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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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7

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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 μ 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 μ 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 ± 0.009). Extracted RNA
291 was stored at -80°C until required.

292 Complementary DNA (cDNA) was prepared from
293 0.3 μ 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 α
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. Log₁₀ 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 foetuses that had relatively lesser
440 weights, and CTMLW foetuses 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 foetuses 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 fetuses
475 indicates there needs to be further investigation into the
476 prenatal development of the hypothalamus and pituitary in
477 growth-restricted pig fetuses 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 PTGFR in foetal ovarian development is not
566 known. One hypothesis could be that the increase in
567 abundance of 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
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914

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 μm .

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 μm .

964