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In vitro-produced horse embryos exhibit a very narrow window of acceptable recipient mare uterine synchrony compared with *in vivo*-derived embryos

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Abstract. In recent years, the number of equine *in vitro*-produced embryos (IVP) has increased markedly; as yet, there are few reports on what constitutes an 'ideal' recipient for an IVP embryo. This study retrospectively investigated the effects of recipient mare oestrous cycle characteristics on the likelihood of pregnancy after transfer of IVP ($n = 264$) and *in vivo*-derived embryos ($n = 262$). IVP embryos tolerated only a narrow window of recipient mare 'synchrony', with transfer on Day 4 after ovulation resulting in a higher likelihood of ongoing pregnancy (69%) than transfer on Days 3, 5 or 6 (53.2%, 41.3% and 23.1% respectively; $P = 0.02$). In contrast, Day 8 *in vivo*-derived embryos tolerated a wide range of uterine (a)synchrony, with no difference in pregnancy or pregnancy loss for recipients that ovulated between Day 4 and Day 9 before transfer. However, transferring *in vivo*-derived embryos to recipients that had a longer oestrus preceding transfer resulted in higher Day 12 and ongoing pregnancy rate ($P < 0.01$). This effect was not significant in IVP embryos. In conclusion, Day 6–8 IVP blastocysts survive best after transfer to Day 4 recipient mares; Day 8 *in vivo*-derived embryos survive equally well in Day 4–9 recipients, but do better in mares that have a long preceding oestrus.

Additional keywords: mare, recipient synchrony, ICSI, embryo.

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Introduction

The availability of suitable recipient mares is a critical aspect of a successful embryo transfer (ET) program, ensuring maximal likelihood of establishing and maintaining pregnancy after transfer of an embryo (Stout 2006). One aspect of recipient 'quality' is an endometrium that creates a uterine environment able to support embryo survival and growth, and a major factor influencing endometrial status and function is the stage of the oestrous cycle (i.e. the number of days after ovulation). Progesterone secreted by the corpus luteum (CL) starting shortly after ovulation (Townson *et al.* 1989) is the principal factor affecting the establishment of endometrial receptivity for the preimplantation embryo, by stimulating or initiating complex changes in the expression of genes involved in the secretion of histotrophic proteins or coding for endometrial receptors (de Ruijter-Villani and Stout 2015).

Preparation of the endometrium to support embryo development has been studied extensively in domestic ruminants (Spencer *et al.* 2016) and to a more limited degree in horses (Gebhardt *et al.* 2012). Interestingly, *in vivo*-derived horse embryos are able to tolerate an unusually large degree of negative uterine asynchrony (i.e. when the recipient mare ovulates on days following donor ovulation), with little difference in the likelihood of pregnancy for embryos transferred into recipient mares that ovulated anywhere between 0 and 5 days after the donor (Wilsher *et al.* 2010; Jacob *et al.* 2012). In contrast, a relatively modest (i.e. >2 days) positive asynchrony (i.e. recipient ovulates before the donor) significantly compromises embryo survival (Wilsher *et al.* 2012). However, the optimisation of uterine receptivity for pregnancy is not dependent solely on the duration of exposure to progesterone; indeed, we recently reported a positive correlation between the duration of a recipient mare's oestrous period immediately before ET and the subsequent likelihood of pregnancy (Cuervo-Arango *et al.* 2018*a*). In short, it appears that there is a positive effect of a relatively long preceding oestrus on the likelihood of pregnancy after ET, presumably because the duration of oestrogen priming influences the ability of the endometrium to create an environment conducive to embryo survival.

During the past decade, *in vitro* production (IVP) of equine embryos by ovum-pick up (OPU) followed by intracytoplasmic sperm injection (ICSI) has become popular among sport horse breeders, and has led to the establishment of several laboratories around the world offering commercial equine OPU-ICSI (Herrera 2018). Compared with embryo flushing, OPU-ICSI has the potential advantage of producing multiple embryos from a single 'out-patient' procedure (Cuervo-Arango *et al.* 2019*a*), with the option of cryopreserving some or all of the embryos

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without negatively affecting the likelihood of ongoing pregnancy following ET (Choi and Hinrichs 2017); cryopreserving embryos allows more efficient management and stricter selection of recipient mares.

Despite the growing numbers of programs offering equine IVP, there is limited information on recipient factors that influence the likelihood of establishing and maintaining pregnancy after transfer of IVP embryos. In this regard, IVP embryos are expected to have different requirements to *in vivo*-derived (flushed) embryos in terms of their 'ideal' uterine environment, primarily because they are transferred at a less advanced developmental stage (i.e. fewer cells (Tremoleda *et al.* 2003) and 'behave like a Day 5–6 embryo' (Claes *et al.* 2019)), when they have yet to develop a confluent capsule (Tremoleda *et al.* 2003; Choi *et al.* 2009) and show delayed upregulation of developmentally important genes (Smits *et al.* 2011). The aims of the present retrospective study were to determine how characteristics of a recipient mare's oestrous cycle (i.e. day after ovulation, length of preceding oestrus) affect the likelihood of pregnancy after transfer of an IVP embryo, and how this compares with the recipient characteristics preferential for establishing pregnancy after transfer of *in vivo*-derived embryos.

Materials and methods

In vivo-derived embryos

In all, 262 Day 8 embryos (where Day 0 is the day of the donor's ovulation) collected by uterine lavage from 153 Warmblood mares aged between 2 and 25 years (mean ($\pm$s.d.) age 11.4 ± 6.5 years) between June 2016 and September 2018 were used in this study. The time interval for the study was selected because from June 2016 all transfers were performed using a speculum and 'Wilsher' forceps (Wilsher and Allen 2004), a technique that was shown to reduce operator effects on ET success (Cuervo-Arango *et al.* 2018*b*).

All embryos recovered were transferred and included in the analysis, regardless of size at collection (mean ($\pm$s.d.) diameter 592 ± 251 μm; range 150–1250 μm). Briefly, the uterus of donor mares was flushed three times with 1 L commercial lactate Ringer's solution (Baxter Nederland) supplemented with 0.5% v/v fetal calf serum. Embryos were located and measured using a dissecting microscope (SZ60; Olympus Nederland) equipped with an eyepiece micrometer. After washing with holding medium (Syngro, Bioniche), all embryos were held in holding medium (Syngro; Bioniche Animal Health) at room temperature for up to 2 h before being transferred transcervically into a recipient mare that had ovulated between 1 day before and 4 days after the donor mare (Day 4 to Day 9 recipient mares).

IVP embryos

In all, 264 ICSI-derived IVP embryos, transferred during the same months as the *in vivo* embryos described above, were included in a parallel analysis. Oocytes were obtained from 136 mares during 2015–18, as described previously (Cuervo-Arango *et al.* 2019*a*). IVM of oocytes, ICSI and embryo culture to produce blastocysts were performed as described by Colleoni *et al.* (2011), using spermatozoa from one of 95 different stallions selected by a mare's owner. Blastocysts were identified 6–8 days after ICSI and cryopreserved by slow freezing in 10% glycerol (Galli *et al.* 2007); blastocysts were subsequently returned to Utrecht University in liquid nitrogen for subsequent ET. Blastocysts that developed on Day 9 or later were discarded.

In preparation for cryopreservation, embryos were removed from culture, transferred to human synthetic oviductal fluid (H-SOF) and allowed to cool to room temperature for 30 min. The embryos were then equilibrated in H-SOF containing 5% glycerol for 5 min, followed by H-SOF containing 10% glycerol for 20 min, before being loaded individually into 0.25-mL straws. The straws were then sealed and placed in a methanol bath at -6.5°C for 5 min. Then, straws were seeded by touching them with a pair of forceps precooled by immersion in liquid nitrogen. Freezing was continued at a rate of -0.5°C per minute down to -35°C, at which point the straws were plunged into liquid nitrogen.

When a suitable recipient was available for ET, IVP embryos were thawed by first holding the straw in air for 8 s, followed by immersion in a water bath at room temperature for 20 s. The glycerol was washed out by successive 5-min incubations in H-SOF containing decreasing percentages of glycerol (8%, 6%, 4% and 2%). Finally, the embryos were held in H-SOF medium before ET within 30 min.

Recipient management

In all, 389 Warmblood mares aged between 3 and 14 years (mean ($\pm$s.d.) age 8.7 ± 4.1 years) were used as recipients. All were either maiden or barren at the time of transfer (i.e. no mares with a foal at foot were included). Mares were maintained either in small groups (5–20 mares) in grass paddocks (*ad libitum* feed and exercise) or in individual boxes with daily exercise in a horse-walker or sand paddock. Hay and concentrated feed were provided three times a day, in addition to *ad libitum* access to fresh water. At any given time, a maximum of 30–40 cyclic recipient mares was housed at the clinic. Once they were confirmed pregnant, mares were transferred to fields in groups, and new groups of 6–10 non-pregnant mares were brought in from the 'winter accommodation' to replenish the recipient pool.

During the first examination after arrival at the clinic, the reproductive tract of recipient mares was evaluated by transrectal palpation and ultrasonography; the cervix was examined *per vaginam* to rule out cervical pathology and help with the staging of the mare's oestrous cycle. Mares were considered to be in anoestrus in the early breeding season (February–April) if luteal tissue was not visible and follicle diameters did not exceed 20 mm for at least 10 days. Anoestrous mares were examined once a week until they entered vernal transition (the presence of 20- to 30-mm follicles with endometrial oedema), after which they were examined two to three times a week in anticipation of the first ovulation of the season.

Mares in dioestrus (the presence of one or more CL, absence of endometrial oedema and a tightly closed cervix) were treated with 75 μg D-cloprostenol (75 μg mL^{-1} D-cloprostenol; Genestranvet; Eurovet Animal Health) to induce oestrus, as long as they did not have a follicle exceeding 28 mm in diameter. Mares with a CL and a follicle >28 mm in diameter were left to enter oestrus spontaneously; treatment of such mares with prostaglandin $F_{2\alpha}$ (PGF) analogues has been associated with ovulation within

a few days, following an abbreviated oestrus and reduced fertility (Newcombe *et al.* 2008; Cuervo-Arango *et al.* 2015; Mateu-Sanchez *et al.* 2016). Once in oestrus, mares were examined every 24 h until ovulation was detected. During these examinations, the intensity of endometrial oedema was scored subjectively on a scale of 0–3 (0, no oedema; 1, some oedema; 2, obvious oedema; 3, a lot of oedema). The 'duration of oestrus with endometrial oedema' was recorded as the interval (in days) from the first day on which the mare showed at least obvious endometrial oedema (score of 2–3) until the day of ovulation (Day 0).

Ovulation was not induced routinely. However, in approximately half the oestrous cycles (245/526), mares with a follicle ≥35 mm in diameter and obvious endometrial oedema were administered 1500 IU, i.v., human chorionic gonadotrophin (Chorulon; Intervet Nederland) to induce ovulation. The decision to induce ovulation was dependent on the veterinary surgeon's preference, and to help ensure adequate donor–recipient synchrony.

ET and donor–recipient synchrony

The days selected for ET (i.e. days of optimal embryo–uterine synchrony) were based on the evidence currently available at the time transfers were performed; *in vivo*-derived embryos were transferred on Days 4–9 (Jacob *et al.* 2012) and IVP embryos were transferred on Days 3–6 (Cuervo-Arango *et al.* 2018*c*). Immediately before transfer, recipient mares were examined by transrectal ultrasonography to confirm the absence of endometrial oedema and intrauterine free fluid, and to confirm the presence of a CL with a toned uterus and cervix.

If considered suitable for ET, a recipient mare was restrained in stocks and sedated with 4 mg, i.v., detomidine hydrochloride (Domosedan; Vetoquinol), regardless of bodyweight. Next, the perineum was thoroughly cleaned with a dilute povidone iodine scrub solution and, after rinsing with clean water, the cutaneous–mucosal border at the entrance to the vestibule was disinfected with a chlorhexidine gluconate–alcohol ketonatus solution (Spervasept Forte; Spervital). No further routine pre- or post-ET supportive treatments were administered to recipient mares. Embryos were transferred transcervically using Wilsher forceps and speculum, as described previously (Cuervo-Arango *et al.* 2018*b*); all transfers were performed by one of four board-certified equine reproduction specialists.

Pregnancy diagnoses

The first pregnancy diagnosis was performed 4 or 7 days after ET for *in vivo*-derived and IVP embryos respectively, because these were the times at which an embryonic vesicle of 5–15 mm in diameter was expected to be present in the uterus (Cuervo-Arango *et al.* 2018*c*). At this time, the diameter of the embryonic vesicle (if present) was measured as the average of two perpendicular measurements at the maximum cross-section of the vesicle, using electronic callipers of the ultrasound machine. A second examination was performed 2–3 weeks later to confirm normal development of the conceptus, including the presence of a heartbeat. At that time, the extent of allantoic development was recorded as the percentage of the conceptus vesicle accounted for by the allantoic cavity (i.e. 50% when the allantoic membrane split the conceptus into two equal halves). A final pregnancy diagnosis was performed at Day 45 of fetal development. Fetal losses between Day 45 and the end of each year were determined by a telephone call to the owner of the pregnant mare. Owner-confirmed pregnancies at the end of each calendar year were referred to as an 'ongoing pregnancy'; gestational ages ranged between 4 and 9 months at that time point. Pregnancy losses were classified according to the gestational period in which they occurred. Embryonic and fetal losses were defined as those that occurred before or after Day 45 respectively.

Statistical analysis

Binary regression models were created for each type of embryo (IVP and *in vivo*-derived embryos) to examine the effects of various factors on the likelihood of pregnancy at the first examination, of ongoing pregnancy and of pregnancy loss. The variables included in the model were donor age, embryo size (*in vivo*-derived embryos only), day of blastocyst formation (IVP embryos only), recipient's length of preceding oestrus, recipient's day after ovulation, use of PGF to induce oestrus and whether or not transfer was performed in the dioestrus of the first recorded ovulation of the year (first vs subsequent ovulations). For *in vivo*-derived embryos, a linear regression model was used to determine the effect of a recipient's day after ovulation on embryonic vesicle growth between ET and the first pregnancy diagnosis 4 days later. Finally, the non-parametric Mann–Whitney test was used to test for differences in embryonic vesicle diameter 7 days after ET of IVP embryos between ongoing and failing pregnancies, and among different recipient day after ovulation groups.

Results

The overall first pregnancy examination (equivalent to Day 12) and ongoing pregnancy rates were 92.3% and 82.4% respectively for *in vivo*-derived ($n = 262$) embryos and 72.3% and 59.1% respectively for IVP embryos ($n = 264$; $P < 0.001$). The rate of embryonic and fetal losses were 7.7% and 4.0% respectively for *in vivo*-derived embryos and 12.7% and 6.8% respectively for IVP embryos ($P > 0.1$). The significance and odds ratios for the variables analysed with regard to pregnancy status following transfer for *in vivo*-derived and IVP embryos are given in Tables 1 and 2, respectively.

The day after ovulation of the recipient mare at the time of ET significantly influenced the likelihood of pregnancy at Day 12 for IVP embryos ($P < 0.001$; Fig. 1), but not for *in vivo*-derived embryos ($P > 0.1$). For IVP embryos, Day 4 recipients had a higher likelihood of ongoing pregnancy (69%) than recipients on other days after ovulation (53.2%, 41.3% and 23.1% for Day 3, 5 and 6 recipients respectively; Table 3). Moreover, the recipient's day after ovulation at transfer of an IVP embryo affected embryonic vesicle diameter 7 days later (Fig. 2). Transfer to a Day 4 recipient resulted in a larger embryonic vesicle ($P < 0.01$) than transfer to a Day 3 or 5 recipient. In addition, failure of an IVP pregnancy was associated with a smaller vesicle at first pregnancy diagnosis ($P < 0.001$; Fig. 2). In contrast, *in vivo*-derived embryos grew at a slower rate during the first 4 days after transfer in Day 4 recipients than in any other group (Fig. 3).

Table 1. Effect of donor mare, embryo and recipient characteristics on pregnancy status following transfer of Day 8 *in vivo*-derived horse embryos
OR, odds ratio; OV, ovulation; PGF, prostaglandin $F_{2\alpha}$

Variable	Day 12 pregnancy		Ongoing pregnancy		Overall losses	
	P-value	OR	*P*-value	OR	*P*-value	OR
Donor mare age	0.63	1.12	0.18	1.21	0.21	0.8
Embryo size	0.03	0.49	0.04	0.57	0.32	0.79
Recipient's oestrus duration	0.003	0.54	0.006	0.72	0.21	1.18
Recipient's day after OV	0.94	0.99	0.49	0.93	0.44	1.1
PGF-induced luteolysis	0.89	1.01	0.87	0.98	0.67	0.97
First OV of year	0.5	1.1	0.46	1.01	0.37	0.94

Table 2. Effect of donor mare, embryo and recipient characteristics on pregnancy status following embryo transfer of *in vitro*-produced equine embryos
OR, odds ratio; OV, ovulation; PGF, prostaglandin $F_{2\alpha}$

Variable	Day 12 pregnancy		Ongoing pregnancy		Overall losses	
	P-value	OR	*P*-value	OR	*P*-value	OR
Donor mare age	0.57	1.02	0.21	1.03	0.25	0.96
Days to blastocyst	0.06	1.78	0.07	1.61	0.51	0.78
Recipient's oestrus duration	0.13	0.91	0.10	0.87	0.21	1.16
Recipient's day after OV	<0.001	2.03	0.02	1.49	0.38	1.31
PGF-induced luteolysis	0.32	1.35	0.74	1.09	0.46	1.36
First OV of year	0.56	0.7	0.59	1.3	0.2	0.44

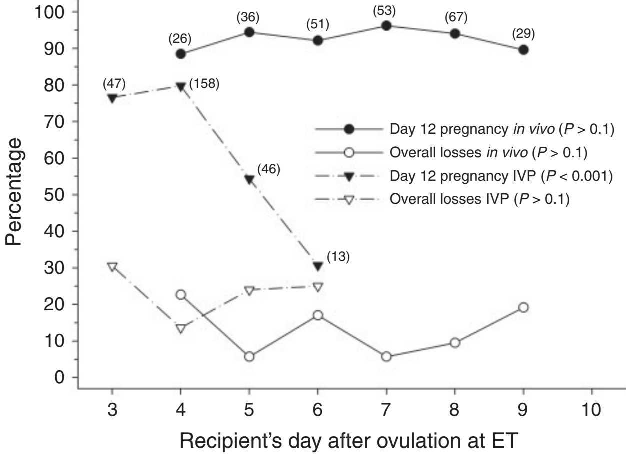

Fig. 1. Percentage of Day 12 pregnancies and subsequent pregnancy losses (from Day 12 to the end of the season) for Day 6–8 frozen–thawed *in vitro*-produced (IVP) equine embryos and Day 8 *in vivo*-derived embryos for the different number of days after ovulation on which they were transferred into a recipient mare. Values in parentheses indicate the number of embryos transferred at each stage.

The age of the donor mare did not affect ($P > 0.1$) the Day 12 pregnancy rate, ongoing pregnancy rate or pregnancy losses for either type of embryo (Tables 1, 2). The diameter of *in vivo*-derived embryos affected the Day 12 pregnancy rate and ongoing pregnancy rate (Table 1) in a similar manner to that reported previously (Cuervo-Arango *et al.* 2019*b*): embryos <300 μm resulted in lower post-ET viability.

The allantoic membrane of *in vivo*-derived embryos took a mean (± s.d.) of 20.4 ± 1.2 days after ET to reach 50% of the embryonic vesicle (i.e. when the allantoic membrane split the conceptus into two equal halves), whereas IVP embryos needed 23.1 ± 2.3 days ($P < 0.05$) following ET to reach similar allantoic development.

In addition, the day at which an IVP embryo was found to have reached the blastocyst stage and was cryopreserved also affected both the likelihood of establishing ($P = 0.06$) and maintaining ($P = 0.07$) pregnancy (Table 2), with embryos that reached the blastocyst stage earlier (Days 6 or 7) more likely to survive after transfer than Day 8 blastocysts.

The length of a recipient mare's oestrus preceding ET had a significant effect on the likelihood of ongoing pregnancy following transfer of *in vivo*-derived embryos ($P = 0.007$), but not IVP embryos. The longer the oestrus, the higher the likelihood of ongoing pregnancy for *in vivo*-derived embryos (Fig. 4). For IVP embryo recipients, a very short preceding (<2 days) oestrus did appear to be disadvantageous, but numbers were too low to verify this statistically.

The type of oestrus (first of the year, induced by PGF or spontaneous) did not affect the likelihood of ongoing pregnancy for either type of embryo (Table 4). As the season advanced, the likelihood of establishing an ongoing pregnancy tended to be lower for IVP embryos ($P = 0.06$) and was significantly lower for *in vivo*-derived embryos ($P = 0.04$; Table 5). This presumably reflected, at least in part, the accompanying decrease in the

length of the preceding oestrus ($P < 0.001$) as the season advanced (Table 5), but may have also reflected other factors contributing to general lower 'quality' of mares left to receive embryos at the end of the season.

Discussion

The large window of uterine asynchrony (principally negative asynchrony) tolerated by *in vivo*-derived equine embryos in a commercial ET program (Jacob *et al.* 2012) was confirmed in the present study, with no significant change in the likelihood of establishing or maintaining pregnancy when Day 8 embryos were transferred into recipient mares anywhere between Day 4 and Day 9 after ovulation. In contrast, IVP embryos proved to be more fastidious with regard to the recipient's stage of the cycle, with as little as a 24 h difference in the recipient mare's uterine stage (i.e. Day 3 or Day 5 vs Day 4 after ovulation) affecting the likelihood of ongoing pregnancy. This was evidenced by both a higher incidence of pregnancy loss after transfer to a Day 3 recipient and a lower initial likelihood of pregnancy in Day 5 or Day 6 recipients. This suggests that, as for *in vivo*-derived embryos, negative uterine asynchrony is more conducive to initial embryo survival than positive asynchrony. Although a larger number of Day 3 transfers is required to confirm the apparent elevated risk of pregnancy loss, this very short window of optimal synchrony for IVP embryos is similar to that reported in other species, such as cattle (Randi *et al.* 2016).

The more narrow window of acceptable uterine synchrony for IVP horse embryos compared with their *in vivo*-derived counterparts may reflect the lower quality of IVP embryos (e.g. higher percentage of apoptotic cells; Tremoleda *et al.* 2003) and a reduced capacity to adjust or to meet the metabolic demands of

Table 3. Effect of recipient mare's day after ovulation at transfer on likelihood of ongoing pregnancy

Data show pregnant recipients/total number of transfers, with the percentage of ongoing pregnancies in parentheses. Within rows, different letters indicate significant differences ($P < 0.05$). ET, embryo transfer; IVP, *in vitro* produced

Embryos	Recipient's day after ovulation at ET						
	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
IVP	25/47 (53.2)[a]	109/158 (69.0)[b]	19/46 (41.3)[a]	3/13 (23.1)[a]	–	–	–
In vivo derived	–	19/26 (73.1)	32/36 (88.9)	39/51 (76.5)	48/53 (90.5)	57/67 (85.1)	21/29 (72.4)

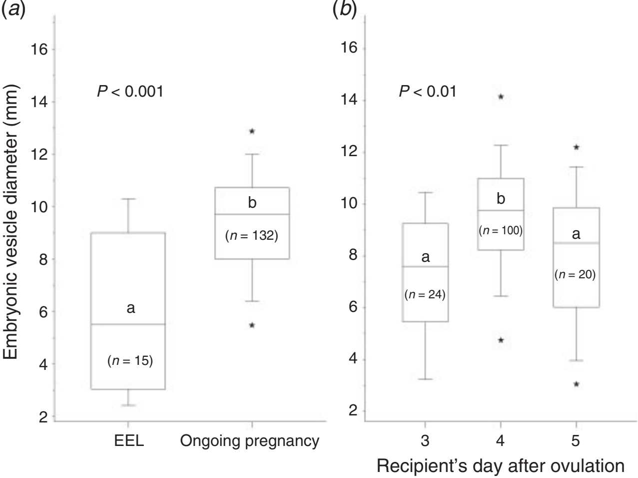

Fig. 2. Box plots of embryonic vesicle diameter 7 days after transfer of Day 6–8 frozen–thawed *in vitro*-produced (IVP) horse embryos. (*a*) Size comparison of vesicles at pregnancy diagnosis (determined 7 days after transfer) between pregnancies that later went on to be classified as either ongoing or lost (EEL: early embryonic loss). Pregnancies that were lost were smaller at the time of first detection ($P < 0.001$). (*b*) Effect of the number of days after recipient ovulation on vesicle size 7 days after transfer. Embryos transferred into mares on Day 4 after ovulation were larger at the first pregnancy examination 7 days after embryo transfer than those transferred on Days 3 and 5 ($P < 0.01$). Boxes show the interquartile range, with median values indicated by horizontal lines; whiskers show the range. Stars indicate conceptus diameter outliers (5th and 95th percentiles). Different letters indicate significant differences ($P < 0.05$).

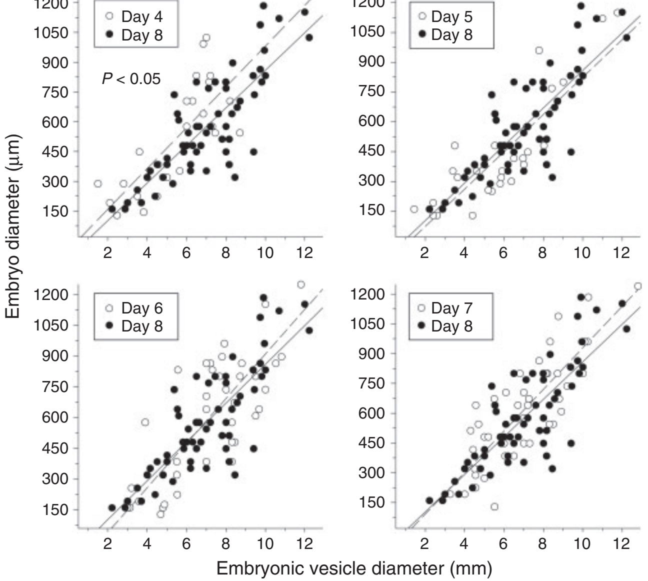

Fig. 3. Scatter plots with regression lines of best fit to show relationships between Day 8 *in vivo* horse embryo diameter at collection and vesicle diameter during ultrasound examination 4 days after transfer. In each case, Day 8 embryos are included as a 'synchronous' reference point. The regression line of best fit for embryos transferred into Day 4 recipients was significantly ($P < 0.05$) to the left of that for Day 8 recipients, indicating that embryos grew more slowly in Day 4 recipients. This was not observed for any other recipient stages ($P > 0.1$).

adjusting to a suboptimal environment (e.g. because of fewer mitochondria (Hendriks *et al.* 2015) or reduced expression of developmentally important genes (Smits *et al.* 2011)). Alternatively, it is possible that the progesterone concentration a uterus is exposed to in early dioestrus may have an effect on the expression of genes and or proteins required to support an embryo (i.e. the difference in progesterone priming effects is more significant between Days 3 and 4 after ovulation than Days 6 and 8). Certainly, progesterone secreted by the CL following ovulation is the primary factor orchestrating the establishment of endometrial receptivity for the preimplantation embryo via complex changes in the expression of genes involved in histotroph secretion (Forde *et al.* 2013). Moreover, the exact duration of endometrial exposure to progesterone is known to critically affect receptivity for embryo development and survival in ruminants (Spencer *et al.* 2016). Further support for the hypothesis that the Day 4 uterus is a more suitable environment for an IVP horse blastocyst was provided by the larger size of embryonic vesicles 7 days after transfer into a Day 4 than either a Day 3 or Day 5 recipient; this presumably reflects a uterine secretome better able to support and promote embryo development and growth.

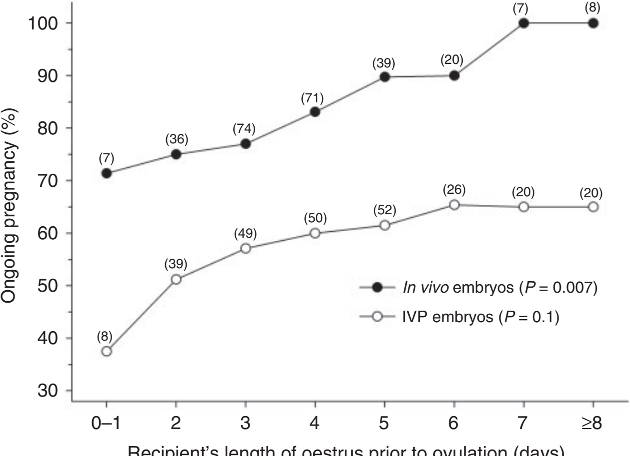

Fig. 4. Ongoing pregnancy rate following embryo transfer of frozen–thawed Day 6–8 *in vitro*-produced (IVP) embryos and Day 8 *in vivo*-derived embryos according to the length of the recipient mare's oestrus (characterised by days of endometrial oedema before the dioestrus in which the embryo was transferred). Values in parentheses indicate the number of transfers for each duration of oestrus. A longer oestrus had a beneficial effect on the likelihood of ongoing pregnancy for *in vivo*-derived ($P = 0.007$) but not IVP ($P = 0.1$) horse embryos.

As anticipated, Day 8 *in vivo*-derived embryos appeared to tolerate negative uterine asynchrony of up to 3 days very well, as evidenced by similar pregnancy and pregnancy loss rates and similar vesicle growth during the first 4 days. The only deviation from the normal pattern was when Day 8 *in vivo*-derived embryos were transferred into Day 4 recipients; although this did not compromise survival, given that the likelihood of pregnancy or pregnancy loss was not affected, the embryos did show a retardation in vesicle growth. A similar delay in embryo vesicle growth and in development of the embryo proper was reported previously for Day 8 embryos transferred into Day 3 recipients (5 days negative asynchrony; Gibson *et al.* 2017); it was hypothesised that the equine embryo is able to slow down or interrupt its development while it 'waits' for the uterus to 'catch up', at which time it resumes a normal growth and development trajectory. This asynchrony-induced delay in embryo development does not appear to compromise embryo survival in a commercial ET program (Jacob *et al.* 2012); similarly, we saw no increase in embryonic or fetal losses for Day 4 recipients of Day 8 *in vivo*-derived embryos.

Although the very short window of optimal synchrony observed for IVP embryos may initially appear to be a

Table 4. Effect of recipient mare's type of oestrus on likelihood of ongoing pregnancy

Data show pregnant recipients/total number of transfers, with the percentage of ongoing pregnancies in parentheses. IVP, *in vitro* produced; OV, ovulation; PGF, prostaglandin $F_{2\alpha}$

Embryos	First OV of year	Spontaneous oestrus (not first OV)	PGF-induced oestrus
IVP	17/28 (60.7)	68/113 (60.2)	71/123 (57.7)
In vivo derived	11/12 (91.7)	102/124 (82.3)	103/126 (81.7)

Table 5. Effect of time of year at embryo transfer on the likelihood of ongoing pregnancy and length of preceding oestrus

Unless indicated otherwise, data are given as mean ± s.d. duration of oestrus (days) or as pregnant recipients/total number of transfers, with percentages in parentheses. IVP, *in vitro* produced; PR, pregnancy

	March–April	May	June	July	August–September	*P*-value
IVP embryos						
Ongoing PR (%)	31/45 (68.9)	32/53 (60.4)	46/79 (58.2)	33/60 (55.0)	14/27 (51.8)	0.06
Oestrus length (days)	5.9 ± 2.3	4.7 ± 2.1	4.0 ± 1.9	3.9 ± 1.6	3.8 ± 1.6	<0.001
In vivo-derived embryos						
Ongoing PR (%)	42/48 (87.5)	53/59 (89.8)	49/58 (84.4)	36/46 (78.3)	36/51 (70.5)	0.04
Oestrus length (days)	4.7 ± 1.9	4.5 ± 1.6	3.8 ± 1.6	3.1 ± 1.3	3.2 ± 1.5	<0.001

disadvantage for the efficient management of recipient mares in a commercial program, it is important to note that all the IVP embryos included in this study were cryopreserved. Although cryopreservation could conceivably have subtly compromised embryo resilience and may have contributed to the reduced ability to tolerate a suboptimal environment, cryopreservation of equine IVP blastocysts appears not to have a detrimental effect on their post-thaw survival, with no difference in foaling rates for freshly transferred or vitrified–warmed IVP embryos (Choi and Hinrichs 2017). Moreover, cryopreserving embryos greatly simplifies the management of a recipient herd, because an embryo may be thawed only when a suitable recipient at the optimal uterine stage (i.e. Day 4 in our program) is available for transfer.

As reported previously (Cuervo-Arango *et al.* 2018*a*), the length of the recipient mare's oestrus (duration of endometrial oedema) before transfer of an *in vivo*-derived embryo affected the likelihood of pregnancy, and indeed the likelihood of an ongoing pregnancy. In this regard, an oestrus characterised by at least 5–6 days of endometrial oedema seems to offer an optimal chance of establishing and maintaining pregnancy. Conversely, we did not find a statistically verifiable effect of the length of the preceding oestrus on the likelihood of pregnancy following transfer of IVP embryos, although a very short (<2 days) oestrus did appear disadvantageous. Because it is not clear why there should be any difference between IVP and *in vivo*-derived embryos, it is possible that any effect of the duration of the preceding oestrus was masked by the marked effect of day after ovulation at transfer on IVP embryo survival after transfer. In an attempt to determine why the duration of the oestrus before ET may affect the likelihood of pregnancy, we recently performed a study in which we administered exogenous oestradiol to anoestrous mares for different durations before the administration of progesterone to simulate the effects of varying lengths of oestrus before ovulation (Silva *et al.* 2019). In endometrial biopsies recovered 4 days after progesterone administration (equating to Days 4–5 after ovulation), we found that anoestrous mares exposed to a long simulated oestrus (7 days) exhibited increased endometrial gene and protein expression for the progesterone-dependent protein P19 (uterocalin; Stewart *et al.* 1995), but reduced expression of fibroblast growth factor 2 (FGF2) compared with mares with short (2 days) or no (0 days) oestradiol priming before progesterone treatment (Silva *et al.* 2019). P19 is a member of the lipocalin family produced by the endometrium and is known to adhere to the capsule and enter the blastocoel fluid (Crossett *et al.* 1998). Although the function of P19 is not known, it has been proposed to be a transport protein capable of carrying lipids and/or small lipophilic molecules through the capsule to the developing embryo (Crossett *et al.* 1998). Similarly, although the exact function of FGF2 is not known, it has been proposed to play a role in the establishment of uterine receptivity to pregnancy, and its expression is known to be modified by oestrogens (de Ruijter-Villani *et al.* 2013). In short, a longer duration of oestrogenic priming before a progesterone rise does appear to modify genes and proteins with the capacity to influence endometrial receptivity to pregnancy. Therefore, it appears advisable, where possible, to select recipient mares that exhibited endometrial oedema for at least 5 days during the oestrus before transfer.

In conclusion, oestrus cycle characteristics of recipient mares significantly affect the likelihood of pregnancy after ET. However, the cycle parameters that most influence the likelihood of establishing and maintaining pregnancy differ between IVP and *in vivo*-derived embryos. Frozen–thawed IVP embryos appear to tolerate only a very narrow window of uterine synchrony and, in our program, Day 4 after ovulation was preferable to any other day. In contrast, *in vivo*-derived Day 8 embryos tolerate a wide range of recipient mare uterine synchrony (Days 4–9 after ovulation) with no apparent effect on pregnancy or pregnancy maintenance, and a modest retardation of embryo growth in Day 4 recipients only. Conversely, *in vivo*-derived embryos survive better in recipients that experienced a relatively long oestrus before transfer, characterised by >5 days of endometrial oedema.

Conflict of interest

The authors declare no conflicts of interest.

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