



# Expression of glucose transporters in the endometrium and early conceptus membranes of the horse



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## ARTICLE INFO

### Keywords:

Glucose transporter  
Pre-implantation  
Histotroph  
Endometrium  
Conceptus

## ABSTRACT

**Introduction:** Glucose is the primary energy substrate for early conceptus development and, for the first 40 days of gestation, the equine conceptus depends solely on glucose available in the histotroph; thereafter, histotrophic glucose provision continues to support transport across the definitive placenta.

**Methods:** To investigate glucose provision routes during early equine pregnancy we examined expression of glucose transporters in conceptus membranes and endometrium recovered on days 7, 14, 21 and 28 after ovulation. To further differentiate the contributions of maternal progesterone priming and conceptus-endometrium crosstalk in regulating glucose transporter expression, day 8 embryos were transferred to recipient mares on day 8 (synchronous) or day 3 (asynchronous) after ovulation; conceptuses and endometrium were recovered 6 or 11 days later.

**Results:** The glucose transporters *SLC2A1*, *2A3*, *2A4*, *2A8*, *2A10* and *5A1* were expressed in equine endometrium. In conceptus membranes, expression of *SLC2A1-3*, *2A5*, *2A8*, *2A10*, *5A1* and *5A11* increased from day 14, and *SLC2A1* protein was highly abundant on the apical trophoblastic membrane and in the endoderm. Asynchronous embryo transfer (ET) resulted in reduced *SLC2A1* expression in both the endometrium and conceptus membranes.

**Discussion:** A wide range of glucose transporters are expressed in the pre-implantation equine conceptus and endometrium, presumably to ensure adequate glucose provision to the developing embryo. Endometrial expression of *SLC2A1* appears to be regulated by a combination of progesterone-priming and conceptus signalling, and its delayed upregulation after asynchronous ET may contribute to the observed delay in conceptus development.

## 1. Introduction

In mammals, glucose is the major energy substrate supporting conceptus development after blastocyst formation [1,2]. While the equine morula uses similar amounts of pyruvate and glucose, glucose uptake increases rapidly during blastocyst expansion such that glucose is the preferred energy substrate thereafter [3]. In sheep and pigs, uterine luminal fluid (ULF) glucose concentrations increase during early pregnancy [4,5], presumably to support conceptus development. In horses, total recoverable glucose in ULF is unaffected by pregnancy status or time after ovulation (5–6 mg [4]) and glucose concentrations in day 11–27 equine yolk-sac fluid are relatively low and stable (0.1–2.7 mM [6,7]). During the same period however, the concentration of fructose, synthesized from glucose by the conceptus, increases markedly in both the ULF (5–60 mg [4]) and yolk-sac fluid (4–62 mM [6,7]). Conceptus fructose production is assumed to depend on maternal glucose transported across the endometrium and conceptus

membranes, a process that could be performed by members of two glucose transporter families; the facilitative transporters (solute carriers 2; *SLC2A*) and the sodium-dependent transporters (solute carriers 5; *SLC5A* [1,8,9]).

Glucose transporter expression in the endometrium and early conceptus is species-specific (See [Supp. Table 1](#) for details), and differences may relate to implantation type and duration of the pre-implantation period. *SLC2A1* (also known as GLUT1 or GT1) supports maintenance glucose uptake in most cell types and is the most abundant glucose transporter in the endometrial stroma of species with invasive implantation, and the endometrial epithelium of species with non-invasive implantation [10,11]. *SLC2A1* is also expressed on the apical membrane of trophoblastic cells in the early conceptus [1,10]. *SLC2A2* (GLUT2) is present in the basolateral membrane of trophoblastic cells, but not in the endometrium [1,12]. However, given its insulin-dependence, high  $K_m$  and low affinity for glucose, *SLC2A2* is unlikely to support glucose uptake from the low glucose environment of the ULF [1].

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Conversely, SLC2A3 (GLUT3 or GT3) is a low  $K_m$ , high affinity transporter that would support glucose transport from the ULF. Moreover, SLC2A3 is present in the apical membrane of trophoblast cells [1] and, since SLC2A3 ablation (by antisense oligonucleotide treatment) inhibits murine blastocyst formation, is known to be required for embryo development [13]. SLC2A4 (GLUT4) and SLC2A8 (GLUT8) are both highly efficient insulin-responsive transporters present in rabbit conceptuses [14]. SLC2A5 (GLUT5) is a low  $K_m$ , high affinity fructose transporter not detected in human endometrium nor human or mouse embryos [15], but present in ruminant conceptuses and endometrium [16–18]. SLC5A1 (SGLT1) and SLC5A11 (SGLT6) are sodium-dependent transporters that transport glucose against a concentration gradient; they were not detected in mouse or human embryos and endometrium [1], but are expressed in ruminant endometrium and conceptuses [5,16,17]. SLC5A9 (SGLT4) and SLC5A10 (SGLT5) are sodium-dependent transporters that have not been detected in the uterus or conceptus of any species.

In various species, maternal progesterone (P4) and conceptus secreted factors such as interferon- $\tau$  (IFN $\tau$ ), prostaglandins (PGs) and estrogens (E2) regulate endometrial expression of certain glucose transporters, and thereby ULF glucose concentrations [5,15,17,19]. In murine and human endometrial stromal cells, SLC2A1 expression and glucose uptake are up-regulated by P4 and down-regulated by E2 [15]. In sheep, maternal progesterone and conceptus IFN $\tau$  and PGs (PGF2 $\alpha$ , PGE2 and PGI2) stimulate endometrial glucose transporter expression (SLC2A1, SLC2A4, SLC2A5, SLC5A1 and SLC5A11) [5,17], and IFN $\tau$  promotes uterine luminal glucose accumulation [17]. Similarly, conceptus E2 upregulates SLC5A1 expression in porcine endometrium [19]. In short, the conceptus actively stimulates endometrial expression of specific glucose transporters, and increases ULF glucose availability [17].

Due to its unusually long pre-implantation period [20,21], the horse conceptus is entirely dependent on histotrophic nutrition for the first 40 days of gestation [22]. During the pre-implantation period, glucose transporters in the endometrium and conceptus membranes must deliver the glucose required for embryonic development. To date, reports of glucose transporter expression during equine pregnancy have been limited to the description of SLC2A1 and SLC2A3 in the placental microcotyledons from day 100 of gestation [23]. As in other species, SLC2A1 was localized to the basolateral membrane of trophoblast and endometrial epithelial cells [2,8], whereas SLC2A3 was present at the apical microvillar junction (interdigitation of trophoblast and uterine epithelium [23,24]). We investigated the expression of glucose transporters (SLC2A1–2A5, SLC2A8, SLC2A10, SLC5A1 and SLC5A9–5A11) in equine conceptus membranes and endometrium during the oestrous cycle and early pregnancy. Previously, we abbreviated the period of endometrial progesterone-priming by transferring day 8 blastocysts (donor mare) into a 5-day negatively asynchronous (day 3) recipient mare. Asynchronous embryo transfer (ET) retarded conceptus development from at least as early as day 14 of pregnancy, and delayed upregulation of expression of various imprinted genes in conceptus membranes [25]. In the present study, we investigated whether delayed conceptus development following asynchronous ET was associated with altered glucose transporter expression.

## 2. Methods

### 2.1. Animals

Animal procedures were approved by Utrecht University's Animal Experimentation Committee (permits 2007. III.02.036 and 2012. III.02.020). Material was recovered from two groups of 18 (study 1) and 22 (study 2) warmblood mares, aged 4–15 years and maintained on pasture. During oestrus, follicle development and ovulation was monitored transrectally using an ultrasound scanner with a 7.5-MHz transducer (MyLab30Vet; Esaote, Maastricht, The Netherlands). When

pregnancy was required, oestrous mares with a dominant follicle  $\geq 35$  mm were inseminated every other day with  $\geq 500 \times 10^6$  sperm cells, until ovulation. Non-pregnant mares required as embryo recipients or for endometrial biopsy, were monitored identically but were not inseminated. For ET, the desired degree of (a)synchrony between donor and recipient mares was ensured by hormone administration to induce luteolysis (PGF2 $\alpha$  analogue) and ovulation (hCG). Day 7 or 8 pregnancy was confirmed by blastocyst recovery during uterine lavage whereas later conceptus presence was diagnosed, and development monitored, by transrectal ultrasonography.

### 2.2. Study design and tissue collection

Study 1 investigated changes in glucose transporter mRNA and protein expression in conceptus membranes and endometrium during early pregnancy and the oestrous cycle. On days 14, 21 and 28, the conceptus ( $n = 4$  per group) was located in the uterine cavity using a video-endoscope, and a PTFE cannula was used to puncture the membranes and aspirate the fluids, after which the conceptus was recovered with a sterile transendoscopic net, as described previously [26,27]. An endometrial biopsy was recovered from the base of a uterine horn on days 7, 14 and 21 after ovulation in cycling mares, and days 7 and 14 in pregnant mares; while on days 21 and 28 of pregnancy, an endoscopically guided biopsy was recovered from the visible hyperemic site of conceptus apposition ( $n = 4$  per group). All endometrial biopsies were recovered using an alligator forceps (141965; Jørgen Kruuse A/S, Langeskov, Denmark).

Study 2 investigated the effect of asynchronous ET on endometrial and conceptus glucose transporter expression. As described previously [25], 20 day 8 blastocysts were collected from the donor mare by uterine lavage and transferred to synchronous (ovulated on the same day) or asynchronous (ovulated 5 days after the donor) recipient mares. Resulting conceptuses ( $n = 5$  per group) were recovered on day 6 or 11 after ET (day 14 or 19 of conceptus development), and an endometrial biopsy ( $n = 5$  per group) was recovered from the base of a uterine horn (day 6 after ET) or the site of conceptus apposition (day 11 after ET).

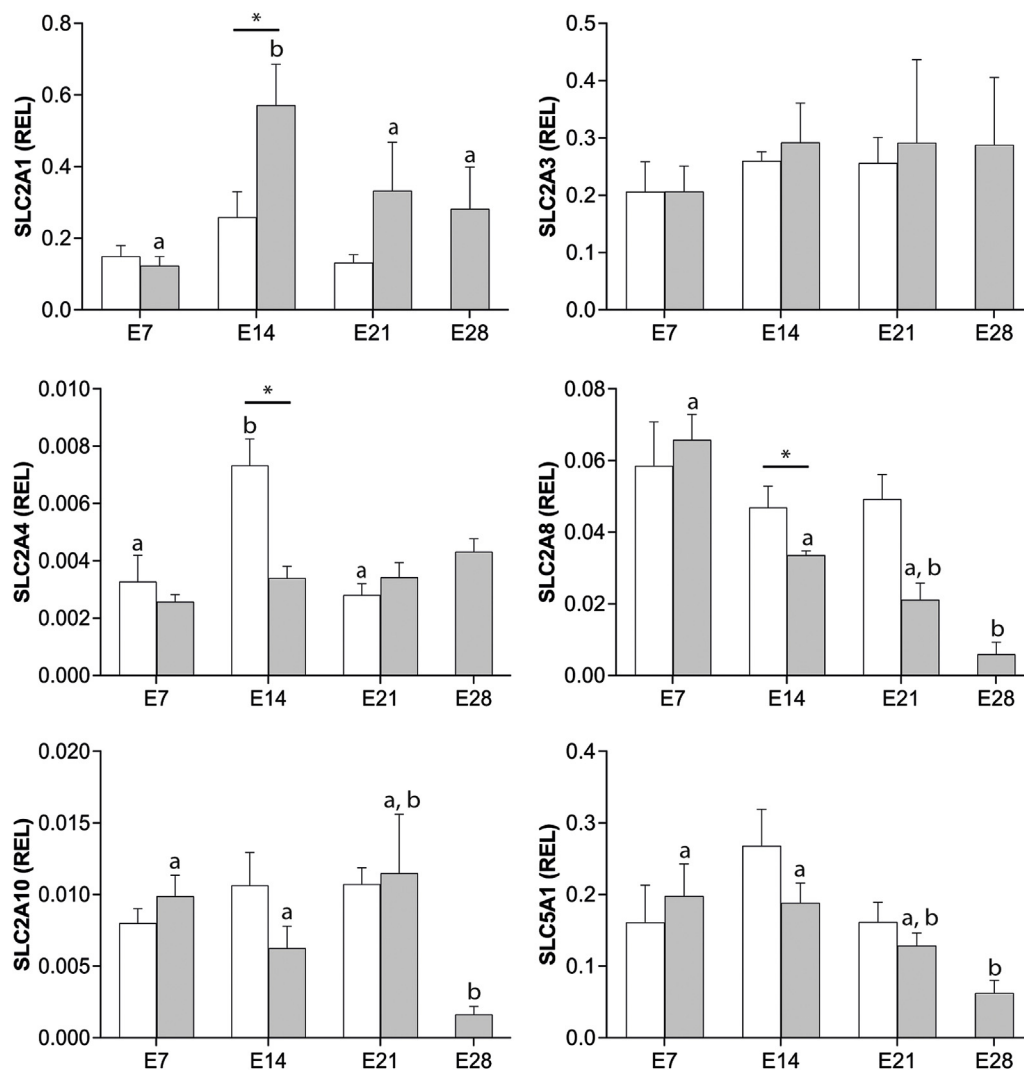
After recovery and washing in 0.9% NaCl, the embryonic disc (day 14) or embryo proper (day 19, 21 and 28; Supp. Fig. 1) were dissected from the membranes (yolk sac) using a stereomicroscope (Olympus SZ-ST; Olympus, Tokyo, Japan); for day 28 conceptuses, the yolk sac (YS) and allantochorion (AC) were separated. All tissues were then divided; one piece was snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  prior to RNA extraction; the other piece was fixed overnight in paraformaldehyde before embedding in paraffin prior to immunohistochemistry.

### 2.3. RNA extraction and cDNA synthesis

Total RNA was extracted using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Venlo, The Netherlands). Samples (30 mg) were homogenized in 600  $\mu\text{l}$  lysis buffer and RNA was eluted with 40  $\mu\text{l}$  RNase-free water. Sample RNA quantity and quality were assessed using a NanoDrop (ND 1000; Isogen Life Sciences, De Meern, The Netherlands) and a BioAnalyzer (Agilent, Palo Alto, CA) respectively; only samples with a RNA integrity index of 7 or higher were used for the present study. DNase I treatment ( $37^\circ\text{C}$  and 10 min at 65  $^\circ\text{C}$ ; 1 IU/ $\mu\text{g}$ ; RNase-free DNase kit; Qiagen) was performed on 1  $\mu\text{g}$  RNA and followed by reverse transcription using Superscript III (Invitrogen, Landsmeer, The Netherlands) in a final volume of 20  $\mu\text{l}$ .

### 2.4. Quantitative RT-PCR

QRT-PCR was performed as described previously [25]. Briefly, primer pair (Eurogentec: Seraing, Belgium; Supp. Table 2) product specificity was assessed by DNA sequencing (ABI PRISM 310 Genetic analyzer; Applied Biosystem, Foster City, USA). For each gene, a 10-fold



**Fig. 1.** Relative gene expression (mean  $\pm$  s.e.m) for glucose transporters in endometrium (E) recovered from cycling (white bars) or pregnant mares (grey bars) on days 7, 14, 21 and 28 after ovulation. Significant differences ( $P < 0.05$ ) between status (cycle versus pregnancy) within a day are indicated by asterisks (\*) whereas differences between days are denoted by different superscripts (a, b, c).

serial dilution of the target gene PCR product, of known quantity, was amplified simultaneously with the samples to make a standard curve, to allow subsequent quantification. PCR was carried out in 15  $\mu$ l reaction mixture containing 1  $\mu$ l sample cDNA and iQ SYBR<sup>®</sup> Green Supermix, on an IQ5 real-time PCR system (Bio-Rad Laboratories; Veenendaal, The Netherlands). Cycle conditions were: 3 min denaturation at 95  $^{\circ}$ C, followed by 40 cycles of amplification (15 s at 95  $^{\circ}$ C, 30 s at the primer-specific annealing temperature and 30 s at 72  $^{\circ}$ C). This was followed by 1 min at 95  $^{\circ}$ C, 1 min at 55  $^{\circ}$ C and finished with a melting curve. Product specificity was evaluated by assessing the melting curve using iQ5 software, and target gene concentrations were quantified using the standard curve. Relative gene expression was calculated as the ratio of target gene mean expression to the geometric mean for the house-keeping genes for endometrium (GAPDH, PGK1 and SRP14) or conceptus membranes (GAPDH, HPRT1, PGK1 and SRP14). The type and the optimum number of reference genes for normalization purposes were selected for both endometrium and conceptus using GeNorm [28].

## 2.5. Immunohistochemistry

Five  $\mu$ m sections of paraffin-embedded tissue were mounted on SuperFrost<sup>®</sup> Plus slides (VWR International, Leuven, Belgium). After deparaffinization (xylene: 2  $\times$  5 min) and rehydration (consecutive

immersion in 100%, 96% and 70% ethanol: 2  $\times$  3 min), endogenous peroxidase activity was blocked by 30 min immersion in 1% H<sub>2</sub>O<sub>2</sub> in methanol. Antigen retrieval was performed by microwaving (750 W) for 15 min in pre-heated Tris/EDTA-buffer (0.01 M Tris; 0.001 M EDTA; 0.05% Tween-20; pH 9.0). Slides were cooled to room temperature (RT) over 30 min and rinsed in PBS-Tween (PBST; 0.05% Tween-20 in PBS: 3  $\times$  5 min). Sections were then incubated for 15 min with goat serum (1:10 in PBS) to block non-specific binding, before overnight incubation at 4  $^{\circ}$ C with SLC2A1 antibody (1:100 dilution of a polyclonal antibody raised in a rabbit against amino acids 218–260 of human SLC2A1: H43 sc-7903; Santa Cruz, Huissen, Netherlands). This antibody recognizes SLC2A1 in equine tissue, and the 42 amino acid sequence targeted by the antibody differs by only one amino acid between human and horse protein. After rinsing with PBST (3  $\times$  5 min), sections were incubated with a secondary, biotinylated goat anti-rabbit antibody for 30 min at RT (BA-1000; diluted 1:250 in PBS: Vector Laboratories Inc., Burlingame, CA), rinsed in PBS (3  $\times$  5 min) and then incubated with Avidin-Biotin-complex (ABC)-peroxidase for 30 min (Vectastain<sup>®</sup> ABC Kit, PK-4000; Vector Laboratories). After washing in PBS (3  $\times$  5 min), slides were incubated for 10 min in freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (45 ml 0.05 M Tris/HCl, pH 7.6; 5 ml DAB and 5  $\mu$ l H<sub>2</sub>O<sub>2</sub>), washed in tap water (5 min), counterstained with haematoxylin (30 s), and washed again with tap water (10 min).

Finally, the sections were dehydrated with ethanol (70%, 96%, 100%; 2 × 3 min) and xylene (2 × 5 min) and mounted under a coverslip with Eukitt™ Mounting Medium (Electron Microscopy Systems, Hatfield, USA). Imaging was performed using an Olympus BX42 microscope with CellB software (Olympus) coupled to a digital camera (ColorViewII, Olympus, Center Valley, USA). For negative controls, the primary antibody was substituted by purified rabbit IgG (1:100 dilution). Staining intensity was evaluated subjectively in 4 categories (-, no staining; +, weak staining; ++, moderate staining; + + +, strong staining).

## 2.6. Statistical analysis

Data were analyzed using SPSS 20 for Windows (SPSS Inc., Chicago, IL, USA). QRT-PCR data were log transformed to obtain normally distributed data sets. Endometrial gene expression was analyzed by two-way ANOVA followed by post-hoc Tukey testing. If a significant interaction between pregnancy status and stage of pregnancy/cycle was apparent, data were sub-divided according to status or stage and re-analyzed by one-way ANOVA. Conceptus membrane expression was analyzed by one-way ANOVA with post-hoc Tukey testing. Data from the ET study was analyzed by two-way ANOVA to investigate effects of pregnancy stage and synchrony; significant effects were verified by independent T-tests. Statistical significance was assumed when  $P < 0.05$ .

## 3. Results

*SLC5A9* and *SLC5A10* were not detected in endometrium or conceptus membranes.

### 3.1. Endometrial glucose transporter expression

*SLC2A2* and *SLC5A11* were not detected in endometrium. *SLC2A5* expression was very low, and *SLC2A3* expression was unaffected by stage of oestrous cycle or early pregnancy. *SLC2A1* expression was higher in pregnant than cycling mares on day 14, and increased from day 7–14 of pregnancy ( $P < 0.05$ ; Fig. 1). *SLC2A4* expression did not increase during pregnancy, but instead peaked on day 14 of dioestrus ( $P < 0.05$ ; Fig. 1). *SLC2A8* expression was lower on day 28 of pregnancy than all other stages ( $P < 0.05$ ), and *SLC2A10* and *SLC5A1* were lower on day 28 than days 7 and 14 of pregnancy ( $P < 0.05$ ); expression of these 3 genes was not affected by oestrous cycle stage (Fig. 1).

### 3.2. Conceptus membrane glucose transporter expression

*SLC2A1*, *SLC2A3* and *SLC2A8* were stably expressed in conceptus membranes between days 14–28, whereas *SLC2A10* expression increased from day 21 ( $P < 0.001$ ) and *SLC2A2* expression peaked on day 21 ( $P < 0.05$ ; Fig. 2). *SLC2A5* and *SLC5A11* were highly expressed in the yolk sac, but marginal in day 28 allantochorion ( $P < 0.001$  and  $P < 0.05$ , respectively) whereas *SLC5A1* was upregulated in day 28 allantochorion ( $P < 0.05$ ; Fig. 2). *SLC2A4* expression was low.

### 3.3. Endometrial and conceptus glucose transporter expression during asynchronous pregnancy

Endometrial expression of most glucose transporters (*SLC2A3*, *SLC2A4*, *SLC2A5*, *SLC2A8*, *SLC2A10*, *SLC5A1*) did not change between days 14 and 19 and was not affected by asynchrony. In fact, only *SLC2A1* expression was affected by asynchrony; on day 14 of conceptus development, *SLC2A1* expression was lower in endometrium from asynchronous (equivalent to day 9) than synchronous mares ( $P < 0.01$ ; Fig. 3A). This difference disappeared by day 19, because *SLC2A1* expression increased in asynchronous ( $P < 0.01$ ) but not synchronous endometrium (Fig. 3A).

In conceptus membranes, expression of *SLC2A4*, *SLC2A10* and *SLC5A1* was low; *SLC2A3* and *SLC2A8* were unaffected by synchrony or stage, just as during normal pregnancy. *SLC2A1*, *SLC2A2*, *SLC2A5* and *SLC5A11* were affected by asynchrony. *SLC2A1* and *SLC5A11* expression decreased in conceptus membranes between days 14 and 19 after synchronous ET only ( $P < 0.05$ ; Fig. 3B). *SLC2A5* was less abundant in asynchronous than synchronous yolk-sac on day 14 ( $P < 0.001$ ); during days 14–19, expression increased in asynchronous ( $P < 0.005$ ) but decreased in synchronous membranes ( $P < 0.05$ ) such that, by day 19, *SLC2A5* expression was higher in asynchronous conceptuses ( $P < 0.05$ ; Fig. 3B). Finally, *SLC2A2* expression increased between days 14 and 19 after synchronous ET only ( $P < 0.001$ ) such that day 19 expression was higher in synchronous conceptuses ( $P < 0.05$ ; Fig. 3B).

### 3.4. *SLC2A1* protein localization

Staining intensity for *SLC2A1* in the endometrium and conceptus membranes is shown in Supp. Table 3. Endometrial *SLC2A1* immunostaining was evident in the cytoplasm of glandular epithelial cells (GE) and the basal membrane of luminal epithelial cells (LE) from day 7 of dioestrus (Fig. 4). Staining intensity increased on days 14 and 21 (oestrus) of the cycle in both LE and GE, and was strongest in the LE on day 21. By contrast, endometrial *SLC2A1* staining intensity decreased with progression of early pregnancy. On day 7 and 14 of gestation, weak to moderate staining was apparent in the GE and basal aspect of the LE, but staining intensity decreased by day 21 and distribution became more sporadic (Fig. 4). Endometrial *SLC2A1* localization after ET was similar; on day 14, however, staining was most prominent in the basal LE of asynchronous endometrium, but the apical LE of synchronous endometrium. By day 19, staining intensity had increased in the GE of asynchronous pregnancies, whereas it was strongest in the LE and the GE after synchronous ET (Fig. 5).

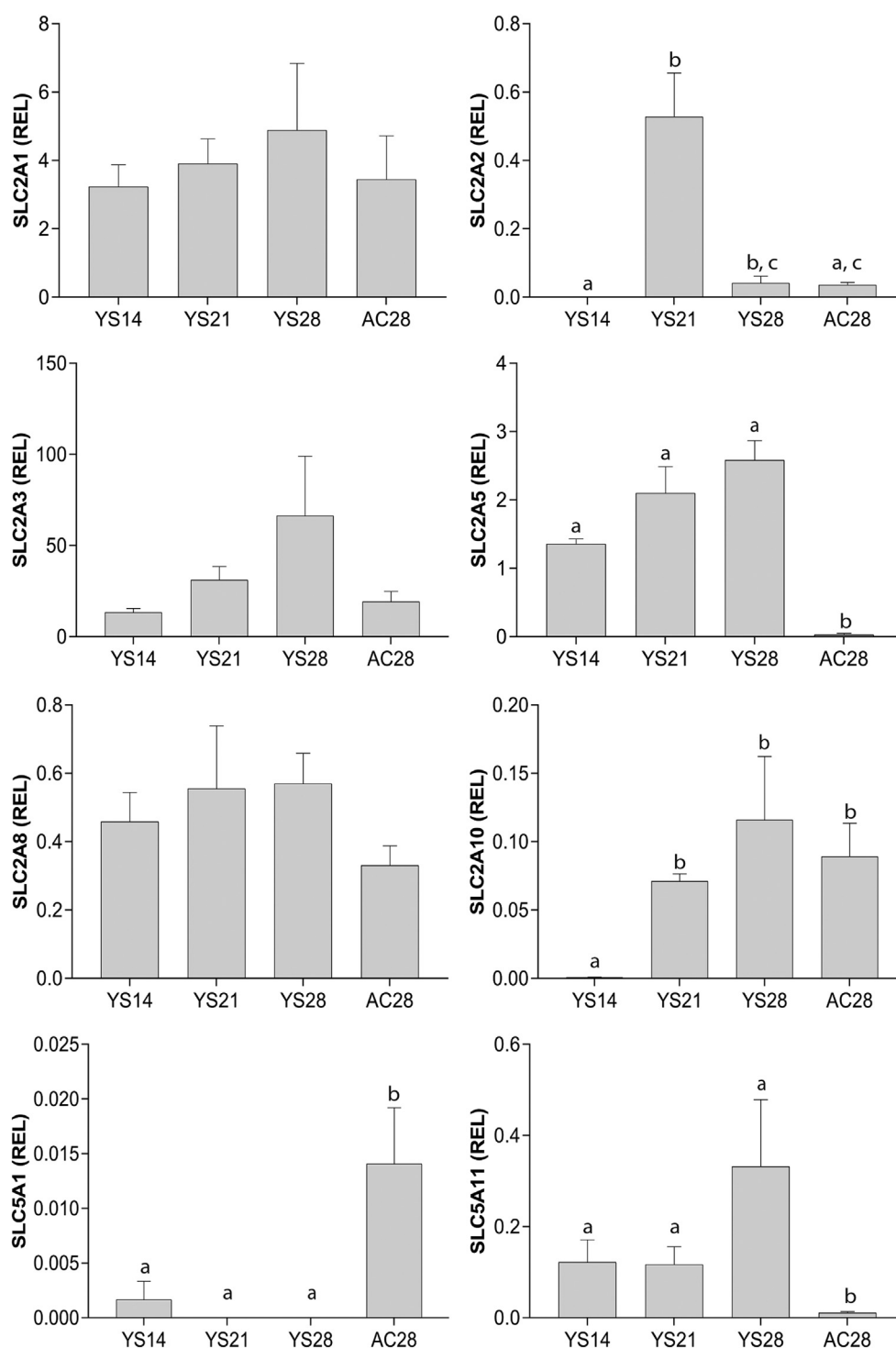
In conceptus membranes, *SLC2A1* protein expression increased during pregnancy (Fig. 4). Moderate staining intensity was observed in the trophoctoderm (TR) and endoderm (EN) on day 14. From day 21, staining intensified in the apical part of the TR in both yolk sac and, on day 28, allantochorion (Fig. 4). *SLC2A1* was not detected in embryonic mesoderm. *SLC2A1* distribution was similar after ET, and staining was more intense in the apical TR of day 19 than day 14 conceptuses (Fig. 5). Staining of both TR and EN was stronger after synchronous than asynchronous ET (Fig. 5).

## 4. Discussion

Glucose is the main energy substrate for post-blastocyst embryo and placental development, and transporters are required to ensure sufficient glucose reaches the conceptus. The array of glucose transporters (facilitative and sodium-dependent) expressed in the endometrium and conceptus membranes during early pregnancy differs between species. Our study indicates that *SLC2A1* and *SLC2A3* are the most highly expressed glucose transporters in equine endometrium and conceptus membranes during early pregnancy. Moreover, asynchronous ET suggests that endometrial *SLC2A1* upregulation depends on a combination of progesterone-priming and conceptus signalling. Surprisingly, *SLC2A1* protein expression in the endometrium from pregnant mares appears to become less abundant from day 7 to day 28. In conceptus membranes, a wide range of glucose transporters was expressed during days 14–28; *SLC2A1* protein was present in the apical part of trophoctoderm cells and in endoderm.

### 4.1. Glucose transporters in equine endometrium

*SLC2A1* and *SLC2A3* were the most highly expressed glucose transporters in equine endometrium. *SLC2A1* is the predominant glucose transporter in human and mouse endometrium, where it is

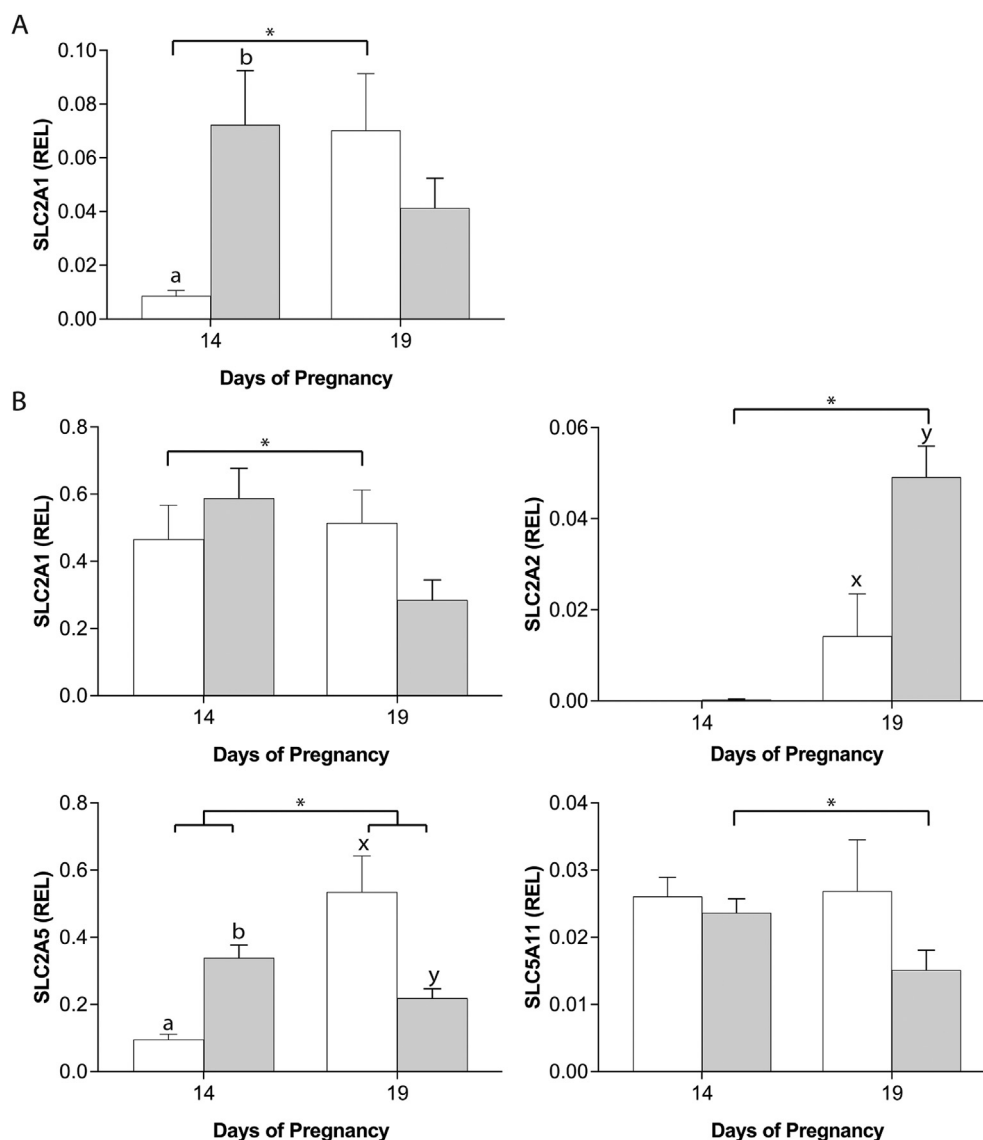


**Fig. 2.** Relative gene expression (mean  $\pm$  s.e.m.) for glucose transporters in equine conceptus membranes on days 14, 21 and 28 of pregnancy (AC = allantochorion; YS = yolk sac). Significant differences between stages are denoted by different superscripts (a, b, c;  $P < 0.05$ ).

essential for decidualization [8,15]. In sheep, *SLC2A1* is up-regulated by maternal P4 and conceptus factors, including IFN $\tau$  and prostaglandins [5,17]. In the horse, it appears that *SLC2A1* expression is also stimulated by conceptus factors following an obligatory period of progesterone-priming, since *SLC2A1* expression increased after day 7 of pregnancy, but not dioestrus, and was lower after asynchronous (shorter period of progesterone priming, but less developed conceptus) than synchronous ET. The delayed upregulation of endometrial *SLC2A1* after asynchronous ET may limit glucose provision and contribute to delayed conceptus development, or be a result of developmental delay.

The reduced intensity of *SLC2A1* staining in endometrium recovered on days 21 and 28 of gestation was unexpected but may indicate a less significant role for this transporter after conceptus fixation, at around day 16, and establishment of a more stable contact between an increasingly vascularized yolk-sac and a more restricted area of endometrium. *SLC2A3* has previously been described at the fetal-maternal interface in man, mouse, rat, cattle and horse [2,11,18,23,29]. In horses, *SLC2A3* was localized to the microcotyledonary microvillus junction (i.e. TR and endometrial LE) from day 100 [23,30]. *SLC2A3* expression during the oestrous cycle and early pregnancy suggests that





**Fig. 3.** Relative gene expression (mean  $\pm$  s.e.m) for glucose transporters in equine endometrium (A) and conceptus membranes (B) collected on days 14 and 19 of conceptus development after asynchronous (white bars) or synchronous (grey bars) embryo transfer. Significant differences ( $P < 0.05$ ) between group (asynchronous versus synchronous) on the same day after transfer are depicted by different superscripts (day 14: a, b; day 19: x, y) whereas differences between day after transfer within a group are indicated by an asterisk (\*).

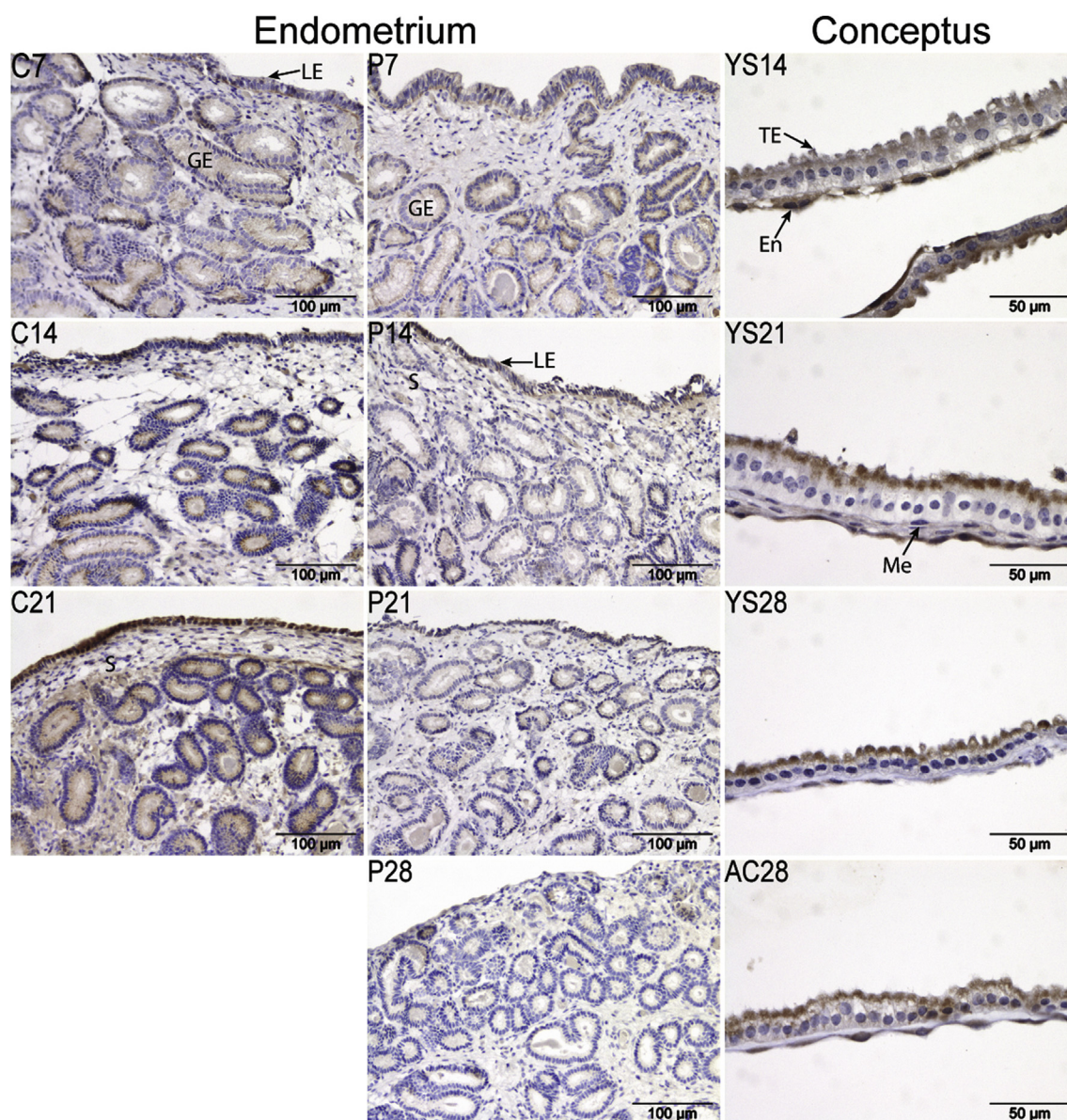
it is also involved in glucose transport during early equine gestation. While SLC2A4 was previously detected in bovine endometrial LE and GE during pregnancy [18], and shown to be P4-responsive in ovine endometrium [5], our data (peak on day 14 of dioestrus) suggest that it is not upregulated by the conceptus or by more extended progesterone priming and, is therefore unlikely to play a major role during early horse pregnancy.

Endometrial expression of the fructose transporter, SLC2A5, differs markedly between species. Endometrial SLC2A5 increased during pregnancy under the influence of conceptus prostaglandins in sheep [17,31], and under the influence of P4 in the pig [32], whereas it was down-regulated during cattle pregnancy [33]. The low SLC2A5 expression in equine endometrium may reflect the likelihood that ULF fructose is primarily derived from conceptus metabolism of glucose. In this respect, Forde et al. proposed that low endometrial SLC2A5 expression would minimize fructose uptake by uterine tissues, and thereby maximize availability for the conceptus [33].

In human and murine endometrial stroma, SLC2A8 and SLC2A10 expression increase during decidualization [8,34]. Moreover, because

decidualization is incomplete in SLC2A8  $^{-/-}$  mice, it has been suggested that SLC2A8 is required to prepare the endometrium for implantation [34]. While ruminants and horses have non-invasive placentae that do not exhibit decidualization, SLC2A8 and SLC2A10 have also been postulated to help prepare the endometrium for implantation [22,35]. While SLC5A1 was the only sodium-dependent glucose transporter expressed in equine endometrium, expression decreased on day 28 of pregnancy. In other species, endometrial SLC5A1 rises during pregnancy under the influence of conceptus IFN $\tau$ , PGE2 and PGF2 $\alpha$  (sheep [5,17]) or oestrogens (pig [19]) and has been proposed to play a specific role in the endometrium of species with an extended pre-implantation.

We propose that SLC2A1 and SLC2A3 are the primary contributors to ULF glucose provision during the first 28 days of equine pregnancy, while SLC2A4, SLC2A8, SLC2A10 and SLC5A1 could further support glucose transport and perhaps play additional or alternative roles in the endometrium.



**Fig. 4.** Immunohistochemical localization of SLC2A1 in endometrium from cycling (C: days 7, 14, 21) and pregnant (P: days 7, 14, 21 and 28) horse mares and conceptuses (days 14, 21 and 28). AC, allantochorion; YS, yolk sac; GE, glandular epithelium; LE, luminal epithelium; S, stroma; TE, trophoblast; En, endoderm; Me: mesoderm. Bar = 100  $\mu$ m (endometrium); 50  $\mu$ m (conceptus).

#### 4.2. Glucose transporters in equine conceptus membranes

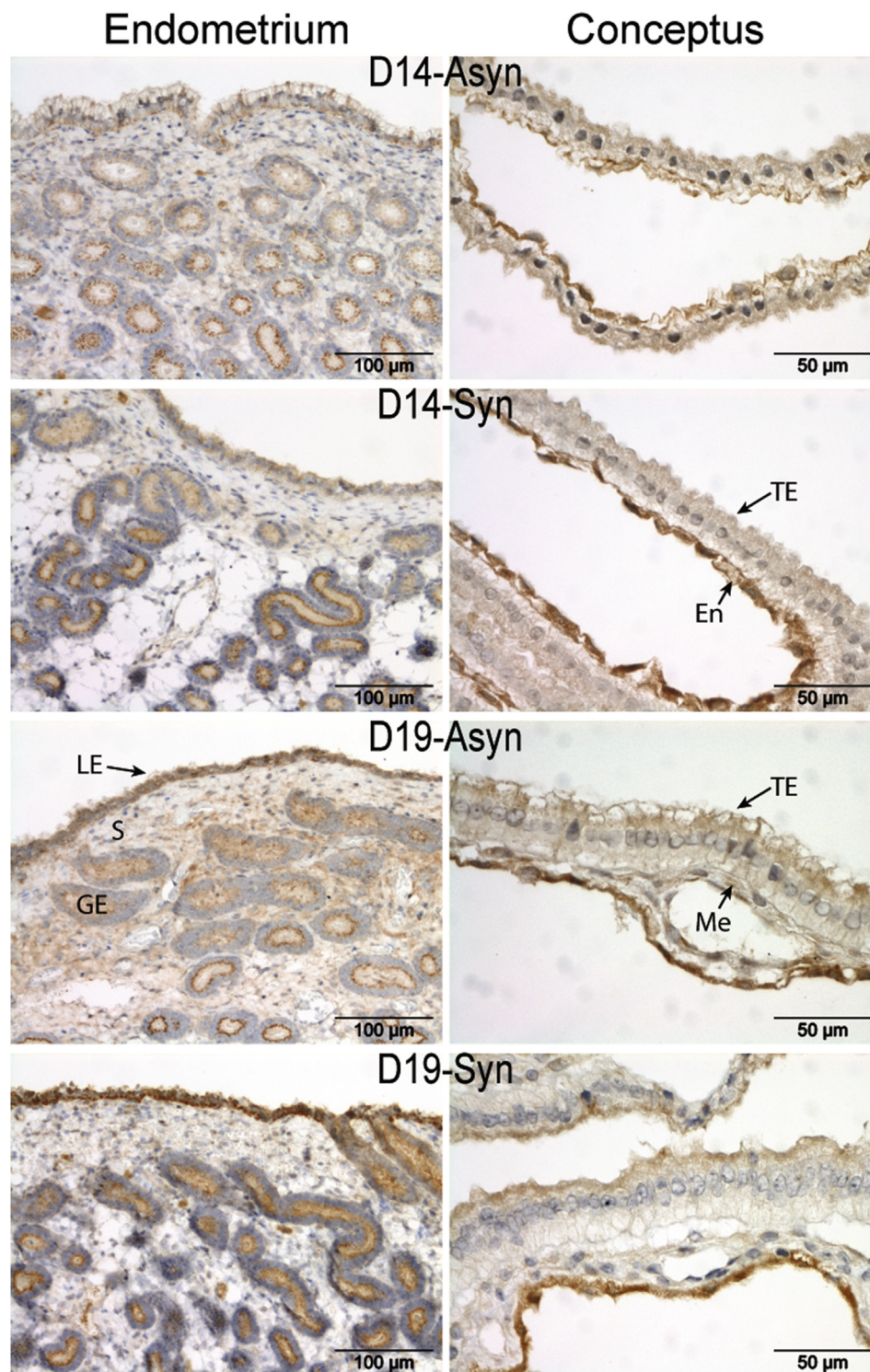
SLC2A1 in the basolateral membrane of trophoblast and inner cell mass (ICM) of pre-implantation human, mouse, rat, rabbit, cow, and pig embryos, has been proposed to transport glucose from the trophoblast into the ICM and blastocoele cavity [1,2,10,12,14,16,36]. We found SLC2A1 in the apical part of equine trophoblast cells and in the endoderm. In human placenta, SLC2A1 was likewise located primarily on the apical membrane of syncytiotrophoblast and cytotrophoblast cells [37], while ovine conceptuses displayed abundant SLC2A1 in the endoderm [5]. The trophoblast-endodermal location of SLC2A1 suggests a role in transporting glucose into the yolk sac, while the reduced SLC2A1 abundance after asynchronous ET could either contribute to or result from delayed conceptus development [25]. In the mouse, rat, rabbit, sheep and cow, SLC2A3 is predominantly located in the apical membrane of trophoblast cells and is thought to transport glucose into the conceptus from the ULF [1,2,5,10,14,16]. During late gestation in the horse, SLC2A3 is also present on the apical membrane

of trophoblast cells [23]. We propose that SLC2A1 and SLC2A3 transport glucose into the yolk-sac and allantoic fluids of early horse conceptuses.

SLC2A2 has previously been reported in murine and bovine embryos [1,10,12,16]. Here, we detected *SLC2A2* expression in equine conceptuses, with a peak on day 21. A similar increase between days 14 and 19 after ET was absent in conceptuses developing in an asynchronous uterus, most probably because of the associated delay in development [25]. The peak of SLC2A2 expression roughly coincides with the time of blastocyst capsule attenuation and disintegration [38].

The fructose transporter, SLC2A5, has been previously reported in 8–16 cell bovine embryos [16] and in day 25 porcine chorion [32]; we found it also to be expressed in equine conceptuses. The horse conceptus synthesises large amounts of fructose, with fructose concentrations increasing first in yolk-sac fluid (days 11–15) and then the ULF (days 14–20 [4,6,7]). In ewes, conceptus fructose production appears to be independent of glucose concentrations in maternal or fetal blood [39], but it is not clear how important fructose is to the conceptus.





**Fig. 5.** Immunohistochemical localization of SLC2A1 in equine endometrium and conceptus membranes recovered on days 14 and 19 of conceptus development after embryo transfer to a synchronous or asynchronous (–5 days) recipient mare. Bar = 100 μm (endometrium); 50 μm (conceptus). *Supp. Fig. 1.* Equine conceptuses, (A) day 7 blastocyst with an inner cell mass; (B) embryonic disc of a day 14 conceptus; (C) embryo proper on day 21 and (D) day 28 embryo surrounded by vascularized membrane.

However, Kim et al. [39] demonstrated that porcine trophoblast cells generate hyaluronic acid from fructose via the hexosamine metabolic pathway, which is important during the formation of the fetal-placental stroma; moreover, various enzymes involved in glucose to fructose conversion (aldose reductase and sorbitol dehydrogenase) and fructose metabolism (ketohexokinase) are expressed by porcine conceptus membranes [32]. Moreover, fructose can stimulate cell

proliferation and mRNA translation via the mTOR pathway [40]. Given the long equine pre-implantation period when the conceptus is entirely dependent on nutrients present in the ULF and yolk-sac fluids, fructose could be important for simply maintaining conceptus growth and development; which might explain the high SLC2A5 expression in horse conceptus membranes. Indeed, retarded conceptuses recovered after asynchronous ET showed an unexpected increase in SLC2A5 expression



from day 14–19, which could represent a response to compensate or ameliorate the developmental delay.

Expression of the insulin-responsive transporter *SLC2A4* was low, but *SLC2A8* expression was high. *SLC2A8* was previously described in mouse and rabbit blastocysts and bovine embryos from the 2–4 cell stage [10,14,16]. To our knowledge, this is the first report of early conceptus *SLC2A10* expression. However, *SLC2A10* has been described as a low  $K_m$ , high affinity transporter in human placenta. The sodium-dependent/active transporters, *SLC5A1* and *SLC5A11*, were expressed in equine conceptuses and have previously been reported in ruminant trophoblast and endoderm [5,16]. Their role in conceptus glucose transport is however controversial, and it has been suggested that they may fulfil alternative functions such as water and ion transport [16].

In conclusion, we show that a variety of glucose transporters are transcribed in equine endometrium and conceptus membranes. *SLC2A1* and *SLC2A3* are likely to be major glucose transporters during early pregnancy since they are highly expressed in both endometrium and conceptus membranes. Glucose transport may be further augmented by *SLC2A4*, *SLC2A8*, *SLC2A10* and *SLC5A1* in the endometrium and *SLC2A2*, *SLC2A5*, *SLC2A8*, *SLC2A10*, *SLC5A1* and *SLC5A11* in conceptus membranes; although some of these transporters may have alternative roles in the preparation for implantation. Additionally, the fructose transporter *SLC2A5* is highly expressed in the conceptus and presumably involved in reuptake of fructose synthesized from ULF glucose, to support further development. Finally, it appears that endometrial *SLC2A1* expression is stimulated by conceptus factors, after a period of progesterone priming. Delayed upregulation of *SLC2A1* in the endometrium may contribute to retarded development of conceptuses transferred into a negatively asynchronous uterus.

## Funding

This work was supported by the European Commission, FP7-PEOPLE-2012-ITN [grant number 317146].

## Conflicts of interest

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

## Acknowledgments

The authors would like to thank Professor Andras Dinnyes, scientific coordinator of EpiHealthNet.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.placenta.2018.06.308>.

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