

# Maternal periconceptional and first trimester protein restriction in beef heifers: effects on placental parameters and fetal and neonatal calf development

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**Abstract.** Few studies have investigated the effects of nutrition during the periconception and early gestation periods on fetal and placental development in cattle. In this study, nulliparous yearling heifers ( $n = 360$ ) were individually fed a diet high or low in protein (HPeri and LPeri) beginning 60 days before conception. From 24 to 98 days after conception, half of each treatment group was changed to the alternative high- or low-protein diet (HPost and LPost) yielding four groups in a  $2 \times 2$  factorial design. A subset of heifers ( $n = 46$ ) was necropsied at 98 days after conception and fetoplacental development assessed. Placentome number and volume decreased in response to LPeri and LPost diets respectively. Absolute lung, pancreas, septum and ventricle weights decreased in LPost versus HPost fetuses, whereas the post-conception diet altered absolute and relative liver and brain weights depending on sex. Similarly, changes in fetal hepatic gene expression of factors regulating growth, glucose output and lipid metabolism were induced by protein restriction in a sex-specific manner. At term, neonatal calf and placental measures were not different. Protein restriction of heifers during the periconception and early gestation periods alters fetoplacental development and hepatic gene expression. These changes may contribute to functional consequences for progeny, but this may not be apparent from gross morphometry at birth.

**Additional keywords:** beef cattle, fetal programming.

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## Introduction

Maternal nutrition during gestation has been reported by many studies to affect fetal development, postnatal growth, metabolism and reproduction in species including humans, laboratory animals and domestic animals of agricultural importance (McMillen and Robinson 2005; Long *et al.* 2009; Sullivan *et al.* 2009a; Micke *et al.* 2010a). The effects of episodes of maternal undernutrition and/or overnutrition have been reported to vary depending on the timing, severity and length of nutrient restriction, as well as the sex of the fetus (Micke *et al.* 2015). Critical windows of vulnerability during fetal development differ for specific tissues and organs (McMillen *et al.* 2001). These periods of vulnerability include folliculogenesis and the preimplantation period

(Velazquez 2015), placental development (Fowden *et al.* 2006) and organogenesis (McMillen *et al.* 2001). During these critical windows, a stimulus or insult such as altered maternal nutrition can result in long-term consequences for the progeny.

In sheep, nutritional restriction during the periconception period has been reported to result in impaired blastocyst development (Borowczyk *et al.* 2006), altered development of the hypothalamic–pituitary–adrenal axis (Edwards and McMillen 2002) and, more recently, altered hepatic insulin signalling and glucocorticoid regulation of hepatic glucose output, as well as hepatic fatty acid metabolism (Nicholas *et al.* 2013; Lie *et al.* 2014). Similarly, it has been established in cattle that the periconception diet affects oocyte quality (Adamiak *et al.*

2005; Adamiak *et al.* 2006) and embryo development (Kruse *et al.* 2017), with implantation occurring between 18 and 22 days post conception (dpc) (Wathes and Wooding 1980). Fetal organogenesis is complete by 42 dpc (DesCôteaux *et al.* 2010) and occurs simultaneously with placental development, preceding the development of bone, muscle and fat tissues (Hubbert *et al.* 1972). Because the growth trajectories for these tissues vary, each tissue is susceptible to maternal dietary perturbations at different gestational stages (McMillen *et al.* 2001; Symonds *et al.* 2012). Although maternal nutrition in mid to late pregnancy may affect fetal growth (Taylor *et al.* 2018) and birthweight (Larson *et al.* 2009; Micke *et al.* 2010b; LeMaster *et al.* 2017), nutritional insults during the first trimester have been reported to affect fetal and postnatal organ development (Long *et al.* 2009, 2010) and tissue gene expression (Long *et al.* 2010; Micke *et al.* 2011). Subsequent effects upon productivity include altered postnatal growth (Micke *et al.* 2010a) and reproductive development (Sullivan *et al.* 2009a; Mossa *et al.* 2013). However, it is acknowledged that the effects of maternal nutrition during the periconception period and early gestation on fetoplacental development in cattle are poorly understood (Mossa *et al.* 2015; Sinclair *et al.* 2016).

The aim of this study was to evaluate the effects of maternal dietary protein intake during the periconception and first trimester periods on fetal and placental development and hepatic gene expression in the offspring of nulliparous adolescent heifers. We hypothesised that restricted maternal protein during the periconception period and early gestation would reduce fetal and placental development, with subsequent deleterious effects on fetal morphometry at 98 dpc and at term. Further, protein restriction would decrease liver growth and mitogenic gene expression, but increase the expression of genes regulating hepatic glucose output and lipid accumulation, thus potentially contributing to the development of altered carcass traits and metabolic dysfunction.

## Materials and methods

### Ethics approval

The use of animals and the procedures performed in this study were in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC 2004) and were approved by the University of South Australia Institute of Medical and Veterinary Science Animal Ethics Committee, Australia (Approval no. 18/11) and The University of Adelaide Animal Ethics Committee, Australia (Approval no. S2012-249).

### Experimental design and animal management

The purpose of this study was to evaluate the effects of maternal dietary protein during the periconception (PERI; -60 to 23 dpc) and first trimester (POST; 24 to 98 dpc) periods on fetal and placental development in nulliparous beef heifers. The maternal dietary protein levels reflected pasture conditions in Australian rangelands without (Low) and with (High) protein supplement.

The study was a two-by-two factorial design. The animals were the heifers and their progeny that have previously been described (Copping *et al.* 2014) with management and diet composition reported in detail by Copping *et al.* (2018).

Briefly, 360 nulliparous Santa Gertrudis (*Bos taurus* × *Bos indicus*) heifers underwent a 60-day acclimatisation period. At 12 months of age, 60 days before AI, heifers were assigned to two equal periconception (PERI; -60 to 23 dpc) treatment groups: high and low protein (HPeri and LPeri). Heifers were fed either a high-protein diet (71 MJ metabolisable energy (ME) and 1.18 kg crude protein (CP) heifer<sup>-1</sup> day<sup>-1</sup>) or a low-protein diet (63 MJ ME, 0.62 kg CP heifer<sup>-1</sup> day<sup>-1</sup>) consisting of a pelleted portion that was measured and fed individually in stalls each day with straw (5% CP) available *ad libitum* in pens. Heifers were oestrous synchronised using a progesterone-based program (Hernandez-Medrano *et al.* 2015) and were inseminated with frozen semen from one Santa Gertrudis bull on Day 0. At 23 dpc half of each nutritional treatment group was swapped to the alternative post-conception treatment (POST; 24–98 dpc), namely either a high-protein diet (HPost; 102 MJ ME, 1.49 kg CP heifer<sup>-1</sup> day<sup>-1</sup>) or a low-protein diet (LPost; 98 MJ ME, 0.88 kg CP heifer<sup>-1</sup> day<sup>-1</sup>), giving rise to four treatment groups in total: HPeri–HPost (HH), HPeri–LPost (HL), LPeri–HPost (LH) and LPeri–LPost (LL).

Pregnancy was confirmed at 36 dpc and the sex of the fetus determined at 60 dpc by rectal ultrasound. At the end of the first trimester (98 dpc), a subset ( $n = 46$  heifers, singleton pregnancy; HH, 6 male, 6 female; HL, 10 male, 5 female; LH, 5 male, 5 female; LL, 4 male, 5 female) were necropsied (Copping *et al.* 2014). The remaining 64 heifers were fed the same diet daily until parturition (99 dpc to term; 79 MJ ME, 0.92 kg CP heifer<sup>-1</sup> day<sup>-1</sup>; formulated to provide additional growth of 0.5 kg day<sup>-1</sup>). Heifers received the pellet portion individually with straw (5% CP) provided *ad libitum* in pens throughout.

At term, individual feeding ceased ( $n = 63$  heifers with singleton calves; HH, 10 male, 8 female; HL, 14 male, 4 female; LH, 11 male, 4 female; LL, 9 male, 3 female). An additional heifer produced twins and was excluded from the analyses reported herein (LL, 2 males). Heifers were weighed at approximately monthly intervals (Copping *et al.* 2020).

### Fetal necropsy

Heifers ( $n = 46$ ) were humanely slaughtered in a commercial abattoir at 98 dpc. The gravid uterus was immediately removed and the total weight of the uterus and fetus determined. The fetus was excised, weighed, measured and then dissected. Measures of fetal biparietal diameter (BPD), crown–nose length (CNL), crown–rump length (CRL) and umbilical cord diameter (UD) were obtained using sliding Vernier callipers. A measure of abdominal circumference (AC) was taken at the level of the umbilical cord using a flexible tape measure.

Fetal brain, heart, kidneys, liver, lung and pancreas were collected. Due to time constraints in handling the fetal tissue, only the left kidney was weighed. The heart was dissected into the atrial cap, septum and left and right ventricles, with each weighed individually. All other fetal organs were weighed complete. Liver samples were snap frozen in liquid nitrogen and stored at -80°C until molecular analysis. Placentomes were dissected from the uterus and all placentomes were counted and weighed. Placentome volume was calculated using saline displacement (Kannekens *et al.* 2006).

### RNA extraction and semiquantitative real-time reverse transcription–polymerase chain reaction

RNA was isolated from the liver (~50 mg) using Qiagen QIAzol Lysis Reagent and Qiagen RNeasy purification columns. RNA was quantified using the ratio of the optical density at 260 and 280 nm and used to calculate the correct dilutions of extracted RNA used for cDNA synthesis. The cDNA was synthesised using the Superscript III First Strand Synthesis System (Invitrogen) according to the manufacturer's instructions with 2 µg total diluted RNA, random hexamers, dNTP, dithiothreitol and Superscript III in a final volume of 20 µL. A no-template control (NTC) containing no RNA transcript and a no-amplification control (NAC) containing no Superscript III were used to check for reagent contamination and genomic DNA contamination respectively, as described previously (Wang *et al.* 2011; Soo *et al.* 2012).

Following Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009), three reference genes, namely peptidyl-prolyl isomerase A (*PPIA*),  $\beta_2$ -microglobulin (*B2M*) and tyrosine 3-monooxygenase (*YWHAZ*; Passmore *et al.* 2009), were chosen from a suite of reference (housekeeper) genes analysed using geNorm component of qBase analysis software (Biogazelle; Hellemans *et al.* 2007). The relative expression of the mRNA transcripts of mitogenic (growth factor) genes (i.e. insulin-like growth factor 1 (*IGF1*), IGF1 receptor (*IGF1R*), insulin-like growth factor 2 (*IGF2*) and IGF2 receptor (*IGF2R*)), glucose transporter 1 (*GLUT1*), genes involved in glucocorticoid signalling and hepatic glucose production (i.e. glucocorticoid receptor (*GR*), 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (*HSD11B1*) and 2 (*HSD11B2*), Forkhead Box O1 (*FOXO1*), and phosphoenolpyruvate carboxykinase-C (*PEPCK-C*)) and genes involved in fatty acid uptake and metabolism (i.e. cluster of differentiation 36, also known fatty acid translocase (*CD36*), fatty acid transport protein 1 (*FATP1*), peroxisome proliferator-activated receptor gamma (*PPAR $\gamma$ ) and alpha (*PPAR $\alpha$ ), retinoid X receptor (*RXR*), peroxisome proliferator activated receptor gamma coactivator 1 alpha (*PGC1a*), acetyl-CoA carboxylase (*ACC*), carnitine palmitoyltransferase I (*CPTI*), 3-phosphoinositide-dependent protein kinase-1 (*PKD1*), -2 (*PKD2*) and -4 (*PKD4*), as well as the reference genes, were measured by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) using Fast SYBR Green Master Mix (Applied Biosystems) in a final volume of 6 µL on a ViiA7 Fast Real-time PCR system (Applied Biosystems), as described previously (Zhang *et al.* 2010; Lie *et al.* 2014). Each qRT-PCR well contained 3 µL of 2 $\times$  Fast SYBR Green Master Mix, 2 µL forward and reverse primers mixed with H<sub>2</sub>O to obtain final primer concentrations given in Table 1 and 1 µL diluted relevant cDNA. Primer sets were originally designed and validated for use in sheep using either ovine or bovine sequences, and thus the product of all sets was sequenced to ensure that it matched the expected sequence (Table 1). Primers were validated to generate a single transcript as confirmed by the presence of an individual double-stranded DNA product of the correct size and sequence. Controls for each primer set containing no cDNA were**

included on each plate to test for reagent contamination (NTC). Melting curve and dissociation curves were also run to check for non-specific product formation (95°C for 15 s, 60°C for 1 min and 95°C for 15 s). Amplification efficiency reactions were performed on five triplicate serial dilutions of cDNA template for each primer set. Amplification efficiencies were determined from the slope of a plot of  $C_t$  (defined as the threshold cycle with the lowest significant increase in fluorescence) against log[cDNA template] (ranging from 1 to 100 ng).  $C_t$  values were in the linear amplification range for all genes. Each sample was run in triplicate for target genes and reference genes (CV <10%). The PCR consisted of 40 cycles at 95°C for 1 s and 60°C for 20 s. The reactions were quantitated by setting the threshold within the exponential growth phase of the amplification curve and obtaining corresponding  $C_t$  values ( $C_t$  of all genes <35). The abundance of each transcript relative to the abundance of stable housekeeping genes (Hellemans *et al.* 2007) was calculated using DataAssist 3.0 analysis software (Applied Biosystems) and expressed as mean normalised expression (Soo *et al.* 2012).

### Neonatal calf measurements

At calving, heifers were monitored visually 24 h day<sup>-1</sup>. Calf measurements were collected within 15 min of birth and before sucking. The whole-body, trunk and cranial measures that were recorded included calf birth bodyweight (BW), CRL, AC, BPD and CNL (Micke *et al.* 2010b). Height was measured from the base of the hoof to the top of the wither. Placentas were collected immediately upon expulsion, assessed for completeness and weighed (Sullivan *et al.* 2009b). If the expulsion time of the placenta exceeded 12 h, the placenta was classified as retained fetal membranes (RFM; Peter 2013). Seven partially eaten placentas and two RFM were excluded from the analyses of all placental measures and calculations. Cotyledons were dissected away from membranes of each placenta, counted and weighed. Cotyledon volume was calculated using saline displacement. Placental efficiency at birth was calculated as the ratio of calf birth BW to placental weight (Leiser *et al.* 1997).

### Statistical analysis

Data were assessed for normality by graphical exploration and square root or Box–Cox transformed (STATA13.1/IC; StataCorp) if required before further analysis. Data are presented as the unadjusted mean  $\pm$  s.e.m. Multifactorial analysis of variance (ANOVA) was used to explore the effects of maternal diet during PERI and POST, progeny sex and their interaction terms on fetal morphology, placental measures and organ weights at 98 dpc, hepatic gene expression, calf and placental measures at term and indices of disproportionate growth in singleton progeny only. Gestation length was included as a covariate for birth measures to account for the length of exposure to the second and third trimester maternal diet. Interactions were explored with one- or two-way ANOVA and Tukey–Kramer post hoc tests as required. A *P* value of <0.05 was considered statistically significant and *P* < 0.10 was considered a tendency.

**Table 1. Primer sequences for quantitative reverse transcription–polymerase chain reaction and amplicon homology to the bovine gene sequence**

Three reference genes were used: peptidylprolyl isomerase A (*PPIA*),  $\beta$ 2-microglobulin (*B2M*) and tyrosine 3-monooxygenase (*YWHAZ*). The genes of interest were the mitogenic genes insulin-like growth factor 1 (*IGF1*), *IGF1* receptor (*IGF1R*), insulin-like growth factor 2 (*IGF2*) and *IGF2* receptor (*IGF2R*), glucose transporter 1 (*GLUT1*), genes involved in glucocorticoid signalling and hepatic glucose production, namely glucocorticoid receptor (*GR*), 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (*HSD11B1*) and 2 (*HSD11B2*), Forkhead Box O1 (*FOXO1*), and phosphoenolpyruvate carboxykinase-C (*PEPCK-C*) and genes involved in fatty acid uptake and metabolism (i.e. cluster of differentiation 36, also known fatty acid translocase (*CD36*), fatty acid transport protein 1 (*FATP1*), peroxisome proliferator-activated receptor gamma (*PPAR* $\gamma$ ) and alpha (*PPAR* $\alpha$ ), retinoid X receptor (*RXR*), peroxisome proliferator activated receptor gamma coactivator 1 alpha (*PGC1* $\alpha$ ), acetyl-CoA carboxylase (*ACC*), carnitine palmitoyltransferase 1 (*CPT1*), 3-phosphoinositide-dependent protein kinase-1 (*PKD1*), -2 (*PKD2*) and -4 (*PKD4*)

Gene	Forward primer		Reverse primer		Amplicon homology with bovine gene sequence (%)
	Sequence (5'–3')	Concentration ( $\mu$ M)	Sequence (5'–3')	Concentration ( $\mu$ M)	
<i>PPIA</i>	TCATTTGCACTGCCAAGACTG	450	TCATGCCCTCTTTCCTTGC	900	97
<i>B2M</i>	CCGCCAGAAGATGGAAAGCCAAAT	450	ACTGATCCTGTCTGTTGGGAGTGA	450	98
<i>YWHAZ</i>	TGTAGGAGCCCGTAGGTCATCT	450	TTCTCTGTATTCTCGAGCCATCT	450	100
<i>IGF1</i>	TTGGTGGATGCTCTCCAGTTC	900	AGCAGCACTCATCCACGATTC	900	99
<i>IGF1R</i>	AAGAACCATGCCTGCAGAAGG	900	GGATTCTCAGGTTCTGGCCATT	900	100
<i>IGF2</i>	GCTTCTGCCTTCTTGGCCTT	900	TCGGTTTATGCGGCTGGAT	900	100
<i>IGF2R</i>	GATGAAGGAGGCTGCAAGGAT	900	CCTGATGCCTGTAGTCCAGCTT	900	100
<i>GLUT1</i>	ATCGTGGCCATCTTTGGCTTTGTG	450	CTGGAAGCACATGCCACAATGAA	450	95
<i>GR</i>	ATGCCCCCAAGTGAACACAGA	900	ATGAACAGAAATGGCAGACATTTTATT	900	98
<i>11<math>\beta</math>HSD1</i>	GCGCCAGATCCCTGTCTGAT	900	AGCGGGATAACCACCTTCTTT	900	99
<i>11<math>\beta</math>HSD2</i>	GAGACATGCCGTTTCCATGC	450	TGATGCTGACCTTGACACCC	450	97
<i>FOXO1</i>	ACACCTTTACAAGTGCCTCTG	450	TAGCCATTGCAGCTGCTCAC	450	100
<i>PEPCK-c</i>	AACTCACGGTCTGCACTCCA	450	GGTCGTGCATGATGACTTTGC	450	100
<i>CD36</i>	TGGTGTGCTAGACATTGGCAAATG	450	TGTTGACCTGCAGCCGTTTTCG	450	100
<i>FATP1</i>	AGCCTGGTCAAGTCTGTCTGGA	450	AGAAGAGTCGATCATCCATGCCCT	450	100
<i>PPAR<math>\gamma</math></i>	ATGTCTCATAATGCCATCAGGTT	900	GATAACAAACGGTGATTTGTCTGTC	300	99
<i>RXR</i>	CATTTTCGACAGGGTGCTG	900	CTTGGCGAACCTTCTG	900	100
<i>PPAR<math>\alpha</math></i>	CGTGTGAACATGACCTAGAAG	900	ACGAAGGGCGGATTGTG	900	95
<i>PGC1<math>\alpha</math></i>	TCTGGAAGTGCAGGCTAACTC	900	GCAAGAGGGCTTCAGCTTTG	900	100
<i>ACC</i>	TGGCCTTACACATGAGATCCAGCA	450	ATGCCATTGTTGGCGATGAGAACC	450	99
<i>CPT1</i>	GATCACGTATCGCCGTAACCT	450	GTTGTTGAACATCCTCTCCATCT	450	98
<i>PKD2</i>	ACTGCAATGTCTCTGAGGTGGTGA	450	GCTGTTTGGAGTTGGATGCGTTGA	450	100
<i>PKD4</i>	GCACCAACGCCTGTGATGGATAAT	50	AGCATCAGTTCCTGATCCTGGCAA	50	100
<i>PKD1</i>	ACGGATCAGGAACCGACACAATGA	900	TGAACATTCTGGCTGGTGAGAGGA	450	98

## Results

### Fetal measurements at 98 dpc

Measures of fetal and placental parameters at 98 dpc are presented in Table 2. Placentome number was decreased ( $P = 0.03$ ) and placentome volume tended to be reduced by the LPeri versus HPeri diet ( $P = 0.06$ ), whereas the LPost diet reduced placentome volume ( $P = 0.01$ ) and tended to reduce weight ( $P = 0.075$ ) compared with the HPost diet. Placental efficiency (fetal weight/placentome weight) did not differ among groups ( $P > 0.10$ ).

As reported by Copping *et al.* (2014), male fetuses were heavier than females overall ( $P < 0.001$ ) and LPost fetuses were lighter than HPost fetuses. Within males and within females, the LPost diet decreased fetal weight by 8% and 10% respectively ( $P < 0.05$ ).

Males had a larger BPD and AC overall at 98 dpc than females ( $P < 0.05$ ) and BPD tended ( $P < 0.1$ ) to be decreased in LPeri versus HPeri fetuses (Copping *et al.* 2014). Abdominal circumference varied with the interaction between PERI and POST diet, and fetal sex ( $P < 0.05$ ). In females only, AC was reduced in fetuses from heifers in the HL compared with HH group (Fig. 1;  $P = 0.03$ ).

Absolute organ and relative weights for fetuses at 98 dpc are reported in Tables 2 and 3 respectively. Compared with males overall, females tended to have reduced absolute fetal brain weight ( $8.59 \pm 0.14$  vs  $8.17 \pm 0.14$  g respectively;  $P = 0.051$ ) but greater brain weight relative to fetal weight ( $26.22 \pm 0.53$  vs  $28.22 \pm 0.63$  g kg<sup>-1</sup> respectively;  $P = 0.005$ ). In addition, compared with males, females had significantly reduced absolute weights of fetal heart ( $2.44 \pm 0.05$  vs  $2.23 \pm 0.06$  g), atrial cap ( $0.72 \pm 0.02$  vs  $0.62 \pm 0.02$  g), lung ( $9.82 \pm 0.19$  vs  $8.87 \pm 0.27$  g) and pancreas ( $0.21 \pm 0.01$  vs  $0.18 \pm 0.01$  g; all  $P < 0.05$ ) and tended to have reduced absolute weights of the liver ( $11.68 \pm 0.26$  vs  $10.84 \pm 0.38$  g;  $P = 0.07$ ) and kidney ( $1.26 \pm 0.04$  vs  $1.14 \pm 0.06$  g;  $P = 0.09$ ). However, organ weights were similar in females and males when assessed relative to fetal BW (all  $P > 0.10$ ).

Absolute brain weight tended ( $P = 0.09$ ) to be reduced in LPeri versus HPeri fetuses. The PERI diet did not affect the absolute or relative weights of any other fetal organ (all  $P > 0.10$ ). In contrast, the POST diet affected the absolute weights of the fetal heart and its structures, along with the

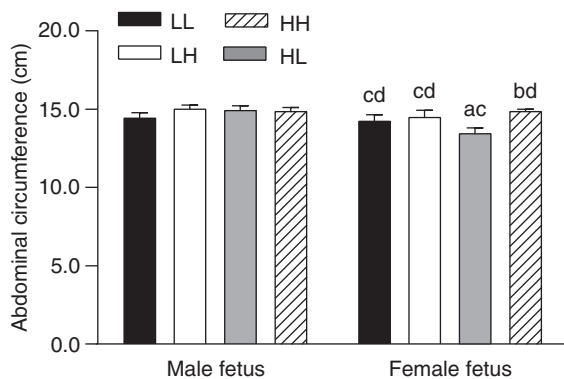


**Table 2.** Measurements and absolute organ weights of fetuses at 98 days post conception (dpc) following exposure to maternal diets low or high in protein during the periconception (PERI; -60 to 23 dpc) and postconception (POST; 24 to 98 dpc) periods

Values are given as the unadjusted mean  $\pm$  s.e.m. Within rows, values with different superscript letters differ significantly ( $P < 0.05$ ). AC, abdominal circumference

PERI POST	Treatment				Sex	<i>P</i> -value		
	Low	High	Low	High		PERI	POST	PERI $\times$ POST
No. fetuses	9	10	15	12				
Gravid uterus (kg)	2.71 $\pm$ 0.06	2.87 $\pm$ 0.11	2.71 $\pm$ 0.09	2.83 $\pm$ 0.09	0.394	0.768	0.132	0.922
AC (cm)	14.39 $\pm$ 0.22	14.80 $\pm$ 0.23	14.48 $\pm$ 0.26	14.92 $\pm$ 0.10	0.007	0.862	0.036	0.709
Left ventricle (g)	0.50 $\pm$ 0.03	0.53 $\pm$ 0.03	0.51 $\pm$ 0.02	0.59 $\pm$ 0.03	0.073	0.326	0.040	0.376
Right ventricle (g)	0.39 $\pm$ 0.02	0.49 $\pm$ 0.02	0.44 $\pm$ 0.02	0.47 $\pm$ 0.03	0.340	0.532	0.016	0.210
Septum (g)	0.50 $\pm$ 0.03	0.58 $\pm$ 0.02	0.54 $\pm$ 0.03	0.58 $\pm$ 0.02	0.202	0.620	0.037	0.568
Atrial cap <sup>A</sup> (g)	0.61 $\pm$ 0.04 <sup>a</sup>	0.75 $\pm$ 0.05 <sup>b</sup>	0.69 $\pm$ 0.03 <sup>ab</sup>	0.65 $\pm$ 0.02 <sup>ab</sup>	0.007	0.539	0.115	0.031
Brain (g)	8.02 $\pm$ 0.12	8.32 $\pm$ 0.24	8.59 $\pm$ 0.16	8.51 $\pm$ 0.23	0.065	0.088	0.502	0.433
Left kidney (g)	1.17 $\pm$ 0.08	1.19 $\pm$ 0.06	1.21 $\pm$ 0.06	1.24 $\pm$ 0.07	0.087	0.637	0.667	0.773
Lung (g)	8.90 $\pm$ 0.16	9.26 $\pm$ 0.43	9.23 $\pm$ 0.33	10.06 $\pm$ 0.31	0.002	0.150	0.040	0.269
Pancreas (g)	0.17 $\pm$ 0.02	0.20 $\pm$ 0.02	0.19 $\pm$ 0.01	0.21 $\pm$ 0.01	0.047	0.660	0.044	0.831
Liver (g)	10.63 $\pm$ 0.68	11.55 $\pm$ 0.51	11.32 $\pm$ 0.39	11.57 $\pm$ 0.34	0.080	0.575	0.182	0.610
No. placentomes	47.44 $\pm$ 3.79	46.50 $\pm$ 4.57	57.47 $\pm$ 5.87	65.00 $\pm$ 7.60	0.095	0.027	0.653	0.609
Placentome weight (g)	158.96 $\pm$ 7.38	172.03 $\pm$ 9.17	164.85 $\pm$ 8.61	191.97 $\pm$ 13.20	0.909	0.250	0.075	0.552
Placentome volume (mL)	146.33 $\pm$ 7.34	151.10 $\pm$ 9.12	149.13 $\pm$ 7.59	185.42 $\pm$ 8.18	0.746	0.062	0.010	0.105

<sup>A</sup>PERI  $\times$  POST  $\times$  sex interaction ( $P < 0.05$ ).



**Fig. 1.** Abdominal circumference in male and female fetuses at 98 days post conception (dpc) from heifers fed diets low (L) or high (H) in protein during the periconception (PERI; -60 to 23 dpc) and postconception (POST; 24 to 98 dpc) periods of gestation. LL, low level of dietary protein in the PERI and POST periods; LH, low level of dietary protein in the PERI period and high level of dietary protein in the POST period; HL, high level of dietary protein in the PERI period and low level of dietary protein in the POST period; HH, high level of dietary protein in the PERI and POST periods. Data are the mean  $\pm$  s.e.m. Different letters above columns indicate significant differences ( $P < 0.05$ ).

weight of the lung and pancreas. Heart weight was reduced in LPost versus HPost fetuses, as reported previously (Hernandez-Medrano *et al.* 2015), along with septum and left and right ventricle weights ( $P < 0.05$ ). Furthermore, atrial cap weight varied with an interaction between maternal nutrition during PERI and POST, with atrial cap weight greater in fetuses exposed to LPeri and HPost diets (LH group) than in fetuses

exposed to a constant low PERI and POST diet (LL group;  $P = 0.03$ ). Absolute lung ( $P = 0.03$ ) and pancreas ( $P = 0.04$ ) weights were also reduced in LPost fetuses compared with HPost fetuses. Overall, absolute liver weight was unaffected by diet, but was reduced in LPost versus HPost males (Fig. 2a;  $P = 0.01$ ) when sexes were analysed separately. Absolute weights of the fetal kidney did not differ due to maternal diet treatment ( $P > 0.10$ ).

Relative fetal brain weight was greater ( $P = 0.007$ ) in LPost than HPost fetuses and within males ( $27.22 \pm 0.64$  vs  $24.94 \pm 0.75$  g kg<sup>-1</sup> respectively;  $P = 0.045$ ), but not females. Relative liver weight varied with an interaction between POST diet and fetal sex ( $P = 0.02$ ). Within females, but not males, relative liver weight was increased in LPost versus HPost fetuses (Fig. 2b;  $P < 0.05$ ). Relative heart, lung, kidney and pancreas weights were unaffected by maternal diet treatment ( $P > 0.10$ ).

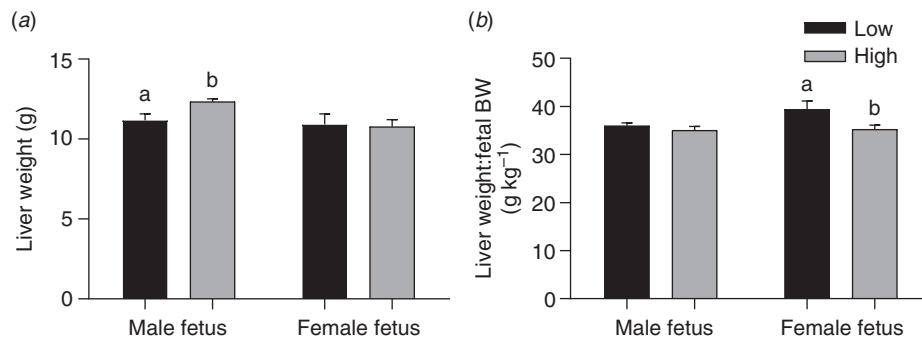
#### Indices of disproportionate growth at 98 dpc

To investigate the effects of maternal diet on proportional growth, BPD : AC, CNL : CRL, CRL : fetal BW and brain : liver ratios were calculated on the 98 dpc fetal measures (Table 4).

Overall, the CRL : fetal BW ratio was less in males than in females ( $55.88 \pm 0.96$  vs  $62.24 \pm 1.17$  mm kg<sup>-1</sup> respectively;  $P < 0.001$ ), but there was no difference in the BPD : AC and CNL : CRL ratios between sexes ( $P > 0.05$ ) and these ratios were not affected by the PERI diet ( $P > 0.05$ ). The LPost diet increased fetal CNL : CRL ( $P = 0.039$ ) and CRL : fetal BW ( $P = 0.003$ ) ratios and tended to increase the BPD : AC ratio compared with the HPost diet ( $P = 0.08$ ). The brain : liver ratio tended to vary with an interaction between POST diet and fetal sex ( $P = 0.07$ ). When sexes were analysed separately, the brain : liver ratio was increased only in male LPost versus

**Table 3. Relative organ weight in fetuses at 98 days post conception (dpc) following exposure to maternal diets low or high in protein during the periconception (PERI; -60 to 23 dpc) and postconception (POST; 24 to 98 dpc) periods**Values are given as the unadjusted mean  $\pm$  s.e.m. BW, bodyweight

PERI POST	Treatment				Sex	P-value		
	Low		High			PERI	POST	PERI $\times$ POST
	Low	High	Low	High				
No. fetuses	9	10	15	12				
Heart weight (g)/fetal BW (kg)	7.37 $\pm$ 0.32	7.66 $\pm$ 0.12	7.57 $\pm$ 0.13	7.46 $\pm$ 0.15	0.167	0.871	0.683	0.211
Brain weight (g)/fetal BW (kg)	27.44 $\pm$ 0.80	26.19 $\pm$ 1.11	28.48 $\pm$ 0.77	26.01 $\pm$ 0.59	0.036	0.374	0.013	0.263
Liver weight (g)/fetal BW (kg) <sup>A</sup>	36.09 $\pm$ 1.76	35.95 $\pm$ 0.92	37.32 $\pm$ 1.13	35.28 $\pm$ 0.65	0.076	0.657	0.294	0.305
Lung weight (g)/fetal BW (kg)	30.44 $\pm$ 0.88	28.85 $\pm$ 0.88	30.34 $\pm$ 0.78	30.75 $\pm$ 0.82	0.519	0.274	0.475	0.287
Pancreas weight (g)/fetal BW (kg)	0.59 $\pm$ 0.05	0.63 $\pm$ 0.04	0.62 $\pm$ 0.04	0.64 $\pm$ 0.04	0.779	0.712	0.486	0.730

<sup>A</sup>POST  $\times$  fetal sex interaction ( $P = 0.07$ ).**Fig. 2.** (a) Absolute and (b) relative liver weight in male and female fetuses at 98 days post conception (dpc) from heifers exposed to maternal diets low (L) or high (H) in protein during the postconception (POST; 24–98 dpc) period. Data are the mean  $\pm$  s.e.m. Different letters above columns indicate significant differences ( $P < 0.05$ ).**Table 4. Indices of disproportionate growth in fetuses at 98 days post conception (dpc) following exposure to maternal diets low or high in protein during the periconception (PERI; -60 to 23 dpc) and postconception (POST; 24 to 98 dpc) periods**Values are given as the unadjusted mean  $\pm$  s.e.m. AC, abdominal circumference; BPD, biparietal diameter; BW, bodyweight; CNL, crown–nose length; CRL, crown–rump length; wt, weight

PERI POST	Treatment				Sex	P-value		
	Low		High			PERI	POST	PERI $\times$ POST
	Low	High	Low	High				
No. fetuses	9	10	15	12				
BPD (mm) : AC (cm)	2.47 $\pm$ 0.04	2.44 $\pm$ 0.06	2.55 $\pm$ 0.03	2.46 $\pm$ 0.03	0.475	0.290	0.206	0.477
CNL (mm) : CRL (mm)	3.17 $\pm$ 0.07	3.04 $\pm$ 0.09	3.23 $\pm$ 0.04	3.13 $\pm$ 0.03	0.143	0.168	0.039	0.940
Brain wt (g) : liver wt <sup>A</sup> (g)	0.77 $\pm$ 0.04	0.73 $\pm$ 0.03	0.77 $\pm$ 0.03	0.73 $\pm$ 0.02	0.373	0.833	0.199	0.900
CRL (mm) : fetal BW (kg)	61.00 $\pm$ 2.01	56.80 $\pm$ 2.02	60.33 $\pm$ 1.75	56.83 $\pm$ 0.95	<0.001	0.759	0.005	0.776

<sup>A</sup>POST  $\times$  fetal sex interaction ( $P = 0.07$ ).HPost fetuses ( $0.78 \pm 0.03$  vs  $0.69 \pm 0.02$  g g<sup>-1</sup> respectively;  $P = 0.03$ ).

#### Hepatic gene expression in fetuses at 98 dpc

Relative hepatic gene expression of signalling factors in the 98 dpc fetal liver varied with an interaction between maternal PERI

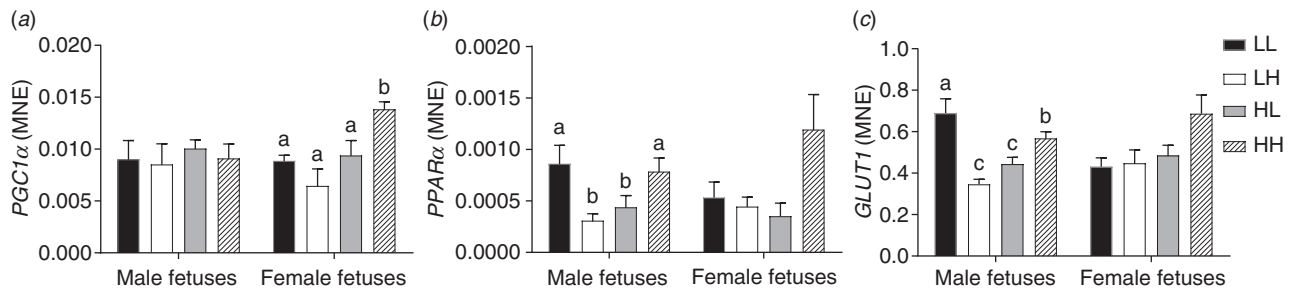
or POST diet and fetal sex for multiple factors ( $P < 0.10$ ). Subsequently, males and females were analysed separately for all factors. The relative hepatic gene expression of signalling factors that were altered by maternal diet ( $P < 0.05$ ) are presented in Table 5, with all signalling factors examined presented in Table S1, available as Supplementary Material to this paper.

**Table 5.** Mean normalised hepatic gene expression of signalling factors in the liver of male and female 98 days post conception (dpc) fetuses exposed to maternal diets low or high in protein during the periconception (PERI; -60 to 23 dpc) and postconception (POST; 24 to 98 dpc) periods of gestation

Data are the mean  $\pm$  s.e.m. Within rows and within sex, values with different superscript letters differ significantly ( $P < 0.05$ ). Mean normalised hepatic gene expression is given as the ratio of the  $\Delta C_t$  for the target gene to the three selected reference genes peptidylprolyl isomerase A (*PPIA*),  $\beta_2$ -microglobulin (*B2M*) and tyrosine 3-monooxygenase (*YWHAZ*). Only signalling factors found to be significantly different are presented in the table; hepatic gene expression of all signalling factors examined is available in Table S1. LL, low level of dietary protein in the PERI and POST periods; LH, low level of dietary protein in the PERI period and high level of dietary protein in the POST period; HL, high level of dietary protein in the PERI period and low level of dietary protein in the POST period; HH, high level of dietary protein in the PERI and POST periods; *FOXO1*, Forkhead Box O1; *GLUT1*, glucose transporter 1; *GR*, glucocorticoid receptor; *IGF1R*, IGF1 receptor; *IGF2*, insulin-like growth factor 2; *PDK1*, 3-phosphoinositide-dependent protein kinase-1; *PGC1 $\alpha$* , peroxisome proliferator activated receptor gamma coactivator 1 alpha; *PPAR $\gamma$* , peroxisome proliferator-activated receptor gamma; *RXR*, retinoid X receptor

PERI diet	Male		Female	
	Low (LL + LH)	High (HH + HL)	Low (LL + LH)	High (HH + HL)
Total no. fetuses <sup>A</sup>	9	16	10	11
<i>IGF2</i>	20.66 $\pm$ 1.69 ( $n = 9$ )	19.89 $\pm$ 1.66 ( $n = 15$ )	18.08 $\pm$ 1.57 <sup>a</sup> ( $n = 9$ )	25.59 $\pm$ 2.25 <sup>b</sup> ( $n = 11$ )
<i>IGF1R</i>	0.053 $\pm$ 0.004 ( $n = 9$ )	0.053 $\pm$ 0.005 ( $n = 16$ )	0.042 $\pm$ 0.006 <sup>a</sup> ( $n = 9$ )	0.057 $\pm$ 0.006 <sup>b</sup> ( $n = 10$ )
<i>FOXO1</i>	0.007 $\pm$ 0.001 ( $n = 9$ )	0.008 $\pm$ 0.001 ( $n = 14$ )	0.007 $\pm$ 0.001 <sup>a</sup> ( $n = 10$ )	0.009 $\pm$ 0.001 <sup>b</sup> ( $n = 10$ )
<i>PDK1</i>	0.014 $\pm$ 0.001 <sup>b</sup> ( $n = 9$ )	0.019 $\pm$ 0.002 <sup>a</sup> ( $n = 16$ )	0.014 $\pm$ 0.002 <sup>a</sup> ( $n = 9$ )	0.021 $\pm$ 0.001 <sup>b</sup> ( $n = 10$ )
<i>GR</i>	0.059 $\pm$ 0.002 ( $n = 7$ )	0.06 $\pm$ 0.06 ( $n = 16$ )	0.053 $\pm$ 0.005 <sup>a</sup> ( $n = 10$ )	0.080 $\pm$ 0.008 <sup>b</sup> ( $n = 11$ )
<i>GLUT1</i>	0.499 $\pm$ 0.067 ( $n = 9$ )	0.490 $\pm$ 0.028 ( $n = 16$ )	0.439 $\pm$ 0.035 <sup>a</sup> ( $n = 9$ )	0.598 $\pm$ 0.062 <sup>b</sup> ( $n = 9$ )
<i>PPAR<math>\gamma</math></i>	0.0034 $\pm$ 0.0006 ( $n = 9$ )	0.0028 $\pm$ 0.0003 ( $n = 16$ )	0.0046 $\pm$ 0.0005 <sup>a</sup> ( $n = 10$ )	0.0029 $\pm$ 0.0003 <sup>b</sup> ( $n = 9$ )
<i>PGC1<math>\alpha</math></i>	0.009 $\pm$ 0.001 ( $n = 9$ )	0.010 $\pm$ 0.001 ( $n = 16$ )	0.008 $\pm$ 0.001 <sup>a</sup> ( $n = 9$ )	0.012 $\pm$ 0.001 <sup>b</sup> ( $n = 10$ )
<i>RXR</i>	0.071 $\pm$ 0.015 ( $n = 9$ )	0.075 $\pm$ 0.007 ( $n = 15$ )	0.107 $\pm$ 0.009 <sup>a</sup> ( $n = 10$ )	0.076 $\pm$ 0.009 <sup>b</sup> ( $n = 10$ )
POST diet	Low (LL + HL)	High (HH + LH)	Low (LL + HL)	High (HH + LH)
Total no. fetuses <sup>A</sup>	14	11	12	10
<i>GLUT1</i>	0.514 $\pm$ 0.043 <sup>a</sup> ( $n = 14$ )	0.467 $\pm$ 0.040 <sup>b</sup> ( $n = 11$ )	0.455 $\pm$ 0.032 ( $n = 9$ )	0.581 $\pm$ 0.068 ( $n = 9$ )
<i>PPAR<math>\gamma</math></i>	0.0029 $\pm$ 0.0003 ( $n = 14$ )	0.0032 $\pm$ 0.0005 ( $n = 11$ )	0.0044 $\pm$ 0.0005 <sup>a</sup> ( $n = 9$ )	0.0032 $\pm$ 0.0004 <sup>b</sup> ( $n = 10$ )

<sup>A</sup>Total numbers included in each group are given. Where animals were excluded from analyses, this is indicated in the appropriate cell per respective gene.



**Fig. 3.** Effects of maternal diet during the periconception (PERI; -60 to 23 days post coitum (dpc)) and postconception (POST; 24 to 98 dpc) periods of gestation on hepatic mRNA expression of (a) peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC1 $\alpha$* ), (b) peroxisome proliferator-activated receptor alpha (*PPAR $\alpha$* ) and (c) glucose transporter 1 (*GLUT1*), normalised against the three reference genes (peptidylprolyl isomerase A (*PPIA*),  $\beta_2$ -microglobulin (*B2M*) and tyrosine 3-monooxygenase (*YWHAZ*)), in fetal bovine liver at 98 dpc in male and female fetuses. LL, low level of dietary protein in the PERI and POST periods; LH, low level of dietary protein in the PERI period and high level of dietary protein in the POST period; HL, high level of dietary protein in the PERI period and low level of dietary protein in the POST period; HH, high level of dietary protein in the PERI and POST periods. Data are the mean  $\pm$  s.e.m. Different letters above columns indicate significant differences ( $P < 0.05$ ).

Relative expression of *IGF2* was decreased in LPeri female fetus only ( $P < 0.05$ ), and no change was observed in LPost fetuses overall or within each sex ( $P > 0.05$ ). Gene expression of the insulin-independent glucose transporter *GLUT1* was increased ( $P < 0.05$ ) in male fetuses exposed to the low diet in both the PERI and POST periods (LL group; Fig. 3;  $P < 0.05$ ), but decreased ( $P \leq 0.05$ ) in LPeri female fetuses. The expression of

the transcription factors regulating the expression of *PEPCK-C*, namely *FOXO1* and *GR* were affected by fetal sex and maternal diet. Overall, *FOXO1* and hepatic *GR* were decreased by the LPeri diet ( $P < 0.05$ ). When sexes were analysed separately, these effects occurred only in female fetuses ( $P < 0.05$ ).

Furthermore, consistent with this change, there was a tendency for decreased *PEPCK-C* expression in LPeri fetuses

**Table 6.** Gestation length, calf height, abdominal circumference (AC) and measurements of placental parameters at term from heifers exposed to maternal diets low (L) or high (H) in protein during the periconception (PERI; –60 to 23 days postconception(dpc)) and postconception (POST; 24 to 98 dpc) periodsValues are given as the unadjusted mean  $\pm$  s.e.m. wt, weight

PERI POST	Treatment				Sex	<i>P</i> -value		
	Low	High	Low	High		PERI	POST	PERI $\times$ POST
No. calves	12	15	18	18				
Gestation length (days)	281.08 $\pm$ 1.82	282.27 $\pm$ 1.36	279.89 $\pm$ 1.03	282.39 $\pm$ 1.20	0.026	0.820	0.091	0.447
Wither height (cm)	76.71 $\pm$ 1.19	77.80 $\pm$ 1.04	78.06 $\pm$ 0.76	75.93 $\pm$ 0.78	0.528	0.975	0.185	0.031
AC (cm)	70.75 $\pm$ 1.30	74.33 $\pm$ 1.39	71.92 $\pm$ 0.71	71.81 $\pm$ 0.93	0.816	0.585	0.242	0.062
Placentas <sup>A</sup>								
No. placentas	11	12	15	16				
Placental wt (kg)	4.95 $\pm$ 0.30	4.88 $\pm$ 0.44	5.08 $\pm$ 0.29	4.66 $\pm$ 0.37	0.388	0.906	0.454	0.608
No. cotyledons	74.55 $\pm$ 7.30	87.17 $\pm$ 5.56	79.13 $\pm$ 4.94	76.38 $\pm$ 3.68	0.787	0.549	0.406	0.149
Cotyledon wt (g)	1660.00 $\pm$ 121.10	1699.00 $\pm$ 96.97	1662.47 $\pm$ 116.55	1645.44 $\pm$ 109.63	0.420	0.821	0.975	0.788
Cotyledon volume (mL)	1610.73 $\pm$ 120.45	1660.83 $\pm$ 87.88	1639.00 $\pm$ 99.61	1637.19 $\pm$ 122.16	0.249	0.976	0.876	0.804
Placental efficiency <sup>B</sup>	6.32 $\pm$ 0.24	7.71 $\pm$ 1.08	6.68 $\pm$ 0.29	7.21 $\pm$ 0.47	0.642	0.863	0.157	0.409

<sup>A</sup>Only data for the 54 heifers with complete placental measurements are presented.<sup>B</sup>Placental efficiency was calculated as the ratio of calf bodyweight at birth (kg) to placental weight (kg).

( $P = 0.06$ ). However, the transcription factors regulating the expression of the fatty acid transporters and lipogenesis, *RXR* and *PPAR $\gamma$* , were increased in LPeri female fetuses ( $P < 0.05$ ), with *PPAR $\gamma$*  also increased in LPost female fetuses ( $P < 0.05$ ), whereas *PPAR $\alpha$*  decreased in male fetuses exposed to the low-protein diet in either the PERI or POST periods (LH and HL groups; Fig. 3;  $P < 0.05$ ). The expression of *PDK1* ( $P < 0.05$ ) was reduced by the LPeri diet overall and in both sexes when analysed separately. Concomitantly, the PPAR coactivator and mitochondrial biogenesis factor, *PGC1 $\alpha$*  ( $P < 0.05$ ), was also decreased in female fetuses exposed to the low protein diet in either the PERI or POST periods (LH and HL) (Fig. 3;  $P < 0.05$ ).

#### Term parameters in neonatal calf and placenta

Placental parameters and placental efficiency at term did not differ according to maternal diet, calf sex or gestation length (Table 6;  $P > 0.10$ ).

Gestation length, calf height and AC at birth are presented in Table 6. Mean calf birth BW was 32.1  $\pm$  0.6 kg (range 20.0–42.8 kg). Gestation length tended to be decreased by LPost compared with HPost ( $P = 0.09$ ), and was longer for male than female calves (282.22  $\pm$  0.77 vs 279.47  $\pm$  1.13 days;  $P = 0.03$ ). Calf height and AC varied with an interaction between PERI and POST diet ( $P < 0.10$ ), but there were no differences between diet groups ( $P > 0.10$ ). As reported previously (Copping *et al.* 2014), calf birth BW and morphology measures of CRL, BPD and CNL were similar between male and female calves ( $P > 0.10$ ) and were not affected by maternal diet overall or within each sex. The inclusion of maternal BW at calving as a covariate in the statistical model did not alter this outcome.

#### Indices of disproportionate growth at birth

Proportional growth at birth, as measured by the BPD:AC (0.204  $\pm$  0.004 cm cm<sup>-1</sup>), CNL:CRL (0.249  $\pm$  0.004 cm cm<sup>-1</sup>) and CRL:birth BW (2.54  $\pm$  0.04 cm kg<sup>-1</sup>) ratios and the

ponderal index, calculated as weight divided by height cubed (69.9  $\pm$  1.0 kg cm<sup>-3</sup>), were unaffected by maternal diet or calf sex ( $P > 0.10$ ).

#### Discussion

This study explored the effects of maternal protein diets during the periconception and first trimester periods in nulliparous beef heifers on fetal and placental development and hepatic gene expression. It is the first study we believe to report fetoplacental effects of the periconception diet in the bovine with a fetal endpoint and to term. Varying levels of maternal dietary protein during the PERI (from –60 to 23 dpc) and POST (from 24 to 98 dpc) periods resulted in altered fetal size and body proportions at 98 dpc but similar birth BW (Copping *et al.* 2014) and morphology at term. This was associated with sex-specific asymmetric development at 98 dpc, a characteristic of intrauterine growth restriction (IUGR; Platz and Newman 2008). Further, it resulted in sex-specific programming effects on genes regulating growth, hepatic glucose output and lipid metabolism in the 98 dpc fetus. Maternal dietary protein restriction, at the moderate level used in this study, is commonly observed in commercial cattle operations (Bortolussi *et al.* 2005). We have previously shown that such restriction alters productivity traits in the progeny (Micke *et al.* 2010a, 2011) and have reported that both postnatal reproductive development (Copping *et al.* 2018) and meat quality traits (Alvarenga *et al.* 2016) were affected in the entire male progeny from the present study. Information presented in this study may provide insights into the underlying molecular pathways that promote susceptibility to increased fat deposition in the growing animal subsequent to protein restriction during early oocyte and/or embryo development.

#### Fetal organ development

The thrifty phenotype hypothesis (Hales and Barker 2001) suggests that gestational undernutrition induces nutrients and



energy to be diverted to ensure the development of critical organs such as the brain at the expense of organs and systems that may be less important for the immediate survival of the fetus (e.g. kidney; Reynolds and Caton 2012). These adaptations have been shown in both human and animal studies to be a contributing factor to the development of metabolic diseases in adult life (McMillen and Robinson 2005) and, in production animals, to affect economically important reproductive (Sullivan *et al.* 2009a; Mossa *et al.* 2013; Copping *et al.* 2018) and carcass (Long *et al.* 2010; Micke *et al.* 2010a; Alvarenga *et al.* 2016) traits.

The periconception period is acknowledged as a particularly sensitive developmental stage (Fleming *et al.* 2015). During the preimplantation period, primary patterns of development are established within the embryo, including the formation of cell lineages (Alberio 2018) and re-establishment of DNA methylation patterns (Dobbs *et al.* 2013). In studies in rodents and ruminants, embryos have been shown to respond to nutritional challenges during this period by permanently changing the pattern of development to protect fetal growth and optimise fitness (Fleming *et al.* 2015; Velazquez 2015). These compensatory changes may, in part, underlie the observed effects of the PERI diet on fetal growth and hepatic gene expression. In the present study, protein restriction during the PERI period had negative effects on embryo survival (Copping *et al.* 2020) and fetal growth (CRL) as early as 36 dpc (Copping *et al.* 2014). This is in agreement with previous studies that have shown that nutritional stress in both the pre- and immediate postinsemination period in beef heifers may affect embryo survival and delay embryo development (Perry *et al.* 2016; Kruse *et al.* 2017). In the 98 dpc fetus, protein restriction during the PERI period had limited effects on organ development, but was associated with changes in fetal hepatic gene expression. In comparison, the POST diet affected the development of several major organs in the 98 dpc fetus, reflecting the differential compensatory effects on fetoplacental growth depending on the timing of the nutritional insult (Nathanielsz 2006).

In the present study, the LPost diet affected the absolute weight of the heart (Hernandez-Medrano *et al.* 2015) and its structures in 98 dpc fetuses, along with absolute liver and lung weights. These data are consistent with earlier female bovine fetal studies (Long *et al.* 2009; Mossa *et al.* 2013). When each fetal sex was analysed separately, the developing cardiovascular system was affected in the female only, associated with altered cardiovascular development that persisted to the neonatal and adolescent period (Hernandez-Medrano *et al.* 2015). These findings correspond with those of an earlier study (Mossa *et al.* 2013).

Absolute liver weight at 98 dpc was reduced by the LPost diet, but only in the male. Relative liver weight was unaffected in the males, but increased in LPost female fetuses. In sheep, maternal nutrient restriction has been shown to not only alter fetal liver growth, but to also be associated with changes in hepatic function (Lie *et al.* 2014). The increased relative liver weight in the LPost female fetus is dissimilar to the observed effects on fetal hepatic gene expression that occurred in the LPeri cohort: in the LPeri female, hepatic gene expression of *IGF2*, along with *GR*, was reduced concomitant with reduced

circulating maternal *IGF2* (Copping *et al.* 2020). This has not been reported previously and may be permissive to the sex-specific fetal growth retardation observed at 36 dpc in the female cohort (K. J. Copping, unpubl. data), the *GR* gene (Nuclear Receptor Subfamily 3 Group C Member 1; *NR3C1*) being a possible candidate for epigenetic regulation of growth in the fetus (Lillycrop *et al.* 2007). Hepatic *GR* plays an important role in embryogenesis and lipid and carbohydrate metabolism. The action of *GR* in the liver is essential for normal body growth because it is a coactivator in growth hormone signalling on its dependent genes, such as *IGF1* and *IGF2* (von Horn 2002). Because the liver is a major target organ for glucocorticoids, the role of hepatic *GR* may be particularly important in regulating sex-specific responses in the progeny (Tronche *et al.* 2004). Similarly, a concomitant effect on *IGF1R* gene expression was seen in the LPeri female cohort in this study.

The LPeri female fetuses also exhibited increased hepatic gene expression of *RXR* and *PPAR $\gamma$* , whereas the LL male fetuses exhibited increased expression of *GLUT1*. The function of *PPAR* is integral to lipid and carbohydrate homeostasis, with altered function related to dyslipidaemia (Brown and Plutzky 2007). Therefore the effects on the female fetal liver may be permissive to increased adipogenesis after exposure to a maternal low-protein diet during the PERI period. This may also hold true for the male fetuses, in which a different mechanism of increased lipogenesis via activation of *GLUT1* was observed; susceptibility to increased postnatal adiposity in the carcass has been noted in 4-month-old sheep following exposure to a low-protein diet during the periconception period (Nicholas *et al.* 2013). The decreased *FOXO1* expression in the LPeri cohort in this study is similar to that reported by Nicholas *et al.* (2013). *FOXO1*, together with *PGC $\alpha$*  and *PDK1*, regulates the expression of the gluconeogenic rate-limiting gene *PEPCK-C*. Both *PDK1* and *PEPCK-C* were downregulated by the LPeri diet in male and female fetuses, although *PGC $\alpha$*  was downregulated by the low protein maternal diet only in female fetuses.

Glucocorticoids regulate the hepatic gluconeogenic rate-limiting genes such as *PEPCK-C* (Yabaluri and Bashyam 2010). The altered maternal metabolite profiles in these protein-restricted yearling heifers (Copping *et al.* 2020) may have led to increased fetal glucocorticoid exposure. Therefore, the observed effects on hepatic genes may reflect a physiological fetal survival mechanism following gestational protein restriction. Elevated glucocorticoids are also associated with reductions in the placental *IGF* system and production of placental hormones (Sferruzzi-Perri *et al.* 2017), as observed in these heifers, attendant to reduced fetal growth.

Protein restriction also affected the development of the fetal pancreas, lung and brain at 98 dpc. Pancreas and lung weights were reduced by the LPost diet. The functional outcomes of these changes in the bovine are unknown, and further histology of both the fetal and adult pancreas and lung is required to facilitate understanding of the relationship between size and function, as explored previously in IUGR sheep (Joyce *et al.* 2001; Limesand *et al.* 2006).

Protein restriction during the POST period increased brain weight assessed relative to fetal weight in male but not female fetuses at 98 dpc, indicative of a brain-sparing effect in this

cohort. Previous studies in sheep have reported similar effects in IUGR fetuses and attributed this to resources being reallocated to spare brain metabolism to the detriment of the development of other organs, namely trunk and abdominal viscera (McMillen *et al.* 2001; Osgerby *et al.* 2002). However, these studies did not differentiate fetuses by sex. Further the brain : liver ratio in this study was higher in LPost male fetuses, providing additional evidence of brain sparing as noted in IUGR models in sheep (Wallace *et al.* 2002; Field *et al.* 2015). Together, the observed differences in fetal measures and indices of growth suggest protein restriction resulted in both sex-specific IUGR and asymmetric development in the fetuses by 98 dpc. Such asymmetric IUGR is reported to be related to uteroplacental insufficiency and redistribution of fetal blood flow (Platz and Newman 2008).

### *Placental parameters*

Similar fetal growth restriction in both sheep (McMillen *et al.* 2001) and cattle (Long *et al.* 2009; Sullivan *et al.* 2009b) has been linked to changes in placental development. The present study reported a similar average placental weight (4.9 kg) to that of dairy cattle (5 kg; Laven and Peters 2001) but higher than the 4.1 and 3.9 kg reported in 2- (Miguel-Pacheco *et al.* 2017) and 3-year-old calving beef heifers (Sullivan *et al.* 2009b). Placental efficiency in the present study, a measure of the capacity of the placenta to support growth of the fetus (Fowden *et al.* 2009), was lower (7.0 : 1) than the 8.5 : 1 reported by Sullivan *et al.* (2009b). This apparent reduction in efficiency may be attributed to the age difference of the heifers, because placental efficiency in beef heifers increases with age (Sullivan *et al.* 2009b) and the present study represents an adolescent pregnancy with competing requirements between the dam and the fetus for nutrients (Wallace *et al.* 2004).

The PERI and POST diets were associated with altered placental parameters at 98 dpc but not at term, analogous to the observed effects in the fetus and term neonate. The reduced number of placentomes at 98 dpc in LPeri heifers and the reduction in placentome volume and weight in LPost heifers suggests an adaptation of the placental phenotype in response to maternal protein restriction and consequent metabolic status of the heifers. Sullivan *et al.* (2009b) reported that restricted maternal dietary protein intake in heifers (between 93 and 180 dpc) was associated with a reduction in cotyledon number at parturition, suggesting that there may be capacity for the bovine placental unit to adjust beyond the first trimester. This is consistent with the reduction in both cotyledon and caruncular weights at 125 dpc noted in placentas from heifers (with female fetuses) that were restricted (50% vs 100%) between 30 and 125 dpc (Zhu *et al.* 2007). Following realimentation in these heifers, only cotyledon weight remained affected at 250 dpc; however, changes in placental vascularity became apparent by 250 dpc (Vonnahme *et al.* 2007). Interestingly, trophoblast volume in heifers that experienced protein restriction during the first trimester was increased at birth (Miguel-Pacheco *et al.* 2017). This adaptation may alter the functional potential of the placenta and thereby enable enhanced nutrient supply to the fetus during later gestation if maternal nutrition increases (Perry *et al.* 1999). Doppler measurements of blood flow and uterine artery

parameters from 120 dpc suggested that vascular supply to the placenta was similarly altered in the study animals once maternal diet treatment ceased (Hernandez-Medrano *et al.* 2015), a scenario also reported in cows realimented following nutritional restriction in early gestation, whereby blood flow to the ipsilateral horn was increased following realimentation but not during the period of restriction (Camacho *et al.* 2014).

As reported, male fetuses were heavier and longer in comparison with female fetuses at 98 dpc, as expected (Eley *et al.* 1978). Although both male and female fetus BW was similarly reduced by the LPost diet (8% and 10% respectively), different effects on fetal morphology and organ size were evident. Because males have higher growth and metabolism than females (Clarke and Mittwoch 1995), this may have altered sensitivity to the diet restriction. This finding is consistent with previous reports of sex-specific effects of dietary perturbation in early gestation on bovine fetal development (Micke *et al.* 2015; Taylor *et al.* 2018). In contrast, studies investigating perturbations occurring during the second trimester in the bovine (Miguel-Pacheco *et al.* 2017) and in the second and third trimesters in humans (Clifton 2010) report that the female is more responsive. The expected sex-differences in calf birth BW were disrupted at term (Copping *et al.* 2014), even though birth BW was normalised following maternal protein restriction ceasing on 98 dpc. It is possible that placental adaptations after restriction ceased were sufficient to meet the lower nutritional requirement of the usually smaller female, but not to completely mitigate the early suppression of growth in the male (Rosenfeld 2015). Although the mechanisms behind this effect are not clear, the reported sex-specific differences in uterine artery flow, Doppler fetoplacental perfusion indices and response of these indices following the maternal dietary interventions in these heifers may have contributed to this finding (Hernandez-Medrano *et al.* 2015). In addition, the sex-specific maternal endocrine adaptations to the maternal diet treatments (Copping *et al.* 2020) may represent a placental signalling mechanism enabling changes in fetoplacental growth, as noted previously in humans (Hodyl *et al.* 2010) and cattle (Micke *et al.* 2015; Taylor *et al.* 2018).

Collectively, this suggests that the maternal dietary treatments between -60 and 98 dpc, followed by feeding to meet nutritional requirement (but not realimentation), altered the fetal growth trajectory in a sex-specific manner. Further studies, including histology and gene expression studies, are required to examine the role of the placenta in this interaction as explored in other species (Hodyl *et al.* 2010; O'Connell *et al.* 2013).

### **Conclusion**

This study demonstrated that protein restriction during the periconception period (from -60 to 23 dpc) and first trimester (from 24 to 98 dpc) decreases early fetal growth, alters placenta parameters and produces asynchronous organ development in the bovine. Similarly, hepatic gene expression is altered in fetal bovine progeny in a sex-specific manner. This may underlie, in part, the sex-specific responses to gestational dietary perturbations reported previously in the adult (Micke *et al.* 2010a, 2011). We propose that an accelerated growth response in restricted

female fetuses, subsequent to the return to 100% of nutrient requirements, reduced the 98 dpc differences and thereby the normal disparity between heifer and bull calf birth BW. Because distinct IUGR was observed at 98 dpc but not at birth, this agrees with previous bovine studies that birth BW is not a satisfactory indicator of IUGR. Thus, quantification of the effects of heifer nutrition programs on long-term productivity of the progeny remains an important area of study.

### Conflicts of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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