Comparison of mRNA for IGFs and their binding proteins in the oviduct during the peri-oestrous period between dairy heifers and lactating cows

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Abstract

The oviduct provides the environment to support gamete maturation, fertilisation and early embryo development. As there is a high incidence of early embryonic death in lactating dairy cows, this study compared expression of IGF family members in the oviduct between lactating Holstein-Friesian dairy cows ($n=16$, 81 ± 2.4 days in milk) and nulliparous heifers ($n=16$, age 1.6 ± 0.07 years) at three stages of the oestrous cycle: A) newly selected dominant follicle in the luteal phase, B) follicular phase before the LH surge and C) pre-ovulatory phase after the LH surge. Expression of *IGF1*, *IGF2*, IGF binding protein 2 (*IGFBP2*), *IGFBP3* and *IGFBP6* mRNA was determined in the ampulla of the oviduct. Oviduct side (ipsilateral or contralateral) with respect to the dominant follicle did not affect gene expression. Expression of *IGF1* and all three *IGFBPs* increased significantly between the luteal and the pre-ovulatory phases, with no further significant alteration post-LH surge. Concentrations of circulating IGF1 were higher in heifers than in cows, as was the mRNA expression of *IGF1*, *IGFBP3* and *IGFBP6*. The pre-LH surge rise in *IGFBP2* mRNA was only observed in heifers. *IGF2* expression was not influenced by either age or stage of cycle. These three *IGFBPs* are generally considered to inhibit IGF action. These results indicate tight regulation of IGF bioavailability in the oviductal environment around oestrus, with pronounced differences between cows and heifers, which are likely to influence early embryonic development. Further studies are required to assess the implications for embryo survival.

Reproduction (2011) 142 457–465

Introduction

Genetic selection of dairy cows for high milk yields has been associated with a reduction in fertility (Veerkamp *et al.* 2001). Less than 40% of inseminations typically result in the birth of a live off spring (Lucy 2001, Santos *et al.* 2004). Early embryonic mortality is a major cause of loss, estimated at about 40% in the first 2 weeks after insemination (Robinson *et al.* 2008). In high-producing dairy cows, there is evidence that a higher proportion of these losses occur before day 8 after insemination (Diskin & Morris 2008). A number of factors may contribute to this high loss rate including poor oocyte quality (Leroy *et al.* 2008), inadequate follicular and luteal steroidogenesis (Robinson *et al.* 2008) and an unfavourable environment within the reproductive tract (Wathes *et al.* 2007a). Fertility is typically somewhat higher in dairy heifers than in mature cows, with first service conception rates of 57% in US Holstein heifers (Kuhn *et al.* 2006) and 67% in British Holstein-Friesian

heifers (Brickell *et al.* 2009). This suggests that a concurrent lactation has a greater negative impact on conception in modern dairy cows than the altered genotype associated with selection for a high yield.

Within the bovine oviduct, the ampulla supports final oocyte maturation, fertilisation, cleavage and development to an 8–16 cell embryo, while the isthmus functions as a sperm reservoir and promotes capacitation (Hunter 1988, Ellington 1991, Buhi 2002, Rodriguez-Martinez 2007). The luminal epithelium contains both ciliated cells and nonciliated, secretory cells, which are responsible for oviduct fluid formation (Yaniz *et al.* 2000). The morphology and activity of the epithelium change during the oestrous cycle (Yaniz *et al.* 2000). Secretory activity increases during the peri-ovulatory period and early luteal phase under the influence of oestrogen (Murray 1995, Buhi *et al.* 2000) but decreases during the luteal phase and pregnancy when there is a prevailing high-progesterone concentration (Nayak *et al.* 1976). Oviductal fluid can enhance sperm survival

(Boilard *et al.* 2004) and fertilisation and provides an environment rich in 'embryotrophic factors' capable of enhancing embryo development (Leese 1988, Lai *et al.* 1996).

The extended insulin-like growth factor (IGF) superfamily includes two ligands (IGF1 and IGF2), two receptors (IGF1R and IGF2R) and at least six binding proteins (IGFBPs; Clemmons 1997). The biologic responses of both IGF1 and IGF2 are mediated predominantly by IGF1R (LeRoith *et al.* 1995), resulting in actions such as differentiation, mitogenesis and inhibition of apoptosis (Ullrich *et al.* 1986, Jones & Clemmons 1995). IGF signalling is influenced by competitive binding to the IGFBPs, which can either inhibit or potentiate IGF activities and thus provide differential regulation of the IGF system between different tissues (Clemmons 1997). Many components of the IGF system are nutritionally regulated (Thissen *et al.* 1994). Dairy cows in early lactation are exposed to a period of negative energy balance (NEB), which may last for up to 20 weeks in high yielding animals (Wathes *et al.* 2007a). During this time, there is altered expression of IGF family members in the liver (Lucy *et al.* 2001, Fenwick *et al.* 2008a), ovary (Llewellyn *et al.* 2007), oviduct (Fenwick *et al.* 2008b) and uterus (Wathes *et al.* 2009a). Together, these changes associated with NEB are not only responsible for a marked peripartum decline in circulating IGF1 concentrations (Butler 2003, Taylor *et al.* 2004) but are also thought to influence the half-life of IGFs in the circulation and the bioavailability of IGF within reproductive tissues. Measurements of circulating IGF1 have, therefore, been used as indicators of metabolic status, which are associated with fertility (Velazquez *et al.* 2008). For example, the interval to first ovulation after calving is shorter in cows with higher IGF1 concentrations (Butler 2003, Patton *et al.* 2007), and multiparous cows with lower IGF1 concentrations both before and immediately after calving experience longer calving to conception intervals and are less likely to conceive (Taylor *et al.* 2004, Wathes *et al.* 2007b).

Previous studies in various species have shown that expression of the IGF system in the oviduct alters according to stage of the oestrous cycle. For example, local production of *IGF1* mRNA increases during oestrus and the early luteal phase in pigs (Simmen *et al.* 1990), sheep (Stevenson & Wathes 1996) and cows (Schmidt *et al.* 1994, Pushpakumara *et al.* 2002). The majority of studies did not detect *IGF1* mRNA in pre-implantation bovine embryos (Yaseen *et al.* 2001, Moore *et al.* 2007, Warzych *et al.* 2007, Wang *et al.* 2009). *IGF2* mRNA was, however, present in bovine pre-implantation embryos as well as IGF2 protein in cumulus cells, oocytes and two-cell stage embryos (Watson *et al.* 1999, Wang *et al.* 2009). *IGF1R* mRNA is expressed in both the muscle and the mucosa of the oviduct (Pushpakumara *et al.* 2002, Fenwick *et al.* 2008b), in spermatozoa (Henricks *et al.* 1998) and in oocytes and embryos at all

stages of development (Watson *et al.* 1999, Wang *et al.* 2009). Together, these data suggest that locally produced and/or systemically derived IGFs may influence the gametes and embryo indirectly through local effects on oviductal secretory activity or directly after release into the lumen (Wathes *et al.* 1998, Watson *et al.* 1999). A key role for the IGFs at this time is supported by *in vitro* studies demonstrating that both IGF1 and IGF2 can stimulate the development of fertilised bovine embryos to the morula and blastocyst stages (Matsui *et al.* 1997, Palma *et al.* 1997, Watson *et al.* 1999, Wang *et al.* 2009, Neira *et al.* 2010). IGF2 may also promote proliferation and metabolic activity of the oviduct (Tiemann & Hansen 1995).

The aim of this study was to investigate the hypothesis that the IGF system in the bovine oviduct is affected not only by time relative to ovulation but also by lactation. The study measured mRNA expression of *IGF1* and *IGF2* and three IGFBPs (*IGFBP2*, *IGFBP3* and *IGFBP6*) at three stages leading up to ovulation in heifers and lactating cows. These binding proteins were selected as we have shown previously that they are: a) expressed in bovine oviduct and b) differentially regulated in the reproductive tract according to energy balance status (Pushpakumara *et al.* 2002, Fenwick *et al.* 2008b).

Results

Results are reported for lactating cows (two to seven lactations, age 5.7 ± 0.5 years, 81 ± 2.4 days in milk; mean $\pm$ s.e.m., $n=16$) and heifers (mean age of 1.6 ± 0.07 years; $n=16$). All animals were synchronised so that samples of ampulla from both the ipsilateral and the contralateral sides with respect to the presence of a dominant follicle could be collected at three stages of the oestrous cycle: A) newly selected dominant follicle on day 14 of the luteal phase, B) pre-ovulatory follicle prior to oestrus and C) luteinising pre-ovulatory follicle, after the LH surge (see Table 1). Stage of the cycle was confirmed by measurement of circulating progesterone concentrations. All animals in group A had elevated values of plasma progesterone of >6 ng/ml (8.1 ± 0.51 ng/ml, mean $\pm$ s.e.m.), whereas concentrations were ≤ 0.22 ng/ml for all animals in groups B and C. The mean IGF1 concentration in blood did not change over time but was significantly higher in heifers than in cows (467 ± 34 and 130 ± 9 ng/ml respectively).

Concentrations of *IGF1*, *IGF2*, *IGFBP2*, *IGFBP3* and *IGFBP6* were measured in all samples as absolute values using reverse transcriptase quantitative PCR (qPCR). In addition, *ACTB* and *RN18S1* were included as reference genes. Across all samples, *IGF1* mRNA expression was considerably higher than that of *IGF2* mRNA (67 ± 3.6 and 2.4 ± 0.2 fg cDNA/50 ng RT RNA respectively, $n=32$). Amongst the IGFBP mRNAs analysed expression of *IGFBP6* $>$ *IGFBP3* $>$ *IGFBP2* (18 ± 3.0 , 7 ± 1.0 and 0.9 ± 0.2 fg cDNA/50 ng RT RNA respectively).

Table 1 Summary scheme of the synchronisation protocol.

<i>n</i>	Group A Day 14 luteal Five cows + six heifers	Group B Pre-LH surge Seven cows + five heifers	Group C Post-LH surge Four cows + five heifers
Days ^a			
-12	Insert CIDR	Insert CIDR	Insert CIDR
-3	Remove CIDR	Remove CIDR	Remove CIDR
-2	i.m. PG	i.m. PG	i.m. PG
0	Check oestrus	Check oestrus	Check oestrus
+8	Reinsert CIDR	Reinsert CIDR	Reinsert CIDR
+13		i.m. PG	i.m. PG
+14	Remove CIDR		
	Slaughter		
+15		Remove CIDR	Remove CIDR
+16		Slaughter	i.m. GnRH
+17			Slaughter

^aRelative to first induced oestrus.

Initial comparison of the gene expression in every animal ($n=32$) between the ipsilateral and the contralateral ampullae with respect to the dominant follicle using paired *t*-tests showed no significant difference for any of the genes studied ($P=0.35$ – 0.94 , data not shown).

Mixed model analysis was then used to compare expression patterns of the genes of interest in the oviduct according to 1) stage of the oestrous cycle, 2) lactation status (cow/heifer) and 3) the stage $\times$ status interaction. There were no differences in *ACTB* or *RN18S1* expression according to stage or status of the animals. There was also no difference in the conclusions reached when values from the IGF-related genes were analysed as either absolute concentrations (fg cDNA/50 ng RT RNA) or relative changes (after normalisation against *ACTB* or *RN18S1*). The original concentration data are, therefore, presented here.

Expression results according to stage of cycle are summarised in Fig. 1. Concentrations of *IGF1* ($P<0.004$) and *IGFBP2* ($P<0.017$) increased from day 14 of the luteal phase to the pre-LH surge and showed a tendency to decrease again after the LH surge. *IGFBP3* ($P<0.017$) and *IGFBP6* ($P<0.016$) also increased from day 14 of the luteal phase to the pre-LH surge, with no further significant alteration after the LH surge. *IGF2* expression remained low at all stages with no change in expression between time points.

Data on the influence of lactation status on expression of IGF family members are summarised in Table 2. Expression of *IGF1*, *IGFBP2*, *IGFBP3* and *IGFBP6* mRNAs was significantly higher in heifers than in cows (fold changes 1.2, 1.8, 2.1 and 1.6, $P=0.033$, $P=0.053$, $P=0.003$ and $P=0.041$ respectively). There was no difference in *IGF2* mRNA expression according to lactation status.

The only gene to show a significant stage $\times$ lactation status interaction was *IGFBP2*. For this, the significant increase in expression before the LH surge occurred only in the heifers (Fig. 2).

Discussion

Fertilisation and early embryo development take place within the ampullary region of the oviduct. The IGF system is an important contributor to this environment. This study compared oviductal expression patterns for IGF family members over the peri-oestrous period and demonstrated that *IGF1*, *IGFBP2*, *IGFBP3* and *IGFBP6* mRNAs increased before the LH surge with differential expression between non-lactating heifers and lactating cows. In contrast, *IGF2* mRNA expression did not alter according to either stage of cycle or age of cow. This study focussed on IGFBPs, based on our previous studies showing their differential expression in the reproductive

Figure 1 Expression levels of (A) *IGF1* and *IGF2* mRNA and (B) *IGFBP2*, *IGFBP3* and *IGFBP6* mRNA (fg/50 ng RT RNA) in the ampullary region of the oviduct of animals on day 14 of the luteal phase ($n=11$), pre-LH surge ($n=12$) and post-LH surge ($n=9$). Values are mean $\pm$ S.E.M. For each gene shown, values with different superscripts are significantly different between times, $b>a$, $P<0.01$, $d>c$ $P<0.05$.

Table 2 Comparison of mRNA expression of IGF family members in the ampullary region of oviducts between cows ($n=16$) and heifers ($n=16$) based on mixed model analysis^a.

Gene	Cow $n=16$	Heifer $n=16$	Fold increase in heifers	P
<i>IGF1</i>	61.6±5.70	71.1±5.06	×1.2	0.033
<i>IGF2</i>	2.4±0.20	2.3±0.37	No change	0.154
<i>IGFBP2</i>	0.6±0.09	1.1±0.30	×1.8	0.053
<i>IGFBP3</i>	4.6±0.75	9.8±1.86	×2.1	0.003
<i>IGFBP6</i>	13.5±2.35	21.7±5.54	×1.6	0.041
<i>RN18S1</i> ^b	1.9×10 ⁶	1.5×10 ⁶	No change	0.786
	±0.38×10 ⁶	±0.26×10 ⁶		
<i>ACTB</i> ^b	1.3×10 ⁴	1.9×10 ⁴	No change	0.350
	±0.31×10 ⁴	±0.38×10 ⁴		

^aValues are mean±s.e.m. and are expressed as fg cDNA/50 ng RNA.

^bIncluded as reference genes for comparison.

tract according to nutritional status (Fenwick *et al.* 2008a, 2008b) and between cows and heifers (Pushpakumara *et al.* 2002). In contrast, expression levels of the *IGF1R* did not differ in these situations and we were previously unable to detect *IGF2R* in bovine oviduct by qPCR (Fenwick *et al.* 2008a, 2008b).

In accordance with previous work (Pushpakumara *et al.* 2002), transcripts for *IGF1* mRNA were highest in the pre-ovulatory stage before the LH surge. In both ovine and bovine ampulla, *IGF1* mRNA was localised mainly to stromal fibroblasts underlying the luminal epithelium in the mucosa and to the surrounding smooth muscle cells (Stevenson & Wathes 1996, Pushpakumara *et al.* 2002). In contrast, *IGF1* protein was localised mainly in the secretory epithelial cells, supporting the likelihood that *IGF1* is exported into the oviductal lumen (Schmidt *et al.* 1994). In the results reported here, expression of *IGF1* mRNA was slightly (1.2-fold) higher in heifers than in cows. We similarly found slightly higher mRNA concentrations in cows in mild compared with severe NEB in early lactation. The bovine oviduct has receptors for oestradiol, GH and glucocorticoids. As their receptor expression was highly correlated with that of *IGF1* mRNA (Fenwick *et al.* 2008b), all three hormones may be involved in regulating local production of *IGF1* within the oviduct.

Oviductal expression of *IGF2* mRNA was much lower than that of *IGF1* mRNA. Xia *et al.* (1996) similarly reported low *IGF2* mRNA expression in bovine oviduct. The lack of difference in *IGF2* mRNA expression between any of the treatment groups agrees with our previous work in the cow (Pushpakumara *et al.* 2002, Fenwick *et al.* 2008b). Studies using *in situ* hybridisation showed that *IGF2* mRNA was predominantly expressed in the muscle layer (Stevenson & Wathes 1996, Pushpakumara *et al.* 2002). Our results, therefore, indicate that *IGF2* production within the mucosa of the ampulla is not acutely regulated. In contrast, Watson *et al.* (1999) reported that bovine oviduct cultures released ten times more *IGF2* than *IGF1* while Pershing *et al.* (2002) found increased oviductal *IGF2* mRNA

expression in the early luteal phase following bST treatment. The potential role of local *IGF2* production within the oviduct, therefore, remains uncertain.

This study showed increased expression of *IGFBP2*, *IGFBP3* and *IGFBP6* mRNA between the luteal stage of the cycle and the onset of the LH surge when oestrus, and therefore mating or artificial insemination, normally occurs. Furthermore, all three *IGFBPs* were influenced by the lactational status: the increase in *IGFBP2* mRNA was only present in heifers and the rise in both *IGFBP3* and *IGFBP6* mRNA expression was likewise greater in heifers than in cows. All three of these binding proteins are expressed in the oviductal mucosa, with *IGFBP2* and *IGFBP3* mRNA also present in the muscle layer (Stevenson & Wathes 1996, Pushpakumara *et al.* 2002, Fenwick *et al.* 2008b). In the bovine uterus, *IGFBP2* mRNA was reported to increase in the late luteal phase (Geisert *et al.* 1991), whereas *IGFBP6* mRNA expression in the endometrium of the ewe was highest around ovulation and the early luteal phase (Gadd *et al.* 2002). In agreement with the findings reported here, *IGFBP3* mRNA expression in the oviduct increased at oestrus in the ewe and cow (Stevenson & Wathes 1996, Pushpakumara *et al.* 2002).

Expression of all of these *IGFBPs* is thus influenced by stage of oestrous cycle as well as nutrition (Thissen *et al.* 1994, Clemmons 1997). We previously reported reduced expression of *IGFBP2*, *IGFBP3* and *IGFBP6* mRNAs in the oviduct when cows were in severe compared with mild NEB after calving (Fenwick *et al.* 2008b). Hepatic down-regulation of *IGFBP3* and *IGFBP6* mRNA in cows in severe NEB was associated with the concurrent down-regulation of the GH receptor (Fenwick *et al.* 2008a). Hepatic *IGFBP3* mRNA expression is regulated concurrently with *IGF1* (Woelfle & Rotwein 2004) and a positive correlation between *IGFBP3* and uterine *IGF1* mRNA levels has also been reported (Pershing *et al.* 2002). In contrast, hepatic *IGFBP2* mRNA expression was increased by severe NEB (Fenwick *et al.* 2008a). Together, these data suggest that there is complex regulation of *IGFBP* expression in the

Figure 2 Two-way interaction from mixed model analysis showing the combined influences of animal status (cow/heifer) and stage of the oestrous cycle on expression of *IGFBP2* mRNA (fg/50 ng RT RNA). The increase in expression pre-LH surge was only found in the heifers, * $P<0.01$.

oviduct by both metabolic status and ovarian steroid hormone environment.

The changes in IGFBP expression shown here imply that the oviduct of the heifer is exposed to higher concentrations of IGFBPs around oestrus than the lactating cow. This study has only measured mRNA, and additional regulation of IGFBP activity can occur at the protein level, for example by proteolysis (Christians & Gruslin 2010). It is not currently known whether any of these binding proteins are secreted into the lumen with the potential then to influence the gametes and embryo directly. However, expression of *IGFBP2* to *IGFBP5* mRNAs has also been detected in bovine embryos from the one cell to the blastocyst stages (Winger *et al.* 1997, Watson *et al.* 1999). The IGFBPs are generally considered to inhibit IGF action, with both IGFBP2 and IGFBP6 having a much higher affinity for IGF2 than IGF1 (Clemmons 1997, Bach 2005), whereas the affinities of IGFBP3 for both IGF1 and IGF2 are similar (Rajaram *et al.* 1997). The evidence presented here, therefore, suggests that the embryos in the heifers will have reduced signalling from IGFs through the IGF1R due to the likely inhibitory effect of each of the locally produced IGFBPs. Whilst this result might be considered unexpected, it concurs with the quiet embryo hypothesis, as reviewed by Leese *et al.* (2008). These authors concluded that the energy requirements of the embryo during the early cleavage stages are quite low. Less viable embryos were metabolically more active with a greater production of reactive oxygen species damaging the DNA and triggering a cellular stress response. The higher IGFBP expression may thus be needed to counteract the higher concentrations of circulating IGF1 present in heifers than cows, which peak at around puberty and then decline (Velazquez *et al.* 2008, Wathes *et al.* 2009b). This suggestion must be considered speculative until further functional studies can be undertaken. Other aspects may be important for fertility, for example the ratio of IGF and IGFBP proteins in the oviductal lumen. In addition, there are clearly other differences in the reproductive systems of cows and heifers. For example, some metabolite concentrations, in particular fatty acid composition, differed between dominant follicles collected from the animals used in this study (Bender *et al.* 2010).

The follicular fluid of the pre-ovulatory follicle contains high concentrations of oestradiol, cortisol, IGFs and other metabolites (Yding Andersen 2002, Mihm & Evans 2008) and these will be released along with the oocyte at ovulation, potentially leading to raised concentrations within the oviduct. The results presented here agree with our previous study in failing to show any differences in expression of IGF family members between the ipsilateral and the contralateral oviducts with respect to the dominant follicle (Fenwick *et al.* 2008b). A previous study compared gene expression in oviduct epithelial cells obtained from Simmental heifers in the early luteal phase using cDNA array hybridisation.

This approach detected 27 genes that were up-regulated in the ipsilateral oviduct and eight in the contralateral oviduct (Bauersachs *et al.* 2003). These included cell surface proteins, enzymes and proteins involved in cell–cell interaction, signal transduction and immune function but not any members of the IGF family.

In summary, this study provides evidence for the dynamic regulation of the IGF system within the oviduct by both lactational status and the systemic endocrine changes associated with the development of a dominant follicle. The oviduct of a growing heifer around the time of oestrus and the early luteal phase will be exposed to high circulating IGF1 from the circulation, slightly raised local production of IGF1, and raised concentrations of IGFBP2, IGFBP3 and IGFBP6, which are together likely to inhibit both IGF1 and IGF2 bioactivity. In contrast, a lactating cow has much lower circulating IGF1 in association with reduced oviductal IGFBP expression. Although oviductal expression of IGF2 is low, IGF2 production by the early embryo itself may also be affected by locally produced IGFBPs. The differences in *IGFBP* mRNA expression reported here indicate that there is tight regulation of IGF bioavailability in the oviductal environment. Further studies are required to assess the implications of these differences in IGFBP expression for development and survival of the embryo.

Materials and Methods

Animals and experimental design

All experimental procedures were conducted in accordance with the European Community Directive, 86-609-EC. The study compared two groups of Holstein-Friesian cattle. The multiparous cow group ($n=16$, range 2–7 lactations) had a mean age of 5.7 ± 0.50 years and were 81 ± 2.4 days in milk (range 68–108 days) at the start of the study, when daily milk yield was 26.2 ± 1.6 kg (mean $\pm$ S.E.M.). The nulliparous heifer group had a mean age of 1.6 ± 0.07 years, $n=16$. The animals were studied at time points with respect to time of calving (cows) or age (heifers) when such animals would normally be bred on commercial dairy farms.

Oestrous cycles were pre-synchronised and all animals were allocated to one of three slaughter time groups: A) animals with a newly selected dominant follicle ($n=11$), B) a differentiated dominant follicle before the LH surge ($n=12$) and C) a pre-ovulatory dominant follicle after the LH ($n=9$). Cows and heifers were selected independently to ensure sufficient distribution between the three groups. Groups A, B and C were achieved using a synchronisation programme summarised in Table 1. All animals were pre-synchronised for 8 days with progesterone via an intra-vaginal device (Eazi-Breed CIDR; Pfizer Pharma GmbH, Karlsruhe, Germany) containing 1.38 g progesterone. After 24 h of CIDR removal, animals received an i.m. injection of 500 μ g of a PGF_{2 α} analogue cloprostenol (Estrumate; Intervet/Schering-Plough Animal Health, Bray, Co., Wicklow, Ireland). Animals were then fitted with heat mount detectors (Kamar; Kamar, Inc., Steamboat Springs, CO, USA).

and observed for oestrus every 8 h between 48 and 72 h after PGF_{2α} administration. Oestrus was confirmed when the animals stood to be mounted by another animal. The day on which standing oestrus first occurred was designated as day 0 in the synchronisation programme. On day 8 of the next cycle, a used CIDR was re-inserted into all animals and ovarian follicle development was monitored daily by ultrasonographic examination (Aloka SSD-900 linear array trans-rectal probe, 7.5-MHz transducer; BCF Ireland Ltd, Fermoy, Ireland). On day 14, the used CIDRs were removed from animals in group A and they were subsequently slaughtered at an abattoir, at which time they presented a clear early dominant follicle whilst being in the mid luteal phase of the cycle. Animals in groups B and C received an i.m. injection of 500 µg cloprostenol on day 13, followed by the removal of the CIDR device 48 h later. The animals in group B were then slaughtered before the onset of the LH surge, between 24 and 30 h post-CIDR removal. The animals in group C were given an i.m. injection of 5 ml GNRH (Receptal, Intervet/Schering-Plough Animal Health, Bray, Co.) 30 h post-CIDR removal, initiating the LH surge. Slaughter was carried out ~18–22 h after the administration of GNRH. Blood samples were obtained from all animals by jugular venipuncture on the day of slaughter for measurement of IGF1 and progesterone. Ovarian characteristics at slaughter were confirmed by measurement of the external diameter of each follicle and assay of oestradiol-17β and progesterone in the follicular fluid as described previously (Bender *et al.* 2010).

Tissue collection

Following slaughter, reproductive tracts were collected within 15 min, oviducts were trimmed free of surrounding connective tissue and an ~10 mm long section of the ampulla from each of the left and right oviducts was dissected and promptly frozen in liquid nitrogen. It was noted at the time of tissue collection which ampulla sample was ipsilateral and which was contralateral to the dominant ovarian follicle present on the ovary of each animal. The samples were then stored at –80 °C.

Hormone assays

Plasma concentrations of IGF1 were determined using a RIA following acid-ethanol extraction as previously described (Beltman *et al.* 2010) using recombinant IGF1 (Upstate, Millipore, Temecula, CA, USA) as the iodination preparation and as standard and 50 µl anti-human (h) IGF1, NHPP-NIDDK AFP 4892898 (National Hormone and Peptide Program, Torrance, CA, USA) dilution 1:750 000 as primary antibody. The intra-assay coefficients of variation (CV, $n=6$) were 17.8, 13.4 and 18.9% for low, medium and high IGF1 quality control sera respectively. The inter-assay CV ($n=5$) for the same control sera were 12.2, 10.1 and 11.3% respectively.

Serum progesterone concentrations were determined using the solid phase RIA Coat-A-Count progesterone kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as previously described (Forde *et al.* 2010). All samples were assayed within one assay. The intra-assay CV ($n=4$) were 6.2, 6.2 and 9% for low-, medium- and high-quality control serum pools respectively.

RNA isolation, RT and qPCR

The procedures of RNA extraction, RT and qPCR were described in our previous studies (Fenwick *et al.* 2008a, 2008b, Wathes *et al.* 2011). Total RNA was extracted from 50 to 100 mg of frozen segments taken from the ampullary region of the oviducts from each cow using TRIzol Reagent (Fisher Scientific UK-Invitrogen) and chloroform and subsequently precipitated using isopropanol with a protocol developed according to ARK Genomics (<http://www.ark-genomics.org/protocols/>). RNA concentration and purity of each sample was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), where all samples had an acceptable 260/280 ratio of absorbance between 1.8 and 2.1.

In the following stage, 1 µg total RNA was treated for genomic DNA carryover with RNase-free DNase according to the guidelines supplied by the manufacturer (Promega Corporation). Subsequently, the DNase-treated RNA was generated into cDNA in 20 µl reactions using random hexamer primers, reverse transcriptase and processed accordingly (Reverse Transcription System Kit; Promega). Selected negative control samples were prepared by including all reagents as above, minus the reverse transcriptase. A mastermix of RT reagents was prepared once to minimise potential variation.

All primer sequences in this study were obtained from published references (Fenwick *et al.* 2008a, 2008b, Anstaett *et al.* 2010). For each gene, PCR conditions were optimised by conventional PCR amplification using Platinum PCR Supermix containing *Taq* polymerase (Invitrogen; Invitrogen Ltd) and the addition of 50–75 ng DNase-treated RT RNA and primers (20 mM); the presence of a single product was confirmed by electrophoresis on a 2% (w/v) agarose gel. Once optimised, external standards were prepared from cDNAs identical to real-time PCR products and purified using QIAquick PCR purification columns (Qiagen). The identity of the cDNA products was confirmed by DNA sequence analysis (Gene-service Ltd, Cambridge, UK) and the precise concentration of purified cDNA product was determined using the NanoDrop ND-1000 spectrophotometer. Eight standards of a tenfold dilution of purified PCR product of each gene ranging between 10 and 1×10^{-6} ng/ml (except *RN18S1* for which the range was from 100 to 1×10^{-5} ng/ml) were diluted in nuclease-free water and included in each assay.

The cDNA concentration of *IGF1*, *IGF2*, *IGFBP2*, *IGFBP3* and *IGFBP6* was determined by a qPCR procedure with a single-plex SYBR Green I assay. *ACTB* and *RN18S1* were tested as reference genes. To minimise variation, all cDNA samples included in each analysis were derived from the same batch. The mastermix was prepared once for each assay in order to avoid pipetting error. The amplification mixes contained 10 µl KAPA SYBR FAST qPCR Kits (GRI, Braintree, Essex, UK), 0.5 µl of 20 µM forward and reverse primers mix, 4.5 µl nuclease-free water and 5 µl unknown sample (50 ng RT RNA). The qPCRs for both standards and samples were run in duplicate in closed white tubes. No template control (NTC) with nuclease-free water was included in every assay. Absolute concentrations of the PCR product were calculated by comparing the C_T values of the unknown samples to the standard curve using the CFX Manager Software Version 1.0.1035.131 (Bio-Rad Laboratories, Inc.).

The expression was estimated as fg cDNA/50 ng RNA. The optimal annealing temperature, the plate reading temperature, the R^2 values relating to the standard curves and the efficiency of the assay for each gene are shown in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

Statistical analysis

Statistical analysis of the data was carried out using SPSS 16.0 for Windows (Chicago, IL, USA). Data were tested for homogeneity of variance using a Levene's test and log transformation was performed if necessary. The data on both heifers and cows were initially analysed by paired *t*-test to compare the transcript number between the ipsilateral oviduct (adjacent to the dominant follicle) and the contralateral oviduct. A linear mixed effect model was then used for analysis. This model included time frame (day 14 luteal phase, pre-LH surge and post-LH surge), cow status (heifer or cow) and their interactions as the fixed effects and animal as the random effect. Data are presented as sample mean $\pm$ S.E.M. Statistical significance was considered at a *P* value of 0.05.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0154>.

Declaration of interest

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

Funding

This work was supported by the Royal Thai Government and Science Foundation Ireland (07/SRC/B1156).

Acknowledgements

We thank F Carter and P Duffy for assistance with the animal work and P Furney for assistance with hormone assays.

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Received 5 May 2011

First decision 2 June 2011

Accepted 5 July 2011