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Peri-conception and first trimester diet modifies reproductive development in bulls

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Abstract. Nutritional perturbation during gestation alters male reproductive development in rodents and sheep. In cattle both the developmental trajectory of the feto–placental unit and its response to dietary perturbations is dissimilar to that of these species. This study examined the effects of dietary protein perturbation during the peri-conception and first trimester periods upon reproductive development in bulls. Nulliparous heifers ($n = 360$) were individually fed a high- or low-protein diet (HPeri and LPeri) from 60 days before conception. From 24 until 98 days post conception, half of each treatment group changed to the alternative post-conception high- or low-protein diet (HPost and LPost) yielding four treatment groups in a 2×2 factorial design. A subset of male fetuses ($n = 25$) was excised at 98 days post conception and fetal testis development was assessed. Reproductive development of singleton male progeny ($n = 40$) was assessed until slaughter at 598 days of age, when adult testicular cytology was evaluated. Low peri-conception diet delayed reproductive development: sperm quality was lowered during pubertal development with a concomitant delay in reaching puberty. These effects were subsequent to lower FSH concentrations at 330 and 438 days of age. In the fetus, the low peri-conception diet increased the proportion of seminiferous tubules and decreased blood vessel area in the testis, whereas low first trimester diet increased blood vessel number in the adult testis. We conclude that maternal dietary protein perturbation during conception and early gestation may alter male testis development and delay puberty in bulls.

Additional keywords: fetal programming, morphology, puberty, testis.

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Introduction

Fetal developmental programming of physiological systems is a well-established concept (McMillen and Robinson 2005). Maternal peri- and post-conception nutrition influences fetal development, which, in turn, can affect postnatal growth, gonad development, gamete quality and hormonal status of the offspring (Sullivan *et al.* 2009*a*, 2010; Micke *et al.* 2010, 2011; Dupont *et al.* 2012; Mossa *et al.* 2013). Seasonal variation in the nutritional value and the quantity of pasture available to pregnant ruminants can occur in grass-fed production systems (Burns *et al.* 2010). Such variation in prenatal nutrition has been shown to affect both testicular development and circulating gonadotrophin levels in the prepubertal bull (Sullivan *et al.* 2010). However, the implications for adult reproductive performance in cattle progeny are not known; this paucity of research on the direct effects of *in utero* nutrition on male progeny postnatal reproductive function and fertility is widely acknowledged (Dupont *et al.* 2012; Chavatte-Palmer *et al.* 2014; Mossa *et al.* 2015; Sinclair *et al.* 2016). Comparable studies in rams have reported effects upon age at puberty (Da Silva *et al.* 2001), testicular weight (Bielli *et al.* 2002), testicular volume (Da Silva *et al.* 2001), Sertoli cell numbers, the diameter of seminiferous tubules (Kotsampasi *et al.* 2009), prepubertal testosterone (Da Silva *et al.* 2001) and pituitary response to gonadotrophin-releasing hormone (GnRH; Kotsampasi *et al.* 2009). Age of puberty in cattle (as in sheep) is considered a driver of efficiency; shortening the generation interval, increasing genetic gain and thereby overall lifetime productivity (Barth and Ominski 2000; Yilmaz *et al.* 2006).

Many studies have shown that folliculogenesis (Fair 2010) and early embryo development are sensitive to perturbations in the maternal environment (Edwards and McMillen 2002;

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Ashworth *et al.* 2009; Mossa *et al.* 2013). The response to such perturbations is orchestrated via the developing placenta (Sullivan *et al.* 2009*b*). As the growth trajectory of the bovine placenta differs from the ovine and rodent models, and is, in fact, more similar to the human (Wooding and Flint 1994), the resultant response of the feto–placental unit also differs (Hernandez-Medrano *et al.* 2015). Correspondingly, unlike altricial or small ruminant models, bovine embryo development occurs at similar developmental time points to the human; organogenesis is complete by 42 days post conception (dpc) (Hopper 2014), with the genital ridges, forming at 27 to 30 dpc (Ross *et al.* 2009). Sertoli cells begin to proliferate between 40 and 50 dpc and play a crucial role in gonad development during fetal life and in postnatal spermatogenesis (Griswold and McLean 2006). Disruptions to the proliferation of fetal Sertoli cells may occur through modifications in the development of the hypothalamic–pituitary–gonadal axis in early fetal life (Klonisch *et al.* 2004; O'Shaughnessy and Fowler 2011) and associated changes in the concentration of hormones including follicle-stimulating hormone (FSH), triiodothyronine (T3), thyroxine (T4) and growth hormone (GH; Dupont *et al.* 2012). Consequently, this may affect development of other testicular cells, leading to altered testicular function in postnatal life (Sharpe *et al.* 2003; Dupont *et al.* 2012).

Spermatogenesis is a complex process of cellular replication and differentiation (Barth and Oko 1989; Wrobel 2000). A suite of molecular pathways is regulated by an interdependent complement of hormones including testosterone, FSH, inhibin and activin, which rise and fall in a specified sequence during prepuberty and peri-puberty to result in functional spermatozoa in the adult bull (Evans *et al.* 1996; Matsuzaki *et al.* 2000; Kaneko *et al.* 2001). This sequence is known to be disrupted by nutritional intervention during the preweaning period (Brito *et al.* 2007*b*, 2007*c*) possibly mediated by metabolic hormones (i.e. insulin-like growth factor 1 (IGF1); Brito *et al.* 2007*a*, 2007*c*; Barth *et al.* 2008) with consequent effects upon the development of spermatogenesis. Previously reported effects of prenatal nutrition also include changes in concentrations of many of the aforementioned hormones (Da Silva *et al.* 2001; Micke *et al.* 2010; Sullivan *et al.* 2010).

The aim of the present study was to examine the effects of dietary protein intake in heifers during the peri-conception period and the first trimester on the reproductive development of their male progeny. We hypothesised that the peri-conception and first trimester low-protein diet would delay puberty with deleterious effects upon testicular development and sperm production and, furthermore, this would be associated with alterations to the hormonal milieu in the developing bull.

Materials and methods

Ethics approvals

All procedures were performed with the prior approval of University of South Australia IMVS Animal Ethics Committee, Australia (Approval number: 18/11), The University of Adelaide, Australia (Approval number: S2012–249), The University of New England, Australia (Approval number AEC14–037) and the University of Nottingham, UK (Approval number 1117 140320).

Experimental design and animal management

The purpose of this experiment was to evaluate the impact of maternal dietary protein during the peri-conception (PERI; −60 to 23 dpc (implantation being 18 to 22 dpc; Wathes and Wooding 1980; Spencer and Hansen 2015)) and first trimester (POST; 24 to 98 dpc) periods in nulliparous beef heifers upon fetal and postnatal reproductive development in the male progeny.

The study was a 2 × 2 factorial design. The animals and fetuses studied were singleton male progeny of 2-year-old heifers that have previously been described (Copping *et al.* 2014). Briefly, 360 nulliparous weaned Santa Gertrudis (*Bos taurus* × *Bos indicus*) heifers were selected on the basis of weight (289.4 ± 23.4 kg) from S. Kidman and Co herds located at 'Glengyle' and 'Morney Plains', south-western Queensland, Australia. Heifers were transported to 'Tungali', Sedan, South Australia (34°29′S, 139°18′E) where they underwent 60 days of acclimatisation before commencement of the study. Heifers that did not acclimatise to the individual feeding were removed from the study.

At ~12 months of age, 60 days before artificial insemination, heifers were stratified by bodyweight and randomly assigned to two equal peri-conception (PERI) treatment groups, high and low protein (HPeri and LPeri). Each heifer was fed a high (71 MJ ME and 1.18 kg crude protein per head per day) or low (63 MJ ME and 0.62 kg crude protein per head per day) protein diet (Table 1) consisting of a pelleted diet supplemented with a commercial vitamin and mineral preparation (Minmix; Ridley Agriproducts) that was individually fed in stalls. Straw (5% crude protein) was available *ad libitum* in pens.

Heifers underwent a progesterone-based oestrus synchronisation program as previously described (Hernandez-Medrano *et al.* 2015) and were artificially inseminated on Day 0 with frozen semen from one Santa Gertrudis bull. At 23 dpc, half of each nutritional treatment group was swapped to the alternative post-conception (POST) treatment, high (HPost: 102 MJ ME and 1.49 kg crude protein per head per day) or low (LPost: 98 MJ ME and 0.88 kg crude protein per head per day; Table 1), giving rise to four groups: HPeri-HPost (HH), HPeri-LPost (HL), LPeri-HPost (LH), LPeri-LPost (LL). Pregnancy was confirmed in 124 heifers at 36 dpc and fetal sex was determined at 60 dpc by transrectal ultrasonography. At 98 dpc a sub-set of heifers ($n = 46$; singleton pregnancy) was humanely slaughtered at a commercial abattoir and fetuses of both sexes ($n = 46$; singletons) collected as described (Copping *et al.* 2014), with the 25 singleton male fetuses reported herein (n: HH = 6, HL = 10, LH = 5, LL = 4). The fetal cohort was randomly selected based on maternal weight and sex of the fetus; however, the HL group had a disproportionate number of male fetuses so a larger number of these animals was available at this point. Fetal gonads were dissected, weighed and collected for histological processing.

From the end of the first trimester of gestation (98 dpc), all heifers were fed the same diet, which was formulated to provide additional growth of 0.5 kg per head per day until parturition (79 MJ ME and 0.92 kg crude protein per head per day; Table 1). Heifers received the pellet portion of their diet individually on a daily basis with straw (5% crude protein) provided *ad libitum* in pens until the animals reached parturition.

Table 1. Ingredients and nutrient content of heifer rations for induction period, the PERI- (−60 to 23 dpc) and POST-conception periods (24 to 98 dpc) and second and third trimester of gestation (99 dpc to term)
L, low; H, high

Ration as fed	Induction	PERI conception		POST conception		Second & third trimester
		L	H	L	H	
Wheat (kg)	0.66	1.81	0.48	2.12	0.56	0.60
Canola meal (kg)	2.23	–	–	–	–	0.89
Soybean meal (kg)		0.48	1.83	0.56	2.14	0.44
Barley Straw (kg)	7[A]	5.5	6.7	10.2	10.7	8.6
Molasses (g)	90	72	72	84	84	60
Biofos MDCP (g)	–	19	–	22	–	–
Salt (g)	15	12	12	14	14	10
Vitamin / trace mineral premix (g)	3	2	2	3	3	2
Dry matter (kg)	9.1[B]	7.2	8.3	11.8	12.3	9.6
Total energy (MJ ME)		63	71	98	102	79
% of energy requirements[C]		85	96	136	142	125
Total crude protein (kg)		0.62	1.18	0.88	1.49	0.92
% of Protein requirements[C]		67	127	72	123	88
% CP (total diet)		8.6	14.2	7.4	12.1	9.6
% Fat[B]		1.5	1.4	1.4	1.4	1.5
% Starch[B]		15.1	4.7	10.9	3.8	4.8
Total calcium (g)		22	26	37	38	33
% of Calcium requirements[C]		110	130	185	190	132
Total phosphorus (g)		17	17	21	21	20
% of Phosphorus requirements[C]		130	130	160	160	125

[A]Assumed value.
[B]Predicted value.
[C]Dietary requirements were calculated using Nutrient Requirements of Domesticated Ruminants (Freer 2007). Input values were based upon nutrient analysis of component ingredients in the total diet, liveweight and age of heifers at each diet change, mature cow weight of 550 kg and the desired growth target. Key assumptions: calculations use the formulated values for pellets and actual values for straw. PERI-conception diet is based upon 340 kg Santa Gertrudis heifer gaining 0.5 kg day^{-1}. POST-conception diet is based upon 400 kg, 60 dpc Santa Gertrudis heifer gaining 0.5 kg day^{-1}. Second and third trimester diet is based upon 480 kg, 200 dpc Santa Gertrudis heifer gaining 0.5 kg day^{-1}.

Sixty-four heifers completed the study and gave birth to 18 live singleton female and 43 live singleton bull calves. Progeny remained with their mothers as one group grazing on improved and native pastures until weaning at 183.3 ± 0.8 days of age. After weaning, progeny were segregated according to sex and grazed improved and native pasture until 507.3 ± 0.8 days of age. Non-castrated male progeny were transported from Sedan, South Australia to the 'Tulimba' Research Feedlot, Kingstown, NSW (30°28′S, 151°11′E) before slaughter at a commercial abattoir on 598.3 ± 0.8 days of age with a final liveweight ± s.d. of 652.3 ± 11.4 (HH), 677.0 ± 10.0 (HL), 678.6 ± 19.1 (LH) and 647.4 ± 15.5 (LL) kg. At slaughter, gonads were dissected, weighed and collected for histological processing. Two progeny were removed from the study after birth, due to poor mothering. An additional animal was a cryptorchid and was excluded from the analysis leaving 40 singleton male progeny that completed the study reported herein (*n*: HH = 10, HL = 14, LH = 8, LL = 8).

Tissue fixation and processing

The complete left testis in the fetus and a 1 cm^3 piece from each testis (same for every sample) in the adult were dissected and fixed overnight in 4% paraformaldehyde diluted in 0.1 M phosphate-buffered saline (PBS; 0.14 M NaCl, 0.03 M NaH_2PO_4, 0.05 M Na_2HPO_4) in a ratio of 1 : 5 (tissue volume : fixative solution volume). Samples were washed three times for 24 h each in PBS. Tissues were processed on an automated tissue processor in the following solutions, 30 min in the case of fetal testis and 1 h for the adult testis per solution: 70% ethanol, 80% ethanol, 95% ethanol, three times in 100% ethanol, two times in 100% xylene and two times in paraffin wax at 60°C under vacuum. Following processing, the testes were orientated and embedded in paraffin wax.

All samples, both adult and fetus, were sectioned at a thickness of 10 μm using a microtome (Leica 5M 2255). The sections were dried onto polysilinated slides (Thermo Scientific) on a hot plate at 42°C for 1 h and then for 24 h at room temperature before histological staining.

Cell counts and proportions

The development of the testis was assessed by the measurement of the following structures: testicular cell number (Sertoli, germ and interstitial cells), seminiferous tubules and blood vessels. These were distinguished within the testis by staining with two techniques: immunohistochemistry using a Novolink Polymer Detection immunostaining kit (Leica Microsystems) with Mis-C20 primary antibody (1 : 1000 dilution; Santa Cruz Biotechnology) and Picrosirius staining (Polysciences, Inc.). Mis-C20

was combined with haematoxylin staining in order to differentiate the Sertoli cells (Mis-C20 stained), germ cells (morphologically apparent by their distinctive cytoplasm) and the morphologically distinct interstitial cells/Leydig cells. In the early stages of testicular development, fetal Sertoli cells are localised in the periphery of the sex cords (Vergouwen *et al.* 1991; Jégou 1992; Abd-Elmaksoud 2005) surrounding the germ cells, which are situated near the centre of the testicular cord (Vergouwen *et al.* 1991; Jégou 1992; Abd-Elmaksoud 2005). Interstitial/Leydig cells distribute across the interstitium between the seminiferous cords (Vergouwen *et al.* 1991; Abd-Elmaksoud 2005). Picrosirius staining was used to assist identification of blood vessel from seminiferous tubules. Following tissue staining, sections were photomicrographed using a DM5000B microscope (Leica Microsystems Inc.) with a Leica CTR5000 light box and Leica DFC420 colour capture camera. The magnification of the eyepiece and lens is stated below for each count using systematic random sampling and stereology methods previously described (Mayhew 1991, 2011). In brief, sections were selected using systematic random sampling (ensuring a minimum of 200 μm between samples to avoid double cell counting). Photomicrographs were captured from each section in a systematic random manner before stereological counting and measurements being undertaken ($n = 5$ sections per sample for cell counts and proportions and $n = 3$ sections per sample for seminiferous tubules and blood vessel measurement). This technique ensured unbiased measurements throughout the tissue. In the fetal testis every testicular cell was identified and manually counted on a total of 420 photomicrographs. Seminiferous tubule numbers and dimensions ($n = 315$ photomicrographs, 20× magnification) and capsular and parenchymal blood vessels ($n = 5670$, photomicrographs at 10× and 40× magnification respectively) were measured manually using an image analysis program (Image-Pro Plus, Version 6.3; Media Cybernetics; n: HH = 5, HL = 7, LH = 5, LL = 4). In the adult testis seminiferous tubules and blood vessels were measured using the same image analysis program ($n = 400$ micrographs, 10× magnification and $n = 800$ photomicrographs, 20× magnification respectively; n: HH = 10, HL = 14, LH = 8, LL = 8). Following calibration of the imaging software, tubules and vessels were manually circumscribed and the average number of tubules and blood vessels per tissue area (referred to throughout as 'number' of blood vessels or seminiferous tubules) was calculated. In addition, the blood vessel and seminiferous tubule areas occupied per tissue section were calculated; this is referred to as blood vessel or tubule 'area' throughout. Importantly all samples were fixed, processed and sectioned in the same manner so that groups could be directly compared.

Animal measures

Liveweight and scrotal circumference

Heifers were visually monitored 24 h a day throughout calving. Progeny birthweight was recorded within 15 min of birth and before first suckling. Liveweight was recorded monthly from birth. Scrotal circumference was assessed monthly from 214.3 ± 0.8 days of age (after weaning) using the Australian Cattle Veterinarians recommended procedure (Beggs *et al.* 2013) with a Reliabull scrotal measuring tape (Lane Manufacturing Inc.).

Blood sampling

Progeny blood samples were collected approximately monthly from weaning until slaughter at 20 months of age. Prior to the commencement of other procedures, samples of whole blood were collected by venipuncture directly into Vacutainer tubes containing lithium–heparin (Becton, Dickinson and Co.). Tubes were rotated by hand for 5 to 10 s and stored on ice before centrifugation (Eppendorf 5702R; Eppendorf Zentrifugen GMBH) at 3000*g* for 10 min at 4°C within 90 min of collection. Plasma was harvested then stored frozen at −80°C until analysis.

Assays

Plasma concentrations of FSH, leptin, IGF1, testosterone, anti-Müllerian hormone (AMH), inhibin and activin A were assayed as detailed.

Plasma FSH was measured in duplicate by a double-antibody radioimmunoassay (Atkinson and Adams 1988) using NIAMDD-oFSH-RP-1 (biopotency 75× NIH-FSH-S1) and NIADDK-anti-oFSH-1 serum. The intra-assay coefficients of variation were 5.7%, 2.7% and 4.4% for control plasma with means of 1.27 ng mL^{-1}, 2.25 ng mL^{-1} and 3.15 ng mL^{-1} respectively. The limit of detection was 0.15 ng mL^{-1}. As the sample levels were 3–4 times higher than the limit of detection they were read in the linear part of the standard curve.

Plasma was assayed for leptin in duplicate by a double-antibody radioimmunoassay (RIA; Blache *et al.* 2000) with samples processed in a single assay. The assay included six replicates of three control samples containing 0.29, 0.71 and 1.68 ng mL^{-1}, which were used to estimate the intra-assay coefficients of variation of 5.4%, 4.4% and 6.6%. The limit of detection was 0.05 ng mL^{-1}.

Plasma testosterone was assayed in duplicate using the reagents of the Immunochem double antibody testosterone RIA kit (MP Biomedical Australia) following the manufacturer's protocol and validated using a serial dilution of two bovine samples. The intra-assay coefficients of variation for quality control samples containing 0.26 ng mL^{-1} and 2.3 ng mL^{-1} were 6.5% and 2.9% respectively. The lowest and highest limits of detection were 0.07 ng mL^{-1} and 6.5 ng mL^{-1} respectively.

Plasma was assayed for IGF1 in duplicate by double-antibody radioimmunoassay with human recombinant IGF1 (ARM4050; Amersham-Pharmacia Biotech) and anti-human IGF1 antiserum (AFP4892898; National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases) following acid–ethanol extraction and cryo-precipitation (Breier *et al.* 1991). The assay was previously validated for bovine samples (Chagas *et al.* 2007). Samples were processed in a single assay. The intra-assay coefficients of variation for control samples containing 51.6 ng mL^{-1} and 253.6 ng mL^{-1} were 6.2% and 5.9% respectively. The limit of detection was 0.1 ng mL^{-1}.

AMH levels were determined using a bovine AMH enzyme-linked immunosorbent assay (ELISA) kit (Ansh Laboratories)

following the manufacturer's protocol. Samples were diluted 15 times using the sample diluents provided in the kit. The intra-assay coefficients of variation for quality control samples containing 290.8 pg mL^{-1} and 875.0 pg mL^{-1} were 2.6% and 3.7% respectively. The limit of detection was 28.4 pg mL^{-1}.

Bovine inhibin levels were measured at the Hudson Institute of Medical Research using a radioimmunoassay employing a rabbit antiserum raised against the α-subunit of bovine inhibin (McLachlan R.I. *et al.* 1986), which detects both inhibin A and B proteins and free inhibin α-subunit (including pro-α-C) in multiple species. Iodinated human recombinant 31-kDa inhibin was used as tracer and 31-kDa human recombinant inhibin was used as standard. Goat anti-rabbit IgG (GAR#12; Hudson Institute) was used as second antibody. The assay has been validated for measurement of inhibin in bovine serum samples and values (in ng mL^{-1}) are expressed relative to the purified human inhibin standard. The intra-assay coefficient of variation was 6.2% and the lowest and highest limits of detection were 0.26 ng mL^{-1} and 8.73 ng mL^{-1} respectively (based on effective dose (ED) 90 and ED10 values).

Total bovine serum activin A concentrations were measured at the Hudson Institute of Medical Research employing a two-site enzyme immunoassay specific for activin A (Knight *et al.* 1996) modified and validated for measurement of bovine serum samples. Human recombinant activin A, which is identical in sequence to bovine activin A, purified as described previously from material provided by Biotech Australia Pty Ltd (Robertson *et al.* 1992), was used as the standard. Values (in pg mL^{-1}) are expressed relative to the purified activin A standard. The mean intra- and inter-assay coefficients of variation for three plates were 5.4% and 7.3% respectively. The lowest and highest limits of detection were 8.84 pg mL^{-1} and 1984 pg mL^{-1} (2 s.d.) respectively.

Semen collection

Semen collection commenced in spring at approximately monthly intervals from 10 months of age until slaughter at 20 months of age. After preliminary stimulation of the ampulla via rectal massage, semen was collected using a standard electro-ejaculation technique (Lane Pulsator IV; Lane Manufacturing Inc.) as previously described (McAuliffe *et al.* 2010; Beggs *et al.* 2013). If an animal did not produce a satisfactory sample within several minutes following electrostimulation, the animal was released and a single further attempt was made after a 10-min interval (Callaghan *et al.* 2016).

Assessment of semen traits was undertaken immediately following collection using established methodology and standards (Entwistle and Fordyce 2003; Fordyce *et al.* 2006) by the same technician blinded to treatment. Briefly, ejaculate density was scored immediately following collection using a 1 (clear to cloudy) to 5 (creamy) scale. A drop of semen was placed on a pre-warmed glass slide (37°C) with a plastic transfer pipette (1 mL) and assessments made of motility (%) and mass motility (or wave motion) using a phase-contrast microscope. Motility was estimated as the percentage of spermatozoa that were progressively motile under their own propulsion (viewed at 400× magnification). Mass motility was assessed under 40× magnification on a 1 (no swirl) to 5 (fast distinct swirl with continuous dark waves) scale (Burns *et al.* 2013; Corbet *et al.* 2013). Animals that did not produce an ejaculate were assigned a value of zero for density, motility and mass motility (Corbet *et al.* 2013). Semen (0.1 mL) was diluted with phosphate-buffered formal saline (4.9 mL) for sperm concentration assessment, with spermatozoa counted in a haemocytometer (Perry *et al.* 1990).

Semen (1 to 2 drops) was placed into phosphate-buffered formal saline (1.0 mL) for assessment of sperm morphology. The morphology of 100 individual spermatozoa in each sample considered to contain sufficient spermatozoa for examination (Burns *et al.* 2013) was assessed using 1000× magnification under differential interference contrast microscopy by an Australian Cattle Veterinarians accredited sperm morphologist blinded to treatment at a commercial third-party pathology laboratory. Morphology traits were individually recorded based on the sperm abnormality format as described (Fordyce *et al.* 2006). The sperm abnormality categories included midpiece abnormalities, knobbed acrosomes, proximal cytoplasmic droplets, abnormal tails and loose heads, pyriform heads, vacuoles and teratoid spermatozoa and swollen acrosomes (Fordyce *et al.* 2006). Total remaining normal spermatozoa were noted as percentage normal spermatozoa per ejaculate at each time point (Entwistle and Fordyce 2003).

Determination of pubertal age and sexual maturity

The threshold used for age at puberty was defined as the first time an ejaculate contained a semen concentration of $\geq 50 \times 10^6$ spermatozoa mL^{-1} with ≥10% motile spermatozoa (Wolf *et al.* 1965). Sexual maturity was characterised as the first time an ejaculate contained ≥70% morphologically normal spermatozoa with semen concentration $\geq 50 \times 10^6$ spermatozoa mL^{-1} (Brito *et al.* 2004).

Statistical analysis

Data were checked for normality and transformed before analysis if required. Data for maternal liveweight, maternal average daily gain, fetal weight, fetal testis weight, testicular cell development, gestation length paired testis weight, age of puberty, age of maturity, inhibin, activin A and AMH were analysed using two-way ANOVA (STATA 13.1; Stata Corp College Station) to determine the effects of maternal diet during PERI and POST periods and their interaction term. Significant interactions were explored with Tukey–Kramer post hoc test as required.

To investigate the interactions between maternal diet (PERI and POST) and time, hormone concentrations (leptin, FSH, IGF1, testosterone), sperm traits and scrotal circumference, linear mixed-effects models were performed, adjusting for repeated measures over time for each of the 40 calves. An autoregressive (1) covariance structure was used as it provided the best fitting model compared with other structures. For sperm morphological abnormalities, generalised estimating equation (GEE) models with a Poisson distribution were performed, adjusting for repeated measures over time for each of the 40 calves. Post hoc comparisons were made for each model: differences of least-squares means for the linear mixed-effects

models and incidence rate ratios (IRR) for the Poisson GEE models. The statistical software used was SAS 9.3 (SAS Institute Inc.). There were no significant interactions between maternal diet during PERI and POST periods for the variables investigated unless expressly stated in the results. Thus, for clarity, the results have been presented as the main effects of PERI and POST maternal diet. Statistical significance is reported at $P < 0.05$ and tendency at $P < 0.10$.

Results

Maternal liveweight

At both the commencement and end of the PERI maternal diet period (−60 to 23 dpc) and the POST maternal diet period (24 to 98 dpc), the liveweights of the heifers were similar (Tables 2 and 3; all $P > 0.10$). There was no interaction of the PERI and POST diet ($P = 0.475$) on liveweight at the end of the POST maternal diet period. Average daily weight gain (ADG) during the PERI diet period was lower ($P < 0.001$) for the LPeri (LL + LH) heifers compared with the HPeri (HH + HL) heifers. ADG during the POST maternal diet period did not differ between LPost (LL + HL) and HPost (HH + LH) groups ($P = 0.164$), nor was there a diet interaction ($P = 0.482$). Heifers that had received the LPeri diet had higher ADG during the POST diet period compared with those that received the HPeri diet (0.36 ± 0.04 vs 0.17 ± 0.04 kg per head per day; $P = 0.002$).

From the end of the POST diet period to late gestation (99 dpc to 256 dpc), during which time all dams received the same diet, maternal liveweights did not differ due to PERI or POST diet, nor was there a diet interaction (all $P > 0.10$; LPeri 506.4 ± 7.3 vs HPeri 507.2 ± 6.3 kg and LPost 507.3 ± 6.1 vs HPost 506.3 ± 7.6 kg). ADG also did not differ (all $P > 0.10$; LPeri 0.63 ± 0.02 vs HPeri 0.64 ± 0.02 and LPost 0.65 ± 0.02 vs HPost 0.61 ± 0.02 kg per head per day). Immediately after calving, a similar pattern was observed whereby maternal liveweights did not differ due to PERI or POST maternal diet, nor was there a diet interaction (all $P > 0.10$; LPeri 456.6 ± 8.3 vs HPeri 464.2 ± 7.7 and LPost 468.6 ± 7.1 vs HPost 452.4 ± 8.5 kg).

Fetal and animal measures

Fetal and gonad weight at 98 dpc

As previously reported (Copping *et al.* 2014), male fetuses from LPost dams were lighter at 98 dpc compared with males from HPost dams (see Table S1, available as Supplementary Material to this paper; $P < 0.05$). There was no effect of PERI diet or the diet interaction term on male fetal weight at 98 dpc. Maternal diet did not influence absolute gonad weight or relative gonad weight at 98 dpc (Table S1).

Birthweight and post-weaning growth

At birth there was no effect of maternal diet upon birthweight or gestation length (Table S2; $P > 0.05$). Increased gestation length was associated with increased birthweight ($r = 0.475$; $P < 0.001$). From weaning until slaughter (600 days), liveweight increased with age ($P < 0.0001$) but did not vary due to maternal diet (data not shown; $P > 0.10$).

Table 2. Maternal liveweight and average daily gain (ADG) at start and end of exposure to diets low (L) or high (H) in protein during the PERI-conception (−60 to 23 dpc) period

Data are mean ± s.e.m. Values with different superscripts differ significantly ($P < 0.05$). LL, low protein maternal diets peri- and post conception; LH, low protein maternal diet in the peri-conception period and high protein post conception; HL, high protein maternal diet in the peri-conception period and low protein post conception; HH, high protein maternal diets peri- and post conception

Parameter	LPeri (LL + LH)	HPeri (HH + HL)
n	16	24
Start weight (kg)	345.6 ± 5.5	335.6 ± 5.3
End weight (kg)	382.9 ± 6.5	395.3 ± 5.5
ADG (kg per head per day)	0.40 ± 0.03^{a}	0.64 ± 0.02^{b}

Table 3. Maternal liveweight and average daily gain (ADG) at start and end of exposure to diets low (L) or high (H) in protein during the POST-conception (24 to 98 dpc) period

Data are mean ± s.e.m. LL, low protein maternal diets peri- and post conception; LH, low protein maternal diet in the peri-conception period and high protein post conception; HL, high protein maternal diet in the peri-conception period and low protein post conception; HH, high protein maternal diets peri- and post conception

Parameter	LPost (LL + HL)	HPost (HH + LH)
n	22	18
Start weight (kg)	391.4 ± 5.5	389.0 ± 6.9
End weight (kg)	405.7 ± 5.0	410.6 ± 5.8
ADG (kg per head per day)	0.20 ± 0.04	0.30 ± 0.04

Scrotal circumference

Scrotal circumference in all treatment groups increased with age (see Fig. S1, available as Supplementary Material to this paper; $P < 0.0001$). There was no overall effect of either maternal diet or gestation length upon progeny scrotal circumference measurements between 214 and 554 days of age (Fig. S1; all $P > 0.10$).

Semen traits

Semen quality parameters

There were maternal nutrition and time effects on a range of semen quality parameters (Fig. 1). There were effects of time ($P < 0.0001$) and PERI maternal diet ($P = 0.0433$) on mass motility, such that bulls from LPeri dams had lower semen mass motility scores compared with bulls from HPeri dams (Fig. 1*a*). There were interactions between POST maternal diet and time for mass motility ($P = 0.0181$), such that bulls from LPost dams had increased mass motility compared with bulls from HPost dams at 554 days of age (Fig. 1*b*; $P = 0.0433$) and tended to be higher at 351 ($P = 0.08$) days of age. There were effects of time

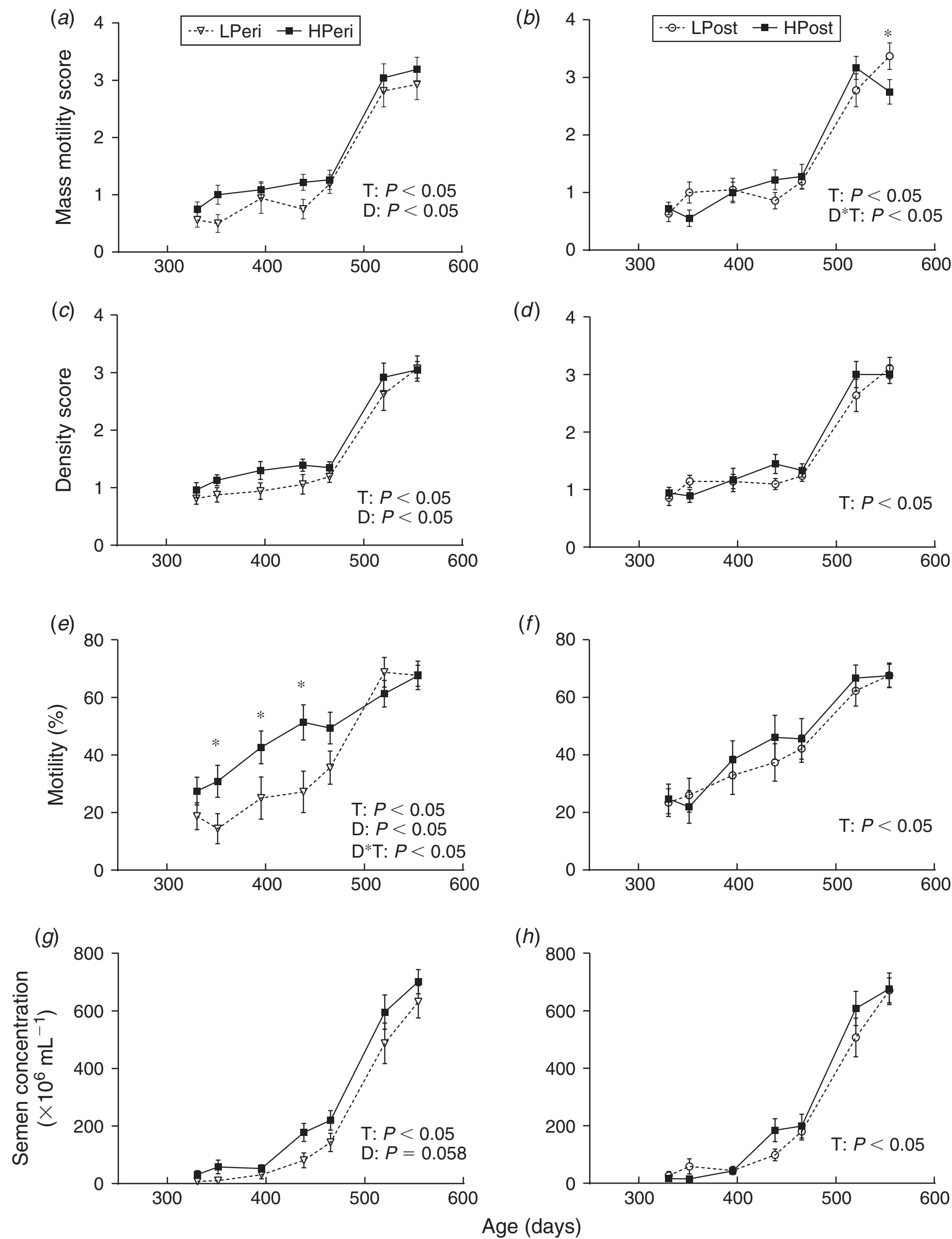

Fig. 1. Semen quality parameters in male progeny between 330 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (*a*, *c*, *e*, *g*) PERI-conception period (−60 to 23 dpc) or (*b*, *d*, *f*, *h*) or POST-conception period (24 to 98 dpc). Data are mean ± s.e.m. D, T, D × T: maternal diet, time and maternal diet-by-time interaction effects respectively. *Values with asterisks indicate significant differences between groups within age; $P < 0.05$.

($P < 0.001$) and PERI maternal diet on semen density (Fig. 1*c*) and sperm motility (Fig. 1*e*). Overall, bulls from LPeri dams had lower sperm density (Fig. 1*c*; $P = 0.04$) and motility (Fig. 1*e*; $P = 0.0217$) compared with bulls from HPeri dams. There was an interaction between PERI maternal diet and time for the motility parameter (Fig. 1*e*; $P = 0.0124$). Bulls from LPeri dams produced ejaculates with reduced motility at 351 ($P = 0.03$), 395 ($P = 0.024$) and 438 ($P = 0.0024$) days of age and tended to have reduced motility at 465 ($P = 0.08$) days of age compared with bulls from HPeri dams. Overall, there were effects of time ($P < 0.001$) on semen concentration and concentration tended to be lower in bulls from LPeri dams (Fig. 1*g*; $P = 0.058$) but there was no interaction of maternal diet and age. The POST maternal diet did not influence density (Fig. 1*d*), motility

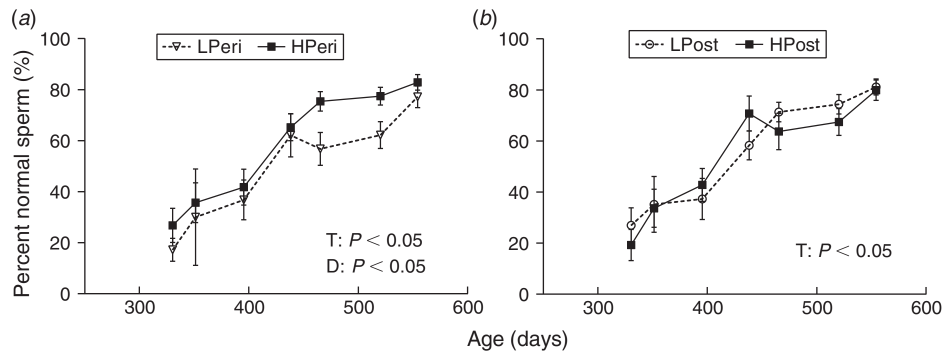

Fig. 2. Percentage normal spermatozoa in male progeny between 330 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (*a*) PERI-conception period (−60 to 23 dpc) or (*b*) POST-conception period (24 to 98 dpc). Data are mean ± s.e.m. D, T: maternal diet and time effects respectively. No significant differences between groups within age ($P > 0.05$).

(Fig. 1*f*) or concentration parameters (Fig. 1*h*), nor was there any difference in any semen quality parameters due to the diet interaction (all $P > 0.05$).

Sperm morphology

There were effects of time ($P < 0.0001$) and PERI maternal diet ($P = 0.0208$) on percentage normal spermatozoa (Fig. 2). Overall, the percentage of normal spermatozoa was lower in bulls from LPeri dams (Fig. 2*a*) compared with HPeri. The reduction in percentage normal spermatozoa within LPeri bulls was consequent to increased levels of sperm abnormalities (Fig. 3).

Specifically, a higher overall incidence of abnormal mid-pieces and knobbed acrosome defects (Fig. 3*a*, *c*; $P < 0.05$) were observed in ejaculates from LPeri bulls. There were interactions between PERI maternal diet and time (Fig. 3*c*; $P = 0.0043$) for knobbed acrosomes with a higher incidence of this defect in ejaculates from LPeri bulls at 438 (IRR = 4.27; $P = 0.0061$), 465 (IRR = 3.60; $P = 0.0156$) and 520 (IRR = 5.39; $P = 0.005$) days of age. There were also interactions between PERI maternal diet and time for abnormal tail and loose head defects (Fig. 3*e*; $P = 0.0024$) such that bulls from LPeri dams produced ejaculates with a higher incidence of abnormal tails and loose heads compared with bulls from HPeri dams at 465 (IRR = 1.87; $P = 0.0394$), 520 (IRR = 2.62; $P = 0.0039$) and 554 days of age (IRR = 2.41; $P = 0.0248$). There was an interaction between PERI maternal diet and age for vacuole and teratoid defects (Fig. 3*g*; $P = 0.0434$); however, there were no differences at any individual age. There was also an interaction between POST maternal diet and time for proximal droplet defects (Fig. 3*j*; $P = 0.0032$); however, once again there were no differences at any individual age. Overall, POST maternal diet increased the incidence of swollen acrosome defects, which was higher overall in ejaculates from bulls with LPost dams than bulls from HPost dams ($P = 0.0352$). POST diet did not influence the incidence of any other sperm defect, nor was there any difference in any sperm defect due to the diet interaction (all $P > 0.05$). There were effects of age overall (Fig. 3; all $P < 0.0001$) for all defects reported. (Data not shown for swollen acrosome and pyriform head defects).

Puberty

Puberty was first reached by a bull at 329 days of age with the final bull reaching the threshold by 521 days of age. Puberty was achieved later in LPeri bulls compared with HPeri bulls (Table 4; $P = 0.049$). There was no difference in puberty due to POST maternal diet or the diet interaction (Table 4; $P > 0.05$).

Sexual maturity

Maturity as assessed using the minimum threshold of 70% normal spermatozoa was not achieved by 17.5% ($n = 7$: LPeri = 4; HPeri = 3; LPost = 4; HPos*t* = 3) of the bulls in this study. The first bull reached the threshold at 330 days of age. Of those bulls that achieved maturity ($n = 33$), there was a tendency for bulls from LPeri dams to reach maturity later than bulls from HPeri dams (466.9 ± 19.0 vs 425.9 ± 12.2 days of age; $P = 0.079$). There were no differences due to POST maternal diet (LPost 435.3 ± 15.6 vs HPost 448.0 ± 15.2 days of age; $P > 0.10$) or the diet interaction ($P > 0.05$).

Paired testes weight

The absolute and relative weights of the paired testes were similar between maternal diet groups at slaughter at 598.3 ± 0.8 days of age (Table S2; $P > 0.05$). Total paired testis weight at slaughter was highly correlated with the final scrotal circumference (Table S2) measured at 554.3 ± 0.8 days ($r = 0.82$; $P < 0.05$) irrespective of maternal diet.

Hormone concentrations

Circulating inhibin and activin A concentrations measured at 3 and 4 months of age were not influenced by either PERI or POST maternal diet (Table 5; $P > 0.10$) or the diet interaction ($P > 0.05$). However, circulating AMH concentrations at 10 months of age were higher in bulls from LPeri dams (Table 5; $P = 0.04$) compared with HPeri bulls and tended to be higher in bulls from LPost dams compared with HPost bulls (Table 5; $P = 0.09$).

There were overall effects of time ($P < 0.001$) on plasma FSH (Fig. 4), IGF1 (Fig. 5) and leptin levels (Fig. 5). Time also tended to influence plasma testosterone concentration (Fig. 4;

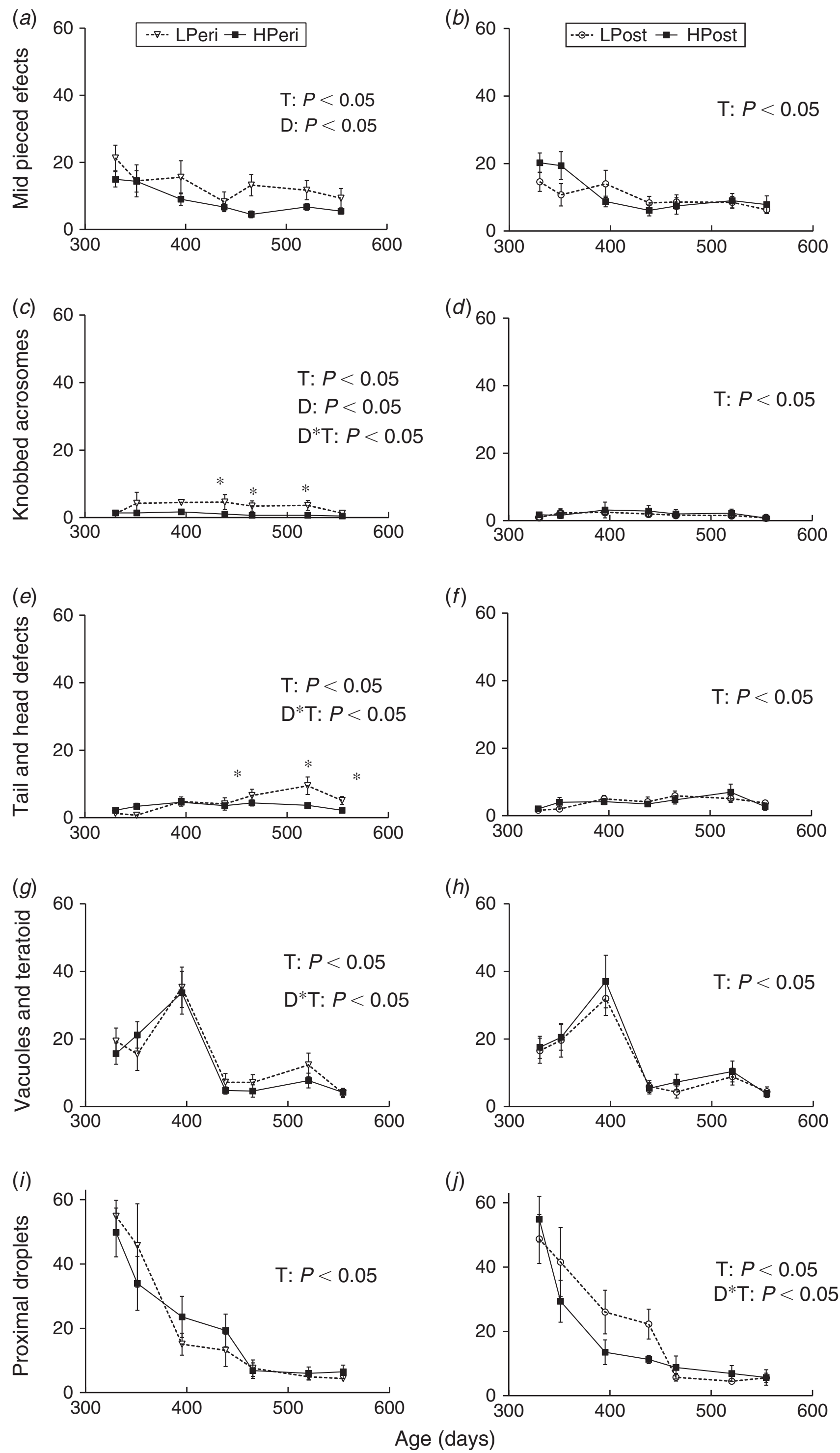

Fig. 3. Sperm morphology defects (count per 100 spermatozoa) in male progeny between 330 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (*a*, *c*, *e*, *g*, *i*) PERI-conception (−60 to 23 dpc) or (*b*, *d*, *f*, *h*, *j*) POST-conception period (24 to 98 dpc). Data are mean ± s.e.m. D, T, D × T: maternal diet, time and maternal diet-by-time interaction effects respectively. *Values with asterisks indicate significant differences between groups within age; $P < 0.05$.

Table 4. Age of puberty in male progeny following exposure to maternal diets low (L) or high (H) in protein during the PERI- (−60 to 23 dpc) and POST-conception (24 to 98 dpc) periods

Data are mean ± s.e.m. Values with different superscripts differ significantly ($P < 0.05$)

Parameter	LPeri (LL + LH)	HPeri (HH + HL)	LPost (LL + HL)	HPost (HH + LH)	*P* value Peri	Post	Peri*Post
n	16	24	22	18			
Age (days)[A]	436.4 ± 10.8[a]	403.3 ± 11.3[b]	414.9 ± 10.9	419.2 ± 13.1	0.049	0.808	0.503

[A]Age at which sperm motility ≥10% and semen concentration ≥50 × 10^6 spermatozoa mL^{-1}.

Table 5. Peripheral inhibin ($ng\,mL^{-1}$) and activin A ($pg\,mL^{-1}$) levels at 3 and 4 months of age and anti-Müllerian hormone (AMH; $ng\,mL^{-1}$) at 10 months of age in male progeny following exposure to maternal diets low (L) or high (H) in protein during the PERI- (−60 to 23 dpc) and POST-conception (24 to 98 dpc) periods

Data are mean ± s.e.m. Values with different superscripts differ significantly ($P < 0.05$)

Parameter	LPeri (LL + LH)	HPeri (HH + HL)	LPost (LL + HL)	HPost (HH + LH)	*P* value Peri	Post	Peri*Post
n	16	24	22	18			
3 months							
Age (days)	124.7 ± 1.4	125.7 ± 0.9	126.4 ± 1.1	124.0 ± 1.0			
Inhibin ($ng\,mL^{-1}$)	7.0 ± 0.3	7.2 ± 0.3	7.1 ± 0.3	7.0 ± 0.3	0.550	0.697	0.177
Activin A ($pg\,mL^{-1}$)	38.1 ± 1.0	43.3 ± 2.2	42.0 ± 2.4	40.3 ± 1.7	0.178	0.955	0.060
4 months							
Age (days)	153.7 ± 1.4	154.7 ± 0.9	155.4 ± 1.1	153.0 ± 1.0			
Inhibin ($ng\,mL^{-1}$)	7.7 ± 0.2	7.6 ± 0.2	7.7 ± 0.2	7.5 ± 0.2	0.720	0.618	0.534
Activin A ($pg\,mL^{-1}$)	36.8 ± 2.0	35.6 ± 1.4	37.2 ± 1.6	34.8 ± 1.8	0.741	0.238	0.110
10 months							
Age (days)	301.7 ± 1.4	302.7 ± 0.9	303.4 ± 1.1	301.0 ± 1.0			
AMH ($ng\,mL^{-1}$)	18.5 ± 0.4[a]	17.3 ± 0.4[b]	18.2 ± 0.4	17.3 ± 0.5	0.039	0.090	0.550

$P = 0.09$). There were interactions between PERI maternal diet and time for FSH (Fig. 4*a*; $P = 0.0435$) such that LPeri bulls had lower circulating FSH at 330 ($P = 0.0317$) and 438 ($P = 0.0147$) days of age and tended to have lower levels at 273 ($P = 0.06$) and 302 ($P = 0.09$) days of age. There were also interactions between POST maternal diet and time for IGF1 (Fig. 5*b*; $P = 0.0127$) such that LPost bulls had higher circulating IGF1 at 465 days of age ($P = 0.004$) compared with HPost bulls. There were no main effects overall of PERI or POST maternal diet or their interaction term on FSH, testosterone, IGF1 or leptin concentrations ($P > 0.10$).

Testis development

The proportions of testicular cells (Sertoli, germ, interstitial/Leydig cells) in 98 dpc fetuses were not altered either by the PERI or POST maternal diet or their interaction term (Table S3; Fig. S2; $P > 0.05$). Seminiferous tubule and blood vessel parameters were altered by dietary treatment (Tables 6 and 7). A higher proportion of seminiferous tubules within the testis (Table 6; $P = 0.04$) due to a greater number of tubules within the tissue (Table 6; $P = 0.04$) were observed in the LPeri diet fetal gonad compared with the HPeri gonad. There were no observed effects of maternal diet in the adult progeny in seminiferous tubule parameters (Table 6; $P > 0.05$).

The LPost fetal gonad displayed decreased numbers of blood vessels within the capsule of the testis (Table 7; $P = 0.02$) whilst the tissue area of blood vessels within the parenchyma of the testis (Table 7; $P = 0.03$) was decreased in the LPeri fetal gonad compared with the HPeri. In the adult testis, the number of blood vessels was increased by the LPost maternal diet (Table 6; $P = 0.03$).

Discussion

This study is the first to our knowledge to investigate the effects of maternal dietary protein during the peri-conception period and early gestation upon bovine male reproductive development. We examined this during fetal development and postnatally through to adulthood. The key findings were that the LPeri dietary treatment in nulliparous heifers altered reproductive development of their male progeny in the early postpubertal period as reflected by differences in reproductive hormones, testicular cytology and sperm production with a subsequent delay in reaching puberty. Increasing protein intake in the peri-conception period may therefore be viable for bull producers as the ability to use yearling bulls reduces production costs and shortens the genetic interval (Barth and Ominski 2000).

Decreased protein intake during early gestation reduced early fetal growth (Copping *et al.* 2014). This *in utero* effect

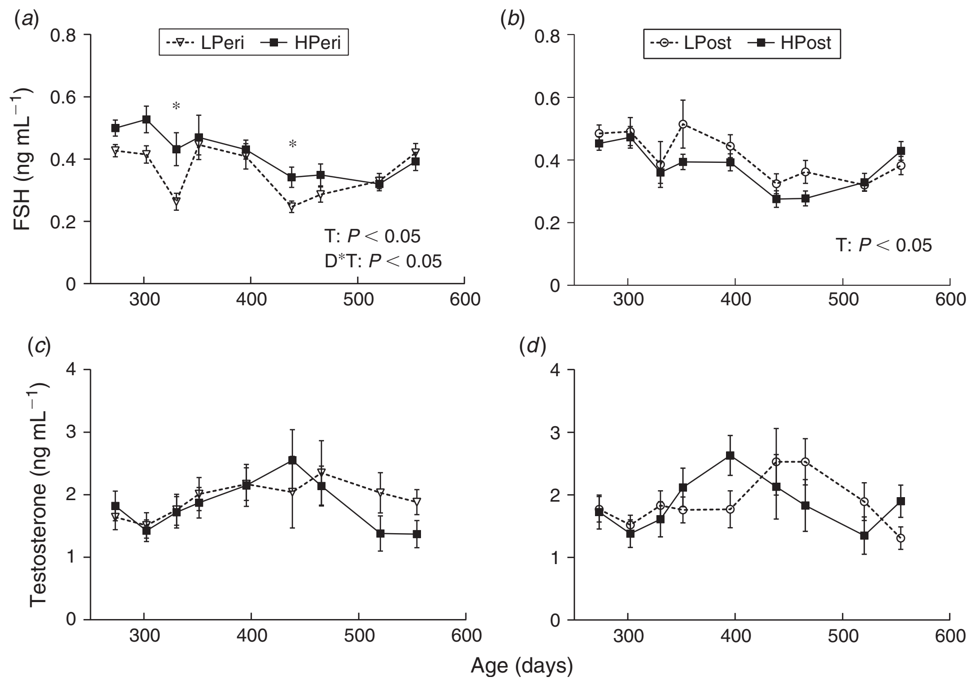

Fig. 4. Circulating concentrations of FSH (ng mL^{-1}) and testosterone (ng mL^{-1}) in male progeny between 273 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (*a*, *c*) PERI-conception period (−60 to 23 dpc) or (*b*, *d*) POST-conception period (24 to 98 dpc). Data are mean ± s.e.m. T, D × T: time and maternal diet-by-time interaction effects respectively. *Values with asterisks indicate significant differences between groups within age; $P < 0.05$.

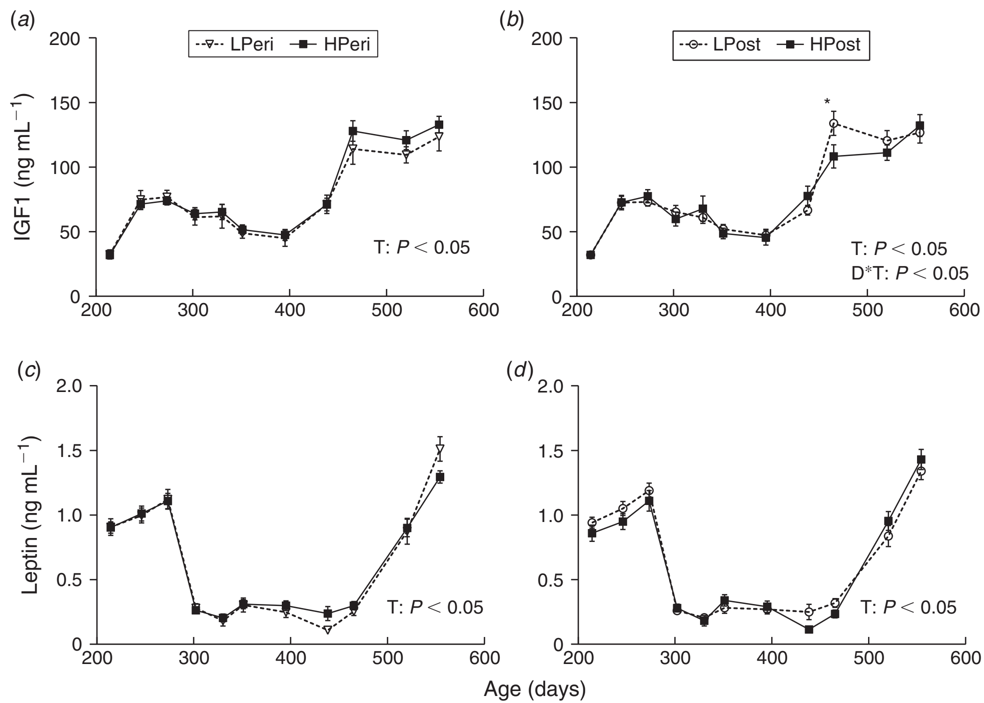

Fig. 5. Circulating concentration of IGF1 (ng mL^{-1}) and leptin (ng mL^{-1}) in male progeny between 214 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (*a*, *c*) PERI-conception period (−60 to 23 dpc) or (*b*, *d*) POST-conception period (24 to 98 dpc). Data are mean ± s.e.m. T, D × T: time and maternal diet-by-time interaction effects respectively. *Values with asterisks indicate significant differences between groups within age; $P < 0.05$.

Table 6. Area, number and percentage coverage of seminiferous tubules and blood vessels in 98-dpc fetus and adult (20-month-old) bulls following exposure to maternal diets low (L) or high (H) in protein during the PERI- (−60 to 23 dpc) and POST-conception (24 to 98 dpc) periods

Data are mean ± s.e.m. Values with different superscripts differ significantly ($P < 0.05$). Sem tubule, seminiferous tubule; BV, blood vessels. Area = average of total area of tissue occupied by blood vessel or tubule

Parameter	LPeri	HPeri	LPost	HPost	*P* value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
Fetal							
n	9	12	11	10			
Sem tubule area (μ^2)	2115.9 ± 214.0	2691.3 ± 653.7	2560.0 ± 723.0	2317.8 ± 200.1	0.691	0.286	0.421
Sem tubule no.	27.1 ± 2.7[a]	20.3 ± 2.0[b]	24.6 ± 2.8	21.8 ± 2.2	0.035	0.166	0.088
Sem tubule %	21.8 ± 1.0[a]	17.9 ± 1.4[b]	18.5 ± 1.7	20.7 ± 0.9	0.041	0.844	0.320
BV area (μ^2)	1042.8 ± 87.4[a]	1457.0 ± 154.1[b]	1221.7 ± 124.1	1343.0 ± 176.3	0.032	0.466	0.139
BV no.	83.6 ± 9.0	65.2 ± 7.6	74.9 ± 8.8	71.07 ± 8.51	0.134	0.531	0.435
BV %	1.9 ± 0.3	2.0 ± 0.3	2.2 ± 0.4	1.7 ± 0.3	0.913	0.318	0.490
Adult							
n	16	24	22	18			
Sem tubule area (μ^2)	3842.3 ± 198.5	3901.1 ± 142.7	3877.2 ± 175.7	3878.0 ± 145.5	0.713	0.890	0.199
Sem tubule no.	15.1 ± 0.5	15.5 ± 0.4	15.6 ± 0.5	15 ± 0.3	0.585	0.611	0.408
Sem tubule %	54.4 ± 1.2	57.0 ± 1.1	56.7 ± 1.2	55.1 ± 1.1	0.146	0.325	0.319
BV area (μ^2)	2451.2 ± 973.8	962.8 ± 229.2	1237.1 ± 486.0	1950.5 ± 731.4	0.704	0.548	0.653
BV no.	2.3 ± 0.2	2.4 ± 0.1	2.6 ± 0.2[c]	2.1 ± 0.1[d]	0.377	0.025	0.621
BV %	2.1 ± 0.7	1.1 ± 0.2	1.4 ± 0.3	1.6 ± 0.5	0.928	0.796	0.828

Table 7. Area, number and proportion of blood vessels within the parenchyma and capsule in 98-dpc fetus following exposure to maternal diets low (L) or high (H) in protein during the PERI- (−60 to 23 dpc) and POST-conception (24 to 98 dpc) periods

Data are mean ± s.e.m. Values with different superscripts differ significantly ($P < 0.05$). BV, blood vessel

Parameter	LPeri	HPeri	LPost	HPost	*P* value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
n	9	12	11	10			
Fetal BV capsule							
BV area	1620.4 ± 163.0	2125.0 ± 302.2	1858.3 ± 287.4	1964.1 ± 261.0	0.273	0.549	0.833
BV no.	73.4 ± 8.0	54.7 ± 7.1	50.5 ± 5.7[c]	76.3 ± 8.2[d]	0.130	0.021	0.495
BV %	9.9 ± 1.1	9.5 ± 1.0	9.0 ± 1.1	10.6 ± 0.9	0.901	0.300	0.719
Fetal BV parenchyma							
BV area	480.6 ± 39.4[a]	715.6 ± 84.6[b]	573.0 ± 68.9	660.9 ± 92.9	0.027	0.309	0.367
BV no.	11.3 ± 2.4	9.6 ± 1.5	10.2 ± 1.8	10.5 ± 2.0	0.540	0.882	0.200
BV %	0.11 ± 0.02	0.16 ± 0.03	0.13 ± 0.02	0.14 ± 0.04	0.328	0.986	0.328

was, however, not discernible in later gross measures such as birthweight or postnatal growth as previously reported in lambs (Kotsampasi *et al.* 2009) or calves (Micke *et al.* 2015) but effects upon postnatal reproductive development were evident: the LPeri diet decreased blood vessel area in the fetal testis. Moreover, seminiferous tubule number and percentage was increased, although this effect was not evident in the adult. In the developing bull, the LPeri maternal diet lowered sperm quality with this effect occurring after lower FSH concentrations in this group at both 330 and 438 days of age compared with the HPeri group.

Nutrition

Variations in natural feed resources in extensive farming systems are common in many countries. In the northern Australian rangelands, protein, rather than energy, is often the major limiting nutrient (Norman 1963) with protein supplementation of replacement heifers a common practice (Bortolussi *et al.* 2005; Burns *et al.* 2010). The dietary protein levels used in the present study therefore reflected pasture conditions in Australian rangelands without (low) and with (high) protein supplement. There was a 1.9- to 2.1-fold difference in crude protein (CP) content and a 1.1-fold difference in energy content between the high and low diets. The ration was as isocaloric as possible for ruminants fed the forage component of the diet under group housing. Dietary fat content was similar and although starch content differed, levels in both low and high diets were moderately low. Protein intake was restricted during both the periconception period and first trimester in the low group whilst both groups received similar energy intake. As the variation in

CP content between the high and low diets was much greater than that in energy, we therefore consider the differences observed in the present study are likely attributable to the effects of protein rather than energy intake.

Testis histology

The lack of effect upon Sertoli, germ and interstitial cells is in contrast to studies that reported a reduction in the number of Sertoli cells in newborn lambs undernourished *in utero* during the second trimester of gestation (Bielli *et al.* 2002; Kotsampasi *et al.* 2009) but concurs with studies that excised the testis at a fetal endpoint (Da Silva *et al.* 2003; Andrade *et al.* 2013).

The observed decrease in vasculature in the LPeri and LPost 98-dpc fetal testis is a novel finding and may reflect a mechanism whereby maternal protein restriction reduces male reproductive function as previously reported (Zambrano *et al.* 2005). Although the observed reduction in parenchymal blood vessel area and in the number of capsular blood vessels in the LPeri and LPost testis respectively was transient (suggesting a compensatory ability of either the fetal or pubertal testis), blood supply affects the physiological function of every organ. The testes are, however, particularly sensitive to alterations in vasculature as minor episodes of ischaemia lead to functional disturbances (Wrobel *et al.* 1981; Polguj *et al.* 2015). Furthermore, the capsule vasculature in ruminants, essential to metabolite and heat exchange (Godinho *et al.* 1973), was observed to be compromised in the LPost cohort. We have previously reported the long-term effects of this protein restriction model upon hypertension in the female cohort (Hernandez-Medrano *et al.* 2015). We propose that this transient vascular perturbation during this critical gestational phase (O'Shaughnessy and Fowler 2011) may lead to testicular oxidative stress as previously reported in a rat model following gestational protein restriction (Rodríguez-González *et al.* 2014). Interestingly this model of gestational protein restriction in the postnatal rat also led to long-term effects upon semen quality and morphology as we similarly report below.

Concomitantly, in the 98-dpc fetus, the LPeri diet caused an increment in the number of seminiferous tubules and the proportion of seminiferous tubules per testis but did not affect tubule area. In combination, these results may indicate that the differentiation and proliferation of testicular cells and the development of the seminiferous tubules is not linked to the development of the blood vessels during the first trimester.

In the adult bulls the number and proportion of seminiferous tubules were unaffected by the dietary regimes, further suggesting that compensation may occur during developmental stages after our dietary intervention either in late gestation or postnatally. A prior study observed reduced seminiferous tubule diameters in bull calves at 5 months of age after supplementation of their mothers' diets with protein (0 to 180 dpc; Sullivan *et al.* 2010). This suggests that compensatory mechanisms occur during the pubertal period.

Postnatal development

In this study an *in utero* LPeri diet increased the age at which bulls reach puberty predicated by the motility, morphology and concentration of spermatozoa produced in the ejaculate (Barth and Oko 1989; Perry *et al.* 1990; Holroyd *et al.* 2002). The higher levels of spermatozoa with non-progressive motility, the overall increased numbers of morphologically abnormal spermatozoa and the tendency for lower concentrations suggest that both epididymal function and spermatogenesis were delayed or disrupted by the LPeri maternal diet. As expected in pubertal bulls, the initial high level of proximal droplets in ejaculates decreased over time (Lunstra and Echternkamp 1982; Barth and Oko 1989; Perry *et al.* 1991; Evans *et al.* 1995) but was not altered by *in utero* diet. Midpiece defects and abnormal heads and tails were increased in the LPeri bulls; both defects are reported to be associated with disturbance of epididymal function (Barth and Bowman 1994). Knobbed acrosomes were similarly increased in the LPeri bulls at 438, 465 and 520 days of age indicating disturbed spermiogenesis during this peripubertal period (Barth and Bowman 1994; Beggs *et al.* 2013). In the present study, the bulls reached puberty at a similar age to that previously reported for *Bos indicus* × *Bos taurus* crossbred bulls (Chase *et al.* 2001; Brito *et al.* 2004) and intermediate to that reported for *Bos taurus* (Lunstra *et al.* 1978; Evans *et al.* 1995) and *Bos indicus* breeds (Fields *et al.* 1982; Aponte *et al.* 2005). The earlier age of puberty observed in the HPeri bull cohort is a desirable production outcome (Barth and Ominski 2000).

There was no effect of maternal dietary treatment upon scrotal circumference or paired testis weight at 600 days. These findings are in agreement with those in rams (6 weeks and 20 months of age) where Rae *et al.* (2002) reported no effect of maternal undernutrition on scrotal circumference. The absence of *in utero* dietary effect upon scrotal circumference concurs with the observed lack of effect upon fetal testis weight and Sertoli cell count at 98 dpc. Consequently, the effects of maternal protein restriction on sperm parameters and age of puberty were considered to be not directly the result of altered Sertoli cell numbers in the developing postnatal animal (Sharpe *et al.* 2003).

The effects on sperm parameters were, however, subsequent to lower FSH concentrations in the LPeri cohort; FSH is an integral part of the hormonal cascade regulating sperm production (Perry *et al.* 1991), epididymal function (Grover *et al.* 2005) and spermatogenesis in the mature bull (Barth and Bowman 1994; Matsuzaki *et al.* 2000; O'Shaughnessy 2014). In ruminants, no comparative studies have documented the associations between prenatal nutrition and sperm abnormalities in the progeny. The results are, however, consistent with those in the adult male rat (90 days of age) where Toledo *et al.* (2011) reported impairment of sperm counts, sperm motility and higher levels of spermatozoa with morphological abnormalities following *in utero* protein restriction (0–21 dpc).

The relationship between reduced FSH and delayed postnatal activation of the reproductive axis observed in the pubertal and postpubertal LPeri cohort concurs with previous research: FSH levels, along with LH, rise transiently between 1 and 4 months of age in the prepubertal bull (Rawlings *et al.* 1978; Evans *et al.* 1996; Moura and Erickson 1997; Kaneko *et al.* 2001; Bagu *et al.* 2006), a rise reported to be associated with the initiation of rapid testis growth (Moura and Erickson 1997). FSH levels then fall,

remaining low during peri-puberty and puberty (Moura and Erickson 1997; Kaneko *et al.* 2001; Brito *et al.* 2007*c*, 2007*d*). In the adult, FSH levels increase as bulls age in association with improvement in sperm quality and quantity (Matsuzaki *et al.* 2000). Thus, the observed lower basal FSH in the LPeri bulls during the pubertal and postpubertal period (330 and 438 days of age) may potentially indicate a hormonal regulation pathway contributing to the delayed elevation of sperm traits discussed above.

Collectively, the lack of effect of maternal diet on testosterone (Rawlings *et al.* 2008), prepubertal inhibin (Kaneko *et al.* 2001) and prepubertal activin A, (Mather *et al.* 1992), all known to be involved in regulation of postnatal FSH secretion in the developing bull, would suggest that the differences in FSH levels associated with the PERI diet were modulated via other pathways. Alternatively, the monthly blood sampling regimen may have been inadequate to detect the effects of maternal diet on testosterone, inhibin or activin A, particularly considering the pulsatile and diurnal nature of testosterone secretion.

The later age of puberty in the LPeri bulls was also associated with higher AMH levels at 10 months. This may suggest a delay in the downregulation of AMH expression that occurs at puberty (Rey and Josso 1996; Rey *et al.* 2003) coincident with Sertoli cell maturation (Sharpe *et al.* 2003). As circulating AMH levels decline sharply in the pubertal bull (Rota *et al.* 2002), it is possible the differences measured at one time point may reflect differences in maturity as opposed to resulting from the dietary perturbation. However, as birthweight and postnatal liveweights were similar, the observed effects on age of puberty are unlikely to have been mediated by the persisting influences of prenatal nutrition on postnatal growth (Micke *et al.* 2010). This is further supported by the lack of maternal dietary effect upon progeny IGF1 and leptin profiles; the relationship between energy homeostasis and puberty being well recognised (Blache *et al.* 2003; Barb and Kraeling 2004; Zieba *et al.* 2005; Brito *et al.* 2007*a*, 2007*c*, 2007*d*; Barth *et al.* 2008). Collectively these observations indicate that postnatal diet and liveweight were not involved in the observed changes in postnatal reproductive development, in contrast to findings reported in prenatally growth-restricted rams (Da Silva *et al.* 2001).

Early maternal undernutrition has been reported to disrupt a range of endocrine pathways with long-term effects on progeny health (McMillen and Robinson 2005; Gardner *et al.* 2006; McMillen *et al.* 2008). Furthermore, previous studies support the concept that early maternal undernutrition impacts hypothalamic and/or pituitary function at later postnatal stages, causing alterations to the endocrine system. These include changes in gonadotrophin profiles (Rae *et al.* 2002), reduced testosterone concentrations and delayed seasonal increase in testosterone (Da Silva *et al.* 2001) as well as altered hypothalamic–pituitary responsiveness to postnatal GnRH challenge in sheep (Kotsampasi *et al.* 2009) and prepubertal bulls (Sullivan *et al.* 2010). In the present study, a GnRH challenge was not undertaken and bulls were allowed to progress through puberty without any exogenous hormonal influence; hence, it is not possible to report on pituitary responsiveness in this study. Further studies are required to explore the role of maternal nutrition on the development and function of the hypothalamic–pituitary–gonadal axis in both the fetal and adult male bovine.

Conclusion

In summary, we have uniquely shown that in the developing bull the LPeri maternal diet delayed the onset of puberty and sexual maturity with negative effects on semen parameters in the early postpubertal period. These effects were subsequent to lower FSH concentrations in the LPeri diet group. The histology of the fetal and adult testis suggests that the early perturbation of the cytology of the testis has been compensated for during later development as no corresponding effects were observed in the adult testis. Whether the effects of this perturbation influenced testicular function through puberty before excision of the testis at 20 months, however, is unknown. The circulating hormone data suggest that the peri-conception diet may have altered the development of the hypothalamic–pituitary–gonadal axis or the receptivity to circulating hormones during the peri-pubertal period.

Whilst this study provides evidence that low maternal dietary protein has a negative impact on reproductive development in the pubertal and postpubertal offspring, some of the mechanisms that mediate this effect remain to be elucidated. Further research in cattle is warranted to enable exploration of causal relationships between gestational nutrition and consequent postnatal male reproductive development of progeny.

Conflicts of interest

The authors declare no conflicts of interest.

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