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Placental alterations in structure and function in intra-uterine growth-retarded horses

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Summary

Background: Following embryo transfer (ET), the size and breed of the recipient mare can affect fetal development and subsequent postnatal growth rate and insulin sensitivity in foals.

Objectives: To investigate placental adaptation in pregnancies where increased or restricted fetal growth was induced through ET between Pony, Saddlebred and Draught horses.

Study design: In vivo experiment.

Methods: Control Pony (P, n = 21) and Saddlebred (S, n = 28) pregnancies were obtained by artificial insemination. Increased pregnancies were obtained by transferring Pony (P-D, n = 6) and Saddlebred (S-D, n = 8) embryos into Draught mares. Restricted pregnancies were obtained by transferring Saddlebred embryos into Pony mares (S-P, n = 6). Placental weight and surface were recorded and samples collected for stereology and analysis of expression of genes involved in placental growth, vascularisation and nutrient transport. Data were analysed by linear model.

Results: S-P foals were growth retarded compared to controls despite increased gestational length. Placental weight was reduced but placental surface density and volume fraction were increased. Placental expression of genes involved in growth and development and nutrient transfer was strongly reduced. In contrast, placental size and weight were increased in enhanced growth P-D and S-D foals. The trophoblastic surface density and the allantoic vessels surface density were decreased in P-D and S-D, respectively, both with very few modifications in gene expression.

Main limitations: Control embryos were produced by artificial insemination whereas experimental embryos were produced by ET.

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Conclusions: Placental structure and gene expression are modified after ET into a smaller or larger breed than that of the embryo. These adaptations contribute to the observed phenotype of foal growth restriction or enhanced growth at birth.

Introduction

Epidemiological studies in humans [1] and experiments in non-equine animals [2–5] have shown that preconceptional and gestational environmental conditions induce physiological adaptations in the offspring that persist postnatally and increase the risks of developing non-communicable diseases at adulthood. A U-shaped curve of risk has been observed, with both individuals born small and large being affected [6,7]. These Developmental Origins of Health and Disease (DOHaD) also apply to the horse [8–11].

The placenta plays a pivotal role in transmitting *stimuli* from the maternal environment to the fetus [12–15]. Placental function varies according to placental size, morphology, blood flow, nutrient transporter abundance and intrinsic nutrient consumption and production [12,13,16]. In equids, placentation is non-invasive, diffuse and epitheliochorial. Microcotyledons with intense branching maximise the contact surface between endometrium and trophoblast for haemotrophic exchanges. Histotrophic exchanges take place in-between microcotyledonary units, where pseudo-stratified trophoblastic cells are specifically adapted to absorb the exocrine material from the uterine glands [17–19].

An estimated 21,359 equine embryos were transferred in the world in 2015 [20]. Recipient mares are mostly chosen for economical or practical (character, availability) reasons and are often of a different size and breed than the embryo. Manipulation of fetal growth through embryo transfers between ponies and Thoroughbreds showed that metabolic and stress responses are affected in growth-enhanced and growth-restricted newborn foals [21,22], with effects on body size persisting up to three years of age [23,24]. Placental structure was pointed out as the primary control mechanism for the observed alterations in foal growth and

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metabolism [23] but functional mechanisms associated with these structural changes were not investigated.

The current study follows up from previous work where pony or Saddlebred embryos were transferred into draught mares and Saddlebred embryos transferred into pony mares [25]. Postnatally, enhanced growth pony foals remained taller than pony controls until 18 months of age whereas restricted Saddlebred foals partially caught up to Saddlebred controls [25,26]. Early insulin-resistance and subsequent lower basal glycaemia were observed in enlarged ponies compared to pony controls. In contrast, restricted Saddlebred foals initially developed increased insulin sensitivity that persisted until 7 months. They had increased basal glucose concentration compared to Saddlebred controls until 18 months of age [25].

Here, we evaluated placental adaptations (placental biometry, histological structure, expression of genes involved in growth, vascularisation and nutrient transfer processes) to possibly relate them to the post-natal development of foals.

Materials and methods

The study was conducted over 2 successive breeding seasons (2011 and 2012 foalings) (Table 1). Welsh Pony mares (P, n = 27) were housed and P embryos (n = 61) were produced in experimental farm 1 (47°32'36.12"N, 0°44'38.96"E, 120 m). Anglo-Arabian Saddlebred (S, n = 28) and draught Breton (D, n = 22) mares were housed and S embryos (n = 48) were produced in the experimental farm 2 (45°34'55.17"N, 1°43'16.29"E, 442 m). Age, parity and size of the mares are shown in Table 1. The experimental protocol is summarised in Figure 1 as described previously [25].

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Control pregnancies

Pony-in-Pony (P-P, n = 21) and Saddlebred-in-Saddlebred (S-S, n = 28) pregnancies were obtained by artificial insemination using semen from one pony (Welsh) and two Saddlebred (Anglo-Arabian) stallions, respectively.

Experimental pregnancies

Pony-in-Draught (P-D, n = 6), Saddlebred-in-Pony (S-P, n = 6) and Saddlebred-in-Draught (S-D, n = 8) pregnancies were obtained by embryo transfer (ET) as described previously [25]. Pony control and experimental foals were produced using the same stallion. Saddlebred embryos were produced by two different stallions depending on the breeding season but both stallions produced experimental and control foals. Briefly, embryos were recovered 7 days after ovulation, washed in Emcare Holding® solution (ICPbio), transported in Equitainer®1 to the other experimental farm (3-4 hours) and transferred immediately into synchronised recipients 5 to 7 days after ovulation. Pregnancy was diagnosed 7 days after ET by ultrasonography.

Management of mares and placental sampling

From ovulation, mares were kept in pasture with free access to mineral salts. From the 5th gestational month, they were housed in boxes and fed a diet based on straw and hay with concentrates in farm 1 and homemade pellets and hay in farm 2, with free access to mineral salts (Supplementary Item1).

At delivery, all placentas (allantochorion) were sampled near the umbilical cord: 1 cm² fragments were fixed in 4% formaldehyde for histological analysis and 3 mm² fragments were snap-frozen in liquid nitrogen for molecular analysis. The foal and the placenta associated to amnion and cord (total fetal membranes - TFM) were weighed the next morning. The gross

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placental surface area was measured by applying a clear Plexiglas sheet marked with 10 cm x 10 cm squares on placentas laid in the “F” configuration, revealing both uterine horns and the uterine body (Fig 2) [23]. Photographs were taken and the placental surface subsequently measured using the ImageJ® software². What corresponded to 10 cm in the picture was set as a pixel scale. The placental surface was subsequently measured as the area calculated by drawing the contour of the placenta, multiplied by 2.

Histological and stereological analyses

Placental samples were embedded in paraffin, cut in 7 µm sections, stained with haematoxylin/eosin and scanned using NanoZoomer Digital Pathology®³. Stereology is a quantitative method that enables the calculation of three dimensional structural quantities (number, length, area and volume) from two-dimensional sectional images. Relative surface (S_v, μ^{-1}) and relative volume ($V_v, \%$) of the different components of the placentas were measured using the One Stop Stereology software (MercatorPro®⁴ [27]).

As presented in Figure 3, the components measured were:

- For the microcotyledonary part: Both the haemotrophic and histotrophic trophoblasts labelled as “trophoblast”, the microcotyledonary vessels and the whole microcotyledons (by adding the measures of the trophoblasts and the microcotyledonary vessels)
- For the allantoic part: the allantoic tissue vessels and the allantoic connective tissue.

Gene expression analysis

Total RNA from placental samples was isolated as previously described [28] and purified using the RNeasy® Mini kit⁵ according to the manufacturer's instructions. Reverse transcription and real time quantitative PCR were performed as previously described [29].

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Gene-specific primers of gene analysed are shown in Table 2. The expression of the following genes was measured:

Growth and development: *Insulin-like growth factor 2 (IGF2)*, a regulator of fetal and placental development, *Insulin-like growth factor 1 receptor (IGF1R)*, a receptor that modulates IGF-2 action, *Insulin-like growth factor 2 receptor (IGF2R)*, a receptor involved in the signalling pathway of IGF2 degradation, *transforming growth factor β 1 (TGF β 1)*, that regulates cell proliferation and differentiation and *H19* that encodes a non-coding RNA acting as a negative regulator of cell proliferation.

Nutrient transfer: *Solute carrier family 2 member 1 (SLC2A1)*, a glucose transporter non-insulin dependent, *Solute carrier family 38 member 1 (SLC38A1)* and *Solute carrier family 38 member 2 (SLC38A2)*, two transporters involved in neutral amino acids transfers, *LPL*, a lipoprotein lipase and *cluster of differentiation 36 (CD36)*, involved in the transfer of phospholipids, lipoproteins and long-chain fatty acids.

Vascularisation *Vascular endothelial growth factor A (VEGFA)*, that promotes angiogenesis and vasculogenesis, *vascular endothelial growth factor receptor 1 (FLT1)* and *vascular endothelial growth factor receptor 2 (KDR)*, receptors of VEGFA that regulate negatively and positively its action, respectively, and *endothelial NOS (eNOS)*, an enzyme that catalyses the production of nitric oxide gaseous vasodilator.

Data were analysed using QbasePLUS^{®6}. Calibrated normalised relative quantities (CNRQ) were calculated as previously described [29]. *Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)*, *Ribosomal Protein L32 (RPL32)* and *Secretory Carrier Membrane Protein 3 (SCAMP3)* [30], as selected by geNorm, were used as reference genes.

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Data analysis

Results are expressed as medians with interquartile range and presented as boxplots (minimum to maximum). Statistical analyses were performed using R software⁷ (version 3.3.0) using linear models to examine associations between breed (P-P vs. S-S); maternal environment (P-P vs. P-D and S-P vs. S-S vs. S-D), age, dam parity and foal sex and feto-placental biometry parameters (foals' birthweight, TFM weight, placental surface area, foal birthweight/placental surface area ratio), volume fraction and surface density of all the components of the placenta (trophoblast, microcotyledonary vessels, microcotyledons, allantoic tissue vessels, allantoic connective tissue) and the placental expression of genes involved in growth and development (*IGF2*, *IGF1R*, *IGF2R*, *TGFβ1*, *H19*), nutrient transfer (*SLC2A1*, *SLC38A1*, *SLC38A2*, *LPL*, *CD36*) and vascularisation (*VEGFA*, *FLT1*, *KDR*, *eNOS*). The effect of the breeding season could not be studied because all S-D foals were born during breeding season 1 (2011), and only one P-D foal was born during breeding season 2 (2012). Effects were considered significant when $p \leq 0.05$.

Results

Median, interquartile range and p-value of statistically significant co-factors are presented in Supplementary Item 2.

Pregnancies (Table 1)

Altogether, 21 P-P and 28 S-S controls and 6 P-D, 6 S-P and 8 S-D experimental foals were delivered spontaneously during spring and summer (6 April – 13 August) with >75% in May/June, all within the normal gestational age range.

Pregnancy length was not statistically different between P-D and P-P nor between S-D and S-S pregnancies. In contrast, pregnancy was 13 days longer in S-P vs. S-S ($p < 0.001$) and 16 days longer in S-P vs. S-D ($p < 0.01$).

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Foals (Figure 4A)

All foals were healthy at birth, but for 3 of 6 S-P foals that needed assistance to stand and suckle immediately after birth and were bottle-fed with horse colostrum and ewe milk until first suckling. These foals presented signs of dysmaturity such as silky hair coat and joint and tendon laxity [31].

P-D foals were heavier than P-P foals (+57.3%, $p < 0.0001$). S-P and S-D foals were respectively lighter (-31.2%, $p < 0.0001$) and heavier (+8.9%, $p < 0.001$) than S-S foals. S-P foals were lighter by 36.8% than S-D foals ($p < 0.0001$).

Placental and fetal membranes biometry (Figures 4B, 4C and 4D)

TFM were heavier ($p < 0.0001$) and the placenta larger ($p < 0.0001$) in P-D compared to P-P. The foal/TFM weight ratio tended to be smaller in P-D compared to P-P groups ($p = 0.07$). TFM weight and placental surface were respectively reduced ($p < 0.001$, $p < 0.0001$, respectively) and increased ($p < 0.01$, $p = 0.02$, respectively) in S-P and S-D compared to S-S controls. Both TFM weight and placental surface area were reduced in S-P compared to S-D ($p < 0.0001$, $p < 0.0001$). Nevertheless, the foal to TFM weight ratio was not different between S-P, S-S and S-D.

Placental morphometry (Supplementary Item 3 and Fig 5)

Only the parameters that differed within a breed are presented in Figure 5. There were no gross or histologic differences observed between controls and experimental groups on histological sections.

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P-D versus P-P controls: The relative trophoblastic surface density was significantly decreased in P-D vs. controls ($p = 0.03$) and the microcotyledonary surface density tended to be reduced in P-D ($p = 0.056$).

S-P versus S-S controls: The relative surface density and volume fraction of microcotyledonary vessels were significantly increased in S-P vs. controls ($p = 0.01$ and $p = 0.03$, respectively). The microcotyledonary surface density tended to be increased in S-P compared to controls ($p = 0.058$).

S-D versus S-S controls: The relative surface density of the allantoic vessels was reduced in S-D compared to S-S placentas ($p = 0.05$). The relative surface density of microcotyledonary vessels and trophoblast were reduced in S-D compared to S-P ($p < 0.01$, $p = 0.02$, respectively), resulting in reduction of the microcotyledonary surface density in S-D versus S-P ($p = 0.01$).

Placental gene expression (Table 3 and Figure 6)

Only the parameters that differed within a breed are presented in Figure 6.

Growth and development: The expression of *H19*, *IGF2*, *IGF1R* and *IGF2R* was decreased in S-P vs. S-S placentas (fold-change = 0.71, $p = 0.03$; fold-change = 0.53, $p < 0.01$; fold-change = 0.67, $p < 0.01$; fold-change = 0.62, $p = 0.05$, respectively). In S-P placentas, the expression of *H19* and *IGF2* was reduced compared to S-D placentas (fold-change = 0.56, $p = 0.02$ and fold-change = 0.63, $p = 0.01$, respectively).

Nutrient transfer: The expression of *SCL38A1* was increased in P-D vs. P-P (fold-change = 1.04, $p = 0.02$). The expression of *SLC2A1*, *SLC38A2* and *LPL* was decreased in S-P vs. S-S (fold-change = 0.71, $p = 0.01$; fold-change = 0.58, 0.03; fold-change = 0.71, $p = 0.02$, respectively). Finally, *CD36* gene expression was not different between groups.

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Vascularisation: *KDR* gene expression was increased in S-D compared to S-S placentas (fold-change = 1.34, $p = 0.04$). The expression of *FLT1* and *KDR* was reduced in S-P vs. S-D (fold-change = 0.76, $p = 0.03$; fold-change = 0.70, $p = 0.05$, respectively).

Discussion

Embryo transfer into a breed of a larger/smaller size induced fetal enhanced growth or growth restriction, respectively, as observed by others [23,32–34]. Enhanced growth was associated with increased placental size and weight in ponies and Saddlebreds, whereas growth restriction was associated with a reduction in both parameters and 3 cases of dysmaturity. In terms of placental features, enhanced growth was associated with a decrease in the surface density of trophoblast in ponies and of allantoic vessels in Saddlebred placentas, both with little gene expression modifications. In contrast, growth restriction was associated with an increase in trophoblast surface density and volume fraction together with a strong reduction in the expression of genes involved in growth and development and nutrient transfer in Saddlebred placentas.

Intrauterine enhanced growth did not affect gestational length in pony and Saddlebred foals. Enhanced growth was associated with heavier TFM and a larger placenta in both groups. These results are consistent with previous studies using between-breed embryo transfer and crossbreeding in horses, pigs and cows [23,33–37]. Placental efficiency (as estimated by the foal/TFM weight ratio) tended to be decreased in P-D foals maybe because placental growth was driven by uterine space. As the power of the statistical test was only 36%, these differences may have become significant if there had been more animals in the P-D group. Placental efficiency was not modified in S-D foals, probably because of the relatively smaller difference between S and D size.

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Pregnancy was longer in restricted S-P, as if time was unsuccessfully given to the foal to try and grow to its genetic potential. In humans, intra uterine growth retardation (IUGR) is defined as a birth weight below the tenth percentile for gestational age [38]. Here, the weight of S-P foals was 7.20 kg below the 10th percentile of S-S foals' birthweight and 8.54 kg below the 10th percentile of birth weight of 115 S-S foals from the same breed born between 2011 and 2015 in the same farm. Restricted S-P foals can thus be clinically considered as IUGR, which could explain the observed dysmaturity (in addition to clinical signs of dysmaturity at birth, foals presented an immature pancreatic function at 3 days of age) [28,30,31]. Indeed, their pancreatic function was immature when evaluated post-natally [25]. In Allen *et al.* experiments, IUGR foals also had symptoms of dysmaturity [23].

IUGR was associated with lighter TFM and a smaller placenta, consistent with other work in horses and pigs [23,35]. The lack of difference in placental efficiency (as estimated by the foal/TFM ratio, as used in other studies in horses and pigs [23,37,39]) between restricted foals and controls indicates that the placenta of Saddlebred foals was unable to overcome maternal uterine size constraints by structural or functional adaptations, which in turn prevented a possible dystocia.

In contrast to what was observed by others [23], trophoblast and microcotyledon surface density were reduced with fetal enhanced growth and increased in cases of IUGR. The reduced density in cases of enhanced growth may reflect placental adaptations to limit fetal growth beyond a certain threshold. This could explain why P-D foals tended to have a lower placental efficiency compared to P-P foals. Moreover, the work of Constancia *et al.* in mice elegantly showed that the placenta will respond to fetal demand and adjust nutrient transfer [40]. In the current situation, however, the demand from the pony foetuses may not have been sufficient to increase placental capacity. Conversely, the increased exchange surface in restricted Saddlebred foetuses may have improved nutrient supply to support fetal development. In their embryo transfer experiments, Allen *et al.* observed increased surface density in case of enhanced growth and reduced density in case of IUGR, using 10 randomly

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collected placental samples [23]. Here, placental structure was only analysed in one area, near the umbilical cord, to improve standardisation (in all the pregnancies, umbilical cord was attached at the base of the pregnant horn). This may be a major limitation of the current work as in humans, microcotyledonary development and gene expression vary between placental areas [41–44], but so far this variability has not been explored in the horse. The sampling in only one part of the placenta may thus not represent the overall placental structure and function. Moreover, both trophoblasts (haemotrophic and histotrophic trophoblasts) have been taken into account for the calculation of the relative surface and volume of the microcotyledons, unlike in the work of Allen *et al.* This difference of measure might also explain the difference of results between both studies.

The difference in gene expression between placentas from enhanced growth and control foetuses can be considered as not biologically relevant as only one gene, *SLC38A1*, was decreased in P-D vs. P-P placentas but with a fold-change of x1.04. Nevertheless, term placentas only reflect the terminal result of the pregnancy and one cannot preclude that adaptations did not take place earlier in pregnancy as shown in response to adverse nutritional conditions in mice [45]. Alternatively, foetal growth may have mainly been driven by the mechanical increase in placental size due to the increased uterine capacity.

In restricted S-P pregnancies, the expression of genes involved in growth and development (*H19*, *IGF2*, *IGF1R* and *IGF2R*) and nutrient transfer (*SLC2A1*, *SLC38A2* and *LPL*) was reduced. These results are well associated with the lowest TFM weight and the growth restriction of the foals at birth. *H19* and *IGF2* are major regulators of placental and fetal growth, especially through *IGF1R* and *IGF2R* [46,47]. A decrease in their expression might then be linked with the IUGR in the S-P group. Moreover, the fact that *H19*, *IGF2* and its receptor *IGF2R* are imprinted genes may have increased their susceptibility to be affected because of their monoallelic expression. As intrauterine growth of foals requires a high amount of glucose [48,49] and as *GLUT1* and *GLUT3* are glucose transporters in the horse placenta [50], a decrease in *SLC2A1* placental expression might also be linked with a lower

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amount of glucose transferred to the foal for growth. These results might also be linked with the delayed pancreatic maturation observed in foals from S-P group, 3 days after birth [25]. Moreover, IGF2 is known to regulate the nutrient availability and to control the expression of glucose and amino acids transporters [46]. Thus, as observed in other species, the decrease in the expression of *IGF2* is linked with the decrease of the expression of *SLC2A1* and *SLC38A1* [46]. Moreover, these results are in agreement with data in other species where IUGR has been associated with similar changes in humans, sheep and rodents [51,52].

One main limitation of this work is that control embryos were produced using artificial insemination. Nevertheless, in horses, data collected from foals and placentas born after ET or *in vivo* produced embryos or artificial insemination did not demonstrate any difference in placental nor foal size and weight at birth [53].

The other limitation is that ponies were not housed in the same farm as Saddlebred and draught mares. Nevertheless, they were all fed concentrates and hay in late gestation and their body weight and body condition remained constant throughout gestation [25]. Data from our own laboratory as well as others indicate that only severe restriction in maternal nutrition affect foal birthweight and placental size [54–58]. Thus, it may be considered that the effects of the different location may be negligible compared to effects due to differences in maternal and fetal size and breed.

Finally, age and parity are known to affect fetal and placental development with smaller foals and placentas being born to young primiparous mares [59,60]. Consequently, parity was always added to the statistical model. Results indicate that even though draught mares were significantly younger than Saddlebred and pony mares and that parity was higher in Saddlebred vs. pony and draught mares, foals born to draught mares were heavier with heavier placentas compared to the other groups. Moreover, when foal weights were corrected for parity (+0.7 kg per parity unit [60]), differences in foal birth weight remained statistically significant.

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Conclusion

Using ET between breeds of different size and weight, it was demonstrated that foal birthweight was directly related to placental weight and gross placental surface. Placental adaptations in restricted pregnancies were not sufficient to overcome the restriction imposed by the reduced maternal size, which also protected the mare and foal from severe risks of dystocia. Conversely, the reduced microcotyledonary exchange surface area in enhanced growth foetuses may also have prevented excess foetal oversize and dystocia.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Animal studies were approved by the local animal care and use committee and received ethical approval from the local ethics committee (approval No5-2013-5).

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Authorship

P. Chavatte-Palmer and A. Tarrade contributed to study design and preparation of the manuscript. P. Peugnet, S. Valentino, M. Robles contributed to study execution, data analysis and interpretation, and preparation of the manuscript. C. Dubois, F. Reigner, M. Dahirel and M-C. Aubrière contributed to study execution. All authors gave final approval of the manuscript.

Manufacturers' addresses

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²National Institute of Health

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⁷www.r-project.org

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Supplementary information

Supplementary Item 1: Nutritional values of pregnant mares' diets.

Supplementary Item 2: Co-factors associated with foal birthweight and placental biometry, morphometry and gene expression.

Supplementary Item 3: Histological sections of term allantochorion.

Figure and table legends

Fig 1: Establishment of control pregnancies by artificial insemination and of experimental pregnancies by embryo transfer.

Fig 2: Measurement of the gross placental surface area. A clear plexiglas sheet marked with 10 cm x 10 cm squares was applied above the placenta

Fig 3: Structure of the horse placenta at term. AC: Allantoic connective tissue, AV: Allantoic vessel, HT: Histotrophic trophoblast.

Fig 4: Foal birth weight (A), total fetal membranes (TFM) weight (B), placental surface area at term (C) and foal/TFM weight ratio (D). Different letters indicate p-value ≤ 0.05 .

Fig 5: Surface density and volume fraction of the different components of the placenta. P-P (n = 19): Pony-in-Pony; P-D (n = 6): Pony-in-Draught; S-P (n = 6): Saddlebred-in-Pony; S-S

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(n = 28): Saddlebred-in-Saddlebred; S-D (n = 8): Saddlebred-in-Draught. Different letters indicate p-value ≤ 0.05 .

Fig 6: Calibrated normalised relative quantity (arbitrary unit) for differentially expressed genes between groups. The graphs present the genes involved in (A): growth and development, (B): nutrient transfer and (C): vascularisation. P-P (n = 19): Pony-in-Pony; P-D (n = 4): Pony-in-Draught; S-P (n = 6): Saddlebred-in-Pony; S-S (n = 21): Saddlebred-in-Saddlebred; S-D (n = 8): Saddlebred-in-Draught. Different letters indicate p-value ≤ 0.05 .

Table 1: Number of pregnancies with maternal age and withers height (median [quartile 1-quartile 3], parity and gestational length within the six groups for each experimental year (2011 and 2012).

Different letters inside each sub-table indicate p-value ≤ 0.05 .

Table 2: Gene-specific primers and accession number or reference.

Table 3: Gene expression within the 6 groups of placentas. A. Calibrated normalised relative quantity (CNRQ) for differentially expressed genes. Data are presented as median [quartile 1 – quartile 3].

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Table 1. Number of pregnancies with maternal age and withers height (median [quartile 1-quartile 3], parity and gestational length within the six groups for each experimental year (2011 and 2012).

Groups	Mares and recipient mares				Pregnancies			
	Age (years)	Withers height (cm)	% primiparous	% multiparous	Foalings - 2011	Foalings - 2012	Foalings - total	Gestational length (days)
Effect of the breed								
P-P	9.0 [5.1-10.1] ^a	126.5 [121.0-129.5] ^a	57 ^a	43 ^a	10	11	21	334 [328 – 340]
S-S	7.2 [4.3-13.0] ^a	159.5 [156.0-163.5] ^b	32 ^b	68 ^b	18	10	28	331 [326 – 335]
Pony embryos transferred into draft mares								
P-P	9.0 [5.1-10.1] ^a	126.5 [121.0-129.5] ^a	57	43	10	11	21	334 [328 – 340]
P-D	4.5 [3.8-6.0] ^b	157.5 [150.5-162.0] ^b	67	33	5	1	6	328 [322 – 332]
Saddlebred embryos transferred into ponies or draft mares								
S-S	7.2 [4.3-13.0] ^a	159.5 [156.0-163.5] ^a	32 ^a	68 ^a	18	10	28	331 [326 – 335] ^b
S-P	8.6 [4.6-9.3] ^{ab}	132.5 [129.8-136.0] ^b	83 ^b	17 ^b	4	2	6	344 [336 – 352] ^a
S-D	3.7 [3.3-4.2] ^b	158.0 [154.3-160.5] ^a	75 ^b	25 ^b	8	0	8	328 [327 – 330] ^b

Different letters inside each sub-table indicate p-value<0.05.

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Table 2. Gene-specific primers and accession number or reference.

	Candidate gene	Forward and reverse primers	Accession number
Growth and development	<i>H19</i>	F 5'-GGACCCCAAGAACCCTCAAG-3' R 5'-GGGACTTGAAGAAGTCCGGG-3'	NR_027326
	<i>IGF2</i>	F 5'-TTTCTTGGCTTTTGCCTCGT-3' R 5'-CCTGCTGAAGTAAAAGCCGC-3'	NM_001114539.1
	<i>IGF1R</i>	F 5'-CGAGAAGACCACCATCAACAAC-3' R 5'-TGGCAGCACTCGTTGTTCTC-3'	XM_001489765.2
	<i>IGF2R</i>	F 5'-GTCGGCTTGCCAGATGAGAT-3' R 5'-TACTGATGGAGACGGCCTCA-3'	XM_001491469.2
	<i>TGFβ1</i>	F 5'-TTGATGTCACCGGAGTCGTG-3' R 5'-CCACGCGGAGTGTGTTATCT-3'	NM_001081849.1
	<i>EGFR</i>	F 5'-GTCTGGAAGTTTGC GGATGC-3' R 5'-CTTGGGCCCATTTCTTGAC-3'	XM_001497730.2
Nutrient transfer	<i>SLC2A1</i>	F 5'-TGTGCTCATGACCATCGCC-3' R 5'-AAGCCAAAGATGGCCACGAT-3'	NM_001163971.1
	<i>SLC38A1</i>	F 5'-AAATGAACTACCCTCCGCCA-3' R 5'-ACACAGAGGGAGAATTATGCCAA-3'	XM_001489402.
	<i>SLC38A2</i>	F 5'-ACAGCTCGAACAGCGACTTCA-3' R 5'-TTCTTCCCCAAATTTCGATTCA-3'	NM_001081849.1
	<i>CD36</i>	F 5'-GGTCTACGCCGTGTTTGGAG-3' R 5'-CCGTGCAGAAGCAGTGGTTA-3'	<u>XM_005609038.1</u>
	<i>LPL</i>	F 5'-AGTTGGGTGCCAAAATTGTG-3' R 5'-GCTTGGTGTACCCCGCAGAC-3'	XM_001489577.2
Vascularization	<i>VEGFα</i>	F 5'-TACCTCCACCATGCCAAGTG-3' R 5'-GTCTCGATTGGACGGCAGTA-3'	<u>NM_001081821.1</u>
	<i>Flt1</i>	F 5'-AGTGTGAGCGGCTCCCTTATG-3' R 5'-ATGCCAAATGCAGATGCTTG-3'	XM_003363176.1
	<i>KDR</i>	F 5'-CAGTGGGCTGATGACCAAGA-3' R 5'-TCCACCGAAGATTCCATGCC-3'	XM_001916946.2
	<i>eNOS</i>	F 5'-TTCGGGAGAGTGAGCTGGTA-3'	XM_001504650.3

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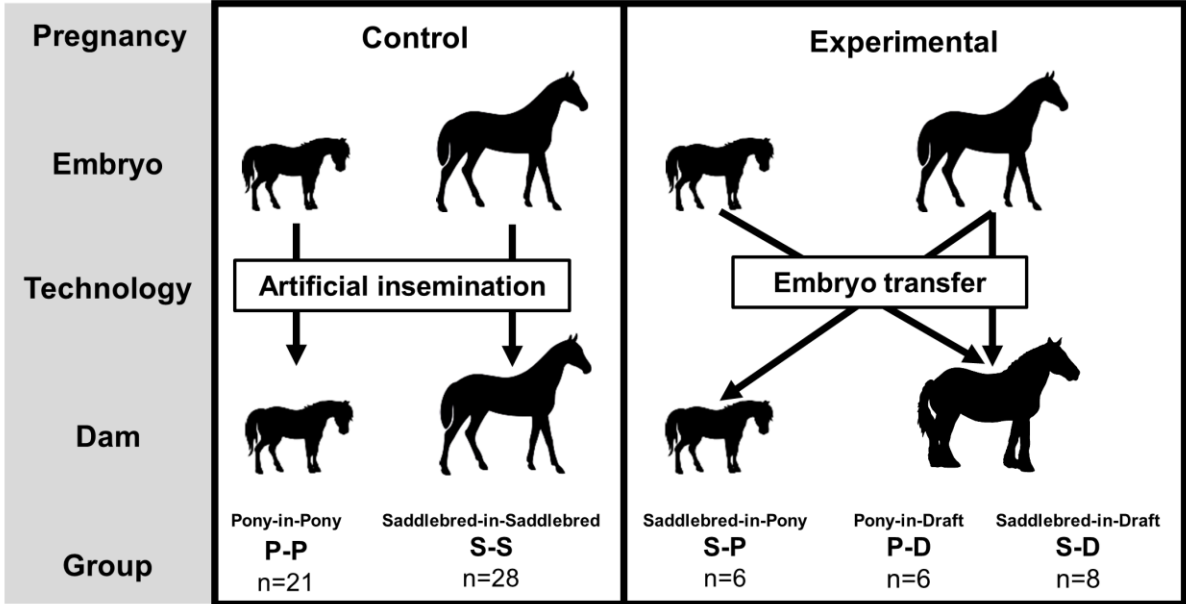
		R 5'-CAATCCCGCGCATCAAAGAC-3'	
<i>Reference genes</i>	<i>GAPDH</i>	F 5'-CGATGGTGAAGGTCGGAGTAA-3' R 5'-TGAAGGGGTCATTGATGGCG-3'	NM_001163856.1
	<i>RPL32</i>	F 5'-TGAAGTGCTGCTCATGTGCA-3' R 5'-GGGATTGGTGATTCTGATGGC-3'	ENSECAG00000007201
	<i>SCAMP3</i>	F 5'-CTGTGCTGGGAATTGTGATG-3' R 5'-ATTCTTGCTGGGCCTTCTG-3'	[25]

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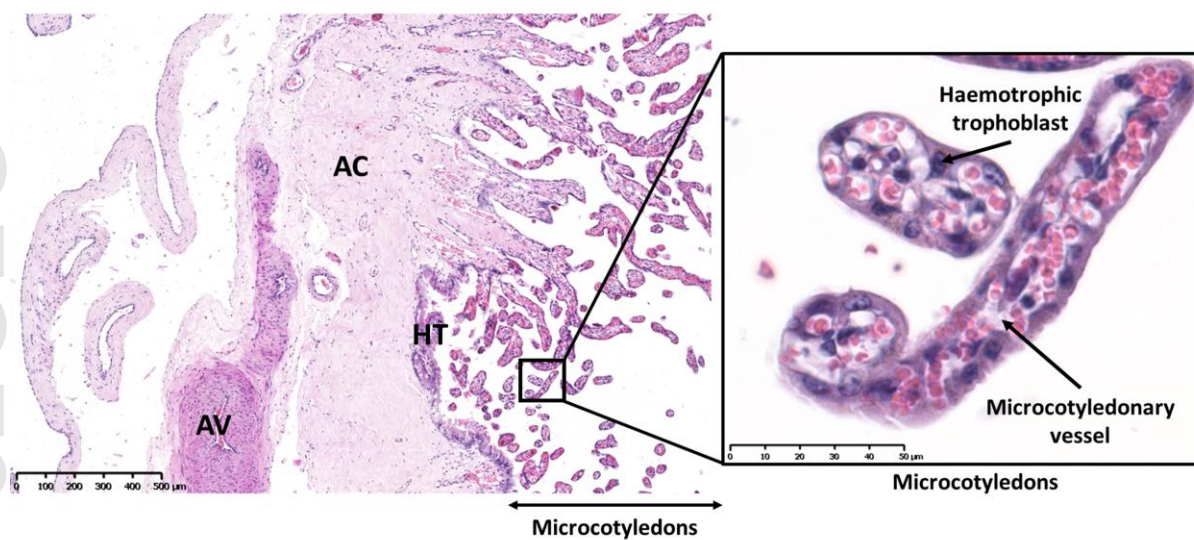
Table 3. Gene expression within the 6 groups of placentas. A. Calibrated normalized relative quantity (CNRQ) for differentially expressed genes. Data are presented as median [quartile 1 – quartile 3].

	Gene	CNRQ				
		P-P	P-D	S-P	S-S	S-D
Growth and development	<i>H19</i>	1.15 [0.51-1.49]	0.86 [0.54-1.24]	0.80 [0.50-1.03]	1.13 [0.91-1.60]	1.43 [0.99-1.74]
	<i>IGF2</i>	0.70 [0.61-0.87]	0.74 [0.56-0.95]	0.55 [0.34-0.66]	1.04 [0.67-1.30]	0.86 [0.65-1.12]
	<i>IGF1R</i>	0.59 [0.48-0.78]	0.56 [0.47-0.68]	0.63 [0.37-0.74]	0.93 [0.61-1.47]	0.65 [0.44-1.30]
	<i>IGF2R</i>	0.62 [0.54-0.66]	0.65 [0.55-1.14]	0.61 [0.41-0.68]	0.97 [0.68-1.31]	0.82 [0.62-1.14]
	<i>TGFB1</i>	0.53 [0.42-0.76]	0.72 [0.26-1.12]	1.23 [0.80-2.30]	1.50 [0.96-2.20]	0.71 [0.63-2.03]
	<i>EGFR</i>	1.50 [1.16-1.78]	1.52 [1.36-2.04]	1.34 [0.52-1.69]	1.18 [0.76-1.42]	0.38 [0.35-0.64]
Nutrient transfer	<i>SLC2A1</i>	0.69 [0.61-0.75]	0.59 [0.57-1.63]	0.68 [0.57-0.75]	0.95 [0.80-1.19]	0.86 [0.66-1.30]
	<i>SLC38A1</i>	0.98 [0.57-1.36]	1.03 [0.58-4.32]	0.61 [0.42-1.36]	0.70 [0.40-1.10]	0.91 [0.63-1.72]
	<i>SLC38A2</i>	0.58 [0.54-0.66]	0.64 [0.56-1.67]	0.58 [0.46-0.65]	1.01 [0.55-1.40]	0.55 [0.49-0.91]
	<i>CD36</i>	0.72 [0.55-0.90]	0.73 [0.58-0.97]	0.59 [0.52-0.72]	0.78 [0.47-1.17]	0.61 [0.49-0.99]
	<i>LPL</i>	0.66 [0.53-0.74]	0.78 [0.74-0.82]	0.77 [0.59-0.88]	1.08 [0.67-1.40]	0.69 [0.50-0.95]
Vascularization	<i>VEGFA</i>	1.22 [0.90-1.40]	0.89 [0.63-1.11]	1.40 [1.05-1.79]	1.23 [0.80-1.57]	0.84 [0.68-1.36]
	<i>Flt-1</i>	0.65 [0.52-0.81]	0.58 [0.45-0.65]	0.62 [0.41-0.76]	0.77 [0.63-1.20]	0.82 [0.74-1.18]
	<i>KDR</i>	0.69 [0.58-0.80]	0.57 [0.46-1.13]	0.65 [0.55-0.81]	0.71 [0.55-0.98]	0.93 [0.75-1.29]
	<i>eNOS</i>	0.65 [0.46-0.80]	0.49 [0.37-5.10]	0.54 [0.35-0.83]	1.13 [0.75-1.68]	0.51 [0.43-2.08]

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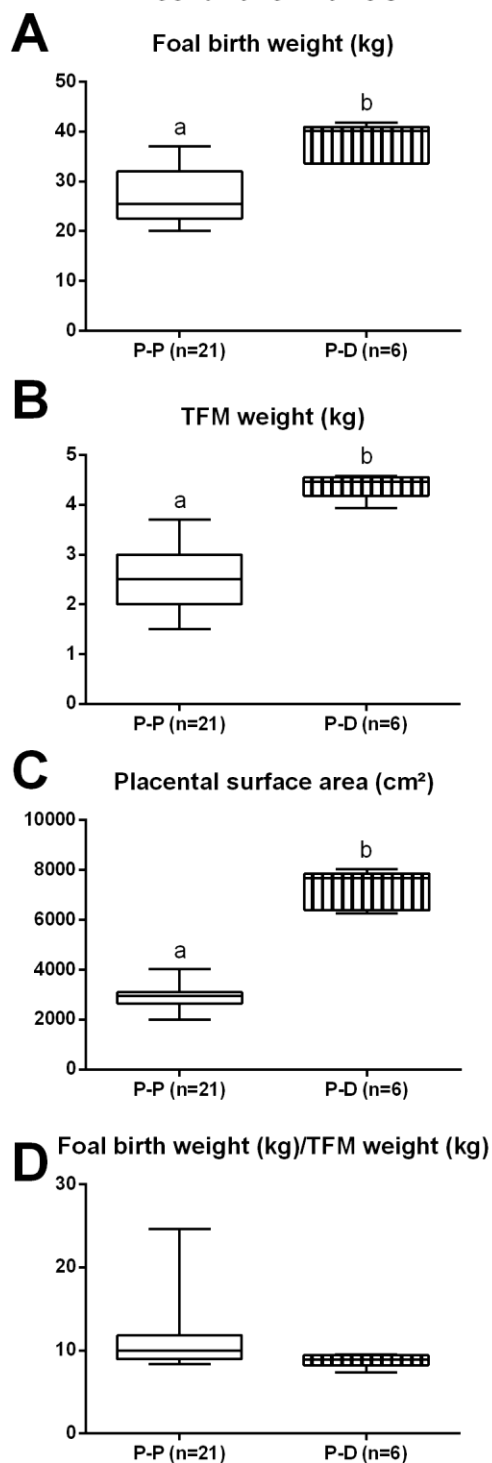


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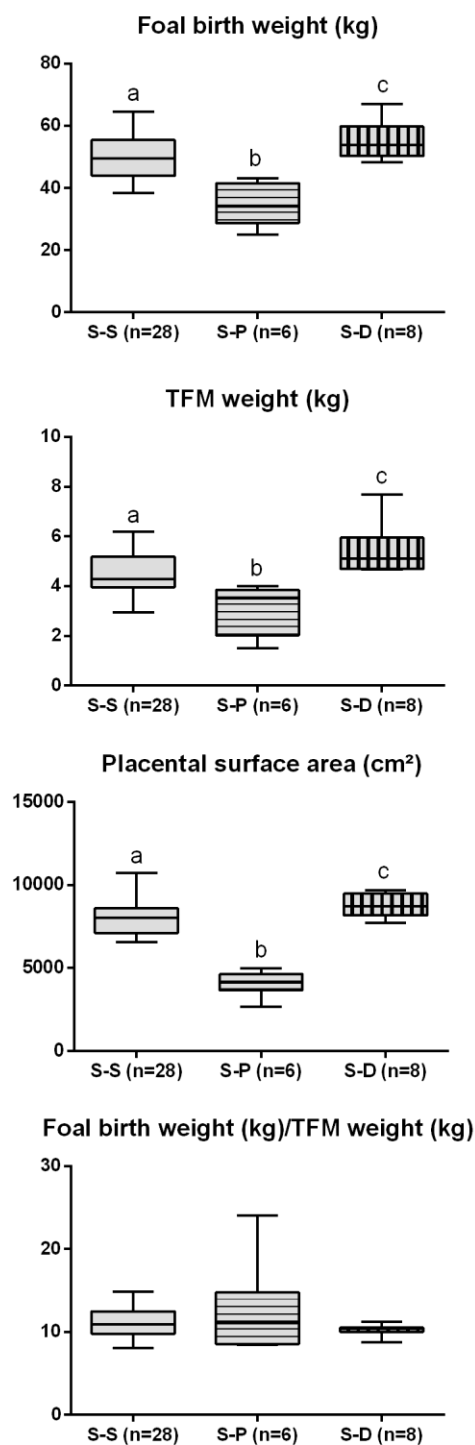


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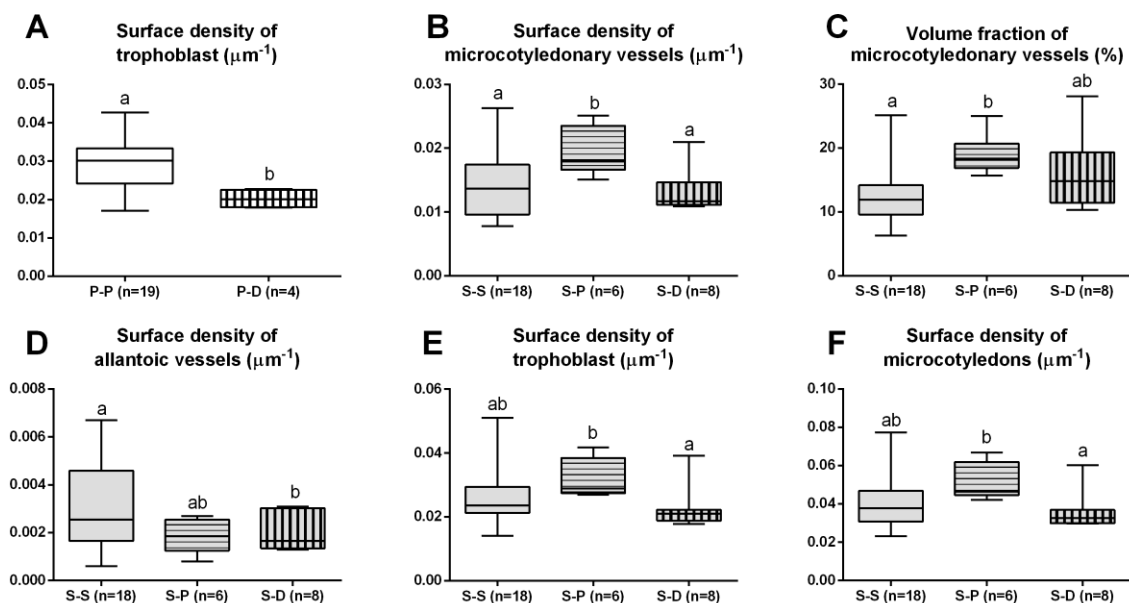
Pony embryos transferred into draft mares



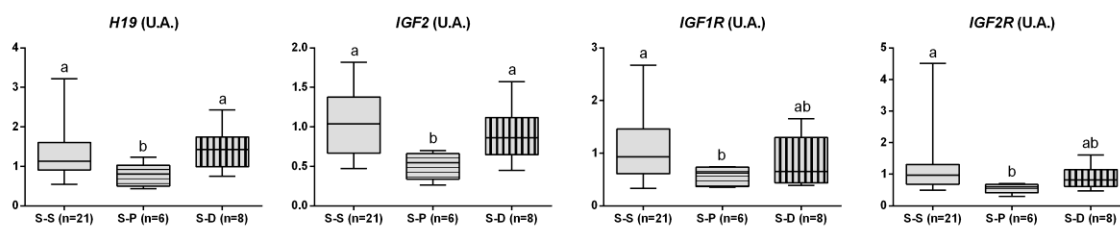
Saddlebred embryos transferred into pony and draft mares



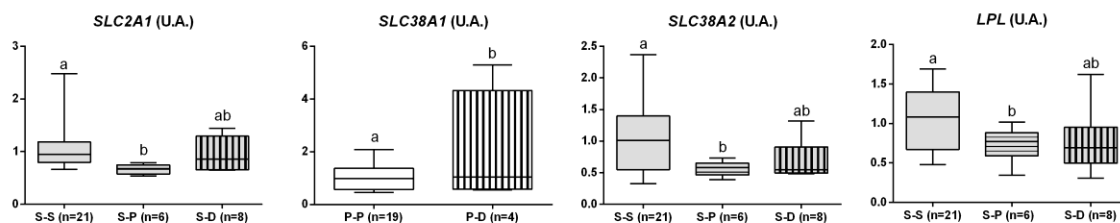
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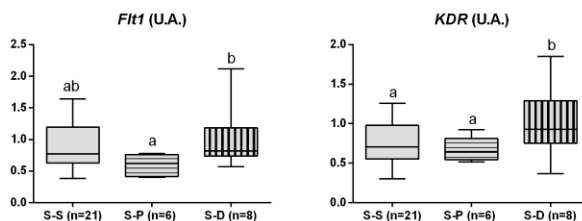
A. Growth and development



B. Nutrient transfer



C. Vascularization



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