

# Influence of energy balance on the somatotrophic axis and matrix metalloproteinase expression in the endometrium of the postpartum dairy cow

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## Abstract

Postpartum dairy cows enter a period of negative energy balance (NEB) associated with low circulating IGF1, during which the uterus must undergo extensive repair following calving. This study investigated the effects of NEB on expression of IGF family members and related genes in the involuting uterus. Cows were allocated to two treatments using differential feeding and milking regimes to produce mild NEB or severe NEB (SNEB). Uterine endometrial samples collected 2 weeks *post partum* were analysed by quantitative PCR. The expression of IGF-binding protein 4 (*IGFBP4*) mRNA increased in the endometrium of SNEB cows, with trends towards increased *IGFBP1* and reduced *IGFBP6* expression. There were no significant differences between treatments in mRNA expression of *IGF1*, *IGF2* or of any hormone receptor studied, but significant correlations across all cows in the expression levels of groups of receptors suggested common regulatory mechanisms: type 1 IGF receptor (*IGF1R*), *IGF2R* and insulin receptor (*INSR*); *GHR* with *ESR1*; and *ESR2* with *NR3C1*. The expression of *IGF1R* and *INSR* also positively correlated with the circulating urea concentration. Matrix metalloproteinases (MMPs) are important in tissue remodelling and can affect IGF signalling via interaction with IGFBPs. The expression levels of *MMP1*, *MMP3*, *MMP9* and *MMP13* mRNAs all showed major upregulation in the endometrium of cows in SNEB and all except *MMP9* were highly correlated with expression of *IGFBP4*. Alpha(2)-HS-glycoprotein (*AHSG*) and *PDK4*, two genes implicated in insulin resistance, were also highly expressed in SNEB. These results suggest that cows in SNEB experience alterations to the IGF and insulin signalling pathways in the postpartum endometrium. This may affect the rate of tissue repair with a possible negative impact on subsequent fertility.

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## Introduction

Most dairy cows mobilise body fat and muscle tissue immediately after calving, as they cannot at this stage meet the energetic demands for milk production entirely from feed intake (Baumann & Currie 1980). High rates of body condition score (BCS) loss in the early postpartum period are associated with a severe negative energy balance (SNEB) status, alterations in blood metabolite and hormone profiles and reduced fertility (Wathes *et al.* 2007a, 2007b, Leroy *et al.* 2008).

After calving, the uterus must undergo extensive remodelling. This involves a major reduction in size, removal of cellular debris and restoration of normal tissue architecture following expulsion of the placenta

(Gier & Marion 1968, Leslie 1983). Most cows also acquire uterine bacterial contamination at calving. This is normally cleared within 2–3 weeks, but about 15% of animals develop a persistent endometritis in the 3–6 week postpartum period (Foldi *et al.* 2006, Sheldon *et al.* 2009). Subclinical endometritis is associated with longer intervals to conception (Fourichon *et al.* 1999, Gilbert *et al.* 2005).

We and others have shown that SNEB in early postpartum dairy cows is also associated with poor subsequent fertility. For example, multiparous cows with a low nadir in circulating insulin-like growth factor 1 (IGF1) in the first 2 weeks *post partum* subsequently failed to conceive (Taylor *et al.* 2004). An increased rate of uterine involution is associated with earlier

resumption of ovarian activity (Mateus *et al.* 2002), which is in turn important for increasing the pregnancy rate to first service (Thatcher *et al.* 2006). Fertility is strongly linked to the animal's health around calving and both retained fetal membranes and excessive lipid mobilisation are associated with reproductive disorders (Lewis 1997). The risk of uterine disease increases in cows with a high liver fat content, with differences becoming apparent around 7–10 days *post partum* (Zerbe *et al.* 2000). It is thus likely that a poor environment within the reproductive tract is a contributing factor to subsequent poor conception rates.

The postpartum uterus is exposed to the prevailing metabolic environment within the animal, including reduced concentrations of glucose and IGF1 and raised concentrations of non-esterified fatty acids (NEFAs) and  $\beta$ -hydroxybutyrate (BHB; Baumann & Currie 1980, Wathes *et al.* 2007a, 2007b). In addition, many members of the somatotrophic axis are expressed locally within the endometrium (Rutanen 1998, Wathes *et al.* 1998). IGF1 and IGF2 can influence proliferation, differentiation and metabolic activities, principally acting through the type 1 IGF receptor (IGF1R). Further control is achieved via competitive binding to the IGF-binding proteins (IGFBPs), whose expression is regulated in a tissue-specific manner and is also influenced by metabolic status (Thissen *et al.* 1994, Clemmons 1997). We have shown previously that *IGF1*, *IGF2* and *IGF1R* are expressed in the postpartum uterus and may therefore play a role in uterine involution (Llewellyn *et al.* 2008).

Insulin concentrations also tend to be lower in postpartum cows (Bell 1995, Wathes *et al.* 2007c). However, the main control of the insulin signalling pathway occurs downstream of the insulin receptor (INSR); impaired signalling is associated with insulin resistance in peripheral tissues (White 2006). Activation of the INSR results in tyrosine phosphorylation of IRS proteins -1 and -2. Phosphorylated IRSs then bind to proteins such as the p85 regulatory subunit of phosphatidylinositol 3-kinase, which has a central role in mediating the metabolic actions of insulin, including glucose uptake (Shepherd 2005, White 2006). Pyruvate dehydrogenase kinase 4 (PD4K) is a mitochondrial protein, which also contributes to the regulation of glucose metabolism by promoting the use of long-chain fatty acids (LCFA) over glucose as an energy substrate when the glucose supply is limited (Holness & Sugden 2003). Alpha(2)-HS-glycoprotein (AHSG) is a plasma protein, produced primarily by the liver, whose circulating concentration is positively associated with insulin resistance and liver fat accumulation in humans (Stefan *et al.* 2006). Circulating AHSG rises during late pregnancy, particularly in women with gestational diabetes (Kalabay *et al.* 2002).

Matrix metalloproteinases (MMPs) are primarily known for their ability to degrade extracellular matrix, but they can also degrade non-matrix proteins (Nagase *et al.* 2006). In relation to the uterus, they have been

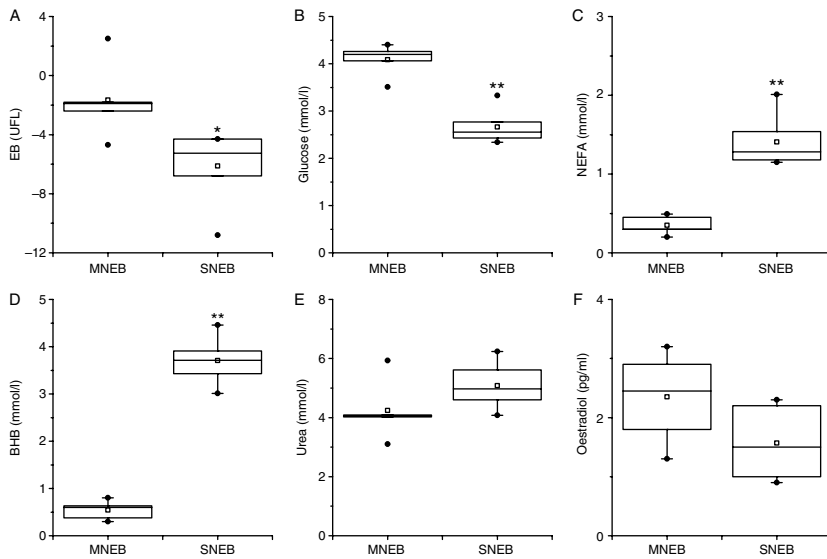
studied most extensively in humans, where increased MMP expression is associated with endometrial breakdown during menstruation (Salamonsen *et al.* 2002).

In this study, we have used a model of differential feeding and milking regimes to produce cows in differing energy balance (EB) status in early lactation (mild NEB (MNEB) or SNEB) as confirmed by markedly divergent metabolic and endocrine profiles. We have previously reported differential expression of the IGF system in both liver and oviduct between the two groups of cows (Fenwick *et al.* 2008a, 2008b). This study extends these findings by examining the expression patterns for members of the somatotrophic axis and associated pathways in the postpartum endometrium. The objective of this study is to test our hypothesis that altered insulin and IGF1 signalling at this critical time may delay uterine repair mechanisms, thus compromising fertility. Uterine tissue samples were collected during the period of postpartum anoestrus before any cows had ovulated, thus avoiding potential differences between animals associated with exposure to luteal progesterone. The study focused on i) the members of the somatotrophic axis (*IGF1*, *IGF2*, *IGFBP1–6* and the acid labile subunit (*IGFALS*); ii) the associated hormone receptors (*IGF1R*, *IGF2R*, *INSR*, GH receptor (*GHR*) and oestradiol receptors (*ESR1* and *ESR2*)); and iii) the glucocorticoid receptor (*Bos taurus* nuclear receptor subfamily 3, group C, member 1, transcript variant 4, *NR3C1*). Some additional candidate genes were selected for analysis based on significant differential expression in a microarray analysis of endometrial samples from the same cows (Wathes *et al.* 2009) together with their known involvement in either glucose metabolism or the IGF system. These were i) the matrix metalloproteinases *MMP1*, *MMP3*, *MMP9* and *MMP13* and ii) *PDK4* and *AHSG*. Gene expression was also related to a variety of endocrine and metabolic measurements made at the time of sample collection.

## Results

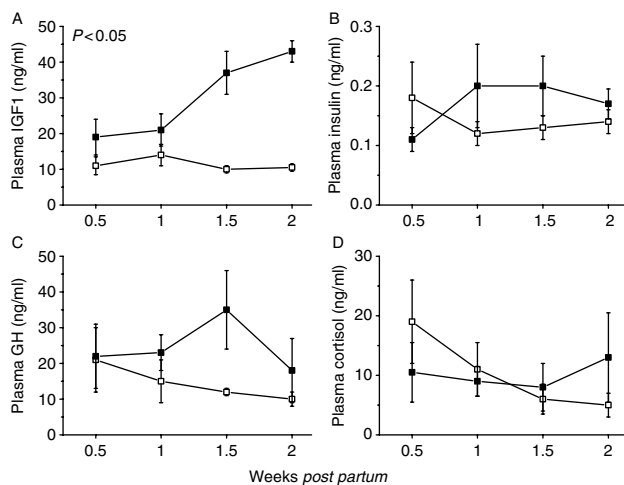
### **Metabolic, hormonal and EB status of cows in MNEB and SNEB**

The EB values and metabolic data from cows at the time of sample collection are summarised in Fig. 1A–E. As reported previously (Fenwick *et al.* 2008a), all cows were in a state of NEB from the beginning of the treatment period to slaughter at  $14 \pm 0.4$  days *post partum*. The SNEB cows experienced a greater reduction in EB values throughout the early postpartum interval leading to a significantly lower mean EB value at the time of tissue collection. Systemic NEFA and BHB values were elevated, while glucose was reduced and urea concentration remained unchanged in SNEB when compared with the MNEB group. Mean plasma IGF1 concentrations were not different between the groups



**Figure 1** Box and whisker plots to show the EB values (A), metabolic status (B–E, glucose, NEFA, BHB and urea) and oestradiol concentrations (F) in blood samples collected pre-slaughter at  $14 \pm 0.4$  days *post partum*. Data are from five cows in MNEB and six in SNEB. The boxes illustrate the median values and the upper and lower quartiles for each group. The mean (open square) and the 99 and 1 percentile values (filled circle) are also shown. There are significant differences between the groups for EB, glucose, NEFA and BHB: \* $P < 0.05$ , \*\* $P < 0.01$ . These results have been reported previously (Llewellyn *et al.* 2007, Fenwick *et al.* 2008a, 2008b).

during the first week of lactation but during the second week IGF1 remained depressed in SNEB cows, whereas concentrations started to increase in MNEB cows: circulating values therefore differed significantly at the time of slaughter (Fig. 2A). Insulin values tended to increase after calving in the MNEB group but decreased in the SNEB group, although the differences were not significant (Fig. 2B). We report here for the first time the circulating values of cortisol and GH: these were both quite variable and did not differ significantly between the groups (Fig. 2C and D). The concentrations of oestradiol were also similar between the two treatment groups (Fig. 1F; Llewellyn *et al.* 2007).



**Figure 2** Circulating values of (A) IGF1, (B) insulin, (C) GH and (D) cortisol during the early postpartum period in dairy cows. Values are least squares means  $\pm$  S.E.M. for the MNEB (filled square;  $n = 5$ ) and SNEB (open square;  $n = 6$ ) groups respectively. Circulating IGF1 concentrations in the 2nd week were significantly higher in the MNEB group but values for insulin, GH and cortisol were not significantly different between the groups (analysed using a repeated measure ANOVA).

### Quantitative gene expression profile in the endometrium

A comparison of the quantitative real-time PCR (qPCR) data for the 25 genes measured in the endometrium according to the EB status is given in Table 1. All IGF family members measured were detectable, but the only gene to show significant differential expression between the groups was *IGFBP4*, which was 2.6-fold higher in the SNEB group ( $P < 0.05$ ). There were, however, trends for *IGFBP1* (higher in SNEB,  $P < 0.06$ ), *IGFBP6* (lower in SNEB,  $P < 0.08$ ) and *IGFALS* (lower in SNEB,  $P < 0.1$ ). The expression of both *IGF1* and *IGF2* mRNAs was numerically higher in MNEB cows, but the differences were not significant. None of the receptors measured (*IGF1R*, *IGF2R*, *INSR*, *GHR*, *NR3C1*, *ESR1* or *ESR2*) differed with the EB status ( $P = 0.26$ – $0.94$ ).

In contrast, there were major differences in expression for all of the MMPs measured: *MMP1*, *MMP3*, *MMP9* and *MMP13* mRNAs showed major upregulation in SNEB cows ( $P < 0.001$ ). *AHSG* mRNA expression was also much higher in SNEB endometrium ( $P < 0.001$ ). *PDK4* was highly expressed and tended to be increased in the endometrium of SNEB cows ( $P = 0.1$ ). The control housekeeping genes (*RPL19*, *GAPDH* and *18S rRNA*) all showed similar levels of expression in the endometrium between the two groups, confirming that equivalent concentrations of input RNA were used.

### Correlation analysis

Correlation analysis was next performed to identify any relationships between the expression patterns of the genes examined. For this purpose, data were combined between both groups of cows. In addition, an analysis of covariance was performed between the pairs of

**Table 1** Comparison of results for qPCR measurements (expressed in fg/ $\mu$ g reverse-transcribed RNA) in the endometrium between postpartum cows in mild (MNEB) or severe negative energy balance (SNEB) after calving.

Gene	MNEB (n=5 <sup>a</sup> )	SNEB (n=6)	P*
IGF family members			
<i>IGF1</i>	16 $\pm$ 4.3	8 $\pm$ 3.2	0.19
<i>IGF2</i>	7.8 $\pm$ 2.03	4.8 $\pm$ 1.21	0.22
<i>IGFBP1</i>	0.5 $\pm$ 0.16	2.1 $\pm$ 0.76	0.06
<i>IGFBP2</i>	1.2 $\pm$ 0.10	0.9 $\pm$ 0.26	0.26
<i>IGFBP3</i>	6.8 $\pm$ 1.46	9.2 $\pm$ 1.65	0.31
<i>IGFBP4</i>	3.5 $\pm$ 0.77	9.2 $\pm$ 3.00	0.05
<i>IGFBP5</i>	4.5 $\pm$ 0.78	6.1 $\pm$ 1.57	0.40
<i>IGFBP6</i>	5.8 $\pm$ 1.12	3.0 $\pm$ 0.89	0.08
<i>IGFALS</i>	0.2 $\pm$ 0.03	0.1 $\pm$ 0.05	0.10
Hormone receptors			
<i>IGF1R</i>	0.7 $\pm$ 0.10	1.1 $\pm$ 0.32	0.35
<i>IGF2R</i>	0.2 $\pm$ 0.04	0.4 $\pm$ 0.14	0.26
<i>INSR</i>	0.6 $\pm$ 0.12	0.9 $\pm$ 0.18	0.28
<i>GHR</i>	6.8 $\pm$ 1.15	5.2 $\pm$ 1.47	0.42
<i>NR3C1</i>	0.9 $\pm$ 0.28	0.8 $\pm$ 0.22	0.91
<i>ESR1</i>	24 $\pm$ 5.1	26 $\pm$ 2.9	0.94
<i>ESR2</i>	2.1 $\pm$ 0.83	1.3 $\pm$ 0.51	0.42
Tissue remodelling			
<i>MMP1</i>	0.2 $\pm$ 0.10	11.1 $\pm$ 6.09	<0.01 <sup>#</sup>
<i>MMP3</i>	0.1 $\pm$ 0.04	1.9 $\pm$ 0.97	<0.01 <sup>#</sup>
<i>MMP9</i>	0.3 $\pm$ 0.11	2.0 $\pm$ 0.55	0.02
<i>MMP13</i>	0.1 $\pm$ 0.03	0.5 $\pm$ 0.25	0.03 <sup>#</sup>
Insulin signalling			
<i>AHSG</i>	0.1 $\pm$ 0.02	1.2 $\pm$ 0.52	<0.01
<i>PDK4</i>	29 $\pm$ 10.0	53 $\pm$ 8.6	0.10
Housekeeping genes			
<i>RPL19</i>	28 $\pm$ 2.3	24 $\pm$ 4.5	0.81
<i>GAPDH</i>	19 $\pm$ 2.6	27 $\pm$ 7.1	0.36
<i>18S rRNA</i>	72 079 $\pm$ 9463	72 187 $\pm$ 9939	0.99

\*Values are presented as mean  $\pm$  s.e.m. Comparison between the groups was by *t*-test, those indicated by <sup>#</sup> used log-transformed data to normalise variances.

<sup>a</sup>Only five cows were included in the MNEB group due to poor RNA quality for one cow.

variables with the treatment (MNEB or SNEB) included as a fixed effect.

This approach revealed three major groupings of genes whose expressions in the endometrium were significantly inter-related (Table 2). The first group included most of those genes for which a main effect of EB treatment had already been detected: *MMP1*, *MMP3*, *MMP13*, *AHSG*, *IGFBP4*, *IGFBP1* and *PDK4*. These correlations remained significant, however, even when the EB status was accounted for in the model. Examples are illustrated in Fig. 3A–D. The group also included the *IGF2R*; although this was not differentially expressed according to the EB group it did show a positive correlation with the expression levels of *MMP3*, *MMP13*, *IGFBP4* and *PDK4* (Fig. 3E). The expression levels of several of these genes (*MMP1*, *MMP3*, *MMP13*, *IGFBP4*, *AHSG*, *PDK4*) also showed a significant positive correlation with the circulating concentration of NEFA at the time of slaughter (Fig. 3F), but not with the other metabolites measured.

The second major group of inter-related genes included *INSR*, *IGF1R*, *IGF2R*, *IGFBP3*, *IGFBP5* and

*PDK4*. Examples are illustrated in Fig. 4A–E. Of these, only *PDK4* showed a trend for differential expression between the EB groups. The expression of *INSR*, *IGF1R*, *IGFBP3* and *IGFBP5* was positively correlated with the circulating urea concentration, although this relationship did not remain significant when the false discovery rate was accounted for (Fig. 4F).

The third group of genes included *IGF1*, *GHR*, *ESR1*, *IGFBP2*, *IGFBP6* and *IGF2*. The expression of these genes did not show any correlation with the metabolites or metabolic hormones measured, although there was a trend towards an EB treatment effect for *IGFBP6*. There were highly significant correlations of *IGF1* mRNA expression with *GHR*, *ESR1*, *IGFBP2* and *IGFBP6*, whereas *IGF2* mRNA expression was only weakly related to *IGFBP2* and *IGFBP6*. Examples are shown in Fig. 5A–D.

Two other sets of genes showed highly significant correlations with each other but not with any of the other genes measured. The two receptors *NR3C1* and *ESR2* were significantly positively related with each other ( $r=0.816$ ,  $P<0.01$ , Fig. 5E) and *MMP9* expression was negatively correlated with *IGFALS* ( $r=-0.897$ ,  $P<0.001$ , Fig. 5F).

## Discussion

This study describes the results from the model that we have developed in which differential feeding and milking regimes were used to produce cows in differing EB status in early lactation (Fenwick *et al.* 2008a, 2008b). Within the members of the somatotrophic axis measured, only *IGFBP4* mRNA expression varied according to the EB status, although there were trends for *IGFBP1*, *IGFBP6* and *IGFALS* mRNAs. It is possible that these differences would have achieved a greater level of significance if more animals had been available for the study. However, several *MMPs* and also *AHSG* expression were highly upregulated in SNEB, and the products of these genes are known to play a key role in controlling the localisation and breakdown of IGFBP protein within tissues.

Uterine involution in dairy cows involves a considerable reduction in size, necrosis of the surface endometrium and extensive restructuring of the extracellular matrix (Gier & Marion 1968, Leslie 1983). Placental tissues remaining in the uterus after calving accumulate as tissue debris in the uterine lumen contributing to a lochial discharge. Following this initial degradation, tissue repair is initiated and the caruncles remodel and regenerate epithelium. Many processes involved in uterine repair *post partum* are thus common to those of wound healing in other tissues, where IGF1 and IGF2 are known to assist the repair mechanisms (Grazul-Bilska *et al.* 2003, Salamonsen 2003). In cows, this process is superficially completed by 3–4 weeks *post partum*, but the deeper layers are not fully restored until 6–8 weeks (Marion & Gier 1959).

**Table 2** Summary of significant Pearson correlations across all 11 cows in the study for relationships between qPCR gene expression values measured in the endometrium or between gene expression and pre-slaughter circulating non-esterified fatty acid (NEFA) and urea concentrations<sup>a</sup>.

Group 1	<i>MMP3</i>	<i>MMP13</i>	<i>AHSG</i>	<i>IGFBP4</i>	<i>IGFBP1</i>	<i>IGF2R</i>	<i>PDK4</i>	NEFA
<i>MMP1</i>	<b>0.979<sup>†</sup> (&lt;0.001)</b>	<b>0.961<sup>†</sup> (&lt;0.001)</b>	<b>0.924<sup>†</sup> (&lt;0.001)</b>	<b>0.931<sup>†</sup> (&lt;0.001)</b>	0.702 (0.016)	NS	0.600 (0.051)	0.693* (0.018)
<i>MMP3</i>		<b>0.982<sup>†</sup> (&lt;0.001)</b>	<b>0.892<sup>†</sup> (&lt;0.001)</b>	<b>0.946<sup>†</sup> (&lt;0.001)</b>	NS	0.675 (0.023)	0.611 (0.046)	0.690* (0.019)
<i>MMP13</i>			<b>0.886<sup>†</sup> (&lt;0.001)</b>	<b>0.918<sup>†</sup> (&lt;0.001)</b>	0.675 (0.046)	0.738* (0.009)	0.675 (0.023)	0.716* (0.013)
<i>AHSG</i>				0.726* (0.006)	0.825 (0.006)	NS	NS	0.682 (0.021)
<i>IGFBP4</i>					NS	0.679 (0.022)	0.728 (0.011)	0.695* (0.018)
<i>IGFBP1</i>						NS	0.697 (0.017)	NS
<i>IGF2R</i>							0.735 (0.01)	NS
<i>PDK4</i>								0.643 (0.033)
Group 2	<i>IGF1R</i>	<i>IGF2R</i>	<i>IGFBP3</i>	<i>IGFBP5</i>	<i>PDK4</i>	Urea		
<i>INSR</i>	<b>0.903<sup>†</sup> (&lt;0.001)</b>	<b>0.812<sup>†</sup> (0.002)</b>	<b>0.857<sup>†</sup> (0.001)</b>	0.754* (0.007)	NS	0.615 (0.044)		
<i>IGF1R</i>		<b>0.901<sup>†</sup> (&lt;0.001)</b>	<b>0.879<sup>†</sup> (&lt;0.001)</b>	<b>0.828<sup>†</sup> (0.002)</b>	0.679 (0.022)	0.643 (0.033)		
<i>IGF2R</i>			<b>0.789<sup>†</sup> (0.004)</b>	<b>0.891<sup>†</sup> (&lt;0.001)</b>	0.735* (0.01)	NS		
<i>IGFBP3</i>				0.683 (0.021)	<b>0.814<sup>†</sup> (0.002)</b>	0.631 (0.037)		
<i>IGFBP5</i>					0.643 (0.033)	0.629 (0.038)		
<i>PDK4</i>						NS		
Group 3	<i>GHR</i>	<i>ESR1</i>	<i>IGFBP2</i>	<i>IGFBP6</i>	<i>IGF2</i>			
<i>IGF1</i>	<b>0.892<sup>†</sup> (&lt;0.001)</b>	0.753 <sup>†</sup> (0.007)	0.731* (0.011)	<b>0.819<sup>†</sup> (0.002)</b>	NS			
<i>GHR</i>		<b>0.797<sup>†</sup> (0.003)</b>	<b>0.839<sup>†</sup> (0.001)</b>	<b>0.802<sup>†</sup> (0.003)</b>	NS			
<i>ESR1</i>			NS	NS	NS			
<i>IGFBP2</i>				<b>0.776* (0.005)</b>	0.634 (0.036)			
<i>IGFBP6</i>					0.669 (0.024)			

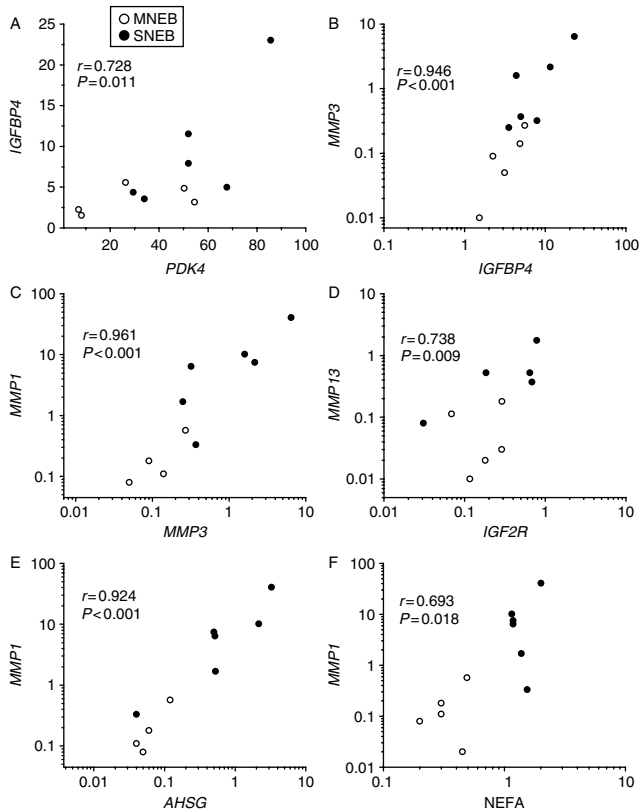
<sup>a</sup>The actual *P* value is given in parentheses. Using a false discovery rate correction for 22 genes (i.e. all genes in the study excluding the housekeeping genes), adjusted 5% significance is at *P*=0.026 and adjusted 1% significance is at *P*=0.005, shown in bold. NS, not significant. The data were also tested using analysis of covariance between the pairs of variables with the treatment (MNEB or SNEB) included as a fixed effect. The significance level for this test is indicated: \**P*<0.05, <sup>†</sup>*P*<0.01 and <sup>‡</sup>*P*<0.001.

Both our own work and that of others have demonstrated that many members of the IGF system are expressed in cyclic and postpartum bovine uterus (Geisert *et al.* 1991, Robinson *et al.* 2000, Pershing *et al.* 2002, Llewellyn *et al.* 2008). Within the postpartum cow, *in situ* hybridisation analysis showed that *IGF1* mRNA was present in the subepithelial stroma, whereas *IGF2* mRNA was confined to the caruncular stroma. *IGFBP2*, *IGFBP4*, *IGFBP5* and *IGFBP6* mRNAs all showed widespread distribution in both the intercaruncular and caruncular stroma in contrast with *IGFBP3* mRNA, which was strongly expressed in the luminal epithelium only (Llewellyn *et al.* 2008). We were unable to detect *IGFBP1* mRNA expression using our *in situ* hybridisation technique (Llewellyn 2008), although it was measurable in this study by qPCR.

The qPCR analysis showed that expression of *IGFBP4* mRNA was higher in the endometrium of cows in SNEB and there were trends towards an increased expression in *IGFBP1* and a reduction in *IGFBP6* and *IGFALS* mRNAs. The result relating to *IGFBP6* was supported by *in situ* hybridisation analysis, which showed a significant reduction in *IGFBP6* mRNA expression in cows in SNEB (Llewellyn 2008). *IGFBP6* mRNA expression in the oviduct also decreased in SNEB relative to MNEB (Fenwick *et al.* 2008b). This limited effect of the EB status on the somatotrophic axis in the uterus contrasted

with the major influence on the liver. Hepatic expressions of *IGF1*, *IGF1R*, *IGF2R*, *IGFBP3*, *-4*, *-5*, *-6*, *IGFALS* and *GHR* mRNAs were all significantly down-regulated in cows in SNEB, whereas expression of *IGFBP2* mRNA increased (Fenwick *et al.* 2008a). In this study, *IGFBP1* mRNA in the endometrium was higher in cows in SNEB, whereas hepatic expression of *IGFBP1* reduced. In the oviduct, expression of *IGFBP2* mRNA decreased in SNEB relative to MNEB, whereas it increased in the liver (Fenwick *et al.* 2008b). Together, these results show clearly that there is differential regulation of components of the IGF system in different tissues by EB in the postpartum cow, even though these tissues are all exposed to the same prevailing endocrine background of elevated BHB and NEFA alongside reduced concentrations of IGF1 and glucose.

*IGF1* mRNA was highly expressed in the postpartum endometrium. In the liver, the main regulator of IGF1 synthesis is GH coupling to GHR (Lucy *et al.* 2001). In rats, GH can also increase the uterine IGF1 expression, although oestradiol seems to be the major regulator of IGF1 expression within the reproductive tract (Murphy & Friesen 1988). We have previously reported increased expression of IGF1 in subepithelial stroma of both sheep and cows at oestrus, with significant correlations with the expression of *ESR1* in the same cells (Stevenson *et al.* 1994, Robinson *et al.* 2000). During NEB the GH-IGF

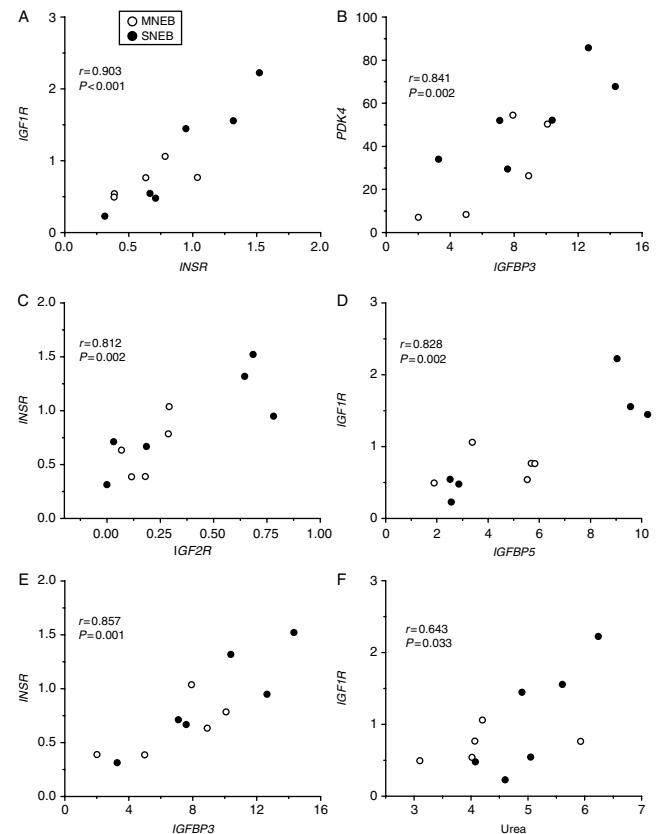


**Figure 3** Scatter plots illustrating significant correlations between gene expression levels (in fg/ $\mu$ g reverse-transcribed RNA; A–E) and NEFA concentrations (in mmol/l; F) for all cows in the study ( $n=11$ ). Examples are taken from Table 2, group 1. Each symbol represents one animal with MNEB cows shown as open symbols and SNEB cows as solid symbols.

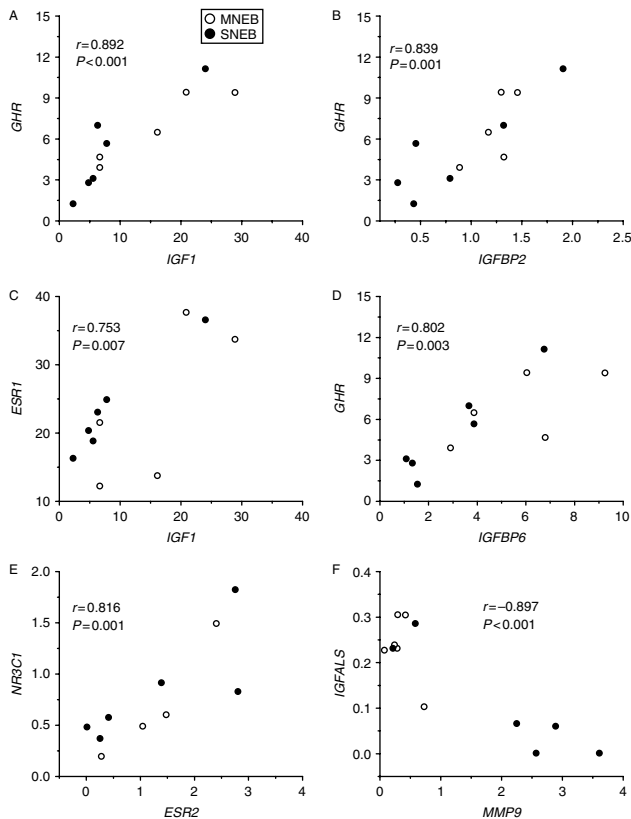
axis uncouples due to downregulation in hepatic GHR (Lucy *et al.* 2001, Fenwick *et al.* 2008a) and this is the major factor causing reduced IGF1 synthesis in the postpartum cow (Kobayashi *et al.* 1999). Pershing *et al.* (2002), however, found no effect of bovine somatotrophin treatment on bovine endometrial *IGF1* expression. In the present experiment, mRNA for both GH and oestradiol receptors was present in the endometrium but there were no differences in circulating GH or oestradiol or between endometrial *GHR*, *ESR1* or *ESR2* mRNA expression between the EB groups. There were, however, highly significant correlations between the expression levels of *IGF1*, *GHR* and *ESR1* in individual cows. It is therefore likely that both oestradiol and GH are involved in regulating endometrial IGF1 synthesis in the postpartum cow. There may also be effects of the EB status on downstream signalling from the receptors that were not investigated in this study.

Both *IGFBP6* and *IGFBP4* mRNAs showed widespread distribution in the endometrial stromal cells from the cows in this study (Llewellyn *et al.* 2008) and both these IGFBPs were regulated by the EB status although in opposite directions. IGFBP4 is the smallest IGFBP: it

binds both IGF1 and IGF2 and is often co-expressed with IGF2 during development (Ning *et al.* 2008). IGFBP4 production increased during decidualisation of human endometrial stromal cells (Ganef *et al.* 2009). Although it is generally thought to inhibit IGF action (Wetterau *et al.* 1999), recent studies revealed that the null mutation of IGFBP4 in mice led to a prenatal growth deficit, suggesting that it may also provide a reservoir of IGF within tissues (Ning *et al.* 2008). IGFBP6 has a much higher affinity for IGF2, generally inhibiting IGF2 activity (Bach 1999, Grellier *et al.* 2002, Bach 2005). Potential regulators of *IGFBP6* expression are retinoic acid and oestrogen (Zhu *et al.* 1993, Bach 1999). The present results also showed a trend towards increased *IGFBP1* mRNA expression in the endometrium of cows in SNEB. In humans, IGFBP1 is a major product of the decidual stroma, where its production is decreased by both insulin and IGFs (Rutanen 1998, Giudice 2006), whereas in sheep *IGFBP1* expression was mainly present in the luminal epithelium, rising during the second half of the luteal phase (Osgerby *et al.* 1999). In rat liver, production of IGFBP1 increases during inflammation



**Figure 4** Scatter plots illustrating significant correlations between gene expression levels (in fg/ $\mu$ g reverse-transcribed RNA; A–E) and urea concentrations (in mmol/l; F) for all cows in the study ( $n=11$ ). Examples are taken from Table 2, group 2. Each symbol represents one animal with MNEB cows shown as open symbols and SNEB cows as solid symbols.



**Figure 5** Scatter plots illustrating significant correlations between gene expression levels (in fg/ $\mu$ g reverse-transcribed RNA) for all cows in the study. Each symbol represents one animal ( $n=11$ ) with MNEB cows shown as open symbols and SNEB cows as solid symbols. For (A–D) examples are taken from Table 2, group 3.

and expression can be stimulated by IL1B (Rutkute & Nikolova-Karakashian 2007). The results suggest that the actions of the IGFs in the postpartum uterus will be highly controlled by the various IGFBPs present and that there is differential regulation of some of the IGFBPs according to the energy status. However, both the actions of the IGFBPs and the precise mechanisms controlling their expression in the endometrium remain to be elucidated.

The expression of *IGFALS* mRNA was also detected in the endometrium, with a trend towards reduced expression in SNEB. *IGFALS* is a glycoprotein, which forms a ternary complex with IGF and IGFBP3 or IGFBP5, prolonging the half-life of IGF in the circulation. In the liver, the expression is regulated by GH and we have previously shown a major downregulation of hepatic *IGFALS* in postpartum cows in concert with decreased expression of *IGF1*, *IGFBP3* and *GHR* mRNAs (Fenwick *et al.* 2008a). Although *IGFALS* is primarily synthesised in the liver, low levels of uterine expression have previously been reported in some extra-hepatic tissues, including the uterus (Lee *et al.* 2001, Li *et al.* 2007). In this study, endometrial *IGFALS* expression

showed a strong negative relationship with *MMP9*, which is generally produced by migratory immune cells (Salamonsen *et al.* 2002), but the significance of this observation and the function of extra vascular *IGFALS* are currently unclear. It seems likely that reduced levels of *IGFALS* would decrease the half-life of locally produced IGFs, although IGF-independent actions are also thought to be possible (Lee *et al.* 2001).

Another putative regulator of the IGF system within the postpartum uterus is insulin. In late pregnancy, falling insulin and elevated placental lactogen stimulate adipose mobilisation, providing nutrients for fetal growth (Bell 1995, Sivan & Boden 2003) and resulting in raised circulating concentrations of NEFAs. Lipid accumulation in muscle inhibits tyrosine phosphorylation of IRS (Kirwan *et al.* 2004) and this contributes to the pregnant mother developing peripheral insulin resistance. In women of normal weight, insulin signalling was restored within a few days of giving birth but this took up to 15 weeks in obese women (Sivan *et al.* 1997). *IGF1* also seems necessary for normal insulin sensitivity (Clemmons 2004). The SNEB cows in this study had significantly higher circulating NEFA and lower *IGF1* after calving; therefore, we propose that they may also be subject to impaired peripheral insulin signalling. *AHSG* is a plasma protein, produced primarily by the liver, whose circulating concentration is positively associated with insulin resistance and hepatic lipidosis in humans (Stefan *et al.* 2006). Local production of *AHSG* mRNA within the uterus was 12-fold higher in cows in SNEB and was also positively correlated to the circulating NEFA concentration, so this could potentially contribute to decreased insulin sensitivity.

Energy requirements of cells can be met by either glucose or LCFA. When glucose is scarce and the supply and oxidation of LCFA is sufficient, then mitochondrial pyruvate dehydrogenase activity is suppressed, limiting the conversion of pyruvate to acetyl-CoA and helping to conserve glucose (Holness & Sugden 2003). *PDK* activity increases in response to starvation or an increased lipid supply, inactivating the pyruvate dehydrogenase complex. *PDK4* is thus thought to act as a marker of lipid status (Holness & Sugden 2003). It is upregulated across many tissues in type 2 diabetes mellitus, with expression regulated by glucocorticoids, retinoic acid and insulin (Rasche *et al.* 2008). The increased expression of *PDK4* mRNA in the endometrium of cows in SNEB and the correlation of *PDK4* expression to the NEFA concentration are therefore consistent with an environment of reduced glucose availability coupled with raised NEFAs.

We also report that the concentrations on endometrial *MMP1*, *MMP3*, *MMP9* and *MMP13* mRNAs were all significantly higher in the SNEB group. Of these, expression levels of *MMP1*, *MMP3* and *MMP13* were all highly correlated with each other, but showed no relationship with *MMP9* expression. In human uterus,

MMP1 and MMP3 are both produced by stromal cells and MMP9 by migratory immune cells (reviewed by Salamonsen *et al.* (2002)). The expression of these MMPs in women increased at menstruation following progesterone withdrawal, when they are thought to be the key players in promoting endometrial breakdown. The increased expression of MMP1, MMP2 and MMP9 was also demonstrated during uterine involution after parturition in the rat (Manase *et al.* 2006). Different MMPs differ with regard to their substrate specificity: MMP1 and MMP13 act principally as collagenases, whereas MMP3 is a stromelysin, which digests extracellular matrix molecules but not collagen and it is also important for pro-MMP activation (Nagase *et al.* 2006). The co-ordinated rise in *MMP1*, *MMP3* and *MMP13* mRNAs shown in this study suggests that these MMPs share common regulatory mechanisms within the uterus. IL1 and TNF are possible candidates, as both were shown to increase MMP1 and MMP3 protein secretion in human uterine fibroblasts (Braundmeier & Nowak 2006).

There is also a considerable body of literature linking the MMPs with IGFBP activity. MMPs can cleave IGFBP1, IGFBP3 and IGFBP5, thus, increasing the bioavailability of IGFs for receptor activation by releasing them from association with the extracellular matrix (Nagase *et al.* 2006). For example, decidualised endometrial cells in first trimester human pregnancy produce both IGFBP1 and MMP3. MMP3 was shown to cleave IGFBP1 into fragments that were unable to bind IGFs, thus, potentially increasing IGF-stimulated placental proliferation (Coppock *et al.* 2004). *MMP* expression in this study also highly correlated with that of *AHSG*. *AHSG* present on the cell surface can anchor other molecules to the plasma membrane (Ochieng *et al.* 1995, Leite-Browning *et al.* 2002). It has been shown to interact with a variety of MMPs (-2, -3, -7 and -9), both activating them and protecting them from autolytic cleavage (Ochieng *et al.* 1995, Ray *et al.* 2003, Kübler *et al.* 2007). Although not studied here, tissue inhibitors of matrix metalloproteinases (TIMPs) are also known to play a key role in regulating MMP activity (Nagase *et al.* 2006).

A number of hormone receptors were measured (*IGF1R*, *IGF2R*, *INSR*, *GHR*, *NR3C1*, *ESR1* and *ESR2*) and in no case did their expression in the endometrium varied according to EB status. Although systemic IGF1 was lower in cows in SNEB, the circulating concentrations of oestradiol, insulin and GH were similar between the EB groups. This suggests that any effects of EB acting through these receptors must be mediated through alteration of the downstream receptor signalling pathways. The receptors did, however, show co-ordinated expression between themselves: *IGF1R*, *IGF2R* and *INSR* were all highly positively correlated with each other, as were *GHR* with *ESR1* and *NR3C1* with *ESR2*. The expression of receptors within each of these three groups is therefore likely to be controlled through common use of transcription factors. The *IGF2R*

is thought to degrade IGF2 and so acts to reduce IGF2 signalling (Kornfeld 1992). The co-expression of *IGF1R* and *IGF2R* is perhaps surprising given their opposing actions, although similar results were found in hepatic tissues (Fenwick *et al.* 2008a).

In the cyclic cow, *IGF1R* is most highly expressed in luminal and glandular epithelial cells, whereas in the postpartum uterus, expression was confined to the stroma (Robinson *et al.* 2000, Llewellyn *et al.* 2008). In early pregnancy in the ewe, *IGF2R* mRNA expression was also mainly localised to the subepithelial stroma (Wathes *et al.* 1998). Transcription of the *IGF1R* is mainly under negative control by several transcriptional repressors, but these can be overcome by a number of transactivators, including *ESR1* (Werner & Roberts 2003). Oestrogen is also known to modulate GH action at the level of receptor expression (Leung *et al.* 2004). In the cyclic and early pregnant cow, *ESR1* in the endometrium is most highly expressed at oestrus; oestradiol is the main upregulator of *ESR1*, with progesterone having an inhibitory effect (Meyer *et al.* 1988, Robinson *et al.* 2001). *GHR* expression in the endometrium of the non-pregnant cow also peaked at oestrus (Rhoads *et al.* 2008) but *GHR* concentrations were higher during pregnancy (Kölle *et al.* 1997). In mice, *ESR2* is more highly expressed in immature than mature uterus with expression negatively regulated by oestradiol. Some evidence suggests a role of *ESR2* in controlling collagen homeostasis (reviewed by Koehler *et al.* (2005)). Glucocorticoid receptors have been identified in the bovine uterus around calving (Schäubli *et al.* 2008). Further work is clearly needed to unravel how the complex interplay of hormone action on the uterus is regulated between different cell types at a transcriptional level.

One novel finding of this study was that, although expression of the *IGF1R* and *INSR* was not altered by the EB status, their expression levels did correlate with the circulating urea concentration, as did expression of *IGFBP3* and *IGFBP5* mRNA. High circulating urea levels in cows are associated with reduced fertility (reviewed by Laven *et al.* (2007)), an effect thought to be mediated in part through a reduced pH in the uterine lumen (Rhoads *et al.* 2004). Blood urea concentrations after calving are influenced by dietary factors, dry matter intake, ill health (in particular liver disease) and the extent of tissue protein catabolism to meet the demands of lactation (Bell 1995, Grummer 1995, Drackley *et al.* 2001). The results presented in this study suggest that the resulting urea concentration may influence the expression of endometrial IGF and *INSRs*. Of potential relevance in this respect is the known inhibitory effect of sulphonylurea drugs in the treatment of non-insulin-dependent diabetes due to their ability to inhibit insulin signalling pathways (Del Prato *et al.* 1991). More recently, diarylurea compounds were identified as being potent inhibitors of *IGF1R* signalling in breast



cancer cells (Gable *et al.* 2006), although the correlation detected in this study between urea and receptor expression was positive rather than negative.

When tissues are injured they must undergo the successive repair phases of degeneration, inflammation, regeneration and fibrosis (Prisk & Huard 2003). In muscle and tendon, local signalling of IGF1 may co-ordinate these responses, allowing better regenerative growth (Dahlgren *et al.* 2005, Mourkioti & Rosenthal 2005). IGF1 signalling has also been implicated in tissue remodelling of the human endometrium (Toyofuku *et al.* 2006). In conclusion, we show in this study that there are alterations in both the IGF bioavailability and in insulin signalling pathways in the endometrium of cows in relation to their EB status in early lactation. Our results reveal a complex network of activity within the postpartum uterus involving MMPs and IGFBPs whose expression is regulated through a combination of inflammatory mediators and metabolic indicators. We suggest that this is likely to affect the ability of the MMPs to promote involution through degradation of the extracellular matrix and will also alter the availability of IGFs to promote proliferation and healing. Delay to the endometrial repair processes when cows are in SNEB may thus be a contributing factor leading to the lower fertility subsequently found in such animals.

## Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich Company Ltd or VWR International Ltd (Poole, Dorset, UK) unless otherwise specified.

### Animals and tissue collection

All procedures were carried out under license in accordance with the European Community Directive, 86-609-EC. From an initial pool of 24 Holstein-Friesian cows with an average previous lactation yield of  $6477 \pm 354$  kg, 12 multiparous cows were randomly allocated 2 weeks prior to expected calving to either a MNEB ( $n=6$ ) or SNEB ( $n=6$ ) treatment, taking account of their parity, BCS and previous lactation yield in a randomised block design. The treatments began in the morning after the second or third milking following parturition. MNEB cows were fed *ad libitum* grass silage with 8 kg/day of a 21% crude protein dairy concentrate and milked once daily; SNEB cows were fed 25 kg/day silage with 4 kg/day concentrate and milked three times daily. Similar treatment groupings were shown in a previous study to produce differences in EB in early lactation (Patton *et al.* 2006).

EB was estimated as the difference between energy intake and the sum of energy for maintenance and milk production, based on measurements of milk yield, milk composition, liveweight and feed intake. The French NE system was used, where 1 unité fourragère lait (UFL) is the net energy for lactation equivalent of 1 kg standard air-dry barley (Jarrige 1989). Samples of the endometrium were collected from all cows following slaughter at  $14 \pm 0.4$  days *post partum* as

described below. This was on days 6–7 of the first follicular wave after calving, before any animal had ovulated, so progesterone levels were still low.

### Blood samples and assays

Blood samples were collected three times weekly from calving (on Mondays, Wednesdays and Fridays) and just prior to slaughter into lithium–heparin primed vials and immediately placed on ice before centrifugation at 2000 g for 10 min. Plasma was decanted and stored at  $-20^\circ\text{C}$  for subsequent analysis. Samples of plasma were analysed for glucose, NEFAs,  $\beta$ -hydroxybutyrate and urea using appropriate kits and an ABX Mira autoanalyser (ABX Mira, Cedex, France). Concentrations of plasma IGF1, insulin and oestradiol were assayed as described and reported previously (Taylor *et al.* 2004, Lewellyn *et al.* 2007, Fenwick *et al.* 2008a).

A double antibody RIA was used for GH determination where bovine GH (a gift from Dr J Gong, Roslin Institute, Edinburgh, UK) was used for iodination and preparation of the standard curve (Taylor *et al.* 2004). The antiserum was raised in a guinea pig against bovine GH and was used at 1:10 000 dilution. The sensitivity of the assay was 1.6 ng/ml. The intra-assay coefficient of variation (CV) was 12.9%. Oestradiol concentrations were assayed using the Estradiol MAIA assay kit (BioStat Diagnostic Systems, Stockport, Cheshire, UK).

Cortisol concentrations were measured by RIA. Aliquots of 100  $\mu\text{l}$  plasma were extracted with 3 ml diethyl ether in glass tubes, the organic phase was dried down and the sample was reconstituted in 300  $\mu\text{l}$  assay buffer. Next, 100  $\mu\text{l}$  aliquots of standard or extracted plasma were incubated with first antibody (100  $\mu\text{l}$  sheep anti-bovine cortisol; Diagnostics Scotland, Pennycuik, UK; diluted 1:1000 in assay buffer) and [1, 2, 6, 7- $^3\text{H}$ ]cortisol (Amersham Biosciences Ltd; 100  $\mu\text{l}$ ,  $\sim 10\,000$  c.p.m.) overnight at  $4^\circ\text{C}$ . Separation was by dextran-charcoal. The intra-assay CV values for samples with a mean of 3.6 and 51.4 cortisol nmol/l of plasma were 5.6 and 6.1% respectively and the sensitivity of the assay was 0.7 nmol/l.

### Uterine tissue collection and RNA isolation

The uterus was opened and samples of intercaruncular endometrial tissue weighing  $\sim 1$  g were dissected from the mid portion of the previously gravid horn  $\sim 1$  cm anterior to the bifurcation of the uterus. These were rinsed in RNase-free phosphate buffer, snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Total RNA was prepared from 200–300 mg fragmented frozen endometrial tissue and homogenised in TRI reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA). RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), where all samples had a 260/280 ratio of absorbance between 1.8 and 2.1.

### Quantitative real-time PCR

Optimised qPCR assays were used to detect gene transcripts. A detailed description of these assays has been published

previously (Fenwick *et al.* 2008a). Total RNA from each sample was treated for potential genomic DNA carryover in a single reaction in accordance with the guidelines supplied by Promega Corporation. From this reaction, precisely 1 µg DNase-treated RNA was reverse transcribed using random hexamer primers and processed accordingly (Reverse Transcription System kit; Promega). A mastermix of reagents was prepared for the above reaction to minimise potential variation. Selected negative control samples were also prepared by

including all reagents as above, minus the reverse transcriptase. Gene symbols, sequence information, accession numbers and expected product lengths are provided in Table 3.

Gene transcripts were quantified as described in detail previously (Fenwick *et al.* 2008a). Standards for qPCR were prepared from purified PCR products that were quantified by spectroscopy (NanoDrop) and diluted over at least eight orders of magnitude. Briefly, for each assay a mastermix was prepared, which contained a final concentration of 1× absolute qPCR

**Table 3** Oligonucleotide primer sequence and expected amplicon size used for real-time PCR assays.

Gene	Primer sequence (5' → 3')	GenBank accession	Product length (bp)
<i>IGF1</i>	For: AGTTGGTGGATGCTCTCCAGT Rev: CACTCATCCACGATTCCTGTC	NM_001077828.1	115
<i>IGF2</i>	For: GCTTCTACTTCAGCCGACCAT Rev: GGCACAGTAAGTCTCCAGCAG	NM_174087.3	110
<i>IGFBP1</i>	For: TCAAGAAGTGAAGGAGCCCT Rev: AATCCATTCTTGTTCAGTTT	NM_174554	127
<i>IGFBP2</i>	For: AGGGTGGCAAACATCACCT Rev: GAAGGCGCATGGTGGAGAT	NM_174555.1	120
<i>IGFBP3</i>	For: ACAGACACCCAGAATTCTCCTC Rev: GTTCAGGAACTTGAGGTGGTTC	NM_174556.1	102
<i>IGFBP4</i>	For: GACCTTACATCATTCCCATCC Rev: AAGCTTCACTCCCGTCTTCC	NM_174557.3	129
<i>IGFBP5</i>	For: CAAGCCAAGATCGAAAGAGACT Rev: AAGATCTTGGGCGAGTAGGTCT	NM_001105327.1	86
<i>IGFBP6</i>	For: GGAGAGAATCCCAAGGAGAGTAA Rev: GAGTGGTAGAGTCCCCGAGT	NM_001040495.1	100
<i>IGFALS</i>	For: CTCTGGCTGGACGCTCTCC Rev: AAGGTCCTCAGCGAGTTGTTT	NM_001075963.1	111
<i>IGF1R</i>	For: GATCCCGTGTCTTCTACGTTC Rev: AAGCCTCCCACTATCAACAGAA	XM_606794.3	101
<i>IGF2R</i>	For: TACAACCTCCGGTGGTACACCA Rev: GGATTTTCGCTAGCCTGGAGAG	NM_174352.2	111
<i>INSR</i>	For: TCCTCAAGGAGCTGGAGGAGT Rev: TTTCTCGAAGGCCTGGGGAT	XM_590552.4	89
<i>GHR</i>	For: ACTTGGGCTAGCAGTGACATTA Rev: TTCCTTTAATCTTTGGAACCTGG	NM_176608.1	101
<i>NR3C1</i>	For: CTGGGGCCAATATAATTGGTAA Rev: TTCTGATCCTGCTGTTGAGAAA	XM_612999.4	124
<i>ESR1</i>	For: TCAGGCTACCATTACGGAGTTT Rev: GTTTTTATCAATCGTGCCTGG	NM_001001443.1	120
<i>ESR2</i>	For: CTTCGTGGAGCTCAGCCTGT Rev: GAGATATTCTTTGTGTTGGAGTTT	NM_174051.3	241
<i>MMP1</i>	For: GGTGAGTTTATAGCTTATGGATTC Rev: TTGAGAGAAGACATCACGGAGA	NM_174112.1	120
<i>MMP3</i>	For: GATGATGAACAATGGACAAAGG Rev: CGAGGGTTCGTAGACTGGGTA	XM_586521.2	134
<i>MMP9</i>	For: GAGGGTAAGGTGCTGCTGCTTC Rev: AAGGTCACGTAGCCACATAGT	NM_174744.2	236
<i>MMP13</i>	For: ACCCTTCCCATGACCTTATCTT Rev: TCTTCCCTGAATCCTCAAAGTG	NM_174389.2	162
<i>AHSG</i>	For: GACTTGCGCCACACTTTTCTC Rev: CTGATTCTCCCTGGGCAAAG	NM_173984.2	137
<i>PK4</i>	For: GGCCTAGTGTGTGGTGCTTC Rev: GAGCCAGAGTCCATAATCAAG	NM_001101883.1	120
<i>GAPDH</i>	For: GGCCTGAACCACGAGAAGTATAA Rev: CCCTCCACGATGCCAAAGT	BC102589	120
<i>18SrRNA</i>	For: CGGCGACGACCCATTCGAAC Rev: GAATCGAACCTGATCCCCGTC	AY779625	99
<i>RPL19</i>	For: TCGATGCCGGAACAC Rev: ATTCTCATCTCTCATCCAG	NM_001040516	119

Gene products analysed are the insulin-like growth factors (*IGF1/IGF2*), IGF-binding proteins 1–6 (*IGFBP1/2/3/4/5/6*) and the acid labile subunit (*IGFALS*); the IGF receptors (*IGF1R, IGF2R*); insulin receptor (*INSR*); GH receptor (*GHR*); glucocorticoid receptor (*NR3C1, Bos taurus* nuclear receptor subfamily 3, group C, member 1, transcript variant 4); oestrogen receptors A and B (*ESR1, ESR2*); matrix metalloproteinases (*MMP-1, -3, -9* and *-13*); Alpha(2)-HS-glycoprotein (*AHSG*); pyruvate dehydrogenase kinase 4 (*PK4*); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); ribosomal protein L19 (*RPL19*).

SYBR Green Mix (ABgene, Epsom, Surrey, UK), 500 nM forward and reverse primers and nuclease-free water. Primer annealing and amplicon-specific melting temperatures were determined using the gradient function of the DNA Engine Opticon 2 thermal cycler (MJ Research, Inc., Waltham, MA, USA). Equivalent amounts of sample cDNA were added to each reaction in duplicate. To minimise variation, all samples included in each analysis were derived from the same RT batch, prepared under the same conditions and were analysed on a single plate. Thermal cycling conditions applied to each assay consisted of an initial Taq activation step at 95 °C for 15 min followed by 38 cycles of denaturation (95 °C), annealing (range 50.0–64.2 °C), extension (72 °C) and an amplicon-specific fluorescence acquisition reading (range 74–84 °C). A melting curve analysis was performed for each amplicon between 50 and 95 °C and as such any smaller non-specific products such as dimers were melted (if present) prior to fluorescence acquisition. All qPCR results were recorded with the Opticon Monitor Analysis Software (V2.02; MJ Research). For comparison of expression data, absolute values were derived from standard curves generated from purified cDNAs identical to amplified products and expressed as fg/μg reverse-transcribed RNA.

### Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS for Windows, V13.0, Chicago, IL, USA). Differences in gene expression at the time of tissue collection between MNEB and SNEB groups were analysed using an independent samples *t*-test. Levene's test was used to account for variance and if homogeneity was not achieved an unequal variance *t*-test was used. The last blood sample measurement from each cow collected pre-slaughter was compared between the groups using *t*-test. For IGF1, GH, insulin and cortisol, a repeated measure ANOVA via a linear mixed effect model was also performed for samples collected during the 2 weeks after calving.

Relationships between absolute levels of expression for all genes measured by qPCR and between gene expression levels and blood hormone and metabolite measurements at slaughter were determined from values pooled across treatment groups. These calculations were also repeated using an analysis of covariance between the pairs of variables with the treatment included as a fixed effect.

Significance has been taken at  $P < 0.05$ , although for the correlation data the rough false discovery rate was also used to adjust the level of significance (Benjamini & Hochberg 1995). For the *t*-tests on the qPCR, data trends are also reported at  $P < 0.10$ .

### Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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