Protein restriction during fetal and neonatal development in the rat alters reproductive function and accelerates reproductive ageing in female progeny

C. Guzmán[1], R. Cabrera[1], M. Cárdenas[1], F. Larrea[1], P. W. Nathanielsz[2] and E. Zambrano[1]

[1]*Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga 15, Tlalpan 14000, México, D.F. México*
[2]*Center for Pregnancy and Newborn Research, University of Texas Health Sciences Center, San Antonio, TX 78229, USA*

Recent studies demonstrate long-term programming of function of specific organ systems resulting from suboptimal environments during fetal life and development up to weaning. Nutrient restriction during pregnancy and lactation impairs overall fetal growth and development. We determined the effects of maternal protein restriction (MPR; 50% normal protein intake) during fetal development and/or lactation in rats on the function and ageing of the reproductive system of female progeny. Rats were fed either a control 20% casein diet (C) or a restricted diet (R) of 10% casein during pregnancy. After delivery mothers received either C or R diet until weaning to provide four groups, CC, RR, CR and RC. We report data on female offspring only. After weaning pups were fed the C diet. MPR increased maternal progesterone, corticosterone, oestradiol and testosterone concentrations at 19 days gestation. Reproductive and somatic phenotype was altered as pup birth weight was decreased, and ano-genital distance was increased by MPR. Pup corticosterone was decreased at 2 days postnatal (PN) life. Vaginal opening and timing of the first oestrus were delayed in RR and CR and these differences were not related to body weight. At 21 days PN oestradiol in RR and CR and progesterone in RR were reduced; at 70 days PN luteinizing hormone (LH) in all restricted groups was reduced in dioestrus while follicle stimulating hormone (FSH) was unchanged. Cycle length increased between 140 days and 1 year in RR and CR but remained unchanged in CC, providing evidence of premature ageing of reproductive function. Fertility rates declined over the same period in the three experimental groups but not CC. MPR in one of the two experimental periods, either pregnancy or lactation, resulted in decreased pup survival compared with CC and RR. These data show that MPR results in delayed sexual maturation and premature ageing of reproductive function.

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Corresponding author E. Zambrano: Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga 15, Tlalpan 14000, México, D.F. México. Email: zamgon@laneta.apc.org

Human epidemiological studies and controlled experimental investigations in several species, such as rodents and sheep, have demonstrated long-term programming of the function of specific organ systems as a result of exposure to a suboptimal environment during early fetal and postnatal development (Hoet & Hanson, 1999; Armitage *et al.* 2004). Nutrient restriction during pregnancy and lactation impairs overall fetal growth and development. A variety of nutrient restriction protocols have been studied ranging from mild 15% reduction in calories (Ozaki *et al.* 2001), through moderate 50% reduction (Ozaki *et al.* 2000) to severe 70% reduction (Woodall *et al.* 1996*a*,*b*; Vickers *et al.* 2001). In the several protein restriction models studied, birth weight is usually lowered (Galler & Tonkiss, 1991; Langley & Jackson, 1994; Holemans *et al.* 1999; Langley-Evans, 2000; Vickers *et al.* 2001). However, some studies report increased birth weight (Langley-Evans *et al.* 1996) or no effect (Langley *et al.* 1994). Birth weight is, however, a crude indicator of the degree of developmental compromise since developing mammals will recruit adaptive mechanisms to protect growth, especially of vital organs, during development.

In sheep reduced lifetime reproductive capacity has been demonstrated in ewes born to mothers undernourished during late pregnancy or the first months of life (Gunn, 1995; Rhind & McNeilly, 1998). Environmental challenges such as prenatal exposure to testosterone impair female reproductive capacity in sheep (Sharma *et al.*

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2002; Savabieasfahani *et al.* 2005). In rodents prepubertal administration of oestradiol disrupts ovarian cyclicity in adult life (Rosa *et al.* 2003). Several studies have addressed the effects of fetal suboptimal nutrition on pubertal onset and development of female reproductive function (Engelbregt *et al.* 2000, 2002; Rae *et al.* 2002; Leonhardt *et al.* 2003). Increased nutritional demand on the mother by increasing litter size also alters sexual development of offspring (Engelbregt *et al.* 2001). However, the impact of maternal protein restriction on several important features of female reproduction such as alteration in cycle length and loss of fertility with ageing have not been studied.

We fed rats either a control 20% casein diet (C) or a restricted diet (R) of 10% casein during pregnancy. After delivery mothers received either the C or the R diet until weaning to provide four groups, CC, RR, CR and RC. After weaning pups were fed the C diet. In this report we present data on female offspring only. Maternal protein restriction altered key components of pregnant maternal steroid endocrinology as well as the endocrinolgy of female progeny. The onset of puberty was delayed and reproductive function aged more rapidly in groups that had been exposed to protein restriction during development. These data indicate that maternal protein restriction during development results in delayed sexual maturation and premature ageing of reproductive function.

Methods

Care and maintenance of animals

Details of protein restriction and generation of the pups have been published previously (Zambrano *et al.* 2005*b*). Briefly, 70 virgin female albino Wistar rats aged between 10 and 12 weeks and weighing 240 ± 20 g (mean ± S.E.M.) were obtained from the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (México City, México). Rats were maintained under controlled lighting (lights on from 07.00 h to 19.00 h at 22–23°C). All procedures were approved by the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán, México City.

Female rats were mated overnight with proven male breeders and the day on which spermatozoa were present in a vaginal smear was designated as the day of conception – day 0. Only rats who were pregnant within 5 days of introduction of the male were retained in the study. Pregnant rats were transferred to individual metabolic cages and allocated at random to one of two groups to be fed either a 20% casein (control diet – C) or 10% casein isocaloric diet (restricted diet – R) (Zambrano *et al.* 2005*b*). Food and water were available *ad libitum* for all animals.

Pregnant and lactating rats were weighed daily through pregnancy and until the pups were removed at weaning. Food was provided in the form of large flat biscuits which were retained behind a grill through which the rats nibbled the food. On day 20 post-conception, pregnant rats were transferred to normal rat cages to provide optimal conditions for delivery, which occurred in the early daylight hours between 09.00 h and 12.00 h on post-conceptual day 22. Day of delivery was considered as day 0 of postnatal life. We report data on female offspring only.

Experiment 1. A group of 30 pregnant rats (13 controls and 17 nutrient restricted, assigned at random) were studied for maternal and neonatal hormonal measurements. Pregnant rats were bled from the tail vein at 19 days gestation between 09.00 h and 10.00 h. Blood was collected into polyethylene tubes and allowed to clot at 4°C for 1 h. The blood samples were centrifuged at 3500 *g* for 15 min at 4°C. Serum samples were kept at −20°C until assayed. These rats were then allowed to deliver vaginally. Pup weight was recorded at birth. Ano-genital distance (AGD) was measured with calipers to enable determination of sex. Our published data indicate that female pups have an ano-genital distance of 1.67 ± 0.13 mm ($n = 291$ pups from 43 litters; mean ± S.E.M.) and males 3.26 ± 0.22 mm ($n = 252$ pups from 43 litters) (Zambrano *et al.* 2006). Thus a value of 2.5 mm is more than 2 S.D. from the mean of either group, and sex was judged according to whether the ano-genital distance was greater than (male) or less than (female) 2.5 mm. Mothers maintained their pups for 2 days postnatally on the diets they ate during pregnancy. On day 2 of postnatal life their pups were rapidly killed by decapitation between 09.00 h and 10.00 h. Trunk blood was collected and processed as described above.

Experiment 2. Sixteen control and 18 nutrient restricted pregnant rats underwent spontaneous vaginal delivery. Pup weight was recorded at birth. Ano-genital distance (AGD) was measured with calipers. To ensure homogeneity of subjects, litters of less than 12 or more than 14 pups were not included in the study. On day 1, litters of 12–14 pups were adjusted to 12 pups for each dam while maintaining as close to a 1 : 1 sex ratio as possible. After removal of five control litters (2 with less than 12 pups and 3 with more than 14) and six protein-restricted litters (3 with less than 12 pups and 3 with more than 14) from the study, pups from 23 mothers were used. Four groups were established: CC ($n = 5$) in which dams who received the control diet during pregnancy continued to be fed the control diet during lactation; RR ($n = 5$) in which dams who had received the restricted diet during pregnancy continued to receive the restricted diet during lactation; CR

($n = 6$) in which dams who received the control diet during pregnancy received the restricted diet during lactation; and RC ($n = 7$) in which dams who received the restricted diet during pregnancy were provided with the control diet during lactation.

Postnatal maintenance

After weaning (postnatal day 21) males and females were separated and the females were maintained in groups of up to six until puberty and fed with control diet *ad libitum*. As the pups grew they were separated into groups of three to four. The pups continued to be weighed daily.

On the days on which blood sampling was performed, rats were rapidly killed by decapitation between 09.00 h and 10.00 h. Trunk blood was collected and processed as described above. Ovaries and uteri were dissected, cleaned of fat and weighed.

Evaluation of reproductive function and assessment of reproductive organs

Onset of puberty in the female pups was defined in two separate ways: as the time of vaginal opening and as the first oestrus. From the day at which vaginal opening occurred, a vaginal smear was obtained every 24 h to establish timing of the first oestrus. Vaginal smears were then continued to 70 days postnatal life and obtained daily again for 10 days at 140 days of age, at 1 year and at 22 months. Cyclicity was recorded following examination of the vaginal smears for the proportions of leucocytes, epithelial cells and cornified cells in the smear. The smears were assessed and cycles identified as described by Rosa *et al.* (2003). Cycles were counted as the interval between pro-oestrus and the next pro-oestrus (Rosa *et al.* 2003). When a cycle had completed less than 4 days at the beginning or the end of an observation period, this cycle was counted as 0.5 of a cycle. The average duration of the cycle was calculated for each animal for each period.

Fertility of the female offspring

Between 150 and 160 days postnatal life, two female rats from the same group were placed in a cage with a single fertile male for 5 days. At 1 year, to allow for longer cycles, the same procedure was conducted but males were left with females for 1 week. Results were expressed as the percentage of female rats per group that became pregnant. Pups from these pregnant rats were then entered in another study.

Radioimmunoassays

Serum FSH and LH concentrations were determined by double-antibody RIA using hormone standards and the specific antibodies provided by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, Baltimore, MD, USA). The volume of samples assayed was l00 μl per tube. Rat FSH and LH were iodinated by the chloramine-T method, following separation of protein bound and free ^{125}I by Sephadex G-100. Results were expressed in terms of NIDDK-rat-FSH-RP2 and NIDDK-rat-LH-RP3. Sensitivity of the RIA was 0.035 and 0.02 ng assay tube^{-1}, respectively; FSH intra- and interassay coefficients of variation were $< 9\%$ and $< 14\%$, respectively, and for LH $< 7\%$ and 12%, respectively. Serum rat prolactin was determined by RIA using commercial kits from Biocode-Hycel, Belgium (AH R011). The sensitivity was 1.07 ng ml^{-1}. Only one assay was performed and the interassay coefficient of variation was $< 6\%$.

Serum progesterone, oestradiol, testosterone and corticosterone concentrations were determined by RIA using commercial kits from DPC Coat-a-count (Diagnostic Product Corporation, Los Angeles, CA, USA) (TKPG2, TKE22, TKTT2 and TKRC1, respectively). Intra- and interassay coefficients of variation were 2% and 4% for progesterone, 3% and 2% for oestradiol, 3% and 5% for testosterone, and 2% and 4% for corticosterone, respectively. Sensitivity of the RIA was 0.02 ng ml^{-1} for progesterone, 0.8 pg ml^{-1} for oestradiol, 0.04 ng ml^{-1} for testosterone and 5.7 ng ml^{-1} for corticosterone.

Statistical analysis

Where more than one animal from any litter was studied, data from animals within the litter were averaged and analysis performed only on numbers of litters. All data are presented as the mean ± S.E.M. Statistical analysis was performed using multiple analysis of variance (ANOVA) followed by Tukey's test. Unpaired Student's t test and the χ^2 test were also used when appropriate; $P \leq 0.05$ was considered significant.

Results

Maternal body weight

Maternal body weights, litter size and percentage of pups of each sex did not differ between groups. Maternal weight gain was significantly lower in the nutrient restricted mothers ($P \leq 0.05$; Table 1).

Maternal endocrinology

At 19 days of gestation, maternal progesterone was elevated in the protein restricted mothers (68.6 ± 6.5 ng ml^{-1}) compared to mothers fed the control diet (50.9 ± 4.4 ng ml^{-1}) ($P \leq 0.05$). Prolactin was unchanged (control mothers: 24.9 ± 1.3 and restricted mothers: 20.3 ± 1.4 ng ml^{-1}). We have previously

Table 1. Maternal body weight (g) at onset of pregnancy and 20 days gestation (dG), and litter size of rats fed with control (20% casein) or restricted (10% casein) diet during pregnancy

	Control ($n = 11$)	Restricted ($n = 12$)
Maternal body weight (g) at onset of pregnancy	243.2 ± 2.93	246.3 ± 7.72
Maternal body weight (g) at 20 dG	384.3 ± 5.36	370.0 ± 6.82
Maternal weight gain (g) at 20 dG	140.9 ± 1.80	123.7 ± 2.56*
% weight gain at 20 dG	57.9 ± 1.18	50.2 ± 1.75*
Litter size	14.3 ± 0.37	13.7 ± 0.37

Unpaired Student's *t* test, mean ± S.E.M.; *n* = mothers or litters;. *$P \leq 0.05$, different from control.

reported that oestradiol, testosterone and corticosterone are elevated in maternal blood by nutrient restriction ($P < 0.05$) (Zambrano *et al.* 2005*b*).

Pup birth weight and morphometric measurements

Body weight of pups of protein restricted mothers had reduced birth weight compared to mothers fed the control diet, reduced abdominal diameter, and increased ratio of head : abdominal diameter, and ano-genital distance was increased by 29% as an absolute measure and 28% relative to body weight (Table 2).

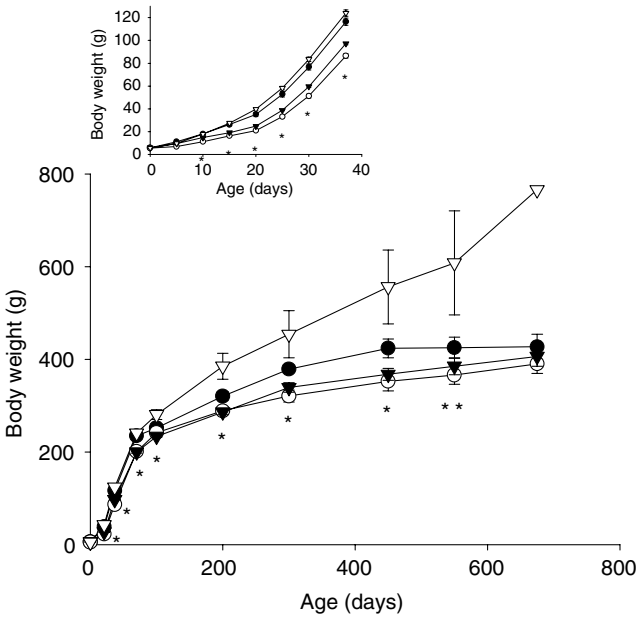

Figure 1. Offspring body weight (g) from birth to 22 months
Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter). CC (●); RR (○); CR (▼); RC (▽). Insert represents 0–37 days enlarged. One way ANOVA followed by Tukey's test, mean ± S.E.M.; $n = 4$–7 litters. At 22 months, only 1 rat from RC group survived. *$P \leq 0.05$ RR and CR different from CC and RC; **$P \leq 0.05$ RC different from CC, RR and CR.

Table 2. Pup weight and morphometric measurments at birth of rats fed the control (20% casein) or restricted (10% casein) diet during pregnancy

	Control ($n = 11$)	Restricted ($n = 12$)
Body weight (g)	6.1 ± 0.08	5.7 ± 0.10**
Lenght (mm)	48.3 ± 0.03	47.8 ± 0.02
Ano-genital distance (mm)	1.4 ± 0.04	1.8 ± 0.05**
Ano-genital distance (mm g^{-1})	0.23 ± 0.01	0.32 ± 0.01**
Head diameter (mm)	10.7 ± 0.07	10.6 ± 0.08
Abdominal diameter (mm)	13.3 ± 0.18	12.6 ± 0.13*
Head : abdominal ratio	0.80 ± 0.01	0.84 ± 0.008*

Unpaired Student's *t* test, mean ± S.E.M.; *n* refers to litters. *$P \leq 0.01$, **$P \leq 0.001$ different from control.

Neonatal endocrinology

At 2 days postnatal age serum corticosterone in pups of control mothers was 132.4 ± 6.95 ng ml^{-1} and in pups of nutrient restricted mothers was 97.6 ± 5.29 ng ml^{-1} ($P \leq 0.001$).

Postnatal growth

Figure 1 shows the body weights of the four groups of offspring from birth to 22 months. Offspring of both groups restricted during lactation were lighter than CC and RC throughout from day 10–450 ($P \leq 0.05$). As they aged, pups from the RC group became heavier than all the other groups ($P \leq 0.05$).

Onset of puberty indicated by vaginal opening and first oestrus

Vaginal opening occurred later in the RR and CR than the CC and RC groups (Tables 3, $P \leq 0.001$). The timing of the first oestrus was similarly delayed (Table 3, $P \leq 0.001$). Pup body weights at the time of vaginal opening and first oestrus were less in the RR and CR groups than CC and RC (Table 3, $P \leq 0.001$).

Ovarian and uterine weight

Ovarian and uterine weights were obtained at 21 days and around 70 days and 22 months of age in dioestrus (Table 4). There were no differences between right and left ovarian weight, and therefore combined ovarian weights are presented. At 21 days ovarian weight was greater in RC than RR and CR ($P < 0.05$). At 70 days ovarian weight was

Table 3. Onset of puberty (d) defined as time of vaginal opening or first oestrus and body weight (g) at onset of puberty of offspring

	CC ($n = 5$)	RR ($n = 5$)	CR ($n = 6$)	RC ($n = 7$)
Age at vaginal opening (d)	37.0 ± 0.42^{a}	40.6 ± 0.39^{b}	38.8 ± 0.25^{b}	36.0 ± 0.37^{a}
Body weight (g) at vaginal opening	115.8 ± 2.53^{a}	101.3 ± 2.42^{b}	105.3 ± 1.31^{b}	118.8 ± 2.71^{a}
Age at first oestrus (d)	37.9 ± 0.47^{a}	40.9 ± 0.39^{b}	39.6 ± 0.33^{b}	36.9 ± 0.44^{a}
Body weight (g) at first oestrus	121.0 ± 3.08^{a}	104.8 ± 2.44^{b}	111.2 ± 1.11^{b}	124.0 ± 2.69^{a}

Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter). One way ANOVA and Tukey's test, mean ± S.E.M.; *n* refers to litters. $P \leq 0.001$, data not sharing a letter are statistically different from other groups.

Table 4. Ovarian and uterine weight (mg) in the four groups of offspring at 21, 70 days and 22 months of postnatal life

	CC	RR	CR	RC
21 days	$n = 5$	$n = 5$	$n = 6$	$n = 7$
Body wt (g)	46.7 ± 0.66^{a}	28.2 ± 0.56^{b}	27.6 ± 1.74^{b}	49.6 ± 1.90^{a}
Ovarian wt (mg)	$9.2 \pm 0.48^{a,b}$	8.0 ± 0.52^{a}	7.0 ± 0.23^{a}	10.8 ± 0.32^{b}
Uterine wt (mg)	20.1 ± 1.15^{a}	12.3 ± 0.67^{b}	$16.0 \pm 0.58^{a,b}$	19.4 ± 1.70^{a}
70 days	$n = 5$	$n = 5$	$n = 5$	$n = 5$
Body wt (g)	242.0 ± 8.88^{a}	206.9 ± 3.51^{b}	205.9 ± 3.37^{b}	251.5 ± 7.98^{a}
Ovarian wt (mg)	62.1 ± 6.81^{a}	43.1 ± 3.90^{b}	$50.0 \pm 2.63^{a,b}$	55.6 ± 2.92^{a}
Uterine wt (mg)	286.0 ± 8.70	320.1 ± 36.01	295.7 ± 14.03	346.0 ± 23.00
22 months	$n = 4$	$n = 5$	$n = 5$	$n = 1$
Body wt (g)	420.4 ± 26.88	402.7 ± 20.70	400.2 ± 20.92	766.0
Ovarian wt (mg)	56.2 ± 2.95	59.8 ± 7.45	66.7 ± 7.43	69.0
Uterine wt (mg)	629.9 ± 45.63	614.2 ± 91.04	499.6 ± 30.33	495.0

Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter). Ovarian weight is expressed as the average of right and left ovaries, RC data at 22 months were not analysed because only one female survived to this age. One way ANOVA and Tukey's test, mean ± S.E.M.; *n* refers to litters. $P \leq 0.05$, data not sharing a letter are statistically different from each other at the same age.

decreased in the RR group compared with CC ($P < 0.05$). There was a tendency to lower ovarian weight at 21 and 70 days in RR and CR groups. No differences were found in ovarian weigh at 22 months. Uterine weight was lower in RR group at 21 days compared with CC and RC.

Hormone concentrations at 21 days, 70 days and 1 year

Hormonal data at 21 days are presented in Fig. 2. Oestradiol was lower in the RR and CR groups compared to CC and RC. Progesterone was only significantly different

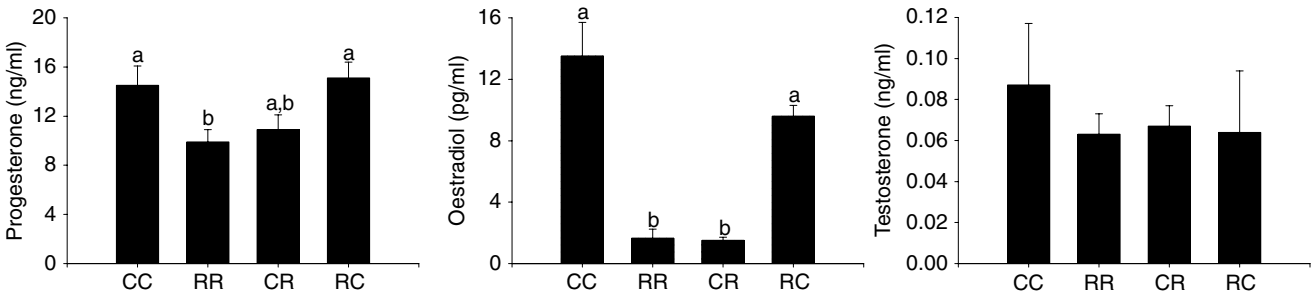

Figure 2. Offspring serum hormone concentrations at 21 days of age
Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter). One way ANOVA and Tukey's test, mean ± S.E.M.; $n = 5$–6 litters. $P \leq 0.05$, data not sharing a letter are statistically different from each other at the same age.

for RR and no differences were observed for testosterone at this age. At 70 days serum LH in dioestrus was lower in all three restricted groups compared with controls (Fig. 3). At one year testosterone and oestradiol were higher at oestrus in RR and CR than CC (Fig. 3). Values at older ages are not presented since animals had varying pregnancy histories.

Analysis of oestrous cycle from vaginal opening to 70 days, 140 days, 1 year and 22 months

Figure 4 shows representative cycles from one animal in each group at 140 days, 1 year and 22 months of postnatal life. Immediately after puberty cycles tend to be short and frequent with no obvious difference between groups (data not shown). Table 5 presents the average cycle length and percentage of animals showing at least one cycle in a 10-day recording period at 140 days and 1 year postnatal age. At 140 days, a time at which fertility was maximal in all groups, cycles were clearly discernible in all animals in all groups and it is of interest that the only significant diference at this age is the longer cycle length in the RC group. By 1 year, cycle length was unchanged in CC but had increased in RR and CR compared with cycle length in the same group at 140 days ($P \leq 0.05$). However, the cycle length did not differ between the three experimental groups and controls. At 22 months postnatal age only one animal in the CC and one animal in the CR group demonstrated any cycles.

Fertility at 150 days and at 1 year

Fertility data are presented in Table 6. At 150 days fertility was maximal in all groups and in agreement with maximal fertility in our institutional records over the last 5 years. Fertility decreased between 150 days and 1 year; at 1 year fertility rate had fallen significantly by 40, 36 and 75% in RR, CR and RC offspring, respectively, compared with 150 days but not CC. At 1 year fertility was reduced in CR and RC compared with controls (Table 6). There were no differences in litter size or sex ratios in any of the pregnancies between groups at either age. Litter size was reduced with age in all groups but the sex ratio was unchanged (Table 7).

Survival rates at 22 months

Figure 5 shows the survival of pups in the four groups. At 21 months survival of RC pups was significantly lower than CC and RR. There was a tendency for RR to survive longer than CR ($P = 0.07$).

Discussion

The goal of these studies was to observe reproductive outcomes of female pups delivered by mothers experiencing maternal protein restriction in two specific windows of their development – pregnancy and lactation.

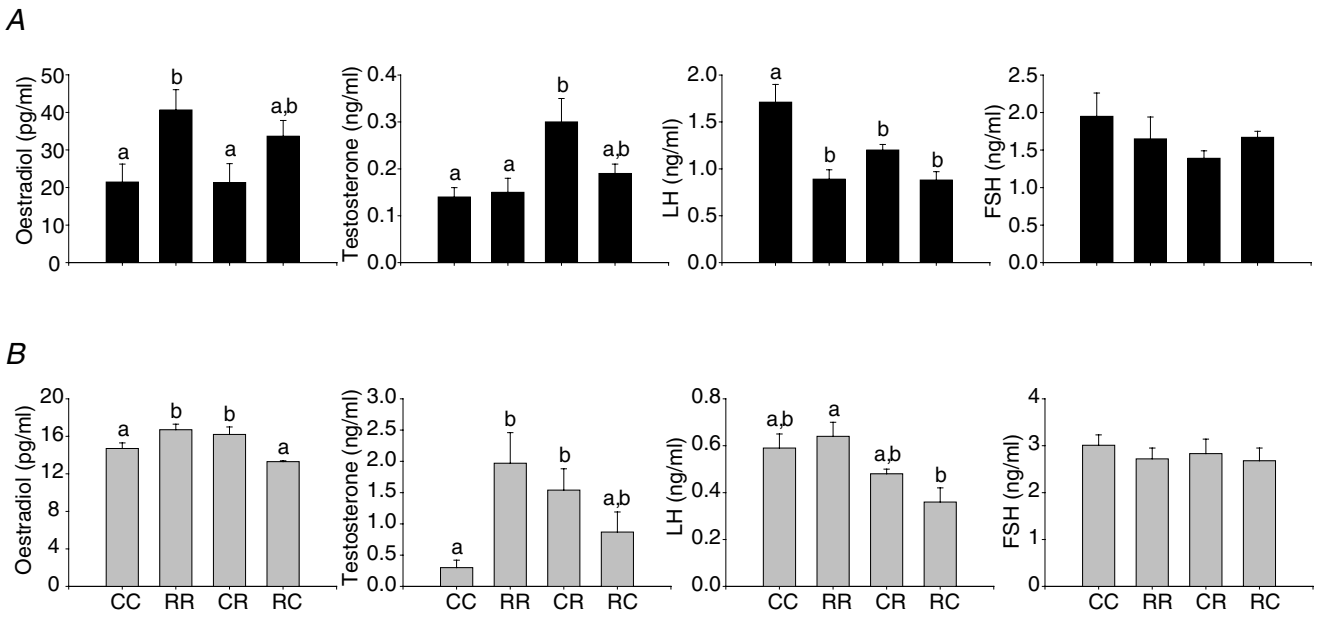

Figure 3. Offspring serum oestradiol and testosterone in oestrus, and LH and FSH in dioestrus at 70 days (*A*, black bars) and one year of age (*B*, grey bars)
Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter). One way ANOVA and Tukey's test, mean ± S.E.M.; $n = 4$–5 litters. $P \leq 0.05$. Note the change in scales to reflect hormone concentration differences at the two ages. Comparisons are only made between groups of the same age. Data not sharing a letter are statistically different from each other at the same age.

Table 5. Cycle length at 140 days and one year of post natal age in offspring

	CC ($n = 5$)	RR ($n = 5$)	CR ($n = 5$)	RC ($n = 4$)
Cycle length (d) at 140 days	4.6 ± 0.16^{a}	4.9 ± 0.14^{a}	4.9 ± 0.33^{a}	6.3 ± 0.46^{b}
% animals with at least one cycle at 140 days	100.0	100.0	100.0	100.0
Cycle length (d) at 1 year	6.7 ± 0.91	$9.7 \pm 0.98^{*}$	$7.5 \pm 0.69^{*}$	7.4 ± 1.70
% animals with at least one cycle at 1 year	100.0	90.0	81.0	75.0

Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter). One way ANOVA and Tukey's test among groups same age, *t* test between same group different age, mean ± S.E.M.; *n* refers to litters. $P \leq 0.05$, data not sharing a letter are statistically different from each other at the same age; $^{*}P \leq 0.01$ different from same group at 140 d.

Each of the three experimental groups we have studied addresses the effects of a different challenge to the developing female rat compared with the CC group. The RR group is composed of offspring restricted for the whole period of pregnancy and lactation and thus there is no recuperation of prenatal restriction during lactation. The CR group provides information on the effects of protein restriction in lactation superimposed on a normal diet during the mother's pregnancy. Our study design addresses different questions from previous investigations of effects of maternal nutrient challenge during pregnancy and lactation on the reproductive capacity of female offspring. For example, Leonhardt *et al.* (2003) studied the effects of a global dietary reduction of 50% on postnatal function of the pituitary gonadal axis and leptin and the timing of puberty in males and females.

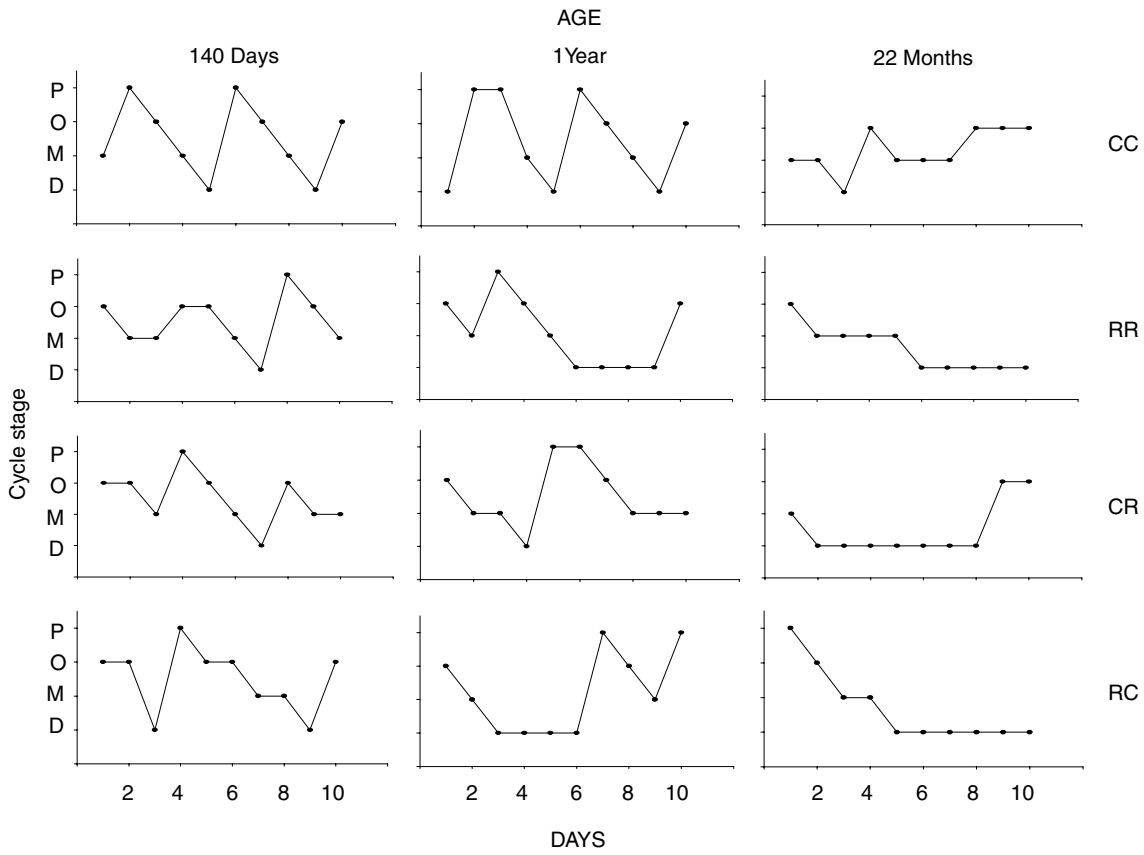

Figure 4. Representative offspring oestrous cycle from one animal in each group at 140 days, 1 year and 22 months of postnatal life
P, pro-oestrus; O, oestrus; M, metoestrus; D, dioestrus. Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter).

Table 6. Fertility rate in the four groups of offspring at 150 days and 1 year of postnatal life

	CC ($n = 5$)	RR ($n = 5$)	CR ($n = 5$)	RC ($n = 4$)
Fertility rate (%) at 150 days	77.7	100.0	86.4	100.0
Fertility rate (%) at 1 year	66.6[a]	60.0[a]*	50.0[b]*	25.0[b]*

Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter). A χ^2 test was performed; data not sharing a letter are statistically different from each other at the same age, * different from same group at 150 d, $P \leq 0.05$. *n* refers to litters.

Table 7. Body weight (g) at onset of pregnancy and 20 days gestation (dG), litter size and sex ratio of pups at 150 days, and 1 year postnatal age

	CC	RR	CR	RC
A. Pregnancy 150 days	$n = 4$	$n = 5$	$n = 5$	$n = 4$
Body weight (g) at onset of pregnancy	293.1 ± 2.5^{a}	263.0 ± 2.7^{b}	258.0 ± 3.4^{b}	337.2 ± 16.1^{a}
Body weight (g) at 20 dG	$383.9 \pm 4.0^{a,c}$	334.7 ± 4.3^{b}	$367.1 \pm 6.1^{a,b}$	402.0 ± 18.2^{c}
Weight gain (g) at 20 dG	90.9 ± 1.3^{a}	71.7 ± 2.1^{b}	109.1 ± 2.5^{c}	65.0 ± 3.0^{b}
% weight gain at 20 dG	31.0 ± 1.8^{a}	$27.3 \pm 1.7^{a,c}$	42.3 ± 2.3^{b}	19.3 ± 1.2^{c}
Litter size	14.1 ± 0.9	13.4 ± 1.1	12.2 ± 0.6	11.8 ± 1.9
B. Pregnancy 1 year	$n = 4$	$n = 4$	$n = 3$	$n = 1$
Body weight (g) at onset of pregnancy	394.2 ± 17.2	364.0 ± 23.1	333.8 ± 19.6	247.3
Body weight (g) at 20 dG	481.7 ± 13.5^{a}	$439.1 \pm 26.5^{a,b}$	401.0 ± 15.6^{b}	361.3
Weight gain (g) at 20 dG	87.4 ± 20.9	75.1 ± 39.4	67.3 ± 16.7	114.0
% weight gain at 20 dG	23.9 ± 7.0	24.0 ± 14.0	24.1 ± 9.8	46.1
Litter size	5.5 ± 1.3*	4.5 ± 2.1*	4.5 ± 1.1*	8.0

RC data at 1 year were not analysed because this group contained only one pregnancy. One way ANOVA followed by Tukey's test for same age different groups, *t* test for same group different age, mean ± S.E.M.; *n* refers to litters. $P \leq 0.05$, data not sharing a letter are statistically different from each other at the same age; *$P \leq 0.05$ different from same group at 150 d.

Birth weight and growth

Maternal nutrient restriction reduced the birth weight of female pups. Other studies of nutrient restriction have produced variable results. In some investigations birth weight was reduced (Galler & Tonkiss, 1991; Vickers *et al.* 2001), in others unchanged (Langley *et al.* 1994) and in some even increased (Langley-Evans *et al.* 1996). Factors that may alter response of the mother and fetus to nutrient restriction include the strain of rat, age of mothers and differences in the diets. It is of interest that the two groups whose mothers were restricted during lactation had lower body weights during the major part of the study but caught up with the body weight of the CC group in later life. We and others have shown that maternal nutrient restriction during lactation increases insulin sensitivity in early postnatal life (Petry *et al.* 1997; Shepherd *et al.* 1997; Zambrano *et al.* 2005*a*, 2006), but these offspring become insulin resistant later in life and put on weight to catch up with the controls.

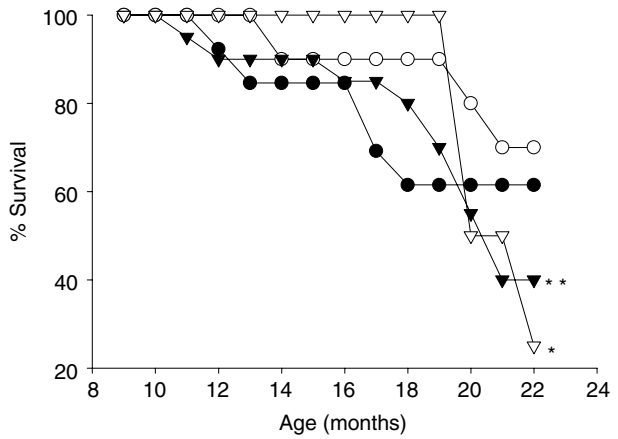

Figure 5. Percentage offspring survival at 22 months
Maternal groups: mothers fed with control (C, 20% casein) or restricted (R, 10% casein) diet during pregnancy (first letter) and lactation (second letter). CC (●); RR (○); CR (▼); RC (▽). A χ^2 test was performed, *$P \leq 0.05$ different from CC and RR; **$P = 0.07$ CR *versus* RR. 100% represents all offspring alive in 5 litters in each group at 8 months of age.

One of the most interesting apsects of developmental programming is the passage of effects transgenerationally. We have shown that the second generation offspring of protein restricted rats have a lower birth weight (Zambrano *et al.* 2005*a*) and similar data are available for the children of the daughters of the Dutch Hunger Winter (Lumey *et al.* 1995). One simple explanation for the growth restriction in the second generation has been that there is increased maternal constraint on fetal growth as a result of altered

uterine development in females nutritionally challenged when they themselves were developing. One study on growth of the fetal liver as a result of maternal protein restriction demonstrated an altered proportion of different cell types with an increase in gluconeogenic hepatic cells (Burns *et al.* 1997). It remains possible that although there is no overall difference in uterine weight, altered growth of specific tissues in the uterus could result from fetal and/or neonatal exposure to maternal protein restriction and play a role in transgenerational passage of epigenetic effects. The differences presented here in the maternal endocrine milieu – elevated maternal progesterone, testosterone, oestradiol and corticosterone – could all affect the growth of uterine, ovarian and neuroendocrine tissues in the female fetuses with consequences in their subsequent pregnancies.

Effects on sexual development and offspring endocrinology

The ano-genital distance in many species (including rats and mice) provides an external marker of sexual differentiation at birth. In the female sheep it has been shown that the ano-genital distance at birth is normally regulated by both oestradiol and testosterone (Kosut *et al.* 1997). Maternal steroids and/or their metabolites can cross the placenta, and thus exposure to endogenous fetal and transplacentally acquired maternal androgens in fetal life may play a role in the increased ano-genital distance we observed in female pups of protein restricted mothers (Graham & Gandelman, 1986). In our study maternal testosterone was elevated and thus may account for the increased ano-genital distance in the pups. We have previously shown an increase in the ano-genital distance at birth in male offspring of protein restricted mothers (Zambrano *et al.* 2005*b*). The elevation of maternal corticosterone in the dams subjected to protein restriction would be anticipated to depress the fetal pituitary-adrenal axis. This is a likely explanation for the lowered concentration of corticosterone observed in the 2-day-old offspring. Increased maternal glucocorticoid levels have been related to delay and decreased function in development of the sexual organs of male offspring (Page *et al.* 2001). Glucocorticoids could be implicated in later reproductive disturbances. It has been reported that dexamethasone inhibits ovarian function in immature female rats and inhibits the differentiation of granulose cells by FSH (Tohei & Kogo, 1999). Leonhardt *et al.* (2002) postulate that increased circulating glucocorticoid levels in female offspring could mediate the impaired development of Graafian follicles induced by maternal protein restriction.

Vaginal opening is considered a good marker of the onset of puberty. We observed significantly delayed vaginal opening in the two groups of rats whose mothers were restricted during lactation. Smith & Waddell (2000) demonstrated that administration of glucocorticoids to pregnant rats resulted in a subsequent delay in the onset of puberty in female pups. We demonstrated an increase in corticosterone levels in the mother restricted during pregnancy (Zambrano *et al.* 2005*b*), and these results may imply that fetal glucocorticoid exposure is an important determinant of the timing of puberty onset in female pups. In agreement with other authors (Engelbregt *et al.* 2000, 2002; Smith & Waddell, 2000) this delay was not related to body weight. Some authors have claimed that it is necessary to reach a particular body weight to initiate puberty (Frisch *et al.* 1977, 1996; Baker, 1985). Our data show that weight of the pup cannot be the only factor in determining the time of vaginal opening and the onset of puberty. Epigenetic factors during development clearly play a role that is independent of pup weight. Undernutrition before and during folliculogenesis delay fetal follicular development in sheep (Rae *et al.* 2001). The consequences of fetal androgen exposure are reflected in a progressive and dramatic impairment of fertility in the ovary-intact ewe, and the reasons for abnormal steroid feedback mechanisms may reside in sexually dimorphic inputs to the GnRH neurones. (Robinson *et al.* 2002). However, the precise mechanism by which maternal protein restriction programmes the delay in the onset of puberty in offspring remains unknown. Changes indicating delayed reproductive maturation – such as delayed testicular descent – occur in male offspring of mothers fed low protein diets (Zambrano *et al.* 2005*b*).

The decreased levels of LH at 70 days in all three groups experiencing maternal protein restriction at some stage of development (FSH was decreased as well but not significantly) and the increases of testosterone levels at 1 year presage potential reproductive problems including changes in the ovarian cycle. These changes could play a role in later fertility problems. This degree of maternal protein restriction leads to similar changes in reproductive hormones in male offspring (Zambrano *et al.* 2005*b*), indicating that a major effect of the challenge imposed on the developing offspring is to alter hypothalamo-pituitary endocrine function.

Cycle length and fertility rate

Our findings agree with the view that exposure to high concentrations of maternal steroids adversely affects both pituitary-gonadal and pituitary-adrenal function in the offspring. For example, it has been reported that maternal adrenocorticotropin injection diminishes reproductive capacity in adult life as shown by behavioural changes such as the frequency of copulation as well as ejaculation (Stylianopoulou, 1983). It was also found that neonatal administration of testosterone affect the sexual behaviour and cyclicity of female offspring (Stylianopoulou *et al.*

1983). Similarly, male offspring of prenatally stressed rats show decreased sexual activity (Anderson *et al.* 1986), delayed sexual maturation and reduced fertility rate (Zambrano *et al.* 2005*b*).

The irregular cycles in the female offspring in all groups from the onset of puberty until 70 days has important implications for studies in which females have been bred at the earliest possible age. The exciting studies of Wallace and colleagues in adolescent sheep indicate that outcomes in offspring in many rodent investigations in the literature may be influenced by maternal age (Wallace *et al.* 2000; Da Silva *et al.* 2001). One intriguing observation from our study is the significantly greater absolute weight gain in the original mothers who became pregnant at 80 days, 141 g in the controls (Table 1) compared with 91 g in the CC offspring in their pregnancies at 150 days (Table 7A) although both pregnancies delivered an average of 14 pups. These findings support the view that the younger pups are still growing during pregnancy. At 1 year maternal weight gain was still approximately 90 g although the total number of pups was only five. The decrease in litter size we observed with maternal age is very similar to that previously reported in Long-Evans rats in which litter size decreased from an average of 12 at 4 months of age to four at 1 year (Matt *et al.* 1986). All of these findings point to the need for control of maternal age, weight and parity in studies of developmental programming resulting from altered maternal nutrition.

Fertility rate was unaffected at 150 days postnatal life but was reduced at 1 year postnatal age in all three groups when compared to their fertility at 150 days. In keeping with this observation, the oestrous cycle length in the RR and CR groups increase between 150 days and 22 months. These findings indicate that some effects of nutrient restriction are not apparent at early stages of life but emerge in later life as do many of the consequences of developmental programming. Although anecdotal, it is of interest that the only female from the RC group who became pregnant was the only female that remained lean in the group. The remaining survivors who did not get pregnant weighed 489 ± 19.2 g ($n = 3$).

Longevity

Studies on the mechanisms of longevity clearly need to incorporate the concept that altered trajectories of ageing begin during fetal and neonatal development. The findings of Ozanne and Hales have introduced the important idea that differences in nutrition at different periods of development can interact to alter life span (Ozanne & Hales, 2004). Thus Gluckman & Hanson (2004) have introduced the idea of the predictive adaptive response in which the organism alters developing processes to enhance its chances of survival in a given environment. When the environment eventually experienced does not match the predicted environment, the organism's phenotype proves to be maladapted and life span is curtailed. The shortest lived group in our study was RC and the longest RR.

In conclusion, the finding that the major persistent effects on growth resulted from nutrient restriction of the mother during lactation is in agreement with the occurrence of the major part of cell division and organ and body growth in the postnatal period in the rat. The demonstration of significant effects on ovarian size, hormone levels and oestrous function and fertility resulting from nutrient restriction during development emphasizes the need for further studies of different exposures and outcomes related to developmental programming of the hypothalamo-pituitary-ovarian axis.

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