Heifer nutrient intake during early- and mid-gestation programs adult offspring adiposity and mRNA expression of growth-related genes in adipose depots

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Abstract

Changes in maternal nutrient intake during gestation alter *IGF* receptor abundance and leptin (*LEP*) mRNA expression in fetal adipose tissue. It is not known whether such changes persist into adult life and whether they are associated with an effect on phenotype. We investigated the effect of high (240%) and low (70%) levels of recommended daily crude protein intake for beef heifers during the first and second trimesters of gestation on singleton progeny ($n=68$): subcutaneous (SC) adipose tissue depth at rump (P8) and rib (RF) sites from 65 until 657 days of age; plasma leptin concentrations from birth until 657 days and expression of *IGF1* and *IGF2*, their receptors (*IGF1R* and *IGF2R*) and *LEP* mRNA in perirenal (PR), omental (OM) and SC adipose tissue at 680 days of age. High-protein diets during the first trimester increased *LEP* and *IGF1* mRNA in PR of males and females, respectively, compared with low-protein diets, and decreased *IGF1R* mRNA in SC of all progeny but increased RF depth of males between 552 and 657 days. High-protein diets compared with low-protein diets during the second trimester increased *IGF1R* mRNA in PR and OM of all progeny; *LEP* mRNA in PR of males; and *IGF2* and *IGF2R* mRNA in OM of all progeny. Conversely, *LEP* mRNA in OM and *IGF2* mRNA in PR of all progeny were decreased following exposure to high- compared with low-protein diets during the second trimester. Heifer diet during gestation has permanent sex- and depot-specific effects on the expression of adipogenic and adipocytokine genes and offspring adiposity.

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Introduction

Adipose tissue has an important regulatory and homeostatic function (Hausman *et al.* 2009) as well as being a significant contributor to the commercial carcass value of beef cattle (Robinson *et al.* 2001). Epidemiological studies have demonstrated a strong association between maternal nutrient intake during gestation and the later risk of obesity and development of the metabolic syndrome during adulthood (McMillen & Robinson 2005). Adipogenesis is induced before birth in a range of species including humans, sheep and cattle, and is regulated by the activation of the insulin-like growth factor (IGF) type 1 receptor (*IGF1R*) via the binding of IGF1 or insulin (Teruel *et al.* 1996). During fetal development, the somatotrophic axis is regulated by fetal nutrient supply (Holt 2002), which is a function of the interplay between maternal nutrient intake and maternal maturity (Wallace *et al.* 2005). In the mature animal, hypertrophy and hyperplasia of adipocytes result in changes to adiposity (Gregoire 2001). This process is regulated by the interaction of IGF1 and leptin (*LEP*), an adipocytokine that controls caloric intake, energy

expenditure (Prins 2002, Louveau & Gondret 2004) and regulates nutritional status effects on reproductive function (Houseknecht *et al.* 1998). Several studies on the effects of fetal nutrient supply during gestation on adipose tissue development in the sheep have demonstrated that the effects are time- and depot-specific. For example, maternal nutrient restriction during the period of maximal placental development results in up-regulation of *IGF1R* and *IGF2R* mRNA abundance in fetal perirenal (PR) adipose tissue (Bispham *et al.* 2003), but placental restriction due to carunclectomy prior to mating results in decreased expression of *IGF1* and *LEP* mRNA in PR (Duffield *et al.* 2008). Conversely, maternal overfeeding during the final month of pregnancy results in up-regulation of *LEP* mRNA expression in fetal PR and subcutaneous (SC) adipose tissue (Muhlhausler *et al.* 2007a). However, the long-term effects of prenatal nutrition on *IGF* and *LEP* mRNA expression in adipose tissue of mature offspring are not clear.

Pasture protein content can vary tenfold throughout the year (Schut *et al.* 2010). In northern Australia, protein supplementation of breeder herds is necessary to

maintain body condition during the dry season (Bortolussi *et al.* 2005), while in Southern Australia, breeder cattle often graze winter and spring pastures that provide greater than the recommended level of protein (Walsh & Birrell 1987). We have previously reported that male fetuses exposed to a low level of maternal nutrient intake during the first trimester were heavier throughout the postweaning period compared with their high-nutrient exposed counterparts. Females, on the other hand, exposed to a high level of maternal nutrient intake during the first trimester of gestation were heavier just prior to slaughter (Micke *et al.* 2010a). Since adipose tissue is an important contributor to traits of economic importance to the beef breeding enterprise such as neonatal survival, carcass value and reproductive performance. We investigated the effect of heifer nutrient intake during the first and second trimesters of gestation on the relative expression of *IGF1*, *IGF2*, *IGF1R*, *IGF2R* and *LEP* mRNA in PR, omental (OM) and SC adipose tissue of their offspring at 680 days (d) of age. We hypothesized that feeding maternal diets low in nutrients during either the first or second trimester of gestation would result in a lower expression of *IGF1*, *IGF2* and *LEP* mRNA and a higher expression of *IGF1R* and *IGF2R* mRNA in the adipose tissue of mature progeny compared with adipose tissue from progeny exposed to maternal diets high in nutrients during these periods.

Results

Postnatal growth and SC adipose tissue accumulation

We have previously reported that in this cohort progeny that were exposed to high levels of maternal nutrition during T2 were 8.3% heavier at birth (HH+LH = 33.05 ± 0.81 kg) compared with their counterparts exposed to low levels of maternal nutrition (HL+LL = 30.76 ± 0.59 kg; $P=0.03$) (Micke *et al.* 2010b). From 191 to 657 d, however, males that were exposed to low levels of maternal nutrition during T1 (LH+LL) were ~4% heavier than their counterparts exposed to high levels of maternal nutrition (HH+HL; $P=0.04$) (Micke *et al.* 2010a). In contrast, females exposed to a high level of maternal nutrition during T1 had 6.9% heavier carcasses (HH+HL: 334.53 ± 5.54 kg) compared with their low-nutrient exposed counterparts (LL+LH: 312.92 ± 6.82 kg; $P=0.04$) (Micke *et al.* 2010a). Males were heavier than females throughout the study ($P<0.01$).

In the current study, the depth of RF adipose tissue from 65 until 657 d tended to depend on maternal nutrition during T1 $\times$ time $\times$ sex ($P=0.07$), and we therefore analyzed each sex separately. For males, the effect of maternal nutrition during T1 changed with time ($P<0.01$) as RF depth was 67% greater from 552 to 657 d following exposure to a high (HH+HL) compared to low (LH+LL) level of maternal nutrition during the first

trimester ($P<0.01$; Fig. 1, Panel A). There was no effect of maternal nutrition during T1 on RF depth of females. There was no effect of maternal nutrition during T1 or T2, sex or gestation length on RF depth relative to liveweight, P8 depth or P8 depth relative to liveweight from 65 until 657 d.

PR adipose tissue mRNA expression

Expression of *IGF1* mRNA in PR adipose tissue depended on the effect of maternal nutrition during T1 $\times$ sex ($P=0.05$), and we therefore analyzed each sex separately. For males, there was no effect of maternal nutrition during T1 (data not shown), but for females, those exposed to a high (HH+HL) compared with low (LL+LH) level of maternal nutrition during the first trimester had 42% greater *IGF1* mRNA expression in PR ($P=0.05$). There was no effect of maternal nutrition during the second trimester on *IGF1* mRNA expression in PR adipose tissue of males or females (data not shown).

Expression of *IGF1R* mRNA in PR adipose tissue of all progeny was 50% greater following exposure to a high (HH+LH) compared with low (LL+HL) level of maternal nutrition during the second trimester of gestation ($P<0.01$; Fig. 2, Panel A). There was no effect of maternal nutrition during the first trimester on *IGF1R* mRNA expression in PR adipose tissue (data not shown).

Expression of *IGF2* mRNA in PR adipose tissue of all progeny was 20% greater following exposure to low (LL+HL) compared with high (HH+LH) levels of maternal nutrition during the second trimester of gestation ($P=0.04$; Fig. 2, Panel B) but there was no effect of maternal nutrition during T1 (data not shown).

Figure 1 Depth of adipose tissue between the 12th and 13th ribs of male (Panels A and C) and female (Panels B and D) progeny during the post-weaning period by trimester treatment group. Asterisks denote a significant effect of maternal nutrition ($P<0.01$).

Figure 2 The expression of *IGF1R* (Panel A) and *IGF2* (Panel B) mRNA relative to *PPIA* mRNA expression in perirenal adipose tissue of cattle at 680 d following exposure to maternal diets high or low in nutrients during the second trimester of gestation. Asterisks denote a significant effect of maternal nutrition ($P < 0.05$).

Expression of *LEP* mRNA in the PR depot depended on maternal nutrition during $T1 \times \text{sex}$ ($P = 0.02$) and maternal nutrition during $T2 \times \text{sex}$ ($P = 0.02$) and we therefore analyzed each sex separately. Males exposed to a high level of maternal nutrition during either the first (HH+HL; $P < 0.01$) or second (HH+LH; $P = 0.04$) trimester of gestation had 116 and 64% greater *LEP* mRNA expression respectively compared with their low-nutrient exposed counterparts (Fig. 3). There was no effect of maternal nutrition during T1 or T2 on PR *LEP* mRNA expression in females (data not shown). There was no effect of maternal nutrition during T1 or T2 on *IGF2R* mRNA expression in PR adipose tissue (data not shown). There was no sex or gestation length effect on *IGF1*, *IGF1R*, *IGF2*, *IGF2R* or *LEP* mRNA expression in PR adipose tissue (data not shown).

Omental adipose tissue mRNA expression

Expression of *IGF1R* ($P = 0.02$), *IGF2* ($P = 0.02$) and *IGF2R* ($P = 0.03$) mRNA in OM adipose tissue were 24, 27 and 17% greater respectively for progeny exposed to a high (HH+LH) compared with low (LL+HL) level of maternal nutrition during the second trimester (Fig. 4, Panels A–C). In contrast, *LEP* mRNA expression in OM adipose tissue was 28% greater in progeny that were exposed to low (LL+HL) compared with high (HH+LH) level of maternal nutrition during the second trimester of gestation ($P = 0.04$; Fig. 4, Panel D). Females had greater *IGF1* mRNA expression compared with males ($P = 0.02$) in OM adipose tissue. There was no effect of gestation length on *IGF1*, *IGF1R*, *IGF2*, *IGF2R* or *LEP* mRNA expression in OM adipose tissue. There was no effect of maternal nutrition during trimester one on the expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA expression, nor of trimester two maternal nutrition on *IGF1* mRNA expression (data not shown).

SC adipose tissue mRNA expression

Expression of *IGF1R* mRNA in SC adipose tissue was 29% greater for progeny that were exposed to a low (LL+LH) compared with high (HH+HL) level of maternal nutrition during the first trimester of gestation ($P = 0.02$; Fig. 5). There were no further effects of maternal nutrition during trimester one or two on the expression of the genes measured (data not shown). Males had greater *IGF2* ($P = 0.04$) and *IGF2R* ($P < 0.01$) mRNA expression in SC adipose tissue compared with females, but there was no effect of gestation length on expression of any of the genes measured in SC adipose tissue.

Progeny plasma leptin

There was no effect of maternal nutrition during T1 or T2, or of gestation length, on plasma leptin concentrations in progeny from birth to 657 d ($P > 0.1$). Females had higher plasma leptin than males throughout the study ($P < 0.01$; Fig. 6).

Relationship between progeny plasma IGF concentrations with IGF and LEP mRNA expression in adipose tissue

Plasma concentrations of IGF1, IGF2 and tIGFBP have been reported (Micke *et al.* 2010a). Significant correlation coefficients for associations between progeny plasma concentrations of IGF1, IGF2 and tIGFBP from birth to 657 d with expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA in PR, OM and SC adipose tissue are shown in Table 1.

Discussion

This study describes the long-term changes in *IGF* and *LEP* mRNA expression in progeny PR, OM and SC adipose tissue, and the relationship to the progression of SC adiposity over time, following exposure to two levels of maternal nutrition during gestation in the bovine.

Figure 3 The expression of *LEP* mRNA relative to *PPIA* mRNA expression in perirenal adipose tissue of males at 680 d following exposure to maternal diets high or low in nutrients during the first and second trimesters of gestation. Asterisks denote a significant effect of maternal nutrition between same trimester treatment groups ($P < 0.05$).

Figure 4 The expression of *IGF1R* (Panel A), *IGF2* (Panel B), *IGF2R* (Panel C) and *LEP* (Panel D) mRNA relative to *PPIA* mRNA expression in omental adipose tissue of cattle at 680 d following exposure to maternal diets high or low in nutrients during the second trimester of gestation. Asterisks denote a significant effect of maternal nutrition ($P < 0.05$).

The main findings are that the effects of maternal diet during the first two trimesters of gestation on expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA in the progeny are depot-specific. This is consistent with there being depot-specific responses of preadipocytes to exogenous factors that regulate preadipocyte differentiation (Soret *et al.* 1999). In our study, PR adipose tissue was susceptible to the programming effects of maternal nutrient intake during the first and second trimesters of gestation, whereas OM adipose tissue was only susceptible during the second trimester. SC adipose tissue was susceptible to the programming effects of maternal nutrient intake during the first trimester of gestation but in a manner which was opposite to either PR or OM adipose tissue. Despite PR and OM adipose tissue being susceptible to the programming effects during the same period of gestation and *IGF1R* being up-regulated by high levels of maternal nutrient intake during the second trimester in both depots, the expression of *IGF2* and *LEP* exhibited differential responses in each depot (see Table 2).

Nutrition

There was a 3.3- to 3.6-fold difference in CP content and 1.2- to 1.3-fold difference in energy content between high- and low-group diets, with both groups receiving above the recommended National Research Council (NRC) energy requirements. The degradable intake protein (DIP) balance during the first trimester was 206 g/day for the high-protein diet group compared with -345 g/day for the low-protein diet group and, similarly during the second trimester, the DIP balance for the high-protein diet group was 214 g/day compared with -464 g/day for the low-protein diet group. Therefore, the difference in CP between the high- and low-protein

diets was much greater than that of energy and the negative DIP balance for the low-protein diet group in both the first and second trimesters, which clearly demonstrates that protein intake was restricted. Although the difference in protein levels between the high (in excess of NRC requirements) and low (below NRC recommended requirements) treatment group diets was large, both the high and low treatment group diets had protein contents that are commonplace under Australia grazing conditions (Schut *et al.* 2010). Thus, the diets used in the current study would be considered relatively normal for breeder herds grazing Australian pastures. Considering the role of protein, especially in respect to the amino acid arginine, in placental angiogenesis and fetal growth (Kwon *et al.* 2004), and the much greater difference in protein compared with energy intake, the differences observed in our study due to the effects of maternal nutrition during the first and second trimesters of gestation are likely attributable to the effects of protein rather than energy intake. It is possible, however, that the over-supply of energy to heifers in the low-protein group during either trimester one or two may have dampened the effects of feeding low-protein diets. In sheep, overfeeding during late gestation increased offspring lipid accumulation in PR and SC adipocytes (Muhlhausler *et al.* 2007a), a change that may have been due to increased expression of *LEP* (Muhlhausler *et al.* 2007a) and adipogenic genes (Muhlhausler *et al.* 2007b). We consider there to be minimal differential environmental effects upon the developing fetus and thus postnatal outcomes measured in the progeny as the heifers in our study were artificially inseminated (AI) on the same day, housed under the same environmental conditions and calved over a short period.

In beef production, as in other production systems that suckle their young for a defined period, effects of prenatal nutrition may include an impact upon lactation. We have previously reported that milk intake was lower ($P = 0.02$) for progeny of heifers that received high-compared with low-protein diets during the first trimester and that milk protein content was also affected by maternal protein intake during the first trimester of gestation (Sullivan *et al.* 2009b). The absence of any significant correlations between *IGF* mRNA expression

Figure 5 The expression of *IGF1R* mRNA relative to expression of *PPIA* mRNA in subcutaneous adipose tissue of cattle at 680 d following exposure to maternal diets high or low in nutrients during the first trimester of gestation. Asterisks denote a significant effect of maternal nutrition ($P < 0.05$).

Figure 6 Plasma leptin concentrations of male and female progeny from birth until 657 days of age. * $P < 0.01$.

and milk production (data not shown) suggests that differences in nutritional intake during the pre-weaning period did not confound our results; however, cross-fostering studies would be required to confirm this. There is a well-established relationship between current nutritional status and circulating IGF concentrations during the postnatal period. Therefore, the lack of a consistent association between adipose tissue mRNA expression and concentrations of IGF1, IGF2 and tIGFBP in progeny plasma suggests that postnatal nutrient intake did not affect mRNA expression in adipose tissue. This finding also supports that the observed changes to mRNA expression were likely of fetal rather than postnatal origin. Interestingly, the significant correlation between *IGF1* mRNA expression in SC adipose tissue and plasma IGF1 concentrations suggests that this depot may be

a key source of endocrine IGF1 during the postnatal period while the perirenal depot contributes to the clearance of IGF2 from circulation.

Changes to gene expression affecting neonatal survival and adiposity

Our results suggest that maternal nutrient intake during the first and second trimesters of gestation may affect the thermoregulatory capacity of the neonate due to its effect on expression of *IGF1* and *IGF1R* mRNA in PR adipose tissue. The PR depot is the largest depot of brown adipose tissue in the newborn calf and plays a fundamental role in neonatal thermogenesis and survival prior to its transition to a white adipose storage depot by the end of the first month of life (Clarke *et al.* 1997). The successful conversion of preadipocytes to mature lipid-containing adipocytes requires IGF1 (Smith *et al.* 1988, Gregoire *et al.* 1998). Up-regulation of *IGF1* mRNA in females following exposure to maternal diets high in nutrients during the first trimester, combined with increased tissue sensitivity to the effects of IGF1 via increased *IGF1R* mRNA, may have enhanced progeny PR adipocyte cell number and, therefore, tissue lipid storage capacity. Importantly, this hypothesis is supported by the concurrent increase in *LEP* mRNA expression in PR adipose tissue of males exposed to a high level of maternal nutrient intake during the second trimester being also of heavier in birth weight than their low-nutrient exposed counterparts (Micke *et al.* 2010b). This is because *LEP* mRNA expression is directly associated with fetal growth (Duffield *et al.* 2008), fetal weight (Yuen *et al.* 1999) and level of maternal nutrient intake during gestation (Muhlhauser *et al.* 2007a).

Table 1 Correlation coefficients for associations of significance between progeny plasma IGF1, IGF2, total IGFBP (tIGFBP) and leptin from birth to 657 d and the relative expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA in perirenal (PR), omental (OM) and subcutaneous (SC) adipose tissue at 680 d of age.

		Age (days)					
Progeny plasma (ng/ml)		0 d	29 d	95 d	191 d	379 d	657 d
SC mRNA expression							
<i>IGF1</i>	IGF1	-0.0947	0.1261	0.0771	0.1220	0.1628	0.3511*
	tIGFBP	0.0125	0.0950	0.0449	0.1928	0.2636 [†]	0.3483*
	Leptin	-0.0858	0.2875 [†]	0.1122	-0.0244	-0.0165	0.0265
<i>IGF1R</i>	tIGFBP	0.3123 [†]	0.0906	0.0165	0.0491	0.1453	0.2715 [†]
	IGF1	0.0080	0.1889	0.1212	0.1805	0.2691 [†]	0.1990
<i>IGF2</i>	tIGFBP	0.3823*	0.1466	0.2766 [†]	0.1945	0.1559	0.2968 [†]
	tIGFBP	0.2599 [†]	0.0553	0.0952	0.0802	0.1412	0.2042
<i>LEP</i>	Leptin	0.1900	0.2690 [†]	-0.1185	-0.0189	-0.2509 [†]	0.1808
PR mRNA expression							
<i>IGF1</i>	IGF2	-0.2794 [†]	-0.1936	-0.0036	0.0149	0.0023	-0.3209 [†]
	IGF2	0.0035	-0.0816	-0.2141	-0.0354	-0.1199	-0.2883 [†]
<i>LEP</i>	tIGFBP	0.0292	0.3263 [†]	0.1073	0.1214	0.2215*	0.0791
	Leptin	-0.0047	0.2409 [†]	0.0181	0.4007*	0.1951	0.0785
OM mRNA expression							
<i>IGF1</i>	tIGFBP	0.2767 [†]	-0.0657	-0.0761	-0.0777	-0.104	0.0386

Probability is indicated by * $P < 0.01$; [†] $P < 0.05$; [‡] $P < 0.1$.

Table 2 Summary of significant effects of maternal nutrient intake during the first and second trimesters of gestation on progeny postnatal phenotype and relative expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA in perirenal (PR), omental (OM) and subcutaneous (SC) adipose tissue of male (M) and female (F) progeny at 680 d.

Trait	Trimester	Treatment group effect	Sex	Mean $\pm$ S.E.M.		Greater (%)	P value of trimester treatment group effect
				High	Low		
Phenotype							
Postweaning weight	1st	Low > high	M		Average	4.1	0.04
	1st	High > low	F		Average	3.2	0.07
Carcass weight (kg)	1st	High > low	F	334.5 $\pm$ 5.5	312.9 $\pm$ 6.8	6.9	0.04
Rib fat depth (552–657 d)	1st	High > low	M		Average	67	<0.01
Birth weight (kg)	2nd	High > low	M + F	33.1 $\pm$ 0.8	30.6 $\pm$ 0.6	8.3	0.03
PR mRNA expression							
<i>IGF1</i>	1st	High > low	F	0.06 $\pm$ 0.007	0.04 $\pm$ 0.004	42	0.05
<i>LEP</i>	1st	High > low	M	0.02 $\pm$ 0.003	0.010 $\pm$ 0.003	116	<0.01
<i>IGF1R</i>	2nd	High > low	M + F	0.008 $\pm$ 0.001	0.005 $\pm$ 0.0004	50	<0.01
<i>LEP</i>	2nd	High > low	M	0.02 $\pm$ 0.004	0.01 $\pm$ 0.003	64	0.04
<i>IGF2</i>	2nd	Low > high	M + F	1.7 $\pm$ 0.1	2.1 $\pm$ 0.1	20	0.04
OM mRNA expression							
<i>IGF1R</i>	2nd	High > low	M + F	0.005 $\pm$ 0.0003	0.004 $\pm$ 0.0002	24	0.02
<i>IGF2</i>	2nd	High > low	M + F	1.7 $\pm$ 0.1	1.3 $\pm$ 0.08	27	0.02
<i>IGF2R</i>	2nd	High > low	M + F	0.0030 $\pm$ 0.0002	0.0025 $\pm$ 0.0001	17	0.03
<i>LEP</i>	2nd	Low > high	M + F	0.07 $\pm$ 0.007	0.09 $\pm$ 0.007	28	0.04
SC mRNA expression							
<i>IGF1R</i>	1st	Low > high	M + F	0.007 $\pm$ 0.0005	0.009 $\pm$ 0.0006	29	0.02

The concomitant up-regulation of *IGF2* mRNA expression in PR adipose tissue following exposure to maternal diets low in nutrients during the second trimester of gestation may reflect a compensatory response by the nutrient-restricted fetus to promote fat deposition and consequent thermogenic capacity, enhancing neonatal survival. However, continued restriction of maternal nutrient supply throughout the second trimester may have limited the ability of the fetus to increase lipid storage as indicated by their comparatively lower *LEP* mRNA expression, despite the provision of adequate nutrients during the final trimester.

The increased expression of *IGF1R*, *IGF2* and *IGF2R* mRNA in OM adipose tissue following exposure to high nutrient diets during the second trimester of gestation suggests an increased rate of proliferation and differentiation of preadipocytes to mature adipocytes, as observed in the PR depot. Given that *LEP* mRNA expression was lower in these same animals, we suggest that despite the potential for a greater number of omental adipocytes available for lipid storage, the amount of lipid stored per cell may have been less than their low-nutrient exposed counterparts. The contrasting effect of second trimester maternal nutrient supply on *LEP* mRNA expression of OM compared with PR adipocytes may represent the higher preference of PR adipose tissue for nutrient partitioning due to its essential role in neonatal survival. However, as OM adipose tissue becomes the largest visceral depot during postnatal life (Cianzio *et al.* 1982) and is more metabolically active than SC adipose tissue (Wajchenberg 2000), changes to OM adipose tissue development may have profound effects on their postnatal metabolism and adipose tissue distribution.

Long-term effects of prenatal nutrition on postnatal adiposity

Exposure to maternal diets low in nutrients during the first trimester of gestation resulted in the expected up-regulation of *IGF1R* mRNA expression in SC adipose tissue of all progeny, but rib site fat depth was greater in males exposed to high maternal nutrient intake in the first trimester despite these same animals being lighter in weight during the post-weaning period (Micke *et al.* 2010a). Reports on the effects of maternal nutrient restriction during gestation on progeny SC fat deposition in the postnatal period are conflicting (Greenwood *et al.* 2006, Stalker *et al.* 2006, Ford *et al.* 2007, Underwood *et al.* 2008, Larson *et al.* 2009). As the difference in SC fat depth of males in our study was present prior to but not at slaughter (Micke *et al.* 2010a), it may indicate that the phenotypic difference observed was transient in nature or too small to accurately reflect postnatal adiposity.

This study has demonstrated long-term effects of maternal nutrient intake during the first two trimesters of gestation on expression of *IGF* and *LEP* genes in progeny adipose tissue that are depot- and sex-specific, a finding that agrees with previous studies (Montague *et al.* 1997, Gardan *et al.* 2006). These changes were not associated with measured commercially important phenotypic changes but may represent fetal adaptations to the nutrient-restricted *in utero* environment that assists with adaptation and survival in the early neonatal period. Furthermore, the observed changes in the expression of *IGF* and *LEP* may be associated with long-term effects on phenotype of cattle that are intensively fed for longer periods of time. Both of these

production traits warrant further investigation. Both of these production traits are of vital economic importance to the beef industry and warrant further investigation.

Materials and Methods

All procedures were performed with the prior approval of, and in accordance with, The University of Queensland Animal ethics committee (approval number SVS/716/06/MLA/AACO) and the Institute of Medical and Veterinary Science (approval number UniSA 07_07).

Animals, experimental design and nutrition

The study utilized a two-by-two factorial design to determine the effects of variations in pasture protein content encountered by gestating breeder herds managed under Australian grazing conditions. The crude protein contents of diets ~4% (low) and 13% (high) on a dry matter basis and were representative of reported variations of 1–17% in legume-based pastures (Schut *et al.* 2010). The two-by-two factorial design allowed investigation into the interaction effects of high and low levels of recommended dietary protein intake during gestation of the beef heifer.

Study animals are the singleton progeny ($n=68$) of composite breed beef heifers (CBX = $\frac{1}{2}$ Senepol $\times$ $\frac{1}{4}$ Brahman $\times$ $\frac{1}{4}$ Charolais; $n=85$ and BeefX = $\frac{1}{2}$ Senepol $\times$ $\frac{1}{4}$ Brahman $\times$ $\frac{1}{8}$ Charolais $\times$ $\frac{1}{8}$ Red Angus; $n=35$) that were AI with frozen semen from a single sire to calve at ~3 years of age (Micke *et al.* 2010b). Dams were divided into four treatment groups on the first day of AI according to stratification by weight within each composite genotype. The four treatment groups determined the level of crude protein (CP) fed to each heifer during the first (T1 = 0–93 d) and second (T2 = 94–180 d) trimesters of gestation, i.e. high high (HH = high level of CP for T1 and T2), high low (HL = high level of CP for T1 and low level of CP for T2), low high (LH = low level of CP for T1 and high level of CP for T2) and low low (LL = low level of CP for T1

and T2). All heifers received the same ration from 181 d until parturition. Details of heifer diets are shown in Table 3. Heifers were individually stall fed until parturition. Seventy-one heifers delivered live calves, gestation length 286 ± 0.5 d; range = 278–298 d (Sullivan *et al.* 2009a). Three progeny were removed from the study after birth: one due to mis-mothering; one pre-weaning from sudden death of unknown causes; and one post-weaning from death due to misadventure. This resulted in 68 progeny distributed across four treatment groups at the completion of the study as follows: HH = 15; HL = 18; LH = 16; LL = 19. No treatments were applied to the progeny. Hereafter, all ages refer to the average age of progeny on the day of sampling.

Postnatally, progeny remained with their mothers on improved and native pastures until weaning at 191 d in accordance with standard beef herd management practice (Meat-and-Livestock-Australia 2004). They were supplemented daily with whole cotton seed (*Gossypium* spp.) allocated at 1 kg/animal while grazing native pastures until 401 d. From 401 d, they were managed as part of a larger group of yearling cattle at Surat, Queensland (27°16'S, 149°07'E) due to unforeseen drought conditions at their property of origin. Each animal was allocated 20 kg/d of a silage-based ration as feed. The ration was 40.7% dry matter and consisted of 85% corn silage (*Zea mays*), 12% whole cottonseed (*Gossypium* spp.) and 3% vitamin and mineral mix. Progeny commenced an intensive feedlot finishing program on 541 d at Dalby, Queensland (27°18'S, 151°26'E), where they remained as one group in their own feedlot pen and were fed commercial feedlot rations prior to commercial slaughter at 680 d. Male and female progeny remained together in the same management group at all times throughout the study.

Males were castrated at 153 d in accordance with standard beef herd management practice (Newman 2007). All progeny were vaccinated against clostridial diseases and leptospirosis (Ultravac 7 in 1: Pfizer Animal Health, West Ryde, NSW, Australia) on 65 and 123 d with a third clostridial vaccine given on 544 d (Ultravac 5 in 1: Pfizer Animal Health). On 379 and 544 d, all progeny were vaccinated against *Bovine herpesvirus*

Table 3 Details of 'high' and 'low' treatment group daily rations fed to dams during each trimester of gestation^a.

Item	Trimester 1 (1–93 d)		Trimester 2 (94–180 d)		Trimester 3 (181 d to term) All
	High	Low	High	Low	
Sorghum (kg)	0.65	1.56	1.00	1.20	1.13
Cottonseed meal (kg)	2.45	0.00	2.50	0.00	1.08
Bambatsi hay (kg)	7.88	2.73	5.79	0.00	0.86
Barley straw (kg)	0.00	5.14	2.21	7.58	7.14
Limestone (kg)	0.07	0.02	0.12	0.06	0.08
Premix (kg ^b)	0.07	0.06	0.10	0.10	0.10
Dry matter intake (DMI) (kg)	9.95	8.64	10.51	8.10	9.39
DMI (g/kg) liveweight ^c	27.81	24.67	25.91	20.18	21.23
Energy, megajoules of metabolisable energy (MJ ME)	76.29	62.54	82.43	63.14	71.45
Energy (%) NRC ^d	243	199	229	176	149
Crude protein (CP) (kg)	1.37	0.41	1.40	0.38	1.06
CP (%) NRC ^d	250	75	228	63	135

^aData are presented on as fed basis/heifer per day. ^bPremix containing 17 g calcium, 9 g phosphorous, 2.91 g magnesium, 5 g sulfur, 27 200 IU vitamin A, 60 mg vitamin E, 70 mg iron, 150 mg zinc, 100 mg manganese, 55 mg copper, 0.5 mg selenium, 3.4 mg cobalt and 4.2 mg iodine per 100 g. ^cAverage liveweight at start of trimester. ^dComparison of ration to National Research Council (NRC) (1996) recommended nutrient requirements for pregnant Brangus replacement heifers bred at 23 months with a mature weight of 475 kg and a calf birth weight of 32 kg.

1 (Rhinogard Intranasal Vaccine (Live); Q-Vax Pty Ltd, Brookfield, QLD, Australia) and *Mannheimia haemolytica* (Bovilis MH; Intervet Australia Pty Limited, Bendigo East, VIC, Australia).

Data and sample collection

Progeny liveweight was measured at regular intervals, blood samples collected at birth (Micke *et al.* 2010b), 29, 94, 191, 379 and 657 d (Micke *et al.* 2010a) and depth of adipose tissue at the rump (P8) and rib (RF) sites measured via real-time ultrasonography (Aloka-500; Aloka, Inc., Tokyo, Japan) using a 20 cm linear probe at 65, 94, 191, 311, 379, 462, 522 and 657 d. The P8 site is located over the *gluteus* muscle on the rump, at the intersection of a line through the pin bone parallel to the spine and its perpendicular through the third sacral crest. The RF site was two-thirds of the distance from the dorsal aspect of *longissimus dorsi* muscle (LD) between the 12th and 13th rib. At slaughter, each animal was killed by captive bolt stunning and exsanguination. Samples of SC at P8 and samples of OM and PR were obtained from each carcass within 20 min of exsanguination, immediately snap frozen in liquid nitrogen and then stored at -80°C until analysis. Standard carcasses (AUS-MEAT 1998) were halved and each side weighed prior to entering the chiller. Carcass weight was calculated as the sum total of the weight of both halves. Assessments of the carcasses were obtained after overnight chilling until the loin temperature was $<11^{\circ}\text{C}$. Fat depth at the P8 and rib sites (AUS-MEAT 1998) were assessed using the Meat Standards Australia (MSA) grading system (MSA 1999).

IGF1, IGF1R, IGF2, IGF2R and LEP mRNA expression in adipose tissue

The relative expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA transcripts in SC, OM and PR adipose tissue of each animal was measured after extraction of total RNA, cDNA synthesis and quantitative real-time PCR (qRT-PCR).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from SC, OM and PR adipose tissue samples as previously described (Duffield *et al.* 2008) and total RNA was purified using the RNeasy Mini Kit (Qiagen) as recommended by the manufacturer. Total RNA was quantified by spectrophotometric measurements at 260 and 280 nm. Complementary DNA was synthesized from 5 μg of total RNA using Superscript III (Invitrogen) by RT (Gentili *et al.* 2006). Controls containing no RNA transcript or no Superscript III were used to test for genomic DNA contamination.

qRT-PCR

The relative expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA transcripts in SC, OM and PR adipose tissue of each animal was measured by qRT-PCR using SYBR Green Master Mix in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using bovine-specific primers validated to generate a single transcript as confirmed by the presence of a single double-stranded DNA product of the correct size (Table 4) and sequence confirmed by using a Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/blast/Blast.cgi, Bethesda, MD, USA). Each qRT-PCR well contained 5 μl Power SYBR Green Master Mix (Applied Biosystems), 1 μl each of forward and reverse primer (Gene-Works, Adelaide, SA, Australia) for the appropriate gene (Table 4), 2 μl water and 1 μl of 50 ng/ μl cDNA to give a total volume of 10 μl . Controls for each primer set containing no cDNA were included on each plate. Three replicates of cDNA from each adipose sample were performed for each gene on each plate. To ensure a consistent result, a quality control (QC) sample was run in triplicate on each plate. 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 the log of the cDNA template concentration (ranging from 1 to 100 ng/ μl). Comparative cycle threshold values were in the linear amplification range, ~ 16 – 26 cycles for all genes. The abundance of each transcript

Table 4 Primer sequence for qRT-PCR.

Primer name	Sequence (from 5' to 3')	Final primer concentration (μM)	Accession number
<i>IGF1</i>			
Forward	TTGGTGGATGCTCTCCAGTTC	9.0	DQ152962
Reverse	AGCAGCACTCATCCACGATTC	9.0	
<i>IGF1R</i>			
Forward	AAGAACCATGCCTGCAGAAGG	9.0	M89789
Reverse	GGATTCTCAGGTTCTGGCCATT	9.0	
<i>IGF2</i>			
Forward	GCTTCTTGCCTTCTTGGCCTT	9.0	AY162434
Reverse	TCGGTTTATGCGGCTGGAT	9.0	
<i>IGF2R</i>			
Forward	GATGAAGGAGGCTGCAAGGAT	9.0	AF327649
Reverse	CCTGATGCCTGTAGTCCAGCTT	9.0	
<i>LEP</i>			
Forward	ATCTCACACACGCAGTCCGT	4.5	NM173928
Reverse	CCAGCAGGTGGAGAAGGTC	4.5	
<i>PPIA</i>			
Forward	CCTGCTTTCACAGAATAATTCCA	9.0	BC105173
Reverse	CATTGCCATGGACAAGATGCCA	9.0	

relative to the abundance of the reference gene peptidylprolyl isomerase A (also known as cyclophilin A or *PPIA*) was calculated using Q-Gen analysis software (Muller *et al.* 2002).

Plasma leptin

Concentrations of leptin in progeny plasma were measured at birth, 29, 94, 191, 379 and 657 d by a competitive ELISA previously described (Kauter *et al.* 2000). The method is briefly described here. The ELISA plate was coated with 7.5 ng bovine recombinant leptin (brLeptin) in 50 µl 0.1 M bicarbonate buffer (pH 9.0) overnight at 37 °C. The plate was blocked with 200 µl 5% skim milk in ELISA buffer for 1 h at 37 °C. The sample was added to the well in a volume of 100 µl and the antiserum in a volume of 50 µl 10% Triton-X 100, 0.5% SDS and 5% sodium deoxycholate, and the plate was incubated overnight at 37 °C. Streptavidin conjugated to alkaline phosphatase (Amrad Biotech, Boronia, VIC, Australia) was incubated for 1 h and the plate developed with *p*-nitrophenylphosphate disodium salt hexahydrate. The plate was washed five times between each step with a washing buffer containing 0.9% saline, 0.05% Triton x 100, using a Titertek Microplate washer (Flow Laboratories, Huntsville, AL, USA). Using brLeptin as standard, the assay has a sensitivity of 0.3 ng/ml with inter- and intra-assay variation of 14 and 6.5% respectively.

Progeny plasma IGF1, IGF2 and total IGF binding proteins

Concentrations of IGF1, IGF2 and total IGF-binding proteins (tIGFBP) were measured in progeny plasma as previously described (Micke *et al.* 2010a), by RIA after separation of IGF and IGFBP by size-exclusion HPLC under acidic conditions. Recovery of ¹²⁵I-IGF1 was 90.1±0.9% for 11 HPLC runs of progeny plasma. Inter-assay coefficient of variation (CV) for HPLC separation and RIA of IGF1 in progeny plasma was 10.9% (*n*=18 assays) and the intra-assay covariance for extraction and assay was 22.0% for a calf QC sample containing 31.4 ng/ml of IGF1. Total IGFBP concentrations were measured by analysis of neutralized fraction 1 in the same assay (Carr *et al.* 1995). Progeny inter-assay CV was 9.7% (*n*=9 assays) and intra-assay covariance for extraction and assay 21.6% for a calf QC sample containing 78.0 ng/ml of IGF2.

Statistical analyses

Liveweight (Micke *et al.* 2010a), ultrasonographic measures of P8 and RF depth, P8 and RF depth relative to liveweight and plasma leptin concentrations were analyzed using repeated measures multifactorial ANOVA to determine the effects of maternal nutrition during T1 and T2, progeny sex and their interaction terms while including gestation length as a covariate. Relative expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA expression in PR, OM and SC adipose tissue, carcass weight and depth of fat at the P8 and RF sites (Micke *et al.* 2010a) were analyzed by multifactorial ANOVA to determine the effects of maternal nutrition in T1 and T2, progeny sex and their interaction terms while including gestation length as a covariate. One-way ANOVA using

Bonferroni adjustment was used to explore significant interactions between maternal nutrition during T1 and T2. Bonferroni adjusted correlation analyses were used to calculate the relationships between relative expression of *IGF1*, *IGF1R*, *IGF2*, *IGF2R* and *LEP* mRNA in each adipose tissue depot with progeny plasma IGF1, IGF2 and tIGFBP concentrations. Data are presented as mean±s.e.m. A probability of 5% (*P*≤0.05) was accepted as the level of significance and trends reported at *P*<0.1. Data were analyzed using Intercooled Stata 9.0 (StataCorp, College Station TX 77845, USA) software.

Declaration 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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