British Journal of Nutrition (2009), **101**, 1020–1030
doi:10.1017/S0007114508057607

Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy

Matthew Harrison and Simon C. Langley-Evans*

University of Nottingham, School of Biosciences, Sutton Bonington, Loughborough LE12 5RD, UK

(Received 6 March 2008 – Revised 15 July 2008 – Accepted 15 July 2008 – First published online 9 September 2008)

Associations between birth weight and CVD in adult life are supported by experiments showing that undernutrition in fetal life programmes blood pressure. In rats, the feeding of a maternal low-protein (MLP) diet during gestation programmes hypertension. The present study aimed to assess the potential for a nutritional insult to impact across several generations. Pregnant female Wistar (F_0) rats were fed a control (CON; *n* 10) or MLP (*n* 10) diet throughout gestation. At delivery all animals were fed a standard laboratory chow diet. At 10 weeks of age, F_1 generation offspring were mated to produce a second generation (F_2) without any further dietary change. The same procedure produced an F_3 generation. Blood pressure in all generations was determined at 4, 6 and 8 weeks of age and nephron number was determined at 10 weeks of age. F_1 generation MLP-exposed offspring exhibited raised ($P<0{\cdot}001$) systolic blood pressure (male 143 (SEM 4) mmHg; female 141 (SEM 4) mmHg) compared with CON animals (male 132 (SEM 3) mmHg; female 134 (SEM 4) mmHg). Raised blood pressure and reduced nephron number was also noted in the F_2 generation ($P<0{\cdot}001$) and this intergenerational transmission occurred via both the maternal and paternal lines, as all three possible offspring crosses (MLP × CON, CON × MLP and MLP × MLP) were hypertensive (132 (SEM 3) mmHg) compared with CON animals (CON × CON; 123 (SEM 2) mmHg). No effect was noted in the F_3 generation. It is concluded that fetal protein restriction may play a critical role in determining blood pressure and overall disease risk in a subsequent generation.

Intergenerational programming: Blood pressure: Epigenetic regulation: Kidney: Pregnancy: Rats

It is well established that diseases in adult life such as hypertension and CVD, and the subsequent development of the metabolic syndrome emerge as a consequence of interplay between genetic and environmental factors. Recent research has concentrated on undernutrition in pregnancy and the role that it may play in the onset of disease[1–3]. It has been postulated that undernutrition programmes long-term changes in gene expression, which alter metabolism in the developing fetus and result in cardiovascular abnormalities in later life[4]. Whilst the origins of the metabolic syndrome are clearly multifactorial, nutrition in early life may have a profound influence upon risk and upon the responses of the individual to environmental and lifestyle-related risk factors in adulthood. The expression of genes that either predispose or protect against these conditions will be further modified by interactions between the genotype, early life nutrition and the postnatal environment[5].

Reports of epidemiological associations between birth weight and CVD[4,6,7] are supported by animal experiments showing that both undernutrition and overnutrition in fetal life can programme adult blood pressure[8–11]. We have demonstrated that, in rats, the feeding of a maternal low-protein diet (MLP) during gestation programmes a lifelong elevation of blood pressure[7,11–14]. Evidence from both human and animal studies indicates that the kidney, and specifically nephron number, may play an important role in the programming of hypertension[15]. In the MLP rat model of nutritional programming, fetal exposure to undernutrition reduces nephron number by as much as 30 %[16].

A number of studies have demonstrated that prenatal undernutrition has the capacity to modulate the epigenetic regulation of gene expression[17,19]. This raises the prospect that periods of undernutrition can establish heritable changes to the epigenome and, as such, the disease programming effects of undernutrition in the fetal period may not be limited to the first generation. Indeed, there is emerging evidence from studies of humans and animals to suggest that transgenerational effects may occur, whereby the consequences of deficits in maternal nutrition are subsequently passed onto the grandchildren[20–22]. The aim of the present study was to assess the potential for both a prenatal insult of maternal protein restriction and a postnatal high-fat (HF) challenge to impact across several generations.

Materials and methods

Animal protocols

The experiments described in the present report were performed under license from the Home Office in accordance with the 1986 Animals (Scientific Procedures) Act. The study

Abbreviations: CON, control; HF, high-fat; MLP, maternal low-protein; NC, nutritional challenge.

* **Corresponding author:** Simon Langley-Evans, fax +44 115 9516122, email Simon.Langley-Evans@Nottingham.ac.uk

used rats of the Wistar strain, and all animals were housed in plastic cages and subjected to a 12 h light–dark cycle at a temperature of 20–22°C. The animals had *ad libitum* access to food and water at all times.

Maternal procedure

Fig. 1 summarises the overall design of the present experiment. Twenty virgin female Wistar rats (Charles River UK Ltd, Margate, Kent, UK) were mated with a single stud male at between 180 and 220 g. Upon confirmation of mating by the presence of a semen plug on the cage floor, the rats were allocated to be fed either an 18 % (w/w) casein (control; CON) or a 9 % (w/w) casein (MLP) diet throughout gestation, as described previously[14]. The diets were isoenergetic, the difference in protein-derived energy between the two diets being made up by the addition of carbohydrate (starch–sucrose, 2:1, w/w). During pregnancy the animals were weighed and food intake was recorded daily. At the time of birth (day 22) the animals were transferred to a standard laboratory chow diet (B&K Universal Ltd, Hull, UK).

F_1 offspring procedure

At birth, litters were sexed and weighed. All litters were culled to eight pups (four male and four female) to ensure a standard plane of nutrition. At approximately 3 weeks of age the offspring were micro-chipped using the Avid micro-chipping system and allocated into two sub-groups, breeders and nutritional challenge (NC) (two males and two females per group). Breeders and one male and one female from the NC group were allocated to the standard chow diet (B&K Universal Ltd). The remaining NC animals were allocated to be fed a HF diet. All animals were housed in

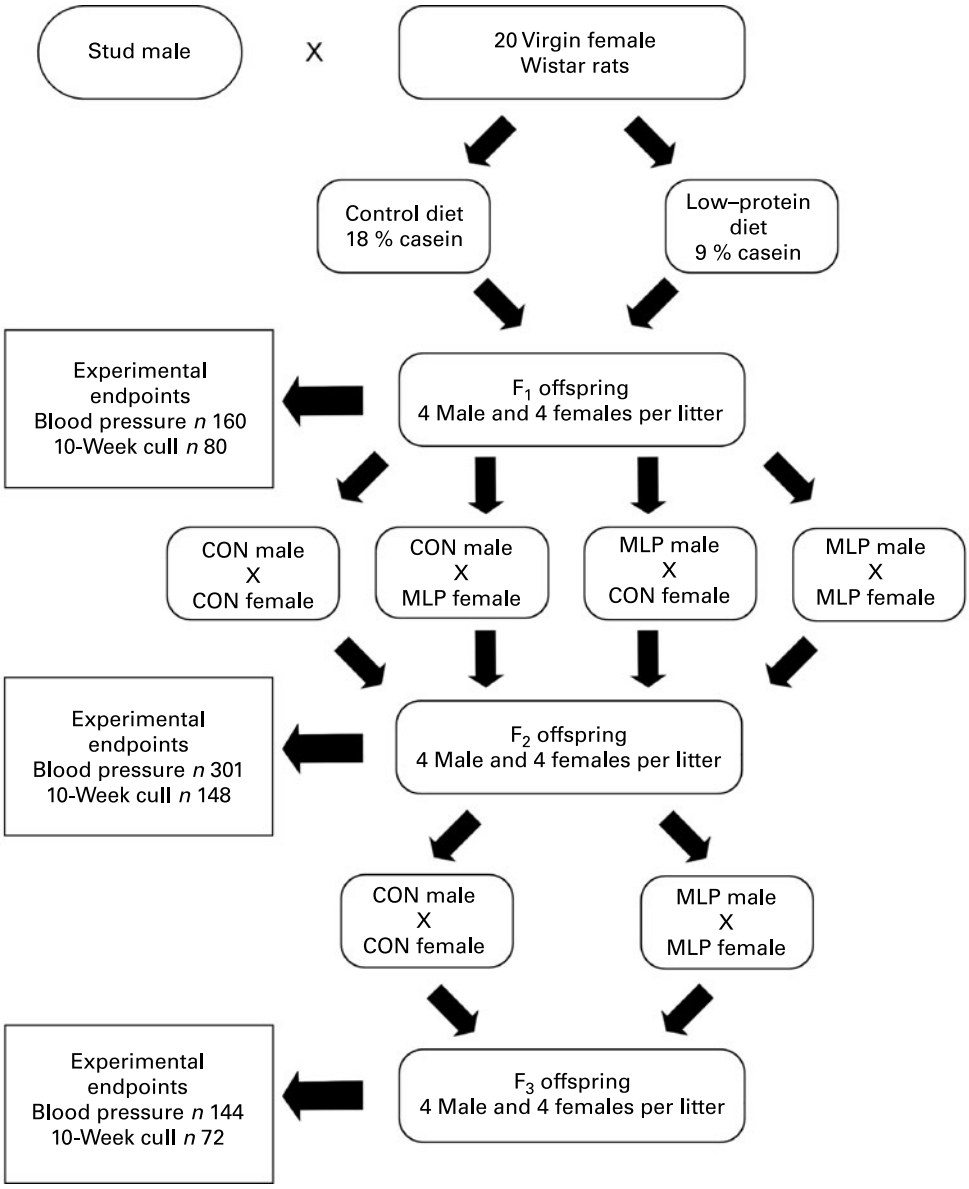


Fig. 1. Schematic showing study design. CON, control; MLP, maternal low-protein; experimental endpoints, determination of body composition.

British Journal of Nutrition

single-sex groups and had *ad libitum* access to food and water at all times.

At approximately 10 weeks of age, two males and two females (breeders sub-group) from each F_1 litter were utilised in a breeding programme for production of the F_2 generation. The four animals from each litter allocated for breeding were crossed with animals from another litter in order to produce the four possible crosses from the F_1 generation (CON male × CON female, MLP male × MLP female, CON male × MLP female and MLP male × CON female). Upon confirmation of mating by the presence of a semen plug on the cage floor the female rats were singly housed and fed the standard chow diet until delivery of the litter.

F_2 offspring procedure

At birth, litters were sexed and weighed. All litters were culled to eight pups (four male and four female) to ensure a standard plane of nutrition. At approximately 3 weeks of age the offspring were micro-chipped and allocated into breeders and NC sub-groups as described above. Breeders and one male and one female from the NC group were allocated to the standard chow diet (B&K Universal Ltd). The remaining NC animals were allocated to be fed a HF diet. The animals were housed in single-sex groups and had *ad libitum* access to food and water at all times.

At approximately 10 weeks of age, one male and one female (breeders sub-group) from each litter were crossed with animals from another litter in order to produce the following crosses based upon the dietary exposures of their parents in the F_1 generation: CON male × CON female and MLP male × MLP female. Upon confirmation of mating by the presence of a semen plug on the cage floor the female rats were singly housed and fed standard chow until delivery of the litter. Male rats were placed back into single-sexed housing with their littermates, until studied later in life.

F_3 offspring procedure

At birth, litters were sexed and weighed. All litters were culled to eight pups (four male and four female) to ensure a standard plane of nutrition. At approximately 3 weeks of age the offspring were micro-chipped and allocated to the standard chow diet (B&K Universal Ltd). One male and one female animal were allocated to be fed a HF diet. The animals were housed in single-sex groups and had *ad libitum* access to food and water at all times.

High-fat feeding procedure

Rats (where possible one male and one female offspring from each NC sub-group per litter in each generation) were allocated to a HF feeding protocol to assess the impact of this NC upon subsequent weight gain and body composition, from approximately 4 weeks of age. The HF diet was identical to that used in our previous work[23] and contained 29·5 % fat (w/w) in the form of lard, and comprised 20 % (w/w) protein (casein). The gross energy content of the diet was 25·12 MJ/kg. A further group (consisting of one male and one female offspring from each NC group per litter in each generation) were fed the standard laboratory chow diet as a control. This diet contained 19 % protein, 4·7 % fat and had a gross energy content of 16·39 MJ/kg. All animals from this group were housed in pairs and provided with *ad libitum* access to the HF diet or standard laboratory chow. At 5, 7 and 9 weeks of age the animals were singly housed and food intake and body weight was monitored for a period of 3 d. Food intake data are shown corrected for body weight as in our previous studies of appetite in this model[24]. This corrects for any influence of body weight upon food intake, and allows comparison between sexes.

Determination of blood pressure

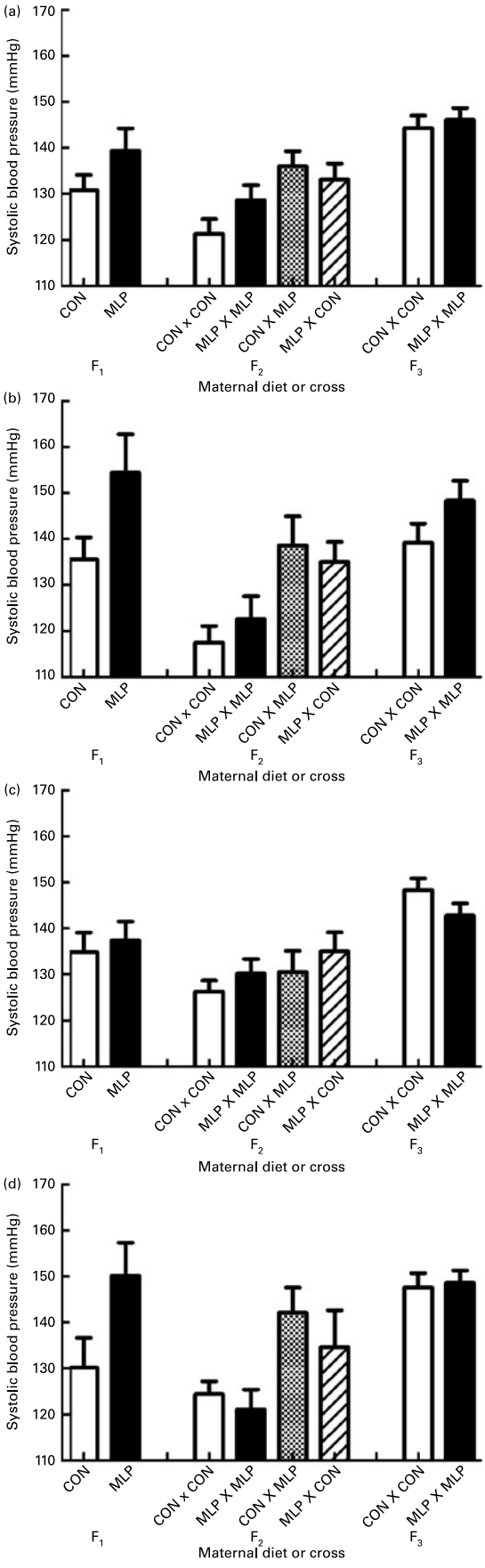
Blood pressure was determined in all animals using an indirect tail-cuff method, as described previously[25]. Measurements were made using the IITC Life Sciences Model 229 Blood Pressure amplifier/pump (IITC Inc., Woodlands, CA, USA). This instrument allows measurements at a temperature of 27°C, thereby avoiding heat stress of the animals. All rats were housed at this temperature, in the room where the measurements were made for at least 2 h before testing. Measurements were made at the same time of day following standard procedures to minimise variation due to diurnal changes in blood pressure. Blood pressure was determined at 4, 6 and 8 weeks of age. Measurements were taken in triplicate and an average value was derived for each animal.

Culling of animals

At approximately 10 weeks of age the NC sub-group animals were killed to coincide with matings for the next generation.(using a rising concentration of CO_2 and cervical dislocation). Liver, heart, kidneys, lungs, spleen, thymus, hippocampus, hypothalamus, gonadal fat and perirenal fat were removed, accurately weighed and then snap-frozen in liquid N_2 and stored at −80°C until used for further analysis. The left kidney was fixed in formalin for later determination of nephron number.

Nephron number determination

Nephron number was determined using an adaptation of the acid maceration method of Welham *et al.* [26]. Although determination of nephron complement via stereology[27] is the gold standard method of analysis, we used maceration in the present study due to the high throughput required. The present results and overall effect match those obtained via stereology in studies of the MLP diet in the F_1 generation[28]. Formalin fixed kidneys were weighed, cut in half and one portion was then incubated in 1 M-hydrochloric acid at 37°C for 30 min. Acid was removed and replaced with 5 ml 50 mM-PBS (pH 7·4). The tissue was then homogenised using a bench-top homogeniser (Polytron) and a further 5 ml PBS were added to give a final volume of 10 ml. The sample was mixed thoroughly by inversion and a 20 µl sample was taken and placed on a microscope slide and overlain with a cover slip. A × 10 objective lens was used to count all glomeruli in the sample. This was carried out in triplicate for each tissue sample. The values



obtained were averaged and used to calculate the total number of glomeruli per kidney using the following equation:

$$\text{Total nephron number} = (\text{kidney weight}/\text{sample weight}) \times \text{average count} \times 500.$$

Determination of circulating metabolites

Cholesterol and TAG assays. Total plasma cholesterol and plasma TAG concentrations were determined using commercially available kits, following the manufacturer's instructions (Thermo Life Sciences, Basingstoke, Hants, UK).

Glucose assay. Plasma glucose concentrations were determined in samples from non-fasted animals, using an adaptation of the glucose oxidase method of Trinder[29]. A standard curve was produced by making serial dilutions of the glucose standard (0–2 μg glucose). Samples were diluted 1:5 with phosphate buffer and loaded with standards in duplicate onto a microtitre plate in 10 μl quantities; 200 μl of glucose reagent was then added to each well. The plate was then incubated at 37°C for 15 min and then read at an absorbance of 620 nm using Magellan, version 4.0 software and plate reader (Sunrise™; Tecan Group Ltd, Männedorf, Switzerland). The inter-assay CV was 2·99 %.

Statistical analysis

All data were analysed using the Statistical Package for Social Sciences (version 14; SPSS, Inc., Chicago, IL, USA). Differences between groups were assessed using a mixed model ANOVA (fixed factors, maternal diet, sex and age), unless otherwise indicated in the text. Values are expressed as mean values with their standard errors. $P<0{\cdot}05$ was considered as significant. As multiple pups from the same dam were used throughout the present study, litter of origin was included as a fixed nested factor in all analyses[30]. Analyses were performed within generations, with no consideration of influences between generations, except for a comparison of blood pressure across the three generations. Within each generation the 18 % casein CON diet (F_1) or the CON × CON breeding cross (F_2 and F_3) was used as the control group.

Results

Birth outcomes

The number of successful pregnancies, litter size, birth weight and the ratio of male and female offspring was unaffected by maternal diet or cross in all three generations when compared with CON animals (data not shown). Average litter size was fourteen, with a ratio of seven males to six female offspring.

Fig. 2. Systolic blood pressure at 8 weeks of age in (a) male offspring fed a standard chow diet, (b) male offspring fed a high-fat diet, (c) female offspring fed the standard chow diet and (d) female offspring fed the high-fat diet. Data are means (n 7 to 35 observations per group), with standard errors represented by vertical bars. CON, maternal control diet; MLP, maternal low-protein diet. ANOVA indicated significant effects of maternal diet (F_1) ($P<0{\cdot}001$) and cross (F_2) ($P<0{\cdot}001$).

Blood pressure

F_1 generation. Systolic blood pressure at 8 weeks of age was significantly increased in males fed either the HF (Fig. 2 (b)) diet (19 mmHg higher) or the standard chow (Fig. 2 (a)) diet (9 mmHg higher) postnatally, in the offspring (F_1) of animals that had been subjected to maternal protein restriction during pregnancy ($P<0{\cdot}001$), when compared with CON animals of the same age. This effect was also noted in the females (Figs. 2 (c) and (d)) with an increase of 20 mmHg in animals on the HF diet and an increase of 3 mmHg ($P<0{\cdot}001$) in animals maintained on the standard chow diet. This trend of increased blood pressure was also apparent at 6 weeks of age ($P<0{\cdot}001$) (data not shown). Blood pressure at 4 weeks of age (data not shown) in the F_1 generation was significantly lower in animals exposed to maternal protein restriction, relative to CON animals ($P<0{\cdot}001$). This result occurred independent of sex, although a postnatal HF diet did substantially reduce systolic blood pressure at this age ($P<0{\cdot}002$).

F_2 generation. The systolic blood pressures of the F_2 generation were significantly influenced by the original F_0 dietary intervention with prenatal MLP exposure of either male or female F_1 parents increasing blood pressure (Figs. 2 (a), (b), (c) and (d)). Systolic blood pressure at 8 weeks of age was significantly increased ($P<0{\cdot}001$) in the offspring from all of the breeding crosses where the parents were originally subjected to protein restriction during pregnancy, when compared with CON animals. The increase in blood pressure was also apparent at 6 weeks of age ($P<0{\cdot}005$; data not shown). There was no effect on systolic blood pressure observed at 4 weeks for this generation. There was no influence of sex or any interaction of postnatal diet upon systolic blood pressure at 8 weeks of age.

F_3 generation. The systolic blood pressures of the F_3 generation at 8 weeks of age were unaffected by the protein restriction of the original F_0 dams. It was noticeable that blood pressures of F_3 animals derived from the F_0 dams fed the CON diet in pregnancy were substantially higher than observed in their F_1 equivalent group (Figs. 2 (a), (b), (c) and (d)).

Nephron number. In animals of the F_1 generation, total nephron number (Fig. 3) was significantly reduced by 33 % in males and 35 % in females among the offspring of rats that were subjected to maternal protein restriction during pregnancy ($P<0{\cdot}001$). The F_2 generation were similarly affected, exhibiting a reduction in nephron number of between 37 and 47 % in males and between 31and 39 % in females ($P<0{\cdot}05$) derived from the breeding crosses where the parents were originally subjected to protein restriction during fetal life. No reduction in nephron number was observed in the F_3 generation.

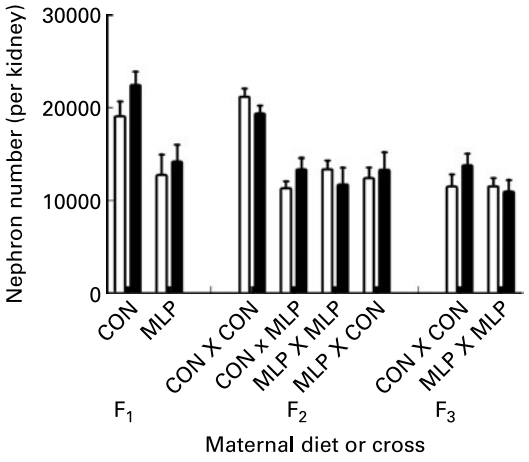


Fig. 3. Nephron number at 10 weeks of age in male (□) and female (■) offspring. Data are means (*n* 8 to 23 observations per group), with standard errors represented by vertical bars. CON, maternal control diet; MLP, maternal low-protein diet. ANOVA indicated significant effects of maternal diet (F_1, $P<0{\cdot}001$) and cross (F_2, $P<0{\cdot}05$).

Body composition

F_1 generation. Among males of the F_1 generation, body weight (Table 1) at cull was similar in CON and MLP-exposed rats that were fed the chow diet in both male and female animals. The HF diet did not increase body weight in the CON or MLP group in females; however, MLP-exposed male animals exhibited a significantly decreased body weight. All organs (data not shown) and fat pads (Table 1) were of similar size relative to body weight in the chow-fed male and female animals, irrespective of maternal dietary exposure. HF feeding in the F_1 generation increased the size of the thymus in males from both the CON and MLP groups, and in the MLP group produced an enlarged spleen. Heart size relative to body weight was significantly reduced by HF feeding (data not shown). HF exposure postnatally in females substantially increased liver ($P<0{\cdot}05$), lung ($P<0{\cdot}05$), spleen ($P<0{\cdot}05$), gonadal fat pad ($P<0{\cdot}05$) and perirenal fat pad ($P<0{\cdot}05$) size relative to body weight in both CON and MLP groups (data not shown).

F_2 generation. F_2 generation body composition did not differ greatly from the F_1 generation. Body weight at cull was similar among all groups of male animals on standard chow and similar to the F_1 generation. HF feeding did not increase body weight in the CON group; however, there was a trend towards a decreased body weight in the MLP-exposed animals although this was not statistically significant. F_2 female body weight did not differ significantly with regards to parental cross in animals exposed to the postnatal chow diet; however, HF feeding postnatally generally decreased body weight (Table 1) in all crosses with the exception of the CON × MLP cross. All organs (data not shown) and fat pads were of similar size relative to body weight in the chow-fed male animals. However, HF feeding (Table 1) in both the males and females significantly decreased perirenal and gonadal fat deposit size relative to body weight in the animals of all cross groups involving a MLP-exposed parent ($P<0{\cdot}05$).

F_3 generation. In the F_3 generation, all organs (data not shown) and fat pads were of similar size relative to body weight in the chow-fed male and female animals. HF feeding decreased body weight, and increased left kidney and heart size relative to body weight relative to their CON counterparts.

Circulating metabolites

Plasma glucose (Table 2) concentrations were unaffected by the original manipulation of the maternal diet, by sex or by HF feeding in any of the three generations. Plasma cholesterol

Table 1. Body fat deposition*

(Mean values with their standard errors)

			Males						Females					
			Body wt (g)		Gonadal fat pad (g/100 g body wt)		Perirenal fat pad (g/100 g body wt)		Body wt (g)		Gonadal fat pad (g/100 g body wt)		Perirenal fat pad (g/100 g body wt)	
Generation	Maternal diet or cross	Postnatal diet	*n*	Mean	SE	Mean	SE	Mean	SE	*n*	Mean	SE	Mean	SE	Mean	SE
F_1	CON	Chow	11	383·7	19·8	0·8	0·1	0·7	0·1	9	246·9	8·4	0·8	0·1	0·6	0·1
	CON	HF	10	381·7	13·6	1·0	0·1	1·0	0·1	10	282·0	23·4	1·1	0·1	0·9	0·1
	MLP	Chow	8	380·8	6·9	0·7	0·0	0·7	0·1	9	243·8	4·6	0·7	0·1	0·5	0·1
	MLP	HF	10	356·9	15·6	0·9	0·1	0·9	0·1	11	240·0	5·8	1·2	0·1	0·9	0·1
F_2	CON × CON	Chow	11	381·3	12·9	0·7	0·0	0·7	0·1	11	249·4	5·7	0·7	0·1	0·5	0·1
	CON × CON	HF	11	381·6	17·6	1·1	0·1	1·1	0·1	11	244·8	7·0	1·1	0·1	0·8	0·1
	CON × MLP	Chow	8	412·9	10·2	0·7	0·1	0·6	0·1	8	239·2	8·3	0·8	0·1	0·5	0·1
	CON × MLP	HF	8	349·3	14·1	0·9	0·1	0·8	0·1	8	244·8	5·0	0·8	0·1	0·7	0·1
	MLP × CON	Chow	6	383·3	18·7	0·7	0·1	0·7	0·1	10	259·0	17·4	0·6	0·1	0·4	0·1
	MLP × CON	HF	7	350·5	10·9	0·9	0·1	0·8	0·2	8	237·0	4·3	0·9	0·1	0·6	0·1
	MLP × MLP	Chow	13	391·2	14·9	0·7	0·0	0·6	0·1	7	250·6	3·9	0·6	0·1	0·5	0·1
	MLP × MLP	HF	10	351·9	10·5	0·9	0·1	0·8	0·1	11	228·3	8·9	0·9	0·1	0·7	0·1
F_3	CON × CON	Chow	11	401·7	8·6	0·8	0·0	0·7	0·1	9	255·9	9·7	0·7	0·1	0·5	0·1
	CON × CON	HF	9	376·1	14·8	1·0	0·1	1·0	0·1	9	236·1	7·5	1·0	0·1	0·7	0·1
	MLP × MLP	Chow	6	411·2	14·2	0·7	0·1	0·6	0·1	8	266·8	18·0	0·7	0·1	0·6	0·1
	MLP × MLP	HF	8	377·8	10·0	0·9	0·1	0·8	0·1	8	238·7	3·6	0·9	0·1	0·7	0·1
P for effect of maternal diet (F_1)				<0·05	–	NS	–	NS	–	–	<0·05	–	NS	–	NS	–
P for effect of postnatal diet (F_1)				NS	–	<0·05	–	<0·05	–	–	NS	–	<0·05	–	<0·05	–
P for interaction of maternal×postnatal diets (F_1)				<0·05	–	NS	–	NS	–	–	<0·05	–	NS	–	NS	–
P for effect of F_2 or F_3 cross				F_3: <0·05	–	F_2: <0·05	–	F_2: <0·05	–	–	F_3: <0·05	–	F_2: <0·05	–	F_2: <0·05	–
P for interaction of F_2 or F_3 cross × postnatal diet				F_3: <0·05	–	F_2: <0·05	–	NS	–	–	F_3: <0·05	–	F_2: <0·05	–	NS	–

CON, control; HF, high-fat; MLP, maternal low-protein.

* For F_2 and F_3 crosses the dietary origin of the male parent is shown before the female parent; for example, CON × MLP indicates a cross between a male exposed to the control diet and a female exposed to the MLP diet *in utero*.

concentrations were also unaffected by maternal diet or cross in all three generations; however, HF feeding substantially increased plasma cholesterol concentrations in both the F_1 and F_3 CON and MLP male groups ($P<0·05$). Plasma TAG concentrations in the F_1 generation were increased with HF feeding in the MLP-exposed male group (0·93 mmol/l) compared with CON animals (0·57 mmol/l) ($P<0·05$). Plasma TAG concentrations were higher in F_2 groups derived from MLP-fed F_0 dams when they were maintained on the standard chow diet, compared with the CON × CON group ($P<0·05$). TAG concentrations were similar in the F_3 generation irrespective of cross.

Food intake

Food intake (Table 3) at 5 and 7 weeks of age (data not shown) was unaffected by maternal diet or cross in the F_1 and F_3 generations although there was an effect of postnatal diet ($P<0·001$) in the F_1 generation with rats consuming roughly double the weight of food per d per kg body weight when maintained on the standard chow diet. In the F_2 generation ($P<0·001$) it was apparent that offspring of the MLP × CON cross had an increased food intake when on a HF diet ($P<0·001$), relative to all other groups at 5 weeks of age. By 9 weeks of age there was an effect of postnatal diet ($P<0·002$) in all three generations whereby animals on standard chow consumed on average more g/d per kg body weight than those fed the HF diet.

Discussion

In the present paper we have considered the potential for intrauterine protein restriction to impact upon blood pressure, renal development and body composition across several generations. We have previously characterised the programming effects of feeding a MLP diet in rat pregnancy upon blood pressure[11,12,14], renal function and development[16,31], body composition[32], feeding behaviour[24], lipid metabolism[23,33] and body fatness[32] in the first generation (F_1). In the present study we have for the first time shown that the effects of a MLP diet during rat gestation may not be limited to the first generation. This unique study has combined measurements of blood pressure with nephron number determination to show that a relatively mild protein restriction has far-reaching consequences for further generations.

Much of the work previously undertaken on transgenerational programming has focused purely on a mechanistic viewpoint, without extensive effort to demonstrate that there is intergenerational transmission of any phenotype[34]. The present paper is the first to examine the interplay between blood pressure and renal structure across several generations, and has demonstrated that the effects of a mild-moderate protein restriction and its resultant phenotype is transgenerationally passed from the F_1 generation to the F_2 generations via both the maternal and paternal lines. It is clear, from our findings on blood pressure and nephron number, that the F_2 offspring of either male or female rats exposed to MLP diets *in utero* develop broadly the same traits as their parents. A major strength of the research design for the present study was the use of males from the F_1 and F_2 generations in the mating crosses to produce the successive generations. This allows us to assess the potential contribution of paternal as well as maternal factors in transgenerational programming. Other studies[35,36] that have found evidence of transgenerational programming have considered only maternal transmission of phenotypes, as they have utilised stud males unexposed to dietary or hormonal challenges *in utero* to breed from programmed females. One of the main problems with such studies is that they cannot conclusively demonstrate that maternal traits are transmitted to the next generation via programmed influences on the ovum as changes to the maternal environment and the composition of proteins in the developing embryo may play a key role. Metabolic traits such as glucose intolerance or cardiovascular traits such as raised blood pressure could be programmed as a response to the prevailing maternal environment during an F_1 pregnancy. For example, it has been previously shown that MLP diets in rat pregnancy and lactation lead to glucose intolerance. F_2 offspring derived from glucose-intolerant females also show this trait, acquired through glucose spill-over across the placenta[37]. In the present study we can at least conclude that male animals must transmit a programming signal to their offspring via alterations to the sperm genome.

The feeding of a MLP diet during rat gestation has been consistently shown to produce a systolic blood pressure rise of between 7 and 30 mmHg[12] by 4 weeks of age. This increase in blood pressure appears permanent, remaining elevated well into adult life[38]. This phenotype was exhibited by the F_1 generation within the present study regardless of their postnatal diet, and was subsequently passed to a second generation (F_2) via both the maternal and paternal lines (as all F_2 crosses involving low protein result in high blood pressure). The decreased blood pressure observed in the F_1 MLP-exposed animals at 4 weeks of age was atypical of previous research using the MLP diet. However, similar outcomes have been observed following maternal restriction of Fe[9,10], where high blood pressure follows a period of lower blood pressure around the time of weaning.

Current thinking is that the transgenerational passage of phenotypic information is likely to involve epigenetic gene regulation. Although the exact mechanism which underpins this phenomenon is poorly understood, DNA methylation[19,36,39,40] and histone modification[17,18] remain key potential candidates. Currently there is a large and varied volume of work within the programming field that is focused on these epigenetic mechanisms[41]. Lillycrop *et al.* [42] have, for example, shown that the MLP diet leads to hypomethylation of specific gene loci and hence leads to their overexpression. There is evidence that these methylation changes also appear in F_2 offspring[41]. Although there is a great deal of controversy surrounding epigenetics it is important to emphasise that the results within the present study show that it is the most likely mechanism through which programming has occurred in the male line. Epidemiological evidence from Swedish cohort studies supports this hypothesis[43]. Arguments suggesting that maternal blood pressure tracking (familial aggregation) may produce these effects fall short of the mark, as high blood pressure was passed down both the maternal and paternal lines. This argument also cannot explain the decrease in nephron number found in both the F_1 and F_2 populations. It has been hypothesised that there may be the potential for some form of selection, whereby only fetuses

Table 2. Circulating metabolites*

(Mean values with their standard errors)

			Males						Female					
			Glucose (mmol/l)		TAG (mmol/l)		Cholesterol (mmol/l)		Glucose (mmol/l)		TAG (mmol/l)		Cholesterol (mmol/l)	
Generation	Maternal diet or cross	Postnatal diet	*n*	Mean	SE	Mean	SE	Mean	SE	*n*	Mean	SE	Mean	SE	Mean	SE
F_1	CON	Chow	11	9·2	1·7	0·7	0·2	1·7	0·1	9	8·9	1·5	0·5	0·1	1·6	0·1
	CON	HF	9	12·3	1·6	0·6	0·1	2·4	0·2	10	9·6	1·2	0·1	0·1	1·5	0·1
	MLP	Chow	8	10·3	1·6	0·5	0·1	1·6	0·2	9	9·9	1·7	0·3	0·1	1·6	0·2
	MLP	HF	9	8·9	0·8	0·9	0·2	2·6	0·2	11	9·7	0·9	0·3	0·1	1·7	0·1
F_2	CON × CON	Chow	11	11·2	1·7	0·9	0·1	1·3	0·2	11	12·0	1·0	0·4	0·1	1·6	0·2
	CON × CON	HF	11	12·9	1·7	0·7	0·1	2·1	0·2	11	11·2	1·1	0·2	0·1	1·8	0·1
	CON × MLP	Chow	8	10·5	1·8	1·2	0·2	1·5	0·1	8	12·4	2·0	0·3	0·1	1·6	0·2
	CON × MLP	HF	8	8·2	1·8	0·7	0·2	2·0	0·2	8	12·2	0·8	0·3	0·1	1·5	0·1
	MLP × CON	Chow	6	13·2	2·0	1·3	0·3	1·4	0·2	10	10·5	2·3	0·6	0·1	1·6	0·2
	MLP × CON	HF	7	9·3	2·4	0·5	0·1	2·1	0·1	8	10·8	2·6	0·3	0·1	1·7	0·2
	MLP × MLP	Chow	13	11·5	1·4	1·0	0·2	2·1	0·4	7	13·7	2·3	0·4	0·2	1·9	0·1
	MLP × MLP	HF	10	10·1	1·3	0·6	0·5	1·5	0·2	11	13·0	1·4	0·4	0·2	1·3	0·4
F_3	CON × CON	Chow	11	11·8	1·0	0·6	0·1	1·5	0·2	9	10·5	1·0	0·3	0·1	1·6	0·2
	CON × CON	HF	9	8·8	1·6	0·9	0·1	2·2	0·2	9	10·0	1·2	0·1	0·0	1·6	0·2
	MLP × MLP	Chow	6	9·0	1·2	0·5	0·1	1·7	0·2	8	11·5	4·0	0·5	0·1	1·4	0·1
	MLP × MLP	HF	8	7·8	1·1	0·7	0·1	2·0	0·2	8	9·5	1·2	0·2	0·1	1·5	0·1
P for effect of maternal diet (F_1)				NS	–	NS	–	NS	–	–	NS	–	NS	–	NS	–
P for effect of postnatal diet (F_1)				NS	–	NS	–	$<0·05$	–	–	NS	–	NS	–	$<0·05$	–
P for interaction of maternal×postnatal diets (F_1)				NS	–	NS	–	NS	–	–	NS	–	NS	–	NS	–
P for effect of F_2 or F_3 cross				NS	–	F_2: $<0·05$	–	NS	–	–	NS	–	F_2: $<0·05$	–	NS	–
P for interaction of F_2 or F_3 cross × postnatal diet				NS	–	NS	–	NS	–	–	NS	–	NS	–	NS	–

CON, control; HF, high-fat; MLP, maternal low-protein.

* For F_2 and F_3 crosses the dietary origin of the male parent is shown before the female parent; for example, CON × MLP indicates a cross between a male exposed to the control diet and a female exposed to the MLP diet *in utero*.

Table 3. Food intake*

(Mean values with their standard errors)

			Males					Females			
			Week 9 (g/d per kg body wt)		Energy (MJ/kg)			Week 9 (g/d per kg body wt)		Energy (MJ/kg)	
Generation	Maternal diet or cross	Postnatal diet	*n*	Mean	SE	Mean	SE	*n*	Mean	SE	Mean	SE
F_1	CON	Chow	11	107·6	11·7	1·8	0·2	9	118·0	8·8	1·9	0·1
	CON	HF	10	64·3	3·0	1·6	0·1	10	83·0	5·5	2·1	0·1
	MLP	Chow	8	98·4	2·9	1·6	0·0	9	118·7	5·0	1·9	0·1
	MLP	HF	10	81·5	12·2	2·0	0·1	11	76·9	6·2	1·9	0·2
F_2	CON × CON	Chow	33	81·0	8·3	1·3	0·1	33	89·5	10·9	1·5	0·2
	CON × CON	HF	11	74·5	15·1	1·9	0·4	11	63·5	2·1	1·6	0·1
	CON × MLP	Chow	24	99·0	3·0	1·6	0·0	24	118·8	4·6	1·9	0·1
	CON × MLP	HF	8	68·7	14·2	1·7	0·4	8	67·6	4·0	1·7	0·1
	MLP × CON	Chow	22	97·4	6·3	1·6	0·1	26	112·5	6·8	1·8	0·1
	MLP × CON	HF	7	55·6	3·2	1·4	0·1	8	59·7	3·1	1·5	0·1
	MLP × MLP	Chow	35	89·6	1·8	1·5	0·0	29	100·4	4·8	1·6	0·1
	MLP × MLP	HF	10	59·8	2·5	1·5	0·1	11	70·2	5·6	1·8	0·1
F_3	CON × CON	Chow	11	109·0	5·8	1·8	0·1	9	121·2	5·9	2·0	0·1
	CON × CON	HF	9	60·6	3·5	1·5	0·1	9	63·7	2·0	1·6	0·1
	MLP × MLP	Chow	6	103·7	3·4	1·7	0·1	8	122·8	4·4	2·0	0·1
	MLP × MLP	HF	8	60·4	4·6	1·5	0·1	8	66·3	4·0	1·7	0·1
P for effect of maternal diet (F_1)				NS	–	NS	–	–	NS	–	NS	–
P for effect of postnatal diet (F_1)				< 0·001	–	NS	–	–	< 0·001	–	NS	–
P for interaction of maternal×postnatal diets (F_1)				NS	–	NS	–	–	NS	–	NS	–
P for effect of F_2 or F_3 cross				NS	–	NS	–	–	NS	–	NS	–
P for interaction of F_2 or F_3 cross × postnatal diet				NS	–	NS	–	–	NS	–	NS	–

CON, control; HF, high-fat; MLP, maternal low-protein.

* For F_2 and F_3 crosses the dietary origin of the male parent is shown before the female parent; for example, CON × MLP indicates a cross between a male exposed to the control diet and a female exposed to the MLP diet *in utero*.

with elevated blood pressures survive the pregnancy insult. This would potentially select for a hypertensive genotype. This 'survivor' effect is not plausible within the present study, as pregnancy outcomes were similar in rats fed the CON and MLP diets.

The kidney plays a major role in homeostatic regulation of blood pressure and, in rats, the MLP diet has been shown to reduce nephron complement by up to 30 %[16,26]. The present study shows that this reduction in nephron number is passed to a second generation via both parental lines. It is hypothesised that to compensate for nephron deficits at birth and to maintain renal haemodynamic functions, blood pressure within the nephrons is increased to maintain glomerular perfusion. Thus a cycle of progressive nephron loss begins whereby increasing blood pressures result in the loss of more nephrons eventually resulting in hypertension[15]. There is, however, emerging evidence that nephron number and the nutritional programming of blood pressure are independent processes acting in a sex-specific manner[44]. Although there is some debate over the relationship between nephron number and subsequent blood pressure, it is clear that the restriction of protein during gestation has the potential to generate a phenotype that features both reduced nephron complement and hypertension and that this phenotype is subsequently passed on to a second generation via both parental lines. Torrens *et al.* [45] have also recently shown, with a design powered only to assess maternal transmission, that high blood pressure can be transmitted to the F_2 generation following maternal protein restriction. Within the study of Torrens *et al.* [45] there was evidence of an impaired vasodilatory response to acetylcholine, suggesting that in addition to renal programming, endothelial dysfunction may contribute to transgenerational phenomena. Both mechanisms are likely to operate in parallel. Further studies are required to identify the primary mechanisms that drive these physiological processes.

An interesting and unexpected result to arise from this investigation was that the blood pressures of CON animals in F_3 were elevated, and nephron counts decreased, compared with the F_1 equivalents. The reason for this is unknown. We would, however, suggest that these intergenerational differences may effectively be due to in-breeding of a small colony in the F_2 and F_3 generations. Furthermore, the fact that the present study shows that the experience of the mother and grandmother of each animal determines blood pressure and nephron complement may be of relevance. The grandmaternal influence on the F_1 generation will have been different to the grandmaternal influence on the F_3 generation, and it is this difference which could explain the changes.

The feeding of a HF diet within the present study was utilised to assess the impact of a 'Western diet' against a background of prenatal undernutrition. Typically a 'Western diet' has a high saturated fat content and is linked with obesity and the later development of adult diseases such as CVD[46,47]. The HF diet mirrored this composition as it had an overall fat content of 29·5 %. Interestingly, rather than becoming obese, the MLP animals within the present study exhibited a lean phenotype consisting of slightly reduced body weight and fat mass compared with chow-fed animals at 10 weeks of age. This result was not entirely unexpected, as our previous work[33] has demonstrated that up to 9 months of age MLP-exposed animals are resistant to obesity and exhibit similar plasma TAG, cholesterol, glucose and insulin concentrations to those of CON animals. By 18 months of age there is an abrupt change in metabolic profile with MLP-programmed hypertriacylglycerolaemia and insulin resistance. This indicates that prenatal protein restriction programmes the development of a metabolic syndrome-like phenotype resistant to obesity that develops with senescence[33]. The present study, due to its complexity, was unable to examine the development of this ageing-related phenotype.

The work within the present study demonstrates that the feeding of a MLP diet throughout gestation results in a phenotype that is transgenerationally passed to a second generation via both the maternal and paternal lines. The impact of the initial period of maternal undernutrition was not observed in the F_3 generation. Further work will take a mechanistic focus and attempt to identify possible gene targets involved in endothelial cell biology. Possible epigenetic modes of inheritance will be analysed in order to understand the molecular machinery behind the phenotype.

Acknowledgements

The present study was supported by the British Heart Foundation (studentship for M. H.). M. H. was responsible for the acquisition of data, statistical analysis, interpretation of data and is an author of the communication. S. L.-E. was responsible for study design, statistical analysis, discussion and interpretation of data and is an author of the communication.

The authors gratefully acknowledge the expert technical support of Mr R. Plant, Mrs C. Armett and Ms S. Kirkland. There are no conflicts of interest to declare.

References

1. Gluckman PD & Hanson MA (2004) Living with the past: evolution, development, and patterns of disease. *Sci Tech Froid* **305**, 1733–1736.
2. Barker DJ, Winter PD, Osmond C, Margetts B & Simmonds SJ (1989) Weight in infancy and death from ischaemic heart disease. *Lancet* **ii**, 577–580.
3. Eriksson J, Forsen T, Tuomilehto J, Osmond C & Barker DJ (2001) Size at birth, childhood growth and obesity in adult life. *Int J Obes* **25**, 735–740.
4. Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA & Robinson JS (1993) Fetal nutrition and cardiovascular disease in adult life. *Lancet* **341**, 938–941.
5. Langley-Evans SC (2006) Developmental programming of health and disease. *Proc Nutr Soc* **65**, 97–105.
6. Barker DJ, Eriksson JG, Forsen T & Osmond C (2002) Fetal origins of adult disease: strength of effects and biological basis. *Int J Epidemiol* **31**, 1235–1239.
7. Langley-Evans SC, Gardner DS & Jackson AA (1996) Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. *J Reprod Fertil* **106**, 307–312.
8. Bergel E & Belizan JM (2002) A deficient maternal calcium intake during pregnancy increases blood pressure of the offspring in adult rats. *BJOG* **109**, 540–545.
9. Crowe C, Dandekar P, Fox M, Dhingra K, Bennet L & Hanson MA (1995) The effects of anaemia on heart, placenta and body weight, and blood pressure in fetal and neonatal rats. *J Physiol* **488**, 515–519.

British Journal of Nutrition

10. Gambling L, Dunford S, Wallace DI, Zurr G, Solanky N, Srai SK & McArdle H (2003) Iron deficiency during pregnancy affects postnatal blood pressure in the rat. *J Physiol* **552**, 603–610.
11. Langley SC & Jackson AA (1994) Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clin Sci (Lond)* **86**, 217–222.
12. Langley-Evans SC, Phillips GJ & Jackson AA (1994) *In utero* exposure to maternal low protein diets induces hypertension in weanling rats, independently of maternal blood pressure changes. *Clin Nutr* **13**, 319–324.
13. Langley-Evans SC, Phillips GJ, Benediktsson R, Gardner DS, Edwards CR, Jackson AA & Seckl JR (1996) Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. *Placenta* **17**, 169–172.
14. Langley-Evans SC, Welham SJ, Sherman RC & Jackson AA (1996) Weanling rats exposed to maternal low-protein diets during discrete periods of gestation exhibit differing severity of hypertension. *Clin Sci (Lond)* **91**, 607–615.
15. Mackenzie HS & Brenner BM (1995) Fewer nephrons at birth: a missing link in the etiology of essential hypertension? *Am J Kidney Dis* **26**, 91–98.
16. Langley-Evans SC, Welham SJ & Jackson AA (1999) Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sci* **64**, 965–974.
17. Jaenisch R & Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* **33**, 245–354.
18. Waterland RA & Jirtle RL (2003) Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol* **23**, 5293–5300.
19. Burdge GC, Hanson MA, Slater-Jefferies JL & Lillycrop KA (2007) Dietary protein restriction of pregnant rats in the F_0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F_1 and F_2 generations. *Br J Nutr* **97**, 435–439.
20. Beach RS, Gershwin ME & Hurley LS (1982) Gestational zinc deprivation in mice: persistence of immunodeficiency for three generations. *Science* **218**, 469–471.
21. James WPT (2002) Will feeding mothers prevent the Asian metabolic syndrome epidemic? *Asia Pac J Clin Nutr* **11**, S516–S523.
22. Pembrey M (1996) Imprinting and transgenerational modulation of gene expression: human growth as a model. *Acta Genet Med Gemellol (Roma)* **45**, 111–125.
23. Erhuma A, Bellinger L, Langley-Evans SC & Bennett AJ (2007) Prenatal exposure to undernutrition and programming of responses to high-fat feeding in the rat. *Br J Nutr* **98**, 517–524.
24. Bellinger L, Lilley C & Langley-Evans SC (2004) Prenatal exposure to a maternal low-protein diet programmes a preference for high-fat foods in the young adult rat. *Br J Nutr* **92**, 513–520.
25. Sherman RC & Langley-Evans SC (1998) Early administration of angiotensin-converting enzyme inhibitor captopril, prevents the development of hypertension programmed by intrauterine exposure to a maternal low-protein diet in the rat. *Clin Sci (Lond)* **94**, 373–381.
26. Welham SJ, Wade A & Woolf AS (2002) Protein restriction in pregnancy is associated with increased apoptosis of mesenchymal cells at the start of rat metanephrogenesis. *Kidney Int* **61**, 1231–1242.
27. Bertram JF (2001) Counting in the kidney. *Kidney Int* **59**, 792–796.
28. Zimanyi MA, Bertram JF & Black JM (2000) Nephron number in the offspring of rats fed a low protein diet during pregnancy. *Image Anal Stereol* **19**, 219–222.
29. Trinder P (1969) Determination of blood glucose using a oxidase-peroxidase system with a non-carcinogenic chromogen. *J Clin Pathol* **22**, 158–161.
30. Festing MF (2006) Design and statistical methods in studies using animal models of development. *ILAR J* **47**, 5–14.
31. Nwagwu MO, Cook A & Langley-Evans SC (2000) Evidence of progressive deterioration of renal function in rats exposed to a maternal low-protein diet in utero. *Br J Nutr* **83**, 79–85.
32. Bellinger L, Sculley DV & Langley-Evans SC (2006) Exposure to undernutrition in fetal life determines fat distribution, locomotor activity and food intake in ageing rats. *Int J Obes (Lond)* **30**, 729–738.
33. Erhuma A, Salter AM, Sculley DV, Langley-Evans SC & Bennett AJ (2007) Prenatal exposure to a low-protein diet programs disordered regulation of lipid metabolism in the aging rat. *Am J Physiol Endocrinol Metab* **292**, E1702–E1714.
34. Anway MD, Rekow SS & Skinner MK (2008) Transgenerational epigenetic programming of the embryonic testis transcriptome. *Genomics* **91**, 30–40.
35. Zambrano E, Martinez-Samayoa PM, Bautista CJ, Deas M, Guillen L, Rodriguez-Gonzalez GL, Guzman C, Larrea F & Nathanielsz PW (2005) Sex differences in transgenerational alterations of growth and metabolism in progeny (F_2) of female offspring (F_1) of rats fed a low protein diet during pregnancy and lactation. *J Physiol* **566**, 225–236.
36. Waterland RA, Travisano M & Tahiliani KG (2007) Diet-induced hypermethylation at agouti viable yellow is not inherited transgenerationally through the female. *FASEB J* **21**, 3380–3385.
37. Reusens B & Remacle C (2001) Intergenerational effect of an adverse intrauterine environment on perturbation of glucose metabolism. *Twin Res* **4**, 406–411.
38. Langley-Evans SC & Jackson AA (1995) Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. *Comp Biochem Physiol A Physiol* **110**, 223–228.
39. Bird A (2002) DNA methylation patterns and epigenetic memory. *Genes Dev* **1**, 6–21.
40. Van den Veyver IB (2002) Genetic effects of methylation diets. *Annu Rev Nutr* **22**, 255–282.
41. Burdge GC, Hanson MA, Slater-Jefferies JL & Lillycrop KA (2007) Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *Br J Nutr* **97**, 1036–1046.
42. Lillycrop KA, Slater-Jefferies JL, Hanson MA, Godfrey KM, Jackson AA & Burdge GC (2007) Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. *Br J Nutr* **97**, 1064–1073.
43. Kaati G, Bygren LO & Edvinsson S (2002) Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. *Eur J Hum Genet* **10**, 682–688.
44. McMullen S & Langley-Evans SC (2005) Maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms. *Am J Physiol Regul Integr Comp Physiol* **288**, R85–R90.
45. Torrens C, Poston L & Hanson MA (2008) Transmission of raised blood pressure and endothelial dysfunction to the F_2 generation induced by maternal protein restriction in the F_0, in the absence of dietary challenge in the F_1 generation. *Br J Nutr* (epublication ahead of print version 28 February 2008).
46. Kris-Etherton P, Eckel RH, Howard BV, St Jeor S & Bazzarre TL (2001) AHA Science Advisory: Lyon Diet Heart Study. Benefits of a Mediterranean-style, National Cholesterol Education Program/American Heart Association Step I Dietary Pattern on cardiovascular disease. *Circulation* **103**, 1823–1825.
47. Adlercreutz H (1990) Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. *Scand J Clin Lab Invest Suppl* **201**, 3–23.