A lectin histochemical study to detect variation in glycosylation at the feto-maternal interface in three interbreeding equine species

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Title: A Lectin Histochemical Study to Detect Variation in Glycosylation at the Feto-maternal Interface in Three Interbreeding Equine Species

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Abstract: Introduction: In this study, we compare glycosylation at the feto-maternal interface in 3 equine species: horse, donkey and zebra, all of which can interbreed to produce hybrids, to assess their glycan similarities and differences.

Methods: Sections cut from 3 specimens of horse (*Equus caballus*) placenta (50, 200 and 280 days gestation), one donkey (*Equus asinus*) placenta (65cm crown-rump length) and 5 specimens of zebra (*Equus quagga*) placentae (81 to 239 days gestation) were stained with a panel of 24 biotinylated lectins using an avidin-peroxidase revealing system.

Results: There were only slight quantitative differences in the lectin histochemistry at the feto-maternal interface between all three specimens; zebra placentae expressed more α 2,6-linked sialic acid, with α 1,2fucosyl residues at the microvillous interface. However, zebra trophoblast showed histological differences from the other two species, with polarised cells, prominent supranuclear Golgi bodies, and fewer intracellular granules.

Discussion: Our findings appear to confirm the hypothesis that closely related, interbreeding species with epitheliochorial placentae express similar glycans at the feto-maternal interface, thereby supporting the existence of a placental glycode. We also observed intraspecies evolutionary diversion to be associated with a different histological architecture and the absence of significant intracellular granules.

Response to Reviewers

Reviewer #1: I do not think that the first sentence in the abstract is justified. It should be removed. All of the important results of the glycoconjugate comparisons have been carried out on mid to late gestation material. They form an important and scientifically useful comparison of species differences and similarities. They measure the expression at the microvillar junction, trophoblast and uterine epithelium together. Noone has shown the expression of glycoconjugates on the separate maternal and embryonic surfaces at implantation to validate or otherwise the "glycocode" hypothesis. The rest of the paper is now modified suitably.

As suggested by this reviewer, we have removed the first sentence and modified the text appropriately: "In this study, we compare glycosylation at the fetomaternal interface in 3 equine species: horse, donkey and zebra, all of which can interbreed to produce hybrids, to assess their glycan similarities and differences."

Reviewer #3: There is an adequate response to the points raised by Reviewer #1 and a rebuttal of the concerns raised by Reviewer #2. I share some of this reviewer's reservations, which arise directly from the interpretation of the lectin histochemistry being dependent on a single author.

Two additional points that must be dealt with before publication:

1) The Figure legends should define the abbreviations used in the figures, e.g. MVM.

This has been addressed, with addition of more labels in Figure 2.

2) The authors are selective in mentioning only embryo transfer from the bovine to sheep or goats, which are classified in separate subfamilies (Bovinae and Antilopinae) by Groves and Grubb and other experts. They ought also to cite the cross between Gaur (*Bos frontalis*) and domestic cattle (*Bos taurus*) that proceeds far past the stage of blastocyst elongation albeit with defects in placentation (Hradecký *Theriogenology* 1988; 30: 593-604). This is far more relevant to the current study of three species of the genus *Equus*.

The authors thank this reviewer for pointing out this important reference and a new sentence has been added (lines 191 – 6): "However, embryo transfer between a gaur (*Bos gaurus*) and domestic Holstein (*Bos taurus*), which have similar gestational lengths, was partially successful [18] with three pregnancies resulting from four embryo transfers. Examination of the placenta of one of these, a dead 9.5 month gaur calf, of small size and with poor muscle development, showed inadequate branching of the chorionic villi and poor penetration into the spaces available in the maternal crypts [19], indicative of a placentation problem in the heifer."

We hope the paper is now acceptable for publication in *Placenta*.

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August 23rd 2017

Dear Martin,

Once again I have pleasure in sending you a newly revised version of our manuscript "**A Lectin Histochemical Study to Detect Variation in Glycosylation at the Feto-maternal Interface in Three Interbreeding Equine Species**" in which we have addressed the comments of the reviewers. I trust it is now acceptable for publication in Placenta. Both my co-authors have approved these changes.

I am keeping very well, thank you, though I have another dose of chemotherapy two days before the meeting. I tried to get it changed but was told it was not in my best interest to do so, so I am hoping I will be fit enough to attend.

All being well, I look forward to meeting you in a few days' time!

Kind regards,

Carolyn Jones, PhD, D.Med.Sc
(Honorary Research Fellow)

Conflict of Interest Declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

No aspect of the work covered in this manuscript has involved human patients.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. A current, correct email address has been provided on the title page of the manuscript.

Carolyn J.P. Jones

Twink Allen

Sandra Wilsher

**A Lectin Histochemical Study to Detect Variation in Glycosylation at the Feto-maternal
Interface in Three Interbreeding Equine Species**

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Abstract

Introduction: In this study, we compare glycosylation at the feto-maternal interface in 3 equine species: horse, donkey and zebra, all of which can interbreed to produce hybrids, to assess their glycan similarities and differences.

Methods: Sections cut from 3 specimens of horse (*Equus caballus*) placenta (50, 200 and 280 days gestation), one donkey (*Equus asinus*) placenta (65cm crown-rump length) and 5 specimens of zebra (*Equus quagga*) placentae (81 to 239 days gestation) were stained with a panel of 24 biotinylated lectins using an avidin-peroxidase revealing system.

Results: There were only slight quantitative differences in the lectin histochemistry at the feto-maternal interface between all three specimens; zebra placentae expressed more α 2,6-linked sialic acid, with α 1,2fucosyl residues at the microvillous interface. However, zebra trophoblast showed histological differences from the other two species, with polarised cells, prominent supranuclear Golgi bodies, and fewer intracellular granules.

Discussion: Our findings appear to confirm the hypothesis that closely related, interbreeding species with epitheliochorial placentae express similar glycans at the feto-maternal interface, thereby supporting the existence of a placental glycode. We also observed intraspecies evolutionary diversion to be associated with a different histological architecture and the absence of significant intracellular granules.

Highlights

- Glycans at the horse, donkey and zebra placental interface shows many similarities
- Zebra trophoblast shows histological polarisation lacking in horse and donkey
- Zebra trophoblast shows a vesicular structure with few intracellular granules
- Despite structural changes placental glycans are similar in zebra, horse and donkey
- Interbreeding Equid species share a placental glycode

A Lectin Histochemical Study to Detect Variation in Glycosylation at the Feto-maternal Interface in Three Interbreeding Equine Species

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32 maternal interface, thereby supporting the existence of a placental glycode. We also
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34 architecture and the absence of significant intracellular granules.

35 **Keywords:** placenta; equine; zebra; glycans; lectin histochemistry

1. Introduction

We have previously examined glycosylation of the feto-maternal interface in the horse (*Equus caballus*) and donkey (*Equus asinus*), which can interbreed [1], and found a general similarity of glycan expression in the two species [2]. This is in contrast to the situation in the Old World dromedary camel (*Camelus dromedarius*) and the New World camelids which cannot freely interbreed and show a different glycosylation pattern at their placental interface [2]. The opportunity to examine placentae of the Common or Plains zebra (*Equus quagga*, formerly *Equus burchelli*), allowed comparison of zebra placental glycosylation with that of the horse and donkey, all three of which can interbreed [2, 3]. It also enabled us to test our previous postulate [4,5] that glycosylation at the feto-maternal interface is regulated by a glycode which prevents attachment and interdigitation of the trophoblast with an inappropriate maternal epithelium and, hence, hybridisation or extragenetic embryo transfer.

2. Materials and methods

2.1. Animals

As described previously [6], the gravid uteri of 5 adult female Common or Plains zebra ranging in estimated gestational age from 81 to 239 days (see Table 1), and culled for management reasons in Zimbabwe, were dissected 90 - 120 min after death and pieces of the placental interface were fixed in 10% neutral buffered formalin (NBF). The fixed tissues were transported to the UK where they were post-fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4 for 3 h at room temperature. After washing 3 times over

24 h in 0.1M sodium cacodylate buffer containing 1mM CaCl₂, the tissues were trimmed and embedded in epoxy resin for lectin histochemistry.

Three resin-embedded specimens of horse placenta at 50, 200 and 280 days gestation (term = 329-342 days) were obtained courtesy of Dr Peter Wooding (University of Cambridge, UK).

The 50 day specimen had been immersion-fixed in 4% (v/v) glutaraldehyde; the placenta at 200 days was fixed by immersion in 3% (w/v) paraformaldehyde with 1% (v/v) glutaraldehyde and the 280 day placenta was perfused from both maternal and fetal sides with the same fixative.

One specimen of donkey placenta from a fetus of 65cm crown-rump length (see Table 1), kindly provided by V. Dantzer (Royal Veterinary and Agricultural University, Denmark) was also used in this study. It had been perfusion-fixed with 3% (v/v) glutaraldehyde in 0.1M phosphate buffer, pH 7.3, with added 2% (w/v) polyvinylpyrrolidone via a small branch of the uterine artery on the maternal side and a branch of the umbilical artery on the fetal side. After immersion fixation for a further two hours it was processed into epoxy resin.

2.2. Lectin histochemistry.

Semithin sections (0.75 µm) were cut on a Reichert OMUIII ultramicrotome and mounted on APES-coated slides. Resin was removed using saturated sodium ethoxide diluted 1 : 1 with absolute ethanol, after which the sections were stained with a carefully selected panel of 24 biotinylated lectins as described previously [7], followed by an avidin-peroxidase revealing system; some were treated with Type VI neuraminidase (Sigma, from *Clostridium perfringens*) in 0.2M acetate buffer, pH 5.5, with 1% (w/v) calcium chloride at 37°C for 2h [8] to cleave off terminal sialic acid. Controls were as described previously. All the specimens

were stained together with selected lectins to avoid any day-to-day variation and to enable between-species comparisons to be made with confidence. See Table 2 for lectin binding specificities and their sources.

Sections were assessed using a ranking system of analysis whereby staining intensity was graded from 0 (negative) to 4 (intensely stained) and intracellular granule density was graded from + (sparse) to +++ (densely packed). With the zebra trophoblast and endometrial epithelium, the amount of staining around vesicular structures was similarly assessed and graded.

3. Results:

3.1. General histology

After apposition and attachment, the trophoblast and maternal uterine epithelium interdigitate with each other via their surface microvilli to form an undulating placental membrane which folds so that trophoblast villi are accommodated within maternal endometrial crypts. Surprisingly, the trophoblast of the Zebra placenta differed from that of the horse and donkey in that the trophoblast lacked the presence of large, irregular granules described previously in later gestation in the horse [9] and tended to be more polarised, with cuboidal cells bearing long microvilli and sub-microvillous granules that were visible when staining with some lectins. Supranuclear Golgi bodies were often detected and the cytoplasm appeared to be composed of a sponge-like substructure with hollow vesicles and occasional solid granules. Due to the inevitable delay in obtaining tissue from culled animals, the fixation was often suboptimal, especially in the early gestation specimens

where the trophoblast had retracted away from the maternal epithelium and the villous core was sometimes lost completely.

The maternal uterine epithelium was generally more flattened and it contained some granules; darkly staining structures adjacent to the nuclei were observed occasionally which may have been Golgi bodies. The microvilli of these maternal epithelial cells were much shorter than those of the trophoblast. The horse and donkey placentae were similar in appearance to one another and in both species the granules in the trophoblast cells seemed randomly dispersed and lacked the polarity seen in the zebra. Some large inclusions were also present, as has been described previously [9, 10]. Figure 1 shows typical fields of the placental barrier of horse, donkey and zebra stained with WFA to show the general characteristics.

3.2. Lectin Histochemistry

In general, there was little change in glycan expression over the period of gestation studied. In many cases the uterine epithelium showed apical microvillous staining when the cytoplasm was unstained, suggesting the possibility that this had resulted from trophoblast microvillous glycan adhering to the epithelium, rather than being synthesised by it.

3.2.1. Trophoblast (see Table 3)

Trophoblast bound several lectins strongly, especially at the earliest stage examined (day 81). The cytosol had a vesiculated appearance (Fig. 2A) and component granules were not easily detectable, even though the vesicular structures bound many lectins more strongly than the background cytosol. The apical membrane, which was clothed in microvilli that were longer than those on the uterine epithelium, was stained intensely by all the lectins

used in the study apart from MAA, though it is possible that with UEA-1 some of the staining was to uterine glycan adhering to microvilli upon post-mortem loosening of the interdigitation (Fig. 2B). The cytosol varied from virtually unstained (MAA), to very pale (IPHA, UEA-1, DBA, VVA, SBA), to more strongly stained as with most other lectins, and invariably the intensity of stain increased considerably just under the microvillous surface. In the later stages of gestation, populations of small, discrete granules could only be detected with certainty when using PSA, ALA (Fig. 2C), GNA and LEA, particularly in the apical regions of the cell, and supranuclear foci of staining, probably Golgi bodies judging by their shape and location, were particularly prominent with ALA, BSA-1B₄, PAA and WGA staining, the latter two both with and without neuraminidase pretreatment (Fig. 2D). This feature was most prominent at the later stages of gestation studied. Cell membranes were stained particularly strongly, especially at the apical tips of the microvilli, with ePHA, MPA (Fig. 2E), DSA, STA, HPA, AHA and ECA (+/-N), WFA, SNA-1 and PAA (+/-N).

3.2.2. Maternal uterine epithelium (see Table 4)

These cells were generally shallower than the trophoblast, and became flatter over the course of gestation. The microvilli were short but they stained strongly with almost all the lectins apart from IPHA and MAA. Occasional paranuclear areas which stained more strongly, and which may have been Golgi bodies, were seen with ePHA, UEA-1, ALA, DBA, VVA, WFA, PAA and WGA (these last two both with and without neuraminidase pretreatment) (Figs 2A, B, C). Cytosol bound most lectins, apart from IPHA, BSA-1B₄ and MAA, though most tailed off in intensity as gestation advanced. With some lectins (ePHA, PSA and SNA-1), occasional deeply stained cells were seen which may have represented a degree of degeneration as reported in a previous study [11]. Lateral cell membranes were

148 strongly stained with UEA-1 (Fig. 2B), DBA, DSA, STA, AHA (+/-N), ECA (+/-N) and WFA (Fig. 1
149 H). The large granules seen in horse and donkey epithelia were absent in Zebra tissues, and
150 GNA staining, indicative of lysosomes, was generally confined to the underside of the
151 microvillous membrane, where it was less intense than the equivalent staining in
152 trophoblast (Fig. 2F).

153 Neuraminidase pretreatment had little effect on lectin staining of both trophoblast and
154 maternal epithelium, though stromal components (vessels and connective tissue) showed
155 stronger staining with AHA and ECA in all three species.

156 Examination of Tables 3 and 4 shows that differences between horse and donkey placental
157 glycosylation were not particularly marked and they were more quantitative than
158 qualitative in nature, with the general patterns of binding being similar in both species.
159 Staining of donkey trophoblast with IPHA and ALA tended to be more intense, while the
160 uterine epithelium showed only minimal differences.

161 In general, the lectin binding characteristics of zebra placenta were not dissimilar to that of
162 horse and donkey, despite the absence of detectable intracellular granules. There was more
163 evidence of binding with SNA-1 in both trophoblast and uterine epithelial cytoplasm, and
164 one feature of the zebra that was absent in horse and donkey was staining of the microvilli
165 with LTA (Fig. 2 G, H); this was more apparent on the long trophoblast microvilli, though
166 uterine microvilli were also bound with the lectin. The trophoblast and uterine epithelial
167 cytoplasm were also weakly stained with LTA, more so than in the horse and donkey. With
168 UEA-1, there was heavy binding by the uterine epithelium in all three species but in the
169 zebra, in areas where the trophoblast and epithelium had separated, there was often patchy
170 staining of the trophoblast microvillous surface (Fig. 2E); it was not possible to say whether

this originated from interdigititation with the uterine epithelium or was produced endogenously, but its superficial nature and lack of cytoplasmic stain suggested the former. There was more binding of DBA and VVA to granules in the trophoblast in horse and donkey although some diffuse binding was present in zebra (Fig. 2 I, J).

4. Discussion:

We have shown previously that genetically related and interbreeding species with an epitheliochorial placenta usually express similar glycans at the feto-maternal interface [2]. Both the maternal and fetal interdigitating surfaces are highly glycosylated and each has its own particular pattern of glycosylation, or glycotype. The glycans on the apposing surfaces are required to be compatible for successful implantation, suggesting the existence of a “glycocode” in each species which may act to prevent interspecies hybridisation [12] and extragenetic embryo transfer. It has been shown, in experiments to produce hybrids between sheep and goat [13, 14] or species of mouse [15,16], that to prevent rejection the implanting blastocysts needed to be manipulated to produce a chimaera in which the outer trophoctoderm cells come from the same species as the recipient. We confirmed this when examining the placenta of a Cama (camel-llama hybrid), in which placental glycosylation resembled that of the maternal (llama) rather than the paternal (camel) species [12]. Furthermore, although bovine embryos can be temporarily transferred to sheep or goat uteri and continue development, albeit at a slower rate than in an homologous recipient, this has only been demonstrated to the point of blastocyst elongation [17], not implantation. However, embryo transfer between a gaur (*Bos gaurus*) and domestic Holstein (*Bos taurus*), which have similar gestational lengths, was partially successful [18] with three pregnancies resulting from four embryo transfers. Examination of the placenta of

one of these, a dead 9.5 month gaur calf, of small size and with poor muscle development, showed inadequate branching of the chorionic villi and poor penetration into the spaces available in the maternal crypts [19], indicative of a placentation problem in the heifer. It was therefore of interest to examine glycosylation at the fetomaternal interface in specimens of the Common zebra (*Equus quagga*) in comparison with other *Equus* species. Horses ($2n = 64$), donkeys ($2n = 62$) and zebras ($2n = 44$) all have different karyotypes and their hybrids exhibit a karyotype that is intermediate between those of the two parental species [1] and which results in infertility. However, the fact that such hybrids can exist implies compatibility between the glycodes of the trophoblast and maternal epithelium in all three species.

This has been confirmed in the present study, with very little qualitative differences in the expression of the various classes of glycan at the feto-maternal interface in all three species. Previous work from our laboratory on *Camelus dromedarius*, a species that does not interbreed with equids [2, 12, 17], shows that the latter have more trophoblastic bi/triantennary bisected and non-bisected N-glycan (ePHA, I-PHA, PSA), less $\alpha 1,2$ -linked fucose (LTA) and more N-acetyl glucosamine oligomers bound by DSA and LEA. Fucose bound by UEA-1, however, was more evident in the camel, as was terminal N-acetyl galactosamine bound by SBA. There was also a complete absence of $\text{GalNAc}\alpha 1,3\text{-(LFuc}\alpha 1,2)\text{Gal}\beta 1,3/4\text{GlcNAc}\beta 1$ bound by DBA in the camel and N-acetyl glucosamine oligomers (PAA) were also more sparsely expressed. The uterine epithelium in the camel also showed differences, with no binding of DBA to $\text{GalNAc}\alpha 1,3\text{-(LFuc}\alpha 1,2)\text{Gal}\beta 1,3/4\text{-GlcNAc}\beta 1$, and lower levels of complex N-glycan bound by PSA, ePHA, IPHA but more $\alpha 1,2$ -linked fucosyl residues (LTA) though, in the camel, more changes were observed with the

217 progression of pregnancy [20] than in the zebra where glycosylation remained relatively
218 uniform.

219 The main difference between the zebra and other equids was found to be the presence of
220 more α 2,6-linked sialic acid (shown by SNA-1) in both the trophoblast and the maternal
221 epithelium, and α 1,2-linked fucosyl residues (LTA) at the microvillous interface in the zebra,
222 which was not evident in the other two species. Fucose in α 1,6-linkage, bound by ALA, is
223 generally distributed ubiquitously in the specimens we have examined, in contrast to that in
224 α 1,2-linkage which is always much more selectively expressed and which appears to be
225 important in separating out different glycotypes [21]. It was surprising, therefore, that the
226 most diversity occurred in the histology of the zebra trophoblast; the degree of polarisation
227 seen there was not evident in either of the other two species, best exemplified by the
228 regular disposition of the purported Golgi bodies as shown particularly well after WGA and
229 PAA staining. The classical ultrastructural studies of equine placenta by Bjorkman and
230 Samuel et al. [9,22, 23] do not mention the location of Golgi bodies in the trophoblast,
231 though small cisternae may be observed in the paper by Samuel et al. [23] lying lateral to
232 the nucleus in a 61 day specimen. Likewise, there was an absence in the zebra of the large,
233 irregular granules seen in horse and donkey trophoblast and described at the ultrastructural
234 level by Samuel et al. [9], which may be lysosomal in nature. A study of the zebra placenta
235 by Kayanja [24] did not report the presence of Golgi bodies in the trophoblast; some small
236 supranuclear dilated vacuoles could be observed in one figure but with poor resolution but
237 significant lysosomal structures were not seen. Even at the earliest stage examined, the
238 cytoplasm of zebra trophoblast was trabecular/vesicular in structure but it exhibited few
239 detectable granules apart from those seen with GNA which binds to lysosomes [25]. The

240 reason for this diversity between zebra trophoblast cellular organisation and that of horse
241 and donkey is not clear but may relate to the packaging and disposal of waste products via
242 lysosomes, those in the zebra being extruded via the apical membrane while remaining
243 intracellular as secondary lysosomes in the other two species. The extrusion process in the
244 zebra trophoblast may have led to the development of more polarisation while, in the horse
245 and donkey, the secondary lysosomes were seen to be lying in random positions within the
246 cell.

247 As with horse and donkey, most classes of glycan were present on zebra trophoblast apart
248 from α 2,3-linked sialic acid (MAA) and GalNAc α 1,3 residues (DBA and VVA). Terminal
249 GalNAc α 1 and tri/tetra-antennary, non- bisected complex N-linked sequences (IPHA) were
250 present but were only weakly expressed. N-acetyl glucosamine residues appeared to be
251 concentrated in the supranuclear Golgi bodies as shown by PAA, WGA, LEA, as was
252 Gal α 1,3Gal β 1,4GlcNAc β 1- bound by BSA-1B4 in later pregnancy, and some α 1,6-linked
253 fucosyl residues (ALA binding). All these were also evident on the microvillous membranes.

254 The uterine epithelium also showed secretory properties, with a glycosylated microvillous
255 membrane and active Golgi bodies which expressed bi-triantennary bisected complex N-
256 glycan (ePHA), N-acetyl galactosamine (DBA, VVA, WFA) as well as N-acetyl glucosamine
257 residues (PAA, WGA) and fucose in α 1,2 and α 1,6 linkage (UEA-1, ALA); other residues were
258 evident but less prominent. In horse and donkey, Golgi bodies could not be distinguished
259 from other glycosylated structures.

260 In summary, our findings confirm the hypothesis put forward in earlier publications that
261 closely related and/or interbreeding species share similar glycosylation patterns at the fetomaternal
262 interface. However, this is the first case in which we have observed intraspecies

263 evolutionary diversion to be associated with the development of a different histological
264 architecture and intracellular granule formation.

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268

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332

333 **Legends to figures**

334 **Figure 1: Semithin sections of horse (A-C,) donkey (D) and zebra (E-H) stained with WFA to**
335 **show general structure.**

336 A) 50 day horse placenta showing simple apposition of the trophoblast (T) to the maternal
337 uterine epithelium (ME). There are numerous pleomorphic granules in the trophoblast while
338 a flatter maternal epithelium contains few granules. MVM: microvillous membrane.

339 B) At 200 days, villi of the microcotyledons contain many small granules, mainly under the
340 microvillous membrane, while in the maternal epithelium the inclusions are less well
341 defined.

342 C) By 280 days, the appearance has not really changed.

343 D) The donkey placenta from a 65cm crown-rump length fetus has more vesicular inclusions
344 but the staining characteristics are similar to those of the horse.

345 E) At 81 days, the zebra trophoblast binds WFA strongly, especially on the microvillous
346 surface, and the vesicular, rather than granular, nature of the cytoplasm can be observed.

347 F) The 81 day zebra maternal epithelium is darkly stained, especially apically, and a uterine
348 gland can be seen. The trophoblast (E) and maternal epithelium (F) have separated from
349 each other.

350 G) At 195 days, the intensity of WFA binding in zebra placenta has decreased in both
351 trophoblast and maternal epithelium, apart from the microvillous membranes. The vesicular
352 nature of the trophoblast, and its lack of intracellular granules, is apparent.

353 H) By 239 days the staining has decreased slightly but there is otherwise little change. T:
354 trophoblast, ME: maternal epithelium, MVM: microvillous membrane. Scale bars: 25µm

355

356 **Figure 2: Characteristics of the zebra placenta.**

357 A) The vesicular nature (arrows) of the trophoblast (T) can be appreciated after staining this
358 195 day placenta with ePHA. Paranuclear structures in the maternal epithelium may be
359 Golgi bodies (G).

360 B) In this 230 day zebra placenta, possible Golgi bodies (G) can again be seen in the
361 maternal epithelium (ME) with UEA-1 staining. Where the trophoblast (T) has retracted
362 from the maternal epithelium, it has a deeply stained microvillous surface (arrowheads),
363 although this may not be endogenous.

364 C) With ALA at 230 days, dark staining granules are evident in the trophoblast (T), especially
365 under the microvillous membrane, while possible Golgi bodies (G) are again visible in the
366 maternal epithelium (ME).

367 D) Regularly spaced, supranuclear structures that are probably Golgi bodies (G) can be seen
368 in the trophoblast of this 195 day placenta after WGA staining with neuraminidase pre-
369 treatment.

370 E) Strong staining of lateral cell membranes can be seen in this 239 day placenta stained
371 with MPA.

372 F) The distribution of lysosomes can be seen after GNA staining of the 195 day placenta; in
373 the maternal epithelium (ME) they are mainly subapical while they are more generally
374 dispersed in the trophoblast (T). Some of the latter appear to be hollow. MVM:
375 microvillous membrane.

376 G) and H) This shows LTA staining in the zebra (G:239 days) and horse (H:280 days)
377 placentae, with absence of stain in the horse microvillous membrane (MVM), but some
378 degree of binding in the zebra, equivalent. ME: maternal epithelium, T: trophoblast.

379 I) and J) DBA staining in the zebra placenta at 239 days compared with that of the horse
380 (280 days) shows similar strong staining of the maternal epithelium (ME), though granular in
381 the horse and more diffuse in zebra, but more stained inclusions in the horse trophoblast
382 (T). MVM: microvillous membrane. Scale bars: 25µm

Table 1: Details of the material used in this study.

<u>Species</u>	<u>Days of gestation</u>	<u>CR length (cm)</u>	<u>Weight of embryo (g)</u>
Horse	50	~43*	N/A
Horse	200	~53*	N/A
Horse	280	~75*	N/A
Donkey	N/A	65cm	N/A
Zebra	81	10	14.3
Zebra	117	24	230
Zebra	195	58	2790
Zebra	230	72	5580
Zebra	239	72	6590

*Estimated from [26] CR = crown-rump; N/A = not available

Table 2: Lectins used in this study and their major specificities

ACRONYM	SOURCE	MAJOR SPECIFICITY
GNA	<i>Galanthus nivalis</i> Snowdrop	Nonreducing terminal a-D-mannose, especially the mannosyl α 1,3mannose linkage
CON A	<i>Canavalia ensiformis</i> Jackbean	α -D-glucosyl and α -D-mannosyl (terminal or 1,2 linked) in high mannose, intermediate and small complex N-linked sequences
PSA	<i>Pisum sativum</i> Garden Pea	α -D-mannose in non-bisected bi/tri-antennary, complex N- linked sequences
e-PHA	<i>Phaseolus vulgaris</i> (erythroagglutinin) Kidney Bean	Bi/tri-antennary bisected complex N-linked sequences
I-PHA	<i>Phaseolus vulgaris</i> (leukoagglutinin) Kidney Bean	Tri/tetra-antennary, non- bisected complex N-linked sequences
UEA-1	<i>Ulex europaeus-1</i> Gorse	H type 2 antigen (α L-Fuc(1,2)Gal β 1,4GlcNAc β 1-) and Le ^y
LTA	<i>Tetragonolobus purpureus</i> Lotus	L-fucosyl terminals (especially where clustered), Fuc α 1,6GlcNAc >Fuc α 1,2-Gal β 1,4(Fuc α 1,3)-GlcNAc β , Le ^{x,y}
ALA	<i>Aleuria aurantia</i> Mushroom	Fucose linked α 1,6- to GlcNAc
DBA	<i>Dolichos biflorus</i> Horse Gram	GalNAc α 1,3(LFuc α 1,2)Gal β 1,3/4GlcNAc β 1-
VVA	<i>Vicia villosa</i> Hairy vetch	GalNAc α 1-Ser/Thr and GalNAc α 1,3Gal β 1-
HPA	<i>Helix pomatia</i> Roman snail	Terminal GalNAc α 1-
WFA	<i>Wisteria floribunda</i> Wisteria	GalNAc α 1,6Gal β 1- >GalNAc α 1,3Gal β 1-
SBA	<i>Glycine max</i> Soybean	Terminal GalNAc α 1- >Gal α 1
AHA	<i>Arachis hypogaea</i> Peanut	Gal β 1,3GalNAc β 1- >Gal β 1,4GlcNAc β 1-
ECA	<i>Erythrina cristagalli</i> Coral Tree	Gal β 1,4GlcNAc β 1-
MPA	<i>Maclura pomifera</i> Osage orange	Gal β 1,3GalNAc α 1- >GalNAc α 1-
BSA-1B ₄	<i>Bandeiraea simplicifolia</i> Griffonia	Gal α 1,3Gal β 1,4GlcNAc β 1-
DSA	<i>Datura stramonium</i> Jimson Weed	β 1,4GlcNAc, N-Acetyl lactosamine >chitotriose
STA	<i>Solanum tuberosum</i> Potato	β 1,4GlcNAc oligomers
LEA	<i>Lycopersicon esculentum</i> Tomato	β 1,4GlcNAc oligomers
PAA	<i>Phytolacca Americana</i> Pokeweed	Similar to WGA
WGA	<i>Triticum vulgaris</i> Wheatgerm	Di-N-acetyl chitobiose, N-acetyl lactosamine (especially if clustered) and some sialyl residues
SNA	<i>Sambucus nigra</i> Elderberry Bark	NeuNAc α 2,6Gal/GalNAc-
MAA	<i>Maackia amurensis</i>	NeuNAc α 2,3Gal β 1-

Table 3: Lectin staining of trophoblast in horse, donkey and zebra.

Key: 0: negative,1: weak, 2: moderate. 3: strong, 4: intense. Granule density from sparse (-/+) to dense (+++). G: possible Golgi bodies

Lectin	Horse						Donkey		Zebra									
	50d (~43cm)		200d (~53cm)		280d (~75cm)		65cm		81d (10cm)		117d (24cm)		195d (58cm)		230d (72cm)		239d (72cm)	
	Cyt	Gran	Cyt	Gran	Cyt	Gran	Cyt	Gran	Cyt	Gran/ves	Cyt	Gran/ves	Cyt	Gran/ves	Cyt	Gran/ves	Cyt	Gran/ves
GNA	1-2	3-4+++	0	3-4++	1	3-4+++	1	3-4+	3	3	1-2	2-3	1-2	3+++	2	3-4++	2	3-4+++
CONA	2	4+++	2	2+++	2	3-4++	2	3++	3	3-4+++	3-4	3-4+++	2	2+++	3-4	3-4+++	2	2++
PSA	1	4++	1	4+	1	4++	1	3-4++	2-3	4++	2	2-3++	2-3	2-3++	2-3	4++	2	3++
ePHA	2	4++	2	2+++	2	4++	2	3-4+	3-4	4+	2-3	2-3++	2-3	2-3++G	3	3++	2-3	2-3++
IPHA	0	2++	0	3+	1	3+	0	3-4+	0-1	1-2++	2	3-4+	1-2	3++	1-2	1-2+	1-2	1-2++
LTA	0	0	0	0	0	0	0	0	2	2+++	2	2+++	2	2+++	2	2++	1-2	1-2+
UEA-1	0	0	0	0	0	0	0	0	1	1++	1	1++	1	1++	1	1++	1	1++
ALA	2	3+++	0	3-4++	1	3-4+++	1	3-4++	3	3-4++	2-3	2-3++G	2-3	2-3++G	2-3	2-3++G	2-3	2-3++G
DBA	0	4++	0	3+	0	4+	0	4-/+	1-2	1-2+	1-2	2+	0-1	1+	1	1-2+	1	1-2+
VVA	0	3-4++	0	3-4+	0	3-4+++	0-1	2-3++	2-3	2-3+	1-2	2+	1-2	2+	1	1-2+	0-1	1-2+
MPA	1	3+++	0	2-4+++	1	3-4+++	1-2	3-4++	3-4	3-4++	2-3	2-3+	2-3	3+	2	2-3+	2-3	3+
BSA-1 _{B4}	1	3-4++	0	3-4+	1	3-4+++	1	3-4++	2-3	2-3++	2	2-3++	1-2	2-3++G	2	2-3++G	2	2-3++G
DSA	1	3-4+++	1	3-4+++	1	3-4+++	1-2	2-3++	2	2-3+++	2-3	3++	2-3	2-3++	2-3	2-3+++	2	2-3++
STA	2	3-4+++	1-2	3-4+++	1-2	3-4+++	2	2-3++	3	3+++	2-3	3++	2-3	2-3++	2-3	2-3+++	2-3	2-3+
LEA	1	3-4+++	0	3-4++	0-1	3-4+++	0	3-4++	2	2-3+++	2	2-3+G	2	2-3++	1-2	2-3++G	2	3++
HPA	0	2-3+++	0-1	3-4++	0-1	2-3++	0-1	2-3+	2-3	2-3+++	2-3	2-3+++	2-3	2-3+++	2	2-3++	2	2-3+
AHA	1	3-4+++	0-1	3-4++	0-1	3-4+++	0-1	3-4++	3	3-4++	2	2-3++	1-2	2+	2	2-3++	2	2-3+
AHA+N	1	3-4++	1	2-3++	1	2=3+++	1	2-3+++	2-3	3-4++	2	2-3++	2	2-3+	2	2-3++	1	1-2+
ECA	1	3-4++	0-1	3-4++	1	3-4+++	0-1	3-4++	2	2-3++	1-2	2-3++	1	1-2+	1-2	2-3+	1	1-2+
ECA+N	0	2-3+	0-1	3-4++	0-1	2-3+++	0-1	2-3++	1-2	2-3++	1-2	2++	1	1-2+	1-2	2-3+	1	1-2+
SBA	1	3-4++	0	2-3++	0-1	2-3++	0	2+	1-2	2-3++	1-2	2-3++	1-2	2+	1	1-2++	1-2	2+
SBA+N	0	3-4++	0-1	2-3++	0-1	2+++	0	2+	1-2	2-3+	1-2	2++	1	1-2+/-	1	1-2++	1	1-2+
WFA	2	3-4+++	1	3-4+++	0-1	3-4++	1	3-4++	2-3	3-4++	2-3	3++	2	2-3+	2	2-3++	2	2-3++
SNA-1	0-1	2-3++	0-1	2+	0	0	0-1	1-2+	3	3-4++	2-3	2-3+++	3	3+++	2-3	2-3+++	3	3+++
SNA-1+N	0-1	2++	0	0	0	0	0	0	2	3++	1	2-3+	3	3+++	2	2+++	3	3+++
MAA	0	0	0	0	0	0	0	0	0-1	1-2++	1	1-2+	1	1+++	0-1	1+++	1	1+++
MAA+N	0	0	0	0	0	0	0	0	0	0-1+	0-1	0-1+	0-1	0-1	0-1+++	0-1+++	0	0-1+++
PAA	1	3-4+++	0-1	2-4++	1	2-4++	0-1	3-4+	3	3-4++	2	2-3++	2	2-3++G	2	2-3++G	2	2-3+G
PAA+N	1	3-4+++	0-1	2-4++	1	2-3++	0	3+	3	3-4++	2	2++	2	2-3++G	2	2-3++G	1-2	2-3+G
WGA	1	3-4++	1	3-4++	1	3-4++	0-1	3+	3	3-4++	2	2-3++	1-2	3-4+G	1-2	2-3+G	1-2	2-3+G
WGA+N	1	3-4+++	0-1	3-4+	0-1	3-4++	1	3+	3	3-4++	2	2-3++	1-2	3-4+G	1-2	2++G	1	2++G

Table 4: Lectin staining of maternal uterine epithelium in horse, donkey and zebra

Key: 0: negative,1: weak, 2: moderate. 3: strong, 4: intense. Granule density from sparse (-/+) to dense (+++). G: possible Golgi bodies

Lectin	Horse						Donkey		Zebra									
	50d (~43cm)		200d (~53cm)		280d (~75cm)		65cm		81d (10cm)		117d (24cm)		195d (58cm)		230d (72cm)		239d (72cm)	
	Cyt	Gran	Cyt	Gran	Cyt	Gran	Cyt	Gran	Cyt	Gran/ves	Cyt	Gran/ves	Cyt	Gran/ves	Cyt	Gran/ves	Cyt	Gran/ves
GNA	1	2-/+	1	4++	1	4++	1	3-4++	1	3-4++	1	1-2++	1-2	3-4++	2	3-4++	2	3-4++
CONA	2	3+/-	2	4-/+	2	4-/+	2	3-4+	3-4	3-4+++	3-4	3-4+++	2	2++	3-4	3-4++	2	2+
PSA	1	3+/-	1	4+	1	4+	1	3-4+	2-3	3+	2	2++	2	2-3+	2	2+	2	2+
ePHA	1	3+	2	4+	2	4+	2	3-4+	3-4	4++G	2	2-3++G	2	2-3+ G	2	3+G	2	3++G
IPHA	0	0	0	2+	0	2+	0	0	0	0-1+	1	1-2+	1	1++	1	1-2+	1	1++
LTA	0	0	0	0	0	0	0	0	2	2+++	2	2+++	2	2++	2	2++	1	1+
UEA-1	1	2-3+	2	3-4++	2	3-4++	0	4++	3	3-4+++	2	2-3++	2-3	2-3++	2-3	2-3++	2-3	2-3++
ALA	2	2	1-2	3-4++	1-2	3-4++	1	3-4+	3	3-4++	2-3	2-3++	2-3	2-3++	2-3	2-3++G	2-3	2-3++
DBA	0	3++	2	3-4+++	2	3-4+++	1	3-4++	2-3	3-4++G	3	3-4+G	2-3	3-4+G	2-3	2-3+G	3	3-4+G
VVA	0	3++	1	3-4+++	1	3-4+++	1	3-4++	2-3	3-4+G	2-3	3-4+G	2-3	3+G	2-3	3+g	2-3	3-4+G
MPA	0	2+	1	3-4++	1	3-4++	2	3-4++	2-3	34+	1-2	2-3+	2-3	3+	2	2-3+	2	2-3+
BSA-1 _{B4}	0	2-/+	0	0	0	0	0	0	2	2-3++	1	1-2++	1	1-2+	0	1-2+	0	0-1+
DSA	1	2++	0	3-4++	0	3-4++	1-2	3-4++	3	3+++	2-3	3++	2	2-3+	2	2-3+	2	2-3+
STA	2	2++	1-2	3-4++	1-2	3-4++	2	3-4++	3	3+++	3	3++	2-3	2-3+	2-3	2-3+	2-3	2-3+
LEA	0	2+	0-1	3-4++	0-1	3-4++	0-1	3-4++	3	3+++	2	2-3+	2	2-3++	1-2	2-3+	2-3	2-3++
HPA	1	3+	0-1	3-4++	0-1	3-4++	0-1	3-4++	3	3+++	2-3	2-3++	2-3	2-3++	2	2-3+	2	2-3+
AHA	0	3++	1	3-4+++	1	3-4+++	0	3-4++	2	3-4++	2	2-3++	1-2	2+	1-2	2+	1-2	2-3+
AHA+N	0	2+	1	3-4+++	1	3-4+++	0	3-4++	2	3-4++	1-2	2-3++	2	2-3+	2	2+	1	1-2+/-
ECA	1	3++	0-1	3-4+++	0-1	3-4+++	0-1	3-4++	2-3	3-4++	1-2	2-3++	0-1	1-2+	2	2-3+	1	1-2+
ECA+N	0	2+	0-1	3-4+++	0-1	3-4+++	0-1	3-4++	2-3	3++	1-2	2++	1-2	2-3+	2	2-3+	1	1-2+
SBA	1	3++	0-1	3-4+++	0-1	3-4+++	0	3-4++	2-3	3++	2-3	2-3++	2	2-3+	1-2	2+	2	2-3++
SBA+N	0	3++	0-1	3-4+++	0-1	3-4+++	0	3-4++	3	3-4+++	2	2-3++	1-2	2-3+	1-2	2+	1-2	2++
WFA	2	2+++	2	3-4+++	2	3-4+++	1-2	3-4++	4	4+++	3-4	4++	3	3-4++G	2-3	2-3+G	3	3-4++G
SNA-1	0-1	2++	1	2-3++	1	2-3++	0-1	2+	3	3-4++	2-3	2-3++	3	3+++	2	2+++	3	3+++
SNA-1+N	0-1	2+	0	0	0	0	0	0	2-3	3-4++	1	2+	3	3+++	2	2+++	3	3+++
MAA	0	0	0	0	0	3-/+	0	0	0	1+	1	1-2+	1	1-2++	0	1++	1	1+++
MAA+N	0	0	0	0	0	0	0	0	0	0	0	0-1+	0-1	0-1+	0	0++	0	0-1+++
PAA	0-1	2-3++	1	3-4+++	1	3-4+++	0-1	3-4++	3-4	4++	1	2++	1	2-3+G	1-2	2+G	1-2	2-3+G
PAA+N	0-1	2+	1	3-4+++	1	3-4+++	0	3-4+	3-4	4++	1-2	2-3+	1-2	2-3+G	1-2	2+G	1-2	2-3+G
WGA	0-1	2-3++	1	3-4++	1	3-4++	0-1	3-4++	3	4++	2	2-3++	1-2	3+G	1-2	2-3+G	1-2	2-3+G
WGA+N	1	2-3++	0-1	3-4++	0-1	3-4++	0-1	2-3++	3	4++	2	2-3++	1	2-3+G	1-2	2++G	1	2+G

Figure
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Figure
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