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► **To cite this version:**

Cindy Riou, Luiz Cordeiro, Lydie Nadal-Desbarats, Nadine Gérard. Differential metabolic composition of the uterine fluid from fertile and subfertile hens after artificial insemination. 2023. hal-04230609

HAL Id: hal-04230609

<https://hal.inrae.fr/hal-04230609>

Preprint submitted on 6 Oct 2023

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1 **Differential metabolic composition of the uterine fluid from fertile and subfertile hens**
2 **after artificial insemination**

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11

12

13 **Abstract**

14 Avian uterine fluid (UF) has been demonstrated to prolong sperm survival, to maintain the
15 fertility potential of the fowl sperm, and to impact the filling rate of sperm storage tubules (SST)
16 from the utero-vaginal junction (UVJ). The purpose of this study was to identify UF metabolites
17 related to long-term sperm storage.

18 Two genetic lines of hens exhibiting long (F+) or short (F-) sperm storage ability were used.
19 Metabolites present in the UF either before or after insemination were identified and quantified
20 using high resolution ¹H-NMR. The effect of the presence of sperm was investigated 24 hours,
21 1 week, 2 weeks and 3 weeks after a single artificial insemination.

22 Thirteen metabolites were identified as discriminating between the UF collected before
23 insemination from the two lines of hens. Among them, formate, organic acids (succinate,
24 fumarate, lactate), dimethylamine and arabinose were significantly more abundant in F+ UF,
25 whereas myo-inositol was significantly more abundant in F- UF (p<0.05). Pathway and
26 metabolite set enrichment analyses revealed that preponderant metabolic pathways were over

27 represented in the F+ UF and involved pyruvate metabolism, citrate cycle and steroid
28 biosynthesis ($p < 0.05$). No metabolite was identified as discriminating in F+ UF, when
29 compared before and after insemination. In contrast in the F- line of hens, 11 metabolites
30 discriminated the UF collected before and after insemination. Actually, we observed that
31 dimethylamine was significantly reduced, while fumarate and myo-inositol metabolites were
32 significantly increased. Moreover, Warburg effect pathway involving glucose to lactate
33 metabolism, was significantly enriched in F- after insemination.

34 Our results indicate that in the hen, the metabolic composition of UF is related to the genetic
35 traits F+/F-, and is differentially modified after insemination in both lines. It could be related
36 to sperm storage. These lead us to conclude that: 1) the UF biochemical composition is
37 associated with the sperm survival ability in SST, 2) is regulated by the presence of sperm, and
38 3) this regulation is related to the sperm survival ability in SST.

39 **Key words:** sperm storage, metabolomic, chicken, uterine fluid, fertility, $^1\text{H-NMR}$

40 **Introduction**

41 The avian female reproductive tract is divided in 5 segments including infundibulum,
42 magnum, white isthmus, uterus and vagina, which are associated with the egg components.
43 After ovulation, the oocyte (yolk) enters the infundibulum where fertilization can take place. It
44 then goes through magnum and isthmus consecutively for the deposition of the vitelline
45 membrane outer layer, egg white (0.5 to 3.5 hours post ovulation) and eggshell membranes (3.5
46 to 5 hours post ovulation), respectively. Eggshell calcification occurs in the uterus within an 18
47 hours period. During this period, the egg mass in the uterus compresses the uterovaginal (UVJ)
48 folds that are positioned towards the uterus (Bakst & Akuffo, 2009). Lastly, the egg passes
49 through the vagina and is laid 24 hours after ovulation.

50 It is well known that female birds are able to produce fertilized eggs for several days, even
51 weeks or months following a single mating (i.e. quail 11 days, hen 3 weeks, turkey 3 months).
52 Actually, after mating sperm ascent the vagina and is stored in specialized structures named
53 uterovaginal sperm storage tubules (UVJ-SST). UVJ-SST have been described in several avian
54 species, and it is well known that they allow the preservation of sperm for long periods
55 (Birkhead & Moller, 1993; Bakst et al., 1994, Brillard, 1993). Sperm cells are gradually released
56 from UVJ-SST to transit to the infundibulum, the fertilization site. Ahammad et al. (2013)
57 demonstrated that the filling of uterovaginal SST is more efficient when insemination is
58 performed at the initialization of eggshell mineralization (i.e. 5-6 hours after ovulation) than
59 the calcifying phase (Ahammad et al., 2013).

60 The mechanisms by which sperm survive and maintain their fertilizing abilities is not clear,
61 and only little is known about the biochemical and/or cellular interactions involved between
62 sperm, uterine fluid (UF) and oviductal cells. Local secretions (Froman, 2003; Bakst & Akuffo,
63 2007; Riou et al., 2017; Riou et al., 2019, 2020) as well as specific interactions between sperm
64 and SST epithelium cells (Tingari & Lake, 1973; Das et al., 2008; Bakst & Bauchan, 2015)
65 may play crucial roles to preserve sperm. Of note that during both storage in UVJ-SST and
66 ascent from the vagina to the infundibulum, sperm are exposed to the UF. It has been shown
67 that the UF collected during the initialization of eggshell mineralization promotes the
68 maintenance of sperm motility and viability in vitro (Brillard et al., 1987; Ahammad et al.,
69 2013). The same authors demonstrated that hens inseminated with sperm exposed to
70 “initialization phase” UF exhibit a longer period of fertile egg production compared to hens
71 inseminated with sperm exposed to “calcifying phase” UF (Brillard et al., 1987; Ahammad et

72 al., 2013). This suggests that UF composition varies throughout the day, in relation to the stage
73 of egg formation, and that some UF components are able to regulate sperm functions such as
74 sperm storage ability and duration. Consequently, we can hypothesize that some UF
75 components or metabolites are essential for sperm functions, such as sperm storage efficiency
76 in birds.

77 UF represents the aqueous environment in which sperm are exposed during storage and
78 transit through the female tract. Its composition has been widely investigated in regards to the
79 egg formation (Gautron et al., 2019). Daily ovulation cycle gives rhythm to the UF protein
80 composition, which is responsible of sequential eggshell deposition (Sun et al., 2013; Marie et
81 al., 2015). Recently, it has been shown that sperm arrival in SST induces a rapid modification
82 of UF protein composition, and of transcriptomic patterns of uterine tissue (Long et al., 2003;
83 Atikuzzaman et al., 2015; Riou et al., 2019). These observations suggest that sperm regulate
84 UF composition.

85 A major physiological role of UF metabolites has been described in relation to the sperm
86 storage process. In quail SST, sperm are stored immobile under low oxygen and high lactic acid
87 concentrations (Matsuzaki et al., 2015) that induces flagellar quiescence. Therefore, it is
88 admitted that the long-term sperm storage in avian species may involve specific metabolic
89 conditions, which enable sperm to survive. We could suggest that these specific metabolic
90 conditions may originate at least in part from UF, and could extend the sperm survival time.

91 In this study, we hypothesized that UF collected during the initiation of eggshell
92 mineralization contains specific metabolites which promote the long-term sperm storage.
93 Therefore, our study aimed to characterize metabolites that are present in UF from fertile (F+)
94 and subfertile (F-) hens during 3 weeks after artificial insemination. This two lines of hens
95 exhibit a long (F+) or short (F-) duration of fertile period, as a consequence of a good or poor
96 ability for a long-term sperm storage in uterovaginal SST, respectively (Brillard et al., 1998).

97 We expected to reveal specific metabolite patterns associated to the long-term sperm storage
98 process. We clearly demonstrated that avian UF contains metabolites which may play key role
99 in the long-term sperm storage process.

100

101

102 **Materials and methods**

103 ***Birds***

104 Sexually mature hens issued from two genetic lines previously selected for their ability to
105 produce normal (F+) or low (F-) numbers of hatched chicks, and to preserve sperm in their SST
106 for a long (F+) or short (F-) period of time, were used in this study. These two lines were
107 different in the duration of their fertile period, the F- line expressing a shorter time potential to
108 lay fertile eggs than the F+ line (Beaumont et al., 1992). Breeding procedures and handling
109 protocols were carried out according to the European Community Council Directives regarding
110 the practice for care and use of animals and of the recommendations of the French Ministry of
111 Agriculture on Animal Experimentation under the supervision of an authorized scientist
112 (Authorization # 37035). Farm facilities (Institut National de la Recherche Agronomique, UE-
113 PEAT, 1295) are recognized as officially authorized to rear and euthanize birds (27-175-1). At
114 47 wks of age, hens were placed in individual cages equipped with automatic devices to record
115 the time of oviposition. They were kept under a 16L: 8D photoperiod, and fed a layer mash *ad*
116 *libitum*. The protocol of bird management and collection was approved by the local ethical
117 comity (Comité d'éthique de Val de Loire n°19) and the French ministry under agreement
118 number # 443.

119 ***Evaluation of fertility performance***

120 The duration of the fertile period within each line was expressed using two definitions:
121 effective duration (De), the number of days after artificial insemination (AI) during which a
122 hen lays 100 % fertile eggs; and maximum duration (Dm), the number of days after AI until the
123 hen lays its last fertile egg. At 50 wks of age, hens were inseminated on two consecutive days
124 (Day 0 and Day 1) with 200×10^6 sperm from mixed ejaculates collected from 6 broiler breeder
125 males on both days. The eggs were identified individually and recorded daily from Day 2 to
126 Day 22. They were stored at room temperature for a maximum of 7 days and incubated for 7
127 days . The percentage of fertile eggs was determined by candling at the end of the incubation
128 period. Eggs primarily classified as infertile were broken up for macroscopic examination of
129 the germinal disc to determine the presence/absence of dead blastodiscs. Comparisons for De,
130 Dm, and fertility, laying and embryo mortality rates between the two lines were computed using
131 a Mann-Whitney test ($P < 0.05$). Sixteen hens (7 F+ and 9 F- hens) were then selected from the
132 60 hens according to the evaluation of fertility performance.

133 ***Uterine fluid (UF) collection***

134 UF collection for hen's that were not subjected to insemination was planned 10h after the
135 previous oviposition and after confirming the presence of an egg in utero. Egg expulsion was
136 carried out by intravenous injection of prostaglandin F2 α at 50 μ g/hen. UF was collected
137 immediately after egg expulsion, into a plastic tube placed at the entrance of the everted vagina
138 before insemination (virginized hens) and 24 hours, 1 week, 2 weeks and 3 weeks after artificial
139 insemination (Gautron et al., 1997). Aliquot of fluid was diluted with PBS (1v:1v) in order to
140 perform ¹H-NMR analysis. All aliquots were rapidly frozen in liquid nitrogen to limit any
141 spontaneous precipitation of calcium carbonate and proteins, and stored at -20°C (Hincke et al.,
142 1999).

143 ***¹H-NMR sample preparation***

144 UF were prepared for the high-resolution proton nuclear magnetic resonance (¹H NMR)
145 analyses by cold methanol precipitation of lipids and proteins. The unfrozen samples were
146 centrifuged at 15,000 g for 10 min at 4 °C. After this, 450 μ L of the supernatants were mixed
147 with 1 mL of methanol and cooled at -20 °C for 20 min. The mixtures were then centrifuged as
148 previously described, and 1,150 μ L of the supernatants were collected in glass tubes for further
149 solvent evaporation in a SpeedVac (ThermoScientific, Villebon sur Yvette, France) at room
150 temperature and conserved at -20 °C.

151 ***¹H-NMR spectroscopy measurements***

152 Before NMR analysis, the extracted UF samples were reconstituted in 220 μ L pH 7.4 potassium
153 phosphate buffer in 99% deuterium oxide (D₂O) with 0.128 mM trimethylsilylpropionic acid
154 (TSP) as an internal reference. After centrifugation at 4,000 g for 15 min at 4 °C to remove any
155 insoluble components, the supernatant was transferred to conventional 3 mm NMR tubes, for
156 NMR analysis. High-resolution ¹H NMR spectra from the UF were acquired on a DRX-600
157 Avance III HD spectrometer (Bruker SADIS, Wissembourg, France), operating at 600.13 MHz,
158 with a TCI cryoprobe. NMR measurements were performed at 298 K. Standard ¹H NMR spectra
159 were acquired using a "cpmgpr-1d" pulse sequence with a relaxation delay of 25 s, an echo time
160 of 80 ms (64 scans), and a time domain of 32,768 points. Water suppression was achieved by
161 presaturation during the relaxation delay. Spectra were processed using Topspin version 3.2
162 software (Bruker Daltonik, Karlsruhe, Germany). The free induction decays (FIDs) were zero-

163 filled to 65,536 data points, which provided sufficient data points for each resonance, and a
164 line-broadening factor of 0.2 Hz was applied before Fourier transformation.

165 *NMR Spectra Post-processing*

166 After manual correction of the phase distortion and baseline on all spectra, the ¹H NMR spectra
167 were imported into AMIX software (version 3.8.4; Bruker). The bin area method was used to
168 segment the spectra between 0.6 and 9 ppm using the intelligent variable size bucketing tool.
169 The spectral region containing water was excluded and a total of 64, 160, and 191 buckets were
170 defined manually for serum, ileal content and caecal content spectra, respectively. Those bin
171 areas corresponding to one or several metabolites were normalised by dividing each integrated
172 segment by the total area of the spectrum on the 3 data sets.

173 *Spectral Assignment*

174 The ¹H NMR spectra, referenced to the internal TSP chemical shift reference, were assigned
175 using spectra from our in-house database and online databases, including LMDb
176 (<http://www.lmdb.ca>) or the Chenomx NMR Suite 7.7 evaluation edition (Chenomx Inc,
177 Edmonton, Canada).

178 *Statistical analysis*

179 Multivariate analysis was performed using SIMCA 13 software (version 13.0, Umetrics,
180 Umeå, Sweden) on the **two datasets, i.e. F+ H-NMR and F- H-NMR**. All data were scaled to
181 unit variance to maximize the separation between F+ and F- lines of hens. OPLS-DA is a
182 method of supervised classification that predicts the categorical factor (defined here by the two
183 chicken lines) by explanatory quantitative variables (defined here by the spectral bins or
184 metabolites). Variation in the spectral bins data X is divided into one predictive component,
185 containing variations correlated with the class identifier Y (F+ or F- line) and single or multiple
186 orthogonal components containing variations orthogonal to the predictive component that do
187 not contribute to discrimination between the defined groups. By separating predictive and non-
188 predictive components, OPLS-DA is more adapted than PLS-DA to predict biological variables
189 that usually contain multiple sources of variation that are potentially not correlated with the
190 predicted variable. The overall quality of the model for the **two datasets** analyzed was judged
191 by cumulative R²Y, defined as the proportion of variance in Y explained by the predictive
192 component of the model and cumulative Q², the class prediction ability of the model obtained

193 by the cross-validation default method (7 fold cross-validation) of SIMCA 13 Software: the
194 higher R2Y (cum) and Q2 (cum) the better the separation between the lines. Cross-validation
195 analysis of variance (CV-ANOVA) was applied to evaluate further the significance of the
196 findings. To improve screening, the minimum of features (spectral bins) needed for optimal
197 classification of the **two OPLS-DA models (F+ H-NMR and F- H-NMR)** was determined by
198 iteratively excluding from the models the variables with low regression coefficients and high
199 standard deviations combined with low VIP (Variable Importance in Projection) until
200 improvement of the predictive component assessed by the maximization of Q2 (cum) and R2Y
201 (cum) was achieved. Metabolites included in the model with a VIP > 1 were considered as
202 important. The variable contribution plot also allowed evaluation of the contribution of each
203 predictor in the model.

204 To identify the most relevant metabolites across the **two datasets**, an alternative model was
205 fitted by multiblock OPLS-DA applied to the spectral bins previously identified by OPLS-DA
206 of **F+ H-NMR and F- H-NMR**, and structured in the two blocks. In practice, a different block
207 was assigned to the variables of each dataset and each block was scaled to avoid the domination
208 of one block over the others. Thereafter, OPLS-DA was performed on the three blocks
209 iteratively, excluding the least relevant variables from the model as described above. The results
210 from the multiblock model were computed with the results from the **two models** performed on
211 each dataset separately.

212 **T-test on the metabolite quantities.** Following the OPLS-DA analyses, the equality of
213 means between the two chicken lines for each discriminant metabolite was tested using
214 univariate statistical analysis, computed with Metaboanalyst, a freely available web-based
215 metabolomics analysis suite (MetaboAnalyst 4.0, <http://www.metaboanalyst.ca/>), consisted of
216 non-parametric t-test (equal variances) at the 95% confidence level.

217 ***Pathway impact analysis***

218 To identify the most significant impact of affected metabolic pathways, the OPLS-DA
219 discriminating metabolites were analyzed using MetaboAnalyst 4.0 software. MetaboAnalyst
220 software derives its predictive ability from the KEGG metabolic pathways database (last
221 updated on October 2019), which includes the *Gallus gallus* library used in this study. The
222 software incorporates several specific algorithms to identify the most significant pathway
223 impact, such as the pathway enrichment analysis (global test), the pathway topology analysis

224 (global test) as well as the log fold change of the differential metabolites. A cutoff was chosen
225 at a raw P-value of 1.0 to retain the most significantly pathway impact.

226 *Metabolites set enrichment analysis*

227 A Metabolite Set Enrichment Analysis (MSEA) was performed using the OPLS-DA
228 discriminating metabolites to identify the biologically meaningful groups of metabolites in
229 terms of pathway, disease and localization. MSEA derived from the gene set enrichment
230 analysis (GSEA) ontology method. MSEA only supports human metabolic data. The metabolite
231 enrichment analysis of each set of metabolites was performed using over-representation
232 analysis (ORA) with the “Pathway-associated metabolite sets (SMPDB)” library containing 99
233 metabolite sets based on normal human metabolic pathways. The ORA was implemented using
234 the hypergeometric test to evaluate whether a particular metabolite set was more highly
235 represented than expected by chance within the given compound list. A cutoff was chosen at
236 a raw P-value of 1.0 to retain the most significantly enriched metabolic pathways.

237 **Results**

238 *Bird fertility traits*

239 Two groups of hens from each genetic line were constituted, F+ (n=7) and F- (n=9). As
240 illustrated in Figure 1 and Table 1, these lines differ mainly in sperm storage ability, i.e. their
241 maximum duration of fertility (Dm). The efficient (De) and maximum (Dm) duration of fertility
242 in the fertile F+ hens were \pm and \pm (days) respectively, nearly times and times higher than in
243 the subfertile F- hens (\pm and \pm days respectively). The fertility rates were $73.2 \pm$ and $28.4 \pm$ %
244 for the two groups on the duration of 3 weeks, respectively (Figure 1). Thus, throughout this
245 manuscript F- and F+ hens are referred to as subfertile and fertile hens. The laying rate and
246 embryo mortality were not significantly different between the two genetic lines (not shown).

247 *¹H NMR Spectroscopic profiles*

248 The annotated representative ¹H NMR spectrum of the oviductal fluid content extract
249 sampled before insemination is shown in Figure 2. The experimental and spectral data were
250 deposited under the accession number MTBLS0000 in the MetaboLights repository hosted by
251 the EMBL-EBI (<http://www.ebi.ac.uk/metabolights/MTBLS00000>)

252 As presented in Figure 3A, the avian UF is mainly composed with organic acids (50%),
253 which include formic acid, succinic acid, fumaric acid, lactic acid, and many others, as well as

254 derivates such as amino acids, peptides and analogues (creatinine, L-alanine). In second place,
255 with 33% of metabolites, is the organic oxygen compounds super class, which includes several
256 carbohydrates and carbohydrates conjugates. Among them, alpha -D-glucose which is the main
257 metabolite of the avian UF (Figure 3B), arabinose, mannose, and glucuronic acid. Alcohol and
258 polyols sub class is represented by myo-inositol and polypropylene glycol. Of note that myo-
259 inositol is the second UF metabolite in terms of concentration (Figure 3B). Organic nitrogen
260 compounds (i.e. amines, amides, nitrosamines, alkyl nitrates, and peroxyacyl nitrates) take 3rd
261 place (6%) and are represented in the avian UF by dimethylamine, which ranks 11th in terms
262 of concentration of UF metabolites (Figure 3B).

263 *Multivariate Analysis of ¹H-NMR Spectral Data*

264 OPLS-DA was carried out on the ¹H-NMR spectral data for (1) the comparison of F+ with
265 F- UF at each time after insemination (genetic effect) and (2) the comparison in both genetic
266 lines of UF collected before insemination (virginized hens) with UF collected at 24 hours or 1
267 week or 2 weeks or 3 weeks after insemination (insemination effect).

268 **Genetic effect:** The 4 models characteristic for the genetic effect are presented Figure 4A.
269 The first model that compares virginized F+ and F- ¹H-NMR spectral data resulted in 1
270 predictive and 1 orthogonal component (1p + 1o) with the predictive ability $Q2(cum) = 0.44$
271 and the overall proportion of the variation in Y explained by the model $R2Y(cum) = 0.69$. The
272 2 genetic lines of hens are well separated, particularly by formic acid and succinic acid
273 concentrations. This is illustrated by the score plot (Figure 4B) and comparisons of formic and
274 succinic acid concentrations that were significantly different between the 2 lines (Figure 4C,
275 $p < 0.05$). The 3 other models also resulted in one predictive and one orthogonal component (1p
276 + 1o) with a specific predictive ability $Q2(cum)$ and a specific overall proportion of the
277 variation in Y explained by the model $R2Y(cum)$, as presented Figure 4A. The quality of
278 prediction of the model $Q2(cum)$ was high for all ¹H-NMR analysis. Of note that no model was
279 found for the comparison of ¹H-NMR spectral data obtained from F+ and F- UF collected 1
280 week after AI (Fig 4A).

281 The corresponding contribution plots generated by SIMCA software were used to identify
282 the metabolites allowing chicken line discrimination with Metaboanalyst 4.0 software. The
283 discriminating metabolites of UF determined by OPLS-DA, their respective VIP reflecting the
284 importance of variables in the OPLS-DA models, F+ /F- fold-change, and their individual t-test
285 P-values are listed in Table 2. The most discriminant metabolites that were significantly

286 different between the two genetic line and characterized by a high VIP value ($VIP > 1.2$) or
287 metabolites with a high fold change value ($FC > 2$) is presented in Figure 5.

288 ***Insemination effect.*** No significant model was found for the comparisons of F+ $^1\text{H-NMR}$
289 spectral data after insemination. In contrast, F- hens displayed significant differences for all
290 comparisons. The 4 models comparing $^1\text{H-NMR}$ spectral data resulted in 1 predictive and 1
291 orthogonal component ($1p + 1o$) with predictive ability and overall proportion of the variation
292 in Y, which values are presented in Figure 6A. The quality of prediction of the model ($Q^2(\text{cum})$)
293 was high for both $^1\text{H-NMR}$ analysis. The corresponding contribution plots generated by
294 SIMCA software were used to identify the metabolites allowing insemination effect
295 discrimination with Metaboanalyst software. The discriminating metabolites of the uterine fluid
296 determined by OPLS-DA, their respective VIP value, “after insemination (24 hours or 1 week
297 or 2 weeks or 3 weeks) /virginized” fold-change, and their individual p-values are listed in
298 Table 3. The most discriminant metabolites that were significantly different between each
299 condition and characterized by high VIP value ($VIP > 1.2$) are presented in Figure 6B.

300 ***Pathway Impact and Metabolite Set Enrichment Analysis***

301 ***Genetic effect.*** To identify the most significant impact of metabolic pathways involved in
302 the genetic line effect, the discriminating metabolites (shown in Table 2) were analyzed using
303 MetaboAnalyst 4.0 software. As shown in Figure 7A, two pathways that are pyruvate
304 metabolism and citrate cycle (TCA cycle) were the most preponderant in F+ and F- UF
305 ($p < 0.05$). Pyruvate metabolism pathway involves several metabolites as lactate, pyruvate and
306 fumarate, which have been observed more abundant in F+ than in F- UF at all time, except at 2
307 weeks after insemination where fumarate was preponderant in F- UF ($p < 0.05$). Citrate cycle
308 pathway involved succinate, citrate, pyruvate and fumarate, which were preponderant in F+ UF
309 comparing to F- ($p < 0.05$).

310 Moreover, a Metabolite Set Enrichment Analysis (MSEA) was performed using all the
311 discriminating metabolites (shown in Table 2) to identify the biologically meaningful groups
312 of metabolites in terms of pathway, disease and localization, that are significantly enriched in
313 the two lines of hens. MSEA only supports human metabolic data. Using the pathway associated
314 metabolites sets (SMPDB) library which contains 99 metabolites sets, our analysis indicated
315 that the most representative metabolic pathways were more abundant in the F+ line and
316 involved pterine and steroid biosynthesis, folate metabolism, androgen, estrogen and
317 androstenedione metabolism, tryptophan metabolism, arginine and proline metabolism and

318 mitochondrial electron transport chain ($p < 0.05$) as illustrated in Figure 7B. Of note that formate
319 is involved in androstenedione and tryptophan metabolism, L-alanine involved in tryptophan
320 metabolism, and succinate and fumarate are involved in mitochondrial electron transport chain,
321 all of them being discriminating metabolites between the two lines of hens.

322 ***Insemination effect.*** Pathway analysis on the discriminating metabolites after insemination
323 in F- UF (shown in Table 3) revealed that three high impact-pathways that are starch and
324 sucrose metabolism, ascorbate and aldarate metabolism and pentose and glucuronate
325 interconversions that were preponderant after insemination (ns) (Figure 7A). These include
326 myo-inositol and UDP-glucuronate which were observed more abundant at each time after
327 insemination, and arabinose which was reduced after insemination ($p < 0.05$, Table 3).

328 MSEA that concerns the insemination effect is illustrated in Figure 7B. It revealed that the
329 10 most representative metabolic pathways after insemination in F- line involved several amino
330 acids metabolism, purine metabolism, citric acid and urea cycles, mitochondrial electron
331 transport chain and Warburg effect ($p < 0.05$) (Figure 7B). Of note that fumarate is involved in
332 mitochondrial electron transport chain and Warburg effect, and that glucose and lactate are
333 involved in Warburg effect. However, we have already observed that fumarate, lactate and
334 glucose metabolites all significantly increased after insemination.

335 **Discussion**

336 We described in our study the metabolomic composition of the UF and analyzed it for three
337 weeks after insemination in two divergent lines of hens that exhibit a long- or short-term sperm
338 storage ability. We demonstrated that UF metabolomic composition is significantly different
339 between the F+ and F- hens (genetic line effect). Despite no significant difference were found
340 for F+ hens, UF metabolomics composition was affected after insemination in F- hens
341 (insemination effect).

342 ***Metabolomic composition of the uterine fluid***

343 Uterine fluid collected from F+ and F- hens was mainly constituted of glucose. It has been
344 shown that UF glucose content varies during the process of egg formation (Brillard et al., 1987;
345 Ahammad et al., 2013). Its presence in sperm extenders has been shown to supply energy to the
346 sperm (van Tienhoven, 1960). In our study, we also identified UDP-glucuronate in the uterine
347 fluid. This metabolite derives from glucose and participates to the constitution of proteoglycans
348 and glycosaminoglycans, which composed the eggshell. Uterine fluid contained others

349 carbohydrates like mannose and arabinose, as well as low molecular weight metabolites
350 including organic cyclohexanol (myo-inositol), creatinine, amino acid (alanine), organic
351 secondary amine (dimethylamine) and several organic acids (lactate, citrate, acetate, formate,
352 glucuronate, pyruvate, succinate, fumarate).

353 *Genetic effect*

354 In our analysis of the genetic effect, no significant SIMCA-models were found when we
355 compared the UF metabolomic composition between the two lines of hens at one week after
356 insemination. Interestingly at 10 days after insemination, it has been previously shown that
357 females F+ inseminated with F+ semen can store more sperm in their SSTs than F- inseminated
358 with F- semen (Brillard et al., 1998). This previous study also demonstrated that at three days
359 after insemination, the number of stored sperm in SSTs is similar in both lines. In the interval
360 between 3-10 days after insemination, capacities of both lines to store sperm in SSTs seems to
361 be similar. This could be partly due to a similar molecular composition, including metabolites,
362 that promotes sperm storage.

363 However, our study showed a clear separation between the F+ and the F- lines even at 24
364 hours, two weeks and three weeks after insemination, suggesting that the selection on
365 reproductive performances has led to significant changes in the metabolomic profile of UF.
366 Among the metabolites discriminant between the two lines, formate, lactate, myo-inositol,
367 fumarate, succinate, dimethylamine and arabinose were the most preponderant.

368 Formate is the simplest carboxylic acid (HCOOH) involved in the particular at the crossroad
369 between cellular and whole body metabolism. Its centrality confers formate with a key role in
370 physiology (Pietzke et al., 2020). In mammals, formate is known to play a significant role in
371 embryonic development. In folate-resistant mouse, formate supplementation rescued neural
372 tube defects (Sudiwala et al., 2016; Kim et al., 2018). Moreover, our Metabolite Set Enrichment
373 Analysis (MSEA) revealed an enrichment in steroid biosynthesis, and androgen, estrogen and
374 androstenedione metabolisms, which all involved formate, suggesting this metabolite could
375 play an important role in hormonal regulation, at least locally. It is of major importance
376 considering that a local hormonal regulation after insemination has been described in turkey
377 and hens (Long et al., 2003; Das, Nagasaka, et al., 2006; Foye-Jackson et al., 2011), and that
378 estrogen play an essential role to maintain SST structures (Yoshimura et al., 2000). Considering
379 that formate were significantly more abundant in F+ UF compared to F-, it could ensure the
380 maintenance of SST structure and promotes long-term sperm storage in F+ hens.

381 Among the organic acids that were discriminant between the two lines, succinate and
382 fumarate were significantly more abundant in F+ UF compared to F-. Succinate and fumarate
383 are well known to be involved in acid citric cycle. Lactate was also significantly more abundant
384 in F+ UF compared to F-. According to Matsuzaki et al. (2015), lactate accumulation in quails
385 SST cells could participate to the regulation of resident sperm motility (Matsuzaki et al., 2015).
386 These authors demonstrated that lactate at physiological concentration inhibits sperm motility
387 *in vitro* by inducing sperm cytoplasmic acidification. Furthermore, they observed that others
388 organic acid such as citrate and malate are also able to inhibit sperm motility (Matsuzaki et al.,
389 2015). Several authors hypothesized a quiescent state of resident sperm in SSTs, with motility
390 and metabolism being reversibly suppressed (Ashizawa et al., 1976; Bakst, 1985; Bakst &
391 Bauchan, 2015; Matsuzaki et al., 2015). Considering our model of hens, higher lactate
392 concentration in F+ UF could be a key factor to allow sperm to be quiescent in sperm reservoir,
393 promoting a long-term sperm storage.

394 Moreover, our pathway impact analysis revealed an enrichment in pyruvate metabolism and
395 citrate cycle (TCA) preponderant in F+ UF. Pyruvate is one of the most important product of
396 the glycolysis. Pyruvate can be further metabolized to lactate that is the final product of aerobic
397 glycolysis or transported into the mitochondria and converted to acetyl-CoA, which participates
398 in the TCA cycle. Thus, the metabolization of pyruvate using glycolysis or TCA cycle may
399 indicate whether cells use more glycolysis or oxidative phosphorylation, respectively.
400 Considering that a majority of metabolites involved in TCA cycle and pyruvate metabolism are
401 more abundant in F+ UF, these two pathways could be more activated in F+ reproductive tract
402 than in F-.

403 Dimethylamine was significantly more abundant in the F+ UF than in the F- UF.
404 Dimethylamine is an organic secondary amine. It is a colorless, liquefied and flammable gas.
405 Dimethylamine have been identified in human seminal plasma and is positively correlated to
406 the sperm motility (Engel et al., 2019).

407 Myo-inositol was a discriminant metabolite preponderant in the uterine fluid of F- hens in
408 comparison with uterine fluid of F+ hens. The presence of myo-inositol has been previously
409 demonstrated in the turkey uterovaginal junction and uterus (van der Horst & Litjens, 1969).
410 The authors described the preservative role of myo-inositol for sperm motility *in vitro* (van der
411 Horst & Litjens, 1969). In mammals, myo-inositol increased the sperm motility (Scarselli et al.,
412 2016).

413 All along the time after insemination, the differences in UF metabolome between both lines
414 exhibiting a short- (F-) and long-term (F+) sperm storage highlights the importance of several
415 metabolites in the long-term sperm storage within SST. Therefore, the follow-up of UF
416 metabolites before and until three weeks after insemination revealed that formate and lactate
417 were constantly preponderant in F+ UF compared to F-UF (fold change: 1.10 to 1.65),
418 suggesting that these metabolites are key factors all along the long-term sperm storage. In
419 contrast, myo-inositol were constantly preponderant in F- UF (fold change: 0.80-0.94). Before
420 insemination and during the first stage of sperm storage (24h after insemination), fumarate and
421 succinate were more abundant in F+ UF compared to F- UF (fold change : 2.12 to 2.46),
422 highlighting the importance of these metabolites at the establishment of the long-term sperm
423 storage. During the late stage of sperm storage (two and three weeks after insemination),
424 dimethylamine and arabinose were preponderant in F+ UF compared to F- UF (fold change:
425 2.02 to 3.34), suggesting that these metabolites could be essential at the late stage of long-term
426 sperm storage.

427 *Insemination effect*

428 In this study, no significant SIMCA-models were found in F+ hens when we compared the
429 effect of insemination after 24 hours, one week, two weeks and three weeks. Although the
430 individual variability, this suggest that the composition of the UF metabolites in F+ hens is
431 relatively stable after artificial insemination. In contrast in F- hens, we demonstrated that the
432 composition of the UF metabolites was significantly modified after artificial insemination. F-
433 line of hens can therefore be considered as a negative control for long-term sperm storage.
434 Fumarate, dimethylamine, myo-inositol and formate were the most significantly modified.
435 While fumarate increased progressively by approximatively threefold during the three weeks
436 after insemination in F- UF, dimethylamine decreased progressively by approximatively
437 threefold. Myo-inositol increased also during the three weeks after insemination (fold change:
438 1.11 to 1.34). Formate increased from the first week after insemination to the third week (fold
439 change: 1.42 to 1.50). Despite its increase, formate amounts were constantly lower in F- UF
440 compared to F+ UF, highlighting its importance for a long-term sperm storage in SSTs.

441 Our MSEA analysis revealed an enrichment in Warburg effect in F- UF. Warburg effect is
442 well known as a modification of the common glycolysis pathway in cancer cells resulting in the
443 high rates of glucose uptake and lactate secretion, even in aerobic condition (DeBerardinis &
444 Chandel, 2020). In non-cancer cells, it have been shown that T lymphocytes induced a fast

445 glucose uptake when activated by CD28 pathway (Frauwirth et al., 2002). The majority of
446 glucose taken by CD28-activated T lymphocytes is metabolized to lactate and release out of the
447 cells (Frauwirth et al., 2002), suggesting that Warburg effect is a physiological phenomenon
448 that is not unique to cancer cells. Interestingly, it is admitted that insemination induces a local
449 immune regulation in hen's utero-vaginal junction, allowing sperm storage process (Das, Isobe,
450 et al., 2006; Atikuzzaman et al., 2017). A close association has been described between sperm
451 storage duration and uterovaginal expression of Transforming growth factor β (TGF- β), which
452 is a pleiotropic cytokine involved in both suppressive and inflammatory immune responses, and
453 TGF- β receptor (T β R) (Das et al.2006). The presence of T β R2 has been identified in the
454 uterovaginal lymphocytes, and it has been proposed that insemination induces an increase of
455 several TGF β s leading to the suppression of lymphocyte activation (Das, Isobe, et al., 2006).
456 In our study, Warburg effect involved several metabolites including glucose, fumarate and
457 lactate. We also found that Warburg effect pathway were enriched in genetic line effect (NS).
458 The metabolization of glucose in regards to immune regulation could be further investigated in
459 the context of sperm storage.

460 Among the metabolites that may have an influence on sperm motility, myo-inositol that
461 was constantly preponderant in F- UF compared to F+ UF, may support sperm motility (van
462 der Horst & Litjens, 1969; Scarselli et al., 2016). Moreover, although lactate amount increased
463 in F-UF after insemination (NS), it was constantly lower in F- UF compared to F+ UF. It is
464 possible that lactate quantity was not efficient in F- UF to promote sperm quiescence, which
465 can explain a short-term sperm storage (Matsuzaki et al., 2015). Moreover, protein acting as
466 sperm motility activator (HSPA8) has been already identified in higher quantity in the F- UF
467 (Riou et al., 2019) supporting evidence of molecules activator of sperm motility in the F- UF
468 that may explain the short-term sperm storage ability in F- hens. The high decrease of
469 dimethylamine in F- UF all along the period after insemination suggest that this metabolite can
470 be essential for long-term sperm storage. Its effect on fowl sperm functions needs to be further
471 investigated.

472 In conclusion, our study revealed that formate, dimethylamine, myo-inositol and organic
473 acids including lactate, succinate and fumarate, were discriminant between the UF from the two
474 genetic lines and represent key metabolites involved in sperm storage duration.

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604

605

606 **Figure legends**

607 Figure 1. histogramme

608 Figure 2 : spectre légendé

609 Figure 3. Metabolites content of UF collected from virginized F+ hens. (A) Proportion of UF
610 metabolites (%) classified in super classes. Two super classes are detailed in sub classes. The
611 lists of metabolites that composed the sub classes are illustrated. (B) Concentration (%) of the
612 metabolites identified in UF.

613 Figure 4. Comparison between F+ and F- ¹H-NMR spectral data. (A) Model characteristics
614 R²Y and Q of the F+ vs F- genetic effect. AI: artificial insemination. (B) Score plot of the
615 model following OPLS-DA, resulted in 1 predictive and 1 orthogonal component (1p + 1o)
616 with the predictive ability $Q^2(\text{cum}) = 0.44$ and the overall proportion of the variation in Y
617 explained by the model $R^2Y(\text{cum}) = 0.69$. (C) Boxplot of formic and succinic acid
618 concentrations in both genetic lines (*: p<0.05).

619 Figure 5. Boxplots of formic acid, D-arabinose, D-lactic acid, myo-inositol, dimethylamine,
620 fumaric acid concentration in each lines at each time of collection (*: p<0.05). 1, virginized; 2,
621 24 hours after insemination; 3, one week after insemination; 4, two weeks after insemination;
622 5, three weeks after insemination. F+ in purple, F- in pink.

623 Figure 6. Comparisons between virginized and inseminated F- hens. (A) Model characteristics
624 R²Y and Q of the insemination effect. AI: artificial insemination. (B) Boxplots of
625 dimethylamine, formic acid, fumaric acid and myo-inositol concentration at each time of
626 collection (*: p<0.05). 1, virginized; 2, 24 hours after insemination; 3, one week after
627 insemination; 4, two weeks after insemination; 5, three weeks after insemination.

628 Figure 7. Summary of pathway impact analysis (A) and metabolic set enrichment analysis
629 (MSEA) (B) of the discriminating metabolites involved in genetic effect. (A) Graphic
630 representation of the impact of pathway involving discriminant metabolites (*p-value <0.05).
631 The x-axis represents the pathway impact, and the y-axis represents the log (p-value). (B) Bar
632 chart of the metabolite sets enrichments. The used metabolite entities were Formic acid,
633 Succinic acid, Fumaric acid, Lactic acid, Glucose, Arabinose, Dimethylamine, Alanine, myo-
634 Inositol, Mannose, Pyruvate and Citric acid. The most significant p-values are in red (p<0.05)
635 while the least significant are in yellow and white.

636 Figure 8. Summary of pathway impact analysis (A) and metabolic set enrichment analysis
637 (MSEA) (B) performed using discriminating metabolites involved in insemination effect in F-
638 UF. (A) Graphic representation of the impact of pathway involving discriminant metabolites
639 (*p-value <0.05). The x-axis represents the pathway impact, and the y-axis represents the log
640 (p-value). (B) Bar chart of the metabolite sets enrichments. The used metabolite entities were
641 Glucose, Mannose, Arabinose, Lactic acid, Creatinine, Citric acid, Acetic acid, Succinic acid,
642 Pyruvic acid, Glucuronic acid, Alanine, Dimethylamine, Formic acid, Fumaric acid, and myo-
643 Inositol. The most significant p-values are in red (p<0.05) while the least significant are in
644 yellow and white.

645

646 **Table 1.** Fertility traits

647

648

649 **Table 2.** Metabolites discriminating the two lines of hens. The uterine fluid metabolomic
650 composition from F+ hens was compared to the ones of F- hens, at each conditions, i.e.
651 virginized, 24 hours, 1 week, 2 weeks or 3 weeks after insemination.

<i>Metabolites</i>	<i>VIP^a</i>	<i>FC^b</i>	<i>P-value^c</i>
Virginized			
Formate	1.52	1.43	0.040
Succinate	1.14	2.46	0.049
Myo-inositol	1.04	0.80	0.189
Fumaric acid	0.97	2.19	0.093
Lactic acid	0.74	1.47	0.232
Glucose	0.59	1.01	0.336
Inseminated, 24 hours after			
Formate	1.75	1.65	0.001
Lactic acid	1.20	1.56	0.0426
Fumaric acid	1.10	2.12	0.228
Glucose	0.98	0.98	0.181
Myo-inositol	0.95	0.94	0.490
Mannose	0.73	0.73	0.755
Inseminated, 2 weeks after			
Arabinose	1.36	2.36	0.035
Dimethylamine	1.34	3.34	0.014
Formate	1.33	1.38	0.051
Unidentified 3.15	1.11	2.22	0.138
Myo-inositol	0.95	0.80	0.073
Citrate	0.89	0.79	0.234
Fumaric acid	0.88	0.55	0.180
Lactic acid	0.81	1.29	0.295
Glucose	0.74	0.97	0.366
Alanine	0.73	1.29	0.445
Inseminated, 3 weeks after			
Myo-inositol	1.27	0.82	0.073
Formate	1.00	1.32	0.149
Pyruvate	0.99	1.62	0.268
Unidentified 3.15	0.96	2.19	0.149
Dimethylamine	0.91	2.02	0.202
Lactic acid	0.24	1.10	0.756

652 ^aVIP = Variable Importance in Projection in the OPLS-DA model.

653 ^bFC = Fold-change ratio (F+/F-). The more highly concentrated metabolites in F+ than in F-
654 line are indicated in bold. ^cWelch's means equality t-test at 95% confidence level.

655 **Table 3.** Metabolites discriminating the effect of insemination at different period of sperm storage
656 in F- hens. The uterine fluid from virginized hens was compared to the uterine fluid from
657 inseminated hens, at 24 hours, 1 week, 2 weeks or 3 weeks after insemination.

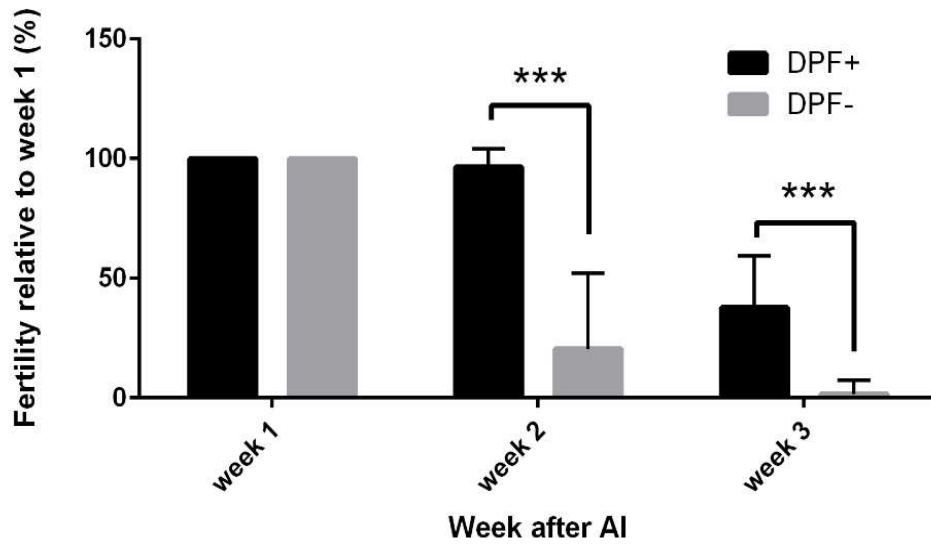
<i>Metabolites</i>	<i>VIP^a</i>	<i>FC^b</i>	<i>P-value^c</i>
Virginized vs Inseminated, 24 hours after			
Fumaric acid	1.69	2.19	0.028
Dimethylamine	1.25	0.59	0.105
Myo-inositol	0.75	1.11	0.279
Glucuronate	0.72	1.17	0.195
Arabinose	0.71	0.76	0.574
Glucose	0.04	0.99	0.960
Virginized vs Inseminated, 1 week after			
Formate	1.38	1.50	0.021
Myo-inositol	1.17	1.37	0.059
Dimethylamine	1.17	0.46	0.046
Fumaric acid	1.17	2.46	0.112
Lactic acid	1.02	1.63	0.093
Unidentified 3.15	0.98	0.47	0.236
Glucose	0.87	1.02	0.114
Arabinose	0.80	0.74	0.312
Virginized vs Inseminated, 2 weeks after			
Fumaric acid	1.38	2.98	0.015
Myo-inositol	1.18	1.34	0.021
Formate	1.13	1.42	0.094
Dimethylamine	1.13	0.44	0.029
Lactic acid	1.06	1.63	0.121
Arabinose	0.95	0.56	0.121
Unidentified 3,15	0.77	0.51	0.613
Mannose	0.65	0.89	0.336
PPG	0.62	0.84	0.534
Virginized vs Inseminated, 3 weeks after			
Fumaric acid	1.31	3.45	0.040
Dimethylamine	1.24	0.34	0.014
Myo-inositol	1.18	1.34	0.014
Formate	0.96	1.45	0.094
Glucuronate	0.90	1.53	0.189
Glucose	0.81	1.02	0.281
Lactic acid	0.81	1.59	0.189

658 ^aVIP = Variable Importance in Projection in the OPLS-DA model.

659 ^bFC = Fold-change ratio (after insemination/ virginized). The more highly concentrated
660 metabolites in inseminated hens than in virginized hens are indicated in bold.

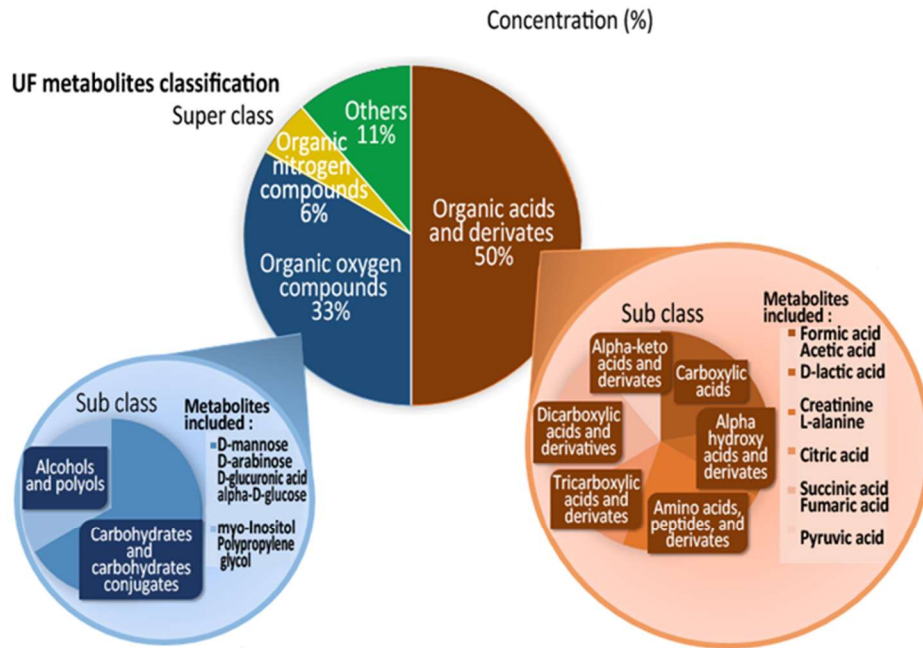
661 ^cWelch's means equality t-test at 95% confidence level.

662



663 Figure 1
664
665

A



B

UF metabolites concentration

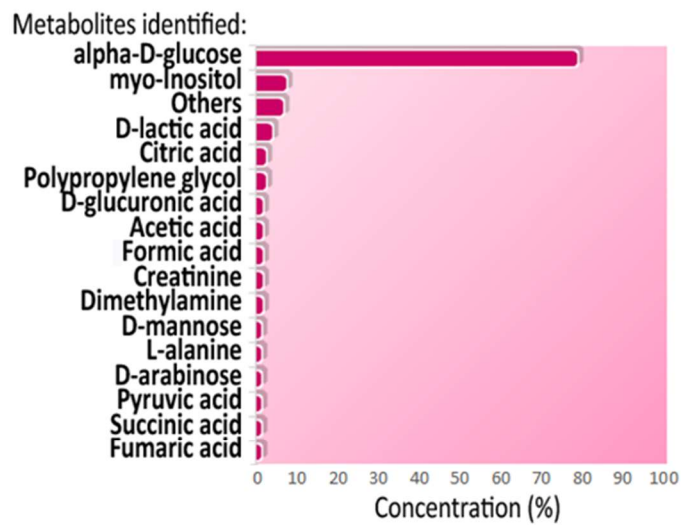
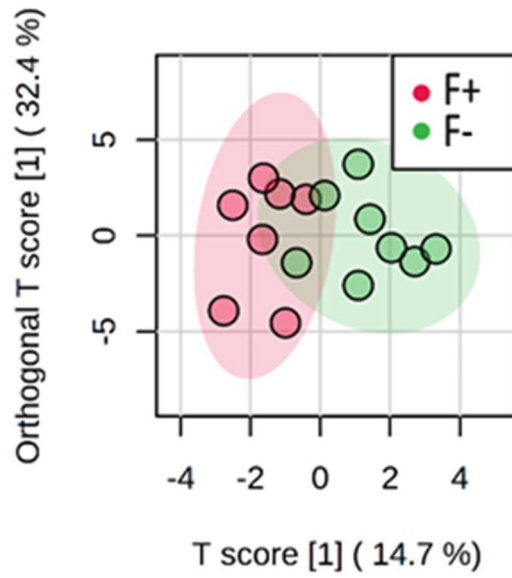
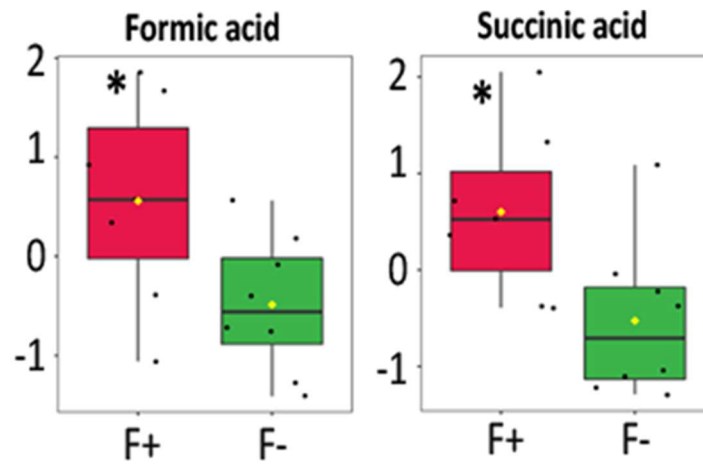


Figure 3

670
671

A

Model characteristics, R2Y and Q	<i>R2Y</i>	<i>Q2</i>
Virginized	0.69	0.44
Inseminated, 24h after AI	0.66	0.44
Inseminated, 2 weeks after AI	0.76	0.52
Inseminated, 3 weeks after AI	0.75	0.38

B**C****Figure 4**672
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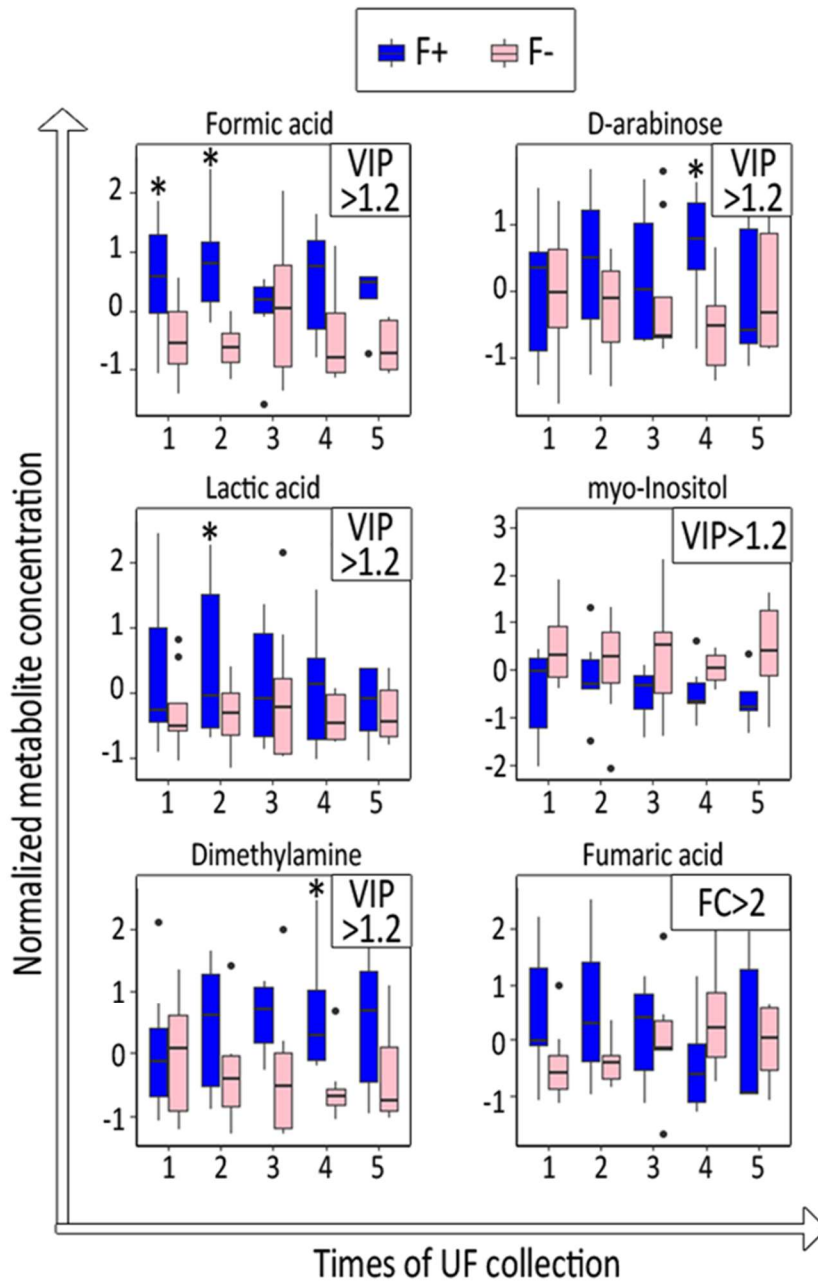
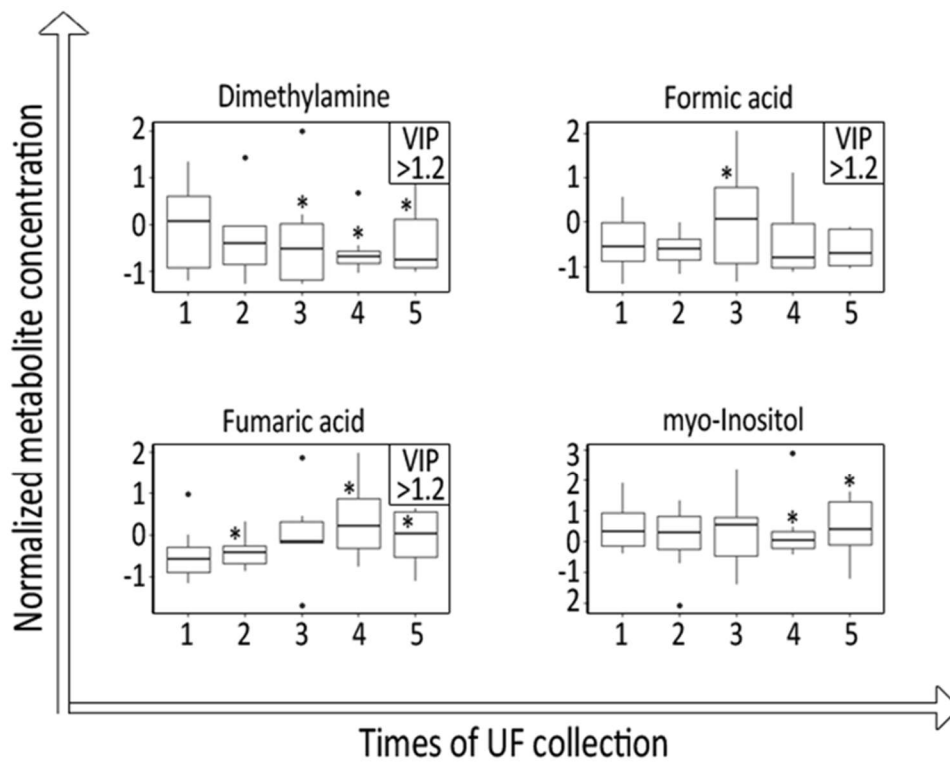


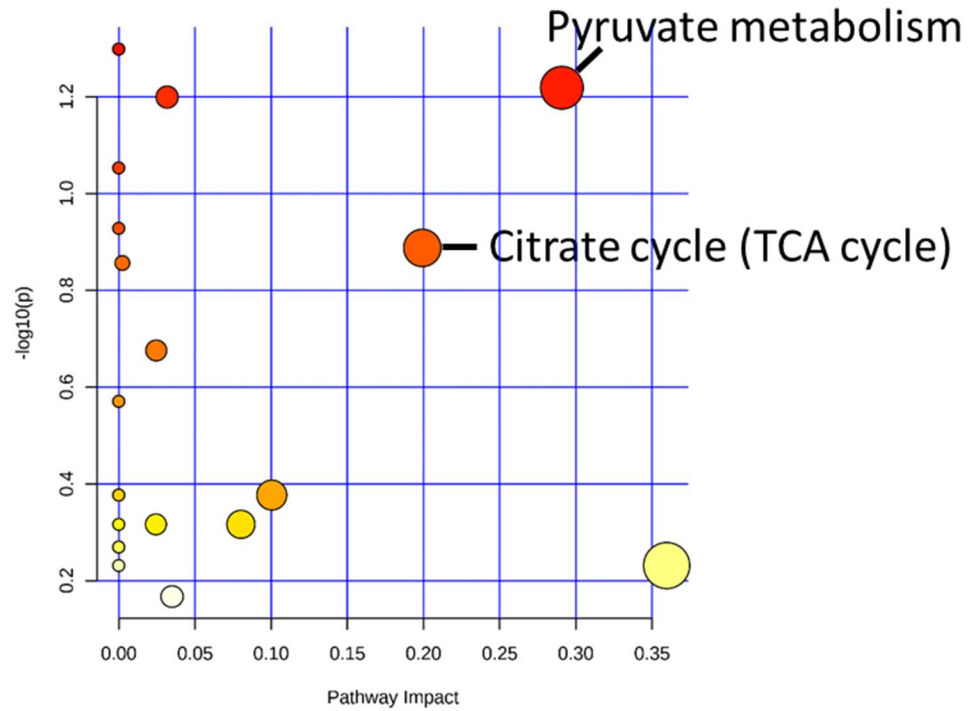
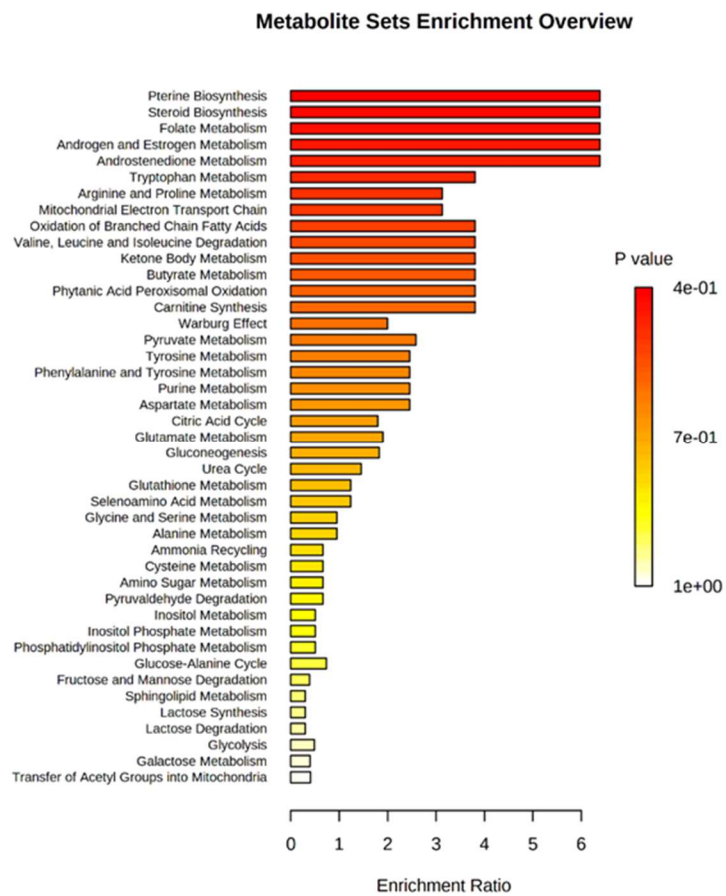
Figure 5

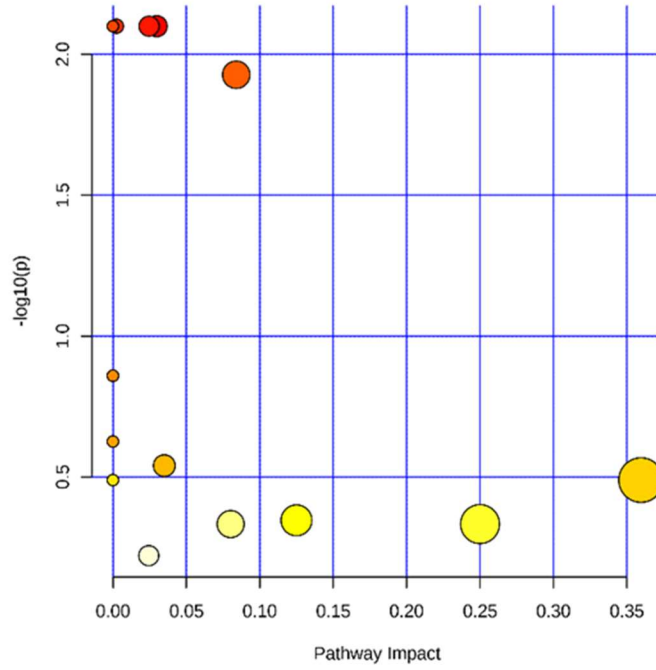
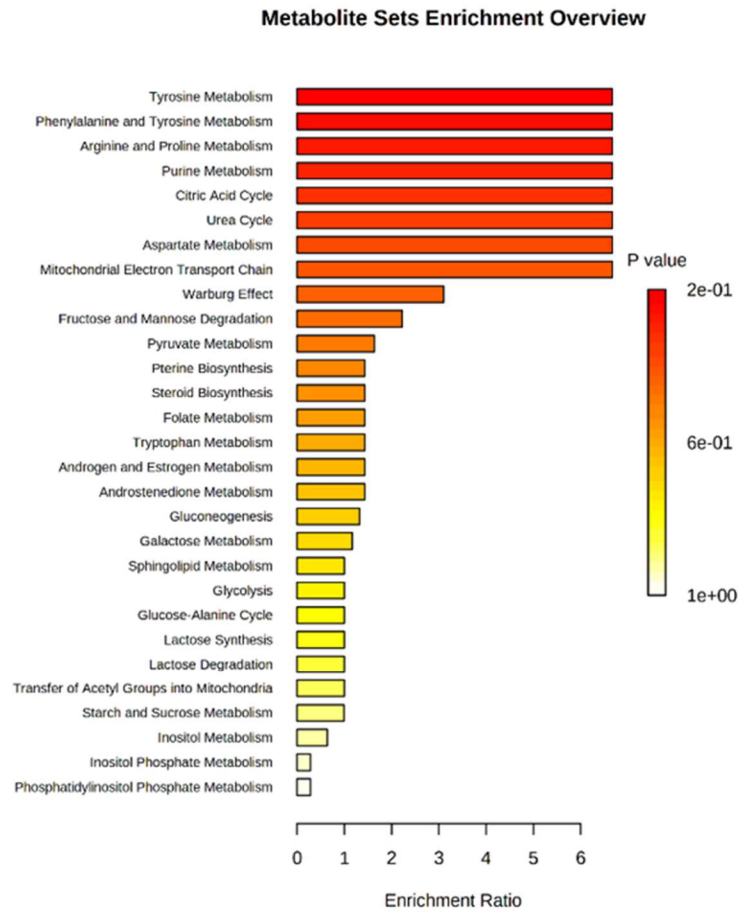
674
675

A

Model characteristics, R2Y and Q	<i>R2Y</i>	<i>Q2</i>
Virginized vs inseminated 24h after AI	0.66	0.27
Virginized vs inseminated 1 week after AI	0.60	0.36
Virginized vs inseminated 2 week after AI	0.63	0.45
Virginized vs inseminated 3 week after AI	0.72	0.50

B**Figure 6**676
677

A**B****Figure 7**

A**B****Figure 8**