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### ► To cite this version:

Emilie Derisoud, Luc Jouneau, Clothilde Gourtay, Anne Margat, Catherine Archilla, et al.. Maternal parity affects Day 8 embryo gene expression in old mares. 2021. hal-04446577

**HAL Id: hal-04446577**

**<https://hal.inrae.fr/hal-04446577v1>**

Preprint submitted on 29 Mar 2024

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# 1                    **Maternal parity affects Day 8 embryo gene expression in old mares**

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## 14                    **Grant support:**

15                    This work was supported by the "Institut Français du Cheval et de l'Équitation" (grant numbers  
16                    CS\_2018\_23, 2018). The National Research Institute for Agriculture, Food and Environment (INRAE)  
17                    department Animal Physiology and Breeding Systems also supported this research.

18

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32 **Running title:**

33 Embryo gene expression in old nulliparous mare

34

35 **Summary sentence:**

36 Mare's parity in old mares impacts the expression of genes related to development and molecule  
37 exchanges in ICM and TE of blastocysts suggesting an adaptation to an altered environment.

38

39 **Keywords:**

40 Blastocyst; RNA sequencing; horse; equine

## 41 **Abstract**

42 As sport career is a priority in most of equine breeds, mares are frequently bred for the first time at an  
43 advanced age. Both age and first gestation were shown to have a deleterious effect on reproduction  
44 outcomes, respectively on fertility and offspring weight but the effect mare's parity in older mares on  
45 embryo quality has never been considered. The aim of this project was to determine the effect of old  
46 mare's nulliparity on gene expression in embryos. Day 8 post ovulation embryos were collected from  
47 old (10-16 years old) nulliparous (ON, N=5) or multiparous (OM, N=6) non-nursing Saddlebred mares,  
48 inseminated with the semen of one stallion. Pure (TE\_part) or inner cell mass enriched (ICMandTE)  
49 trophoblast were obtained by embryo bisection and paired end, non-oriented RNA sequencing  
50 (Illumina, NextSeq500) was performed on each hemi-embryo. To discriminate gene expression in the  
51 ICM from that in the TE, deconvolution (DeMixT R package) was used on the ICMandTE dataset.  
52 Differential expression was analyzed (DESeq2) with embryo sex and diameter as cofactors using a false  
53 discovery rate <0.05 cutoff. Although the expression of only a few genes was altered by mare's  
54 nulliparity (33 in ICM and 23 in TE), those genes were related to nutrient exchanges and responses to  
55 environment signaling, both in ICM and TE, suggesting that the developing environment from these  
56 mares are not optimal for embryo growth. In conclusion, being nulliparous and old does not seem to  
57 be the perfect match for embryonic development in mares.

## 58 **Introduction**

59 In the equine industry, mares are bred until an advanced age for economic and sentimental reasons.  
60 Depending on the breed, mares older than 10 years old represent between 37 and 63% of the  
61 broodmares [1–3]. In addition, 4% of Thoroughbred mares in the UK are older than 18 years old at the  
62 time of covering [1].

63 Several reproductive parameters are affected by age and mares older than 10 years old can be  
64 considered as already old for reproduction as their fertility has already started to progressively decline  
65 [for review 4]. Oocytes are particularly affected by maternal age with alterations of spindle stability  
66 [5–7], altered gene expression [8,9], and altered metabolism [8,10–12] being reported, all suggesting  
67 that oocyte developmental potential is reduced in old mares. The resulting embryos were smaller at  
68 the same developmental age in most studies [13–23] with altered gene expression [23] and  
69 metabolism [11,12], suggesting impaired development that has mainly been related to the oocyte  
70 quality. The reproductive tract, however, is also affected by maternal age. Indeed, more oviductal  
71 masses [24], uterine morphological degenerations such as cysts [25–30] and fibrosis [31–33] as well as  
72 more endometritis [19,25,34,35] are observed in old mares.

73 In most of farm animals, female parity, defined as the number of pregnancies that reached a viable  
74 gestational age (stillbirth and live birth included), is highly correlated with age as to remain profitable  
75 and stay in the farm, females must produce offspring regularly. In horses, however, the sport career is  
76 prioritized and depending on discipline and breed, can last up to 15 years or even more, as in  
77 warmblood dressage and show jumping. In Finn horse and Standardbred, nulliparous mares  
78 represented, respectively, 20.5% and 15.5% of mares that were bred in Finland [2]. To the authors'  
79 knowledge, the effect of nulliparity/primiparity on oocyte and embryo quality as well as fetal  
80 development has not been explored. In any case, the reproductive tract is affected by mare parity.  
81 Indeed, the ventral position of the uterus [36] and the number of vascular degenerations in the  
82 endometrium [37] have been positively correlated with the number of foals. Furthermore, cervical

83 dilatation is poorer in nulliparous vs multiparous mares [38] and it was suggested that uterine  
84 clearance was impeded. Primiparity, however, does not affect the prevalence of endometritis [34].  
85 Both age and parity thus affect mare reproductive efficiency but the cumulative effect of nulliparity  
86 and aging has not been explored.  
87 The aim of this study was to determine the effect of maternal nulliparity in old mares on embryo gene  
88 expression at the blastocyst stage. Old (>10 years) nulliparous and multiparous mares were  
89 inseminated with the same stallion semen. Blastocysts were collected and bisected to separate the  
90 pure trophoblast (TE\_part) from the inner cell mass enriched hemi-embryo (ICMandTE). Gene  
91 expression was analyzed by RNA-seq in each compartment.

92

## 93 **Materials and methods**

### 94 *Ethics*

95 The experiment was performed at the experimental farm of IFCE (“Institut Français du Cheval et de  
96 l’Equitation – La jumenterie du Pin” research agreement D61-157-2 valid until November 2023). The  
97 protocol was approved by the local animal care and use committee and by the regional ethical  
98 committee (“Comité d’Éthique Normand en Matière d’Expérimentation Animale”, approved under N°  
99 CEEA - 54 in the National Registry of French Ethical Committees for animal experimentation) under  
100 protocol number APAFIS#20857-2019051709319621 v3. All experiments were performed in  
101 accordance with the European Union Directive 2010/63EU.

102

### 103 *Embryo collection*

104 Twenty-five multiparous mares (mostly Selle Français breed with some French Anglo-Arabian,  
105 Standardbred and Saddlebred) aged from 10 to 16 years old were included in this study. Multiparous

106 mares were defined as dams that had already foaled at least once while nulliparous mares were  
107 defined as mares that have never foaled before the experiment. During the experimental protocol,  
108 mares were managed in two herds, independent of mare group, in natural pastures 24h/day with free  
109 access to water. The experiments took place from July 8<sup>th</sup> to August 13<sup>th</sup>, 2019. All mares remained  
110 healthy during this period.

111 Mares were allocated to one of 2 groups according to their parity: nulliparous (ON, n = 11) and  
112 multiparous mares (OM, n = 14). During the experimentation, mare's withers' height and weight were  
113 measured. Characteristics of all mares and mares that produced an embryo are detailed in Table 1.

114 The mares' estrous period was monitored routinely in the morning by ultrasound with a 50-60Hz trans-  
115 rectal transducer. During estrus, ovulation was induced with a single injection of human chorionic  
116 gonadotropin (i.v.; 1500IU; Chorulon® 5000; MSD Santé animale, France) as soon as one ovarian follicle  
117 > 35mm in diameter was observed, together with marked uterine edema. Ovulation usually takes place  
118 within 48h, with > 80% occurring 25 to 48h after injection [39,40]. At the same time and one day later,  
119 mares were inseminated once with fresh or refrigerated semen containing at least 1 billion motile  
120 spermatozoa from a single fertile stallion. Ovulation was checked every 12-24 hours by  
121 ultrasonography. If no embryo was recovered, the procedure could be repeated once more.

122 Embryos were collected by non-surgical uterine lavage using prewarmed (37°C) lactated Ringer's  
123 solution (B.Braun, France) and EZ-Way Filter (IMV Technologies, France) on the morning, 8 days post  
124 ovulation. At Day 14 post ovulation, a pregnancy diagnosis was performed for each mare and they  
125 were treated with luproliol, an analogue of prostaglandin F2 $\alpha$  (i.m; 7.5 mg; Prosolvin, Virbac, France).

126 When an embryo was collected, a blood sampling was performed at the same time on heparin tube.  
127 Plasma was recovered after centrifugation (3500 rpm, 10min at 4°C). Progesterone was measured in  
128 plasma using ELISA assay as previously described [41,42].

129

130

131 *Embryo bisection and RNA extraction*

132 Using a binocular magnifying glass, collected embryos were immediately photographed with a size  
133 standard to subsequently determine embryo diameter using ImageJ® software (version 1.52a; National  
134 Institutes of Health, Bethesda, MD, USA). Then embryos were washed 4 times in commercially  
135 available Embryo holding medium (IMV Technologies, France) at 34°C and bisected with a microscalpel  
136 under binocular magnifying glass to obtain a trophoblast (TE\_part) and an inner cell mass enriched  
137 (ICMandTE) hemi-embryo. At this stage, the TE\_part is composed of trophectoderm and endoderm  
138 whereas the ICM is composed of epiblast cells. Directly after bisection, RNA extraction of each hemi-  
139 embryo was started in extraction buffer (PicoPure RNA isolation kit, Applied Biosystems, France) for  
140 30 min at 42°C prior to storage at - 80°C. RNA was extracted later on from each hemi-embryo using  
141 PicoPure RNA isolation kit (PicoPure RNA isolation kit, Applied Biosystems, France), which included a  
142 DNase treatment, following the manufacturer's instructions. RNA quality and quantity were assessed  
143 with the 2100 Bioanalyzer system using RNA 6000 Pico kit (Agilent Technologies, France) according to  
144 the manufacturer's instructions.

145

146 *RNA sequencing*

147 Five nanograms of total RNA were mixed with ERCC spike-in mix (ThermoFisher Scientific, France)  
148 according to manufacturer's recommendations. Messenger RNAs were reverse transcribed and  
149 amplified using the SMART-Seq V4 ultra low input RNA kit (Clontech, France) according to the  
150 manufacturer recommendations. Nine PCR cycles were performed for each hemi-embryo. cDNA  
151 quality was assessed on an Agilent Bioanalyzer 2100, using an Agilent High Sensitivity DNA Kit (Agilent  
152 Technologies, France). Libraries were prepared from 0.15 ng cDNA using the Nextera XT Illumina library  
153 preparation kit (Illumina, France). They were pooled in equimolar proportions and sequenced (Paired



154 end 50-34 pb) on NextSeq500 instrument, using a NextSeq 500 High Output 75 cycles kit (Illumina,  
155 France). Demultiplexing was performed with bcl2fastq2 version 2.2.18.12 (Illumina, France) and  
156 adapters were trimmed with Cutadapt version 1.15 [43]. Only reads longer than 10pb were kept.

157

### 158 *RNA mapping and counting*

159 As previously described [23], alignment was performed using STAR version 2.6 [44] on previously  
160 modified Ensembl 99 EquCab3.0 assembly and annotation. Genes were then counted with  
161 FeatureCounts [45] from Subreads package version 1.6.1.

162

### 163 *Availability of data and materials*

164 The RNA sequencing data supporting the conclusions of this article are available in the GEO  
165 repository, [accession: GSE188866;  
166 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE188866>].

167

168

### 169 *Data analysis*

170 All statistical analyses were performed by comparing ON to OM (set as reference group) using R version  
171 4.0.2 [46] on Rstudio software version 1.3.1056 [47].

172 Embryo were sexed using *X Inactive Specific Transcript (XIST)* expression as previously described [23].  
173 Seven embryos were determined as females (4 in the ON group and 3 in the OM group) while 4 were  
174 considered as males (1 in the ON group, and 3 in the OM group).

175

### 176 *Embryo recovery and fertility rate, embryo diameter and total RNA content analysis*

177 Embryo recovery rates (ERR) were calculated as the number of attempts with at least one embryo  
178 collected/total number of attempts. Furthermore, fertility was calculated as the sum of embryo  
179 collections with at least one embryo and the number of positive pregnancy checks at Day 11 after a  
180 negative embryo collection on the total number of attempts. Both were analyzed using the Exact Fisher  
181 test to determine if maternal parity in old mares influenced embryo recovery and the probability of  
182 leaving an embryo in the uterus after uterine flush.

183 For total RNA content analyses, as embryos were bisected without strict equality for each hemi-  
184 embryo, a separate analysis of ICMandTE and TE\_part RNA quantities would not have been  
185 meaningful. Thus, ICMandTE and TE\_part RNA quantities were summed up. With embryo diameter,  
186 both variables were analyzed using a linear model of nlme package version 3.1-148 [48] including  
187 maternal age and embryo sex, followed by 1000 permutations using PermTest function from pgirmess  
188 package version 1.6.9 [49]. Variables were kept in models when statistically significant differences  
189 were observed. Differences were considered as significant for  $p < 0.05$ .

190

### 191 ***Deconvolution of gene expression in ICMandTE using DeMixT***

192 The deconvolution method has already been described in equine embryos [23]. Briefly, this method  
193 enables the estimation of the relative gene expression of TE and ICM cell types within the hemi-embryo  
194 ICMandTE which is composed of both trophoblast and inner cell mass in unknown relative proportions.  
195 After filtering out all genes with at least 3 null count values in at least one group (ON or OM) per hemi-  
196 embryo (ICMandTE or TE\_part), removing genes with a null variance in TE\_part and adding the value  
197 “1” to all count values in ICMandTE and TE\_part datasets, deconvolution was performed using the  
198 DeMixT R package version 1.4.0 [50,51]. Output datasets were DeMixT\_ICM\_cells and  
199 DeMixT\_TE\_cells, corresponding to the deconvoluted gene expression in ICM cells and TE cells of  
200 ICMandTE, respectively.

201 At the end of deconvolution, a quality check was automatically performed by the DeMixT R package  
202 with the TE\_part used as reference for DeMixT\_TE\_cells. Genes were automatically filtered out if the  
203 difference between average deconvoluted expression of reference cells in mixed samples and average  
204 expression of reference cells > 4.

205 Outputs of DeMixT\_ICM\_cells vs DeMixT\_TE\_cells, DeMixT\_ICM\_cells vs TE\_part and ICMandTE vs  
206 TE\_part were compared with Deseq2 version 1.28.1 [52] to confirm that the deconvolution was  
207 effective at separating gene expression. To check if deconvolution was efficient, as previously  
208 described [23], the expression of several genes proper to ICM and TE cells in equine embryos identified  
209 using literature search [53] was compared before and after deconvolution. Results of these analyses  
210 were represented through manually drawn Venn diagrams as well as principal component analysis  
211 graphics of individuals, using ggplot2 version 3.3.3 [54] and factoextra version 1.0.7 [55].

212

### 213 ***Maternal parity comparison for gene expression***

214 All genes with an average expression <10 counts in both ON and OM per hemi-embryo (ICM or TE)  
215 were filtered out on the DeMixT\_ICM\_cells and TE\_part datasets. Differential analyses were  
216 performed with Deseq2 version 1.28.1 [52] with the OM group as reference, without independent  
217 filtering and taking into account embryo diameter and sex in the model. Genes were considered  
218 differentially expressed (DEG) for FDR < 0.05 after Benjamini-Hochberg correction (also known as false  
219 discovery rate, FDR).

220 Equine Ensembl IDs were converted into Human Ensembl IDs and Entrez Gene names using gorth  
221 function in gprofiler2 package version 0.1.9 [56]. Genes without Entrez Gene names using gprofiler2  
222 were manually converted when Entrez Gene names were available, using Ensembl web search function  
223 [57]. GO molecular function and GO Biological process annotations were obtained from Uniprot  
224 website.

225

## 226 ***Gene set enrichment analyses (GSEA)***

227 After log transformation using RLOG function of DESeq2 version 1.28.1, gene set enrichment analyses  
228 (GSEA) were performed on expressed genes using GSEA software version 4.0.3 (Broad Institute, Inc.,  
229 Massachusetts Institute of Technology, and Regents of the University of California) [58,59] to identify  
230 biological gene sets disturbed by maternal parity. Molecular Signatures Databases [60] version 7.1 (C2:  
231 KEGG: Kyoto Encyclopedia of Genes and Genomes; REACTOME, C5: BP: GO biological process) were  
232 used to identify most perturbed pathways. Pathways were considered significantly enriched for  
233  $FDR < 0.05$ . When the normalized enrichment score (NES) was positive, the gene set was enriched in  
234 the ON group while when NES was negative, the gene set was enriched in the OM group.

235

## 236 **Results**

### 237 *Embryo recovery rates, diameter, total RNA content and quality and progesterone* 238 *concentrations*

239 Altogether, 32 embryo collections were performed (14 in ON and 18 in OM, 8 mares being flushed  
240 twice) and 15 embryos were obtained (6 from 5 ON mares and 9 from 8 OM mares). Two mares (one  
241 in each parity group) produced twin embryos.

242 Positive embryo collection rate was 36% and 44% in ON and OM, respectively and did not differ  
243 between groups ( $p = 0.72$ ). The embryo recovery rate was 43% and 50% in ON and OM, respectively  
244 and did not differ between groups ( $p = 0.30$ ). At the Day 14 pregnancy check, embryos were found in  
245 3 OM mares (from 2 to 4 foalings) and none was found in the ON group. Fertility, calculated combining  
246 positive embryo collections and Day 14 pregnancy diagnosis, was 36% and 61% in ON and OM,  
247 respectively, and did not differ between groups ( $p = 0.29$ ).

248 Altogether, 7 and 11 double ovulations were observed, respectively, in ON and OM. The embryo  
249 recovery rate per ovulation at the time of embryo collection and after Day 14 pregnancy check were  
250 not different according to group (respectively, 29% and 29% in ON and 31% and 41% in OM,  $p = 1$  and  
251  $p = 0.39$ ).

252 All embryos were expanded blastocysts grade I or II according to the embryo classification of McKinnon  
253 and Squires [61]. For each twin collection, one embryo was large and the other was small (766 $\mu$ m and  
254 295 $\mu$ m; 829 $\mu$ m and 481 $\mu$ m). For both, as only one embryo per mare was required, only the largest  
255 embryo of the twins was chosen for further analysis. Altogether, all ON embryos but only 6 OM  
256 embryos out of 8 collected were RNA sequenced. The smallest OM embryo (480 $\mu$ m) and another one  
257 randomly chosen (907 $\mu$ m) of diameter were not sequenced.

258 In embryos selected for RNA sequencing, embryo diameter ranged from 562  $\mu$ m to 1426  $\mu$ m, with no  
259 effect of group on embryo diameter ( $p = 0.18$ ). Female embryos, however, were significantly smaller  
260 than male embryos (in average  $764 \pm 223$   $\mu$ m and  $1046 \pm 287$   $\mu$ m,  $p < 0.05$ ) without interaction  
261 between maternal parity and embryo sex. RNA yield per embryo ranged from 25.2 ng to 624 ng and  
262 was not related to parity ( $p = 0.43$ ) nor embryo sex ( $p = 0.08$ ).

263 The median RNA Integrity Number (RIN) was 9.7 (8.9 - 10 range). Between 34.6 and 54.1 million reads  
264 per sample were obtained after trimming. On average, 74.10% of the reads were mapped on the  
265 modified EquCab 3.0 using STAR and 67.07% were assigned to genes by featureCounts.

266 Except one old multiparous mare that had a progesterone plasma concentration of 3.9 ng/ml,  
267 progesterone concentrations in plasma were  $> 4$  ng/ml for all mares (range from 8.3 to 25.6ng/ml with  
268 an average of 13.7ng/ml) and were not affected by mares' parity ( $p = 0.66$ ).

269

270 *Deconvolution of gene expression to discriminate ICM and TE gene expression in ICMandTE*

271 *hemi-embryos*

272 After selecting genes with less than 3 non null count values in at least one group (ON or OM) per hemi-  
273 embryo (ICMandTE or TE\_part), 16,803 genes were conserved for deconvolution. In addition, nine

274 genes were removed because their variance was null in the TE\_part, as DeMixT does not allow the use  
275 of genes with a null variance in the pure sample. For these genes, the mean count in ICMandTE samples  
276 was lower or equal to 10 counts. One further gene was removed during the deconvolution because  
277 the deconvolution quality for this gene was not sufficient. Therefore, at the end of the deconvolution  
278 algorithm, 16,793 genes were available for differential analysis.

279 Before deconvolution, 303 genes were differentially expressed (FDR < 0.05) between the ICMandTE  
280 and the TE\_part (Figure 1A). After deconvolution, the comparison between DeMixT\_ICM\_cells and  
281 DeMixT\_TE\_cells yielded 7,116 differentially expressed genes while the comparison DeMixT\_ICM\_cells  
282 vs TE\_part yielded 5,615 differentially expressed genes, with 5,103 in common (74%). Moreover, all  
283 but one of the initially 303 differentially expressed genes before deconvolution were also identified as  
284 differentially expressed in both post-deconvolution analyses. On the PCA graph of individuals, Axis 1  
285 (21.8% of variance) separated well groups according to data origin. ICMandTE and TE\_part were  
286 separated on axis 1 but very close before deconvolution (Figure 1B). DeMixT\_TE\_cells and TE\_part  
287 were partly superposed, indicating that datasets before and after deconvolution have a similar global  
288 gene expression; whereas the DeMixT\_ICM\_cells group is clearly separated from both, indicating that  
289 the deconvolution effectively enabled the separation of gene expression in the two cell types.

290 Only 5 of the 12 genes previously reported as more expressed in the ICM [53] were also identified  
291 more expressed in the ICMandTE vs TE\_part comparison (Table 2). After deconvolution (comparison  
292 DeMixT\_ICM\_cells vs TE\_part), 10 out of 12 of these genes were observed differentially expressed with  
293 9 effectively more expressed in the ICM. The expression of *Undifferentiated Embryonic Cell*  
294 *Transcription Factor 1, UTF1*, however, was identified decreased in the DeMixT\_ICM\_cells, in contrast  
295 to the only published report [53]. In the TE, no gene previously identified was observed differentially  
296 expressed in the comparison ICMandTE vs TE\_part, *i.e.*, before deconvolution. After deconvolution,  
297 the expression of 3 of the 7 reported genes were found increased in TE\_part compared to  
298 DeMixT\_ICM\_cells.

299 These results indicated that a better qualification of genes expressed by ICM cells was enable by the  
300 deconvolution. Thus, for further analyses, TE\_part and DeMixT\_ICM\_cells datasets have been studied.  
301

### 302 *Differential gene expression in deconvoluted ICM cells*

303 After the filtering out of genes without an average expression  $\geq 10$  counts in at least one maternal age  
304 group/hemi-embryo, 13,910 genes were considered as expressed in the ICM cells from ON or OM  
305 embryos. Only 33 genes were differentially expressed (23 downregulated and 10 upregulated in ON)  
306 (Figure 2 and Additional file 1). Respectively, 20 and 5 genes out of the down- and upregulated genes  
307 were associated to a protein known and described in human. These 25 genes are presented in Table  
308 3. Down regulated genes in the ICM of old nulliparous mares were involved in RNA processing and  
309 transcription, immunity, nervous system development, lipid/protein transport, lipid metabolism,  
310 chromatin remodeling, DNA repair, cell cycle, signaling and adhesion whereas up-regulated genes  
311 were related to different biological processes such as ion transport, regulation of transcription and  
312 lipid/protein/carbohydrate catabolism.

313

### 314 *Differential gene expression in the TE part*

315 In the TE, 13,322 genes were considered as expressed in OM or ON. Twenty-three were differentially  
316 expressed (Additional file 2) with 16 genes being downregulated and 7 being up regulated in ON  
317 (Figure 2). Respectively, 14 and 6 out of the down- and upregulated genes were associated to a known  
318 protein in human. Moreover, despite the filtering, 2 down-regulated genes in ON (LIM and cysteine  
319 rich domains 1, *LMCD1*; lysophosphatidic acid receptor 4, *LPAR4*) were only expressed in one embryo  
320 and were not considered for further analysis. The remaining 19 genes are presented in Table 4. Down-  
321 regulated genes in the TE of old nulliparous mares were mainly involved in spindle organization,  
322 chromatin remodeling and cellular process, transcription and DNA repair, cell polarity, adhesions,  
323 junctions and signaling, extracellular matrix organization, ion homeostasis, glycerol metabolism,

324 glycolysis, immunity, gastrulation and placenta development while up-regulated genes were related  
325 to ion binding, cell death, lipid metabolism, protein maturation and membrane organization

326

### 327 *Gene set enrichment analysis in deconvoluted ICM cells*

328 After Entrez Gene ID conversion, 12,287 genes were considered expressed in ICM cells. Only one GO  
329 Biological Process and one KEGG pathways were disturbed by maternal parity in ICM cells (Additional  
330 file 3 & Table 5). The GO BP “Cytoplasmic microtubule organization” was enriched in the ICM cells from  
331 OM embryos (NES = -2.17). The KEGG pathway “Neuroactive ligand receptor interaction” was enriched  
332 in ICM cells from ON embryos (NES = 1.98). Detailed examination indicated that genes involved in this  
333 enrichment were related to biological regulation, signaling and response.

334

### 335 *Gene set enrichment analysis in TE*

336 After Entrez Gene ID conversion, 11,993 genes were considered expressed in TE from ON or OM  
337 embryos. All the 2, 2 and 8 perturbed gene sets from GO BP, KEGG and REACTOME, respectively, were  
338 enriched in the TE of embryos from OM mares (NES < -1.8; Additional file 4 & Table 5). In the TE, several  
339 gene sets were related to extracellular matrix organization and function. Others were involved in  
340 NOTCH signaling pathway. The last two pathways were involved in histidine metabolism and  
341 cholesterol transport.

342

## 343 **Discussion**

344 Maternal parity in mares older than 10 years old did not influence embryo recovery rates. This rate,  
345 however, seems lower in old nulliparous compared to old multiparous mares. The effect of mare  
346 nulliparity on the fertility in mares older than 10 years old have never been explored.

347 For 3 mares, a developing embryo was observed at 14 days post ovulation, demonstrating that  
348 embryos were not recovered with the flushing. This happened only in multiparous mares and increased



349 occurrence in multiparous mares has not been described to the author's knowledge yet. Uterine size  
350 as well as histological and morphological degenerations in uterus could explain this result. After  
351 foaling, uterine size decreases quickly during the first week post-partum but the complete involution  
352 of both uterine horns seems to end only after twenty to thirty days [62,63] whereas histology of the  
353 uterine body endometrium returns to normal by 7 to 10 days post-partum [31] . Ageing and the  
354 multiplication of foalings, with repetition of uterine extensions and involutions may affect the uterus's  
355 ability to involute. Indeed, clinically the authors have observed that a larger volume of fluid is required  
356 to flush the uterus of multiparous mares compared to young mares. Histologically, however, parity is  
357 correlated with the presence of elastosis in the myometrial vessels [37]. These alterations may be  
358 associated to alterations of uterine contractility and decrease fluid clearance in multiparous mares,  
359 both factors that could affect embryo recovery.

360 In both ICM and TE, only a few genes were affected by maternal parity. Some downregulated genes in  
361 embryos collected from ON mares were involved in common functions between ICM and TE such as  
362 regulation of transcription, cell cycle and development, cell organization and immunity. Up-regulated  
363 genes in the ICM of ON embryos were related to ion transport, regulation of transcription and  
364 catabolism of lipids, proteins and carbohydrates. In the TE, downregulated genes in ON embryos were  
365 related to glycerol metabolism, glycolysis and directly involved in gastrulation and placental formation  
366 while upregulated genes were related to cell death, lipid metabolism, protein maturation, membrane  
367 organization and as in ICM, ion binding.

368 In the GSEA analysis, only two gene sets were perturbed in the ICM in relation to maternal parity in  
369 old mares with one related to microtubule organization being enriched in ON and the other one being  
370 enriched in OM embryos and related membrane receptors. No gene set was enriched in the TE of ON  
371 mares but enriched pathways in OM embryos were mainly related to extracellular matrix organization  
372 and cell differentiation, mainly related to NOCTH signaling pathway.

373 Of particular interest, the gene SLX4 Structure-Specific Endonuclease Subunit (*SLX4*), the regulatory  
374 subunit of structure-specific endonucleases that are required for repair of DNA lesions, is down-

375 regulated in the ICM of embryos from ON mares. In TE, moreover, part of the post-replicative DNA  
376 mismatch repair system, MutS Homolog 3 (*MSH3*) is downregulated in embryos from ON mares. These  
377 results indicate that DNA repair systems in both ICM and TE are affected by maternal parity.  
378 Interestingly, the gene Gamma-aminobutyric acid type A receptor subunit rho1, *GABRR1*, was not  
379 expressed in embryos collected from multiparous mares while in nulliparous mares, 4/5 embryos  
380 expressed this gene in the ICM. *GABRR1* encodes for a Cl<sup>-</sup> channel receptor involved in the gamma-  
381 aminobutyric acid (GABA) pathway. Work on mouse embryonic and peripheral neural crest stem cells  
382 have shown that GABA receptors negatively affect preimplantation embryonic growth by negatively  
383 controlling cell proliferation, being involved in DNA damage checkpoint and by increasing cellular  
384 arrest in the S phase [64]. Alterations in the embryo environment because of mare parity could  
385 therefore modify DNA lesion repair and therefore, cell proliferation, suggesting that embryo growth is  
386 reduced in nulliparous mares.

387 Several gene sets related to NOTCH signaling pathways were also enriched in the TE of OM embryos  
388 and Notch Receptor 1 (*NOTCH1*) always contributed to those enrichments. NOTCH signaling pathway  
389 is essential for proper development, with *NOTCH1* being required for cell proliferation in early bovine  
390 embryos [65]. The enrichment of this pathway in embryos from multiparous mares therefore suggests  
391 that cell proliferation is slowed down in embryos from nulliparous mares

392 The expression of SET domain containing 5 (*SETD5*) signal transducer and activator of transcription 5A  
393 (*STAT5A*), PCF11 cleavage and polyadenylation factor subunit (*PCF11*), ribosomal protein L36 (*RPL36*),  
394 exosome component 7 (*EXOSC7*), H2A clustered histone 20 (*H2AC20*) were downregulated in the ICM  
395 and H4 clustered histone 3 (*H4C3*) were downregulated in the TE of embryos collected from nulliparous  
396 mares. These genes are all involved in transcription and/or translation, suggesting that the expression  
397 of genes in embryos from nulliparous mares was altered. Of particular interest, *SETD5* et *STAT5A* are  
398 known to be, respectively, key regulators of methylation and signaling via cytokines, both gene  
399 expressions being essential for embryo development [77,78].

400 In the TE, the gene encoding for eomesodermin (*EOMES*) is downregulated in embryos collected from  
401 ON mares. This gene controls the formation of germ cell layers and is involved in the differentiation of  
402 the trophoblast in the mouse [66] while in human, cattle and pigs, *EOMES* is not expressed in the  
403 preimplantation embryos [67–69]. In horses, *EOMES* is suggested to also be a marker of induced  
404 trophoblast cells but its role has never been explored [70]. If its role is similar as in mouse, this down-  
405 regulation could lead to a reduced differentiation of cells in the trophoblast, that could impair its  
406 principal function, i.e., the regulation of exchanges with the maternal environment.

407 As the external part of the embryo is exclusively composed of trophoblast in the mare at the studied  
408 developmental stage, poorer maternal-embryo exchanges through the trophoblast in nulliparous  
409 mares could explain the defects observed in both compartments. Indeed, the reduced gene expression  
410 of TRIO and F-Actin Binding Protein (*TRIOBP*) in the ICM as well as actin beta (*ACTB*) and microtubule  
411 associated protein 1B (*MAP1B*) in the TE of equine embryos seems to fit the hypothesis that molecule  
412 transfer is altered in embryos from ON mares. In polarized epithelial cells, such as the trophoblast,  
413 cytoskeleton is essential for the communication with the extracellular environment (for review [71]).  
414 The protein encoded by *ACTB* is a direct component of the cytoskeleton and the one encoded by  
415 *MAP1B* is a molecule responsible for the stabilization of microtubules [72]. *TRIOBP* regulates actin  
416 cytoskeletal organization and the formation of a Tara and TRIO complex coordinates actin remodeling  
417 which is essential for exchanges [73]. In addition, the extracellular matrix (ECM) is very important for  
418 embryo development and embryo-maternal exchanges (for a review [74]). ECM relative gene sets  
419 appeared to be altered by maternal parity in old mares with several gene sets enriched in the TE of  
420 embryos from ON mares. The expression of the ADAM metallopeptidase domain 19 gene (*ADAM19*),  
421 moreover, was reduced in the TE of ON embryos. This gene encodes for a transmembrane glycoprotein  
422 that is essential for tight junction formation. Tight junctions formation and integrity are essential for  
423 blastocyst development in mouse and pigs [75]. The reduction of the expression of *ADAM19* in the TE  
424 of embryos from old nulliparous mares, could therefore support the hypothesis that embryo integrity  
425 is altered, leading to alteration of ion and nutrient exchanges in both TE and ICM. Alterations of

426 cytoskeleton, ECM and integrity of the TE in ON embryos, probably related to adaptation to the embryo  
427 environment, could affect embryo-maternal exchanges and consequently embryonic development.  
428 The lipid metabolism is particularly important for mammalian embryo development as it is an  
429 important source of energy for growth (for review [79]). In horses, the embryo is particularly reliant  
430 on its environment as it develops free inside the mare's uterus until around 35 days post ovulation,  
431 when it finally starts to implant. Several genes related to lipid metabolism and transport were affected  
432 in the ICM by maternal parity. Among them, carnitine palmitoyltransferase 2 (*CPT2*) is downregulated  
433 in the ICM of ON embryos. The protein encoded by this gene catalyzes the oxidation of fatty acids in  
434 the mitochondria. In mouse embryos, *CTP2* transcription is essential and could be used as a marker for  
435 future implantation [80]. Therefore, the reduced expression of this gene could indicate that embryo  
436 development is reduced because of reduced lipid oxidation in old nulliparous mares. This could be the  
437 result or at least related to reduced lipolysis via lipase or a defect in the transport of lipids. Both  
438 functions are altered in the ICM of ON embryos. Indeed, lipase E (*LIPE*), also known as hormone-  
439 sensitive lipase, and apolipoprotein B (*APOB*), involved in the transport of lipids, are downregulated in  
440 the ICM of ON embryos. *APOB* has been identified as very important for equine embryo development  
441 as its expression is increased by a factor of 200 between day 8 and day 14 [81]. Furthermore, the  
442 production of the protein apolipoprotein B by the endoderm is required for the development of  
443 preimplantation mouse embryos [82]. In the equine species, *APOB* expression is dysregulated in Day 8  
444 embryos when mares do not produce enough progesterone [83]. It has been observed that the risk of  
445 embryo mortality is increased in mares producing less than 4 ng/ml of progesterone through the post  
446 ovulation period [84]. Progesterone, indeed, is mandatory for embryo development by modulating its  
447 environment to ensure pregnancy [85]. Reduced expression of *APOB* suggests that the expression of  
448 this gene is particularly dependent on the embryo environment. Here, except one, close to the cut-off  
449 value, all mares produced sufficient progesterone for normal gestation at 8 days post ovulation and  
450 there was no difference in progesterone concentration according to mare parity. It could be

451 hypothesized, however, that the environment in the uterus of old nulliparous mares could lead to the  
452 difference of expression of this gene, independently of progesterone production.

453

## 454 **Conclusion**

455 Mare's parity, especially in mares older than 10 years old, has never been considered for the study of  
456 embryos. Here, however, it has been shown that mare's parity in old mares affects the expression of  
457 genes in ICM and TE of blastocysts. Although only the expression of a few genes is altered by mare's  
458 parity, some of these genes are particularly important for embryo growth and development. Genes  
459 related to nutrient exchanges and responses to environment signaling in both ICM and TE are  
460 particularly affected by the nulliparity of mares, suggesting that the developing environment from  
461 these mares are not optimal for embryonic growth. It is not possible to conclude, however, if  
462 differences in oocyte quality also play a role in those observations. More work on the oocyte, oviductal  
463 and uterine environment are needed to elucidate the exact mechanism leading to the present  
464 observations in Day 8 embryos.

465 In the present experiment, the destruction of the embryos made it impossible to predict their  
466 individual chance of implantation. The observed alterations suggest that implantation defects may be  
467 present in the embryos of old nulliparous mares. It is often assumed that nulliparous mares have a  
468 better uterine environment, more favorable to the development and implantation of an embryo than  
469 multiparous mares. In this study, however, being nulliparous and old does not seem to be the perfect  
470 match for embryonic development. If embryos succeed to implant, the apparent lower quality of  
471 embryos in nulliparous mares may accentuate differences in placentation and development observed  
472 in nulliparous mares, exacerbating the observed phenotype of smaller and lighter foals at birth.

473

## 474 **Acknowledgments**

475 The authors are grateful to the staff of the Institut Français du Cheval et de l'Équitation (IFCE)  
476 experimental farm (Jumenterie du Haras du Pin, Exmes, France) for care and management of animals.  
477 We acknowledge the high-throughput sequencing facility of I2BC for its sequencing and bioinformatics  
478 expertise. The bioinformatics analyses were performed thanks to Core Cluster of the Institut Français  
479 de Bioinformatique (IFB) (ANR-11-INBS-0013). The authors thank A.L. Lainé and the staff of the  
480 Phenotyping-Endocrinology laboratory for the hormonal assays. Many thanks to Matthias Zytnicki and  
481 Christophe Klopp for their advice on RNA-seq de novo analysis. Many thanks to Pablo Ross who kindly  
482 provided the coordinates for the XIST gene.

483

## 484 **Conflict of interest**

485 The authors declare that they have no conflict of interests.

486

## 487 **Author contributions**

488 PCP obtained the funding. PCP and VD conceived the project. FDG, LB, VD and PCP supervised the  
489 study. ED, CG, AM, CA, ND, NP, FDG, VD and PCP adapted the methodology for the project. ED, CG,  
490 AM, CA and YJ performed the experiments. AM, CG, CA, ND, NP, MD, LB and FDG provided the  
491 resources. ED, LJ, YJ and RL performed data curation. ED and LJ analyzed the data. ED wrote the original  
492 draft. All authors read, revised, and approved the submitted manuscript.

493

494

495

496 **List of abbreviations**

497 DEG: differential expressed genes

498 DeMixT\_ICM\_cells: deconvoluted gene expression in ICM cells

499 DeMixT\_TE\_cells: deconvoluted gene expression in TE cells

500 ECM: Extracellular matrix

501 ERR: embryo collection rate

502 FDR: false discovery rate

503 GO BP: Gene Ontology biological process

504 GO: Gene Ontology

505 GSEA: gene set enrichment analyses

506 ICM: inner cell mass

507 ICMandTE: inner cell mass enriched hemi-embryo

508 ICSI: intracytoplasmic sperm injection

509 KEGG: Kyoto Encyclopedia of Genes and Genomes

510 Log2FC: log2 fold change

511 NES: normalized enrichment score

512 OM: old multiparous mares

513 ON: old nulliparous mares

514 TE: trophoblast

515 TE\_part: pure trophoblast hemi-embryo

516 XIST: X inactive Specific Transcript

517



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765

## 766 Figure legends

767 Figure 1: Gene expression in ICM and TE before and after deconvolution using DeMixT

768 A) Venn diagrams of the differential gene expression in ICMandTE vs TE\_part (before deconvolution),  
769 DeMixT\_ICM\_cells vs DeMixT\_TE\_cells (after deconvolution) and DeMixT\_ICM\_cells vs TE\_part (gene  
770 expression of ICM after deconvolution vs gene expression in TE\_part without deconvolution); B)  
771 Principal Component Analysis of gene expression of DeMixT\_ICM\_cells, DeMixT\_TE\_cells, ICMandTE  
772 and TE part datasets.

773 Deconvolution was used to isolate gene expression of ICM and TE cells in ICMandTE hemi-embryos.  
774 ICMandTE: inner cell mass + trophoblast; TE\_part: pure trophoblast. Here trophoblast represents  
775 trophectoderm + endoderm.

776

777 Figure 2: Analysis of differentially expressed genes (DEG) in embryos according to maternal parity

778 A) representation of down- (blue) and upregulated (red) DEG in ICM (from DeMixT\_ICM\_cells data  
779 obtained after deconvolution of ICMandTE using DeMixT R package [50,51]) and TE (from TE\_part  
780 dataset) of embryos from ON vs OM.

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

783

784 Additional file 1:

785 Supp1.xlsx

786 Differential gene analysis using DeSeq2 in DeMixT\_ICM\_cells of equine embryo at Day 8 post-ovulation  
787 according to mares parity

788 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene  
789 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis  
790 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in ICM (after gene  
791 expression deconvolution of ICMandTE using DeMixT) of ON and OM embryos

792 ICM: Inner cell mass; ON: Old nulliparous mares; OM: old multiparous mares

793

794 Additional file 2:

795 Supp2.xlsx

796 Differential gene analysis using DeSeq2 in TE\_part of equine embryo at Day 8 post-ovulation according  
797 to mares parity

798 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene  
799 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis  
800 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in TE\_part of ON and OM  
801 embryos

802 ICM: Inner cell mass; ON: Old nulliparous mares; OM: old multiparous mares

803

804 Additional file 3:

805 Supp3.xlsx

806 Gene set enrichment analysis results on gene expression of DeMixT\_ICM\_cell of embryos from old  
807 nulliparous and multiparous mares

808 Gene Set Enrichment Analysis results (database, pathway name, size, enrichment score without and  
809 with normalization, p-value and FDR corrected q-value) for GO biological process, KEGG and  
810 REACTOME databases on genes expressed in ICM (after gene expression deconvolution of ICMandTE  
811 using DeMixT).

812 ICM: Inner cell mass

813

814 Additional file 4:

815 Supp4.xlsx

816 Gene set enrichment analysis results on gene expression of TE\_part of embryos from old nulliparous  
817 and multiparous mares

818 Gene Set Enrichment Analysis results (database, pathway name, size, enrichment score without and  
819 with normalization, p-value and FDR corrected q-value) for GO biological process, KEGG and  
820 REACTOME databases on genes expressed in TE\_part

821 TE: trophoblast



822 **Tables**

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

Characteristics	Nulliparous (ON)		Multiparous (OM)	
	All mares (n = 11)	Embryo donors (n = 5)	All mares (n = 14)	Embryo donors (n = 6)
Breed	SF n = 9; ST n = 1; SB n = 1	SF n = 4; ST n = 1	SF n = 7; AA n = 4; ST n = 2	SF n = 5; AA n = 1
Age (in years)	12.7 ± 2.0	13.6 ± 2.2	13.4 ± 2.1	13.5 ± 2.4
Parity (number of foalings)	0	0	1.7 ± 1.3	1.8 ± 1.6
Weight (in kg)	663.3 ± 49.1	644.8 ± 53.3	641.0 ± 76.1	658.0 ± 81.7
Withers' height (in cm)	168.2 ± 5.8	165.8 ± 7.6	163.6 ± 4.1	164.0 ± 5.1
Progesterone concentration (in ng/ml)	-	12.7 ± 5.8	-	14.5 ± 7.3

824 All mares were at least 10 years old.

825 AA: Anglo Arab or Anglo-Arabian type; SF: Selle Français section A or B; ST: Standardbred; SB:

826 Saddlebred. Age, parity, weight, and height are presented as mean ± SD

827

828 Table 2: Comparison of selected genes expression 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
<b>ICM</b>	<b>SOX2</b>	ENSECAG00000010653	3.82	0.069	5.08	1.02E-04
	<b>NANOG</b>	ENSECAG00000012614	3.01	0.017	4.26	2.50E-06
	<b>SPP1</b>	ENSECAG00000017191	4.02	8.39E-09	3.99	2.19E-05
	<b>LIN28B</b>	ENSECAG00000020994	2.75	0.045	3.64	5.14E-06
	<b>SMARCA2</b>	ENSECAG00000024187	0.10	1.00	0.64	0.398
	<b>POU5F1 (OCT4)</b>	ENSECAG00000008967	0.40	0.046	0.96	1.6E-07
	<b>ID2</b>	ENSECAG00000008738	0.31	0.409	0.82	1.01E-04
	<b>DNMT3B</b>	ENSECAG00000012102	0.37	0.034	0.87	2.10E-09
	<b>DPPA4</b>	ENSECAG00000013271	0.38	0.089	0.87	5.8E-06
	<b>SALL4</b>	ENSECAG00000018533	0.09	0.829	0.29	1.41E-03
	<b>KLF4</b>	ENSECAG00000010613	0.05	0.999	-0.13	0.848
	<b>UTF1</b>	ENSECAG00000039888	-0.18	0.973	-0.91	3.28E-04
<b>TE</b>	<b>TFAP2A</b>	ENSECAG00000017468	-0.14	0.700	-0.19	0.259
	<b>CDX2</b>	ENSECAG00000027754	-0.10	0.924	-0.24	0.032
	<b>ELF3</b>	ENSECAG00000014608	-0.19	0.830	-0.67	1.73E-04
	<b>GATA2</b>	ENSECAG00000016768	-0.04	0.998	-0.11	0.614
	<b>GATA3</b>	ENSECAG00000024574	-0.11	0.789	-0.08	0.756
	<b>TEAD4</b>	ENSECAG00000011303	-0.11	0.692	-0.22	8.81E-03
	<b>FREM2</b>	ENSECAG00000020410	-0.01	1.000	0.24	0.439

829 Gene expressions were obtained from RNA of 11 equine embryos bisected in two hemi-embryos: one  
830 part was composed only of trophoblast (TE), TE\_part, while the other part was composed of TE and  
831 inner cell mass (ICM), ICMandTE. As it is impossible to estimate the proportion of each cell in  
832 ICMandTE, a deconvolution algorithm (package DeMixT) was used to estimate gene expression of  
833 these different kind of cells. DeMixT\_ICM\_cells dataset corresponds to the deconvoluted gene  
834 expression of ICM cells from ICMandTE. Log2 fold change (log2FC) and padj (adjusted p-value with  
835 Benjamini-Hochberg correction) were obtained with Deseq2 package. TE\_part is the reference group

836 in both analyses: when  $\log_2$  fold changes ( $\log_2FC$ )  $> 0$ , gene is more expressed in ICMandTE or  
837 DeMixT\_ICM\_cells while when  $\log_2FC < 0$ , gene is more expressed in TE\_part. Green is used to  
838 represent gene differentially expressed in the present study. Orange is used to represent genes that  
839 have been previously identified as predominant in the ICM [53] but which are identified here as  
840 predominant in the TE.

841

842

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

Ensembl Name	Entrez Gene ID	Description	GO Molecular function	GO Biological process	log2 Fold Change	Adjusted p-value
ENSECAG00000002796	GABRR1	Gamma-aminobutyric acid type A receptor subunit rho1	Chloride channel Ion channel	Ion transport Chloride transmembrane transport Gamma-aminobutyric acid signaling pathway Regulation of membrane potential Signal transduction	5.19	0.004
ENSECAG00000007423	ZKSCAN2	zinc finger with KRAB and SCAN domains 2	DNA-binding transcription factor activity, RNA polymerase II-specific Metal ion binding	Regulation of transcription by RNA polymerase II	4.42	0.008
ENSECAG00000012957	RHAG	Rh associated glycoprotein	Ammonium transmembrane transporter activity Ankyrin binding Leak channel activity	Ammonia transport Cellular ion homeostasis Bicarbonate transport Carbon dioxide transport	4.25	0.024
ENSECAG00000017370	PPT1	palmitoyl-protein thioesterase 1	Hydrolase	Nervous system development Lipid catabolism process Protein transport Protein catabolic process	1.97	0.008
ENSECAG00000023194	XYLB	xylulokinase	ATP binding Kinase Transferase	Carbohydrate metabolism Xylose metabolism	0.63	0.009
ENSECAG00000036419	RPL36	ribosomal protein L36	Ribonucleoprotein Ribosomal protein	Translation Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay rRNA processing	-0.36	0.001
ENSECAG00000012792	MIF	macrophage migration inhibitory factor	Cytokine Isomerase	Immunity Inflammatory response Innate immunity	-0.39	0.001
ENSECAG00000007113	ATXN10	ataxin 10	Enzyme binding	Nervous system development Cilium assembly Neuron projection development	-0.46	3.78E-08

ENSECAG00000020469	VPS51	VPS51 subunit of GARP complex		Lipid transport Protein transport Transport Autophagy Brain morphogenesis Golgi organization	-0.56	0.024
ENSECAG00000006539	EXOSC7	exosome component 7	RNA-binding	rRNA processing	-0.65	0.002
ENSECAG00000042897	H2AC20	H2A clustered histone 20	DNA-binding	Chromatin silencing	-0.76	0.007
ENSECAG00000020116	RABEP2	rabaptin, RAB GTPase binding effector protein 2	Growth factor activity GTPase activator activity	Cilium biogenesis/degradation Endocytosis Protein transport Transport	-0.76	0.009
ENSECAG00000018683	PCF11	PCF11 cleavage and polyadenylation factor subunit	mRNA processing	mRNA 3'-end processing mRNA cleavage mRNA polyadenylation mRNA splicing, via spliceosome termination of RNA polymerase II transcription	-0.78	0.010
ENSECAG00000012328	STAT5A	signal transducer and activator of transcription 5A	Activator DNA-binding	Transcription Transcription regulation	-0.89	0.001
ENSECAG00000014662	SLX4	SLX4 structure-specific endonuclease subunit	DNA damage DNA recombination DNA repair	DNA repair	-0.90	0.020
ENSECAG00000028573	TRIOBP	TRIO and F-actin binding protein	Actin-binding	Cell cycle Cell division Mitosis	-0.99	0.014
ENSECAG00000016843	SETD5	SET domain containing 5	Chromatin regulator Methyltransferase Transferase	Transcription Transcription regulation Histone H3-K36 trimethylation Regulation of histone acetylation Regulation of chromatin organization	-1.02	0.023

ENSECAG00000008726	NPY	neuropeptide Y	Neuropeptide Calcium channel regulator activity G protein-coupled receptor activity Hormone activity signaling receptor binding	Adult feeding behavior G protein-coupled receptor signaling pathway Innate immune response Central nervous system neuron development	-1.27	0.001
ENSECAG00000021312	ABHD14A	abhydrolase domain containing 14A	Hydrolase		-1.30	0.007
ENSECAG00000030141	PXMP2	peroxisomal membrane protein 2	Transporter	Protein import into peroxisome membrane	-1.37	0.019
ENSECAG00000014057	ASB1	ankyrin repeat and SOCS box containing 1	Developmental protein	Ubiquitine-like conjugation pathway	-1.42	0.006
ENSECAG00000022130	CPT2	carnitine palmitoyltransferase 2	Acyltransferase	Fatty acid metabolism Lipid metabolism Transport	-1.52	0.004
ENSECAG00000015074	LIPE	lipase E, hormone sensitive type	Hydrolase	Cholesterol metabolism Lipid degradation Lipid metabolism Steroid metabolism Sterol metabolism	-1.53	0.018
ENSECAG00000008600	APOB	apolipoprotein B	Heparin-binding	Cholesterol metabolism Lipid metabolism Lipid transport Steroid metabolism Sterol metabolism Transport	-1.99	0.041
ENSECAG00000011240	CNTN1	Contactin 1	Carbohydrate binding	Cell adhesion Notch signaling pathway	-2.36	0.001

844 Log2 Fold-Change > 0 indicates up-regulation of the gene in embryos from old nulliparous mares; Log2 Fold-Change < 0 indicates down-regulation of the gene in embryos  
845 from old nulliparous mares

846

847 Table 3: Up- and down-regulated genes coding for a protein in the trophoblast part of equine embryos according to old mare parity

Ensembl Name	Entrez Gene ID	Description	GO Molecular function	GO Biological process	log2 Fold Change	Adjusted p-value
ENSECAG00000022403	<i>EFCAB2</i>	EF-hand calcium binding domain 2	Calcium ion binding		2.70	0.029
ENSECAG00000012686	<i>SNCAIP</i>	synuclein alpha interacting protein	Binding	Amyloid fibril formation Cell death Dopamine metabolic process Regulation of inclusion body assembly Regulation of neurotransmitter secretion	0.98	0.012
ENSECAG00000001080	<i>PLPP5</i>	phospholipid phosphatase 5	Hydrolase	Phospholipid dephosphorylation Phospholipid metabolic process	0.86	0.003
ENSECAG00000012682	<i>NFU1</i>	NFU1 iron-sulfur cluster scaffold	Iron ion binding	Iron-sulfur cluster assembly Protein maturation by iron-sulfur cluster transfer	0.61	0.024
ENSECAG00000004829	<i>SYNJ1</i>	synaptojanin 1	Hydrolase RNA-binding	Endocytosis Brain development Membrane organization Phosphatidylinositol metabolic process Inositol phosphate metabolic process	0.57	0.043
ENSECAG00000019000	<i>CHPT1</i>	choline phosphotransferase 1	Transferase	Lipid metabolism Phospholipid metabolism	0.52	0.026
ENSECAG00000009962	<i>MSH3</i>	mutS homolog 3	DNA-binding	DNA damage DNA repair	-0.41	0.030
ENSECAG00000024071	<i>MZT2B</i>	mitotic spindle organizing protein 2B			-0.43	0.029

ENSECAG00000015935	<i>ACTB</i>	Actin beta	BindingStructural constituent of cytoskeleton	Apical protein localization ATP-dependent chromatin remodeling Axonogenesis Cell junction assembly Cell motility Establishment or maintenance of cell polarity Membrane organization Morphogenesis of a polarized epithelium Negative regulation of protein binding Positive regulation of gene expression, epigenetic Protein deubiquitination Regulation of cyclin-dependent protein Regulation of transepithelial transport	-0.44	0.001
ENSECAG00000003599	<i>GK</i>	glycerol kinase	Kinase Transferase	Glycerol metabolism Phosphorylation Triglyceride biosynthetic process	-0.48	0.040
ENSECAG00000009054	<i>CKB</i>	creatine kinase B	Kinase Transferase	Cellular chloride ion homeostasis Cerebellum development Creatine metabolic process	-0.95	0.040
ENSECAG000000037199	<i>H4C3</i>	H4 clustered histone 3	DNA-binding	DNA-templated transcription, initiation Double-strand break repair Mitotic chromosome condensation Negative regulation of gene expression, epigenetic	-1.06	6.03E-09
ENSECAG000000020400	<i>ENO3</i>	enolase 3	Lyase	Glycolysis Aging Gluconeogenesis Skeletal muscle tissue regeneration	-1.31	0.030
ENSECAG000000039059	<i>CD99</i>	CD99 molecule (Xg blood group)		Cell adhesion Diapedesis Positive regulation of neutrophil extravasation Positive regulation of T cell extravasation	-1.33	0.001



ENSECAG00000012921	<i>ALPK2</i>	alpha kinase 2	Serine/threonine-protein kinase Transferase	Establishment of cell polarity Heart morphogenesis Negative regulation of Wnt signaling pathway involved in heart development Regulation of apoptotic process Regulation of gene expression	-2.23	0.001
ENSECAG00000017922	<i>ADAM19</i>	ADAM metalloproteinase domain 19	Hydrolase Metalloprotease	Amyloid precursor protein catabolic process Extracellular matrix organization Membrane protein ectodomain proteolysis Placenta development Positive regulation of cell-cell adhesion mediated by cadherin Positive regulation of gene expression Protein processing	-2.65	0.047
ENSECAG00000021052	<i>EOMES</i>	eomesodermin	Activator Developmental protein DNA-binding	Adaptive immunity Differentiation Gastrulation Transcription Transcription regulation	-2.88	0.029
ENSECAG00000008777	<i>MAP1B</i>	microtubule associated protein 1B	Actin binding Phospholipid binding Microtubule binding	Cellular process Dendrite development Mitochondrion transport among microtubule	-4.61	0.009

848 Log2 Fold-Change > 0 indicates up-regulation of the gene in embryos from old nulliparous mares; Log2 Fold-Change < 0 indicates down-regulation of the gene in embryos  
849 from old nulliparous mares

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851 Table 5: Enriched pathway in ICM and TE of embryos according to maternal parity in old mares

	Database	Geneset name	Number of genes	NES	FDR q-val	Subset of genes that contributes most to the enrichment result
ICM	GO BP	CYTOPLASMIC MICROTUBULE ORGANIZATION	45	-2.17	0.013	<i>SLAIN2, CCDC88A, EZR, CHP1, DYNC1H1, IFT172, KATNA1, TUBGCP6, TUBGCP4, FIGN, HOOK3, CLASP1, DLG1, DST, TUBGCP5, NUMA1, WDR73, CCDC88B, SLK, CCDC88C</i>
	KEGG	NEUROACTIVE LIGAND RECEPTOR INTERACTION	65	1.98	0.019	<i>GABRR1, GPR50, SCTR, GLP1R, CRHR2, P2RY11, GABRA5, PLG, MCHR2, ADORA2B, EDNRA, GABRA4, GHR, LPAR4, F2RL1, GRIA2, NPY1R, GABRG1, CHRNA5, GABRG2, GH2, F2R, CHRNA3, CGA, ADORA2A, CNR2, GRIN2C, CHRNA2, HCRTR2, NR3C1</i>
TE	GO BP	REGULATION OF GLIAL CELL DIFFERENTIATION	35	-2.21	0.008	<i>PTN, SLC45A3, NF1, DAG1, PRPF19, LIN28A, CXCR4, TNFRSF1B, LDLR, ID2, TGFB1, EPHA4, BIN1, BMP2, MDK, NOTCH1, TMEM98</i>
	REACTOME	ECM PROTEOGLYCAN	46	-2.20	0.002	<i>BCAN, ITGA7, COL6A6, HSPG2, COL4A5, COL5A2, ITGAV, NCAN, DAG1, TNXB, LAMB1, COL1A2, PTPRS, LAMA1, TGFB3, AGRN, MATN4, COL4A1, TGFB1, COL9A2, SPARC, VCAN, COL1A1, NCAM1, COL4A2, TNC</i>
	GO BP	POSITIVE REGULATION OF GLIAL CELL DIFFERENTIATION	23	-2.12	0.018	<i>PTN, DAG1, PRPF19, CXCR4, TNFRSF1B, ID2, TGFB1, BIN1, BMP2, MDK, NOTCH1</i>
	REACTOME	INTEGRIN CELL SURFACE INTERACTIONS	50	-2.11	0.004	<i>COL3A1, COL4A6, ITGA5, ITGA7, FBN1, ICAM3, COL6A6, BSG, HSPG2, CD47, COL4A5, COL5A2, ITGAV, CDH1, ICAM1, SPP1, COL1A2, AGRN, COL4A1, FGA, COL9A2, COL1A1, COL4A2, TNC, ITGA3, COL18A1, FGB</i>
	REACTOME	EXTRACELLULAR MATRIX ORGANIZATION	178	-1.99	0.018	<i>CAPN15, COL4A6, ADAMTS2, ITGA5, MMP9, MMP11, ITGB4, MFAP3, CASK, BCAN, CAPNS1, ITGA7, P3H3, ADAMTS1, FBN1, ICAM3, COL6A6, BSG, HSPG2, CD47, LTBP2, ADAMTS18, COL4A5, CAPN1, DMD, TIMP1, COL5A2, ITGAV, LAMB3, PLG, ACTN1, NCAN, DAG1, CDH1, CTSD, MMP2, TNXB, LAMB1, ICAM1, SPP1, COL1A2, SPOCK3, LAMC2, PTPRS, LAMA1, PXDN, TGFB3, EFEMP1, FBLN2, AGRN, COL14A1, MATN4, COL4A1, FURIN, TGFB1, FGA, ADAMTS5, COL9A2, LTBP3, SPARC, SERPINH1, DST, VCAN, COL1A1, PLOD3, <b>ADAM19</b>, NCAM1, MMP14, BMP2, COL4A2, PLEC, TNC, ADAM8, ITGA3, COL18A1, FGB</i>
	REACTOME	LAMININ INTERACTIONS	23	-1.98	0.015	<i>NID2, COL4A6, ITGB4, ITGA7, HSPG2, COL4A5, ITGAV, LAMB3, LAMB1, LAMC2, LAMA1, COL4A1, COL4A2, ITGA3, COL18A1</i>
	KEGG	HISTIDINE METABOLISM	18	-1.93	0.033	<i>HNMT, ALDH3A1, ALDH7A1, HAL, AMDHD1, UROC1, HEMK1, FTCD, MAOB</i>
REACTOME	REGULATION OF BETA CELL DEVELOPMENT	22	-1.91	0.038	<i>HNF4A, CREBBP, HNF1B, HNF1A, FOXA2, NR5A2, MAML1, AKT2, PKLR, PDX1, NOTCH1</i>	

REACTOME	NR1H3 NR1H2 REGULATE GENE EXPRESSION LINKED TO CHOLESTEROL TRANSPORT AND EFFLUX	28	-1.90	0.034	<i>EEPD1, ARL4C, TNRC6A, GPS2, NCOR2, APOC2, RXRB, KDM4A, MOV10, PLTP, NR1H2, APOE, TNRC6B, ABCA1, RXRA</i>
REACTOME	NOTCH3 INTRACELLULAR DOMAIN REGULATES TRANSCRIPTION	16	-1.90	0.031	<i>STAT1, KAT2A, CREBBP, WWC1, PTCRA, MAML1, PBX1, NOTCH1</i>
REACTOME	DEGRADATION OF THE EXTRACELLULAR MATRIX	76	-1.89	0.028	<i>FBN2, COL3A1, CAPN15, COL4A6, MMP9, MMP11, BCAN, CAPNS1, ADAMTS1, FBN1, COL6A6, BSG, HSPG2, ADAMTS18, COL4A5, CAPN1, TIMP1, COL5A2, LAMB3, PLG, CDH1, CTSD, MMP2, LAMB1, SPP1, COL1A2, SPOCK3, LAMC2, COL14A1, COL4A1, FURIN, ADAMTS5, COL9A2, COL1A1, MMP14, COL4A2, ADAM8, COL18A1</i>
KEGG	ECM RECEPTOR INTERACTION	50	-1.86	0.037	<i>COL3A1, COL4A6, ITGA5, ITGB4, ITGA7, COL6A6, HSPG2, CD47, COL5A2, ITGAV, LAMB3, DAG1, TNXB, LAMB1, SPP1, COL1A2, LAMC2, LAMA1, AGRN, COL4A1, COL1A1, COL4A2, TNC, ITGA3</i>

852 ICM: Inner cell mass; TE: Trophoblast; Genes in bold have also been identified as differentially expressed. Gene sets in blue are related to the extracellular matrix organization

853 and function while ones in green represent gene sets related to NOTCH signaling pathway.

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