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# The Mare as a Model for Luteinized Unruptured Follicle Syndrome: Intrafollicular Endocrine Milieu.

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1 **The mare as a model for luteinized unruptured follicle syndrome:**  
2 **intrafollicular endocrine milieu**

3

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23

**Abstract**

Luteinized unruptured follicle (LUF) syndrome is a recurrent anovulatory dysfunction that affects up to 23% of women with normal menstrual cycles and up to 73% with endometriosis. Mechanisms underlying the development of LUF syndrome in mares were studied to provide a potential model for human anovulation. The effect of extended increase in circulating LH achieved by administration of recombinant equine LH (reLH) or a short surge of LH and decrease in progesterone induced by prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) on LUF formation (Experiment 1), identification of an optimal dose of COX-2 inhibitor (flunixin-meglumine, FM; to block the effect of prostaglandins) for inducing LUFs (Experiment 2), and evaluation of intrafollicular endocrine milieu in LUFs (Experiment 3) were investigated. In Experiment 1, mares were treated with reLH from Days 7–15 (Day 0 = ovulation),  $PGF_{2\alpha}$  on Day 7, or in combination. In Experiment 2, FM at doses of 2.0 or 3.0 mg/kg every 12 h and hCG (1500 IU) were administered after a follicle  $\geq 32$  mm was detected. In Experiment 3, FM at a dose of 2.0 mg/kg every 12 h plus hCG was used to induce LUFs and investigate the intrafollicular endocrine milieu. No LUFs were induced by reLH or  $PGF_{2\alpha}$  treatment; however, LUFs were induced in 100% of mares using FM. Intrafollicular  $PGF_{2\alpha}$  metabolite (PGFM),  $PGF_{2\alpha}$ , and  $PGE_2$  were lower and the ratio of  $PGE_2:PGF_{2\alpha}$  was higher in the Induced LUF group. Higher levels of intrafollicular E2 and total primary sex steroids were observed in the Induced LUF group along with a tendency for higher levels of GH, cortisol, and T; however, LH, PRL, VEGF-A, and NO did not differ between groups. In conclusion, this study reveals part of the intrafollicular endocrine milieu and the association of prostaglandins in LUF formation, and indicates that the mare might be an appropriate model for studying the poorly understood LUF syndrome.

46

## 47 **Introduction**

48 Anovulation is one of the main causes of infertility in women and females of many domestic  
49 species. One of the types of anovulatory dysfunction is luteinized unruptured follicle (LUF)  
50 syndrome, which has been reported in women (Marik & Hulka 1978, Koninckx *et al.* 1981,  
51 Hamilton *et al.* 1985, Hulka 1985, Katz 1988, Check 2007), mares (Kaiser *et al.* 1999, Gastal *et*  
52 *al.* 2006, Ginther *et al.* 2007a, Cuervo-Arango & Newcombe 2009), cattle (Peter 2004), llamas  
53 (Adams *et al.* 1991), rhinoceroses (Stoops *et al.* 2004), and elephants (Lueders *et al.* 2011).  
54 LUFs, also known as hemorrhagic anovulatory follicles (HAFs) in veterinary medicine, occur  
55 when the preovulatory follicle fails to rupture or ovulate and the antrum gets increasingly filled  
56 with blood. LUF/HAF is the most common form of anovulation in mares. LUF syndrome during  
57 the breeding season is considered a serious economic concern for the equine industry. Similarly,  
58 anovulation can have significant financial implications for women undergoing assisted  
59 reproductive techniques (Eijkemans *et al.* 2005). Therefore, it is important to investigate the  
60 mechanisms of development of LUF syndrome. Greater knowledge about the pathophysiology of  
61 LUFs can be applied to prevent their occurrence and thus to develop safe and effective  
62 treatments to optimize reproductive health in both animals and humans.

63 The ultrasonographic morphological characteristics of naturally occurring or induced LUFs  
64 are similar in women (Priddy *et al.* 1990, Zaidi *et al.* 1995) and mares (Coetsier & Dhont 1996,  
65 Cuervo-Arango & Newcombe 2012). LUF formation involves the development of well-  
66 vascularized luteal tissue, as indicated by echotexture and color Doppler signals in both women  
67 and mares (Zaidi *et al.* 1995, Ginther *et al.* 2007a). In the absence of ovulation, the antrum of the  
68 follicle becomes permeated with blood, which appears as echogenic foci and fibrin-like strands  
69 on a B-mode ultrasonogram. Due to similarities in antral follicular dynamics (Ginther *et al.*

70 2004, Baerwald 2009, Gastal 2011) and LUF morphology between species, we have proposed  
71 the mare as an appropriate model for understanding LUF syndrome in women.

72 The reported incidence of LUFs in women is highly variable. LUFs have been documented to  
73 occur in 11-23% of women with normal menstrual cycles (Vanrell *et al.* 1982, Kerin *et al.* 1983,  
74 Dal *et al.* 2005), 13-73% of women with endometriosis (Kaya & Oral 1999), and 4-58% of  
75 women with unexplained infertility (Koninckx & Brosens 1983, Kugu *et al.* 1991). In addition to  
76 this, LUFs have been reported in women with pelvic inflammatory disease (Hamilton *et al.*  
77 1986) and inflammatory arthritis (Smith *et al.* 1996). LUFs are highly repeatable across cycles  
78 (79-90%), resulting in recurrent anovulation (Hamilton *et al.* 1986, Qublan *et al.* 2006) and  
79 infertility.

80 In cycling mares, the incidence of LUFs is also highly variable (5-25%; Lefranc & Allen  
81 2003, Ginther *et al.* 2008a, Cuervo-Arango & Newcombe 2009, 2010). A 5% incidence of LUFs  
82 has been reported during the early ovulatory season, followed by 20% during the late  
83 reproductive season (Gastal *et al.* 1998). Similarly, LUF syndrome has been shown to occur  
84 more often in older mares and be recurrent in some individuals (>50% of the estrous cycles),  
85 encompassing much or all of the breeding season (Ginther *et al.* 2007a, Cuervo-Arango &  
86 Newcombe 2009, 2010). Therefore, recurrent LUFs result in prolonged periods of anovulation  
87 and long interovulatory intervals (Ginther *et al.* 2007a).

88 The systemic and intrafollicular endocrine milieu associated with LUF syndrome is poorly  
89 understood. Knowledge about the endocrine changes associated with LUF formation is lacking  
90 (Hamilton *et al.* 1985, Ginther *et al.* 2007). The use of luteinizing hormone (LH) during early  
91 proestrus in rodents has successfully induced LUFs (Plas-Roser *et al.* 1985, Mattheij & Swarts  
92 1995). Similarly, the use of luteinizing hormone releasing hormone, human chorionic

93 gonadotropin (hCG), and human menopausal gonadotropin has been associated with LUFs in  
94 women (Bergquist & Lindgren 1983, Ghanem *et al.* 2009), and hCG in guinea pigs (Westfahl  
95 1988). Continued investigations are required to determine the effects of chronic administration of  
96 LH on the incidence of LUFs in mares and women. It has been well documented that a PGF<sub>2</sub>α  
97 injection causes immediate release of LH, resulting in induction of ovulation in several species  
98 such as cows (Hafs *et al.* 1975), sows (Srikandakumar & Downey 1989), and mares (Gastal *et al.*  
99 2005). In a recent study, administration of PGF<sub>2</sub>α with or without complete ablation of antral  
100 follicles increased LH concentrations early in the ovulatory wave and also during the  
101 preovulatory period and were associated with a high incidence of LUFs in mares (Ginther *et al.*  
102 2008b). Although it is not known exactly what physiological mechanism (interference with  
103 luteinization or maturation of granulosa cells) might be disturbed when LH levels are affected at  
104 the beginning of the follicular wave or during the ovulatory period, there is enough evidence to  
105 allow the test of a hypothesis of LH participation in LUF formation.

106       The advances in knowledge regarding LUF syndrome in women and animals have been slow,  
107 in part because of the difficulty of predicting the occurrence of such ovarian structures.  
108 Therefore, the use of pharmacologically induced LUFs is a promising way to study this  
109 syndrome. Use of pharmacological agents in fertility or superovulatory treatments has increased  
110 the occurrence of LUFs and/or anovulatory follicles in women (Martinez *et al.* 1991, Ghanem *et*  
111 *al.* 2009) and mares (Lefranc & Allen 2003, Ginther *et al.* 2008a, Cuervo-Arango & Newcombe  
112 2010, Meyers-Brown *et al.* 2011). Similarly, intrafollicular or systemic administration of  
113 prostaglandin inhibitors has been shown to cause luteinization of follicles in rats (Armstrong &  
114 Grinwich 1972), rabbits (Salhab *et al.* 2003), mares (Watson & Sertich 1991, Ginther *et al.*  
115 2008a, Cuervo-Arango & Domingo-Ortiz 2011), and women (Killick & Elstein 1987, Priddy *et*

116 *al.* 1990, Jesam *et al.* 2010, 2014). In mares, flunixin meglumine (FM) at a dose rate of 1.7 or 2.0  
117 mg/kg body weight has been shown to induce LUFs in 73-83% of mares, respectively (Cuervo-  
118 Arango *et al.* 2011, Cuervo-Arango & Domingo-Ortiz 2011). Furthermore, intrafollicular  
119 administration of prostaglandins has successfully prevented FM-induced LUF formation,  
120 allowing subsequent ovulation and conception in mares (Martínez-Boví & Cuervo-Arango  
121 2015). In women, NSAIDs have been used experimentally to inhibit ovulation, and a dose-  
122 dependent effect has been observed (Athanasίου *et al.* 1996, Bata *et al.* 2006, Jesam *et al.* 2010,  
123 2014). Therefore, pharmacological approaches to inhibit ovulation and induce LUF formation,  
124 such as the use of COX-2 inhibitors, may serve as an effective model to elucidate the  
125 pathophysiology of LUF syndrome.

126 The objectives of the experiments conducted in this study were to investigate: the role of  
127 reLH and PGF<sub>2</sub>α in the formation of LUFs (Experiment 1), the optimum dose of FM required to  
128 experimentally induce LUFs (Experiment 2), and the intrafollicular endocrine milieu  
129 (Experiment 3) in induced LUFs in the mare. The hypotheses tested were that: (1) reLH, when  
130 administered during diestrus alone or along with PGF<sub>2</sub>α, would increase the incidence of LUFs;  
131 (2) higher doses of FM would increase the incidence of LUFs; (3) inhibition of intrafollicular  
132 prostaglandin synthesis would be associated with LUF formation; (4) intrafollicular  
133 prostaglandins (PGF<sub>2</sub>α and PGE<sub>2</sub>) concentrations would be decreased during systemic FM  
134 treatment; (5) imbalance in the intrafollicular endocrine milieu is associated with LUF formation.

135

## 136 **Materials and Methods**

137

### 138 ***Animals***



139 Mares ( $n = 36$ ) were evaluated during two ovulatory seasons (April to October) in the northern  
140 hemisphere and handled in accordance with the United States Department of Agriculture Guide  
141 for Care and Use of Agricultural Animals in Research. This study was approved by the Southern  
142 Illinois University Institutional Animal Care and Use Committee (IACUC, 10-041). The mares  
143 were Quarter-Horse type breed, 5–15 years of age, weighed 400–550 kg, had docile  
144 temperament, and did not present any apparent abnormality of the reproductive tract as  
145 determined by ultrasonographic examination. Mares were reused in subsequent experiments after  
146 ultrasonographically confirming at least two normal ovulatory cycles in between experiments.  
147 All mares had good body condition score (average score ~6-7; score 1 = emaciated and score 9 =  
148 obese; Henneke *et al.* 1983) throughout the study. Mares were kept under natural light in pasture  
149 with free access to water and trace-mineralized salt.

150

### 151 ***Experiment 1. Effect of reLH and PG on LUF formation***

152

#### 153 *Animals and treatments*

154 On Day 7 (Day 0 = ovulation), mares ( $n = 30$ ) were randomly divided into six different treatment  
155 groups to receive either saline, PGF<sub>2</sub> $\alpha$  (5 mg i.m.; Lutalyse, Pfizer Animal Health, Kalamazoo,  
156 MI, USA), reLH (0.5 mg or 1.0 mg i.v.; AspenBio Pharma Inc., Castle Rock, CO), or a  
157 combination of PGF<sub>2</sub> $\alpha$  and reLH. The groups were: Control (10 ml saline, i.v.), LH 0.5 (0.5 mg  
158 of reLH), LH 0.5 + PG (0.5 mg reLH and 5 mg PGF<sub>2</sub> $\alpha$ ), PG (5 mg PGF<sub>2</sub> $\alpha$ ), LH 1.0 (1 mg reLH),  
159 and LH 1.0 + PG (1 mg reLH and 5 mg PGF<sub>2</sub> $\alpha$ ). Mares were treated with PGF<sub>2</sub> $\alpha$  (5 mg/mare)  
160 only once on Day 7 and with reLH once every day from Days 7–15.

161

162 *Ultrasonographic examinations and end points*

163 Transrectal ultrasonographic examinations were performed daily from Day 0 until 4 days after  
164 the next ovulation or the beginning of LUF formation. A LUF was detected ultrasonographically  
165 by the presence of a thick and echoic follicle wall, suggestive of luteinization, decreased  
166 turgidity, echoic foci and/or fibrin septae in the antrum, a gel-like substance in antrum, and/or  
167 pronounced serration of the granulosa layer around the whole follicle, as previously described  
168 (Ginther *et al.* 2006, Cuervo-Arango & Newcombe 2012). A series of comparative ultrasound  
169 images of LUFs in women and mares is given for illustration purposes regarding the similarities  
170 shown by both species (Fig. 1). For transrectal scanning, a duplex B-mode (gray-scale) and  
171 pulsed-wave color-Doppler ultrasound instrument (Aloka SSD-3500; Aloka America,  
172 Wallingford, CT, USA) equipped with a finger-mounted 3.5–10 MHz convex-array transducer  
173 was employed. The color-flow mode was used to display follicle, corpus luteum (CL), and LUF  
174 blood flow as previously described (Ginther *et al.* 2007a, 2007b). Constant color-gain, velocity,  
175 and filter settings were used for all Doppler examinations. The entire follicle, CL, and LUF were  
176 scanned in a gradual, steady motion several times.

177 Follicle diameter was calculated from the average of height and width of the antrum at the  
178 apparent maximal area from two frozen images. The largest follicle was measured on each day of  
179 examination. CL diameter was measured throughout the study. In addition, endometrial  
180 echotexture was scored from 1 to 4 (minimal to maximal) during each examination, based on the  
181 extent of anechoic areas of the endometrial folds (Ginther & Pierson 1984). Follicle wall blood  
182 flow was quantified in follicles  $\geq 28$  mm until ovulation or LUF formation, using color-flow  
183 Doppler as previously described in mares (Acosta *et al.* 2004) and women (Campbell *et al.*  
184 1993).

185

186 ***Experiment 2. Optimum dose of flunixin-meglumine (FM) to induce LUF***

187

188 *Animals and treatments*

189 At the beginning of estrus, cycling mares ( $n = 18$ ) with a growing follicle  $\geq 32$  mm (Hour 0), in  
190 the presence of endometrial edema (echotexture score  $\geq 3$ ) were administered 1500 IU of hCG  
191 (Chorulon®, i.v.; Intervet Inc, Millsboro, DE). Immediately, mares were randomly divided ( $n =$   
192 6 mares/group) into three treatment groups (FM-2, FM-3, and Control), and treatments were  
193 started. All groups were treated every 12 h with FM or saline until Hour 36. FM-2 and FM-3  
194 groups received 2.0 or 3.0 mg/kg of body weight of FM (Flunixiject™, i.v.; Henry Schein®  
195 Animal Health, Dublin, OH), respectively, and the Control group received 10 ml of saline (i.v.)  
196 solution. Mares were monitored for any adverse effects from the above mentioned doses of FM,  
197 as the normal dose was exceeded (1.1 mg/kg of body weight per day).

198

199 *Ultrasound evaluations*

200 Mares were scanned every other day from Day 12 after ovulation until a follicle reached 25–27  
201 mm in diameter; subsequently, scans were conducted daily. Mares with a  $\geq 32$ -mm follicle were  
202 randomly allocated to a treatment group and scans were performed: every 12 h from Hours 0–36,  
203 every 2 h between Hours 36–48, and every 12 h from Hours 60–96. Mares with more than one  
204  $\geq 32$ -mm follicle at the beginning of treatment were not included in the study.

205 Follicle diameter, follicle blood flow, LUFs, and endometrial echotexture were evaluated  
206 using the same ultrasound methodology as in Experiment 1. The thickness of the follicle wall  
207 (granulosa layer) was determined by averaging measurements made on three different locations

208 (Gastal *et al.* 1998, 2006). In addition, the following qualitative B-mode characteristics of the  
209 preovulatory follicle were recorded from Hour 0 until ovulation or LUF formation: 1) presence  
210 of echoic foci floating in the antrum, 2) detection of follicle wall serration (i.e., irregular or  
211 notched surfaces of the granulosa layer; Gastal *et al.* 2007), and 3) loss of spherical shape.

212

### 213 ***Experiment 3. Systemic and intrafollicular hormones and growth factors***

214

#### 215 *Animals and treatments*

216 Cycling mares ( $n = 23$ ) with a growing follicle  $\geq 28$ -mm were scanned daily after Day 15 using  
217 B-mode and color-Doppler ultrasonography (Gastal *et al.* 1998, 2006) until a follicle  $\geq 32$  mm  
218 was detected. When the largest follicle reached  $\geq 32$  mm (Hour 0) and the score for endometrial  
219 echotexture was between 3 and 4 (estrus-like), mares were randomly assigned into two treatment  
220 groups: Control ( $n = 11$ ) and Induced LUF ( $n = 12$ ). Mares received an injection of 1500 IU of  
221 hCG and were treated twice daily with saline (10 ml, i.v.; Control group) or FM (2.0 mg FM/kg  
222 of body weight; Induced LUF group) until 36 h after hCG injection.

223

#### 224 *Collection of follicular fluid and end points*

225 Follicular fluid was collected from the preovulatory follicle in the Induced LUF and the Control  
226 groups by ultrasound-guided transvaginal follicle aspiration at Hour 36 after hCG injection as  
227 described (Gastal *et al.* 1995, 1999b). The aspirated follicular-fluid was immediately processed  
228 in a refrigerated centrifuge (1500 xg for 10 min), and 10 ml of the supernatant was stored at  
229  $-20^{\circ}\text{C}$  until hormone assays were performed. Follicle diameter, follicle blood flow, and  
230 endometrial echotexture were measured, as described in Experiment 1.

231

232 ***Blood samples and hormone assays***

233 Jugular blood samples were collected in heparinized tubes, immediately placed in ice water bath,  
234 processed in a refrigerated centrifuge (1500 xg for 10 min), decanted, and stored (-20°C) until  
235 analyzed. For Experiment 1, samples were collected daily between Days 7–15. For Experiment  
236 2, samples were collected every 12 h between Hours 0–36 and then every 2 h until ovulation or  
237 LUF formation. For Experiment 3, samples were collected every 12 h between Hours 0–36. For  
238 Experiment 1, systemic LH was assayed from Days 7–9 to investigate the effect of treatment on  
239 the increase in circulating LH, and progesterone (P4) was assayed from Days 7–15. For  
240 Experiment 2, systemic PGFM (prostaglandin F<sub>2α</sub> metabolite) and P4 were assayed from Hours  
241 0–38. For Experiment 3, PGFM was assayed in both plasma and follicular fluid; additionally,  
242 follicular fluid was assayed for PGF<sub>2α</sub>, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), estradiol 17-β (E2), P4,  
243 testosterone (T), LH, nitric oxide (NO), vascular endothelial growth factor-A (VEGF-A),  
244 cortisol, prolactin (PRL), and growth hormone (GH). Furthermore, the ratios of PGE<sub>2</sub>:PGF<sub>2α</sub>,  
245 E2:P4, E2:T, and P4:T were calculated. Total primary sex steroids were calculated by combining  
246 the E2, P4, and T.

247 Plasma P4 concentrations were determined using a solid-phase radioimmunoassay kit  
248 containing antibody-coated tubes and I<sup>125</sup>-labeled P4 (Coat-A Count Progesterone, Diagnostic  
249 Products Corporation, Los Angeles, CA, USA) as described and validated for mare plasma  
250 (Ginther *et al.* 2005). Plasma LH was assayed using an equine ELISA kit (Endocrine  
251 Technologies, INC., Fremont, CA, USA). Plasma and follicular fluid PGFM, PGF<sub>2α</sub>, PGE<sub>2</sub>, and  
252 E2 concentration was determined using ELISA kits (Neogen Co., Lexington, KY, USA) after  
253 extraction with diethyl-ether, as previously described (Ginther *et al.* 2010). Intrafollicular LH,

254 GH, cortisol, PRL, and testosterone concentrations were determined by ELISA kits (Endocrine  
255 Technologies, INC., Fremont, CA, USA). Intrafollicular NO was estimated using a colorimetric  
256 kit (Cayman Chemical Company, Ann Arbor, MI, USA). Intrafollicular VEGF-A was assayed by  
257 ELISA kit (Kingfisher Biotech, Inc., Saint Paul, MN, USA). All the assays were performed  
258 following the manufacturers' protocol and were validated for equine follicular fluid by using  
259 multiple dilutions and pilot assays to determine the optimal dilutions required for the hormone  
260 concentration to be within the detection range of the assay. The intra-assay CVs and sensitivities  
261 for the different hormone assays were as follows: P4, 8.7% (experiment 1), 6.3% (experiment 2),  
262 6.6% (experiment 3), and 0.02 ng/ml; PGFM, 6.3% (experiment 2) and 3.2% (experiment 3), and  
263 20 pg/ml; PGF<sub>2</sub> $\alpha$ , 6.1% and 2 pg/ml; PGE<sub>2</sub>, 2.6% and 0.1 ng/ml; E2, 4.9% and 0.1 ng/ml; NO,  
264 5.1% and 20  $\mu$ M/ml; LH, 4.5% (plasma) and 8.9% (follicular fluid), and 0.25 ng/ml; GH, 5.2%  
265 and 0.25 ng/ml; cortisol, 9.5% and 1 ng/ml; testosterone, 6.1% and 0.1 ng/ml; and VEGF-A,  
266 3.6% and 28.5 pg/ml.

267

### 268 *Statistical analyses*

269 The Shapiro-Wilk test was used for testing normal distribution of the data. Data not normally  
270 distributed were transformed to log or rank before any statistical analyses. Dixon's test was used  
271 to identify outlier observations, which were excluded from any statistical analyses. Sequential  
272 data were analyzed by one-way ANOVA for main effects of group, time, and group by time  
273 interaction. The analyses were done using SAS PROC MIXED (Version 9.2; SAS Institute, Inc.)  
274 with a REPEATED statement to minimize autocorrelation between sequential measurements.  
275 When a group effect or interaction was obtained, differences among groups within time points  
276 were further analyzed. Tukey's test was used between time points within a group to identify

277 significant differences. A probability of  $P < 0.05$  indicated that a difference was significant and  
278  $P > 0.05$  or  $< 0.1$  indicated that results tended to be different. Data are presented as mean  $\pm$   
279 S.E.M.

280

## 281 **Results**

282

### 283 *Experiment 1. Effect of reLH and PG on LUF formation*

284 No LUFs were observed in this experiment, regardless of treatment. More specifically, reLH  
285 (0.5–1.0mg) given for 9 days during the diestrous phase and/or PG administered on Day 7 did  
286 not induce LUF formation. Furthermore, reLH had no effect on any other endpoint. The diameter  
287 of the largest follicle increased ( $P < 0.0001$ ) for all groups, but no overall group effect was  
288 observed during the treatment period (data not shown). A group-by-day interaction ( $P < 0.02$ )  
289 was potentially caused by the greater diameters in the PG groups when compared with the  
290 Control and LH groups. After combining the reLH (LH 0.5 + LH 1.0) groups and PG (LH  
291 0.5+PG, LH 1.0+PG, and PG) groups, a larger ( $P < 0.05$ ) follicle diameter was seen on Days  
292 13–16 for the PG group when compared with the Control and LH groups (Fig. 2A). The growth  
293 rate of the largest follicle from Days 7–16 was greater ( $P < 0.001$ ) in the PG group than in the  
294 Control and reLH groups (Table 1). In addition, maximum follicle diameter during the treatment  
295 period and on Day 16 was greater ( $P < 0.01$ ) in the PG group compared to the Control and reLH  
296 groups. The percentage of blood flow of the dominant follicle 3 days before ovulation did not  
297 differ ( $P > 0.05$ ) among groups.

298  $\text{PGF}_{2\alpha}$  had a marked effect on CL diameter and half-life, which affected the plasma P4  
299 concentrations and shortened the interovulatory interval (IOI; Table 1). As expected, CL

300 diameter and P4 concentration decreased ( $P < 0.007$ ) faster in PG-treated groups, compared to  
301 the other groups (data not shown). The Control and reLH groups did not differ throughout  
302 treatment, whereas PG-treated groups had a smaller CL diameter on Day 12 ( $P < 0.0001$ ).  
303 Therefore, the reLH (0.5 and 1.0 mg) groups and the PG groups were combined for further  
304 analyses (Fig. 2B). The Control and reLH-treated groups had a larger ( $P < 0.0001$ ) CL diameter  
305 and greater P4 concentration from Days 9–15 than the PG group (Fig. 2B, C). The reLH  
306 treatment had no effect on CL diameter or P4 concentration when compared to the Control  
307 group. P4 concentration was greater ( $P < 0.0002$ ) on Day 12 and lower ( $P < 0.004$ ) on the day of  
308 the beginning of luteolysis in the Control and reLH groups compared to the PG-treated group  
309 (Table 1). Also, the mean day of the beginning of luteolysis (day before P4 was  $< 1.0$  ng/ml) was  
310 earlier ( $P < 0.0001$ ) in the PG-treated group compared to the Control and reLH-treated groups  
311 (Table 1). Endometrial echotexture was greater ( $P < 0.002$ ) in the PG ( $3.3 \pm 0.1$ ) versus Control  
312 ( $2.9 \pm 0.1$ ) and reLH ( $2.8 \pm 0.1$ ) groups. Plasma LH concentrations from Days 7–9 increased ( $P$   
313  $< 0.03$ ) for reLH ( $3.7 \pm 0.7$  ng/ml) and PG ( $3.0 \pm 0.6$  ng/ml) groups; however, an increase was  
314 not observed in the Control group ( $1.8 \pm 0.2$  ng/ml).

315

### 316 ***Experiment 2. Optimum dose of flunixin-meglumine to induce LUF***

317 In the Control group, ovulation was detected at  $38.7 \pm 0.7$  h (range, 36 to 40 h) in all mares. In  
318 FM groups, both doses (2 and 3 mg/kg of body weight) resulted in induction of LUFs in 100% of  
319 the animals; no complications were observed in any animal after FM treatment. LUFs were first  
320 observed in treated mares at  $49.2 \pm 1.9$  h (range, 44 to 60 h). No difference was observed in the  
321 time of first detection of LUFs and plasma PGFM concentration between FM treated groups.  
322 Therefore, FM groups were combined for further analyses (i.e., Induced LUF group). The plasma



323 PGFM concentration was lower ( $P < 0.05$ ) in the Induced LUF group when compared to the  
324 Control group at Hour 24 (Fig. 3). Plasma P4 concentration was not different ( $P > 0.05$ ) between  
325 groups from Hour 0 to 36 (data not shown).

326 Follicle diameters of Induced LUF and Control groups were not different ( $P > 0.05$ ) between  
327 Hours 0–38. However, Induced LUFs grew to a larger ( $P < 0.009$ ) diameter from Hours 0–60  
328 (Fig. 4A). Follicle wall serration, follicle wall thickness, and follicular shape (round/irregular)  
329 were not different between Induced LUF and Control groups up to Hour 38. The first increase ( $P$   
330  $< 0.05$ ) in follicle wall serration occurred between Hours 0–24 in both groups (Fig. 4B). Follicle  
331 wall thickness and follicle wall serration continued to increase between Hours 38–60 for the  
332 Induced LUF group (Fig. 4B, C).

333 Follicle blood flow (Fig. 5D) increased differentially between groups after the beginning of  
334 treatment (time effect,  $P < 0.001$ ; interaction,  $P < 0.02$ ). The follicle blood flow of the largest  
335 follicle was greater ( $P < 0.02$ ) in the Induced LUF group at Hour 38 than in the Control group.  
336 Follicle blood flow increased earlier in the Control group (Hour 12) compared to the Induced  
337 LUF group (Hour 24). The number of echoic foci in the follicular fluid before ovulation or LUF  
338 formation was lower ( $P < 0.0001$ ) in the Control group ( $0.8 \pm 0.3$ ) than in the Induced LUF  
339 group ( $3.5 \pm 0.2$ ). Endometrial echotexture (overall score,  $3.8 \pm 0.1$ ) was not different between  
340 groups.

341

### 342 ***Experiment 3. Systemic and intrafollicular hormones and growth factors***

343 Plasma PGFM concentration was lower ( $P < 0.0002$ ) at Hour 24 in the Induced LUF group  
344 versus the Control group (Fig. 5A). Overall, the follicle diameter tended ( $P < 0.07$ ) to be greater  
345 in the Induced LUF group when compared with the Control group at Hours 0–36 (Fig. 5B). The

346 follicle diameter was greater in the Induced LUF group at Hour 24 ( $P < 0.02$ ) and Hour 36 ( $P <$   
347  $0.05$ ). Follicle diameters at Hours 12, 24, and 36 increased in both groups, when compared to  
348 Hour 0. Follicle blood flow did not differ between groups (Fig. 5C), but increased earlier in the  
349 Control group (Hour 12) compared to the Induced LUF group (Hour 24). In addition,  
350 endometrial echotexture did not differ between groups; however, a faster decrease ( $P < 0.05$ ) in  
351 edema score was detected between Hours 24–36 in the Control group (Fig. 5D).

352 Differences in follicular fluid hormone concentrations were observed between the Control  
353 and Induced LUF groups (Fig. 6). PGFM concentration was lower ( $P < 0.004$ ) in the Induced  
354 LUF group ( $49.6 \pm 2.0$  pg/ml) versus the Control group ( $102.3 \pm 20.5$  pg/ml; Fig. 6A).  $\text{PGF}_2\alpha$   
355 was lower ( $P < 0.0006$ ) in the Induced LUF group ( $0.013 \pm 0.005$  ng/ml) compared to the  
356 Control group ( $22.4 \pm 6.1$  ng/ml; Fig. 6B). Also,  $\text{PGE}_2$  concentration was lower ( $P < 0.004$ ) in  
357 the Induced LUF group ( $0.35 \pm 0.05$  ng/ml) compared to the Control group ( $24.7 \pm 8.5$  ng/ml;  
358 Fig. 6C). The  $\text{PGE}_2:\text{PGF}_2\alpha$  ratio was higher ( $P < 0.03$ ) in Induced LUF ( $93.0 \pm 43.0$ ) compared  
359 to Control ( $3.6 \pm 1.5$ ) mares (Fig. 6D).

360 Differences in follicular fluid primary sex steroids and their ratios were detected between  
361 treatment groups (Fig. 7). Intrafollicular  $\text{E}_2$  concentration was greater ( $P < 0.02$ ) in the Induced  
362 LUF group ( $1993.0 \pm 325.6$  ng/ml) compared to the Control group ( $1114.3 \pm 173.0$  ng/ml).  $\text{P}_4$   
363 was not different between groups (Fig. 7B); however, T concentration tended ( $P < 0.1$ ) to be  
364 greater in the Induced LUF group (Fig. 7C). Furthermore, the total primary sex steroid  
365 concentration was greater ( $P < 0.009$ ) in the Induced LUF group ( $3340.6 \pm 350.4$  ng/ml) versus  
366 the Control group ( $2047.4 \pm 320.4$  ng/ml; Fig. 7D). The ratios of  $\text{E}_2:\text{P}_4$  and  $\text{P}_4:\text{T}$  tended ( $P <$   
367  $0.1$ ) to be lower in the Induced LUF group (Fig. 7E, F); however, the  $\text{E}_2:\text{T}$  ratio was not different  
368 between groups (Fig. 7G).

369 The follicular fluid LH, PRL, VEGF-A, and NO concentrations did not differ between the  
370 Control and Induced LUF groups (Fig. 8A, B, E, F). However, the concentration of cortisol  
371 tended ( $P < 0.09$ ) to be higher in the Induced LUF group ( $6.8 \pm 1.9$  ng/ml) compared to the  
372 Control group ( $3.2 \pm 0.5$  ng/ml; Fig. 8D). Also, the follicular GH levels tended ( $P < 0.07$ ) to be  
373 higher in the Induced LUF ( $0.59 \pm 0.05$  ng/ml) versus the Control group ( $0.48 \pm 0.05$  ng/ml; Fig.  
374 8C).

375

### 376 **Discussion**

377 The pathophysiologic mechanisms underlying LUF syndrome in mares, women, or other species  
378 are not known. This series of experiments was conducted to gain insight about the effects of  
379 exogenous and endogenous LH and  $\text{PGF}_2\alpha$  on LUF formation, as well as the effects on  
380 intrafollicular prostaglandins and other hormones in induced LUFs when a COX-2 inhibitor  
381 (FM) was used. This study is apparently the first to report and compare a wide range of  
382 intrafollicular biomarkers between ovulatory follicles and LUFs. Thus, the results herein  
383 presented are clinically relevant to veterinary and human medicine.

384 Low levels of follicular fluid prostaglandins and PGFM in mares with induced LUFs were  
385 the most consistent and novel finding in this study (Experiment 3). Our hypotheses that  
386 intrafollicular prostaglandins would be inhibited by systemic FM administration and that  
387 inhibition of intrafollicular prostaglandin synthesis would be associated with LUF formation  
388 were therefore substantiated. Intrafollicular  $\text{PGF}_2\alpha$ , PGFM, and  $\text{PGE}_2$  were consistently  
389 decreased in mares of the Induced LUF group. Similar results have been shown in the follicular  
390 fluid of women treated with various NSAIDs (Priddy *et al.* 1990). Prostaglandins are vital for the  
391 ovulatory process in vertebrates (reviewed in Murdoch *et al.* 1993). It has been reported that

392 intrafollicular prostaglandins increased 36 h after hCG treatment in mares (Watson & Sertich  
393 1991, Sirois & Dore 1997). A recent study (Martínez-Boví & Cuervo-Arango 2015)  
394 demonstrated the importance of prostaglandins in the ovulation process in mares by using  
395 intrafollicular injection of a supra-physiological cocktail of PGE<sub>2</sub> and PGF<sub>2</sub>α (500 μg and 125  
396 μg, respectively) to reverse the anovulatory effects of FM. Another novel finding from our study  
397 was the increased intrafollicular PGE<sub>2</sub>:PGF<sub>2</sub>α ratio in mares with induced LUFs. High  
398 intrafollicular PGE<sub>2</sub>:PGF<sub>2</sub>α ratio has been associated with lower pregnancy rates in humans  
399 (Smith *et al.* 1991). These findings corroborate the role of lowered intrafollicular prostaglandins  
400 in LUF formation and support the concept that an altered PGE<sub>2</sub>:PGF<sub>2</sub>α ratio may be caused by a  
401 compensatory mechanism when prostaglandin synthetase is blocked. These results highlight the  
402 importance of the mare as a possible animal model to study LUF syndrome in women.

403 Increased follicular fluid E2 in mares with induced LUFs was another novel finding in this  
404 study. We have previously reported that mares had higher E2 concentrations 3 days before the  
405 beginning of LUF formation (Ginther *et al.* 2007a). Likewise, higher plasma E2 concentrations  
406 have been described in women with spontaneous LUFs (Hamilton *et al.* 1985). However, no  
407 differences in intrafollicular E2 concentrations were previously documented in women following  
408 NSAID treatment (Priddy *et al.* 1990). Estradiol-mediated stimulation of PGE<sub>2</sub> synthesis in  
409 preovulatory follicles in mice (Toda *et al.* 2012), may explain the increased PGE<sub>2</sub>:PGF<sub>2</sub>α ratio in  
410 mares of the Induced LUF group. Furthermore, intrafollicular total primary sex steroids were  
411 higher in the Induced LUF group, although no differences were found in P4 concentrations.  
412 Greater overall steroid concentrations can be due to low levels of intrafollicular prostaglandins  
413 since prostaglandins decrease intracellular transport of cholesterol, reduce cellular cholesterol  
414 uptake, and lower the activity of steroidogenic enzymes (reviewed in Niswender *et al.* 2000).

415 Higher intrafollicular E2 levels in Induced LUF mares were consistent with a more differentiated  
416 endometrium with higher uterine edema.

417 LH, VEGF-A, NO, and PRL do not seem to be involved in LUF formation as the levels were  
418 not different between Induced LUF and Control groups. Cortisol and GH levels only tended to be  
419 higher in the Induced LUF group. Cortisol is thought to be involved in controlling the  
420 inflammatory process of ovulation (Espey & Lipner 1994, Andersen 2002). The role of cortisol  
421 in LUF formation is unclear at this point. It is possible that over-inhibition of the inflammatory  
422 process results in anovulation. Growth hormone has been shown to increase steroidogenesis in  
423 granulosa cells, and GH receptors (GHR) have been detected in granulosa cells in mice (Silva *et al.*  
424 *al.* 2009).

425 In this study, higher doses of FM were used in an attempt to increase the incidence of LUFs.  
426 Our hypothesis was not substantiated because both 2 mg and 3mg doses of FM induced LUFs in  
427 all mares (100% success rate). Nevertheless, the use of FM and hCG provides a reliable model  
428 for the study of LUF syndrome (Experiment 2). FM treatments were administered every 12 h and  
429 no adverse effects were noted in any animal. The percentage of LUFs in our study was higher  
430 than the 83% incidence of LUFs reported following FM treatment in mares (Cuervo-Arango &  
431 Domingo-Ortiz 2011) and the 36% incidence following treatment with higher doses of COX-2  
432 inhibitor (meloxicam) in women (Jesam *et al.* 2014). The increased incidence of LUFs in the  
433 present study was, in part, attributed to a higher dose of FM in our study compared to previous  
434 study (Cuervo-Arango *et al.* 2011), and/or to a lower dose (1500 IU) of hCG in our study versus  
435 2500 IU in a previous study (Cuervo-Arango *et al.* 2011, Cuervo-Arango & Domingo-Ortiz  
436 2011). Therefore, it seems that for proper experimental induction of LUFs, an optimum balance

437 between ovulatory stimulus (hCG) and an adequate decrease of intrafollicular prostaglandin (by  
438 the use of COX-2 inhibitor) must be achieved.

439 The systemic concentration of PGFM was reduced in FM treated mares at Hour 24 in  
440 Experiment 2 and at Hours 12 and 24 in Experiment 3. These results were consistent with  
441 previous reports in mares (Ginther *et al.* 2011, Cuervo-Arango *et al.* 2011). Plasma PGFM  
442 concentrations are indicative of systemic PGF<sub>2</sub>α concentrations, since PGF<sub>2</sub>α is rapidly  
443 metabolized in the body (Shrestha *et al.* 2012). Lower systemic PGFM levels were also an  
444 indicative of the effectiveness of FM treatment in blocking prostaglandin synthesis in our study.

445 In Experiment 1, we aimed to induce LUFs by injecting reLH between Days 7–15 with or  
446 without treating with PGF<sub>2</sub>α on Day 7. However, our hypothesis was not supported because  
447 reLH did not induce any LUFs. Furthermore, no differential effect on dominant follicle growth,  
448 CL development, or plasma P4 was seen by the use of reLH. PGF<sub>2</sub>α decreased the CL lifespan  
449 and plasma P4, and shortened the IOIs. LH has been shown to be important for the establishment  
450 of follicle dominance in mares (Gastal *et al.* 1999a, 2000). Daily injections of eLH at the  
451 preovulatory phase, after a 32-mm follicle was detected, failed to induce anovulatory follicles in  
452 mares (Schauer *et al.* 2013). Similarly, in our study, the reLH treatment did not affect follicular  
453 growth before and after deviation phases, nor did it induce anovulation (LUF). Although the  
454 results of our experiment are not similar to what has been described in women (Bergquist &  
455 Lindgren 1983) and rats (Mattheij & Swarts 1995), it seems to be premature to assume that LH  
456 does not affect LUF formation in mares. The dose and frequency of LH administrations were not  
457 evaluated in our study. Continued titration studies are required to fully understand the role of LH  
458 in LUF formation.

459 In summary, COX-2 inhibitors used in conjunction with hCG can be used to  
460 pharmacologically induce LUFs with 100% success in mares. We postulated that LUFs result  
461 from decreased intrafollicular prostaglandin concentrations and/or altered prostaglandin  
462 synthesis, as indicated by disparity in PGE<sub>2</sub>:PGF<sub>2</sub>α ratio. Increased intrafollicular E2 was  
463 associated with LUF formation; however, further studies are necessary to ascertain the cause-  
464 effect relationship and also to understand the role of testosterone, cortisol, and GH. The effect of  
465 LH on LUF formation remains unclear. This study further encourages the use of intrafollicular  
466 versus systemic biomarkers for evaluating ovulatory disorders. Finally, results from this study  
467 suggest the use of the mare as a potential model for investigating anovulatory infertility in  
468 women.

469

#### 470 **Declaration of interest**

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

473

474

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505 **References**

- 506 **Acosta TJ, Gastal EL, Gastal MO, Beg MA & Ginther OJ** 2004 Differential blood flow  
507 changes between the future dominant and subordinate follicles precede diameter changes  
508 during follicle selection in mares. *Biology of Reproduction* **71** 502-507.
- 509 **Adams GP, Sumar J & Ginther OJ** 1991 Hemorrhagic ovarian follicles in llamas.  
510 *Theriogenology* **35** 557-568.
- 511 **Andersen C** 2002 Possible new mechanism of cortisol action in female reproductive organs:  
512 physiological implications of the free hormone hypothesis. *Journal of Endocrinology* **173**  
513 211-217.
- 514 **Armstrong DT & Grinwich DL** 1972 Blockade of spontaneous and LH-induced ovulation in  
515 rats by indomethacin, an inhibitor of prostaglandin biosynthesis. I. *Prostaglandins* **1** 21-  
516 28.
- 517 **Athanasίου S, Bourne TH, Khalid A, Okokon EV, Crayford TJ, Hagstrom HG, Campbell**  
518 **S & Collins WP** 1996 Effects of indomethacin on follicular structure, vascularity, and  
519 function over the periovulatory period in women. *Fertility and Sterility* **65** 556-560.
- 520 **Baerwald AR** 2009 Human antral folliculogenesis: what we have learned from the bovine and  
521 equine models. *Animal Reproduction* **6** 20-29.
- 522 **Bata MS, Al-Ramahi M, Salhab AS, Gharaibeh MN & Schwartz J** 2006 Delay of ovulation  
523 by meloxicam in healthy cycling volunteers: A placebo-controlled, double-blind,  
524 crossover study. *Journal of Clinical Pharmacology* **46** 925-932.
- 525 **Bergquist C & Lindgren PG** 1983 Ultrasonic measurement of ovarian follicles during chronic  
526 LRH agonist treatment for contraception. *Contraception* **28** 125-133.

- 527 **Campbell S, Bourne TH, Waterstone J, Reynolds KM, Crayford TJ, Jurkovic D, Okokon**  
528 **EV & Collins WP** 1993 Transvaginal color blood flow imaging of the periovulatory  
529 follicle. *Fertility and Sterility* **60** 433-438.
- 530 **Check JH** 2007 Ovulation disorders: Part II. Anovulation associated with normal estrogen.  
531 *Clinical and Experimental Obstetrics and Gynecology* **34** 69-72.
- 532 **Coetsier T & Dhont M** 1996 Complete and partial luteinized unruptured follicle syndrome after  
533 ovarian stimulation with clomiphene citrate/human menopausal gonadotrophin/human  
534 chorionic gonadotrophin. *Human Reproduction* **11** 583-587.
- 535 **Cuervo-Arango J, Beg MA & Ginther OJ** 2011 Follicle and systemic hormone  
536 interrelationships during induction of luteinized unruptured follicles with a prostaglandin  
537 inhibitor in mares. *Theriogenology* **76** 361-373.
- 538 **Cuervo-Arango J & Domingo-Ortiz R** 2011 Systemic treatment with high dose of flunixin-  
539 meglumine is able to block ovulation in mares by inducing hemorrhage and luteinisation  
540 of follicles. *Theriogenology* **75** 707-714.
- 541 **Cuervo-Arango J & Newcombe JR** 2009 The effect of cloprostenol on the incidence of  
542 multiple ovulation and anovulatory hemorrhagic follicles in two mares: A case report.  
543 *Journal of Equine Veterinary Science* **29** 533-539.
- 544 **Cuervo-Arango J & Newcombe JR** 2010 Risk factors for the development of haemorrhagic  
545 anovulatory follicles in the mare. *Reproduction in Domestic Animals* **45** 473-480.
- 546 **Cuervo-Arango J & Newcombe JR** 2012 Ultrasound characteristics of experimentally induced  
547 luteinized unruptured follicles (LUF) and naturally occurring hemorrhagic anovulatory  
548 follicles (HAF) in the mare. *Theriogenology* **77** 514-524.

- 549 **Dal J, Vural B, Caliskan E, Ozkan S & Yucesoy I** 2005 Power Doppler ultrasound studies of  
550 ovarian, uterine, and endometrial blood flow in regularly menstruating women with  
551 respect to luteal phase defects. *Fertility and Sterility* **84** 224-227.
- 552 **Eijkemans MJ, Polinder S, Mulders AG, Laven JS, Habbema JD & Fauser BC** 2005  
553 Individualized cost-effective conventional ovulation induction treatment in  
554 normogonadotrophic anovulatory infertility (WHO group 2). *Human Reproduction* **20**  
555 2830-2837.
- 556 **Espey LL & Lipner H** 1994 Ovulation. In *The Physiology of Reproduction*, pp. 725-813.
- 557 **Gastal EL** 2011 Ovulation: Part 2. Ultrasonographic morphology of the preovulatory follicle. In  
558 *Equine Reproduction*, pp. 2032-2054.
- 559 **Gastal EL, Bergfelt DR, Nogueira GP, Gastal MO & Ginther OJ** 1999a Role of luteinizing  
560 hormone in follicle deviation based on manipulating progesterone concentrations in  
561 mares. *Biology of Reproduction* **61** 1492-1498.
- 562 **Gastal EL, Gastal MO & Ginther OJ** 1998 The suitability of echotexture characteristics of the  
563 follicular wall for identifying the optimal breeding day in mares. *Theriogenology* **50**  
564 1025-1038.
- 565 **Gastal EL, Gastal MO & Ginther OJ** 2006 Relationships of changes in B-mode echotexture  
566 and colour-Doppler signals in the wall of the preovulatory follicle to changes in systemic  
567 oestradiol concentrations and the effects of human chorionic gonadotrophin in mares.  
568 *Reproduction* **131** 699-709.
- 569 **Gastal EL, Gastal MO & Ginther OJ** 2007 Serrated granulosa and other discrete ultrasound  
570 indicators of impending ovulation in mares. *Journal of Equine Veterinary Science* **26** 67-  
571 73.

- 572 **Gastal EL, Gastal MO, Nogueira GP, Bergfelt DR & Ginther OJ** 2000 Temporal  
573 interrelationships among luteolysis, FSH and LH concentrations and follicle deviation in  
574 mares. *Theriogenology* **53** 925-940.
- 575 **Gastal EL, Gastal MO, Wiltbank MC & Ginther OJ** 1999b Follicle deviation and  
576 intrafollicular and systemic estradiol concentrations in mares. *Biology of Reproduction* **61**  
577 31-39.
- 578 **Gastal EL, Kot K & Ginther OJ** 1995 Ultrasound-guided intrafollicular treatment in mares.  
579 *Theriogenology* **44** 1027-1037.
- 580 **Gastal EL, Rodrigues BL, Gastal MO, Beg MA & Ginther OJ** 2005 Responsiveness of the  
581 early corpus luteum to PGF2 $\alpha$  and resulting progesterone, LH, and FSH interrelationships  
582 in mares. *Animal Reproduction* **2** 240-249.
- 583 **Ghanem MES, Sadek EM, Helal AS, Al-Boghdady LA, Emam ME & Bakr NI** 2009 Effect  
584 of stimulation protocol on the risk of luteinized unruptured follicle (LUF) in polycystic  
585 ovarian syndrome (PCOS): does LUF affect luteal phase profile? *Fertility and Sterility* **92**  
586 99.
- 587 **Ginther OJ, Cuervo-Arango J & Beg MA** 2011 Disruption of periovulatory FSH and LH  
588 surges during induced anovulation by an inhibitor of prostaglandin synthesis in mares.  
589 *Animal Reproduction Science* **126** 91-95.
- 590 **Ginther OJ, Gastal EL, Gastal MO & Beg MA** 2005 Regulation of circulating gonadotropins  
591 by the negative effects of ovarian hormones in mares. *Biology of Reproduction* **73** 315-  
592 323.
- 593 **Ginther OJ, Gastal EL, Gastal MO & Beg MA** 2006 Conversion of a viable preovulatory  
594 follicle into a hemorrhagic anovulatory follicle in mares. *Animal Reproduction* **3** 29-40.

- 595 **Ginther OJ, Gastal EL, Gastal MO & Beg MA** 2007a Incidence, endocrinology, vascularity,  
596 and morphology of hemorrhagic anovulatory follicles in mares. *Journal of Equine*  
597 *Veterinary Science* **27** 122-131.
- 598 **Ginther OJ, Gastal EL, Gastal MO, Bergfelt DR, Baerwald AR & Pierson RA** 2004  
599 Comparative study of the dynamics of follicular waves in mares and women. *Biology of*  
600 *Reproduction* **71** 1195-1201.
- 601 **Ginther OJ, Gastal EL, Gastal MO, Utt MD & Beg MA** 2007b Luteal blood flow and  
602 progesterone production in mares. *Animal Reproduction Science* **99** 213-220.
- 603 **Ginther OJ, Gastal MO, Gastal EL, Jacob JC & Beg MA** 2008a Induction of haemorrhagic  
604 anovulatory follicles in mares. *Reproduction Fertility and Development* **20** 947-954.
- 605 **Ginther OJ, Jacob JC, Gastal MO, Gastal EL & Beg MA** 2008b Follicle and systemic  
606 hormone interrelationships during spontaneous and ablation-induced ovulatory waves in  
607 mares. *Animal Reproduction Science* **106** 181-187.
- 608 **Ginther OJ & Pierson RA** 1984 Ultrasonic anatomy and pathology of the equine uterus.  
609 *Theriogenology* **21** 505-516.
- 610 **Ginther OJ, Shrestha HK, Fuenzalida MJ, Shahiduzzaman AK & Beg MA** 2010  
611 Characteristics of pulses of 13,14-dihydro-15-keto-prostaglandin f2alpha before, during,  
612 and after spontaneous luteolysis and temporal intrapulse relationships with progesterone  
613 concentrations in cattle. *Biology of Reproduction* **82** 1049-1056.
- 614 **Hafs HD, Louis TM, Stellflug JN, Convey EM & Britt JH** 1975 Blood LH after PGF2alpha in  
615 diestrous and ovariectomized cattle. *Prostaglandins* **10** 1001-1009.

- 616 **Hamilton CJ, Evers JL & Hoogland HJ** 1986 Ovulatory disorders and inflammatory adnexal  
617 damage: a neglected cause of the failure of fertility microsurgery. *British Journal of*  
618 *Obstetrics and Gynaecology* **93** 282-284.
- 619 **Hamilton CJ, Wetzels LC, Evers JL, Hoogland HJ, Muijtjens A & de Haan J** 1985 Follicle  
620 growth curves and hormonal patterns in patients with the luteinized unruptured follicle  
621 syndrome. *Fertility and Sterility* **43** 541-548.
- 622 **Henneke DR, Potter GD, Kreider JL & Yeates BF** 1983 Relationship between condition  
623 score, physical measurements and body fat percentage in mares. *Equine Veterinary*  
624 *Journal* **15** 371-372.
- 625 **Hulka JF** 1985 Luteinized unruptured follicle syndrome. *Fertility and Sterility* **44** 853-854.
- 626 **Jesam C, Salvatierra AM, Schwartz JL & Croxatto HB** 2010 Suppression of follicular  
627 rupture with meloxicam, a cyclooxygenase-2 inhibitor: potential for emergency  
628 contraception. *Human Reproduction* **25** 368-373.
- 629 **Jesam C, Salvatierra AM, Schwartz JL, Fuentes A & Croxatto HB** 2014 Effect of oral  
630 administration of a continuous 18 day regimen of meloxicam on ovulation: experience of  
631 a randomized controlled trial. *Contraception* **90** 168-173.
- 632 **Kaiser B, Koene M, Swagemakers J, Bader H & Hoppen HO** 1999 Diagnosis, therapy and  
633 endocrinologic parameters of persistent follicles in mares in comparison with  
634 preovulatory follicles. *Tierarztl Praxis Ausgabe G Grosstiere Nutztiere* **27** 180-186.
- 635 **Katz E** 1988 The luteinized unruptured follicle and other ovulatory dysfunctions. *Fertility and*  
636 *Sterility* **50** 839-850.

- 637 **Kerin JF, Kirby C, Morris D, McEvoy M, Ward B & Cox LW** 1983 Incidence of the  
638 luteinized unruptured follicle phenomenon in cycling women. *Fertility and Sterility* **40**  
639 620-626.
- 640 **Killick S & Elstein M** 1987 Pharmacologic production of luteinized unruptured follicles by  
641 prostaglandin synthetase inhibitors. *Fertility and Sterility* **47** 773-777.
- 642 **Koninckx PR & Brosens IA** 1983 The luteinized unruptured follicle syndrome. *Fertility and*  
643 *Sterility* **39** 249-250.
- 644 **Koninckx PR, Brosens IA, Verhoeven G & De Moor P** 1981 Increased postovulatory plasma  
645 follicle stimulating hormone levels in the luteinized unruptured follicle syndrome: a role  
646 for inhibin? *British Journal of Obstetrics and Gynaecology* **88** 525-529.
- 647 **Kugu K, Taketani Y, Kohda K & Mizuno M** 1991 Exaggerated prolactin response to  
648 thyrotropin-releasing hormone in infertile women with the luteinized unruptured follicle  
649 syndrome. *Archives Gynecology Obstetrics* **249** 27-31.
- 650 **Lefranc A-C & Allen WR** 2003 Incidence and morphology of anovulatory haemorrhagic  
651 follicles in the mare. *Pferdeheilkunde* **19** 611-612.
- 652 **Lueders I, Taya K, Watanabe G, Yamamoto Y, Yamamoto T, Kaewmanee S, Niemuller C,**  
653 **Gray C, Streich WJ & Hildebrandt TB** 2011 Role of the double luteinizing hormone  
654 peak, luteinizing follicles, and the secretion of inhibin for dominant follicle selection in  
655 Asian elephants (*Elephas maximus*). *Biology of Reproduction* **85** 714-720.
- 656 **Marik J & Hulka J** 1978 Luteinized unruptured follicle syndrome: a subtle cause of infertility.  
657 *Fertility and Sterility* **29** 270-274.
- 658 **Martínez-Boví R & Cuervo-Arango J** 2015 Intrafollicular treatment with PGE2 and PGF2 $\alpha$   
659 inhibits the formation of luteinised unruptured follicles (LUF) and restores normal

- 660 ovulation in mares treated with flunixin-meglumine. *Equine Veterinary Journal* (doi:  
661 10.1111/evj.12396).
- 662 **Martinez AR, Bernardus RE, Kucharska D & Schoemaker J** 1991 Urinary luteinizing  
663 hormone testing and prediction of ovulation in spontaneous, clomiphene citrate and  
664 human menopausal gonadotropin-stimulated cycles. A clinical evaluation. *Acta*  
665 *Endocrinologica (Copenh)* **124** 357-363.
- 666 **Mattheij JA & Swarts HJ** 1995 Induction of luteinized unruptured follicles in the rat after  
667 injection of luteinizing hormone early in pro-oestrus. *European Journal of Endocrinology*  
668 **132** 91-96.
- 669 **Meyers-Brown G, Bidstrup LA, Famula TR, Colgin M & Roser JF** 2011 Treatment with  
670 recombinant equine follicle stimulating hormone (reFSH) followed by recombinant  
671 equine luteinizing hormone (reLH) increases embryo recovery in superovulated mares.  
672 *Animal Reproduction Science* **128** 52-59.
- 673 **Murdoch WJ, Hansen TR & McPherson LA** 1993 A review--role of eicosanoids in vertebrate  
674 ovulation. *Prostaglandins* **46** 85-115.
- 675 **Niswender GD, Juengel JL, Silva PJ, Rollyson MK & McIntush EW** 2000 Mechanisms  
676 controlling the function and life span of the corpus luteum. *Physiological Reviews* **80** 1-  
677 29.
- 678 **Peter AT** 2004 An update on cystic ovarian degeneration in cattle. *Reproduction in Domestic*  
679 *Animals* **39** 1-7.
- 680 **Plas-Roser S, Kauffmann MT & Aron C** 1985 Prostaglandins involvement in the formation of  
681 luteinized unruptured follicles in the cyclic female rat. *Prostaglandins* **29** 243-253.



- 682 **Priddy AR, Killick SR, Elstein M, Morris J, Sullivan M, Patel L & Elder M** 1990 The effect  
683 of prostaglandin synthetase inhibitors on human preovulatory follicular fluid  
684 prostaglandin, thromboxane, and leukotriene concentrations. *Journal of Clinical*  
685 *Endocrinology and Metabolism* **71** 235-242.
- 686 **Qublan H, Amarin Z, Nawasreh M, Diab F, Malkawi S, Al-Ahmad N & Balawneh M** 2006  
687 Luteinized unruptured follicle syndrome: incidence and recurrence rate in infertile  
688 women with unexplained infertility undergoing intrauterine insemination. *Human*  
689 *Reproduction* **21** 2110-2113.
- 690 **Salhab AS, Amro BI & Shomaf MS** 2003 Further investigation on meloxicam contraceptive  
691 in female rabbits: luteinizing unruptured follicles, a microscopic evidence. *Contraception*  
692 **67** 485-489.
- 693 **Schauer SN, Guillaume D, Decourt C, Watson ED, Briant C & Donadeu FX** 2013 Effect of  
694 luteinizing hormone overstimulation on equine follicle maturation. *Theriogenology* **79**  
695 409-416.
- 696 **Shrestha HK, Beg MA, Burnette RR & Ginther OJ** 2012 Plasma clearance and half-life of  
697 prostaglandin F<sub>2</sub>α: a comparison between mares and heifers. *Biology of*  
698 *Reproduction* **87** 18, 11-16.
- 699 **Silva JR, Figueiredo JR & van den Hurk R** 2009 Involvement of growth hormone (GH) and  
700 insulin-like growth factor (IGF) system in ovarian folliculogenesis. *Theriogenology* **71**  
701 1193-1208.
- 702 **Sirois J & Dore M** 1997 The late induction of prostaglandin G/H synthase-2 in equine  
703 preovulatory follicles supports its role as a determinant of the ovulatory process.  
704 *Endocrinology* **138** 4427-4434.

- 705 **Smith EM, Anthony F & Masson GM** 1991 Follicular fluid prostaglandin E<sub>2</sub>:prostaglandin F<sub>2</sub>  
706 alpha ratio in relation to outcome of matched oocyte. *Human Reproduction* **6** 519-523.
- 707 **Smith G, Roberts R, Hall C & Nuki G** 1996 Reversible ovulatory failure associated with the  
708 development of luteinized unruptured follicles in women with inflammatory arthritis  
709 taking non-steroidal anti-inflammatory drugs. *British Journal of Rheumatology* **35** 458-  
710 462.
- 711 **Srikandakumar A & Downey BR** 1989 Induction of ovulation in gilts with cloprostenol.  
712 *Theriogenology* **32** 445-449.
- 713 **Stoops MA, Pairan RD & Roth TL** 2004 Follicular, endocrine and behavioural dynamics of the  
714 Indian rhinoceros (*Rhinoceros unicornis*) oestrous cycle. *Reproduction* **128** 843-856.
- 715 **Toda K, Ono M, Yuhki K, Ushikubi F & Saibara T** 2012 17beta-Estradiol is critical for the  
716 preovulatory induction of prostaglandin E<sub>2</sub> synthesis in mice. *Molecular and Cellular*  
717 *Endocrinology* **362** 176-182.
- 718 **Vanrell JA, Balasch J, Fuster JS & Fuster R** 1982 Ovulation stigma in fertile women. *Fertility*  
719 *and Sterility* **37** 712-713.
- 720 **Watson ED & Sertich PL** 1991 Concentrations of arachidonate metabolites, steroids and  
721 histamine in preovulatory horse follicles after administration of human chorionic  
722 gonadotrophin and the effect of intrafollicular injection of indomethacin. *Journal of*  
723 *Endocrinology* **129** 131-139.
- 724 **Westfahl PK** 1988 Circulating sex steroids after induction of luteinized unruptured follicles in  
725 adult guinea pigs. *Steroids* **51** 101-114.

726 **Zaidi J, Jurkovic D, Campbell S, Collins W, McGregor A & Tan SL** 1995 Luteinized  
727 unruptured follicle: morphology, endocrine function and blood flow changes during the  
728 menstrual cycle. *Human Reproduction* **10** 44-49.

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749 **Figure Legends**

750

751 **Figure 1.** A series of comparative ultrasound images of preovulatory follicles and LUFs in four  
752 women (A–D) and one mare (E–H). Images were obtained before ovulation in women (A) and a  
753 mare (E), and various stages of LUF formation. Large diameter, thicker and echogenic follicle  
754 wall (luteinized; long arrows), and echoic foci and fibrin-like strands (short arrows) in the  
755 follicle antrum can be observed in different degrees in LUFs in women (B–D) and mare (F–H).

756

757 **Figure 2.** Mean ( $\pm$  S.E.M.) diameters of the largest follicle and CL, and corresponding  
758 concentrations of P4 for Days 7–15 after ovulation (Day 0) for the Control, LH combined, and  
759 LH + PG combined groups. Arrow indicates beginning of treatments on Day 7. The probabilities  
760 for a group effect (G), day effect (D), and group-by-day interaction (GD) are shown. An asterisk  
761 (\*) indicates days of a significant difference ( $P < 0.05$ ) between groups and a pound mark (#)  
762 indicates a difference that approached significance ( $P < 0.1$ ).

763

764 **Figure 3.** Mean ( $\pm$  S.E.M.) PGF<sub>2</sub> $\alpha$  metabolite (PGFM) concentration for the Induced LUF group  
765 (flunixin meglumine treatments combined) versus the Control group (saline). The probabilities  
766 for a group effect (G), hour effect (H), and group-by-hour interaction (GH) are shown. An  
767 asterisk (\*) indicates hours of a significant difference ( $P < 0.05$ ) between groups.

768

769 **Figure 4.** Mean ( $\pm$  S.E.M.) follicle diameter, follicle wall serration, follicle wall thickness, and  
770 follicle blood flow for the Induced LUF group (flunixin meglumine treatments combined) versus  
771 the Control group (saline). The probabilities for a group effect (G), hour effect (H), and group-

772 by-hour interaction (GH) are shown for Hours 0–38. An asterisk (\*) indicates the first increase  
773 ( $P < 0.05$ ) within a group.

774

775 **Figure 5.** Mean ( $\pm$  S.E.M.) plasma PGF<sub>2</sub> $\alpha$  metabolite (PGFM) concentration, follicle diameter,  
776 follicle blood flow, and endometrial echotexture score for the Induced LUF group (flunixin  
777 meglumine treatment) versus the Control group (saline). The probabilities for a group effect (G),  
778 hour effect (H), and group-by-hour interaction (GH) are shown. An asterisk (\*) indicates hours  
779 of a significant difference ( $P < 0.05$ ) between groups, and a pound mark (#) indicates a  
780 difference that approached significance ( $P < 0.1$ ) between groups.

781

782 **Figure 6.** Mean ( $\pm$  S.E.M.) follicular fluid concentrations of PGF<sub>2</sub> $\alpha$  metabolite (PGFM), PGF<sub>2</sub> $\alpha$ ,  
783 PGE<sub>2</sub>, and PGE<sub>2</sub>:PGF<sub>2</sub> $\alpha$  ratio for the Induced LUF group (flunixin meglumine treatment) versus  
784 the Control group (saline). Bars with different superscripts within an endpoint are different ( $P <$   
785 0.05).

786

787 **Figure 7.** Mean ( $\pm$  S.E.M.) follicular fluid concentrations of E2, P4, T, and total primary sex  
788 steroids for the Induced LUF group (flunixin meglumine treatment) versus the Control group  
789 (saline). Bars with different superscripts within an endpoint are different ( $P < 0.05$ ), and a pound  
790 mark (#) indicates a difference that approached significance ( $P < 0.1$ ) between groups. NS, non-  
791 significant.

792

793 **Figure 8.** Mean ( $\pm$  S.E.M.) follicular fluid concentrations of LH, cortisol, GH, VEGF-A, and NO  
794 for the Induced LUF group (flunixin meglumine treatment) versus the Control group (saline).

795 Bars with a pound mark (#) indicate a difference that approached significance ( $P < 0.1$ ) between  
796 groups.

797