



## Original Research Article

## Saliva and plasma metabolome changes during anoestrus, the oestrous cycle and early gestation in the mare: A pilot study

Ghylène Goudet<sup>a,\*</sup>, Stéphane Beauclercq<sup>b</sup>, Cécile Douet<sup>a</sup>, Fabrice Reigner<sup>c</sup>, Stéfan Deleuze<sup>d</sup>, Lydie Nadal-Desbarats<sup>e</sup><sup>a</sup> INRAE, CNRS, IFCE, Université de Tours, PRC, 37380, Nouzilly, France<sup>b</sup> BOA, INRAE, Université de Tours, 37380, Nouzilly, France<sup>c</sup> PAO, INRAE, 37380, Nouzilly, France<sup>d</sup> Faculté de Médecine Vétérinaire, Département des Sciences Cliniques, Clinique Equine, Université de Liège, B-4000, Liège, Belgium<sup>e</sup> UMR 1253, iBrain, INSERM, Université de Tours, 37000, Tours, France

## ARTICLE INFO

## Keywords:

Equine  
Nuclear magnetic resonance spectroscopy  
Seasonal anoestrus  
Follicular phase  
Luteal phase  
Pregnancy

## ABSTRACT

Successful reproductive management of domestic mammals depends primarily upon timely identification of oestrous cycle stages. There is a need to develop an alternative non-invasive, welfare-friendly, accurate and reliable method to identify reproductive cycle stages. This is of particular interest for horse breeders, because horses are high-value farm animals that require careful management and individual monitoring. Saliva sampling is non-invasive, painless and welfare-friendly. Thus, we performed a metabolomic analysis of equine saliva during different reproductive stages to identify changes in the salivary metabolome during anoestrus, the oestrous cycle and early gestation. We compared the saliva and plasma metabolomes to investigate the relationship between the two fluids according to the physiological stage. We collected saliva and plasma samples from six mares during seasonal anoestrus, during the follicular phase 3 days, 2 days and 1 day before ovulation and the day when ovulation was detected, during the luteal phase 6 days after ovulation, and during early gestation 18 days after ovulation and insemination. Metabolome analysis was performed by proton-nuclear magnetic resonance spectroscopy. We identified 58 and 51 metabolites in saliva and plasma, respectively. The levels of four metabolites or groups of metabolites in saliva and five metabolites or groups of metabolites in plasma showed significant modifications during the 4 days until ovulation, *ie* 3 days prior to and on the day of ovulation. The levels of 11 metabolites or groups of metabolites in saliva and 17 metabolites or groups of metabolites in plasma were significantly different between the seasonal anoestrus and the ovarian cyclicity period. The physiological mechanisms involved in the onset of ovarian cyclicity and in ovulation induced modifications of the metabolome both in plasma and saliva. The metabolites whose salivary levels changed during the reproductive cycle could be potential salivary biomarkers to detect the reproductive stage in a welfare friendly production system. In particular, we propose creatine and alanine as candidate salivary biomarkers of ovulation and of the onset of ovarian cyclicity, respectively. However, extensive validation of their reliability is required. Our study contributes to extend to domestic mammals the use of saliva as a non-invasive alternative diagnostic fluid for reproduction in a welfare-friendly production system.

## 1. Introduction

Successful reproductive management of domestic mammals depends primarily upon timely identification of oestrous cycle stages. The available methods for oestrous cycle stage detection include examining animal behaviour signs, ultrasonography, per rectal examination, and

evaluation of hormone plasma levels. However, these methods are either invasive or lack accuracy. Hence, there is a need to develop new alternative approaches that are non-invasive, welfare-friendly, accurate and reliable to help breeders identify reproductive cycle stages.

This issue is of particular interest for horse breeders, because horses are high-value farm animals that require careful management and

\* Corresponding author. PRC, INRAE centre Val de Loire, 37380, Nouzilly, France.

E-mail addresses: [ghylene.goudet@inrae.fr](mailto:ghylene.goudet@inrae.fr) (G. Goudet), [beauclercq.stephane@courrier.uqam.ca](mailto:beauclercq.stephane@courrier.uqam.ca) (S. Beauclercq), [cecile.douet@inrae.fr](mailto:cecile.douet@inrae.fr) (C. Douet), [fabrice.reigner@inrae.fr](mailto:fabrice.reigner@inrae.fr) (F. Reigner), [s.deleuze@uliege.be](mailto:s.deleuze@uliege.be) (S. Deleuze), [lydie.nadal@univ-tours.fr](mailto:lydie.nadal@univ-tours.fr) (L. Nadal-Desbarats).

<https://doi.org/10.1016/j.theriogenology.2024.08.007>

Received 29 February 2024; Received in revised form 5 August 2024; Accepted 6 August 2024

Available online 8 August 2024

0093-691X/© 2024 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

individual monitoring. They are seasonal breeders with a winter anoestrus followed by a transitional phase bringing the mare back to cyclic activity in spring and summer [1]. Oestrus detection requires the presence of a stallion since oestrous behaviour is characterised by increased interest in stallions and proceptive behaviour in response to the stallion, with a high individual variability and a low accuracy [1]. Breeders generally prefer transrectal ultrasonography and evaluation of hormone levels in the plasma for accurate oestrous cycle stage evaluation, although these methods are invasive. Given the long gestation length in mares (320–360 days), there is not much time for mating when breeders expect one foal per year [2]. Moreover, racehorses born in the northern hemisphere are administratively considered born on January 1st; thus, conception early in the year is advantageous to young horses competing in the same age category. For these reasons, reproduction management at the beginning of the breeding season is critical for horse breeders.

Saliva is emerging as an ideal biofluid with diagnostic value because of its easy, painless and non-invasive collection and its cost-effectiveness. Saliva is a complex biological fluid composed of secretions from the major salivary glands - the parotid, submandibular, and sublingual glands - and minor glands including the labial, buccal, lingual, and palatal tissues, as well as an abundance of molecules from the plasma [3]. Saliva can thus be used to determine different systemic events and/or physiological states in an animal due to systemic origin of some of its bioanalytes.

Analyses of saliva composition using omics approaches such as proteomics, steroidomics and metabolomics, have been used to help to identify salivary biomarkers of the physiological status in horses [4,5], cows [6], goats [7], sows [8,9], and women [10]. They are of interest for species for which there is difficulty in oestrus detection such as buffalos [11]. Moreover, salivary analysis could represent a suitable non-invasive method to assess reproductive activity in wild and/or threatened species [12]. Metabolomics is of particular interest because metabolites are end-products of intracellular biochemical reactions organised in pathways whose activity can be modified by physiological changes, such as hormone-induced modifications during the oestrous cycle. The metabolome, which includes low-molecular-weight compounds such as peptides, lipids, carbohydrates, amino acids, vitamins and minerals, is thus the signature of a physiological state.

In the present study, we aimed to identify changes in the metabolome of equine saliva during reproductive stages by using proton-nuclear magnetic resonance ( $^1\text{H}$  NMR) spectroscopy, with a focus on identifying specific metabolites whose levels significantly fluctuate during anoestrus, the oestrous cycle and early gestation. We compared the saliva and plasma metabolomes to investigate the relationship between these two fluids according to the physiological stage. The identification of metabolites whose levels change during the reproductive cycle that could be potential salivary biomarkers to detect the reproductive stage in a welfare-friendly production system is discussed.

## 2. Materials and methods

### 2.1. Animals, housing, saliva and blood samples collection

Six healthy Welsh type pony mares aged 5–9 years old and weighing 277–390 kg were used. They were reared at the experimental farm Unité Expérimentale de Physiologie Animale de l'Orfrasière (UEPAO; <https://doi.org/10.15454/1.5573896321728955E12>) from Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) in France. From December to March, mares were penned in groups in an indoor stall on a straw bedding under natural daylight with free access to an outdoor paddock during the day. They were fed 0.6 kg of concentrate (20 % oats, 18 % wheat straw, 16 % wheat bran, 15 % barley, 12 % alfalfa; Eperon INRA, Axereal, Saint-Germain de Salles, France). Straw, salt licks (Sodical, Levallois-Perret, France) and water were available *ad libitum*. From April to November, they lived in groups

in the pasture with salt licks and water available *ad libitum*.

All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the French Ministry of Agriculture and with the approval of the ethical review committee (Comité d'Éthique en Expérimentation Animale Val de Loire n°019) on 10 December 2021, under number APAFIS #34147–202111261451233 v2. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with European Union Directive 2010/63/EU for animal experiments.

Saliva and blood samples were collected at seven physiological stages from each mare:

- during seasonal anoestrus,
- during the follicular phase, 3 days, 2 days and 1 day before ovulation, and the day when ovulation was detected,
- during the luteal phase, 6 days after ovulation,
- during early gestation, 18 days after ovulation and artificial insemination.

For saliva and blood samples collection during seasonal anoestrus, the anoestrus period was assessed based on plasma progesterone analysis using weekly blood collection from December to February. The anoestrus period was ascertained by plasmatic progesterone concentrations lower than 1 ng/mL once a week and no increase over 1 ng/mL from December to the end of February. Plasmatic progesterone concentrations were measured with an enzyme-linked immunosorbent assay [13] as described previously [14]. Saliva and blood samples were collected during seasonal anoestrus on February 7.

For saliva and blood samples collection during the follicular and luteal phases, ovarian activity was assessed by routine transrectal ultrasound scanning using an EXAPad device (IMV imaging, Angoulême, France) with a 7.5 MHz liner probe. No saliva and blood samples were collected during the first oestrous cycle of the season to avoid the transitional phase [1]. Saliva and blood samples collected during the follicular and luteal phases were recovered between 27 April and 20 June. Samples collection during the follicular phase started when the largest follicle reached 28 mm and was performed every day until detection of natural ovulation (mean  $\pm$  standard error of the mean [SEM] = 8.6  $\pm$  0.6 days). Only samples collected 3 days, 2 days and 1 day before ovulation and the day when ovulation was detected were analyzed. Sample collection during the luteal phase was performed 6 days after ovulation, at the time of the maximum progesterone concentration [1,15]. Saliva and blood samples collected during gestation were recovered between 6 June and 3 August. For this purpose, during the second oestrous cycle of each mare, when the largest follicle reached 33 mm, mares were inseminated with fresh equine semen from a Welsh pony stallion from our experimental stud (400  $\times$  10<sup>6</sup> spermatozoa in 10 mL of extender (INRA96®, IMV Technologies, France)) [16]. A second insemination was performed the day after if the mare did not ovulate. The pregnancy was confirmed by visualisation of the embryonic vesicle 14 days after ovulation using transrectal ultrasound scanning. If the pregnancy was confirmed, then saliva and blood were collected 18 days after ovulation, to search for early salivary biomarkers of gestation, because progesterone levels decrease 14 days following ovulation in non-pregnant mares and remain high in pregnant mares. The presence of the embryonic vesicle was ascertained again 18 days after ovulation using transrectal ultrasound scanning. If the pregnancy was not confirmed 14 days after ovulation, then the mare was inseminated during the following oestrous cycle.

Saliva and blood samples were collected from each mare in the morning between 9 a.m. and 10 a.m. to avoid circadian variations of steroid secretions. Mares were kept in a stall without straw 1 h before sampling, to avoid contamination of saliva with food. Saliva was collected first, using a cotton swab (Sarstedt Salivette® ref. 51.1534.500; Sarstedt, Nümbrecht, Germany). The cotton swab was held with forceps and the mare was allowed to chew on it until it was

soaked. Saliva collection was well tolerated by the mares, restraint with a halter was not required. The cotton swab was immediately centrifuged at  $3000\times g$  for 5 min at room temperature. The recovered saliva was aliquoted and stored at  $-80\text{ }^{\circ}\text{C}$  until analysis. After saliva collection, blood samples were collected from the external jugular vein by experienced operators, using Vacutainer® heparinised tubes and 20G needles to ensure a smooth procedure and animal welfare. In our conditions, blood collection was well tolerated by the mares, restraint with a halter was not needed of the horse needed. Blood samples were then immediately centrifuged at  $4000\times g$  for 10 min at room temperature. The recovered plasma was aliquoted and stored at  $-80\text{ }^{\circ}\text{C}$  until analysis.

A portion of each saliva and plasma samples was used for steroidome analysis using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) in a previous study [4]. The remaining samples were used in the present study for metabolome analysis using  $^1\text{H}$  NMR spectroscopy.

## 2.2. Metabolome analysis by $^1\text{H}$ NMR spectroscopy

The 42 saliva samples were prepared for  $^1\text{H}$  NMR by precipitation of proteins using methanol. Briefly, 200  $\mu\text{L}$  of saliva was mixed with 400  $\mu\text{L}$  of cold methanol and vortexed. The mixtures were cooled at  $-20\text{ }^{\circ}\text{C}$  for 20 min before centrifugation at  $15,000\times g$  for 15 min at  $4\text{ }^{\circ}\text{C}$ . The supernatants were recovered and transferred to glass tubes for further evaporation of the solvent. For the 42 plasma samples, a modified Folch's method [17] (200  $\mu\text{L}$  of plasma, 300  $\mu\text{L}$  of cold methanol and 500  $\mu\text{L}$  of cold chloroform) was used to extract metabolites and to eliminate proteins and lipids from the plasma in order to avoid overlap of the metabolites of interest with broad lipid and protein signals. The samples were vortexed for 1 min. The polar fractions, containing the metabolites, were separated after centrifugation at  $15,000\times g$  for 15 min at  $4\text{ }^{\circ}\text{C}$  and 300  $\mu\text{L}$  of the supernatants (the polar fractions) were collected in glass tubes. The solvent of saliva supernatants and plasma polar fractions were evaporated in a SpeedVac (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) for 2 h at  $35\text{ }^{\circ}\text{C}$ , followed by another 2 h at room temperature before storage at  $-20\text{ }^{\circ}\text{C}$  until analysis. Five quality control samples for the saliva and plasma experiments were prepared by pooling a fraction of each sample together (either saliva or plasma) and were further processed with all the other samples.

Before  $^1\text{H}$  NMR analyses, the dried residues were dissolved in 210  $\mu\text{L}$  of phosphate buffer (200 mM, pH 7.4) prepared in Deuterium Oxide ( $\text{D}_2\text{O}$ ) supplemented with 152  $\mu\text{M}$  (final concentration) of 3-trimethylsilylpropionic acid (TSP) as an internal reference and transferred to 3 mm NMR tubes.

$^1\text{H}$  NMR spectra of the saliva and plasma samples were acquired at 298 K on an AVANCE III HD 600 MHz system (Bruker Biospin, Karlsruhe, Germany) equipped with a Bruker 5 mm TCI CryoProbe with Z-gradient.  $^1\text{H}$  NMR spectra were recorded with the 'noesypr1d' pulse sequence with a relaxation delay of 20 s, on a sweep width of 12 ppm, 64 k data points, an acquisition time of 4.56 s, with 64 transients, and 8 dummy scans. Sample shimming was performed automatically on the  $\text{D}_2\text{O}$  signal. The phasing of the spectra was corrected in TopSpin 3.6.3 (Bruker Biospin, Karlsruhe, Germany).

The phased spectra were imported into NMRProcFlow [18] for baseline correction and manual bucketing into 86 and 113 regions containing the signal of one or several metabolites with a variation inferior to 30 % across the quality controls for the saliva and plasma samples, respectively. The data tables for the saliva and plasma samples generated were normalised by the constant sum method before statistical analysis.

The  $^1\text{H}$  NMR spectra, referenced to the internal TSP chemical shift reference, were assigned using spectra from our in-house database and online databases, including HMDB (<http://www.hmdb.ca>) [19] or the Chemomx NMR Suite 8.8 (Chemomx Inc, Edmonton, Canada).

## 2.3. Metabolite set enrichment analysis

To identify the most represented chemical classes in the NMR-detected metabolomes of the mare saliva and plasma, a metabolite set enrichment analysis [20] was performed on the metabolites identified in the saliva and plasma samples using Metaboanalyst, a freely available web-based metabolomics analysis suite (MetaboAnalyst 5.0, <http://www.metaboanalyst.ca>) [21]. The metabolite enrichment analysis of each set of metabolites was performed using over-representation analysis (ORA) with the 'main chemical class metabolite sets' provided, containing 464 metabolite sets. The ORA was implemented using the hypergeometric test to evaluate whether a particular metabolite set was more highly represented than expected by chance within the given compound list. The p-value from the hypergeometric test were corrected for type I errors arising from multiple comparisons by the false discovery rate (FDR) method.

## 2.4. Statistical analysis

The significance of the differences in peak area for each bucket (metabolites) between the different reproductive stages were tested by repeated-measures one-way analysis of variance (ANOVA) in GraphPad Prism 9 (GraphPad Software, La Jolla California, USA), using the method of Geisser and Greenhouse to correct for possible violations of the assumption of sphericity [22]. ANOVA was followed by the Tukey post hoc multiple comparison test. A p-value  $<0.05$  was considered to indicate a significant difference.

## 3. Results

### 3.1. $^1\text{H}$ NMR spectroscopic profiles

Annotated representative  $^1\text{H}$  NMR spectra of the saliva and plasma extracts are shown in Fig. 1A and B, respectively. The experimental and spectral data have been deposited with the DOI <https://doi.org/10.5281/zenodo.7806212> in the Zenodo repository (<https://zenodo.org>) hosted by the European Organization for Nuclear Research (CERN).

The  $^1\text{H}$  NMR spectra of the saliva extracts (Fig. 1A) allowed the identification of 86 spectral regions or buckets; 80 buckets were assigned to one or more metabolites, corresponding to 58 known metabolites (Fig. 2). Following metabolite set enrichment analysis, we identified 10 compound classes as enriched in the saliva with a p-value, corrected for multiple testing by FDR, inferior to 0.05 (Fig. 3A). Amino acids were the most significantly enriched class (FDR-corrected p-value =  $6.72 \times 10^{-21}$ ; alanine, aspartic acid, betaine, creatine, glutamic acid, glycine, isoleucine, leucine, ornithine, phenylalanine, sarcosine, threonine, tyrosine, valine). Fatty acids were the second main class of metabolites significantly enriched (FDR-corrected p-value =  $6.01 \times 10^{-14}$ ). The contents of this class can be further divided into short chain fatty acids (SCFA) and derivatives (2-oxobutyrate, 3-hydroxyisobutyrate, 3-hydroxyisovalerate, 4-aminobutyrate, 5-aminopentanoate, acetate, acetoacetate, butyrate, isovalerate, propionate), saturated fatty acids (caprate, caprylate, isocaproate), pyruvate, and succinate. Acids involved in the energy production via the tricarboxylic acid (TCA) cycle (malate, oxoglutarate, succinate) were the third class of metabolites enriched with a p-value adjusted of  $9.06 \times 10^{-8}$ , followed by disaccharides ( $1.26 \times 10^{-4}$ ; maltose, sucrose), phenylpropanoids ( $9.77 \times 10^{-4}$ ; 3-phenylpropionate, 2-phenylpropionate), pyrimidines (0.00691; thymine, uracil), cholines (0.0262; choline), sulfonic acids (0.0262; taurine), hydroxy acids (0.0262; 3-hydroxybutyrate), and short-chain acids and derivatives (0.0413; pyruvate).

The  $^1\text{H}$  NMR spectra of the plasma polar fraction (Fig. 1B) allowed the identification of 113 spectral regions, 95 were assigned to one or more metabolites, corresponding to 51 known metabolites (Fig. 2). Eight chemical classes were over-represented (FDR-corrected p-value  $<0.05$ ; Fig. 3B) in the mare plasma metabolome. As in saliva, amino acids were

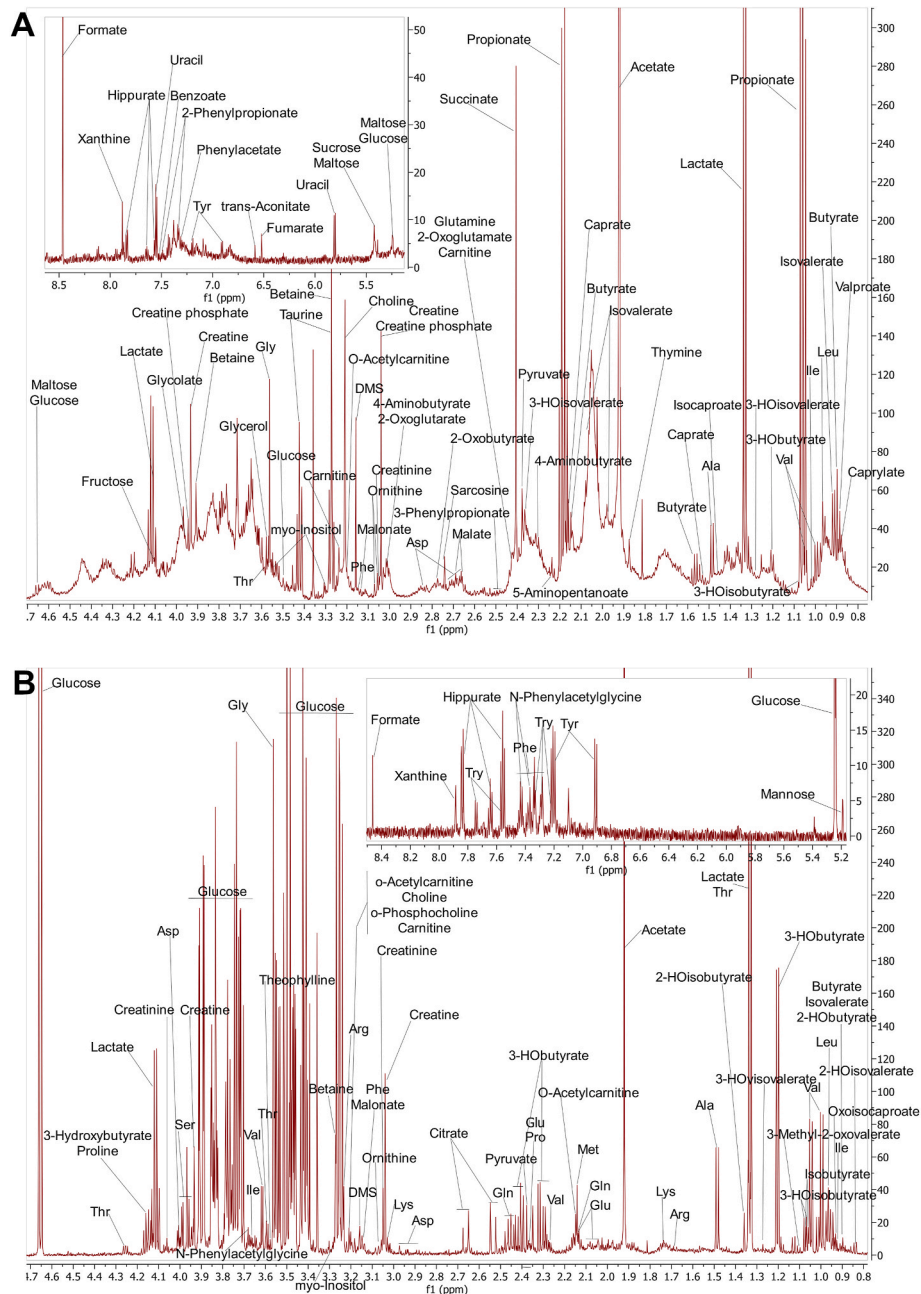


Fig. 1. Annotated representative  $^1\text{H}$  Nuclear Magnetic Resonance spectroscopy spectra of mares saliva (A) and plasma (B) extracts.

the most significantly enriched class ( $2.82 \times 10^{-35}$ ; Alanine, Arginine, Aspartic acid, betaine, creatine, Glutamine, Glutamic acid, Glycine, Isoleucine, Leucine, Lysine, Methionine, N Phenylacetylglutamine, Ornithine, Phenylalanine, Proline, Threonine, Tryptophan, Tyrosine, Valine), followed by fatty acids and conjugates ( $2.25 \times 10^{-6}$ ; 2 hydroxyisobutyrate, 2 oxyisovalerate, 3 hydroxyisobutyrate, 3 hydroxyisovalerate, acetate, butyrate, isobutyrate, isovalerate, pyruvate). Most of these fatty acids are short chain fatty acids also found in saliva. Choline compounds ( $2.24 \times 10^{-5}$ ; choline, phosphorylcholine) were more represented in plasma than in saliva. Purines (0.0103; theophylline, xanthine), monosaccharides (0.0103; glucose, mannose) and primary alcohols (0.0176; ethanol) were found only in plasma. Hydroxy acids (0.0302; 3-hydroxybutyrate) and short-chain acids and derivatives (0.0462; pyruvate) were classes enriched in the two biological compartments (saliva and plasma). As in the saliva spectra, the signal of protons from several metabolites overlap in 34 buckets; 65 regions

contained the signal of a single metabolite, which correspond to 39 unique metabolites.

Among metabolites, we identified 32 in both saliva and plasma, 26 only in saliva and 19 only in plasma (Fig. 2).

### 3.2. The metabolome and reproductive stages

In **saliva**, 14 metabolites or groups of metabolites with overlapping signals showed significant differences between the seven physiological stages. Fig. 4 shows their spectral areas normalised to the total area of all the integrated spectral regions.

The salivary concentrations (in arbitrary units [AU]) of 11 metabolites or groups of metabolites were significantly different between seasonal anoestrus and another or several other stages: the levels of betaine, myo-inositol, alanine, and the groups of metabolites containing lactate + fructose, alanine + glucose + glycerol + maltose, or malate +

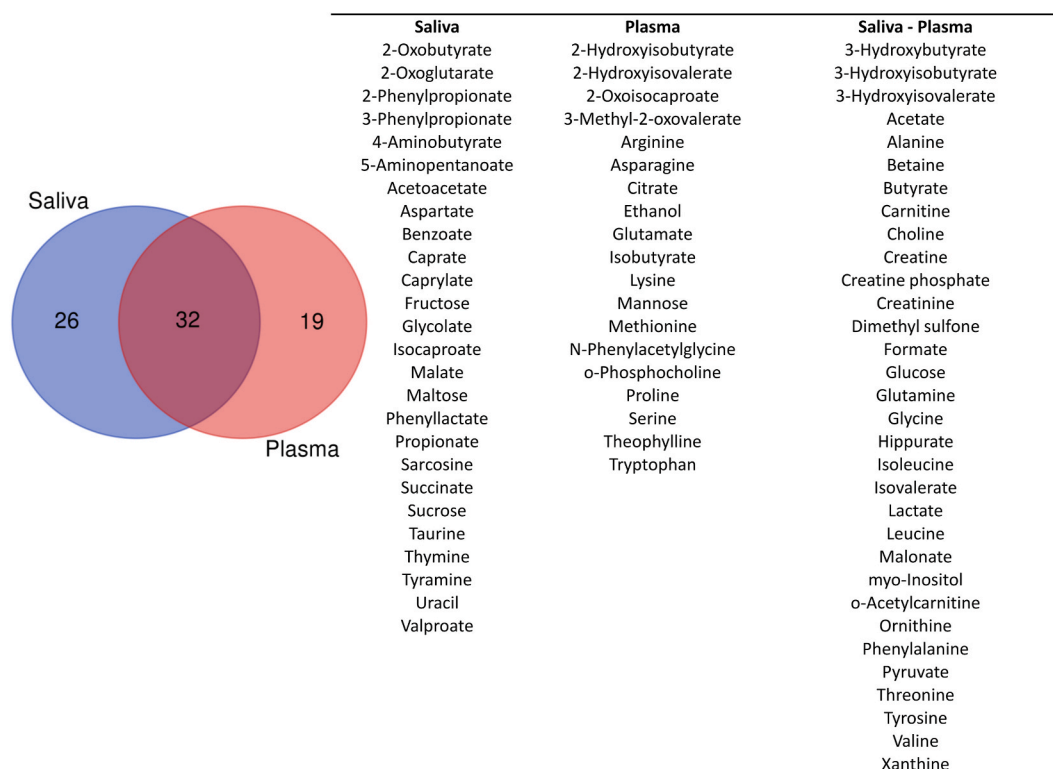


Fig. 2. Venn diagram and list of mares saliva, plasma and common metabolites.

aspartate were significantly higher during the anoestrus stage than during another or several other physiological stages. On the contrary, the levels of sarcosine, isocaproate, 3-hydroxyisobutyrate, and the groups of metabolites containing 4-aminobutyrate + 2-oxoglutarate or glutamine + 2-oxoglutarate + carnitine were significantly lower during the anoestrus stage than during another or several other physiological stages.

The salivary concentrations (in AU) of four metabolites or groups of metabolites showed significant modifications during the 4 days until ovulation, *ie* 3 days prior to and on the day of ovulation. The salivary level of creatine tended to decrease during these 4 days, and was significantly higher 2 days before ovulation compared with the day when ovulation was detected. The level of the group of metabolites containing malate + aspartate tended to decrease during the 3 days before ovulation, with a significant difference between 3 days and 1 day before ovulation. On the contrary, the levels of the group containing 2-phenylpropionate + phenyllactate or 4-aminobutyrate + 2-oxoglutarate increased significantly between 3 days and 2 days before ovulation.

The salivary level of formate was significantly different between the follicular phase (1 day before ovulation) and early gestation (18 days after ovulation and insemination). The salivary level of 3-hydroxyisobutyrate was significantly increased between the day of ovulation and the luteal phase.

In the **plasma**, 21 metabolites and groups of metabolites with overlapping signals showed significant differences between the seven physiological stages. Fig. 5 shows their normalised spectral intensities to the total area of all the integrated spectral regions.

The plasma levels of 17 metabolites or groups of metabolites were significantly different between the seasonal anoestrus and another or several other stages. The levels of tryptophan, mannose, theophylline, carnitine, creatinine, glutamine, alanine, and the groups of metabolites containing tryptophan + creatinine, phenylalanine + an unknown metabolite, or glutamate + glutamine + *o*-acetylcarnitine + methionine were higher during the anoestrus stage than during another or several other physiological stages. On the contrary, the levels of hippurate,

tyrosine, threonine, valine, 3-hydroxyisobutyrate, leucine, and the group containing asparagine + an unknown metabolite were lower during the anoestrus stage than during another or several other physiological stages.

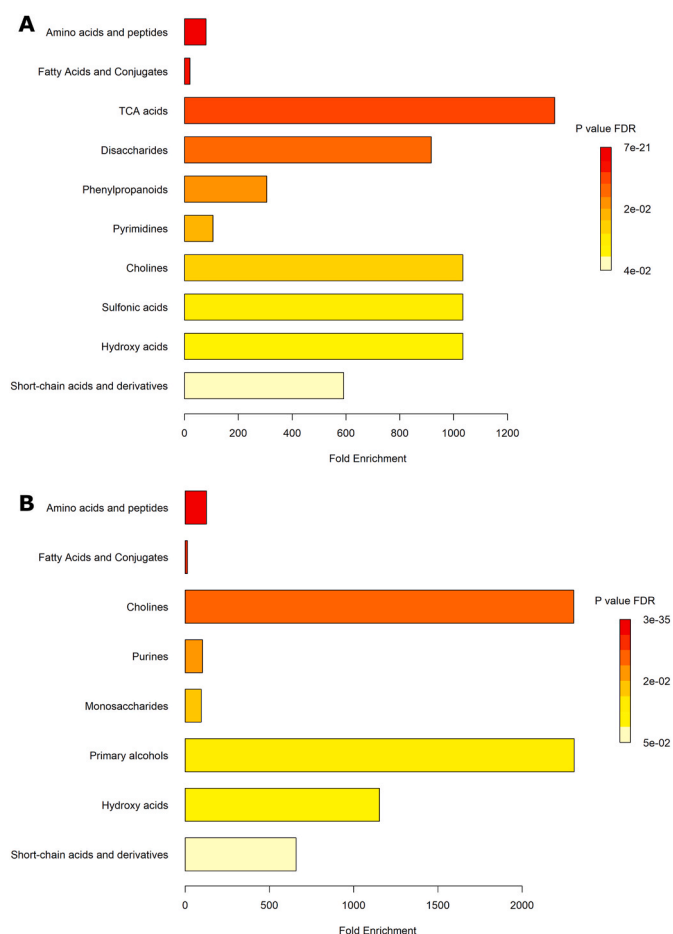
The plasma levels of five metabolites or groups of metabolites showed a significant decrease during the 4 days until ovulation, *ie* 3 days prior to and on the day of ovulation: serine between 3 days and 2 days before ovulation, dimethyl sulfone between 2 days before ovulation and the day when ovulation was detected, valine and the group of metabolites containing serine + phenylalanine + asparagine between 3 days and 1 day before ovulation, and the group containing phenylalanine + an unknown metabolite between 3 days before ovulation and the day when ovulation was detected.

The plasmatic level of 3-hydroxyisovalerate was significantly different between the day before ovulation and the luteal phase.

#### 4. Discussion

Practical non-invasive tools for the identification of reproductive stages of female animals are of great interest for breeders. Saliva collection is a non-invasive, painless, easy and inexpensive technique. Moreover, saliva contains secretions from the salivary glands as well as molecules from the blood which enter via passive and active transport. Thus, saliva may reflect plasma modifications during the reproductive cycle. In our study, the use of  $^1\text{H}$  NMR to analyse multiple metabolites with high specificity and sensitivity provided opportunities to characterise, for the first time, a panel of metabolites and the changes in their saliva and plasma levels during seasonal anoestrus, the ovarian cycle and early gestation in mares. This study allowed the identification of metabolites whose salivary and plasma levels were significantly different between these physiological stages.

We identified 32 metabolites in both saliva and plasma, 26 only in saliva and 19 only in plasma. Metabolites can enter saliva from the blood through transcellular and paracellular routes. The transcellular route involves passive intracellular diffusion and active transport across cell



**Fig. 3.** Metabolite set enrichment analysis of the metabolome of mares saliva (A) and plasma (B). The metabolite set enrichment analysis was performed on the  $^1\text{H}$  Nuclear Magnetic Resonance spectroscopy detected metabolome of the mares saliva (A) and plasma (B). The horizontal bars represent the fold enrichment of the main identified chemical classes compared with a random distribution. The two most significant chemical classes were ‘amino acids and peptides’ and ‘fatty acids and conjugates’ for both saliva and plasma.

membranes, while the paracellular route involves extracellular ultra-filtration through the spaces between cells. Additionally, metabolites can be locally produced by the major salivary glands, which include the parotid, submandibular, and sublingual glands, as well as multitudes of minor glands including labial, buccal, lingual, and palatal tissues [3]. Most of the common metabolites between saliva and plasma covering, in particular, amino acid pathways (alanine, betaine, glutamine, glycine, threonine, ornithine) or energy metabolism (creatine, glucose, lactate, pyruvate, phosphocreatine) could support the use of saliva as a non-invasive proxy of plasma for diagnostic purposes.

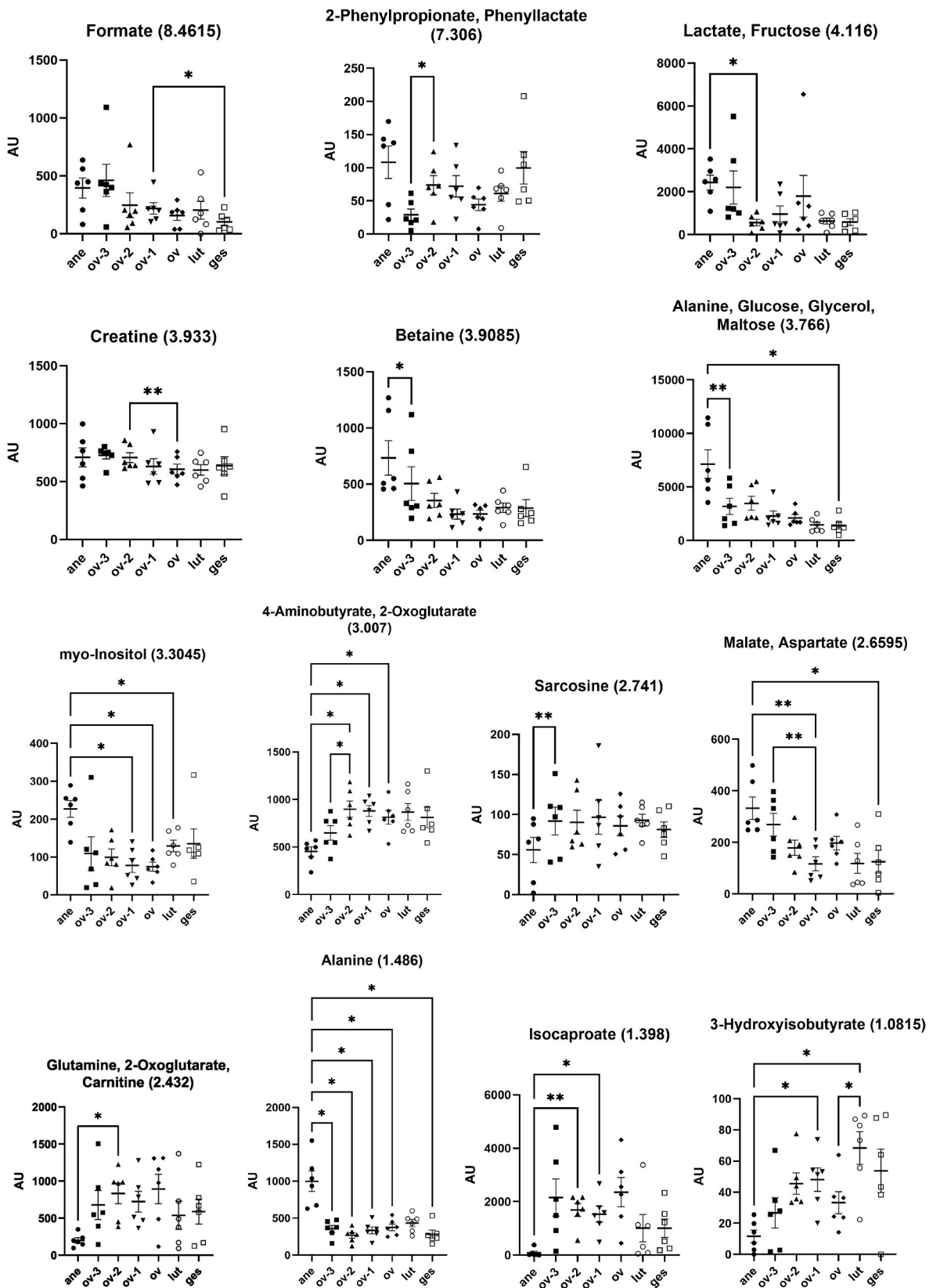
#### 4.1. Metabolome modifications during the four days until ovulation

In plasma, the levels of five metabolites or groups of metabolites showed a significant decrease during the 4 days until ovulation, *ie* 3 days prior to and on the day of ovulation, showing that the physiological mechanisms involved in the ovulation process are related to modifications of the plasma metabolome in the mare. Moreover, in saliva, the levels of four metabolites or groups of metabolites showed significant modifications during these 4 days. Thus, the physiological mechanisms involved in ovulation induced not only modifications of the plasma metabolome, but also modifications of the saliva metabolome, although the affected metabolites are different. Most of the differential metabolites in the plasma were amino acids, whereas in saliva the differential

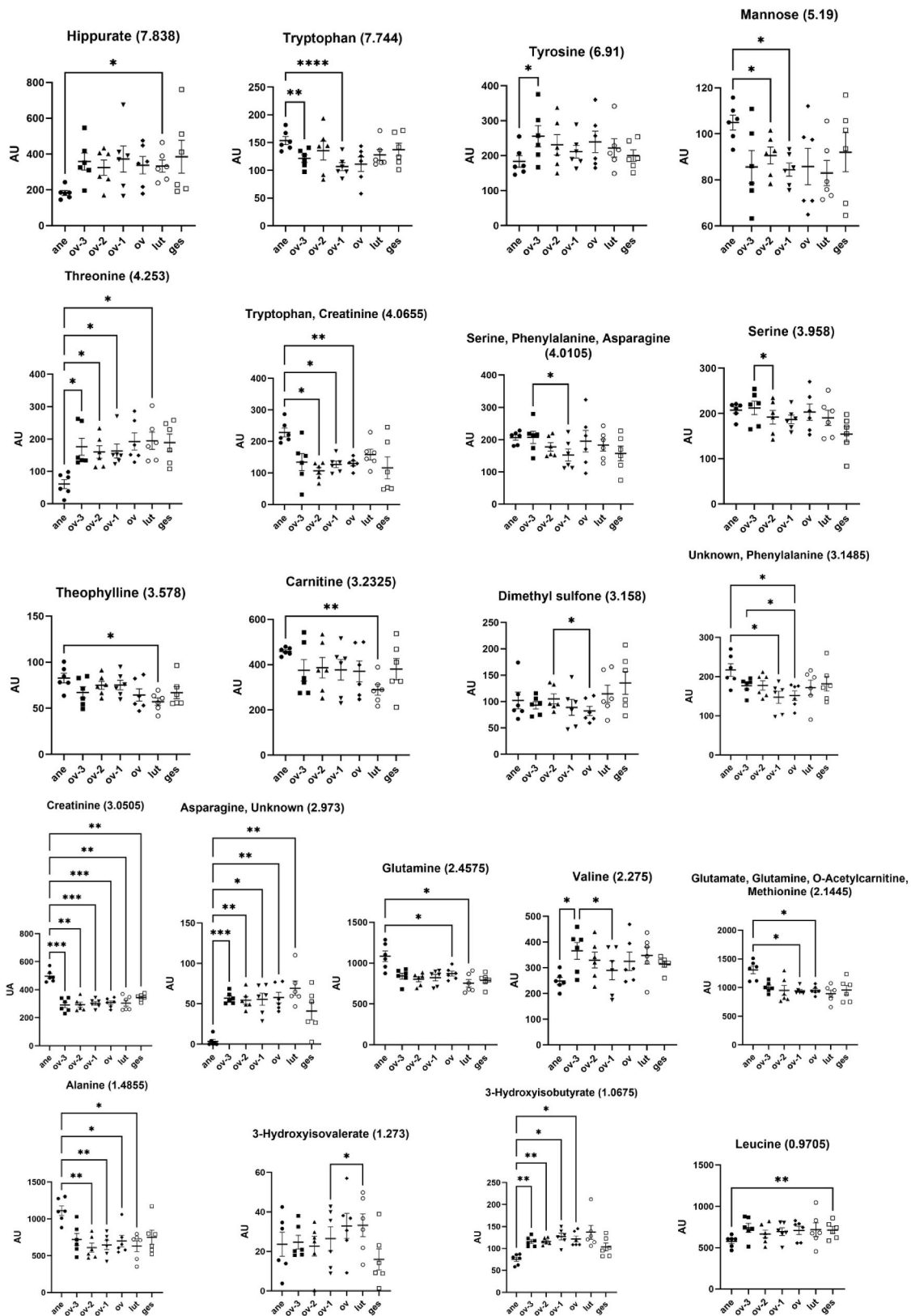
metabolites were associated with energy metabolism, including oxidative phosphorylation, the Krebs cycle, and aerobic glycolysis pathways. The differential metabolites in saliva could come from secretions from the major and minor salivary glands [3].

In mammals, ovulation is triggered by systemic modifications linked to secretion of gonadotropins and steroid hormones and local processes such as steroidogenesis, prostaglandin synthesis and proteolysis leading to follicle rupture, a crucial process for successful ovulation [23]. Multiple endocrine, paracrine and autocrine signaling pathways regulate ovulation. In mares, plasma LH concentrations, assayed by radioimmunoassay, peak one day after ovulation [15]. Evaluation of steroid concentrations in plasma of cyclic mares using enzyme immunoassay and radioimmunoassay showed that progesterone concentrations increase from the day of ovulation to 6 days post-ovulation and then decrease, whereas 17 $\beta$ -oestradiol concentrations reach a peak 2 days before ovulation and then decrease [1,15]. Using GC-MS/MS analysis of the plasma steroidome in mares, we confirmed the decrease in 17 $\beta$ -oestradiol concentrations before ovulation, and we showed that dehydroepiandrosterone concentrations decreased significantly on the last day before ovulation [4]. In the present study, using  $^1\text{H}$  NMR analysis of the plasma metabolome, we showed that the levels of serine (proteinogenic amino acid), dimethyl sulfone (organic sulphur compound belonging to sulfones, source of sulphur) and valine (proteinogenic amino acid) decreased before ovulation. Thus, these two steroids and three metabolites could be potential candidate biomarkers for the detection of ovulation. However, the individual data overlapped and the differences were relatively small. Serine, valine and sulphur consumption may increase throughout the ovulatory process initiated by LH, to support the protein synthesis essential for ovulation, as observed in hamsters [24]. Similarly, valine has been linked to potential increase in ovulation rate in ewes [25]. Additionally, sulphur plays a critical role in protecting against oxidative stress, which is vital for the quality of ovulation [26]. However, the analysis of these biomarkers relies on invasive and stressful serial blood sampling, highlighting the need for non-invasive, welfare-friendly techniques for the detection of ovulation.

In saliva, the modifications of the 17 $\beta$ -oestradiol and dehydroepiandrosterone plasma levels before ovulation were not reflected [4]. However, in the present study, we observed significant modifications of the saliva metabolome during the days preceding ovulation. The creatine level significantly decreased from two days before ovulation to the day when ovulation was detected, suggesting that a drop in the creatine salivary level could allow one to detect ovulation. Moreover, creatine has been shown to be related to the ovulation process in human and mouse: it was detected in human follicular fluid after induction of ovulation [27] and in mouse follicular and oviductal fluids with a significant increase around ovulation [28]. Components of the creatine pathway may contribute to a global metabolic effect during ovulation; however, the mechanisms remain unknown. Moreover, estrogen and progesterone have been shown to increase creatine kinase activities and the expression of arginine-glycine aminotransferase, the rate-limiting enzyme in creatine synthesis, which is crucial for cellular energy homeostasis through the regeneration of ATP [29]. Thus, we propose creatine as a potential salivary biomarker of ovulation, providing that further experiments with more animals and salivary sampling points during the ovarian cyclicity phase are performed, to ascertain its reliability. Moreover, absolute salivary concentrations of creatine are within the detection range of commercial colorimetric assay kits (Goudet, personal communication), which represent inexpensive and fast assays with possible application in the field. We identified other metabolites whose salivary levels varied significantly before ovulation. The level of the group of metabolites containing malate (an intermediate of the Krebs cycle) and aspartate (proteinogenic and glucogenic amino acid) decreased significantly before ovulation, potentially supporting a modification in the energy and protein metabolism during ovulation as hypothesized for the plasma. The levels of the groups containing 2-phenylpropionate and phenyllactate or 4-aminobutyrate (GABA) and



**Fig. 4.** Spectral intensities of the 14 metabolites or groups of metabolites from mares saliva. The spectral intensities of the 14 metabolites or groups of metabolites – presented as name (chemical shift) – from mares saliva show significant differences between the seven physiological stages, normalised to the total area of all the integrated spectral regions. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ . ane: seasonal anoestrus; ov-3: 3 days before ovulation; ov-2: 2 days before ovulation; ov-1: 1 day before ovulation; ov: the day when ovulation was detected; lut: luteal phase 6 days after ovulation; ges: early gestation 18 days after ovulation and artificial insemination.



**Fig. 5.** Spectral intensities of the 21 metabolites or group of metabolites from mares plasma. The spectral intensities of the 21 metabolites or group of metabolites – presented as name (chemical shift) - from mares plasma show significant differences between the seven physiological stages, normalised to the total area of all the integrated spectral regions. \*: p < 0.05; \*\*: p < 0.01. ane: seasonal anoestrus; ov-3: 3 days before ovulation; ov-2: 2 days before ovulation; ov-1: 1 day before ovulation; ov: the day when ovulation was detected; lut: luteal phase 6 days after ovulation and artificial insemination; ges: early gestation 18 days after ovulation and artificial insemination.



2-oxoglutarate increased significantly before ovulation. Interestingly, both 4-aminobutyrate and 2-oxoglutarate are substrate of the GABA transaminase, an enzyme from the GABAergic system, and propionate inhibits GABA transaminase [30]. The GABA system has been shown to be significantly modulated across the menstrual cycle by gonadal steroids in human [31] and may be involved in the regulation of ovarian blood flow and hormone secretion [32]. However, <sup>1</sup>H NMR analysis did not allow us to assign these buckets to a single metabolite because the signals of these metabolites overlapped. The level of these metabolites has to be evaluated separately with another technique to ascertain whether one or both could be potential biomarkers of ovulation. Finally, the formate salivary level tended to decrease from 3 days before ovulation to the day when ovulation was detected, although no significant differences were observed. Because 17β-oestradiol synthesis through testosterone aromatisation generates formate [33], the decrease in formate levels may be related to the decrease in 17β-oestradiol levels before ovulation. Thus, formate levels could be related to the modifications of steroidogenesis leading to ovulation.

We have shown that significant modifications of the saliva metabolome occur before ovulation in mares. In humans, saliva is now widely used as an alternative diagnostic fluid to search for biomarkers, especially for non-invasive detection of ovulation: salivary proteome profile of women during menstrual cycle allowed the identification of 16 differentially expressed proteins during the ovulatory phase [10]. Our study will contribute to extend to domestic mammals the use of saliva as an alternative diagnostic fluid.

#### 4.2. Metabolome modifications between seasonal anoestrus and the ovarian cyclicity phase

The levels of 13 metabolites and four groups of metabolites in plasma, and six metabolites and five groups of metabolites in saliva, were significantly different during anoestrus than during another or several other stages of the ovarian cyclicity period. These data show that the transition from seasonal anoestrus to ovarian cyclicity induces modifications of the metabolome both in plasma and saliva.

During seasonal anoestrus, modifications of plasma concentrations of gonadotropin and steroid hormones have been shown. Plasma concentrations of LH are low and LH pulses are almost undetectable, whereas mean plasma concentrations of FSH are relatively constant throughout the year [1]. Plasma concentrations of progesterone and most of the dihydro-, tetrahydro- and hexahydro-progesterone are lower during seasonal anoestrus than during the luteal phase [4]. Plasma concentrations of dehydroepiandrosterone, estrone and 17β-oestradiol are lower during seasonal anoestrus than during the days before ovulation [4]. Moreover, in the present study, we observed significant differences between seasonal anoestrus and the other physiological stages for the plasma levels of proteinogenic amino acids such as tryptophan, glutamine, alanine, tyrosine, threonine, valine and leucine, for the plasma level of mannose, which is involved in protein glycosylation, and for the plasma level of creatinine, a by-product of the phosphorylation of creatine, that could indicate modifications of the protein turnover [34]. Amino acid are essential not only for building proteins but also for regulating cell metabolism, proliferation, differentiation and growth. Their bioavailability and metabolism, along with those of other macronutrients, are crucial for reproductive physiology [35]. The concentration of specific amino acids varies with the stage of follicular development, the species and bio-fluid [35]. These variations could also be related to differences in protein breakdown or synthesis, digestion in the digestive tract, or differential utilization in pathways. Some of the identified metabolites could be of particular interest for candidate biomarkers because their levels were significantly different between anoestrus and all or most of the other physiological stages: creatinine level was significantly higher in anoestrus than in all the other physiological stages with no overlap of individual data, alanine level was significantly higher during anoestrus than in most of the other stages,

threonine and 3-hydroxyisobutyrate (catabolic intermediate of valine) levels were significantly lower than in most of the other stages. The variation in creatinine levels during anoestrus provides further evidence that creatine and energy metabolisms are also implicated in the reproductive cycle. Therefore, these significant alterations of the plasma levels of amino acids and metabolites involved in protein turnover could be the signature of the transition between seasonal anoestrus and the ovarian cyclicity period.

Significant differences in the saliva metabolome were also observed between seasonal anoestrus and the ovarian cyclicity period, mostly modulation of the glycine and methionine metabolism. Levels of betaine (osmolyte and methyl groups donor), myo-inositol (osmolyte involved in energy metabolism) and alanine were higher, whereas levels of sarcosine (alanine isomer), isocaproate (branched-chain saturated fatty acid) and 3-hydroxyisobutyrate were lower during anoestrus than during another or several other physiological stages. Similar data were obtained in the blood of dairy cows: plasma levels of betaine and alanine were significantly higher in cows with inactive ovaries compared with cows in oestrus [36]. Moreover, antioxidant and methyl donor properties of betaine have been shown in the rat ovary [37]. On the contrary, serum levels of myo-inositol were significantly lower in cows with inactive ovaries compared with cows in oestrus [38]. Moreover, myo-inositol has been shown to strengthen aromatase and FSH receptor expression and modulate ovary steroidogenesis [39]. Thus, these metabolites could be interesting biomarkers of ovaries activity both in equine and bovine species. Alanine is of particular interest because its levels during seasonal anoestrus were significantly higher than during most of the other physiological stages in both saliva and plasma. Moreover, alanine absolute concentrations in equine saliva are within the detection range of commercial colorimetric assay kits (Goudet, personal communication), which open opportunities for inexpensive and fast assays in the field. We also identified other metabolites whose salivary levels varied significantly between seasonal anoestrus and the ovarian cyclicity period. However, these buckets could not be assigned to a single metabolite because the signals of several metabolites overlapped. The levels of these metabolites have to be evaluated separately with another assay to ascertain whether one or more are potential biomarkers of the transition between anoestrus and ovarian cyclicity period.

In summary, our study has revealed that significant modifications of the saliva metabolome occur between the seasonal anoestrus and the ovarian cyclicity period. We have proposed potential salivary biomarkers to differentiate mares in anoestrus from cyclic mares, providing that additional experiments with more animals and more salivary sampling points are performed to validate their reliability. Validation of these salivary biomarkers would allow us to consider the development of non-invasive tools to detect the entry of mares into the reproductive period; that would be of particular interest for horse breeding.

#### 4.3. Metabolome modifications during early gestation

Two metabolites and two groups of metabolites in saliva as well as two metabolites in plasma showed significantly different levels in samples collected during early gestation, all except one being significantly different from anoestrus stage. Since their levels were not different between ovarian cyclicity period and early gestation, they could not be used as biomarkers of gestation. However, in a previous study, the analysis of saliva steroidome showed that pregnenolone could be a potential salivary biomarker of gestation, since its concentrations during early gestation were significantly higher than during anoestrus and follicular phase and tended to be higher than during luteal phase [4].

## 5. Conclusion

This <sup>1</sup>H NMR analysis of the saliva and plasma metabolome during seasonal anoestrus, the follicular and luteal phases and early gestation,

allowed us to identify metabolites whose saliva and plasma levels vary significantly between the targeted physiological stages. We found that the physiological mechanisms involved in the transition from seasonal anoestrus to ovarian cyclicity and in ovulation induce modifications of the metabolome in both plasma and saliva. Based on our results, we have identified potential candidate biomarkers to detect ovulation or the transition from seasonal anoestrus to ovarian cyclicity. In particular, creatine and alanine could be proposed for candidate salivary biomarkers of ovulation and of the onset of ovarian cyclicity, respectively. However, extensive validation of their reliability is required by the examination of more animals and more time points. Salivary biomarkers would be of particular interest because saliva collection is a non-invasive, painless and easy sampling method allowing the collection of repeated samples, that would allow to develop diagnostic tests in the field in a welfare-friendly production system. Our study contributes to extend to domestic mammals the use of saliva as an alternative diagnostic fluid for reproduction.

#### Ethics declarations and approval for animal experiments

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 with the approval of the ethical review committee (Comité d’Éthique en Expérimentation Animale Val de Loire n°019) on 10 December 2021 (number APAFIS #34147–202111261451233 v2). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

#### Consent for publication

Not applicable.

#### Funding

This work was supported by INRAE, Institut Français du Cheval et de l’Équitation (IFCE) and University of Liège.

#### Data availability statement

The experimental and spectral data have been deposited under the DOI <https://doi.org/10.5281/zenodo.7806212> in the Zenodo repository (<https://zenodo.org>) hosted by the European Organization for Nuclear Research (CERN).

#### CRedit authorship contribution statement

**Ghylene Goudet:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Stéphane Beauclercq:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Conceptualization. **Cécile Douet:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Fabrice Reigner:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Conceptualization. **Stéfan Deleuze:** Writing – review & editing, Validation, Conceptualization. **Lydie Nadal-Desbarats:** Writing – review & editing, Validation, Project administration, Methodology, Formal analysis, Conceptualization.

#### Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use generative AI or AI-assisted technologies in the writing process.

#### Declaration of competing interest

None.

#### Acknowledgements

We would like to thank the staff of the experimental stud: Amandine Gesbert, Philippe Barrière, Thierry Blard and Yvan Gaude. We thank [Proof-Reading-Service.com](https://www.proofreading-service.com) for editing and proofreading.

#### List of abbreviations

|                    |                                                                                         |
|--------------------|-----------------------------------------------------------------------------------------|
| ane                | seasonal anoestrus                                                                      |
| UEPAO              | Unité Expérimentale de Physiologie Animale de l’Orfrasière                              |
| AU                 | arbitrary unit                                                                          |
| FDR                | false discovery rate                                                                    |
| GC-MS/MS           | gas chromatography coupled to tandem mass spectrometry                                  |
| ges                | early gestation 18 days after ovulation and artificial insemination                     |
| <sup>1</sup> H NMR | <sup>1</sup> H nuclear magnetic resonance spectroscopy                                  |
| INRAE              | Institut National de la Recherche pour l’Agriculture, l’Alimentation et l’Environnement |
| lut                | luteal phase 6 days after ovulation                                                     |
| ORA                | over-representation analysis                                                            |
| ov-3               | 3 days before ovulation                                                                 |
| ov-2               | 2 days before ovulation                                                                 |
| ov-1               | 1 day before ovulation                                                                  |
| ov                 | the day when ovulation was detected                                                     |
| SCFA               | short chain fatty acids                                                                 |
| TCA                | tricarboxylic acid                                                                      |
| TSP                | 3-trimethylsilylpropionic acid                                                          |

#### References

- [1] Aurich C. Reproductive cycles of horses. *Anim Reprod Sci* 2011;124:220–8.
- [2] Davies Morel MCG, Newcombe JR, Holland SJ. Factors affecting gestation length in the Thoroughbred mare. *Anim Reprod Sci* 2002;74:175–85.
- [3] Lee YH, Wong DT. Saliva: an emerging biofluid for early detection of diseases. *Am J Dent* 2009;22:241–8.
- [4] Goudet G, Douet C, Pianos A, Haddad L, Reigner F, Deleuze S, et al. Saliva and plasma steroidome in mare during reproductive stages: a GC-MS/MS study. *Frontiers Animal Sci.* 2022;3.
- [5] Bazzano M, Laghi L, Zhu C, Lotito E, Sgariglia S, Tesi B, et al. Exercise induced changes in salivary and serum metabolome in trained standardbred. *Metabolites* 2020;10. Assessed by (1)H-NMR.
- [6] Mojsym W, Wawrzykowski J, Jamioł M, Chrobak Ł, Kankofer M. Comparative analysis of saliva and plasma proteins patterns in pregnant cows-preliminary studies. *Animals : an open access J MDPI* 2022;12.
- [7] Fatet A, Nadal-Desbarats L, Boissard K, Antar C, Freret S, Pellicer-Rubio M-T, et al. Characterization of serum metabolome changes during the 5 weeks prior to breeding in female goat kids. *Livest Sci* 2021;250:104572.
- [8] Goudet G, Nadal-Desbarats L, Douet C, Savoie J, Staub C, Venturi E, et al. Salivary and urinary metabolome analysis for pre-puberty-related biomarkers identification in porcine. *Animal :Int J Animal biosci* 2019;13:760–70.
- [9] Goudet G, Prunier A, Nadal-Desbarats L, Grivault D, Ferchaud S, Pianos A, et al. Steroidome and metabolome analysis in gilt saliva to identify potential biomarkers of boar effect receptivity. *Animal : Int J Animal biosci* 2021;15:100095.
- [10] Saibaba G, Rajesh D, Muthukumar S, Sathiyarayanan G, Aarthy AP, Archunan G. Salivary proteome profile of women during fertile phase of menstrual cycle as characterized by mass spectrometry. *Gynecology and minimally invasive therapy* 2021;10:226–34.
- [11] Singh LK, Pandey M, Baithalu RK, Fernandes A, Ali SA, Jaiswal L, et al. Comparative proteome profiling of saliva between estrus and non-estrus stages by employing label-free quantitation (Lfq) and tandem mass tag (TMT)-LC-MS/MS analysis: an approach for estrus biomarker identification in *Bubalus bubalis*. *Front Genet* 2022;13.
- [12] Kersey DC, Dehnhard M. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. *Gen Comp Endocrinol* 2014;203:296–306.
- [13] Canepa S, Laine A-L, Bluteau A, Fagu C, Flon C, Monniaux D. Validation d’une méthode immunoenzymatique pour le dosage de la progestérone dans le plasma des ovins et des bovins. *Cahier des Techniques de l’INRA* 2008;64:19–30.
- [14] Panzani D, Di Vita M, Lainé A-L, Guillaume D, Rota A, Tesi M, et al. Corpus luteum vascularization and progesterone production in autumn and winter cycles of the mare: relationship between ultrasonographic characteristics of corpora lutea and

- plasma progesterone concentration in the last cycles before anestrous. *J Equine Vet Sci* 2017;56:35–9.
- [15] Ginther OJ, Gestal EL, Gestal MO, Beg MA. Regulation of circulating gonadotropins by the negative effects of ovarian hormones in mares. *Biol Reprod* 2005;73:315–23.
- [16] Pillet E, Batellier F, Duchamp G, Furstoss V, Vern YLE, Kerboeuf D, et al. Freezing stallion semen in INRA96®-based extender improves fertility rates in comparison with INRA82. *Dairy Sci Technol* 2008;88:257–65.
- [17] Martias C, Gatién J, Roch L, Baroukh N, Mavel S, Lefèvre A, et al. Analytical methodology for a metabolome atlas of goat's plasma, milk and feces using (1)H-NMR and UHPLC-HRMS. *Metabolites* 2021;11.
- [18] Jacob D, Deborde C, Lefebvre M, Maucourt M, Moing A. NMRProcFlow: a graphical and interactive tool dedicated to 1D spectra processing for NMR-based metabolomics. *Metabolomics* 2017;13:36.
- [19] Wishart DS, Guo A, Oler E, Wang F, Anjum A, Peters H, et al. HMDB 5.0: the human metabolome database for 2022. *Nucleic Acids Res* 2022;50:D622. d31.
- [20] Xia J, Wishart DS. MSEA: a web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. *Nucleic Acids Res* 2010;38:W71–7.
- [21] Pang Z, Chong J, Zhou G, de Lima Morais DA, Chang L, Barrette M, et al. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. *Nucleic Acids Res* 2021;49:W388. w96.
- [22] Greenhouse SW, Geisser S. On methods in the analysis of profile data. *Psychometrika* 1959;24:95–112.
- [23] Tokmakov AA, Stefanov VE, Sato KI. Dissection of the ovulatory process using ex vivo approaches. *Front Cell Dev Biol* 2020;8:605379.
- [24] Alleva JJ, Bonventre PF, Lamanna C. Inhibition of ovulation in hamsters by the protein synthesis inhibitors diphtheria toxin and cycloheximide. *Proc Soc Exp Biol Med* 1979;162:170–4.
- [25] Downing JA, Joss J, Scaramuzzi RJ. A mixture of the branched chain amino acids leucine, isoleucine and valine increases ovulation rate in ewes when infused during the late luteal phase of the oestrous cycle: an effect that may be mediated by insulin. *J Endocrinol* 1995;145:315–23.
- [26] Zaha I, Muresan M, Tulcan C, Huniadi A, Naghi P, Sandor M, et al. The role of oxidative stress in infertility. *J Personalized Med* 2023;13:1264.
- [27] Castiglione Morelli MA, Iuliano A, Schettini SCA, Petruzzi D, Ferri A, Colucci P, et al. Metabolic changes in follicular fluids of patients treated with recombinant versus urinary human chorionic gonadotropin for triggering ovulation in assisted reproductive technologies: a metabolomics pilot study. *Arch Gynecol Obstet* 2020;302:741–51.
- [28] Umehara T, Kawai T, Goto M, Richards JS, Shimada M. Creatine enhances the duration of sperm capacitation: a novel factor for improving in vitro fertilization with small numbers of sperm. *Hum Reprod* 2018;33:1117–29.
- [29] Smith-Ryan AE, Cabre HE, Eckerson JM, Candow DG. Creatine supplementation in women's health: a lifespan perspective. *Nutrients* 2021;13:877.
- [30] Morland C, Frøland AS, Pettersen MN, Storm-Mathisen J, Gundersen V, Rise F, et al. Propionate enters GABAergic neurons, inhibits GABA transaminase, causes GABA accumulation and lethargy in a model of propionic acidemia. *Biochem J* 2018;475:749–58.
- [31] Epperson CN, Haga K, Mason GF, Sellers E, Gueorguieva R, Zhang W, et al. Cortical  $\gamma$ -aminobutyric acid levels across the menstrual cycle in healthy women and those with premenstrual dysphoric disorder: a proton magnetic resonance spectroscopy study. *Arch Gen Psychiatr* 2002;59:851–8.
- [32] Erdő SL, Varga B, Horváth E. Effect of local GABA administration on rat ovarian blood flow, and on progesterone and estradiol secretion. *Eur J Pharmacol* 1985;111:397–400.
- [33] Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* 2004;25:947–70.
- [34] Biolo G, Toigo G, Ciocchi B, Morena G, Situlin R, Vasile A, et al. Relationship between whole-body protein turnover and serum creatinine in chronically uremic patients. *Miner Electrolyte Metab* 1998;24:267–72.
- [35] Gao H. Amino acids in reproductive nutrition and health. *Adv Exp Med Biol* 2020;1265:111–31.
- [36] Song YX, Hu P, Bai YL, Zhao C, Xia C, Xu C. Plasma metabolic characterisation of dairy cows with inactive ovaries and oestrus during the peak of lactation. *J Veterinary Res* 2019;63:359–67.
- [37] Alirezai M, Niknam P, Jelodar G. Betaine elevates ovarian antioxidant enzyme activities and demonstrates methyl donor effect in non-pregnant rats. *Int J Pept Res Therapeut* 2012;18.
- [38] Zhao C, Bai Y, Fu S, Wu L, Xia C, Xu C. Comparison of metabolic alterations in serum and milk whey between inactive ovaries and estrus dairy cows. *Front Vet Sci* 2020;7:609391.
- [39] Bizzarri M, Monti N, Piombarolo A, Angeloni A, Verna R. Myo-inositol and D-chiro-inositol as modulators of ovary steroidogenesis: a narrative review. *Nutrients* 2023;15.