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# Speed of *in vitro* embryo development affects the likelihood of foaling and the foal sex ratio*

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**Abstract.** The success of *in vitro* embryo production (IVEP) in horses has increased considerably during recent years, but little is known about the effect of the speed of *in vitro* embryo development. Blastocysts ($n = 390$) were produced by intracytoplasmic sperm injection of IVM oocytes from warmblood mares, cryopreserved, thawed and transferred into recipient mares on Days 3, 4, 5 or 6 after ovulation. The time required for *in vitro*-produced (IVP) embryos to reach the blastocyst stage was recorded (Day 7 vs Day 8). The likelihood of foaling was affected by the speed of *in vitro* embryo development and recipient day after ovulation at transfer. The odds ratio for foaling was ~0.63 for transfer of Day 8 (46%) compared with Day 7 (56%) IVP blastocysts. The highest likelihood of pregnancy (72%) and foaling (60%) was observed when IVP blastocysts were transferred to recipient mares on Day 4 after ovulation. Finally, the sex (colt : filly) ratio was higher after transfer of Day 7 (71% : 29%) than Day 8 (54% : 46%) IVP blastocysts, suggesting that the speed of embryo development is sex dependent. In conclusion, the speed of *in vitro* embryo development in our IVEP system affects the likelihood of foaling and the sex of the foal.

**Additional keywords:** equine, intracytoplasmic sperm injection (ICSI), *in vitro* embryo production (IVEP), pregnancy.

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## Introduction

The success of equine *in vitro* embryo production (IVEP) has increased considerably over the past decade, leading to the commercialisation of this technology. Currently, IVEP by intracytoplasmic sperm injection (ICSI) of IVM equine oocytes is successful (i.e. at least one embryo per ovum pick-up (OPU)–ICSI treatment) in approximately 60% of donor warmblood mares (Claes *et al.* 2016). Nevertheless, *in vitro* culture of embryos to the blastocyst stage remains the limiting step in equine IVEP. The mean percentage of injected IVM oocytes that develop into a blastocyst, generally referred to as the blastocyst rate, is low and variable in horses, ranging from 16% to 38% (Galli *et al.* 2014), and there are several factors, such as breed, ICSI technique, IVM and culture media, that could contribute to this wide variation in blastocyst rate. Some of the between-laboratory differences in blastocyst rate are probably explained by the maximum time allowed for injected oocytes to reach the blastocyst stage, which ranges between 8 and 12 days after ICSI. Unfortunately, this time factor is rarely mentioned when the blastocyst rate is defined in clinical or experimental studies. In humans, the speed of *in vitro* embryo development is of crucial importance for subsequent success (Lewin *et al.* 1994; Lundin *et al.* 2001). More specifically, the speed of *in vitro* embryo development has been used as a marker for embryo quality, and subsequently as a predictor of pregnancy (Lewin *et al.* 1994; Lundin *et al.* 2001) and birth (Lundin *et al.* 2001). The likelihood of pregnancy and birth is approximately 10% higher for *in vitro*-produced (IVP) human embryos with a fast cleavage rate (i.e. early cleavage) than those with a slow cleavage rate (Lundin *et al.* 2001). It has also been reported that early cleavage of IVP horse embryos is associated with an increased likelihood of obtaining a pregnancy (Carnevale and Sessions 2012). Based on these findings, we hypothesised that the time required to reach the blastocyst stage, referred to as 'the speed of *in vitro*

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embryo development', affects the likelihood of both establishing pregnancy and, ultimately, foaling.

The speed of *in vitro* embryo development has not only been reported to affect the likelihood of ongoing pregnancy, but is also sex dependent in some species. Male bovine embryos produced by IVF develop faster *in vitro* than female embryos (Avery *et al.* 1991; Xu *et al.* 1992); almost 75% of Day 7 IVP bovine embryos are male, compared with only 20% of Day 8 IVP embryos (Avery *et al.* 1991). In addition, several studies have shown that the sex ratio of calves in IVF programs is skewed, with a higher proportion of males than females (Reichenbach *et al.* 1992; Agca *et al.* 1998). Unlike in cattle, it is not known whether the sex ratio of foals produced by IVEP is affected by the speed of *in vitro* embryo development or whether it is skewed towards males. Furthermore, with the exception of anecdotal data, there is little published information about foaling parameters with regard to gestation length and the health of newborn foals produced by ICSI of IVM equine oocytes. Therefore, the objectives of the present study were to: (1) examine the effect of the speed of *in vitro* embryo development on the likelihood of pregnancy and foaling, as well as on the sex ratio of foals; and (2) record the gestation length and describe the health of newborn foals produced by ICSI.

## Materials and methods

Data for the present retrospective study were derived from the clinical equine IVEP program at Utrecht University. The embryos were produced from oocytes shipped overnight from Utrecht University (Netherlands) to Avantea, in Italy, and returned as frozen embryos to Utrecht. The age (in years) of the oocyte donor mare and the year of IVEP (2014–17) were recorded.

### In vitro *production of equine blastocysts*

#### *Oocyte collection*

Immature oocytes were collected by transvaginal aspiration (TVA) of antral follicles from 163 donor mares at any stage of the oestrous cycle, as described by Claes *et al.* (2019). Mares were premedicated with detomidine hydrochloride (0.01 mg kg$^{-1}$, i.v.), butorphanol tartrate (0.01 mg kg$^{-1}$), flunixin meglumine (1.1 mg kg$^{-1}$, i.v.) and broad-spectrum antibiotics (6.6 mg kg$^{-1}$, i.v., gentamycin sulfate, 20 000 IU kg$^{-1}$, i.m., benzylpenicillin); epidural anaesthesia (8 mL of 2% lignocaine) was induced before TVA. Antral follicles >5 mm were punctured using a 12-gauge double-lumen needle and flushed 8–10 times with medium (Euroflush, IMV Technologies) supplemented with heparin sodium (20 IU mL$^{-1}$) to prevent blood clotting. Recovered follicular and flushing fluids were filtered (70-μm filter) and the fluid residue in the filter was poured into a sterile Petri dish. Oocytes were retrieved using a dissecting microscope, washed three times with modified HEPES-buffered synthetic oviductal fluid (mH-SOF), placed into mH-SOF and shipped overnight at 22°C in prewarmed polystyrene organ transplant boxes to the assisted reproductive technology laboratory (Avantea, Italy) for the *in vitro* production of blastocysts.

#### *IVM of oocytes, ICSI and* in vitro *culture of embryos*

*In vitro* processes (IVM, ICSI and *in vitro* culture (IVC)) were performed as described previously (Colleoni *et al.* 2011). The holding time of oocytes at 22°C before IVM ranged from 18 to 24 h. On arrival at the laboratory, immature oocytes were washed in mH-SOF and placed into IVM medium for 26–28 h in an incubator (38.5°C, 5% $CO_2$ in air). The IVM medium was a mixture (1 : 1) of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM; Life Technologies, ThermoFisher) supplemented with 0.1 IU mL$^{-1}$ FSH and 0.1 IU mL$^{-1}$ LH (Meropur; Ferring), epidermal growth factor (50 ng mL$^{-1}$; Peprotech), 1 mM sodium pyruvate (Sigma-Aldrich), 10% fetal calf serum (Life Technologies, ThermoFisher) and insulin–transferrin–sodium selenite (BD Bioscience). After 26–28 h, oocytes were transferred to mH-SOF supplemented with 2.5 μg mL$^{-1}$ hyaluronidase (Sigma-Aldrich) for 5 min and then pipetted in mH-SOF to remove cumulus cells. Only MII oocytes underwent ICSI. Frozen semen from 67 warmblood stallions, selected by the mare owner, was used for ICSI. After thawing, 100–150 μL semen was layered beneath 900 μL Tyrode's albumin–lactate–pyruvate (TALP) and placed upright in an incubator (38.5°C, 1 h) for sperm selection by swim-up. Approximately 800 μL was aspirated from the top, diluted with TALP (4 mL) and centrifuged (400*g*, 10 min, room temperature). Alternatively, density gradient centrifugation (45–90% Percoll; Sigma-Aldrich) was used to select motile, morphologically normal spermatozoa. After centrifugation, a modified SOF-IVF medium containing penicillamine, hypotaurine and epinephrine (PHE) was used to resuspend the semen pellet (Lazzari *et al.* 2002). Piezo pulses were used to immobilise a morphologically normal, motile spermatozoon and Piezo-driven ICSI was performed. IVC of injected MII oocytes was performed in a modified SOF-IVC medium supplemented with 6 mg mL$^{-1}$ bovine serum albumin (BSA; free fatty acid, Lampire Biological Products) and minimum essential medium (MEM) amino acids (Life Technologies, ThermoFisher; Lazzari *et al.* 2002) in 4-well dishes with 0.5 mL medium, without oil. Two days after ICSI, cleavage was assessed and non-cleaved embryos were removed from the dishes. Approximately half the modified SOF-IVC medium was replaced on Day 4 with SOF-IVC and on Day 6 with DMEM-F12 supplemented with 10% fetal calf serum (FCS). Blastocyst formation was assessed once daily from Day 6 to Day 9 (where Day 0 is the day of ICSI). Embryos were classified as blastocysts when a layer of aligned trophoblast cells was clearly visible, often accompanied by an increase in embryo diameter and thinning of the zona pellucida (Fig. 1). The speed of *in vitro* embryo development, defined as the interval between ICSI and blastocyst formation according to the parameter described in the previous sentence (Day 7 vs Day 8) was recorded. Day 9 blastocysts were not used for cryopreservation.

#### *Cryopreservation and thawing of equine IVP blastocysts*

IVP blastocysts ($n = 390$) were washed in mH-SOF, placed in mH-SOF containing increasing percentages of glycerol (5% glycerol for 5 min followed by 10% glycerol for 20 min) at room temperature and then loaded into 0.25-mL straws before the end of the last incubation step. Straws containing an IVP blastocyst were placed into a precooled (−6.5°C) alcohol

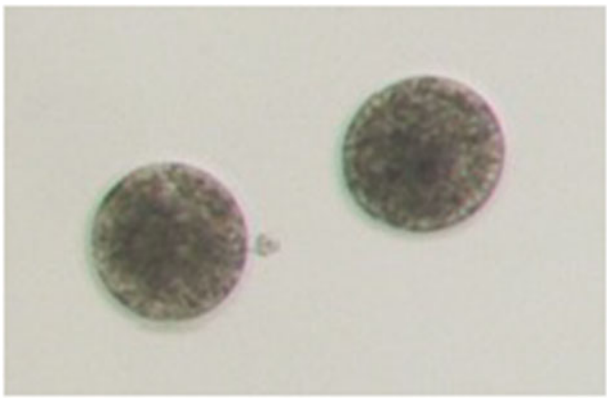

**Fig. 1.** Two *in vitro*-produced blastocysts in which a layer of trophoblast cells is clearly visible.

bath programmable freezing machine (Biocool IV; FTS Systems) and subjected to the following freezing protocol: −6.5°C for 5 min, manual seeding, −6.5°C for 5 min, cooling (−0.5°C $min^{-1}$) to −32°C and plunging of straws directly into liquid nitrogen (−196°C).

The cryopreserved IVP blastocysts were thawed by taking the straw out of the liquid nitrogen, holding it at room temperature for 8 s and then immersing it in a water bath (22–24°C) for 20–30 s. The outside of the straw was dried with tissue paper containing ethanol and the contents of the straw were expelled. The IVP blastocyst was washed in mH-SOF containing decreasing amounts of glycerol (8%, 6%, 4%, 2%; 5 min each) and subsequently held in mH-SOF until embryo transfer (ET). The IVP blastocyst was loaded into a 0.5-mL straw, which was placed into a Cassou gun (IMV technologies) for ET.

### *ET and the likelihood of pregnancy and foaling*

Most of the recipient mares were warmblood mares <14 years of age. Recipient mares were examined by ultrasonography once daily during late oestrus to determine the day of ovulation. Cryopreserved–thawed IVP blastocysts ($n = 390$) were transferred into sedated (0.067 mg $kg^{-1}$, i.v., detomidine hydrochloride) recipient mares on Days 3, 4, 5 or 6 after ovulation. Recipient mares were examined 7–10 days after ET for pregnancy, and pregnant recipient mares were examined at 2-week intervals until Day 42 of gestation. Pregnancy loss before Day 42 of gestation was defined as embryo loss, whereas pregnancy loss after Day 42 was defined as fetal loss. The owner of the embryo was contacted the year after ET to obtain the date of birth, the sex of the foal and the health of the foal over the first 24 h. Gestation length was calculated as the number of days between the day of ET and the birth of the foal + 5 days (estimated age of the IVP embryo at the time of ET).

## Statistical analysis

Logistic regression was used to examine the relationship between the speed of *in vitro* embryo development (Day 7 vs Day 8), recipient mare day of ovulation (Days 3, 4, 5 or 6), age of the donor mare (years), year of IVEP (2014–17) and the likelihood of pregnancy, pregnancy loss and foaling. Logistic regression was also used to examine the association between the speed of *in vitro* embryo development (Day 7 vs Day 8), year of IVEP (2014–17), age of the donor mare (years) and the sex of the foal. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for all significant variables in the logistic regression models. Statistical analyses were performed using JMP Version 14.0 (SAS Institute). $P < 0.05$ were considered significant, whereas P-values between 0.05 and 0.1 were considered to indicate a trend.

## Results

The overall likelihood of pregnancy at 10–14, 28 and 42 days of development was 62% (242/390), 56% (218/390), and 54% (210/390) respectively. The incidence of embryo and fetal loss was 13.2% (32/242) and 7.0% (17/242) respectively, yielding a likelihood of foaling of 49% (193/390).

The likelihood of establishing pregnancy (at 10–14 days) was affected by the recipient mare day of ovulation ($P = 0.01$) and tended to be affected by the speed of *in vitro* embryo development ($P = 0.06$) and the age of the donor mare ($P = 0.08$), but not by the year of IVEP ($P = 0.4$). The likelihood of foaling was affected by the speed of *in vitro* embryo development ($P = 0.03$) and the day of ovulation of the recipient mare ($P = 0.02$), and tended to be affected by the age of the donor mare ($P = 0.06$) but not by the year of IVEP ($P = 0.5$). The likelihood of establishing early pregnancy tended ($P = 0.06$) to be higher after transfer of Day 7 than Day 8 IVP blastocysts. The likelihood of foaling was 10% higher after transfer of Day 7 than Day 8 IVP blastocysts; the odds of foaling after transfer of Day 8 IVP blastocysts were approximately 0.63-fold those for Day 7 IVP blastocysts (Table 1). The likelihood of establishing pregnancy or foaling was higher after transfer of IVP blastocysts into recipients on Day 4 after ovulation than on Days 5 or 6 after ovulation (Table 2). Transfer of IVP blastocysts on Days 5 and 6 after ovulation significantly decreased the odds of obtaining an early pregnancy or of foaling, with the largest difference between Days 4 and 6 after ovulation. Furthermore, there was a tendency to lower odds of establishing early pregnancy after transfer of IVP blastocysts on Day 6 after ovulation than on Days 3 or 5 (Table 3).

Of the 193 foals born, 190 were healthy, two were stillborn and one needed intensive neonatal care. The mean (± s.d.) gestation length after transfer of IVP equine blastocysts was 339 ± 9 days. The sex (colt : filly) ratio was skewed towards males, with 61% of the foals born being colts ($n = 117$) and 39% being fillies ($n = 76$). The sex of the foal was affected by the year of IVEP ($P < 0.003$) and by the speed of *in vitro* embryo development ($P = 0.01$), but not by the age of the donor mare ($P = 0.4$). The odds of obtaining a colt were 20-fold (95% CI 2.1–197.9), 5-fold (95% CI 2.2–13.5) and 4-fold (95% CI 1.6–9.7) higher in 2014 ($n = 7$ foals, 6 colts), 2015 ($n = 76$ foals, 54 colts) and 2016 ($n = 76$ foals, 47 colts) respectively than in 2017 ($n = 34$ foals, 10 colts). The sex (colt : filly) ratio was higher after transfer of Day 7 than Day 8 IVP blastocysts (Fig. 2). The odds of obtaining a colt versus a filly were 2.28-fold higher (95% CI 1.19–4.39) after transfer of Day 7 than Day 8 IVP blastocysts.

**Table 1. Likelihood of early pregnancy, pregnancy loss and foaling in relation to the speed of *in vitro* embryo development (Day 7 vs Day 8 *in vitro*-produced blastocysts)**

Unless indicated otherwise, data are given as percentages, with *n*/*N* in parentheses, where *n* is number of pregnancies and *N* is number of transferred IVP blastocysts. Within rows, different superscript letters indicate significant differences ($P < 0.05$). CI, confidence interval; OR, odds ratio (Day 8 vs Day 7); *P*-value, the influence of speed of *in vitro* embryo development on the reproductive parameter.

| Reproductive parameter | Speed of *in vitro* embryo development | | OR (95% CI) | *P*-value |
|---|---|---|---|---|
| | Day 7 | Day 8 | | |
| Likelihood of early pregnancy | $68^{A}$ (95/140) | $59^{A}$ (147/250) | 0.65 (0.42–1.02) | 0.06 |
| Likelihood of pregnancy loss | $16.8^{A}$ (16/95) | $22.4^{A}$ (33/147) | – | 0.3 |
| Likelihood of foaling | $56^{A}$ (79/140) | $46^{B}$ (114/250) | 0.63 (0.41–0.96) | 0.03 |

**Table 2. Likelihood of early pregnancy, pregnancy loss and foaling in relation to the day of ovulation of the recipient mare (Days 3, 4, 5 and 6)**

Unless indicated otherwise, data are given as percentages, with *n*/*N* in parentheses, where *n* is number of pregnancies and *N* is number of transferred IVP blastocysts. Within rows, different superscript letters indicate significant differences ($P < 0.05$). *P*-value, the influence of that day of ovulation had on the reproductive parameter.

| Reproductive parameter | Day of ovulation (recipient mare) | | | | *P*-value |
|---|---|---|---|---|---|
| | Day 3 | Day 4 | Day 5 | Day 6 | |
| Likelihood of early pregnancy | $67^{A,B}$ (16/24) | $72^{A}$ (78/108) | $58^{B}$ (138/234) | $42^{B}$ (10/24) | 0.01 |
| Likelihood of pregnancy loss | $19^{A}$ (3/16) | $17^{A}$ (13/78) | $22^{A}$ (31/138) | $20^{A}$ (2/8) | 0.8 |
| Likelihood of foaling | $54^{A,B}$ (13/24) | $60^{A}$ (65/108) | $46^{B}$ (107/234) | $33^{B}$ (8/24) | 0.02 |

**Table 3. Odds ratios (ORs) and 95% confidence intervals (CIs) of pregnancy and foaling with regard to the day of ovulation of the recipient mare**

Only ORs with a *P*-value <0.1 are shown

| Reproductive parameter | Day of ovulation (recipient mares) | | OR (95% CI) | *P*-value |
|---|---|---|---|---|
| Likelihood of early pregnancy | Day 3 | Day 6 | 0.35 (0.11–1.13) | 0.08 |
| | Day 4 | Day 5 | 0.55 (0.33–0.90) | 0.02 |
| | Day 4 | Day 6 | 0.26 (0.10–0.66) | 0.005 |
| | Day 5 | Day 6 | 0.48 (0.20–1.13) | 0.09 |
| Likelihood of foaling | Day 4 | Day 5 | 0.55 (0.34–0.88) | 0.01 |
| | Day 4 | Day 6 | 0.32 (0.12–0.81) | 0.02 |

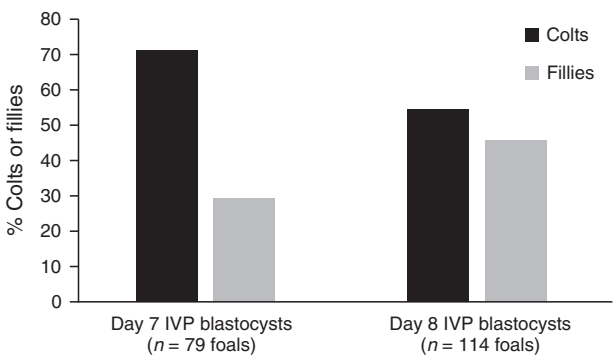


**Fig. 2.** Percentages of colts and fillies born after transfer of Day 7 and Day 8 *in vitro*-produced (IVP) blastocysts. In all, 79 and 114 foals were born after transfer of Day 7 and Day 8 IVP blastocysts respectively. The odds of obtaining a colt versus a filly were twice as high after transfer of Day 7 compared with Day 8 IVP blastocysts.

## Discussion

To the best of the authors' knowledge, the present study is the first reporting the effects of the speed of *in vitro* embryo development on pregnancy outcome and the sex ratio of foals after transfer of a large number of frozen–thawed IVP equine blastocysts. The likelihood of foaling, and of obtaining a colt, was higher after transfer of a Day 7 IVP blastocyst than a Day 8 IVP embryo. Furthermore, IVEP was associated with a skewed sex ratio among the foals (61% colts vs 39% fillies), but did not appear to affect the gestation length or health of the newborn foals (compared with other reproductive techniques).

The likelihood of pregnancy and foaling after transfer of frozen–thawed IVP embryos was 62% and 49% respectively, which is roughly comparable to fresh IVP embryos (McCue *et al.* 2016) but still lower than reported for an ET program using freshly transferred *in vivo*-derived horse embryos (Cuervo-Arango *et al.* 2018*a*). The two most important factors affecting foaling outcome

were the speed of *in vitro* embryo development and the day of ovulation of the recipient mare at the time of ET. The likelihood of foaling was 10% higher after transfer of Day 7 than Day 8 IVP blastocysts, indicating that rapid *in vitro* development of IVP embryos results in a more favourable pregnancy outcome. This, in turn, implies that the speed of *in vitro* embryo development reflects embryo quality, and could be used as a marker for the quality of equine IVP blastocysts. Data in other species suggest that a timely development of embryos to the blastocyst stage is of crucial importance because both unusually fast or slow developing embryos are associated with lower implantation rates (Gutiérrez-Adán *et al.* 2015). In order to obtain equine IVP blastocysts of good quality, it is of crucial importance that laboratories offering ICSI commercially set a maximum or cut-off time for IVP embryos to reach the blastocyst stage. Although no Day 9 IVP blastocysts were transferred in the present study, it is tempting to speculate that the likelihood of pregnancy will decrease even further after Day 8. The underlying reason for the lower likelihood of foaling after transfer of Day 8 IVP blastocysts is not known. However, it has been shown in humans that the incidence of chromosomal abnormalities in IVP embryos increases with the time required for IVP embryos to develop into a blastocyst; the incidence of euploidy is higher for human IVP embryos reaching the blastocyst stage on Day 5 than for Day 6 or Day 7 embryos (Shah *et al.* 2016; Barash *et al.* 2017). Similarly, Chen *et al.* (2014) reported that aneuploidy has a negative effect on *in vitro* embryo development, delaying the time to blastocyst formation. Therefore, a higher incidence of chromosomal aberrations among Day 8 equine IVP blastocysts could have contributed to the lower likelihood of foaling observed in this study, but the incidence of aneuploidy in horse embryos at different developmental stages has not been studied. Finally, chromosomal abnormalities in IVP embryos may be of greater importance than the speed of *in vitro* embryo development because the pregnancy outcome is similar for Day 5 and Day 6 human IVP blastocysts, provided that the embryos are euploid (Taylor *et al.* 2014). Another factor that could contribute to the lower likelihood of foaling after transfer of Day 8 IVP blastocysts is the number of cells within the IVP blastocyst. The number of cells in bovine IVP embryos is closely correlated with the time required for early IVP embryos to develop into a blastocyst; Day 8 IVP blastocysts contained fewer cells in the inner cell mass than Day 7 IVP blastocysts (van Soom *et al.* 1997). However, whether this finding can be extrapolated to horses remains to be determined.

In addition to the speed of *in vitro* embryo development, the day after ovulation at which an embryo is transferred to the recipient mare has a strong effect on the likelihood of pregnancy and foaling. To obtain the highest likelihood of pregnancy and foaling using our IVEP system, IVP embryos should be transferred into recipient mares on Day 4 after ovulation. A more advanced uterine environment appears to be associated with a reduced likelihood of pregnancy and foaling. Thus, in contrast with flushed fresh *in vivo* embryos, the time window in which frozen–thawed IVP blastocysts can be transferred into recipient mares is very narrow (Cuervo-Arango *et al.* 2018*b*). This can be explained by the fact that cryopreservation *per se* is an 'insult' to the embryo, which, as a result, requires some time after thawing and transfer to resume development.

As in cattle, the sex ratio of foals after transfer of Day 7 and Day 8 IVP blastocysts appears to be skewed towards males (~60% colts). Preliminary data on Day 9 embryos indicates a prevalence of female embryos (C. Galli, unpubl. obs.). Interestingly, this skewed sex ratio was more obvious after transfer of Day 7 IVP blastocysts, when more than 70% of the foals born were colts. Therefore, selecting fast-growing IVP embryos for transfer not only affects pregnancy outcome, but also favours the production of colts. Because the sex ratio of the foals is affected by the speed of *in vitro* embryo development using our IVEP system, it is plausible that *in vitro* embryo development after ICSI is sex dependent, with male horse embryos developing more rapidly *in vitro* than female horse embryos. The sex ratio of IVP blastocysts can also be affected by the IVC medium. It is well established that glucose in the IVC medium has a negative effect on the cleavage and formation of female bovine blastocysts (Gutiérrez-Adán *et al.* 2001; Peippo *et al.* 2001). Male embryos cleave earlier than female embryos and are more likely to develop into a blastocyst in a glucose-enriched medium, whereas female embryos cleave earlier than male embryos in a glucose-deprived culture medium (Peippo *et al.* 2001). This, in turn, indicates that the metabolism of glucose differs between male and female embryos. One of the enzymes that plays an important role in the metabolism of glucose is glucose-6-phosphate dehydrogenase (G6PD). G6PD is encoded by a gene on the X chromosome and regulates the pentose phosphatase pathway (Kimura *et al.* 2005). G6PD is more abundantly expressed in female than male bovine embryos (Peippo *et al.* 2002), and inhibition of G6PD results in a more balanced sex ratio of embryos (Kimura *et al.* 2005). The sex ratio of foals was also affected by the year of IVP, with the odds of obtaining a colt decreasing each year. This observation is intriguing because no changes have been made to the IVC medium over the years studied. It has been shown in cattle that the sex ratio of embryos can be affected by the composition of the IVM medium (Marei *et al.* 2018) and the bull used for IVF (Alomar *et al.* 2008), but whether there is a similar effect in horses remains to be determined. Finally, whether this skewed sex ratio can also be observed in other equine IVEP systems using different maturation and culture media and conditions is unknown. Although the present study did not include a control population for comparison, IVEP does not appear to have an effect on the gestation length or health of newborn foals. The gestation length after transfer of IVP blastocysts was 339 days, which is similar to the gestation length of 338 days reported previously for warmblood mares (Kuhl *et al.* 2015). Finally, nearly all the newborn foals were healthy, indicating that ICSI and IVC of embryos can be considered a safe technology for producing foals.

In conclusion, the likelihood of pregnancy and foaling using our IVEP system is highest when IVP blastocysts are transferred into recipients on Day 4 after ovulation. The speed of *in vitro* embryo development or the time required for injected oocytes to reach the blastocyst stage has an effect on the likelihood of foaling, and therefore could be used as marker for the quality of IVP equine blastocysts. Furthermore, the skewed sex ratio of foals using our IVEP system suggests that *in vitro* embryo development is sex dependent in horses, with more rapid development linked to male embryos. Finally, IVEP in horses

does not appear to have an effect on gestation length or the health of newborn foals.

## Conflicts of interest

The authors declare no conflicts of interest.

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