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Maternal Undernutrition in Cows Impairs Ovarian and Cardiovascular Systems in Their Offspring¹

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ABSTRACT

Severe prenatal undernutrition is usually associated with low birth weights in offspring and disorders including hypertension, obesity, and diabetes. Whether alterations in maternal nutrition insufficient to impair birth weight or prenatal growth impact the cardiovascular, stress, or metabolic systems is unknown. In addition, little is known about the effects of maternal dietary restriction on development of the reproductive system in mammals. Here, we use the bovine model, which has a gestational length and birth rate similar to humans, to show that offspring from nutritionally restricted dams (during the first trimester) were born with identical birth weights and had similar postnatal growth rates (to 95 wk of age), puberty, glucose metabolism, and responses to stress compared to offspring from control mothers. However, an increase in maternal testosterone concentrations was detected during dietary restriction, and these dams had offspring with a diminished ovarian reserve (as assessed by a reduction in antral follicle count, reduced concentrations of anti-Müllerian hormone, and increased follicle-stimulating hormone concentrations), enlarged aorta, and increased arterial blood pressure compared with controls. Our study links transient maternal undernutrition and enhanced maternal androgen production with a diminished ovarian reserve as well as potential suboptimal fertility, enlarged aortic trunk size, and enhanced blood pressure independent of alterations in birth weight, postnatal growth, or stress response and glucose tolerance. The implications are that relatively mild transient reductions in maternal nutrition during the first trimester of pregnancy (even those that do not affect gross development) should be avoided to ensure healthy development of reproductive and cardiovascular systems in offspring.

arterial blood pressure, developmental origins of health and disease, fetal development, follicular development, ovarian reserve, ruminants

INTRODUCTION

It is well documented that the environment encountered during fetal and neonatal life exerts a profound influence on physiological function and risk of disease in adult life [1, 2]. Specifically, animal studies demonstrate a direct association between nutrient imbalance during fetal life and later disease states, including hypertension, diabetes, obesity, and renal disease [3]. However, little is known about the effects of maternal diet on the development of the fetal reproductive system, especially in single-ovulating species like humans and cattle. Female reproductive capacity in mammals is defined by the number and quality of primordial follicles developed in the ovary during the neonatal period [4]. In sheep, nutrient restriction during different critical windows of gestation is associated with delayed ovarian follicular development in the fetus [5, 6], but no data are available on the effects of prenatal nutrient restriction on follicular development during adulthood. In addition, studies that have restricted maternal nutrition during gestation resulted in concomitant effects on offspring growth and measures of their health [7], and these two factors have not been separated.

The primary aim of this study was to test the hypothesis that nutritional restriction during early pregnancy (to 60% of maternal requirements and that does not impact offspring growth) has permanent effects on the establishment of the ovarian reserve (defined as total number of morphologically healthy follicles and oocytes in ovaries) in offspring. We used a bovine model because of its long gestation (9 mo) and well-defined postnatal characteristics of ovarian follicle growth and development [8]. We focused on the first trimester of gestation because the differentiation of oogonia into primordial cells, which activates and initiates an irreversible process of follicular growth [9], starts between 90 and 140 days of fetal life in cattle [10–12] and because the first trimester of pregnancy coincides with the peak in number of germ cells in fetal ovaries [13]. Like humans, cattle have multiple “waves” [14] of growth and atresia of antral follicles during a reproductive cycle. Based on previous studies, we used antral follicle count (AFC), anti-Müllerian hormone (AMH), and follicle-stimulating hormone (FSH) concentrations as reliable biomarkers for the size of the ovarian reserve [15–19].

In addition, based on the extensive literature on the long-term consequences of poor maternal nutrition on the cardiovascular system of the offspring [20] and glucose metabolism [21], we tested the hypothesis that dietary nutritional restriction (to 60% of maintenance) during the first trimester of pregnancy increases arterial blood pressure and impairs glucose metabolism in the offspring.

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MATERIALS AND METHODS

Maternal Nutritional Treatments, Estrous Synchronization, and Artificial Insemination

Animal experimentation was in compliance with the University College Dublin Animal Research Ethics Committee, the Cruelty to Animal Act (Ireland, 1876), and European Union Directive 86/609/EC.

Crossbred beef heifers that would subsequently be pregnant with a single female calf were individually fed at either 1.2 (control [C], $n = 13$) or 0.6 (nutrient restricted [NR], $n = 10$) of their maintenance (M) energy requirements, starting 11 days before artificial insemination up to Day 110 of gestation. The diet was a commercial concentrate feed containing 15% crude protein (C, 4 kg/day; NR, 2 kg/day) and straw (C, 3 kg/day; NR, 1.5 kg/day). From Day 111, all animals were group fed and received a 1.4 M diet until calving. All heifers were artificially inseminated with sex-sorted semen from a single sire and were blocked by AFC for assignment to groups.

Body weight was measured monthly until Day 110 of gestation, and thereafter every fortnight until calving. Eleven days before artificial insemination and on Days 43, 108, 163, and 275 of pregnancy, ultrasonography was performed (Aquila) to measure the depth of the *Musculus longissimus dorsi* and the depth of the fat in the lumbar and rump areas [22]. To determine whether nutrition restriction had a negative impact on maternal endocrine function, which may in turn affect fetal development, peripheral concentrations of testosterone, progesterone, estradiol, and cortisol were measured regularly during pregnancy.

Calving and Phenotypic Measurements of the Offspring

To investigate whether nutrient restriction influenced offspring growth, fetal size was assessed using ultrasonography on Day 94 of gestation. Fetal development was monitored using transrectal ultrasonography (Voluson; GE Healthcare) in a subset of animals (C, $n = 11$; NR, $n = 10$). Twenty-three (C, $n = 13$; NR, $n = 10$) single female calves were born, and their biometrical measurements were recorded at birth, every fortnight until puberty, and monthly until slaughter.

Follicle Numbers and Circulating FSH and AMH Concentrations

We used AFC (total number of antral follicles >3 mm in diameter growing during a follicular wave; cattle have two or three waves 7 to 10 days in length per 21-day estrous cycle, similar to that in female humans), AMH, and FSH concentrations as reliable biomarkers for the ovarian reserve to determine if maternal nutrient restriction impacted the ovarian reserve [15–17, 19]. The total number of antral follicles ≥ 3 mm in diameter growing during a follicular wave [14] was assessed at 7, 18, and 35 wk of age with transrectal ultrasonography (Aloka) [16, 17, 23]. When animals were sexually mature (48.1 ± 0.5 wk, 341.4 ± 6.2 kg), daily transrectal ultrasonography was performed for an entire interovulatory interval (or 21-day estrous cycle) at 56 wk of age and for the first wave of follicular development at 86 wk of age.

At 18, 56, and 86 wk of age, blood was collected every 8 h for 6 to 10 days to determine serum FSH concentrations at the time of emergence of a first follicular wave. To measure serum AMH concentrations [15], serum samples were collected on a random day of the wave during each period of ultrasonographic examination.

Arterial Blood Pressure

To determine the long-term effects of maternal undernutrition during gestation on the cardiovascular system in the offspring, arterial blood pressure was measured with the tail-cuff system every fortnight from 20 wk of age to puberty, and monthly thereafter until slaughter, using a noninvasive blood pressure monitor (Cardell Veterinary Monitor 9401BP, SHARN Veterinary).

Validation of the Arterial Blood Pressure Monitor

The accuracy of the blood pressure monitor was tested by measuring blood pressure simultaneously, both inside the carotid artery and with the monitor, in six nonlactating dairy cows. Twenty-four hours prior to the procedure, all animals received a dose of long-acting antibiotic (15 mg/kg amoxicillin i.m.; Betamox LA; Norbrook), which was repeated after 48 h. The animals were sedated with xylazine (2.5 mg/50 kg i.m.; Chanzine 2%; Chanelle) and locally anesthetized with lidocaine (Norocaine; Norbrook). Sterile surgical catheters (6 Fr, 18 cm; Vygon) were inserted aseptically into the carotid artery of each animal and flushed with a heparinized solution (100 IU/ml in NaCl 0.9%).

Catheters were connected to a precalibrated pressure transducer attached at heart level and linked to a Mac-Lab data-acquisition system (MatLab, Mathworks), and recordings of real-time systemic intra-arterial systolic and diastolic blood pressure were taken for approximately 10 min. During the same period, systolic and diastolic blood pressures were measured using the blood pressure monitor at intervals of approximately 1 min. The cuff was positioned around the tail, and all measurements were recorded with the animals standing. After blood pressure assessment, catheters were removed and all animals were injected with carprofen (1.4 mg/kg; Norocarp LA; Norbrook), a nonsteroidal anti-inflammatory drug, to reduce inflammation and swelling.

Intravenous Glucose Tolerance Test, Age at Puberty, and Stress Test

To investigate whether offspring metabolism was influenced by nutrient restriction, calves were challenged with an i.v. glucose tolerance test at 30 wk of age [24].

To determine age at puberty (defined as the age at which the first of three consecutive samples containing >1 ng/ml of progesterone was collected [25]), circulating progesterone concentrations were assessed in blood samples collected twice per week starting at 42 wk of age.

At 94 wk of age, the effects of short-term exposure to an acute stressor (transport for 20 min in a livestock trailer through an unfamiliar environment) on circulating cortisol concentrations and blood pressure were assessed [26].

Slaughter

At 95 wk of age, all heifers were slaughtered in a commercial abattoir. Liver, kidneys (C, $n = 9$; NR, $n = 9$), and heart were weighed, and the circumference of aortic root was measured. Pairs of ovaries were weighed and measured (C, $n = 13$; NR, $n = 9$), the number of visible antral follicles was counted, and the diameters of the corpus luteum and preovulatory follicles were recorded.

Glomeruli Count

Several studies report that maternal undernutrition impairs number of renal glomeruli, which is linked with increased arterial blood pressure [27]; thus, to investigate the possible causes of the increased blood pressure in NR offspring, kidney glomeruli number was estimated using a modified protocol from Gopalakrishnan [28] in a subset of heifers (C, $n = 6$; NR, $n = 7$).

Hormone Analyses

Tubes containing either heparin or fluoride oxalate were immediately placed on ice, centrifuged at $1500 \times g$ at 4°C for 20 min, and plasma was recovered, whereas additive-free blood tubes were refrigerated (4°C) for 24 h before being centrifuged. Both plasma and serum samples were stored at -20°C until assayed.

Total testosterone was measured in duplicate following ether extraction of plasma samples (1 ml) with commercially available radioimmunoassay kits (Cruinn Diagnostics Limited) [29, 30]. Inter- and intra-assay coefficients of variation (CVs) were 17.8% and 17.8% for low reference samples (mean, 0.2 ng/ml), 11.6% and 11.7% for medium (mean, 1.0 ng/ml), and 8.5% and 8.3% for high reference samples (mean, 5.8 ng/ml), respectively. The lower limit of detection was 0.03 ng/ml.

Cortisol was measured in duplicate in plasma samples with commercially available radioimmunoassay kits (Cruinn Diagnostics Limited) [31]. Sensitivity of the cortisol assay was 0.2 $\mu\text{g/dl}$; the intra- and interassay CVs were 29.2% and 21.5% for low reference samples (mean, 0.34 $\mu\text{g/dl}$), 13.5% and 10.7% for medium (mean, 1.38 $\mu\text{g/dl}$), and 8.9% and 9.2% for high reference samples (mean, 4.81 $\mu\text{g/dl}$), respectively.

Estradiol concentrations were measured in duplicate in plasma samples with commercially available microbial adherence immobilization assay (MAIA) kits (Adaltis) [32]. Sensitivity of the assay was 0.3 pg/ml. The intra- and interassay CVs were 7% and 61.4% for low reference samples (mean, 0.43 pg/ml), 11.4% and 18.1% for medium (mean, 3.3 pg/ml), and 10.4% and 12.9% for high reference samples (mean, 6.4 pg/ml), respectively.

Serum progesterone concentrations were measured using a time-resolved fluorescent immunoassay with an AutoDELFA Progesterone kit (Perkin Elmer). The sensitivity of the assay was 0.01 ng/ml. For maternal samples, the interassay CVs were 24.8% (low), 18.5% (medium), and 9.6% (high). The intra-assay CVs were 9.5%, 8.4%, and 5.1%, respectively.

For samples collected from the offspring to assess puberty onset, the interassay CVs were 27.1% (low), 3.3% (medium), and 21.3% (high). The intra-assay CVs were 25.8%, 8%, and 7.6%, respectively.

FSH concentrations were determined in duplicate using a validated radioimmunoassay [33] with a sensitivity of 0.025 ng/ml. The interassay CVs were 15.4% (low), 12% (medium), and 15.5% (high). The intra-assay CVs were 11.8%, 12%, and 15.6%, respectively.

Serum AMH concentrations were measured with an AMH Gen II ELISA kit (Beckman-Coulter) with a sensitivity of 0.004 ng/ml [15]. The CV for AMH kit controls at 2.87 ± 0.049 ng/ml and 7.97 ± 0.286 ng/ml was 4.5% and 9.5%, respectively. Concentrations of AMH in bovine serum from low-AFC (0.038 ± 0.004 ng/ml) and high-AFC (0.485 ± 0.021 ng/ml) cows also served as controls for seven assays, and the CVs were 22.5% and 10.5%, respectively.

Serum insulin concentrations were determined using a two-site fluoroimmunoassay (AutoDELFIA insulin; Perkin Elmer Life Sciences). Sensitivity of the assay was 0.5 μ IU/ml; intra- and interassay CVs were 7.9% and 13.1% for low reference samples (mean, 4.98 μ IU/ml), 6.9% and 6.3% for medium (mean, 10.24 μ IU/ml), and 7.8% and 6.9% for high reference samples (mean, 29.35 μ IU/ml), respectively.

Plasma glucose concentrations were determined by enzymatic analysis using Randox Imola Clinical Biochemistry Analyzer (Randox Laboratories Ltd.). The mean interassay CVs for glucose samples containing low (5.8 ± 0.1 mmol/L) and high (15.5 ± 0.2 mmol/L) concentrations were 1.6% and 1.3%, respectively. The sensitivity of the assay was 0.64 mmol/L.

Statistical Analysis

Normality of distribution of data for all dependent variables was determined using the UNIVARIATE procedure of SAS (version 9.1; SAS Institute). For nonnormally distributed variables, data were log transformed before the analysis. Actual values are reported in the text. Data collected from mothers pregnant with a single female calf were divided into three periods: collected 1) before conception (and the start of the differential nutritional regime), 2) from Day 7 to Day 110 of gestation, and 3) after Day 110 of gestation. Separate analyses were performed for different time periods. Body weight, depth of back muscle and fat, and peripheral concentrations of cortisol, testosterone, estradiol, and progesterone (dependent variables) were analyzed using a repeated-measures ANOVA (MIXED procedure) in SAS with the terms for group (C vs. NR), day of gestation, and their interaction included in the model. AFC, size of the dominant follicle, and blood pressure (systolic, diastolic, mean) in the offspring at different ages were analyzed using the MIXED procedure of SAS and Tukey critical difference test, with the terms for group (C vs. NR), time (day of wave, day of estrous cycle, weeks of age), and their interaction included in the model. Differences between groups in body measurements (recorded on Day 94 of gestation, at birth, and at slaughter); age and body weight at puberty; maximum follicular diameter; peak, nadir, and mean AFC at each age; and glomeruli number in the offspring were tested for significance using a one-way ANOVA. Differences between groups in mean level of assistance required at calving scores were analyzed with Fisher exact test. The agreement of the systolic and diastolic measurements with the monitor with the corresponding simultaneous intra-arterial blood pressure values was analyzed according to the Bland-Altman method [34]. Area under the response curve for insulin and glucose concentrations for the i.v. glucose tolerance test was calculated using Sigma Plot, version 8.0 (SYSTAT). Significance was set at $P < 0.05$. Results are reported as the mean \pm SEM.

RESULTS

Maternal Body Weight, Back Fat, and Hormonal Concentrations During Gestation

Fifteen days before artificial insemination, C and NR heifers that would subsequently be pregnant with a single female calf weighed 472.5 ± 6.0 and 471.5 ± 6.9 kg, respectively. Differential feeding resulted in a lower ($P < 0.0001$) maternal body weight in the NR compared to the C group (Fig. 1a). By Day 110 of gestation (end of nutritional restriction), heifers in the C group had gained on average 32.2 ± 3.9 kg, while those in the NR group had lost 52.2 ± 3.7 kg, compared to their preconception weights. From Day 110 to calving, maternal body weight was lower ($P < 0.0001$) in the NR compared to the C group (Fig. 1a). Depth of fat tissue before conception was similar between groups in the lumbar and rump areas (lumbar area: C, 0.37 ± 0.04 ; NR, 0.28 ± 0.04 cm; Fig. 1b; rump area: C, 0.41 ± 0.04 ; NR, 0.31 ± 0.05 cm) and was constantly lower in the NR versus C group ($P < 0.01$) both during and after the individual feeding period. Depth of *Musculus*

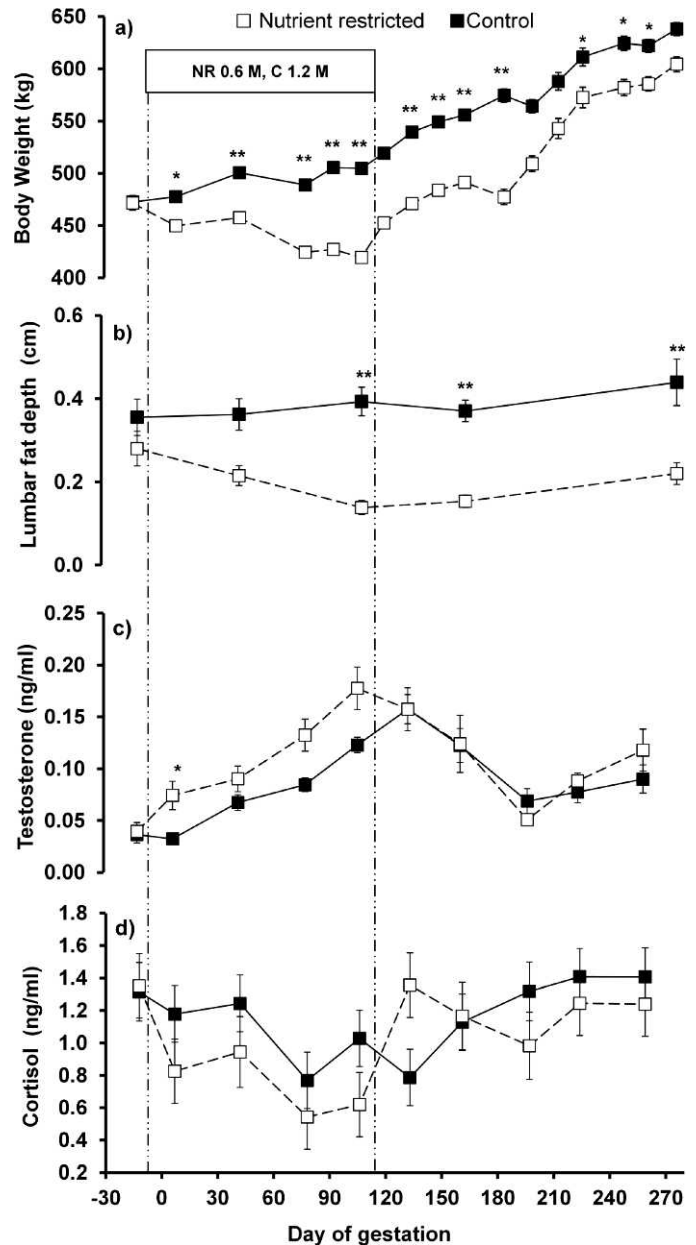


FIG. 1. Effects of nutritional restriction during the first trimester of pregnancy in beef heifers. Variations in maternal body weight (a), depth of fat in the lumbar region (b), circulating testosterone (c), and cortisol concentrations (d) during gestation in cattle pregnant with single female fetuses. NR ($n = 10$, open symbols) animals were individually fed at 0.6, while C ($n = 13$, solid symbols) animals were fed at 1.2 M from Day -11 to Day 110 of gestation, respectively. Each symbol represents the daily mean (\pm SEM) value for animals in the NR and C groups. Asterisks indicate differences (* $P < 0.05$, ** $P < 0.01$) between groups at that time point.

longissimus dorsi was similar between groups during the first 110 days of gestation (C, 6.0 ± 0.07 ; NR, 5.7 ± 0.14 cm), while during the remainder of pregnancy, muscular depth was smaller ($P < 0.05$) in the NR compared to the C group (C, 6.02 ± 0.09 ; NR, 5.66 ± 0.12 cm).

Circulating testosterone concentrations were similar between groups before conception and after the period of differential feeding, but higher ($P < 0.05$) in the NR compared to C group during the first 110 days of gestation (Fig. 1c). Peripheral cortisol concentrations decreased ($P < 0.01$) during the first 110 days of gestation in all pregnant females, but did not differ between groups either during or after the period of

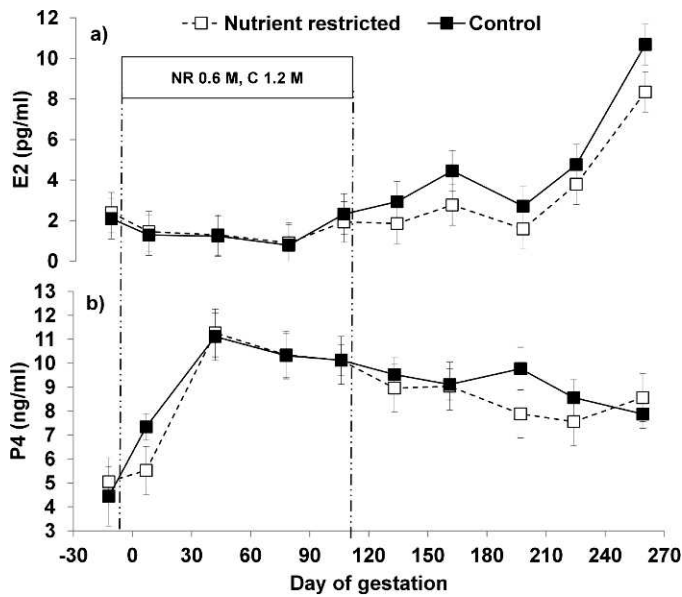


FIG. 2. Effects of nutritional restriction during the first trimester of pregnancy on circulating estradiol (a) and progesterone (b) concentrations in beef heifers. NR ($n = 10$, open symbols) heifers were individually fed at 0.6 M while C ($n = 13$, solid symbols) heifers were fed at 1.2 M from Day -11 to Day 110 of gestation, respectively. Each symbol represents the daily mean (\pm SEM) value for heifers in each group.

differential feeding (Fig. 1d). No difference was detected between groups in serum estradiol concentrations before conception or during the first 110 days of gestation, but C heifers had higher estradiol concentrations ($P < 0.05$) in the second and third trimester of pregnancy (Fig. 2a). Circulating progesterone concentrations increased ($P < 0.01$) in the first trimester of pregnancy and stayed constant thereafter, but were similar between groups before conception and throughout gestation (Fig. 2b).

Calving and Phenotypic Measurements of the Offspring

On Day 94 of gestation, measurements taken using ultrasonography showed that fetal skeletal and cardiac development were not influenced by maternal diet, whereas the aortic root diameter was greater ($P < 0.05$) in fetuses of NR compared to C heifers (Table 1).

At birth, gestational length and placental weight were similar between groups, and maternal nutritional regime did not affect body weight, back length, or body mass index of the offspring (Table 1). Postnatal growth was similar between groups in terms of body weight, back length, and other biometrical measurements recorded throughout their life. At slaughter at 95 wk of age, body weight and the weights of the major organs were similar for C and NR heifers, while aortic root circumference was larger ($P < 0.05$) in NR versus the C group (Table 1).

Age at Puberty

Maternal nutritional restriction during early pregnancy did not influence age or weight at puberty of the female offspring (Table 1).

Antral Follicle Count

Antral follicle count during follicular waves was lower at 7 ($P < 0.01$) and 18 ($P < 0.05$) wk of age (before puberty) and

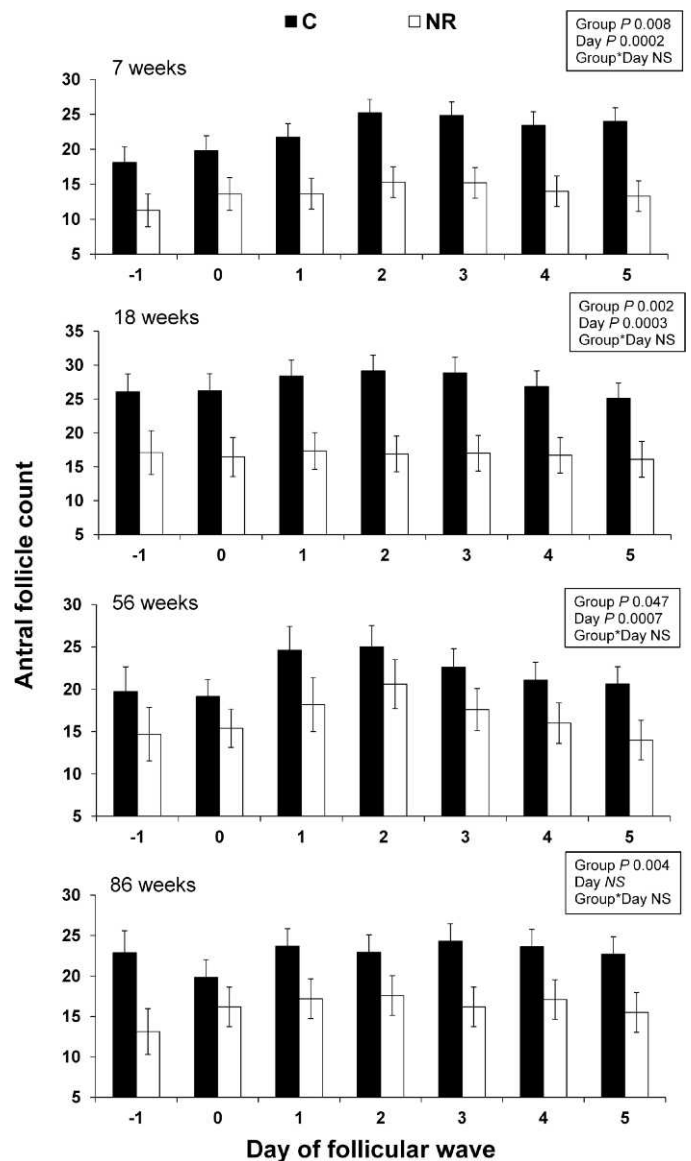


FIG. 3. AFC, assessed by daily ultrasonography, during waves of follicular development at different ages in offspring of cows in the NR ($n = 10$, open symbols) and C ($n = 13$, solid symbols) groups. NR mothers were individually fed 0.6 M, while C mothers were fed 1.2 M from Day -11 to Day 110 of gestation. At 7 and 18 wk, heifers were not sexually mature, while at 56 and 86 wk, data were collected during the first follicular wave of the estrous cycle. Data were aligned relative to the day of wave emergence. Each symbol represents the daily mean (\pm SEM) AFC. Probabilities for the main effects of the repeated-measures ANOVA are given. NS, not significant.

during the first follicular wave of the estrous cycle at 56 ($P < 0.05$) and 86 wk ($P < 0.01$) of age in the NR compared to C group (Fig. 3). However, wave length and maximum follicular diameter were similar between groups at all ages (Table 2). At 56 wk of age, AFC was lower ($P < 0.05$) in the NR versus C group during all of the follicular waves in the estrous cycle, while estrous cycle length and number of follicular waves per cycle were similar between groups (Table 2). Moreover, peak, nadir, and mean AFC were higher ($P < 0.05$) in the C compared to the NR group at all ages (Table 2).

TABLE 1. Effect of maternal nutritional restriction on fetal and postnatal growth of their female offspring.

Parameter	Control	Nutrient restricted
Number of female offspring	13	10
Day 94 of gestation		
Thorax max width (cm) ^a	3.7 ± 0.1	3.6 ± 0.3
Femoral length (cm) ^a	1.4 ± 0.1	1.4 ± 0.1
Cardiac length (cm) ^a	2.5 ± 0.1	2.3 ± 0.1
Cardiac width (cm) ^a	1.8 ± 0.1	1.6 ± 0.1
Diameter of aortic root (mm) ^a	2.6 ± 0.1	3.1 ± 0.2*
Birth		
Gestational length (day)	280.5 ± 1.4	281.1 ± 1.4
Placental weight (kg) ^a	5.6 ± 0.6	5.3 ± 0.5
Body weight (kg)	40.5 ± 1.0	39.5 ± 2.0
Back length (cm)	58.2 ± 0.9	55.9 ± 1.5
BMI (kg/m ²) ^b	72.4 ± 1.6	76.5 ± 3.5
Puberty		
Age (wk)	48.0 ± 0.7	48.1 ± 0.8
Body weight (kg)	343.5 ± 8.8	338.6 ± 9.0
Post mortem		
Age (wk)	94.8 ± 0.2	94.8 ± 0.2
Body weight (kg)	585.8 ± 10.6	582.9 ± 17.4
Carcass weight (kg)	294.7 ± 5.2	290.5 ± 8.0
Liver weight (kg)	6.4 ± 0.2	6.4 ± 0.3
Total kidneys weight (g) ^a	977.5 ± 13.0	1023.4 ± 49.1
Heart (g)	1969.2 ± 43.4	1948.8 ± 82.6
Circumference of aorta at base (mm)	87.4 ± 2.2	96.3 ± 3.0*
Ovaries postmortem ^a		
Weight (g)	14.9 ± 0.7	16 ± 1.3
Volume (cm ³)	6.8 ± 0.5	8.0 ± 0.9
Visible antral follicles (n)	47.1 ± 4.8	32 ± 5.7*
CL diameter (mm)	13.7 ± 0.7	15.3 ± 1.4
Preovulatory follicle diameter (mm)	14.1 ± 0.3	14.3 ± 0.5

^a Data available from a subset of animals, details in the *Materials and Methods*.

^b Body mass index (BMI) was calculated as weight/height at withers².

* $P < 0.05$.

FSH and AMH Concentrations

Serum FSH concentrations at the time of follicular wave emergence (first day of a follicular wave) were similar between groups at 18 wk of age (before puberty, Fig. 4a), whereas at 56 wk (during an estrous cycle) the NR group had higher ($P < 0.05$) circulating FSH concentrations compared to those of the C group at the time of wave emergence ($P < 0.05$, Fig. 4b) and during the follicular wave ($P < 0.05$).

Circulating AMH concentrations decreased with age ($P < 0.0001$) in both groups, but were higher ($P = 0.0039$) in the C compared to NR group (Fig. 4c).

Validation of the Arterial Blood Pressure Monitor

The mean systolic (\pm SD) was 203 ± 13.9 (CV, 6.8%) and 154.1 ± 10.5 (CV, 6.8%) mm Hg when measured intra-arterially and with the cuff system, respectively (blood pressure readings $n = 62$). The mean intra-arterial diastolic pressure was 188.9 ± 16.2 mm Hg (CV, 8.6%), whereas the mean cuff diastolic was 118.8 ± 9.2 mm Hg (CV, 7.7%). Values obtained with the two methods were correlated for both systolic ($r = 0.77$; $P < 0.001$) and diastolic ($r = 0.69$; $P < 0.001$). The mean difference (\pm SD) between the intra-arterial and cuff system was 14.5 ± 10.4 and 35.3 ± 7.9 mm Hg for systolic and diastolic, respectively; 98% of the systolic and diastolic blood pressure readings fell within the limits of agreement (mean difference ± 2 SD) in the Bland-Altman plots.

Arterial Blood Pressure, Renal Glomeruli Number, and Response to Stress

Heifers in the NR group had higher ($P < 0.01$) systolic, diastolic, and mean arterial blood pressure during the study (20 to 85 wk of age; Fig. 5). Systolic ($P < 0.001$) and diastolic ($P = 0.007$) blood pressure decreased with age. No difference was observed between groups in number of renal glomeruli at slaughter (C, 2741 ± 136 ; NR, 2560 ± 262) or in peripheral cortisol concentrations in response to acute stress (area under the curve: C, 15.6 ± 0.6 ; NR, 15.7 ± 0.9).

Glucose Metabolism

There was no effect of maternal nutritional level on offspring for baseline glucose concentrations (C, 4.6 ± 0.1 ; NR, 4.5 ± 0.1), for the area under the response curve coefficient for glucose disappearance (C, 1723.6 ± 33.2 ; NR, 1745.9 ± 36.4), or for insulin concentrations in serum (C, 1839.1 ± 237.1 ; NR, 1890.9 ± 320.3) in response to an i.v. glucose tolerance test at 30 wk of age.

DISCUSSION

The main findings of this study are that maternal nutrient restriction during the first third of gestation resulted in higher maternal circulating testosterone concentrations during gestation and resulted in female offspring with smaller ovarian reserves, enlarged aortas, and increased blood pressure but with identical prenatal growth, birth weights, and postnatal growth rates compared to controls. This is the first study to show an effect of maternal nutrition on the ovarian reserve, aortic trunk

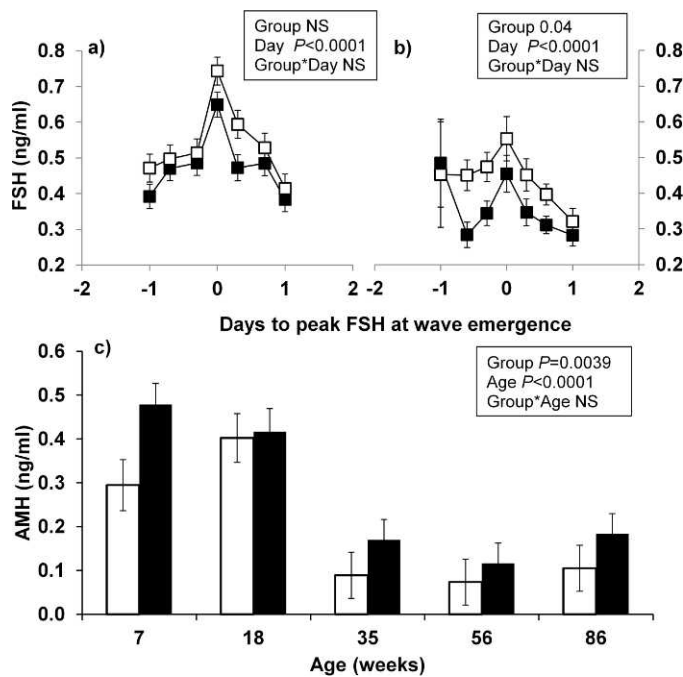


FIG. 4. Circulating FSH concentrations at the time of emergence of a follicular wave at 18 (a) and 56 (b) wk of age and AMH concentrations (c) in offspring of cows in the NR ($n = 10$, open symbols or white bars) and C ($n = 13$, solid symbols or black bars) groups (mean \pm SEM). NR mothers were individually fed 0.6 M, while C mothers were fed 1.2 M from Day -11 to Day 110 of gestation. Data were aligned relative to peak FSH concentrations at wave emergence (first day when the dominant follicle of the examined wave was detected and measured ≥ 4 mm in diameter). Probabilities for the main effects of the repeated-measures ANOVA are given. NS, not significant.

size, and blood pressure that is not concomitant with effects on fetal size, postnatal growth, stress response, or glucose tolerance. Mammals such as cattle [13, 35], pigs [36], sheep [37], and humans [38] are born with a highly variable number of morphologically healthy follicles and a pool of oocytes that is never replenished. For the first time, we provide evidence that maternal undernutrition during early pregnancy contributes to this high variation, though pre- or postnatal growths were not altered as in other studies [39, 40]. This conclusion is supported by the findings in offspring of NR mothers that circulating AMH concentrations were consistently lower from 4 mo to 1.8 yr of age, and circulating FSH concentrations were higher after puberty. It has been well established that AFC reliably predicts the number of morphologically healthy follicles in the ovarian reserve of cattle [15] and that FSH concentrations are higher in age-matched cattle with low versus a high AFC [16, 17, 19]. Finally, a high positive correlation has been documented between circulating AMH concentrations and follicle numbers in cattle [15, 41] and other species, including humans [42]. Taken together, these data indicate an impairment of the ovarian reserve in heifers born in the current study to NR dams.

Such findings have relevance for both the dairy industry and for human reproduction, since there is increasing direct and indirect evidence for an association between the size of the ovarian reserve and fertility in both species [43, 44]. Moreover, both cattle and women with a relatively low AFC have numerous phenotypic characteristics usually associated with infertility [45], such as poor responsiveness to superovulation [46, 47], low circulating progesterone concentrations [18, 48],

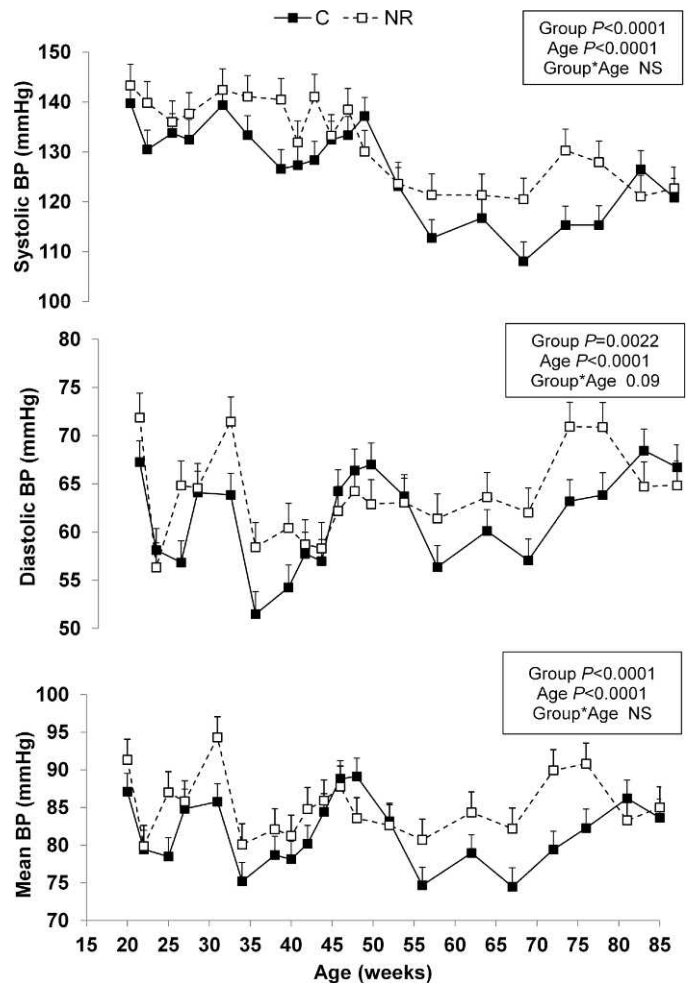


FIG. 5. Arterial blood pressure in offspring of cows in the NR ($n = 10$, open symbols) and C ($n = 13$, solid symbols) groups throughout life. NR mothers were individually fed 0.6 M, while C mothers were fed 1.2 M from Day -11 to Day 110 of gestation. Each symbol represents the mean (\pm SEM). Probabilities for the main effects of the repeated-measures ANOVA are given. NS, not significant.

and diminished endometrial growth during estrous cycles [18, 49].

The reported increase in blood pressure in offspring of NR compared to C dams is in accordance with the extensive epidemiological studies in humans and experimental studies in other animal models [2], but for the first time the present study experimentally demonstrates this independent of effects on offspring growth and in cattle, which is the only animal model studied with similar gestation length to humans. Moreover, we provide evidence for a simultaneous effect of transient maternal nutrient restriction during early pregnancy (without impacting birth weight or postnatal growth) on reproductive and cardiovascular systems in offspring. It is not known if both effects were mediated by the same factors, but both the ovaries and aorta are derived from common embryonic tissue [50]. Several studies report that maternal undernutrition impairs number of renal glomeruli and that this is linked with increased arterial blood pressure [27], but only when fetal growth is impaired. In our study, neither fetal growth nor renal glomeruli numbers were affected by maternal nutrition, but blood pressure was. Mothers were exposed to relatively mild undernutrition during the first trimester of gestation compared with other animal models [27], plus fetal growth occurs

TABLE 2. Peak, nadir, and mean antral follicle count (AFC) at different ages in offspring of cows in the nutrient restricted (n = 10) and control (n = 13) groups.

Parameter	Age (wk)	Control	Nutrient restricted
Peak AFC	7	30.3 ± 2.9	17.8 ± 1.4**
	18	33.8 ± 3.5	20.5 ± 1.6**
	35	27.8 ± 2.6	20.6 ± 1.7**
	56	32.5 ± 2.5	25.9 ± 2.4
	86	30.5 ± 2.8	20.9 ± 2.5**
Nadir AFC	7	18.15 ± 1.8	10 ± 0.7**
	18	19.7 ± 2.1	11.7 ± 1**
	35	19.9 ± 2	12.9 ± 0.8**
	56	14.5 ± 1.4	9.7 ± 0.9**
	86	16.3 ± 1.5	10.9 ± 1.2*
Mean AFC	7	23.8 ± 2.1	14.1 ± 0.9**
	18	26 ± 2.8	16.2 ± 1.1**
	35	23.9 ± 2.2	16.6 ± 1.2**
	56	21.9 ± 2	17.5 ± 1.9
	86	23.6 ± 1.9	15.8 ± 1.8*
Wave length (day)	7	7.5 ± 0.3	8.3 ± 0.5
	18	7.8 ± 0.4	8 ± 0.6
	56	8 ± 0.5	7.9 ± 0.5
Maximum follicular diameter (mm)	7	10.8 ± 0.2	10.5 ± 0.3
	18	10.4 ± 0.2	10.1 ± 0.4
	56	13.1 ± 0.5	12.1 ± 0.5
	86	13.5 ± 0.4	13.4 ± 0.4
Preovulatory follicle diameter (mm)			
First preovulatory follicle	56	15.2 ± 0.5	15.7 ± 0.5
Second preovulatory follicle	56	14.4 ± 0.5	14.6 ± 0.7
	86	15.6 ± 0.6	14.4 ± 0.6
Cycle length (day)	56	21.2 ± 0.4	20.6 ± 0.6
Heifers with 2 waves/cycle (n)	56	7	6
Heifers with 3 waves/cycle (n)	56	6	3

* $P < 0.05$ and ** $P < 0.01$ compared to control at the same age.

predominantly during the last trimester of pregnancy in cattle. This may explain why, in the present study, there was no difference in fetal development (birth weight) at Day 90 of gestation, and consequently no catch-up growth was detected. However, the same level of undernutrition imposed during different windows of gestation may result in different effects on offspring [51].

Interestingly, NR heifers had a larger aortic root during both fetal and adult life. In humans, aortic root dilatation can lead to serious complications, including aortic valvular insufficiency [52] and aortic dissection and rupture [53]. To our knowledge, this is the first time that an increase in aortic diameter, without a reduction in birth weight, is reported in offspring of nutrient-restricted mothers. Aortic root dilatation can result from both inherited disorders [54] and hypertension. In this study, the aortic enlargement was first detected in the NR group during fetal life; thus, we speculate that the condition is congenital and not caused by postnatal high blood pressure. In low-birth weight, hypertensive rodents, maternal food restriction resulted in decreased angiogenesis and increased peripheral vascular resistance [55]. Similarly, in our study, the aortic enlargement coupled with increased blood pressure may be explained by higher peripheral resistance due to decreased angiogenesis. This is supported by the finding that an enlarged aorta is associated with increased vascular stiffness and increase in blood pressure [54]. In addition, during organogenesis, part of the splanchnopleura transforms into a morphologically distinct structure consisting of the dorsal aorta, genital ridges, and mesonephroi [50]. This common origin could explain the reported effects of nutrient restriction on the cardiovascular and reproductive system.

The finding that fetal size on Day 94 of gestation, birth weight, and postnatal growth were not affected by nutritional restriction during early pregnancy suggests that the impairment

of the ovarian reserve and the increase in blood pressure in offspring are not a direct result of a reduction in metabolic energy, but may have been mediated by other factors. In sheep, for example, prenatal testosterone treatment from Day 30 to 90 of gestation causes a significant reduction in total number of follicles per animal on Day 140 of gestation and at 10 mo of age [56, 57]. Moreover, a decrease in proportion of primordial follicles in the ovarian reserve coupled with an increase in the percentage of growing follicles occurs in both studies [56, 57], suggesting that an increase in rate of follicular recruitment occurs in offspring of testosterone-treated ewes. Female sheep and rats exposed prenatally to testosterone also show an increase in arterial blood pressure [58, 59]. These phenotypical similarities between offspring of testosterone-treated and undernourished mothers indicate that the increase in maternal testosterone concentrations observed in this study is a possible mediator of the effects of maternal undernutrition. Therefore, we speculate that the 66% increase in maternal testosterone concentration detected during the first trimester of gestation in our study enhanced follicular recruitment, leading to early follicular depletion of the follicular reserve, enlarged the aorta, and caused mild hypertension, without influencing fetal growth. This conclusion is also indirectly supported by previous reports showing that testosterone addition to culture medium of ovarian cortex from 5- to 8-mo old bovine fetuses or injections into adult monkeys increases growth of preantral follicles [60, 61].

The mechanism whereby transient nutrient restriction enhanced testosterone concentrations during early pregnancy is unclear. Approximately 65% of serum testosterone is bound to sex hormone-binding globulin (SHBG) [62]; consequently, bioavailable testosterone or free testosterone concentrations have been inversely related to the levels of SHBG [63]. In humans, diet interventions that reduce caloric intake and lead to

significant weight loss increase SHBG levels, regardless of diet composition, but the literature remains inconclusive as to whether diet plays a direct role in the regulation of androgens [64]. Thus, it is unclear whether the higher serum testosterone concentrations in this study originate from an enhanced production or from a greater bioavailability. Although the source of its enhanced concentration is unclear (ovaries, placenta, or adrenals), the etiological role of testosterone is confirmed by the lack of difference in the concentrations of the other steroids: estradiol, progesterone, and cortisol.

Another hypothesis is that nutritional restriction, and the consequent weight loss, during the first trimester of gestation were associated with an activation of the stress axis in the offspring, thus affecting fetal outcomes [65, 66]. However, cortisol concentrations at baseline and in response to an acute stressor were similar between groups in postnatal offspring. Thus, if the stress axis was affected by maternal undernutrition, this happened only during fetal life, and the effect was transient and not permanent after birth. In addition, maternal cortisol concentrations were not influenced by nutrition, indicating that undernourished mothers were not stressed. Hence, based on the current literature [65, 66], we can speculate that fetal stress may have had long-term consequences in the offspring, but we have no evidence to support or reject this hypothesis.

Finally, we propose cattle as a novel biomedical model to study developmental programming in humans. In spite of a different placental structure, cattle and humans have similar gestational length, are generally monotonous, and availability of bovine sex-sorted semen eliminates the confounding effect of fetal sex [67]. In addition, cattle are polyestrous, have multiple follicular waves during reproductive cycles, and are single-ovulating, thus they represent an excellent experimental model to elucidate the causes and mechanisms whereby the high variation in the ovarian reserve may alter ovarian function in women.

Our study shows that maternal undernutrition in early pregnancy, which did not affect birth weight, body growth, glucose metabolism, or stress response, resulted in female offspring with a smaller ovarian follicular reserve, enlarged aorta, and increased arterial blood pressure compared to offspring from adequately fed mothers. The bovine maternal undernutrition model may serve as a unique model to determine the mechanisms causing simultaneous alterations in the ovarian reserve, aortic diameter, and blood pressure without affecting offspring growth.

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REFERENCES

- McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev* 2005; 85: 571–633.
- Barker DJ. The origins of the developmental origins theory. *J Intern Med* 2007; 261:412–417.
- Langley-Evans SC. Developmental programming of health and disease. *Proc Nutr Soc* 2006; 65:97–105.
- Zuckerman S. The number of oocytes in the mature ovary. *Recent Prog Horm Res* 1951; 6:63–108.
- Rae MT, Palassio S, Kyle CE, Brooks AN, Lea RG, Miller DW, Rhind SM. Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction* 2001; 122:915–922.
- Borwick SC, Rhind SM, McMillen SR, Racey PA. Effect of undernutrition of ewes from the time of mating on fetal ovarian development in mid gestation. *Reprod Fertil Dev* 1997; 9:711–715.
- Reynolds LP, Caton JS. Role of the pre- and post-natal environment in developmental programming of health and productivity. *Mol Cell Endocrinol* 2012; 354:54–59.
- Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *J Reprod Fertil* 1992; 94:177–188.
- Webb R, Campbell BK. Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. *Soc Reprod Fertil Suppl* 2007; 64:141–163.
- Russe I. Oogenesis in cattle and sheep. *Bibl Anat* 1983; 24:77–92.
- Yang MY, Fortune JE. The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during mid-gestation and is associated with meiotic arrest of oocytes. *Biol Reprod* 2008; 78: 1153–1161.
- Tanaka Y, Nakada K, Moriyoshi M, Sawamukai Y. Appearance and number of follicles and change in the concentration of serum FSH in female bovine fetuses. *Reproduction* 2001; 121:777–782.
- Erickson BH. Development and radio-response of the prenatal bovine ovary. *J Reprod Fert* 1966; 10:97–105.
- Evans AC. Characteristics of ovarian follicle development in domestic animals. *Reprod Domest Anim* 2003; 38:240–246.
- Ireland JL, Scheetz D, Jimenez-Krassel F, Themmen AP, Ward F, Lonergan P, Smith GW, Perez GI, Evans AC, Ireland JJ. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biol Reprod* 2008; 79: 1219–1225.
- Burns DS, Jimenez-Krassel F, Ireland JL, Knight PG, Ireland JJ. Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. *Biol Reprod* 2005; 73:54–62.
- Ireland JJ, Ward F, Jimenez-Krassel F, Ireland JL, Smith GW, Lonergan P, Evans AC. Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. *Hum Reprod* 2007; 22:1687–1695.
- Jimenez-Krassel F, Folger JK, Ireland JL, Smith GW, Hou X, Davis JS, Lonergan P, Evans AC, Ireland JJ. Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during estrous cycles in cattle. *Biol Reprod* 2009; 80:1272–1281.
- Mossa F, Jimenez-Krassel F, Walsh S, Berry DP, Butler ST, Folger J, Smith GW, Ireland JL, Lonergan P, Ireland JJ, Evans AC. Inherent capacity of the pituitary gland to produce gonadotropins is not influenced by the number of ovarian follicles > or = 3 mm in diameter in cattle. *Reprod Fertil Dev* 2010; 22:550–557.
- McMillen IC, MacLaughlin SM, Muhlhauser BS, Gentili S, Duffield JL, Morrison JL. Developmental origins of adult health and disease: the role of periconceptional and foetal nutrition. *Basic Clin Pharmacol Toxicol* 2008; 102:82–89.
- Langley-Evans SC, McMullen S. Developmental origins of adult disease. *Med Princ Pract* 2010; 19:87–98.
- Kelly AK, McGee M, Crews DH Jr, Fahey AG, Wylie AR, Kenny DA. Effect of divergence in residual feed intake on feeding behavior, blood metabolic variables, and body composition traits in growing beef heifers. *J Anim Sci* 2010; 88:109–123.
- Evans AC, Adams GP, Rawlings NC. Follicular and hormonal development in prepubertal heifers from 2 to 36 weeks of age. *J Reprod Fertil* 1994; 102:463–470.
- Grunberg W, Donkin SS, Constable PD. Periparturient effects of feeding a low dietary cation-anion difference diet on acid-base, calcium, and phosphorus homeostasis and on intravenous glucose tolerance test in high-producing dairy cows. *J Dairy Sci* 2011; 94:727–745.
- Romano MA, Barnabe VH, Kastelic JP, de Oliveira CA, Romano RM. Follicular dynamics in heifers during pre-pubertal and pubertal period kept under two levels of dietary energy intake. *Reprod Domest Anim* 2007; 42: 616–622.
- Wagenmaker ER, Breen KM, Oakley AE, Tilbrook AJ, Karsch FJ. The estrous cycle of the ewe is resistant to disruption by repeated, acute psychosocial stress. *Biol Reprod* 2010; 82:1206–1215.
- Bagby SP. Maternal nutrition, low nephron number, and hypertension in later life: pathways of nutritional programming. *J Nutr* 2007; 137: 1066–1072.
- Gopalakrishnan GS, Gardner DS, Dandrea J, Langley-Evans SC, Pearce S, Kurlak LO, Walker RM, Seetho IW, Keisler DH, Ramsay MM,

- Stephenson T, Symonds ME. Influence of maternal pre-pregnancy body composition and diet during early-mid pregnancy on cardiovascular function and nephron number in juvenile sheep. *Br J Nutr* 2005; 94: 938–947.
29. Ronayne E, Enright WJ, Roche JF. Effects of continuous administration of gonadotropin-releasing hormone (GnRH) or a potent GnRH analogue on blood luteinizing hormone and testosterone concentrations in prepubertal bulls. *Domest Anim Endocrinol* 1993; 10:179–189.
30. Mossa F, Jimenez-Krassel F, Folger JK, Ireland JL, Smith GW, Lonergan P, Evans AC, Ireland JJ. Evidence that high variation in antral follicle count during follicular waves is linked to alterations in ovarian androgen production in cattle. *Reproduction* 2010; 140:713–720.
31. Fisher AD, Crowe MA, Alonso de la Varga ME, Enright WJ. Effect of castration method and the provision of local anesthesia on plasma cortisol, scrotal circumference, growth, and feed intake of bull calves. *J Anim Sci* 1996; 74:2336–2343.
32. Prendiville DJ, Enright WJ, Crowe MA, Vaughan L, Roche JF. Immunization of prepubertal beef heifers against gonadotropin-releasing hormone: immune, estrus, ovarian, and growth responses. *J Anim Sci* 1995; 73:3030–3037.
33. Crowe MA, Padmanabhan V, Hynes N, Sunderland SJ, Enright WJ, Beitins IZ, Roche JF. Validation of a sensitive radioimmunoassay to measure serum follicle-stimulating hormone in cattle: correlation with biological activity. *Anim Reprod Sci* 1997; 48:123–136.
34. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; 1:307–310.
35. Erickson BH. Development and senescence of the postnatal bovine ovary. *J Anim Sci* 1966; 25:800–805.
36. Black JL, Erickson BH. Oogenesis and ovarian development in the prenatal pig. *Anat Rec* 1968; 161:45–55.
37. McNatty KP, Smith P, Hudson NL, Heath DA, Tisdall DJ, O WS, Braw-Tal R. Development of the sheep ovary during fetal and early neonatal life and the effect of fecundity genes. *J Reprod Fertil Suppl* 1995; 49:123–135.
38. Block E. Quantitative morphological investigations of the follicular system in women; variations at different ages. *Acta Anat (Basel)* 1952; 14: 108–123.
39. Bernal AB, Vickers MH, Hampton MB, Poynton RA, Sloboda DM. Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. *PLoS One* 2010; 5: e15558.
40. Murdoch WJ, Van Kirk EA, Vonnahme KA, Ford SP. Ovarian responses to undernutrition in pregnant ewes, USA. *Reprod Biol Endocrinol* 2003; 1: 6.
41. Rico C, Fabre S, Medigue C, di Clemente N, Clement F, Bontoux M, Touze JL, Dupont M, Briant E, Remy B, Beckers JF, Monniaux D. Anti-mullerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. *Biol Reprod* 2009; 80:50–59.
42. La Marca A, Pati M, Orvieto R, Stabile G, Carducci Arsenio A, Volpe A. Serum anti-mullerian hormone levels in women with secondary amenorrhea. *Fertil Steril* 2006; 85:1547–1549.
43. Mossa F, Walsh SW, Butler ST, Berry DP, Carter F, Lonergan P, Smith GW, Ireland JJ, Evans AC. Low numbers of ovarian follicles ≥ 3 mm in diameter are associated with low fertility in dairy cows. *J Dairy Sci* 2012; 95:2355–2361.
44. Holte J, Brodin T, Berglund L, Hadziosmanovic N, Olovsson M, Bergh T. Antral follicle counts are strongly associated with live-birth rates after assisted reproduction, with superior treatment outcome in women with polycystic ovaries. *Fertil Steril* 2011; 96:594–599.
45. Ireland JJ, Smith GW, Scheetz D, Jimenez-Krassel F, Folger JK, Ireland JLH, Mossa F, Lonergan P, Evans ACO. Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-Mullerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. *Reprod Fertil Dev* 2011; 23:1–14.
46. Singh J, Dominguez M, Jaiswal R, Adams GP. A simple ultrasound test to predict the superstimulatory response in cattle. *Theriogenology* 2004; 62: 227–243.
47. Beckers NG, Macklon NS, Eijkemans MJ, Fauser BC. Women with regular menstrual cycles and a poor response to ovarian hyperstimulation for in vitro fertilization exhibit follicular phase characteristics suggestive of ovarian aging. *Fertil Steril* 2002; 78:291–297.
48. Pritts EA, Atwood AK. Luteal phase support in infertility treatment: a meta-analysis of the randomized trials. *Hum Reprod* 2002; 17:2287–2299.
49. Raga F, Bonilla-Musoles F, Casan EM, Klein O, Bonilla F. Assessment of endometrial volume by three-dimensional ultrasound prior to embryo transfer: clues to endometrial receptivity. *Hum Reprod* 1999; 14: 2851–2854.
50. Kumaravelu P, Hook L, Morrison AM, Ure J, Zhao S, Zuyev S, Ansell J, Medvinsky A. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* 2002; 129:4891–4899.
51. Ellis-Hutchings RG, Zucker RM, Grey BE, Norwood J Jr, Richards JH, Lau C, Rogers JM. Altered health outcomes in adult offspring of Sprague Dawley and Wistar rats undernourished during early or late pregnancy. *Birth Defects Res B Dev Reprod Toxicol* 2010; 89:396–407.
52. Guiney TE, Davies MJ, Parker DJ, Leech GJ, Leatham A. The aetiology and course of isolated severe aortic regurgitation: a clinical, pathological, and echocardiographic study. *Br Heart J* 1987; 58:358–368.
53. Pressler V, McNamara JJ. Aneurysm of the thoracic aorta. Review of 260 cases. *J Thorac Cardiovasc Surg* 1985; 89:50–54.
54. Erbel R, Eggebrecht H. Aortic dimensions and the risk of dissection. *Heart* 2006; 92:137–142.
55. Khorram O, Khorram N, Momeni M, Han G, Halem J, Desai M, Ross MG. Maternal undernutrition inhibits angiogenesis in the offspring: a potential mechanism of programmed hypertension. *Am J Physiol Regul Integr Comp Physiol* 2007; 293:R745–R753.
56. Steckler T, Wang J, Bartol FF, Roy SK, Padmanabhan V. Fetal programming: prenatal testosterone treatment causes intrauterine growth retardation, reduces ovarian reserve and increases ovarian follicular recruitment. *Endocrinology* 2005; 146:3185–3193.
57. Smith P, Steckler TL, Veiga-Lopez A, Padmanabhan V. Developmental programming: differential effects of prenatal testosterone and dihydrotestosterone on follicular recruitment, depletion of follicular reserve, and ovarian morphology in sheep. *Biol Reprod* 2009; 80:726–736.
58. Sathishkumar K, Elkins R, Yallampalli U, Balakrishnan M, Yallampalli C. Fetal programming of adult hypertension in female rat offspring exposed to androgens in utero. *Early Hum Dev* 2011; 87:407–414.
59. King AJ, Olivier NB, Mohankumar PS, Lee JS, Padmanabhan V, Fink GD. Hypertension caused by prenatal testosterone excess in female sheep. *Am J Physiol Endocrinol Metab* 2007; 292:E1837–E1841.
60. Vendola K, Zhou J, Wang J, Famuyiwa OA, Bievre M, Bondy CA. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. *Biol Reprod* 1999; 61:353–357.
61. Yang MY, Fortune JE. Testosterone stimulates the primary to secondary follicle transition in bovine follicles in vitro. *Biol Reprod* 2006; 75: 924–932.
62. Allen NE, Appleby PN, Davey GK, Key TJ. Lifestyle and nutritional determinants of bioavailable androgens and related hormones in British men. *Cancer Causes Control* 2002; 13:353–363.
63. Vermeulen A. Physiology of the testosterone-binding globulin in man. *Ann N Y Acad Sci* 1988; 538:103–111.
64. Morisset AS, Blouin K, Tchernof A. Impact of diet and adiposity on circulating levels of sex hormone-binding globulin and androgens. *Nutr Rev* 2008; 66:506–516.
65. Entringer S, Buss C, Swanson JM, Cooper DM, Wing DA, Waffarn F, Wadhwa PD. Fetal programming of body composition, obesity, and metabolic function: the role of intrauterine stress and stress biology. *J Nutr Metab* 2012; 2012:632548.
66. Weinstock M. The long-term behavioural consequences of prenatal stress. *Neurosci Biobehav Rev* 2008; 32:1073–1086.
67. Gilbert JS, Nijland MJ. Sex differences in the developmental origins of hypertension and cardiorenal disease. *Am J Physiol Regul Integr Comp Physiol* 2008; 295:R1941–R1952.