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Fetal programming in 2-year-old calving heifers: peri-conception and first trimester protein restriction alters fetal growth in a gender-specific manner

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Abstract. Protein restriction in early bovine gestation affects post-natal reproduction and production traits in progeny. This experiment evaluated the effects of dietary protein restriction during the peri-conception period and first trimester in yearling heifers on conceptus growth and development; this period of dietary intervention being earlier than any previous bovine fetal programming studies. Three-hundred and sixty primiparous 12-month-old Santa Gertrudis heifers were individually fed high [14% crude protein (CP)] or low (7% CP) diets for 60 days before conception. At 23 days post-conception (dpc), each high (HPERI) or low (LPERI) group was again split into high (HPOST) or low (LPOST) protein groups yielding four treatment groups in a 2 × 2 factorial design. From the end of the first trimester of gestation (98dpc), the pregnant heifers were individually fed a 12% CP diet until parturition. Forty-six fetuses were excised at 98dpc. Sixty-four heifers went on to calve. Conceptus development was assessed via transrectal ultrasound from 36dpc, fetal necropsy at 98dpc and live calf measures at term. At 36dpc, HPERI diet increased fetal crown–rump length (CRL) ($P < 0.05$) and at the 60dpc scan, biparietal diameter (BPD) tended to be increased by HPOST diet ($P < 0.1$) though the greater effect upon BPD was still the HPERI diet ($P < 0.05$). At 60dpc, BPD in the male fetus was affected by the peri-conception diet ($P < 0.05$), while in females, BPD was not different among nutritional groups. These ultrasound measures of fetal growth were validated by measures of the excised fetus at 98dpc. Fetal weight was heavier ($P < 0.01$) in those whose mothers were fed the HPOST diet than their LPOST counterparts. Males fetuses were heavier than female fetuses ($P < 0.001$). Fetal CRL was increased by HPERI diet ($P < 0.05$) and tended to be increased by HPOST diet ($P < 0.1$). Fetal BPD tended to be increased by HPERI diet ($P < 0.1$). In males, BPD tended to be increased in those fetuses whose mothers were fed HPERI ($P < 0.1$). For females, maternal nutrition during PERI or POST did not affect BPD at 98dpc ($P > 0.1$). At term, no dietary effect on birthweight was observed ($P > 0.1$) and males were not heavier than females ($P > 0.1$). These results suggest that maternal protein intake during the peri-conception (–60 to 23dpc) and first trimester (24–98dpc) may influence early conceptus growth and development in the bovine. The long-term effects on offspring metabolism and post-natal development of this dietary intervention are yet to be determined.

Additional keywords: beef, embryo, fetus, nutrition, oocyte.

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Introduction

Protein is the major limiting nutrient in range beef cattle in Australia. It has previously been established that protein restriction in early gestation may affect post-natal reproduction and production traits in progeny (Sullivan *et al.* 2009, 2010; Micke *et al.* 2010*a*, 2011; Mossa *et al.* 2013). Further, studies have also shown effects of maternal diet upon fetal growth to 123 days post-conception (dpc) after dietary intervention 30–123dpc (Long *et al.* 2009) although this effect was no longer apparent at birth (Long *et al.* 2012).

Critical windows exist during oocyte, embryo and fetal development where decreased maternal dietary protein may reset early cell lineages (Wu *et al.* 2006). Maternal nutrition affects both the number of oocytes that ovulate and their quality (Ashworth *et al.* 2009), which has then been associated with differences in embryo survival (Borowczyk *et al.* 2006). The gender of an embryo has also been shown to affect the susceptibility of the embryo to altered nutrition before mating (Vinsky *et al.* 2006). Gender-specific differences in embryo DNA methylation pattern (Dobbs *et al.* 2013), expression of key developmental genes (Kwong *et al*. 2006), fetal and placental perfusion (Prior *et al*. 2013) post-natal carcass traits (Micke *et al*. 2011) have all been reported. Intriguingly, neonatal and fetal death in cattle is increased if the fetus is male, as with most mammalian species.

The aim of this study is to further evaluate the critical windows during development where dietary protein may alter fetal development. By examining the effects of protein diets similar to those experienced in range conditions in supplemented and non-supplemented breeders during the period from oocyte development in the ovary to the end of the first trimester we aim to establish the effects of such restriction.

This paper focuses on the effects of protein restriction in early life upon conceptus growth and development. We hypothesise that low maternal protein during the peri-conception period and the first trimester will reduce fetal growth in a gender dependent manner.

Materials and methods

This project was approved by the University of South Australia IMVS Animal Ethics Committee (Approval number 18/11) on 11 March 2011.

Experimental animals

Three-hundred and sixty primiparous 12-month-old Santa Gertrudis (*Bos taurus* × *Bos indicus*) heifers were selected based on weight and age from SK Kidman herds at Glengyle and Morney Plains, south-western Queensland. All heifers were vaccinated against viral and bacterial diseases on two occasions 4 weeks apart before transport. The heifers were transported to Sedan, South Australia to purpose-built, shaded feedlot pens, acclimatised to diet, and trained to individual stall feeding.

At 12 months of age, 60 days before artificial insemination (AI), the heifers were randomly assigned to two equal groups stratified by weight. Each heifer was individually fed a high [14% crude protein (CP)] or low (7% CP) protein diet consisting of pellet diet fed in stalls and straw (5% CP) *ad libitum* in pens. The digestible energy content of the diet was as similar as possible in the ruminant and supplemented with a vitamin and mineral commercial preparation (Table 1).

Heifers underwent an 8-day progesterone-based oestrous synchronisation program. On Day 0, heifers were AI sequentially with frozen semen from one low birthweight Santa Gertrudis bull, selected on the basis of estimated breeding value.

At 23dpc each high (HPERI) or low (LPERI) group was again split into high (HPOST) or low (LPOST) protein groups yielding four treatment groups [high high (HH), high low (HL), low high (LH), low low LL)] in a 2 × 2 factorial design. Detailed composition of the heifer rations is given in Table 1.

Pregnancy was positively diagnosed in 124 heifers (HH = 33; HL = 37; LH = 30; LL = 28) at 36dpc via transrectal ultrasound and non-pregnant heifers were removed from the trial. At 60dpc fetuses were sexed via ultrasonography enabling equal numbers of sex fetuses to be excised at slaughter from each of the four treatment groups. From the end of the first trimester of gestation (98dpc), the remaining pregnant heifers were individually fed at the NRC recommended ration for weight containing 12% CP until parturition.

Table 1. Ingredients and nutrient content of heifer rations for peri-conception and during the first trimester of gestation

	Induction	Peri-conception		First trimester	
		High	Low	High	Low
Ration as fed					
Wheat (kg)	0.66	0.48	1.81	0.56	2.12
Canola meal (kg)	2.23	–	–	–	–
Soybean meal (kg)	–	1.83	0.48	2.14	0.56
Barley straw (kg)	7[B]	6.7	5.5	10.7	10.2
Molasses (g)	90	72	72	84	84
Biofos MDCP (g)	–	–	19	–	22
Salt (g)	15	12	12	14	14
Vitamin/trace mineral premix (g)	3	2	2	3	3
Dry matter	9.1[C]	8.3	7.2	12.3	11.8
Total energy (MJ ME)	–	71	63	102	98
% of energy requirements[A]	–	96	85	142	136
Total crude protein (kg)	–	1.18	0.62	1.49	0.88
% of protein requirements[A]	–	127	67	123	72
% CP (total diet)	–	14.2	8.6	12.1	7.4
Total calcium (g)	–	26	22	38	37
% of calcium requirements[A]	–	130	110	190	185
Total phosphorus (g)	–	17	17	21	21
% of phosphorus requirements[A]	–	130	130	160	160

[A]Dietary requirements were calculated using Nutrient Requirements of Domesticated Ruminants (2007, CSIRO). 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.
[B]Assumed value.
[C]Predicted value.

Fetal calf measurements

Fetuses were measured using transrectal ultrasound (Sonosite M-Turbo; Sonosite Inc., Bothell, Washington, DC, USA) at 36, 60 and 95dpc. Fetal sex was determined at 60dpc by rectal ultrasound. Measurements of the conceptus were taken at the time of ultrasonsography (Micke *et al*. 2010*b*). All fetal body measurements were in centimetres. Crown–rump length (CRL) was measured from a lateral view of the fetus from the tip of nose to the base of the tail. Biparietal diameter (BPD) was measured from a dorso-ventral view of the cranium perpendicular to the sagittal crest at the widest span between the most lateral parts of the parietal bone.

Fetal excision 98dpc

A subset of heifers (HH = 12; HL = 15; LH = 10; LL = 9) were killed in a commercial abattoir at 98dpc and used for human consumption. Tissues for examination were immediately collected from the uterus and fetus after removal from the kill floor. The fetus was excised from the uterus, cord blood collected, weighed, measured and then dissected. Sex of fetus was recorded during dissection. Measures of BPD and CRL were obtained from fetuses using sliding calipers. The remaining heifers were taken to term.

Calf measurements

At calving, heifers (*n* = 64; 19 female, 45 male) were monitored individually and assistance provided where necessary. Calves were collected within 15 min of birth, before sucking, and sex, birthweight and CRL recorded.

Statistical analyses

Multifactorial ANOVA using STATA SE version 11 (Stata Corporation, College Station, TX, USA) was used to interpret the main effects of peri- and post-conception diet, sex and their interaction terms on ultrasound measures of embryo and fetal body dimensions from 36dpc, fetal measures at 98dpc and live calf measures at term. Individual calf was considered the experimental unit, with gestation length as a co-variate for birth measures only. Males and females were analysed together and independently. A probability of 5% ($P < 0.05$) was accepted as the level of significance and trends reported as $P < 0.1$. Data presented as mean ± standard errors of the mean.

Results

Fetal measures *in utero*

At 36dpc high protein (14% CP) preconception diets increased fetal crown–rump length (CRL) compared with low protein (7% CP) ($P < 0.05$) (Fig. 1) and by 60dpc BPD tended to be increased by diet post-conception ($P < 0.1$) though the greater effect upon BPD was still peri-conception diet ($P < 0.05$) (Fig. 1). In males, BPD was increased in those fetuses whose mothers were fed HPERI ($P < 0.05$). For females, maternal nutrition during PERI or POST did not affect BPD at 60dpc ($P > 0.1$).

Weight gain during peri- and post-conception

Initial weight of the heifers at acclimatisation was 350 kg. The average daily gain of heifers was 0.4 kg/day during the high and 0.2 kg/day during the low protein treatment periods (Fig. 2).

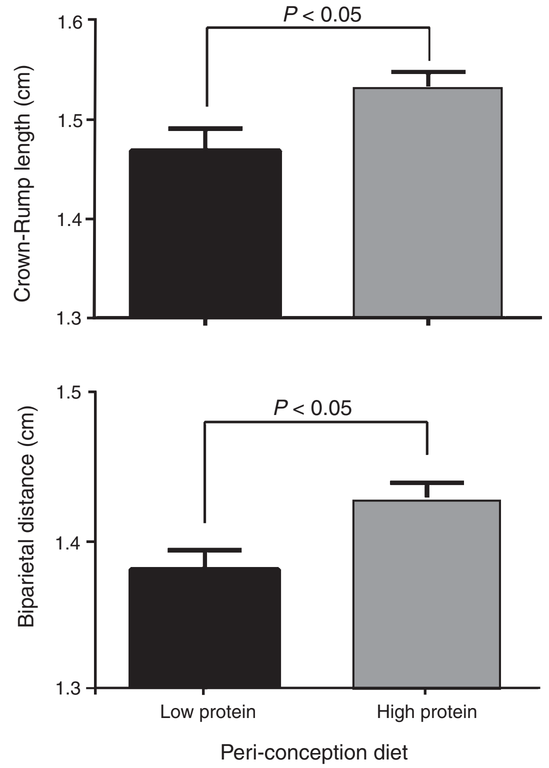

Fig. 1. The effects of peri-conception dietary protein; high (14% CP), low (7% CP) upon fetal growth at 36 and 60 gd (days gestation). Values are unadjusted means ± s.e.m.

Fetal size at 98dpc

In measures of the excised fetus at 98dpc, fetal weight was heavier ($P < 0.01$) in those whose mothers were fed HPOST diet (HH + LH = 326.3 ± 6.7 g) compared to their low counterparts (HL + LL = 301.2 ± 7.6 g). Male fetuses were heavier than female fetuses ($P < 0.001$). Fetal CRL at 98dpc was increased by HPERI diet compared to LPERI ($P = < 0.05$) and by this stage of development also tended to be increased by HPOST diet compared to LPOST ($P < 0.1$). Fetal BPD tended to be increased by HPERI diet ($P < 0.1$). In males, BPD tended to be increased in those fetuses whose mothers were fed the HPERI diet compared to LPERI ($P < 0.1$). For females, maternal nutrition during PERI or POST did not affect BPD at 98dpc ($P > 0.1$).

Calf size at birth

There was no effect of dietary treatment on birthweight, CRL or head measures (all $P > 0.1$). Gestation length did however affect birthweight ($P < 0.05$). Male calves were not larger or heavier at birth than female calves ($P > 0.1$).

Discussion

The present study demonstrates a significant difference in bovine conceptus size as early as 36 days post-conception in

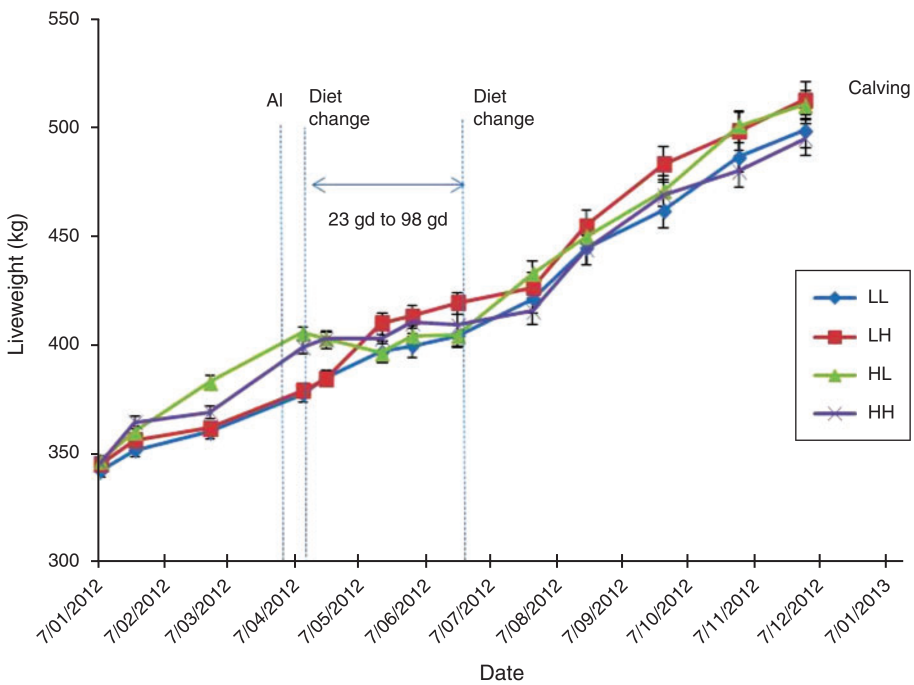

Fig. 2. Liveweight over time of heifers in each dietary treatment group. HH = high protein peri-conception and first trimester, LL = low protein peri-conception and first trimester, HL = high protein peri-conception and low protein first trimester, LH = low protein peri-conception and high protein first trimester.

response to maternal dietary protein restriction during peri-conception and first trimester. Further, this effect was gender specific there being a greater effect of early dietary intervention on measures of male fetal growth at least until the end of the first trimester.

Nutritional intervention

The 360 heifers were individually fed for a total of 14 months (including acclimatisation) and inseminated via fixed time AI over 1.5 days. Thus, the nutritional regimens were instigated at exactly the same time points in gestation and were controlled with heifers being fed individually from 60 days before AI and throughout gestation until term. This is similar to previous fetal programming studies reported by (Sullivan *et al*. 2009; Micke *et al*. 2010*a*) but not others (Cafe *et al*. 2006; Greenwood and Cafe 2007; Hickson *et al*. 2008; Long *et al*. 2009, 2012). Furthermore, in these latter three studies, realimentation of the restricted heifers was applied so that all calved at similar weight. In this study the high protein diet group had average daily gain of 0.4 kg/day and low protein 0.2 kg/day over the treatment period. After this time they continued to be individually fed throughout gestation at positive weight gain [unlike studies with negative gain in the low treatment (Cafe *et al*. 2006)]; there being also no attempt, to attain similar liveweight at parturition. This difference in (1) nutritional window of dietary intervention and (2) severity and period of restriction applied, may explain the apparently conflicting results in fetal development reported from bovine fetal programming studies.

Sexual dimorphism

This period of dietary intervention is earlier than any previous studies investigating bovine fetal growth trajectory in utero. The sexual dimorphism observed, such that the effect peri-conception protein restriction effect was greater in the male, supports our previous findings in beef cattle (Micke *et al*. 2011, 2014). This suggests that the male embryo is more susceptible to early gestational intervention than the female, as seen in the *in vitro* embryo (Bermejo-Alvarez *et al*. 2011), and that this effect continues until at least 98dpc. The reason for this gender difference may be that the pattern of DNA methylation during embryo development in the two sexes is different over time making susceptibility to epigenetic change both gender and time dependent (Dobbs *et al*. 2013). We note however, that the expected gender effect on birthweight where the male is larger and heavier than the female was not observed in this study.

Birthweight

The finding that maternal peri-conception and first trimester nutrition did not affect birthweight at term may be an important dystocia management consideration when considering timing of supplementation to heifers. This is in agreement with previous reports following first trimester intervention (Micke *et al*. 2010*b*) and between 30 and 123dpc (Long *et al*. 2009). If restriction is applied during the second trimester (Micke *et al*. 2010*b*), however, or second and third trimester (Cafe *et al*. 2006) birthweight has reportedly been affected.

The lack of effect on gross morphology at birth, however, does not necessarily reflect the impact of early gestational intervention upon long-term growth, development and health in the offspring (Wu *et al*. 2006) as we have previously reported. In sheep, peri-conceptional undernutrition does not necessarily result in altered birthweight but has been associated with altered fetal growth trajectory and altered post-natal growth, metabolic and endocrine regulation (Jaquiery *et al*. 2011). The post-natal effects of similar periods of peri-conceptional undernutrition in the bovine are less well understood. Further studies of the post-natal development of the progeny are under way.

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