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1 **Serum concentration of mineralocorticoids, glucocorticoids and sex-steroids, in peripartum**
2 **bitches**

3 Milani Chiara¹, Rota Ada^{2*}, Ulf Olsson³, Paganotto Alessandra⁴, Hölst Bodil S⁵

4 ¹-Department of Animal Medicine, Production and Health, Padova, Italy

5 ²-Department of Veterinary Sciences, Torino, Italy

6 ³-Department of Energy and Technology, Applied Statistics and Mathematics, SLU, Uppsala, e-

7 mail: ulf.olsson@slu.se

8 ⁴-Private practitioner, France

9 ⁵-Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala,

10 Sweden

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14

15 ***Corresponding Author:**

16 Ada Rota, BScAgrSc, DVM, PhD, ECAR Dipl.

17 Department of Veterinary Sciences-University of Torino

18 Largo Paolo Braccini, 2-5

19 10095 Grugliasco (TO) Italy

20 Email: ada.rota@unito.it

21

22 **Abstract**

23 The aim of the work was to describe the profile of steroid hormones in the peripartum period of the
24 bitch. Twenty-five healthy pregnant bitches presented for clinical pregnancy monitoring and
25 parturition assistance were included in the study. A blood sample was collected for routine
26 progesterone assay and serum was stored at -20°C. The day of parturition and the number of born
27 puppies was registered. Concentrations of corticosteroids, androgens, progestogens, estrogens, for a
28 total number of 17 different hormones, were measured using the ultra-performance supercritical fluid
29 chromatography – tandem mass spectrometry (UPSFC-MS/MS) method. Data were analysed using a
30 repeated measure, mixed model approach, that took into account day (from day -4 to day +2 from
31 parturition), age, parity (primiparous vs pluriparous), number of delivered puppies (<4 vs 4-8 vs >8),
32 and interactions between the factors. Day related to parturition significantly affected the concentration
33 of progesterone ($p<0.001$), testosterone ($p<0.001$), 17α -hydroxyprogesterone ($p=0.0002$), and
34 cortisone ($p=0.006$). Estrogen concentration did not show any significant variation over time.
35 Testosterone and androstenedione showed an abrupt decline on the day of parturition. The
36 concentration of all glucocorticoids increased the day before parturition. Age or parity were not
37 significantly associated with any of the steroids. Litter size significantly affected concentrations of
38 aldosterone ($p=0.02$) and etiocholanolone ($p=0.01$). Aldosterone concentrations were higher in litters
39 with 4-8 pups than in litters with more than 8 pups ($p=0.02$). None of the steroids measured in our
40 study, with the already known exception of progesterone, shows potential to be clinically useful in
41 predicting the onset of parturition in the bitch.

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46 **Keywords**

47 Dog, peri-partum, hormones, corticosteroids, sex-steroids

48 **1. Introduction**

49

50 Canine reproductive endocrinology has interesting features. Unlike the situation in most other
51 species, no specific factor related to maternal recognition of pregnancy has been described. Moreover,
52 progesterone concentrations are similar in pregnant and non-pregnant bitches, and corpora lutea are
53 the sole source of this hormone during pregnancy. In pregnant bitches, progesterone concentrations
54 drop at parturition, whereas in non-pregnant ones, concentrations decrease gradually over a longer
55 time [1]. Not only progesterone concentrations change in relation to parturition. In sheep and
56 primates, estrogens have been shown to play a crucial role in the onset of parturition because they
57 'activate' the myometrium that acquires the capacity to respond to the stimuli that lead to contraction
58 and labor [2]. A prepartal increase of estrogen levels is common in several species such as sheep,
59 goats, and humans [3]. In dogs, estradiol concentrations around parturition were described to be
60 constant [4] or even to decrease prior to parturition [5-7].

61 Changes in a panel of steroids concentrations, measured using liquid chromatography - tandem mass
62 spectrometry (LC-MS/MS), have been described in bitches during the first weeks of pregnancy. [8].
63 LC-MS/MS is often considered the gold standard for steroid hormone assay due to its high sensitivity
64 and specificity, and it has the advantage of allowing simultaneous analysis of several steroids from a
65 small sample volume [9]. As this method allows simultaneous analysis of several steroids, not only
66 information is achieved on specific steroids but differences in enzyme activity may also be estimated.
67 Analytical methods for improving steroid profiling are continuously developed, and a recently
68 described method in human medicine is the ultra-performance supercritical fluid chromatography –
69 tandem mass spectrometry (UPSFC-MS/MS), with even higher sensitivity than LC-MS/MS, and
70 short analytical duration [10].

71 Endocrinology of canine parturition has not been extensively investigated, and many aspects of this
72 species may not be inferred by investigations in other species, given the strong peculiarities of canine
73 reproductive physiology. Steroid profiling of the time around parturition using UPSFC-MS/MS can

74 lead to deeper understanding of the events leading to parturition in the dog. Knowledge on the
75 variation of steroid concentrations is also of value for diagnostic purposes, including prediction of
76 parturition, and is of potential value when choosing appropriate treatments.

77 The objective of this work was to assess the endocrine changes associated with parturition in dogs by
78 measuring serum concentration of steroids using UPSFC-MS/MS in healthy animals around natural
79 parturition.

80

81 **2.Materials and Methods**

82

83 *2.1 Animals and samples*

84

85 The study was performed in accordance with the guidelines for the care and use of animals of the
86 Department of Veterinary Science of the University of Turin and of the Department of Animal
87 Medicine Production and Health of Padova. Informed consent to use the stored samples was obtained
88 from dog owners. Approval by the Ethical and Animal Welfare Committee of the Department of
89 Veterinary Science of the University of Turin was obtained (1057/27/05/2020).

90 Twenty-five healthy bitches belonging to various breeds [Staffordshire Bull Terrier (N=5), Flat
91 Coated Retriever (N=4), Boxer (N=4), Jack Russell Terrier (N=2), Bouvier des Flandres (N=2),
92 Australian Shepherd (N=2), and one each of the following: American Staffordshire Terrier,
93 Bloodhound, Bassett Hound, Labrador Retriever, Golden Retriever, Samoyed] and ranging in age
94 from 2 to 8 years (mean \pm SD 4.0 \pm 1.6) were included in the study. The bitches were presented to the
95 veterinary teaching hospitals of the University of Padova or Torino for pregnancy monitoring and
96 parturition assistance, in the period from June 2017 to October 2017. Blood was sampled by cephalic
97 venipuncture for routine progesterone assay and routine biochemistry evaluation. The number of
98 samples varied among bitches according to clinical needs. Serum remnants obtained after
99 centrifugation at 1700 G were stored frozen at -20°C. Only normal pregnancies and bitches having

100 normal parturition events were considered in the study. For each pregnancy, the parturition date and
101 the number of delivered puppies were recorded.

102 Stored samples collected between four days before and two days after parturition were selected for
103 analysis.

104

105 *2.2 Hormone analysis*

106

107 An analysis of steroid hormones was performed by supercritical fluid chromatography–tandem mass
108 spectrometry (SFC–MS/MS) on an Acquity UPC² (Waters Corporation, Milford, MA, USA) system
109 coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The SFC
110 system was equipped with a binary solvent delivery pump, an autosampler, a column oven and a back
111 pressure regulator. Separation of the seventeen steroids: androgens [androsterone, androstenedione,
112 dehydroepiandrosterone (DHEA), etiocholanolone, testosterone]; corticosteroids (aldosterone,
113 cortisol, cortisone, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol); estrogens (estrone and
114 estradiol), and progestins (17 α -Hydroxyprogesterone, pregnanolone, pregnenolone, progesterone)
115 was accomplished within Acquity UPC² BEH column (150 mm \times 3.0 mm, 1.7 μ m particle size;
116 Waters, Milford, MA, USA). It was kept at 40 °C and at a mobile phase flow rate of 2 mL/min. The
117 gradient program was started with 98% A (CO₂) and 2% B (0.1% formic acid in methanol/isopropanol
118 (1:1)), linearly increased to 17% B over 3 min, held at 17% B for 0.5 min, followed by a linear
119 gradient down to 2% B over 0.5 min. Finally it was held for 1 min at 2% B for the elution of ionic
120 liquids out of the instrument, resulting in a total separation time of 5 min. The back pressure was set
121 to 1500 psi and the injection volume was 1.0 μ L. Elution from the SFC system into the MS system
122 was aided by a make-up solvent (0.1% formic acid in methanol) at a flow rate of 0.4 mL/min. Mass
123 spectrometric detection was performed using electrospray ionization in the positive ionization mode
124 (ESI+) with a capillary voltage of 2.8 kV, cone voltage of 30 V, and source offset of 30 V. Nitrogen
125 and argon (0.15 mL/min) served as the desolvation gas and the collision gas, respectively.

126 Desolvation temperature was maintained at 500 °C, and source temperature was set to 150 °C.
127 Desolvation gas flow and cone gas flow were maintained at a rate of 150 L/h and 750 L/h,
128 respectively. The nebulizer gas flow was set to 7.0 bar (101.5 psi). Collision energy was varied to
129 optimize product ion formation. The data acquisition range was set for m/z 100-600. Standard
130 solutions of the steroids at 10 µg/mL were introduced to the source at 10 µL/min using IntelliStart™
131 in infusion mode. Mass spectra for each analyte were recorded in MS and MS/MS mode. The
132 quantification was based on a multiple reaction monitoring (MRM) method and collision energy and
133 scan dwell time were set according to Table 1. MS/MS conditions and the method were confirmed
134 by individual analysis of the standard steroids (50 ng/mL). Data were acquired, analyzed and
135 processed with Waters MassLynx NT4.1 software. Quantification of steroids was performed using
136 the corresponding IS.

137

138 *2.3 Statistical analysis*

139

140 The data were analyzed as repeated-measures data. A mixed model approach [11,12], as implemented
141 in the Mixed procedure of the SAS (2014) [13] system was used. The relations between time points
142 within dog were modeled using an autoregressive covariance structure.

143 The fixed part of the models included Day (related to parturition), Age, Parity (primiparous vs
144 pluriparous), Number of delivered pups (<4 vs 4-8 vs >8), and interactions between these. Several
145 different models were tested. The selection of interactions to include was made based on the Akaike
146 Information Criterion (AIC;[14]), and based on this the interaction between Day and Number of
147 delivered pups was included. The assumptions underlying the analyses were checked using diagnostic
148 plots. The plots suggested that a logarithmic transformation of the steroid concentrations was
149 warranted, and all steroids were logarithmized. Within each analysis, post-hoc pairwise comparisons
150 were adjusted for multiplicity using Tukey's method. The level of statistical significance was set at
151 $p < 0.05$. No corrections for multiplicity between the 17 analyses were made. For significant variables

152 diagnostic plots were checked. If plots did not visualize associations, they were not considered
153 biologically significant and were not considered further.

154

155 **3. Results**

156

157 In total, 57 samples were analyzed, 1-5 samples for each bitch. The median age of the bitches was 4
158 years (inter-quartile range, IQR, 3-5 years). Eleven of the 25 bitches were primiparous. Three bitches
159 had a litter size of less than 4 pups, 15 had litters with 4 to 8 pups, and 7 had more than eight pups.
160 The number of samples for each of the seven days of observation, and the median serum concentration
161 of all steroids each day are shown in Table 1. Age or parity were not significantly associated with any
162 of the steroids. Litter size significantly affected concentrations of aldosterone ($p=0.02$) and
163 etiocholanolone ($p=0.01$), Figure 1. Aldosterone concentrations were higher in litters with 4-8 pups
164 than in litters with more than 8 pups ($p=0.02$).

165 Day related to parturition significantly affected the concentration of progesterone ($p<0.001$),
166 testosterone ($p<0.001$), 17α -hydroxyprogesterone ($p=0.0002$), and cortisone ($p=0.006$), Figure 2,
167 Table 1. The concentrations of androsterone, androstenedione, cortisol, corticosterone, 11-
168 deoxycorticosterone, 11-deoxycortisol, DHEA, pregnanolone, pregnenolone, estrone and estradiol
169 were not related to any of the investigated factors. Statistically significant associations that were not
170 apparent on diagnostic plots and therefore were not considered further included an interaction
171 between day related to parturition and litter size for cortisone ($p=0.04$) and 17α -hydroxyprogesterone
172 ($p=0.04$), as well as age of the bitch and the concentration of aldosterone ($p=0.04$).

173

174 **4. Discussion**

175

176 The present work offers a complete picture of steroid hormone concentrations around parturition in
177 bitches, adding information to the endocrinology of this event. The canine species shows peculiarities
178 also for the mechanisms of parturition onset as for other known aspects of reproductive physiology.
179 It is well known that progesterone, exclusively of luteal origin, declines before parturition [1] and our
180 data confirm that the hormone reaches basal values on the day of parturition, significantly decreasing
181 over the last two days. The progesterone derivative 17α -hydroxyprogesterone showed a later decline,
182 from the day of parturition onwards. The other derivative of progesterone, pregnanolone, tended to
183 decline even later, one day after parturition. Only the progesterone precursor pregnenolone did not
184 show any significant variation in the period of observation.

185 The decline in progesterone concentration preceding parturition in the dog is due to prostaglandin-
186 induced luteolysis. An increase of prostaglandin concentration has been observed 24 hours before
187 parturition, corresponding to prepartal luteolysis [7,15-17]. In women and sheep an increase of
188 glucocorticoids, of fetal origin, and occurring when the fetal pituitary-adrenal axis approaches
189 maturity, causes intrauterine prostaglandin production both directly and indirectly, through the
190 stimulation of placental estradiol synthesis [3]. As shown in sheep, glucocorticoids stimulate the
191 activity of placental cytochrome P450 17α hydroxylase (CYP17A) and cytochrome P450
192 (P450arom), which results in placental steroid production in favor of estrogens [18]. During
193 pregnancy, the placenta becomes the primary site of estrogen synthesis in many species, although
194 species-specific differences exist in the placental suppliers of the C19 precursors (DHEA, and its
195 sulfoconjugate DHEA-S). Estrogens origin from fetal and/or maternal adrenal cortex in humans [19]
196 and fetal gonads in horses [20]. In goat placentae obtained postpartum, enzymes responsible for the
197 synthesis of estrogens from C21 steroids (pregnenolone, progesterone) have been identified [21]. The
198 dog placenta does not synthesize estrogen [22]. The localization of aromatase, the estrogen convertase
199 enzyme, in all cells throughout canine corpora lutea in late gestation, confirms that corpora lutea are
200 the principal site of estrogen production in dogs [23]. Neither DHEA nor pregnenolone concentration
201 was affected by day of parturition in our investigation. We did not observe an increase in estrogen

202 concentration around parturition, and neither estrone nor estradiol showed significant variations over
203 time.

204 Our results are thus in agreement with previous studies in dogs, not showing the sharp increase in
205 serum estrogen concentration observed in several other species preceding parturition [4,23]. Some
206 studies have described a prepartum decline of estradiol concentrations in the dog, concomitant with
207 the decline in progesterone [5-7]. Failure to detect a prepartum decline in estrogen concentration in
208 our work may be due to the fact that the bitches reached basal estrogen concentrations earlier during
209 gestation. However, Onclin et al. (2002) and Hoffman et al. (1994) [5,6] reported the decrease during
210 the corresponding time of gestation as the present study.

211 Estrone was not detected from any sample by Hoffman et al. (1994)[5], whereas elevated serum
212 estrone concentrations throughout the canine gestation, followed by a decline at parturition, were
213 found by Chakraborty (1987) [24], both studies using radioimmunoassays (RIA).

214 Estrogens are considered to have a vital function at parturition because they are involved in dilation
215 of uterine cervix and promote uterine contractions, increasing uterine sensitivity to oxytocin [2]. Since
216 estrogen concentrations do not increase but, either remain stable or even decline in the dog, it can be
217 assumed that changes in hormonal ratios (progesterone/estradiol) instead of changes in absolute
218 concentrations may play a role at parturition.

219 In the present study, testosterone and androstenedione showed a similar profile, with an abrupt decline
220 on the day of parturition. The variation over time was significant for testosterone but not for
221 androstenedione. A larger variation in concentrations of androstenedione compared to testosterone
222 concentrations may contribute to this. Similar variations, with a sharp decline at parturition, have
223 been described previously, with changes being significant for androstenedione but not for testosterone
224 [25]. Androgen convertase enzyme has not been detected in canine corpora lutea during the late stage
225 of gestation, and consequently, corpora lutea are not a site of androgen production by conversion of
226 pregnenolone and progesterone [23]. The sharp decrease in androgen concentrations at parturition is
227 therefore not related to a decreased production by the corpora lutea.

228 The concentration of all glucocorticoids increased the day preceding parturition, although the effect
229 of time was significant only for cortisone. A large individual variation and relatively few dogs,
230 leading to a low statistical power, may contribute to the lack of significance for the other
231 glucocorticoids. Previous investigations have described an increase in cortisol concentration shortly
232 before parturition [7,26] or at the time of parturition [5,16]. Olsson et al. (2003) [27] detected a
233 significant increase only when fetal membranes of the first pup were visible.

234 The peak glucocorticoid levels in women and bitches at parturition have been interpreted as a result
235 of maternal and fetal stress with the onset of labor rather than as a signal triggering parturition [5,28].
236 A great increase of the glucocorticoid receptor *GR/NR3C1* is observed during prepartum luteolysis
237 but not following antigestagen-treatment, suggesting that glucocorticoids are not required to start the
238 signalling cascade leading to the onset of parturition [29]. In many mammalian species, the increase
239 of plasma cortisol at the end of gestation is of fetal origin, but studies in the dog on this topic are
240 lacking [29].

241 Maternal concentration of mineralcorticoids (aldosterone, 11-deoxycorticosterone) increases during
242 pregnancy in women but there is not a peak at the beginning of delivery [28,30], and the
243 concentrations had returned to basal values at 2-5 days postpartum [30]. The aldosterone
244 concentrations measured in our study were generally low, in the lower range of the values for normal
245 dogs [31] and there was no effect of time. Aldosterone protects against sodium losses and
246 extracellular fluid volume depletion. The significantly lower aldosterone concentrations in larger
247 litters may be related to a lower efficiency of the renin-angiotensin system, or to a more efficient
248 sodium retention and potassium excretion by the kidneys in case of higher number of fetuses.

249

250 **5. Conclusion**

251

252 The picture that we can draw from our data is a prepartum decline of progesterone followed by the
253 abrupt decline of its derivative 17 α -hydroxyprogesterone, of testosterone and of its precursor

254 androstenedione on the day of parturition; an increase of cortisone on the day before parturition and
255 a similar trend for the other glucocorticoids and for 11-deoxycorticosterone, and the absence of
256 variation over time of estrogen concentration and of aldosterone. None of the steroids measured in
257 our study, with the already known exception of progesterone, shows potential to be clinically useful
258 in predicting the onset of parturition in the dog.

259

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261

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265 Sweden.

266

267 **Credits to authors**

268

269 Milani Chiara : study design, data collection and interpretaion, contributed in writing and editing
270 the work; Rota Ada: paper writing; data collection and interpretation, funding; Olsson Ulf: data
271 analysis, tables and figure editing; Paganotto Alessandra: data collection, editing the work; Strom
272 BH: data analysis and interpretation; funding; contributed in writing and editing the work.

273

274 **References**

275

276 [1] Concannon PW. Reproductive cycles of the domestic bitch. *Anim Reprod Sci.* 2011;124:200-10.

277 [2] Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term

278 and preterm. *Endocr Rev.* 2000;21(5):514-50.

- 279 [3] Whittle WL, Patel FA, Alfaidy N, Holloway AC, Fraser M, Gyomorey S, et al. Glucocorticoid
280 regulation of human and ovine parturition: the relationship between feral hypothalamic–pituitary–
281 adrenal axis activation and intrauterine prostaglandin production. *Biol Reprod* 2001;64:1019–32.
- 282 [4] Edqvist LE, Johansson ED, Kasstrom H, Olsson SE, Richkind M. Blood plasma levels of
283 progesterone and oestradiol in the dog during the oestrous cycle and pregnancy. *Acta Endocrinol*
284 (Copenh) 1975;78:554–64.
- 285 [5] Hoffmann B, Höveler R, Nohr B, Hasan SH. Investigations on hormonal changes around
286 parturition in the dog and the occurrence of pregnancy-specific non conjugated oestrogens. *Exp Clin*
287 *Endocrinol* 1994;102:185-9.
- 288 [6] Onclin K, Murphy B, Verstegen JP. Comparisons of estradiol, LH and FSH patterns in pregnant
289 and nonpregnant beagle bitches. *Theriogenology* 2002;57:1957–72.
- 290 [7] Baan M, Taverne, MAM, de Gier J, Kooistra HS, Kindahl H, Dieleman SJ, Okkens AC.
291 Hormonal changes in spontaneous and aglépristone-induced parturition in dogs. *Theriogenology*
292 2008;69:399-407.
- 293 [8] Holst BS, Kushnir MM, Bergquist J. Liquid chromatography-tandem mass spectrometry (LC-
294 MS/MS) for analysis of endogenous steroids in the luteal phase and early pregnancy in dogs: a pilot
295 study. *Vet Clin Pathol.* 2015;44:552-8.
- 296 [9] Field HP. Tandem mass spectrometry in hormone measurement. *Methods in molecular biology*
297 (Clifton, NJ). 2013;1065:45-74.
- 298 [10] deKock N, Acharya SR, Ubhayasekera S, Bergquist J. A Novel targeted analysis of peripheral
299 steroids by ultra-performance supercritical fluid chromatography hyphenated to tandem mass
300 spectrometry. *Scientific Reports.* 2018;8:169-93
- 301 [11] Littell, R., Milliken, G., Stroup, W. Wolfinger, R. and Schabenberger O. (2006): *SAS for*
302 *mixed models*, second ed. Cary, N. C., SAS Institute Inc.
- 303 [12] Fitzmaurice, G. M., Laird, N. M. and Ware, J. H. (2004): *Applied longitudinal analysis.* New
304 York, Wiley.

- 305 [13] SAS Institute Inc. (2014): SAS/Stat User's Guide. Version 9.4. Cary, N. C., SAS Institute Inc.
- 306 [14] Akaike, H. (1976): An information criterion (AIC). *Math. Sci.* 14(153):5–9.1976 or
307 1973???OPPURE: Akaike, H. (1973). Information theory and an extension of the maximum
308 likelihood principle. In B. N. Petrov and F. Csaki (Eds.), Second international symposium on
309 information theory (pp. 267-281). Budapest: Akademiai Kiado.
- 310 [15] Concannon PW, Isaman L, Frank DA, Michel FJ, Currie WB. Elevated concentrations of
311 13,14-dihydro-15-keto-prostaglandin F-2 alpha in maternal plasma during parturition and
312 parturition in dogs (*Canis familiaris*). *J Reprod Fertil* 1988;84:71–7.
- 313 [16] Veronesi MC, Battocchio M, Marinelli L, Faustini M, Kindahl H, Cairoli F. Correlations
314 among body temperature, plasma progesterone, cortisol and prostaglandin F2alpha of the
315 periparturient bitch. *J Vet Med A Physiol Pathol Clin Med.* 2002;49(5):264-8.
- 316 [17] Kowalewski MP, Beceriklisoy HB, Pfarrer C, Aslan S, Kindahl H, Kücükaslan I, et al. Canine
317 placenta: a source of prepartal prostaglandins during normal and antiprogestin-induced parturition.
318 *Reproduction* 2010;139:655-64.
- 319 [18] Mason JI, France JT, Magness RR, Murry BA & Rosenfeld CR. Ovine placental steroid 17 alpha-
320 hydroxylase/C-17,20-lyase, aromatase and sulphatase in dexamethasone-induced and natural
321 parturition. *J Endocrinol* 1989;122:351–359.
- 322 [19] Kaludjerovic J and Ward WE The interplay between estrogen and fetal adrenal cortex *Journal of*
323 *Nutrition and Metabolism* 2012, Article ID 837901, 12 pages
- 324 [20] Legacki EL, Ball BA, Corbin CJ, Loux SC, Scoggin KE, Stanley SD, Conley AJ. Equine fetal
325 adrenal, gonadal and placental steroidogenesis. *Reproduction* 2017;154(4):445-54.
- 326 [21] Flint AP, Kingston EJ, Robinson JS, Thorburn GD. Initiation of parturition in the goat:
327 evidence for control by foetal glucocorticoid through activation of placental C21-steroid
328 17alpha hydroxylase. *J Endocrinol* 1978;78:367–78.
- 329 [22] Ryan KJ. Endocrine control of gestational length. *Am J Obstet Gynec* 1971;109:299-306.

- 330 [23] Nishiyama T, Tsumagari S, Ito M, Kimura J, Watanabe G, Taya K, et al. Immunohistochemical
331 study of steroidogenic enzymes in the ovary and placenta during pregnancy in the dog. *Anat Histol*
332 *Embryol* 1999;28:125–9.
- 333 [24] Chakraborty P.K. Reproductive hormone concentrations during estrus, pregnancy, and
334 pseudopregnancy in the Labrador bitch. *Theriogenology* 1987;27:827-40.
- 335 [25] Concannon PW, Castracane VD. Serum androstenedione and testosterone concentrations during
336 pregnancy and nonpregnant cycles in dogs. *Biol Reprod.* 1985;33(5):1078-83.
- 337 [26] Concannon PW, Butler WR, Hansel W, Knight PJ, Hamilton JM. Parturition and lactation in the
338 bitch: serum progesterone, cortisol and prolactin. *Biol Reprod.* 1978;19(5):1113-8.
- 339 [27] Olsson K, Bergström A, Kindahl H, Lagerstedt AS. Increased plasma concentrations of
340 vasopressin, oxytocin, cortisol and the prostaglandin F2alpha metabolite during labour in the dog. *Acta*
341 *Physiol Scand* 2003;179:1–7.
- 342 [28] Dörr HG, Heller A, Versmold HT, Sippell WG, Herrmann M, Bidlingmaier F, Knorr D.
343 Longitudinal study of progestins, mineralocorticoids, and glucocorticoids throughout human
344 pregnancy. *J Clin Endocrinol Metab.* 1989;68(5):863-8.
- 345 [29] Gram A, Trachsel A, Boos A, Kowalewski MP. Elevated utero/placental GR/NR3C1 is not
346 required for the induction of parturition in the dog. *Reproduction* 2016;152:303-11.
- 347 [30] Ledoux F, Genest J, Nowaczynski W, Kuchel O, Lebel M. Plasma progesterone and
348 aldosterone in pregnancy. *Can Med Assoc J.* 1975;112(8):943-7.
- 349 [31] Golden DL, Lothrop CD Jr. A retrospective study of aldosterone secretion in normal and
350 adrenopathic dogs. *J Vet Intern Med.* 1988;2(3):121-5.

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356 Figure 1. Box-plot representation of the impact of litter size (<4, 4-8, >8 pups) on aldosterone
357 (p=0.02) and etiocholanolone (p=0.01) concentration levels across N samples. The top and bottom
358 of each box are the 25th and 75th percentiles, and the line inside the box is the median concentration
359 levels. The top whiskers are the minimum and maximum sample levels, excluding outliers, which are
360 represented by filled rounds.

361 Figure 2. Box-plot representation of the impact of the peripartum period ranging from -4 to +2 d
362 from parturition (=day 0) on progesterone (p<0.001), 17- α hydroxyprogesterone (p=0.0002),
363 testosterone (p<0.001), and cortisone (p=0.006) concentration levels across N samples. The top and
364 bottom of each box are the 25th and 75th percentiles, and the line inside the box is the median
365 concentration levels. The top whiskers are the minimum and maximum sample levels, excluding
366 outliers, which are represented by filled rounds.

367 Table 1. Median serum concentration and inter-quartile range of the steroids in the four days
368 preceding parturition, on parturition day (day 0) and corticosteroids, androgens, progestogens and
369 estrogens in the two peripartum period (-4 days; +2 days following; day 0= parturition) in bitches
370 with normal pregnancies.

371 * Indicates the significant effect of time in the concentration trend of the corresponding hormone in
372 the row, p<0.05.

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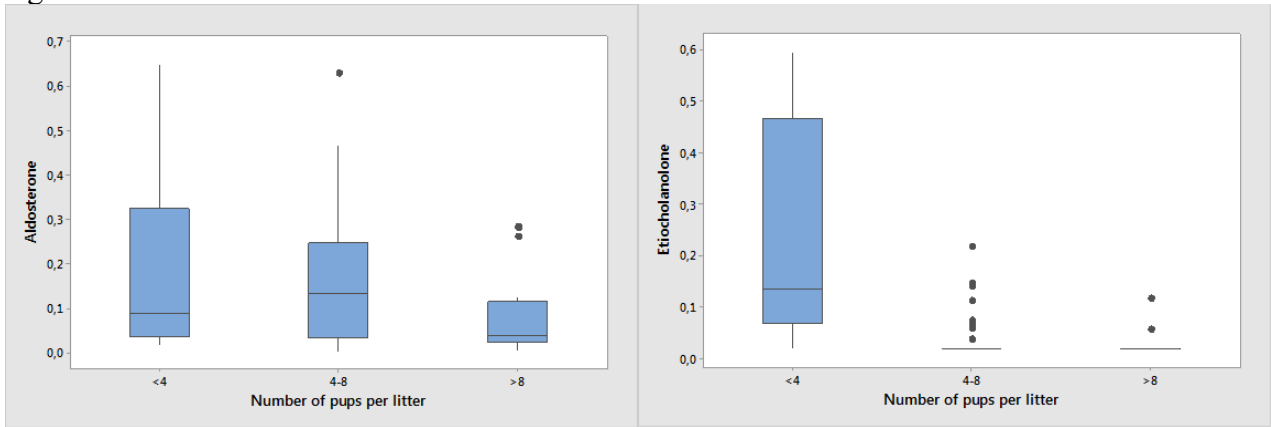
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385 Figure 1.



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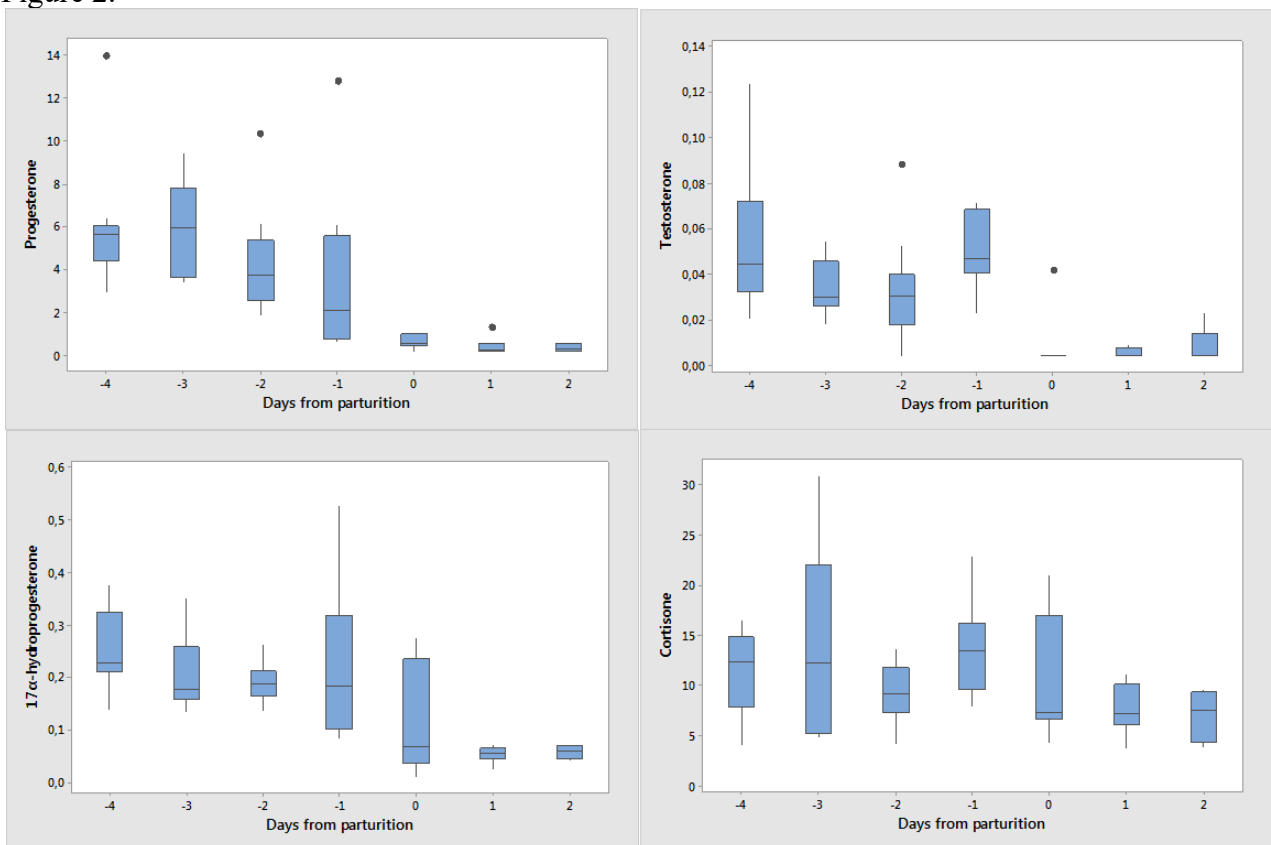
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Figure 2.



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	Day -4 n = 9	Day -3 n = 9	Day -2 n = 10	Day -1 n = 8	Day 0 n = 7	Day 1 n = 8	Day 2 n = 6
Corticosteroids (ng/ml)							
Aldosterone	0,06 (0,04-0,2)	0,03 (0,02-0,2)	0,1 (0,03- 0,3)	0,1 (0,02- 0,2)	0,1 (0,04- 0,5)	0,05 (0,03-0,3)	0,02 (0,004- 0,3)
Cortisol	34.5 (22- 70.5)	29.7 (13.7- 37.1)	22.2 (16.0- 30.8)	42.6 (19.2- 73.2)	37.1 (16.4- 47.8)	18.8 (15.4- 24.6)	25.5 (16.3- 32.0)
Cortisone*	12.3 (7.8- 14.8)	12.2 (5.2- 22.0)	9.1 (7.3- 11.8)	13.4 (9.6- 16.3)	7.3 (6.6- 17.0)	7.2 (6.0- 10.1)	7.5 (4.3- 9.4)
Corticosterone	1,9 (1,0- 3,5)	1,3 (0,9- 1,6)	1,1 (1,0- 1,5)	2,2 (1,2- 4,8)	1,2 (1,0- 4,5)	0,9 (0,5- 1,0)	0,9 (0,6- 1,9)
11-deoxycorticosterone	0,9 (0,7- 1,1)	1,0 (0,9- 1,5)	0,9 (0,6- 1,7)	1,4 (0,9- 2,0)	1,1 (0,6- 2,9)	1,2 (0,9- 1,8)	1,0 (0,5- 1,5)
11-deoxycortisol	2,5 (1,5- 4,5)	1,9 (1,5- 3,4)	1,4 (0,9- 2,1)	5,0 (1,7- 6,8)	1,4 (0,8- 8,3)	1,2 (0,7- 1,8)	1,4 (0,6- 1,6)
Androgens (ng/ml)							
Androsterone	0.02 (0.02- 0.04)	0.02 (0.02- 0.06)	0.02 (0.02- 0.05)	0.02 (0.02- 0.02)	0.02 (0.02- 0.02)	0.02 (0.02- 0.04)	0.02 (0.02-0.1)
Androstenedione	0.1 (0.05- 0.2)	0.2 (0.06- 0.6)	0.1 (0.03- 0.9)	0.2 (0.1- 0.4)	0.03 (0.03- 0.06)	0.03 (0.03-0.1)	0.06 (0.03-0.2)
Dehydroepiandrosterone	0.09 (0.02-0.3)	0.2 (0.2- 0.4)	0.2 (0.1- 0.2)	0.3 (0.05- 0.4)	0.3 (0.1- 0.7)	0.2 (0.1- 0.2)	0.1 (0.1- 0.3)
Etiocholanolone	0.02 (0.02-0.1)	0.02 (0.02- 0.02)	0.02 (0.02- 0.06)	0.02 (0.02-0.2)	0.02 (0.02- 0.08)	0.02 (0.02- 0.02)	0.02 (0.02- 0.09)
Testosterone*	0.04 (0.03- 0.07)	0.03 (0.03- 0.05)	0.03 (0.02- 0.04)	0.05 (0.04- 0.07)	0.005 (0.004- 0.004)	0.005 (0.004- 0.008)	0.005 (0.004- 0.01)
Progestogens (ng/ml)							
17 α -hydroxyprogesterone*	0.2 (0.2- 0.3)	0.2 (0.2- 0.3)	0.2 (0.2- 0.2)	0.2 (0.1- 0.3)	0.07 (0.04-0.2)	0.06 (0.04- 0.07)	0.06 (0.04- 0.07)
Pregnanolone	0.08 (0.04-0.1)	0.09 (0.05-0.1)	0.05 (0.03- 0.07)	0.05 (0.03- 0.06)	0.05 (0.008- 0.07)	0.008 (0.008- 0.02)	0.01 (0.008- 0.02)
Pregnenolone	3.2 (2.1- 8.1)	4.1 (2.1- 5.5)	1.9 (1.1- 2.7)	3.2 (2.1- 8.4)	2.4 (1.6- 6.2)	2.2 (1.0- 4.2)	3.2 (0.8- 3.9)
Progesterone*	5.6 (4.4- 6.1)	5.9 (3.6- 7.8)	3.7 (2.5- 5.3)	2.1 (0.8- 5.5)	0.5 (0.4- 1.0)	0.2 (0.2- 0.5)	0.3 (0.2- 0.5)
Estrogens (ng/ml)							
Estrone	0.05 (0.004- 0.08)	0.05 (0.03-0.1)	0.02 (0.004- 0.08)	0.06 (0.04-0.1)	0.06 (0.03-0.1)	0.006 (0.004- 0.05)	0.06 (0.004- 0.1)
Estradiol	0.4 (0.005- 0.5)	0.1 (0.004- 0.6)	0.02 (0.002- 0.8)	0.1 (0.05- 0.2)	0.1 (0.002- 0.1)	0.06 (0.004- 0.1)	0.002 (0.002- 0.6)

398 *: Significant effect of time