This is a repository copy of Sexually dimorphic gene expression in bovine conceptuses at the initiation of implantation.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/103097/

Version: Accepted Version

Article:

https://doi.org/10.1095/biolreprod.116.139857

© 2016 by the Society for the Study of Reproduction, Inc. This is an author produced version of a paper published in Biology of Reproduction. Uploaded in accordance with the publisher's self-archiving policy.

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.
Sexually dimorphic gene expression in bovine conceptuses at the initiation of implantation.

Niamh Forde,1,2*, Veronica Maillo3, Peadar O’Gaora4, Constantine A. Simintiras5, Roger G. Sturmey5, Alan D Ealy6, Thomas E. Spencer7, Alfonso Gutierrez-Adan3, Dimitrios Rizos3,

Patrick Lonergan1.

1School of Agriculture and Food Science, 4School of Biomolecular and Biomedical Sciences, University College Dublin, Belfield, Dublin 4, Ireland.
2Current address: Division of Reproduction and Early Development, Leeds Institute of Cardiovascular and Molecular Medicine, School of Medicine, University of Leeds, Clarendon Way, Leeds, LS2 9JT, United Kingdom.
3Departamento de Reproducción Animal, INIA, Madrid, Spain.
4Center for Cardiovascular and Metabolic Research, Hull York Medical School, University of Hull, Hull, HU6 7RX, United Kingdom.
6Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 24060, USA.
7Division of Animal Sciences, University of Missouri, Columbia, Missouri, 65211, USA.

Funding: This work was funded by grants from Science Foundation Ireland grant number 13/IA/1983 and the Spanish Ministry of Science and Innovation (AGL2012-37510 and AGL2012-39652-C02-01).
ABSTRACT

In cattle, maternal recognition of pregnancy occurs on Day 16 via secretion of interferon tau (IFNT) by the conceptus. The endometrium can distinguish between embryos with different developmental competencies. In eutherian mammals, X-chromosome inactivation (XCI) is required to ensure an equal transcriptional level of most X-linked genes for both male and female embryos in adult tissues, but this process is markedly different in cattle than mice. We examined how sexual dimorphism affected conceptus transcript abundance and amino acid composition as well as the endometrial transcriptome during the peri-implantation period of pregnancy. Of the 5132 genes that were differentially expressed on Day 19 in male compared to female conceptuses, 2.7% were located on the X-chromosome. Concentrations of specific amino acids were higher in the uterine luminal fluid with male compared to female conceptuses, while female conceptuses had higher transcript abundance of specific amino acid transporters (SLC6A19 and SLC1A35). Of note, the endometrial transcriptome was not different in cattle gestating a male or a female conceptus. These data support the hypothesis that, far from being a blastocyst specific phenomenon, XCI is incomplete before and during implantation in cattle. Despite differences in transcript abundance and amino acid utilization in male versus female conceptuses, the sex of the conceptus itself does not elicit a different transcriptomic response in the endometrium.
INTRODUCTION

In cattle, pregnancy recognition both at the physiological [1, 2] and transcriptomic level [3, 4] is initiated on day 16 in order to prevent release of luteolytic pulses of prostaglandin F2 alpha from the endometrium, corpus luteum regression, and subsequent return to cyclicity. Subtle responses of the endometrium to the conceptus can be detected as early as day 13 [5], but the major transcriptomic response to the pregnancy recognition signal in cattle, interferon tau (IFNT) from the cells of the conceptus trophectoderm, occurs at day 16 [3]. Additional studies have demonstrated a high degree of similarity in the changes in the endometrial transcript abundance between pregnant and cyclic endometrium as pregnancy progresses from Day 15 through to Day 20 i.e. during the period of maternal recognition of pregnancy [3, 4, 6-9]. The endometrial transcriptomic response to early pregnancy is quite specific to the type of conceptus present, such that by Day 18 and 20 the response signature can distinguish between in vivo, in vitro and cloned embryos and thus the developmental outcome of the embryo [10, 11].

In eutherian mammals, X-chromosome inactivation (XCI) is required in females to ensure an equal transcriptional level of most X-linked genes for both males and females. In female (XX) preimplantation embryos, both X-chromosomes are transcriptionally active from embryonic genome activation (EGA) until a long non-coding RNA (X-inactive specific transcript: XIST) mediates the inactivation of one of them. Okamoto et al. [12] revealed substantial diversity in the timing and regulation of XCI initiation between mice, in which this phenomenon has been studied in most detail, and rabbits and humans. For example, XIST transcript abundance is not imprinted in rabbit and human embryos, and the choice of which X chromosome to inactive seems to occur downstream of XIST upregulation and X-chromosome coating, which differs significantly from the processes in the mouse.
We found that the process of XCI in the bovine differs markedly from that of the mouse. Similar to the situation reported in humans, XCI in cattle is far from being accomplished at the blastocyst stage [13]. Furthermore, abundance of many X-linked transcripts which escaped XCI in the bovine blastocyst were effectively equalized among sexes in Day 14 elongated conceptuses [14]. This mirrors the situation in the rabbit late blastocyst, and suggests that a large component of XCI occurs after the differentiation of TE/ICM lineages, but before gastrulation, indicating a significant amount of discord between the mouse and many other mammalian species. In addition, we found sexually dimorphic differences exist between male and female embryos in terms of developmental rate [15], and Day 7 blastocyst transcriptome [13] as well as amino acid turnover [16], with as many as one third of all actively expressed transcripts in the blastocyst being determined by the sex of the embryo [13].

In this study, we hypothesized that sex-related differences in transcript abundance remain throughout conceptus elongation and that male and female embryos elicit a different response in the endometrium. Thus, the aims of this study were to: (i) examine the effect of conceptus sex on conceptus transcript abundance and amino acid utilization at Day 19; (ii) compare the temporal changes in conceptus transcript abundance between Day 7 and Day 19; and (iii) determine whether male and female embryos on Day 19 elicit a different response from the endometrium.

MATERIALS AND METHODS

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EC and were sanctioned by the Animal
Research Ethics Committee of University College Dublin. Unless otherwise stated, all chemicals and reagents were sourced from Sigma (Dublin, Ireland).

Animal model and sample collection

The estrous cycles of crossbred beef heifers (n=30) were synchronized using an 8-day controlled internal drug release (CIDR) device (1.38 g P4; Pfizer Animal Health, Sandwich, Kent, UK) placed intra-vaginally. One day prior to CIDR removal, a 2 ml intramuscular injection of a prostaglandin F$_2$α analogue (Estrumate, Intervet, Dublin, Ireland; equivalent to 0.5 mg cloprostenol) was administered. All heifers were then observed at 4 h intervals and only those observed in standing estrus (=Day 0) were inseminated with semen from a proven sire. All heifers were slaughtered on Day 19 following estrus corresponding to the initiation of implantation in cattle. Thirty minutes after slaughter each uterine horn was flushed with 10 ml of PBS and the presence of a conceptus was observed under a stereo-microscope. Only those heifers from which a conceptus was recovered were further processed for tissue collection (n=24). Each conceptus was dissected into 4 pieces, 3 containing only extra-embryonic tissue (EET) and one containing the embryonic disc along with associated trophectoderm cells, and immediately snap-frozen in liquid nitrogen. The uterine luminal flush samples were then placed into 1ml aliquots and snap frozen in liquid nitrogen prior to amino acid analysis. The uterine horn ipsilateral to the corpus luteum was opened longitudinally and intercaruncular endometrium from the mid-part of the horn was dissected away from the underlying myometrium and snap frozen in liquid nitrogen. All samples were stored at -80 °C prior to processing.

Conceptus sexing
DNA was extracted from a sample of EET cells from each conceptus with phenol/chloroform treatment and finally re-suspended in 200 µL of milliQ water. Two microliters of each sample were used to perform embryo sexing by PCR amplification of sex-specific polymorphic fragments in the amelogenin gene as previously described [17].

**RNA extraction and microarray hybridization**

Total RNA was extracted from the EET cells from confirmed female (n=5) and male (n=5) conceptuses as well as their corresponding intercaruncular endometrial tissue (100 mg), using Trizol reagent as per manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Following on column DNase digestion and RNA clean up, (Qiagen, Crawley, West Sussex, UK) both the quality and quantity of the RNA was determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a NanoDrop 1000 (Thermo Fischer Scientific Inc. Wilmington, DE, USA), respectively. A subset of samples (both conceptus and corresponding intercaruncular endometrial tissue) with an RNA Integrity Number of greater than 8.0, were randomly chosen for microarray analysis (n=5 per tissue type). Transcriptomic analysis was carried out using the Affymetrix GeneChip® Bovine Genome Array. Two micrograms of total RNA were converted to cDNA via first and second strand synthesis using the GeneChip® Expression 3’-Amplification One-Cycle cDNA Synthesis kit. Biotin-labeled cRNA was synthesized from double-stranded cDNA using the GeneChip® Expression 3’-Amplification in vitro transcription (IVT) Labeling Kit. cRNA quality was assessed on the Agilent 2100 Bioanalyzer and 25 µg of cRNA was fragmented using 5X Fragmentation buffer in RNase-free water contained within the GeneChip® Sample Cleanup Module at 94 ºC for 35 min and quality accessed again on the Agilent 2100 Bioanalyzer. Fifteen µg of fragmented cRNA and hybridization cocktail were added to the GeneChip® Bovine Genome Array and hybridized for 16 h at 45 ºC. Each array was then washed and stained on the
GeneChip® fluidics station 450 using the appropriate fluidics script and once completed the array was inserted into the Affymetrix autoloader carousel and scanned using the GeneChip® Scanner 3000.

The raw signal intensities were read into R and pre-processed using functions of both affy and GCRMA packages of the BioConductor project [18]. Hierarchical clustering analysis was performed to determine the greatest source of variation in the tissue samples. Lists of differentially expressed genes (DEGs) were determined by the Limma package [19] employing linear modeling and an empirical Bayes framework to shrink the variance of measurements on each probe set. A modified t-test was then carried out and all $p$ values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate method.

**Analysis of Interferon Tau (IFNT) in the uterine luminal fluid**

Concentrations of IFNT in the ULF recovered from heifers with a male (n=11) or female (n=11) conceptus was carried out using a cytopathic antiviral assay [20, 21] as previously reported [22]. Samples were examined in duplicate by titrating in a 96-well plate (1:3 serial dilution). Madin-Darby Bovine Kidney (MDBK) cells were added and incubated in medium (DMEM containing 10% FBS) at 37 °C in 5% CO$_2$ in humidified air. After 24 h, cells were challenged with vesicular stomatitis virus for 1 h. Thereafter virus was removed and cells were incubated with growth medium (DMEM containing 10% FBS) for 18 h. Cell viability was determined after fixation (75% ETOH) using 0.5% [w/v] Gentain-violent. The ability of samples to prevent lysis by 50% was compared with a recombinant human IFN alpha standard (EMD Biosciences; 3.8 x 10$^8$ IU/mg). Data are presented as IU of antiviral activity per ml of conditioned medium. Unconditioned medium (blanks) did not contain antiviral activity.
**Analysis of amino acid content of uterine luminal fluid**

The amino acid composition of uterine luminal fluid (n=11 samples hosting a male and n=11 samples hosting a female conceptus) was quantitatively analyzed by High Performance Liquid Chromatography (HPLC) as previously described [16]. Briefly, amino acids in ULF were derivatised with O-Phthaldialdehyde (OPA) reagent, supplemented with 1 mg/ml 2-mercaptoethanol. Derivitised samples were subjected to reverse phase chromatography using an Agilent 1100 Series HPLC system coupled with a Phenomenex HyperClone® 5 mm C-18 ODS 250 x 4.6 mm column (Phenomenex, Macclesfield, UK). A gradient elution with two buffers: (A) 80% 83 mM sodium acetate, 19.5% methanol, 0.5% tetrahydrofuran, and (B) 80% methanol and 20% 83 mM sodium acetate was used to separate OPA-amino acid conjugates at 30 °C with a flow rate of 1.3 ml/min for 60 min per sample. Concentrations of amino acids in the ULF (µM) were determined by comparing sample peak areas to those from certified standards.

**Quantitative real-time PCR (qRT-PCR) analysis**

One thousand nanograms of total RNA from the conceptus and corresponding endometrial tissue of n=5 male and n=5 female conceptuses were subjected to reverse transcription reaction using Superscript III (Applied Biosystems, Foster City, CA, USA) and random hexamers as per manufacturer’s instructions. Primers for microarray validation were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries where possible. Primers for amino acid transporters have been previously reported [23]. Each qRT-PCR reaction was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems) with 5 ng of cDNA, optimized primer concentrations (S8 Table), and 7.5 µl FAST Sybergreen mastermix (Applied Biosystems) in a final reaction volume of 15 µl. The cycling conditions for all qRT-PCR reactions were as 2
min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. All reactions were carried out in duplicate, with the inclusion of a dissociation curve to ensure specificity of amplification as well as no template controls. A standard curve was included for each gene of interest as well as for the normalizer gene to obtain primer efficiencies. All raw cycle threshold values were then imported into qbase\textsuperscript{plus} software (Biogazelle, Zwijnaarde, Belgium) where data were calibrated, normalized and expression values for each gene were determined in arbitrary units (calibrated normalized relative quantities, CNRQ).

**Overrepresented gene ontology terms, upstream regulators, and pathway analysis**

To further interrogate the differences in transcript abundance associated with the sex of the conceptus, the list of DEGs was subjected to analysis using the functional annotation tool in DAVID (http://david.abcc.ncifcrf.gov/) to generate overrepresented biological processes and molecular functions. In addition, Ingenuity Pathway Analysis (IPA) was performed (http://www.ingenuity.com) to identify overrepresented pathways associated with the sex of the conceptus i.e. those with a larger number of differentially expressed genes that one would expect by chance. IPA was also used to assess whether or not the sex of the conceptus modified upstream regulators of the DEGs identified between male and female conceptuses. For all overrepresented gene ontologies, pathways and upstream regulators a p-value for a given function was calculated by considering the number of functional analysis molecules that participate in that function e.g. ligand, receptor etc. and the total number of molecules that are known to be associated with that function/pathway in the DAVID/Ingenuity Knowledge Base. GO terms, pathways and/or upstream regulators with p-value less than 0.05 were considered significant (i.e. more differentially expressed genes associated with these than would be expected by chance).
RESULTS

Correspondence analysis revealed segregation between the different tissue types (i.e. endometrial tissue data clustered together and conceptus tissue data clustered together) (Figure 1A). Conceptus sex did not affect the overall transcriptional profile of the endometrium, but did affect its global transcript abundance in the conceptus (Figure 1B).

Differential transcript abundance in Day 19 male and female conceptuses

In Day 19 conceptuses, the abundance of 5132 transcripts were significantly different between males and females (P<0.05); 2498 were increased in male compared to female conceptuses while 2634 genes were increased in female compared to male conceptuses. Full gene descriptions, associated p-values, and log2 fold change differences are given in S1 Table. Of the transcripts whose abundance was highest in male conceptuses to the greatest extent, zinc finger protein 665-like (2.63 log2 fold change increase in male), lumican (2.34), zinc finger protein 107 (2.33), mirror-image polydactyly gene 1 protein-like (2.20), microRNA mir-2284d (2.03), unknown gene (2.01), zinc finger protein 208 (1.95), microRNA mir-2399 (1.95), CDC-like kinase 1 (1.91) and zinc finger protein 91-like (1.87), none were located on the X chromosome. Transcripts whose abundance was increased in female conceptuses compared to male to the greatest extent included XIST (4.65 log2 fold increase in female compared to male conceptuses), immunoglobulin light chain VJ region (2.44), intestine-specific transcript 1 protein (2.17), synapsin I (1.91), uncharacterized protein C12orf54 homolog (1.84), TIMP metallopeptidase inhibitor 1 (1.69), pancreatic progenitor cell differentiation and proliferation factor homolog (zebrafish) (1.5), KxDL motif containing 1 (1.48), histone cluster 1, H3a-like 23 (1.47) and mucin 15, cell surface associated (1.45). Of these, X (inactive)-specific transcript, synapsin I and TIMP metallopeptidase inhibitor 1 are
located on the X chromosome. Of the total number of DEGs identified between male and female conceptuses, 140 were located on the X chromosome and 78 were increased in the female conceptuses while 62 were increased in the male conceptus on Day 19 of pregnancy. No probes on the microarray were located on the Y chromosome.

Gene Ontology (GO) and Ingenuity pathway analysis of overrepresented biological processes, molecular functions, pathways, and upstream regulators of differentially abundant transcripts in male compared to female conceptuses on Day 19.

In total, sex of the conceptus significantly affected 102 biological processes more than would be expected by chance with 68 molecular functions associated with conceptus sex on Day 19. A full list of the overrepresented biological processes and molecular functions and their associated genes are given in S2 Table and S3 Table. Ingenuity pathway analysis identified 407 pathways that were overrepresented in this list of differentially abundant transcripts, the details of which are available in S4 Table. Included in these pathways were those associated with cell cycle progression (Role of CHK Proteins in Cell Cycle Checkpoint Control 32 DEGs: Cell Cycle Control of Chromosomal Replication 14 DEGs: Cell Cycle: G2/M DNA Damage Checkpoint Regulation 22 DEGs), Embryonic cell lineage (Mouse Embryonic Stem Cell Pluripotency 36 DEGs: Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency 40 DEGs: Telomerase Signaling 35 DEGs: Human Embryonic Stem Cell Pluripotency 32 DEGs: DNA Methylation and Transcriptional Repression Signaling 8 DEGs) as well as pathways involved in conceptus-maternal interactions (Role of PKR in Interferon Induction and Antiviral Response 18 DEGs: GM-CSF Signaling 24 DEGs: Androgen Signaling 39 DEGs: Glucocorticoid Receptor Signaling 81 DEGs: HGF Signaling 37 DEGs: MIF Regulation of Innate Immunity 11 DEGs: MIF-mediated Glucocorticoid Regulation 8 DEGs).
Interestingly, 23 of these pathways were associated with amino acid degradation, biosynthesis and signaling. In total, 66 DEGs were involved in the mTOR signaling pathway, six were involved in Tryptophan Degradation X, five involved in Methionine Degradation I, five in the pathway of Phenylalanine Degradation IV, with seven, five and two DEGs associated with the pathways of Isoleucine Degradation I, Leucine Degradation I and Lysine Degradation II, respectively.

Analysis of upstream regulators of the DEGs in male compared to female conceptuses revealed that 55 of these DEGs were identified as significant upstream regulators of other DEGs due to sex of the conceptus (S5 Table). Moreover, a significant proportion of these were transcriptional regulators (16 in total). Additional molecules identified as upstream regulators of genes that were different by virtue of conceptus sex included the amino acids serine and glutamine as well as interferon alpha.

*Differences in amino acid composition of uterine luminal fluid (ULF) recovered from uteri with male or female conceptuses*

To assess whether conceptus sex affected the amino acid composition of ULF we examined the amino acid content of ULF recovered from heifers with either a male or a female conceptus on Day 19. Of the 18 amino acids analyzed in the ULF containing female or male conceptuses, the concentrations of arginine, asparagine, glutamine, histidine, isoleucine, lysine, methionine, and tryptophan were significantly higher (P<0.05: Figure 2) in the ULF containing a male compared to a female conceptus on Day 19. Interestingly, amino acid concentrations were consistently higher in the ULF hosting a male compared to a female conceptus on Day 19.
Quantitative real-time PCR validation of microarray data and expression of amino acid transporters in the endometrium and conceptus on Day 19

Consistent with the microarray analysis, there was no difference in the abundance of any of 26 amino acid transporters analyzed between endometria from cattle gestating a male compared to a female conceptus (P>0.05). Expression of \( XIST \) was higher (P<0.001) in the endometrium of all animals analyzed compared to conceptus expression, but did not differ between groups (P>0.05).

Comparison of transcript abundance between male and female conceptuses revealed a higher expression of \( XIST \) in female compared to male conceptuses (P=0.01) with a large range in expression values in female conceptuses (range 0.118-2.62 CNRQ) compared to males (range: 0.004-0.016 CNRQ) while abundance in the endometrial tissue was substantially higher (range: 12.35-33.37 CNRQ). The expression of the amino acid transporters \( SLC6A19 \) and \( SLC1A3 \) was significantly higher in male compared to female conceptuses on Day 19 (Figure 3).

Comparison of sexually dimorphic transcript abundance in Day 19 conceptuses with that in the Day 7 blastocyst

In order to identify the temporal changes in sexually dimorphic gene expression that occur between the blastocyst stage (Day 7) and implantation (Day 19), the same pre-processing and stringency measures were applied to previously published data from our group [13]. This resulted in the identification of 2,295 DEGs between male and female Day 7 blastocysts, 1,176 of which were higher in the male and 1,119 were higher in female blastocysts. Of these, 7.1 % of DEGs (163) in the blastocyst linked with sex were located on the X chromosome in comparison to the 2.7 % of X-chromosome associated genes on Day 19 of conceptus development (Figure 4). A similar number of DEGs were identified on each
chromosome as a proportion of the total number of genes on each chromosome. A comparison of these DEGs revealed 1392 were only differentially abundant on Day 7 (659 increased in male, 904 increased in female embryos) while 4239 genes were altered on Day 19 (2,418 increased in male and 2,210 increased in female conceptuses). Only 862 genes were differentially abundant in the embryo/conceptus on both days 7 and 19 (Figure 5A).

These transcripts separated into 4 main categories; 164 DEGs were affected in the same way i.e. increased in male embryo compared to the female embryo on both Day 7 and 19, while 203 were increased in the female embryo/conceptus on both days. In contrast, 161 transcripts were increased in female embryos on Day 7 and decreased on Day 19 compared to male embryos, while for 334 genes the expression pattern was opposite i.e. decreased in female embryos on Day 7 and increased on Day 19 compared to male embryos (Figure 5B: Full information on fold change and P-value are given in S6 Table). The location of these temporally altered transcripts was notable, as 21.2% of these DEGs that were increased in female compared to male embryos on Day 7 and 19 and 14.9% of these DEGs that were increased in female embryos on Day 7 and decreased in female embryos on Day 19 were located on the X chromosome (S7 Table).

**Endometrial transcriptomic response to a male vs female conceptus**

Despite major differences in day 19 conceptus gene expression, no differences in the endometrial transcriptomic response to a male or female conceptus were observed (p>0.05: data accessible in Gene Expression Omnibus (GEO) database GSE75754). Consistent with this finding, the amount of IFNT present in the uterine flush from which a male (335,687±68,118 AU) or female (279,413±55,797 AU) conceptus was recovered was not different (P>0.05).
DISCUSSION

This study builds on the knowledge that one-third of transcripts present at the blastocyst stage of development in cattle are regulated by the sex of the embryo [13] as well as the novel hypothesis that the endometrium is a biosensor of the developmental competency or origin of the embryo [24] by addressing whether this sexual dimorphism is maintained throughout the elongation period of conceptus development and/or whether the endometrium is sensitive to the sex of the developing conceptus on Day 19. Using large-scale transcriptomic analysis, we have determined that more transcripts are differentially abundant between male and female conceptuses on Day 19 than in blastocysts on Day 7, but some differences (approximately 7%) are maintained between these two distinct morphological stages of embryo/conceptus development. Proportionally, more transcripts on autosomal chromosomes are modulated by sex as opposed to just the X-chromosome on Day 19 compared to Day 7. Despite these differences, the endometrium does not respond differently to the presence of a male or a female conceptus, at least not at the transcriptome level after pregnancy recognition has occurred and production of IFNT is not different. However, sex of the conceptus does affect the availability of amino acids in the ULF, likely due to the different requirements of the conceptuses for amino acids in a sex-dependent manner.

The significant number of DEGs between male and female conceptuses was a surprising finding for a number of reasons. Firstly, a number of studies in the literature have found little differences in transcript abundance between male and female conceptuses. While the lack of a significant effect of sex on gene expression is somewhat at odds with the current study, the design of these studies was very different and the platforms used were not always comparable. The study design in the paper of Degrelle et al., (2012) [25] was radically different the present study. They compared Day 18 somatic cell nuclear transfer (SCNT)-derived conceptuses (n=30) to those derived by AI (n=10) or IVP (n=10). All of the SCNT
conceptuses were female one-half of which showed signs of atypical elongation and gastrulation. To generate SCNT and IVP conceptuses, 5-6 blastocysts were transferred per recipient. For the AI controls, animals underwent a mild superovulation protocol (600 IU PMSG) and they also used a different microarray platform. The absence of a sex effect was based on comparison of conceptuses derived from AI (males versus females) as well as females only (AI-IVP-SCNT). Similarly, Betscha et al., (2013) [26] compared gene expression in Day 16 conceptuses derived by AI, SCNT or IVP bovine using the Affymetrix bovine genome array. In this case, all SCN embryos were male. While the authors failed to detect differential gene expression amongst the AI and IVP embryos this was based on analysis of only one female and two male embryos in each case. Valour et al., (2014) [27] investigated the effect of dam physiological status on embryo development and conceptus gene expression in growing heifers and postpartum cows using a homemade 22K bovine oligonucleotide probe array. Sex of the conceptus explained 3.1% of the variability observed in the overall gene expression pattern, respectively. Conversely, the physiological status of the dams represented 23.7% of the variability. However, in these studies both the experimental design and platform differed from this study.

Secondly, the X-chromosome that is silenced is always the paternal X-chromosome in extra-embryonic membranes i.e. in the EET (reviewed by [28]). There are substantial data in the literature demonstrating a sex-dependent rate of growth/development. However, these data refer to development to the blastocyst stage only, and to our knowledge cell number in elongated conceptuses has not been quantified. In as much as was possible to assess, conceptus length was not different between male and female conceptuses in this study. This is difficult to measure accurately at Day 19 because of the fragile nature of the conceptus at this stage and the fact that the conceptuses are often tangled due to the flushing recovery technique. However, two points would suggest that male conceptuses were not advanced
compared to females. Firstly, IFNT content of uterine lumen fluid, which is highly correlated with conceptus length and trophectoderm cell number [29] was not different between males and females. Secondly, the expression of a number of marker genes of gastrulation in the EET cells (as detailed in the study by Degrelle et al., [25] - specifically the genes CPA3, CALM1, and HNRNPDL) were not differentially expressed between male and female conceptuses in this study (S1 Table). Despite the fact that the proportion of DEGs located on the X-chromosome was lower than those previously reported in the bovine blastocyst [13], similar numbers of DEGs were located on the X chromosome. Sexual dimorphic biological pathways in Day 19 conceptuses included cell cycle progression and chromosomal replication as well as stem cell pluripotency. Also overrepresented are genes involved in the pathways of glucocorticoid receptor signaling, androgen signaling and MIF signaling. These data are interesting in the context of a recent study by Dobbs et al. [30] that found a sex-dependent response of the embryo to colony-stimulating factor 2. Thus, embryo trophic factors produced by male and female conceptuses on Day 19 may affect pathways in the conceptus itself in a sex-specific manner.

Comparisons of transcripts affected by sex of the embryo at Day 7 to those affected in the Day 19 conceptus revealed that only a small proportion are conserved between these distinct morphological time-points (Figure 4). The timing and mechanism of XCI differs between germ line and somatic cells [12] [28] and this may account for the seemingly small overlap in sex-regulated genes. In particular, tissue analyzed on Day 7 consists of a mixture of approximately 2:1 trophoblast to inner cell mass whereas on Day 19 only trophoblast cells were analyzed. Within these 864 genes, less than half displayed a similar expression pattern on both days of pregnancy. Interestingly, a number of genes were decreased on both days 7 and 19 in the female compared to male embryo/conceptus i.e. when X-chromosome inactivation has occurred, given increased expression of XIST in the female embryo [14] and
conceptus (this study). Evidence from other species shows that coordinate with XCI, portions of autosomal chromosomes can become inactivated during this process (reviewed by [31]). The decreased transcript abundance in female conceptuses on Day 19 may be autosomal genes inactivated during the process of XCI. In addition, it is possible that the transcripts only decreased on Day 19 in female conceptuses may be a late or delayed consequence of XCI. Some caution is needed with the interpretation of these comparisons however, given the two data sets are derived from somewhat different sources. The Day 7 blastocyst data were derived from in vitro fertilized embryos produced with sex-sorted semen. The current data were derived from AI with conventional (non-sex-sorted semen).

In contrast to the sex-induced differences in the conceptus, no differences in the endometrial transcriptomic response to either a male or female conceptus were detectable on Day 19. As has been shown on Day 18 [11] and Day 20 [10] in cattle and in humans [32], the endometrium does respond differently to conceptuses of differing quality and trajectories with regard to pregnancy outcome [10, 11]. Therefore, the lack of differences observed in the endometrial transcriptomic response to male and female conceptuses may simply reflect a lack of difference in developmental competency of a male versus a female conceptus after Day 19. It is interesting, however, that a number of the DEGs between male and female conceptuses are also expressed in the endometrium (e.g. MIF, OXT, SI1). In addition, we know from previously reported studies that the endometrium expresses receptors for some conceptus-derived ligands but the fact remains that no differences in the pregnancy recognition signal occur, at least in the intercaruncular endometrium. However, it is possible that there may be protein differences or differences in the post-translational modifications of proteins in the endometrium in response to male and female conceptuses and could be an avenue of future study.
Both male and female Day 8 blastocysts [33] as well as Day 14 conceptuses [14] display differences in the type of IFNT transcripts expressed; however, there was no difference in the abundance of IFNT in the ULF containing male or female conceptuses on Day 19. Given that IFNT is predominantly responsible for the pregnancy recognition response in the endometrium of cattle, as there are no differences between male and female abundance of IFNT, this may explain in part, why there is no difference in the transcriptomic response of the endometrium to the conceptus. Differences in amino acid composition in the ULF on Day 19 were interesting. In a previous study by Sturmey et al., [16], male in vivo-derived blastocysts had a lower depletion of amino acids and lower amino acid turnover compared to female blastocysts i.e. increased amounts of amino acids in the media similar to increased amino acids in the ULF in this study. If increased amino acids in the ULF are indicative of reduced uptake by the Day 19 conceptus (likely given there are no differences in amino acid transporters in the endometrium) then it is reasonable that male conceptuses do not utilize amino acids to the same extent as female conceptuses. Differences in the amino acid composition of the ULF hosting cloned and in vitro produced conceptuses on Day 18 have been observed [34] but this is coordinate with reduced expression of amino acid transporters in the endometrium (likely due to the different endometrial response these conceptuses elicit [11]). In the present study differences in amino acid composition were not due to differences in transcript abundance for the amino acid transporters in the endometrium. Thus, despite a similar uterine environment male and female conceptuses utilize this environment in a sex-specific manner in vivo similar to the phenomenon observed in vitro with regard to amino acid uptake [16] as well as other energy substrates. Indeed, concentrations of the neutral amino acids asparagine, glutamine, and methionine were higher in the ULF hosting male compared to female conceptuses. Male conceptuses exhibited increased expression of the neutral amino acid transporter SLC6A19. This increased
expression of the transporter in the male conceptuses may not necessarily translate into increased neutral amino acid uptake. Alternatively, this could be increased transcript abundance that may translate into increased amino acid uptake by the male conceptus at a later time point i.e. after Day 19 when these samples were taken.

In conclusion, the results of this study support the hypothesis that XCI is incomplete during the initiation of implantation in cattle. There is also significant sexual dimorphism in terms of amino acid consumption as well as gene expression in the conceptus at Day 19. Yet despite the significant difference in gene expression changes, the sex of the conceptus itself does not elicit a significantly different response in the transcriptome of the endometrium, at least on Day 19. Moreover, given the fact the conceptus is exposed to the same maternal environment (as there is no difference in endometrial response) we propose that conceptuses of different sexes utilize the same uterine environment but in a sex-dependent fashion.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the help of present and previous graduate students during sample collection. This work was funded by grants from Science Foundation Ireland grant number 13/IA/1983 and the Spanish Ministry of Science and Innovation (AGL2012-37510 and AGL2012-39652-C02-01).

REFERENCES


FIGURE LEGENDS

Figure 1. Correspondence analysis indicating the source of greatest variation in the overall transcriptional profile. Each dot represents an individual microarray for an individual sample. (A) A clear segregation in the expression profiles of the different tissue types, i.e. endometrium and conceptus, was observed, but sex of the conceptus affected gene expression in the conceptus (right) but not the endometrium (left). (B) Differences in overall gene expression profiles in male and female conceptus when plotted alone.

Figure 2. Concentrations (µM±SEM) of amino acids in the uterine luminal fluid (ULF) recovered on Day 19 of pregnancy from heifers with a male (black bars) or female (open bars) conceptus present (n=11 per sex). Significant differences in amino acid concentrations from the uterus containing a male versus a female conceptus are noted with an asterisk (*) when P<0.05.

Figure 3. Analysis of transcript expression for amino acid transporters and selected transcripts from the microarray study qRT-PCR in male and female conceptuses on Day 19 of pregnancy. All expression values are given as mean calibrated, normalized, relative expression values in arbitrary units (AU±SEM) for n=5 male (closed bars) and n=5 female (open bars) conceptuses on Day 19. Significant differences in expression between male and female conceptuses are noted by an asterisk (*) when P<0.05.

Figure 4. Graph depicting the number of genes on each chromosome as well as the frequency of chromosomal location of genes identified as differentially expressed between male and
female conceptuses on Day 19 of pregnancy (this study) and embryos on Day 7 of pregnancy [13].

Figure 5. Venn diagram showing (A) the overlap in differentially expressed genes as well as (B) the direction of fold change between male and female embryos using the same analysis criteria (Day 7 [13]) and male and female conceptuses (Day 19: This study). All data was subjected to the same stringent data pre-processing prior to generation and comparison of lists of differentially expressed genes.

SUPPLEMENTARY TABLE LEGENDS

S1 Table. Gene symbol, Affymetrix probe ID, gene description, P-value and log2 fold change for genes differentially expressed in male versus female conceptuses on Day 19 of pregnancy. Fold change is given relative to expression in the male conceptus i.e. a positive value indicates increased expression in the male, a negative value indicates increased expression in female conceptuses.

S2 Table. Biological processes overrepresented in the list of differentially expressed genes (DEGs) associated with sex of the conceptus on Day 19 of pregnancy. All biological processes are significantly overrepresented in each sample set, i.e. more genes detected in a specific biological process than would be expected by chance. Columns give the gene ontology (GO) term as determined by http://david.abcc.ncifcrf.gov/, the p-value associated with a given biological process, number of DEGs in that GO term (count), and the number of
genes in the biological process (size) as well as the full description and details of genes associated with a given biological process.

**S3 Table.** Molecular Functions overrepresented in the list of differentially expressed genes (DEGs) associated with sex of the conceptus on Day 19 of pregnancy. All molecular functions are significantly overrepresented in each sample set, i.e. more genes detected in a specific molecular function than would be expected by chance. Columns give the gene ontology (GO) term as determined by http://david.abcc.ncifcrf.gov/, the p-value associated with a given molecular function, number of DEGs in that GO term (count), and the number of genes in the molecular function (size) as well as the full description and details of genes associated with a given molecular function.

**S4 Table.** Ingenuity Pathway Analysis (IPA) of overrepresented pathways in the list of differentially expressed genes (DEGs) associated with sex of the conceptus on Day 19 of pregnancy. All pathways are significantly overrepresented in each sample set, i.e. more genes detected in a specific pathway than would be expected by chance.

**S5 Table.** Ingenuity pathway analysis of up-stream regulators of differently expressed genes between male and female conceptuses on Day 19 of pregnancy

**S6 Table A.** Genes up-regulated in male compared to female embryos on Day 7 and Day 19 of pregnancy, **S6B.** Genes up-regulated in female compared to male embryos on Day 7 and Day 19 of pregnancy, **S6C.** Genes down-regulated in female embryos on Day 7 and up-
regulated on Day 19 compared to male embryos and S6D. Genes up-regulated in female embryos on Day 7 and down-regulated on Day 19 compared to male embryos.

**S7 Table.** Chromosomal location for all differently expressed genes that are identified on Day 7 and Day 19 between male and female embryos and conceptuses.

**S8 Table.** Primer information used for quantitative real time PCR analysis of candidate genes. All primers were used at a concentration of 300 nM in a final reaction volume of 15 µl. A dissociation curve was included to ensure specificity of each primer pair.
Figure 1(A). (B).
Average (±SEM) calibrated normalized relative gene expression values on Day 19 of pregnancy

- Endometrium Female conceptus present
- Endometrium Male conceptus present
- Female Conceptus
- Male Conceptus

P=0.1
P<0.05

Genes:
- LOC788423
- XIST
- SLC6A19
- SLC1A3
Figure 4.