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Negative uterine asynchrony retards early equine conceptus development and upregulation of placental imprinted genes

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ABSTRACT

Introduction: Placental imprinted genes appear to be sensitive indicators of an inappropriate pre-implantation environment. This study examined the effects of negative uterine asynchrony after embryo transfer (ET) on early horse embryo development, and yolk-sac membrane expression of DNA methyltransferases (DNMTs) and equine specific placental imprinted genes.
Methods: Day 8 embryos were transferred to recipient mares on day 8 (synchronous) or day 3 (asynchronous) after ovulation, and conceptuses were recovered 6 or 11 days later (day 14 or 19 of development).
Results: Day 14 conceptuses recovered from an asynchronous uterus had a smaller embryonic disc, in which primitive streak development was visibly retarded compared to conceptuses from a synchronous uterus. Similarly, length, somite number and organogenesis were retarded in day 19 embryos after asynchronous ET. Maternal (*GRB10*, *H19*, *IGF2R*, *PHLDA2*) and paternal (*IGF2*, *INSR*, *PEG3*, *PEG10*, *DIO3*, *NDN*, *SNRPN*) imprinted genes and DNMTs (*DNMT1*, *3A* and *3B*) were all up-regulated between day 14 and 19 of pregnancy and, for most, mRNA expression was higher in synchronous than asynchronous day 19 yolk-sac membrane. Expression of the paternally imprinted gene HAT1 increased between day 14 and 19 of pregnancy, but was not affected by the asynchrony.
Discussion: Conceptus development and upregulation of DNMTs and imprinted genes were delayed rather than dysregulated after transfer into a negatively asynchronous uterus. We propose that this ability to 'reset' conceptus development to uterine stage is an adaptation that explains why horse embryos are unusually tolerant of asynchrony after ET.

1. Introduction

Early equine pregnancy is characterized by a long pre-implantation period of approximately 40 days, after which implantation (meaning formation of the definitive allantochorion placenta) involves the non-invasive interdigitation of allantochorion villi with maternal endometrium which is shortly preceded by an additional 'implantation-like' event, the invasion of the specialized trophectoderm cells of the chorionic girdle into the endometrium [1]. In the horse industry, embryo transfer is a common practice and often, due to shortage of recipients, non-optimally synchronized recipient mares are used. Good synchrony between a donor and a recipient mare is considered to have been obtained when the recipient ovulates 1 day before to 3 days after the donor [2]; however, increasing the degree of negative asynchrony does not seem to be harmful to the conceptus and could be beneficial in a commercial setup, requiring fewer recipient mares to be available [3].

Several studies in domestic animals have shown that conceptus-endometrial asynchrony alters growth and development of the conceptus, with growth accelerated in a more advanced uterus and retarded in a negatively asynchronous uterus; these changes are associated with a higher incidence of early embryonic loss [4–7]. One notable exception is the equine conceptus which, although showing a significant retardation in development, can tolerate up to five days of negative uterine asynchrony without a marked loss of viability [3,8].

Progesterone secreted by the corpus luteum stimulates and maintains endometrial functions critical for conceptus growth and development [9]. The time of onset, absolute concentration and duration of exposure of the uterus to progesterone seem to play

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causative roles in accelerating or decelerating conceptus development, at least in the cow and sheep [10–12]. The exact mechanism by which progesterone priming of the uterus controls conceptus growth is however unclear, although it appears that lengthening the period of progesterone exposure increases glucose and amino acid transport in ewes, which in turn stimulates conceptus growth and development [13].

Imprinted genes have been proposed to act as nutrient sensors regulating placental growth and nutrient transport to the fetus, and consequently stimulating or constraining fetal growth [14–16]. In general, imprinted genes are highly expressed in placental tissue [17,18], in a parent-of-origin specific manner, and it is believed that imprinting has evolved to regulate the 'evolutionary conflict' between maternal and paternal interests with regard to the allocation of resources to the developing embryo [19]. Indeed, paternally expressed genes generally enhance fetal growth, whereas maternally expressed genes restrict it [20]. Moreover, recent studies have shown that environmental factors can affect early embryo development and expression of developmentally important and/or placentally imprinted genes, presumably by epigenetic dysregulation [18,21,22]. Imprinted genes may, therefore, be particularly sensitive to epigenetic alterations as a result of an inappropriate or altered environment during early embryo development [21]. Recently, Wang and co-workers [23] used donkey-horse hybrid conceptuses to verify genes displaying parent-of-origin differential expression in early equine placenta, and identified 15 ancient imprinted genes.

The large degree of asynchrony tolerated by the equine conceptus offers a unique model to study the possible involvement of dysregulated imprinted gene expression in altered early conceptus development in an 'out of phase' uterine environment. The current study therefore used embryo transfer to evaluate the effect of negative uterine asynchrony on early embryo development and to examine whether altered development was associated with changes in expression of DNA methyltransferases and/or imprinted genes linked to placental growth and development.

2. Materials and methods

2.1. Animals

All animal procedures were approved by Utrecht University's Animal Experimentation Committee (permit number 2012.III.02.020). A total of 22 warmblood mares, aged between 4 and 15 years of age, were managed at pasture with *ad libitum* access to grass and water. During oestrus, mares were monitored by transrectal ultrasonography (MyLab30 ultrasound machine equipped with a 7.5 MHz linear transducer; Esoate, Maastricht, The Netherlands) [24]. When the dominant follicle exceeded 35 mm in diameter, donor mares were inseminated with a minimum of 500×10^6 sperm cells from a single fertile stallion; insemination was repeated every second day until ovulation was detected. In order to obtain the correct degree of synchrony or asynchrony, all mares (donors and recipients) were treated with a PGF2α analogue (37.5 μg D-cloprostenol; Genestranvet: Eurovet Animal Health B.V., Bladel, The Netherlands) to induce luteolysis and human chorionic gonadotrophin to ensure the desired timing of ovulation (hCG; 1500 i.u. Chorulon: Intervet, Boxmeer, The Netherlands).

2.2. Embryo collection and embryo transfer

On day 8 after ovulation, embryos were recovered from donor mares by uterine lavage, as described previously [25]. Recovered embryos were washed in holding medium (Syngro; Bioniche Animal Health, Pullman, WA, USA) and their diameter was measured using a stereomicroscope (Olympus SZ-ST; Olympus, Tokyo, Japan) equipped with an eye-piece micrometre. Only grade 1–2 embryos [26] were used for embryo transfer (ET). In order to produce the desired 20 pregnancies, a total of 26 day 8 embryos were transferred, as previously described [25], to recipient mares that had either ovulated on the same day as the donor (synchronous; n = 13), or 5 days after the donor mare (asynchronous; n = 13, Fig. 1). Transfer to synchronous mares resulted in 10 pregnancies out of 13 transfers (76%); 12 mares out of 13 were pregnant after asynchronous embryo transfer (92%) but 2 conceptuses were lost during collection.

2.3. Conceptus and endometrium collection

Before recovery, embryonic vesicles were measured in 2 perpendicular directions by transrectal ultrasonography. Day 14 conceptuses were recovered 6 days after ET by uterine lavage via a sterile endotracheal tube with an internal diameter of 19 mm [27]. Day 19 conceptuses were recovered 11 days after ET using an endoscopically-guided net, after puncture of the membranes and aspiration of the yolk-sac fluid with a sharpened PTFE catheter [24]. Conceptuses were washed in large amounts of 0.9% NaCl. Under a stereomicroscope, the capsule was removed and the embryonic disc region or embryonic body were dissected from the conceptus membranes using microsurgical scissors. On day 14, the remaining conceptuses membranes consisted of bilaminar yolk-sac (endoderm and trophectoderm). For day 19 conceptuses, in which extra-embryonic mesoderm has extended out such that part of the yolk-sac membranes are trilaminar, it was often difficult to definitively distinguish between bilaminar and trilaminar yolk-sac membranes using a dissecting microscope; for this reason, all extra-embryonic membranes were stored together. Yolk-sac membranes were snap frozen in liquid nitrogen and stored at −80 °C.

2.4. Microscopic analysis of conceptus development

After dissection, the embryo proper was examined under the stereomicroscope (magnification of x30). The embryonic disc of day 14 conceptuses was measured, the area calculated and the developmental stage of the primitive streak was noted. For day 19 conceptuses, the embryo's length was measured, the number of pairs of somites counted and flexion of the embryo, development of the neural tube and a primitive heart structure, and membrane vascularity were recorded.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Venlo, The Netherlands). Yolk-sac membranes (30 mg) were homogenized in 600 μl lysis buffer and total RNA was eluted with 40 μl RNase-free water, after which RNA concentration and integrity were measured as previously described [24]. Reverse transcription was performed using Superscript III (Invitrogen) as previously described [24], in a total volume of 20 μl made up of 10 μl of sample containing 1000 ng of RNA which was treated with DNAse I (30 min at 37 °C followed by 10 min at 65 °C; 1 IU/μg of RNA; RNAse-Free DNase set, Qiagen).

2.6. Quantitative RT-PCR

The qRT-PCR protocol was described previously [24]. Primers were produced at Eurogentec (Seraing, Belgium) and specificity was tested by DNA sequencing (ABI PRISM 310 Genetic analyzer; Applied Bio-system, Foster City, CA). Real-time PCR was carried out in 15 μl of reaction mix including 7.5 μl of IQ SYBR® Green Supermix

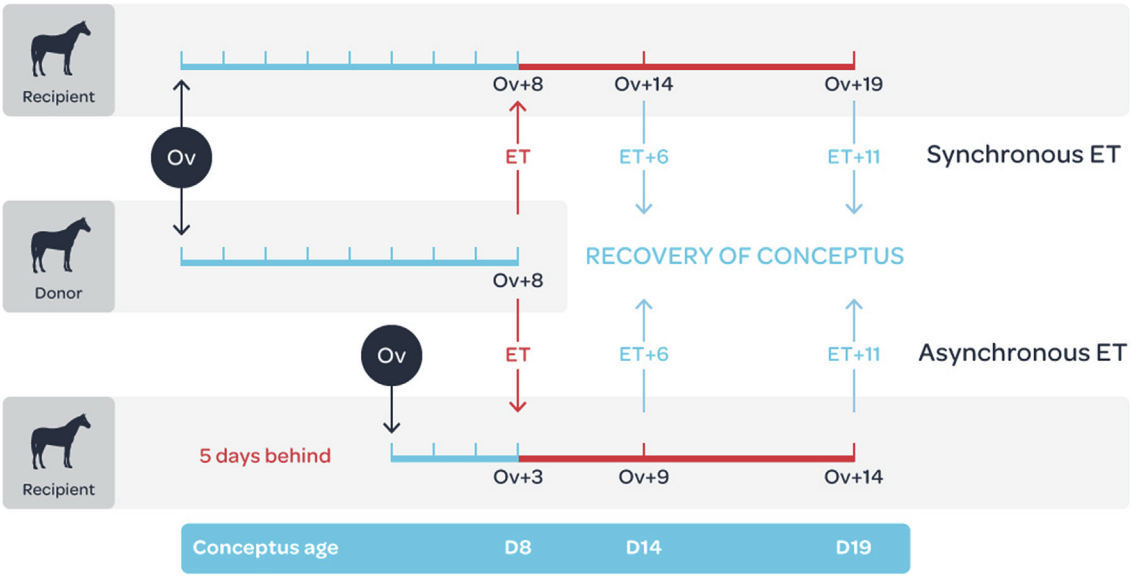

Fig. 1. Equine asynchronous embryo transfer model. Recipient mares ovulated on the same day (synchronous) or 5 days later (asynchronous) than the donor mare. Day 8 embryos were transferred to recipient mares and subsequently recovered 6 or 11 days after embryo transfer (Day 14 or 19 of conceptus development). Endometrial stage is determined by the ovulation date of the recipient mare (Ov: ovulation; ET: embryo transfer).

(BioRad), 0.5 mM of primer (forward and reverse; Supp. table 1), and 1 μl of cDNA, on an IQ5 Real-Time PCR detection System (BioRad; Veenendaal, The Netherlands). Cycle conditions were; denaturation for 3 min at 95 °C, followed by 40 cycles of amplification (15 s at 95 °C, 30 s at primer specific annealing temperature and 30 s at 72 °C). For each gene, a melting curve and standard curve were performed to verify product specificity and enable expression quantification. Relative gene expression was expressed as the ratio of target gene expression to the geometric mean of three housekeeping genes (GAPDH, HPRT1 and SRP14), selected after stability evaluation using GeNorm [28].

2.7. Statistical analysis

All data were analyzed using SPPS Statistics 20 for Windows (SPSS Inc., Chicago, IL). Normally distributed QRT-PCR datasets were obtained by logarithmic transformation. A two-way ANOVA was performed to examine the impact of stage of pregnancy and synchrony of ET; when a significant effect was found, independent T-tests were used to further examine the source. One-way ANOVA was performed to compare embryo development at a given stage. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Conceptus development

3.1.1. Ultrasonography

At day 14 of development, asynchronous conceptus vesicles had a smaller diameter (mean ± SEM) than synchronous conceptuses (11.3 ± 0.6 mm vs. 18.2 ± 1.1 mm; $P < 0.005$: Suppl. Fig. 1). Vesicle diameter increased from day 14–19 of conceptus age in both synchronous and asynchronous groups ($P < 0.005$), but was no longer significantly different between the groups at day 19 (26.5 ± 2.2 mm vs. 30.8 ± 2.2 mm: Suppl. Fig. 1).

3.1.2. Day 14 conceptuses (6 days post ET)

Although all conceptuses had developed an embryonic disc by day 14 (10/10), only the synchronous conceptuses exhibited a visible primitive streak (sync: 5/5 vs. asyn: 0/5 Fig. 2A–B). Moreover, the embryonic disc area was smaller in the asynchronous group (0.48 ± 0.08 mm^2) than the synchronous group (1.57 ± 0.08 mm^2; $P < 0.001$: Fig. 2C).

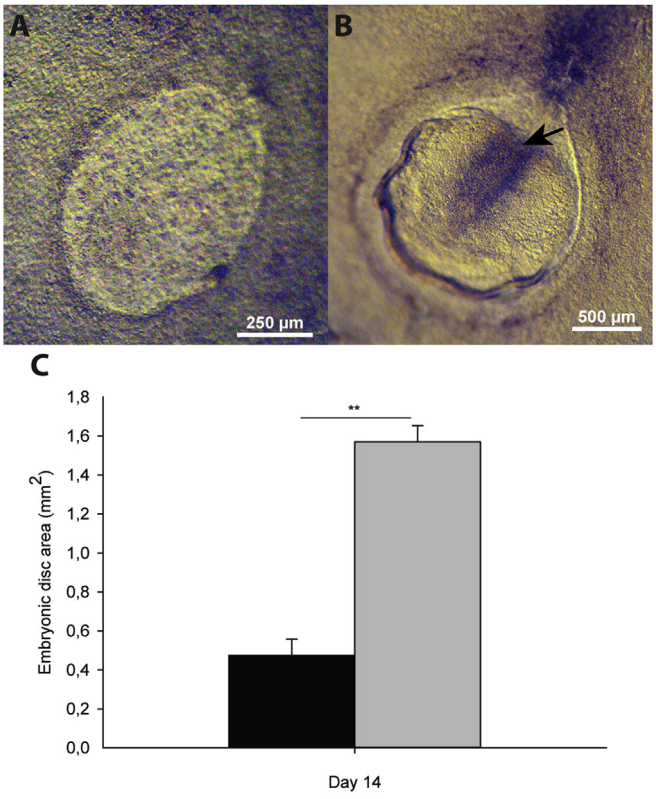

Fig. 2. Development of horse embryos on day 14 (ET+6), following asynchronous (−5 days) or synchronous embryo transfer on day 8. Microscopic appearance of the embryonic disc after asynchronous (A) or synchronous (B) embryo transfer. The arrow indicates the primitive streak. (C) Mean (±s.e.m) area of the embryonic disc of day 14 embryos from asynchronous (black bars) or synchronous (grey bars) embryo transfer (**: $P < 0.001$, $n = 5$).

3.1.3. Day 19 conceptuses (11 days post ET)

By day 19, all of the embryos had undergone neurulation (10/10); however, the process was not as advanced in the asynchronous group (Fig. 3A–B) in which the neural folds delimiting the neural groove had formed, but only just started to fuse from the embryo's mid-point (5/5; Fig. 3A). By contrast, synchronous embryos had a fully formed neural tube with a closed cranial neuropore, and had undergone flexure (5/5 compared to 0/5 for asynchronous embryos). Only embryos from the synchronous group displayed a primitive heart, brain structures and vascularized (trilaminar) conceptus membranes (extra-embryonic mesoderm; 5/5 versus 0/5; Fig. 3B). The length of the embryonic body in the asynchronous group was half of that in the synchronous group (2.55 ± 0.19 mm vs. 5.05 ± 0.23 mm; $P < 0.001$: Fig. 3C) and, while somites were visible in both groups, the number of somite pairs was significantly reduced in the asynchronous compared to the synchronous embryos (7.5 ± 1.2 vs. 21.7 ± 0.3; $P < 0.001$: Fig. 3C).

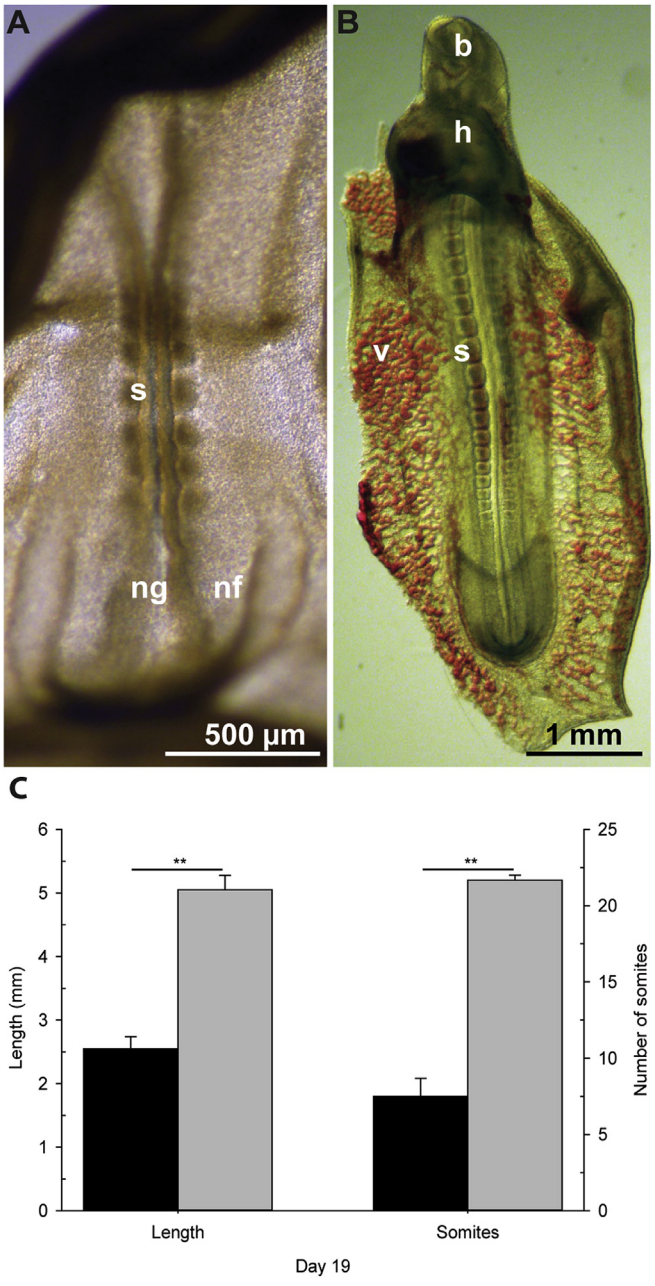

Fig. 3. Development of horse embryos on day 19 (ET+11), following asynchronous (−5 days) or synchronous embryo transfer on day 8. Microscopic appearance of the embryo proper after asynchronous (A) or synchronous (B) embryo transfer. b = brain: h = heart: nf = neural fold: ng = neural groove: v = vascularization. (C) The mean (±s.e.m) length (left) and number of somites (right) of day 19 embryos resulting from asynchronous (black bars) or synchronous (grey bars) embryo transfer (**$P < 0.001$, n = 5).

3.2. qRT-PCR

3.2.1. Expression of paternally expressed genes

There was no single common pattern for the effects of conceptus age and embryo-uterus synchrony on the expression of paternally expressed genes in yolk-sac membranes. However, for the majority of paternally expressed genes (*DIO3*, *IGF2*, *INSR*, *PEG3*, *PEG10*, *NDN*, *SNRPN*: $P < 0.05$: Fig. 4), gene expression increased with the stage of embryo development (i.e. was higher at day 19 than 14). One gene, *HAT1*, was not affected by the stage of embryo development, but was more highly expressed in synchronous than asynchronous pregnancies at both days 14 and 19 ($P < 0.05$). Expression of *IGF2*, *INSR*, *PEG3* and *PEG10* mRNA was higher in synchronous than asynchronous conceptuses at day 19 ($P < 0.05$) and, for *IGF2*, *PEG3* and *PEG10* there was also a combined effect of pregnancy stage and synchrony status ($P < 0.005$). For *DIO3*, *NDN* and *SNRPN* there was no difference in expression between synchronous and asynchronous pregnancies.

3.2.2. Expression of maternally expressed genes

All of the maternally expressed genes examined (*GBR10*, *H19*, *IGF2R* and *PHLDA2*) showed an increase in yolk-sac mRNA transcript abundance between days 14 and 19 of development ($P < 0.05$: Fig. 5), although for *IGF2R* the difference only reached statistical significance for the synchronous conceptuses. Expression of *GRB10* was not affected by embryo-uterine synchrony, whereas expression of *H19* and *PHLDA2* was higher in the synchronous group at day 19 ($P < 0.05$).

3.2.3. Expression of DNMTs

The expression of *DNMT1*, *DNMT3A* and *DNMT3B* was affected by stage of conceptus age for synchronous conceptuses only ($P < 0.05$: Fig. 6). In all cases, expression increased between day 14 and day 19; moreover, on day 19 but not day 14, mRNA levels were significantly higher in synchronous than in asynchronous conceptuses ($P < 0.05$).

4. Discussion

As in previous studies, early equine embryos transferred to a severely negatively asynchronous uterus were able to adapt and survive without an obvious reduction in their viability, in as much as they were able to establish pregnancy albeit with an obvious retardation in the rate of development [3,8,29]. Moreover, the delay in development was detectable at the level of the transcriptome in that expression of imprinted genes and DNMTs in yolk-sac membrane of conceptuses recovered from an asynchronous uterus was altered, in most cases this involved a reduction presumed to result from delayed upregulation.

The delayed development of asynchronous conceptuses was quantifiable by ultrasonography as a reduced diameter of the embryonic vesicle on day 14 of conceptus age. By day 19, vesicle diameter was no longer statistically different, in part because of intra-group variability, but also because this is the period (days 17–27) in which the vesicle loses its perfectly spherical shape and enters the 'plateau phase', when it ceases to show a marked day-to-day increase in diameter [30]. Nevertheless, the reduced diameters

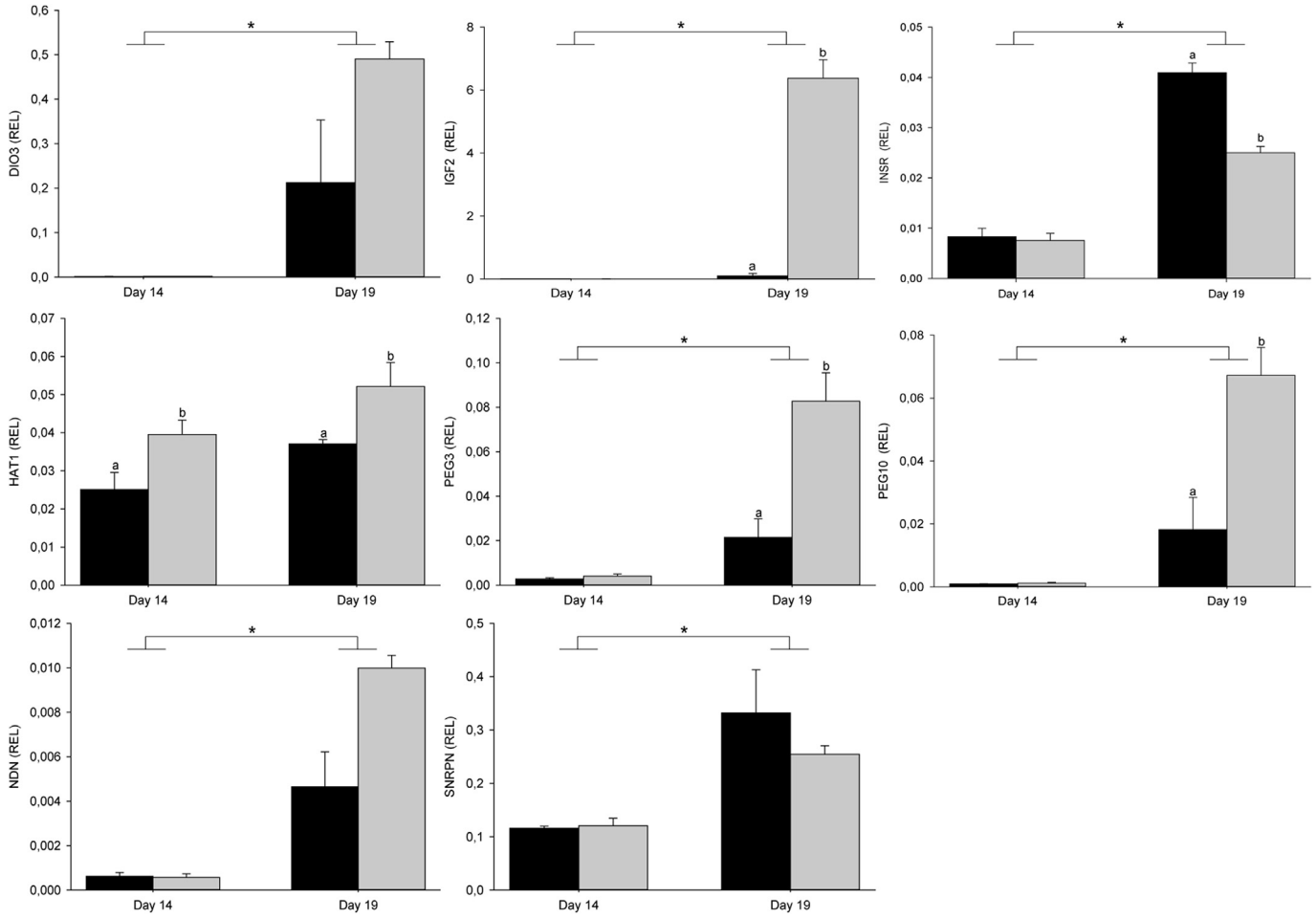

Fig. 4. Relative gene expression (mean ± s.e.m) for paternally expressed genes in equine yolk-sac membranes at days 14 and 19 of conceptus development, after asynchronous (- 5 days: black bars) or synchronous (grey bars) embryo transfer. Significant differences ($P < 0.05$) between conditions (synchronous versus asynchronous) within a pregnancy stage are depicted by different superscripts (a, b) whereas between pregnancy stage differences are indicated by an asterisk (*).

observed by ultrasonography paralleled the developmental delay of approximately four days reported by Wilsher et al. [8] after conceptuses were transferred to a six day negatively asynchronous uterus. In the current study, asynchronous embryos also underwent a delay in the processes of gastrulation, neurulation and organogenesis. In horse conceptuses, the embryonic disc is first visible on day 10 of gestation, with the primitive streak arising at the posterior pole of the embryonic disc on day 11–12 and advancing to reach the anterior pole by day 14 [31,32]. Here, day 14 synchronous conceptuses presented a primitive streak extending towards the anterior pole of the embryonic disc, as expected for this developmental stage, whereas asynchronous conceptuses had yet to develop a visible primitive streak and more closely resembled 'normal' day 11 conceptuses [31,32]. Day 19 synchronous embryos had undergone flexure, contained an average of 21 pairs of somites bordering a closed neural tube, and had a heart and vascularized extra-embryonic membranes, as anticipated for day 19 embryos [30–33]. By contrast, asynchronous embryos displayed an average of only 7 pairs of somites alongside neural folds that were still fusing at the mid-point, and had neither a discernible head nor heart structures, thus resembling a 16 day embryo from a normal pregnancy [30–32]. Overall, transfer of an embryo to a recipient mare that was 5 days negatively asynchronous resulted in a developmental retardation of approximately 3 days.

The imprinted genes studied here are all expressed in both mouse and horse placenta; in mice, *GRB10*, *H19*, *IGF2R*, *PHLDA2*, *IGF2*, *PEG3* and *PEG10* are all considered essential for placental function and fetal growth because gene knock-out resulted either in fetal and placental overgrowth or restriction [14,15,17]. The genes *DIO3*, *NDN*, *SNRPN*, *HAT1* and *INSR* all encode for proteins regulating DNA or RNA processing, or cell metabolism, and have therefore also been proposed to affect placenta and fetal development [17,23,34]. Similar to other species with delayed implantation (e.g. cattle and sheep), a marked up-regulation in the expression of the selected imprinted genes in equine yolk-sac membranes occurred in the period soon after blastocyst formation, namely days 14 and 19 [35,36]. By day 19, both bilaminar and trilaminar yolk-sac are present as the extra-embryonic mesoderm extends outwards, which presumably contributed to the changes observed in gene expression ascribed to development i.e. day 14 membranes were largely bilaminar while later membranes would have a higher proportion of more developed trilaminar membrane. In ruminants, a comparable up-regulation takes place just before trophoblast interdigitation with the endometrium [35] and, while some imprinted genes are expressed earlier in development in ruminants and pigs (namely the blastocyst stage), monoallelic expression doesn't occur until the time of trophoblast apposition and adhesion to the endometrial epithelium [35,37,38]. Although we didn't investigate the precise timing, by analogy to other species we speculate that monoallelic expression of imprinted genes in equine

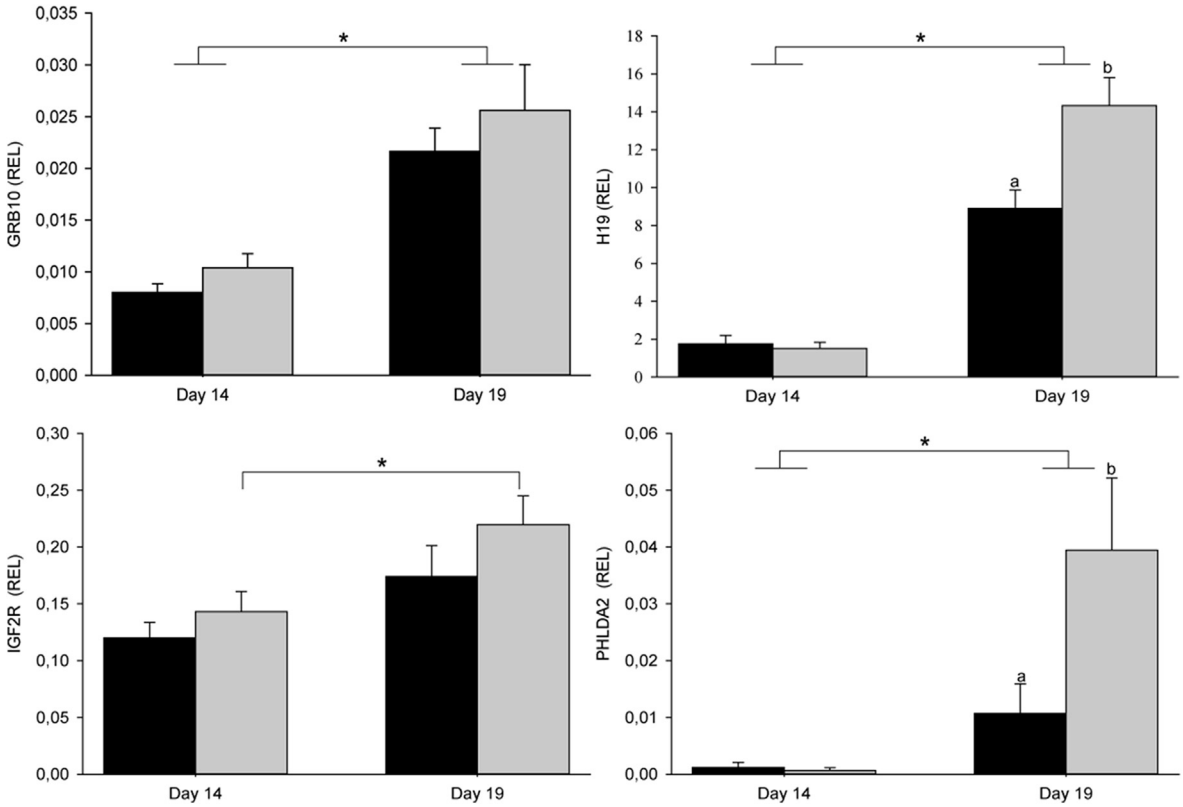

Fig. 5. Relative gene expression (mean ± s.e.m) for maternally expressed genes in equine yolk-sac membranes at day 14 and 19 of conceptus age, after asynchronous (−5 days: black bars) or synchronous (grey bars) embryo transfer. Significant differences ($P < 0.05$) between conditions (synchronous versus asynchronous) within a pregnancy stage are depicted by different superscripts (a, b) whereas between pregnancy stage differences are indicated by an asterisk (*).

yolk-sac membrane is delayed until implantation is initiated by the disintegration of the embryonic capsule (days 21–23 of pregnancy) [29,39]. Certainly, by day 33, imprinted genes display a parent-of-origin monoallelic expression in equine trophoblast [23].

As suggested above, the increase in expression of imprinted genes observed from day 14 to day 19 in conceptus membranes is likely to be a normal developmental stage involved in preparing the trophoblast for implantation and formation of a definitive placenta. Moreover, most of the imprinted genes studied, independent of their imprinting status (maternal or paternal), suffered delayed up-regulation in conceptuses transferred to an asynchronous environment, which may simply reflect developmental retardation. On the other hand, imprinted genes are known to be susceptible to epigenetic modification in response to environmental perturbations during early pregnancy [18,21,22]. To examine whether there was any rationale to suspect epigenetic alteration, we also examined expression of DNA methyltransferase genes, which regulate gene function by maintaining genome methylation (*DNMT1*) or establishing *de novo* methylations (*DNMT3a* and *DNMT3b*) [40]. As observed for many of the imprinted genes, *DNMT1, DNMT3a* and *DNMT3b* all showed lower expression in day 19 asynchronous conceptuses; we therefore conclude that it is unlikely that the reduced expression of imprinted genes observed in asynchronous conceptuses is caused primarily by silencing of imprinted genes by DNA methylation.

Thus, the reduced expression of the imprinted genes and the DNMTs in conceptuses exposed to an asynchronous uterus is more likely to be a factor of the delayed conceptus development, i.e. the reduction in gene expression is a consequence, rather than a cause, of retarded conceptus development. Other mechanisms, driven primarily by the maternal endometrium are therefore likely to be responsible for the delay in conceptus development. Indeed, the major difference between the synchronous and the asynchronous condition is the duration of exposure of the endometrium to progesterone by the time of embryo transfer. Progesterone stimulates and maintains uterine endometrial function necessary for conceptus growth and development [9], and available data suggests that progesterone affects conceptus development indirectly by altering endometrial gene expression, resulting in changes in histotroph composition [11,41,42]. In cattle and sheep, early exposure to progesterone accelerates embryo development, whereas reduced concentrations of progesterone during the early luteal phase retards the development of cattle embryos [11,12,41,42]. Overall, the results of the current study suggest that the horse conceptus can sense endometrial stage (perhaps via changes in histotroph composition, although this remains to be proven) and adapt its development appropriately, in this case slowing down to wait for optimal uterine conditions. This phenomenon could be a less extreme form of the changes that underlie embryonic diapause in some species of mouse, deer, bear and seal, the mechanisms of which have been proposed to be evolutionarily conserved in the blastocysts of domestic species that do not normally undergo diapause, e.g. the sheep [43]; to confirm this suspicion, specific markers for embryonic diapause need to be analyzed.

In conclusion, an asynchronous uterine environment delays equine conceptus development at the gene, cell, tissue and whole conceptus level. However, it appears that the conceptus can adapt to its asynchronous environment and, after a suitable delay, return to a normal developmental trajectory. Since embryos from many species may conserve the ability to undergo developmental delay but do not survive uterine asynchrony, it is tempting to speculate that the horse uterus has a greater ability to maintain embryo

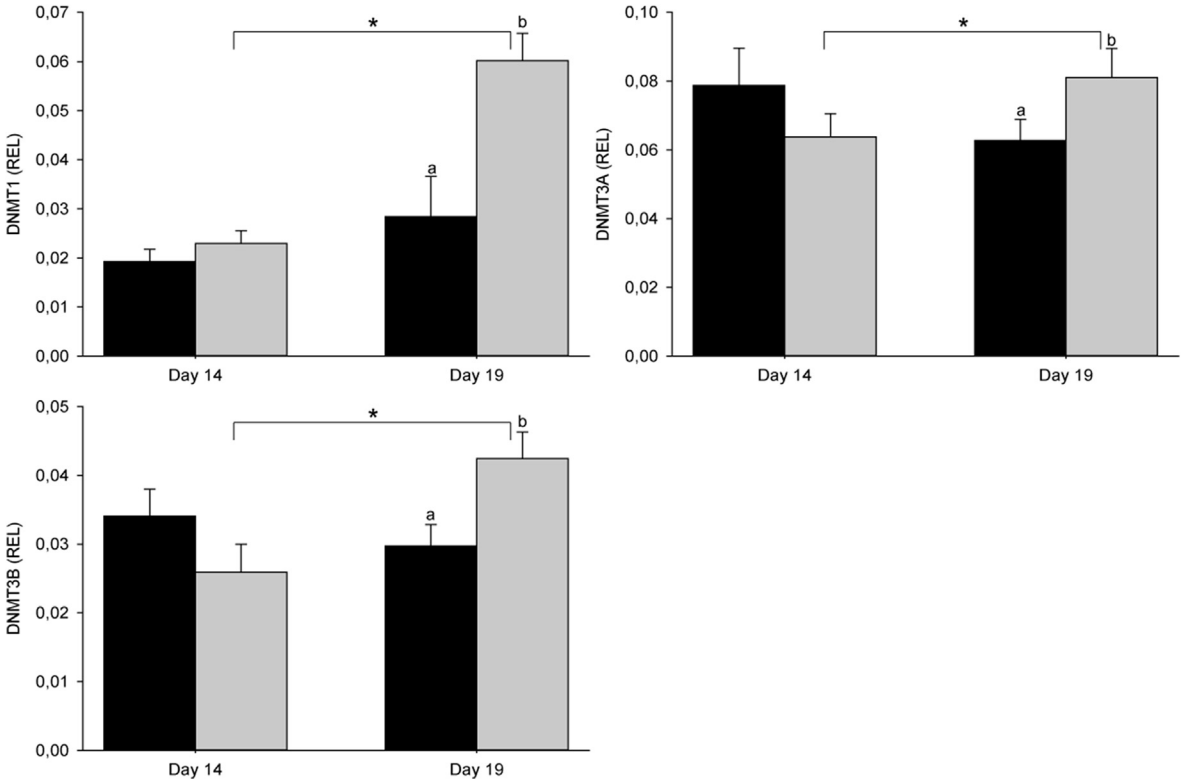

Fig. 6. Relative gene expression (mean ± s.e.m) for selected DNMTs in equine yolk-sac membranes at day 14 and 19 of conceptus age, after asynchronous (−5 days: black bars) or synchronous (grey bars) embryo transfer (ET+6 and ET+9). Significant differences ($P < 0.05$) between conditions (synchronous versus asynchronous) within a pregnancy stage are depicted by different superscripts (a, b) whereas between pregnancy stage differences are indicated by an asterisk (*).

viability while the latter waits for endometrial conditions to 'catch up', than the uterus of most other domestic species. In this respect, the horse appears to be an interesting animal to study the effects of maternal uterine regulation of embryo and placental development. Although expression of imprinted genes was altered by an asynchronous environment and conceptus development was delayed, negative asynchronous embryo transfer does not appear to compromise either the establishment of pregnancy or, other than a delay roughly corresponding with the delayed development, the expression of placental imprinted genes and DNMT's. We cannot, however, rule out the possibility that an asynchronous maternal environment does alter aspects of epigenetic programming in a way that may affect embryonic or fetal and placental development later in pregnancy, or indeed offspring health and development postnatally.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2017.07.007.

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