Title: Stage-specific metabolomic changes in equine oviductal fluid: New insights into the equine fertilization environment

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ABSTRACT

A repeatable protocol for equine in vitro fertilization (IVF) has remained elusive possibly due to suboptimal composition of IVF media. Hence, we aimed to analyse the metabolome of equine oviductal fluid (OF) at the pre- (PRE) and post-ovulatory (PST) stages using proton magnetic resonance spectroscopy (1H-NMR). Oviductal fluid from eight PRE and six PST mares were used to prepare five samples per group and 18 metabolites were identified. The five metabolites with the highest concentrations were lactate, myoinositol, creatine, alanine and carnitine. In addition, glucose concentration was 0.18 ± 0.04 (mM ± SD) in OF samples from PRE and 0.57 ± 0.2 in OF samples from PST while lactate reached 54.7 ± 10.7 in OF samples from PRE and 69.2 ± 7.3 in OF samples from PST ($p>0.05$). Only fumarate and glycine showed significant differences in their concentrations between OF from PRE and PST samples, showing higher concentrations in PST samples. Furthermore, we incubated stallion spermatozoa with different concentrations of PST OF (0, 0.0625, 0.125, 0.25, 0.5 or 1 %; v:v). After 4h of sperm incubation, protein tyrosine phosphorylation (PY) by western blotting and sperm motility were evaluated. An increase in PY was observed in two of the three stallions used in the 0.125 % OF treatment but no differences were observed in spermatozoa motility. These results show for the first time information on the metabolomics of equine OF at different stages of the oestrous cycle and the regulatory role of OF on PY in equine spermatozoa.

Keywords: Equine, oviductal fluid, spermatozoa, protein tyrosine phosphorylation, IVF.

1. INTRODUCTION

Equine assisted reproductive techniques have been utilized increasingly in the last two decades, but a repeatable equine *in vitro* fertilization (IVF) protocol has not yet been

established [1]. Many different approaches have been used to try to enhance equine IVF rates, including extensive research on spermatozoa culture conditions and IVF media. Partial zona pellucida (ZP) removal of oocytes resulted in fertilization [2], demonstrating that the barrier to IVF was likely the inability of spermatozoa to penetrate the ZP. Attempts to overcome this have included exposure of equine oocytes to porcine oviductal fluid [3], fetuin [4] or equine follicular fluid during their maturation [5,6], although none have proved successful. Regarding spermatozoa, induction of capacitation has been attempted using different incubation media such as modified Whitten's (MW) medium [7], human tubular fluid (HTF, [8]), Tissue Culture Medium 199 (TCM-199) [9] or specific incubation conditions including alkaline medium or medium without calcium [10–13]. While some aspects associated with spermatozoa capacitation in other species have been reported in treated equine spermatozoa, including an increase in protein tyrosine phosphorylation [7,9–11,13], changes in membrane fluidity [14] or increased intracytoplasmic calcium and pH [15], no method for functional capacitation (induction of the ability to penetrate and fertilize oocytes) has been developed. Sporadically, equine oocyte fertilization has been reported (ranging 29-36%) using calcium ionophore, caffeine, heparin, progesterone or follicular fluid (FF) [1,16]. While equine IVF does not result in repeatable embryo production, it has been demonstrated that *in vitro* matured oocytes can be transferred to the oviduct of an inseminated recipient mare resulting in embryo development [6,17].

Fertilization and early embryo development occur in the oviduct, a highly specialized structure of the female reproductive tract [18]. The oviduct is lined with an epithelium and contains oviductal fluid (OF), which is composed in part by secretions of these cells and in part by blood plasma filtrate. Oviductal fluid composition depends on the species studied and vivid differences appear in descriptions of fluid from mice, cows and pigs [19–21]. Composition of oviductal fluid has shown to be complex and differ from those of the uterine fluid and plasma,

92 containing hormones, proteins, growth factors and metabolites that vary depending on the
93 stage of the estrus cycle [20,22] and also on the presence of gametes or embryos [23].

94 In the horse, some studies on the OF composition have been reported, characterizing
95 the proteins present [24] as well as the concentration of glucose and some ions [25].
96 Unfortunately, in these works the fluid was collected from long-term indwelling catheters,
97 which may cause inflammation and thus alter the nature of the recovered fluid [26] and no
98 information is available regarding equine OF composition using other systems. As fertilization
99 occurs readily *in vivo*, research detailing OF composition would allow it to be effectively
100 mimicked *in vitro,* and thus may offer a method to increase rates of equine *in vitro* fertilization.
101 In this regard, HTF used for human IVF, and synthetic oviductal fluid (SOF) used in mice were
102 designed after in-depth study of oviductal fluid components [27,28]. The composition of the
103 fertilization medium directly influences fertilization success in domestic species. For example,
104 in cattle, the presence of glucose impedes spermatozoa capacitation and fertilization, and the
105 addition of heparin increases it [29], while in pigs, glucose and caffeine appear to be required
106 to attain fertilization *in vitro* [30].

107 We hypothesized that equine IVF failure could be related to suboptimal composition of
108 the fertilization medium. Thus, we analyzed the metabolome of equine oviductal fluid,
109 recovered immediately post-mortem from mares. The OF was classified as preovulatory or
110 postovulatory and its metabolic composition was determined by proton nuclear magnetic
111 resonance spectroscopy (^{1}H-NMR). In addition, the effect of different concentrations of native
112 equine OF were tested *in vitro* to establish if OF induces an increase in protein tyrosine
113 phosphorylation (an indicator of sperm capacitation in other species) or changes in
114 spermatozoa kinematics compatible with induction of hyperactivation.

115

116 **2. MATERIALS AND METHODS**

117 **2.1. Collection of oviductal fluid**

118

119 Oviducts were obtained immediately post-mortem at a commercial slaughterhouse, on five
120 separate days. At the time of evisceration, the entire reproductive tract was extracted and
121 carefully inspected. The ovaries were examined and those tracts having a preovulatory follicle
122 ≥ 35 mm in diameter (as confirmed after opening the follicle using a scalpel blade), associated
123 with uterine edema on examination of the opened endometrial surface (vivid endometrial
124 folds with a gelatinous appearance) were sampled as preovulatory (PRE). When the ovaries
125 had evidence of a recent ovulation, as confirmed after sectioning of the ovary to examine the
126 presence of a corpus hemorrhagicum or juvenile corpus luteum (CL) identified as a luteal
127 structure with a large, red central clot and a luteinized wall that was still visibly crenulated, the
128 reproductive tracts were classified as postovulatory (PST) and also harvested. The oviduct and
129 attached ovary containing the preovulatory follicle or the CL were separated from the uterus
130 distally to the uterotubal junction, the ovary was dissected, and the oviduct was carefully dried
131 with a tissue and placed in a Petri dish within approximately 30 min of slaughter. A non-
132 heparinized hematocrit capillary tube [Merck, Madrid, Spain] attached to a 5 mL syringe by a
133 silicone tube was inserted into the ampulla of the oviduct through the infundibular opening.
134 Gentle aspiration was performed to recover fluid, and the fluid retrieved was expressed into
135 500-µL tubes. Aspiration was repeated for a total of at least 3 times per oviduct. Some
136 additional samples were obtained from the contralateral oviduct of some mares for use in
137 assigning the chemical shift for each metabolite during analysis. The retrieved fluid was pooled
138 and then centrifuged for 2 min in a microcentrifuge at room temperature (RT) to remove large
139 cellular masses. The supernatant was retrieved, transferred to a clean tube and placed on dry
140 ice until its arrival at the laboratory (4-5 hours). Once at the laboratory, the oviductal fluid was
141 centrifuged at 16000 *g* at 4°C for 20 min, and the supernatant was transferred to a clean tube.

142 The volume obtained was measured using a micropipette. The samples were then kept at -80
143 °C until analysis.

144

145 **2.2. Oviductal fluid analysis by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR)**

146

147 *2.2.1. Sample preparation*

148

149 Samples of OF from oviducts from eight mares at the preovulatory stage and six mares at the
150 postovulatory stage were used. Samples with volume < 10 µL were pooled; thus, for PRE OF
151 analysis, 3 individual samples, 1 pool of 2 mares and 1 pool of 3 mares were used (n = 5
152 samples); at the postovulatory stage, 4 individual samples and 1 pool from 2 mares were
153 analyzed (n = 5 samples), for a total of 10 samples analyzed. Pooled OF was obtained by mixing
154 equal volumes of OF from the different oviducts. On the day of analysis, samples were thawed
155 for 30 min slowly on ice. An aliquot of 10 µL was transferred into a 1.5-mL microcentrifuge
156 tube. The sample was diluted in 189 µL of 0.2 M potassium phosphate buffer in deuterium
157 oxide (D_2O) with a pH of 7.4 ± 0.5 (mean ± SEM) and 1.11 µL of DSS (4,4-dimethyl-4-
158 silapentane-1-sulfonic acid), to reach a final volume of 200 µL. Samples were briefly vortexed,
159 and 200 µL of the OF/buffer mixture was pipetted into a 3-mm NMR tube. In all cases, sample
160 preparation was manually done at 298 K (kelvin). Additionally, a sample with 40 µL of oviductal
161 fluid (OF) obtained from a random oviduct (contralateral to the ovulation site) was prepared.
162 This sample was prepared diluting the 40 µL of OF with 159 µL of 0.2 M potassium phosphate
163 buffer in deuterium oxide (D_2O) with a pH of 7.4 ± 0.5 and 1.11 µL of DSS, to have a final
164 volume of 200 µL. This sample was used to identify the chemical shifts for each metabolite in
165 study (Supplemental Files 1 and 2); the chemical shift is the resonant frequency of a nucleus

166 relative to a standard in a magnetic field and is diagnostic of the structure of a molecule
167 allowing for an accurate metabolite identification [31].

168

169 *2.2.2. NMR measurements*

170

171 Samples were measured in an 800-MHz Bruker spectrometer [AVANCE III, Bruker Biosciences,
172 Madrid, Spain] equipped with a ^{1}H detected cryoprobe with z-gradient and automatic tuning
173 and matching unit. Optimization of experimental conditions included automated tuning and
174 matching, automated locking and automated shimming using TopShim. The 90° hard pulse was
175 optimized to be sample-specific and the presaturation field strength was adjusted to 25 Hz. To
176 minimize the interference of the water content in the NMR spectrum, solvent suppression
177 techniques were applied.

178 For each sample, one-dimensional (1D) ^{1}H-NMR spectra were collected using a Carr-
179 Purcell-Meiboom-Gill (CPMG) pulse sequence; 2D *J*-resolved included 800 x 40 points. Data
180 analysis was done using the TopSpin 3.5 software [Bruker Biospin GmbH, Billerica, MA, USA].
181 Free induction decays were multiplied by an exponential function equivalent to 0.3 Hz line-
182 broadening before applying a Fourier transformation. All transformed spectra were
183 automatically corrected for phase and baseline distortions and referenced to the DSS singlet at
184 0 ppm for further analysis.

185 The 2D *J*res experiment was also routinely included in the acquisition package, along
186 with the 1D ^{1}H-NOESY. This experiment separates J-couplings and chemical shifts in the 2D
187 plane and provides a useful and simplified proton-decoupled projection spectrum. A standard
188 pulse sequence with a water peak suppression was used. After 16 dummy scans, 2 free

induction decays (FIDs) were accumulated into 8 k x 40 data points at a spectral width of 16 ppm.

For assignment purposes, a battery of experiments including 2D-^{1}H, ^{13}C-HSQC, 2D-^{1}H, ^{1}H-TOCSY and 2D, ^{1}H, ^{1}H-NOESY were recorded in a Bruker Avance III 800 MHz spectrometer.

2.3. Semen collection and processing

Semen was collected from three fertile stallions, aged 5 to 15 years, housed at the Centro de Selección y Reproducción Animal (CENSYRA), using an artificial vagina [Hannover model, Minitüb, Landshut, Germany]. All experimental procedures were performed according to institutional and European regulations. A nylon in-line filter [Animal Reproduction Systems, Chino, CA, USA] was used to eliminate the gel fraction. The base medium used for sperm incubation was modified Whitten´s (MW) medium, consisting of 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose (anhydrous), 22 mM HEPES, 25 mM bicarbonate, 2.4 mM calcium chloride, 2.4 mM sodium lactate, 0.02 % of polivinylalcohol (v:v), and 1.0 mM pyruvic acid and adjusted to a pH of 7.25. Bicarbonate was added 1 h prior to the experiment and the medium was maintained at 37 °C until the beginning of the experiment when the pH was checked again and adjusted if necessary.

After sperm collection, the spermatozoa-rich fraction was diluted 1:5 (v:v) in MW medium without calcium and bicarbonate and subsequently processed as previously described protocol [10]. In brief, ejaculates were centrifuged 10 min at 400 *g* at RT, the supernatant was discarded, and the pellet was re-suspended and centrifuged again at 400 *g* for 10 min at RT. Finally, the spermatozoa were suspended in complete MW (with bicarbonate and calcium) containing 0, 0.0625, 0.125, 0.25, 0.5 or 1 % postovulatory oviductal fluid (v:v) and incubated at 37°C in air for 4 h at a final concentration of 30 x 10^6 spermatozoa per mL in a volume of 500

214 µL in 5-mL round-bottom plastic tubes from [BD Falcon™ , San Jose, CA, USA]. One single
215 sample of postovulatory OF retrieved from 1 individual mare was used to supplement MW in
216 all the experiments.

217

218 **2.4. Western blotting for tyrosine-phosphorylated proteins**

219

220 After incubation in the absence or presence of OF, spermatozoa were processed as previously
221 described [10]. Briefly, spermatozoa were centrifuged at 5000 *g* for 3 min at RT and washed in
222 phosphate buffered saline (PBS). After centrifugation, the supernatant was removed, and the
223 pellet was resuspended in Laemmli Sample Buffer (2×) [Bio-Rad, Hercules, CA, USA]. Samples
224 were then centrifuged at 10000 *g* for 10 min at 4°C and the protein concentration of the
225 supernatant was determined using a Bio-Rad DC Protein Assay [Bio-Rad, Hercules, CA, USA]
226 according to the manufacturer's instructions. After protein determination, 2-mercaptoethanol
227 (2.5%) was added to the spermatozoa lysates before heating for 5 min at 95°C. Aliquots of
228 lysates containing the designated amount of protein (15 µg) were loaded in 10 %
229 polyacrylamide gels and separated according to their molecular weight by SDS-PAGE
230 electrophoresis. The proteins were then transferred to Immobilon-P PVDF membranes
231 [Millipore, Billerica, MA, USA]. Membranes were blocked for 1 h at RT using 3 % BSA in a Tris-
232 buffer saline-tween 20 solution (TBS-T) containing 20 mM Tris/HCl pH 7.5, 500 mM NaCl, and
233 0.1 % (v:v) Tween 20 [Sigma-Aldrich, Madrid, Spain]. Immunodetection of tyrosine-
234 phosphorylated proteins was performed using the anti-phosphotyrosine monoclonal antibody
235 (clone 4G10) [Millipore, Billerica, MA, USA] diluted 1:5000 in 3 % BSA in TBS-T overnight at 4°C.
236 As loading control, the constitutive α-tubulin protein was used; the designated membranes
237 were incubated using a primary anti- α-tubulin antibody diluted 1:5000 (v:v) in 3% BSA in TBS-T
238 at 4°C overnight [Santa Cruz Biotechnology Inc, CA, USA]. After incubation with the primary

antibody, membranes were washed 10 min with TBS-T. Then, the membranes were incubated for 45 min at RT with a secondary anti-mouse antibody conjugated to horseradish peroxidase-coupled IgG antibody [Santa Cruz Biotechnology Inc., CA, USA] diluted 1:5000 in TBS-T at RT for 45 min. Following secondary antibody incubation, the membranes were washed for 30 min in TBS-T, then incubated for 5 min with Supersignal™ West Pico Kit [Thermo Fisher Scientific Inc., Waltham, MA, USA] and exposed to Hyperfilm™ ECL [Amersham, Arlington Heights, IL, USA]. Densitometric analysis for all tyrosine-phosphorylated bands in each treatment was performed using Gel-Pro Analyzer™ ver. 4.0.

2.5 Sperm motility assays

Sperm motility was evaluated objectively by a CASA system [ISAS®, Proiser R+D, Paterna, Valencia, Spain]. After sperm incubation, two microliters of the sample were placed in a pre-warmed counting chamber [Leja; Luzernestraat, The Netherlands] and analyzed using a microscope [Nikon Eclipse 50i] equipped with a 10 x negative-phase contrast objective and a heated stage at 37 °C. Analysis was based on the examination of 25 consecutive digitalized images and at least 300 spermatozoa per sample were analyzed. After acquiring four representative fields in a random distribution, total motility (TM), progressive motility (PM), curvilinear velocity (VCL in µm/s) and lateral head displacement (ALH in µm) were recorded [32]. Spermatozoa with an average path velocity (VAP) > 10 µm/s were considered mobile. Spermatozoa with straightness (STR) > 75 % were considered progressive.

2.6. Statistical analyses

A Shapiro-Wilk test was used to determine the distribution of the data. When two treatments were compared a Student t-test was used; for non-normally distributed samples, a Mann-Whitney Rank Sum test was used. Differences in motility parameters among treatments concentrations were determined by a one-way analysis of variance. The significance level was set at $p < 0.05$.

3. RESULTS

3.1.Oviductal fluid retrieval

Oviductal fluid was recovered from oviducts of 29 mares. The volume of oviductal fluid recovered varied from 2 to 65 µL for PRE mares (14.2 ± 16.5 µL, mean ± SD; n = 13) and 2 to 40 µL (8.8 ± 9.5 µL; n = 16) for PST mares. The volume of OF retrieved did not differ significantly between the preovulatory and postovulatory stages ($p > 0.05$).

3.2.Metabolite identification

The chemical shift assignment for each metabolite was performed using a 40-µL sample obtained from an oviduct contralateral to the ovulation site; the spectra of the PRE and PST were contrasted against the identified. The list of the chemical shift for each metabolite is provided in **Supplemental file 1**. The measured concentrations of 20 metabolites, selected based on a previous report [20,32] and the known identification capabilities of the NMR facilityare presented in **Table 1**. Pyruvate and succinate are characterized by one single peak

with the same chemical shift and cannot be discriminated; therefore, they are presented as the sum of both metabolites. Significantly higher concentrations of fumarate and glycine were detected in the PST fluid ($p < 0.05$); the concentration of the remaining metabolites did not differ significantly. In our study the mean glucose concentrations were not significantly different between PRE and PST samples; however, it has to be mentioned that glucose was detected but non-quantifiable in three out of five of the preovulatory samples submitted while it was clearly detected in all at the postovulatory samples. Phosphocholine could not be quantified in three of the postovulatory samples and in one preovulatory sample.

3.3. Protein tyrosine phosphorylation analyses

To determine the effect of equine OF on protein tyrosine phosphorylation (PY) in equine spermatozoa, different concentrations of postovulatory oviductal fluid were added to MW medium supplemented with calcium and bicarbonate. The maximum increase in protein tyrosine phosphorylation was observed for the 0.125 % (v:v) OF dosage in two of the three stallions evaluated (**Figure 1**); higher dosages (0.25-1 %) did not induce a greater increase in protein PY in equine spermatozoa.

3.4 Motility assays

To further understand if OF could influence sperm motility or induce sperm hyperactivation sperm motility s was evaluated by a CASA system. In our setting, no statistically significant differences were detected between the control group and the different OF concentrations in none of the following parameters: total motility, progressive motility, VCL and ALH ($p > 0.05$; Figure 2).

4. DISCUSSION

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313 The results of this study present the first examination of metabolites contained in the
314 periovulatory equine oviduct. In previous reports in which the oviducts of live mares were
315 cannulated during complete oestrous cycles, the volume of fluid obtained was significantly
316 higher during estrus than after estrus (23 *vs.* 8 µL/mare/hour) [24,25]. However, cannulation
317 of the oviduct may be associated with artifactual changes in composition of the recovered
318 fluid, because of inflammation or contamination associated with the catheter and its
319 placement [25,26]. Our data may support the observation of increased fluid in estrus, as we
320 recovered more oviductal fluid from PRE than from PST mares (14.2 µL *vs.* 8.8 µL) however,
321 statistically significant differences were not detected ($p>0.05$).

322 The metabolite concentration of OF, as determined in this study, can be compared with that of
323 media that have been used for experiments in the horse in which *in vitro* fertilization has been
324 reported: Menezo B2 [33,34]; MW: Whitten's medium [35–37]; Tyrode's albumin lactate
325 pyruvate (TALP) [34,38,39]. In all these media, glucose is added at about 5 mM; lactate is
326 present at concentrations ranging from 0.45 to 21.6 mM, and pyruvate ranges from 0-5 mM. A
327 major difference between the concentrations of these common energy sources in these media
328 and that determined in our study is that the concentration of glucose in OF is lower (0.18 ±
329 0.04 and 0.57 ± 0.2 mM preovulatory *vs.* postovulatory) and the concentration of lactate in OF
330 is higher (54.66 ± 10.7 and 69.25 ± 7.3 mM preovulatory *vs.* postovulatory). Notably, in our
331 samples, no measurable glucose was found in 3 of 5 PRE oviductal samples. It is known that
332 lactate increases in post-mortem tissue due to enhanced anaerobic metabolism so our glucose
333 and lactate measurements may not be entirely representative of the actual content in the live
334 mare [40]. However, the extremely high lactate concentration (55–70 mM) cannot be readily
335 explained as metabolism of the typical amount of available plasma glucose (~5 mM); thus it
336 appears to reflect an intrinsically high level in the OF due to a high activity of lactic acid

dehydrogenase as previously demonstrated [41]. Lactate and other metabolites can be transported from blood into the oviductal lumen unchanged (2 5% of total lactate present in OF) and lactate is also synthetized in the oviductal lumen from vascular glucose (75 % of lactate present in OF) [21,42]; hence, OF is not just a transudate of plasma and its composition cannot be estimated by the metabolite concentration in the bloodstream or any other reproductive fluid [22,42]. This observation has also been corroborated in the proteins present in OF in mares, which differ from the ones present in plasma [24], highlighting once more the need of a profound study of equine OF composition.

Only one previous report has presented glucose concentration in mare OF [25]. These authors reported glucose concentrations of 0.24 mM (4.3 mg/100 mL) during estrus and 0.33 mM (5.9 mg/100 mL) in non-estrous mares; our values are in agreement with these findings. Interestingly, glucose determination in the report by Campbell et al. (1979) was done by spectrophotometry whereas in our work, NMR was used. These data highlight that the concentration of glucose used in equine IVF media exceeds the physiologic oviductal concentrations, and thus, the composition of equine IVF media needs to be readjusted. Interestingly, the concentration of glucose found in the present work and in the study of Campbell et al. (1979) differs from the PRE and PST glucose concentrations reported in other species) such as cows [20], sows [21] and mice [19] oviductal fluid (0.97 to 2.5 mM and 3.1 to 4.79 mM, respectively); only the postovulatory concentration of glucose reported in pigs (0.43 mM) by Nichol et al. (1992) parallels the ones reported in the present work. This indicates that equine gamete requirements for successful IVF may be unique and could possibly be hindered by using media compositions extrapolated from other species. It must be noted that the media utilized for “successful” equine IVF summarized above were supplemented with additives (such as calcium ionophore or follicular fluid) aimed at stimulating spermatozoa capacitation. Currently, no single medium has been demonstrated to be superior for *in vitro* fertilization in horses.

Comparison between preovulatory and postovulatory OF composition in mares resulted in statistically significant differences for amounts of glycine and fumarate, but not for the rest of the investigated metabolites (**Table 1**). In cows, glycine concentration was significantly higher in preovulatory OF compared to the postovulatory stage [20]; however we found the opposite: glycine was over twice as high in PST than in PRE OF contrasting with a previous report in mares in which glycine concentration did not vary throughout the estrous cycle [43] (**Table 1**). This amino acid has been described to act as an osmolyte and play a role in intracellular pH regulation in embryos, and serve as an energy source for bovine spermatozoa [22,43]. Hence, the increased concentration in postovulatory OF may respond to the specific requirements that the gametes need to achieve fertilization and the fine pH regulation that equine sperm require to capacitate [11–13]; furthermore, it is known that mammalian sperm possess a glycine receptor involved in the acrosome reaction, so glycine could be playing a core role at this level as well explaining its rise in PST compared to PRE samples [41].

Regarding fumarate, Lamy et al. (2018) recently reported its presence in bovine oviductal fluid for the first time and its concentration did not vary between the preovulatory and postovulatory stages, which differs from our findings. However, aside from its role in the Krebs cycle, the exact role that fumarate may play in the oviduct in the periovulatory stage is unclear and needs to be further studied.

This is the first report of the metabolomic profile in equine OF. For this reason, the investigated components as well as their respective chemical shift assignment are presented (**Supplemental File 1**). Aside from the metabolic significance, surprising metabolites such as histamine are present in mare OF and may have an important physiological role. Histamine is an inflammatory mediator that may be brought to the oviduct by mast cells and may be involved in regulation of muscular contractions and smooth muscle electrophysiology [44]; this component has also been described in OF in cows [42,44] and its presence was in part linked

388 to contamination by blood cells. However, in our work, collection of the OF was performed
389 avoiding blood contamination and thus, in view of our data and that provided by Lamy et al.
390 (2018), histamine could be an essential component of mammalian OF. Histamine increases
391 intracellular calcium in human spermatozoa [45], and has been shown to play a role in
392 promoting oocyte calcium transients at fertilization in sea urchin [46]. The suppressive action
393 of anti-histamine administration on spermatozoa quality has been investigated (review, [47]),
394 but little work has been done on the direct effect of histamine at the time of fertilization.

395 Regarding myoinositol, it has been demonstrated that this molecule exerts positive
396 effects on spermatozoa count and volume when orally administered to oligospermic men [48]
397 improving oocyte fertilization rates and embryo quality after intracytoplasmic spermatozoa
398 injection (ICSI) [49]. In addition, supplementation of culture media with myoinositol promotes
399 the completion of preimplantation development of bovine, rabbit and mouse embryos [50].
400 Myoinositol is present in the reproductive tract in cows [51] and female rats, and its
401 concentration has been reported to be consistently higher in the oviduct compared to blood
402 serum [52]. Interestingly, the transcriptome of fertile stallions is significantly enriched in
403 metabolic pathways related to D-myoinositol phosphate metabolism compared to subfertile
404 stallions, indicating that this metabolite also plays an important role in equine fertility [53]. All
405 these data suggest that myoinositol may play a key role during gamete co-incubation and
406 subsequent embryo development in horses. More studies are needed to completely
407 understand the role of this and other OF metabolites in equine assisted reproduction.

408 As above stated, the presence of glycine receptors/Cl^- channels in human, porcine and
409 mouse spermatozoa and their involvement in acrosome reaction regulation and ZP interaction
410 have been demonstrated [54]. β-alanine is a well-known agonist of the glycine receptor.
411 Notably, β-alanine induces the acrosome reaction in capacitated hamster spermatozoa [55].
412 Glycine and alanine supplementation of embryo culture media increases bovine embryo

413 development *in vitro,* their combination with oviductal cell monolayers yields a superior
414 blastocyst rate [56]. Addition of creatine to IVF medium in mice has shown to increase ATP
415 levels in capacitated spermatozoa and to improve fertilization rates [57]. Thus, all these factors
416 appear to warrant investigation as to their effects on both equine spermatozoa capacitation
417 and *in vitro* fertilization.

418 To determine if native OF induces protein tyrosine phosphorylation in equine
419 spermatozoa *in vitro,* as described in bulls and boars [58–60], equine spermatozoa were
420 incubated with varying concentrations of OF ranging from 0 to 1 %. Our results showed that
421 the maximum effect in two of the three stallions used was reached at a low dosage (0.125 %).
422 Differences among males in their sperm protein PY patterns have previously been described in
423 stallion spermatozoa subjected to capacitating conditions [7,11,61] and boars when
424 spermatozoa were capacitated with 20 % homologous preovulatory OF [59]. In comparison,
425 equine OF induce PY in equine spermatozoa at very low concentrations (Figure 1), and thus
426 might constitute a valuable supplement to equine IVF or embryo culture media, as described in
427 other species [60,62]. In our conditions, OF addition did not induce changes in sperm motility
428 or in hyperactivated motility (VCL > 300 μm/s and ALH > 12 μm [32,63]) disregarding the OF
429 dose used (Figure 2). These data are in agreement with previous reports that describe that PY
430 induction in horses is not related to the acquisition of hyperactivated motility and vice versa
431 [11,14,37]. Nevertheless, the fact that no variations among treatments in the percentage of
432 totally motile spermatozoa were observed demonstrates that OF is not exerting a toxic effect,
433 at least at the concentrations used in the present work.

434 The limitations of the present study include the low number of mares from which OF
435 was harvested as well as the variability of the biological samples obtained; therefore, an exact
436 range of metabolite concentration in equine OF cannot be fully established. Additionally,
437 individual stallions may also respond differently in terms of protein tyrosine phosphorylation

to the oviductal fluid of individual mares and a higher number of mares and stallions should be used. However, equine OF obtention is very difficult due to the scarcity of mares slaughtered and the small amount of sample retrieved per oviduct, hence, the above-mentioned limitations should be considered in light of these obstacles.

5. CONCLUSIONS

Our data are the first reporting equine OF metabolomics. This information is valuable to critically assess the composition of media used for equine IVF. In addition, our preliminary finding that low dosages of oviductal fluid promote protein tyrosine phosphorylation in equine spermatozoa open new avenues in the search for a method to induce spermatozoa capacitation for equine IVF. In view of our findings, it is likely that media that have been used in attempts at equine IVF could be lacking some core metabolites that may play important roles in equine spermatozoa capacitation, acrosome reaction, or gamete interaction. Our data provide new insights in the comparative composition of oviductal fluid in mammals and reveal information that could help to improve our understanding in spermatozoa, zygote and embryo requirements, to increase the efficiency of assisted reproduction biotechnologies in horses.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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6. REFERENCES

[1] Leemans B, Gadella BM, Stout TAE, De Schauwer C, Nelis H, Hoogewijs M, et al. Why doesn't conventional IVF work in the horse? The equine oviduct as a microenvironment for capacitation/fertilization. Reproduction 2016;152:R233–45. https://doi.org/10.1530/REP-16-0420.

[2] Choi YH, Okada Y, Hochi S, Braun J, Sato K, Oguri N. In vitro fertilization rate of horse oocytes with partially removed zonae. Theriogenology 1994;42:795–802. https://doi.org/10.1016/0093-691X(94)90448-R.
[3] Douet C, Parodi O, Martino NA, Lacalandra GM, Nicassio M, Reigner F, et al. Exposure to follicular fluid during oocyte maturation and oviductal fluid during post-maturation does not improve in vitro embryo production in the horse. Zygote Camb Engl 2017;25:612–30. https://doi.org/10.1017/S096719941700048X.
[4] Dell'Aquila ME, De Felici M, Massari S, Maritato F, Minoia P. Effects of fetuin on zona pellucida hardening and fertilizability of equine oocytes matured in vitro. Biol Reprod 1999;61:533–40.
[5] Dell'Aquila ME, Cho YS, Minoia P, Traina V, Lacalandra GM, Maritato F. Effects of follicular fluid supplementation of in-vitro maturation medium on the fertilization and development of equine oocytes after in-vitro fertilization or intracytoplasmic sperm injection. Hum Reprod Oxf Engl 1997;12:2766–72.
[6] Hinrichs K, Love CC, Brinsko SP, Choi YH, Varner DD. In vitro fertilization of in vitro-matured equine oocytes: effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilization in vivo after oviductal transfer. Biol Reprod 2002;67:256–62.
[7] McPartlin LA, Littell J, Mark E, Nelson JL, Travis AJ, Bedford-Guaus SJ. A defined medium supports changes consistent with capacitation in stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and high rates of acrosomal exocytosis. Theriogenology 2008;69:639–50. https://doi.org/10.1016/j.theriogenology.2007.11.016.
[8] Arroyo-Salvo C, Sanhueza F, Fuentes F, Treulén F, Arias ME, Cabrera P, et al. Effect of human tubal fluid medium and hyperactivation inducers on stallion sperm capacitation and hyperactivation. Reprod Domest Anim 2019;54:184–94. https://doi.org/10.1111/rda.13328.
[9] González-Fernández L, Macedo S, Lopes JS, Rocha A, Macías-García B. Effect of Different Media and Protein Source on Equine Gametes: Potential Impact During In Vitro Fertilization. Reprod Domest Anim Zuchthyg 2015;50:1039–46. https://doi.org/10.1111/rda.12634.
[10] Gonzalez-Fernandez L, Macias-Garcia B, Loux SC, Varner DD, Hinrichs K. Focal Adhesion Kinases and Calcium/Calmodulin-Dependent Protein Kinases Regulate Protein Tyrosine Phosphorylation in Stallion Sperm. Biol Reprod 2013;88:138–138. https://doi.org/10.1095/biolreprod.112.107078.
[11] González-Fernández L, Macías-García B, Velez IC, Varner DD, Hinrichs K. Calcium-calmodulin and pH regulate protein tyrosine phosphorylation in stallion sperm. Reprod Camb Engl 2012;144:411–22. https://doi.org/10.1530/REP-12-0067.
[12] Leemans B, Gadella BM, Stout TAE, Nelis H, Hoogewijs M, Van Soom A. An alkaline follicular fluid fraction induces capacitation and limited release of oviduct epithelium-bound stallion sperm. REPRODUCTION 2015;150:193–208. https://doi.org/10.1530/REP-15-0178.
[13] Macías-García B, González-Fernández L, Loux SC, Rocha AM, Guimarães T, Peña FJ, et al. Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. Reprod Camb Engl 2015;149:87–99. https://doi.org/10.1530/REP-14-0457.
[14] Leemans B, Stout TAE, De Schauwer C, Heras S, Nelis H, Hoogewijs M, et al. Update on mammalian sperm capacitation: how much does the horse differ from

other species? Reproduction 2019;157:R181–97. https://doi.org/10.1530/REP-18-0541.
[15] Loux SC, Macías-Garcia B, González-Fernández L, Canesin HD, Varner DD, Hinrichs K. Regulation of Axonemal Motility in Demembranated Equine Sperm1. Biol Reprod 2014;91. https://doi.org/10.1095/biolreprod.114.122804.
[16] Lange-Consiglio A, Cremonesi F. 163 HYPERACTIVATION OF STALLION SPERM IN FOLLICULAR FLUID FOR IN VITRO FERTILIZATION OF EQUINE OOCYTES. Reprod Fertil Dev 2012;24:193. https://doi.org/10.1071/RDv24n1Ab163.
[17] Preis KA, Carnevale EM, Coutinho da Silva MA, Caracciolo di Brienza V, Gomes GM, Maclellan LJ, et al. In vitro maturation and transfer of equine oocytes after transport of ovaries at 12 or 22 degrees C. Theriogenology 2004;61:1215–23. https://doi.org/10.1016/j.theriogenology.2003.06.008.
[18] Pérez-Cerezales S, Ramos-Ibeas P, Acuña OS, Avilés M, Coy P, Rizos D, et al. The oviduct: from sperm selection to the epigenetic landscape of the embryo†. Biol Reprod 2018;98:262–76. https://doi.org/10.1093/biolre/iox173.
[19] Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. Reproduction 1990;88:361–8. https://doi.org/10.1530/jrf.0.0880361.
[20] Lamy J, Gatien J, Dubuisson F, Nadal-Desbarats L, Salvetti P, Mermillod P, et al. Metabolomic profiling of bovine oviductal fluid across the oestrous cycle using proton nuclear magnetic resonance spectroscopy. Reprod Fertil Dev 2018;30:1021. https://doi.org/10.1071/RD17389.
[21] Nichol R, Hunter RH, Gardner DK, Leese HJ, Cooke GM. Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period. J Reprod Fertil 1992;96:699–707.
[22] Hugentobler SA, Diskin MG, Leese HJ, Humpherson PG, Watson T, Sreenan JM, et al. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. Mol Reprod Dev 2007;74:445–54. https://doi.org/10.1002/mrd.20607.
[23] Hunter RHF. Components of oviduct physiology in eutherian mammals. Biol Rev 2012;87:244–55. https://doi.org/10.1111/j.1469-185X.2011.00196.x.
[24] Willis P, Sekhar KNC, Brooks P, Fayrer-Hosken RA. Electrophoretic characterization of equine oviductal fluid. J Exp Zool 1994;268:477–85. https://doi.org/10.1002/jez.1402680608.
[25] Campbell DL, Douglas LW, Ramge JC. Cannulation of the equine oviduct and chemical analysis of oviduct fluid. Theriogenology 1979;12:47–59. https://doi.org/10.1016/0093-691X(79)90009-8.
[26] Leese HJ, Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear S-L, et al. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. Reprod Fertil Dev 2008;20:1–8.
[27] Quinn P. Review of media used in ART laboratories. J Androl 2000;21:610–5.
[28] Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 1985;44:493–8.
[29] Parrish JJ, Susko-Parrish JL, First NL. Capacitation of Bovine Sperm by Heparin: Inhibitory Effect of Glucose and Role of Intracellular pH1. Biol Reprod 1989;41:683–99. https://doi.org/10.1095/biolreprod41.4.683.

583 [30] Coy P, Gadea J, Romar R, Matas C, Garcia E. Effect of in vitrofertilization
584 medium on the acrosome reaction, cortical reaction, zona pellucida hardening and
585 in vitro development in pigs. Reproduction 2002:279–88.
586 https://doi.org/10.1530/rep.0.1240279.
587 [31] Keeler J. Understanding NMR spectroscopy. Chichester, England ; Hoboken, NJ:
588 Wiley; 2005.
589 [32] Rathi R, Colenbrander B, Bevers MM, Gadella BM. Evaluation of In Vitro
590 Capacitation of Stallion Spermatozoa1. Biol Reprod 2001;65:462–70.
591 https://doi.org/10.1095/biolreprod65.2.462.
592 [33] Palmer E, Bézard J, Magistrini M, Duchamp G. In vitro fertilization in the horse.
593 A retrospective study. J Reprod Fertil Suppl 1991;44:375–84.
594 [34] Zhang JJ, Muzs LZ, Boyle MS. In vitro fertilization of horse follicular oocytes
595 matured in vitro. Mol Reprod Dev 1990;26:361–5.
596 https://doi.org/10.1002/mrd.1080260411.
597 [35] Ambruosi B, Accogli G, Douet C, Canepa S, Pascal G, Monget P, et al. Deleted in
598 malignant brain tumor 1 is secreted in the oviduct and involved in the mechanism
599 of fertilization in equine and porcine species. Reprod Camb Engl 2013;146:119–
600 33. https://doi.org/10.1530/REP-13-0007.
601 [36] Lange-Consiglio A, Corradetti B, Perrini C, Bizzaro D, Cremonesi F. Leptin and
602 leptin receptor are detectable in equine spermatozoa but are not involved in in vitro
603 fertilisation. Reprod Fertil Dev 2016;28:574. https://doi.org/10.1071/RD14130.
604 [37] McPartlin LA, Suarez SS, Czaya CA, Hinrichs K, Bedford-Guaus SJ.
605 Hyperactivation of stallion sperm is required for successful in vitro fertilization of
606 equine oocytes. Biol Reprod 2009;81:199–206.
607 https://doi.org/10.1095/biolreprod.108.074880.
608 [38] Alm H, Torner H, Blottner S, Nürnberg G, Kanitz W. Effect of sperm
609 cryopreservation and treatment with calcium ionophore or heparin on in vitro
610 fertilization of horse oocytes. Theriogenology 2001;56:817–29.
611 [39] Dell'aquila ME, Fusco S, Lacalandra GM, Maritato F. In vitro maturation and
612 fertilization of equine oocytes recovered during the breeding season.
613 Theriogenology 1996;45:547–60.
614 [40] Tews JK, Carter SH, Roa PD, Stone WE. FREE AMINO ACIDS AND RELATED
615 COMPOUNDS IN DOG BRAIN: POST-MORTEM AND ANOXIC CHANGES,
616 EFFECTS OF AMMONIUM CHLORIDE INFUSION, AND LEVELS DURING
617 SEIZURES INDUCED BY PICROTOXIN AND BY PENTYLENETETRAZOL.
618 J Neurochem 1963;10:641–53.
619 [41] Ménézo Y, Guérin P, Elder K. The oviduct: a neglected organ due for re-
620 assessment in IVF. Reprod Biomed Online 2015;30:233–40.
621 https://doi.org/10.1016/j.rbmo.2014.11.011.
622 [42] Leese HJ. The formation and function of oviduct fluid. J Reprod Fertil
623 1988;82:843–56.
624 [43] Engle CC, Foley CW, Plotka ED, Witherspoon DM. Free amino acids and protein
625 concentrations in reproductive tract fluids of the mare. Theriogenology
626 1984;21:919–30. https://doi.org/10.1016/0093-691X(84)90385-6.
627 [44] Leese H, Tay J, Reischl J, Downing S. Formation of Fallopian tubal fluid: role of a
628 neglected epithelium. Reproduction 2001:339–46.
629 https://doi.org/10.1530/rep.0.1210339.
630 [45] Gupta A, Khosla R, Gupta S, Tiwary AK. Influence of histamine and H1-receptor
631 antagonists on ejaculated human spermatozoa: role of intrasperm Ca2+. Indian J
632 Exp Biol 2004;42:481–5.

[46] Leguia M, Wessel GM. The histamine H1 receptor activates the nitric oxide pathway at fertilization. Mol Reprod Dev 2006;73:1550–63. https://doi.org/10.1002/mrd.20586.
[47] Mondillo C, Varela ML, Abiuso AMB, Vázquez R. Potential negative effects of anti-histamines on male reproductive function. Reproduction 2018;155:R221–7. https://doi.org/10.1530/REP-17-0685.
[48] Palmieri M, Papale P, Della Ragione A, Quaranta G, Russo G, Russo S. *In Vitro* Antioxidant Treatment of Semen Samples in Assisted Reproductive Technology: Effects of Myo-Inositol on Nemaspermic Parameters. Int J Endocrinol 2016;2016:1–5. https://doi.org/10.1155/2016/2839041.
[49] Condorelli RA, La Vignera S, Mongioì LM, Vitale SG, Laganà AS, Cimino L, et al. Myo-inositol as a male fertility molecule: speed them up! Eur Rev Med Pharmacol Sci 2017;21:30–5.
[50] Bevilacqua A, Carlomagno G, Gerli S, Montanino Oliva M, Devroey P, Lanzone A, et al. Results from the International Consensus Conference on myo-inositol and D-chiro-inositol in Obstetrics and Gynecology – assisted reproduction technology. Gynecol Endocrinol 2015;31:441–6. https://doi.org/10.3109/09513590.2015.1006616.
[51] Tríbulo P, Balzano-Nogueira L, Conesa A, Siqueira LG, Hansen PJ. Changes in the uterine metabolome of the cow during the first 7 days after estrus: TRÍBULO ET AL. Mol Reprod Dev 2019;86:75–87. https://doi.org/10.1002/mrd.23082.
[52] Lewin LM, Yannai Y, Melmed S, Weiss M. myo-inositol in the reproductive tract of the female rat. Int J Biochem 1982;14:147–50.
[53] Suliman Y, Becker F, Wimmers K. Implication of transcriptome profiling of spermatozoa for stallion fertility. Reprod Fertil Dev 2018;30:1087. https://doi.org/10.1071/RD17188.
[54] Bray C, Son J-H, Kumar P, Harris JD, Meizel S. A role for the human sperm glycine receptor/Cl(-) channel in the acrosome reaction initiated by recombinant ZP3. Biol Reprod 2002;66:91–7.
[55] Llanos MN, Ronco AM, Aguirre MC, Meizel S. Hamster sperm glycine receptor: evidence for its presence and involvement in the acrosome reaction. Mol Reprod Dev 2001;58:205–15. https://doi.org/10.1002/1098-2795(200102)58:2<205::AID-MRD10>3.0.CO;2-J.
[56] Moore K, Bondioli KR. Glycine and alanine supplementation of culture medium enhances development of in vitro matured and fertilized cattle embryos. Biol Reprod 1993;48:833–40.
[57] 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 Oxf Engl 2018;33:1117–29. https://doi.org/10.1093/humrep/dey081.
[58] Kumaresan A, Johannisson A, Humblot P, Bergqvist A-S. Effect of bovine oviductal fluid on motility, tyrosine phosphorylation, and acrosome reaction in cryopreserved bull spermatozoa. Theriogenology 2019;124:48–56. https://doi.org/10.1016/j.theriogenology.2018.09.028.
[59] Kumaresan A, Johannisson A, Humblot P, Bergqvist A-S. Oviductal fluid modulates the dynamics of tyrosine phosphorylation in cryopreserved boar spermatozoa during capacitation. Mol Reprod Dev 2012;79:525–40. https://doi.org/10.1002/mrd.22058.
[60] Lopera-Vasquez R, Hamdi M, Maillo V, Lloreda V, Coy P, Gutierrez-Adan A, et al. Effect of bovine oviductal fluid on development and quality of bovine embryos

produced in vitro. Reprod Fertil Dev 2017;29:621–9. https://doi.org/10.1071/RD15238.
[61] Pommer AC, Rutllant J, Meyers SA. Phosphorylation of Protein Tyrosine Residues in Fresh and Cryopreserved Stallion Spermatozoa under Capacitating Conditions1. Biol Reprod 2003;68:1208–14. https://doi.org/10.1095/biolreprod.102.011106.
[62] Avilés M, Gutiérrez-Adán A, Coy P. Oviductal secretions: will they be key factors for the future ARTs? MHR Basic Sci Reprod Med 2010;16:896–906. https://doi.org/10.1093/molehr/gaq056.
[63] Loux SC, Crawford KR, Ing NH, González-Fernández L, Macías-García B, Love CC, et al. CatSper and the Relationship of Hyperactivated Motility to Intracellular Calcium and pH Kinetics in Equine Sperm1. Biol Reprod 2013;89. https://doi.org/10.1095/biolreprod.113.111708.

FIGURE LEGENDS

Figure 1. Effect of different concentrations of equine postovulatory oviductal fluid on protein tyrosine phosphorylation on spermatozoa from three different stallions (A, B and C). Spermatozoa were incubated in MW medium supplemented with bicarbonate and calcium, with added oviductal fluid, for 4 h at 37°C in air. Densitometric analysis (fold increase in arbitrary units compared to control) for all tyrosine-phosphorylated bands is reported under each lane.

Figure 2. Effect of different concentrations of equine postovulatory oviductal fluid on sperm motility parameters. Spermatozoa were incubated in MW medium supplemented with bicarbonate and calcium, with added oviductal fluid, for 4 h at 37°C in air. Total motility (TM; panel A), progressive motility (PM; panel B), curvilinear velocity (VCL; panel C) and the amplitude of the lateral head displacement (ALH; panel D) were evaluated. No statistically significant differences were found among groups ($p > 0.05$).

Supplemental File 1. Chemical shift assignment, multiplicity and number of contributing protons for the identified metabolites.

713 **Supplemental File 2.** Metabolite assignment on 1D ^{1}H NMR spectra of an OF sample. (A)
714 chemical shift region from 0.9 to 2 ppm, (B) chemical shift region from 2.6 to 3.8 ppm, (C)
715 chemical shift region from 3.84 to 4.35 ppm, (D) chemical shift region from 5.10 to 5.65 ppm.