Steroidome and metabolome analysis in gilt saliva to identify potential biomarkers of boar effect receptivity

Ghylène Goudet, Armelle Prunier, Lydie Nadal-Desbarats, Doryan Grivault, Stéphane Ferchaud, A. Pianos, L. Haddad, F. Montigny, Cécile Douet, Jonathan Savoie, et al.

Animal 15 (2021) 100095

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Steroidome and metabolome analysis in gilt saliva to identify potential biomarkers of boar effect receptivity

G. Goudet [a,*], A. Prunier [b], L. Nadal-Desbarats [c], D. Grivault [d], S. Ferchaud [d], A. Pianos [e], L. Haddad [e], F. Montigny [c], C. Douet [a], J. Savoie [f], F. Maupertuis [g], A. Roinsard [h], S. Boulot [i], P. Liere [e]

[a] PRC, INRAE, CNRS, IFCE, Université de Tours, 37380 Nouzilly, France
[b] PEGASE, INRAE, Institut Agro, 35590 Saint-Gilles, France
[c] UMR 1253, iBrain, Université de Tours, Inserm, 37000 Tours, France
[d] GENESI, INRAE, 86480 Rouillé, France
[e] U1195 INSERM Université Paris Saclay, 94276 Kremlin Bicêtre, France
[f] PAO, INRAE, 37380 Nouzilly, France
[g] Chambre d'agriculture Pays de la Loire, 44150 Ancenis, France
[h] ITAB, 49105 Angers, France
[i] IFIP Institut du Porc, 35650 Le Rheu, France

ARTICLE INFO

Article history:
Received 2 June 2020
Received in revised form 15 September 2020
Accepted 21 September 2020
Available online 16 December 2020

Keywords:
Male effect
Metabolites
Porcine
Saliva
Steroids

ABSTRACT

Optimal management of gilt reproduction requires oestrus synchronization. Hormonal treatments are used for this purpose, but there is a growing demand for non-hormonal alternatives, especially in organic farms. The boar effect is an important alternative opportunity to induce and synchronize oestrus without hormones. Before puberty, gilts exhibit a 'waiting period' during which boar exposure could induce and synchronize the first ovulation. We searched for salivary biomarkers of this period of boar effect receptivity to improve detection of the gilts to stimulate with the perspective of enhancing the efficacy of the boar effect. Saliva samples were collected from 30 Large-White × Landrace crossbred gilts between 140 and 175 days of age. Gilts were exposed twice a day to a boar and subjected to oestrus detection from 150 to 175 days of age. Among the 30 gilts, 10 were detected in oestrus 4 to 7 days after the first introduction of the boar and were considered receptive to the boar effect, 14 were detected in oestrus more than 8 days after first boar contact, and six did not show oestrus and were considered non-receptive. Saliva samples from six receptive and six non-receptive gilts were analyzed for steroidome and for metabolome using gas chromatography coupled to tandem mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy, respectively. Four saliva samples per gilt were analyzed: 25 days and 11 days before boar introduction, the day of boar introduction, 3 days later for receptive gilts or 7 days later for non-receptive gilts. Twenty-nine steroids and 31 metabolites were detected in gilt saliva. Salivary concentrations of six steroids and three metabolites were significantly different between receptive and non-receptive gilts: progesterone and glycolate 25 days before boar introduction, 3α5β20α- and 3β5α20β-hexahydroprogesterone, dehydroepiandrosterone, androstenediol, succinate, and butyrate 11 days before boar introduction, and 3β5α-tetrahydroprogesterone on the day of boar introduction. Thus, nine potential salivary biomarkers of boar effect receptivity were identified in our experimental conditions. Further studies with higher numbers of gilts and salivary sampling points are necessary to ascertain their reliability.

Implications

The boar effect is a tool to stimulate puberty and synchronize oestrus cycles without hormones. This pilot study identifies potential salivary biomarkers of the period during which gilts are receptive to the boar effect. These biomarkers would help to guide the choice of the optimal timing to present the boar to prepubertal gilts, and thus enhance the efficacy of the boar effect. Enhancing the development of non-hormonal and non-invasive tools for oestrus induction and synchronization will lead to a decrease of the use of synthetic progestogens and a reduction of environment contamination and would be a major advance for gilt management in organic farms where synthetic hormones are forbidden.

* Corresponding author at: PRC, INRAE, 37380 Nouzilly, France.
E-mail address: ghylene.goudet@inrae.fr (G. Goudet).

https://doi.org/10.1016/j.animal.2020.100095

Introduction

The pig industry is of great economic importance, but its sustainability is questioned. Hormonal treatments, based mainly on progestogens, are generally used to synchronize oestrus and ovulation for gilt batch management. However, the wide use of synthetic progestogens results in environmental pollution from animal manure and urine that contain steroids and their metabolites. These molecules have recognized adverse effects on human and wildlife endocrine systems (Liu et al., 2012a, 2012b and 2015; Zhang et al., 2019). The development of environmental friendly production systems is an important issue. In organic farms, synthetic hormones are prohibited, and farmers face a challenge to effectively conduct reproduction of sows in batches and to maintain synchrony. Batch management is needed for all-in/all-out rearing and separation of age groups for biosecurity, easy cross-fostering, labor planning, and management. Hence, the development of non-hormonal tools for estrous synchronization in gilts is an important issue for improving sustainability of livestock production.

Socio-sexual signals, such as the male effect, offer alternatives to hormonal techniques. Twice daily boar exposure is able to stimulate and synchronize oestrus in gilts (Hughes et al., 1990; Hughes and Thorogood, 1999). Before puberty, gilts exhibit a 'waiting period' defined by the development of ovarian follicles but a relatively low gonadotrophin secretion (Camous et al., 1985; Prunier et al., 1993). During the 'waiting period', external stimulations, such as boar exposure, could easily trigger the first ovulation and hence synchronize gilts (Camous et al., 1985; Hughes et al., 1990; Prunier et al., 1993). The discovery of a biomarker that can identify this receptive period in gilts would guide the choice of the optimal timing to present the boar to prepubertal gilts, and thus enhance the efficacy of the boar effect. Unlike blood, saliva is easily accessible and can be collected in a noninvasive manner without stress or pain. Saliva is now widely used as an alternative diagnostic fluid for the search of biomarkers in human (Choi et al., 2019; Klimiuk et al., 2019; Liang and Lu, 2019). Thus, we intended to search for biomarkers of the period of boar effect receptivity in gilt saliva for a welfare and environment friendly production system.

Omics approaches are powerful tools to identify large numbers of biomolecules in body fluids. Steroidomics allows the detection of a whole panel of steroid hormones and their precursors and metabolites. Progestogens and estrogens are produced by the ovaries, and their level varies with sexual maturation in mammals. Therefore, they could be relevant biomarkers of the period of receptivity to the male effect. We previously developed an analytical protocol, based on gas chromatography coupled to tandem mass spectrometry analysis (**GC–MS/MS**) for screening and quantification of steroid hormones and their precursors and metabolites (progestogens, androgens, estrogens, gluco- and mineralocorticoids, steroid sulfates) in small volumes of biological samples (Liere et al., 2004 and 2009; Goudet et al., 2019b). Gas chromatography coupled to tandem mass spectrometry is the analytical technology of reference to determine steroids in biological matrix thanks to its high specificity, sensitivity, precision, and accuracy. Metabolomics is the non-targeted identification and quantification of low-molecular weight compounds depicting a specific physiological state. ^{1}H nuclear magnetic resonance (**NMR**) is one of the principal analytical techniques for metabolome analysis, and we recently developed NMR analysis of pig saliva metabolome (Goudet et al., 2019a). This is a stable, accurate, robust, and repeatable technique. Our objective was to use both GC–MS/MS and NMR to search for salivary biomarkers of boar effect receptivity with the perspective of enhancing the efficacy of the boar effect.

Material and methods

Animals, housing, and sample collection

This experiment was conducted from August to October in our experimental farm (GENESI, INRAE, France). We performed all procedures on animals in accordance with the guiding principles for the care and use of animals in research facilities and the animal welfare and ethic committee approved the work. Thirty 120- to 123-day-old Large-White × Landrace crossbred gilts were penned in groups of six females (floor area for six gilts: 35m^2) on straw bedding under natural daylight. They were fed with 2 kg concentrate (Arrive Nutrition Animale, 85250 Saint Fulgent, France) per animal once a day and had free access to water. Trans-abdominal ultrasonography was carried out once a week until 148–151 days of age to assess the physiological status and ascertain that all females were immature based on uterus and ovaries development, as previously described (Goudet et al., 2019a).

Starting at 120–123 days of age, salivary samples were collected for each gilt in the morning before feeding, twice a week. Saliva samples were collected using a cotton swab (Sarstedt Salivette® ref. 51.1534, Sarstedt, Nümbrecht, Germany) held gently with forceps in the gilt mouth that chewed until it was soaked. Saliva samples were brought to the lab on ice, centrifuged at 3000 g for 5 min at 4 °C, within 30 min after collection, aliquoted, and stored at −80 °C until analysis. We used one aliquot for metabolomics analysis and the other for steroidomics analysis. Starting at 152–155 days of age, gilts were exposed twice a day to a mature boar in the gilt pen to induce the male effect. The boar was in contact with the group of six gilts for 30 min and a technician ensured that each gilt was in full physical contact with the boar for 5 min. At the same time, gilts were subjected to the back pressure test to detect oestrus behavior. Gilts were considered to be in oestrus when standing completely still and locked up in response to the back pressure test during boar exposure. Gilts that expressed oestrus within 7 days after the first introduction of the boar were considered receptive to the boar effect. Gilts were slaughtered at 175–178 days of age for puberty confirmation and the genital tract was carefully examined as previously described (Goudet et al., 2019a). For each gilt, saliva was analyzed at four different times: 25 days before boar introduction to search for very early candidates (B-25; 126–129 days of age), 11 days before boar introduction to search for early candidates (B-11; 140–143 days), the day of boar introduction to search for simultaneous candidates (B0; 151–154 days), 3 days later for receptive gilts to investigate biomarker concentration just after the boar effect (B3; 154–157 days) or 7 days later for non-receptive gilts to search for the emergence of delayed candidates (B7; 158–161 days).

Steroidomics analysis by gas chromatography coupled to tandem mass spectrometry

The analytical protocol for steroid analysis in gilt saliva by GC–MS/MS was previously described by Goudet and collaborators (Goudet et al., 2019b) and was used here with minor modifications. Steroids were extracted from gilt saliva (329–496 µl) with 5 ml of MeOH. Internal standards were added into the saliva extract for endogenous steroid quantification. All the targeted steroids and their respective internal standards are indicated in Table 1. The parameters of purification, fractionation, derivatization, and analytical steps of the GC–MS/MS protocol were described previously (Goudet et al., 2019b).

Metabolomics analysis using ^{1}H-nuclear magnetic resonance

The ^{1}H NMR analysis of saliva samples was described previously by Goudet and collaborators (Goudet et al., 2019a). Briefly, saliva samples were thawed at room temperature and 150 µl were added to 100 µl of 0.2 M potassium phosphate buffer in deuterium oxide (**D_2O**) 99%. Eight µl of 3-trimethylsilylpropionic acid (0.05% wt in D_2O) were added to samples as an internal reference. The ^{1}H NMR spectra were obtained with a DRX-600 Bruker spectrometer (Bruker, Sadis, Wissembourg, France) and post-processed as previously described

Table 1
Targeted steroids analyzed by gas chromatography coupled to tandem mass spectrometry in gilts saliva and their abbreviation.

Targeted steroids	Abbreviation	Internal standard (IS)	IS amount	Detected
C21-Steroids				
Pregnenolone	PREG	Epietiocholanolone	2 ng	Yes
Pregnenolone sulfate	PREGS	$^{2}H_{2}^{13}C_{2}$-PREGS	2 ng	Yes
20α-dihydropregnenolone	20α-DHPREG	Epietiocholanolone	2 ng	Yes
17α-hydroxypregnenolone	*17α-OH PREG*	$^{13}C_{3}$-17α-OH PROG	2 ng	*No*
Progesterone	PROG	$^{13}C_{3}$-PROG	2 ng	Yes
5α-dihydroprogesterone	5α-DHPROG	$^{2}H_{6}$-5α-DHPROG	2 ng	Yes
5β-dihydroprogesterone	5β-DHPROG	$^{2}H_{6}$-5α-DHPROG	2 ng	Yes
20α-dihydroprogesterone	20α-DHPROG	19-nor-PROG	2 ng	Yes
20β-dihydroprogesterone	20β-DHPROG	19-nor-PROG	2 ng	Yes
3α5α-tetrahydroprogesterone	3α5α-THPROG	Epietiocholanolone	2 ng	Yes
3α5β-tetrahydroprogesterone	3α,5β-THPROG	Epietiocholanolone	2 ng	Yes
3β5α-tetrahydroprogesterone	3β5α-THPROG	Epietiocholanolone	2 ng	Yes
5α20α-tetrahydroprogesterone	*5α20α-THPROG*	Epietiocholanolone	2 ng	*No*
3α5α20α-hexahydroprogesterone	3α5α20α-HHPROG	Epietiocholanolone	2 ng	Yes
3α5β20α-hexahydroprogesterone	3α5β20α-HHPROG	Epietiocholanolone	2 ng	Yes
3β5α20α-hexahydroprogesterone	3β5α20α-HHPROG	Epietiocholanolone	2 ng	Yes
3β5α20β-hexahydroprogesterone	3β5α20β-HHPROG	Epietiocholanolone	2 ng	Yes
17α-hydroxyprogesterone	17α-OH PROG	$^{13}C_{3}$-17α-OH PROG	2 ng	Yes
16α-hydroxyprogesterone	*16α-OH PROG*	$^{13}C_{3}$-17α-OH PROG	2 ng	*No*
Deoxycorticosterone	*DOC*	$^{13}C_{3}$-DOC	2 ng	*No*
5α-dihydrodeoxycorticosterone	*5α-DHDOC*	19-nor-PROG	2 ng	*No*
5β-dihydrodeoxycorticosterone	*5β-DHDOC*	19-nor-PROG	2 ng	*No*
3α5α-tetrahydrodeoxycorticosterone	*3α5α-THDOC*	Epietiocholanolone	2 ng	*No*
3β5α-tetrahydrodeoxycorticosterone	*3β5α-THDOC*	Epietiocholanolone	2 ng	*No*
3α5β-tetrahydrodeoxycorticosterone	*3α5β-THDOC*	Epietiocholanolone	2 ng	*No*
Corticosterone	*B*	$^{2}H_{8}$-B	5 ng	*No*
5α-dihydrocorticosterone	*5α-DHB*	$^{2}H_{8}$-B	5 ng	*No*
5β-dihydrocorticosterone	*5β-DHB*	$^{2}H_{8}$-B	5 ng	*No*
3α5α-tetrahydrocorticosterone	*3α5α-THB*	$^{2}H_{8}$-B	5 ng	*No*
3β5α-tetrahydrocorticosterone	*3β5α-THB*	$^{2}H_{8}$-B	5 ng	*No*
3α5β-tetrahydrocorticosterone	*3α5β-THB*	$^{2}H_{8}$-B	5 ng	*No*
C19-Steroids				
Dehydroepiandrosterone	DHEA	Epietiocholanolone	2 ng	Yes
Dehydroepiandrosterone sulfate	DHEAS	$^{2}H_{6}$-DHEAS	5 ng	Yes
Androstenediol	ADIOL	Epietiocholanolone	2 ng	Yes
Androstenedione	ADIONE	$^{13}C_{3}$-ADIONE	2 ng	Yes
5α-androstane-3,17-dione	5α-DHADIONE	$^{2}H_{6}$-5α-DHPROG	2 ng	Yes
5β-androstane-3,17-dione	5β-DHADIONE	$^{2}H_{6}$-5α-DHPROG	2 ng	Yes
Epiandrosterone	Epiandrosterone	Epietiocholanolone	2 ng	Yes
Etiocholanolone	Etiocholanolone	Epietiocholanolone	2 ng	Yes
Testosterone	T	$^{13}C_{3}$-T	2 ng	Yes
5α-dihydrotestosterone	5α-DHT	Epietiocholanolone	2 ng	Yes
5β-dihydrotestosterone	*5β-DHT*	Epietiocholanolone	2 ng	*No*
3α5α-tetrahydrotestosterone	*3α5α-THT*	Epietiocholanolone	2 ng	*No*
3β5α-tetrahydrotestosterone	*3β5α-THT*	Epietiocholanolone	2 ng	*No*
3α5β-tetrahydrotestosterone	*3α5β-THT*	Epietiocholanolone	2 ng	*No*
C18-Steroids				
Oestrone	E1	$^{13}C_{3}$-E1	2 ng	Yes
17β-oestradiol	17β-E2	$^{13}C_{3}$-17β-E2	2 ng	Yes
Oestriol	E3	$^{13}C_{3}$-E3	1 ng	Yes
2-methoxyoestradiol	*2-ME2*	$^{12}H_{5}$-2ME2	1 ng	*No*

The internal standards (IS) and their respective amounts added in the saliva extract are indicated for each targeted steroid. Steroids are classified according to their chemical structure, i.e. their carbon number. Among the 49 targeted steroids, 29 were detected and quantified. Steroids in italics were not detected.

(Goudet et al., 2019a). The NMR assignment was done using spectra online databases as HMDB (http://www.hmdb.ca) and ChenomX NMR Suite 8.1 evaluation edition (ChenomX, Inc., Edmonton, Canada).

Statistical analysis

The normality of the data was checked with the Kolmogorov–Smirnov test. The comparison of the absolute concentrations of steroids and metabolites between receptive and non-receptive gilts was performed using repeated measures two-way ANOVA, followed by a post-hoc Fisher multiple comparisons test, using GraphPad Prism version 6 (GraphPad Software, La Jolla California, USA) to unveil potential differences at each time point. A difference between receptive and non-receptive gilts was considered to be significant if $P < 0.05$ and to tend to be different if $P < 0.1$.

Results

Reproductive response to the male effect and sample selection

Trans-abdominal ultrasonography showed that all 30 females were immature before introducing the male at B0. Ten gilts (10/30, 33%) started to show a standing oestrus response to the male effect 4 to 7 days after the first introduction of the boar and were considered receptive to the boar effect (Fig. 1). Fourteen gilts started to show a standing oestrus response more than 8 days after the first introduction of the boar (Fig. 1). Analysis of the genital tracts at slaughter confirmed that all these gilts had ovulated (presence of early corpora lutea). Six gilts (6/30, 20%) did not show oestrus behavior and were considered non-receptive to the boar effect. Their genital tract was still immature at slaughter (no corpus luteum was observed and uterine horns were not developed). Saliva samples from the six non-receptive gilts and

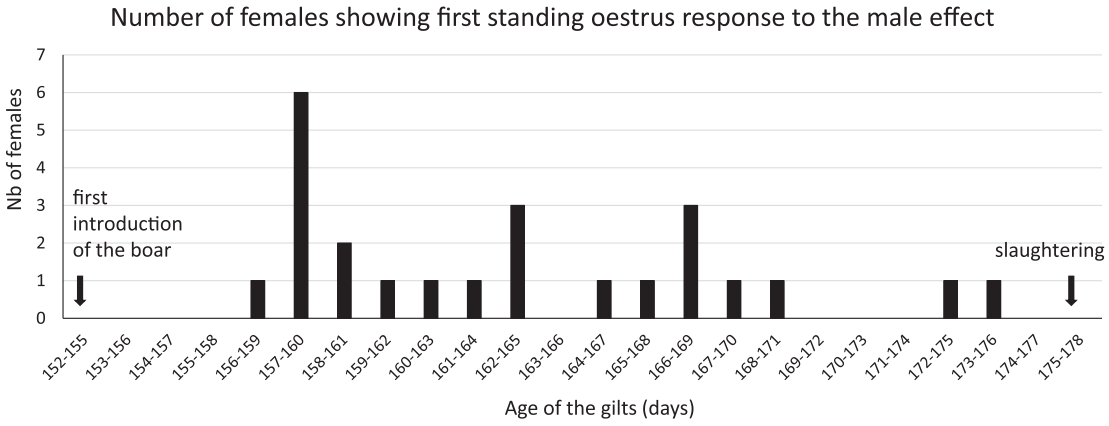

Fig. 1. Number of females showing first standing oestrus response after being in contact twice a day with a boar.

the six receptive gilts showing standing oestrus response 5 days after boar introduction were analyzed for steroidome by GC–MS/MS and for metabolome by ^{1}H NMR spectroscopy.

Steroidome analysis in gilt saliva

Among the 49 targeted steroids, GC–MS/MS analysis allowed the detection of 29 steroids in saliva (Table 2). Salivary concentrations were significantly different between receptive and non-receptive gilts for six steroids (Table 2, Fig. 2). The concentration of progesterone (**PROG**) was significantly higher in receptive than in non-receptive gilts 25 days before boar introduction (B-25) ($P < 0.05$; Fig. 2). The concentrations of 3α5β20α-hexahydroprogesterone (**HHPROG**) and 3β5α20β-HHPROG, dehydroepiandrosterone (**DHEA**), and androstenediol (**ADIOL**) were significantly higher in receptive than in non-receptive gilts 11 days before boar introduction (B-11) ($P < 0.05$; Fig. 2). The concentration of 3β5α-tetrahydroprogesterone (**THPROG**) was significantly higher in receptive than in non-receptive gilts on the day of boar introduction (B0) ($P < 0.05$; Fig. 2). The salivary concentration of the other 23 steroids was not significantly different between receptive and non-receptive gilts (Table 2, Supplementary Figure S1).

When combining progesterone and its 5α/β-reduced metabolites (5α-dihydroprogesterone (**DHPROG**), 5β-DHPROG, 3α5α-THPROG, 3α5β-THPROG, 3β5α-THPROG, 3α5α20α-HHPROG, 3α5β20α-HHPROG, 3β5α20α-HHPROG, and 3β5α20β-HHPROG), the concentration tended to be higher in receptive than in non-receptive gilts (Table 2, Fig. 3). However, the concentration of the 5α-reduced metabolites of PROG (5α-DHPROG, 3α5α-THPROG, 3β5α-THPROG, 3α5α20α-HHPROG, 3β5α20α-HHPROG, and 3β5α20β-HHPROG) was significantly higher in receptive than in non-receptive gilts 11 days before boar introduction (B-11) ($P < 0.05$; Table 2, Fig. 3). On the contrary, no difference was found for the 5β-reduced metabolites of PROG between the two groups of gilts (Table 2, Fig. 3).

Metabolome analysis in gilt saliva

Saliva metabolome ^{1}H NMR analysis allowed the identification of 35 spectral regions. Among them, 31 metabolites were identified as single metabolite (formate, benzoate, sucrose, glucose, tartrate, for example) or an overlapping of metabolites (kynurenine_benzoate, and glucose_lactose, for example) (Table 3). The metabolites included organic acids (formate, benzoate, pyruvate, tartrate, acetate, malonate, butyrate, lactate, succinate, propionate), amino acids (alanine, threonine, valine, leucine), organic compounds (betaine, creatine, choline), and sugars (sucrose, glucose, lactose).

Salivary concentrations were significantly different between receptive and non-receptive gilts for three metabolites (Table 3, Fig. 4). The concentration of glycolate was significantly lower in receptive than in non-receptive gilts 25 days before boar introduction (B-25) ($P < 0.05$; Fig. 4). Eleven days before boar introduction (B-11), the concentration of succinate was significantly lower and the concentration of butyrate was significantly higher in receptive than in non-receptive gilts ($P < 0.01$; Fig. 4). The salivary concentrations of the other 28 metabolites were not significantly different between receptive and non-receptive gilts (Table 3, Supplementary Figure S2).

Discussion

Our objective was to identify salivary biomarkers of the period of boar effect receptivity to improve the detection of gilts sensitive to the effect that are characterized by an early puberty attainment. We used omics approaches, steroidomics and metabolomics, to search for such salivary candidate biomarkers.

Daily exposure of pre-pubertal gilts to a mature boar (the boar effect) is regularly used to induce and synchronize puberty (Pearce and Hughes, 1985). Twice daily boar exposure significantly increased the proportion of gilts reaching puberty in the first 15 days compared with either once-daily boar contact or no boar contact (Hughes and Thorogood, 1999). Moreover, full physical contact with the boar is more effective as a puberty stimulation technique than fence-line contact. Indeed, the boar effect is mediated through the synergistic actions of visual, tactile, olfactory, and auditory stimuli, and only mature boars are able to induce puberty in gilts (Hughes et al., 1990). Thus, in our study, gilts were exposed twice a day to a mature boar introduced in their pen to obtain optimal conditions for the boar effect. Boar exposure started when the gilts were 152 to 155-day-old, in order to obtain two groups of gilts, the gilts receptive to the boar effect and the non-receptive ones. This allowed us to obtain 33% of the gilts showing standing oestrus response in the first 7 days, when they were 154 to 160-day-old (receptive group), and 20% of the gilts showing no oestrus behavior until experiment end at 175–178 days of age (non-receptive group). In our previous study, gilts without boar contact reached puberty at a mean age of 189 ± 1.5 days (Goudet et al., 2019b). Thus, in our conditions, boar exposure was able to stimulate first oestrus for gilts receptive to boar effect.

The response of the gilts to boar contact was reported to be variable, reflecting internal (breed, age, live weight, backfat depth) and management factors (nutrition, housing and climatic environment) (Hughes et al., 1990). These factors are mediated via the endocrine reproductive axis and reflected in hormonal changes that precede puberty (Evans and O'Doherty, 2001). Our hypothesis was that these changes could be detected in the composition of gilt saliva.

In a previous study, we used a GC–MS/MS steroidomic approach to identify and quantify steroid hormones and their precursors and

Table 2
Steroids profiling in non-receptive and receptive gilt saliva collected 25 days and 11 days before boar introduction (B-25 and B-11), the day of boar introduction (B0), 3 days later for receptive gilts (B3), or 7 days later for non-receptive gilts (B7). Results are presented as mean (ng/ml) ± SEM. See Table 1 for the steroid definitions. The comparison of the absolute concentrations of steroids between receptive and non-receptive gilts was performed using repeated measures two-way ANOVA, followed by a post hoc Fisher multiple comparisons test to detect the significant differences at each time point between the two groups of gilts.

Steroids	Groups ($n = 6$)	Mean concentration (ng/ml) ± SEM			
		B-25	B-11	B0	B3B7
C21-Steroids					
PREG	Non-receptive	0.70 ± 0.115	0.45 ± 0.068	0.465 ± 0.081	0.553 ± 0.067
	Receptive	0.555 ± 0.052	0.478 ± 0.057	0.587 ± 0.095	0.553 ± 0.080
PREGS	Non-receptive	0.061 ± 0.011	0.064 ± 0.019	0.060 ± 0.019	0.070 ± 0.016
	Receptive	0.069 ± 0.012	0.062 ± 0.019	0.094 ± 0.019	0.086 ± 0.037
20α-DHPREG	Non-receptive	0.049 ± 0.009	0.047 ± 0.008	0.053 ± 0.008	0.057 ± 0.007
	Receptive	0.054 ± 0.005	0.060 ± 0.006	0.054 ± 0.008	0.069 ± 0.012
PROG	Non-receptive	0.210 ± 0.032[a]	0.184 ± 0.043	0.254 ± 0.040	0.271 ± 0.077
	Receptive	*0.369 ± 0.065*[b]	0.255 ± 0.030	0.284 ± 0.045	0.324 ± 0.052
5α-DHPROG	Non-receptive	0.402 ± 0.048	0.267 ± 0.045	0.322 ± 0.053	0.375 ± 0.115
	Receptive	0.470 ± 0.082	0.347 ± 0.042	0.412 ± 0.065	0.538 ± 0.100
5β-DHPROG	Non-receptive	0.032 ± 0.009	0.024 ± 0.007	0.049 ± 0.012	0.035 ± 0.017
	Receptive	0.021 ± 0.006	0.023 ± 0.002	0.084 ± 0.026	0.042 ± 0.012
20α-DHPROG	Non-receptive	0.044 ± 0.013	0.025 ± 0.008	0.029 ± 0.003	0.033 ± 0.006
	Receptive	0.058 ± 0.015	0.034 ± 0.009	0.036 ± 0.003	0.025 ± 0.005
20β-DHPROG	Non-receptive	0.023 ± 0.006	0.013 ± 0.004	0.020 ± 0.004	0.012 ± 0.005
	Receptive	0.032 ± 0.013	0.016 ± 0.005	0.015 ± 0.004	0.025 ± 0.006
3α5α-THPROG	Non-receptive	0.036 ± 0.013	0.042 ± 0.017	0.098 ± 0.044	0.061 ± 0.028
	Receptive	0.049 ± 0.014	0.116 ± 0.074	0.065 ± 0.023	0.035 ± 0.015
3α5β-THPROG	Non-receptive	0.126 ± 0.065	0.166 ± 0.050	0.190 ± 0.053	0.123 ± 0.048
	Receptive	0.168 ± 0.062	0.162 ± 0.066	0.140 ± 0.037	0.135 ± 0.049
3β5α-THPROG	Non-receptive	0.022 ± 0.004	0.017 ± 0.003	0.016 ± 0.005[a]	0.021 ± 0.003
	Receptive	0.024 ± 0.006	0.048 ± 0.029	*0.132 ± 0.102*[b]	0.019 ± 0.004
3α5α20α-HHPROG	Non-receptive	0.034 ± 0.009	0.026 ± 0.008	0.027 ± 0.010	0.033 ± 0.010
	Receptive	0.030 ± 0.009	0.065 ± 0.040	0.023 ± 0.007	0.029 ± 0.012
3α5β20α-HHPROG	Non-receptive	0.012 ± 0.002	0.010 ± 0.003[a]	0.009 ± 0.003	0.010 ± 0.003
	Receptive	0.013 ± 0.005	*0.031 ± 0.018*[b]	0.013 ± 0.003	0.010 ± 0.002
3β5α20α-HHPROG	Non-receptive	0.012 ± 0.002	0.013 ± 0.004	0.011 ± 0.003	0.015 ± 0.003
	Receptive	0.014 ± 0.004	0.022 ± 0.010	0.018 ± 0.003	0.012 ± 0.003
3β5α20β-HHPROG	Non-receptive	0.015 ± 0.004	0.012 ± 0.003[a]	0.015 ± 0.003	0.013 ± 0.003
	Receptive	0.033 ± 0.013	*0.050 ± 0.034*[b]	0.025 ± 0.005	0.019 ± 0.003
17α-OH PROG	Non-receptive	0.045 ± 0.008	0.035 ± 0.008	0.044 ± 0.009	0.042 ± 0.018
	Receptive	0.031 ± 0.006	0.018 ± 0.007	0.039 ± 0.010	0.037 ± 0.008
PROG + 5α/β-metabolites	Non-receptive	0.902 ± 0.111	0.760 ± 0.157	0.990 ± 0.165	0.958 ± 0.237
	Receptive	1.191 ± 0.172	1.119 ± 0.264	1.196 ± 0.183	1.165 ± 0.198
5α-metabolites of PROG	Non-receptive	0.336 ± 0.125	0.234 ± 0.060[a]	0.337 ± 0.098	0.348 ± 0.188
	Receptive	0.619 ± 0.111	*0.648 ± 0.180*[b]	0.676 ± 0.124	0.654 ± 0.118
5β-metabolites of PROG	Non-receptive	0.170 ± 0.162	0.201 ± 0.054	0.248 ± 0.063	0.168 ± 0.047
	Receptive	0.203 ± 0.068	0.215 ± 0.080	0.237 ± 0.058	0.187 ± 0.053
C19-Steroids					
DHEA	Non-receptive	0.097 ± 0.007	0.073 ± 0.010[a]	0.083 ± 0.012	0.106 ± 0.008
	Receptive	0.083 ± 0.010	*0.262 ± 0.177*[b]	0.090 ± 0.012	0.094 ± 0.023
DHEAS	Non-receptive	0.082 ± 0.033	0.080 ± 0.025	0.106 ± 0.013	0.124 ± 0.016
	Receptive	0.096 ± 0.019	0.087 ± 0.031	0.128 ± 0.061	0.090 ± 0.018
ADIOL	Non-receptive	0.051 ± 0.004	0.062 ± 0.012[a]	0.055 ± 0.006	0.058 ± 0.007
	Receptive	0.047 ± 0.008	*0.102 ± 0.032*[b]	0.047 ± 0.006	0.038 ± 0.004
ADIONE	Non-receptive	0.220 ± 0.036	0.152 ± 0.061	0.186 ± 0.042	0.172 ± 0.035
	Receptive	0.388 ± 0.127	0.187 ± 0.029	0.186 ± 0.052	0.245 ± 0.050
5α-DHADIONE	Non-receptive	0.105 ± 0.020	0.112 ± 0.019	0.108 ± 0.021	0.112 ± 0.028
	Receptive	0.095 ± 0.021	0.115 ± 0.019	0.120 ± 0.025	0.105 ± 0.014
5β-DHADIONE	Non-receptive	0.218 ± 0.049	0.216 ± 0.040	0.205 ± 0.038	0.209 ± 0.045
	Receptive	0.212 ± 0.048	0.184 ± 0.030	0.269 ± 0.061	0.172 ± 0.019
Epiandrosterone	Non-receptive	0.013 ± 0.002	0.012 ± 0.003	0.007 ± 0.001	0.012 ± 0.001
	Receptive	0.013 ± 0.003	0.021 ± 0.009	0.010 ± 0.002	0.011 ± 0.002
Etiocholanolone	Non-receptive	0.040 ± 0.009	0.077 ± 0.021	0.069 ± 0.016	0.045 ± 0.013
	Receptive	0.058 ± 0.019	0.134 ± 0.081	0.065 ± 0.013	0.041 ± 0.005
T	Non-receptive	0.019 ± 0.002	0.016 ± 0.004	0.016 ± 0.003	0.014 ± 0.002
	Receptive	0.040 ± 0.022	0.022 ± 0.004	0.014 ± 0.002	0.013 ± 0.002
5α-DHT	Non-receptive	0.007 ± 0.002	0.011 ± 0.001	0.009 ± 0.001	0.014 ± 0.001
	Receptive	0.007 ± 0.001	0.075 ± 0.067	0.006 ± 0.001	0.008 ± 0.001
C18-Steroids					
E1	Non-receptive	0.033 ± 0.007	0.023 ± 0.004	0.040 ± 0.008	0.043 ± 0.008
	Receptive	0.039 ± 0.005	0.027 ± 0.005	0.032 ± 0.004	0.038 ± 0.008
17β-E2	Non-receptive	0.033 ± 0.004	0.023 ± 0.005	0.028 ± 0.005	0.029 ± 0.004
	Receptive	0.027 ± 0.004	0.025 ± 0.003	0.025 ± 0.004	0.025 ± 0.004
E3	Non-receptive	0.019 ± 0.004	0.009 ± 0.002	0.013 ± 0.003	0.016 ± 0.002
	Receptive	0.021 ± 0.006	0.016 ± 0.004	0.016 ± 0.004	0.015 ± 0.004

The significant differences are indicated in italics. Statistical significance between receptive and non-receptive gilts: [a,b] for $P < 0.05$.

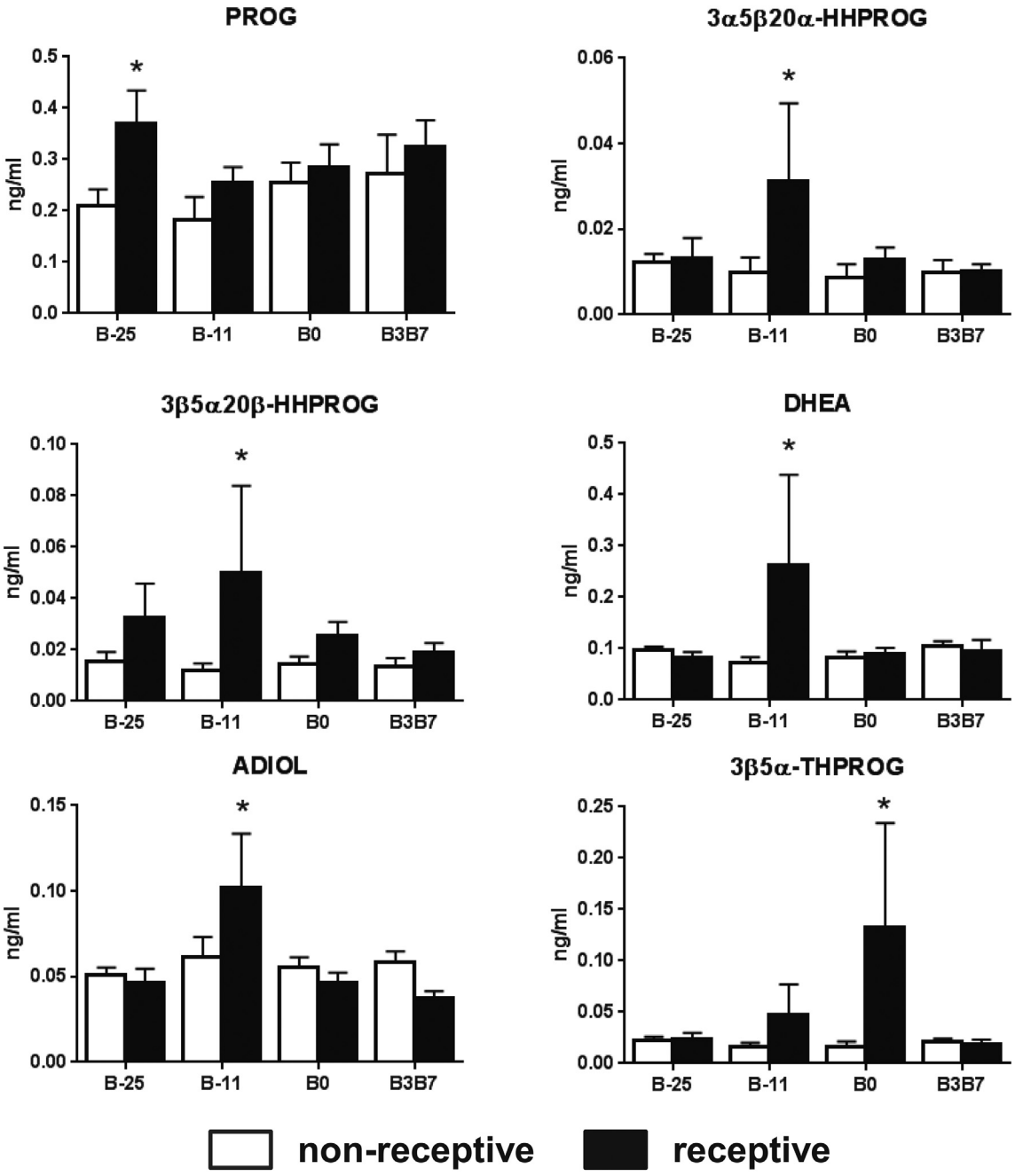

Fig. 2. Gas chromatography coupled to tandem mass spectrometry measurements of the concentrations of progesterone, 3α5β20α-hexahydroprogesterone, 3β5α20β-hexahydroprogesterone, dehydroepiandrosterone, androstenediol, and 3β5α-tetrahydroprogesterone (ng/ml ± SEM) in saliva of receptive and non-receptive gilts collected 25 days and 11 days before boar introduction (B-25 and B-11), the day of boar introduction (B0), 3 days later for receptive gilts (B3), or 7 days later for non-receptive gilts (B7). For each steroid, the y-axis was standardized to the maximal concentration, so that the variations are easy-to-read. PROG: progesterone; HHPROG: hexahydroprogesterone; DHEA: dehydroepiandrosterone; ADIOL: androstènediol; THPROG: tetrahydroprogesterone. *: statistical differences between receptive and non-receptive gilts for each collection day, $P < 0.05$.

metabolites in saliva of Large-White gilts (Goudet et al., 2019b). This methodology allowed the detection of 28 steroids in saliva from immature to pubertal gilts (Goudet et al., 2019b). Here, thanks to this sensitive, precise, and selective analytical methodology suitable for quantifying numerous steroids in small individual samples, we detected 29 among the 49 targeted steroids in Large-White × Landrace gilts saliva. Most of the steroids were identified in both studies, except for 20β-DHPROG, 3β5α20β-HHPROG, 17α-OH PROG, and oestriol detected in the present study but not in the previous one. On the contrary, 5α20α-THPROG, deoxycorticosterone (DOC), and 3α5α-tetrahydrotestosterone (THT) identified in the previous study were not detected in the present one. Steroids concentrations were within the same range in both studies supporting that GC–MS/MS analysis is a reproducible technology and a powerful tool to establish a salivary steroidome in the porcine species.

We developed previously a metabolomic analysis using ^{1}H NMR in saliva from immature to pubertal Large-White gilts (Goudet et al., 2019a). This methodology allowed the detection of 23 metabolites in gilts saliva and 17 of them were identified (Goudet et al., 2019a). Thus, ^{1}H NMR has been shown to be an efficient technique for non-targeted identification of low-molecular-weight metabolites that can represent a signature of a physiological state. In the present study, we identified 31 low-molecular-weight metabolites in Large-White ×

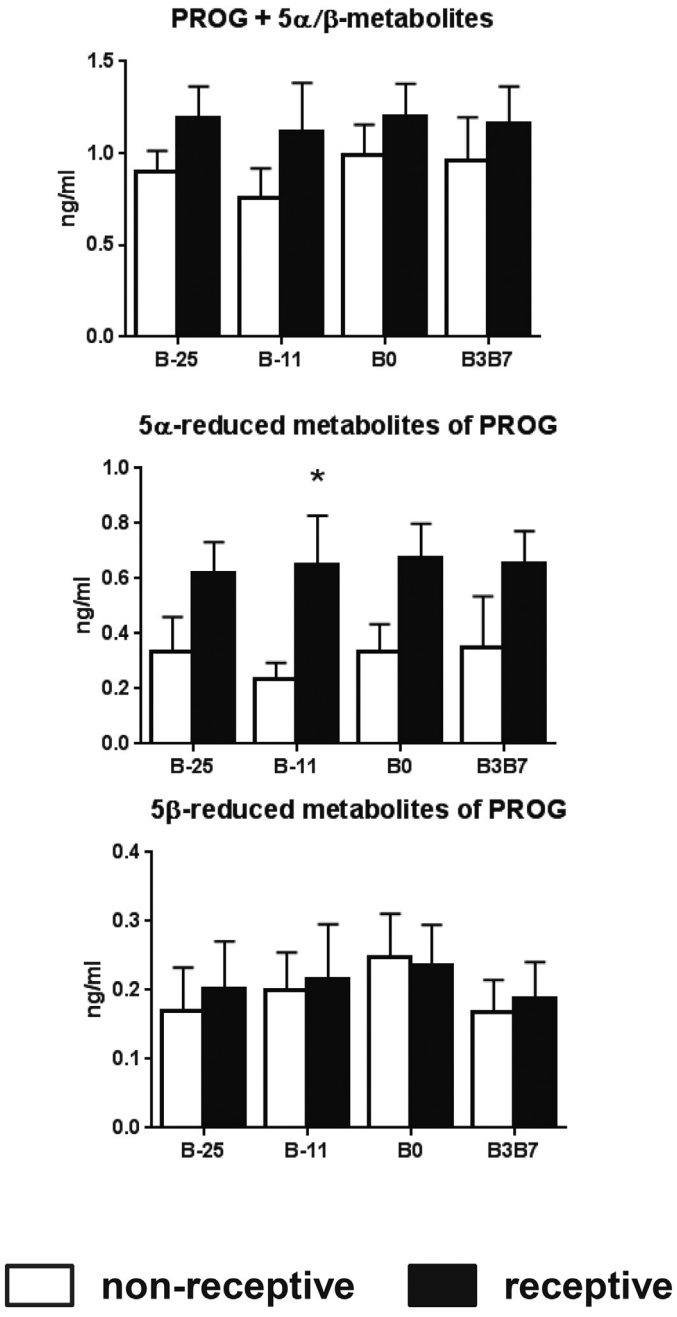

Fig. 3. Gas chromatography coupled to tandem mass spectrometry measurements of the concentrations of progesterone and its 5α- and 5β-reduced metabolites, of 5α-reduced metabolites of progesterone and 5β-reduced metabolites of progesterone (ng/ml ± SEM) in saliva of receptive and non-receptive gilts collected 25 days and 11 days before boar introduction (B-25 and B-11), the day of boar introduction (B0), 3 days later for receptive gilts (B3), or 7 days later for non-receptive gilts (B7). For each group of steroids, the y-axis was standardized to the maximal concentration, so that the variations are easy-to-read. PROG: progesterone. *: statistical differences between receptive and non-receptive gilts for each collection day, $P < 0.05$.

Landrace gilts saliva. Most metabolites identified in the previous study were also detected in the present study, such as formate, sucrose, betaine, choline, malonate, succinate, acetate, butyrate, alanine, and propionate.

In the present study, we used omics approaches to look for potential biomarkers that could differentiate gilts receptive to boar effect from non-receptive gilts. Saliva samples were analyzed 25 days before boar introduction to search for very early candidates, 11 days before boar introduction to search for early candidates and the day of boar introduction to search for simultaneous candidates. Moreover, saliva was analyzed 3 days after boar introduction for receptive gilts to investigate biomarker concentration just after the boar effect, and 7 days after boar introduction for non-receptive gilts to search for the emergence of delayed candidates. In the present study, the concentrations of three metabolites and six steroids were significantly different in receptive gilts compared to non-receptive gilts several days before or on the day of boar introduction. They could be potential salivary biomarkers to detect gilts receptive to boar effect.

Among metabolites, the concentrations of glycolate, succinate, and butyrate were significantly different between receptive and non-receptive gilts 25 and 11 days before boar introduction. In our previous study, succinate and butyrate were detected in gilts saliva before puberty, but their concentrations did not significantly change before puberty (Goudet et al., 2019a). Thus, they may not be reliable markers of the sexual maturation. Moreover, the salivary concentrations of glycolate, succinate, and butyrate showed small differences between receptive and non-receptive gilts. Hence, these metabolites may probably not be reliable candidates to identify biomarkers of the period of boar effect receptivity.

Among steroids, the concentration of four C21-steroids, PROG and three of its reduced metabolites (3α5β20α-HHPROG, 3β5α20β-HHPROG, and 3β5α-THPROG) was significantly higher in receptive gilts compared to non-receptive ones at B-25, B-11, B-11, and B0 respectively. Moreover, the concentration of the combined 5α-reduced metabolites of PROG at B-11 was three times higher in receptive gilts than in non-receptive gilts. We previously showed that the concentration of most of the PROG metabolites, as well as the concentration of the combined 5α-metabolites, increased 3 weeks before puberty, suggesting that they are reliable markers of the sexual maturation in gilts (Goudet et al., 2019b). Progesterone is indeed a sex steroid hormone produced in ovaries and playing a critical role in the modulation of female reproductive physiology and sexual behavior (Graham and Clarke, 1997). Thus, higher levels of PROG and some of its main metabolites in the saliva of receptive gilts could reflect an earlier sexual maturation, which could be related to an earlier receptivity to the boar effect. Consequently, PROG and some of its metabolites could be reliable biomarkers of the period of boar effect receptivity. Interestingly, the relative high concentrations of PROG, 5α-DHPROG, and the 5α-reduced metabolites of PROG (0.2 to 0.6 ng/ml) make feasible an analysis with a commercial technique at low cost. Thus, these biomarkers fit the criteria for a possible application in the field.

Two C-19 precursors of androgens, DHEA and ADIOL, had higher concentrations in receptive gilts at B-11. In our previous study, DHEA was also identified as a potential biomarker of gilts receptivity to the boar effect (Goudet et al., 2019b). However, DHEA concentrations in receptive gilts at B-11 were highly variable and very low. Thus, DHEA suffering from a high inter-individual variability may not be a reliable biomarker to be used in the field. Similarly, ADIOL concentrations were higher in receptive gilts but highly variable, making it also an unreliable candidate. Thus, among the nine potential candidates identified in gilt saliva, PROG and some of its 5α-reduced metabolites are possibly the most reliable biomarkers of the period of boar effect receptivity.

No differences were observed in the steroid and metabolite salivary concentrations between receptive and non-receptive gilts at B3B7. It should be remembered that plasma progesterone starts to increase about 3 days after the first day of oestrus (Knox et al., 2003), and plasma oestradiol has been shown to rise 1 to 4 days before the first day of oestrus (Knox et al., 2003). The receptive gilts may have been sampled just before an expected increase. Moreover, the steroid synthesis in adrenals could interfere with the ovarian secretion such as there are no differences between salivary levels of steroids between gilts in oestrus

Table 3
Metabolites profiling in non-receptive and receptive gilt saliva (arbitrary unit ± SEM) collected 25 days and 11 days before boar introduction (B-25 and B-11), the day of boar introduction (B0), 3 days later for receptive gilts (B3), or 7 days later for non-receptive gilts (B7). The comparison of metabolites between receptive and non-receptive gilts was performed using repeated measures two-way ANOVA, followed by a post hoc Fisher multiple comparisons test to detect the significant differences at each time point between the 2 groups of gilts.

Metabolites	Groups ($n = 6$)	Mean (arbitrary unit) ± SEM			
		B-25	B-11	B0	B3B7
Formate	Non-receptive	0.0659 ± 0.0061	0.0528 ± 0.0075	0.0472 ± 0.0049	0.0629 ± 0.0038
	Receptive	0.0592 ± 0.006	0.0638 ± 0.0096	0.0449 ± 0.0052	0.0552 ± 0.0098
Kynurenine benzoate	Non-receptive	0.0024 ± 0.001	0.0011 ± 0.0002	0.0017 ± 0.0004	0.0011 ± 0.0002
	Receptive	0.0025 ± 0.0004	0.0018 ± 0.0003	0.002 ± 0.0003	0.0019 ± 0.0003
Benzoate uracil	Non-receptive	0.0029 ± 0.0008	0.0013 ± 0.0003	0.0022 ± 0.0004	0.0017 ± 0.0003
	Receptive	0.003 ± 0.0004	0.0021 ± 0.0003	0.0027 ± 0.0004	0.0026 ± 0.0003
Benzoate	Non-receptive	0.0023 ± 0.001	0.0009 ± 0.0002	0.0014 ± 0.0004	0.001 ± 0.0002
	Receptive	0.0024 ± 0.0004	0.0016 ± 0.0003	0.0018 ± 0.0003	0.0016 ± 0.0002
Phenyl lactate	Non-receptive	0.0048 ± 0.0008	0.0042 ± 0.0004	0.0042 ± 0.0002	0.0034 ± 0.0003
	Receptive	0.0046 ± 0.0007	0.0045 ± 0.0006	0.0046 ± 0.0003	0.004 ± 0.0003
Kynurenine	Non-receptive	0.0022 ± 0.0004	0.0016 ± 0.0002	0.0021 ± 0.0002	0.0019 ± 0.0002
	Receptive	0.002 ± 0.0003	0.0018 ± 0.0002	0.0022 ± 0.0003	0.002 ± 0.0002
Glucose lactose	Non-receptive	0.0087 ± 0.0009	0.0116 ± 0.0019	0.013 ± 0.0015	0.011 ± 0.0009
	Receptive	0.0105 ± 0.0014	0.0103 ± 0.0005	0.0138 ± 0.001	0.0118 ± 0.0016
Glucose	Non-receptive	0.0166 ± 0.002	0.014 ± 0.0028	0.0143 ± 0.0031	0.0197 ± 0.0009
	Receptive	0.016 ± 0.0019	0.0107 ± 0.0022	0.0165 ± 0.0022	0.0128 ± 0.0045
Glycolate	Non-receptive	0.0204 ± 0.001[a]	0.018 ± 0.0005	0.0191 ± 0.0005	0.0213 ± 0.0004
	Receptive	*0.018 ± 0.0006*[b]	0.0183 ± 0.0011	0.018 ± 0.0006	0.0196 ± 0.0011
Creatine	Non-receptive	0.0169 ± 0.0005	0.0176 ± 0.0015	0.0188 ± 0.0009	0.0196 ± 0.0012
	Receptive	0.0154 ± 0.0006	0.0159 ± 0.0005	0.0185 ± 0.0011	0.0185 ± 0.0011
Betaine	Non-receptive	0.0376 ± 0.0032	0.0359 ± 0.0052	0.0489 ± 0.0051	0.0442 ± 0.0038
	Receptive	0.0402 ± 0.0058	0.0393 ± 0.0038	0.0539 ± 0.0019	0.0498 ± 0.0047
Dimethyl sulfone	Non-receptive	0.0086 ± 0.0005	0.0156 ± 0.0019	0.0122 ± 0.0016	0.0103 ± 0.0012
	Receptive	0.0075 ± 0.0007	0.0135 ± 0.0014	0.0118 ± 0.0021	0.0126 ± 0.0014
Malonate	Non-receptive	0.0028 ± 0.0002	0.0021 ± 0.0002	0.0024 ± 0.0001	0.0031 ± 0.0003
	Receptive	0.0024 ± 0.0003	0.0026 ± 0.0002	0.0023 ± 0.0001	0.0025 ± 0.0003
Creatine	Non-receptive	0.0043 ± 0.0005	0.0043 ± 0.0004	0.0049 ± 0.0003	0.0052 ± 0.0008
	Receptive	0.0047 ± 0.0006	0.0043 ± 0.0005	0.0055 ± 0.001	0.0048 ± 0.0007
Succinate	Non-receptive	0.0156 ± 0.0007	0.018 ± 0.0009[a]	0.0134 ± 0.0008	0.0139 ± 0.0006
	Receptive	0.0142 ± 0.0008	*0.015 ± 0.0007*[b]	0.0143 ± 0.0008	0.0131 ± 0.0008
Propionate butyrate	Non-receptive	0.0625 ± 0.0042	0.0583 ± 0.003	0.0648 ± 0.0027	0.0565 ± 0.0033
	Receptive	0.07 ± 0.0059	0.0674 ± 0.0063	0.0632 ± 0.0031	0.0586 ± 0.0019
Butyrate	Non-receptive	0.0284 ± 0.0021	0.0249 ± 0.0016[a]	0.0309 ± 0.0025	0.027 ± 0.0018
	Receptive	0.0334 ± 0.0028	*0.036 ± 0.0058*[b]	0.0313 ± 0.0022	0.029 ± 0.001
Alanine	Non-receptive	0.0227 ± 0.0027	0.019 ± 0.0018	0.0261 ± 0.002	0.0218 ± 0.0025
	Receptive	0.0221 ± 0.0034	0.0231 ± 0.0026	0.0261 ± 0.0018	0.0228 ± 0.0026
Lactate threonine	Non-receptive	0.0356 ± 0.002	0.0496 ± 0.0057	0.0384 ± 0.0021	0.0305 ± 0.0024
	Receptive	0.0375 ± 0.002	0.0437 ± 0.0041	0.0434 ± 0.002	0.0368 ± 0.0029
3OHisoValerate	Non-receptive	0.0068 ± 0.0003	0.0063 ± 0.0003	0.0074 ± 0.0005	0.0068 ± 0.0005
	Receptive	0.0067 ± 0.0004	0.0065 ± 0.0001	0.007 ± 0.0005	0.0072 ± 0.0004
Propionate	Non-receptive	0.0259 ± 0.0034	0.0222 ± 0.003	0.0237 ± 0.0013	0.0204 ± 0.0017
	Receptive	0.0329 ± 0.005	0.0235 ± 0.0006	0.0226 ± 0.0017	0.0265 ± 0.0019
Valine	Non-receptive	0.0074 ± 0.001	0.0055 ± 0.0008	0.0083 ± 0.0012	0.0075 ± 0.0009
	Receptive	0.0073 ± 0.0012	0.007 ± 0.0005	0.0083 ± 0.0011	0.0091 ± 0.0008
Leucine	Non-receptive	0.0302 ± 0.0034	0.0247 ± 0.0025	0.034 ± 0.0038	0.0312 ± 0.0038
	Receptive	0.029 ± 0.0048	0.0308 ± 0.0019	0.0349 ± 0.0041	0.0376 ± 0.0039
Choline	Non-receptive	0.0097 ± 0.0017	0.0132 ± 0.0014	0.0083 ± 0.0012	0.0079 ± 0.0012
	Receptive	0.0103 ± 0.0014	0.0097 ± 0.0007	0.0097 ± 0.0019	0.0067 ± 0.001
Pyruvate 3OHisovalerate	Non-receptive	0.0075 ± 0.0023	0.0112 ± 0.004	0.0093 ± 0.0018	0.0079 ± 0.0009
	Receptive	0.0052 ± 0.0008	0.006 ± 0.0012	0.0073 ± 0.0016	0.005 ± 0.0005
Carnitine	Non-receptive	0.0134 ± 0.0013	0.0132 ± 0.0014	0.0144 ± 0.001	0.015 ± 0.0013
	Receptive	0.0118 ± 0.0008	0.0121 ± 0.0008	0.0134 ± 0.0009	0.0117 ± 0.0009
Acetate	Non-receptive	0.2405 ± 0.0124	0.2287 ± 0.0124	0.2359 ± 0.0094	0.2142 ± 0.0112
	Receptive	0.2538 ± 0.0178	0.2578 ± 0.0073	0.2417 ± 0.0068	0.2673 ± 0.0121
Sucrose	Non-receptive	0.01 ± 0.0017	0.0219 ± 0.0054	0.0216 ± 0.0039	0.0315 ± 0.0151
	Receptive	0.0168 ± 0.0038	0.0121 ± 0.0017	0.0224 ± 0.004	0.013 ± 0.0014
Glycerol glycine	Non-receptive	0.0348 ± 0.0029	0.0346 ± 0.0016	0.0266 ± 0.0016	0.0344 ± 0.004
	Receptive	0.0287 ± 0.0031	0.0299 ± 0.0018	0.0249 ± 0.0019	0.0266 ± 0.0037
Glycerol	Non-receptive	0.0257 ± 0.002	0.0256 ± 0.001	0.0207 ± 0.0009	0.0234 ± 0.0014
	Receptive	0.022 ± 0.0023	0.0221 ± 0.0013	0.0193 ± 0.001	0.0202 ± 0.0023
Tartrate	Non-receptive	0.0057 ± 0.0006	0.005 ± 0.0009	0.0048 ± 0.0006	0.0053 ± 0.0005
	Receptive	0.0042 ± 0.0004	0.0044 ± 0.0003	0.0043 ± 0.0004	0.0046 ± 0.0006

The significant differences are indicated in italics. Statistical significance between receptive and non-receptive gilts: [a,b] for $P < 0.05$.

and those that remain immature. Indeed, mammals can synthesize PROG and its reduced metabolites and androgens in adrenals. However, as shown in Fig. 3, we observed a tendency for a persistent difference of PROG and its 5α- and 5β-reduced metabolites concentrations, and specifically the 5α-reduced metabolites of PROG, between receptive and non-receptive gilts. The increased levels of 5α-reduced metabolites of PROG in saliva of receptive gilts at all the investigated times strongly suggest that the ovary is the main source of progestogens and support the view that they could be biomarkers of boar effect receptivity in gilts characterized by an early puberty attainment.

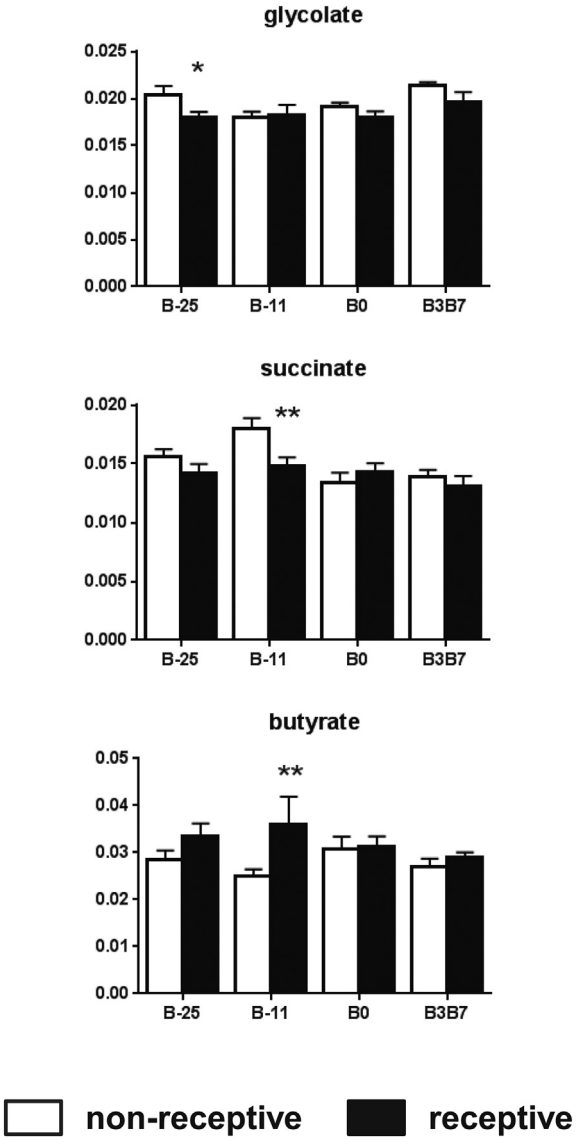

Fig. 4. ^{1}H nuclear magnetic resonance spectroscopy analysis of the concentrations of glycolate, succinate, and butyrate (arbitrary unit ± SEM) in saliva of receptive and non-receptive gilts collected 25 days and 11 days before boar introduction (B-25 and B-11), the day of boar introduction (B0), 3 days later for receptive gilts (B3), or 7 days later for non-receptive gilts (B7). For each metabolite, the y-axis was standardized to the maximal concentration, so that the variations are easy-to-read. * and **: statistical differences between receptive and non-receptive gilts for each collection day, $P < 0.05$ and $P < 0.01$.

Many hormones (steroids, amines, peptides, proteins) are present in saliva. In order to know if the steroidome and metabolome are correlated between the saliva and plasma, it is important to know how steroids and metabolites are carried from blood to saliva and if steroids can be locally synthesized and/or metabolized in salivary glands. Lipid-soluble steroids (Riad-Fahmy et al., 1982) and amines (Reiter, 1986) can be rapidly transferred from blood into saliva through the lipophilic layers of the capillaries and glandular epithelial cells, and thus travel by passive diffusion along a concentration gradient. Consequently, the salivary concentration of steroids is close to the plasma concentration in the absence of in situ metabolism. A second mechanism concerns lipid-insoluble conjugated steroids such as steroid sulfates (pregnenolone sulfate, dehydroepiandrosterone sulfate) that cross the glandulary epithelium via the tight junctions. In this case, the steroid concentration is much lower in saliva as compared to plasma. Usually, steroids are not metabolized to polar, water-soluble metabolites by the salivary glands, in contrast to what occurs in kidneys. Furthermore, only steroids without binding proteins can be transferred from blood to saliva. The salivary concentration of steroids mirrors the plasma concentration of biologically active unbound steroids. The steroid concentration in saliva generally represents around 10% unbound steroids and around 2–5% of the total steroid concentration in plasma (Wood, 2009). The presence of binding proteins in plasma (Siiteri et al., 1982) and of steroidogenic enzymes, such as 11β-hydroxysteroid dehydrogenase type 2 and 17-hydroxydehydrogenase in salivary glands have been shown (Swinkels et al., 1992; Stewart et al., 1995). However, the correlation between salivary and plasma free steroids concentration is generally quite good (Wood, 2009).

Salivary hormone analyses have been used for clinical and basic research. For example, the salivary analyses in humans for cortisol in psychology, stress research and in the diagnosis in Cushing syndrome, and of androgens and 17α-hydroxyprogesterone (**OHPROG**) in congenital adrenal hyperplasia have been investigated (Gröschl, 2008). Sex steroids, progestogens, estrogens, and androgens have also been successfully analyzed in saliva. Salivary PROG (Gröschl et al., 2001) and 17β-oestradiol (Bao et al., 2003) concentrations allowed differentiation of the follicular and luteal phases of the menstrual cycle in women. The significant difference of concentration of both steroids between the two phases consistently allowed an assessment of the ovarian function. Testosterone measurement in saliva has also been successfully used in fertility research in men and salivary testosterone concentration was proposed as a biomarker in the diagnosis of male androgen deficiency (Arregger et al., 2007). Saliva samples are also used in veterinary medicine in fertility research and breeding of valuable and endangered animals. Studies in mammals such as guinea pigs, marmosets, and dolphins have demonstrated the advantages of saliva collection relative to blood collection to assess glucocorticoids and mineralocorticoids (Gröschl, 2008). The reliable assessment of the ovarian cycle has been performed by the salivary steroid analysis and pregnancy was detected by the salivary concentration of 20α-DHPROG in rhinoceros (Gröschl, 2008). Finally, other omics approaches may be of interest to search for salivary biomarkers of the period of boar effect receptivity. For example, global proteomic analysis of water buffalo saliva was used to identify candidate biomarkers for the detection of oestrus (Shashikumar et al., 2018).

Further studies are necessary to confirm the reliability of our candidate biomarkers of boar effect receptivity. There are some important points to be taken into consideration. First, concentrations of steroids in gilt saliva were low. This could explain the high variability inherent to the GC–MS/MS measurements due to a low signal-to-noise ratio. However, we cannot exclude the existence of important inter-individual differences between gilts. Indeed, the pubertal response of the gilts to boar contact was reported to be variable, reflecting internal physiologic and management factors that have a relevant impact on the endocrine reproductive axis and consequently on steroid level changes preceding puberty (Evans and O'Doherty, 2001). Given the overall high variability, the results of this pilot study performed on a limited number of animal should be substantiated by a further study on a larger number of gilts. Moreover, a larger number of sampling points to collect saliva and a more precise longitudinal study before boar exposure may be of interest.

In conclusion, we demonstrated that saliva is an interesting alternative diagnostic fluid for the identification of the physiological stages of the gilts in an animal welfare-friendly production system. The advantages of salivary hormone analysis are the noninvasive stress-free collection procedure avoiding an adrenal stress response and a short-interval sampling. We demonstrated here that a complete steroidome and metabolome could be reliably described by the very specific and sensitive GC–MS/MS and ^{1}H NMR methods. The present study shows that the period during which gilts become receptive to boar effect is characterized by specific changes of the steroidome and metabolome in the saliva. Three metabolites and six steroids were

identified as salivary biomarker candidates. Among them, we suggest that PROG and some of its reduced metabolites could be the most reliable candidates to identify biomarkers of the period of boar effect receptivity. Further studies are necessary to confirm this hypothesis.

Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2020.100095.

Ethics approval

All procedures on animals were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the French Ministry of Agriculture and under the supervision of the ethical review and welfare committee.

Data and model availability statement

None of the data were deposited in an official repository.

Author ORCIDs

Ghylène Goudet: 0000-0001-7805-6985.

Author contributions

Ghylène Goudet: Conceptualization, methodology, validation, formal analysis, investigation, writing, visualization, supervision, funding acquisition. Armelle Prunier: Conceptualization, methodology, formal analysis, writing, funding acquisition. Lydie Nadal-Desbarats: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing, visualization. Doryan Grivault: Methodology, validation, investigation, resources. Stéphane Ferchaud: Conceptualization, methodology, validation, resources, writing, funding acquisition. Antoine Pianos: Methodology, validation, investigation, resources. Léna Haddad: Methodology, validation, investigation, resources. Frédéric Montigny: Methodology, validation, investigation, resources. Cécile Douet: Methodology, investigation. Jonathan Savoie: Conceptualization, methodology, funding acquisition. Florence Maupertuis: Conceptualization, methodology, writing, funding acquisition. Antoine Roinsard: Conceptualization, writing, funding acquisition. Sylviane Boulot: Conceptualization, methodology, writing, funding acquisition. Philippe Liere: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing, visualization.

Declaration of interest

None.

Acknowledgements

We would like to thank the staff of the experimental pigsty and Massimiliano Beltramo for critical reading of the manuscript.

Financial support statement

This work was supported by INRAE, Comité Interne en Agriculture Biologique (CIAB), programme AgriBio4.

References

Arregger, A.L., Contreras, L.N., Tumilasci, O.R., Aquilano, D.R., Cardoso, E.M., 2007. Salivary testosterone: a reliable approach to the diagnosis of male hypogonadism. Clinical Endocrinology 67, 656–662.

Bao, A.M., Liu, R.Y., van Someren, E.J., Hofman, M.A., Cao, Y.X., Zhou, J.N., 2003. Diurnal rhythm of free estradiol during the menstrual cycle. European Journal of Endocrinology 148, 227–232.

Camous, S., Prunier, A., Pelletier, J., 1985. Plasma prolactin, LH, FSH and estrogen excretion patterns in gilts during sexual development. Journal of Animal Science 60, 1308–1317.

Choi, S.-W., Kim, M.-S., Kwon, D.Y., Lee, H.-J., Park, S.-J., Chun, S., Shin, P.-K., 2019. Circulating extracellular MicroRNAs in blood and saliva are a new source of biomarkers in the assessment of health effects of traditional Korean diet (P15-003-19). Current Developments in Nutrition 3 (suppl. 1), P15-003-19.

Evans, A.C.O., O'Doherty, J.V., 2001. Endocrine changes and management factors affecting puberty in gilts. Livestock Production Science 68, 1–12.

Goudet, G., Nadal-Desbarats, L., Douet, C., Savoie, J., Staub, C., Venturi, E., Ferchaud, S., Boulot, S., Prunier, A., 2019a. Salivary and urinary metabolome analysis for pre-puberty-related biomarkers identification in porcine. Animal 13, 760–770.

Goudet, G., Liere, P., Pianos, A., Fernandez, N., Cambourg, A., Savoie, J., Staub, C., Venturi, E., Douet, C., Ferchaud, S., Maupertuis, F., Roinsard, A., Boulot, S., Prunier, A., 2019b. Evolution of steroid concentrations in saliva from immature to pubertal gilts for the identification of biomarkers of gilts receptivity to boar effect. Livestock Science 228, 5–17.

Graham, J.D., Clarke, C.L., 1997. Physiological action of progesterone in target tissues. Endocrine Reviews 18, 502–519.

Gröschl, M., 2008. Current status of salivary hormone analysis. Clinical Chemistry 54, 1759–1769.

Gröschl, M., Rauh, M., Schmid, P., Dörr, H.G., 2001. Relationship between salivary progesterone, 17-hydroxyprogesterone, and cortisol levels throughout the normal menstrual cycle of healthy postmenarcheal girls. Fertility and Sterility 76, 615–617.

Hughes, P.E., Thorogood, K.L., 1999. A note on the effects of contact frequency and time of day of boar exposure on the efficacy of the boar effect. Animal Reproduction Science 57, 121–124.

Hughes, P.E., Pearce, G.P., Paterson, A.M., 1990. Mechanisms mediating the stimulatory effects of the boar on gilt reproduction. Journal of Reproduction and Fertility. Supplement 40, 323–341.

Klimiuk, A., Maciejczyk, M., Choromańska, M., Fejfer, K., Waszkiewicz, N., Zalewska, A., 2019. Salivary redox biomarkers in different stages of dementia severity. Journal of Clinical Medicine 8, 840.

Knox, R.V., Vatzias, G., Naber, C.H., Zimmerman, D.R., 2003. Plasma gonadotropins and ovarian hormones during the estrous cycle in high compared to low ovulation rate gilts. Journal of Animal Science 81, 249–260.

Liang, D., Lu, H., 2019. Salivary biological biomarkers for Alzheimer's disease. Archives of Oral Biology 105, 5–12.

Liere, P., Pianos, A., Eychenne, B., Cambourg, A., Liu, S., Griffiths, W., Schumacher, M., Sjovall, J., Baulieu, E.E., 2004. Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain. Journal of Lipid Research 45, 2287–2302.

Liere, P., Pianos, A., Eychenne, B., Cambourg, A., Bodin, K., Griffiths, W., Schumacher, M., Baulieu, E.E., Sjovall, J., 2009. Analysis of pregnenolone and dehydroepiandrosterone in rodent brain: cholesterol autoxidation is the key. Journal of Lipid Research 50, 2430–2444.

Liu, S., Ying, G.-G., Zhang, R.-Q., Zhou, L.-J., Lai, H.-J., Chen, Z.-F., 2012a. Fate and occurrence of steroids in swine and dairy cattle farms with different farming scales and wastes disposal systems. Environmental Pollution 170, 190–201.

Liu, S., Ying, G.-G., Zhou, L.-J., Zhang, R.-Q., Chen, Z.-F., Lai, H.-J., 2012b. Steroids in a typical swine farm and their release into the environment. Water Research 46, 3754–3768.

Liu, S.-S., Ying, G.-G., Liu, Y.-S., Yang, Y.-Y., He, L.-Y., Chen, J., Liu, W.-R., Zhao, J.-L., 2015. Occurrence and removal of progestagens in two representative swine farms: effectiveness of lagoon and digester treatment. Water Research 77, 146–154.

Pearce, G.P., Hughes, P.E., 1985. The influence of daily movement of gilts and the environment in which boar exposure occurs on the efficacy of boar-induced precocious puberty in the gilt. Animal Production 40, 161–167.

Prunier, A., Chopineau, M., Mounier, A.M., Mormede, P., 1993. Patterns of plasma LH, FSH, oestradiol and corticosteroids from birth to the first oestrous cycle in Meishan gilts. Journal of Reproduction and Fertility 98, 313–319.

Reiter, R.J., 1986. Normal patterns of melatonin levels in the pineal gland and body fluids of humans and experimental animals. Journal of Neural Transmission. Supplementum 21, 35–54.

Riad-Fahmy, D., Read, G.F., Walker, R.F., Griffiths, K., 1982. Steroids in saliva for assessing endocrine function. Endocrine Reviews 3, 367–395.

Shashikumar, N.G., Baithalu, R.K., Bathla, S., Ali, S.A., Rawat, P., Kumaresan, A., Kumar, S., Maharana, B.R., Singh, G., Puneeth Kumar, D.S., Singh, S.K., Lathwal, S.S., Jaiswal, L., Mohanty, T.K., Mohanty, A.K., 2018. Global proteomic analysis of water buffalo (*Bubalus bubalis*) saliva at different stages of estrous cycle using high throughput mass spectrometry. Theriogenology 110, 52–60.

Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J., Kuhn, R.W., 1982. The serum transport of steroid hormones. Recent Progress in Hormone Research 38, 457–510.

Stewart, P.M., Whorwood, C.B., Mason, J.I., 1995. Type 2 11 beta-hydroxysteroid dehydrogenase in foetal and adult life. Journal of Steroid Biochemistry and Molecular Biology 55, 465–471.

Swinkels, L.M., van Hoof, H.J., Ross, H.A., Smals, A.G., Benraad, T.J., 1992. Low ratio of androstenedione to testosterone in plasma and saliva of hirsute women. Clinical Chemistry 38, 1819–1823.

Wood, P., 2009. Salivary steroids. Annals of Clinical Biochemistry 46, 427.

Zhang, J.N., Yang, L., Zhang, M., Liu, Y.S., Zhao, J.L., He, L.Y., Zhang, Q.Q., Ying, G.G., 2019. Persistence of androgens, progestogens, and glucocorticoids during commercial animal manure composting process. Science of the Total Environment 665, 91–99.