilatation to ACh and ISO. Such effects have been shown to associate with aspects of cardiovascular disease in the rat following maternal LPD during pregnancy (Brawley *et al.* 2003; Torrens *et al.* 2003; Torrens *et al.* 2006). These data indicate that reduced vascular responsiveness in males may be mediated through endothelial-dependent mechanisms, possibly associated with nitric oxide and/or  $\beta$ -adrenoceptor pathways (Brawley *et al.* 2003; Torrens *et al.* 2003; Torrens *et al.* 2006).

Our other studies to evaluate cardiovascular phenotype have focused on the female kidney, which has been shown to be reduced in size with reduced nephron number as a result of gestational maternal diet restriction in several species (Zandi-Nejad *et al.* 2006; Bagby, 2007), including the mouse (Hoppe *et al.* 2007). Compromised kidney development during late gestation may reflect a fetal ‘strategy’ to protect vital organ development such as the brain and heart at the expense of abdominal organs in response to restricted nutrient availability (McMillen and Robinson, 2005; Bagby, 2007). Whilst reduced kidney growth or body weight in offspring coincides with reduced nephron number and hypertension in several (Langley-Evans *et al.* 1999; Bagby, 2007) but not all (Woods *et al.* 2005) models of gestational diet restriction, our findings are unusual in that maternal LPD female offspring exhibited reduced kidney size coupled with *increased* nephron number. These changes in kidney size also altered the relationship with Life SBP. Given that kidney development occurs late in gestation and that our dietary challenge actually precedes the initiation of the developmental programme, it is possible that the kidney phenotype of increased nephron number may reflect a compensatory response to the maternal challenge. Indeed, the negative correlation observed between Life SBP and kidney nephron number identified in the female LPD group supports this contention. However, further molecular and biochemical studies on kidney morphogenesis and postnatal function in response to protein restriction during oocyte maturation are required to interpret more fully the kidney phenotype and its relationship with cardiovascular health.

The diverse outcomes identified in offspring following maternal LPD during oocyte maturation indicate that nutrition prior to conception is critical in the setting of homeostatic regulators affecting health and disease risk. Whilst the discrete nature of the dietary challenge allows us to focus on the health implications for a specific period of the reproductive cycle, and also to make comparisons between different periods (discussed below), we need to consider two alternative interpretations of our outcomes. First, the outcomes may also reflect a persistence of the dietary challenge on maternal environment and physiology beyond that of oocyte maturation into early development. Second, the outcomes may also reflect changes in maternal environment induced by the stress of the diet change itself. We cannot exclude either of these factors contributing to outcomes but, nevertheless, it would be reasonable to conclude that even if they were, then a changed diet during oocyte maturation would still be the upstream mediator, a factor pertinent in any extrapolation to the human situation and the importance of dietary practice prior to conception. However, we feel that these two indirect components are likely to be minimal for the following reasons. First, in our previous mouse diet analysis focusing on the preimplantation period (Watkins *et al.* 2008), the outcomes identified compared to here were distinct but with some shared responses (discussed below) suggesting that the discrete nature of the challenges were having specific effects at that time rather than persisting longer. Second, in that study, embryo transfer was conducted of LPD embryos into NPD recipients to test directly whether a persistent effect on the maternal environment was contributory to outcomes. This revealed that the LPD-induced effect on the embryo was preserved in the absence of a maternal LPD environment after transfer, confirming the derivation of the outcome to maternal diet during preimplantation development. Whilst a similar strategy of analysing embryo transfers in the current study would notionally confirm the origin of our findings, and will be considered for future studies, the transfer procedure and in vitro treatment involved may generate confounding stress-related responses as these would need to be conducted on the stage immediately following oocyte maturation, the period of fertilisation-mediated signalling of embryonic genome activation known for its environmental sensitivity (Gardner and Lane, 1996; Qiu *et al.* 2003). Indeed, in an earlier study where we investigated the effects of embryo culture on postnatal outcomes, blastocyst transfer itself was found to affect outcomes but minimally compared to the embryo culture challenge (Watkins *et al.* 2007).

Comparison of the postnatal phenotypes induced in mice in response to maternal LPD experienced during oocyte maturation here with that during preimplantation embryo development (Watkins *et al.* 2008) gives further insight into potential mechanisms. Whilst hypertension in male and female offspring and behavioural changes, predominantly in female offspring, are evident in both models, the major distinction is in the effect on fetal and postnatal growth, only evident in response to maternal LPD during preimplantation development (Watkins *et al.* 2008). Whilst, in the current study, we are challenging a different phase in the reproductive cycle (gamete versus preimplantation embryo development), and therefore cannot discount the effect this may have upon postnatal development, one important distinction between the two models is the presence of the paternal genome at the time of dietary challenge during the preimplantation period. Genomic imprinting has been identified a critical role in controlling growth of the conceptus during pregnancy by, for example, modulating placental efficiency for maternal-fetal nutrient delivery and coordinating growth factor ligand-receptor interactions (Angiolini *et al.* 2006; Smith *et al.* 2006). Thus, paternal and maternal alleles of imprinted genes may confer opposing effects in such mechanisms, reflecting the need for the paternal genome to promote fetal growth and the maternal genome to temper this to protect maternal health for later pregnancies. The ‘conflict hypothesis’ embodies these divergent genomic strategies (Angiolini *et al.* 2006; Smith *et al.* 2006). Changes in the expression (but to date not changes in DNA methylation status) of imprinted genes have been identified in preimplantation embryos and their derivative tissues in response to maternal LPD (Kwong *et al.* 2006). Moreover, changes in expression and/or DNA methylation status of imprinted genes have been shown following embryo in vitro culture treatments (Doherty *et al.* 2000; Khosla *et al.* 2001; Young *et al.* 2001; Mann *et al.* 2004). Thus, the distinct effects on growth control mediated by maternal LPD during oocyte maturation versus preimplantation development may reflect differential effects on the paternal genome.

In conclusion, we show that oocyte maturation in mice is sensitive in vivo to maternal dietary protein level with behavioural and cardiovascular markers of postnatal health being adversely affected in conditions of protein undernutrition. These novel findings support the contention that periconceptual environment including both before and after fertilization, and both in vitro and in vivo, associates

with adult health and disease risk. Whilst our data here using an animal model cannot be extrapolated directly to the human, we consider animal models as useful in identifying potential relationships that merit separate investigation across species including the human. Given that preimplantation programming of adult disease has been shown to comprise a complex of mechanisms acting at epigenetic, physiological and cellular levels (Fernandez-Gonzalez *et al.* 2004), further studies are required to elucidate how oocyte maturation may be affected by maternal diet with such long-lasting consequences.

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## **Figure legends**

### **Figure 1**

Maternal low protein diet (LPD) during oocyte maturation causes an elevation in birth weight in male offspring at a trend level ( $P = 0.073$ ) independent of maternal origin and gestational litter size. Mean ( $\pm$  SEM) for males ( $n = 82-96$ ) and females ( $n = 101-105$ ) from 19 litters per treatment group.

### **Figure 2**

Maternal LPD during oocyte maturation has no effect on postnatal growth. Mean ( $\pm$  SEM) body mass for (A) male ( $n = 42-60$ ) and (B) female ( $n = 48-57$ ) offspring for 3-28 weeks from 19 litters per treatment group.

### **Figure 3**

Maternal LPD during oocyte maturation leads to changes in postnatal behaviour. Mean open field behaviour for (A) male ( $n = 52-59$  per treatment) and (B) female ( $n = 54-57$ ) offspring from 19 litters. \* denotes  $P < 0.01$  independent of maternal origin and gestational litter size. ‡ denotes difference at a trend level ( $P = 0.071$ ).

### **Figure 4**

Maternal LPD during oocyte maturation induces an elevation in postnatal SBP. Mean ( $\pm$  SEM) SBP of (A) male ( $n = 45-59$  per treatment) and (B) female ( $n = 51-56$ ) offspring from 19 litters per treatment group. \* denotes  $P < 0.05$  independent of maternal origin and gestational litter size. ‡ denotes difference at a trend level ( $P = 0.09$ ).

### **Figure 5**

Maternal LPD during oocyte maturation reduces the vasoreactivity of isolated male offspring mesenteric arteries at 22 weeks. Cumulative additions of (A) phenylephrine (PE) and, after pre-constriction with PE of the vasodilators (B) acetylcholine (ACh), (C) isoprenaline (ISO) and (D) sodium nitroprusside (SNP). No difference is observed in responsiveness to PE or SNP between treatments, however, LPD arteries display attenuated responses to ACh and ISO when compared to NPD arteries. Values are

mean  $\pm$  SEM; n = 7 males, each from different litters, from each treatment group; \*P <0.015.

### **Figure 6**

Maternal LPD during oocyte maturation alters the correlation between organ:body weight ratios and Life SBP in offspring. (A, B) LPD males have a positive correlation (solid line) between both left and right kidney:body weight ratio and Life SBP (P <0.015) which differs from NPD males (dashed line, P <0.015). (C) LPD males have a positive correlation (solid line) between heart:body weight ratio and Life SBP (P =0.004) which differed from that of NPD males (dashed line, P =0.05). All differences were independent of maternal origin and gestational litter size.

### **Figure 7**

Maternal LPD during oocyte maturation increases kidney mean glomerular number (GN) in female LPD offspring at 28 weeks. (A) GN in whole left kidney and GN/gm kidney mass and GN/cm<sup>3</sup> kidney tissue. Values are mean  $\pm$  SEM, n = 6 kidneys per treatment, \*P <0.05 independent of maternal origin and gestational litter size. ‡P = 0.091.



Table 1. Litter size and birth criteria ( $\pm$  SEM) of offspring from LPD and NPD treatment groups.

	Litter number	Gestation length, days	Litter size at birth	Offspring number	Male/female ratio
NPD	19	20	$10.6 \pm 0.366$	201	$1.341 \pm 0.403$
LPD	19	20	$11.12 \pm 0.621$	211	$0.996 \pm 0.114$

Table 2. Mean organ weight (g) and organ:body weight ratio of (A) male (n = 27-42 per treatment) and (B) female (n = 30-39 per treatment) offspring. \* denotes P = 0.031 independent of maternal origin and litter size; ‡ denotes difference at a trend level P <0.075.

(A) Male

Organ weights (g)

	Liver	Left Kidney	Right Kidney	Heart	Lung	Brain	Cerebellum
NPD	2.246 ± 0.044	0.330 ± 0.008	0.341 ± 0.009	0.220 ± 0.004	0.219 ± 0.005	0.455 ± 0.005	0.056 ± 0.002
LPD	2.172 ± 0.063	0.323 ± 0.011	0.34 ± 0.011	0.218 ± 0.004	0.215 ± 0.005	0.458 ± 0.005	0.058 ± 0.001

Organ:body weight ratio (x 100)

	Liver	Left Kidney	Right Kidney	Heart	Lung	Brain	Cerebellum
NPD	4.780 ± 0.053	0.702 ± 0.014	0.725 ± 0.017	0.469 ± 0.008	0.468 ± 0.012	0.977 ± 0.0017	0.120 ± 0.003
LPD	4.740 ± 0.138	0.706 ± 0.025	0.734 ± 0.026	0.479 ± 0.013	0.473 ± 0.014	1.010 ± 0.028	0.127 ± 0.004

(B) Female

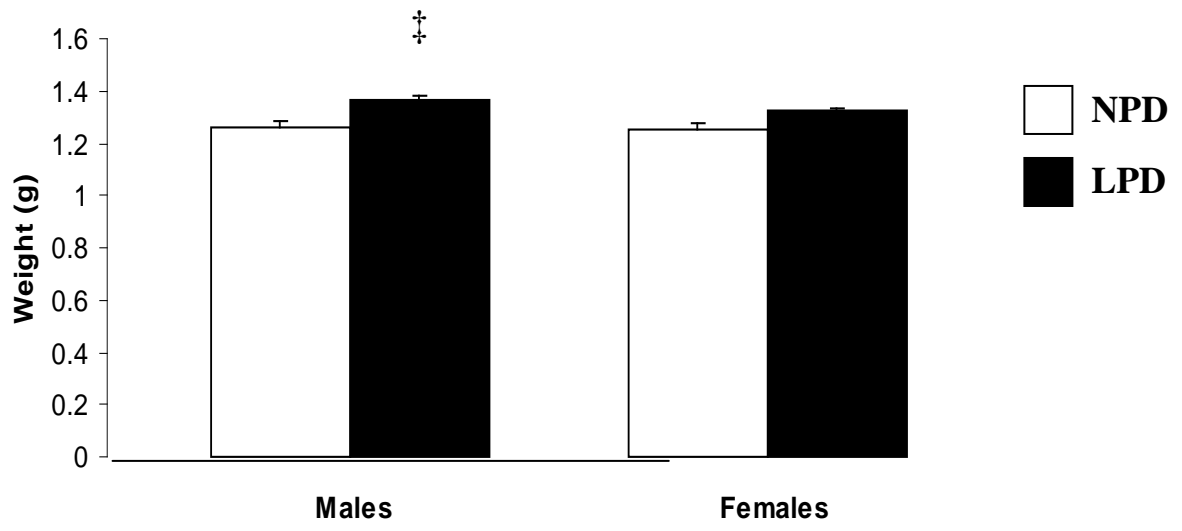
Organ weights (g)

	Liver	Left Kidney	Right Kidney	Heart	Lung	Brain	Cerebellum
NPD	1.824 ± 0.05	0.197 ± 0.006	0.204 ± 0.004	0.176 ± 0.003	0.196 ± 0.005	0.471 ± 0.009	0.058 ± 0.001
LPD	1.833 ± 0.03	0.189 ± 0.003	0.192 ± 0.003‡	0.174 ± 0.003	0.197 ± 0.004	0.466 ± 0.001	0.058 ± 0.001

Organ:body weight ratio (x100)

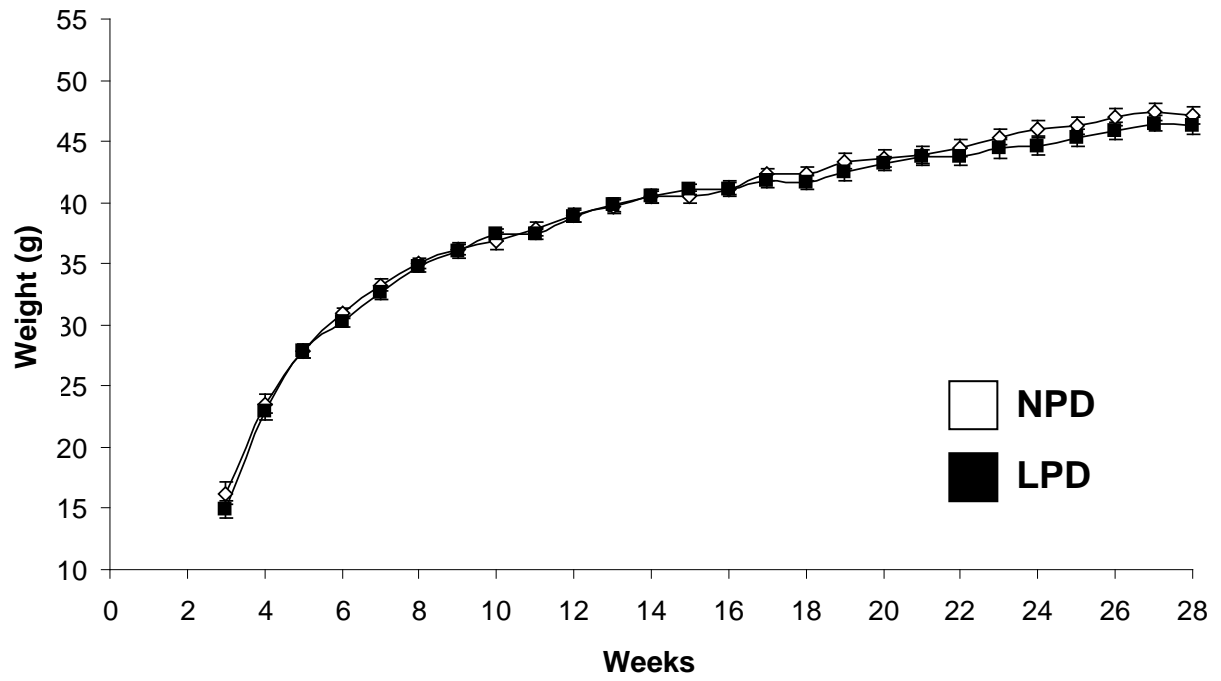
	Liver	Left Kidney	Right Kidney	Heart	Lung	Brain	Cerebellum
NPD	4.500 ± 0.080	0.494 ± 0.015	0.510 ± 0.015	0.442 ± 0.012	0.493 ± 0.011	1.200 ± 0.032	0.148 ± 0.004
LPD	4.410 ± 0.143	0.455 ± 0.016‡	0.462 ± 0.017*	0.422 ± 0.016	0.475 ± 0.017	1.170 ± 0.031	0.146 ± 0.004

**Figure 1**

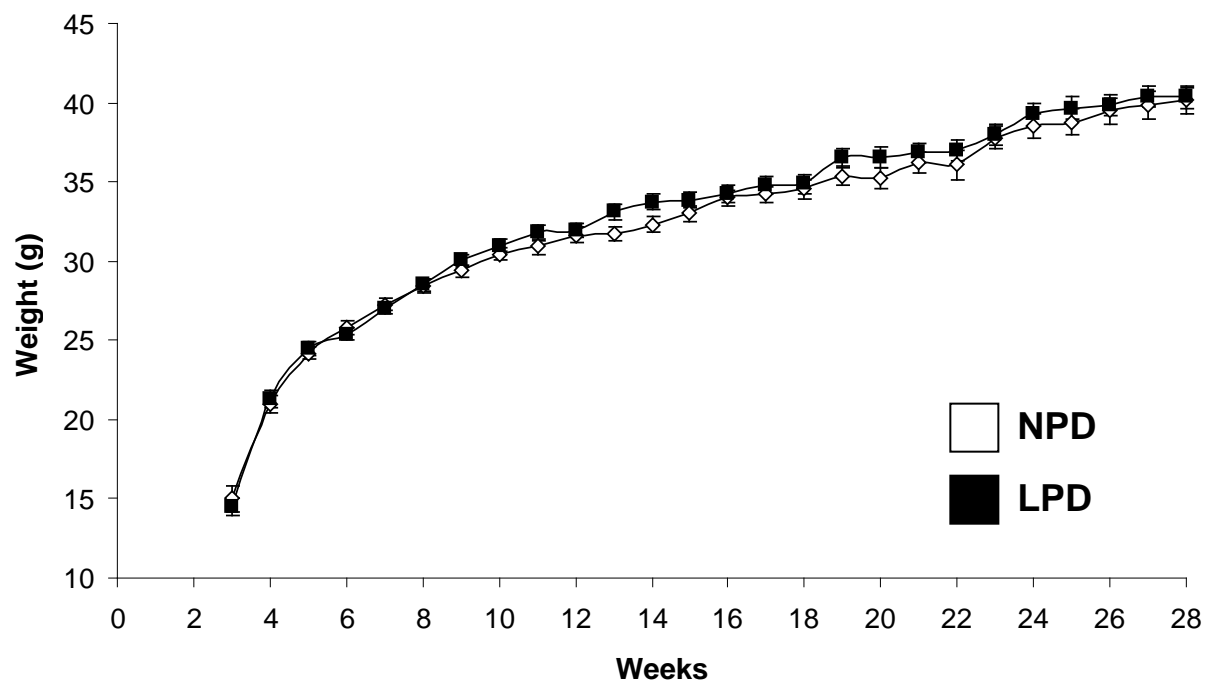


**Figure 2**

(A) Males

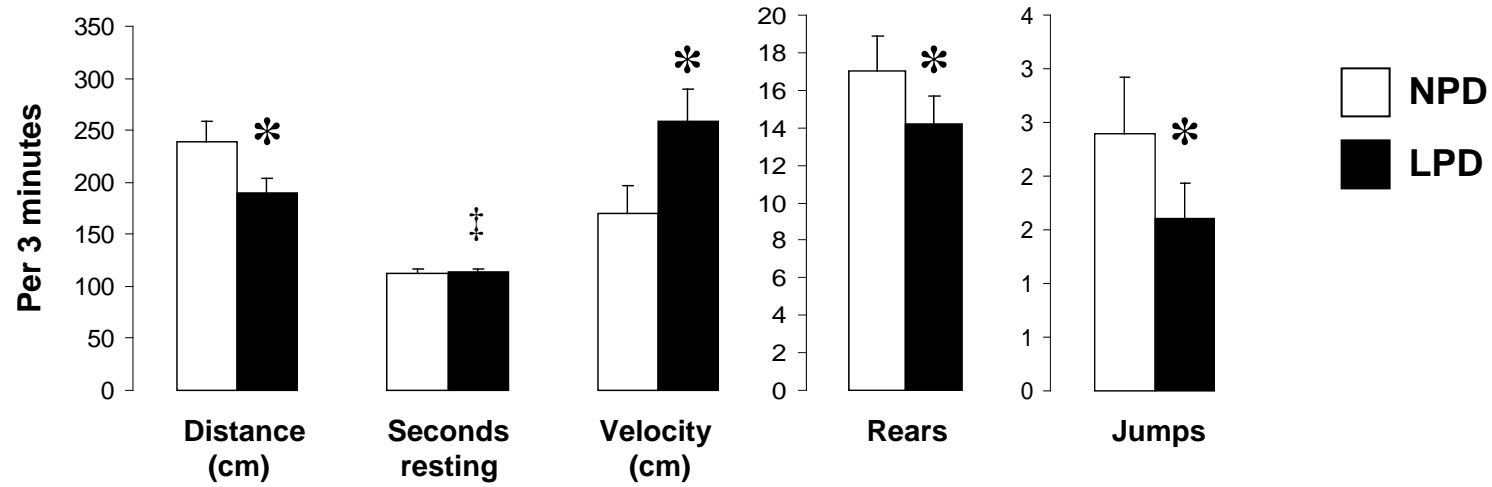


(B) Females

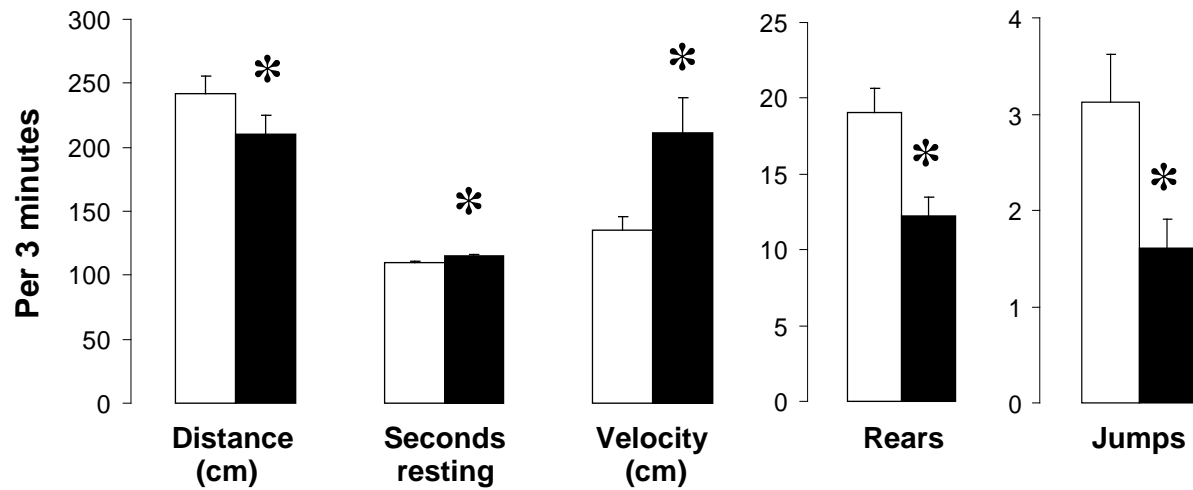


**Figure 3**

(A) Males

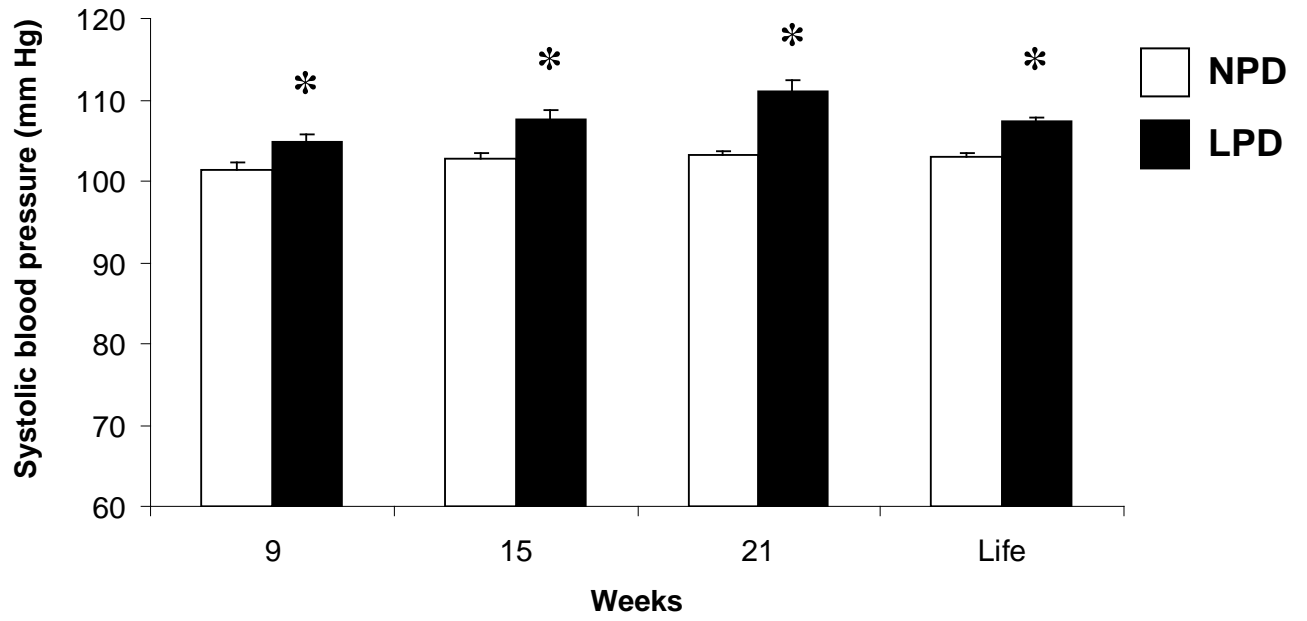


(B) Females

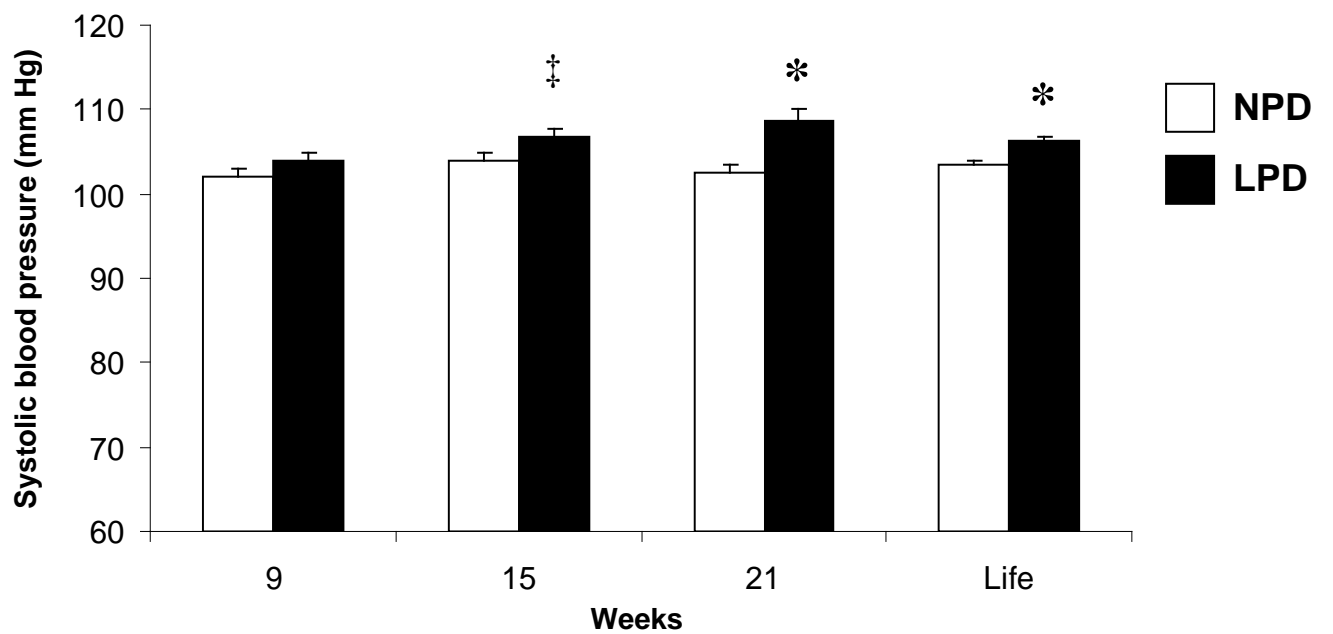


**Figure 4**

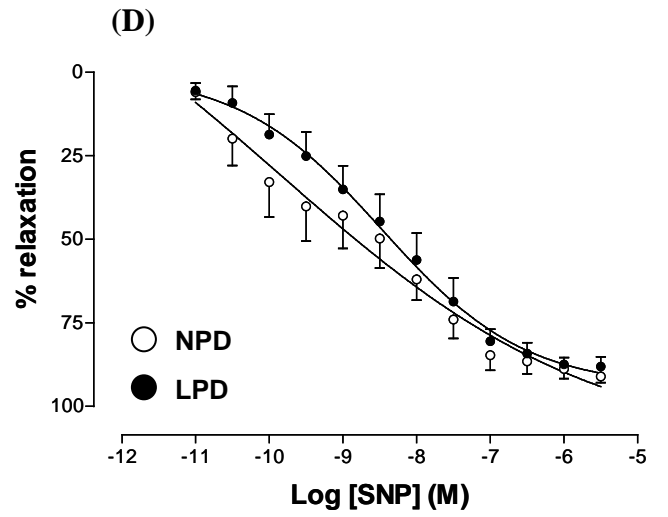
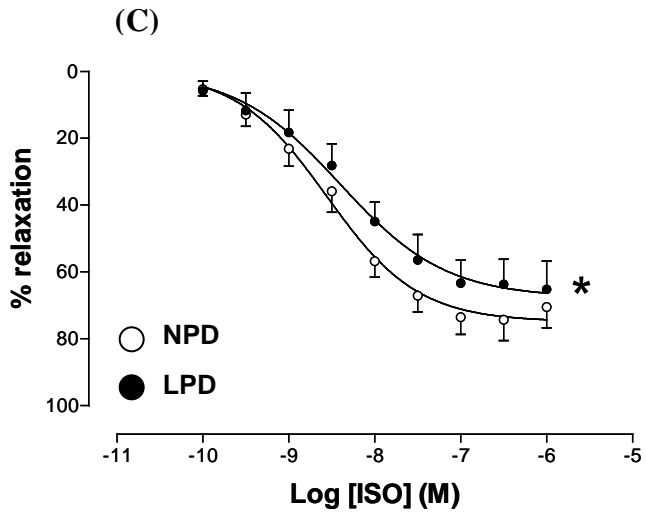
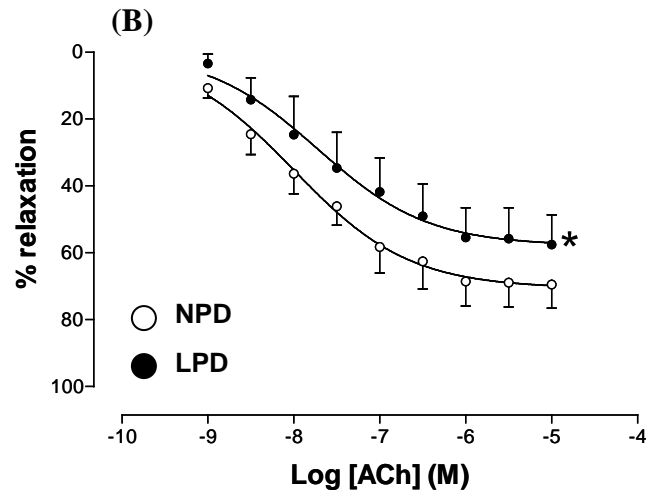
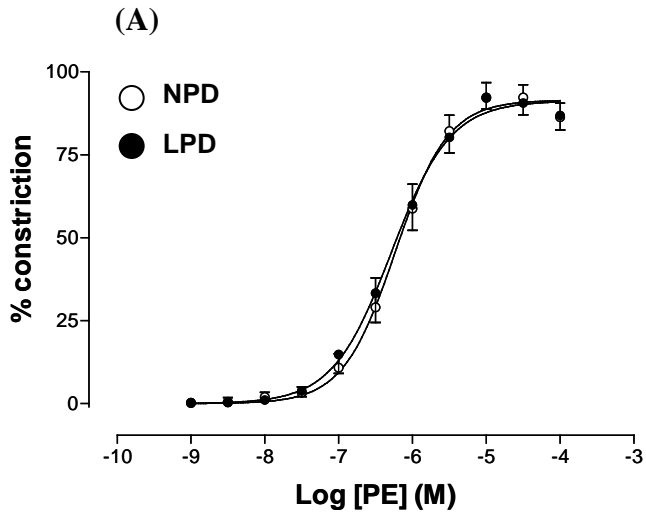
(A) Males



(B) Females



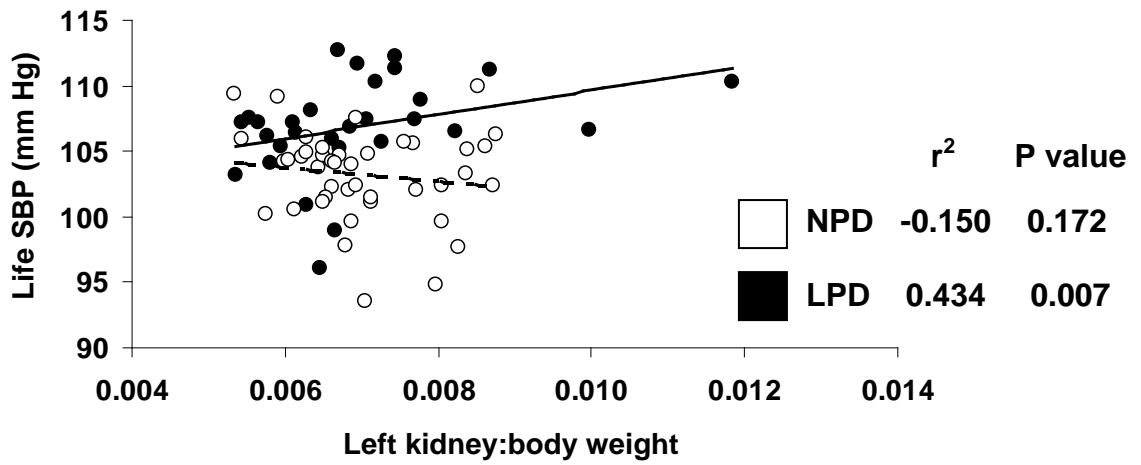
**Figure 5**



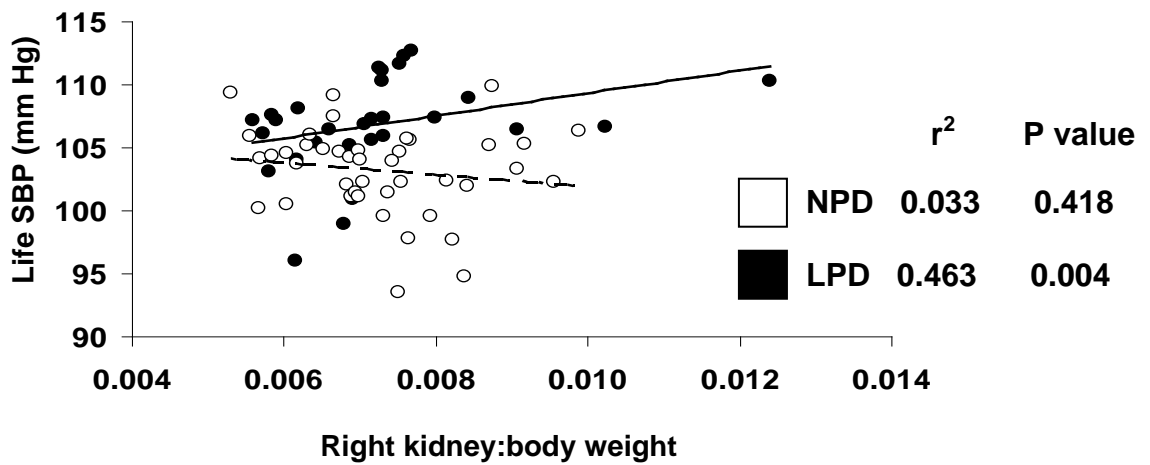


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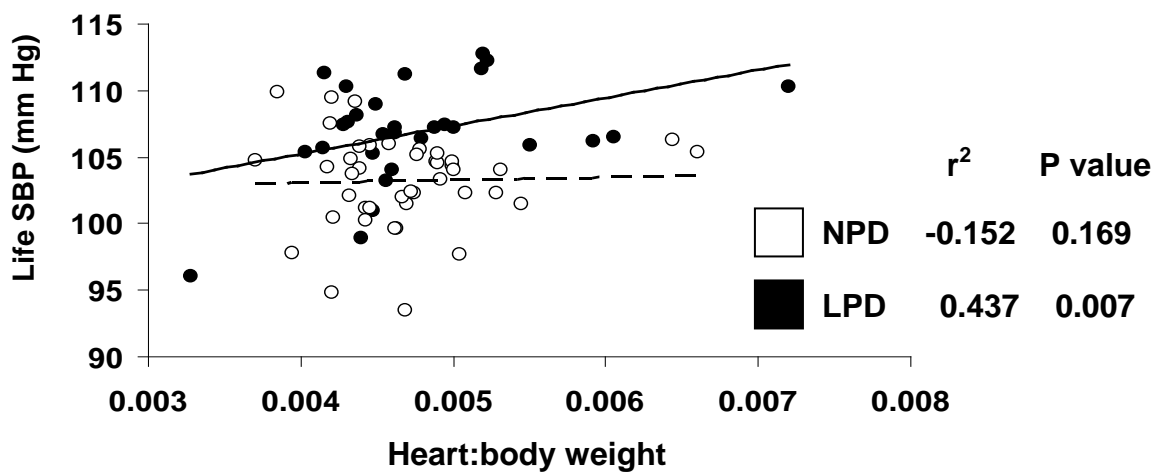
(A)



(B)

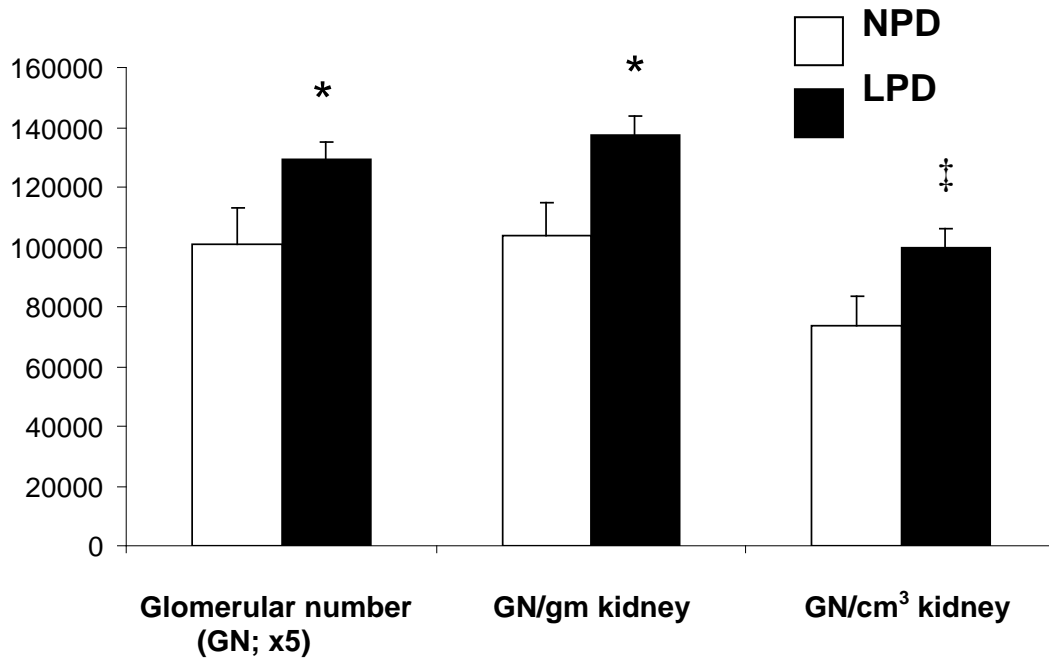


(C)



**Figure 7**

(A)



(B)

