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Amino acid transporter expression in the endometrium and conceptus membranes during early equine pregnancy

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Abstract. Maternally derived amino acids (AA) are essential for early conceptus development, and specific transporters enhance histotrophic AA content during early ruminant pregnancy. In the present study we investigated AA transporter expression in early equine conceptuses and endometrium, during normal pregnancy and after induction of embryo–uterus asynchrony. 'Normal' conceptuses and endometrium were recovered on Days 7, 14, 21 and 28 after ovulation. To investigate asynchrony, Day 8 embryos were transferred to recipient mares on Day 8 or Day 3, and conceptuses were recovered 6 or 11 days later. Endometrial expression of AA transporters solute carrier family 38 member 2 (*SLC38A2*), solute carrier family 1 members 4 and 5 (*SLC1A4* and *SLC1A5*) increased during early pregnancy, whereas solute carrier family 7 member 8 (SLC7A8), solute carrier family 43 member 2 (SLC43A2) and solute carrier family 7 member 1 (SLC7A1) *SLC7A8*, *SLC43A2* and *SLC7A1* expression decreased and the expression of solute carrier family 1 member 1 (*SLC1A1*) and solute carrier family 7 member 2 (*SLC7A2*) was unaffected. In conceptus membranes, most transporters studied were upregulated, either after Day 14 (solute carrier family 7 member 5 – *SLC7A5*, *SLC38A2*, *SLC1A4*, *SLC1A5* and *SLC7A1*) or Day 21 (*SLC43A2* and *SLC7A2*). Asynchronous ET indicated that endometrial *SLC1A5*, *SLC1A1* and *SLC7A8* are primarily regulated by conceptus factors and/or longer exposure to progesterone. In conclusion, AA transporters are expressed in early equine conceptus membranes and endometrium in specific spatiotemporal patterns. Because conceptuses express a wider range of transporters than the endometrium, we speculate that the equine yolk sac has recruited AA transporters to ensure adequate nutrient provision during an unusually long preimplantation period.

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Introduction

The preimplantation period in horses is unusually long, with placental interdigitation not starting until Day 40–42 of pregnancy (Allen *et al.* 1973; Allen and Wilsher 2009), and is characterised by a high incidence of pregnancy failure (5–23%; Morris and Allen 2002). During this period, conceptus–endometrium communication coordinates mechanisms that control maternal recognition of pregnancy and corpus luteum (CL) maintenance, as well as the growth and development of the conceptus (yolk sac (YS) expansion, electrolyte and fluid accumulation, blastocyst capsule growth and subsequent dissolution, gastrulation, neurulation and early organogenesis) and the conceptus membranes (vascularisation, chorioallantois development) in preparation for implantation (Oriol *et al.* 1993; Waelchli *et al.* 1997; Budik *et al.* 2008; Allen and Wilsher 2009; Gaivão *et al.* 2014; Stout 2016). Prior to placental interdigitation, the equine conceptus relies entirely on histotroph for nutrient provision (Bazer *et al.* 2009; Wilsher and Allen 2009). In sheep, human and mouse, the ability to transport and/or secrete nutrients from the endometrial luminal and glandular epithelia to the uterine cavity is regulated by both progesterone produced by the CL and conceptus secretions (Filant and Spencer 2014). Deficiencies in the histotroph during the preimplantation period, for example due to chronic endometrial degeneration, have been proposed as important contributors to pregnancy loss in aged mares (Morris and Allen 2002; Allen *et al.* 2007).

Amino acids (AAs) are present in the histotroph and play a major role in conceptus development because they are essential for protein synthesis and cellular functions (Van Winkle 2001; Hyde *et al.* 2003; Martin *et al.* 2003; Bazer *et al.* 2015). Non-essential AA supplementation during mouse embryo culture increases the likelihood of blastocyst formation and implantation after embryo transfer (ET; Van Winkle 2001). In addition, essential AAs promote cell growth and division in the inner cell mass of the mouse blastocyst and are involved in critical signalling pathways, such as activation of mammalian target of rapamycin (mTOR; serine–threonine kinase; Martin *et al.* 2003). In the cow, sheep and pig, total AA content in the uterine fluid increases significantly during early pregnancy (Gao *et al.* 2009*a*; Forde *et al.* 2014; Bazer *et al.* 2015).

In the endometrium and conceptus membranes, AA transit is mediated by transporters classified into systems on the basis of both their sodium dependency (Table 1) and their preference for

Table 1. Selected amino acid transporter systems

Selection based on previous studies (Moe 1995; Regnault *et al.* 2002; Grillo *et al.* 2008; Gao *et al.* 2009*b*; Forde *et al.* 2014; Bazer *et al.* 2015*b*). Solute carrier family members: SLC38A2, SLC1A4, SLC1A5, SLC7A5, SLC7A8, SLC43A2, SLC1A1, SLC7A1, SLC7A2. Abbreviations: SNAT2, sodium-coupled neutral amino acid transporter 2; ASCT1, alanine-serine-cysteine transporter 1; ASCT2, alanine-serine-cysteine transporter 2 ; LAT1, large neutral amino acids transporter small subunit 1; LAT2, large neutral amino acids transporter small subunit 2; LAT4, large neutral amino acids transporter small subunit 4 ; EAAT3, excitatory amino acid transporter 3; CAT1, cationic amino acid transporter 1; CAT2, cationic amino acid transporter 2; CAT3, cationic amino acid transporter 3; Ala, alanine; Gly, glycine; Gln, glutamine; Pro, proline; Ser, serine; Cys, cysteine; Leu, leucine; Ile, isoleucine; Val, valine; Met, methionine; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine; Asp, aspartate; Glu, glutamate; Arg, arginine; His, histidine; Lys, lysine

Type	System	Type	Gene	Associated proteins	Amino acids
Neutral	A	Na^+	*SLC38A2*	SNAT2	Ala, Gly, Gln, Pro, Ser
	ASC	Na^+	*SLC1A4*,	ASCT1	Ala, Cys, Ser
			SLC1A5	ASCT2	
	L	Exchanger	*SLC7A5*	LAT1	Leu, Ile, Val, Met, Phe, Trp, Tyr
			SLC7A8	LAT2	
			SLC43A2	LAT4	
Anionic (acidic)	ASC	Na^+	*SLC1A4*	ASCT1	Asp, Glu
			SLC1A5	ASCT2	
	X_{AG}^-	Na^+	*SLC1A1*	EEAT3	Asp, Glu
Cationic (basic)	y^+	Uniport	*SLC7A1*	CAT1	Arg, His, Lys
			SLC7A2	CAT2	
			SLC7A3	CAT3	

neutral, anionic or cationic substrates (Moe 1995; Battaglia and Regnault 2001; Gao *et al.* 2009*b*, 2009*c*). Systems A, ASC and L preferably transport neutral AAs, systems ASC and X_{AG}^- primarily transport anionic AAs and the y^+ system is used for the transport of cationic AAs. The sodium-dependent system A transports primarily small neutral AAs (Ala, Gly) via one main transporter, sodium-coupled neutral amino acid transporter 2 (SNAT2), encoded by the solute carrier (SLC) family 38 member 2 (*SLC38A2*) gene (Regnault *et al.* 2002; Cleal and Lewis 2008; Grillo *et al.* 2008; Gao *et al.* 2009*b*). The L system, which consists of three isoforms, large neutral amino acids transporter small subunit 1, 2 and 4 (LAT1, LAT2 and LAT4; encoded by solute carrier family 7 member 5, solute carrier family 7 member 8 and solute carrier family 43 member 2, *SLC7A5*, *SLC7A8* and *SLC43A2* respectively), has a high affinity for leucine and other branched chain AAs (Regnault *et al.* 2002; Cleal and Lewis 2008; Grillo *et al.* 2008; Gao *et al.* 2009*b*; Lager and Powell 2012; Bazer *et al.* 2015). The sodium-dependent ASC system transports neutral AAs (Ala, Ser, Cys) and some anionic AAs (Asp, Glu) and consists of two members, alanine-serine-cysteine transporter 1 and 2 (ASCT-1 and ASCT-2;encoded by solute carrier family 1 member 4 and 5, *SLC1A4* and *SLC1A5*, respectively, Regnault *et al.* 2002; Cleal and Lewis 2008; Grillo *et al.* 2008; Gao *et al.* 2009*b*; Bazer *et al.* 2015). The X_{AG}^- system is the main transport system for anionic AAs (Asp, Glu) in the placenta, and one of the associated proteins is excitatory amino-acid transporter 3 (EAAT3; encoded by solute carrier family 1 member 1, *SLC1A1*; Battaglia and Regnault 2001; Cleal and Lewis 2008; Grillo *et al.* 2008; Bazer *et al.* 2015). Finally, the major system for cationic AA transport is the y^+ passive system, which comprises three transporters: cationic amino acid transporter 1, 2 and 3 (CAT1, CAT2 and CAT3; encoded by solute carrier family 7 member 1, 2 and 3, *SLC7A1*, *SLC7A2* and *SLC7A3*, respectively, Battaglia and Regnault 2001; Cleal and Lewis 2008; Grillo *et al.* 2008; Gao *et al.* 2009*c*).

Although more than 20 AA transporters have been described in the endometrium and placenta of women and domestic species (cow, sheep, pig; Van Winkle 2001; Regnault *et al.* 2002; Martin *et al.* 2003; Grillo *et al.* 2008; Gao *et al.* 2009*b*, 2009*c*; Lager and Powell 2012; Forde *et al.* 2014), little is known about AA transport during the equine preimplantation period. Therefore, the aim of the present study was to investigate the expression and localisation of specific AA transporters in equine endometrium and conceptus membranes during the oestrous cycle and early pregnancy. Moreover, because equine embryos possess the unique ability to tolerate a large degree of negative uterine asynchrony without a marked loss of viability, we used an asynchronous ET model to shorten the period of preparatory endometrial progesterone exposure and thereby determine whether the resulting delayed conceptus development was associated with a reduction in the expression of specific AA transporters.

Materials and methods

Animals

All animal procedures were approved by Utrecht University's Animal Experimentation Committee (Permit numbers 2007. III.02.036 and 2012.III.02.020). The material used for this study was collected and used for two previous studies from 18 (Experiment 1; de Ruijter-Villani *et al.* 2015*b*) and 22 (Experiment 2; Gibson *et al.* 2017) warmblood mares aged between 4 and 15 years and maintained on pasture with *ad libitum* access to grass, hay and water. As described in the previous studies, the oestrous cycles of the mares were monitored by transrectal

palpation and ultrasonography using a scanner equipped with a 7.5-MHz linear transducer (MyLab30Vet; Esaote). Mares were examined three times a week during early oestrus and daily once the dominant follicle exceeded 30 mm in diameter. When a pregnancy was required, and the dominant follicle exceeded 35 mm in diameter, 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 observed. Non-pregnant mares used as recipients for ET or for endometrium collection were monitored similarly until ovulation, but were not inseminated. For the ET experiments, to ensure the desired degree of synchrony or asynchrony between the donor and recipient mares, all cycles were manipulated by hormone administration to induce luteolysis (prostaglandin $F_{2\alpha}$ analogue) and ovulation (human chorionic gonadotrophin) as described by Gibson *et al.* (2017). Pregnancy status and conceptus development were monitored by transrectal ultrasonography during Days 14–28 of pregnancy or on Day 7 by recovery of a blastocyst by uterine lavage.

Experimental design and tissue collection

Experiment 1 examined temporal expression of AA transporter mRNA and temporal and spatial expression of protein in equine conceptus membranes and endometrium during early pregnancy and the oestrous cycle.

Conceptuses were recovered 14, 21 or 28 days after ovulation ($n = 4$ per group) using an endoscopically guided net after puncture of the membrane and aspiration of the embryonic fluids, as described by de Ruijter-Villani *et al.* (2015*b*). Endometrial biopsies were harvested at the base of one uterine horn in cyclic mares on Days 7, 14 and 21 after ovulation and in pregnant mares on Days 7 and 14 of pregnancy (before conceptus fixation; $n = 4$ per group). On Days 21 and 28 of pregnancy (after conceptus fixation), endometrial biopsies were harvested at the site of conceptus apposition. Recovered conceptuses and endometrial biopsies were washed in large volumes of 0.9% NaCl. Using a stereomicroscope (Olympus SZ-ST), the embryonic disc region (Day 14) or embryonic body (Days 21 and 28) were dissected from the conceptus membranes, and the YS and allantochorion (AC) were further separated in Day 28 conceptuses, as described previously (de Ruijter-Villani *et al.* 2015*b*).

Experiment 2 investigated the effects of a shortened exposure of the endometrium to endogenous progesterone (by negatively asynchronous ET) on the abundance and distribution of AA transporters in equine endometrium and conceptus membranes. The tissues collected and used for Experiment 2 were derived from a previous study (Gibson *et al.* 2017). On Day 8 after ovulation, blastocysts were collected from donor mares by uterine lavage, as described by Gibson *et al.* (2017). Twenty Day 8 embryos were transferred to recipient mares that either ovulated on the same day as the donor mare (synchronous; $n = 10$ per group) or 5 days after the donor (negatively asynchronous; $n = 10$ per group). Conceptuses were recovered on Days 14 or 19 of conceptus development. Day 14 conceptuses were collected 6 days after ET by uterine lavage using a sterile endotracheal tube ($n = 5$ synchronous and asynchronous conceptuses each). Day 19 conceptuses were recovered 11 days after ET using an endoscopically guided net after puncture of the membrane and aspiration of the YS fluids ($n = 5$ synchronous and asynchronous conceptuses each). Following conceptus recovery, an endometrial biopsy was collected using alligator forceps (141965; Jørgen Kruuse). On Day 14 of conceptus development, biopsies were recovered at the base of one uterine horn, and on Day 19 they were recovered at the site of conceptus apposition ($n = 5$ per group). Conceptuses and endometrial biopsies were washed in large volumes of 0.9% NaCl. Under a stereomicroscope, the embryonic disc region (Day 14) or embryonic body (Day 19) were dissected from the YS membranes. The Day 14 YS membrane comprised trophectoderm and endoderm cells, whereas the intervening mesoderm was evident as a thickening of the trilaminar part of the Day 19 YS.

All tissues were cut into two pieces; one piece was snap frozen in liquid nitrogen and stored at −80°C before RNA extraction, whereas the other piece was fixed overnight in paraformaldehyde and then embedded in paraffin for immunohistochemistry.

RNA extraction and cDNA synthesis

Total RNA was isolated using the AllPrep DNA/RNA/Protein Mini kit (Qiagen), following the manufacturer's instructions. Thirty milligrams of endometrium or conceptus membranes was homogenised in 600 μL lysis buffer and total RNA was eluted with 40 μL RNAse-free water. Total RNA quantity and quality were determined by spectrometry (NanoDrop ND 1000; Isogen Life Sciences) using an Agilent BioAnalyzer 2100 with an RNA 6000 Nano Chip, according to the manufacturers' instructions. Total RNA (1 μg) was treated with DNase I for 30 min at 37°C and 10 min at 65°C (1 IU μg^{-1}; RNAse-free DNase kit; Qiagen) and was followed by reverse transcription. The reaction was performed with 1 μg total RNA in a final reaction volume of 20 μL containing 1× First Strand Buffer (Invitrogen), 10 mM dithiothreitol (Invitrogen), 0.5 mM dNTPs (Promega), 1.8 U mL^{-1} Random primer (Invitrogen), 0.4 U μL^{-1} RNAsin (Promega) and 7.5 U μL^{-1} Superscript III (Invitrogen). The reaction was incubated for 60 min at 50°C and for 5 min at 80°C. Negative reverse transcription products were prepared from 0.5 μg RNA using the same protocol but omitting the Superscript III.

Quantitative real-time polymerase chain reaction

All primer pairs (Table 2) were designed using PerlPrimer software (ver. 1.1.14, based on the equine coding sequence and were produced by Eurogentec (Seraing, Belgium). For each primer pair, the gene was amplified from the endometrium or conceptus membrane cDNA to obtain a polymerase chain reaction (PCR) product. The PCR product was purified and the quantity of product was measured using a NanoDrop ND 100 (Isogen Life Science), whereas specificity was assessed by DNA sequencing (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). For each target gene, a 10-fold serial dilution of the target gene PCR product was amplified simultaneously with the samples to establish a standard curve, which was used to quantify sample expression. PCRs were performed in a 15-μL reaction mixture containing 1 μL cDNA sample, 0.05 mM

Table 2. Details of equine primer pairs used in the present study
Abbreviation: Ta, annealing temperature

Gene symbol	Gene name	Primer sequence (5′–3′)	Ta (°C)	Product size (bp)	Accession no.
SLC38A2	Solute carrier family 38 member 2	Forward: CAGCCTGACACAACCAGCGGC Reverse: GGGTGACAGCCACTAACACAGCC	62	377	XM_001489523.3
SLC7A5	Solute carrier family 7 member 5	Forward: GAAAGGTGACATCTCCAATCTG Reverse: GTGATAGTTCCCGAAGTCCA	60	297	XM_001916639.1
SLC7A8	Solute carrier family 7 member 8	Forward: GTGCGTGCCCCAGCAAAAGTG Reverse: AGGCGAAGGAGCCCTGAAGGAA	60	244	XM_003363550.1
SLC43A2	Solute carrier family 43 member 2	Forward: CATCCACTCTGCCGTCGGGG Reverse: GCCCAGCAGCCCCACATTCA	60	181	XM_001502290.1
SLC1A4	Solute carrier family 1 member 4	Forward: TGTGTGGCCGCGGTGTTCAT Reverse: GGCGACGTCTCCTCCTCCGA	63	365	XM_001493515.3
SLC1A5	Solute carrier family 1 member 5	Forward: TCAGCCTGCCGGTTCACGAC Reverse: TCCTGCCCCAAAGGCGTCAC	60	104	XM_001917363.1
SLC1A1	Solute carrier family 1 member 1	Forward: CATAGAAGTTGAAGACTGGGA Reverse: TATCAGTGGGAGAACTATAAGG	58	102	XM_001492215.2
SLC7A1	Solute carrier family 7 member 1	Forward: TGCCGGTACTCCCCGTCCTG Reverse: CCCAGGGACGCCTCCTCACT	60	154	XM_001492839.1
SLC7A2	Solute carrier family 7 member 2	Forward: CTTGTGCTGTTTCTTGTTCTC Reverse: TGCCATCCAAATGCTAAATCTG	61	188	XM_001488803.2
SLC7A3	Solute carrier family 7 member 3	Forward: ATGCTGTGGCAGGCACTCCG Reverse: AACGTGCTGCCCACACCCAG	63	137	XM_001491455.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward: AGGCCATCACCATCTTCCAG Reverse: ACCGGAGTCCATCACGATGC	53	112	NM_001081838.1
HPRT1	Hypoxanthine phosphoribosyltransferase	Forward: GAGATGTGATGAAGGAGATGG Reverse: CTTTCCAGTTAAAGTTGAGAGG	58	232	XM_001490189.2
PGK1	Phosphoglycerate kinase 1	Forward: CTGTGGGTGTATTTGAATGG Reverse: GACTTTATCCTCCGTGTTCC	54	151	XM_001502668.3
SRP14	Signal recognition particle 14	Forward: CTGAAGAAGTATGACGGTCG Reverse: CCATCAGTAGCTCTCAACAG	55	101	XM_001503583.2

primer (forward and reverse; Table 2) and 7.5 μL iQ SYBR Green Supermix (Bio-Rad Laboratories) on an IQ5 real-time PCR detection system (Bio-Rad Laboratories). Cycle conditions were as follows: denaturation for 3 min at 95°C, followed by 40 cycles of amplification (15 s at 95°C, 30 s at the primer-specific annealing temperature and 30 s at 72°C). This was followed by 1 min at 95°C, 1 min at 55°C and finished with a melting curve. Product specificity was evaluated by reading the melting curve with iQ5 optical system software (ver. 2.1, Bio-Rad Laboratories), and target gene concentrations were quantified by reference to their specific standard curve (starting quantity value). Finally, the stability of four potential reference genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate kinase 1 (*PGK1*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and signal recognition particle 14 (*SRP14*) was evaluated using GeNorm (Vandesompele *et al.* 2002).

Western blot

Approximately 100 mg endometrium was lysed in 200 μL RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate; Pierce Thermo Scientific) supplemented with 2 μL protease inhibitor (Thermo Scientific) and following the protocol described by de Ruijter-Villani *et al.* (2015*a*). Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories). Either 80, 40, 20 or 10 μg protein was loaded per well and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% SDS–polyacrylamide gel, and then transferred to nitrocellulose membranes (Trans-Blot1; Bio-Rad Laboratories), as described previously (de Ruijter-Villani *et al.* 2015*a*). The membranes were then rinsed in Tris-buffered saline with 0.05% Tween-20 (TBST; ICN) and blocked with 5% non-fat dry milk in TBST (blocking buffer) for 1 h, followed by overnight incubation at 4°C with the primary antibody diluted 1 : 500 in blocking buffer (rabbit polyclonal anti-SLC43A2 antibody (ab107426; Abcam), rabbit polyclonal anti-SLC7A2 antibody (NBP1-59872; Novus Biologicals), rabbit polyclonal anti-SLC1A5 antibody (NBP1-59732; Novus Biologicals)). After three washing steps with blocking buffer, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (31460; Pierce Biotechnology) diluted 1 : 10 000 in blocking buffer. Subsequently, membranes were washed four times in blocking buffer, three times in TBST and once in Tris-buffered saline; this was followed by visualisation of the antibody–protein complexes using Immun-Star1 chemiluminescent substrate (Bio-Rad Laboratories) and exposure to X-ray film (Fuji).

Immunohistochemistry

For each paraffin block, 5-μm sections were mounted on SuperFrost Plus slides (VWR International). The sections were deparaffinised and rehydrated by immersion in xylene (2 × 5 min) followed by decreasing concentrations of ethanol (EtOH; 100%, 96%, 70%: 2 × 3 min; Klinipath) and then rinsed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by immersing the sections in 1% H_2O_2 in methanol (Merck) for 30 min. For LAT4 (SLC43A2) and ASCT2 (SLC1A5), antigen retrieval was performed by microwaving (at 750 W) for 15 min in preheated Tris–EDTA buffer (0.01 M Tris base (Merck), 0.001 M EDTA (Sigma-Aldrich), 0.05% Tween-20, pH 9.0). For CAT2 (SLC7A2), antigen retrieval was performed by microwaving (at 840 W) for 15 min in preheated citrate-buffer (0.2% citrate (Merck), pH 6.0). After microwaving, the slides were cooled to room temperature over 30 min and then rinsed in PBS–0.05% Tween (PBST; 3 × 5 min). To block non-specific binding, sections were incubated with goat serum (diluted 1 : 10 in PBS) for 15 min at room temperature, then incubated with the specific primary antibody overnight at 4°C. The primary antibodies used were a rabbit polyclonal anti-SLC43A2 antibody (diluted 1 : 200 in PBS; ab107426; Abcam), a rabbit polyclonal anti-SLC7A2 antibody (diluted 1 : 100 in PBS; NBP1-59872; Novus Biologicals) or a rabbit polyclonal anti-SLC1A5 antibody (diluted 1 : 200 in PBS; NBP1-59732; Novus Biologicals). After rinsing in PBST (3 × 5 min), sections were incubated with the secondary biotinylated goat anti-rabbit antibody (diluted 1 : 250 in PBS; BA-1000; Vector Laboratories) for 30 min at room temperature. Sections were rinsed in PBS (3 × 5 min), then incubated with avidin-biotin-complex (ABC)–peroxidase (Vectastain ABC Kit; PK-4000; Vector Laboratories) for 30 min at room temperature. After washing in PBS (3 × 5 min), the slides were incubated in freshly prepared 3,3′-diaminobenzidine tetrahydrochloride solution (45 mL of 0.05 M Tris/HCl, pH 7.6 (Merck), 5 mL diaminobenzidine and 5 μL H_2O_2 (Merck)) for 10 min. The slides were then washed for 5 min under running tap water, after which the nuclei were counterstained with haematoxylin (30 s; Merck) and slides were washed again for 10 min under running tap water. Finally, the sections were dehydrated in EtOH (70%, 96%, 100%; 2 × 3 min) followed by xylene (2 × 5 min) and mounted under a coverslip with Eukitt Mounting Medium (Electron Microscopy Systems). Sections were imaged with a digital camera (ColorViewII; Olympus) coupled to a microscope (Olympus BX42) using CellB software (Olympus). For negative controls, the primary antibody was replaced by purified rabbit IgG at the same final concentration.

Statistical analysis

All data were analysed using SPSS 20 for Windows (IBM Corp.). Quantitative real-time PCR data were first subjected to logarithmic transformation to obtain normally distributed datasets. Results obtained from endometrium were analysed by two-way analysis of variance (ANOVA) followed by a post hoc Tukey test. If a significant interaction was found between pregnancy status and stage of pregnancy or cycle, data were split according to the status or the stage and analysed by one-way ANOVA, followed by a Tukey test. Data obtained from conceptus membranes were analysed by one-way ANOVA followed by post hoc Tukey testing. For the asynchrony study, the data were analysed by two-way ANOVA to examine the effect of stage of pregnancy and synchrony of ET; if a significant effect was found, an independent *t*-test was performed for verification. Statistical significance was assumed at two-tailed $P < 0.05$.

Results

Based on published literature from humans, mice and farm animal species (Van Winkle 2001; Regnault *et al.* 2002; Martin *et al.* 2003; Grillo *et al.* 2008; Gao *et al.* 2009*b*, 2009*c*; Lager and Powell 2012; Forde *et al.* 2014), we selected 10 AA transporters, representative of the main transport systems present during early pregnancy. Expression and localisation of the transporter protein was only possible for a small number of transporters because of a lack of antibodies validated to cross-react with equine tissues. On the basis of mRNA expression results, three transporters were selected for immunohistochemical studies. The antibodies used for immunohistochemistry were tested by western blot using equine endometrial tissue and gave one or two distinct bands close to the predicted size or similar to that seen on western blot of human tissue (Fig. 1 and Table S1, available as Supplementary Material to this paper).

Expression of AA transporters in the endometrium during the oestrous cycle and early pregnancy

Neutral AA transporters

Expression of neutral AA transporters was affected by stage (days of cycle or pregnancy) for *SLC7A8* (LAT2), *SLC43A2* (LAT4) and *SLC38A2* (SNAT2) and by the interaction between stage and pregnancy status (pregnant vs cycling) for *SLC7A8* and *SLC38A2* (Fig. 2). Endometrial expression of *SLC7A5* (LAT1) was low (data not shown). Endometrial *SLC7A8* (LAT2) expression was stable during the cycle, but decreased progressively as pregnancy proceeded ($P < 0.05$), whereas *SLC43A2* (LAT4) mRNA expression showed a temporal down-regulation as both the cycle and pregnancy progressed. Gene expression for *SLC38A2* (SNAT2) was stable during the cycle but increased as pregnancy progressed ($P < 0.01$).

Immunostaining for LAT4 (*SLC43A2*) was detected in the cytoplasm of glandular epithelial cells, luminal epithelial cells and, to a lesser extent, in the stroma (Fig. 3*a*). As found for *SLC43A2* mRNA levels, a stronger signal for LAT4 protein was detected in the endometrium on Days 7 and 14 (dioestrus) than on Day 21 (oestrus) of the cycle. In pregnant mares, LAT4 protein was more abundant on Days 7, 21 and 28 than on Day 14 of gestation.

Anionic AA transporters

Endometrial mRNA expression for the anionic AA transporters *SLC1A1* (EAAT3), *SLC1A4* and *SLC1A5* (ASCT1–2) was affected by stage and by the interaction between stage and pregnancy status (Fig. 2). Expression of *SLC1A1* (EAAT3) peaked on Day 14 and had decreased again by Day 21 of the cycle ($P < 0.01$). *SLC1A1* expression was stable during pregnancy, and was higher in the endometrium from pregnant than

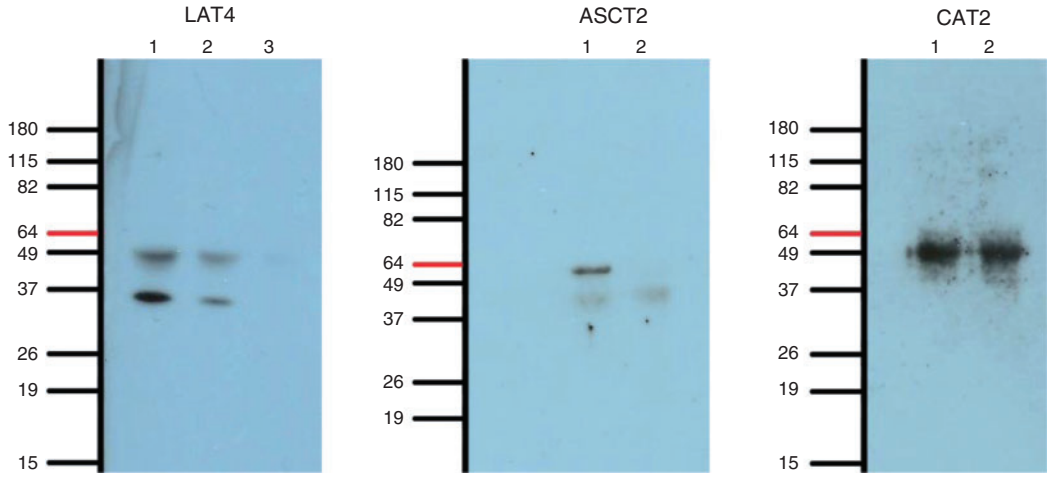

Fig. 1. Western blot analysis using anti-human large neutral amino acids transporter small subunit 4 (LAT4), alanine-serine-cysteine transporter 2 (ASCT2) and cationic amino acid transporter 2 (CAT2) antibodies on equine endometrial protein extracts (LAT4: 1 = 80 μg, 2 = 40 μg, 3 = 20 μg; ASCT2 and CAT2: 1 = 20 μg, 2 = 10 μg). The molecular weights are indicated on the left. LAT4: band at ~50 kDa; ASCT2: band between 49 and 64 kDa; CAT2: clear single band at around 50–60 kDa.

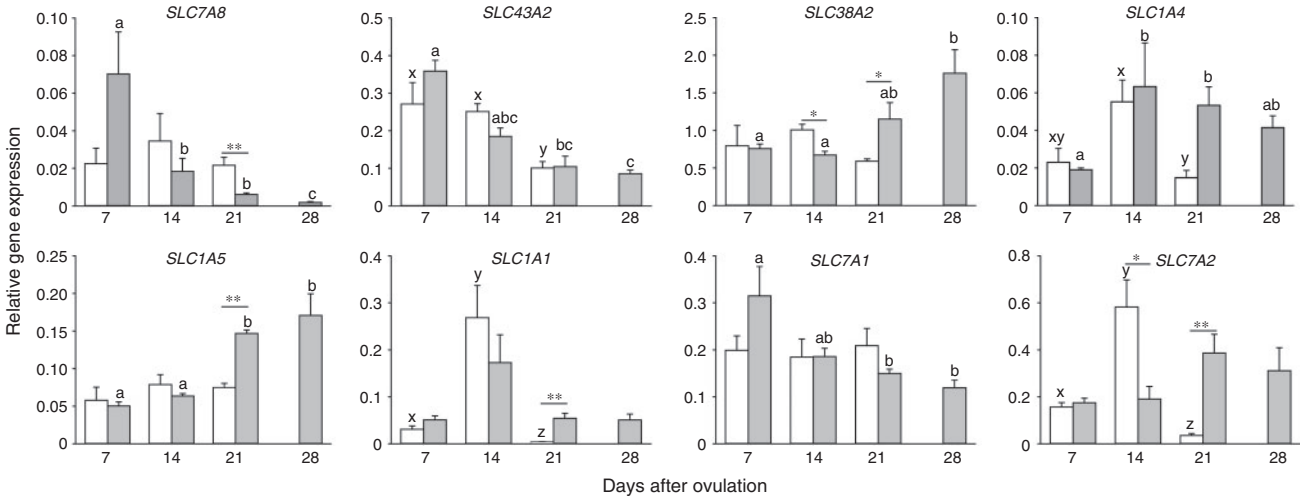

Fig. 2. Relative gene expression of eight amino acid transporters in equine endometrium from cyclic (□) or pregnant (■) mares on various days after ovulation. Values are calculated as the ratio of the target gene mean value to the geometric mean for the reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate kinase 1 (*PGK1*) and signal recognition particle 14 (*SRP14*). Data are the mean ± s.e.m. Asterisks indicate significant differences between condition (cyclic vs pregnant) within the same day (*$P < 0.05$, **$P < 0.01$). Different letters above columns indicate significant ($P < 0.05$) differences between days (x, y, z for cyclic mares; a, b, c for pregnant mares). *SLC7A8*, solute carrier family 7 member 8; *SLC38A2*, solute carrier family 38 member 2; *SLC1A5*, solute carrier family 1 member 5; *SLC7A1*, solute carrier family 7 member 1; *SLC43A2*, solute carrier family 43 member 2; *SLC1A4*, solute carrier family 1 member 4; *SLC1A1*, solute carrier family 1 member 1; *SLC7A2*, solute carrier family 7 member 2.

cyclic mares on Day 21. Although *SLC1A4* mRNA levels (ASCT1) were downregulated on Day 21 of the cycle, both *SLC1A4* and *SLC1A5* (ASCT2) were upregulated during pregnancy ($P < 0.05$). ASCT2 (*SLC1A5*) immunostaining was detected in the cytoplasm of endometrial glandular epithelial cells and luminal epithelial cells, but not in the stroma (Fig. 3*b*). During the oestrous cycle, endometrial expression of ASCT2 protein increased after Day 7 and was localised in the cytoplasm of luminal and glandular epithelium. Similarly, expression of ASCT2 protein increased in the luminal and glandular epithelium as gestation advanced.

Cationic AA transporters

Endometrial mRNA expression for the cationic AA transporters *SLC7A1* and *SLC7A2* (CAT1–2) was affected by stage and by the interaction between stage and pregnancy status

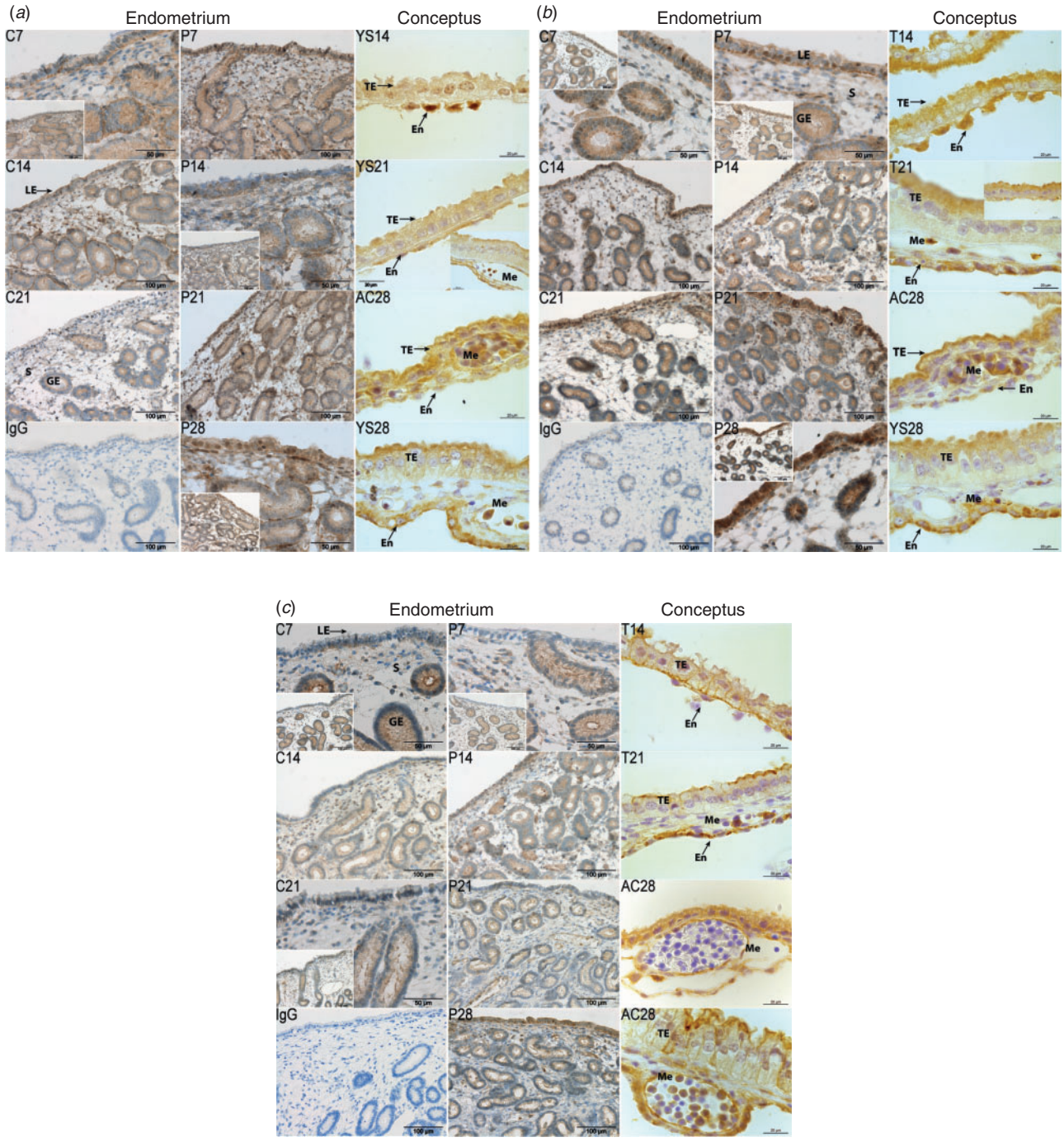

Fig. 3. Immunohistochemical localisation of (*a*) large neutral amino acids transporter small subunit 4 (LAT4;solute carrier family 43 member 2, *SLC43A2*), (*b*) alanine-serine-cysteine transporter 2 (ASCT2;solute carrier family 1 member 5, *SLC1A5*) and (*c*) cationic amino acid transporter 2 (CAT2; solute carrier family 7 member 2, *SLC7A2*) in endometrium from cyclic (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, trophectoderm; En, endoderm; Me, mesoderm; IgG, immunoglobulin.

(Fig. 2). The transporter *SLC7A3* (CAT3) was not expressed in the endometrium (data not shown). Expression of *SLC7A1* (CAT1) was stable during the cycle, whereas it decreased as pregnancy progressed ($P < 0.01$). Endometrial *SLC7A2* (CAT2) gene expression followed a similar pattern of expression to the anionic AA transporter *SLC1A1* with a transient peak

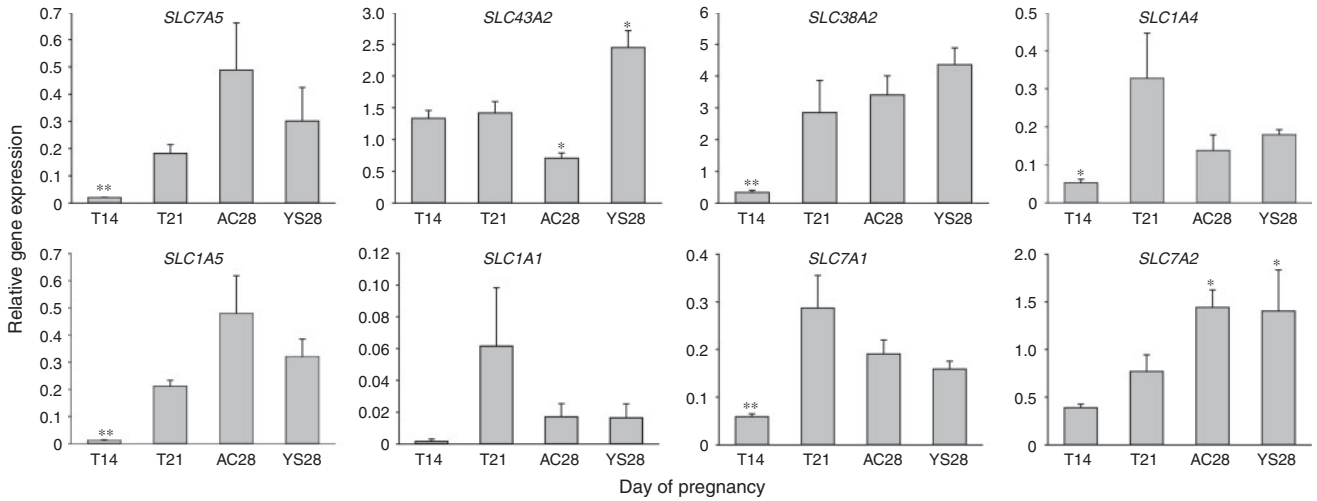

Fig. 4. Relative gene expression of eight amino acid transporters in equine conceptuses in the trophectoderm (T), yolk sac (YS) and allantochorion (AC) on various days of pregnancy. Values are calculated as the ratio of the target gene mean value to the geometric mean for the reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), phosphoglycerate kinase 1 (*PGK1*) and signal recognition particle 14 (*SRP14*). Data are the mean ± s.e.m. Asterisks indicate significant differences between pregnancy stage (*$P < 0.05$, **$P < 0.01$). Solute carrier family members: *SLC7A5*, *SLC43A2*, *SLC38A2*, *SLC1A4*, *SLC1A5*, *SLC1A1*, *SLC7A1*, *SLC7A2*.

on Day 14 of the cycle and higher expression on Day 21 of pregnancy compared with Day 21 of the cycle ($P < 0.01$). CAT2 (*SLC7A2*) immunostaining was primarily localised to the cytoplasm of the glandular epithelium at all stages (Fig. 3*c*). During the cycle, CAT2 showed a weak signal in the luminal epithelium and the signal was stronger in the cytoplasm of the glandular epithelium on Day 7 compared with Days 14 and 21. During early pregnancy, moderate staining was visible in the glandular epithelium, a weak signal was detected in the luminal epithelium and intense staining was observed in the apical part of the luminal epithelium by Day 28.

Expression of AA transporters in early conceptus membranes

Neutral AA transporters

Expression for the neutral AA transporters *SLC7A5* (LAT1) and *SLC38A2* (SNAT2) was higher after Day 14 of pregnancy ($P < 0.01$; Fig. 4). *SLC7A8* (LAT2) was not expressed in conceptus membranes (data not shown). For *SLC43A2* (LAT4), mRNA levels increased in Day 28 YS but were reduced in the AC ($P < 0.05$; Fig. 4). LAT4 protein was detected from Day 14 in the cytoplasm of trophectoderm cells and more strongly in endoderm cells (Fig. 3*a*). Day 21 bilaminar and trilaminar membranes showed similar staining in the apical part of the trophectoderm and endoderm cells, and LAT4 was also evident in the mesoderm and the endothelium. Staining was stronger in the mesoderm, endoderm and apical aspect of the trophectoderm of Day 28 AC and YS.

Anionic AA transporters

As found for the neutral transporters, mRNA expression for the anionic AA transporters *SLC1A4* (ASCT1; $P < 0.05$) and *SLC1A5* (ASCT2, $P < 0.001$) increased after Day 14 of pregnancy in conceptus membranes (Fig. 4). In contrast, *SLC1A1* (EAAT3) was not affected by the stage of pregnancy (Fig. 4). Correspondingly, immunostaining for ASCT2 (*SLC1A5*) protein was moderate on the apical surface of trophectoderm cells and in the cytoplasm of endoderm cells from Day 14 of pregnancy and increased in intensity by Days 21 and 28 (Fig. 3*b*). On Day 21, no differences in staining pattern between bilaminar and trilaminar YS were evident, and there was little staining apparent in the mesoderm. On Day 28, ASCT2 staining was more intense at the apical side of the YS endoderm and only sporadically visible in mesodermal cells.

Cationic AA transporters

The cationic AA transporter *SLC7A3* (CAT3) was not expressed (data not shown), *SLC7A1* (CAT1) increased after Day 14 of pregnancy ($P < 0.01$) and *SLC7A2* (CAT2) increased after Day 21 of pregnancy ($P < 0.05$; Fig. 4) in conceptus membranes. As for the gene expression, CAT2 protein expression increased slightly during pregnancy in the cytoplasm of trophectoderm and endoderm cells (Fig. 3*c*). On Day 14 of pregnancy, moderate staining was detected in the basolateral membrane of the trophectoderm, and from Day 21 strong staining was detected in the apical part of the trophectoderm and the endoderm, with no differences between bilaminar and trilaminar YS. CAT2 expression was also evident in the endothelium and other mesodermal cells.

Effect of asynchronous ET on AA transporter expression in endometrium and conceptus membranes

In a previous study, we showed that transferring Day 8 blastocysts into a 5-day negatively asynchronous recipient mare delayed conceptus development by approximately 3 days (Gibson *et al.* 2017). Indeed, conceptuses transferred to a negatively asynchronous uterus had a smaller embryonic disc on

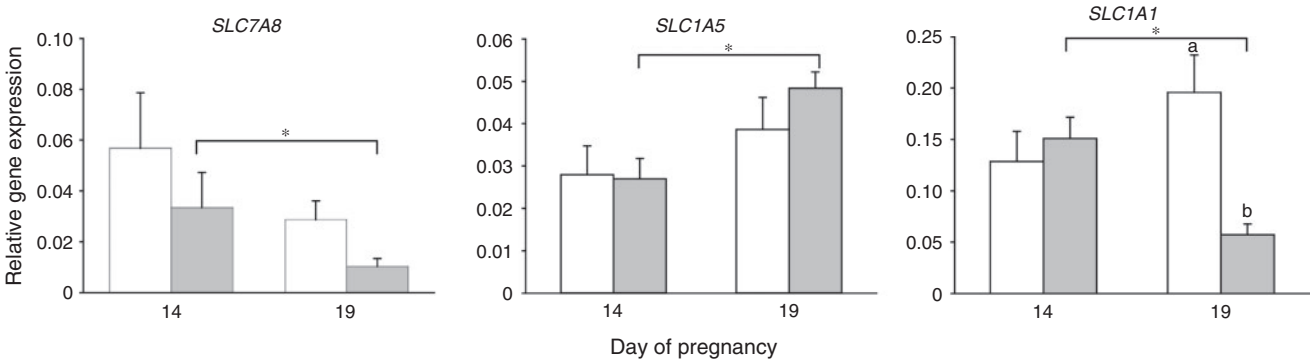

Fig. 5. Relative gene expression for three amino acid transporters in equine endometrium on Days 14 and 19 of pregnancy after asynchronous (□) or synchronous (■) embryo transfer. Values are calculated as the ratio of the target gene mean value to the geometric mean for the reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate kinase 1 (*PGK1*) and signal recognition particle 14 (*SRP14*). Data are the mean ± s.e.m. Asterisks indicate significant differences ($P < 0.05$) within a group. Different letters above columns indicate significant ($P < 0.05$) differences between groups (asynchronous vs synchronous) within the same day. Solute carrier family members: *SLC7A8*, *SLC1A5*, *SLC1A1*.

Day 14 of pregnancy, and the crown–rump length, somite number and organogenesis were retarded in Day 19 asynchronous compared with synchronous embryos.

Endometrium

As expected, expression of AA transporters after synchronous ET was similar to the expression during normal pregnancy in that the neutral AA transporter *SLC7A8* was downregulated ($P < 0.05$) and the anionic AA transporter *SLC1A5* was upregulated ($P < 0.05$) between Days 14 and 19 of synchronous transfer (Fig. 5). In contrast, after negative asynchronous ET, gene expression was stable between Days 14 and 19, suggesting a delay in *SLC7A8* downregulation and *SLC1A5* upregulation (Fig. 5). Interestingly, *SLC1A1* (EAAT3), which did not change in expression during normal pregnancy, was downregulated between Days 14 and 19 of synchronous pregnancies ($P < 0.05$) but not after asynchronous transfer (Fig. 5) such that on Day 19 of conceptus development *SLC1A1* was more highly expressed in the endometrium of asynchronous than synchronous pregnancies ($P < 0.01$). The AA transporters *SLC43A2*, *SLC38A2*, *SLC1A4*, *SLC7A1* and *SLC7A2*, which showed stable expression between Days 14 and 21 of normal pregnancy, were also stable after synchronous ET; moreover, asynchronous ET did not affect their expression (data not shown).

Although *SLC43A2* gene expression was not affected by time after ET or synchrony, protein expression was affected by asynchrony (Fig. 6*a*). In this respect, at Day 14 of conceptus development, LAT4 staining was weaker in the glandular and luminal epithelium and stroma of asynchronous endometrium, whereas at Day 19 of conceptus development staining appeared to be stronger in the luminal epithelium and weaker in the stroma after asynchronous ET. Day 14 synchronous and Day 19 asynchronous endometrium, both characterised by the same time interval after ovulation (14 days), showed a similar localisation of LAT4, although the intensity appeared to be weaker after asynchronous ET. On Days 14 and 19 of pregnancy, ASCT2 (*SLC1A5*) staining was more intense in the luminal and glandular epithelium of synchronous compared with asynchronous endometrium; moreover, staining intensity increased between Days 14 and 19 in both groups (Fig. 6*b*). Staining intensity was weaker in Day 19 asynchronous endometrium than in Day 14 synchronous endometrium (both recovered on Day 14 after recipient mare ovulation). It appears that the upregulation of ASCT2 protein expression was delayed after asynchronous ET.

Conceptus membranes

Expression of the AA transporters in conceptus membranes was mostly upregulated between Days 14 and 19 of pregnancy, regardless of the group (synchronous or asynchronous; *SLC7A5*, *SLC38A2*, *SLC1A4*, *SLC1A5*, *SLC1A1* and *SLC7A1*; $P < 0.05$; Fig. 7); that is, it followed the same trend observed during normal pregnancy. The exception was *SLC43A2*, which showed a downregulation between Days 14 and 19 of pregnancy in the synchronous group only (Fig. 7). Nevertheless, conceptuses transferred to an asynchronous uterus had lower mRNA expression on both Days 14 and 19 of development (*SLC1A4*, *SLC7A1*; $P < 0.05$) or had a lower expression on Day 14 (*SLC38A2*; $P < 0.05$) or on Day 19 (*SLC7A5*, *SLC1A5*, and *SLC1A1*; $P < 0.05$) of development only. This indicates a delayed upregulation for most AA transporters after asynchronous ET (Fig. 7). Here the exception was *SLC1A5* (ASCT2), which showed a higher mRNA expression on Day 14 of pregnancy after asynchronous compared with synchronous ET ($P < 0.05$); and although *SLC1A5* expression increased between Days 14 and 19 of pregnancy in synchronous pregnancies, it decreased after asynchronous ET ($P < 0.05$). Finally, *SLC7A2* (CAT2) gene expression was not affected by asynchronous ET (data not shown).

In conceptus membranes, LAT4 (*SLC43A2*) and ASCT2 (*SLC1A5*) proteins were detected in trophectodermal, endodermal and mesodermal cells, and staining intensity increased with stage (Fig. 6*a*, *b*). From Day 14 of pregnancy, LAT4 was detected in the cytoplasm of the endoderm and the apical part of the trophectoderm in both synchronous and asynchronous conceptuses, and staining intensity increased by Day 19 of pregnancy, with no effect of asynchrony (Fig. 6*a*). On Day 14 of pregnancy, staining for ASCT2 (*SLC1A5*) was weaker in the endoderm of asynchronous compared with synchronous

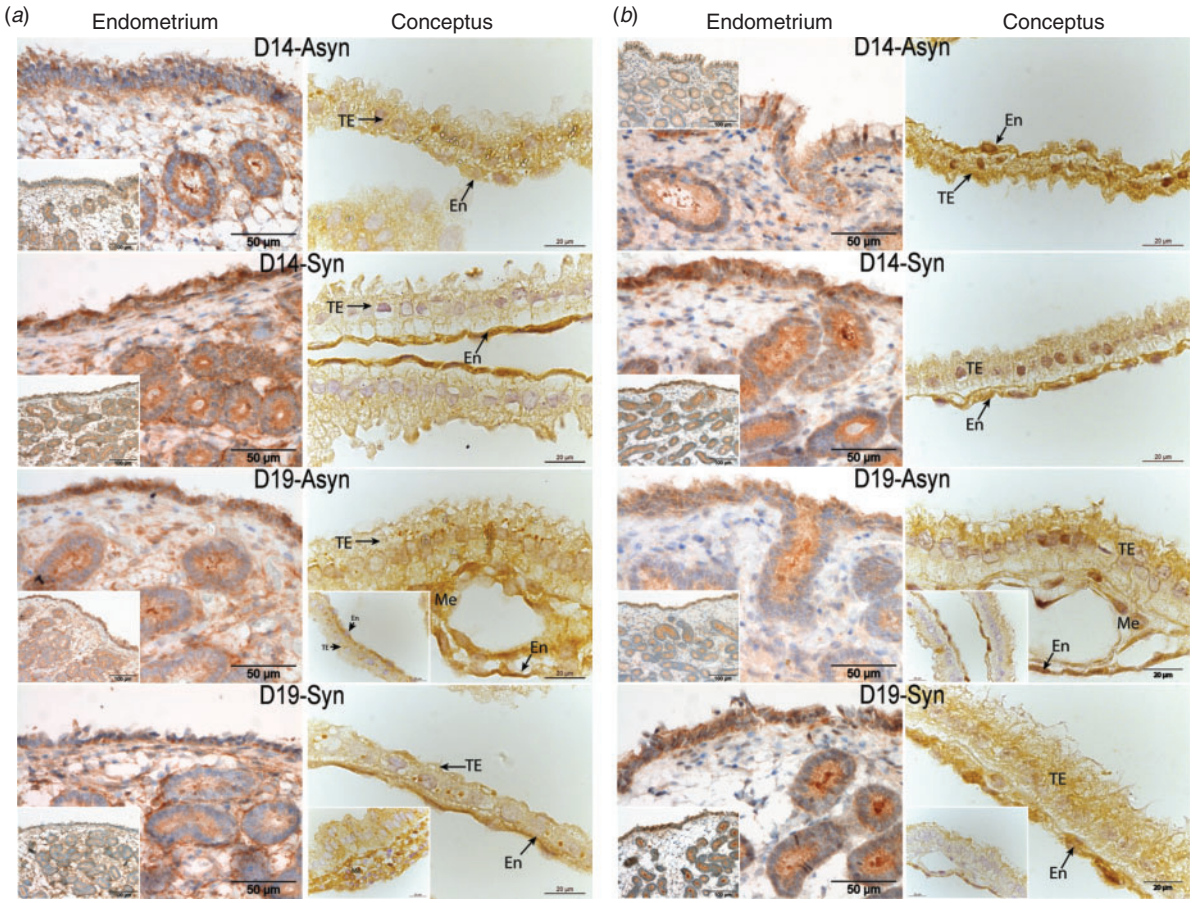

Fig. 6. Immunohistochemical localisation of (*a*) large neutral amino acids transporter small subunit 4 (LAT4;solute carrier family 43 member 2, *SLC43A2*) and (*b*) alanine-serine-cysteine transporter 2 (ASCT2;solute carrier family 1 member 5, *SLC1A5*) in equine endometrium and conceptus membranes after asynchronous (Asyn) or synchronous (Syn) embryo transfer. Protein localisation was performed on Days 14 and 19 of pregnancy after asynchronous or synchronous embryo transfer. TE, trophectoderm; En, endoderm; Me: mesoderm.

conceptuses. At Day 19, staining for ASCT2 was intense in the apical part of the trophectoderm, in the endoderm and the mesoderm, but did not seem to differ between groups (Fig. 6*b*). By Day 19 of pregnancy, protein expression for LAT4 and ASCT2 did not vary appreciably between conditions (synchronous vs asynchronous) and staining did not seem to differ between Day 19 bilaminar and trilaminar YS.

Discussion

As in other species, various AA transport systems are present in the equine endometrium and conceptus membranes during early pregnancy. In the endometrium, the expression of these transporters changes over the course of the oestrous cycle and during early pregnancy, presumably reflecting effects of both ovarian steroid hormones and the presence of a conceptus. In the conceptus membranes, a range of AA transporters is progressively upregulated during early pregnancy.

Major transporters in equine endometrium

In equine endometrium, neutral (*SLC7A8*, *SLC43A2*, *SLC38A2*), anionic (*SLC1A4*, *SCL1A5*, *SLC1A1*) and cationic AA transporters (*SLC7A1*, *SCL7A2*) were all expressed during the oestrous cycle and early pregnancy. However, we observed different patterns of expression, suggesting different regulatory mechanisms and functions of different transporters. *SLC1A4* appeared to be responsive primarily to the ovarian steroid environment because it increased during both late dioestrus and pregnancy (progesterone dominance), but fell during oestrus (Day 21 of the cycle; oestrogen dominance). The expression of *SLC38A2* and *SLC1A5* increased in the endometrium during early pregnancy despite little change during the oestrous cycle, suggesting that it is stimulated primarily by the presence of the conceptus, although it is also conceivable that an extended duration of exposure to progesterone plays a role. In contrast, *SLC7A8*, *SLC43A2* and *SLC7A1* were downregulated during early pregnancy, whereas *SLC1A1* and *SLC7A2* were not affected by pregnancy stage. The neutral AA transporter *SLC38A2*, part of System A, can transport two AAs, glutamine and proline (Regnault *et al.* 2002; Cleal and Lewis 2008; Grillo *et al.* 2008), that are necessary for conceptus development (Wu 2009; Bazer *et al.* 2015). In the cow, the neutral AAs are the most abundant AAs in the histotroph, and endometrial *SLC38A2* expression increases during pregnancy (Forde *et al.* 2014). The

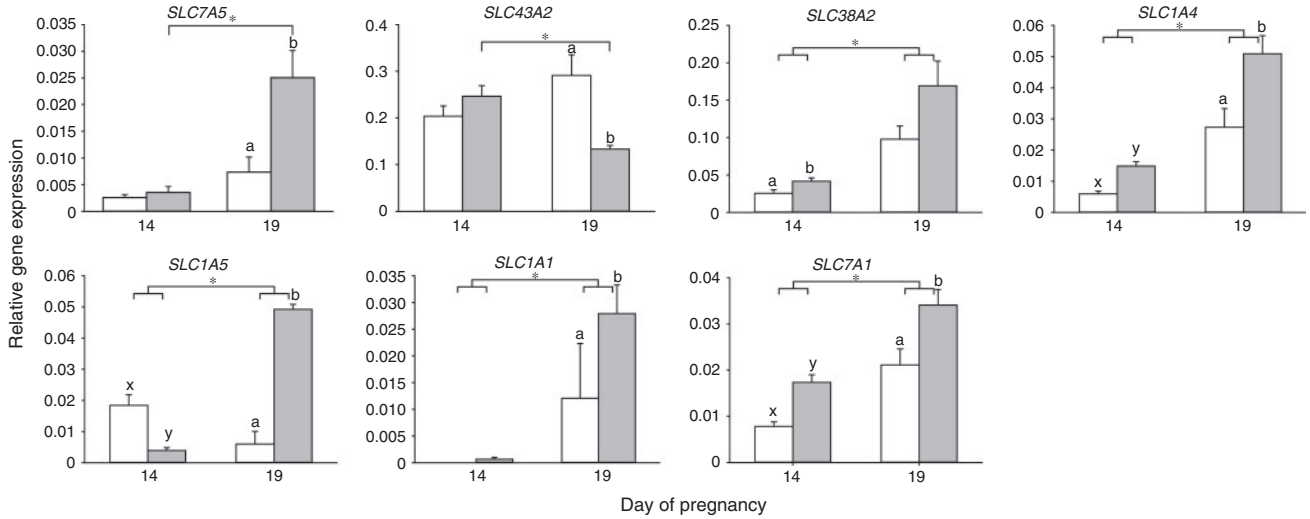

Fig. 7. Relative gene expression for seven amino acid transporters in equine conceptus membranes on Days 14 and 19 of pregnancy, after asynchronous (□) or synchronous (■) embryo transfer. Values are calculated as the ratio of the target gene mean value to the geometric mean for the reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), phosphoglycerate kinase 1 (*PGK1*) and signal recognition particle 14 (*SRP14*). Data are the mean ± s.e.m. Asterisks indicate significant differences ($P < 0.05$) between pregnancy stages within a group. Different letters above columns indicate significant ($P < 0.05$) differences between groups (asynchronous vs synchronous) within the same day. Solute carrier family members: *SLC7A5*, *SLC43A2*, *SLC38A2*, *SLC1A4*, *SLC1A5*, *SLC1A1*, *SLC7A1*.

results of the present study suggest that *SLC38A2* is the principle player in neutral AA transport across the endometrium during the preimplantation period and that the conceptus plays an essential role in upregulating its expression. *SLC1A4* and *SLC1A5* are transporters from the ASC system that facilitate the transport of the anionic AAs aspartate and glutamate (Regnault *et al.* 2002; Gao *et al.* 2009*b*). Both these transporters were upregulated in the endometrium during early horse pregnancy; between Days 7 and 14 for *SLC1A4* and between Days 14 and 21 for *SLC1A5* (which was coupled with an increase in expression of ASCT2 protein in the luminal and glandular epithelium of pregnant endometrium). Expression of these transporters similarly increased during ovine pregnancy (Gao *et al.* 2009*b*) and the upregulation was accompanied by an increase in total recoverable aspartate and glutamate in the uterine fluids from pregnant compared with cyclic ewes (Gao *et al.* 2009*b*). Because acidic AAs are essential for early conceptus development, it seems reasonable to conclude that *SLC1A4* and *SLC1A5* play crucial roles in delivering them to the uterine lumen during early gestation.

Major transporters in equine conceptus membranes

In contrast with other domestic species, most AA transporters studied were upregulated in equine conceptus membranes, either after Day 14 of pregnancy (*SLC7A5*, *SLC38A2*, *SLC1A4*, *SLC1A5*, *SLC7A1*) or after Day 21 (*SLC43A2*, *SLC7A2*), although *SLC1A1* was stable during pregnancy. Upregulation of the *SLC1A5*, *SLC43A2* and *SLC7A2* genes was coupled with an increase in expression of ASCT2, LAT4 and CAT2 protein, mostly in the endoderm and on the apical surface of the trophectoderm, suggesting that these transporters help deliver amino acids from the uterine lumen into trophectoderm cells, although they do not seem to be involved with transport across the basolateral membrane. Because the transporters were also expressed in the endoderm, the AAs could be transported to or from the YS cavity. Further transport within the conceptus could be supported by LAT4 and CAT2 because they are expressed in mesodermal cells. Although the neutral AA transporters *SLC7A5*, *SLC43A2* and *SLC38A2* were expressed in ruminant conceptuses, only *SLC43A2* was upregulated during early pregnancy (Gao *et al.* 2009*b*; Forde *et al.* 2014). Conversely, although the neutral AA transporter *SLC7A8* was expressed in cow and ewe conceptuses (Gao *et al.* 2009*b*; Forde *et al.* 2014), it was not detected in equine conceptus membranes. Although all the anionic AA transporters studied (*SLC1A4*, *SLC1A5*, *SLC1A1*) were also expressed in ruminant conceptuses, only S*LC1A4* expression increased during pregnancy, whereas *SLC1A5* and *SLC1A1* decreased after Day 13 of pregnancy in cattle (Gao *et al.* 2009*b*; Forde *et al.* 2014). Finally, the expression of the cationic AA transporters *SLC7A1* and *SLC7A2* was upregulated in horse conceptus membranes, whereas their expression remained relatively low between Days 13 and 18 of pregnancy in ewe and cow conceptuses (Gao *et al.* 2009*c*).

Because the horse has a longer preimplantation period (~40 days; Allen and Wilsher 2009) than other domestic species (15–16 days in the sheep; 19–20 days in the cow; Sandra *et al.* 2011), the horse conceptus presumably depends on histotrophic nutrition for longer. It is therefore reasonable to speculate that the horse embryo expresses a greater range of AA transporters at elevated levels to ensure adequate AA provision during the long preimplantation period. Furthermore, YS AA transporter upregulation occurred primarily between Days 14 and 21 of pregnancy, suggesting an increase in gene expression to support rapid conceptus development. Leading up to and during this

period, the horse embryo undergoes critical developmental events, including Na^+/K^+-ATPase pump- and aquaporin-driven rapid expansion, electrolyte and fluid accumulation (Days 7–16; Waelchli *et al.* 1997; Budik *et al.* 2008), growth of the acellular glycoprotein capsule (Days 7–18; Oriol *et al.* 1993), fixation at the base of one of the uterine horns (Day 16; Ginther 1998), gastrulation (from Days 11–12), neurulation (from Day 13) and organogenesis (Ginther 1998; Acker *et al.* 2001; Walter *et al.* 2010; Gaivão *et al.* 2014; Gibson *et al.* 2017). Conceptus expansion, fixation and the growth and then attenuation and loss of the capsule affect fluid, ion and protein expression and abundance in the conceptus (Waelchli *et al.* 1996, 1997; Budik *et al.* 2008); the equine conceptus therefore presumably requires increasing amounts or changing profiles of AAs to support protein synthesis and cellular processes (Van Winkle 2001; Hyde *et al.* 2003; Martin *et al.* 2003; Stout 2016).

Transporters regulated by progesterone or conceptus factors

It has been reported previously that AA transporters in the endometrium can be regulated by either progesterone or a combination of progesterone and factors secreted by the conceptus, such as interferon (IFN) τ in ruminants (Gao *et al.* 2009*b*, 2009*c*; Forde *et al.* 2014). In ruminant endometrium, *SLC43A2*, *SLC7A1*, *SLC7A2*, *SLC1A1*, *SLC1A4* and *SLC1A5* are all upregulated by progesterone, whereas increased *SLC38A2* expression required a low progesterone environment (Gao *et al.* 2009*a*, 2009*b*; Forde *et al.* 2014). Furthermore, *SL7A2* and *SLC1A5* were stimulated by IFNτ in the ovine endometrium (Gao *et al.* 2009*a*, 2009*b*).

We used asynchronous ET to modify the duration of endometrial progesterone exposure in the presence of an embryo. Equine conceptuses transferred to a negatively asynchronous uterus of more than 5 days exhibit an ultrasonographically visible delay in development from as early as Day 14 of pregnancy (Wilsher *et al.* 2010; Gibson *et al.* 2017), accompanied by a smaller embryonic disc with a delay in primitive streak development; by Day 19 they exhibit retarded crown–rump length, somite number and organogenesis compared with conceptuses recovered from a synchronous uterus.

We found that endometrial mRNA and protein expression for *SLC1A5* (ASCT2) increased between Days 14 and 19 of pregnancy after synchronous, but not after asynchronous ET; *SLC1A5* (ASCT2) expression did not change during the oestrous cycle. Therefore, it appears that *SLC1A5* expression in equine endometrium is primarily upregulated by the presence of a conceptus (and its secreted factors), although it may still require a background of continuous progesterone secretion. In this regard, in ruminants upregulation of *SLC1A5* depends primarily on progesterone but is further stimulated by IFNτ (Gao *et al.* 2009*b*; Forde *et al.* 2014).

SLC7A8 was stable during the oestrous cycle but was downregulated during normal equine pregnancy and between Days 14 and 19 after synchronous ET. This suggests that conceptus secreted factors play the primary role in downregulating the expression of endometrial *SLC7A8* in the horse. Endometrial *SLC1A1* was upregulated between Days 7 and 14 of the oestrous cycle, and then decreased between Days 14 and 21; although a similar trend appeared during normal pregnancy, suggesting downregulation after a longer period of exposure to progesterone, the downregulation was less profound than during oestrus. Nevertheless, the suggestion that a longer period of exposure to progesterone may play a role in *SLC1A1* downregulation was supported by the decrease in expression between Days 14 and 19 of pregnancy after synchronous but not asynchronous ET. Alternatively, conceptus factors, such as oestrogens produced in abundance by the early equine conceptus (Zavy *et al.* 1979), could contribute to the pregnancy-associated decrease in *SLC1A1* and *SLC7A8*. These results contrast with the cow, where *SLC1A1* was stimulated by progesterone and *SLC7A8* was not affected by either progesterone or IFNτ treatment (Gao *et al.* 2009*b*).

SLC7A5, *SLC38A2*, *SLC1A4*, *SLC1A5*, *SLC1A1* and *SLC7A1* expression in conceptus membranes on Days 14 and/or 19 of conceptus development was reduced after transfer to a negatively asynchronous uterus; this delayed upregulation of gene expression for a range of AA transporters could be a contributor to retarded conceptus development after asynchronous ET. However, it is more likely that the delayed upregulation of the AA transporters is a consequence of retarded conceptus development, similar to the delayed upregulation of expression observed for several imprinted genes after asynchronous ET (Gibson *et al.* 2017).

The larger range and more frequent upregulation of AA transporters in the conceptus membranes compared with the endometrium may indicate that AA transport across the YS wall is the primary limiting step in embryonic AA supply during early pregnancy in the mare. In other species, histotrophic AA content increases during early pregnancy to support conceptus growth and development (Gao *et al.* 2009*a*; Groebner *et al.* 2011; Forde *et al.* 2014), and in humans, where fetal AA concentrations are usually higher than in the maternal circulation, the expression and activity of AA transporters is considered to be the rate limiting step in AA transport through the syncytiotrophoblast (Jones *et al.* 2007; Lager and Powell 2012).

In conclusion, a wide range of AA transporters is expressed in the endometrium and conceptus membranes during early equine pregnancy in a specific spatiotemporal manner determined by a combination of maternal progesterone and conceptus-secreted factors. In this regard, asynchronous ET suggested that *SLC1A5*, *SLC7A8* and *SLC1A1* expression in the endometrium is primarily regulated by conceptus-secreted factors, although it may also depend on prolonged exposure to progesterone. Proper coordination between the endometrium and the conceptus undoubtedly regulates expression of the AA transporters in a manner necessary to support normal development. Finally, more AA transporters were expressed in early equine YS membranes than in the endometrium. Because the preimplantation period is unusually long in the horse, and the conceptus is entirely dependent on histotrophic nutrition for the first 40 days of its development, we propose that the equine conceptus membranes have recruited additional AA transporters to satisfy the nutrient provision required to support embryo and placental growth and development.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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