Serum concentration of mineralocorticoids, glucocorticoids, and sex steroids in peripartum bitches

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

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Abstract

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

Keywords

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

1. Introduction

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

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

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

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

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

2.Materials and Methods

2.1 Animals and samples

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

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

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

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

2.2 Hormone analysis

An analysis of steroid hormones was performed by supercritical fluid chromatography–tandem mass spectrometry (SFC–MS/MS) on an Acquity UPC^2 (Waters Corporation, Milford, MA, USA) system coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The SFC system was equipped with a binary solvent delivery pump, an autosampler, a column oven and a back pressure regulator. Separation of the seventeen steroids: androgens [androsterone, androstenedione, dehydroepiandrosterone (DHEA), etiocholanolone, testosterone]; corticosteroids (aldosterone, cortisol, cortisone, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol); estrogens (estrone and estradiol), and progestins (17α-Hydroxyprogesterone, pregnanolone, pregnenolone, progesterone) was accomplished within Acquity UPC^2 BEH column (150 mm × 3.0 mm, 1.7 μm particle size; Waters, Milford, MA, USA). It was kept at 40 °C and at a mobile phase flow rate of 2 mL/min. The gradient program was started with 98% A (CO_2) and 2% B (0.1% formic acid in methanol/isopropanol (1:1)), linearly increased to 17% B over 3 min, held at 17% B for 0.5 min, followed by a linear gradient down to 2% B over 0.5 min. Finally it was held for 1 min at 2% B for the elution of ionic liquids out of the instrument, resulting in a total separation time of 5 min. The back pressure was set to 1500 psi and the injection volume was 1.0 μL. Elution from the SFC system into the MS system was aided by a make-up solvent (0.1% formic acid in methanol) at a flow rate of 0.4 mL/min. Mass spectrometric detection was performed using electrospray ionization in the positive ionization mode (ESI+) with a capillary voltage of 2.8 kV, cone voltage of 30 V, and source offset of 30 V. Nitrogen 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

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

3. Results

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

5. Conclusion

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

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

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Credits to authors

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

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

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

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

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

Figure 1.

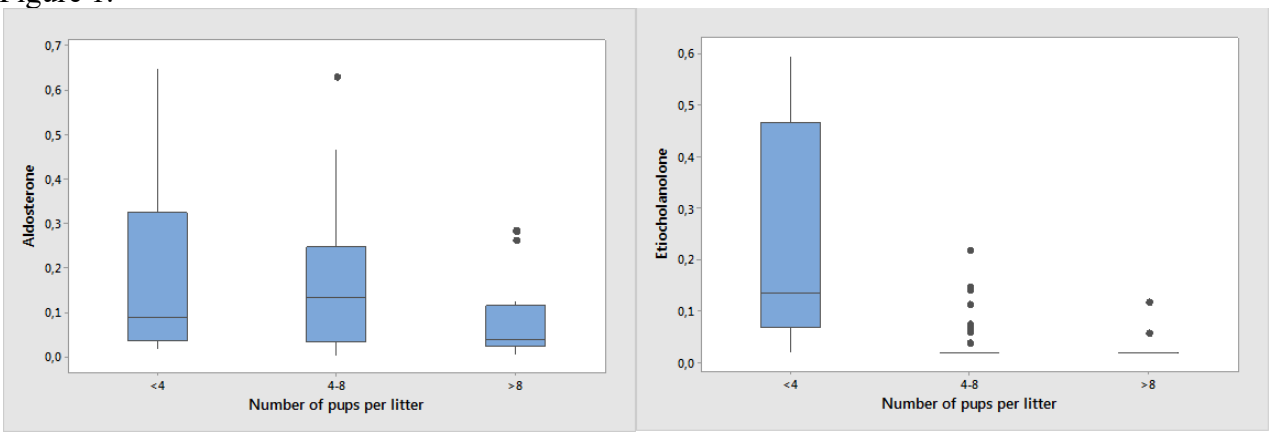
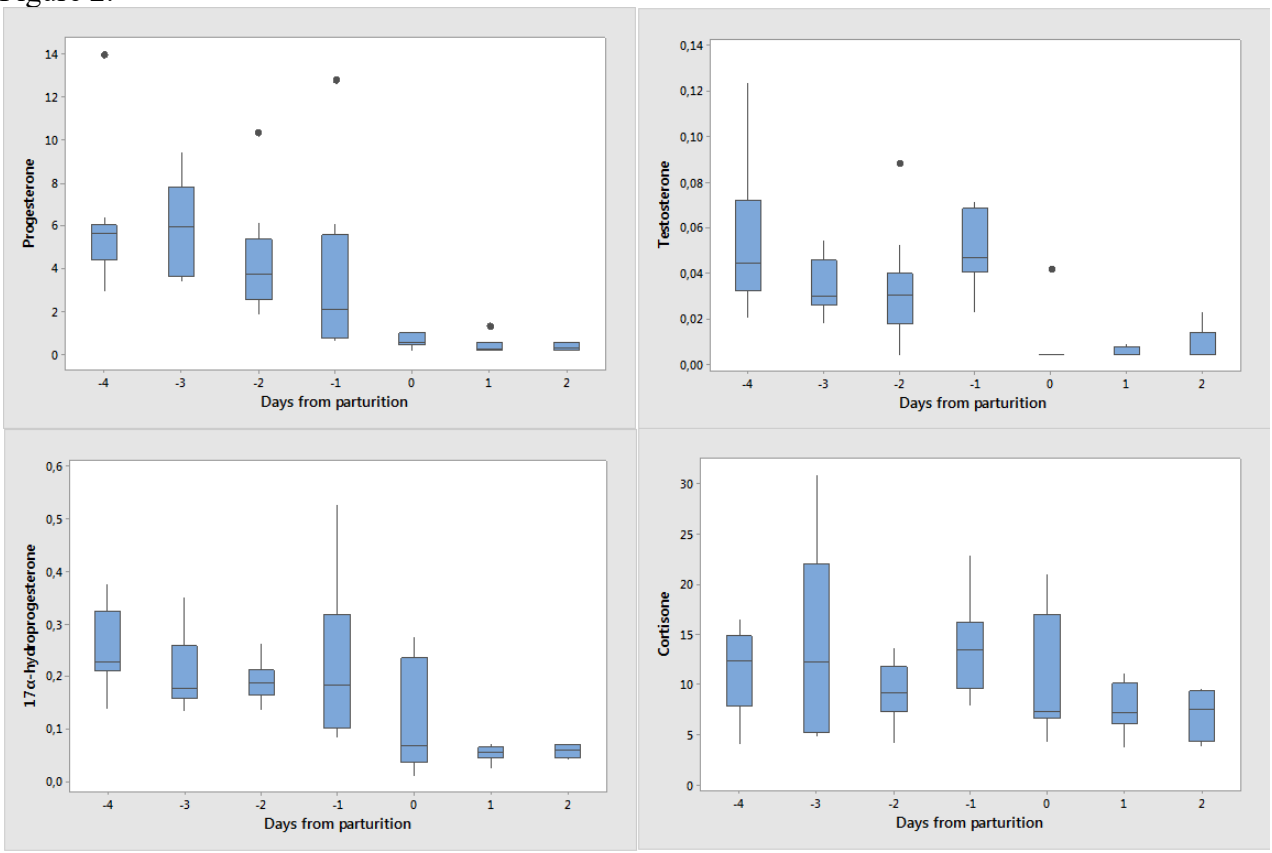


Figure 2.



396 Table 1.

397

	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)							
17α-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