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Identification of large offspring syndrome during pregnancy through ultrasonography and maternal blood transcriptome analyses

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In vitro production (IVP) of embryos in cattle can result in large/abnormal offspring syndrome (LOS/AOS) which is characterized by macrosomia. LOS can cause dystocia and lead to the death of dam and calf. Currently, no test exists to identify LOS pregnancies. We hypothesized that fetal ultrasonography and/or maternal blood markers are useful to identify LOS. Bovine fetuses were generated by artificial insemination (control) or IVP. Fetal ultrasonographies were taken on gestation D55 (D55) and fetal collections performed on D56 or D105 (gestation in cattle ≈ D280). IVP fetuses weighing ≥ 97 percentile of the control weight were considered LOS. Ultrasonography results show that the product of six D55 measurements can be used to identify extreme cases of LOS. To determine whether maternal blood can be used to identify LOS, leukocyte mRNA from 23 females was sequenced. Unsupervised hierarchical clustering grouped the transcriptomes of the two females carrying the two largest LOS fetuses. Comparison of the leukocyte transcriptomes of these two females to the transcriptome of all other females identified several misregulated transcripts on gestation D55 and D105 with *LOC783838* and *PCDH1* being misregulated at both time-points. Together our data suggest that LOS is identifiable during pregnancy in cattle.

Large offspring syndrome (LOS) is an overgrowth condition observed in ruminant fetuses and neonates[1,2]. LOS was first reported in 1991 in a cloned calf produced via nuclear transfer[3]. Later, in 1995, overgrowth was reported as a result of non-invasive in vitro production (IVP) procedures[4]. At that time, the overgrown animals were called "large calves" and the syndrome was coined LOS[5]. As the use of IVP in ruminants increased during the next decade, so did the number of LOS reports[6–11]. The main characteristic of LOS is overgrowth, which in some instances can result in calves weighing twice the average birthweight of their breed[4]. However, LOS in ruminants is a complex disorder with other phenotypes observed including visceromegaly, macroglossia, increased incidence of hydro-allantois, abnormal limbs and spinal cord, ear malformation, hypoglycemia, and umbilical hernia[6,7,9–14]. Because of these varied phenotypes, this syndrome is also known as abnormal offspring syndrome (AOS)[11].

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Even though it is now clear that LOS/AOS is a multi-locus loss-of-imprinting (i.e., epigenetic) condition[14], it is still not known what triggers LOS and which assisted reproductive technology procedure (e.g., in vitro maturation of oocytes, in vitro fertilization, in vitro embryo culture of embryos or embryo transfer) is involved. Several reported cases of LOS in the literature were produced using serum supplementation during oocyte maturation and/or during embryo culture, which suggest that serum may be a factor promoting the syndrome[1,2,4,15,16]. Serum has been experimentally determined to cause LOS in sheep[16–18] and bovine offspring derived from embryos cultured in serum containing medium can develop LOS[2]. In addition, the syndrome can also occur in fetuses and calves derived from embryos cultured without serum supplementation[19,20] and, more recently, we have documented that this syndrome occurs spontaneously in cattle produced by natural or artificial insemination[21–23]. The latter is of interest as there is a similar loss-of-imprinting overgrowth syndrome in humans, namely Beckwith–Wiedemann syndrome, which occurs naturally, and its incidence is increased in children conceived by assisted reproduction[24].

Due to its large size, LOS can cause dystocia and, sometimes, cesarean section is needed for delivery[25]. Even if the newborn calf survives the difficult birth, the enlarged tongue or extreme body weight make suckling difficult, thus increasing the chances of postnatal death[26]. In addition to the possible death of calves and cows, other financial losses are incurred due to veterinary costs[22] and the associated negative economic impact in terms of losses in milk, fat, and protein yields in the subsequent lactation[27,28]. For example, two independent LOS cases have been recently reported with total estimated losses of approximately $30,000 each[22]. These monetary losses could have been minimized if the early identification of LOS was possible. To date, however, no test exists to predict LOS pregnancies in cattle. As IVP is the current method of choice to improve genetic merit of the offspring in the cattle industry[29,30] it is of particular importance to find biomarkers to identify fetal overgrowth early during gestation to help producers decide whether to terminate the pregnancy or prepare for a difficult birth.

Ultrasound is a valuable non-invasive and repeatable tool that has been widely used in cattle to determine fetal growth[7,31,32], fetal age[33], fetal sex[34] and clinical pathologies such as mummified fetuses or endometritis[35]. In addition, blood biomarkers have been successfully used as a non-invasive method to determine pregnancy status. For example, *ISG-15* mRNA from pregnant cattle leukocytes[36] and pregnancy associated glycoproteins from bovine maternal blood serum are useful markers of early pregnancy[37]. Whether maternal blood components can be used to identify LOS early in pregnancy is not known.

For our study, we hypothesized that LOS can be identified during pregnancy in cattle by use of ultrasonography and/or maternal blood leukocyte mRNA biomarkers. The approach was to generate embryos by artificial insemination (AI; control) or by in vitro procedures previously shown by us to generate overgrown fetuses and to perform ultrasonographic measurements of those fetuses at day 55 (D55) of gestation and transcriptome analysis of maternal blood leukocytes on D55 and D105 of gestation.

Materials and methods

The study is reported in accordance with ARRIVE guidelines[38].

Heifers. All animal procedures were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of the University of Missouri (Protocol #9455). All animals were kept at the University of Missouri South Farm Research Center in Columbia.

Angus crossbred heifers of approximately 18–20 months of age were synchronized and selected for breeding using the 14-day controlled internal drug release (CIDR®, Zoetis, Kalamazoo, MI) prostaglandin and timed artificial insemination protocol (Supplemental Fig. 1).

In vitro and in vivo production of embryos and embryo transfer. Media and procedures were as previously described by us[2,39]. Briefly, *Bos taurus taurus* (*B. t. taurus;* Angus/Angus-Crossbred) oocytes were harvested from slaughterhouse ovaries. Oocytes were removed from maturation medium after ~ 21 h of culture and inseminated with semen from one *B. t. indicus* male (Brahman breed [JDH MR MANSO 7 960958 154BR599 11200 EBS/INC CSS 2]). Putative zygotes were stripped of cumulus cells by five minutes of vigorous vortexing at approximately 18 h after insemination and then cultured in KSOM supplemented with amino acids in a humidified atmosphere containing 5% O_2, 5% CO_2, and 90% N_2. On D5 after insemination, the culture medium was supplemented with 10% (v/v) estrus cow serum (collected and prepared in house and previously used in Ref.[2]) and embryos returned to the incubator. On D7, blastocyst-stage IVP embryos were selected, washed in BioLife Holding & Transfer Medium (AgTech; Manhattan, KS), and loaded in groups of two into 0.25 cc yellow, direct transfer and irradiated straws (AgTech). Blastocysts were transferred to synchronized recipient females on D7 after estrus (Supplemental Fig. 1).

Rationale for experimental design. This experiment is a part of a large-scale study aimed at the identification of epigenetic misregulations in LOS (as defined by IVP fetuses weighing ≥ 97 percentile of the weight of the control fetuses). Based on our previous results for pregnancy rates from IVP embryos, percent LOS on D105, and transcriptome and methylome analyses[40], we calculated that four LOS males and four LOS females would be required to achieve 90% power. Therefore, we transferred two embryos per heifer (as we did in Ref.[2]) to achieve that goal. In our previous study[2], body weight did not differ between singletons and twins on D105 of gestation. It should be noted that twinning occurs in cattle[41]. In addition, the rationale for generating *Bos taurus indicus* × *Bos taurus taurus* F1 fetuses in this study is that since LOS is a loss-of-imprinting condition, allele sequence differences are required to characterize parental-specific genomic (mis)regulation in the F1. This has been a useful breeding scheme to enhance the list of imprinted genes in bovine and to identify loss-of-imprinting in LOS[40,42].

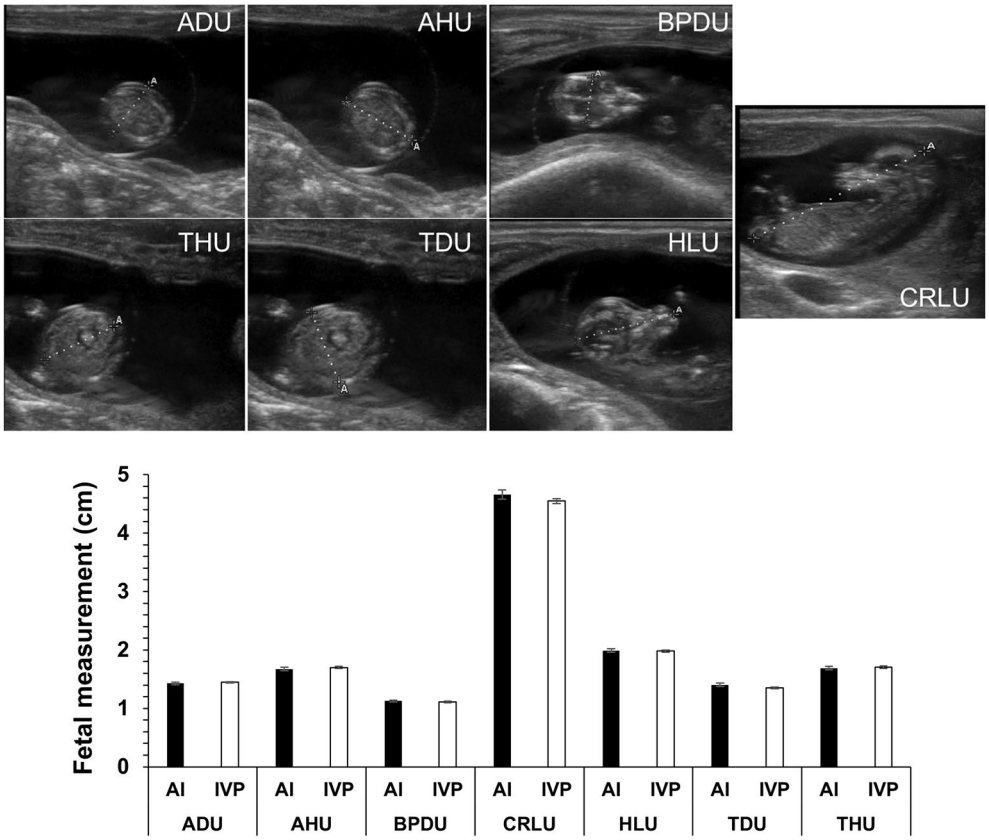

Figure 1. D55 fetal morphometry. *Top* Sagittal views of the ultrasound images taken on D55 of gestation. *U* ultrasound, *AH* abdominal height, *AD* abdominal diameter, *BPD* biparietal diameter, *CRL* crown rump length, *HL* head length, *TD* thoracic diameter, *TH* thoracic height. *Bottom* Comparison of ultrasound measurements between all the AI (n = 26) and IVP (n = 89) fetuses in this study. No statistical differences were detected between groups. *AI* artificial insemination (i.e., control), *IVP* in vitro produced embryos.

D55 and D77 fetal ultrasound morphometry. At D55 of pregnancy, presumed pregnant animals were checked for the presence of a fetus/es by transrectal ultrasonography using a SonoSite EDGE ultrasound machine equipped with a L52 10.0–5.0 megahertz linear-array transducer (Supplemental Fig. 1). Images acquired by ultrasound were analyzed using software resident on the machine. Ultrasound measurements taken were abdominal height, abdominal diameter, thoracic diameter, thoracic height, crown rump length, head length, and biparietal diameter (Fig. 1A). Fetal sex was determined by ultrasonography on D77 of gestation (Supplemental Fig. 1). Furthermore, fetal morphometry was assessed on D77 in a subset of 18 animals (6 AI and 12 IVP animals). Ultrasound measurements taken on D77 were abdominal height, abdominal diameter, thoracic diameter, thoracic height, crown rump length, head length, and biparietal diameter.

Surgical fetal collections of D56 and D105 fetuses. Heifers (n = 51 for D56; n = 48 for D105) were fasted at least 12 h prior to surgery. Fetuses were surgically retrieved to preserve nucleic acid integrity. All surgical procedures were performed by a licensed veterinarian.

Collection of fetal tissues and fetal measurements of D56 and D105 fetuses. Collected fetuses and their fetal membranes were weighed, fetal morphometry was assessed, and abnormal phenotypes were noted. Measurements were crown-rump length, heart girth, forelimb length, biparietal length, abdominal height, head length and thoracic height. Subsequently, all tissues (i.e. pancreas, kidney, liver, ears, skeletal muscle, heart, diaphragm, tongue, buccal mucosa, umbilicus, placenta [cotyledons and intercotyledon], brain, reproductive tract, gonad, skin, stomach, intestine, lung, spleen, tail, leftover carcass) were dissected and divided in two and immediately frozen in liquid nitrogen. For all collections, the same person measured and weighed all fetuses and fetal membranes, and another person (veterinary anatomic pathologist) dissected all the tissues. The average time from excision of the fetus from the uterus to when all tissues were frozen in liquid nitrogen was approximately 18 min. All tissues were stored at − 86 °C until further use.

Image analysis of crown rump length and umbilicus diameter in D105 fetuses. Measurement of D105 fetuses' crown rump length (from the top of the head to base of the tail) and diameter of the umbilicus (at the base of the umbilicus where it protrudes from the body) were measured using the ImageJ's[43] freehand line

function using a lateral side picture of the D105 fetuses. The surface where the fetuses laid was squared (each square = 2.54 cm) and was used to convert all measurements to cm, and then a ratio of umbilicus diameter to crown rump length was determined.

Maternal blood collection and processing. Maternal blood was collected via tail venipuncture on D55 and D105 of pregnancy into K3 EDTA tubes (BD, Franklin Lakes). Blood processing was done as described earlier in Ref.[44]. Briefly, blood containing tubes were centrifuged at 1200×*g* for 20 min at 4 °C. The buffy coat was transferred to 15 ml centrifuge tubes containing 12 ml of red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.0). White blood cells (WBC = leukocytes) containing tubes were briefly vortexed, incubated at room temperature for 5 min, and later centrifuged at 300×*g* for 10 min at 4 °C. After discarding the supernatant, the WBC pellet was washed once in 5 ml red blood cell lysis buffer and then with 5 ml ice-cold 1× Dulbecco's phosphate buffered saline, with centrifugation at 300×*g* at 4 °C for 5 min at each wash. After discarding the supernatant, the WBC pellet containing tubes were placed immediately on dry ice and then stored at − 86 °C until use.

Selection of samples for RNA sequencing (RNAseq). The WBC samples of 23 D105 pregnant heifers were selected for RNAseq on the basis of their group (AI or IVP), weight of IVP fetuses (≥ 97 percentile of AI weight = IVP-LOS or < 97 percentile of AI weight = IVP-Normal), fetal sex, and whether the females carried one or two fetuses in the IVP group. In addition, the D55 WBC samples of the same 23 females were also used for transcriptome analyses (Supplemental Fig. 2).

RNA isolation and transcriptome analyses. Total RNA was isolated using Trizol™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and stored at − 86 °C until use.

Library preparation and transcriptome sequencing. RNA processing and sequencing was performed by BGI Americas Corporation (Cambridge, MA). Libraries were sequenced using the DNBSEQ-G400 platform to generate 20 million 100 bp paired end reads.

RNAseq data analysis. Base quality and adapter contamination was assessed with FastQC and low quality (Pred < 20) raw reads trimmed using DynamicTrim. Trimmed reads less than 60 bases in length were removed with SolexaQA++ LengthSort function[45]. Retained paired-end reads were aligned to the bovine reference genome, ARS-UCD1.2[46] with HISAT2 v2.1.0[47] with the parameter adjustment (–mp 6,6; –score-min L,0,-0.2; –known-splicesite-infile) included to improve specificity. Total read counts for each gene were calculated by using the HTseq-count default union-counting module[48] using NCBI (GCF_002263795.1_ARS-UCD1.2) RefSeq gene set.

Hierarchical clustering. Raw read counts were normalized and the dist(method = 'euclidean') function was used to compute the distances between the rows of normalized count data. Finally, unsupervised hierarchical clustering was applied using the average linkage method and the base R hclust function to build the dendrogram.

Differential expression analysis. Differential expression analysis was performed using edgeR v3.32.1[49] and DESeq2 v1.30.1[50]. Likelihood ratio tests were done using both packages (edgeR using glmLRT and DESeq2 using DESeq(test = LRT)). For edgeR, the raw read counts were normalized using RUVseq[51]. The betweenLaneNormalization function of EDAseq v2.24.0 was used to adjust for sequencing depth and then upper quantile normalization was done to remove unwanted variation[52]. The trimmed means of M values (TMM) was also used for edgeR analysis. For DESeq2, the median of ratios method of normalization was used using estimateSizeFactors and assigning the normalization factors back to our count matrix. Pairwise comparisons between each treatment (Control-AI vs IVP-Normal, IVP-Normal vs IVP-LOS, and Control-AI vs IVP-LOS) group at each time point (D55 and D105) were performed. In addition, we compared the transcriptome of the dams carrying the two largest IVP-LOS individuals (#604 and #664) against all other animals for D55 and D105. Furthermore, a pairwise comparison of WBC transcriptomes of females carrying two vs one fetuses was done to account for differential expression due to multiple fetuses (i.e. increased fetal mass). Genes with a false discovery rate (FDR; EdgeR) or adjusted P-value (padj; DEseq2) ≤ 0.05 were classified as significant. Significant genes from both packages were overlapped in order to generate a list of candidate genes for downstream analysis.

cDNA synthesis and quantitative RT-PCR (qRT-PCR). Total RNA was treated with DNase (Fischer Scientific, Waltham, MA) and used as template to synthesize cDNA in a 20 µl reaction using oligo dT and Superscript IV (Invitrogen, Carlsbad, CA) as recommended by the manufacturer.

Arginine and Serine Rich Coiled-Coil 1 (RSRC1) and *Transcription Termination Factor 1* (TTF1) were used as test genes to corroborate RNA sequencing results based on their upregulation in the WBC transcriptome of the two females carrying the two largest LOS fetuses. Endogenous transcripts used as normalizers were: *Ecdysoneless Cell Cycle Regulator* (*ECD*), *Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, Beta* (*NFKBIB*), and *VPS35 Endosomal Protein Sorting Factor Like* (*VPS35L*). These were chosen based on their constancy of expression in the RNAseq result (i.e., EdgeR FDR = 1; DESeq2 padj > 0.8 and coefficient of variation ≤ 0.10 across all 23 D105 WBC samples. In addition, these transcripts were also chosen based on availability of intron-spanning TaqMan probes (ABI, Foster City, CA) for bovine. TaqMan probe information is as follows: *ECD* (Bt03235022_m1), *NFKBIB* (Bt03247668_m1), *RSRC1* (Bt03236115_m1), *TTF1* (Bt03266651_m1), *VPS35L*

(Bt03269522_m1). The mRNA levels of the target genes for the pregnant females carrying IVP-LOS and the two largest LOS fetuses (cow #604 and #664) relative to the combined AI and IVP-Normal groups was calculated using the comparative cycle threshold (C_T) method. Briefly, the C_T for each sample was normalized to the geometric mean of the three endogenous reference genes. The average C_T was calculated by averaging the C_T of all independent samples excluding those from the females carrying the two largest LOS fetuses. The comparative C_T method ($\Delta\Delta C_T$) was used to compare the values of #604 and #664 against the average C_T for all other samples. Fold difference is used for data representation.

Statistical analyses. All analyses include an IVP-Normal group to remove potential confounding effects of method of conception when analyzing variables. The morphometric data were analyzed by analysis of variance using the general linear model procedure using SAS software v9.4 (SAS Institute, Cary, NC). Dependent variables were all fetal measurements, and the independent variables were the group and day of collection (AI, IVP-Normal, IVP-LOS). Differences were considered statistically significance when $p < 0.10$.

Results

Pregnancy rate. The overall pregnancy rates on D55 were 61.1% for the AI group and 43.0% for the IVP group. For the D56 fetal collection set, there were 14 singleton pregnancies in the AI group, while, for the IVP group, there were 19 singleton pregnancies and 12 females carried two conceptuses. For D105 fetal collection set, there were 12 singleton pregnancies in the AI group, while, for the IVP group, there were 26 singleton pregnancies and 10 females carried two conceptuses.

D55 fetal ultrasonographies. Fetal ultrasonographies were taken on gestation D55 to determine if LOS could be detected by this stage of pregnancy. Figure 1 shows the average measurements of all fetuses collected in this study (AI = 26, IVP = 89). There were no differences in any of the fetal ultrasonographic measurements between the AI and IVP groups. Head length was greater in male fetuses (n = 69; mean ± SEM; $p < 0.04$; 2.01 ± 0.02) when compared to female fetuses (n = 43; 1.94 ± 0.03). There was also a sex effect on crown rump length ($p = 0.06$) with males (4.64 ± 0.05) being longer than females (4.46 ± 0.06). This effect was more pronounced within the IVP group ($p < 0.03$; 4.62 ± 0.05 and 4.42 ± 0.07, for males (n = 53) and females (n = 34), respectively).

Fetal collections and LOS determination. Fetal overgrowth is the defining phenotype of LOS, and weight at surgical collection was used to define LOS using criteria described earlier by us[2]. A fetus was categorized as overgrown if its weight was ≥ 97 percentile weight of the AI in a sex-specific manner. We chose ≥ 97 percentile of control weight as threshold to ascribe LOS as this has previously been used to describe BWS[53], the counterpart syndrome in humans.

D56 fetuses. Fetal weight was not significantly different between singletons and twins (mean ± SEM; 8.94 ± 0.28 g and 8.79 ± 0.23 g, respectively). Fetal weight was similar between the AI and IVP fetuses (mean ± S.D.; 8.66 ± 1.39 and 8.95 ± 1.43, respectively). For the AI group, there were eight males and six females. The average weight for the males was 8.78 ± 1.33 g (weight range: 6.21–10.27 g; Fig. 2) while the average weight for the females was 8.50 ± 1.59 g (weight range: 6.20–10.22 g). For the IVP group, we collected 21 males and 22 females. The average weight for the males was 9.52 ± 1.53 g (weight range = 6.94–13.77 g) while the average weight for the females was 8.40 ± 1.11 g (weight range = 5.55–10.31 g). Fetuses weighing ≥ 97 percentile of controls (male = 10.18 g and female = 10.16 g) in the IVP group were considered LOS (Fig. 2). In total, there were eight IVP-LOS males (weight range = 10.23–13.77 g) and one IVP-LOS female (weight = 10.31 g). All IVP fetuses weighing < 97 percentile weight of controls were referred to "IVP-Normal" (males—n = 13 [weight range: 6.94–9.98 g] females—n = 21 [weight range: 5.55–9.8 g]). Besides heavier body weight, other phenotypes observed in the IVP group were focal hemorrhage on the brain and abdominal wall defects (Fig. 2C). The fetal measurements at collection for the AI, IVP-Normal and IVP-LOS groups are summarized in Fig. 3. Only one female in the IVP group was considered LOS, therefore no comparisons were made between it and the other groups.

D105 fetuses. Fetal weight was not significantly different between singletons and twins (mean ± SEM; 532.78 ± 20.02 g and 494.80 ± 29.69 g, respectively). Fetal weight was similar between the AI and IVP fetuses (mean ± S.D.; 466.00 ± 55.62 and 532.15 ± 134.80 g, respectively). For the AI group, we collected eight males and four females. The average weight for the males was 494.29 ± 44.05 g (mean ± S.D.; weight range: 442–550 g; note: there is a missed observation in this group) while the average weight for the females was 416.50 ± 36.01 g (weight range: 388–468 g; Fig. 4). For the IVP group, we collected 33 males and 13 females. The average weight for the males was 526.39 ± 123.00 g (weight range = 366–1080 g) while the average weight for the females was 546.77 ± 167.77 g (weight range = 318–986 g). Fetuses weighing ≥ 97 percentile of controls (male = 548.92 g and female = 463.14 g) in the IVP group were considered LOS (Fig. 4).

In this study, it appears that female fetuses were preferentially lost between D56 and D105, especially in the IVP group. On D56, we collected 6 females and 8 males in the AI group and 22 females and 21 males in the IVP group. On D105 we collected 4 females and 8 males in the AI group and 13 females and 33 males in the IVP group, which is different than the expected 1:1 ratio ($p < 0.01$). Although the reason for the loss of female fetuses between D56 and D105 is unknown, this was far more pronounced in the IVP group. The deficiency of female fetuses was not statistically significant in the AI group ($p = 0.20$) but was highly significant in the IVP group ($p < 0.003$) when compared to the binomial distribution with the expected 1:1 sex ratio.

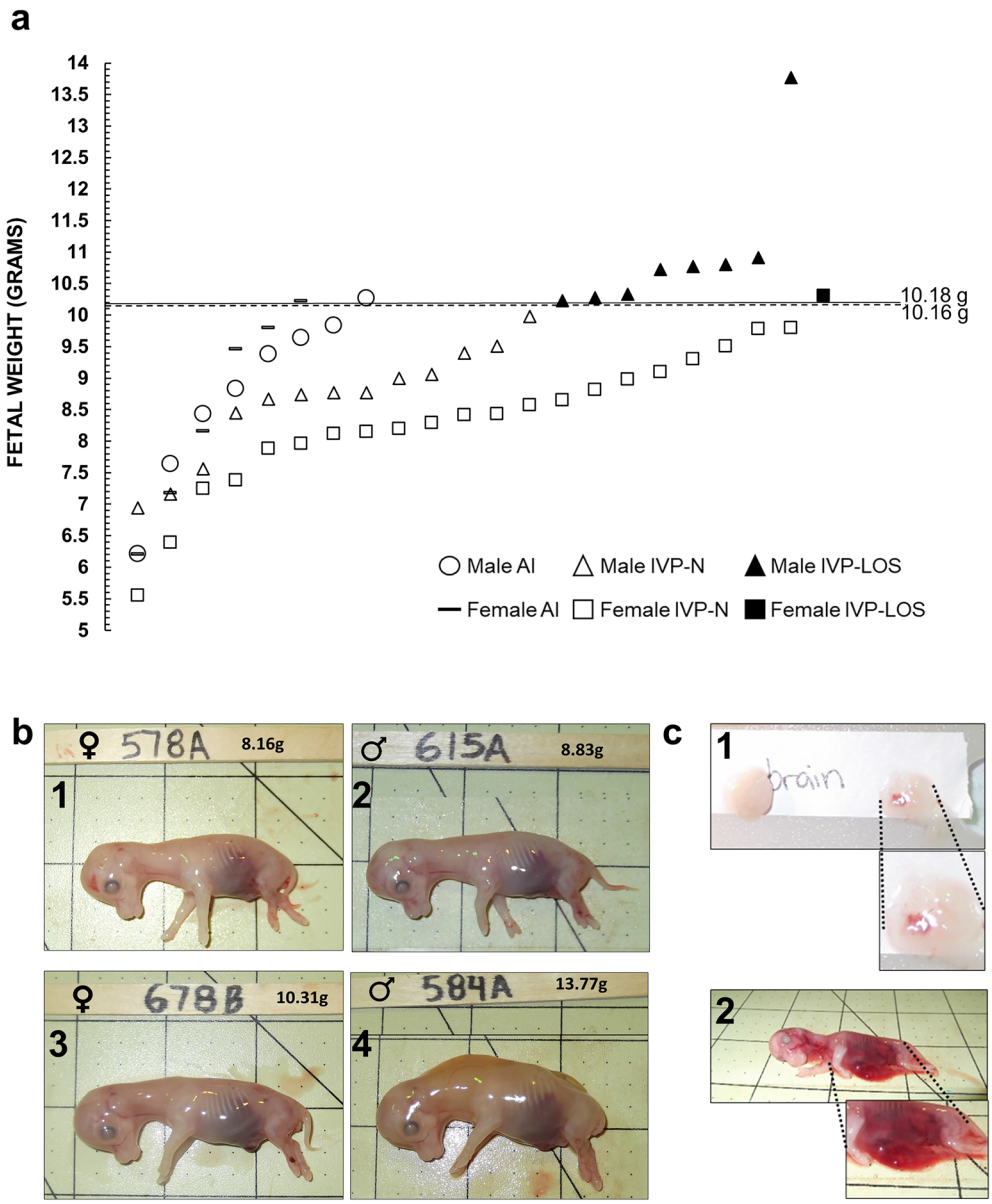

Figure 2. D56 fetal collections. **(a)** Fetal weight at D56 of gestation. The X axis has no actual implication and is used to scatter the spots representing each fetus for ease of visualization. The sex of the fetuses and the way they were generated is shown at the bottom of the figure. The lines represent the 97 percentile of AI D56 fetal weight (i.e., male—10.18 g, female—10.16 g). **(b)** The pictures show D56 fetuses in the control **(b1,b2)** and IVP-LOS group **(b3,b4)**. Also, females **(b1,b3)** and males **(b2,b4)**. **(b1)** AI-578A (control female weighing 8.16 g which is approximate average weight of female control fetuses). **(b2)** AI-615A (control male weighing 8.83 g which is the approximate average weight of male control fetuses). **(b3,b4)** Show the heaviest LOS (IVP-LOS 678B: female weighing 10.31 g and IVP-LOS 584A: male weighing 13.77 g). Each square on the background = 2.54 cm. **(c)** Secondary phenotypes observed in D56 LOS fetuses—focal hemorrhage on the brain **(c1)** and abdominal wall defects **(c2)**. *LOS* large offspring syndrome, *AI* artificial insemination (i.e., control), *IVP* in vitro produced embryos.

In total, there were eight IVP-LOS males (weight range = 552–1080 g) and nine IVP-LOS females (weight range = 468–986 g). All IVP fetuses weighing < 97 percentile weight of controls were referred to "IVP-Normal" (males—n = 25 [weight range: 366–542 g] females—n = 4 [weight range: 318–448 g). Besides heavier body weight, other phenotypes observed in the IVP group were long protruding tongue (Fig. 4C.3), large organs (heart, kidney, lung, pancreas), hepatic cyst, abdominal ascites, gelatinous material in the peritoneal cavity and organs (Fig. 4C.1–2) and skull asymmetry (Fig. 4C.4). In addition, large umbilicus (Fig. 4B.3) were observed at collection in two of the female IVP-LOS fetuses (fetus number 656 and 604B), however, the ratio of the umbilicus to the crown-rump length (as determined by image analysis) was similar between groups, although the largest female (604B) had at least a 40% wider base of the umbilicus when compared to all other fetuses (Fig. 4B.3). There was

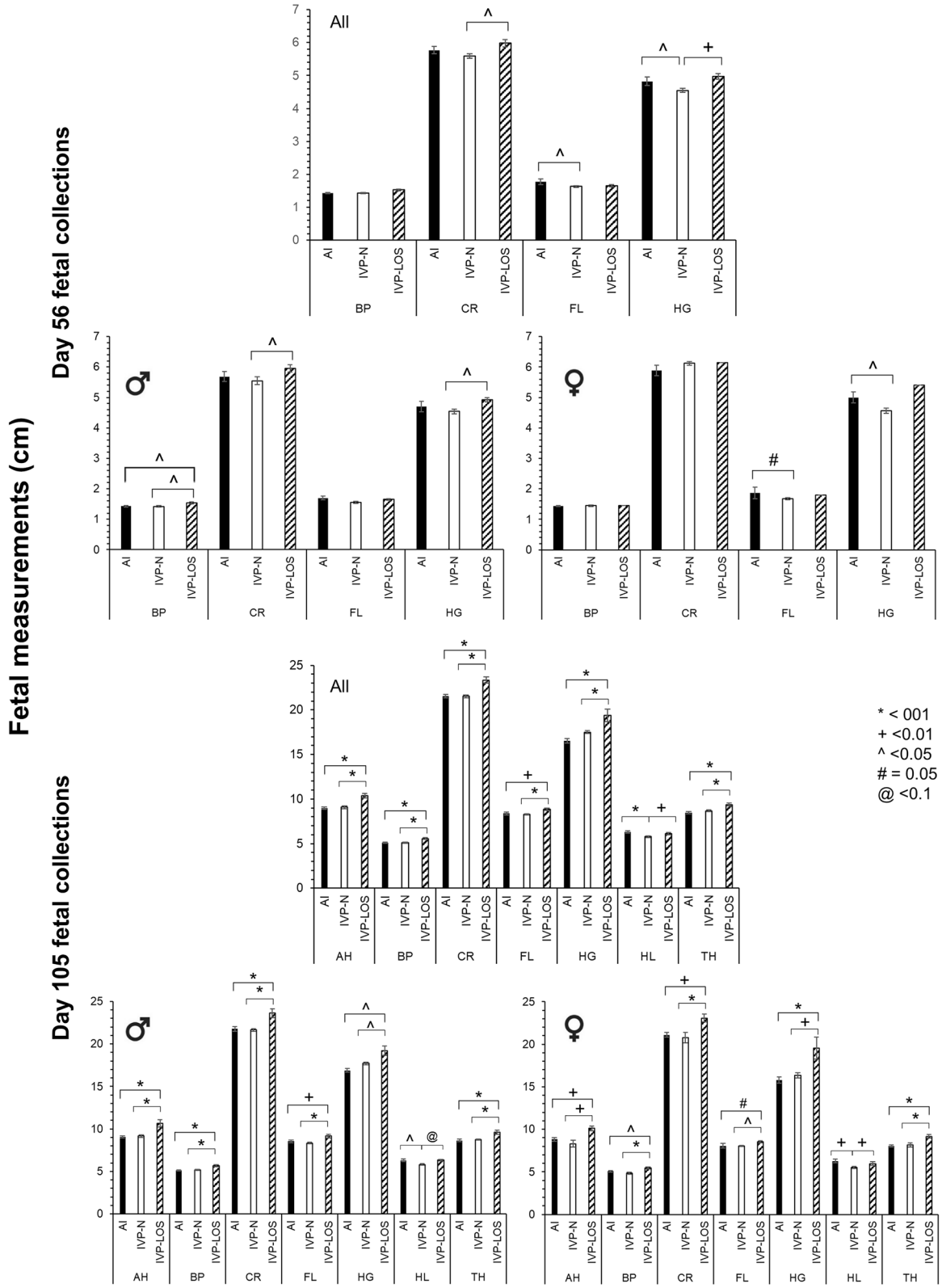

Figure 3. Fetal measurements at collection. Top three panels—D56 fetal measurements at collection (n = 6 females and 8 males in the AI group and 22 females [1 LOS] and 21 males [8 LOS] in the IVP group). Bottom three panels—D105 fetal measurements at collection (n = 4 females and 8 males in the AI group and 13 [9 LOS] females and 33 males [8 LOS] in the IVP group). For each day set, the top graph includes both sexes and the bottom two graphs are separated by sex, males on the left and females on the right. *BP* biparietal diameter, *CR* crown-rump length, *FL* forelimb length, *HG* hearth girth, *AH* abdominal height, *HL* head length, *TH* thoracic height. Data are represented as average ± SEM. Lines going over three bars are used to represent statistical differences between the first and the third bar.

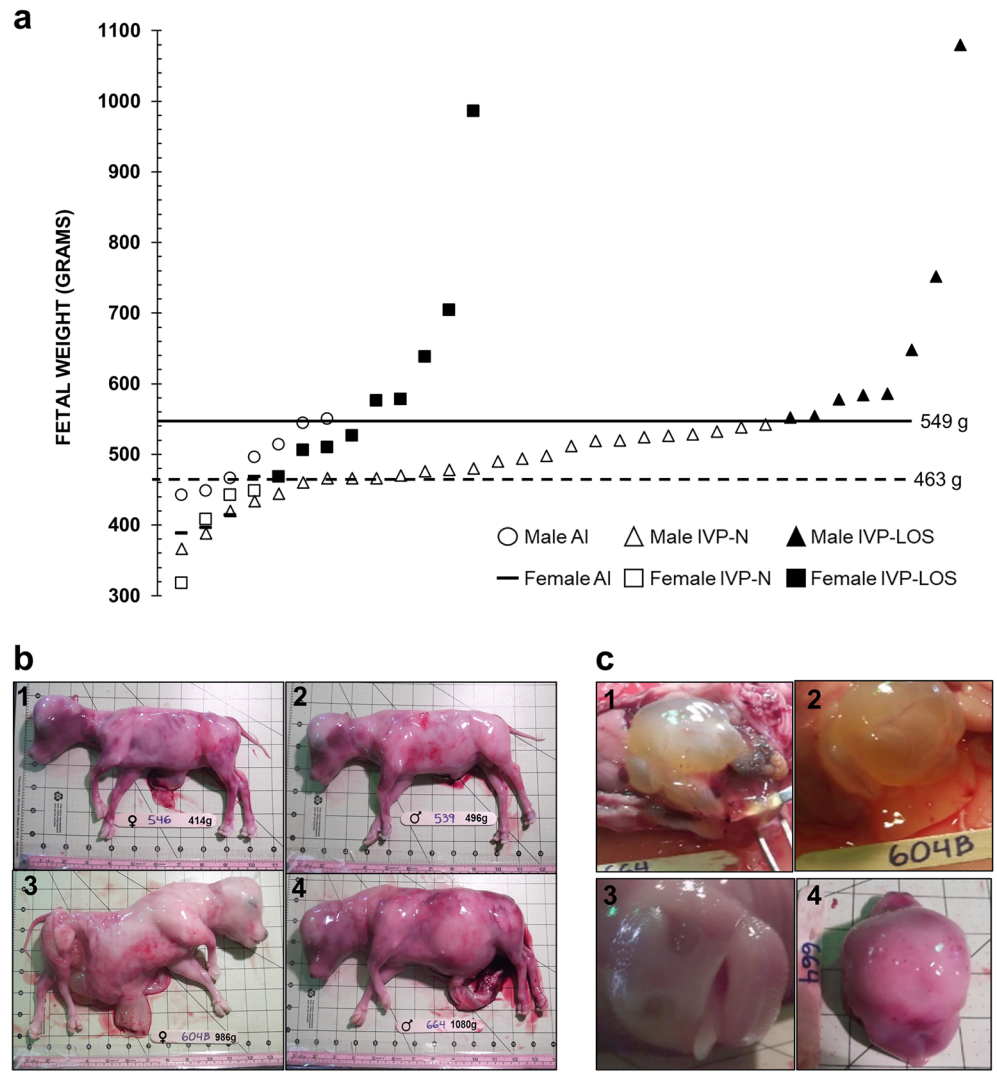

Figure 4. D105 fetal collections. **(a)** Fetal weight at D105 of gestation. The X axis has no actual implication and is used to scatter the spots representing each fetus for ease of visualization. The sex of the fetuses and the way they were generated is shown at the bottom of the figure. The lines represent the 97 percentile of AI D105 fetal weight (i.e., male—549 g, female—463 g). **(b)** The pictures show D105 fetuses in the control **(b1,b2)** and IVP-LOS group **(b3,b4)**. Also, females **(b1,b3)** and males **(b2,b4). (b1)** AI-546 (control female weighing 414 g which is approximate average weight of female control fetuses). **(b2)** AI-539 (control male weighing 496 g which is the approximate average weight of male control fetuses). **(b3,b4)** Show the heaviest LOS (IVP-LOS 604B: female **(b3)** weighing 986 g and IVP-LOS 664: male **(b4)** weighing 1080 g). Each square on the background = 2.54 cm. **(c)** Secondary phenotypes observed in D105 LOS fetuses—gelatinous material in the peritoneal cavity and organs **(c1,c2)** long protruding tongue **(c3)**, skull asymmetry **(c4)**. *AI* artificial insemination (i.e., control), *IVP* in vitro produced embryos.

a group (i.e. AI, IVP-Normal, IVP-LOS) effect for crown-rump length, abdominal height, biparietal diameter, forelimb length, heart girth, thoracic height and head length ($p < 0.002$) and sex effects for crown-rump length, abdominal height, biparietal diameter, forelimb length, and thoracic height ($p < 0.05$). The fetal measurements at collections are summarized in Fig. 3.

Associations between D55 fetal ultrasonographies and collection measurements. *D55 ultrasonographic measurements on fetuses collected on D56.* The summary of D55 ultrasonographic measurements for fetuses collected on D56 may be found in Fig. 5. Female specific analysis for the IVP-LOS group was not performed as only one female in the IVP group weighed more than the ≥ 97 percentile threshold used to ascribe overgrowth. No differences were observed for sex, group, or their interaction for abdominal diameter, abdominal height, biparietal diameter, thoracic diameter, or crown-rump length. Head length was greater in males than females ($p < 0.006$; Mean ± SEM; 2.05 ± 0.02 and 1.93 ± 0.03 for males and females, respectively). A group by sex interaction was detected for thoracic height in which the males of the IVP-Normal group were smaller

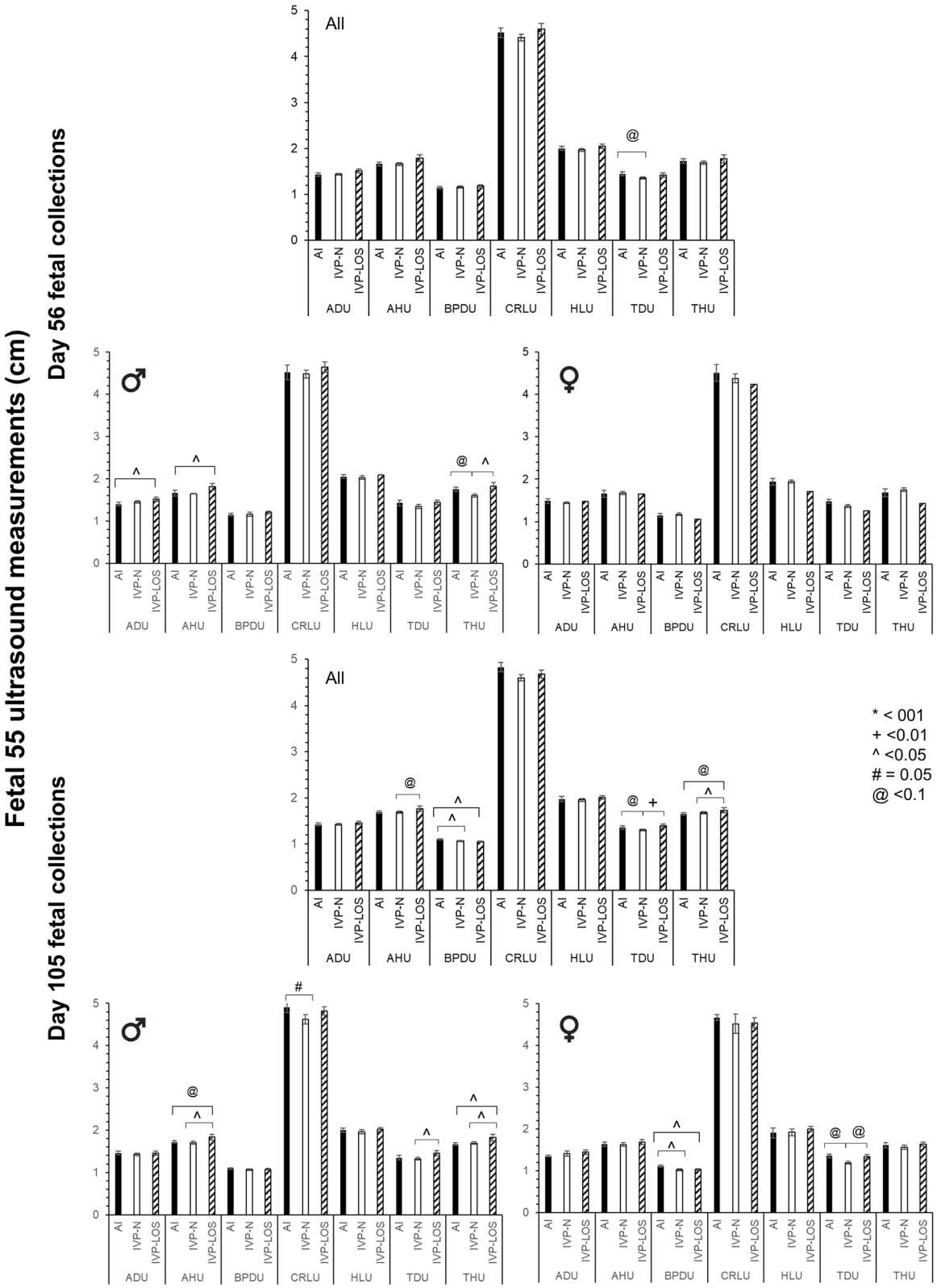

Figure 5. D55 fetal ultrasound measurements of fetuses collected on D55 and D105. Top three panels—D55 ultrasonographic measurements of fetuses collected on D56. n = 6 females and 8 males in the AI group and 22 females [1 LOS] and 21 males [8 LOS] in the IVP group. Bottom three panels—D55 ultrasonographic measurements of fetuses collected on D105. For each day set, the top graph includes both sexes and the bottom two graphs are separated by sex, males on the left and females on the right. n = 4 females and 8 males in the AI group and 13 [9 LOS] females and 33 males [8 LOS] in the IVP group. *U* ultrasound, *AH* abdominal height, *AD* abdominal diameter, *BPD* biparietal diameter, *CRL* crown rump length, *HL* head length, *TD* thoracic diameter, *TH* thoracic height. Data are represented as average ± SEM. For D56, there was only one female considered LOS, hence the lack of error bars. Lines going over three bars are used to represent statistical differences between the first and the third bar.

than males in the AI and IVP-LOS groups ($p < 0.03$; Mean ± SEM; 1.60 ± 0.04, 1.75 ± 0.05 and 1.83 ± 0.08 for IVP-Normal, AI and IVP-LOS groups, respectively).

A slight positive correlation was observed between the D55 ultrasonographic measurements and the D56 fetal weight for abdominal diameter (0.40; $p < 0.003$), abdominal height (0.36; $p < 0.007$), crown-rump length (0.34; $p < 0.01$), head length (0.32; $p < 0.02$), and thoracic height (0.25; $p = 0.06$), while no correlations were observed between biparietal diameter or thoracic diameter measurements. When males and females were analyzed separately, positive correlations were observed for abdominal diameter (0.57; $p < 0.002$), abdominal height (0.64; $p < 0.0003$), crown-rump length (0.36; $p = 0.06$), and thoracic height (0.54; $p < 0.004$) in males. However, no correlations were found between any of the D55 ultrasonographic measurements and D56 fetal weight in females.

D56 ultrasonographic measurements on fetuses collected on D105. The summary of D55 ultrasonographic measurements for fetuses collected on D105 may be found in Fig. 5. No differences were observed for sex, group, or their interaction for abdominal diameter and head length. Females were smaller than males in their crown-rump length ($p = 0.08$), abdominal height ($p < 0.04$), thoracic diameter ($p = 0.07$), and thoracic height ($p < 0.008$).

A moderate positive correlation was observed between the D55 ultrasonographic measurements and the D105 fetal weight for abdominal diameter (0.57; $p < 0.0001$) and abdominal height (0.58; $p < 0.0001$). A slight positive correlation was observed between fetal weight and crown-rump length (0.27; $p < 0.04$), head length (0.33; $p < 0.02$), thoracic diameter (0.34; $p < 0.02$), and thoracic height (0.49; $p < 0.0002$) while no correlation was observed for biparietal diameter. For males, there was a moderate positive correlation between fetal weight and abdominal diameter (0.52; $p < 0.0009$), abdominal height (0.57; $p < 0.0002$), and thoracic height (0.56; $p < 0.0003$) and slight positive correlation for thoracic diameter (0.36; $p < 0.03$). For females, there was a moderate positive correlation between fetal weight and abdominal diameter (0.67; $p < 0.005$), abdominal height (0.67; $p < 0.005$), and head length (0.59; $p < 0.02$).

D77 ultrasonographic measurements on fetuses collected D105. An attempt was made to determine fetal morphometry on the subset of D77 pregnant group (AI = 6; IVP = 12), however this was not possible or reliable for many of the samples as the fetus was too large to do accurate measurements (data not shown).

Are D55 ultrasonographic measurements useful to identify LOS? Overall, no single ultrasonographic measurement can explain LOS on D105 of gestation. We tested various combinations of measurements and identified a strong positive correlation between D105 fetal weight and the product of the D55 ultrasonographic measurements for abdominal diameter, abdominal height, crown-rump length, head length, thoracic height, and thoracic diameter (0.76; $p < 0.007$ and 0.72; $p < 0.0001$ for AI and IVP fetuses, respectively; Fig. 6). The highest number resulting from the multiplication of the beforementioned ultrasonographic measurements was 79.92 for the AI (control) group. The D105 IVP fetuses were compared to that threshold and all except the two most extreme LOS cases (fetus 604B and 664 weighing 986 and 1080 g, respectively) were on or below the threshold. Similar comparisons were made for the set of fetuses collected on D56. While the correlation was also strong (0.75; $p < 0.003$), the threshold for the AI was higher (91.32) than for that obtained for the D105 AI fetuses. The correlation decreased to 0.46 ($p < 0.03$) for the IVP group indicating more variability in fetal weight at this stage and perhaps inclusion of fetuses that will be lost later during pregnancy or will have a differential rate of growth after this stage. In this group, only the heaviest IVP fetus was above the 100-threshold used in the D105 group.

Maternal blood transcriptome analysis. Only reads which aligned to known genes of the bovine reference genome assembly ARS-UCD1.2 using NCBI (GCF_002263795.1_ARS-UCD1.2) were used in the present study. The results of all transcriptome analyses generated in this study may be found in Supplementary Table 1.

Unsupervised hierarchical clustering of the normalized read counts showed 18 of 46 samples (ie. D55 and D105 WBC transcriptomes of the same 23 females) clustered by animal (Supplementary Fig. 3). In other words, the D55 and D105 samples from 9 of the heifers grouped together by individual and this was irrespective of treatment group (AI, IVP-Normal or IVP-LOS). In addition, the analysis separated the females carrying the two largest D105 IVP-LOS fetuses (dam #604 and #664) from the rest of the animals (Supplementary Fig. 3). The day-specific unsupervised hierarchical clustering analyses may be found in Supplementary Fig. 4. Figure 7 shows the results for differentially expressed genes identified during the pairwise comparisons between each treatment group at each time point (D55 and D105). In addition, a pairwise comparison of WBC transcriptomes of females carrying two fetuses vs one was done to account for differential expression due to multiple fetuses (i.e. increased fetal mass). Furthermore, we compared the transcriptome of the dams carrying the two largest IVP-LOS individuals (#604 and #664) against all other animals for D55 and D105. Overall, for the D55 comparison, there were 13 differentially expressed genes identified by EdgeR and 8 identified by DESeq2 and for D105 there were 31 differentially expressed genes identified by EdgeR and 4451 by DESeq2. Data show that a large number of genes identified as differentially expressed are uncharacterized transcripts ("LOC"). Maternal WBC transcriptome analyses found that *LOC783838* and *PCDH1* were identified as differentially expressed in the extreme cases of LOS on gestation D55 and D105 by both EdgeR and DESeq2 statistical packages (Fig. 7). In addition, transcript levels of *ACTA2*, *KDM5A*, *MAN1A2*, *MIR2376*, *PRRC2C*, *RSBN1*, *S100A14*, *SRPK2*, and *TTF1* were identified as differentially expressed in the females carrying the two largest fetuses on D105. For qRT-PCR corroborations, we focused on two genes upregulated in the two largest LOS fetuses when compared to all other fetuses, and whose intron-spanning TaqMan probes were readily available, namely *TTF1* and *RSRC1*. *TTF1* was identified as differentially expressed by both EdgeR and DESeq2 statistical packages and *RSRC1* was identified as differentially expressed only by DESeq2. *RSRC1* was used for corroborations even though it was

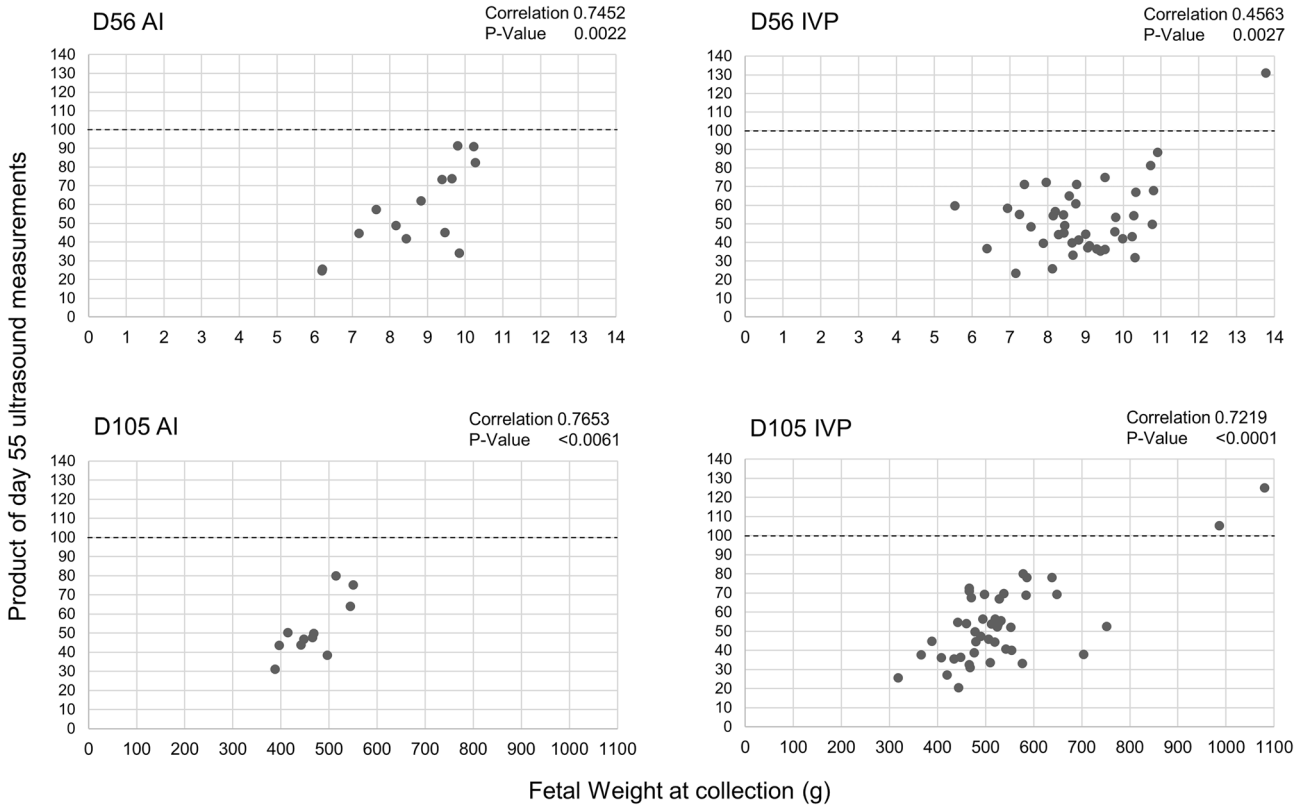

Figure 6. Correlation between fetal weight at collection and the product of D55 ultrasonographic measurements. Ultrasonographic measurements included = abdominal diameter, abdominal height, crown-rump length, head length, thoracic height, and thoracic diameter. *AI* artificial insemination (i.e., control), *IVP* in vitro produced embryos. The dashed line on the 100 mark is used to separate the two largest LOS fetuses on day 105 (bottom right panel) and drawn on the other three panels for comparisons.

only identified by one package due to the limitation of available Taqman probes of other genes in Fig. 7. Data show that the pattern of expression for these genes is similar between RNAseq and qRT-PCR results (Fig. 8).

Discussion

The goal of the present study was to determine the usefulness of D55 of gestation fetal morphometry and D55 and D105 maternal leukocyte transcriptome for the identification of congenital fetal overgrowth in cattle. In total, 20.9% (9/43) of the D56 and 36.9% (17/46) of the D105 collected IVP fetuses were considered LOS (≥ 97 percentile of the sex-specific weight of controls). The study revealed that the product of the D55 ultrasonographic measurements for abdominal diameter, abdominal height, crown-rump length, head length, thoracic height, and thoracic diameter may be used as an indicator of extreme cases of LOS. In addition, we identified that LOS fetuses had a different growth pattern from AI and IVP-Normal fetuses after D55 of gestation and that LOS fetuses had several developmental abnormalities such as hemihyperplasia (asymmetric growth), enlarged tongue, brain hemorrhage, enlarged umbilical cord, abdominal ascites, and abdominal wall defect. Maternal leukocyte transcriptome analyses identified that *ADGRE3*, *LOC107131273*, *LOC783838*, *PCDH1*, and *RAB34*, were differentially expressed on D55 in the females carrying the two largest LOS fetuses. On D105 of pregnancy, the leukocyte transcriptomes of the same females had differential expression of *ACTA2*, *KDM5A*, *LOC783838*, *MAN1A2*, *MIR2376*, *PCDH1*, *PRRC2C*, *RSBN1*, *S100A14*, *SRPK2*, and *TTF1* when compared to all other animals.

Collection of fetuses was done on D56 of gestation since organogenesis has been shown to be completed before this day in cattle[54]. The reason behind this decision was to answer other aspects of the project, which are beyond the scope of the current study, such as questions regarding the epigenetic mechanisms associated with abnormal organ formation in LOS; information that will be used to identify etiologies of fetal overgrowth syndrome in cattle and human (i.e. BWS). Furthermore, fetuses were also collected on D105 as we have previously shown that fetal overgrowth is evident at this stage of gestation[2]. In humans, 97 percentile criteria is used to describe macrosomia in newborn babies[53]. Since macrosomia is the main characteristic of LOS and one characteristic that can result in dystocia, we used this weight to ascribe LOS in the present study, similar to what we have done previously.

A greater than two-times increase in fetal weight was observed in two of the D105 IVP-LOS fetuses in the current study, an observation also reported by others[4], suggesting that if those fetuses were allowed to go to term, dystocia would be probable and assisted delivery or caesarean section would be required. Dystocia as a result of LOS can lead to neonatal death[4] and/or death of cows[22]. Further, dystocia associated with stillbirth[55], is a major contributor to perinatal mortality in cattle[56], and has also been shown to increase the chances of metritis[57], and

	AII, 1 Fetus vs. AII, 2 Fetuses	IVP-N, 1 Fetus vs. IVP-N, 2 Fetuses	AI vs. IVP-N	AI vs. IVP-LOS	IVP-N vs. IVP-LOS	AI & IVP-N vs. IVP-LOS	604 & 664 vs. AII IVP	604 & 664 vs. AII
D55	no genes-both	CACNA1B	LOC104969863	LOC100849069	BOLA.DQB	LOC100849069	ADGRE3	ADGRE3
		ALAS2	LOC107132678		CELA1		HAP1	LOC107131273
		FBN1			RBM44		LOC783838	LOC783838
		LOC112445594					PCDH1	PCDH1
		LOC112446750					RAB34	RAB34
		LOC112448774						
		SH3GL3						
D105	ABCB4	CACNA1B	no genes-both	LOC100849069	BOLA.DQB	HAL	LOC112442223	ACTA2
	COTL1	DMBT1		LOC107131224	CXCL8	LOC100849069	MAN1A2	KDM5A
	GABARAPL2	FBN1			EFHB	LOC101906317		LOC783838
	GALR1	HIST1H1C			LOC100335553	LOC104969458		MAN1A2
	GJA10	LOC101903853			LOC101905499	LOC104973781		MIR2376
	IL4I1	LOC101904916			LOC104974259	LOC104974259		PCDH1
	LOC281376	LOC104973604			LOC107132224	LOC104975299		PRRC2C
	LOC782527	LOC107131271			LOC107132917	LOC107132783		RSBN1
	NRIP3	LOC112443184			LOC112442735	LOC107132917		S100A14
	PLPPR5	LOC112447617			LOC112447323	LOC112443717		SRPK2
	S100A1				PCDH8	LOC112445870		TTF1
	SH3GL3				RBM44	LOC112448536		
	TMEM232				SLC20A2	LOC617224		
	X44257.1				SLC22A14	SLC22A14		

Figure 7. Differentially expressed genes in D55 and D105 maternal blood leukocyte transcriptomes. Blood was collected from the same females on D55 and D105 of gestation. The selection of the dams for this study was based on the group, weight, sex, and number of fetuses at D105 collection. Shown are the genes identified by both EdgeR and DESeq2 as statistically different ($p < 0.05$) in the named comparisons. *AI* artificial insemination (i.e., control), *IVP-N* embryos were produced by in vitro procedures that were < 97% of the control's weight at D105. *IVP-LOS* embryos were produced by in vitro procedures and were ≥ 97% of the control's weight at D105. "604 & 664" are the numbers of the heifers carrying the two largest LOS fetuses. Gene names starting with "LOC" are uncharacterized transcripts. The "No gene-both" designation notes that genes were identified as differentially expressed by either EdgeR or DESeq2 but not both.

lameness[28]. Furthermore, dystocia can have an adverse impact on milk production[58] and increase the calving to conception interval in dairy cows[59]. Thus, overgrown fetuses can have negative economic consequences to cattle producers[22] and identification of those large calves during early pregnancy would help overcome these problems.

In the current study, we performed fetal ultrasonographic measurements at D55 for fetuses that were collected on D56 or D105 of gestation. Based on our findings, it is evident that the ≥ 97 percentile AI weight criteria used to assign a fetus as being LOS while useful at D105, it is not appropriate at gestation D56. From the findings of the correlation analysis between fetal weight and the product of six ultrasound measurements, we hypothesize that fetuses are lost after this day of pregnancy and/or will have subsequent disproportionate growth. This is in accordance to previous work which showed that smaller in vitro or cloned fetuses in the first trimester resulted in heavier fetuses at term[7,60]. Further, Bertolini and coinvestigators also suggested that in vitro produced fetuses show early growth retardation and then follow acceleration in fetal growth at later stages of gestation, showing biphasic growth pattern[7].

Previous research reported that larger biparietal diameter might be a useful measurement to identify LOS at D63 of gestation in cloned LOS fetuses[61] and that a smaller crown rump length might be a useful measurement to identify LOS at D58 of pregnancy in in vitro produced LOS fetuses[7]. However, those measurements were not found to be indicators for LOS in our current study. Possibilities for the discrepancies in our findings, are; (1) different definition of LOS (we used ≥ 97% weight of the control fetuses while Bertolini used fetuses that were 33% heavier than controls for comparisons[7], (2) improvement in ultrasonography technology allowing for more accurate measurements, and (3) number of observations (113 fetuses in our study vs. 34 fetuses in Ref.[60]).

When we tried to do fetometry at D77 of gestation, we were only able to measure head length with some accuracy. All other measurements were not reliable, indicating that D77 ultrasonography might be too late to try to accurately identify LOS. Given the allometric growth that occurs after D56 and the fact that D77 is too late to predict LOS by ultrasonography, and that fetal sex is most accurately predicted between D60–80[34], we suggest that future studies focus on ~ D65 as a target day to identify LOS in a sex-specific manner.

Larger than normal umbilicus and presence of large amounts of fluid-gelatinous material in the abdominal cavity were observed in largest/heaviest D105 IVP-LOS fetuses. This is similar to what Constant et al.[60] reported in D220 fetuses produced by somatic cell nuclear transfer. In that study, the authors suggested that a large umbilical cord and abdominal ascites were not the result of fetal overgrowth per se, but rather a consequence of placental dysfunction, which in turn led to placental overgrowth. Contrary to this, other studies have shown

				Cow ID	647	533	546	517	648	609	590	606	519	699	560	633	688	628	637	616	663	512	656	602	557	664	604
				Group	AI	AI	AI	AI	AI	AI	AI	AI	IVP N	IVP N	IVP N	IVP N	IVP N	IVP N	IVP LOS	IVP LOS	IVP LOS	IVP LOS	IVP LOS	IVP LOS	IVP LOS	IVP LOS	IVP LOS
				Sex	F	F	F	M	M	F	M	M	F	F	M	M	F, M	M, M	M	F	F	M	F	M	F, M	M	M, F
				# Fetuses	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	2	1	2
				Fetal Wt	396	388	414	442	466	468	544	550	408	442	480	538	444 448	434 532	586	638	578	648	704	752	506* 556*	1080	584 986*
		DESeq2 padj	EdgeR FDR	CV	Normalized Read Counts								Normalized Read Counts						Normalized Read Counts								
Normalizers	ECD	0.83	1	0.08	24	24	24	26	23	23	22	22	25	23	25	20	24	17	21	25	25	24	22	24	24	23	22
	NFKBIB	0.98	1	0.10	33	39	31	35	39	31	33	33	34	34	35	39	37	37	31	26	27	31	31	36	37	33	31
	VPS35L	0.95	1	0.09	53	47	63	59	57	56	49	53	59	53	56	43	56	47	56	57	57	59	59	52	58	52	53
Test genes	RSRC1	3.2E-15	0.370	0.45	14	15	13	19	13	12	11	13	21	15	16	16	13	16	10	16	13	14	17	15	22	44	35
	TTF1	3.0E-23	0.027	0.44	68	72	61	95	60	73	70	65	62	71	72	73	57	82	57	63	59	78	66	80	97	206	168

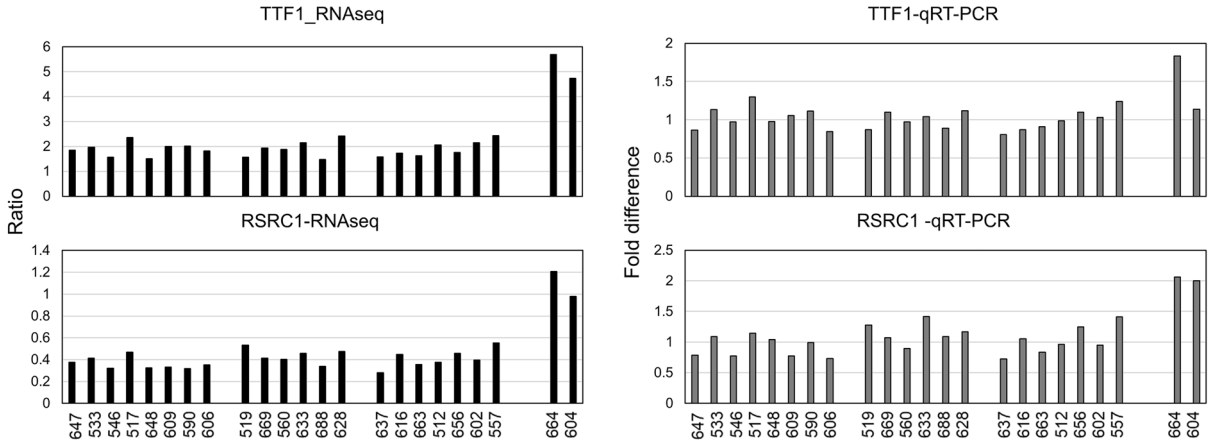

Figure 8. qRT-PCR corroborations of genes identified as differentially expressed in dams carrying the two largest LOS fetuses on D105 gestation. *Top* Table showing all information pertaining the samples for the genes chosen from the transcriptome for qRT-PCR corroborations. Three transcripts, namely *ECD*, *NFKBIB* and *VPS35L* were chosen as normalizers as their coefficient of variation (CV) across all 23 samples was ≤ .10. Test genes chosen were *TTF1* (identified as differentially expressed by DESeq2 statistical software) and *RSRC1* (identified as differentially expressed by DESeq2 and EdgeR statistical software packages). The table is organized by total fetal mass in increasing order. # of fetuses indicates how many fetuses the dam was carrying at the time of collection. *F* female fetus, *M* male fetus, *AI* artificial insemination (i.e. control). *IVP-N* embryos were produced by in vitro procedures that were < 97% of the control's weight at D105. Asterisk—denotes the LOS of the pair. *Bottom* Bar graphs showing RNAseq results represented as sample ratio of the named gene from the mean of the three normalizers and qRT-PCR results represented as fold difference. For qRT-PCR, the geometric mean of three endogenous transcripts, namely *ECD*, *NFKBIB* and *VPS35L* were used to normalize the levels of *TTF1* and *RSRC1*. Data are represented as fold difference from the average of 21 samples (average fold difference = 1; not including 604 and 664). Transcripts of *TTF1* and RSRC1 for 604 and 664 were compared to the normalized average of 21 samples for each test gene ($\Delta\Delta C_T$).

an association of placental defects in cloned fetuses, with their loss during early pregnancy as a result of growth retardation[62,63]. Taken together, these studies suggest that fetuses with severe placental defects may be lost during early pregnancy and if those fetuses with placental defects survive, they could have higher placental and fetal growth at later stages of pregnancy through compensatory mechanisms. In the current study, the conceptuses were surgically removed to allow rapid collection of tissues in order to preserve nucleic acid integrity for other aspects of the project, therefore, even though we collected the placentas, we were not able to make thorough morphological assessments of this tissue. Regardless, no obvious placental abnormalities were evident at collection for IVP conceptuses.

Enlarged tongue was also observed in D105 IVP-LOS which is similar to previous findings in our laboratory[2,64] and comparable to what has been observed in a similar congenital overgrowth condition in humans, namely Beckwith–Wiedemann Syndrome[65]. Large tongues can lead to difficulty in suckling and increase the chances of prenatal death[26]. In addition, the largest D105 IVP-LOS fetus showed brachycephaly and asymmetrical growth of the cranium, an interesting finding given that one characteristic of BWS is hemihyperplasia[65]. These similarities demonstrates that BWS and LOS are the same syndrome, as previously reported by us[2], and that they share similar misregulated developmental epi(genetic) mechanisms associated with asymmetrical growth.

In our study, we also had the objective of determining if maternal blood could be used as a biomarker to identify LOS on D55 and/or D105 of pregnancy. For this, we analyzed leukocyte transcriptome of 23 females carrying D105 AI, IVP-Normal and IVP-LOS fetuses. We also analyzed the D55 leukocyte transcriptomes of the same females. Our initial approach was to do an unsupervised hierarchical clustering of de-identified samples to determine if obvious difference existed between the females carrying LOS fetuses when compared to the other two groups. Surprisingly, the transcriptome of 18/46 samples clustered together by animal (i.e., n = 9) regardless of pregnancy stage (i.e., D55 and D105). Another interesting point is that the experiment was done

over three seasons (Autumn, Spring and Summer) with a range in temperature of − 22 °C to 33 °C, and this was not detected in the transcriptome given the clustering by individual. Further, given the design of the study, in which we transferred two embryos per recipient heifer, some pregnancies in the D55 and D105 IVP groups had two fetuses, however, the unsupervised hierarchical clustering did not cluster animals by number of fetuses. The unsupervised hierarchical clustering, did however, cluster the D105 transcriptomes of the females carrying the two largest LOS from all other females.

For the maternal leukocyte transcriptome analysis, we focused on the two females that carried the largest LOS fetuses as those were the ones that separated by hierarchical clustering and the ones that would most likely cause a difficult birth. Analyses identified *ADGRE3*, *LOC107131273*, *LOC783838*, *PCDH1*, and *RAB34*, as being different on D55 and *ACTA2*, *KDM5A*, *LOC783838*, *MAN1A2*, *MIR2376*, *PCDH1*, *PRRC2C*, *RSBN1*, *S100A14*, *SRPK2*, and *TTF1* on D105 when compared to all other animals. For qRT-PCR corroborations we focused on two transcripts whose TaqMan probes were readily available, namely *TTF1* which was identified as differentially expressed on D105 by EdgeR and DESeq2 statistical packages as well as *RSRC1* which was identified as differentially expressed by DESeq2 only. Analyses show consistency of expression between RNAseq and qRT-PCR results. Other genes will be tested in the future as assays become available.

Our study has several limitations. As it pertains to fetal ultrasonographies, published work shows that fetal growth varies among different breeds[66] and that size difference can be noticeable as early as 3 months of gestation, therefore it is possible that fetal growth patterns may vary as early as D55 of gestation among different breeds; however, there is lack of published data showing that on D55 of gestation, fetuses from different breeds differ in size. Future research would have to address this question. Here, we also did sex-specific analyses for all our variables, including for ultrasonographic measurements. Since in our experiment, the number of male fetuses was unexpectedly higher in the IVP group at D105, female specific ultrasonographic data analysis may be limiting. Finally, and importantly, it should be noted that during transcriptome analysis, we only used the known (mostly coding) group of bovine transcripts for this study and that the non-coding and novel transcript portion of the transcriptome remains unexplored. Future work will focus on these types of transcripts.

In summary, here we document initial efforts to identify LOS during the first trimester of pregnancy in cattle. We found that the product of the D55 ultrasonographic measurements for abdominal diameter, abdominal height, crown-rump length, head length, thoracic height, and thoracic diameter may be useful to identify the largest fetuses, whereas maternal leukocyte transcriptome analyses suggest *LOC783838* and *PCDH1* as potential markers for extreme cases of LOS on gestation D55 and D105. In addition, transcript levels of *ACTA2*, *KDM5A*, *MAN1A2*, *MIR2376*, *PRRC2C*, *RSBN1*, *S100A14*, *SRPK2*, and *TTF1* may also serve as biomarkers on D105 of pregnancy for extreme cases. Further, our analysis identified several genes that were misregulated in all LOS fetuses when compared to fetuses of normal weight produced by IVP. Future work will query the usefulness of these genes to predict milder cases of LOS. Finally, the long-term goal of this research is to identify the best time of pregnancy to capture LOS by ultrasonography and train a model using fetuses that will be allowed to go to term to determine the best maternal blood markers to identify fetal overgrowth in cattle.

Data availability

The raw FASTQ files are publicly available at Gene Expression Omnibus (GEO accession no. GSE179946).

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References

1. Walker, S. K., Hartwich, K. M. & Seamark, R. F. The production of unusually large offspring following embryo manipulation: Concepts and challenges. *Theriogenology* **45**, 111–120 (1996).
2. Chen, Z., Robbins, K. M., Wells, K. D. & Rivera, R. M. Large offspring syndrome: A bovine model for the human loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann. *Epigenetics* **8**, 591–601. https://doi.org/10.4161/epi.24655 (2013).
3. Willadsen, S. M. *et al.* The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. *Theriogenology* **35**, 161–170 (1991).
4. Behboodi, E. *et al.* Birth of large calves that developed from in vitro-derived bovine embryos. *Theriogenology* **44**, 227–232. https://doi.org/10.1016/0093-691x(95)00172-5 (1995).
5. Young, L. E., Sinclair, K. D. & Wilmut, I. Large offspring syndrome in cattle and sheep. *Rev. Reprod.* **3**, 155–163. https://doi.org/10.1530/ror.0.0030155 (1998).
6. Bertolini, M. & Anderson, G. B. The placenta as a contributor to production of large calves. *Theriogenology* **57**, 181–187. https://doi.org/10.1016/s0093-691x(01)00665-3 (2002).
7. Bertolini, M. *et al.* Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. *Theriogenology* **58**, 973–994. https://doi.org/10.1016/s0093-691x(02)00935-4 (2002).
8. Hiendleder, S. *et al.* Tissue-specific elevated genomic cytosine methylation levels are associated with an overgrowth phenotype of bovine fetuses derived by in vitro techniques. *Biol. Reprod.* **71**, 217–223. https://doi.org/10.1095/biolreprod.103.026062 (2004).
9. Hiendleder, S. *et al.* Tissue-specific effects of in vitro fertilization procedures on genomic cytosine methylation levels in overgrown and normal sized bovine fetuses. *Biol. Reprod.* **75**, 17–23. https://doi.org/10.1095/biolreprod.105.043919 (2006).
10. Miles, J. R., Farin, C. E., Rodriguez, K. F., Alexander, J. E. & Farin, P. W. Angiogenesis and morphometry of bovine placentas in late gestation from embryos produced in vivo or in vitro. *Biol. Reprod.* **71**, 1919–1926. https://doi.org/10.1095/biolreprod.104.031427 (2004).
11. Farin, P. W., Piedrahita, J. A. & Farin, C. E. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology* **65**, 178–191. https://doi.org/10.1016/j.theriogenology.2005.09.022 (2006).
12. McEvoy, T. G., Sinclair, K. D., Broadbent, P. J., Goodhand, K. L. & Robinson, J. J. Post-natal growth and development of simmental calves derived from in vivo or in vitro embryos. *Reprod. Fertil. Dev.* **10**, 459–464. https://doi.org/10.1071/rd98126 (1998).
13. Hiendleder, S. *et al.* Maternal-fetal transplacental leakage of mitochondrial DNA in bovine nuclear transfer pregnancies: Potential implications for offspring and recipients. *Cloning Stem Cells* **6**, 150–156. https://doi.org/10.1089/1536230041372391 (2004).
14. Chen, Z. *et al.* Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 4618–4623. https://doi.org/10.1073/pnas.1422088112 (2015).

15. van Wagtendonk-de Leeuw, A. M., Aerts, B. J. & den Daas, J. H. Abnormal offspring following in vitro production of bovine preimplantation embryos: A field study. *Theriogenology* **49**, 883–894. https://doi.org/10.1016/s0093-691x(98)00038-7 (1998).
16. Thompson, J. G., Gardner, D. K., Pugh, P. A., McMillan, W. H. & Tervit, H. R. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biol. Reprod.* **53**, 1385–1391. https://doi.org/10.1095/biolreprod53.6.1385 (1995).
17. Sinclair, K. D. *et al.* Aberrant fetal growth and development after in vitro culture of sheep zygotes. *J. Reprod. Fertil.* **116**, 177–186. https://doi.org/10.1530/jrf.0.1160177 (1999).
18. Rooke, J. A. *et al.* Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. *Theriogenology* **67**, 639–647. https://doi.org/10.1016/j.theriogenology.2006.09.040 (2007).
19. Bonilla, L., Block, J., Denicol, A. C. & Hansen, P. J. Consequences of transfer of an in vitro-produced embryo for the dam and resultant calf. *J. Dairy Sci.* **97**, 229–239. https://doi.org/10.3168/jds.2013-6943 (2014).
20. Siqueira, L. G. *et al.* Colony-stimulating factor 2 acts from days 5 to 7 of development to modify programming of the bovine conceptus at day 86 of gestationdagger. *Biol. Reprod.* **96**, 743–757. https://doi.org/10.1093/biolre/iox018 (2017).
21. Li, Y., Donnelly, C. G. & Rivera, R. M. Overgrowth syndrome. *Vet. Clin. N. Am. Food Anim. Pract.* **35**, 265–276. https://doi.org/10.1016/j.cvfa.2019.02.007 (2019).
22. Rivera, R. M., Donnelly, C. G., Patel, B. N., Li, Y. & Soto-Moreno, E. J. Abnormal offspring syndrome. In *Bovine Reproduction* (ed. Hopper, R. M.) 876–895 (Wiley, 2021).
23. Li, Y., Sena Lopes, J., Fuster, P. C. & Rivera, R. M. Spontaneous and ART-induced large offspring syndrome: Similarities and differences in DNA methylome. *Epigenetics*. https://doi.org/10.1080/15592294.2022.2067938 (2022).
24. Mussa, A. *et al.* Assisted reproductive techniques and risk of Beckwith-Wiedemann syndrome. *Pediatrics*. https://doi.org/10.1542/peds.2016-4311 (2017).
25. Kruip, T. A. M. & den Daas, J. H. G. In vitro produced and cloned embryos: Effects on pregnancy, parturition and offspring. *Theriogenology* **47**, 43–52 (1997).
26. Hansen, P. J. & Block, J. Towards an embryocentric world: The current and potential uses of embryo technologies in dairy production. *Reprod Fertil. Dev.* **16**, 1–14. https://doi.org/10.10371/RD03073 (2004).
27. Dematawewa, C. M. & Berger, P. J. Effect of dystocia on yield, fertility, and cow losses and an economic evaluation of dystocia scores for Holsteins. *J. Dairy Sci.* **80**, 754–761. https://doi.org/10.3168/jds.s0022-0302(97)75995-2 (1997).
28. Linden, T. C., Bicalho, R. C. & Nydam, D. V. Calf birth weight and its association with calf and cow survivability, disease incidence, reproductive performance, and milk production. *J. Dairy Sci.* **92**, 2580–2588. https://doi.org/10.3168/jds.2008-1603 (2009).
29. Kasinathan, P. *et al.* Acceleration of genetic gain in cattle by reduction of generation interval. *Sci. Rep.* **5**, 8674. https://doi.org/10.1038/srep08674 (2015).
30. Fleming, A., Abdalla, E. A., Maltecca, C. & Baes, C. F. Invited review: Reproductive and genomic technologies to optimize breeding strategies for genetic progress in dairy cattle. *Arch. Anim. Breed.* **61**, 43–57. https://doi.org/10.5194/aab-61-43-2018 (2018).
31. Curran, S., Pierson, R. A. & Ginther, O. J. Ultrasonographic appearance of the bovine conceptus from days 20 through 60. *J. Am. Vet. Med. Assoc.* **189**, 1295–1302 (1986).
32. Kastelic, J. P., Curran, S., Pierson, R. A. & Ginther, O. J. Ultrasonic evaluation of the bovine conceptus. *Theriogenology* **29**, 39–54 (1988).
33. Kahn, W. Sonographic fetometry in the bovine. *Theriogenology* **31**, 1105–1121. https://doi.org/10.1016/0093-691x(89)90494-9 (1989).
34. Christmas, R. A. & Colloton, J. *Ultrasound Determination of Fetal Gender* 2nd edn, 303–307 (Saunders Elsevier, 2007).
35. Fissore, R. A., Edmondson, A. J., Pashen, R. L. & Bondurant, R. H. The use of ultrasonography for the study of the bovine reproductive tract. II. Non-pregnant, pregnant and pathological conditions of the uterus. *Anim. Reprod. Sci.* **12**, 167–177 (1986).
36. Yoshino, H. *et al.* A predictive threshold value for the diagnosis of early pregnancy in cows using interferon-stimulated genes in granulocytes. *Theriogenology* **107**, 188–193. https://doi.org/10.1016/j.theriogenology.2017.11.014 (2018).
37. Green, J. A. *et al.* The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. *Theriogenology* **63**, 1481–1503. https://doi.org/10.1016/j.theriogenology.2004.07.011 (2005).
38. Percie du Sert, N. *et al.* Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. *PLoS Biol.* **18**, e3000411. https://doi.org/10.1371/journal.pbio.3000411 (2020).
39. Tribulo, P., Rivera, R. M., Ortega Obando, M. S., Jannaman, E. A. & Hansen, P. J. Production and culture of the bovine embryo. *Methods Mol. Biol.* **2006**, 115. https://doi.org/10.1007/978-1-4939-9566-0_8 (2019).
40. Chen, Z., Hagen, D. E., Ji, T., Elsik, C. G. & Rivera, R. M. Global misregulation of genes largely uncoupled to DNA methylome epimutations characterizes a congenital overgrowth syndrome. *Sci. Rep.* **7**, 12667. https://doi.org/10.1038/s41598-017-13012-z (2017).
41. Silva del Rio, N., Stewart, S., Rapnicki, P., Chang, Y. M. & Fricke, P. M. An observational analysis of twin births, calf sex ratio, and calf mortality in Holstein dairy cattle. *J. Dairy Sci.* **90**, 1255–1264. https://doi.org/10.3168/jds.s0022-0302(07)71614-4 (2007).
42. Chen, Z. *et al.* Global assessment of imprinted gene expression in the bovine conceptus by next generation sequencing. *Epigenetics* **11**, 501–516. https://doi.org/10.1080/15592294.2016.1184805 (2016).
43. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675. https://doi.org/10.1038/nmeth.2089 (2012).
44. Ortega, M. S. *et al.* Influences of sire conception rate on pregnancy establishment in dairy cattle. *Biol. Reprod.* **99**, 1244–1254. https://doi.org/10.1093/biolre/ioy141 (2018).
45. Cox, M. P., Peterson, D. A. & Biggs, P. J. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinform.* **11**, 485. https://doi.org/10.1186/1471-2105-11-485 (2010).
46. Rosen, B. D. *et al.* De novo assembly of the cattle reference genome with single-molecule sequencing. *Gigascience*. https://doi.org/10.1093/gigascience/giaa021 (2020).
47. Kim, D., Paggi, J. M., Park, C., Bennett, C. & Salzberg, S. L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* **37**, 907–915. https://doi.org/10.1038/s41587-019-0201-4 (2019).
48. Anders, S., Pyl, P. T. & Huber, W. HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169. https://doi.org/10.1093/bioinformatics/btu638 (2015).
49. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: A bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140. https://doi.org/10.1093/bioinformatics/btp616 (2010).
50. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550. https://doi.org/10.1186/s13059-014-0550-8 (2014).
51. Risso, D., Ngai, J., Speed, T. P. & Dudoit, S. Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat. Biotechnol.* **32**, 896–902. https://doi.org/10.1038/nbt.2931 (2014).
52. Risso, D., Schwartz, K., Sherlock, G. & Dudoit, S. GC-content normalization for RNA-Seq data. *BMC Bioinform.* **12**, 480. https://doi.org/10.1186/1471-2105-12-480 (2011).
53. Weksberg, R., Shuman, C. & Beckwith, J. B. Beckwith-Wiedemann syndrome. *Eur. J. Hum. Genet.* **18**, 8–14. https://doi.org/10.1038/ejhg.2009.106 (2010).
54. McGeady, T. A. (ed.) *Veterinary Embryology* (Blackwell Pub, 2006).

55. Lombard, J. E., Garry, F. B., Tomlinson, S. M. & Garber, L. P. Impacts of dystocia on health and survival of dairy calves. *J. Dairy Sci.* **90**, 1751–1760. https://doi.org/10.3168/jds.2006-295 (2007).
56. Mee, J. F. Why do so many calves die on modern dairy farms and what can we do about calf welfare in the future? *Animals (Basel)* **3**, 1036–1057. https://doi.org/10.3390/ani3041036 (2013).
57. Giuliodori, M. J. *et al.* Metritis in dairy cows: Risk factors and reproductive performance. *J. Dairy Sci.* **96**, 3621–3631. https://doi.org/10.3168/jds.2012-5922 (2013).
58. Rajala, P. J. & Grohn, Y. T. Effects of dystocia, retained placenta, and metritis on milk yield in diary cows. *J. Dairy Sci.* **81**, 3172–3181. https://doi.org/10.3168/jds.s0022-0302(98)75883-7 (1998).
59. Gaafar, H. M., Shamiah Sh, M., El-Hamd, M. A., Shitta, A. A. & El-Din, M. A. Dystocia in Friesian cows and its effects on postpartum reproductive performance and milk production. *Trop. Anim. Health Prod.* **43**, 229–234. https://doi.org/10.1007/s11250-010-9682-3 (2011).
60. Constant, F. *et al.* Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. *Biol. Reprod.* **75**, 122–130. https://doi.org/10.1095/biolreprod.106.051581 (2006).
61. Chavatte-Palmer, P. *et al.* Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. *Theriogenology* **66**, 829–840. https://doi.org/10.1016/j.theriogenology.2006.01.061 (2006).
62. Stice, S. L., Strelchenko, N. S., Keefer, C. L. & Matthews, L. Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. *Biol. Reprod.* **54**, 100–110. https://doi.org/10.1095/biolreprod54.1.100 (1996).
63. Hill, J. R. *et al.* Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol. Reprod.* **63**, 1787–1794. https://doi.org/10.1095/biolreprod63.6.1787 (2000).
64. Li, Y., Lopes, J. S., Fuster, P. C. & Rivera, R. M. Spontaneous and ART-induced large offspring syndrome: Similarities and differences in DNA methylome. *BioRxiv*. https://doi.org/10.1101/2022.02.07.479430 (2022).
65. Brioude, F. *et al.* Expert consensus document: Clinical and molecular diagnosis, screening and management of Beckwith-Wiedemann syndrome: An international consensus statement. *Nat. Rev. Endocrinol.* **14**, 229–249. https://doi.org/10.1038/nrendo.2017.166 (2018).
66. Mao, W. H. *et al.* Growth- and breed-related changes of fetal development in cattle. *Asian-Aust. J. Anim. Sci.* **21**, 640–647 (2008).

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Author contributions

R.M.R. designed the experiment. R.M.R., B.N.P., A.K.G., D.E.H., E.J.S.M., Y.L., C.K., C.M., F.W.I.I.I., E.J., Y.X., P.T., E.E.C., A.R.B.R., P.J.H., Z.W., C.M.S., N.M., C.G.E. conducted the experiment and collected data. R.M.R. wrote the original draft and all authors contributed to the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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