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## Associations between foetal size and ovarian development in the pig

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1 **Running Title:** Porcine fetal size and ovarian development

2

3 Associations between foetal size and ovarian development in the pig

4

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## 13 **ABSTRACT**

14 It is estimated that intra-uterine growth restricted piglets  
15 represent 25% of the total number of piglets born. Growth  
16 restricted female piglets have impaired reproductive  
17 performance postnatally, however, when during gestation  
18 this phenotype arises is not known. With this study, the aim  
19 was to improve the understanding of foetal ovarian  
20 development in normal and small fetuses throughout  
21 gestation. Female Large White X Landrace fetuses were  
22 obtained at gestational day (GD) 45, 60 and 90 ( $n = 5-6$   
23 litters/GD). Histological analysis of GATA4 stained foetal  
24 ovaries at GD60 and 90 indicated there were fewer primary  
25 follicles ( $P \leq 0.05$ ) in the fetuses weighing the least  
26 compared to those with a weight similar to the mean for the  
27 litter (CTMLW) at GD90. Plasma oestradiol concentrations  
28 were less in the fetuses with lesser weights compared  
29 with greater weight fetuses at GD90 ( $P \leq 0.05$ ). The RNA  
30 was extracted from ovaries of the lesser weight and  
31 CTMLW fetuses at GD45, 60 and 90 and qPCR was  
32 performed to quantify relative abundance of 12 candidate  
33 mRNAs for which encoded proteins modulate ovarian  
34 function and development. Gestational changes in relative  
35 abundances of *CD31*, *PTGFR*, *SPP1* and *VEGFA* mRNA  
36 transcripts were observed. Relative abundance of *KI67* ( $P$   
37 = 0.066) and *P53* ( $P \leq 0.05$ ) was less in ovaries of the

38 lesser weight compared to CTMLW foetuses at GD60.  
39 There was a lesser relative abundance of *PTGFR* mRNA  
40 transcript in ovaries from the foetuses with lesser weight  
41 compared to CTMLW foetuses at GD45 and 60 ( $P \leq 0.05$ ).  
42 These findings indicate that postnatal differences in  
43 reproductive potential of growth restricted females are  
44 programmed early in gestation. It is hoped that further  
45 investigation will improve the understanding of the  
46 relationship between prenatal reproductive development  
47 and postnatal reproductive performance.

48

49 **Keywords:** Foetal Growth; Intrauterine Growth Restriction  
50 (IUGR); Porcine; Ovary.

## 51 **1. Introduction**

52           The number of primordial follicles present in the  
53 ovary at birth is considered to define a female's  
54 reproductive potential, a hypothesis first suggested in the  
55 19th century (Waldeyer, 1870). Numerous processes must  
56 occur in the ovary during foetal development for there to  
57 be adequate formation of the pool of primordial follicles  
58 including proliferation and apoptosis of the germ cells,  
59 formation of oogonial nests followed by follicular formation  
60 and growth, accompanied by initiation of meiosis.

61           Impaired foetal growth is associated with impaired  
62 reproductive potential post-puberty, in both sexes and in  
63 multiple species. In an experimentally induced model of  
64 intrauterine growth restriction (IUGR), there were less  
65 ovarian follicles during the late gestation period in sheep  
66 foetuses (Da Silva et al., 2002). The lesser follicle numbers  
67 in ewes with a lesser foetal body weight included mainly  
68 the primordial follicle pool, suggesting that the impaired  
69 ovarian function observed postnatally may be associated  
70 with defects in foetal ovarian development.

71           Significant variation in birth weight is observed in  
72 piglets, with small outliers being a problem to the industry.  
73 It is estimated that IUGR piglets contribute to 25% of the  
74 total number of piglets born (Wu et al., 2010). Many  
75 definitions for IUGR exist including piglets weighing less

76 than 1.1 kg at birth, weighing less than two standard  
77 deviations of the mean body weight for age, the smallest  
78 of each litter or a small weight statistical outlier from the  
79 population, or weighing less than two-thirds of the mean  
80 litter weight (Ashworth et al., 2001; Royston et al., 1982;  
81 van der Lende et al., 1990; Wu et al., 2010). It has been  
82 suggested that IUGR in the piglet may be programmed  
83 from an early stage of gestation, with marked within-litter  
84 variation in foetal size observed from as early as  
85 gestational day 30 to 35 (Finch et al., 2002; Foxcroft et al.,  
86 2006; Foxcroft and Town, 2004; Pettigrew et al., 1986;  
87 Wise et al., 1997).

88         At birth, IUGR gilts have delayed initiation of  
89 follicular development, with more primordial follicles,  
90 accompanied by fewer primary and secondary follicles  
91 compared to normally developing littermates (Da Silva-  
92 Buttkus et al., 2003). Gilts with lesser growth rates are  
93 older when first mating occurs (Tummaruk et al., 2000),  
94 which results in fewer litters produced by sows in pork  
95 production enterprises. It has been suggested that in  
96 addition to having an effect on reproductive performance  
97 during their first pregnancy, gilts which have lesser birth  
98 weights will also have impaired first parity reproductive  
99 performance (lesser pregnancy rates and litter sizes) and  
100 also at their second parity (Hoving et al., 2010).

101           Dysregulation of the foetal hypothalamic-pituitary-  
102 gonadal axis can alter the production of androgens and  
103 oestrogens, in turn causing defective reproductive  
104 organogenesis and altered development of the foetus  
105 (Brooks and Thomas, 1995). In mammals, an aberrant  
106 oestrogen milieu during foetal development can have  
107 marked effects on postnatal ovarian function (Abbott et al.,  
108 2006). Considering the functions of oestrogens in the  
109 regulation of oestrous cycles, oestradiol concentrations in  
110 gilts during the prepubertal period can be used as a  
111 biomarker for reproductive efficiency (Steel et al., 2018).

112           Impaired reproductive performance is one of the  
113 primary reasons for the removal of sows from commercial  
114 herds (Sasaki and Koketsu, 2011). Considering the  
115 prevalence of small birthweight piglets, and the severity of  
116 this reproductive phenotype, it is important that there be  
117 improvement in understanding of the mechanisms  
118 regulating foetal ovarian development to improve  
119 reproductive efficiency. Whilst in previous studies the  
120 temporal changes in follicle types during gestation have  
121 been elucidated (Oxender et al., 1979; Pontelo et al.,  
122 2018), there is a limited understanding of the expression  
123 profiles of genes, as indicated by relative abundance of  
124 mRNA transcripts, associated with ovarian development  
125 and function.

126           There are marked changes in the structure of the  
127 foetal ovary during gestation that affect female fertility  
128 postnatally. In the present study, the temporal relative  
129 abundance profiles of mRNA transcripts with central  
130 functions in apoptosis, proliferation and the ovarian  
131 extracellular matrix was determined. Even though it is  
132 widely recognized that angiogenesis is essential for organ  
133 development and growth, and that angiogenesis has  
134 important effects in the postnatal ovary, little is known  
135 regarding vascularisation of the foetal ovary. Considering  
136 this, the encoded proteins of several of the candidate  
137 genes investigated in the present study have central  
138 functions in the regulation of angiogenesis.

139           In the present study, the aim was to improve  
140 understanding of temporal changes in ovarian gene  
141 expression, as indicated by relative abundances of mRNA  
142 transcripts, and histology in fetuses with relatively  
143 average and lesser than average birth weights as  
144 compared with the entire litter. It was hypothesised that the  
145 developmental trajectory of ovarian development of the  
146 fetuses with lesser weights deviates from that of ovaries  
147 from average-sized female fetuses.

148



## 149 **2. Materials and methods**

150 All procedures were performed with approval from  
151 The Roslin Institute (University of Edinburgh) Animal  
152 Welfare and Ethical Review Board and in accordance with  
153 the U.K. Animals (Scientific Procedures) Act, 1986.

### 154 *2.1. Experimental animals and sample collection*

155 Large White X Landrace gilts (age 11 - 14 months)  
156 were observed daily for signs of oestrus and were housed  
157 in groups of 6 to 8 animals per pen. Oestrous cyclicity and  
158 ovarian function were controlled in accordance with routine  
159 normal practice at The Roslin Institute Large Animal Unit.  
160 All gilts were inseminated twice daily for the duration of  
161 oestrus with semen from one of four Large White sires. The  
162 sires used were equally distributed between gilts at the  
163 different gestational days (GD) of interest to minimize any  
164 effect of sire. The first day of insemination was assigned  
165 as GD0. Gilts were humanely killed with sodium  
166 pentobarbitone 20% w/v (Henry Schein Animal Health) at  
167 a dose of 0.4 ml/kg by intravenous injection via a cannula  
168 inserted in the ear vein at GD45, GD60 and GD90 ( $n =$  five  
169 – eight gilts/GD). Immediately before euthanasia, cardiac  
170 puncture was performed using an EDTA coated syringe to  
171 collect maternal blood from five gilts of the GD90 group.

172 Following confirmation of death, mid-ventral  
173 incisions were made so that there was access to the

174 reproductive tract. The tract was removed from the body  
175 cavity and placed in a dissecting tray. Ovaries were  
176 removed from the uterus and the number of corpora lutea  
177 was quantified. The percentage prenatal survival was  
178 calculated by dividing the number of live foetuses by the  
179 number of corpora lutea and multiplying this value by 100.  
180 Both uterine horns were dissected, from the ovary towards  
181 the cervix. Foetal sex was determined morphologically.  
182 Cardiac puncture was performed using an EDTA coated  
183 syringe to collect blood samples from the female foetuses  
184 with the relatively lesser, average (CTMLW) and relatively  
185 greater weights when there was consideration of the  
186 individual foetal weights for the entire litter at GD90 ( $n =$   
187 five litters; Supplementary Table 1). Plasma was obtained  
188 from the maternal and foetal blood samples by  
189 centrifugation and samples were stored at  $-20\text{ }^{\circ}\text{C}$  until  
190 required. At GD45, GD60 and GD90 ( $n =$  five or six  
191 litters/GD), ovaries from the foetuses that had relatively  
192 lesser, CTMLW and relatively greater weights were  
193 dissected, weighed (GD60 and GD90), and one ovary from  
194 each foetus was snap frozen in liquid nitrogen and stored  
195 at  $-80\text{ }^{\circ}\text{C}$  until relevant evaluations occurred. The other  
196 ovary was fixed in Bouin's for histological analysis.

197           Comparisons were made between the female  
198 foetuses that had relatively lesser, CTMLW and relatively

199 greater weights for plasma oestradiol concentrations and  
200 ovarian weight. Considering that the majority of litters have  
201 a negatively skewed distribution of birth weights (Milligan  
202 et al., 2002; Quesnel et al., 2008), and considering the  
203 greater prevalence of the lesser birth weight outliers, the  
204 focus for the remainder of the analyses was the lighter and  
205 CTMLW foetal comparisons.

## 206 *2.2. Histological analysis*

207 Ovarian samples from GD60 ( $n =$  seven CTMLW  
208 foetuses; eight lesser weight foetuses) and GD90 ( $n =$  six  
209 from both the CTMLW and lesser weight foetuses) were  
210 used. Whole ovaries were fixed with Bouin's overnight at  
211 room temperature and changed daily for approximately 1  
212 week in 70% ethanol (Genta Medical). The ovaries were  
213 then transferred into labelled tissue processing cassettes  
214 (Simport) and processed using a tissue processor  
215 (ASP3005, Leica) by passing through graded ethanol  
216 (70%, 95%, and 99%; Genta Medical) and xylene (Genta  
217 Medical). The samples were embedded in paraffin wax  
218 (Fisher Scientific), and 5  $\mu$ m sections were cut and placed  
219 on polysine microscope slides (Fisher Scientific).

220 Following dewaxing and heat-induced epitope  
221 retrieval in 0.01 M sodium citrate (Vector Laboratories),  
222 endogenous peroxidase activity was blocked by incubating  
223 slides with 0.3% hydrogen peroxide (Sigma Aldrich) in

224 methanol. Non-specific binding sites were blocked by  
225 incubation with normal goat serum (Vectastain Elite ABC  
226 kit; Vector Laboratories). Sections were incubated with a  
227 primary antibody for GATA binding protein 4 (GATA4; sc-  
228 9053; Santa Cruz) at a 1:200 dilution, anti-CD31 antibody  
229 (ab28364; Abcam) at a 1:100 dilution (GD90 ovaries only)  
230 or with rabbit immunoglobulin G (RIgG; Vector  
231 Laboratories; equivalent total protein concentration) as a  
232 negative control. Sections were stained with GATA4 as  
233 this protein is exclusively expressed by somatic cells, and  
234 not by germ cells (McCoard et al., 2001). The slides were  
235 incubated in a humidified chamber at 4°C overnight,  
236 washed in phosphate buffered saline (PBS), and incubated  
237 for 30 min at room temperature with a biotinylated anti-  
238 rabbit IgG secondary antibody (Vectastain Elite ABC kit;  
239 Vector Laboratories) at a dilution of 1:200 in PBS  
240 containing 1.5% normal goat serum. Sections were  
241 incubated with Vectastain Elite ABC reagent (Vectastain  
242 Elite ABC kit; Vector Laboratories) for 30 min, before  
243 incubation with the Novared peroxidase substrate (Vector  
244 Laboratories) for 5 min. Sections were counterstained with  
245 haematoxylin and dehydrated in a graded series of ethanol  
246 and xylene (70%, 95%, and 99% ethanol; 99% ethanol 1:1  
247 with xylene, and absolute xylene; Genta Medical). The

248 sections were imaged using the NanoZoomer slide  
249 scanner (Hamamatsu).

### 250 *2.2.1. Image analysis*

251 All image analyses were performed using ImageJ.  
252 Six non-overlapping images from both the GATA4 and  
253 CD31 stained sections were taken at x 20 magnification.  
254 For each ovary, two GATA4 stained sections were  
255 analysed which were a minimum of 15 serial sections apart  
256 from one another from the middle of the ovary. Using the  
257 GATA4 stained sections, the number of oogonia (GD60  
258 and GD90), primordial (GD60 and GD90), and primary  
259 (GD90) follicles were quantified within each image. The  
260 data were then expressed as number of oogonial  
261 nest/follicles per  $100000\mu\text{M}^2$ . The number of oogonia per  
262 germ cell nest was counted. Only a few primary and  
263 secondary follicles were observed at GD60 and GD90,  
264 respectively, so further analyses on these follicle types at  
265 these gestational days could not be performed.

### 266 *2.3. Plasma oestradiol quantification*

267 Oestradiol concentrations were determined in  
268 maternal plasma samples at GD90 ( $n = 5$ ) and in foetal  
269 plasma samples from the relatively lesser, CTMLW and  
270 relative greater weight female foetuses at GD90 ( $n = 5$   
271 litters) in a single ELISA validated for use with samples of  
272 pigs (Abscitech; EK0373), as per the manufacturer's

273 instructions. The detection range of the assay was 20  
274 pg/ml to 1,600 pg/ml, with a sensitivity of 25 pg/ml.

275 *2.4. Analysis of relative abundances of mRNA transcripts*  
276 *for candidate genes using qPCR*

277 The relative abundance of mRNA transcripts for  
278 candidate genes was investigated using qPCR in ovarian  
279 samples from the foetuses that were of relatively lesser  
280 weights and CTMLW at GD45, GD60 and GD90 ( $n =$  five  
281 – six litters/GD).

282 *2.4.1. Total RNA extraction and cDNA synthesis*

283 The RNA was extracted from 20 to 50  $\mu$ g of snap-  
284 frozen ovarian samples as described previously  
285 (Stenhouse et al., 2019, 2018a, 2018b). The RNA was  
286 quantified, and the quality assessed  
287 spectrophotometrically using a Nanodrop ND-1000  
288 (Labtech International Ltd.) and electrophoretically using a  
289 Tapestation 2200 (Agilent Technologies; RNA Integrity  
290 Number Equivalent - RINe;  $9.209 \pm 0.009$ ). Extracted RNA  
291 was stored at  $-80^{\circ}\text{C}$  until required.

292 Complementary DNA (cDNA) was prepared from  
293 0.3  $\mu$ g of RNA with SuperScript III reverse transcriptase  
294 (Life Technologies) following the manufacturer's  
295 instructions. Each reaction contained 250 ng random  
296 primers (Promega) and 40 units RNaseIn (Promega).  
297 Negative controls without reverse transcriptase were

298 included to assess for genomic contamination and all  
299 cDNA was stored at -20°C until required.

300 *2.4.2. Quantification of relative abundance of mRNA transcripts*  
301 *for candidate genes in ovarian samples*

302 Quantitative PCR was performed using a  
303 Stratagene MX3000 instrument using SensiFAST® SYBR  
304 Lo-ROX (Bioline) utilising cDNA from foetal ovaries at  
305 GD45, GD60 and GD90. All qPCRs were conducted at an  
306 annealing temperature of 60°C and dissociation curves  
307 consisting of single peaks were generated. The relative  
308 abundance of mRNA transcripts for candidate genes was  
309 quantified: BCL-2-associated X protein (*BAX*) (Zhao et al.,  
310 2014), B-cell lymphoma 2 (*BCL2*), platelet endothelial cell  
311 adhesion molecule (*CD31*), Doublesex and Mab-3 Related  
312 Transcription Factor 1 (*DMRT1*), GATA Binding Protein 4  
313 (*GATA4*), hypoxia inducible factor 1 alpha subunit (*HIF1A*)  
314 (Oliver et al., 2011), insulin like protein 3 (*INSL3*), *KI67*,  
315 tumour suppressor protein 53 (*P53*), prostaglandin F2  $\alpha$   
316 receptor (*PTGFR*) (Kaczynski and Waclawik, 2013) ,  
317 secreted phosphoprotein 1 (*SPP1*) (Hernández et al.,  
318 2013), and vascular endothelial growth factor A (*VEGFA*)  
319 (Oliver et al., 2011). Two reference genes were used:  
320 TATA box binding protein 1 (*TBP1*) (Nygard et al., 2007)  
321 and Topoisomerase II Beta (*TOP2B*) (Erkens et al., 2006).  
322 These reference genes were identified as having stable

323 mRNA in ovarian samples by geNORM V3.5 (Ghent  
324 University Hospital, Centre for Medical Genetics). The  
325 primer sequences for all genes investigated are detailed in  
326 Supplementary Table 2.

327 Serial dilutions of pooled cDNA ranging from 1:5 to  
328 1:640 in nuclease-free water were used as standards.  
329 Sample cDNA was diluted 1:25 and 2 µl of sample,  
330 standard or control were added per well. Each plate  
331 contained duplicate wells of a no template control,  
332 standards, sample cDNA and reverse transcriptase  
333 blanks. SensiFAST® SYBR Lo-ROX supermix (5 µl), 10  
334 µM forward and reverse primer stock (0.4 µl each) and  
335 water (2.2 µl).

336 Data were analysed using qbase+ software V3.0  
337 (Biogazelle). A target and procedural specific strategy was  
338 utilised and the results, normalised to the geometric mean  
339 of two reference genes using the  $2^{-\Delta\Delta CT}$ , were scaled to the  
340 minimum sample. The mean slope, intercept, PCR  
341 efficiency and  $R^2$  values are detailed in Supplementary  
342 Table 3.

### 343 2.5. Statistical analysis

344 All statistical analyses were performed using  
345 GenStat 13.1 (VSN International Ltd.). Mean values were  
346 calculated for each individual sample for each variable  
347 investigated and the normality of the distribution of the data



348 was assessed using an Anderson-Darling test. If there was  
349 a  $P$  value of  $<0.05$ , the data were not considered to have  
350 a normal distribution. Log<sub>10</sub> and square root  
351 transformations were conducted to achieve normality of  
352 the distribution of the data where required. Outlier data  
353 points identified using a ROUT outlier test were excluded.

354       Where data had a normal distribution, ANOVA for  
355 GD or foetal size was conducted, with a block for gilt to  
356 account for the common maternal environment. When  
357 results with use of an ANOVA indicated there was  
358 significance, a *post-hoc* Tukey test was performed. Where  
359 data were not normally distributed, the Kruskal-Wallis and  
360 Mann Whitney tests were performed where appropriate.  
361 Analyses for foetal size were performed overall and within  
362 GD of interest. A two-way ANOVA with a block for gilt to  
363 account for the common maternal environment was  
364 performed to assess the presence of GD by foetal size  
365 interactions. To determine associations between litter size,  
366 percentage prenatal survival and percentage of males in  
367 the litter, and plasma oestradiol, Pearson's correlations  
368 were performed. Pearson's correlations were also  
369 performed for the comparison of foetal and ovarian weight.  
370 In all cases, significance was considered when there were  
371 mean differences with a  $P<0.05$ , with there being

372 considered to be a trending towards differences when the  
373  $P$  was  $> 0.05 < 0.1$  and not significant when  $P > 0.1$ .

### 374 **3. Results**

#### 375 *3.1. Ovarian weight associations with foetal size at GD60* 376 *or GD90*

377 Paired ovarian weight (Fig. 1A) and paired ovarian  
378 weight as a percentage of foetal weight (Fig. 1B) were not  
379 different between the female foetuses with the relatively  
380 lesser, CTMLW and relatively greater weights at GD60 or  
381 GD90. Paired ovarian weight ( $P < 0.001$ ; Fig. 1A) was  
382 greater at GD90 compared to GD60. In contrast, paired  
383 ovarian weight as a percentage of foetal weight ( $P < 0.01$ )  
384 was less at GD90 compared to GD60. Results from  
385 regression analyses indicated there was a positive  
386 association between paired ovarian and foetal weight at  
387 GD60 ( $P < 0.05$ ;  $R^2 = 0.370$ ; Fig. 1C), but not GD90 (Fig.  
388 1D). There were no associations between ovarian weight  
389 as a percentage of body and foetal weights at GD60 or  
390 GD90 (data not presented).

#### 391 *3.2. Composition of follicular pool in ovaries from the* 392 *foetuses with lesser weight compared to the CTMLW at* 393 *GD90*

394 The GATA4 stained ovaries were used for  
395 quantification of oogonia and follicles in ovaries collected

396 at GD60 and GD90 ovaries (Supplementary Fig. 1).  
397 Numbers of oogonial nests (Fig. 1E), primordial follicles  
398 (Fig. 1F) and oogonia per germ cell nest (Fig. 1H) were  
399 affected by gestational day ( $P < 0.05$ ) but not by foetal size.  
400 In contrast, there were fewer primary follicles in the  
401 fetuses with lesser weight compared to those that were  
402 CTMLW at GD90 (Fig. 1G;  $P < 0.05$ ).

### 403 *3.3. Foetal plasma oestradiol concentration associations* 404 *with foetal size at GD90*

405 There were no associations between maternal  
406 plasma oestradiol concentrations and number of live  
407 fetuses (Fig. 2A). There was a trend towards an inverse  
408 association between maternal plasma oestradiol  
409 concentration and percentage prenatal survival at GD90  
410 ( $R^2 = 0.6702$ ;  $P = 0.09$ ; Fig. 2B). Plasma oestradiol  
411 concentrations were less in the lesser compared to the  
412 greater weight female fetuses at GD90 ( $P \leq 0.05$ ; Fig.  
413 2C).

### 414 *3.4. Temporal changes in relative abundances of ovarian* 415 *CD31, PTGFR, SPP1 and VEGFA mRNA transcripts*

416 There were temporal changes in relative  
417 abundance of mRNA transcripts of several candidate  
418 genes investigated in ovaries collected at GD45, GD60  
419 and GD90 (Table 1). There was a lesser relative  
420 abundance of *CD31* mRNA transcript in ovaries between

421 GD60 and GD90 ( $P \leq 0.05$ ; Table 1).  
422 Immunohistochemical evaluations for CD31 protein in  
423 ovaries at GD90 confirmed that CD31 is exclusively  
424 present in endothelial cells in the foetal ovary  
425 (Supplementary Fig. 2). Greater relative abundances of  
426 ovarian *PTGFR* mRNA at GD90 than GD60 ( $P \leq 0.001$ ;  
427 Table 1). In contrast, there was a lesser relative  
428 abundance of ovarian *SPP1* mRNA transcript at GD60  
429 than GD90 ( $P \leq 0.01$ ; Table 1). There was a trend towards  
430 a gestational day effect on the relative abundance of  
431 *VEGFA* mRNA ( $P = 0.086$ ; Table 1). There were no  
432 temporal changes in the relative abundance of *BAX*, *BCL2*,  
433 *DMRT1*, *GATA4*, *HIF1A*, *INSL3*, *KI67* or *P53* mRNA  
434 transcripts (Table 1).

### 435 3.5. Foetal size associations with relative abundance of 436 *KI67*, *P53* and *PTGFR* mRNA transcripts

437 The relative abundance of mRNA transcripts for the  
438 candidate genes were quantified and compared between  
439 ovaries from the fetuses that had relatively lesser  
440 weights, and CTMLW fetuses within GD (Fig. 3). At  
441 GD60, there was a trend towards a lesser relative  
442 abundance of *KI67* mRNA transcript in ovarian samples  
443 from the fetuses that had relatively lesser weights as  
444 compared with CTMLW ( $P = 0.066$ ; Fig. 3I). Similarly, there  
445 was a lesser relative abundance of *P53* mRNA transcript

446 in ovarian samples from fetuses that had relatively lesser  
447 weights than CTMLW at GD60 ( $P \leq 0.05$ ; Fig. 3J). The  
448 relative abundance of *PTGFR* mRNA transcript was less  
449 in ovaries of fetuses that had relatively lesser weights  
450 than the fetuses with CTMLW at both GD45 and GD60  
451 ( $P \leq 0.05$ ; Fig. 3K). There were GD by foetal size  
452 interactions for *DMRT1* ( $P < .05$ ; Fig. 3E) and *PTGFR*  
453 ( $P = 0.01$ ; Fig. 3K) mRNA transcripts. There were no other  
454 GD x foetal size interactions. There were associations  
455 between foetal size and relative abundances of ovarian  
456 *BAX* (Fig. 3A), *BCL2* (Fig. 3B), *CD31* (Fig. 3D), *GATA4*  
457 (Fig. 3F), *HIF1A* (Fig. 3G), *INSL3* (Fig. 3H), *SPP1* (Fig. 3L)  
458 or *VEGFA* (Fig. 3M) mRNA transcripts.

#### 459 **4. Discussion**

460 Improved understanding of the mechanisms and  
461 timing of developmental changes in the foetal ovary is  
462 important for improving knowledge of how female pigs  
463 reach their reproductive potential postnatally. This study  
464 was conducted to determine whether there were  
465 differences in foetal ovarian gene expression, as evaluated  
466 by determination of relative abundances of mRNA  
467 transcripts for genes of interest, between littermate  
468 fetuses of different size from as early as GD45.

469 Dysregulation of the hypothalamic-pituitary-gonadal  
470 axis can alter the production of androgens and oestrogens,

471 in turn causing defective reproductive organogenesis and  
472 altered development of the foetus (Brooks and Thomas,  
473 1995). The lesser circulating oestradiol concentrations on  
474 GD90 in the lesser compared to greater weight foetuses  
475 indicates there needs to be further investigation into the  
476 prenatal development of the hypothalamus and pituitary in  
477 growth-restricted pig foetuses at multiple stages of  
478 gestation.

479 Primordial germ cells have been observed in the  
480 foetal pig ovary from as early as GD18. By GD30, germ  
481 cells are undergoing proliferation (Black and Erickson,  
482 1968) which allows for an increase to maximal numbers by  
483 GD50 (Black and Erickson, 1968). Considering the marked  
484 differences that have been reported regarding variation in  
485 foetal size of pigs from this early stage of gestation (Finch  
486 et al., 2002; Foxcroft et al., 2006; Foxcroft and Town, 2004;  
487 Pettigrew et al., 1986; Wise et al., 1997), and the marked  
488 developmental changes of the ovary during early and mid-  
489 gestation, it was hypothesized that that there would be  
490 differences in relative abundances of mRNA transcripts in  
491 ovaries for candidate genes involved in extracellular-matrix  
492 remodelling, proliferation and apoptosis as gestational  
493 stages advanced and between littermates of different  
494 weight.

495           The SPP1 protein, also known as osteopontin, is an  
496 extra-cellular matrix (ECM) protein which functions by  
497 binding to integrin receptors present on the cell surface to  
498 promote cellular adhesion and communication  
499 (Humphries, 2006). As indicated by the follicular counts in  
500 the present study (Figure 1) and evaluations of GATA4  
501 stained sections (Supplementary Figure 1), there were  
502 marked changes in the structure of the ovary during  
503 gestation. There are these developmental changes in the  
504 ovary so that essential functions can occur that are related  
505 to female fertility postnatally. Considering the important  
506 functions of this extracellular matrix protein in other tissues  
507 (Fisher et al., 2001; Johnson et al., 2014, 2003), the  
508 gestational changes in relative abundance of *SPP1* mRNA  
509 transcript were expected. Further studies should be  
510 performed to characterise the functions of SPP1, integrins,  
511 and other ligands of the integrin receptors such as  
512 fibronectin, in the foetal ovary, improving the  
513 understanding of the mechanisms involved in foetal  
514 ovarian development.

515           In the current study, there was a marked decrease  
516 in relative abundance of *Ki67* mRNA transcript in ovaries  
517 of the fetuses with lesser weights compared to the  
518 CTMLW at GD60. There was a similar pattern at GD45  
519 although there was not statistical significance. Growth-

520 restricted gilts at birth have delayed initiation of follicular  
521 development, with more primordial follicles, and fewer  
522 primary and secondary follicles compared to foetuses from  
523 gilts fed a diet meeting nutritional requirements for growth  
524 (Da Silva-Buttkus et al., 2003). Although not statistically  
525 significant, Da Silva-Buttkus et al. reported that there was  
526 a larger number of oogonial nests at birth in ovaries of  
527 foetuses from growth restricted animals. In the present  
528 study, there was a lesser number of primary follicles in the  
529 ovary of the foetuses of lesser weight compared with those  
530 with CTMLW at GD90, indicating there were differences in  
531 follicle number at this stage of gestation that were also  
532 present at the time of birth. Together, the histological and  
533 mRNA transcript abundance data warrant further  
534 examination into germ and somatic cell proliferation and  
535 degeneration throughout gestation, with a particular focus  
536 between days 45 and 90 of gestation.

537         Even though it is recognised that angiogenesis is  
538 essential for organ development and growth, and the  
539 processes of angiogenesis are integral for postnatal  
540 ovarian development, little is known regarding  
541 vascularisation of the foetal ovary. In the present study  
542 there was a decrease in the relative abundance of *CD31*  
543 mRNA transcript between GD60 and GD90. The CD31  
544 protein is an endothelial cell marker, present at the



545 junctions between endothelial cells (Kim et al., 2013;  
546 Mamdouh et al., 2003). Furthermore, the CD31 protein is  
547 thought to be involved in angiogenesis by regulating  
548 endothelial cell migration (Cao et al., 2009, 2002; DeLisser  
549 et al., 1997; Matsumura et al., 1997; Yang et al., 1999;  
550 Zhou et al., 1999). Results from immunohistochemical  
551 evaluations for CD31 in the foetal ovaries at GD90  
552 confirmed that CD31 is present in endothelial cells of the  
553 foetal ovary and there are indications that the foetal ovary  
554 has undergone marked vascularisation prior to this stage  
555 of development. It is hoped that further studies will be  
556 conducted to elucidate the timing and pattern of  
557 vascularisation in the foetal ovary.

558         The function of  $\text{PGF2}\alpha$  in the development and  
559 maintenance of the corpora lutea (CL) is well  
560 characterised. There is no question as to whether  $\text{PGF2}\alpha$   
561 is essential for luteolysis and the establishment of  
562 pregnancy (Geisert and Bazer, 2015; McCracken et al.,  
563 1999; Moeljono et al., 1977; Przygodzka et al., 2016,  
564 2015; Waclawik et al., 2017; Ziecik et al., 2017). The  
565 function of the  $\text{PTGFR}$  in foetal ovarian development is not  
566 known. One hypothesis could be that the increase in  
567 abundance of  $\text{PTGFR}$  mRNA in the foetal ovary with  
568 advancing gestational day is indicative that there are  
569 ovarian developmental changes occurring in preparation

570 for postnatal ovarian functions. Binding of  $\text{PGF2}\alpha$  to the  
571 *PTGFR* results in increased production of VEGFA by the  
572 endometrium, increasing angiogenesis (Kaczynski et al.,  
573 2016). Considering the functions of  $\text{PGF2}\alpha$  in  
574 angiogenesis, it could also be hypothesized that this  
575 increase in abundance of *PTGFR* in late gestation may be  
576 a mechanism to further increase vascularization of the  
577 foetal ovary during this period of marked remodelling and  
578 growth. Because there was a decrease in the relative  
579 abundance of *PTGFR* mRNA transcript in the foetuses that  
580 were of a lesser weight compared to those of average  
581 weight at GD45 and GD60, this may warrant further  
582 investigation into angiogenesis in growth-restricted foetal  
583 ovaries during these stages of development. This study, to  
584 our knowledge, is the first in which there is evaluation of  
585 an association between foetal size and the relative  
586 abundance of *PTGFR* mRNA transcript in the foetal ovary  
587 of pigs. There should be further investigation of the protein  
588 to identify which cell types produce *PTGFR* and to  
589 elucidate the functions of  $\text{PGF2}\alpha$  in gonadal development.

590         Growth restriction leads to a phenotype where pig  
591 foetuses are of a lesser weight, with impaired adaptation  
592 to extra-uterine conditions, lesser rates of pre-weaning  
593 survival, altered postnatal growth trajectories, undesirable  
594 carcass qualities and impaired reproductive performance

595 post-puberty (Wu et al., 2006). Male piglets are often  
596 considered to be at a disadvantage from a product  
597 productivity perspective postnatally (Baxter et al., 2012),  
598 and results from recent investigations assessing IUGR  
599 pigs have indicated female piglets are more likely to  
600 undergo compensatory growth than male IUGR piglets  
601 (Gonzalez-Bulnes et al., 2012). In the current study,  
602 several differences in gene expression, as evidenced by  
603 relative abundances of mRNA transcripts, were observed  
604 at GD45 or GD60 but not GD90. This could be interpreted  
605 either that there are changes that occur in the early foetal  
606 ovary which program the postnatal impaired reproductive  
607 phenotype in small gilts or that whilst there are marked  
608 changes in the foetal ovary early in development, there  
609 appears to be compensation in ovarian development as  
610 gestational stage advances.

611

## 612 **5. Conclusions**

613         These findings indicate there are differences in  
614 reproductive potential of lesser birthweight females  
615 postnatally that are programmed early in gestation. It is  
616 hoped that further investigation will improve the  
617 understanding of the association between prenatal  
618 reproductive development and postnatal reproductive  
619 performance.

620

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624

**625 Author contribution statement**

626 CS, CJA, and FXD devised the experiment. CS, CJA and  
627 COH collected samples. CS and YCA performed the  
628 experiments. CS, YCA and CJA analysed the data. COH  
629 provided technical support. CS and CJA prepared the final  
630 manuscript. All authors approved the manuscript.

631

**632 Competing interest statement**

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

636

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646 **References**

647 Abbott, D.H., Padmanabhan, V., Dumesic, D.A., 2006.

648 Contributions of androgen and estrogen to fetal  
649 programming of ovarian dysfunction. *Reprod. Biol.*

650 *Endocrinol.* 4, 1–8. <https://doi.org/10.1186/1477->

651 7827-4-17

652 Ashworth, C.J., Finch, A.M., Page, K.R., Nwagwu, M.O.,

653 McArdle, H.J., 2001. Causes and consequences of

654 fetal growth retardation in pigs. *Reprod. Suppl.* 58,

655 233–46.

656 Baxter, E.M., Jarvis, S., Palarea-Albaladejo, J., Edwards,

657 S.A., 2012. The weaker sex? the propensity for male-

658 biased piglet mortality. *PLoS One* 7, e30318.

659 <https://doi.org/10.1371/journal.pone.0030318>

660 Black, J.L., Erickson, B.H., 1968. Oogenesis and ovarian

661 development in the prenatal pig. *Anat. Rec.* 161, 45–

662 55. <https://doi.org/10.1002/ar.1091610105>

663 Brooks, A., Thomas, G., 1995. Ontogeny and function of

664 the pituitary-gonadal axis during fetal development in

665 sheep. *Reprod. Domest. Anim.* 30, 158–162.

- 666 Cao, G., Fehrenbach, M.L., Williams, J.T., Finklestein,  
667 J.M., Zhu, J.X., DeLisser, H.M., 2009. Angiogenesis  
668 in platelet endothelial cell adhesion molecule-1-null  
669 mice. *Am. J. Pathol.* 175, 903–915.  
670 <https://doi.org/10.2353/ajpath.2009.090206>
- 671 Cao, G., O'Brien, C.D., Zhou, Z., Sanders, S.M.,  
672 Greenbaum, J.N., Makrigiannakis, A., DeLisser, H.M.,  
673 2002. Involvement of human PECAM-1 in  
674 angiogenesis and in vitro endothelial cell migration.  
675 *Am. J. Cell Physiol.* 282, C1181–C1190.  
676 <https://doi.org/10.1152/ajpcell.00524.2001>
- 677 Da Silva-Buttkus, P., Hurk, R.V.D., Velde, E.R., Taverne,  
678 M.A.M., 2003. Ovarian development in intrauterine  
679 growth-retarded and normally developed piglets  
680 originating from the same litter. *Reproduction* 126,  
681 249–258.
- 682 Da Silva, P., Aitken, R.P., Rhind, S.M., Racey, P.A.,  
683 Wallace, J.M., 2002. Impact of maternal nutrition  
684 during pregnancy on pituitary gonadotrophin gene  
685 expression and ovarian development in growth-  
686 restricted and normally grown late gestation sheep  
687 fetuses. *Reproduction* 123, 769–777.
- 688 DeLisser, H.M., Christofidou-Solomidou, M., Strieter, R.M.,  
689 Burdick, M.D., Robinson, C.S., Wexler, R.S., Kerr,

- 690 J.S., Garlanda, C., Merwin, J.R., Madri, J.A., Albelda,  
691 S.M., 1997. Involvement of endothelial PECAM-  
692 1/CD31 in angiogenesis. *Am. J. Pathol.* 151, 671–677.
- 693 Erkens, T., Van Poucke, M., Vandesompele, J., Goossens,  
694 K., Van Zeveren, A., Peelman, L.J., 2006.  
695 Development of a new set of reference genes for  
696 normalization of real-time RT-PCR data of porcine  
697 backfat and longissimus dorsi muscle, and evaluation  
698 with PPARGC1A. *BioMed Cent. Biotechnol.* 6, 1–8.  
699 <https://doi.org/10.1186/1472-6750-6-41>
- 700 Finch, A.M., Anitpatis, C., Pickard, A.R., Ashworth, C.J.,  
701 2002. Patterns of fetal growth within Large White X  
702 Landrace and Chinese Meishan gilt litters at three  
703 stages of gestation. *Reprod. Fertil. Dev.* 14, 419–425.
- 704 Fisher, L.W., Torchia, D.A., Fohr, B., Young, M.F.,  
705 Fedarko, N.S., 2001. Flexible Structures of SIBLING  
706 Proteins, Bone Sialoprotein, and Osteopontin.  
707 *Biochem. Biophys. Res. Commun.* 280, 460–465.  
708 <https://doi.org/10.1006/bbrc.2000.4146>
- 709 Foxcroft, G.R., Dixon, W.T., Novak, S., Putman, C.T.,  
710 Town, S.C., Vinsky, M.D.A., 2006. The biological  
711 basis for prenatal programming of postnatal  
712 performance in pigs. *J. Anim. Sci.* 84, E105–E112.
- 713 Foxcroft, G.R., Town, S.C., 2004. Prenatal Programming

- 714 of Postnatal Performance – the Unseen Cause of  
715 Variance. *Adv. Pork Prod.* 15, 4–10.
- 716 Geisert, R.D., Bazer, F.W., 2015. Regulation of  
717 Implantation and Establishment of Pregnancy in  
718 Mammals. [https://doi.org/10.1007/978-3-319-15856-](https://doi.org/10.1007/978-3-319-15856-3)  
719 3
- 720 Gonzalez-Bulnes, A., Ovilo, C., Lopez-Bote, C., Astiz, S.,  
721 Ayuso, M., Perez-Solana, M., Sanchez-Sanchez, R.,  
722 Torres-Rovira, L., 2012. Gender-specific early  
723 postnatal catch-up growth after intrauterine growth  
724 retardation by food restriction in swine with  
725 obesity/leptin resistance. *Reproduction* 144, 269–278.  
726 <https://doi.org/10.1530/REP-12-0105>
- 727 Hernández, S.C., Hogg, C.O., Billon, Y., Sanchez, M.P.,  
728 Bidanel, J.P., Haley, C.S., Archibald, A.L., Ashworth,  
729 C.J., 2013. Secreted phosphoprotein 1 expression in  
730 endometrium and placental tissues of hyperprolific  
731 large white and meishan gilts. *Biol. Reprod.* 88, 1–7.  
732 <https://doi.org/10.1095/biolreprod.112.104679>
- 733 Hoving, L.L., Soede, N.M., Graat, E.A.M., Feitsma, H.,  
734 Kemp, B., 2010. Effect of live weight development and  
735 reproduction in first parity on reproductive  
736 performance of second parity sows. *Anim. Reprod.*  
737 *Sci.* 122, 82–89.



- 738 <https://doi.org/10.1016/j.anireprosci.2010.07.009>
- 739 Humphries, J.D., 2006. Integrin ligands at a glance. *J. Cell*  
740 *Sci.* 119, 3901–3903.  
741 <https://doi.org/10.1242/jcs.03098>
- 742 Johnson, G.A., Burghardt, R.C., Bazer, F.W., 2014.  
743 Osteopontin: A leading candidate adhesion molecule  
744 for implantation in pigs and sheep. *J. Anim. Sci.*  
745 *Biotechnol.* 5, 1–14. [https://doi.org/10.1186/2049-](https://doi.org/10.1186/2049-1891-5-56)  
746 [1891-5-56](https://doi.org/10.1186/2049-1891-5-56)
- 747 Johnson, G.A., Burghardt, R.C., Bazer, F.W., Spencer,  
748 T.E., 2003. Osteopontin: Roles in Implantation and  
749 Placentation. *Biol. Reprod.* 69, 1458–1471.  
750 <https://doi.org/10.1095/biolreprod.103.020651>
- 751 Kaczynski, P., Kowalewski, M.P., Waclawik, A., 2016.  
752 Prostaglandin F<sub>2</sub>α promotes angiogenesis and  
753 embryo-maternal interactions during implantation.  
754 *Reproduction* 151, 539–552.  
755 <https://doi.org/10.1530/REP-15-0496>
- 756 Kaczynski, P., Waclawik, A., 2013. Effect of conceptus on  
757 expression of prostaglandin F<sub>2</sub>α receptor in the  
758 porcine endometrium. *Theriogenology* 79, 784–790.  
759 <https://doi.org/10.1016/j.theriogenology.2012.12.003>
- 760 Kim, M., Park, H.J., Seol, J.W., Jang, J.Y., Cho, Y.S., Kim,  
761 K.R., Choi, Y., Lydon, J.P., Demayo, F.J., Shibuya,

- 762 M., Ferrara, N., Sung, H.K., Nagy, A., Alitalo, K., Koh,  
763 G.Y., 2013. VEGF-A regulated by progesterone  
764 governs uterine angiogenesis and vascular  
765 remodelling during pregnancy. *EMBO Mol. Med.* 5,  
766 1415–1430.  
767 <https://doi.org/10.1002/emmm.201302618>
- 768 Mamdouh, Z., Chen, X., Plerini, L.M., Maxfield, F.R.,  
769 Muller, W.A., 2003. Targeted recycling of PECAM  
770 from endothelial surface-connected compartments  
771 during diapedesis. *Nature* 421, 748–753.  
772 <https://doi.org/10.1038/nature01300>
- 773 Matsumura, T., Wolff, K., Petzelbauer, P., 1997.  
774 Endothelial cell tube formation depends on cadherin 5  
775 and CD31 interactions with filamentous actin. *J.*  
776 *Immunol.* 158, 3408–16.
- 777 McCoard, S.A., Wise, T.H., Fahrenkrug, S.C., Ford, J.J.,  
778 2001. Temporal and Spatial Localization Patterns of  
779 Gata4 During Porcine Gonadogenesis. *Biol. Reprod.*  
780 65, 366–374.
- 781 McCracken, J.A., Custer, E.E., Lamsa, J.C., 1999.  
782 Luteolysis : A Neuroendocrine-Mediated Event.  
783 *Physiol. Rev.* 79, 263–324.
- 784 Milligan, B.N., Fraser, D., Kramer, D.L., 2002. Within-litter  
785 birth weight variation in the domestic pig and its

- 786 relation to pre-weaning survival, weight gain, and  
787 variation in weaning weights. *Livest. Prod. Sci.* 76,  
788 181–191.
- 789 Moeljono, M.P., Thatcher, W.W., Bazer, F.W., Frank, M.,  
790 Owens, L.J., Wilcox, C.J., 1977. A study of  
791 prostaglandin F<sub>2</sub>alpha as the luteolysin in swine: II  
792 Characterization and comparison of prostaglandin F,  
793 estrogens and progesterin concentrations in utero-  
794 ovarian vein plasma of nonpregnant and pregnant  
795 gilts. *Prostaglandins* 14, 543–555.
- 796 Nygard, A.B., Jørgensen, C.B., Cirera, S., Fredholm, M.,  
797 2007. Selection of reference genes for gene  
798 expression studies in pig tissues using SYBR green  
799 qPCR. *BioMed Cent. Mol. Biol.* 8, 1–6.  
800 <https://doi.org/10.1186/1471-2199-8-67>
- 801 Oliver, G.A., Novak, S.A., Patterson, J.L.A., Pasternak,  
802 J.A.A., Paradis, F.A., 2011. Restricted feed intake in  
803 lactating primiparous sows . II . Effects on subsequent  
804 litter sex ratio and embryonic gene expression.  
805 *Reprod. Fertil. Dev.* 23, 899–911.
- 806 Oxender, W.D., Colenbrander, B., van deWiel, D.F.M.,  
807 Wensing, C.J.G., 1979. Ovarian Development in Fetal  
808 and Prepubertal Pigs. *Biol. Reprod.* 21, 715–721.
- 809 Pettigrew, J., Cornelius, S., Moser, R., Heeg, T., Hanke,

- 810 H., Miller, K., Hagen, C., 1986. Effects of oral doses  
811 of corn oil and other factors on preweaning survival  
812 and growth of piglets . J. Anim. Sci. 62, 601–12.
- 813 Pontelo, T.P., Miranda, J.R., Felix, M.A.R., Pereira, B.A.,  
814 da Silva, W.E., Avelar, G.F., Mariano, F.C.M.Q.,  
815 Guimarães, G.C., Zangeronimo, M.G., 2018.  
816 Histological characteristics of the gonads of pig  
817 fetuses and their relationship with fetal anatomical  
818 measurements. Res. Vet. Sci. 117, 28–36.  
819 <https://doi.org/10.1016/j.rvsc.2017.11.005>
- 820 Przygodzka, E., Kaczmarek, M.M., Kaczynski, P., Ziecik,  
821 A.J., 2016. Steroid hormones, prostanoids, and  
822 angiogenic systems during rescue of the corpus  
823 luteum in pigs. Reproduction 151, 135–147.  
824 <https://doi.org/10.1530/REP-15-0332>
- 825 Przygodzka, E., Witek, K.J., Kaczmarek, M.M.,  
826 Andronowska, A., Ziecik, A.J., 2015. Expression of  
827 factors associated with apoptosis in the porcine  
828 corpus luteum throughout the luteal phase of the  
829 estrous cycle and early pregnancy: Their possible  
830 involvement in acquisition of luteolytic sensitivity.  
831 Theriogenology 83, 535–545.  
832 <https://doi.org/10.1016/j.theriogenology.2014.10.016>
- 833 Quesnel, H., Brossard, L., Valancogne, A., Quiniou, N.,

- 834           2008. Influence of some sow characteristics on within-  
835           litter variation of piglet birth weight. *Animal* 2, 1842–  
836           1849. <https://doi.org/10.1017/S175173110800308X>
- 837   Royston, J.P., Fleckneww, P.A., Wootton, R., 1982. New  
838           Evidence that the Intra-Uterine Growth Retarded  
839           Piglet is a Member of a Discrete Subpopulation. *Biol.*  
840           *Neonate* 42, 100–104.
- 841   Sasaki, Y., Koketsu, Y., 2011. Reproductive profile and  
842           lifetime efficiency of female pigs by culling reason in  
843           high-performing commercial breeding herds. *J. Swine*  
844           *Heal. Prod.* 19, 284–291.
- 845   Steel, A., Athorn, R.Z., Grupen, C.G., 2018. Anti-Müllerian  
846           hormone and Oestradiol as markers of future  
847           reproductive success in juvenile gilts. *Anim. Reprod.*  
848           *Sci.*                    195,                    197–206.  
849           <https://doi.org/10.1016/j.anireprosci.2018.05.024>
- 850   Stenhouse, C., Hogg, C.O., Ashworth, C.J., 2019.  
851           Association of foetal size and sex with porcine feto-  
852           maternal interface integrin expression. *Reproduction.*  
853           <https://doi.org/10.1530/REP-18-0520>
- 854   Stenhouse, C., Hogg, C.O., Ashworth, C.J., 2018a.  
855           Associations between fetal size, sex and both  
856           proliferation and apoptosis at the porcine feto-  
857           maternal interface. *Placenta* 70, 15–24.

- 858 <https://doi.org/10.1016/j.placenta.2018.08.006>
- 859 Stenhouse, C., Hogg, C.O., Ashworth, C.J., 2018b.  
860 Associations between fetal size, sex and placental  
861 angiogenesis in the pig. *Biol. Reprod.* 100, 239–252.
- 862 Tummaruk, P., Lundeheim, N., Einarsson, S., Dalin, A.M.,  
863 2000. Factors influencing age at first mating in  
864 purebred Swedish Landrace and Swedish Yorkshire  
865 gilts. *Anim. Reprod. Sci.* 63, 241–253.  
866 [https://doi.org/10.1016/S0378-4320\(00\)00184-6](https://doi.org/10.1016/S0378-4320(00)00184-6)
- 867 van der Lende, T., Hazeleger, W., de Jager, D., 1990.  
868 Weight distribution within litters at the early foetal  
869 stage and at birth in relation to embryonic mortality in  
870 the pig. *Livest. Prod. Sci.* 26, 53–65.  
871 [https://doi.org/10.1016/0301-6226\(90\)90055-B](https://doi.org/10.1016/0301-6226(90)90055-B)
- 872 Waclawik, A., Kaczmarek, M.M., Blitek, A., Kaczynski, P.,  
873 Ziecik, A.J., 2017. Embryo-maternal dialogue during  
874 pregnancy establishment and implantation in the pig.  
875 *Mol. Reprod. Dev.* 84, 842–855.  
876 <https://doi.org/10.1002/mrd.22835>
- 877 Waldeyer, W., 1870. *Eierstock und Ei* [Ovary and egg.  
878 Leipzig: Engelmann.
- 879 Wise, T., Roberts, A.J., Christenson, R.K., 1997.  
880 Relationships of Light and Heavy Fetuses to Uterine  
881 Position, Placental Weight, Gestational Age, and Fetal

- 882 Cholesterol Concentrations. *J. Anim. Sci.* 75, 2197–  
883 2207.
- 884 Wu, G., Bazer, F.W., Burghardt, R.C., Johnson, G.A., Kim,  
885 S.W., Li, X.L., Satterfield, M.C., Spencer, T.E., 2010.  
886 Impacts of amino acid nutrition on pregnancy outcome  
887 in pigs: mechanisms and implications for swine  
888 production. *J. Anim. Sci.* 88, E195–E204.  
889 <https://doi.org/10.2527/jas.2009-2446>
- 890 Wu, G., Bazer, F.W., Wallace, J.M., Spencer, T.E., 2006.  
891 Board-invited review: intrauterine growth retardation:  
892 implications for the animal sciences. *J. Anim. Sci.* 84,  
893 2316–37. <https://doi.org/10.2527/jas.2006-156>
- 894 Yang, S., Graham, J., Kahn, J.W., Schwartz, E.A.,  
895 Gerritsen, M.E., 1999. Functional roles for PECAM-1  
896 (CD31) and VE-Cadherin (CD144) in tube assembly  
897 and lumen formation in three-dimensional collagen  
898 gels. *Am. J. Pathol.* 155, 887–895.  
899 [https://doi.org/10.1016/S0002-9440\(10\)65188-7](https://doi.org/10.1016/S0002-9440(10)65188-7)
- 900 Zhao, Y.Y., Wang, W., Li, X.X., Chen, X., Yu, P., Wang,  
901 J.J., Xu, Y.X., 2014. Effect of BMPRII gene silencing  
902 by siRNA on apoptosis and steroidogenesis of porcine  
903 granulosa cells. *Genet. Mol. Res.* 13, 9964–9975.  
904 <https://doi.org/10.4238/2014.November.28.1>
- 905 Zhou, Z., Christofidou-Solomidou, M., Garlanda, C.,

906 DeLisser, H.M., 1999. Antibody against murine  
907 PECAM-1 inhibits tumor angiogenesis in mice.  
908 *Angiogenesis* 3, 181–188. <https://doi.org/255492> [pii]  
909 Ziecik, A.J., Przygodzka, E., Kaczmarek, M.M., 2017.  
910 Chapter 12: Corpus Luteum Regression and Early  
911 Pregnancy Maintenance in Pigs, in: *The Life Cycle of*  
912 *the Corpus Luteum*. pp. 227–248.  
913 <https://doi.org/10.1007/978-3-319-43238-0>

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## 915 **Figure Legends**

916 **Fig. 1.** Foetal ovarian weights and histological analyses at  
917 GD60 and GD90; Foetal paired ovarian weight (A) and  
918 paired ovarian weight as a percentage of body weight (B),  
919 number of oogonia (E), number of primordial follicles (F),  
920 number of primary follicles (G), and number of nuclei per  
921 oogonia (H) were compared between fetuses of different  
922 size; Mean values presented.  $n = 4-7$  fetuses per group;  
923 Error bars represent S.E.M.; The association between  
924 foetal weight and paired ovarian weight at GD60 (C) and  
925 GD90 (D) was assessed.

926

927 **Fig. 2.** Maternal and foetal plasma oestradiol  
928 concentrations on GD90; Maternal plasma oestradiol  
929 concentrations were correlated with (A) number of live



930 fetuses and (B) percentage prenatal survival; Foetal  
931 plasma oestradiol concentrations were compared between  
932 fetuses of different size (C); Letters indicate that group  
933 means differ from one another ( $P < 0.05$ );  $n = 4-5$  fetuses  
934 per group; Error bars represent S.E.M.

935

936 **Fig. 3.** Candidate relative abundances of ovarian mRNA  
937 transcripts in fetuses of different sizes at days 45, 60 and  
938 90 of pregnancy; Relative abundance mRNA transcripts  
939 for *BAX* (A), *BCL2* (B) *BAX:BCL2* Ratio (C), *CD31* (D),  
940 *DMRT1* (E), *GATA4* (F), *HIF1A* (G), *INSL3* (H), *KI67* (I),  
941 *P53* (J), *PTGFR* (K), *SPP1* (L) and *VEGFA* (M) in the  
942 lesser weight to mean litter weight (CTMLW) foetal ovaries  
943 at gestational days 45, 60 and 90;  $n = 3-6$  fetuses per  
944 group; Error bars represent S.E.M.;  $*P < 0.05$ .

945

946 **Supplementary Fig. 1.** Representative Images of GATA4  
947 immunohistochemistry in foetal ovaries;  
948 Immunohistochemistry results indicated that GATA4  
949 protein is present in the somatic cells in both the fetuses  
950 of lesser weight (D and F) and those closest to mean litter  
951 weight (CTMLW) (C and E) at both gestational day (GD)  
952 60 (C and D) and 90 (E and F); Rabbit IgG controls at an  
953 equivalent protein concentration were utilised as a  
954 negative control (A and B); Scale bars represent 100  $\mu\text{m}$ .

955

956 **Supplementary Fig. 2.** Representative Images of CD31

957 Immunohistochemistry in foetal ovaries;

958 Immunohistochemistry confirmed that CD31 protein is

959 present in endothelial cells in the ovary of both the lesser

960 (C) and closest to mean litter weight (CTMLW) (B) fetuses

961 at gestational day (GD) 90; Rabbit IgG controls at an

962 equivalent protein concentration were utilised as a

963 negative control (A); Scale bars represent 100  $\mu$ m.

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