



Title	Characterisation of endometrial gene expression and metabolic parameters in beef heifers yielding viable or non-viable embryos on Day 7 after insemination
Authors(s)	Beltman, Marijke Eileen, Forde, Niamh, Furney, P., Carter, F., Roche, J. F., Lonergan, Patrick, Crowe, Mark
Publication date	2010-07
Publication information	Beltman, Marijke Eileen, Niamh Forde, P. Furney, F. Carter, J. F. Roche, Patrick Lonergan, and Mark Crowe. "Characterisation of Endometrial Gene Expression and Metabolic Parameters in Beef Heifers Yielding Viable or Non-Viable Embryos on Day 7 after Insemination." CSIRO PUBLISHING, July 2010. https://doi.org/10.1071/RD09302 .
Publisher	CSIRO PUBLISHING
Item record/more information	http://hdl.handle.net/10197/4650
Publisher's version (DOI)	10.1071/RD09302

Downloaded 2026-05-02 00:24:20

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd_oa)



© Some rights reserved. For more information

Characterisation of endometrial gene expression and metabolic parameters in beef heifers yielding viable or non-viable embryos on Day 7 after insemination

Beltman, M.E¹, Forde, N. ¹, Furney, P.¹, Carter, F. ¹, Roche, J.F. ¹, Lonergan, P. ^{1,2}, Crowe, M.A. ^{1,2}

¹*UCD Veterinary Sciences Centre, School of Agriculture, Food Science and Veterinary Medicine, and* ²*Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland.*

Corresponding Author: Mark A. Crowe, Room 249, Veterinary Sciences Centre, UCD School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

Phone: +353-1-7166255

Fax: +353-1-7166253

Email: mark.crowe@ucd.ie

Abridged title: Endometrial gene expression and metabolic parameters in subfertile beef heifers.

Abstract

The objective of this study was to compare the hormonal, metabolic characteristics and endometrial gene expression profiles in beef heifers yielding either a viable or a degenerate embryo on Day 7 after insemination as a means to explain differences in embryo survival. Oestrus was synchronised in cross bred beef heifers (n=145) using a CIDR-prostaglandin protocol and heifers (n=102) detected in standing oestrus (within 24-48 h post CIDR removal) were inseminated 12-18 h after detection of oestrus (Day 0) with frozen-thawed semen from a single ejaculate of a bull with proven fertility. Blood samples were collected from Day 4 to Day 7 post oestrus to measure progesterone (on Days 4, 5 and 7), insulin and IGF-I (on Days 4 and 6) and urea concentrations (on Day 7). All animals were slaughtered on Day 7. Uterine pH was also determined. Animals from which an embryo was recovered were classified as either having a viable embryo (morula/blastocyst stage); n=32, or a retarded embryo (arrested at 2- to 16-cell stage); n=19. The overall recovery rate was 64%. There was no significant difference in blood parameters measured or uterine pH at slaughter between the heifers with either a viable or retarded embryo. The relative abundance of 9 transcripts out of 53 tested from the endometrial tissue was different between heifers with a viable embryo or retarded embryo: *MOGAT1*, *PFKFB2*, *LYZ2*, *SVS8*, *UHRF1*, *PTGES*, *AGPAT4*, *DGKA* and *HGPD*. Both *LYZ2* and *UHRF1* are associated with regulation of the immune system. *PFKFB2* is a mediator in glycolysis and *MOGAT*, *AGPAT4* and *DGKA* belong to the triglyceride synthesis pathway. *PTGES* and *HGPD* belong to the prostaglandin pathway. Both of these metabolic pathways are important for early embryonic development. In conclusion, retarded embryo development in this study was not related to serum progesterone, IGF-I, insulin or urea concentrations nor to uterine pH at slaughter. However, altered expression of genes involved in the prostaglandin and triglyceride pathways, as well

as 2 genes that are closely associated with the regulation of immunity in the endometrium may indicate a uterine component to the retardation of the embryos in these beef heifers.

Introduction

Embryo mortality in cattle, reflected in reduced conception rate/calving rate per service, is a major cause of economic loss for the farming industry. In heifers, only 60% of single inseminations lead to a successful full term pregnancy despite a fertilisation rate of 90-95% (Diskin and Morris 2008). Most of the embryonic loss in such heifers occurs during the period between fertilisation and maternal recognition of pregnancy (Day 16) (Dunne *et al.* 2000). Several studies have shown that progesterone concentrations in this critical period of embryo development have a significant effect on the development of the embryo/conceptus (Diskin *et al.* 2002; Garrett *et al.* 1988; Mann *et al.* 2003; Starbuck *et al.* 1999). Low concentrations of progesterone in the initial days following conception (Day 3-8) are associated with smaller embryos on Day 16 of pregnancy which may produce insufficient interferon- τ to suppress the luteolytic secretion of prostaglandin F 2α (Mann and Lamming 2001). Similarly, a delay in the post-ovulatory increase in progesterone has been associated with a decreased pregnancy rate in heifers (Diskin *et al.* 2002). Several studies have reported a positive association between elevated progesterone in the early post conception period and an advancement of conceptus elongation in cattle (Carter *et al.* 2008; Garrett *et al.* 1988; Mann *et al.* 1999; Satterfield *et al.* 2006).

Other components of the reproductive axis that have been associated with a decreased fertility in cattle are insulin and insulin-like growth factor I (IGF-I). Insulin regulates the expression of IGF binding proteins (IGFBPs) which are responsible for the bioavailability of

IGFs (Royal *et al.* 2000). IGFBPs are expressed in the pre-ovulatory follicles as well as in the oviduct and endometrium (Armstrong *et al.* 2002; Velazquez *et al.* 2008; Wathes *et al.* 1998). Together with IGF, they play an important role in regulating the development of the embryo and the uterus (Wathes *et al.* 1998). During the pre-implantation period the embryo is dependent on secretions from the endometrial glands for its metabolic requirements. In this period IGFs are also expressed in the conceptus (Watson *et al.* 1992) and the histotroph (Ko *et al.* 1991). In the endometrium IGF has an effect on the secretion of early embryotrophic factors by the endometrial glands (Webb *et al.* 1999). As the action of the IGFs is modulated by the IGFBPs, these BPs have a major role in regulating the passage of IGF between the maternal tissues and the early embryo during the period of early embryonic development (Wathes *et al.* 1998). Several studies have concentrated on the presence of specific IGFBPs during early embryonic development. IGFBP-1, which can have an inhibitory effect on IGF action, was found solely in the luminal epithelium of the endometrium of sheep and cattle where it was thought to be regulated not only by progesterone but also by the conceptus itself during the early stages of pregnancy (Osgerby *et al.* 1999; Robinson *et al.* 2000). In cattle, other members of the IGFBP family, such as IGFBP-2 and -3 were also influenced by the presence of an embryo (Robinson *et al.* 2000). A recent study (Simmons *et al.* 2009) has shown that IGFBP-1 seems to be a major marker for conceptus elongation in both cattle and sheep.

Rapidly digestible feedstuffs fed to cattle can also negatively impact fertility via altering circulating concentrations of ammonia/urea. Highly digestible feedstuffs generally contain high levels of rumen degradable protein, which are converted into ammonia and urea (Canfield *et al.* 1990). The liver converts ammonia to urea but in some cases the ammonia production can exceed this clearance process. Both high concentrations of ammonia and urea

can have a severe negative effect on the follicle and embryo (McEvoy *et al.* 1997; Sinclair *et al.* 2000; Sinclair *et al.* 1999).

Apart from these systemic mediators of fertility, local regulators in the reproductive tract are equally important with regard to successful reproductive outcomes. The oviduct is the first area of the reproductive tract through which the embryo passes on its way to the uterus and therefore can have an important impact on the development of the embryo (Wolf 2003; Fenwick *et al.* 2008). In beef heifers however, there is clear evidence that the transcriptional profile of the uterine endometria is altered at different stages of the oestrous cycle (Mitko *et al.* 2008) and during specific developmental stages of early pregnancy (Forde *et al.* 2009a). Moreover, these changes in endometrial gene expression can be affected by the quality of the conceptus present (Bauersachs *et al.* 2009; Mansouri-Attia *et al.* 2009). In addition, systemic factors such as elevated concentrations of serum progesterone (Forde *et al.* 2009a), disease (Gabler *et al.* 2009; Herath *et al.* 2009) as well as dietary status (Coyne *et al.* 2008; Wathes *et al.* 2009) all alter gene expression in the uterine endometrium. These studies indicate that alterations in endometrial gene expression are required to provide an optimum uterine environment for the developing embryo/conceptus. Also they demonstrate that alterations by systemic parameters can enhance and / or impair the alterations in endometrial gene expression required for the successful establishment and maintenance of early pregnancy.

The objective of this study was to describe the relationship between systemic (endocrine/physiological) or uterine (endometrial gene expression) dysfunction in the initial period of embryonic development from fertilisation to blastocyst formation in subfertile beef heifers.

Materials and Methods

All experimental procedures involving animals were approved by the University's Animal Research Ethics Committee and were licensed by the Department of Health and Children, Ireland, in accordance with the cruelty to animals act (Ireland 1876) and European Community Directive 86/609/EC.

Animal management and treatments

Cross-bred beef heifers (n=145), approximately 2 years old and weighing 524 ± 5.5 kg housed in a slatted floor facility in a commercial feedlot were used. All heifers were housed under the same management conditions with *ad-libitum* access to a total mixed ration designed to achieve an average live-weight gain of 1.3 kg/heifer/day. Oestrus (Day 0) was synchronised by insertion of a Controlled Internal Drug Release (CIDR, 1.36g Progesterone, Pfizer UK) device intravaginally for 8 days with a 2 ml injection of PGF₂ α analogue (Prosolvin, Intervet Ireland Ltd., Dublin, Ireland) given on Day 7. Heifers were checked for signs of oestrus 4 times per day commencing 36 h after CIDR removal. Twelve to eighteen hours after onset of oestrus (Day 0) only those heifers recorded in standing oestrus within a narrow window (n=102) were inseminated with frozen-thawed semen from a single ejaculate of a bull with proven fertility.

Blood sampling and tissue collection

Jugular blood samples were collected on Days 4, 6 and 7 post-oestrus from all heifers. Blood samples were stored at room temperature for 1 h and at 4°C for a further 16 h. Serum was decanted after centrifugation for 20 minutes at 1,600 x g and stored at -20°C until subsequent analyses. All heifers were slaughtered on Day 7 of pregnancy. Within 30 min of slaughter the reproductive tract of all heifers was flushed with 20 ml of 10 mM Tris (pH 7.2, Sigma,

Dublin, Ireland). Heifers from which an embryo was recovered were assigned to either (i) the viable group (V) when the embryo was at the correct developmental stage for age (i.e. morula/early blastocyst), or (ii) the retarded group (R) when the embryo was arrested at the 2- to 16-cell stage. Heifers from which an unfertilised oocyte was recovered or from which no structure was recovered were omitted from the study. Uterine pH was determined at slaughter using a calibrated electronic pH meter (Hanna Instruments pH 210 Microprocessor pH meter). Strips of endometrium were taken from the tip of the uterine horn ipsilateral to the corpus luteum using a curved scissors. Tissue was stored in 1:5 w/v of RNAlater (Sigma, Dublin, Ireland), transported to the laboratory on ice and stored at 4°C. Excess RNAlater was removed 24 h later and endometrial tissue was transferred into RNase/DNase free tubes and stored at -80°C until RNA extraction.

Metabolic and hormonal parameters

Serum progesterone concentrations were measured in all heifers on Days 4, 6 and 7 post oestrus. Insulin and IGF-I were measured on Days 4 and 6. Urea was measured on Day 7 only. Both serum progesterone and insulin concentrations were measured using a time-resolved fluorescentimmunoassay (FIA) with an AutoDELFIA™ Progesterone kit and an AutoDELFIA™ Insulin kit (Perkin Elmer, Wallac Oy, Turku, Finland), respectively, as previously described (Carter *et al.* 2008; Ting *et al.* 2004). All samples were assayed within a single assay with a sensitivity of 0.01 ng/ml for the progesterone assay and 0.1 µIU/ml for the insulin assay. The intra-assay coefficients of variation (% CV) were 4.6, 5.5 and 4.6% for high, medium and low progesterone quality control sera, respectively. For the insulin quality control sera the intra-assay CVs were 3.4, 3.9 and 8.0% for high, medium and low sera respectively.

Serum concentrations of IGF-I were determined using a RIA following acid-ethanol extraction using a modification of the assay previously described by Echterkamp *et al.* 1990. Recombinant IGF-I (Upstate, Millipore, Temecula, CA, USA) was iodinated using a chloramine T and sodium metabisulphite method as described previously (Spicer *et al.* 1988, Echterkamp *et al.* 1990). Recombinant human IGF-I (Upstate, Millipore, Temecula, CA, USA) was used to generate the standard curve. One hundred μ l of serum was extracted in borosilicate tubes in duplicate with 400 μ l of acid-alcohol and incubated at 4°C for 16 hours. The tubes were centrifuged for 30 minutes at 1600 x g, neutralized with 100 μ l of 0.855 M Tris base and centrifuged for a further 30 minutes at 1600 x g. A one hundred μ l aliquant of the supernatant was pipetted into polypropylene tubes and diluted 1:10 with assay buffer (0.25% BSA; IGF-1 free; 0.4% sodium phosphate monobasic, 0.04% protamine sulphate, 0.3% EDTA disodium salt, 0.02% sodium azide). Fifty μ l of primary antibody (anti-hIGF-I, NHPP-NIDDK AFP 4892898; National Hormone & Peptide Program, Torrance, CA, USA; dilution 1:750000) was added to each tube and they were incubated at room temperature for 1 hour. 100 μ l of iodinated IGF-I (approx 10,000 CPM) was added. Following incubation at 4°C overnight, 50 μ l of second antibody anti-rabbit IgG (Sac-cel, AA-SAC1, Immunodiagnostic Systems, Bolden, UK) was added. The tubes were incubated at room temperature for 30 minutes before 250 μ l of distilled water was added to each tube. Tubes were centrifuged at 1600 x g for 15 minutes and the supernatant was aspirated and counted using a gamma counter (Wizard 1470, Wallac/Perkin Elmer, Turku, Finland). Recovery of added mass of IGF-I standard to serum indicated 83 % recovery. Parallelism studies indicated that the standard curve and diluted serum samples were parallel. Cross reactivity studies on the antibody revealed ~100% cross reactivity with IGF-I, 0.001% and 0.88% cross reactivity with IGF-II and insulin. The sensitivity of the assay was 6 pg/tube (6 ng/ml). The intra assay

CV's were 11.9, 9.4 and 9.6% for high, medium and low IGF-I quality control sera, respectively.

Urea was measured using a Randox RX ImolaTM multichannel autoanalyser via an enzymatic kinetic method using urease following the manufacturer's instructions. The minimum detectable concentration was 1.46 mmol/l and the intra-assay coefficients of variation were 3.82, 2.98 and 1.59% for high, medium and low quality control sera, respectively.

RNA extraction and quantitative realtime PCR (Q-RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. One ml of Trizol was added to 100 mg of tissue and homogenised using the Qiagen TissueLyser (Qiagen, Crawley, West Sussex, UK). Following homogenisation, bromo-3-chloro-2-propanol (BCP) and isopropanol (Sigma, Dublin, Ireland) steps were performed and RNA was resuspended in 100 µl of RNase/DNase free water. RNA cleanup and on-column DNase digestion were performed using the Qiagen mini kit (Qiagen, UK). Total RNA quality and quantity were determined using the Agilent bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Four micrograms of total RNA were converted to cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. cDNA was quantified and diluted to give a final concentration of 10 ng/µl.

Quantitative real-time PCR (Q-RT-PCR) was performed on candidate endometrial genes previously identified as differentially expressed between Days 5 and 7 of normal pregnancy and genes up regulated in pregnant animals under the influence of progesterone (Forde et al. 2009a; Forde et al. 2009b) as well as the genes from the IGF family (Tables 2-5). Based on the initial results, 2 metabolic pathways were further characterised (the prostaglandin and the

triglycerol synthesis (TAG) pathway). All primers were designed using Primer 3 online software (<http://frodo.wi.mit.edu/primer3/>) and subsequently entered in the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure specificity. Q-RT-PCR was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). Each reaction consisted of 20ng cDNA, forward and reverse primers at 300/300 nmol concentration, 10µl SYBRgreen mastermix (Applied Biosystems, USA) with a final reaction volume of 20µl made up with RNase DNase free water. All reactions were carried out in duplicate and cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. A dissociation curve was included to ensure specificity of amplification and the normaliser gene used was *ACTB*, a previously validated normaliser gene in bovine endometrial gene expression analysis (Forde et al. 2009a).

Statistical analyses

Total area under the curve (AUC) was calculated for progesterone concentrations of each individual heifer in both groups and subsequently analysed using ANOVA with Bonferroni for multiple variance using SPSS 15.0 for Windows. Differences between treatment groups for all other variables were also analysed using ANOVA with Bonferroni for multiple variance using SPSS 15.0 for Windows. Transcript abundance was determined relative to the normaliser gene (*ACTB*) using the Δ Ct method as previously described (Livak and Schmittgen 2001) and relative abundance differences between groups were compared using the Students t-test as described by Snedecor and Cochran (1989).

Results

Embryo recovery and uterine pH

Following flushing of uteri, structures (oocytes/embryos) were recovered from 64% of the heifers of which 32 were classified as viable (i.e. correct stage of development for day) and 19 were classified as retarded (i.e. arrested stage of development). The remaining recovered structures (n=14, 14%) were single-celled unfertilised oocytes. There was no significant difference between uterine pH recorded at time of slaughter between heifers yielding viable (6.59 ± 0.03) and retarded (6.58 ± 0.02) embryos.

Hormonal and metabolic parameters

Serum progesterone concentrations are shown in Figure 1. There were no differences in area under the curve (AUC) for serum progesterone concentrations between both groups of heifers from Day 4 to Day 7. IGF-I concentrations and insulin concentrations did not differ between the groups on Day 4 and Day 6 (Table 1). On Day 7 of pregnancy, urea concentrations were not different between the viable (5.99 ± 0.20 nmol/l) and retarded groups (5.69 ± 0.34 nmol/l).

Quantitative Realtime PCR (Q-RT-PCR)

Of the 53 genes analysed, 9 were significantly differentially expressed in the endometrium of heifers yielding viable or retarded embryos (Table 2 and 5). Of these 9 genes, expression of *MOGAT1*, *PFKB2*, *LYZ2*, *SVS8*, *UHRF1*, *HGPD* and *DGKA* was lower in heifers that had V embryos compared with those having R embryos, while *PTGES* and *AGPAT4* expression was higher in heifers with V embryos compared with those having R embryos (Figures 2-4).

Discussion

Results of this study reveal that beef heifers with a high proportion of retarded embryos had no significant differences in serum progesterone concentrations compared with beef heifers with normal embryos. These results indicate that low progesterone is not a contributing factor to early embryo loss in these heifers who were fed on a diet to achieve high growth rates. This is in contrast to earlier research indicating a negative effect of low concentrations of progesterone on early embryonic development (Diskin *et al.* 2002; Mann *et al.* 1999), but supported by earlier research by Roche *et al.* (1985) which reported a difference in progesterone concentration between pregnant and non pregnant heifers. However, the effect of progesterone appears to play a role early in the establishment of pregnancy (between days 3-7) and to affect conceptus development rather than survival (Beltman *et al.* 2009; Carter *et al.* 2008).

Other hormones that contribute to reproductive efficiency and that are influenced by metabolic activity are insulin and IGF-I. Insulin is influenced by starch and fat content in the diet and together with IGF-I plays an important role in ovarian follicular development (Garnsworthy *et al.* 2008; Gong *et al.* 2002). IGF-I, which is also produced by the liver, is used as a measurement for long term nutritional status in cattle. Studies in cattle have shown that IGF-I affects oocyte development and has a direct effect on early embryonic development (Armstrong *et al.* 2002; Webb *et al.* 1999). However, neither systemic insulin nor IGF-I concentrations were different between the heifers having a viable compared with a retarded embryo in this study. A recent study in sheep describes that local concentrations of IGFBP1 (which controls the bioavailability of IGF-I) in the uterine lumen contribute to conceptus development particularly to trophoblast migration (Simmons *et al.* 2009). None of the genes from the IGF family were significantly different between heifers with a retarded embryo and heifers with a viable embryo in this study, leading to the conclusion that in these

heifers the retardation of growth of the embryo was not the result of different expression of genes of the IGF family in the endometrium.

Increased plasma and milk urea concentrations are closely correlated to a decrease in fertility in post partum dairy cows (Butler 1998). High blood urea concentrations in heifers (Westwood *et al.* 1998) are thought to be due to high dietary protein, with a direct relationship between urea concentrations and uterine pH (Butler 2000). In our study, urea concentrations were not different between the two groups of heifers and this was consistent with the findings of Gath *et al.* (1999) who reported no effects of urea on early embryonic development between heifers fed a high energy, high urea diet when compared with heifers fed a low energy, urea diet. Also, several studies have demonstrated that differences in uterine pH in cattle commonly occur around oestrus and ovulation and not on day 7 (Elrod and Butler 1993; Perry and Perry 2008). In this study, uterine pH measured on Day 7 in both groups was not significantly different. Collectively, these data suggest that the sub fertility in beef heifers fed to achieve high growth rates (~1.2-1.3 kg / day) can not be attributed to the measured systemic effects. In this study sub-fertility may more likely be mediated by local events in the reproductive tract.

While the oviductal environment has an important role in early embryonic development (Pushpakumara, *et al.* 2002; Wolf 2003, Fenwick *et al.* 2008) the main focus of this study was on the uterine component that affects early embryonic development. Alterations in the local uterine microenvironment can be one of the potential factors contributing to the subfertility in these heifers. Recent studies have characterised the transcriptomic changes that occur across the different stages of early pregnancy as well as progesterone-induced genes associated with advanced conceptus development (Forde *et al.* 2008; Forde *et al.* 2009a). Thus, it was important to determine whether the expression of key genes associated with

'normal' and advanced embryo/conceptus development was different in the endometria of retarded compared with viable heifers on Day 7 of pregnancy in this experiment.

From the list of 53 initial candidate genes selected for Q-RT-PCR analysis, the relative abundance of 44 genes was not different between heifers with a viable embryo and heifers with a retarded embryo. Possible reasons for this may be that i) the genes studied were not affected, but other genes in the specific biochemical pathways targeted may be involved or ii) the pathways that these genes are involved in are not different between heifers with viable vs. retarded embryos. Further characterisation of these pathways in the future could potentially help determine which of the reasons mentioned above is the most correct.

The relative abundance of the remaining 9 genes was significantly different between the heifers with V and R embryos. 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (*PFKFB2*) is an ubiquitous enzyme that is found in all types of tissues and is one of the signal molecules of the glycolysis pathway where it acts as a signal to stimulate glycolysis when glucose is available (Rider *et al.* 2004). *PFKFB2* was up regulated in endometria of heifers with retarded embryo development compared with the heifers having a viable embryo. This seems counterintuitive as one would assume that increased glucose would be beneficial to the embryo. However, it is known that during pre-blastocyst stages of embryonic development, utilisation of glucose as an energy source is not possible and that only the stages from blastocysts onwards can benefit from increased glucose concentrations (Leese and Barton 1984; Thompson *et al.* 1992). Increased glycolysis in the endometria of heifers with a retarded embryo would therefore not be beneficial but its actual function and relevance in the uterine endometrium is unknown (Gao *et al.* 2009).

Seminal vesicle secretion 8 (*SVS8*) is a gene that is associated with secretion of seminal plasma. Seminal plasma has an important role in maintaining sperm motility during storage in the oviduct and contains multiple proteins that are associated with bull fertility (Gwathmey

et al. 2006; Killian *et al.* 1993). SVS8 was up regulated in R heifers compared with V heifers but the role of SVS8 in the endometrium at this stage of embryonic development is, as yet, unknown.

Lysozyme 2 (*LYZ2*) is a member of the lysozyme family which consists of enzymes that have an important role in the innate immune system by attacking invading bacteria (Gordon *et al.* 1979). As the immune system has an important role in early embryonic development, the increased expression of *LYZ2* in the R heifers could mean that the retarded embryo may have triggered an immune response.

Ubiquitin-like with PHD and ring finger domains 1 (*UHRF1*) was also up regulated in the endometrium of R heifers when compared with V heifers. The most prominent function of ubiquitin is labeling proteins for proteasomal degradation. As such it is involved in the immune response, development and programming of cell death (Hershko and Ciechanover 1998). Therefore the up regulation of this enzyme in the R heifers could be related with the retardation of the embryo through degradation of proteins required for early embryo development although further study would be required. Interestingly, both *LYZ2* and *UHRF1* are involved in immune function and the whole process of embryonic development in the early stages depends on a tight modulation and regulation of the maternal immune system in order to allow the development of the semi-allograph embryo (Hansen 1997; Leung *et al.* 2000).

The remaining genes which had a relative abundance that was significantly different between both groups of heifers belonged to the prostaglandin synthesis pathway and the triglyceride pathway, respectively. The production of prostaglandin F₂α (PGF₂α) is responsible for luteolysis and prostaglandin E₂ (PGE₂) is involved in maternal recognition of pregnancy (Arosh *et al.* 2002; Shelton *et al.* 1990) and as such these pathways are of major importance in early embryonic development. Triglycerides are not only important in membrane synthesis

but are also suggested to have a role in early embryonic development as an energy source (Ferguson and Leese 2006; Forde *et al.* 2009a).

From the prostaglandin pathway, two genes had a relative abundance that was significantly different between groups: prostaglandin E synthase (*PTGES*) and hydroxyprostaglandin dehydrogenase 15-(NAD) (*HPGD*). *PTGES* converts PGH_2 to PGE_2 and together with prostaglandin-endoperoxide synthase 2 (*COX2*) is responsible for the production of PGE_2 in the bovine endometrium (Arosh *et al.* 2002). *HPGD* catabolises $\text{PGF}2\alpha$ into 15-keto $\text{PGF}2\alpha$ which is the first step of the biological inactivation of mainly $\text{PGF}2\alpha$ but also PGE_2 (Parent *et al.* 2006).

PTGES was down regulated in the endometria of heifers with a retarded embryo. As PGE_2 has embryotrophic capacities (Arosh *et al.* 2004, Ulbrich *et al.* 2009), a decrease in its expression in the endometrium could have contributed to the decreased fertility in these animals. The expression of *HGPD* was significantly higher in the endometria of heifers with a retarded embryo and thus could inhibit $\text{PGF}2\alpha$ which would be considered beneficial to the developing embryo, but can also inhibit PGE_2 and as such have a negative effect on the development of the early embryo. These results indicate that prostaglandin synthesis pathways are important and active before maternal recognition of pregnancy.

The remaining 3 genes that had a relative abundance significantly different between the two groups of heifers belonged to the triglyceride synthesis pathway. Triglycerides are not only important for membrane synthesis but are also suggested to have a major role in both oocyte maturation and early embryo development as an energy source (Ferguson and Leese 2006). Of significantly different the genes in this pathway, monoacylglycerol O-acyltransferase 1 (*MOGAT1*), 1-acylglycerol-3-phosphate O-acyltransferase 4 (*AGPAT4*) and diacylglycerol

kinase (*DGKA*) were significantly different between R heifers and V heifers. All of these genes encode enzymes that are involved in processes that either synthesise or metabolise diacylglycerol or DAG. DAG is the precursor of physiologically important lipids such as triacylglycerol and phospholipids, which store energy and play a role in the formation and functioning of cellular membranes and is also well known as an intracellular signaling molecule. *MOGATI* catalyses the synthesis of diacylglycerol (DAG) from monoacylglycerol (MAG) (Yen *et al.* 2002). *AGPAT* is involved in the conversion from lysophosphatic acid into phosphatidic acid (PA) (Coleman and Lee 2004), which is a major constituent of cell membranes as well as a signaling lipid. Diacylglycerol kinase (*DGKA*) regulates signal transduction by modulating the balance between the two signaling lipids DAG and PA. *DGKA* serves as a key attenuator of DAG during signaling functions. It is difficult to define the exact contribution of *DGKA* to the regulation of the DAG levels since there are many other enzymes involved in the metabolisation of DAG. *DGKA* however is thought to be important for the depletion of DAG in important intercellular signaling complexes (Kanoh *et al.* 2002).

Even though *MOGATI* was significantly more abundant in the endometria of heifers with a retarded embryo and *AGPAT 4* was downregulated in these animals, *DGKA* was upregulated and as this enzyme has a role in regulating the availability of diacylglycerides further downstream, this could lead to decreased availability of diacylglycerides and as such a potentially decreased output of triacylglycerides into the lumen of the endometrium. As previous studies (Ferguson and Leese 2006) have shown that triacylglycerides can be used as an energy source in early embryonic development up to blastocyst stage, this could have contributed to the decreased fertility in heifers with retarded embryos.

In conclusion, none of the metabolic/hormonal parameters measured were different between the heifers with viable embryos or retarded embryos and were therefore not a contributing factor to the sub-fertility exhibited in this group of beef heifers. There were a number of genes involved in both the prostaglandin and triglyceride synthesis pathways which displayed altered expression patterns in viable compared with retarded groups. The altered expression of genes involved in the prostaglandin and triglyceride pathways, as well as two genes that are closely associated with the regulation of the immune system in the endometrium suggest that a deficiency in the endometrial gene expression pattern may be an important component of the cause of the sub-fertility observed in these beef heifers.

Acknowledgements

This work was supported by Science Foundation Ireland Strategic Research Cluster grant code 07/SRC/B1156 (the opinions, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of Science Foundation Ireland). The authors thank Pat Duffy, Mary Wade and Mary Duane for their technical assistance, Michael Garrett for help with the urea analysis, Dr. John Browne for help with the transcriptomic analyses and the IAD Castlebellingham for access to experimental animals. The authors would like to thank Dr. L.J. Spicer for assistance with establishment of the IGF-1 iodination and RIA procedures and Dr. Parlow the National Hormone & Peptide Program (NHPP) for supplying the anti-hIGF-1, NHPP-NIDDK. Finally, the authors like to thank Pfizer Animal Health (Sandwich, UK) for providing the CIDRs for this study.

References

Armstrong D., Baxter G., Hogg C., Woad K. (2002) Insulin-like growth factor (IGF) system in the oocyte and somatic cells of bovine preantral follicles. *Reproduction* **123**, 789-797.

Arosh J. A., Parent J., Chapdelaine P., Sirois J., Fortier M. A. (2002) Expression of Cyclooxygenases 1 and 2 and Prostaglandin E Synthase in Bovine Endometrial Tissue During the Estrous Cycle. *Biol. Reprod.* **67**, 161-169.

Arosh, J. A., S. K. Banu, S. Kimmins, P. Chapdelaine, L. A. MacLaren and M. A. Fortier (2004). "Effect of interferon- τ on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: Evidence of polycrine actions of prostaglandin e₂." *Endocrinol.* **145**, 5280-5293

Bauersachs S., Ulbrich S. E., Zakhartchenko V., Minten M., Reichenbach M., Reichenbach H.-D., Blum H., Spencer T. E., E W. (2009) The endometrium responds differently to cloned versus fertilized embryos. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 5681-6.

Beltman M. E., Lonergan P., Diskin M. G., Roche J. F., Crowe M. A. (2009) Effect of progesterone supplementation in the first week post conception on embryo survival in beef heifers. *Theriogenology* **71**, 1173-1179.

Butler W. R. (1998) Review: Effect of Protein Nutrition on Ovarian and Uterine Physiology in Dairy Cattle. *J. Dairy Sci.* **81**, 2533-2539.

Butler W. R. (2000) Nutritional interactions with reproductive performance in dairy cattle. *Anim. Reprod. Sci.* **60-61**, 449-457.

Canfield R. W., Sniffen C. J., Butler W. R. (1990) Effects of excess degradable protein on postpartum reproduction and energy balance in dairy cattle. *J. Dairy Sci.* **73**, 2342-2349.

Carter F., Forde N., Duffy P., Wade M., Fair T., Crowe M. A., Evans A. C. O., Kenny D. A., Roche J. F., Lonergan P. (2008) Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reprod. Fertil. Dev.* **20**, 368-375.

Coleman R. A., Lee D. P. (2004) Enzymes of triacylglycerol synthesis and their regulation. *Prog. Lipid Res.* **43**, 134-176.

Coyne G. S., Kenny D. A., Childs S., Sreenan J. M., Waters S. M. (2008) Dietary n-3 polyunsaturated fatty acids alter the expression of genes involved in prostaglandin biosynthesis in the bovine uterus. *Theriogenology* **70**, 772-782.

Diskin M. G., Kenny D. A., Dunne L. D., Sreenan J. M. (2002) Systemic progesterone pre- and post- AI and embryo survival in heifers. In 'Irish Agricultural Research Forum'. Tullamore, Ireland. (Ed. MG Diskin) p. 27

Diskin M. G., Morris D. G. (2008) Embryonic and Early Foetal Losses in Cattle and Other Ruminants. *Reprod. Domest. Anim.* **43**, 260-267.

Dunne L. D., Diskin M. G., Sreenan J. M. (2000) Embryo and foetal loss in beef heifers between day 14 of gestation and full term. *Anim. Reprod. Sci.* **58**, 39-44.

Echternkamp S., Spicer L., Gregory K., Canning S., Hammond J. (1990) Concentrations of insulin-like growth factor-I in blood and ovarian follicular fluid of cattle selected for twins. *Biol. Reprod.* **43**, 8-14.

Elrod C. C., Butler W. R. (1993) Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. *J. Anim. Sci.* **71**, 694-701.

Fenwick, M. A., S. Llewellyn, R. Fitzpatrick, D. A. Kenny, J. J. Murphy, J. Patton and D. C. Wathes (2008). "Negative energy balance in dairy cows is associated with specific changes in igf-binding protein expression in the oviduct." *Reproduction* **135**, 63-75.

Ferguson E., Leese H. (2006) A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. *Mol. Reprod. Dev.* **73**, 1195-1201.

Forde N., Carter F., Fair T., Crowe M. A., Evans A. C., Spencer T. E., Bazer F. W., O'Gaora P., McBride R., Boland M. P., Lonergan P., Roche J. F. (2008) Effect of pregnancy and progesterone on gene expression in the uterine endometrium of cattle. *Biol. Reprod.* **78**, 60-61.

Forde N., Carter F., Fair T., Crowe M. A., Evans A. C. O., Spencer T. E., Bazer F. W., McBride R., Boland M. P., O'Gaora P., Lonergan P., Roche J. F. (2009a) Progesterone-Regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biol. Reprod.*, **81**, 784-794.

Forde N., McGettigan P. A., Browne J. A., Carter F., Loftus B. J., Roche J. F., Lonergan P., Crowe M. A., Evans A. C. O. (2009b) Pregnancy-Specific Endometrial Gene Expression at the Initiation of Conceptus Elongation in Cattle. *Biol. Reprod.* **81**, 303-.

Gabler C., Drillich M., Fischer C., Holder C., Heuwieser W., Einspanier R. (2009) Endometrial expression of selected transcripts involved in prostaglandin synthesis in cows with endometritis. *Theriogenology* **71**, 993-1004.

Gao H., Wu G., Spencer T. E., Johnson G. A., Bazer F. W. (2009) Select Nutrients in the Ovine Uterine Lumen. II. Glucose Transporters in the Uterus and Peri-Implantation Conceptuses. *Biol. Reprod.* **80**, 94-104.

Garnsworthy P. C., Lock A., Mann G. E., Sinclair K. D., Webb R. (2008) Nutrition, metabolism, and fertility in dairy cows: 1. Dietary energy source and ovarian function. *J. Dairy Sci.* **91**, 3814-3823.

Garrett J. E., Geisert R. D., Zavy M. T., Morgan G. L. (1988) Evidence of maternal regulation of early conceptus growth and development in beef cattle. *J. Reprod. Fertil.* **84**, 437-446.

- Gath V., Lonergan P., Boland M. P., O'Callaghan D. (1999) Effects of diet type on establishment of pregnancy and embryo development in beef heifers. *Theriogenology* **51**, 224-224.
- Gong J. G., Lee W. J., Garnsworthy P. C., Webb R. (2002) Effect of dietary-induced increases in circulating insulin concentrations during the early postpartum period on reproductive function in dairy cows. *Reproduction* **123**, 419-427.
- Gordon L., Douglas S., Kay N., Yamada O., Osserman E., Jacob H. (1979) Modulation of neutrophil function by lysozyme. Potential negative feedback system of inflammation. *J. Clin. Invest.* **64**, 226-232.
- Gwathmey T. M., Ignatz G. G., Mueller J. L., Manjunath P., Suarez S. S. (2006) Bovine Seminal Plasma Proteins PDC-109, BSP-A3, and BSP-30-kDa Share Functional Roles in Storing Sperm in the Oviduct. *Biol. Reprod.* **75**, 501-507.
- Hansen P. J. (1997) Interactions between the immune system and the bovine conceptus. *Theriogenology* **47**, 121-130.
- Herath S., Lilly S., Santos N., Gilbert R., Goetze L., Bryant C., White J., Cronin J., Sheldon I. M. (2009) Expression of genes associated with immunity in the endometrium of cattle with disparate postpartum uterine disease and fertility. *Reprod. Biol. Endocrinol.* **7**, 55.
- Hershko A., Ciechanover A. (1998) The Ubiquitin System. *Annu. Rev. Biochem.* **67**, 425-479.
- Kanoh H., Yamada K., Sakane F. (2002) Diacylglycerol Kinases: Emerging Downstream Regulators in Cell Signaling Systems. *J. Biochem.* **131**, 629-633.
- Killian G., Chapman D., Rogowski L. (1993) Fertility-associated proteins in Holstein bull seminal plasma. *Biol. Reprod.* **49**, 1202-1207.
- Ko Y., Lee C., Ott T., Davis M., Simmen R., Bazer F., Simmen F. (1991) Insulin-like growth factors in sheep uterine fluids: concentrations and relationship to ovine trophoblast protein-1 production during early pregnancy. *Biol. Reprod.* **45**, 135-142.
- Leese H. J., Barton A. M. (1984) Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J. Reprod. Fertil.* **72**, 9-13.
- Leung S., Derecka K., Mann G., Flint A., Wathes D. (2000) Uterine lymphocyte distribution and interleukin expression during early pregnancy in cows. *J. Reprod. Fertil.* **119**, 25-33.
- Livak K. J., Schmittgen T. D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $^{-[\Delta\Delta]CT}$ Method. *Methods* **25**, 402-408.
- Mann G. E., Green M. P., Sinclair K. D., Demmers K. J., Fray M. D., Gutierrez C. G., Garnsworthy P. C., Webb R. (2003) Effects of circulating progesterone and insulin on early embryo development in beef heifers. *Anim. Reprod. Sci.* **79**, 71-79.

Mann G. E., Lamming G. E. (2001) Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* **121**, 175-180.

Mann G. E., Lamming G. E., Robinson R. S., Wathes D. C. (1999) The regulation of interferon tau production and uterine hormone receptors during early pregnancy. *J. Reprod. Fertil. Suppl.* **54**, 317-328.

Mansouri-Attia N., Sandra O., Aubert J., Degrelle S., Everts R. E., Gerault-Deville C., Heyman Y., Galio L., Hue I., Yang X., X.C. T., Lewin H. A., Renard J. P. (2009) Endometrium as an early sensor of in vitro embryo manipulation technologies. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 5687-92.

McEvoy T. G., Robinson J. J., Aitken R. P., Findlay P. A., Robertson I. S. (1997) Dietary excesses of urea influence the viability and metabolism of preimplantation sheep embryos and may affect fetal growth among survivors. *Anim. Reprod. Sci.* **47**, 71-90.

Mitko K., Ulbrich S. E., Wenigerkind H., Sinowatz F., Blum H., Wolf E., Bauersachs S. (2008) Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. *Reproduction* **135**, 225-240.

Osgerby J., Gadd T., Wathes D. (1999) Expression of insulin-like growth factor binding protein-1 (IGFBP-1) mRNA in the ovine uterus throughout the oestrous cycle and early pregnancy. *J. Endocrinol.* **162**, 279-287.

Parent M., Madore E., MacLaren L. A., Fortier M. A. (2006) 15-Hydroxyprostaglandin dehydrogenase in the bovine endometrium during the oestrous cycle and early pregnancy. *Reproduction* **131**, 573-582.

Perry G. A., Perry B. L. (2008) Effects of standing estrus and supplemental estradiol on changes in uterine pH during a fixed-time artificial insemination protocol. *J. Anim. Sci.* **86**, 2928-2935.

Pushpakumara, P. G., R. S. Robinson, K. J. Demmers, G. E. Mann, K. D. Sinclair, R. Webb and D. C. Wathes (2002). "Expression of the insulin-like growth factor (igf) system in the bovine oviduct at oestrus and during early pregnancy." *Reproduction* **123**, 859-868.

Rider M. H., Bertrand L., Vertommen D., Michels P. A., Rousseau G. G., Hue L. (2004) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. *Biochem. J.* **381**, 561-579.

Robinson R., Mann G., Gadd T., Lamming G., Wathes D. (2000) The expression of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy. *J. Endocrinol.* **165**, 231-243.

Roche J., Ireland J., Boland M., McGeady T. (1985) Concentrations of luteinising hormone and progesterone in pregnant and non-pregnant heifers. *Vet. Rec.* **116**, 153-155.

Royal M. D., Darwash A. O., Flint A. P. F., Webb R., Woolliams J. A., Lamming G. E. (2000) Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. *Anim. Sci.* **70**, 487-501.

Satterfield M. C., Bazer F. W., Spencer T. E. (2006) Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biol. Reprod.* **75**, 289-296.

Shelton K., Parkinson T. J., Hunter M. G., Kelly R. W., Lamming G. E. (1990) Prostaglandin E-2 as a potential luteotrophic agent during early pregnancy in cattle. *J Reprod Fertil* **90**, 11-17.

Simmons R. M., Erikson D. W., Kim J., Burghardt R. C., Bazer F. W., Johnson G. A., Spencer T. E. (2009) Insulin-like Growth Factor Binding Protein One in the Ruminant Uterus: Potential Endometrial Marker and Regulator of Conceptus Elongation. *Endocrinology*, en.2009-0060.

Sinclair K. D., Kuran M., Gebbie F. E., Webb R., McEvoy T. G. (2000) Nitrogen metabolism and fertility in cattle: II. Development of oocytes recovered from heifers offered diets differing in their rate of nitrogen release in the rumen. *J. Anim Sci.* **78**, 2670-2680.

Sinclair K. D., Kuran M., Sorohan P., Staines M. E., McEvoy T. G. (1999) Dietary nitrogen metabolism, folliculogenesis and IVP in cattle. *Theriogenology* **51**, 309-309.

Snedecor G. W., Cochran W. G. (1989) 'Statistical Methods.' Ames Iowa State University Press: Iowa

Spicer L. J., Echtenkamp S. E., Canning S. F., Hammond J. M. (1988) Relationship between concentrations of immunoreactive insulin-like growth factor-I in follicular fluid and various biochemical markers of differentiation in bovine antral follicles. *Biology of Reproduction* **39**, 573-580.

Starbuck G. R., Darwash A. O., Lamming G. E. (1999) The importance of progesterone during early pregnancy in the dairy cow. *Cattle Pract.* **7**, 397-399.

Thompson J. G., Simpson J. G., Pugh P. A., Tervit H. R. (1992) Requirement for glucose during in vitro culture of sheep preimplantation embryos. *Mol. Reprod. Dev.* **31**, 253-257.

Ting S. T. L., Earley B., Crowe M. A. (2004) Effect of cortisol infusion patterns and castration on metabolic and immunological indices of stress response in cattle. *Domest. Anim. Endocrinol.* **26**, 329-349.

Ulbrich, S. E., K. Schulke, A. E. Groebner, H. D. Reichenbach, C. Angioni, G. Geisslinger and H. H. D. Meyer (2009). "Quantitative characterization of prostaglandins in the uterus of early pregnant cattle." *Reproduction* **138**, 371-382.

Velazquez M. A., Spicer L. J., Wathes D. C. (2008) The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. *Domest. Anim. Endocrinol.* **35**, 325-342.

Wathes D. C., Cheng Z., Chowdhury W., Fenwick M. A., Fitzpatrick R., Morris D. G., Patton J., Murphy J. J. (2009) Negative energy balance alters global gene expression and immune responses in the uterus of postpartum dairy cows. *Physiol. Genomics*, 00064.2009.

Wathes D. C., Reynolds T. S., Robinson R. S., Stevenson K. R. (1998) Role of the Insulin-Like Growth Factor System in Uterine Function and Placental Development in Ruminants. *J. Dairy Sci.* **81**, 1778-1789.

Watson A. J., Hogan A., Hahnel A., Schultz G. A., Wiemer K. E. (1992) Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. *Mol. Reprod. Dev.* **31**, 87-95.

Webb R., Garnsworthy P. C., Gong J. G., Robinson R. S., Wathes D. C. (1999) Consequences for reproductive function of metabolic adaptation to load. *BSAS, Occasional Publication No. 24*, 99-112.

Westwood C. T., Lean I. J., Kellaway R. C. (1998) Indications and implications for testing of milk urea in dairy cattle: A quantitative review. Part 2. Effect of dietary protein on reproductive performance. *N. Z. Vet. J.* **46**, 123-130.

Wolf, E., G.J. Arnold, S. Bauersachs, H.M. Beier, H. Blum, R. Einspanier, T. Fröhlich, A. Herrler, S. Hiendleder, S. Kölle, K. Prella, H-D. Reichenbach, M. Stojkovic, H. Wenigerkind, F. Sinowatz, (2003). "Embryo-maternal communication in bovine - strategies for deciphering a complex cross-talk." *Reprod. Domest. Anim* **38**, 276-289.

Yen C.-L. E., Stone S. J., Cases S., Zhou P., Farese R. V. (2002) Identification of a gene encoding MGAT1, a monoacylglycerol acyltransferase. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 8512-8517.

1 Figure 1: Mean (\pm SEM) serum progesterone concentrations of all heifers in the
2 experiment. Blood samples were taken on Days 4, 6 and 7. AUC was not different
3 between the heifers with a viable embryo and the heifers with a retarded embryo on
4 Day 7.

5

6

7 Figure 2a-d: Gene expression relative to ACTB of candidate genes not belonging to
8 either the TAG synthesis pathway or the prostaglandin pathway with significant
9 differences between heifers with a viable embryo and heifers with a retarded embryo.
10 * depicts significant difference ($P < 0.05$) between the 2 groups of heifers. SEM is
11 displayed in the error bars.

12

13 Figure 3a+b: Gene expression relative to ACTB of all genes all genes from the
14 prostaglandin pathway with significant differences between heifers with a viable
15 embryo and heifers with a retarded embryo. * depicts significant difference ($P < 0.05$)
16 between the 2 groups of heifers. SEM is displayed in the error bars.

17

18 Figure 4a-c: Gene expression relative to ACTB of all genes all genes from the TAG
19 synthesis pathway with significant differences between heifers with a viable embryo
20 and heifers with a retarded embryo. * depicts significant difference ($P < 0.05$) between
21 the 2 groups of heifers. SEM is displayed in the error bars.

22

23