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1                    **Evolution of steroid concentrations in saliva from immature to pubertal gilts**  
2                    **for the identification of biomarkers of gilts receptivity to boar effect.**

3

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23

24 **Short title:** Salivary steroidome analysis in gilts

25

26 **Abstract**

27 Estrus synchronization is necessary for management of gilt reproduction in pig farms. It is usually  
28 achieved by using synthetic progestagens, but there is increasing demand for non-hormonal  
29 alternative tools with the prospect of sustainability of livestock production. Moreover, in organic  
30 farms, synthetic hormones are not allowed. Before reaching puberty, gilts exhibit a “waiting period”  
31 during which external stimulations, such as boar exposure, could trigger and synchronize the first  
32 ovulation. However, practical non-invasive tools for detection of the “waiting period” in pig farms  
33 are lacking. During this period, estrone levels in urine are high, but urine sampling is difficult in  
34 group-housed females. Our objective was to identify among steroids potential biomarkers of this  
35 “waiting period” through saliva monitoring from immature to pubertal gilts using gas  
36 chromatography coupled to tandem mass spectrometry.

37 Starting between 144 to 147 days of age, six Large White gilts were submitted to ultrasound  
38 puberty diagnosis 3 times a week until first ovulation. Urine and saliva samples were collected to  
39 analyze weekly estrone and steroidome respectively, until puberty. Urinary estrone concentration  
40 significantly increased 2 weeks before first ovulation occurring between 182 and 192 days of age.  
41 The period with increasing estrone levels was considered as the “waiting period”. Steroidome  
42 analysis allowed identifying and quantifying 28 steroids in 500  $\mu$ l of gilts saliva. Significant  
43 decrease of dehydroepiandrosterone and significant increase of 5 $\alpha$ -dihydroprogesterone and 17 $\beta$ -  
44 estradiol were detected 2 weeks before puberty, suggesting that these steroids could be potential  
45 biomarkers of the “waiting period”.

46 These results show that painless sampling of saliva could be a non-invasive welfare-friendly tool for  
47 the identification of the physiological hormonal status of the gilts and possibly the optimal time for  
48 application of the boar effect, a solution to synchronize puberty without exogenous hormones.

49  
50 **Keywords:** steroidome, saliva, puberty, porcine, male effect, GC-MS/MS

51  
52 **Introduction**

53 In pig farms, batch management of sows has developed extensively due to advantages in labor  
54 planning, possibilities of piglet adoption when litter size is excessive, all-in all-out practices and  
55 health management. The importance of the control of estrous cycle has increased in recent years  
56 because batch management requires groups of service-ready females for artificial insemination at  
57 planned times. Use of synthetic progestagens alone or in combination with gonadotropins has been  
58 commonly used to synchronize estrous cycle in gilts in swine farms. However, the wide use of  
59 synthetic progestagens is a source of steroids releasing into the environment (Liu et al., 2012a; Liu  
60 et al., 2012b; Liu et al., 2015). This results in contamination of environment by steroids and their  
61 metabolites from animal manure and urine. Thus, our aim is to develop an alternative strategy for  
62 hormonal pharmacological tools for estrous synchronization in gilts.

63 Regular boar contact, called the boar effect, is able to stimulate and synchronize puberty attainment  
64 in gilts, although its success has been shown to be variable (Hughes et al., 1990). Part of this  
65 variability can be linked to differences in sexual maturity, with an effect of age and growth rate of  
66 the gilts, breed, season, and physiological stage of the gilts at onset of boar exposure (Hughes et al.,  
67 1990; Paterson et al., 1991; Kemp et al., 2005; van Wettere et al., 2006; Amaral Filha et al., 2009;  
68 Magnabosco et al., 2014). Before puberty, gilts exhibit a “waiting period”, defined by a relatively  
69 high ovarian follicles development but relatively low gonadotrophin secretion (Camous et al., 1985;  
70 Prunier et al., 1993). During this “waiting period”, external stimulations, such as boar exposure,  
71 could induce and synchronize first ovulation (Hughes et al., 1990). Targeting more effectively the  
72 period when gilts are able to respond to boar exposure would enhance the efficacy of the boar  
73 effect. Thus, the identification of biomarkers of this receptivity period would allow the detection of  
74 optimal time for application of the boar effect in pre-pubertal gilts.

75 Steroid hormones are major actors of sexual maturation in mammals such as humans (Mauras et al.,  
76 1996; Mauras, 2001; Castellano and Tena-Sempere, 2016), ruminants (Kinder et al., 1995) and pig  
77 (Prunier et al., 1993; Kanematsu et al., 2006). Therefore, they could be relevant biomarkers of the  
78 receptivity period. An increase of urinary estrone levels has been observed during the “waiting

79 period” (Camous et al., 1985) and high urinary estrone concentrations could be biomarkers of the  
80 receptivity period. However, urine sampling is difficult in group-housed females. On the contrary,  
81 saliva can be sampled easily without pain or stress. Several reports have shown that steroid  
82 hormones could be detected in saliva in human (Manh et al., 2013; Kido et al., 2014; Majewska et  
83 al., 2014). In large non-human mammals, salivary concentrations of steroid hormones have never  
84 been analyzed, except for cortisol in bovine (Moya et al., 2014; Schwinn et al., 2018), equine  
85 (Diego et al., 2016; Schwinn et al., 2018), porcine (Devillers et al., 2004; Jama et al., 2016; Casal et  
86 al., 2017) and donkey (Rota et al., 2018) and testosterone in porcine (Escribano et al., 2015).

87 The combination of gas chromatography (GC) or liquid chromatography with mass spectrometry  
88 (MS) or tandem mass spectrometry (MS/MS) has provided reference methods for steroids analysis  
89 both in clinical studies and in fundamental research. Thanks to their analytical specificities, these  
90 technologies permit to reach extreme sensitivity down to the attomoles and have the advantage to  
91 accurately measure several steroids in a biological tissue or fluid of small size, and to establish  
92 extended steroid profiles. In particular, GC-MS/MS allows measuring a very large number of  
93 structurally similar steroids such as stereoisomers and enantiomers (Schumacher et al., 2015; Lamy  
94 et al., 2016; Zhu et al., 2017). We developed an analytical protocol, based on GC-MS/MS analysis  
95 coupled to a multi-step sample workup for screening and quantification of steroid hormones and  
96 their precursors and metabolites in small volumes of biological samples (Liere et al., 2004; Liere et  
97 al., 2009).

98 Thus, the aim of the present study was to identify potential salivary biomarkers of the receptivity  
99 period to boar effect through screening and quantification of steroids and their  
100 precursors/metabolites in saliva from immature to pubertal gilts using the GC-MS/MS analytical  
101 technology.

102

## 103 **Materials and methods**

### 104 ***Ethics approval statement***

105 All procedures on animals were performed in accordance with the “guiding principles for the care  
106 and use of animals in research facilities” from the French Ministry of Agriculture and Research.  
107 The protocol was approved by the ethics committee “Comité d’Ethique en Expérimentation  
108 Animale Val de Loire n° 19”.

### 109 *Animals, housing and sample collection*

110 This experiment was conducted from November to December in our experimental farm (PAO,  
111 INRA, Nouzilly, France). Nine Large-White 144 to 147-day-old gilts were penned in a group on a  
112 concrete floor without straw under natural daylight. They had free access to water and were fed  
113 with concentrate once a day (2.4 kg/animal/day; porc Elevage INRA, Sanders Ouest, 35370  
114 Etreilles, France; 16 % crude protein, 2 % crude fat content, 7.1 % cellulose, 6.9 % minerals, 0.62 %  
115 phosphore, 1.15 % calcium, 0.25 % sodium, 0.8 % lysine, 0.26 % methionine). No boar contact and  
116 no modification of the housing or feeding were performed during the experiment.

117 Starting at 144-147 days of age, trans-abdominal ultrasonography was carried out three times a  
118 week to assess the physiological reproductive status, immature versus pubertal, until puberty  
119 detection based on uterus and ovaries development, as previously described (Goudet et al., 2019).  
120 Females were considered pubertal when the ultrasonographic images were characteristic of cyclic  
121 females with well-defined sections of the uterus and ovulations were detected. Females were  
122 considered immature when the uterine images were dark and homogeneous.

123 Starting at 144-147 days of age, urinary and salivary samples were collected in the morning before  
124 feeding (7 o'clock), three times a week, on the day of ultrasonography, until puberty detection.  
125 Urine samples were collected using a container placed under the urine stream, when possible. Urine  
126 samples were obtained one of the two collection attempts. Saliva samples were collected using a  
127 cotton swab for saliva collection (Sarstedt Salivette® ref. 51.1534, Sarstedt, Nümbrecht, Germany)  
128 held gently with forceps in the gilt mouth that was chewing until it was soaked. Saliva samples were  
129 obtained at each collection attempt, 550 µl to 2.25 ml were collected. Urinary and saliva samples

130 were brought to the lab on ice, centrifuged at 3000 g for 5 min at 4°C, within 30 minutes after  
131 collection. The recovered saliva and urine were aliquoted and stored at -80°C until analysis.

132 Gilts were slaughtered 7 days after ultrasonographic puberty detection for puberty confirmation and  
133 genital tract was carefully examined as previously described (Goudet et al., 2019).

134 Urine samples were used for urinary oestrone assay, as mentioned in a previous experiment (Goudet  
135 et al., 2019). Briefly, urinary oestrone concentrations were measured using the DetectX-Estrone-3-  
136 sulfate enzyme immunoassay kit (Arbor Assays) and urinary creatinine concentrations were  
137 measured using the Creatinine Assay kit (R&D Systems) in order to calculate the oestrone-to-  
138 creatinine ratio and take into account urine dilution.

139 Part of the saliva samples was used for metabolome analysis using <sup>1</sup>H-nuclear magnetic resonance  
140 spectroscopy in a previous experiment (Goudet et al., 2019). The other part was used in the present  
141 study for steroidome analysis using GC-MS/MS technology.

#### 142 *Steroids measurement by GC-MS/MS*

143 Steroids were extracted from the saliva (400-550 µl) of gilts with methanol. The saliva was spiked  
144 with all the following internal standards for steroid quantification: 2 ng of <sup>2</sup>H<sub>6</sub>-5α-DHP (CDN  
145 Isotopes, Sainte Foy la Grande, France) for 5α/β-dihydroprogesterone (5α/β-DHP) and 5α/β-  
146 dihydroandrostenedione (5α/β-ADIONE), 2 ng of <sup>2</sup>H<sub>4</sub>-pregnenolone sulfate (PREGS) for PREGS,  
147 5 ng of <sup>2</sup>H<sub>4</sub>-dehydroepiandrosterone sulfate (DHEAS) for DHEAS, 2 ng of <sup>13</sup>C<sub>3</sub>-testosterone  
148 (Isoscience, PA, USA) for testosterone, 2 ng of <sup>13</sup>C<sub>3</sub>-androstenedione (ADIONE) for ADIONE, 2  
149 ng of <sup>13</sup>C<sub>3</sub>-progesterone (P) for P, 2 ng of <sup>13</sup>C<sub>3</sub>-17β-estradiol for 17β-estradiol, 2 ng of <sup>13</sup>C<sub>3</sub>-estrone  
150 for estrone, 2 n of <sup>2</sup>C<sub>3</sub>-deoxycorticosterone (DOC) for DOC, 2 ng of 19nor-PROG for 20α-DHP  
151 and 5α-DHDOC (dihydrodeoxycorticosterone), 2 ng of epietiocholanolone (Steraloids, Newport,  
152 Rhode Island) for androstenediol (ADIOL), etiocholanolone, epiandrosterone, 5α-  
153 dihydrotestosterone (5α-DHT), 3α/β5α-tetrahydrotestosterone (THT), pregnenolone (PREG), 20α-  
154 dihydropregnenolone (20α-DHPREG) 3α/β5α/β-tetrahydroprogesterone (THP), 5α20α-THP,  
155 3α/β5α/β-hexahydroprogesterone (HHP) and 3α5α-THDOC, 2 ng of <sup>13</sup>C<sub>3</sub>-17α-OH PROG for 17α-

156 OH PROG, 17 $\alpha$ -OH PREG and 16 $\alpha$ -OH PROG, and 1 ng of <sup>2</sup>H<sub>5</sub>-2-methoxyestradiol (2-ME2) for  
157 2-ME2. Samples were purified and fractionated by solid-phase extraction with the recycling  
158 procedure (Liere et al., 2004; Liere et al., 2009). Briefly, the extracts were dissolved in 1 ml MeOH  
159 and applied to the C18 cartridge (500 mg, 6 ml, International Sorbent Technology, IST), followed  
160 by 5 ml of MeOH/H<sub>2</sub>O (85/15). The flow-through, containing the free and sulfated steroids, was  
161 collected and dried. After a previous re-conditioning of the same cartridge with 5 ml H<sub>2</sub>O, the dried  
162 samples were dissolved in MeOH/H<sub>2</sub>O (2/8) and re-applied. The cartridge was then washed with 5  
163 ml H<sub>2</sub>O and sulfated and unconjugated steroids were eluted with 5 ml MeOH/H<sub>2</sub>O (1/1) and 5 ml  
164 MeOH/H<sub>2</sub>O (9/1), respectively.

165 The unconjugated steroids-containing fraction was then filtered and further purified and  
166 fractionated by HPLC. The HPLC system was composed of a WPS-3000SL analytical autosampler  
167 and a LPG-3400SD quaternary pump gradient coupled with a SR-3000 fraction collector  
168 (Thermoscientific, USA). The HPLC separation was achieved with a Lichrosorb Diol column (25  
169 cm, 4.6 mm, 5  $\mu$ m) in a thermostated block at 30°C. The column was equilibrated in a solvent  
170 system of 90 % hexane and 10 % of a mixture composed of hexane/isopropanol (85/15). Elution  
171 was performed at a flow-rate of 1 ml/min, first with 90 % hexane and 10 % of hexane/isopropanol  
172 (85/15) for 8 min, then with a linear gradient to 100 % of hexane/isopropanol (85/15) in 2 min. This  
173 mobile phase was kept constant for 10 min and a linear gradient to 100 % MeOH was applied. The  
174 column was washed with MeOH for 15 min.

175 Two fractions were collected from the HPLC system: 5 $\alpha$ / $\beta$ -DHPRG and 5 $\alpha$ / $\beta$ -ADIONE were  
176 eluted in the first HPLC fraction (3-13 min) and were next silylated with 50  $\mu$ l of a mixture N-  
177 methyl-N-trimethylsilyltrifluoroacetamide/ammonium iodide/dithioerythritol (1000:2:5 vol/wt/wt)  
178 for 15 min at 70°C. The second fraction (13-25 min) containing PREG, 20 $\alpha$ -DHPREG, P, DOC,  
179 testosterone and their precursors and reduced metabolites, 17 $\beta$ -estradiol and estrone, was  
180 derivatized with 25  $\mu$ l heptafluorobutyric anhydride (HFBA) and 25  $\mu$ l anhydrous acetone for 1h at  
181 20°C. Both fractions were dried under a stream of N<sub>2</sub> and resuspended in hexane.



182 GC-MS/MS analysis of the extracts was performed using an AI 1310 autosampler, a Trace 1310 gas  
183 chromatograph (GC), and a TSQ 8000 tandem mass spectrometer (MS/MS) (Thermo Fisher  
184 Scientific San Jose, CA) using Argon as collision gas. Injection was performed in the splitless mode  
185 at 250°C (1 min of splitless time). The temperature of the gas chromatograph oven was initially  
186 maintained at 50°C for 1 min and ramped between 50 to 200°C at 20°C/min, then ramped to 300°C  
187 at 5°C/min and finally ramped to 350°C at 30°C/min. The helium carrier gas flow was maintained  
188 constant at 1 ml/min during the analysis. The transfer line and ionization chamber temperatures  
189 were 330°C and 180°C, respectively. Electron impact ionization was used for mass spectrometry  
190 with ionization energy of 70 eV. GC-MS/MS signals were evaluated using a computer workstation  
191 by means of the software Excalibur<sup>®</sup>, release 3.0 (ThermoScientific, USA). Identification of steroids  
192 was supported by their retention time and according two or three transitions. The precise retention  
193 time and the MS/MS parameters of the 35 targeted steroids in gilt saliva are reported in Table 1.  
194 Quantification was performed according to the transition giving the more abundant product ion with  
195 a previously established calibration curve. The calibration curves were prepared by using 100 µl of  
196 gilt saliva by adding increasing concentrations of pure targeted steroids (5 calibration points with  
197 steroids concentrations ranging from 0.100 to 5 ng/ml) with fixed amounts of internal standards, as  
198 described in the analytical work up. Then, steroids were extracted, purified, fractionated and  
199 derivatized according to the procedure described in the paper. For each calibration curve, a  
200 regression line was calculated using least squares methodology. The calibration curve is considered  
201 as satisfactory when the concentrations of the standards were within 15 % of the nominal  
202 concentration.

203 The analytical protocol has been validated for all the targeted steroids by using 1 ml from a pool of  
204 male rat plasma. The parameters of validation included the limit of detection, linearity, accuracy,  
205 intra- and inter-assay precisions and were all described by Lamy et al (Lamy et al., 2016). The limit  
206 of detection was determined as the lowest amount of compounds that can be measured by GC-  
207 MS/MS with a signal-to-noise ratio greater than 3. It ranged from 5 pg/ml to 0.05 pg/ml. The

208 linearity was assessed by analysing increasing amounts of rat plasma extracts (20, 50, 100 and 200  
209  $\mu$ l) in triplicate. The linearity was satisfactory for all the steroids with a coefficient of correlation  
210 ranging from 0.99 to 0.995. Five aliquots of 1 ml of rat plasma were used at two different times to  
211 determine the inter-assay coefficient of variation that was estimated at roughly 5-10 % for the  
212 targeted steroids. The accuracy of the assay was evaluated by determining the analytical recovery,  
213 which was defined as  $C/(C_0+S) \times 100(\%)$ . C is the concentration of the steroid in the spiked plasma  
214 extract (100  $\mu$ l),  $C_0$  is the concentration of a steroid in the unspiked plasma extract (100  $\mu$ l) and S is  
215 the spiked concentration. The accuracy was in the range of 94-106 %.

216 Stable isotope dilution analysis combined with extensive sample purification/fractionation and  
217 derivatization, sample dilution and the highly specific GC/MS/MS analysis (high resolution gas  
218 chromatography coupled to multiple reaction monitoring) are satisfactory to avoid any potential  
219 matrix effects with gilt saliva for steroid analysis (Anastassiades et al., 2003).

#### 220 *Statistical analysis*

221 The comparison of concentrations of steroids determined by GC-MS/MS between weeks was  
222 performed using repeated measures one-way ANOVA, followed by a post-hoc Tukey's multiple  
223 comparisons test, using GraphPad Prism version 6 (GraphPad Software, La Jolla California, USA).

224

## 225 **Results**

### 226 *Uterine tract development and urinary estrone levels*

227 As mentioned in our previous experiment (Goudet et al., 2019), six gilts presented first pubertal  
228 ultrasonic images of the uterus and ovary at 182, 189, 190, 190, 191 and 192 days of age (day of  
229 puberty detection) and puberty was ascertained at slaughter. Two other gilts were still pre-pubertal  
230 at slaughter, and first ovulation was missed for one gilt. Only samples from the six gilts whose  
231 onset of puberty was detected at 182 to 192 days were analyzed.

232 The period from 144-147 days of age to the day of puberty detection (Day 0) was divided into 5  
233 weeks named week-5 to week-1 relatively to the day of puberty detection. For each week, one of

234 the three collected samples was chosen for analyses, so that both urinary and saliva were available  
235 on the same day. Then, for each gilt, six salivary samples were analyzed: one from each week, from  
236 week-5 to week-1, and one from the day of puberty detection. The mean age of the gilts ( $\pm$  sem) on  
237 the day of saliva sampling was 154 days ( $\pm$  0.9) for week-5, 163 days ( $\pm$  0.5) for week-4, 170 days  
238 ( $\pm$  0.5) for week-3, 178 days ( $\pm$  0.5) for week-2, 186 days ( $\pm$  1.4) for week-1, and 189 days ( $\pm$  1.5)  
239 for the day of puberty detection.

240 As mentioned in our previous experiment (Goudet et al., 2019), mean urinary estrone  
241 concentrations  $\pm$  sem were  $12.9 \pm 2.5$  ng/ml (week-5),  $10.4 \pm 2.7$  ng/ml (week-4),  $13.4 \pm 4.1$  ng/ml  
242 (week-3),  $12.7 \pm 2.3$  ng/ml (week-2),  $62.5 \pm 31.1$  ng/ml (week-1) and  $121.3 \pm 63.1$  ng/ml on the day  
243 of puberty detection (Day 0). The estrone-to-creatinine ratio was low from week-5 to week-2 and  
244 increased significantly ( $P < 0.05$ ) from week-2 to the day of puberty detection. The period with  
245 increasing estrone levels was considered as the “waiting period”.

#### 246 *Salivary steroidome analysis*

247 Steroids concentrations were analyzed in six salivary samples from the six gilts, from week-5 to the  
248 day of puberty detection. Among the 35 targeted steroids, GC-MS/MS analysis allowed the  
249 detection of 28 steroids in 500  $\mu$ l of saliva (Table 2). Seven targeted steroids were not detected in  
250 saliva :  $17\alpha$ -OH PREG,  $16\alpha$ -OH PROG,  $17\alpha$ -OH PROG,  $5\alpha$ -DHDOC,  $3\alpha5\alpha$ -THDOC,  $3\beta5\alpha$ -  
251 THT, 2-ME2 (Table 2).

252 The salivary concentration of pregnenolone, the precursor of all steroid hormones in mammals,  
253 tended to slightly increase from  $0.9 \pm 0.16$  ng/ml (week-5 and week-4) to  $1.47 \pm 0.15$  ng/ml (week-  
254 1) but without reaching statistical significance (Figure 1). Both direct metabolites of pregnenolone,  
255 pregnenolone sulfate (PREGS) and  $20\alpha$ -dihydropregnenolone ( $20\alpha$ -DHPREG), were also detected  
256 in gilts saliva. PREGS concentrations showed important but non-significant variations ranging from  
257  $0.67 \pm 0.19$  to  $2.08 \pm 0.53$  ng/ml, whereas  $20\alpha$ -DHPREG concentrations were lower and showed  
258 little variations around 0.1 ng/ml (Figure 1).

259 Dehydroepiandrosterone (DHEA) is also a metabolite of pregnenolone. Its concentrations showed a  
260 significant decrease from week-3 ( $0.26 \pm 0.02$  ng/ml) to week-2 ( $0.11 \pm 0.01$  ng/ml,  $p < 0.01$ ), to  
261 week-1 ( $0.12 \pm 0.01$  ng/ml,  $p < 0.01$ ) and to the day of puberty detection ( $0.09 \pm 0.01$  ng/ml,  $p <$   
262  $0.01$ ) (Figure 1). DHEA sulfate concentrations tended to decrease from week-5 ( $0.58 \pm 0.15$  ng/ml)  
263 to week-4 ( $0.22 \pm 0.03$  ng/ml) and then tended to increase progressively to the day of puberty  
264 detection ( $0.70 \pm 0.22$  ng/ml) (Figure 1).

265 Concentrations of deoxycorticosterone, progesterone and some of its reduced metabolites ( $3\alpha5\alpha$ -  
266 THPROG,  $3\beta5\alpha$ -THPROG,  $5\alpha20\alpha$ -THPROG,  $3\alpha5\alpha20\alpha$ -HHPROG,  $3\alpha5\beta20\alpha$ -HHPROG,  
267  $3\beta5\alpha20\alpha$ -HHPROG) were lower than 0.2 ng/ml in saliva, whereas concentrations of the other  
268 progesterone metabolites ( $5\alpha$ -DHPROG,  $5\beta$ -DHPROG,  $20\alpha$ -DHPROG,  $3\alpha5\beta$ -THPROG) ranged  
269 between 0.1 and 3.9 ng/ml (Figures 2 and 3). The concentrations of progesterone and all of its  
270 reduced metabolites (dihydro-, tetrahydro- and hexahydroprogesterone) tended to decrease between  
271 week-5 and week-4. Then progesterone levels tended to increase between week-3 and the day of  
272 puberty detection and most of its metabolites showed similar pattern ( $5\alpha$ -DHPROG,  $5\beta$ -DHPROG,  
273  $3\alpha5\beta$ -THPROG,  $3\beta5\alpha$ -THPROG,  $5\alpha20\alpha$ -THPROG,  $3\alpha5\alpha20\alpha$ -HHPROG,  $3\alpha5\beta20\alpha$ -HHPROG,  
274  $3\beta5\alpha20\alpha$ -HHPROG). Concentrations of  $5\alpha$ -DHPROG were significantly different between week-3  
275 ( $0.32 \pm 0.05$  ng/ml) and week-2 ( $0.66 \pm 0.11$  ng/ml,  $p < 0.05$ ) as well as between week-4 ( $0.32 \pm$   
276  $0.06$  ng/ml) and week-1 ( $1.06 \pm 0.16$  ng/ml) (Figure 2). Concentrations of  $3\beta5\alpha$ -THPROG were  
277 significantly different between week-1 vs week-4 (Figure 3,  $p < 0.05$ ). Concentrations of  $5\alpha20\alpha$ -  
278 THPROG were significantly different between week-2 vs week-4 (Figure 3,  $p < 0.05$ ).

279 The concentrations of androgens androstenediol, androstenedione,  $5\alpha$ -ADIONE,  $5\beta$ -ADIONE,  
280 epiandrosterone and etiocholanolone ranged from 0.01 to 0.6 ng/ml. Except for androstenediol, all  
281 of these androgens showed a similar pattern. They tended to decrease from week-5 to week-4 and  
282 increase gradually to the day of puberty detection (Figure 4). Androstenediol tended to increase  
283 from week-5 to week-1 and decrease on the day of puberty detection (Figure 4).

284 Testosterone levels were low and did not change significantly with time as for its reduced  
285 metabolites 5 $\alpha$ -DHT and 3 $\alpha$ 5 $\alpha$ -THT (Figure 5).

286 Estrone salivary levels significantly decreased from week-5 ( $0.079 \pm 0.012$  ng/ml) to week-4 ( $0.015$   
287  $\pm 0.007$  ng/ml,  $p < 0.01$ ) and increased progressively to the day of puberty detection ( $0.063 \pm 0.021$   
288 ng/ml) (Figure 5). The concentrations of the most potent estrogen 17 $\beta$ -estradiol were low from  
289 week-5 ( $0.035 \pm 0.006$  ng/ml) to week-3 ( $0.011 \pm 0.001$  ng/ml), significantly higher on week-2  
290 ( $0.047 \pm 0.005$  ng/ml,  $p < 0.01$ ) and week-1 ( $0.055 \pm 0.007$  ng/ml,  $p < 0.01$ ) and decreased on the  
291 day of puberty detection ( $0.026 \pm 0.006$  ng/ml) (Figure 5).

292 When combining progesterone and its 5 $\alpha$ / $\beta$ -reduced metabolites (5 $\alpha$ -DHPROG + 5 $\beta$ -DHPROG +  
293 20 $\alpha$ -DHPROG + 3 $\alpha$ ,5 $\alpha$ -THPROG + 3 $\alpha$ ,5 $\beta$ -THPROG + 3 $\beta$ ,5 $\alpha$ -THPROG + 5 $\alpha$ ,20 $\alpha$ -THPROG +  
294 3 $\alpha$ ,5 $\alpha$ ,20 $\alpha$ -HHPROG + 3 $\alpha$ ,5 $\beta$ ,20 $\alpha$ -HHPROG + 3 $\beta$ ,5 $\alpha$ ,20 $\alpha$ -HHPROG), concentrations tended to  
295 decrease between week-5 ( $3.48 \pm 0.92$  ng/ml) and week-4 ( $0.89 \pm 0.17$  ng/ml), remained low on  
296 week-3 ( $1.58 \pm 0.33$  ng/ml), and increased progressively from week-2 ( $3.47 \pm 0.98$  ng/ml) to the  
297 day of puberty detection ( $8.44 \pm 2.9$  ng/ml) (Figure 6). A similar pattern was observed when the 5 $\alpha$ -  
298 reduced metabolites of progesterone were pooled (5 $\alpha$ -DHPROG + 3 $\alpha$ ,5 $\alpha$ -THPROG + 3 $\beta$ ,5 $\alpha$ -  
299 THPROG + 5 $\alpha$ ,20 $\alpha$ -THPROG + 3 $\alpha$ ,5 $\alpha$ ,20 $\alpha$ -HHPROG + 3 $\beta$ ,5 $\alpha$ ,20 $\alpha$ -HHPROG) with a significant  
300 increase from week-3 ( $0.42 \pm 0.07$  ng/ml) to week-2 ( $0.81 \pm 0.12$  ng/ml,  $p < 0.05$ ), to week-1 ( $1.26$   
301  $\pm 0.19$  ng/ml) and to the day of puberty detection ( $1.95 \pm 0.67$  ng/ml). The salivary concentrations  
302 of 5 $\beta$ -metabolites of progesterone (5 $\beta$ -DHPROG + 3 $\alpha$ ,5 $\beta$ -THPROG + 3 $\alpha$ ,5 $\beta$ ,20 $\alpha$ -HHPROG) also  
303 showed the same tendency with increasing levels from week-3 ( $0.45 \pm 0.14$  ng/ml) to the day of  
304 puberty detection ( $5.22 \pm 1.8$  ng/ml) (Figure 6).

305

## 306 Discussion

307 Studies about endocrine events during sexual maturation often include analyses of changes in the  
308 concentrations of steroid hormones in plasma (Prunier et al., 1993; Kanematsu et al., 2006; Noguchi  
309 et al., 2010). However, blood sampling is invasive, stressful and difficult in the pig. On the

310 contrary, saliva sampling is an interesting alternative since it is easy to collect, without pain or  
311 stress. Steroid hormones enter saliva by different mechanisms depending on whether they are  
312 charged or neutral, free or bound to proteins. Furthermore, the presence of some steroidogenic  
313 enzymes in saliva may complicate the relationship between the salivary and plasma steroids. Thus,  
314 salivary steroid levels do not always reflect plasma levels (Lewis, 2006). However, some reports  
315 have shown that the determination of progesterone,  $17\alpha$ -hydroxyprogesterone and  $17\beta$ -estradiol in  
316 saliva may be useful for assessing the ovarian function and in particular, salivary samples have been  
317 shown to allow the differentiation between the follicular and luteal phase in women (Groschl et al.,  
318 2001; Bao et al., 2003). Saliva sampling might have some disadvantages such as lower hormones  
319 concentrations compared to blood or urine, but this inconvenience can be solved by highly sensitive  
320 detection techniques. Gas chromatography coupled to tandem mass spectrometry (GC-MS/MS)  
321 associated with a well-validated extraction, fractionation and purification procedure is a sensitive,  
322 precise and selective technique suitable for quantifying numerous steroid hormones and their  
323 precursors and metabolites in small volumes of biological fluids with high sensitivity and accuracy  
324 (Liere et al., 2004; Liere et al., 2009). The analytical protocol used in this study was validated by  
325 using mice brain extracts which represent the more challenging complex matrix in an analytical  
326 point of view (Liere and Schumacher, 2015; Schumacher et al., 2015). This protocol included the  
327 addition of stable isotope internal standards in the extraction step in the beginning of the analytical  
328 procedure combined with subsequent multiple purification and fractionation steps such as Solid  
329 Phase Extraction and High Performance Liquid Chromatography in order to have a very reliable  
330 protocol in terms of steroids identification and quantification whatever the studied biological  
331 matrix: brain, plasma (Zhu et al., 2017), oviductal fluid (Lamy et al., 2016), saliva. Many groups  
332 having a great expertise in the analytical field have reported that the matrix effect could be bypassed  
333 by using highly specific GC-MS/MS stable isotope dilution analysis combined with upstream  
334 extensive sample purification/fractionation, derivatization and sample dilution (Schenck and  
335 Lehotay, 2000; Anastassiades et al., 2003; Mastovska et al., 2005; Chamkasem and Harmon, 2016)

336 indicating that our GC-MS/MS protocol was suitable and satisfactory to avoid any potential matrix  
337 effects with gilt saliva for steroid analysis (Anastassiades et al., 2003).

338 Thus, in the present study, we were able to characterize the steroid profile in gilt saliva from  
339 immature to pubertal stage by using GC-MS/MS analysis. This technique allowed us the detection  
340 of 28 steroids in 500  $\mu$ l of saliva.

341 Conversion of cholesterol to pregnenolone is the first step in the synthesis of all steroid hormones in  
342 gonads and adrenal cortex (Payne and Hales, 2004; Robic et al., 2014). Pregnenolone can then be  
343 converted to  $17\alpha$ -OHPREG and  $20\alpha$ -DHPREG or sulfated to pregnenolone sulfate (Payne and  
344 Hales, 2004; Schuler et al., 2014). In our study, pregnenolone and pregnenolone sulfate levels  
345 ranged between 0.7 and 2 ng/ml,  $20\alpha$ -DHPREG levels were lower than 0.11 ng/ml and  $17\alpha$ -  
346 OHPREG was undetectable in gilts saliva. In human, similar levels were observed in pre-pubertal  
347 girls (Majewska et al., 2014). To our knowledge, the salivary concentrations of these steroid  
348 hormones had never been analyzed in large domestic mammals. Plasma concentrations of  
349 pregnenolone, pregnenolone sulfate,  $20\alpha$ -DHPREG and  $17\alpha$ -OHPREG were analyzed in cyclic  
350 gilts (Stone and Seamark, 1985), cyclic ewes (McKay et al., 1987) and women (Deng et al., 2017).  
351 Pregnenolone levels in gilts saliva were similar to plasma levels in cyclic gilts or ewes, but  
352 pregnenolone sulfate,  $20\alpha$ -DHPREG and  $17\alpha$ -OHPREG levels in gilts saliva were lower than  
353 plasma levels in gilts, ewes and women. In our study, pregnenolone, pregnenolone sulfate and  $20\alpha$ -  
354 DHPREG concentrations did not significantly vary between weeks. They were not influenced by  
355 the entrance into the “waiting period”, evidenced by increasing urinary estrone levels. Thus, these  
356 precursors of steroid hormones may not be relevant biomarkers of the period of receptivity to the  
357 boar effect.

358 DHEA is synthesized from  $17\alpha$ -OHPREG in gonads and adrenal cortex, and sulfated to DHEAS in  
359 adrenals (Payne and Hales, 2004; Robic et al., 2014). In gilts saliva, DHEA levels were around 0.3  
360 ng/ml from week-5 to week-3 and significantly dropped to 0.1 ng/ml from week-2 to the day of  
361 puberty detection. DHEAS levels did not significantly vary between weeks. Salivary concentrations

362 of DHEA and DHEAS had never been analyzed in large domestic mammals. In human saliva from  
363 pre-pubertal girls, DHEA levels were similar to levels from young gilts, but DHEAS levels were  
364 higher (Majewska et al., 2014). Plasma from cyclic gilts **contains** 0.03 ng/ml of DHEA, suggesting  
365 that DHEA levels continue to decrease after puberty (Stone and Seamark, 1985). In our study,  
366 DHEA levels in gilts saliva significantly dropped between week-3 and week-2, around 175 days  
367 old. The drop of DHEA levels is concomitant with the increase of urinary oestrone concentration  
368 and could correspond to the beginning of the “waiting period” during which gilts are receptive to  
369 the boar effect. Thus, DHEA may be a potential biomarker of the period of receptivity to the boar  
370 effect. Further experiments comparing gilts that respond or not to the boar effect are in progress to  
371 confirm that DHEA is a relevant biomarker.

372 Progesterone is synthesized from pregnenolone in gonads and adrenal cortex and is ubiquitously  
373 converted to its reduced metabolites (dihydroprogesterone, tetrahydroprogesterone,  
374 hexahydroprogesterone) (Payne and Hales, 2004; Meffre et al., 2007; Zhu et al., 2017). The use of  
375 GC-MS/MS as a tool to analyze multiple steroids with very closed chemical structures with high  
376 specificity and sensitivity provides opportunities to explore for the first time the presence and  
377 concentration of progesterone metabolites in saliva of large domestic animals. In our study,  
378 progesterone levels from gilts saliva ranged between 0.02 ng/ml and 0.2 ng/ml. Saliva progesterone  
379 was analyzed in some domestic and wild animals with concentrations ranging from 0.05 to 0.1  
380 ng/ml in cyclic cows (Gao et al., 1988), from 2 to 8.5 ng/ml in cyclic buffaloes (Lasheen et al.,  
381 2018), and 2.4 ng/ml in adult female alpacas (Volkery et al., 2012). No data are available in saliva  
382 from non-pubertal females. Blood progesterone concentrations in pre-pubertal gilts were similar to  
383 saliva levels (Kolesarova et al., 2010; Zhuo et al., 2014). Thus, salivary progesterone levels could  
384 reflect the circulating level, and salivary progesterone could be a relevant biomarker for assessing  
385 ovarian function, as shown in human (Lewis, 2006). In our study, concentrations of the  
386 progesterone metabolites ranged between 0.001 and 4 ng/ml. In human saliva of pre-pubertal girls,  
387  $3\alpha5\alpha$ -THPROG levels were similar to levels in gilts saliva (Majewska et al., 2014). Recent studies



388 have analyzed the concentration of progesterone metabolites in oviductal fluid of cows (Lamy et al.,  
389 2016), in plasma of mare (Legacki et al., 2016), in brain and plasma rats and mice (Meffre et al.,  
390 2007; Zhu et al., 2017). However, to our knowledge, no data are available on the saliva of domestic  
391 mammals. In our study, progesterone levels from gilts saliva tended to increase between week-3 and  
392 week-2, close to the beginning of the “waiting period”, suggesting a relationship between salivary  
393 progesterone levels and ovarian activity. However, this increase was not significant. Interestingly,  
394 most of the progesterone metabolites showed a similar increase from week-3 to the day of puberty  
395 detection ( $5\alpha$ -DHPROG,  $5\beta$ -DHPROG,  $3\alpha5\beta$ -THPROG,  $3\beta5\alpha$ -THPROG,  $5\alpha20\alpha$ -THPROG,  
396  $3\alpha5\alpha20\alpha$ -HHPROG,  $3\alpha5\beta20\alpha$ -HHPROG,  $3\beta5\alpha20\alpha$ -HHPROG) meaning that they are reliable  
397 markers of the ovarian progesterone synthesis. Moreover,  $5\alpha$ -DHPROG concentrations  
398 significantly increased from week-3 to week-2, at the beginning of the “waiting period”, and  
399 remained high until the day of puberty detection. When adding together the concentrations of all  
400  $5\alpha$ -metabolites of progesterone and all  $5\beta$ -metabolites of progesterone, we showed an increase of  
401 the concentrations between week-3 and week-2, and this increase was significant for the  $5\alpha$ -  
402 metabolites of progesterone. These metabolites analyzed all together represent an interesting target  
403 for investigating the ovarian function. Thus, the  $5\alpha$ -metabolites of progesterone and  $5\alpha$ -DHPROG  
404 could be potential biomarkers of the “waiting period” during which gilts are receptive to the boar  
405 effect. Further experiments are in progress to confirm that this biomarker could be relevant.

406 Androstenediol is synthesized from DHEA while androstenedione is synthesized from both  $17\alpha$ -  
407 OHPROG and DHEA in gonads and adrenal cortex (Payne and Hales, 2004; Robic et al., 2014;  
408 Kovac et al., 2016). Androstenedione is then reduced to  $5\alpha$ -ADIONE which is converted to  
409 epiandrosterone, and  $5\beta$ -ADIONE which is converted to etiocholanolone (Anizan et al., 2011; Luu-  
410 The, 2013; Robic et al., 2014). Their concentrations in gilts saliva ranged from 0.01 to 0.63 ng/ml.  
411 To our knowledge, the salivary concentration of these steroid hormones had never been analyzed in  
412 domestic mammals. In human saliva from pre-pubertal girls, similar levels were observed for  
413 androstenediol, androstenedione, etiocholanolone and epiandrosterone (Majewska et al., 2014).

414 Plasma from cyclic gilts contained 0.15 ng/ml androstenedione (Stone and Seamark, 1985), whereas  
415 salivary levels on the day of puberty detection reached 0.6 ng/ml, suggesting a decrease after  
416 puberty. Finally, our data showed a progressive increase from week-4 to puberty for  
417 androstenedione and its metabolites. Androstenedione is a precursor of  $17\beta$ -estradiol in ovaries and  
418 its increase could also reflect the beginning of the ovarian maturation. But the sensitivity as a  
419 biomarker appears to be too low so that androstenedione may not be a relevant biomarker of the  
420 period of receptivity to the boar effect.

421 Testosterone is synthesized from androstenedione and androstenediol in testis (Robic et al., 2014;  
422 Kovac et al., 2016). It is then reduced to dihydrotestosterone which can be converted to  
423 tetrahydrotestosterone (Meffre et al., 2007; Robic et al., 2014; Kovac et al., 2016). In our study,  
424 testosterone levels in gilts saliva were low. Testosterone levels were evaluated in the saliva of  
425 young gilts (< 150 days old) (Escribano et al., 2014), heifers (10-11 months old) (Geburt et al.,  
426 2015), and young girls (< 1 year old) (Fang et al., 2017). A correlation was shown between saliva  
427 and blood concentration of testosterone in young girls (Fang et al., 2017). However, no data were  
428 available on the evolution of testosterone levels during pre-pubertal development of female  
429 mammals, whether in saliva or in blood. Since no significant changes of testosterone,  $5\alpha$ -DHT and  
430  $3\alpha,5\alpha$ -THT levels were observed around the beginning of the “waiting period”, these steroid  
431 hormones were not considered to be relevant biomarkers of the period of receptivity to the boar  
432 effect.

433 Estrone is synthesized mainly from androstenedione and converted to estradiol in ovaries (Payne  
434 and Hales, 2004; Robic et al., 2014). In gilts saliva, estrone and  $17\beta$ -estradiol levels ranged between  
435 0.01 and 0.08 ng/ml, with a decrease from week-5 to week-4 and a progressive increase to week-1.  
436 In human saliva from young girls, estradiol and estrone concentrations reached similar levels (Fang  
437 et al., 2017). To our knowledge, the salivary concentration of these steroid hormones has never  
438 been analyzed in domestic mammals, except in saliva from buffalo females during estrus and  
439 diestrus (Ravinder et al., 2016). Serum  $17\beta$ -estradiol concentrations in gilts (0.02 ng/ml before

440 puberty and 0.03 ng/ml at puberty) (Cosgrove et al., 1993; Li et al., 2016) were close to salivary  
441 levels measured in our study. Moreover, a correlation has been shown between saliva and serum  
442 concentrations of estradiol and estrone in adult women and young girls (Dielen et al., 2017; Fang et  
443 al., 2017). Finally, the increase of estradiol levels before puberty was also observed in serum from  
444 young girls (Sehested et al., 2000) and plasma from heifers (Melvin et al., 1999). Thus, salivary  
445 17 $\beta$ -estradiol might be considered as a surrogate marker for blood estradiol. In our study, 17 $\beta$ -  
446 estradiol levels in gilts saliva significantly increased between week-3 and week-2, around 175 days  
447 old. Since this period was considered as the beginning of the “waiting period”, the increase in  
448 estradiol levels could be related to the “waiting period” during which gilts are receptive to the boar  
449 effect. Thus, 17 $\beta$ -estradiol may be a potential biomarker of the period of receptivity to the boar  
450 effect.

451 Salivary biomarkers of the period of gilts receptivity to the boar effect, such as steroids whose  
452 concentration significantly vary at the beginning of this period, would be of great help to improve  
453 detection of the gilts to stimulate. Overall, our study showed that the beginning of the “waiting  
454 period”, during which gilts are sensitive to boar exposure, is concomitant with a significant decrease  
455 of salivary DHEA levels, a significant increase of 5 $\alpha$ -DHPROG concentrations, and a significant  
456 increase of 17 $\beta$ -estradiol levels. This increase of estradiol levels could coincide with the appearance  
457 of antral follicles in the porcine ovary, with increasing estrogen production (Camous et al., 1985).  
458 In prepubertal heifers, increasing circulating concentrations of 17 $\beta$ -estradiol were associated with  
459 development of ovarian follicles (Bergfeld et al., 1994). Since DHEA is the precursor of androgens  
460 such as androstenedione, which is the main precursor of estrone and estradiol, as mentioned above,  
461 the decrease of DHEA levels may be linked to an increase of its consumption for the synthesis of  
462 estradiol. The increase of the concentration of progesterone and its metabolites also suggests a  
463 relationship with the increase of ovarian activity, since luteinization of follicles and increase of  
464 circulating progesterone levels of ovarian origin has been observed during the peri-pubertal period  
465 in heifers and ewes (Kinder et al., 1995). Thus, the combined determination of DHEA, 17 $\beta$ -

466 estradiol, 5 $\alpha$ -dihydroprogesterone and/or 5 $\alpha$ -reduced metabolites of progesterone in gilts saliva  
467 appears to give a precise “picture” of the ovarian function and thus could help to identify the  
468 “waiting period” during which gilts are receptive to boar effect. However, their low concentrations  
469 in saliva require expensive and sensitive analysis and may limit their use in pig farms. Moreover,  
470 whether these potential steroidal biomarkers are relevant to identify the period of gilts receptivity to  
471 boar effect has to be checked. For this purpose, experiments comparing gilts that respond or not to  
472 boar exposure are in progress. They will help to confirm relevant biomarkers of the period of gilts  
473 receptivity to boar effect.

474

## 475 **Conclusion**

476 These results show that painless sampling of saliva could be a non-invasive welfare-friendly tool for  
477 the identification of the physiological hormonal status of the gilts. DHEA, 17 $\beta$ -estradiol, 5 $\alpha$ -  
478 dihydroprogesterone and 5 $\alpha$ -reduced metabolites of progesterone could be potential salivary  
479 biomarkers of the receptivity period to boar effect. They could allow the identification of the  
480 optimal time for application of the boar effect. Further studies are in progress to ascertain their  
481 relevance. These studies participate in the development of non-hormonal alternative breeding tools  
482 for optimal management of gilt reproduction.

483

## 484 **List of non-standard abbreviations**

485 GC: gas chromatography

486 MS: mass spectrometry

487 MS/MS: tandem mass spectrometry

488 PREG : Pregnenolone

489 PREGS : Pregnenolone sulfate

490 20 $\alpha$ -DHPREG : 20 $\alpha$ -dihydropregnenolone

491 17 $\alpha$ -OH PREG : 17 $\alpha$ -hydroxypregnenolone

- 492 PROG : Progesterone
- 493 5 $\alpha$ -DHPROG : 5 $\alpha$ -dihydroprogesterone
- 494 5 $\beta$ -DHPROG : 5 $\beta$ -dihydroprogesterone
- 495 20 $\alpha$ -DHPROG : 20 $\alpha$ -dihydroprogesterone
- 496 3 $\alpha$ ,5 $\alpha$ -THPROG : 3 $\alpha$ ,5 $\alpha$ -tetrahydroprogesterone (allopregnanolone)
- 497 3 $\alpha$ ,5 $\beta$ -THPROG : 3 $\alpha$ ,5 $\beta$ -tetrahydroprogesterone (pregnanolone)
- 498 3 $\beta$ ,5 $\alpha$ -THPROG : 3 $\beta$ ,5 $\alpha$ -tetrahydroprogesterone (iso-allopregnanolone)
- 499 5 $\alpha$ ,20 $\alpha$ -THPROG : 5 $\alpha$ ,20 $\alpha$ -tetrahydroprogesterone
- 500 3 $\alpha$ ,5 $\alpha$ ,20 $\alpha$ -HHPROG : 5 $\alpha$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol (3 $\alpha$ ,5 $\alpha$ ,20 $\alpha$ -hexahydroprogesterone)
- 501 3 $\alpha$ ,5 $\beta$ ,20 $\alpha$ -HHPROG : 5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol (3 $\alpha$ ,5 $\beta$ ,20 $\alpha$ -hexahydroprogesterone)
- 502 3 $\beta$ ,5 $\alpha$ ,20 $\alpha$ -HHPROG : 5 $\alpha$ -pregnane-3 $\beta$ ,20 $\alpha$ -diol (3 $\beta$ ,5 $\alpha$ ,20 $\alpha$ -hexahydroprogesterone)
- 503 16 $\alpha$ -OH PROG : 16 $\alpha$ -hydroxyprogesterone
- 504 17 $\alpha$ -OH PROG : 17 $\alpha$ -hydroxyprogesterone
- 505 DOC : Deoxycorticosterone
- 506 5 $\alpha$ -DHDOC : 5 $\alpha$ -dihydrodeoxycorticosterone
- 507 3 $\alpha$ ,5 $\alpha$ -THDOC : 3 $\alpha$ ,5 $\alpha$ -tetrahydrodeoxycorticosterone (allotetrahydrodeoxycorticosterone)
- 508 DHEA : Dehydroepiandrosterone
- 509 DHEAS : Dehydroepiandrosterone sulfate
- 510 ADIOL : Androstenediol ( $\Delta$ 5-androstene 3 $\beta$ ,17 $\beta$ -diol)
- 511 ADIONE : Androstenedione ( $\Delta$ 4-androstene 3,17-dione)
- 512 5 $\alpha$ -ADIONE : 5 $\alpha$ -androstane-3,17-dione
- 513 5 $\beta$ -ADIONE : 5 $\beta$ -androstane-3,17-dione
- 514 Epiandrosterone : 5 $\alpha$ -androstane-3 $\beta$ -ol-17-one
- 515 Etiocholanolone : 5 $\beta$ -androstane-3 $\alpha$ -ol-17-one
- 516 T : Testosterone

517 5 $\alpha$ -DHT : 5 $\alpha$ -dihydrotestosterone

518 3 $\alpha$ ,5 $\alpha$ -THT : 3 $\alpha$ ,5 $\alpha$ -tetrahydrotestosterone

519 3 $\beta$ ,5 $\alpha$ -THT : 3 $\beta$ ,5 $\alpha$ -tetrahydrotestosterone

520 E1 : Estrone

521 17 $\beta$ -E2 : 17 $\beta$ -estradiol

522 2-ME2 : 2-methoxyestradiol

523

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526

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528

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530

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533

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709

## 710 **Figure captions**

711 Figure 1. GC-MS/MS measurements of pregnenolone, pregnenolone sulfate, 20 $\alpha$ -  
712 dihydropregnenolone, dehydroepiandrosterone and dehydroepiandrosterone sulfate concentrations  
713 (ng/ml  $\pm$  sem) in gilts saliva from week-5 to the day of puberty detection. For each steroid the y-  
714 axis was standardized to the maximal concentration, so that the variations are easy-to-read.  
715 Statistical differences between weeks were analyzed with one-way repeated measures ANOVA  
716 followed by Tukey post-tests. \* significantly different from week-5, # from week-4, \$ from week-3.  
717 \* # \$ : P < 0.05, \$\$ P < 0.01.

718

719 Figure 2. GC-MS/MS measurements of progesterone, 5 $\alpha$ -dihydroprogesterone, 5 $\beta$ -  
720 dihydroprogesterone, 20 $\alpha$ -dihydroprogesterone, 3 $\alpha$ 5 $\alpha$ -tetrahydroprogesterone and 3 $\alpha$ 5 $\beta$ -  
721 tetrahydroprogesterone concentrations (ng/ml  $\pm$  sem) in gilts saliva from week-5 to the day of  
722 puberty detection. For each steroid the y-axis was standardized to the maximal concentration, so  
723 that the variations are easy-to-read. Statistical differences between weeks were analyzed with one-  
724 way repeated measures ANOVA followed by Tukey post-tests. # significantly different from week-  
725 4, \$ from week-3. # \$ : P < 0.05.

726

727 Figure 3. GC-MS/MS measurements of 3 $\beta$ 5 $\alpha$ -tetrahydroprogesterone, 5 $\alpha$ 20 $\alpha$ -  
728 tetrahydroprogesterone, 3 $\alpha$ 5 $\alpha$ 20 $\alpha$ -hexahydroprogesterone, 3 $\alpha$ 5 $\beta$ 20 $\alpha$ -hexahydroprogesterone,  
729 3 $\beta$ 5 $\alpha$ 20 $\alpha$ -hexahydroprogesterone and deoxycorticosterone concentrations (ng/ml  $\pm$  sem) in gilts  
730 saliva from week-5 to the day of puberty detection. For each steroid the y-axis was standardized to  
731 the maximal concentration, so that the variations are easy-to-read. Statistical differences between

732 weeks were analyzed with one-way repeated measures ANOVA followed by Tukey post-tests. #  
733 significantly different from week-4,  $P < 0.05$ .

734

735 Figure 4. GC-MS/MS measurements of androstenediol, androstenedione,  $5\alpha$ -androstane-3,17-  
736 dione,  $5\beta$ -androstane-3,17-dione,  $5\alpha$ -androstane-3 $\beta$ -ol-17-one and  $5\beta$ -androstane-3 $\alpha$ -ol-17-one  
737 concentrations (ng/ml  $\pm$  sem) in gilts saliva from week-5 to the day of puberty detection. For each  
738 steroid the y-axis was standardized to the maximal concentration, so that the variations are easy-to-  
739 read. Statistical differences between weeks were analyzed with one-way repeated measures  
740 ANOVA followed by Tukey post-tests.

741

742 Figure 5. GC-MS/MS measurements of testosterone,  $5\alpha$ -dihydrotestosterone,  $3\alpha,5\alpha$ -  
743 tetrahydrotestosterone, estrone and  $17\beta$ -estradiol concentrations (ng/ml  $\pm$  sem) in gilts saliva from  
744 week-5 to the day of puberty detection. For each steroid the y-axis was standardized to the maximal  
745 concentration, so that the variations are easy-to-read. Statistical differences between weeks were  
746 analyzed with one-way repeated measures ANOVA followed by Tukey post-tests. \* significantly  
747 different from week-5, # from week-4, \$ from week-3. \*\* ## \$\$  $P < 0.01$ .

748

749 Figure 6. GC-MS/MS measurements of progesterone and its main reduced metabolites ( $5\alpha$ -  
750 DHPROG +  $5\beta$ -DHPROG +  $3\alpha,5\alpha$ -THPROG +  $3\alpha,5\beta$ -THPROG +  $3\beta,5\alpha$ -THPROG +  $5\alpha,20\alpha$ -  
751 THPROG +  $3\alpha,5\alpha,20\alpha$ -HHPROG +  $3\alpha,5\beta,20\alpha$ -HHPROG +  $3\beta,5\alpha,20\alpha$ -HHPROG),  $5\alpha$ -  
752 metabolites ( $5\alpha$ -DHPROG +  $3\alpha,5\alpha$ -THPROG +  $3\beta,5\alpha$ -THPROG +  $5\alpha,20\alpha$ -THPROG +  
753  $3\alpha,5\alpha,20\alpha$ -HHPROG +  $3\beta,5\alpha,20\alpha$ -HHPROG) and  $5\beta$ -metabolites ( $5\beta$ -DHPROG +  $3\alpha,5\beta$ -  
754 THPROG +  $3\alpha,5\beta,20\alpha$ -HHPROG) concentrations (ng/ml  $\pm$  sem) in gilts saliva from week-5 to the  
755 day of puberty detection. The y-axis was standardized to the maximal concentration, so that the  
756 variations are easy-to-read. Statistical differences between weeks were analyzed with one-way

757 repeated measures ANOVA followed by Tukey post-tests. \* significantly different from week-5, #  
 758 from week-4, \$ from week-3. \* # \$ : P < 0.05.

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764 **Tables**

765

766 Table 1 : GC-MS/MS parameters used for steroid identification and quantification

Steroids (Molecular weight)	Derivatized steroids (molecular weight)	Retention time (min.)	Precursor ions (m/z)	Product ions (m/z)	Collision energy (eV)
<b>HPLC fraction # 1</b>					
5 $\alpha$ -DHPROG (316)	5 $\alpha$ -DHPROG-3,20-TMS <sub>2</sub> (460)	16.37	460	445	10
5 $\beta$ -DHPROG (316)	5 $\beta$ -DHPROG-3,20-TMS <sub>2</sub> (460)	15.03	445	355	12
5 $\alpha$ -ADIONE (288)	5 $\alpha$ -ADIONE-3,17-dione (432)	18.23	432	275	18
5 $\beta$ -ADIONE (288)	5 $\beta$ -ADIONE-3,17-dione (432)	16.85	432	417	8
<b>HPLC fraction # 2</b>					
PREG (316)	PREG-3-HFB (512)	23.31	298	283	8
20 $\alpha$ -DHPREG (318)	20 $\alpha$ -DHPREG-3,20-HFB <sub>2</sub> (710)	19.85	496	211	10
17 $\alpha$ -OH PREG (332)	17 $\alpha$ -OH PREG-HFB (528)	21.88	467	253	8
PROG (314)	PROG-3-HFB (510)	23.42	510	147	10
17 $\alpha$ -OH PROG (330)	17 $\alpha$ -OH PROG-HFB (526)	21.96	465	369	14
16 $\alpha$ -OH PROG (330)	16 $\alpha$ -OH PROG-HFB (526)	21.49	722	493	10
20 $\alpha$ -DHPROG (316)	20 $\alpha$ -DHPROG-3,20-HFB <sub>2</sub> (708)	20.38	708	693	12
3 $\alpha$ 5 $\alpha$ -THPROG (318)	3 $\alpha$ 5 $\alpha$ -THPROG-3-HFB (514)	22.22	496	481	10
3 $\alpha$ 5 $\beta$ -THPROG (318)	3 $\alpha$ 5 $\beta$ -THPROG-3-HFB (514)	22.40	496	481	12
3 $\beta$ 5 $\alpha$ -THPROG (318)	3 $\beta$ 5 $\alpha$ -THPROG-3-HFB (514)	23.73	496	467	8
5 $\alpha$ 20 $\alpha$ -THPROG (318)	5 $\alpha$ 20 $\alpha$ -THPROG-3-HFB (514)	25.06	514	231	10
3 $\alpha$ 5 $\alpha$ 20 $\alpha$ -HHPROG (320)	3 $\alpha$ 5 $\alpha$ 20 $\alpha$ -HHPROG-3,20-HFB <sub>2</sub> (712)	19.19	712	429	10
3 $\alpha$ 5 $\beta$ 20 $\alpha$ -HHPROG (320)	3 $\alpha$ 5 $\beta$ 20 $\alpha$ -HHPROG-3,20-HFB <sub>2</sub> (712)	19.41	712	429	10
3 $\beta$ 5 $\alpha$ 20 $\alpha$ -HHPROG (320)	3 $\beta$ 5 $\alpha$ 20 $\alpha$ -HHPROG-3,20-HFB <sub>2</sub> (712)	20.66	712	429	10
11-deoxycorticosterone (330)	11-deoxycorticosterone-3,21-HFB <sub>2</sub> (722)	23.91	722	707	12
5 $\alpha$ -DHDOC (332)	5 $\alpha$ -DHDOC-HFB (528)	28.34	301	255	6

3 $\alpha$ 5 $\alpha$ -THDOC (334)	3 $\alpha$ 5 $\alpha$ -THDOC-3,21-HFB <sub>2</sub> (726)	22.74	499	257	6
DHEA (288)	DHEA-3HFB (484)	21.15	270	199	8
ADIOL (290)	ADIOL-HFB <sub>2</sub> -3,17-HFB <sub>2</sub> (682)	17.57	468	453	10
ADIONE (286)	ADIONE-3-HFB (482)	21.20	482	268	8
Epiandrosterone (290)	Epiandrosterone-HFB (486)	21.61	442	213	16
Etiocolanolone (290)	Etiocolanolone-HFB (486)	20.33	442	213	16
Testosterone (288)	Testosterone-3,17-HFB <sub>2</sub> (680)	17.61	680	665	12
5 $\alpha$ -DHTestosterone (290)	5 $\alpha$ -DHTestosterone-17-HFB (486)	22.16	414	399	10
3 $\alpha$ 5 $\alpha$ -THT (292)	3 $\alpha$ 5 $\alpha$ -THT-3,17-HFB <sub>2</sub> (684)	16.42	455	241	8
3 $\beta$ 5 $\alpha$ -THT (292)	3 $\beta$ 5 $\alpha$ -THT-3,17-HFB <sub>2</sub> (684)	17.94	470	241	14
17 $\beta$ -estradiol (272)	17 $\beta$ -estradiol-3,17-HFB <sub>2</sub> (664)	18.32	664	237	10
Estrone (270)	Estrone-3-HFB (466)	21.79	466	422	10
2-ME2 (302)	2-ME2-3,17-HFB <sub>2</sub> (694)	21.19	694	267	10

**Steroid sulfates fraction**

PREGS, Na (418)	PREG-3-HFB (512)	23.31	298	283	8
DHEAS, Na (390)	DHEA-3HFB (484)	21.15	270	199	8

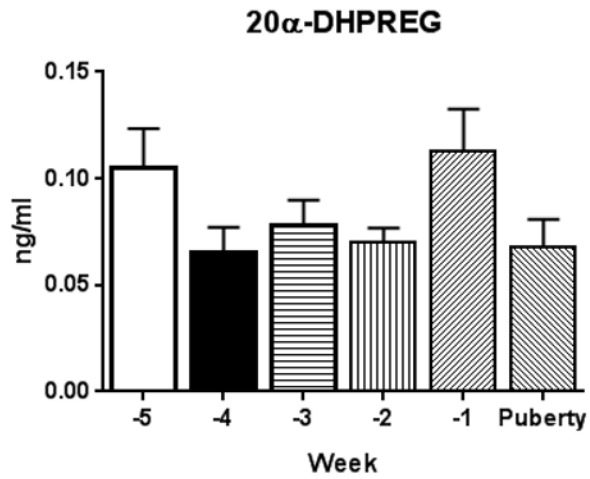
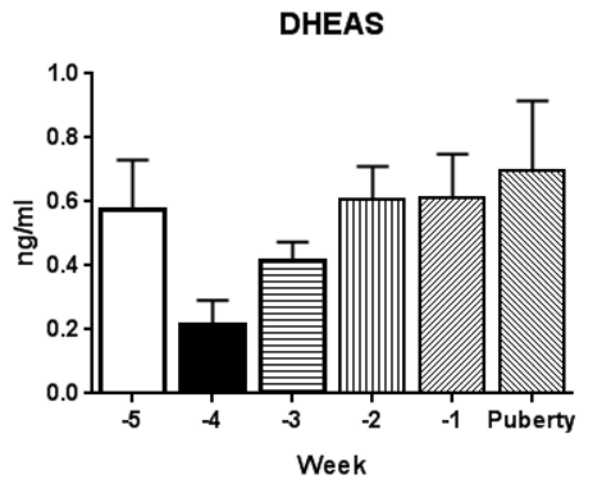
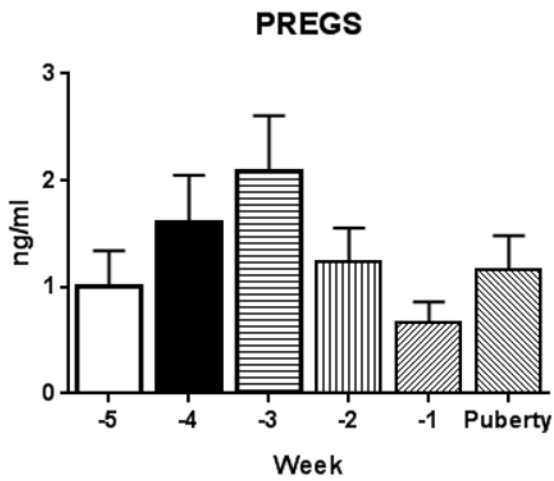
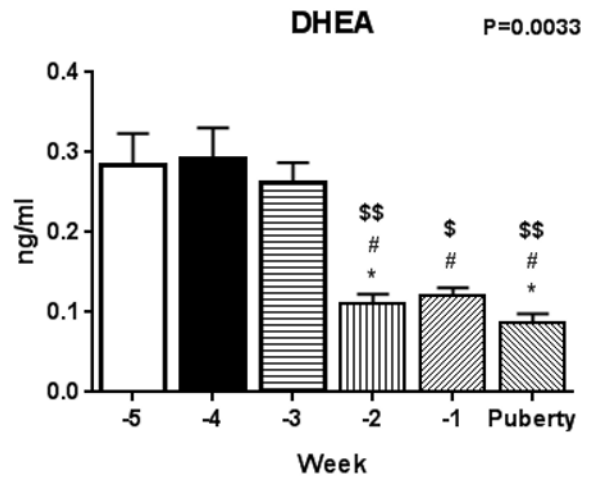
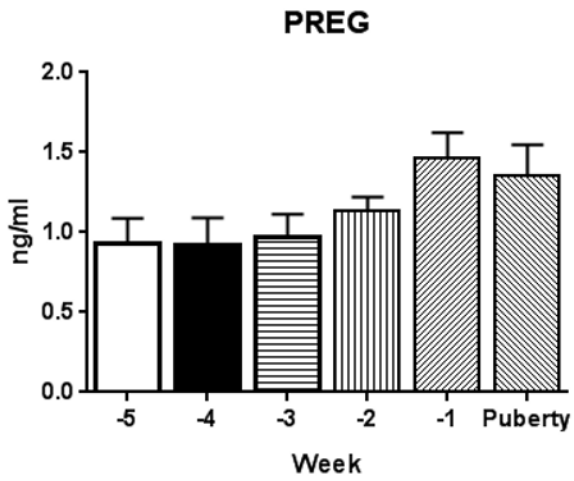
767

768 Table 2 : targeted steroids analyzed by GC-MS/MS in gilt saliva and their abbreviation. Steroids are  
769 classified according to their chemical structure, i.e. their carbon number. Among the 35 targeted  
770 steroids, 28 steroids were detected and quantified.

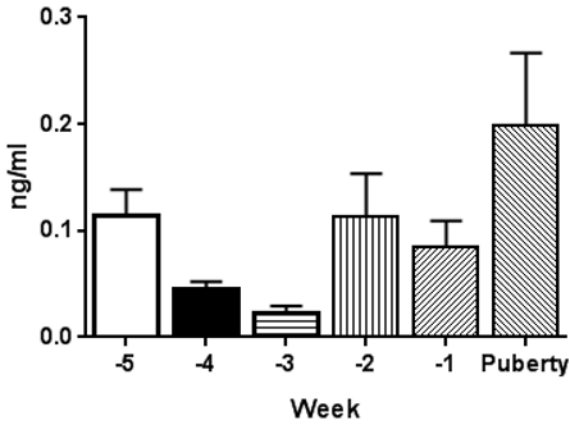
Targeted steroids	Abbreviation	Detected
<b>C21-Steroids</b>		
Pregnenolone	PREG	Yes
Pregnenolone sulfate	PREGS	Yes
20 $\alpha$ -dihydropregnenolone	20 $\alpha$ -DHPREG	Yes
17 $\alpha$ -hydroxypregnenolone	17 $\alpha$ -OH PREG	No
Progesterone	PROG	Yes
5 $\alpha$ -dihydroprogesterone	5 $\alpha$ -DHPROG	Yes
5 $\beta$ -dihydroprogesterone	5 $\beta$ -DHPROG	Yes
20 $\alpha$ -dihydroprogesterone	20 $\alpha$ -DHPROG	Yes
3 $\alpha$ ,5 $\alpha$ -tetrahydroprogesterone (allopregnanolone)	3 $\alpha$ ,5 $\alpha$ -THPROG	Yes
3 $\alpha$ ,5 $\beta$ -tetrahydroprogesterone (pregnanolone)	3 $\alpha$ ,5 $\beta$ -THPROG	Yes
3 $\beta$ ,5 $\alpha$ -tetrahydroprogesterone (iso-allopregnanolone)	3 $\beta$ ,5 $\alpha$ -THPROG	Yes
5 $\alpha$ ,20 $\alpha$ -tetrahydroprogesterone	5 $\alpha$ ,20 $\alpha$ -THPROG	Yes
5 $\alpha$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol (3 $\alpha$ ,5 $\alpha$ ,20 $\alpha$ -hexahydroprogesterone)	3 $\alpha$ ,5 $\alpha$ ,20 $\alpha$ -HHPROG	Yes
5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol (3 $\alpha$ ,5 $\beta$ ,20 $\alpha$ -hexahydroprogesterone)	3 $\alpha$ ,5 $\beta$ ,20 $\alpha$ -HHPROG	Yes
5 $\alpha$ -pregnane-3 $\beta$ ,20 $\alpha$ -diol (3 $\beta$ ,5 $\alpha$ ,20 $\alpha$ -hexahydroprogesterone)	3 $\beta$ ,5 $\alpha$ ,20 $\alpha$ -HHPROG	Yes
16 $\alpha$ -hydroxyprogesterone	16 $\alpha$ -OH PROG	No
17 $\alpha$ -hydroxyprogesterone	17 $\alpha$ -OH PROG	No
Deoxycorticosterone	DOC	Yes
5 $\alpha$ -dihydrodeoxycorticosterone	5 $\alpha$ -DHDOC	No

<i>3<math>\alpha</math>5<math>\alpha</math>-tetrahydrodeoxycorticosterone (allotetrahydrodeoxycorticosterone)</i>	<i>3<math>\alpha</math>,5<math>\alpha</math>-THDOC</i>	<i>No</i>
<b>C19-Steroids</b>		
Dehydroepiandrosterone	DHEA	Yes
Dehydroepiandrosterone sulfate	DHEAS	Yes
Androstenediol ( $\Delta$ 5-androstene 3 $\beta$ ,17 $\beta$ -diol)	ADIOL	Yes
Androstenedione ( $\Delta$ 4-androstene 3,17-dione)	ADIONE	Yes
5 $\alpha$ -androstane-3,17-dione	5 $\alpha$ -ADIONE	Yes
5 $\beta$ -androstane-3,17-dione	5 $\beta$ -ADIONE	Yes
5 $\alpha$ -androstane-3 $\beta$ -ol-17-one	Epiandrosterone	Yes
5 $\beta$ -androstane-3 $\alpha$ -ol-17-one	Etiocholanolone	Yes
Testosterone	T	Yes
5 $\alpha$ -dihydrotestosterone	5 $\alpha$ -DHT	Yes
3 $\alpha$ ,5 $\alpha$ -tetrahydrotestosterone	3 $\alpha$ ,5 $\alpha$ -THT	Yes
<i>3<math>\beta</math>,5<math>\alpha</math>-tetrahydrotestosterone</i>	<i>3<math>\beta</math>,5<math>\alpha</math>-THT</i>	<i>No</i>
<b>C18-Steroids</b>		
Estrone	E1	Yes
17 $\beta$ -estradiol	17 $\beta$ -E2	Yes
<i>2-methoxyestradiol</i>	<i>2-ME2</i>	<i>No</i>

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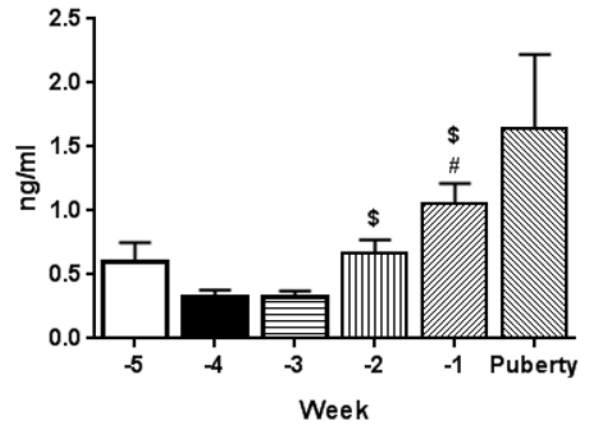


### PROG



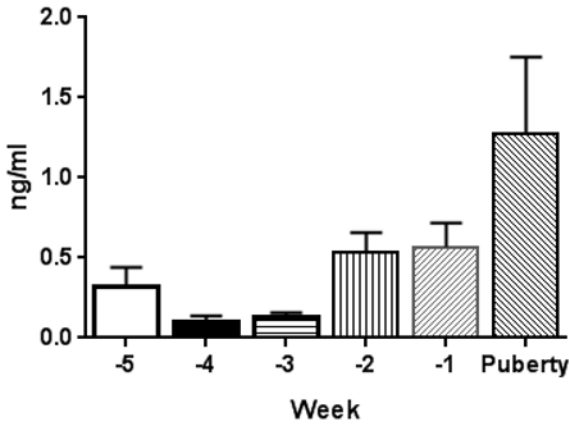
P=0.08

### 5 $\alpha$ -DHPROG

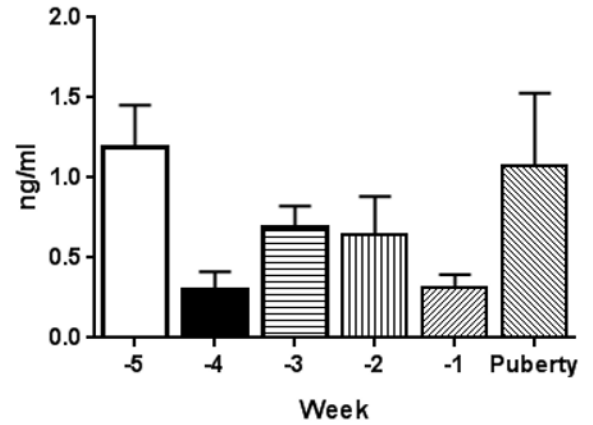


P=0.09

### 5 $\beta$ -DHPROG

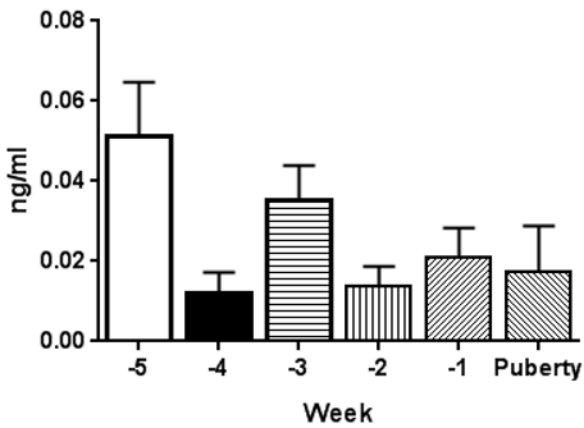


### 20 $\alpha$ -DHPROG



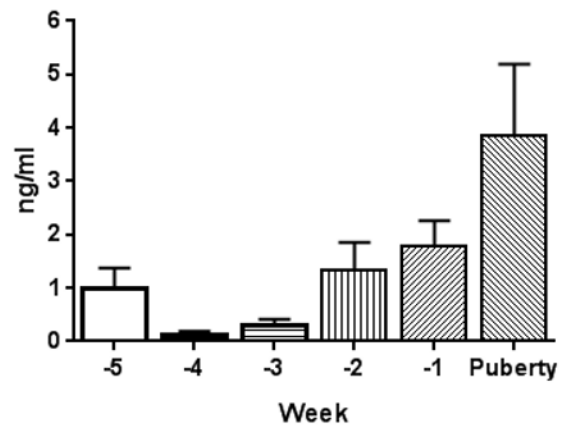
P=0.08

### 3 $\alpha$ 5 $\alpha$ -THPROG



P=0.06

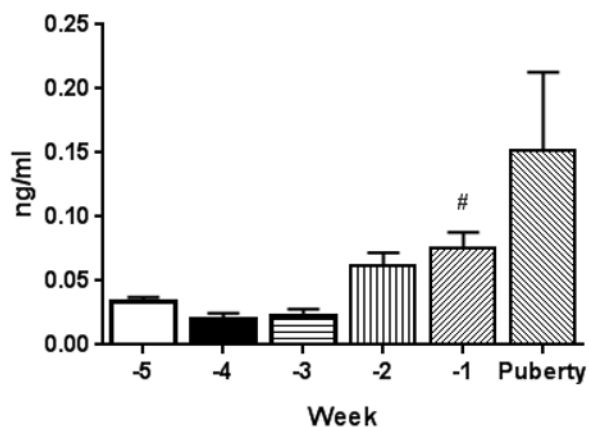
### 3 $\alpha$ 5 $\beta$ -THPROG





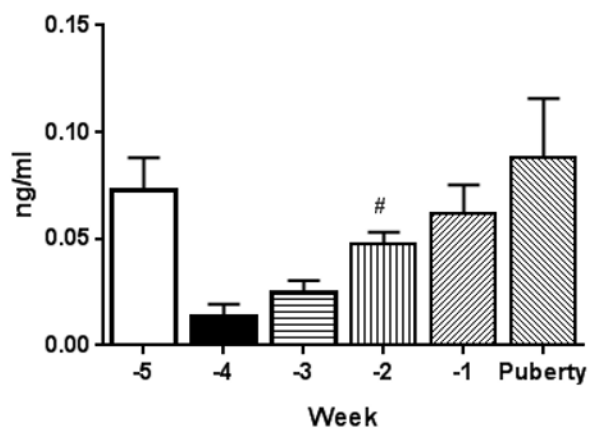
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### 3 $\beta$ 5 $\alpha$ -THPROG

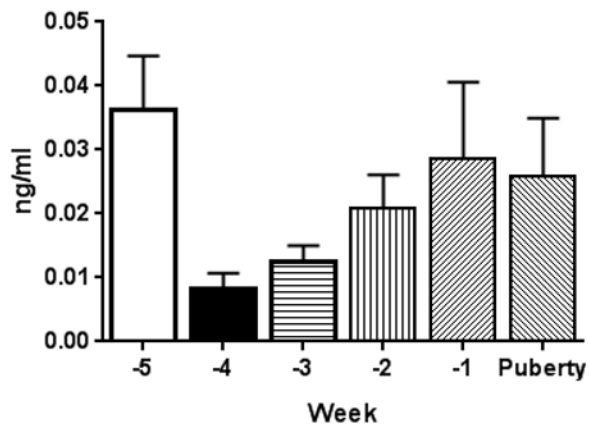


P=0.08

### 5 $\alpha$ 20 $\alpha$ -THPROG

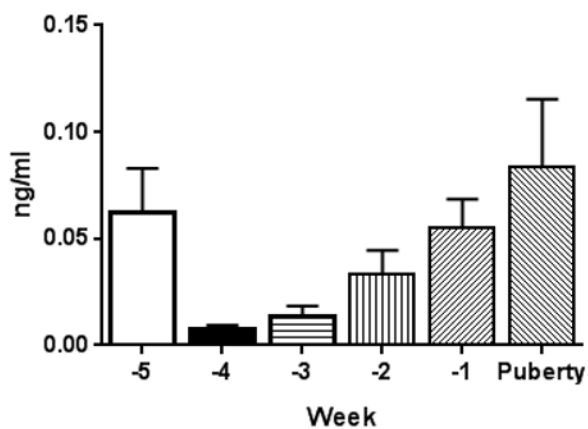


### 3 $\alpha$ 5 $\alpha$ 20 $\alpha$ -HHPROG

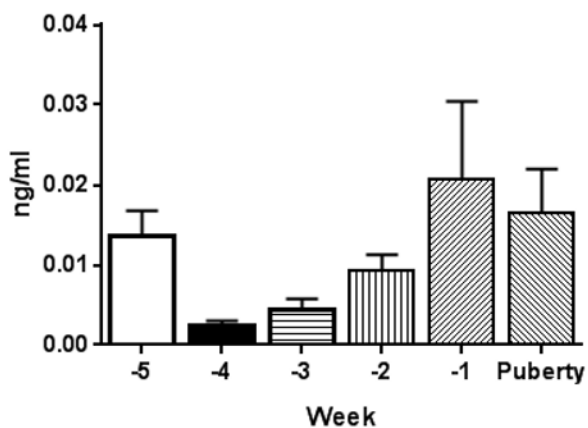


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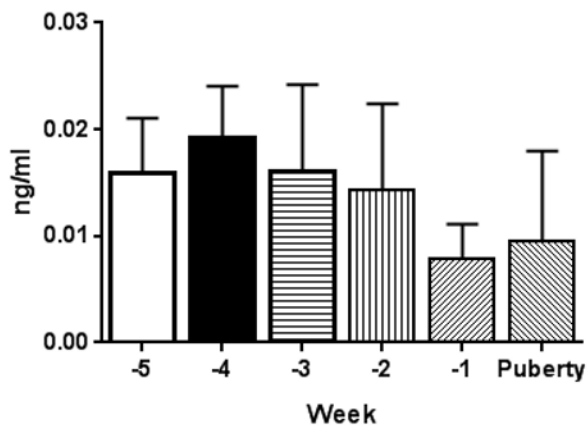
### 3 $\alpha$ 5 $\beta$ 20 $\alpha$ -HHPROG

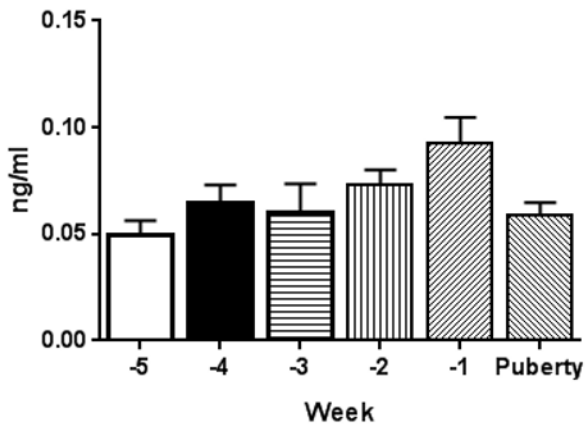
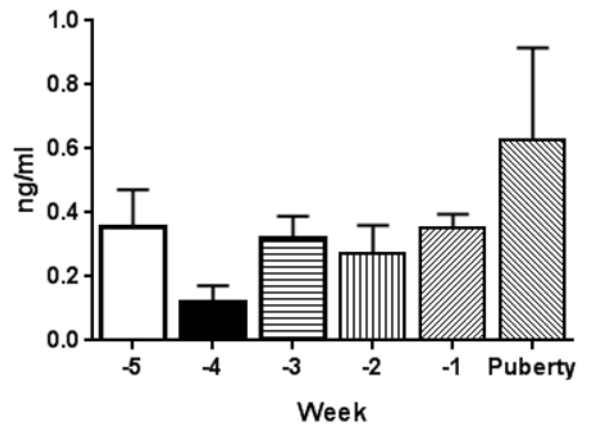
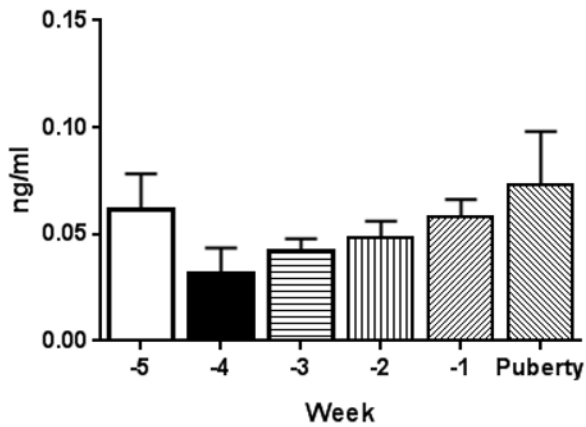
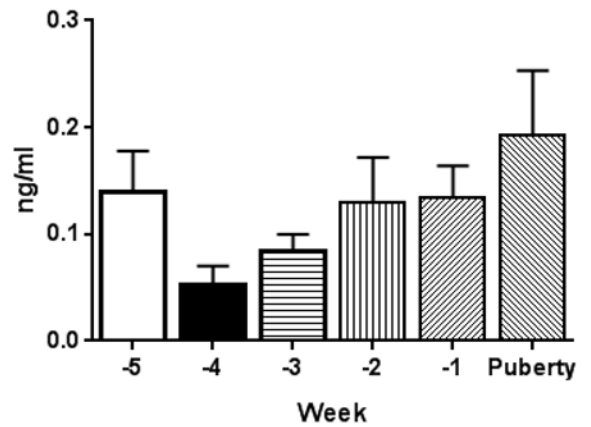
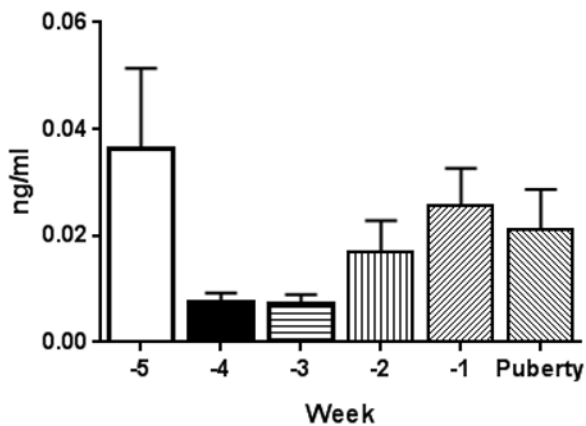
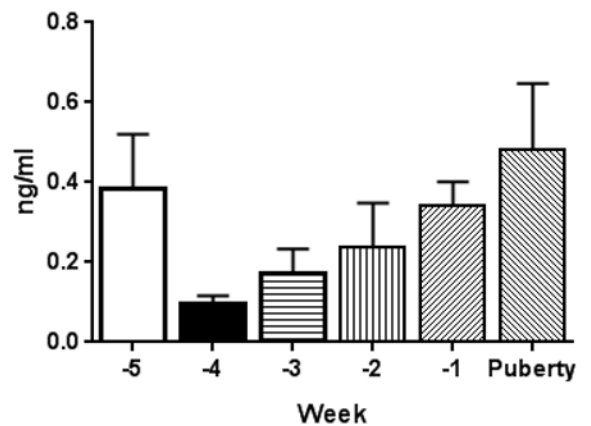


### 3 $\beta$ 5 $\alpha$ 20 $\alpha$ -HHPROG

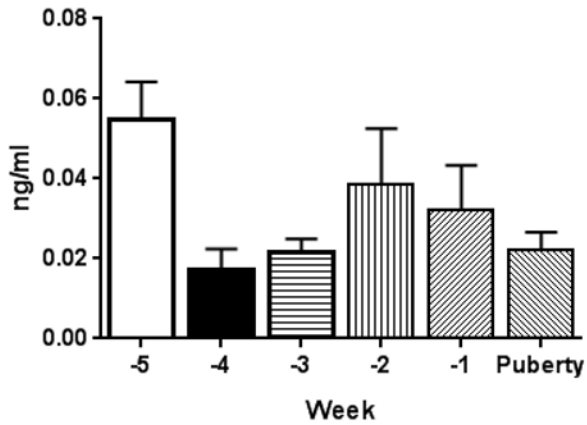


### DOC

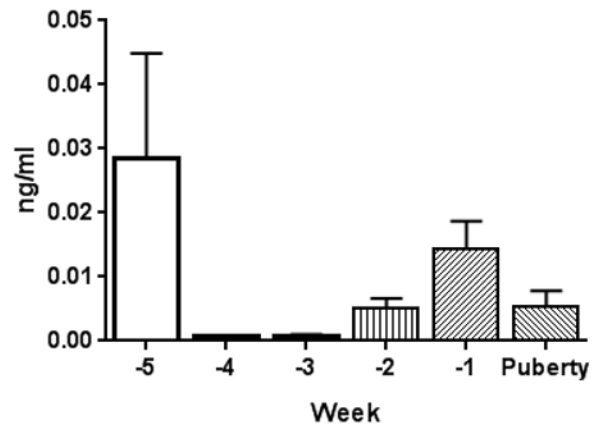


**ADIOL****ADIONE****5 $\alpha$ -ADIONE****5 $\beta$ -ADIONE****Epiandrosterone****Etiocholanolone**

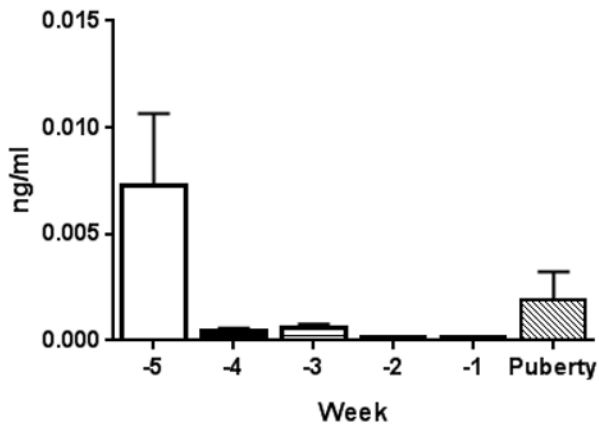
### TESTOSTERONE



### 5 $\alpha$ -DHT

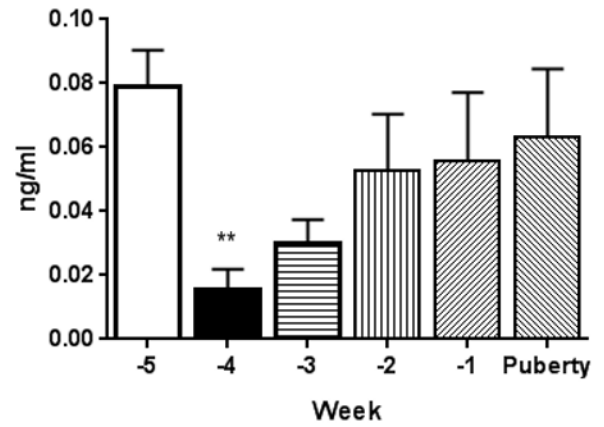


### 3 $\alpha$ 5 $\alpha$ -THT



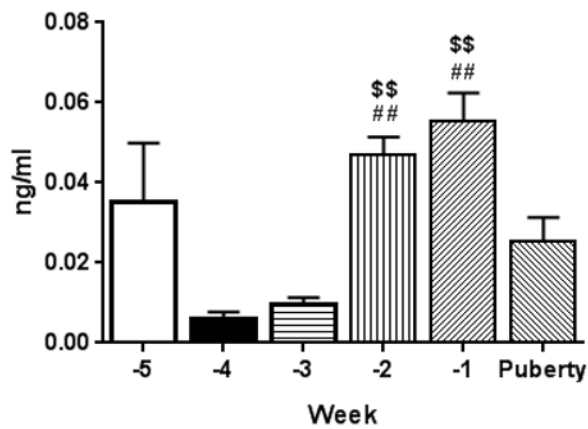
### ESTRONE

P=0.10



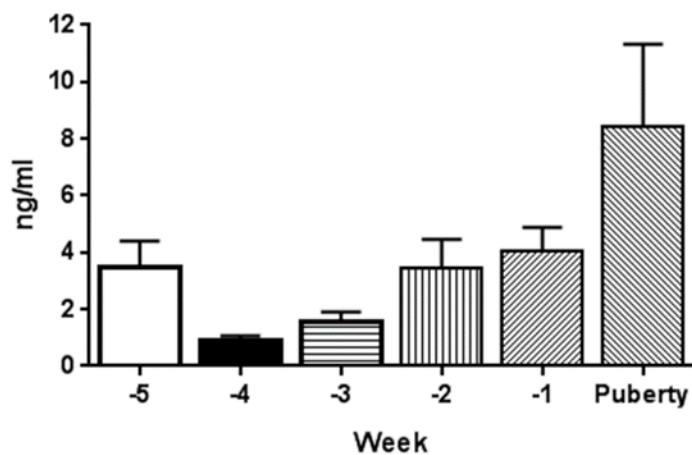
### 17 $\beta$ -ESTRADIOL

P=0.02



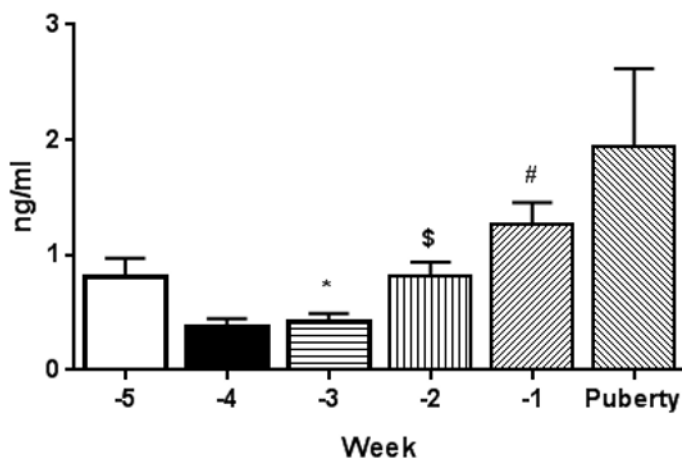
P=0.08

### PROG + 5 $\alpha$ / $\beta$ -metabolites



P = 0.09

### 5 $\alpha$ -metabolites of PROG



P = 0.06

### 5 $\beta$ -metabolites of PROG

