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Metabolic profiling of preovulatory follicular fluid in jennies

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ABSTRACT

Follicular fluid is formed from the transudation of theca and granulosa cells in the growing follicular antrum. Its main function is to provide an optimal intrafollicular microenvironment to modulate oocyte maturation. The aim of this study was to determine the metabolomic profile of preovulatory follicular fluid (PFF) in jennies. For this purpose, PFF was collected from 10 follicles of five jennies in heat. Then, PFF samples were analysed by nuclear magnetic resonance (NMR) and heteronuclear single quantum correlation (2D $^{1}H/^{13}C$ HSQC). Our study revealed the presence of at least 27 metabolites in the PFF of jennies (including common amino acids, carboxylic acids, amino acid derivatives, alcohols, saccharides, fatty acids, and lactams): 3-hydroxybutyrate, acetate, alanine, betaine, citrate, creatine, creatine phosphate, creatinine, ethanol, formate, glucose, glutamine, glycerol, glycine, hippurate, isoleucine, lactate, leucine, lysine, methanol, phenylalanine, proline, pyruvate, threonine, tyrosine, valine, and τ-methylhistidine. The metabolites found here have an important role in the oocyte development and maturation, since the PFF surrounds the follicle and provides it with the needed nutrients. Our results indicate a unique metabolic profile of the jennies PFF, as it differs from those previously observed in the PFF of the mare, a phylogenetically close species that is taken as a reference for establishing reproductive biotechnology techniques in donkeys. The metabolites found here also differ from those described in the TCM-199 medium enriched with fetal bovine serum (FBS), which is the most used medium for *in vitro* oocyte maturation in equids. These differences would suggest that the established conditions for *in vitro* maturation used so far may not be suitable for donkeys. By providing the metabolic composition of jenny PFF, this study could help understand the physiology of oocyte maturation as a first step to establish *in vitro* reproductive techniques in this species.

1. Introduction

Metabolomics is a modern field in analytical biochemistry that can be understood as the final step in the "omics" cascade (Dettmer and Hammock, 2004; Jurowski et al., 2017). Whereas genomics investigates the complete genome to decipher gene function, transcriptomics gives a more functional focus by measuring only the expressed genes and proteomics tries to comprehend the role of translated proteins, metabolomics represents the ultimate answer of an organism to environmental influences, genetic alterations, or disease (Dettmer and Hammock, 2004).

Metabolomics examines small molecules (<1000 Da), the metabolites, which are the end products of cellular processes that are affected by the physiological state of a cell at any given time (Dumesic et al., 2015). In the case of ovarian cell types, their metabolites are found in a complex dynamic biological fluid that surrounds the developing oocyte:

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the so-called follicular fluid (FF) (Singh and Sinclair, 2007; Nel-Themaat and Nagy, 2011; Mariani and Bellver, 2018). The FF is formed from the transudation of theca and granulosa cells in the growing follicular antrum (Guerreiro et al., 2018) and contains a variety of molecules, such as steroid hormones, polysaccharides, proteins, reactive oxygen species (ROS), antioxidants, and metabolites (Mariani and Bellver, 2018). The function of these components is to provide an optimal intrafollicular microenvironment to modulate oocyte and follicular maturation (Bianchi et al., 2016), facilitate essential communication between somatic and germ cells to acquire oocyte competence (Mariani and Bellver, 2018), protect follicular cells from oxidative damage (Ambekar et al., 2013; Freitas et al., 2017; Gupta et al., 2011) and contribute to the oocyte extrusion during ovulation (Richards et al., 1998). The composition and quantity of FF within the follicle changes during the different follicular developmental stages, from initial growth to atresia (Freitas et al., 2017; Gupta et al., 2011; Yang et al., 2020; Wise, 1987; Fortune et al., 2004), whereas other factors associated to FF variations are animal age (Takeo et al., 2017; Bender et al., 2010), follicular size and day of oestrous (Gupta et al., 2011; Wise, 1987).

Because the FF constitutes the immediate microenvironment of the oocyte, it provides the nutrients, growth factors and hormones necessary to promote its growth and maintain its viability (Dumesic et al., 2015). Thus, the composition of FF significantly affects follicular growth (Freitas et al., 2017; Gupta et al., 2011), the quality and quantity of matured oocytes (Dumesic et al., 2015; Guerreiro et al., 2018; Takeo et al., 2017), *in vitro* fertilization rate (Nagy et al., 2019; Botero-Ruiz et al., 1984) and pregnancy outcome after embryo transfer (Boakari et al., 2021) by accumulating products from all types of metabolism (Yang et al., 2020). In fact, multiple FF components have been identified as biomarkers of oocyte developmental competence (Kreiner et al., 1987; Revelli et al., 2009; O'Gorman et al., 2013) and *in vitro* embryo development (Kafi et al., 2017; Sinclair et al., 2008).

In equids, the production of *in vivo*-derived embryos from donors is limited by the lack of efficiency in superovulation treatments (Squires et al., 2003; Smits et al., 2012; Meyers-Brown et al., 2011), thus reducing the number of embryos available to one per oestrus cycle (Smits et al., 2012; Goudet et al., 2015; Deleuze et al., 2018). In addition, the large size of recovered *in vivo* embryos makes them "bad candidates" for cryopreservation (Choi and Hinrichs, 2017; Herrera et al., 2015). *In vivo*-derived embryos are normally recovered after 7–8 days from ovulation in the blastocyst stage and have a diameter of >300 μm, due to a blastocoelic cavity full of fluid that makes more difficult the exchange of water by cryoprotectants (Squires et al., 2003; Deleuze et al., 2018; Choi and Hinrichs, 2017; Herrera et al., 2015; Hinrichs and Hinrichs, 2012). For this reason, the very first step for IVP of embryos from donors is widely documented in horses: the ultrasound-guided oocyte retrieval, or Ovum Pick Up (OPU) (Kanitz et al., 1995; Purcell et al., 2007; Jacobson et al., 2010; Galli et al., 2007; Spacek and Carnevale, 2018; Dell'Aquila et al., 1997; Caillaud et al., 2008; Douet et al., 2017). After collection and IVM, oocytes are then fertilized by intracytoplasmic sperm injection (ICSI), which in horses is very efficient and the only option, as, to date, conventional IVF does not work in this species (Choi and Hinrichs, 2017; Herrera et al., 2015; Jacobson et al., 2010; Galli et al., 2007; Spacek and Carnevale, 2018; Dell'Aquila et al., 1997; Caillaud et al., 2008; Douet et al., 2017; Leemans et al., 2016). OPU and ICSI are, therefore, established procedures that have been widely implemented to produce horse embryos *in vitro*, both for scientific purposes and breeding from infertile and sporting mares (Galli et al., 2014). While, as it is one of the key steps for embryo IVP, much research has been conducted regarding the IVM of horse oocytes (Jacobson et al., 2010; Galli et al., 2007; Spacek and Carnevale, 2018; Dell'Aquila et al., 1997; Caillaud et al., 2008; Fernández-hernández et al., 2020; Lewis et al., 2020a; Lewis et al., 2020b; Walter et al., 2019; Ascari et al., 2017; Pereira et al., 2019), little has been made in the case of donkeys (Zhao et al., 2011).

Based on its properties and role in physiological conditions, several attempts to use FF for the *in vitro* maturation (IVM) of oocytes in different species have been made (Douet et al., 2017; Zhao et al., 2020; Horikawa et al., 2019; Cruz et al., 2014; Somfai et al., 2012; Azari-Dolatabad et al., 2021; Tesarik and Mendoza, 1995; Tesarik and Mendoza, 1997). It has been demonstrated that supplementing the media used for IVM with FF resulted in improved oocyte quality and maturation rates (Zhao et al., 2020; Horikawa et al., 2019; Azari-Dolatabad et al., 2021), sperm penetration (Somfai et al., 2012), and subsequent embryonic development (Horikawa et al., 2019; Cruz et al., 2014; Azari-Dolatabad et al., 2021). In bovine, Azari-Dolatabad et al. (Azari-Dolatabad et al., 2021) observed that these beneficial effects rely upon the amount of FF added to the IVM medium, the composition of that medium (serum *vs.* serum-free), and the type of culture system (group *vs.* individual). In horses, supplementing the IVM medium with FF also has a positive effect (Spacek and Carnevale, 2018; Dell'Aquila et al., 1997; Caillaud et al., 2008; Douet et al., 2017); however, whether this is also the case of the donkey - a species that, despite being phylogenetically close to the horse, has a different reproductive strategy, remains unaddressed.

Most wild equids are currently endangered or threatened, while many breeds of domestic horses and donkeys are in risk of extinction. This situation led to the development of various conservation programs and to focus on the need of storing genomic resources (Douet et al., 2017). This, added to the growing demand for new uses (milk production, cosmetics, forestry, rural tourism, leisure…), promotes an increasing interest in developing studies to understand and improve donkey reproductive performance (Martini et al., 2018; Miró et al., 2020).

Despite advances in donkey sperm cryopreservation, the use of frozen-thaw semen for artificial insemination is limited by the low pregnancy rates obtained in jennies (Miró et al., 2020; Álvarez et al., 2019; Vidament et al., 2009; Canisso et al., 2011; Oliveira et al., 2012). Thus, the development of other reproductive biotechnologies such as *in vitro* production (IVP) of embryos could be important for breeding in this species. In addition, IVP of embryos and their cryopreservation would allow the preservation of the male and female genetics, being a fast method to restore a breed (Douet et al., 2017; Woelders et al., 2012). Although the first experiments of oocyte IVM (Goudet et al., 2015; Deleuze et al., 2018; Zhao et al., 2011; Abdoon et al., 2014), *in vitro* fertilization (IVF) (Goudet et al., 2015; Deleuze et al., 2018) and even vitrification (Douet et al., 2019) were performed in jennies, maturation and sperm penetration rates are still very low and no IVP embryos have been obtained yet.

For the above reasons, further knowledge on the metabolic profile of donkey follicular fluid is important for the development of reproductive biotechnologies such as IVM, IVF and IVP of embryos in this species. As far as we are concerned, there are no previous reports on the composition of jenny FF. Thus, the objective of this study was to describe, for the first time, the metabolic profile of preovulatory follicular fluid (PFF) collected from Catalonian jennies, as a preliminary step to set up better conditions for IVM in this species.

2. Materials and methods

2.1. Chemicals and suppliers

Unless otherwise stated, all chemicals and reagents were purchased from Merck (Merck KgaA, Darmstadt, Germany).

2.2. Animals and samples

FF samples were obtained from 10 preovulatory follicles of five Catalonian jennies (two follicles per jenny) between 3 and 12 years old. Animals were kept in paddocks, fed with grain forage, straw and hay, provided with water *ad libitum*, and housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola

del Vallès, Spain), which works under strict health control and animal welfare protocols. This Service already works under the approval of the *Generalitat de Catalunya* (Spain), and the study was approved by the Ethics Committee of the Autonomous University of Barcelona (Code: CEEAH 1424).

The reproductive tract of jennies was carefully examined by ultrasound prior to the extraction of PFF. Samples were obtained from follicles ≥35 mm in diameter accompanied by uterine edema and without corpus luteum in the ovaries, which was considered preovulatory according to the described by Taberner et al. (Taberner et al., 2008) for this breed. By echo-guided flank puncture using a 20-mL plastic syringe attached to a 20-g hypodermic needle and ovary displacement to the flank by transrectal palpation, approximately 15 mL of PFF per follicle were aspirated. Then, the total volume of PFF was split into 1-mL aliquots and kept in Eppendorf tubes at −80 °C until further use.

2.3. Preparation of PFF samples for NMR

An aliquot of each PFF sample was thawed on ice, vortexed and centrifuged through 0.5-mL Amicon® ultracentrifuge filters at 14,000 ×*g* and 4 °C for 90 min to remove protein and cell debris. These filters had been previously washed four times by adding 500 μL PBS and centrifuging at 14,000 ×*g* and 4 °C for 20 min.

After centrifugation, 200 μL of each eluted PFF sample were diluted with 100 μL PBS (containing 10% D_2O with 0.33% DSS; pH 7.4) and 300 μL D_2O. Samples were then transferred to 5-mm Wilmad® NMR tubes for NMR profile acquisition.

2.4. NMR spectra

To obtain ^1H NMR spectra, a Bruker 600-MHz AVANCE III NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at a ^1H frequency of 600.13 MHz and 300 K with a previous equilibration time (10 min), was used. The 1D-^1H-nuclear Overhauser effect spectroscopy (1D-NOESY) pulse sequence from the Bruker library was used (noesygppr1d). The parameters applied were: (1) mixing time: 100 ms (d8); (2) recovery delay: 2 s (d1); (3) 90° pulse: 10.42 μs (p1); (4) spectral width: 7211.539 Hz; (5) spectral size: 32 k; (6) number of scans: 256; and (7) acquisition time: 2.27 s.

For metabolite identification, a 2D $^1H/^{13}C$ HSQC (Heteronuclear Single Quantum Correlation) was acquired in a representative sample. A Bruker 500-MHz AVANCE-III NMR spectrometer equipped with a triple channel cryoprobe and z-gradient was used. The experiment was run using a slight modification of the original pulse sequence hsqcf3gpph19 from Bruker library; this modification consisted of adding water presaturation during the recovery delay and changing the original f3 channel (usually used for ^{15}N) to f2 channel for ^{13}C. Relevant acquisition parameters were (1) recovery delay d1 = 1 s; (2) constant for JCH evolution = 140 Hz; (3) Spectral size = 2 K × 128; (4) number of scans = 400; (5) Spectral widths = 15.6 ppm for ^1H and 100 ppm for ^{13}C.

See Supplementary Material for ^1H and HSQC spectral images and metabolite assignment.

2.5. Data processing and analysis

Spectra were processed and analysed through the Chenomx 8.0 profiler software (Chenomx Inc., Edmonton, Canada). This software contains tools for automatic phase, baseline correction, reference calibration and a library of metabolites for spectral profiling. The concentration of each metabolite identified in SP was calculated based on DSS concentration (0.216 mmol/L).

2.6. Statistical analyses

The statistical package R (V 4.0.3, R Core Team; Vienna, Austria) was used to run statistical analyses, whereas the GraphPad Prism software (V 8.4.0, GraphPad Software LLC; San Diego, CA, USA) was used to design the graphics. Concentrations of each metabolite identified in jenny PFF were analysed by descriptive statistics, where the mean, SEM, and range were established.

3. Results

3.1. Metabolites identified in jenny PFF

The rationale behind studying the PFF metabolome is supported by the crucial function of this fluid, as it supports oocyte maturation, ovulation and fertility, and also plays a role in embryo development and implantation (Singh and Sinclair, 2007; Guerreiro et al., 2018; Fortune et al., 2004; Revelli et al., 2009).

The present work investigated for the first time the metabolic profile of jennies' PFF using ^1H NMR, followed by a heteronuclear simple quantum correlation for metabolite identification purposes. Our study revealed the presence of at least 27 metabolites (see Table 1), including common amino acids, carboxylic acids, amino acid derivatives, alcohols, saccharides, fatty acids, and lactams. A correlation heat map (see Supplementary Fig. 1) revealed that most metabolites correlated positively with each other across the 10 PFF samples, except for ethanol with methanol, lactate and glycerol, which presented a negative correlation.

Representative ^1H NMR spectra with expanded areas for metabolite assignment acquired at 600.13 MHz is shown in Supplementary Fig. 2. Expanded area of $^1H/^{13}C$ HSQC NMR and metabolite assignment of a representative sample acquired at 500 MHz equipped with a cryprobe is available in Supplementary Fig. 3.

3.2. Comparison of jenny preovulatory follicular fluid metabolites with those found in the mare and in TCM-199 medium

Table 2 shows the comparison of metabolites described in mare PFF by Fernández-Hernández et al. (Fernández-hernández et al., 2020) and those included in the composition of commercial TCM-199 medium with those found in our study in jenny. Of the 27 metabolites identified in jenny PFF, 12 coincided with those described in the mare. These metabolites were acetate, alanine, citrate, creatine, creatine phosphate, glucose, glycine, isoleucine, lactate, leucine, threonine, and valine. Concentrations of acetate, citrate and glucose did not differ greatly from the mean reported in the mare. For the rest of metabolites, there were marked differences of a lower concentration in the PFF of the donkey than in the mare, except for valine, which has a much higher concentration in the donkey.

On the other hand, eight of the 27 metabolites identified in jenny PFF coincided with those included in the composition of TCM-199. These metabolites were acetate, alanine, glucose, glycine, isoleucine, leucine, threonine and valine. While the concentrations of alanine and glucose in jenny PFF did not differ greatly from those in TCM-199, those of the remaining metabolites, except for acetate and valine, are considerably lower in jenny PFF than in TCM-199.

4. Discussion

In equids, the production of *in vivo*-derived embryos from donors is limited by the lack of efficiency in superovulation treatments (Squires et al., 2003; Smits et al., 2012; Meyers-Brown et al., 2011), thus reducing the number of embryos available to one per oestrus cycle (Smits et al., 2012; Goudet et al., 2015; Deleuze et al., 2018). In addition, the large size of recovered *in vivo* embryos makes them "bad candidates" for cryopreservation (Choi and Hinrichs, 2017; Herrera et al., 2015). *In vivo*-derived embryos are normally recovered after 7–8 days from ovulation in the blastocyst stage and have a diameter of >300 μm, due to a blastocoelic cavity full of fluid that makes more difficult the exchange of water by cryoprotectants (Squires et al., 2003; Deleuze et al., 2018; Choi and Hinrichs, 2017; Herrera et al., 2015; Hinrichs and

Table 1
Mean ± SEM and range of concentrations, chemical nature, KEGG pathways, cell localisation and identification (PubChem/KEGG database) of metabolites in jenny preovulatory follicular fluid (PFF) samples ($n = 10$).

Metabolite	Mean (mM) ± SEM (mM)	Range (mM)	Chemical nature[1]	KEGG pathways[1]	Cellular localisation[2]	PubChem CID[2]/KEGG entry[1]
3-Hydroxybutyrate	0.414 ± 0.048	0.242–0.655	Hydroxy fatty acid	Intermediate metabolite Regulatory signaling	–	3,541,112 -
Acetate	1.333 ± 0.183	0.707–2.234	Organic monocarboxylic acid	Glycolysis/Gluconeogenesis Pyruvate metabolism	–	175 C00033
Alanine	0.355 ± 0.044	0.204–0.593	Common amino acid	Biosynthesis of amino acids Protein digestion and absorption	Cytoplasm Lysosome Mitochondria Peroxisome Extracellular	602 C00041
Betaine	0.106 ± 0.019	0.038–0.213	Amino acid derivative	Glycine, serine and threonine metabolism ABC transporters	–	247 C00719
Citrate	0.343 ± 0.034	0.225–0.544	Tricarboxylic acid	Citrate cycle (TCA cycle, Krebs cycle) Reductive citrate cycle (Arnon-Buchanan cycle)	–	31,348 C00158
Creatine	0.111 ± 0.010	0.061–0.175	Composed amino acid	Metabolic pathways (ATP) Biosynthesis of amino acids[2]	Cytoplasm Mitochondria Extracellular	586 C00300
Creatine phosphate	0.089 ± 0.012	0.050–0.168	Phosphoamino acid	Rapid source of energy (ADP➔ATP)[2]	Mitochondria	9,548,602 C02305
Creatinine	0.063 ± 0.010	0.029–0.129	Lactam	Creatine pathway	Cytoplasm	588 C00791
Ethanol	0.794 ± 0.144	0.467–2.033	Primary alcohol	Glycolysis / Gluconeogenesis Pyruvate metabolism Inflammatory mediator regulation of TRP channels	Cytoplasm Peroxisome Extracellular	702 C00469
Formate	0.815 ± 0.088	0.453–1.188	Organic monocarboxylic acid	Pyruvate metabolism Degradation of aromatic compounds	–	283 C00058
Glucose	4.820 ± 0.424	2.954–6.612	Monosaccharide	Glycolysis / Gluconeogenesis Starch and Sucrose metabolism Amino sugar and Nucleotide sugar metabolism	–	5793 C00031
Glutamine	0.465 ± 0.050	0.245–0.735	Common amino acid	Nucleotide metabolism ABC transporters / GABAergic synapse Protein digestion and absorption	Extracellular Mitochondria	5961 C00303
Glycerol	0.915 ± 0.129	0.377–1.756	Sugar alcohol	Regulation of lipolysis Thermogenesis Metabolic pathways	Extracellular Mitochondria	753 C00116
Glycine	0.436 ± 0.053	0.257–0.694	Common amino acid	Protein digestion and absorption ABC transporters Glutathione metabolism Biosynthesis of amino acids and cofactors	Extracellular Lysosome Mitochondria Peroxisome	750 C00037
Hippurate	0.053 ± 0.007	0.020–0.081	Carboxylic acid	Metabolic pathways	Cytoplasm	464 C01586
Isoleucine	0.139 ± 0.012	0.089–0.199	Common amino acid	Protein digestion and absorption ABC transporters Biosynthesis of amino acids	Cytoplasm Extracellular Mitochondria	6303 C00407
Lactate	2.091 ± 0.345	1.306–4.270	Hydroxycarboxylic acid	Glycolysis / Gluconeogenesis cAMP signaling pathway Glucagon signaling pathway	–	91,435 C00186
Leucine	0.179 ± 0.018	0.101–0.248	Common amino acid	2-Oxocarboxylic acid metabolism Biosynthesis of amino acids ABC transporters Protein digestion and absorption	Extracellular Mitochondria	6106 C00123
Lysine	0.131 ± 0.013	0.076–0.193	Common amino acid	2-Oxocarboxylic acid metabolism Biosynthesis of amino acids ABC transporters Protein digestion and absorption	Cytoplasm Extracellular Mitochondria	5962 C00047

(*continued on next page*)

Table 1 (*continued*)

Metabolite	Mean (mM) ± SEM (mM)	Range (mM)	Chemical nature[1]	KEGG pathways[1]	Cellular localisation[2]	PubChem CID[2]/KEGG entry[1]
					Nucleus	
					Peroxisome	
Methanol	0.241 ± 0.090	0.043–0.815	Primary alcohol	Carbon metabolism	Endoplasmic reticulum	887
				Degradation of aromatic compounds	Extracellular	C00132
				Metabolic pathways	Lysosome	
Phenylalanine	0.065 ± 0.007	0.036–0.094	Common amino acid	Phenylpropanoid biosynthesis	–	155,178
				Biosynthesis of amino acids		C00079
				ABC transporters		
				Protein digestion and absorption		
Proline	0.321 ± 0.036	0.167–0.484	Common amino acid	Aminoacyl-tRNA biosynthesis	Cytoplasm	145,742
				Biosynthesis of amino acids	Endoplasmic reticulum	C00148
				ABC transporters	Extracellular	
				Protein digestion and absorption	Lysosome	
					Mitochondria	
Pyruvate	0.198 ± 0.020	0.122–0.299	Carboxylic acids	Glycolysis / Gluconeogenesis	–	107,735
				Citrate cycle (TCA cycle)		C00022
				Pentose phosphate pathway		
				Biosynthesis of amino acids		
				AMPK signaling pathway		
Threonine	0.147 ± 0.027	0.033–0.293	Common amino acid	Aminoacyl-tRNA biosynthesis	Cytoplasm	6288
				Biosynthesis of amino acids	Extracellular	C00188
				ABC transporters		
				Protein digestion and absorption		
Tyrosine	0.076 ± 0.009	0.043–0.120	Common amino acid	Aminoacyl-tRNA biosynthesis	Cytoplasm	6057
				2-Oxocarboxylic acid metabolism	Extracellular	C00082
				Biosynthesis of amino acids	Mitochondria	
				Protein digestion and absorption		
Valine	0.422 ± 0.038	0.258–0.594	Common amino acid	Aminoacyl-tRNA biosynthesis	Extracellular	6287
				2-Oxocarboxylic acid metabolism	Mitochondria	C00183
				Biosynthesis of amino acids		
				ABC transporters		
				Protein digestion and absorption		
τ-Methylhistidine	0.059 ± 0.005	0.033–0.082	Modified amino acid	β-alanine metabolism	Cytoplasm	C03298
				ABC transporters / GABAergic synapse		64,969
				Protein digestion and absorption		
				Histidine Metabolism		

[1] KEGG Compound Database: https://www.kegg.jp/kegg/compound/.
[2] PubChem: https://pubchem.ncbi.nlm.nih.gov/.

Table 2
Presence and concentration of metabolites identified in jenny and mare preovulatory follicular fluids (PFF), and in TCM-199 medium.

Metabolite	Jenny PFF (mM)	Mare PFF (mM)[1]	TCM-199 medium (mM)[2]
3-Hydroxybutyrate	0.414	–	–
Acetate	1.333	1.500	0.610
Acetylcarnitine	–	0.370	–
Alanine	0.355	1.100	0.280
Aspartate	–	2.700	0.220
Betaine	0.106	–	–
Carnitine	–	0.090	–
Choline	–	0.030	0.003
Citrate	0.343	0.400	–
Creatine	0.111	0.360	–
Creatine phosphate	0,089	0.360	–
Creatinine	0.063	–	–
Ethanol	0.794	–	–
Formate	0.815	–	–
Fumarate	–	0.050	–
Glucose	4.820	4.300	5.550
Glucose-1-phosphate	–	6.900	–
Glutamine	0.465	–	–
Glycerol	0.915	–	–
Glycine	0.436	3.200	0.670
Hippurate	0.053	–	–
Histamine	–	0.250	–
Histidine	–	0.050	0.100
Isoleucine	0.139	0.600	0.300
Lactate	2.091	27.300	–
Leucine	0.179	0.500	0.460
Lysine	0.131	–	–
Methanol	0.241	–	–
Phenylalanine	0.065	–	–
Proline	0.321	–	–
Pyruvate	0.198	–	–
Pyruvate + Succinate	–	0.160	–
Threonine	0.147	0.350	0.250
Tyrosine	0.076	–	–
Valine	0.422	0.130	0.210
τ-Methylhistidine	0.059	–	–

[1] Concentration mean of metabolites that were described in mare preovulatory follicular fluid (PFF) by Fernández-Hernández et al. (Fernández-hernández et al., 2020) and concurred with those found in our study in jennies.

[2] Concentration mean of metabolites included in the commercial TCM-199 medium (manufacturer's specifications) that coincided with those found in our study in jennies.

Hinrichs, 2012). For this reason, the very first step for IVP of embryos from donors is widely documented in horses: the ultrasound-guided oocyte retrieval, or Ovum Pick Up (OPU) (Kanitz et al., 1995; Purcell et al., 2007; Jacobson et al., 2010; Galli et al., 2007; Spacek and Carnevale, 2018; Dell'Aquila et al., 1997; Caillaud et al., 2008; Douet et al., 2017). After collection and IVM, oocytes are then fertilized by intracytoplasmic sperm injection (ICSI), which in horses is very efficient and the only option, as, to date, conventional IVF does not work in this species (Choi and Hinrichs, 2017; Herrera et al., 2015; Jacobson et al., 2010; Galli et al., 2007; Spacek and Carnevale, 2018; Dell'Aquila et al., 1997; Caillaud et al., 2008; Douet et al., 2017; Leemans et al., 2016). OPU and ICSI are, therefore, established procedures that have been widely implemented to produce horse embryos *in vitro*, both for scientific purposes and breeding from infertile and sporting mares (Galli et al., 2014). While, as it is one of the key steps for embryo IVP, much research has been conducted regarding the IVM of horse oocytes (Jacobson et al., 2010; Galli et al., 2007; Spacek and Carnevale, 2018; Dell'Aquila et al., 1997; Caillaud et al., 2008; Fernández-hernández et al., 2020; Lewis et al., 2020a; Lewis et al., 2020b; Walter et al., 2019; Ascari et al., 2017; Pereira et al., 2019), little has been made in the case of donkeys (Zhao et al., 2011).

To the best of our knowledge, there are no previous studies about the composition of follicular fluid in donkeys. Yet, several works investigated the composition of PFF in mares (Fernández-hernández et al., 2020; Gérard et al., 2002; Gérard et al., 2015), the phylogenetically closest species, which has been used as a model for applying assisted reproduction techniques in donkey and the rest of wild equids (Smits et al., 2012). A recent report revealed the presence of 22 metabolites in mare PFF (Fernández-hernández et al., 2020), in contrast with the 27 detected herein in that of jenny (see Table 2). The most interesting finding is that only 12 metabolites found in jenny PFF coincided with those reported in that of the mare (Fernández-hernández et al., 2020). To note, pyruvate peak was overlapped with that of succinate peak in the NMR spectrum of mare PFF, these two metabolites being reported together in a single concentration (Fernández-hernández et al., 2020). This did not happen in our study, where the presence of pyruvate in jenny PFF was confirmed by heteronuclear single quantum correlation. Moreover, concentrations of metabolites found in both mare and jenny PFF were, except valine, lower in the former than in the latter (Fernández-hernández et al., 2020). These results point to clear differences between the metabolic profile of PFF of the jenny compared to the mare. These variations are attributable to the fact that, despite donkey and horse being phylogenetically close, both species differ for a long time having their own reproductive strategy, which would support that the composition of PFF could be species-specific (Canisso et al., 2019). Another explanation could be related to the source of PFF samples. In the study of mare PFF, the authors mentioned the presence of some intracellular metabolites, such as glucose 1-phosphate and carnitine (Fernández-hernández et al., 2020). The identification of these metabolites in the follicular fluid could be due to release following cell damage, as samples were obtained from slaughterhouse ovaries. Cellular disruption could also explain the high levels of lactate reported by Fernández-Hernández et al. (Fernández-hernández et al., 2020), almost 13 times higher than those observed in our study. The time interval between animal death and harvesting of PFF samples could lead to *post-mortem* accumulation of lactate (Tews et al., 1963), as reported by a previous study carried out with equine oviductal fluid (González-Fernández et al., 2020).

TCM-199 is the basic culture medium most used for the formulation of IVM media in horses and in the attempts carried out so far in donkeys (Goudet et al., 2015; Deleuze et al., 2018; Zhao et al., 2011; Abdoon et al., 2014; Douet et al., 2019). When comparing our results to the metabolites described in TCM-199 culture medium (see Table 2), we detected that only 11 of the 27 metabolites found in jenny PFF were present in TCM-199 medium. In addition, acetate and valine concentrations were considerably higher in donkey PFF than in TCM-199 medium, whereas glycine, isoleucine, leucine, and threonine were less concentrated in the former than in the latter. Although the TCM-199 medium is routinely enriched with fetal bovine serum (FBS) to perform oocyte IVM (Goudet et al., 2015; Deleuze et al., 2018; Zhao et al., 2011; Abdoon et al., 2014; Douet et al., 2019), this supplement only provides eight additional metabolites (Caseiro et al., 2018). According to the metabolites described in two available commercial FBS formulations by Caseiro et al. (Caseiro et al., 2018), supplementation with FBS would provide a series of metabolites. While 18 of these metabolites are already present in jenny PFF and 7 of them are contained in TCM-199, 8 identified here in jennies' PFF are missing from TCM-199 + FBS. These metabolites are betaine, citrate, creatine phosphate, creatinine, glycerol, hippurate, methanol and pyruvate. Furthermore, three of the eight metabolites added through FBS supplementation (isoleucine, leucine, and threonine) are in excess in TCM-199 compared to jenny PFF (see Table 2). These differences suggest that the established maturation medium used for IVM of horse oocytes (*i.e.*, TCM-199 + FBS) may not be suitable for donkeys, which could contribute to explain the poor results hitherto obtained (Goudet et al., 2015; Deleuze et al., 2018; Zhao et al., 2011; Abdoon et al., 2014; Douet et al., 2019).

In vitro-matured mare oocytes present significant alterations in the proteome and metabolome of their cumulus-oophorous complexes

(COCs) in respect to the *in vivo*-matured (Walter et al., 2019). Among others, there are differences in the physiological pathways responsible for oxygen supply, oxidative phosphorylation and metabolism of glucose, fatty acids, amino acids, and other substances. This previous research supports that a physiological approach should be adopted in the improvement of IVM of oocytes in equids. Formulating IVM media that brings conditions more similar to the *in vivo* scenario *(*Walter et al., *2019)* could be an option, while another possibility would be adding PFF to the IVM medium in the case of donkeys.

The metabolites present in the jenny PFF that are missing in routine IVM media (TCM-199 supplemented with FBS) are pyruvate, citrate, betaine, creatine phosphate, creatinine, glycerol, hippurate, and methanol. Pyruvate plays a crucial role in oocyte metabolism (Lewis et al., 2020a) and, in bovine, its concentration in the medium is known to increase as the oocyte undergoes IVM (Uhde et al., 2018). Follicular environment in mammalian ovaries relies mainly on anaerobic glycolysis to satisfy demands of metabolic energy during oocyte maturation, consuming glucose and producing lactate as a the end-product (Dumesic et al., 2015), which was also found in jenny PFF. In a finely regulated cooperation within the COC, glucose is metabolized into pyruvate *via* glycolysis in the cumulus cells, as the oocyte has low capacity for glucose oxidation because of the lack of phosphofructokinase activity (Cetica et al., 2002). Then, this pyruvate is transferred to the oocyte, where it enters mitochondria and is converted into acetyl CoA, which, *via* the tricarboxylic acid (TCA) cycle, is used to produce the ATP molecules needed for maturation, fertilization and early embryo development (Dumesic et al., 2015; Cetica et al., 2001; Biggers et al., 1967; Warzych and Lipinska, 2020). Furthermore, pyruvate acts as an antioxidant by neutralizing ROS through its α-keto carboxylate structure (Guarino et al., 2019). In an approach to understand the basic physiology of COC and the effect of *in vitro* conditions to that physiology, Lewis et al. (Lewis et al., 2020a) extensively examined *in vitro* metabolism of equine COC. They found that adding 0.15 mM pyruvate to the IVM medium led to a rise in glucose consumption without altering maturation rate, O_2 consumption or mitochondrial function, suggesting that the current IVM systems of equine gametes fail to satisfy the unique metabolic needs of the equine oocyte (Lewis et al., 2020a). Thus, while further insights into donkey COC metabolic dynamics are needed, the addition of pyruvate at an adequate concentration for donkey IVM media should be envisaged.

On the other hand, **citrate** is an intermediate metabolite produced during glucose/pyruvate metabolism in the TCA cycle (Fernández-hernández et al., 2020). Citrate acts as a linkage between CTA cycle, β-hidroxibutirate and lipid metabolism in FF (Piñero-Sagredo et al., 2010; Guo et al., 2018). Furthermore, citrate has been found to act as a key substrate epigenetic modifications in the oocyte (Gu et al., 2015; Harvey, 2019), while it seems to be play an important role during early embryogenesis, as a positive correlation between citrate concentration in PFF and the number of embryos produced per matured oocyte in humans (Castiglione Morelli et al., 2019). He et al. (He et al., 2022) recently revealed that the adverse impact of autophagy impairment on oocyte quality is mediated by downregulated citrate levels, which once enhanced by exogenous supplementation could significantly restore oocyte maturation. They also observed that the decreased autophagy levels in cumulus cells, typical of maternal aging, are related to lower levels of citrate in human PFF, and that elevated citrate levels in porcine PFF can promote oocyte maturation (He et al., 2022). Therefore, the lack of citrate in the IVM conditions currently used in donkeys should be addressed by incorporating it in the media.

Betaine (*N*,*N*,*N*-trimethylglycine) is an amino acid derivative that has two known important roles in mammals: cell volume homeostasis, which has been proven in mouse embryos, and to be a source of methyl groups, becoming a key methyl donor in blastocysts (Corbett et al., 2014). Supplementation of IVM media with 16 mM betaine significantly boosts the levels of nucleic acid N^6-methyladenosine epigenetic modification, which is important for meiotic maturation (Wang et al., 2018), in porcine COCs. Based on the physiological importance of betaine, it should be taken into account in a future reformulation of IVM media in donkeys.

Creatine and **creatine phosphate** constitute the system creatine/phosphocreatine that has the ability to buffer the high-energy phosphate, thus contributing to intracellular energy homeostasis (Jomura et al., 2021). While both creatine and creatine phosphate are subproducts of glycine and arginine metabolism, arginine has not been detected in the PFF of jennies or mares (see Table 2). In human oocytes, the reduction of arginine in the last six hours of IVM is known to be related to a greater development potential of oocytes (Hemmings et al., 2013; Houghton et al., 2002). Thus, a possible explanation for the absence of arginine in the PFF, but the presence of its products, would be that a high metabolization rate of this amino acid occurs in oocytes that are undergoing maturation. In this case, it would be convenient to maintain the supply of arginine in the culture media, as it does TCM-199 (Fernández-hernández et al., 2020).

Creatinine levels found in jennies' PFF is considerably lower than those found in mares (see Table 2). While **creatinine** levels in buffalo FF significantly increase at the meta estrous stage compared to the diestrus, these levels do not differ between medium and large sized follicles (Abd Ellah et al., 2010). Although creatinine levels in FF have been suggested to result of an inward movement from blood, the relationship between concentration of creatinine in the FF and oocyte viability remains unknown (Abd Ellah et al., 2010).

While this metabolite was not observed in mare's PFF by Fernández-Hernández (Fernández-hernández et al., 2020), a considerable concentration of glycerol was detected in jennies' PFF (see Table 2). **Glycerol** was also identified in human PFF, which concentration was correlated with the found in blood plasma (Józwik et al., 2007). The biological function of glycerol, together with myo-inositol, is to provide the essential alcohol moieties required for the synthesis of cell membrane phospholipids (Fung and Stryer, 1978). Having this key role, it seems preferable that there is bioavailability of glycerol in the IVM media rather than the cell synthesizing glycerol from glucose *via* glycolysis in an excess of glucose scenario (Rotondo et al., 2017).

No information on the role of **hippurate** or **methanol** in the PFF of mares or other species is found in the literature. The concentration of hippurate detected in jenny PFF was the lowest (0.053 mM) from all the metabolites. Although this low value points to a residual importance of hippurate, it could have some specific role in jenny PFF. Methanol, as indicated in Table 1, is a subproduct of either carbon metabolism or the degradation of aromatic compounds. As methanol was not found in mare PFF, one could suggest that this metabolite has a particular significance in jennies.

It is worth mentioning that, among the 27 metabolites identified in jennies PFF, almost 41% were common amino acids (alanine, phenylalanine, glutamine, glycine, isoleucine, leucine, lysine, proline, threonine, tyrosine, and valine), and around 15% were modified amino acids or amino acid derivatives (creatine, creatine phosphate, τ-methylhistidine and betaine) (See Fig. 1). The biological functions of these biomolecules in the mammalian oocyte are diverse. In addition to being basic substrates for protein synthesis (Collado-Fernandez et al., 2013), which is very active during oocyte maturation (Tomek et al., 2002), amino acids play important roles in cell function (Sturmey et al., 2008), such as energy substrates and regulators of osmolarity and pH (Collado-Fernandez et al., 2013). In this sense, it has been reported that the amino acid profile is a good predictor of oocyte development competence and embryo quality (Collado-Fernandez et al., 2013). In this study, we could observe glutamine, glycine and alanine as the most concentrated amino acids present in donkey PFP. Glutamine and glycine have been reported to be required for *de novo* synthesis of purine and pyrimidine nucleotides for mRNA synthesis (Collado-Fernandez et al., 2013). In bovine FF, contrary to the fatty acids profile that did not have predictive value, some amino acids have been identified as useful markers of the oocyte *in vitro* competence, as their presence in the PFF was associated with good quality morphological aspect and subsequent development until the

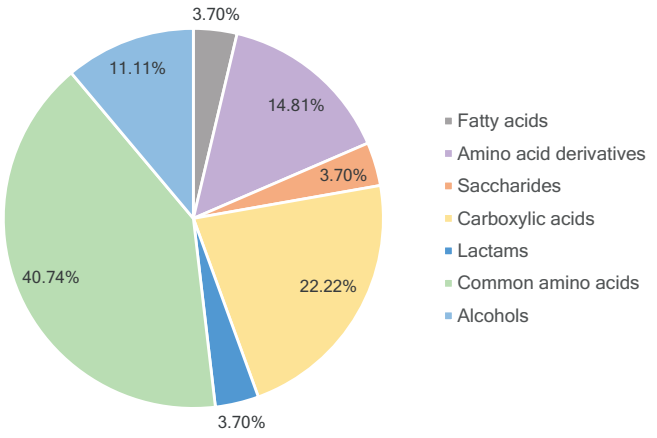

Fig. 1. Chemical nature of the 27 metabolites found in jennies preovulatory follicular fluid (PFF), and proportion of metabolites found in jennies PFF according to their chemical nature.

blastocysts stage (Sinclair et al., 2008; Matoba et al., 2014). Likewise, in cattle, the concentrations of leucine and cysteine in the follicular fluid have been related to *in vivo* fertility *(*Moore et al., *2017)*.

The metabolic profile of donkey PFF reported in this study is the first attempt to address that composition in this species, while also trying to optimize the media for IVM of oocyte. Our results showed clear differences in the metabolites found in jenny PFF compared to those found in that of the mare, and to those of the maturation medium composed by TCM-199 supplemented with FBS. These divergences indicate that both equine species differ in the composition of their PFF and point to the scope for improvement in the IVM media used up to now in the jenny, towards the optimization of IVM rates and obtaining IVP embryos, which has not yet been achieved in donkeys. Further research should be focused on elucidating the PFF metabolome across the different follicular stages, to learn more about the dynamics during the maturation process *in vivo*. It is also important to consider that the metabolites are only part of the different components of PFF, such as hormones, proteins, antioxidants, *etc.*, what should be addressed in subsequent studies. In addition, further knowledge on the components of the follicular fluid would contribute to the identification of biomarkers of oocyte *in vitro* competence and female fertility.

5. Conclusion

In conclusion, the present study provides novel information on the PFF metabolic profile in jennies. Our study revealed the presence of at least 27 metabolites, being amino acids, carbohydrates, and intermediate metabolites found in a higher proportion. As these metabolites have a key role in the oocyte development and maturation, the profile found here should be considered to formulate novel oocyte *in vitro* maturation media.

Results here show that jennies have their own metabolic profile. This profile is different from that found in the mare, the closest phylogenetic species, pointing to a species-specific composition of the fluid. The variations found between the PFF samples, and the media used for the IVM of their oocytes, indicate that there is still room for an improvement of jennies' IVM conditions and support that knowledge on the composition of jenny PFF is an important step in that direction. Future research in the hormonal, antioxidant and protein composition of PFF is guaranteed.

Author contribution

Conceptualization, J.C. and J.M.; Data curation, J.C.,.I.M-R. and I.Y-O.; Formal analysis: J.C. and I.Y.-O.; Funding acquisition: J.M.; Investigation: J.C., I.M.-R.; I.Y.-O.; Y.M.-O.; A.B.-F.; P.N. and J.M.; Methodology, J.C., I.M.-R.; I.Y.-O.; Y.M.-O.; A.B.-F.; M.Y. and J.M.; Project administration, M.Y. and J.M.; Resources, P.N.; M.Y. and J.M.; Supervision, M.Y. and J.M.; Writing - original draft: J.C. and I.M.; Writing - review & editing: M.Y. and J.M.

P.N. NMR data acquisition and analysis.

Declaration of Competing Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported herein.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.10.026.

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