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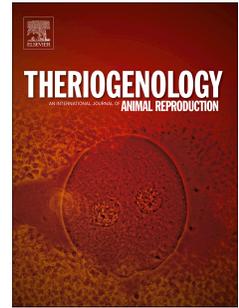
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1 **Maternal parity affects placental development, growth and metabolism of**
2 **foals until 1 year and a half**

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21 Abstract

22 Primiparous mares are known to produce smaller foals than multiparous mares. This difference
23 seems to be partly explained by the reduced exchange surface and volume of the placental villi in
24 primiparous compared to multiparous placentas. The effect of maternal parity on foals' post-natal
25 growth, metabolism and sexual maturation, however, has been given little consideration. The
26 objectives of this work were to analyse placental biometry and structure at term, growth of foals and
27 yearlings, their metabolism and testicular maturation at one year of age.

28 Twenty multiparous mares (M), aged over 6 years and 12 primiparous mares (P), aged up to 5 years
29 were artificially inseminated with the same stallion and monitored the same way until foaling. At
30 birth, foals and placentas were measured and placentas were sampled above at the umbilical cord
31 insertion, as well as in the pregnant and the non-pregnant horn to perform stereological analyses.
32 Foals were weighed and measured until 540 days of age. At 120 and 360 days of age, an Intravenous
33 Glucose Tolerance Test was performed on foals and yearlings. At 360 days of age, the males were
34 castrated and testicular maturation analysed by RT-qPCR.

35 At birth, P dams produced lighter and smaller foals and placentas. The foal birth weight to placental
36 surface ratio was lower in the P compared to the M group. P Foals remained lighter than M foals until
37 360 days of age and smaller until at least 540 days of age. At 120 days of age, P foals had a higher
38 glucose tolerance than M foals, and then may be less mature than M foals in terms of the control of
39 their glucose homeostasis. At 360 days of age, the testicles of prepubertal P stallions were less
40 mature in the P vs the M group.

41 In conclusion, primiparous dams produce intrauterine growth restricted, less mature and smaller
42 foals compared to multiparous dams with altered metabolism and growth until at least 540 days of
43 age. These differences could affect the sport career of these foals, especially if it begins at an early
44 age.

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52 Chamberet, France) for care and management of animals.

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71 **1. Introduction**

72 Studies in human populations and experimental works in non-equine animals have demonstrated
73 that the maternal environment affects fetoplacental development and can increase the risk of
74 developing non-communicable diseases at adulthood [1–4]. This concept is referred to as the
75 Developmental Origins of Health and Disease (DOHaD). In the horse, intra uterine growth restriction
76 (IUGR), experimentally induced by embryo transfers of Saddlebred horses into pony mares, was
77 shown to impair fetoplacental development and postnatal growth of the offspring until 3 years of
78 age, to alter glucose metabolism until weaning and to increase the risk of developing
79 osteochondrosis at 6 months of age [5–7].

80 Incidentally, primiparous mares are known to produce lighter and smaller foals at birth compared to
81 multiparous mares [8–16]. These foals remain smaller until one year of age [9,12,14,16]. They have
82 lighter, smaller and less voluminous placentas [10,11,16] with a reduced microscopic area of
83 fetomaternal contact compared to multiparous placentas [10,16]. Thus, Wilsher and Allen, 2003,
84 suggested that the uterus of the mare needs to be ‘primed’ by a first pregnancy to achieve its full
85 potential [10].

86 Primiparous dams seem to produce fewer winners in flat races compared to horses born to
87 multiparous mares [17] and this difference might be explained by the fact that horses born to
88 primiparous dams are smaller. Indeed, taller horses perform better than smaller horses in flat races

89 [18,19]. Moreover, it has been shown in the United States that the heaviest and tallest yearlings are
90 sold at better auction prices than the smaller yearlings [20].

91 According to the DOHaD concept, *in utero* growth retardation might affect more than the size of the
92 foal until weaning, *i.e.*, metabolism, gonadal maturation and disease susceptibility could also be
93 affected [21]. The present study aims to investigate the effects of maternal parity on placental
94 structure at term, growth of foals until 540 days, carbohydrate metabolism of foals until 360 days,
95 testicular maturation and osteochondrosis at 360 days of age.

96 **2. Material and methods**

97 **2.1. Animals**

98 **2.1.1. Ethical statement**

99 Animal studies were approved by the local animal care and use committee and received ethical
100 approval from the local ethics committee ("Comité Régional d'Ethique pour l'Expérimentation
101 Animale du Limousin") under number 5-2013-5.

102 **2.1.2. Management of broodmares and foals**

103 Thirty-two Anglo-Arabian mares raised in the "Institut Français du Cheval et de l'Équitation"
104 experimental farm (Chamberet, France, 45° 34'55.17"N, 1°43'16.29"E, 442 m) were used, including
105 20 multiparous mares (group M, median parity: 2; range min 1.0 to max 4, median age: 8; range min
106 6 to max 18) and 12 primiparous mares (group P, median age: 4; range min 4 to max 5). Pregnancies
107 were obtained in all mares by artificial insemination (from May 25th to July 2nd) using semen from one
108 single Anglo-Arabian stallion. Broodmare characteristics are detailed in Table 1.

109 From insemination, pregnant mares were managed in one same herd in pasture 24h/day with free
110 access to water and mineral salts (Krouner Rumi, CTH, France). From October 28th (median
111 gestational day 146; range 118 to 156), the mares were housed in individual boxes in the same barn.

112 They were fed cracked barley twice a day, hay and haylage and vitamin and minerals supplement
113 (Excel Prima S, Chauveau Nutrition, France) with free access to water until foaling (Supplementary
114 Table 1).

115 All foals were born in spring (from April 8th to June 6th). Three days after foaling, mares and foals
116 returned in pasture and were managed in one same herd until weaning between October 15th and
117 November 26th (median age: 185; range 172 to 195) by complete, abrupt separation of mares and
118 foals. Foals were then housed in open stalls and fed twice a day with hay and homemade pellets
119 containing barley, oat, soybean cake, bran, molasses and vitamins and minerals with free access to
120 water (Supplementary Table 2). Six months after weaning, at 360 days of age, all yearlings returned
121 to pasture 24h/day with free access to water and vitamin and minerals supplement (Supplementary
122 Table 3).

123 **2.1.3. Body measurements**

124 At birth, every 30 days until 180 days and every 90 days until 540 days, foals and yearlings were
125 weighed and withers' height and thoracic perimeter measured.

126 **2.2. Placentas**

127 **2.2.1. Placental biometry and sampling**

128 At delivery, the placenta (allantochorion) was weighed. The macroscopic placental surface area was
129 measured by applying a clear Plexiglas sheet marked with 10cmx10cm squares above the placenta
130 [22]. Placentas were laid in the "F" configuration, revealing both uterine horns and the uterine body.
131 Photographs were taken and the placental surface subsequently measured using the ImageJ®
132 software (National Institute of Health). The foal birth weight to placental weight ratio was calculated.
133 Placental volume was evaluated using a graduated water container and measuring the volume of
134 water displaced after immersing the placenta into the water.

135 Placental samples were collected within 30 minutes after delivery from three anatomical regions:
136 one from <10 cm above the umbilical cord insertion, one from the center of the pregnant horn and
137 one from the non-pregnant horn. Three aliquots of each region were then fixed in 4% formaldehyde
138 for histological analysis.

139 **2.2.2. Histological and stereological analyses**

140 Samples were embedded in paraffin. Sections (7 μ m) were stained with hematoxylin/eosin and
141 scanned using NanoZoomer Digital Pathology[®] (Hamamatsu Photonics).

142 Surface densities (Sv) and Volume fractions (Vv) of the different components of the allantochorion,
143 *i.e.*, composing the microcotyledons and the allantois, were quantified by One stop stereology using
144 the Mercator[®] software (ExploraNova, France, [23]).

145 As presented in Figure 1, the components measured were:

- 146 - **In the microcotyledonary region:** haemotrophic trophoblast, microcotyledonary vessels,
147 connective tissue and microcotyledons as the sum of all microcotyledonary components.
- 148 - **In the allantoic region:** histotrophic trophoblast and allantoic vessels. The chorionic
149 mesoderm and allantoic connective tissue were merged as allantoic connective tissue.

150 Vv and Sv were multiplied by the total volume of the placenta to obtain an estimation of the absolute
151 volume (cm³) and surface (cm²) of the components of the allantochorion. Results from the different
152 parts of the placenta were pooled to be analysed as one sample representative of the whole organ.

153 **2.3. Endocrinology**

154 *Evaluation of glucose metabolism*

155 Intravenous Glucose Tolerance Tests (IVGTT) were performed at median ages 120 and 360 days. The
156 procedure and calculations were previously described [8]. Briefly, foals were muzzled to prevent
157 suckling 6 h before the procedure. They were infused with glucose (0.25 g/kg, 30% glucose IV in one

158 jugular vein) over 2 min. Samples were collected from the contralateral jugular vein into EDTA-coated
159 tubes at -5 min and 2, 3, 5, 7, 9, 12, 15, 30, 60 and 120 min after the injection started. Blood glucose
160 concentration was immediately analysed using a glucometer (Freestyle optium, Abbott, USA). Plasma
161 was separated after 10 min centrifugation at 5500 rpm and stored in several aliquots at -20°C until
162 insulin assay. Plasma insulin concentrations were measured using a previously validated human
163 insulin AlphaLISA immunoassay kit (PerkinElmer, USA) [24].

164 *Evaluation of thyroid function*

165 At birth before suckling and at 3, 30, 90 and 180 days of age (subsequent to a 4h fasting period at 3
166 days of age and 6h fasting at the subsequent time points), jugular vein blood samples were collected
167 in EDTA tubes. Plasma was separated after 10 min centrifugation at 5500 rpm and stored at -20°C
168 until assay.

169 Total T3 and total T4 were assayed using MP Biomedicals (Solon, USA) radiimmunoassay kits in
170 aliquots of 25 or 10 µl and 50 to 200µl, for T4 and T3, respectively. These assays were validated for
171 horse serum by measuring T3 and T4 in horse plasma spiked with four different concentrations
172 levels: 0, 20, 50 and 100ng/ml and 0, 1, 2.5 and 5ng/ml for T4 and T3, respectively. For T4 assay,
173 mean inter- and intra-assay CVs were 16.0% and 5.7%, respectively and mean exactitude according
174 to expected concentrations in spiked samples was -12.4%. For T3, mean inter- and intra-assay CVs
175 were 9.4 and 13.4%, respectively and mean exactitude according to expected concentrations in
176 spiked samples was -14.5%.

177 **2.4. Testicles**

178 **2.4.1. Tissue sampling and treatment**

179 Testicles from prepubertal stallions (group M: n=9 and group P: n=9; median age: 324.5 days, range
180 298-357 days) were collected during routine castration. Castrations were timed so that the age of the

181 animals at the time of castration was not different between groups. Testicular samples were snap
182 frozen on dry ice and stored at -80°C until mRNA extraction.

183 2.4.2. Quantitative real time PCR

184 Total mRNA was extracted with a Tri-Reagent kit (Sigma-Aldrich, France) according to the
185 manufacturer's instructions. Reverse transcription and PCR reactions were performed as previously
186 described [24]. The relative gene expression was normalized with *GAPDH* RNA expression. The genes
187 analysed were markers of testicular maturity (*Cyp19*, *Connexin 43 (Cx43)*, *Androgen Receptor (AR)*,
188 *Steroidogenic Acute Regulatory Protein (STAR)*), markers of immaturity (*Anti-Müllerian hormone*
189 (*AMH*)) and genes involved in the establishment of the blood-testis barrier (*Occludin*, *N Cadherin*, *β*
190 *Catenin*). Primer sequences are presented in Table 2.

191 2.5. Statistical analyses

192 Results are expressed as median [quartile 1-quartile 3] and presented as boxplots (median and
193 interquartile range (IQR)). Statistical analyses were performed using the R software ([http://www.r-](http://www.r-project.org)
194 [project.org](http://www.r-project.org), version 3.4.0.).

195 Data were analysed using a mixed linear model (*nlme* package [25]) followed by a type 3 ANOVA (*car*
196 package [26]) :

- 197 - **Feto-placental biometry:** Parity of the mare (P or M) and sex of the foal (female or male)
198 were considered as fixed effects. The mare's withers' height at insemination was considered
199 as a random effect. The age of mare could not be included in the statistical model because
200 nearly all primiparous dams were 4 years old (11/12).
- 201 - **Stereology results:** Parity of the mare (P or M) and placental region (CO, PH or NPH) were
202 considered as fixed effects. The mare was considered as a random effect. When results
203 differed between placental regions, a post-hoc Tuckey test was applied using the *lsmeans*

204 package. The interaction between placental region and parity was not statistically significant
205 and was subsequently removed from the model.

206 - **Foal and yearling measurements** and metabolic data were analysed using a mixed quadratic
207 model considering parity of the mare (P or M), the age of the foal (from 30 days of age) and
208 the interaction parity:age of the foal as fixed effects and the foal as random effect. When
209 parity:age interaction was statistically significant, a post-hoc Tuckey test was applied using
210 the *lsmeans* package [27].

211 - **Thyroid hormone results** were analysed using a mixed linear model considering the parity of
212 the mare (P or M), the age of the foal and the interaction parity:age of the foal as fixed
213 effects. The foal itself was considered as random effect. When parity:age interaction was
214 statistically significant, a post-hoc Tuckey test was applied using the *lsmeans* package.

215 Metabolic tests results were analysed with a fixed linear model followed by a type 3 ANOVA
216 considering parity of the mare (P or M).

217 The sex of the foal was removed from the metabolic tests and thyroid hormone concentrations
218 models as it had no effect on these parameters. Maternal weight at insemination and parturition had
219 no statistically significant effect on any measured parameter and was thus not included in any of the
220 models.

221 Testicular data were analysed using a principal component analysis (PCA) of the FactoMineR package
222 [28].

223 Effects were considered significant when $p < 0.05$.

224 **3. Results**

225 **3.1. Feto-placental biometry and placental structure at birth**

226 **3.1.1. Feto-placental biometry**

227 Statistically significant results are presented in Figure 2.

228 Gestation length was reduced by 8 days in P compared with M mares ($p < 0.01$).

229 At birth, P foals were lighter (-8.3kg, $p<0.001$), smaller (-5cm, $p<0.01$) and had a reduced thoracic
230 perimeter ($p<0.010$) than M foals. P placentas were lighter (-510g, $p<0.01$) and less voluminous (-
231 585.6cm², $p<0.01$). Moreover, the foal birth weight to placental surface ratio was reduced in P
232 compared to M groups (-0.24g/cm², $p<0.01$). P placentas tended to have a lower placental surface
233 area than M placentas (-4.75cm, $p=0.087$). There was no effect of maternal parity on placental
234 efficiency.
235 Gestation length was reduced by 2 days in mares carrying females comparing with males ($p=0.046$).
236 Sex of foal had no effect on other parameters measured.

237 **3.1.2. Placental structure**

238 Stereological results are presented in Table 3.

239 - **In the microcotyledonary region:** the volume and surface of microcotyledonary connective
240 tissue was reduced in P compared to M placentas (-30.0cm³, -18.2%, $p=0.03$; -0.62.10⁵cm², -
241 27.7%, $p=0.01$, respectively).

242 The volume of microcotyledonary connective tissue (-6.31cm³, -4.1%, $p<0.001$) and that of
243 microcotyledons (-46.1cm³, -5.5%, $p=0.02$) was reduced in female compared to male
244 placentas.

245 - **In the allantoic region:** the volume of allantoic connective tissue (-602.8cm³, -35.7%,
246 $p<0.001$), the surface of histotrophic trophoblast (-0.13.10⁵cm², -18.8%, $p=0.04$) and the
247 surface of allantoic vessels (-0.21.10⁵cm², -27.8%, $p<0.01$) were reduced in P compared to M
248 placentas.

249

250 **3.2. Growth of foals and yearlings**

251 Measurements of foals and yearlings are presented in Figure 3.

252 P foals and yearlings were lighter than M foals and yearlings until 360 days of age ($p<0.01$).
253 Moreover, P foals were smaller with a reduced thoracic perimeter compared to M foals until 540
254 days of age ($p<0.0001$, $p<0.0001$, respectively).

255 **3.3. Glucose metabolism**

256 Results for metabolic tests are presented in Figure 4.

257 At 120 days of age, there was no difference in glucose and insulin concentrations after fasting. The
258 area under curve for glucose was reduced in P compared to M foals ($p=0.02$). There was no
259 difference for maximum glucose concentration, area under curve for insulin and maximum insulin
260 concentration between groups.

261 At 360 days of age, there was no difference in glucose and insulin concentrations after fasting. The
262 area under curve for glucose tended to be reduced in P vs M foals ($p=0.09$). There was no difference
263 for maximum glucose concentration, area under curve for insulin and maximum insulin concentration
264 between groups.

265 **3.4. Thyroid endocrinology**

266 Maternal parity did not affect plasma T3 nor T4 concentrations until weaning. Both T3 and T4
267 concentrations decreased with age until 30 days of age ($p<0.0001$, $p<0.0001$, respectively).
268 Moreover, the interaction parity:age was statistically significant ($p=0.04$) for plasma T3, with P foals
269 having a higher plasma T3 concentrations at birth compared to M foals ($p<0.01$).

270 **3.5. Testicular maturation**

271 Principal component analysis of testicular maturation is shown in Figure 5.

272 The 2nd dimension (accounting for 22.69% of the variance) discriminated P from M prepubertal
273 stallions (F test, $p=0.03$) and was associated with a decreased expression of *Cyp 19* ($p<0.001$) and *AR*
274 ($p<0.0001$) and an increased expression of *β Catenin* ($p<0.0001$) in P compared with M yearlings.

275 4. Discussion

276 In the present study, primiparous dams produced lighter and smaller foals and placentas at term
277 compared to multiparous dams. The surface of allantoic components was reduced in primiparous
278 compared to placentas of multiparous placentas. The foal birth weight to placental ratio was reduced
279 in the primiparous compared to the multiparous group. Foals born to primiparous mares were also
280 lighter until 360 days of age and smaller until 540 days of age compared to foals born to multiparous
281 mares. Glucose tolerance was higher in foals born to primiparous dams vs foals born to multiparous
282 dams at 120 days of age and tended to be higher at 360 days of age. At 360 days of age, the testicles
283 of prepubertal stallions born to primiparous dams had less mature compared to prepubertal stallions
284 born to multiparous dams.

285 As observed previously in other breeds (Thoroughbred, Brazilian Sport Horse, Mangalarga Paulista
286 and Quarter Horse), foals born to primiparous dams were lighter and smaller at birth compared with
287 foals born to multiparous dams [10–12,15,16]. In humans, intra uterine growth retardation (IUGR) is
288 defined as a birth weight below the tenth percentile for gestational age [29]. Here, the weight of P
289 foals was 3.1kg below the 10th percentile of M foals' birth weight. Moreover, the weight of P foals
290 was 3.1kg below the 10th percentile of the 89 foals of the same breed born between 2011 and 2015
291 in the same farm. Thus, in the light of this definition, P foals could be considered as IUGR.

292 In the present study, the gestation length of primiparous dams was shorter than the gestation length
293 of multiparous dams. This could partly explain the difference in foal birthweight. Nevertheless, when
294 the theoretical *in utero* fetal growth is calculated using the equation provided by the National
295 Research Council (NRC, [30]), we observe that the difference between groups should have been only
296 of 4.3 kg, *i.e.*, that P foals should have weighed a mean 48.7kg at birth instead of 44.3kg if they had
297 had the same *in utero* growth as M foals but were born 8 days earlier. Moreover, in other studies,
298 gestation length was either not affected by maternal parity or it was observed that primiparous dams
299 had longer gestations than multiparous dams [9,16,31,32]. As a matter of fact, gestation length has

300 been shown to be influenced by numerous factors such as foal sex, month of birth, year of breeding
301 (climate, photoperiod), age, genetics, breed and nutritional state [31–35]. To conclude, the
302 decreased birth weight of foals born to primiparous dams is unlikely to be fully related to the
303 reduced gestation length observed in the present study.

304 We also confirm studies indicating that primiparous mares produce lighter placentas than
305 multiparous mares [10–12,16]. As defined by Wilsher and Allen, 2003, the foal birth weight to
306 placental surface can be used as an index to estimate placental efficiency [10]. Using this index, we
307 show in the present study that primiparous dams have a reduced placental efficiency compared to
308 multiparous dams while other authors observed the opposite, comparing primiparous dams of all
309 ages to young multiparous dams (5 to 9 years) [10,16]. Here, all primiparous dams were 4 to 5 years
310 old while the multiparous dams were more than 6 years of age. It has been observed previously that
311 the placentas of younger mares (5 to 9 years of age) were less efficient than those of older mares
312 [10]. Thus, the age difference between primiparous and multiparous mares in the present study may
313 explain these observations.

314 The surface and volume of microcotyledonary villi have been previously shown to be reduced in
315 primiparous vs multiparous placentas based on measurements performed in the placental body or in
316 the whole placenta [10,16]. In the present study, we observed a difference only for
317 microcotyledonary connective tissue. The increased connective tissue in multiparous mares may be
318 explained by the age of mares as fibrosis become more important with age and particularly the
319 number of foalings [16]. We also observed that the surface of allantoic components (histotrophic
320 trophoblast, vessels and connective tissue) was reduced in P compared to M placentas. Nevertheless,
321 when comparing the surface and volume of microcotyledons between young multiparous mares
322 (aged 5-9 years) and primiparous mares, we did not observe any statistically significant difference. It
323 has been observed that the diameter of the uterine arteries was much smaller in primiparous

324 compared to multiparous mares [13]. These observations may be related to the decreased
325 vascularisation of the allantois observed in the placentas of P mares.

326 The histotrophic trophoblast, located within the areolae, is involved in the exchange of nutrients that
327 are produced by the uterine glands [36]. This tissue has been shown not to have precisely the same
328 function as the haemotrophic trophoblast: for example, it expresses the calcium transporter while
329 the haemotrophic trophoblast does not [37]. Data presented here show that histotrophic nutrition is
330 more severely affected than haemotrophic nutrition in primiparous mares, possibly because of a
331 difference in endometrial gland function, as proposed by Wilsher and Allen, (2003) [10].

332 These differences were observed within all placental regions and may help explaining the observed
333 decreased placental efficiency in primiparous mares as well as the smaller and lighter placentas and
334 foals at birth.

335 P foals were smaller than M foals until at least 540 days of age. A similar difference was observed
336 until 360 days of age in various breeds [9,12,16]. The difference has been reported not to be present
337 any more in the Brazilian Sport Horse and the Mangalarga Paulista breed at 2 years of age [16]. These
338 results are of importance for the equine industry as horses bred for flat races are sold at auction at
339 around one year of age and begin their career early, at around two years of age. It has been shown
340 that taller horses are sold a better price at auction than smaller horses and that withers' height might
341 be related to racing performance [18–20].

342 To our knowledge, the glucose metabolism of foals and yearlings born to primiparous or multiparous
343 dams has not been investigated previously. At 120 days of life, there was no difference for insulin
344 secretion between groups but P foals had an accelerated glucose clearance rate. According to George
345 et al., (2009), insulin sensitivity decreases with foal age and immature foals may be more insulin
346 sensitive until at least 160 days of age [38]. Here, P foals were more insulin sensitive and may be less
347 mature than M foals until at least 120 days of age. Moreover, it has been shown that dysmature
348 foals, piglets and lambs were more insulin sensitive in early life but that their insulin sensitivity had

349 decreased at adulthood [6,39–44]. The difference in insulin sensitivity observed at 120 days of age
350 remained only as a tendency at 360 days of age, following the same trend as other IUGR individuals.
351 In addition to the difference in height, this difference in glucose metabolism may affect the short-
352 term (racing) or long-term (show jumping, eventing...) performance of horses.

353 Thyroid hormones are involved in bone growth, energy metabolism and thermoregulation. As
354 observed earlier, thyroid hormones concentrations were elevated at birth and decreased with age
355 [6,45–49]. An elevated T3 concentration in the days following birth has been associated with
356 dysmaturity and IUGR in horses [6]. The increased plasma T3 concentrations observed in P foals at
357 birth may thus be associated with delayed maturity in these foals, possibly due to the decreased
358 histotrophic nutrition capacity and vascularisation of the placenta. Moreover, increased plasma T3
359 concentrations are observed with catch-up growth in foals born dysmature [6]. As P foals did not
360 catch-up to M foals up to 450 days of age, we did not expect to observe a difference in plasma T3
361 concentrations between groups during post-natal growth in the present study.

362 To our knowledge, the testicular maturation of prepubertal stallions born to primiparous or
363 multiparous dams has not been investigated. Puberty in stallions lasts around 1 year, from
364 approximately 1 to 2 years of age [50,51]. Concomitantly, the testicular sensibility to androgens
365 increases with testicular maturation [52]. In a previous study, using testicles of prepubertal stallions
366 of the same age and the same breed, we observed that the expression of *Cyp19* and *AR* was
367 correlated with the higher histological grades for testicular maturity (opened tubules) while the
368 expression of *N Cadherin*, *Occludin* and *β Catenin* was associated with the lower grades of maturity
369 (tubules without a lumen) [24]. Here, maternal multiparity was associated with increased testicular
370 expression of *Cyp19* and *AR* while maternal primiparity was associated with increased *β -catenin*
371 expression. Thus, the testes of P colts were likely less mature than those of M colts. It has been
372 shown that IGF-1 stimulates Sertoli cell proliferation in mice [53]. We also know that foals born to
373 primiparous dams have decreased IGF-1 serum concentrations compared to foals born to
374 multiparous dams until at least 4 months of age [14]. Reduced IGF-1 stimulation of Sertoli cells may

375 putatively explain the delayed testicular maturity in P foals. This hypothesis is in agreement with
376 observations of delayed puberty and testicular development and long-term alterations in testicular
377 function in IUGR pigs and rats [54,55].

378 5. Conclusion

379 In conclusion, primiparous mares produced Small for Gestational Age (SGA) foals with signs of
380 delayed maturity such as decreased plasma triiodothyronine concentrations. These differences at
381 birth were associated with a decreased foal birth weight to placental surface ratio and reduced
382 histotrophic nutrition capacity in placentas of primiparous mares. Foals born to primiparous mares
383 remained smaller until at least 540 days of age. Their testicular maturation and possibly the
384 maturation of the glucose homeostasis regulation system were delayed.

385 These alterations of long-term development may affect the selling price and the short- and long-term
386 sport performance of horses.

387 6. Bibliography

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530

Figure 1: Structure of the horse placenta at term and placental components measured by stereology.

AC: Allantoic connective tissue, AV: Allantoic vessel, HT : Histotrophic trophoblast.

Figure 2: Gestation length and feto-placental biometry at birth between foals born to multiparous (n=20) and primiparous (n=12) mares. A. Gestation length. B. Foal birth weight. C. Foal wither's height. D. Foal thoracic perimeter. E. Placental weight. F. Placental volume. G. Foal birth weight/placental surface area ratio. Different letters correspond to statistically different results ($p < 0.05$), the use of the same letter corresponds to statistically non-different results ($p > 0.05$).

Figure 3: Growth of foals and yearlings born to multiparous (n=20) or primiparous (n=12) dams until 1.5 years of age. A. Body weight. B. Wither's height. C. Thoracic perimeter. Values marked with an asterisk are significantly different ($p < 0.05$).

Figure 4: Intravenous glucose tolerance test result of foals and yearlings born to multiparous (n=20) or primiparous (n=12) dams at 120 and 360 days of age. A. Area under the curve of glucose at 120 days of age. B. Area under the curve of insulin at 12 days of age. C. Area under the curve of glucose at 360 days of age. D. Area under the curve of insulin at 360 days of age.

Figure 5: Principal component analysis of expression of genes involved in testicular maturity (*Cyp19*, *Connexin 43 (Cx43)*, *Androgen Receptor (AR)*, *Steroidogenic Acute Regulatory Protein (STAR)*), testicular immaturity (*Anti-Müllerian hormone (AMH)*) and genes involved in the establishment of the blood-testis barrier (*Occludin*, β *catenin* and *N Cadherin*). A. Graph of variables. The expression of *Cyp19* ($p < 0.0001$) and *AR* ($p < 0.0001$) is positively correlated with the 2nd dimension while the expression of β -*catenin* ($p < 0.0001$) is negatively correlated with the 2nd dimension. B. Graph of individuals. Despite the fact that ellipses overlap, there is a statistically significant difference between M and P testicles, separated by the 2nd dimension ($p = 0.045$).

Table 1. Maternal age, parity, withers' height, weight at insemination and weight at parturition (median [quartile 1-quartile 3]) and % of male and female foals within groups of multiparous and primiparous mares.

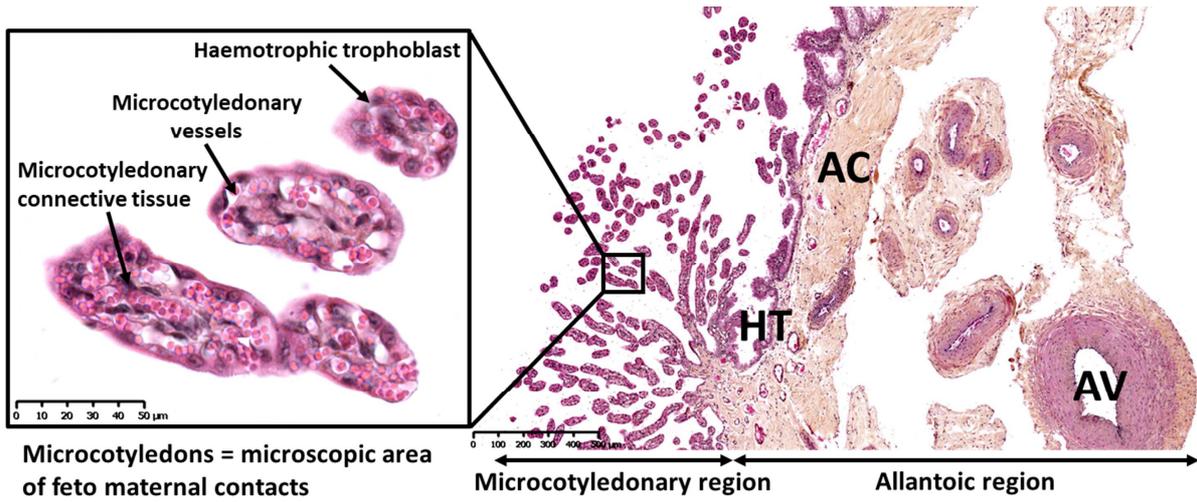
Group	Age (years)	Parity	Withers' height (cm)	Weight at insemination (kg)	Weight at parturition (kg)	% males	% females
Multiparous (n=20)	8.1 [7.1-9.2]	2.0 [1.0-2.25]	161 [160-164]	597.5 [572.6-626.4]	649.5 [622.5-667.5]	45	55
Primiparous (n=12)	4.1 [4.1-4.1]	0.0 [0.0-0.0]	159 [153-164]	523.9 [478.9-550.7]	612.2 [550.0-631.5]	75	25

Table 2. Primers sequences used for amplification of equine products in RT-qPCR assays in testicles of prepubertal colts.

Gene	GenBank accession number	Primer	Size of PCR product (pb)
<i>AMH</i>	JF330269,1	Forward: 5' - CCATCTGGAGGAGCCAAC - 3' Reverse: 3' - CCCGTGACAGTGACCTCAG - 5'	80
<i>Androgen Receptor</i>	NM_001163891,1	Forward: 5' - AGTACTCCTGGATGGGGCTT - 3' Reverse: 3' - TGTACATCCGGGACTTGTGC - 5'	132
<i>B Catenine</i>	NM_001122762,1	Forward: 5' - ACAATGGCTACTCAAGCCGA - 3' Reverse: 3' - CCAGTGACTAACAGCCGCTT - 5'	78
<i>Connexin 43</i>	XM_005596901,1	Forward: 5' - CTCTTCACCAACCGCTCCT - 3' Reverse: 3' - CTGTCTCCGGTAACCAGCTT - 5'	68
<i>Cyp 19</i>	NM_001081805,1	Forward: 5' - AAAGCCACCCGGTTCCTAAC - 3' Reverse: 3' - CCTTGATCTCAGGCGAAGCA - 5'	119
<i>GAPDH</i>	AF157626	Forward: 5' - CCAGAACATCATCCCTGCTT - 3' Reverse: 3' - CGTATTTGGCAGCTTTCTCC - 5'	158
<i>N Cadherine</i>	XM_001495782,3	Forward: 5' - GCCATTCAGACTGACCCGAA - 3' Reverse: 3' - CTGCAGCGACAGTAAGGACA - 5'	100
<i>Occludin</i>	XM_001504044,3	Forward: 5' - AAGCTTCCATGTCAGTGCCTT - 3' Reverse: 3' - TTGCTTGGTGC GTAATGATTGG - 5'	84
<i>STAR</i>	NM_001081800,1	Forward: 5' - TGGCCTATATCCAGCAGGGA - 3' Reverse: 3' - AATACCTTGCCCACGTCTGG - 5'	142

Table 3. Volume and surface of the different components of the placenta measured using the stereology method in term placentas of primiparous (n=11) or multiparous (n=17) mares.

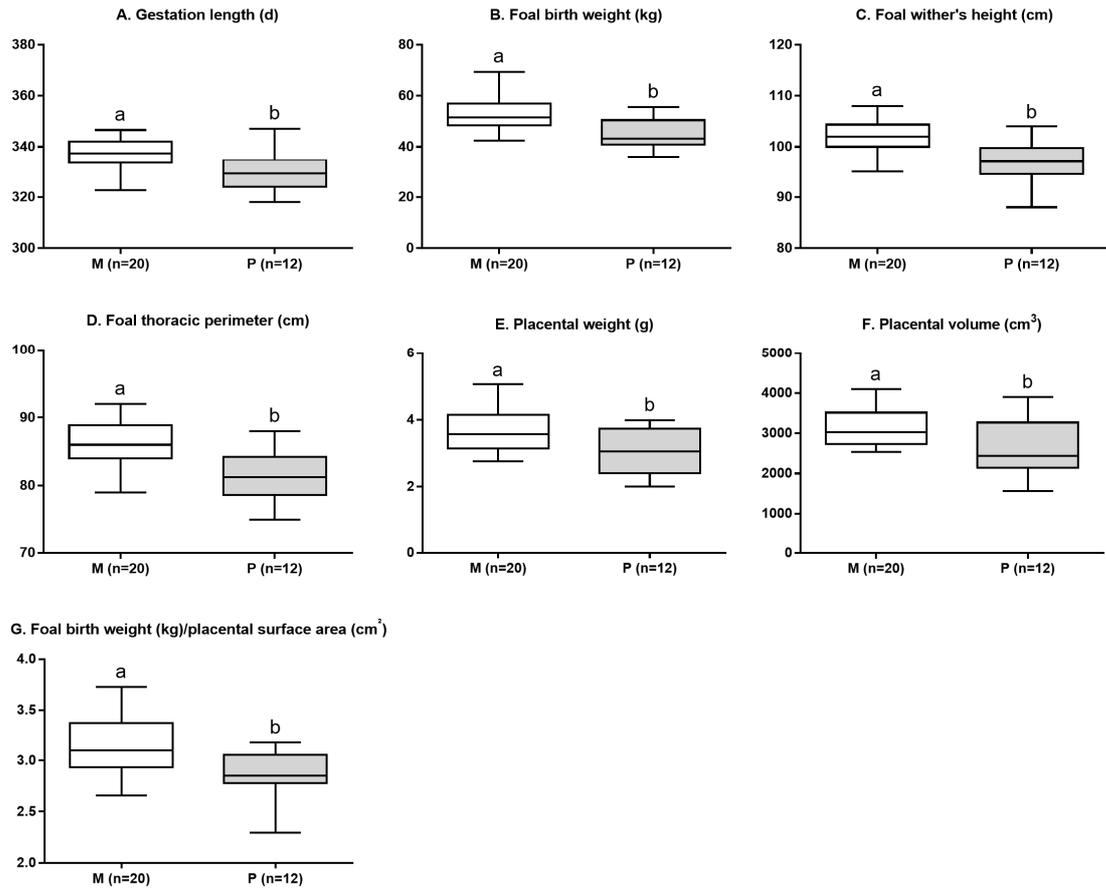
	Primiparous (n=11)	Multiparous (n=17)	P value
Volume (cm³)			
Microcotyledonary region			
Haemotrophic trophoblast	424.5 [302.7-561.2]	347.1 [259.4-508.4]	NS
Vessels	272.1 [192.9-501.5]	307.7 [209.3-373.8]	NS
Connective tissue	134.7 [86.2-159.5]	164.7 [138.7-220.5]	0.03
Microcotyledon	855.0 [555.2-1265.1]	830.0 [685.0-1020.6]	NS
Allantoic region			
Histotrophic trophoblast	134.5 [106.1-158.5]	116.6 [101.3-159.0]	NS
Vessels	343.5 [233.6-540.8]	353.2 [263.9-468.7]	NS
Connective tissue	1084.2 [1043.1-1364.8]	1687.0 [1492.3-1924.1]	<0.001
Surface (10⁵ cm²)			
Microcotyledonary region			
Haemotrophic trophoblast	4.35 [3.45-8.41]	5.62 [5.00-7.02]	NS
Vessels	2.75 [2.35-5.35]	4.36 [3.75-5.18]	NS
Connective tissue	1.62 [1.10-2.02]	2.25 [1.73-2.73]	0.01
Microcotyledon	8.73 [6.77-15.98]	12.69 [11.00-14.01]	NS
Allantoic region			
Histotrophic trophoblast	0.55 [0.44-0.65]	0.68 [0.59-0.94]	0.04
Vessels	0.54 [0.39-0.63]	0.75 [0.63-1.35]	<0.01
Connective tissue	1.08 [0.95-1.73]	1.58 [1.36-2.18]	NS

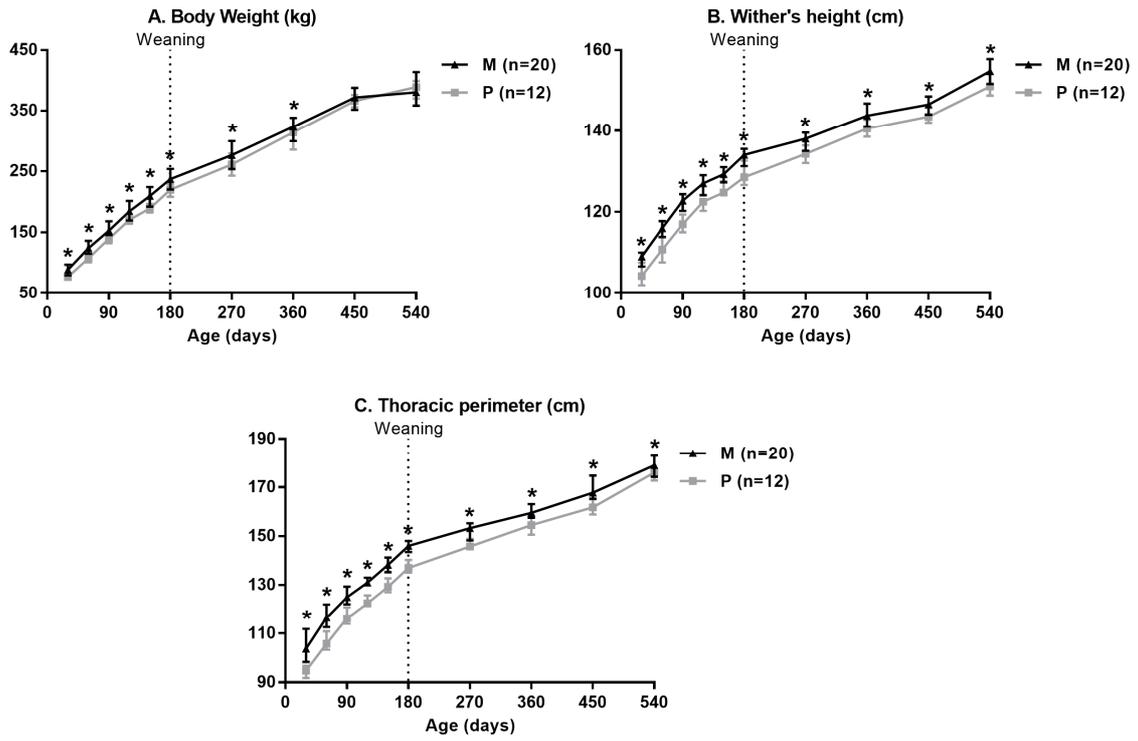


Microcotyledons = microscopic area of fetal maternal contacts

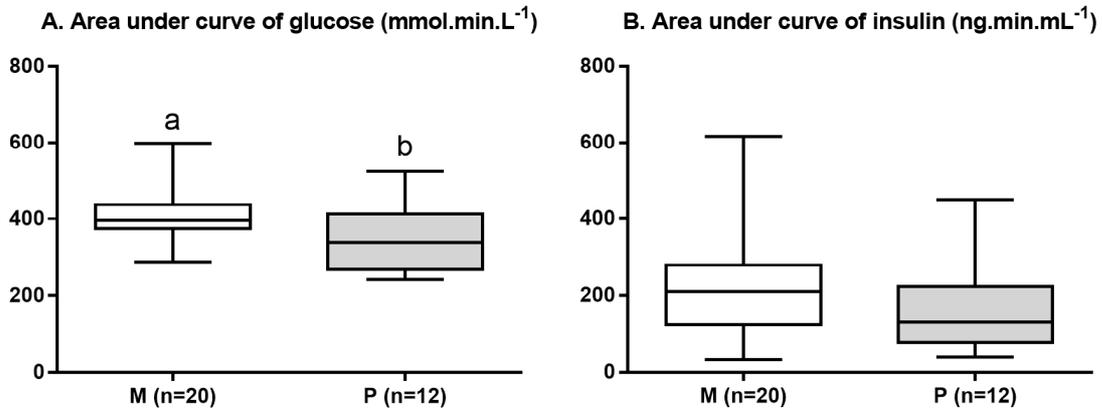
Microcotyledonary region

Allantoic region

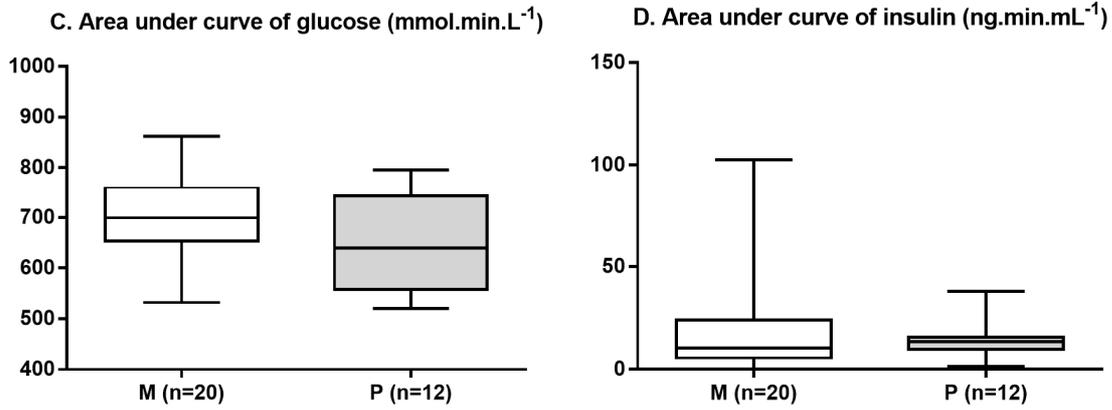




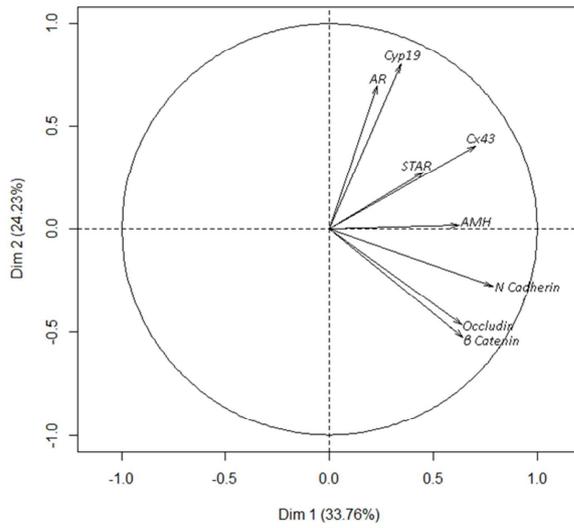
120 days of age



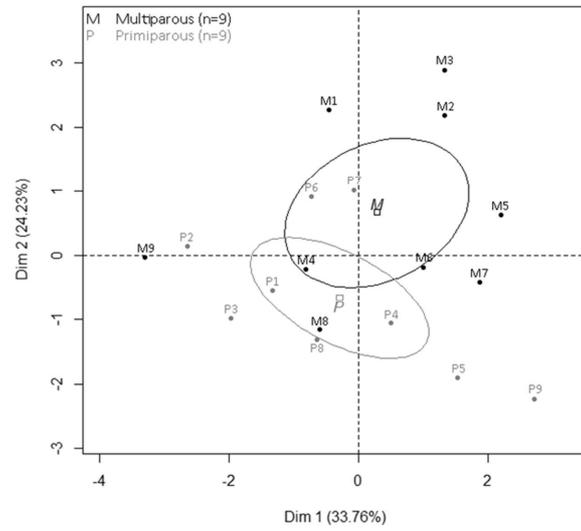
360 days of age



A. Graph of variables



B. Graph of individuals



- Placentas of primiparous mares are lighter with a reduced capacity of histotrophic nutrition at term
- Foals born to primiparous mares are lighter until 12 months of age and smaller until 18 months of age
- Foals born to primiparous mares have less mature metabolic and male reproductive functions

ACCEPTED MANUSCRIPT