



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

Journal:	<i>Reproduction</i>
Manuscript ID	REP-15-0415.R2
mstype:	Research paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Gregson, Emma; University of Nottingham, School of Biosciences Webb, Robert; University of Nottingham, School of Biosciences Sheldrick, Elizabeth; University of Nottingham, School of Biosciences Campbell, Bruce; University of Nottingham, Human Development Mann, George E; University of Nottingham, School of Biosciences; University of Nottingham, School of Biosciences Liddell, Susan; University of Nottingham, School of Biosciences Sinclair, Kevin D; University of Nottingham, Biosciences;
Keywords:	Corpus luteum, Follicle, Insulin-like growth factor, Oestrus cycle, Gene expression

SCHOLARONE™
Manuscripts

Only

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

3

4 E Gregson¹, R Webb¹, EL Sheldrick¹, BK Campbell², GE Mann¹, S Liddell¹ and KD Sinclair^{1*}

5

6 ¹School of Biosciences, University of Nottingham, Loughborough, UK, LE12 5RD

7 ²School of Clinical Sciences, University of Nottingham, Nottingham, UK, NG7 2RD

8 *Corresponding author: kevin.sinclair@nottingham.ac.uk

9

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

34 Introduction

35

36 Since the introduction of ovulation synchronization into mainstream reproductive management of
37 cattle (Pursley *et al.*, 1995) a plethora of studies have examined in detail the benefits of follicle
38 synchrony in reproductive management programs (Bisinotto *et al.*, 2014; Wiltbank and Pursley,
39 2014). However, it has been shown that ovulation of follicles that have not undergone normal
40 physiological maturation can lead to suboptimal luteal function compared to spontaneous
41 ovulation. For example, following synchronization Perry *et al.* (2005) found that ovulation of
42 smaller follicles (presumed to be short of full maturity) resulted in decreased pregnancy rates.
43 This was associated with lower oestradiol (E2) on the day of insemination together with impaired
44 subsequent luteal function. In contrast, they reported no effect of ovulatory follicle size when
45 ovulation occurred spontaneously. Furthermore, Bisinotto *et al.* (2010) found differences in
46 pregnancy rate following artificial insemination (AI) according to wave of the ovulated follicle,
47 with higher pregnancy rates following ovulation of a second than a first wave dominant follicle
48 (DF). However, less is known about the impact of ovulatory control programs on the detailed
49 molecular control mechanisms underpinning the adequacy of the ovulatory follicle and resulting
50 corpus luteum (CL).

51 The expression of several genes involved with ovulation, luteinisation and CL function is under
52 endocrine control. Production of the prostaglandin PGF_{2α}, for instance, has been shown to be
53 regulated by progesterone (P4) (Sharzynski and Okuda, 1999; Okuda *et al.*, 2004). A first wave
54 DF undergoes selection during a period of low circulating P4 whereas, during later follicular
55 waves, DF selection occurs during the luteal phase of the oestrous cycle in the presence of higher
56 concentrations of circulating P4 (Savio *et al.*, 1988; Ginther *et al.*, 1989). There is evidence that
57 P4 supplementation prior to induced ovulation (around the time of ovulatory DF selection) can
58 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 \pm 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

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 (≥ 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 (± 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 (± 2 mg) 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

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/ μ l 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°C for 10 min using a thermal cycler (BioRad, Hemel-Hempstead, UK) prior to
158 RT and subsequent transcript expression analysis.

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 ($P_{(1)} \leq \dots \leq P_{(m)}$) were ordered along with
221 their respective null hypotheses ($H_{(1)}, \dots, H_{(m)}$), 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 $H_{(1)}, \dots, H_{(k)}$. 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 \pm 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***

258 Total P4 content (amount of P4 per CL), P4 production (amount of P4 produced per unit of tissue
259 cultured = $P4_{\text{tissue}} + P4_{\text{media}} - P4_{\text{initial tissue}}$) 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.

298 ***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).

302 ***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 ($0.267 \pm$
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. Quinital-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 $\text{PGF}_{2\alpha}$
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

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 (Echternkamp 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.

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

474 **Acknowledgements:** Technical assistance was provided by Neil Saunders, Dr Kamila Derecka,
475 Marcus Mitchell, Morag Hunter, Ralph Hourd, Tim Allen, Linda Staniforth and the BSU staff at
476 the University of Nottingham.

477 **Funding:** This research did not receive any specific grant from any funding agency in the public,
478 commercial or not-for-profit sector. EG was supported by a Doctoral Account from the
479 University of Nottingham.

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**

483

484 **Agricultural and Food Research Council (AFRC)** 1993 *Energy and Protein Requirements of*
485 *Ruminants*. An Advisory Manual Prepared by the AFRC Technical Committee on Responses to
486 Nutrients. Wallingford, UK: CAB International.

487 **Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Woad KJ, Webb R &**
488 **Sinclair KD** 2001 Effect of dietary energy and protein on bovine follicular dynamics and embryo
489 production in vitro: associations with the ovarian insulin-like growth factor system. *Biology of*
490 *Reproduction* **64**(6) 1624-1632.

491 **Azhar S, Tsai L, Medicherla S, Chandrasekher Y, Giudice L & Reaven E** 1998 Human
492 granulosa cells use high density lipoprotein cholesterol for steroidogenesis *The Journal of*
493 *Clinical Endocrinology and Metabolism* **83** 983-91.

494 **Bisinotto RS, Chebel RC & Santos JEP** 2010 Follicular wave of the ovulatory follicle and not
495 cyclic status influences fertility of dairy cows. *Journal of Dairy Science* **93** 3578-3587.

496 **Bisinotto RS, Ribeiro ES & Santos JEP** 2014 Synchronisation of ovulation for management of
497 reproduction in dairy cows. *Animal* **8** *Supplement 1*: 151-159.

498 **Byers M, Kuiper GG, Gustafsson JA & Park-Sarge OK** 1997 Estrogen receptor-beta mRNA
499 expression in rat ovary: down-regulation by gonadotropins. *Molecular Endocrinology* **11**(2) 172-
500 182.

501 **Colazo MG, Dourey A, Rajamahendran R & Ambrose DJ** 2013 Progesterone
502 supplementation before timed AI increased ovulation synchrony and pregnancy per AI, and

503 supplementation after timed AI reduced pregnancy losses in lactating dairy cows. *Theriogenology*
504 **79** 833-841.

505 **Costello LM, O'Boyle P, Diskin MG, Hynes AC & Morris DG** 2014 Insulin-like growth factor
506 and insulin-like growth factor-binding proteins in the bovine uterus throughout the oestrous
507 cycle. *Reproduction, Fertility and Development* **26** 599-608.

508 **Echternkamp SE, Howard HJ, Roberts AJ, Grizzle J & Wise T** 1994 Relationships among
509 concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding
510 proteins in ovarian follicular fluid of beef cattle. *Biology of Reproduction* **51** 971-81.

511 **Funston RN, Seidel GE Jr, Klindt J & Roberts AJ** 1996 Insulin-like growth factor I and
512 insulin-like growth factor-binding proteins in bovine serum and follicular fluid before and after
513 the preovulatory surge of luteinizing hormone. *Biology of Reproduction* **55** 1390-1396.

514 **Ginther OJ, Kastelic JP & Knopf L** 1989 Composition and characteristics of follicular waves
515 during the bovine oestrous cycle. *Animal Reproduction Science* **20** 187-200.

516 **Glister C, Groome NP & Knight PG** 2006 Bovine follicle development is associated with
517 divergent changes in 22hydroxyl-A, inhibin A and follistatin and the relative abundance of
518 different follistatin isoforms in follicular fluid. *Journal of Endocrinology* **188** 215–225.

519 **Guzmán A, Macías-Valencia R, Fierro-Fierro F, Gutiérrez CG & Rosales-Torres AM** 2015
520 The corpora lutea proangiogenic state of VEGF system components is turned to antiangiogenic at
521 the later phase of the oestrous cycle in cows. *Animal* **9** 301-307.

522 **Hastie PM & Haresign W** 2006 A role for LH in the regulation of expression of mRNAs
523 encoding components of the insulin-like growth factor (IGF) system in the ovine corpus luteum.
524 *Animal Reproduction Science* **96**(1-2) 196-209.

525 **Hayashi KG, Matsui M, Acosta TJ, Kida K & Miyamoto A** 2006 Effect of the dominant
526 follicle aspiration before or after luteinizing hormone surge on the corpus luteum formation in the
527 cow. *Journal of Reproduction and Development* **52**(1) 129-135.

528 **Ireland JJ, Murphee RL & Coulson PB** 1980 Accuracy of predicting stages of bovine estrous
529 cycle by gross appearance of the corpus luteum. *Journal of Dairy Science* **63** 155-160.

530 **Kanakkaparambil R, Singh R, Li D, Webb R & Sinclair KD** 2009 B-vitamin and
531 homocysteine status determines ovarian response to gonadotropin treatment in sheep. *Biology of*
532 *Reproduction* **80** 743-752.

533 **Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson DM &**
534 **Rosenfeld RG** 1996 Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory
535 dynamics. *The International Journal of Biochemistry and Cell Biology* **28**(6) 619-637.

536 **Lowman BG, Scott NA & Somerville SH** 1976 Condition scoring of cattle. *Bulletin, East of*
537 *Scotland College of Agriculture* No. 6.

538 **Mamluk R, Chen D, Greber Y, Davis JS & Meidan R** 1998 Characterization of messenger
539 ribonucleic acid expression for prostaglandin F2 alpha and luteinizing hormone receptors in
540 various bovine luteal cell types. *Biology of Reproduction* **58**(3) 849-56.

541 **McCarthy SD, Roche JF & Forde N** 2012 Temporal changes in endometrial gene expression
542 and protein localization of members of the IGF family in cattle: effects of progesterone and
543 pregnancy. *Physiological Genomics* **44**(2) 130-140.

544 **Minegishi T, Nakamura Y, Yamashita S & Omori Y** 2007 The effect of splice variant of the
545 human luteinizing hormone (LH) receptor on the expression of gonadotropin receptor. *Molecular*
546 *and Cellular Endocrinology* **260-262** 117-125.

547 **Monget P, Pisselet C & Monniaux D** 1998 Expression of insulin-like growth factor binding
548 protein-5 by ovine granulosa cells is regulated by cell density and programmed cell death in vitro.
549 *Journal of Cellular Physiology* **177**(1) 13-25.

550 **Müller T, Gromoll J & Simoni M** 2003 Absence of exon 10 of the human luteinizing hormone
551 (LH) receptor impairs LH, but not human chorionic gonadotrophin action. *The Journal of*
552 *Clinical Endocrinology & Metabolism* **88**(5): 2242-2249.

553 **Murdoch WJ & Van Kirk EA** Luteal dysfunction in ewes induced to ovulate early in the
554 follicular phase 1998 *Endocrinology* **139**(8) 3480-3484.

555 **Nakamura K, Yamashita S, Omori Y & Minegishi T** 2004 A splice variant of the human
556 luteinizing hormone (LH) receptor modulates expression of the wild-type human LH receptor.
557 *Molecular Endocrinology* **18**(6) 1461-1470.

558 **Ndiaye K, Fayad T, Silversides DW, Sirois J & Lussier JG** 2005 Identification of
559 downregulated messenger RNAs in bovine granulosa cells of dominant follicles following
560 stimulation with human chorionic gonadotropin. *Biology of Reproduction* **73**(2) 324-333.

- 561 **Neuvians TP, Pfaffl MW, Berisha B & Schams D** 2003 The mRNA expression of the members
562 of the IGF-system in bovine corpus luteum during induced luteolysis. *Domestic Animal*
563 *Endocrinology* **25**(4) 359-372.
- 564 **Nio-Kobayashi J, Trendell J, Giakoumelou S, Boswell L, Nicol L, Kudo M, Sakuragi N,**
565 **Iwanaga T & Duncan WC** 2015 Bone morphogenetic proteins are mediators of luteolysis in the
566 human corpus luteum. *Endocrinology* **156**(4) 1494-503.
- 567 **Niswender GD, Davies TL, Griffith RJ, Bogan RL, Monser K, Bott RC, Bruemmer JE &**
568 **Nett TM** 2007 Judge, jury and executioner: the autoregulation of luteal function. *Reproduction in*
569 *Domestic Ruminants VII; Society of Reproduction and Fertility* **64** 191-206.
- 570 **Nogueira MFG, Buratini Jr J, Price CA, Castilho ACS, Pinto MGL & Barros CM** 2007
571 Expression of LH receptor mRNA splice variants in bovine granulosa cells: changes with follicle
572 size and regulation by FSH in vitro. *Molecular Reproduction and Development* **74** 680-686.
- 573 **O'Hara L, Scully S, Maillo V, Kelly AK, Duffy P, Carter F, Forde N, Rizos D & Lonergan P**
574 2012 Effect of follicular aspiration just before ovulation on corpus luteum characteristics,
575 circulating progesterone concentrations and uterine receptivity in single-ovulating and
576 superstimulated heifers. *Reproduction* **143**(5) 673-82.
- 577 **Okuda K, Korzekwa A, Shibaya M, Murakami S, Nishimura R, Tsubouchi M, Woclawek-**
578 **Potocka I & Skarzynski DJ** 2004 Progesterone is a suppressor of apoptosis in bovine luteal cells
579 *Biology of Reproduction* **71** 2065-2071.

580 **Perry GA, Smith MF, Lucy MC, Green JA, Parks TE, MacNeil MD, Roberts AJ & Geary**
581 **TW** 2005 Relationship between follicle size at insemination and pregnancy success. Proceedings
582 of the National Academy of Sciences of the United States of America **102** 5268-5273.

583 **Pursley JR, Mee MO & Wiltbank MC** 1995 Synchronization of ovulation in dairy cows using
584 PGF2 α and GnRH. *Theriogenology* **44** 915–923.

585 **Quintal-Franco JA, Kojima FN, Melvin EJ, Lindsey BR, Zanella E, Fike KE, Wehrman**
586 **ME, Clopton DT & Kinder JE** 1999 Corpus luteum development and function in cattle with
587 episodic release of luteinizing hormone pulses inhibited in the follicular and early luteal phases of
588 the estrous cycle. *Biology of Reproduction* **61** 921-926.

589 **Rai AJ, Kamath RM, Gerald W & Fleisher M** 2009 Analytical validation of the GeXP
590 26hydroxyl and design of a workflow for cancer-biomarker discovery using multiplexed gene-
591 expression profiling. *Analytical and Bioanalytical Chemistry* **393** 1505-1511.

592 **Reiner A, Yekutieli D & Benjamini Y** 2003 Identifying differentially expressed genes using
593 false discovery rate controlling procedures. *Bioinformatics* **19**(3) 368-375.

594 **Robinson RS Nicklin LT Hammond AJ Schams D Hunter MG & Mann GE** 2007 FGF2 is
595 more dynamic than VEGFA during the follicle-luteal transition in the cow. *Biology of*
596 *Reproduction* **77** 28-36.

597 **Robert C, Gagné D, Lussier JG, Bosquet D, Barnes FL & Sirard MA** 2003 Presence of LH
598 receptor mRNA in granulosa cells as a potential marker of oocyte developmental competence and
599 characterization of bovine splicing isoforms. *Reproduction* **125** 437-446.

- 600 **Savio JD, Keenan L, Boland MP & Roche JF** 1988 Pattern of growth of dominant follicles
601 during the oestrous cycle in heifers *Journal of Reproduction and Fertility* **83** 663-671.
- 602 **Sharma SC, Clemens JW, Pisarska MD & Richards JS** 1999 Expression and function of
603 estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin.
604 *Endocrinology* **140**(9) 4320-4334.
- 605 **Skarzynski DJ & Okuda K** 1999 Sensitivity of bovine corpora lutea to prostaglandin F2a is
606 dependent on progesterone, oxytocin, and prostaglandins. *Biology of Reproduction* **60** 1292-1298.
- 607 **Webb R & Campbell BK** 2007 Development of the dominant follicle: mechanisms of selection
608 and maintenance of oocyte quality. *Soc Reprod Fertil Suppl.* **64** 141-163.
- 609 **Wiltbank MC, Souza AH, Carvalho PD, Bender RW & Nascimento AB** 2011 Improving
610 fertility to timed artificial insemination by manipulation of circulating progesterone
611 concentrations in lactating dairy cattle. *Reproduction, Fertility and Development* **24** 238-243.
- 612 **Wiltbank MC & Pursley JR** 2014 The cow as an induced 27hydroxyl²⁷: Timed AI after
613 synchronization of ovulation. *Theriogenology* **81** 170-185.
- 614 **Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC & Sinclair**
615 **KD** 2010 Dietary omega-3 and -6 polyunsaturated fatty acids affect the composition and
616 development of sheep granulosa cells, oocytes and embryos. *Reproduction* **139** 57-69.
- 617 **Wu Y, Gutierrez MR, Sciabica KE & Luo J** 2008 Quantitative multiplexed gene expression:
618 an analysis of sensitivity, accuracy and precision. *Technical Information Bulletin, B2008-8704*,
619 Beckman Coulter Inc.

620 **Yoshioka S, Abe H, Sakumoto R & Okuda K** 2013 Proliferation of luteal steroidogenic cells in
621 cattle. *PloS One* **8** e84186.

622 **Yuan W & Lucy MC** 1996 Messenger ribonucleic acid expression for growth hormone receptor,
623 luteinizing hormone receptor, and steroidogenic enzymes during the estrous cycle and pregnancy
624 in porcine and bovine corpora lutea. *Domestic Animal Endocrinology* **13** 431-44.

625

For Review Only

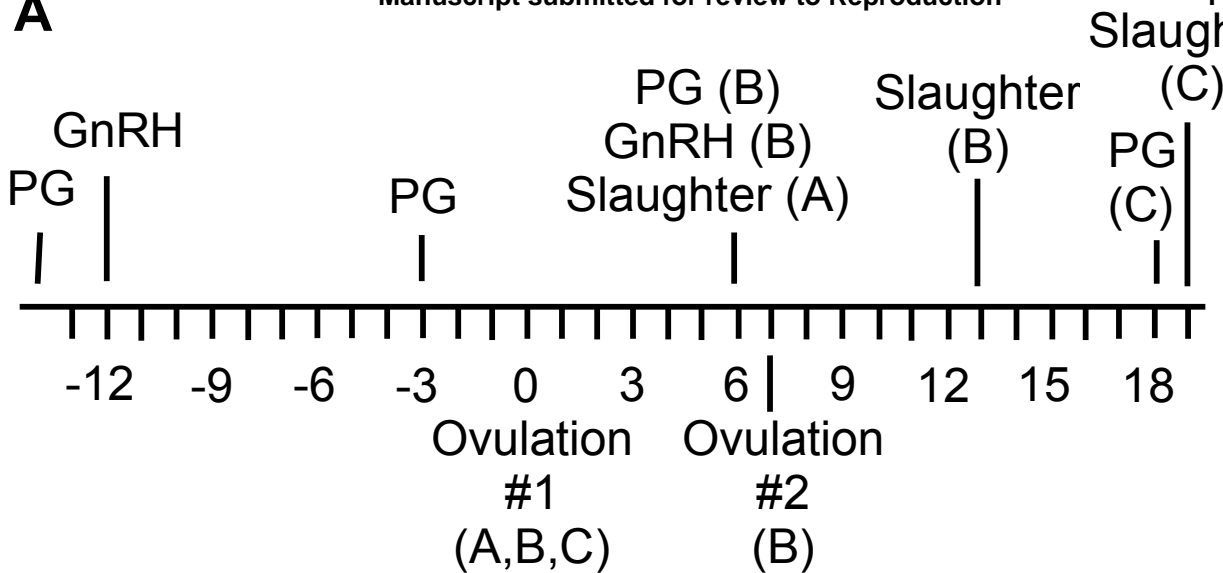
1 **Figure 1** Oestrous cycle manipulation timeline (A). Oestrous cycles of 24 Hereford x Holstein
2 heifers were synchronised using prostaglandin (PG) and gonadotrophin releasing hormone
3 (GnRH). Animals were given further injections according to their treatment group, indicated by
4 letters A to C 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.

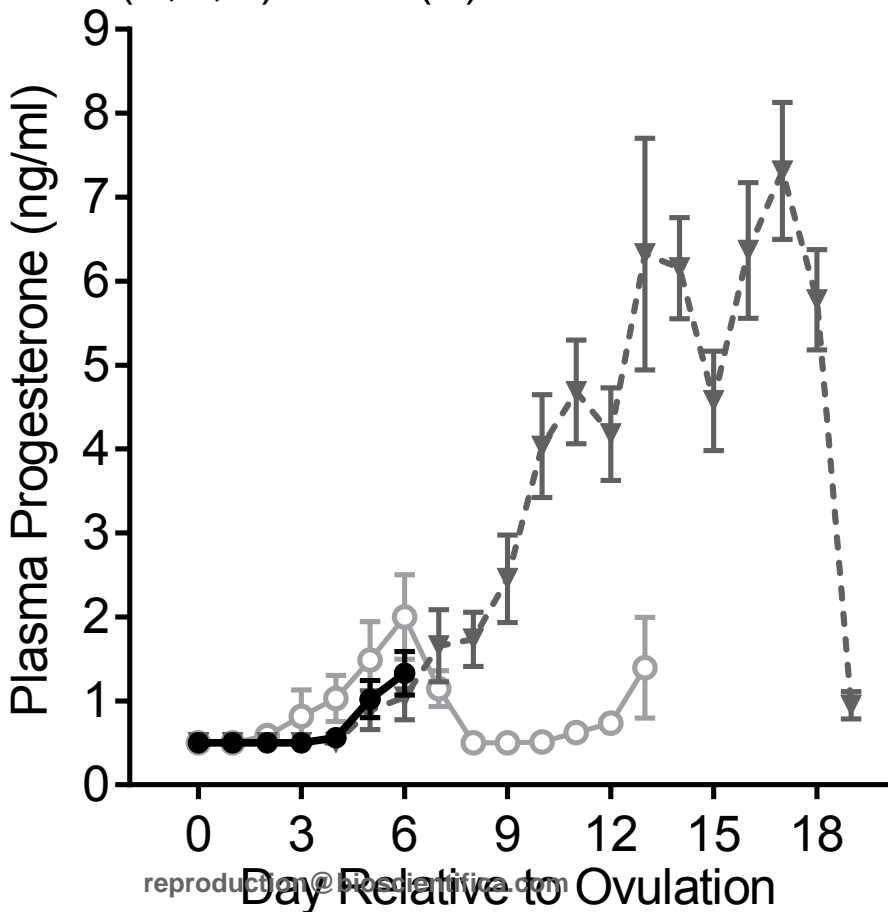
14 **Figure 3** Follicular fluid IGFBP2 protein concentration (from cycle-stage determined abattoir
15 derived ovaries) was greater ($P < 0.001$) in early follicular- than early luteal-phase dominant
16 follicles (A). IGFBP2 was quantified by Western blotting. B, A typical gel for follicular fluid
17 from two early luteal- (EL1 & EL2) and two early follicular (EF1 & EF2) phase-dominant
18 follicles, with alternative lanes left blank (-).

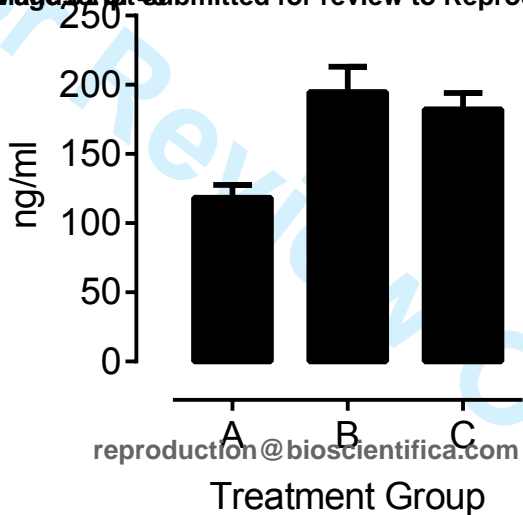
19

A



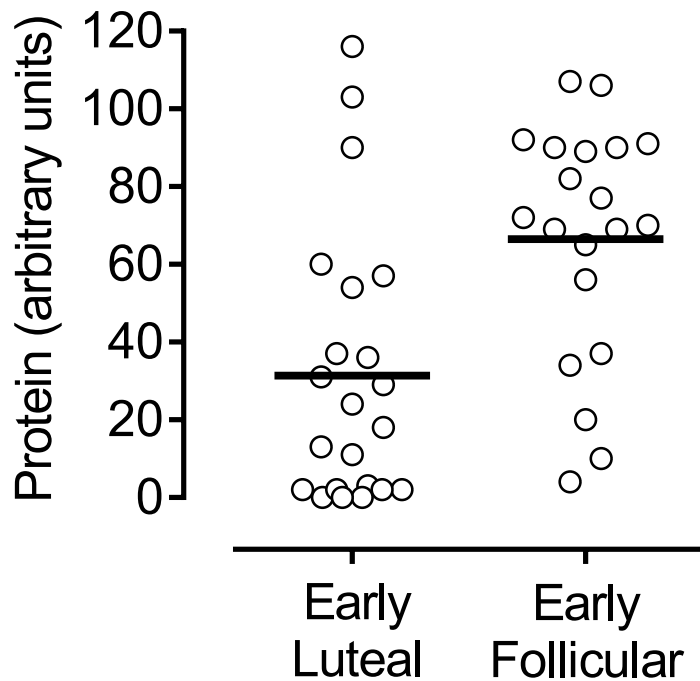
B





reproduction@bioscientifica.com

A



B

marker EL1 - EL2 - EF1 - EF2

55kDa

35kDa

25kDa

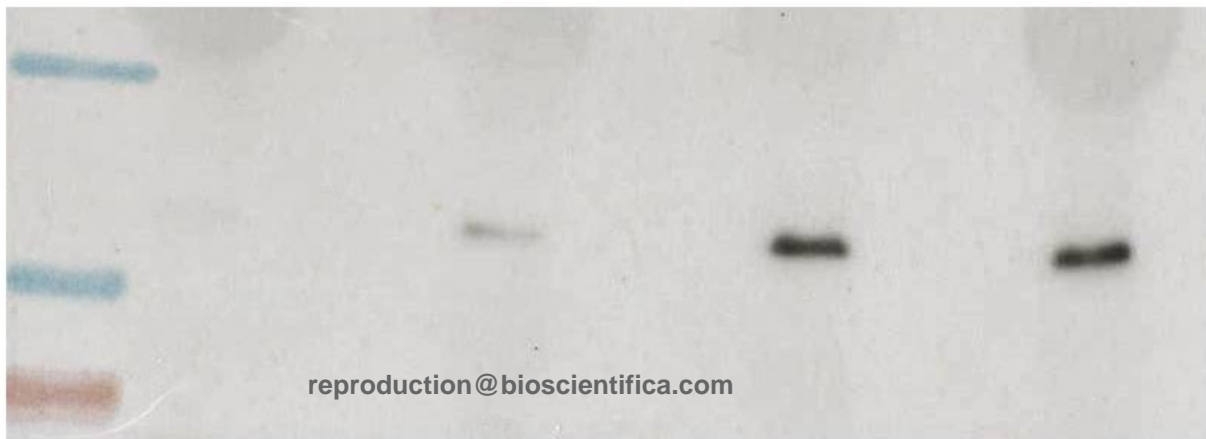


Table 1A. Transcripts quantified in bovine dominant follicles and corpora lutea by GeXP.

Gene	Full Name	Accession Number	Primers
TGF-β Superfamily			
<i>AMH</i>	anti-Mullerian hormone	NM_173890	F: aggtgacctatagaatacgtgagctgagcgtagacct R: gtacgactcactatagggagacaggctgatgaggagctt
<i>BMP2</i>	bone morphogenetic protein (BMP) 2	NM_001099141	F: aggtgacctatagaataacttttgacaccagggttg R: gtacgactcactatagggactaatccgacatgcctctt
<i>BMP4</i>	BMP 4	NM_001045877	F: aggtgacctatagaatagcttcaccacgaagaacat R: gtacgactcactatagggatagctgtgatgagggtcc
<i>BMP6</i>	BMP 6	XM_869844.3	F: aggtgacctatagaatatgtcatgtggcattttgtt R: gtacgactcactatagggaaccaacacaggagaagtggc
<i>BMPR1A</i>	BMP receptor, type IA	NM_001076800	F: aggtgacctatagaatagtggtgtgtgcatacgtgc R: gtacgactcactatagggaatggctttatgcgattgg
<i>BMPR1B</i>	BMP receptor, type IB	NM_001105328	F: aggtgacctatagaataatggaacagcagaggaatgc R: gtacgactcactatagggaagtccacggagaagaaa
<i>BMPR2</i>	BMP receptor, type II	XM_617592	F: aggtgacctatagaatacctgtcacacaataggcgtg R: gtacgactcactatagggactggacatcgaatgctcaga
<i>INHHA</i>	inhibin, alpha	NM_174094	F: aggtgacctatagaatatagtcaccctcccagtttc R: gtacgactcactatagggaggttggcaccatctcatic
<i>INHBA</i>	inhibin, beta A	NM_174363	F: aggtgacctatagaataccaaagaaggcagtgacctg R: gtacgactcactatagggaagctggagacaggaagatg
<i>INHBB</i>	inhibin, beta B	NM_176852	F: aggtgacctatagaataagatcatcagcttcgccg R: gtacgactcactatagggacttcaggtagaccacaggc
Insulin/IGF family			
<i>IGF1</i>	insulin-like growth factor (IGF) 1	NM_001077828	F: aggtgacctatagaatagaagatgcccatcacatcct R: gtacgactcactatagggagcctcctcagatcacagctc
<i>IGF1R</i>	IGF 1 receptor	XM_606794.3	F: aggtgacctatagaatacaaggcaatctgctcatca

			R: gtacgactcactataggaagttccccttagctgctcc
<i>IGF2</i>	IGF 2	NM_174087	F: aggtgacctatagaataacagcgagacctgacagaa
			R: gtacgactcactataggagacgggtgactctgtgtg
<i>IGF2R</i>	IGF 2 receptor	NM_174352	F: aggtgacctatagaataggaccttctacctgagcgtg
			R: gtacgactcactataggagttctggagctgaaaggtcg
<i>IGFBP2</i>	IGF binding protein 2	NM_174555	F: aggtgacctatagaatacaagggtggcaaacatcac
			R: gtacgactcactataggagaggtgtacaggccatgct
<i>IGFBP4</i>	IGF binding protein 4	NM_174557	F: aggtgacctatagaatacaggctccccttactcctc
			R: gtacgactcactataggaccttctccatcaggcacat
<i>IGFBP5</i>	IGF binding protein 5	NM_001105327.1	F: aggtgacctatagaatagatcgaagagactcccgtg
			R: gtacgactcactataggagtcagcttcttctcggtc
<i>INSR</i>	insulin receptor	XM_590552	F: aggtgacctatagaataaaagaggccccttaccagaa
			R: gtacgactcactataggatgtacggcgttcatcagaaa
Steroidogenic mediators			
<i>CYP11A1</i>	cytochrome P450, family 11, subfamily A, polypeptide 1	NM_176644.2	F: aggtgacctatagaataaagttgacccaaccaggtg
			R: gtacgactcactataggagtgccacgtcaccgatatg
<i>CYP17A1</i>	cytochrome P450, family 17, subfamily A, polypeptide 1	NM_174304	F: aggtgacctatagaataagacaaccaaaggcattg
			R: gtacgactcactataggaggcaggatcctcattcttga
<i>CYP19A1</i>	cytochrome P450, family 19, subfamily A, polypeptide 1	NM_174305	F: aggtgacctatagaataaagccaagagcaacaagcat
			R: gtacgactcactataggaatttggcgctaattccaaga
<i>ESR1</i>	estrogen receptor 1	NM_001001443	F: aggtgacctatagaataggtgtacatggacagcagca
			R: gtacgactcactataggatccaggtaataggcacctg
<i>ESR2</i>	estrogen receptor 2	NM_174051	F: aggtgacctatagaatagacagaccacaagcccaaat
			R: gtacgactcactataggagttcacgccaaggactctt
<i>HSD3B1</i>	2hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	NM_174343.2	F: aggtgacctatagaatagcagaaaaccaaggagtgga
			R: gtacgactcactataggaatcaccttgtctgtcccctg
<i>PGR</i>	progesterone receptor	XM_583951.4	F: aggtgacctatagaatagtctcgtctacggggac

			R: gtacgactcactataggattgtacaggacgcactccag
<i>SCARB1</i>	scavenger receptor class B, member 1	NM_174597.2	F: aggtgacctatagaataacaaactgggaacatccagc
			R: gtacgactcactataggagatgggatgagcagtagga
<i>LRP8</i>	low density lipoprotein receptor-related protein 8	NM_001097565.1	F: aggtgacctatagaataccctgcaagggttcattgtat
			R: gtacgactcactataggagaaaatggcctcattctcca
<i>SHBG</i>	sex hormone-binding globulin	NM_001098858	F: aggtgacctatagaataccagagtcattggaggcta
			R: gtacgactcactataggagatcccaagtcgaaactca
<i>STAR</i>	steroidogenic acute regulatory protein	NM_174189.2	F: aggtgacctatagaatacctactgccaggaaagatgc
			R: gtacgactcactatagggaagaacctaggagagagccgc
Cytokines			
<i>IL1B</i>	interleukin (IL) 1, beta	NM_174093.1	F: aggtgacctatagaatatgaaccatcaacgaaatga
			R: gtacgactcactataggatggatgtttccatctcccat
<i>IL2</i>	IL 2	NM_180997.1	F: aggtgacctatagaatacaaacgggtgcactacttca
			R: gtacgactcactataggagaaatccttgatctctctgggg
<i>IL6</i>	IL 6	NM_173923.2	F: aggtgacctatagaataagctctcattaagcgcatgg
			R: gtacgactcactataggatctcgatctttgtcttcag
<i>IL8</i>	IL 8	NM_173925	F: aggtgacctatagaataaccaatggaacgaggctctg
			R: gtacgactcactataggacctacaccagaccacacag
<i>KITLG</i>	KIT ligand	NM_174375	F: aggtgacctatagaataagcattgccagcattctttt
			R: gtacgactcactataggagaaactgttaccgccaatgt
<i>MIF</i>	macrophage migration inhibitory factor	NM_001033608.1	F: aggtgacctatagaatacaacttctgcacatgaacg
			R: gtacgactcactataggacgtttattgctcctccagg
<i>PTPRC</i>	protein tyrosine phosphatase receptor type C	BC148881	F: aggtgacctatagaatacggagatgcaggatcaaact
			R: gtacgactcactataggaccagatcatctccagaaa
Apoptotic regulators			
<i>CCND2</i>	cyclin D2	NM_001076372.1	F: aggtgacctatagaataagcagtagcgtcaggaccag
			R: gtacgactcactatagggaagagaaggagagagcggattg
<i>CFLAR</i>	CASP8 and FADD-like apoptosis regulator	NM_001012281.1	F: aggtgacctatagaataactaaggctccagaatggcag

			R: gtacgactcactataggagcttgacttcatagcccagg
<i>GADD45B</i>	growth arrest and DNA-damage-inducible, beta	NM_001040604.1	F: aggtgacctatagaatatcacgaacctcacacagac R: gtacgactcactataggagctgtttccgcagcaagttt
Angiogenic regulators			
<i>HIF1A</i>	hypoxia-inducible factor 1, alpha subunit	NM_174339.3	F: aggtgacctatagaatatgcctctgaaactccaaagc R: gtacgactcactatagggactggggcctgtaaagaaa
<i>VEGFA</i>	vascular endothelial growth factor A	NM_174216.1	F: aggtgacctatagaataagcaaggcaagaaaatccct R: gtacgactcactatagggatcctggtgagacgtctggtt
Miscellaneous			
<i>FGF1</i>	fibroblast growth factor 1	NM_174055	F: aggtgacctatagaatagtaacgcgcttctaataatgcc R: gtacgactcactatagggaatgagagggaatcatgccag
<i>FSHR</i>	FSH receptor	NM_174061	F: aggtgacctatagaataatgtttccaggagcctct R: gtacgactcactatagggatgaccctagcctgagtcac
<i>SFRS9</i>	splicing factor, arginine/serine-rich 9	NM_001083398	F: aggtgacctatagaataatgcctgcgtaaactgg R: gtacgactcactatagggaattcccaccacgtctctag
<i>PGF2AR</i>	Prostaglandin F2 α receptor	BD187584	F: aggtgacctatagaatatgccactttttctaggcag R: gtacgactcactatagggaatggcattgcaacaacaatga
House-keeping genes			
<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase	NM_001034034	F: aggtgacctatagaatacacacctcaagattgtcagca R: gtacgactcactataggaggtcataagtccctccacga
<i>H2AZ</i>	H2A histone family, member Z	NM_174809.2	F: aggtgacctatagaatatccagtggttgattccag R: gtacgactcactatagggatttggttggtgaaagctaa
<i>RPLP0</i>	ribosomal protein, large, P0	NM_001012682.1	F: aggtgacctatagaataactgtctgaaaaggtaaggc R: gtacgactcactataggagactcctccgactcctcctt

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

Product name	Product Location	Primers
<i>LHCGRex2</i>	exons 2-4	F: aggtgacctatagaatacacctatctccctatcaaagtaatcc R: gtacgactcactatagggacgaggagatttgtaaagcgc
<i>LHCGRex8</i>	exons 8-11	F: aggtgacctatagaatagagctgaaggaaaatgcacg R: gtacgactcactatagggaggagtgtcttggttaagcaga
<i>LHCGRex11</i>	within exon 11	F: aggtgacctatagaatatgttaggcacatcaggcaaa R: gtacgactcactatagggacctgttcattgattggaag

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

B. Thecal cells

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

*Values are mean \pm 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.**

For Review Only

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

7 Values are mean \pm 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.

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.

References

- Berisha B, Sinowatz F & Schams D** 2004 Expression and localization of fibroblast growth factor (FGF) family members during the final growth of bovine ovarian follicles. *Molecular Reproduction and Development* **67** 162-171.
- Bromfield JJ & Sheldon IM** 2011 Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression in vitro. *Endocrinology* **152** 5029-5040.
- Bornstein SR, Rutkowski H & Vrezas I** 2004 Cytokines and steroidogenesis. *Molecular Cellular Endocrinology* **215** 135-141.

Erickson GF, Shimasaki S 2003 The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. *Reproductive Biology & Endocrinology* **1** 9.

Glister C & Knight PG 2010 Changes in expression of bone morphogenetic proteins (BMPs), their receptors and inhibin co-receptor betaglycan during bovine antral follicle development: inhibin can antagonize the suppressive effect of BMPs on thecal androgen production. *Reproduction* **140** 699-712.

Glister C, Satchell L, Bathgate RA, Wade JD, Dai Y, Ivell R, Anand-Ivell R, Rodgers RJ & Knight PG 2013 Functional link between bone morphogenetic proteins and insulin-like peptide 3 signaling in modulating ovarian androgen production. *Proceedings of the National Academy of Sciences of United States of America* **110** E1426-1435

Jiang Z & Price CA 2012 Differential actions of fibroblast growth factors on intracellular pathways and target gene expression in bovine ovarian granulosa cells. *Reproduction* **144** 625-632.

Kayani AR, Glister C & Knight PG 2009 Evidence for an inhibitory role of bone morphogenetic protein(s) in the follicular–luteal transition in cattle. *Reproduction* **137** 67-78.

Knight PG & Glister C 2006 TGF-beta superfamily members and ovarian follicle development. *Reproduction* **132** 191-206.

Petroff MG, Petroff BK & Pate JL 1999 Expression of cytokine messenger ribonucleic acids in the bovine corpus luteum. *Endocrinology* **140** 1018-1021.

Rico C, Fabre S, Médigue C, di Clemente N, Clément F, Bontoux M, Touzé JL, Dupont M, Briant E, Rémy B, Beckers JF & Monniaux D 2009 Anti-mullerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. *Biology of Reproduction* **80** 50-59.

Richards JS, Liu Z, Shimada M 2008 Immune-like mechanisms in ovulation. *Trends Endocrinology & Metabolism* **19** 191–196

Rico C, Médigue C, Fabre S, Jarrier P, Bontoux M, Clément F & Monniaux D 2011 Regulation of anti-Müllerian hormone production in the cow: a multiscale study at endocrine, ovarian, follicular, and granulosa cell levels. *Biology of Reproduction* **84** 560-571.

Takahashi M, Hayashi M, Manganaro TF & Donahoe PK 1986 The ontogeny of Mullerian inhibiting substance in granulosa cells of the bovine ovarian follicle. *Biology of Reproduction* **35** 447–453.

Telleria CM, Ou J, Sugino N, Ferguson S & Gibori G 1998 The expression of interleukin-6 in the pregnant rat corpus luteum and its regulation by progesterone and glucocorticoid. *Endocrinology* **139** 3597-3605.

Vigier B, Picard JY, Tran D, Legeai L & Josso N 1984 Production of anti-Müllerian hormone: another homology between Sertoli and granulosa cells. *Endocrinology* **114** 1315–1320.

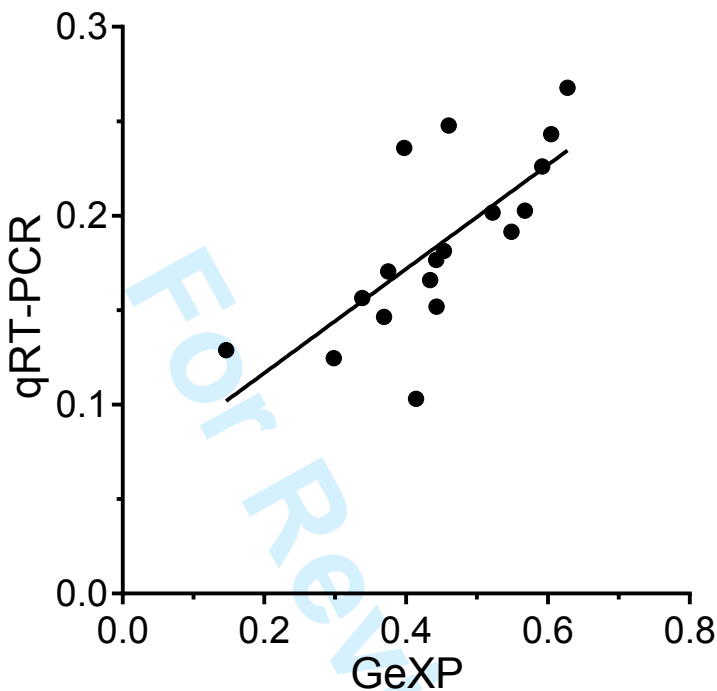
Supplementary Figure 1 A comparison of GeXP and qRT-PCR determined *ESR2* (A) and *HIF1A* (B) transcript expression in granulosa cells from bovine dominant ovarian follicles collected from a local abattoir. Expression is given in arbitrary fluorescence units relative to house-keeping gene expression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

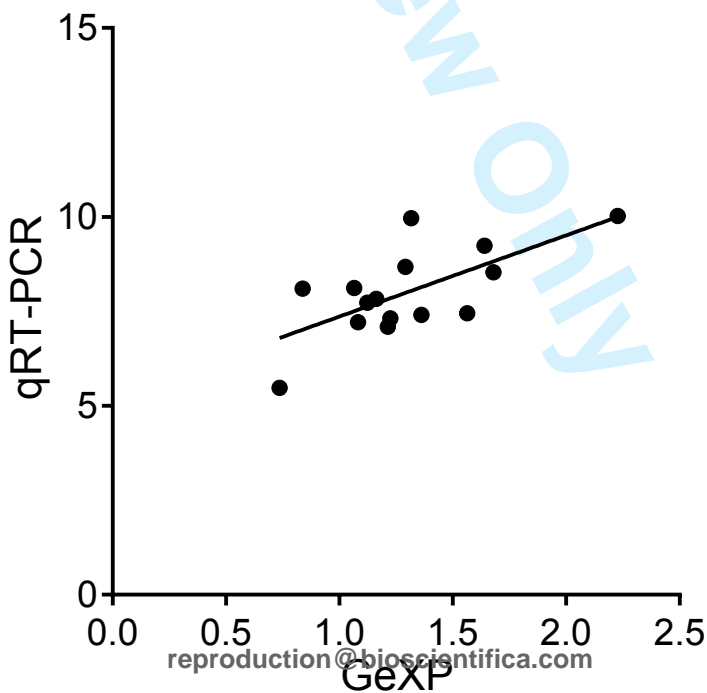
Supplementary Figure 3. Relationships between ovulatory dominant follicle (DF) and 6-day old *corpus luteum* (CL) diameter (A), and CL diameter and progesterone (P4) synthesis (B). All DFs were scanned on experimental Day -1 (●) and the resulting CL were measured on experimental Day 6 by ultrasonography (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.

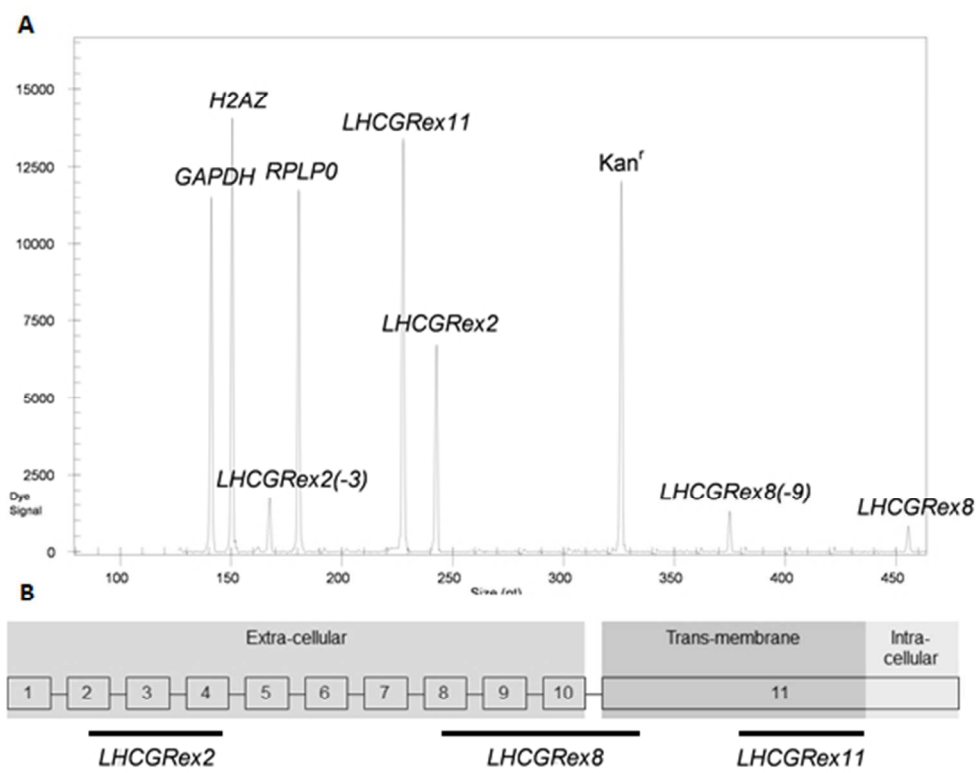
Supplementary Figure 4. Ovarian follicular growth and plasma progesterone (P4) concentrations (ng/ml) in eight Group C Hereford x Holstein heifers following synchronised oestrus (ovulation occurred between 11 am on Day -1 and 11 am on Day +1), as determined by trans-rectal ultrasonography. Heifers 230 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

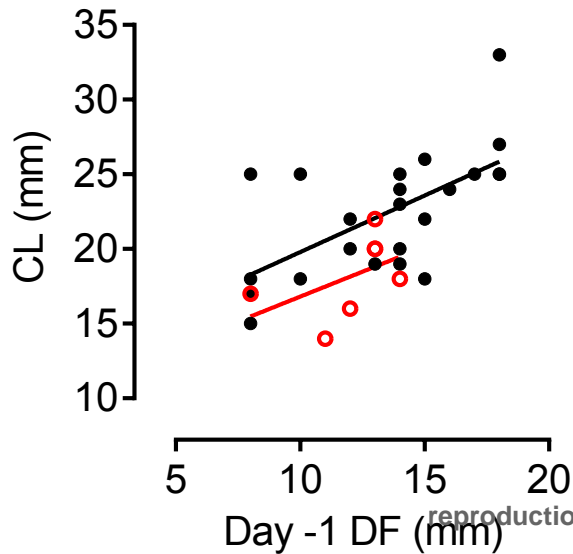




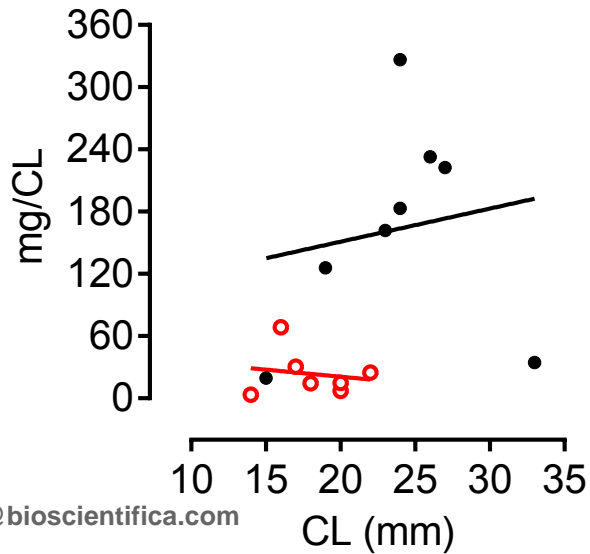
152x121mm (96 x 96 DPI)

Only

A. Follicle vs CL diameter

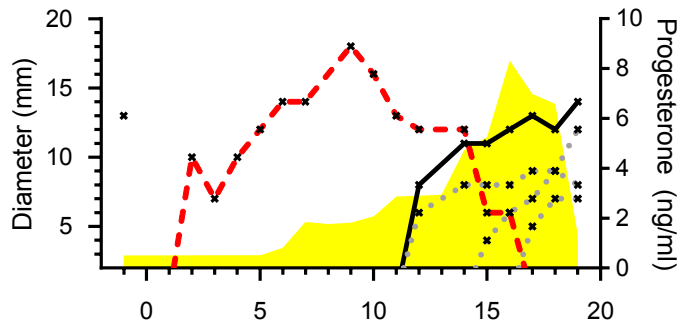
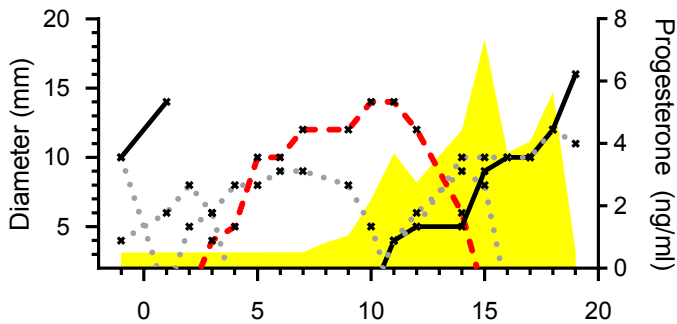


B. Progesterone synthesis



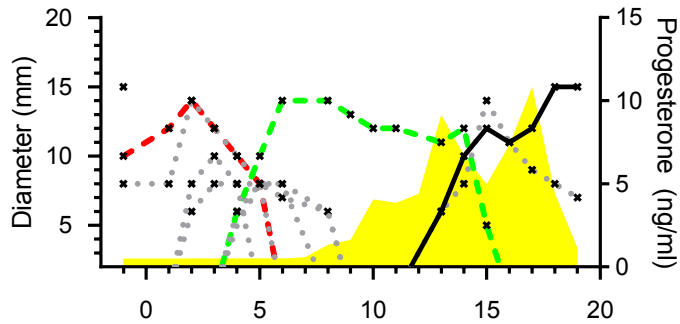
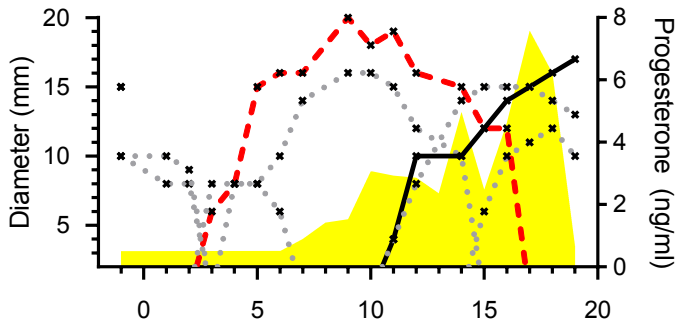
A. Animal 230

B. Animal 231



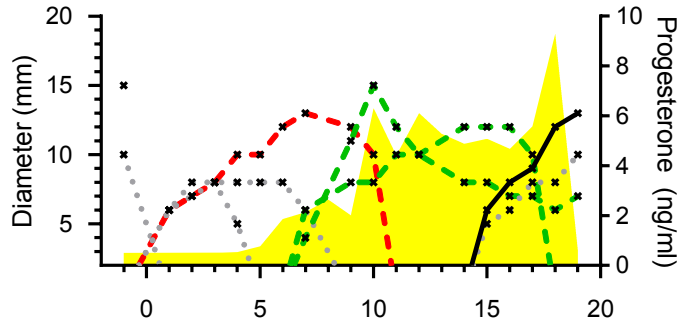
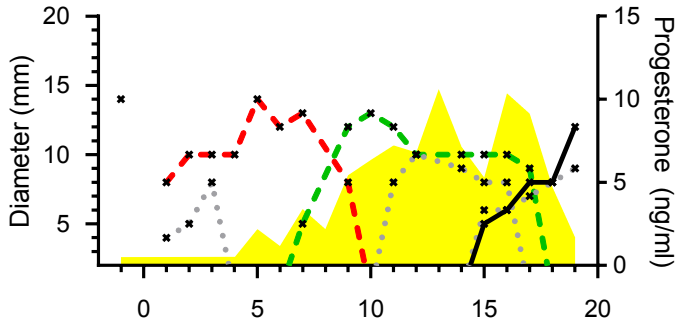
C. Animal 289

D. Animal 404



E. Animal 409

F. Animal 239



G. Animal 260

H. Animal 220

