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Short title: Salivary steroidome analysis in gilts

Abstract

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

Starting between 144 to 147 days of age, six Large White gilts were submitted to ultrasound puberty diagnosis 3 times a week until first ovulation. Urine and saliva samples were collected to analyze weekly estrone and steroidome respectively, until puberty. Urinary estrone concentration significantly increased 2 weeks before first ovulation occurring between 182 and 192 days of age. The period with increasing estrone levels was considered as the "waiting period". Steroidome analysis allowed identifying and quantifying 28 steroids in 500 µl of gilts saliva. Significant decrease of dehydroepiandrosterone and significant increase of 5α-dihydroprogesterone and 17β-estradiol were detected 2 weeks before puberty, suggesting that these steroids could be potential biomarkers of the "waiting period".

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

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

Introduction

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

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

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

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

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

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

Materials and methods

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 Etrelles, 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

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

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

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

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

Steroids measurement by GC-MS/MS

Steroids were extracted from the saliva (400-550 µl) of gilts with methanol. The saliva was spiked with all the following internal standards for steroid quantification: 2 ng of $^{2}H_{6}$-5α-DHP (CDN Isotopes, Sainte Foy la Grande, France) for 5α/β-dihydroprogesterone (5α/β-DHP) and 5α/β-dihydroandrostanedione (5α/β-ADIONE), 2 ng of $^{2}H_{4}$-pregnenolone sulfate (PREGS) for PREGS, 5 ng of $^{2}H_{4}$-dehydroepiandrosterone sulfate (DHEAS) for DHEAS, 2 ng of $^{13}C_{3}$-testosterone (Isoscience, PA, USA) for testosterone, 2 ng of $^{13}C_{3}$-androstenedione (ADIONE) for ADIONE, 2 ng of $^{13}C_{3}$-progesterone (P) for P, 2 ng of $^{13}C_{3}$-17β-estradiol for 17β-estradiol, 2 ng of $^{13}C_{3}$-estrone for estrone, 2 n of $^{2}C_{3}$-deoxycorticosterone (DOC) for DOC, 2 ng of 19nor-PROG for 20α-DHP and 5α-DHDOC (dihydrodeoxycorticosterone), 2 ng of epietiocholanolone (Steraloids, Newport, Rhode Island) for androstenediol (ADIOL), etiocholanolone, epiandrosterone, 5α-dihydrotestosterone (5α-DHT), 3α/β5α-tetrahydrotestosterone (THT), pregnenolone (PREG), 20α-dihydropregnenolone (20α-DHPREG) 3α/β5α/β-tetrahydroprogesterone (THP), 5α20α-THP, 3α/β5α/β-hexahydroprogesterone (HHP) and 3α5α-THDOC, 2 ng of $^{13}C_{3}$-17α-OH PROG for 17α-

OH PROG, 17α-OH PREG and 16α-OH PROG, and 1 ng of $^{2}H_5$-2-methoxyestradiol (2-ME2) for 2-ME2. Samples were purified and fractionated by solid-phase extraction with the recycling procedure (Liere et al., 2004; Liere et al., 2009). Briefly, the extracts were dissolved in 1 ml MeOH and applied to the C18 cartridge (500 mg, 6 ml, International Sorbent Technology, IST), followed by 5 ml of MeOH/H_2O (85/15). The flow-through, containing the free and sulfated steroids, was collected and dried. After a previous re-conditioning of the same cartridge with 5 ml H_2O, the dried samples were dissolved in MeOH/H_2O (2/8) and re-applied. The cartridge was then washed with 5 ml H_2O and sulfated and unconjugated steroids were eluted with 5 ml MeOH/H_2O (1/1) and 5 ml MeOH/H_2O (9/1), respectively.

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

Two fractions were collected from the HPLC system: 5α/β-DHPROG and 5α/β-ADIONE were eluted in the first HPLC fraction (3-13 min) and were next silylated with 50 µl of a mixture N-methyl-N-trimethylsilyltrifluoroacetamide/ammonium iodide/dithioerythritol (1000:2:5 vol/wt/wt) for 15 min at 70°C. The second fraction (13-25 min) containing PREG, 20α-DHPREG, P, DOC, testosterone and their precursors and reduced metabolites, 17β-estradiol and estrone, was derivatized with 25 µl heptafluorobutyric anhydride (HFBA) and 25 µl anhydrous acetone for 1h at 20°C. Both fractions were dried under a stream of N_2 and resuspended in hexane.

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

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

linearity was assessed by analysing increasing amounts of rat plasma extracts (20, 50, 100 and 200 µl) in triplicate. The linearity was satisfactory for all the steroids with a coefficient of correlation ranging from 0.99 to 0.995. Five aliquots of 1 ml of rat plasma were used at two different times to determine the inter-assay coefficient of variation that was estimated at roughly 5-10 % for the targeted steroids. The accuracy of the assay was evaluated by determining the analytical recovery, which was defined as $C/(C_0+S)x100(\%)$. C is the concentration of the steroid in the spiked plasma extract (100 µl), C_0 is the concentration of a steroid in the unspiked plasma extract (100 µl) and S is the spiked concentration. The accuracy was in the range of 94-106 %.

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

Statistical analysis

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

Results

Uterine tract development and urinary estrone levels

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

The period from 144-147 days of age to the day of puberty detection (Day 0) was divided into 5 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 (± sem) on
237 the day of saliva sampling was 154 days (± 0.9) for week-5, 163 days (± 0.5) for week-4, 170 days
238 (± 0.5) for week-3, 178 days (± 0.5) for week-2, 186 days (± 1.4) for week-1, and 189 days (± 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 ± sem were 12.9 ± 2.5 ng/ml (week-5), 10.4 ± 2.7 ng/ml (week-4), 13.4 ± 4.1 ng/ml
242 (week-3), 12.7 ± 2.3 ng/ml (week-2), 62.5 ± 31.1 ng/ml (week-1) and 121.3 ± 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 µl of saliva (Table 2). Seven targeted steroids were not detected in
250 saliva : 17α-OH PREG, 16α-OH PROG, 17α-OH PROG, 5α-DHDOC, 3α5α-THDOC, 3β5α-
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 ± 0.16 ng/ml (week-5 and week-4) to 1.47 ± 0.15 ng/ml (week-
254 1) but without reaching statistical significance (Figure 1). Both direct metabolites of pregnenolone,
255 pregnenolone sulfate (PREGS) and 20α-dihydropregnenolone (20α-DHPREG), were also detected
256 in gilts saliva. PREGS concentrations showed important but non-significant variations ranging from
257 0.67 ± 0.19 to 2.08 ± 0.53 ng/ml, whereas 20α-DHPREG concentrations were lower and showed
258 little variations around 0.1 ng/ml (Figure 1).

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

Concentrations of deoxycorticosterone, progesterone and some of its reduced metabolites (3α5α-THPROG, 3β5α-THPROG, 5α20α-THPROG, 3α5α20α-HHPROG, 3α5β20α-HHPROG, 3β5α20α-HHPROG) were lower than 0.2 ng/ml in saliva, whereas concentrations of the other progesterone metabolites (5α-DHPROG, 5β-DHPROG, 20α-DHPROG, 3α5β-THPROG) ranged between 0.1 and 3.9 ng/ml (Figures 2 and 3). The concentrations of progesterone and all of its reduced metabolites (dihydro-, tetrahydro- and hexahydroprogesterone) tended to decrease between week-5 and week-4. Then progesterone levels tended to increase between week-3 and the day of puberty detection and most of its metabolites showed similar pattern (5α-DHPROG, 5β-DHPROG, 3α5β-THPROG, 3β5α-THPROG, 5α20α-THPROG, 3α5α20α-HHPROG, 3α5β20α-HHPROG, 3β5α20α-HHPROG). Concentrations of 5α-DHPROG were significantly different between week-3 (0.32 ± 0.05 ng/ml) and week-2 (0.66 ± 0.11 ng/ml, $p < 0.05$) as well as between week-4 (0.32 ± 0.06 ng/ml) and week-1 (1.06 ± 0.16 ng/ml) (Figure 2). Concentrations of 3β5α-THPROG were significantly different between week-1 vs week-4 (Figure 3, $p < 0.05$). Concentrations of 5α20α-THPROG were significantly different between week-2 vs week-4 (Figure 3, $p < 0.05$).

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

Testosterone levels were low and did not change significantly with time as for its reduced metabolites 5α-DHT and 3α5α-THT (Figure 5).

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

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

Discussion

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

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

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

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

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

DHEA is synthesized from 17α-OHPREG in gonads and adrenal cortex, and sulfated to DHEAS in adrenals (Payne and Hales, 2004; Robic et al., 2014). In gilts saliva, DHEA levels were around 0.3 ng/ml from week-5 to week-3 and significantly dropped to 0.1 ng/ml from week-2 to the day of 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 3α5α-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 (5α-DHPROG, 5β-DHPROG, 3α5β-THPROG, 3β5α-THPROG, 5α20α-THPROG,
396 3α5α20α-HHPROG, 3α5β20α-HHPROG, 3β5α20α-HHPROG) meaning that they are reliable
397 markers of the ovarian progesterone synthesis. Moreover, 5α-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 5α-metabolites of progesterone and all 5β-metabolites of progesterone, we showed an increase of
401 the concentrations between week-3 and week-2, and this increase was significant for the 5α-
402 metabolites of progesterone. These metabolites analyzed all together represent an interesting target
403 for investigating the ovarian function. Thus, the 5α-metabolites of progesterone and 5α-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 17α-
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 5α-ADIONE which is converted to
409 epiandrosterone, and 5β-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).

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

Testosterone is synthesized from androstenedione and androstenediol in testis (Robic et al., 2014; Kovac et al., 2016). It is then reduced to dihydrotestosterone which can be converted to tetrahydrotestosterone (Meffre et al., 2007; Robic et al., 2014; Kovac et al., 2016). In our study, testosterone levels in gilts saliva were low. Testosterone levels were evaluated in the saliva of young gilts (< 150 days old) (Escribano et al., 2014), heifers (10-11 months old) (Geburt et al., 2015), and young girls (< 1 year old) (Fang et al., 2017). A correlation was shown between saliva and blood concentration of testosterone in young girls (Fang et al., 2017). However, no data were available on the evolution of testosterone levels during pre-pubertal development of female mammals, whether in saliva or in blood. Since no significant changes of testosterone, 5α-DHT and 3α5α-THT levels were observed around the beginning of the "waiting period", these steroid hormones were not considered to be relevant biomarkers of the period of receptivity to the boar effect.

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

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

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

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

Conclusion

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

List of non-standard abbreviations

GC: gas chromatography

MS: mass spectrometry

MS/MS: tandem mass spectrometry

PREG : Pregnenolone

PREGS : Pregnenolone sulfate

20α-DHPREG : 20α-dihydropregnenolone

17α-OH PREG : 17α-hydroxypregnenolone

PROG : Progesterone

5α-DHPROG : 5α-dihydroprogesterone

5β-DHPROG : 5β-dihydroprogesterone

20α-DHPROG : 20α-dihydroprogesterone

3α,5α-THPROG : 3α,5α-tetrahydroprogesterone (allopregnanolone)

3α,5β-THPROG : 3α,5β-tetrahydroprogesterone (pregnanolone)

3β,5α-THPROG : 3β,5α-tetrahydroprogesterone (iso-allopregnanolone)

5α,20α-THPROG : 5α,20α-tetrahydroprogesterone

3α,5α,20α-HHPROG : 5α-pregnane-3α,20α-diol (3α,5α,20α-hexahydroprogesterone)

3α,5β,20α-HHPROG : 5β-pregnane-3α,20α-diol (3α,5β,20α-hexahydroprogesterone)

3β,5α,20α-HHPROG : 5α-pregnane-3β,20α-diol (3β,5α,20α-hexahydroprogesterone)

16α-OH PROG : 16α-hydroxyprogesterone

17α-OH PROG : 17α-hydroxyprogesterone

DOC : Deoxycorticosterone

5α-DHDOC : 5α-dihydrodeoxycorticosterone

3α,5α-THDOC : 3α5α-tetrahydrodeoxycorticosterone (allotetrahydrodeoxycorticosterone)

DHEA : Dehydroepiandrosterone

DHEAS : Dehydroepiandrosterone sulfate

ADIOL : Androstenediol (Δ5-androstene 3β,17β-diol)

ADIONE : Androstenedione (Δ4-androstene 3,17-dione)

5α-ADIONE : 5α-androstane-3,17-dione

5β-ADIONE : 5β-androstane-3,17-dione

Epiandrosterone : 5α-androstane-3β-ol-17-one

Etiocholanolone : 5β-androstane-3α-ol-17-one

T : Testosterone

5α-DHT : 5α-dihydrotestosterone

3α,5α-THT : 3α,5α-tetrahydrotestosterone

3β,5α-THT : 3β,5α-tetrahydrotestosterone

E1 : Estrone

17β-E2 : 17β-estradiol

2-ME2 : 2-methoxyestradiol

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Figure captions

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

Figure 2. GC-MS/MS measurements of progesterone, 5α-dihydroprogesterone, 5β-dihydroprogesterone, 20α-dihydroprogesterone, 3α5α-tetrahydroprogesterone and 3α5β-tetrahydroprogesterone concentrations (ng/ml ± sem) in gilts saliva from week-5 to the day of puberty detection. For each steroid the y-axis was standardized to the maximal concentration, so that the variations are easy-to-read. Statistical differences between weeks were analyzed with one-way repeated measures ANOVA followed by Tukey post-tests. # significantly different from week-4, $ from week-3. # $: $P < 0.05$.

Figure 3. GC-MS/MS measurements of 3β5α-tetrahydroprogesterone, 5α20α-tetrahydroprogesterone, 3α5α20α-hexahydroprogesterone, 3α5β20α-hexahydroprogesterone, 3β5α20α-hexahydroprogesterone and deoxycorticosterone concentrations (ng/ml ± sem) in gilts saliva from week-5 to the day of puberty detection. For each steroid the y-axis was standardized to 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, 5α-androstane-3,17-
736 dione, 5β-androstane-3,17-dione, 5α-androstane-3β-ol-17-one and 5β-androstane-3α-ol-17-one
737 concentrations (ng/ml ± 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, 5α-dihydrotestosterone, 3α5α-
743 tetrahydrotestosterone, estrone and 17β-estradiol concentrations (ng/ml ± 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 (5α-
750 DHPROG + 5β-DHPROG + 3α,5α-THPROG + 3α,5β-THPROG + 3β,5α-THPROG + 5α,20α-
751 THPROG + 3α,5α,20α-HHPROG + 3α,5β,20α-HHPROG + 3β,5α,20α-HHPROG), 5α-
752 metabolites (5α-DHPROG + 3α,5α-THPROG + 3β,5α-THPROG + 5α,20α-THPROG +
753 3α,5α,20α-HHPROG + 3β,5α,20α-HHPROG) and 5β-metabolites (5β-DHPROG + 3α,5β-
754 THPROG + 3α,5β,20α-HHPROG) concentrations (ng/ml ± 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)
HPLC fraction # 1					
5α-DHPROG (316)	5α-DHPROG-3,20-TMS_2 (460)	16.37	460	445	10
5β-DHPROG (316)	5β-DHPROG-3,20-TMS_2 (460)	15.03	445	355	12
5α-ADIONE (288)	5α-ADIONE-3,17-dione (432)	18.23	432	275	18
5β-ADIONE (288)	5β-ADIONE-3,17-dione (432)	16.85	432	417	8
HPLC fraction # 2					
PREG (316)	PREG-3-HFB (512)	23.31	298	283	8
20α-DHPREG (318)	20α-DHPREG-3,20-HFB_2 (710)	19.85	496	211	10
17α-OH PREG (332)	17α-OH PREG-HFB (528)	21.88	467	253	8
PROG (314)	PROG-3-HFB (510)	23.42	510	147	10
17α-OH PROG (330)	17α-OH PROG-HFB (526)	21.96	465	369	14
16α-OH PROG (330)	16α-OH PROG-HFB (526)	21.49	722	493	10
20α-DHPROG (316)	20α-DHPROG-3,20-HFB_2 (708)	20.38	708	693	12
3α5α-THPROG (318)	3α5α-THPROG-3-HFB (514)	22.22	496	481	10
3α5βTHPROG (318)	3α5β-THPROG-3-HFB (514)	22.40	496	481	12
3β5α-THPROG (318)	3β5α-THPROG-3-HFB (514)	23.73	496	467	8
5α20α-THPROG (318)	5α20α-THPROG-3-HFB (514)	25.06	514	231	10
3α5α20α-HHPROG (320)	3α5α20α-HHPROG-3,20-HFB_2 (712)	19.19	712	429	10
3α5β20α-HHPROG (320)	3α5β20α-HHPROG-3,20-HFB_2 (712)	19.41	712	429	10
3β5α20α-HHPROG (320)	3β5α20α-HHPROG-3,20-HFB_2 (712)	20.66	712	429	10
11-deoxycorticosterone (330)	11-deoxycorticosterone-3,21-HFB_2 (722)	23.91	722	707	12
5α-DHDOC (332)	5α-DHDOC-HFB (528)	28.34	301	255	6

3α5α-THDOC (334)	3α5α-THDOC-3,21-HFB_2 (726)	22.74	499	257	6
DHEA (288)	DHEA-3HFB (484)	21.15	270	199	8
ADIOL (290)	ADIOL-HFB_2-3,17-HFB_2 (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
Etiocholanolone (290)	Etiocholanolone-HFB (486)	20.33	442	213	16
Testosterone (288)	Testosterone-3,17-HFB_2 (680)	17.61	680	665	12
5α-DHTestosterone (290)	5α-DHTestosterone-17-HFB (486)	22.16	414	399	10
3α5α-THT (292)	3α5α-THT-3,17-HFB_2 (684)	16.42	455	241	8
3β5α-THT (292)	3β5α-THT-3,17-HFB_2 (684)	17.94	470	241	14
17β-estradiol (272)	17β -estradiol-3,l7-HFB_2 (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_2 (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

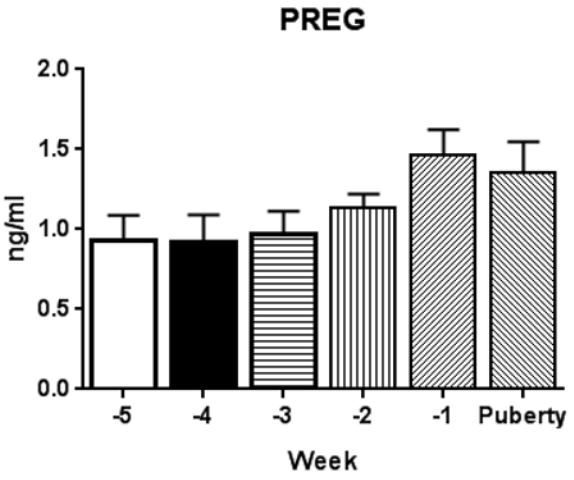
767

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
C21-Steroids		
Pregnenolone	PREG	Yes
Pregnenolone sulfate	PREGS	Yes
20α-dihydropregnenolone	20α-DHPREG	Yes
17α-hydroxypregnenolone	*17α-OH PREG*	*No*
Progesterone	PROG	Yes
5α-dihydroprogesterone	5α-DHPROG	Yes
5β-dihydroprogesterone	5β-DHPROG	Yes
20α-dihydroprogesterone	20α-DHPROG	Yes
3α,5α-tetrahydroprogesterone (allopregnanolone)	3α,5α-THPROG	Yes
3α,5β-tetrahydroprogesterone (pregnanolone)	3α,5β-THPROG	Yes
3β,5α-tetrahydroprogesterone (iso-allopregnanolone)	3β,5α-THPROG	Yes
5α,20α-tetrahydroprogesterone	5α,20α-THPROG	Yes
5α-pregnane-3α,20α-diol (3α,5α,20α-hexahydroprogesterone)	3α,5α,20α-HHPROG	Yes
5β-pregnane-3α,20α-diol (3α,5β,20α-hexahydroprogesterone)	3α,5β,20α-HHPROG	Yes
5α-pregnane-3β,20α-diol (3β,5α,20α-hexahydroprogesterone)	3β,5α,20α-HHPROG	Yes
16α-hydroxyprogesterone	*16α-OH PROG*	*No*
17α-hydroxyprogesterone	*17α-OH PROG*	*No*
Deoxycorticosterone	DOC	Yes
5α-dihydrodeoxycorticosterone	*5α-DHDOC*	*No*

3α5α-tetrahydrodeoxycorticosterone (allotetrahydrodeoxycorticosterone)	*3α,5α-THDOC*	*No*
C19-Steroids		
Dehydroepiandrosterone	DHEA	Yes
Dehydroepiandrosterone sulfate	DHEAS	Yes
Androstenediol (Δ5-androstene 3β,17β-diol)	ADIOL	Yes
Androstenedione (Δ4-androstene 3,17-dione)	ADIONE	Yes
5α-androstane-3,17-dione	5α-ADIONE	Yes
5β-androstane-3,17-dione	5β-ADIONE	Yes
5α-androstane-3β-ol-17-one	Epiandrosterone	Yes
5β-androstane-3α-ol-17-one	Etiocholanolone	Yes
Testosterone	T	Yes
5α-dihydrotestosterone	5α-DHT	Yes
3α,5α-tetrahydrotestosterone	3α,5α-THT	Yes
3β,5α-tetrahydrotestosterone	*3β,5α-THT*	*No*
C18-Steroids		
Estrone	E1	Yes
17β-estradiol	17β-E2	Yes
2-methoxyestradiol	*2-ME2*	*No*

771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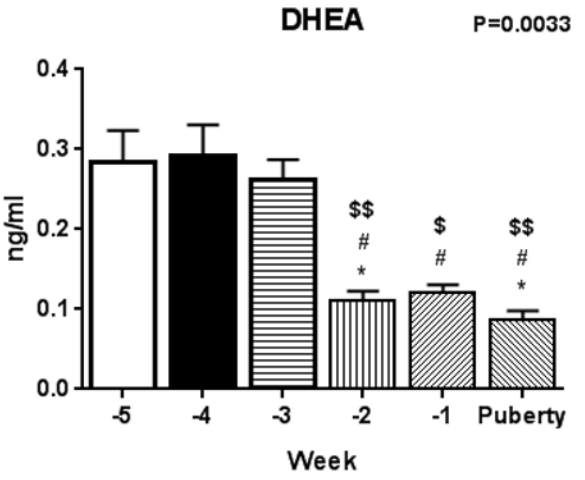
Week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*
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*
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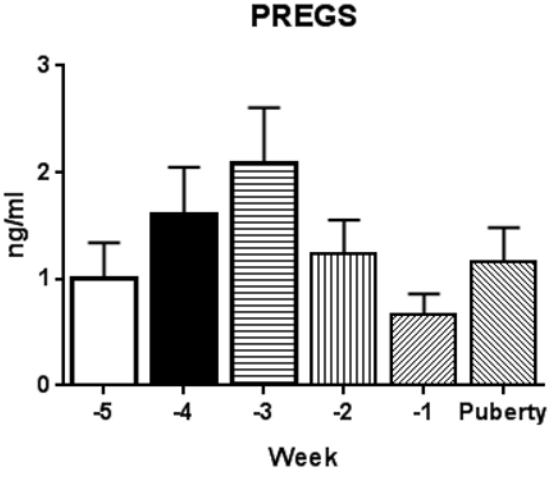
-1
Puberty
Week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ng/ml
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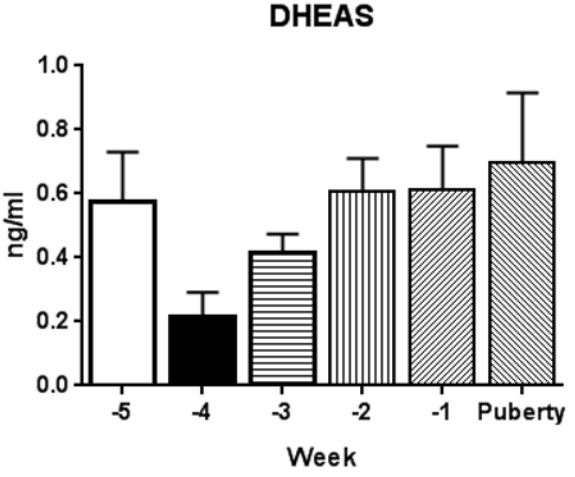
Puberty
Week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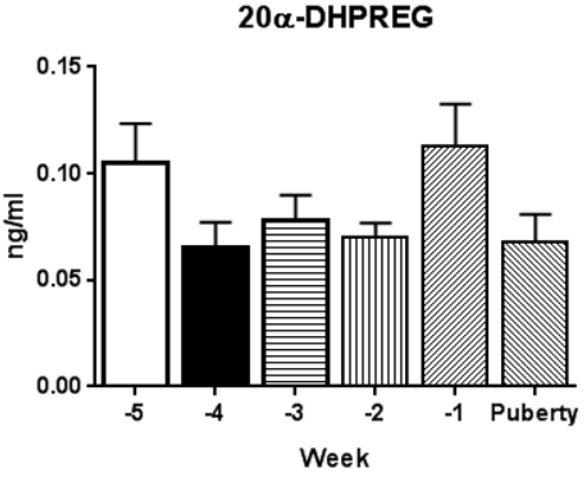
Puberty
Week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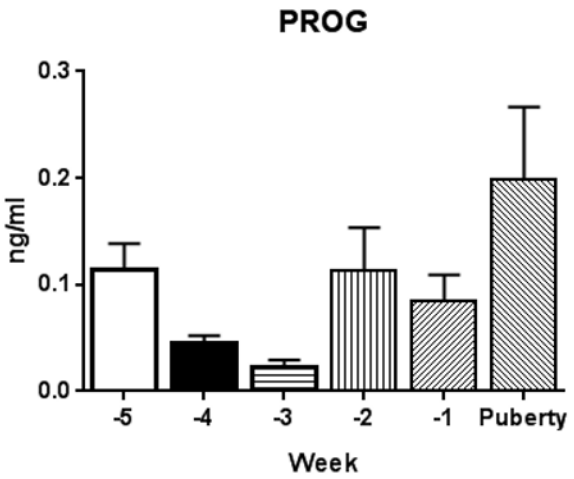
-1
Puberty
Week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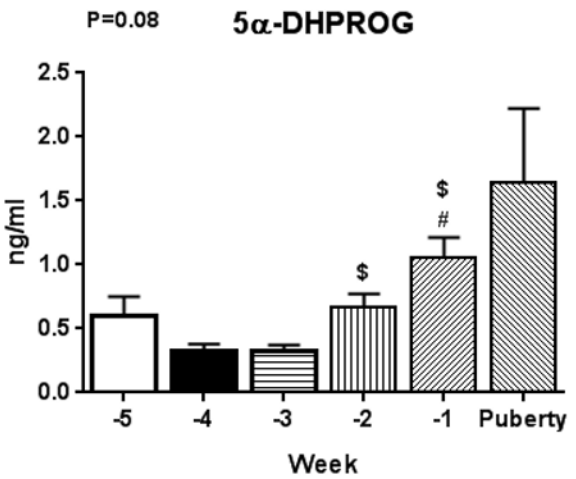
Puberty
Week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-2
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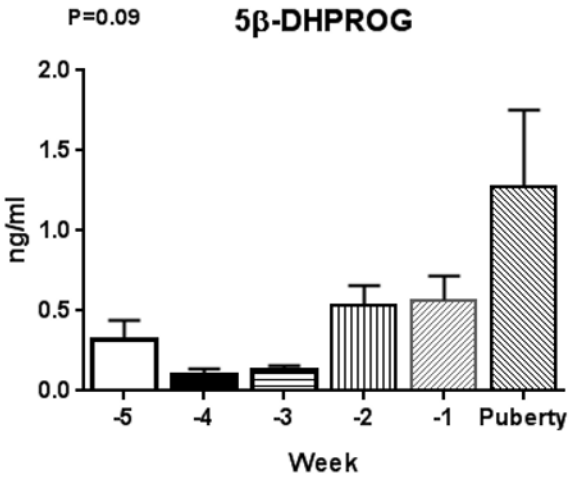
Puberty
Week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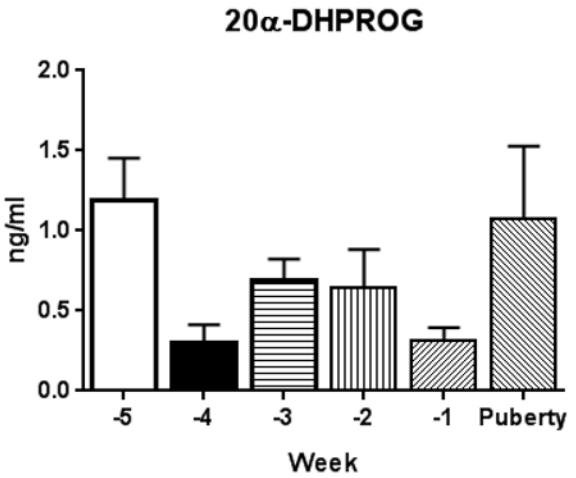
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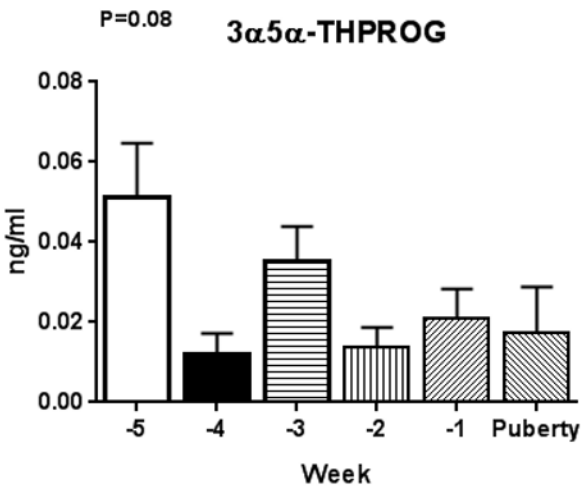
-1
Puberty
Week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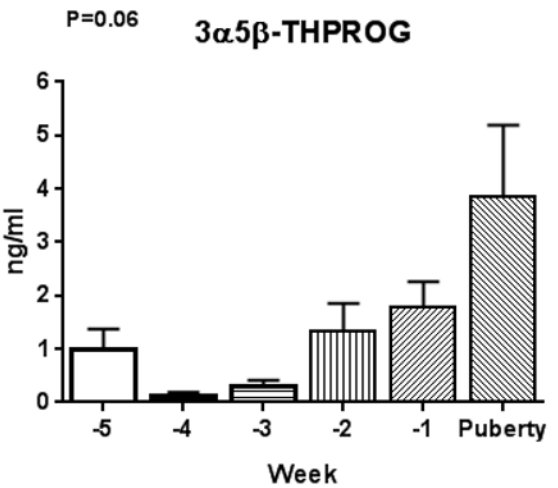
Puberty
Week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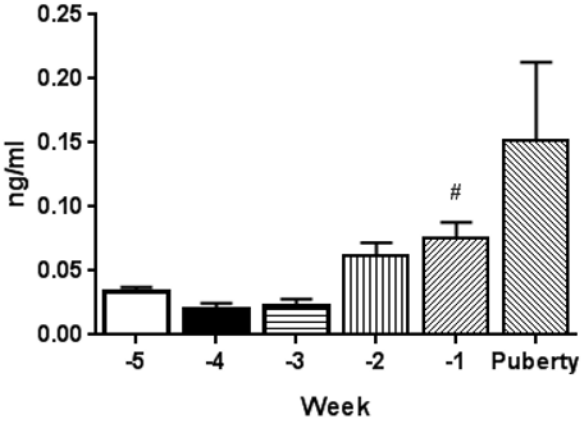

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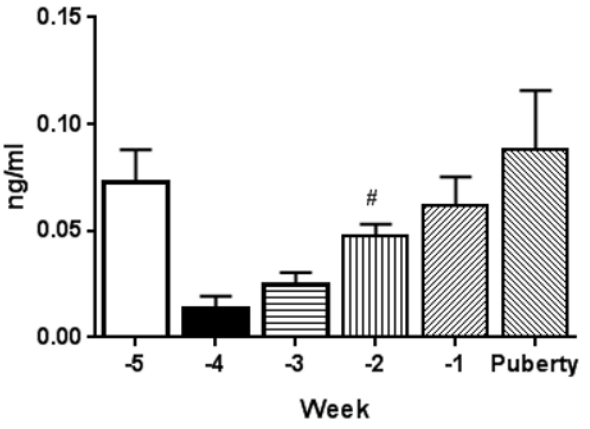

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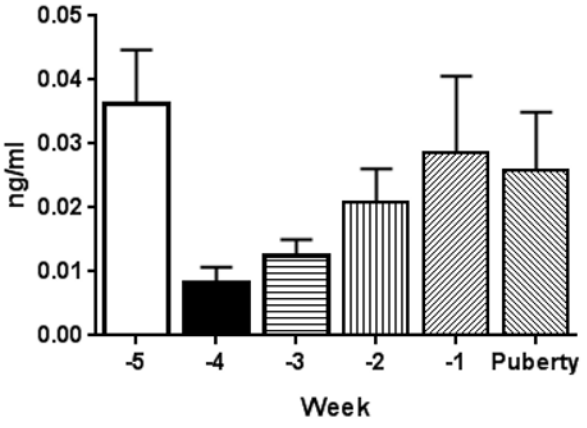

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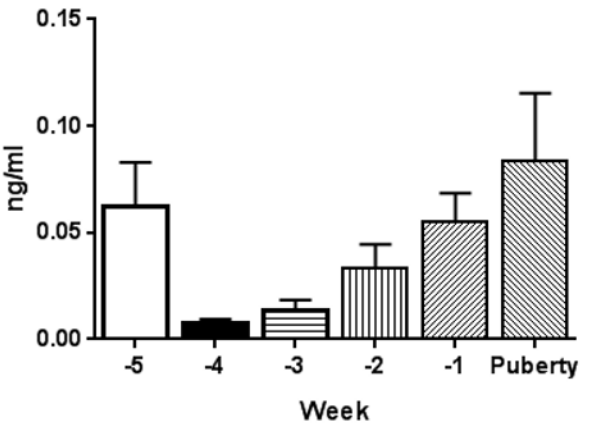

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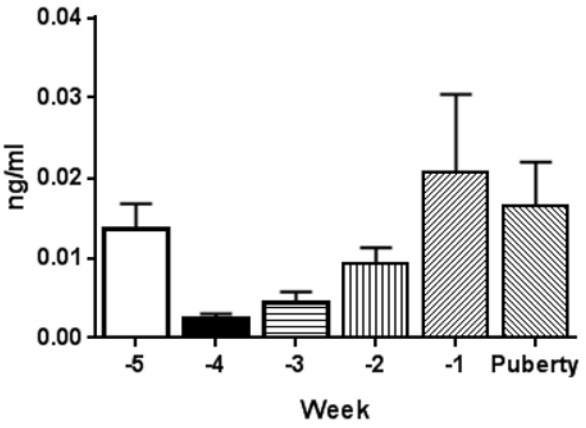

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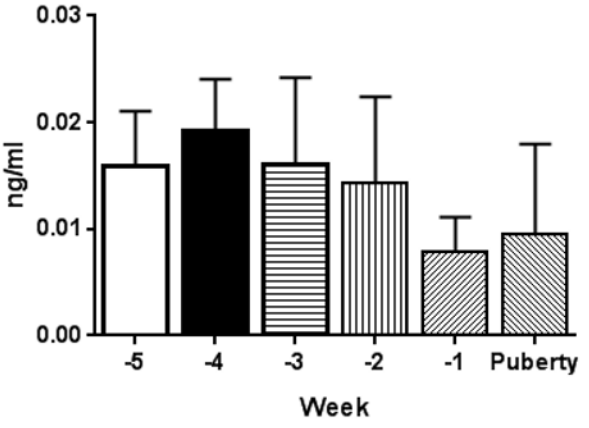
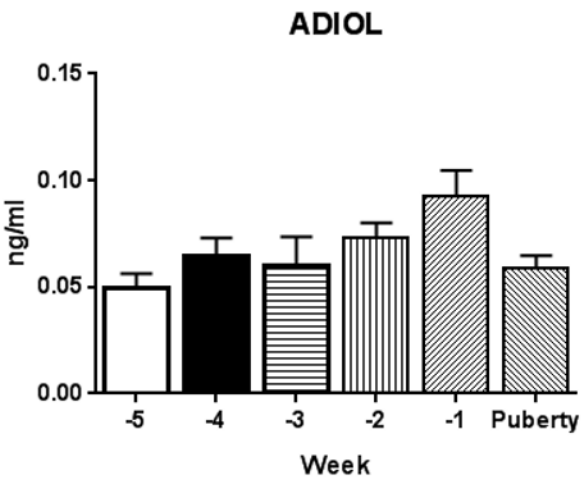
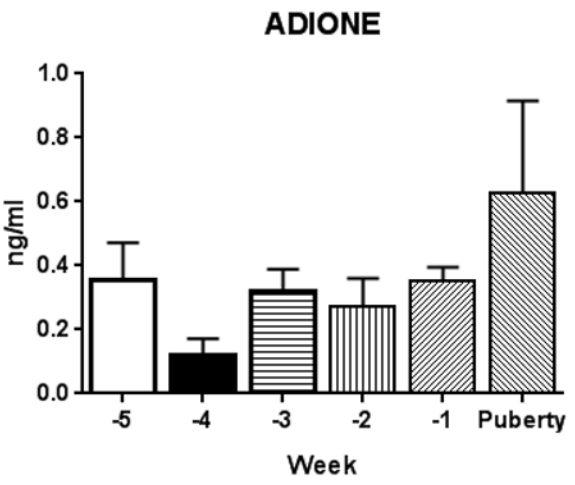
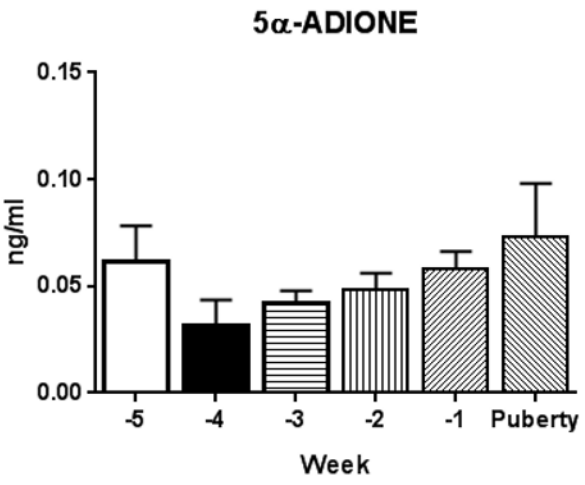
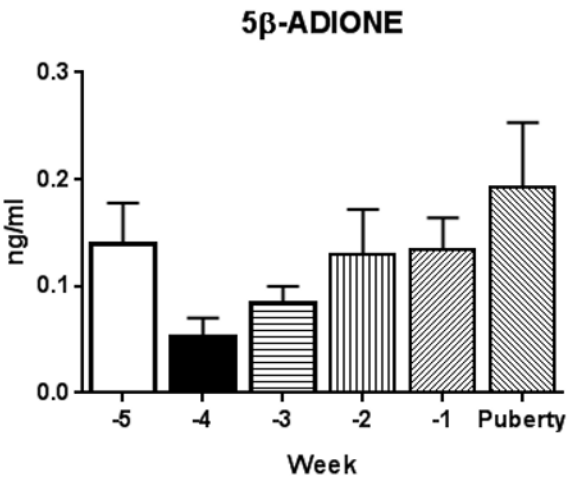
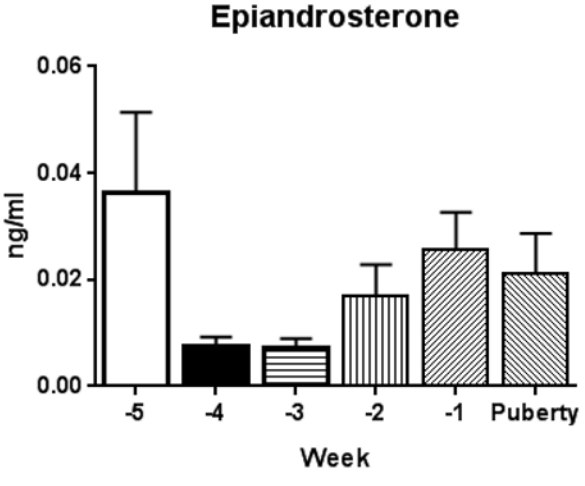


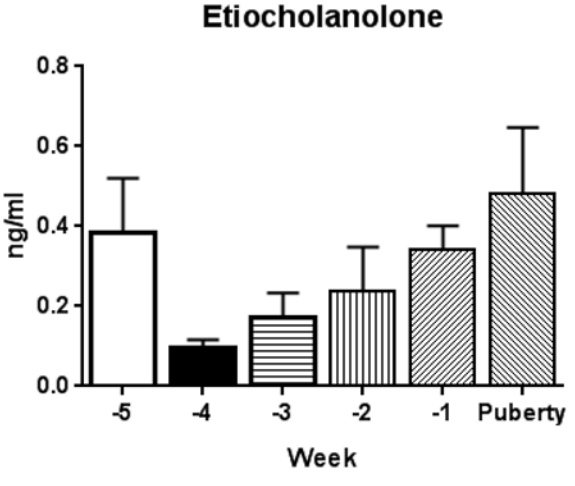

ADIOL
0.15
0.10
0.05
0.00
ng/ml
-5
-4
-3
-2
-1
Puberty
Week

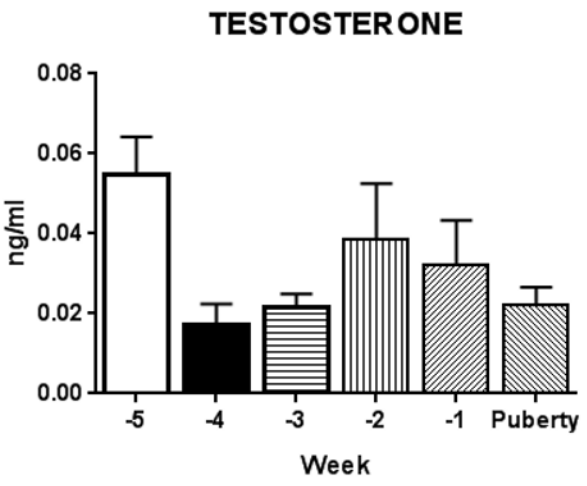

ADIONE
1.0
0.8
0.6
0.4
0.2
0.0
ng/ml
-5
-4
-3
-2
-1
Puberty
Week

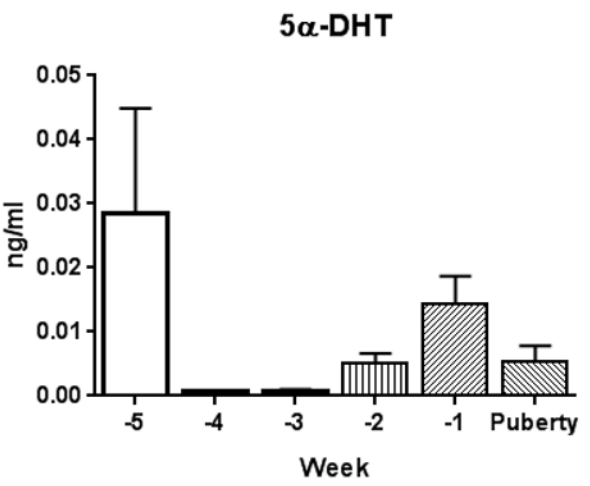

5α-ADIONE
0.15
0.10
0.05
0.00
ng/ml
-5
-4
-3
-2
-1
Puberty
Week

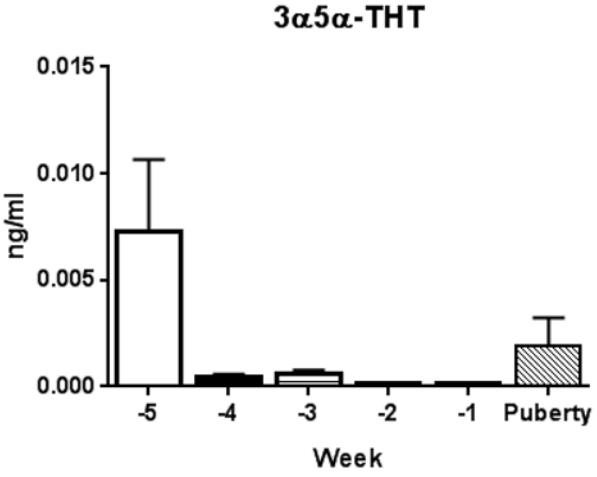

5β-ADIONE
0.3
0.2
0.1
0.0
ng/ml
-5
-4
-3
-2
-1
Puberty
Week

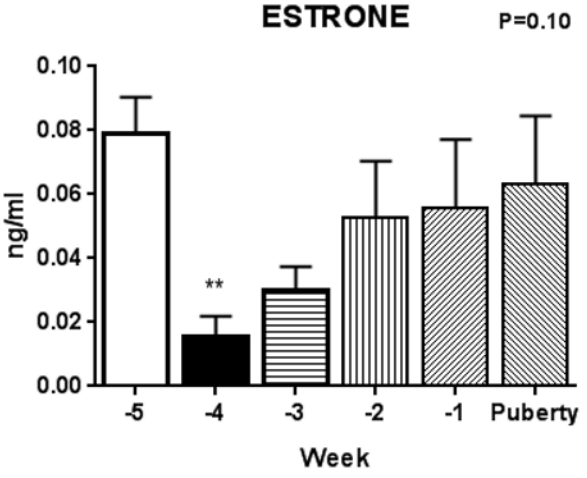

Epiandrosterone
0.06
0.04
0.02
0.00
ng/ml
-5
-4
-3
-2
-1
Puberty
Week

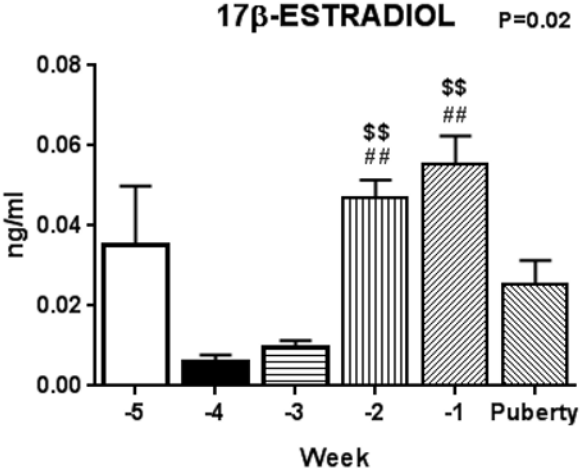

Etiocholanolone
0.8
0.6
0.4
0.2
0.0
ng/ml
-5
-4
-3
-2
-1
Puberty
Week


TESTOSTERONE
0.08
0.06
0.04
0.02
0.00
ng/ml
-5
-4
-3
-2
-1
Puberty
Week

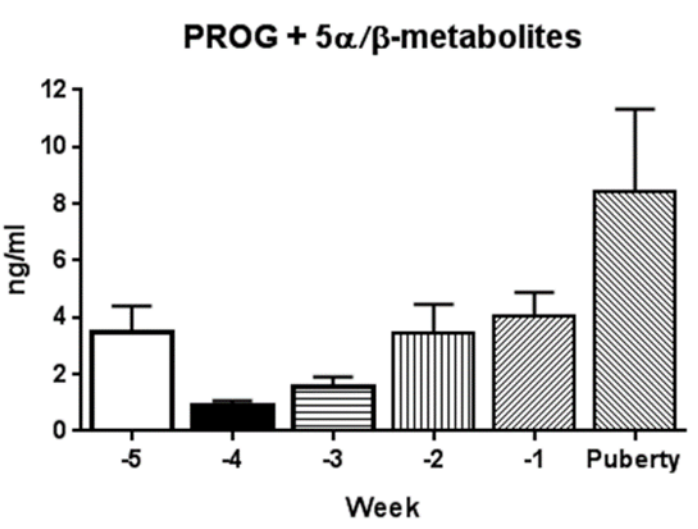

5α-DHT
0.05
0.04
0.03
0.02
0.01
0.00
ng/ml
-5
-4
-3
-2
-1
Puberty
Week


3α5α-THT
0.015
0.010
0.005
0.000
ng/ml
-5
-4
-3
-2
-1
Puberty
Week

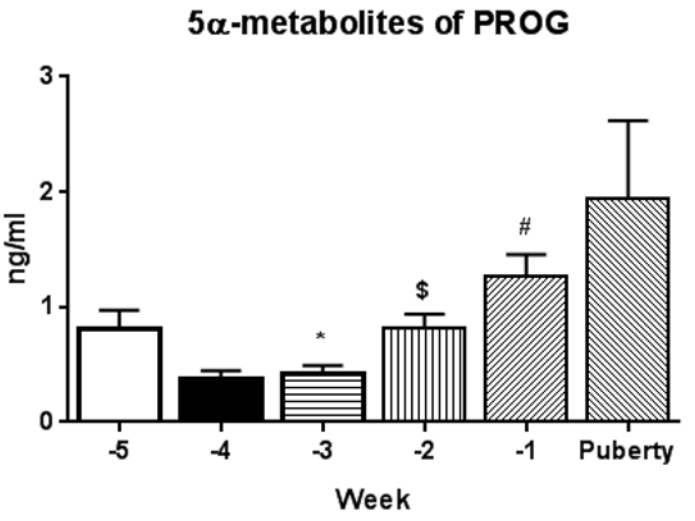

ESTRONE
P=0.10
0.10
0.08
0.06
0.04
0.02
0.00
ng/ml
**
-5
-4
-3
-2
-1
Puberty
Week


17β-ESTRADIOL
P=0.02
0.08
0.06
0.04
0.02
0.00
ng/ml
$$
##
$$
##
-5
-4
-3
-2
-1
Puberty
Week

P=0.08



P = 0.09



P = 0.06