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Nulliparity affects the expression of a limited number of genes and pathways in Day 8 equine embryos

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► To cite this version:

E. Derisoud, L. Jouneau, Catherine Archilla, Y. Jaszczyszyn, R. Legendre, et al.. Nulliparity affects the expression of a limited number of genes and pathways in Day 8 equine embryos. 2024. hal-04446593

HAL Id: hal-04446593

<https://hal.inrae.fr/hal-04446593>

Preprint submitted on 31 May 2024

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30 **Abstract**

31 Nulliparous mares produce lighter and smaller foals compared to mares having previously foaled,
32 with effects observed at least until 4 months of age. The need for a first gestation priming for the
33 uterus to reach its full capacity has been proposed to explain this observation. Embryo
34 developmental defects could be hypothesized but effects of maternal parity on the embryo have only
35 been described once, in old mares, thus combining effects of parity and old age. The aim of this study
36 was to determine effects of mare parity on embryo gene expression. Day-8 post ovulation blastocysts
37 were collected from young (5/6 years old) nulliparous (YN, N=6) or multiparous (YM, N=4) non-
38 nursing Saddlebred mares, inseminated with the semen of one stallion. Pure (TE_{part}) or inner-cell-
39 mass-enriched (ICMandTE) trophoblast were obtained by embryo bisection for RNA sequencing
40 (paired end, non-oriented, Illumina, NextSeq500). Deconvolution was performed on the ICMandTE
41 dataset. Differential expression, with embryo sex and diameter as cofactors and gene set enrichment
42 analysis (GO BP, KEGG, REACTOME databases) were performed using a false discovery rate <0.05
43 cutoff. Only a few genes were altered (ICM: n=18; TE: n=6) but several gene sets were perturbed
44 (ICM: n=62; TE: n=50) by maternal parity. In YM, only pathways related to transcription, RNA
45 processing and vesicle transport functions were enriched in the ICM whereas only pathways related
46 to RNA localization were enriched in TE. In YN, while only gene sets related to ribosomes and
47 extracellular matrix were enriched in the ICM, functions related to energy and lipid metabolism, lipid
48 transport and interleukin-1 signaling were enriched in the TE. In conclusion, several genes and
49 pathways are affected in embryos collected from nulliparous mares, with different effects on TE and
50 ICM. Embryo development is altered in nulliparous mares, which could partially explain the term
51 phenotype. Whether differences in gene expression result/induce poor embryo-maternal
52 communication remains to be determined.

53

54 **Keywords:**

55 Blastocyst; RNA sequencing; horse; mare; periconception; equine

56

57 **1. Introduction**

58 In mammalian species, including the horse, it is now well established that the periconceptual and
59 gestational maternal environment affect intra and extra-uterine growth and offspring long-term
60 health [1,2]. These observations fall within the context of the Developmental Origins of Health and
61 Diseases (DOHaD).

62 In horses, maternal parity defined as the number of gestations that produced a viable fetus (live or
63 stillborn foal), is one of the main factors affecting the foal intra-uterine development. Indeed, foals
64 born to primiparous mares (mares that have not foaled before) are lighter and smaller at birth and
65 remain smaller until 18 months and lighter until 4 month of age compared to controls born to
66 multiparous dams [3–13]. Their insulin sensitivity is higher than that of foals born to multiparous
67 mares, and these data suggest that the normal decrease in insulin sensitivity observed in relation
68 with foal age is delayed [13]. Similarly, testicular maturation is also delayed in foals born to
69 primiparous mares [13]. These alterations in morphology and physiology of foals born to primiparous
70 dams seem to be related to poorer performances in show jumping or on the racecourse than those
71 of subsequent foals born to the same mare [14,15].

72 For a long time, these differences in mares' first born foals have been attributed to the need for a
73 first gestation priming for the uterus to be able to reach its optimal size and vascularisation and fully
74 support feto-placental developmental needs [16]. Indeed, primiparous mares produced lighter and
75 less voluminous placentas than multiparous ones [8,12,13,16]. In horses, placentation is diffuse and
76 the epitheliochorial placenta is in contact with the entire surface of the uterus [17,18]. Most feto-
77 maternal exchanges occur through branched vascular structures that form interdigitations with the
78 mare endometrium, called microcotyledons, that maximize nutrient exchanges by increasing feto-
79 maternal contact surface [17–19]. Reduced placental volume and weight are associated with reduced
80 foal development in first born foals and suggest that primiparity could be a form of intra-uterine
81 growth restriction in horses.

82 The placenta derives from the equine embryo trophoblast. Its later efficacy is conditioned by proper
83 implantation and development. Implantation takes place around 35-38 days post ovulation [20].
84 Prior to that, the equine embryo develops free in the uterus and depends on direct support of
85 uterine secretions for its development. Impaired pre-implantation development in nulliparous mares
86 could play a role in the reduced size of both term placenta and newborn foal. The few existing
87 studies that consider maternal parity on fertility effects are controversial. While some found that
88 parity did not affect fertility [21–27], others reported that mares that have never foaled have
89 reduced embryo and fetal mortality compared to mares that previously foaled [27–33]. Confounding
90 effects of maternal parity and age is probably the source of those discrepancies. Indeed, in a recent
91 epidemiological study considering the effect of parity only in mares older than 10 years, there is a
92 cumulative negative effect of nulliparity and aging on the rates of pregnancy at 14 days post-
93 ovulation (ED and PCP, personal communication). Maternal age have been shown to affect oocyte
94 and embryo developmental capacities (for review [34]) as well as gene expression in Day 8 embryos
95 [35]. At the opposite, only one study considered the effect of maternal parity on preimplantation
96 embryo and showed alterations of the expression of genes related to embryo development and
97 exchanges with the environment were observed [36]. This study, however, only considered mares
98 older than 10 years, in which uterine degenerative changes had probably occur. As maternal age
99 affects embryo gene expression, it is important to consider maternal parity in young mares. At this
100 time, there is no study considering the effect of parity on gene expression of embryos in young
101 mares.

102 The aim of this study was to determine the effect of maternal nulliparity in young mares on embryo
103 gene expression at the blastocyst stage. Young (5-6 years old) nulliparous and multiparous mares
104 were inseminated with semen of the same stallion. Day-8 blastocysts were collected, measured and
105 bisected to separate the pure trophoblast (TE_part) from the inner cell mass enriched hemi-embryo
106 (ICMandTE). Gene expression was analyzed by RNA-seq in each compartment.

107

108 **2. Materials and methods**

109 *2.1. Ethics*

110 The experiment was performed at the experimental farm of IFCE (research agreement C1903602
111 valid until March 22, 2023). The protocol was approved by the local animal care and use committee
112 (“Comité des Utilisateurs de la Station Expérimentale de Chamberet”) and by the regional ethical
113 committee (“Comité Régional d’Ethique pour l’Expérimentation Animale du Limousin”, approved
114 under N° C2EA - 33 in the National Registry of French Ethical Committees for animal
115 experimentation) under protocol number APAFIS#14963-2018050316037888 v2. All experiments
116 were performed in accordance with the European Union Directive 2010/63EU. The authors complied
117 with the ARRIVE guidelines.

118

119 *2.2. Embryo collection*

120 Twenty-one non-nursing mares (mostly French Anglo-Arabian with some Selle Français) aged from 5
121 to 6 years old were included in this study. Mares were allocated to one of 2 groups according to their
122 parity: nulliparous (YN, n = 10) and multiparous mares (YM, n = 11). Multiparous mares were defined
123 as dams that had already foaled at least once while nulliparous mares were defined as mares that
124 had never foaled before the experiment. During the experimental protocol, mares were managed in
125 one herd in natural pastures 24h/day with free access to water with no nutritional supplementation
126 but for salt blocks. The experiments took place from April 1st to May 3rd, 2019. All mares remained
127 healthy during this period. During the experimentation, mare’s withers’ height and weight were
128 measured. Characteristics of all mares and mares that produced an embryo are detailed in Table 1.

129 Mares were monitored as previously described [35]. Briefly, the mares’ estrous period was
130 monitored routinely by ultrasound with a 5MHz trans-rectal transducer. During estrus, ovulation was

131 induced with a single injection of human chorionic gonadotropin (i.v.; 750 - 1500IU; Chorulon® 5000;
132 MSD Santé animale, France) as soon as one ovarian follicle >35mm in diameter was observed,
133 together with marked uterine edema. Ovulation usually takes place within 48h, with > 80% occurring
134 25 to 48h after injection [37,38]. At the same time, mares were inseminated once with fresh or fresh
135 overnight cooled semen containing at least 1 billion motile spermatozoa from a single fertile stallion.
136 Ovulation was confirmed within the next 48 hours by ultrasonography.

137 Embryos were collected by non-surgical uterine lavage using prewarmed (37°C) lactated Ringer's
138 solution (B.Braun, France) and EZ-Way Filter (IMV Technologies, France) 10 days after insemination,
139 i.e., approximately 8 days post ovulation. Just after embryo collection, mares were treated with
140 luprotiol an analogue of prostaglandin F₂α (i.m; 7.5 mg; Prosolvin, Virbac, France).

141 The aim of the embryo collection was to obtain 5 embryos/group with each embryo coming from a
142 different mare. Therefore, some mares that failed to produce an embryo at their first attempt were
143 bred again for a second attempt.

144

145 *2.3. Embryo bisection and RNA extraction*

146 Using a binocular magnifying glass, collected embryos were immediately photographed with a size
147 standard to subsequently determine embryo diameter using ImageJ® software (version 1.52a;
148 National Institutes of Health, Bethesda, MD, USA). Embryos were then washed 4 times in
149 commercially available Embryo holding medium (IMV Technologies, France) at 34°C and bisected
150 with a microsurgical scalpel under binocular magnifying glass to obtain a trophoblast (TE_part) and an inner
151 cell mass enriched (ICMandTE) hemi-embryo. At this stage, the TE_part is composed of
152 trophectoderm and endoderm whereas the ICM is composed of epiblast layered on the internal side
153 by endoderm cells [39,40]. Immediately after bisection, RNA extraction of each hemi-embryo was
154 started in extraction buffer (PicoPure RNA isolation kit, Applied Biosystems, France) for 30 min at

155 42°C prior to storage at -80°C. RNA was extracted later from each hemi-embryo using PicoPure RNA
156 isolation kit (PicoPure RNA isolation kit, Applied Biosystems, France), which included a DNase
157 treatment, following the manufacturer's instructions. RNA quality and quantity were assessed with
158 the 2100 Bioanalyzer system using RNA 6000 Pico kit (Agilent Technologies, France) according to the
159 manufacturer's instructions.

160

161 *2.4. RNA sequencing*

162 Five nanograms of total RNA were mixed with ERCC spike-in mix (ThermoFisher Scientific, France)
163 according to manufacturer's recommendations. Messenger RNAs were reverse transcribed and
164 amplified using the SMART-Seq V4 ultra low input RNA kit (Clontech, France) according to the
165 manufacturer recommendations. Nine PCR cycles were performed for each hemi-embryo. cDNA
166 quality was assessed on an Agilent Bioanalyzer 2100, using an Agilent High Sensitivity DNA Kit
167 (Agilent Technologies, France). Libraries were prepared from 0.15 ng cDNA using the Nextera XT
168 Illumina library preparation kit (Illumina, France). They were pooled in equimolar proportions and
169 sequenced (Paired end 50-34 pb) on NextSeq500 instrument, using a NextSeq 500 High Output 75
170 cycles kit (Illumina, France). Demultiplexing was performed with bcl2fastq2 version 2.2.18.12
171 (Illumina, France) and adapters were trimmed with Cutadapt version 1.15 [41]. Only reads longer
172 than 10pb were kept.

173

174 *2.5. RNA mapping and counting*

175 As previously described [35], alignment was performed using STAR version 2.6 [42] on previously
176 modified Ensembl 99 EquCab3.0 assembly and annotation. Genes were then counted with
177 FeatureCounts [43] from Subreads package version 1.6.1.

178

179 **2.6. Data analysis**

180 All statistical analyses were performed by comparing YN to YM (YM set as reference group) using R
181 version 4.0.2 [44] on Rstudio software version 1.3.1056 [45].

182 Embryo were sexed using *X Inactive Specific Transcript (XIST)* expression as previously described [35].
183 Six embryos were determined as female (2 in the YN group and 4 in the YM group) while 5 were
184 considered as male (4 in the YN group, and 1 in the YM group).

185

186 **2.6.1. Embryo recovery and fertility rate, embryo diameter and total RNA content**
187 **analysis**

188 Embryo recovery rates (ERR) per mare and per ovulation were calculated as the number of attempts
189 with at least one embryo collected/total number of attempts. Both were analyzed using the Exact
190 Fisher test to determine if maternal parity influenced embryo recovery.

191 For total RNA content analyses, as embryos were bisected without strict equality for each hemi-
192 embryo, a separate analysis of ICMandTE and TE_part RNA quantities would not have been
193 meaningful. Thus, ICMandTE and TE_part RNA quantities were summed up. RNA quantity and
194 embryo diameter were analyzed using a linear model of nlme package version 3.1-148 [46] including
195 maternal parity and embryo sex, followed by 1000 permutations using PermTest function from
196 pgirmess package version 1.6.9 [47]. Variables were kept in the subsequent models when statistically
197 significant differences were observed. Differences were considered as significant for $p < 0.05$.

198

199 **2.6.2. Deconvolution of gene expression in ICMandTE using DeMixT**

200 The deconvolution method has already been described in equine embryos [35]. Briefly, this method
201 enables the estimation of the relative gene expression of TE and ICM cell types within the hemi-

202 embryo ICMandTE which is composed of both trophoblast and inner cell mass in unknown relative
203 proportions. After filtering all genes with 3 non-null count values in at least one group (YN or YM) per
204 hemi-embryo (ICMandTE or TE_part), removing genes with a null variance in TE_part and adding the
205 value “1” to all count values in ICMandTE and TE_part datasets, deconvolution was performed using
206 the DeMixT R package version 1.4.0 [48,49]. Output datasets were DeMixT_ICM_cells and
207 DeMixT_TE_cells, corresponding to the deconvoluted gene expression in ICM cells and TE cells of
208 ICMandTE, respectively.

209 At the end of deconvolution, a quality check was automatically performed by the DeMixT R package
210 with the TE_part used as reference for DeMixT_TE_cells. Genes were automatically filtered out if the
211 difference between average deconvoluted expression of reference cells in mixed samples and
212 average expression of reference cells > 4.

213 Outputs of DeMixT_ICM_cells vs DeMixT_TE_cells, DeMixT_ICM_cells vs TE_part and ICMandTE vs
214 TE_part were compared with Deseq2 version 1.28.1 [50] to confirm that the deconvolution was
215 effective at separating gene expression. To check if deconvolution was efficient, as previously
216 described [35], the expression of several genes proper to ICM and TE cells in equine embryos
217 identified using literature search [51] was compared before and after deconvolution. Results of these
218 analyses were represented through manually drawn Venn diagrams as well as principal component
219 analysis graphics of individuals, using ggplot2 version 3.3.3 [52] and factoextra version 1.0.7 [53].

220

221 **2.6.3. Maternal parity comparison for gene expression**

222 All genes with an average expression <10 counts in both YN and YM per hemi-embryo (ICM or TE)
223 were filtered out on the DeMixT_ICM_cells and TE_part datasets. Differential analyses were
224 performed with Deseq2 version 1.28.1 [50] with the YM group as reference, without independent
225 filtering. Genes were considered differentially expressed (DEG) for FDR <0.05 after Benjamini-
226 Hochberg correction (also known as false discovery rate, FDR). As ovulation was checked only every

227 48h and because embryos growth is exponential in the uterus, embryo diameter was considered as a
228 cofactor in the model as well as embryo sex.

229 Equine Ensembl IDs were converted into Human Ensembl IDs and Entrez Gene names using gorth
230 function in gprofiler2 package version 0.1.9 [54]. Genes without Entrez Gene names using gprofiler2
231 were manually converted when Entrez Gene names were available, using Ensembl web search
232 function [55]. GO molecular function and GO Biological process annotations of genes were obtained
233 from Uniprot website.

234

235 **2.6.4. Gene set enrichment analyses (GSEA)**

236 After log transformation using RLOG function of DESeq2 version 1.28.1, gene set enrichment analyses
237 (GSEA) were performed on expressed genes using GSEA software version 4.0.3 (Broad Institute, Inc.,
238 Massachusetts Institute of Technology, and Regents of the University of California) [56,57] to identify
239 biological gene sets disturbed by maternal parity. Molecular Signatures Databases [58] version 7.1
240 (C2: KEGG: Kyoto Encyclopedia of Genes and Genomes; REACTOME, C5: BP: GO biological process)
241 were used to identify most perturbed pathways. Pathways were considered significantly enriched for
242 $FDR < 0.05$. When the normalized enrichment score (NES) was positive, the gene set was enriched in
243 the YN group while when NES was negative, the gene set was enriched in the YM group.

244 If applicable, as previously described in equine embryos [35], enriched terms from GO BP, KEGG and
245 REACTOME databases were represented using SUMER analysis from SUMER R package version 1.1.5
246 and using FDR q-values [59]. Results were represented with graphs modified using Cytoscape version
247 3.8.2 [60]. In these graphs, gene sets are represented by nodes and the gene set size is represented
248 by the size of the node. Node shape represents the gene set database (GO BP, KEGG or REACTOME).
249 Blue nodes represent gene sets enriched in YN ($NES > 0$) while green nodes represent gene sets
250 enriched in YM ($NES < 0$). Edge width represents the level of connection between representative

251 gene sets (thinner edges represent the first clustering while thicker edges represent the second
252 clustering of the affinity propagation algorithm).

253 As SUMER is not able to consider only genes that participate to enrichment in GSEA, pathways with
254 genes in common are grouped together, although genes in common are not the ones that participate
255 to the enrichment. To better understand groups, therefore, authors, first recovered genes that were
256 enriched in each pathway of a common group of gene sets according to SUMER analysis. Then, they
257 only considered genes in common between pathways in one group to better qualify the function that
258 was altered by maternal parity.

259

260 **3. Results**

261 *3.1. Embryo recovery rates, diameter, total RNA content and quality and progesterone* 262 *concentrations*

263 Altogether, 25 embryo collections were performed (13 in YN and 12 in YM, 4 mares being flushed
264 twice) and 12 embryos were obtained (7 from 6 YN mares and 5 from 5 YM mares). One young
265 nulliparous mare produced twin embryos.

266 Embryo recovery rate per mare was 46% and 42% in YN and YM, respectively and did not differ
267 between groups ($p = 1$).

268 Altogether, 1 and 2 double ovulations were observed, respectively, in YN and YM. The embryo
269 recovery rate per ovulation at the time of embryo collection was not different according to group
270 (50% in YN and 36% in YM, $p = 0.70$).

271 All embryos were expanded blastocysts grade I or II according to the embryo classification of
272 McKinnon and Squires [61]. For the twin collection, embryos diameters were 580 μ m and 591 μ m. As
273 only one embryo per mare was required, the 580 μ m diameter was randomly chosen for further
274 analysis. Altogether, only 6 YN and all 5 YM embryos collected were RNA sequenced. Embryo
275 diameter ranged from 457 μ m to 2643 μ m, with no effect of group on embryo diameter ($p = 0.18$). In

276 embryos selected for RNA sequencing, there was no effect of embryo sex on its size ($p = 0.63$). RNA
277 yield per embryo ranged from 12.0 ng to 2915.5 ng and was not related to parity ($p = 0.07$) nor
278 embryo sex ($p = 0.77$).

279 The median RNA Integrity Number (RIN) was 9.6 (8.9 - 10 range). Between 39.7 and 69.5 million
280 reads per sample were obtained after trimming. On average, 70.94% of the reads were mapped on
281 the modified EquCab 3.0 using STAR and 66.45% were assigned to genes by featureCounts.

282

283 *3.2. Deconvolution of gene expression to discriminate ICM and TE gene expression in* 284 *ICMandTE hemi-embryos*

285 After selecting genes with more than 3 non null count values in at least one group (YN or YM) per
286 hemi-embryo (ICMandTE or TE_part), 16,901 genes were conserved for deconvolution. In addition,
287 67 genes were removed because their variance was null in the TE_part. For these genes, the mean
288 count in ICMandTE samples was above 110 counts. The deconvolution quality of all gene was
289 sufficient. Therefore, at the end of the deconvolution algorithm, 16,834 genes were available for
290 differential analysis.

291 Before deconvolution, 681 genes were differentially expressed ($FDR < 0.05$) between the ICMandTE
292 and the TE_part (Fig. 1a). After deconvolution, the comparison between DeMixT_ICM_cells and
293 DeMixT_TE_cells yielded 6,171 differentially expressed genes while the comparison
294 DeMixT_ICM_cells vs TE_part yielded 5,262 differentially expressed genes, with 4713 genes in
295 common with the previous comparison (70%). Moreover, 677 of the initially 681 differentially
296 expressed genes before deconvolution were also identified as differentially expressed in both post-
297 deconvolution analyses. Only in the comparison DeMixT_ICM_cells vs TE_part, 3 among the 4
298 remaining genes were identified. On the PCA graph of individuals, ICMandTE and TE_part were partly
299 overlapping (Fig 1b). DeMixT_TE_cells and TE_part superposed well, suggesting that datasets before
300 and after deconvolution have a similar global gene expression; whereas the DeMixT_ICM_cells group
301 is clearly separated from others on Axis 1 (22.3% of variance), indicating that the deconvolution

302 effectively enabled the separation of gene expression of the two cell types in the mixed part
303 (ICMandTE).

304 On the 12 genes previously identified by Iqbal et al. as more expressed in the ICM [51], one had to be
305 removed before deconvolution because its variance in the TE was zero (*ENSECAG00000010653*,
306 annotated as SRY-Box Transcription Factor 2, *SOX2*). On the 11 remaining genes, 4 were also more
307 expressed in the ICMandTE vs TE_part comparison (Table 2). After deconvolution (comparison
308 DeMixT_ICM_cells vs TE_part), 10 out of 11 of these genes were effectively more expressed in the
309 ICM. Iqbal et al. identified 7 genes that were more expressed in the TE. One of those genes was
310 differentially expressed in the comparison ICMandTE vs TE_part, *i.e.*, before deconvolution. After
311 deconvolution, the expression of 3 of the 7 reported genes, different from the only gene identified
312 before deconvolution, were increased in the TE_part compared to the DeMixT_ICM_cells.

313 All of these results validate the deconvolution procedure and justify the use of data from the
314 DeMixT_ICM_cells file. In the following results, the TE_part was used as representative of TE and
315 DeMixT_ICM_cells was used as representative of gene expression in the ICM.

316

317 *3.3. Sample selection*

318 One embryo (YM) was larger than 2000 μm while all other embryos were smaller than 1400 μm in
319 diameter (Supplementary Figure 1). Embryo size has been shown to affect equine embryo gene
320 expression [62]. Thus, the analysis was performed both with or without this large embryo to check if
321 results were affected. All but one differential expressed genes identified with the largest embryo
322 were also found differentially expressed without it (Supplementary Figure 2). Nevertheless, to limit
323 size effect, the analyses described below are those without the largest embryo, where only 6 YN and
324 4 YM embryos were analyzed. The results of the differential analyses that were performed including
325 the 2643 μm large YM embryo are shown in Supplementary Tables 1 and 2.

326

327 *3.4. Differential gene expression in deconvoluted ICM cells*

328 After retaining only genes with an average expression ≥ 10 counts in at least one maternal parity
329 group andhemi-embryo, 14,418 genes were considered as expressed in the YN or YM embryos ICM
330 cells. Only 18 genes were differentially expressed (12 downregulated and 6 upregulated in YN) (Fig. 2
331 and Supplementary table 3). Respectively, 11 and 5 genes out of the down- and upregulated genes
332 were associated to a protein known and described in human. These 16 genes an gene sets
333 determined from Uniprot in wich they are susceptible to play a role are presented in Table 3.

334

335 *3.5. Differential gene expression in the TE part*

336 In the TE, 13,203 genes were considered as expressed in YN or YM. Only 6 were differentially
337 expressed (Supplementary table 4) with half being down and up- regulated in YN (Fig. 2). Except one
338 that was a long noncoding RNA, all other genes were associated to a known protein in human. These
339 genes are presented in Table 4 with the pathways in which they are susceptible to play a role.

340

341 *3.6. Gene set enrichment analysis in deconvoluted ICM cells*

342 After Entrez Gene ID conversion, 12,892 genes were considered expressed in ICM cells. Fifty-eight GO
343 Biological Process and 4 KEGG pathways were disturbed by maternal parity in ICM cells
344 (Supplementary table 5). After SUMER analysis, 2 and 27 gene sets, respectively enriched in YN and
345 YM, were represented (Fig. 3). They were clustered in 8 groups. The group enriched in YM and
346 clustered under the term “DNA recombination” was composed of genes related to the maintenance
347 of DNA integrity, chromosome segregation and recombination. Enriched in YM groups “NCRNA
348 metabolic process” and ‘Peptidyl lysine trimethylation” contained both, genes related to methylation
349 and transcription. The only gene set enriched in YN in the group “NCRNA metabolic process” was
350 mainly enriched by genes encoding for a subunit of ribosomes that were common with other gene
351 sets enriched in YM. Genes related to ribosomes were, however, not participating in gene set
352 enrichment of other pathways in this cluster. “multi organism localization”, “RNA splicing” and “RNA
353 localization” clusters were composed of genes involved in RNA maturation and transport. The last

354 group enriched in YM was clustered under the term “vesicle targeting” and was containing genes
355 related to intracellular transport. In this group, the pathway “Golgi vesicle transport” included a DEG
356 that was Vacuolar Protein Sorting-Associated Protein 52 Homolog (*VPS52*), up-regulated in the ICM
357 of embryos from YM mares. The only one group enriched in YN was composed of one pathway
358 named “ECM receptor interaction” in which genes related to extracellular matrix (ECM) were
359 observed.

360

361 *3.7. Gene set enrichment analysis in TE*

362 After Entrez Gene ID conversion, 11,889 genes were considered expressed in TE from YN or YM
363 embryos. Altogether, 50 gene sets from GO BP, KEGG and REACTOME were perturbed (23 GO BP, 7
364 KEGG and 20 REACTOME) by maternal parity in young mares (Supplementary table 6). After SUMER
365 analysis, 36 gene sets were represented (Fig. 3) and were clustered in 8 groups. Among them, 7 were
366 enriched in YN. The first group was composed of one gene set named “cardiac septum
367 morphogenesis”. Most genes that participated to the enrichment of this pathway were related to
368 transcriptional factors. The second group was composed of 3 pathways and clustered under the term
369 “negative regulation of secretion”. Genes that participated most to the enrichment of these
370 pathways were related to the innate immunity, more particularly to the production and transport of
371 the interleukin 1 beta (IL1B). Altogether, 3 groups were related to the production of energy inside
372 the cell. Indeed, the cluster under the term “Parkinsons disease” was actually composed of genes
373 with an enriched expression that were related to oxidative phosphorylation. One group was
374 “Hydrogen peroxide metabolic process” where genes that participated the most to the enrichment
375 were involved in the degradation of hydrogen peroxide. The last group involved in energy production
376 was clustered under the term “valine, leucine and isoleucine degradation”. Genes that participated
377 the most to the enrichment of these pathways were directly involved in the beta oxidation of fatty
378 acid. The group under the term “positive regulation of lipid transport” was composed of genes
379 related to the regulation of the transport of lipids and cholesterol. The last group enriched in YN was

380 clustered under the term “eukaryotic translation elongation” and was mostly enriched due to
381 components of the ribosomes that mostly participate in these enrichments. Moreover, the
382 REACTOME pathway “RRNA modification in the nucleus and cytosol” was clustered with this group
383 because of genes that encoded for ribosome components. These genes were, however, not enriched
384 in this particular pathway. Genes that participate to its enrichment, nevertheless, were related to
385 ribosome biogenesis. The only one group enriched in YM was represented by the term “Regulation of
386 glucokinase by glucokinase regulatory protein”. These pathways were mostly enriched in YM because
387 of genes that encode for nucleoporin subunits.

388

389 **4. Discussion**

390 Maternal parity in young mares slightly affected both ICM and TE gene expression without affecting
391 embryo recovery rates nor growth. Although only a few genes were affected by maternal parity, up
392 regulated genes in the ICM of embryos from young nulliparous were involved in lipid, amine and
393 creatinine metabolism, positive regulation of transcription, growth factor signaling and
394 morphogenesis while down regulated were related to extracellular matrix (ECM) disassembly,
395 reactive oxygen species (ROS) metabolism, transcription regulation, endocytosis, protein transport,
396 protein metabolism, MAP kinase signalization and cell differentiation. In the TE, only five known
397 genes were observed differentially expressed. One of the 3 up regulated genes in the TE of embryos
398 from YN was involved in the cell response to hypoxia while the 2 others encode for ion binding
399 proteins. Down regulated genes in the TE of embryos from YN mares were related to prostaglandin
400 metabolism and amino acid/creatinine exchange. Interestingly, while gene set enrichment analysis
401 showed almost only pathway enrichment in YM in the ICM, in the TE, almost all pathways were
402 enriched in YN embryos. In the ICM, after SUMER analysis, the pathways enriched in YM were related
403 to DNA modification, RNA production and maturation and cell transport while gene sets related to
404 the extracellular matrix function and ribosome were enriched in ICM of YN embryos. In the TE, gene

405 sets enriched in embryos from YN were related to immunity, growth factor signaling,
406 phosphorylation oxidative, metabolism of reactive oxygen species, beta oxidation and transport of
407 lipids and ribosome while gene sets enriched in embryos from YM were related to nucleoporins.

408

409 In the ICM of embryos from YM mares, enriched gene sets were mostly related to DNA conformation
410 and methylation changes as well as RNA formation, transport and maturation. These results suggest
411 that transcription and regulating pathways are less active in embryos from nulliparous mares. These
412 results are comforted by the fact that teneurin transmembrane protein 3 (*TENM3*) is downregulated
413 in embryos from nulliparous mares. This gene is part of the teneurin family, which encodes for
414 transmembrane proteins that are essential for embryo morphogenesis and nervous system
415 development. The knockdown of these genes in mice and drosophila leads to embryo lethality (for
416 review [63]). Altogether, these results suggested that ICM growth and development would be poorer
417 in embryo from nulliparous compared to multiparous mares but, here, no difference in embryo size
418 had been observed. One hypothesis could be because only ICM seemed affected or because, embryo
419 size at a same age is highly variable as shown in several studies [64–68], although ovulation check
420 was performed twice daily [69]. This huge variation could hide size differences in studies.

421 In the TE, the gene named "family with sequence similarity 162 member A" (*FAM162A*), also known
422 as E2-Induced Gene 5 Protein (*E2IG5*) or growth and transformation-dependent protein (*HGTD-P*)
423 was up-regulated in embryos from nulliparous mares. This gene is one of the hypoxia inducible
424 factors (HIF)-activated downstream gene and is normally responsible of the activation of
425 mitochondrial proapoptotic cascades when overexpressed [70]. As energy production processes
426 (protein and lipid oxidation, oxidative phosphorylation and related regulatory pathways) were
427 enriched in embryos from nulliparous mares, it seemed unlikely that *FAM162A* up-regulation in
428 embryos from nulliparous mares was a response to hypoxic environment. Nevertheless, it could be
429 hypothesized that the uterine environment of embryos may vary according to mares' parity, partly
430 due to reduced uterine blood perfusion in nulliparous mares. To the authors' knowledge, there is no

431 study on the effect of nulliparity on uterine vascularization in young mares. It has nonetheless been
432 shown that *FAM162A* expression is increased in intestinal and uterine cervical cancer [71,72] its
433 overexpression enhanced cell proliferation processes, suggesting a non-elucidated positive role in
434 tumor development [72]. As in tumor, here, *FAM162A* could play a role in cell proliferation of equine
435 embryos but the process remains to be elucidated. This could also indicate that proliferation in
436 equine embryos differ according to mares' parity, maybe as a response to their environment.

437 In the TE of embryos from nulliparous mares, pathways related to oxidative phosphorylation were
438 enriched. The enrichment of the expression of genes involved in these pathways could indicate that
439 the production of ATP from oxidative phosphorylation is up regulated in TE of embryos from
440 nulliparous mares in comparison to the ones from multiparous mares. This up-regulation of oxygen
441 oxidation in mitochondria could be harmful for TE cells as oxidative phosphorylation is accompanied
442 by the production of reactive oxygen species (ROS) and particularly of hydrogen peroxide (for review
443 [75]). Pathways related to hydrogen peroxide metabolic processes, however, were also enriched in
444 the TE of embryos from nulliparous mares, showing that there is an up-regulation of the control of
445 ROS such as hydrogen peroxide. The up regulation of both oxidative phosphorylation and regulation
446 of ROS pathways suggests that there is an increased production of energy that is not harmful
447 because well controlled in the TE of embryos from nulliparous mares.

448 At this developmental stage in equine embryos, 40 to 50% of glucose uptake is oxidized in the
449 mitochondria, probably to meet the high energy demand of ionic transport associated with the
450 important growth of both blastocoelic cavity and trophoblast [76]. Here, however, the enrichment in
451 oxidative phosphorylation was not accompanied by an enrichment in glucose metabolism nor
452 transport pathways but pathways linked to beta-oxidation of lipids and degradation of amino acids
453 were enriched. This suggests that glycolysis is not affected by maternal parity but, to meet energy
454 requirements, embryos from nulliparous mares use fatty acids and/or amino acids whilst embryos
455 from multiparous mares do not need more energy than already provided and therefore, do not
456 require the degradation of these substrates. The increased catabolism of amino acids and lipids could

457 be detrimental for embryo development as the first are required for protein synthesis and the latter
458 are mandatory for hormone production (for review [77,78]). Pathways related to amino acid
459 degradation, however, were mostly enriched in genes involved in beta-oxidation of fatty acids.
460 Moreover, pathways related to translation and protein maturation but not pathways related to
461 amino acids transport were enriched in embryos from nulliparous mares. Altogether, these results
462 suggest that only lipid catabolism is enriched in the TE of nulliparous mares' embryos.

463 In addition, pathways related to the transport of lipids and cholesterol were enriched in the TE of
464 nulliparous mares' embryos compared to those of multiparous mares. One hypothesis to explain
465 these results could be that there is a higher energy demand in embryos from nulliparous mares and
466 that they would compensate by degrading more lipids for oxidative phosphorylation, which requires
467 more lipids to be obtained from the external environment. Another possibility is that the lipid
468 composition of the uterine environment is altered in nulliparous mares, possibly due to immature
469 uterine glands, leading to increased absorption by the embryo, that would stimulate beta-oxidation
470 and thus oxidative phosphorylation. Indeed, the metabolism of lipids was also shown to be perturbed
471 in blastocysts at the same developmental stage according to maternal parity in old mares [36]. To the
472 authors' knowledge, there is no study on the effects of maternal parity in any species on uterine fluid
473 composition and how it could interfere with embryo gene expression. Although it is more likely that
474 there are modifications in the uterine environment according to mare's parity, the present results
475 cannot conclude about the origin of the altered embryo metabolism.

476 As a confirmation of increased lipid transport, retinol binding protein 1 (*RBP1*) was up regulated in
477 the ICM of embryos from nulliparous mares. Retinol is well known to be an important regulator of
478 vertebrate development (for review [79]). In bovine, the addition of retinol to the maturation and
479 culture medium of oocytes and embryos increased the blastocyst rate [80]. RBP transports the
480 hydrophobic retinol in physiological fluids such as plasma [81] or uterine fluids. Pig conceptuses at
481 the time of elongation produce RBP in large amounts, suggesting that retinol is important for embryo
482 development [82]. In horses, the expression of *RBP* increased in the endometrium during diestrus

483 under steroid regulation but did not vary according to the presence of an embryo or not [83].
484 Although underlying mechanisms are missing, *RBP1* could, however, play an important role in equine
485 early embryo development by transporting retinol to the embryo. The increased expression of RBP in
486 the ICM of embryos from nulliparous mares could be a response to a reduced availability of retinol in
487 the close environment of the embryo or an increased requirements of retinol from embryos of
488 nulliparous mares.

489 Nutrient and ion exchanges were also modified by maternal parity in the TE. Indeed, solute carrier
490 family 47 member 1 (*SLC47A1* also known as *MATE1*), the solute transporter for molecules such as
491 creatinine or guanidine, was down regulated in the TE of embryos from nulliparous mares. In
492 addition, the expression of EF-hand calcium binding domain 11 (*EFCAB11*) and GTP binding protein 8
493 (*GTPBP8*) was increased in the TE of embryos from nulliparous mares compared to that of
494 multiparous mares. These results could indicate a perturbed transport of different molecules in the
495 TE of embryos from nulliparous mares.

496 These modifications of cell metabolism in the TE were associated with an alteration of pathways
497 related to immunity, especially those linked to interleukin 1 beta (*IL1B*), being enriched in embryos
498 from nulliparous mares. In cattle, it has been suggested that the early bovine embryo interacts with
499 the dam's immune system through processes involving *IL1* [84]. In horses, maternal recognition of
500 pregnancy (MRP) is thought to take place between 10-13 days post ovulation (for review [85]). At 19-
501 and 25-days, but not at 13 days post ovulation, expression of the *IL1 receptor antagonist* has been
502 shown to be markedly increased in the endometrium of pregnant compared to cyclic mares,
503 suggesting that the endometrium regulates the *IL1* signal and that *IL1* plays a role in MRP in equine
504 [86]. The expression of *IL1B* is increased in the luminal epithelium of pregnant vs cyclic mares at 10-
505 13 days post ovulation, confirming the involvement of *IL1B* signaling process in MRP [87]. Here,
506 embryos were collected earlier from the assumed MRP period but the observed differences in the
507 *IL1B* signaling pathway could indicate that embryo-maternal communication and possibly MRP are
508 affected by maternal parity.

509 Furthermore, related to lipid metabolism and IL1B signaling, peroxiredoxin like 2B (*PRXL2B*), also
510 known as Prostamide/Prostaglandin F Synthase, was downregulated in the TE of nulliparous mares'
511 embryos. This gene encodes for an enzyme that has been shown to catalyze the reduction of
512 prostamide H₂ to prostamide F_{2α} as well as the reduction of prostaglandin H₂ (PGH₂) to prostaglandin
513 F_{2α} (PGF_{2α}) [88]. In bovine, IL1B upregulates PGF_{2α} and prostamide secretion by in vitro cultured
514 endometrial cells [89]. In horses, PGF_{2α} is secreted by the uterus to provoke the corpus luteum
515 luteolysis (for review [90]). It has been shown that the suppression of the pulsatile secretion of PGF_{2α}
516 from the endometrium is responsible for the maintenance of pregnancy [91] and that *in vitro*, PGF_{2α}
517 production is significantly reduced when endometrial explants are co-cultured with embryonic
518 tissues [92]. From the oviduct stages, equine embryos are able to produce prostaglandins [93–95].
519 Prostaglandins produced by the embryo, however, do not reach the blood circulation in sufficient
520 amount to induce luteolysis [91]. It has been shown that these prostaglandins are required for
521 myometrial contractions that participate in the migration of the equine embryo at the time of MRP
522 [96]. By impeding the movement of the embryo, one study observed that equine embryo migration
523 through at least 2/3 of the uterus is required to prevent luteolysis [97]. Moreover, the use of an
524 intra-uterine device to imitate the physical presence of an embryo, allowed to prevent the luteolysis
525 [98]. A recent study, nevertheless, observed that the contact of a substance/object is not sufficient to
526 reduce PGF secretion from the endometrium, suggesting that embryo secretions are required for
527 luteolysis [99]. Therefore, although MRP is thought to begin 2 days later, the present study shows
528 that MRP might be delayed or disturbed in nulliparous mares.

529

530 **5. Conclusion**

531 So far, the effect of mare's parity on embryo gene expression had never been considered. The
532 present study shows that mare's parity affects the expression of genes in both ICM and TE of
533 blastocysts. Only the expression of few genes is altered but several important functions for embryo

534 development are affected by mare's parity. Indeed, nulliparity in young mares particularly alters the
535 expression of genes related to transcription and RNA processing in the ICM and embryo-maternal
536 communication in the TE, suggesting embryo adaptation to an environment that is different in
537 nulliparous vs multiparous mares. Individual chances of implantation for each embryo could not be
538 predicted by the results of this study. Until today, the capacity of uterus to enlarge and support
539 pregnancy was the only suggested explanation for the lighter and smaller foal and placenta at birth in
540 nulliparous mares. The present results indicate differences in embryo-maternal communication long
541 before implantation that could alter the embryo development as well as maternal recognition of
542 pregnancy.

543 **Data Availability Statement**

544 The RNA sequencing data supporting the conclusions of this article are available in the GEO
545 SuperSeries [accession: GSE193676;
546 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193676>], containing repositories
547 [accession: GSE162893; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162893>] and
548 [accession: GSE193675; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193675>].

549

550 **Conflict of interest**

551 The authors declare no conflicts of interest.

552

553 **Declaration of funding**

554 This work was supported by the "Institut Français du Cheval et de l'Équitation" (grant numbers
555 CS_2018_23, 2018). The National Research Institute for Agriculture, Food and Environment (INRAE)
556 department Animal Physiology and Breeding Systems also supported this research.

557

558 **Acknowledgments**

559 The authors are grateful to the staff of the Institut Français du Cheval et de l'Équitation (IFCE)
560 experimental farm (Plateau technique de la Valade, Chamberet, France) for care and management of
561 animals. We acknowledge the high-throughput sequencing facility of I2BC for its sequencing and
562 bioinformatics expertise. The bioinformatics analyses were performed thanks to Core Cluster of the
563 Institut Français de Bioinformatique (IFB) (ANR-11-INBS-0013). Many thanks to Matthias Zytnecki and
564 Christophe Klopp for their advice on RNA-seq de novo analysis. Many thanks to Pablo Ross who
565 kindly provided the coordinates for the XIST gene.

566

567 **Author contributions**

568 PCP obtained the funding. PCP and VD conceived the project. VD and PCP supervised the study. ED,
569 CA, ND, NP, VD and PCP adapted the methodology for the project. ED, CA, JAR and YJ performed the
570 experiments. CA, ND, NP and MD provided the resources. ED, LJ, YJ and RL performed data curation.
571 ED and LJ analyzed the data. ED wrote the original draft. All authors read, revised, and approved the
572 submitted manuscript.

573 **List of abbreviations**

- 574 DEG: differential expressed genes
- 575 DeMixT_ICM_cells: deconvoluted gene expression in ICM cells
- 576 DeMixT_TE_cells: deconvoluted gene expression in TE cells
- 577 ECM: Extracellular matrix
- 578 ERR: embryo collection rate
- 579 FDR: false discovery rate
- 580 GO BP: Gene Ontology biological process
- 581 GO: Gene Ontology
- 582 GSEA: gene set enrichment analyses
- 583 ICM: inner cell mass
- 584 ICMandTE: inner cell mass enriched hemi-embryo
- 585 ICSI: intracytoplasmic sperm injection
- 586 IL1B: Interleukin 1 beta
- 587 KEGG: Kyoto Encyclopedia of Genes and Genomes
- 588 Log2FC: log2 fold change
- 589 NES: normalized enrichment score
- 590 OM: old multiparous mares
- 591 ON: old nulliparous mares
- 592 TE: trophoblast

593 TE_part: pure trophoblast hemi-embryo

594 XIST: X inactive Specific Transcript

595

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873

874 **Figure legends**

875 Fig. 1: Gene expression in ICM and TE before and after deconvolution using DeMixT

876 a) Venn diagram of genes differentially expressed in the different analyses: ICMandTE vs TE_part
877 (before deconvolution), DeMixT_ICM_cells vs DeMixT_TE_cells (after deconvolution) and
878 DeMixT_ICM_cells vs TE_part (gene expression of ICM after deconvolution vs gene expression in
879 TE_part without deconvolution); b) Principal Component Analysis of gene expression from
880 DeMixT_ICM_cells, DeMixT_TE_cells, ICMandTE and TE part datasets.

881 Deconvolution was used to isolate gene expression of ICM and TE cells in ICMandTE hemi-embryos.
882 ICMandTE: inner cell mass + trophoblast; TE_part: pure trophoblast. Here trophoblast represents
883 trophectoderm + endoderm.

884

885 Fig. 2: Analysis of differentially expressed genes (DEG) in embryos according to maternal parity

886 A) representation of down- (blue) and up- (red) regulated DEG in ICM (from DeMixT_ICM_cells data
887 obtained after deconvolution of ICMandTE using DeMixT R package [48,49]) and TE (from TE_part
888 dataset) of embryos from ON vs OM.

889 DEG: Differentially Expressed Genes (FDR < 0.05); TE: Trophoblast; ICM: Inner Cell Mass; ON: Old
890 nulliparous mares; OM: Old multiparous mares

891

892 Fig. 3: SUMER clustering of GSEA terms clustering of the most perturbed terms in the ICM and TE of
893 embryos according to mares' parity

894 Nodes represent altered gene sets in the ICM and TE (FDR <0.05). Node size represents the gene set
895 size. Node shape represents the gene set database: GO BP (circle) or KEGG (diamond) or REACTOME
896 (square). Gene sets are represented in blue if enriched (NES >0) in young nulliparous mares' embryos
897 and in green if enriched (NES <0) in young multiparous mares' embryos. The lighter the color, the
898 more the NES is close to 0. Edges represent the level of connection between representative gene
899 sets. This graph was performed using SUMER R package [59] and modified using cytoscape 3.8.2 [60]

900 GSEA: Gene set enrichment analysis; ICM: Inner cell mass; TE: trophoblast; FDR: False Discovery Rate;

901 GO BP: Gene Ontology Biological Process; Kyoto Encyclopedia of Genes and Genomes; NES:

902 Normalized Enrichment Score

903 **Supplementary material**

904 Supplementary Figure 1:

905 SupFig1_Embryosize.tif

906 Plot of equine individual embryo according to their size

907

908 Supplementary Figure 2:

909 SupFig2_comp_with_without.png

910 Venn diagrams of differential analyses on equine embryo gene expression according to maternal
911 parity in the inner cell mass (ICM) and the trophoblast part (TE) with or without the largest embryo
912 (2643 μ m in diameter)

913

914 Supplementary Table 1:

915 Sup1_ICM_Diff_avecYME5.csv

916 Differential gene analysis using DeSeq2 in DeMixT_ICM_cells of equine embryo at Day 8 post-
917 ovulation according to mares' parity with the large embryo

918 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene
919 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis
920 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in ICM (after gene
921 expression deconvolution of ICMandTE using DeMixT) of YN and YM embryos

922 ICM: Inner cell mass; YN: young nulliparous mares; YM: young multiparous mares

923

924 Supplementary Table 2:

925 Sup2_TE_Diff_avecYME5.csv

926 Differential gene analysis using DeSeq2 in TE_part of equine embryo at Day 8 post-ovulation
927 according to mares' parity with the large embryo

928 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene
929 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis
930 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in TE_part of YN and YM
931 embryos

932 TE: trophoblast; YN: young nulliparous mares; YM: young multiparous mares

933

934

935 Supplementary Table 3:

936

937 Sup3_ICM_Diff_sansYME5.csv

938 Differential gene analysis using DeSeq2 in DeMixT_ICM_cells of equine embryo at Day 8 post-
939 ovulation according to mares' parity without the largest embryo

940 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene
941 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis
942 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in ICM (after gene
943 expression deconvolution of ICMandTE using DeMixT) of YN and YM embryos

944 ICM: Inner cell mass; YN: young nulliparous mares; YM: young multiparous mares

945

946 Supplementary Table 4:

947 Sup4_TE_Diff_sansYME5.csv

948 Differential gene analysis using DeSeq2 in TE_part of equine embryo at Day 8 post-ovulation
949 according to mares' parity with the largest embryo

950 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene
951 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis
952 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in TE_part of YN and YM
953 embryos

954 TE: trophoblast; YN: young nulliparous mares; YM: young multiparous mares

955

956 Supplementary Table 5:

957 Sup5_ICM_GSEA_sansYME5.csv

958 Gene set enrichment analysis results on gene expression of DeMixT_ICM_cells of embryos from
959 young nulliparous and multiparous mares

960 Gene Set Enrichment Analysis results (database, pathway name, size, enrichment score without and
961 with normalization, p-value and FDR corrected q-value) for GO biological process, KEGG and
962 REACTOME databases on DeMixT_ICM_cells gene expression table. These results did not include
963 YM_E5, the embryo larger than 2,000µm. ICM: Inner cell mass

964

965 Supplementary Table 6:

966 Sup6_TE_GSEA_sansYME5.csv

967 Gene set enrichment analysis results on gene expression of TE_part of embryos from young
968 nulliparous and multiparous mares

969 Gene Set Enrichment Analysis results (database, pathway name, size, enrichment score without and
970 with normalization, p-value and FDR corrected q-value) for GO biological process, KEGG and
971 REACTOME databases on TE_part gene expression table. These results did not include YM_E5, the
972 embryo larger than 2,000 μ m.

973

974 **Tables**

975 Table 1: Mares' characteristics at embryo collection time.

| Characteristics | Nulliparous (YN) | | Multiparous (YM) | |
|-----------------------------|--------------------|---------------------|----------------------------|---------------------|
| | Total (n = 10) | With embryo (n = 6) | Total (n = 11) | With embryo (n = 5) |
| Breed | AA n = 7; SF n = 3 | AA n = 5; SF n = 1 | AA n = 9; SF n = 1; SB n=1 | AA |
| Age (in years) | 6.00 ± 0.00 | 6.00 ± 0.00 | 6.00 ± 0.00 | 6.00 ± 0.00 |
| Parity (number of foalings) | 0 ± 0.00 | 0 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 |
| Weight (in kg) | 535.66 ± 30.72 | 544.55 ± 30.50 | 536.62 ± 44.12 | 524.02 ± 61.39 |
| BCS (scale 1-5) | 2.35 ± 0.32 | 2.21 ± 0.25 | 2.16 ± 0.28 | 2.3 ± 0.33 |
| Withers' height (in cm) | 159.70 ± 3.33 | 161.67 ± 2.50 | 157.95 ± 3.74 | 157.40 ± 5.41 |

976 AA: Anglo Arab or Anglo-Arabian type; SF: Selle Français section A or B; SB: Saddlebred. Age, parity,

977 weight, and height are presented as mean ± SD

978

979 Table 2: Comparison of the expression of selected genes previously identified as specific to TE or ICM
 980 in equine embryos [51], before and after deconvolution

| | Gene name | Ensembl ID | ICMandTE vs TE_part | | DeMixT_ICM_cells vs TE_part | |
|------------|----------------------|--------------------|---------------------|----------|-----------------------------|----------|
| | | | log2FC from DeSeq2 | padj | log2FC from DeSeq2 | padj |
| ICM | SOX2 | ENSECAG00000010653 | - | - | - | - |
| | NANOG | ENSECAG00000012614 | 5.78 | 6.93E-58 | 7.09 | 9.24E-81 |
| | SPP1 | ENSECAG00000017191 | 4.52 | 2.21E-12 | 5.86 | 6.46E-21 |
| | LIN28B | ENSECAG00000020994 | 3.21 | 6.84E-13 | 4.44 | 2.00E-26 |
| | SMARCA2 | ENSECAG00000024187 | 1.01 | 0.139 | 1.71 | 8.10E-05 |
| | POU5F1 (OCT4) | ENSECAG00000008967 | 0.62 | 8.32E-04 | 1.25 | 1.22E-06 |
| | ID2 | ENSECAG00000008738 | 0.33 | 0.604 | 0.52 | 0.025 |
| | DNMT3B | ENSECAG00000012102 | 0.49 | 0.056 | 1.04 | 5.66E-07 |
| | DPPA4 | ENSECAG00000013271 | 0.39 | 0.545 | 0.87 | 2.63E-04 |
| | SALL4 | ENSECAG00000018533 | 0.21 | 0.177 | 0.60 | 1.50E-16 |
| | KLF4 | ENSECAG00000010613 | 0.03 | 0.995 | -0.39 | 0.526 |
| | UTF1 | ENSECAG00000039888 | 0.34 | 0.726 | 0.66 | 0.047 |
| TE | TFAP2A | ENSECAG00000017468 | -0.21 | 0.010 | -0.07 | 0.42 |
| | CDX2 | ENSECAG00000027754 | -0.21 | 0.290 | -0.37 | 2.87E-04 |
| | ELF3 | ENSECAG00000014608 | -0.12 | 0.864 | -0.19 | 0.148 |
| | GATA2 | ENSECAG00000016768 | -0.10 | 0.913 | -0.05 | 0.75 |
| | GATA3 | ENSECAG00000024574 | -0.18 | 0.28 | -0.10 | 0.325 |
| | TEAD4 | ENSECAG00000011303 | -0.19 | 0.206 | -0.31 | 9.58E-06 |
| | FREM2 | ENSECAG00000020410 | -0.06 | 0.970 | 0.13 | 0.474 |

981

982 Gene expressions were obtained from RNA of 11 equine embryos bisected in two hemi-embryos:
 983 one part was composed only of trophoblast (TE), TE_part, while the other part was composed of TE
 984 and inner cell mass (ICM), ICMandTE. As it is impossible to estimate the proportion of each cell in
 985 ICMandTE, a deconvolution algorithm (package DeMixT) was used to estimate gene expression of
 986 these different kind of cells. DeMixT_ICM_cells dataset corresponds to the deconvoluted gene

987 expression of ICM cells from ICMandTE. Log2 fold change (log2FC) and padj (adjusted p-value with
988 Benjamini-Hochberg correction) were obtained with Deseq2 package. TE_part is the reference group
989 in both analyses: when log2 fold changes (log2FC)>0, the gene is more expressed in the ICMandTE or
990 DeMixT_ICM_cells, while when log2FC<0, the gene is more expressed in the TE_part.

991 Green is used to represent genes differentially expressed in the present study. Orange is used to
992 represent genes that have been previously identified as predominant in the ICM [51] but that are
993 identified here as predominant in the TE.

994

995

6 Table 3: Up- and down-regulated genes coding for a protein in the inner cell mass of equine embryos according to mare parity

| Ensembl Name | Entrez Gene ID | Description | GO Molecular function | GO Biological Process | log2 Fold Change | padj |
|--------------------|----------------|--|--|---|------------------|----------|
| ENSECAG00000029895 | <i>MAGEB16</i> | MAGE family member B16 | Tumor antigen | | -3.24 | 0.013 |
| ENSECAG00000017619 | <i>NOXO1</i> | NADPH oxidase organizer 1 | Phospholipid binding Superoxide-generating NADPH oxidase activator activity | Extracellular matrix disassembly Positive regulation of cell killing Regulation of hydrogen peroxide metabolic process Superoxide metabolic process Regulation of respiratory burst | -2.67 | 0.034 |
| ENSECAG00000023392 | <i>ZBTB8A</i> | zinc finger and BTB domain containing 8A | Metal ion binding DNA binding | Regulation of transcription by RNA polymerase II Transcription regulation | -2.02 | 5.84E-05 |
| ENSECAG00000019702 | <i>VPS52</i> | VPS52 subunit of GARP complex | Syntaxin binding | Ectodermal cell differentiation Embryonic ectodermal digestive tract development Endocytic recycling Lysosomal transport Protein transport | -1.26 | 0.001 |
| ENSECAG00000011960 | <i>DESI1</i> | desumoylating isopeptidase 1 | Hydrolase Identical protein binding | Protein desumoylation Protein export from nucleus Protein modification by small protein removal | -1.23 | 0.001 |
| ENSECAG00000020433 | <i>TENM3</i> | teneurin transmembrane protein 3 | Cell adhesion molecule binding Protein heterodimerization activity Protein homodimerization activity | Cell adhesion Differentiation Neuron development Signal transduction | -1.00 | 0.015 |

| | | | | | | |
|---------------------|--------------|---|--|--|-------|-------|
| ENSECAG00000015867 | <i>PAQR3</i> | progesterone and adipoQ receptor family member 3 | Signaling receptor activity | Negative regulation of MAP kinase activity Negative regulation of neuron projection development Negative regulation of protein phosphorylation | -0.84 | 0.018 |
| ENSECAG00000004931 | <i>MARS2</i> | methionyl-tRNA synthetase 2, mitochondrial | Aminoacyl-tRNA synthetase Ligase ATP binding | Protein biosynthesis Methionyl-tRNA aminoacylation tRNA aminoacylation for protein translation | -0.82 | 0.020 |
| ENSECAG000000034815 | <i>VHL</i> | Von Hippel-Lindau tumor suppressor | Enzyme binding Transcription factor binding | Ubl conjugation pathway Cell morphogenesis Negative regulation of apoptotic process Negative regulation of cell population proliferation Negative regulation of gene expression Negative regulation of transcription by RNA polymerase II Positive regulation of cell differentiation Positive regulation of transcription Protein stabilization Proteolysis | -0.81 | 0.018 |
| ENSECAG00000007262 | <i>EEA1</i> | early endosome antigen 1 | GTP-dependant protein binding Protein homodimerization activity | Early endosome to late endosome transport Endocytosis Vesicle fusion | -0.79 | 0.015 |
| ENSECAG00000009590 | <i>GSTCD</i> | glutathione S- transferase C- terminal domain containing | | | -0.72 | 0.037 |

| | | | | | | |
|--------------------|---------------|---|---|--|------|-------|
| ENSECAG00000014243 | <i>GATM</i> | glycine amidinotransferase | Amidinotransferase activity | Creatine metabolic process Multicellular organism development | 0.73 | 0.018 |
| ENSECAG00000041817 | <i>RBP1</i> | retinol binding protein 1 | Retinoid binding | Lipid homeostasis Retinoic acid biosynthetic process | 1.13 | 0.020 |
| ENSECAG00000010447 | <i>EFEMP1</i> | EGF containing fibulin extracellular matrix protein 1 | Calcium ion binding Epidermal growth factor receptor binding | Embryonic eye morphogenesis Epidermal growth factor receptor signaling pathway Regulation of transcription, DNA-templated | 1.95 | 0.008 |
| ENSECAG00000010385 | <i>MET</i> | MET proto-oncogene, receptor tyrosine kinase | ATP binding Protein phosphatase binding Transmembrane receptor protein tyrosine kinase activity | Branching morphogenesis of an epithelial tube Cell migration Cell surface receptor signaling pathway MAPK cascade Phagocytosis Positive regulation of microtubule polymerization Positive regulation of transcription by RNA polymerase II | 2.83 | 0.013 |
| ENSECAG00000022277 | <i>MAOA</i> | Monoamine oxidase A | Monoamine oxidase activity Oxidoreductase | Cellular biogenic amine metabolic process Cytokine-mediated signaling pathway Dopamine catabolic pathway Positive regulation of signal transduction | 4.51 | 0.001 |

7

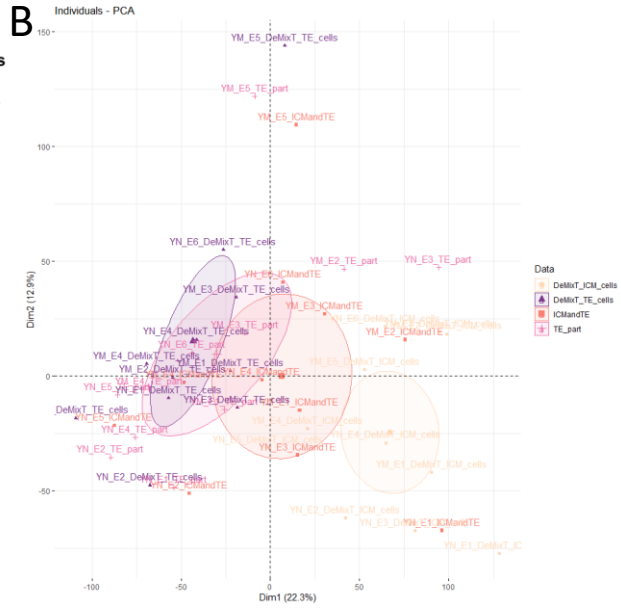
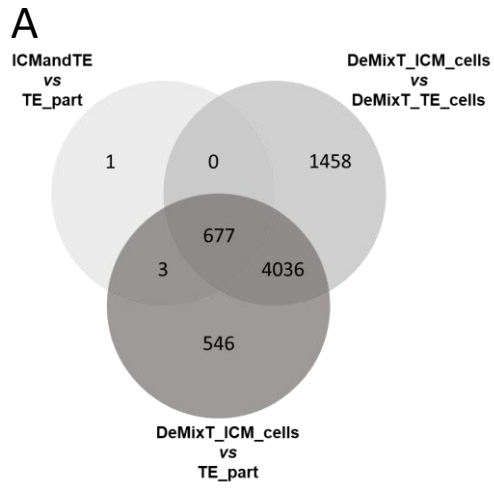
8 Log2 Fold-Change<0 indicates down-regulation of the gene in embryos from nulliparous mares, also indicated in green; Log2 Fold-Change>0 indicates up-regulation of the
9 gene in embryos from nulliparous mares, also indicated in blue.

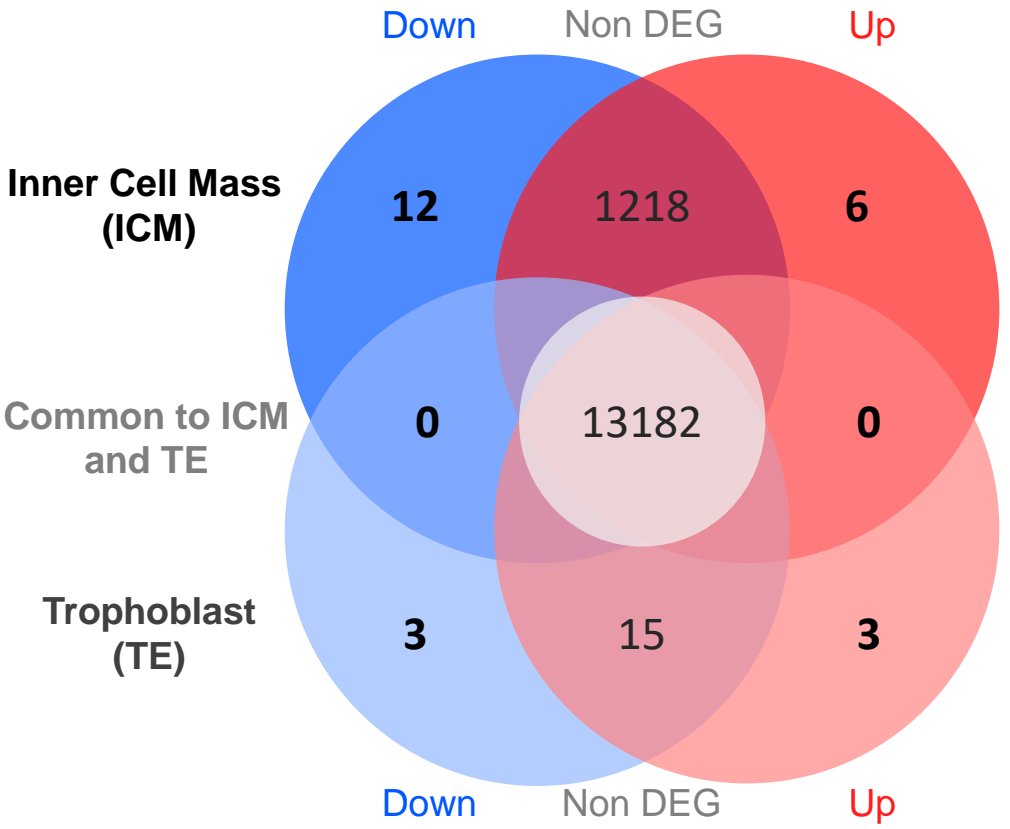
0 Table 4: Up- and down-regulated genes coding for a protein in the trophoblast part of equine embryos according to mare parity

| Ensembl Name | Entrez Gene ID | Description | GO Molecular function | GO Biological Process | log2 Fold Change | padj |
|--------------------|----------------|--|---|--|------------------|-------|
| ENSECAG00000008177 | <i>PRXL2B</i> | peroxiredoxin like 2B | Antioxydant activity Prostaglandin-F synthase activity | Prostaglandin biosynthetic process | -2.15 | 0.006 |
| ENSECAG00000012493 | <i>SLC47A1</i> | solute carrier family 47 member 1 | Amide transmembrane transporter activity L-amino acid transmembrane transporter activity | Amino acid import across plasma membrane L-arginine import across plasma membrane Organic cation transport | -1.47 | 0.001 |
| ENSECAG00000018727 | <i>EFCAB11</i> | EF-hand calcium binding domain 11 | Calcium ion binding | | 0.51 | 0.046 |
| ENSECAG00000020415 | <i>FAM162A</i> | family with sequence similarity 162 member A | | Cellular response to hypoxia Positive regulation of apoptotic process Positive regulation of release of cytochrome c from mitochondria | 0.65 | 0.041 |
| ENSECAG00000012554 | <i>GTPBP8</i> | GTP binding protein 8 (putative) | GTP binding Metal ion binding | | 0.93 | 0.044 |

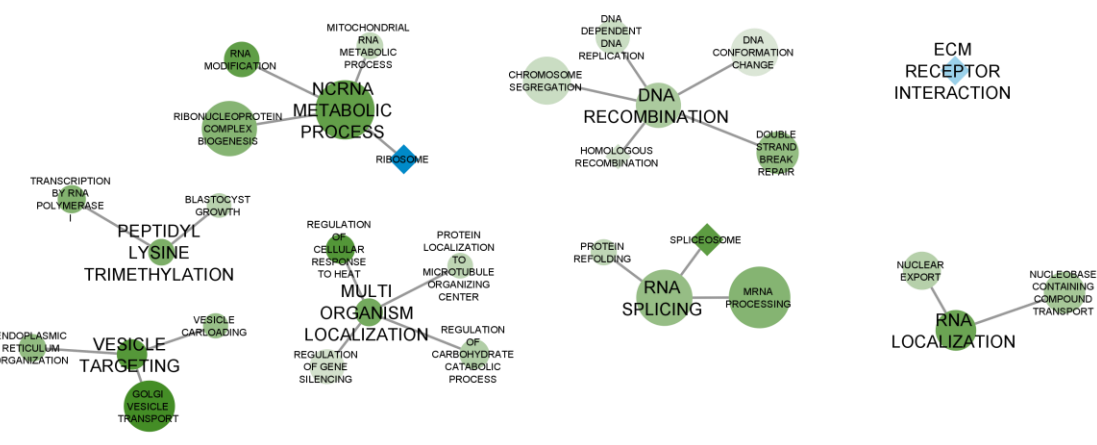
1

2 Log2 Fold-Change<0 indicates down-regulation of the gene in embryos from nulliparous mares, also indicated in green; Log2 Fold-Change>0 indicates up-regulation of the
 3 gene in embryos from nulliparous mares, also indicated in blue.





Inner cell mass



Trophoblast

