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Reproduction

Molecular determinants of a competent bovine corpus luteum: first vs final wave dominant follicles

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1 **Molecular determinants of a competent bovine *corpus luteum*: first vs final wave dominant**

2 **follicles**

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10 **Short title (maximum 46 characters, including spaces)**

11

12 Dominant follicles and *corpus luteum* competence.

13 **Abstract**

14 Reproductive management in cattle requires the synchrony of follicle development and oestrus

15 prior to insemination. However, the ovulation of follicles that have not undergone normal

16 physiological maturation can lead to suboptimal luteal function. Here we investigated the

17 expression of a targeted set of 47 genes in (a) a first-wave vs final-wave dominant follicle (DF;

18 the latter destined to ovulate spontaneously), and (b) 6-day old corpora lutea (CLs) following

19 either spontaneous ovulation, or induced ovulation of a first-wave DF, to ascertain their

20 functional significance for competent CL development. Both the mass and progesterone

21 synthesising capacity of a CL formed following induced ovulation of a first-wave DF were

22 impaired. These impaired CLs had reduced expression of steroidogenic enzymes (e.g. *STAR* and

23 *HSD3B1*), luteotrophic receptors (*LHCGR*) and angiogenic regulators (e.g. *VEGFA*), and

24 increased expression of *BMP2* (linked to luteolysis). Relative to final-wave DFs, characteristic

25 features of first-wave DFs, included reduced oestradiol concentrations and a reduced

26 oestradiol:progesterone ratio in the face of increased expression of key steroidogenic enzymes

27 (i.e. *CYP11A1*, *HSD3B1* and *CYP19A1*) in granulosa cells; and reduced expression of the HDL

28 receptor *SCARB1* in thecal cells. Transcripts for further components of the TGF and IGF systems

29 (e.g. *INHA*, *INHBA*, *IGF2R* and *IGFBP2*) varied between first- and final-wave DFs. These results

30 highlight the importance of hormones such as progesterone interacting with local components of

31 both the TGF and IGF systems to affect the maturation of the ovulatory follicle and functional

32 competency of the subsequent CL.

33

Introduction

Since the introduction of ovulation synchronization into mainstream reproductive management of cattle (Pursley *et al*., 1995) a plethora of studies have examined in detail the benefits of follicle synchrony in reproductive management programs (Bisinotto *et al*., 2014; Wiltbank and Pursley, 2014). However, it has been shown that ovulation of follicles that have not undergone normal physiological maturation can lead to suboptimal luteal function compared to spontaneous ovulation. For example, following synchronization Perry *et al*. (2005) found that ovulation of smaller follicles (presumed to be short of full maturity) resulted in decreased pregnancy rates. This was associated with lower oestradiol (E2) on the day of insemination together with impaired subsequent luteal function. In contrast, they reported no effect of ovulatory follicle size when ovulation occurred spontaneously. Furthermore, Bisinotto *et al*. (2010) found differences in pregnancy rate following artificial insemination (AI) according to wave of the ovulated follicle, with higher pregnancy rates following ovulation of a second than a first wave dominant follicle (DF). However, less is known about the impact of ovulatory control programs on the detailed molecular control mechanisms underpinning the adequacy of the ovulatory follicle and resulting corpus luteum (CL).

The expression of several genes involved with ovulation, luteinisation and CL function is under endocrine control. Production of the prostaglandin $PGF_{2\alpha}$, for instance, has been shown to be regulated by progesterone (P4) (Sharzynski and Okuda, 1999; Okuda *et al*., 2004). A first wave DF undergoes selection during a period of low circulating P4 whereas, during later follicular waves, DF selection occurs during the luteal phase of the oestrous cycle in the presence of higher concentrations of circulating P4 (Savio *et al*., 1988; Ginther *et al*., 1989). There is evidence that P4 supplementation prior to induced ovulation (around the time of ovulatory DF selection) can increase pregnancy per AI (Wiltbank *et al*., 2011; Colazo *et al*., 2013), which is likely due to the

59   beneficial effects of P4 supplementation on the development of the first wave DF (Bisinotto *et*

60   *al.*, 2010).

61   With the foregoing discussion in mind, our hypothesis was that the hormonal milieu within which

62   a DF develops affects its ability to form a viable CL, that this is related to the expression of genes

63   with key roles in regulating DF development, subsequent luteinisation and CL function, and that

64   the expression of these genes differs between first and final wave DFs. It was also hypothesised

65   that the CL formed following induced ovulation of a first wave DF would be smaller and less

66   capable of P4 production than those formed following spontaneous ovulation, and that this would

67   be associated with altered expression of genes involved in cellular differentiation, tissue growth

68   and steroidogenesis.

69   To test these hypotheses we conducted an experiment which involved 24 cyclic virgin heifers

70   where we compared the expression of a targeted set of genes (Table 1), with established

71   physiological effects within the bovine ovary, in follicles and CLs of differing size at contrasting

72   stages of the oestrous cycle. Specifically, we wanted to compare the molecular characteristics of

73   (a) a first-wave DF to that of a final-wave DF destined to ovulate spontaneously, and (b) a 6-day

74   old CL following spontaneous ovulation to a 6-day old CL following induced ovulation of a first-

75   wave DF. These data were related to quantitative measures of steroidogenesis and local and

76   systemic growth factor and hormone concentrations.

77

78   **Materials and Methods**

79

80   **Sample Collection**

81   Twenty-four post-pubertal Hereford x Holstein heifers (mean ± SEM live weight of 417.5 ± 7.3

82   kg and body-condition score (BCS) of 2.53 ± 0.05 units; Lowman et al. (1976)) were allocated to

83   one of three treatment groups (A-C) according to live weight and BCS, giving eight animals per

4

84 treatment. Animals were group housed on straw bedding and given *ad libitum* access to water and

85 hay. Mineralised concentrates were given twice daily at a rate of 5 kg per animal per day, rising

86 to 6 kg as the animals gained weight in line with their metabolisable energy and protein

87 requirements (AFRC, 1993). All procedures were performed under the auspices of the Animal

88 Scientific Procedures Act (1986) and approved by the University of Nottingham ethical review

89 committee.

90 Oestrous cycles were synchronised initially using two intra-muscular prostaglandin (PG)

91 injections (2 ml Estrumate; Intervet UK Ltd., Milton Keynes, UK) given 11 days apart. An intra-

92 muscular injection of GnRH (2.5 ml Receptal; Intervet UK Ltd., Milton Keynes, UK) was given

93 48 hours after the initial dose of PG (Figure 1). Timing of ovulation was confirmed by transrectal

94 real time B-mode ultrasonography using an Aloka SSD-500v scanner (Aloka Co. Ltd., Tokyo,

95 Japan) equipped with a 5-MHz linear array on nominal Day -1 and +1 of the anticipated day of

96 ovulation. We (Sinclair and Mann, unpublished data) have previously observed that trans-rectal

97 ovarian ultrasonography on the expected day of ovulation can delay or inhibit this event in some

98 animals. Heifers in group A were slaughtered on Day 6 after synchronised ovulation (Day 0) to

99 recover a first-wave DF and a 6-day old, spontaneous CL. Animals in group B were given 5 ml

100 GnRH and 2 ml PG on Day 6 to cause ovulation of the first-wave DF and regression of the

101 spontaneous CL, then slaughtered on Day 13 to retrieve a 6-day old, induced CL and a DF.

102 Animals in group C were given 2 ml PG on Day 18 and slaughtered on Day 19 to retrieve a final-

103 wave DF and a regressing CL. All animals were blood sampled daily by jugular venipuncture and

104 samples were analysed for plasma P4 and insulin-like growth factor 1 (IGF-1). Additional blood

105 samples were taken from group B at 0, 1 and 2 hours after GnRH injection on Day 6 for plasma

106 LH analysis. To monitor ovarian follicular development and to confirm cyclicity, animals in

107 group C underwent transrectal ultrasonography daily, except on the day of expected ovulation.

108 All other animals underwent transrectal ultrasonography on the days prior to and following

109 expected ovulations (including that following initial synchronisation; Day 0) and on the day prior

110 to slaughter.

111 Animals were blood sampled prior to transportation to an on-site abattoir for slaughter. Ovaries

112 from each animal were recovered, transferred to the laboratory within 10 minutes of slaughter

113 and processed immediately. The largest follicle ($\geq$ 11 mm) was dissected from each pair of

114 ovaries. Follicular fluid was aspirated from this large follicle (presumed to be a DF), the largest

115 subordinate follicle (SF) and a selection of smaller subordinate follicles (2-6 mm) from each pair

116 of ovaries and stored at -20°C. Granulosa cells were then scraped from the DF and washed in

117 PBS before storage at -80°C in RLT+ lysis buffer (Qiagen, Crawley, UK). The thecal sheet was

118 then peeled away from the DF wall using a pair of fine forceps, washed in PBS and stored at

119 -80°C in RLT+ lysis buffer (Qiagen, Crawley, UK).

120 CLs were dissected from ovaries, measured, weighed and then divided into three sections. The

121 first section was minced using a scalpel blade, washed in PBS then centrifuged at 1,500 g for 3

122 minutes. RLT+ lysis buffer (Qiagen, Crawley, UK) was added to the cell pellet, which was stored

123 at -80°C to be homogenised immediately prior to RNA extraction. The second section was

124 minced and washed in PBS then divided further to give three 25 mg ($\pm$ 2 mg) samples per animal,

125 which were snap frozen in liquid nitrogen and stored at -80°C prior to P4 analysis by ELISA. The

126 third section was also minced, washed and divided to give three 25 mg ($\pm$ 2mg) samples per

127 animal. These samples were re-suspended in 2 ml culture medium (M199 containing 0.068 mM

128 L-glutamine) and incubated at 38°C for 30 min in a shaking water bath at 70 strokes per minute.

129 They were then centrifuged at 1,500g for 5min and the tissue and spent media snap frozen

130 separately in liquid nitrogen and stored at -80°C prior to P4 analysis by ELISA.

131 **Hormone Assays**

132 A commercially available ELISA kit (Ridgeway Science, St. Briavels, UK) was used to measure

133 P4 in follicular fluid, blood plasma, spent culture media and CL extracts as previously described

6

134   (Wonnacott *et al.*, 2010). CL tissue samples were ethanol extracted prior to P4 assay. 5 ml

135   double-distilled ethanol was added to each sample, on ice. The samples were homogenised for 30

136   seconds (Polytron PT400; Kinematica, Lucerne, Switzerland), evaporated to dryness using a

137   speedvac (Savant DNA 110; Thermo Fisher Scientific, Cramlington, UK) and then re-dissolved

138   in 1 ml PBS. Plasma standards and quality controls (QCs) (Ridgeway Science, St. Briavels, UK)

139   were used when analysing blood plasma samples and buffer standards and QCs (Ridgeway

140   Science, St. Briavels, UK) were used when analysing all other samples, with intra- and inter-

141   assay coefficients of variation of 6.69% and 5.68%, respectively.

142   Oestradiol was measured by radioimmunoassay, as previously described (Kanakkaparambil *et al.*,

143   2009). The intra- and inter-assay coefficients of variation for this assay were 4.63% and 11.63%

144   respectively. A commercially available bovine ELISA kit (LH Detect; ReproPharm, France) was

145   used to measure LH in blood plasma. The intra- and inter-assay coefficients of variation for this

146   assay were 5.49% and 15.24% respectively.

147   A commercially available kit was used to measure IGF1 in blood plasma (DRG Instruments

148   GmbH, Marburg, Germany) from day 0 and the day of slaughter (refer to Figure 1). No sample

149   dilution was necessary. The intra- and inter-assay coefficients of variation for this assay were

150   4.43% and 7.21% respectively.

151   **Transcript Expression**

152   RNA extraction was performed using a commercially available kit (RNeasy mini kit; Qiagen,

153   Crawley, UK) and RNA concentration was determined using a NanoDrop ND-1000 UV-vis

154   spectrophotometer (Thermo Fisher Scientific, Cramlington, UK). Samples were diluted in RNase

155   free water to a concentration of 20 ng/µl prior to a further gDNA removal step using another

156   commercially available kit (TURBO DNA-free; Ambion, Huntingdon, UK). mRNA was

157   denatured at $70^{o}C$ for 10 min using a thermal cycler (BioRad, Hemel-Hempstead, UK) prior to

158   RT and subsequent transcript expression analysis.

7

159   Expression of 47 genes (Table 1A) known to regulate DF and CL function was quantified using

160   the GenomeLab GeXP Genetic Analysis System (Beckman Coulter Inc., High Wycombe, UK).

161   This method utilises gene specific primers that have a universal sequence tag. Forward universal

162   primers within the PCR buffer are fluorescently labelled, allowing detection and quantification of

163   up to 30 size separated products within a single PCR reaction (Wu *et al*., 2008; Rai *et al*., 2009).

164   Transcripts were divided arbitrarily between two multiplex reactions. Due to the size and relative

165   importance of the *LHCGR* transcript in ovulation and luteinisation, a separate multiplex reaction

166   was designed to amplify several regions of the mRNA (Table 1b).

167   RT-PCR reactions were performed using reagents (including an internal standard) and software

168   provided by Beckman Coulter Inc. (High Wycombe, UK). For each of three different multiplex

169   reactions, reverse primers (Sigma-Genosys Ltd., Poole, UK) were mixed together at

170   concentrations ranging from 60 to 1,500 nM in 10 mM Tris-HCl solution, pH 8.0 (Sigma-

171   Aldrich, Poole, UK) and forward primers (Sigma-Genosys Ltd., Poole, UK) were mixed together

172   at a concentration of 200 nM each using the same solution. Reverse primer concentrations were

173   adjusted to allow for variation in initial concentrations of mRNA templates and primer

174   efficiencies. Forward primers contained universal sequence tags used for amplification after the

175   first few cycles of PCR. For primer sequences see Tables 1A and 1B. RT was carried out

176   including the mixture of reverse primers, and 35 cycles of PCR were carried out including the

177   mixture of forward primers, as per the manufacturer's instructions. The resulting PCR products

178   were diluted 1:30 in water and 2µl of diluted sample was mixed with 0.5µl DNA size standard-

179   400 and 37.5µl sample loading solution in an appropriate well of a 96-well electrophoresis plate

180   and covered with mineral oil. The plate was then placed in a GeXP Genetic Analysis System

181   which separates the PCR products by capillary electrophoresis.

182   Data was checked using the fragment analysis module of the GenomeLab GeXP system software

183   and any samples lacking a peak from the internal standard, Kan[r], were repeated. The fragment

184   data and peak area was then imported into the eXpress Analysis module of eXpress Profiler

185   software, where fragments are linked with gene information giving expression, in arbitrary

186   fluorescence units, for each transcript within each well. This was then exported into Microsoft

187   Excel and transcript expression was normalised within each sample by dividing the target

188   expression by the average expression of the three control genes, giving target expression relative

189   to *GAPDH*, *H2AZ* and *RPLP0* in relative fluorescence units. Although the GeXP multiplex

190   technology is tried and tested, by way of validation in our hands we compared expression of a

191   number of genes using quantitative real-time PCR and GeXP (Supplementary Figure 1).

192   **Western Blotting**

193   In support of the transcript data that emerged from this study, and given that the follicular fluids

194   collected were committed fully to steroid analyses, additional pairs of bovine ovaries were

195   collected from a local abattoir, retaining individual animal identity, and classified according to

196   stage of the oestrous cycle by assessing gross morphology of the CL, based on the observations

197   and classification of Ireland *et al*. (1980). Pairs of ovaries presenting healthy, non-atretic follicles

198   were classified as originating from either the early follicular- or early luteal-phases. The largest

199   follicle (10-14 mm) per pair of ovaries was dissected, aspirated and granulosa cells scraped and

200   washed as described previously.

201   Follicular fluid samples (5 μl), in Laemmli buffer were subjected to electrophoresis on 10% SDS-

202   polyacrylamide gels. Proteins were blotted onto nitrocellulose membrane (Optitran BA-S 83,

203   Schleicher & Schuell). Membranes were incubated for 60 min at 21°C with blocking solution

204   (PBS, pH 7.4 with 0.05% Tween-20 and 3% non-fat milk powder) and then incubated overnight

205   at 4°C in the same solution containing the specific primary antiserum (rabbit anti-IGFBP-2,

206   Upstate Biotechnology) diluted 1:1500. The membranes were washed three times with PBS-

207   Tween and then incubated with HRP-labelled anti-rabbit IgG (BioRad) diluted 1:25000 in

208   blocking solution for 60 min at 21°C. Membranes were washed twice for ten minutes with PBS-

209   Tween and once with PBS. The bands were visualised using enhanced chemiluminescence (ECL,

210   GE Healthcare) and detected on BioMax Light film (Carestream). Bands were quantified using

211   Image J software.

212   **Statistical analysis**

213   All statistical analysis was performed using Genstat version 11.1.0.1504 (VSN International Ltd.,

214   Hemel-Hempstead, UK). Necessary transformations of P4 and E2 data were determined by Box-

215   Cox analysis. Analysis of variance (ANOVA) was used to compare E2 concentrations, P4

216   concentrations, and gene expression between stages of the oestrous cycle. DF size, CL weight

217   and CL size were also compared by ANOVA. For transcript analyses a common approach in

218   simultaneous testing is the Benjamini and Hochberg linear step-up false discovery rate (FDR)

219   controlling procedure (Reiner et al., 2003). For such data an FDR of 0.25 (q) is typically applied

220   to avoid a high proportion of false negatives. P values ($P_{(1)} \leq \ldots \leq P_{(m)}$) were ordered along with

221   their respective null hypotheses ($H_{(1)}, \ldots, H_{(m)}$), and ranked P_i were compared to the critical value

222   q.i/m. In this analysis k = max i for which $P_i \leq$ q.i/m. We then rejected $H_{(1)}, \ldots, H_{(k)}$. Treatment

223   comparisons were then made using the least significant difference test.

224

225   **Results**

226

227   *Ovarian follicle and CL development*

228   Shortly after the onset of oestrous synchrony (i.e. Day -13, Figure 1A), ultrasound scanning

229   confirmed that a CL was present in all 8 heifers allocated to Group A, 6/8 heifers allocated to

230   Group B, and 6/8 heifers allocated to Group C. Ultrasound scanning on Day -13 (one day prior to

231   GnRH) further confirmed the presence of follicles ≥ 8 mm in diameter in all 24 heifers (size

232   range 8 to 18 mm). One of the two heifers in Group B that didn't have a CL present at the onset

233   of synchrony subsequently failed to ovulate at Day 0 (Figure 1A), so this animal was removed

234   from any further analysis. All other animals ovulated between 11 am on Day -1 and 11 am on

235   Day +1 as expected, and diameter of the ovulatory follicle (i.e. its last recorded diameter as

236   determined by ultrasound scanning) did not differ between groups (mean ± SEM of 13.4 ± 2.62

237   mm). Similarly, DF and CL diameter on Day 5 did not differ between groups (mean values of

238   11.6 ± 2.02 mm and 18.7 ± 4.32 mm respectively). Plasma P4 concentrations were also found not

239   to differ between groups prior to Day 6 (Figure 1B). Of the seven animals remaining in Group B,

240   all underwent luteal regression, resulting in a decrease in plasma P4 (Figure 1B), and ovulated

241   between 11 am on Day 6 and 11 am on Day 8. Response to GnRH was supported by an

242   immediate increase (P<0.001) in plasma LH (from 1.0 ± 0.64 pg/ml at the time of GnRH

243   administration to 8.7 ± 0.83 ng/ml two hours later), followed by disappearance of the DF within

244   48 h. Ovulatory-follicle diameter was compared between the initial, synchronised ovulation

245   (Groups A, B and C; Day 0) and induced ovulation (Group B; Day 7), but no significant

246   difference was detected (13.4 vs 12.0 mm (P=0.09) measured on Days -1 and 6 respectively;

247   Supplementary Figure 3A). Furthermore, when it came to slaughter there was no difference in DF

248   diameter between groups (Table 2A). However, CLs collected from Group B at slaughter were

249   smaller (P = 0.021) and weighed less (P = 0.049) than those collected from group A (Table 2A).

250   Of the 8 heifers in Group C, 3 had a two-wave cycle, 4 had a three-wave cycle, and one had a

251   four-wave cycle. For DFs at slaughter in these animals, the time interval from initial visualisation

252   (≤ 2 mm) to slaughter was 8.25 ± 0.48 vs 6.0 ± 0.57 days (P = 0.024) for two- and three-wave

253   cycles respectively. There was no significant difference in diameter of the DF between two- vs

254   three-wave cycles (15.5 ± 1.29 vs 14.0 ± 2.16 mm). Similarly, there was no difference in FF

255   steroid concentrations between these two groups (E2: 402 ± 172 vs 412 ± 156 ng/ml. P4: 69 ± 13

256   vs 83 ± 46 ng/ml).

257   ***CL progesterone producing capacity***

11

258   Total P4 content (amount of P4 per CL), P4 production (amount of P4 produced per unit of tissue

259   cultured $= P4_{tissue} + P4_{media} - P4_{initial\ tissue}$) and P4 synthetic capacity (P4 production corrected for

260   total CL weight) were greater ($P = 0.035$, < 0.001 and < 0.001 respectively) for Group A than for

261   either Groups B or C (Table 2A). Furthermore analyses indicated that diameter of the follicle

262   destined to ovulate was positively ($P = 0.001$) correlated with diameter of the resulting CL six

263   days after ovulation for DFs scanned on Day -1, but not for DFs scanned on Day 6 (i.e. Group B)

264   (Supplementary Figure 3A). However, diameter of the resulting CL was not correlated with its P4

265   synthetic capacity for either Group A or B treatments (Supplementary Figure 3B), indicating that

266   size of these structures alone does not explain CL functionality.

267   *Follicular fluid hormone concentrations*

268   As one might expect, follicular-fluid P4 concentration was greater ($P<0.05$) in small follicles than

269   in DFs, and E2 concentration was greater ($P < 0.001$) in DFs than in small follicles (Table 2B).

270   There was a strong indication ($P = 0.058$) that P4 concentrations were greater in small follicles

271   from Group A than in small follicles from Groups B and C. There was also a strong indication (P

272   $= 0.056$) that E2 concentrations were lower in follicular fluids from DFs in Group A than in

273   follicular fluids from either Groups B or C. This observation was supported by a lower ($P =$

274   0.054) E2:P4 ratio in DF fluids from Group A compared to Groups B and C.

275   *Transcript expression*

276   Transcripts for *AMH*, *BMP2*, *BMP6*, *ESR1*, *FGF1*, *IGF2*, *CYP17A1*, *IL2*, *IL6*, *MIF* and *PGR*

277   were not detected in granulosa cells. Similarly transcripts for *AMH*, *BMP2*, *BMP6*, *FGF1*,

278   *CYP19A1*, *IL2*, *IL6*, *MIF* and *VEGFA* mRNA were not detected in thecal cells; and transcripts for

279   *AMH*, *BMP6*, *FGF1*, *IL2*, *IL6*, *INHA*, *LRP8* and *PGR* mRNA were not detected within the CL.

280   Although expressed in our mixed population of ovarian cells during GeXP platform development,

281   the following genes were not expressed in any of our experimental cell types: *AMH, BMP6,*

282   *FGF1, IL2* and *IL6* (see Supplementary Materials and Results).

283   In granulosa cells, expression of *INHA, INHBA, CYP11A1, CYP19A1, ESR2, HSD3B1, HIF1A*

284   and *PGF2AR* was greater (P < 0.05) in Group A (first wave DF) than Group C (final wave DF)

285   (Table 3A). In thecal cells, expression of *IGF2R, IGFBP2, SCARB1* and *PTPRC* was lower (P <

286   0.05) for Group A than for Group C (Table 3B). Interestingly, thecal cell *SCARB1* expression was

287   lower (P < 0.05) in Group A than Group B, and expression of *PGF2AR* was only detectable in

288   thecal cells from Group B (data not shown). *LHCGR* splice variant expression within granulosa

289   and also thecal cells of the DF did not differ with stage of the oestrous cycle.

290   Many more of our selected transcripts were differentially expressed in the CL (Table 4) than in

291   either granulosa or thecal cells (Table 3). For the CL, the greatest differences in transcript

292   expression were between Groups A and C; transcript expression for Group B often was

293   intermediate to these contrasting levels. Given that the comparison of particular interest lies

294   between Groups A and B, it is noteworthy that *BMP2* and *IGFBP5* expression was lower in CLs

295   from Group A than from Group B. In contrast, expression of *IGFBP4, HSD3B1, STAR, KITLG,*

296   *GADD45B, VEGFA, PGF2AR, LHCGRex2, -ex2(-3), -ex8* and *–ex8(-9)* was greater for Group A

297   than Group B.

Plasma IGF1 concentration

299   At Day 0 (see Figure 1), plasma IGF1 concentration was 148 ± 47 ng/ml and did not differ

300   between treatment groups. At the point of slaughter, however, plasma IGF1 was significantly

301   lower (P = 0.001) in heifers from Group A than from Groups B and C (Figure 2).

IGF2R and IGFBP2 expression in supplementary abattoir ovaries

303   In granulosa cells harvested from cycle-stage determined abattoir-derived ovaries, relative

304   expression of *IGF2R* and *IGFBP2* was greater (P = 0.004) in cells from early follicular-phase

305   (similar to Group C) than early luteal-phase (similar to Group A) dominant follicles (0.267 ±

306   0.022 vs 0.188 ± 0.018 for *IGF2R*; 0.631 ± 0.060 vs 0.353 ± 0.050 for *IGFBP2*). In agreement

307 with transcript abundance, the concentration of IGFBP2 protein in follicular fluid was greater (P

308 < 0.001) from early follicular- than early luteal-phase DFs (Figure 3).

309

310 **Discussion**

311

312 This study reports a number of key findings. Both the mass and P4 synthesising capacity of a CL

313 formed following induced ovulation of a first-wave DF (i.e. CLs from Group B in this study)

314 were reduced relative to a CL formed following spontaneous ovulation (i.e. CLs from Group A).

315 Indeed, the P4 synthetic capacity of these induced (i.e. Group B) CLs was similar to that of a

316 regressing CL (i.e. Group C) during the pro-oestrous phase of the cycle and, at a molecular level,

317 they were characterised as having reduced expression of steroidogenic enzymes (i.e. *STAR and*

318 *HSD3B1*) involved in cholesterol transfer into mitochondria and conversion of pregnenolone to

319 progesterone. These induced CLs were further characterised as having reduced expression of

320 *LHCGR* (required for luteal support; Niswender et al., 2007) and *VEGFA* (a key angiogenic

321 regulator; Robinson et al., 2007), together with increased expression of *BMP2* (linked to

322 luteolysis in regressing CLs; Nio-Kobayashi et al., 2015).

323 Regarding follicular development, relative to final wave DFs (i.e. those from Group C at Day 19),

324 key functional features of first wave DFs (i.e. those from Group A at Day 6, coinciding with

325 GnRH treatment in Group B) included reduced E2 concentrations and a reduced E2:P4 ratio.

326 These differences occurred in the face of increased transcript expression of key steroidogenic

327 enzymes (i.e. *CYP11A1* (encoding cholesterol side-chain cleavage), *HSD3B1* and *CYP19A1*

328 (encoding aromatase)) in granulosa cells; and reduced expression of *SCARB1* (which facilitates

329 cellular cholesterol uptake from high-density lipoproteins; Azhar et al., 1998) in thecal cells. Also

330 different between these two DF groups were transcripts for two inhibin/activin subunits (i.e.

331 *INHA* and *INHBA*), which were both increased in first wave relative to final wave DFs.

332   Importantly, given that background plasma and follicular-fluid P4 levels were similar between

333   Groups A and B (Figure 1B and Table 2B), it is noteworthy that transcript expression for a range

334   of genes in granulosa and thecal cells from both Groups A and B were also similar, highlighting

335   the importance of P4 as a regulator of follicular maturation. Finally, it is also worth noting the

336   differences in transcript expression of *IGF2R* and *IGFBP2* in both granulosa and thecal cells, and

337   protein expression of IGFBP2 in follicular fluid, between first- and final-wave DFs (i.e. Group A

338   vs Group C). These were consistently lower in first- relative to final-wave DFs, when circulating

339   levels of IGF1 were also at their lowest (Figure 3). These differences seem to be of key

340   significance given that these IGF family members each serve to regulate the bioavailability of

341   both IGF-1 and -2 within the ovarian follicle (Webb and Campbell, 2007). However, the issue of

342   proximity to PG administration cannot be discounted. Indeed, *PGF2AR* expression was lower in

343   Group C ovarian cells (i.e. granulosa and luteal) than in Group A, with Group B in between. This

344   could be due to direct or indirect actions of PG.

345   Collectively, these results indicate an important role of P4 during terminal follicle maturation that

346   determines subsequent luteal competence, although the effects of endogenous LH, which are well

347   established (e.g. Qunintal-Franco et al., 1999) but not determined in this study, and differences in

348   the nature and timing of pharmacological intervention (i.e. PG relative to endogenous or

349   administered GnRH) between groups cannot be discounted. Indeed, in sheep Murdoch and Van

350   Kirk (1998) found that premature induction of ovulation (i.e. 12 h vs 36 h after PG-induced

351   luteolysis) compromised the formation of a functionally competent CL. In the current study,

352   follicles that gave rise to less competent CLs were less oestrogenic than those that gave rise to

353   more competent CLs, and the data point to underlying contributions by components of both the

354   TGF and IGF systems.

355   ***The 'final-wave' dominant follicle***

356   In the current study one could consider the DF that ovulated around Day 0 to be representative of

357   a 'final wave' DF, although it is recognised that this follicle did not occur in a natural,

358   uncontrolled oestrous cycle, but rather in one where both follicle and CL development were

359   regulated and synchronised (Figure 1). This was necessary for experimental purposes as it

360   standardised follicle development to a more precisely timed ovulation. From the perspective of

361   assisted reproduction, it is also representative of protocols routinely used for oestrous

362   synchronisation. Furthermore, ovulation of the resultant DF was induced by the endogenous

363   surge of LH that followed the second prostaglandin treatment; and thus more closely resembles

364   the natural ovulatory process than that represented by the GnRH-induced ovulation of a Day 6

365   DF. However, this 'final-wave' DF (i.e. destined to ovulate around Day 0) probably developed

366   under a low P4 environment (not determined), given that PG administration preceded GnRH

367   treatment during the initial synchrony programme (Figure 1 A). In contrast, the DFs harvested

368   from Group C heifers on Day 19 (24 h after PG) better represent the normal final-wave, pre-

369   ovulatory follicle.

370   *Corpus luteum*

371   In bovine assisted reproduction, either follicle ablation or aspiration (to recover ova) close to the

372   anticipated time of ovulation leads to the formation of small CLs, with reduced capacity to

373   produce and secrete P4 (O'Hara et al., 2012). This reduction in P4 secretion is, in turn, associated

374   with reduced expression of *LHCGR* in luteal tissue. These authors commented that this may be

375   due in part to removal of a variable number of granulosa cells that would otherwise have

376   contributed to luteal formation; although given the preferential localisation of LHCGR to small

377   (i.e. theca derived) luteal cells (Yuan and Lucy, 1996; Mamluk et al., 1998) it is uncertain if this

378   alone could account for reduced *LHCGR* expression. The study of Hayashi et al. (2006),

379   however, highlighted the importance of appropriate LH priming prior to GnRH induced ovulation

380   for the formation of functionally competent CLs. In the current study, DFs from Group C best

381   represent 'final-wave' (pre-ovulatory) DFs, but the timing of their collection (i.e. 24 h post PG

382   administration; Figure 1) probably precluded exposure to surge levels of LH. In contrast, the

383   'final-wave' DF that gave rise to a Day 6 CL (Group A) will probably have been 'older' and

384   larger at the point of ovulation, and almost certainly would have been exposed to higher levels of

385   LH; although these parameters were not determined. Therefore, whilst molecular features of

386   Group C relative to Group A DFs (discussed later) provide important information on factors

387   regulating subsequent CL function, they probably don't represent the complete picture.

388   *Molecular basis of luteal support and steroidogenesis*

389   The reduced capacity of induced (Group B) CLs to produce P4 is consistent with the reduced

390   expression of *STAR* and *HSD3B1* observed (Table 4). Reduced expression of transcripts for

391   *IGFBP4* and increased expression of transcripts for *IGFBP5* in Group C (regressing), relative to

392   Group A (developing), CLs is consistent with earlier reports of CL demise following PGF$_{2\alpha}$

393   induced luteolysis in cattle (Neuvians et al., 2003) and sheep (Hastie and Haresign, 2006).

394   Whereas IGFBP4 generally inhibits IGF action, IGFBP5 is known to have both IGF-dependent

395   and independent effects, but generally is associated with growth arrest and apoptosis (Kelley et

396   al., 1996; Monget et al., 1998). What's interesting in the current study is that transcript expression

397   for these two binding proteins in Group B CLs more closely matches that of Group C than Group

398   A CLs which, when considered with the P4 data in Table 2, lends further support to the

399   functional inadequacy of these induced CLs. Closer inspection of Table 4 data, however,

400   indicates that there are numerous molecular differences between Group B and C CLs, not least of

401   which is transcript expression for steroidogenic enzymes and key cytokines, indicating that whilst

402   these CLs may be developmentally compromised, they nevertheless retained some residual

403   function.

404   VEGFA is a potent mitogen that promotes the growth, migration and permeability of vascular

405   endothelial cells in CLs throughout the luteal phase (Robinson et al., 2007). Levels of this protein

17

406 within the CL peak at around Day 15 of the oestrous cycle, but decline on luteolysis as witnessed

407 in the current study (Table 4) and by Guzman et al. (2014). This latter study also demonstrated

408 that there are both pro- and anti-angiogenic isoforms of VEGFA in the bovine CL, and that

409 immediately prior to luteolysis there is an increase in anti-angiogenic isoforms. With respect to

410 the various isoforms identified by Guzman et al (2014), we can deduce from the primers designed

411 for the current study that we amplified the single isoform 205; which has only been described as

412 pro-angiogenic. Increased expression of this isoform in Group A CLs, relative to Groups B and C

413 CLs, further serves to confirm their viability.

414 Several alternatively spliced variants of the *LHCGR* gene have also been reported in the bovine

415 ovary, but only a couple of these variants with open reading frames over the entire sequence are

416 capable of producing a fully functional receptor (Robert et al., 2003). The variants reported

417 include a complete deletion of exon 10 and/or partial deletion of exon 11; and there is also a loss

418 of exon 3 in bovine granulosa cells (Nogueira et al., 2007). In humans, a splice variant lacking

419 exon 10 produces a protein capable of binding hCG, but not LH (Müller et al., 2003) and, in

420 keeping with a further human splice variant lacking exon 9, can form complexes with other

421 LHCGR isoforms to reduce overall receptor expression and cAMP accumulation (Nakamra et al.,

422 2004; Ndiaye et al., 2005; Minegishi et al., 2007). Primer design in the current study (Table 1)

423 allowed us to confirm expression of *LHCGR* transcripts lacking exons 3 and 9, but we were

424 unable to detect transcripts lacking exon 10 in any of the somatic (i.e. granulosa, thecal and

425 luteal) cells studied in the ovary. Relative to CLs from Group A (formed from 'final-wave' DFs),

426 expression of all *LHCGR* variants was reduced in CLs from Group B (derived from first-wave

427 DFs), and was barely detectable in regressing CLs (Group C) (Table 4). Based on quantitative

428 measurements of *LHCGR* expression within the bovine CL during a regular oestrous cycle

429 (Yoshioka et al., 2013), we surmise that Group B CLs in the current study were more similar to

430 regular Day 2-3 CLs than Day 5-7 CLs. This point is consistent with reduced levels of P4

431 production by Group B relative to Group A CLs (Table 2A).

432 *Molecular features of DFs that give rise to CLs*

433   In the current study Group C DFs were more oestrogenic than Group A DFs (Table 2B), but

434   transcript expression for *ESR2,* three steroidogenic enzymes (*CYP11A1, HSD3B1* and *CYP19A1*),

435   and subunits for inhibin-A and activin-A were decreased (Table 3A). These features are

436   consistent with a number of previous observations. For example, increasing levels of LH (Byers

437   et al., 1997) and ovarian oestrogens (Sharma et al., 1999) are each known to down-regulate *ESR2*

438   expression in granulosa cells and, in cattle, levels of inhibin-A and activin-A in follicular fluid

439   are reduced in large (13-20 mm) follicles with high (> 5) compared to low (< 5) E2:P4 ratios

440   (Glister et al., 2006). Peripheral (Armstrong et al., 2001) and follicular (Echternkamp et al., 1994)

441   concentrations of IGF1 also increase under these oestrogen-dominated conditions (Figure 3) as

442   animals enter the follicular phase. Expression of uterine IGFBP2 mRNA and protein increases

443   towards the late luteal phase and is thought to be under the regulation of P4 (McCarthy et al.,

444   2012; Costello et al., 2014). Elevated expression of IGFBP2 transcripts in Group C DFs (Table 3)

445   and IGFBP2 protein in follicular-phase fluids (Figure 3) is consistent with these observations but,

446   on first inspection, is somewhat at odds with earlier studies which indicate that IGFBP2 levels in

447   follicular fluids decrease in large oestrogen-active and pre-ovulatory follicles (Enchternkamp et

448   al., 1994; Funston et al., 1996). However, in contrast to previous work, the current study

449   compared DFs of equivalent size but at different stages of the oestrous cycle. Furthermore, it is

450   noteworthy that (i) follicles were harvested in both luteal and early follicular phases, and (ii)

451   E2:P4 ratios were only slightly greater for Group C than Group A DFs (Table 2B) and were

452   similar for abattoir derived early luteal- and early follicular-phase DFs (1.27 ± 0.96 vs 2.2 ± 1.50

453   respectively). Western blot analyses also revealed proteolytic fragments of IGFBP2 in early

454   follicular-phase DFs (data not shown), suggesting initial stages of degradation at the onset of this

455   oestrogen-dominated period. Collectively, these data suggest the presence of an active IGF

456   regulatory system in final wave DFs to tightly control cellular responses to increased circulating

457   IGF1.

19

458   **Conclusions**

459   The foregoing discussion focused on differences in functional competency and transcript

460   expression of CLs derived following induced and spontaneous ovulations, together with

461   differences in transcript expression of DFs that give rise to these structures. This study confirms

462   that induced ovulation of a first-wave DF results in the formation of a smaller CL with

463   functionally lower P4 production than one formed following spontaneous ovulation. Furthermore,

464   these smaller induced CLs were characterised as having reduced expression of transcripts

465   required for luteal support, angiogenesis and steroidogenesis, together with increased expression

466   of transcripts associated with luteolysis. Importantly, these differences in CL function were not

467   related to size of the ovulated DF, but were associated with their steroidogenic activity.

468   Transcript expression differed between first- and final-wave DFs, and was associated with

469   peripheral and local levels of P4 and components of the IGF system. These data indicate that

470   these separate follicular systems interact to affect maturation of the ovulatory follicle transiting

471   from di-oestrus to pro-oestrus in a manner that subsequently alters the functional competency of

472   the CL.

473

480   **Declaration of interests:** There is no conflict of interest that could be perceived as prejudicing

481   the impartiality of the research reported.

482   **References**

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625

1   **Figure 1** Oestrous cycle manipulation timeline (**A**). Oestrous cycles of 24 Hereford x Holstein

2   heifers were synchronised using prostaglandin (PG) and gonadotrophin releasing hormone

3   (GnRH). Animals were given further injections according to their treatment group, indicated by

4   letters A to C in brackets. Group A animals were slaughtered at Day 6 to recover a first wave

5   dominant follicle (DF) and 6-day old corpus luteum (CL); Group B animals ovulated on Day 7

6   and were slaughtered on Day 13 to recover a 6-day old induced CL; Group C animals were

7   slaughtered on Day 19 to recover a final wave DF. Plasma progesterone was monitored from Day

8   0 to slaughter for Group A (closed circles), B (open circles) and C (triangles) (**B**).

9   **Figure 2** Plasma IGF1 concentrations at the point of slaughter for 24 Hereford x Holstein heifers.

10   Animals were synchronised (see Figure 1) then Group A animals slaughtered at Day 6 (after

11   initial synchronised ovulation); Group B animals were induced to ovulate on Day 7 and

12   slaughtered on Day 13; Group C animals were slaughtered on Day 19. Plasma IGF1

13   concentrations were lower (P = 0.001) in Group A than in Group B and C animals.

14   **Figure 3** Follicular fluid IGFBP2 protein concentration (from cycle-stage determined abattoir

15   derived ovaries) was greater (P < 0.001) in early follicular- than early luteal-phase dominant

16   follicles (**A**). IGFBP2 was quantified by Western blotting. **B,** A typical gel for follicular fluid

17   from two early luteal- (EL1 & EL2) and two early follicular (EF1 & EF2) phase-dominant

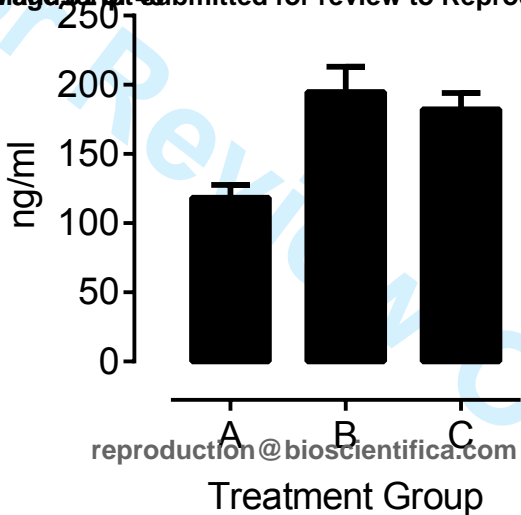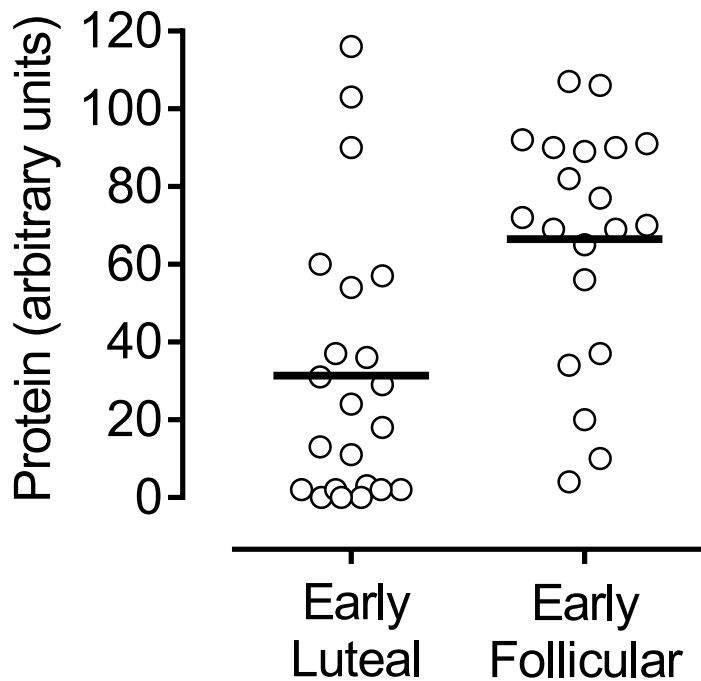18   follicles, with alternative lanes left blank (-).
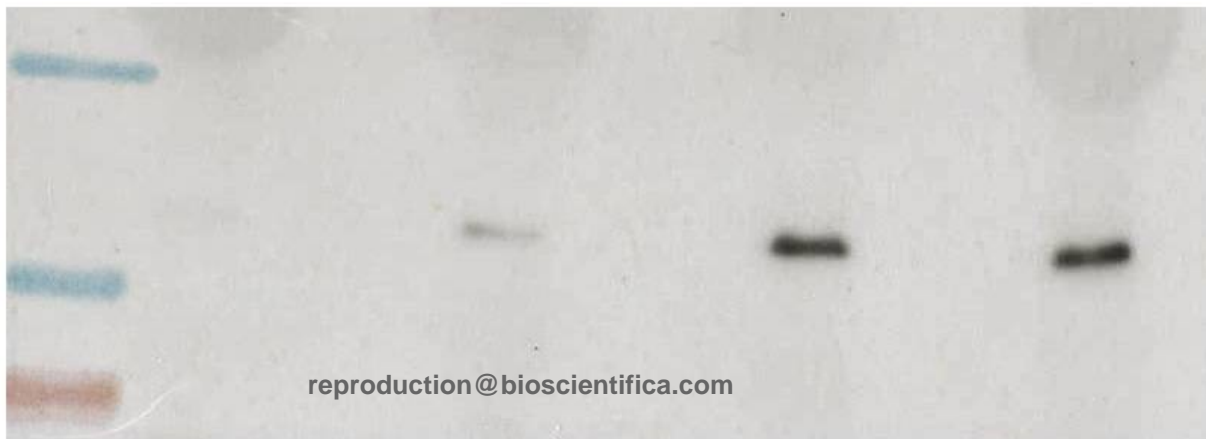
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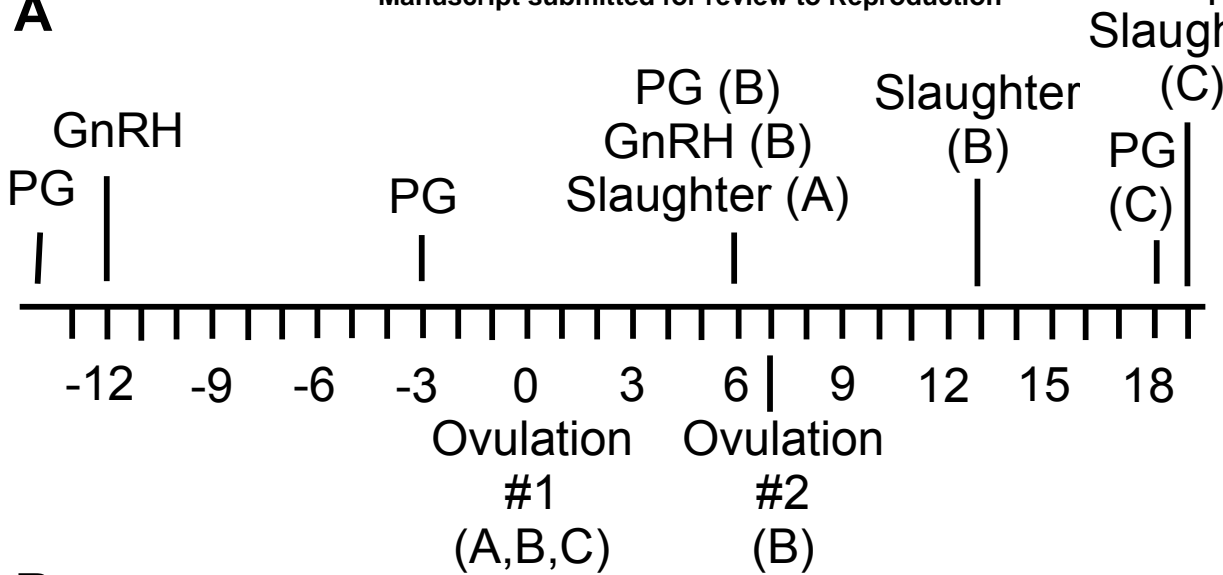
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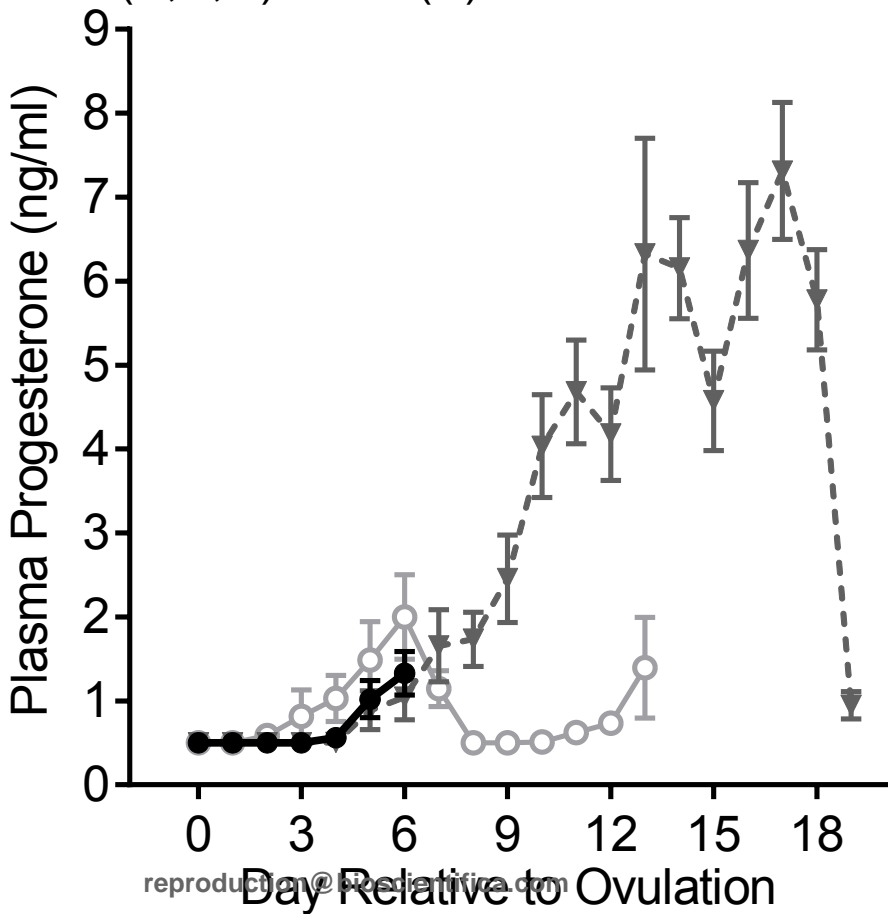
A

B

Table 1A. *Transcripts quantified in bovine dominant follicles and corpora lutea by GeXP.*

Gene	Full Name	Accession Number	Primers
TGF-β Superfamily			
AMH	anti-Mullerian hormone	NM_173890	F: aggtgacactatagaatacgtgagctgagcgtagacct
			R: gtacgactcactatagggagacaggctgatgaggagctt
BMP2	bone morphogenetic protein (BMP) 2	NM_001099141	F: aggtgacactatagaataacttttggacaccaggttgg
			R: gtacgactcactatagggactaatccgcacatgcctctt
BMP4	BMP 4	NM_001045877	F: aggtgacactatagaatagcttccaccacgaagaacat
			R: gtacgactcactatagggatagtcgtgtgatgaggtgcc
BMP6	BMP 6	XM_869844.3	F: aggtgacactatagaatatgtcatgtgggcattttgtt
			R: gtacgactcactatagggaaccaacacaggagaagtggc
BMPR1A	BMP receptor, type IA	NM_001076800	F: aggtgacactatagaatagtgtgtgtgtgcatacgtgc
			R: gtacgactcactatagggaaatggcttttatgcgattgg
BMPR1B	BMP receptor, type IB	NM_001105328	F: aggtgacactatagaataatggaacagcagaggaatgc
			R: gtacgactcactatagggaaagtgccacggagaagaaaa
BMPR2	BMP receptor, type II	XM_617592	F: aggtgacactatagaatacctgtcacacaataggcgtg
			R: gtacgactcactatagggactggacatcgaatgctcaga
INHA	inhibin, alpha	NM_174094	F: aggtgacactatagaatatagtgcaccctcccagtttc
			R: gtacgactcactatagggaggttgggcaccatctcatac
INHBA	inhibin, beta A	NM_174363	F: aggtgacactatagaataccaaagaaggcagtgacctg
			R: gtacgactcactatagggaagctggagacagggaagatg
INHBB	inhibin, beta B	NM_176852	F: aggtgacactatagaataagatcatcagcttcgccg
			R: gtacgactcactatagggacttcaggtagagccacaggc
Insulin/IGF family			
IGF1	insulin-like growth factor (IGF) 1	NM_001077828	F: aggtgacactatagaatagaagatgcccatcacatcct
			R: gtacgactcactatagggagcctcctcagatcacagctc
IGF1R	IGF 1 receptor	XM_606794.3	F: aggtgacactatagaatacaaaggcaatctgctcatca

			R: gtacgactcactatagggaagttcccctctagctgctcc
IGF2	IGF 2	NM_174087	F: aggtgacactatagaataacagcgagacacttgcagaa
			R: gtacgactcactatagggagacggtggtgactctgtgtg
IGF2R	IGF 2 receptor	NM_174352	F: aggtgacactatagaataggaccttctacctgagcgtg
			R: gtacgactcactatagggagttctggagctgaaaggtcg
IGFBP2	IGF binding protein 2	NM_174555	F: aggtgacactatagaatacaagggtggcaaacatcac
			R: gtacgactcactatagggagagaggttgtacaggccatgct
IGFBP4	IGF binding protein 4	NM_174557	F: aggtgacactatagaatacaggctcccctttactcctc
			R: gtacgactcactatagggaccttctccatcaggcacat
IGFBP5	IGF binding protein 5	NM_001105327.1	F: aggtgacactatagaatagatcgaaagagactcccgtg
			R: gtacgactcactatagggagtcagcttcttctgcggtc
INSR	insulin receptor	XM_590552	F: aggtgacactatagaataaaagaggccccttaccagaa
			R: gtacgactcactatagggatgtacggcgttcatcagaaa

Steroidogenic mediators

CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	NM_176644.2	F: aggtgacactatagaataaagtttgacccaaccaggtg
			R: gtacgactcactatagggagtgtccacgtcaccgatatg
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	NM_174304	F: aggtgacactatagaataagacaaccaaaagggcattg
			R: gtacgactcactatagggaggcaggatcctcattcttga
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	NM_174305	F: aggtgacactatagaataaagccaagagcaacaagcat
			R: gtacgactcactatagggaatttggcgctaattccaaga
ESR1	estrogen receptor 1	NM_001001443	F: aggtgacactatagaataggtgtacatggacagcagca
			R: gtacgactcactatagggatccaggtaatagggcacctg
ESR2	estrogen receptor 2	NM_174051	F: aggtgacactatagaatagacagaccacaagcccaaat
			R: gtacgactcactatagggagtttcacgccaaggactctt
HSD3B1	2ydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	NM_174343.2	F: aggtgacactatagaatagcagaaaaccaaggagtgga
			R: gtacgactcactatagggaatcaccttgtctgtcccctg
PGR	progesterone receptor	XM_583951.4	F: aggtgacactatagaatagttctcgctctacggggac

			R: gtacgactcactatagggattgtacaggacgcactccag
SCARB1	scavenger receptor class B, member 1	NM_174597.2	F: aggtgacactatagaataacaaactgggaacatccagc
			R: gtacgactcactatagggagatggggatgagcagtagga
LRP8	low density lipoprotein receptor-related protein 8	NM_001097565.1	F: aggtgacactatagaataccctgcaagggttcatgtat
			R: gtacgactcactatagggagaaaatggcctcattctcca
SHBG	sex hormone-binding globulin	NM_001098858	F: aggtgacactatagaatacccagagtcattggaggcta
			R: gtacgactcactatagggagatcccaagtccgaaactca
STAR	steroidogenic acute regulatory protein	NM_174189.2	F: aggtgacactatagaatacctactgccaggaaagatgc
			R: gtacgactcactatagggaagaacctaggagagagccgc

Cytokines

IL1B	interleukin (IL) 1, beta	NM_174093.1	F: aggtgacactatagaatatgaacccatcaacgaaatga
			R: gtacgactcactatagggatggatgtttccatctcccat
IL2	IL 2	NM_180997.1	F: aggtgacactatagaatacaaacggtgcacctacttca
			R: gtacgactcactatagggagaatccttgatctctctgggg
IL6	IL 6	NM_173923.2	F: aggtgacactatagaataagctctcattaagcgcatgg
			R: gtacgactcactatagggatctgcgatctttgcttcag
IL8	IL 8	NM_173925	F: aggtgacactatagaataaccaatggaaacgaggtctg
			R: gtacgactcactatagggacctacaccagacccacacag
KITLG	KIT ligand	NM_174375	F: aggtgacactatagaataagcattgccagcattctttt
			R: gtacgactcactatagggagaactgttacccgccaatgt
MIF	macrophage migration inhibitory factor	NM_001033608.1	F: aggtgacactatagaatacaacttctgcgacatgaacg
			R: gtacgactcactatagggacgtttattgctccttccagg
PTPRC	protein tyrosine phosphatase receptor type C	BC148881	F: aggtgacactatagaatacggagatgcaggatcaaact
			R: gtacgactcactatagggacccagatcatcctccagaaa

Apoptotic regulators

CCND2	cyclin D2	NM_001076372.1	F: aggtgacactatagaataagcagtaccgtcaggaccag
			R: gtacgactcactatagggaagagaaggagagagcggattg
CFLAR	CASP8 and FADD-like apoptosis regulator	NM_001012281.1	F: aggtgacactatagaatactaaggctccagaatggcag

			R: gtacgactcactatagggagcttgacttcatagcccagg
GADD45B	growth arrest and DNA-damage-inducible, beta	NM_001040604.1	F: aggtgacactatagaatatcacgaaccctcacacagac
			R: gtacgactcactatagggagtgttttccgcagcaagttt

Angiogenic regulators

HIF1A	hypoxia-inducible factor 1, alpha subunit	NM_174339.3	F: aggtgacactatagaatatgcctctgaaactccaaagc
			R: gtacgactcactatagggactggggcatggtaaaagaaa
VEGFA	vascular endothelial growth factor A	NM_174216.1	F: aggtgacactatagaataagcaaggcaagaaaatccct
			R: gtacgactcactatagggatcctggtgagacgtctggtt

Miscellaneous

FGF1	fibroblast growth factor 1	NM_174055	F: aggtgacactatagaatagtaacgcgcttctaaatgcc
			R: gtacgactcactatagggaatgagagggaatcatgccag
FSHR	FSH receptor	NM_174061	F: aggtgacactatagaataatgttttccagggagcctct
			R: gtacgactcactatagggatgacccctagcctgagtcat
SFRS9	splicing factor, arginine/serine-rich 9	NM_001083398	F: aggtgacactatagaataatatgccctgcgtaaactgg
			R: gtacgactcactatagggaattcccaccacctgtctcag
PGF2AR	Prostaglandin F2α receptor	BD187584	F: aggtgacactatagaatatgcccacttttttctaggcag
			R: gtacgactcactatagggaatggcattgcaaacaaatga

House-keeping genes

GAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_001034034	F: aggtgacactatagaatacaccctcaagattgtcagca
			R: gtacgactcactatagggaggtcataagtccctccacga
H2AZ	H2A histone family, member Z	NM_174809.2	F: aggtgacactatagaatatccagtgttggtgattccag
			R: gtacgactcactatagggatttggttggttggaaagctaa
RPLP0	ribosomal protein, large, P0	NM_001012682.1	F: aggtgacactatagaatacttgctgaaaaggtcaaggc
			R: gtacgactcactatagggagactcctccgactcctcctt

Table 1B. Primers designed to amplify regions of the luteinizing hormone/chorionic gonadotrophin receptor (*LHCGR*) (NM_174381) by GeXP

Product name	Product Location	Primers
LHCGRex2	exons 2-4	F: aggtgacactatagaatacacctatctccctatcaaagtaatcc
		R: gtacgactcactatagggacgagggagatttgtaaacgc
LHCGRex8	exons 8-11	F: aggtgacactatagaatagagctgaaggaaaatgcacg
		R: gtacgactcactatagggaggagtgtcttgggtaagcaga
LHCGRex11	within exon 11	F: aggtgacactatagaatatgttaggcacatcaggcaaa
		R: gtacgactcactatagggaccatgttcatggattggaag

Table 2. Structures present on the ovaries of heifers at slaughter. Group A were slaughtered at day 6 after synchronised ovulation; Group B were given prostaglandin and GnRH on day 6 to induce CL regression and ovulation on day 7 and were slaughtered on day 13; Group C were slaughtered on day 19.

A. Size of the dominant follicle (DF), and size and mass of the corpus luteum (CL), together with progesterone production

	Treatment Group			Probability
	A (n=8)	B (n=7)	C (n=8)	
DF				
Diameter (mm)	15.12 ± 0.85	16.57 ± 0.61	14.75 ± 0.65	-
CL				
Diameter (mm)	23.88 ± 1.89[a]	18.14 ± 1.03[b]	21.00 ± 0.53[ab]	0.021
Mass (g)	6.36 ± 1.47[a]	2.94 ± 0.44[b]	3.92 ± 0.29[ab]	0.049
Total P4 content (mg)	209 ± 81[a]	31 ± 8[b]	49 ± 8[b]	0.035
P4 production (ng/25 mg tissue)	771 ± 161[a]	191 ± 54[b]	102 ± 35[b]	<0.001
P4 synthetic capacity (mg/CL)	163 ± 36[a]	23 ± 8[b]	17 ± 6[b]	<0.001

B. Oestradiol and progesterone concentrations in follicular fluids from dominant (DF), largest subordinate (SF) and a selection of small (2-6mm) subordinate follicles

	Follicle Class	Treatment Group			Probability
		A (n=8)	B (n=7)	C (n=8)	
E2 (ng/ml)	DF	181.4 ± 103.5	576.9 ± 109.6	407.2 ± 109.5	0.056
	Small	0.60 ± 0.75	2.56 ± 0.75	1.43 ± 0.76	-
	SF	7.93 ± 7.42	3.51 ± 9.82	15.12 ± 7.42	-
P4 (ng/ml)	DF	81.5 ± 11.3	67.3 ± 13.0	75.7 ± 11.3	-
	Small	284.3 ± 56.3	197.5 ± 56.5	85.7 ± 56.3	0.058
	SF	274.9 ± 116.2	234.5 ± 124.2	275.6 ± 116.2	-
E2:P4 Ratio	DF	2.55 ± 1.60	8.98 ± 1.65	6.01 ± 1.65	0.054
	Small	0.004 ± 0.003	0.022 ± 0.008	0.020 ± 0.007	-
	SF	0.25 ± 0.20	0.009 ± 0.17	0.40 ± 0.21	-

Values are given as mean ± SE, letters in superscript indicate significant differences (P<0.05).

Table 3. Transcript expression in first wave (A), first wave in the presence of a sub-functional corpus luteum (B) and final wave (C) bovine dominant follicles. In general, only transcripts that differed significantly between treatment groups are shown.

A. Granulosa cells

Transcript	Treatment Group			Probability
	A (n=8)	B (n=7)	C (n=8)	
TGF-β Superfamily				
INHA	0.281 ± 0.026^a	0.272 ± 0.046^a	0.132 ± 0.043^b	0.020
INHBA	0.948 ± 0.060^a	0.769 ± 0.093^{ab}	0.500 ± 0.132^b	0.015
Insulin/IGF family				
IGF2R	0.100 ± 0.012	0.092 ± 0.009	0.159 ± 0.037	0.122
IGFBP2	0.200 ± 0.029	0.166 ± 0.014	0.353 ± 0.086	0.059
Steroidogenic mediators				
CYP11A1	0.545 ± 0.048^a	0.462 ± 0.050^a	0.273 ± 0.079^b	0.015
CYP19A1	2.955 ± 0.145^a	2.411 ± 0.121^a	1.195 ± 0.315^b	<0.001
ESR2	0.231 ± 0.029^a	0.199 ± 0.025^{ab}	0.123 ± 0.024^b	0.024
HSD3B1	0.229 ± 0.028^a	0.205 ± 0.025^{ab}	0.112 ± 0.038^b	0.036
SCARB1	0.433 ± 0.062	0.492 ± 0.062	0.2839 ± 0.066	0.083
Angiogenic regulators				
HIF1A	0.984 ± 0.053^a	0.951 ± 0.064^a	0.668 ± 0.091^b	0.009
Miscellaneous				
PGF2AR	0.034 ± 0.004^a	0.025 ± 0.007^{ab}	0.013 ± 0.005^b	0.038
SFRS9	0.601 ± 0.037	0.569 ± 0.031	0.493 ± 0.032	0.079

B. Thecal cells

Transcript	Treatment Group			Probability
	A (n=8)	B (n=7)	C (n=8)	
Insulin/IGF family				
IGF2R	0.080 ± 0.009^a	0.102 ± 0.014^{ab}	0.136 ± 0.012^b	0.011
IGFBP2	0.211 ± 0.028^a	0.216 ± 0.045^a	0.366 ± 0.053^b	0.030
Steroidogenic mediators				
SCARB1	0.272 ± 0.048^a	0.535 ± 0.073^b	0.505 ± 0.099^b	0.047
Cytokines				
PTPRC	0.044 ± 0.006^a	0.048 ± 0.007^a	0.094 ± 0.017^b	0.011

Values are mean ± SE in arbitrary fluorescence units relative to the control genes GAPDH, H2AZ *and* RPLP0*. Letters in superscript indicate significant differences (P<0.05). Reported transcripts (other than IGF2R in granulosa cells) lie within the FDR threshold of 0.25.*

1   **Table 4.** Transcript expression in six-day old spontaneous (A), six day old induced (B) and 19
2   day old regressing (C) bovine corpora lutea. Only transcripts that differed between treatment
3   groups are shown. Abundance of three regions (*LHCGRex11, LHCGRex2* and *LHCGRex8*) with
4   two splice variants lacking exon three (*LHCGRex2(-3)*) and exon nine (*LHCGRex8(-9)*) of the
5   *LHCGR* transcript is given.
6

Transcript	Treatment Group			Probability
	A (n=8)	B (n=7)	C (n=8)	
TGF-β Superfamily				
BMP2	0.003 ± 0.002[a]	0.021 ± 0.006[b]	0.020 ± 0.006[b]	0.037
INHBA	0.017 ± 0.006[a]	0.039 ± 0.013[ab]	0.063 ± 0.009[b]	0.009
INHBB	0.016 ± 0.004[a]	0.038 ± 0.016[ab]	0.063 ± 0.012[b]	0.027
Insulin/IGF family				
IGFBP4	0.228 ± 0.037[a]	0.116 ± 0.016[b]	0.134 ± 0.031[b]	0.038
IGFBP5	0.266 ± 0.022[a]	0.550 ± 0.086[b]	0.635 ± 0.121[b]	0.016
Steroidogenic mediators				
CYP11A1	0.933 ± 0.065[a]	0.720 ± 0.126[a]	0.466 ± 0.036[b]	0.002
HSD3B1	0.637 ± 0.032[a]	0.415 ± 0.126[b]	0.078 ± 0.024[c]	<0.001
SCARB1	1.722 ± 0.092[a]	1.493 ± 0.210[a]	1.038 ± 0.085[b]	0.005
STAR	1.350 ± 0.061[a]	0.837 ± 0.185[b]	0.266 ± 0.053[c]	<0.001
Cytokines				
IL1B	0.307 ± 0.027[a]	0.246 ± 0.026[ab]	0.182 ± 0.025[b]	0.011
IL8	0.039 ± 0.010[a]	0.062 ± 0.013[a]	0.166 ± 0.038[b]	0.004
KITLG	0.046 ± 0.008[a]	0.024 ± 0.004[b]	0.002 ± 0.004[c]	<0.001
MIF	0.037 ± 0.003[a]	0.034 ± 0.006[a]	0.012 ± 0.004[b]	<0.001
Apoptotic regulators				
GADD45B	0.444 ± 0.028[a]	0.295 ± 0.040[b]	0.231 ± 0.034[b]	<0.001
Angiogenic regulators				
HIF1A	0.674 ± 0.034[a]	0.612 ± 0.039[ab]	0.441 ± 0.047[b]	0.002
VEGFA	0.037± 0.009[a]	0.009 ± 0.005[b]	ND	0.002
Miscellaneous				
PGF2AR	0.500 ± 0.045[a]	0.304 ± 0.056[b]	0.144 ± 0.036[c]	<0.001
SFRS9	0.579 ± 0.046	0.621 ± 0.027	0.479 ± 0.042	0.075
Luteinizing hormone receptor variants				
LHCGRex2	0.392 ± 0.054[a]	0.218 ± 0.085[b]	0.003 ± 0.002[c]	<0.001
LHCGRex2(-3)	0.059 ± 0.012[a]	0.017 ± 0.010[b]	ND	<0.001
LHCGRex8	0.012 ± 0.007	ND	ND	-
LHCGRex8(-9)	0.019 ± 0.009	ND	ND	-
LHCGRex11	0.428 ± 0.048[a]	0.281 ± 0.137[a]	0.001 ± 0.001[b]	0.003

7   *Values are mean ± SE in arbitrary fluorescence units relative to the control genes* GAPDH,
8   H2AZ *and* RPLP0. *Letters in superscript indicate significant differences (P<0.05), ND means*
9   *none detected. Reported transcripts lie within the FDR threshold of 0.25.*

10

Supplementary Information

Supplementary Materials and Results

To develop the GeXP platform for our study, RNA was extracted from a mixture of cells (i.e. aspirated and scrapped bovine follicles of varying size, plus sonicated CL tissue). These mixed cell populations included oocytes. This phase of the study concerned GeXP primer design. All genes in the master list (Table 1 of manuscript) were detected.

For GeXP validation, *ESR2* and *HIF1A* were chosen at random and quantified by quantitative, real time PCR (qRT-PCR) and by GeXP. Granulosa cells were collected from a selection of 10-14 mm diameter, abattoir derived bovine dominant follicles. 12μl of mRNA, extracted and purified as described in the materials and methods, was denatured at 70°C for 10 minutes using a thermal cycler (BioRad, Hemel-Hempstead, Hertfordshire, UK). Reverse transcription (RT) was performed at 37°C for 60 minutes using an Omniscript RT Kit (Qiagen Ltd., Crawley, West Sussex, UK) with RNase inhibitor (Bioline Ltd., London, UK). 1μl of the resulting cDNA was added to a PCR reaction mixture including SYBR Green (*Applied Biosystems, Warrington, Cheshire, UK)* and 20μM forward and reverse primers. The PCR reaction was performed within a LightCycler 480 (Roche Diagnostics GmBH, Mannheim, Germany) using the following program: Quantification – 1 cycle of 95°C for 10 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 90 seconds, 72°C for 10 seconds; Melting – 95°C for 5 seconds; 65°C for 1 minute; Cooling – 40°C for 30 seconds. LightCycler 480 software was used to normalise expression relative to the house-keeping gene *H2AZ*, interpret and analyse the results. The same samples were also quantified by GeXP, as described in the materials and methods.

GeXP and qRT-PCR determined expression of *ESR2* and *HIF1A* was correlated (R^2=0.515, P<0.001 and R^2=0.453, P=0.006, respectively) (Supplementary Figure 1) so confirming GeXP as a suitable method for the quantification of gene expression.

Genes not expressed in experimental cells

Transcripts for all genes described in Table 1 of the manuscript (including those listed below) were expressed in a mixed population of cells during GeXP platform development. These cells were harvested from a mixed sample of abattoir derived CLs and follicles at various antral-stages of development (from 2 mm in diameter). Below we describe expression patterns and functions for those genes not detected in our experimental granulosa, theca and luteal cells. In some cases this may have arisen as a consequence of primer design and splice variant expression, because bovine variants for these genes are poorly described in the literature.

AMH: Important during pre-antral follicle development, regulating both the transition from primordial to primary follicle stages, and the response to FSH (Knight and Glister, 2006). Transcript expression for *AMH* is restricted to granulosa cells (Vigier et al., 1984; Takahashi et al., 1986) and declines beyond the early antral stages of follicle development, and is further reduced in granulosa cells from atretic follicles (Rico et al., 2009). Interestingly, blood AMH concentrations are also at their lowest between Days 4 and 6 of the oestrous cycle (Rico et al., 2011) when Group A animals were slaughtered in our study. Collectively these results may

explain why we did not detect *AMH* transcripts in selected somatic cells of the bovine ovary in the current study.

BMP6: Expressed and active in bovine granulosa and thecal cells at least up to approximately 6 mm in diameter where it can attenuate the actions of both FSH and forskolin *in vitro* (Kayani et al., 2009; Glister et al., 2013). *BMP6* mRNA is lost during selection of the dominant follicle in the rat (Erickson and Shimasaki, 2003), although transcripts for this transforming growth factor-beta superfamily member have been detected within the bovine CL (Kayani et al., 2009) and follicle up to 18 mm in diameter (Glister et al., 2010). Established actions of BMP6 on bovine ovarian follicular cells have largely been confined to *in vitro* culture with cells from follicles < 6 mm in diameter. Inability to detect transcripts for *BMP6* in the current study could have been due to low expression, especially in granulosa cells (Glister et al., 2010).

FGF1: Transcripts for this fibroblast growth factor have previously been reported in theca and granulosa cells from antral (5 to 14 mm) follicles derived from abattoir recovered bovine ovaries (Berisha et al., 2004). Expression was relatively greater in theca than granulosa cells in that study and did not vary significantly between follicle size classes. FGF1 is generally known to exert mitogenic, anti-apoptotic and angiogenic effects in a variety of tissues. In cultured bovine granulosa cells (harvested from 2-5 mm follicles) FGF1 increased expression of Sprouty family members (*SPRY2* and *SPRY4*), as well as orphan nuclear receptors (*NR4A1* and *NR4A3*), thereby confirming functional activity in these cells (Jiang and Price, 2012). Inability to detect transcripts for *FGF1* in the current study could have also been due to low expression, again especially in granulosa cells.

IL6 and IL2: Interleukin-6 is a pro-inflammatory cytokine involved in a variety of roles (including anti-apoptotic) within the ovary associated with ovulation, CL formation and demise (Bornstein et al., 2004; Richards et al., 2008). IL6 is produced predominantly by macrophages and activated T cells within the CL, particularly during luteolysis. *IL6* mRNA expression is often barely detectable in the CL, with inhibition stemming from locally produced progesterone (Telleria et al., 1998). Similarly, Petroff et al. (1999) failed to detect transcripts for *IL2* in the bovine CL at various stages of the luteal phase. However, interleukins are inducible. For example, exposure of bovine granulosa cells to lipopolysaccharide led to a rapid and sustained increase in transcripts for *IL6* in cultured bovine granulosa cells (Bromfield and Sheldon, 2011). It's possible that the necessary conditions for induction may have been absent in our cells.

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Supplementary Figure 1 A comparison of GeXP and qRT-PCR determined *ESR2* (A) and *HIF1A* (B) transcript expression in granulosa cells from bovine dominant ovarian follicles collected from a local abattoir. Expression is given in arbitrary fluorescence units relative to house-keeping gene expression (*GAPDH*, *H2AZ* and *RPLP0* for GeXP; *H2AZ* for qRT-PCR).

Supplementary Figure 2 *LHCGR* transcripts, control genes (*GAPDH, H2AZ* and *RPLP0*) and internal standard (Kan^r) peaks detected using GeXP in granulosa cells from a sample of abattoir derived early luteal dominant follicles (A). Smaller fragments of the *LHCGR* with missing exons (indicated in brackets) were identified alongside three complete fragments (B) (not to scale).

Supplementary Figure 3. Relationships between ovulatory dominant follicle (DF) and 6-day old *corpus luteum* (CL) diameter (**A**), and CL diameter and progesterone (P4) synthesis (**B**). All DFs were scanned on experimental Day -1 (●) and the resulting CL were measured on experimental Day 6 by ultrasonography (Groups B and C) or following dissection (Group A). DFs present in Group B were scanned on experimental Day 6 (○) and the resultant CL measured following dissection on experimental Day 13. Day -1 DFs were positively correlated (r = 0.63; P = 0.001) with Day 6 CL diameter, whereas Day 6 DFs were not significantly correlated with Day 13 CL diameter. There was no significant difference in mean diameter between Day -1 and Day 6 DFs (13.4 vs 12.0 mm; P = 0.09). There was no relationship (r = 0.17; NS) between CL diameter and P4 synthesis for either Group A or B treatments.

Supplementary Figure 4. Ovarian follicular growth and plasma progesterone (P4) concentrations (ng/ml) in eight Group C Hereford x Holstein heifers following synchronised oestrus (ovulation occurred between 11 am on Day -1 and 11 am on Day +1), as determined by trans-rectal ultrasonography. Heifers 230 and 260 had not ovulated when initially scanned early on Day +1 but had later that morning. Solid black lines indicate ovulated dominant follicles (DFs) (heifers 230 and 260) and DFs present at slaughter. Coloured dashed lines indicate other DFs. Grey dotted lines indicate all other follicles greater than 4 mm in diameter. Plasma P4 concentrations are illustrated by yellow shading.
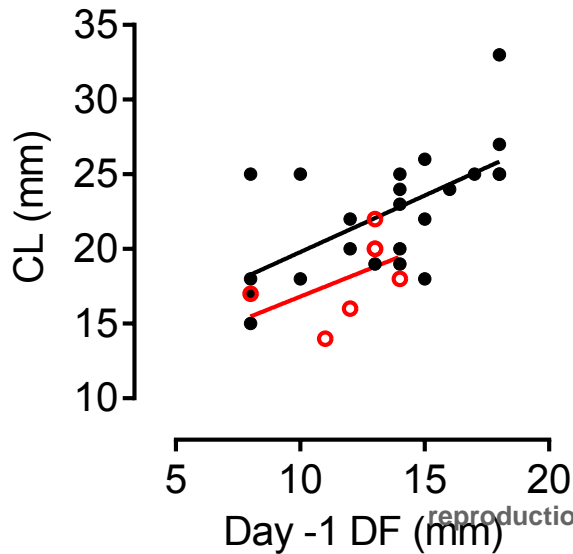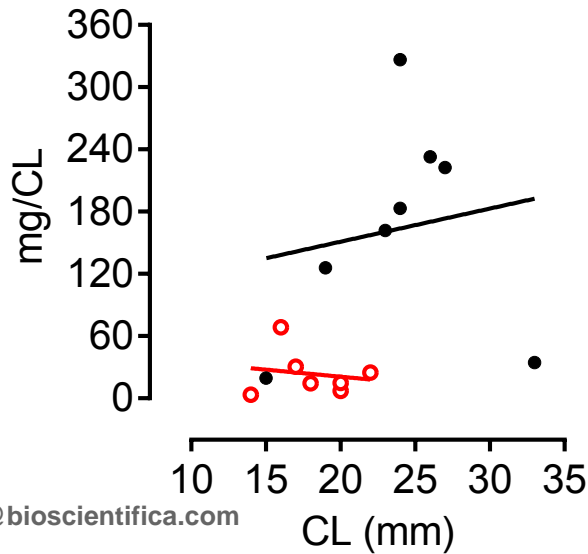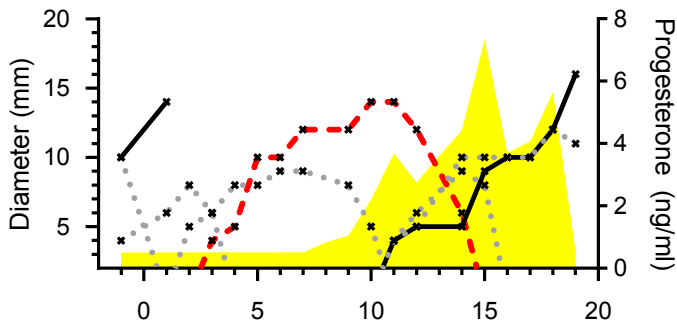
A. ESR2
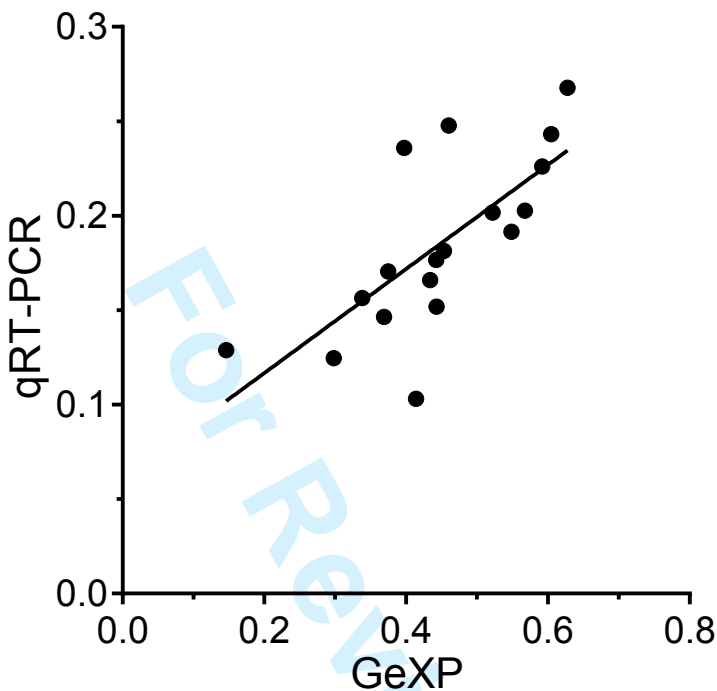
B. HIF1A
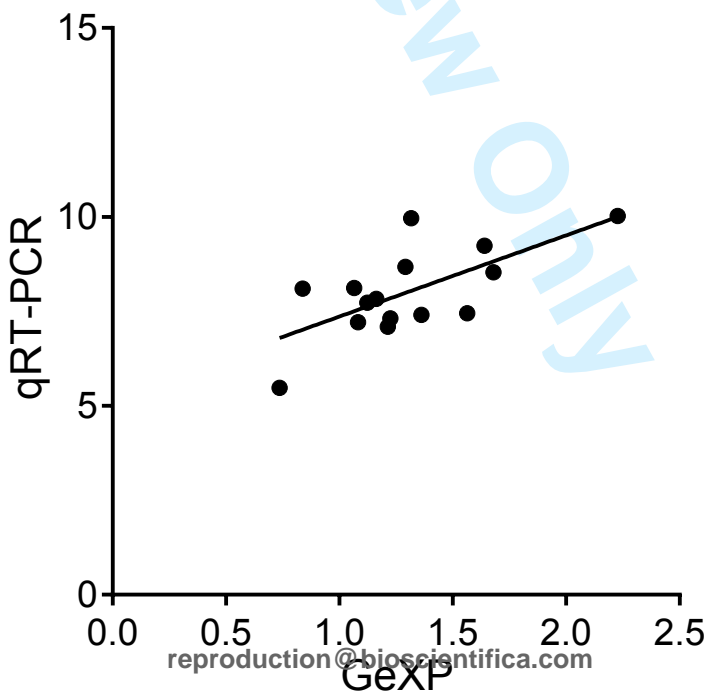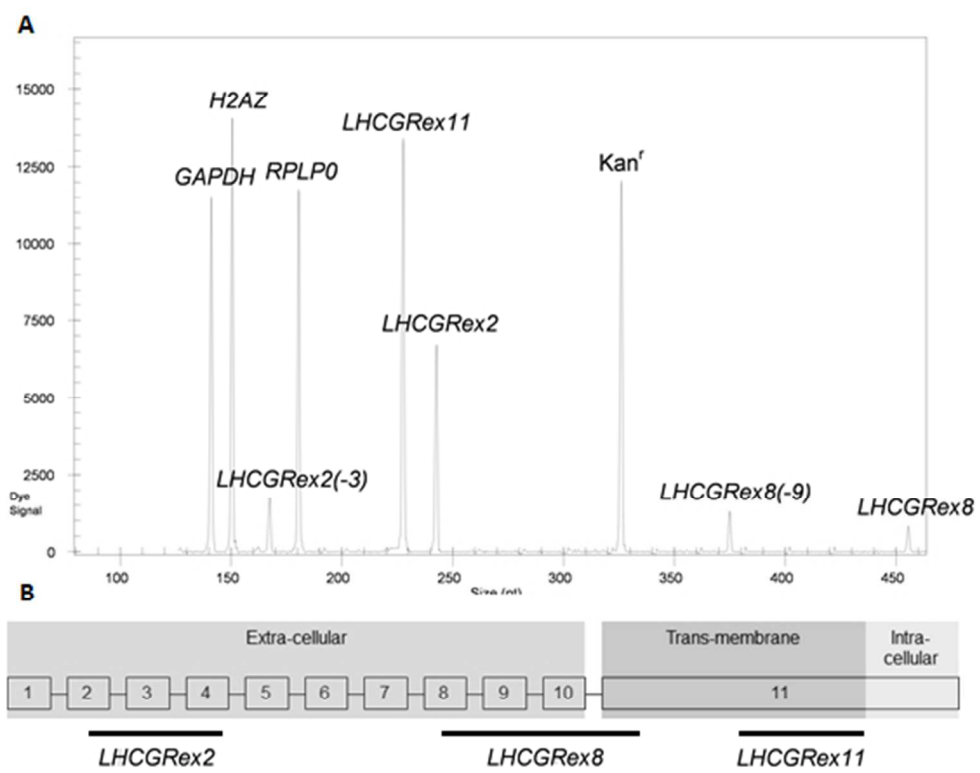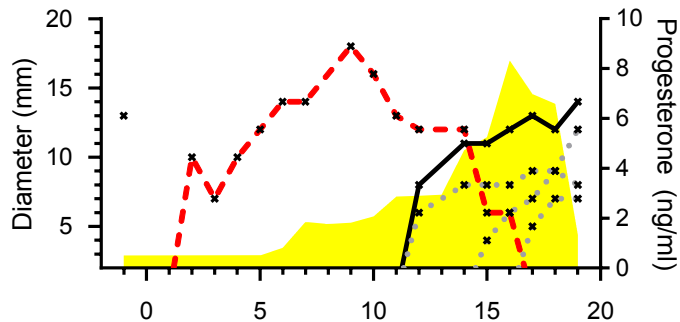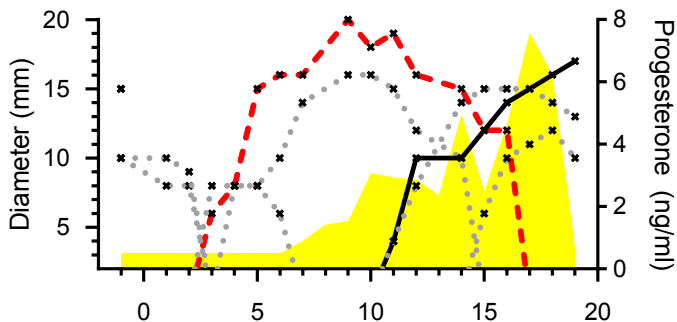
152x121mm (96 x 96 DPI)
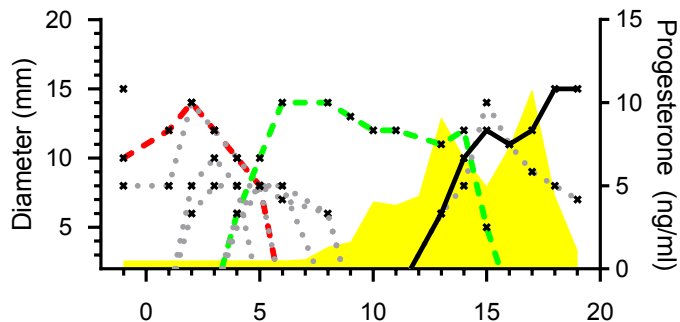
A. Follicle vs CL diameter

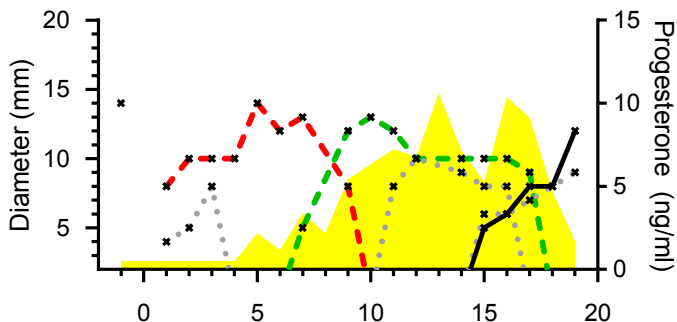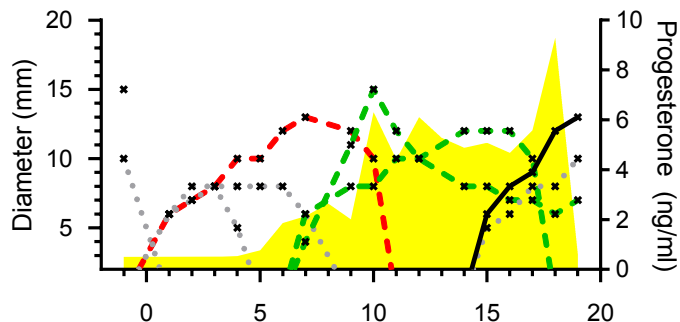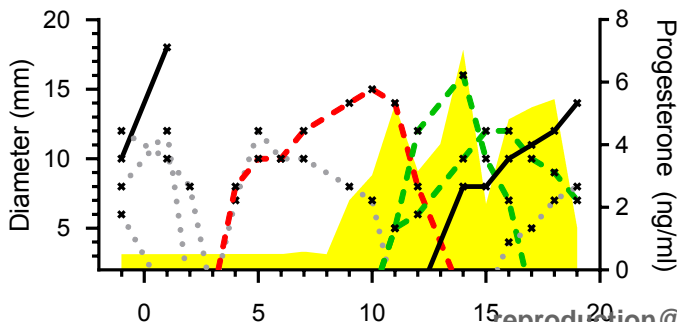B. Progesterone synthesis

A. Animal 230
B. Animal 231
C. Animal 289
D. Animal 404
E. Animal 409
F. Animal 239
G. Animal 260
H. Animal 220

Experimental Day