Effect of negative energy balance on the insulin-like growth factor system in pre-recruitment ovarian follicles of *post partum* dairy cows

S Llewellyn, R Fitzpatrick¹, D A Kenny², J J Murphy³, R J Scaramuzzi and D C Wathes

Reproduction, Genes and Development Group, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts AL9 7TA, UK, ¹Animal Production Research Centre Mellows Campus, Athenry, Co. Galway, Ireland, ²Faculty of Agriculture, University College Dublin, Dublin, Ireland and ³Teagasc Dairy Production Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

Correspondence should be addressed to S Llewellyn; Email: sllewellyn@rvc.ac.uk

Abstract

Post partum negative energy balance (NEB) in dairy cattle is associated with a delayed return to ovarian cyclicity and reduced fertility. This study compared the IGF system of pre-recruitment ovarian follicles between cows in mild ($n=6$) or severe ($n=6$) NEB during early lactation. Ovaries were collected in the second week *post partum*, when circulating concentrations of IGF-I and glucose were lower ($P<0.01$) in severe NEB cows. mRNA expression for IGF-II, type 1 IGF receptor (IGF-1R) and IGF-binding proteins (IGFBP)-1 to IGFBP-6 was determined by *in situ* hybridisation in individual follicles using radiolabelled oligonucleotide probes. Follicles were classified as very small (1–2.5 mm) or small (2.5–5 mm) and healthy or atretic. Relative mRNA concentrations were measured as optical density (OD) units using image analysis. Thecal IGF-II mRNA expression was highest in very small, healthy follicles ($P<0.05$). Granulosa cell IGFBP-2 was the only component to change with EB status, with higher mRNA expression in mild compared with severe NEB cows ($P<0.05$). IGFBP-1 and IGFBP-3 mRNA expression were undetectable. IGF-1R, IGFBP-4 and IGFBP-5 mRNA expression were not significantly altered by follicle size or health, but IGFBP-5 tended to increase in atretic follicles. The pattern of IGFBP-6 mRNA expression in theca paralleled that of IGF-II mRNA, with higher ($P<0.05$) levels in healthy, very small follicles. In conclusion, the reduced expression of IGFBP-2 mRNA in severe NEB cows may alter the bioavailability of circulating IGF-I and locally produced IGF-II to modulate the pre-recruitment stages of follicles required to maintain normal *post partum* ovarian cyclicity.

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Introduction

Dairy cow fertility has declined over the last 50 years, representing a major source of economic loss to the agricultural industry worldwide (Royal *et al.* 2000). An early return to ovarian cyclicity after calving can improve reproductive efficiency by promoting a short interval to first service, coupled with good conception rates (Butler & Smith 1989). During the early *post partum* period, the energy demand for maintenance and production exceeds that of dietary energy intake and dairy cows enter a state of negative energy balance (NEB) during which they mobilise body reserves (Bauman & Currie 1980). One effect of NEB on fertility is to delay the interval from calving to first ovulation (Beam & Butler 1999). The initiation of follicle waves after calving occurs regardless of NEB (Beam & Butler 1999), but the number of follicles growing and their development to

larger sizes is decreased when poor body condition is combined with low nutritional levels (Perry *et al.* 1991).

The metabolic hormones insulin-like growth factor-I (IGF-I) and insulin are both likely candidates for mediating the effects of NEB on reproductive function. Circulating IGF-I concentrations fall sharply in the first week after calving. The extent of the fall has been related to both the interval to first ovulation and to conception (Beam & Butler 1999, Taylor *et al.* 2004). In the cow, the concentration of IGF-I in the follicular fluid of a dominant follicle reflects systemic IGF-I concentrations (Funston *et al.* 1996), whereas IGF-II is derived locally from theca cells (Perks *et al.* 1999, Armstrong *et al.* 2000). The bovine ovary can respond to IGFs via type 1 IGF receptors (Perks *et al.* 1999). IGF-I enhances the ovarian action of gonadotrophins (Adashi 1998) and also stimulates follicle cell proliferation and oestradiol

production directly (Gutierrez *et al.* 1997). The local production of IGF-II supports follicle growth (Kamada *et al.* 1992) and steroidogenesis (Spicer *et al.* 2004).

Insulin also acts directly on antral follicle growth and function (Hamilton *et al.* 1999), appearing a less-potent mitogen of bovine granulosa cells than IGF-I (Spicer & Echternkamp 1995), but a similarly potent stimulator of oestradiol production (Gutierrez *et al.* 1997). Circulating insulin concentrations may (Gong *et al.* 2002) or may not (Meikle *et al.* 2004) be related to the time of re-initiation of ovarian cyclicity after parturition, but could act indirectly to influence ovarian activity, as insulin is a primary regulator of circulating IGF-I concentrations via effects on expression of GH receptors in the liver (Butler *et al.* 2003).

The bioactivity of both IGF-I and IGF-II in the ovary is regulated by the IGF-binding proteins (IGFBPs). In the ovine and bovine ovary, IGFBP-2 and IGFBP-5 are synthesised locally in the follicle wall by granulosa cells, and IGFBP-4 and IGFBP-6 in theca cells (Armstrong *et al.* 1998). Concentrations of IGFBP-2, IGFBP-4 and IGFBP-5 in follicular fluid decrease during growth and development of dominant follicles in cows (Spicer & Echternkamp 1995, Stewart *et al.* 1996). Dominance is associated with the inhibition of IGFBP mRNA expression (Webb *et al.* 1999) and post-translational modification via proteolytic degradation of both locally produced and systemically derived IGFBPs (Spicer 2004). The production of IGFBPs is thus a key factor controlling follicle growth and atresia (Bao & Garverick 1998). Expression of follicular IGF-1R, IGFBP-2 and IGFBP-4 mRNA are reported to be nutritionally sensitive (Munoz-Gutierrez *et al.* 2004) influencing the bioavailability of intrafollicular IGF and thus the sensitivity of follicles to gonadotrophins (Adashi 1998). We have recently shown in the liver that severe NEB specifically attenuates expression of IGF-I and IGFBP-3 to IGFBP-6 mRNA, while increasing IGFBP-2 synthesis (Fenwick *et al.* 2006). This profile fits a scenario of globally suppressed IGF-I availability, which likely contributes to an altered IGF environment in peripheral tissues involved in reproduction.

Development of a preovulatory follicle in the cow is estimated to take in excess of 40 days from the early antral stage to ovulation (Lussier *et al.* 1987). From antrum acquisition at around 300 µm, growth to 5 mm takes more than 30 days (Lussier *et al.* 1987) and from 5 mm follicles are recruited into a wave of gonadotrophin-dependent growth lasting 4–6 days that selects one follicle for dominance, while subordinate follicles regress (Rajakoski 1960, Fortune *et al.* 2001). Since the service period usually begins at about 50–60 days after calving, follicles that are due to ovulate at this time undergo their earlier stages of development during the NEB nadir. To the best of our knowledge, no previous studies have explored the effect of NEB on expression of the ovarian IGF system in lactating dairy cows. This study

investigated the hypothesis that the early stages of follicular growth may be influenced by alterations to the follicular IGF system when cows are in NEB. Such changes might influence the fate of dominant follicles in the *post partum* period, contributing to longer anoestrus intervals seen in association with NEB. They might also affect later fertility via changes to oocyte quality (Britt 1994). The objective was to compare patterns of mRNA expression for the IGF system within pre-recruitment follicles during the time of NEB in two groups of cows managed to achieve a mild or severe negative energy balance status during early lactation.

Materials and Methods

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, 12 multiparous cows were blocked 2 weeks prior to expected calving according to parity, body condition score and previous lactation yield, and randomly allocated to either a mild (mNEB) or severe NEB (sNEB) treatment. Treatments began on 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 thrice daily. Similar treatments have been shown in a previous study to produce differences in EB in early lactation (Patton *et al.* 2006).

Energy balance 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). Milk yield (kg) was recorded daily at the morning, evening and night milkings using electronic milk meters (Dairy Master, Causeway, Co. Kerry, Ireland). Milk composition (fat, protein and lactose) was determined twice weekly from successive morning, evening and night milk samples by automated infrared absorption analysis using a Milkoscan 605 (Foss Electric, Hillerød, Denmark). Forage and concentrate intakes were recorded electronically using the Griffith Elder feeding system. Samples of grass silage offered were taken twice weekly. Concentrate samples were taken once weekly. The dry matter intake was calculated on a daily basis. Cow body weight (kg) was recorded immediately post-calving and once weekly thereafter. The dry cows were weighed before feeding in the morning and the lactating cows were weighed after morning milking, before feeding.

The diameter of the first dominant follicle was determined for each cow from day 7 *post partum* via

daily transrectal ovarian ultrasonography (Aloka SSD-900, Aloka Ltd, Tokyo, Japan, 7.5 MHz transducer). Cows were slaughtered on day 6–7 of the first follicular wave after calving. This design was chosen to collect tissue at a fixed time in relation to the stage of ovarian activity. The ovary contralateral to that containing the dominant follicle was bisected and the two halves were then immediately frozen using isopentane and liquid nitrogen, and stored at -80°C .

Blood samples were obtained by jugular venipuncture and collected into vials containing lithium heparin as an anticoagulant. Thrice weekly sampling took place after morning milking and before feeding. The samples were placed on ice packs and were centrifuged at 2000 *g* for 10 min immediately after sampling. The plasma was decanted and stored at -20°C until analysis. Daily samples obtained from day 7 *post partum* until slaughter were analysed for oestradiol-17 β . Samples collected pre-slaughter were analysed for IGF-I, insulin, glucose, urea, β -hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA).

In situ hybridisation procedure

All chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) or VWR International Ltd (Poole, Dorset, UK) unless otherwise specified. Serial sections of 10 μm were cut from each ovary using a cryostat. The sections were thaw-mounted onto Super-Frost Plus or POLYSINE microscope slides, fixed in 4% w/v paraformaldehyde in 0.01 M PBS (pH 7.0) for 5 min at room temperature, then washed thrice with 0.01 M PBS (3 min each), dehydrated in 70% (5 min) and 95%

(1 min) ethanol and stored at 4°C in 95% ethanol until used for *in situ* hybridisation.

The method was based upon that described previously by McMullen *et al.* (2005). All probes used were single-stranded oligonucleotides of 45 bases in length (Table 1). The oligonucleotide probes (5 ng) were end-labelled with [^{35}S]dATP (Amersham Biosciences UK Ltd) using terminal deoxynucleotidyl transferase (Promega UK Ltd) at 34°C for 1 h. The labelled probe was diluted to a final concentration of 100 000 c.p.m. $100\ \mu\text{l}^{-1}$ in hybridisation buffer (50% v/v deionised formamide, $4\times$ SSC, 25 mM sodium phosphate, 1 mM sodium pyrophosphate, $5\times$ Denhardt's solution, 0.2 mg/ml denatured salmon sperm DNA, 120 $\mu\text{g/ml}$ sodium heparin, 100 $\mu\text{g/ml}$ polyadenylic acid and 100 mg/ml dextran sulphate) and 100 μl was added to each slide. The slides were incubated in a humidified box overnight at 42, 45 or 52°C (Table 1). Following incubation, the slides were washed in a solution of $1\times$ SSC, 2 g/l sodium thiosulphate at room temperature for 30 min, then in fresh $1\times$ SSC, 2 g/l sodium thiosulphate at 60°C for 60 min. The slides were then rinsed successively for 1 min in solutions of $1\times$ SSC, $0.1\times$ SSC, 75% ethanol and 95% ethanol and air-dried. The slides were exposed to β -max hyperfilm (Kodak BioMax MR Film) for either 4 or 5 days (Table 1).

All ovarian sections treated with a particular probe were hybridised in the same batch. Sense probes, which were identical in sequence to the respective mRNA targets, were always included as negative controls and any signal from these was regarded as non-specific. Each batch also contained an appropriate positive control tissue, based on previous studies. These were cross-sections of uterus from an oestrous ewe for the type 1 IGF receptor (Stevenson

Table 1 The sense sequences of oligonucleotide probes used for *in situ* hybridisation analysis, their similarity to the equivalent bovine genome and the exposure times for X-ray films and photographic emulsions.

Probe	Sense sequence	Hybridisation temperature ($^{\circ}\text{C}$)	Exposure time (days)		Homology to bovine gene (%)
			X-ray film	Emulsions	
IGF-II	95–139 of ovine <i>IGF-II</i> gene (O'Mahoney & Adams 1989) 5'-CCAGCGAGACTCTGTGCGGCGGGAGCTGGTGACACCTCCAGT-3'	52	4	30	100
IGF-1R	352–396 of human <i>IGF-1R</i> mRNA (Stoeltzing <i>et al.</i> 2003) 5'-CTCACGGTCATCCGCGGCTGGAACTCTTCTACAACCTACGCCCTG-3'	42	4	42	97 ^a
IGFBP-1	779–823 of bovine <i>IGFBP-1</i> gene (Sneyers <i>et al.</i> 1991) 5'-GGAGAGCCTGGGCTCTGTGGTGTGTCTACCCTTGGAGTGGAAG-3'	45	5	^b	100
IGFBP-2	452–496 of ovine <i>IGFBP-2</i> liver mRNA (Delhanty & Han 1992) 5'-GCGCCAGCCCGAGCAGGTTCAGACAATGGCGAGGAGCACTCTG-3'	45	4	42	97
IGFBP-3	532–576 bovine <i>IGFBP-3</i> mRNA (Erondur <i>et al.</i> 1997) 5'-GAGTCGGAAGAAGACCACAGCATGGGGAGCAGACAGAACCAAGGCT-3'	45	4	28	100
IGFBP-4	901–945 of bovine <i>IGFBP-4</i> mRNA (Moser <i>et al.</i> 1992) 5'-AAGACGGGAGTGAAGCTTCCGGGGGGCTGGAGCCGAAGGGGGAG-3'	45	5	42	100
IGFBP-5	291–335 of bovine <i>IGFBP-5</i> gene (Moser <i>et al.</i> 1992) 5'-CTACTCGCCCAAGATCTTCCGGCCCAAGCACACCCGCATCTCCGA-3'	42	4	28	100
IGFBP-6	292–336 of bovine <i>IGFBP-6</i> gene (Moser <i>et al.</i> 1992) 5'-CTCTACGTGCCTAATTGTGACCATAGGGGCTTCTACCGGAAGCGG-3'	42	5	42	100

^aThe bovine *IGF-1R* gene sequence corresponding to nucleotides 352–396 of the human *IGF-1R* gene is not completely known, but predicted to be 97% homologous.

^bAutoradiography revealed no hybridisation so emulsions were not prepared.

et al. 1994), IGFBP-1 (Osgerby *et al.* 1999) and IGFBP-6 (Gadd *et al.* 2002); ovine placenta for IGF-II and IGFBP-2, IGFBP-3 and IGFBP-4 (McMullen *et al.* 2005) and ovine intercotyledonary tissue for IGFBP-5 (Osgerby *et al.* 2004). We did not investigate IGF-I mRNA localisation as we and others have previously shown that IGF-I mRNA is weakly expressed in the bovine ovary (Perks *et al.* 1999, Armstrong *et al.* 2000).

To aid cellular localisation of hybridised probes, selected slides were subsequently coated with photographic emulsion (LM1; Amersham Biosciences UK Ltd) according to the manufacturer's instructions and stored for 28, 30 or 42 days (Table 1) at 4 °C in the dark. The slides were developed in 20% phenisol (ILFORD Imaging UK Ltd, Knutsford, Cheshire, England) fixed in 1.9 M sodium thiosulphate and counterstained with haematoxylin and eosin. All other slides were also stained with haematoxylin and eosin to aid follicular characterisation.

Follicular characterisation

The follicles were characterised according to their size (very small, 1–2.5 mm and small, 2.5–5 mm) and stage of atresia. No follicles examined in this study exceeded 5 mm in diameter. The stage of atresia was assessed by morphological criteria (Irving-Rodgers *et al.* 2001) from the haematoxylin- and eosin-stained sections. Follicles classified as non-atretic had a regular granulosa layer with cells tightly packed and 'healthy' as indicated by a rounded, regular cell outline. Atretic follicles were marked by a convoluting and/or ruptured basement membrane, an irregular antral surface or granulosa cells of the most basal layer separated from each other and from the basal lamina.

Optical density measurements

The relative expression of mRNA for components of the ovarian IGF system was quantified from the autoradiographs using the public domain NIH Image program (available through the NIH website – <http://www.nih.gov>), which calculated the average OD over the selected area of film based on a linear grey scale of 0.01–2.71. For each ovary, individual follicles were identified and using the 'freehand selection tool', follicle walls were measured separately for mRNA expression of the gene of interest. Autoradiographs were scanned into a computer and images projected on screen so that areas of interest were selected and the OD measured. Readings were obtained from at least two sections per tissue for each of the antisense (AS) and sense (S) probes. The background OD, from a blank area of film, was also measured and subtracted from OD measurements of both AS and S. Finally, the S values were subtracted from AS values to give an average OD value for specific hybridisation (Perks *et al.* 1995). The detection limit was taken as an OD value of 0.01.

Metabolite and hormone assays

Blood plasma was analysed for glucose, NEFA, BHB and urea concentrations using appropriate kits (Randox Laboratories Ltd, Crumlin, Co. Antrim, UK) and a Mira autoanalyzer (HORIBA ABX, Montpellier, France). Measurement of plasma oestradiol concentrations used the Estradiol MAIA assay kit (BioStat Diagnostic Systems, Stockport, Cheshire, UK) based on the method used by Prendiville *et al.* (1995). The intra-assay coefficients of variation (CV) for samples with a mean of 1.1 and 4.6 oestradiol pg/ml of plasma were 15.8 and 7.3% respectively. The inter-assay CV for the same samples were 0.6 and 5.6% respectively.

Plasma insulin was assayed by a double antibody RIA (Williams *et al.* 2001). Briefly, 100 µl aliquots of plasma and standard were incubated with first antibody (50 µl guinea pig anti-bovine insulin; Sigma-Aldrich Company Ltd; diluted 1:100 in assay buffer) overnight at 4 °C. Bovine insulin (Sigma-Aldrich Company Ltd) was iodinated with ¹²⁵I (Amersham Biosciences UK Ltd; 100 µl, approx 17 000 c.p.m.) was added to each tube; tubes were vortexed and incubated for an additional 24 h at 4 °C. Second antibody (100 µl; anti-guinea pig; SAC-CEL; IDS Ltd, Boldon, Tyne & Wear, UK) was added to each tube, vortexed and incubated at room temperature for 30 min. Bound and free fractions were separated by centrifugation, the supernatants decanted and counts per minute in the pellets were determined with a gamma counter. The intra-assay CV for samples with a mean of 0.09 insulin ng/ml of serum was 0.11% and the sensitivity was 0.05 ng/ml.

Plasma IGF-I was measured using human OCTEIA IGF-I kits (IDS Ltd; human and bovine IGF-I only differ by 1 amino acid) as described previously (Swali & Wathes 2006). The intra-assay CV was 2.1% and the sensitivity was 1.9 ng/ml. All samples for both insulin and IGF-I were measured in the same assays.

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences version 13.0. Data for plasma metabolite and hormone concentrations at the time of tissue collection were analysed using Student's *t*-test. Repeated measures ANOVA was used to determine the effect of treatment on daily UFL and plasma oestradiol concentrations. For mRNA expression in the ovary, the effects of treatment, follicle size and follicle health on the proportion of follicles expressing mRNA for each probe were analysed by the χ^2 -test. Levels of mRNA expression for each probe were compared between treatments, with follicle health (healthy or atretic) and size (very small (1–2.5 mm) or small (2.5–5 mm)) as factors, by linear mixed model analysis. Follicle and cow were entered as random effects. For this purpose, data from follicles in which

a particular probe showed no detectable specific hybridisation (OD of <0.01) were excluded from the analysis. Results were considered statistically significant when $P < 0.05$.

Results

Energy balance and plasma measurements

Results are reported for the day of tissue collection. This occurred on days 14 ± 0.73 post partum ($n=6$, range 11–16) in the mNEB group and days 14 ± 0.68 post partum ($n=6$, range 12–16) in the sNEB group. Over the 2-week treatment period from day 2 post-calving until tissue collection, cows in the sNEB group had significantly ($P=0.02$) lower EB levels (Fig. 1). Body condition score was unchanged. Measurements of plasma metabolite and hormone concentrations are reported in Table 2. Circulating concentrations of glucose and IGF-I were higher ($P < 0.01$) in the mNEB cows, whereas BHB and NEFA were lower ($P < 0.01$). Urea tended to be higher ($P=0.08$) in the sNEB group. While the mean concentration of plasma insulin was numerically lower in sNEB cows, the difference was not significant (Table 2). At the time of tissue collection, mean circulating oestradiol concentrations (Table 2) and dominant follicle size (mNEB: 15.0 ± 0.69 mm, sNEB: 12.2 ± 1.25 mm) did not vary significantly between treatment groups.

Follicle numbers and characteristics

A total of 108 pre-recruitment follicles from 12 ovaries were analysed by *in situ* hybridisation. The spatial distribution of mRNA-encoding components of the IGF system within these follicles is shown in Figs 2–4. The proportion of follicles showing detectable hybridisation for each probe (OD of AS-S value of >0.01 from the autoradiographs) is summarised in Table 3 according to

Figure 1 Effect of milking frequency and diet on energy balance (EB) in post partum dairy cows. mNEB (■) = milked once daily on *ad libitum* grass silage with 8 kg/day concentrate; sNEB (□) = milked thrice daily on 25 kg/day silage with 4 kg/day concentrate. Values are mean $\pm$ S.E.M., $n=6$ cows per group. Over the 2-week treatment period, there was a significant ($P=0.02$) difference in mean EB between treatments. UFL, unité fourragère lait.

Table 2 Plasma hormone and metabolite concentrations at the time of tissue collection in post partum dairy cows maintained under mild or severe negative energy balance (NEB).

	Mild NEB	Severe NEB	P
IGF-I (ng/ml)	51.0 ± 8.44	10.6 ± 1.09	<0.01
Insulin (ng/ml)	0.223 ± 0.0582	0.130 ± 0.0193	0.178
Oestradiol (pg/ml)	2.24 ± 0.326	1.57 ± 0.240	0.137
Glucose (mmol/l)	4.10 ± 0.126	2.66 ± 0.147	<0.01
NEFA (mmol/l)	0.553 ± 0.216	1.41 ± 0.136	<0.01
BHB (mmol/l)	0.593 ± 0.0965	3.71 ± 0.201	<0.01
Urea (mmol/l)	3.98 ± 0.460	5.08 ± 0.310	0.079

Values are mean $\pm$ S.E.M., $n=6$ cows per group. Tissue was collected on approximately day 14 post partum.

follicle size and health. The only significant difference in proportion of follicles showing detectable hybridisation according to the EB treatment group was for IGFBP-6 mRNA (described below). For those follicles with detectable expression with a particular probe, the concentrations of mRNA in OD units are summarised in Table 4 according to size (very small (1–2.5 mm) or small (2.5–5 mm)), health and energy balance classifications. These data were analysed in a mixed model analysis and the significant effects of each factor and their two-way interactions are summarised in Table 5. The results are discussed below according to probe.

Expression of IGF-II mRNA

IGF-II mRNA was localised to theca cells from both healthy and atretic very small and small follicles (Fig. 2) in 97% of follicles examined (Table 3). There was no effect of EB treatment on the expression of thecal IGF-II mRNA (Table 5). Levels of IGF-II mRNA expression were however affected by an interaction between follicle health and size. Expression was higher in healthy than atretic follicles ($P < 0.05$) and within healthy follicles, expression was higher ($P < 0.01$) in very small than small follicles (Fig. 5B).

Expression of type 1 IGF receptor mRNA

IGF-1R mRNA was detected in granulosa cells (Fig. 2). Of 97 follicles examined, 26 showed no detectable IGF-1R mRNA expression and EB treatment did not affect the proportion of follicles expressing IGF-1R mRNA. Follicle health and size affected the proportion of follicles expressing IGF-1R mRNA when including all cows from both treatment groups in the statistical analysis (Table 3). There was a greater ($P < 0.01$) proportion of small than very small follicles expressing IGF-1R mRNA and it was detectable in more healthy than atretic follicles ($P < 0.01$). When IGF-1R mRNA was detectable, the concentrations measured were not affected by EB treatment, follicle size or health (Table 5).

Figure 2 Expression of mRNA for the ovarian IGF system in *post partum* dairy cows in negative energy balance. Autoradiographic localisation of mRNA for (A and B) theca IGF-II and (E and F) granulosa IGF-1R. Photographs are from corresponding sections of ovary coated with photographic emulsion and counterstained with haematoxylin and eosin; (C and D) IGF-II and (G and H) IGF-1R mRNA expression. Examples of antisense (A, C, E, and G) and sense (B, D, F, and H) probes are illustrated. Arrows on autoradiographs denote regions showing specific hybridisation. Follicles are very small (1–2.5 mm) healthy (VSH), very small atretic (VSA) or small (2.5–5 mm) atretic (SA). The regressing corpus luteum of pregnancy (corpus albicans, CA), ovarian stroma (S), and granulosa (G) and theca (T) follicle layers are also shown. (A and E) Scale bars=5 mm; (C and G) Scale bars=100 µm.

Expression of IGFBP-1 and IGFBP-3 mRNA

The expression of IGFBP-1 and IGFBP-3 mRNA could not be detected in any follicles from either treatment group, regardless of follicle health or size, despite expression being detected in ovine oestrus uterus and placentome used respectively, as positive control tissues (data not shown).

Figure 3 Expression of mRNA for the ovarian IGF system in *post partum* dairy cows in negative energy balance. Autoradiographic localisation of mRNA for (A and B) granulosa IGFBP-2 and (E and F) theca IGFBP-4. Photographs are from corresponding sections of ovary coated with photographic emulsion and counterstained with haematoxylin and eosin; (C and D) IGFBP-2 and (G and H) IGFBP-4 mRNA expression. Examples of antisense (A, C, E, and G) and sense (B, D, F, and H) probes are illustrated. Arrows on autoradiographs denote regions showing specific hybridisation. Follicles are very small (1–2.5 mm) healthy (VSH) or small (2.5–5 mm) atretic (SA). The regressing corpus luteum of pregnancy (corpus albicans, CA), ovarian stroma (S) and granulosa (G) and theca (T) follicle layers are also shown. (A and E) Scale bars=5 mm; (C and G) Scale bars=100 µm.

Expression of IGFBP-2 mRNA

IGFBP-2 mRNA was localised to granulosa cells (Fig. 3). Of 92 follicles examined, 47% did not express IGFBP-2 mRNA. Neither EB treatment nor follicle health affected the proportion of follicles expressing IGFBP-2 mRNA. When including all cows from both treatment groups in

Figure 4 Expression of mRNA for the ovarian IGF system in *post partum* dairy cows in negative energy balance. Autoradiographic localisation of mRNA for (A and B) granulosa IGFBP-5 and (E and F) thecal IGFBP-6. Photographs are from corresponding sections of ovary coated with photographic emulsion and counterstained with haematoxylin and eosin; (C and D) IGFBP-5 and (G and H) IGFBP-6 mRNA expression. Examples of antisense (A, C, E and G) and sense (B, D, F and H) probes are illustrated. Arrows on autoradiographs denote regions showing specific hybridisation. Follicles are very small (1–2.5 mm) atretic (VSA), small (2.5–5 mm) healthy (SH) or small atretic (SA). The regressing corpus luteum of pregnancy (corpus albicans, CA), ovarian stroma (S) and granulosa (G) and theca (T) follicle layers are also shown. (A and E) Scale bars = 5 mm; (C and G) Scale bars = 100 µm.

the statistical analysis (Table 3), a larger proportion of small than very small follicles were expressing IGFBP-2 mRNA ($P=0.01$). Follicular IGFBP-2 mRNA expression was the only member of the IGF family influenced by the cow's EB status. Concentrations of granulosa IGFBP-2 mRNA were higher ($P<0.05$) in mNEB than in sNEB cows (Table 5 and Fig. 5A).

Expression of IGFBP-4 mRNA

IGFBP-4 mRNA was detected in theca cells (Fig. 3) with 27% of 92 follicles examined showing no detectable expression (Table 3). The proportion of follicles expressing IGFBP-4 mRNA was unaffected by EB treatment, follicle size or follicle health. There were similarly no significant effects of EB group, follicle size or health on the measured concentrations of IGFBP-4 mRNA (Table 5).

Expression of IGFBP-5 mRNA

IGFBP-5 mRNA was expressed by granulosa cells (Fig. 4). Of 105 follicles examined, 50% did not express IGFBP-5 mRNA and the proportion of follicles expressing IGFBP-5 mRNA did not vary between EB treatment groups. Follicle size and health affected the proportion of follicles expressing IGFBP-5 mRNA when including all cows from both treatment groups in the statistical analysis (Table 3). There was a greater ($P<0.01$) proportion of small than very small follicles expressing IGFBP-5 mRNA and it was detectable in more atretic than healthy follicles ($P<0.01$). Concentrations of IGFBP-5 mRNA were not affected by EB treatment or follicle size, but there was a trend ($P=0.063$) for increased expression in atretic follicles (Tables 4 and 5).

Expression of IGFBP-6 mRNA

IGFBP-6 mRNA was detected in theca cells (Fig. 4). Of 96 follicles examined, 32% showed no detectable expression of IGFBP-6 mRNA (Table 3). EB status had an effect on the proportion of follicles expressing IGFBP-6 mRNA. Within very small atretic follicles, the sNEB cows were more likely to have detectable expression than the mNEB cows (69% of 13 follicles examined compared with 28% of 18 follicles examined, $P<0.01$). Overall, the proportion of healthy follicles expressing IGFBP-6 mRNA was greater ($P=0.01$) than atretic follicles but follicle size had no effect (Table 3). When IGFBP-6 mRNA was detectable, there was no effect of EB treatment on its expression (Table 5). Concentrations of IGFBP-6 mRNA expression were however affected by an interaction between follicle health and size. Within healthy follicles, IGFBP-6 expression was higher ($P<0.05$) in very small than small follicles (Fig. 5C).

Discussion

The aim of this work was to investigate the hypothesis that severe NEB may compromise the early stages of follicular growth via alterations to the follicular IGF system. The two EB treatments produced the desired differences in the depth of negative energy balance at

Table 3 The percentage of follicles examined with detectable (optical density > 0.01) mRNA expression for members of the insulin-like growth factor (IGF) family in ovaries from *post partum* dairy cows, classified according to size (very small, 1–2.5 mm; small, 2.5–5 mm) and health.

Follicle classification	IGF-II	IGF-1R	IGFBP-2	IGFBP-4	IGFBP-5	IGFBP-6
Very small	95% (64)	67% (55) ^b	37% (51) ^b	66% (47)	39% (61) ^b	66% (56)
Small	100% (44)	81% (42) ^a	73% (41) ^a	80% (45)	66% (44) ^a	70% (40)
Healthy	100% (39)	91% (35) ^c	54% (35)	85% (33)	26% (38) ^d	88% (34) ^a
Atretic	96% (69)	63% (62) ^d	53% (57)	66% (59)	64% (67) ^c	56% (62) ^b
No expression	3% (108)	27% (97)	47% (92)	27% (92)	50% (105)	32% (96)

The number of follicles examined in each category is given in parenthesis. Within columns, proportions differ significantly where indicated by different superscripts: a > b, c > d, $P \leq 0.01$. Tissues were collected on day 6–7 of the first follicular wave, which occurred on approximately day 14 after calving.

slaughter. This resulted in higher concentrations of plasma IGF-I and glucose in mNEB cows, while sNEB cows had higher systemic concentrations of BHB and NEFA. There was no effect of treatment on body condition score as reported by Patton *et al.* (2006), who showed that similar EB treatments delayed both the interval to first ovulation and the proportion of cows ovulating their first dominant follicle. In this study, EB status had a clear effect on follicular IGFBP-2 mRNA expression, which was significantly reduced in sNEB cows. More sNEB cows also expressed IGFBP-6 mRNA in their very small atretic follicles. Although no other clear effects of EB status were detected, all except one of the cows were still in NEB at the time of ovarian collection, and this may have influenced all the cows to a similar extent.

The samples were obtained during the first follicular wave, which occurred in the second week after calving. The follicles which were examined did not exceed 5 mm in diameter, consistent with the data of Dufour & Roy

(1985) who reported that the follicular population in early *post partum* dairy cows consisted predominantly of follicles < 4 mm in diameter. These smaller follicles, at the pre-recruitment stage of development (Fortune *et al.* 2001), may emerge in succeeding follicle growth waves and potentially progress to dominance before and during the service period. The prevailing follicular IGF system at the time of collection may therefore affect *post partum* ovarian cyclicity and follicular and/or oocyte quality, with consequences for subsequent conception rates.

IGF-II mRNA was expressed by theca cells, confirming earlier observations in both the bovine (Armstrong & Webb 1997, Perks *et al.* 1999) and the ovine (Perks *et al.* 1995) ovary. The higher concentrations of IGF-II mRNA found in healthy than atretic follicles may indicate that IGF-II is important for follicular survival. IGF-II mRNA expression also decreased with increasing follicle diameter (Perks *et al.* 1999). Since smaller ovarian follicles have a greater capacity to proliferate whereas larger follicles have greater steroidogenic ability (Spicer

Table 4 Expression of the ovarian insulin-like growth factor (IGF) system in granulosa and theca cell layers of bovine follicles classified according to energy balance group, size and health.

	Follicle classification							
	Health				Size			
	<i>n</i>	Healthy	<i>n</i>	Atretic	<i>n</i>	Very small	<i>n</i>	Small
IGF-II (theca)								
MNEB	14	0.88±0.096	37	0.71±0.043	30	0.90±0.048	21	0.69±0.094
SNEB	25	0.86±0.057	29	0.72±0.048	31	0.82±0.047	23	0.76±0.058
IGF-1R (granulosa)								
MNEB	11	0.16±0.032	22	0.17±0.017	18	0.20±0.019	15	0.14±0.031
SNEB	21	0.20±0.020	17	0.16±0.023	19	0.18±0.023	19	0.18±0.021
IGFBP-2 (granulosa)								
MNEB	7	0.18±0.024	18	0.14±0.015	10	0.17±0.018	15	0.15±0.021
SNEB	12	0.11±0.017	12	0.11±0.022	9	0.10±0.023	15	0.12±0.015
IGFBP-4 (theca)								
MNEB	8	0.19±0.028	18	0.18±0.020	11	0.21±0.024	15	0.16±0.025
SNEB	20	0.21±0.018	21	0.18±0.018	20	0.19±0.018	21	0.20±0.018
IGFBP-5 (granulosa)								
MNEB	5	0.38±0.219	21	0.67±0.089	12	0.73±0.121	14	0.32±0.203
SNEB	5	0.23±0.182	22	0.53±0.086	12	0.39±0.132	15	0.38±0.152
IGFBP-6 (theca)								
MNEB	10	0.21±0.055	15	0.24±0.038	13	0.27±0.040	12	0.18±0.054
SNEB	20	0.27±0.037	20	0.30±0.032	24	0.31±0.030	16	0.26±0.038

All values are optical density units (mean ± s.e.m.) measured for follicle walls expressing the gene of interest. Very small, 1–2.5 mm; small, 2.5–5 mm. Data were analysed using a mixed model analysis and the results of the analysis are summarised in Table 5.

Table 5 Mixed model analysis of energy balance treatment, follicle size and follicle health effects and their two-way interactions on expression of mRNA for components of the follicular insulin-like growth factor (IGF) system in the ovaries of *post partum* dairy cows.

	<i>P</i> values					
	IGF-II	IGF-1R	IGFBP-2	IGFBP-4	IGFBP-5	IGFBP-6
EB	0.916	0.603	0.012	0.680	0.363	0.174
Size	0.046	0.209	0.820	0.416	0.178	0.098
Health	0.022	0.578	0.477	0.264	0.063	0.531
EB × size	0.217	0.274	0.396	0.121	0.205	0.596
EB × health	0.831	0.307	0.398	0.583	0.982	0.957
Size × health	0.009	0.578	0.410	0.323	0.751	0.026

Effects following a trend ($P < 0.1$) or statistically significant ($P < 0.05$ or < 0.01) are indicated in bold.

& Echternkamp 1995), the higher concentration of IGF-II mRNA in healthy follicles of 1–2.5 mm implies that IGF-II may play a greater role in follicle cell proliferation than oestradiol production. Both IGF-I and IGF-II have been shown to stimulate follicle growth *in vitro* (Kamada *et al.* 1992, Gutierrez *et al.* 1997) and so the lower concentration of circulating IGF-I in sNEB could detrimentally affect folliculogenesis (Lucy *et al.* 1992). Previous work has shown that cows with low circulating concentrations of IGF-I during the first 2 weeks *post partum* subsequently have poor conception rates to first service (Beam & Butler 1999, Pushpakumara *et al.* 2003), whereas those returning to positive energy balance by week 3 *post partum* have higher follicular fluid IGF-I concentrations and produce more good quality oocytes (Kendrick *et al.* 1999).

The IGF-1R was localised to follicle granulosa cells, confirming earlier observations in the cow (Perks *et al.* 1999), whereas in the ovine ovary we have detected expression in both granulosa and theca layers (Munoz-Gutierrez *et al.* 2004). IGF-1R mRNA expression did not vary with follicle size (Stewart *et al.* 1996, Perks *et al.* 1999), which also contrasts with the ewe, where concentrations decreased with increasing follicle diameter (Perks *et al.* 1995). We found a clear reduction in the proportion of atretic follicles expressing IGF-1R mRNA compared with healthy follicles. A reduction in the number of IGF-1R in the follicle wall would be likely to reduce IGF-I and IGF-II stimulated follicle growth and function in atretic follicles (Monget *et al.* 1993). sNEB did not down-regulate IGF-1R mRNA expression, implying that IGF-stimulated ovarian function is not arrested despite concurrent body catabolism. Hamilton *et al.* (1999) similarly found that granulosa cells from nutritionally induced anoestrus cows retained the capacity to respond to IGF-I *in vitro*.

The expression of IGFBP-2 mRNA was confined to granulosa cells (Armstrong *et al.* 1998, Munoz-Gutierrez *et al.* 2004). Whilst there was no effect of EB on the proportion of follicles expressing IGFBP-2 mRNA, EB status was the main factor influencing the concentration, with higher expression in the mNEB group. Munoz-Gutierrez *et al.* (2004) also found that granulosa IGFBP-2 mRNA was nutritionally regulated in the ewe, with

concentrations increasing in response to glucose infusion. Work *in vitro* showed that the production of IGFBP-2 by cultured ovine and bovine granulosa cells was dependent on and stimulated by the presence of IGF-I in the culture medium (Armstrong *et al.* 1996, Voge *et al.* 2004). The reduced expression of granulosa IGFBP-2 mRNA in the sNEB group may therefore have been caused by the lower glucose and/or IGF-I concentrations found in these cows. Neither follicle health nor size affected the expression of IGFBP-2 mRNA. Armstrong *et al.* (1998) also found no difference in granulosa IGFBP-2 mRNA expression in 1–8 mm healthy bovine follicles, but reported that levels of gene expression decreased with atresia. In contrast, de la Sota *et al.* (1996) found increased levels of IGFBP-2 in large atretic follicles collected in the mid-luteal phase. As IGFBP-2 is generally considered to inhibit the action of the IGFs (Monget *et al.* 2003), then assuming the reduced expression of IGFBP-2, mRNA leads to lower intrafollicular IGFBP-2 in sNEB, the availability of free IGF-I and IGF-II may be increased for IGF-stimulated follicle growth and oestradiol production in small follicles (Monget *et al.* 1993). Indeed, levels of free IGF must increase as follicle size increases (Monget *et al.* 2003), in order to enhance follicular response to follicle-stimulating hormone (FSH; Mihm *et al.* 2000) and increase follicular oestradiol secretion (Gutierrez *et al.* 1997). This might represent a mechanism to support the continued growth of antral follicles despite NEB or severe chronic undernutrition (Rhodes *et al.* 1995).

IGFBP-4 mRNA localisation in theca cells is in agreement with other studies (Armstrong *et al.* 1998). Treatment did not affect concentrations of IGFBP-4 mRNA, implying that transcription of this IGF-binding protein is resistant/insensitive to severe negative energy balance. Levels of expression did not vary with follicle size, concurring with work by Armstrong *et al.* (1998) in the cow. We also found no effect of follicle health on concentrations of IGFBP-4 mRNA, which is dissimilar to other studies in the cow and ewe that report lower IGFBP-4 mRNA in atretic follicles when data is combined from small, medium and large follicle sizes (Armstrong *et al.* 1998, Hastie & Haresign 2006). The survival of growing follicles becomes increasingly

Figure 5 Summary of factors affecting follicular expression of components of the follicular IGF system in *post partum* dairy cows. Values are mean $\pm$ S.E.M. optical density (OD) units measured from autoradiographs. (A) The effect of energy balance status on IGFBP-2 mRNA expression in the granulosa; mNEB, $n=25$; sNEB, $n=24$; $P<0.05$. (b) The effects of size (■ very small, VS; □ small, S) and health (healthy, H or atretic, A) on IGF-II mRNA expression in the theca. VSH, $n=30$; VSA, $n=31$; SH, $n=9$ and SA, $n=35$. There was a significant effect of health ($a>b$, $P<0.05$). Within healthy follicles, $VS>S$, $*P<0.05$. (c) The effect of size and health on IGFBP-6 mRNA expression in the theca. VSH $n=23$, VSA $n=14$, SH $n=7$, SA $n=21$. Within healthy follicles, $VS>S$, $*P<0.05$.

dependent upon luteinizing hormone (LH; Mihm & Bleach 2003), and thereby expression of the LH receptor (LHR) and thecal IGFBP-4 production *in vitro* is also stimulated by LH (Armstrong *et al.* 1996, 1998). It may be that expression of IGFBP-4 mRNA and thecal LHR mRNA (Stewart *et al.* 1996) are associated.

The expression of follicular IGFBP-5 mRNA was localised to granulosa cells, supporting the detection of bovine granulosa IGFBP-5 mRNA with RT-PCR (Schams *et al.* 1999). The proportion expressing IGFBP-5 mRNA was significantly greater in atretic than healthy follicles. In addition, concentrations of IGFBP-5 mRNA also tended to be greater in atretic follicles, as reported for the ovine ovary (Hastie & Haresign 2006). Assuming IGFBP-5 message leads to IGFBP-5 protein that reduces IGF ligand bioavailability, the subsequent decrease in IGF-stimulated follicular development is probably a factor in follicular atresia. Other studies similarly report higher IGFBP-5 protein in oestrogen-inactive, atretic and subordinate follicles compared with oestrogen-active, healthy and dominant follicles (de la Sota *et al.* 1996, Santiago *et al.* 2005).

The localisation of IGFBP-6 mRNA in the ovary is poorly documented, but this study detected IGFBP-6 mRNA in theca cells, which agrees with Schams *et al.* (1999). IGFBP-6 has a markedly higher affinity for IGF-II (Baxter 2000). The higher IGFBP-6 expression in healthy follicles 1–2.5 mm in diameter than follicles 2.5–5 mm in diameter parallels that of IGF-II mRNA and may thus contribute to the control of IGF-II-stimulated folliculogenesis.

IGF-binding proteins probably act to inhibit the activities of IGFs, but they may also enhance IGF action by storing IGFs intracellularly (Blum *et al.* 1989) or facilitating transport of IGFs into their target tissues (Hall *et al.* 1987). IGFBP-2 and IGFBP-4 have been found within the extracellular matrix surrounding granulosa cells (Glazyrin *et al.* 1996), where they may act as a reservoir to sustain controlled delivery of IGFs to the IGF-1R (Clemmons 1998). NEB may thus influence the storage of IGFs within the ovary by influencing the association of IGFs with the extracellular matrix. Another possibility, which we did not examine, is that EB status might alter the action of specific IGFBP proteases that are needed to access the extracellular store of IGFs (Hill *et al.* 1989), thus influencing the bioavailability of IGF for follicle growth and function (Armstrong *et al.* 1998). The ability of the low molecular weight binding proteins to cross the endothelial barrier (Bar *et al.* 1990) would suggest that follicular IGFs may transport IGFs from the vasculature into theca and granulosa cells, further increasing the intraovarian concentration of IGFs. It seems then that the lower concentration of IGFBP-2 in sNEB, whilst increasing the bioavailability of IGFs within follicle cells, could impact negatively upon the transport and storage of IGFs within the ovary. There is also increasing evidence that some of the IGFs may exert

IGF-independent effects on target cells (Rajaram *et al.* 1997); IGFBP-3, for example, has been shown to inhibit proliferation of breast cancer cells (Oh *et al.* 1993), while IGFBP-5 possesses transcription-regulatory activity (Xu *et al.* 2004) and may promote cell proliferation in osteoblasts (Andress & Birnbaum 1992, Mohan *et al.* 1995). In the ovary, IGFBPs may thus act directly to inhibit granulosa steroidogenesis (Bicsak *et al.* 1990).

In summary, we have shown that the concentration of mRNA for IGFBP-2 and the pattern of IGFBP-6 mRNA expression in pre-recruitment ovarian follicles are influenced by EB status in the *post partum* dairy cow. Expression of mRNA for IGF-1R, IGF-II, IGFBP-4 and IGFBP-5 did not follow the same trend, indicating that regulation of the ovarian IGF system is mediated by more than one mechanism. Future studies should develop a better understanding of the mechanisms underlying the effects of NEB on the IGF system, which in turn are likely to influence the timing of the return to ovarian cyclicity after parturition.

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