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Article

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

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Abstract:

The use of assisted reproductive technologies (ART) 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 ART. Fetal ultrasonographies were taken on gestation day 55 (D55) and fetal collections performed on D56 or D105 (gestation in cattle ≈280 days). ART 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.

Keywords: Large offspring syndrome, abnormal offspring syndrome, assisted reproductive technologies, fetal morphometry, maternal blood biomarker, Beckwith-Wiedemann Syndrome.

INTRODUCTION

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 assisted reproductive technology (ART) procedures[4]. At that time, the overgrown animals were called "large calves" and the syndrome was coined LOS[5]. As the use of ART 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].

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 ART (e.g., *in vitro* maturation of oocytes, *in vitro* fertilization, *in vitro* embryo culture 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 ART [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 ART 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 of gestation (gestation length in cattle ≈ 280 days) and transcriptome analysis of maternal blood leukocytes on days 55 and 105 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

120 release (CIDR®, Zoetis, Kalamazoo, MI) prostaglandin and timed artificial insemination
121 protocol (**Supplemental Figure 1**).

122 ***In vitro* and *in vivo* production of embryos and embryo transfer**

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

138 **Rationale for experimental design**

139 This experiment is a part of a large-scale study aimed at the identification of
140 epigenetic misregulations in LOS (as defined by ART fetuses weighing ≥97 percentile of
141 the weight of the control fetuses). Based on our previous results for pregnancy rates

from ART 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 [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* x *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].

D55 and D77 fetal ultrasound morphometry

At day 55 (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 Figure 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 (**Figure 1A**). Fetal sex was determined by ultrasonography on D77 of gestation (**Supplemental Figure 1**). Furthermore, fetal morphometry was assessed on D77 in a subset of 18 animals (6 AI and 12 ART 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, umbiliculs, 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 minutes. 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

188 squared (each square = 2.54 cm) and was used to convert all measurements to cm, and
189 then a ratio of umbilicus diameter to crown rump length was determined.

190 **Maternal blood collection and processing**

191 Maternal blood was collected via tail venipuncture on D55 and D105 of
192 pregnancy into K3 EDTA tubes (BD, Franklin Lakes). Blood processing was done as
193 described earlier in [44]. Briefly, blood containing tubes were centrifuged at 1200 x *g* for
194 20 minutes at 4°C. The buffy coat was transferred to 15 milliliters centrifuge tubes
195 containing 12 milliliters of red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1
196 mM EDTA, pH 7.0). White blood cells (WBC = leukocytes) containing tubes were briefly
197 vortexed, incubated at room temperature for 5 minutes, and later centrifuged at 300 x *g*
198 for 10 minutes at 4°C. After discarding the supernatant, the WBC pellet was washed
199 once in 5 milliliters red blood cell lysis buffer and then with 5 milliliters ice-cold 1X
200 Dulbecco's phosphate buffered saline, with centrifugation at 300 x *g* at 4°C for 5
201 minutes at each wash. After discarding the supernatant, the WBC pellet containing
202 tubes were placed immediately on dry ice and then stored at -86°C until use.

203 **Selection of samples for RNA sequencing (RNAseq)**

204 The WBC samples of 23 D105 pregnant heifers were selected for RNAseq on the
205 basis of their group (AI or ART), weight of ART fetuses (≥97 percentile of AI weight =
206 ART-LOS or <97 percentile of AI weight = ART-normal), fetal sex, and whether the
207 females carried one or two fetuses in the ART group. In addition, the D55 WBC
208 samples of the same 23 females were also used for transcriptome analyses
209 (Supplemental Figure 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 100bp 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,

232 the raw read counts were normalized using RUVseq[51]. The betweenLaneNormalization
233 function of EDAseq v2.24.0 was used to adjust for sequencing depth and then upper
234 quantile normalization was done to remove unwanted variation[52]. The trimmed means of
235 M values (TMM) was also used for edgeR analysis. For DESeq2, the median of ratios
236 method of normalization was used using estimateSizeFactors() and assigning the
237 normalization factors back to our count matrix. Pairwise comparisons between each
238 treatment (Control-AI vs ART-Normal, ART-Normal vs ART-LOS, and Control-AI vs
239 ART-LOS) group at each time point (D55 and D105) were performed. In addition, we
240 compared the transcriptome of the dams carrying the two largest ART-LOS individuals
241 (#604 and #664) against all other animals for D55 and D105. Furthermore, a pairwise
242 comparison of WBC transcriptomes of females carrying two vs one fetuses was done to
243 account for differential expression due to multiple fetuses (i.e. increased fetal mass).
244 Genes with a false discovery rate (FDR; EdgeR) or adjusted P-value (padj; DEseq2) ≤
245 0.05 were classified as significant. Significant genes from both packages were
246 overlapped in order to generate a list of candidate genes for downstream analysis.

247 **cDNA synthesis and quantitative RT-PCR (qRT-PCR)**

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

251 *Arginine and Serine Rich Coiled-Coil 1* (RSRC1) and *Transcription Termination*
252 *Factor 1* (TTF1) were used as test genes to corroborate RNA sequencing results based
253 on their upregulation in the WBC transcriptome of the two females carrying the two
254 largest LOS fetuses. Endogenous transcripts used as normalizers were: *Ecdysoneless*

255 *Cell Cycle Regulator* (*ECD*), *Nuclear Factor of Kappa Light Polypeptide Gene Enhancer*
256 *in B-Cells Inhibitor, Beta* (*NFKBIB*), and *VPS35 Endosomal Protein Sorting Factor Like*
257 (*VPS35L*). These were chosen based on their constancy of expression in the RNAseq
258 result (i.e., EdgeR FDR = 1; DESeq2 padj > 0.8 and coefficient of variation ≤ 0.10
259 across all 23 D105 WBC samples. In addition, these transcripts were also chosen
260 based on availability of intron-spanning TaqMan probes (ABI, Foster City, CA) for
261 bovine. TaqMan probe information is as follows: *ECD* (Bt03235022_m1), *NFKBIB*
262 (Bt03247668_m1), *RSRC1* (Bt03236115_m1), *TTF1* (Bt03266651_m1), *VPS35L*
263 (Bt03269522_m1). The mRNA levels of the target genes for the pregnant females
264 carrying ART-LOS and the two largest LOS fetuses (cow #604 and #664) relative to the
265 combined AI and ART-normal groups was calculated using the comparative cycle
266 threshold (C_T) method. Briefly, the C_T for each sample was normalized to the geometric
267 mean of the three endogenous reference genes. The average C_T was calculated by
268 averaging the C_T of all independent samples excluding those from the females carrying
269 the two largest LOS fetuses. The comparative C_T method ($\Delta\Delta C_T$) was used to compare
270 the values of 604 and 664 against the average C_T for all other samples. Fold difference
271 is used for data representation.

272 **Statistical analyses**

273 All analyses include an ART-normal group to remove potential confounding
274 effects of method of conception when analyzing variables. The morphometric data were
275 analyzed by analysis of variance using the general linear model procedure using SAS
276 software v9.4 (SAS Institute, Cary, NC). Dependent variables were all fetal
277 measurements, and the independent variables were the group and day of collection (AI,

ART-normal, ART-LOS). Differences were considered statistically significance when $p<0.10$.

RESULTS

Pregnancy rate

The overall pregnancy rates on day 55 were 61.1% for the AI group and 43.0% for the ART group. For the D56 fetal collection set, there were 14 singleton pregnancies in the AI group, while, for the ART 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 ART group, there were 26 singleton pregnancies and 10 females carried two conceptuses.

Day 55 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, ART=89). There were no differences in any of the fetal ultrasonographic measurements between the AI and ART 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 ART group ($P < 0.03$; 4.62 ± 0.05 and 4.42 ± 0.07, for males (n=53) and females (n=34), respectively).

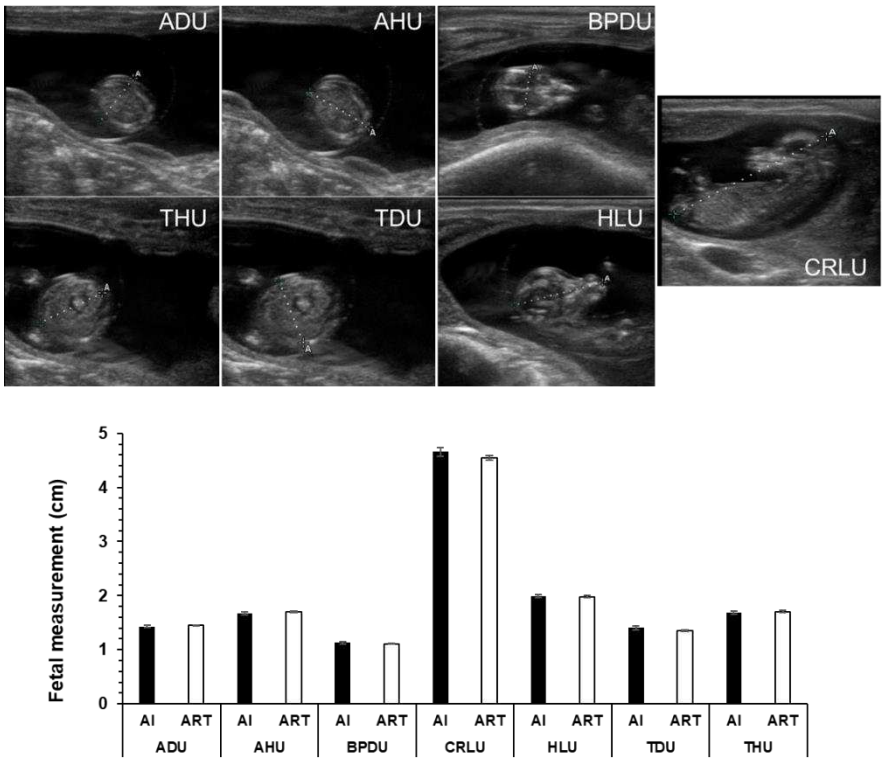

Figure 1. Fetal morphometry of fetuses collected on day 105 gestation. Top. sagittal views of the ultrasound images taken on day 55 of gestation. U: ultrasound. AH: abdominal height. AD: abdominal diameter. BPD: biparietal diameter. CRL: crown rump length. HL: head length. TD: thoracic diameter. TH: thoracic height. **Bottom:** Comparison of ultrasound measurements between all the AI (n=26) and ART (n=89) fetuses in this study. No statistical differences were detected between groups**.** AI: artificial insemination (i.e., control). ART: assisted reproductive technology (*i.e. in vitro* produced embryos).

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.

316 ***Day 56 fetuses***

317 Fetal weight was not significantly different between singletons and twins (mean ±
318 SEM; 8.94 ± 0.28 g and 8.79 ± 0.23 g, respectively). Fetal weight was similar between
319 the AI and ART fetuses (mean ± S.D.; 8.66 ± 1.39 and 8.95 ± 1.43, respectively). For
320 the AI group, there were eight males and six females. The average weight for the
321 males was 8.78 ± 1.33 g (weight range: 6.21 - 10.27 g; Figure 2) while the average
322 weight for the females was 8.50 ± 1.59 g (weight range: 6.20 - 10.22 g). For the ART
323 group, we collected 21 males and 22 females. The average weight for the males was
324 9.52 ± 1.53 g (weight range = 6.94 - 13.77 g) while the average weight for the females
325 was 8.40 ± 1.11 g (weight range = 5.55 - 10.31 g). Fetuses weighing ≥97 percentile of
326 controls (male = 10.18 g and female = 10.16 g) in the ART group were considered LOS
327 (Figure 2). In total, there were eight ART-LOS males (weight range = 10.23 - 13.77 g)
328 and one ART-LOS female (weight = 10.31 g). All ART fetuses weighing <97 percentile
329 weight of controls were referred to "ART-normal" (males – n=13 [weight range: 6.94 –
330 9.98 g] females – n=21 [weight range: 5.55 - 9.8 g]). Besides heavier body weight,
331 other phenotypes observed in the ART group were focal hemorrhage on the brain and
332 abdominal wall defects (Figure 2C). The fetal measurements at collection for the AI,
333 ART-normal and ART-LOS groups are summarized in Figure 3. Only one female in the
334 ART group was considered LOS, therefore no comparisons were made between it and
335 the other groups.

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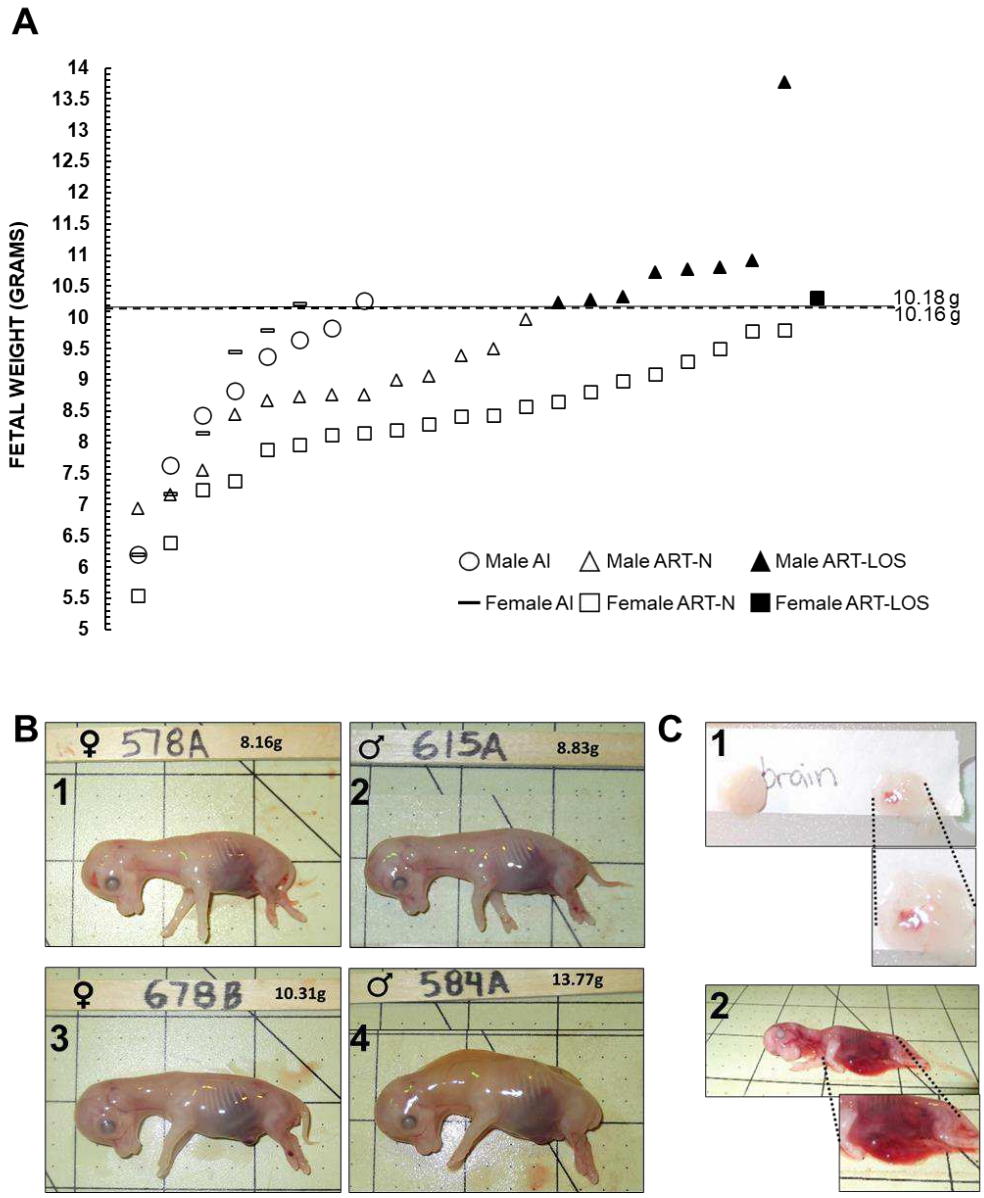
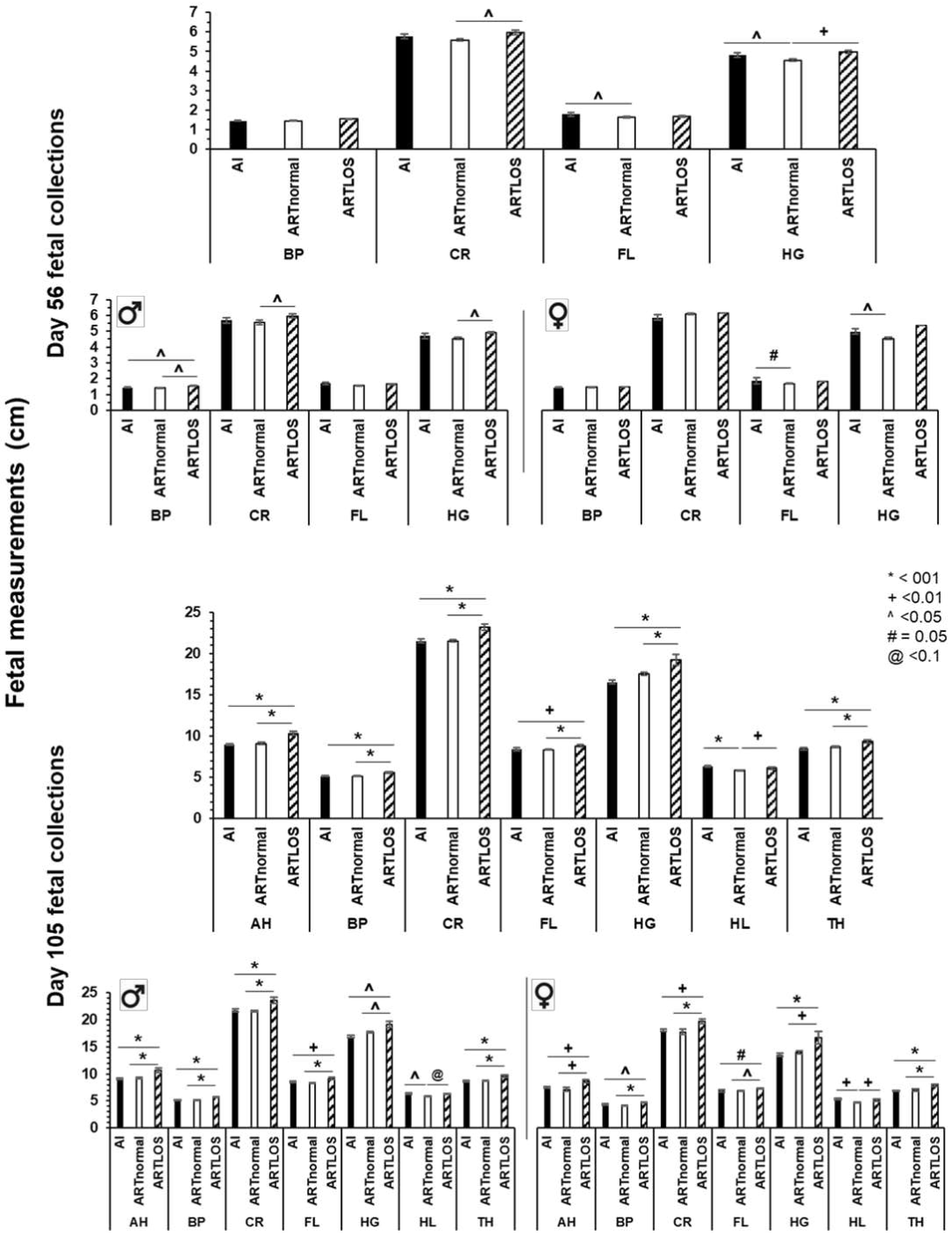


Figure 2. Day 56 fetal collections. (A) Fetal weight at D56 of gestation. The X axis has no actual implication and is used to scatter the spots representing each fetus for ease of visualization. The sex of the fetuses and the way they were generated is shown at the bottom of the figure. The bold line represents 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 ART-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 and B4 show the heaviest LOS (ART-LOS 678B: female weighing 10.31 g and ART-LOS 584A: male weighing 13.77 g). Each square on the background = 2.54 cm. (C) Secondary phenotypes observed in day 56 LOS fetuses - focal hemorrhage on the brain (C1) and abdominal wall defects (C2). LOS: large offspring syndrome. AI: artificial insemination (i.e., control). ART: assisted reproductive technologies (i.e., *in vitro* produced embryos).



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

Day 105 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 ART 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; Figure 4). For the ART 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 ART group were considered LOS (Figure 4).

In this study, it appears that female fetuses were preferentially lost between D56 and D105, especially in the ART group. On day 56, we collected 6 females and 8 males in the AI group and 22 females and 21 males in the ART group. On D105 we collected 4 females and 8 males in the AI group and 13 females and 33 males in the ART 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 ART group. The deficiency of female fetuses was not statistically significant in the AI group ($p = 0.20$) but was highly significant in the ART group ($p < 0.003$) when compared to the binomial distribution with the expected 1:1 sex ratio.

In total, there were eight ART-LOS males (weight range = 552 - 1080 g) and nine ART-LOS females (weight range = 468 - 986 g). All ART fetuses weighing <97 percentile weight of controls were referred to “ART-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 ART group were long protruding tongue (Figure 4C.3), large organs (heart, kidney, lung, pancreas), hepatic cyst, abdominal ascites, gelatinous material in the peritoneal cavity and organs (Figure 4C.1-2) and skull asymmetry (Figure 4C.4). In addition, large umbilicus (Figure 4B.3) were observed at collection in two of the female ART-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 (Figure 4B.3). There was a group (i.e. AI, ART-normal, ART-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 Figure 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 Figure 5. Female specific analysis for the ART-LOS group was

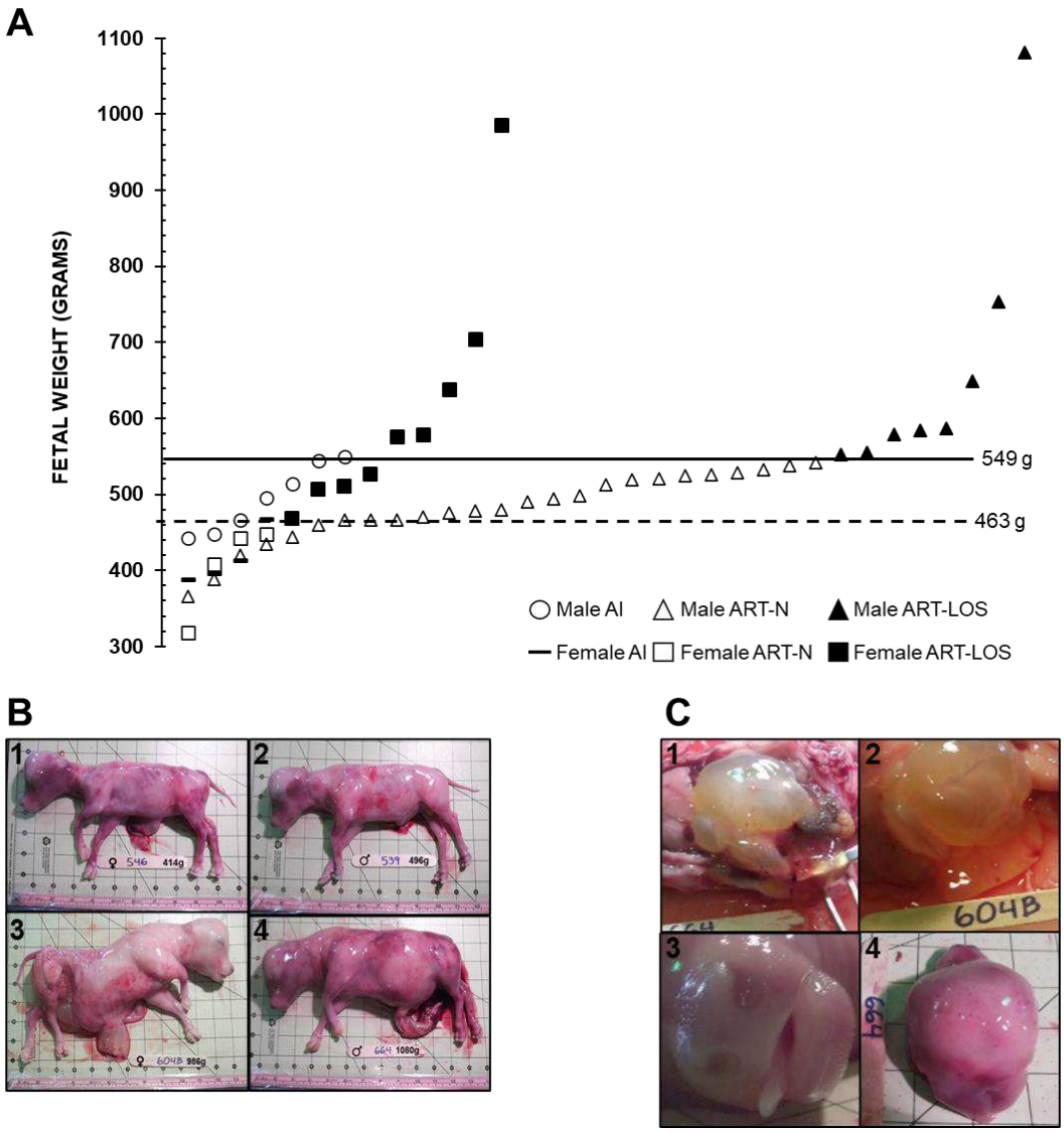


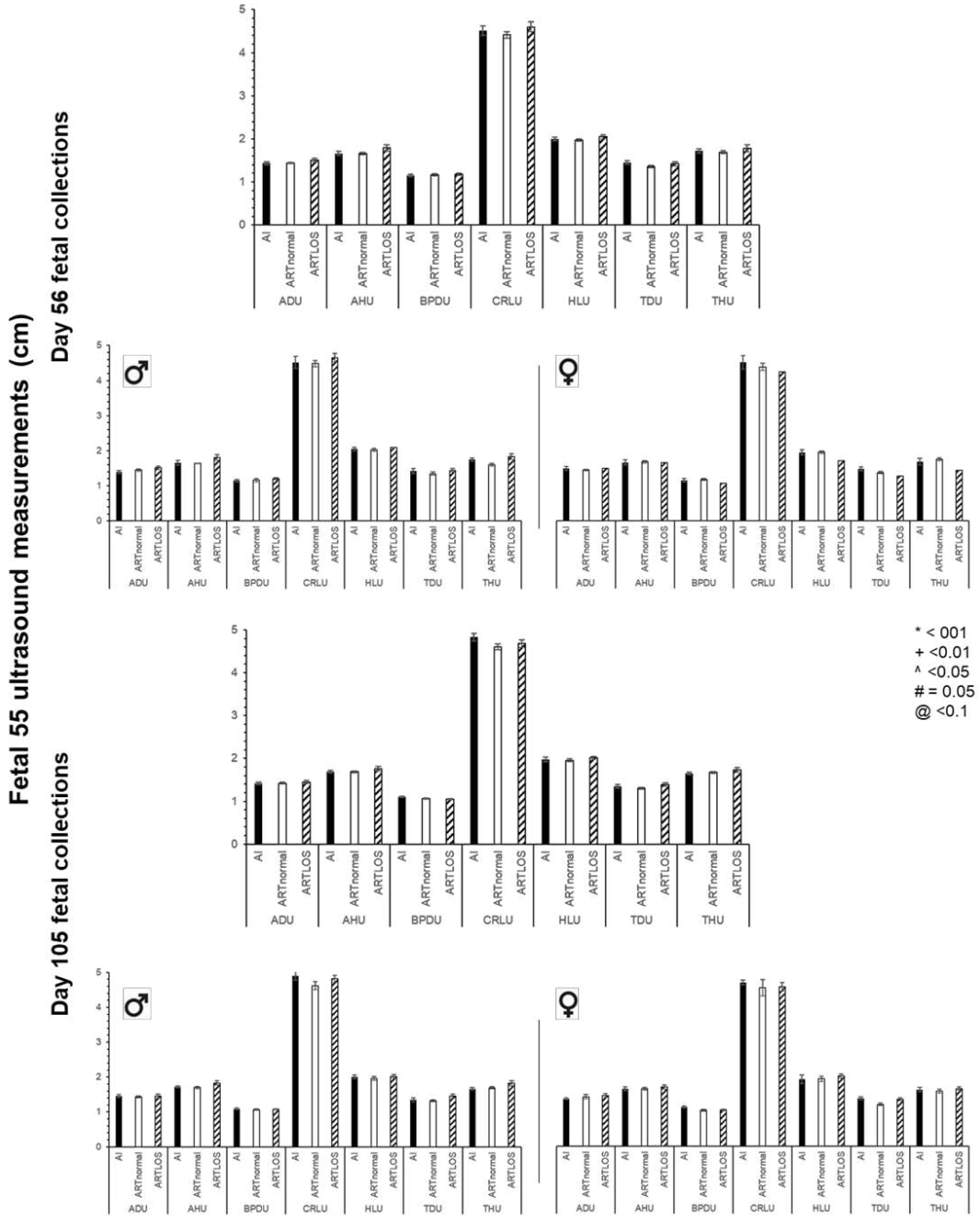
Figure 4. Day 105 fetal collections. (A) Fetal weight at D105 of gestation. The X axis has no actual implication and is used to scatter the spots representing each fetus for ease of visualization. The sex of the fetuses and the way they were generated is shown at the bottom of the figure. The bold line represents 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 ART-LOS group (B3-B4). Also, females (B1, B3) and males (B2, B4). B1 = AI-546 (control female weighing 414 grams which is approximate average weight of female control fetuses). B2 = AI-539 (control male weighing 496 grams which is the approximate average weight of male control fetuses). B3 and B4 show the heaviest LOS (ART-LOS 604B: female weighing 986 grams and ART-LOS 664: male weighing 1080 g). Each square on the background = 2.54 cm. **(C)** Secondary phenotypes observed in day 105 LOS fetuses - gelatinous material in the peritoneal cavity and organs (C1-2) long protruding tongue (C3), skull asymmetry (C4). AI: artificial insemination (i.e., control). ART: assisted reproductive technologies (*i.e., in vitro* produced embryos).

not performed as only one female in the ART 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 ART-normal group were smaller than males in the AI and ART-LOS groups ($p < 0.03$; Mean ± SEM; 1.60 ± 0.04, 1.75 ± 0.05 and 1.83 ± 0.08 for ART-normal, AI and ART-LOS groups, respectively).

A slight positive correlation was observed between the day 55 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 Figure 5. No differences were observed for sex, group, or their interaction for abdominal diameter and head length. Females were smaller than males



445 **Figure 5. Group-specific day 55 fetal ultrasound measurements.** Top three panels – D55
446 ultrasonographic measurements of fetuses collected on D56. n= 6 females and 8 males in the
447 AI group and 22 females [1 LOS] and 21 males [8 LOS] in the ART group. Bottom three panels
448 – D55 ultrasonographic measurements of fetuses collected on D105. For each day set, the top
449 graph includes both sexes and the bottom two graphs are separated by sex, males on the left
450 and females on the right. n= 4 females and 8 males in the AI group and 13 [9 LOS] females and
451 33 males [8 LOS] in the ART group. U: ultrasound. AH: abdominal height. AD: abdominal
452 diameter. BPD: biparietal diameter. CRL: crown rump length. HL: head length. TD: thoracic
453 diameter. TH: thoracic height. Data are represented as average ± SEM. For D56, there was
454 only one female considered LOS, hence the lack of error bars. Lines going over three bars are
455 used to represent statistical differences between the first and the third bar.

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 day 77 pregnant group (AI= 6; ART=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 day 105 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 ART fetuses, respectively; Figure 6). The highest number resulting from the multiplication of the beforementioned ultrasonographic measurements was 79.92 for the AI (control) group. The D105 ART 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 ART 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 ART 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 in 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 Figure 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, ART-normal or ART-LOS). In addition, the analysis separated the

females carrying the two largest D105 ART-LOS fetuses (dam #604 and #664) from the rest of the animals (Supplementary Figure 3). The day-specific unsupervised hierarchical clustering analyses may be found in Supplementary figure 4.

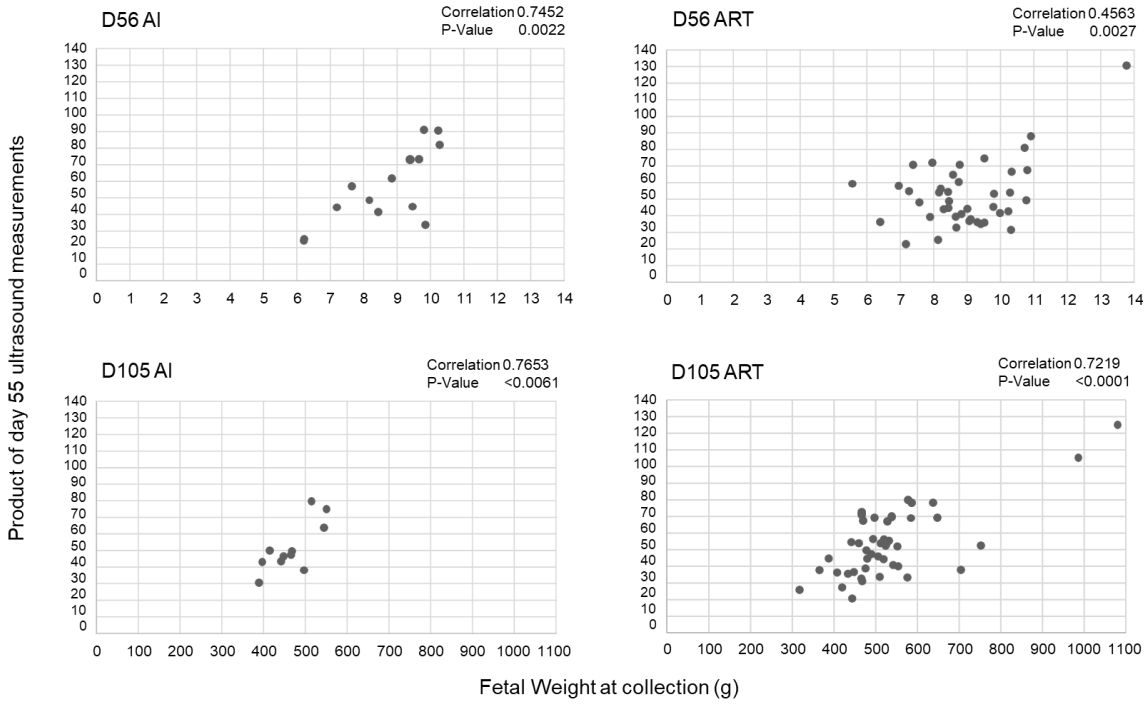


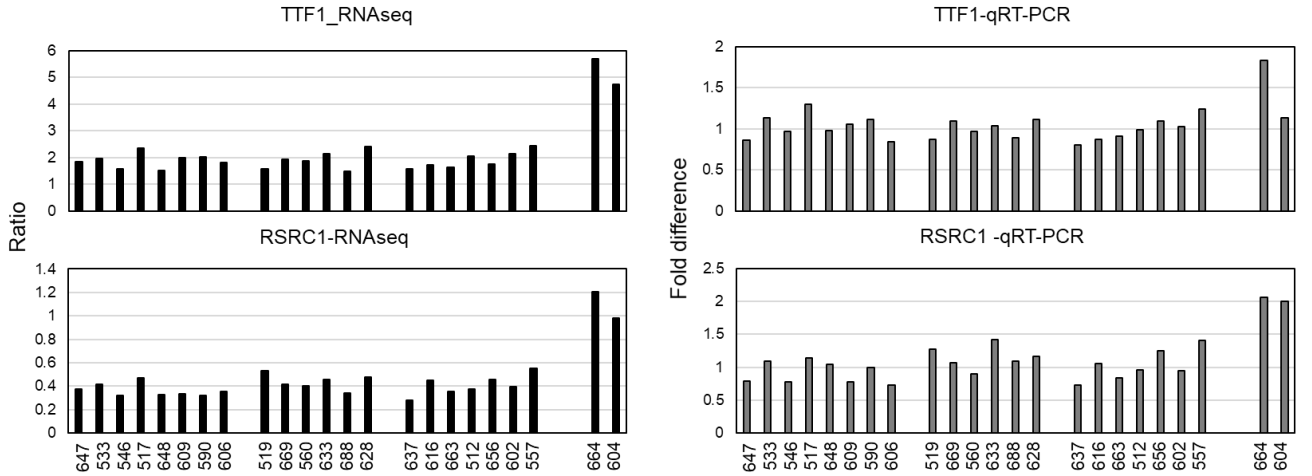
Figure 6. Correlation between fetal weight at collection and the product of D55 ultrasonographic measurements. **(A)** D56 AI group. **(B)** D56 ART group. **(C)** D105 AI group. **(D)** D105 ART group. Ultrasonographic measurements included = abdominal diameter, abdominal height, crown-rump length, head length, thoracic height, and thoracic diameter. AI: artificial insemination (i.e., control). ART: assisted reproductive technologies (*i.e. in vitro* produced embryos). The line on the bottom right figure is used to separate the two largest LOS fetuses (above the line) from all other fetuses (below the line).

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 ART-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 4,451 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 days 55 and 105 by both EdgeR and DESeq2 statistical packages (Figure 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 by DESeq2. Data show that the pattern of expression for these genes is similar between RNAseq and qRT-PCR results (Figure 8).

	AII, 1 Fetus vs. AII, 2 Fetuses	ART-N, 1 Fetus vs. ART-N, 2 Fetuses	AI vs. ART-N	AI vs. ART-LOS	ART-N vs. ART-LOS	AI & ART-N vs. ART-LOS	604 & 664 vs. AII ART	604 & 664 vs. AII
Day 55	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						
Day 105	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 WBC transcriptomes. 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). ART-N = embryos were produced by *in vitro* procedures that were <97% of the control's weight at D105. ART-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.

				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	ART N	ART N	ART N	ART N	ART N	ART N	ART LOS	ART LOS	ART LOS	ART LOS	ART LOS	ART LOS	ART LOS	ART LOS	ART 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



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557 **Figure 8. Normalized read counts and qRT-PCR corroborations of D105 maternal**
558 **transcriptomes. Top.** Table showing all information pertaining the samples and the
559 transcriptome's statistics for the genes chosen for qRT-PCR corroborations. The table is
560 organized by total fetal mass in increasing order. # of fetuses indicates how many fetuses the
561 dam was carrying at the time of collection. F = female fetus. M = male fetus. AI = artificial
562 insemination (*i.e.* control). ART-N = embryos were produced by *in vitro* procedures that were
563 <97% of the control's weight at D105. ART-LOS embryos were produced by *in vitro*
564 procedures and were ≤97% of the control's weight at D105. CV = coefficient of variation of all
565 23 samples. Asterisk – denotes the LOS of the pair. **Bottom**. Bar graphs showing RNAseq
566 results represented as sample ratio of the named gene from the mean of the three normalizers
567 and qRT-PCR results represented as fold difference. For qRT-PCR, the geometric mean of
568 three endogenous transcripts, namely *ECD*, *NFKBIB* and *VPS35L* were used to normalize the
569 levels of *TTF1* and *RSRC1*. Data are represented as fold difference from the average of 21
570 samples (average fold difference = 1; not including 604 and 664). Transcripts of *TTF1* and
571 RSRC1 for 604 and 664 were compared to the normalized average of 21 samples for each test
572 gene ($\Delta\Delta C_T$).

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575

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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, 20.93% (9/43) of the D56 and 36.95% (17/46) of the D105 collected ART 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 ART-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

600 fetal overgrowth syndrome in cattle and human (i.e. BWS). Furthermore, fetuses were
601 also collected on D105 as we have previously shown that fetal overgrowth is evident at
602 this stage of gestation[2]. In humans, 97 percentile criteria is used to describe
603 macrosomia in newborn babies[53]. Since macrosomia is the main characteristic of LOS
604 and one characteristic that can result in dystocia, we used this weight to ascribe LOS in
605 the present study, similar to what we have done previously.

606 A greater than two-times increase in fetal weight was observed in two of the
607 D105 ART-LOS fetuses in the current study, an observation also reported by others[4],
608 suggesting that if those fetuses were allowed to go to term, dystocia would be probable
609 and assisted delivery or caesarean section would be required. Dystocia as a result of
610 LOS can lead to neonatal death[4] and/or death of cows[22]. Further, dystocia associated
611 with stillbirth[55], is a major contributor to perinatal mortality in cattle[56], and has also been
612 shown to increase the chances of metritis[57], and lameness[28]. Furthermore, dystocia
613 can have an adverse impact on milk production[58] and increase the calving to conception
614 interval in dairy cows[59]. Thus, overgrown fetuses can have negative economic
615 consequences to cattle producers[22] and identification of those large calves during early
616 pregnancy would help overcome these problems.

617 In the current study, we performed fetal ultrasonographic measurements at D55
618 for fetuses that were collected on D56 or D105 of gestation. Based on our findings, it is
619 evident that the ≥97 percentile AI weight criteria used to assign a fetus as being LOS
620 while useful at D105, it is not appropriate at gestation D56. From the findings of the
621 correlation analysis between fetal weight and the product of six ultrasound
622 measurements, we hypothesize that fetuses are lost after this day of pregnancy and/or

623 will have subsequent disproportionate growth. This is in accordance to previous work
624 which showed that smaller *in vitro* or cloned fetuses in the first trimester resulted in
625 heavier fetuses at term[7,60]. Further, Bertolini and coinvestigators also suggested that *in*
626 *vitro* produced fetuses show early growth retardation and then follow acceleration in
627 fetal growth at later stages of gestation, showing biphasic growth pattern[7].

628 Previous research reported that larger biparietal diameter might be a useful
629 measurement to identify LOS at day 63 of gestation in cloned LOS fetuses[61] and that a
630 smaller crown rump length might be a useful measurement to identify LOS at day 58 of
631 pregnancy in *in vitro* produced LOS fetuses[7]. However, those measurements were not
632 found to be indicators for LOS in our current study. Possibilities for the discrepancies in
633 our findings, are; 1) different definition of LOS (we used ≥97% weight of the control
634 fetuses while Bertolini used fetuses that were 33% heavier than controls for
635 comparisons[7], 2) improvement in ultrasonography technology allowing for more
636 accurate measurements, and 3) number of observations (113 fetuses in our study vs.
637 34 fetuses in[60]).

638 When we tried to do fetometry at day 77 of gestation, we were only able to
639 measure head length with some accuracy. All other measurements were not reliable,
640 indicating that day 77 ultrasonography might be too late to try to accurately identify
641 LOS. Given the allometric growth that occurs after D56 and the fact that day 77 is too
642 late to predict LOS by ultrasonography, and that fetal sex is most accurately predicted
643 between days 60-80[34], we suggest that future studies focus on day ~65 as a target day
644 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 ART-LOS fetuses. This is similar to what Constant *et al*.[60] reported in day 220 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 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 ART conceptuses.

Enlarged tongue was also observed in D105 ART-LOS which is similar to previous findings in our laboratory[2,23] and comparable to what has been observed in a similar congenital overgrowth condition in humans, namely Beckwith-Wiedemann Syndrome[64]. Large tongues can lead to difficulty in suckling and increase the chances of prenatal death[26]. In addition, the largest D105 ART-LOS fetus showed brachycephaly and asymmetrical growth of the cranium, an interesting finding given that

668 one characteristic of BWS is hemihyperplasia[64]. These similarities demonstrates that
669 BWS and LOS are the same syndrome, as previously reported by us[2], and that they
670 share similar misregulated developmental epi(genetic) mechanisms associated with
671 asymmetrical growth.

672 In our study, we also had the objective of determining if maternal blood could be
673 used as a biomarker to identify LOS on D55 and/or D105 of pregnancy. For this, we
674 analyzed leukocyte transcriptome of 23 females carrying D105 AI, ART-normal (weight)
675 and ART-LOS fetuses. We also analyzed the D55 leukocyte transcriptomes of the
676 same females. Our initial approach was to do an unsupervised hierarchical clustering of
677 de-identified samples to determine if obvious difference existed between the females
678 carrying LOS fetuses when compared to the other two groups. To our surprise, the
679 transcriptome of 18/46 samples clustered together by animal (i.e., n=9) regardless of
680 pregnancy stage (i.e., D55 and D105). Another interesting point is that the experiment
681 was done over three seasons (Autumn, Spring and Summer) with a range in
682 temperature of -30°C to 43°C, and this was not detected in the transcriptome given the
683 clustering by individual. Further, given the design of the study, in which we transferred
684 two embryos per recipient heifer, some pregnancies in the D55 and D105 ART groups
685 had two fetuses, however, the unsupervised hierarchical clustering did not cluster
686 animals by number of fetuses. The unsupervised hierarchical clustering, did however,
687 cluster the D105 transcriptomes of the females carrying the two largest LOS from all
688 other females.

689 For the maternal leukocyte transcriptome analysis, we focused on the two
690 females that carried the largest LOS fetuses as those were the ones that separated by

691 hierarchical clustering and the ones that would most likely cause a difficult birth.
692 Analyses identified *ADGRE3*, *LOC107131273*, *LOC783838*, *PCDH1*, and *RAB34*, as
693 being different on D55 and *ACTA2*, *KDM5A*, *LOC783838*, *MAN1A2*, *MIR2376*, *PCDH1*,
694 *PRRC2C*, *RSBN1*, *S100A14*, *SRPK2*, and *TTF1* on D105 when compared to all other
695 animals. For qRT-PCR corroborations we focused on two transcripts whose TaqMan
696 probes were readily available, namely *TTF1* which was identified as differentially
697 expressed on D105 by EdgeR and DESeq2 statistical packages as well as *RSRC1*
698 which was identified as differentially expressed by DESeq2. Analyses show
699 consistency of expression between RNAseq and qRT-PCR results. Other genes will be
700 tested in the future as assays become available.

701 Our study has several limitations. As it pertains to fetal ultrasonographies,
702 published work shows that fetal growth varies among different breeds[65] and that size
703 difference can be noticeable as early as 3 months of gestation, therefore it is possible
704 that fetal growth patterns may vary as early as D55 of gestation among different breeds;
705 however, there is lack of published data showing that on D55 of gestation, fetuses from
706 different breeds differ in size. Future research would have to address this question.
707 Here, we also did sex-specific analyses for all our variables, including for
708 ultrasonographic measurements. Since in our experiment, the number of male fetuses
709 was unexpectedly higher in the ART group at D105, female specific ultrasonographic
710 data analysis may be limiting. Finally, and importantly, it should be noted that during
711 transcriptome analysis, we only used the known (mostly coding) group of bovine
712 transcripts for this study and that the non-coding and novel transcript portion of the
713 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 days 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. In addition, our analysis identified several genes that were misregulated in all LOS fetuses when compared to fetuses of normal weight produced by ART, 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.

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Authors contributions

RMR designed the experiment. RMR, BNP, AKG, DEH, EJSM, YL, CK, CM, FWIII, EJ, YX, PT, EEC, ARBR, PJH, ZW, CMS, NM, CGE conducted the experiment and collected data. RMR wrote the original draft and all authors contributed to the manuscript.

Data Availability Statement

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

Competing Interests Statement

There are no competing interests.

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