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Manuscripts

1 **Environmental constraints and pathologies that modulate**
2 **equine placental genes and development**

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18 Abstract

19 Equine placental development is a long process with unique features. Implantation occurs around 40 days
20 of gestation (dpo) with the presence of a transient invasive placenta from 25-35dpo to 100-120dpo. The
21 definitive non-invasive placenta remains until term (330d). This definitive placenta is diffuse and
22 epitheliochorial, exchanging nutrients, gas and waste with the endometrium through microvilli, called
23 microcotyledons. These are lined by an external layer of haemotrophic trophoblast. Moreover,
24 histotrophic exchange remains active through the histotrophic trophoblast located along the areolae.
25 Placental development is dependent on the maternal environment that can be affected by several factors
26 (*e.g.*, nutrition, metabolism, age, embryo technologies, pathologies) that may affect foetal development
27 as well as long-term offspring health. The first section of the reviews focuses on normal placental
28 development as well as definitive placental structure. Differences between the various regions of the
29 placenta are also highlighted. The latter sections provide an overview of the effects of the maternal
30 environment and reproductive pathologies, respectively, on trophoblast/placental gene expression and
31 structure. So far, only pre-implantation and late gestation/term data are available, which demonstrate an
32 important placental plasticity in response to environmental variation, with genes involved in oxidative
33 stress and tissue differentiation mostly involved in the pre-implantation period, whereas genes involved
34 in foeto-placental growth and nutrient transfers are mostly perturbed at term.

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40 Introduction

41 Gestation in the horse takes approximately 11 months, however large variations in the duration of
42 pregnancy exist between individual mares and between individual pregnancies. Foeto-maternal
43 exchanges are first carried out by a choriovitelline tissue apposition, which then switches to an
44 allantochorion around 25 days post-ovulation (dpo). The allantochorion develops close interactions with
45 the entire endometrium through tree-like structures called microcotyledons and enables nutritional
46 exchanges as well as endocrine support for the pregnancy. In addition to these structures, a subpopulation
47 of trophoblastic cells, unique to the horse, differentiate into transient, invasive trophoblast known as
48 chorionic girdle. Chorionic girdle trophoblast gives rise to the endometrial cups which secrete equine
49 Chorionic Gonadotropin (eCG) from 38 to around 120 days of pregnancy.

50 Microcotyledons branch-out and grow throughout the entire gestation period, allowing the equine
51 placenta to be a particularly adaptable organ. The maternal environment, however, such as nutrition,
52 metabolism and pathologies, can alter placental development and in turn affect the development of the
53 foal.

54 This review starts by briefly covering the normal development of placenta from fertilization, as well as the
55 detailed structure of the definitive organ. This information is provided for the reader to be able to
56 understand the following sections of the review. The second sections focus on how the maternal
57 environment from early gestation (breed, parity, nutrition, assisted reproductive technologies) and
58 pathology (early gestation pathologies, placental retention, hydrops, placentitis) affect placental gene
59 expression and structure. The latter parts of the review mostly focus on late gestation because studies
60 performed in early gestation remain scarce in equidae, and, to our knowledge, none exist for mid-
61 gestation. Because paternal contribution has been recently reviewed elsewhere (Wang *et al.*, 2013; Dini
62 *et al.*, 2021), it is not considered here. For information concerning placental gene expression in normal

63 pregnancies according to cell lineages or time of gestation, refer to the review from Loux et al (XX). In
64 response to nutritional challenges, changes in gene expression mainly affect genes involved in growth and
65 development, nutrient transfer and vascularisation of the trophoblast and are accompanied with
66 concomitant alterations of cotyledonary exchange surfaces.

67

68 **Morphological structure of the placenta throughout gestation**

69 ***Before implantation***

70 The early stages of placental development have been thoroughly described (Allen and Wilsher, 2009).
71 After fertilization, the embryo remains in the oviduct until 6-7 days post-ovulation (dpo) and, at the
72 blastocyst stage, trophoblast cells secrete threonine rich glycoproteins forming a capsule that persists
73 intact until 20-21 dpo (Flood *et al.*, 1982; Oriol *et al.*, 1993; Albiñ *et al.*, 2003). The embryonic vesicle is
74 mobile in the uterus until 16-17 dpo (around 22 mm diameter) when increased myometrial tone prevents
75 embryonic movements. After disintegration of the capsule, trophoblastic cells on the outer layer of the
76 choriovitelline membrane become exposed to luminal epithelium and to endometrial gland secretions,
77 allowing establishment of the first form of adherence of the conceptus to the endometrium and favouring
78 histotrophic nutrition (van Niekerk and Allen, 1975).

79 The allantois originates from the posterior end of the embryonic gut and develops rapidly until it
80 surrounds the embryo and fuses with the chorion to form the allantochorion that will become the main
81 nutritional placenta. At 45 dpo, the allantois has completely replaced the yolk sac, which is incorporated
82 into the umbilical cord that elongates from 40 dpo (Ginther, 1992). Meanwhile, organogenesis takes place
83 and is complete at 35 dpo when the embryo becomes a foetus (van Niekerk and Allen, 1975).

84 ***Implantation and the endometrial cups***

85 Invasive trophoblast cells differentiate from 25 to 35 dpo from the chorionic tissue in the zone of
86 apposition between allantois and yolk sac, forming a cellular girdle of about 0.5 to 1 cm width around the
87 conceptus. The binucleate cells become hyperplastic, develop pseudopods allowing adherence to the
88 endometrium and start migrating at 35-37 dpo along the uterine glands into the endometrial stroma until
89 40-42 dpo. At this stage, these binucleated cells round off and enlarge to form the endometrial cups, i.e.,
90 concave horse-shoe shape structures at the endometrial surface (Allen and Wilsher, 2009). Maximal size
91 and endocrine activity are reached at 70 dpo, followed by gradual degeneration from 80 dpo to complete
92 loss at 100-120 dpo (Antczak *et al.*, 2013). Endometrial cups secrete equine Chorionic Gonadotropin
93 hormone (eCG), possessing FSH- and LH-like properties, that induces the development of secondary
94 corpora lutea (Stewart *et al.*, 1976). They persist until the allantochorion placenta is able to produce
95 enough progestogens to maintain gestation (Ganjam *et al.*, 1975).

96 ***Development of the non-invasive placenta***

97 At 35-38 dpo, the allantois has vascularized more than 90% of the chorion. At 40 dpo, the trophoblast of
98 the allantochorion begins to form microvilli enabling intimate contact with the endometrium (Allen *et al.*,
99 1973). Foetal villi are formed over the whole surface of the conceptus, with the exception of uterine
100 glands' apertures, where the trophoblast develops as a dome called areola, consisting of columnar cells
101 with high absorptive capacity (Allen and Wilsher, 2009). From 60 dpo, villi lengthen and branch intensively,
102 with secondary branches appearing at 100 dpo, to result in microcotyledons around 120 dpo (Samuel *et*
103 *al.*, 1974) that continue to grow until term (Macdonald *et al.*, 2000). Moreover, foeto-maternal exchanges
104 are enhanced as gestation progresses, with the distance between foetal vessels and trophoblast being
105 reduced (fetal vessels have migrated into the trophoblast by 250 dpo) due to the merging of fetal vessels'

106 basal lamina with that of the trophoblastic cells and the reduction of maternal epithelium thickness
107 (Samuel *et al.*, 1976).

108 ***Definitive placentation***

109 In the horse, the definitive placenta is diffuse, with villi covering the whole surface of the uterus by term,
110 and epitheliochorial, because allantochorion is simply affixed over the endometrium. Thus, between 5
111 and 6 cell layers separate maternal from foetal blood, depending on foetal vessel location. The definitive
112 allantochorion is composed of i) the allantois, consisting of an epithelium and a loose, highly vascularized,
113 connective tissue (allantoic mesoderm) on the foetal side (Steven, 1982) and ii) the chorion, consisting in
114 a tightly vascularized connective tissue (chorionic mesoderm) and a pseudostratified epithelium, iii) the
115 histotrophic trophoblast located along the areolae and specialized in absorption of exocrine secretions of
116 the uterine glands (histotrophic nutrition), and iv) microcotyledons (haemotrophic trophoblast,
117 connective tissue and foetal vessels) that insert into the endometrial microcaruncles (**Figure 1**).
118 Microcotyledons maximise the contact area between maternal and foetal epithelia for nutrient and waste
119 exchange, which is further enhanced by the epithelial cells' membrane villi present on the
120 microcotyledons, the haemotrophic trophoblast and on the endometrial epithelial cells (Samuel *et al.*,
121 1976; Steven, 1982) (**Figure 1.B**). At term, the placental structure was shown to differ between uterine
122 regions (corresponding to the uterine body, the pregnant or the non-pregnant horn). In Thoroughbred
123 placentas, the placental villous height was shown to be reduced at the tip of the pregnant horn (but not
124 the base) and allantoic vessels to be larger in the pregnant compared to the non-pregnant horn and the
125 placental body (Oikawa *et al.*, 1990). Moreover, in Brazilian Sport Horse and Mangalarga Paulista
126 placentas, pregnant horn microcotyledons were shown to be less voluminous than that of non-pregnant
127 horn and placental body, with less haemotrophic trophoblast and the volume of the microcotyledonary

128 vessels was increased in the placental body compared to both pregnant and non-pregnant horns
129 (Meirelles *et al.*, 2017).

130 In a recent stereological analysis of 43 placentas collected from French Anglo-Arabian mares, we
131 confirmed the presence of important morphological differences between pregnant horn, non-pregnant
132 horn and the zone of insertion of the umbilical cord (personal data, Robles, Chavatte-Palmer, Aïoun,
133 Geeverding, Dubois, Wimmel) (**Figure 2, Material and Methods available in supplementary data 1**). First,
134 the volume of allantoic connective tissue (allantoic and chorionic mesoderms) was increased in the
135 pregnant compared to the non-pregnant horn and umbilical cord insertion area whereas the surface of
136 the allantoic connective tissue was decreased in the non-pregnant compared to the pregnant horn and
137 umbilical cord insertion area. Nevertheless, the microcotyledonary volume was increased in the non-
138 pregnant compared to the pregnant horn and umbilical cord insertion area, due to an increased volume
139 of haemotrophic trophoblast and microcotyledonary connective tissue, as well as of the
140 microcotyledonary and haemotrophic trophoblast surfaces. These structural differences may result from
141 increased stretching of the pregnant horn due to foetal volume. Moreover, the volume and surface of
142 microcotyledonary vessels were increased at the site of umbilical cord insertion, compared to other
143 locations. Finally, transmission and scanning electronic microscopy confirm the stereology results and
144 show that membrane villi of the haemotrophic trophoblast cells appeared to be less developed in the
145 pregnant compared to the non-pregnant horn (**Figure3**). Despite being diffuse and covering the whole
146 uterus, the equine placental structure differs between sites, which could also be observed for gene and
147 protein expression. But the latter needs to be confirmed in new studies. Therefore, especially for term
148 placenta studies, it is important to describe where placental samples were sampled as differences in
149 adaptation to maternal environment might also be observed between sites.

150 Environmental and maternal factors that impact gene expression

151 The summary of the effects of the maternal environment on placental gene expression is presented in
152 Table 1.

153 **During early gestation: before implantation**

154 ***Asynchronous embryo transfer***

155 Asynchronous embryo transfer corresponds to the transfer of an embryo in a more advanced (positive
156 asynchrony) or delayed (negative asynchrony) uterine environment. Horse embryos tolerate 5 days or
157 more negative asynchrony but are very sensitive to positive uterine asynchrony (Jacob *et al.*, 2012).
158 Moreover, negative asynchrony has been shown to delay embryo development in early gestation (Gibson
159 *et al.*, 2017), as demonstrated through both transcriptomic and candidate gene analyses (Table 1).
160 Transcriptomic analysis showed underrepresentation of functional terms involved in embryo
161 development (central nervous system, metanephros, retina layer formation) in 14-day-old bilaminar yolk-
162 sacs (endoderm and trophoctoderm) and of cell adhesion (cell adhesion, cell-matrix adhesion, regulation
163 of cell shape, positive regulation of cell migration) as well as protein kinase activity and inflammatory
164 response in 19-days old trilaminar yolk-sacs (endoderm, trophoctoderm and mesoderm) (Gibson *et al.*,
165 2020). Finally, using a gene-candidate analysis, decreased expression of genes involved in placental
166 function and foetal growth (**maternal expression:** *H19*, *Insulin-like growth factor 2 receptor (IGF2R)*,
167 *Pleckstrin Homology Like Domain Family A Member 2 (PHLDA2)*, **paternal expression:** *Paternally-expressed*
168 *gene 3 protein (PEG3)* and *Retrotransposon-derived protein (PEG10)*), DNA/RNA processing and cell
169 metabolism (*Insulin receptor (INSR)*) and control of epigenetic regulations (*Histone Acetyltransferase 1*
170 (*HAT1*), *DNA (cytosine-5)-methyltransferase 1 (DNMT1)*, *DNA (cytosine-5)-methyltransferase 3A*
171 (*DNMT3A*), *DNA (cytosine-5)-methyltransferase 3B (DNMT3B)*) was observed in 19 dpo asynchronous
172 compared to synchronous trilaminar yolk-sacs (Gibson *et al.*, 2017). **If more research is needed to**
173 **understand the set up and the role of epigenetic modifications in placental and embryo development in**

174 the horse, these results show that DNA methylation and histone modifications are likely involved in the
175 delayed development observed in asynchronous concepti. Moreover, the difference in expression of
176 imprinted genes highlight their role in early gestation and embryo development and as sensitive markers
177 of early conceptus environment and placental development as observed in other studies (Dini *et al.*,
178 2021).

179 **Obesity**

180 Horses are grazing animals, so their body condition varies during the year, depending on the energy
181 content of the pasture (Dawson and Hone, 2012). Horses are considered overweight when their body
182 condition score exceeds 6 (1-9 scale) (Henneke *et al.*, 1984) or 3.5 (0-5 scale) (Carroll and Huntington,
183 1988; Martin-Rosset *et al.*, 2008) and obese when their body condition is higher than 7/9 or 4/5. Obesity
184 has been shown to affect both placental structure and function in rodents, humans and non-human
185 primates (Shook *et al.*, 2020).

186 To our knowledge, only one study focussing on the effects of obesity on placental function has been
187 performed in the horse (Sessions-Bresnahan *et al.*, 2018). In light breed mares, the effect of maternal
188 obesity (defined as Body Condition Score (BCS)>7 and body fat>10%, increased leptin and insulin serum
189 concentration, and insulin resistance) at ovulation was explored in the trophoblast at 16 dpo using a
190 candidate gene approach (Table 1). The expression of genes involved in fatty acids transport was altered
191 (increased expression of *fatty acid synthase (FASN)* and *long chain fatty acid transport protein 1 (SLC27A1)*
192 whereas the expression of *cluster of differentiation 36 (CD36)* was reduced). The expression of genes
193 involved in oxidative and mitochondrial stress inhibition was also altered (increased expression of
194 *glutaredox-1 (GPX1)* while *thioredoxin-2 (TXN2)* expression was reduced), as well as the expression of
195 genes involved in inflammation (decreased expression of *interleukin-6 signal transducer (IL6ST)*) in obese
196 compared to normal pregnancies (Sessions-Bresnahan *et al.*, 2018). These results were associated with a

197 tendency for a decrease in global DNA methylation in the embryonic discs of the obese group, which
198 confirms that the alteration of maternal environment in obese mares can induce epigenetic modifications
199 in the conceptus. Indeed, the expression of genes involved in fatty acids transport, inflammation, growth
200 factor signalling and/or endoplasmic reticulum stress was altered in D8 embryos and D16 embryonic discs
201 as well as in the endometrium collected from D16 contralateral uterine horns. Finally, obesity was
202 associated with an inflamed uterine environment that altered conceptus gene expression until at least 16
203 dpo. Effects of obesity on placental function later during pregnancy and at term remain, to our knowledge,
204 unexplored in the equine species. Nevertheless, maternal obesity throughout gestation was shown to
205 promote chronic inflammation and affect glucose metabolism, as well as osteoarticular development in
206 offspring until at least 18 months of age (Robles *et al.*, 2018a).

207 ***Omega-3 fatty acids supplementation***

208 Omega-3 fatty acids are essential fatty acids known to have immunomodulatory and anti-inflammatory
209 effects in horses (Warren and Vineyard, 2013). Fresh grass is rich in omega-3 fatty acids (Dewhurst *et al.*,
210 2001). Cereals, conversely, are rich in omega-6 fatty acids, known for their pro-inflammatory effects (Ryan
211 *et al.*, 2007). Modern diets of horses, which are mainly composed of cereals and conserved forages, are
212 far richer in omega-6 than pasture, which means that supplementation of horse feeds with omega-3
213 sources such as linseed, for instance, may have positive effects on horse health. Because of their
214 immunomodulatory effects, but also their roles in neurodevelopment and metabolism as shown in other
215 species (Buddington *et al.*, 2018; Madore *et al.*, 2020; Shrestha *et al.*, 2020), these fatty acids may affect
216 embryo implantation as well as placental and foetal development.

217 Overweight light breed mares (mean BCS of 7/9) were supplemented with an algae-derived product, rich
218 in omega-3 fatty acids (especially in docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids), from 45
219 days before ovulation (Jacobs *et al.*, 2018). Mares supplemented with omega-3 had increased

220 incorporation of DHA and total omega-3 fatty acids in the endometrium compared to control mares.
221 Moreover, gene expression of markers of trophoctoderm differentiation (*Trans-acting T-cell-specific*
222 *transcription factor (GATA3)*, *Transcription factor AP-2 alpha (TFAP2A)* and *E74 Like ETS Transcription*
223 *Factor 3 (ELF3)*) as well as primitive endoderm differentiation (*GATA binding protein 4 (GATA4)* and *GATA*
224 *binding protein 6 (GATA6)*) was increased in D12.5 conceptuses from omega-3 supplemented mares
225 compared to control (Table 1) (Jacobs *et al.*, 2018).

226 Thus, omega-3 fatty acids supplementation could help improve early placental development, at least in
227 overweight animals, but these effects need to be confirmed in other studies to determine if they translate
228 to changes in protein expression and consequently wider benefits for the developing fetus. Effects of
229 omega-3 supplementation on late-pregnancy and term placental function and structure, as well as on
230 development of the offspring, remain to be investigated.

231 **During late gestation: last third of gestation**

232 ***Effect of breed***

233 The different breeds of horses have been either selected by humans to fulfil specialized activities, such as
234 racing (Thoroughbred), sport (warmblood horses) or traction (draft horses) for example. Some have been
235 adapted to harsh environments, such as severe winter (ponies) or dry and hot environments (Arabians
236 and Iberian horses). From these specificities, different sizes and formats, as well as metabolic specificities,
237 were derived. For instance, ponies and Iberian horses have been shown to be more insulin resistant than
238 Saddlebred horses (Bamford *et al.*, 2014; Peugnet *et al.*, 2014).

239 In alignment with adult size, ponies are born lighter than Thoroughbred and French Anglo-Arabian horses,
240 that are born lighter than draft horses. These differences between breeds are correlated with placental
241 surface area (Allen *et al.*, 2002; Robles *et al.*, 2018b) and associated with variation in placental function.

242 The placenta of ponies appears to be the most efficient, as the birthweight to placental surface area ratio
243 is increased compared to French Anglo-Arabians and draft horses. Moreover, the surface density (surface
244 per volume unit, μm^{-1}) and volume fraction (volume per volume unit, %) of microcotyledonary vessels are
245 increased in pony compared to French Anglo-Arabian placentas (Robles *et al.*, 2018b). Surface density and
246 surface of microcotyledons, however, were shown to be reduced in pony compared to Thoroughbred
247 term placentas (Allen *et al.*, 2002) (**Figure 4.A**). This could mean that despite a decreased placental
248 exchange surface in ponies, there was a placental vascular compensation that could explain the increased
249 placental efficiency observed in the small breed compared to bigger horses. Moreover, the expression of
250 genes involved in growth and development is reduced in pony compared to Saddlebred and draft (*insulin-*
251 *like growth factor 2 (IGF2)* and its receptor *IGF2R*), and in Saddlebred compared to draft placentas (*IGF2*)
252 (Table 1). The expression of genes involved in nutrient transfer is reduced in pony compared to Saddlebred
253 placentas (*solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1)*, *sodium-coupled*
254 *neutral amino acid transporter 2 (SLC38A2)*, *lipoprotein lipase (LPL)*). Finally, genes involved in
255 vascularization are expressed less in pony and Saddlebreds compared to draft placentas (*Fms related*
256 *tyrosine kinase 1 (FLT1)*, *kinase insert domain receptor (KDR)*) (Robles *et al.*, 2018b). These latter results
257 indicate that genes directly or indirectly involved in foetal growth are less expressed in pony placentas
258 compared to larger breeds, which is in agreement with the smaller size of the breed.

259 Altogether, these results highlight the fact that there are differences between breeds in terms of placental
260 structure and function, at least at term, and that results observed in one breed may not be reproducible
261 in another one. Uterine size and maternal metabolism thus affect foeto-placental development and
262 should be considered when designing a study. To our knowledge, early-pregnancy placental development
263 has not been compared between breeds.

264 ***Effect of maternal parity and age***

265 In the equine species, maternal parity has been shown to have strong effects on birth weight, growth and
266 metabolism of foals. Primiparous mares produce lighter and smaller foals until at least 18 months of age
267 (Wilsher and Allen, 2003; Elliott *et al.*, 2009; Meirelles *et al.*, 2017; Robles *et al.*, 2018b, c). In addition, the
268 maturation of metabolic and male reproductive functions appears to be delayed in these foals (Robles *et*
269 *al.*, 2018c). As a result, foals born to primiparous dams might be slightly less performant than foals born
270 to multiparous mares, at least in racing (Barron, 1995) and show jumping (Palmer *et al.*, 2018).

271 Indeed, first pregnancies are different from subsequent ones. The uterine artery diameter and blood flow
272 volume is reduced in mares that foaled two foals or less compared to mares with a higher parity (Klewitz
273 *et al.*, 2015). In terms of placental structure, haemotrophic (microcotyledons) (Wilsher and Allen, 2003;
274 Meirelles *et al.*, 2017) and histotrophic (Robles *et al.*, 2018c) exchange surfaces are reduced in term
275 placentas of primiparous compared to multiparous dams (**Figure 4.B**). Finally, the expression of genes
276 involved in growth and development, such as *insulin-like growth factor 1 receptor (IGF1R)*, *transforming*
277 *growth factor beta 1 (TGFβ1)* and *epidermal growth factor receptor (EGFR)* and in vascularization, such as
278 *vascular endothelial growth factor A (VEGFα)* is reduced in primiparous compared to multiparous term
279 placentas (Robles *et al.*, 2018c, 2019) (Table 1). Conversely, the expression of genes involved in protein
280 metabolism and transport (*arginase 2 (ARG2)*, *ornithine decarboxylase 1 (ODC1)* and *high affinity cationic*
281 *amino acid transporter 1 (SLC7A1)*) is increased in primiparous compared to multiparous term placentas,
282 which could reduce the foeto-maternal amino acid transfer capacity in primiparous placentas (Robles *et*
283 *al.*, 2019). These results are consistent with the effects of primiparity observed on uterine blood flow
284 (Klewitz *et al.*, 2015) and placental morphometry at birth (Wilsher and Allen, 2003; Elliott *et al.*, 2009;
285 Meirelles *et al.*, 2017; Robles *et al.*, 2018c).

286 Effects of decreased placental development and function may be enhanced by a decreased uterine ability
287 to expand, as measured through a decreased placental gross surface at birth in primiparous pregnancies

288 (Wilsher and Allen, 2003), but also by an inability of primiparous dams to adapt their metabolism to
289 pregnancy (Robles *et al.*, 2019). Generally, in late gestation, pregnant mares become less glucose tolerant
290 and produce less insulin in response to an increased glycaemia, in order to maximize the placental transfer
291 of glucose to the foetus (Fowden *et al.*, 1984). Higher postprandial insulin secretion was observed in
292 primiparous vs multiparous mares, however, and it was hypothesized that these elevated insulin
293 concentrations could reduce glucose availability for transfer to the foetus (Robles *et al.*, 2019). Finally, a
294 reduced global DNA methylation was observed in placentas of primiparous compared to multiparous
295 dams, suggesting that epigenetic modifications may also play a role in parity-related variations in placental
296 development (Robles *et al.*, 2019).

297 Maternal parity is positively correlated to maternal age, and as a result, maiden mares are also often
298 young mares. Maternal age has also been shown to affect the weight of placenta and foal at birth as young
299 (<9 years old) and old (>16 years old) mares produce lighter foals than mares aged between 9 and 16 years
300 (Wilsher and Allen, 2003; Elliott *et al.*, 2009). These observations have been correlated to placental
301 structure as microcotyledonary surface density decreases as maternal age increases (Wilsher and Allen,
302 2003). Moreover, together with mare ageing, the endometrium undergoes fibrotic degenerative changes
303 that can lead to endometriosis and therefore affect placental development. To our knowledge, the effect
304 of maternal age on placental gene expression has not been studied. Effects of both age and parity on
305 placental and embryo/foetal development remain to be investigated.

306 ***Effect of assisted reproductive technologies (ART)***

307 ART are increasingly used to produce foals from high genetic value mares (**Figure 5**) in order to increase
308 the number of foals born to a mare each year. This enables the production of foals while the mare
309 continues to compete and the production of foals from infertile mares. To our knowledge, apart from
310 equine pregnancies following nuclear transfer where placental abnormalities have been described

311 (Johnson *et al.*, 2010; Pozor *et al.*, 2016), reports of developmental or pregnancy issues following ART in
312 the horse are non-existent, despite the fact that ART have been shown to be associated with
313 developmental and pregnancy issues in human and other animals (Duranton and Chavatte-Palmer,
314 2018). In the horse, commercial ART (embryo transfer (ET) and intracytoplasmic sperm injection (ICSI))
315 was shown to have no effect on the placental expression of 19 genes involved in growth and development,
316 vascularization and nutrient transfer, nor on placental morphology (Valenzuela *et al.*, 2018).

317 When embryo transfers are performed between breeds of different size, however, effects on placental
318 structure and function are observed. Pony embryos transferred to Thoroughbred or draft mares were
319 born heavier, with a heavier and larger placenta than pony embryos carried by pony mares (Allen *et al.*,
320 2002; Robles *et al.*, 2018d). These differences in foeto-placental biometry were associated with different
321 placental structure alterations, depending on whether the embryo was transferred into a Thoroughbred
322 or a draft mare (**Figure 4.C**):

323 - Thoroughbred: The surface density and surface of microcotyledons was increased in transferred
324 compared to pony-in-pony placentas (Allen *et al.*, 2002).

325 - draft: The surface density of trophoblastic tissues (haemotrophic and histotrophic trophoblasts) was
326 decreased in transferred compared to pony-in-pony placentas (Robles *et al.*, 2018d).

327 The gene expression of *sodium-coupled neutral amino acid transporter 1 (SLC38A1)*, involved in amino
328 acid transfer, was increased in term placentas of pony embryos transferred to draft mares. Despite
329 modifications in placental structure, the placental expression of genes involved in growth and
330 development or vascularization did not differ between both groups (Robles *et al.*, 2018d).

331 In contrast, French Anglo-Arabian/Thoroughbred embryos transferred into pony mares were born lighter,
332 with a lighter placenta than that of Saddlebred-in-Saddlebred foals (Allen *et al.*, 2002; Robles *et al.*,

333 2018d). These differences were associated to alterations in placental structure that differed depending
334 on whether the embryo was transferred into a Thoroughbred or a French Anglo-Arabian mare (**Figure**
335 **4.C**):

336 - Thoroughbred: The surface density and surface of microcotyledons was decreased in transferred
337 compared to control placentas (Allen *et al.*, 2002).

338 - French Anglo-Arabian: The surface density and volume fraction of microcotyledonary vessels were
339 increased in placentas of transferred compared to control embryos (Robles *et al.*, 2018d).

340 Moreover, the expression of genes involved in growth and development (**H19, IGF2 IGF2R and IGF1R**) and
341 nutrient transfer (**SLC2A1, SLC38A2 and LPL**) were decreased in placentas of transferred compared to
342 control embryos (Robles *et al.*, 2018d). These results may result from vascular placental adaptations in
343 order to ensure nutrient transfers to the foetus despite the limitation imposed by uterine size.

344 These results coincided, however, with differences in methodology (differences in parity, body condition
345 score, feeding and housing between groups) at least in the Anglo-Arabian study (Peugnet *et al.*, 2014).

346 These differences need therefore to be confirmed and further studied.

347 ***Effect of maternal nutrition and metabolism***

348 **Effects of starch and insulin resistance**

349 Horses are monogastric herbivorous animals that have evolved to eat grass all day (Martin-Rosset *et al.*,
350 1978). In modern practice, horses are housed in boxes or in small pastures and are often fed conserved
351 forages that do not have the quality of fresh grass. To meet their needs in energy and proteins, they are
352 also fed with concentrated feeds, composed of cereals, rich in starch and sugar. Supplementation with
353 starch-rich concentrates has been associated with metabolic alterations, such as insulin resistance, in both

354 pregnant (Peugnet *et al.*, 2015) and non-pregnant horses (Bamford *et al.*, 2016). Moreover, these diets
355 are associated with increased risks of developing osteochondrosis lesions in growing foals (Vander Heyden
356 *et al.*, 2013; Peugnet *et al.*, 2015), as well as decreased colostrum quality at birth (Caure and Lebreton,
357 2004; Thorson *et al.*, 2010; Robles, 2017).

358 An increase in allantoic arteriole vascular wall thickness (**Figure 4.D**) and a decrease in microcotyledonary
359 vessels volume have been observed in term placentas of mares fed with cereals (flattened barley,
360 3.5kg/day, last 4.5 months of gestation) compared to mares fed with forages only (Robles *et al.*, 2018e).
361 These vascular alterations were also linked to modifications in the enrichment of gene sets involved in
362 placental vascularization, inflammation, coagulation and collagen production in mares fed with
363 concentrates (Robles *et al.*, 2018e). Placental inflammation may therefore also be involved in the
364 mediation of the effects of cereal feeding in broodmares on development of osteoarticular diseases in
365 foals.

366 Insulin resistance is also associated with chronic inflammation. Moreover, increased vascular wall
367 thickness (vascular fibrosis) of allantoic arterioles as well as fibrosis in microcotyledons were reported in
368 term placentas from laminitic broodmares (Pazinato *et al.*, 2017).

369 **Effects of maternal undernutrition**

370 Maternal moderate undernutrition does not seem to affect foals birthweight, nor placental
371 measurements and efficiency (Henneke *et al.*, 1984; Peugnet *et al.*, 2015; Robles *et al.*, 2018e). Despite
372 the lack of effect on placental gross morphology at term, placentas from slightly underfed mares fed with
373 forage only (very poor in soluble carbohydrates but rich in fibres) had an enrichment in gene sets involved
374 in amino acids, folate and anions transport, and amino acids, fatty acids, cholesterol and folate catabolism
375 compared to mares fed at 100% of their needs but with concentrates in addition to forage (Robles *et al.*,
376 2018e). This observation indicates that placental metabolism is able to adapt to the source of energy.

377 Placental cells seem to rely primarily on the amino acids and fatty acids catabolism to produce energy
378 when the mare is underfed but with forage only. These placental adaptations could then partly explain
379 why moderate undernutrition did not affect the weight of foals at birth but affected their long-term
380 development (Peugnet *et al.*, 2015; Robles *et al.*, 2017).

381 **Effects of L-arginine supplementation**

382 L-arginine is an essential amino acid during pregnancy and growth in the horse. Compared to other
383 species, the milk of mares is particularly rich in L-arginine, which indicates that L-arginine needs are high
384 in growing foetuses and foals. In the horse, L-arginine supplementation during pregnancy (100 g/day, for
385 21 days before foaling) has been shown to increase uterine artery blood flow in the non-pregnant horn
386 and to decrease the gestational length without affecting, however, foal and placental biometry at birth
387 (Mortensen *et al.*, 2011). In this study, mares' parity was not provided, although effects of L-arginine
388 supplementation may differ between primiparous and multiparous dams. Indeed, L-arginine
389 supplementation (100 g/day during the last 4.5 months of gestation) was shown to affect the metabolism
390 of primiparous but not multiparous mares, leading to an increased metabolic adaptation to pregnancy in
391 supplemented compared to control primiparous mares (Robles *et al.*, 2019). These modifications of
392 maternal environment may have also affected placental function as expression of **SLC2A1 and CD36**, were
393 increased in arginine supplemented primiparous placentas, compared to placentas from control
394 primiparous mares (Robles *et al.*, 2019).

395 **Pathologies that impact on placental gene expression**

396 **Early pregnancy**

397 Pathologies of pregnancy can lead to pregnancy failure at any stage between fertilisation and parturition.
398 Early pregnancy loss (Early Pregnancy Loss, EPL) is the most common occurrence in the mare and includes

399 both embryo loss prior to clinical detection and the 6-12% clinically confirmed pregnancies that are lost
400 within two months of conception (Bosh *et al.*, 2009; Rose *et al.*, 2018). For many years, the
401 characterisation of gene expression in the placentae of failed early pregnancies (EPL) was hampered by a
402 lack of materials and methodologies. This has now been partly overcome (Rose *et al.*, 2016), although
403 data indicated that the quality of the placental tissue was, in the majority of cases, only suitable for
404 genomic studies and to some degree histological analysis. Applying these methodologies to develop a
405 large biobank of EPL tissue, Shilton *et al.*, 2020 reported aneuploidy in the allantochorion or chorion of
406 22% of EPLs, suggesting this condition is a common reason for pregnancy to fail before 65 days.
407 Aneuploidy is a genetic imbalance that arises due to loss or gain of a whole chromosome. Of 55 EPL
408 assessed, trisomy was identified in 16.3% and monosomy in 5.4% of placental membranes (Shilton *et al.*,
409 2020) with the latter almost certainly lethal with no surviving monosomies reported. The phenotype of
410 mammalian trisomies is more varied: some are associated with miscarriage alone, whilst others, like the
411 well reported human trisomies 13, 18, 21, can result in the birth of a live infant. Preliminary data in the
412 mare suggests the size of the chromosome may dictate whether the trisomy is embryonic lethal or can
413 produce a greater spectrum of phenotypes (Shilton *et al.*, 2020). It is plausible that such copy number
414 changes disrupts gene expression in the placenta, although this needs to be confirmed experimentally.

415 A second pathology of early pregnancy is endometritis that is associated with approximately 5-10% of EPL
416 cases in well managed mares (Ricketts, 2003; Rose *et al.*, 2018). Whilst changes in endometrial gene
417 expression in response to the presence of endometritis have been reviewed (Morris *et al.*, 2020), the
418 genes and signalling pathways within the placental membranes following infection have not been
419 investigated which is in stark contrast to the extensive work on placentitis in late gestation, discussed
420 below. Clearly there is still much to do to better understand the key genes and signals in the early placenta
421 that can lead to EPL. The recent description of defects in the brain and cardiovascular systems of equine
422 embryos/foetuses from clinical cases of EPL (Kahler *et al.*, 2020), along with mounting data linking such

423 defects to placental aberrations in rodent models (Woods *et al.*, 2018), certainly suggest this is an avenue
424 worth pursuing.

425 **Placentitis**

426 Placental infection (placentitis) is one of the major causes of late-term abortion in mares, responsible for
427 the loss of 1-5% of all pregnancies (Lyle, 2014). Despite its prevalence, most published gene-related
428 research has focused on experimental models rather than clinical cases. This has the advantage of
429 facilitated sampling and a better-defined timeline; however, the course of disease can differ significantly,
430 with a more acute, severe reaction seen in response to induced placentitis. As an inflammatory disease,
431 much of the early work focused on the pro-inflammatory cytokine response, identifying an early up-
432 regulation of *interleukin 6 (IL-6)* and *interleukin 8 (IL-8)* at the cervical star (LeBlanc *et al.*, 2002) and
433 *interleukin 18 (IL-18)*, *interleukin 15 (IL-15)* and *interferon γ (INF γ)* in the placental body (Lyle *et al.*, 2009).
434 The balance of cytokines has since been revealed to be more complex, with the maternal response being
435 primarily pro-inflammatory, while modulated by the foetus through production of *interleukin 10 (IL-10)*
436 and *IL-6* (Fedorka *et al.*, 2019). The myometrial and cervical response to placentitis are quite similar to the
437 allantochorion, with a similar up-regulation of both pro-inflammatory and modulatory cytokines
438 (*interleukin 1* \square) *IL-1* \square and *IL-8* in both, *IL-6*, *tumor necrosis factor α (TNF α)* and *interleukin 10 (IL-10)* in
439 cervix) (El-Sheikh Ali *et al.*, 2019; Fernandes *et al.*, 2020).

440 Use of NGS identified toll-like receptor signalling as an important pathway for the myometrial immune
441 response, as well as 8 transcripts which were placentitis specific (*Matrix Metalloproteinase 1 (MMP1)*,
442 *Matrix Metalloproteinase 8 (MMP8)*, *S100 Calcium Binding Protein A8 (S100A8)*, *S100 Calcium Binding*
443 *Protein A9 (S100A9)*, *Peptidase Inhibitor 3 (PI3)*, *apolipoprotein B mRNA-editing enzyme-catalytic*
444 *polypeptide-like 3Z1b (APOBEC3Z1B)*, *resistin (RETN)* and *C-X-C Motif Chemokine Ligand 2 (CXCL2)*). These
445 genes are all involved in regulation of immune and inflammatory processes and may be involved in

446 placentitis myometrial activation and initiation of preterm birth. This work also proposed *Signal*
447 *Transducer And Activator Of Transcription 1 (STAT1)* as an up-stream regulator for placentitis, while *Signal*
448 *Transducer And Activator Of Transcription 3 (STAT3)* may regulate transcriptional changes in the pre-term
449 period (El-Sheikh *et al.*, 2020a).

450 The endocrine response to placentitis is well-characterized, however, the underlying gene-level changes
451 have only recently come under scrutiny. Expression of *Steroid 5 Alpha-Reductase 1 (SRD5A1)* and *Aldo-*
452 *keto reductase family 1 member C23 (AKR1C23)*, key progesterone metabolizing enzymes, were reduced
453 in the allantochorion, corresponding to a decrease in 5 α -reduced progestogens in the same tissues.
454 Likewise, aromatase (*CYP19A1*) expression trended down in the allantochorion, a trend accompanied by
455 lower estrone and oestrogen receptor (*ESR1*) concentrations (El-Sheikh *et al.*, 2020b). A tissue-specific
456 response is evident, as *ESR1* is instead up-regulated in cervical stroma during placentitis, as are
457 prostaglandin synthases *prostaglandin E synthase (PTGES)* and *Prostaglandin-Endoperoxide Synthase 2*
458 *(PTGS2)* (Fernandes *et al.*, 2020). These recent studies showcase the multi-faceted nature of placentitis,
459 including robust maternal and foetal immune responses far beyond the placenta, with each tissue
460 exhibiting its own unique response to placentitis.

461 **Hydrops**

462 Hydrops occurs when excess foetal fluid builds up during gestation. It can affect either the allantoic
463 (hydroallantois) or amniotic (hydroamnios) fluid cavity. Although little information exists on the aetiology
464 of hydrops, hydroallantois appears to be associated with impaired placental angiogenesis. Not only do
465 hydroallantoic placentae exhibit reduced capillary density, but also gene expression changes are indicative
466 of altered angiogenesis and hypoxic changes. Specifically, *hypoxia-induced gene Hypoxia-inducible factor*
467 *1-alpha (HIF1A)* is up-regulated, as is *VEGF*, a known inducer of vascular permeability. Meanwhile, *VEGF*
468 receptors *VEGFR1* and *VEGFR2* are downregulated. Evidence for the involvement of oestrogen

469 dysfunction also exists, as both *CYP19A1* and oestrogen receptors *ESR1* and *ESR2* are down-regulated (Dini
470 *et al.*, 2020). Although preliminary, this work sheds light on underlying gene dysregulation associated with
471 hydroallantois, as well as highlighting potential consequences for insufficient angiogenesis during
472 placentation.

473 **Placental retention**

474 Placental retention is defined as a failure to expel foetal membranes within 3 hours of delivery. This
475 condition is common in the horse, especially in draft horse breeds, but its aetiology is still poorly
476 understood. Placental retention causes endometritis and can lead to life threatening diseases such as
477 endotoxemia, laminitis and septicaemia. Histologically, at term, placental retention has been associated
478 with fibrosis in the microcotyledons, overdeveloped stromal connective tissue in the non-pregnant horn
479 (retained horn), oversized histotrophic epithelium and sparser and shorter villi (microcotyledons) in draft
480 mares' placentas (Rapacz *et al.*, 2012; Rapacz-Leonard *et al.*, 2015). These structural alterations were
481 associated with a higher apoptotic index in both haemotrophic and histotrophic trophoblasts, as well as
482 in both luminal and glandular endometrial epitheliums (Paździor *et al.*, 2012). Increased gene and protein
483 expression of matrix metalloproteinase-2 (MMP-2) were also observed, together with increased gene
484 expression of matrix metalloproteinase-9 (MMP-9) and decreased expression of Tissue inhibitor of
485 metalloproteinases 2 (TIMP-2) in retained compared to control allantochorions (Rapacz-Leonard *et al.*,
486 2015). Moreover, MMP-2 activity was reduced in retained compared to control placentas. Gene
487 expression of *IL-1 β* and *IL-6* (as well as protein expression of IL-6) were also increased in retained
488 allantochorions (Jaworska and Janowski, 2019). Gene expression of prostaglandin endoperoxide synthase-
489 2 (*PTGS2*), involved in prostaglandin production, was however decreased in retained placenta which could
490 contribute to the observed imbalance in inflammatory processes and tissue remodelling in this pathology
491 (Rapacz-Leonard *et al.*, 2020). Finally, protein expression of oxytocin receptors (OXTR) was greatly

492 reduced in retained placentas in case of secondary atony, but this difference was not observed in retained
493 allantochorion without secondary atony (Rapacz-Leonard *et al.*, 2015; Rapacz-Leonard *et al.*, 2020). From
494 these results, it can be concluded that abnormal placental development results from abnormal epithelial
495 regeneration, as well as abnormal extracellular matrix remodelling and inflammatory signalling.

496 ***Streptococcus equi* infection**

497 *Streptococcus equi*, or strangles, is highly contagious disease observed worldwide in horses (Mitchell *et*
498 *al.*, 2021). The condition is characterized by the development of abscesses in the head and neck lymph
499 nodes, pharyngitis, as well as fever and lethargy (Boyle *et al.*, 2018). In one study, young (3-4 years old)
500 primiparous mares were infected with *Streptococcus equi* between 14 and 161 days of gestation. No
501 antibiotics were administered. In addition to other symptoms (such as fever, nasal discharges, inflamed
502 and swollen lymph nodes and depression), this infection led to a severe undernutrition for a median of 3
503 weeks. Infected mares had a decreased placental microcotyledonary surface (**Figure 4.D**), leading to a
504 decreased birth weight of their foals. Placental function, however, was not investigated in this study
505 (Wilsher and Allen, 2006).

506

507 **Conclusion and final remarks**

508 Despite all the new information that has been provided during the last decade, a lot of unknowns remain
509 concerning factors affecting placental development in horses. So far, apart from one study that performed
510 RNA sequencing (RNAseq) to analyse differences in gene expression between two nutritional conditions,
511 all reports comparing gene expression in equine placenta in response to environment at large used a RT-
512 qPCR candidate gene approach, thus greatly limiting the possibility to uncover unexpected mechanisms
513 that could be highlighted by high throughput approaches. Even though RT-qPCR approaches provide

514 valuable information as gene expression is compared between equal total RNA concentrations per
515 sample, variations in placental size and in gene expression between placental zones may affect total
516 placenta expression and the conclusions that can be drawn from results. Moreover, there might be a
517 change in cell composition between samples that is not controllable in the field but could be corrected
518 with the use of trophoblastic cells marker genes for example (see Loux et al., XX for more information on
519 gene expression between cell lines). It is thus always important to combine gene expression approaches,
520 whether limited to a few candidates or unbiased through high throughput strategies, with physiological
521 and structural observations.

522 In any case, RNseq approaches are essential because they provide an unbiased analysis necessary for
523 understanding i) the pathogenesis of placental disease in order to help the design of therapeutic
524 treatments and ii) the processes leading to the programming of foal's health in order to elaborate
525 strategies to maximise health, welfare, performance and longevity in horses. Additionally, more refined
526 methodologies such as single cell RNAseq would be useful to determine the respective contribution of
527 different cell lines in placental gene expression.

528

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530 The authors have no interest conflicts to declare.

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For Review Only

1 **Environmental constraints and pathologies that modulate**
2 **equine placental genes and development**

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18 **Abstract**

19 Equine placental development is a long process with unique features. Implantation occurs around 40 days
20 of gestation (dpo) with the presence of a transient invasive placenta from 25-35dpo to 100-120dpo. The
21 definitive non-invasive placenta remains until term (330d). This definitive placenta is diffuse and
22 epitheliochorial, exchanging nutrients, gas and waste with the endometrium through microvilli, called
23 microcotyledons. These are lined by an external layer of haemotrophic trophoblast. Moreover,
24 histotrophic exchange remains active through the histotrophic trophoblast located along the areolae.
25 Placental development is dependent on the maternal environment that can be affected by several factors
26 (*e.g.*, nutrition, metabolism, age, embryo technologies, pathologies) that may affect foetal development
27 as well as long-term offspring health. The first section of the reviews focuses on normal placental
28 development as well as definitive placental structure. Differences between the various regions of the
29 placenta are also highlighted. The latter sections provide an overview of the effects of the maternal
30 environment and reproductive pathologies, respectively, on trophoblast/placental gene expression and
31 structure. So far, only pre-implantation and late gestation/term data are available, which demonstrate an
32 important placental plasticity in response to environmental variation, with genes involved in oxidative
33 stress and tissue differentiation mostly involved in the pre-implantation period, whereas genes involved
34 in foeto-placental growth and nutrient transfers are mostly perturbed at term.

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40 Introduction

41 Gestation in the horse takes approximately 11 months, however large variations in the duration of
42 pregnancy exist between individual mares and between individual pregnancies. Foeto-maternal
43 exchanges are first carried out by a choriovitelline tissue apposition, which then switches to an
44 allantochorion around 25 days post-ovulation (dpo). The allantochorion develops close interactions with
45 the entire endometrium through tree-like structures called microcotyledons and enables nutritional
46 exchanges as well as endocrine support for the pregnancy. In addition to these structures, a subpopulation
47 of trophoblastic cells, unique to the horse, differentiate into transient, invasive trophoblast known as
48 chorionic girdle. Chorionic girdle trophoblast gives rise to the endometrial cups which secrete equine
49 Chorionic Gonadotropin (eCG) from 38 to around 120 days of pregnancy.

50 Microcotyledons branch-out and grow throughout the entire gestation period, allowing the equine
51 placenta to be a particularly adaptable organ. The maternal environment, however, such as nutrition,
52 metabolism and pathologies, can alter placental development and in turn affect the development of the
53 foal.

54 This review starts by briefly covering the normal development of placenta from fertilization, as well as the
55 detailed structure of the definitive organ. This information is provided for the reader to be able to
56 understand the following sections of the review. The second sections focus on how the maternal
57 environment from early gestation (breed, parity, nutrition, assisted reproductive technologies) and
58 pathology (early gestation pathologies, placental retention, hydrops, placentitis) affect placental gene
59 expression and structure. The latter parts of the review mostly focus on late gestation because studies
60 performed in early gestation remain scarce in equidae, and, to our knowledge, none exist for mid-
61 gestation. Because paternal contribution has been recently reviewed elsewhere (Wang *et al.*, 2013; Dini

62 *et al.*, 2021), it is not considered here. For information concerning placental gene expression in normal
63 pregnancies according to cell lineages or time of gestation, refer to the review from Loux et al (XX).

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65 **Morphological structure of the placenta throughout gestation**

66 ***Before implantation***

67 The early stages of placental development have been thoroughly described (Allen and Wilsher, 2009).
68 After fertilization, the embryo remains in the oviduct until 6-7 days post-ovulation (dpo) and, at the
69 blastocyst stage, trophoblast cells secrete threonine rich glycoproteins forming a capsule that persists
70 intact until 20-21 dpo (Flood *et al.*, 1982; Oriol *et al.*, 1993; Albiñ *et al.*, 2003). The embryonic vesicle is
71 mobile in the uterus until 16-17 dpo (around 22 mm diameter) when increased myometrial tone prevents
72 embryonic movements. After disintegration of the capsule, trophoblastic cells on the outer layer of the
73 choriovitelline membrane become exposed to luminal epithelium and to endometrial gland secretions,
74 allowing establishment of the first form of adherence of the conceptus to the endometrium and favouring
75 histotrophic nutrition (van Niekerk and Allen, 1975).

76 The allantois originates from the posterior end of the embryonic gut and develops rapidly until it
77 surrounds the embryo and fuses with the chorion to form the allantochorion that will become the main
78 nutritional placenta. At 45 dpo, the allantois has completely replaced the yolk sac, which is incorporated
79 into the umbilical cord that elongates from 40 dpo (Ginther, 1992). Meanwhile, organogenesis takes place
80 and is complete at 35 dpo when the embryo becomes a foetus (van Niekerk and Allen, 1975).

81 ***Implantation and the endometrial cups***

82 Invasive trophoblast cells differentiate from 25 to 35 dpo from the chorionic tissue in the zone of
83 apposition between allantois and yolk sac, forming a cellular girdle of about 0.5 to 1 cm width around the
84 conceptus. The binucleate cells become hyperplastic, develop pseudopods allowing adherence to the
85 endometrium and start migrating at 35-37 dpo along the uterine glands into the endometrial stroma until
86 40-42 dpo. At this stage, these binucleated cells round off and enlarge to form the endometrial cups, i.e.,
87 concave horse-shoe shape structures at the endometrial surface (Allen and Wilsher, 2009). Maximal size
88 and endocrine activity are reached at 70 dpo, followed by gradual degeneration from 80 dpo to complete
89 loss at 100-120 dpo (Antczak *et al.*, 2013). Endometrial cups secrete equine Chorionic Gonadotropin
90 hormone (eCG), possessing FSH- and LH-like properties, that induces the development of secondary
91 corpora lutea (Stewart *et al.*, 1976). They persist until the allantochorion placenta is able to produce
92 enough progestogens to maintain gestation (Ganjam *et al.*, 1975).

93 ***Development of the non-invasive placenta***

94 At 35-38 dpo, the allantois has vascularized more than 90% of the chorion. At 40 dpo, the trophoblast of
95 the allantochorion begins to form microvilli enabling intimate contact with the endometrium (Allen *et al.*,
96 1973). Foetal villi are formed over the whole surface of the conceptus, with the exception of uterine
97 glands' apertures, where the trophoblast develops as a dome called areola, consisting of columnar cells
98 with high absorptive capacity (Allen and Wilsher, 2009). From 60 dpo, villi lengthen and branch intensively,
99 with secondary branches appearing at 100 dpo, to result in microcotyledons around 120 dpo (Samuel *et*
100 *al.*, 1974) that continue to grow until term (Macdonald *et al.*, 2000). Moreover, foeto-maternal exchanges
101 are enhanced as gestation progresses, with the distance between foetal vessels and trophoblast being
102 reduced (fetal vessels have migrated into the trophoblast by 250 dpo) due to the merging of fetal vessels'
103 basal lamina with that of the trophoblastic cells and the reduction of maternal epithelium thickness
104 (Samuel *et al.*, 1976).

105 ***Definitive placentation***

106 In the horse, the definitive placenta is diffuse, with villi covering the whole surface of the uterus by term,
107 and epitheliochorial, because allantochorion is simply affixed over the endometrium. Thus, between 5
108 and 6 cell layers separate maternal from foetal blood, depending on foetal vessel location. The definitive
109 allantochorion is composed of i) the allantois, consisting of an epithelium and a loose, highly vascularized,
110 connective tissue (allantoic mesoderm) on the foetal side (Steven, 1982) and ii) the chorion, consisting in
111 a tightly vascularized connective tissue (chorionic mesoderm) and a pseudostratified epithelium, iii) the
112 histotrophic trophoblast located along the areolae and specialized in absorption of exocrine secretions of
113 the uterine glands (histotrophic nutrition), and iv) microcotyledons (haemotrophic trophoblast,
114 connective tissue and foetal vessels) that insert into the endometrial microcaruncles (**Figure 1**).
115 Microcotyledons maximise the contact area between maternal and foetal epithelia for nutrient and waste
116 exchange, which is further enhanced by the epithelial cells' membrane villi present on the
117 microcotyledons, the haemotrophic trophoblast and on the endometrial epithelial cells (Samuel *et al.*,
118 1976; Steven, 1982) (**Figure 1.B**). At term, the placental structure was shown to differ between uterine
119 regions (corresponding to the uterine body, the pregnant or the non-pregnant horn). In Thoroughbred
120 placentas, the placental villous height was shown to be reduced at the tip of the pregnant horn (but not
121 the base) and allantoic vessels to be larger in the pregnant compared to the non-pregnant horn and the
122 placental body (Oikawa *et al.*, 1990). Moreover, in Brazilian Sport Horse and Mangalarga Paulista
123 placentas, pregnant horn microcotyledons were shown to be less voluminous than that of non-pregnant
124 horn and placental body, with less haemotrophic trophoblast and the volume of the microcotyledonary
125 vessels was increased in the placental body compared to both pregnant and non-pregnant horns
126 (Meirelles *et al.*, 2017).

127 In a recent stereological analysis of 43 placentas collected from French Anglo-Arabian mares, we
128 confirmed the presence of important morphological differences between pregnant horn, non-pregnant
129 horn and the zone of insertion of the umbilical cord (personal data, Robles, Chavatte-Palmer, Aïoun,
130 Geeverding, Dubois, Wimmel) (**Figure 2, Material and Methods available in supplementary data 1**). First,
131 the volume of allantoic connective tissue (allantoic and chorionic mesoderms) was increased in the
132 pregnant compared to the non-pregnant horn and umbilical cord insertion area whereas the surface of
133 the allantoic connective tissue was decreased in the non-pregnant compared to the pregnant horn and
134 umbilical cord insertion area. Nevertheless, the microcotyledonary volume was increased in the non-
135 pregnant compared to the pregnant horn and umbilical cord insertion area, due to an increased volume
136 of haemotrophic trophoblast and microcotyledonary connective tissue, as well as of the
137 microcotyledonary and haemotrophic trophoblast surfaces. These structural differences may result from
138 increased stretching of the pregnant horn due to foetal volume. Moreover, the volume and surface of
139 microcotyledonary vessels were increased at the site of umbilical cord insertion, compared to other
140 locations. Finally, transmission and scanning electronic microscopy confirm the stereology results and
141 show that membrane villi of the haemotrophic trophoblast cells appeared to be less developed in the
142 pregnant compared to the non-pregnant horn (**Figure3**). Despite being diffuse and spread over the whole
143 uterus, the respective tissue proportions in the placental structure differ between sites. So far, differences
144 in gene and protein expression according to placental location have not been confirmed. Therefore,
145 especially for term placenta studies, it is important to describe where the placenta was sampled as
146 differences in adaptation to maternal environment might exist between locations (pregnant horn, non-
147 pregnant horn or placental body).

148 **Environmental and maternal factors that impact gene expression**

149 The summary of the effects of the maternal environment on placental gene expression is presented in
150 Table 1.

151 **During early gestation: before implantation**

152 ***Asynchronous embryo transfer***

153 Asynchronous embryo transfer corresponds to the transfer of an embryo in a more advanced (positive
154 asynchrony) or delayed (negative asynchrony) uterine environment. Horse embryos tolerate 5 days or
155 more negative asynchrony but are very sensitive to positive uterine asynchrony (Jacob *et al.*, 2012).
156 Moreover, negative asynchrony has been shown to delay embryo development in early gestation (Gibson
157 *et al.*, 2017), as demonstrated through both transcriptomic and candidate gene analyses (Table 1).
158 Transcriptomic analysis showed underrepresentation of functional terms involved in embryo
159 development (central nervous system, metanephros, retina layer formation) in 14-day-old bilaminar yolk-
160 sacs (endoderm and trophoctoderm) and of cell adhesion (cell adhesion, cell-matrix adhesion, regulation
161 of cell shape, positive regulation of cell migration) as well as protein kinase activity and inflammatory
162 response in 19-days old trilaminar yolk-sacs (endoderm, trophoctoderm and mesoderm) (Gibson *et al.*,
163 2020). Finally, using a gene-candidate analysis, decreased expression of genes involved in placental
164 function and foetal growth (maternal expression: *H19*, *Insulin-like growth factor 2 receptor (IGF2R)*,
165 *Pleckstrin Homology Like Domain Family A Member 2 (PHLDA2)*, paternal expression: *Paternally-expressed*
166 *gene 3 protein (PEG3)* and *Retrotransposon-derived protein (PEG10)*), DNA/RNA processing and cell
167 metabolism (*Insulin receptor (INSR)*) and control of epigenetic regulations (*Histone Acetyltransferase 1*
168 (*HAT1*), *DNA (cytosine-5)-methyltransferase 1 (DNMT1)*, *DNA (cytosine-5)-methyltransferase 3A*
169 (*DNMT3A*), *DNA (cytosine-5)-methyltransferase 3B (DNMT3B)*) was observed in 19 dpo asynchronous
170 compared to synchronous trilaminar yolk-sacs (Gibson *et al.*, 2017). If more research is needed to
171 understand the role of epigenetic modifications in embryo and placental development in the horse, these

172 results show that DNA methylation and histone modifications are likely involved in the delayed
173 development observed in asynchronous concepti. Moreover, the difference in expression of imprinted
174 genes highlight their role as sensitive markers of early conceptus environment and placental
175 development, as observed in other studies (Dini *et al.*, 2021).

176 **Obesity**

177 Horses are grazing animals, so their body condition varies during the year, depending on the energy
178 content of the pasture (Dawson and Hone, 2012). Horses are considered overweight when their body
179 condition score exceeds 6 (1-9 scale) (Henneke *et al.*, 1984) or 3.5 (0-5 scale) (Carroll and Huntington,
180 1988; Martin-Rosset *et al.*, 2008) and obese when their body condition is higher than 7/9 or 4/5. Obesity
181 has been shown to affect both placental structure and function in rodents, humans and non-human
182 primates (Shook *et al.*, 2020).

183 To our knowledge, only one study focussing on the effects of obesity on placental function has been
184 performed in the horse (Sessions-Bresnahan *et al.*, 2018). In light breed mares, the effect of maternal
185 obesity (defined as Body Condition Score (BCS)>7 and body fat>10%, increased leptin and insulin serum
186 concentration, and insulin resistance) at ovulation was explored in the trophoblast at 16 dpo using a
187 candidate gene approach (Table 1). The expression of genes involved in fatty acids transport was altered
188 (increased expression of *fatty acid synthase (FASN)* and *long chain fatty acid transport protein 1 (SLC27A1)*
189 whereas the expression of *cluster of differentiation 36 (CD36)* was reduced). The expression of genes
190 involved in oxidative and mitochondrial stress inhibition was also altered (increased expression of
191 *glutaredox-1 (GPX1)* while *thioredoxin-2 (TXN2)* expression was reduced), as well as the expression of
192 genes involved in inflammation (decreased expression of *interleukin-6 signal transducer (IL6ST)*) in obese
193 compared to normal pregnancies (Sessions-Bresnahan *et al.*, 2018). These results were associated with a
194 tendency for a decrease in global DNA methylation in the embryonic discs of the obese group, which

195 confirms that the alteration of maternal environment in obese mares can induce epigenetic modifications
196 in the conceptus. Indeed, the expression of genes involved in fatty acids transport, inflammation, growth
197 factor signalling and/or endoplasmic reticulum stress was altered in D8 embryos and D16 embryonic discs
198 as well as in the endometrium collected from D16 contralateral uterine horns. Finally, obesity was
199 associated with an inflamed uterine environment that altered conceptus gene expression until at least 16
200 dpo. Effects of obesity on placental function later during pregnancy and at term remain, to our knowledge,
201 unexplored in the equine species. Nevertheless, maternal obesity throughout gestation was shown to
202 promote chronic inflammation and affect glucose metabolism, as well as osteoarticular development in
203 offspring until at least 18 months of age (Robles *et al.*, 2018a).

204 ***Omega-3 fatty acids supplementation***

205 Omega-3 fatty acids are essential fatty acids known to have immunomodulatory and anti-inflammatory
206 effects in horses (Warren and Vineyard, 2013). Fresh grass is rich in omega-3 fatty acids (Dewhurst *et al.*,
207 2001). Cereals, conversely, are rich in omega-6 fatty acids, known for their pro-inflammatory effects (Ryan
208 *et al.*, 2007). Modern diets of horses, which are mainly composed of cereals and conserved forages, are
209 far richer in omega-6 than pasture, which means that supplementation of horse feeds with omega-3
210 sources such as linseed, for instance, may have positive effects on horse health. Because of their
211 immunomodulatory effects, but also their roles in neurodevelopment and metabolism as shown in other
212 species (Buddington *et al.*, 2018; Madore *et al.*, 2020; Shrestha *et al.*, 2020), these fatty acids may affect
213 embryo implantation as well as placental and foetal development.

214 Overweight light breed mares (mean BCS of 7/9) were supplemented with an algae-derived product, rich
215 in omega-3 fatty acids (especially in docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids), from 45
216 days before ovulation (Jacobs *et al.*, 2018). Mares supplemented with omega-3 had increased
217 incorporation of DHA and total omega-3 fatty acids in the endometrium compared to control mares.

218 Moreover, gene expression of markers of trophoctoderm differentiation (*Trans-acting T-cell-specific*
219 *transcription factor (GATA3), Transcription factor AP-2 alpha (TFAP2A) and E74 Like ETS Transcription*
220 *Factor 3 (ELF3)*) as well as primitive endoderm differentiation (*GATA binding protein 4 (GATA4) and GATA*
221 *binding protein 6 (GATA6)*) was increased in D12.5 conceptuses from omega-3 supplemented mares
222 compared to control (Table 1) (Jacobs *et al.*, 2018).

223 Thus, omega-3 fatty acids supplementation could help improve early placental development, at least in
224 overweight animals, but these effects need to be confirmed in other studies to determine if they translate
225 to changes in protein expression and consequently wider benefits for the developing fetus. Effects of
226 omega-3 supplementation on late-pregnancy and term placental function and structure, as well as on
227 development of the offspring, remain to be investigated.

228 **During late gestation: last third of gestation**

229 ***Effect of breed***

230 The different breeds of horses have been either selected by humans to fulfil specialized activities, such as
231 racing (Thoroughbred), sport (warmblood horses) or traction (draft horses) for example. Some have been
232 adapted to harsh environments, such as severe winter (ponies) or dry and hot environments (Arabians
233 and Iberian horses). From these specificities, different sizes and formats, as well as metabolic specificities,
234 were derived. For instance, ponies and Iberian horses have been shown to be more insulin resistant than
235 Saddlebred horses (Bamford *et al.*, 2014; Peugnet *et al.*, 2014).

236 In alignment with adult size, ponies are born lighter than Thoroughbred and French Anglo-Arabian horses,
237 that are born lighter than draft horses. These differences between breeds are correlated with placental
238 surface area (Allen *et al.*, 2002; Robles *et al.*, 2018b) and associated with variation in placental function.
239 The placenta of ponies appears to be the most efficient, as the birthweight to placental surface area ratio

240 is increased compared to French Anglo-Arabians and draft horses. Moreover, the surface density (surface
241 per volume unit, μm^{-1}) and volume fraction (volume per volume unit, %) of microcotyledonary vessels are
242 increased in pony compared to French Anglo-Arabian placentas (Robles *et al.*, 2018b). Surface density and
243 surface of microcotyledons, however, were shown to be reduced in pony compared to Thoroughbred
244 term placentas (Allen *et al.*, 2002) (**Figure 4.A**). This could mean that despite a decreased placental
245 exchange surface in ponies, there was a placental vascular compensation that could explain the increased
246 placental efficiency observed in the small breed compared to bigger horses. Moreover, the expression of
247 genes involved in growth and development is reduced in pony compared to Saddlebred and draft (*insulin-*
248 *like growth factor 2 (IGF2)* and its receptor *IGF2R*), and in Saddlebred compared to draft placentas (*IGF2*)
249 (Table 1). The expression of genes involved in nutrient transfer is reduced in pony compared to Saddlebred
250 placentas (*solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1)*, *sodium-coupled*
251 *neutral amino acid transporter 2 (SLC38A2)*, *lipoprotein lipase (LPL)*). Finally, genes involved in
252 vascularization are expressed less in pony and Saddlebreds compared to draft placentas (*Fms related*
253 *tyrosine kinase 1 (FLT1)*, *kinase insert domain receptor (KDR)*) (Robles *et al.*, 2018b). These latter results
254 indicate that genes directly or indirectly involved in foetal growth are less expressed in pony placentas
255 compared to larger breeds, which is in agreement with the smaller size of the breed.

256 Altogether, these results highlight the fact that there are differences between breeds in terms of placental
257 structure and function, at least at term, and that results observed in one breed may not be reproducible
258 in another one. Uterine size and maternal metabolism thus affect foeto-placental development and
259 should be considered when designing a study. To our knowledge, early-pregnancy placental development
260 has not been compared between breeds.

261 ***Effect of maternal parity and age***

262 In the equine species, maternal parity has been shown to have strong effects on birth weight, growth and
263 metabolism of foals. Primiparous mares produce lighter and smaller foals until at least 18 months of age
264 (Wilsher and Allen, 2003; Elliott *et al.*, 2009; Meirelles *et al.*, 2017; Robles *et al.*, 2018b, c). In addition, the
265 maturation of metabolic and male reproductive functions appears to be delayed in these foals (Robles *et*
266 *al.*, 2018c). As a result, foals born to primiparous dams might be slightly less performant than foals born
267 to multiparous mares, at least in racing (Barron, 1995) and show jumping (Palmer *et al.*, 2018).

268 Indeed, first pregnancies are different from subsequent ones. The uterine artery diameter and blood flow
269 volume is reduced in mares that foaled two foals or less compared to mares with a higher parity (Klewitz
270 *et al.*, 2015). In terms of placental structure, haemotrophic (microcotyledons) (Wilsher and Allen, 2003;
271 Meirelles *et al.*, 2017) and histotrophic (Robles *et al.*, 2018c) exchange surfaces are reduced in term
272 placentas of primiparous compared to multiparous dams (**Figure 4.B**). Finally, the expression of genes
273 involved in growth and development, such as *insulin-like growth factor 1 receptor (IGF1R)*, *transforming*
274 *growth factor beta 1 (TGF β 1)* and *epidermal growth factor receptor (EGFR)* and in vascularization, such as
275 *vascular endothelial growth factor A (VEGF α)* is reduced in primiparous compared to multiparous term
276 placentas (Robles *et al.*, 2018c, 2019) (Table 1). Conversely, the expression of genes involved in protein
277 metabolism and transport (*arginase 2 (ARG2)*, *ornithine decarboxylase 1 (ODC1)* and *high affinity cationic*
278 *amino acid transporter 1 (SLC7A1)*) is increased in primiparous compared to multiparous term placentas,
279 which could reduce the foeto-maternal amino acid transfer capacity in primiparous placentas (Robles *et*
280 *al.*, 2019). These results are consistent with the effects of primiparity observed on uterine blood flow
281 (Klewitz *et al.*, 2015) and placental morphometry at birth (Wilsher and Allen, 2003; Elliott *et al.*, 2009;
282 Meirelles *et al.*, 2017; Robles *et al.*, 2018c).

283 Effects of decreased placental development and function may be enhanced by a decreased uterine ability
284 to expand, as measured through a decreased placental gross surface at birth in primiparous pregnancies

285 (Wilsher and Allen, 2003), but also by an inability of primiparous dams to adapt their metabolism to
286 pregnancy (Robles *et al.*, 2019). Generally, in late gestation, pregnant mares become less glucose tolerant
287 and produce less insulin in response to an increased glycaemia, in order to maximize the placental transfer
288 of glucose to the foetus (Fowden *et al.*, 1984). Higher postprandial insulin secretion was observed in
289 primiparous vs multiparous mares, however, and it was hypothesized that these elevated insulin
290 concentrations could reduce glucose availability for transfer to the foetus (Robles *et al.*, 2019). Finally, a
291 reduced global DNA methylation was observed in placentas of primiparous compared to multiparous
292 dams, suggesting that epigenetic modifications may also play a role in parity-related variations in placental
293 development (Robles *et al.*, 2019).

294 Maternal parity is positively correlated to maternal age, and as a result, maiden mares are also often
295 young mares. Maternal age has also been shown to affect the weight of placenta and foal at birth as young
296 (<9 years old) and old (>16 years old) mares produce lighter foals than mares aged between 9 and 16 years
297 (Wilsher and Allen, 2003; Elliott *et al.*, 2009). These observations have been correlated to placental
298 structure as microcotyledonary surface density decreases as maternal age increases (Wilsher and Allen,
299 2003). Moreover, together with mare ageing, the endometrium undergoes fibrotic degenerative changes
300 that can lead to endometriosis and therefore affect placental development. To our knowledge, the effect
301 of maternal age on placental gene expression has not been studied. Effects of both age and parity on
302 placental and embryo/foetal development remain to be investigated.

303 ***Effect of assisted reproductive technologies (ART)***

304 ART are increasingly used to produce foals from high genetic value mares (**Figure 5**) in order to increase
305 the number of foals born to a mare each year. This enables the production of foals while the mare
306 continues to compete and the production of foals from infertile mares. To our knowledge, apart from
307 equine pregnancies following nuclear transfer where placental abnormalities have been described

308 (Johnson *et al.*, 2010; Pozor *et al.*, 2016), reports of developmental or pregnancy issues following ART in
309 the horse are non-existent, despite the fact that ART have been shown to be associated with
310 developmental and pregnancy issues in human and other animals (Duranton and Chavatte-Palmer,
311 2018). In the horse, commercial ART (embryo transfer (ET) and intracytoplasmic sperm injection (ICSI))
312 was shown to have no effect on the placental expression of 19 genes involved in growth and development,
313 vascularization and nutrient transfer, nor on placental morphology (Valenzuela *et al.*, 2018).

314 When embryo transfers are performed between breeds of different size, however, effects on placental
315 structure and function are observed. Pony embryos transferred to Thoroughbred or draft mares were
316 born heavier, with a heavier and larger placenta than pony embryos carried by pony mares (Allen *et al.*,
317 2002; Robles *et al.*, 2018d). These differences in foeto-placental biometry were associated with different
318 placental structure alterations, depending on whether the embryo was transferred into a Thoroughbred
319 or a draft mare (**Figure 4.C**):

320 - Thoroughbred: The surface density and surface of microcotyledons was increased in transferred
321 compared to pony-in-pony placentas (Allen *et al.*, 2002).

322 - draft: The surface density of trophoblastic tissues (haemotrophic and histotrophic trophoblasts) was
323 decreased in transferred compared to pony-in-pony placentas (Robles *et al.*, 2018d).

324 The gene expression of *sodium-coupled neutral amino acid transporter 1 (SLC38A1)*, involved in amino
325 acid transfer, was increased in term placentas of pony embryos transferred to draft mares. Despite
326 modifications in placental structure, the placental expression of genes involved in growth and
327 development or vascularization did not differ between both groups (Robles *et al.*, 2018d).

328 In contrast, French Anglo-Arabian/Thoroughbred embryos transferred into pony mares were born lighter,
329 with a lighter placenta than that of Saddlebred-in-Saddlebred foals (Allen *et al.*, 2002; Robles *et al.*,

330 2018d). These differences were associated to alterations in placental structure that differed depending
331 on whether the embryo was transferred into a Thoroughbred or a French Anglo-Arabian mare (**Figure**
332 **4.C**):

333 - Thoroughbred: The surface density and surface of microcotyledons was decreased in transferred
334 compared to control placentas (Allen *et al.*, 2002).

335 - French Anglo-Arabian: The surface density and volume fraction of microcotyledonary vessels were
336 increased in placentas of transferred compared to control embryos (Robles *et al.*, 2018d).

337 Moreover, the expression of genes involved in growth and development (*H19*, *IGF2*, *IGF2R* and *IGF1R*) and
338 nutrient transfer (*SLC2A1*, *SLC38A2* and *LPL*) were decreased in placentas of transferred compared to
339 control embryos (Robles *et al.*, 2018d). These results may result from vascular placental adaptations in
340 order to ensure nutrient transfers to the foetus despite the limitation imposed by uterine size.

341 These results coincided, however, with differences in methodology (differences in parity, body condition
342 score, feeding and housing between groups) at least in the Anglo-Arabian study (Peugnet *et al.*, 2014).

343 These differences need therefore to be confirmed and further studied.

344 ***Effect of maternal nutrition and metabolism***

345 **Effects of starch and insulin resistance**

346 Horses are monogastric herbivorous animals that have evolved to eat grass all day (Martin-Rosset *et al.*,
347 1978). In modern practice, horses are housed in boxes or in small pastures and are often fed conserved
348 forages that do not have the quality of fresh grass. To meet their needs in energy and proteins, they are
349 also fed with concentrated feeds, composed of cereals, rich in starch and sugar. Supplementation with
350 starch-rich concentrates has been associated with metabolic alterations, such as insulin resistance, in both

351 pregnant (Peugnet *et al.*, 2015) and non-pregnant horses (Bamford *et al.*, 2016). Moreover, these diets
352 are associated with increased risks of developing osteochondrosis lesions in growing foals (Vander Heyden
353 *et al.*, 2013; Peugnet *et al.*, 2015), as well as decreased colostrum quality at birth (Caure and Lebreton,
354 2004; Thorson *et al.*, 2010; Robles, 2017).

355 An increase in allantoic arteriole vascular wall thickness (**Figure 4.D**) and a decrease in microcotyledonary
356 vessels volume have been observed in term placentas of mares fed with cereals (flattened barley,
357 3.5kg/day, last 4.5 months of gestation) compared to mares fed with forages only (Robles *et al.*, 2018e).
358 These vascular alterations were also linked to modifications in the enrichment of gene sets involved in
359 placental vascularization, inflammation, coagulation and collagen production in mares fed with
360 concentrates (Robles *et al.*, 2018e). Placental inflammation may therefore also be involved in the
361 mediation of the effects of cereal feeding in broodmares on development of osteoarticular diseases in
362 foals.

363 Insulin resistance is also associated with chronic inflammation. Moreover, increased vascular wall
364 thickness (vascular fibrosis) of allantoic arterioles as well as fibrosis in microcotyledons were reported in
365 term placentas from laminitic broodmares (Pazinato *et al.*, 2017).

366 **Effects of maternal undernutrition**

367 Maternal moderate undernutrition does not seem to affect foals birthweight, nor placental
368 measurements and efficiency (Henneke *et al.*, 1984; Peugnet *et al.*, 2015; Robles *et al.*, 2018e). Despite
369 the lack of effect on placental gross morphology at term, placentas from slightly underfed mares fed with
370 forage only (very poor in soluble carbohydrates but rich in fibres) had an enrichment in gene sets involved
371 in amino acids, folate and anions transport, and amino acids, fatty acids, cholesterol and folate catabolism
372 compared to mares fed at 100% of their needs but with concentrates in addition to forage (Robles *et al.*,
373 2018e). This observation indicates that placental metabolism is able to adapt to the source of energy.

374 Placental cells seem to rely primarily on the amino acids and fatty acids catabolism to produce energy
375 when the mare is underfed but with forage only. These placental adaptations could then partly explain
376 why moderate undernutrition did not affect the weight of foals at birth but affected their long-term
377 development (Peugnet *et al.*, 2015; Robles *et al.*, 2017).

378 **Effects of L-arginine supplementation**

379 L-arginine is an essential amino acid during pregnancy and growth in the horse. Compared to other
380 species, the milk of mares is particularly rich in L-arginine, which indicates that L-arginine needs are high
381 in growing foetuses and foals. In the horse, L-arginine supplementation during pregnancy (100 g/day, for
382 21 days before foaling) has been shown to increase uterine artery blood flow in the non-pregnant horn
383 and to decrease the gestational length without affecting, however, foal and placental biometry at birth
384 (Mortensen *et al.*, 2011). In this study, mares' parity was not provided, although effects of L-arginine
385 supplementation may differ between primiparous and multiparous dams. Indeed, L-arginine
386 supplementation (100 g/day during the last 4.5 months of gestation) was shown to affect the metabolism
387 of primiparous but not multiparous mares, leading to an increased metabolic adaptation to pregnancy in
388 supplemented compared to control primiparous mares (Robles *et al.*, 2019). These modifications of
389 maternal environment may have also affected placental function as expression of *SLC2A1* and *CD36*, were
390 increased in arginine supplemented primiparous placentas, compared to placentas from control
391 primiparous mares (Robles *et al.*, 2019).

392 **Pathologies that impact on placental gene expression**

393 **Early pregnancy**

394 Pathologies of pregnancy can lead to pregnancy failure at any stage between fertilisation and parturition.
395 Early pregnancy loss (Early Pregnancy Loss, EPL) is the most common occurrence in the mare and includes

396 both embryo loss prior to clinical detection and the 6-12% clinically confirmed pregnancies that are lost
397 within two months of conception (Bosh *et al.*, 2009; Rose *et al.*, 2018). For many years, the
398 characterisation of gene expression in the placentae of failed early pregnancies (EPL) was hampered by a
399 lack of materials and methodologies. This has now been partly overcome (Rose *et al.*, 2016), although
400 data indicated that the quality of the placental tissue was, in the majority of cases, only suitable for
401 genomic studies and to some degree histological analysis. Applying these methodologies to develop a
402 large biobank of EPL tissue, Shilton *et al.*, 2020 reported aneuploidy in the allantochorion or chorion of
403 22% of EPLs, suggesting this condition is a common reason for pregnancy to fail before 65 days.
404 Aneuploidy is a genetic imbalance that arises due to loss or gain of a whole chromosome. Of 55 EPL
405 assessed, trisomy was identified in 16.3% and monosomy in 5.4% of placental membranes (Shilton *et al.*,
406 2020) with the latter almost certainly lethal with no surviving monosomies reported. The phenotype of
407 mammalian trisomies is more varied: some are associated with miscarriage alone, whilst others, like the
408 well reported human trisomies 13, 18, 21, can result in the birth of a live infant. Preliminary data in the
409 mare suggests the size of the chromosome may dictate whether the trisomy is embryonic lethal or can
410 produce a greater spectrum of phenotypes (Shilton *et al.*, 2020). It is plausible that such copy number
411 changes disrupts gene expression in the placenta, although this needs to be confirmed experimentally.

412 A second pathology of early pregnancy is endometritis that is associated with approximately 5-10% of EPL
413 cases in well managed mares (Ricketts, 2003; Rose *et al.*, 2018). Whilst changes in endometrial gene
414 expression in response to the presence of endometritis have been reviewed (Morris *et al.*, 2020), the
415 genes and signalling pathways within the placental membranes following infection have not been
416 investigated which is in stark contrast to the extensive work on placentitis in late gestation, discussed
417 below. Clearly there is still much to do to better understand the key genes and signals in the early placenta
418 that can lead to EPL. The recent description of defects in the brain and cardiovascular systems of equine
419 embryos/foetuses from clinical cases of EPL (Kahler *et al.*, 2020), along with mounting data linking such

420 defects to placental aberrations in rodent models (Woods *et al.*, 2018), certainly suggest this is an avenue
421 worth pursuing.

422 **Placentitis**

423 Placental infection (placentitis) is one of the major causes of late-term abortion in mares, responsible for
424 the loss of 1-5% of all pregnancies (Lyle, 2014). Despite its prevalence, most published gene-related
425 research has focused on experimental models rather than clinical cases. This has the advantage of
426 facilitated sampling and a better-defined timeline; however, the course of disease can differ significantly,
427 with a more acute, severe reaction seen in response to induced placentitis. As an inflammatory disease,
428 much of the early work focused on the pro-inflammatory cytokine response, identifying an early up-
429 regulation of *interleukin 6 (IL-6)* and *interleukin 8 (IL-8)* at the cervical star (LeBlanc *et al.*, 2002) and
430 *interleukin 18 (IL-18)*, *interleukin 15 (IL-15)* and *interferon γ (INF γ)* in the placental body (Lyle *et al.*, 2009).
431 The balance of cytokines has since been revealed to be more complex, with the maternal response being
432 primarily pro-inflammatory, while modulated by the foetus through production of *interleukin 10 (IL-10)*
433 and *IL-6* (Fedorka *et al.*, 2019). The myometrial and cervical response to placentitis are quite similar to the
434 allantochorion, with a similar up-regulation of both pro-inflammatory and modulatory cytokines
435 (*interleukin 1 β*) *IL-1 β* and *IL-8* in both, *IL-6*, *tumor necrosis factor α (TNF α)* and *interleukin 10 (IL-10)* in
436 cervix) (El-Sheikh Ali *et al.*, 2019; Fernandes *et al.*, 2020).

437 Use of NGS identified toll-like receptor signalling as an important pathway for the myometrial immune
438 response, as well as 8 transcripts which were placentitis specific (*Matrix Metalloproteinase 1 (MMP1)*,
439 *Matrix Metalloproteinase 8 (MMP8)*, *S100 Calcium Binding Protein A8 (S100A8)*, *S100 Calcium Binding*
440 *Protein A9 (S100A9)*, *Peptidase Inhibitor 3 (PI3)*, *apolipoprotein B mRNA-editing enzyme-catalytic*
441 *polypeptide-like 3Z1b (APOBEC3Z1B)*, *resistin (RETN)* and *C-X-C Motif Chemokine Ligand 2 (CXCL2)*). These
442 genes are all involved in regulation of immune and inflammatory processes and may be involved in

443 placentitis induced myometrial activation and initiation of preterm birth. This work also proposed *Signal*
444 *Transducer And Activator Of Transcription 1 (STAT1)* as an up-stream regulator for placentitis, while *Signal*
445 *Transducer And Activator Of Transcription 3 (STAT3)* may regulate transcriptional changes in the pre-term
446 period (El-Sheikh *et al.*, 2020a).

447 The endocrine response to placentitis is well-characterized, however, the underlying gene-level changes
448 have only recently come under scrutiny. Expression of *Steroid 5 Alpha-Reductase 1 (SRD5A1)* and *Aldo-*
449 *keto reductase family 1 member C23 (AKR1C23)*, key progesterone metabolizing enzymes, were reduced
450 in the allantochorion, corresponding to a decrease in 5 α -reduced progestogens in the same tissues.
451 Likewise, aromatase (*CYP19A1*) expression trended down in the allantochorion, a trend accompanied by
452 lower estrone and oestrogen receptor (*ESR1*) concentrations (El-Sheikh *et al.*, 2020b). A tissue-specific
453 response is evident, as *ESR1* is instead up-regulated in cervical stroma during placentitis, as are
454 prostaglandin synthases *prostaglandin E synthase (PTGES)* and *Prostaglandin-Endoperoxide Synthase 2*
455 (*PTGS2*) (Fernandes *et al.*, 2020). These recent studies showcase the multi-faceted nature of placentitis,
456 including robust maternal and foetal immune responses far beyond the placenta, with each tissue
457 exhibiting its own unique response to placentitis.

458 **Hydrops**

459 Hydrops occurs when excess foetal fluid builds up during gestation. It can affect either the allantoic
460 (hydroallantois) or amniotic (hydroamnios) fluid cavity. Although little information exists on the aetiology
461 of hydrops, hydroallantois appears to be associated with impaired placental angiogenesis. Not only do
462 hydroallantoic placentae exhibit reduced capillary density, but also gene expression changes are indicative
463 of altered angiogenesis and hypoxic changes. Specifically, *hypoxia-induced gene Hypoxia-inducible factor*
464 *1-alpha (HIF1A)* is up-regulated, as is *VEGF*, a known inducer of vascular permeability. Meanwhile, *VEGF*
465 receptors *VEGFR1* and *VEGFR2* are downregulated. Evidence for the involvement of oestrogen

466 dysfunction also exists, as both *CYP19A1* and oestrogen receptors *ESR1* and *ESR2* are down-regulated (Dini
467 *et al.*, 2020). Although preliminary, this work sheds light on underlying gene dysregulation associated with
468 hydroallantois, as well as highlighting potential consequences for insufficient angiogenesis during
469 placentation.

470 **Placental retention**

471 Placental retention is defined as a failure to expel foetal membranes within 3 hours of delivery. This
472 condition is common in the horse, especially in draft horse breeds, but its aetiology is still poorly
473 understood. Placental retention causes endometritis and can lead to life threatening diseases such as
474 endotoxemia, laminitis and septicaemia. Histologically, at term, placental retention has been associated
475 with fibrosis in the microcotyledons, overdeveloped stromal connective tissue in the non-pregnant horn
476 (retained horn), oversized histotrophic epithelium and sparser and shorter villi (microcotyledons) in draft
477 mares' placentas (Rapacz *et al.*, 2012; Rapacz-Leonard *et al.*, 2015). These structural alterations were
478 associated with a higher apoptotic index in both haemotrophic and histotrophic trophoblasts, as well as
479 in both luminal and glandular endometrial epitheliums (Paździor *et al.*, 2012). Increased gene and protein
480 expression of matrix metalloproteinase-2 (MMP-2) were also observed, together with increased gene
481 expression of matrix metalloproteinase-9 (MMP-9) and decreased expression of Tissue inhibitor of
482 metalloproteinases 2 (TIMP-2) in retained compared to control allantochorions (Rapacz-Leonard *et al.*,
483 2015). Moreover, MMP-2 activity was reduced in retained compared to control placentas. Gene
484 expression of *IL-1 β* and *IL-6* (as well as protein expression of IL-6) were also increased in retained
485 allantochorions (Jaworska and Janowski, 2019). Gene expression of prostaglandin endoperoxide synthase-
486 2 (*PTGS2*), involved in prostaglandin production, was however decreased in retained placenta which could
487 contribute to the observed imbalance in inflammatory processes and tissue remodelling in this pathology
488 (Rapacz-Leonard *et al.*, 2020). Finally, protein expression of oxytocin receptors (OXTR) was greatly

489 reduced in retained placentas in case of secondary atony, but this difference was not observed in retained
490 allantochorion without secondary atony (Rapacz-Leonard *et al.*, 2015; Rapacz-Leonard *et al.*, 2020). From
491 these results, it can be concluded that abnormal placental development results from abnormal epithelial
492 regeneration, as well as abnormal extracellular matrix remodelling and inflammatory signalling.

493 ***Streptococcus equi* infection**

494 *Streptococcus equi*, or strangles, is a highly contagious disease observed worldwide in horses (Mitchell *et*
495 *al.*, 2021). The condition is characterized by the development of abscesses in the head and neck lymph
496 nodes, pharyngitis, as well as fever and lethargy (Boyle *et al.*, 2018). In one study, young (3-4 years old)
497 primiparous mares were infected with *Streptococcus equi* between 14 and 161 days of gestation. No
498 antibiotics were administered. In addition to other symptoms (such as fever, nasal discharges, inflamed
499 and swollen lymph nodes and depression), this infection led to a severe undernutrition for a median of 3
500 weeks. Infected mares had a decreased placental microcotyledonary surface (**Figure 4.D**), leading to a
501 decreased birth weight of their foals. Placental function, however, was not investigated in this study
502 (Wilsher and Allen, 2006).

503

504 **Conclusion and final remarks**

505 Despite all the new information that has been provided during the last decade, a lot of unknowns remain
506 concerning factors affecting placental development in horses. So far, apart from one study that performed
507 RNA sequencing (RNAseq) to analyse differences in gene expression between two nutritional conditions,
508 all reports comparing gene expression in equine placenta in response to environment at large used a RT-
509 qPCR candidate gene approach, thus greatly limiting the possibility to uncover unexpected mechanisms
510 that could be highlighted by high throughput approaches. Even though RT-qPCR approaches provide

511 valuable information as gene expression is compared between equal total RNA concentrations per
512 sample, variations in placental size and in gene expression between placental zones may affect total
513 placenta expression and the conclusions that can be drawn from results. Moreover, there might be a
514 change in cell composition between samples that is not controllable in the field but could be corrected
515 with the use of trophoblastic cells marker genes for example (see Loux et al., XX for more information on
516 gene expression between cell lines). It is thus always important to combine gene expression approaches,
517 whether limited to a few candidates or unbiased through high throughput strategies, with physiological
518 and structural observations.

519 In any case, RNseq approaches are essential because they provide an unbiased analysis necessary for
520 understanding i) the pathogenesis of placental disease in order to help the design of therapeutic
521 treatments and ii) the processes leading to the programming of foal's health in order to elaborate
522 strategies to maximise health, welfare, performance and longevity in horses. Additionally, more refined
523 methodologies such as single cell RNAseq would be useful to determine the respective contribution of
524 different cell lines in placental gene expression.

525

526 **Declaration of interest**

527 The authors have no interest conflicts to declare.

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529 MdM

530 SL

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532 PCP

533

534

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For Review Only

Figure 1: Structure of the equine placenta at term. (A) Hematoxylin-eosin-saffron staining of the whole placenta with both allantois and microcotyledons. Details on microcotyledonary (B) and allantoic (C) structures with hematoxylin-eosin-saffron staining or semi-thin sections with toluidin blue staining.

Figure 2: Differences in placental structure between pregnant horn (PH, n=43), non-pregnant horn (NPH, n=43) and umbilical cord insertion area (UC, n=43) at term.

(A) Volume of allantoic and microcotyledonary components (cm³). (B) Surface of allantoic and microcotyledonary components (10⁵ cm²). (C) Haematoxylin-eosin-saffron staining of term placentas sampled in the pregnant horn, the non-pregnant horn and above the umbilical cord insertion
AM= allantoic mesoderm, CM=chorionic mesoderm, M=microcotyledons. Allantois connective tissue=AM+CM

Different superscript letters indicate a significant difference between placental regions (p<0.05)

Figure 3: Transmission (A, B) and scanning (C, D) electron microscopy of haemotrophic trophoblast cells and placental villi (microcotyledons) in pregnant (A, C) and non-pregnant (B, D) horns (Credits: Josiane Aioun and Audrey Geeverding).

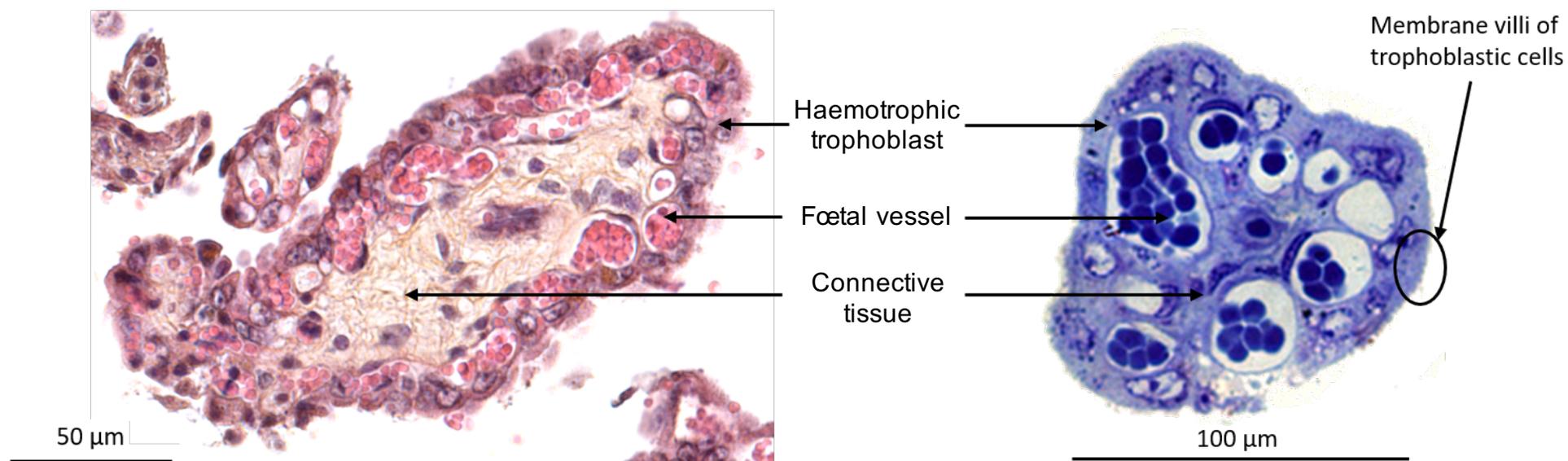
Figure 4: Differences in placental structure as measured using the stereology method according to breed (A), parity (B), embryo transfer between breeds (C) and maternal nutrition and metabolism (D). Results were compiled from (A) Robles et al., 2018b, (B) Robles et al., 2018c and 2019, (C) Allen et al., 2002, Robles et al., 2018d, and (D) Robles et al., 2018e, Wilsher and Allen, 2006.

Figure 5: Worldwide number of equine embryo transfers between 2000 and 2018 (derived from Data Retrieval Committee Reports of the International Embryo Technology Society (International Embryo Technology Society (IETS))

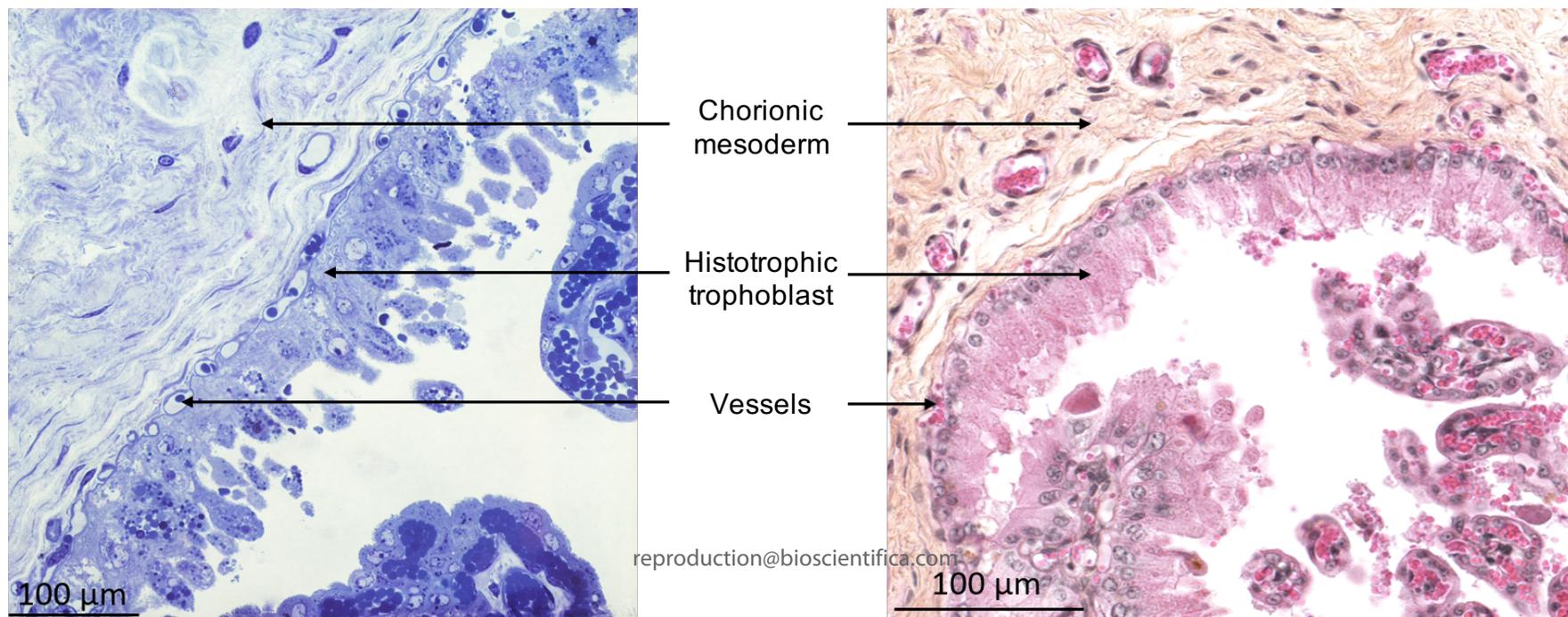
A. Placental structure

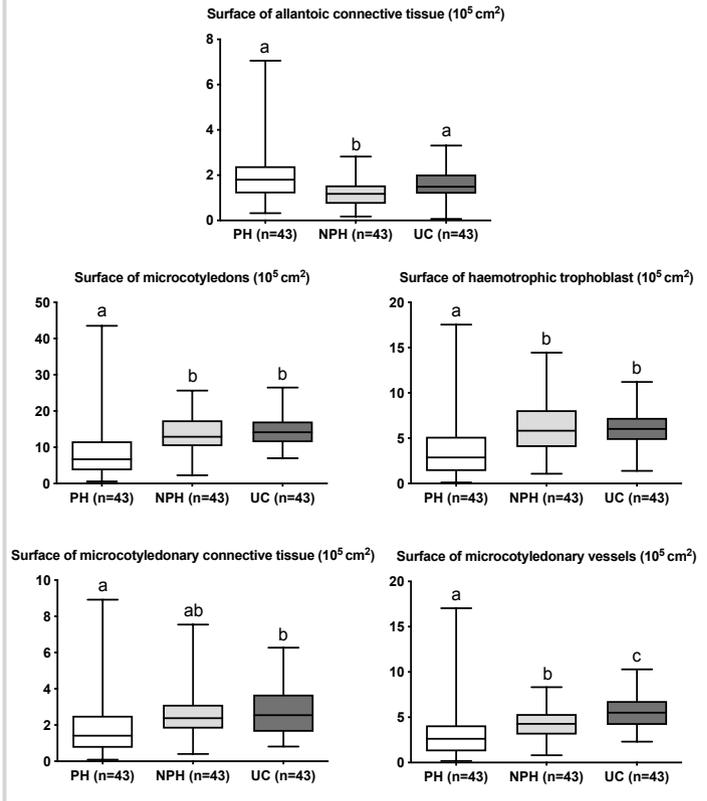
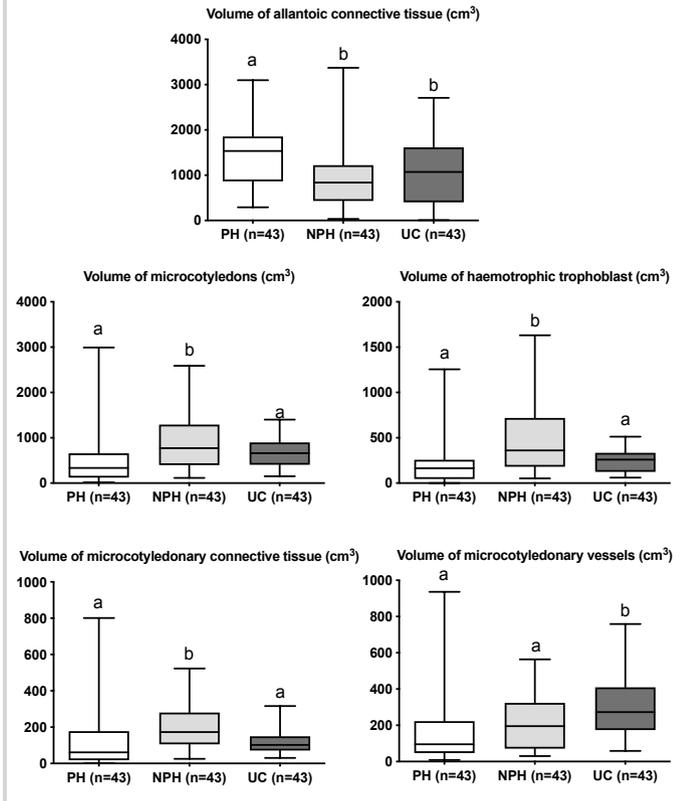


B. Microcotyledons



C. Chorionic mesoderm and histotrophic trophoblast



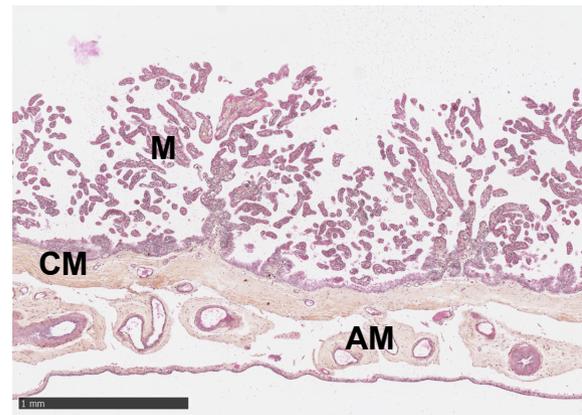
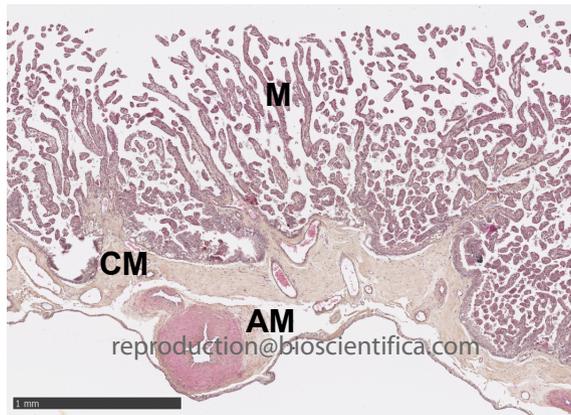
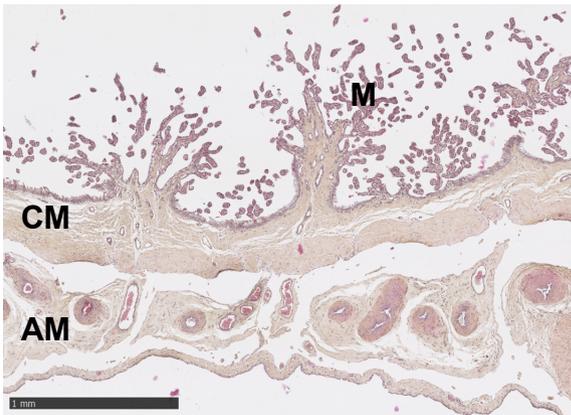


C. Histology

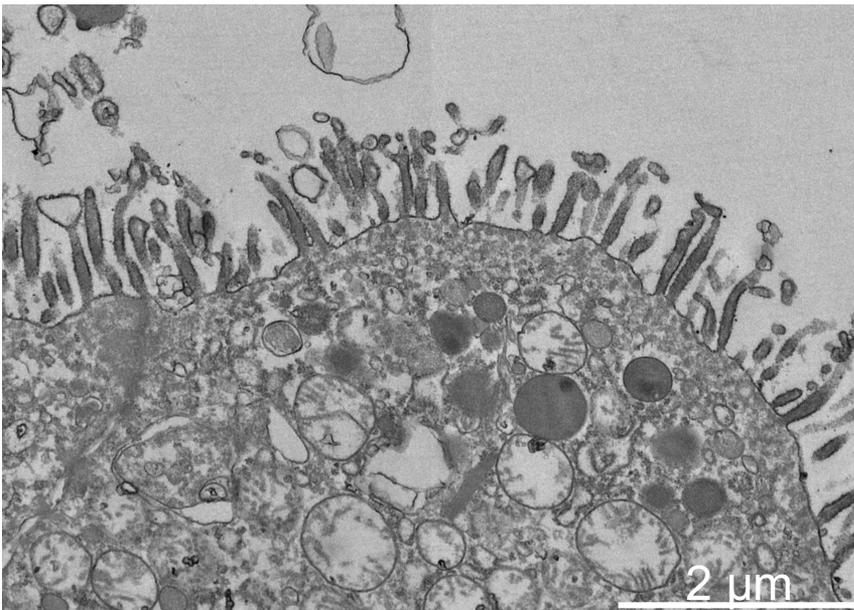
Pregnant horn

Non-pregnant horn

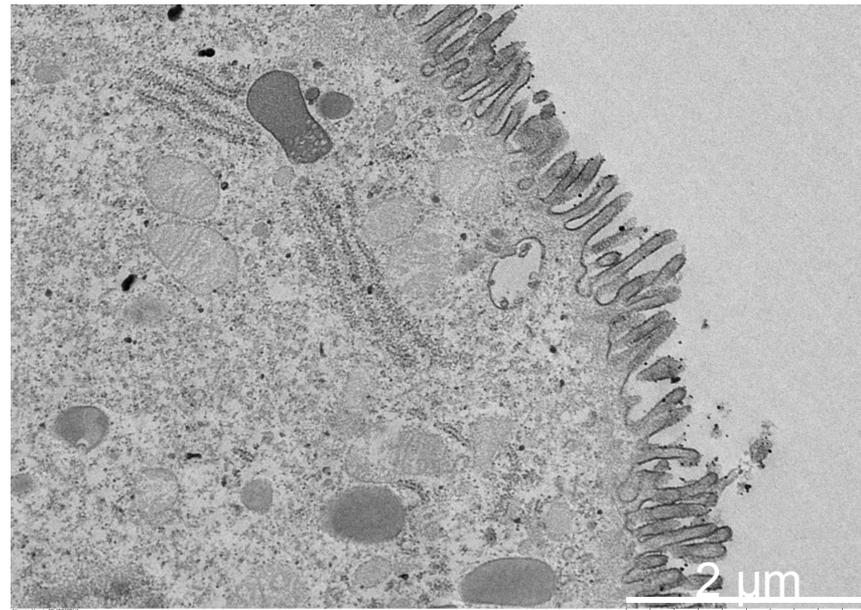
Umbilical cord insertion



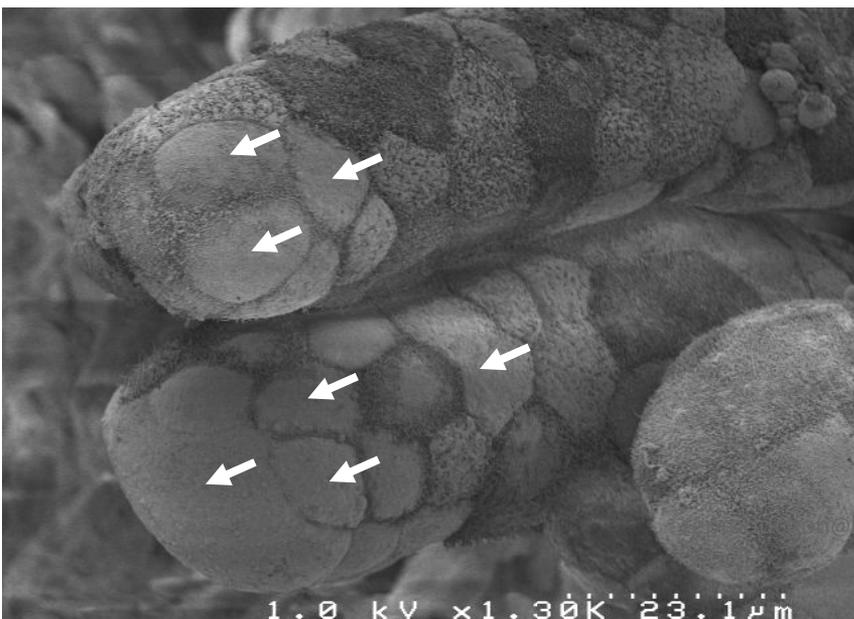
A. Pregnant horn (transmission)



B. Non-pregnant horn (transmission)



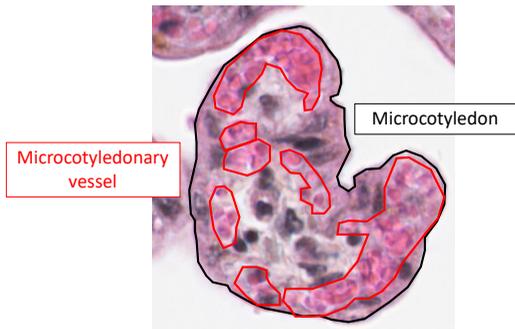
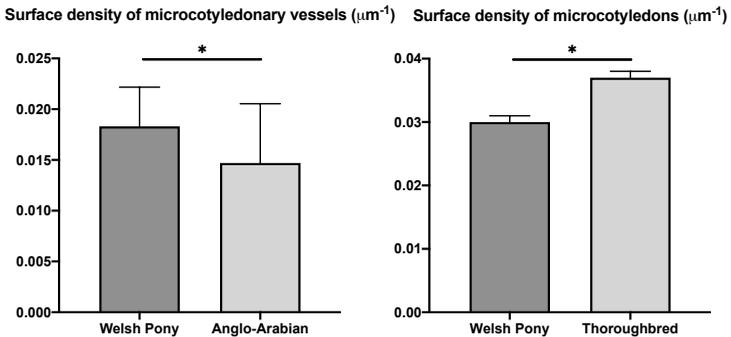
C. Pregnant horn (scanning)



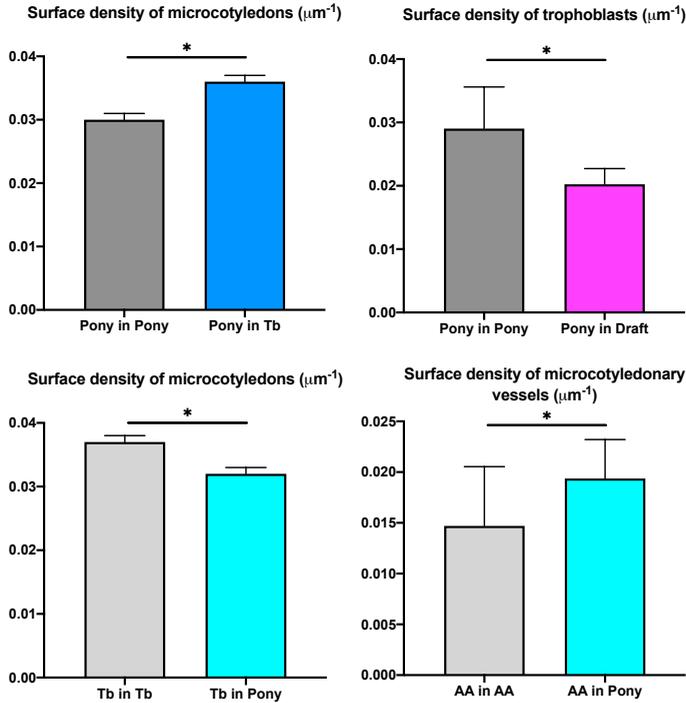
D. Non-pregnant horn (scanning)



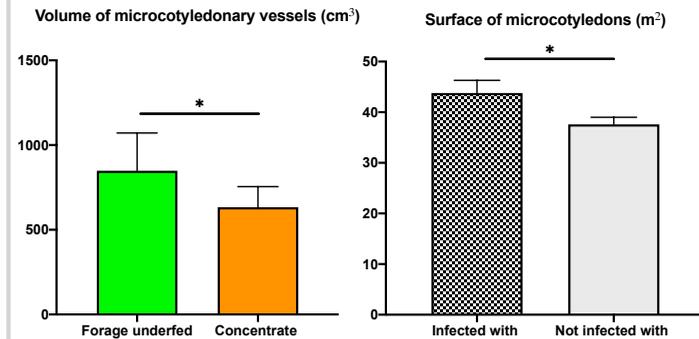
A. Effect of breed



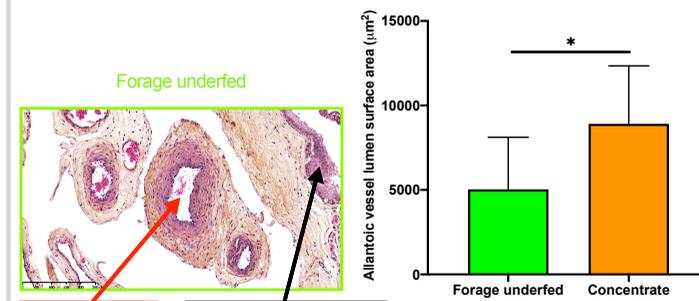
C. Effect of assisted reproductive technologies (ART)



D. Effect of maternal nutrition and metabolism

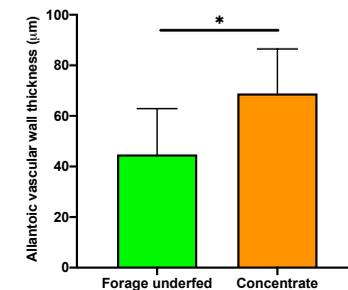


Lumen area and vascular wall thickness of allantoic vessels

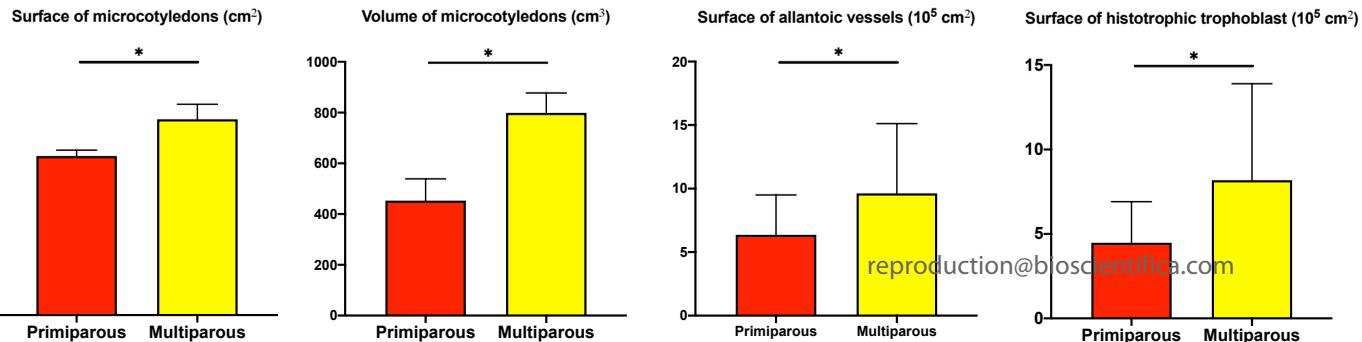


Allantoic vessel

Histotrophic trophoblast



B. Effect of maternal parity and age



Worldwide number of
equine embryo transfers
(fresh and frozen)

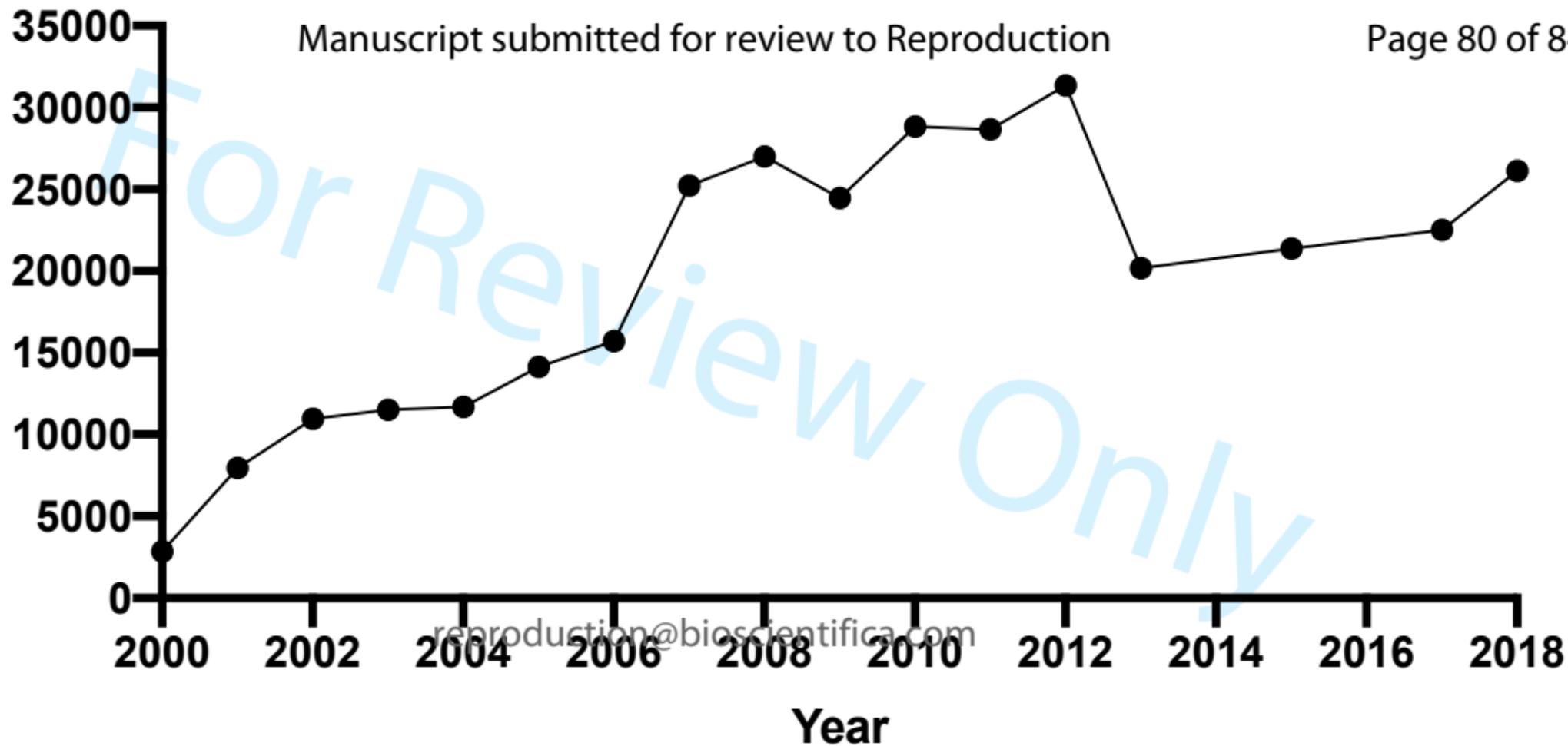


Table 1. Summary of the effects of maternal environment on placental gene expression.

Differences are expressed compared to control.

Maternal factor	Tissue	Gene expression	Gene category	Method	Source
Early gestation					
Obesity	D16 trophoctoderm	↗ FASN ↗ SLC27A1 ↘ CD36	Fatty acid transport	RT-qPCR	(Sessions-Bresnahan et al., 2018)
		↗ GPX1 ↗ TXN2	Oxidative and mitochondrial stress		
		↘ IL6ST	Inflammation		
Omega-3	D12.5 conceptus	↗ GATA3 ↗ TFAP2A ↗ ELF3	Trophoctoderm differentiation	RT-qPCR	(Jacobs et al., 2018)
		↗ GATA4 ↗ GATA6	Primitive endoderm differentiation		
Asynchronous embryo transfer	D19 trilaminar yolk-sacs	↘ H19 ↘ IGFR2 ↘ PHLDA2 ↘ PEG3 ↘ PEG10	Placental function and fetal growth	RT-qPCR	(Gibson et al., 2017)
		↘ INSR	DNA/RNA processing and cell metabolism		
		↘ HAT1 ↘ DNMT1 ↘ DNMT3 ↘ DNMT3B	Control of epigenetic regulations		
At term					
Breed pony vs draft and Saddlebred	Term placenta	↘ IGF2 ↘ IGF2R	Growth and development	RT-qPCR	(Robles et al., 2018a)
		↘ SLC2A1 ↘ SLC38A2 ↘ LPL	Nutrient transfer		
↘ FLT1 ↘ KDR		Vascularization			
Breed Saddlebred vs draft		↘ IGF2	Growth and development		
		↘ KDR	Vascularization		
Primiparity	Term placenta	↘ IGF1R ↘ TGFβ1 ↘ EGFR	Growth and development	RT-qPCR	(Robles et al., 2018b)
		↘ VEGFα	Vascularization		

Commercial ET and ICSI	Term placenta	No difference		RT-qPCR	(Valenzuela et al., 2017)
Pony embryos transferred into draft mares	Term placenta	↗SLC38A1	Nutrient transfer	RT-qPCR	(Robles et al., 2018e)
Saddlebred embryos transferred into pony mares		↘H19 ↘IGF2 ↘IGF1R ↘IGF2R	Growth and development		
		↘SLC2A1 ↘SLC38A2 ↘LPL	Nutrient transfer		
L-arginine supplementation	Term placenta	↗SLC2A1 ↗CD36	Nutrient transfer	RT-qPCR	(Robles et al., 2019)

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Supplementary data

Material and Methods for analysis of structural differences between placental regions

Animals

Forty-three French Anglo-Arabian mares were raised in the “Institut Français du Cheval et de l'Équitation” experimental farm (Chamberet, France, 45°34'55.17"N, 1°43'16.29"E, 442 m) in 2012-2013 (n=7), 2013-2014 (n=20), 2015-2016 (n=4) and 2016-2017 (n=12). All mares were vaccinated and dewormed as for standard care. Each year, during the first 8 months of gestation, mares were housed in pasture as a single heard. From the 8th month of gestation, they were housed individually in boxes and fed forage (hay + haylage) and flattened barley (+ minerals and vitamins supplement) until foaling. Mares pregnant in 2015-2016 received a mix of flattened barley, and ground barley + molasses pellets. The same stallion was used for the foaling years 2012-2013 and 2013-2014, another stallion was used for foaling years 2015-2016 and another for foaling years 2016-2017. All stallions were French Anglo-Arabians.

Placental sampling and microscopy

Mares foaled and delivered naturally. Placental samples including the chorionic villi and the underlying allantoic membrane were collected within 30 min of delivery procedure in the pregnant horn, the non-pregnant horn and above the umbilical cord insertion. Aliquots (N=3 for each analysis, 1 cm² samples) were fixed in 4% formaldehyde, then embedded in paraffin. Sections (7 µm) were stained with hematoxylin/eosin/saffron and scanned using NanoZoomer Digital Pathology® (Hamamatsu Photonics). Surface densities (Sv) and Volume fractions (Vv) of the different components of the allantochorion, *i.e.*, microcotyledons and allantois, were quantified by One stop stereology using the Mercator® software (ExploraNova, France).

The following components were measured:

- In the microcotyledonary region: haemotrophic trophoblast, microcotyledonary vessels, connective tissue and microcotyledons as the sum of all microcotyledonary components.
- In the allantoic region: histotrophic trophoblast and allantoic vessels. The chorionic and allantoic mesoderms were merged as allantoic connective tissue.

Vv and Sv were multiplied by the total volume of the placenta to obtain an estimation of the absolute volume (cm³) and surface (cm²) of the components of the allantochorion. Placental volume was evaluated using a graduated water container, by measuring the volume of water displaced after immersing the placenta into the water.

During the foalings of year 2016, pregnant horn and non-pregnant horn samples (3mm²) were also fixed in 2.5% glutaraldehyde in 0.2M cacodylate buffer, pH 7.2.

For transmission electron microscopy, samples were rinsed in cacodylate buffer 0.1M with sucrose 0.2M, then immersed in filtered 0.5% Oolong tea extract, post-fixed in 1% osmium tetroxide (O₂O₄) and 1.5% potassium ferrocyanide and dehydrated in alcohol and acetone at 37°C using a KOS microwave (microwave Histo STATION, Milestone medicals, USA). Samples were embedded in Epon resin (Epon 812, EMS, Delta microscopies, France) at 45°C. Placental sections, contrasted with lead citrate, were observed on a Hitachi HT-7700 (Hitachi, Japan) transmission electron microscope (TEM) at a tension of 80kv.

For scanning electron microscopy, samples were dehydrated in ethanol in increasing concentrations (until 100%). Once dehydrated, liquid CO₂ was injected at 37°C and 80-85 bars. After dehydration, samples were coated with platinum (800V, 10mA, 220sc) and observed on a Hitachi S4500 FEG (Hitachi, Japan) at a tension of 2kV.

Statistical analyses

Data were analysed using a mixed linear model (nlme package) followed by a type 3 Anova (car package) considering region of sampling, parity of the mare and the year of foaling as fixed effects. The mare was considered as random effect. Sex of the foal had no effect on measured parameters and was therefore removed from the model. Because of the multiple analyses, p-values obtained with the Anova were corrected using the FDR method. When placental region effect was statistically significant, a pairwise post hoc Tukey test was applied using the emmeans package. Results are presented as median, first and third quartiles and minimum and maximum (boxplot).

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