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2 **Running title: Toll-like receptors in the bovine corpus luteum**

3 **Toll-like receptor and related cytokine mRNA expression in bovine corpora lutea**
4 **during the estrous cycle and pregnancy**

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27

28 Abstract

29 Improving our understanding of the mechanisms controlling the corpus luteum (CL) and
30 its role in regulating the reproductive cycle should lead to improvements in the
31 sustainability of today's global animal industry. The corpus luteum (CL) is a transient
32 endocrine organ composed of a heterogeneous mixture steroidogenic, endothelial and
33 immune cells, and it is becoming clear that immune mechanisms play a key role in CL
34 regulation especially in luteolysis. Toll-like receptors (TLR) mediate innate immune
35 mechanisms via the production of pro-inflammatory cytokines, especially within various
36 tissues, although the role of TLR within CL remains unknown. Thus, the objectives of
37 this study were to characterize TLR mRNA expression in the CL during the estrous cycle
38 and in pregnancy day 30-50, and to examine the role of TLR signaling in luteal cells.
39 Corpora lutea were collected at various stages of the cycle and pregnancy and analyzed
40 for TLR and cytokine mRNA expression. In addition, luteal cells were cultured with the
41 TLR4 ligand (lipopolysaccharide, LPS) for 24 h to evaluate the role of TLR4 in
42 regulating luteal function. Toll-like receptors 1, 2, 4, 6, tumor necrosis factor alpha
43 (TNF), interferon gamma (IFNG), and interleukin (IL)12, mRNA expression was greatest
44 in regressing CL compared with earlier stages ($P < 0.05$), whereas no change was observed
45 for IL6 mRNA expression. Cytokine mRNA expression in cultured luteal cells was not
46 altered by LPS. Based on these data, one or more of the TLRs found within the CL may
47 play a role in luteolysis, perhaps via pro-inflammatory cytokine mRNA expression.

48

49 **Keywords:** Toll-like Receptors, Corpus Luteum, Cytokines, Luteal Immune Response

50

51 **Introduction:**

52 Understanding the complex molecular regulatory mechanisms involved in the
53 maintenance or regression (luteolysis) of the bovine corpus luteum (CL) is essential to
54 the development of improved methods for enhancing reproductive efficiency of dairy
55 cattle, especially since continued secretion progesterone by the CL is critical for
56 embryonic survival and the successful outcome of pregnancy. Luteolysis has been
57 described as a pro-inflammatory event (Walusimbi and Pate, 2013), and there is strong
58 evidence to show that the pro-inflammatory cytokines (e.g. Tumor Necrosis Factor (TNF)
59 Interferon gamma (IFNG) and Interleukin 1 beta (IL1B)) display elevated levels of
60 mRNA expression in bovine CL undergoing natural or prostaglandin (PG) F-2 α -induced
61 luteolysis (Petroff et al. 2001; Neuvians et al., 2004). Furthermore TNF and IFNG
62 treatment of bovine luteal cells in vitro inhibited progesterone production in a dose
63 dependent manner, indicating that these cytokines have luteolytic actions (Petroff et al.,
64 2001; Skarzynski et al., 2008; Walusimbi and Pate, 2013). Lastly, TNF and IFNG have
65 been shown to act synergistically to promote luteal cell death (Petroff et al., 2001;
66 Walusimbi and Pate, 2013). One ligand/receptor system that regulates inflammation in
67 tissues are the Toll-like Receptors (TLR) and their ligands. In view of their involvement
68 in promoting inflammation and our need to investigate further the control of
69 inflammation during luteolysis in the bovine CL, we chose to examine the mRNA
70 expression of these receptors in bovine CL throughout the estrous cycle and during
71 pregnancy.

72 Toll-like receptors are critical components of the innate-immune system, allowing
73 host cells to recognize and mount an appropriate response (e.g. inflammation) to

74 microbial pathogens, that the host organism may encounter (Aflatoonian and Fazeli 2008;
75 Kawai and Akira 2010). Toll-like receptors are members of the pattern recognition
76 receptor (PRR) family that have evolved to recognize protein domains/sequences on
77 various pathogens, which are known as pathogen-associated molecular patterns (PAMPs)
78 (Aflatoonian and Fazeli 2008; Kawai and Akira 2010). There are currently 10 (human,
79 bovine) or 12 (mouse) different TLR, each of which has a relatively well-defined
80 function (Aflatoonian and Fazeli 2008; Davies et al. 2008; Kawai and Akira 2010). Toll-
81 like receptors 1, 2, 4 and 6 are associated with the plasma membrane and enable
82 recognition of a variety of PAMPs produced by bacteria, viruses, fungi or parasites.
83 Additionally, the lipopolysaccharide (LPS) and triacyl lipopeptides produced by gram
84 negative bacteria such as E. coli is recognized by TLR4, and by TLR1 and TLR2
85 heterodimers, respectfully, and diacyl lipopeptides of gram positive bacteria are
86 recognized by TLR2 and TLR6 heterodimers (Aflatoonian and Fazeli 2008; Kawai and
87 Akira 2010). On the other hand, TLR3, 7 and 8 are intracellular receptors and recognize
88 internalized bacterial or viral nucleic acids (Aflatoonian and Fazeli 2008; Kawai and
89 Akira 2010). Little is known about the function of, or ligand for, TLR 10, but it is
90 homologous to TLR2 and is likely a TLR2-associated receptor (Aflatoonian and Fazeli,
91 2008). However, TLR (e.g TLR2 and 4) activation has also been shown to occur in
92 response to the products of normal cellular degradation, such as heat shock proteins
93 (HSP) and high mobility group box 1 (HMGB1) proteins, and thus TLR-mediated pro-
94 inflammatory cytokine production may occur quite independently of microbial infection
95 (Kawai and Akira, 2010). Furthermore, since luteolysis involves widespread cellular
96 degeneration, it is likely that the regressing CL during the estrous cycle would represent a

97 rich source of potential endogenous ligands to activate TLRs expressed within this tissue
98 in otherwise healthy animals.

99 The responses of Toll-like receptors to microbial pathogens have been studied in
100 reproductive tissues such as the bovine uterus (endometrium) and ovary (Battaglia et al.
101 2000; Sheldon et al. 2002; Herath et al. 2007; Davies et al, 2008; Sheldon et al., 2009).
102 These investigators demonstrated several TLRs in the endometrium and examined their
103 role in response to pathogens in cattle with uterine infections (e.g. endometritis, metritis
104 or pyometra). Lipopolysaccharide (LPS), produced by *E. coli*, a common causative agent
105 of uterine infections in dairy cows, binds to TLR4 on uterine epithelial cells. This induces
106 a pro-inflammatory cascade of cytokines leading to altered prostaglandin (PG) production
107 (increased PGE-2:PGF-2 α ratio) by the uterus (Davies et al, 2008; Sheldon et al., 2009)
108 which causes delayed luteolysis and extended cycles in animals with uterine infections
109 (Davies et al, 2008; Sheldon et al., 2009; Saut et al., 2014). In other studies it was shown
110 that LPS concentrations in peripheral blood (Mateus et al. 2003), and within the ovarian
111 follicular fluid (Herath et al. 2007), are elevated in animals with uterine infections,
112 suggesting that LPS *may* also have direct effects on ovarian function. Indeed, estrogen
113 production and follicular growth is reduced in animals with uterine infections or in
114 response to LPS infusion (Battaglia et al. 2000; Sheldon et al. 2002; Herath et al. 2007).
115 It is also known that smaller follicles produce smaller CL with lower serum progesterone
116 concentrations (Perry et al. 2005; Robinson et al. 2005), which may help to explain lower
117 than normal luteal phase progesterone concentrations observed in animals with uterine
118 infections (Sheldon et al. 2009). Furthermore, LPS has been shown to exert direct actions
119 on the CL as shown by studies in which bovine luteal cells were cultured with increasing

120 doses of LPS, resulting in increased progesterone secretion (Grant et al. 2007). Taken
121 together, these data suggest that in cows with uterine infections, microbial pathogens
122 such as LPS, may also exert direct effects on the CL.

123 In view of the importance of improving our understanding about the role of
124 cytokines and their role in the inflammatory luteolytic process, the overall goal of the
125 current study was to examine the potential roles that TLR may have within the CL during
126 the bovine estrous cycle versus pregnancy, with particular emphasis on their possible
127 roles, via pro-inflammatory actions, in the control of luteolysis. This study was designed
128 to carry out the following ***Primary Objectives:***

- 129 1) To determine the steady state levels of mRNA expression of multiple TLR and
130 cytokines in the bovine CL collected at different stages of the estrous cycle and
131 pregnancy, and
- 132 2) To examine the effects of LPS on cytokine mRNA expression by bovine luteal cells in
133 culture.

134

135 **Materials and Methods:**

136 **Tissue Collection**

137 Ovaries were collected from a local abattoir (Martin's Abattoir and Wholesale
138 Meats, Godwin, NC). Approval to obtain specimens from this official establishment was
139 granted by the North Carolina Department of Agriculture and Consumer Services Meat
140 and Poultry Inspection Division. Corpora lutea (CL; n=51) were collected and catalogued
141 by stage (I, II, III, IV, or Pregnant (P)) based on appearance, ovarian and reproductive
142 tract morphology (Ireland et al. 1980). Corpora lutea collected at stage I (~days 1-4:

143 n=7), stage II (~days 5-10: n=11), stage III (~days 11-17: n=11), stage IV (~days 18-20:
144 n=13), and from pregnancy (~ 30-50 days: n=7), were used for these experiments.
145 Corpora lutea of pregnancy were confirmed by the presence of embryos in the uterus.
146 Stage of pregnancy was estimated by conceptus size or crown-rump length measurement
147 (by Dr. Peter Farin). Following collection, luteal tissues were frozen at -80°C until
148 analysis for mRNA or progesterone concentrations. Additional mid-stage (stage II; n=6)
149 CL were collected and placed into ice-cold Hams F-12 media (Gibco, Invitrogen
150 Corporation, Carlsbad, CA, USA) during transport to the laboratory to be dissociated for
151 culture experiments.

152 **Dissociation of corpora lutea**

153 Corpora lutea were collected and dissociation of luteal tissue was performed
154 according to Pate (1993). Luteal tissue was minced and placed in 24 mM HEPES-
155 buffered Ham's F-12 culture medium (Gibco, Invitrogen) containing 0.5% BSA (Sigma-
156 Aldrich, St. Louis, MO, USA), 20 µg/ml gentamicin (Gentamicin Reagent Solution;
157 Invitrogen), and 2000 U/g tissue collagenase type I (Worthington Biochemical
158 Corporation, Lakewood, NJ, USA). Pate (1993) demonstrated that this method of luteal
159 cell isolation results in highly enriched populations of small and large luteal cells.
160 Smaller cell types, endothelial cells, fibroblasts, immune cells, are removed during the
161 slower speed centrifugation process (Pate 1993; Poole and Pate, 2012). Enrichment of
162 small and large luteal cells as previously described (Pate, 1993; Poole and Pate, 2012)
163 was confirmed in this set of experiments. Following dissociation, luteal cells were re-
164 suspended in Ham's F-12 culture medium and cell viability was determined via standard
165 viability stain (trypan blue; Sigma-Aldrich). Cells were placed in a 0.5% trypan blue

166 solution, and counted on a hemacytometer according to Pate (1993) with cell viability
167 routinely \geq 80% live cells.

168 **Luteal cell culture**

169 Luteal cells (1.0×10^6 cells/ml) were plated to approximately 75% confluency in
170 24 well plates in 24 mM HEPES-buffered Ham's F-12 culture medium (Gibco,
171 Invitrogen) containing 5% FCS (Sigma–Aldrich), 20 μ g/ml gentamicin (Gentamicin
172 Reagent Solution; Invitrogen) and incubated for 24 h at 37°C and 5% CO₂ in air. After
173 24 hr, media was replaced to remove dead cells and debris. Luteal cells were treated with
174 LPS (TLR ligand tested; cat # L3024; Sigma–Aldrich) at 0, 0.01, 0.1, 1 μ g/ml
175 concentrations and were incubated at 37°C and 5% CO₂ in air, for additional 24 hours.
176 These LPS doses have been shown to be effective in increasing progesterone secretion by
177 bovine luteal cells in culture (Grant et al. 2007). After culture, media were removed and
178 luteal cells were harvested to quantify cytokine mRNA by quantitative PCR (qPCR).
179 Culture experiments were repeated a total of three times using CL from different animals.

180 **Total RNA extraction and PCR**

181 A. RNA Extraction and semi-quantitative PCR - bovine CL whole tissue samples

182 RNA was extracted from luteal tissue (approximately 100 mg) using 1ml of
183 TRIzol (Sigma–Aldrich) and homogenization as described by Crosier et al., (2002).
184 Following RNA isolation, RNA pellets were allowed to dry at room temperature for 5
185 minutes and were then dissolved in nuclease free diH₂O (Sigma–Aldrich) in volumes
186 ranging from 10-60 μ l based on pellet size. The concentration of RNA, and the 260nm :
187 280nm OD ratio, were determined by Nanodrop 2000 Spectrophotometer (Thermo
188 Scientific, Waltham, MA, USA). RNA samples were then subjected to DNase treatment

189 followed by cDNA synthesis and semi-quantitative PCR as described previously (Crosier
190 et al. 2002). For optimum synthesis efficiency, 1 µg of RNA was reversed transcribed in
191 a total volume of 20 µl per reaction in accordance with manufacturers' recommendations.
192 RNA was determined to be of high quality based on a 260:280 ratio of 1.8 or more, and
193 this was confirmed by the presence of clear 28S and 18S rRNA bands on denaturing
194 agarose gels stained with ethidium bromide. Primers for this procedure were synthesized
195 based upon the published GenBank sequences to produce the expected product sizes (see
196 Table 1). All primers were validated for semi-quantitative (sq) RT-PCR by running each
197 primer at 20, 25, 30, 35 and 40 cycles and choosing a cycle number that corresponded to
198 the linear range of amplification (36 cycles for all primer sets, except for the
199 housekeeping gene, Beta Actin (ACTB) which was 33 cycles). Once primers were
200 validated, sq RT-PCR was carried out using 2 µl of the cDNA product and the Taq PCR
201 Mastermix® Kit (Qiagen Sciences, Valencia, CA, USA). Thermocycler conditions were
202 as follows: 3 minute initial denaturation 94°C, followed by 33 or 36 cycles: denaturation,
203 30 seconds 94°C; annealing, 1 minute 50°C; extension, 1 minute 72°C; followed by a 5
204 minute 72°C final extension. Amplicons were separated on 2% agarose gels and stained
205 with ethidium bromide. Signal intensities were quantified using Lab-Works imaging
206 system (UVP Imaging Company, Upland, CA, USA). Values were calculated as the
207 ratio of amplicon band intensity/ACTB band intensity; ACTB was used as a
208 housekeeping gene since its levels of mRNA expression did not vary with stage of
209 estrous cycle.

210 B. RNA Extraction and quantitative (Q)-PCR - bovine CL whole tissue and cell culture
211 samples

212 Total RNA was extracted and purified from luteal tissues at various stages of the
213 estrous cycle/pregnancy and the cultured luteal cells, using the RNeasy® mini kit
214 (Qiagen Sciences, Valencia, CA, USA) according to manufacturer's recommendations.
215 DNase treatment (Turbo DNA-free kit, Life Technologies, Carlsbad, CA, USA) was
216 performed and RNA concentrations and purity determined using the Nanodrop 2000
217 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Random-primed, reverse-
218 transcribed (RT) cDNA synthesis in 20 µl reactions were performed using the iScript
219 cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's
220 recommendations. For optimum synthesis efficiency, 1 µg of RNA was reversed
221 transcribed in a total volume of 20 µl per reaction in accordance with manufacturers'
222 recommendations. Forward and reverse primers directed toward *Bos taurus* TLR 1, 2, 4
223 and 6, TGFB, IFNG, TNF, IL6 and IL12A (Table 2; Integrated DNA Technologies,
224 Coralville, IA, USA) were designed specifically for Q-PCR. Final concentrations (300
225 nM) of the forward and reverse primers were determined for each primer pair based upon
226 optimal amplification efficiency (> 95%). Following the RT reaction, Q-PCR was
227 performed on the LightCycler 480 II (Roche Diagnostics, Indianapolis, IN, USA) using
228 the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with
229 2 µl of the cDNA product in a 20 µl reaction volume under the following conditions: 95
230 °C for 5 min, 40 cycles of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 60 s, followed by
231 an extra elongation of 5 min at 72 °C. Annealing temperature was optimized for each
232 primer set. Primers were optimized using a temperature gradient from 51° to 61°C and
233 checked for specificity based on the appearance of a single band via 1.2% agarose gel in
234 1X TAE buffer. Optimal annealing temperature for all primers was 57°C with a 300 nM

235 primer concentration. Melting curves were generated for each run. The Q-PCR
236 amplification products were electrophoretically separated on 1.5% agarose gels and
237 visualized with ethidium bromide under UV light to ensure a specific band corresponded
238 to the size of the expected cDNA fragment. Specific bands were extracted and purified
239 using QIAquick gel extraction kit (Qiagen Sciences, Valencia, CA, USA). Following
240 extraction, Q-PCR products were sequenced to confirm product specificity. A control
241 sample that was not reverse transcribed was used to confirm that the product obtained
242 was not amplified from genomic DNA. The Ct (the cycle number at which the
243 fluorescence exceeds a threshold level) was determined for each reaction (run in
244 triplicate) using the LightCycler 480 software and quantification was accomplished by
245 normalization of Q-PCR data for each TLR and cytokine to that of ribosomal protein L19
246 (*RPL19*) RNA (housekeeping gene), using the $\Delta\Delta C_t$ method (Livak and Schmittgen
247 2001). Ribosomal protein L19 was shown to be a constitutively expressed gene across all
248 CL from different stages of the cycle and pregnancy. Q-PCR data from the luteal cell
249 cultures studies were expressed as fold-difference compared with the control treatment (0
250 $\mu\text{g/ml}$ of LPS).

251 **Progesterone Radioimmunoassay**

252 The method for the extraction and assay of CL progesterone concentrations was
253 adapted from previously published methods (Estill et al. 1995; Garverick et al., 1985).
254 Briefly, approximately 100 mg of luteal tissue was immersed in 10ml of cold 100%
255 ethanol and homogenized. After centrifugation, the ethanol extract was dried under
256 nitrogen and re-constituted in radioimmunoassay buffer (phosphate buffered saline plus
257 0.1% gelatin; PBS-gel; Estill et al, 1995). CL samples and progesterone standards (25-

258 2000 pg/ml) were assayed in duplicate using the Coat-a-count RIA kit (Coat-a-count,
259 Diagnostic Products Corporation, Los Angeles, CA, USA) following the manufacturers
260 instructions (Richards et al., 1994; Estill et al., 1995). All samples were run in a single
261 assay and the intra-assay coefficient of variation was 6.3%. The sensitivity of this assay
262 was 10 pg progesterone per tube (Richards et al., 1994). This assay has also been
263 validated in our laboratory for use with bovine serum in a single assay (Whisnant and
264 Burns, 2002; Lyons et al., 2016). Data are reported as nanograms of progesterone/mg
265 luteal tissue.

266 **Statistical Analysis:**

267 All statistical analyses were performed using the mixed model of SAS (Statistical
268 Analysis System Institute, Cary, NC, USA). mRNA expression data were log-
269 transformed and analyzed using covariate analysis, with RPL19 as the covariate for Q-
270 PCR and ACTB as the covariate for semi-quantitative PCR. One-way ANOVA was
271 performed to determine differences among stages of luteal development for progesterone
272 concentrations. In addition, a correlation analysis (PROC CORR) was conducted to
273 determine the relationship between the mRNA expression of TLR and pro-inflammatory
274 cytokines within bovine luteal tissue. Cell culture experiments were independently
275 replicated with cells from different animals a minimum of three times unless otherwise
276 stated. Additionally, the cell culture experiment was analyzed for linear, quadratic, and
277 cubic relationships using a PROC GLM with orthogonal contrast for respective
278 relationships. Differences were considered significant at $p < 0.05$, and a statistical
279 tendency was declared when $0.05 < p < 0.1$.

280 **Results:**

281 Progesterone Concentrations within bovine CL:

282 Whole tissue CL progesterone concentrations were significantly decreased to 6.5
283 \pm 2.2 ng/mg in stage IV CL, from ~ 30-40 ng/mg observed in CL from stages I, II, III,
284 and in pregnancy ($p < 0.001$). These CL progesterone concentrations are in agreement with
285 those published by Garverick and colleagues (Garverick et al., 1985) in the bovine CL,
286 who used a similar CL extraction procedure for progesterone.

287 TLR 1, 2, 4 and 6 mRNA expression:

288 The steady state mRNA expression levels of Toll-like receptors 1, 2, 4 and 6
289 mRNAs were examined in whole tissue CL samples via Q-PCR (Fig. 1). Toll-like
290 receptor 1 mRNA expression was significantly increased in stage IV compared to stages
291 I, II and III ($p < 0.01$). Similar stage dependent profiles were seen for *TLR2*, *TLR4* and
292 *TLR6* with a significantly increased at stage IV compared to stages I, II, III, and
293 pregnancy ($p < 0.01$; $p < 0.05$; $p < 0.05$; respectfully). There was also a tendency for
294 pregnancy to show increased *TLR6* mRNA expression over stage II ($p < 0.1$).

295 TLR3, 7, 8 and 10, mRNA expression:

296 Analysis of steady state mRNA levels of *TLR3*, 7, 8 and 10 was carried out by
297 semi-quantitative (sQ) PCR, and the data are summarized in Table 3. No significant
298 differences were observed between the stages of the cycle or pregnancy for any of these
299 TLRs. *TLR3* mRNA expression at stage IV tended to be lower ($p < 0.1$) than stage III.
300 *TLR8* mRNA expression tended to be lower in pregnancy compared to stage IV ($p < 0.1$).

301 MD2, CD14 and CD45 mRNA expression:

302 An examination of the expression levels of *MD2*, *CD14* and *CD45* mRNAs was
303 also carried out by semi-quantitative (sQ) PCR (Table 3). Lymphocyte antigen (*MD2*)

304 and *CD14* were examined since they represent critical components of *TLR4* signaling.
305 Expression of *MD2* was significantly increased at stage III compared to stages I, II, IV
306 and pregnancy ($p < 0.05$). *CD14* showed a similar increase (significant) at stage III
307 compared to stage IV ($p < 0.01$). Cluster of Differentiation (CD) 45 (non-specific marker
308 of leukocytes) mRNA expression was significantly increased ($p = 0.01$) from stage I to
309 stage III and there was a tendency ($p < 0.1$) for stage III to be increased compared to stage
310 II. Expression of *CD45* at Stage IV was significantly decreased compared to stages II, III,
311 and pregnancy ($p < 0.05$).

312 Cytokine mRNA expression:

313 Interferon gamma, *IL6*, *IL12* and *TNF* mRNA expression was also examined in
314 whole tissue CL samples by Q-PCR (Fig. 2). IFNG was significantly increased at stage
315 IV compared to stages I, II, III, and pregnancy ($p < 0.05$). Interleukin 12 mRNA
316 expression displayed a similar profile with a significant increase observed at stage IV vs.
317 stages I, II, III, and pregnancy ($p < 0.01$). Tumor necrosis factor mRNA expression also
318 showed a significant increase at stage IV compared with stages I, II, III, and pregnancy
319 ($p < 0.01$). The cytokine *IL6* tended to be decreased in pregnancy compared to stage I
320 ($p < 0.1$) but showed no other stage dependent changes. There was no stage-dependent
321 differential expression seen for TGF β mRNA expression (Fig. 2). An analysis of
322 correlation between TLRs and proinflammatory cytokines (IFNG, *IL6*, *IL12*, and *TNF*)
323 mRNA expression in bovine CL tissue revealed a significant positive correlation ($p <$
324 0.001) between TLR1, 2, 4, and 6 and IFNG, *IL12*, and *TNF* mRNA expression (Table 4
325 and Fig. 3). No significant correlations were found between TLR1, 2, 4, and 6 and *IL6*
326 (Table 4).

327 Cytokine mRNA expression in bovine luteal cells treated with LPS in culture

328 Luteal cells were dissociated and cultured for 24h with LPS (0, 0.01, 0.1,
329 1 µg/ml). Cytokine (*TNF*, *TGFβ*, *IFNG*, *IL6*, and *IL12*) mRNA expression did not
330 significantly differ in response to increasing LPS doses ($p>0.05$; Fig. 4), even though
331 there were numerical increases observed in *IFNG* and *IL6* mRNA expression with
332 increasing LPS dose. Additional analyses to test for a linear, quadratic, or cubic
333 relationships for cytokine mRNA expression relative to LPS doses were carried out, but
334 did not show any significance ($p>0.05$).

335

336 **Discussion:**

337 The major focus of this study was to improve our understanding of role of TLR in
338 the pro-inflammatory/luteolytic process during the estrous cycle in normal animals.
339 However, the data described here may also be viewed from the perspective that in the CL
340 of cows with uterine infections, TLRs may also respond to the direct actions microbial
341 agents, leading to inappropriate inflammatory pathways that may have adverse impacts
342 on the CL, reproductive cyclicity and pregnancy in these animals.

343 The studies described above provide compelling evidence for the expression of
344 mRNAs for 8 different TLRs (i.e. *TLR1*, 2, 3, 4, 6, 7, 8 and 10) within the bovine CL by
345 PCR analysis. *TLR1-TLR10* mRNAs were also found to be expressed in bovine follicles
346 raising the possibility that TLR mRNA expression continues following ovulation and
347 during luteinization of the follicle into a fully functioning CL (Herath et al. 2007; Price et
348 al. 2013).

349 Stage IV CL are essentially CL undergoing luteal regression as confirmed by the
350 reduced CL progesterone concentrations seen at this stage, which prompts us to suggest
351 that the increase of *TLR1*, 2, 4 and 6 mRNA expression (Figure 1) may be associated
352 with, or in some way involved in, the process of luteolysis potentially through the up-
353 regulation of pro-inflammatory cytokines (*IFNG*, *TNF* and *IL12*; Figure 2). The
354 correlation data (Table 4 and Fig. 3) showing significant positive correlations between
355 *TLR1*, 2, 4 and 6 mRNAs and these cytokines, support the notion that these TLRs may
356 function to promote pro-inflammatory cytokine secretion during luteolysis in the cow.
357 Luteolysis has been described as a pro-inflammatory event (Walusimbi and Pate, 2013),
358 and the significantly elevated mRNA expression of pro-inflammatory cytokines *TNF*,
359 *IFNG* and *IL12* mRNA observed in this study in stage IV CL, support this notion.
360 Numerous studies have demonstrated that *TNF* and *IFNG* act as luteolytic, and
361 (cooperatively) as cytotoxic, agents within the bovine CL (Petroff et al. 2001; Skarzynski
362 et al. 2008; Walusimbi and Pate, 2013), and these cytokines have been shown by others
363 to display elevated mRNA expression in regressing bovine CL (Petroff et al. 2001).
364 Furthermore, Neuvians and colleagues (Neuvians et al., 2004) have shown that *PGF2 α*
365 treatment of cows increased *TNF*, *IFNG*, and *IL1B* mRNA expression, and that *TNF* and
366 *IFNG* treatment of bovine luteal cells in vitro inhibited progesterone production in a dose
367 dependent manner. Interleukin 12 (*IL12*), which was also elevated in regressing CL in
368 our studies, is a classical pro-inflammatory cytokine, and was shown to promote natural
369 killer cell differentiation, leading to increased *IFNG* mRNA expression (Yang et al.
370 2011). Since in our studies both *IL12* and *IFNG* were elevated, a similar relationship may
371 also exist in the bovine CL. It is worth considering what ligands might be involved in

372 activating these TLRs in the bovine CL of a normal healthy animal, in the absence of
373 ligands (e.g. LPS) generated by bacteria or viruses. It has been suggested that the
374 breakdown products of luteal tissue and dead or dying cells, such as those of the
375 extracellular matrix, heat-shock proteins and high mobility group box 1 (HMGB1)
376 proteins, may activate TLR (e.g TLR2 and 4) and thus promote pro-inflammatory
377 cytokine production (Kawai and Akira, 2010). Since stage IV represents regressing (i.e.
378 degenerating) CL, it is likely that this would represent a rich source of potential
379 endogenous ligands to activate TLRs expressed within this tissue.

380 The results presented here for *TLR 2* and *4* are consistent with a recent publication
381 showing that these two *TLR* are expressed in the bovine CL, and that their mRNA
382 expression was increased in CL collected at mid and late stages of the estrous cycle,
383 compared with those collected during the early cycle (Luttgenau et al. 2016). These
384 investigators were also able to demonstrate the mRNA expression of *TLR 2* and *4* at the
385 protein level using immunohistochemistry, and they observed immunoreactivity in both
386 endothelial and luteal cells, providing some critical insights into the cellular location of
387 these *TLRs* (Luttgenau et al. 2016).

388 Even though it is clear that *TLR 2* and *4* are expressed on luteal and endothelial
389 cells in non-regressing CL (Luttgenau et al. 2016), it is possible that as luteolysis
390 proceeds, at least some of the increased *TLR2* and *4* mRNA's observed in the regressing
391 (stage IV) bovine CL described above, may be associated with increased immune cell
392 (e.g. macrophages, T-cells, and neutrophils) infiltration into the CL (Walusimbi and Pate
393 2013). In the present study, *CD45*, which is a common leukocyte marker (Aflatoonian
394 and Fazeli 2008; Kawai and Akira 2010), increased at stage III (vs. stage I), but

395 decreased again at stage IV, while *TLR1*, 2, 4 and 6 mRNAs were at their maximal levels
396 in CL undergoing luteal regression (stage IV). Thus, while the increased mRNA
397 expression of *CD45* from the developing CL (stage I) to a fully functional CL (stage III)
398 may be explained by the increased infiltration of immune cells into the CL during this
399 period, the decline in *CD45* mRNA observed in stage IV CL, is difficult to explain since
400 this is a time when immune cell infiltration would be maximal (Walusimbi and Pate
401 2013).

402 Further evidence to indicate that TLR4 in bovine CL is functional, comes from
403 our data showing that two critical components of TLR4 signaling in response to its ligand
404 LPS, namely *MD2* and *CD14*, were also expressed at mRNA level in the bovine CL.
405 *MD2*, which is a TLR4 co-receptor, is located found on the external cell membrane and
406 aids in the binding of ligand (e.g. LPS) to the extracellular domain of the TLR4 dimer
407 (Hirata et al. 2005). Cluster of differentiation (CD) 14 also plays a role in the binding of
408 LPS to TLR4, and is an essential component of the functional TLR4 complex (Kawai and
409 Akira 2010). The mRNA expression of *CD14* and *MD2* mRNAs seen in fully functional
410 CL (stage III) were significantly elevated compared with early (stage I) and late (IV)
411 stages, suggesting that these components are expressed prior to the elevated mRNA
412 expression of TLR4 seen at stage IV, to prepare the TLR4-receptor-signaling complex for
413 a critical role in luteal regression.

414 It should be noted that *TLR2* and 4, and all cytokine mRNAs were found to be
415 decreased in pregnancy vs. stage IV CL. The general pattern of reduced pro-
416 inflammatory TLR and cytokine mRNA expression profiles during gestation may be

417 needed to prevent luteolysis, and ensure sufficient progesterone secretion to maintain
418 pregnancy and embryo survival (Aflatoonian and Fazeli 2008).

419 Even though the anti-inflammatory cytokine *TGFB* was expressed in the bovine
420 CL, it did not show a differential mRNA expression patterns based on CL stage. *TGFB*
421 may have a role in the CL throughout the estrous cycle and pregnancy to counter any
422 unexpected inflammatory signals that may threaten CL maintenance, and thus may play a
423 critical role in the ongoing regulation of luteal lifespan. Alternatively, since *PGF2 α*
424 treatment of bovine luteal endothelial cells induced the mRNA expression of *TGFB*, it
425 has been suggested that *TGFB* may play a role in disassembly of the microvasculature
426 during luteal regression (Maroni and Davis 2011).

427 In the current study, the intracellular TLRs (*TLR3*, *TLR7*, and *TLR8*) did not
428 display significantly different mRNA expression profiles at different stages of the estrous
429 cycle or pregnancy. These TLRs recognize viral or bacterial nucleic acids (Kawai and
430 Akira 2010), and given the location of the CL, the likelihood of luteal cells coming into
431 direct contact with bacteria or viruses is probably very small. Thus, the function of these
432 TLR within the CL is unknown and warrants further investigation. However, it has been
433 suggested that DNA arising from dead or dying cells may bind these TLR and activate
434 their intracellular pathways (Kawai and Akira 2010). Thus since luteolysis results in
435 wholesale death of multiple cells types, these TLR may also play an important role within
436 the regressing CL.

437 In the culture studies, we used dissociated bovine luteal cells from mid-cycle
438 (stage II) CL to examine a functional role of *TLR4*, using its ligand LPS, on cytokine
439 production, as a model for the pro-inflammatory response seen during luteolysis.

440 However, multiple analyses of linear, quadratic, and cubic relationships between different
441 LPS treatment doses were unable to reveal any statistically significant changes in
442 cytokine mRNA expression, even though some numerical increases in *IFNG* and *IL6*
443 mRNA expression were evident. There are several possible explanations for the apparent
444 lack of an effect of LPS on luteal cell cytokine mRNA expression. Firstly, while the CL
445 used for these studies were stage II, to maximize the yield of viable luteal cells and their
446 survival during the culture, *TLR4* levels (mRNA expression) were quite low at these
447 stages, and only increased in stage IV CL. It is possible that while a more robust response
448 to LPS may be observed in stage IV cells in vitro, obtaining sufficient viable and culture-
449 healthy cells to carry out this study would probably represent an insurmountable
450 challenge. Secondly, the CL is a heterogeneous tissue composed of multiple cell types
451 such as large luteal cells, small luteal cells, fibroblasts, endothelial cells and immune cells
452 (Skarzynski et al. 2008), many of which may express *TLR4* and be targets for LPS
453 (Aflatoonian and Fazeli, 2008; Kawai and Akira 2010; Luttgenu et al. 2016). Variations
454 in the viability or proportion of any of these cell types could contribute to the variability
455 seen in these cultures, which did show some evidence of a numerical increase in *IFNG*
456 and *IL6* at the highest dose, but were not significant. It is worth noting that in recent
457 studies, the effects of LPS on bovine (mid-cycle) CL function were examined in vivo,
458 and decreased enzymes associated with progesterone secretion and elevated *IL1B* and
459 *TNF* mRNA expression were observed (Luttgenau et al. 2016), suggesting that the bovine
460 luteal TLR4 receptor acts as a functional receptor.

461 In conclusion, we have presented evidence for the mRNA expression of several
462 *TLR*, TLR4 accessory proteins (CD14 and MD2) and cytokine mRNAs in bovine CL,

463 which appear to be maximal in mid (stage III; CD14 and MD2) and late (stage IV; TLR
464 and cytokines) CL. These findings suggest an involvement of one or more of the TLR in
465 the process of luteolysis, and perhaps in the increased mRNA expression of pro-
466 inflammatory cytokines, which also play a role in luteolysis. Finally, in view of the
467 prevalence of infectious diseases such as uterine infections (Sheldon et al. 2009) and
468 mastitis (Barker et al.1998) in dairy cattle, the data presented in this manuscript also raise
469 the possibility that TLR on the bovine CL may mediate adverse effects of infectious
470 pathogens, leading to infertility or sub-fertility.

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479 NC, for their assistance with tissue collection.

480 **Conflict of interest**

481 None of the authors have any conflict of interest to declare.

482

483 **Author contributions**

484 J E Gadsby, IM Sheldon and DH Poole designed the study; AM Tyson, HA Faircloth, M
485 D'Annibale-Tolhurst, J Chang, and PW Farin carried out the study and collected the data;

486 J E Gadsby, D. H. Poole and A.M. Tyson Nipper, analyzed the data and wrote the
487 manuscript

488

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579 **FIGURE LEGEND:**

580 **Table 1:** Primer Sequences for semi-quantitative PCR

581 **Table 2:** Primer Sequences for quantitative real-time PCR

582 **Table 3:** mRNA expression, via semi-quantitative PCR, of various toll like receptors
583 (TLR3, 7, 8, and 10) and their co-activators, Lymphocyte antigen (MD2) and Cluster
584 of Differentiation (CD) 14, and 45, at various stages of luteal development (I = days
585 1-4: n=7, II = days 5-10: n=11, III = days 11-17: n=11, IV = days 18-20 of the estrous
586 cycle: n=13, and P = pregnancy: n=9). Data are represented as least squared means±
587 SEM and different letters within a row indicate significant differences (P<0.05),
588 whereas * within a row indicates statistical tendencies (0.1<P>0.05).

589

590 **Table 4:** Correlation table between toll like receptors (TLR) 1, 2, 4 and 6 mRNA
591 expression and proinflammatory cytokines, interferon gamma (IFNG), interleukin (IL) 6,
592 12, and tumor necrosis factor alpha (TNF), mRNA expression. * indicates statistically
593 significant correlations (P<0.001).

594

595 **Figure 1:** Toll like receptor (*TLR*)1, 2, 4 and 6 mRNA expression, via quantitative Real-
596 Time PCR, at various stages of luteal development (I = days 1-4: n=7, II = days 5-10:
597 n=11, III = days 11-17: n=11, IV = days 18-20 of the estrous cycle: n=13, and P =
598 pregnancy: n=9). Expression of *TLR*1, 2, 4 and 6 increased in the regressing CL (days
599 18-20 of the estrous cycle) compared to other stages of the estrous cycle. Data are
600 presented as least-square means ± SEM. Different letters within a specific gene indicate
601 significant differences (P<0.05).

602

603 **Figure 2:** Interferon gamma (*IFNG*), Interleukin (*IL*) 6, 12, Transforming Growth Factor
604 beta (*TGFB*) and Tumor necrosis factor alpha (*TNF*) mRNA expression, via quantitative
605 Real-Time PCR, at various stages of luteal development (I = days 1-4: n=7, II = days 5-
606 10: n=11, III = days 11-17: n=11, IV = days 18-20 of the estrous cycle: n=13, and P =
607 pregnancy: n=9). Expression of *TNF*, *IFNG*, and *IL12* increased in the regressing CL
608 (days 18-20 of the estrous cycle) compared to other stages of the estrous cycle. Data are
609 presented as least-square means \pm SEM. Different letters within a specific gene indicates
610 significant differences ($p < 0.05$), whereas * within a specific gene indicates statistical
611 tendencies ($0.1 < p > 0.05$).

612

613 **Figure 3:** Correlation between toll like receptor (TLR) 4 mRNA expression and
614 proinflammatory cytokines, interferon gamma (*IFNG*), interleukin (*IL*) 6, 12, and tumor
615 necrosis factor alpha (*TNF*), mRNA expression. Expression of *TNF*, *IFNG*, and *IL12*
616 displayed a positive relationship with TLR4, whereas this relationship was not observed
617 with IL6 mRNA expression.

618

619 **Figure 4:** Interferon gamma (*IFNG*), Interleukin (*IL*) 6, 12, tumor necrosis factor alpha
620 (*TNF*) and transforming growth factor beta (*TGFB*) mRNA expression, via quantitative
621 Real-Time PCR, in luteal cells cultured in the presence of either 0, 0.01, 0.1, or 1 $\mu\text{g/ml}$
622 Lipopolysaccharide (LPS) for 24 hours. No differences were observed in cytokine
623 expression following treatment is increasing concentrations of LPS (n=3). Data are
624 presented as least-square means \pm SEM.

625 **Table 1:** Primer Sequences for semi-quantitative PCR

Target	GenBank accession no.	Primer Sequence	Cycle	Amplicon s
<i>ACTB</i>	NM_173979.3	Fwd 5' ATC GGC AAT GAG CGG TTC C-3' Rev 5' GTG TTG GCG TAG AGG TCC TTG-3'	33	143
<i>CD14</i>	NM_174008.1	Fwd 5' GGG TAC TCT CTG CTC AAG GAA C-3' Rev 5' CTT GGG CAA TGT TCA GCA C-3'	36	199
<i>CD45</i>	AJ400864.1	Fwd 5' CTC GAT GTT AAG CGA GAG GAA T-3' Rev 5' TCT TCA TCT TCC ACG CAG TCT A-3'	36	185
<i>MD2</i>	NM_001046517.1	Fwd 5' GGG AAG CCG TGG AAT ACT CTA T-3' Rev 5' CCC CTG AAG GAG AAT TGT ATT G-3'	36	204
<i>TLR3</i>	NM_001008664.1	Fwd 5' GAT GTA TCA CCC TGC AAA GAC A-3' Rev 5' TGC ATA TTC AAA CTG CTC TGC T-3'	36	195
<i>TLR7</i>	NM_001033761.1	Fwd 5' TCT TGA GGA AAG GGA CTG GTT A-3' Rev 5' AAG GGG CTT AAG GAA TAT C-3'	36	205
<i>TLR8</i>	NM_001033937.1	Fwd 5' TAA CCT TCG GAA TGT CTC CAG T-3' Rev 5' GTG GGA AAT TCT GTT TCG ACT C-3'	36	232
<i>TLR10</i>	NM_001076918.2	Fwd 5' ATG GTG CCA TTA TGA ACC CTA C-3' Rev 5' CAC ATG TCC CTC TGG TGT CTA A-3'	36	239

626

627

628 **Table 2:** Primer Sequences for quantitative real-time PCR

Target	GenBank accession no.	Primer Sequence	Amplicon
<i>IFNG</i>	NM_174086	Fwd 5' GAT CTG GAT TCT GAG CCA CTA C-3' Rev 5' GCC AGG TAT AAG GTG AGA TGA G-3'	175
<i>IL6</i>	NM_173923.2	Fwd 5' CAA GGA GAC ACT GGC AGA AA-3' Rev 5' CAG TGG TTC TGA TCA AGC AAA TC-3'	107
<i>IL12A</i>	U14416	Fwd 5' TCA AGC TCT GCA TCC TTC TTC-3' Rev 5' GGT TAT GAG AGA CCT CAG CAT TC-3'	254
<i>RPL19</i>	NM_001040516	Fwd 5' ATC GAT CGC CAC ATG TAT CA-3' Rev 5' GCG TGC TTC CTT GGT CTT AG-3'	227
<i>TGFB</i>	M36271	Fwd 5' CGT CAG CTC TAC ATT GAC TTC C-3' Rev 5' GGA CCT TGC TGT ACT GTG TAT C-3'	205
<i>TNF</i>	NM_173966	Fwd 5' TCT ACT CAC AGG TCC TCT TCA G-3' Rev 5' GAT GTT GAC CTT GGT CTG GTA G-3'	235
<i>TLR1</i>	NM_001046504.1	Fwd 5' ATT TCT TGC CAC CCT ACT CTG-3' Rev 5' GTT GAG ACA TGT TGC CAA ACT C-3'	100
<i>TLR2</i>	NM_174197.2	Fwd 5' GCA CTT CAA CCC TCC CTT TA-3' Rev 5' GTT CTC CGA AAG CAC AAA GAT G-3'	127
<i>TLR4</i>	NM_174198.6	Fwd 5' TCT ACT GCA GCC AGG ATG AA-3' Rev 5' GTA GTG AAG GCA GAG CTG AAA-3'	92
<i>TLR6</i>	NM_001001159.1	Fwd 5' GAC TCT CAA GCA TTT AGA CCT CTC-3' Rev 5' GCA AGT GAG CAA TGG GTA GTA-3'	146

629

630

631 **Table 3:**

Target	Stage of Luteal Development					P Value
	I	II	III	IV	P	
<i>CD14</i>	24.6±6.1 ^{ab}	20.4±3.0 ^{ab}	32.4±5.9 ^{ab}	16.1±4.5 ^a	22.5±8.2 ^{ab}	0.0074
<i>CD45</i>	16.7±2.1 ^a	23.2±2.6 ^{ab}	31.3±4.4 ^b	17.5±4.0 ^a	25.0±4.2 ^{ab}	0.0036
<i>MD2</i>	13.2±1.6 ^a	10.4±1.4 ^a	21.5±3.3 ^b	13.0±3.1 ^a	10.5±2.5 ^a	0.0376
<i>TLR3</i>	39.2±11.9	40.6±2.3	42.8±5.3 [*]	26.8±2.8 [*]	40.1±3.6	0.082
<i>TLR7</i>	10.4±1.6	11.7±1.5	16.4±7.8	14.3±12.9	10.5±6.8	>0.05
<i>TLR8</i>	10.9±2.2	15.5±5.0	18.6±2.3	22.8±8.0 [*]	9.8±3.1 [*]	0.084
<i>TLR10</i>	14.4±3.2	8.3±2.9	8.7±4.2	9.3±7.0	10.1±2.7	>0.05

632

633 **Table 3:** mRNA expression, via semi-quantitative PCR, of various toll like receptors (TLR3,
634 7, 8, and 10) and their co-activators, Lymphocyte antigen (MD2) and Cluster of
635 Differentiation (CD) 14, and 45, at various stages of luteal development (I = days 1-4: n=7, II
636 = days 5-10: n=11, III = days 11-17: n=11, IV = days 18-20 of the estrous cycle: n=13, and P
637 = pregnancy: n=9). Data are represented as least squared means± SEM and different letters
638 within a row indicate significant differences (P<0.05), whereas * within a row indicates
639 statistical tendencies (0.1<P>0.05).

640

641

642 **Table 4:**

	Proinflammatory Cytokines			
	IFNG	IL6	IL12	TNF
TLR1	0.77*	-0.02	0.75*	0.78*
TLR2	0.71*	-0.01	0.70*	0.75*
TLR4	0.61*	0.09	0.60*	0.65*
TLR6	0.80*	0.05	0.77*	0.80*

643

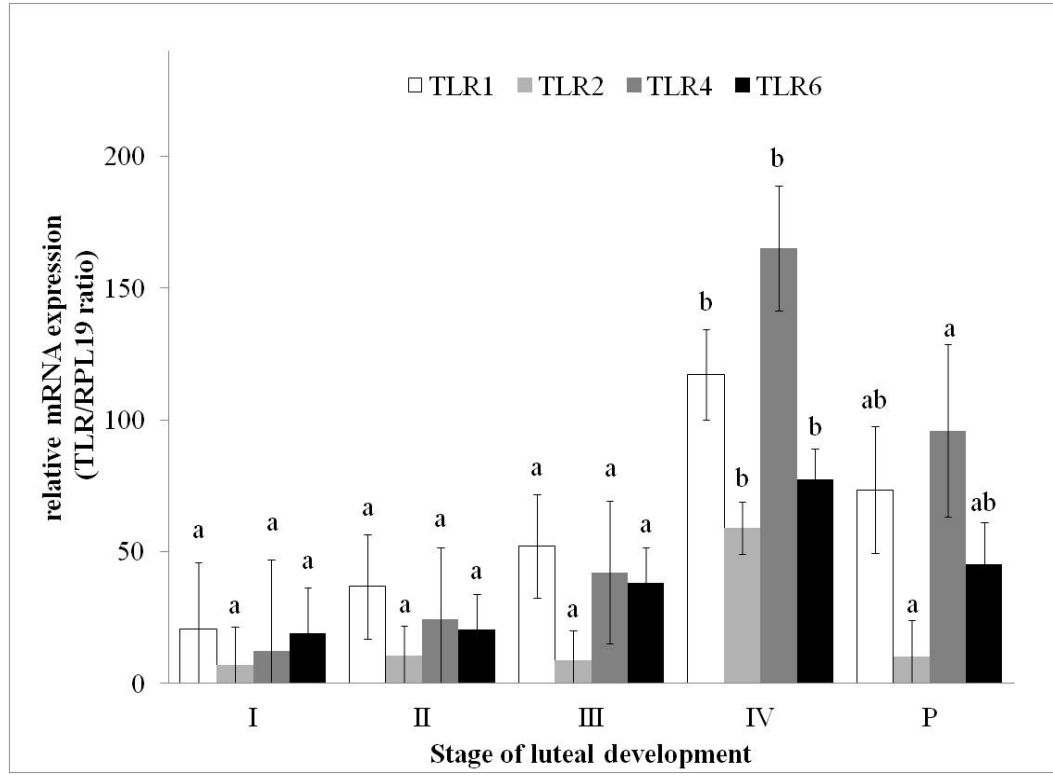
644 **Table 4:** Correlation table between toll like receptors (TLR) 1, 2, 4 and 6 mRNA expression and
 645 proinflammatory cytokines, interferon gamma (IFNG), interleukin (IL) 6, 12, and tumor necrosis
 646 factor alpha (TNF), mRNA expression. * indicates statistically significant correlations

647 (P<0.001).

648

649

650 **Figure 1**

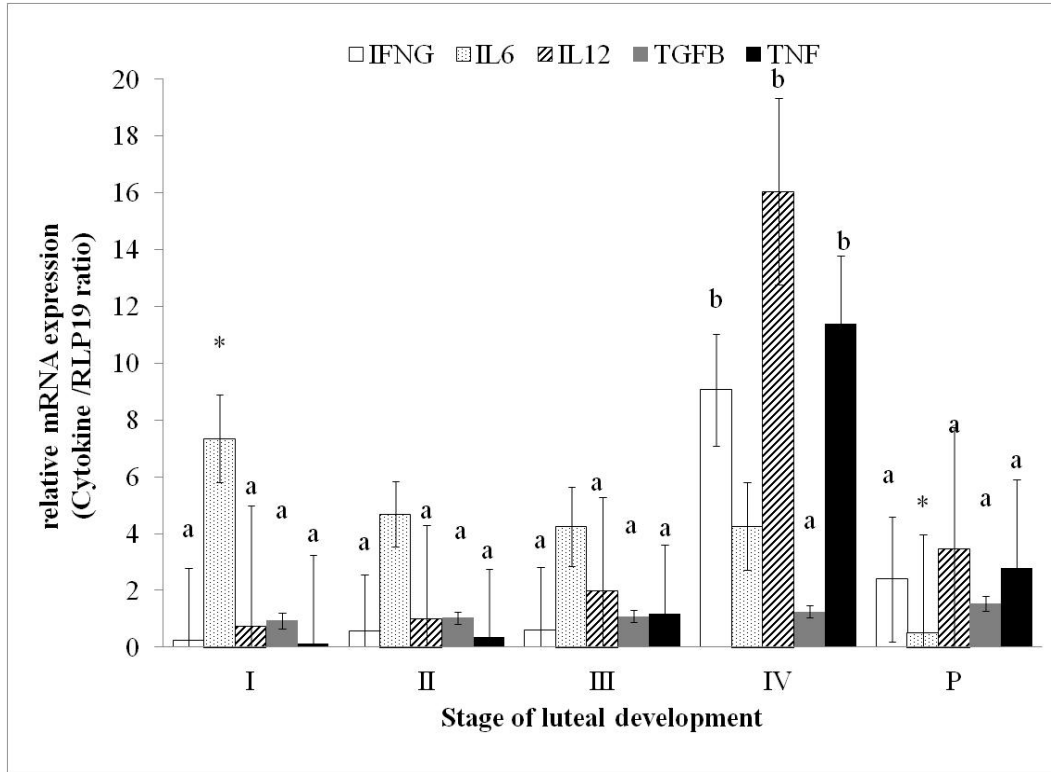


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652

653 **Figure 1:** Toll like receptor (*TLR*)1, 2, 4 and 6 mRNA expression, via quantitative Real-Time
654 PCR, at various stages of luteal development (I = days 1-4: n=7, II = days 5-10: n=11, III = days
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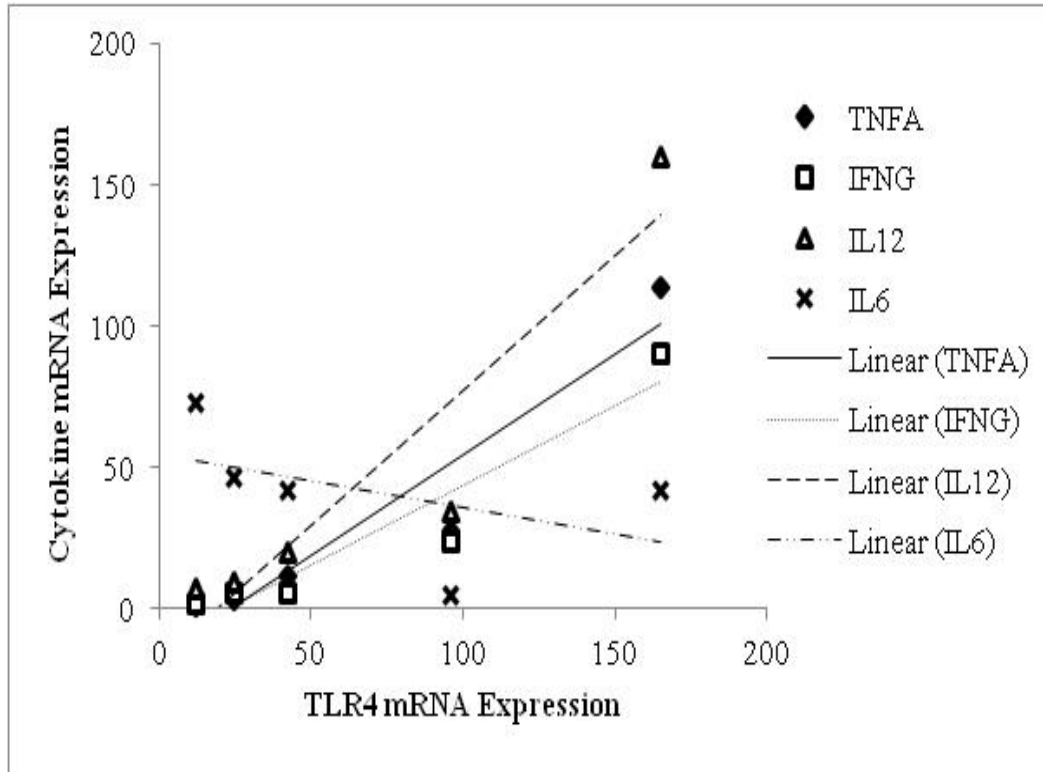
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 669 specific gene indicates statistical tendencies ($0.1 < p > 0.05$).

670

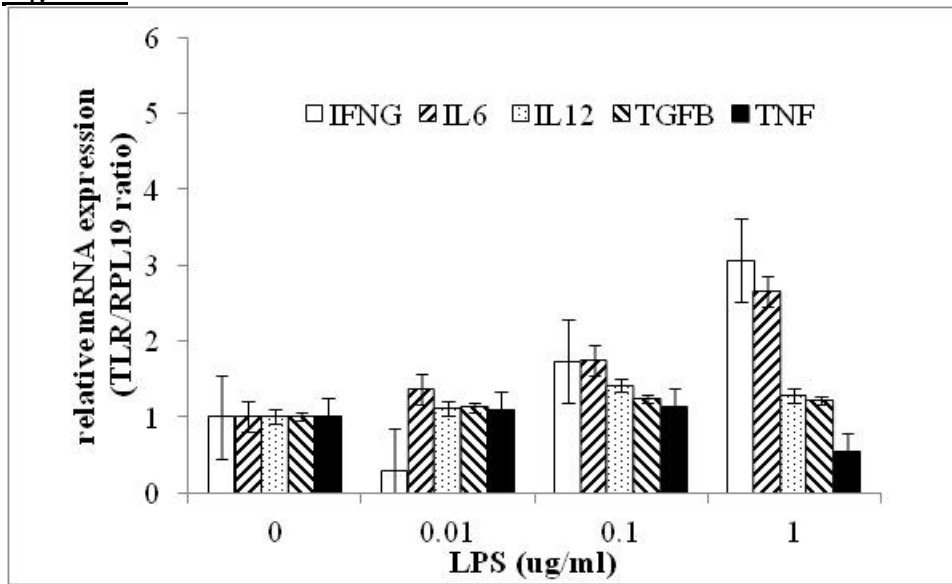
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689 (LPS) for 24 hours. No differences were observed in cytokine expression following treatment is
690 increasing concentrations of LPS (n=3). Data are presented as least-square means ± SEM.

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693

